Microbial Community Dynamics in Biofilters of Recirculating Aquaculture Systems

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Departamento de Biologia – Faculdade de Ciências da Universidade do Porto
2023

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Acknowledgements

Throughout my dissertation, I have received significant support and assistance, and I am deeply grateful for it. Firstly, I would like to express my gratitude to Joana Pereira Fernandes, my supervisor, whose expertise was invaluable in formulating the research questions and providing valuable guidance throughout my studies. She equipped me with the necessary tools to choose the right direction and pushed me to sharpen my thinking, elevating my work to a higher level. I am grateful for her patient support and for the numerous opportunities she provided me to further my research.

I would also like to thank my tutors, Catarina Magalhães and Ana Paula Mucha, for their dedicated involvement in every step of the process and their valuable guidance throughout my studies. Without their assistance, I would not have been able to successfully complete my dissertation.

Moreover, I want to express my gratitude to everyone at CIIMAR for providing me with the necessary facilities and equipment to carry out my research. The opportunity to conduct my research at CIIMAR was an amazing experience, and I am thankful for it.

I would like to acknowledge everyone at the EcoBioTec Laboratory at CIIMAR, especially Diana Almeida, for her invaluable guidance and advising. She provided precious feedback that helped me address several issues, and I am grateful for her input. I would also like to thank Rafaela Mendes, Inês Ribeiro, Maria Bôto, and Miguel Semedo for their continuous support and willingness to help me throughout my research.

I am also extremely grateful to Isidro Blanquet for his kind guidance to the Safiestela compound and for the opportunity to visit the enterprise.

I would like to express my heartfelt gratitude to my parents for their wise counsel and sympathetic ear. Despite the distance and homesickness during the pandemic, they were always there supporting me, and I am grateful for the opportunities they have granted me. Finally, I want to thank my friends for providing me with happy distractions that allowed me to rest my mind outside of my research and for welcoming me to this new challenge in my life. I could not have completed this dissertation without their support.

The research of this thesis was supported by the project 39948_FeedMi, supported by Portugal and the European Union through FEDER/ERDF, CRESC Algarve 2020 and NORTE 2020, in the framework of Portugal 2020.

Resumo

A produção de peixes em aquacultura tem vindo a registar um aumento significativo para dar resposta à crescente procura de alimentos devido ao crescimento exponencial da população mundial. Os sistemas de recirculação em aquacultura (RAS, do inglês Recirculating Aquaculture Systems) foram concebidos como uma abordagem mais sustentável para a produção intensiva de peixes. Estes sistemas permitem a reutilização da água, reduzindo o desperdício e preservando a saúde dos peixes. Um exemplo dessa tendência é a Safiestela, uma divisão do grupo Seaeight localizada na Póvoa de Varzim, que se concentra na produção do linguado *Solea senegalensis*, uma espécie marinha altamente rentável quando cultivada em sistemas RAS.

Esse tipo de sistema envolve várias etapas de tratamento da água, sendo a utilização de biofiltros um dos elementos essenciais. Nos sistemas RAS, a eliminação dos níveis de NH_4^+ é de extrema importância, já que esse composto nocivo está presente na água devido aos processos metabólicos dos peixes. A nitrificação microbiana nos biofiltros é a técnica mais amplamente usada para reduzir os níveis de NH_4^+. Essas comunidades microbianas desempenham um papel crucial, especialmente por meio das bactérias nitrificantes, que realizam a oxidação sequencial do NH_4^+ (ião amónio) em NO_2^- (nitrito) e NO_3^- (nitrato), substância menos tóxica. A manutenção do funcionamento ideal dessa comunidade microbiana representa um desafio constante na gestão dos sistemas RAS, com o objetivo de assegurar um ambiente saudável tanto para o ecossistema como para os peixes. É importante notar que quaisquer mudanças abruptas que possam ocorrer nos sistemas de produção ou nos parâmetros físico-químicos da água (salinidade, temperatura, pH, etc.) podem afetar significativamente a comunidade bacteriana presente no biofiltro. A ativação da comunidade do biofiltro é necessária sempre que uma nova unidade é iniciada ou após a limpeza ou manutenção de um biofiltro. Durante a ativação, os biofiltros passam por um processo de colonização até que a comunidade bacteriana se estabilize, levando a uma eficiência potencialmente menor do biofiltro durante esse período.

Compreender as mudanças dinâmicas e a maturação dessas comunidades bacterianas durante o processo de ativação do biofiltro é essencial para otimizar o desempenho dos RAS.

Este estudo investiga as mudanças dinâmicas nas comunidades microbianas em três biofiltros de RAS marinho (dois biofiltros com uma diferença de um mês e meio de reativação, e um biofiltro completamente operacional). Especificamente, examinámos as alterações nas abundâncias relativas durante os períodos de crescimento da

comunidade, incluindo a inicialização, monitorização inicial e verificação após um ano de operação do sistema.

Para investigar a composição do biofiltro, utilizámos sequenciação de DNA de larga escala e a região V4-V5 do gene 16S rRNA para analisar e comparar as comunidades microbianas nos biofiltros. Não foram identificados perfis taxonómicos comparáveis entre os dois biofiltros reativados e o biofiltro em funcionamento pleno. Verificou-se que ao longo do tempo, as comunidades microbianas nos dois biofiltros ativados passaram por mudanças que fizeram com que as abundâncias de diferentes grupos taxonómicos se tornassem mais semelhantes entre os biofiltros. Essas mudanças levaram a uma composição de comunidade parcialmente semelhante nos biofiltros, indicando que, apesar das diferenças iniciais, os sistemas reativados convergiram para uma configuração microbiana semelhante ao longo do tempo.

Após um período de ativação de cerca de 60 dias, observámos comunidades microbianas altamente diversas, com uma baixa percentagem de nitrificantes. No entanto, as baixas concentrações de amónia e nitrito, juntamente com o rápido aumento nas concentrações de nitrato um ano após a reativação, indicaram uma tendência de diminuição e a consolidação de boas condições de nitrificação em ambos os sistemas. Isso levou-nos a supor que, ao longo do tempo, as comunidades bacterianas nos biofiltros passam por mudanças dinâmicas de adaptação e mudança com o propósito de tornar o sistema de biofiltros mais eficiente. Os nossos resultados demonstram uma diversidade significativa de espécies nos três biofiltros, apresentando distribuições únicas entre as comunidades microbianas em cada sistema. Os filos dominantes em todos os biofiltros incluem Proteobacteria e Bacteroidota. Verificou-se a presença de bactérias nitrificantes como *Nitrospira* (NOB) e *Nitrosomonas* (AOB), respetivamente dos filos Nitrospirota e Proteobacteria, mas apenas um ano após a reativação.

Palavras-chave: Sistemas de Recirculação em Aquacultura (RAS), biofiltros, ativação de biofiltros, dinâmica da comunidade microbiana, parâmetros físico-químicos, AOB, NOB, nitrificação, diversidade microbiana, sustentabilidade da aquacultura.

Abstract

The production of fish in aquaculture has been experiencing a significant increase to meet the growing demand for food due to the exponential growth of the world population. Recirculating Aquaculture Systems (RAS) have been designed as a more sustainable approach to intensive fish production. These systems allow for water reuse, reducing waste and preserving fish health. An example of this trend is Safiestela, a division of the Seaeight group located in Póvoa de Varzim, focusing on the production of Senegalese sole, a highly profitable marine species when cultivated in RAS.

Such a system involves several water treatment stages, with the use of biofilters being one of the essential elements. In RAS, the removal of NH_4^+ levels is extremely important as this harmful compound is present in water due to fish metabolic processes. Microbial nitrification in biofilters is the most widely used technique to reduce NH_4^+ levels. These microbial communities play a crucial role, especially through nitrifying bacteria, which sequentially oxidize NH_4^+ (ammonium ion) to NO_2^- (nitrite) and NO_3^- (nitrate), a less toxic substance. Maintaining the ideal function of this microbial community represents a constant challenge in RAS management to ensure a healthy environment for both the ecosystem and the fish. It is important to note that any abrupt changes that may occur in production systems or physicochemical water parameters (salinity, temperature, pH, etc.) can significantly affect the bacterial community present in the biofilter. Activation of the biofilter community is required whenever a new unit is started or after cleaning or maintaining a biofilter. During activation, biofilters undergo a colonization process until the bacterial community stabilizes, potentially leading to lower biofilter efficiency during this period.

Understanding the dynamic changes and maturation of these bacterial communities during the biofilter activation process is essential for optimizing RAS performance. This study investigates the dynamic changes in microbial communities in three marine RAS biofilters (two biofilters with a difference of one and a half months in reactivation and one fully operational biofilter). Specifically, we examined changes in relative abundances during community growth periods, including startup, initial monitoring, and verification after one year of system operation.

To investigate the biofilter's composition, we used high-throughput DNA sequencing and the V4-V5 region of the 16S rRNA gene to analyze and compare microbial communities in the biofilters. Different taxonomic profiles were identified among the two reactivated biofilters and the fully operational biofilter. Over time, microbial communities in the two reactivated biofilters underwent changes that made the abundances of different

taxonomic groups become more similar between the biofilters. These changes led to a partially similar community composition in the biofilters, indicating that, despite initial differences, the reactivated systems converged to a similar microbial configuration over time.

After an approximately 60-day startup period, we observed highly diverse microbial communities with a low percentage of nitrifiers. However, the decreasing concentrations of ammonia and nitrite, coupled with the rapid increase in nitrate concentrations one year after reactivation, indicated a trend towards improvement, establishing favorable nitrification conditions in both systems. This led us to assume that, over time, bacterial communities in the biofilters undergo dynamic changes, adapting and shifting to enhance the efficiency of the biofilter system. Our findings demonstrate significant species diversity in all three biofilters, with unique distributions among microbial communities in each system. Dominant phyla in all biofilters include Proteobacteria and Bacteroidota. The presence of nitrifying bacteria such as *Nitrospira* (NOB) and *Nitrosomonas* (AOB), belonging to the phyla Nitrospirota and Proteobacteria, respectively, was observed, but only one year after reactivation.

Keywords: recirculating aquaculture systems (RAS), biofilters, biofilter reactivation process, microbial community dynamics, physicochemical parameters, AOB, NOB, nitrification, microbial diversity, aquaculture sustainability.

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List of Abbreviations

AOA	AMMONIA-OXIDIZING ARCHAEA
AOB	AMMONIA-OXIDIZING BACTERIA
DADA2	DIVISIVE AMPLICON DENOISING ALGORITHM 2
DNA	DEOXYRIBONUCLEIC ACID
ENM	ESTRATÉGIA NACIONAL PARA O MAR (NATIONAL STRATEGY FOR THE SEA IN PORTUGAL)
FAO	FOOD AND AGRICULTURE ORGANIZATION
NGS	NEXT-GENERATION SEQUENCING
NOB	NITRITE-OXIDIZING BACTERIA
PCR	POLYMERASE CHAIN REACTION
PEAP	PLANO ESTRATÉGICO PARA A AQUICULTURA PORTUGUESA
RAS	RECIRCULATING AQUACULTURE SYSTEM
rRNA	RIBOSOMAL RIBONUCLEIC ACID
SILVAngs	DATABASE FOR RRNA GENE SEQUENCES, INCLUDING THOSE OBTAINED FROM NEXT-GENERATION SEQUENCING (NGS) TECHNOLOGIES
TAN	TOTAL AMMONIA NITROGEN

1. Motivation and Main Findings

The increasing global demand for fish has led to the development of intensive aquaculture systems (FAO, 2022), the water recirculation systems (RAS) meet the need for large-scale production, by improving efficiency and sustainability (Timmons et al., 2002). RAS allows for water reuse, minimizing waste, and reducing the environmental impact associated with conventional open-water aquaculture (Masser et al., 1999). Among the key components of RAS, biofilters play a crucial role in water treatment, as they host microbial communities responsible for nitrification (Li, Q. et al., 2022), converting toxic nitrogen compounds such as ammonia (NH_4^+) and nitrite (NO_2^-) into less harmful forms like nitrate (NO_3^-) (Aalto et al., 2022a; Daims et al., 2001). Biofilter microbial communities perform various services, not only removing toxic metabolic but also products organic waste (Rieder et al., 2023). The effectiveness of biofilters in RAS is essential for ensuring water quality, fish health, and overall RAS performance (Tal et al., 2003). Maintaining high water quality is important for these systems, especially in compartments such as tanks, purifiers, and biofilters, which hold diverse and complex microbial communities (Martins et al., 2010). However, the specific composition and diversity of these communities, including potentially harmful pathogenic bacteria, are not well understood and continue to pose a challenge (Schreier et al., 2010). Additionally, global studies exploring RAS microbial community patterns and keystone taxa are scarce, leading to incomplete characterization of the overall microbial dynamics in RAS environments (Rieder et. al., 2023). In-depth studies to analyze these communities can provide valuable insights for modelling the appropriate microbiome in RAS systems.

During the biofilter activation phase, which can be time-consuming, concerns arise about the potential accumulation of toxic compounds in the system, which may affect fish health and compromise the RAS stability (Bastos Almeida et al., 2022). Therefore, understanding the dynamics of bacterial communities during the biofilter activation process is crucial for optimizing their efficiency and promoting biofilter stability (Burut-Archanai et al., 2021). Moreover, the structure and function of bacterial communities in biofilters can be influenced by various physicochemical factors, such as pH, temperature, dissolved oxygen (DO), and nutrient availability (Keller and Zengler, 2004; Chen et al., 2006). The crucial role of temperature, salinity, and pH in the selection and performance of the microbial groups involved in the nitrogen transformations like the nitrifying bacteria (AOB and NOB) cannot be overlooked. Hence, it is imperative to comprehend the connection between physicochemical factors and the composition of bacterial communities to foster stability and efficiency in RAS biofilters.

The present study aims to investigate the prokaryotic community changes during the biofilter activation process in three recirculating aquaculture systems. By analyzing microbial diversity through high-throughput sequencing of the 16S rRNA gene, we seek to identify key bacterial species present in the biofilters and understand how physico-chemical factors may influence the composition of these communities.Prior to this study, research on prokaryotic communities in RAS has primarily focused on general microbial composition, nitrifying bacteria, and their roles in nitrogen removal (Li, Q. et al., 2022; Blancheton et al., 2013), and Almeida et al. (2021) have emphasized the dominance of the phylum Proteobacteria (reclassified as Pseudomonadota) in RAS setups. This finding is consistent with the well-known fact that Proteobacteria is the largest and most diverse phylum, encompassing various functional types of bacteria (Gupta, 2000). Regarding genera, *Nitrospira* and *Nitrosomonas*, are thought to be the primary nitrifiers present in RAS biofilters (Bartelme et al., 2017) and are essential for the conversion of ammonia (NH_4^+) to nitrite (NO_2^-) and further to nitrate (NO_3^-) during the nitrification process. While these studies have provided valuable insights into the functioning of biofilters and nitrification, they often lack a detailed investigation into the temporal changes and dynamics of these microbial communities during the crucial period of biofilter reactivation. The novelty of this study lies in its comprehensive examination of these temporal changes and dynamics, specifically focusing on the crucial phase of biofilter reactivation.

This work introduces a novel approach by investigating the dynamic changes in bacterial communities during the biofilter activation process in two comparable RAS over a year after activation. Utilizing high-throughput sequencing of the 16S rRNA gene (Vieira, 2017; Hastuti et al., 2019), the study provides a detailed analysis of microbial diversity at both the phylum and genus levels, with a particular focus on key genera involved in nitrification and system stability. The identification of five common genera, namely *Thiothrix*, *Nitrospira*, *Nitrosomonas*, *Maribacter*, and *Blastopirellula*, with only minor differences in their relative abundances between the two systems, indicates a significant convergence of bacterial community composition over time. The similarity in abundance values implies that these genera may have similar ecological roles or responses to the environmental conditions in both RAS systems. It is possible that the two systems share comparable environmental conditions, such as temperature, pH, nutrient levels, and organic loading, which promote the growth of these particular bacterial genera. However, the exact reasons for this convergence of abundance values require further investigation and analysis. Factors such as the type of fish species, feed composition, and water management practices may also play a role in shaping the microbial community

dynamics. This temporal aspect of microbial community dynamics, specifically after a year of biofilter reactivation, has not been extensively explored in previous research, making this study a valuable contribution to the field. This thesis offers valuable insights into the factors influencing the evolution of microbial colonization in the two studied RAS compared to another one operating in continuous mode. Through meticulous data analysis and interpretation, the research sheds light on the mechanisms driving the observed convergence of bacterial community composition in the biofilters of the studied RAS setups. By contrasting the findings with the continuous RAS, the study provides a comprehensive understanding of how different operational modes may influence microbial community dynamics in RAS improving significantly our knowledge on biofilter performance and stability. Finally, this study represents a significant advancement in our knowledge of microbial community dynamics in RAS biofilters and provides a solid foundation for future aquaculture practices, guiding the development of more efficient and sustainable biofiltration strategies.

2. State of the Art

2.1. Aquaculture

Aquaculture is the term used for the cultivation of aquatic species with the aim of producing commercial food products efficiently and profitably. In order to cope with the increasing demand of fish, aquaculture has become one of the fastest growing food production sectors in recent years.

Aquaculture is currently considered to increase global food and nutrition security (FAO, 2022). The year 2022 marks the contribution of FAO statistical system in the fields of fisheries and aquaculture for the years1950-2020, which resulted in the longest time series of data ever published (FAO, 2022). From an almost residual global aquaculture production in the 1950s, it increased in 2020 to a record 122.6 million tons (Figure 1). This means that this sector of food production from animal origin (marine and inland waters) has grown very quickly in the last 70 years. Although the supply of fish has remained at a relatively stable level since the 1990s (about 80 million tons), the demand for fish and fishery products continues to grow as part of healthy human food. Aquaculture is expected to expand its current production by 2050 (FAO, 2022).

On the other hand, the intensive fishing that has been practiced in recent decades has led to the depletion of marine stocks, which is why more attention has been given to aquaculture production. Based on the FAO assessment, the fraction of fish stocks in biologically sustainable areas declined to 64.6% in 2019, which it is 1.2% lower than in 2017. This fraction was 90% in 1974. Overall, many of the global marine stocks are already classified as overexploited (FAO, 2022). Therefore, implementation of effective measures and careful fisheries management are needed to control catches in the world's oceans.

The growth and rapid development of farms with intensive and hyper-intensive production emerged in response to the growing demand for fish, enabling recruitment alternatives that exhibit very high production rates, but which have a high environmental impact. These production units, although producing large quantities of fish, they release effluents to the outside that contain high concentrations of waste, which contributed to environmental pollution (Bohnes and Laurent, 2021). Thus, different solutions have been adopted to reduce or eliminate the impact on ecosystems. These solutions basically consist of treating wastewater, recycling it and sending it to waste treatment plants. Therefore, it is not surprising that the current and future expansion of fisheries

production, through aquaculture, will have to make efforts to combine the best conditions for fish production, mitigating the risk and impact on the environment (Tal et al., 2009).

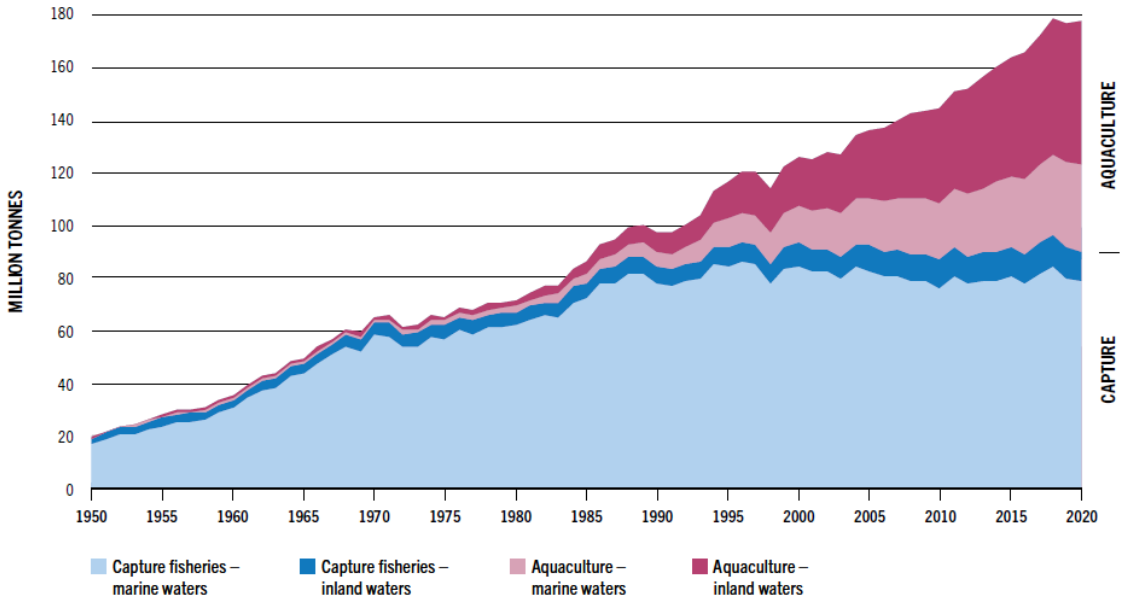


Figure 1 - World capture fisheries and aquaculture production (FAO, 2022).

In Portugal, until the 1970s, the cultivation of marine species was carried out in estuarine waters and coastal lagoons, many of which reused saline industry infrastructure in extensive production regimes (PEAP, 2014-2020). As in Portugal the catches became insufficient for the high demand of fish, the growth of aquaculture was promoted to balance things, a measure that is currently included in the scope of the National Strategy for the Sea in Portugal until 2030 (ENM 2021-2030).

2.2. Recirculating Aquaculture Systems (RAS)

Recirculating Aquaculture Systems (RAS) are sustainable processes for the intensive fish production and has raised as an alternative to traditional aquaculture systems (Aich et al., 2020). RAS systems rely on several processes that support life in the culture tanks, namely in terms of purifying the circulating water and processing the waste that accumulates during fish growth, since, under ideal conditions, the RAS system does not require water replenishment (Bregnballe, 2022; Ruiz et al., 2019).

The implementation of the RAS allows a better control of biological and environmental parameters (Rurangwa et al., 2014), as it reuses the water that circulates in the system and treats it to maintain a good water quality for the cultivated species. The reuse of water through integrated waste management and nutrient recycling allows a more

controlled and economic intensive production conditions, with less associated environmental impact (Duarte et al, 2019; Almeida et al., 2021). Nonetheless, the most significant drawback of the RAS is the potential deterioration of water quality if the treatment processes within the system are not adequately controlled (Ruiz et al., 2019). The RAS technology is highly suitable for species that require improved water quality including those of extreme sensitivity to outbreaks of infectious diseases such as vibriosis (caused by several species of the genus Vibrio, especially *Vibrio anguillarum*) and flexibacteriosis (caused by *Tenacibaculum maritimum)* (Lopez et al., 2022; Mabrok et al., 2016).

Fish excrete a variety of nitrogenous wastes through urine and feces (Takeuchi, 2017). Additionally, NH_4^+ is a by-product of the metabolism of feed proteins assimilated by fish (Hall et al., 1992; Takeuchi, 2017), and it is also produced by the bacterial decomposition of organic matter present in the system (Figure 2). Uneaten feed also causes high levels of nitrogenous waste (Foore, 2016). In intensive aquaculture systems, the widespread issue of nitrogen accumulation, mainly in the form of ammonia, poses a significant challenge. However, bacteria present in RAS water play relevant roles in maintaining water quality in aquaculture systems. For example, beneficial nitrifying bacteria such as *Nitrosomonas* and *Nitrobacter* are responsible for converting ammonia to nitrite and then to nitrate, respectively, through the nitrification process (Figure 2). These nitrate-producing bacteria play a vital role in reducing ammonia levels, as excessive ammonia can be harmful to fish (Takeuchi, 2017). Therefore, biofilters are used for the removal of nitrogen compounds using nitrifying bacteria (Masser et al., 1999; Hagopian and Riley, 1998; Takeuchi, 2017; Schuster and Stelz, 1998).

The toxicity levels of ammonia (NH_3-N/NH_4^+) and nitrites (NO_2^-) vary according to fish species, as well as specific conditions in the aquatic environment. In RAS, where the concentration of these substances can be monitored and controlled, acceptable toxicity levels are usually kept within safe ranges for fish. As a typical guideline, salmonids are considered to have an upper safe limit for NH_3-N of 0.0125 mg/L (Timmons et al., 2018). Nitrate is non-toxic at levels normally found in a RAS unit (>200 mg/L) (Losordo et al., 1998). Regarding nitrites, toxicity is related to their ability to interfere with oxygen transport in fish blood, leading to conditions known as methemoglobinemia. Nitrite concentrations above 0.5 mg/L are considered highly toxic (Gomez Isaza et al., 2021).

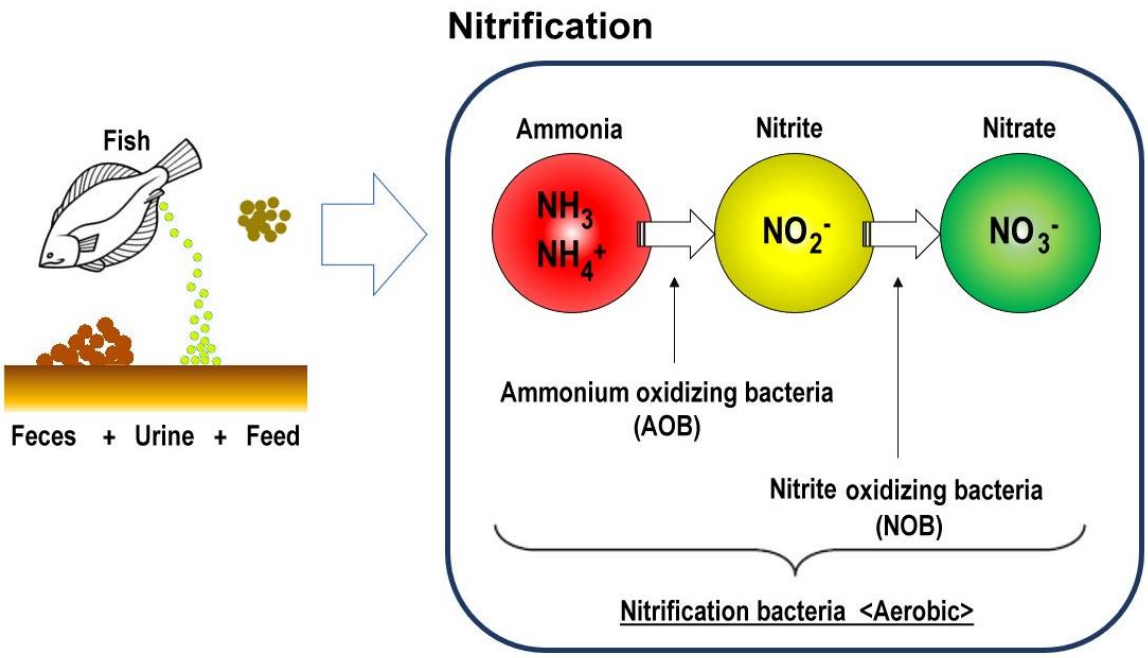


Figure 2 - Schematic diagram of flow of nitrification by bacteria. Adapted from (Takeuchi, 2017).

The accumulation of organic matter to RAS culture tanks (faeces and feed), can benefit heterotrophic bacteria, that could outperform and compete with the nitrifying bacteria (Foore, 2016), increasing the Biological Oxygen Demand (BOD). Heterotrophic bacteria grow considerably faster than nitrifying bacteria when quantities of organic matter are high and will predominate over nitrifying bacteria in competition for space and oxygen inside the biofilters. Any disturbance of the natural microbial balance can induce the growth of opportunistic pathogenic bacteria in the biofilter (Blancheton et al., 2013).

A RAS production unit configures a synergy of several biological and mechanical processes (Figure 3). The water from the tank is pumped and recirculated through a biofilter treatment system that “removes” ammonia and other waste products.

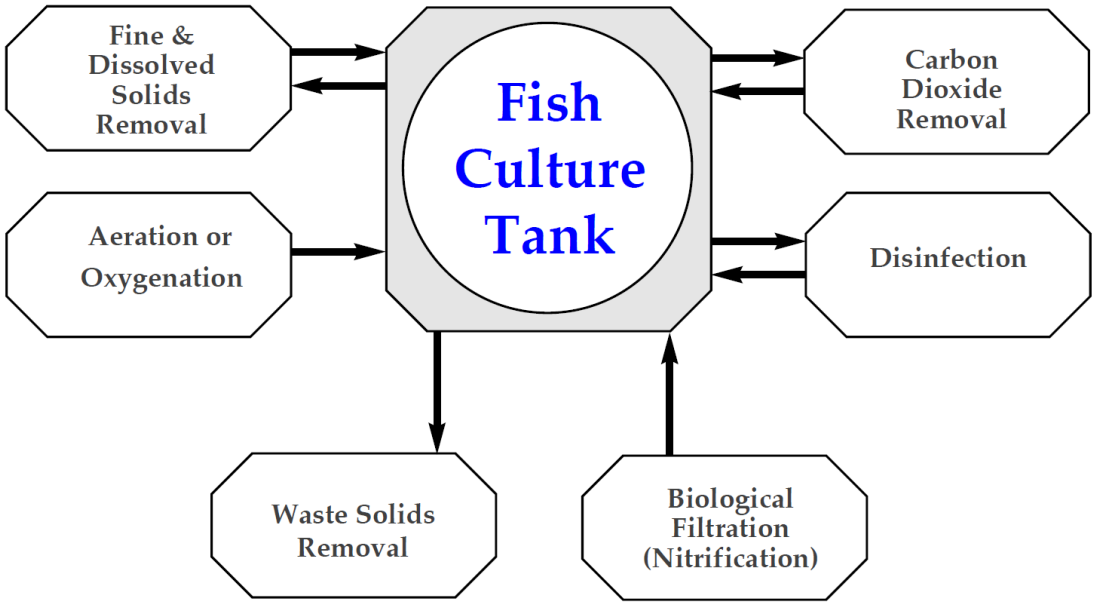


Figure 3 - Principal components used in RAS systems, adapted from (Losordo et al., 1998).

From an operational perspective, subdividing biofilters into multiple tanks can reduce the risk of complete failure (Masser et al., 1999). Failures in biofilters are often associated with factors such as insufficient or imbalanced microbial communities, improper maintenance, and inadequate water quality control. Therefore, having multiple tanks allows for better management and risk mitigation (Masser et al., 1999). The biological filter is designed to allow maximum contact of the culture tank water with the biofilter carriers that contains the bacterial communities (Takeuchi, 2017). The water runs through the biofilter carriers that allows the contact with the bacterial biofilm. In addition, a variety of aeration or oxygenation devices are integrated into the system to add atmospheric oxygen to the water or to release excess carbon dioxide from the water (Losordo et al., 1998; Espinal and Matulić, 2019).

To safeguard the health and well-being of species in RAS, including delicate species like Sea Bass (*Dicentrarchus labrax*), it may be necessary to implement additional disinfection measures in the RAS environment. Delicate species are particularly vulnerable to diseases and infections (Yardimci and Timur, 2016). Hence, implementing appropriate disinfection protocols and closely monitoring water quality becomes crucial in maintaining a clean and disease-free culture system for all the species present. By adopting these measures, aquaculture practitioners can effectively mitigate the risk of disease outbreaks and ensure the well-being of all the species in the RAS. Therefore, cleaning devices such as UV (ultraviolet) light and ozone generators are also used for

water disinfection in the RAS system. Additionally, various mechanical systems for monitoring and controlling physicochemical parameters (dissolved oxygen, temperature, ozone, UV light, pH, and salinity) are also integral components of the RAS system (Schreier et al., 2010).

In the investigation conducted by Li, H. et al. (2023), an examination of the factors influencing RAS operation is undertaken. The study not only delves into the mechanisms through which these factors impact the organisms being cultivated but also explores their effects on microbial community composition. These factors encompass a range of environmental conditions, spanning temperature, dissolved oxygen levels, pH, salinity, as well as diverse feeding strategies and disinfection methods. The findings suggest that these factors can potentially have negative effects on the operation of the RAS and the quality of the products (Li, H. et al., 2023). The harmonious interaction of various factors enables intensive fish production to be compatible with environmental sustainability while also enhancing fish health. Temperature, in particular, plays a crucial role in influencing the overall well-being of fish. Even a small temperature variation can significantly impact their physiological processes (Crawshaw, 2011). Extreme temperatures can lead to physiological stress and decreased growth rates (Islam et al., 2022). When fish are exposed to temperatures outside their preferred range, a cascade of physiological changes occurs. For instance, warmer temperatures can boost their metabolic rates (Liu et al., 2019), leading to increased energy demands and greater oxygen requirements. In contrast, colder temperatures may slow down metabolism, affecting growth rates and feeding behavior (Volkoff and Rønnestad, 2020). Temperature not only impacts fish growth directly but also affects their appetites, digestive functions, and feeding abilities (Volkoff and Rønnestad, 2020). Additionally, temperature fluctuations can weaken the fish's immune system, making them more susceptible to diseases and infections (Mugwanya et al., 2022). Temperature also plays a vital role in shaping the microbial community composition within the biofilter of the RAS. Extreme temperatures, whether too high or too low, can inhibit essential processes like nitrification, leading to a negative impact on water quality (Godoy-Olmos et al., 2019).

Dissolved oxygen (DO) is also a critical factor in RAS that significantly impacts system capacity and yield (Timmons et al., 2001). Insufficient DO levels can lead to suffocation, affecting feeding, reproduction, and overall behaviour (Li, H. et al., 2023). Moreover, a decrease in dissolved oxygen (DO) content has been shown to significantly reduce the proportion of operational taxonomic units of nitrifying bacteria, particularly inhibiting the growth of heterotrophic nitrifying bacteria (Li, H. et al., 2023; Wei et al., 2019). On the

other hand, an increase in DO content (above 5 mg/L) has been found to benefit the removal of ammonia, nitrate, and total nitrogen in the RAS (Li, H. et al., 2023; Wei et al., 2019). Nitrification, an aerobic process, is heavily dependent on the availability of oxygen for efficient control, emphasizing the crucial role of dissolved oxygen management in the water (Tryggvason, 2016). Successful nitrification requires effective circulation to supply oxygen to all parts of the biofilm (Foore, 2016). Additionally, higher C/N ratios can lead to competition among various heterotrophic organisms, not exclusively denitrifiers, for disposable oxygen, nutrients, and space within nitrifying biofilters. This competition can inhibit nitrification and increase the occurrence of denitrification (Li, C. et al., 2019). Hence, maintaining a balance between autotrophic nitrifiers and other heterotrophic bacteria becomes critical for ensuring the efficient nitrification processes in the biofilters.

Another critical water quality parameter in aquaculture is the pH level, which reflects the activity of hydrogen ions (H^+) in water. Ideally, the pH range for successful aquaculture should be between 6.0 and 9.0 (Li, H. et al., 2023). Maintaining the pH level close to 7 can be particularly beneficial as it helps alleviate stress in fish by reducing the levels of un-ionized ammonia (Masser et al., 1999). Acidic conditions with a pH below 5 can cause acidosis in fish, leading to organ dysfunction and reduced oxygen supply. Low pH also damages gills, sensory organs, and reproduction. Conversely, pH levels above 9 can be corrosive to fish, causing harm to their bodies and gills (Li, H. et al., 2023). Furthermore, it is essential to consider the impact of pH on the nitrification process within the biofilter. At low pH levels, the nitrification process can be inhibited, affecting the system's overall efficiency. On the other hand, alkaline environments with pH levels between 7.0 and 9.0 promote the reproduction of nitrifying bacteria, enhancing the removal of ammonia and nitrite (Li, H. et al., 2023). In RAS the pH levels can be influenced by various factors, including CO_2 emission from biological respiration and the acid generated during nitrification. These processes can lead to a decrease in water pH, affecting the biofilter's performance (Li, H. et al., 2023).

Salinity is another crucial indicator of water quality in brackish water or seawater RAS. Salinity exerts a significant impact on the composition of microorganisms within RAS biofilters (Bakke et al., 2017). Research has shown that as salinity increases, the diversity of AOB decreases (Deng et al., 2017), indicating that certain ammonia oxidizing bacteria species may be less adapted to higher salinity conditions. Additionally, the population of freshwater nitrifying bacteria declines and becomes almost entirely suppressed at a salinity level of 15 PSU (Practical Salinity Units), suggesting that these freshwater nitrifiers are not well-suited to higher salinity environments (Deng et al., 2017).

On the other hand, salt-tolerant nitrifying bacteria can maintain their nitrification activity across a wide range of salinities. These salt-tolerant nitrifiers are better adapted to marine environments with higher salinity levels and play a crucial role in maintaining nitrification efficiency in Marine RAS setups (Hüpeden et al., 2020). Furthermore, elevated salinity levels have been observed to reduce the abundance of pathogenic microorganisms, such as pathogenic Vibrio species, in aquaculture ponds (Li, H. et al., 2023). This reduction effectively lowers the risk of disease outbreaks and contributes to maintaining the health and quality of the cultured fish (Li, H. et al., 2023). In addition, various factors can have cascading effects on the prokaryotic communities that inhabit the biofilter in RAS. Changes in pH and salinity, for example, can alter the microbial composition and activity within the biofilter. These changes may, in turn, impact the efficiency of ammonia and nitrite removal, which can subsequently affect water quality and fish health (Almeida et al., 2021). Ozone is another parameter that can influence the microbial composition within the RAS biofilter. Since it is commonly used as a disinfection method to control pathogens and maintain water quality, it may also have unintended consequences on the biofilter's microbial community (Gonçalves and Gagnon, 2011). High ozone levels can lead to the destruction of beneficial bacteria, disrupting the stability and function of the biofilter. This can potentially affect the overall efficiency of ammonia and nitrite removal, leading to potential water quality issues and compromised fish health (Gonçalves and Gagnon, 2011).

2.2.1. Biofilter Community and Nitrification

Biofilter are the unit responsible for the biological filtration, where nitrification process plays a main role, and by which ammonia that is released from the fish is transformed into a non-toxic form of nitrate (Ruiz et al., 2019). Firstly, ammonia is oxidized to nitrite (NO_2^-) by ammonia-oxidizing bacteria (AOB). Additionally, ammonia-oxidizing archaea (AOA) are active participants in this conversion process (Ruiz et al., 2019). Then, nitrite is further oxidized to nitrate (NO_3^-) by nitrite-oxidizing bacteria (NOB), as shown in Figure 2. The biofilter compartments are filled by an accurate volume of matrices that function as platforms/substrates (plastic/biofilm carriers) in which the bacterial community adheres, facilitating the formation of biofilm layers that colonize the substrate, usually of highest specific surface (Masser et al., 1999).

Microorganisms can be found floating freely in the water body or arranged in complex, highly organized aggregates called biofilms (Ribeiro, 2008). It is widely documented that bacteria in aquatic environments easily adhere to any solid support that is in contact with water (Ribeiro, 2008). In particular, the use of artificial substrates (Figure 4) significantly

enhances the colonization of nitrifying bacteria in recirculating aquaculture systems (RAS) (Duarte et al., 2019; Masłoń et al., 2015). These substrates provide a surface for the bacteria to grow and establish their colonies. Such carriers come in various types (Takeuchi, 2017), with the more popular ones often referred to as biofilters or plastic carriers, typically made of polypropylene. Other different types of biological filters are the submerged fixed substrate biofilters (static bed), which exhibit high purification rates but requires periodic maintenance.

Figure 4 - Typical biofilter carriers used in RAS (Credit image: https://www.alibaba.com/product-detail/Moving-Bed-Biofilm-aquarium-filter-media_62178259394.html).

Research has demonstrated that the presence of biofilms on biofilter media plays a crucial role in creating a favourable environment for nitrifying bacteria (Ribeiro, 2008). The nitrifying bacteria responsible for this transformation colonize the biofilter and form a biofilm on the surface of plastic carriers. This biofilm houses a diverse community of different bacterial genera, contributing to the efficient nitrification process (Chen et al., 2006). These biofilms provide an ideal surface for nitrifiers to colonize and carry out their metabolic processes efficiently. As a result, the formation of biofilms has significantly contributed to enhancing the overall performance of biofilters in RAS setups. Exploration of alternative biofilter media has further expanded our understanding of nitrifying bacteria dynamics in RAS. Biochar and Zeolite, as novel media options, have demonstrated remarkable capabilities in enhancing the growth of nitrifying bacteria and consequently boosting biofilter efficiency (Paul and Hall, 2021). These alternative media provide unique surface characteristics and chemical properties that support the attachment and proliferation of nitrifying microorganisms, leading to improved ammonia and nitrite removal from the system. Imbalances or disruptions in the factors influencing nitrifying

bacteria can indeed affect their activity and survival, potentially leading to biofilter issues. In such instances, reactivation becomes necessary, often involving water changes in the RAS to restore optimal conditions and ensure the biofilter functions properly. However, it is essential to note that the need for reactivation is not always due to failure; it can also be a necessary step during the establishment of a new biofilter in a RAS. This process is critical for cultivating a healthy population of nitrifying bacteria capable of efficiently removing ammonia and nitrite from the system. According to Masser et al. (1999), this activation period typically lasts for at least one month. The requirement of a one-month duration for biofilter activation, as noted by Burut-Archanai et al. (2021), poses a significant disadvantage as it can lead to production interruptions in RAS. Dealing with this challenge and expediting the process can be quite demanding.

Researchers have explored various methods to expedite the establishment of nitrifying bacteria in new biological filters. One approach involves pre-activating the biofilters by introducing commercial nitrifying bacteria and a synthetic growth medium (Masser et al., 1999). Alternatively, they have utilized a "seed" of nitrifying bacteria from an existing, well-established filter to jumpstart the nitrification process in the new filter. This seed can be obtained by transferring medium from a mature and fully operational biofilter in an older RAS unit to inoculate the biofilters in the newest RAS unit (Roalkvam et al., 2019). Additionally, reducing normal stocking and feeding rates is also recommended as a way to address the one-month duration for biofilter activation, which may otherwise cause production interruptions in RAS (Roalkvam et al., 2021). For example, research on the reactivation of biofilters in a marine RAS for Atlantic salmon post-smolt can be facilitated by low stocking density, which allows for low and steady concentrations of ammonium, nitrite, and organic load, stimulating the rapid development of a nitrifying population (Roalkvam et al., 2021).

The duration of the reactivation period lacks consensus among researchers. In the past, older studies from the 1970s, such as Carmignani and Bennett (1977) and Collins et al. (1975), reported diverse timeframes for the two-step oxidation of ammonia to nitrate in a reactivated biological filter, ranging from 28 to 60 days and occurring under temperatures between 21 and 26°C. Currently, there are still conflicting estimates from different sources regarding the reactivation period. While some adhere to the norm of approximately one month, others suggest a much broader range (e.g., 4–8 weeks) to establish a healthy and viable population of both ammonia-oxidizing and nitrite-oxidizing bacteria (Kuhn et al., 2010). This variability is influenced by factors such as water temperature, pH levels, and the types and quantities of bacteria present in the system

(Aalto et al., 2022a). Moreover, some sources acknowledge that the startup period duration is a complex interplay of multiple factors (Padeniya et al., 2022). This suggests that no single fixed timeframe can be universally applied to all RAS setups, as the reactivation process's duration can vary depending on the specific conditions and characteristics of each system. As of now, uncertainty remains regarding the most accurate estimate for the reactivation period. The disparity in findings suggests that additional research is needed to gain a comprehensive understanding of the factors influencing the duration of the reactivation process in biological filters.

The usual start-up characteristics for bringing a new biofilter up to full capacity are depicted in Figure 5 (Takeuchi, 2017). A typical startup curve for a biological filter shows a gradual increase in ammonia and nitrite levels in the water as the nitrifying bacterial population slowly grows and establishes in the biofilter.

During the initial days to weeks of the startup process, ammonia and nitrite levels may spike as the bacterial population is still low, and they are not yet efficient in converting ammonia to nitrite and nitrite to nitrate. As the nitrifying bacterial population grows, ammonia and nitrite levels will gradually decrease, and nitrate levels will increase (Timmons et al. 2002). This indicates the successful colonization and activation of the biofilter, leading to its full capacity and effective removal of nitrogenous compounds from the water in RAS. Within the biofilms attached to the carriers' surface, autotrophic nitrifiers and heterotrophic bacteria coexist, playing vital roles in converting ammonium and nitrite, as well as breaking down dissolved and particulate organic matter (Qi et al., 2022).

Table 1 provides a comprehensive overview of the generic composition of the microbiome found in RAS biofilters, along with the primary reactions occurring within them.

It's important to note that this Table 1 does not encompass the latest findings regarding comammox bacteria, such as *Nitrospira inopinata*, and other recently identified bacterial species within the nitrogen cycle (Lu et al., 2020). Recent research has unveiled new insights into the diverse bacterial community involved in nitrification, potentially influencing the current understanding of processes within these systems (Bartelme et al., 2017).

Primary activities associated with RAS biofiltration units and participating microorganisms

Process	Reaction	Microorganism * Marine
Nitrification Ammonium oxidation	$NH_4^+ + 1.5O_2 \rightarrow NO_2^- + 2H^+ + H_2O$	*Nitrosomonas sp.* *Nitrosomonas cryotolerans* *Nitrosomonas europaea* *Nitrosomonas cinnybus/nitrosa* *Nitrosococcus mobilis*
Nitrite oxidation	$NO_2^- + H_2O \rightarrow NO_3^- + 2H^+ + 2e^-$	*Nitrospira marina** *Nitrospira moscoviensis**
Denitrification Autotrophic (sulfide-dependent)	$S^{2-} + 1.6NO_3^- + 1.6H^+ \rightarrow$ $SO_4^{2-} + 0.8N_2(g) + 0.8H_2O$	*Thiomicrosporia denitrificans* *Thiothrix disciformis** *Rhodobacter litoralis** *Hydrogenophaga sp.*
Heterotrophic	$5CH_3COO^- + 8NO_3^- + 3H^+ \rightarrow$ $10HCO_3^- + 4N_2(g) + 4H_2O$	*Pseudomonas sp.* *Pseudomonas fluorescens* *Pseudomonas stutzeri* *Paracoccus denitrificans*

* Microorganisms identified solely on the basis of partial 16S rRNA gene or functional gene sequences.

Table 1 - Main bacterial reactions inside a biological marine filter (Adapted from Schreier et al., 2010).

Some genera of bacteria, including *Nitrosomonas*, *Nitrosococcus*, *Nitrosospira*, Nitrosolobus, and *Nitrosovibrio*, are typically involved in the first step of nitrification, which is the oxidation of ammonia to nitrite. The second step, oxidation of nitrite to nitrate, involves genera like *Nitrobacter, Nitrococcus, Nitrospira,* and *Nitrospina* (Hagopian and Riley, 1998; Foore, 2016). In a study conducted by Almeida et al. (2021), the genus *Nitrosomonas* and *Nitrospira* were identified in a RAS sole farm, with *Nitrosomonas* classified as AOB and *Nitrospira* as NOB. These two genera have a relatively low specific growth rate and compete with faster and more efficient-growing species for resources like oxygen, ammonia, and carbon dioxide (Prosser, 1989; Roalkvam et al., 2021).

The diversity of biofilters can vary from one RAS to another, as each fish species fosters its unique microbial community (Schreier et al., 2010). Consequently, notable differences have been observed in microbial communities among different RAS systems (Li, Q. et al., 2022)

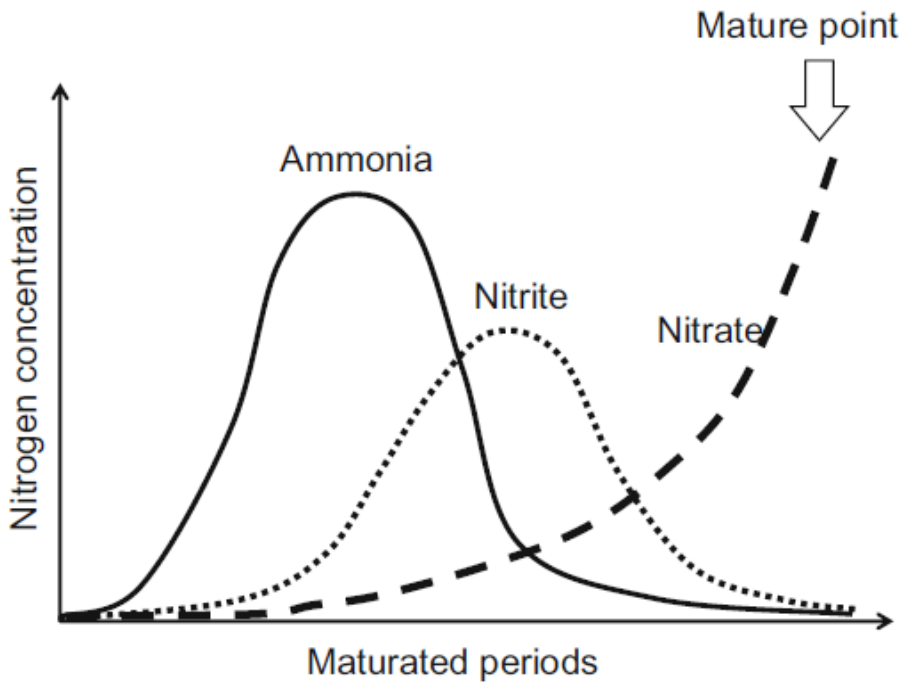


Figure 5- Schematic diagram of maturation point of biofilter medium in biofilter tank, according to the three forms of nitrogen (ammonia, nitrite, and nitrate) (Takeuchi, 2017).

2.2.2. Key Challenges in Biofilter Reactivation

Although biofilm carriers inoculated with commercial inoculants are often used during biofilter start-up to provide an initial bacterial culture (Foore, 2016; Brailo et al., 2019), this process can be costly, as it involves purchasing and transporting the inoculated biofilm carriers and pose potential risks in RAS due to the need for a time of adaptation to each fish culture, which remains largely unknown in many cases. Commercial inoculants typically contain a mixture of autotrophic nitrifiers, such as *Nitrosomonas* and *Nitrobacter*, which are essential for the nitrification process in RAS. However, each aquaculture system can have unique environmental conditions, including water quality parameters, temperature, and feed composition, which may influence the success of the introduced bacterial culture. Without a comprehensive understanding of how the introduced bacteria will interact and adapt to the specific RAS conditions, there is a risk of potential imbalances in the microbial community (Foore, 2016; Brailo et al., 2019).

To overcome these challenges an alternative strategy for biofilter reactivation involves utilizing microbial populations from existing operational biofilters (Roalkvam et al., 2019).

The utilization of microbial populations from operational biofilters has been widely applied as start-up cultures for activation proposals (Kumar et al., 2013) and involves the use of biofilm carriers that have matured within the same RAS unit. In a previous study, researchers demonstrated the effectiveness of transferring biofilm carriers from a mature

RAS biofilter that had been operating for 1.5 years after activation. These carriers, despite having a lower abundance of nitrifiers (15.2% relative abundance), proved to be more efficient in activating nitrification compared to a specific commercial inoculum, where 63.7% of the microbial community was classified as nitrifying. The findings highlight the potential advantages of utilizing naturally colonized biofilm carriers over commercial inoculums for biofilter reactivation in marine RAS (Roalkvam et al., 2019). Notably, the main nitrifiers found on the colonized biofilm carriers belonged to the genera *Nitrospira*, *Nitrosomonas*, and *Nitrosococcus* (Roalkvam et al., 2019) that are well-known for their involvement in the key steps of nitrification.

Developing a bacterial consortium is another strategy used to optimize the performance of biofilters in RAS by harnessing the synergistic interactions among different bacterial species. A bacterial consortium is a mixed population of bacteria that work collaboratively to carry out specific biological processes, such as nitrification, denitrification, and organic matter decomposition. The process of developing a bacterial consortium involves carefully selecting and combining bacterial strains with complementary functions. For example, the consortium may consist of autotrophic nitrifiers, which convert ammonia to nitrite, and nitrite-oxidizing bacteria (NOB), responsible for the conversion of nitrite to nitrate. Additionally, heterotrophic bacteria can be included to facilitate the breakdown of dissolved and particulate organic matter. Previous studies have focused on nitrifying consortia, particularly *Nitrosomonas*, *Nitrobacter*, and *Nitrospira* (Tal et al., 2003). The research conducted by Kumar et al. (2009a, 2009b, 2009c) is particularly noteworthy for developing nitrifying bacterial consortia (NBC) that have shown promising results and practical applicability in RAS. Specifically, their work has been successful in activating and enhancing the performance of bioreactors in larval rearing systems of *Penaeus monodon* and *Macrobrachium rosenbergii* (Kumar et al., 2009b, 2009c).

Despite considerable research on these nitrifying consortia, the mechanism of action in terms of bacterial synergy and performance remains poorly understood due to multiple dynamic interactions among microbial communities and their environment. In the past century, AOB were commonly considered the primary microorganisms responsible for ammonia oxidation (Ferrera and Sánchez, 2016). However, recent advances in molecular biology techniques have revealed the presence of numerous archaea distributed throughout marine environments that also exhibit the physiological metabolic capability for ammonia oxidation (Schleper, 2010). Moreover, the conversion process also involves AOA from the phylum Thaumarchaeota, encompassing genera such as *Nitrosoarchaeum*, *Nitrosopumilus*, and *Nitrososphaera* (Ruiz et al., 2019). Furthermore,

microorganisms known as commamox, specifically from the *Nitrospira genera*, has brought attention to their role in facilitating complete nitrification (Daims et al., 2015). The coexistence and interactions between these diverse microorganisms have a significant impact on the overall efficiency and stability of the nitrification process in RAS. Therefore, the process can be done in one-step oxidation of ammonia to nitrate (Van Kessel et al. 2015).

2.3. Tools to Characterize the Microbial Community

Characterizing the microbial communities is crucial for understanding their functions, responses to environmental changes, and potential implications for RAS operations. One of the primary obstacles arises from the immense diversity of microbial populations present in these systems (Kirk et al. 2004). RAS environments are teeming with various microorganisms, each performing distinct ecological functions. Capturing a comprehensive profile of this diverse community requires specialized techniques and analytical tools. Additionally, some bacteria, notably NOB like *Nitrospira*, are difficult, if not impossible, to cultivate in laboratory settings (Daims et al., 2016). Earlier studies on microbial communities in biofilters primarily relied on traditional cultivation and enrichment techniques (Keller and Zengler, 2004; Hugenholtz et al., 1998). However, recent advances in molecular-based techniques have revolutionized our understanding of these communities by providing a more comprehensive and detailed view of their microbial diversity, biological activities, and internal interactions. Molecular methods have particularly shed light on the bacterial profile of biofilters, allowing for a deeper exploration of AOB and NOB involved in the nitrification process. However, it is essential to carefully evaluate and optimize the various methodological steps, such as DNA extraction and PCR amplification, before conducting molecular characterization of microbial communities (Cabrol et al., 2012).

Until recent years, the microbial community composition in the biological filters of newly operational recirculating aquaculture systems (RAS) received limited attention due to sequencing limitations. However, the emergence of Next-Generation Sequencing (NGS) technologies has revolutionized the field, making it more feasible and informative to characterize biofilter communities (Almeida et al., 2021; Brailo et al., 2019). With NGS, researchers can now obtain a comprehensive picture of the microbial diversity and dynamics within biofilters, enabling a better understanding of their role in supporting water quality and fish health in RAS operations. These advancements in sequencing techniques have opened new avenues for studying and optimizing the microbial

communities in biofilters, ultimately contributing to the sustainable and efficient management of RAS in aquaculture. Two commonly used approaches are short- and long-read sequencing, which primarily focus on the 16S gene.

Short-amplicon sequencing employs primers that target specific variable regions of the genes. However, a major drawback of this method is the limited resolution for species identification. Additionally, the choice of primers can introduce biases favouring certain taxonomic groups (Rieder et al., 2023). On the other hand, long-amplicon sequencing targets all variable regions of the 16S rRNA gene, leading to enhanced resolution for species identification and reducing biases associated with primer selection. This wider gene segment coverage helps mitigate primer bias, resulting in a more comprehensive, less distorted view of microbial community composition and diversity. Both short- and long-amplicon 16S rRNA gene sequencing primarily concentrate on bacteria and may not detect other critical microbes, such as archaea, that also may be present in the RAS (Rieder et al., 2023). Nevertheless, specific 16S rRNA gene primers, such as Arch344F and Arch915R, are carefully designed to exclusively target Archaea (Ohene-Adjei et al., 2007). By utilizing NGS technologies, such as Illumina sequencing (e.g., Illumina MiSeq®), researchers can gain valuable insights into the composition and dynamics of microbial communities in RAS of marine environments (Chiu and Miller, 2016). NGS generates a vast amount of data from 16S rRNA gene sequencing, enabling the identification of taxonomic profiles and bacterial species that are difficult to cultivate and may exist in low abundance within the community. NGS allows for the analysis of DNA sequences directly from environmental samples, providing a more comprehensive understanding of microbial diversity and functioning.

Recent research has shed light on nitrifying bacteria diversity and dynamics within RAS biofilters, alongside factors impacting their growth and colonization. Variations in fish species, water temperature, and water source have been shown to influence biofilter microbial communities (Dahle et al., 2022; Almeida et al., 2021). Scientists have explored probiotics and microbial supplements to enhance beneficial bacteria growth and water quality (Menanteau-Ledouble et al., 2020). Studies have also investigated spatial nitrifying bacteria distribution in biofilters and their interactions with other microbes. Notably, coexistence of ammonia-oxidizing and nitrite-oxidizing bacteria in distinct biofilter layers hints at mutualistic relationships (Duarte et al., 2019). Molecular techniques have revealed novel nitrifiers, e.g., *Pantoea calida*, *Pseudomonas stutzerii*, and *Halomonas sp*. in mud crab RAS systems (Hastuti et al., 2019). Furthermore, DNA amplification and sequencing unveiled microbial diversity, including archaea and

heterotrophic bacteria, underscoring biofilter communities' complexity (Huang et al., 2018).

3. Objectives

In this study, we investigated the microbial succession of two biofilters in a large-scale industrial marine RAS for *Solea senegalensis* cultivation. The two biofilters had different start-up reactivation periods, and we aimed to compare the establishment of the nitrifying population in each biofilter and reveal how the development of microbial communities is affected by time and variations in physicochemical parameters. We analysed colonizing biocarriers of the two reactivated biofilters and compared them with a fully working biofilter using metabarcoding targeting 16S rRNA gene to obtain microbiome profiling. We focused on the structure and stability of microbial communities over different biofilter activation times. The results of this study will be relevant to decrease the activation period of new biofilters, ensure the welfare of the cultivated species, and improve the economic profitability of the aquaculture system. Understanding the development and dynamics of nitrifying bacteria during biofilter reactivation can facilitate identification of factors that promote their growth and reduce the waiting time before resuming normal activity levels after RAS start-up. We examine the diversity of species and their respective environmental optima to enhance the resilience of biofilm function under fluctuating production and environmental conditions. The new knowledge generated may have an impact on the efficiency of the biofilter, by preventing harmful concentrations of ammonia and nitrite and contributing to the sustainable development of the aquaculture industry. By identifying factors that promote the growth of the nitrifying population, this study also offers valuable insights to reduce the activation period of biofilters while ensuring the welfare of cultivated species and economic profitability of aquaculture systems.

4. Materials and Methods

4.1. RAS Design

Safiestela Aquafarming Investments, Lda is a sustainable aquaculture production unit located in Estela, Portugal, operating under the RAS regime and specializing in the hatchery of *Solea senegalensis*. The unit comprises four main integrated elements: Pre-Ongrowing (PO), Weaning (WE), Brooding Stock (BS), and an Open System (OS), as previously described by Almeida et al. (2021). While the PO, BS, and WE elements operate with water recirculation, both RAS1 (associated with PO) and RAS3 (associated with WE) systems are actively in operation, as indicated. The OS, however, operates without water recirculation.

During the PO stage, as reported by the aquaculture unit, the fish are usually between 2-10 g in weight and are grown in a nursery or hatchery unit with low stocking density. The purpose of this stage is to provide the optimal conditions to promote the fish growth and health, and to prevent diseases, in which the water quality is closely monitored and maintained.

The WE stage precedes the PO stage and involves transferring the fish to a larger grow-out RAS system, where they continue to grow to a larger size. This stage requires careful monitoring of water quality, feeding regimes, and growth rates to ensure optimal fish growth and welfare. The fish are weaned onto a new diet and transferred to a higher stocking density, which increases their growth rate.

Both the PO and WE stages are crucial in ensuring a successful RAS production cycle and the growth of healthy fish. Proper management of water quality, feeding regimes, and stocking densities are important factors that contribute to the success of these stages.

The PO element of the system includes a biological biofilter, which is the focus of this study on reactivation. In addition to RAS1, another PO system, RAS2, has been activated to enable performance comparison. It's important to note that these two RAS systems for PO are distinct, differing not only in reactivation time but also in their operational characteristics, despite a difference in reactivation time of approximately 1.5 months. RAS1 and RAS2 operate in parallel but with a time lag.

Furthermore, it's worth mentioning that RAS3 is integrated into the WE element and serves as a reference biofilter that is already activated and functioning effectively. A schematic layout of the configuration is presented in Figure 6.

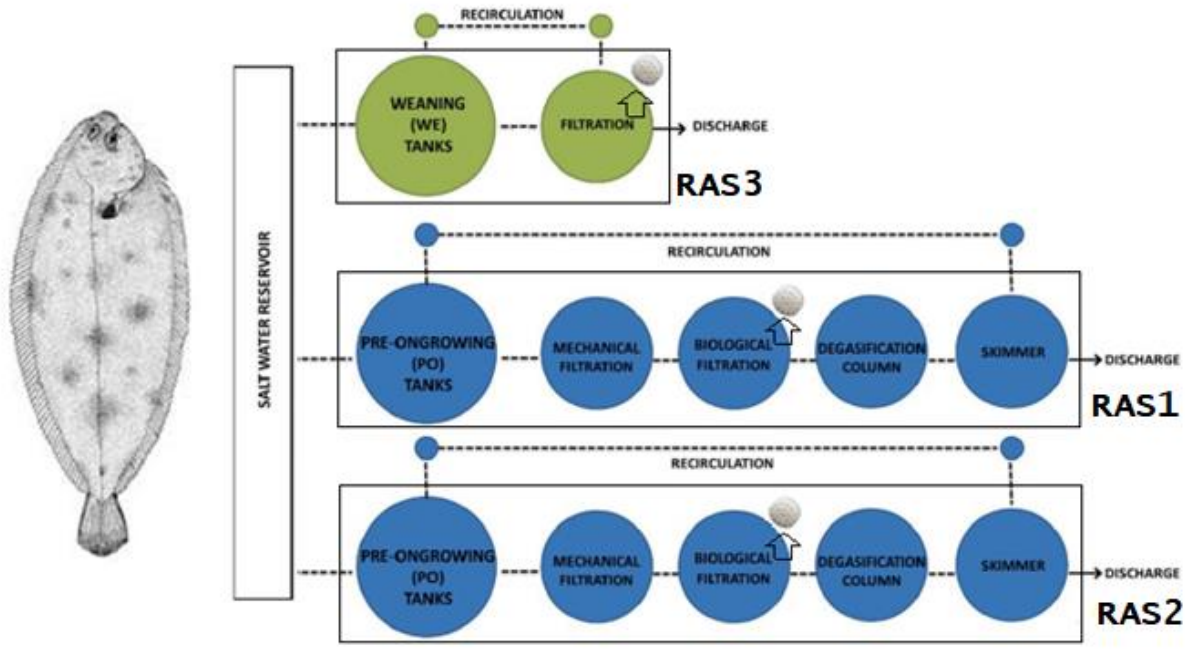


Figure 6 - Layout of the aquaculture unit studied. The pre-ongrowing (PO) and weaning (WE) systems operate with water recirculation. Water sampling points were inside the biofilter and are indicated with arrow (and a BB picture). RAS1 and RAS2 are replicas of the blue line (PO). These two RAS are working in parallel but with a difference of one month and half concerning its activation start. RAS3 (the green line) is fully working (i.e., is well established). Adapted from Almeida et al., 2021.

4.2. Sampling: Biofilms and Physical-chemical Parameters

Biofilter carriers were collected weekly from RAS1 and RAS2, with a month and a half of activation difference, for a period of two months (from 4/09/2020 to 23/10/2020). In addition, biofilter carrier samples from RAS3 were also collected in the same period of time. The performance of the biofilter was investigated for RAS1, RAS2 and RAS3 Samples were collected from RAS1 after 22 days of activation, and from RAS2 after 66 days RAS2 of activation.

Biofilm carriers from a fully functioning biofilter (RAS3) were also investigated, and these samples were collected in the same sampling days as RAS 1 and RAS2. . After 1 year, new samples were collected from all 3 RAS systems to characterize the microbial community. A scheme of the sampling (biological material) procedure is exibithed in Figure 7.

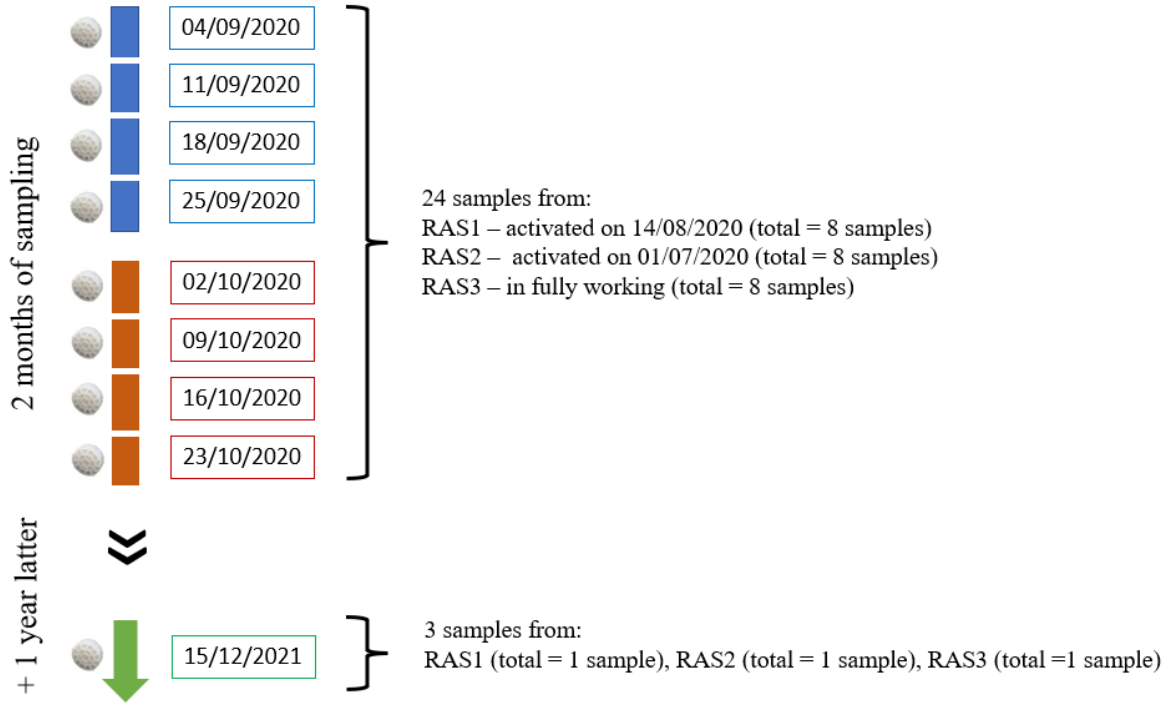


Figure 7 - Sequential schema of the sampling procedure for biological material. September 4, 2020, corresponds to day 22 for RAS 1, while for RAS 2, it marks day 66, denoting the reactivation days for each respective biofilter.

All samples collected were stored, inside Falcon® conical centrifuge tubes, (identified by the date of collection and RAS number) and transferred to the lab for further analysis. They were preserved at -80ºC until processing.

The Physical-chemical parameters were monitored daily by the aquaculture company, and they were provided to be used in this study. Salinity, pH and temperature, concentration of ammonia (total ammonia nitrogen (TAN)), nitrite-N (NO2--N), and nitrate-N (NO3--N)) were the principal monitored parameters. During this period, the temperature was maintained within the range of 18–19 °C, and the pH level was regulated between 7 and 8 through the controlled automatic dosing of NaHCO3 to ensure consistent environmental conditions.

4.3. DNA Extraction and PCR

DNA extraction from biofilter carriers was carried out using the DNeasy PowerSoil Kit (Qiagen, Germany). Before initiating the extraction protocol, a batch of 3 to 4 colonized biofilter carriers underwent centrifugation in 15 mL tubes for 15 minutes at maximum speed (4350 rpm), followed by a quick vortex and an additional 5-minute centrifugation. After this initial step, the extracted DNA was quantified using the Qubit® dsDNA HS

Assay Kit (Invitrogen) and a Qubit 3.0 fluorometer (Invitrogen), following the protocol provided by the supplier. To facilitate cell lysis for both biofilter carriers and tank biofilm samples, additional beads were added, as described in Almeida et al., 2021.

4.4. PCR Validation

Polymerase chain reaction (PCR) tests were conducted in the laboratory to ensure the DNA amplification on the target regions. Thus, the hypervariable V4-V5 regions of the 16S rRNA gene were amplified using the primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 926R (5'-CCGYCAATTYMTTTRAGTTT-3'). For that, PCR was performed in reactions of 10 µl containing 1-3 ul of DNA template, 1 µL of each primer, and 5 µL of DreamTaq master mix 2X (Thermo Scientific™, Waltham, MA). Moreover, sterile water was added to the samples with 1 – 2 µL of DNA template to complete reaction volume. The PCR reaction program was conducted as described in the Table 2.

Step	Temperature (ºC)	Time	Cycles
Denaturation	95	3min	
Denaturation	95	30 sec	
Annealing	52	40 sec	35
Elongation	72	10sec	
Final elongation	72	5 min	

Table 2 - The PCR program for amplification of bacterial 16S rDNA hypervariable region V4 to V5

The PCR products were visualized in a 1.5 % agarose gel.

4.5 Next-Generation Sequencing and Bioinformatic Analysis

In the process of analyzing microbial communities, DNA samples were subjected to PCR amplification targeting the hypervariable V4V5 region of the 16S rRNA gene. This amplification was carried out using specific primers and KAPA HiFi HotStart PCR Kit, following the manufacturer's suggestions. Each sample underwent an initial PCR reaction, which included 0.3 µM of each PCR primer: forward primer 515F-Y 5'-GTGYCAGCMGCCGCGGTAA -3' and reverse primer 926R 5'-CCGYCAATTYMTTTRAGTTT -3' (Parada, Needham, & Fuhrman, 2016), along with 2.5 µL of template DNA in a total volume of 25 µL. The PCR conditions involved a 3-minute

denaturation at 95°C, followed by 25 cycles of 98°C for 20 s, 50°C for 30 s, and 72°C for 30 s, followed by a final extension at 72°C for 5 minutes.

To prepare the samples for Illumina Sequencing, a limited-cycle PCR reaction was performed to add sequencing adapters and dual indexes to the amplified target region, following the recommendations from Illumina (2013). Throughout these procedures, negative PCR controls were included to ensure the accuracy of the amplification.

Following the PCR amplification, the PCR products were purified and normalized using the SequalPrep 96-well plate kit from ThermoFisher Scientific, located in Waltham, USA (Comeau, Douglas, & Langille, 2017). The purified samples were then pooled and subjected to pair-end sequencing on the Illumina MiSeq® sequencer. This sequencing was performed using the MiSeq Reagent Kit v3 (600 cycles), following the manufacturer's instructions provided by Illumina, based in San Diego, CA, USA.

The sequencing and data processing took place at Genoinseq, situated in Cantanhede, Portugal. Sequence data was extracted from the Illumina MiSeq® System in fastq format and subsequently underwent quality filtering using PRINSEQ version 0.20.4 (Schmieder & Edwards, 2011). This filtering step aimed to remove sequencing adapters, trim bases with an average quality lower than Q25 in a window of 5 bases and eliminate reads with less than 100 bases.

Furthermore, the forward and reverse reads were merged by overlapping paired-end reads using AdapterRemoval version 2.1.5 (Schubert, Lindgreen, & Orlando, 2016) with default parameters. Finally, for taxonomic attribution, the SILVAngs version 1.9.8 database was employed during the analysis.

4.6. Downstream Data Analysis

This followed downstream data analysis pipeline allowed us to explore and assess various aspects of microbial community diversity, structure, and significance.

In the analysis of community composition, we initially performed a transformation to relative abundance for each sample. This preprocessing step was conducted using the R package phylosEq (v1.36.0). Specifically, at the genus level, we included taxa contributing between 2% and 5% of the relative abundance in each sample.

The downstream data analysis phase encompassed several analytical steps using a combination of software tools and packages. The ASV (Amplicon Sequence Variant)

table served as the foundation for these analyses. The procedures were executed using R version 4.1.0 (R Core Team, 2021) in conjunction with the vegan package (v2.5–5).

To assess alpha diversity, we computed the Observed ASVs and Shannon diversity indexes based on the ASV table. For the evaluation of beta diversity, we employed the unweighted UniFrac metric, which involved the generation of a distance matrix. Notably, all values were normalized to the lowest number of sequences in the samples.

The results of these diversity analyses were visually represented using two techniques: nonmetric multiple dimension analysis (NMDS) and hierarchical cluster analysis (HCA). Both methods utilized the Bray-Curtis dissimilarity index. The R packages phylosEq (v1.36.0), ggplot (v3.3.6), vegan (v2.6–4), scales (v1.2.1), dplyr (v1.0.10), and phangorn (v2.10.0) were employed for these visualizations.

In order to determine the statistical significance of variations in prokaryotic community structure across different systems and variables, the Adonis test for beta group significance was conducted. This analysis utilized the Bray-Curtis distance matrix within the R environment (version 4.1.0) with the vegan package (v2.6–4). Statistical significance was established with a threshold of p-values lower than 0.05.

5. Results and Discussion

5.1. Physicochemical Parameters

Physicochemical parameters, setting the stage for a comprehensive understanding of this study before delving into the analysis of the microbial community. These parameters serve as the bedrock upon which the ecological dynamics are built. Within the context of this study, the presentation of data is approached through a dual lens: both quantitative tables and graphical representations. While tables offer a structured, comprehensive overview of the physicochemical parameters across various sampling dates, graphs provide a dynamic visualization of how these parameters evolve over time.

Table 3 provides detailed data concerning water quality in the recirculating aquaculture systems (RAS1, RAS2, and RAS3) spanning an eight-week period from September 4th, 2020, to October 23rd, 2020, as well as a subsequent evaluation on December 15th, 2021. The parameters under scrutiny encompass temperature, salinity, pH, ammonia nitrogen (NH_3-N), nitrite nitrogen (NO_2-N), nitrate nitrogen (NO_3-N), un-ionized ammonia (NH_3), ozone concentration (O_3), bromine concentration (Br), and feed supplied. The inclusion of data from December 2021 facilitates a longitudinal perspective, allowing for the assessment of the sustained performance of the biofilters and the identification of potential trends or fluctuations in water quality parameters over time. This strategic approach lays the groundwork for understanding the intricate interplay between physicochemical conditions and microbial communities. As we transition into the community analysis, it will become evident how these parameters fundamentally shape microbial populations and interactions within the studied aquatic ecosystems, enriching our comprehension of these environments.

System	Sampling date	Temp. (ºC)	Salin. (mg/L)	pH	NH_3-N (mg/L)	NO_2-N (mg/L)	NO_3-N (mg/L)	NH_3 un-ionized (mg/L)	O_3 Nm3/hr	Br (mg/L)	Feed supplied (Kg)
RAS1	04/09/2020	19,4	35	7,99	0,88	1,286	4,80	0,032	no data	0,08	13,6
RAS1	11/09/2020	19,1	36	7,92	0,20	0,750	5,40	0,006	no data	0,09	19,3
RAS1	18/09/2020	19,8	35	7,63	0,18	0,271	7,60	0,003	no data	0,03	21,6
RAS1	25/09/2020	19,1	35	7,76	0,13	0,327	5,2	0,003	no data	0,05	33,9
RAS1	02/10/2020	18,5	37	7,78	0,21	0,284	5,10	0,004	400	0,03	36,7
RAS1	09/10/2020	18,6	37	7,54	0,41	0,197	5,10	0,005	1100	0,05	41,1
RAS1	16/10/2020	17,1	37	7,65	0,43	0,143	5,70	0,005	1100	0,05	42,8
RAS1	23/10/2020	18,3	35	7,55	0,58	0,179	5,70	0,007	1100	0,09	40,0
RAS1	15/12/2021	19,1	37	7,92	0,04	0,089	no data	no data	1040	0,06	39,5
RAS2	04/09/2020	19,5	35	7,47	0,04	2,845	9,8	0,000	no data	0,1	35,1
RAS2	11/09/2020	19	37	7,52	0,14	3,351	9,5	0,002	no data	0,11	36,1
RAS2	18/09/2020	19,8	35	7,78	0,00	1,499	8,7	0,000	1300	0,05	37,3
RAS2	25/09/2020	19,3	35	7,62	0,17	2,616	9,4	0,003	1100	0,05	39,3
RAS2	02/10/2020	18,4	37	7,6	0,12	2,603	8,0	0,002	1000	0,04	42,1

RAS2	09/10/2020	18,7	37	7,73	0,04	1,243	8,0	0,001	900	0,07	35,9
RAS2	16/10/2020	17,1	37	7,35	0,06	0,568	7,8	0,000	900	0,05	37,6
RAS2	23/10/2020	18,2	35	7,51	0,06	0,527	7,8	0,001	900	0,05	33,5
RAS2	15/12/2021	19,4	37	7,98	0,02	0,053	no data	no data	400	0,03	14,9
RAS3	04/09/2020	23,6	36	7,80	0,29	0,054	no data	0,009	50	0,05	6,0
RAS3	11/09/2020	24,4	36	8,07	0,11	0,036	no data	0,007	50	0,1	6,6
RAS3	18/09/2020	20,5	35	7,94	0,31	0,040	no data	0,011	150	0,02	7,5
RAS3	25/09/2020	20,1	35	7,88	0,14	0,023	no data	0,004	150	0,04	5,3
RAS3	02/10/2020	20,2	36	7,83	0,25	0,050	no data	0,007	180	0,03	0,0
RAS3	09/10/2020	19,8	36	8,00	0,16	0,031	no data	0,006	180	0,01	7,3
RAS3	16/10/2020	19,1	37	7,68	0,29	0,132	no data	0,005	200	0,03	8,1
RAS3	23/10/2020	19,9	35	7,87	2,5	0,053	no data	0,071	200	0,07	7,5
RAS3	15/12/2021	19	37	8,29	0	0,025	no data	no data	140	0,04	11,7

Table 3 - Data for the water in which the samples collected from the units (RAS1, RAS2 and RAS3) were immersed. Data includes pH, temperature (°C), salinity; the nutrients (mg/L) (NH_4^+-N), nitrite (NO_2^--N), nitrate (NO_3^--N) and NH3 unionized, O3 (ozone), Br (Bromine) and feed supplied.

To prevent toxic levels for the fish, ammonia and nitrite concentrations were closely monitored daily in all systems. Nitrate concentration was only measured in RAS1 and RAS2 to confirm its formation. Changes in pH, temperature, and salinity throughout the sampling period for each water system can be observed in Figure 8-a), Figure 8-b), and Figure 8-c), respectively. The mean values and their standard deviation are provided in Table 4.

It was evident that there are some variations in the water quality parameters across the three studied RAS. For instance, RAS1 and RAS2 show relatively stable water quality parameters, while RAS3 shows more variability. While the precise causes of these variations cannot be conclusively determined from the data in the table alone, several factors may contribute to these differences. Regarding the temperature, RAS3 showed fluctuations between 19.1°C to 24.4°C, which is significantly higher compared to the other two systems. In RAS1 and RAS2 the temperature ranged from 17,1 ºC to 19,8 ºC throughout the sampling period, with the highest temperature recorded in both RAS1 and RAS2 on September 18th, 2020 (Figure 8-a). The temperature was relatively stable over the eight-week period, and the values on December 15th, 2021, were similar to those recorded in September and October 2020. These variations in water quality parameters could be influenced by operational factors within the RAS systems themselves. Differences in feed composition, feeding regimes, or maintenance practices may result in fluctuations in these parameters. While the continuous operation of RAS3 might suggest that it could be relatively stable compared to RAS1 and RAS2, which were reactivated, the reality is that we have observed greater fluctuations in water quality parameters in RAS3. This apparent contradiction may indicate that factors other than operational continuity are playing a significant role in the water parameter fluctuations.

Furthermore, the specific microbial communities in each RAS may influence water quality dynamics uniquely.

During the first three weeks of sampling (from September 4th to September 18th 2020), the temperature in RAS3 was higher than in RAS1 and RAS2, ranging from 20,5°C to 24.4°C. In fact, during summer, temperatures can rise to levels that are not suitable for the aquatic organisms that live in RAS. As a result, heatwaves can cause peak temperatures that require increased water exchange rates to decrease the temperature and maintain a suitable environment for the fishes and microorganisms. Increased water exchange rates can help remove the excess heat generated in the RAS by transferring it to the new incoming water, which is typically at a lower temperature.

For the pH values RAS3 showed ranges from 7.68 to 8.07, which is higher than RAS1 and RAS2.

Throughout the experiment, the average pH in RAS3 was slightly higher (pH = 7.9) than in RAS1 (pH = 7.7) and RAS2 (pH = 7.6). As the bacterial community needs to adapt to the working operating conditions, the optimum pH range for nitrification can vary from 7.0 to 9.0 (Chen et al., 2006), which falls within our system range. It is important to note that rapid changes in pH greater than 0.5 units within a short period of time can stress the biofilter and require time for the community to adjust to the new environmental conditions (Ebeling et al., 2012). Whenever there is a change in pH, it takes about 2 weeks to reverse the situation, as clearly illustrated in Figure 8-b. Based on the data provided in the table, it is evident that whenever there is a change in pH, such as the transition from 7.99 to 7.92 in RAS1 between September 4th, 2020, and September 11th, 2020, it typically takes approximately 2 weeks for the pH levels to revert to their previous state. This temporal pattern is consistent across the studied RAS systems, as demonstrated by the values and trends in the table. Nevertheless, the pH was relatively stable over the eight-week period, with a slight increase observed in RAS3 on December 15th, 2021. Overall, RAS1 and RAS2 appear to maintain relatively stable pH levels throughout the sampling period. In contrast, RAS3 shows more pronounced fluctuations in pH, with values ranging from 7.68 to 8.29. It's important to note that RAS3's pH data for December 15th, 2021, were unavailable, making it challenging to assess the long-term trend.

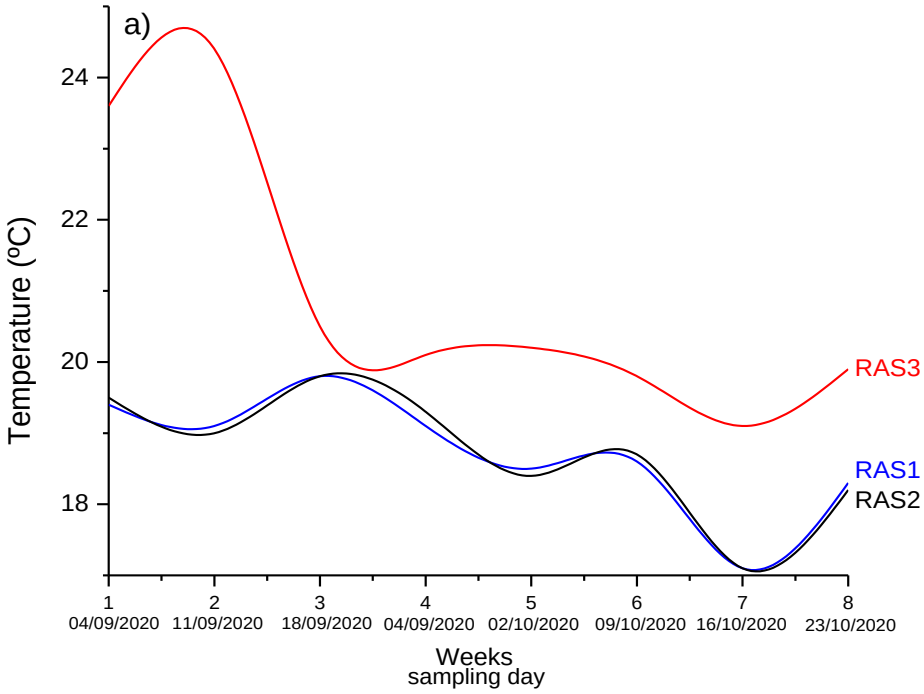
Based on the provided data, there are indeed indications of variations in pH that appear to be related to temperature changes, particularly in RAS3. For example, in RAS3, between September 4th, 2020 (temperature of 23.6°C) and September 11th, 2020 (temperature of 24.4°C), there is an increase in pH from 7.80 to 8.07. Similarly, when the

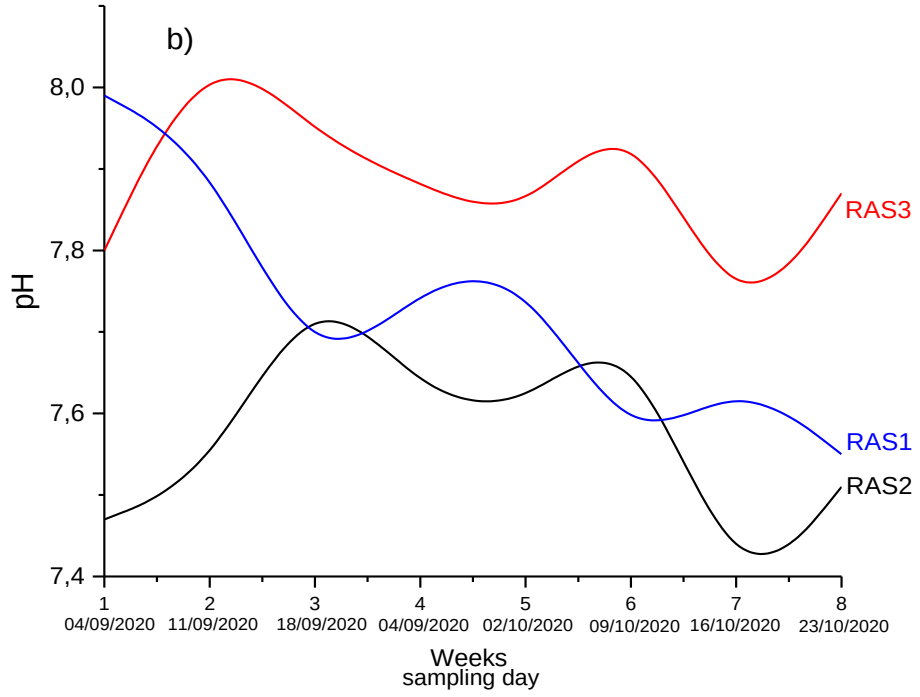
temperature in RAS3 decreases from 20.5°C on September 18th, 2020, to 20.1°C on September 25th, 2020, the pH decreases from 7.94 to 7.88.

These fluctuations in pH correspond to changes in temperature within RAS3. However, it's important to note that this correlation may not be a direct causation, as multiple factors can influence pH levels in aquatic systems.

The temperature in RAS3 remained relatively high for several weeks before gradually decreasing, while the pH levels remained elevated throughout this period until the final sampling date on December 15th, 2021. The cause of this prolonged elevation in temperature and pH is not entirely clear, but it may be attributed to factors such as summer heatwaves, potential equipment malfunctions, or other issues within the system.

Salinity exhibits a remarkable consistency across all three RAS systems. Throughout the majority of the sampling period, encompassing RAS1, RAS2, and RAS3, salinity levels are consistently maintained within the range of 35 to 37 mg/L. It is important to note, however, that an unusual reading of 2.5 mg/L was recorded in RAS3 on October 23rd, 2020, which is clearly an outlier compared to the otherwise stable salinity levels. Conversely, both RAS1 and RAS2 consistently maintain stable salinity levels over the observed time frame. In the year 2020, all three RAS systems consistently operated within the salinity range of 35 to 37 mg/L, displaying minor fluctuations within this range from week to week, as illustrated in Figure 8-c. A closer examination of the data reveals that by December 15th, 2021, salinity levels had equilibrated at 37 mg/L across all the studied RAS systems, demonstrating a consistent and sustained pattern over time.


a)
Temperature (ºC)
24
22
20
18
RAS3
RAS1
RAS2
1
04/09/2020
2
11/09/2020
3
18/09/2020
4
04/09/2020
5
02/10/2020
6
09/10/2020
7
16/10/2020
8
23/10/2020
Weeks
sampling day


b)
pH
8,0
7,8
7,6
7,4
RAS3
RAS1
RAS2
1
04/09/2020
2
11/09/2020
3
18/09/2020
4
04/09/2020
5
02/10/2020
6
09/10/2020
7
16/10/2020
8
23/10/2020
Weeks
sampling day

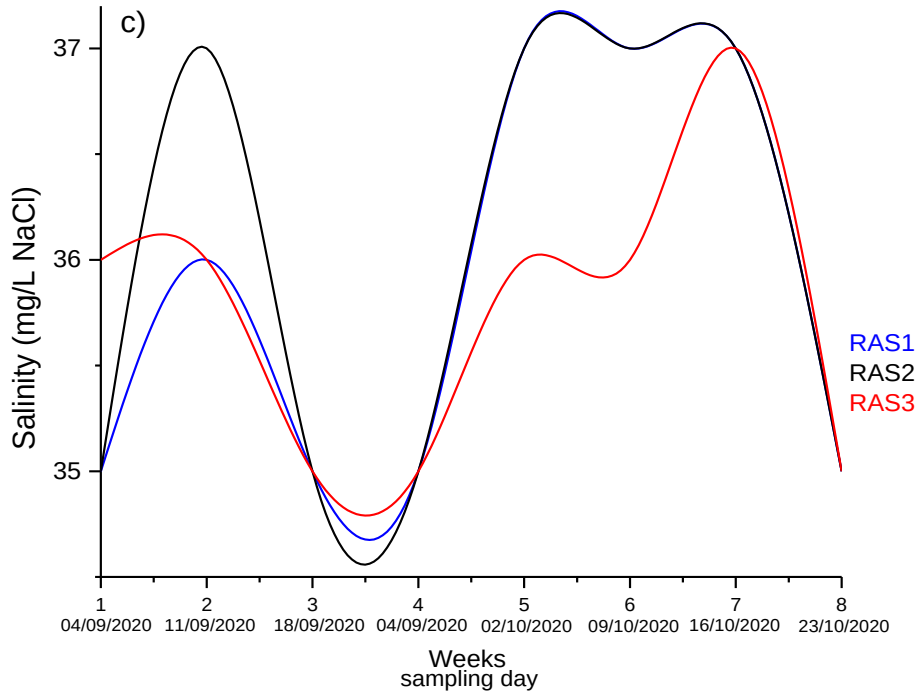


Figure 8 - Water physicochemical parameters were registered on a daily sampling for RAS1, RAS2 and RAS3. The parameters for a) Temperature, b) pH, and c) Salinity, show adjusted spline curves across all biofilters, using all available points.

The mean values, as presented in Table 4, play a pivotal role in understanding the central tendencies of crucial parameters within each RAS under investigation. These averages serve as valuable reference points, summarizing the typical conditions observed during the comprehensive sampling period, providing insights that enrich our understanding of the system dynamics.

Examining the mean temperature values, it is evident that RAS1 and RAS2 consistently maintained relatively similar average temperatures, hovering around 18.7°C and 18.8°C, respectively. In contrast, RAS3 exhibited a notably higher average temperature of 21°C, signifying relatively warmer conditions on average within this particular system.

Regarding salinity, the mean values demonstrate a remarkable consistency in all three RAS systems, as salinity levels predominantly fell within the range of 35.8 to 36 mg/L-NaCl. Although RAS3 exhibited a slightly lower average salinity compared to RAS1 and RAS2, the variations are relatively minor, reflecting the overall stability of salinity levels.

The pH values, as indicated by the mean values, reveal distinct characteristics among the RAS systems. RAS3 exhibited the highest average pH of 7.9, suggesting slightly more alkaline conditions on average. In contrast, RAS1 and RAS2 displayed lower

average pH values of 7.7 and 7.6, respectively, indicating slightly more acidic conditions on average within these systems.

These mean values, woven into our analysis, offer a concise summary of the central tendencies for temperature, salinity, and pH within each RAS system. They provide critical insights into the typical environmental conditions these systems experienced throughout the study period, enriching our understanding of the dynamic interplay between physicochemical parameters and microbial communities.

	System	N total	Mean	Stand. Dev.	Minimum	Median	Maximum
Temperature (ºC)	RAS1	8	18,7	0,826	17,1	18,9	19,8
	RAS2	8	18,8	0,860	17,1	18,9	19,8
	RAS3	8	21	1,937	19,1	20,2	24,4
Salinity (mg/L-NaCl)	RAS1	8	35,9	0,991	35	35,5	37
	RAS2	8	36	1,069	35	36	37
	RAS3	8	35,8	0,707	35	36	37
pH	RAS1	8	7,7	0,166	7,54	7,71	7,99
	RAS2	8	7,6	0,140	7,35	7,56	7,78
	RAS3	8	7,9	0,121	7,68	7,88	8,07

Table 4- Mean values and their standard deviation for the physicochemical parameters of the water.

In the study conducted by Almeida et. al., (2021), it was noted that the salinity levels were proposed to be maintained at lower values in the Pre-Ongrowing system, with variations ranging from 15 to 19, while in the Weaning system, the salinity levels ranged between 35 and 37. These two distinct systems, Pre-Ongrowing (comprising RAS 1 and RAS2) and Weaning (represented by RAS 3), were designed to cater to the specific needs of the cultured aquatic organisms at different developmental stages.

However, upon closer examination of the data provided in the present study (Table 3), it becomes apparent that the recorded salinity levels in RAS 1, RAS2, and RAS3 are almost identical, with an average salinity of 36 mg/L. This observation raises questions regarding the apparent discrepancy between the proposed salinity management plan and the actual recorded salinity levels in the systems.

Usually, as already referred in 2.2.1, the biofilters take a time to start functioning property after the bacteria population is established. Examining the data from the Table 3 regarding NH_3-N concentrations in the three RAS systems (RAS1, RAS2, and RAS3) over time, several observations can be made:

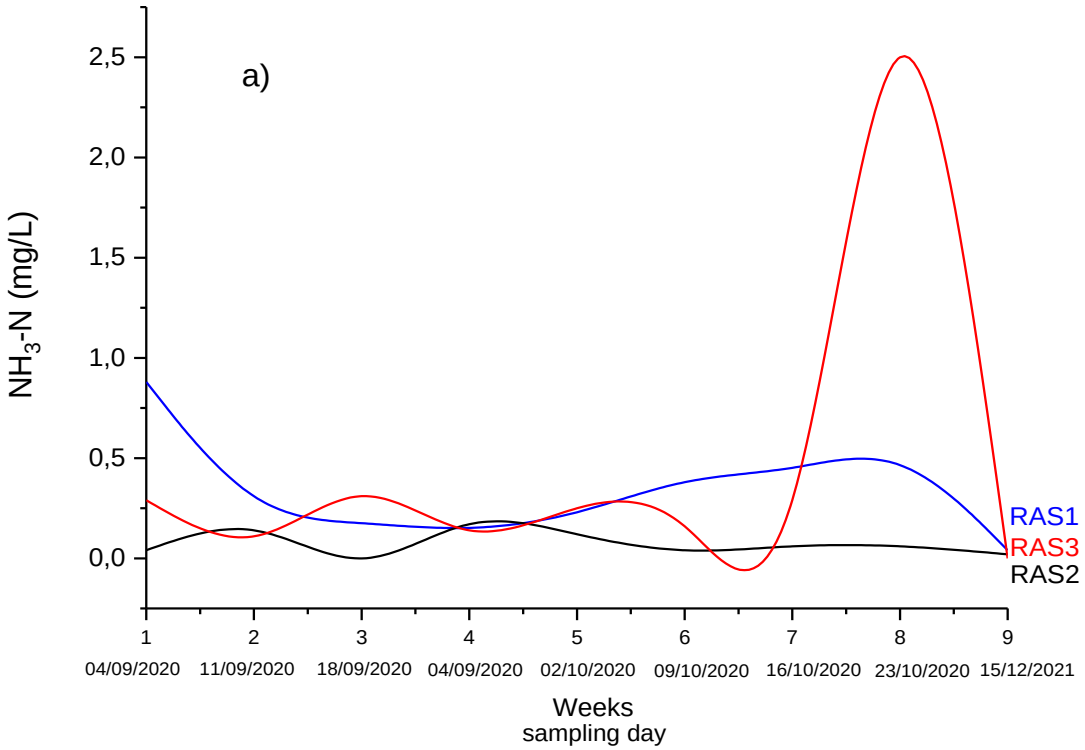
- In RAS1 the initial NH_3-N concentration on 04/09/2020 was 0.88 mg/L. Over the following weeks, the NH_3-N concentration fluctuated but generally decreased over time, reaching 0.04 mg/L on 15/12/2021.
- RAS2 started with a very low NH_3-N concentration of 0.04 mg/L on 04/09/2020. The concentration remained relatively low throughout the monitoring period, decreasing slightly to 0.02 mg/L on 15/12/2021.
- RAS3 had an initial NH_3-N concentration of 0.29 mg/L on 04/09/2020. The NH_3-N concentration showed fluctuations over time, with some increases and decreases. Notably, on 23/10/2020, the NH_3-N concentration spiked to 2.5 mg/L but decreased afterward. On 15/12/2021, it was 0 mg/L.

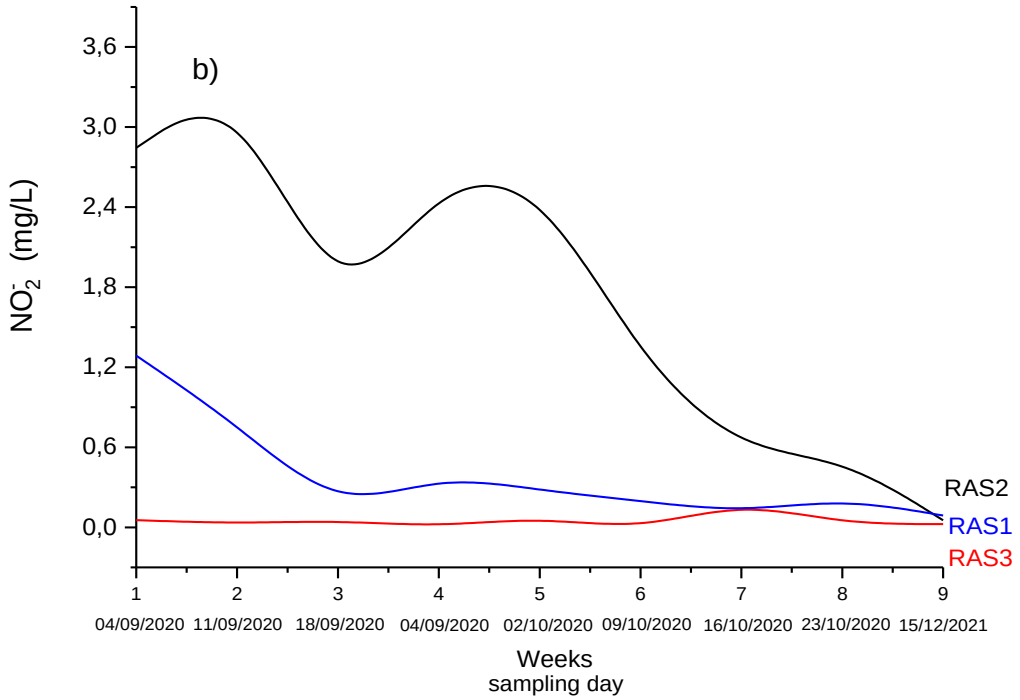
NH_3-N concentrations in RAS2 were consistently lower compared to RAS1. Notably, the initial concentration in RAS2 was considerably lower than in RAS1, indicating a well-established and properly functioning system from the start. Since RAS2 was first activated before RAS1, it was expected to have a more stable performance with lower initial ammonia concentrations.

In RAS1, ammonia concentrations initially showed a decreasing trend and remained stable after the second week. There was a slight inclination for the levels to rise from week 6 onwards. In RAS1, there was a peak in ammonia concentration 20 days after activation (week 1, in Figure 9a), followed by a significant decrease over the next 3 weeks, reaching a minimum of 0.13 mg/L-N. The concentration then increased again but remained lower than the maximum value of 0,88 mg/L at week 1. Compared to RAS2, which was activated a month and a half earlier, RAS1 exhibit superior ammonia concentrations. This observation aligns with the general understanding that a well-established system typically exhibits fewer fluctuations in ammonia levels, as seen in RAS2.

In the initial stages, RAS3 maintained ammonia levels below 0.5 mg/L, exhibiting stability with low ammonia concentrations during the first 6 weeks. However, an unexpected surge in NH_3-N was observed on October 23rd, after 7 weeks, as illustrated in Figure 9-a. This unanticipated increase in NH_3-N levels can be attributed to a chemical reaction involving bromide ions (Br^-) and ozone. This reaction can result in the creation of bromate ions (BrO_3^-), recognized as a disinfection byproduct that might contribute to the elevation in NH_3-N levels.

The changes in concentrations of ammonia, nitrite and nitrate for all biofilters are exhibited, respectively in Figure 9-a), Figure 9-b) and Figure 9-c).


a)
NH_3-N (mg/L)
2,5
2,0
1,5
1,0
0,5
0,0
RAS1
RAS3
RAS2
1
2
3
4
5
6
7
8
9
04/09/2020
11/09/2020
18/09/2020
04/09/2020
02/10/2020
09/10/2020
16/10/2020
23/10/2020
15/12/2021
Weeks
sampling day


b)
NO_2^- (mg/L)
3,6
3,0
2,4
1,8
1,2
0,6
0,0
RAS2
RAS1
RAS3
1
2
3
4
5
6
7
8
9
04/09/2020
11/09/2020
18/09/2020
04/09/2020
02/10/2020
09/10/2020
16/10/2020
23/10/2020
15/12/2021
Weeks
sampling day

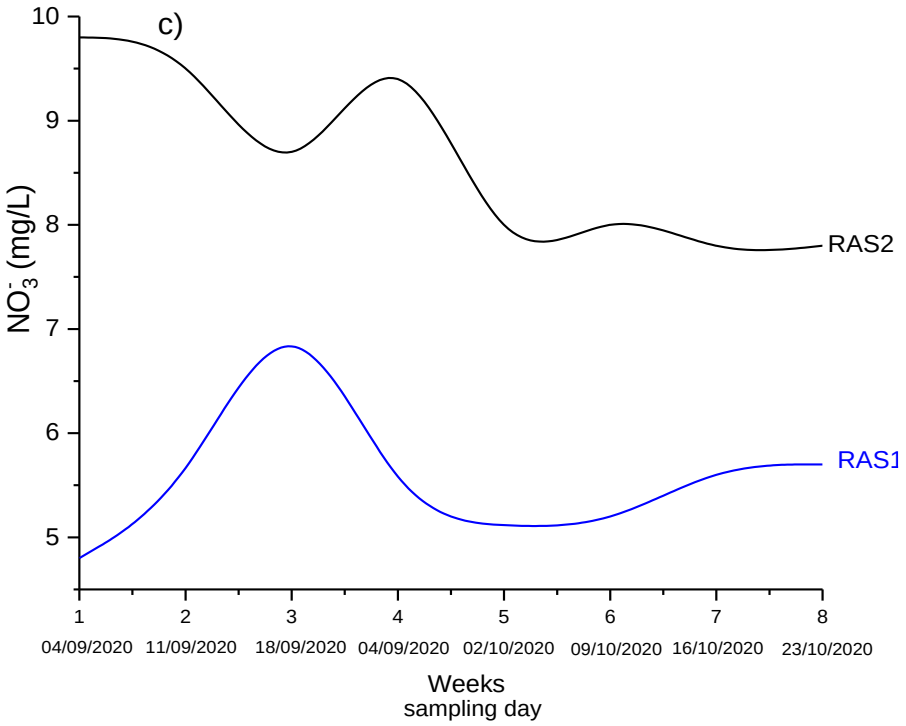


Figure 9 – Changes of the concentrations of a) ammonium, b) nitrite and c) nitrate on a daily sampling for RAS1, RAS2 and RAS3, depicted through adjusted spline curves across all biofilters, using all available points.

To further elucidate this occurrence, it's important to note that bromide ions (Br^-) are naturally present in seawater, including marine RAS systems, albeit typically in low concentrations. Ozone is a common tool employed in RAS for disinfection and the removal of organic carbon. Recommended flow rates of 10-15 g ozone per kg of feed are often used to minimize organic buildup (Gonçalves and Gagnon, 2011). In addition, ozone can improve water quality by reducing ammonia and nitrite (Sharrer and Summerfelt, 2007). When exposed to ozone, bromide is oxidized to HOBr, which reacts quickly with many inorganic compounds like ammonia (Heeb et al., 2014). Analysis of Figure 10 reveals a consistent correlation between a low bromine concentration and a high ozone flow rate within the system, and conversely. However, on October 23rd were detected the presence of both elevated ozone levels and bromide ions, and so a chemical reaction can occur, resulting in the formation of bromate ions (BrO_3^-): $O_3 + Br^- \rightarrow BrO_3^-$

Bromate ions can be categorized as part of the group known as "ozone-produced oxidants" (OPO). This categorization is based on the chemical oxidation process depicted above, wherein bromide ions are transformed into bromate ions. Elevated levels of bromate ions can indirectly affect NH_3-N concentrations within RAS3 due to their interference with the nitrification process. Nitrification, responsible for converting toxic

ammonia (NH_3) into less harmful nitrate (NO_3^-), may be hindered by the presence of bromate ions. Schroeder et al., (2015), reported a reduction in nitrifying activity as OPO concentration increased for *Nitrosomonas marina*.

Given that dominant OPOs are known for their strong bactericidal properties (Schroeder et al., 2015), it's expected that they could have a detrimental impact on nitrifying biofilter bacteria. Consequently, the potential presence of bromate ions can inhibit the activity of these bacteria, impeding the efficient conversion of ammonia and resulting in an increase in ammonia levels (NH_3-N).

Unfortunately, the data provided to conduct this study does not include information regarding concentrations of nitrate (NO_3^-) in RAS3.

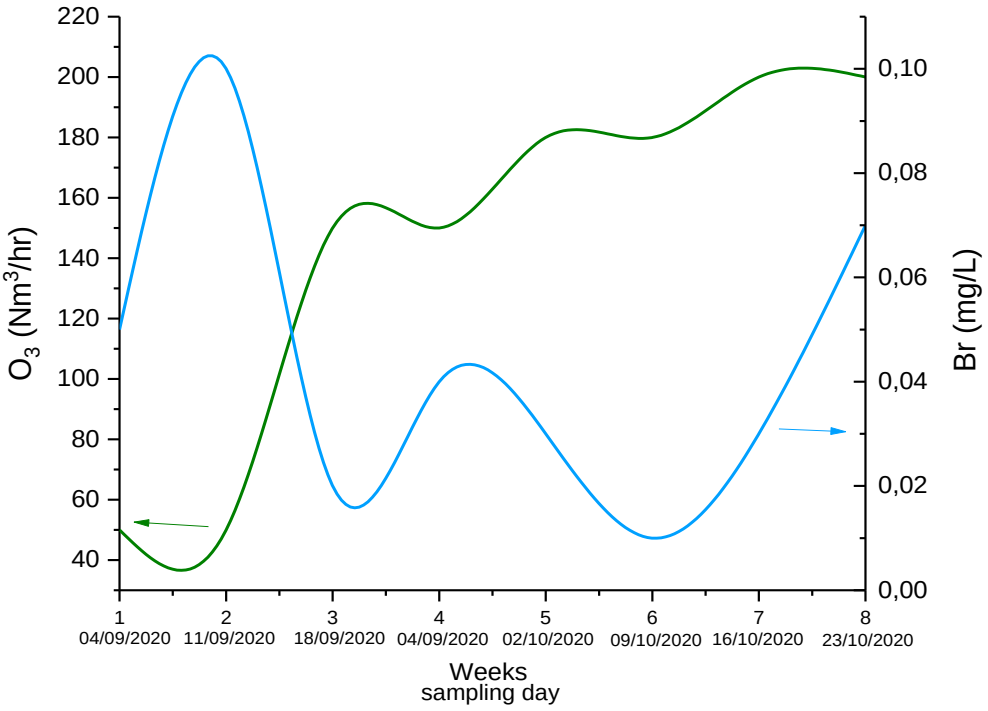


Figure 10 - Changes of the o ozone caudal (O3) and bromine (Br) concentrations on a daily sampling for RAS3. The curves depicted were derived utilizing spline interpolation, incorporating all available data points.

The effects on nitrification during biofilter reactivation were also evaluated by monitoring ammonia converted to nitrite, and nitrite converted to nitrate over time (Figure 9-b) and Figure 9-c), respectively. NO_2-N concentration is another important parameter in aquaculture, as nitrite is also toxic to fish at high concentrations, namely above 0.5 mg/L (Gomez Isaza et al., 2021). In RAS1 and RAS2, NO2-N concentrations displayed fluctuations. Elevated levels of NO_2-N were initially observed during the first sampling in

September in both RAS1 and RAS2. Throughout the monitoring period, NO_2 concentrations in RAS1 and RAS2 remained notably higher than 0.5 mg/L, as indicated in Table 3, except for December.

In RAS1, NO_2-N concentrations reached a maximum value of 1,286 mg/L on 04/09/2020, followed by a subsequent decrease. This observation may indicate the system's capability to maintain water quality and effectively reduce nitrite levels. Additionally, this data correlates with the survival of the fish cultures in the tanks, as observed during the facility visit.

In RAS1, as depicted in Figure 9-b, there was a notable decrease in nitrite concentration up to week 3, followed by an increase in nitrate concentration. Subsequently, it decreased again after week 6. The rise in nitrite concentration may imply incomplete nitrification towards nitrate. This can happen due to variations in the populations of ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) during the early stages of biofilter establishment (Timmons et al. 2002).

On the other hand, in RAS2, nitrite concentrations consistently decreased after week 4 and reached their lowest point in December, remaining above 0.5 mg/L throughout, except for December. In contrast, RAS3 maintained very low nitrite concentrations, almost at residual levels, which remained constant throughout the experiment.

To provide specific data, in RAS1, the NO_2-N concentration decreased from 1.286 mg/L on 04/09/2020 to 0.089 mg/L on 15/12/2021. In RAS2, the NO_2-N concentration also decreased from 3.351 mg/L on 11/09/2020 to 0.053 mg/L on 15/12/2021. In RAS3, the NO_2-N concentration on 15/12/2021 was exceptionally low, measuring only 0.025 mg/L.

NO_3-N concentration is less critical for fish health. In RAS1, there was an initial increase until 09/18/2020, followed by relative stability until 10/23/2020. In RAS2, there was a consistent decrease until 10/23/2020. However, the absence of data points after this date limits the analysis of long-term trends for both systems.

5.2. Microbial Community Analysis

To analyse the data sets generated by amplicon sequencing variants (ASVs), α-diversity was determined using the observed ASVs and exponential Shannons index. The phylogenetic relatedness between different bacterial communities was assessed using a nonmetric multidimensional scaling (NMDS) approach based on Bray-Curtis similarities.

The variability of observed ASVs in the bacterial communities was higher in the RAS3 biofilter compared to the other two. However, there were exceptions on 2Oct20 and 23Oct20, where the observed diversity in RAS3 dropped to very low values compared to the higher ones of the other days of sampling. The Observed ASVs and Shannon index calculated for each sample were plotted in Figure 11.

Since the start of the study, the observed species values in RAS3 have shown high instability with significant fluctuations, ranging from about 1140 on 4Sept2020 to a minimum near 500 on 2Oct20, followed by a rise above 1000 and then a sharp drop to nearly 600 on 23Oct2020. By 15Dec2021, the observed species in RAS3 had increased considerably, reaching a maximum above 1200, surpassing the values from 2020. Similarly, RAS1 also displayed oscillations in the observed species values, albeit with a lower amplitude. Starting at 500 on 4Sept2020, the observed species in RAS1 peaked at about 730 on 16Oct20, and a year later, the values were slightly above 650. In RAS2, the amplitude of oscillations was in between RAS3 and RAS1. At the beginning of the study on 4Sept2020, RAS2 had 730 observed species, which reached a maximum close to 900. However, a year later, the observed species in RAS2 decreased to around 500. This suggests that the fluctuations in RAS2 were not as extreme as those in RAS3 but were more pronounced than in RAS1.

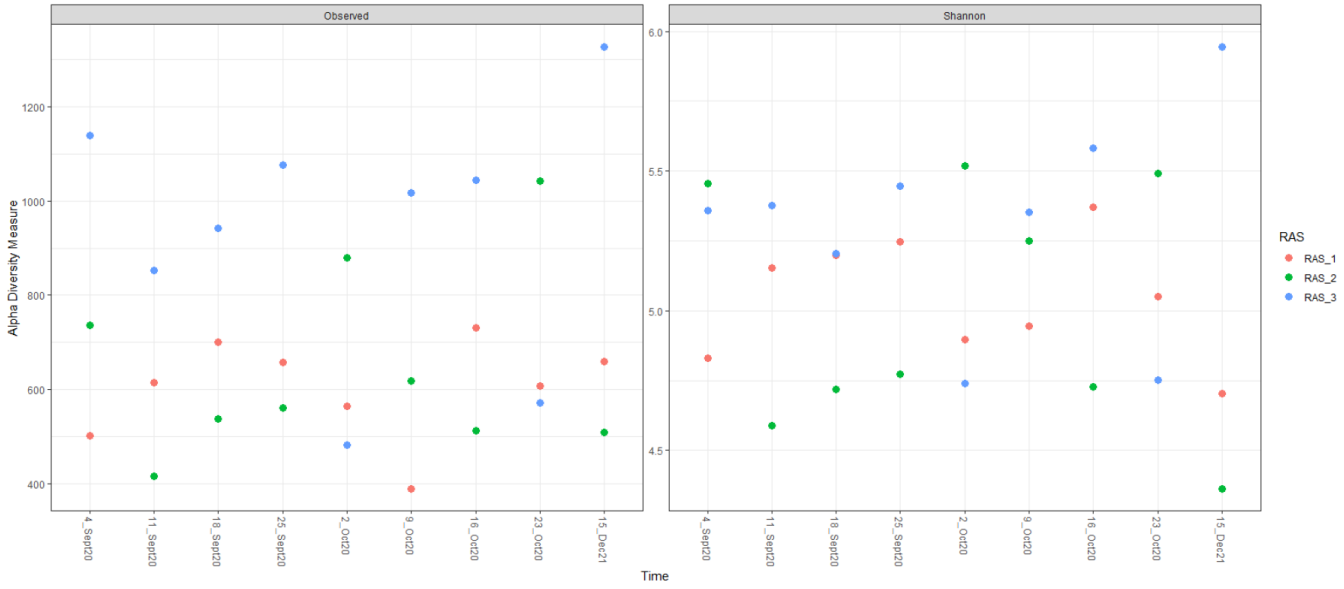


Figure 11 - Observed ASVs and Shannon index calculated for each system (RAS1, RAS2, RAS3).

The results of the Shannon index analysis indicated that the samples from all RAS exhibited a scattered distribution pattern. The highest value, close to 6.0 bits/ind, was

recorded in RAS3 (15Dec21), while the lowest value, approximately 4.4 bits/ind, was found in RAS2 (15Dec21). This suggests varying distribution conditions where specific communities are exposed to more favourable environmental factors, supporting higher microbial growth and survival. Additionally, when the number of species is relatively higher and constant, as in RAS3, the Shannon index values remain in the range of 5.25 to 6 bits/ind for the overall species.

The high abundance of the Observed ASVs can be attributed to its distribution in a stable biofilter, like RAS3, which is in full working condition and considered a reference in this study. This behaviour indicates that the bacterial community is more likely to stay stable in a biofilter that is in full operation. In all RAS, the fluctuation between high and low densities shows that the bacterial community is related to seasonal factors over time. So, the Shannon index values remain constant in RAS3 even when the Observed ASVs is high and constant. This is because RAS3 has a stable biofilter that is in full working condition, which provides favourable environmental conditions for the growth and survival of a larger number of microorganisms. As a result, the high abundance of the Observed ASVs in RAS3 can be attributed to its distribution in a stable biofilter.

This behaviour indicates that the bacterial community is more likely to stay stable in a biofilter that is in full operation. However, in all RAS, the bacterial community fluctuates between high and low densities due to seasonal factors over time.

The NMDS plot (Figure12) indicates that the microbial community composition in all RAS changed over time and showed a significant dissimilarity in the prokaryotic community.

During the 8-week period in 2020, the microbial communities in the biofilters seemed to have developed distinct groups. Initially, RAS1 and RAS2 exhibited highly similar communities, forming a distinct cluster from the established community in RAS3 during the same period.

As time progressed, a different pattern emerged, revealing mixed clusters among the microbial communities of RAS1, RAS2, and RAS3. Notably, samples from RAS3 were detected within the cluster initially associated with 'RAS1+RAS2', while samples from RAS2 were observed within the cluster categorized as RAS3.

By December 15, 2021, microbial communities sampled from RAS1 and RAS2 displayed reduced similarity compared to those from 2020, as they did not cluster with the previously exhibited ones.

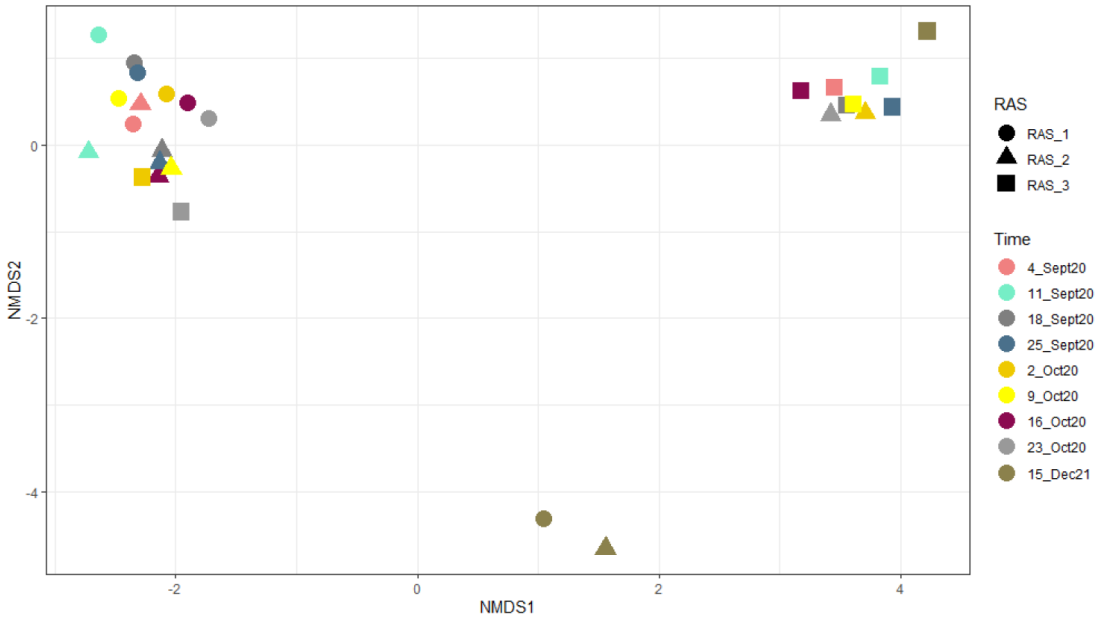


Figure 12 - Beta-diversity Visualized Using Non-Metric Multidimensional Scaling (NMDS) Plot with Bray-Curtis Dissimilarity Distances. NMDS of bacterial communities over time were studied in the three RAS: RAS1, RAS2 and RAS3 biofilters.

The microbial communities in RAS2 changed little during the 2020 sampling period, but a comparison of the communities on 11Sept2020 and 2Oct2020 revealed completely different communities in carriers. This indicates that the similarity between the samples was lost in just two weeks.

Later, on 23Oct2020, the existing microbial community in RAS2 moved away again from what existed in the first days and was closer to the community shown on 2Oct2020. In contrast, the samples from RAS3 exhibited minimal variation among themselves, remaining clustered within the same cluster, predominantly showcasing the characteristics of RAS3, and maintaining proximity over time. However, the similarity was lost after one year, likely due to the longer time span, indicating a successful period in service.

In 2020, the communities in unit RAS3 were more similar to each other than to those in 2021, which were more dispersed. In units RAS1 and RAS2, the communities were quite similar and very distant from those of RAS3.

There was a greater proximity between the communities of RAS2 in the period from 18Sept20 to 16Oct20 than those from the beginning of sampling (4Sept20 and 11Sept20), which were more dispersed. In RAS1, the communities over time were much more dispersed among themselves, with situations of greater proximity between 18Sept20 and 25Sept20, and also between 2Oct20 and 23Oct20.

It is worth noting that in RAS2, on 2Oct20 and 23Oct20, the communities were closer to those found in RAS3, which is the biofilter that was in full operation. However, bacterial communities in RAS1 didn't consistently mirror those found in RAS3; nevertheless, there were occasions where their proximity was observed during specific weeks. This indicates that the microbial communities in biofilters are affected by operational conditions and their stability is dependent on various factors, including the presence of favourable environmental conditions for microbial growth and survival.

Understanding the microbial community composition in biofilters is essential for efficient and sustainable operation of RAS. To achieve this, ASVs were classified by phylum and genus using a database of 16S rRNA gene sequencing data. Our study revealed significant differences in the microbial community composition among RAS1, RAS2, and RAS3 biofilters. The bacterial community compositions were analysed at both phylum and genus levels for all 27 biofilm carriers.

The primary objective was to determine if there were any similarities in the bacterial communities between RAS1 and RAS2 biofilters, as they used the same medium and had similar physicochemical conditions. Furthermore, we compared the evolution of bacterial communities in RAS1 and RAS2 to the established community in RAS3, which had been in full operation for a longer period. In this study, we characterized the microbial taxa in the communities at both phylum and genus levels to provide a comprehensive understanding of the microbial community composition in the biofilters. Figure 13 displays the relative abundance (>2%) of bacterial phyla over time in RAS1, RAS2, and RAS3.

Only 12 phyla were detected since the figure was generated for phyla with abundance >2%. Bacteroidota and Proteobacteria were the dominant phyla in all biofilters, which aligns with previous research (Almeida et al., 2021). The most abundant phyla varied across samples, including Bacteroidota (up to 49.03% in RAS2 on 18Sep20), Proteobacteria (up to 50.96% in RAS2 on 11Sep20), Planctomycetota (up to 15.38% in RAS1 on 23Oct20), Chloroflexi (up to 12.5% in RAS1 on 2Oct20), and Verrucomicrobiota (up to 8.65% in RAS2 on 23Oct20).

Other phyla appeared sporadically and in lower abundance over time in the different biofilters. The relative abundance of the dominant phyla varied among the different biofilters.

Although not dominant, Nitrospirota is a significant phylum of bacteria in RAS1 and even more abundant in RAS2 after one year of operation (15Dec21). Nitrospirota was also found in RAS3 throughout the study, but with a low relative abundance.

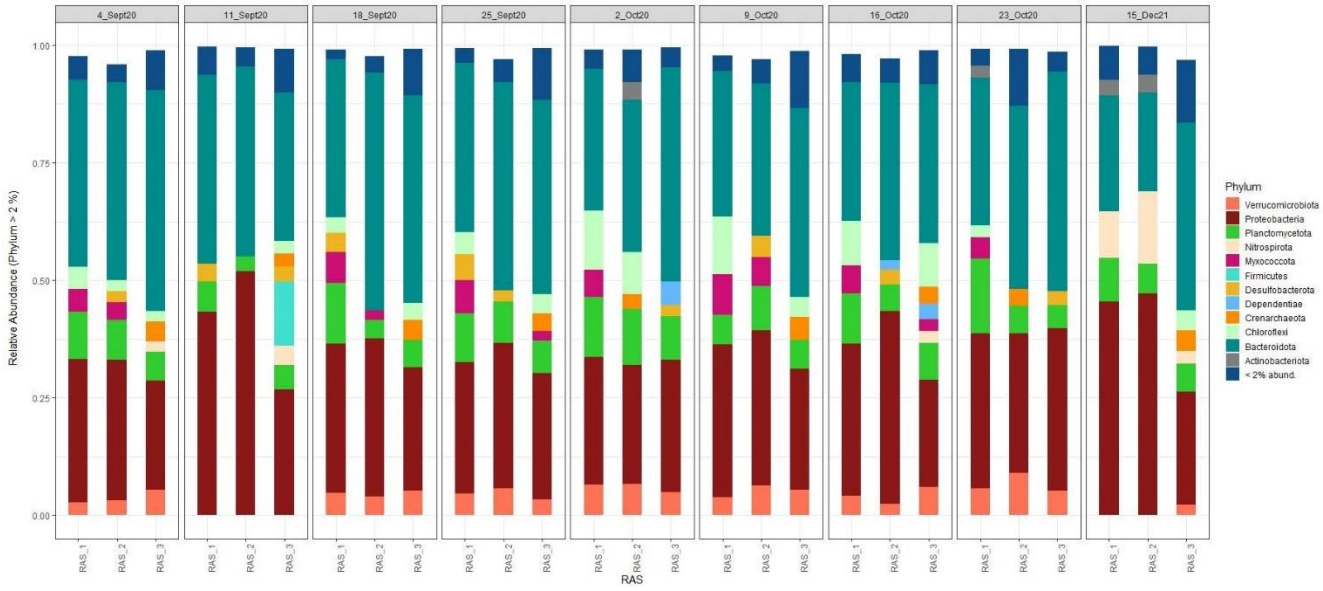


Figure 13 - Relative abundances (RA) of phylum over time in RAS1, RAS2 and RAS3. The relative abundance of phylum is above 2 % in at least one sample. Relative abundances are displayed in the stacked bar graphs. The taxonomy of each of the top of most abundant ASVs is detailed based on its closest match in the DADA2 database. Any phylum below 2% RA within a sample do not show on the plot.

While comparing the microbial communities across the time periods, there seems to be an impression of similarity among them. However, despite their apparent similarity, nuanced differences exist within these communities across various time frames. The bacterial communities at the phylum level in RAS1 and RAS2 are considered developing communities since they are based on the activation of the respective biofilters. This is an important indication for the future as it suggests that the population in these systems tends to adapt considerably to the environment.

In contrast, the population in RAS3 should be considered more stable. After one year of activity, on 15Dec21, the communities in RAS1 and RAS2 converged to a similar phyla diversity (detecting 5 common phyla), despite the respective populations evolving differently. The only difference between them was in their relative abundance (as shown in Table 5).

Phylum (>2%)	Rel. Abundance (%) (15Dec21)	
	RAS1	RAS2
Proteobacteria	44,23	46,15
Planctomycetota	9,62	5,76
Nitrospirota	9,62	15,38
Bacteroidota	24,03	25
Actinobacteriota	2,88	3,8
< 2% abund.	6,73	5,76

Table 5 –Relative abundances of common phyla from RAS1 and RAS2 purification plants when analysed 1 year after their respective biofilters reactivation.

The results suggest that the phyla listed in Table 5 are the most stable and well-adapted to the system's operating conditions over time. Proteobacteria, Planctomycetota, Nitrospirota, Bacteroidota (synonym Bacteroidetes), and Actinobacteriota are all phyla of bacteria commonly found in marine RAS (Kyritsi et al., 2023).

Proteobacteria, as demonstrated in Almeida et al. (2021), stands out as the predominant phylum within RAS, fulfilling a vital function as nitrifying bacteria—an indispensable component for the effective functioning of RAS. Bacteroidetes, on the other hand, contain species that are specialized in the degradation of complex polymers and protein-rich substances. They tend to be attached to particles or surfaces and commonly colonize organic matter particles. For instance, within the Bacteroidetes phylum, recent findings have unveiled a significant contribution to nitrous oxidation-reduction, the final step of denitrification (Rieder et al., 2023).

The ratio of Proteobacteria to Bacteroidetes in RAS1 and RAS2 is 1.840 and 1.846, respectively. This ratio could potentially serve as a significant indicator of the overall health of the RAS. These microbial groups are crucial in preserving the stability of the aquatic environment. A consistent and balanced ratio between these groups over time may signify a more stable and healthier RAS environment. Additionally, comparing the Proteobacteria to Bacteroidetes ratio in RAS3 on 4Sep20 and 15Dec21, which were 1.99 and 1.36, respectively, reveals a decline in Proteobacteria abundance over time. This decrease allows other phyla to occupy that ecological niche. Hence, monitoring changes in this ratio could offer valuable insights into the general health and stability of the RAS.

A study conducted by Martins et al., (2013) aimed to determine the bacterial communities in a recirculating aquaculture system (RAS) for *Solea senegalensis*, at various stages of the RAS including water and biofilter media. The findings demonstrated a varied bacterial community in the RAS, with Proteobacteria and Bacteroidetes being the most dominant phyla, similar to the current study.

In the study, the relative phylum abundances (>1%) were analysed to observe variations in bacterial communities over time between all RAS. Figure 14 represents the 18 phyla detected in the 27 samples. The relative phylum abundances (Phylum >1%) detected 6 more phyla than Phylum >2%, which are NB1-j, Gemmatimonadota, Dadabacteria, Campilobacterota, Bdellovibrionota, and Acidobacteriota. However, these phyla had a very low relative abundance, ranging from under 1% to near 2%. These phyla are less commonly detected in marine RAS compared to Proteobacteria and Bacteroidetes. Gemmatimonadota has been found in high relative abundance in a marine RAS with ozonation and may play a role in nutrient cycling and carbon fixation (Aalto et al., 2022b).

Dadabacteria is a relatively newly discovered phylum, and its role in RAS is not yet well understood. Campylobacterota includes several potential human and animal pathogens and has not been yet described in marine RAS studies. Bdellovibrionota is a group of bacteria that prey on other bacteria and has been found in some marine RAS studies, but their significance in these systems is not yet known (Qu et al., 2021).

The role of Acidobacteria is mainly related to their contribution to carbon and nitrogen cycles in environments with limited nutrients (oligotrophic conditions). They also possess genes that enable them to break down specific substances, such as polysaccharides and aromatic compounds. However, due to their slow growth rate and lower energy generation in their metabolism, they may not compete well with other microorganisms, which could explain their relatively low abundance in this microbial community (Nguyen et al., 2019)

As expected, both Bacteroidota and Proteobacteria dominated phylum (>1%)-level ASV affiliation for all RAS throughout the study. Members of the Verrucomicrobiota and Planctomycetota were also consistently detected across all samples, ranging between 1-9% of all ASVs for each sample. These last are well-suited for existence within marine biofilms. They possess a specialized holdfast structure that facilitates surface attachment and exhibit rapid adaptability to changes in their environment, as documented by Kallscheuer et al. (2020). Members of the Verrucomicrobiota phylum are known for their diverse metabolic capabilities, including the ability to degrade complex organic compounds and participate in nutrient cycling (Herlemann et al., 2013). In marine RAS, Verrucomicrobiota may play a role in maintaining water quality and processing organic matter. Verrucomicrobia have shown a great potential for using a wide variety of carbon sources (Herlemann et al., 2013). This could potentially be explained by a higher quantity of nutrients in the feed provided or a less efficient particle removal process, which rapidly increases the concentration of organic matter.

Schreier et al. (2010) provided a comprehensive overview of the microbial community composition in RAS biofilters, which includes Actinobacteria, Bacteroidetes/Chlorobi, Firmicutes, Nitrospirae, Planctomycetales, and Proteobacteria. At the phylum level, the microbial community composition of this study closely aligns with the findings of Schreier et al. (2010), as well with the research of Chen et al. (2019) that was found a bacterial community of bioreactor mainly dominated by Proteobacteria, followed with Planctomycetes, Bacteroidetes, Actinobacteria and Verrucomicrobia phylum. Proteobacteria stands out as the most dominant phylum in RAS (Chen et al., 2019).

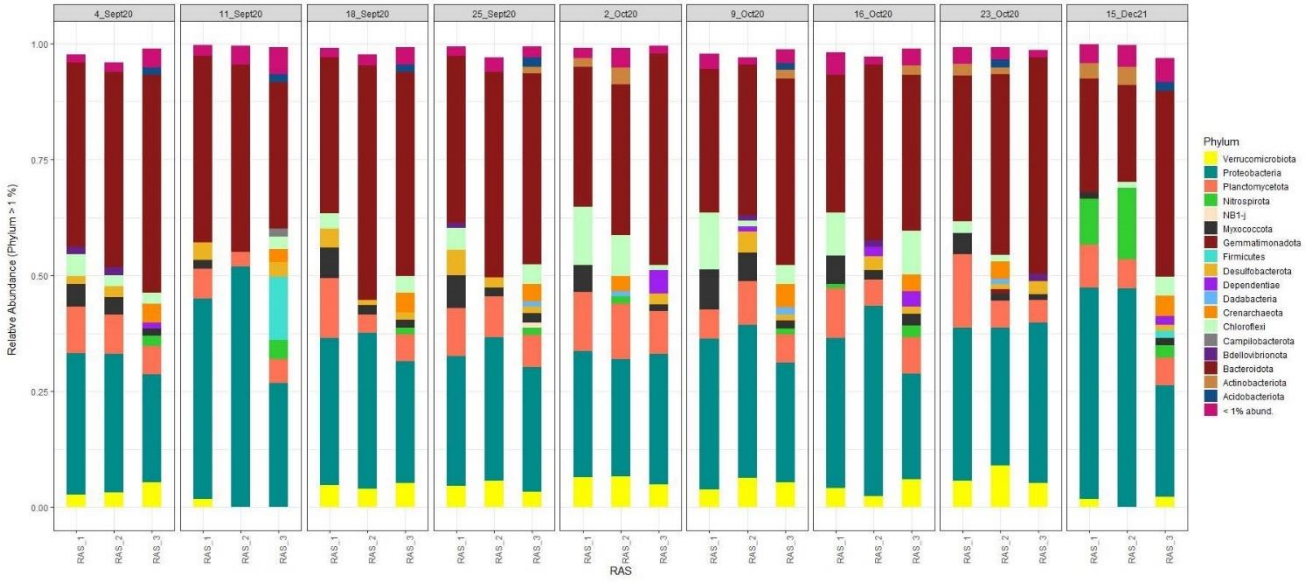


Figure 14 - Relative abundances (RA) of phylum by RAS over time. The relative abundance of phylum is above 1 % in at least one sample. Relative abundances are displayed in the stacked bar graphs. The taxonomy of each of the top of most abundant ASVs is detailed based on its closest match in the DADA2 database. Any phylum below 1% RA within a sample do not show on the plots.

Both Figures 13 and 14 shown that there was a considerable difference in the microbial community's composition between the RAS1 and RAS2 systems compared to the RAS3 system. Thus, when analysing the systems individually in terms of relative abundance of the most dominant phyla, it is observed that Proteobacteria is lower prevalent than Bacteroidota in RAS3, while in RAS1 the two phyla occurring at similar relative abundance and in RAS2 there is a slight increase in Proteobacteria compared to Bacteroidota. In addition to the dominant phyla, there is the presence of a diverse community of phyla that presents its specificities depending on the type of biofilter. Thus, in RAS1, it can be said that the phyla Planctomycetota, Myxococcota and Chloroflexi dominate.

The dominance of Planctomycetota, Myxococcota, and Chloroflexi in a bacterial community suggests its adaptation to low-nutrient conditions. Planctomycetota has diverse metabolic abilities, including nitrogen fixation, which is important in nutrient-limited environments. Myxococcota is associated with the degradation of complex organic matter, and Chloroflexi can degrade a wide range of organic compounds. Chloroflexi and Planctomycetota were in greater abundance under low-N condition (Chai et al., 2023). Low-nutrient conditions can result from factors such as high-water exchange rates, low feed input, or efficient nutrient removal by the biofilter and feeding regime affects nitrogen balance (Mes et al., 2023). On the other hand, Table 3 shows that RAS1 had an average feed supply of 29.5 kg over eight weeks, while RAS2 had an average feed supply of 37.2 kg. Therefore, it can be inferred that RAS1 can be characterized as experiencing lower nutrient conditions compared to RAS2.

In RAS2 the presence of Planctomycetota is still noted, although with lower relative abundance. The same is true for Myxococcota and Chloroflexi.

Chloroflexi is also an anaerobic bacterium, which is notably in more abundance in RAS1. Since the increase of dissolved oxygen (DO) inhibits the growth and metabolism of Chloroflexi (Lee et al., 2014), RAS1 has probably low oxygen conditions, which can benefit heterotrophic bacteria, that could then outperform and compete with the nitrifying bacteria (Foore, 2016). As nitrification is an aerobic process, oxygen is a key factor in the nitrification control with NOBs being more sensitive to low concentrations of O_2 than AOBs. As the nitrifying bacteria are inoperative whenever DO concentrations in the Biofilter are low (Chen in al. 2006), and although the growth rate of *Nitrosomonas* improves above 2 mg/L, this is not the case in *Nitrobacter* which are more sensitive to DO and showed a rate of reduced growth with DO less than 4 mg/L (Chen et al. 2006).

This can explain the high abundance of the phyla Planctomycetes in RAS1. *Planctomycetes* usually oxidise NH_3 through the Anammox process, i.e., occurs at low O_2 concentrations (Prosser, 2007). This supports the hypothesis that RAS1 is very likely to have a low concentration of DO.

Furthermore, RAS1 was the last activated biofilter, separating the first sampling day from the biofilter start date, only about 20 days elapsed. Thus, it is not surprising that RAS1 initially has higher ammonia levels than the other two biofilters (Figure 9-a). The reasons for this behaviour could be the lack of time given for the development of RAS1.

In addition, seasonal changes in temperature and salinity within the tanks can also affect nitrification. RAS2 exhibits slightly greater phyla diversity than RAS1, but with reduced

relative abundance. However, the presence of Desulfobacterota in RAS2 stands out. RAS3 is the system that exhibits the greatest multiplicity of phyla in addition to the dominant phyla. It is also in RAS3 that all 19 identified phyla are found, especially those that were not found in the other systems (RAS1 and RAS2), namely Firmicutes and NB1-j, or are residual in these systems, such as Dadabacteria, Dependentiae and Acidobacteriota (residual in RAS2 and absent in RAS1).

The observation of high abundance of Firmicutes in RAS3 on September 11th, 2020, when the water temperature rises to 24.4 ºC, could suggest a potential relationship between the abundance of Firmicutes and water quality in RAS, particularly under elevated water temperatures. However, further research and investigation are warranted to better understand the role of Firmicutes in RAS systems and their potential impact on water quality parameters.

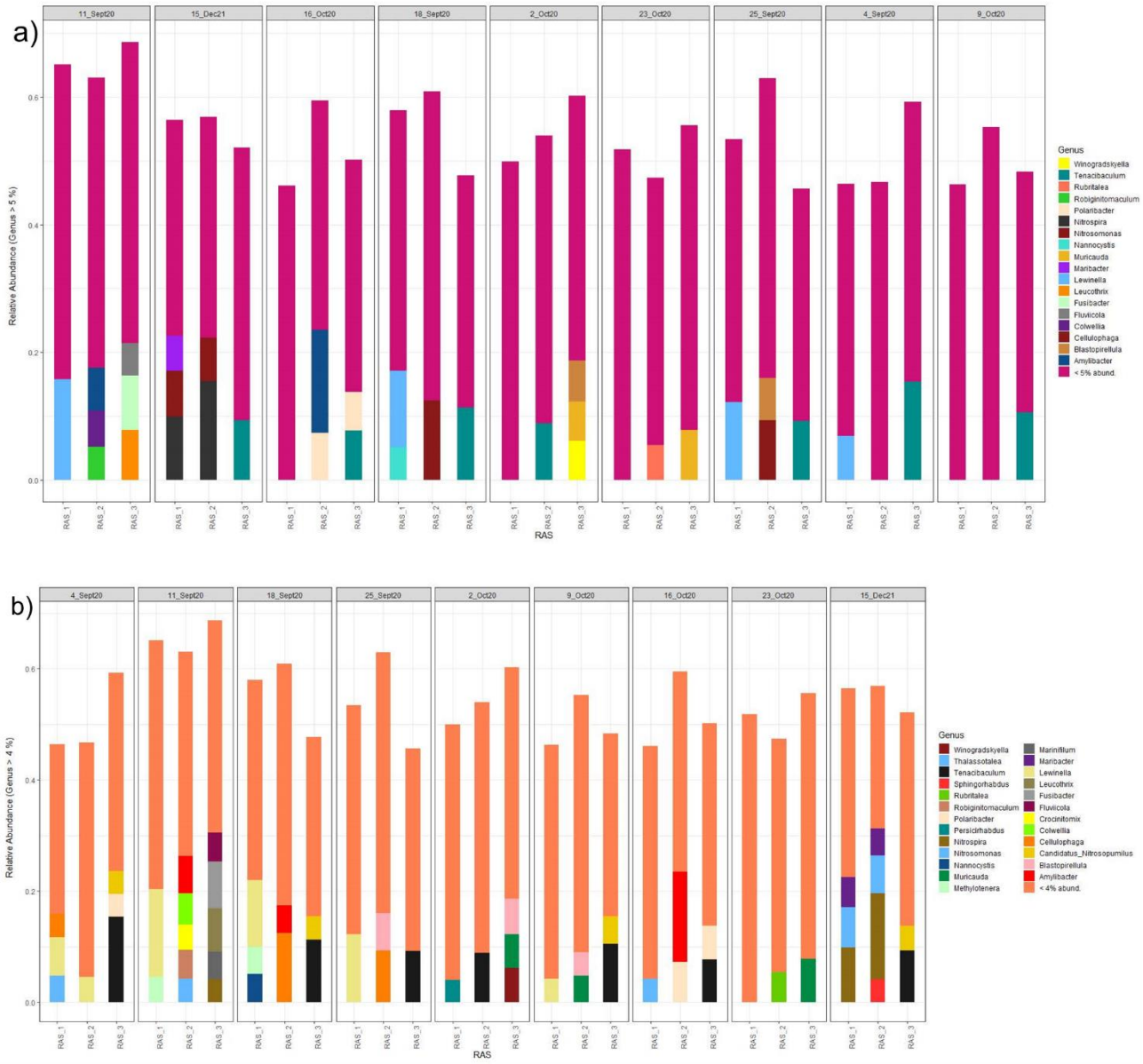
The abundance of certain taxa in an ecosystem is often influenced by their suitability to local environmental conditions. Dominant taxa, which possess characteristics that allow them to thrive in the specific environment, can play significant roles in shaping biological relationships within the ecosystem. Furthermore, analysis of Figure 9b) on October 23rd, 2020, reveals a decrease in nitrite levels, indicating effective nitrification, where ammonia is converted into nitrite and then into nitrate by certain microorganisms, in the ecosystem. This efficient nitrification process may be attributed to the activity of dominant taxa that are well-adapted to the environment, while low abundant taxa may have a lesser role in this process.

These findings suggest that the relative abundance and activity of dominant and low abundant taxa in the ecosystem can provide insights into its functioning. In the phylum analysis of RAS1 and RAS2 systems, a taxonomic profile similar to the reference system (RAS3) was observed, as most sequences align with the same phyla.

Although the sampled biofilter communities appeared similar at the phylum level, more distinct profiles were observed when displayed at the genus level. The composition of prokaryotic communities at the genus level is presented in Figures 15-a), 15-b), 15-c), and 15-d), where different thresholds of relative abundances were applied (>5%, >4%, >3%, and >2% respectively). The results showed that varying numbers of genera (18, 25, 29, and 40) were detected under each threshold.

For instance, when the threshold was set at 5%, there were instances where no genera appeared, as seen in RAS1 and RAS2 on December 15th, 2021. Similarly, for RAS1 on October 16th, 2020, no genera were detected at the 5% threshold, but the genus

Nitrosomonas was detected at the 4% threshold, suggesting the importance of choosing an appropriate threshold. Interestingly, the threshold of 2% allowed for the detection of six additional genera that were not detected at higher thresholds. Furthermore, it was observed that none of the cases reached 100% filled, indicating that the samples were highly diversified with multiple genera but low dominance.



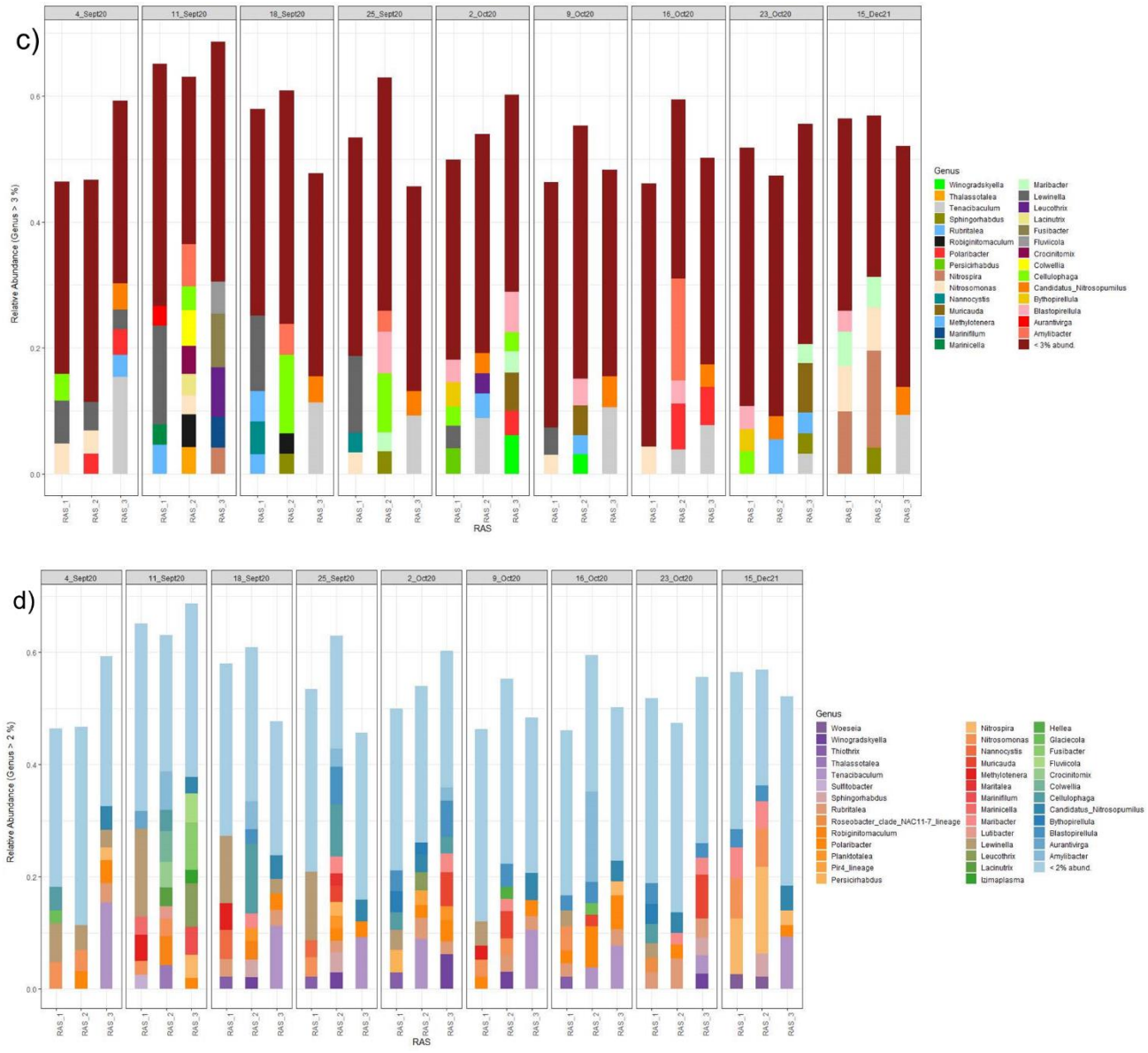


Figure 15 - Relative abundances (RA) of genus on RAS1, RAS2 and RAS3, by sampling day. The relative abundance of genus is above 5%, 4%, 3% and 2%, respectively in a), b), c) and d), in at least one sample. Relative abundances are displayed in the stacked bar graphs.

Considering that more detailed taxonomic information can provide a better understanding of the biological community in the biofilters, the data presented in Figure 15-d, with a threshold of >2%, offers a more detailed analysis of the community composition at the genus level. Based on these results, a total of 40 genera were detected, providing valuable insights into the diversity of microorganisms in the biofilters. The total relative abundance of microorganisms at genus level accounted for 11% (RAS2, 4Sep20) to 42% (RAS2, 25Sep20). The relative abundance of genus < 2% is higher, ranging from 20% (RAS2, 25Sep20) to 35% (RAS2, 4Sep20). The most abundant genera in the set of the three studied biofilters are *Nitrospira* (up to 16%), which belongs

to the phylum Nitrospirota, and genera *Tenacibaculum* and *Lewinella* (both up to 15%), *Cellulophaga* (up to 12%), and *Muricauda* (up to 8%), which belong to the phylum Bacteroidetes. Moreover, *Fusibacter* (up to 9%), a genus of bacteria within the phylum Bacillota, and *Polaribacter* (up to 8%) from the Bacteroidetes phylum were also among the most abundant. Additionally, the genera *Leucothrix*, belonging to the phylum Proteobacteria, and *Amylibacter*, belonging to the phylum Pseudomonadota, were present in relatively high abundance (7%) on only one day (11Sep20), specifically in RAS3 and RAS2, respectively. Out of the most abundant taxa at the genus level, *Colwellia* (up to 6%), *Blastopirellula* (up to 6%), *Winogradskyella* (up to 6%), *Rubritalea* (up to 5%) were present in almost samples. According to the literature, the genera normally associated with biofiltration in RAS in biofilter carriers are *Nitrospira* (NOB), *Nitrosomonas* (AOB) and *Thiothrix* (sulfide-dependent autotrophic denitrification) (Almeida et al., 2021, Bastos Almeida et al., 2021). These were significantly detected in RAS1 and RAS2, in 15Dec21. Its abundance is dominant only 1 year after the biofilter activation. Prior to that mark, *Nitrospira* and *Nitrosomonas* are below the abundance of the most representative genera found in these biofilters. The most dominant taxonomic groups detected in the total of the three systems, are presented in Tables 6, 7 and 8, respectively to RAS1, RAS2 and RAS3.

RAS1: Sampling Day	**Phylum**	**Genus**
4Sep20	Bacteroidetes	*Lewinella*
11Sep20	Bacteroidetes	*Lewinella*
18Sep20	Bacteroidetes	*Lewinella*
25Sep20	Bacteroidetes	*Lewinella*
2Oct20	Verrucomicrobia	*Persicirhabdus*
9Oct20	Bacteroidetes	*Lewinella*
16Oct20	Pseudomonadota	*Roseobacter_clade_NAC11-17_lineage*
23Oct20	Planctomycetota	*Blastopirellula*
15Dec21	Nitrospirota	*Nitrospira*

Table 6**–** Most dominant taxonomic groups (phylum and genus) detected in RAS1 (data from Figure 15d)

RAS2: Sampling Day	**Phylum**	**Genus**
4Sep20	Bacteroidetes	*Lewinella*
11Sep20	Bacteroidetes Pseudomonadota Pseudomonadota	*Polaribacter* *Colwellia* *Amylibacter*
18Sep20	Bacteroidetes	*Cellulophaga*
25Sep20	Bacteroidetes	*Cellulophaga*
2Oct20	Bacteroidetes	*Tenacibaculum*
9Oct20	Bacteroidetes	*Muricauda*
16Oct20	Bacteroidetes	*Polaribacter*
23Oct20	Verrucomicrobia	*Rubritalea*
15Dec21	Nitrospirota	*Nitrospira*

Table 7**–** Most dominant taxonomic groups (phylum and genus) detected in RAS2 (data from Figure 15d)

RAS3: Sampling Day	Phylum	Genus
4Sep20	Bacteroidetes	*Tenacibaculum*
11Sep20	Proteobacteria Pseudomonadota	*Leucothrix* *Amylibacter*
18Sep20	Bacteroidetes	*Tenacibaculum*
25Sep20	Bacteroidetes	*Tenacibaculum*
2Oct20	Bacteroidetes	*Muricauda*
9Oct20	Bacteroidetes	*Tenacibaculum*
16Oct20	Bacteroidetes	*Polaribacter* *Tenacibaculum*
23Oct20	Bacteroidetes	*Muricauda* *Tenacibaculum*
15Dec21	Bacteroidetes	*Tenacibaculum*

Table 8 **–** Most dominant taxonomic groups (phylum and genus) detected in RAS3 (data from Figure 15d)

The convergence of the composition of the bacterial community of RAS1 and RAS2 at the phylum level is also verified in the genus, since there is a similarity of taxonomic composition after 1 year of the biofilter activation, 5 common genera detected, diverging only slightly in their relative abundance, and only 1 genus not common (Table 9).

Genus (>2%)	Rel. Abundance (%) (15Dec21)	
	RAS1	RAS2
Thiothrix	3	2
Nitrospira	10	16
Nitrosomonas	7	6
Maribacter	5	6
Blastopirellula	4	3
Sphingorhabus	0	5
< 2% abund.	30	21

Table 9**–**Relative abundances of different genera from RAS1 and RAS2 purification plants when analysed 1 year after their respective biofilters activation (data from Fig.15d)

Upon closer examination of the Table 9, it appears that the abundance values for the genera *Thiothrix*, *Nitrospira*, *Nitrosomonas*, *Maribacter*, *Blastopirellula*, and *Sphingorhabus* are indeed quite similar between RAS1 and RAS2. This indicates a convergence or similarity in the abundance of these genera in both RAS systems.

This similarity in abundance values could potentially suggest that these genera may have similar ecological roles or responses to the environmental conditions in both RAS1 and RAS2. However, it's important to conduct further research and analysis to understand the underlying reasons for this convergence of values and its implications for the microbial community dynamics in these RAS systems. The findings suggest that there is a consistent pattern of convergence in the composition of bacterial communities in the RAS during periods of community growth. This could be considered a positive aspect in the context of RAS operation, as it indicates a stabilization and establishment of a

common composition of bacteria, which may have beneficial effects on water quality, fish health, and system performance.

As mentioned earlier, the genus *Tenacibaculum* has been identified as a potential pathogen that can negatively impact the culture of several commercially valuable fish species, such as the *Senegalese sole*, leading to significant losses in aquaculture worldwide. The high abundance of *Tenacibaculum* in RAS3, as indicated by our study, suggests that this genus is widespread in the system throughout the monitoring period.

This finding is in line with previous studies conducted at the same facility, which reported similar results (Almeida et al., 2021; Bastos Almeida et al., 2022). The persistent presence of *Tenacibaculum* in RAS3, from the beginning of sampling to the end of the study, highlights the need for further investigation and management strategies to mitigate its potential impact on fish health and production in RAS systems.

5.3. Conclusions

Solea senegalensis is commonly cultured in Recirculating Aquaculture Systems. In these systems, biofilters are essential for maintaining water quality and providing a suitable environment for fish growth. Biofilters consist of a fixed-film system in which beneficial bacteria grow on surfaces to convert toxic nitrogen compounds, such as ammonia and nitrite, into less harmful nitrate. To support the growth of these bacteria, carriers or biofilm substrates are added to the biofilters.

The biofilm that develops on the carriers in the biofilters plays a crucial role in maintaining water quality in RAS. The bacteria in the biofilm create a diverse microbial community that performs a range of functions, including the breakdown of organic matter, the conversion of nitrogen compounds, and the removal of pathogens from the water.

However, the composition of the biofilm community can be affected by several factors, including water temperature, among others.

Our study reveals that the biofiltration of water in the studied RAS system for *Solea senegalensis* cultivation is governed by a highly dynamic bacterial community. The composition and performance of these communities are greatly influenced by physical-chemical parameters that maintain optimal water conditions for fish health, such as salinity, temperature, and pH. Additionally, other factors like ozone and food quantity can also impact the microbiome of the system.

Analyzing the changes in relative abundances over time, there's notable initial variability during phases like the inception and early monitoring stages of community growth. This variability gradually diminishes, resulting in a partially similar composition between the two studied systems. Throughout the start-up, early monitoring, and after 1 year of operation, this initial variability converges both at the phylum and genus levels, partially sharing the composition between the two activated systems. A more pronounced convergence trend is observed after a year of stabilization, indicating greater similarity between the systems in this longer period, contrasting with the variability observed in short time intervals (weeks).

The development of the nitrifying community in the biofilm and the nitrifying capacity were compared in the two systems. We observed after around 60 days of start-up, that the biofilms of the carriers have very diverse microbial communities and a low proportion of nitrifiers. However, low concentrations of ammonia and nitrite with rapidly increasing nitrate concentrations indicated that complete nitrification was established in both systems. Hence, we hypothesized that bacterial communities in the biofilters exhibit dynamic changes leading to an optimized state. To investigate this, we utilized high-throughput DNA sequencing to analyze and compare the microbial communities in two biofilters of a commercial aquaculture recirculation system that were activated with a time difference of one and a half months. These results were then compared with a fully operational biofilter.

Our findings showed a rich species diversity in all three biofilters, with distinct microbial community relationships in each system. Proteobacteria and Bacteroidota were identified as the dominant phyla across all biofilters. Notably, the presence of Nitrospirota, which includes nitrifying bacteria like *Nitrospira*, was only observed in biofilters that were operational for more than one year after activation. The genera of ammonia and nitrite oxidizing bacteria were also detected, albeit sporadically and not in all samples.

Furthermore, our results revealed a lack of consistent patterns in the behavior of the microbiome. Only the results obtained at the end of one year provided insight into the trend of the microbiome, which did not align with the background data. This suggests that it remains uncertain at what point after reactivation the microbiome begins to exhibit a mature composition, indicating the need for further research to better understand the dynamics and maturation of bacterial communities in biofilters. Nevertheless, this study highlights the potential for microbiome modulation in aquaculture systems.

6. Suggestions to Future Work

Following the present work, further studies must be conducted to enhance the application of a bacterial consortium in RAS biofilms, namely:

- Taxonomic identification of bacterial isolates obtained from enrichment with biofilter matrix to provide a comprehensive understanding of the microbial community composition and diversity, which is essential for further characterization and selection of potential isolates for the consortium.
- Carrying out specific assays to test the ability of the isolates of interest to oxidize ammonia (NH_4^+-N) and nitrite (NO_2^--N), which are key steps in the nitrification process. These assays can be conducted in controlled laboratory conditions to determine the nitrification potential of the isolates.
- Biofilter colonization tests using the most promising isolates. This involves inoculating the biofilters with the selected isolates and monitoring their establishment and growth over time. This will help determine the effectiveness of the isolates in colonizing the biofilter matrix and contributing to the nitrification process.

In addition, concept proof studies can be conducted to validate the results obtained. This can involve comparing the microbial community composition and nitrifying potential of the isolates obtained from NGS with those isolated in the laboratory. Feasibility tests can also be conducted in a production environment to simulate the formulation obtained in the laboratory and assess its performance in real-world conditions. These studies will provide valuable information on the practical applicability and effectiveness of the developed microbial consortium in commercial systems.

The suggested future work aims to advance the understanding of bacterial consortia development, their nitrifying potential, colonization ability, and performance in commercial systems. These studies will contribute to the development of biotechnological tools to optimize biofilter activation and improve the efficiency of nitrogen removal in RAS and other related applications.

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