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Modelling biofilm microbial community to optimize water quality and fish health in a marine Recirculating Aquaculture System

Diana Almeida

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This Thesis includes one scientific paper published in an international peer-review journal originating from part of the results obtained in the experimental work, referenced:

Almeida, D.B., Magalhães, C., Sousa, Z., Borges, M.T., Silva, E., Blanquet, I., Mucha, A.P., 2021. Microbial community dynamics in a hatchery recirculating aquaculture system (RAS) of sole (*Solea senegalensis*). Aquaculture. 539, 736592.

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Summary

Recirculating aquaculture systems (RAS) have been developed to reduce the environmental impact of fish production by reducing water usage. They rely on water recirculation to optimize waste management, and so, thrive to make intensive fish production compatible with environmental sustainability. To reuse the water, these systems rely on a series of treatment steps, with each step reducing the need for water exchange. This type of system has been suggested for sole (*Solea senegalensis*) production as a promising strategy for aquaculture diversification in the south of Europe. However, this species is highly vulnerable to disease outbreaks, mainly caused by pathogenic bacteria, particularly *Tenacibaculum maritimum*.

Inside a RAS, the main waste produced is ammonia, released into the water by the fish as a metabolite from protein catabolism. The maximization of the microbial nitrification process within the biofilter is the most common treatment used to control ammonia concentrations. Because RAS systems depend on this beneficial microbial community, they pose unique challenges in microbial community management, to maintain key functional roles, both in the environment and in the fish host.

This thesis aims to deepen the knowledge of RAS prokaryotic community composition and interactions, particularly in two fronts: a deep characterization of the composition and dynamics of the prokaryotic community along the different RAS compartments. Also, we want to know how these communities relate with the fish host and how they developed throughout its life cycle and production stages. Afterwards, we will use this knowledge to develop biotechnology tools to improve production sustainably. These will include two specific tools, the first, active nitrifying bacterial formulation, adapted to a marine RAS environment, to shorten biofilter stabilization times or be a re-enforcement in dysbiosis events that compromise efficiency. The second tool is a therapeutical alternative to antibiotics for pathogen outbreak control, particularly *Tenacibaculum maritimum*. This work was performed in partnership with the sole hatchery (Safiestela S.A.), located in Estela, Portugal.

For the first objective, we investigated the dynamics of the prokaryotic community of a sole hatchery RAS. We collected samples from different matrices and from several compartments of a commercial RAS. These samples were then used for total DNA isolation and V4-V5 region of the 16S rRNA gene was sequenced using Illumina MiSeq® platform and the output analysed with the DADA2 pipeline using the SILVAngs database. We found a highly dynamic prokaryotic community, sensitive to water parameters such

as salinity and pH, that developed different profiles in the different compartments and matrices of the same aquaculture unit. By defining relevant target groups and then performeing cluster identification using network tools we found a positive correlation between the NOB *Nitrospira*, with both the AOB *Nitrosomonas* and the AOA Candidatus *Nitrosopumilus*. We also found sub-communities of *Nitrospira*, with no AOA or AOB correlations, which suggested a separate functional role of complete ammonia oxidation to nitrate. Furthermore, two taxa commonly associated with pathogenic outbreaks, *Tenacibaculum* and *Vibrio*, had a significant positive interaction in one of the systems that may prove relevant in disease preventions and to improve outbreak predictions.

Microbial community management in RAS pose unique challenges, as they must simultaneously deal with the environment (water, tank biofilm and biofilter carriers) but also with the host community. So, we also focused on making an evaluation of the community inherited from the broodstock, and the community acquired at different production stages, particularly regarding key target groups: potentially probiotic and pathogenic. Fish tissue samples were collected from -2 days after hatching (DAH) up to 145 DAH, including the live feed introduced in the first stages (from larvae to weaning). For these different tissue samples, total DNA was isolated, V6-V8 region of the 16S rRNA gene sequenced and the output analysed with the DADA2 pipeline, and taxonomic attribution with SILVAngs. We found that there were few genera inherited, but most become part of the overall core microbiome. The community inherited vertically from the eggs into larvae and juveniles included two genera of potentially probiotic bacteria (*Bacillus* and *Enterococcus*), also detected in later stages, with others being acquired, mainly when feed is introduced. *Tenacibaculum* and *Vibrio* are also already present in the eggs, and *Photobacterium* and *Mycobacterium* seem to be acquired at 49 and 119 DAH respectively. We found significant co-occurrences between the potentially pathogenic genera *Tenacibaculum* and *Photobacterium*.

Using the knowledge acquired on the RAS and host microbiome characterization, we began our work in prokaryotic community modulation to improve water quality and fish welfare through bacterial formulation. To track nitrifying activity, isolates have been obtained from enrichment protocols and, in the future, we will proceed with the genetic identification. To inhibit pathogenic outbreaks, we identified some bacterial strains with anti-*T. maritimum* activity, with three already been classified as *Pseudoalteromonas* sp., a promising finding for controlling outbreaks in RAS since this genus has already been identified as having anti-*Vibrio* activity. With more isolates yet to be tested, these experiments are opening new scientific questions to be answered in future studies.

In conclusion, this thesis demonstrated that longitudinal studies, both in the environment and fish microbiome are of paramount importance in aquaculture, particularly to detect temporal changes both in the routine husbandry environment and as the fish progresses in the production stages. Also, through the combination of culture dependent and independent approaches, this thesis brings us closer to reaching a set of possible solutions to potentiate the nitrifying community of the biofilter for increased water quality, and solutions to optimize fish health by preventing or controlling disease outbreaks in the reared fish.

Keywords: Recirculating Aquaculture Systems (RAS), aquaculture microbiome, biofilter, *Solea senegalensis*, nitrifying microorganisms, *Tenacibaculum maritimum*

Resumo

Os sistemas de recirculação em aquacultura (no inglês, RAS) foram desenvolvidos para reduzir o impacto ambiental da produção de peixes, reduzindo o consumo de água e a carga do efluente. Estes sistemas recorrem à recirculação de água para otimizar a gestão de resíduos e, assim, tornar a produção intensiva de peixes compatível com a sustentabilidade ambiental. Para reutilizar a água, estes sistemas contam com uma série de etapas de tratamento, em que cada etapa reduz a necessidade de troca de água. Este tipo de sistema foi sugerido para a produção de linguado (*Solea senegalensis*) como uma estratégia promissora para a diversificação da aquacultura no sul da Europa. No entanto, esta espécie é altamente vulnerável a surtos de doenças, principalmente causadas por bactérias patogénicas, nomeadamente a *Tenacibaculum maritimum*.

Dentro de um RAS, o principal resíduo produzido é o azoto amoniacal, libertado na água pelos peixes, sendo um metabolito do catabolismo das proteínas. A otimização do processo de nitrificação microbiana dentro do biofiltro é o tratamento mais comum para controlar as concentrações de azoto amoniacal. Como os sistemas RAS dependem dessa comunidade microbiana benéfica, representam desafios únicos na sua gestão, de modo a manter os principais papéis funcionais, tanto no ambiente quanto no peixe.

Esta tese visa aprofundar o conhecimento sobre a composição e interação da comunidade procariótica em RAS, particularmente em duas frentes: primeiro fazendo uma caracterização profunda da composição e da dinâmica da comunidade procariótica ao longo dos diferentes compartimentos RAS. Em segundo lugar, queremos saber como estas comunidades se relacionam com o peixe e como se desenvolveram ao longo de seu ciclo de vida e fases de produção. Depois, usaremos este conhecimento para desenvolver ferramentas de biotecnologia para melhorar a produção de forma sustentável. Estes incluirão duas ferramentas específicas, a primeira, uma formulação bacteriana nitrificante ativa, adaptada a um ambiente RAS marinho, para encurtar os tempos de estabilização do biofiltro ou funcionar como um reforço em eventos de disbiose que comprometam a sua eficiência. A segunda ferramenta é uma alternativa terapêutica aos antibióticos para o controlo de surtos de agentes patogénicos, particularmente a *Tenacibaculum maritimum*. Este trabalho foi realizado em parceria com uma maternidade de linguados (Safiestela S.A.), localizada na Estela, Portugal.

Para o primeiro objetivo, investigamos a dinâmica da comunidade em RAS. Recolhemos amostras de diferentes matrizes e de vários compartimentos. Essas amostras foram

usadas para o isolamento total de DNA e a região V4-V5 do gene 16S rRNA foi sequenciada usando a plataforma Illumina MiSeq® e o resultado analisado com a pipeline DADA2 e a base de dados SILVAngs. Encontrámos uma comunidade procariótica altamente dinâmica, sensível a parâmetros da água como salinidade e pH, que desenvolveu diferentes perfis nos vários compartimentos e matrizes de uma mesma unidade aquícola. Ao definir grupos-alvo relevantes e, em seguida, ao realizar a identificação de clusters com ferramentas de networks, encontrámos uma correlação positiva entre a NOB *Nitrospira*, tanto com a AOB *Nitrosomonas* quanto com a AOA *Candidatus Nitrosopumilus*. Também encontramos sub-comunidades de *Nitrospira*, sem correlações AOA ou AOB, o que sugere um papel funcional separado da oxidação completa do azoto amoniacal a nitrato. Além disso, dois géneros regularmente associados a surtos patogénicos, *Tenacibaculum* e *Vibrio*, tiveram uma interação positiva significativa num dos sistemas que podem ser relevantes na prevenção de doenças e melhoramento das previsões de surtos.

A gestão da comunidade microbiana em RAS apresenta desafios únicos, pois deve lidar simultaneamente com o meio ambiente (água, biofilme dos tanques e matrizes de biofiltro), mas também com a comunidade hospedeira. Assim, também nos focámos em fazer uma avaliação da comunidade herdada dos reprodutores e da comunidade adquirida em diferentes fases de produção, principalmente em relação aos principais grupos-alvo: potencialmente probióticos e patogénicos. Amostras de tecido dos peixes foram recolhidas a partir de -2 dias após a eclosão (no inglês DAH) até 145 DAH, incluindo o alimento vivo introduzido nas primeiras fases (da larva ao desmame). Para essas diferentes amostras de tecido, o DNA total foi isolado, a região V6-V8 do gene 16S rRNA sequenciada e o resultado analisado com a pipeline DADA2, atribuição taxonómica com SILVAngs. Descobrimos que havia poucos géneros herdados, mas a maioria torna-se parte do microbioma "*core*" geral. A comunidade herdada incluiu dois géneros de bactérias potencialmente probióticas (*Bacillus* e *Enterococcus*) com outros que são adquiridos posteriormente, principalmente quando a ração é introduzida. *Tenacibaculum* e *Vibrio* que também já estão presentes nos ovos, e *Photobacterium* e *Mycobacterium* que parecem ser adquiridos aos 49 e 119 DAH respetivamente. Encontrámos coocorrências significativas entre os géneros potencialmente patogénicos *Tenacibaculum* e *Photobacterium*.

Utilizando o conhecimento adquirido sobre o RAS e a caracterização do microbioma do peixe, iniciámos o nosso trabalho de modulação das comunidades procarióticas para melhorar a qualidade da água e o bem-estar dos peixes através de uma formulação

bacteriana. Para detetar a atividade nitrificante, foram obtidos isolados de protocolos de enriquecimento e, futuramente, prosseguiremos com a identificação genética. Para inibir surtos patogénicos, identificámos algumas estirpes bacterianas com atividade anti-*T. maritimum*, três destes já classificados como *Pseudoalteromonas* sp., um resultado promissor para o controlo de surtos em RAS, uma vez que este género já foi identificado como tendo atividade anti-*Vibrio*. Com mais isolados ainda a serem testados, esses ensaios estão a colocar novas questões científicas para serem respondidas em estudos futuros.

Concluindo, esta tese demonstrou que estudos longitudinais, tanto no ambiente como no microbioma dos peixes, são de importância prioritária na aquacultura, principalmente para detetar mudanças temporais tanto no ambiente de produção de rotina como à medida que o peixe avança nas etapas de desenvolvimento. Além disso, através da combinação de abordagens dependentes e independentes da cultura bacteriana, esta tese aproxima-se de um conjunto de possíveis soluções para potencializar a comunidade nitrificante do biofiltro e para aumentar a qualidade da água, assim como soluções para aumentar o bem-estar dos peixes, controlando surtos de doenças nas áreas de cultivo.

Palavras-chave: sistemas de aquacultura de recirculação, microbioma de aquacultura, biofiltro, microorganismos nitrificantes, *Solea senegalensis, Tenacibaculum maritimum*

INDEX

List of Abbreviations

AOA: Ammonia Oxidizing Archaea

AOB: Ammonia Oxidizing Bacteria

CO_2: Carbon Dioxide

DAH: Days After Hatching

DNA: DeoxyriboNucleic Acid

NGS: Next Generation Sequencing

NH_3; Ammonia (also NH_3-N)

NH_4^+: Ammonium

NO_2: Nitrite (also NO_2-N)

NO_3: Nitrate (also NO_3-N)

NOB: Nitrite Oxidizing Bacteria

RAS: Recirculating Aquaculture System

rRNA: Ribosomal RiboNucleic Acid

CHAPTER 1

Introduction

1. Introduction

1.1. Aquaculture

1.1.1 World, European, and Portuguese Aquaculture

Fish is essential to a nutritious diet in many areas across the world. Its production was estimated at 179 million tonnes in 2018, of these, 82 million tonnes were supplied by aquaculture (**Figure 1**). Of the total, 156 million tonnes for human consumption, equivalent to an estimated annual supply of 20.5 kg per capita, rising from 9.0 kg (live weight equivalent) in 1961 to 20.3 kg in 2017. To try to answer to this increase, developments in aquaculture have been made so it can increase its contribute to fish availability, leading to improved nutrition and food security and to minimize the need to source fishing stocks as an answer to the increased consumer demand (Stankus, 2021).

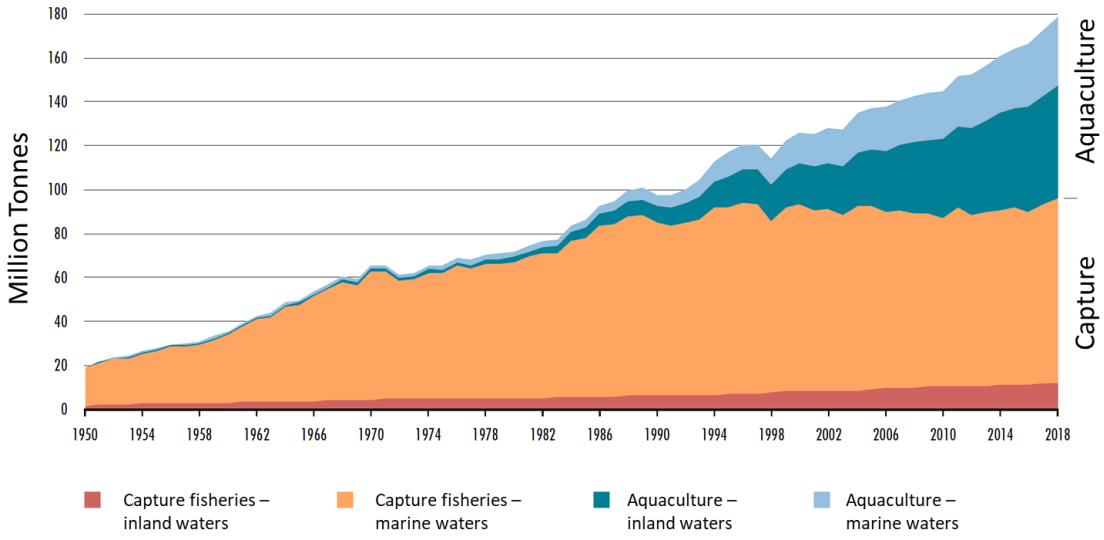

Figure 1: World capture fisheries and aquaculture production, excluding animals, crocodiles, alligators and caimans, seaweeds, and other aquatic plants. **Source:** Food and Agriculture Organization of the United Nations (FAO).

In Portugal, the “Estratégia Nacional para o Mar (2021-2030)” identifies aquaculture as a development priority, particularly its sustainable development. Aquaculture production in 2019 reached 14.291 tonnes (**Figure 2**), with an added value of 118.3 million euros, this represented an increased in 2.6% in quantity, and 18.5% in value, in comparison with the previous year. Sole represents 1.9% of the total production (Guerra et al., 2019).

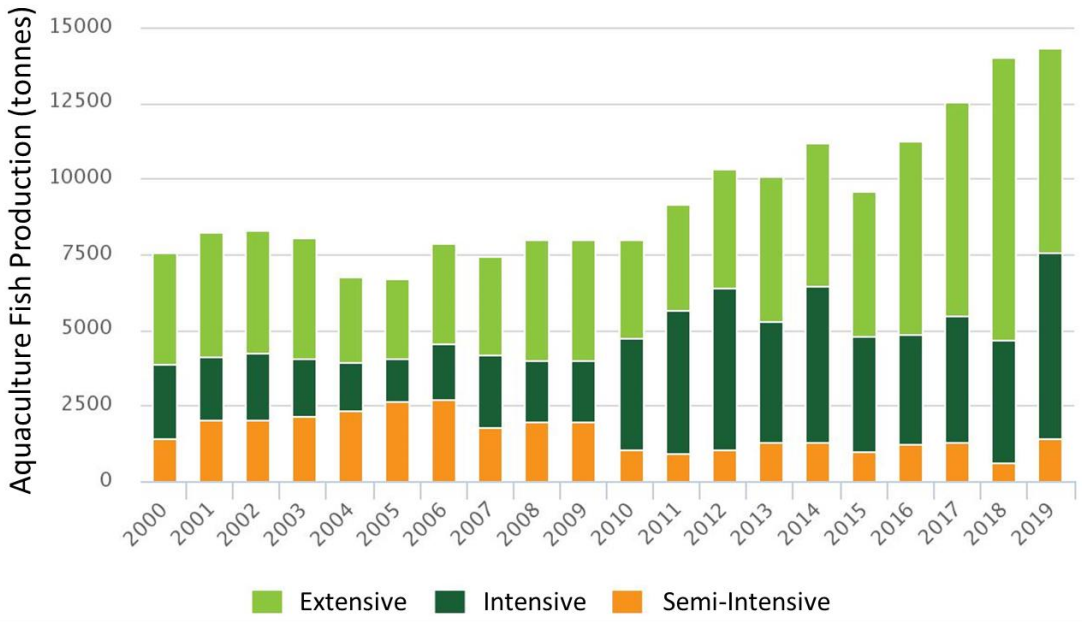

Figure 2: Progression of aquaculture production regimes in Portugal. **Adapted from**: Instituto Nacional de Estatística/ Direção-Geral de Recursos Naturais, Segurança e Serviços Marítimos, 2021

This global increase, also observed nationally, shows the increase in production efficiency, which also translates into improved sustainability, both environmental and economic. Aquaculture technologies are working in reducing their carbon footprint, their use of water and land resources and have improved feed management strategies (Boyd et al., 2020), with sustainable intensive aquaculture contributing to satisfy the global demand for fish.

1.1.2 Sole Aquaculture and Hatcheries

The sole (*Solea senegalensis*) has been identified as a suitable candidate species for aquaculture diversification in the south of Europe. Particularly in Portugal and Spain, where it is a common high-value flatfish commonly reared in extensive aquacultural production, although it poses some challenges in disease mortality, pigmentation abnormalities and malformations (Dinis et al., 1999). With the increase in production, it is important to understand with maximum detail the biology of the species for production improvements. For example, the study and integration of the cytogenetic and physical maps of sole have just recently been published (Merlo et al., 2020).

However, intensive production has been slow to take off, which has been attributed to serious disease problems, high mortality at weaning, variable growth, and poor juvenile quality (Morais et al., 2016). Diseases in this species are mainly caused by pathogenic bacteria: the most notorious being tenacibaculosis (formerly flexibacteriosis, fin rot or black patch necrosis), others include photobacteriosis (formerly pasteurellosis) and vibriosis (Morais et al., 2016). Tenacibaculosis, caused by the bacteria *Tenacibaculum maritimum*, has been described as highly contagious and the cause of significant mortality (Toranzo et al., 2005) with the only treatments available being formalin and antibiotics. Member of the family Flavobacteriaceae, phylum Bacteroidetes (Suzuki et al., 2001), it affects a variety of feral, captive, and cultured fish species worldwide (Pérez-Pascual et al., 2017).

1.1.3 Recirculating Aquaculture Systems

Recirculating aquaculture systems (RASs) are intensive fish production systems, with reduced use of water and land, that allow to produce food with a minimum ecological impact. Because they operate in indoor controlled environment, they are not only minimally affected by climatic factors, but are also water efficient, highly productive, not associated with several environmental impacts (habitat destruction, water pollution and eutrophication and biotic depletion) and adverse ecological effects on biodiversity due to escapes (disease outbreaks, and parasite transmission) (Ahmed & Turchini, 2021). The biggest drawback of these systems is the high energy needed, and because of this, the design of an economic and environmentally sustainable RAS should find a compromise between water use, waste discharge, energy consumption and productivity (Badiola et al., 2018). New developments focus on technical improvements of the recirculation loops and recycling of nutrients for a circular economy with the incorporation of wetlands or algae integrated production (Martins et al., 2010).

Recirculating aquaculture systems (RAS) partially re-use water after a series of treatment steps, with each step reducing the need for water exchange (Rosenthal, 1986). With this, they offer conditions of reduced water consumption, waste management, nutrient recycling, better hygiene and disease management, biological pollution control (no escapees) and are sometimes referred to as ‘urban’ aquaculture, enabling the production of seafood products near markets and reducing carbon dioxide (CO_2) emissions associated with food transport (Martins et al., 2010).

In a general treatment design (**Figure 3**), the water flows from the fish culture tank through systems that remove the dissolved solids (mechanical filter), convert ammonia

to nitrate (biofilter), add oxygen and remove proteins (aeration/skimmer), remove carbon dioxide (degasification) and optionally: the water is disinfected before returning to the culture tank (chemical treatment or ultra-violet light). A control system monitors water quality, and a biosecurity program is also imposed to prevent losses due to disease outbreaks (Ebeling et al., 2002).

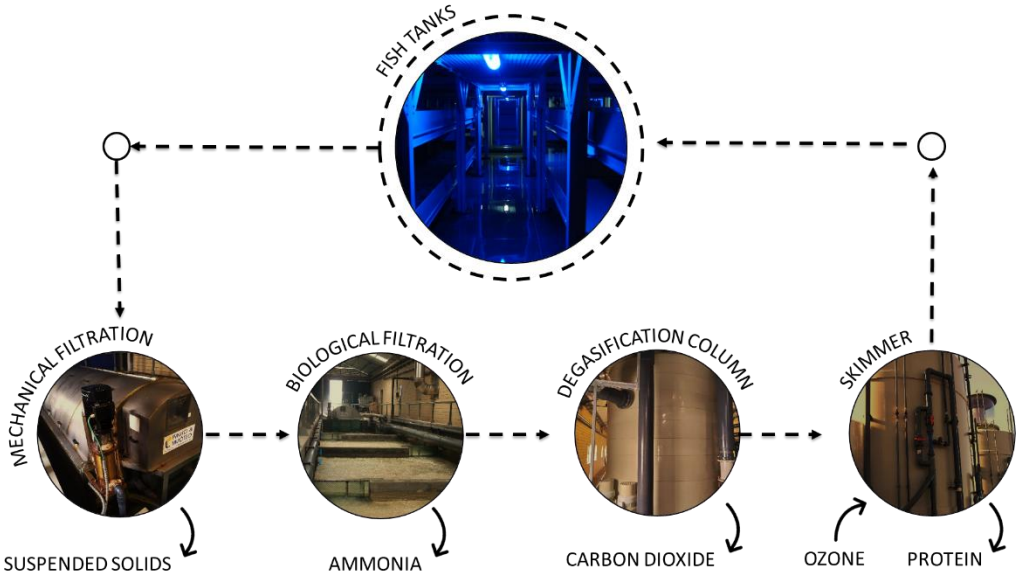

Figure 3: Schematic of a classical recirculating aquaculture system (RAS).

Biological filtration is responsible for the oxidation of the main metabolite released into the water by the fish (ammonia). It occurs inside the biofilter, a unit used to optimize the naturally occurring microbial nitrogen cycle, where ammonia is oxidized to nitrite and subsequently to nitrate (Davidson et al., 2017). Both ammonia and its subsequent component in the nitrification process, nitrite, are toxic to aquatic organisms and cannot accumulate within a RAS environment at the cost of negative impacts in animal health, growth, and survival rates. (Ebeling et al., 2002).

1.1.4 Biofilter

The biofilter (**Figure 4**), responsible for the biological treatment of the water, has been one of the main challenges in RAS optimization: the beneficial bacterial community (or microbiome) is in large part unculturable (Streit & Schmitz, 2004) and the interactions between individuals are complex (Ruan et al., 2015). In this component, the nitrogen cycle pathway responsible for ammonia oxidation (nitrification) is mediated by a microbial community that is considered a vital component of RAS (Ebeling et al., 2002). Equipped

with heavy aeration and plastic media, that provides an increase in superficial area available, it provides an environment where the beneficial microbial community grows with minimal manipulation. However, previous studies (Emparanza, 2009) indicate a sensitivity to variations in the production system, responsible for unstable culture conditions.

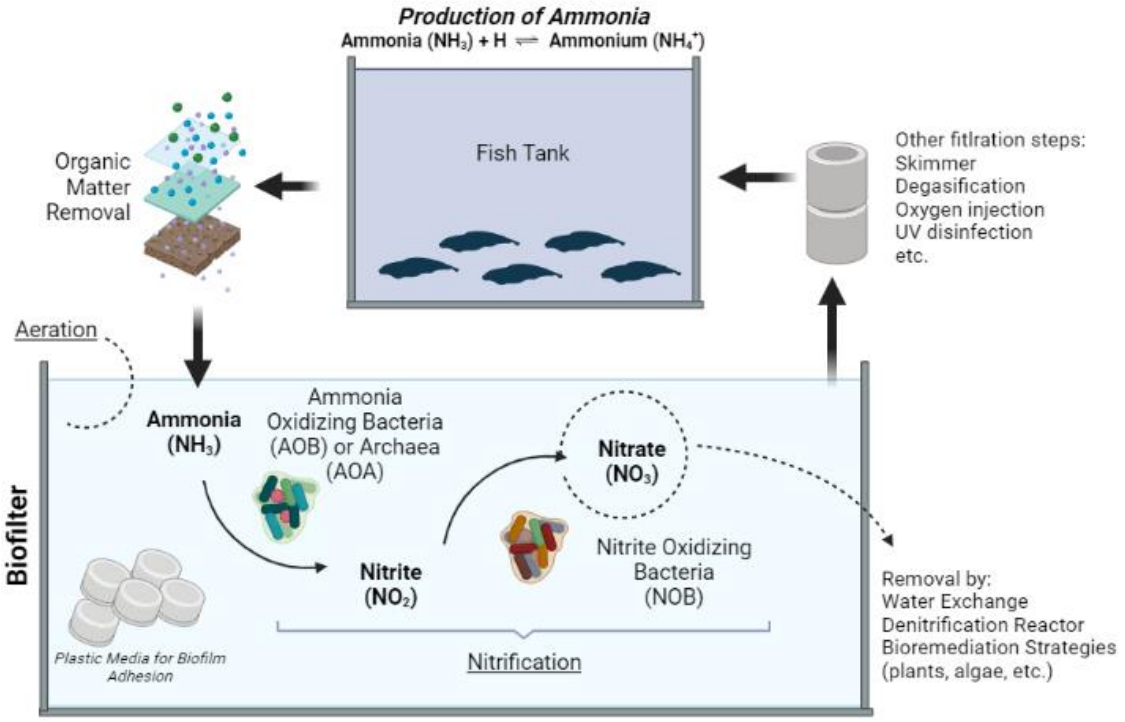

Figure 4: Schematic of the ammonia removal process, in a recirculating aquaculture system (RAS), through nitrification by ammonia oxidizing bacteria and archaea and nitrite oxidizing bacteria in the biofilter, inside a RAS system. Water recirculation flow is represented by the bold arrows. Created with BioRender.

Nitrogen is an essential nutrient for all organisms, and fish expel ammonia (a nitrogenous compound) through gill diffusion, urine and faces causing a nitrogen enrichment in the aquaculture water (Penczak et al., 1982). In the naturally occurring nitrogen cycle there are two phylogenetically distinct groups that preform nitrification, the successive transformation of ammonia to nitrite to nitrate: these are the ammonia oxidizing bacteria (AOB) (Suzuki et al., 1974), that consume the un-ionized ammonia (NH_3) to nitrite (NO_2), and the nitrite oxidizing bacteria (NOB) that consume nitrite to nitrate (NO_3) (Watson & Waterbury, 1971). It is also possible in RAS to couple a denitrification component to achieve total nitrogen removal with a 50% efficiency (Nootong et al., 2013). Additionally, there are ammonia oxidizing archaea (AOA), more efficient at removing very low concentrations of ammonia, even out-competing with AOBs in these conditions (Hatzenpichler, 2012).

Some genera that have already been identified as the key players in nitrification processes are the AOB *Nitrosococcus*, *Nitrospira* and *Nitrosomonas* (Head et al., 1993; Koops et al., 1990; Pommerening-Röser et al., 1996), and the NOB attributed to the *Nitrospirae* and *Nitrospinae* phylum, the latter being the dominant in marine environments (Levipan et al., 2014; Semedo et al., 2021). *Nitrospira*, commonly associated with NOB activity, has also been found to be able to perform complete nitrification (Daims et al., 2015). Archaea have also been identified in the nitrifying process, these include *Thaumarchaeota* (Bartelme et al., 2017) and *Nitrosopumilus* (Brown et al., 2013).

Nitrogen Cycle: "*Although men and other land animals live in an ocean of air that is 79 percent nitrogen, their supply of food is limited more by the availability of fixed nitrogen (...). By "fixed" is meant nitrogen incorporated in a chemical compound that can be utilized by plants and animals. (...) One might think that fixation would merely be termed nitrification, to indicate the addition of nitrogen to some other substance, but nitrification is reserved for a specialized series of reactions in which a few species of microorganisms oxidize the ammonium ion (NH_4^+) to nitrite (NO_2^-) or nitrite to nitrate (NO_3^-). When nitrites or nitrates are reduced to gaseous compounds such as molecular nitrogen (N_2) or nitrous oxide (N_2O) the process is termed denitrification*." (Delwiche, 1970) - first description found in the literature

Within the biofilter community, other beneficial secondary processes are also carried out such as: sulphide-oxidizing activity (Cytryn et al., 2005) and heterotrophic nitrification (Borges et al., 2008). The first removes the toxic form of sulphide from the water, which can originate from bacterial activity in sulfur-rich waters in anaerobic conditions (Bagarinao, 1993; Letelier-Gordo et al., 2020). The latter is the oxidation of organic nitrogen compounds to nitrite and nitrate by chemo-organotrophic microorganisms, with their development dependent on the oxidation of organic substrates (Bock, 1976). This process, however, is slower than autotrophic nitrification, although heterotrophic bacteria out-compete the slow-growing autotrophs (Prosser, 1990). Some heterotrophic nitrifying bacteria have already been identified, such as the genera *Bacillus*, *Paracoccus*, *Pseudomonas*, *Thermus* and *Azoarcus*, but reports about the heterotrophic activity of marine strains are still rare (Preena et al., 2021). Another part of this community, a result of undifferentiated bacterial growth, is the opportunist pathogen bacteria (Blancheton et al., 2013).

The biggest challenge in biofilter management, is that both AOB and NOB are slow growing autotrophic bacteria, which translates in long biofilter activation periods with high variations in water quality (Chen et al., 2006). As a strategy to shorten this period,

commercial formulations of bacterial inocula have been developed, which usually include microbial consortiums containing AOB and NOB, with promising results (Patil et al., 2021). With the success of the biofilter in removing the most toxic forms of nitrogen being highly dependent on the stable interactions between the microbial communities (Preena et al., 2021), microbiome studies in RAS are of paramount importance for future modulation strategies.

Microbiome: "*A characteristic microbial community occupying a reasonably well-defined habitat which has distinct physio-chemical properties. The term thus not only refers to the microorganisms involved but also encompasses their theatre of activity*." (Whipps et al., 1988) – first description found in the literature

1.2. Microbiome

In general, the microbiome (by definition includes both the bacterial communities and their activity) is deeply influenced by the variety of ecological processes that affect community development (Goldford et al., 2018), such as selective pressures and nutrient availability, as is within a microbiome that bacteria communicate and trade metabolites and services (Marx, 2009). Considering the impact of bacterial communities in RAS, studies that evaluate their potential as reservoir for pathogenic bacterial strains (Rud et al., 2017) and influence in the water quality and health of the fish (Blancheton et al., 2013) are of paramount importance. However, in aquaculture environment, and RAS in particular, microbiome studies with cutting edge sequencing technology have not been extensively applied. This approach is an essential tool to monitor the complex network of the microbial roles that maintain a healthy aquaculture system, and to better understand pathogen outbreaks (Martínez-Porchas & Vargas-Albores, 2017). Imbalances in the microbiome (called dysbiosis) have linked a loss of beneficial organisms or loss of diversity with a consequent expansion of pathogenic species (Infante-Villamil et al., 2020).

Dysbiosis: "*Perturbations to the structure of complex commensal communities (referred to as dysbiosis) can lead to deficient education of the host immune system and subsequent development of immune mediated diseases.*" (Petersen & Round, 2014) - first description found in the literature

Next-Generation Sequencing (NGS) technologies have been developed to tackle the complexities of genomes, providing vast quantities of data, but with higher associated error rates and with shorter read lengths than traditional Sanger sequencing platforms,

requiring careful examination of the results (Goodwin et al., 2016). NGS relies on the amplification and sequencing of targeted genetic elements, or amplicon sequencing of taxonomic marker genes such as the 16S rRNA gene in bacteria, that provides a census of a community (Rosen et al., 2015).

16S rRNA gene: "*The (…)16S rRNA gene sequences (…) has been by far the most common housekeeping genetic marker used (…) to study bacterial phylogeny and taxonomy, for a number of reasons (…): (i) its presence in almost all bacteria, often existing as a multigene family, or operons; (ii) the function of the 16S rRNA gene over time has not changed, suggesting that random sequence changes are a more accurate measure of time (evolution); and (iii) the 16S rRNA gene (1,500 bp) is large enough for informatics purposes.*" Janda & Abbott (2007) based on Patel (2001) - first description found in the literature

Inferring biological variation from amplicon sequencing poses a challenge. This is performed either by the construction of Operational Taxonomic Units (OTUs: sequencing reads that are assigned to the closest taxa from a reference database by less than a fixed dissimilarity threshold) (Caporaso et al., 2010) or Amplicon Sequence Variants (ASV: amplicon sequence variants that are inferred without imposing any arbitrary threshold). In order to divide amplicon reads into partitions, reference free, consistent with the error model, this last method follows a workflow of quality filtering, dereplication, sample inference, chimera identification and removal, and only merging paired-end reads at the end (Callahan et al., 2016). One of the workflows available, DADA2, generates ASV levels providing an increased sensitivity in obtaining abundancy tables with a single-base resolution even at high abundance ratios (Prodan et al., 2020).

1.2.1. The Environment Community

In the studies focusing on the environment RAS microbiome (water, biofilter carriers or tank wall biofilm), this is usually dominated by the phyla Proteobacteria and Bacteroidetes (Brailo et al., 2019; Hüpeden et al., 2020; Xue et al., 2017). The traditionally nitrifying phylum Nitrospirae has been reported, but with relatively low abundance in RAS biofilters (Brailo et al., 2019; Huang et al., 2016; Ruan et al., 2015). When studying the environmental microbiome, and shifts in its composition, it is also important to monitor the parameters that may influence its dynamics. The water column parameters, such as pH, turbidity, total ammonia nitrogen, nitrites, and CO_2, have all been linked to variability in the prokaryotic community profile in a RAS (Matos et al., 2011). Differences in microbial diversity between systems and matrices (water and biofilm) were reported by Rud et al. (2017) and biofilms as highly species-rich

ecosystems was described by Hüpeden et al. (2020). Providing a membrane filtration to the water treatment was found to improve water quality with smaller and shorter bacterial blooms, generally lower densities of bacteria, and more diverse microbial communities (Fossmark et al., 2020). The lack of more definition (to confirm these tendencies) and diversity of factors in these interactions, highlights the need for regular monitoring of microbial community dynamics in RAS, as an important tool for aquaculture planning and management.

Within the environmental parameters, salinity is the one whose impact on the microbial community is more extensively studied and already documented to cause shifts in microbial diversity (Rud et al., 2017). Particularly relevant is the effects on nitrifying activity, as it has been described that ammonia is converted to nitrate and nitrite with an efficiency of up to 92% with salinity of 35 (Gao et al., 2020) but inhibition occurs between salinity levels 15 to 25, resulting in nitrite accumulation. In the same study, *Nitrosomonas* and *Nitrospira* were the dominant nitrifying genus, even with the significant impact of salinity in the community. It has also been reported that bacterial communities were stable between 12 to 26 salinity increase (Fossmark et al., 2021). With the same dominating nitrifying groups in the biofilters, Brailo et al. (2019) found that populations were strongly influenced by a few dominant individuals, with a significant population of AOB and NOB in the nitrification biofilter.

One of the strategies found to shorten biofilter activation times is the use of commercial inoculum, but a study found that most bacteria in a commercial inocula (usually freshwater species) was not capable of adapting to a marine environment (Brailo et al., 2019), and another (von Ahnen et al., 2019) described a decrease in nitrate removal efficiency with an increase in salinity. However, Navada et al. (2019) described that nitrifying bacteria were only temporarily inhibited by the salinity increase, regaining activity when adapted to the altered environmental conditions. Recently (Fossmark et al., 2021), it was described that an adaptation with a salinity increase of 1 per day is possible if the biofilter has been previously exposed to osmotic stress. Furthermore, it also found that different compositions of bacterial communities may exhibit the same nitrification activity. Regarding salinity interactions with the nitrifying community, it was observed that the nitrifying genera can have relative low abundancy in the community, and only a few species can perform this role at different salinities. However, these individuals are adapted to the environment and cannot cope with a fast salinity increase. Considering that commercial inocula are usually composed of freshwater species, they should be subjected to an acclimatization of a salinity increase of 1 per day before

application to marine biofilters. However, this is not feasible for marine environments (with salinities around 35) since it would take more than a month to perform.

Studies describing the early colonization of the biofilter are still rare. For this work, we found only very recent one (Drønen et al., 2022), where early colonizing microorganisms steadily dominated the biofilter over time with a slow development from a few to several dominating groups with very high relative abundance. This slow development coincided with a lengthy biofilter maturation with the *Nitrospira* strain in the starter culture not adapting to marine salinity, a problem described earlier as well (Brailo et al., 2019). This study termed environmental microrganisms those that were not associated with the starter culture and only became prominent by the end of the trial.

Aside from the beneficial community, some potential opportunistic bacteria are also found in the RAS environment. At the family level: Flavobacteria, Vibrionaceae (Xue et al., 2017) and Alteromonadaceae (Rud et al., 2017); and at the genus level: *Aliivibrio*, and *Polaribacter* (Rud et al., 2017). Particularly relevant for this work, groups commonly associated with disease outbreaks in sole are the *Tenacibaculum* genus (Gourzioti et al., 2016), *Vibrio* (Austin, 2010) and *Photobacterium* (Toranzo et al., 2005). The first two have also been linked in a pathogenic dysbiosis event (Wynne et al., 2020).

Opportunist pathogen: "*Opportunist organisms have three main characteristics: (1) they are usually organisms of low pathogenicity, (2) they cause serious infections mainly when the host's defence mechanisms against infection are impaired, and (3) they can behave as conventional pathogens but under opportunistic conditions may cause atypical clinical presentations or disseminated lesions.*" (Shanson, 2014) - first description found in the literature

1.2.2 The Host Community

The microbiome, as the previous section indicates, forms specific ecological niches, with dynamic and interactive prokaryotic communities integrated in macro-ecosystems (such as eukaryotic hosts) and becoming crucial for their health (Berg et al., 2020). Due to this dynamic nature, the microbiome evolves throughout the development of its host and early bacterial colonization can be heavily shaped by diet and environmental conditions (Bledsoe et al., 2016; Wilkes Walburn et al., 2019). In fish, live feed may play a key role in early development stages (larvae) as latent vectors (Califano et al., 2017). For example, studies have linked live feed, particularly brineshrimp, as a potential infection vector of the *Vibrio* genus (Montanari et al., 1999; Olafsen, 2001).

RAS can also play a part in shaping the fish microbiome: Steiner et al. (2021) found that the microbiome of Chinook salmon in RAS are different from fish farmed in open pens (although with similar key taxa), finding that the gut microbiome was shaped by the environment (water, feed). Although, another study (Yu et al., 2022) reported that no significant differences were found in the bacterial community of the fish gut between open pens and RAS, attributing the genetic background as the main driver for bacterial diversity. Even though, this same study found that there were more common microorganisms between the gut and the seawater than between the gut and the formulated feed. In eel (Hossain et al., 2021), it was described that it was the host physiology itself combined with rearing conditions that shaped the intestinal microbiome. Another study (Fossmark et al., 2021) found similar findings, with the faecal microbiome of the fish showing a high inter-individual variation, suggesting that stochastic processes (or random variation) affect the community structure as much as environmental conditions.

As a potential illustration of the role played by the microbiome in strengthening the fish health, Dahle et al. (2020) found that disinfection systems in the RAS loop affected the gut microbiome and the gill health negatively. The occurrence of disease in a Yunlong Grouper farm was also linked to changes in the intestinal microbiome structure (Ma et al., 2019) and general low microbial diversity was described (Si et al., 2021) in infected tissues identifying *Edwardsiella* spp. as the main pathogen in diseased turbot. In aquaculture, there is also a beneficial microbial community that includes several species that have been identified as probiotics. These are bacteria linked with several health benefits such as improved productivity, resistance to diseases and immune functions that are mainly present in the gut and water (El-Saadony et al., 2021).

Probiotic: "*Live microorganisms which when administered in adequate amounts confer a health benefit on the host*." (Hill et al., 2014) - first description found in the literature

1.2.3 Studying Community Interactions

As mentioned earlier, ecological processes can affect early microbiome community development with selective pressures and nutrient availability generating cross-feeding networks for primary production and nutrient recycling (Marx, 2009). There are a multitude of interactions within a RAS microbiome, but nitrification is probably the most important. Aside from the environmental parameters that influence the nitrification process, the coordination of the two consecutive steps between the AOB and NOB

metabolisms is essential (Costa et al., 2006). Another important aspect of community interactions is the search currently in place to find substitutes for the use of antibiotics as sustainable preventive measures, mainly using probiotic bacteria, as it has been suggested that their activity against pathogenic bacteria could be a solution for inhibition of the expression of virulence factors in fish pathogens (Bentzon-Tilia et al., 2016).

A useful tool to infer these complex taxonomic architectures are network models (Goldford et al., 2018) that attribute to targeted taxa (nodes or individuals) the connections between them (edges or links) (Newman, 2003). The biotic relationship is inferred using correlation coefficients between taxonomic unit pairs such as Spearman Coefficient (Spearman, 1987; Xia et al., 2011). Previous studies in freshwater RAS have used this method to demonstrate that interactions decrease in complexity during the biofilter start-up, after an initial peak (Jiang et al., 2019). Biofilters have been found to have more nodes but less interactions than biofloc reactors used in wastewater treatment (Deng et al., 2019). These, in turn, have been found to be more complex than water (Wei et al., 2020). Competitive interaction between taxa responsible for ammonia removal and nitrate removal processes has also been described (Deng et al., 2021), an interaction justifiable by different niche requirement. Network studies can also highlight seasonal differences in bacterial community interactions (Lin et al., 2019).

Although not many network analysis have been performed for environmental communities, a bacterial-fungal network analysis indicated that inter-domain associations were important for composite degradation and denitrification (Qi et al., 2020). Prevalence and relative abundance analysis can also be a useful tool to determine microbiome interactions. One study found that the core Chinook salmon microbiome was made up of less than 10 bacterial genera, but most of them were only present in a few or even individual fish, suggesting again a host-specific niche formation (Steiner et al., 2021).

1.3. Main challenges

RAS Microbiome studies can help to find alternative biotechnology tools to answer some of the most pressing issues faced by producers. Particularly, the activation, or re-activation, of the biofilter and pathogen control. Long activation periods are still the limiting factor in starting water recirculation, with the only viable solution being to increase new water flow into the system. Shortening this period would mean less water consumption and subsequent waste. Another challenge is pathogen outbreaks that could lead to high mortality events, and even not considering the great impact on fish welfare,

this still means great economic, and ultimately food, loss. The development of tools to help mitigate these events, while also making fish production more sustainable and environment friendly, are in line with the United Nations Sustainable Development Goal (SDG) 12: “Ensure sustainable consumption and production patterns”.

We believe that by deepening our knowledge in the prokaryotic interactions that rule the production systems, we can begin to pin those that potentiate nitrifying activity and the ones that inhibit pathogen species colonization and development, finding ways to modulate the community. With this knowledge, we can start to sketch two sets of tools to improve production sustainably. The first is an active nitrifying bacterial formulation, adapted not only to a marine environment, but to the complex and stable dynamics of RAS. This tool could shorten biofilter stabilization times or be a re-enforcement in dysbiosis events that compromise efficiency, reducing water consumption and waste. The second tool, or even sets of tools, is a therapeutical alternative to antibiotics in pathogen control, particularly *Tenacibaculum maritimum*. Not only will it contribute to the SDG 12, but it can also impact SDG 14: “Life below water” with the minimization, or even suspension, of antibiotic administration. In fact, new European Union Regulation (EU) 2019/4 on Medicated Feed (European Commission, 2019) will prohibit, by 2022, all forms of routine antibiotic use in farming, including preventative group treatments, before the appearance of clinical signs.

1.4. Aim and outline of the PhD Thesis

This Animal Science Doctoral Program was performed in an industrial setting with a commercial sole (*Solea senegalensis*) hatchery, Safiestela S.A.. To answer some of the challenges pointed by the production, the research in this thesis presents an integrated approach to the characterization of a sole hatchery RAS microbiome, aiming at the modulation of the prokaryotic community to optimize water quality and fish welfare. Both culture independent and culture dependent techniques were used to fulfil the specific objectives:

1. Investigate the dynamics of the prokaryotic community along the different RAS system compartments and its relationship with key physicochemical factors.
2. Performing an in-depth analysis of the interactions that form the network between individuals of these communities.
3. Extensive description of the fish microbiome, with emphasis on its succession throughout the production cycle. This strategy will be the one that best translates into applicable, and valuable, strategies for the company. Through a clear

definition of the inherited and acquired community in the different tissues analysed (gill, intestine, fin, and mucus), we can help shape new husbandry strategies.

4. Finally, the valorisation of the acquired knowledge of the community to develop new biotechnology tools to kick-start marine RAS biofilters and find alternative therapies for pathogen control. These include marine nitrifying bacterial formulations for biofilter activations and potential probiotic bacteria with anti-*T. maritimum* activity.

The thesis starts with a global overview of the state-of-the-art, to review the role of aquaculture both in the world food production, but also in the national scenario in Portugal, and how both have evolved in recent years. Literature review focuses of the *Solea senegalensis* aquaculture, specifically what challenges hinder production development, what tools are already available and how can they be improved.

In **Chapter 2**, we try to answer to the first objective, describing the dynamics of the prokaryotic community of a sole hatchery RAS in relation to the variability of water physical-chemical parameters, a description that is still not widely performed in the literature. We did this by collecting samples from different matrices and compartments of a commercial sole hatchery operating in RAS, which were then used for total DNA isolation and sequencing of the V4-V5 region of the 16S rRNA gene using Illumina MiSeq® platform and the output analysed with the DADA2 pipeline using the SILVAngs database. In **Chapter 3**, we completed the characterization with the in-depth analysis of the network interactions, to answer the second objective. Using the same amplicon library, we began by defining relevant target groups and then performed cluster identification using co-variance and co-occurrence tools.

Regarding the third objective, considering that microbial community management in RAS poses unique challenges, as they must simultaneously deal with the environment but also with the host community, in **Chapter 4** we tried to get some light into the microbiome-host interactions, by describing the fish prokaryotic community, its development throughout the production cycle, making a clear a definition as possible of the community inherited from the broodstock, and the community acquired at different production stages. We targeted specific groups: potential probiotic and pathogenic bacteria and used fish tissue samples from -2 days after hatching (DAH) and up to 145 DAH, including the live feed of the first stages (from larvae to weaning). Total DNA from these samples was isolated from the different tissues, the V6-V8 region of the 16S rRNA

gene was sequenced and, as before, the output was then analysed with the DADA2 pipeline, and taxonomic attribution.

With the background knowledge of these chapters, in **Chapter 5** we began our work in prokaryotic community modulation strategies to improve water quality though bacterial inocula of a nitrifying community and to inhibit pathogenic outbreaks in the system using heterotrophic strains. These studies are not yet completed, but promising preliminary results are discussed.

We conclude with a summary of the main findings, outline the main take home messages and present possible outlines for future studies to continue this investigation in the Final Remarks and Future Perspectives chapter.

1.5 References

Ahmed, N., Turchini, G.M., 2021. Recirculating aquaculture systems (RAS): Environmental solution and climate change adaptation. Journal of Cleaner production. 297, 126604.

Austin, B., 2010. *Vibrios* as causal agents of zoonoses. Veterinary microbiology. 140, 310-317.

Bartelme, R. P., McLellan, S. L., & Newton, R. J., 2017. Freshwater recirculating aquaculture system operations drive biofilter bacterial community shifts around a stable nitrifying consortium of ammonia-oxidizing archaea and comammox Nitrospira. Frontiers in microbiology, 8, 101.

Badiola, M., Basurko, O., Piedrahita, R., Hundley, P., Mendiola, D., 2018. Energy use in recirculating aquaculture systems (RAS): a review. Aquacultural engineering. 81, 57-70.

Bagarinao, T.U., 1993. Sulfide as a toxicant in aquatic habitats. SEAFDEC Asian Aquaculture. 15, 2-4.

Bentzon-Tilia, M., Sonnenschein, E.C., Gram, L., 2016. Monitoring and managing microbes in aquaculture–Towards a sustainable industry. Microbial biotechnology. 9, 576-584.

Berg, G., Rybakova, D., Fischer, D., Cernava, T., Vergès, M.-C.C., Charles, T., Chen, X., Cocolin, L., Eversole, K., Corral, G.H., 2020. Microbiome definition re-visited: old concepts and new challenges. Microbiome. 8, 1-22.

Blancheton, J.P., Attramadal, K.J.K., Michaud, L., d'Orbcastel, E.R., Vadstein, O., 2013. Insight into bacterial population in aquaculture systems and its implication. Aquacultural engineering. 53, 30-39.

Bledsoe, J.W., Peterson, B.C., Swanson, K.S., Small, B.C., 2016. Ontogenetic characterization of the intestinal microbiota of channel catfish through 16S rRNA gene sequencing reveals insights on temporal shifts and the influence of environmental microbes. PloS one. 11, e0166379.

Bock, E., 1976. Growth of *Nitrobacter* in the presence of organic matter. II. Chemoorganotrophic growth of *Nitrobacter agilis*. Archives of Microbiology. 108, 305-312.

Borges, M.-T., Sousa, A., De Marco, P., Matos, A., Hönigová, P., Castro, P.M., 2008. Aerobic and anoxic growth and nitrate removal capacity of a marine denitrifying bacterium isolated from a recirculation aquaculture system. Microbial ecology. 55, 107-118.

Boyd, C.E., D'Abramo, L.R., Glencross, B.D., Huyben, D.C., Juarez, L.M., Lockwood, G.S., McNevin, A.A., Tacon, A.G., Teletchea, F., Tomasso Jr, J.R., 2020. Achieving sustainable aquaculture: Historical and current perspectives and future needs and challenges. Journal of the World Aquaculture Society. 51, 578-633.

Brailo, M., Schreier, H.J., McDonald, R., Maršić-Lučić, J., Gavrilović, A., Pećarević, M., Jug-Dujaković, J., 2019. Bacterial community analysis of marine recirculating aquaculture system bioreactors for complete nitrogen removal established from a commercial inoculum. Aquaculture. 503, 198-206.

Brown, M. N., Briones, A., Diana, J., & Raskin, L., 2013. Ammonia-oxidizing archaea and nitrite-oxidizing nitrospiras in the biofilter of a shrimp recirculating aquaculture system. FEMS microbiology ecology, 83(1), 17-25.

Califano, G., Castanho, S., Soares, F., Ribeiro, L., Cox, C.J., Mata, L., Costa, R., 2017. Molecular taxonomic profiling of bacterial communities in a gilthead seabream (*Sparus aurata*) hatchery. Frontiers in microbiology. 8, 204.

Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A., Holmes, S.P., 2016. DADA2: High-resolution sample inference from Illumina amplicon data. Nature methods. 13, 581-583.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Peña, A.G., Goodrich, J.K., Gordon, J.I., 2010. QIIME allows analysis of high-throughput community sequencing data. Nature methods. 7, 335-336.

Chen, S., Ling, J., Blancheton, J.-P., 2006. Nitrification kinetics of biofilm as affected by water quality factors. Aquacultural engineering. 34, 179-197.

Commission, E., 2019. Regulation (EU) 2019/4 of the European Parliament and of the Council of 11 December 2018 on the manufacture, placing on the market and use of medicated feed, amending regulation (EC) NO 183/2005 of the European Parliament and of the Council and repealing Council Directive 90/167/EEC (text with EEA relevance). Official Journal of the Eurean Union. 4.

Costa, E., Pérez, J., Kreft, J.-U., 2006. Why is metabolic labour divided in nitrification? Trends in microbiology. 14, 213-219.

Cytryn, E., Minz, D., Gelfand, I., Neori, A., Gieseke, A., de Beer, D., van Rijn, J., 2005. Sulfide-oxidizing activity and bacterial community structure in a fluidized bed reactor from a zero-discharge mariculture system. Environmental science & technology. 39, 1802-1810.

Dahle, S.W., Bakke, I., Birkeland, M., Nordøy, K., Dalum, A.S., Attramadal, K.J., 2020. Production of lumpfish (*Cyclopterus lumpus* L.) in RAS with distinct water treatments: Effects on fish survival, growth, gill health and microbial communities in rearing water and biofilm. Aquaculture. 522, 735097.

Daims, H., Lebedeva, E.V., Pjevac, P., Han, P., Herbold, C., Albertsen, M., Jehmlich, N., Palatinszky, M., Vierheilig, J., Bulaev, A., 2015. Complete nitrification by *Nitrospira* bacteria. Nature. 528, 504-509.

Davidson, J., Good, C., Williams, C., Summerfelt, S.T., 2017. Evaluating the chronic effects of nitrate on the health and performance of post-smolt Atlantic salmon *Salmo salar* in freshwater recirculation aquaculture systems. Aquacultural engineering. 79, 1-8.

Delwiche, C.C., 1970. The nitrogen cycle. Scientific American. 223, 136-147.

Deng, Y., Ruan, Y., Ma, B., Timmons, M.B., Lu, H., Xu, X., Zhao, H., Yin, X., 2019. Multi-omics analysis reveals niche and fitness differences in typical denitrification microbial aggregations. Environ Int. 132, 105085.

Deng, Y., Ruan, Y., Taherzadeh, M.J., Chen, J., Qi, W., Kong, D., Ma, B., Xu, X., Lu, H., 2021. Carbon availability shifts the nitrogen removal pathway and microbial community in biofilm airlift reactor. Bioresour Technol. 323, 124568.

Dinis, M.T., Ribeiro, L., Soares, F., Sarasquete, C., 1999. A review on the cultivation potential of *Solea senegalensis* in Spain and in Portugal. Aquaculture. 176, 27-38.

Drønen, K., Roalkvam, I., Dahle, H., Olsen, A., Nilsen, H., Wergeland, H., 2022. Microbiome dataset from a marine recirculating aquaculture system (RAS) for salmon post-smolt production in Norway. Data in brief. 40, 107767.

Ebeling, J.M., Timmons, M.B., Wheaton, J.M., Summerfelt, S.T., Vinci, B.J., 2002. Recirculating aquaculture, 4th ed. Ithaca Publishing Company.

Emparanza, E.J., 2009. Problems affecting nitrification in commercial RAS with fixed-bed biofilters for salmonids in Chile. Aquacultural engineering. 41, 91-96.

Fossmark, R.O., Attramadal, K.J., Nordøy, K., Østerhus, S.W., Vadstein, O., 2021. A comparison of two seawater adaptation strategies for Atlantic salmon post-smolt (*Salmo salar*) grown in recirculating aquaculture systems (RAS): Nitrification, water and gut microbiota, and performance of fish. Aquaculture. 532, 735973.

Fossmark, R.O., Vadstein, O., Rosten, T.W., Bakke, I., Košeto, D., Bugten, A.V., Helberg, G.A., Nesje, J., Jørgensen, N.O., Raspati, G., 2020. Effects of reduced organic matter loading through membrane filtration on the microbial community dynamics in recirculating aquaculture systems (RAS) with Atlantic salmon parr (*Salmo salar*). Aquaculture. 524, 735268.

Gao, Y., Wang, X., Li, J., Lee, C.T., Ong, P.Y., Zhang, Z., Li, C., 2020. Effect of aquaculture salinity on nitrification and microbial community in moving bed bioreactors with immobilized microbial granules. Bioresource technology. 297, 122427.

Goldford, J.E., Lu, N., Bajić, D., Estrela, S., Tikhonov, M., Sanchez-Gorostiaga, A., Segrè, D., Mehta, P., Sanchez, A., 2018. Emergent simplicity in microbial community assembly. Science. 361, 469-474.

Goodwin, S., McPherson, J.D., McCombie, W.R., 2016. Coming of age: ten years of next-generation sequencing technologies. Nature Reviews Genetics. 17, 333-351.

Gourzioti, E., Kolygas, M., Athanassopoulou, F., Babili, V., 2016. Tenacibaculosis in aquaculture farmed marine fish. Journal of the Hellenic Veterinary Medical Society. 67, 21-32.

Guerra, M., Ribeiro, R., Rodrigues, S., 2019. Relatório do estado do ambiente. Agência Portuguesa do Ambiente.

Hatzenpichler, R., 2012. Diversity, physiology, and niche differentiation of ammonia-oxidizing archaea. Applied and environmental microbiology. 78, 7501-7510.

Head, I.M., Hiorns, W.D., Embley, T.M., McCarthy, A.J., Saunders, J.R., 1993. The phylogeny of autotrophic ammonia-oxidizing bacteria as determined by analysis of 16S ribosomal RNA gene sequences. Microbiology. 139, 1147-1153.

Hill, C., Guarner, F., Reid, G., Gibson, G.R., Merenstein, D.J., Pot, B., Morelli, L., Canani, R.B., Flint, H.J., Salminen, S., 2014. Expert consensus document: The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nature reviews Gastroenterology & hepatology.

Hossain, M., Dai, J., Qiu, D., 2021. European eel (*Anguilla anguilla*) GI tract conserves a unique metagenomics profile in the recirculation aquaculture system (RAS). Aquaculture International. 29, 1529-1544.

Huang, Z., Wan, R., Song, X., Liu, Y., Hallerman, E., Dong, D., Zhai, J., Zhang, H., Sun, L., 2016. Metagenomic analysis shows diverse, distinct bacterial communities in biofilters among different marine recirculating aquaculture systems. Aquaculture international. 24, 1393-1408.

Hüpeden, J., Wemheuer, B., Indenbirken, D., Schulz, C., Spieck, E., 2020. Taxonomic and functional profiling of nitrifying biofilms in freshwater, brackish and marine RAS biofilters. Aquacultural Engineering. 90, 102094.

Infante-Villamil, S., Huerlimann, R., Jerry, D.R., 2020. Microbiome diversity and dysbiosis in aquaculture. Reviews in Aquaculture.

Janda, J.M., Abbott, S.L., 2007. 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, and pitfalls. Journal of clinical microbiology. 45, 2761-2764.

Jiang, W., Tian, X., Li, L., Dong, S., Zhao, K., Li, H., Cai, Y., 2019. Temporal bacterial community succession during the start-up process of biofilters in a cold-freshwater recirculating aquaculture system. Bioresour Technol. 287, 121441.

Koops, H.-P., Böttcher, B., Möller, U., Pommerening-Röser, A., Stehr, G., 1990. Description of a new species of *Nitrosococcus*. Archives of Microbiology. 154, 244-248.

Letelier-Gordo, C.O., Aalto, S.L., Suurnäkki, S., Pedersen, P.B., 2020. Increased sulfate availability in saline water promotes hydrogen sulfide production in fish organic waste. Aquacultural Engineering. 89.

Levipan, H.A., Molina, V., Fernandez, C., 2014. *Nitrospina*-like bacteria are the main drivers of nitrite oxidation in the seasonal upwelling area of the E astern South Pacific (Central Chile~ 36° S). Environmental Microbiology Reports. 6, 565-573.

Lin, Y., Zhao, D., Zeng, J., Cao, X., Jiao, C., 2019. Network analysis reveals seasonal patterns of bacterial community networks in lake taihu under aquaculture conditions. Water. 11, 1868.

Ma, C., Chen, C., Jia, L., He, X., Zhang, B., 2019. Comparison of the intestinal microbiota composition and function in healthy and diseased Yunlong Grouper. AMB Express. 9, 1-11.

Martínez-Porchas, M., Vargas-Albores, F., 2017. Microbial metagenomics in aquaculture: a potential tool for a deeper insight into the activity. Reviews in Aquaculture. 9, 42-56.

Martins, C.I.M., Eding, E.H., Verdegem, M.C.J., Heinsbroek, L.T.N., Schneider, O., Blancheton, J.-P., d'Orbcastel, E.R., Verreth, J.A.J., 2010. New developments in recirculating aquaculture systems in Europe: A perspective on environmental sustainability. Aquacultural engineering. 43, 83-93.

Marx, C.J., 2009. Getting in touch with your friends. Science. 324, 1150-1151.

Matos, A., Borges, M.T., Peixe, C., Henriques, I., Pereira, C.M., Castro, P.M., 2011. A molecular and multivariate approach to the microbial community of a commercial shallow raceway marine recirculation system operating with a moving bed biofilter. Aquaculture Research. 42, 1308-1322.

Merlo, M.A., Portela-Bens, S., Rodríguez, M.E., García-Angulo, A., Cross, I., Arias-Pérez, A., García, E., Rebordinos, L., 2020. A comprehensive integrated genetic map of the complete karyotype of *Solea senegalensis* (Kaup 1858). Genes. 12, 49.

Montanari, M.P., Pruzzo, C., Pane, L., Colwell, R.R., 1999. *Vibrios* associated with plankton in a coastal zone of the Adriatic Sea (Italy). FEMS Microbiology Ecology. 29, 241-247.

Morais, S., Aragão, C., Cabrita, E., Conceição, L.E., Constenla, M., Costas, B., Dias, J., Duncan, N., Engrola, S., Estevez, A., 2016. New developments and biological insights into the farming of *Solea senegalensis* reinforcing its aquaculture potential. Reviews in Aquaculture. 8, 227-263.

Navada, S., Vadstein, O., Tveten, A.-K., Verstege, G.C., Terjesen, B.F., Mota, V.C., Venkataraman, V., Gaumet, F., Mikkelsen, Ø., Kamstra, A., 2019. Influence of rate of salinity increase on nitrifying biofilms. Journal of Cleaner Production. 238, 117835.

Newman, M.E., 2003. The structure and function of complex networks. SIAM review. 45, 167-256.

Nootong, K., Nurit, S., Powtongsook, S., 2013. Control of inorganic nitrogen and suspended solids concentrations in a land-based recirculating aquaculture system. Engineering Journal. 17, 49-60.

Olafsen, J.A., 2001. Interactions between fish larvae and bacteria in marine aquaculture. Aquaculture. 200, 223-247.

Patel, J.B., 2001. 16S rRNA gene sequencing for bacterial pathogen identification in the clinical laboratory. Molecular diagnosis. 6, 313-321.

Patil, P.K., Antony, L., Avunje, S., Viswanathan, B., Lalitha, N., Jangam, A.K., Kumar, D., Solanki, H.G., Reddy, M.A., Alavandi, S.V., 2021. Bioaugmentation with nitrifying and denitrifying microbial consortia for mitigation of nitrogenous metabolites in shrimp ponds. Aquaculture. 541, 736819.

Penczak, T., Galicka, W., Molinski, M., Kusto, E., Zalewski, M., 1982. The enrichment of a mesotrophic lake by carbon, phosphorus and nitrogen from the cage aquaculture of rainbow trout, *Salmo gairdneri.* Journal of Applied Ecology, 371-393.

Petersen, C., Round, J.L., 2014. Defining dysbiosis and its influence on host immunity and disease. Cellular microbiology. 16, 1024-1033.

Pommerening-Röser, A., Rath, G., Koops, H.-P., 1996. Phylogenetic diversity within the genus *Nitrosomonas*. Systematic and Applied Microbiology. 19, 344-351.

Preena, P.G., Rejish Kumar, V.J., Singh, I.S.B., 2021. Nitrification and denitrification in recirculating aquaculture systems: the processes and players. Reviews in Aquaculture. 13, 2053-2075.

Prodan, A., Tremaroli, V., Brolin, H., Zwinderman, A.H., Nieuwdorp, M., Levin, E., 2020. Comparing bioinformatic pipelines for microbial 16S rRNA amplicon sequencing. Plos one. 15, e0227434.

Prosser, J.I., 1990. Autotrophic nitrification in bacteria. Advances in microbial physiology. 30, 125-181.

Qi, W., Taherzadeh, M.J., Ruan, Y., Deng, Y., Chen, J.-S., Lu, H.-F., Xu, X.-Y., 2020. Denitrification performance and microbial communities of solid-phase denitrifying reactors using poly (butylene succinate)/bamboo powder composite. Bioresource technology. 305, 123033.

Rosen, M.J., Davison, M., Bhaya, D., Fisher, D.S., 2015. Fine-scale diversity and extensive recombination in a quasisexual bacterial population occupying a broad niche. Science. 348, 1019-1023.

Rosenthal, H., 1986. Flow-through and Recirculation Systems: Report of the Working Group on Terminology, Format, and Units of Measurement. Food & Agriculture Org.

Ruan, Y.-J., Guo, X.-S., Ye, Z.-Y., Liu, Y., Zhu, S.-M., 2015. Bacterial community analysis of different sections of a biofilter in a full-scale marine recirculating aquaculture system. North American Journal of Aquaculture. 77, 318-326.

Rud, I., Kolarevic, J., Holan, A.B., Berget, I., Calabrese, S., Terjesen, B.F., 2017. Deep-sequencing of the bacterial microbiota in commercial-scale recirculating and semi-closed aquaculture systems for Atlantic salmon post-smolt production. Aquacultural Engineering. 78, 50-62.

Semedo, M., Lopes, E., Baptista, M.S., Oller-Ruiz, A., Gilabert, J., Tomasino, M.P., Magalhães, C., 2021. Depth Profile of Nitrifying Archaeal and Bacterial Communities in the Remote Oligotrophic Waters of the North Pacific. Frontiers in microbiology. 12, 319.

Shanson, D.C., 2014. Microbiology in clinical practice. Butterworth-Heinemann.

Si, Y., Wen, J., Xu, Y., Roengjit, P., Wang, H., 2021. Rapid pathogen discovery in diseased turbot (*Scophthalmus maximus*) using 16S rRNA high throughput sequencing. Aquaculture Reports. 21, 100835.

Spearman, C., 1987. The proof and measurement of association between two things. The American journal of psychology. 100, 441-471.

Stankus, A., 2021. State of world aquaculture 2020 and regional reviews: FAO webinar series. FAO Aquaculture Newsletter, 17-18.

Steiner, K., Heasman, K., Laroche, O., Pochon, X., Preece, M., Bowman, J.P., Walker, S.P., Symonds, J.E., 2021. The microbiome of Chinook salmon (*Oncorhynchus tshawytscha*) in a recirculation aquaculture system. Aquaculture. 534, 736227.

Streit, W.R., Schmitz, R.A., 2004. Metagenomics–the key to the uncultured microbes. Current opinion in microbiology. 7, 492-498.

Suzuki, I., Dular, U., Kwok, S., 1974. Ammonia or ammonium ion as substrate for oxidation by *Nitrosomonas europaea* cells and extracts. Journal of Bacteriology. 120, 556-558.

Toranzo, A.E., Magariños, B., Romalde, J.L., 2005. A review of the main bacterial fish diseases in mariculture systems. Aquaculture. 246, 37-61.

von Ahnen, M., Aalto, S.L., Suurnäkki, S., Tiirola, M., Pedersen, P.B., 2019. Salinity affects nitrate removal and microbial composition of denitrifying woodchip bioreactors treating recirculating aquaculture system effluents. Aquaculture. 504, 182-189.

Watson, S.W., Waterbury, J.B., 1971. Characteristics of two marine nitrite oxidizing bacteria, *Nitrospina gracilis* nov. gen. nov. sp. and *Nitrococcus mobilis* nov. gen. nov. sp. Archiv für Mikrobiologie. 77, 203-230.

Wei, G., Shan, D., Li, G., Li, X., Tian, R., He, J., Shao, Z., 2020. Prokaryotic communities vary with floc size in a biofloc-technology based aquaculture system. Aquaculture. 529.

Whipps, J., Lewis, K., Cooke, R., 1988. Mycoparasitism and plant disease control. Fungi in biological control systems, 161-187.

Wilkes Walburn, J., Wemheuer, B., Thomas, T., Copeland, E., O'Connor, W., Booth, M., Fielder, S., Egan, S., 2019. Diet and diet-associated bacteria shape early microbiome

development in Yellowtail Kingfish (*Seriola lalandi*). Microbial biotechnology. 12, 275-288.

Wynne, J.W., Thakur, K.K., Slinger, J., Samsing, F., Milligan, B., Powell, J.F.F., McKinnon, A., Nekouei, O., New, D., Richmond, Z., Gardner, I., Siah, A., 2020. Microbiome Profiling Reveals a Microbial Dysbiosis During a Natural Outbreak of Tenacibaculosis (*Yellow Mouth*) in Atlantic Salmon. Front Microbiol. 11, 586387.

Xia, L.C., Steele, J.A., Cram, J.A., Cardon, Z.G., Simmons, S.L., Vallino, J.J., Fuhrman, J.A., Sun, F., 2011. Extended local similarity analysis (eLSA) of microbial community and other time series data with replicates, BMC systems biology. Springer, pp. 1-12.

Xue, S., Xu, W., Wei, J., Sun, J., 2017. Impact of environmental bacterial communities on fish health in marine recirculating aquaculture systems. Veterinary microbiology. 203, 34-39.

Yu, C., Zhang, C., Salisu, A., Wang, Y., 2022. Comparison of the Intestinal Bacteria Between Black Seabass Centropristis striata Reared in Recirculating Aquaculture System and Net Pen. Current Microbiology. 79, 1-10.

CHAPTER 2

[Published]

Microbial community dynamics in a hatchery recirculating aquaculture system (RAS) of sole (*Solea senegalensis*)

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2. Microbial community dynamics in a hatchery recirculating aquaculture system (RAS) of sole (*Solea senegalensis*)

2.1 Abstract:

Recirculating aquaculture systems (RAS) have been developed to reduce the aquaculture environmental impact and control rearing conditions. They allow water reuse by managing waste and nutrient recycling, consequently making intensive fish production compatible with environmental sustainability. A key aspect of these systems is the water treatment performed by the beneficial bacterial community of the biofilter. In this study we aim to investigate the dynamics of the prokaryotic community of a sole (*Solea senegalensis*) hatchery RAS in relation to the variability of water physical-chemical parameters. Samples from different matrices (water, biofilter and tank wall biofilm) were collected from several compartments of a commercial RAS. Total DNA was isolated from the different matrices and the V4-V5 region of the 16S rRNA gene was sequenced using Illumina MiSeq® platform and the output analysed in the DADA2 pipeline using the SILVAngs database. Overall, the prokaryotic communities were dominated by Proteobacteria (12-89%) and Bacteroidetes (8-86%) and a total of 58 genera contributed with more than 3% of the relative abundance across the different samples. The most abundant genera were *Tenacibaculum*, *Sulfitobacter*, *Leucothrix*, *Novosphingobium*, *Marinicella*, *Pseudoalteromonas*, *Polaribacter_2*, *Schleiferia* and *Algibacter*. The prokaryotic community shifts were found to be modelled by water parameters such as salinity and pH. This study provides new knowledge on the prokaryotic community composition in different units of recirculating systems, essential for the understanding of the microbial community balance in aquaculture and represents an important tool for overall aquaculture system management.

2.2. Introduction

Recirculating aquaculture systems (RAS) are a promising technology of fish production. They reduce aquaculture environmental impact by saving water usage via optimizing waste management and nutrient recycling (Piedrahita, 2003). Thus, they make intensive fish production compatible with environmental sustainability. Water recirculation relies on the stability of physical, chemical, and biological processes to diminish the environmental impact with an optimized effluent treatment (Martins et al., 2010). However, there is still a knowledge gap in the understanding of how the beneficial

bacterial community treats RAS wastewater, in part because most of the bacteria in a given environment are unculturable (Streit & Schmitz, 2004), but also because of the complexity of the interactions occurring in a dynamic setting (Ruan et al., 2015).

Inside an aquaculture unit, a main waste produced is ammonia. It is released into the water by the fish as a metabolite from protein catabolism, and so, ammonia concentrations must be closely monitored and kept at trace concentrations (below 0.0125 mg/L for NH3-N and 1 mg/L for total ammonia nitrogen), since it is toxic to fish (Ebeling et al., 2002, 2018). Ammonia toxicity increases with pH values because it increases its most toxic form NH_3-N (Thurston et al., 1981). The most commonly used treatment is maximizing the microbial nitrification process within the biofilter, where ammonia is oxidized to nitrite and subsequently nitrite is oxidized to nitrate, a metabolite with a reduced toxicity, with safe levels until concentrations of 100 mg/L (Davidson et al., 2017; Pereira et al., 2017; Pierce et al., 1993). These processes, which are part of the naturally occurring nitrogen cycle, are performed by a microbial community, incubated in the biofilm of a biofilter, which is considered a vital component of an aquaculture wastewater treatment (Ebeling et al., 2002, 2018). This compartment is usually subjected to aerification and equipped with plastic biofilter media (which provides a substantial increase in superficial area available), where beneficial microbial communities grow with minimal manipulation. Biofilters are not easily controlled considering the complex interactions of the microbial community within itself and with the environment (Schreier et al., 2010). Previous studies (Emparanza, 2009; Suhr & Pedersen, 2010) indicate that RAS biofilters, in particular the nitrification process, are sensitive to large variations in daily feeding, fish density, oxygen concentration, and variable daily water exchanges, since they cause unstable culture conditions.

Some of the main genera that have been identified as performing the nitrification process in biofilters, are *Nitrosomonas* (Foesel et al., 2008; Paungfoo et al., 2007) and *Nitrosococcus* (Foesel et al., 2008) for ammonia oxidation, *Nitrospira* (Foesel et al., 2008; Tal et al., 2003b) for nitrite oxidation. Nevertheless, these biofilters perform other beneficial secondary processes to maintain the water quality of RAS such as autotrophic, sulphide-dependent, denitrification by the genera *Thiomicrosporia*, *Thiothrix* and *Rhodobacter* (Cytryn et al., 2005a; Cytryn et al., 2005b) and heterotrophic denitrification by *Pseudomonas* (Borges et al., 2008).

A healthy and stable microbiological community is essential in a RAS water treatment, and consequentially, to fish welfare. As a result of insufficient knowledge, the biofilter is a sector for optimal, but undifferentiated, bacterial growth. Therefore, without selection

of the bacteria incubated, there is a risk that disruptions in the system may cause pathogenic outbreaks by opportunist bacteria (Blancheton et al., 2013).

Nowadays, Next Generation Sequencing (NGS) technologies give the potential for a deeper understanding of the microbial diversity and interactions in complex aquaculture systems (Martínez-Porchas & Vargas-Albores, 2017). Previous works that implemented this method for fish water samples include pyrosequencing to characterize bacterioplankton in a semi-intensive system of Seabream (*Sparus aurata*) (Duarte et al., 2019b) and in Sole (*Solea senegalensis*) (Duarte et al., 2019a). This technique has also been used to study the prokaryotic communities in a Tongue sole (*Cynoglossus semilaevis*) RAS (Ruan et al., 2015). Aquaculture microbial community studies in the past have also been done using traditional microbiological techniques (Michaud et al., 2009) or molecular fingerprinting analysis like the characterization of the microbial communities in moving bed bioreactors using denaturing gradient gel electrophoresis (DGGE) in a Seabream (*Sparus aurata*) RAS system (Tal et al., 2003a) and Turbot (Scophthalmus maximus) RAS (Matos et al., 2011). Traditionally NGS studies process raw sequencing data into biologically meaningful information in the form of OTU-level (Operational Taxonomical Units) abundancy tables, but recently workflows at the Amplicon Sequence Variants (ASV) level, were found to offer superior resolution, better specificity and allowed for easier inter-study integration (Prodan et al., 2020). So far, only one paper has used this workflow for aquaculture samples (Wynne et al., 2020).

Although NGS provides the opportunity to examine the microbial community at a high taxonomy sensitivity level, there are still few applications in microbial aquaculture studies, particularly in environmental samples (water and biofilm). Analysis of the biofilter microbial communities by this technique have been done in cultures of flow-through fish farm for Lumpfish (*Cyclopterus lumpus* L.) (Roalkvam et al., 2019), Atlantic salmon (*Salmo salar*), Pacific white shrimp (*Litopenaeus vannamei*), half-smooth Tongue sole (*Cynoglossus semilaevis*) and Turbot (*Scophthalmus maximus*) (Huang et al., 2016). Rud et al. (2017) also characterized water and biofilter microbial communities in an Atlantic salmon semi-closed containment system alongside a RAS, and Bakke et al. (2017) described the microbial community dynamics in three large-scale RAS, for the same species. Aquaculture microbial studies using cutting edge sequencing technology have not been extensively applied, granting they will provide an essential tool to understand this fast-growing industry. This is an ideal approach to monitor the complex network of the microbial roles in maintaining a healthy aquaculture system and to control and/or predict potential pathogen outbreaks (Martínez-Porchas & Vargas-Albores,

2017). In fact, imbalances in the microbiome (dysbiosis) have been characterized by loss of beneficial organisms or loss of diversity with a consequent expansion of pathogenic species (Infante-Villamil et al., 2020). Matos et al. (2011) also found the importance of several water column parameters (e.g. pH, turbidity, TAN, NO_2-N, TSS and CO_2) in explaining variability in the prokaryotic community profile in a shallow raceway marine recirculation system. Thus, monitoring the microbial community dynamics in RAS represents an important tool for aquaculture planning and management.

In this study we performed a spatial and temporal characterization of the dynamics of the prokaryotic microbiome in a Sole (*Solea senegalensis*) hatchery RAS unit. For this purpose, samples of water, biofilter carriers and tank wall biofilm were collected to isolate total DNA and subsequently sequencing the 16S rRNA gene using Illumina MiSeq® platform. This study will represent a reference map of a RAS microbial community, relevant to outline the profile of potential opportunistic agents, to provide new knowledge on their spatial-temporal patterns and susceptibility to cope with physical-chemical shifts within aquaculture RAS units.

2.3. Methods

2.3.1 Study site and sampling

This study was performed in partnership with an aquaculture production unit, a sole hatchery (Safiestela Sustainable Aquafarming Investments, Lda.), located in Estela, Portugal. This unit is composed by four systems: pre-ongrowing (PO), weaning (WE), Brooding Stock (BS) and an Open System (OS). A scheme of the recycled water treatment processes for this system is presented in **Figure 1**. The pre-ongrowing is the largest area in the production unit with a fish density of 2.5 to 5 kg/m2, total water volume of 370 m^3 and a water recirculation rate of 400%/h. After the PO outlet pipe (sampling site A), the first step in the wastewater treatment is the mechanical filtration by a rotary drum filter (mainly for particulate organic matter removal) followed by the biofilter (Moving Bed Biofilter Reactor type, with an approximate total dimension of 150 m^3), filled with plastic carriers, which is sampling site B. The degasification column follows, where water trickles down and sampling point C is at the bottom of this column. Water then passes through the skimmer (where ozone is added) before returning to the tanks (tank inlet is sampling site D). The weaning system follows the same recycled water treatment processes, except for the dimension of the biofilter (approximately 25 m^3) and total water volume in the tanks (60 m^3). Sampling sites for WE are at the tank outlet (G) and inlet (H). In both systems a regime of, approximately, 2% biomass/day is followed. The

Brooding Stock system operates in RAS and water was sampled once at the tank outlet. Water from the Open System, used for the earlier and more sensitive life stages, was also collected; this water is also used for water turnover in the RAS systems. The freshwater used for regular cleaning maintenance was also collected. These last three sampling sites (Brooding Stock, Open System and Freshwater) were collected only once to confirm if they could influence the main systems under study. At the other sampling sites, the sampling program was performed at six different times, according to **Table 1**. Samples were always collected between 8 and 10 am, during the daily regular maintenance of the production systems. During the sampling program, no bacterial outbreaks were reported by the aquaculture management, and thus this study was performed during a stable and healthy period.

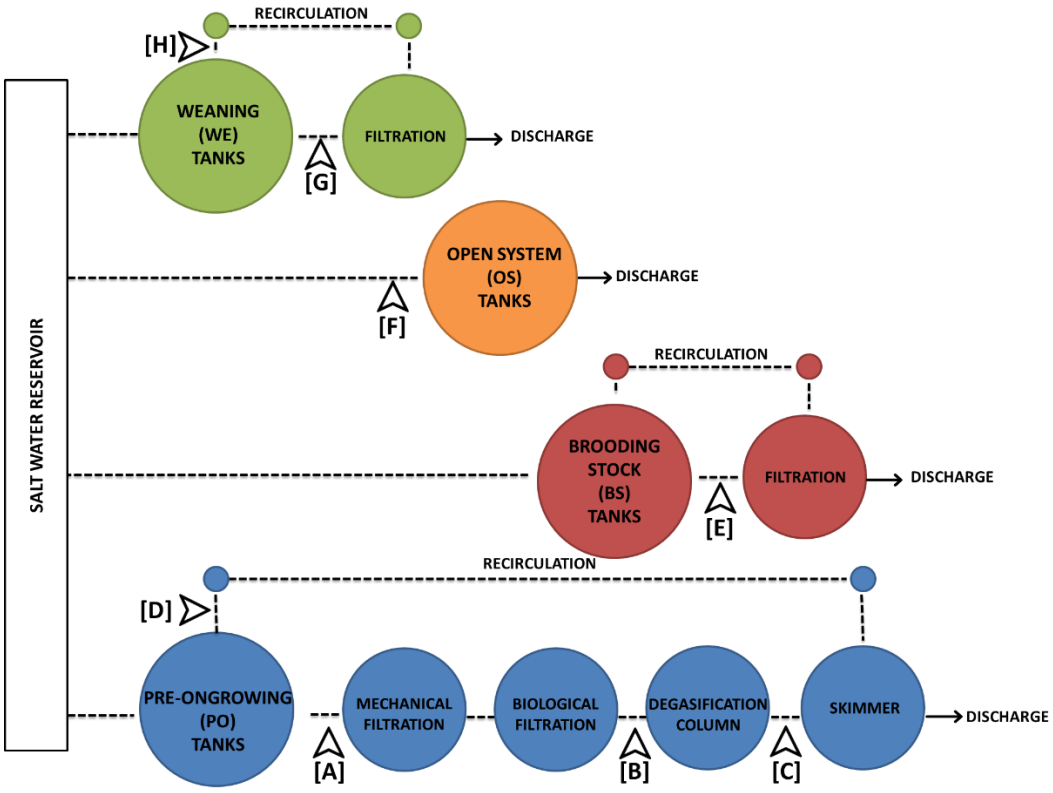

Figure 1: Representation of the aquaculture unit studied. The pre-ongrowing (PO), brooding stock (BS) and weaning (WE) systems all operate with water recirculation. There is also one open system (OS) without recirculation. Water sampling points (A-H) are marked with arrows. FW is located outside the facility and OS, BS and FW were sampled only once.

Table 1: Data for the samples collected across the different systems of the aquaculture unit (pre-ongrowing (PO), brooding stock (BS), weaning (WE), open system (OS) and fresh water (FW) in different matrices (water (A-H), tank biofilm (BF) and biofilter carriers (BB)). Data includes pH, temperature (ºC), salinity, transmittance at 400 and 600 nm and redox potential (mV). Also, the nutrients (mg/L) ammonia (NH_4-N), nitrite (NO_2-N), nitrate (NO_3-N) and phosphate (PO_4-P)

System	Matrix	Samples	Date	pH	Temperature	Salinity	NH_4-N	NO_2-N	NO_3-N	PO_4-P	Transmittance		Redox
					ºC		mg/L	mg/L	mg/L	mg/L	400 nm	500 nm	mV
PO	W	PO_A_1	17/may/18	8.19	20.1	17	0.38	0.45	52.88	4.7	88.1	90.9	242
PO	W	PO_A_2	31/jul/18	7.42	21.6	15	0.43	0.64	57.24	2.6	86.6	91.1	237
PO	W	PO_A_4	20/mar/19	7.7	20.3	19	0.33	0	19.06	0.09	90.2	92.8	243
PO	W	PO_A_5	27/mar/19	7.62	20	19	0.42	0.01	35.19	0.09	NA	NA	171
PO	W	PO_A_6	10/apr/19	7.64	20.2	17	0.3	0.09	10.58	0.16	91.9	94.8	224
PO	W	PO_B_2	31/jul/18	7.42	21.6	15	0.29	0.71	75.54	3.02	86.6	91.1	237
PO	W	PO_B_4	20/mar/19	7.7	20.3	19	0.14	0.21	20.68	0.09	90.2	92.8	243
PO	W	PO_C_2	31/jul/18	7.42	21.6	15	0.24	0.46	83.02	2.74	86.6	91.1	237
PO	W	PO_C_4	20/mar/19	7.7	20.3	19	0.31	0.17	42.93	0.11	90.2	92.8	243
PO	W	PO_D_2	31/jul/18	7.42	21.6	15	0.27	0.4	65.42	2.43	86.6	91.1	237
PO	W	PO_D_4	20/mar/19	7.7	20.3	19	0.23	0.33	34.58	0.07	90.2	92.8	243
PO	W	PO_D_5	27/mar/19	7.62	20	19	0.4	0.04	8.87	0.29	NA	NA	171
PO	W	PO_D_6	10/apr/19	7.64	20.2	17	0.33	0.28	38.67	0.13	91.9	94.8	224
PO	BB	PO_BB_3	20/dec/18	7.61	20.2	NA	NA	NA	NA	NA	NA	NA	225
PO	BB	PO_BB_4	20/mar/19	7.7	20.3	19	0.14	0.21	20.68	0.09	90.2	92.8	243
PO	BB	PO_BB_5	27/mar/19	7.62	20	19	0.42	0.01	35.19	0.09	NA	NA	171
PO	BB	PO_BB_6	17/may/19	7.64	20.2	17	0.3	0.09	10.58	0.16	91.9	94.8	224
PO	BF	PO_BF_3	20/dec/18	7.61	20.2	NA	NA	NA	NA	NA	NA	NA	225
PO	BF	PO_BF_4	20/mar/19	7.7	20.3	19	0.33	0	19.06	0.09	90.2	92.8	243
PO	BF	PO_BF_5	27/mar/19	7.62	20	19	0.42	0.01	35.19	0.09	NA	NA	171
PO	BF	PO_BF_6	10/apr19	7.64	20.2	17	0.3	0.09	10.58	0.16	91.9	94.8	224
WE	W	WE_G_2	31/jul/18	7.51	21.5	35	0.5	0.38	17.02	2.13	97.9	99	178
WE	W	WE_G_4	20/mar/19	7.67	20.2	37	0.4	0.04	8.87	0.29	99.5	100.5	160
WE	W	WE_G_5	27/mar/19	7.71	20	37	0.19	0.4	11.76	0.16	NA	NA	154
WE	W	WE_G_6	10/apr/19	7.67	20.1	35	0.19	0.22	13	0.26	98.7	99.4	169
WE	W	WE_H_2	31/jul/18	7.51	21.5	35	0.18	0.19	10.92	1.33	97.9	99	178
WE	W	WE_H_4	20/mar/19	7.67	20.2	37	0.15	0.12	10.98	0.3	99.5	100.5	160
WE	W	WE_H_5	27/mar/19	7.71	20	37	0.46	0.07	10.38	0.21	NA	NA	154
WE	W	WE_H_6	10/apr/19	7.67	20.1	35	0.62	0.07	13	0.31	98.7	99.4	169
WE	BB	WE_BB_5	27/mar/19	7.71	20	37	0.46	0.07	10.38	0.21	NA	NA	154
WE	BB	WE_BB_6	10/apr/19	7.67	20.1	35	0.62	0.07	13	0.31	98.7	99.4	169
WE	BF	WE_BF_3	20/dec/18	7.4	20.1	35	NA	NA	NA	NA	NA	NA	224
WE	BF	WE_BF_5	27/mar/19	7.71	20	37	0.19	0.4	11.76	0.16	NA	NA	154
WE	BF	WE_BF_6	10/apr/19	7.67	20.1	35	0.19	0.22	13	0.26	98.7	99.4	169
BS	W	BS_E_2	31/jul/18	7.71	20.3	35	0.008	0.11	11.33	1.02	NA	NA	NA
FW	W	FW_I_2	31/jul/18	NA	NA	NA	0.01	0.15	67.71	0.01	NA	NA	NA
OS	W	OS_F_2	31/jul/18	NA	NA	NA	0	0.01	3.08	0.11	NA	NA	NA

Water microbiome communities were collected by concentrating the planktonic biomass in a Sterivex filter unit (Millipore Merck KGaA, Darmstadt, Germany, SVGS01015, pore size 0.22 µm). Because the samples had different amounts of organic and bacterial loads, a limit for the filtration was imposed of either 2 L or 2 hours of filtration. The time limit was imposed to guarantee that the permanence of the samples at room temperature did not significantly affect the microbial composition of the samples. The Sterivex filter unit was then stored at -80 ºC until analysis. Biofilm from the tank walls was collected using disposable and sterile spatulas and placed in sterile micro tubes (2 mL). Five microbially colonized biofilter carriers were directly collected into sterile 15 mL falcon tubes. All the biological samples collected were stored at -80 ºC until use.

2.3.2 Physical-Chemical parameters

For inorganic nutrient analysis, water was filtrated through nitrocellulose membrane filters with a 0.45 µm pore size (M0475545, PRAT DUMAS France) and stored at -20 ºC.

Water concentrations of ammonia, nitrite, nitrate, and phosphate were evaluated by spectrophotometry using standard previously described methodologies. Briefly, ammonia was quantified using the Grasshoff & Johannsen (1972) method, an adaptation of Koroleff (1970). Nitrite and phosphate were analyzed following Grasshoff et al. (2009) and nitrate was quantified using the reduction with cadmium technique according to Jones (1984).

Water salinity, temperature and pH were provided by the aquaculture management, which performs daily measurements in the production unit.

2.3.3 DNA Extraction

Total DNA extraction from water samples was performed using the DNeasy PowerWater Sterivex DNA Isolation Kit (QIAGEN, Merck KGaA, Darmstadt, Germany), following the manufacturer instructions. In tank walls biofilm and biofilter carrier samples, the DNA was isolated with DNeasy Power Soil Kit (QIAGEN, Merck KGaA, Darmstadt, Germany) with some adaptations to the manufacturer protocol. For the colonized biofilter carriers, before starting the extraction protocol, these were centrifuged inside 15 mL tubes for 15 min at maximum speed (4300 g), followed by a quick vortex and additional 5 min centrifugation. For both biofilter carriers and tank biofilm samples, additional beads were added to facilitate cell lysis. All DNA samples were quantified using QubitTM dsDNA HS Assay Kit with a QubitTM 4 fluorometer (Q32854, Invitrogen, Thermo Fisher Scientific).

2.3.4 Sequencing and Bioinformatic analysis

The 16S rRNA gene was amplified for the hypervariable V4-V5 region with the primer pair 515YF (5′ - GTGYCAGCMGCCGCGGTAA - 3′) and Y926R-jed (5′ - CCGYCAATTYMTTTRAGTTT - 3′), designed by Caporaso et al. (2011); Caporaso et al. (2012) and later modified by Apprill et al. (2015); Parada et al. (2016). Both primers (515YF/Y926R-jed) have a degeneracy to cover a broad spectrum of diversity, specifically the Crenarchaeota/Thaumarchaeota (degeneracy at 515YF) phylum and the marine and freshwater clade SAR11 (alphaproteobacterial class; degeneracy at Y926R-jed) (http://www.earthmicrobiome.org) (Apprill et al., 2015; Parada et al., 2016) .

The initial PCR reaction included 12.5 ng of DNA template in a total volume of 25 µL. The PCR protocol involved a 3 min denaturation step at 95 ºC, followed by 25 cycles of 98 ºC for 20 s, 60 ºC for 30 s and 72 ºC for 30 s and a final extension at 72 ºC for 5 min. Negative controls were included in all PCR amplification procedures. PCR products were then one-step purified and normalized using SequalPrep 96-well plate kit (ThermoFisher Scientific, Waltham, USA) (Comeau et al., 2017), pooled and pair-end sequenced in the Illumina MiSeq® sequencer with the V3 chemistry, according to manufacturer instructions (Illumina, San Diego, CA, USA) at Genoinseq (Cantanhede, Portugal). Sequence data was processed at Genoinseq (Cantanhede, Portugal). Raw reads were extracted from Illumina MiSeq® System in fastq format and quality-filtered with PRINSEQ version 0.20.4 (Schmieder & Edwards, 2011) to remove sequencing adapters, reads with less than 100 bases and trim bases with an average quality lower than Q25 in a window of 5 bases. The forward and reverse reads were merged by overlapping paired-end reads with AdapterRemoval version 2.1.5 (Schmieder & Edwards, 2011) using default parameters.

To obtain the amplicon sequence variant (ASV) table, the DADA2 pipeline was implemented on our multi-sample dataset. This was done using R 3.6.1 (R Core Team, 2019) and the package dada2 (v1.12.1). For taxonomic attribution, the SILVAngs version 132 (Quast et al., 2012) database was used.

2.3.5 Downstream data analysis

The ASV table (without normalization) was used to obtain the Observed ASVs and Shannon alpha diversity indexes, (Spellerberg & Fedor, 2003) using R 3.6.1 (R Core Team, 2019) and the package vegan (v2.5-5). For beta diversity the unweighted UniFrac metric (Lozupone & Knight, 2005) was estimated with the generation of a distance matrix

(values were normalized to the lowest number of sequences in samples) and visualized using the nonmetric multiple dimension analysis (NMDS) methods (Clarke, 1993) and hierarchical cluster analysis (HCA) (Köhn & Hubert, 2014), both with the Bray-Curtis dissimilarity index (Bray & Curtis, 1957) with the R packages phyloseq (v1.27.6), ggplot (v3.2.1), vegan (v2.5-5), dplyr (v0.8.3), scales (v1.0.0) and reshape2 (v1.4.3).

To test the significance of differences in the prokaryotic community structure between systems and variables, the Adonis test for beta group significance was performed, using a Bray-Curtis distance matrix (Anderson, 2006; Bray & Curtis, 1957) in R 3.6.1 (R Core Team, 2019) with vegan (v2.5-5). It was considered significant p-values lower than 0.05.

For the community composition analysis, a pre-treatment of transformation to relative abundance was performed for each sample with the R package phyloseq (v1.27.6). For analysis at the genus level, only the taxa that contributed with more than 3% of the relative abundance for each sample was included.

Functional predictions (KEGG - Kyoto Encyclopedia of Genes and Genomes) orthologs and Enzyme Classification (Kanehisa & Goto, 2000) from 16S rRNA gene amplicon sequences was performed using PICRUSt2 (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) (Douglas et al., 2019) with the tools EPA-NG (Barbera et al., 2019), Gappa (Czech et al., 2019) for phylogenetic placement of reads; castor (Louca & Doebeli, 2018) for hidden state prediction and MinPath (Ye & Doak, 2009) for pathway inference. In this study relevant KEGG orthology (KOs) related with genes implicated in nitrogen cycle transformations were considered, such as K00362 (nirB), K00363 (nirD), K03385 (nrfA), K00366 (nirA) for dissimilatory nitrite reduction to ammonia; K00370 (narG), K00371 (narH), K00374 (narI), K02567 (napA), K02568 (napB), K00367 (narB), K00372 (nasA) for dissimilatory nitrate reduction to nitrite; K00368 (nirK) for dissimilatory nitrite reduction to nitric oxide; K04561 (norB) and K02305 (norC) for dissimilatory nitric oxide reduction to nitrous oxide; K00376 (nosZ) for dissimilatory nitrous oxide reduction to dinitrogen; K10944 (amoA), K10945 (amoB), K10946 (amoC) for oxidation of ammonia into nitrite; K10535 (hao) for hydroxylamine reduction to nitrite; K00370 (nxrA), K00371 (nxrB) for nitrite oxidation into nitrate. A synthesis of the KOs and gene codified proteins and associated reactions and pathways can be found in **Table S10**.

Significant interactions within taxonomic groups, and between taxonomic groups and physicochemical parameters, as well as between the predicted KEGGS and

physicochemical parameters were generated. These tests were performed using the R packages Hmisc (v4.1.1) and corrplot (v0.84).

2.4. Results

In the sequencing data set generated for all samples, the minimum read count per sample (number of sequences after trimming) was 10396, the mean per sample was 28850 and the max sample read count was 70487, the complete list of read counts per sample is presented in **Table S1**.

2.4.1 Prokaryotic community alpha and beta diversity

The Observed ASVs and Shannon index calculated for each sample are presented in **Table S1** and were plotted in **Figure 2**. Both the Observed ASVs and the Shannon index indicated that biofilm samples (both biofilter carriers and tank biofilm) presented a higher diversity than water samples. Samples from the different matrices of the PO system have shown variability in alpha diversity (observed ASVs), with water samples (80-202) presenting lower diversity compared with tank biofilm samples (313-440) and biofilter carriers (167-648). In the WE system, tank biofilm (265-275) and biofilter carriers (385-466) were also found to be more diverse than the WE water samples (291-546).

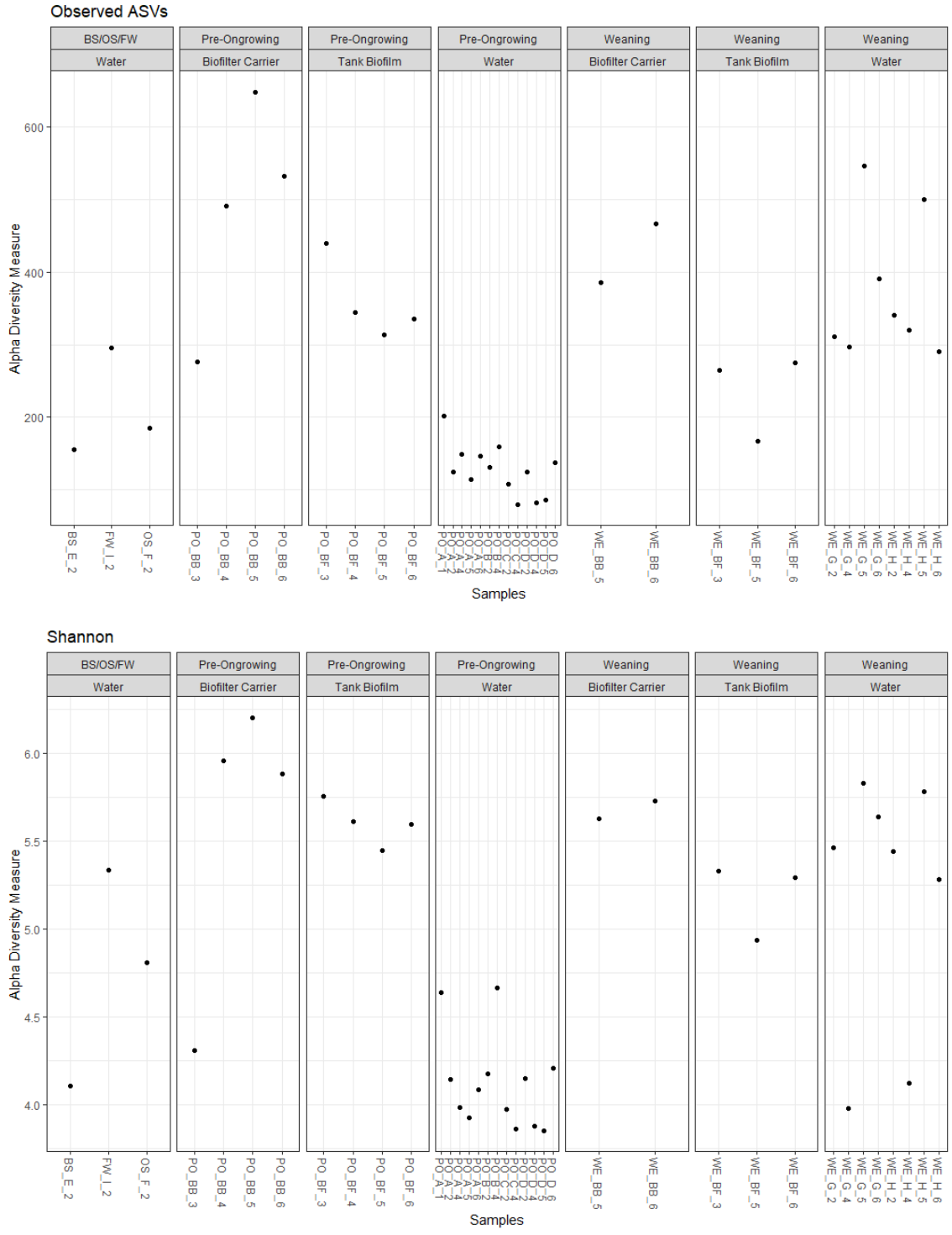

Figure 2: Alpha diversity (without normalization) represented by the Observed ASVs and the Shannon diversity index for all samples separated by matrix (biofilter carriers, BB; tank biofilm, BF and water, A-H) and system (pre-ongrowing, PO; weaning, WE; Breeding Stock, BS; Fresh Water, FW; Open System, OS).

At beta diversity level, the Bray-Curtis dissimilarity index visualized through NMDS (**Figure 3A**) revealed high prokaryotic community dissimilarity between samples from the PO and the WE system, as well as when compared to the samples from the other compartments (Open System, Brooding Stock and Freshwater). Also, within the PO system, there is a separation between water samples and samples from tank biofilm and biofilter, while samples from WE were grouped together. The hierarchical cluster dendrogram (**Figure 3B**), confirmed that the water samples of the PO are clustered together, while the biofilm samples (biofilter carriers and tank biofilm) of this system are clustered with the biofilm samples from the WE system.

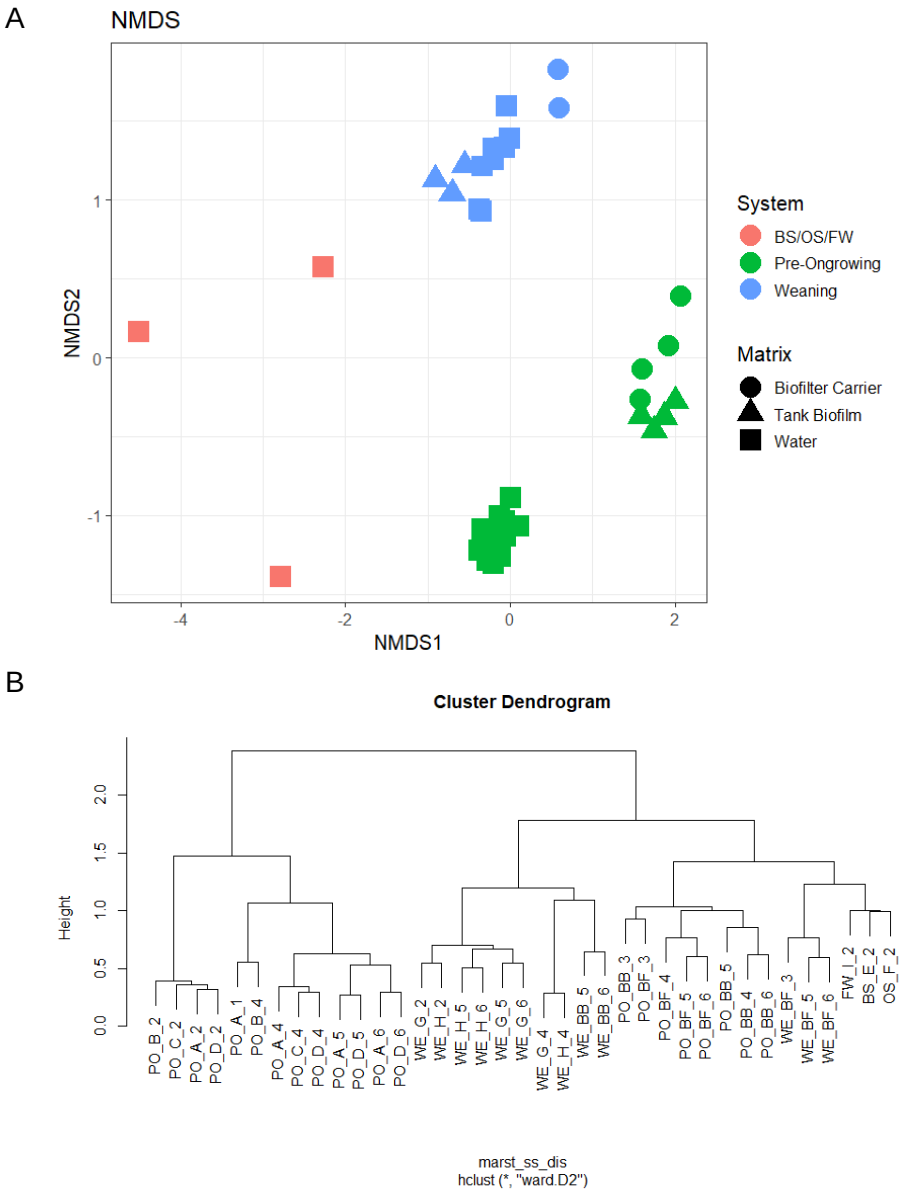

Figure 3: [**A**] Unconstrained Ordination with Non-Metric Multidimensional Scaling (NMDS) and [**B**] Hierarchical Cluster Dendogram, both using the Bray-Curtis dissimilarity index between samples. Representation is performed considering the matrixes in study (water (W or A-H), tank biofilm (BF) and biofilter carriers (BB)) and the system that they belong to (pre-ongrowing (PO), weaning (WE), brooding stock (BS), fresh water (FW) and open system (OS)).

To test the significance of the prokaryotic community dissimilarity between systems (PO, WE, Brooding Stock, Open System and Freshwater) the Adonis test was performed, using the Bray-Curtis dissimilarity index for the distance matrix. Results showed that prokaryotic community structure varies significantly between the different systems ($p < 0.001$). Therefore, the subsequent analysis was performed in separate for the PO and WE systems. Brooding Stock, Open System and Freshwater were not considered henceforth since they were sampled only once.

The differences in the prokaryotic community structure vary significantly with the matrices (i.e. water, tank biofilm and biofilter carriers) in the PO system (p-value < 0.001). The same trend was observed in the WE system, with significant differences within the different types of matrices ($p < 0.001$).

2.4.2 Taxonomic profiles of the prokaryotic communities

Taxonomy profiles, at phylum level, across the samples are represented in **Figure 4**. Overall, the prokaryotic communities were dominated by Proteobacteria (12-89%) and Bacteroidetes (8-86%). In the PO system, water samples were dominated by Proteobacteria (73-89%) followed by Bacteroidetes (9-24%) and Patescibacteria (up to 12%); while the biofilms (both biofilter carrier and tank biofilm) were dominated by Proteobacteria (37-73%) and Bacteroidetes (15-48%), followed by Verrucomicrobia (3-8%), Planctomycetes (up to 10%) and Actinobacteria (up to 3%). In the WE water samples, Bacteroidetes (40-86%) dominated the community followed by Proteobacteria (15-50%) and Chloroflexi (up to 17%) and the same trend was observed in the biofilm samples (biofilter carrier and tank biofilm) with Bacteroidetes (24-52%), Proteobacteria (35-54%), Planctomycetes (up to 8%), Verrucomicrobia (3-7%) and Actinobacteria (up to 2%) dominating the community in this order. In the WE biofilter carrier samples there is also a relevant presence of Chloroflexi (9-18%). In the Open System, the water supply used for water renovation in the recirculating systems, at the only time of sampling, Proteobacteria (78%) was the most abundant phylum followed by Bacteroidetes (8%), with other relevant phylum such as Cyanobacteria (4%), Actinobacteria (3%) and Planctomycetes (2%).

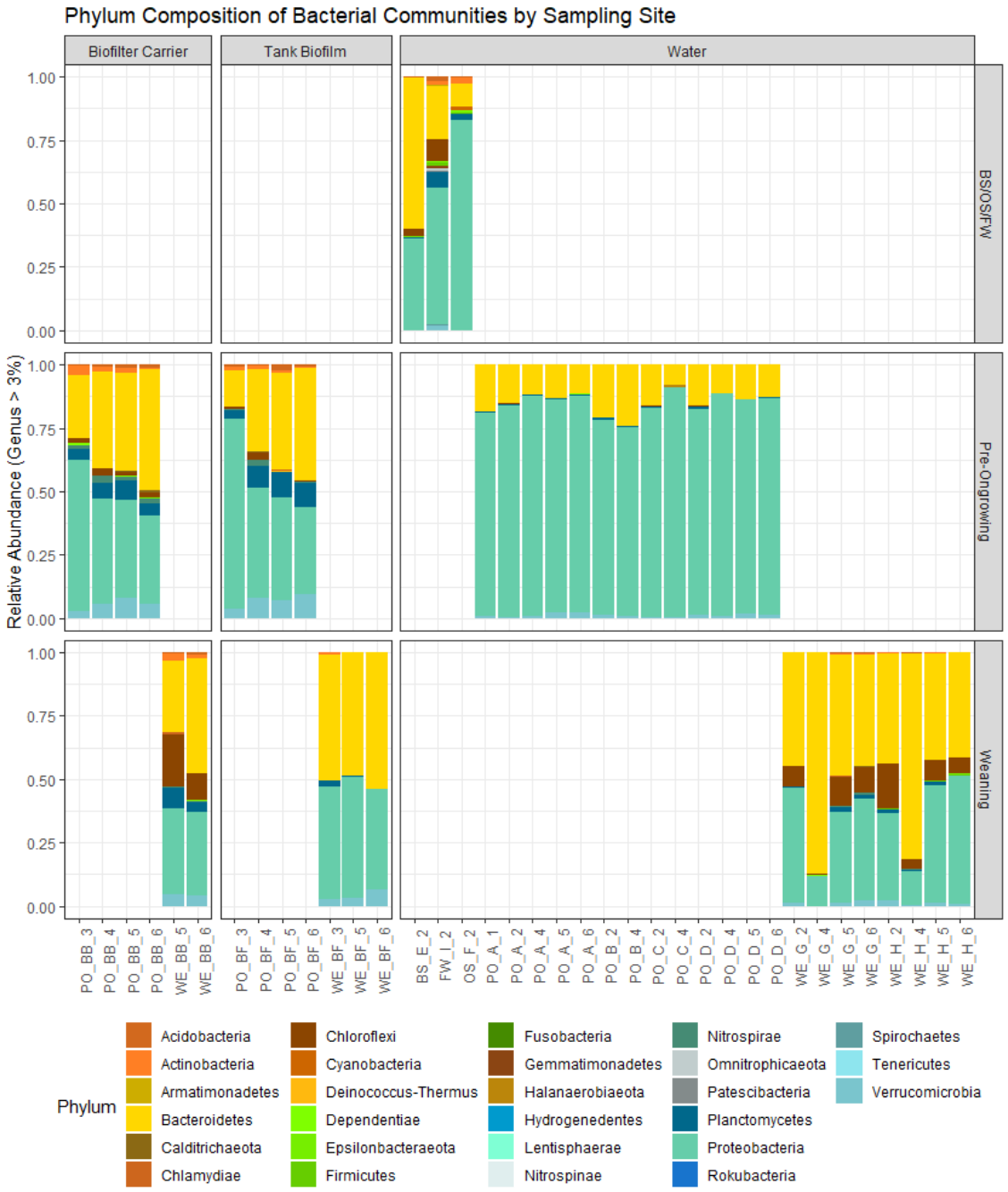

Figure 4: Relative phylum composition of the bacterial community by sampling site. Representation is performed considering the matrixes under study (water (A-H), tank biofilm (BF) and biofilter carriers (BB)) and the system that they belong to (pre-ongrowing (PO), weaning (WE), brooding stock (BS), fresh water (FW) and open system (OS)).

The prokaryotic community composition at the genus level is represented in **Figure 5**, results showed that a total of 58 genera contributed with more than 3% of the relative abundance across the different samples. The most abundant genera in the aquaculture unit are *Tenacibaculum* (up to 82%), *Sulfitobacter* (up to 56%) *Leucothrix* (up to 53%), *Novosphingobium* (32%), *Marinicella* (up to 27%), *Pseudoalteromonas* (up to 25%), *Polaribacter_2* (17%), *Schleiferia* (12%) and *Algibacter* (11%).

The pre-ongrowing system (PO), was dominated in the water samples by *Sulfitobacter* (2-56%), followed by *Leucothrix* (2-53%), *Pseudoalteromonas* (4-25%), *Polaribacter_4* (up to 8%) *Thalassotalea* (3-8%) and *Francisella* (up to 7%) (**Table S2**). It is interesting to note that the biofilm samples in this system (biofilter carrier and tank biofilm) have not shown a set of highly dominating genera, with a variable representation of *Marinicella* (up to 27%), *Polaribacter_2* (up to 17%), *Schleiferia* (up to 12%), *Algibacter* (up to 11%) and *Lewinella* (up to 10%) (**Table S3**).

The *Tenacibaculum* (17-82%) genus dominated the WE water samples (**Table S4**), followed by *Pseudoalteromonas* (up to 16%), *Leucothrix* (up to 12%) and *Polaribacter_4* (up to 9%). Tenacibaculum (up to 39%) is also the dominating genus in the biofilm samples (biofilter carrier and tank biofilm) (**Table S5**), followed by *Polaribacter_2* (up to 15%), *Oleispira* (up to 8%), *Rubritalea* (3-8%), *Leucothrix* (up to 7%), *Fluviicola* (up to 7%), *Polaribacter_4* (2-6%) and *Blastopirellula* (up to 6%). The same tendency of a variable representation of several genera, contrary to a dominance of a few, is observed in the biofilm samples of the WE system.

Prokaryotic community compositions for the Brooding Stock, Open System and Freshwater water samples are presented in **Table S6**. To note that the most abundant genera in the Open System are *Novosphingobium* (32%), *Pseudoalteromonas* (7%), *Thalassospira* (6%) and *Halomonas* (5%).

From the genera commonly associated with biofiltration activity in RAS, some were found in biofilter carriers of the studied systems, such as *Nitrospira* (nitrification) (up to 4%) in WE biofilter carriers, and *Nitrosomonas* (nitrification) (up to 7%) and *Thiothrix* (sulfide-dependent autotrophic denitrification) (up to 7%) in PO biofilter carriers. The later, *Thiothrix*, was also found in the tank biofilm of the WE system).

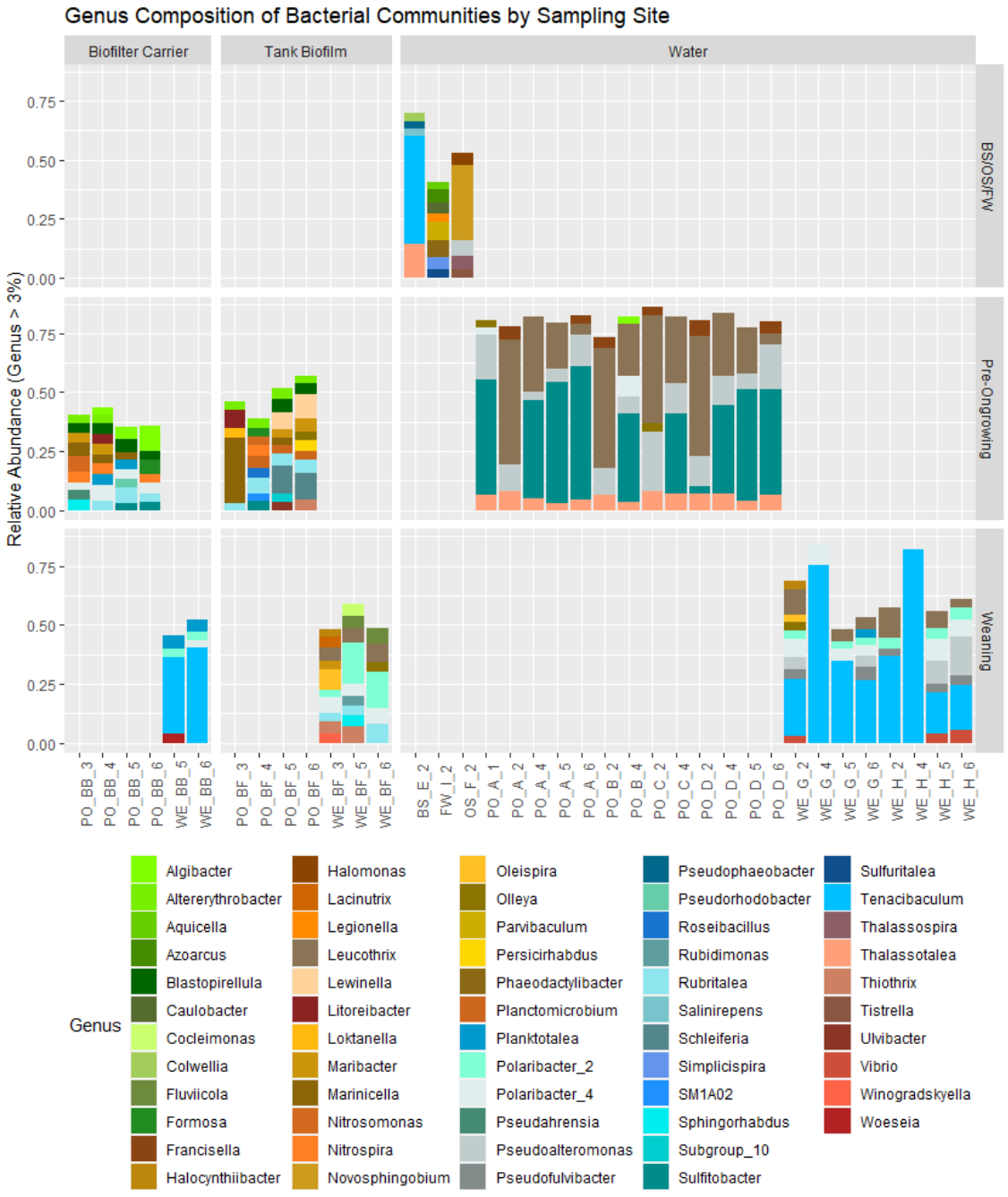

Figure 5: Relative genus composition, with ASVs that contributed with more than 3% of the relative abundance, of the bacterial community by sampling site. Representation is performed considering the matrixes under study (water (A-H), tank biofilm (BF) and biofilter carriers (BB)) and the system that they belong to (pre-ongrowing (PO), weaning (WE), brooding stock (BS), fresh water (FW) and open system (OS)).

At sampling time 4, in the PO system, samples from water, tank biofilm and biofilter carriers were collected (**Table 1**). This allows for a more complete overview of the prokaryotic community structure within the system. At the phylum level, water samples (PO_A_4, PO_D_4) were almost completely composed of the phylum Bacteroidetes and Proteobacteria, while the tank biofilm (PO_BF_4) and biofilter carrier (PO_BB_4) also had a Planctomycetes and Verrucomicrobia with abundances higher than 3%. At the genus level, the water samples have a homogeneous composition, with *Leucothrix*, *Pseudoalteromonas*, *Sulfitobacter* and *Thalassotalea* representing almost the totality of the prokaryotic composition detected (83% for sample PO_A_4 and 84% for PO_D_4). The tank biofilm and biofilter carrier samples showed higher prokaryotic diversity (**Table S1**) with a high number of rare OTUs (<3% abundance).

Temporal variation was also considered since some sampling points were characterized several times during the studied period. Using as an example PO_A, that was sampled at 5 different times, it was possible to observe a cyclic variation of the community, with the PO_A_1 (collected in May, 2018) and the PO_A_6 (collected in April, 2019) with very similar communities (**Figure 5**). Between these two samples there were shifts in the most abundant genera, specially Leucothrix which starts with a relatively low abundance at PO_A_1 of 2%, reaching 53% two months later (PO_A_2) and then has a steady decline over the next year to back at 5% (PO_A_6). In general, the same pattern of variation was observed for the other PO water samples (**Figure 5**).

2.4.3 Predicted Functional profile within the nitrogen dissimilatory metabolism

The predictive functional nitrogen cycle profile of the RAS prokaryotic community was analyzed by using PICRUSt2 and the absolute values for the different nitrogen cycle KOs across all samples are presented in Tables S7, S8 and S9. Results clearly showed different patterns of distribution of the genes involved in nitrogen processes across the different samples (Figure 6). This in silico analysis suggested that biofilter carriers and tank biofilm samples were more enriched than the water samples in nitrogen cycle genes with high prevalence of the genes involved in the ammonia oxidation (hao, amoA, amoB, amoC), nitrite oxidation (nxrA, nxrB) as well as in the complete denitrification process, including nitrate reduction (narG, narH, narI), nitrite reduction (nirK) and nitrous oxide reduction (nosZ) into N2 (Figure 6). Genes involved in the Dissimilatory Nitrate Reduction to Ammonia (DNRA) were also found to be enriched in biofilm samples (nirB, nirA, nirD, nrfA). Contrary, water samples from pre-ongrowing system (PO) showed no signal in

terms of the genes involved on ammonia oxidation within nitrification process and very weak signal on the genes involved in the denitrification pathway (Figure 6). Here the more relevant genes were the ones involved in the nitrite reduction to ammonia during DNRA and the nitrate (napA, napB) and nitric oxide (norB) reductase genes (Figure 6). Regarding the water samples from the weaning system, they have a mixed prevalence of the genes analysed, with abundant ammonia oxidation genes, although not as high as in biofilter carriers and tank biofilm samples (**Figure 6**). The same was observed for most of the genes involved in the denitrification process, except nirB, nirD (DNRA) and nasA (nitrite reduction) that presented lower abundancies relatively to PO biofilter carrier samples.

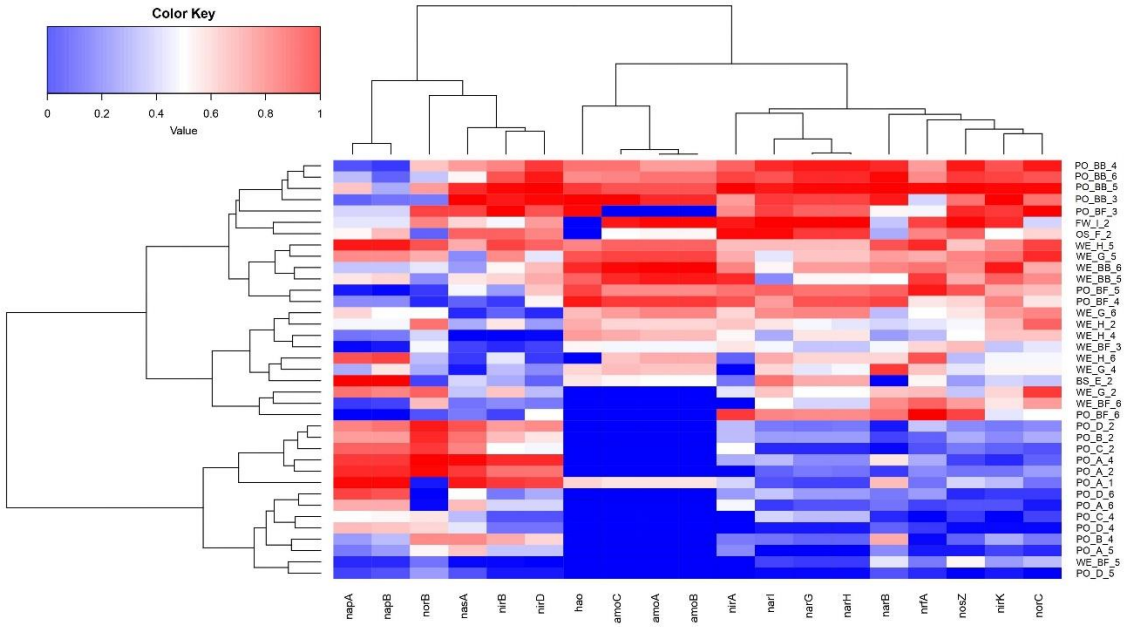

Figure 6: Heatmap plot of the Functional predictions (KEGG (Kyoto Encyclopedia of Genes and Genomes) orthologs and Enzyme Classification (Kanehisa and Goto 2000)) from amplicon sequences related with genes of the nitrogen cycle and their correlations in the different sampling sites. Representation is performed considering the matrixes under study (water (A-H), tank biofilm (BF) and biofilter carriers (BB)) and the system that they belong to (pre-ongrowing (PO), weaning (WE), brooding stock (BS), fresh water (FW) and open system (OS)).

Spearman correlations between the KOs analyzed revealed a dominance of positive relationships between the genes involved in the nitrogen cycle (Figure 7), with strong significant relationships between the genes that are involved in the codification of the same enzyme, as expected (e.g. amoA, amoB, amoC). Also, significant relationships were observed between the genes involved in the two steps nitrification process (ammonia oxidation – hao, amoA, amoB, amoC; nitrite oxidation - nxrA, nxrB) and the different steps of denitrification pathway (nitrate reduction - narB, narG, narH, narI; nitrite

reduction – nirK; nitric oxide reduction – norC; nitrous oxide reduction – nosZ), suggesting that nitrification and denitrification pathways are tightly coupled.

Interestingly, there were two exceptions to the prevalence of positive correlations observed between the relative abundance of the genes NapAB (dissimilatory nitrate reductase) with NarB (assimilatory nitrate reductase), and with the gene involved in the dissimilatory nitrous oxide reduction to dinitrogen (nosZ), that showed a significant negative relationship (**Figure 7**).

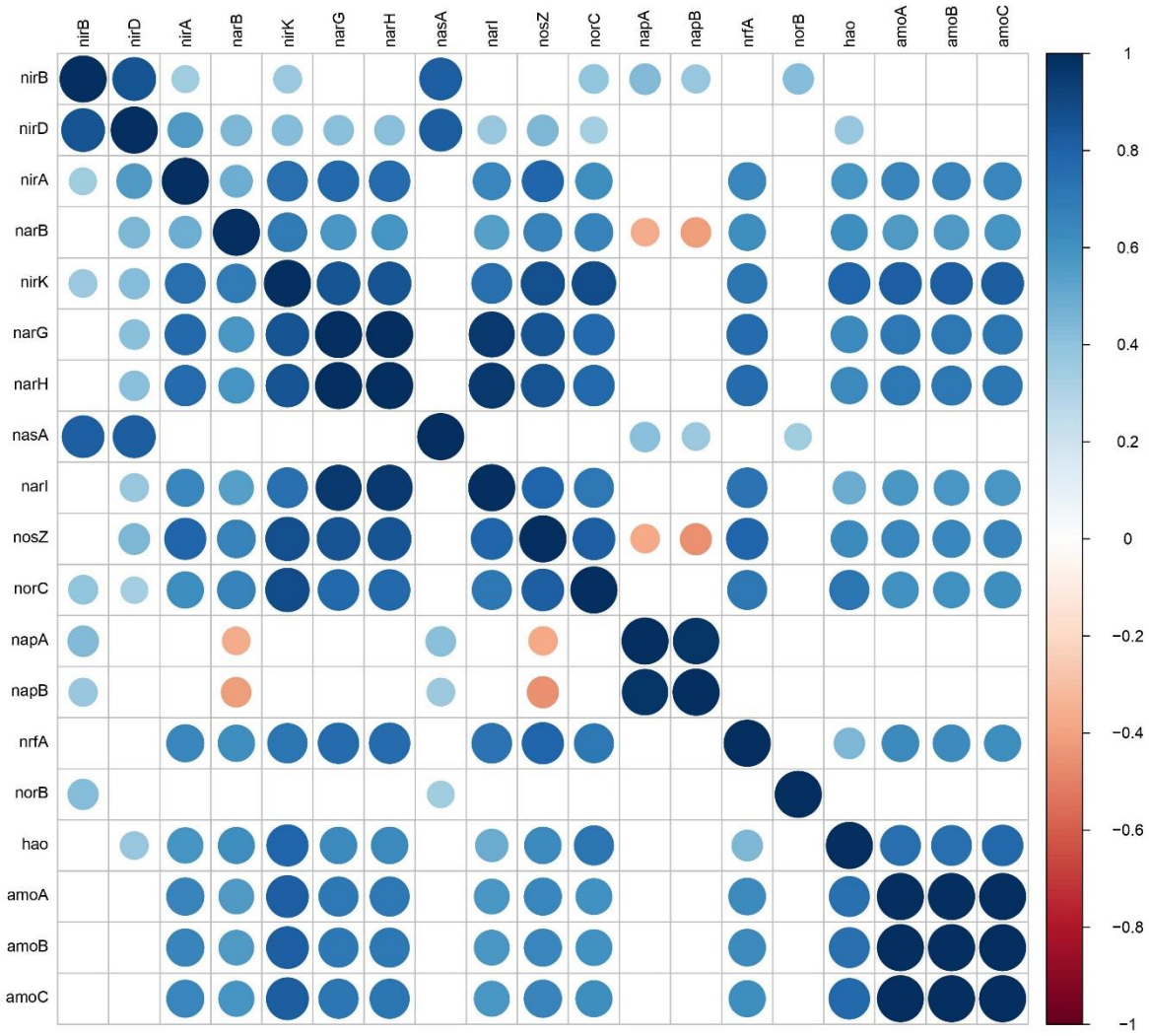

Figure 7: Significant correlations (Spearman correlations) between the Functional predictions (KEGG (Kyoto Encyclopedia of Genes and Genomes) orthologs and Enzyme Classification (Kanehisa and Goto 2000)) from amplicon sequences related with genes of the nitrogen cycle.

2.4.4 Prokaryotic taxa and predicted functions vs. water physico-chemical properties

Physical-chemical parameters measured are reported on **Table 1**. Overall, there were minimal changes registered within the system, pH varied between 7.4 and 8.2, temperature 20 and 21.6 ºC, ammonia (NH_3-N) 0 to 0.6 mg/L, nitrite (NO_2-N) 0 to 0.7 mg/L and phosphate (PO_4-P) 0 to 4.7 mg/L. The exception is nitrate (NO_3-N) with values between 3.1 (in the new seawater entering the system) and 75.5 mg/L (after the biofilter of pre-ongrowing). Salinity was purposely maintained at low values in the pre-ongrowing system, varying between 15 and 19 in this system and between 35 and 37 in the weaning system. Transmittance varied between 86.6 to 99.5 at 400 nm and 90.9 to 100.5 at 500 nm; redox potential between 154 to 243 mV.

The Adonis test was performed, using the Bray-Curtis dissimilarity index for the distance matrix, to identify possible relationships between the physico-chemical parameters measured and the variability of the prokaryotic community across the samples analyzed (**Table 2**). Because the community proved to be significantly different between systems ($p < 0.001$), samples were also analyzed in separate between PO and WE for the subsequent analysis. Results showed that the prokaryotic community structure is dependent on matrix (water, biofilter carrier and tank biofilm) for both RAS systems (PO: $p < 0.001$, $R2 = 0.37$; WE: $p < 0.001$, $R2 = 0.42$). In the PO system it is also dependent on salinity (p-value = 0.002, R2 = 0.30), the same was not observed for the WE system (p-value = 0.467, R2 = 0.08).

Table 2: Beta-diversity analysis with Adonis test for beta group significance, with a Bray-Curtis distance matrix. The null hypothesis is that there is no interaction between Pre-Ongrowth (PO) and weaning (WE) systems water or biofilm (Matrix) samples prokaryotic diversity and the Salinity (‰), pH, Temperature (ºC), nutrients (ammonia, nitrite, nitrate and phosphate in mg/L), turbidity (transmittance 400 nm, 500 nm) and Redox.

		Subset		
Variable		All samples	PO	WE
System	R2	0.18579		
	p	< 0.001		
Matrix	R2	0.17095	0.36859	0.4161
	p	< 0.001	< 0.001	< 0.001
Salinity	R2	0.36696	0.29731	0.08319
	p	< 0.001	0.002	0.467
pH	R2	0.46084	0.37858	0.26102
	p	< 0.001	0.002	0.322
Temperature	R2	0.26226	0.30017	0.30408
	p	0.002	0.01	0.122
NH_4^+-N	R2	0.5597	0.5437	0.55537
	p	0.272	0.907	0.722
NO_2^--N	R2	0.5392	0.57477	0.55379
	p	0.551	0.975	0.727
NO_3^--N	R2	0.5677	0.57477	0.52675
	p	0.551	0.976	0.897
PO_4^--P	R2	0.50957	0.50819	0.59844
	p	0.552	0.847	0.955
400 nm	R2	0.36912	0.30268	0.30406
	p	< 0.001	0.012	0.11
500 nm	R2	0.36912	0.30268	0.30406
	p	< 0.001	0.013	0.115
Redox	R2	0.1282	0.05696	0.1016
	p	< 0.001	0.303	0.226

Adonis test revealed that prokaryotic diversity varies significantly with the temperature in the PO system (p-value < 0.01, R2 = 0.30), but this trend was not observed in WE system (p-value = 0.122, R2 = 0.30). The same was true for the water pH, which significantly influenced the prokaryotic community structure in PO system (p-value = 0.002, R2 = 0.38) but not in the WE system (p-value = 0.122, R2 = 0.26). For transmittance, which is an indicative of water turbidity, we found that Adonis results were significant in PO (T400nm p-value = 0.012, R2 = 0.30, T500nm p-value = 0.013, R2 = 0.30), while Redox were found to do not significantly influence the prokaryotic community structure of both systems.

To understand how the physico-chemical properties are related to the distribution of the most abundant genus, Spearman correlations analysis was also performed (**Figure 8**) for the genera that represented more than 3% of the prokaryotic community of all samples. Results revealed that pH is an important variable in driving the distribution of three dominant genera (*Marinicella*, *Oleispira* and *Candidatus Nitrosopumilus*). Temperature were positive related with the occurrence of *Leucothrix*, *Thalassotalea* and *Pseudoalteromonas* and negative related with *Polaribacter* 2, *Sphingorhabdus*, *Candidatus Nitrosopumilus*, *Fluviicola*, *Winogradskyella* and *Rubidimonas*. Salinity was also found to influence positively the occurrence of *Tenacibaculum*, *Polaribacter* 2, *Planktotalea*, *Halocynthiibacter*, *Rubritalea*, *Thiothrix*, *Pseudofulvibacter*, *Candidatus Nitrosopumilus* and *Winogradskyella* and influence negatively the occurrence of *Sulfitobacter*, *Leucothrix*, *Thalassotalea*, *Pseudoalteromonas*, *Algibacter*, *Olleya* and *Marinicella* (Figure 8). In the PO water samples, the *Leucothrix* genus dominated when salinity was at 15, but shifted when there was an increase to 17-19 and the dominating genus was *Sulfitobacter*, this tendency can be seen on **Figure S1**.

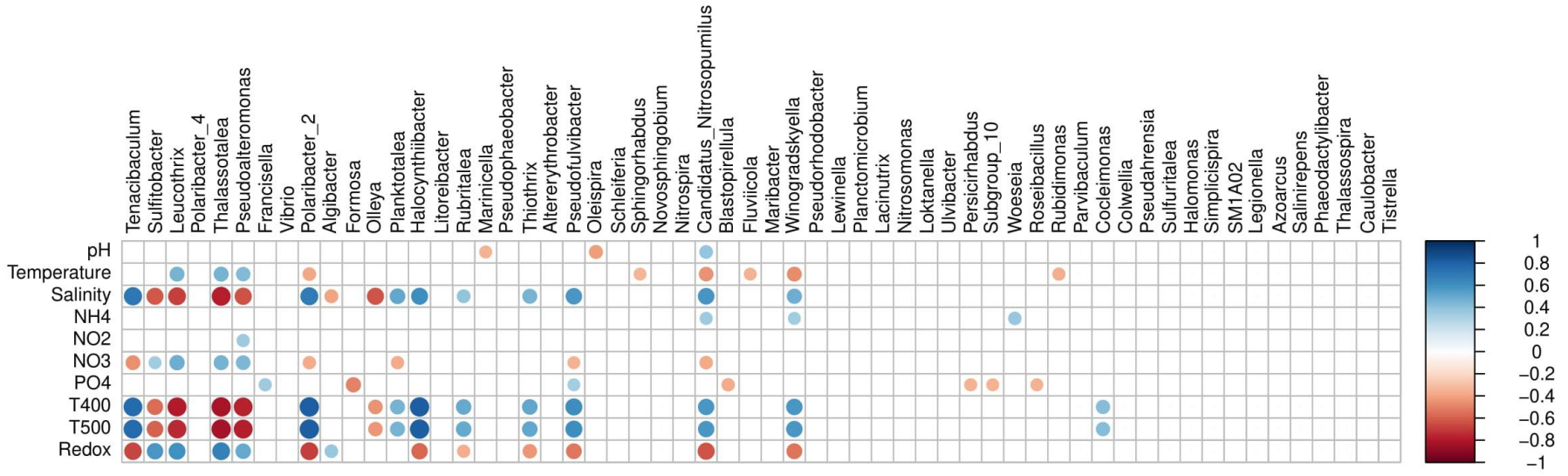

Figure 8: Significant correlations (Spearman correlations) between the 58 genera with abundancy higher than 3% (of both pre-ongrowing and weaning) and the environmental factors pH, temperature, salinity, ammonia (NH_4), nitrite (NO_2), nitrate (NO_3) and phosphate (PO_4).

Regarding the significant correlations with the water nutrients, NH_3-N was positively correlated with the genera *Polaribacter* 4, *Vibrio*, *Rubidimonas* and *Cocleimonas*. On the contrary, NO_2-N only had negative correlations with the genera *Sphingorhabdus* and *Cocleimonas*. The NO_3-N concentration in the water had a negative correlation with *Tenacibaculum*, and positive with *Leucothrix*, *Thalassotalea* and *Pseudoalteromonas*. There was only one significant negative correlation with PO_4-N, with the genus *Formosa*.

Relationships between in silico analysis of the genes involved in nitrogen cycle and the environmental parameters monitored for this study are represented in **Figure 9**. Salinity was the variable that most positively influenced the occurrence of nitrogen metabolic genes, although water column temperature and NO3- concentrations are mostly negatively related with the distribution of the nitrogen cycle genes.

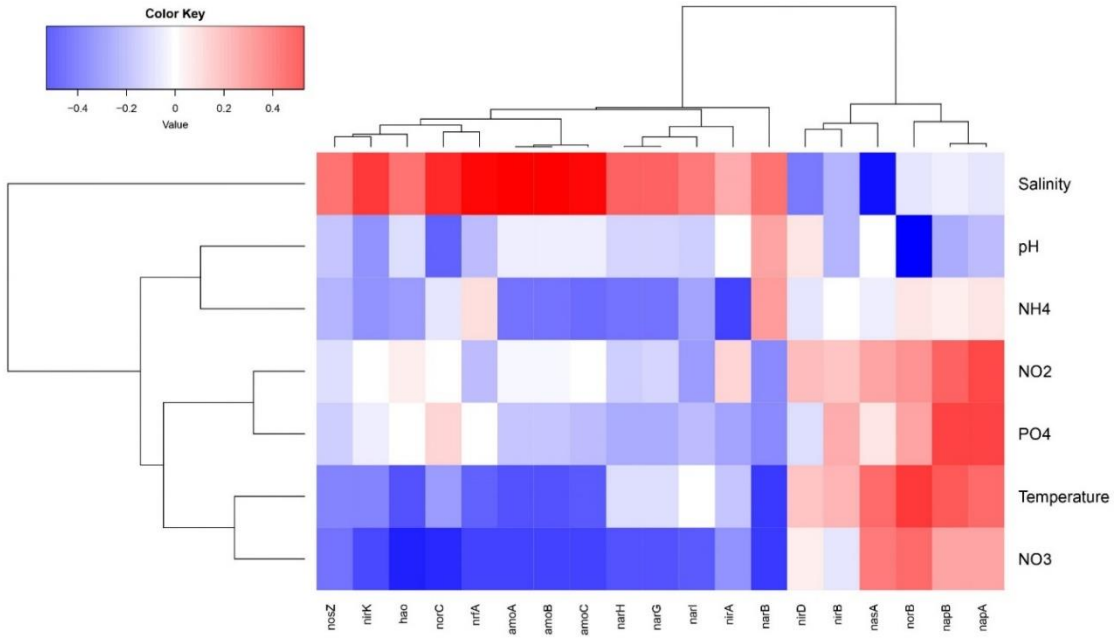

Figure 9: Heatmap plot of the Functional predictions (KEGG (Kyoto Encyclopedia of Genes and Genomes) orthologs and Enzyme Classification (Kanehisa and Goto 2000)) from amplicon sequences related with genes of the nitrogen cycle and their correlations with the environmental factors pH, temperature, salinity, ammonia (NH_4), nitrite (NO_2), nitrate (NO_3) and phosphate (PO_4).

2.5. Discussion

2.5.1 Diversity and Taxonomic Profiles of RAS Prokaryotic Community

This study was performed to investigate prokaryotic community dynamics in a sole hatchery RAS in relation to water physical-chemical parameters. In general, the phyla Bacteroidetes and Proteobacteria dominate the prokaryotic communities in all samples, which is in agreement with previous findings from other aquaculture systems (Brailo et al., 2019; Huang et al., 2016; Matos et al., 2011; Rud et al., 2017). In addition, the prokaryotic communities of the sole hatchery aquaculture unit, were richer in the tank biofilm and in the biofilter carriers compared with the water, with a higher prevalence of rare ASVs (<3%). This difference proved to be significant, and it goes in accordance with recent findings in Atlantic salmon RAS (Bakke et al., 2017) and in a flow-through fish farm for lumpfish (Roalkvam et al., 2019). Comparing the two main systems from RAS unit, PO and WE, the later had higher prokaryotic diversity (in terms of Observed ASVs and Shannon index). Previous findings suggest a correlation of the microbiome of an aquaculture and animal performance, with high bacterial alfa-diversity and number of rare groups being associated with higher growth rates and health (Boutin et al., 2013; Infante-Villamil et al., 2020; Trinh et al., 2017). Most of these studies have been using OTU levels to describe prokaryotic community diversity, but recently it has been found that DADA2 generated ASV levels provided the best sensitivity in obtaining abundancy tables (Prodan et al., 2020) including the ability to differentiate sequences at single-base resolution even at high abundance ratios. Thereby this approach was used in our study and is becoming an innovation in aquaculture prokaryotic community studies (Wynne et al., 2020).

Communities in different RAS water compartments of the same system (e.g. A, B, C, D in PO) were found to be very similar, but differed significantly across the different matrices (water, tank biofilm and biofilter carriers), this is in accordance with the findings of Bakke et al. (2017). However, it was not found that tank biofilm and biofilter carriers were dissimilar between systems, as was described in the later study. It was interesting to observe a clear and significant shift in the water prokaryotic communities across the four different systems operating in the same aquaculture unit, as seen at sampling time 2 (when all four were collected). These units are supplied by the same water reservoir (which originates in a direct catchment system in the nearby shore). This goes in accordance with the findings of (Bartelme et al., 2019) that suggested the occurrence of individual aquatic microbiome assemblage that across the different RAS units. The original seawater is represented by the sample OS_F_2, the one that supplies the open

system (and used for water turnover in the RAS). The OS_F_2 water sample was dominated by *Novosphingobium* (32%) and the second dominating genus was *Pseudoalteromonas* with only 7%. In the remaining samples, even those collected at the same time, the genus *Novosphingobium* was not detected although *Pseudoalteromonas* increases in such a way becoming the dominating genus in some samples (PO_A_1, PO_A_6, PO_D_6). The different conditions within the aquaculture unit could promote the observed shifts in the prokaryotic community. These results seem to diverge from previous findings (Brailo et al., 2019), where it was concluded that environmental sea water can be a natural enhancer of the microbial community. Here we found that it has a minimal contribution to the established community, at least in these stable systems with years of operation.

The *Flavobacteriales* family has already been reported as a dominating group in RAS compartments and biofilters (Martins et al., 2010; Ruan et al., 2015; Rud et al., 2017), and in our study the more relevant representatives were *Tenacibaculum* and *Polaribacter* 4. It has been linked as an indicator of health in the production of the shrimp *Penaeus vannamei* (Zhang et al., 2014). The genus *Polaribacter* has been also previously reported as dominant in RAS (Martins et al., 2013; Ruan et al., 2015; Rud et al., 2017). The genus *Tenacibaculum* is more commonly known as encompassing opportunistic pathogen species (Toranzo et al., 2005), but this genus has been described as having high degradation activity against a wide range of N-acylhomoserine lactones, that mediate quorum sensing systems which control virulence factors of many species of fish-pathogenic bacteria, such as *Edwardsiella tarda* (Romero et al., 2014). The high representation of *Tenacibaculum* found in WE has also been reported in the water of a flow-through fish farm of Lumpfish (Roalkvam et al., 2019). In our study it was found that *Tenacibaculum* prevailed in the biofilm carriers of the biofilter, but not so much in the tank biofilm. It is important to note that no outbreaks have been reported by the aquaculture management during the period of this study. Granting this, the high occurrences of *Tenacibaculum*, without the development of the disease, could be due to the low rearing time of the fishes in this system (90 days) compared with the PO (180 days), where past outbreaks have been reported. Furthermore, recent studies in a natural outbreak in Atlantic salmon (Wynne et al., 2020) found that opportunistic taxa (such as *Vibrio* spp.) could influence the progression of tenacibaculosis, a multifactorial disease, characterized by a profound dysbiosis of the microbial community. The hypothesis that some strains of the *Tenacibaculum* genus, despite its infamy in aquaculture, could be part of a healthy RAS microbial community and pathogen

controllers, should be tested in future studies, together with the characterizations of possible taxa that potentiate or inhibits its disease potential.

The genus *Leucothrix*, a chemoheterotrophic, colorless sulfur-oxidizing group (Brock, 1981), was found to dominate PO water and BB. This genus has been identified in a lumpfish (*Cyclopterus lumpus* L.) aquaculture flow-through system in Norway, during healthy conditions (Roalkvam et al., 2019). While *Leucothrix* genus has been reported to form dense biofilms that harm egg production (Sadusky & Bullis, 1994), here this genus was found in trace amounts in the water that supplies the egg incubator system (OS).

Dominant in the water of PO, but also present in WE, *Pseudoalteromonas* has been described in sole RAS hatcheries as a dominant clade by DGGE (Duarte et al., 2019a). *Pseudoalteromonas*, *Polaribacter* and *Algibacter* closest relative 16S rRNA gene sequences were also found in the isolates obtained in the RAS turbot system (Matos et al., 2011). A strain of this genus has been tested with promising results as being protective against *Vibrio* infections in European Abalone (*Haliotis tuberculate*) by Offret et al. (2019).

Genera commonly associated with biofiltration activity in RAS (Espinal & Matulić, 2019), such as *Nitrospira* (nitrificaton), *Nitrosomonas* (nitrification) and *Thiothrix* (sulfide-dependent autotrophic denitrification) were found in the WE and PO biofilter. Even though they were not the utmost representative genus found in these matrices. In agreement functional predictions of KEGG (Kyoto Encyclopedia of Genes and Genomes) orthologs related with Nitrogen cycle genes revealed that biofilter carriers (BB) and tank biofilm (BF) samples were enriched in nitrogen cycle genes with high prevalence of the genes involved in the ammonia oxidation (hao, amoA, amoB, amoC), nitrite oxidation (nxrA, nxrB) and in denitrification pathway (narG, narH, narI, nirK, nosZ). This trend of higher representativeness of Nitrogen taxa affiliates in biofilm and biofilter samples has been previously observed in other studies (Brailo et al., 2019; Chen et al., 2006). In addition, we also found that Spearman correlations between the nitrogen cycle KOs were predominantly positive, suggesting that the taxa involved on nitrogen processes, like nitrification and denitrification pathways, are tightly coupled. However, it should be considered, that functional predictions of KEGG orthologs are unable to provide information about gene expression or metabolic activity and it is limited by the available sequences in the reference database and genome annotations.

For the remaining dominating genera, *Thalassotalea*, found mainly in the BS and PO water, has been previously isolated from a yellow grouper (*Epinephelus awoara*) RAS system (Hou et al., 2015). *Rubritalea*, presented throughout the PO and WE, has also been detected with NGS in the same study. *Marinicella* found in a tank biofilm sample, has also been detected in an experimental RAS biofilter (Ruan et al., 2015). *Francisella*, present throughout WE and PO has been detected by PCR in a nile tilapia (*Oreochromis niloticu*s L.) production (Sebastião et al., 2017). *Lewinella*, found in the tank biofilm and in the biofilter of both WE and PO, has also been detected in bioflocs (Luo et al., 2019) and *Oleispira*, punctually present in WE and PO, has been detected by DGGE in a sole hatchery RAS (Duarte et al., 2019a).

2.5.2 Influence of Water Parameters on Prokaryotic Community

Regarding the physical-chemical parameters, evaluated at the same time as sample collection for prokaryotic community analysis, it was found that in the PO, the only system that suffered changes in salinity (although purposely for disease outbreak prevention), the prokaryotic communities changed significantly with fluctuations in this parameter, justifying 30% of the variation. Bakke et al. (2017) also found that salinity appeared to structure the microbial community. In the PO, temperature was found to be a significant driver of microbial community compositions explaining 30% of the variability. However, this parameter was relatively stable, as it is expected in a commercial RAS, so this outcome may be indirectly due to other factors. It should also be considered that recently published studies (Duarte et al., 2019b) in a sea bass (*Dicentrarchus labrax*), although from a semi-intensive aquaculture system (and not RAS), similarly found that temperature and salinity were significant drivers of the overall microbial community composition.

In the PO system, pH has also proven to significantly cause fluctuations in the system prokaryotic community (justifying 38% of the variability), while ammonia (NH_3-N) nitrite (NO_2-N), nitrate (NO_3-N) and phosphate (PO_4-N) have not. It is expected that the microbial community responds to fluctuations in nutrient load, but although there are studies proving this to be true in aquaculture wastewater effluents (Olsen et al., 2017), studies in RAS are still scarce. It must be considered that regular maintenance ensures physico-chemical parameters are kept at with minimal deviation (and close to null values in nutrient load), although this may also mean that these small variations have caused a selective pressure in the community, and it may be very sensible to alterations. Also, Matos et al. (2011) found that in a turbot RAS, using multivariate canonical correspondence analysis of PCR-DGGE, the most common aquaculture water quality

descriptors (pH, turbidity, TAN, NO_2-N, TSS, CO2) explained 70% of the DGGE pattern variability. Therefore, more studies must be accomplished, and with a large set of parameters, to describe prokaryotic community variations and drivers since evidence suggest that variability is caused by a set of factors with complex interactions (Ruan et al., 2015).

For the WE, the same parameters were tested for significant interaction (temperature, pH, NH_3-N, NO_2-N, NO_3-N and PO_4-P) and relationships with prokaryotic community were also found to be non-significant. This can be attributed to the same control as mentioned earlier or a much smaller set of samples, thus insufficient for a complete characterization. It must also be considered that the groups in study do not have the same dispersion, results provided by ADONIS could be influenced by unbalanced sampling and that this method may fail to detect a multivariate effect unless it is expressed in high-variance taxa (Warton et al., 2012).

Of interest is the negative relation of *Tenacibaculum* with pH and NO_3-N and positive relation with salinity. This relation with salinity is in accordance with previous findings showing that most *Tenacibaculum* species have high salinity ranges (Avendaño-Herrera et al., 2006). Manipulation of this parameter was also suggested as a way to reduce *Tenacibaculum maritimum* infection in salmonids (Soltani & Burke, 1995). It is also interesting to observe the significant positive correlations with nitrate (NO_3-N) of the genera *Leucothrix*, *Thalassotalea* and *Pseudoalteromonas*. These three genera could be promising for future studies, along with *Sulfitobacter*, for sulphur was not monitored in this study, but these results may hint that there is also some sulphate reduction activity (Krishnani et al., 2010). Which is relevant because high sulphate concentrations can be problematic in salt water aquaculture, as hydrogen sulphide (H_2S) production can originate from bacterial activity in sulfur-rich waters and cause high mortality rates (Bagarinao, 1993). However, it can be controlled by preventing anaerobic conditions to form within the RAS (Letelier-Gordo et al., 2020).

2.6. Conclusion and future perspectives

In this study we demonstrated that the studied RAS sole hatchery is dominated by a highly dynamic prokaryotic community. The RAS prokaryotic community appears to be sensitive to the physical-chemical changes within the different compartments and matrices of the same aquaculture unit, developing different profiles with the same water source. This may indicate that the structure of these communities is potentially modelled by parameters such as salinity, temperature, and pH. Furthermore, it shows potential for

fine tuning when designing modulation protocols, without compromising the fish welfare, since the community appears to be sensible to small variations. Because of these, dynamic, closed systems (operating in the same RAS unit) could develop different prokaryotic communities even if supplied by the same reservoir. These results are relevant not only to characterize this unit, but also contributes to the overall understanding of the microbial community dynamic and complex interactions in stable aquaculture systems. Further studies must be focused on which set of factors cause variations in the prokaryotic community, with the potential to favour a healthy state, mainly through an increase in diversity. This may be a challenge in RAS environments where there is a stabilization of key parameters as temperature, pH, and salinity. Fluctuations are undesirable, and potentially harmful for fish welfare. However, this study shows a potential for microbiome modulation with minimal parameter variation, since in two relatively stable systems, pH and temperature appear to cause significant variability in the prokaryotic community. Future studies are fundamental to identify how the key players in maintaining a healthy RAS system can be used to achieve a healthy prokaryotic community when imbalances or fish disease outbreaks occur.

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Supplementary data in this chapter can be found in Appendix A.

2.8. References

Anderson, M.J., 2006. Distance-based tests for homogeneity of multivariate dispersions. Biometrics. 62, 245-253.

Apprill, A., McNally, S., Parsons, R., Weber, L., 2015. Minor revision to V4 region SSU rRNA 806R gene primer greatly increases detection of SAR11 bacterioplankton. Aquatic Microbial Ecology. 75, 129-137.

Avendaño-Herrera, R., Toranzo, A.E., Magariños, B., 2006. Tenacibaculosis infection in marine fish caused by *Tenacibaculum maritimum*: a review. Diseases of aquatic organisms. 71, 255-266.

Bagarinao, T.U., 1993. Sulfide as a toxicant in aquatic habitats. SEAFDEC Asian Aquaculture. 15, 2-4.

Bakke, I., Åm, A.L., Kolarevic, J., Ytrestøyl, T., Vadstein, O., Attramadal, K.J.K., Terjesen, B.F., 2017. Microbial community dynamics in semi-commercial RAS for production of Atlantic salmon post-smolts at different salinities. Aquacultural Engineering. 78, 42-49.

Barbera, P., Kozlov, A.M., Czech, L., Morel, B., Darriba, D., Flouri, T., Stamatakis, A., 2019. EPA-ng: massively parallel evolutionary placement of genetic sequences. Systematic biology. 68, 365-369.

Bartelme, R.P., Smith, M.C., Sepulveda-Villet, O.J., Newton, R.J., 2019. Component Microenvironments and System Biogeography Structure Microorganism Distributions in Recirculating Aquaculture and Aquaponic Systems. Msphere. 4, e00143-00119.

Blancheton, J.P., Attramadal, K.J.K., Michaud, L., d'Orbcastel, E.R., Vadstein, O., 2013. Insight into bacterial population in aquaculture systems and its implication. Aquacultural engineering. 53, 30-39.

Borges, M.-T., Sousa, A., De Marco, P., Matos, A., Hönigová, P., Castro, P.M., 2008. Aerobic and anoxic growth and nitrate removal capacity of a marine denitrifying bacterium isolated from a recirculation aquaculture system. Microbial ecology. 55, 107-118.

Boutin, S., Bernatchez, L., Audet, C., Derome, N., 2013. Network analysis highlights complex interactions between pathogen, host and commensal microbiota. PLoS One. 8, e84772.

Brailo, M., Schreier, H.J., McDonald, R., Maršić-Lučić, J., Gavrilović, A., Pećarević, M., Jug-Dujaković, J., 2019. Bacterial community analysis of marine recirculating aquaculture system bioreactors for complete nitrogen removal established from a commercial inoculum. Aquaculture. 503, 198-206.

Bray, J.R., Curtis, J.T., 1957. An ordination of the upland forest communities of southern Wisconsin. Ecological monographs. 27, 326-349.

Brock, T.D., 1981. The genus *Leucothrix*, The prokaryotes. Springer, pp. 400-408.

Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Lozupone, C.A., Turnbaugh, P.J., Fierer, N., Knight, R., 2011. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. Proceedings of the national academy of sciences. 108, 4516-4522.

Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Huntley, J., Fierer, N., Owens, S.M., Betley, J., Fraser, L., Bauer, M., 2012. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. The ISME journal. 6, 1621.

Chen, S., Ling, J., Blancheton, J.-P., 2006. Nitrification kinetics of biofilm as affected by water quality factors. Aquacultural engineering. 34, 179-197.

Clarke, K.R., 1993. Non-parametric multivariate analyses of changes in community structure. Australian journal of ecology. 18, 117-143.

Comeau, A.M., Douglas, G.M., Langille, M.G., 2017. Microbiome helper: a custom and streamlined workflow for microbiome research. MSystems. 2, e00127-00116.

Cytryn, E., van Rijn, J., Schramm, A., Gieseke, A., de Beer, D., Minz, D., 2005a. Identification of bacteria potentially responsible for oxic and anoxic sulfide oxidation in biofilters of a recirculating mariculture system. Appl. Environ. Microbiol. 71, 6134-6141.

Cytryn, E., Minz, D., Gelfand, I., Neori, A., Gieseke, A., de Beer, D., van Rijn, J., 2005b. Sulfide-oxidizing activity and bacterial community structure in a fluidized bed reactor from

a zero-discharge mariculture system. Environmental science & technology. 39, 1802-1810.

Czech, L., Barbera, P., Stamatakis, A., 2019. Genesis and Gappa: Processing, analyzing and visualizing phylogenetic (placement) data. BioRxiv, 647958.

Dahle, S.W., Bakke, I., Birkeland, M., Nordøy, K., Dalum, A.S., Attramadal, K.J., 2020. Production of lumpfish (*Cyclopterus lumpus* L.) in RAS with distinct water treatments: Effects on fish survival, growth, gill health and microbial communities in rearing water and biofilm. Aquaculture. 522, 735097.

Davidson, J., Good, C., Williams, C., Summerfelt, S.T., 2017. Evaluating the chronic effects of nitrate on the health and performance of post-smolt Atlantic salmon *Salmo salar* in freshwater recirculation aquaculture systems. Aquacultural engineering. 79, 1-8.

Douglas, G.M., Maffei, V.J., Zaneveld, J., Yurgel, S.N., Brown, J.R., Taylor, C.M., Huttenhower, C., Langille, M.G., 2019. PICRUSt2: An improved and extensible approach for metagenome inference. BioRxiv, 672295.

Duarte, L.N., Coelho, F.J., Oliveira, V., Cleary, D.F., Martins, P., Gomes, N.C., 2019a. Characterization of bacterioplankton communities from a hatchery recirculating aquaculture system (RAS) for juvenile sole (*Solea senegalensis*) production. PloS one. 14, e0211209.

Duarte, L.N., Coelho, F.J.R.C., Cleary, D.F.R., Bonifácio, D., Martins, P., Gomes, N.C.M., 2019b. Bacterial and microeukaryotic plankton communities in a semi-intensive aquaculture system of sea bass (*Dicentrarchus labrax*): A seasonal survey. Aquaculture. 503, 59-69.

Ebeling, J.M., Timmons, M.B., Wheaton, J.M., Summerfelt, S.T., Vinci, B.J., 2002, 2018. Recirculating aquaculture, 4th ed. Ithaca Publishing Company.

Emparanza, E.J., 2009. Problems affecting nitrification in commercial RAS with fixed-bed biofilters for salmonids in Chile. Aquacultural engineering. 41, 91-96.

Espinal, C.A., Matulić, D., 2019. Recirculating Aquaculture Technologies, Aquaponics Food Production Systems. Springer, pp. 35-76.

Foesel, B.U., Gieseke, A., Schwermer, C., Stief, P., Koch, L., Cytryn, E., De La Torré, J.R., Van Rijn, J., Minz, D., Drake, H.L., 2008. *Nitrosomonas* Nm143-like ammonia oxidizers and *Nitrospira* marina-like nitrite oxidizers dominate the nitrifier community in a marine aquaculture biofilm. FEMS Microbiology Ecology. 63, 192-204.

Grasshoff, K., Johannsen, H., 1972. A new sensitive and direct method for the automatic determination of ammonia in sea water. ICES Journal of Marine Science. 34, 516-521.

Grasshoff, K., Kremling, K., Ehrhardt, M., 2009. Methods of seawater analysis. John Wiley & Sons.

Hou, T.-T., Liu, Y., Zhong, Z.-P., Liu, H.-C., Liu, Z.-P., 2015. *Thalassotalea marina* sp. nov., isolated from a marine recirculating aquaculture system, reclassification of *Thalassomonas eurytherma* as *Thalassotalea eurytherma* comb. nov. and emended description of the genus *Thalassotalea*. International journal of systematic and evolutionary microbiology. 65, 4710-4715.

Huang, Z., Wan, R., Song, X., Liu, Y., Hallerman, E., Dong, D., Zhai, J., Zhang, H., Sun, L., 2016. Metagenomic analysis shows diverse, distinct bacterial communities in biofilters among different marine recirculating aquaculture systems. Aquaculture international. 24, 1393-1408.

Infante-Villamil, S., Huerlimann, R., Jerry, D.R., 2020. Microbiome diversity and dysbiosis in aquaculture. Reviews in Aquaculture.

Jones, M.N., 1984. Nitrate reduction by shaking with cadmium: alternative to cadmium columns. Water Research. 18, 643-646.

Kanehisa, M., Goto, S., 2000. KEGG: kyoto encyclopedia of genes and genomes. Nucleic acids research. 28, 27-30.

Köhn, H.F., Hubert, L.J., 2014. Hierarchical cluster analysis. Wiley StatsRef: statistics reference online, 1-13.

Koroleff, F., 1970. Direct determination of ammonia in natural waters as indophenol blue. Information on techniques and methods for seawater analysis, 19-22.

Krishnani, K.K., Kathiravan, V., Natarajan, M., Kailasam, M., Pillai, S., 2010. Diversity of sulfur-oxidizing bacteria in greenwater system of coastal aquaculture. Applied biochemistry and biotechnology. 162, 1225-1237.

Letelier-Gordo, C.O., Aalto, S.L., Suurnäkki, S., Pedersen, P.B., 2020. Increased sulfate availability in saline water promotes hydrogen sulfide production in fish organic waste. Aquacultural Engineering. 89.

Louca, S., Doebeli, M., 2018. Efficient comparative phylogenetics on large trees. Bioinformatics. 34, 1053-1055.

Lozupone, C., Knight, R., 2005. UniFrac: a new phylogenetic method for comparing microbial communities. Appl. Environ. Microbiol. 71, 8228-8235.

Luo, G., Chen, X., Tan, J., Abakari, G., Tan, H., 2019. Effects of carbohydrate addition strategy and biofloc levels on the establishment of nitrification in biofloc technology aquaculture systems. Aquaculture, 734441.

Martínez-Porchas, M., Vargas-Albores, F., 2017. Microbial metagenomics in aquaculture: a potential tool for a deeper insight into the activity. Reviews in Aquaculture. 9, 42-56.

Martins, C.I.M., Eding, E.H., Verdegem, M.C.J., Heinsbroek, L.T.N., Schneider, O., Blancheton, J.-P., d'Orbcastel, E.R., Verreth, J.A.J., 2010. New developments in recirculating aquaculture systems in Europe: A perspective on environmental sustainability. Aquacultural engineering. 43, 83-93.

Martins, P., Cleary, D.F., Pires, A.C., Rodrigues, A.M., Quintino, V., Calado, R., Gomes, N.C., 2013. Molecular analysis of bacterial communities and detection of potential pathogens in a recirculating aquaculture system for Scophthalmus maximus and *Solea senegalensis.* PloS one. 8, e80847.

Matos, A., Borges, M.T., Peixe, C., Henriques, I., Pereira, C.M., Castro, P.M., 2011. A molecular and multivariate approach to the microbial community of a commercial shallow raceway marine recirculation system operating with a moving bed biofilter. Aquaculture Research. 42, 1308-1322.

Michaud, L., Lo Giudice, A., Troussellier, M., Smedile, F., Bruni, V., Blancheton, J.-P., 2009. Phylogenetic characterization of the heterotrophic bacterial communities

inhabiting a marine recirculating aquaculture system. Journal of applied microbiology. 107, 1935-1946.

Offret, C., Rochard, V., Laguerre, H., Mounier, J., Huchette, S., Brillet, B., Le Chevalier, P., Fleury, Y., 2019. Protective efficacy of a *Pseudoalteromonas* strain in European abalone, *Haliotis tuberculata*, infected with *Vibrio harveyi* ORM4. Probiotics and antimicrobial proteins. 11, 239-247.

Olsen, L.M., Hernández, K.L., Van Ardelan, M., Iriarte, J.L., Bizsel, K.C., Olsen, Y., 2017. Responses in bacterial community structure to waste nutrients from aquaculture: an *in situ* microcosm experiment in a Chilean fjord. Aquaculture Environment Interactions. 9, 21-32.

Parada, A.E., Needham, D.M., Fuhrman, J.A., 2016. Every base matters: assessing small subunit rRNA primers for marine microbiomes with mock communities, time series and global field samples. Environmental microbiology. 18, 1403-1414.

Paungfoo, C., Prasertsan, P., Burrell, P.C., Intrasungkha, N., Blackall, L.L., 2007. Nitrifying bacterial communities in an aquaculture wastewater treatment system using fluorescence in situ hybridization (FISH), 16S rRNA gene cloning, and phylogenetic analysis. Biotechnology and bioengineering. 97, 985-990.

Pereira, A., Carvalho, A.P., Cruz, C., Saraiva, A., 2017. Histopathological changes and zootechnical performance in juvenile zebrafish (*Danio rerio*) under chronic exposure to nitrate. Aquaculture. 473, 197-205.

Piedrahita, R.H., 2003. Reducing the potential environmental impact of tank aquaculture effluents through intensification and recirculation. Aquaculture. 226, 35-44.

Pierce, R.H., Weeks, J.M., Prappas, J.M., 1993. Nitrate toxicity to five species of marine fish. Journal of the World Aquaculture Society. 24, 105-107.

Prodan, A., Tremaroli, V., Brolin, H., Zwinderman, A.H., Nieuwdorp, M., Levin, E., 2020. Comparing bioinformatic pipelines for microbial 16S rRNA amplicon sequencing. Plos one. 15, e0227434.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., Glöckner, F.O., 2012. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic acids research. 41, D590-D596.

Roalkvam, I., Drønen, K., Dahle, H., Wergeland, H., 2019. Microbial communities in a flow-through fish farm for lumpfish (*Cyclopterus lumpus* L.) during healthy rearing conditions. Frontiers in microbiology. 10, 1594.

Romero, M., Muras, A., Mayer, C., Buján, N., Magariños, B., Otero, A., 2014. In vitro quenching of fish pathogen *Edwardsiella tarda* AHL production using marine bacterium *Tenacibaculum* sp. strain 20J cell extracts. Diseases of aquatic organisms. 108, 217-225.

Ruan, Y.-J., Guo, X.-S., Ye, Z.-Y., Liu, Y., Zhu, S.-M., 2015. Bacterial community analysis of different sections of a biofilter in a full-scale marine recirculating aquaculture system. North American Journal of Aquaculture. 77, 318-326.

Rud, I., Kolarevic, J., Holan, A.B., Berget, I., Calabrese, S., Terjesen, B.F., 2017. Deep-sequencing of the bacterial microbiota in commercial-scale recirculating and semi-closed aquaculture systems for Atlantic salmon post-smolt production. Aquacultural Engineering. 78, 50-62.

Sadusky, T.J., Bullis, R.A., 1994. Experimental disinfection of lobster eggs infected with *Leucothrix mucor.* The Biological Bulletin. 187, 254-255.

Schmieder, R., Edwards, R., 2011. Quality control and preprocessing of metagenomic datasets. Bioinformatics. 27, 863-864.

Schreier, H.J., Mirzoyan, N., Saito, K., 2010. Microbial diversity of biological filters in recirculating aquaculture systems. Current opinion in biotechnology. 21, 318-325.

Sebastião, F., Pilarski, F., Kearney, M., Soto, E., 2017. Molecular detection of *Francisella noatunensis* subsp. *orientalis* in cultured Nile tilapia (*Oreochromis niloticus* L.) in three Brazilian states. Journal of fish diseases. 40, 1731-1735.

Soltani, M., Burke, C., 1995. Responses of fish-pathogenic Cytophaga/Flexibacter-like bacteria (CFLB) to environmental conditions. Bulletin of the European Association of Fish Pathologists (United Kingdom).

Spellerberg, I.F., Fedor, P.J., 2003. A tribute to Claude Shannon (1916–2001) and a plea for more rigorous use of species richness, species diversity and the 'Shannon–Wiener' Index. Global ecology and biogeography. 12, 177-179.

Streit, W.R., Schmitz, R.A., 2004. Metagenomics–the key to the uncultured microbes. Current opinion in microbiology. 7, 492-498.

Suhr, K., Pedersen, P.B., 2010. Nitrification in moving bed and fixed bed biofilters treating effluent water from a large commercial outdoor rainbow trout RAS. Aquacultural engineering. 42, 31-37.

Tal, Y., Watts, J.E., Schreier, S.B., Sowers, K.R., Schreier, H.J., 2003a. Characterization of the microbial community and nitrogen transformation processes associated with moving bed bioreactors in a closed recirculated mariculture system. Aquaculture. 215, 187-202.

Tal, Y., Watts, J.E.M., Schreier, S.B., Sowers, K.R., Schreier, H.J., 2003b. Characterization of the microbial community and nitrogen transformation processes associated with moving bed bioreactors in a closed recirculated mariculture system. Aquaculture. 215, 187-202.

Thurston, R.V., Russo, R.C., Vinogradov, G.A., 1981. Ammonia toxicity to fishes. Effect of pH on the toxicity of the unionized ammonia species. Environmental science & technology. 15, 837-840.

Toranzo, A.E., Magariños, B., Romalde, J.L., 2005. A review of the main bacterial fish diseases in mariculture systems. Aquaculture. 246, 37-61.

Trinh, L.T.T., Bakke, I., Vadstein, O., 2017. Correlations of age and growth rate with microbiota composition in Atlantic cod (*Gadus morhua*) larvae. Scientific Reports. 7.

Warton, D.I., Wright, S.T., Wang, Y., 2012. Distance-based multivariate analyses confound location and dispersion effects. Methods in Ecology and Evolution. 3, 89-101.

Wynne, J.W., Thakur, K.K., Slinger, J., Samsing, F., Milligan, B., Powell, J.F.F., McKinnon, A., Nekouei, O., New, D., Richmond, Z., Gardner, I., Siah, A., 2020. Microbiome Profiling Reveals a Microbial Dysbiosis During a Natural Outbreak of Tenacibaculosis (Yellow Mouth) in Atlantic Salmon. Front Microbiol. 11, 586387.

Ye, Y., Doak, T.G., 2009. A parsimony approach to biological pathway reconstruction/inference for genomes and metagenomes. PLoS computational biology. 5.

Zhang, D., Wang, X., Xiong, J., Zhu, J., Wang, Y., Zhao, Q., Chen, H., Guo, A., Wu, J., Dai, H., 2014. Bacterioplankton assemblages as biological indicators of shrimp health status. Ecological Indicators. 38, 218-224.

CHAPTER 3

[Submitted]

The network of nitrifying and pathogenic prokaryotic interactions in a recirculating aquaculture system of a sole (*Solea senegalensis*) hatchery

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3. The network of nitrifying and pathogenic prokaryotic interactions in a recirculating aquaculture system of a sole (*Solea senegalensis*) hatchery

3.1 Abstract

Prokaryotic interactions in recirculating aquaculture environmental communities may play a crucial role in driving their functional potential. However, these interactions are often neglected. The aim of this work is to detect prokaryotic interactions in RAS, through the definition of relevant taxa and cluster identification using co-variance and co-occurrence tools. Amplicon sequence variants were obtained from the water, tank biofilm, and biofilters of two systems, pre-ongrowing and weaning, and the study focuses on two microbial target groups, the potentially pathogenic and the nitrifying prokaryotes. No significant negative correlations were found with any target ASVs, indicating a mostly cooperative environment. As expected, ammonia oxidizing bacteria (*Nitrosomonas*) and archaea (*Candidatus Nitrosopumilus*) were found to be positively interacting with the nitrite oxidizing bacteria *Nitrospira*. However, no interactions were found between them, and results hint at a niche differentiation based on ammonia competition. *Nitrospira* also showed subcommunities with no AOA or AOB correlations, hinting at a separate functional role of complete ammonia oxidation to nitrate for some *Nitrospira* ASVs. Two taxa commonly associated with pathogenic outbreaks, *Tenacibaculum* and *Vibrio*, had a significant positive interaction in one of the systems. With no outbreaks reported, this association may prove relevant in disease preventions and to improve outbreak predictions.

3.2. Introduction

Solea senegalensis, has been proposed as a candidate species with high potential for aquaculture diversification in the south of Europe (Morais et al., 2016). With disease outbreaks identified as one of the main challenges in sole farms (Howell, 1997), the industry has turned to Recirculating Aquaculture Systems (RAS), that enable a tight control on the environmental parameters and eliminate contact with wild species. This type of system has the additional advantage of making intensive aquaculture compatible with environmental sustainability objectives, since it allows a reduction in water usage and an improvement in waste management and nutrient recycling (Martins et al., 2010; Piedrahita, 2003).

Monitoring the composition of bacterial communities established in aquaculture systems is of paramount importance to evaluate their potential to serve as reservoirs for pathogenic bacterial strains (Canada et al., 2020; Rud et al., 2017). This community influences the water quality and health of the fish being reared in the system (Blancheton et al., 2013) with a complex network of microbial roles (Martínez-Porchas & Vargas-Albores, 2017). Alterations to this structure, translated in changes in bacterial diversity, have been linked to animal performance and health (Infante-Villamil et al., 2020). One of the main outcomes, dysbiosis, has been defined as a shift in the structure of a commensal community that may result in perturbations in the immune system and mediated diseases (Petersen & Round, 2014). Among the diverse roles microbial communities play in RAS, the presence/absence of pathogens and active nitrogen cycling are crucial for fish health and well-being.

Despite technological improvements, disease outbreaks are still one of the great concerns in sole aquaculture management, particularly of the bacterial disease flexibacteriosis (*Tenacibaculum maritimum*) (Toranzo et al., 2005). A previous study (Wynne et al., 2020) reported high relative abundance of *T. maritimum* in healthy fish and suggested that this is a complex multifactorial disease, and the interactions with other taxa may be a key role in disease progression.

A vital feature of a RAS is the conversion of ammonia (a metabolite from protein catabolism) to nitrate in the nitrification process, performed by a microbial community incubated in the biofilter carriers (Ebeling et al., 2002). Nitrification is the naturally occurring pathway where ammonia oxidizing bacteria (AOB) oxidize ammonia to nitrite, and coupled to this reaction, nitrite oxidizing bacteria (NOB) oxidize nitrite to nitrate (Sharma & Ahlert, 1977), thus removing ammonia from the system. Some of the main genera that have been identified as performing the nitrification process in marine biofilters are the AOB *Nitrosomonas* (Foesel et al., 2008; Paungfoo et al., 2007) and *Nitrosococcus* (Foesel et al., 2008) and the NOBs *Nitrospira* (Foesel et al., 2008; Tal et al., 2003). Previous studies (Brailo et al. 2019) have found, through Next Generation Sequencing (NGS) techniques, bacteria with the capacity to carry out ammonia and nitrite oxidation in a RAS nitrification biofilter. Active biofilters have also shown to possess a more diverse community when compared with water samples from the same systems (Almeida et al., 2021; Ruan et al., 2015). One of the challenges in managing a RAS microbial community is the competition between chemoautotrophic nitrifiers (such as AOB and NOB) and heterotrophs for oxygen, nutrients, and space (Michaud et al., 2006) in the biofilter. A disbalance could lead to an increase in ammonia and nitrite

concentrations in detriment of the nitrifying populations (Blancheton et al. 2013). As a sector for optimal, but undifferentiated, bacterial growth, there is a risk that disruptions in the system may cause pathogenic outbreaks by opportunist bacteria (Blancheton et al., 2013).

In general, microbiomes are strongly influenced by the multiplicity of ecological processes that affect community assembly (Goldford et al., 2018), such as selective pressures and nutrient availability, generating cross-feeding networks. In these, microbes communicate and trade metabolites and services (Marx, 2009). RAS develop complex and dynamic microbial ecosystems, with differential composition between systems and matrices (Almeida et al., 2021). Co-variance network models are useful to infer the complex taxonomic architectures of these communities in their multitude of ecological processes (Goldford et al., 2018) by attributing to targeted taxa (nodes) the connections between them (edges) (Newman, 2003). Previous studies in freshwater RAS have used this method to conclude that interactions decrease in complexity during the biofilter start-up, after an initial peak (Jiang et al., 2019). Biofilters have been found to have more nodes but less interactions than biofloc reactors used in wastewater treatment (Deng et al., 2019). These, in turn, have been found to be more complex than water (Wei et al., 2020). Interestingly, a recent study revealed a competitive interaction between taxa responsible for ammonia removal and nitrate removal processes (Deng et al., 2021), justifiable by different niche requirement.

In this work, we targeted our networking analysis to two groups as important model microorganisms for RAS sustainability: potentially pathogenic and nitrifying prokaryotes. For this analysis, hub taxa are defined as highly connected with other taxa (Faust et al., 2012) and cosmopolitan taxa have a wide-spread occurrence across different environments, linked to a tendency to form positive connections (Faust et al., 2015).

The biotic relationships between microorganisms present in these communities may play a crucial role in driving their functional potential. However, these interactions are often neglected in engineered environments. The aim of this work is to detect prokaryotic interactions among relevant taxa in RAS, using network correlations and cluster identification.

3.3. Methods

3.3.1 Sample Collection And DNA Extraction

This study was performed in partnership with an aquaculture production unit, a sole hatchery (Safiestela S.A.), located in Estela, Portugal. The analysis was performed using the dataset from Almeida et al. (2021), details about the equipment used for physical, chemical, and biological description of the collected samples are presented in the mentioned paper. Samples of water column, tank biofilm and biofilter carrier were collected from the two parallel recirculating systems: pre-ongrowing (PO) and weaning (WE) (**Figure 1**). Total DNA was isolated from the water column using the DNeasy PowerWater Sterivex DNA Isolation Kit (QIAGEN, Merck KGaA, Darmstadt, Germany), following the manufacturer instructions. In tank wall biofilm and biofilter carrier samples, the DNA was isolated with DNeasy Power Soil Kit (QIAGEN, Merck KGaA, Darmstadt, Germany) with some adaptations to the manufacturer protocol. For the biofilter carriers, before starting the extraction protocol, these were centrifuged inside 15 mL tubes for 15 min at maximum speed (4300 g), followed by a quick vortex and additional 5 min centrifugation. For both biofilter carriers and tank biofilm samples, additional beads were added to facilitate cell lysis.

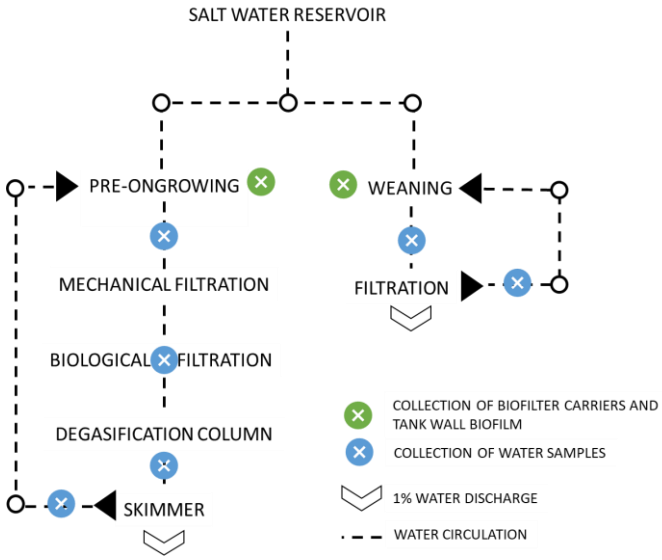

Figure 1: Representation of the aquaculture unit in study. The pre-ongrowing and weaning operate with water recirculation.

The V4-V5 region of the 16S rRNA gene (Apprill et al., 2015; Caporaso et al., 2011; Caporaso et al., 2012; Parada et al., 2016) was used for the NGS Analysis. The PCR protocol involved a 3 min denaturation step at 95 ◦C, followed by 25 cycles of 98 ◦C for

20 s, 60 ◦C for 30 s and 72 ◦C for 30 s and a final extension at 72 ◦C for 5 min. PCR products were purified and normalized using SequalPrep 96-well plate kit (ThermoFisher Scientific, Waltham, USA)(Comeau et al., 2017), pooled and pair-end sequenced in the Illumina MiSeq® sequencer at Genoinseq (Cantanhede, Portugal). Sequence data was pre-processed at Genoinseq (Cantanhede, Portugal), by removing sequences from Illumina MiSeq® System in fastq format and quality filtered with PRINSEQ version 0.20.4 (Schmieder & Edwards, 2011) removing sequencing adapters and reads with less than 100 bases and trim bases with an average quality lower than Q25 in a window of 5 bases. Forward and reverse reads were merged by overlapping paired-end reads with AdapterRemoval version 2.1.5 using default parameters.

3.3.2 Bioinformatic and Statistics

To obtain the amplicon sequence variant (ASV) table, the DADA2 pipeline was implemented on our filtered sequences dataset. This was done using R 3.6.1 (R Core Team, 2019) and the package dada2 (v1.12.1). For taxonomic attribution, the SILVA database version 132 was used (Quast et al., 2012). Taxa classified at the Kingdom level as Eukaryota, at the Order level as Chloroplast and at the Family level as Mitochondria were removed. The water column, tank biofilm and biofilter carrier overall prokaryotic diversity and composition analysis is described in Almeida et al. (2021), as part of the dynamic of the RAS microbial community in relation to the variability of water physical-chemical parameters.

The biotic relationship between microorganisms was inferred using eLSA software (Xia et al., 2013; Xia et al., 2011) and the Spearman correlation coefficient (SCC) between ASVs pairs (Spearman, 1987). To avoid misleading correlations, the ASV table was pre-filtered to remove low frequency ASVs (not present in at least three samples). Samples were split by system (pre-ongrowing and weaning) before the correlation analysis to avoid spurious autocorrelations. The correlations set obtained for each system was trimmed to include only significant interactions (SCC > |0.7|, p-value < 0.001). Network vertexes were selected based on a significant correlation with one (or more) of the ASVs belonging to the targeted pathogenic and nitrifying genera (taxa identification described below). Correlation networks were plotted in the R environment version 4.1.2 (R Core Team, 2021) using iGraph v. 1.2.7 ;(Csardi & Nepusz, 2006) and sub-communities were identified with the Louvain algorithm (Blondel et al., 2008). Vertexes with 0 significant correlations (degrees) were removed.

As mentioned in the introduction, this study was based on the definition of two target groups in a recirculating aquaculture system, the potentially pathogenic and nitrifying, the later subdivided into ammonia oxidizing bacteria (AOB), nitrite oxidizing bacteria (NOB) and ammonia oxidizing archaea (AOA). The selection was based on the taxonomic attribution. Potentially pathogenic bacteria are those belonging to either the *Tenacibaculum* or *Vibrio* genus. For the nitrification target group, taxa were identified at different taxonomic levels to include unclassified sequences, as described by (Semedo et al., 2021): Thaumarchaeota, Nitrospinae, Nitrospirae, and Nitrospinota were selected at the phylum level; Nitrosomonadaceae and Nitrosococcaceae at the family level; and *Nitrosococcus*, *Nitrospirae*, *Nitrobacter*, *Candidatus Nitrotoga*, *Nitrotoga*, *Nitrospina*, *Nitrococcus*, *Nitrolancea*, *Candidatus Nitromaritima*, and *Nitromaritima* at the genus level. However, only members of the families Nitrosococcaceae, Nitrosomonadaceae and Nitrospiraceae; and the Nitrososphaeria class were identified. To evaluate the effect of the system and matrix variables on the abundance response, a two-way ANOVA test was performed, also in the R environment.

3.4. Results

The dataset generated for the downstream analysis has a minimum read count per sample (after trimming) of 10,396, a mean per sample of 28,850 and a max sample read count of 70,487. The complete list of read counts per sample and observed ASVs, before and after filtering (for nitrifying and pathogenic genera), can be found in **Table S1**.

3.1 Nitrifying and Potentially Pathogenic Genus Distribution

The relative distribution of the two target groups is presented in **Figure 2**, mean values per system and matrix can be found in **Table S2**. The potentially pathogenic genus *Tenacibaculum* (that includes a total of 81 ASVs) is most predominant in the weaning samples across all matrices (p-value < 0.05), while *Vibrio* (13 ASVs) seems to be associated with the water matrix, regardless of the RAS system (p-value < 0.05). The AOB *Nitrosomonas* (24 ASVs) is more present in the biofilm samples (both in the biofilter carriers and tank wall; p-value < 0.05), as well as the NOB *Nitrospira* (28 ASVs, p-value p-value < 0.05) and the AOA *Candidatus Nitrosopumilus* (11 ASVs, p-value p-value < 0.05). In these matrices, the average relative abundance of putative AOB and NOB are 3 and 2 times higher in the pre-ongrowing than the weaning system, respectively, while AOAs are 30 times higher in the weaning (being almost absent in the pre-ongrowing). Computed two-way ANOVA test p-values can be found in Table S3. The matrix appears to influence taxa presence and abundance. The genera *Vibrio*, *Nitrospira* and

Nitrosomonas show a cosmopolitan nature between systems, as they show a similar distribution between them. With the inverse behaviour, *Tenacibaculum* and *Candidatus Nitrosopumilus* are shaped by the systems.

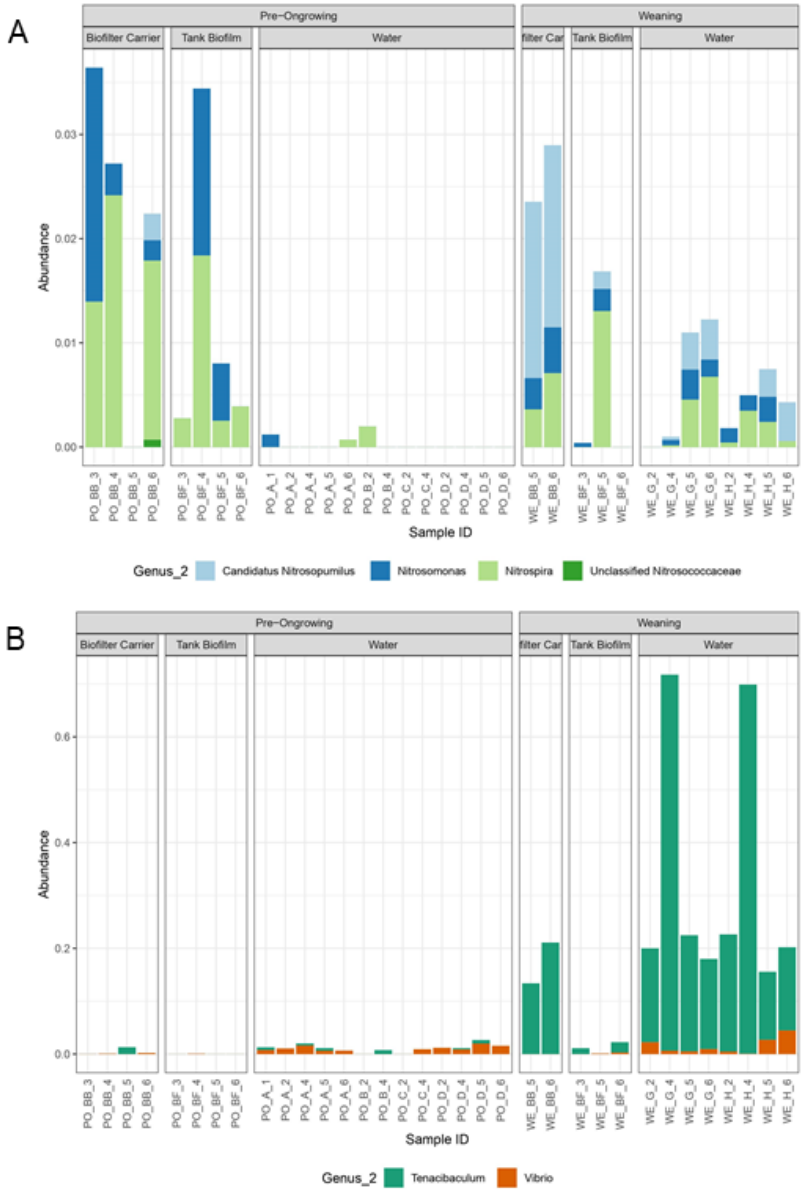

Figure 2: Relative distribution of the functional groups, genera associated with nitrification (A), the ammonia oxidizing bacteria (AOB, *Nitrosomonas*, Unclassified *Nitrosococcaceae*), nitrite oxidizing bacteria (NOB, *Nitrospira*) and ammonia oxidizing archaea (AOA, Candidatus *Nitrosopumilus*); and genera with potentially pathogenic activity (B, *Vibrio* and *Tenacibaculum*).

3.4.2 Inter-ASVs Interactions in The Pre-Ongrowing System

The spearman correlation network in the pre-ongrowing is composed by 343 ASVs (nodes/taxa) and 743 links (significant correlations) (**Figure 3**). Links were selected based on a significant correlation with one (or more) of the ASVs belonging to pathogenic or nitrifying genera. Nine subcommunities were identified with the multi-level modularity optimization algorithm. In this network, there is one genus associated with AOB, *Nitrosomonas*, with 2 ASVs and 86 correlations, and one genus associated with NOB, *Nitrospira*, with 7 ASVs and 629 correlations. The two genera associated with pathogenic outbreaks are also present in the pre-ongrowing network. *Vibrio* is present with 3 ASVs and 26 correlations, while *Tenacibaculum* has 2 ASVs with a total of 13 correlations. The remaining ASVs are classified between 110 genera in total. The complete summary of the pre-ongrowing community can be found in the **Table S4-A**, with the number of ASVs from the assigned ASV, respective number of correlations and the mean number of correlations per node.

No ASV was found to be functionally exclusive, that is, correlated only with a specific target group, and a total of 15 different phyla correlated strongly with our target groups in the pre-ongrowing. Of these, Proteobacteria and Bacteroidetes had the highest number of correlations (respectively, 361 and 182). In unclassified genera, the families Rhodobacteraceae (85 correlations), Saprospiraceae (43 correlations) and Flavobacteriaceae (39 correlations) had the highest number of correlations, and of the classified genera, Sphingorhabdus (24 correlations), Litoreibacter (22 correlations) and Blastopirellula (17 correlations) had a higher number of correlations.

The sub-communities III to IX have an individualist nature between the functions studied, meaning that only one genus from the assigned groups is present. The reverse behaviour (where there is a cluster of positive correlations between target groups, collectivist) can be found in Community I, composed of an interaction between two *Nitrospira* ASVs and in community II between four *Nitrospira* and one *Nitrosomonas*.

Although the target groups ASVs have different correlation patterns, this was not a simple consequence of different groupings by system or matrix (barplots can be consulted in **Table S5**)**.**

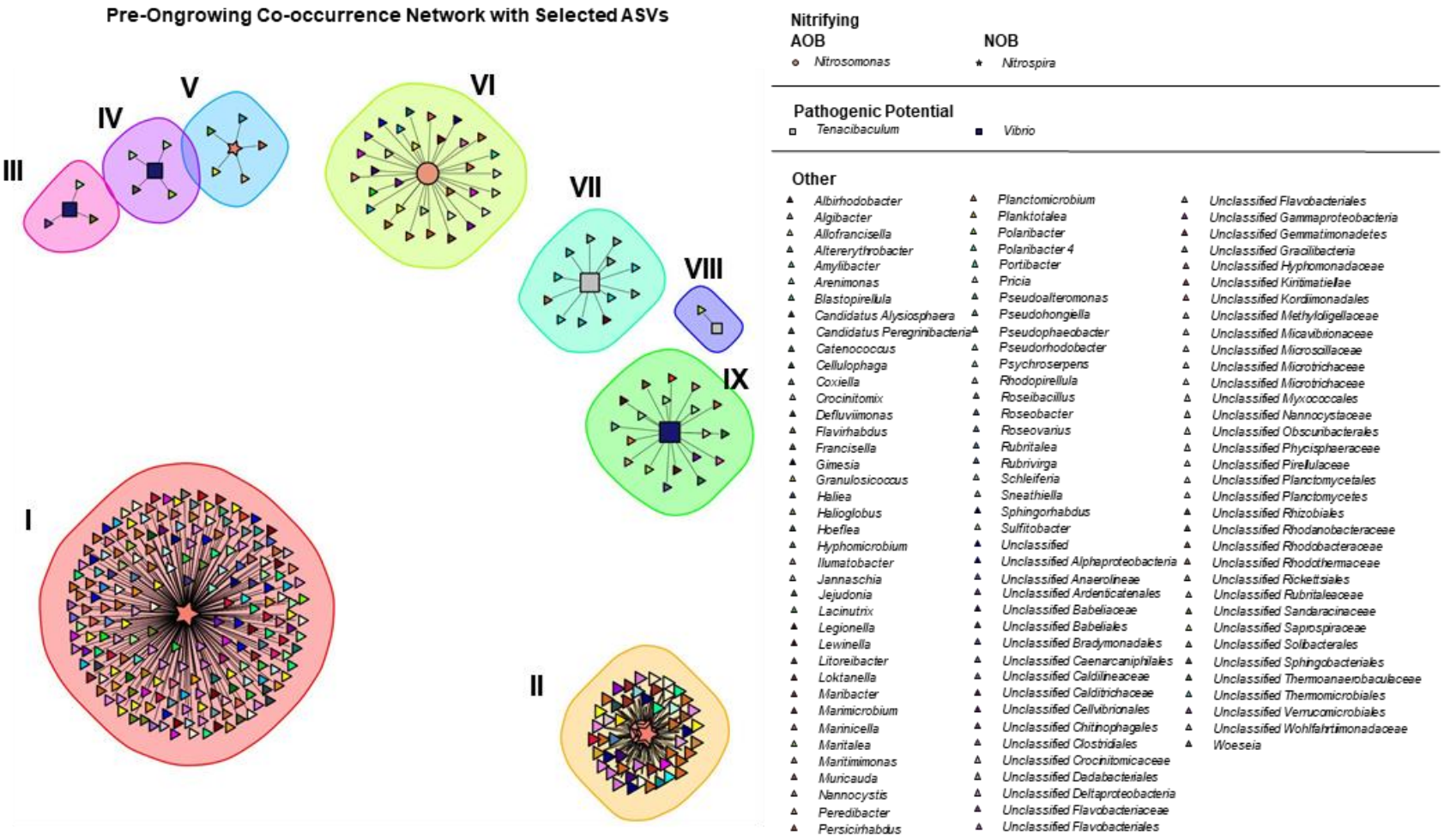

Figure 3: Correlations network in pre−ongrowing samples, coloured by genus and shapes by function: pathogenic (squares), nitrifying oxidizing bacteria (stars), ammonia oxidizing bacteria (circles) and other (triangles).

3.4.3 Inter-ASVs Interactions in The Weaning System

3.3 Inter-ASVs Interactions in The Weaning System

The weaning spearman correlation network, composed by 391 ASVs with 1164 correlations is presented in (**Figure 4**). Vertexes were selected with the same criteria as the previous system. In this system, there are three kinds of nitrifying ASVs: AOAs, AOBs, and NOB. The AOA *Candidatus Nitrosopumilus*, of the Nitrosopumilaceae family, has 6 ASVs with 351 total correlations. There is only one ASV belonging to the AOB genus *Nitrosomonas* with a total of 6 correlations. *Nitrospira*, the only NOB genus detected in this network, has 4 ASVs with 208 total correlations. As for the potentially pathogenic genera, *Tenacibaculum* is present with 56 ASVs and a total of 812 correlations and *Vibrio* with 5 ASVs, 38 total correlations. The complete summary of the community can be found in the **Table S4-B**.

No ASVs were found to be functionally exclusive, as before, correlating only with a specific target group, and a total of 12 different phyla correlated strongly with our target groups. Of these, Proteobacteria and Bacteroidetes had the highest number of correlations (respectively, 438 and 193), as seen in the pre-ongrowing. In unclassified genera, the families Ardenticatenaceae (90 correlations), Rhodobacteraceae (63 correlations), Bradymonadales (40 correlations) and Flavobacteriaceae (40 correlations) had the highest number of correlations with the selected target groups. Of the classified genera, Polaribacter 2 (23 correlations), Pseudoalteromonas (17 correlations) and Thalassobius (17 correlations) had the highest number of correlations.

Nineteen subcommunities were identified with the multi-level modularity optimization algorithm. Fifteen subcommunities did not have correlations between target groups (V to XI, XIII-XVII and XIX), while eight subcommunities did (I to IV, XII and XVIII). The cluster of the subcommunities I and II is composed of three *Vibrio* ASVs, and 40 *Tenacibaculum* ASVs. There are two subcommunities composed of exclusively inter-*Tenacibaculum* ASVs (not counting ASVs with function “other”), III and XII, with four and three ASVs, respectively. In the subcommunity XVIII, two *Nitrospira* ASVs interact with one *Tenacibaculum*. The only exclusively nitrifying target group subcommunity is IV with five *Candidatus Nitrosopumilus* (AOA) ASVs and one *Nitrospira* (NOB).

Only three ASVs had significant strong interactions in both networks, all three of them had opposite natures between systems. Two *Vibrio* ASVs (126 and 221) had no correlation with other target groups in the pre-ongrowing but had significant correlations in the weaning (with *Tenacibaculum* ASVs). The only ASV attributed to *Nitrosomonas* in both networks (323) also had no correlations with other target groups in the weaning but did have them in the pre-ongrowing (with *Nitrospira*).

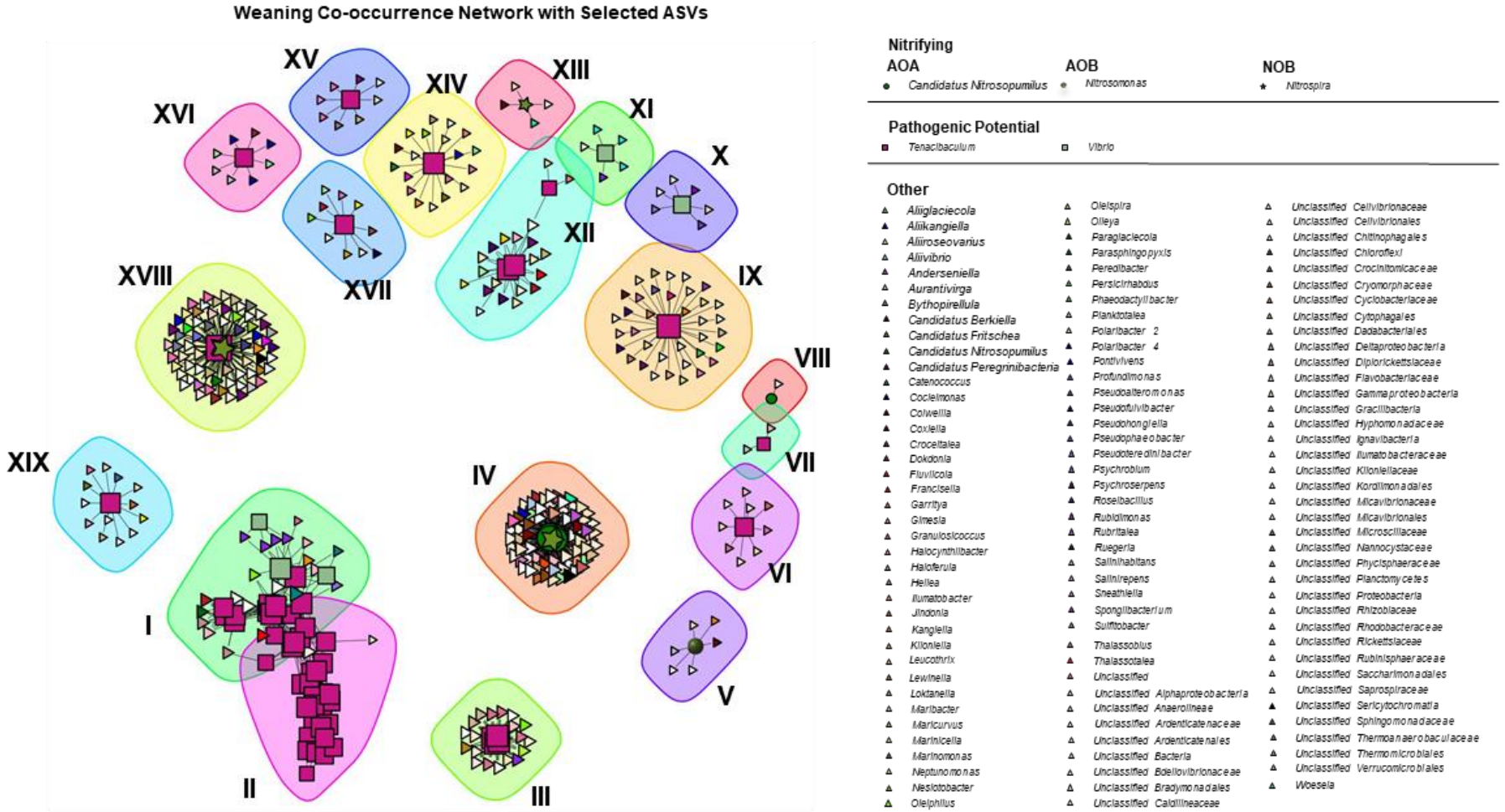

Figure 4: Correlations network in weaning samples, coloured by Genus and shapes by function: pathogenic (squares), nitrifying oxidizing bacteria (stars), ammonia oxidizing bacteria (circles), ammonia oxidizing archaea (spheres) and other (triangles).

3.5. Discussion

A previous study from our research group has generically characterized these two parallel recirculating systems two systems (pre-ongrowing and weaning) (Almeida et al., 2021), reporting that the prokaryotic communities of the sole hatchery aquaculture unit were richer in the tank biofilm and in the biofilter carriers compared with the water. Also, that the weaning had a higher prokaryotic diversity than the pre-ongrowing. In this study, we found that predominancy alters between ASVs. The potentially pathogenic *Tenacibaculum* is most abundant in the weaning system and *Vibrio* is mostly associated with water samples. The nitrifying genera (*Nitrosomonas*, *Nitrospira* and *Candidatus Nitrosopumilus*) are more predominant in the biofilms (biofilter carriers and tank wall biofilm). Additionally, the AOA *Candidatus Nitrosopumilus* appears to dominate the ammonia-oxidizing community in the weaning system, while the *Nitrosomonas* AOB are more abundant in the pre-ongrowing system.

Overall, with the lack of negative correlations, the dynamics of this prokaryotic communities appears to be of a cooperative nature. Negative correlations are associated with competition between microbes, antibiotic interactions, and lack of cooperative secretions (Fiegna & Velicer, 2005), but no significant negative correlation was found in the present study. As a RAS system, the stability of the environmental factors most likely contributes to this dynamic and a cooperative network may be a desirable trait because it promotes overall metabolic efficiency. But it has been hypothesised that it may also have a destabilizing impact due to the coupling effect where if one ASV decreases, it will also destabilize the others (Coyte et al., 2015) and so it may be beneficial to add competitors or promote diversity.

Going forward, we also need to consider the potential biases in community network studies. These are usually associated with an unequal amount of abundance-yielding material (that can be sequencing depth or a varying number of samples, or both) per sample/condition that can lead to artefactual correlations between ASVs with low-abundance with the ASVs that dominate the community (Faust & Raes, 2012). This bias results in misleading positive correlations, and it comes from a technical aspect of NGS studies, where more ASVs are detected in deeply sequenced samples, which causes a co-variation with sequencing depth (Faust et al., 2015). One way to detect this bias is by identifying cosmopolitan ASVs with a wide-spread occurrence across the samples (Faust et al., 2015), which is the case for some of the ASVs in our target groups: *Vibrio*, *Nitrosomonas* and *Nitrospira*.

The ASVs that had the highest number of correlations with our selected target groups in both networks (pre-ongrowing and weaning) were unclassified members of the families Rhodobacteraceae, Saprospiraceae, Flavobacteriaceae, Ardenticatenaceae and Bradymonadales. The diverse family Rhodobacteraceae includes both phototrophs and chemotrophs, with aerobic or facultative anaerobic metabolism (Garrity et al., 2015), mainly involved in sulphur and carbon biogeochemical cycling (Pujalte et al., 2014). Members of this family have been previously isolated from RAS biofilters (Foesel et al., 2011), which might indicate a role in sulphur and carbon nutrient recycling assigned to this family in our communities. The strictly heterotrophic and aerobic family Saprospiraceae, although without an obvious role in the community, has also been isolated from activated sludge from nutrient removal plants (Xia et al., 2008). The family Flavobacteriaceae is a diverse bacterial family (Bernardet et al., 2002) that includes the common *Solea senegalensis* pathogen *Tenacibaculum maritimum* (Avendaño-Herrera et al., 2006). The presence of unclassified members of this family could be explained by the variety of its members, including several non-pathogenic member of the genus *Tenacibaculum*. The family Ardenticatenaceae has only one thermophilic, chemoheterotrophic genus, Ardenticatena (Kawaichi et al., 2013) and, as with the family Rhodobacteraceae, likely also plays a role of sulphur and carbon nutrient recycling within the community of our RAS as well. Finally, Bradymonadales is a group of bacterial opportunistic predators in saline environments (Mu et al., 2020), which could be important in regulating global nutrient cycling in these networks as well as in creating ecological niches for interacting bacteria.

Aside from the families mentioned above, of the classified genera that dominated the correlations in our networks, *Sphingorhabdus* has previously been identified in saline bioreactors (Gao et al., 2020), but its functional role is not clear. *Litoreibacter* was isolated from seawater around an aquaculture site (Kanamuro et al., 2021) and has been positively correlated with *Tenacibaculum* (Liu et al., 2020), although in the present study it is only positively correlated with *Nitrosomonas* and *Nitrospira* (within the target groups selected). This genus is part of the Rhodobacteraceae family and as mentioned before, this family has a role as phototrophs and chemotrophs. *Blastopirellula* has been detected in marine RAS biofilters (Hüpeden et al., 2020) as a carbon degrading heterotroph. *Polaribacter* is a dominating bacteria in RAS compartments and biofilters (Rud et al., 2017) in marine RAS, and has been identified as a fish pathogen in the Norwegian Fish Health Report of 2014 (Bornø & Lie, 2015). However, we did not find references associating it with sole diseases, so its presence is potentially innocuous in our communities. The genus *Pseudoalteromonas* has been identified as a probiotic in

aquaculture with anti-*Vibrio* activity (Handayani et al., 2021), although our study found no negative correlations between them. Finally, *Thalassobius* has also been described in marine RAS communities (Michaud et al., 2009), with species characterized as aerobic chemoorganotrophic marine bacteria (Pujalte et al., 2018), justifying their role in this environment.

3.5.1. Nitrifying Prokaryotes

Regarding the nitrifying ASVs, overall, these were more abundant in biofilm samples (biofilter carriers and tank wall biofilm), where, in the pre-ongrowing system, there is one subcommunity of four *Nitrospira* ASVs interacting with one *Nitrosomonas*. Since *Nitrospira* are commonly associated with nitrite oxidation (Daims et al., 2015) and *Nitrosomonas* with ammonia oxidation (Head et al., 1993), the later result is to be expected. Ecological and metabolic cooperation between AOB and NOB is widely recognized. However, three cases of *Nitrospira* ASVs with no correlations with other target groups were found, two in the pre-ongrowing and one in the weaning system. This genus has also been reported as able to perform the complete nitrification of ammonia to nitrate (Daims et al., 2015) and this could be an indication of some ASVs that perform the complete nitrification process (comammox) in both systems studied in the present work. In the pre-ongrowing system, no interactions were found with pathogenic species, but it might be due to their relative low abundance. Further nitrifying ASVs with no correlations with other target groups are present in the weaning system, one *Candidatus Nitrosopumilus* (AOA) and one *Nitrosomonas* (AOB), and considering an eventual competition for ammonia, it is interesting to find no negative correlations between them (although the bias mentioned earlier should be considered). Previous studies demonstrated that AOAs and AOBs differentiate by niche based on ammonia concentration, where AOA outcompete AOB at relatively low concentrations (Hatzenpichler, 2012). Although the current system is overall characterized by low ammonia concentrations, the pre-ongrowing system has a higher fish density and may experience more frequent peaks of ammonia (Almeida et al., 2021). Therefore, we hypothesise that this might be the reason why we only found positive correlation between AOA and NOB in the weaning system, and only found correlations between AOB and NOB in the pre-ongrowing, the system where no AOAs were detected.

A non-conventional interaction was found between *Nitrospira* and *Tenacibaculum* in the weaning system. Nevertheless, *Tenacibaculum* is the most abundant type of node in the network (56 total) with the most correlations (812) and *Nitrospira*, although not high in abundance, is rich in interactions (208, third highest) and so an interaction between these

two groups is most likely inevitable and may not translate in a direct biological meaning. Although an argument could be made if, as a biofilm promoting genus (Romero et al., 2010), *Tenacibaculum* may promote the establishment of the autotroph and slow growing *Nitrospira*. Confirming this speculation, however, would require more empirical data and further analysis to test a causal relationship.

Only one nitrifying ASV (classified as *Nitrosomonas*) had significant strong interactions in the networks of the two systems, with opposite natures in each. *Nitrosomonas* had no correlations with other target groups in the weaning and but did so in the pre-ongrowing with *Nitrospira*. This is most likely justified by the increased abundance of *Nitrospira* in the system were *Nitrosomonas* shows a cooperative nature. A manifestation of the bias mentioned earlier.

3.5.2. Pathogenic Potential

Two ASVs, commonly associated with pathogenic outbreaks, had a significant positive interaction in the weaning system: *Tenacibaculum* and *Vibrio*. Previously (Wynne et al., 2020), a similar association was observed between them in fish displaying clinical signs of yellow mouth syndrome. Keeping in mind the bias already mentioned, and the particularly high abundance of *Tenacibaculum* in this dataset, we speculate an association between these two genera in the surrounding environment of healthy fish as well. The specific interactions between these two genera may prove relevant in disease preventions and are deserving of further studies, particularly with experimental data.

These genera are also present in individual clusters. There are five *Vibrio* clusters (three in the pre-ongrowing and two in weaning), with no correlation with other target groups, and 12 *Tenacibaculum* clusters (two in the pre-ongrowing and ten in the weaning). Their distribution is mainly in the weaning samples where *Tenacibaculum* shows a higher prevalence than in pre-ongrowing, which might be another illustration of the positive bias of network NGS studies. Only two *Vibrio* ASVs had significant strong interactions simultaneously in the networks of the two systems, and with opposite natures. *Vibrio* had no correlations with other target groups in the pre-ongrowing but did so in the weaning with *Tenacibaculum*; as previously described, this is most likely justified by the increased abundance and diversity of *Tenacibaculum* in the weaning system.

3.6. Conclusion

Some ASVs in our target groups showed a cosmopolitan nature, with wide distribution in the dataset (*Vibrio*, *Nitrosomonas* and *Nitrospira*), while others showed a behaviour shaped by the system variable (*Tenacibaculum* and *Candidatus Nitrosopumilus*). All the significant interactions found were positive. Although studies like ours are characterized by positive interaction biases, it is expected that RAS have a cooperative prokaryotic network, considering the environmental stability associated. Even if this environment promotes functional optimization, the coupling effect should also be considered as an event of one ASV destabilization may also destabilize others. Ammonia oxidizing bacteria and archaea were found to be positively interacting with nitrite oxidizing bacteria, as anticipated. Two ASVs commonly associated with pathogenic outbreaks had a significant positive interaction in one of the systems, even with no outbreaks reported. Future studies should focus on this interaction as it may prove relevant in disease preventions.

Supplementary data in this chapter can be found in Appendix B.

3.7. References

Almeida, D.B., Magalhães, C., Sousa, Z., Borges, M.T., Silva, E., Blanquet, I., Mucha, A.P., 2021. Microbial community dynamics in a hatchery recirculating aquaculture system (RAS) of sole (*Solea senegalensis*). Aquaculture. 539, 736592.

Apprill, A., McNally, S., Parsons, R., Weber, L., 2015. Minor revision to V4 region SSU rRNA 806R gene primer greatly increases detection of SAR11 bacterioplankton. Aquatic Microbial Ecology. 75, 129-137.

Avendaño-Herrera, R., Toranzo, A.E., Magariños, B., 2006. Tenacibaculosis infection in marine fish caused by *Tenacibaculum maritimum*: a review. Diseases of aquatic organisms. 71, 255-266.

Bernardet, J.-F., Nakagawa, Y., Holmes, B., 2002. Proposed minimal standards for describing new taxa of the family Flavobacteriaceae and emended description of the family. International Journal of Systematic and Evolutionary Microbiology. 52, 1049-1070.

Blancheton, J.P., Attramadal, K.J.K., Michaud, L., d'Orbcastel, E.R., Vadstein, O., 2013. Insight into bacterial population in aquaculture systems and its implication. Aquacultural engineering. 53, 30-39.

Blondel, V.D., Guillaume, J.-L., Lambiotte, R., Lefebvre, E., 2008. Fast unfolding of communities in large networks. Journal of statistical mechanics: theory and experiment. 2008, P10008.

Bornø, G., Lie, L.M., 2015. Fish Health Report 2014. Veterinary Institute, Harstad.

Canada, P., Pereira, A., Nogueira, N., Png-Gonzalez, L., Andrade, C., Xavier, R., 2020. Analysis of bacterial microbiome associated with nylon and copper nets in an aquaculture context. Aquaculture. 516, 734540.

Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Lozupone, C.A., Turnbaugh, P.J., Fierer, N., Knight, R., 2011. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. Proceedings of the national academy of sciences. 108, 4516-4522.

Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Huntley, J., Fierer, N., Owens, S.M., Betley, J., Fraser, L., Bauer, M., 2012. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. The ISME journal. 6, 1621.

Comeau, A.M., Douglas, G.M., Langille, M.G., 2017. Microbiome helper: a custom and streamlined workflow for microbiome research. MSystems. 2, e00127-00116.

Coyte, K.Z., Schluter, J., Foster, K.R., 2015. The ecology of the microbiome: networks, competition, and stability. Science. 350, 663-666.

Csardi, G., Nepusz, T., 2006. The igraph software package for complex network research. InterJournal, complex systems. 1695, 1-9.

Daims, H., Lebedeva, E.V., Pjevac, P., Han, P., Herbold, C., Albertsen, M., Jehmlich, N., Palatinszky, M., Vierheilig, J., Bulaev, A., 2015. Complete nitrification by *Nitrospira* bacteria. Nature. 528, 504-509.

Deng, Y., Ruan, Y., Ma, B., Timmons, M.B., Lu, H., Xu, X., Zhao, H., Yin, X., 2019. Multi-omics analysis reveals niche and fitness differences in typical denitrification microbial aggregations. Environ Int. 132, 105085.

Deng, Y., Ruan, Y., Taherzadeh, M.J., Chen, J., Qi, W., Kong, D., Ma, B., Xu, X., Lu, H., 2021. Carbon availability shifts the nitrogen removal pathway and microbial community in biofilm airlift reactor. Bioresour Technol. 323, 124568.

Ebeling, J.M., Timmons, M.B., Wheaton, J.M., Summerfelt, S.T., Vinci, B.J., 2002. Recirculating aquaculture, 4th ed. Ithaca Publishing Company.

Faust, K., Raes, J., 2012. Microbial interactions: from networks to models. Nature Reviews Microbiology. 10, 538-550.

Faust, K., Sathirapongsasuti, J.F., Izard, J., Segata, N., Gevers, D., Raes, J., Huttenhower, C., 2012. Microbial co-occurrence relationships in the human microbiome. PLoS computational biology. 8, e1002606.

Faust, K., Lima-Mendez, G., Lerat, J.-S., Sathirapongsasuti, J.F., Knight, R., Huttenhower, C., Lenaerts, T., Raes, J., 2015. Cross-biome comparison of microbial association networks. Frontiers in microbiology. 6, 1200.

Fiegna, F., Velicer, G.J., 2005. Exploitative and hierarchical antagonism in a cooperative bacterium. PLoS biology. 3, e370.

Foesel, B.U., Drake, H.L., Schramm, A., 2011. *Defluviimonas denitrificans* gen. nov., sp. nov., and *Pararhodobacter aggregans* gen. nov., sp. nov., non-phototrophic Rhodobacteraceae from the biofilter of a marine aquaculture. Systematic and applied microbiology. 34, 498-502.

Foesel, B.U., Gieseke, A., Schwermer, C., Stief, P., Koch, L., Cytryn, E., De La Torré, J.R., Van Rijn, J., Minz, D., Drake, H.L., 2008. *Nitrosomonas* Nm143-like ammonia oxidizers and *Nitrospira marina*-like nitrite oxidizers dominate the nitrifier community in a marine aquaculture biofilm. FEMS Microbiology Ecology. 63, 192-204.

Gao, Y., Wang, X., Li, J., Lee, C.T., Ong, P.Y., Zhang, Z., Li, C., 2020. Effect of aquaculture salinity on nitrification and microbial community in moving bed bioreactors with immobilized microbial granules. Bioresource technology. 297, 122427.

Garrity, G.M., Bell, J.A., Lilburn, T., 2015. Helicobacteraceae fam. nov. Bergey's Manual of Systematics of Archaea and Bacteria, 1-1.

Goldford, J.E., Lu, N., Bajić, D., Estrela, S., Tikhonov, M., Sanchez-Gorostiaga, A., Segrè, D., Mehta, P., Sanchez, A., 2018. Emergent simplicity in microbial community assembly. Science. 361, 469-474.

Handayani, D.P., Isnansetyo, A., Istiqomah, I., Jumina, J., 2021. Anti-Vibrio activity of *Pseudoalteromonas xiamenensis* STKMTI. 2, a new potential vibriosis biocontrol bacterium in marine aquaculture. Aquaculture Research.

Hatzenpichler, R., 2012. Diversity, physiology, and niche differentiation of ammonia-oxidizing archaea. Applied and environmental microbiology. 78, 7501-7510.

Head, I.M., Hiorns, W.D., Embley, T.M., McCarthy, A.J., Saunders, J.R., 1993. The phylogeny of autotrophic ammonia-oxidizing bacteria as determined by analysis of 16S ribosomal RNA gene sequences. Microbiology. 139, 1147-1153.

Howell, B.R., 1997. A re-appraisal of the potential of the sole*, Solea solea* (L.), for commercial cultivation. Aquaculture. 155, 355-365.

Hüpeden, J., Wemheuer, B., Indenbirken, D., Schulz, C., Spieck, E., 2020. Taxonomic and functional profiling of nitrifying biofilms in freshwater, brackish and marine RAS biofilters. Aquacultural Engineering. 90, 102094.

Infante-Villamil, S., Huerlimann, R., Jerry, D.R., 2020. Microbiome diversity and dysbiosis in aquaculture. Reviews in Aquaculture.

Jiang, W., Tian, X., Li, L., Dong, S., Zhao, K., Li, H., Cai, Y., 2019. Temporal bacterial community succession during the start-up process of biofilters in a cold-freshwater recirculating aquaculture system. Bioresour Technol. 287, 121441.

Kanamuro, M., Sato-Takabe, Y., Muramatsu, S., Hirose, S., Muramatsu, Y., Takaichi, S., Hanada, S., 2021. *Litoreibacter roseus* sp. nov., a novel bacteriochlorophyll a-containing bacterium. International Journal of Systematic and Evolutionary Microbiology. 71, 004679.

Kawaichi, S., Ito, N., Kamikawa, R., Sugawara, T., Yoshida, T., Sako, Y., 2013. *Ardenticatena maritima* gen. nov., sp. nov., a ferric iron-and nitrate-reducing bacterium of the phylum 'Chloroflexi' isolated from an iron-rich coastal hydrothermal field, and description of *Ardenticatenia classis* nov. International journal of systematic and evolutionary microbiology. 63, 2992-3002.

Liu, Q., Zhi, Y., He, Y., Ren, Z., Chen, H., Yang, R., 2020. Changes in phycospheric and environmental microbes associated with an outbreak of yellow spot disease on Pyropia yezoensis. Aquaculture. 529, 735651.

Martínez-Porchas, M., Vargas-Albores, F., 2017. Microbial metagenomics in aquaculture: a potential tool for a deeper insight into the activity. Reviews in Aquaculture. 9, 42-56.

Martins, C.I.M., Eding, E.H., Verdegem, M.C.J., Heinsbroek, L.T.N., Schneider, O., Blancheton, J.-P., d'Orbcastel, E.R., Verreth, J.A.J., 2010. New developments in recirculating aquaculture systems in Europe: A perspective on environmental sustainability. Aquacultural engineering. 43, 83-93.

Marx, C.J., 2009. Getting in touch with your friends. Science. 324, 1150-1151.

Michaud, L., Blancheton, J.-P., Bruni, V., Piedrahita, R., 2006. Effect of particulate organic carbon on heterotrophic bacterial populations and nitrification efficiency in biological filters. Aquacultural engineering. 34, 224-233.

Michaud, L., Lo Giudice, A., Troussellier, M., Smedile, F., Bruni, V., Blancheton, J.-P., 2009. Phylogenetic characterization of the heterotrophic bacterial communities inhabiting a marine recirculating aquaculture system. Journal of applied microbiology. 107, 1935-1946.

Morais, S., Aragão, C., Cabrita, E., Conceição, L.E., Constenla, M., Costas, B., Dias, J., Duncan, N., Engrola, S., Estevez, A., 2016. New developments and biological insights into the farming of *Solea senegalensis* reinforcing its aquaculture potential. Reviews in Aquaculture. 8, 227-263.

Mu, D.-S., Wang, S., Liang, Q.-Y., Du, Z.-Z., Tian, R., Ouyang, Y., Wang, X.-P., Zhou, A., Gong, Y., Chen, G.-J., 2020. *Bradymonabacteria*, a novel bacterial predator group with versatile survival strategies in saline environments. Microbiome. 8, 1-15.

Newman, M.E., 2003. The structure and function of complex networks. SIAM review. 45, 167-256.

Parada, A.E., Needham, D.M., Fuhrman, J.A., 2016. Every base matters: assessing small subunit rRNA primers for marine microbiomes with mock communities, time series and global field samples. Environmental microbiology. 18, 1403-1414.

Paungfoo, C., Prasertsan, P., Burrell, P.C., Intrasungkha, N., Blackall, L.L., 2007. Nitrifying bacterial communities in an aquaculture wastewater treatment system using fluorescence in situ hybridization (FISH), 16S rRNA gene cloning, and phylogenetic analysis. Biotechnology and bioengineering. 97, 985-990.

Petersen, C., Round, J.L., 2014. Defining dysbiosis and its influence on host immunity and disease. Cellular microbiology. 16, 1024-1033.

Piedrahita, R.H., 2003. Reducing the potential environmental impact of tank aquaculture effluents through intensification and recirculation. Aquaculture. 226, 35-44.

Pujalte, M.J., Lucena, T., Rodrigo-Torres, L., Arahal, D.R., 2018. Comparative Genomics of *Thalassobius* Including the Description of *Thalassobius activus* sp. nov., and *Thalassobius autumnalis* sp. nov. Frontiers in Microbiology. 8, 2645.

Pujalte, M.J., Lucena, T., Ruvira, M.A., Arahal, D.R., Macián, M.C., 2014. The family rhodobacteraceae. Springer.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., Glöckner, F.O., 2012. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic acids research. 41, D590-D596.

Romero, M., Avendaño-Herrera, R., Magariños, B., Cámara, M., Otero, A., 2010. Acylhomoserine lactone production and degradation by the fish pathogen *Tenacibaculum maritimum*, a member of the Cytophaga–Flavobacterium–Bacteroides (CFB) group. FEMS microbiology letters. 304, 131-139.

Ruan, Y.-J., Guo, X.-S., Ye, Z.-Y., Liu, Y., Zhu, S.-M., 2015. Bacterial community analysis of different sections of a biofilter in a full-scale marine recirculating aquaculture system. North American Journal of Aquaculture. 77, 318-326.

Rud, I., Kolarevic, J., Holan, A.B., Berget, I., Calabrese, S., Terjesen, B.F., 2017. Deep-sequencing of the bacterial microbiota in commercial-scale recirculating and semi-closed aquaculture systems for Atlantic salmon post-smolt production. Aquacultural Engineering. 78, 50-62.

Schmieder, R., Edwards, R., 2011. Quality control and pre-processing of metagenomic datasets. Bioinformatics. 27, 863-864.

Semedo, M., Lopes, E., Baptista, M.S., Oller-Ruiz, A., Gilabert, J., Tomasino, M.P., Magalhães, C., 2021. Depth Profile of Nitrifying Archaeal and Bacterial Communities in the Remote Oligotrophic Waters of the North Pacific. Frontiers in microbiology. 12, 319.

Sharma, B., Ahlert, R., 1977. Nitrification and nitrogen removal. Water Research. 11, 897-925.

Spearman, C., 1987. The proof and measurement of association between two things. The American journal of psychology. 100, 441-471.

Tal, Y., Watts, J.E.M., Schreier, S.B., Sowers, K.R., Schreier, H.J., 2003. Characterization of the microbial community and nitrogen transformation processes associated with moving bed bioreactors in a closed recirculated mariculture system. Aquaculture. 215, 187-202.

Team, R.C., 2021. R: A language and environment for statistical computing.

Toranzo, A.E., Magariños, B., Romalde, J.L., 2005. A review of the main bacterial fish diseases in mariculture systems. Aquaculture. 246, 37-61.

Wei, G., Shan, D., Li, G., Li, X., Tian, R., He, J., Shao, Z., 2020. Prokaryotic communities vary with floc size in a biofloc-technology based aquaculture system. Aquaculture. 529.

Wynne, J.W., Thakur, K.K., Slinger, J., Samsing, F., Milligan, B., Powell, J.F.F., McKinnon, A., Nekouei, O., New, D., Richmond, Z., Gardner, I., Siah, A., 2020. Microbiome Profiling Reveals a Microbial Dysbiosis During a Natural Outbreak of Tenacibaculosis (Yellow Mouth) in Atlantic Salmon. Front Microbiol. 11, 586387.

Xia, L.C., Ai, D., Cram, J., Fuhrman, J.A., Sun, F., 2013. Efficient statistical significance approximation for local similarity analysis of high-throughput time series data. Bioinformatics. 29, 230-237.

Xia, L.C., Steele, J.A., Cram, J.A., Cardon, Z.G., Simmons, S.L., Vallino, J.J., Fuhrman, J.A., Sun, F., 2011. Extended local similarity analysis (eLSA) of microbial community and other time series data with replicates, BMC systems biology. Springer, pp. 1-12.

Xia, Y., Kong, Y., Thomsen, T.R., Halkjær Nielsen, P., 2008. Identification and ecophysiological characterization of epiphytic protein-hydrolyzing Saprospiraceae ("*Candidatus Epiflobacter*" spp.) in activated sludge. Applied and environmental microbiology. 74, 2229-2238.

CHAPTER 4

The development of the sole microbiome in a hatchery life cycle, from egg to juvenile

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4. The development of the sole microbiome in a hatchery life cycle, from egg to juvenile

4.1 Abstract

Recirculating aquaculture systems (RAS) pose unique challenges in microbial community management since they rely on a stable community with key target groups, both in the RAS environment as in the host (in this case, *Solea senegalensis*). Our goal was to determine how much of the microbiome of the fish is inherited from the brooding stock, and how much is acquired during the fish life cycle in an aquaculture production batch, especially regarding potential probiotic and pathogenic groups. Our work comprises fish tissue samples from -2 days after hatching (DAH) and up to 145 DAH, and includes the live feed introduced in the first stages (from larvae to weaning). Total DNA was isolated from the different tissues, 16S rRNA gene was sequenced (V6-V8 region) using the Illumina MiSeq® platform. The output was analysed with the DADA2 pipeline, and taxonomic attribution with SILVAngs version 138.1. In non-metric multidimensional scaling analysis (NMDS) distribution of the Bray-Curtis dissimilarity index, both age and life cycle stage appeared to be drivers of the prokaryotic community dissimilarity. However, only life cycle stage was significant with homogeneity of dispersion in the Adonis test. To try to distinguish the inherited (present since the egg stage) from the acquired (detected at some later stage in the production) community, different tissues were analysed at 49, 119 and 146 DAH (gill, intestine, fin and mucus). Only a few genera were inherited (when compared with the egg and larvae community), but those that were inherited accompany the fish microbiome through the life cycle. Determining the inherited and acquired community can have direct impact in husbandry strategies and we highlight the need for these types of study in commercial aquacultures. Two genera of potentially probiotic bacteria (*Bacillus* and *Enterococcus*) are already present in the eggs, others are acquired later, in particular when feed is introduced. The potential pathogenic genera *Tenacibaculum* and *Vibrio* are inherited in the eggs, *Photobacterium* and *Mycobacterium* seem to be acquired at 49 and 119 DAH respectively. Significant co-occurrence was found between *Tenacibaculum* and both *Photobacterium* and *Vibrio*. Significantly negative correlations exist between Vibrio and *Streptococcus*, *Bacillus*, *Limosilactobacillus* and *Gardnerella*. Our work reinforces the importance of these types of life cycle studies (with samples from different stages in production for a temporal dynamic). We still need more information on this topic as repetition of patterns in different settings is essential to confirm our findings, and to find

new patterns. Aside from this, works like ours can make scientific advancements while providing improvements in production husbandry strategies.

4.2 Introduction

Recirculating aquaculture systems (RAS) have been developed to reduce water usage trough waste management, and so, making intensive fish production compatible with environmental sustainability (Piedrahita, 2003). However, these types of systems pose unique challenges in microbial community management, being extremely demanding to maintain a stable and healthy microbial community within the RAS environment (Martins et al., 2013; Schreier et al., 2010).

Microbiomes usually form specific communities in different physical and biological environments, with a dynamic and interactive nature crucial for the functioning and health of their hosts (Berg et al., 2020). Due to their dynamic nature, bacterial colonization in its host can be heavily influenced by diet and environmental conditions (Bledsoe et al., 2016; Wilkes Walburn et al., 2019). In fish, this translates, for example, in the role live feed plays in early development stages (Califano et al., 2017) as latent vectors for potential pathogenic bacteria from the *Vibrio* genus (Montanari et al., 1999; Olafsen, 2001). The gut microbiome has already been extensively studied due to its role in reinforcing the digestive and immune system of the fish (Talwar et al., 2018). The nature of the fish diet affects gut microbiome composition, thus different diets applied to the different stages of fish development, are expected to influence gut microbial communities during its life cycle (Stephens et al., 2016). In aquaculture the richness of the fish diet is higher in later stages (with commercial feed) which is conflicting with the importance of early bacterial colonization (Yukgehnaish et al., 2020). The fish microbiome development can also be affected by the environmental conditions with fish developing different profiles when they transition to RAS (Steiner et al., 2021).

There is a multiplicity of ecological processes in microbiomes that affect community assembly (Goldford et al., 2018), such as selective pressures and nutrient availability, which causes cross-feeding networks with microbes communicating and trading metabolites and services (Marx, 2009). In aquaculture, and RAS in particular, life cycle studies are still rare, although they are required to detect temporal changes of the microbiome along farming cycles to identify the core taxa for future modulation (Infante-Villamil et al., 2020).

As mentioned above, microbiome studies are important to better understand how pathogen outbreaks occur and identify dysbiosis events. The community in RAS, particularly in the biofilter (a sector for optimal but undifferentiated bacterial growth used for ammonia removal from the system), influences the farmed fish that is in constant contact with the water, with its own prokaryotic community (Verschuere et al., 2000) that also provides continuity between different physical and biological environments (host and biofilter, for example). Therefore, in this complex and interactive environment, there is a risk that disruptions may cause pathogenic outbreaks by opportunist bacteria (Blancheton et al., 2013). Groups commonly associated with disease outbreaks in sole are the *Tenacibaculum* genus (Gourzioti et al., 2016), *Vibrio* (Austin, 2010) and *Photobacterium* (Toranzo et al., 2005). The first two have also been linked in a pathogenic dysbiosis event (Wynne et al., 2020). The Mycobacteriaceae family also includes a large number of pathogenic bacteria for a number of organisms, including fish (Delghandi et al., 2020).

The prokaryotic community can also result in improved nutrition and effective disease control by inhibiting potential fish pathogens (Irianto & Austin, 2002). In aquaculture, several microbial species, mainly present in the fish gut and water, have already been identified as potentially probiotics with several health benefits such as improved fish productivity, resistance to diseases and increased immune functions (El-Saadony et al., 2021). Microbiome studies can then help to guide the best practices to promote the persistence of these agents (Borges et al., 2021). Some of the bacterial orders already identified as having potentially probiotic interest are Lactobacillales (Alonso et al., 2019) and Bifidobacteriales (Quigley, 2017). Additionally, the genera *Bacillus* (Kuebutornye et al., 2020), *Roseobacter*, *Phaeobacter*, *Paenibacillus*, *Pseudoalteromonas*, *Alteromonas*, *Pseudomonas*, *Aeromonas*, *Arthrobacter*, *Clostridium* (Ringø, 2020), *Saccharomyces* (Gaggìa et al., 2010), *Streptomyces* (Tan et al., 2019) and *Shewanella* (de La Banda et al., 2010) have also been linked to this activity.

Our goal in this paper is to start filling the gap on the microbiota analysis during fish life cycle in aquaculture. That is, to characterize the prokaryotic community along a farming cycle, accompanying a batch form egg to the pre-ongrowing stage. In this study we were able to evaluate the temporal microbiota shifts across sole life cycle, providing a reference microbiota map for this species at different stages of development. In addition, we were able to determine how much of the sole microbiome is inherited from the brooding stock, and how much is acquired in the different production stages. This work improved the background knowledge needed to develop future microbiome modulation

in sole production. Additionally, the results presented here can have a direct impact on the production husbandry strategies.

4.3 Methods

4.3.1. Sample Collection

This study was performed in partnership with an aquaculture production unit, who provided the samples, a sole hatchery (Safiestela Sustainable Aquafarming Investments, Lda.), located in Estela, Portugal. Fish density throughout the systems was 2.5 to 5 kg/m2, the pre-ongrowing system operates in a recirculating aquaculture system (RAS), from egg to weaning fish are kept in a flow-through water system. A description of the age, system, life cycle stage and feed, is presented in **Figure 1**. A feeding regime of, approximately, 2% biomass/day was followed. Fish were fed rotifers from 2 to 5 days after hatching (DAH) and brineshrimp from 7-30 DAH. Commercial Feed (CF) A, for flatfish larvae without potentially probiotic disclaimed was introduced at 31 and replaced by CF B, for nursery with supplemented potentially probiotic *Pediococcus acidilactici*, at 120 DAH. For this study, a production batch was accompanied throughout the development stages, samples were collected in duplicate. Eggs were collected at -2 DAH, larvae at 2 and 14 DAH. For juveniles, the separate tissues were collected for microbiome characterization (caudal fin, gills, mucus and intestine) at the weaning system and at the beginning and end of the pre-ongrowing. Live feed samples were also collected in duplicate. Information about temperature, salinity, and pH (at the sampling time) from where samples were collected can be found in **Table S1**.

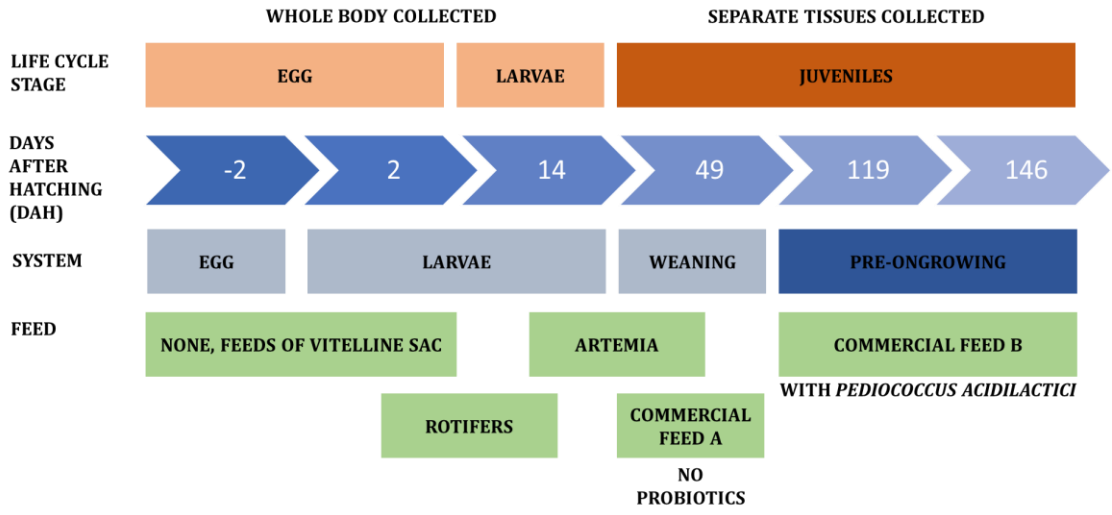


Figure 1: A resume of the age at which fish samples were collected, the system they were collected from, the life cycle stage associated and feed.

4.3.2. DNA Extraction and Sequencing

Total DNA was isolated from the different matrices (eggs, larvae, caudal fin, gills, mucus and intestine, live feed), in duplicate, with DNeasy Power Soil Kit (QIAGEN, Merck KGaA, Darmstadt, Germany). Samples were prepared for Illumina Sequencing by 16S rRNA gene amplification of the bacterial community. The DNA was amplified for the hypervariable V6-V8 region with specific primers and further reamplified in a limited-cycle PCR reaction to add sequencing adapters and dual indexes. First PCR reactions were performed for each sample using KAPA HiFi HotStart PCR Kit according to manufacturer suggestions, 0.3 μM of each PCR primer: forward primer B969F 5'-ACGCGHNRAACCTTACC -3' and reverse primer BA1406R 5'-ACGGGCRGTGWGTRCAA -3' (Michl et al., 2019) and 50 ng of template DNA in a total volume of 25 μL. The PCR conditions involved a 3 min denaturation at 95 ºC, followed by 35 cycles of 98 ºC for 20 s, 60 ºC for 30 s and 72 ºC for 30 s and a final extension at 72 ºC for 5 min. Second PCR reactions added indexes and sequencing adapters to both ends of the amplified target region according to manufacturer's recommendations (Illumina, 2013). Negative PCR controls were included for all amplification procedures. PCR products were then one-step purified and normalized using SequalPrep 96-well plate kit (ThermoFisher Scientific, Waltham, USA) (Comeau et al., 2017), pooled and pair-end sequenced in the Illumina MiSeq® sequencer with the V3 chemistry, according to manufacturer's instructions (Illumina, San Diego, CA, USA) at Genoinseq (Cantanhede, Portugal).

4.3.3. Upstream Analysis

To obtain a amplicon sequence variant (ASV) table, the DADA2 pipeline (Callahan et al., 2016) was implemented on our dataset. This was done using the R environment version 4.1.2 (R Core Team, 2019) with the package dada2 (v1.16.0). Primer removal was performed within the pipeline of DADA2 using the filterAndTrim function. Sequence filtering, trimming, error rates learning, dereplication, chimera removal and amplicon sequence variant (ASV) inference were performed with default settings. For taxonomic attribution, the SILVAngs version 138.1 (Quast et al., 2012) database was used. Taxa classified at the Kingdom level as Eukaryota, at the Order level as Chloroplast and at the Family level as Mitochondria were removed.

4.3.4. Downstream Analysis

For the general prokaryotic community analysis, the package phyloseq (v1.38.0) and ggplot2 (v3.3.5) were used for data handling and visualization. Alfa diversity was calculated using the Observed ASVs metric and the Shannon index (Spellerberg & Fedor, 2003) with vegan (v2.5-7). Beta diversity was calculated with Bray-Curtis dissimilarity index (Anderson, 2006; Bray & Curtis, 1957) and plotted with non-metric multidimensional scaling (NMDS), this was also performed for the target groups subsets (potentially pathogenic and potentially probiotic). Dissimilarity results were tested by permutational multivariate ANOVA (PERMANOVA) using the Adonis function (vegan) for beta group significance (p-values lower than 0.05) the parameters age (DAH), sample type (egg, larvae, fin, gills, mucus and intestine), life cycle stage (egg, larvae, juveniles) and system (egg, larvae, weaning, pre-ongrowing) were tested.

By core microbiome, we consider the prokaryotic genera that are common in all samples from sole life cycle, and in the samples of live feed (brineshrimp and rotifers), for a shared core microbiome analysis (Neu et al., 2021), with an abundance higher than 75% at least one sample, using the microbiome R package (v. 1.16.0). Additionally, venn diagrams were performed to analyse the membership of shared taxa across the sole life cycle with tissue samples being separated by life cycle stages. Venn diagrams were obtained using the venn R package (v. 1.10) to display the number of shared and exclusive taxa between whole body samples (egg and larvae) and each fish tissue (fin, gill, intestine, mucus) at different ages (49, 119, and 146 days).

To explore our target groups, potential probiotic and potentially pathogenic prokaryotic organisms, these groups were identified at different taxonomic levels to mitigate the effects of unclassified sequences and (in the case of probiotics) to potentially find new promising genera for further studies. For the potential probiotic group, we selected all genera from the order Lactobacillales (Alonso et al., 2019) and Bifidobacteriales (Quigley, 2017), and also the genera *Bacillus* (Kuebutornye et al., 2020), *Roseobacter*, *Phaeobacter*, *Paenibacillus*, *Pseudoalteromonas*, *Alteromonas*, *Pseudomonas*, *Aeromonas*, *Arthrobacter*, *Clostridium* (Ringø, 2020), *Saccharomyces* (Gaggìa et al., 2010), *Streptomyces* (Tan et al., 2019) and *Shewanella* (de La Banda et al., 2010). The genera *Tenacibaculum* (Gourzioti et al., 2016), *Vibrio* (Austin, 2010), *Photobacterium* (Toranzo et al., 2005) and *Mycoplasma* (Delghandi et al., 2020) were selected as potentially pathogenic as it was demonstrated in previous studies. A correlation matrix between our target groups was also built with significant correlations (Spearman pairwise, p-value < 0.05) using the R packages Hmisc (v4.1.1) and corrplot (v0.84).

4.4 Results

The 16S rRNA gene sequencing dataset used had a minimum and maximum read counts per sample (after trimming) of 7776 and 84097, respectively. The mean read counts for all samples was 32709, the complete list of read counts per sample is presented in **Table S2**.

4.4.1. General Prokaryotic Community

The most abundant Phylum were Proteobacteria (42-91%), Bacteroidetes (or Bacteroidota, 2-40%) and Firmicutes (0-39%), the complete distribution at this taxonomic level can be found in **Figure S2** and at the genus level (abundance >1%) in **Figure S3**. Overall, alpha diversity indexes do not appear to be influenced by the different phases of fish life cycle or type of fish tissue at the juvenile stage (**Figure S1** and **Table S2**). The NMDS distribution of the Bray-Curtis dissimilarity index had a stress value of 0.166 and is plotted in **Figure 2**. It shows an apparent grouping by age and life cycle stage. All the parameters tested, age (DAH), sample type (egg, larvae, fin, gills, mucus and intestine), life cycle stage (egg, larvae, juveniles) and system (egg, larvae, weaning, pre-ongrowing), had significant p-values in the Adonis test, but only the life cycle stage had a non-significant homogeneity of dispersion test. Complete results for Adonis test can be found in **Table S3**.

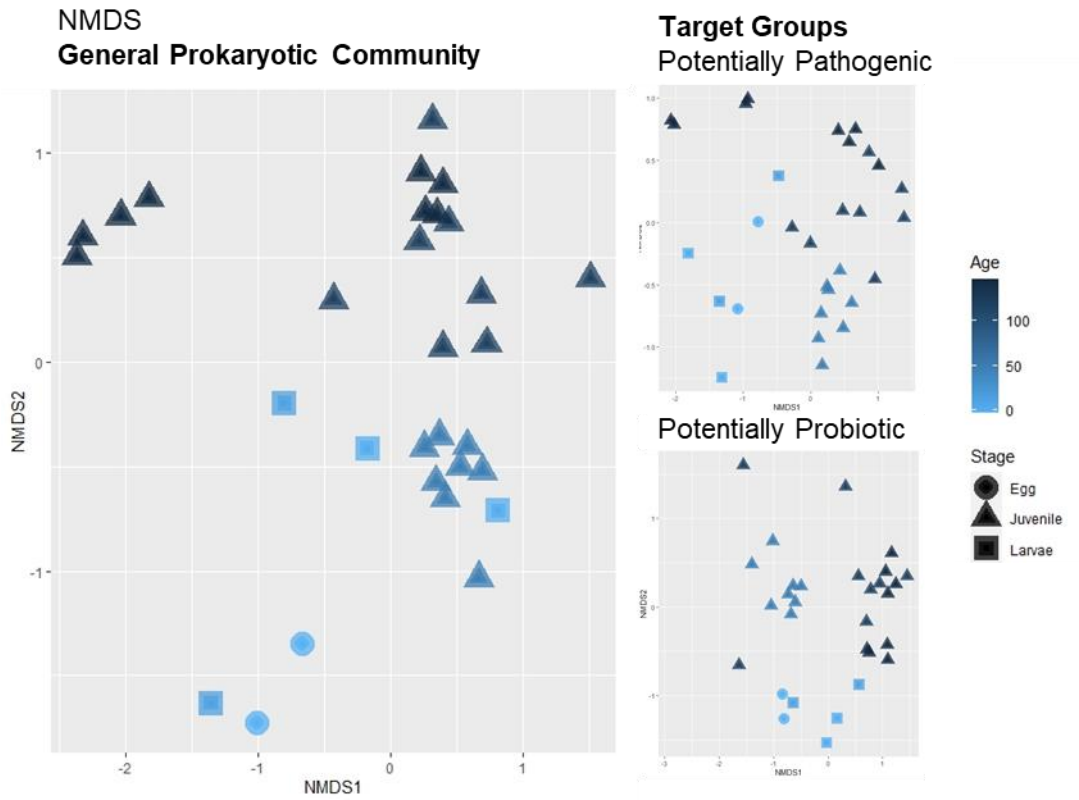


Figure 2: Beta-diversity calculated with Bray-Curtis dissimilarity index and plotted with non-metric multidimensional scaling (NMDS) was performed for the general prokaryotic community and for the subsets of the target groups (potentially pathogenic and potentially probiotic).

The core microbiome, at the genus level, can be consulted in **Figure 3**. Twelve genera are part of this core microbiome, *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*, *Vibrio*, *Pseudoalteromonas*, *Tenacibaculum*, *Cutibacterium*, *Methylobacterium-Methylorubrum*, *Delftia*, *Pseudomonas*, *Paracoccus*, *Peredibacter*, *Acinetobacter* and *Halomonas.*

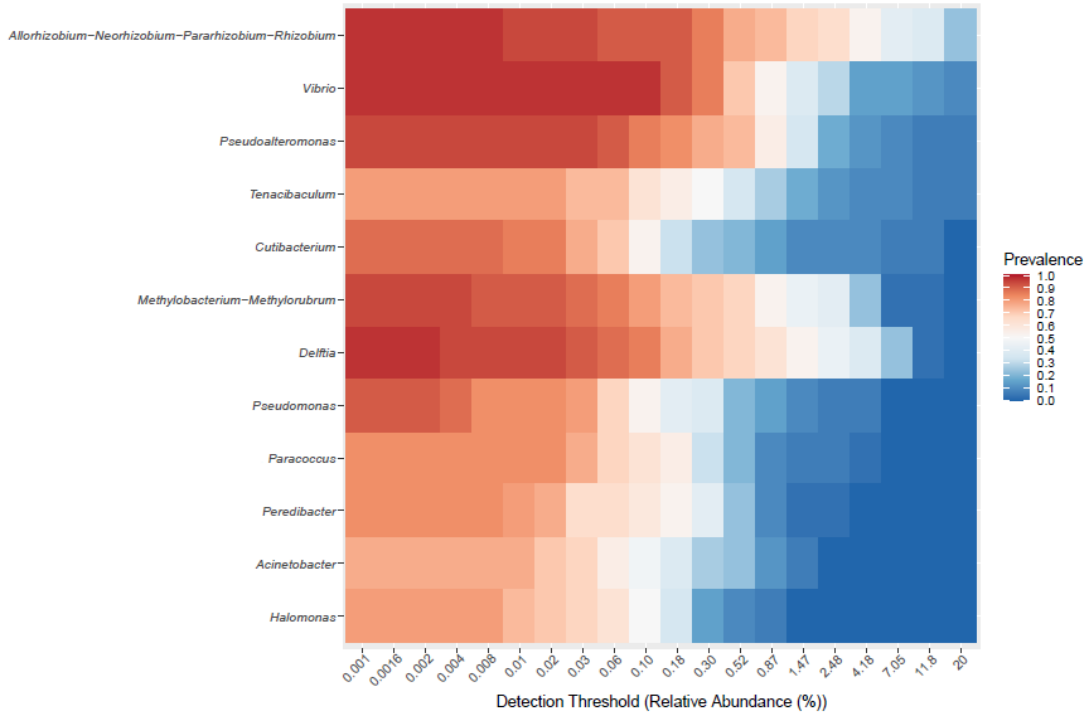


Figure 3: Members of the core microbiota were determined as prokaryotic genera that are common in all samples from sole life cycle, and in the samples of live feed (brineshrimp and rotifers), for a shared core microbiome analysis, with an abundance higher than 75% at least one sample.

Venn diagrams (**Figure 4**) were used to distinguish the inherited from the acquired community along the fish life cycle, by analysing the shared genera across samples. In the caudal fin prokaryotic community, there are ten genera (that represent 2.9% of the genera in this analysis) that are present across the entire life cycle (*Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*, *Cutibacterium*, *Delftia*, *Halomonas*, *Marinobacter*, *Methylobacterium-Methylorubrum*, *Pseudoalteromonas*, *Sulfitobacter*, Unclassified Cryomorphaceae, *Vibrio*). In the gills, there are eight genera (2.2%) present across all samples (*Halomonas*, *Marinobacter*, *Phaeobacter*, *Pseudoalteromonas*, *Roseovarius*, *Tenacibaculum*, Unclassified Cryomorphaceae, *Vibrio*). A total of eleven genera (2.7%) were present across the whole samples in the intestine (*Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*, *Colwellia*, *Cutibacterium*, *Delftia*, *Methylobacterium-Methylorubrum*, *Octadecabacter*, *Pseudoalteromonas*, *Roseovarius*,

Tenacibaculum, *Vibrio*, *Yoonia-Loktanella*) and ten genera (2.9%) in the mucus (*Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*, *Delftia*, *Halomonas*, *Marinobacter*, *Methylobacterium-Methylorubrum*, *Pseudoalteromonas*, *Roseovarius*, *Tenacibaculum*, *Vibrio*, *Yoonia-Loktanella*). There is no apparent tendency in the number of exclusive genus per tissue, with numbers varying between ages.

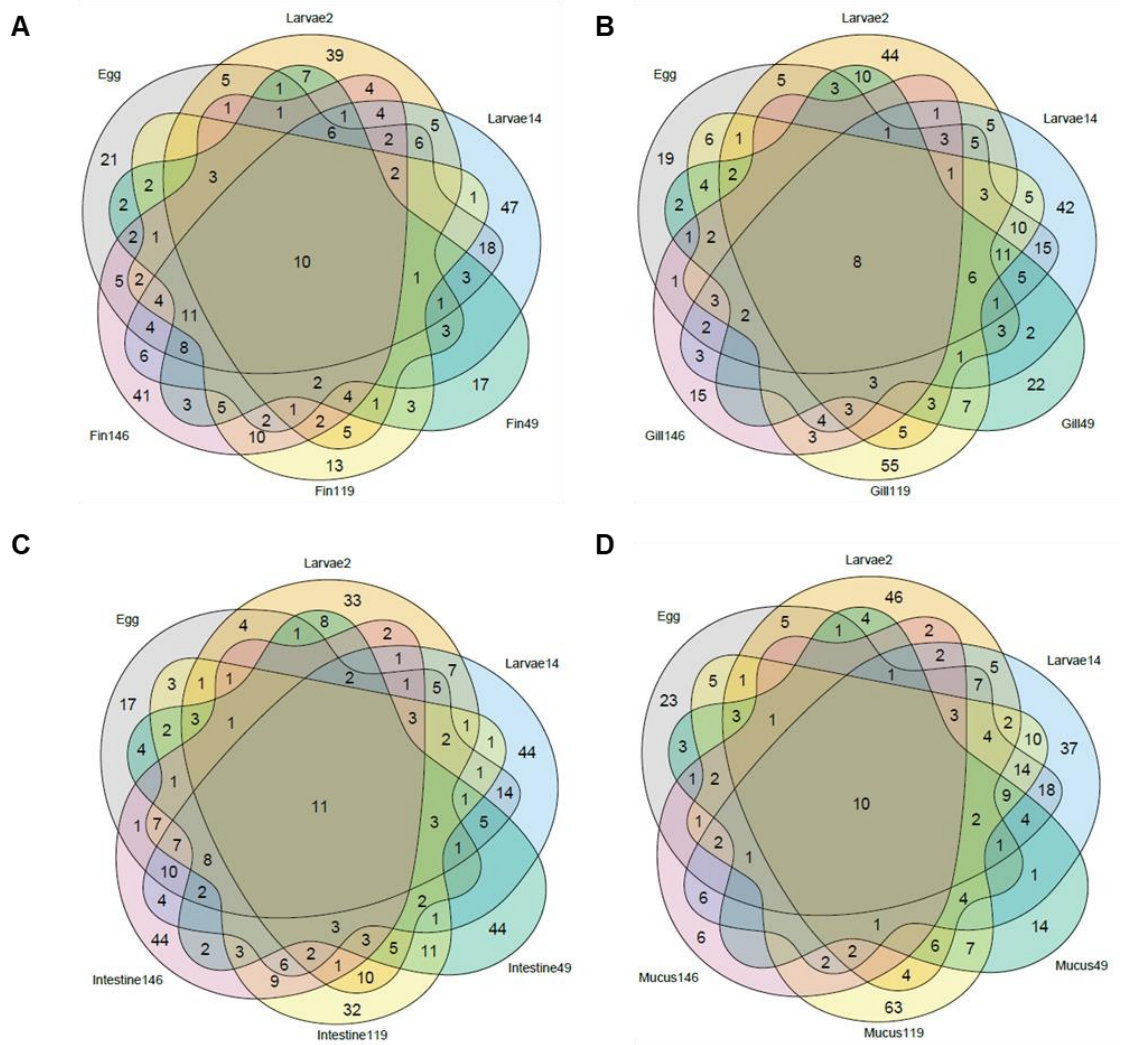


Figure 4: Venn diagram of the shared genera between whole body samples (Egg and Larvae) and different types of tissue collected from later stages of the sole: fin (A), gill (B), intestine (C) and mucus (D).

4.4.2. Target prokaryotic Groups

Relative abundance of genus distribution of the target groups can be seen in **Figure 5** and **Table S4**. For the genera associated with potential potentially probiotic bacteria, it was observed that sequences from *Bacillus*, *Enterococcus*, *Phaeobacter*, *Pseudoalteromonas*, *Pseudomonas* and *Shewanella* were already present in the eggs. *Shewanella* disappears at 2 DAH and was only detected again when commercial feed was introduced. *Bacillus* and *Enterococcus* also drop below the detection limit (no

sequences obtained) at 14 DAH, and only the first re-emerges when commercial feed is introduced. The remaining three genera were present throughout the life of the sole in the hatchery. Results showed that more potentially probiotic genera were introduced after hatching, at 2 DAH. At this stage of the life cycle the genera *Alteromonas*, *Sreptococcus*, *Gardnerella*, *Sreptomyces*, *Pedococcus*, *Granulicatela*, *Lactobacilus* emerged, but only the first was detected at 14 DAH (aside from *Phaeobacter*, *Pseudoalteromonas*, *Pseudomonas*). However, most of the other (except for *Gardnerella*) re-emerged when commercial feed was introduced. At this stage, 19 new genera appeared for the first time: *Weissella*, *Vagococcus*, *Aerophaera*, *Roseobacter*, *Latilactobacillus*, *Limosilactobacillus*, *Aeromonas*, *Bifidobacterium*, *Lactococcus*, *Leuconostoc*, *Carnobacterium*, *Ligilactobacillus*, *Desemzia*, *Brochothrix*, *Loigolactobacillus*, *Lactiplantibacillus*, *Dellaglioa*, *Liquorilactobacillus* and *Facklamia*. At 146 DAH only 7 potential potentially probiotic genera were detected: *Pseudoalteromonas*, *Shewanella*, *Phaeobacter*, *Pseudomonas*, *Alteromonas*, *Roseobacter* and *Aeromonas*.

In respect to the potentially pathogenic genera, *Tenacibaculum* and *Vibrio* accompany the fish microbiome through its development, from egg to 146 DAH. *Photobacterium* and *Mycoplasma* were introduced respectively at 49 DAH and 119 DAH. *Photobacterium* was also detected in brineshrimp and *Mycoplasma* was detected in both brineshrimp and rotifer samples. Regarding richness of our target groups (observed ASVs per sample, results in Figure S4), with high sample variability, there is no obvious trend. However, it is worth noticing an increase in the number of potentially probiotic genera in the intestine samples after day 49. Despite the no changes observed in total relative abundance of this group, the number of potentially probiotic genera increased from 4 at the end of the larval stage (14 DAH) to 22 and 21, in the intestine at day 49 and 119 respectively, and then back to 4 at 146 DAH.

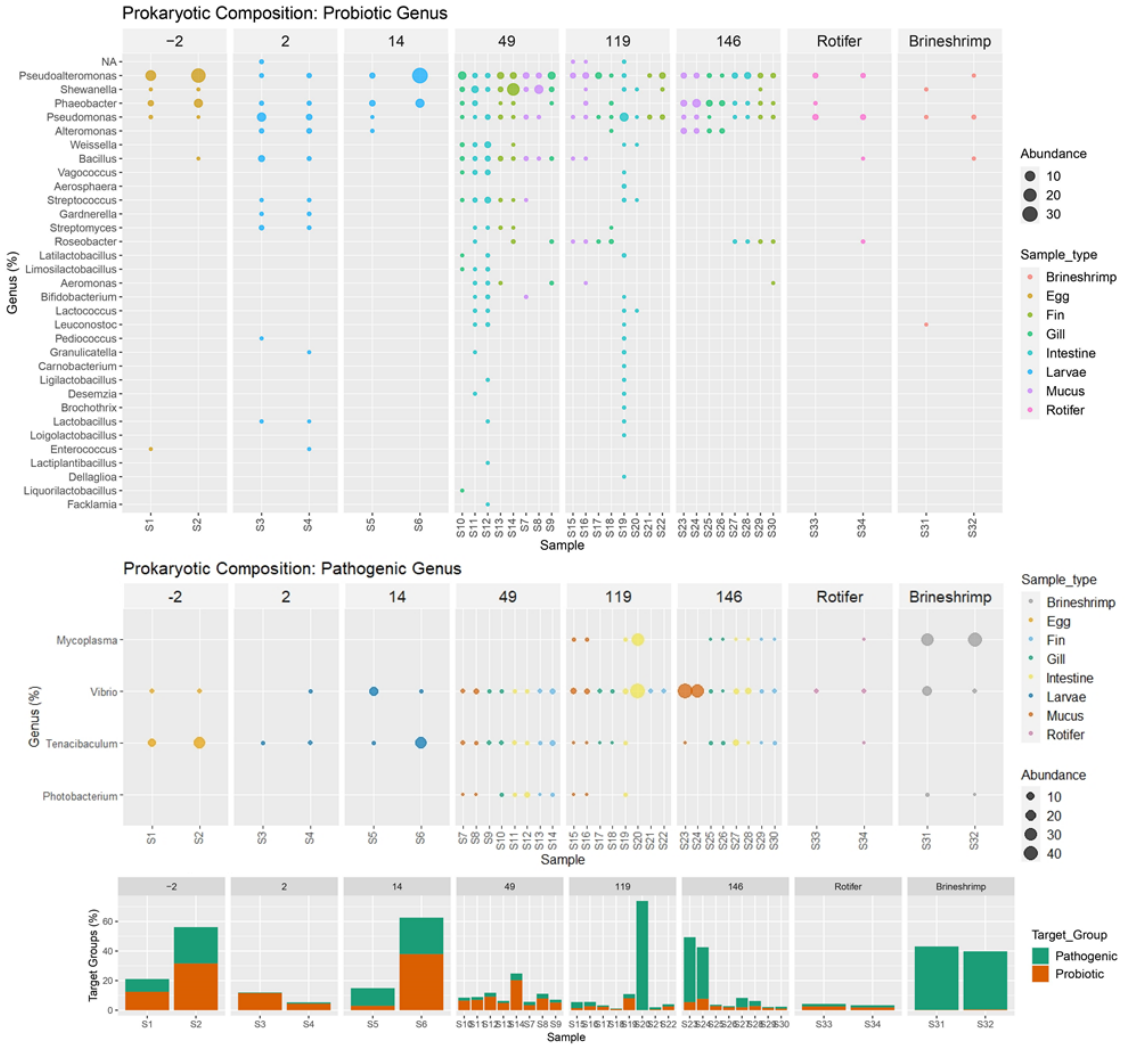


Figure 5: Relative genus distribution of the target groups (probiotic and potentially pathogenic) ordered by age and coloured by sample type (Egg, Larvae, Gill, Intestine, Mucus, Fin, Rotifer or Brineshrimp) and with a bar plot summary of overall target group composition by sample. Samples with no detectable abundance of each functional group have been removed.

The spearman correlation matrix between potentially probiotic and pathogenic genera can be found in **Figure 6**. There are no correlations between the potential pathogenic genera. Almost all correlations between potentially probiotic taxa are positive, despite two exceptions (*Alteromonas* with *Shewanella* and *Pseudomonas* with *Phaeobacter*). There are two positive correlations between *Tenacibaculum* and potentially probiotic taxa (*Pseudoalteromonas* and *Phaeobacter*) and one negative with *Pseudomonas*. There are only negative correlations between Vibrio and six potentially probiotic taxa (*Streptococcus*, *Bacillus*, *Streptomyces*, *Limosilactobacillus*, *Gardnerella* and *Lactobacilus*). Only two potentially probiotic taxa have negative correlations with *Mycoplasma*, *Pseudoalteromonas* and *Streptomyces*. Finally, *Photobacterium* has two negative correlations with *Phaeobacter* and *Alteromonas* and 14 positive correlations

with potentially probiotic taxa (*Shewanella*, *Streptococcus*, *Weissella*, *Bacillus*, *Vagococcus*, *Leuconostoc*, *Aeromonas*, *Lactococcus*, *Limosilactobacillus*, *Bifidobacterium*).

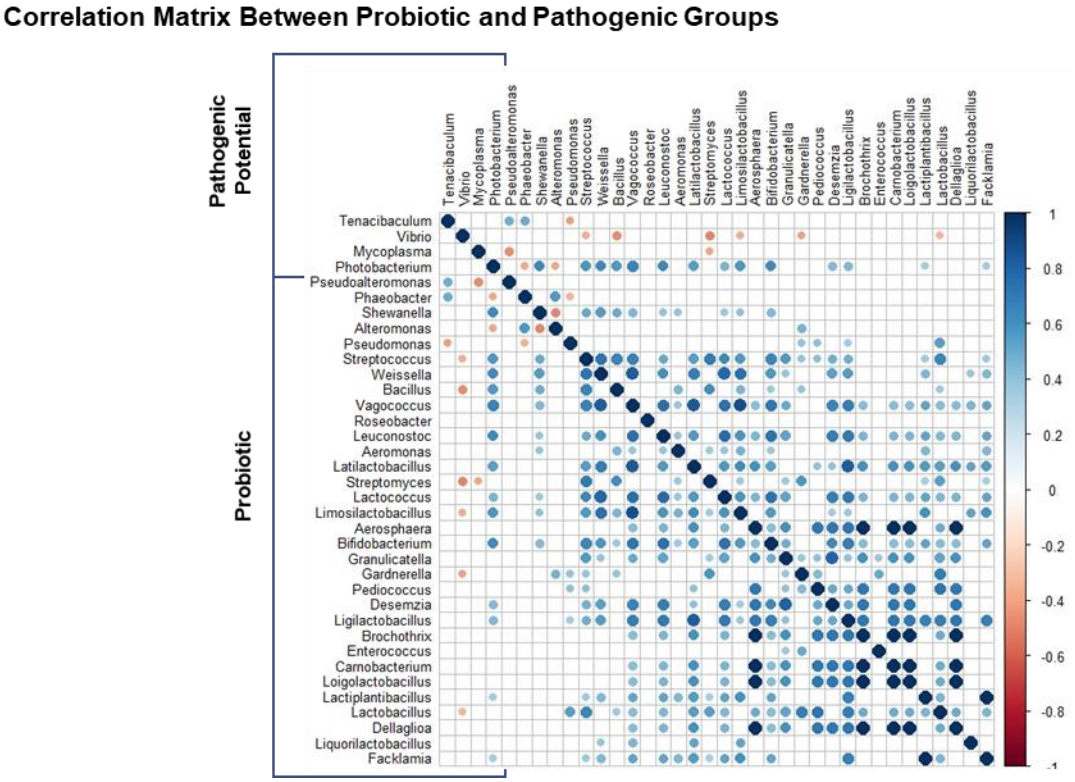


Figure 6: Species-species interactions representation by a correlation matrix of significant interactions ($p < 0.05$) between genera of the target groups: probiotic and pathogenic using Spearman pairwise correlation coefficient.

4.5 Discussion

Recirculating aquaculture systems have a unite challenge in managing a stable and functional microbial community (Martins et al., 2013; Schreier et al., 2010), with communities that are crucial for the health of the host (Berg et al., 2020) that can be heavily influenced by diet and environmental conditions (Bledsoe et al., 2016; Wilkes Walburn et al., 2019). To fill the gap in life cycle studies, crucial to improve microbiome managing strategies, we characterized the prokaryotic community along a farming cycle, form egg to the pre-ongrowing juveniles, evaluating the temporal microbiota shifts.

We found that alfa-diversity indexes did not change throughout development, although previous studies refer to a loss of bacterial species diversity when artificial feeding is introduced (Ringø et al., 2006). When analysing the structure of the prokaryotic communities through beta-diversity (NMDS), there seems to be a grouping by age and

by stage of the life cycle of the fish samples. When testing the significance of the variables, age (DAH), sample type (egg, larvae, caudal fin, gills, mucus and intestine), life cycle stage (egg, larvae, juveniles) and system (egg, larvae, weaning, pre-ongrowing) proved to be significant with the Adonis test. However, only the life cycle stage had no significant homogeneity of dispersion test, so it is the only factor that provides confidence on the Adonis test. It must also be considered that test results may fail to detect a multivariate effect unless it is expressed in high-variance taxa (Warton et al., 2012).

The most abundant phyla in this dataset, Proteobacteria, Bacteroidetes and Firmicutes are commonly found to be the most abundant in aquaculture systems (Bledsoe et al., 2016; Wilkes Walburn et al., 2019). At the genus level, it appears that there are no dominating genera across the general prokaryotic community and there is some variability in the relative abundance of genera detected between duplicates of the same sample. This variability in the prokaryotic community composition may be a consequence of the formation of different physical and biological environments with specific prokaryotic communities as described by other studies (Sylvain et al., 2020; Zhang et al., 2019).

The term “core microbiome” has become widely used in microbial ecology and it describes the set of microbial taxa that characterize a host or environment of interest (Neu et al., 2021). In this work we used a shared core microbiome analysis to infer possible conserved ecological roles and found that it was composed of twelve genera, four of them (*Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*, *Vibrio*, *Pseudoalteromonas* and *Tenacibaculum*) present in all the samples. As mentioned before, two of them are potentially pathogenic (*Vibrio* and *Tenacibaculum*). One thing to keep in mind is that both the live feed and the border fish tissues collected are in permanent contact with the water, and when studying these frontier environments it is complex to disentangle the host from the environment community, indeed they have already been identified in the water, tank biofilm and biofilter carriers in this aquaculture unit (Almeida et al., 2021).

Using venn diagrams, we found that the inherited community had very few genera represented (2.2-2.9%), all of them included *Tenacibaculum* and *Vibrio*. However, to our knowledge, this is the first time this type of characterization is performed in an aquaculture setting, other studies might be helpful since we know that in migratory wild salmon, there is a microbiota community destabilisation in migratory phases of the life cycle (Llewellyn et al., 2016). Another study found that, although deep-sea anglerfishes microbiome is dominated by the same genera from larvae to adult, its characteristic

bacterial bioluminescent symbionts were not present in the early stages and were acquired from the environment (Freed et al., 2019).

Two target groups were selected (potentially pathogenic and probiotic) as having the most impact during the fish life cycle in an aquaculture production batch. We found an increase in potentially probiotic genera when commercial feed was introduced in the diet, but interestingly most of the genera found were not reported as supplements in either of the feed formulations (except for *Pediococcus*). A theory could be that components in these formulations may act as prebiotics, that is, nutrients that are not digested by the fish that may fortify certain components of the intestinal flora by stimulating the growth and the activity of bacteria (Ringø et al., 2010). Indeed, prebiotic supplementation has shown potential as a strategy to overcome chronic stress-induced disease susceptibility in farmed S. senegalensis (Azeredo et al., 2019). Although reaching its highest number at 119 DAH, the number of potentially probiotic genera drops abruptly at 146 DAH with no change in the feed, raising the question if it was a consequence of husbandry or an unsuccessful establishment of the potential probiotic community. We should note, also, that most of the prokaryotic diversity is found in the rare biosphere (Pascoal et al., 2020), a genetic pool mostly undetected with the sequencing depth applied in this study, and some rare taxa can remain rare while others may grow abundant when the conditions change. This shift from undetectable to detectable groups may happen when the production alters the diet (specially between feeds), as the nutrients available change, diversity of certain genera increases momentarily and then declines with the stabilizing environmental conditions, explaining the drop of potentially probiotic genera at 146 DAH. Much like in the human gut microbiome, a diverse diet provides a competitive advantage to low abundant taxa, and the more diverse the microbiome, the more adaptable it will be to perturbations (Heiman & Greenway, 2016). Studies in fish also found that the gut microbiome is shaped by the environment, both by water and by formulated feed (Steiner et al., 2021). However, high inter-individual variation suggests that the host physiology itself may affect the community structure as much as environmental conditions (Fossmark et al., 2021; Hossain et al., 2021). In our study it has also been observed that some genera associated with nitrifying activity increased when fish were introduced to RAS during the pre-ongrowing production stage and other studies (van Kessel et al., 2016) have also found colonization of this group in fish tissue under similar conditions. Most probably this is a consequence of nitrifying groups circulating from biofilters to the different compartments of the RAS unit, where they were found to occur (Almeida et al., 2021)

For the potentially pathogenic genera, *Tenacibaculum* and *Vibrio*, they appear to be inherited from the brood stock, accompanying the fish microbiome through its development, from egg to 146 DAH. The other two, *Photobacterium* and *Mycoplasma*, appear to be introduced later in production. In this study they have been identified in the rotifers and brineshrimp and thus the live feed could be a potential vector as has been previously demonstrated (Hurtado et al., 2020). This early diet driven microbiome development can have a significant impact in the future fish microbiome (Wilkes Walburn et al., 2019). Differentiating which pathogenic genera are inherited from those that the fish acquires throughout production is paramount. By identifying where in the production, the fish is exposed to these groups, husbandry improvements can be implemented to control them. However, if these pathogens are inherited from a wild broodstock, it may be difficult to safely remove them in a sustainable way. However, it is important to have in mind that the genera included in this study are potentially pathogenic and not composed solely by pathogenic species. In fact, the genus *Vibrio* is an important ecological marker, as it is widely abundant in riverine, estuarine, and marine aquatic environments (Colwell, 2006) and one of the most diverse marine bacterial genera (Gomez-Gil et al., 2014). In the case of *Tenacibaculum*, out of 28 total species (Parte et al., 2020), only seven are generally associated with disease outbreaks: *T. maritimum, T. soleae, T. discolor, T. gallaicum, T. dicentrarchi, T. finnmarkense, T. ovolyticum* (Fernández-Álvarez & Santos, 2018).

In the correlation matrix, we found that six genera with potential probiotic activity were significantly negatively correlated with *Vibrio*, two of them, *Bacillus* and *Streptomyces* have already been described as potential inhibitors of *Vibrio* pathogen species (Tan et al., 2019; Vaseeharan & Ramasamy, 2003). Only one genus had a negative correlation with *Tenacibaculum* (*Pseudomonas*), two with *Mycoplasma* (*Pseudoalteromonas*, *Streptomyces*) and two with *Photobacterium* (*Phaeobacter* and *Alteromonas*). For this analysis, we must recognize the potential biases in NGS community correlation studies that may result in misleading positive correlations, derived from the fact that more taxa are detected in deeply sequenced samples and therefore taxa co-vary with sequencing depth (Faust et al., 2015). Attesting to this, the correlation matrix shows a positive interaction between *Shewanella* and *Photobacterium*, however it had been amply reported that the first increases resistance to the later (de La Banda et al., 2010; García de la Banda et al., 2012; Vidal et al., 2016). Also, it is relevant to note that *Photobacterium* has a total of 14 positive correlations with potentially probiotic taxa, which might also be a consequence of the positive bias. A similar observation occurred between *Phaeobacter* and *Tenacibaculum* (Tesdorpf et al., 2022). With the limits of these

techniques, it is unreliable to distinguish between positive bias from non-specific potentially probiotic activity with positive correlations between pathogenic and potentially probiotic taxa in our data (*Phaeobacter* and *Vibrio*) or cases like *Bacillus* that shows a negative correlation with *Vibrio* but a positive one with *Photobacterium*. The genera *Streptococcus*, *Phaeobacter* and *Liminosilactobacillus* also have a similar behaviour. Four genera had exclusive negative correlations with the potential pathogenic bacteria (*Alteromonas*, *Pseudomonas*, *Gardnerella*, *Lactobacillus*), therefore these might be the most promising for future empirical studies.

4.6. Conclusion

This work aimed at extensively defining the host community in a RAS, particularly to accompany how the fish microbiome develops throughout the production cycle. Through a description of the inherited and acquired community in the different tissues analysed (gill, intestine, fin, and mucus) at different production (egg, larvae, weaning, pre-ongrowing) and life (egg, larvae, juvenile) stages, we hope to promote the emergence of life cycle studies in aquaculture and to underscore its applicability.

We found that the prokaryotic community was significantly altered throughout the *Solea senegalensis* early development, establishing in different physical and biological environments. Two potentially probiotic genera were inherited from the broodstock (*Bacillus* and *Enterococcus*) but the main increase in potentially probiotic abundance and diversity occurs when feed is introduced in the diet (at the weaning stage), although we did not find a successful establishment of this community in the following fish development stages (146 DAH). Regarding the potentially pathogenic genera, two appear to be inherited (*Tenacibaculum* and *Vibrio*), and two are suggested to be acquired during production (*Photobacterium* and *Mycoplasma*). These results are relevant, because acquired potentially pathogenic groups may be prophylactically treated with improvement in husbandry conditions, but those that are inherited from wild broodstock may be difficult to safely eradicate in a sustainable aquaculture setting, which highlights the need for further studies to provide a deep taxonomic analysis and distinguish the pathogenic strains within this potential pathogenic genus. In the correlation analysis, we found a dynamic prokaryotic community where the potentially pathogenic genus *Tenacibaculum* positively correlates with another (*Photobacterium*), while at the same time, six genera were found with negative correlations with the genus Vibrio, one with *Tenacibaculum*, two with *Photobacterium* and another two with *Mycoplasma*. These genera with negative correlations with potentially pathogenic groups may be a promising source of future probiotic studies.

Our study has conducted a comprehensive description of the prokaryotic community in different life cycle stages of the *Solea senegalensis*, to our knowledge, the first of its kind. By analysing the composition of this community, particularly with the definition of key target groups and the definition of the inherited and acquired community in this production cycle, we have highlighted the importance of whole life cycle studies to understand the vulnerability of the stages of fish production with a direct impact in husbandry strategies. The shifts in the composition of key components of *Solea senegalensis* gut microbiome during its life cycle, open important questions related to the functional significance of the observed taxonomic changes in terms of potentially probiotic activity and pathogenic incidences in the life cycle of this fish that must be explored in future investigations.

Supplementary data in this chapter can be found in Appendix C.

4.7. References

Almeida, D.B., Magalhães, C., Sousa, Z., Borges, M.T., Silva, E., Blanquet, I., Mucha, A.P., 2021. Microbial community dynamics in a hatchery recirculating aquaculture system (RAS) of sole (*Solea senegalensis*). Aquaculture. 539, 736592.

Alonso, S., Castro, M.C., Berdasco, M., de la Banda, I.G., Moreno-Ventas, X., de Rojas, A.H., 2019. Isolation and partial characterization of lactic acid bacteria from the gut microbiota of marine fishes for potential application as probiotics in aquaculture. Probiotics and antimicrobial proteins. 11, 569-579.

Anderson, M.J., 2006. Distance-based tests for homogeneity of multivariate dispersions. Biometrics. 62, 245-253.

Austin, B., 2010. Vibrios as causal agents of zoonoses. Veterinary microbiology. 140, 310-317.

Azeredo, R., Machado, M., Martos-Sitcha, J.A., Martínez-Rodríguez, G., Moura, J., Peres, H., Oliva-Teles, A., Afonso, A., Mancera, J.M., Costas, B., 2019. Dietary tryptophan induces opposite health-related responses in the Senegalese sole *(Solea senegalensis)* reared at low or high stocking densities with implications in disease resistance. Frontiers in Physiology. 10, 508.

Berg, G., Rybakova, D., Fischer, D., Cernava, T., Vergès, M.-C.C., Charles, T., Chen, X., Cocolin, L., Eversole, K., Corral, G.H., 2020. Microbiome definition re-visited: old concepts and new challenges. Microbiome. 8, 1-22.

Blancheton, J.P., Attramadal, K.J.K., Michaud, L., d'Orbcastel, E.R., Vadstein, O., 2013. Insight into bacterial population in aquaculture systems and its implication. Aquacultural engineering. 53, 30-39.

Bledsoe, J.W., Peterson, B.C., Swanson, K.S., Small, B.C., 2016. Ontogenetic characterization of the intestinal microbiota of channel catfish through 16S rRNA gene sequencing reveals insights on temporal shifts and the influence of environmental microbes. PloS one. 11, e0166379.

Borges, N., Keller-Costa, T., Sanches-Fernandes, G.M., Louvado, A., Gomes, N.C., Costa, R., 2021. Bacteriome Structure, Function, and Probiotics in Fish Larviculture: The Good, the Bad, and the Gaps. Annual Review of Animal Biosciences. 9, 423-452.

Bray, J.R., Curtis, J.T., 1957. An ordination of the upland forest communities of southern Wisconsin. Ecological monographs. 27, 326-349.

Califano, G., Castanho, S., Soares, F., Ribeiro, L., Cox, C.J., Mata, L., Costa, R., 2017. Molecular taxonomic profiling of bacterial communities in a gilthead seabream (*Sparus aurata*) hatchery. Frontiers in microbiology. 8, 204.

Callahan, B.J., Sankaran, K., Fukuyama, J.A., McMurdie, P.J., Holmes, S.P., 2016. Bioconductor workflow for microbiome data analysis: from raw reads to community analyses. F1000Research. 5.

Colwell, R., 2006. A global and historical perspective of the genus *Vibrio*. The biology of Vibrios, 1-11.

Comeau, A.M., Douglas, G.M., Langille, M.G., 2017. Microbiome helper: a custom and streamlined workflow for microbiome research. MSystems. 2, e00127-00116.

de La Banda, I.G., Lobo, C., León-Rubio, J.M., Tapia-Paniagua, S., Balebona, M.C., Moriñigo, M.A., Moreno-Ventas, X., Lucas, L.M., Linares, F., Arce, F., 2010. Influence of two closely related probiotics on juvenile Senegalese sole (*Solea senegalensis*, Kaup 1858) performance and protection against *Photobacterium damselae* subsp. *piscicida*. Aquaculture. 306, 281-288.

Delghandi, M.R., El-Matbouli, M., Menanteau-Ledouble, S., 2020. Mycobacteriosis and infections with non-tuberculous mycobacteria in aquatic organisms: A review. Microorganisms. 8, 1368.

El-Saadony, M.T., Alagawany, M., Patra, A.K., Kar, I., Tiwari, R., Dawood, M.A., Dhama, K., Abdel-Latif, H.M., 2021. The functionality of probiotics in aquaculture: An overview. Fish & Shellfish Immunology.

Faust, K., Lima-Mendez, G., Lerat, J.-S., Sathirapongsasuti, J.F., Knight, R., Huttenhower, C., Lenaerts, T., Raes, J., 2015. Cross-biome comparison of microbial association networks. Frontiers in microbiology. 6, 1200.

Fernández-Álvarez, C., Santos, Y., 2018. Identification and typing of fish pathogenic species of the genus *Tenacibaculum*. Applied microbiology and biotechnology. 102, 9973-9989.

Fossmark, R.O., Attramadal, K.J., Nordøy, K., Østerhus, S.W., Vadstein, O., 2021. A comparison of two seawater adaptation strategies for Atlantic salmon post-smolt (*Salmo salar*) grown in recirculating aquaculture systems (RAS): Nitrification, water and gut microbiota, and performance of fish. Aquaculture. 532, 735973.

Freed, L.L., Easson, C., Baker, L.J., Fenolio, D., Sutton, T.T., Khan, Y., Blackwelder, P., Hendry, T.A., Lopez, J.V., 2019. Characterization of the microbiome and bioluminescent symbionts across life stages of Ceratioid Anglerfishes of the Gulf of Mexico. FEMS microbiology ecology. 95, fiz146.

Gaggìa, F., Mattarelli, P., Biavati, B., 2010. Probiotics and prebiotics in animal feeding for safe food production. International journal of food microbiology. 141, S15-S28.

García de la Banda, I., Lobo, C., Chabrillón, M., León-Rubio, J.M., Arijo, S., Pazos, G., María Lucas, L., Moriñigo, M.Á., 2012. Influence of dietary administration of a probiotic strain *Shewanella putrefaciens* on Senegalese sole (*Solea senegalensis*, Kaup 1858) growth, body composition and resistance to *Photobacterium damselae* subsp *piscicida*. Aquaculture Research. 43, 662-669.

Goldford, J.E., Lu, N., Bajić, D., Estrela, S., Tikhonov, M., Sanchez-Gorostiaga, A., Segrè, D., Mehta, P., Sanchez, A., 2018. Emergent simplicity in microbial community assembly. Science. 361, 469-474.

Gomez-Gil, B., Thompson, C., Matsumura, Y., Sawabe, T., Iida, T., Christen, R., Thompson, F., Sawabe, T., 2014. The family Vibrionaceae. The prokaryotes. 9, 659-747.

Gourzioti, E., Kolygas, M., Athanassopoulou, F., Babili, V., 2016. Tenacibaculosis in aquaculture farmed marine fish. Journal of the Hellenic Veterinary Medical Society. 67, 21-32.

Heiman, M.L., Greenway, F.L., 2016. A healthy gastrointestinal microbiome is dependent on dietary diversity. Molecular metabolism. 5, 317-320.

Hossain, M., Dai, J., Qiu, D., 2021. European eel (*Anguilla anguilla*) GI tract conserves a unique metagenomics profile in the recirculation aquaculture system (RAS). Aquaculture International. 29, 1529-1544.

Hurtado, L., Miranda, C.D., Rojas, R., Godoy, F.A., Añazco, M.A., Romero, J., 2020. Live feeds used in the larval culture of red cusk eel, *Genypterus chilensis*, carry high levels

of antimicrobial-resistant bacteria and antibiotic-resistance genes (ARGs). Animals. 10, 505.

Illumina, I., 2013. 16S Metagenomic sequencing library preparation. Preparing 16S Ribosomal RNA Gene Amplicons for the Illumina MiSeq System, 1-28.

Infante-Villamil, S., Huerlimann, R., Jerry, D.R., 2020. Microbiome diversity and dysbiosis in aquaculture. Reviews in Aquaculture.

Irianto, A., Austin, B., 2002. Probiotics in aquaculture. Journal of fish diseases. 25, 633-642.

Kuebutornye, F.K., Abarike, E.D., Lu, Y., Hlordzi, V., Sakyi, M.E., Afriyie, G., Wang, Z., Li, Y., Xie, C.X., 2020. Mechanisms and the role of probiotic *Bacillus* in mitigating fish pathogens in aquaculture. Fish physiology and biochemistry, 1-23.

Llewellyn, M.S., McGinnity, P., Dionne, M., Letourneau, J., Thonier, F., Carvalho, G.R., Creer, S., Derome, N., 2016. The biogeography of the Atlantic salmon (*Salmo salar*) gut microbiome. The ISME journal. 10, 1280-1284.

Martins, P., Cleary, D.F., Pires, A.C., Rodrigues, A.M., Quintino, V., Calado, R., Gomes, N.C., 2013. Molecular analysis of bacterial communities and detection of potential pathogens in a recirculating aquaculture system for *Scophthalmus maximus* and *Solea senegalensis*. PloS one. 8, e80847.

Marx, C.J., 2009. Getting in touch with your friends. Science. 324, 1150-1151.

Michl, S.C., Beyer, M., Ratten, J.-M., Hasler, M., LaRoche, J., Schulz, C., 2019. A diet-change modulates the previously established bacterial gut community in juvenile brown trout (*Salmo trutta*). Scientific reports. 9, 2339.

Montanari, M.P., Pruzzo, C., Pane, L., Colwell, R.R., 1999. Vibrios associated with plankton in a coastal zone of the Adriatic Sea (Italy). FEMS Microbiology Ecology. 29, 241-247.

Neu, A.T., Allen, E.E., Roy, K., 2021. Defining and quantifying the core microbiome: Challenges and prospects. Proc Natl Acad Sci U S A. 118.

Olafsen, J.A., 2001. Interactions between fish larvae and bacteria in marine aquaculture. Aquaculture. 200, 223-247.

Parte, A.C., Carbasse, J.S., Meier-Kolthoff, J.P., Reimer, L.C., Göker, M., 2020. List of Prokaryotic names with Standing in Nomenclature (LPSN) moves to the DSMZ. International Journal of Systematic and Evolutionary Microbiology. 70, 5607.

Pascoal, F., Magalhaes, C., Costa, R., 2020. The Link Between the Ecology of the Prokaryotic Rare Biosphere and Its Biotechnological Potential. Front Microbiol. 11, 231.

Piedrahita, R.H., 2003. Reducing the potential environmental impact of tank aquaculture effluents through intensification and recirculation. Aquaculture. 226, 35-44.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., Glöckner, F.O., 2012. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic acids research. 41, D590-D596.

Quigley, E.M.M., 2017. Bifidobacteria as Probiotic Organisms: An Introduction, The microbiota in gastrointestinal pathophysiology. Elsevier, pp. 125-126.

Ringø, E., 2020. Probiotics in shellfish aquaculture. Aquaculture and Fisheries. 5, 1-27.

Ringø, E., Sperstad, S., Myklebust, R., Refstie, S., Krogdahl, Å., 2006. Characterisation of the microbiota associated with intestine of Atlantic cod (*Gadus morhua* L.): the effect of fish meal, standard soybean meal and a bioprocessed soybean meal. Aquaculture. 261, 829-841.

Ringø, E., Olsen, R., Gifstad, T., Dalmo, R., Amlund, H., Hemre, G.I., Bakke, A., 2010. Prebiotics in aquaculture: a review. Aquaculture Nutrition. 16, 117-136.

Schreier, H.J., Mirzoyan, N., Saito, K., 2010. Microbial diversity of biological filters in recirculating aquaculture systems. Current opinion in biotechnology. 21, 318-325.

Spellerberg, I.F., Fedor, P.J., 2003. A tribute to Claude Shannon (1916–2001) and a plea for more rigorous use of species richness, species diversity and the 'Shannon–Wiener'Index. Global ecology and biogeography. 12, 177-179.

Steiner, K., Heasman, K., Laroche, O., Pochon, X., Preece, M., Bowman, J.P., Walker, S.P., Symonds, J.E., 2021. The microbiome of Chinook salmon (*Oncorhynchus tshawytscha*) in a recirculation aquaculture system. Aquaculture. 534, 736227.

Stephens, W.Z., Burns, A.R., Stagaman, K., Wong, S., Rawls, J.F., Guillemin, K., Bohannan, B.J., 2016. The composition of the zebrafish intestinal microbial community varies across development. The ISME journal. 10, 644-654.

Sylvain, F.-É., Holland, A., Bouslama, S., Audet-Gilbert, É., Lavoie, C., Val, A.L., Derome, N., 2020. Fish skin and gut microbiomes show contrasting signatures of host species and habitat. Applied and environmental microbiology. 86, e00789-00720.

Talwar, C., Nagar, S., Lal, R., Negi, R.K., 2018. Fish gut microbiome: current approaches and future perspectives. Indian journal of microbiology. 58, 397-414.

Tan, L.T.-H., Lee, L.-H., Goh, B.-H., 2019. The bioprospecting of anti-*Vibrio Streptomyces* species: Prevalence and applications. Progress In Microbes & Molecular Biology. 2.

Tesdorpf, J.E., Geers, A.U., Strube, M.L., Gram, L., Bentzon-Tilia, M., 2022. Roseobacter group probiotics exhibit differential killing of fish pathogenic *Tenacibaculum* species. Applied and Environmental Microbiology, aem. 02418-02421.

Toranzo, A.E., Magariños, B., Romalde, J.L., 2005. A review of the main bacterial fish diseases in mariculture systems. Aquaculture. 246, 37-61.

van Kessel, M.A., Mesman, R.J., Arshad, A., Metz, J.R., Spanings, F.T., van Dalen, S.C., van Niftrik, L., Flik, G., Wendelaar Bonga, S.E., Jetten, M.S., 2016. Branchial nitrogen cycle symbionts can remove ammonia in fish gills. Environmental microbiology reports. 8, 590-594.

Vaseeharan, B., Ramasamy, P., 2003. Control of pathogenic *Vibrio* spp. by *Bacillus subtilis* BT23, a possible probiotic treatment for black tiger shrimp *Penaeus monodon*. Letters in applied microbiology. 36, 83-87.

Verschuere, L., Rombaut, G., Sorgeloos, P., Verstraete, W., 2000. Probiotic bacteria as biological control agents in aquaculture. Microbiology and molecular biology reviews. 64, 655-671.

Vidal, S., Tapia-Paniagua, S.T., Moriñigo, J.M., Lobo, C., de la Banda, I.G., del Carmen Balebona, M., Moriñigo, M.Á., 2016. Effects on intestinal microbiota and immune genes of *Solea senegalensis* after suspension of the administration of *Shewanella putrefaciens* Pdp11. Fish & shellfish immunology. 58, 274-283.

Warton, D.I., Wright, S.T., Wang, Y., 2012. Distance-based multivariate analyses confound location and dispersion effects. Methods in Ecology and Evolution. 3, 89-101.

Wilkes Walburn, J., Wemheuer, B., Thomas, T., Copeland, E., O'Connor, W., Booth, M., Fielder, S., Egan, S., 2019. Diet and diet-associated bacteria shape early microbiome development in Yellowtail Kingfish (*Seriola lalandi*). Microbial biotechnology. 12, 275-288.

Wynne, J.W., Thakur, K.K., Slinger, J., Samsing, F., Milligan, B., Powell, J.F.F., McKinnon, A., Nekouei, O., New, D., Richmond, Z., Gardner, I., Siah, A., 2020. Microbiome Profiling Reveals a Microbial Dysbiosis During a Natural Outbreak of Tenacibaculosis (Yellow Mouth) in Atlantic Salmon. Front Microbiol. 11, 586387.

Yukgehnaish, K., Kumar, P., Sivachandran, P., Marimuthu, K., Arshad, A., Paray, B.A., Arockiaraj, J., 2020. Gut microbiota metagenomics in aquaculture: Factors influencing gut microbiome and its physiological role in fish. Reviews in Aquaculture. 12, 1903-1927.

Zhang, Z., Li, D., Xu, W., Tang, R., Li, L., 2019. Microbiome of co-cultured fish exhibits host selection and niche differentiation at the organ scale. Frontiers in microbiology. 10, 2576.

CHAPTER 5

Preliminary studies on exploring microbiome-based solutions to improve water quality and fish welfare in RAS

5. Preliminary studies on exploring microbiome-based solutions to improve water quality and fish welfare in RAS

5.1 Introduction

Accounting to its importance in providing a nutritious diet, fish consumption has risen from 9.0 kg (live weight equivalent) in 1961 to 20.3 kg in 2017 (Stankus, 2021). To minimize the need to resource to fishing stocks, developments have been made in aquaculture technologies to contribute to an increment on fish availability. Recirculating Aquaculture Systems (RAS) are a technology, that optimize waste management (using a series of water treatment processes) to reduce water consumption and subsequent waste (Piedrahita, 2003). For species such as the sole (*Solea senegalensis*), identified as a suitable candidate species for aquaculture diversification, RAS have been suggested to help face some of the main challenges in disease mortality, pigmentation abnormalities and malformations (Dinis et al., 1999). However, intensive production has been slow to take off, mostly attributed to serious disease problems (Morais et al., 2016). Tenacibaculosis, caused by the bacteria *Tenacibaculum maritimum*, has been described as highly contagious and the cause of significant mortality with the only treatments available being formalin and antibiotics (Toranzo et al., 2005).

RAS are an intensive fish production, operating in a controlled environment, with little impact of weather conditions, highly productive and water efficient. Usually associated with lower environmental impacts, their biggest drawback is the high energy needed, and because of this, main developments focus on technical improvements of the recirculation loops and recycling of nutrients (Martins et al., 2010).

Inside RAS, the main waste produced is ammonia, released into the water as a fish metabolite derived from the protein catabolism (Ebeling et al., 2002). Both ammonia and its subsequent component in the nitrification process, nitrite, are toxic to aquatic organisms and cannot accumulate within a RAS environment at the cost of negative impacts in animal health, growth, and survival rates (Ebeling et al., 2002). Inside the water treatment station of the recirculation loop, a beneficial microbial community is responsible for the removal of these compounds. The beneficial microbial community is growing in the biofilter, a station equipped with heavy aeration and plastic media to provide a large superficial area for the biofilm to adhere to (Ebeling et al., 2002). However, the complexity of the microbial interactions within this beneficial community

(Schreier et al., 2010) and its sensitive to variations in culture conditions (Emparanza, 2009), make it challenge to manage. This challenge includes to find the conditions to optimize the naturally occurring nitrogen cycle, particularly microbial nitrification, where ammonia is oxidized to nitrite and subsequently to nitrate (Davidson et al., 2017).

The key bacteria groups in RAS biofilters responsible for the nitrification processes are Ammonia Oxidizing Bacteria (AOB) found in the *Nitrosococcus* genus (Koops et al., 1990) and *Nitrospira* and *Nitrosomonas* cluster (Head et al., 1993; Pommerening-Röser et al., 1996); and Nitrite Oxidizing Bacteria (NOB) attributed to the Nitrospirae and Nitrospinae phylum (Levipan et al., 2014; Semedo et al., 2021). Within the genera usually associated with NOB, *Nitrospira* has been described as able to perform complete nitrification (Daims et al., 2015), that is, the conversion of ammonia subsequently into nitrite and nitrate.

The technological improvement of the recirculation loop focuses on the biofilter management, that needs to go through long activation periods with high variations in water quality (Chen et al., 2006). To try to shorten this period, bacterial formulations are being developed and commercialized, which usually include microbial consortiums containing AOB and NOB (Patil et al., 2021). However, studies have found that most bacteria in these inocula, typically from freshwater environments, are not capable of adapting to the marine environment (Brailo et al., 2019) and colonization of the biofilter is mostly due to the environmental microbial communities (Drønen et al., 2022).

Another challenge in microbial community management in RAS, is that the unspecific promotion of bacterial growth in the biofilter can also translate in the development of potential opportunistic bacteria. Specifically for the *S. senegalensis*, the species *T. maritimum* (Gourzioti et al., 2016), *Photobacterium damsela*, *Vibrio harveyi* and *V. parahaemolyticus* are of particular concern (Morais et al., 2016). In aquaculture, there are bacteria that have been correlated with health benefits (probiotics) such as improved productivity, resistance to diseases and immune functions (El-Saadony et al., 2021) administered either in the feed (Gatesoupe, 1999) or in the water (Jahangiri & Esteban, 2018). Because of the European Union Regulation (EU) 2019/4 on Medicated Feed (European Commission, 2019) that prohibits the routine antibiotic use in animal farming, there is a wide search for probiotics targeting disease control by inhibiting the growth of specific species such as *Vibrio* (Gao et al., 2017; Handayani et al., 2021; Tan et al., 2019), *Photobacterium* (de La Banda et al., 2010), *Listonella anguillarum* or *Edwardsiella tarda* (Wanka et al., 2018). Regarding *Tenacibaculum maritimum*, same probiotic candidates have been isolated from the digestive system of three temperate flatfish

species (Wanka et al., 2018). The current challenges are to find bacteria adapted to a large range of environment conditions (Pérez-Sánchez et al., 2014) and to learn the exact mechanisms of action of the used probiotics (El-Saadony et al., 2021).

Our goal with this work was to develop biotechnological tools to modulate the bacterial community of RAS to improve or maintain water quality and fish welfare, particularly for a *S. senegalensis* hatchery. To accomplish this, first we try to isolate the nitrifying autotrophic and the heterotrophic culturable community the hatchery biofilter compartment. The nitrifying community is then used for the preliminary studies in developing a formulation to kick-start or to re-establish the biofilter activity. The heterotrophic bacteria were tested against the potentially pathogenic bacteria *T. maritimum*, to investigate new biotechnological tools for outbreak prevention.

5.2 Materials & Methods

5.2.1 Direct Isolation of Bacteria

This study was performed in partnership with an aquaculture production unit, a sole hatchery (Safiestela S.A.), located in Estela, Portugal. For both the nitrifying autotrophic and the heterotrophic culturable community, water and biofilter carrier samples were collected from the RAS. Using Saline Solution (0.85% NaCl), biofilter carries were mixed in a 1.5 mL falcon tube to release the biofilm. Both the water samples, and the biofilter carrier solution were then spread in ten-fold dilutions in the selected media.

Selective solid media for the direct isolation of nitrifying bacteria were chosen to select AOB (ammonia oxidizing bacteria) and NOB (nitrite oxidizing bacteria) as described in Elbanna et al. (2012). For AOB, the formulation was 0.5 g $(NH_4)SO_4$, 1 g K_2HPO_4, 0.03 g $FeSO_4 \cdot 7H_2O$, 0.3 g $MgSO_4 \cdot 7H_2O$, 1 g $CaCO_3$, 15 g Agar, diluted in either marine water (AM) or deionized water (AD - in which salinity was adjusted by adding 30 g NaCl). For NOB, the formulation was 0.5 g $NaNO_2$, 1 g K_2HPO_4, 0.03 g $FeSO_4 \cdot 7H_2O$, 0.1 g $MgSO_4 \cdot 7H_2O$, 1 g $CaCO_3$, 0.3 g $CaCl_2$, 15 g Agar, diluted in either marine water (NM) or deionized water (ND, with salinity adjustment).

For the heterotrophic culturable community, culture media were selected based on a previous characterization of the microbial community of the biofilter by Illumina sequencing (Almeida et al., 2021). To maximize both the number and the diversity of the bacterial strains obtained for our library, and have a representation of the endemic community, we focused on the genera *Tenacibaculum*, *Sulfitobacter*, *Leucothrix*, *Pseudoalteromonas* and *Algibacter*. The commercial medium Marine Agar (Condalab,

Madrid Spain, ref. 1059.00) was selected for the genus *Sulfitobacter* (Yoon et al., 2007), *Algibacter* (Park et al., 2013), *Leucothrix* (Zhang et al., 2015) and *Pseudoalteromonas* (Zhang et al., 2016). The medium M1 (10 g of soluble starch, 4 g yeast extract, 2 g de peptone per 1 L of marine water) was chosen for its similarity with mediums used for the isolation of *Tenacibaculum* species (Pazos et al., 1996). Commercial medium Reasoner's 2A (VWR International, Pensilvânia, EUA, ref. 84671.0500) and the medium SCN (Starch Casein Nitrate, 10 g of soluble starch, 0.3 g of casein, 2 g of K_2HPO_4, 2 g of KNO_3, 2 g of NaCl, 0.05 g of $MgSO_4.7H_2O$, 0.02 g of $CaCO_3$, 0.01 g of $FeSO_4.7H_2O$, and 17 g of agar, per L of distilled water) were also selected, the first for *Leucothrix* isolation (Baek et al., 2018) and the second has a similar formulation to another one used for *Pseudoalteromonas* isolation (Xu et al., 2010).

Incubation of the cultures was performed at room temperature (~22 °C) and at 28 °C. All the bacterial strains with different morphological features were selected and purified, and cultures were kept until no new colonies were detected. All the isolated bacteria were purified and cryopreserved at -80°C with cryopreserving media (Glycerol 25% solution), for further biotechnological applications.

5.2.2 Enrichment of nitrifying bacteria

Due to the difficulty in obtaining nitrifying bacteria through direct isolation, an enrichment protocol using microcosms was assembled. A concise illustration of the set up can be consulted in **Figure 1**. Enrichment was performed in three separate conditions: nitrite formulation using $NaNO_2$ and ammonia formulation in two forms, NH_4Cl and $(NH_4)_2SO_4$ (to test eventual differences in bioavailability). Nitrite and ammonia enrichment must be performed separately since nitrite is susceptible to inhibition by ammonia (Park & Bae, 2009).

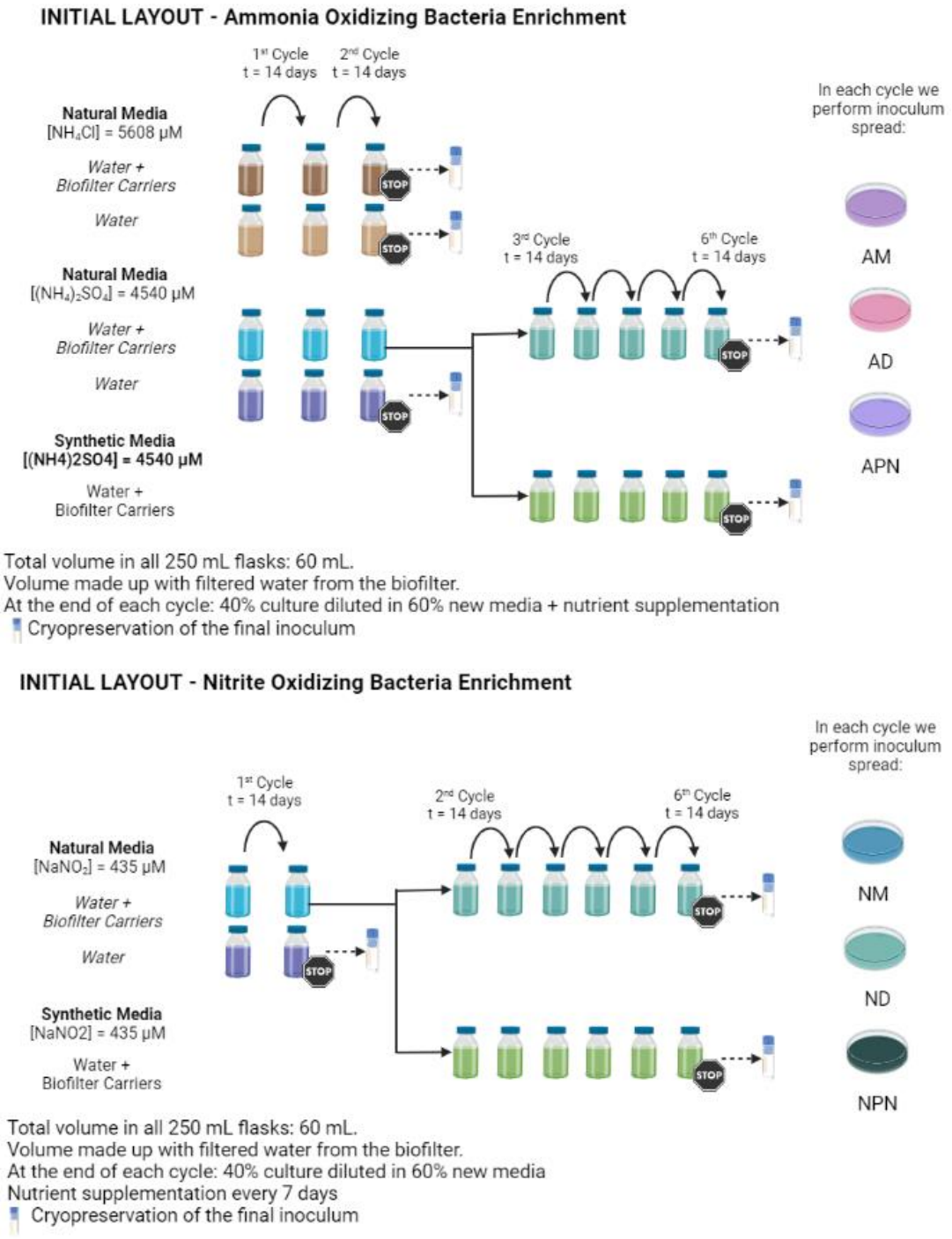


Figure 1: Illustration of the enrichment set up using microcosms for ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB). Created using BioRender.

Briefly, each microcosms consisted of 60 mL of media in 250 mL flasks, which were supplemented with the selected nutrient. The experiments were conducted in cycles of 14 days and part of the culture was diluted into a renewed media in a 2:3 (culture:media) proportion. During the experiment, ammonium supplementation was performed every 14 days, while nitrite supplementation was performed at every 7 days. Initially six

microcosms were assembled using three separate nutrient supplementations: 435 µM $NaNO_2$, 4540 µM $(NH_4)_2SO_4$, 5608 µM NH_4Cl and using filtered water from the biofilter with or without biofilter carriers included. To test if the ammonium complex could affect its availability (or cause competition with other groups such as sulphide-degrading bacteria) microcosms were kept under both conditions (4540 µM $(NH_4)_2SO4$ and 5608 µM NH_4Cl) during 2 cycles (28 days) and then, based on similar ammonia removal rates, the form $(NH_4)_2SO_4$ was selected for further enrichment.

For the natural media renewal, filtered water from the biofilter was used. To purify the water, a series of filtrations were employed, first through 1,6 µm class fibre filters (VWR International, Pensilvânia, EUA, ref. VWRI516-0861), followed by 0.45 µm mixed cellulose ester membrane filters (Whatman®, Maidstone, UK, ref. ME 25/21) and finally 0.22 µm mixed cellulose esters membrane filter (Millipore™, Massachusetts, EUA, ref. GSWP04700). This way we made sure that most of the dissolved organic matter and bacteria was removed from the natural media.

Microcosms were tested with and without biofilter carriers, to confirm that the main ammonia/nitrite removal capacity was not provided by the biofilter carriers. This experiment was also carried out for 2 cycles (28 days). At the end of the second cycle, the remaining microcosms (435 µM $NaNO_2$ and 4540 µM $(NH_4)_2SO_4$, each with biofilter carriers) were subdivided into natural and synthetic media. The later was adapted from Lipponen et al. (2002): to a common base formulation of 0.1 g K_2HPO_4, 35 g NaCl, 0.04 g $MgSO_4 \cdot 7H_2O$, 0.015 g $CaCl_2$ and 1 mL Trace Solution (3.370 g EDTA, 2.703 g $FeCl_2 \cdot 6H_2O$, 0.1362 g $MnCl_2 \cdot 4H_2O$, 0.024 g $NiCl_2 \cdot 6H_2O$, 0.024 g $CoCl_2 \cdot 6H_2O$, 0.017 g $CuCl_2 \cdot 2H_2O$, 0.068 g $ZnCl_2$, 0.024 g $Na_2MoO_4 \cdot 2H_2O$ and 0.062 g H_3BO_3 per litre of deionized water) either 0.330 g $(NH_4)SO_4$ (APN) or 0.0345 g $NaNO_2$ (NPN) are added, per litre of deionized water.

The enriched cultures were incubated at room temperature, in dark and static conditions. At the end of each cycle, culture samples were collected and spread in ten-fold dilutions into the media AM, NM, AD and ND, as well as an adaptation of the liquid media NPN e APN to solid media (with 16 g agar). Samples were also collected for ammonia and nitrite determination by spectrophotometry using standard previously described methodologies: ammonia was quantified using the Grasshoff & Johannsen (1972) method for ammonia (NH_3-N) and Grasshoff et al. (2009) for nitrite quantification. As each enrichment procedure was terminated, inocula samples were cryopreserved in cryotubes at -80 °C with cryopreserving media (Glycerol 25% solution).

5.2.3 Screening assays for inhibition of *T. maritimum*

The library of heterotrophic culturable community previously isolated (5.2.1) was partially tested to assess their potential to inhibit *T. maritimum*. This was done using two approaches: cross streak and diffusion disc inhibition tests, adapted from Yoshida et al. (2009).

Cross-streak inhibition tests were performed by streaking a *T. maritimum* strain vertically on the plate and then cross streaking with the test strain. After incubation for 48 hours at 28 °C, inhibition (if successful) is observed if the *T. maritimum* line growth is interrupted. In parallel, diffusion disc inhibition tests are performed by strain growth in liquid media, the complementation of the cross-streak test with this procedure is important to also obtain the metabolites released into the media (which might not be as well represented in plate growth) that might themselves have inhibition activity. Pure colonies of bacterial isolates were grown in liquid media during 48 hours at room temperature, after which they are centrifuged to collect the supernatant for the assay. The *T. maritimum* inoculum (grown in a MA plate for 48 hours at 28 °C) is dissolved in the liquid media and adjusted to an optical density ranging between 0.8-1.2 (λ= 600 nm) and evenly spread on the plate. Discs are then placed regularly over the *T. maritimum* spread and inoculated with the test strain supernatant (15 µL), along with negative (sterile culture media) and positive controls (Enrofloxacin, Sigma-Aldrich, Missouri, EUA, ref. 17849). After two days of incubation at 28 °C, potential inhibition halos are measured.

The most promising bacterial isolates were taxonomically identified using 16S rRNA gene sequencing. Genomic DNA was extracted using a commercial kit (E.Z.N.A.® Bacterial DNA Kit, Omega Bio-tek, Inc., Norcross, USA) followed by the amplification of the V1–V9 regions with the universal primers 27 F (5′- AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-CGGTTACCTTGTTACGACTT-3′) (Lane, 1991). This is performed with a polymerase chain reaction (PCR) of a solution containing 1 µL of DNA template, 2 mM of each primer and MyTaq™ Mix (Meridian Bioscience, Cincinnati, Ohio, EUA). The PCR reaction program was an initial denaturation at 95 °C for 2 min, followed by 30 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, and a final extension at 72 °C for 7 min. PCR products were visualized to confirm quality in a 1.5 % electrophoresis agarose gel and quantified using Qubit® dsDNA HS Assay Kit and Qubit 3.0 fluorometer (Invitrogen). Purification and sequencing of the amplicons was performed by GenCore, I3S (Instituto de Investigação e Inovação em Saúde), in Porto, Portugal. Raw sequences were

analysed using Geneious 11.1.4 software (Biomatters Ltd, Auckland, New Zealand) and the consensus sequences were submitted to GenBank for taxonomic identification (Nucleotide Blast), in both nucleotide collection and 16S ribosomal RNA sequences. To confirm identification, sequences were also submitted to the EzBioCloud 16S database (Yoon et al., 2017) and the Ribosomal Database Project (RDP) (Cole et al., 2014).

5.3 Results

5.3.1 Direct Isolation of Bacteria

The total number of isolates from the biofilter (biofilter carriers and circulating water), for each media incubated at different temperatures (28°C and room temperature) are presented in **Figure 2-A**. From the direct serial spreading of our samples, we obtained a total of 250 heterotrophic culturable isolates were obtained, with more isolates obtained at 28 °C (152) than at room temperature (98). In both temperatures there were more isolates obtained from the biofilter carriers (85 at 28°C and 65 at room temperature) than from the water (68 at 28°C and 33 at room temperature). For nitrifying media, only 11 isolates were successfully obtained from direct isolation, and all of them from the media AM, with biofilter carrier samples, 3 at room temperature and the remaining 8 at 28 °C.

A

	Room Temperature		28 ºC		
Non-Nitrifying	**Water**	**Biofilter Carriers**	**Water**	**Biofilter Carriers**	**Total**
R2A	26	31	30	13	100
SCN	7	34	13	20	74
MA	0	0	6	33	39
M1	0	0	19	18	37
Total per matrix	33	65	68	84	250
Nitrifying	**Water**	**Biofilter Carriers**	**Water**	**Biofilter Carriers**	**Total**
AM	0	3	0	8	11
Total per matrix	0	3	0	8	11

B

		Biofilter		Biofilter	
Nitrifying	**Water**	**Carriers**	**Water**	**Carriers**	**Total**
AM	6	0	0	0	6
APN	8	5	0	0	13
NPN	2	5	0	0	7
Total per matrix	16	10	0	0	26

C

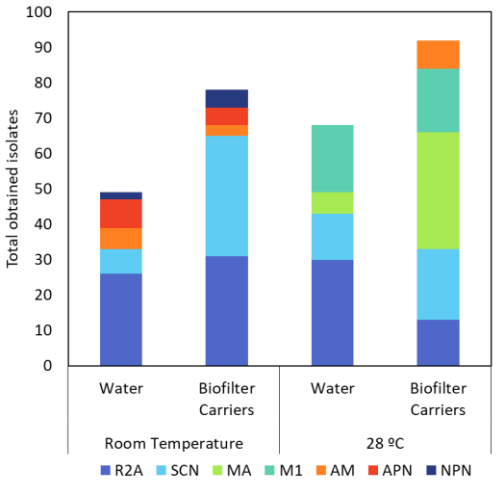


Figure 2: Table and bar plot of the isolates obtained from direct isolation plus isolation at the end of the enrichment for each media, temperature, and matrix (water, biofilter carriers).

5.3.2 Enrichment of Nitrifying Bacteria

Due to the difficulty in obtaining isolates from the nitrifying media in direct isolation, enrichments of nitrifying bacteria, using water and biofilter carriers from the RAS, was performed.

During the ammonia enriched cultures (Figure 3-A), in the first two cycles, it was observed that the ammonia removal rates were very similar between both matrices in each form of ammonia supplementation. For $(NH_4)_2SO_4$: we obtained removal rates of 22% with only biofilter water and 28% with the biofilter carriers; for NH_4Cl: 17% with only biofilter water and 14% with the biofilter carriers, all rates by the end of the second cycle. So $(NH_4)_2SO_4$ with biofilter carriers was selected for further studies and split into natural and synthetic media. After the second cycle, activity was lost, with ammonia removal successively diminishing with removal rates between 0 and 10%. After the 5th cycle, we decided to terminate the protocol due to the lack of promising results.

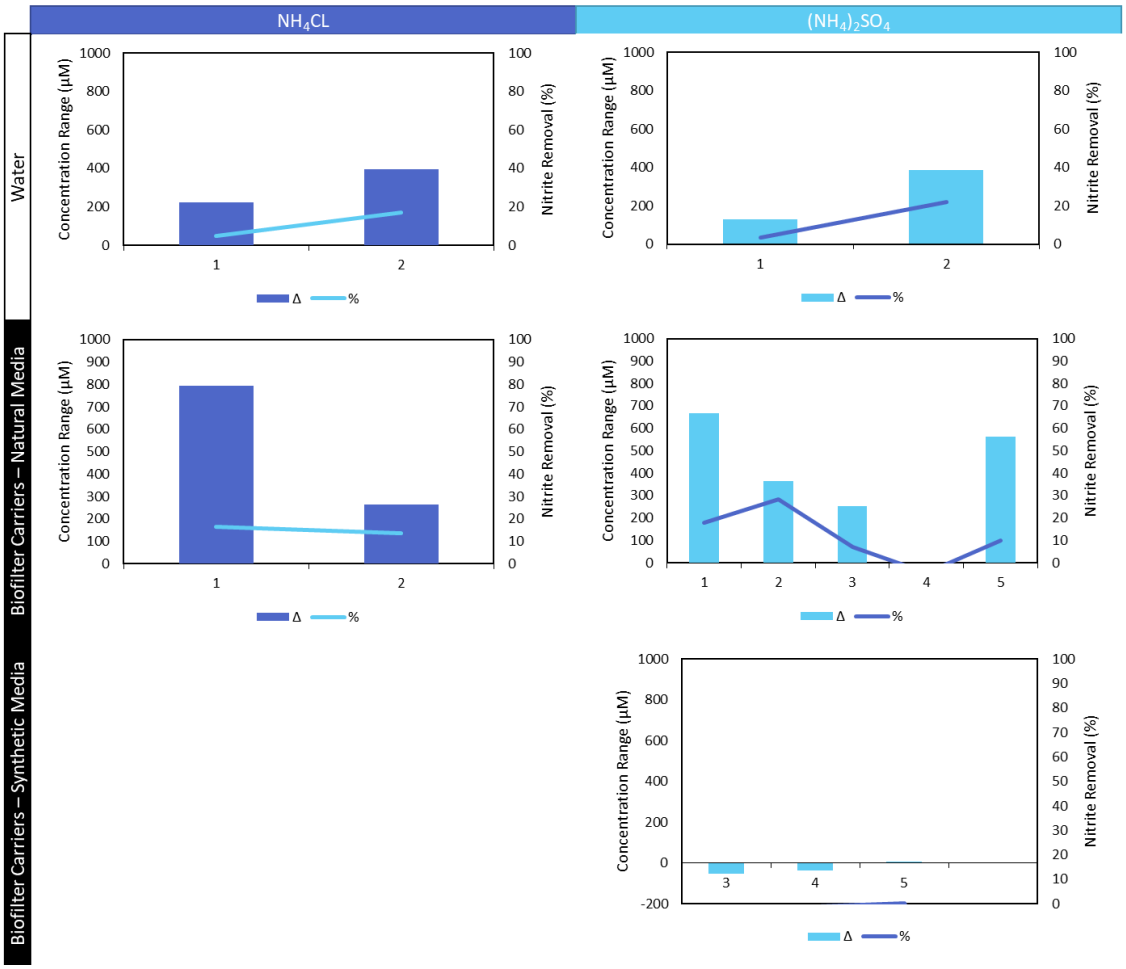


Figure 3: Ammonia removal efficiency of the enriched cultures measured both as depletion in ammonia concentration (concentration range Δ) and rate of removal (%) for each cycle (1-6). Two forms of ammonia were tested ($(NH_4)_2SO_4$, and NH_4Cl) and two initial conditions (using only water from the biofilter and using water and biofilter carriers) to which later was added a second condition (Synthetic Media), with the duplication of the biofilter containing enrichment. Nutrient supplementation and media renewal was performed every 14 days.

As to the $NaNO_2$ enrichment (Figure 3-B), the initial biofilter water condition did not present any nitrite removal activity, so it was terminated. The biofilter carrier conditions, however, 100% nitrite removal activity in the first two weeks (t = 1 and t =1.5), which was

kept relatively stable even after the split into natural and synthetic media. By the end of the experiment, the natural, biofilter containing, condition completed 12 weeks (with weekly media renewals) of nearly complete nitrite removal and the synthetic biofilter containing condition completed 10 weeks in the same conditions.

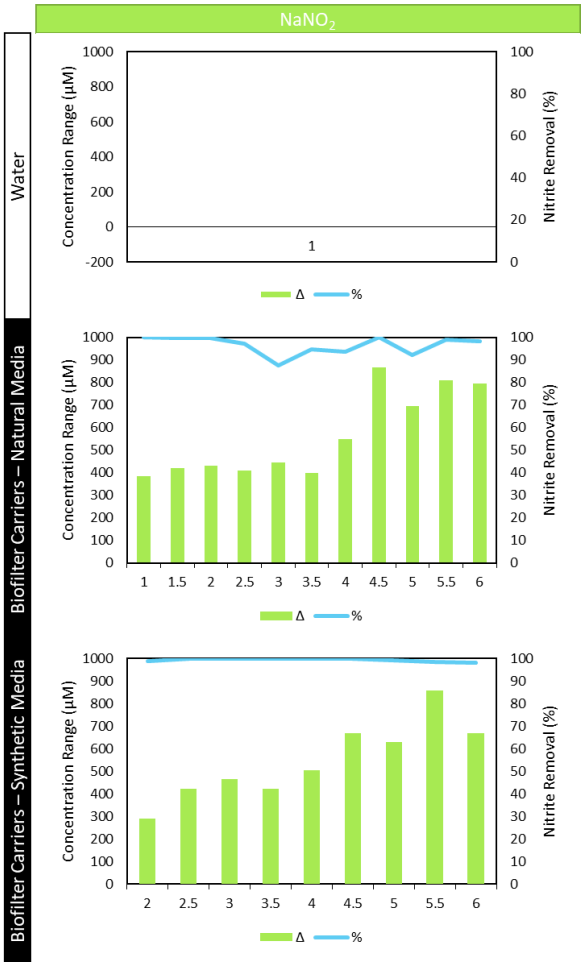


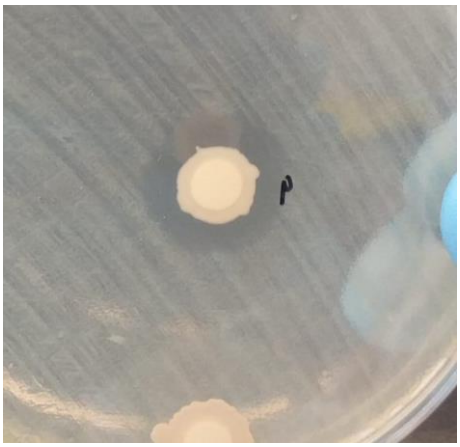
Figure 4: Nitrite removal efficiency of the enriched cultures measured both as depletion in ammonia concentration (concentration range Δ) and rate of removal (%) every 7 days (half a cycle, 1-6). Two initial conditions were tested (using only water from the biofilter and using water and biofilter carriers) to which later was added a second condition (Synthetic Media), with the duplication of the biofilter containing enrichment. Nutrient supplementation was performed every 7 days and media renewal every 14 days.

At the end of each cycle, isolation was performed leading to an additional 26 strains to be isolated (**Figure 2-B**). Of these, 16 were obtained from the enrichment using only water from the biofilter and 10 were obtained from the enrichment with the biofilter carriers. The media APN and NPN were the most successful in the isolation. The combination of these isolates with those obtained by direct isolation make a total of 37 nitrifying bacterial strains, 30 were obtained from ammonia media, and 7 from nitrite media. (**Figure 2-C**).

5.3.3 Screening Assays for Inhibition Of *T. Maritimum*

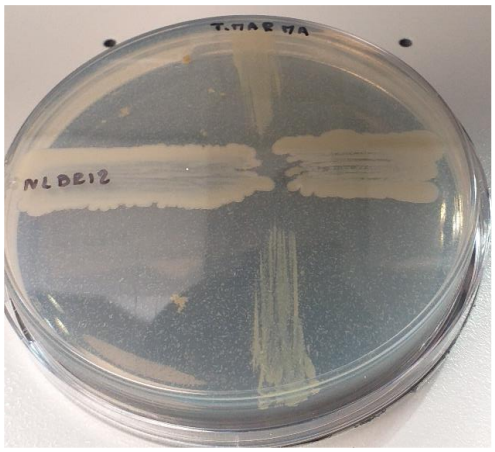
Cross streak and diffusion disc inhibition tests (**Figure 4**) were performed using a library of heterotrophic culturable community against a strain of *Tenacibaculum maritimum*. So far (**Figure 5**), a total of 84 isolates were tested, of these 11 are incomplete (with either the diffusion disc or the cross-streak result being inconclusive). Specifically, a total of 12 isolates tested positive and 70 negative in the cross-streak test, and 13 positive and 62 negative in the diffusion disk. So far, in the cross-streak test, every media had isolates with positive inhibition, except for SCN. In the diffusion disc test, this media also has not yet produced an isolate with positive inhibition and R2A has the most strains with anti-*T. maritimum* activity (11). Three isolates with positive results for both cross-streak and diffusion disc inhibition test, have already been identified as *Pseudoalteromonas* sp..

Diffusion Disc



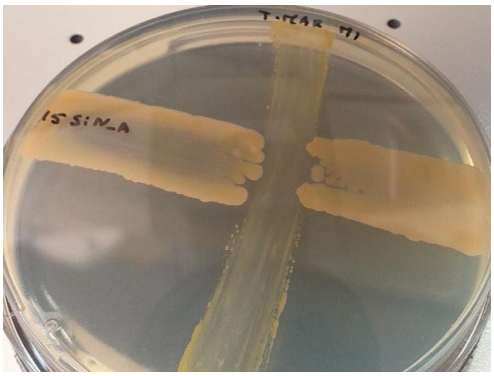
Example of positive inhibition.

Cross Streak



Example of positive inhibition.

Example of negative inhibition.



Example of negative inhibition.

Figure 5: Example photographs of the cross streak and diffusion disc *Tenacibaculum maritimum* inhibition tests.

	Cross Streak		Diffusion Disc	
Media	**Negative**	**Positive**	**Negative**	**Positive**
M1	25	4	25	1
MA	19	5	18	1
R2A	15	3	8	11
SCN	11	0	11	0
Total	70	12	62	13

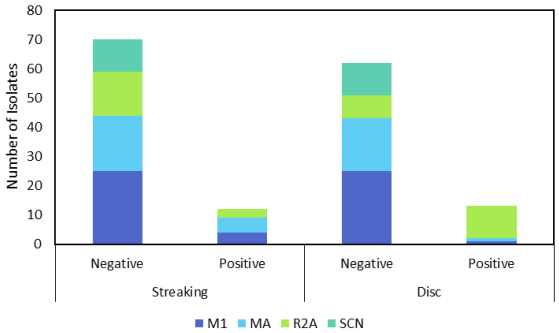


Figure 6: Results for the cross streak and diffusion disc *Tenacibaculum maritimum* inhibition tests, separated by the isolate original media. Isolates came from a previously obtained library of the non-nitrifying culturable community. A total of 85 isolates were tested, with 19 incomplete (one of the tests was inconclusive).

5.4 Discussion

In this study on microbiome-based solutions to improve water quality and fish welfare in RAS, we successfully obtained a considerable library of 250 isolates of the heterotrophic community with the direct isolation from the water and biofilter carriers of the RAS. However, no direct nitrifying bacteria were successfully isolated, which we attributed to the difficulty in identifying the colonies in a media with high turbidity due to the $CaCl_2$ added, and to the introduction of organic material when adding agar (Schmidt & Belser, 1983). Because of this, we established enrichment protocols to obtained them, though time consuming, they have been described as an efficient method to obtain nitrifying bacteria (MacFarlane & Herbert, 1984). After the enrichment protocol was successfully established, we managed to obtain 37 isolates. Attesting to the higher diversity in the biofilter carriers previously described in this system (Almeida et al., 2021). Also, more isolates were obtained from this matrix for the heterotrophic community.

Even though we managed to obtain isolates from the AOB enrichment, we can only consider a success the enrichment of NOB bacteria due to the nutrient removal activity that accompanied these experiments. In the AOB enrichment, after an initial promising activity, removal activity disappeared and even became negative with ammonia levels increasing in the system. This suggests an inability for this bacteria to replicate in the conditions used in this study. We know that AOB activity decreases at concentrations higher than 400 mg/L NH_4-N (or 23 mM NH_4-N), which is much higher than the one we used, but this activity is inhibited by salinity (Claros et al., 2010) and is dependent on the AOB species, so this value could be lower in the current conditions. As seen in **Chapter 2** of this thesis, NH_3-N concentrations in this unit are usually between 0-0.6 mg/L (0-0.3 µM), so the rapid increase to 4540 µM NH_3-N may have compromised the AOB community from the biofilter.

The NOB enrichment, however, successively kept close to 100% removal efficiencies. We believe that the main reason for this discrepancy between the AOB and NOB enrichment was a result of the 10x higher ammonia concentration used in the first. This concentration was selected based on previous literature (Elbanna et al., 2012; Lipponen et al., 2002), but it was probably too high for a nitrifying community as mentioned before. Future studies will be performed with lower ammonia concentrations, similar to those used for the NOB enrichment. These are the first steps in developing an active nitrifying bacterial formulation, adapted to a marine aquaculture. Such formulation will be useful to shorten biofilter stabilization times or to act as a re-enforcement for recovery of dysbiosis events that compromise biofilter efficiency.

AOB and NOB enrichments are not new or rare, although difficult to implement and reproduce. For example, in the enrichment cultures of AOB collected from different areas of Salar de Huasco (a high altitude, saline, pH-neutral water) with 10 mM NH_4^+-N, a few enrichment cultures observed AOB growth (Dorador et al., 2008). But studies with successful enrichments abound, such as the AOB enrichment with ammonia oxidised to nitrite in 10 days of Baskaran et al. (2020) or the 288% and 181% improvement in ammonium and nitrite removal by enriched microbial communities (when compared to the original biofilter community) of Neissi et al. (2022). However, very few studies attest to the failed attempts at enriching (and purifying) nitrifying bacteria (characterized by their low growth rates and low representation in the prokaryotic community) and to the particularly laborious, frequent unsuccessful, experiments. Recently a study (Herbet et al., 2022) found that, although more than 8 out of 10 researchers have produced negative results and consider them useful to their scientific community, only 12.5% of them had them published in a scientific journal. As mentioned in the paper, negative results remain mostly internal and informal, rarely going outside the walls of the laboratory.

After obtaining the heterotrophic library, we tested for anti-*T. maritimum* activity. Three of the isolates that tested positive for both cross-streak and diffusion disc assay have been identified in this study as *Pseudoalteromonas* sp.. These results are very promising, as this genera have also been identified as having anti-*Vibrio* activity (Handayani et al., 2021). And both *Vibrio* and *Tenacibaculum* have been linked in previous NGS studies (Almeida et al., 2021), which might be further indication of a complex interaction between the species from these two genera.

Overall, we can determine that the enrichment is the most promising protocol for nitrifying bacteria in this RAS, but in further studies ammonia concentration needs to be adapted. The most promising results are, by far, the anti-*T. maritimum* activity identified in some

of the library isolates. These strains also have a good potential for follow up studies, particularly if there is a particular compound produced by the strains that causes the inhibition, it can prove to be an alternative to antibiotics. The identification of *Pseudoalteromonas* sp. As potential inhibitor of *T. maritimum* may be particularly relevant since a previous study (see chapter 4 of this thesis) it was attributed a positive co-relation between this *Pseudoalteromonas* and *Tenacibaculum* genera in the context of a complex community. Although further studies can be performed to test this hypothesis. A therapeutical alternative to antibiotics in pathogen control is relevant because the use of this therapies will, by 2022, under new European Union Regulation (EU) 2019/4 on Medicated Feed (European Commission, 2019) be prohibited in all forms of routine antibiotic use in farming, including preventative group treatments, before the appearance of clinical signs.

5.5 Conclusion

This chapter aimed to present the preliminary results of the tests to obtain the biotechnological tools to modulate the bacterial community of a RAS. To improve or maintain the water quality, we need to develop a formulation of a nitrifying community adapted to a marine environment. Our attempts in enriching nitrifying bacteria had mixed success rates, with the AOB enrichment losing bacterial activity after two cycles, but the NOB enrichment successively kept close to 100% removal efficiencies during the 12 weeks duration of the natural media experiment, and the 10 week for the synthetic media. The NOB enrichment will follow for community characterization, while for the AOB enrichment, new experiments will be tested using lower ammonia concentrations.

We have obtained a total of 37 isolates from both direct isolation and enrichment protocols, the following stage of this experiment will be to identify these isolates (though sanger sequencing of the 16S gene) and characterize the original (and enriched) communities using illumine sequencing to determine if the enrichment was successful. To improve the fish welfare, aside from the stabilization of the water quality of the previous point, so far, 12 isolates have shown anti-*T. maritimum* activity in the cross-streak assay and 13 in the diffusion disk. The remaining isolates will also be tested in the future, with the identification of all isolates that show positive inhibition in either test. These isolates will be a promising starting point to find new biological tools for outbreak prevention serving as potential probiotics or to elucidate new bioactive compounds, suitable to be used as alternatives to antibiotics.

5.6 References

Almeida, D.B., Magalhães, C., Sousa, Z., Borges, M.T., Silva, E., Blanquet, I., Mucha, A.P., 2021. Microbial community dynamics in a hatchery recirculating aquaculture system (RAS) of sole (*Solea senegalensis*). Aquaculture. 539, 736592.

Baek, K., Choi, A., Lee, Y.M., Lee, H.K., Cho, J.-C., 2018. *Leucothrix arctica* sp. nov., isolated from Arctic seawater. International Journal of Systematic and Evolutionary Microbiology. 68, 3851-3855.

Baskaran, V., Patil, P.K., Antony, M.L., Avunje, S., Nagaraju, V.T., Ghate, S.D., Nathamuni, S., Dineshkumar, N., Alavandi, S.V., Vijayan, K.K., 2020. Microbial community profiling of ammonia and nitrite oxidizing bacterial enrichments from brackishwater ecosystems for mitigating nitrogen species. Scientific Reports. 10, 1-11.

Brailo, M., Schreier, H.J., McDonald, R., Maršić-Lučić, J., Gavrilović, A., Pećarević, M., Jug-Dujaković, J., 2019. Bacterial community analysis of marine recirculating aquaculture system bioreactors for complete nitrogen removal established from a commercial inoculum. Aquaculture. 503, 198-206.

Chen, S., Ling, J., Blancheton, J.-P., 2006. Nitrification kinetics of biofilm as affected by water quality factors. Aquacultural engineering. 34, 179-197.

Claros, J., Jiménez, E., Borrás, L., Aguado, D., Seco, A., Ferrer, J., Serralta, J., 2010. Short-term effect of ammonia concentration and salinity on activity of ammonia oxidizing bacteria. Water Science and Technology. 61, 3008-3016.

Cole, J.R., Wang, Q., Fish, J.A., Chai, B., McGarrell, D.M., Sun, Y., Brown, C.T., Porras-Alfaro, A., Kuske, C.R., Tiedje, J.M., 2014. Ribosomal Database Project: data and tools for high throughput rRNA analysis. Nucleic acids research. 42, D633-D642.

Commission, E., 2019. Regulation (EU) 2019/4 of the European Parliament and of the Council of 11 December 2018 on the manufacture, placing on the market and use of medicated feed, amending regulation (EC) NO 183/2005 of the European Parliament and of the Council and repealing Council Directive 90/167/EEC (text with EEA relevance). Official Journal of the Eurean Union. 4.

Daims, H., Lebedeva, E.V., Pjevac, P., Han, P., Herbold, C., Albertsen, M., Jehmlich, N., Palatinszky, M., Vierheilig, J., Bulaev, A., 2015. Complete nitrification by *Nitrospira* bacteria. Nature. 528, 504-509.

Davidson, J., Good, C., Williams, C., Summerfelt, S.T., 2017. Evaluating the chronic effects of nitrate on the health and performance of post-smolt Atlantic salmon *Salmo salar* in freshwater recirculation aquaculture systems. Aquacultural engineering. 79, 1-8.

de La Banda, I.G., Lobo, C., León-Rubio, J.M., Tapia-Paniagua, S., Balebona, M.C., Moriñigo, M.A., Moreno-Ventas, X., Lucas, L.M., Linares, F., Arce, F., 2010. Influence of two closely related probiotics on juvenile Senegalese sole (*Solea senegalensis*, Kaup 1858) performance and protection against *Photobacterium damselae* subsp. piscicida. Aquaculture. 306, 281-288.

Dinis, M.T., Ribeiro, L., Soares, F., Sarasquete, C., 1999. A review on the cultivation potential of *Solea senegalensis* in Spain and in Portugal. Aquaculture. 176, 27-38.

Dorador, C., Busekow, A., Vila, I., Imhoff, J.F., Witzel, K.-P., 2008. Molecular analysis of enrichment cultures of ammonia oxidizers from the Salar de Huasco, a high altitude saline wetland in northern Chile. Extremophiles. 12, 405-414.

Drønen, K., Roalkvam, I., Dahle, H., Olsen, A., Nilsen, H., Wergeland, H., 2022. Microbiome dataset from a marine recirculating aquaculture system (RAS) for salmon post-smolt production in Norway. Data in brief. 40, 107767.

Ebeling, J.M., Timmons, M.B., Wheaton, J.M., Summerfelt, S.T., Vinci, B.J., 2002. Recirculating aquaculture, 4th ed. Ithaca Publishing Company.

El-Saadony, M.T., Alagawany, M., Patra, A.K., Kar, I., Tiwari, R., Dawood, M.A., Dhama, K., Abdel-Latif, H.M., 2021. The functionality of probiotics in aquaculture: An overview. Fish & Shellfish Immunology.

Elbanna, K., El-Shahawy, R., Atalla, K., 2012. A new simple method for the enumeration of nitrifying bacteria in different environments. Plant, Soil and Environment. 58, 49-53.

Emparanza, E.J., 2009. Problems affecting nitrification in commercial RAS with fixed-bed biofilters for salmonids in Chile. Aquacultural engineering. 41, 91-96.

Gao, X.-Y., Liu, Y., Miao, L.-L., Li, E.-W., Hou, T.-T., Liu, Z.-P., 2017. Mechanism of anti-Vibrio activity of marine probiotic strain *Bacillus pumilus* H2, and characterization of the active substance. AMB Express. 7, 1-10.

Gatesoupe, F.J., 1999. The use of probiotics in aquaculture. Aquaculture. 180, 147-165.

Gourzioti, E., Kolygas, M., Athanassopoulou, F., Babili, V., 2016. Tenacibaculosis in aquaculture farmed marine fish. Journal of the Hellenic Veterinary Medical Society. 67, 21-32.

Grasshoff, K., Johannsen, H., 1972. A new sensitive and direct method for the automatic determination of ammonia in sea water. ICES Journal of Marine Science. 34, 516-521.

Grasshoff, K., Kremling, K., Ehrhardt, M., 2009. Methods of seawater analysis. John Wiley & Sons.

Handayani, D.P., Isnansetyo, A., Istiqomah, I., Jumina, J., 2021. Anti-Vibrio activity of Pseudoalteromonas xiamenensis STKMTI. 2, a new potential vibriosis biocontrol bacterium in marine aquaculture. Aquaculture Research.

Head, I.M., Hiorns, W.D., Embley, T.M., McCarthy, A.J., Saunders, J.R., 1993. The phylogeny of autotrophic ammonia-oxidizing bacteria as determined by analysis of 16S ribosomal RNA gene sequences. Microbiology. 139, 1147-1153.

Herbet, M.E., Leonard, J., Santangelo, M.G., Albaret, L., 2022. Dissimulate or disseminate? A survey on the fate of negative results. Learned Publishing. 35, 16-29.

Jahangiri, L., Esteban, M.Á., 2018. Administration of probiotics in the water in finfish aquaculture systems: a review. Fishes. 3, 33.

Koops, H.-P., Böttcher, B., Möller, U., Pommerening-Röser, A., Stehr, G., 1990. Description of a new species of Nitrosococcus. Archives of Microbiology. 154, 244-248.

Lane, D., 1991. 16S/23S rRNA sequencing. Nucleic acid techniques in bacterial systematics, 115-175.

Levipan, H.A., Molina, V., Fernandez, C., 2014. *Nitrospina*-like bacteria are the main drivers of nitrite oxidation in the seasonal upwelling area of the Eastern South Pacific (Central Chile~ 36° S). Environmental Microbiology Reports. 6, 565-573.

Lipponen, M.T., Suutari, M.H., Martikainen, P.J., 2002. Occurrence of nitrifying bacteria and nitrification in Finnish drinking water distribution systems. Water Research. 36, 4319-4329.

MacFarlane, G., Herbert, R., 1984. Comparative study of enrichment methods for the isolation of autotrophic nitrifying bacteria from soil, estuarine and marine sediments. FEMS microbiology letters. 22, 127-132.

Martins, C.I.M., Eding, E.H., Verdegem, M.C.J., Heinsbroek, L.T.N., Schneider, O., Blancheton, J.-P., d'Orbcastel, E.R., Verreth, J.A.J., 2010. New developments in recirculating aquaculture systems in Europe: A perspective on environmental sustainability. Aquacultural engineering. 43, 83-93.

Morais, S., Aragão, C., Cabrita, E., Conceição, L.E., Constenla, M., Costas, B., Dias, J., Duncan, N., Engrola, S., Estevez, A., 2016. New developments and biological insights into the farming of *Solea senegalensis* reinforcing its aquaculture potential. Reviews in Aquaculture. 8, 227-263.

Neissi, A., Rafiee, G., Rahimi, S., Farahmand, H., Pandit, S., Mijakovic, I., 2022. Enriched microbial communities for ammonium and nitrite removal from recirculating aquaculture systems. Chemosphere. 295, 133811.

Park, S., Bae, W., 2009. Modeling kinetics of ammonium oxidation and nitrite oxidation under simultaneous inhibition by free ammonia and free nitrous acid. Process Biochemistry. 44, 631-640.

Park, S.C., Hwang, Y.M., Lee, J.H., Baik, K.S., Seong, C.N., 2013. *Algibacteragarivorans* sp. nov. and *Algibacteragarilyticus* sp. nov., isolated from seawater, reclassification of *Marinivirga aestuarii* as *Algibacteraestuarii* comb. nov. and emended description of the genus *Algibacter*. International journal of systematic and evolutionary microbiology. 63, 3494-3500.

Patil, P.K., Antony, L., Avunje, S., Viswanathan, B., Lalitha, N., Jangam, A.K., Kumar, D., Solanki, H.G., Reddy, M.A., Alavandi, S.V., 2021. Bioaugmentation with nitrifying and denitrifying microbial consortia for mitigation of nitrogenous metabolites in shrimp ponds. Aquaculture. 541, 736819.

Pazos, F., Santos, Y., Macias, A., Núñez, S., Toranzo, A., 1996. Evaluation of media for the successful culture of *Flexibacter maritimus*. Journal of Fish Diseases. 19, 193-197.

Pérez-Sánchez, T., Ruiz-Zarzuela, I., de Blas, I., Balcázar, J.L., 2014. Probiotics in aquaculture: a current assessment. Reviews in Aquaculture. 6, 133-146.

Piedrahita, R.H., 2003. Reducing the potential environmental impact of tank aquaculture effluents through intensification and recirculation. Aquaculture. 226, 35-44.

Pommerening-Röser, A., Rath, G., Koops, H.-P., 1996. Phylogenetic diversity within the genus *Nitrosomonas*. Systematic and Applied Microbiology. 19, 344-351.

Schmidt, E.L., Belser, L.W., 1983. Nitrifying bacteria. Methods of Soil Analysis: Part 2 Chemical and Microbiological Properties. 9, 1027-1042.

Schreier, H.J., Mirzoyan, N., Saito, K., 2010. Microbial diversity of biological filters in recirculating aquaculture systems. Current opinion in biotechnology. 21, 318-325.

Semedo, M., Lopes, E., Baptista, M.S., Oller-Ruiz, A., Gilabert, J., Tomasino, M.P., Magalhães, C., 2021. Depth Profile of Nitrifying Archaeal and Bacterial Communities in the Remote Oligotrophic Waters of the North Pacific. Frontiers in microbiology. 12, 319.

Stankus, A., 2021. State of world aquaculture 2020 and regional reviews: FAO webinar series. FAO Aquaculture Newsletter, 17-18.

Tan, L.T.-H., Lee, L.-H., Goh, B.-H., 2019. The bioprospecting of anti-Vibrio Streptomyces species: Prevalence and applications. Progress In Microbes & Molecular Biology. 2.

Toranzo, A.E., Magariños, B., Romalde, J.L., 2005. A review of the main bacterial fish diseases in mariculture systems. Aquaculture. 246, 37-61.

Wanka, K.M., Damerau, T., Costas, B., Krueger, A., Schulz, C., Wuertz, S., 2018. Isolation and characterization of native probiotics for fish farming. Bmc Microbiology. 18, 1-13.

Xu, X.-W., Wu, Y.-H., Wang, C.-S., Gao, X.-H., Wang, X.-G., Wu, M., 2010. *Pseudoalteromonas lipolytica* sp. nov., isolated from the Yangtze River estuary. International journal of systematic and evolutionary microbiology. 60, 2176-2181.

Yoon, J.-H., Kang, S.-J., Oh, T.-K., 2007. *Sulfitobacter marinus* sp. nov., isolated from seawater of the East Sea in Korea. International journal of systematic and evolutionary microbiology. 57, 302-305.

Yoon, S.-H., Ha, S.-M., Kwon, S., Lim, J., Kim, Y., Seo, H., Chun, J., 2017. Introducing EzBioCloud: a taxonomically united database of 16S rRNA gene sequences and whole-genome assemblies. International journal of systematic and evolutionary microbiology. 67, 1613.

Yoshida, K., Nasu, Y., Shitami, N., Toyoda, H., Takemura, H., Oomori, K., 2009. A novel convenient method for high bacteriophage titer assay, Nucleic acids symposium series. Oxford University Press, pp. 315-316.

Zhang, D.-C., Liu, Y.-X., Huang, H.-J., Wu, J., 2016. *Pseudoalteromonas profundi* sp. nov., isolated from a deep-sea seamount. International Journal of Systematic and Evolutionary Microbiology. 66, 4416-4421.

Zhang, Z., Gao, X., Wang, L., Zhang, X.-H., 2015. *Leucothrix pacifica* sp. nov., isolated from seawater, and emended description of the genus *Leucothrix*. International Journal of Systematic and Evolutionary Microbiology. 65, 2397-2402.

CHAPTER 6

Final Remarks and Future Perspectives

6.1 Final Remarks and Future Perspectives

6.1. Current challenges of RAS microbial communities

To maintain fish welfare in an aquaculture production, particularly in Recirculating Aquaculture Systems (RAS), it is essential to find new and alternative biotechnology tools to both maintain the water quality and to provide disease resistance. In practice, this means that we need to find a tool that provides the necessary boost to the biofilter in response to destabilizing events, to make sure it maintains its function of removing toxic compounds from the water (such as ammonia). To provide disease resistance, we need to find antibiotic alternatives to prevent disease associated stress and high mortality events. These tools may be relevant to help the aquaculture sector to achieve a more sustainable fish production, in line with the United Nations Sustainable Development Goal (SDG) 12: “Ensure sustainable consumption and production patterns”.

To answer to the long activation times of the biofilter (or re-activation in case of destabilization), as this period is essential to manage water consumption and subsequent waste, we need to find an active nitrifying bacterial formulation, adapted to the marine environment and to the complex dynamics of RAS. This is particularly relevant as most bacteria commercial inocula are usually composed of freshwater species, who are not capable of adapting to a marine environment (Brailo et al., 2019). Therefore, we need to characterize the community in a marine RAS to identify the key players in maintaining water quality. Then, we need to isolate them from the community to start the marine biofilter inocula formulations.

At the same time, we need to tackle the challenge of pathogen outbreaks. By deepening our knowledge of the composition and interactions that rule a RAS microbiome, particularly the prokaryotic community, we can begin to highlight the bacteria and/or interactions that inhibit pathogen species colonization and development. The most promising route is to identify probable probiotics, bacteria that provide health benefits, including resistance to diseases, that are usually associated with the gut and water microbiome (El-Saadony et al., 2021). This is particularly relevant as the use of antibiotic administration in all forms of routine antibiotic (including preventative group treatments) before the appearance of clinical signs, will be prohibited by 2022, in the new European Union Regulation (EU) 2019/4 on Medicated Feed (European Commission, 2019) .

6.2. The composition and dynamics of the environmental community of a RAS

Studies aimed at characterizing the environmental RAS community using cutting edge technology, such as Next Generation Sequencing (NGS) are still not extensively applied. This type of work has the potential to disentangle the complex network of the microbial roles to explore what can be done to control and predict potential pathogen outbreaks (Martínez-Porchas & Vargas-Albores, 2017).

NGS techniques can be a tool for aquaculture planning and management, and we have used it to perform a spatial and temporal characterization of the dynamics of the prokaryotic microbiome in a Sole (*Solea senegalensis*) hatchery. By collecting samples of water, biofilter carriers and tank wall biofilm and then isolating the total DNA for 16S rRNA gene sequencing using the Illumina MiSeq® platform, we have made a map of the RAS prokaryotic community. This map showed a highly dynamic prokaryotic community, sensitive to the physical-chemical changes within the different compartments and matrices of the same aquaculture unit. This behaviour was already hinted by a previous study (Borges et al., 2008), but the exclusive use of classical microbiological tools (culture dependent) prevented the complete characterization of the prokaryotic community. Even within the same production unit, distinct systems developed different profiles, indicating a high sensitivity to even small variations (in salinity, temperature, and pH) and unravelling a potential for fine tuning in future modulation protocols. This is important to not compromise the fish welfare with high physical-chemical fluctuation.

In completing this study, we learned that in the future we must focus on identifying more factors that cause variations in the prokaryotic community, so we can develop modulation protocols to favour a healthy environmental microbiome, mainly through an increase in diversity. Maintaining a diverse community is important, as high alpha-diversity indexes have been associated with better health and higher growth rates in fish (Infante-Villamil et al., 2020). However, this is a challenge in a RAS environment, where there is a stabilization of key parameters (temperature, pH, and salinity) as fluctuations are undesirable, and potentially harmful for fish welfare.

6.3. In-depth analysis of the complex interactions within the prokaryotic community

As mentioned before, understanding the prokaryotic interactions in a RAS environment is essential to determine which bacteria may play a crucial role in driving the functional

potential of the community. To do this, we used network tools based on co-variance and co-occurrence, and began to label some constituents as belonging to two key target groups: potentially pathogenic and nitrifying prokaryotes. We found no negative correlations with our target bacteria, although NGS network studies are characterized by positive biases. These are mainly caused by variation in sequencing depth, which can result in some misleading positive correlations (Faust et al., 2015).

Among the nitrifying communities, ammonia oxidizing bacteria (*Nitrosomonas*) and archaea (*Candidatus Nitrosopumilus*) were positively interacting with the nitrite oxidizing bacteria *Nitrospira*. Not only that, but results hint at an environment differentiation based on ammonia competition, where ammonia oxidizing archaea (AOA) outcompete ammonia oxidizing bacteria (AOB) at relatively low concentrations. This had been previously described in a similar environment (Hatzenpichler, 2012), and we now reinforce it using NGS techniques. Attesting to this, in the environment were AOA positively correlate with nitrite oxidizing bacteria (NOB), the later has no corresponding correlation with AOBs. These correlations are only found in the environment where no AOAs were detected, between the genera *Nitrospira* and *Nitrosomonas*. Within the communities observed, *Nitrospira* also presented itself in subcommunities with no AOA or AOB correlations, suggesting a separate functional role of complete ammonia oxidation to nitrate for some *Nitrospira* ASVs. With this behaviour previously described (Daims et al., 2015), we now theorize that it may be a part of the RAS biofilter bacterial activity.

Some genera in the RAS prokaryotic community were also labelled as potentially pathogenic, and within these, we found that two taxa commonly associated with pathogenic outbreaks, *Tenacibaculum* and *Vibrio*, had a significant positive interaction in one of the systems. This interaction has previously been described in fish infected with *Tenacibaculum maritimum* (Wynne et al., 2020), but this is the first time that is described in the environmental community of a RAS (water, biofilter carriers and tank wall biofilm). Due to the fact that during our study no outbreaks were reported by the production, this association may prove relevant in disease preventions and to improve outbreak predictions.

Going forward, and while studies like ours are subjected to positive biases, we must expect a cooperative prokaryotic network to establish in the stable RAS environment. The need to optimize functional roles promotes this interaction, but we must also take into consideration the coupling effect in an event of community destabilization, where the fluctuation of one taxa may destabilize the entire community.

6.4. The fish microbiome development in a production cycle

Microbiomes are characterized by their dynamic and interactive nature, crucial for the functioning and health of their host (Berg et al., 2020) with a constitution that evolves from early bacterial colonization and has an important role in reinforcing the immune system of the fish. This development is affected by environmental conditions, from diet to production systems and the continuity between different environmental niches provided by the water in circulation.

In our work, we tried to extensively describe the fish prokaryotic community of a RAS. By describing the inherited and acquired community in the different tissues analysed (gill, intestine, fin, and mucus) at different production systems (egg, larvae, weaning, pre-ongrowing) and life cycle stages (egg, larvae, juvenile), we found that the prokaryotic community is significantly altered throughout the fish early development, with the establishment of niche habitats. Few genera are inherited from the broodstock, but the ones that do, become part of the core community, and maintain high relative abundances through the production. Beneficial bacterial genera (potentially probiotic) may be inherited from the broodstock, but it is the introduction of commercial feed in the weaning stage that causes the main increase in potentially probiotic genera abundance. Although, it appears that this diversity may not be successfully installed without a diverse diet.

Similarly, to the potential probiotic genera, potentially pathogenic genera can be inherited from the broodstock, or acquired in the production routine. The risk of introducing these genera may be minimized through the application of life cycle studies, as we have now some confirmation that the most likely vector is the live feed. However, if inherited from wild broodstock, it may be difficult to safely eradicate them in a sustainable aquaculture setting.

Our work reinforced the importance of these types of life cycle studies. Recurrent evaluation of variation patterns in different settings with different microbiomes is essential to confirm our findings, and to find new patterns. It is important to highlight that these may be a great opportunity to ally scientific findings with direct applications to the aquaculture production units, in which this work has necessarily to be performed.

6.5. Valorisation of the acquired knowledge in new biotechnology tools

We have begun to work on translating the main findings of previous studies into new biotechnology tools to modulate the RAS prokaryotic community. There are two key

functions we want to obtain: to improve water quality through bacterial inoculum nitrifying community boost and to inhibit pathogenic outbreaks in the system.

For the first challenge, improving water quality, we need to develop a formulation of a nitrifying bacteria adapted to a marine aquaculture. We began by trying to obtain them using direct isolation protocols, but with insufficient results. Afterwards, we tried enrichment protocols, which provided more interesting results. We were able to track nitrifying activity, successively keeping removal efficiencies close to 100% during the 12 weeks of the natural media experiment, and 10 week for the synthetic media. Although we still need to improve the ammonia enrichment protocol. From both techniques, we have obtained a total of 37 isolates, that are in identification process.

For the second challenge, inhibit pathogenic outbreaks, we started by obtaining a library of 250 isolates of the heterotrophic community. This was performed by direct isolation with different culture media, using samples from the biofilter of the RAS. Part of these isolates were already tested, and 12 bacterial strains have presented anti-*T. maritimum* activity in the cross-streak assay and 13 in the diffusion disk. Three strains have already been identified as *Pseudoalteromonas* sp., a promising finding as this genus has already been identified as also possessing anti-*Vibrio* activity (Handayani et al., 2021). This is also relevant in the context the complex interaction between the genera *Vibrio* and *Tenacibaculum* described in our previous network approach. There are still more isolates to be tested, but this initial proof of concept has established the potential for future studies.

6.6. Main conclusions

At the end of this work, we can outline the following take home messages:

- Fish welfare, from higher growth rate to disease prevention, starts with maintaining a highly diverse community within the traditionally stable RAS environment. Fine tuning manipulation of environmental parameters may be a tool to put differential selective pressures in the microbiome.
- The cooperative prokaryotic networks of the nitrifying community are extremely complex, with AOB-NOB and AOA-NOB classical interactions co-existing with the *Nitrospira* ability for complete nitrification.
- We have added to the (still few) number of studies correlating different pathogenic bacteria, finding both *Tenacibaculum*-*Vibrio* and *Tenacibaculum*-*Photobacterium* positive interactions.

- The fish host community is constituted by both inherited and acquired genera, with the inherited community constituting an important part in the core microbiome, maintain high relative abundances through the production.
- Both potentially pathogenic and probiotic groups may be inherited or acquired in the production.
- Early development of the microbiome is key and initial diet must be richer than the current paradigm of live feed, which has the added risk of being a vector to potentially pathogenic bacteria.
- Three bacterial strain (*Pseudoalteromonas*) have been identified as having anti-*Tenacibaculum maritimum* activity, and we are likely to find more in future works.

6.7. Future Approaches

The two types of studies, culture dependent and independent, that have been explored in this thesis need to be further expanded to other production units, as these may have different constitutions in the microbiome. Longitudinal studies, both in the environment and fish microbiome must be of paramount importance in aquaculture NGS studies, particularly to detect temporal changes both in the routine husbandry environment and as the fish progresses in the production stages. For our case study, future work should include a more diverse characterization. To our knowledge, no wide-ranging microbiome studies have been published for RAS, that is, we need to keep characterizing the prokaryotic community as it evolves, but we must also include other components of the microbiome. These includes eukaryotic organisms such as fungi, as they have been associated with antibacterial activity (Özkaya et al., 2017), and microalgae, which also have ammonia-removal potential (Milhazes-Cunha & Otero, 2017) and may play a key role in the microbial network of the system while competing with nitrifying prokaryotes.

Nitrifying bacteria are still a challenge to successfully isolate and manipulate in vitro. In the future we must keep optimizing the enrichment procedures, both for the unsuccessful AOB enrichment, but also to establish a protocol that may shorten the activation period. As for disease prevention, going forward, we must elucidate the interactions between potentially pathogenic genera. More NGS studies (particularly with new technologies such as PacBio, highly accurate long-read sequencing for almost-complete 16S sequencing) as well as empirical evidence (such as anti-activity plate assays or fish challenges) are needed to confirm that these interactions may play a key role in disease development. The most promising isolates with anti-*T. maritimum* activity should be

further tested. There are two roads to do this, they can either be tested to be integrated in probiotic formulations or to produce bioactive compounds, suitable to be used as alternatives to antibiotics.

The results obtained from this thesis bring us closer to reaching a set of possible solutions to potentiate the nitrifying community of the biofilter for increased water quality, and solutions for providing a degree of disease resistance in the reared fish. This work also emphasizes the importance of having Research and Development departments in aquaculture productions working together with research groups. In these partnerships, as the science progresses, the productions not only have a microbial characterization that would otherwise not be part of the routine, but also the tools developed are tailor made for them.

6.8. References:

Berg, G., Rybakova, D., Fischer, D., Cernava, T., Vergès, M.-C.C., Charles, T., Chen, X., Cocolin, L., Eversole, K., Corral, G.H., 2020. Microbiome definition re-visited: old concepts and new challenges. Microbiome. 8, 1-22.

Borges, M.-T., Sousa, A., De Marco, P., Matos, A., Hönigová, P., Castro, P.M., 2008. Aerobic and anoxic growth and nitrate removal capacity of a marine denitrifying bacterium isolated from a recirculation aquaculture system. Microbial ecology. 55, 107-118.

Brailo, M., Schreier, H.J., McDonald, R., Maršić-Lučić, J., Gavrilović, A., Pećarević, M., Jug-Dujaković, J., 2019. Bacterial community analysis of marine recirculating aquaculture system bioreactors for complete nitrogen removal established from a commercial inoculum. Aquaculture. 503, 198-206.

European Commission, 2019. Regulation (EU) 2019/4 of the European Parliament and of the Council of 11 December 2018 on the manufacture, placing on the market and use of medicated feed, amending regulation (EC) NO 183/2005 of the European Parliament and of the Council and repealing Council Directive 90/167/EEC (text with EEA relevance). Official Journal of the Eurean Union. 4.

Daims, H., Lebedeva, E.V., Pjevac, P., Han, P., Herbold, C., Albertsen, M., Jehmlich, N., Palatinszky, M., Vierheilig, J., Bulaev, A., 2015. Complete nitrification by *Nitrospira* bacteria. Nature. 528, 504-509.

El-Saadony, M.T., Alagawany, M., Patra, A.K., Kar, I., Tiwari, R., Dawood, M.A., Dhama, K., Abdel-Latif, H.M., 2021. The functionality of probiotics in aquaculture: An overview. Fish & Shellfish Immunology.

Faust, K., Lima-Mendez, G., Lerat, J.-S., Sathirapongsasuti, J.F., Knight, R., Huttenhower, C., Lenaerts, T., Raes, J., 2015. Cross-biome comparison of microbial association networks. Frontiers in microbiology. 6, 1200.

Handayani, D.P., Isnansetyo, A., Istiqomah, I., Jumina, J., 2021. Anti-*Vibrio* activity of *Pseudoalteromonas xiamenensis* STKMTI. 2, a new potential vibriosis biocontrol bacterium in marine aquaculture. Aquaculture Research.

Hatzenpichler, R., 2012. Diversity, physiology, and niche differentiation of ammonia-oxidizing archaea. Applied and environmental microbiology. 78, 7501-7510.

Infante-Villamil, S., Huerlimann, R., Jerry, D.R., 2020. Microbiome diversity and dysbiosis in aquaculture. Reviews in Aquaculture.

Martínez-Porchas, M., Vargas-Albores, F., 2017. Microbial metagenomics in aquaculture: a potential tool for a deeper insight into the activity. Reviews in Aquaculture. 9, 42-56.

Milhazes-Cunha, H., Otero, A., 2017. Valorisation of aquaculture effluents with microalgae: the integrated multi-trophic aquaculture concept. Algal Research. 24, 416-424.

Özkaya, F.C., Peker, Z., Camas, M., Sazak Camas, A., Altunok, M., 2017. Marine Fungi Against Aquaculture Pathogens and Induction of the Activity via Co-Culture. CLEAN–Soil, Air, Water. 45, 1700238.

Wynne, J.W., Thakur, K.K., Slinger, J., Samsing, F., Milligan, B., Powell, J.F.F., McKinnon, A., Nekouei, O., New, D., Richmond, Z., Gardner, I., Siah, A., 2020. Microbiome Profiling Reveals a Microbial Dysbiosis During a Natural Outbreak of Tenacibaculosis (Yellow Mouth) in Atlantic Salmon. Front Microbiol. 11, 586387.

Appendixes

Appendix A: Supplementary Materials Chapter 2

Microbial community dynamics in a hatchery recirculating aquaculture system (RAS) of sole (*Solea senegalensis*)

Table S1: Total number of sequences obtained for each sample after sequencing (Input - raw sequences), and number of sequences after each DADA2 treatment: after filtering out low-quality sequencing reads (Filtered), trimming the reads to a consistent length (Denoised) and after the removal of the chimeras (Nonchim). Alpha diversity is represented with total read counts (observed ASVs) and the Shannon index, of the microbial community found in samples of the aquaculture unit in the different systems (pre-ongrowing (PO), brooding stock (BS), weaning (WE) and open system (OS)), different matrixes (water (A-H), biofilter (BB) and tank biofilm (BF)) at different times (1-6).

Sample	Input	Filtered	Denoised	Nonchim	Observed ASVs	Shannon
PO_A_1	76002	70771	66144	30916	202	4.64
PO_A_2	38892	36688	32935	18692	125	4.15
PO_A_4	58436	55203	50380	32772	149	3.98
PO_A_5	39250	37040	32927	20526	114	3.93
PO_A_6	42687	40236	35327	24456	146	4.08
PO_B_2	33411	31639	27698	16417	131	4.18
PO_B_4	33110	31688	28529	14379	159	4.66
PO_C_2	37330	35199	31752	16331	108	3.97
PO_C_4	18751	17740	15449	10396	80	3.86
PO_D_2	40408	38152	33800	19157	125	4.15
PO_D_4	22570	21263	18590	12619	82	3.88
PO_D_5	23415	21885	18967	12232	86	3.85
PO_D_6	40652	38421	33986	21652	138	4.21
PO_BB_3	44611	42640	37776	29513	276	4.31
PO_BB_4	66002	62156	48086	36788	491	5.96
PO_BB_5	120356	116370	96982	63691	648	6.21
PO_BB_6	78610	74852	57898	44448	532	5.88
PO_BF_3	95851	90342	75675	46562	440	5.76
PO_BF_4	37657	35497	27760	22316	344	5.61
PO_BF_5	47270	44676	34767	25339	313	5.45
PO_BF_6	58435	55462	43427	30480	335	5.60
WE_G_2	49596	46829	38469	25074	311	5.46
WE_G_4	118383	114323	108247	70487	297	3.98
WE_G_5	69404	66145	53815	39847	546	5.83
WE_G_6	47431	44873	33288	25128	391	5.64
WE_H_2	65184	60910	50116	33379	340	5.44
WE_H_4	72027	69204	61089	45394	320	4.12
WE_H_5	85662	81564	64497	43372	500	5.78
WE_H_6	46856	44818	36358	23593	291	5.28
WE_BB_5	66615	63737	55301	36498	385	5.63
WE_BB_6	75800	73003	62092	42855	466	5.73
WE_BF_3	39754	37567	30157	20101	265	5.33

WE_BF_5	20331	19121	15271	11961	167	4.94
WE_BF_6	65623	62512	53250	30103	275	5.29
BS_E_2	67559	64294	57424	22801	155	4.11
FW_I_2	73077	68488	46322	33390	296	5.34
OS_F_2	26994	25253	18855	13792	185	4.81

Table S2: Relative microbial community diversity in the water (sample points A, B, C and D) of the pre-ongrowing system (PO). Pruned for genera that contributed with more than 3% in the sum of the samples.

	Samples												
Genus	PO_A_1	PO_A_2	PO_A_4	PO_A_5	PO_A_6	PO_B_2	PO_B_4	PO_C_2	PO_C_4	PO_D_2	PO_D_4	PO_D_5	PO_D_6
Tenacibaculum	0.61	0.00	0.44	0.66	0.00	0.00	0.90	0.00	0.00	0.00	0.28	0.88	0.00
Sulfitobacter	48.91	2.81	41.60	51.20	56.14	2.76	37.58	2.11	33.56	3.05	37.77	47.18	44.60
Leucothrix	2.48	53.16	31.92	19.75	4.99	51.09	21.75	45.95	28.13	51.02	26.53	19.59	4.47
Polaribacter_4	3.07	1.27	2.59	2.18	2.12	1.46	8.91	0.82	1.89	1.75	2.68	2.87	2.34
Thalassotalea	6.68	8.15	5.15	3.14	4.68	6.57	3.30	8.24	7.29	7.06	6.85	3.96	6.72
Pseudoalteromonas	18.57	11.00	3.55	5.63	13.37	11.22	7.33	24.74	13.00	12.83	12.31	6.62	18.87
Francisella	0.45	5.59	1.10	1.25	3.31	4.48	0.70	3.55	2.15	6.59	1.81	2.71	5.26
Vibrio	0.90	1.26	1.76	0.85	0.81	0.00	0.00	0.00	0.98	1.43	0.95	2.58	2.00
Polaribacter_2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Algibacter	1.70	1.16	1.66	2.11	0.99	1.27	3.51	0.68	1.64	1.23	1.74	2.00	1.39
Formosa	0.00	0.00	0.74	1.40	0.65	0.00	0.00	0.00	0.00	0.00	0.61	0.55	0.72
Olleya	3.05	2.33	1.50	2.18	1.40	2.67	1.21	3.60	1.34	2.19	1.64	2.52	2.42
Planktotalea	0.00	0.00	0.60	0.00	0.40	0.00	0.59	0.00	0.59	0.00	0.61	0.86	0.22
Halocynthiibacter	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Litoreibacter	0.00	0.31	0.21	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.13
Rubritalea	0.77	0.48	0.72	2.21	2.03	1.19	0.70	0.56	0.38	0.87	0.83	2.22	1.07
Marinicella	0.31	0.51	0.27	0.30	0.43	0.54	0.10	0.48	0.15	0.00	0.31	0.23	0.22
Pseudophaeobacter	0.41	1.05	0.54	0.00	0.48	0.84	0.56	0.60	0.41	1.44	0.62	0.54	0.43
Thiothrix	0.12	0.25	1.28	1.53	2.04	0.00	1.13	0.00	1.01	0.00	1.32	0.00	1.96
Altererythrobacter	0.00	0.32	0.00	0.00	0.00	0.28	0.46	0.16	0.00	0.35	0.00	0.00	0.00
Pseudofulvibacter	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Oleispira	0.00	0.00	0.00	0.00	0.07	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Schleiferia	0.00	0.00	0.06	0.00	0.12	0.23	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Sphingorhabdus	0.05	0.00	0.05	0.26	0.47	0.00	0.44	0.00	0.00	0.00	0.00	0.08	0.47
Novosphingobium	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Nitrospira	0.00	0.00	0.00	0.00	0.09	0.24	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Candidatus_ Nitrosopumilus	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Blastopirellula	0.33	0.42	0.24	0.16	0.35	0.17	0.11	0.44	0.13	0.58	0.11	0.00	0.33
Fluviicola	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Maribacter	0.46	0.00	0.00	0.09	0.35	0.54	0.37	0.37	0.00	0.24	0.24	0.00	0.03
Winogradskyella	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Pseudorhodobacter	0.00	0.00	0.00	0.00	0.00	0.10	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Lewinella	0.84	0.15	0.04	0.00	0.11	0.32	0.13	0.12	0.10	0.21	0.00	0.10	0.00
Planctomicrobium	0.21	0.00	0.12	0.18	0.43	0.08	0.14	0.00	0.00	0.16	0.00	0.00	0.19
Lacinutrix	0.44	0.26	0.00	0.00	0.00	0.35	0.00	0.47	0.00	0.39	0.00	0.00	0.00
Nitrosomonas	0.14	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Loktanella	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Ulvibacter	0.35	0.20	0.00	0.00	0.00	0.24	0.00	0.00	0.00	0.13	0.00	0.00	0.00
Persicirhabdus	0.20	0.00	0.04	0.00	0.26	0.00	0.00	0.00	0.00	0.18	0.00	0.00	0.00
Subgroup_10	0.00	0.00	0.00	0.00	0.05	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Woeseia	0.00	0.00	0.00	0.00	0.09	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Roseibacillus	0.00	0.00	0.07	0.22	0.30	0.16	0.00	0.00	0.00	0.13	0.00	0.00	0.27
Rubidimonas	0.05	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Parvibaculum	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Cocleimonas	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Colwellia	0.10	0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Pseudahrensia	0.00	0.00	0.04	0.00	0.00	0.03	0.09	0.00	0.00	0.00	0.00	0.00	0.00
Sulfuritalea	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Halomonas	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.11	0.00	0.00	0.00	0.00
Simplicispira	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
SM1A02	0.00	0.12	0.00	0.00	0.00	0.14	0.00	0.00	0.00	0.09	0.00	0.00	0.00
Legionella	0.00	0.00	0.06	0.08	0.11	0.00	0.17	0.00	0.00	0.00	0.00	0.00	0.00
Azoarcus	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Salinirepens	0.00	0.00	0.00	0.00	0.06	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Phaeodactylibacter	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Thalassospira	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Caulobacter	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Tistrella	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Table S3: Relative microbial community diversity in the tank biofilm and biofilter (sample points BF and BB respectively) of the pre-ongrowing system (PO). Pruned for genera that contributed with more than 3% in the sum of the samples.

	Samples							
Genus	PO_BB_3	PO_BB_4	PO_BB_5	PO_BB_6	PO_BF_3	PO_BF_4	PO_BF_5	PO_BF_6
Tenacibaculum	0.00	0.00	1.67	0.00	0.00	0.00	0.00	0.00
Sulfitobacter	0.00	2.92	0.91	3.42	1.27	3.85	2.89	0.48
Leucothrix	0.00	0.88	6.01	0.24	1.48	1.22	0.00	2.02
Polaribacter_4	3.45	6.61	5.22	4.55	0.69	0.37	2.52	1.44
Thalassotalea	2.00	0.15	0.00	0.75	0.79	0.71	0.00	0.07
Pseudoalteromonas	0.99	0.00	0.54	0.00	0.41	0.55	0.04	0.27
Francisella	1.50	0.24	0.00	2.97	0.05	1.08	0.18	0.63
Vibrio	0.08	0.16	0.00	0.43	0.00	0.19	0.00	0.00
Polaribacter_2	0.00	0.00	17.43	0.00	0.00	0.00	0.00	0.00
Algibacter	0.10	3.08	0.00	10.65	0.12	2.33	2.63	0.34
Formosa	0.00	2.62	0.00	5.82	0.00	3.22	0.57	0.00
Olleya	0.00	0.81	1.33	0.89	0.00	0.00	1.57	3.66
Planktotalea	1.13	4.75	2.40	2.18	1.67	0.28	0.00	0.00
Halocynthiibacter	0.00	0.00	1.61	0.00	0.00	0.00	0.00	0.00
Litoreibacter	1.80	3.79	0.00	1.51	7.24	2.52	2.86	2.81
Rubritalea	2.35	3.98	4.12	3.71	2.99	6.46	5.47	5.45
Marinicella	5.44	3.72	0.00	2.86	27.33	1.53	3.36	2.47
Pseudophaeobacter	0.08	1.21	0.00	1.63	0.45	0.55	0.21	0.14
Thiothrix	0.00	0.69	7.07	0.19	0.32	1.18	1.01	4.28
Altererythrobacter	3.76	3.79	0.66	1.94	3.96	4.35	4.44	3.27
Pseudofulvibacter	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Oleispira	0.00	0.00	2.15	0.00	0.58	0.00	0.00	0.00
Schleiferia	0.00	2.26	0.00	1.92	0.00	1.00	11.72	11.34
Sphingorhabdus	4.71	2.97	4.67	1.69	0.30	1.04	1.45	1.50
Novosphingobium	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Nitrospira	4.29	4.38	0.00	3.60	0.46	4.27	0.45	0.67
Candidatus_Nitrosopumilus	0.00	0.00	0.00	0.53	0.00	0.00	0.00	0.00
Blastopirellula	3.87	4.83	0.51	3.65	1.37	1.43	5.86	4.61
Fluviicola	0.00	0.00	4.95	0.00	0.00	0.00	0.00	0.00
Maribacter	4.32	4.80	2.82	2.73	1.22	2.94	3.81	5.68
Winogradskyella	0.00	0.00	1.88	0.00	0.00	0.00	0.00	0.00
Pseudorhodobacter	1.93	2.21	0.00	1.97	0.98	0.66	0.00	0.00
Lewinella	0.91	1.04	0.19	1.89	1.27	1.52	6.71	9.76
Planctomicrobium	1.97	2.21	0.00	1.94	1.05	5.17	3.18	3.40
Lacinutrix	0.13	0.62	2.47	1.71	0.76	0.09	0.57	0.73
Nitrosomonas	6.91	0.55	0.00	0.41	0.00	3.72	0.98	0.00
Loktanella	0.08	1.35	0.00	0.77	4.41	0.00	0.21	0.00
Ulvibacter	0.00	0.17	0.08	0.00	0.13	0.00	3.29	2.40
Persicirhabdus	1.29	0.58	0.00	1.72	0.00	2.83	1.46	4.72
Subgroup_10	1.06	1.26	0.00	1.21	1.10	1.10	3.68	1.35

Woeseia	1.79	1.00	0.00	1.52	0.12	1.50	0.24	0.32
Roseibacillus	0.00	2.17	0.00	2.10	1.06	4.07	2.58	2.48
Rubidimonas	0.00	0.39	4.06	0.12	0.00	0.00	0.00	0.00
Parvibaculum	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Cocleimonas	0.00	0.00	5.39	0.00	0.00	0.00	0.00	0.00
Colwellia	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Pseudahrensia	3.66	1.16	0.00	0.51	0.27	0.00	0.33	0.49
Sulfuritalea	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Halomonas	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Simplicispira	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
SM1A02	0.00	0.74	0.00	0.48	0.17	3.17	1.59	2.11
Legionella	0.00	0.27	0.00	0.27	0.21	0.30	0.00	0.00
Azoarcus	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Salinirepens	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Phaeodactylibacter	0.00	0.17	0.00	0.65	0.00	0.00	0.00	0.08
Thalassospira	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Caulobacter	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Tistrella	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Table S4: Relative microbial community diversity in the water (sample points G and H) of the weaning system (WE). Pruned for genera that contributed with more than 3% in the sum of the samples.

	Samples							
Genus	WE_G_2	WE_G_4	WE_G_5	WE_G_6	WE_H_2	WE_H_4	WE_H_5	WE_H_6
Tenacibaculum	23.96	75.21	34.18	26.18	36.72	82.15	17.47	18.89
Sulfitobacter	0.80	0.20	1.46	1.24	0.87	0.12	0.93	0.72
Leucothrix	10.95	0.55	5.34	4.73	12.39	1.67	7.56	3.65
Polaribacter_4	7.39	8.86	5.36	4.53	2.40	1.96	8.93	7.21
Thalassotalea	0.39	0.05	0.17	0.26	0.28	0.00	0.44	0.70
Pseudoalteromonas	5.27	1.29	1.71	4.52	2.18	0.31	10.14	16.16
Francisella	1.86	0.19	1.64	2.67	1.77	0.54	0.96	2.66
Vibrio	3.02	0.67	0.78	1.45	0.77	0.08	3.65	5.35
Polaribacter_2	3.70	2.45	2.99	3.32	4.51	0.72	4.39	5.26
Algibacter	0.36	0.00	0.36	0.46	0.40	0.00	0.39	0.41
Formosa	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Olleya	3.43	0.94	0.87	0.80	1.41	0.00	1.93	2.49
Planktotalea	1.31	0.92	1.90	3.74	0.00	0.75	1.34	1.68
Halocynthiibacter	3.70	0.39	1.38	2.04	1.94	0.44	1.54	1.98
Litoreibacter	0.00	0.12	0.27	0.40	0.00	0.00	0.24	0.24
Rubritalea	1.40	0.04	1.34	2.32	2.24	0.27	1.06	0.60
Marinicella	1.05	0.01	1.75	1.97	1.61	0.32	1.50	0.50
Pseudophaeobacter	0.60	0.00	1.08	0.97	1.25	0.31	1.13	1.50
Thiothrix	1.35	0.27	1.80	1.45	1.33	0.23	1.70	1.37
Altererythrobacter	0.00	0.07	0.27	0.14	0.27	0.13	0.20	0.05
Pseudofulvibacter	4.21	0.23	2.87	5.68	3.11	0.00	3.51	4.26
Oleispira	3.10	0.29	0.21	0.85	1.90	0.03	0.59	2.55
Schleiferia	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Sphingorhabdus	0.00	0.36	0.14	0.15	0.00	0.06	0.14	0.00
Novosphingobium	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Nitrospira	0.00	0.02	0.71	1.04	0.07	0.41	0.33	0.07
Candidatus_Nitrosopumilus	0.00	0.03	0.55	0.59	0.00	0.00	0.36	0.45
Blastopirellula	0.03	0.02	0.03	0.32	0.27	0.10	0.07	0.00
Fluviicola	0.00	0.00	0.32	0.12	0.00	0.00	0.52	0.36
Maribacter	0.20	0.00	0.48	0.63	0.19	0.23	0.34	0.22
Winogradskyella	0.34	0.48	0.39	0.73	0.56	0.26	0.88	1.03
Pseudorhodobacter	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Lewinella	0.56	0.00	1.08	1.15	1.09	0.31	0.45	0.11
Planctomicrobium	0.36	0.00	0.71	0.44	1.01	0.16	0.42	0.00
Lacinutrix	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Nitrosomonas	0.00	0.05	0.45	0.25	0.23	0.18	0.33	0.00
Loktanella	0.78	0.05	0.00	0.15	0.18	0.00	0.44	0.00
Ulvibacter	0.00	0.02	0.00	0.00	0.00	0.00	0.14	0.11

Persicirhabdus	0.00	0.00	0.14	0.16	0.00	0.00	0.08	0.00
Subgroup_10	0.02	0.05	0.48	0.41	0.00	0.25	0.07	0.00
Woeseia	0.00	0.00	0.75	0.75	0.00	0.18	0.40	0.07
Roseibacillus	0.32	0.00	0.28	0.37	0.71	0.09	0.14	0.06
Rubidimonas	0.00	0.00	0.31	0.40	0.18	0.09	0.11	0.00
Parvibaculum	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Cocleimonas	0.00	0.28	0.05	0.06	0.00	0.00	0.08	0.03
Colwellia	0.34	0.08	0.36	0.36	0.21	0.03	0.91	0.67
Pseudahrensia	0.00	0.00	0.38	0.20	0.05	0.13	0.06	0.00
Sulfuritalea	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Halomonas	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Simplicispira	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
SM1A02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Legionella	0.00	0.00	0.12	0.00	0.00	0.01	0.00	0.00
Azoarcus	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Salinirepens	0.00	0.00	0.00	0.00	0.00	0.00	0.12	0.00
Phaeodactylibacter	0.00	0.00	0.11	0.10	0.00	0.01	0.05	0.00
Thalassospira	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Caulobacter	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Tistrella	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Table S5: Relative microbial community diversity in the tank biofilm and biofilter (sample points BF and BB respectively) of the weaning system (WE). Pruned for genera that contributed with more than 3% in the sum of the samples.

	Samples				
Genus	WE_BB_5	WE_BB_6	WE_BF_3	WE_BF_5	WE_BF_6
Tenacibaculum	31.09	38.96	1.60	0.00	2.69
Sulfitobacter	2.28	0.49	2.46	3.07	0.95
Leucothrix	1.29	1.97	5.83	0.64	7.42
Polaribacter_4	2.47	3.15	6.47	4.24	6.48
Thalassotalea	0.00	0.04	0.18	0.37	0.00
Pseudoalteromonas	0.00	0.00	1.03	0.10	1.54
Francisella	0.00	0.67	0.00	0.24	0.00
Vibrio	0.00	0.00	0.00	0.25	0.40
Polaribacter_2	3.50	3.56	3.18	0.00	15.16
Algibacter	1.19	2.10	0.00	4.95	0.00
Formosa	0.00	0.14	0.00	1.81	0.00
Olleya	0.00	0.66	1.13	0.31	4.19
Planktotalea	5.45	4.89	2.11	3.61	2.39
Halocynthiibacter	0.00	0.88	3.19	0.00	2.36
Litoreibacter	0.57	0.34	0.00	1.70	0.88
Rubritalea	2.58	2.66	3.82	6.48	8.04
Marinicella	0.41	0.51	0.18	3.28	0.00
Pseudophaeobacter	0.00	0.27	0.00	2.18	0.00
Thiothrix	0.54	0.50	5.28	0.66	1.66
Altererythrobacter	0.74	0.09	0.93	2.65	0.83
Pseudofulvibacter	0.00	0.51	2.66	0.00	0.00
Oleispira	0.05	0.00	8.38	0.00	0.17
Schleiferia	0.00	0.00	0.15	1.98	0.00
Sphingorhabdus	0.59	0.82	2.23	1.57	1.19
Novosphingobium	0.00	0.00	0.00	0.00	0.00
Nitrospira	0.84	1.31	0.00	2.76	0.00
Candidatus_Nitrosopumilus	3.93	3.23	0.00	0.36	0.00
Blastopirellula	2.17	0.52	1.56	5.78	0.00
Fluviicola	0.00	0.00	1.22	0.00	7.02
Maribacter	1.15	0.98	3.69	2.92	1.09
Winogradskyella	0.00	0.58	3.64	0.00	2.01
Pseudorhodobacter	0.00	0.00	0.00	3.56	0.00
Lewinella	0.59	0.72	1.25	1.37	0.19
Planctomicrobium	1.35	0.59	0.24	2.66	0.00
Lacinutrix	0.00	0.00	4.27	0.36	0.66
Nitrosomonas	0.70	0.81	0.06	0.45	0.00
Loktanella	0.42	0.00	0.75	0.46	1.34
Ulvibacter	0.27	0.00	0.00	0.17	0.28
Persicirhabdus	0.68	0.48	0.00	2.39	0.00
Subgroup_10	0.26	0.27	0.00	1.15	0.00
Woeseia	3.66	2.31	0.07	1.35	0.00

Roseibacillus	0.58	0.75	0.00	2.32	0.06
Rubidimonas	0.14	0.05	1.96	0.33	1.48
Parvibaculum	0.00	0.00	0.00	0.00	0.00
Cocleimonas	0.18	0.61	2.47	0.00	1.88
Colwellia	0.00	0.00	0.21	0.00	0.12
Pseudahrensia	1.97	0.63	0.29	1.16	0.16
Sulfuritalea	0.00	0.00	0.00	0.00	0.00
Halomonas	0.00	0.00	0.00	0.00	0.00
Simplicispira	0.00	0.00	0.00	0.00	0.00
SM1A02	0.10	0.00	0.00	0.35	0.00
Legionella	0.18	0.12	0.00	0.19	0.00
Azoarcus	0.00	0.00	0.00	0.00	0.00
Salinirepens	0.00	0.00	0.24	0.00	0.00
Phaeodactylibacter	0.00	0.00	0.00	0.42	0.00
Thalassospira	0.00	0.00	0.00	0.00	0.00
Caulobacter	0.00	0.00	0.00	0.00	0.00
Tistrella	0.00	0.00	0.00	0.00	0.00

Table S6: Relative microbial community diversity in the breeding stock (BS), freshwater reservoir (FW) and open system (OS, saltwater reservoir). Pruned for genera that contributed with more than 3% in the sum of the samples.

	Samples		
Genus	BS_E_2	FW_I_2	OS_F_2
Tenacibaculum	45.00	0.65	1.32
Sulfitobacter	0.40	0.47	0.00
Leucothrix	2.07	0.00	0.15
Polaribacter_4	0.00	0.00	0.00
Thalassotalea	14.43	0.00	0.00
Pseudoalteromonas	2.49	0.00	6.77
Francisella	0.05	0.00	0.08
Vibrio	0.00	0.18	0.00
Polaribacter_2	0.16	0.00	0.00
Algibacter	0.00	0.00	0.00
Formosa	0.00	0.00	0.00
Olleya	0.00	0.12	0.00
Planktotalea	1.13	0.00	0.00
Halocynthiibacter	0.00	0.00	0.00
Litoreibacter	0.00	0.00	0.00
Rubritalea	0.00	0.00	0.00
Marinicella	0.48	0.00	0.00
Pseudophaeobacter	3.01	0.00	0.00
Thiothrix	0.56	0.00	0.00
Altererythrobacter	0.00	0.00	0.00
Pseudofulvibacter	1.27	0.00	0.00
Oleispira	0.63	0.00	0.00
Schleiferia	0.00	0.00	0.00
Sphingorhabdus	0.00	0.00	1.17
Novosphingobium	0.00	1.95	31.65
Nitrospira	0.00	0.30	0.00
Candidatus_Nitrosopumilus	0.10	0.00	0.00
Blastopirellula	0.11	0.00	0.83
Fluviicola	0.00	0.74	0.00
Maribacter	0.00	0.00	0.00
Winogradskyella	0.00	0.00	0.00
Pseudorhodobacter	0.00	1.23	0.00
Lewinella	0.11	0.00	0.00
Planctomicrobium	0.00	0.00	0.00
Lacinutrix	0.00	0.00	0.00
Nitrosomonas	0.09	0.00	0.00
Loktanella	2.54	0.00	0.00
Ulvibacter	0.00	0.00	0.00
Persicirhabdus	0.00	0.00	0.00
Subgroup_10	0.00	2.74	0.00
Woeseia	0.00	0.00	0.00

Roseibacillus	0.00	0.00	0.00
Rubidimonas	0.00	0.00	0.00
Parvibaculum	0.00	7.54	0.92
Cocleimonas	0.00	0.00	0.00
Colwellia	3.60	0.00	0.15
Pseudahrensia	0.00	0.00	0.00
Sulfuritalea	0.00	3.62	0.00
Halomonas	0.00	0.00	5.17
Simplicispira	0.00	5.05	0.00
SM1A02	0.03	2.30	0.00
Legionella	0.00	3.24	0.23
Azoarcus	0.00	5.29	0.00
Salinirepens	3.18	0.00	0.00
Phaeodactylibacter	0.00	6.91	0.00
Thalassospira	0.00	0.00	5.68
Caulobacter	0.00	4.66	0.00
Tistrella	0.00	0.00	3.66

Table S7: Functional predictions (KEGG (Kyoto Encyclopedia of Genes and Genomes) orthologs from amplicon sequences related with genes of the nitrogen cycle for the Pre-Ongrowing (PO) system.

	Samples												
KO	PO_A_1	PO_A_2	PO_A_4	PO_A_5	PO_A_6	PO_B_2	PO_B_4	PO_C_2	PO_C_4	PO_D_2	PO_D_4	PO_D_5	PO_D_6
K00362	11014	7966	12232	5369	5447	6596	6679	6118	4483	7129	4555	3373	4766
K00363	11119	7876	12304	5297	5509	6411	6624	6101	4210	7248	4542	3290	4764
K03385	26	0	14	9	33	19	8	40	0	19	0	0	11
K00366	943	230	748	412	392	239	965	108	226	208	319	282	393
K00368	195	118	22	47	61	146	154	91	5	122	7	12	27
K00370	25	54	75	0	29	76	36	16	86	40	12	8	76
K00371	25	36	75	0	29	76	36	16	86	40	12	8	76
K00374	10039	7623	11445	4897	5038	6173	5612	6003	3967	6982	4193	3004	4272
K02567	25	30	75	0	18	53	36	16	86	40	12	8	76
K02568	450	191	85	37	88	313	170	199	85	234	22	13	71
K00367	88	146	73	27	15	151	96	64	51	128	12	8	28
K00372	1981	1069	1062	329	617	650	399	863	530	797	560	273	865
K10944	1981	1069	1062	329	607	650	399	863	530	797	560	273	865
K10945	71	83	93	12	31	66	12	44	9	94	29	29	84
K10946	700	6202	6241	1943	648	5125	2669	4848	2062	5753	2106	1221	563
K00376	35	0	0	0	0	0	0	0	0	0	0	0	0
K10535	35	0	0	0	0	0	0	0	0	0	0	0	0
K04561	35	0	0	0	0	0	0	0	0	0	0	0	0
K02305	70	0	0	0	0	0	0	0	0	0	0	0	0

KO	PO_BB_3	PO_BB_4	PO_BB_5	PO_BB_6	PO_BF_3	PO_BF_4	PO_BF_5	PO_BF_6
K00362	12361	7532	2813	9094	14530	4300	4930	4392
K00363	13679	11474	2506	12428	8781	6309	6664	6279
K03385	128	315	0	346	188	330	297	347
K00366	3243	2689	597	5174	736	1635	1429	1422
K00368	4610	1763	151	1844	1918	1235	801	406
K00370	859	1368	23	1131	612	651	490	414
K00371	859	1380	23	1131	612	651	490	414
K00374	11527	5427	1827	4294	6997	3133	4264	3668
K02567	507	630	23	498	503	285	440	325
K02568	1452	3035	736	2428	3026	891	1701	1900

K00367	914	1847	357	1131	3637	732	848	660
K00372	292	277	182	489	498	339	129	47
K10944	275	187	182	274	498	279	75	36
K10945	110	348	71	361	161	233	573	937
K10946	1161	2163	1142	1322	4343	837	862	946
K00376	779	93	0	72	350	319	109	0
K10535	599	93	0	166	0	319	109	0
K04561	599	93	0	166	0	319	109	0
K02305	1380	243	0	238	0	638	218	0

Table S8: Functional predictions (KEGG (Kyoto Encyclopedia of Genes and Genomes) orthologs from amplicon sequences related with genes of the nitrogen cycle for the Weaning (WE) system.

	Samples												
KO	WE_BB_5	WE_BB_6	WE_BF_3	WE_BF_5	WE_BF_6	WE_G_2	WE_G_4	WE_G_5	WE_G_6	WE_H_2	WE_H_4	WE_H_5	WE_H_6
K00362	6407	6224	4094	13081	4884	6595	5234	7293	4498	6309	2801	9548	5493
K00363	6966	6825	3934	19519	4620	5121	4663	5650	3785	4717	2585	7911	3848
K03385	350	192	55	1004	0	32	0	109	56	84	45	94	3
K00366	714	1401	616	5830	1323	813	2528	1027	539	587	419	1522	794
K00368	1548	2177	212	2446	498	530	494	1313	896	681	668	1217	440
K00370	172	390	107	1636	109	152	135	284	441	151	221	331	263
K00371	172	390	107	1636	109	152	135	266	433	131	221	325	263
K00374	4316	3687	2999	9151	3177	4053	1916	3677	2464	3883	1529	5229	2582
K02567	49	112	107	923	109	152	124	104	286	119	72	238	251
K02568	1076	1157	1050	3963	1083	438	512	1209	808	636	693	975	355
K00367	887	849	583	2177	872	1207	700	1245	892	992	817	1177	621
K00372	553	495	107	556	196	828	462	712	555	511	301	1245	864
K10944	553	495	107	378	196	787	542	661	529	515	278	1260	885
K10945	534	382	247	810	420	286	250	317	183	152	93	564	427
K10946	1197	1450	1567	2659	2492	3141	1243	2531	1664	3053	1435	3221	1315
K00376	102	172	7	116	0	0	35	103	36	38	64	87	0
K10535	677	857	7	208	0	0	56	230	120	38	64	183	79
K04561	677	857	7	208	0	0	56	230	120	38	64	183	79
K02305	779	1029	14	324	0	0	91	333	156	76	128	270	79

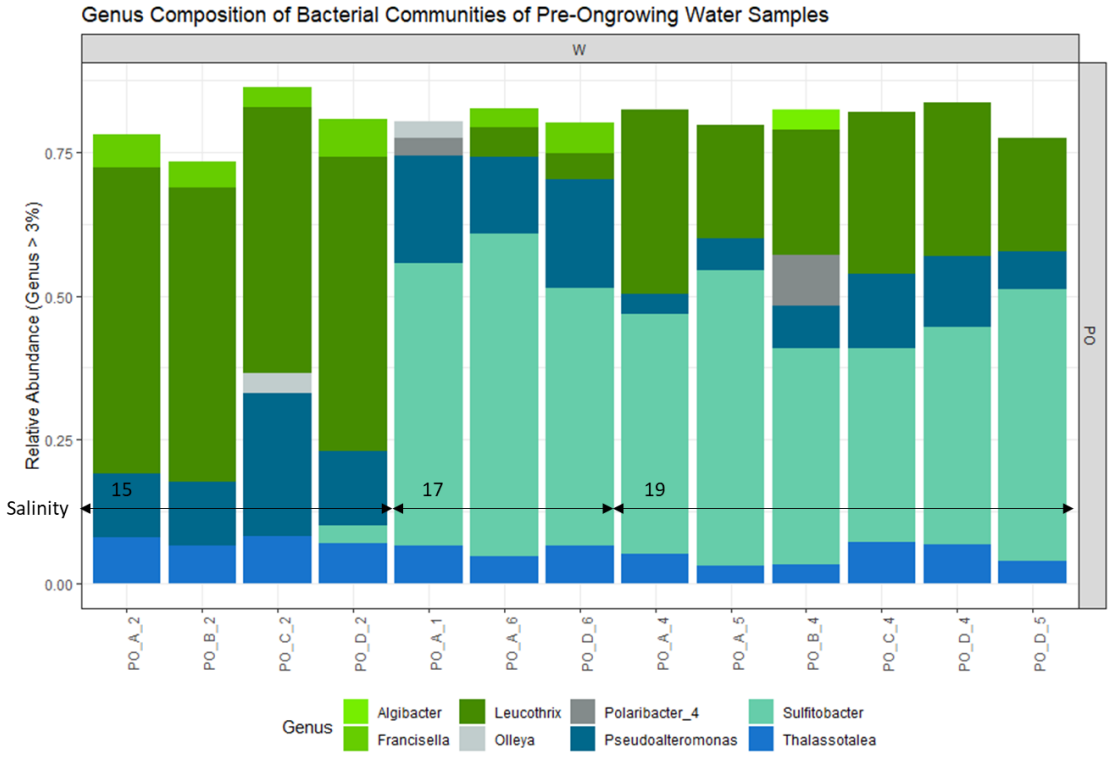
Table S9: Functional predictions (KEGG (Kyoto Encyclopedia of Genes and Genomes) orthologs from amplicon sequences related with genes of the nitrogen cycle for the Fresh Water (FW), Breeding Stock (BS) and Open (OS) systems.

KO	FW_I_2	BS_E_2	OS_F_2
K00362	5154	5913	8073
K00363	4297	7014	7445
K00366	8	461	597
K00367	22	540	523
K00368	276	2118	441
K00370	369	2628	1115
K00371	369	2572	1115
K00372	4089	4880	6741
K00374	369	2525	1117
K00376	266	3067	1560
K02305	556	559	814
K02567	2782	507	548
K02568	2782	507	570
K03385	192	435	379
K04561	884	2800	1074
K10535	12	0	0
K10944	25	786	34
K10945	25	786	34
K10946	37	885	34

Table S10: Description of the functional predictions (KEGG (Kyoto Encyclopedia of Genes and Genomes) orthologs, the gene they represent, the protein, associated reaction, and pathway.

KO	Gene	Protein	Reaction	Pathway
K00362	nirB	NirBD	DNRA	Dissimilatory nitrite reduction to ammonia
K00363	nirD	NirBD	DNRA	Dissimilatory nitrite reduction to ammonia
K03385	nrfA	NrfAH	DNRA	Dissimilatory nitrite reduction to ammonia
K00366	nirA	NirA	DNRA	Dissimilatory nitrite reduction to ammonia
K00368	nirK	NirK	Denitrification	Dissimilatory nitrite reduction to nitric oxide
K00370	narG	NarGHI	Denitrification	Dissimilatory nitrate reduction to nitrite
K00371	narH	NarGHI	Denitrification	Dissimilatory nitrate reduction to nitrite
K00374	narI	NarGHI	Denitrification	Dissimilatory nitrate reduction to nitrite
K02567	napA	NapAB	Denitrification	Dissimilatory nitrate reduction to nitrite
K02568	napB	NapAB	Denitrification	Dissimilatory nitrate reduction to nitrite
K00367	narB	NarB	Denitrification	Dissimilatory nitrate reduction to nitrite
K00372	nasA	NasAB	Denitrification	Dissimilatory nitrate reduction to nitrite
K10944	amoA	AmoABC	Nitrificatio	Oxidation of ammonia into nitrite
K10945	amoB	AmoABC	Nitrification	Oxidation of ammonia into nitrite
K10946	amoC	AmoABC	Nitrification	Oxidation of ammonia into nitrite
K00376	nosZ	NosZ	Denitrificaion	Dissimilatory nitrous oxide reduction to dinitrogen
K10535	hao	HAO	Nitrification	Hydroxylamine to nitrite
K04561	norB	NorBC	Denitrification	Dissimilatory nitric oxide reduction to nitrous oxide
K02305	norC	NorBC	Denitrification	Dissimilatory nitric oxide reduction to nitrous oxide

Image S1: Genus composition of the bacterial communities of the Pre-Ongrowing water samples, ordered according to sample salinity.



Appendix B: Supplementary Materials Chapter 3

The network of nitrifying and pathogenic prokaryotic interactions in a recirculating aquaculture system of a sole (Solea senegalensis) hatchery

Table S1: Number of sequences obtained from sequencing (DADA2 input), after upstream processing (DADA2 output) and observed ASVs of the original dataset. Number of sequences left after functional groups filtering (Filtering output) and observed ASVs for each sub-dataset. Samples are named by and system (Pre-Ongrowing, PO; Weaning, WE), matrix (biofilter carriers, BB; tank biofilm, BF and water, A-H) and then numbered.

	Original Dataset			**Filter: Pathogenic Genus**		**Filter: Nitrifying Genus**	
Sample	**DADA2 input**	**DADA2 output**	**Observed ASVs**	**Filtering output**	**Observed ASVs**	**Filtering output**	**Observed ASVs**
PO_A_1	76002	30916	202	373	2	35	1
PO_A_2	38892	18692	125	143	1	0	0
PO_A_4	58436	32772	149	422	6	0	0
PO_A_5	39250	20526	114	146	2	0	0
PO_A_6	42687	24456	146	101	1	11	1
PO_B_2	33411	16417	131	0	0	23	2
PO_B_4	33110	14379	159	105	2	0	0
PO_C_2	37330	16331	108	0	1	0	0
PO_C_4	18751	10396	80	70	1	0	0
PO_D_2	40408	19157	125	157	2	0	0
PO_D_4	22570	12619	82	97	1	0	0
PO_D_5	23415	12232	86	213	0	0	0
PO_D_6	40652	21652	138	232	1	0	0
PO_BB_3	44611	29513	276	7	0	971	15
PO_BB_4	66002	36788	491	27	0	831	11
PO_BB_5	120356	63691	648	132	0	0	0
PO_BB_6	78610	44448	532	75	1	825	13
PO_BF_3	95851	46562	440	0	1	109	1
PO_BF_4	37657	22316	344	16	2	685	13
PO_BF_5	47270	25339	313	0	3	159	2
PO_BF_6	58435	30480	335	0	3	89	1
WE_G_2	49596	25074	311	4686	27	0	0
WE_G_4	118383	70487	297	48434	32	67	3
WE_G_5	69404	39847	546	8009	4	392	6
WE_G_6	47431	25128	391	3923	1	267	5
WE_H_2	65184	33379	340	6222	6	50	2
WE_H_4	72027	45394	320	29900	53	213	4
WE_H_5	85662	43372	500	5619	48	270	4
WE_H_6	46856	23593	291	4272	51	91	2
WE_BB_5	66615	36498	385	4549	41	800	9

WE_BB_6	75800	42855	466	8264	49	1135	13
WE_BF_3	39754	20101	265	195	46	7	1
WE_BF_5	20331	11961	167	64	48	921	12
WE_BF_6	65623	30103	275	536	48	0	0

Table S2: Summary statistics of the computed mean and standard deviation (SD) of the relative abundance (%) of the functional groups by system (System and Matrix).

	System	Pre-Ongrowing			Weaning		
	Matrix	Biofilter Carrier	Tank Biofilm	Water	Biofilter Carrier	Tank Biofilm	Water
Tenacibaculum	Mean	0.33	0	0.24	17.2	1.03	31.1
	SD	0.65	0	0.29	5.45	0.98	24.5
Vibrio	Mean	0.08	0.02	0.87	0	0.14	1.5
	SD	0.09	0.04	0.63	0	0.15	1.51
Nitrosomonas	Mean	0.69	0.54	0.01	0.37	0.08	0.13
	SD	1.05	0.76	0.03	0.10	0.11	0.11
Unclassified Nitrosococcaceae	Mean	0.02	0	0	0	0	0
	SD	0.04	0	0	0	0	0
Nitrospira	Mean	1.38	0.69	0.02	0.54	0.44	0.23
	SD	1.02	0.77	0.06	0.25	0.75	0.25
Candidatus Nitrosopumilus	Mean	0.06	0	0	1.72	0.06	0.18
	SD	0.13	0	0	0.04	0.10	0.18

Table S3: Computed two-way ANOVA test p-values regarding the effect of the system and matrix variables on the abundance response.

	Tenacibaculum	*Vibrio*	*Nitrosomonas*	*Unclassified Nitrosococcaceae*	*Nitrospira*	*Candidatus Nitrosopumilus*
System	**6.31×10^{-11}**	0.268798	0.38608	0.417	0.579293	**8.26×10^{-7}**
Matrix	**6.08×10^{-4}**	**3.29×10^{-4}**	**0.00687**	0.102	**3.14×10^{-4}**	**3.50×10^{-5}**
System:Matrix	**6.53×10^{-4}**	0.523316	0.14669	0.289	0.051149	**4.17×10^{-5}**

Table S4-A: Summary of the Pre-Ongrowing community with n representing the number of ASVs from the assigned genus, links the number of correlations connecting to those taxa, and then the mean number of correlations per node (mean_degree) and corresponding standard deviation (SD).

Genus	n	links	mean_degree	SD
Albirhodobacter	1	2	2	NA
Algibacter	4	4	1	0
Allofrancisella	2	2	1	0
Altererythrobacter	5	9	1.8	0.447213595499958
Amylibacter	2	10	5	0
Arenimonas	1	1	1	NA
Blastopirellula	9	17	1.88888888888889	0.333333333333333
Candidatus Alysiosphaera	1	2	2	NA
Candidatus Peregrinibacteria	1	2	2	NA
Catenococcus	1	1	1	NA
Cellulophaga	2	4	2	0
Coxiella	1	2	2	NA
Crocinitomix	1	1	1	NA
Defluviimonas	4	11	2.75	1.5
Flavirhabdus	2	4	2	0
Francisella	1	1	1	NA
Gimesia	1	1	1	NA
Granulosicoccus	1	5	5	NA
Haliea	2	3	1.5	0.707106781186548
Halioglobus	1	5	5	NA
Hoeflea	1	5	5	NA
Hyphomicrobium	1	2	2	NA
Ilumatobacter	1	2	2	NA
Jannaschia	1	2	2	NA
Jejudonia	1	1	1	NA
Lacinutrix	2	3	1.5	0.707106781186548
Legionella	1	1	1	NA
Lewinella	6	10	1.66666666666667	0.516397779494322
Litoreibacter	5	22	4.4	1.34164078649987
Loktanella	2	4	2	0
Maribacter	7	17	2.42857142857143	1.13389341902768
Marimicrobium	1	2	2	NA
Marinicella	7	12	1.71428571428571	0.487950036474267
Maritalea	1	2	2	NA
Maritimimonas	1	2	2	NA

Muricauda	4	8	2	0
Nannocystis	2	2	1	0
Nitrosomonas	2	86	43	15.556349186104
Nitrospira	7	629	89.8571428571429	80.0008928521605
Peredibacter	1	1	1	NA
Persicirhabdus	3	4	1.33333333333333	0.577350269189626
Planctomicrobium	3	5	1.66666666666667	0.577350269189626
Planktotalea	5	10	2	0
Polaribacter	1	1	1	NA
Polaribacter 4	4	4	1	0
Portibacter	1	2	2	NA
Pricia	1	2	2	NA
Pseudoalteromonas	2	2	1	0
Pseudohongiella	1	2	2	NA
Pseudophaeobacter	1	2	2	NA
Pseudorhodobacter	5	13	2.6	1.34164078649987
Psychroserpens	1	2	2	NA
Rhodopirellula	1	2	2	NA
Roseibacillus	6	12	2	0
Roseobacter	3	9	3	1.73205080756888
Roseovarius	1	2	2	NA
Rubritalea	8	13	1.625	0.517549169506766
Rubrivirga	1	2	2	NA
Schleiferia	2	4	2	0
Sneathiella	1	5	5	NA
Sphingorhabdus	6	24	4	1.54919333848297
Sulfitobacter	3	5	1.66666666666667	0.577350269189626
Tenacibaculum	2	13	6.5	7.78174593052022
Unclassified	14	22	1.57142857142857	0.513552591013095
Unclassified Alphaproteobacteria	1	2	2	NA
Unclassified Anaerolineae	9	17	1.88888888888889	0.333333333333333
Unclassified Ardenticatenales	2	4	2	0
Unclassified Babeliaceae	1	1	1	NA
Unclassified Babeliales	3	11	3.66666666666667	2.3094010767585
Unclassified Bradymonadales	3	6	2	0
Unclassified Caenarcaniphilales	1	1	1	NA
Unclassified Caldilineaceae	1	2	2	NA
Unclassified Calditrichaceae	1	2	2	NA

Unclassified Cellvibrionales	1	2	2	NA
Unclassified Chitinophagales	6	12	2	1.549193338482 97
Unclassified Clostridiales	1	1	1	NA
Unclassified Crocinitomicaceae	1	2	2	NA
Unclassified Dadabacteriales	3	9	3	1.732050807568 8
Unclassified Deltaproteobacteria	4	11	2.75	1.5
Unclassified Flavobacteriaceae	1 5	39	2.6	1.242118006816 24
Unclassified Flavobacteriales	2	4	2	0
Unclassified Gammaproteobacteria	9	30	3.333333333333 33	1.581138830084 19
Unclassified Gemmatimonadetes	1	2	2	NA
Unclassified Gracilibacteria	1	1	1	NA
Unclassified Hyphomonadaceae	3	6	2	0
Unclassified Kiritimatiellae	1	2	2	NA
Unclassified Kordiimonadales	2	2	1	0
Unclassified Methyloligellaceae	1	2	2	NA
Unclassified Micavibrionaceae	1	1	1	NA
Unclassified Microscillaceae	2	3	1.5	0.707106781186548
Unclassified Microtrichaceae	4	13	3.25	2.061552812808 83
Unclassified Myxococcales	4	11	2.75	1.5
Unclassified Nannocystaceae	1	5	5	NA
Unclassified Obscuribacterales	1	1	1	NA
Unclassified Phycisphaeraceae	3	6	2	0
Unclassified Pirellulaceae	1	2	2	NA
Unclassified Planctomycetales	1	2	2	NA
Unclassified Planctomycetes	2	3	1.5	0.707106781186548
Unclassified Rhizobiales	1	1	1	NA
Unclassified Rhodanobacteraceae	2	6	3	2.828427124746 19
Unclassified Rhodobacteraceae	3 1	85	2.741935483870 97	1.632334564062 24
Unclassified Rhodothermaceae	1	5	5	NA
Unclassified Rickettsiales	1	2	2	NA
Unclassified Rubritaleaceae	1	1	1	NA
Unclassified Sandaracinaceae	1	2	2	NA
Unclassified Saprospiraceae	2 0	43	2.15	1.039989878493 26
Unclassified Solibacterales	1	2	2	NA
Unclassified Sphingobacteriales	2	3	1.5	0.707106781186548
Unclassified Thermoanaerobaculaceae	4	8	2	0
Unclassified Thermomicrobiales	1	5	5	NA

Unclassified Verrucomicrobiales	6	13	2.16666666666667	1.47196014438797
Unclassified Wohlfahrtiimonadaceae	1	2	2	NA
Vibrio	3	26	8.66666666666667	8.9628864398325
Woeseia	1	2	2	NA

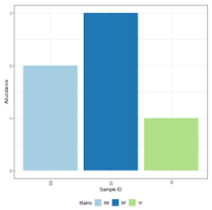
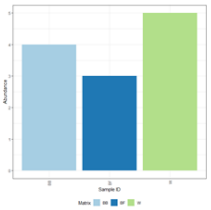
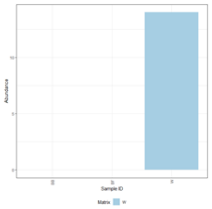
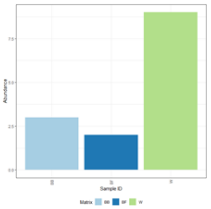
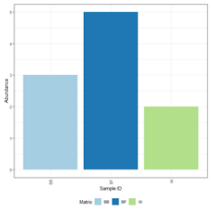
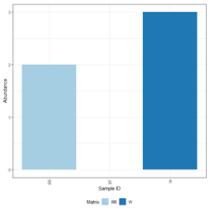
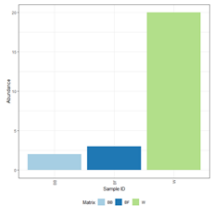
Table S4-B Summary of the Weaning community with n representing the number of ASVs from the assigned genus, links the number of correlations connecting to those taxa, and then the mean number of correlations per node (mean_degree) and corresponding standard deviation.

Genus	n	link	mean_degree	SD
Aliiglaciecola	1	3	3	NA
Aliikangiella	1	1	1	NA
Aliiroseovarius	2	2	1	0
Aliivibrio	3	3	1	0
Anderseniella	1	1	1	NA
Aurantivirga	2	2	1	0
Bythopirellula	1	1	1	NA
Candidatus Berkiella	4	14	3.5	2.88675134594813
Candidatus Fritschea	1	1	1	NA
Candidatus Nitrosopumilus	6	351	58.5	28.1691320420065
Candidatus Peregrinibacteria	1	3	3	NA
Catenococcus	2	11	5.5	4.94974746830583
Cocleimonas	1	1	1	NA
Colwellia	1	1	1	NA
Coxiella	1	1	1	NA
Croceitalea	2	9	4.5	2.12132034355964
Dokdonia	3	7	2.33333333333333	1.52752523165195
Fluviicola	1	1	1	NA
Francisella	2	6	3	1.4142135623731
Garritya	1	1	1	NA
Gimesia	1	1	1	NA
Granulosicoccus	2	12	6	0
Halocynthiibacter	1	1	1	NA
Haloferula	1	6	6	NA
Hellea	1	1	1	NA
Ilumatobacter	1	1	1	NA
Jindonia	1	6	6	NA
Kangiella	4	8	2	1.15470053837925
Kiloniella	1	1	1	NA
Leucothrix	1	2	2	NA
Lewinella	3	10	3.33333333333333	0.577350269189626
Loktanella	1	1	1	NA
Maribacter	1	6	6	NA
Maricurvus	4	7	1.75	0.957427107756338
Marinicella	8	12	1.5	0.925820099772551
Marinomonas	1	1	1	NA
Neptunomonas	2	2	1	0
Nesiotobacter	1	3	3	NA
Nitrosomonas	1	6	6	NA
Nitrospira	4	208	52	32.0312347560939
Oleiphilus	2	4	2	1.4142135623731
Oleispira	3	8	2.66666666666667	1.52752523165195

Olleya	1	4	4	NA
Paraglaciecola	1	4	4	NA
Parasphingopyxis	1	3	3	NA
Peredibacter	1	1	1	NA
Persicirhabdus	1	1	1	NA
Phaeodactylibacter	1	1	1	NA
Planktotalea	1	1	1	NA
Polaribacter 2	7	23	3.28571428571429	2.56347977784662
Polaribacter 4	5	12	2.4	2.19089023002066
Pontivivens	1	3	3	NA
Profundimonas	1	1	1	NA
Pseudoalteromonas	7	17	2.42857142857143	1.13389341902768
Pseudofulvibacter	5	9	1.8	0.836660026534076
Pseudohongiella	2	6	3	0
Pseudophaeobacter	1	1	1	NA
Pseudoteredinibacter	1	3	3	NA
Psychrobium	2	4	2	1.4142135623731
Psychroserpens	2	4	2	1.4142135623731
Roseibacillus	1	1	1	NA
Rubidimonas	1	1	1	NA
Rubritalea	2	12	6	0
Ruegeria	5	11	2.2	0.836660026534076
Salinihabitans	1	3	3	NA
Salinirepens	1	1	1	NA
Sneathiella	1	1	1	NA
Spongiibacterium	1	3	3	NA
Sulfitobacter	2	9	4.5	2.12132034355964
Tenacibaculum	56	812	14.5	9.17902747868997
Thalassobius	5	17	3.4	1.34164078649987
Thalassotalea	1	1	1	NA
Unclassified	19	34	1.78947368421053	1.35724178507659
Unclassified Alphaproteobacteria	8	31	3.875	2.03100960115899
Unclassified Anaerolineae	5	12	2.4	1.34164078649987
Unclassified Ardenticatenaceae	19	90	4.73684210526316	1.93913225906039
Unclassified Ardenticatenales	2	4	2	1.4142135623731
Unclassified Bacteria	1	1	1	NA
Unclassified Bdellovibrionaceae	1	4	4	NA
Unclassified Bradymonadales	8	40	5	1.92724822331886
Unclassified Caldilineaceae	2	6	3	0
Unclassified Cellvibrionaceae	1	1	1	NA
Unclassified Cellvibrionales	1	1	1	NA
Unclassified Chitinophagales	6	7	1.16666666666667	0.408248290463863
Unclassified Chloroflexi	3	18	6	0
Unclassified Crocinitomicaceae	1	1	1	NA
Unclassified Cryomorphaceae	2	7	3.5	3.53553390593274
Unclassified Cyclobacteriaceae	2	4	2	1.4142135623731

Unclassified Cytophagales	1	6	6	NA
Unclassified Dadabacteriales	3	18	6	0
Unclassified Deltaproteobacteria	7	19	2.71428571428571	1.25356634105602
Unclassified Diplorickettsiaceae	3	8	2.66666666666667	1.52752523165195
Unclassified Flavobacteriaceae	14	40	2.85714285714286	1.91581044739026
Unclassified Gammaproteobacteria	4	6	1.5	1
Unclassified Gracilibacteria	1	1	1	NA
Unclassified Hyphomonadaceae	2	7	3.5	0.707106781186548
Unclassified Ignavibacteria	2	7	3.5	3.53553390593274
Unclassified Ilumatobacteraceae	1	6	6	NA
Unclassified Kiloniellaceae	1	3	3	NA
Unclassified Kordiimonadales	2	7	3.5	0.707106781186548
Unclassified Micavibrionaceae	1	1	1	NA
Unclassified Micavibrionales	2	6	3	0
Unclassified Microscillaceae	1	3	3	NA
Unclassified Nannocystaceae	1	3	3	NA
Unclassified Phycisphaeraceae	3	13	4.33333333333333	2.88675134594813
Unclassified Planctomycetes	1	3	3	NA
Unclassified Proteobacteria	4	13	3.25	3.30403793359983
Unclassified Rhizobiaceae	3	5	1.66666666666667	1.15470053837925
Unclassified Rhodobacteraceae	21	63	3	2.25831795812724
Unclassified Rickettsiaceae	4	11	2.75	2.3629078131263
Unclassified Rubinisphaeraceae	4	14	3.5	2.08166599946613
Unclassified Saccharimonadales	4	19	4.75	2.5
Unclassified Saprospiraceae	7	24	3.42857142857143	2.07019667802706
Unclassified Sericytochromatia	2	9	4.5	2.12132034355964
Unclassified Sphingomonadaceae	1	3	3	NA
Unclassified Thermoanaerobaculaceae	1	3	3	NA
Unclassified Thermomicrobiales	1	3	3	NA
Unclassified Verrucomicrobiales	1	1	1	NA
Vibrio	5	38	7.6	5.31977443130816
Woeseia	2	7	3.5	3.53553390593274

Table S5: Distribution of the functional groups ASVs, divided by network sub-community nature (individualist nature between the functions studied, that is, only one genus from the assigned groups is present, and collectivist, where there is a cluster of positive correlations between target groups collectivist) and system, grouped by matrix (Biofilter Carriers, BB; Tank Wall Biofilm, BF and Water, W).

		Pre-Ongrowing	Weaning
Individualists	Nitrifying		
	Pathogenic		
Collectivists	Nitrifying		
	Pathogenic	NA	

Appendix C: Supplementary Materials Chapter 4

The development of the sole microbiome in a hatchery life cycle, from egg to juvenile

Table S1: Temperature (ºC), pH and salinity (‰) recorded in the hatchery at collection dates.

dah	-2	2	14	49	119	146
System	Egg	Larvae	Larvae	Weaning	Pre-Ongrowing	Pre-Ongrowing
Temperature	19.7	19.1	19.5	19.7	20.5	19.9
pH	8.48	8.41	8.43	7.75	7.68	7.75
Salinity	35	35	36	35	36	35

Table S2: Total number of sequences obtained for each sample after sequencing (Input - raw sequences), and number of sequences after each DADA2 treatment: after filtering out low-quality sequencing reads (Filtered), trimming the reads to a consistent length (Denoised), after merging (Merged) and after the removal of the chimeras (Nonchim). Alpha diversity is represented with total read counts (Observed) and the Shannon index, of the microbial community.

Sample	Age	Sample_ty	Stage	System	Input	Filtered	denoisedF	denoisedR	Merged	conchim	Observed	Shannon
1	-2	Egg	Egg	Incubator	94251	76569	74166	76043	65797	57331	413	4.232478093
2	-2	Egg	Egg	Incubator	124118	97929	96442	97295	90485	72105	233	3.256452763
3	2	Larvae	Larvae	Larvae	68227	31864	30210	29959	19668	18728	188	3.655039415
4	2	Larvae	Larvae	Larvae	119526	47300	43423	44722	25513	24071	303	3.741506749
5	14	Larvae	Larvae	Larvae	168295	109465	105917	106970	91004	80637	519	3.791124722
6	14	Larvae	Larvae	Larvae	144234	112234	110000	111366	103729	84491	376	3.417163682
7	49	Mucus	Juvenile	Weaning	130489	57254	53827	53541	42076	40838	457	3.982772279
8	49	Mucus	Juvenile	Weaning	70189	33506	30507	30398	19859	19371	273	3.83366613
9	49	Gill	Juvenile	Weaning	66801	31770	28803	28397	21042	21131	328	4.299422418
10	49	Gill	Juvenile	Weaning	95048	53681	52025	52066	40939	38167	284	4.136828938
11	49	Intestine	Juvenile	Weaning	94346	43273	41662	42398	34210	30171	253	3.621919237
12	49	Intestine	Juvenile	Weaning	53208	36470	35815	36255	34003	32118	191	3.562540383
13	49	Fin	Juvenile	Weaning	116665	47538	45868	45747	36780	35464	263	4.111897329
14	49	Fin	Juvenile	Weaning	115389	54734	52535	52920	41327	36489	309	3.971197752
15	119	Mucus	Juvenile	Pre-Ongrc	77469	28520	26590	26459	19946	19317	343	4.344098441
16	119	Mucus	Juvenile	Pre-Ongrc	182975	102470	98446	100500	82145	72944	662	4.359896058
17	119	Gill	Juvenile	Pre-Ongrc	91379	64720	63338	63575	52907	46176	349	2.649029141
18	119	Gill	Juvenile	Pre-Ongrc	59545	47802	46750	47578	42897	41286	381	4.586588454
19	119	Intestine	Juvenile	Pre-Ongrc	77178	42546	41057	42176	34916	32518	365	4.747917956
20	119	Intestine	Juvenile	Pre-Ongrc	51665	45802	45660	45741	45450	39788	56	2.081627932
21	119	Fin	Juvenile	Pre-Ongrc	93971	44099	42579	42185	31330	29817	268	3.656934209
22	119	Fin	Juvenile	Pre-Ongrc	61893	42968	41587	41065	34077	33524	206	3.273297514
23	146	Mucus	Juvenile	Pre-Ongrc	61199	54768	53949	54272	49978	44113	95	2.603469774
24	146	Mucus	Juvenile	Pre-Ongrc	61585	53819	52888	53180	48632	42741	113	2.771637534
25	146	Gill	Juvenile	Pre-Ongrc	121053	108050	106548	107800	101100	77262	118	2.791317431
26	146	Gill	Juvenile	Pre-Ongrc	68277	64203	62924	63940	57332	42221	109	3.034621006
27	146	Intestine	Juvenile	Pre-Ongrc	71037	51315	47329	50425	37341	30437	417	4.794762197
28	146	Intestine	Juvenile	Pre-Ongrc	89575	56985	52974	55076	41826	36498	488	4.825869999
29	146	Fin	Juvenile	Pre-Ongrc	80104	54529	52135	53718	45519	41668	414	4.644292281
30	146	Fin	Juvenile	Pre-Ongrc	59219	41914	40081	41100	34141	30751	367	4.469813349
31	Brineshrir	Brineshrir	Brineshrir	Brineshrir	55758	31621	30317	30516	23377	19685	143	2.959426511
32	Brineshrir	Brineshrir	Brineshrir	Brineshrir	60167	53086	52598	52720	47987	45875	118	2.577497761
33	Rotifer	Rotifer	Rotifer	Rotifer	40682	20106	18706	19007	12022	11362	235	4.356682378
34	Rotifer	Rotifer	Rotifer	Rotifer	104716	58763	56349	57056	46133	42820	416	4.652477122

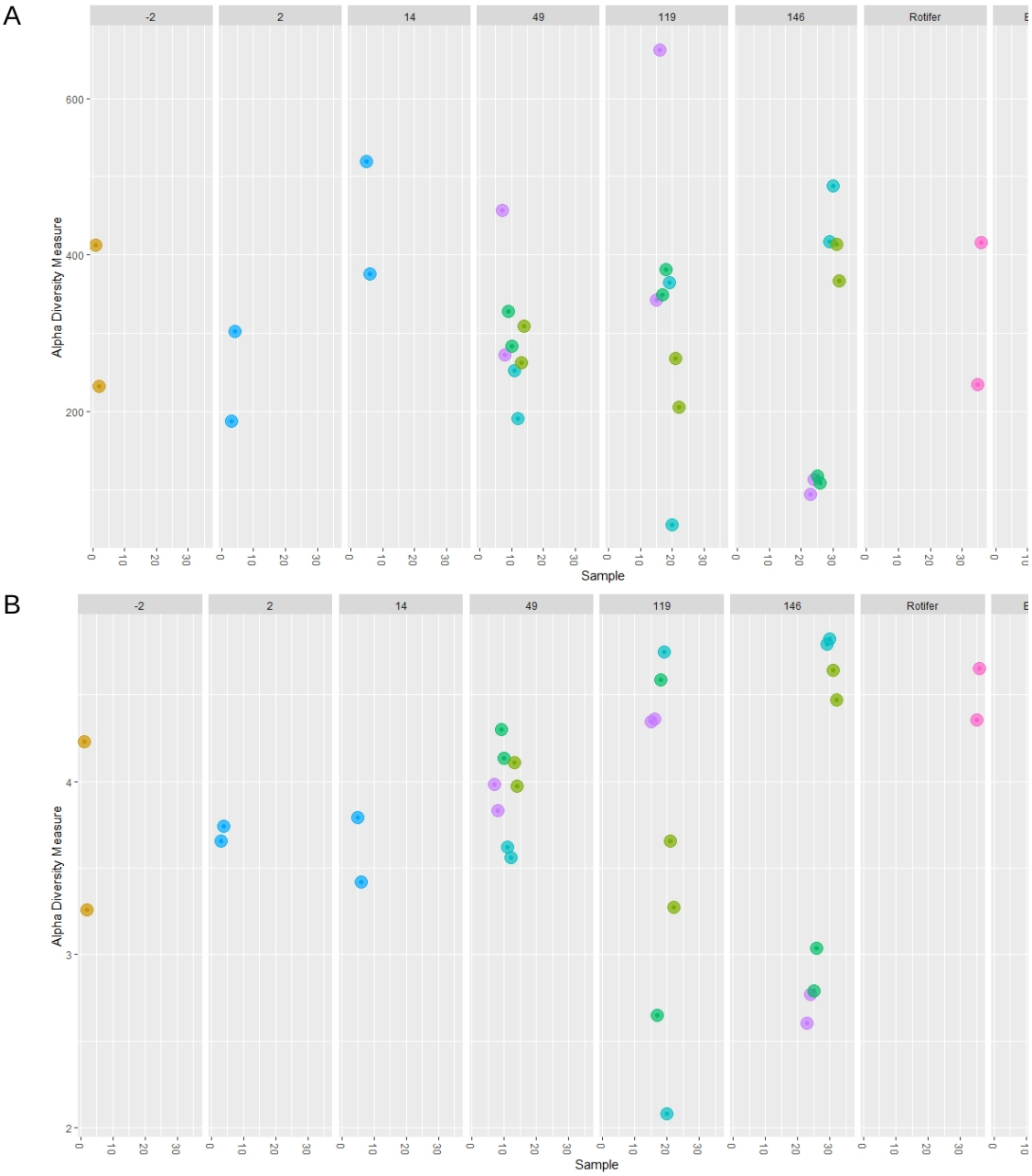


Figure S1: Alfa diversity indexes, Observed ASVs (A) and Shannon (B) grouped by age and by sample type.

Table S3: Results for the Adonis test for beta group significance with a Bray-Curtis distance matrix. The null hypothesis is that there is no interaction between our test variables and prokaryotic diversity. The null hypothesis that groups have the same dispersion was also tested with the homogeneity of dispersion.

Variable	p-value	R^2	Homogeneity of dispersion test
Age	<0.001	0.112	0.019
Sample_type	0.030	0.225	0.041
Stage	0.024	0.111	0.097
System	<0.001	0.248	0.001

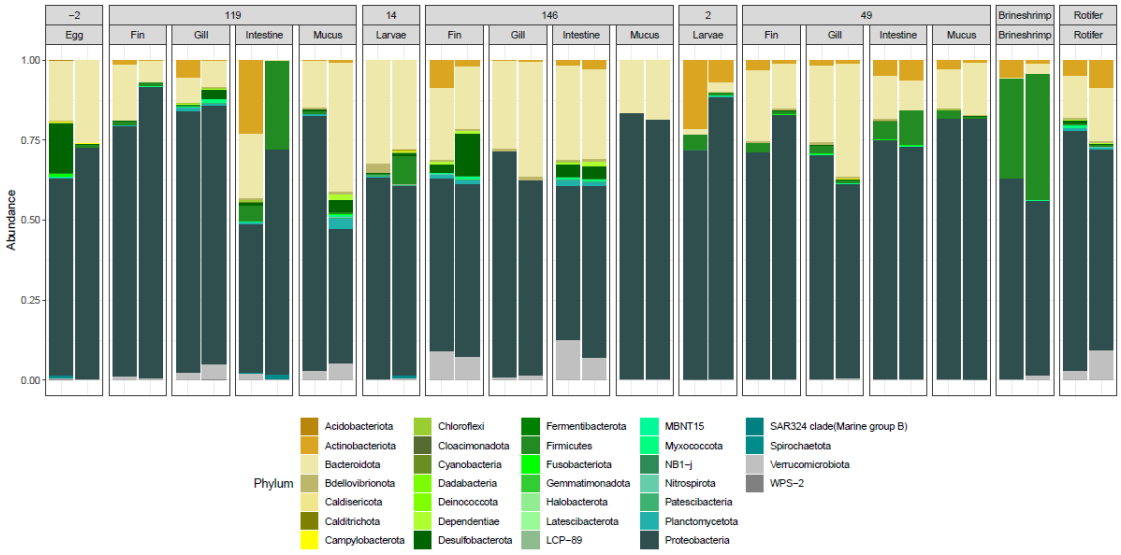


Figure S2: Relative phylum composition of the prokaryotic community ordered by age and sample type.

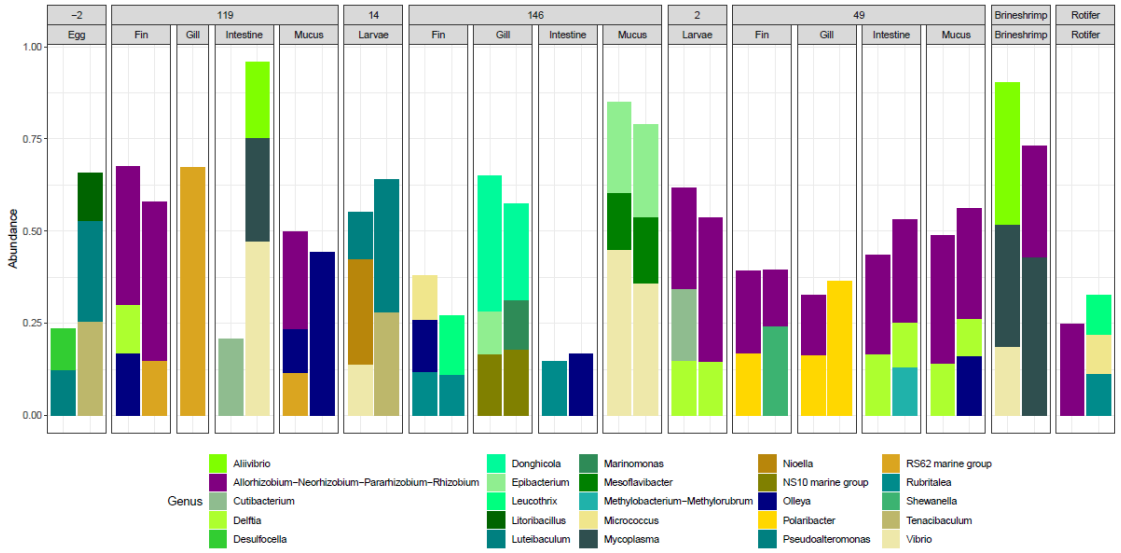


Figure S3: Relative genus composition of the prokaryotic community (abundance >0.01) ordered by age and sample type.

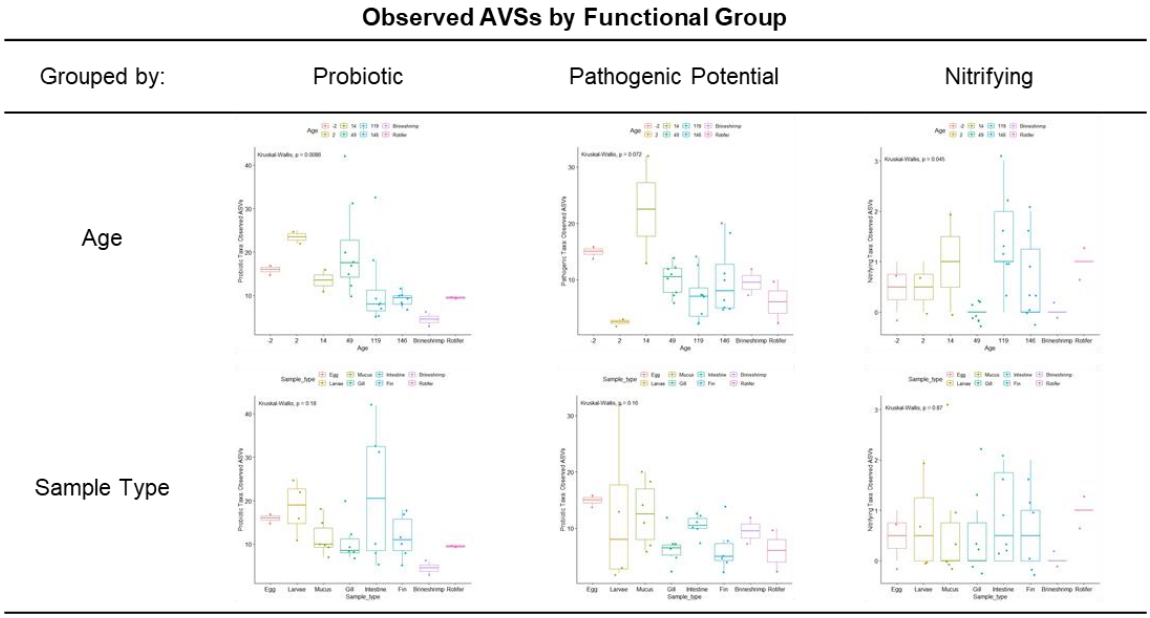


Figure S4: Estimated richness using the functional groups (Probiotic, Pathogenic Potential and Nitrifying) Observed ASVs grouped by age (from -2 to 146 days after hatching) and by sample type (egg, larvae, fin, gill, intestine, mucus). Rotifer and brineshrimp samples are also added.

Table S4: Relative genus distribution of the functional groups (probiotic, potentially pathogenic and nitrifying) ordered by sample.

Abundancy (%)	Sample S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16	S17	S18	S19	S20	S21	S22	S23	S24	S25	S26	S27	S28	S29	S30	S31	S32	S33	S34
Probiotic Genus																																		
Pseudoalteromonas	10.95	25.44	0.31	0.59	1.17	31.59	1.97	1.27	3.84	4.76	0.72	0.67	2.63	2.29	1.12	2.01	2.11	0.09	0.27	0.00	0.18	1.86	1.36	1.13	0.45	0.09	1.40	2.42	0.85	0.68	0.00	0.06	1.04	0.61
Phaeobacter	1.37	6.14	0.39	0.39	1.64	6.37	0.00	0.00	0.06	0.22	0.87	0.00	0.02	0.10	0.00	0.31	0.00	0.19	0.00	0.00	0.00	0.00	2.17	5.24	2.01	1.20	0.29	0.20	0.25	0.08	0.00	0.00	0.10	0.00
Shewanella	0.02	0.02	0.00	0.00	0.00	0.00	0.47	6.42	0.72	0.62	3.01	0.41	0.87	17.37	0.00	0.01	0.00	0.00	0.18	0.02	0.00	0.11	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.06	0.00	0.00	0.00
Alteromonas	0.00	0.00	0.58	0.86	0.10	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.00	0.00	0.00	0.00	1.55	0.90	0.36	0.73	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Pseudomonas	0.12	0.00	6.75	1.66	0.02	0.00	0.41	0.13	0.00	0.07	0.06	0.77	0.40	0.06	0.08	0.16	0.06	0.06	6.11	0.01	0.37	0.60	0.35	0.39	0.01	0.00	0.10	0.08	0.20	0.18	0.09	0.35	1.44	1.11
Streptococcus	0.00	0.00	0.09	0.14	0.00	0.00	0.03	0.00	0.00	0.09	0.47	1.86	0.13	0.01	0.00	0.00	0.00	0.00	0.18	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Weissella	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.28	0.37	2.05	0.00	0.02	0.00	0.00	0.00	0.00	0.01	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Bacillus	0.00	0.01	2.25	0.31	0.00	0.00	0.42	0.09	0.21	0.31	0.41	1.05	0.70	0.16	0.06	0.06	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.06	0.00	0.02
Vagococcus	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.06	0.45	0.86	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Roseobacter	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.17	0.00	0.06	0.00	0.00	0.23	0.07	0.12	0.11	0.38	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.14	0.10	0.18	0.11	0.00	0.00	0.00	0.11
Leuconostoc	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.07	0.22	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00
Aeromonas	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.16	0.00	0.14	0.24	0.06	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00
Latilactobacillus	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.17	0.00	0.00	0.00	0.00	0.00	0.00	0.21	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Streptomyces	0.00	0.00	0.67	0.18	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.06	0.09	0.01	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Lactococcus	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.12	0.12	0.00	0.00	0.00	0.00	0.00	0.00	0.06	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Limosilactobacillus	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.05	0.23	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Aerosphaera	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.32	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Bifidobacterium	0.00	0.00	0.00	0.00	0.00	0.00	0.09	0.00	0.00	0.00	0.02	0.28	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Granulicatella	0.00	0.00	0.00	0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.16	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Gardnerella	0.00	0.00	0.14	0.20	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Pediococcus	0.00	0.00	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.10	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Unclassified	0.00	0.00	0.31	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Unclassified	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.09	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Desemzia	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.06	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Ligilactobacillus	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.00	0.00	0.00	0.00	0.00	0.00	0.06	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Brochothrix	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Enterococcus	0.01	0.00	0.00	0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Carnobacterium	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.06	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Loigolactobacillus	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Lactiplantibacillus	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Lactobacillus	0.00	0.00	0.06	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Dellaglioa	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Liquorilactobacillus	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Facklamia	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Pathogenic Genus																																		
Tenacibaculum	7.81	23.71	0.39	0.74	0.44	24.17	1.38	0.40	1.51	1.09	0.95	0.25	0.30	2.04	0.12	0.07	0.03	0.08	0.41	0.00	0.00	0.00	0.03	0.00	0.57	0.58	3.55	0.11	0.07	0.37	0.00	0.00	0.00	0.08
Vibrio	0.80	0.85	0.00	0.16	11.51	0.46	0.76	2.74	0.44	0.50	0.33	0.35	1.05	2.35	3.67	2.67	0.92	0.30	1.78	46.34	1.44	1.45	43.90	34.93	0.28	0.13	2.76	3.26	0.55	0.86	15.32	0.78	1.69	1.40
Mycoplasma	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.19	0.14	0.00	0.00	0.39	27.46	0.00	0.00	0.00	0.00	0.02	0.02	0.05	0.07	0.04	0.03	27.37	38.50	0.00	0.02
Photobacterium	0.00	0.00	0.00	0.00	0.00	0.00	0.13	0.06	0.00	0.41	0.68	2.05	0.05	0.16	0.13	0.03	0.00	0.00	0.26	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.25	0.07	0.00	0.00
Nitrifying Genus																																		
Nitrospira	0.02	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.02	0.13	0.13	0.00	0.02	0.02	0.00	0.00	0.00	0.00	0.02	0.12	0.09	0.00	0.00	0.00	0.00	0.04
Unclassified Nitrosomonadac	0.00	0.00	0.00	0.10	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Nitrosomonas	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.01	0.00	0.00	0.00	0.05	0.00
Unclassified Nitrosococcacea	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00