Graduate thesis

NTNU
Norwegian University of Science and Technology
Faculty of Natural Sciences
Department of Biotechnology and Food Science

Stian Ringbakken Stenhaug

The effect of organic load on the rearing water and biofilter biofilm microbiota across the freshwater and brackish water phases in RAS with Atlantic salmon (*Salmo salar*)

Graduate thesis in Chemical engineering and biotechnology
Supervisor: Ingrid Bakke
Co-supervisor: Simen Fredriksen
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Abstract

Atlantic salmon aquaculture is experiencing significant growth in land-based recirculating aquaculture systems (RAS), particularly for smolt production. The biofilter within RAS plays a crucial role in maintaining ideal water quality and fish health through the activities of microorganisms. However, it is not well understood how biofilter function and the microbial communities associated with RAS water and biofilter biofilm are influenced by organic load.

This thesis aimed to investigate the freshwater and brackish water phase of Atlantic salmon RAS and the effect of high organic load on the microbial communities in the biofilter biofilm and rearing water. To address this aim, six identical RAS were examined, with three RAS operated with high organic load and three RAS operated with low organic load. Daily measurements of water quality, including nitrogenous substances, were conducted. Subsequently, a total of 206 samples from the biofilter and RAS water were subjected to microbial community analysis by Illumina sequencing of the variable V3-V4 regions of the 16S rRNA gene amplicons, followed by data processing and statistical analyses.

The biofilter biofilm and water-suspended microbiota differed significantly between the RAS operated at high organic load and the RAS operated at low organic load during the brackish water phase, indicating that the organic load played a role in shaping the biofilm microbiota. In general, the dominant orders characterizing the microbial community composition in the RAS water samples were *Rhodobacterales*, *Flavobacteriales*, *Burkholderiales* and *Cytophagales*. The microbial community composition in biofilm carriers of both the RAS operated at high organic load and the RAS operated at low organic load was mainly characterized by the orders *Rhodobacterales*, *Flavobacteriales*, *Saprospirales* and *Burkholderiales*. Additionally, this study explored the differences in microbial compositions between 0.2 µm and 8 µm filtered water samples, revealing significant distinctions in these microbial communities' freshwater phase. Notably, the amplicon sequence variants (ASV) contributing the most to these dissimilarities were classified as *Lacihabitans*, *Comamonadaceae* and *Rhodobacteraceae*.

Further research in this field will enhance our understanding of the intricate relationships between organic load, microbial communities, and the performance of RAS in Atlantic salmon production.

Sammendrag

Oppdrett av Atlantisk laks opplever for tiden betydelig vekst innenfor landbaserte resirkulerende akvakultursystemer, spesielt for smoltproduksjon. Biofilteret i RAS spiller en avgjørende rolle i å opprettholde ideell vannkvalitet og fiskehelse gjennom aktiviteten til mikroorganismer. Imidlertid vet vi lite om hvordan biofilterfunksjonen og de mikrobielle samfunnene forbundet med RAS-vann og biofilterbiofilm påvirkes av organisk belastning.

Denne avhandlingen hadde som mål å undersøke ferskvanns- og brakkvannsfasen i RAS for Atlantisk laks og effekten av høy organisk belastning på de mikrobielle samfunnene i biofilterbiofilm og oppdrettsvann. For å oppnå dette ble seks identiske RAS undersøkt, hvorav tre RAS ble drevet under forhold med høy organisk belastning og tre RAS ble drevet under forhold med lav organisk belastning. Daglige målinger av vannkvalitet, inkludert nitrogenholdige stoffer, ble utført. Deretter ble totalt 206 prøver fra biofilteret og RAS-vannet analysert ved bruk av mikrobiell samfunnsanalyse ved Illumina-sekvensering av variable V3-V4 regioner av 16S rRNA-genampliconer, etterfulgt av databehandling og statistiske analyser.

Biofilterbiofilmen og suspendert vann mikrobiota viste betydelige forskjeller mellom RAS drevet med høy organisk belastning og RAS drevet med lav organisk belastning i brakkvannsfasen, noe som tyder på at organisk belastning spilte en rolle i utformingen av biofilmens mikrobiota. Generelt ble de dominerende ordener som karakteriserte den mikrobiell samfunnsstruktur i RAS-vannprøvene klassifisert som *Rhodobacterales*, *Flavobacteriales*, *Burkholderiales* og *Cytophagales*. Den mikrobielle sammensetningen i biofilmbærere av både RAS operert med høy organisk belastning og RAS operert med lav organisk belastning var hovedsakelig preget av ordener *Rhodobacterales*, *Flavobacteriales*, *Saprospirales* og *Burkholderiales*. I tillegg utforsket denne studien forskjellene i mikrobiell sammensetning mellom 0,2 µm og 8 µm filtrerte vannprøver, og avslørte betydelige forskjeller i disse mikrobielle samfunnene i ferskvannsfasen. ASV-ene som bidro mest til disse ulikhetene ble klassifisert som *Lacihabitans*, *Comamonadaceae* og *Rhodobacterace*.

Ytterligere forskning på dette feltet vil øke vår forståelse av de intrikate sammenhengene mellom organisk belastning, mikrobielle samfunn og ytelsen til RAS i produksjon av Atlantisk laks.

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1 Introduction

Aquaculture has become one of the fastest-growing food-producing sectors globally, with Norway being at the forefront [1]. With the goal of minimizing environmental impact whilst increasing production, parts of Norwegian aquaculture have evolved from traditional open-net pens to more advanced closed systems, such as recirculating aquaculture systems (RAS). There are several benefits to RAS, including better control over production parameters, enhanced biosecurity and reduced environmental impact [2]. There are still numerous challenges that need to be addressed. These challenges include maintaining appropriate water quality, ensuring the effective removal of organic waste and managing microbial communities [3]. This thesis endeavors to shed light on these critical issues, with a specific focus on unraveling the intricate interplay between organic load and microbial communities within the context of RAS. The primary focus is to contribute to the development of efficient and sustainable aquaculture practices that meet the growing demand for seafood while reducing environmental impacts.

1.1 Atlantic salmon aquaculture and biology

The life of *Salmo salar*, commonly known as Atlantic salmon, involves several distinct developmental stages, including egg, alevin, fry, parr, smolt and adult salmon (Figure 1.1). Atlantic salmon is an anadromous species that undergo a metamorphosis called smoltification characterized by behavioral, morphological and physiological changes, to adapt from freshwater to seawater [4] [5].

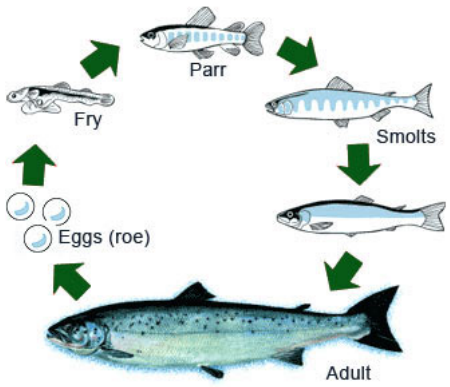


Figure 1.1: Life cycle of Atlantic salmon from eggs to adult. The picture is adapted from MESA [6].

Atlantic salmon aquaculture commences with the hatching of salmon eggs in freshwater land-based hatcheries. Once hatched, the fry are nurtured in freshwater tanks until they reach the smolt stage. Careful monitoring of the water quality, temperature and feeding of the fish is important in the freshwater tanks to ensure the fish's optimum growth and well-being [5]. When the fish reach the smolt stage, typically at around 100 grams, they are relocated to seawater pens [7]. The salmon are harvested when they reach market size, which usually takes about

12 to 18 months after smoltification[8]. Recently, the industry has increased the residence time in land-based systems, until the salmon reach post-smolt stage (up to 250-1000 grams), to enhance the fish's growth and resilience[9].

There are several advantages to Atlantic salmon aquaculture. It provides a reliable and sustainable source of high-quality seafood, reducing the pressure on wild fish stocks[10]. It also creates jobs and economic opportunities in coastal communities. However, Atlantic salmon aquaculture also faces challenges, including concerns about the impact on the environment and the risk of disease transmission between farmed and wild salmon populations. Lice infestations also pose significant challenges in Atlantic salmon aquaculture. The lice feed on the blood and mucus of the fish, leading to skin lesions, impaired swimming ability, and increased susceptibility to infections[11]. Salmon farmers work to mitigate these risks by employing responsible and sustainable farming practices, such as using environmentally friendly feed, minimizing the use of antibiotics and the use of lumpfish (*Cyclopterus lumpus*) for sea-lice control[12][13][14].

1.2 Recirculating aquaculture system (RAS)

RAS is a highly controlled, closed-water system designed for growing aquatic animals, such as fish, crustaceans and mollusks in a sustainable and efficient manner. Unlike traditional aquaculture systems, like flow-through systems that rely on large volumes of water exchange with the environment, RAS recirculates and treats the water, enabling smaller water volumes and reducing water waste[2](Figure 1.2). Effective water treatment is an essential aspect for the reuse of water in RAS. The water treatment loop typically commences with a particle removal step removing larger suspended solids such as fish feces and excess feed. Subsequently, a biofilter, containing beneficial bacteria, facilitates the conversion of toxic ammonia to nitrate, a less hazardous substance to the cultured species[15]. The next stage involves a degassing process, aimed at removing carbon dioxide, followed by aeration to elevate the dissolved oxygen levels. Additionally, the pH and alkalinity are modulated to maintain optimal levels for ideal water quality. In some RAS, a disinfection process utilizing either ultraviolet irradiation or ozone gas is implemented to avoid disease outbreaks caused by pathogenic viruses, fungi, or bacteria[16]. To obtain optimal water quality and oxygen levels the water is continuously filtrated and treated. This creates ideal conditions for the health and growth of cultured species[2].

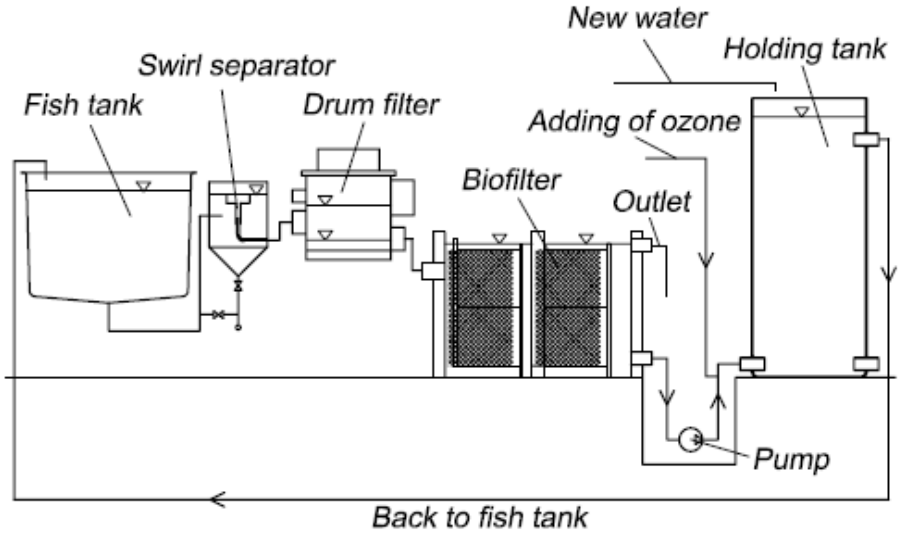


Figure 1.2: Flow chart of the re-use plant at the Norwegian University of Life Sciences. The system consists of a fish tank, followed by the water treatment unit with a swirl separator, drum filter, submerged biofilter with bioblocks, a pump and addition of ozone to disinfect and oxygenate the water. The figure is adapted from Lekang et al.[2].

There are several advantages of RAS compared to traditional flow-through systems. The high water efficiency of up to 100% recirculation makes the system more environmentally sustainable, with an opportunity to create ideal conditions for water quality allowing precise control of temperature, oxygen levels, turbidity and other aspects affecting the health of the cultured species. The isolated environment in RAS also reduces the risk of disease transmission from wild animals and contaminated water. The highly controlled system facilitates improved growth rates and increased production efficiency and is operational in any climate with year-round production. However, the initial investment cost of RAS components and the operational cost of the water treatment system and pumps are higher than those of a flow-through system[2] [15]. RAS has emerged as the most prevalent approach to producing Atlantic salmon smolts in Norway. The multitude of benefits associated with RAS has resulted in a steep upsurge in its adoption, with nearly all new land-based aquaculture systems in Norway presently employing RAS technology[7].

Removal of suspended, settleable and dissolved solids is considered one of the more critical processes in closed aquaculture systems. Solids in RAS comprise a variety of materials, including fish waste, uneaten feed, feces, dead organisms, algae, bacteria, organic debris and inorganic particles. These solids can vary in size, shape and composition. The presence of solids introduces a profusion of nutrients into the water, significantly contributing to carbonaceous biochemical oxygen demand - the oxygen required to degrade carbon-based compounds. Optimally, the solids are extracted from the rearing tank as soon as possible[15].

1.2.1 Biofilters in RAS

Biofilters are an essential component in RAS used to facilitate the conversion of nitrogenous compounds, specifically ammonia. Ammonia is a toxic byproduct from fish metabolism, which relatively low concentrations (>2 mg/L) can be detrimental to aquatic life. There are different types of biofilters, but most RAS utilize fixed-film biofilters, that contain plastic, rocks, or sand to provide surface area for the growth and colonization of bacterial biofilms. The type of material can impact the surface area-to-volume ratio, oxygen transfer efficiency and the hydraulic retention time of the biofilter. The main function of the biofilter is to create ideal conditions for nitrifying bacteria to grow[16].

Submerged biofilters such as moving bed bioreactors (MBBR) and fixed bed bioreactors (FBBR) are commonly used in RAS for production of Atlantic salmon smolts. In MBBR, a biofilm develops on biofilm carriers suspended in the water. The biofilm carriers are kept in continuous motion by the up-flowing water current and aeration (Figure 1.3). This movement induces turbulence, which promotes a self-cleaning property as the excess biofilm is knocked off. The particles released then enter the RAS. In contrast, the FBBR uses fixed media to provide surface area for biofilm growth, while water flows through the fixed biofilter. Particles often accumulate in the fixed media. Therefore, the filter must be back-washed frequently to avoid clogging. Establishing a functional and stable biofilter requires a minimum of 6 weeks[16].

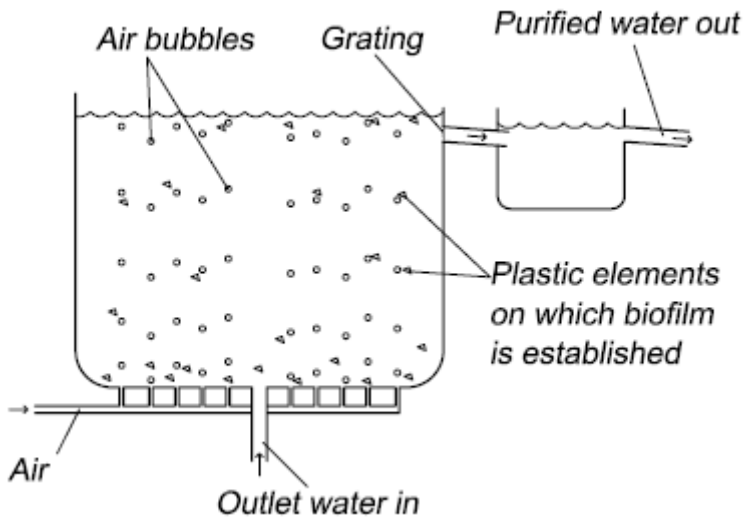


Figure 1.3: A moving bed biofilter showcasing the distribution of biofilm carriers. The figure is adapted from Lekang et al.[2].

1.3 Microbial communities in RAS

The water quality and fish health in RAS are highly dependent on microorganisms present in the water and biofilms throughout the system. Microorganisms

enter the system through various pathways, including feed, air, make-up water, employees and from fish. The microbial composition varies from RAS to RAS and is dependent on several factors affecting selection pressure in the system. The RAS microbiology is a complex ecosystem, where the different sources of bacteria and the selection pressure acting on the microbial communities in the system contribute to unique microbiota. The selection pressure is affected by the operational routines and system design[17]. Bacteria play a vital role in degrading organic matter and other pollutants in the water, with two groups widely discussed in RAS: autotrophic nitrifying bacteria and heterotrophic bacteria. The autotrophic nitrifying bacteria are mainly present in the biofilter and oxidize ammonia to nitrite and then to nitrate, which is less toxic to fish. The heterotrophic bacteria are distributed throughout the whole system, decomposing organic matter[18]. However, there are several heterotrophs that affect physiochemical and microbial water quality, with some causing diseases in fish. The presence of harmful bacteria such as some species of *Vibrio* and *Aeromonas* can be detrimental to fish health and growth[17]. Although many studies have been conducted on microbial communities in RAS, little is still known about the interaction of bacteria associated with fish, biofilm microbiota and the bacteria suspended in the water.

1.3.1 Bacteria associated with RAS biofilter

Biofiltration is a fundamental part of the water treatment process in RAS. The nitrification process that takes place in the biofilter ensures proper chemical water quality by converting ammonia into less harmful nitrate. The ammonia in the system originates from fish excretion and bacterial decomposition of organic matter. The intricate partnership between heterotrophs and autotrophic nitrifiers create a stable biofilm on the biofilm carriers, which is essential for biofilter efficiency[19]. The biofilm performance is influenced by various parameters such as pH, temperature, dissolved oxygen, salinity, substrate, alkalinity and turbulence. Therefore, monitoring the activity of the nitrifiers by measuring the concentration of nitrogenous compounds is important to understand and optimize the biofilter[19].

In water treatment systems, the term total ammonia nitrogen (TAN) represents the summation of ammonium and ammonia present in aqueous solutions (Eq. 1.1). The threshold of TAN should be below 2 mg/L according to regulations in Norwegian freshwater RAS smolt production[20]. The equilibrium in Eq. 1.1 is primarily dictated by the pH. An increase in pH above 7 shifts the equilibrium towards ammonia, which is the more toxic compound[21]. Exposure to elevated levels of ammonia can induce detrimental effects on stress hormones, osmoregulation, respiration and the tissue of the liver, kidney and gills[20].

$$NH_3 + H^+ \rightleftharpoons NH_4^+ \tag{1.1}$$

Nitrification is generally viewed as a two-step process, involving the oxidation of ammonium to nitrite by ammonia oxidizing bacteria (AOB) in the first step

(Eq. 1.2) and the oxidation of nitrite to nitrate by nitrite oxidizing bacteria (NOB) in the second step (Eq. 1.3)[2]. The enzymatic conversion of ammonia to nitrite is accomplished by the enzymes ammonia monooxygenase and hydroxylamine dehydrogenase in RAS in AOBs such as *Nitrosomonas*, *Nitrosovibrio* and *Nitrolobus*. In contrast, in RAS NOBs such as *Nitrobacter*, *Nitrotoga* and *Nitrospira* use the enzyme nitrite oxidoreductase to oxidize nitrite to nitrate[22]. Some species of nitrifying bacteria exhibit varying preferences and can only be located in specific environments, whilst others coexist in more common habitats. In marine environments, freshwater species from two or three genera of NOBs have been identified, whilst in RAS NOBs from *Nitrobacter*, *Nitrospira* and *Nitrotoga* have been reported to coexist[22].

$$NH_4^+ + 1.5O_2 \rightarrow NO_2^- + H_2O + 2H^+ \tag{1.2}$$

$$NO_2^- + 0.5O_2 \rightarrow NO_3^- \tag{1.3}$$

The rapid growth of heterotrophic bacteria may cause competition with nitrifiers for oxygen and space, leading to suboptimal water quality. Nevertheless, heterotrophic bacteria are essential for biofilm stability, providing protection to nitrifiers from environmental stressors[17]. Heterotrophic bacteria are found to dominate the microbial communities in RAS, including the nitrifying biofilters[23]. The relationship between carbon and nitrogen, known as the C/N ratio, plays a crucial role in the activity and efficiency of heterotrophic bacterial communities and nitrification processes within a submerged biofilter. The C/N ratio determines the availability and competition between organic carbon and inorganic nitrogen compounds[24]. When the C/N ratio is high, nitrifying bacteria face competition from heterotrophic bacteria for limited space and oxygen within the biofilm of the biofilter. On the other hand, a low C/N ratio is necessary for nitrifying bacteria to dominate. However, the impact of the C/N ratio on nitrification efficiency varies depending on the system and the characteristics of available carbon. Easily degradable dissolved organic carbon is quickly decomposed by heterotrophs, whilst larger and less degradable particles accumulate in the system if not removed. Studies have shown that a C/N ratio of 0.5 can reduce TAN (Total Ammonia Nitrogen) removal rates by 30% compared to a C/N ratio of 0, and higher C/N ratios can further decrease the efficiency by 50%[24].

In addition to bacteria, ammonia-oxidizing archaea (AOA), containing the same enzymes as the AOBs have been found in RAS biofilters, but how much they contribute to ammonia-oxidizing in RAS is still unclear[22]. The AOAs include genera such as *Nitrososphaera*, *Nitrosopumilus* and *Nitrosoarchaeum*. Furthermore, certain bacteria with the ability to carry out both steps of nitrification, known as complete ammonia oxidizers (COMAMMOX), have been identified. Notably, COMAMMOX species such as *Nitrospira nitrificans* and *Nitrospira nitrosa* have been detected within the biofilters of RAS[25][26][27].

Incomplete nitrification in RAS presents a considerable hazard to Atlantic salmon, as it results in the build-up of nitrite and consequent mass mortality among the

fish population. Nitrite out-competes chloride in the gills during uptake and prolonged exposure can lead to the oxidation of haemoglobin to methaemoglobin, reducing the blood oxygen transport of the fish[28]. Several factors influence nitrite toxicity in fish such as pH, temperature, fish species and time of exposure. However, the concentration of chloride in the rearing water is considered to be the factor that influences the toxicity of nitrite the most. Notably, seawater exhibits a 50-100 fold lower lethal nitrite concentration compared to freshwater[28]. In freshwater production of Atlantic salmon, the Norwegian Food Safety Authority has suggested that the nitrite concentration should be below 0.1 mg/L[20].

1.3.2 Water suspended bacteria

The bacteria suspended in the RAS water influence the chemical water quality and can potentially colonize the fish, affecting its health. These bacteria decompose easily degradable organic carbon from waste products such as excess feed and fish excrement, contributing to the nutrient balance in the water. The rearing water mainly contains non-pathogenic heterotrophic bacteria dominated by *Alphaproteobacteria* and *Gammaproteobacteria*[17][29][30][31]. The fish are directly affected by the water suspended bacteria. In some cases, the bacteria contribute to improved fish health and positively affect the digestive system and the development of morphology in fish larvae[32][33]. However, pathogenic heterotrophs and blooms of opportunistic bacteria can affect the fish negatively, especially if the fish have suboptimal rearing conditions or are under stress[34].

The heterotrophs contribute heavily to the reduction of chemical water quality through the generation of CO_2 and various other metabolic by-products. Therefore, keeping the amount of dissolved organic carbon low is critical to control the bacterial composition[17]. Microbial community management can be achieved through r/K-selection, a method that categorizes bacteria into two groups: r- and K-selected bacteria[35][34]. K-selected bacteria thrive in crowded environments with bacterial densities close to the carrying capacity, which is the maximum amount of bacterial biomass that a system can sustain. These bacteria specialize in competing for resources but have lower growth rates compared to r-selected bacteria. r-selected bacteria, on the other hand, have higher growth rates but lack the competitive abilities of K-selected bacteria. However, they quickly colonize unexploited environments that are far from the carrying capacity if sufficient resources are available[34]. r-selected bacteria often affect RAS negatively due to a higher probability of opportunistic and pathogenic bacteria[36]. K-selected microbial communities are generally more stable than r-selected communities and have been implied to be more compatible with RAS.

1.4 Organic load in RAS

In RAS, the organic load in a system refers to the amount of organic matter present in the water. As mentioned, the organic load significantly affects the microbial communities and is introduced to the system through the input water,

fish feed and feces. The particles exist in different forms and quantities and various techniques have been devised to define the amount of suspended particles. Total suspended solids (TSS) is a method used to quantify the number of particles stopped by a fiberglass filter with a pore size of 0.45 µm. Another method for measurement is turbidity, which measures the light-scattering properties of water, which is influenced by the amount of suspended particles in the water. Turbidity is a cheaper and less timely method compared to TSS[37]. However, turbidity is less accurate because it only measures the total amount of light scattered by the particles and not the actual number of particles. Nonetheless, turbidity can be a useful indicator of particle concentration and water quality[38].

Particles are classified by size, where particles smaller than 0.001 µm are classified as dissolved/soluble, particles between 0.001-1 µm are colloidal, those between 1-100 µm are supercolloidal and particles larger than 100 µm are settleable[2]. The larger particles are removed by a mechanical drum filter or in the swirl separator in the RAS loop. The swirl separator utilizes centrifugal forces to separate particles that are denser than water from the water, where the dense particles sink to the bottom of the tank. Particles that are too small for the particle removal steps can also settle in the fish tank or along any surface in the RAS loop, creating a biofilm on the surface. The organic matter can also settle in the biofilter and become feed for the heterotrophic bacteria[2]. Previous studies have shown that the biofilter contains the primary source of particulate organic carbon for heterotrophic growth and therefore, the main source of heterotrophic bacteria in RAS[24]. Additionally, the abundance of heterotrophic bacteria suspended in the water is correlated with the composition of microbial communities present on the packing media of the biofilter[39] [24].

1.4.1 Problems related to high TSS

High levels of total suspended solids (TSS) and turbidity pose several challenges for Atlantic Salmon production in RAS. The presence of suspended solids can decrease water quality by reducing light penetration and altering temperatures[40]. High TSS will also lead to decreased dissolved oxygen levels, due to oxygen consumption by in-situ decomposition and the subsequent growth of heterotrophs[40]. Significant gaps in our understanding persist regarding the impact of organic load on microbial communities within RAS, particularly concerning the intricate dynamics involving particle load, dissolved organic matter and growth of heterotrophs.

TSS can also have direct effects on the fish, including gill clogging and irritation[41], decreased feeding activity and growth rates and increased stress levels resulting in disease susceptibility[42]. However, available evidence suggests that TSS levels up to 25 mg/L have no harmful effects on fish[43]. To ensure safe aquaculture practices, The European Inland Fishery Advisory Commission (FIFAC) recommends keeping the TSS concentration below 15 mg/L in inland fisheries for general intensive aquaculture[44], whilst other sources suggest a range of 20-

40 mg/L for freshwater fish[45]. Exposure to high levels of TSS may prove fatal for the fish and lower levels may affect the physiology and behavior of salmon resulting in sub-lethal effects[43].

The impact of TSS on fish is influenced by four main factors: The duration of exposure to TSS, the concentration of TSS, the particle-size distribution and the composition of TSS.[38] Moreover, the effects of TSS vary based on the life stage of the fish, further complicating the understanding of TSS's effects in aquaculture[46].

1.5 Methods to study microbial communities

Studying microbial communities is necessary to fully understand their role in ecosystems and how they interact with other organisms. In recent years, the field of microbial ecology has been revolutionized by the emergence of sequencing technologies, specifically Illumina sequencing of 16S rRNA amplicons. This advanced method has significantly enhanced the ability to analyze microbial communities in a cost-effective manner, thanks to its high-throughput sequencing (HTS) capabilities. It is possible to differentiate between different microbial taxa due to the hypervariable region of the 16S rRNA gene providing enough sequence, resulting in an accurate identification of the community composition. HTS includes several sequencing technologies that can conduct parallel sequencing of different DNA molecules simultaneously with high-throughput, unlike the more traditional Sanger sequencing with low-throughput[47]. The 16S rRNA gene, which is present in all prokaryotes, comprises nine variable regions (V1-V9) and nine conserved regions. These variable regions within the 16S rRNA gene are valuable for species identification and evaluating microbial community diversity, as they exhibit dissimilarity among distinct bacterial species. Conversely, the conserved regions serve as targets for broad-coverage PCR primers, enabling comprehensive amplification of the gene[48]. The 16S rRNA gene is the "gold standard" as a marker gene in studies about microbial diversity, phylogeny and taxonomy.

Illumina sequencing comprises four main steps, including library preparation, bridge amplification, sequencing and data analysis. During the library preparations, the sample DNA is fragmented and specialized adapters are ligated to both fragments ends. During the cluster generation process, the DNA library is loaded onto a flow cell, onto which surface-bound oligos, are complementary to the library adapters. The fragments of DNA in the library are then captured by these oligos and bridge amplification is used to generate distinct, clonal clusters. Once the cluster generation process is completed, the resulting templates are then prepared for sequencing. The sequencing employs the Synthesis method, in which fluorescent-labeled nucleotides are added in a sequential manner, emitting light upon their incorporation. The resulting light pulses are simultaneously captured and analyzed to determine the sequence. Finally, in the data analysis, the obtained sequences are quality filtered, followed by alignment and/or assembling

with bioinformatic software[49].

Illumina sequencing of 16S rRNA gene amplicons offers several advantages compared to other sequencing technologies, one being the substantial reference data for 16s rRNA sequences that provide high-resolution taxonomic information about microbial communities. The method is also scalable and can analyze a wide range of microbial communities in different environments, from soil to water and human microbiomes[50]. However, the method also has some limitations. The presence of PCR bias and varying amounts of the 16S rRNA gene in different bacterial genomes can result in over- or under-representation of certain taxa in the final data. Furthermore, it is possible that the method inaccurately differentiates between closely related taxa, resulting in incorrect taxonomic assignment[51].

1.6 Study Aim

This master's thesis is a component of the MikroRAS project, a collaborative research effort involving NIVA, NTNU, and UIB. Led by NIVA in Bergen, the project aims to investigate the impact of smoltification and increased salinity on the microbiomes of salmon's feces, skin, and gills under varying organic load levels. Additionally, it examines the influence of organic load before, during, and after smoltification on salmon's microbiome, mucosal health, and welfare. Moreover, it explores the effects of organic load and increased salinity on physicochemical properties, microbial water quality, nitrification processes, and the microbiota associated with biofilms and particles. The aim of this master's thesis is to investigate the freshwater and brackish water phase of Atlantic salmon RAS and the effect of high organic load on the microbial communities in the biofilter biofilm and rearing water.

Specifically, this study will look into these objectives:

1. To evaluate the extraction of DNA from Atlantic salmon skin and gut, biofilm carriers and water filters from RAS using the NAxtra^{TM} Fish total nucleic basic extraction kit.
2. To inspect how the concentration of nitrogenous substances varies throughout the freshwater and brackish water phases in RAS.
3. To examine how organic load influences the biofilter microbiota and the water-suspended microbiota.
4. To investigate how the biofilter and water-suspended microbiota changed from freshwater to brackish water.
5. To assess the potential differences in microbial communities associated with two fractions of the water particles; larger and smaller than 8 µm.
6. To analyze the temporal changes in microbial communities in RAS water and biofilter biofilm.

2 Methods

This master thesis is a part of the collaboration project MikroRAS and also a continuation of a project thesis by Stenhaug et al.[52] on the effect of high and low organic load on bacterial concentration and water quality. The project thesis explained the RAS facility and experimental design in more detail, including physicochemical water quality throughout the experiment. Specifically, how and why the nitrogenous substances vary with different TSS. The experimental design and description of the RAS facility are summarized below.

2.1 Experimental design

The experiment was conducted in six identical RAS at Marineholmen RASLab in Bergen, Norway. Prior to fish stocking, each of the six RAS with a volume of 2.5 m^3 was randomly assigned to one of two treatments: RAS operating at high organic load (H-RAS) or RAS operating at low organic load (L-RAS). RAS4, RAS6 and RAS9 were designated as H-RAS and were operated at 12-15 mg TSS/L. RAS5, RAS7 and RAS8 were designated as L-RAS and were operated at 1-2 mg TSS/L. This thesis includes the eight first sampling times of the experiment, ranging from 08.08.2022 (Day 1) to 06.12.22 (Day 121), where samples for microbial community analysis were collected and fish health was monitored (Figure 2.2).

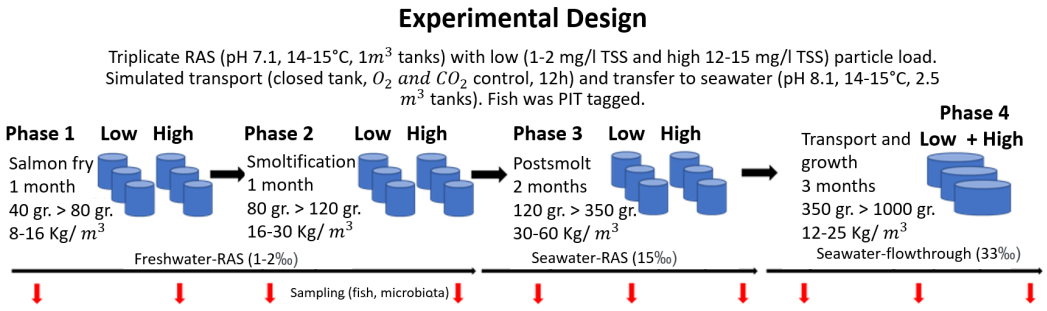


Figure 2.1: Experimental design with Phase 1: Salmon fry in freshwater, Phase 2: Smoltification in freshwater, Phase 3: postsmolt in brackish water, Phase 4: postsmolt in seawater. The 6 cylinders in Phase 1-3 represent the 6 RAS, where 3 were operated at low organic load, and 3 were operated at high organic load. The three cylinders in Phase 4 represent three flow-through systems used to simulate open-net pen fish farms.

The experiment can be divided into four distinct phases (Figure 2.1). Phase 1 was the freshwater stage, which housed salmon fry weighing between 40-80 g. On Day 1, the fish were weighed, and each system was stocked with an initial biomass of 7.7 kg of 39.5 g of Atlantic salmon, resulting in a fish density of 7.7 kg/m^3. Phase 2 was dedicated to the smoltification process, which was induced by altering the lighting conditions to continuous light (24 hours per day). At

this stage, the fish weighed between 80-120 g. Phase 3 involved post-smolt and a change to brackish water with a weight range of 120-350 g. On day 72, the freshwater biofilter media was replaced by biofilter media (described below) that had been adapted to brackish water at Marineholmen RASlab. Finally, Phase 4 comprised post-smolt fish in seawater weighing approximately 350-1000g. Phase 1 and Phase 2 of the study spanned a duration of one month each, while Phase 3 extended over a period of two months. Phase 4, encompassing three months, and the subsequent transition to flow-through systems (FTS), are excluded from the analysis conducted in this thesis.

Two weeks before the start of the trial, Atlantic salmon (*Salmo salar*) were stocked in each system to acclimate to the new environmental conditions. The fish were fed daily and on-demand with 15% extra feed based on RASLab feeding tables using Skretting Nutra RC 2 or 3 mm pellets. To quantify the daily feed intake, the feed that was not consumed and accumulated at the bottom of the swirl separator, where feces and leftover feed are separated, was collected. The collected feed was then subjected to overnight drying in an oven. The difference between the weight of the feed provided and the dried weight of the collected feed was calculated to determine the daily feed intake. To minimize variation in cumulative feed burden (CFB, kg feed/m3 make-up water) between H-RAS and L-RAS, make-up water was added on-demand. Feces collected from the swirl separator of H-RAS were placed back daily into their corresponding RAS by manual re-addition upstream of the biofilters to achieve the planned TSS level.

2.2 Description of RAS facility

Each RAS consisted of a 1 m^3 fish tank, followed by a 464 mm diameter swirl separator, a 40 µm drum filter, a moving bed biofilter of 0.58 m^3 filled to 65% with 625 m^2/m^3 mature biomedia, as described in detail below, (KSK Saddle Chips 1.0, KSK Aqua Aps, Skive, Denmark), and a trickling filter. Salinity, pH, temperature, and dissolved oxygen were automatically controlled using a control system (Georg Fischer AS, Rud, Norway). Additionally, daily monitoring of these parameters was performed using a multiprobe handheld device (WTW Multi 3620 IDS, Xylem, Washington DC, USA) equipped with sensors for salinity (Tetracon 925), temperature, dissolved oxygen (FDO 925 optical oxygen IDS sensor), and pH (VWR pH pen).

The quantification of ammonium, nitrite, and nitrate concentrations in the swirl separator water was conducted by NIVA using Spectroquant analytical techniques. Specifically, the Spectroquant Ammonium Test (Supelco), Spectroquant Nitrite Test (Supelco), Spectroquant Nitrate Test (Supelco) and Spectroquant Nitrate Test in seawater (Supelco) were employed for the measurement of the aforementioned compounds. The Spectroquant tests utilize a photometric method, whereby ammonia, nitrite and nitrate react with a reagent and are subsequently measured photometrically against a reagent blank to determine their respective concentrations in the water sample[52].

Prior to being placed in the experimental RAS, the biomedia was matured for 2-4 weeks in freshwater or brackish water maturation tanks and fed NH4Cl (CAS nr. 12125-02-9, Hjelle Kjemi, Bergen, Norway) and NaHCO3 (CAS nr. 144-55-8, Hjelle Kjemi, Bergen, Norway) daily. Water flow to the unstocked fish tanks was maintained at 55.9 ± 1.8 L/h, resulting in a hydraulic retention time of 18 h. Water intake was automatically controlled based on salinity setpoints defined in the different trial phases. To adjust pH and alkalinity an automatic dosing system with $NaHCO_3$ was used.

2.3 Sampling

In this study, various measurements and samplings were conducted at RASlab in Bergen, by employees and students from Ilab, NIVA, UiB, and NTNU on all of the sampling dates (Figure 2.2), with the assistance of S. R. Stenhaug at sampling time T1 and T4. Sampling for microbial analysis was conducted from T0 on 08.08.2022 until T7 on 06.12.22. In this master's project, microbial community analysis was performed on four different sample types (Table 2.1) and a total of 173 samples via Illumina sequencing of the variable V3-V4 regions of the 16S rRNA gene amplicons. Initially, the total number of samples was 206; however, 33 of these samples were excluded due to reasons such as missing samples, insufficient DNA extraction, and failed sequencing. An overview of all the samples used for analysis with the corresponding sample name, sampling time, RAS tank and sample type are shown in Table B.1.

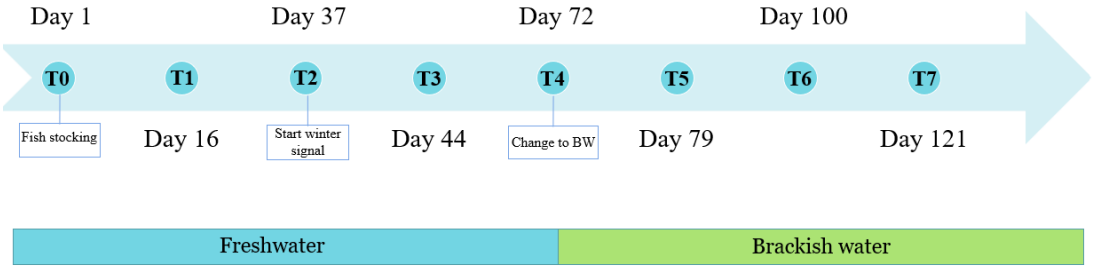


Figure 2.2: Experimental timeline including eight sampling times (T0-T7), and corresponding days from the first sampling time at Day 1. Important information such as fish stocking, changing the light conditions, and changing salinity is indicated.

2.3.1 Biofilm samples from biocarriers

Ten biocarriers were systematically collected for microbial community analyses from each RAS biofilter during all the sampling times and were collected from a depth several centimeters below the water surface. Following collection, all samples were immediately frozen at -80 ℃ and stored until subsequent analysis. Biocarriers from each of the six RAS at each sampling time were later shipped from Bergen to NTNU in Trondheim.

2.3.2 Water samples

Water samples for microbial community analyses were collected from the swirl separator of all RAS at all sampling times. The swirl separator was chosen as the sampling point because the water is equivalent to the rearing tank water and can be extracted without disturbing the fish. Prior to sampling, the bottles were rinsed with the water being sampled. Each water sample was then filtered through an 8 µm filter using a Büchner funnel, followed by a second filtration through a 0.2 µm filter. Both filters were then collected and stored in cryotubes at -80 °C. To prevent cross-contamination, the equipment was washed with ethanol and Milli-Q water between each new water sample.

Table 2.1: The different sample types used and the corresponding abbreviation. The biofilm was taken from biocarriers in the biofilter, and the water was collected from the swirl separator and later filtrated. The abbreviations are used to simplify reading and analyzing the results.

Sample type	Abbreviation
Biofilm	X1
Biofilm replicate	X2
Water sample 0.2 µm	Y
Water sample 8 µm	Z

2.4 Microbial community analysis

2.4.1 DNA extraction

DNA was extracted from all sample types (Table 2.1) utilizing the NAxtra^{TM} Fish total nucleic basic extraction kit (Lybe Scientific AS). The NAxtra extraction kit was developed by researchers at NTNU as an alternative to commercially available DNA extraction kits for SARS-CoV-2 testing and has recently adapted to other scientific applications. To date, the NAxtra kit has not been extensively tested on fish tissue or other aquaculture-related samples. Hence, this study includes preliminary testing of the kit on fish gut, fish skin, and biofilm from RAS biocarriers.

To prepare the biofilm samples from the biocarriers, the biofilm was manually scraped off two biocarriers using a pipette. This was conducted for biofilm samples from each RAS at each sampling time. The scraped biofilm was then suspended in an Eppendorf tube containing 1-2 mL of DNase-free water. The sample was subjected to centrifugation at 10,000 rpm for 5 minutes, resulting in the separation of the supernatant and the formation of a pellet. The DNA extraction process was performed using the pellet. To ensure replicability and account for variability, this entire procedure was replicated for two additional biocarriers from the same RAS and sampling time, thus creating another replicate under similar conditions. This comprehensive procedure was repeated for all RAS and sampling times included in the study[52].

The extraction process was carried out according to the manufacturer's instructions (Appendix C) with the following deviations. Step 1 was modified by using 0.1 mm glass beads (Precellys). In step 3 precellys 24 tissue homogenizer was used with 5000 rpm x30 s, followed by a 25-second pause and another round with 5000 rpm x 30 s, and in step 5 a 60 ℃ heating cabinet without shaking was used. From step 8 the Thermo Scientific™ KingFisher™ Flex Purification System was utilized with a script provided by the manufacturer. PCR-grade water was used as elution buffer. Following the DNA extraction, the concentration of the extracted DNA was determined using a NanoDrop Spectrophotometer.

2.4.2 Polymerase Chain Reaction (PCR)

The V3-V4 regions of the bacterial 16S rRNA gene were amplified from the extracted DNA through polymerase chain reaction (PCR) using the primers ill341F_Kl and ill805R primers (Table 2.3). The reaction mixture was composed of 5 µL 5x Phusion buffer HF (7.5 mM $MgCl_2$) (Thermo Scientific), 0.4 µL of ill341Fkl (10 µM), 0.4 µL ill805R (10 µM), 0.5 µL dNTP (10 mM)(Thermo Scientific), 0.18 µL Phusion Hot Start DNA polymerase (2 units/µL) (Thermo Scientific), 1 µL template, and DNA-free H_2O adjusted to the final reaction volume of 25 µL[52]. A T100™ Thermal Cycler (BioRad) was utilized for the amplification with the cycling conditions according to Table 2.2.

Table 2.2: Temperature cycling condition in the thermal cycler used to amplify the V3-V4 regions of the 16S rRNA gene.

Step	Temperature [°C]	Time	
Denaturation	98	2 min	
Denaturation	98	15 s	
Annealing	55	20 s	x38 cycles
Elongation	72	20 s	
Final elongation	72	5 min	
cooling	4	1 min	

Table 2.3: Primer name and nucleotide sequence of the PCR primers used to amplify the V3-V4 region of 16S rRNA gene. Target sequences of the primers are shown in bold.

Primer name	Nucleotide sequence	Target region
Ill341F_Kl	5'-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG NNNN **CCT ACG GGN GGC WGC AG**-3'	V3
Ill805R	5'- GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G NNNN **GAC TAC NVG GGT ATC TAA KCC**-3'	V4

2.4.3 Agarose gel electrophoresis

The PCR products' quality and quantity were evaluated by performing agarose gel electrophoresis. An agarose solution (1%) containing 50 mM GelRed (Biotium) was prepared and poured into a gel chamber containing a gel comb. The

gel was allowed to solidify for 15-20 minutes before 5 µL of each PCR product mixed with 1 µL 6x DNA Loading dye (Thermo Scientific) was loaded into the gel wells. GeneRuler™ 1 kb Plus DNA Ladder (Thermo Scientific) was loaded at each end of the gel as a size marker. The gels were run at 110V for 60 minutes for the large gels, and 90V for 45 minutes for the smaller gels and then visualized under UV light[52].

2.4.4 Preparation of Illumina amplicon sequencing library

Amplicon library preparation following successful PCR amplification was conducted following the Norwegian Sequencing Centres instructions. The PCR products were normalized and purified using a Sequal Prep Normalization Plate Kit (Invitrogen) following the manufacturer's protocol (Appendix D) utilizing 15 µL of the PCR product for each sample. The next step was the indexing PCR, where a unique combination of a forward and a reverse sequence index was added to each normalized PCR product using the Nextera XT Index Kit Set A and Set D (Illumina), giving each sample a unique barcode. Subsequently, 2.5 µL of the forward index and 2.5 µL of the reverse index was added to a PCR reaction mixture comprised of 5 µL 5x Phusion buffer HF (7.5 mM $MgCl_2$)(Thermo Scientific), 0.5 µL dNTP (10 mM)(Thermo Scientific), 0.19 µL Phusion Hot Start DNA polymerase (2 units/µL)(Thermo Scientific), 2.5 µL template and DNA-free H_2O up to a total reaction volume of 25 µL. The PCR reactions were run for 9 cycles on a T100™ Thermal Cycler (BioRad) with the cycling conditions shown in Table 2.4. The PCR products' quality and quantity were then evaluated by performing agarose gel electrophoresis.

Table 2.4: Temperature cycling condition in the thermal cycler used to amplify the V3-V4 regions of the 16S rRNA gene.

Step	Temperature [°C]	Time	
Denaturation	98	2 min	
Denaturation	98	15 s	
Annealing	55	20 s	x9 cycles
Elongation	72	20 s	
Final elongation	72	5 min	
cooling	4	1 min	

The indexed PCR products were then normalized and purified another time with the Sequal Prep Normalization Plate Kit (Invitrogen) using 10 µL of each sample. The resulting samples were pooled into one sample and concentrated using Amicon Ultra 0.5 centrifugal filter devices (30K membrane, Merck Millipore) as per the manufacturer's protocol (Appendix E) with the following deviations: For step 4 the samples were centrifuged at 14,000 G for 10 minutes and the washing step was performed twice with 500 µL sterile filtrated 1x TE buffer. The concentrated pooled sample was analyzed for concentration and purity using NanoDrop™ One (Thermo Scientific) and was further examined by gel electrophoresis. Since un-

specific PCR products, probably representing primer dimers, were observed in the gel, the product with the desired band length was excised from the agarose gel. The excised product was purified with the QIAquick PCR Purification Kit (QIAGEN) following the manufacturer's protocol (Appendix F) to obtain a pure product.

The amplicon library was sent to the Norwegian Sequencing Centre (NCS) for sequencing on one MiSeq run (Illumina, San Diego, CA) with V3 reagents (Illumina).

2.4.5 Processing of sequencing data

The Illumina sequencing data were processed using the USEARCH pipeline (version 11; https://www.drive5.com/usearch/) by I. Bakke. In brief, the Fastq_mergepairs command was employed for the merging of paired reads, primer sequence trimming, and filtering out reads with less than 400 base pairs. Quality trimming was performed using the Fastq_filter command, with an expected error threshold of 1. Amplicon sequencing variants (ASVs) were generated using Unoise3. Taxonomy assignment was performed using the Sintax script [53] with a confidence value threshold of 0.8 and the RDP reference data set (version v18).

The ASV table obtained from the sequencing data was subjected to manual inspection to remove ASVs representing salmon genes. Additionally, 4 ASVs identified as dominating in negative control for DNA extraction were also excluded. Tables representing the community composition at the various taxonomic levels were generated based on this ASV table using the Taxa_summary command in Usearch. Subsequently, the resulting ASV table was normalized by rarefying the read numbers per sample to 9005 by S. Fredriksen.

2.4.6 Statistical analysis

To determine the similarity between community profiles, the Bray-Curtis similarity index was computed using PAST software (version 4.12). The resultant matrix of Bray-Curtis similarities was exported to Microsoft Excel for further analysis. The Bray-Curtis similarity index is employed in ecological studies to assess beta diversity by considering both species presence and relative abundance within samples [54] [55] [56]. Bray-Curtis similarities range between 0 and 1, where 0 denotes communities that are entirely dissimilar (with no shared ASVs), and 1 represents identical communities. To visualize beta diversity, Principal Coordinate Analysis (PCoA) was performed using Bray-Curtis similarities. PCoA plots depict samples based on a distance matrix, where samples are ordinated such that the distances between them correspond to their Bray-Curtis similarity. The two coordinates showcasing the biggest variations in the distance matrix are then visualized onto a two-dimensional plot where samples with similar microbial communities are located closer to each other than those with less similar microbial communities [57]. Separate box plots with Pairwise Bray-Curtis dissimilarity analysis and visualization were conducted by S. Fredriksen.

One-way PERMANOVA (permutational multivariate analysis of variance) using Bray-Curtis similarities was employed to examine the presence of statistically significant differences in community profiles between sample groups[58]. The threshold for significance was set at a p-value below 0.05. In cases where multiple groups were compared, one-way PERMANOVAs with Bonferroni-corrected p-values were utilized.

To examine the alpha diversity of biofilm and water sample communities, Shannon's diversity and ASV richness were calculated. PAST software was employed for this analysis, as it provides a comprehensive set of diversity indices and statistical tools for ecological studies. The alpha diversities obtained in PAST were then exported to Excel to create box plots.

To determine which taxa contributed the most to differences in microbial composition a Wilcoxon test was performed by S. Fredriksen on the USEARCH data. The ASVs with the lowest p-value (although not necessarily $p < 0.05$ with FDR multiple testing correction) with the Wilcoxon rank-sum test within each sampling time were later visualized in a box plot. A SIMPER (Similarity Percentage) analysis[59] based on Bray-Curtis values was also conducted in PAST to identify the ASVs that contributed the most to the dissimilarities in microbial community composition between the H-RAS and L-RAS samples.

3 Results

3.1 Preliminary testing of NAxtra™ Fish total nucleic basic extraction kit

The success of PCR amplification of the V3-V4 region of the 16S rRNA gene from DNA samples of Atlantic salmon has previously been limited within the research group ACMS at NTNU. This difficulty is likely due to the presence of inhibitors in the DNA extract and/or the low amount of bacterial DNA relative to host DNA. Therefore, a key aspect of this study was to test the NAxtra™ Fish total nucleic basic extraction kit (Lybe Scientific AS) to overcome these challenges.

The initial assessment involved the implementation of three variations of the NAxtra protocol. The first variant, referred to as Method 1 (M1), followed the procedure outlined in Subsection 2.4.1. Method 2 (M2) was performed similarly to M1, but excluded the addition of Proteinase K in the lysis step, while Method 3 (M3) omitted both Proteinase K and tissue disruption using a Homogenizer. These three methods were evaluated using diverse sample types, including a biofilm sample obtained from a RAS biofilm carrier, filtrated water samples from RAS, as well as skin and gut samples from Atlantic salmon. Subsequent to DNA extraction, PCR amplification of the V3-V4 region of the 16S rRNA gene was carried out on the total DNA extracts following the steps described in Subsection 2.4.2, and the resultant products were visualized using agarose gel electrophoresis. Notably, the biofilm and filtrated water samples resulted in expected-sized products (500-600 bp), both by using the M1 and M2 protocols, while some products are observed for the gut sample in the M3 protocol (Figure 3.1). Overall, the outcomes demonstrated low or negligible product yield for the fish samples across the tested methods. Importantly, the DNA extraction positive control (KP) and the PCR positive control (PC), executed on a biofilm sample previously yielding successful PCR amplification, validated the functionality of the kit. Conversely, the negative control for the DNA extraction kit (KB) and the PCR negative template control (NTC) exhibited no detectable PCR product, indicative of an absence of DNA contamination.

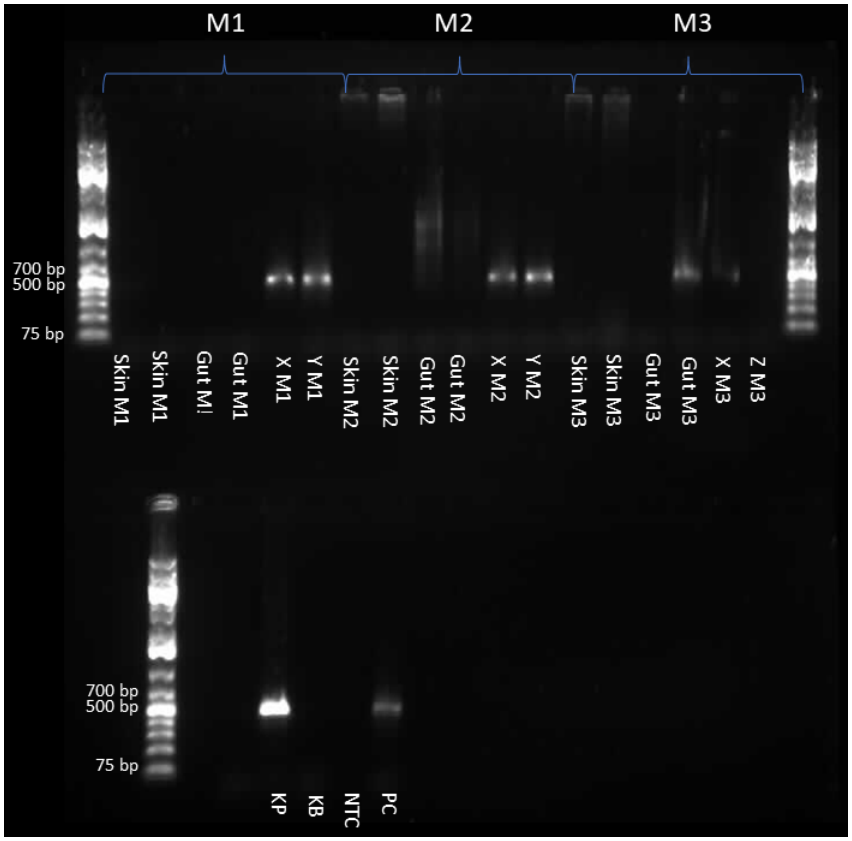


Figure 3.1: Agarose gel (1%) showing PCR products representing the V3-V4 region of the 16S rRNA gene obtained with the primers ill341F_Kl and ll805R. Samples represent DNA extracted with the NAxtra^{TM} Fish total nucleic basic extraction kit (Lybe Scientific AS) from biofilm carriers (X), 0.2 µm filtrated water (Y), 8 µm filtrated water (Z), gut and skin samples from Atlantic salmon with the three lysis protocols M1, M2, and M3. The negative DNA extraction control (KB) and the PCR non-template control (NTC) served as negative controls to assess potential contamination. The kit positive control (KP) and the PCR positive control (PC) were included using a sample that had previously demonstrated successful PCR amplification. Three different DNA extraction methods were evaluated: M1 representing the original protocol, M2 excluding Proteinase K addition, and M3 omitting both Proteinase K and tissue disruption using a Homogenizer.

Lybe Scientific AS created a new lysis buffer for the NAxtra^{TM} Fish total nucleic basic extraction kit to enhance the extraction of bacterial DNA from fish samples. In the next step of the study, DNA extraction from gut and skin samples was performed using three different lysis protocols (M1, M2, and M3) with both the new and old lysis buffers for comparative analysis. Subsequently, PCR amplification targeting the V3-V4 region of the 16S rRNA gene was carried out on all extracted samples, with three dilutions of each DNA extract employed as templates (undiluted, 1/10, 1/100). The resulting PCR products were visualized using agarose gel electrophoresis (Figure 3.2).

In the gel image corresponding to the PCR product obtained with the protocol using the old lysis buffer (top gel), Method 2 displayed detectable PCR products

in the expected band length for the diluted 1/100 skin sample, undiluted gut sample, and diluted 1/100 gut sample. For the protocol using the new lysis buffer (bottom gel), PCR products were observed for the skin sample with 1/10 diluted DNA extract, the skin sample with 1/100 diluted DNA extract, and the gut sample with 1/10 diluted DNA extract in Method 1. However, the two skin samples exhibited unspecified PCR products with larger than expected products (Figure 3.2). In Method 2, PCR product was evident for the gut sample with 1/10 diluted DNA extract. Additionally, in Method 3, PCR products were observed for the undiluted skin sample and the gut sample with 1/10 diluted DNA extract. Overall, the protocol utilizing the new lysis buffer and Method 1 showed promising results.

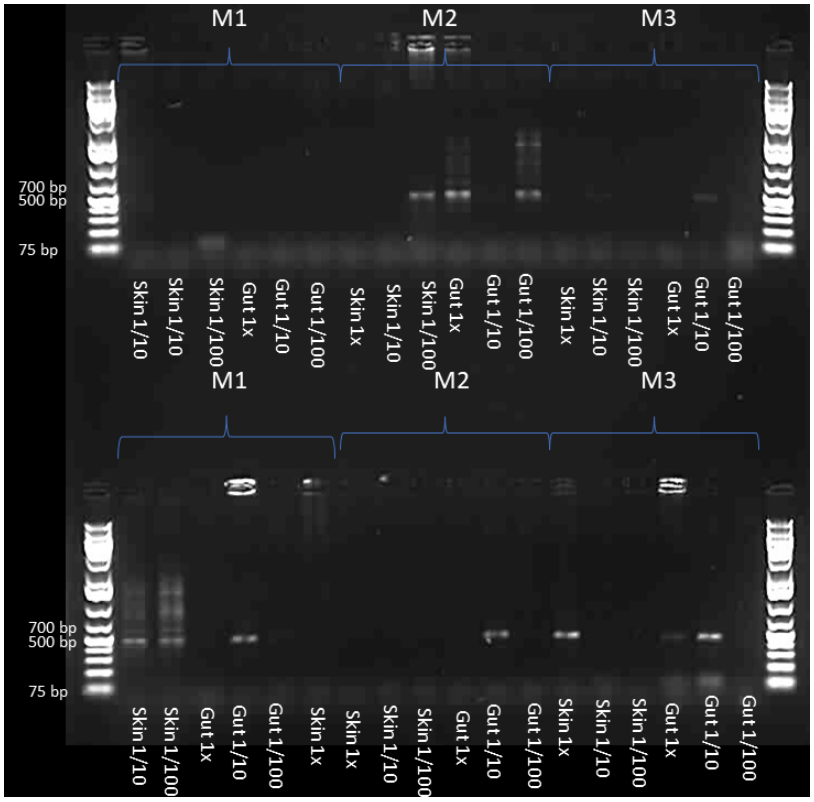
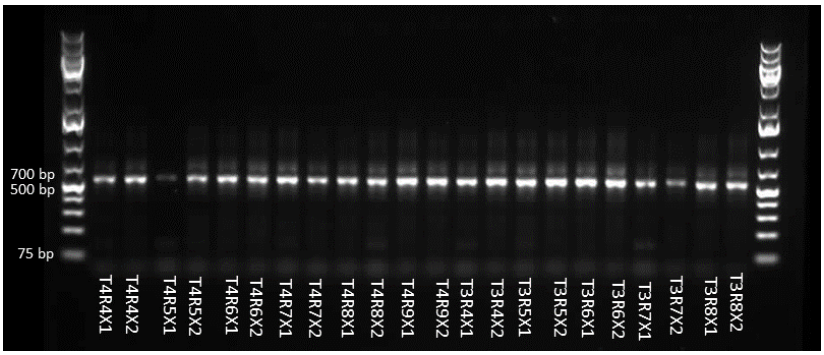


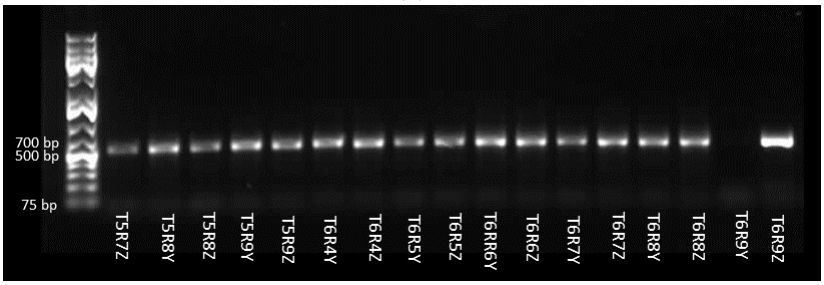
Figure 3.2: Agarose gel (1%) showing PCR products representing the V3-V4 region of the 16S rRNA gene obtained with the primers ill341F_Kl and ll805R. Samples represent DNA extracted with the NAxtra^{TM} Fish total nucleic basic extraction kit (Lybe Scientific AS) from skin and gut samples from Atlantic salmon with the three lysis protocols M1, M2, and M3. The extracted DNA was prepared in three dilutions as the template for PCR: undiluted, 1/10 dilution and 1/100 dilution. Three different DNA extraction methods were evaluated: M1 representing the original protocol, M2 excluding Proteinase K addition, and M3 omitting both Proteinase K and tissue disruption using a Homogenizer. Additionally, two different lysis buffers were tested. The upper part of the gel utilized the original lysis buffer provided by Lybe Scientific AS, while the lower part of the gel employed a lysis buffer optimized by Lybe Scientific AS for fish samples.

This assessment provided valuable insights into the performance of the proto-

cols using different lysis buffers, dilutions and extraction methods employed in the NAxtra^{TM} Fish total nucleic basic extraction kit (Lybe Scientific AS). The findings showed that the newly optimized protocol and lysis buffer resulted in successful PCR amplification of the target region in diverse sample types, including biofilm, water, and fish samples. Based on these results, the NAxtra kit incorporating the new and optimized lysis buffer and the M1 protocol was selected for amplification of the V3-V4 region of the 16S rRNA gene for all water and biofilm samples in the current research project (Figure 3.3).



(a)



(b)

Figure 3.3: Agarose gel (1%) showing PCR products representing the V3-V4 region of the 16S rRNA gene obtained with the primers ill341F_Kl and ll805R. Samples represent DNA extracted with the NAxtra^{TM} Fish total nucleic basic extraction kit (Lybe Scientific AS) from biofilm and water samples from RAS. Each sample is named with a sampling time (T), RAS unit (R) and sample type (X1, X2, Y, Z). T4R4X1 is a biofilm sample from RAS4 taken at sampling time T4. (a) shows biofilm samples from sampling times T4 and T3. (b) shows RAS water samples from sampling time T5 and T6 filtrated with 0.2 µm (Y) or 8 µm filters (Z).

3.2 Water quality and production data

The water quality parameters and production data obtained from the three RAS operated at high organic load (RAS4, RAS6 and RAS9) and the three RAS operated at low organic load (RAS5, RAS7 and RAS8) provide valuable insights into the performance and overall health of the Atlantic salmon (Table 3.1). In this study, NIVA and Ilab monitored various water quality parameters, including dissolved oxygen levels, temperature, pH, ammonia, nitrite, nitrate, and suspended solids throughout the production cycle. The comprehensive analysis of

the Physicochemical water quality data was presented in the project thesis by S. R. Stenhaug[52] and is summarized within this subsection.

Table 3.1: Tank water parameters during the different phases of the experiment. Day 1-37: Freshwater, Day 37-72: Smoltification in freshwater, Day 72-121: Postsmolt in brackish water.

Parameter	**Day 1-37**	**Day 37-72**	**Day 72-121**
Temperature [°C]	12.3 ± 0.8	12.3 ± 0.8	12.3 ± 0.8
Salinity [ppt]	1.4 ± 0.1	1.4 ± 0.1	15.0 ± 0.9
pH	7.2 ± 0.3	7.2 ± 0.3	7.8 ± 0.3
Dissolved oxygen	93.0 ± 5.6%	93.0 ± 5.6%	93.0 ± 5.6%
Water flow [L/h]	55.9 ± 1.8	55.9 ± 1.8	55.9 ± 1.8
Light (light/dark)	12h/12h	24h/0h	24h/0h

Table 3.2 presents the quantification of total suspended solids (TSS) expressed in terms of the dry weight of particles in all the examined RAS. Consistent with expectations, a statistically significant difference in TSS concentration (t-test, p=0.04) was observed between L-RAS (0.7-1.54 mg/L TSS) and H-RAS (1.88-8.06 mg/L TSS). The TSS was only measured during the start-up phase of the project, but turbidity measurements throughout the experiment showed similar results.

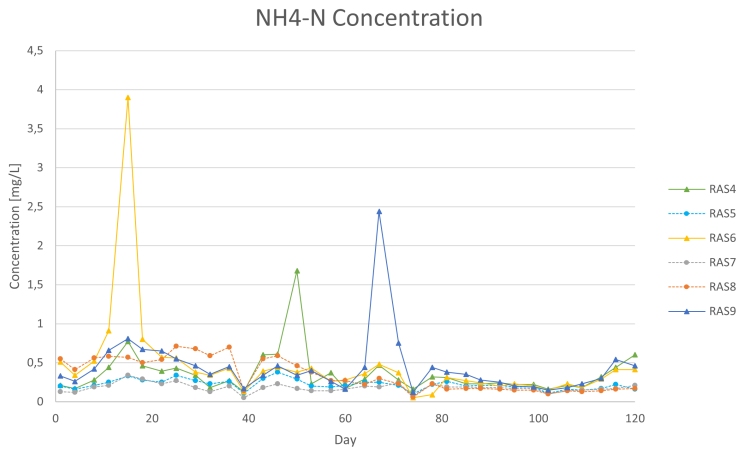
Table 3.2: Total suspended solids [mg/L] in the rearing tanks of RAS 4-9. RAS4, RAS6 and RAS9 were operated at high organic load, whilst RAS5, RAS7 and RAS8 were operated at low organic load[52].

Date	RAS4	RAS5	RAS6	RAS7	RAS8	RAS9
16.08.2022	2.64	1.3	2.46	1.54	1.8	2.06
19.08.2022	3.6	1.7	8.06	1.28	1.02	2.12
31.08.2022	2	0.7	6.58	1.02	0.88	1.88
Average	2.7	1.2	5.7	1.3	1.2	2.0

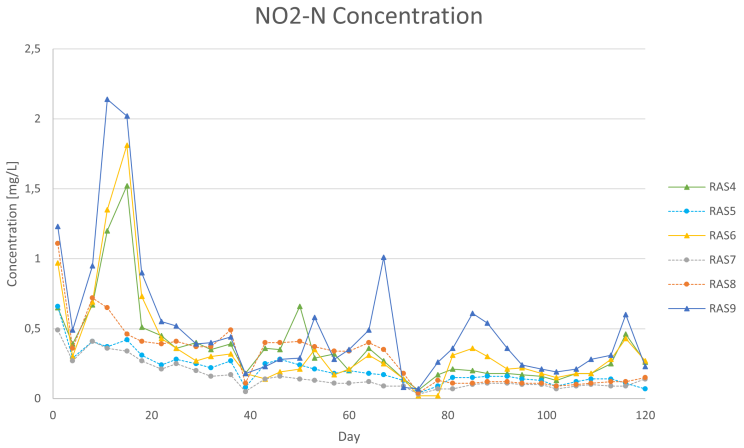
The concentration of nitrogenous substances in all RAS was measured throughout the experiment (Figure 3.4). Firstly, the comparison of TAN concentrations (Figure 3.4a) between H-RAS and L-RAS reveals no apparent differences from day 0 until day 100. However, in the last 20 days of the experiment, the TAN concentrations differ between H-RAS and L-RAS. Across all RAS, TAN concentration remained stable and below 0.5 mg/L, which is within the recommended Norwegian threshold of <2 mg/L[16]. However, two notable spikes were observed: one in RAS6 (Day 15) following an increase in salinity, with a TAN concentration reaching 3.9 mg/L, and another in RAS9 (Day 67) where the concentration peaked at 2.44 mg/L. Additionally, RAS4 experienced a spike with a TAN concentration of 1.68 mg/L, which still falls within the recommended limit. Notably, on day 41, the TAN concentration decreased in all RAS due to a 2-day feeding interruption.

The concentrations of nitrite (Figure 3.4b) also remained relatively stable and below 0.5 mg/L for the majority of the experiment. However, during the initial 20 days and the final 20 days, H-RAS exhibited higher nitrite concentrations compared to L-RAS. A t-test confirmed that the differences in nitrite concentration between H-RAS and L-RAS were significant for the initial 20 days ($p=0.0001$) and the final 20 days ($p=0.0001$). On day 41, nitrite concentrations in all RAS were low, likely influenced by the two-day feed interruption.

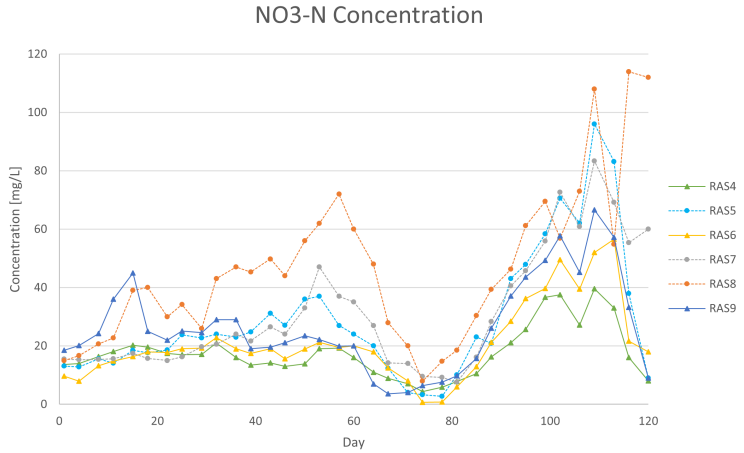
The nitrate concentrations generally remained below 60 mg/L until the last 30 days when it increased towards 100 mg/L in all RAS, albeit with some variations among the systems (Figure 3.4c). The three H-RAS exhibited similar nitrate concentrations throughout the experiment. RAS9 and RAS8 displayed nitrate concentrations over twice as high as the other RAS, ranging from 40-45 mg/L compared to 15-20 mg/L between day 2 and day 12. RAS9 stabilized after the start-up phase, while RAS8 continued to increase steadily throughout the experiment. In all RAS, nitrate concentration decreased below 10 mg/L towards T5 as a result of significantly increased daily water usage. After the biofilter change on day 72, the nitrite increased for the next 40 days in all RAS, with L-RAS having the highest values[52].



(a)



(b)



(c)

Figure 3.4: Ammonium (a), nitrite (b) and nitrate (c) concentrations from day 0 (08.08.2022) until day 120 (06.12.22) in RAS 4-9. Circles and dotted lines indicate RAS operated at low organic load, whilst triangle and full lines indicate RAS operated at high organic load [52].

3.3 Microbial Community Analysis

To characterize the microbial communities present in biofilm carriers and water samples of the recirculating aquaculture systems, a comprehensive microbial community analysis using Illumina sequencing of the V3-V4 region of the bacterial 16S rRNA gene was conducted. To assess potential variations in community profiles among different sample types, sampling times, and water salinities (freshwater and brackish water), a Principal Coordinate Analysis (PCoA) based on Bray-Curtis similarities was performed (Figure 3.5). The PCoA plot revealed that the freshwater (red) and brackish water (blue) communities clustered in different areas of the plot, with the coordinate 2 axis clearly separating the two groups. A one-way PERMANOVA test confirmed that the microbiota profiles of the two sample groups were significantly different (p=0.0001). During the freshwater phase, the biofilm carrier microbiota exhibited significant differences from those of the water samples (one-way PERMANOVA: p=0.0001), as also indicated in the PCoA ordination where most of the biofilm carrier microbiota clustered separately from the water samples. Notably, the T0 biofilm carrier samples displayed significantly different microbiota apart from the remaining samples in a one-way PERMANOVA test (p=0.0001), where the remaining FW biofilm samples formed a distinct cluster apart from the T0 biofilm carrier samples in the PCoA plot. In contrast, during the brackish water phase, samples from the same sampling time tended to cluster together in the PCoA ordination regardless of the sample type, indicating temporal changes in the microbial communities. A one-way PERMANOVA test showed a significant difference in microbiota between each sampling time in T5-T7 for all three sample types(p=0.0001).

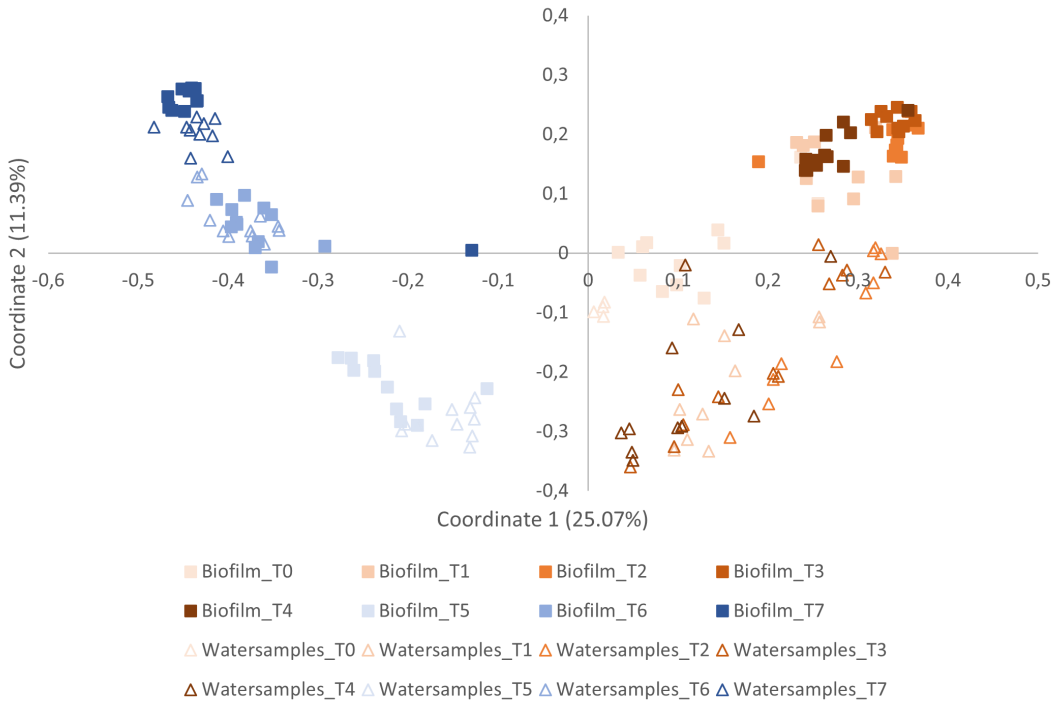


Figure 3.5: PCoA ordination based on Bray-Curtis similarities of microbial community profiles from biofilm carriers and water samples from RAS 4-9 during a time period of 121 days. All samples colored blue are from the brackish water phase, whilst all samples colored red are from the freshwater phase. The color gradient indicates time, with weak colors indicating the early stages and darker colors indicating the later stages. The biofilm carrier samples are all filled squares and the water samples are outlined triangles.

3.3.1 Temporal variations in microbial community composition

Analysis of the Bray-Curtis dissimilarities was used to explore the temporal development of the biofilm and water communities (Figure 3.6). The microbial communities in water samples exhibited lower compositional variation over time than those in biofilm samples. The microbial communities in both the biofilm carriers and the water samples demonstrated similar temporal change in microbial community composition. Notably, a difference in the microbiota was observed when assessing the Bray-Curtis dissimilarity of T0 compared to the freshwater stages (T1-T4) and the brackish water stages (T5-T7). Furthermore, within each sampling time, considerable Bray-Curtis dissimilarities were observed within the microbial communities in both biofilm and water samples, underlining the heterogeneity within the microbial communities.

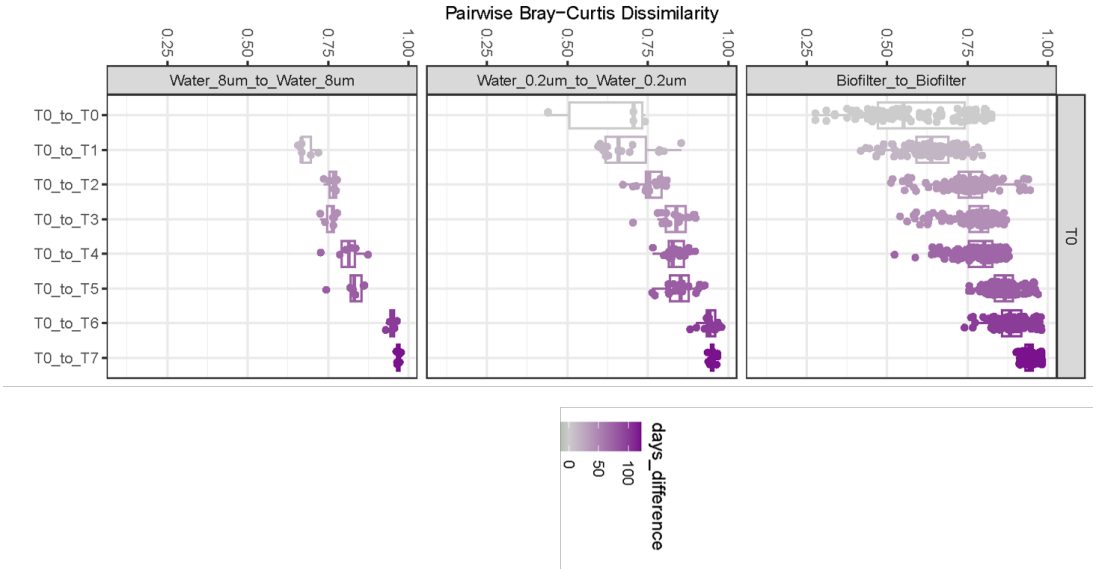


Figure 3.6: Pairwise Bray-Curtis dissimilarity analysis results for biofilm and water samples between sampling time T0 and the remaining seven different sampling times (T1-T7). The color in the figure represents the time difference, with darker colors indicating later stages and lighter purple colors indicating earlier stages. The y-axis represents the sampling times (T0-T7) and which sampling times have been compared, while the x-axis represents the pairwise dissimilarity. Each point in the graph corresponds to the Bray-Curtis dissimilarity value for a specific pair of samples.

An analysis was conducted on the biofilm and water samples, employing pairwise Bray-Curtis dissimilarity to examine the changes in microbial community composition between consecutive sampling times (Figure 3.7). The analysis revealed substantial variations in microbial composition over time. The dissimilarity between sampling times T2 and T3 reached its lowest level across all sample types, with biofilm samples exhibiting pairwise Bray-Curtis dissimilarity as low as 0.25. As anticipated, the most pronounced differences were observed in the final sampling times, corresponding to the transition of the RAS from freshwater to brackish water. However, there are also large differences within the three final sampling times, where no major conditional changes occur.

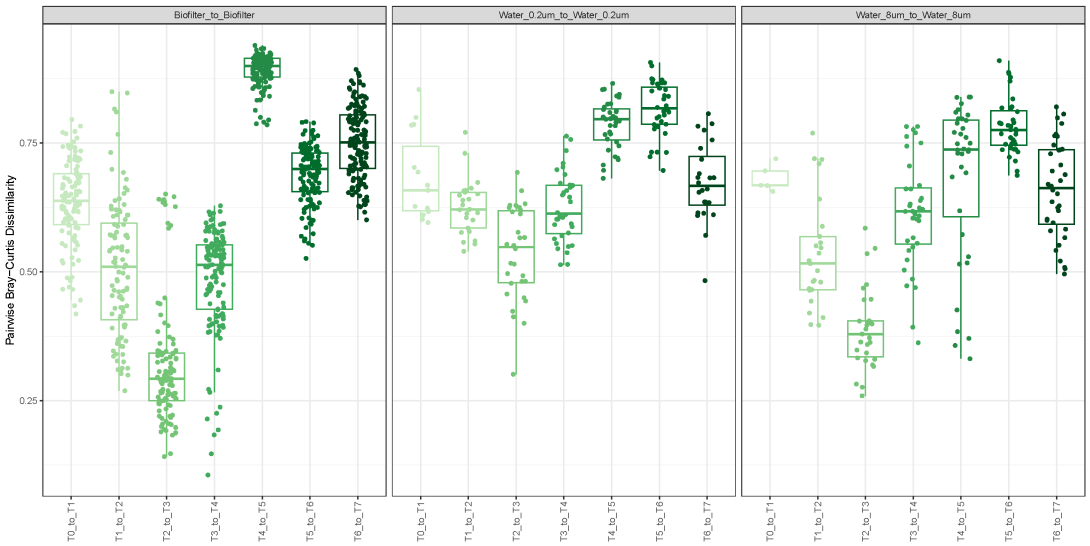


Figure 3.7: Microbial community composition of one sample time compared to the following sample time based on pairwise Bray-Curtis dissimilarity analysis results for biofilm and water samples. The color in the figure represents the time difference, with darker green colors indicating later stages. The x-axis represents the sampling times (T0-T7) and which sampling times have been compared, while the y-axis represents the pairwise dissimilarity. Each point in the graph corresponds to the Bray-Curtis dissimilarity value for a specific pair of samples.

A comparative analysis based on pairwise Bray-Curtis dissimilarity was conducted between different sample types to assess their dissimilarities (Figure 3.8). The biofilm carrier samples exhibited substantial dissimilarities when compared to the 0.2 µm water samples, with dissimilarity ranging from 0.7 to 0.8. However, in T7, the Bray-Curtis dissimilarity was lower between biofilm and water samples, with a Bray-Curtis dissimilarity of approximately 0.55, indicating more similar microbial communities throughout the system. When comparing the 8 µm filtered water samples with the biofilm, notable temporal differences were still observed. However, the microbial communities at sampling times T3 and T7 displayed Bray-Curtis dissimilarity values of around 0.50, indicating more similar microbial communities. The two water communities also had considerable Bray-Curtis dissimilarities at each sampling time, with T7 being the only instance where the Bray-Curtis dissimilarity dropped below 0.5, indicating a higher level of similarity in microbial composition than all the previous sampling times.

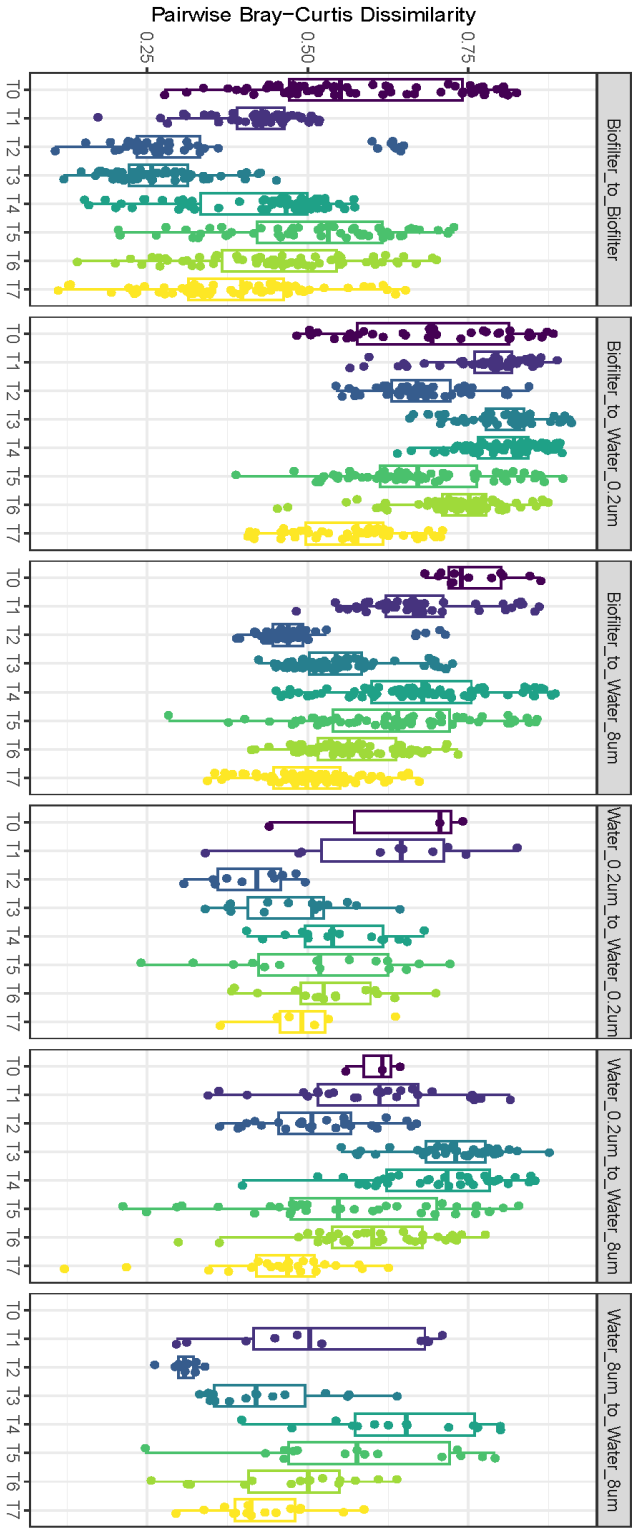


Figure 3.8: Pairwise Bray-Curtis dissimilarity within and between sample types at each sampling time, with samples collected at eight different sampling times (T0-T7). The x-axis represents the sampling times (T0-T7) and is visualized with different colors, while the y-axis represents the pairwise dissimilarity. Each point corresponds to the Bray-Curtis dissimilarity value for a specific pair of samples.

3.3.2 Microbial communities in RAS water

The taxonomic community compositions for water samples obtained from the three H-RAS and the three L-RAS were analyzed at the order level (Figure 3.9). Overall, the community compositions showed similarities among samples from different RAS units at each sampling time; however, notable variations in composition were observed over time, especially after the biofilter change (T5). The dominant orders in the water samples were *Rhodobacterales*, *Flavobacteriales*, *Burkholderiales* and *Cytophagales*. A substantial proportion of the community consisted ASVs unclassified at the order level, making them the third most abundant group. The orders *Nitrospirales* and *Nitrosomonadales*, representing nitrifying bacteria, were also detected in the water samples, albeit at relatively low average abundances of 0.2% and 0.03%, respectively.

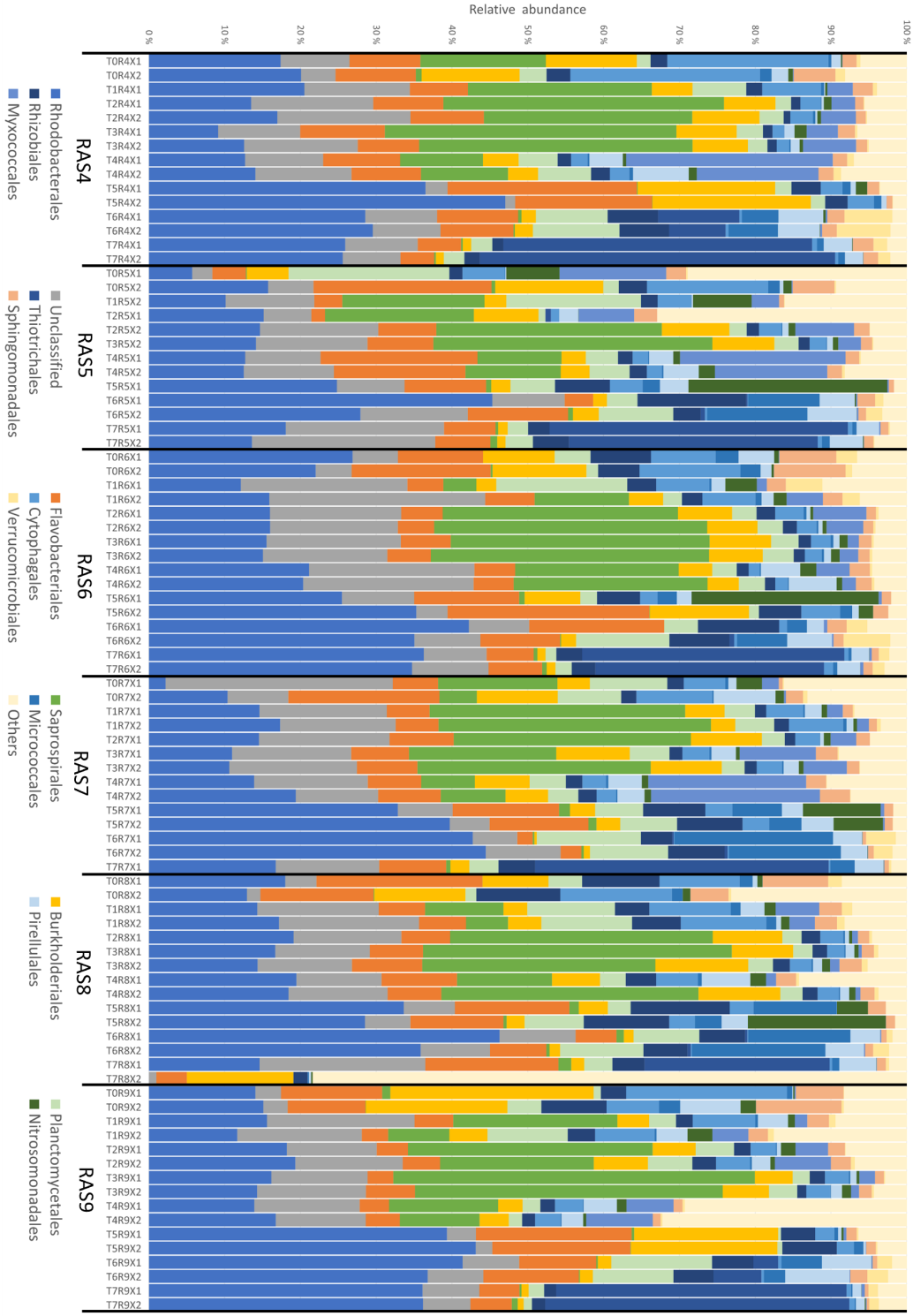


Figure 3.9: Microbial community composition at order level for water samples from RAS 4-9. Orders with an average relative abundance of less than 0.1% in all samples are included in "Other". RAS4, RAS6 and RAS9 were operated at high organic load, whilst RAS5, RAS7 and RAS8 were operated at low organic load. Each sample is named with a sampling time (T), RAS unit (R) and sample type Y (0.2 µm filtrated water sample) or Z (8 µm filtrated water sample).

Freshwater suspended microbiota

To investigate potential dissimilarities in the microbial community composition among freshwater samples collected from H-RAS and L-RAS at different sampling times, a PCoA ordination based on Bray-Curtis similarities was performed (Figure 3.10). The PCoA ordination revealed that the microbiota in the water samples collected at T0 and T1 clustered apart from the remaining sampling times. Statistical analysis using one-way PERMANOVA test ($p = 0.0007$) confirmed the presence of significant dissimilarities in the microbiota between T0 and T1 in comparison to the other sampling times. These dissimilarities suggest temporal variations in the microbial community composition during the initial stages of the experiment. The PCoA ordination does not indicate any observable differences between the H-RAS and L-RAS freshwater microbiota.

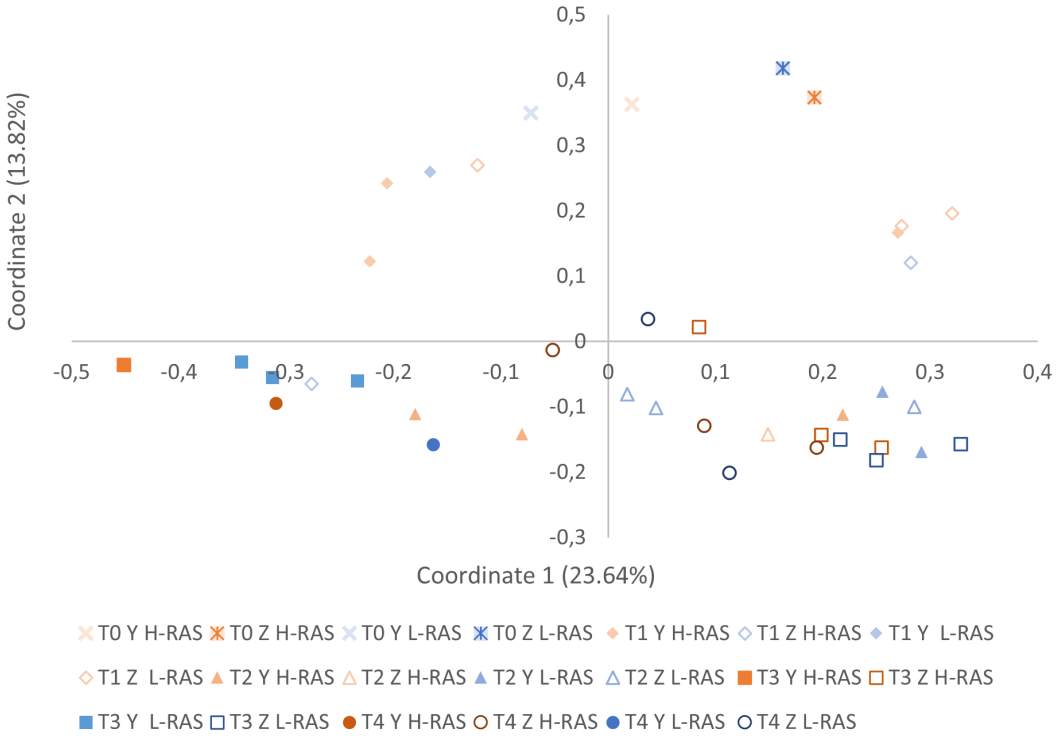


Figure 3.10: PCoA ordination based on Bray-Curtis similarities of microbial community profiles from freshwater samples from RAS 4-9 including sampling times T0-T4. The samples are sorted by time, TSS and sample type. Different symbols indicate different sampling times, symbols with fill indicate 0.2 µm filtered water samples (Y) and symbols without fill indicate 8 µm filtered water samples (Z). All samples colored blue are from L-RAS, whilst all samples colored red are from H-RAS. The color gradient indicates time, with weak colors indicating the early stages and darker colors indicating the later stages.

In order to identify the ASVs that contributed the most to the dissimilarities be-

tween H-RAS and L-RAS freshwater-suspended microbiota, a SIMPER analysis based on Bray-Curtis similarities was employed. The analysis revealed that a cumulative contribution of nearly 30% was attributed to ten ASVs (Table A.1). ASV2, representing the genus *Haliscomenobacter*, emerged as the most influential ASV, singularly accounting for 3.86% of the dissimilarity. Interestingly, the average relative abundance of ASV2 was found to be higher in L-RAS compared to H-RAS. ASV8 (*Arcicella*) ranked as the second most influential ASV, making a cumulative contribution of 3.81% and exhibiting the highest relative abundance in L-RAS. Conversely, ASV20 (*Comamonadaceae*) ranked as the fourth most influential ASV, with three times as high relative abundance in H-RAS compared to L-RAS. ASV42 (*Parcubacteria genera incertae sedis*), was on the other hand, approximately equally abundant in L-RAS and H-RAS (Table A.1).

Brackish water suspended microbiota

During the later sampling times and the brackish water phase, differences in the water microbiota between H-RAS and L-RAS became evident in all sampling times (Figure 3.11), and a one-way PERMANOVA test ($p<0.0128$) confirmed that the differences were significant. At T5, directly after the change of the biofilters, the microbial communities in the water samples H-RAS and L-RAS exhibited a relatively similar composition. Conversely, at T6 and T7, clear differences in microbial community composition between H-RAS and L-RAS were observed, with distinct clustering patterns emerging (Figure 3.11). A one-way PERMANOVA test also confirmed significant differences between sampling times ($p=0.0003$).

In order to assess the ASVs that contributed the most to dissimilarities between the microbial communities between H-RAS and L-RAS in brackish water-suspended microbiota, a SIMPER analysis based on Bray-Curtis similarities was performed. Interestingly, ASV6 (*Rhodobacteraceae*) emerged as the sole ASV that contributed to one of the biggest dissimilarities in microbiota between H-RAS and L-RAS in both freshwater and brackish water conditions (Table A.2). In brackish water, ASV5 (*Leucothrix*) exhibited the highest contribution, accounting for 6.12% of the dissimilarity between H-RAS and L-RAS. Notably, ASV5 was found to be twice as abundant in H-RAS compared to L-RAS. ASV10 (*Rhodobacteraceae*) and ASV24 (*Saprospirales*) ranked as the second and third most influential ASVs, respectively, with both being more abundant in L-RAS, which also were clearly more abundant in L-RAS. On the other hand, ASV21 (*Rhodobacteraceae*), ASV6 (*Rhodobacteraceae*), ASV47 (*Flavobacterium*) and ASV39 (*Leucothrix*) were more abundant in H-RAS (Table A.2).

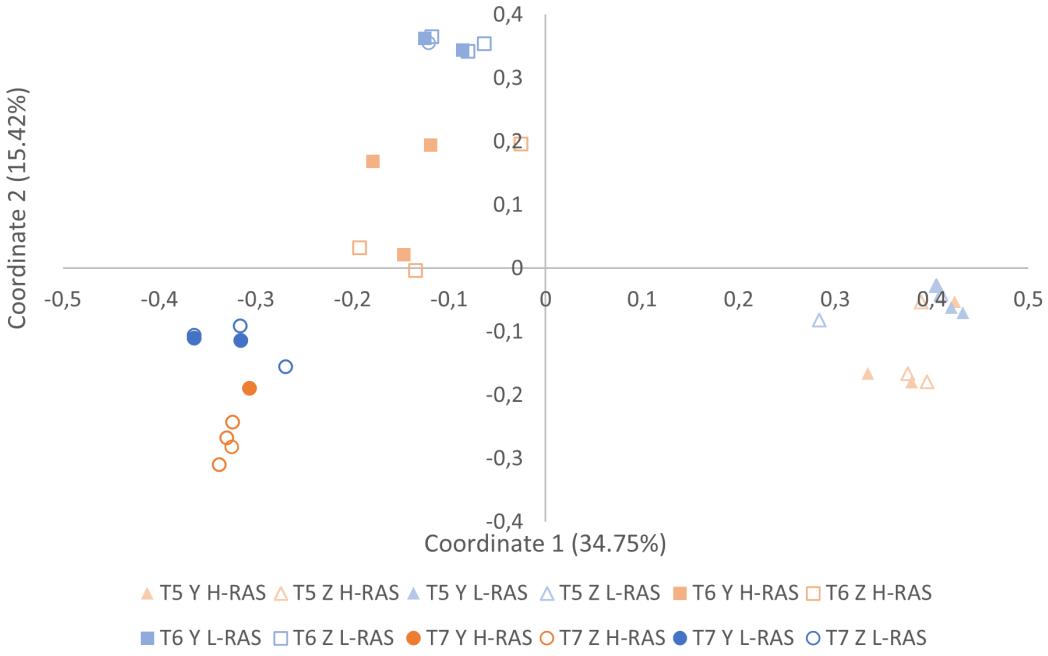


Figure 3.11: PCoA ordination based on Bray-Curtis similarities of microbial community profiles from brackish water samples from RAS 4-9 including the three final sampling times T5-T7. The samples are sorted by time, TSS and sample type. Different symbols indicate different sampling times, symbols with fill indicate 0.2 µm water samples (Y) and symbols without fill indicate 8 µm water samples (Z). All samples colored blue are from L-RAS, whilst all samples colored red are from H-RAS. The color gradient indicates time, with weak colors indicating the early stages and darker colors indicating the later stages.

0.2 µm and 8 µm filter microbial water communities

Throughout the experiment, each water sample was subjected to sequential filtration using an 8 µm filter followed by a subsequent filtration of the effluent using a 0.2 µm filter. To explore the dissimilarities between microbial communities collected on the 0.2 µm and 8 µm filtered water samples, an additional PCoA ordination based on Bray-Curtis similarities was performed (Figure 3.12). In the freshwater phase, differences were observed between the water communities of the 0.2 µm and 8 µm filters, primarily separated by the separation along the coordinate 2 axis, with a few notable outliers. A one-way PERMANOVA test($p = 0.0001$) confirmed significant dissimilarities in the microbiota between 0.2 µm and 8 µm filtered freshwater samples. However, during the T5-T7 period, the two communities displayed greater similarity, and no significant differences were observable between the 0.2 µm and 8 µm filter microbiota (one-way PERMANOVA: $p=0.1074$).

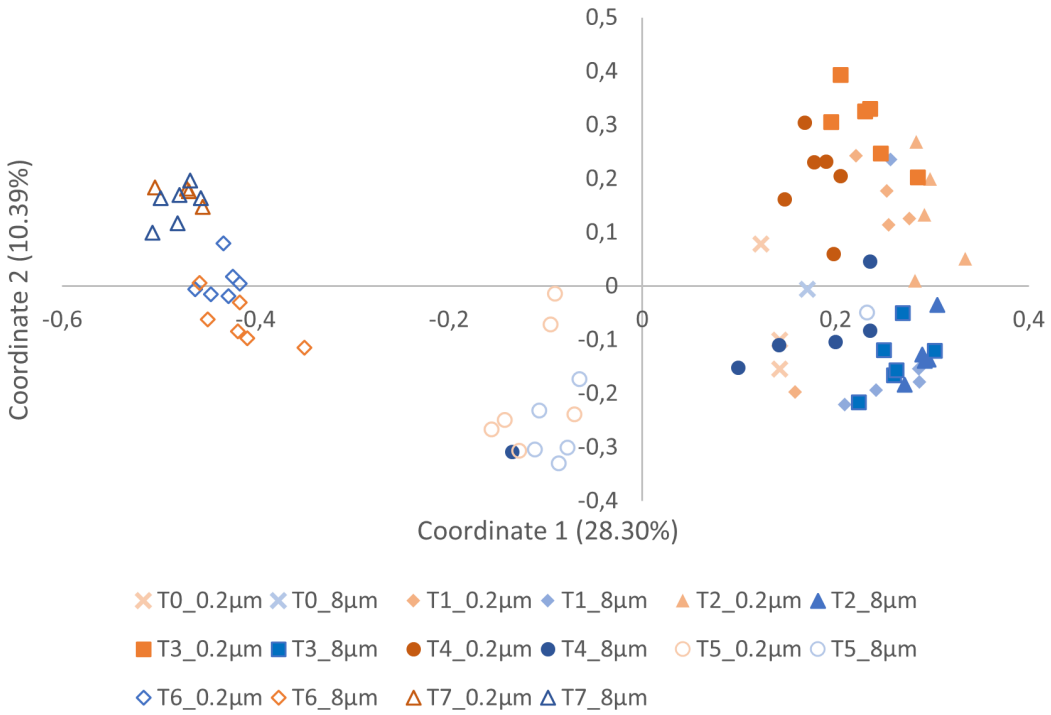
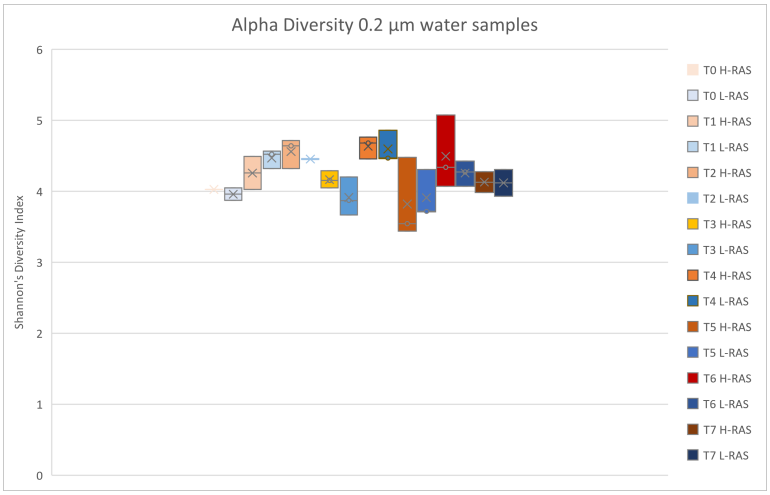
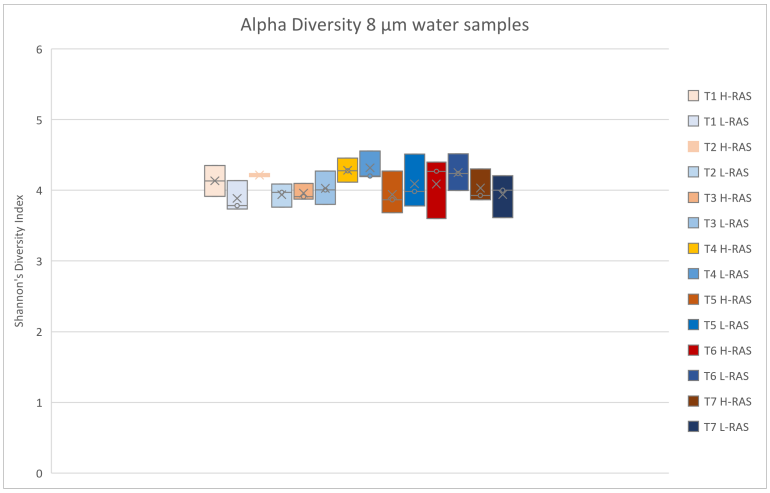


Figure 3.12: PCoA ordination based on Bray-Curtis similarities of microbial community profiles from water samples filtrated with 0.2 µm and 8 µm filters from RAS 4-9 for sampling times T0-T7. The samples are sorted by time and sample type, where different symbols indicate different sampling times. All samples colored blue are from 8 µm water samples, whilst all samples colored red are from 0.2 µm water samples. The color gradient indicates time, with weak colors indicating the early stages and darker colors indicating the later stages.

The alpha diversity for the 0.2 µm filtered water samples exhibited some variation, but no obvious differences in both L-RAS and H-RAS (Figure 3.13a), indicating a fluctuating diversity over time. Conversely, the 8 µm water samples demonstrated relatively stable alpha diversity levels over time (Figure 3.13b).



(a)



(b)

Figure 3.13: Box plot showing Shannon's diversity index for (a) 0.2 µm filter and (b) 8 µm filter water samples. Shannon's diversity index is visualized for all H-RAS and L-RAS at each sample time. The alpha diversity indices were based on the normalized ASV-table and calculated using the Shannon diversity index. All samples colored blue are from L-RAS, whilst all samples colored red are from H-RAS. The color gradient indicates time, with weak colors indicating the early sampling times and darker colors indicating the later sampling times.

A box plot depicting the differential abundance of ASVs contributing most to the difference between the 0.2 µm and the 8 µm water samples at each sampling time (Figure 3.14). Notably, several ASVs exhibited high abundance in both sample types during the freshwater phase (T0-T5) but still contributed to the differences in relative abundance in the two sample types, including ASV13 (*Lacihabitans*) and ASV127 (*Rhodobacteraceae*). Conversely, ASV1288 (*Gammaproteobacteria*) appeared to be exclusively present in the 0.2 µm water samples during the freshwater phase. In the brackish water phase, ASV635 (*Maricaulis*) and ASV811 (*Francisellaceae*) demonstrated higher abundance in the 0.2 µm water samples, while ASV109 (*Mariniblastu*) displayed increased abundance at T6 specifically in the 8 µm water samples.

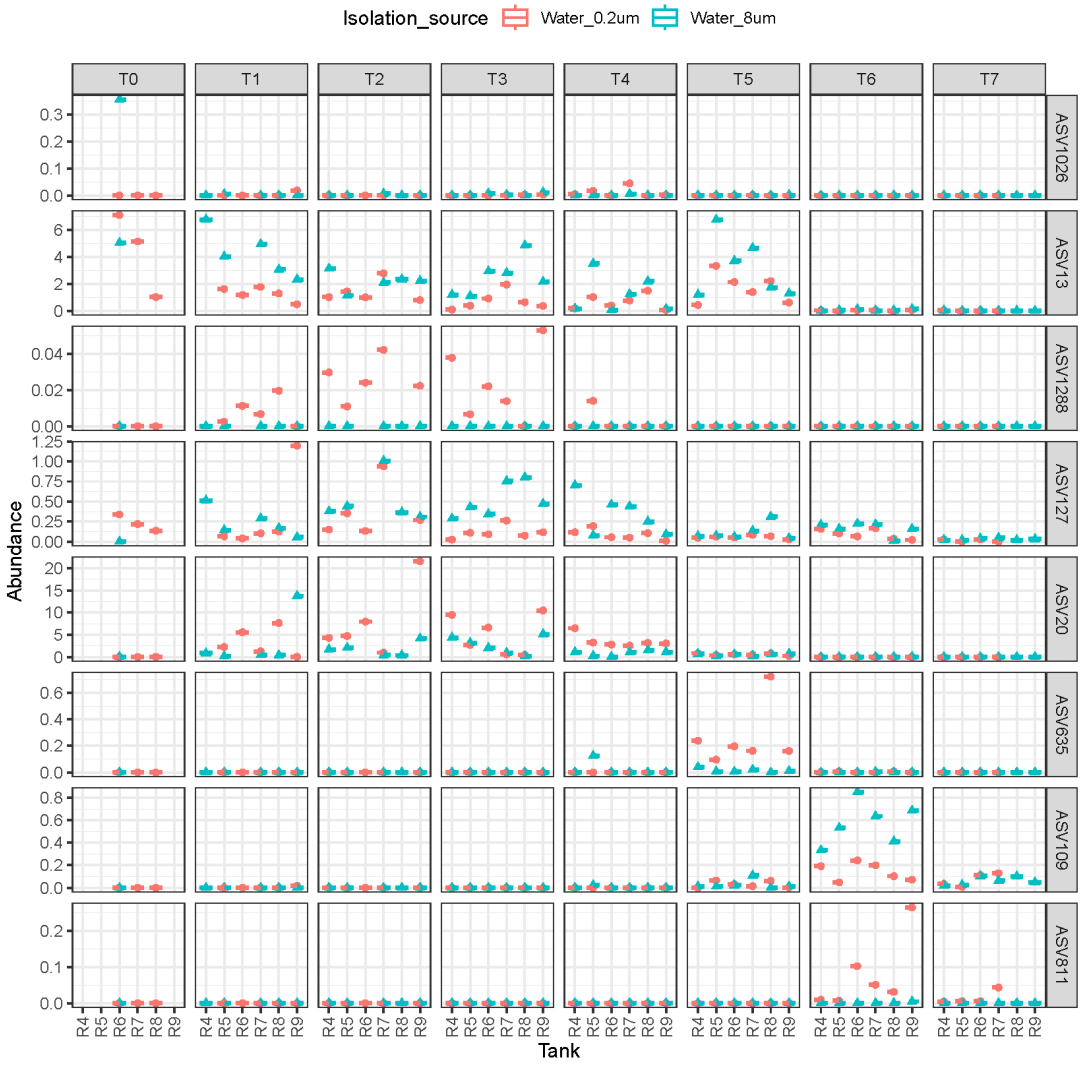


Figure 3.14: Box plot showcasing the ASVs with the lowest p-value with Wilcoxon rank-sum test within between the 0.2 µm (Red) and the 8 µm (Blue) water samples at each sampling time, although not necessarily $p < 0.05$ with FDR multiple testing correction (the diagonal shows the ASV most significantly different at that sampling time). The y-axis is the abundance of the ASVs in percentage. Note that the y-axis scale is different for each ASV. The x-axis represents RAS4-9 where RAS 4, 6 and 9 are operated at high organic load and RAS 5, 7 and 8 are operated at low organic load. The ASVs were classified at the lowest level obtained: ASV1026 = g: *Micrococcus*, ASV13 = g: *Lacihabitans* , ASV1288 = c: *Gammaproteobacteria*, ASV127 = f: *Rhodobacteraceae* , ASV20 = f: *Comamonadaceae*, ASV635 = g: *Maricaulis*, ASV109 = g: *Mariniblastu*, ASV811 = f: *Francisellaceae*.

3.3.3 Microbial communities in biofilm carriers

The composition of microbial communities in biofilm samples from the biofilter exhibited notable variation over time, particularly following the biofilter change at T5 (Figure 3.15). Upon examining the community composition at the order level, *Rhodobacterales*, *Flavobacteriales*, *Saprospirales*, and *Burkholderiales* were identified as the most prevalent orders (Figure 3.7). ASVs unclassified at the order level accounted for the second largest group, representing 11% of the total relative abundance. While the dominant orders exhibited similar levels across most RAS at a given time, significant changes in composition were observed over time. Notably, the nitrite-oxidizing order *Nitrospirales* ranked 18th in terms of relative abundance (0.7%) when sorted by the average relative abundance across all sampling times. The ammonia-oxidizing order *Nitrosomonadales* had an average relative abundance of 2.7% and ranked 12th among the most common orders.

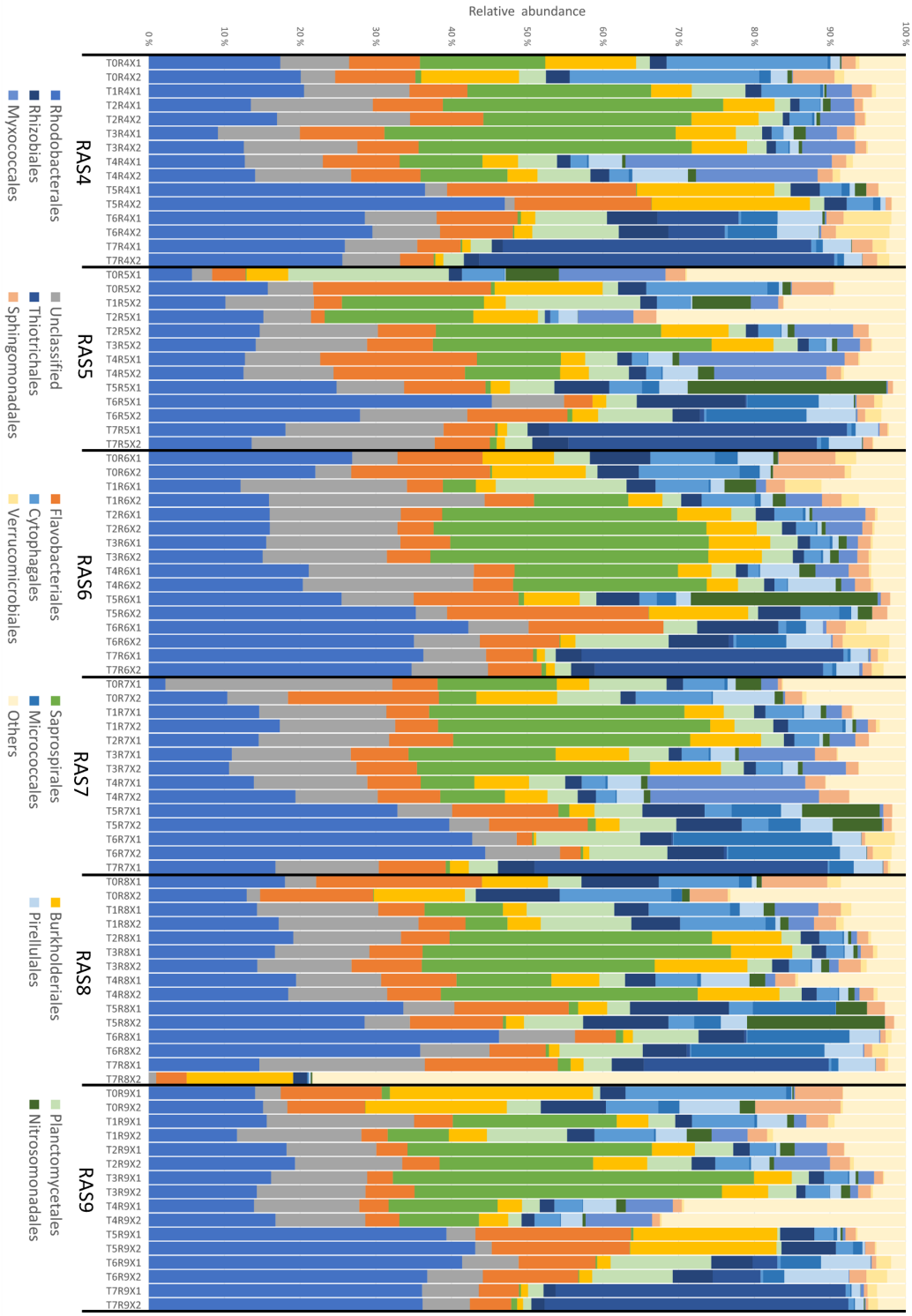


Figure 3.15: Microbial community composition at order level for biofilm carrier samples from RAS 4-9. Orders with an average relative abundance of less than 0.1% in all samples are included in "Other". RAS4, RAS6 and RAS9 were operated at high organic load, whilst RAS5, RAS7 and RAS8 were operated at low organic load. Each sample is named with a sampling time (T), RAS unit (R) and sample type X1 (biofilm) and X2 (biofilm replicate).

Shannon's diversity index was calculated to assess the average diversity across the eight sampling times (T0-T7) for both H-RAS and L-RAS (Figure 3.13). Notably, the alpha diversity in the biofilm carriers displayed certain variations in both L-RAS and H-RAS, suggesting temporal fluctuations in diversity over the course of the study.

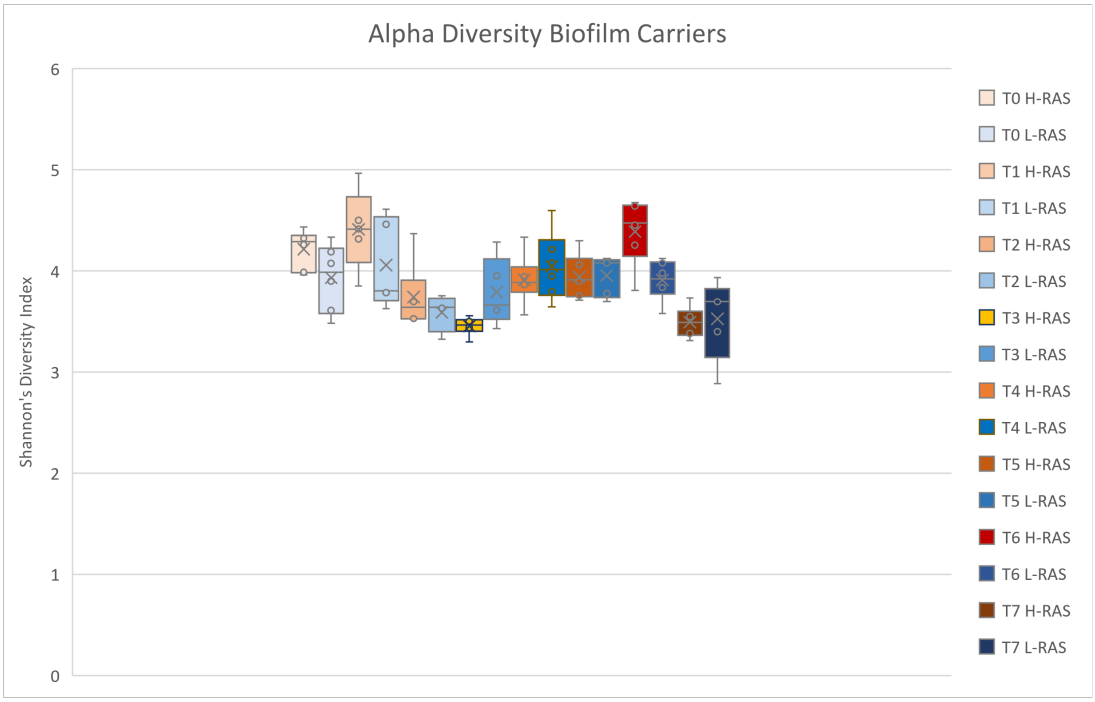


Figure 3.16: Box plot showing Shannon's diversity index for the biofilm carriers. Shannon's diversity index is shown for all H-RAS and L-RAS at each sample time. The alpha diversity indices were based on the normalized ASV-table and calculated using the Shannon diversity index. All samples colored blue are from L-RAS, whilst all samples colored red are from H-RAS. The color gradient indicates time, with weak colors indicating the early sampling times and darker colors indicating the later sampling times.

A box plot depicting the differential abundance of ASVs that contribute to the most pronounced dissimilarities between H-RAS and L-RAS in biofilm carrier samples across various sampling times (Figure 3.17). The relative abundance of ASV649 (*Chitinophagaceae*), ASV13 (*Lacihabitans*), and ASV127 (*Rhodobacteraceae*) are similarly abundant and appear to exhibit temporal variation in both H-RAS and L-RAS. Additionally, ASV364 (*Simplicispira*) is exclusively present in H-RAS, with notably higher abundance in T5. In the brackish water phase (T5-T7), both ASV10 (*Rhodobacteraceae*) and ASV11 (*Pseudorhodobacter*) are observed in both H-RAS and L-RAS, but their abundances vary. Notably, ASV11 (*Pseudorhodobacter*) belongs to the family *Rhodobacteraceae*. Interestingly, ASV2018 (*Leucothrix*) is detected in T7 and present in all three L-RAS.

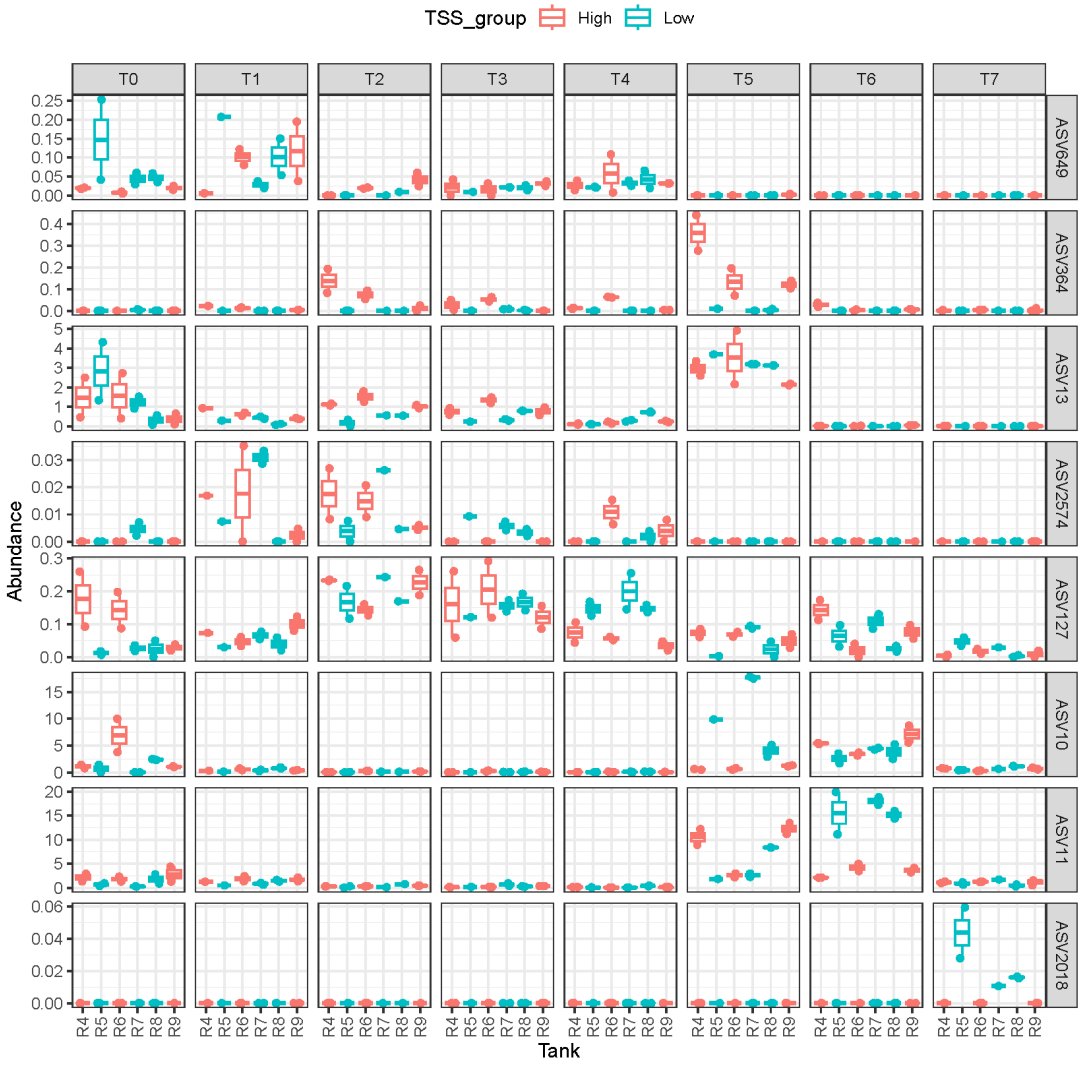


Figure 3.17: Box plot showcasing the ASVs with lowest p-value at each sampling time between RAS operated at high TSS (Red) and RAS operated at low TSS (Blue) from the biofilm carrier samples at each sampling time (the diagonal shows the ASV most significantly different at that sampling time). The y-axis is the abundance of the ASVs in percent. Note that the y-axis scale is different for each ASV. The x-axis represents RAS4-9 where RAS 4, 6 and 9 are operated at high organic load and RAS 5, 7 and 8 are operated at low organic load. The ASVs were classified at the lowest level obtained: ASV649 = f: *Chitinophagaceae*, ASV364 = g: *Simplicispira*, ASV13 = g: *Lacihabitans* , ASV2574 = g: *Flavobacterium*, ASV127 = f: *Rhodobacteraceae* , ASV10 = f: Rhodobacteraceae, ASV11 = g: *Pseudorhodobacter*, ASV2018 = g: *Leucothrix*.

Freshwater biofilm microbiota

In order to assess potential differences in the microbial community composition between biofilm carrier samples from H-RAS and L-RAS, as well as across different sampling times, a PCoA ordination based on Bray-Curtis similarities was employed (Figure 3.18). The objective was to determine any discernible patterns or variations in community structure among the samples. The analysis revealed no significant dissimilarities between H-RAS and L-RAS in terms of microbial community composition (one-way PERMANOVA: $p=0.5055$). However, a significant temporal pattern was observed, indicating noteworthy alterations in community composition over time with a one-way PERMANOVA test ($p<0.0112$) confirming the visual differences in the PCoA ordination.

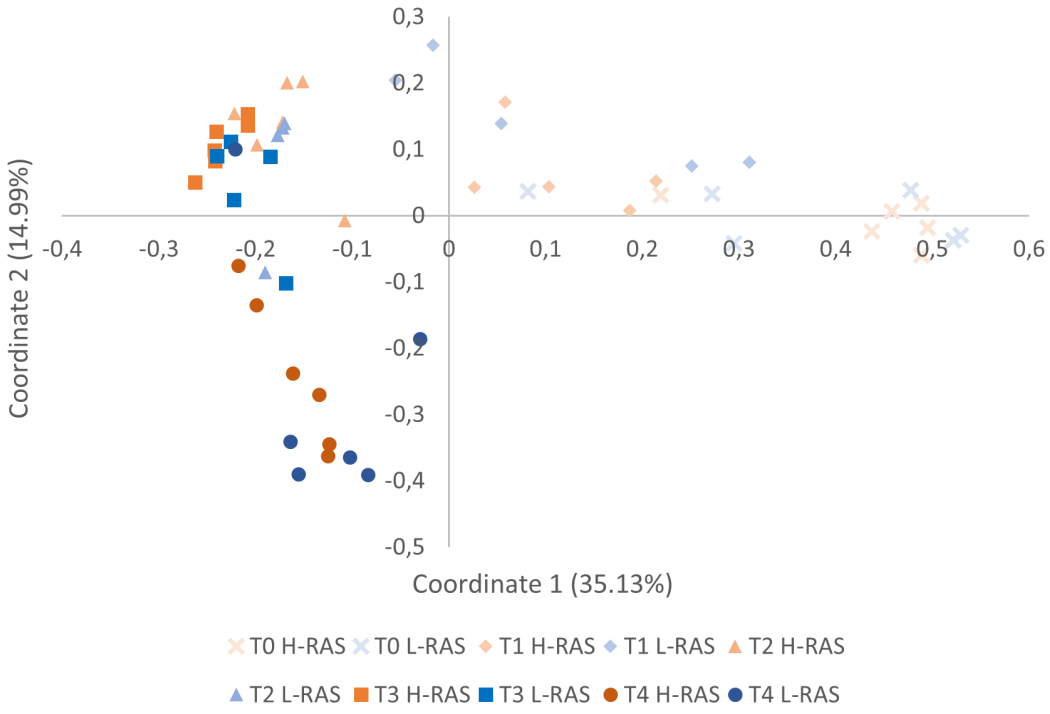


Figure 3.18: PCoA ordination based on Bray-Curtis similarities of microbial community profiles from freshwater biofilm carrier samples from RAS 4-9 including sampling times T0-T4. The samples are sorted by time and TSS, where different symbols indicate different sampling times. All samples colored blue are from L-RAS, whilst all samples colored red are from H-RAS. The color gradient indicates time, with weak colors indicating the early stages and darker colors indicating the later stages.

Brackish biofilm microbiota

Analogous to the observed pattern in the brackish water samples, the biofilm carrier samples exhibit dissimilarities between the H-RAS and L-RAS during the brackish water phase (Figure 3.19). A one-way PERMANOVA test ($p<0.0136$) confirmed that the differences were significant. These disparities highlight the

potential impact of varying salinity levels on the microbial community composition within biofilm carriers. Furthermore, significant dissimilarities were also observed across different sampling times, indicating temporal dynamics and temporal-specific influences on the microbial community structure (one-way PERMANOVA: $p<0.0001$).

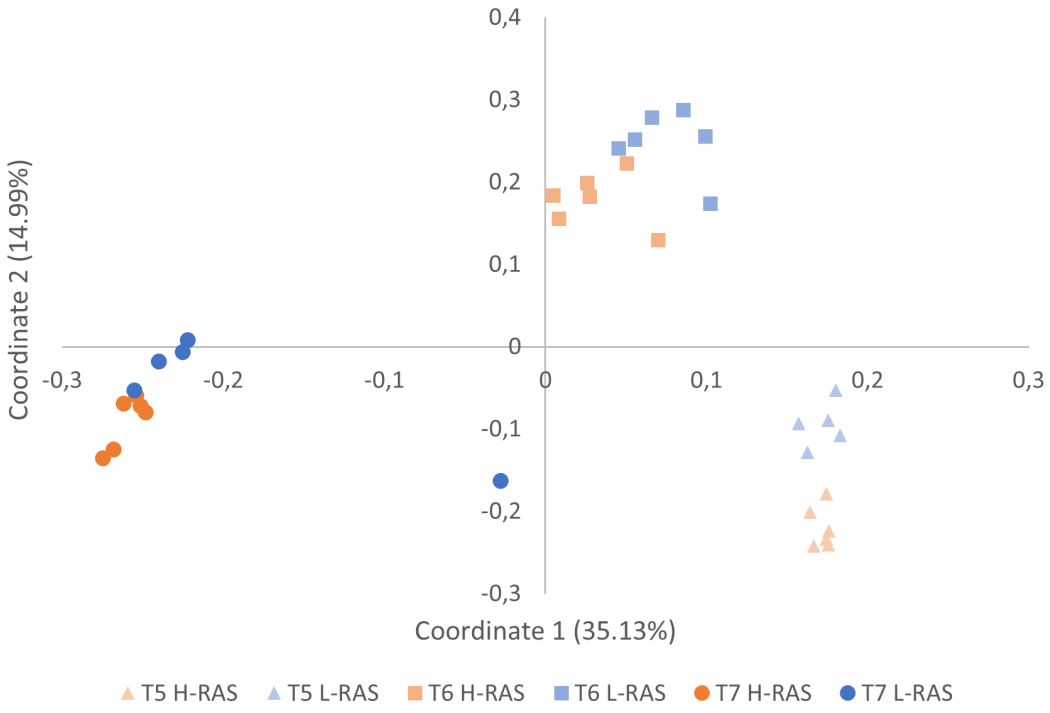


Figure 3.19: PCoA ordination based on Bray-Curtis similarities of microbial community profiles from brackish biofilm carrier samples from RAS 4-9 including the three final sampling times T5-T7. The samples are sorted by time and TSS, where different symbols indicate different sampling times. All samples colored blue are from L-RAS, whilst all samples colored red are from H-RAS. The color gradient indicates time, with weak colors indicating the early stages and darker colors indicating the later stages.

Nitrifiers in the biofilter biofilm

Nitrifying bacteria ASVs were identified through manual examination of the normalized ASV-table. A total of 14 ASVs were determined to represent nitrifying bacteria. The relative abundances of these nitrifying bacterial ASVs exhibited variations throughout the 121-day sampling period in the biofilm samples from the biofilter (Figure 3.20). Among the nitrifiers, the genus *Nitrosomonas*, comprising ammonia-oxidizing bacteria, displayed the highest abundance. Furthermore, the nitrite-oxidizing genus *Nitrospira* emerged as the second most dominant genus among the nitrifiers across the majority of the sampling times.

The most pronounced variations were observed during T5 across the majority of the RAS, coinciding with the transition of the biofilter to brackish water. Notably, ASV 23, belonging to the *Nitrosomonadaceae* family, exhibited an increase

in abundance immediately following the conversion, but its presence diminished rapidly before the subsequent sampling periods. The community composition observed during T5 demonstrated a resemblance to that of the maturation tank (Table 3.3).

Table 3.3: Microbial composition at lowest obtained taxonomy of the brackish water biofilter maturation tank. The lowest obtained taxonomy is given, either at family (f) or phylum (p) level.

ASV ID	**Abundance**
ASV23 f: *Nitrosomonadaceae*	31%
ASV112 p: *Nitrospirae*	2%
ASV55 p: *Nitrospirae*	42%

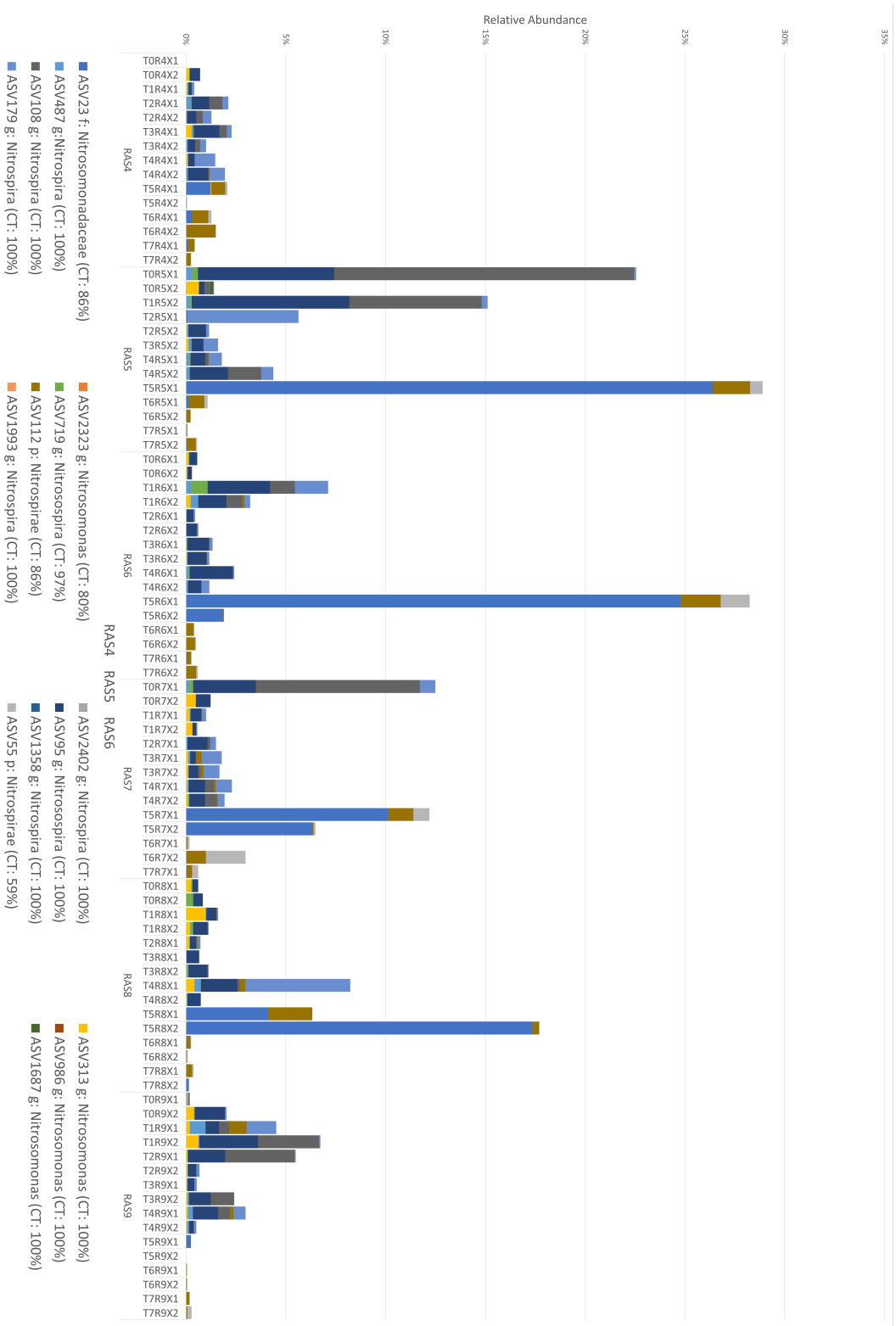


Figure 3.20: Relative abundance of ASVs classified as nitrifiers in each RAS throughout the experiment in the biofilm carrier samples. The lowest obtained taxonomy is given, either at the family (f), genus (g), or phylum (p) level. Each sample is named with a sampling time (T), RAS unit (R) and sample type (X1, X2). CT = confidence threshold.

4 Discussion

4.1 Evaluation of NAxtra^{TM} Fish total nucleic basic extraction kit

PCR amplification of regions of the 16S rRNA gene in Atlantic salmon microbiota samples has posed challenges in previous studies conducted by the "Analysis and Control of Microbial Systems" research group. The presence of contaminating DNA in DNA extraction kits has been reported in previous studies [60] [61], which may pose a challenge if the target DNA concentration is low. Another obstacle is the co-amplification of host DNA due to primer homology with regions of the 18S rRNA gene and mt 12S rRNA gene of Atlantic salmon. When the fraction of bacterial DNA is low, it may be out-competed during amplification. There is also a plausible indication that PCR inhibitors are present in the DNA extracts derived from the fish sample (Personal communication I. Bakke, 12.06.23). Notably, other aspects of the MikroRAS project will investigate the characterization of microbial communities in gut, skin and gill samples obtained from Atlantic salmon, thereby augmenting the requirement for a versatile DNA extraction kit.

To address these issues, the NAxtra^{TM} Fish total nucleic basic extraction kit (Lybe Scientific) was evaluated alongside various PCR protocols to optimize the yield of PCR products from individual gut and skin samples obtained from Atlantic salmon. However, the initial trials of PCR amplification of the V3-V4 regions of the 16S rRNA gene did not result in the expected PCR product. The difficulties encountered are believed to arise from several factors, including the presence of unknown inhibitors in the DNA extracts, low bacterial DNA concentration relative to host DNA in the samples, DNA contamination in the extraction kits and PCR reagents, and co-amplification of salmon rRNA genes when employing universal primers.

To obtain better results another trial was performed with a new lysis buffer and diluted samples, to mitigate the high concentration of Atlantic salmon DNA and to dilute potential PCR inhibitors. This resulted in the appearance of weak bands within the anticipated region for 1/10 and 1/100 diluted skin samples and for 1/10 diluted gut samples. Further experimentation is required to establish the efficacy of these modifications, but the application of the NAxtra kit showed promise as a cost-effective and superior alternative for the research group. The findings also shed light on the efficacy of the newly optimized protocol and lysis buffer in facilitating the PCR amplification of the target region in diverse sample types, including biofilm, water and fish samples.

4.2 Chemical water quality

Throughout the fish experiment at Marineholmen RASLab, the physicochemical parameters remained generally stable in all RAS. The fish were maintained at a temperature of 12.3 $\pm$ 0.8 ℃, which falls within the recommended tempera-

ture range but is slightly below the temperature suggested for optimal growth at 15-16 °C[20]. The pH, which maintained an average of 7.5, consistently remained within the recommended range of 6.5-8 throughout the duration of the experiment.[62][52]. However, the two variables that exhibited differences between the H-RAS and L-RAS were as anticipated turbidity and TSS, with significantly higher TSS levels observed in the H-RAS (Table 3.2). The reintroduction of feces and feed into the rearing tanks, as expected, created an environment with elevated turbidity and TSS levels.

Concentration of nitrogenous compounds

The total ammonia nitrogen (TAN) concentration remained relatively stable in all RAS throughout the duration of the experiment, with minor variations observed (Figure 3.4a). It has been proposed that the TAN removal rate is expected to decrease as the carbon-to-nitrogen (C/N) ratio increases[24]. The observed rise in TAN can be attributed to the rapid growth of heterotrophic bacteria in the outer layers of the biofilm, which out-compete the nitrifying bacteria for oxygen and physical space, particularly when the organic matter concentration is high[24][2]. However, in this experiment, the H-RAS exhibited similar TAN concentrations to L-RAS, suggesting that the biofilter could adjust and function even with higher organic load[52]. Another contributing factor to the stability of the biofilter could be attributed to the early degradation of easily degradable organic matter. Subsequently, the reintroduced organic particles to the system primarily consisted of complex substrates that posed challenges for the majority of heterotrophs in terms of degradation. All RAS experienced a decline in TAN concentration, reaching its lowest point around day 41 following a two-day stop in feeding. Previous studies have shown a direct correlation between reduced feeding and a decrease in TAN levels, as lower protein decomposition leads to a reduced influx of ammonia into the system[63][2].

The nitrite concentrations remained relatively stable across all RAS throughout the experiment (Figure 3.4b). However, during the initial phases of both the freshwater (day 0-20) and brackish water (day 72-92) biofilter, the H-RAS had considerably higher nitrite concentrations compared to the L-RAS. Establishing a functional and stable biofilter typically requires a minimum of 6 weeks[16]. However, in this study, the biomedia matured for 2-4 weeks prior to being introduced into the RAS. There are several potential reasons for the variation in nitrite concentration between the H-RAS and L-RAS. More TAN might be produced in the RAS with high organic matter, and subsequently also more nitrite. Additionally, it appears that the nitrite-oxidizing bacteria (NOB) took longer to establish and achieve optimal efficiency in the H-RAS, potentially due to competition for space and oxygen from heterotrophic bacteria[64]. As a result, the biofilter experienced a decrease in nitrite-oxidizing bacteria abundance, leading to nitrite accumulation for a certain period of time[52].

The three H-RAS exhibited similar trends in nitrate concentration compared to each other, while the three L-RAS demonstrated varying nitrate levels with

several large spikes in nitrate concentration throughout the experiment (Figure 3.4c). In particular, RAS8 had considerably higher nitrate concentrations than all other systems. This discrepancy is likely attributable to RAS8's higher water recirculation rate and correspondingly lower water usage[52]. In RAS, nitrate concentration is regulated through water exchange. Without sufficient water exchange, nitrate accumulates within the system, with RAS8 reaching a maximum concentration of 115 mg/L. A study conducted on post-smolt Atlantic salmon reported no discernible negative effects on the fish when exposed to nitrate concentrations of 100 mg/L NO_3^--N[65], suggesting that the nitrate concentration in this experiment remained within acceptable limits. However, it is possible that the elevated nitrate levels may have had some impact on the salmon during the final 10 days of the experiment. The impact of high nitrate concentrations on Atlantic salmon is not widely studied, but in rainbow trout, high exposure has previously resulted in abnormal swimming behavior and mildly reduced survival[66].

4.3 Analysis of microbial communities

4.3.1 Microbial communities in the freshwater phase compared to the brackish water phase

The microbial communities exhibited significant differences between the freshwater and brackish water phases, as anticipated due to the change of biofilter (Figure 3.5). In the freshwater phase, the biofilm carrier samples differed significantly from the water samples, whereas in the brackish water phase, distinguishing between these sample types was more challenging. Several factors could account for the changes in microbial composition between the sample types under different salinity conditions, including nutrient availability, substrate preference, environmental conditions, and successional changes.

In this experiment, the change of biofilter between the freshwater phase and the brackish water phase had a pronounced impact on the microbial communities. However, it is important to acknowledge that the change in salinity likely exerted an influence on the microbiota throughout the RAS as well, contributing to the observed dissimilarities in microbial community composition between salinities (Figure 3.7). Previous studies have shown that salinity is a critical environmental factor that appears to structure microbial communities in RAS[67]. Changes in salinity could have altered the physicochemical properties of the water, including nutrient availability, dissolved oxygen concentration and ion concentrations. These changes, in turn, could have impacted the growth and metabolism of different microbial taxa in the RAS. Additionally, salinity variations can selectively favor certain microbial populations while inhibiting others, leading to shifts in community composition[68].

4.3.2 Comparison of microbial communities in biofilm carrier samples and water samples

Significant differences were observed between the microbial communities in the biofilm carrier samples and water samples. Generally, the availability of nutrients, and the nutrient composition experienced by bacteria in the systems, will differ between the biofilm community in the biofilter and the microbial community suspended in the water. Biofilm carriers provide surfaces for microbial attachment, allowing certain microorganisms to establish themselves and form unique communities. The water, on the other hand, may have had a different nutrient profile, retention time and lower organic matter content, leading to a distinct microbial composition[69]. Microbes within biofilms often exhibit preferences for specific substrates, which could influence microbial composition. In the freshwater phase, the biofilm carriers might have provided favorable substrates for certain microbial species that are less abundant or absent in the rearing water. This could have led to a divergence in microbial communities between the two sample types[70][71][72].

The order *Rhodobacterales* was consistently the most abundant across all sample types. Previous studies have also *Rhodobacterales* as highly prevalent in both RAS production of Atlantic salmon in brackish water[73] and in seawater RAS for Lumpsucker (*Cyclopterus lumpus*)[74].

Upon comparing the microbial communities in the water samples with those in the biofilm samples, it was observed that the 8 µm filter water samples exhibited higher similarities with the biofilm samples (Figure 3.8). This discrepancy in dissimilarities could be attributed to the retention of larger detached particles from the biofilm on the 8 µm filter, leading to certain shared microbiota between the biofilm and the filtered water samples (Personal communication, I. Bakke, 01.06.23).

4.3.3 Microbial communities in RAS water

Differences in rearing water microbial communities in H-RAS and L-RAS

The microbial communities in the RAS water freshwater phase (T0-T4) show no clear differences between H-RAS and L-RAS (Figure 3.10). However, significant differences between H-RAS and L-RAS became apparent in the brackish water phase (T5-T7)(Figure 3.11). This observation could be explained by the microbiota in the water requiring more time to adapt to the high TSS conditions. The long adaption time could be due to the organic carbon responsible for the TSS differences in the freshwater phase being only present in non-degradable forms. As a result, the bacteria were unable to utilize the carbon, leading to similar microbial composition between H-RAS and L-RAS[75][76][77]. At sampling time T5, the microbial communities in the water samples exhibited a relatively similar composition among all systems, which can be attributed to the recent biofilter

exchange, where all RAS obtained mature biomedia from the same maturation tank, which resulted in a similar distribution of microbial taxa within the both H-RAS and L-RAS.

0.2 µm and 8 µm filter water microbial communities

In the freshwater phase, the microbiota associated with 0.2 µm and 8 µm water samples showed significant differences, but their dissimilarity decreased in the brackish water phase. The most significant contributing ASVs that account for the disparities between the two sample types are primarily observed during the freshwater phase (Figure 3.14). Although *Rhodobacteraceae* was the most abundant order in the water samples, it also played a substantial role in the differences between the 0.2 µm and 8 µm filter water microbial communities. *Rhodobacteraceae* was commonly found in both sample types throughout the entire 120-day experiment, but its abundance varied considerably between different sampling times, with the early stages having higher abundance in the 0.2 µm water samples and later stages being more abundant in the 8 µm water samples. ASV1288 (*Gammaproteobacteria*) appeared to be highly abundant in the 0.2 µm water samples during the freshwater phase. *Gammaproteobacteria* encompass a diverse group of heterotrophic bacteria, including several genera and species that can potentially infect humans, but also several commensals[78]. Many aquatic animal pathogens are also classified within the Gram-negative *Gammaproteobacteria* class[79]. However, due to the large diversity within this class, it is challenging to determine if these bacteria were indeed negatively impacting the RAS system.

In the brackish water phase, the 0.2 µm filtered water samples at sampling time T5 exhibited around 20% higher abundance than the 8 µm filtered water samples of ASV635, classified as *Maricaulis*, which belongs to the halophilic *Caulobacteria* group known for their significance in carbon cycling[80]. At T6, the 0.2 µm water samples displayed a high abundance of ASV811, classified as *Francisellaceae*. *Francisellaceae* consists of various species that have been extensively studied due to their potential to cause human infections[78]. Furthermore, in the 8 µm filtered water samples, ASV109, classified as *Mariniblastus*, demonstrated increased abundance specifically at T6. *Mariniblastu* belongs to the family *Planctomycetes*, which plays a significant role in global carbon and nitrogen cycles. Many species within this phylum are capable of anaerobic ammonium oxidation, commonly known as anammox[81][82].

In general, the microbial community composition in RAS water was predominantly characterized by the orders *Rhodobacterales*, *Flavobacteriales*, *Burkholderiales* and *Cytophagales*. Among these, the order Flavobacteriales has been frequently associated with healthy aquatic organisms in the water environment but also includes some pathogenic species[83]. It has been reported that *Flavobacteriales* possess specialized abilities in degrading complex organic matter and biopolymers, such as cellulose and chitin. This suggests that members of this bacterial taxa might have a positive impact on improving the quality of rearing water[84][85][86].

4.3.4 Microbial communities in biofilm carriers in H-RAS and L-RAS

Similar to the RAS water, the biofilm carrier samples in the freshwater phase (T0-T4) did not exhibit clear differences between H-RAS and L-RAS (Figure 3.18). However, significant differences between H-RAS and L-RAS biofilter communities became apparent in the brackish water phase (T5-T7)(Figure 3.19). An examination of the ASVs contributing to the major differences between biofilm carrier samples in H-RAS and L-RAS revealed the presence of ASV649 (*Chitinophagaceae*), ASV13 (*Lacihabitans*), and ASV127 (*Rhodobacteraceae*) in both H-RAS and L-RAS, albeit at different sampling times (Figure 3.17). *Chitinophagaceae* is commonly found in freshwater RAS biofilters[87] and has been associated with beneficial effects on the health of mariculture animals by secreting antibiotics and inhibiting the growth of pathogens[88][89].

The highly abundant genus *Lacihabitans* in both H-RAS and L-RAS in T0-T5 represents anaerobic functional bacteria that potentially contribute to antibiotic removal[90]. Anaerobic bacteria may grow in the parts of the biofilm that have less access to oxygen. ASV364 (*Simplicispira*), which is most abundant in H-RAS and present in both freshwater and brackish water phases, has been strongly correlated with electrogenic sulfide oxidation (e-SOx), however, no studies were conducted on sulfide oxidation in this thesis. *Simplicispira* is known to be aerobic and possesses genes related to motility and chemotaxis[91][92]. ASV11 (*Pseudorhodobacter*) exhibits increased abundance in both H-RAS and L-RAS during the brackish water phase. This chemoorganotrophic bacterium has been associated with promoting plant growth and protecting plants against root pathogens[93].

Interestingly, ASV2018 (*Leucothrix*) is detected in T7 and present in all three L-RAS and not in any of the H-RAS. *Leucothrix*, a filamentous bacterium, is very common in seawater systems[94] and proliferates whenever nutrients are present in the water[95]. Infection from *Leucothrix* can hinder embryonic development, cause mortality in eggs or larvae, or contaminate hatched larvae from infected egg masses. Previous studies have reported that reducing the dissolved oxygen concentration from 2 mg/L to approximately 1.5 mg/L led to significant growth of *Leucothrix*[96][97][98]. All three L-RAS showed some reduction in dissolved oxygen leading up to T7, but nothing significant.

The microbial community composition in biofilm carriers of both H-RAS and L-RAS was predominantly characterized by the orders *Rhodobacterales*, *Flavobacteriales*, *Saprospirales*, and *Burkholderiales*. *Saprospirales* exhibited a high abundance primarily in the freshwater phase. Within the *Saprospirales* order, certain members have been key contributors to organic matter degradation and nutrient cycling processes, as evidenced by their genomic characteristics[99]. These bacteria possess the ability to efficiently degrade complex organic compounds, such as cellulose and chitin, which can contribute to the overall breakdown of organic matter in the system[100][101]. This can help maintain water quality by reducing the accumulation of organic waste and preventing potential issues related to

water pollution. Additionally, some *Saprospirales* bacteria have been associated with the removal of toxic compounds from wastewater treatment plants, such as ammonia and nitrite, through their metabolic activities[102].

4.3.5 Nitrifiers in the biofilter biofilm

The biofilm communities of the biofilter in the analyzed system contained 15 distinct ASVs associated with nitrification processes. These ASVs were classified within the genus *Nitrosomonas*, *Nitrospira*, the family *Nitrosomonadaceae*, and the phylum *Nitrospirae*. These nitrifying bacteria are commonly encountered in RAS biofilters[22] and are considered to be the primary contributors to nitrification within RAS biofilters[87]. The abundances of nitrifiers varied throughout the experiment (Figure 3.20). A substantial increase in the abundance of nitrifiers was observed at T5, which coincided with the transition of the biofilter to brackish water conditions. This observation implies that the abundance of nitrifying bacteria in the maturation tank was influenced by the elevated concentration of TAN, which was substantially lower in the experimental RAS. Consequently, the reduced TAN levels in the RAS contributed to the stabilization of nitrifier populations and their associated abundance by the next sampling time.

The freshwater phase encompassed a diverse range of nitrifying bacteria, while the brackish water phase exhibited reduced diversity, primarily characterized by the prevalence of ASVs affiliated with the phylum *Nitrospirae* during T6 and T7. The relatively low abundance of ammonia-oxidizing bacteria detected in the brackish water phase could potentially be attributed to the presence of complete ammonia oxidizers (COMAMMOX)[87]. However, this was not tested or analyzed in this thesis. It is also plausible that ammonia-oxidizing archaea (AOA) might have been present in the biofilm of the biofilter[87] but remained undetected due to the PCR primers not targeting archaea. The nitrifying communities in RAS are highly complex, and the biofilter can harbor a wide array of nitrifying microorganisms, thereby posing challenges for comprehensive analysis.

4.4 Future Work and Perspectives

The investigation of microbial communities in Atlantic salmon RAS has provided valuable insights into the dynamics and responses of these communities to high and low organic load conditions. However, there are several avenues for future research that can further enhance our understanding of the complex interactions between microbial communities and organic load in RAS.

One avenue for further exploration involves investigating the temporal dynamics of microbial communities under different organic load conditions through more extensive and prolonged experiments. By extending the duration of the study, it is possible to obtain a more comprehensive understanding of how these communities evolve and stabilize over time. In the current investigation, notable differences in microbial composition between high and low organic load RAS

emerged only towards the end of the experiment. Conducting a longer experiment would validate these findings and potentially unveil patterns of community succession and identify potential microbial indicators that can serve as markers for the health status of the RAS system.

Exploring alternative methods to introduce increased organic load to the system could be a viable approach. Increasing the feed-loading rate, rather than reintroducing waste, may offer a more straightforward means of introducing easily degradable organic carbon into the system. This, in turn, could potentially induce additional shifts in the microbial community composition, whilst also being relevant to commercial RAS.

In addition to the scope of this thesis, the MikroRAS project aims to investigate and characterize the impact of high and low organic loads on the smoltification process and overall fish development in Atlantic salmon. This will be accomplished through the comprehensive analysis of salmon microbiomes obtained from fecal, skin, and gill samples. Furthermore, the project seeks to examine the influence of organic load on the composition of salmon microbiomes, as well as its implications for mucosal health, welfare and performance, even beyond the initial exposure to marine environments. By conducting such investigations, MikroRAS endeavors to expand our understanding of the complex interplay between organic loads, microbial communities and various physiological parameters in Atlantic salmon aquaculture.

5 Conclusion

This thesis investigated the water and biofilter microbial communities in RAS operated at high and low organic load in freshwater and brackish water.

The main findings of this master's thesis were:

1. The NAxtra^{TM} Fish total nucleic basic extraction kit (Lybe Scientific AS) resulted in the successful extraction of DNA from Atlantic salmon biofilm carriers and water filters from RAS, which was used as a template in a subsequent successful PCR amplification of the V3-V4 region of the 16S rRNA gene. However, it is worth noting that the extracted DNA from gut and skin samples of Atlantic salmon did not yield successful PCR amplification, necessitating further investigation and additional testing to identify potential factors impeding PCR amplification success.

2. Nitrite concentrations in H-RAS were considerably higher than that of L-RAS during the start-up phase of both freshwater and brackish water biofilter, indicating that the biofilters in H-RAS required a longer time to stabilize.

3. The biofilter microbiota differed significantly between H-RAS and L-RAS during the brackish water phase, indicating that the organic load influenced the biofilm microbiota. ASVs classified as *Lacihabitans*, *Rhodobacteraceae*, and *Pseudorhodobacter* were mainly more abundant in H-RAS and contributed most to this difference in biofilter communities.

4. The water-suspended microbiota differed significantly between H-RAS and L-RAS in the brackish water phase. The ASV classified as *Leucothrix* contributed to over 6% of the differences and was twice as abundant in H-RAS compared to L-RAS. On the other hand, the ASVs classified as *Rhodobacteraceae* and *Saprospirales* were more than 5 times as abundant in L-RAS compared to H-RAS and contributed to around 6% of the total differences.

5. The biofilter and water-suspended microbiota were significantly different between the freshwater phase and the brackish water phase. The order *Saprospirales* exhibited high abundance in both the biofilter and water-suspended microbiota during the freshwater stage. On the other hand, *Rhodobacterales* showed a pronounced increase from the freshwater to brackish water phase in both sample types.

6. The 0.2 µm and 8 µm microbial water communities were significantly different in freshwater while more similar in brackish water. The ASVs contributing to the biggest differences were classified as *Lacihabitans* and *Rhodobacteraceae*, which were mainly more common in the 8 µm microbial water communities and *Comamonadaceae* which was more common in the 0.2 µm filter microbial communities.

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Appendices

A SIMPER analysis

The top ten ASVs contributing to the dissimilarity between microbial communities between H-RAS and L-RAS for both freshwater and brackish water suspended microbiota were identified through SIMPER analysis using Bray-Curtis similarities and are summarized in Table A.1 and Table A.2.

Table A.1: The top ten ASVs contributing to the dissimilarity between microbial communities between H-RAS and L-RAS in freshwater-suspended microbiota were identified through SIMPER analysis using Bray-Curtis similarities. The relative mean abundances of these ASVs were calculated by dividing the OTU mean abundance by the average number of normalized reads in each sample. The taxonomy assigned to these ASVs represents the lowest taxonomic level obtained, which can be at the phylum (p), family (f), or genus (g) level.

ASV ID	Taxonomy	Contrib. %	Cumulative %	Mean H-RAS	Mean L-RAS
ASV2	g:Haliscomenobacter	3.86	3.86	312	426
ASV8	g:Arcicella	3.81	7.66	364	507
ASV16	g:Pseudarcobacter	3.59	11.25	268	353
ASV20	f:Comamonadaceae	3.21	14.46	442	146
ASV6	f:Rhodobacteraceae	3.10	17.56	456	355
ASV9	f:Rhodobacteraceae	2.87	20.43	282	307
ASV15	p:Verrucomicrobia	2.08	22.51	169	215
ASV12	p:Bacteroidetes	1.95	24.46	185	230
ASV42	g:Parcubacteria genera incertae sedis	1.91	26.37	163	143
ASV18	g:Chryseobacterium	1.66	28.03	162	247

Table A.2: The top ten ASVs contributing to the dissimilarity between microbial communities between H-RAS and L-RAS in brackish water suspended microbiota were identified through SIMPER analysis using Bray-Curtis similarities. The relative mean abundances of these ASVs were calculated by dividing the OTU mean abundance by the average number of normalized reads in each sample. The taxonomy assigned to these ASVs represents the lowest taxonomic level obtained, which can be at the phylum (p), order (o), family (f), or genus (g) level

ASV ID	Taxonomy	Contrib. %	Cumulative %	Mean H-RAS	Mean L-RAS
ASV5	g:Leucothrix	6.12	6.12	785	386
ASV10	f:Rhodobacteraceae	3.60	9.72	126	517
ASV24	o:Saprospirales	3.23	12.95	61	441
ASV28	p:Proteobacteria	2.95	15.90	236	302
ASV21	f:Rhodobacteraceae	2.83	18.73	345	155
ASV6	f:Rhodobacteraceae	2.61	21.35	319	150
ASV47	g:Flavobacterium	2.60	23.94	321	113
ASV29	g:Flavobacterium	1.83	25.77	70	219
ASV39	g:Leucothrix	1.82	27.59	236	38.2
ASV41	g:Hydrogenophaga	1.59	29.19	196	113

B Overview of all samples

Table B.1: Overview of all samples with the corresponding sample name, sampling time, RAS tank and sample type used for analysis in this master's thesis. R4, R5, R6, R7, R8, and R9 are abbreviations for RAS4-9. X1 and X2 are biofilm samples from the biofilter, the Y samples are 0.2 µm filtered water samples and the Z samples are 8 µm filtered water samples.

Sample name	Sampling time	Tank	Sample type
T0R4X1	T0	R4	X1
T0R4X2	T0	R4	X2
T0R5X1	T0	R5	X1
T0R5X2	T0	R5	X2
T0R6X1	T0	R6	X1
T0R6X2	T0	R6	X2
T0R7X1	T0	R7	X1
T0R7X2	T0	R7	X2
T0R8X1	T0	R8	X1
T0R8X2	T0	R8	X2
T0R9X1	T0	R9	X1
T0R9X2	T0	R9	X2
T1R4X1	T1	R4	X1
T1R5X2	T1	R5	X2
T1R6X1	T1	R6	X1
T1R6X2	T1	R6	X2
T1R7X1	T1	R7	X1
T1R7X2	T1	R7	X2
T1R8X1	T1	R8	X1
T1R8X2	T1	R8	X2
T1R9X1	T1	R9	X1
T1R9X2	T1	R9	X2
T4R4X1	T4	R4	X1

Continued on next page

Table B.1: Overview of all samples with the corresponding sample name, sampling time, RAS tank and sample type used for analysis in this master's thesis. R4, R5, R6, R7, R8, and R9 are abbreviations for RAS4-9. X1 and X2 are biofilm samples from the biofilter, the Y samples are 0.2 µm filtered water samples and the Z samples are 8 µm filtered water samples. (Continued)

Sample name	Sampling time	Tank	Sample type
T4R4X2	T4	R4	X2
T4R5X1	T4	R5	X1
T4R5X2	T4	R5	X2
T4R6X1	T4	R6	X1
T4R6X2	T4	R6	X2
T4R7X1	T4	R7	X1
T4R7X2	T4	R7	X2
T4R8X1	T4	R8	X1
T4R8X2	T4	R8	X2
T4R9X1	T4	R9	X1
T4R9X2	T4	R9	X2
T3R4X1	T3	R4	X1
T3R4X2	T3	R4	X2
T3R5X2	T3	R5	X2
T3R6X1	T3	R6	X1
T3R6X2	T3	R6	X2
T3R7X1	T3	R7	X1
T3R7X2	T3	R7	X2
T3R8X1	T3	R8	X1
T3R8X2	T3	R8	X2
T3R9X1	T3	R9	X1
T3R9X2	T3	R9	X2
T5R4X1	T5	R4	X1
T5R4X2	T5	R4	X2
T5R5X1	T5	R5	X1
T5R6X1	T5	R6	X1

Continued on next page

Table B.1: Overview of all samples with the corresponding sample name, sampling time, RAS tank and sample type used for analysis in this master's thesis. R4, R5, R6, R7, R8, and R9 are abbreviations for RAS4-9. X1 and X2 are biofilm samples from the biofilter, the Y samples are 0.2 µm filtered water samples and the Z samples are 8 µm filtered water samples. (Continued)

Sample name	Sampling time	Tank	Sample type
T5R6X2	T5	R6	X2
T5R7X1	T5	R7	X1
T5R7X2	T5	R7	X2
T5R8X1	T5	R8	X1
T5R8X2	T5	R8	X2
T5R9X1	T5	R9	X1
T5R9X2	T5	R9	X2
T6R4X1	T6	R4	X1
T6R4X2	T6	R4	X2
T6R5X1	T6	R5	X1
T6R5X2	T6	R5	X2
T6R6X1	T6	R6	X1
T6R6X2	T6	R6	X2
T6R7X1	T6	R7	X1
T6R7X2	T6	R7	X2
T6R8X1	T6	R8	X1
T6R8X2	T6	R8	X2
T6R9X1	T6	R9	X1
T6R9X2	T6	R9	X2
T7R4X1	T7	R4	X1
T7R4X2	T7	R4	X2
T7R5X1	T7	R5	X1
T7R5X2	T7	R5	X2
T7R6X1	T7	R6	X1
T7R6X2	T7	R6	X2
T7R7X1	T7	R7	X1

Continued on next page

Table B.1: Overview of all samples with the corresponding sample name, sampling time, RAS tank and sample type used for analysis in this master's thesis. R4, R5, R6, R7, R8, and R9 are abbreviations for RAS4-9. X1 and X2 are biofilm samples from the biofilter, the Y samples are 0.2 µm filtered water samples and the Z samples are 8 µm filtered water samples. (Continued)

Sample name	Sampling time	Tank	Sample type
T7R8X1	T7	R8	X1
T7R8X2	T7	R8	X2
T7R9X1	T7	R9	X1
T7R9X2	T7	R9	X2
T8R4X1	T2	R4	X1
T8R4X2	T2	R4	X2
T8R5X1	T2	R5	X1
T8R5X2	T2	R5	X2
T8R6X1	T2	R6	X1
T8R6X2	T2	R6	X2
T8R7X1	T2	R7	X1
T8R8X1	T2	R8	X1
T8R9X1	T2	R9	X1
T8R9X2	T2	R9	X2
T1R4Z	T1	R4	Z
T1R5Y	T1	R5	Y
T1R5Z	T1	R5	Z
T1R6Y	T1	R6	Y
T1R7Y	T1	R7	Y
T1R7Z	T1	R7	Z
T1R8Y	T1	R8	Y
T1R8Z	T1	R8	Z
T1R9Y	T1	R9	Y
T1R9Z	T1	R9	Z
T2R4Y	T2	R4	Y
T2R4Z	T2	R4	Z

Continued on next page

Table B.1: Overview of all samples with the corresponding sample name, sampling time, RAS tank and sample type used for analysis in this master's thesis. R4, R5, R6, R7, R8, and R9 are abbreviations for RAS4-9. X1 and X2 are biofilm samples from the biofilter, the Y samples are 0.2 µm filtered water samples and the Z samples are 8 µm filtered water samples. (Continued)

Sample name	Sampling time	Tank	Sample type
T2R5Y	T2	R5	Y
T2R5Z	T2	R5	Z
T2R6Y	T2	R6	Y
T2R7Y	T2	R7	Y
T2R7Z	T2	R7	Z
T2R8Z	T2	R8	Z
T2R9Y	T2	R9	Y
T2R9Z	T2	R9	Z
T3R4Y	T3	R4	Y
T3R4Z	T3	R4	Z
T3R5Y	T3	R5	Y
T3R5Z	T3	R5	Z
T3R6Y	T3	R6	Y
T3R6Z	T3	R6	Z
T3R7Y	T3	R7	Y
T3R7Z	T3	R7	Z
T3R8Y	T3	R8	Y
T3R8Z	T3	R8	Z
T3R9Y	T3	R9	Y
T3R9Z	T3	R9	Z
T4R4Y	T4	R4	Y
T4R4Z	T4	R4	Z
T4R5Y	T4	R5	Y
T4R5Z	T4	R5	Z
T4R6Y	T4	R6	Y
T4R6Z	T4	R6	Z

Continued on next page

Table B.1: Overview of all samples with the corresponding sample name, sampling time, RAS tank and sample type used for analysis in this master's thesis. R4, R5, R6, R7, R8, and R9 are abbreviations for RAS4-9. X1 and X2 are biofilm samples from the biofilter, the Y samples are 0.2 µm filtered water samples and the Z samples are 8 µm filtered water samples. (Continued)

Sample name	Sampling time	Tank	Sample type
T4R7Y	T4	R7	Y
T4R7Z	T4	R7	Z
T4R8Y	T4	R8	Y
T4R8Z	T4	R8	Z
T4R9Y	T4	R9	Y
T4R9Z	T4	R9	Z
T5R4Y	T5	R4	Y
T5R4Z	T5	R4	Z
T5R5Y	T5	R5	Y
T5R5Z	T5	R5	Z
T5R6Y	T5	R6	Y
T5R6Z	T5	R6	Z
T5R7Y	T5	R7	Y
T5R7Z	T5	R7	Z
T5R8Y	T5	R8	Y
T5R8Z	T5	R8	Z
T5R9Y	T5	R9	Y
T5R9Z	T5	R9	Z
T6R4Y	T6	R4	Y
T6R4Z	T6	R4	Z
T6R5Y	T6	R5	Y
T6R5Z	T6	R5	Z
T6R6Y	T6	R6	Y
T6R6Z	T6	R6	Z
T6R7Y	T6	R7	Y
T6R7Z	T6	R7	Z

Continued on next page

Table B.1: Overview of all samples with the corresponding sample name, sampling time, RAS tank and sample type used for analysis in this master's thesis. R4, R5, R6, R7, R8, and R9 are abbreviations for RAS4-9. X1 and X2 are biofilm samples from the biofilter, the Y samples are 0.2 µm filtered water samples and the Z samples are 8 µm filtered water samples. (Continued)

Sample name	Sampling time	Tank	Sample type
T6R8Y	T6	R8	Y
T6R8Z	T6	R8	Z
T6R9Y	T6	R9	Y
T6R9Z	T6	R9	Z
T7R4Y	T7	R4	Y
T7R4Z	T7	R4	Z
T7R5Y	T7	R5	Y
T7R5Z	T7	R5	Z
T7R6Y	T7	R6	Y
T7R6Z	T7	R6	Z
T7R7Y	T7	R7	Y
T7R7Z	T7	R7	Z
T7R8Z	T7	R8	Z
T7R9Z	T7	R9	Z
M2	-	M2	X
T0R6Y	T0	R6	Y
T0R6Z	T0	R6	Z
T0R7Y	T0	R7	Y
T0R8Y	T0	R8	Y
T7R8X2	T7	R8	X2
T7R9X1	T7	R9	X1

C Protocol NAxtra^{TM} Fish total nucleic basic extraction kit

Lybe Scientific AS

Erling Skjalgssons gate 1
7030 Trondheim

Preliminary NAxtra™ Protocol for fish tissue.

1. Put the tissue sample ~2x2x2 mm in a homogenization tube containing homogenization beads (I have used 10-15 beads 1,4 per tube)
2. Add **315 µL** NAxtra Lysis Buffer (TEST BUFFER) and 5 µL Proteinase K (20 mg/mL).
3. Disrupt the tissue in Homogenizer at hand til tissue is fully disrupted. (I have used the MAGnalyzer at 5000 rpm for 50 sec.)
4. Quick spin down to reduce the foaming.
5. After disruption of tissue and centrifugation put the tubes on heat-block 50-60˚C for 10 min. (I have used 60 ˚C with 1400 rpm heat block with shaking).
6. At the end of incubation remove the tubes from heat-block and add **400 µL** NAxtra™ Beadmix (**380 µL 2-propanol + 20 µL NAxtra™ Magnetic Beads**)
7. Mix bye vortexing and let bind to beads for 5 min.
8. Put tubes on magnet and let sit to liquid is clear and remove supernatant.
9. Add **600 µL** 100% 2-propanol and resuspend beads bye vortexing.
10. Put tubes on magnet and let sit to liquid is clear and remove supernatant.
11. Add **600 µL** 80% EtOH, fully resuspend beads bye vortexing.
12. Put tubes on magnet and let sit to liquid is clear and remove supernatant.
13. Repeat step 10-12.
14. Add **600 µL** 70% EtOH, fully resuspend beads bye vortexing.
15. Put tubes on magnet and let sit to liquid is clear and remove supernatant.
16. Let the bead pellet dry at room temperature for 5 min.
17. Remove tubes from magnet.
18. Add **30-100 µL** preheated (60 ˚C) Elution Buffer (ddH2O can be used, but I found that TE Buffer (10 mM Tris-HCl pH 7,5-8) work best) and fully resuspend bead pellet bye pipetting and vortexing.
19. Put tubes on magnet and let sit to liquid is clear. Move the cleared eluate to a new tube.

On King Fisher FLEX you can run either Total nucleic acid extraction and run the NAxtra_KFF_PostDNase FISH.bdz script.

If you want to treat the samples with DNase you first run the NAxtra_KFF_PreDNase FISH.bdz with 50 µL elution volume, treat the eluate containing magnetic beads with 50 µL DNase solution for 10 min. Then transfer the DNase treated sample with magnetic beads to a new plate and run the NAxtra_KFF_PostDNase FISH.bdz script.

D SequalPrep™ Normalization Plate (96) Kit(Invitrogen)

invitrogen™

SequalPrep™ Normalization Plate (96) Kit

Catalog no: A10510-01 **Store at room temperature (15–30ºC)**

Contents and Storage

The components included with the SequalPrep™ Normalization Plate (96) Kit are listed in the table below. Sufficient reagents are included to perform 10 × 96 purification/normalization reactions. Upon receipt, **store all components at room temperature (15–30°C)**. Store plates for up to 6 months.

Components	Quantity
SequalPrep™ Normalization Plate (96)	2 bags of 5 plates each
SequalPrep™ Normalization Binding Buffer	40 ml
SequalPrep™ Normalization Wash Buffer	50 ml
SequalPrep™ Normalization Elution Buffer (10 mM Tris-HCl, pH 8.5)	40 ml

Description

The SequalPrep™ Normalization Plate Kit allows simple, one-step, high-throughput amplicon purification and normalization of PCR product concentration (2–3 fold range) via a limited binding capacity solid phase. Each well of the SequalPrep™ Normalization Plate can bind and elute ~25 ng of PCR amplicon. Eluted PCR amplicon can be subsequently pooled and subjected to a variety of massively parallel sequencing analyses. The SequalPrep™ Normalization Plate is compatible with any automated liquid handling workstations without the need for shakers, magnets, or vacuum. The SequalPrep™ Normalization Plate Kit when used with SequalPrep™ Long PCR Kit provides a complete PCR enrichment and amplicon normalization system that is designed to complement amplicon sequencing workflows such as next-generation sequencing.

The conventional next generation sequencing workflows require laborious sample prep methods consisting of amplicon purification, quantitation, and manual normalization to adjust amplicon concentration. The SequalPrep™ Normalization Plate Kit eliminates the tedious amplicon quantitation and manual normalization steps.

SequalPrep™ Normalization Plate Kits utilize ChargeSwitch® Technology that provides a switchable surface charge depending on the pH of the surrounding buffer to facilitate nucleic acid purification. Under low pH conditions, the positive surface charge of the ChargeSwitch® coating binds the negatively charged nucleic acid backbone. Proteins and other contaminants (such as short oligonucleotide primers) are not bound and are simply washed away.

System Overview

The SequalPrep™ Normalization Plate Kit is a solid phase, high-throughput amplicon purification and normalization system in a 96-well plate format. PCR products (5–25 µl) are added to a SequalPrep™ Normalization Plate well and mixed with the Binding Buffer. DNA binding to the plate is performed at room temperature for 1 hour. The wells are washed with Wash Buffer to efficiently remove contaminants. Purified PCR products are eluted using 20 µl Elution Buffer at normalized concentrations.

System Specifications

Starting Material:	At least 250 ng PCR product (amplicon) per well
DNA Fragment Size:	100 bp to 20 kb
Elution Volume:	20 µl
DNA Yield:	Up to 25 ng per well
Normalization Range:	2–3-fold
Plate Dimensions:	Standard SBS (Society for Biomolecular Screening) footprint, semi-skirted 96-well plate
Plate Capacity:	0.2 ml

Accessory Products

The following products may be used with the SequalPrep™ Normalization Plate Kit. For details, visit www.invitrogen.com.

Product	Quantity	Catalog no.
SequalPrep™ Normalization Wash Buffer	4 × 50 ml	A10510-03
SequalPrep™ Long PCR Kit with dNTPs	1,000 units	A10498
Platinum® PCR Supermix	100 reactions	11306-016
Platinum® PCR Supermix High Fidelity	100 reactions	12532-016
Quant-iT™ PicoGreen® dsDNA Assay Kit	1 kit	P7589
PureLink™ Foil Tape	50 tapes	12261-012
E-Gel® 96 gels 1% (or 2%)	8 gels	G7008-01 (G7008-02)

Part no: 100003531 Rev. date: 5 May 2008

General Guidelines

- Wear a laboratory coat, disposable gloves, and eye protection when handling reagents and plate.
- Always use proper aseptic techniques when working with DNA and use only sterile, DNase-free tips to prevent DNase contamination.
- If you are using only part of the plate for DNA purification, cover unused wells with the Plate Seal and leave them attached while purifying DNA in the other wells. The plates can be stored at room temperature for up to 6 months.
- The SequalPrep™ Normalization Plates are compatible for use with automated liquid handling workstation; the workstation must be capable of handling and manipulating 96-well plates.
- If you are using automated liquid handling workstations for purification, you may need additional Wash Buffer depending on your type of workstation. See previous page for Wash Buffer ordering information.

Generating PCR Amplicon

You can generate the PCR amplicon using a method of choice. General recommendations for generating PCR amplicons are listed below:

- To obtain the best results, we recommend using the SequalPrep™ Long PCR Kit with dNTPs (page 1) which provides a robust system for long-range, high-fidelity PCR for use in next-generation sequencing applications.
- Other commercially available PCR supermixes and enzymes such as Platinum® PCR Supermix (page 1), Platinum® PCR Supermix High Fidelity (page 1), or equivalent are suitable for use.
- Perform PCR in a separate plate. **Do not** use the SequalPrep™ Normalization Plate to perform PCR.
- You need at least 250 ng amplicon per well to use with the SequalPrep™ Normalization Plate (see below).

Sample Amount

To achieve robust normalization, we recommend adding at least 250 ng/well of amplicon. This input amount is easily achieved using only a fraction of most PCR amplification reactions. An average efficiency PCR (20 µl reaction volume) produces product in the range of 25–100 ng/µl, allowing you to purify 5–10 µl using the SequalPrep™ system.

Elution Options

Depending on the nature of the downstream application and target nucleic acid concentrations desired, the SequalPrep™ kit offers the flexibility to elute purified DNA in a variety of options.

The **standard elution** method described in the protocol below is designed to elute purified DNA from each well using 20 µl elution volume to obtain each amplicon at a concentration of 1–2 ng/µl.

The **optional sequential elution** method is designed to sequentially elute multiple rows or columns using the same 20 µl of elution buffer to obtain higher amplicon concentrations. The amplicon concentrations will be additive as sequential wells are eluted. For example, dispense 20 µl of elution buffer into the first column (A1–H1), mix well, and incubate for 5 minutes at room temperature. Then, simply move this column of elution buffer to the next column (A2–H2), and again incubate for 5 minutes. Continue this step to obtain your specific elution needs for the downstream application of choice.

Materials Needed

- PCR reactions containing amplicons of the desired length (see **Generating PCR Amplicon**, above)
- DNase-free, aerosol barrier pipette tips
- *Optional:* automated liquid handling workstation capable of handling and manipulating 96-well plates
- *Optional:* PureLink™ Foil Tape (see previous page)

Binding Step

1. Transfer the desired volume of PCR product (5–25 µl PCR reaction mix, at least 250 ng amplicon/well) from the PCR plate into the wells of the SequalPrep™ Normalization plate.
2. Add an equivalent volume of SequalPrep™ Normalization Binding Buffer.

   For example: To purify 10 µl of PCR product, add 10 µl SequalPrep™ Normalization Binding Buffer.
3. Mix completely by pipetting up and down, or seal the plate with PureLink™ Foil Tape (page 1), vortex to mix, and briefly centrifuge the plate.
4. Incubate the plate for 1 hour at room temperature to allow binding of DNA to the plate surface. Mixing is not necessary at this stage.

   Note: Incubations longer than 60 minutes do not improve results. However, depending on your workflow you may perform overnight incubation at room temperature for the binding step.
5. **Optional:** If >25 ng DNA/well yield is desired, transfer the amplicon/Binding Buffer mixture from Step 4 to another, fresh well/plate to sequentially bind more DNA. Perform DNA binding at room temperature for 1 hour.

   Note: After binding is complete, you can remove the amplicon/Binding Buffer mixture from the well and store at –20ºC for up to 30 days to perform additional purifications at a later time.
6. Proceed to **Washing Step**, next page.

Washing Step

1. Aspirate the liquid from wells. Be sure not to scrape the well sides during aspiration.

   Note: If you wish to store the amplicon/Binding Buffer mixture for additional purifications at a later time, aspirate the liquid from wells into another plate and store at –20ºC for up to 30 days.

2. Add 50 µl SequalPrep™ Normalization Wash Buffer to the wells. Mix by pipetting up and down twice to improve removal of contaminants.
3. Completely aspirate the buffer from wells and discard.

   To ensure complete removal of wash buffer and maximize elution efficiency, you may need to invert and tap the plate on paper towels depending on the pipetting technique or instrument used. A small amount of residual Wash Buffer (1–3 µl) is typical and does not affect the subsequent elution or downstream applications.

4. Proceed to **Elution Step**, below.

Elution Step

Review **Elution Options** (previous page).

1. Add 20 µl SequalPrep™ Normalization Elution Buffer to each well of the plate.

   Note: Do not use water for elution. If you need to elute in any other buffer, be sure to use a buffer of pH 8.5–9.0. If the pH of the buffer is <8.5, the DNA will not elute efficiently.

2. Mix by pipetting up and down 5 times or seal the plate with PureLink™ Foil Tape (page 1), vortex to mix, and briefly centrifuge the plate. Ensure that the buffer contacts the entire plate coating (up to 20 µl level).
3. Incubate at room temperature for 5 minutes.
4. Transfer and pool the purified DNA as desired or store the eluted DNA at 4°C (short-term storage) or –20°C (long-term storage) until further use.

Expected Yield and Concentration

The expected DNA concentration is 1–2 ng/µl when using 20 µl elution volume. The expected DNA yield is ~25 ng/well normalized.

Optional: DNA Quantitation

The SequalPrep™ Normalization Plate Kit is designed to eliminate the quantitation and manual dilution steps typically performed for normalization in next-generation sequencing workflows. You can pool the eluted amplicon and use the pooled amplicons directly for your downstream applications without DNA quantitation.

However, if your downstream application requires DNA quantitation, you may determine the yield of the eluted amplicon using Quant-iT™ PicoGreen® dsDNA Assay Kit (page 1). We **do not** recommend using UV spectrophotometric measurements (A_{260}/A_{280} nm), as this method is inaccurate for low DNA concentrations.

Downstream Applications

The SequalPrep™ Normalization Plate Kit is designed to produce purified PCR products with normalized concentrations and substantially free of salts and contaminating primers. PCR amplicons purified from this system can be used individually or pooled in any downstream application for which normalization is an important sample preparation criterion such as next generation sequencing applications.

Pooled amplicons purified using the SequalPrep™ Normalization Plate Kit have produced successful data from massively parallel sequencing-by-synthesis on the Illumina/Solexa Genome Analyzer indicating that the amplicon purity is suitable for other next-generation sequencing platforms (Roche/454 FLX, Applied Biosystems SOLiD™ system). For detailed sample preparation guidelines, refer to the instrument manufacturer's recommendations.

Continued on next page

Troubleshooting

Problem	Cause	Solution
Low DNA yield	Insufficient starting material	Be sure to input at least 250 ng amplicon per well for best results.
	PCR conditions not optimal	Check amplicon on gel to verify the PCR product prior to purification. Use SequalPrep™ Long Polymerase (page 2) for best results.
	Incorrect binding conditions	Be sure to add an equivalent volume of SequalPrep™ Normalization Binding Buffer, mix completely, and incubate for 1 hour during the Binding Step.
	Incorrect elution conditions	Use 20 µl SequalPrep™ Normalization Elution Buffer for elution and ensure that the buffer contacts the entire plate coating (up to 20 µl level). **Do not** use any water for elution.
DNA degraded	DNA contaminated with DNase	Follow the guidelines on page 2 to prevent DNase contamination.
Poor normalization	Insufficient starting material	Be sure to input at least 250 ng amplicon per well for best results.
	Inconsistent pipetting or handling	Avoid introducing bubbles while pipetting and do not scratch the plate surface while pipetting. To avoid pipetting inconsistencies, we recommend using automated liquid handling workstations.
	Incorrect binding conditions	Be sure to add an equivalent volume of SequalPrep™ Normalization Binding Buffer, mix completely, and incubate for 1 hour during the Binding Step.
	Too much (>3 µl) wash buffer remaining	Completely remove wash buffer and if needed, invert and tap the plate on paper towels to remove any remaining wash buffer.

Quality Control

The Certificate of Analysis provides quality control information for this product, and is available by product lot number at www.invitrogen.com/cofa. Note that the lot number is printed on the kit box.

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E Amicon Ultra-0.5 Centrifugal Filter Devices User Guide

Millipore®

User Guide

Amicon® Ultra-0.5 Centrifugal Filter Devices

for volumes up to 500 µL

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not for use in diagnostic procedures.

Introduction

Amicon® Ultra-0.5 centrifugal filter devices provide fast ultrafiltration, with the capability for high concentration factors and easy concentrate recovery from dilute and complex sample matrices. The vertical design and available membrane surface area provide fast sample processing, high sample recovery (typically greater than 90% of dilute starting solution), and the capability for 30-fold concentration. Typical processing time is 10 to 30 minutes depending on Molecular Weight Cut off (MWCO). Solute polarization and subsequent fouling of the membrane are minimized by the vertical design, and a physical deadstop in the filter device prevents spinning to dryness and potential sample loss. Efficient recovery of the concentrated sample (retained species) is achieved by a convenient reverse spin step after collecting the filtrate. Amicon® Ultra-0.5 devices are supplied non-sterile and are for single use only.

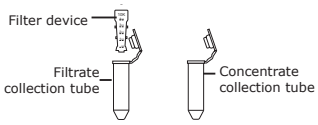
The Amicon® Ultra-0.5 product line includes 5 different cutoffs (Molecular Weight Cut Off, MWCO). These devices are for research use only and not for use in diagnostic procedures.

- Amicon® Ultra 3K device — 3,000 MWCO
- Amicon® Ultra 10K device — 10,000 MWCO
- Amicon® Ultra 30K device — 30,000 MWCO
- Amicon® Ultra 50K device — 50,000 MWCO
- Amicon® Ultra 100K device — 100,000 MWCO

Applications

- Concentration of biological samples containing antigens, antibodies, enzymes, nucleic acids (DNA/RNA samples, either single- or double-stranded), microorganisms, column eluates, and purified samples
- Purification of macromolecular components found in tissue culture extracts and cell lysates; removal of primer, linkers, or molecular labels from a reaction mix, and protein removal prior to HPLC
- Desalting, buffer exchange, or diafiltration

Materials Supplied



The Amicon® Ultra-0.5 device is supplied with two microcentrifuge tubes. During operation, one tube is used to collect filtrate, the other to recover the concentrated sample.

Required Equipment

Centrifuge with fixed angle rotor that can accommodate 1.5 mL microcentrifuge tubes

CAUTION: To avoid damage to the device during centrifugation, check clearance before spinning.

Suitability

Preliminary recovery and retention studies are suggested to ensure suitability for intended use. See the "How to Quantify Recoveries" section.

Device Storage

Store at room temperature.

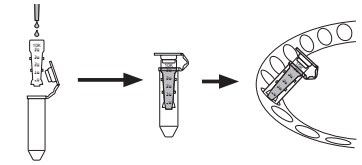
Prerinsing

The ultrafiltration membranes in Amicon® Ultra-0.5 devices contain trace amounts of glycerine. If this material interferes with analysis, pre-rinse the device with buffer or Milli-Q® water. If interference continues, rinse with 0.1 N NaOH followed by a second spin of buffer or Milli-Q® water.

CAUTION: Do not allow the membrane in Amicon® Ultra filter devices to dry out once wet. If you are not using the device immediately after pre-rinsing, leave fluid on the membrane until the device is used.

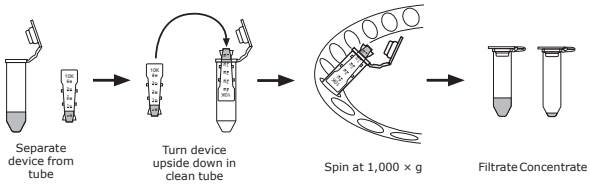
How to Use Amicon® Ultra-0.5 Centrifugal Filter Devices

1. Orient membrane panel facing up.
2. Insert the Amicon® Ultra-0.5 device into one of the provided microcentrifuge tubes.
3. Add up to 500 µL of sample to the Amicon® Ultra filter device and cap it.
4. Place capped filter device into the centrifuge rotor, aligning the cap strap toward the center of the rotor; counterbalance with a similar device.
5. Spin the device at 14,000 × g for approximately 10–30 minutes depending on the MWCO of the device used. Refer to Figure 1 and Table 2 for typical spin times.



6. Remove the assembled device from the centrifuge and separate the Amicon® Ultra filter device from the microcentrifuge tube.
7. To recover the concentrated solute, place the Amicon® Ultra filter device upside down in a clean microcentrifuge tube. Place in centrifuge, aligning open cap towards the center of the rotor; counterbalance with a similar device. Spin for 2 minutes at 1,000 × g to transfer the concentrated sample from the device to the tube. The ultrafiltrate can be stored in the centrifuge tube.

   NOTE: For optimal recovery, perform the reverse spin immediately.



Protocol: QIAquick PCR Purification using a Microcentrifuge

This protocol is designed to purify single- or double-stranded DNA fragments from PCR and other enzymatic reactions using the QIAquick PCR Purification Kit or the QIAquick PCR & Gel Cleanup Kit. For cleanup of other enzymatic reactions, follow the protocol as described for PCR samples or use the MinElute Reaction Cleanup Kit. Fragments ranging from 100 bp to 10 kb can be purified from primers, nucleotides, polymerases and salts using QIAquick spin columns in a microcentrifuge.

Important points before starting

- Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).
- All centrifugation steps are carried out at 17,900 x *g* (13,000 rpm) in a conventional tabletop microcentrifuge at room temperature (15–25°C).
- Add 1:250 volume pH Indicator I to Buffer PB (i.e., add 120 µl pH Indicator I to 30 ml Buffer PB or add 600 µl pH Indicator I to 150 ml Buffer PB). The yellow color of Buffer PB with pH Indicator I indicates a pH ≤7.5.
- Add pH Indicator I to entire buffer contents. Do not add pH Indicator I to buffer aliquots.
- If the purified PCR product is to be used in sensitive microarray applications, it may be beneficial to use Buffer PB without the addition of pH Indicator I.

Procedure

1. Add 5 volumes of Buffer PB to 1 volume of the PCR sample, and then mix. It is not necessary to remove mineral oil or kerosene.

   For example, add 500 µl of Buffer PB to 100 µl PCR sample (not including oil).
2. If pH Indicator I has been added to Buffer PB, check that the mixture's color is yellow.

   If the color of the mixture is orange or violet, add 10 µl of 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn yellow.

3. Place a QIAquick spin column in a provided 2 ml collection tube.
4. To bind DNA, apply the sample to the QIAquick column and centrifuge for 30–60 s.
5. Discard flow-through. Place the QIAquick column back into the same tube.

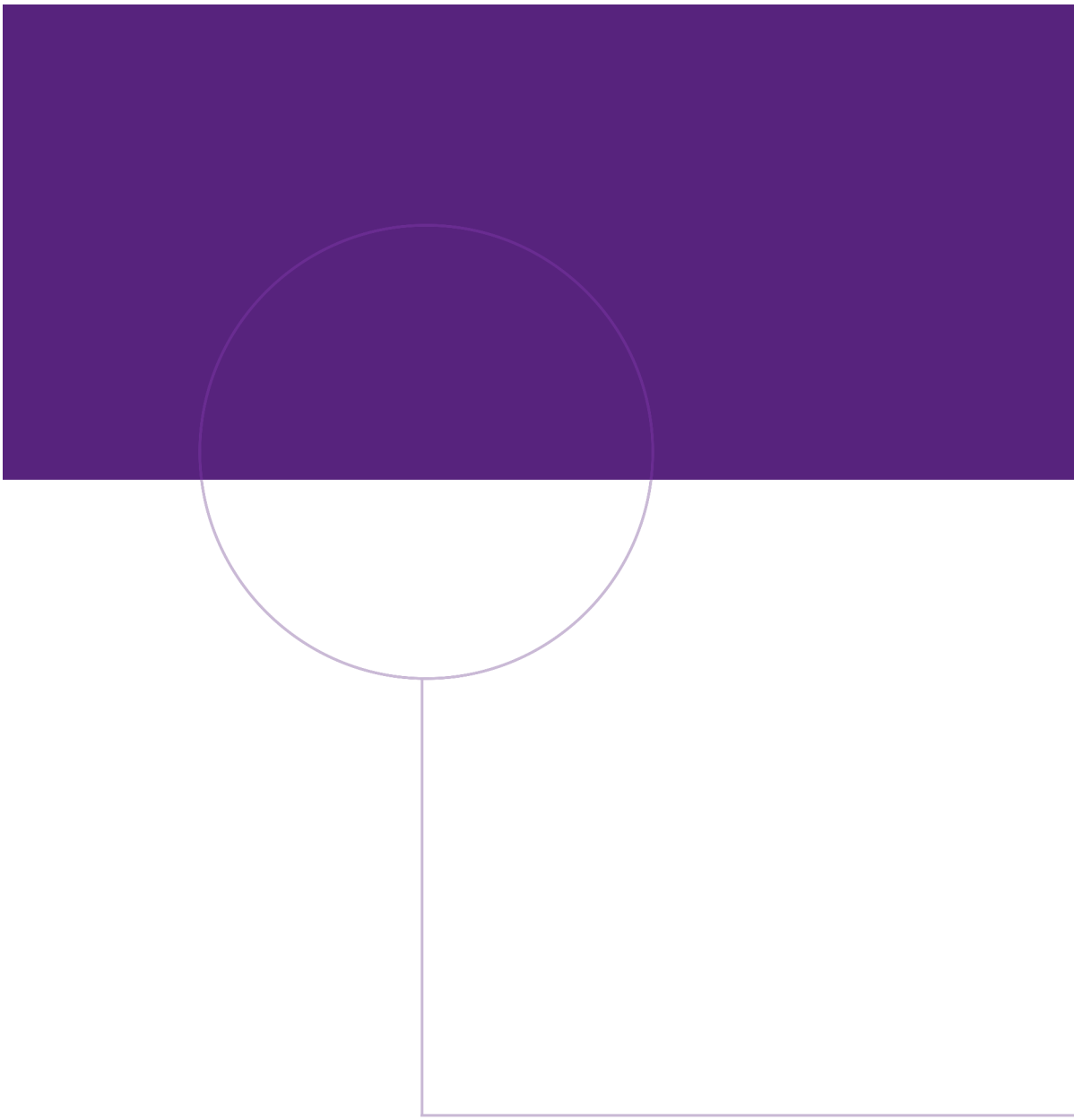
   Collection tubes are reused to reduce plastic waste.
6. To wash, add 0.75 ml Buffer PE to the QIAquick column and centrifuge for 30–60 s.
7. Discard flow-through and place the QIAquick column back into the same tube. Centrifuge the column for an additional 1 min.

   IMPORTANT: Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.
8. Place QIAquick column in a clean 1.5 ml microcentrifuge tube.
9. To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water (pH 7.0–8.5) to the center of the QIAquick membrane and centrifuge the column for 1 min. Alternatively, for increased DNA concentration, add 30 µl elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge.

   IMPORTANT: Ensure that the elution buffer is dispensed directly onto the QIAquick membrane for complete elution of bound DNA. The average eluate volumes are 48 µl from 50 µl elution buffer volume and 28 µl from 30 µl elution buffer.

   Elution efficiency is dependent on pH. Maximum elution efficiency is achieved between pH 7.0 and 8.5. When using water, make sure that the pH value is within this range, and store DNA at –30°C to –15°C because DNA may degrade in the absence of a buffering agent. The purified DNA can also be eluted in TE buffer (10 mM Tris·Cl, 1 mM EDTA, pH 8.0), but the EDTA may inhibit subsequent enzymatic reactions.
10. If the purified DNA is to be analyzed on a gel, add 1 volume Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting it up and down before loading the gel.

    Loading Dye contains 3 marker dyes – bromophenol blue, xylene cyanol and orange G – that facilitate estimation of DNA-migration distance and optimization of the agarose gel run time. Refer to Table 2 (page 17) to identify the dyes according to migration distance and agarose gel percentage and type.



NTNU

Norwegian University of
Science and Technology