Masteroppgave

NTNU
Norges teknisk-naturvitenskapelige universitet
Fakultet for naturvitenskap
Institutt for bioteknologi og matvitenskap

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Investigating the influence of biofilter-biofilm bacterial communities on the microbiota of rearing water and Atlantic salmon fry in RAS

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Veileder: Ingrid Bakke
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Trondheim, May 2023
Anna Aasen

Abstract

Recirculating aquaculture systems (RAS) provide numerous advantages, including water conservation, reduced environmental impact, and improved control over water quality parameters, ultimately enhancing fish growth. One key benefit of RAS is the ability to manage water quality to create an optimal environment for cultured organisms. However, our understanding of microbial community dynamics in RAS remains limited due to a lack of research. Additionally, the biofilter, a crucial component of RAS responsible for removing toxic nitrogenous substances excreted by fish, serves as a significant reservoir for bacteria. The impact these bacteria in the biofilter-biofilm have on the microbiota of the rearing water and the fish is not well known.

This study aimed to investigate the influence of biofilter-biofilm bacterial communities on the microbiota of rearing water and Atlantic salmon fry in RAS. This was done by rearing Atlantic salmon fry in two identical laboratory-scaled RAS operating under identical conditions, differing only in the biofilm carriers used in their biofilters. The biofilm carriers were obtained from two commercial RAS located in distinct geographical regions. After 30 days of RAS operation, the biofilm carriers were exchanged between the systems. Although the planned duration of the experiment was 60 days, an unexpected power outage the experiment ended after 40 days. Throughout the experiment, samples of biofilm carriers, suspended water, and salmon fry were collected and subjected to bacterial community analysis using Illumina sequencing of 16S rDNA amplicons.

The findings revealed significant differences between the bacterial communities of the rearing water and salmon fry compared to the biofilter-biofilm microbiota throughout the experiment. However, both the water and biofilm carriers exhibited a high abundance of shared taxa, such as *Rhodobacterales* and *Burkholderiales*. Moreover, the water microbiota demonstrated greater similarity to the biofilter-biofilm microbiota within its respective system. While the microbiota of the salmon fry significantly differed from the water and biofilter-biofilm microbiota, notable changes were observed in the skin microbiota during the experiment, indicating a potential influence by the biofilter. This study has demonstrated the biofilter's influence on the microbiota of the water and salmon fry in laboratory-scaled RAS.

Sammendrag

Resirkulerende akvakultursystemer (RAS) gir mange fordeler, blant annet vannsparing, redusert miljøpåvirkning og bedre kontroll over vannkvalitetsparametere, noe som til slutt fører til bedre vekst hos oppdrettsfisken. En viktig fordel med RAS er evnen til å regulere vannkvaliteten og skape optimale forhold for de oppdrettede organismene. Dessverre er vår kunnskap om mikrobielle samfunnsdynamikker i RAS begrenset på grunn av manglende forskning. I tillegg fungerer biofilteret, en essensiell komponent i RAS som fjerner giftige nitrogenforbindelser utskilt av fisken, som et betydelig reservoir for bakterier. Vi vet imidlertid lite om innvirkningen av disse bakteriene i biofilter-biofilm på mikrobiotaen i oppdrettsvannet og hos fisken.

Målet med denne studien var å undersøke hvordan biofilter-biofilm-bakteriesamfunnene påvirker mikrobiotaen i oppdrettsvannet og hos atlantisk lakseyngel i RAS. Vi oppdrettet atlantisk lakseyngel i to identiske laboratoriebaserte RAS-anlegg som opererte under samme forhold, med den eneste forskjellen i biofilm-bærerne som ble brukt i biofilterne. Disse biofilm-bærerne ble hentet fra to kommersielle RAS-anlegg som var lokalisert i ulike geografiske områder. Etter 30 dagers drift ble biofilm-bærerne byttet mellom systemene. Selv om eksperimentet var planlagt å vare i 60 dager, ble det avsluttet etter 40 dager på grunn av en uventet strømbrudd. I løpet av eksperimentet ble prøver av biofilm-bærerne, suspendert vann og lakseyngel samlet inn, og bakteriesamfunnsanalyse ble utført ved hjelp av Illumina-sekvensering av 16S rDNA-ampliconer.

Resultatene viste betydelige forskjeller mellom de bakterielle samfunnene i oppdrettsvannet og lakseyngelen sammenlignet med biofilter-biofilm-mikrobiotaen gjennom hele eksperimentet. Imidlertid hadde både vannet og biofilm-bærerne en høy andel av delte taksonomiske grupper, som *Rhodobacterales* og *Burkholderiales*. Videre viste vannets mikrobiota større likhet med biofilter-biofilm-mikrobiotaen innenfor sitt eget system. Selv om mikrobiotaen hos lakseyngelen var signifikant forskjellig fra vannet og biofilter-biofilm-mikrobiotaen, ble det observert betydelige endringer i hudens mikrobiota i løpet av eksperimentet, noe som tyder på en potensiell påvirkning fra biofilteret. Denne studien har bidratt til å belyse biofilterets innvirkning på mikrobiotaen i vannet og hos lakseyngelen.

Abbreviations

AmoA	Ammonia monooxygenase
AOA	Ammonia Oxidizing Archea
AOB	Ammonia Oxidizing Bacteria
Anova	Analysis of variance
ASV	Amplicon sequence variants
cDNA	Complementary DNA
Comammox	Complete ammonia oxidizer
DNA	Deoksyribonuclease
DO	Dissolved oxygen
dNTP	Deoxyribonucleotide triphosphates
Lab-scale RAS	Laboratory scaled recirculating aquaculture system
NOB	Nitrogen Oxidizing Bacteria
NSC	Norwegian sequencing center
NTC	Non-template control
PCR	Polymerase chain reaction
PCoA	Principal coordinate analysis
PERMANOVA	Non-parametric multivariate analysis of variance
RAS	Recirculating Aquaculture System
RDP	Ribosomal Database Project
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SD	Standard deviation
SIMPER	Similarity Percentage
TAN	Total Ammonia Nitrogen
TDS	Total dissolved solids
v3	Variable region 3
v4	Variable region 4

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1 Introduction

1.1 The aquaculture industry

The global supply of seafood is undergoing a significant shift towards aquaculture as the catch of wild species in various regions remains stagnant. In 2019, the volume of farmed salmonids accounted for one-third of the total wild salmonids caught[1]. Aquaculture is believed to be an important contributor to nourishing the fast-growing human population in a more sustainable manner compared to land-based livestock production[1]. With the world's population projected to reach 8.6 billion people by 2030 and 9.8 billion by 2050[2], the importance of aquaculture becomes even more apparent. Today only 7% of the total protein sources humans consume is fish, even though 70% of the Earth's surface is covered by oceans. Pork, poultry, and beef are the largest sources of animal protein[1].

The aquaculture industry consists of farming numerous amounts of different species in water. Multiple species of fish, shellfish, and aquatic plants are bred, raised, and harvested in different aquatic environments[3]. The various ways or *systems* of farming in water are classified by these terms; the type of rearing facility, the amount of water exchange, the intensity of culture, and the farming method which is a combination of the previous terms. Rearing structures such as ponds, cages, pens, raceways, and tanks are utilized based on the requirements of the target species. The amount of water exchange in the facility can be open, static, semi-closed, or "fully" closed (recirculating). Culture intensity is classified as intensive, semi-intensive, or extensive. For instance, Atlantic salmon is commonly reared using an open and intensive farming method in sea cages during its final life stage, after it has transitioned to saltwater[4].

Despite the promising and growing future of aquaculture, the industry faces several concerns and challenges. Key issues include fish escaping from sea cages, the discharge of waste into the environment, and the significant demand for fish meal and fish oil[5][6]. When farmed fish escape, there is a risk of interbreeding with wild populations, potentially resulting in a hybrid population. The introduction of farmed species' genes can negatively impact the survival chances of hybrids in the wild, as they may possess traits that are not well-adapted to the natural environment. To mitigate this risk, some aquaculturists employ sterilization techniques to minimize the production of hybrid generations[7]. Additionally, the discharge of waste and effluents from intensive fish farming can contribute to eutrophication by over-nourishing the recipient water body and sediment[8].

The industry's sustainability has become a significant public concern in light of these issues[5]. Recirculating aquaculture systems (RAS) offer a potential solution to address these concerns. In RAS, fish farming takes place on land, and the majority of water used in the system is recycled, significantly reducing environmental impact compared to traditional flow-through systems. RAS minimizes the potential for eutrophication and significantly reduces waste volumes through enhanced nutrient recycling[9].

1.2 Farming of Atlantic salmon

The family *Salmonidae* comprises several fish species commonly referred to as salmon[10]. These species include salmon, trout, whitefish, and grayling. Due to their reliance on cold and clean water, these fish species are particularly vulnerable to the impacts of climate change[11].

Among marine fish, the Atlantic salmon (Salmo salar) is the most extensively farmed globally, with a production exceeding four million metric tons in 2021[12][13]. It represents the fastest-growing food production system worldwide[14], and almost all available Atlantic salmon in the market is of farmed origin[1]. Norway, as the largest salmon farming nation, accounted for approximately 50% of global salmon production in 2015[furuset˙a˙furuset˙these˙2022][15]. In fact, according to a 2022 report by the Federation of European Aquaculture Producers (FEAP), Norway contributed 58% of Europe's total fish production through aquaculture[16].

However, Norway's salmon production growth has reached a stagnation point, primarily due to limited available licensed sites for sea cage farming and increased pressure to control pathogens and parasites, notably the ectoparasite sea lice (*Lepeophtheirus salmonis*)[17].

1.2.1 The life and production cycle of Atlantic salmon

Atlantic salmon, an anadromous species, exhibits a dual habitat lifestyle, inhabiting both freshwater and saltwater environments. During their life cycle, they undergo physiological changes to transition from freshwater to saltwater habitats[18]. In the wild, the natural lifespan of an Atlantic salmon ranges from 3 to 8 years, whereas in commercial production, the typical lifespan is around 3 years. The production process for salmon consumption generally comprises three stages: the freshwater stage, lasting 10-16 months; the seawater stage, lasting 12-24 months; and the final stage involving primary processing and packaging[1] The different stages in commercial production is shown in Figure 1.2.1.

The Atlantic salmon's life cycle begins as eggs, also known as "ova." Over time, the developing embryos exhibit increased visibility of their eyes, and as the yolk sac's food is consumed, more movement is observed. The next life stage, known as "yolk-sac fry" or "alevins," corresponds to hatching, during which the fry still receive nourishment from the attached yolk sac[19][20]. Eventually, the yolk-sac fry absorb the yolk sac and, in the wild, swim up from their river nests to the surface to gulp air. At this point, they are referred to as fry. Fry possess eight fins and swim against the current. As they continue to develop, they transform into "parr," exhibiting distinct stripes that serve as camouflage. It is during this life stage that they undergo a physiological process called "smoltification" to adapt to seawater, transitioning into "smolt" status. Smoltification involves changes in salt-regulating mechanisms, enabling smolt to swim with the current. In the wild, smolt leaves the rivers and migrates to the sea, eventually reaching full maturity as adult salmon[19].

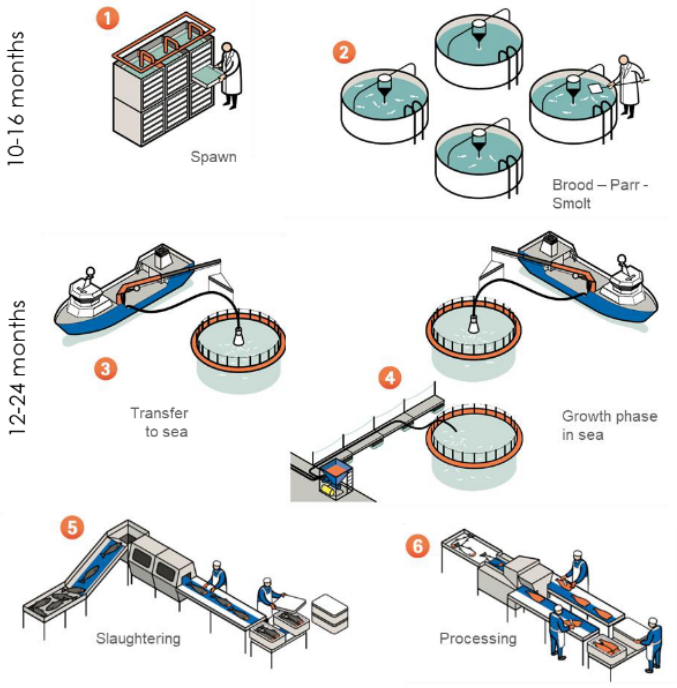


Figure 1.2.1: The production cycle of farmed Atlantic salmon. Figure taken from MOWI[20].

The production cycle of Atlantic salmon in aquaculture begins with carefully selecting broodstock from production stocks in seawater and moving them to freshwater tanks or cages. The eggs extracted from the broodstock undergo stripping and are then fertilized with milt. Subsequently, the fertilized eggs are water hardened, disinfected, and placed in trays or silo systems. Within these hatchery trays, the eggs hatch, giving rise to yolk-sac fry. To simulate their natural nesting environment in the wild, a stony substrate is provided for the yolk-sac fry. The incubation of the eggs and yolk-sac fry occurs at a water temperature typically exceeding 10 °C. Once the yolk sac is consumed, the alevins swim up and become ready for their initial feeding. The fry continue to grow in tanks utilizing either flow-through or water-recirculating systems, or alternatively in lake cage systems, until they reach the smolt stage. Upon confirming their smoltification, the fish are transferred to ocean-based cages[21].

In Norway, the release of smolt into the sea transpires throughout the year, although the primary periods for release are spring and autumn[22]. Multiple factors are taken into consideration when selecting the sea site, including water temperature, flow rates, salinity, proximity to other fish farms or wild fisheries, and adherence to licensing regulations[21]. Harvesting of the fish occurs after a period of 12-24 months in the seawater phase, once they have attained the appropriate size for harvesting. Subsequently, the harvested fish are transported to processing plants for slaughter[22].

In the field of salmon farming, two prominent environmental and economic challenges are sea lice infestation and fish escapes in the open sea. Sea lice pose a significant threat to salmon as they feed on the fish's skin and blood, while also spreading easily among the population. To remove sea lice, chemical treatments are commonly employed, alongside

the use of cleaner fish such as lumpfish and wrasse, which help remove the lice. However, the chemicals utilized in these treatments can lead to environmental pollution and weaken the salmon. Furthermore, there have been instances of salmon lice developing resistance to many of the chemical therapeutics. The welfare and mortality rates of cleaner fish within salmon cage systems are also concerning. A study conducted by Geitung et al.[23] during a four-month autumn-winter period reported a mortality rate of 57% for wrasse and 27% for lumpfish. Although improved farming equipment and facility design can reduce the likelihood of fish escapes, the risk cannot be entirely eliminated[24][23].

As an alternative, land-based recirculating aquaculture systems (RAS) are gaining popularity as replacements for traditional sea cages. These systems are utilized to produce various stages of salmon, including smolts, post-smolts, and even fish up to market size. Currently, the focus in Norway lies on designing or considering facilities intended to produce post-smolts weighing up to 1 kg[17].

1.3 Recirculating Aquaculture Systems (RAS)

In Recirculating Aquaculture Systems (RAS) the water is re-used several times within the system, in contrast to flow-through systems where the water is only used once[25]. The main advantage of RAS is that it allows for the management of water quality to create the desired target environment for the organism being cultured. This means that the type of organism being grown is not limited by the environment, as the water quality can be controlled to meet their needs[26]. Water quality parameters such as pH, temperature, dissolved oxygen, carbon dioxide, total ammonia nitrogen (TAN) levels, suspended solids, and salinity can be controlled, making it possible to optimize growth and feed utilization for the given farmed species[27]. RAS can re-use up to up to 99% of their water, making it possible to establish them in places with limited amounts of new water[25], which is an important motivation for utilizing these systems in aquaculture[28], considering limited resources of fresh water is a huge problem in the world[25]. The use of RAS in Norway has been driven by several factors, such as the increasing demand for high quality seafood and the desire to reduce environmental impact and improve biosecurity[28].

Even though RAS have a lot of positive outlooks, there are some drawbacks, such as operation and investment costs. The need for a high level of biological and technological knowledge to operate the system increases the operation costs, along with higher equipment maintenance compared to flow-through systems[25]. RAS also have a high energy requirement which impacts the operational costs of the systems and the environment, by releasing more CO_2 if fossil fuels are used [9]. Focusing on the investment costs of building the systems, they are higher compared to other aquaculture production systems[29]. Additionally, due to the high re-use of water in these facilities multiple water treatment components are needed to prevent the accumulation of harmful and toxic substances secreted by the fish and bacteria. Making the system more advanced and complex than traditional flow-through systems [25].

1.3.1 RAS design and water treatment components

RAS consist of multiple operating units, corresponding to specific treatment processes such as oxygen supply, carbon dioxide removal, biofilter for removal of toxic nitrogenous substances, particle removal, and if necessary, disinfection unit. The design of a RAS facility varies and is often defined by financial constraints and availability of resources[26].

In Figure 1.3.1 a RAS design is illustrated, and the different units are marked with numbers 1-10. Following the flow of the water from the fish culture tanks (1) that are oxygenated and aerated (2), the outlet water flows through a mechanical filtration unit (3) to remove settable and suspended waste solids. Toxic ammonia is then converted into much less toxic nitrate by nitrifying bacteria in the biofilter (4). The water is then pumped (5) from the sump into the gas management unit (6) for the removal of carbon dioxide. Finally, the water is disinfected with UV and ozone if necessary (7). The water quality and the operation of the units are monitored and controlled by different process controls (8), which will notify if water quality parameters move outside acceptable ranges. Automatic feeders (9) are installed at each of the fish tanks, and in this figure, there is a fish handling station (10)[30][26].

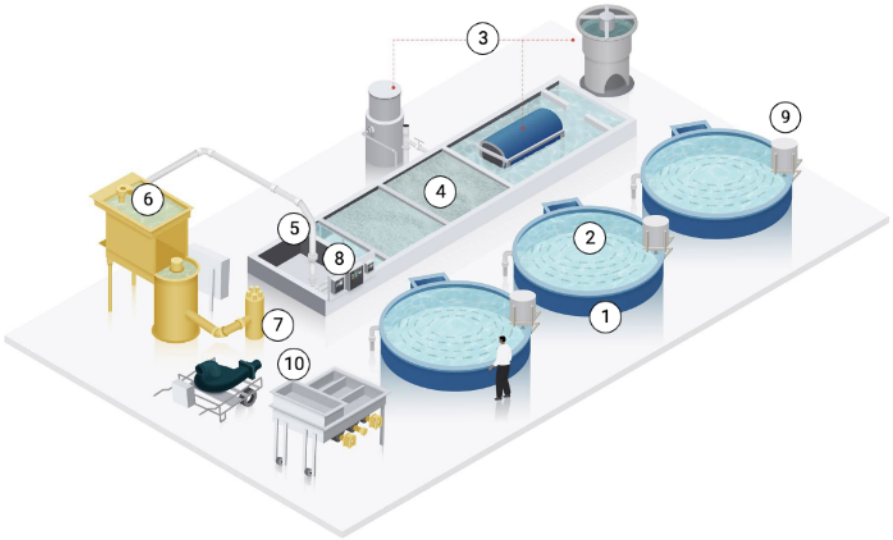


Figure 1.3.1: An example of a RAS design. Modified figure from InnovaSea[30]. 1: The fish culture tanks, 2: Aeration and oxygenation, 3: Mechanical filtration by drum filters or disc filters, 4: Biological filtration, 5: Pumps, 6: Gas Management, 7: UV and ozone disinfection, 8: Monitoring and controls, 9: automatic feeder and 10: Station to handle the fish.

1.3.2 Fish metabolism and water quality in RAS

During fish metabolism excretion products are released into the rearing water, such as CO_2 and ammonia (Figure 1.3.2). Accumulation of these products is toxic to the fish and must therefore be removed from the system by different water treatment technologies[31]. Toxic nitrogenous components containing ammonia are released from the fish into the

rearing water from their urine, feces, gill diffusion, and gill cation exchange[32]. Feces and uneaten feed will also increase ammonia levels through bacterial decomposition[25].

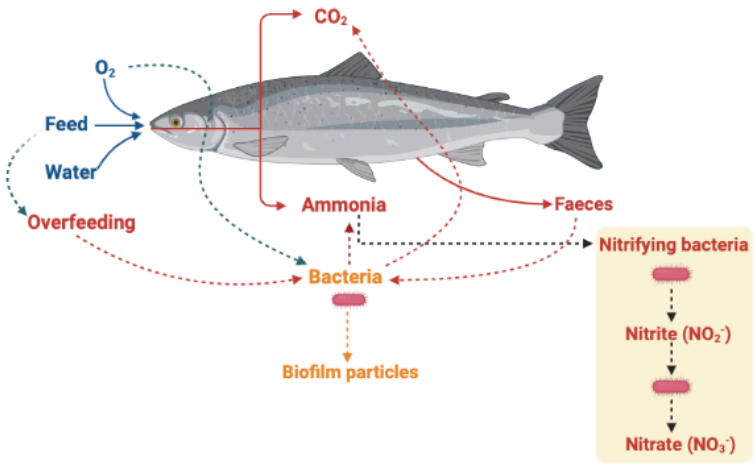


Figure 1.3.2: The formation and accumulation of harmful and toxic substances in RAS. Modified figure from Fjellheim et al.[31]. Created in biorender.

The build-up of TAN is a significant concern in RAS. TAN is the sum of unionized ammonia (NH_3) and ionized ammonia (NH_4^+). The concentration of the two forms of ammonia in water is dependent on pH, temperature, and salinity, as they exist in equilibrium[32]. The fraction of toxic unionized ammonia increases rapidly with increased temperature and pH[33]. NH_3 passes easily through the gills and is extremely toxic to fish. Due to the charged nature of NH_4^+ ions and the charged surface of fish gills, there is a hindrance to their diffusion into fish tissues, resulting in reduced toxicity compared to NH_3[34]. According to the Norwegian regulation limits, the TAN concentration in freshwater RAS when producing Atlantic salmon must be under 2 mg/L[35].

1.3.3 Biological water treatment

The biological filtration system, *the biofilter*, is an essential water treatment component in RAS that removes the toxic nitrogenous waste produced by the fish, bacteria, and uneaten feed[31]. There are two distinct types of biofilter technologies used in RAS: fixed film (attached growth) and suspended growth. Fixed film biofilters utilize media to provide a surface for microorganisms to attach and grow, while suspended growth biofilters keep sludge and microorganisms suspended in the water. Biofiltration in RAS has largely been directed towards aerobic fixed film biofilters[36].

Normally different kinds of submerged biofilters are used in RAS facilities. Submerged biofilters can be fixed bed- or moving bed biofilters, both made up of support media selecting for biofilm formation. A fixed bed biofilter is stationary, leading to a collection of organic material and the biofilm will continuously grow on the filter material surface. Therefore, this type of biofilter effectively removes smaller particles as well. As a result

of this, the filter surface needs to be cleaned frequently, to maintain good nitrification. Moving bed biofilters are self-cleaning since the support media is mixed by aeration and will therefore graze against each other, preventing biofilm buildup. Because of this, they submit small particles to the water, in contrast to the fixed bed that removes small particles. Submerged biofilters are aerated since oxygen is needed for nitrification to take place[31].

1.3.4 Nitrifying bacteria in the biofilter

A RAS system contains diverse microniches that harbor various microbial communities, predominantly comprising heterotrophic bacteria, with a subset of chemo-autotrophic bacteria playing a crucial role in nitrification within the biofilter[29]. Microorganisms live in biofilms on the carriers in the biofilter, on pipes, vessels, tank surfaces, and naturally in the water[37]. They retrieve their preferential oxygen and nutrient conditions in the different parts of the system[29]. The biofilter is the second largest habitat for microorganisms, the largest being the farmed animals[38].

Within a biofilter, the heterotrophic bacteria compete with the autotrophic bacteria. The heterotrophic bacteria are r-strategists, meaning they have a short generation time and they require a high amount of nutrients, while the chemo-autotrophic bacteria involved in the nitrification are K-strategists, so-called specialists. These bacteria have a long generation time, thus a slow growth rate and they can live with a low amount of nutrients. In a stable RAS, the environment will be unfavourable to the r-strategists, which are often opportunistic bacteria, and the K-strategists will outcompete the r-strategists[25].

Heterotrophic bacteria derive energy and carbon from organic compounds derived from uneaten feeds, dead organisms, and fish excreta. On the other hand, chemo-autotrophic bacteria utilize CO2 as their carbon source and derive energy by oxidizing inorganic nitrogen, iron, or sulfur[37][33][29]. In a RAS, the aerobic chemolithoautotrophic *nitrifying* bacteria are crucial for removing the nitrogenous waste products excreted by the fish and bacteria. These nitrifying bacteria utilize oxygen as their electron acceptor and obtain energy by oxidizing the reduced toxic nitrogen compounds ammonia (NH_3) and nitrite (NO_2^-) into the less toxic compound nitrate (NO_3^-) during a process called *nitrification*[39]. In freshwater RAS rearing Atlantic salmon the NO_2^--N level should be below 0.1 mg/L[35].

The growth dynamics on the biofilter-biofilm carriers contribute to the segregation of heterotrophic and nitrifying bacteria. Heterotrophs tend to thrive in the outer layer of the biofilm, where oxygen and nutrient concentrations are highest. Conversely, nitrifiers prefer the deeper layers of the biofilm. However, an excessive growth of heterotrophic bacteria can lead to an unfavorable outcome as they outcompete the nitrifiers for oxygen[40]. In addition, the presence of a high organic load and elevated carbon-to-nitrogen (C/N) ratio in the surrounding environment favors the growth of heterotrophic bacteria, thereby negatively impacting the nitrification process[29].

Furthermore, there is limited knowledge about how the microbiota of the biofilter influences both the microbiota of the suspended water and the reared species in RAS. One potential mechanism through which the biofilter can affect the water microbiota is by altering the chemical water quality, consequently influencing the selection pressure on bacteria present in the water. Additionally, biofilm detachment can directly influence the water microbiota by releasing bacteria into the water column. In principle, all bacteria present in the water have the potential to interact with the fish microbiota.

Nitrification

Nitrification consists of two reactions usually performed by two different groups of microorganisms, that carry out complementary metabolisms[39] (Equation 1). The initial step of oxidizing ammonia to nitrite is performed by *ammonia oxidizing bacteria* (AOB), such as *Nitromonas* and *ammonia oxidizing archea* (AOA) such as *Nitrosopumilus* (Equation 2). Subsequently, nitrite is converted to nitrate by *Nitrite oxidizing bacteria* (NOB) such as *Nitrobacter* (Equation 3). These two groups of organisms live in tight association within their habitat[41].

$$NH_4^+{}_{(toxic)} \xrightarrow{AOB} NO_2^-{}_{(toxic)} \xrightarrow{NOB} NO_3^-{}_{(less-toxic)} \tag{1}$$

$$NH_4^+ + 1,5O_2 \rightarrow NO_2^- + 2H^+ + H_2O \tag{2}$$

$$NO_2^- + 0,5O_2 \rightarrow NO_3^- \tag{3}$$

$$NH_4^+ + 2O_2 \rightarrow NO_3^- + 2H^+ + H_2O \tag{4}$$

Multiple factors such as the TAN concentration, oxygen level, amount of organic material, turbulence, temperature, pH, and alkalinity affect nitrification. Normally the nitrification rate will increase proportionally with the TAN concentration. At high TAN concentrations, the nitrification capacity will be maximized and the nitrification rate is constant, thus independent of the TAN concentration. Access to oxygen is a demanding factor for the nitrifying bacteria since they need oxygen to oxidize ammonia and nitrite into nitrate. Organic material will give growth of heterotrophic bacteria, which can out-compete the nitrifiers and thereby decrease the nitrification rate. Further, turbulence in the water can affect how TAN and nitrite are transferred between the water and the bacteria in the biofilm. Additionally, and important factor affecting nitrification is temperature, even though nitrifiers can adapt to different temperatures, the nitrification rate increases with increasing temperature. Other water quality factors affecting nitrification are pH and alkalinity. The optimum pH range for nitrifying bacteria is between 7 and 9, and alkalinity values lower than 45 mg/L $CaCO_3$ are limiting[31].

1.4 Methods to determine bacterial communities and their diversity

Microorganisms have over billions of years evolved into every possible niche on the planet. They have reshaped the oceans and atmosphere, and given rise to favorable conditions for multicellular organisms. It is only within the last decade that we have begun to look deeply into the world of microorganisms[42]. For nearly 300 years, the study of microorganisms was based on morphology, growth, and some biochemical profiles[43]. Then, in the late 1970s, the study and classification of microorganisms were totally transformed by the introduction of using ribosomal RNA genes as molecular markers for classification by Carl Woese, in addition to the Sanger automated sequencing by Fred Sanger. Some years later, in the 1980s/early 1990s molecular tools such as nucleic acid hybridization, polymerase chain reaction (PCR), DNA- cloning, and sequencing became more available, making it possible to study microbial communities without the limitation of cultivation techniques[44]. At present time sequencing has shifted from Sanger sequencing technology to next-generation sequencing (NGS)[45]. NGS is also referred to as deep or massively parallel sequencing and is capable of generating millions of sequence reads per run[46]. The current dominating NGS method is Illumina sequencing [47],[48].

Illumina sequencing

Illumina sequencing is a high throughput sequencing (HTS) technology and utilizes a method called sequencing by synthesis (SBS). The first step is library preparation where purified DNA is fragmented into smaller pieces and unique primers are attached to the ends of each fragment. These unique primer- or adaptor sequences contain the information needed for sequencing as well as an index to identify the sample. In the next step, the DNA fragments are bound to a flow cell as hairpin loops. Each unique library fragment is then amplified by bridge amplification using PCR where the 3' end of the DNA fragment is denatured and replaced by the complementary strand. After rounds of copying and denaturation, a cluster of thousands of copies of clonally amplified fragments is generated. The fragments are then ready for sequencing. Sequencing primer, DNA polymerase, and fluorescent nucleotides are passaged through the flow cell. The nucleotides are labeled with different color fluorescent tags and contain a reversible terminator. One nucleotide is added to the complementary strand within each cluster for each cycle with an SBS approach and a camera detects the color of each cluster. Cleavage of the fluorescent label then permits the next nucleotide to be added. The number of cycles to produce the final read length is specified in advance. This is the end for single-read sequencing, but sequencing can also be done from both ends (paired-end sequencing), using a second primer to the newly synthesized DNA strand[46][47].

Microbial diversity

Begon et al. defined microbial communities as a group of microorganisms that coexist in the same space and time[49]. Metagenomics is the study of the entire nucleotide sequences from all the microorganisms in an environmental sample[45] [50]. Compared to phylogenetic studies, metagenomics provides a much broader description since it offers

access to the functional gene composition of the community. This is in contrast to phylogenetic studies which are often based on the diversity of one gene, such as the 16S rRNA gene[45][51]. In spite of this, determining the microbial diversity using the 16S rRNA gene is widely used, and is referred to as Amplicon sequencing[43]. The procaryotic 16S rRNA gene has nine variable regions distributed between conserved regions and is almost 1500 bp long. Most frequently the small, but highly variable regions of the gene, often the v3-V4 region, are looked at to assess taxonomic relationships[52].

The description of microbial diversity is dependent on the phylogenic interference derived from highly conserved genes that are vertically inherited, such as the 16S rRNA gene sequences. In microbial ecology, the most common diversity unit is the operational taxonomic unit (OTU), which clusters sequences with typically $>$97% sequence similarity of the 16S rRNA gene[42]. Methods identifying the amplicon sequence variants (ASVs) can also be utilized. ASV methods are capable of identifying biological sequences within a sample and distinguishing between sequence variants that differ by as little as one nucleotide. Compared to OTU methods, ASV methods have better resolution, sensitivity, and specificity. In addition, they are reusable across studies and not constrained by incomplete reference databases[53].

In determining the diversity of a community, there are several concepts and metrics to consider. The species/OTU richness concept provides information on the number of species present in a specified region, while evenness measures the relative abundance of each species. If all species are similarly distributed, the evenness is high. However, to better describe and compare communities, other metrics such as Alpha (α), Beta (β), and Gamma (γ) diversity are used. Alpha diversity measures the diversity within a single sample or community[43], while Gamma diversity measures the overall diversity across communities. Beta diversity, on the other hand, compares the differentiation between species communities[54] and can be measured using various indexes such as the Bray-Curtis dissimilarity indices[43].

1.5 Aims and objectives

This study aimed to *examine the biofilter-biofilm communities influence on the microbiota of the rearing water and the Atlantic salmon fry in RAS*. The study was performed in collaboration with Ph.D. student Sujan Khadka who is participating in a project funded by the European Union's Horizon 2020 research and innovation program called *RASOPTA*. The research program aims to identify and bridge gaps in existing knowledge in fish production of species important for nutrition and economy in Europe[55]. His Ph.D. project focuses on water quality in RAS and will examine interactions between microbes in the water and fish.

In previous studies, PCR amplification of bacterial 16S rDNA from Atlantic salmon has been challenging, and the quality of the DNA/RNA extracts is assumed to be a possible reason for this. Therefore, a sub-aim of this study is to determine *the DNA and RNA-extraction kit resulting in the most optimal nucleic acid extracts from Atlantic yolk-sac fry samples with the use of KingFisher™ Flex Purification System for downstream amplification of bacterial 16S rDNA.*

The specific objectives of this master project were to:

- Examine which DNA and RNA extraction kit yields the most optimal nucleic acid extracts from Atlantic salmon yolk-sac fry for successful amplification of bacterial 16S rDNA
- Conduct a study in two lab-scaled RAS with Atlantic salmon fry
- Modify the PCR amplification protocol for more optimal amplification of bacterial 16S rDNA from Atlantic salmon fry
- Characterize the bacterial communities in biofilter-biofilm carriers, rearing water, and Atlantic salmon fry
- Determine if the bacterial communities of biofilters originating from hatcheries at different geographical locations are significantly different
- Evaluate if the microbiota of Atlantic salmon fry and water are influenced by the biofilter's microbiota

2 Materials and method

2.1 Evaluating DNA/RNA extraction kits for fish samples

In the ACMS group, there have been challenges regarding PCR amplification of bacterial 16S rDNA from DNA extracts originating from fish gut and skin samples. This might be related to the quality of the DNA extracts. To evaluate the significance of the specific DNA/RNA extraction kit on the subsequent PCR, four different DNA/RNA extraction kits were evaluated: MagAttract® PowerSoil® Pro DNA kit with KingFisher® (Kit A; QIAGEN), 96 MagBead DNA kit (Kit B; ZymoBIOMICS™), MagBead DNA/RNA kit (Kit C; ZymoBIOMICS™), Quick-DNA/RNA™ MagBead (Kit D; ZymoBIOMICS™) on KingFisher™ Flex Purification System (Thermo Fisher Scientific). Details about the extraction kits are given in (Table 2.1.1). All the kits are able to extract DNA, but only kits C and D can be used to extract both DNA and RNA. In this experiment, samples of salmon yolk sac fry (skin and gut) from a previous master project were utilized.

Table 2.1.1: The four DNA/RNA extraction kits evaluated (Kit A-D). All kits are DNA extraction kits, while kits C and D are additionally RNA extraction kits.

Kit	Manufacturer	Kit name	Catalog No.	Nucleic acid
A	QIAGEN	MagAttract® PowerSoil® Pro DNA kit	47109	DNA
B	ZymoBIOMICS™	96 MagBead DNA kit	D4308	DNA
C	ZymoBIOMICS™	MagBead DNA/RNA kit	R2135	DNA+RNA
D	ZymoBIOMICS™	Quick-DNA/RNA™ MagBead	R2130	DNA+RNA

2.1.1 DNA/RNA-extraction

Three gut replicates and three skin replicates of salmon yolk sac fry from previous ACMS projects were utilized for the nucleic acids extraction for each of the kits. In addition, a negative (DNA-free water)- and positive control (bacteria culture in liquid media) were included. As an approximation for skin, whole salmon yolk sac fry where the gut had been dissected was used. The manufacturer's protocols for the kits (Appendix B) and their script on KingFisher™ Flex Purification System were used to perform the DNA or the DNA/RNA extractions with a few deviations, such as the homogenization method and the elution volume was set to 100 μL. The 32 samples were homogenized in solution supplied with the relevant kit, using Precellys® 24 tissue homogenizer (Bertin Technologies) with 0.1 mm glass beads (Bertin Technologies) at 5500 rpm for 30 seconds with two repetitions and a 15-second pause between each repetition. The samples were then centrifuged at 10 000 x g for 1 minute and their supernatant was used for the DNA or DNA/RNA extraction using KingFisher™ Flex Purification System. The extraction protocol for Kit C (Appendix B.3) involves first extracting DNA from the samples, then extracting RNA from the original sample used for DNA extraction (the sample plate), by the use of DNase treatment as a step on KingFisher. Kit D, extracts RNA from the co-purified DNA+RNA

elution plate by DNase treatment on Kingfisher (Appendix B.4.1). The extracted DNA and RNA samples were transferred into 1.5 ml Eppendorf tubes and stored at -20^oC until further analysis.

2.1.2 Nucleic acid measurements

Prior to the PCR amplification of 16S rRNA gene, the concentration and the purity of isolated DNA from all samples were measured on NanoDrop™ One Microvolume UV-Vis Spectrophotometer (Thermo Scientific™) following the manufacturer's protocol[56]. The nucleic acid absorbance values at 260 nm and 280 nm were used to calculate the purity ratios. For DNA an A260/A280 purity ratio around 1.8 is generally accepted as "pure"[56].

Both the DNA- and RNA concentration were measured in the RNA extracts using Qubit 3 (Thermo Fisher Scientific), following the belonging protocol[57]. To determine the DNase treatment, the DNA concentration was measured before and after DNase treatment, while RNA was measured after the treatment.

2.1.3 PCR amplification of 16S rDNA

The DNA and RNA extracts were used as templates for PCR amplification of the v3+v4 regions of the 16S rRNA gene. The RNA extracts were converted into cDNA by reverse transcription before PCR amplification.

cDNA synthesis
The extracted RNA by kits C and D were converted into cDNA by reverse transcription using iSpcript™ cDNA Synthesis Kit (BIO-RAD) (Appendix C). The RNA extracts were transcribed to cDNA twice, once using 2 μL RNA as template, and once using 4 μL RNA as template. In addition to the RNA templates each reaction consisted of 4 μL 5x iScript Reaction Mix, 1 μL iScript Reverse Transcriptase, and nuclease-free water adjusted to reach the desired total volume of 20 μL. The reaction mix was incubated in T100™ Thermal Cycler (BIO-RAD), where the first step in the protocol was priming at 25^oC for 5 minutes, then reverse transcription at 46^oC for 20 minutes and RT inactivation at 95^oC for 1 minute.

PCR
To amplify the v3+v4 regions of the 16S rRNA gene from all samples the primers Ill 341F_KI and Ill 805R (Table 2.1.2) were utilized. Each PCR reaction consisted of 1 μL template, 1x Phusion buffer HF (Thermo Scientific™), 0.3 mM of each primer (Sigma-Aldrich), 200 μM dNTP (Thermo Scientific™) and 0.02 units/μL Phusion Hot Start Polymerase (Thermo Scientific™) in a total volume of 25 μL. The expected length of the PCR products was 540 nt. All extracted DNA and cDNA samples were used as templates and amplified in three parallels; undiluted, 1:10, and 1:100 dilution. Cycling conditions for the PCR reactions were denaturation at 98^oC for two minutes, then they were run for 35 cycles with denaturation for 15 seconds at 98^oC, annealing for 20

seconds at 55^oC, followed by elongation at 72^oC for 20 seconds. After the 35 cycles, 72^oC were held for 5 minutes followed by 4^oC for 1 minute.

Table 2.1.2: The primers utilized for amplification of the V3 + V4 region of the 16S rRNA gene during PCR. The target sequences are in bold.

Primer	Nucleotide sequence	Target region
Ill-341F_KI	5'-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG NNNN **CCT ACG GGW GGC AGC AG**-3	V3
Ill-805R	5'-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G NNNN **GAC TAC NVG GGT ATC TAA KCC**-3'	V4

2.1.4 Agarose Gel Electrophoresis

To examine the size and quality of the PCR products 1% agarose gel electrophoresis was performed in 1x Tris-acetate-EDTA (TAE) buffer (Appendix A.2). The 1% agarose solution was prepared by dissolving agarose in 1xTAE buffer by heating it in a microwave. When casting the gel, 5 μL GelRed® (Qiagen) was added per 100 ml agarose solution, for visualization of the PCR bands after the gel electrophoresis. The PCR products (5 μL) were mixed with 6x DNA loading dye (Thermo Scientific™) (1 μL) before being loaded into the gel wells. To roughly determine the size of the DNA fragments 6 μL GeneRuler™ 1 kb Plus DNA Ladder (Thermo Scientific™) was used as a size marker. The agarose gel electrophoresis was run for 30 minutes at 90V to 1 hour at 110V, depending on the size of the electrophoresis chamber. When the electrophoresis was completed, the gel was photographed under UV light in G:box (Syngene) with GeneSnap (Syngene).

2.2 Lab-scaled RAS experiment for the rearing of Atlantic salmon fry

This experiment was performed in collaboration with Ph.D. student Sujan Khadka, participating in a project called RASOPTA. The aim of this experiment was to examine *to which extent the biofilter-biofilm communities influence the microbiomes of the rearing water and the Atlantic salmon fry in RAS*.

In the experiment Atlantic salmon fry were reared in two lab-scale RAS at NTNU Sealab under identical conditions, using different biofilter-biofilm carriers in the systems. It has previously been shown that biofilm communities in biofilters differ between geographical locations[58]. Hence, the biofilm carriers utilized in this experiment originated from two different geographically located Lerøy facilities. The biofilter-biofilm carriers originated from Lerøy's hatchery department in Belsvik located in Trøndelag and their department in Kjærelva located in Vestlandet. The Atlantic salmon fry was supplied by the hatchery in Belsvik. In parallel with this experiment, another Ph.D. and master's student performed an experiment with two identical lab-scaled RAS. The four RAS were operated and maintained in cooperation by the two Ph.D. candidates and the two master students.

2.2.1 Experimental design

To examine the impact biofilm carriers have on the microbiota of rearing water and salmon fry, two identical lab-scale RAS were operated under identical conditions, but with different biofilm carriers in the biofilters. During the experiment, the Belsvik- and Kjærelva biofilm carriers were swapped between the systems (Figure 2.2.1). The experiment was originally planned to last for two months, one month before, and one month after the swap. Day_0 of the experiment was defined as the day the salmon fry and the biofilm carriers were moved into the systems. The two lab-scale RAS were labeled RAS_A and RAS_B and they were loaded with biofilm carriers from Belsvik and Kjærelva, respectively. After one month (30 days), the biofilm carriers were swapped between the systems, then the Kjærelva carriers were placed in the biofilter in RAS_A, and the Belsvik carriers were placed in the biofilter in RAS_B. The systems were supposed to run for one more month after the swap, but due to an unexpected power outage, the experiment had to be ended 10 days after the swap. The systems were maintained in the morning and afternoons, and the power outage happened after the routine morning maintenance, when no one was present. When the Ph.D. responsible for maintenance that day came back in the afternoon, approximately 6 hours since the morning shift, the salmon fry had died from oxygen deficiency.

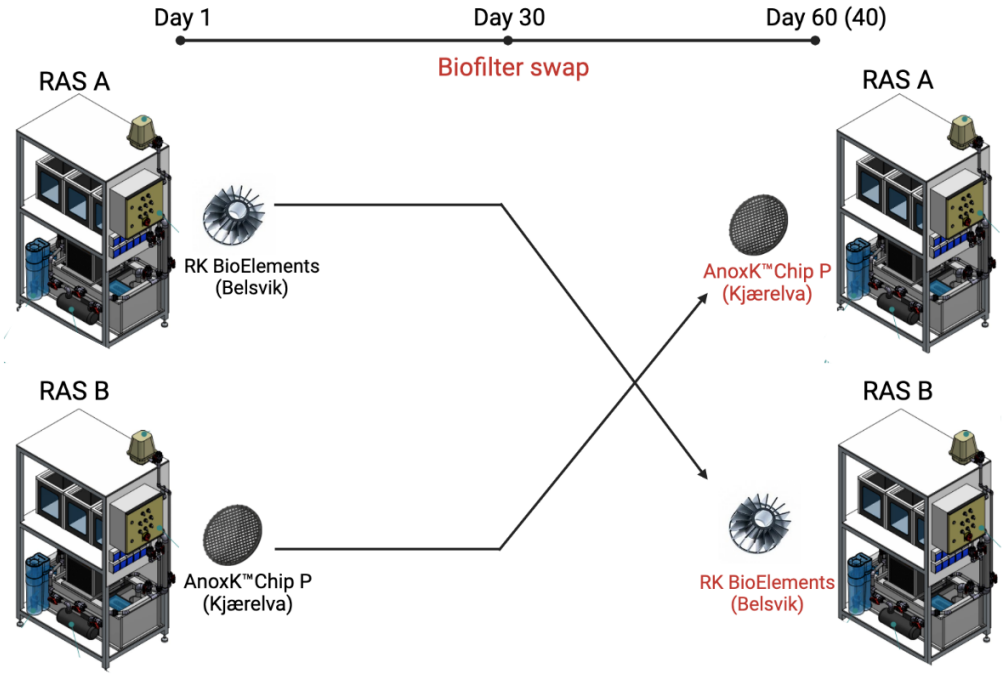


Figure 2.2.1: The experimental design for the rearing of Atlantic fry in lab-scale RAS using two different biofilters. Created at *Biorender.com*.

2.2.2 The acclimization stage

Lerøy department Belsvik provided 1400 salmon fry with an average weight of 3.7 grams on September 29, 2023, which were later moved into the RAS. To ensure their adaptation to the new environment, the fry was kept in four 200L flow-through tanks for 37 days, during which they were hand-fed twice daily, and several water quality parameters were monitored. The measurements included daily monitoring of dissolved oxygen (DO) levels and every-other-day monitoring of pH, ammonium, nitrite, and nitrate levels. More details about how the water quality parameters were measured are provided in section 2.2.6.

The biofilm carriers from Belsvik were also provided on 29.09.2023 while the Kjærelva carriers were provided on 31.10.2023. Until the experiment started the biofilm carriers were kept in separate aerated 100L buckets filled with water, and 80 ml of 10gTAN/L solution was added daily (Appendix A.3). The pH of the water in the buckets was regulated by the TAN solution's pH and by adding bicarbonate. TAN solution with pH 5 and pH 7 was utilized, depending on the pH measured.

The salmon fry was kept in the flow-through tanks longer than planned due to reconstructing the two lab-scale RAS. This had to be done since the fish tanks were under-dimensioned in relation to the total system volume and also the biofilter volume in the original design. For there to be enough ammonia for the nitrifying community in the biofilter, the fish biomass needed to be larger, and consequently the fish tanks. Because of this the fish tanks including the inlet and outlet pipes were re-designed and constructed.

2.2.3 Design and dimensions of the lab-scale RAS

The experimental lab-scale RAS employed in this study had a total volume of 165L and featured three 30L fish tanks, as depicted in Figure 2.2.2. The system was comprised of various water treatment units. Mechanical filtration involved the utilization of a long or short 100μL filtration sock for the removal of particles. To address the removal of nitrogenous compounds, an aerated moving bed biofilter was implemented. Specifically, 25L of the Belsvik biofilm carriers and 20.8L of the Kjærelva biofilm carriers were loaded into the systems. The biofilter unit had a capacity of 45L, resulting in filling degrees of 55% and 46.2% for the Belsvik and Kjærelva carriers, respectively. Detailed information regarding the biofilm carriers is provided in Section 2.2.4. The water within the system was tempered to 11^oC by the heater/chiller, and a protein skimmer was incorporated to remove smaller particles. As a final step of the water treatment, before the water returned to the tanks, a trickling filter was included for the removal of CO_2. Flow meters allowed for the regulation of water flow, and an electrical panel facilitated control over the system's different units. Notably, there was no disinfection step in the RAS loop by the UV lamp in this experiment.

The feeding systems consisted of automatic feeders laying on transparent lids with holes on top of each tank. The lid had holes for the feed to drop, for the aeration tube, and for the water inlet pipe.

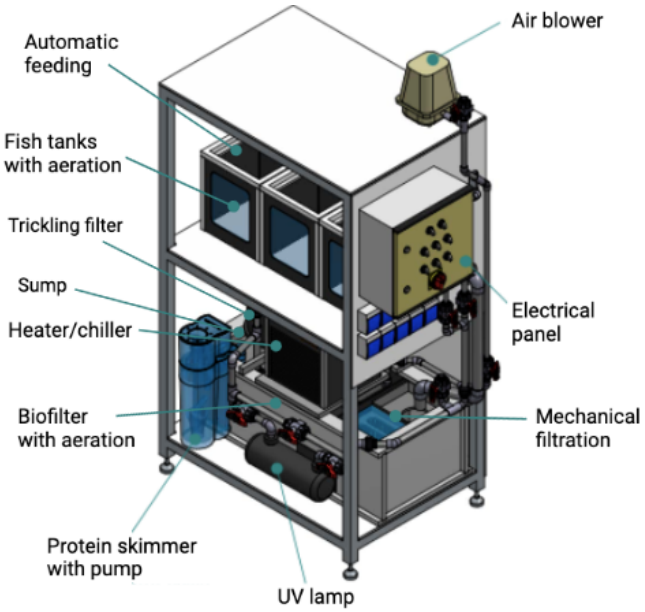
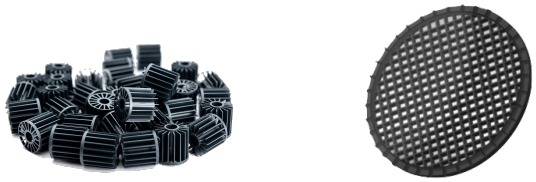


Figure 2.2.2: Illustration of the RAS utilized in the lab-scale RAS experiment. The system was provided by the manufacturer Spranger Kunstoffe GmbH (Appendix K). The illustration does not show the tank outlets situated at the bottom of the tanks, and the UV lamp was not utilized in this experiment. The figure was modified on Biorender.com.

2.2.4 The biofilm carriers

The biofilm carriers originating from Belsvik were *RK BioElements* with a surface area of 750 m^2/m^3[59], while the biofilm carriers originating from Kjærelva was*AnoxK™Chip P* with a surface area of 900 m^2/m^3 (Figure 2.2.3). Product specifications are found in Appendix L. At Day_0 of the experiment 25L of the Belsvik biofilm carriers were loaded into the biofilter in RAS_A and 20.8L of Kjærelva biofilm carriers were loaded into the biofilter in RAS_B. The different volumes corresponded to the same biofilm carrier surface area.

The difference in volume was due to their unequal biofilm carrier surface area. To obtain similar conditions when examining the biofilter-biofilm communities' possible influence on the water and salmon skin and intestine microbiota the total biofilm-carrier surface area was equal in the two systems.



(a) **(b)**

Figure 2.2.3: The two biofilm carrier models utilized in the lab-scale RAS experiment. a) the RK BioElements from Belsvik and b) the AnoxK™Chip P from Kjærelva.

2.2.5 Rearing of the Atlantic salmon fry

The Atlantic salmon supplied by Lerøy's hatchery in Belsvik was reared in the two lab-scale RAS for 40 days, from 06.11.2023 to 16.12.2023. On Day_0 of the experiment, 52 salmon fry was loaded into each of the six tanks, which gave an average total biomass of 348 grams per tank. The average weight of each salmon fry was 6,7 g. The feed utilized in the experiment was developed for Atlantic salmon fry, manufactured by EWOS®.

The feeding load in each tank was calculated based on the fish biomass in each tank. During the first three weeks, the feeding load was around 1.5% of the fish biomass per tank. It was then decreased to around 1% of the biomass as a measure to reduce the organic loading in the RAS. Appendix H provides an overview of the daily feeding load throughout the experiment. The fish were hand fed during the first week of the experiment before automatic feeders of the type *Fish Mate F14 Automatic Fish Feeders*[8] were installed. The feed was dropped from the automatic feeders into the tanks approximately eight times in 24 hours. To evaluate the growth of the fish during the experiment, they were weight and measured at the sampling points.

The fish was reared with constant light, replicating the conditions at the Belsvik hatchery. Biosecurity measures were implemented, including the use of shoe covers and new lab coats when working in the room where the RAS were located. However, there were no

sensors or alarms connected to the RAS during the experiment. Any fish moralities were manually removed from the tanks using a fishing net.

2.2.6 Maintenance of the systems and water quality measurements

To maintain the water quality, a set of routines and measurements were followed. Daily routines included exchanging approximately 5% of the total water volume (8L), loading feed in the automatic feeders, changing the mechanical filter socks, and measuring water quality. During water exchange, particles in the fish tanks and in the mechanical filter tank were manually siphoned out. In the tanks, water temperature (^oC) and dissolved oxygen (DO, mg/L) were measured daily with ProfiLine Oxi 3310 IDS DO Meter (WTW). The water exchange of 5% every day started routinely about one week into the experiment, and on multiple days more than 5% water was exchanged due to problems with the systems leading to water leakage. The amount of water exchanged every day of the experiment is shown in Appendix I.

Various parameters were measured in water collected from the sump, upstream of the fish tanks. Every day total dissolved solids (TDS, ppm), conductivity (mS/cm), and pH were measured by Pocket Pro+ Multi 2 Tester (HACH®). Additionally, every other day, alkalinity (mg $CaCO_3$ L^-1) was measured using JBL ProAquaTest KH following the manufacturer's protocol (Appendix J.2), and NH_4^+, NO_2^-, and NO_3^- were measured using API® freshwater master test kit according to the protocol of the kit (Appendix J.1).

2.2.7 Sampling for microbiota analysis of biofilm carriers, rearing water, and salmon fry

During the experiment sampling for microbial community analysis was conducted at three time points (Figure 2.2.1). The first sampling was performed on Day_1 of the experiment, followed by the second sampling on Day_30 before the biofilm carriers were swapped between the systems. The third and final sampling was originally planned to occur one month after the swap, however, due to the power outage, the third sampling took place at this timepoint, Day_40, 10 days after the swap.

To conduct the microbiome analysis, samples were taken of biofilm carriers, rearing water in the fish tanks, and the skin, intestine, and gills of the salmon fry. At each of the three sampling times, six samples were prepared for both the rearing water and the biofilters from both of the systems. Dissection was performed on seven fish for each system; three replicates from the first tank and two replicates from the following tanks. The samples were stored in dry ice during the sampling procedure and stored at -80^oC after completion.

For the analysis of the bacterial communities, 132 of the collected samples were selected (Table 2.2.1). This amounted to a total of 24 replicates of the biofilter-biofilm carriers. Of the salmon fry skin, intestine, and gill samples, a total of 72 replicates were selected for

analysis. Next, 36 samples were selected for bacterial community analysis of the rearing water. More details are given in Table 2.2.1.

Table 2.2.1: The number of samples from the lab-scale RAS experiment selected for microbial community analysis.

Sample Type	Number of samples for analysis per RAS, per sampling day	Total number of samples for analysis
Biofilm carriers	4	24
Fish skin	4	24
Fish intestine	4	24
Fish gill	4	24
Rearing water	6	36
Total number of samples for analysis		**132**

The biofilm carrier samples were collected by taking out one biofilm carrier at a time and rinsing it with autoclaved Milli-Q® water before cutting it with scissors on a petri-dish. One-fourth of the carriers were conserved in autoclaved Precellys tubes with preloaded 0.1 mm glass beads (Bertin Technologies) for later homogenization. A replicate for each sample was collected as well, and stored in cryotubes. Between each replicate, the equipment was disinfected with ethanol (70%).

The preparation of samples for microbial analysis of the rearing water consisted of pushing tank water through a 0,2μm filter with a 20 ml syringe until it was clogged, or pushing a maximum of 100 ml through if not clogged earlier. Two filters were preserved per tank, also here in autoclaved Precellys tubes with preloaded 0.1 mm glass beads (Bertin Technologies).

The fish for dissection were retracted and subjected to anesthesia in pools, one tank at a time. As the initial anesthesia 50 mg/L Tricaine Methanesulfonate (MS-222) (FINQUEL® vet) buffered with an equal amount of sodium bicarbonate (ScanAqua AS) was used. Once the fish had no movement, which usually took around 10 minutes, they were transferred into 150 mg/L Tricaine Methanesulfonate with the same concentration of sodium bicarbonate (ScanAqua AS). Approximately 15 minutes later, when the fish were euthanized, they were weighed and measured individually. Their gills and skin were rinsed with autoclaved Milli-Q® water before dissection. Thereafter, their gills, gut, and a patch of skin were dissected out and put in Precellys tubes with preloaded 0.1 mm glass beads (Bertin Technologies).

2.2.8 DNA isolation using KingFisher

The first step in establishing the microbial composition of the samples was to isolate the total DNA using KingFisher™ Flex Purification System combined with the

ZymoBIOMICS™ 96 MagBead DNA kit (D4308). This DNA extraction kit was evaluated as most optimal for extracting DNA from yolk sac salmon fry for later amplification of the 16S rRNA gene during the first part of this master project (section 4.1.1). The protocol for the DNA extraction kit (Appendix B.2) and its customized script on KingFisher™ were used to perform the DNA isolation, with a few deviations, as described in section 2.1.1. The DNA extracts were stored at -20^oC.

2.2.9 Illumina library preparation

As the first step in preparing the Illumina library, the v3+v4 region of the bacterial 16S rRNA gene was amplified for the biofilm carriers, salmon fry, and water samples on T100™ Thermal Cycler (BIO-RAD) as described in section 2.1.3. The negative kit control for the extraction kit was also used as a template, to examine possible bacterial DNA contamination from the kit or during the DNA extraction. Additionally, a non-template PCR control and a positive PCR control were always included. The number of cycles and the annealing temperature for each PCR run are specified with the results. Agarose gel electrophoresis (1%) of the PCR products was performed to examine their size and quality.

In the first round of PCR, all the DNA extracts from salmon fry skin, intestine, and gill samples were diluted 1:10, since this was found to be the most optimal dilution when testing the DNA/RNA extraction protocols and the subsequent PCR amplification of the 16S rDNA by the use of KingFisher™ Flex Purification System. For the biofilter-biofilm carrier and water samples, 1μL undiluted DNA extracts were used as templates. Samples that did not yield desired PCR products were diluted 1:10 prior to a second round of PCR, with the same PCR conditions. It appeared to be problematic to achieve PCR products from the salmon fry samples, especially from the skin and gill samples. Multiple PCR reactions and cycling conditions were therefore tested to optimize bacterial DNA amplification from these samples. These results are described in section 3.2.

In the next step of the amplicon library preparation, 15μL of the PCR products were normalized and purified using the SequalPrep™ Normalization Plate (96) Kit (invitrogen™) as described by the manufacturer (Appendix E). Following this, indexing PCR was performed, where every normalized sample was marked with a unique combination of forward and reverse sequence barcodes (indexes), provided by the Nextera XT DNA Library Preparation Kit (Illumina). Each of the indexing PCR reactions consisted 1x Phusion HF Buffer, 200μM dNTP, 0.02 units/μL Phusion Hot Start Polymerase, 2.5μL of each indexing primer, comprising a unique pair for every PCR sample (2.5μL), and DNA-free water to reach the desired total volume of 25 μL. The same cycling conditions as the first PCR on T100™ Thermal Cycler (BIO-RAD) were used, except here only 12 cycles were run and the annealing temperature was 57^oC. To examine the indexed PCR products 1% agarose gel electrophoresis was performed.

Senior laboratory engineer Amalie Johanne Horn Mathisen performed the next and final steps to prepare the samples for Illumina sequencing. First, the indexed PCR products

were normalized and purified using the SequalPrep™ Normalization Plate (96) Kit (invitrogen™) as previously described. Once normalized, the individual samples were pooled together and concentrated using AmiconUltra 0.5 centrifugal filter devices (30K membrane, Merch Millipore), following the protocol outlined in Appendix F. An additional washing step was performed in step 4 of the protocol by adding 500μL TE-buffer (Appendix A.2.1) to the pooled sample and centrifuging it at 14 000 x g for 10 minutes. This additional washing step was repeated once more. To assess the concentration and purity of the concentrated product, NanoDrop™ One Microvolume UV-Vis Spectrophotometer (Thermo Scientific™) was used. Additionally, the size and quality of the product were evaluated by running a 1% agarose gel. As a method to remove primer dimers in the sample, the product was extracted and purified from the gel by following the protocol in Appendix G, not including step 5. The purified product was then stored at -20^oC until it was sent to the Norwegian Sequencing Centre (NGS) for Illumina sequencing.

2.2.10 Sequencing data processing

The Illumina sequencing data were processed using the USEARCH pipeline (version 11; https://www.drive5.com/usearch)[60]. First, by using the mergepair command the forward and reverse sequences were merged, and any too-short sequences and primers were removed by using the fastqmergepairs command. Next, the fastq_filter command was used to perform quality filtering with an expected threshold error of 1. During this step, the Fastq files, one for each sample, were converted to fasta files. Following this, all the fasta files were pooled and sorted by size, and identical sequences were removed. In the last step, the Unoise3 command[61] was used to eliminate chimera sequences and to generate amplicon sequencing variants. The recommended minimum abundance threshold of 8 reads (in the complete data set) was used. This resulted in the creation of an ASV table. Taxonomy assignment to the ASVs was performed using the Sintax script[62] with a confidence level of 0.80, and the Ribosomal database project (RDP) reference dataset (version 18) was used for reference. The Sintax file and ASV table were combined in Excel for further analysis.

During the further analysis of the ASV table in Microsoft Excel, 44 non-bacterial ASVs were removed, such as those representing Atlantic salmon, chloroplasts, archaea, and eukaryotes. Additionally, ASVs found in the negative control for the DNA extraction were eliminated, which amounted to four ASVs that were not abundant in the samples. The resulting ASV table was then normalized to 16 400 reads per sample by calculating the fraction of ASVs in each sample, multiplying it by 16 400, and rounding the numbers to integers. This normalization process was conducted so that all samples had the same sequencing depth. The normalized ASV table was used for all downstream analyses.

2.2.11 Statistical analysis

PAST (version 4.0)[63] was used to calculate both the alpha and beta diversity indices from the normalized ASV table. The alpha diversity indices Chao-1, observed ASV richness, and Shannon's diversity were transported to Microsoft Excel and used for further evaluation of richness and evenness in sample groups. Observed ASV richness represents the number of ASVs detected in each sample, while Chao-1 provides a theoretical estimate of the total number of ASVs, including those that may not have been detected, giving a more complete estimate of ASV richness[64]. Shannon's diversity index considers both the number of ASVs and their relative abundance, providing information about both richness and evenness[65]. Evenness reflects how evenly or unevenly the ASV abundance is distributed in a sample or community.

For beta diversity analyses, Bray-Curtis similarities were calculated and a matrix of similarities between 0 and 1 was exported to Microsoft Excel for further analysis. Similarity value of 1 equals complete similarity while 0 equals complete dissimilarity[66]. Principal Coordinate Analysis (PCoA) ordination plots were created based on the Bray-Curtis similarities to visualize beta diversity. In a PCoA, each sample is represented as a point in a multidimensional space, where coordinates 1 and 2 are defined as the coordinates contributing the most to the variation between the samples. This can be used to project a two-dimensional plot, where the distance between the samples reflects their degree of similarity or dissimilarity. These plots can offer insight into the community structure and can visualize patterns in the overall community composition[63].

One-way PERMANOVA (permutational multivariate analysis of variance) based on Bray-Curtis similarities was used to check for statistically significant differences in community profiles between sample groups. Bonferroni corrected p-values were used when comparing more than two groups.

SIMPER (Similarity Percentage) based on Bray-Curtis similarities was used to identify which ASVs contributed to the detected difference in community profiles between sample groups[67].

To evaluate potential significant differences in alpha diversities between sample groups, a two-sample t-test or Anova (single factor) was calculated in Microsoft Excel. Prior to the t-test, an F-test was performed to examine if the sample groups had equal or unequal variances. A significance level of 0.05 was used. Based on the F-test, t-tests assuming equal or unequal variances were chosen.

A phylogenetic analysis was performed to examine the relationship between the ASVs that were classified as Nitrospira to previously sequenced Nitrospira 16S rRNA gene sequences from the biofilter samples. Sequences for representative Nitrospira were retrieved from the Ribosomal Database Project (RDP) browser [68] and from NCBI Genbank. The tree was inferred by using the maximum likelihood method in MEGA-X [69] with the Tamura-Nei model for sequence evolution [70].

3 Results

3.1 DNA/RNA extraction for bacterial 16S rDNA amplification from Atlantic salmon yolk-sac fry

This experiment aimed to determine the DNA- and/or RNA-extraction kit providing the most optimal nucleic acid extracts from Atlantic salmon yolk sac fry, with the use of KingFisher™ Flex Purification System, for downstream amplification of bacterial 16S rDNA. In this evaluation, 4 different DNA/RNA extraction kits were used for the isolation of DNA/RNA from gut and skin samples of three individual Atlantic salmon yolk sac fry. The concentration and purity of the extracted DNA and RNA were measured on Nanodrop, and thereafter was the 16S rRNA gene amplified by PCR. Prior to PCR, RNA was converted into cDNA by reverse transcription.

3.1.1 DNA extraction

When measuring the concentration and purity of the extracted DNA on NanoDrop™ One Microvolume UV-Vis Spectrophotometer, the concentrations of the DNA extracts for the gut samples were generally lower than those for the skin samples (Table 3.1.1). This could be expected since whole salmon yolk sac fry without the gut was an approximation for the skin samples. However, Kit B resulted in DNA extracts where all the concentrations were more similar across sample types. Samples extracted with Kit A exhibited the highest average concentrations of DNA, with an average concentration of 22.67 ng/μL for gut samples, and 273 ng/μL for skin samples. The samples extracted with Kit B had the lowest average DNA concentrations with 16.23 ng/μL for gut samples and 18.37 ng/μL for skin samples. In addition to resulting in the highest DNA concentrations, the samples extracted by Kit A had the purest DNA extracts (A260/A280 ratio of 1.8). The DNA extracts obtained by Kit C did also have high purity (close to 1.8), while DNA extracts obtained by Kit B showed A260/A280 purity ratios lower than 1.8. Not as pure extracts were observed for Kit D, where the DNA extracts had purity ratios higher than 1.8.

Table 3.1.1: The DNA concentration (ng/μL) and A260/280 purity rations of the DNA extracts obtained with the kits (A-D) determined by NanoDrop™ One.

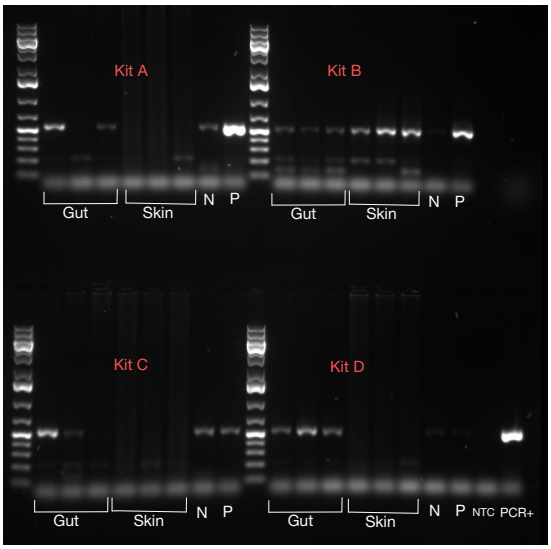
	Kit A		Kit B		Kit C		Kit D	
Samples	**DNA**	**A260/280**	**DNA**	**A260/280**	**DNA**	**A260/280**	**DNA**	**A260/280**
Gut 1	12.8	1.78	8.9	1.71	5.5	1.81	7.4	2.10
Gut 2	15.6	1.83	29.6	1.69	9.5	1.90	51.7	1.94
Gut 3	39.6	1.81	10.2	1.67	12.0	1.71	27.9	1.93
Skin 1	281.8	1.85	21.5	1.76	135.6	1.87	257.0	1.93
Skin 2	292.0	1.85	27.4	1.79	168.4	1.89	110.2	1.88
Skin 3	247.7	1.85	6.2	1.59	181.0	1.89	314.0	1.92

PCR was used to determine which extraction kit obtained the most optimal DNA extracts for amplification of the v3+v4 region of the 16S rRNA gene. An annealing temperature of 55℃ and 35 cycles was used (section 2.1.3). Three different dilutions of the DNA extracts were tested (undiluted, 1:10- and 1:100 dilution), and agarose gel electrophoresis was performed to determine the size and quality of the PCR products.

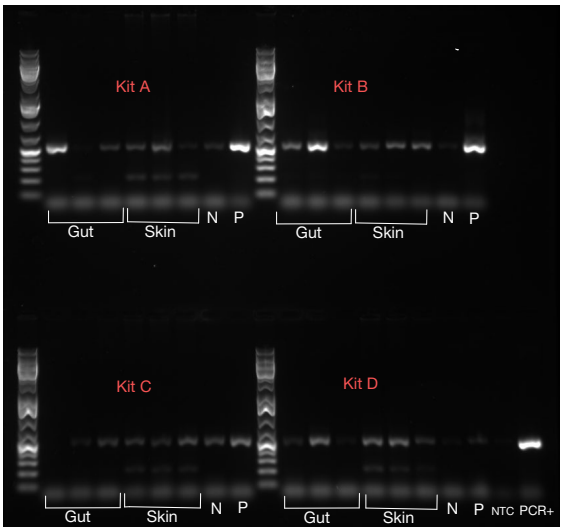
The undiluted DNA extracts (Figure 3.1.1a) resulted in PCR products at the expected length for all extracts obtained by Kit B, where the DNA extracts from skin samples gave the most PCR product compared to the DNA extracts from gut samples. For Kit D, only PCR products from the gut DNA extracts were detected. Shorter PCR products were also produced for Kit A, Kit C and especially Kit B, probably representing primer dimers or other unspecific DNA fragments. Weak PCR products are detected for the negative control of the DNA extraction at the desired length from both Kit A and Kit C, suggesting bacterial DNA contamination.

PCR of the 1:10 diluted DNA extracts resulted in PCR products for multiple samples by all kits and fewer unspecific PCR products (Figure 3.1.1b). No unspecific PCR products were observed for Kit B, while a few were observed for the other kits, although the unspecific PCR products were weaker than for the PCR with the undiluted DNA extracts. As the only kit, the DNA extracts from Kit B resulted in PCR products of the expected length for all samples, although with varied strengths. PCR products from the negative control of Kit A and C were still observed. Diluting the DNA extract 1:100 resulted in PCR products of the expected length from the negative control of all the kits (Figure 3.1.1c). It also resulted in generally weaker PCR products than the previous dilution. However, the unspecific PCR products were no longer observed. Additionally, a weak PCR product was observed for the NTC.

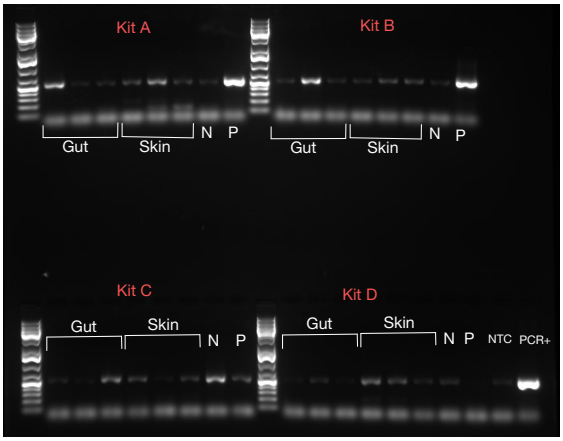
Of the four DNA-extraction kits, Kit A resulted in the purest DNA extracts with the highest DNA concentrations. However, when testing the DNA extractions from all kits by PCR, Kit B resulted in successful PCR amplification for the largest number of samples. Diluting the DNA extracts prevented the formation of unspecific PCR products, but 1:100 dilution resulted in PCR products for the negative kit control for all kits. In summary, Kit B, using 1:10 diluted DNA extracts as PCR template, resulted in PCR products for all six samples, and no unspecific PCR products were observed.



(a)



(b)



(c)

Figure 3.1.1: Agarose gel showing PCR products of the V3+V4 regions of the 16S rRNA gene from yolk-sac fry DNA extracts. Amplification was performed with 35 cycles and an annealing temperature of 55℃ using primers III-341F_KI and III-805R (Table 2.1.2). DNA extracts obtained with kits A-D (Table 2.1.1) from three salmon yolk sac fry gut and skin samples were used as templates. The PCR products were obtained by using a) undiluted DNA extracts as templates, b) 1:10 diluted DNA extracts as templates, and c) 1:100 diluted DNA extracts as templates. Negative and positive control was included for each extraction kit. Additionally, a non-template PCR control (NTC) and a positive PCR control (PCR+) were included.

3.1.2 RNA extraction

Kits C and D were compared to evaluate whether RNA extracts could be used to generate 16S rDNA amplicons and to see which of the kits resulted in the most successful PCR. The RNA extracts by Kit C were obtained from the sample plate by KingFisher which was the same plate DNA was extracted from. The RNA extracts obtained by Kit D were obtained from the co-purified DNA + RNA elution plate.

To examine the effect of the DNase treatment on the extracts, the DNA concentration was determined before and after DNase treatment, while RNA was measured after the treatment, in the RNA extracts (Table 3.1.2). For Kit C, the average DNA concentration after DNase treatment (2.55 ng/μL) was higher than before the DNase treatment (2.14 ng/μL). The DNase treatment of the extracts obtained by Kit D decreased the average DNA concentration from 8.03 ng/μL to 1.29 ng/μL. The final RNA concentration was 13 times higher than the final DNA concentration. In contrast, the final RNA concentration in extracts by Kit C was detected to be 5.5 times higher than the final DNA concentration. DNA was not completely removed after the DNase treatment for either of the kits.

Table 3.1.2: DNA concentrations for RNA extracts obtained by Kit C and Kit D before and after DNase treatment, and RNA concentrations after DNase treatment. The concentrations were determined using Qubit 3.

	Kit C			**Kit D**		
	Before DNase treatment	After DNase treatment		Before DNase treatment	After DNase treatment	
Sample	DNA (ng/μL)	DNA (ng/μL)	RNA (ng/μL)	DNA (ng/μL)	DNA (ng/μL)	RNA (ng/μL)
Gut 1	0.14	0.45	3.0	4.4	0.27	4.34
Gut 2	0.15	1.69	7.8	7.32	1.52	10.8
Gut 3	0.29	1.44	6.8	6.84	1.08	4.32
Skin 1	3.40	3.67	22.0	13.80	1.86	33.6
Skin 2	6.08	4.16	21.6	7.80	1.31	8.04
Skin 3	2.81	3.9	23.6	8.04	1.69	38.8

Due to the remaining DNA in the RNA extracts, PCR was performed using the DNase-treated RNA extracts before cDNA synthesis to examine if the DNA would amplify, which could potentially influence the later PCR based on cDNA. No PCR products were obtained where non-diluted and 1:10 diluted RNA extracts by Kit C and Kit D were used as templates (Figure 3.1.2). For comparison, cDNA was used as templates in identical PCR reactions and resulted in a few PCR products from both kits (Figure 3.1.2).

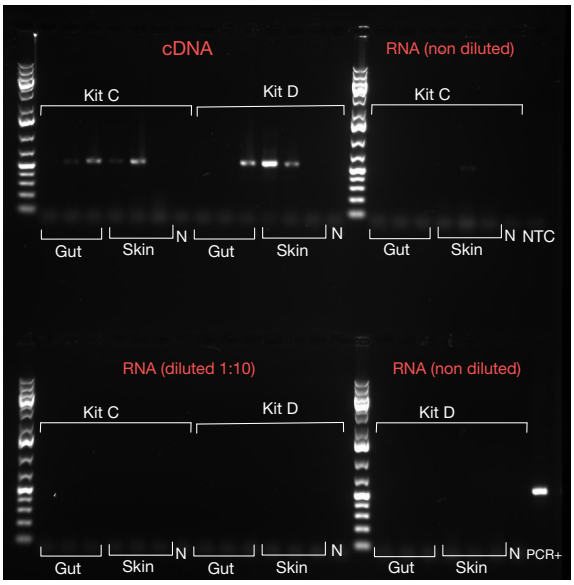


Figure 3.1.2: Agarose gel showing PCR products of the V3+V4 regions of the 16S rRNA gene where cDNA, undiluted RNA, and diluted (1:10) RNA from RNA extracts isolated by Kit C and Kit D 2.1.1) were used as templates. Amplification was performed with 35 cycles and an annealing temperature of 55℃ using primers III-341F KI and III-805R (Table 2.1.2). RNA extracts of three replicates of gut and skin samples from salmon yolk-sac fry were used as templates. Negative and positive control was included for each extraction kit. Additionally, a non-template PCR control (NTC) and a positive PCR control (PCR+) were included.

All RNA extracts obtained by the two kits were reverse transcribed to cDNA using both 2μL and 4μL RNA as templates, and the cDNA was further used as templates in PCR (Figure 3.1.3). Similar amounts of PCR products were obtained from cDNA using either volume of RNA as templates. From Kit D, the highest number of detectable PCR products were obtained, while Kit C resulted in fewer and weaker PCR products. The cDNA samples using 2μL of RNA as templates were also diluted 1:10 before being used as templates in the PCR. This yielded only two weak PCR products.

In summary, the RNA extracts obtained with Kit D yielded the most successful PCR amplification of the v3+v4 region of the 16S rRNA gene.

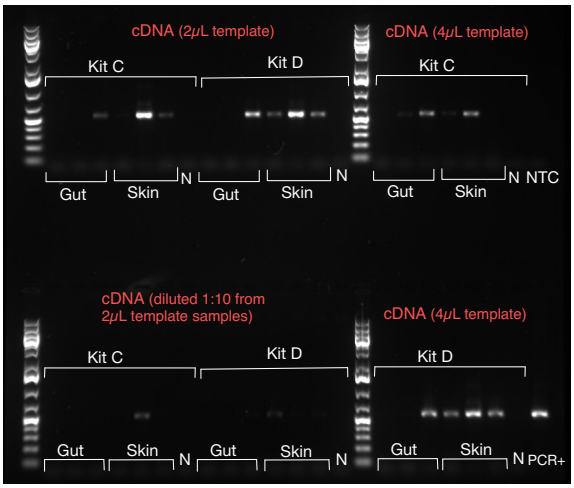


Figure 3.1.3: Agarose gel showing PCR products of the V3+V4 regions of the 16S rRNA gene using cDNA (2μL, undiluted and 1:10 dilution), and 4μL RNA obtained from salmon yolk-sac fry as templates. Amplification was performed with 35 cycles and an annealing temperature of 55℃ using primers Ill-341F KI and Ill-805R (Table 2.1.2). RNA extracts of three replicates of gut and skin samples from salmon yolk-sac fry were used as templates. Negative and positive control was included for each extraction kit. Additionally, a non-template PCR control (NTC) and a positive PCR control (PCR+) were included.

3.2 Optimization of the PCR protocol for amplifying bacterial 16S rDNA from the lab-scale RAS salmon fry samples

Amplification of the v3+v4 region of the 16S rRNA gene from the extracted DNA samples of the Atlantic salmon fry from the lab-scale RAS experiment was proven to be challenging. This has also been indicated in previous projects within the ACMS research group, where the amplification of bacterial DNA from Atlantic salmon has been difficult. This can be due to inhibitors in the DNA extract, or it can also be due to low levels of bacterial DNA compared to host DNA.

Based on the obtained results of the DNA-extraction kits evaluation described in section 3.1, extraction kit B (96 MagBead DNA kit, ZymoBIOMICS™) was used to extract DNA from samples collected in the lab-scale RAS experiment (Table 2.2.1). The DNA concentrations and A260/280 purity ratios of a selection of DNA extracts of the Atlantic salmon fry samples were determined using NanoDrop™ One Microvolume UV-Vis Spectrophotometer[56]. The concentration and purity were determined for three of each of the salmon sample types (intestine, skin, and gill) extracted by 6 MagBead DNA kit (Kit B; ZymoBIOMICS™) (Table 3.2.1). The skin and gill samples yielded the purest DNA extracts (A260/A280 ratio of 1.8), and also the highest DNA concentrations.

Table 3.2.1: DNA concentrations and A260/280 purity ratios of a random selection of DNA extracts from the lab-scaled RAS Atlantic salmon fry samples (section 2.2.7). The concentrations were determined with NanoDrop™ One.

Samples	DNA (ng/μL)	A260/280
Intestine 1	1.0	1.63
Intestine 2	5.6	1.36
Intestine 3	0.6	1.85
Skin 1	71.2	1.89
Skin 2	50.6	1.83
Skin 3	55.2	1.88
Gill 1	102.5	1.87
Gill 2	92.0	1.85
Gill 3	95.8	1.86

Prior to PCR amplification of bacterial 16S rDNA from DNA extracts from all salmon fry samples, PCR was optimized for a selection of samples. Various PCR conditions were examined, such as the annealing temperature, number of cycles, template volume and concentration, and addition of $MgCl_2$.

First, PCR for a selection of skin, intestine, and gill samples was performed at 55℃ annealing temperature, 35 cycles, and undiluted DNA extracts were used as templates. This resulted in non-detectable amounts of PCR product for any of the samples (results not shown). Next, the effect of diluting the DNA extracts used as templates and increasing the number of cycles was investigated. PCR was performed at 55℃ annealing temperature, 38 cycles, and as templates 2μL of DNA extracts diluted 1:10

and 1:100 of three- skin, intestine, and gill samples were used. No detectable amounts of PCR product were observed for any of the samples (results not shown). Further, the effect of adding 0.5 mM $MgCl_2$ was examined with PCR at 55℃ annealing temperature, 38 cycles, and 2μL of the diluted DNA extracts (1:10 and 1:100) as templates. Neither this resulted in successful PCR amplification of 16S rDNA (results not shown).

Next, an increase in the annealing temperature was explored. PCR at 56^oC annealing temperature, 38 cycles, and 1:10 diluted DNA extracts from skin, intestine, and gill samples were used as templates. This resulted in PCR products of the expected length for all the intestine samples (Figure 3.2.1). These PCR products were utilized for the next steps in the preparation of the Amplicon library for Illumina sequencing. However, the PCR resulted in strong primer dimer formation for three intestine samples from RAS_A (d1). Due to this, these three samples were discarded from further analysis of the bacterial community composition.

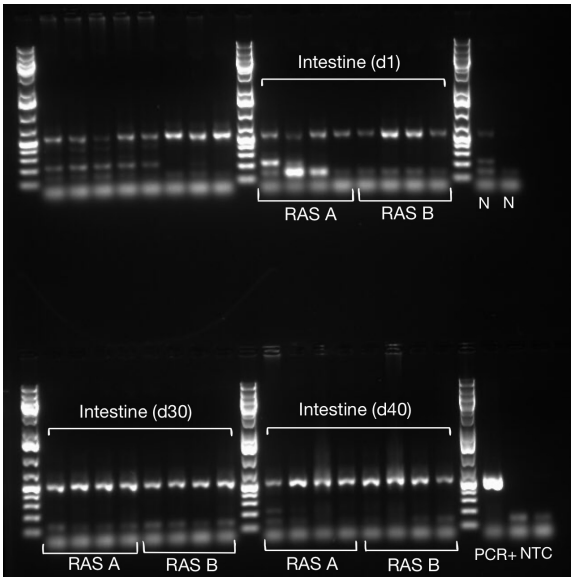


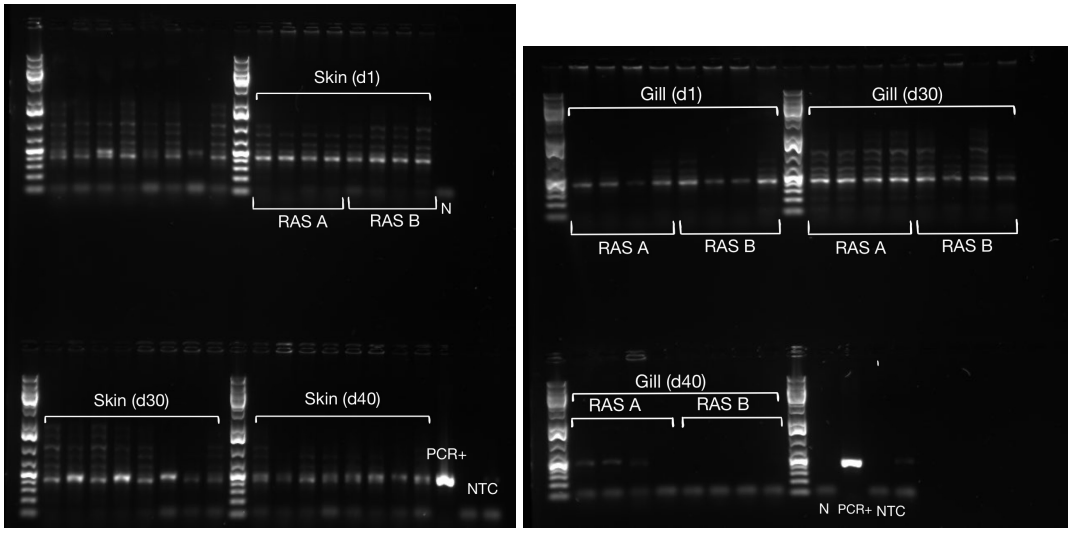
Figure 3.2.1: Agarose gel electrophoresis of PCR products obtained by primers Ill-341F_KI and Ill-805R (Table 2.1.2) of the v3+v4 regions of the 16S rRNA gene from DNA extracts of the lab-scale RAS salmon fry intestine. The PCR products were obtained by using 1:10 diluted DNA extracts of the salmon fry intestine, and four intestine samples from each RAS (A and B) at three different sampling time points (d1, d30, and d40) were included. The PCR reaction was run for 38 cycles and an annealing temperature of 56℃. Two negative controls for the extraction kit are included and for the PCR two non-template controls (NTC) and one positive PCR control (PCR+) are included.

PCR amplification of bacterial 16S rDNA from skin and gill DNA extracts was still not successful. Therefore, PCR using primers without Illumina adapter sequences (341F and 805R) was evaluated to avoid the problem of primer dimer formation and obtain PCR products with the desired length. PCR was conducted at 56^oC annealing temperature, 38 cycles, and 1:10 diluted DNA extracts from skin and gill samples used as templates. This resulted in successful PCR amplification of 16S rDNA (Figure 3.2.2). Varied amounts of PCR products of DNA extract from all skin samples were obtained

(Figure 3.2.2a). The majority of DNA extracts of gill samples collected on Day_40 did not yield any detectable PCR product (Figure 3.2.2b), and the gill samples were therefore discarded and not analyzed further.

To summarize, the optimization of the PCR protocol resulted in PCR products of the DNA extracts of intestine samples by performing PCR at 56^oC annealing temperature, 38 cycles, and 1:10 diluted DNA extracts. PCR products of only 16S rDNA target sequence of the DNA extracts from skin and gill samples were obtained by PCR using primers without Illumina adapter sequences.

To continue the preparation of the amplicon library for Illumina sequencing, Illumina adapter sequences needed to be added to the PCR products of the DNA extracts from the skin samples. These PCR products were normalized using the SequalPrep™ Normalization Plate (96) Kit (Invitrogen™) and then amplified by a short PCR (12 cycles) by the use of Illumina primers Ill-341F_KI and Ill-805R. PCR products were acquired from all skin samples, except one sample from RAS_A on Day_30.



(a) **(b)**

Figure 3.2.2: Agarose gel electrophoresis of PCR products of the v3+v4 regions of the 16S rRNA gene obtained by primers 341_F and 805R from DNA extracts of salmon fry skin and gill. The PCR products were obtained by using a) 1:10 diluted DNA extracts of skin samples as templates and b) 1:10 diluted DNA extracts of gill samples as templates. The PCR reactions consisted of 38 cycles and 56℃ annealing temperature. Four replicates of skin and gill samples from each RAS (A and B) at three different sampling times (d1, d30, and d40) are included. Additionally, negative control for the extraction kit, non-template control (NTC), and positive control (PCR+) for the PCR are included.

3.3 Rearing of Atlantic salmon in lab-scale RAS

To examine the biofilter-biofilm bacterial community's influence on the microbiota of the water and salmon fry, the salmon fry was reared in identical RAS with different biofilm carriers for 40 days. On Day_30 of the experiment, the biofilm carriers originating from Belsvik located in the biofilter in RAS_A was swapped with the biofilm carriers originating from Kjærelva located in the biofilter in RAS_B (See Figure 2.2.1). Samples for bacterial community analysis were collected on Day_1, Day_30 (before the biofilter swap), and Day_40 of the experiment.

3.3.1 Water quality

During the 40 days lab-scale RAS experiment of rearing Atlantic salmon fry in two identical RAS with different biofilm carriers in the biofilters, multiple water quality parameters were measured (Table 3.3.1). Dissolved oxygen (DO, mg/L), pH, and temperature in both RAS were stable and within the recommended threshold values for Atlantic salmon rearing throughout the experiment. The average values were 9.00 (mg/L) for DO, 7.00 for pH, and an average temperature of 11℃. Total dissolved solids (TDS, mg/L) and conductivity (uS/cm) were similar in both systems and the TDS concentration increased from around 200 mg/L to over 300 mg/L, and the conductivity increased from around 370 uS/cm to around 500 uS/cm in both RAS over the duration of the project (Table 3.3.1). The concentration of the nitrogenous waste components total ammonia nitrogen (TAN, mg/L) and nitrite (mg/L) was low throughout the period. The highest concentration of TAN detected was 0.38 mg/L in RAS_A and 0.25 mg/L in RAS_B, while the highest nitrite concentration was 0.1 mg/L in both systems. Nitrate (mg/L) increased in the period before the biofilter swap and reached a concentration of 640 mg/L in both RAS on Day_19. After this day, the nitrate concentration decreased in both systems, most likely due to high water exchange in this period.

Table 3.3.1: Water quality parameters measured in RAS_A and RAS_B before and after biofilter swap between the systems (average ± SD). All variables were measured in the sump (see Figure 2.2.2), except the dissolved oxygen and temperature which were measured in the rearing tanks.

Period	**Before swap (day 0-30)**		**After swap (day 31-40)**	
System	**RAS_A**	**RAS_B**	**RAS_A**	**RAS_B**
DO (mg/L)	9,26 ± 0.50	9,39 ± 0,39	8,79 ± 0,42	9,00 ± 0,56
pH	7,16 ± 0,38	6,96 ± 0,53	6,89 ± 0,24	6,94 ± 0,28
Temperature (℃)	11,52 ± 0,33	11,50 ± 0,27	11,83 ± 0,09	11,82 ± 0,16
Alkalinity (mg/L)	56,32 ± 21,23	57,53 ± 17,46	51,78 ± 13,72	51,78 ± 13,72
TDS (mg/L)	197,11 ± 102,96	205,94 ± 62,75	337,56 ± 78,41	328,00 ± 89,48
Conductivity (uS/cm)	365,13 ± 141,52	376,06 ±73,18	494,56 ± 89,98	506,22 ±139,99
TAN (mg/L)	0.08 ± 0,13	0,01 ± 0,06	0,00 ± 0,00	0,00 ± 0,00
Nitrite (mg/L)	0,00 ± 0,00	0,005 ± 0,02	0,00 ± 0,00	0,00 ± 0,00
Nitrate (mg/L)	154,63 ± 163,43	171,76 ± 162,26	45 ± 19,15	35,00 ± 20,82

During the experiment, approximately 5% of the total water in both RAS was exchanged every day, with the exception of unforeseen events. An ongoing problem in both systems was clogging of the mechanical filter at night, causing water to flow out of the system, and resulting in higher water exchange due to addition of makeup water. Between 15 and 30 liters of water had to be added to the systems on several occasions from Day_10 to Day_32 of the experiment to compensate for water loss (Appendix I). The systems were treated identically and new water was added according to which system lost the most. Later in the experiment, on Day_26, the water in the tanks of RAS_A occasionally overflowed. This issue persuaded until Day_38 when accumulated particles were removed from pipes in both of the systems.

Overall, the water quality in the systems was generally satisfying and relatively similar across RAS_A and RAS_B until the experiment ended on Day_40. As earlier described in section 2.2.1 the systems crashed on Day_40 due to an unexpected power outage. Tragically, this led to no addition of oxygen to the water, and the fish died because of oxygen deficiency.

3.3.2 Fish performance

The specific growth rate (SGR) based on the weight (g) of the reared Atlantic salmon fry from the start of the experiment (Day_0) to the end of the experiment (Day_40) was between 1.26-1.46% for each tank (Table 3.3.2). There was no significant difference in SGR between RAS_A and RAS_B (one-way ANOVA $p>0.05$). The weight of the salmon fry varied in the tanks. On Day_40 the sampled fish were between 7.93 and 16.28 grams and between 8.6 and 16.3 cm long. No sores or abnormalities were observed on the salmon fry during the experiment. Additionally, there was no mortality before the power outage on Day_40, when all fish died due to oxygen deficiency.

Table 3.3.2: The total fish biomass in each tank and the average individual weight based on the total biomass at the start of the experiment (Day_0) and the end of the experiment (Day_30). Also, the number of salmon fry in each tank at the start and the end of the experiment, and the SGR (%).

	Start of the experiment Day_0			**End of the experiment Day_40**			
RAS fish tank	Number of fish	Biomass(g)	Weight per fish (g)	Number of fish	Biomass(g)	Weight per fish (g)	**SGR (%)**
A1	51	349.63	6.86	44	498.56	11.33	**1.26**
A2	52	358.99	6.90	41	491.42	11.99	**1.38**
A3	52	343.9	6.61	42	491.22	11.67	**1.43**
B1	52	354.23	6.81	46	510.56	11.10	**1.22**
B2	52	336.15	6.46	42	486.76	11.59	**1.46**
B3	52	342.09	6.58	46	526.16	11.44	**1.38**

3.4 Bacterial community analysis for biofilter, water, and salmon fry samples from the lab-scale RAS

Samples for microbial community analysis were collected at three different time points throughout the experiment. The first sampling occurred on Day_1, followed by the second sampling on Day_30 before the biofilm carriers were swapped between the systems. Finally, the third sampling was conducted 40 days into the experiment. From RAS_A and RAS_B, a total of 132 samples were collected from the biofilm carriers, tank water, and salmon fry for analysis. However, the v3 + v4 region of the 16S rRNA gene could not be amplified from all of the samples, resulting in 104 samples being sent for Illumina sequencing. After Illumina sequencing, a total of 4589 ASVs were identified and used to generate an ASV table, where 48 ASVs were removed, resulting in 4541 ASVs, that were normalized to 16400 reads per sample to get comparable measures.

3.4.1 Alpha diversity

The average of the alpha diversity measures, the estimated richness (Chao-1), and the observed richness were determined for groups of samples (Figure 3.4.1).

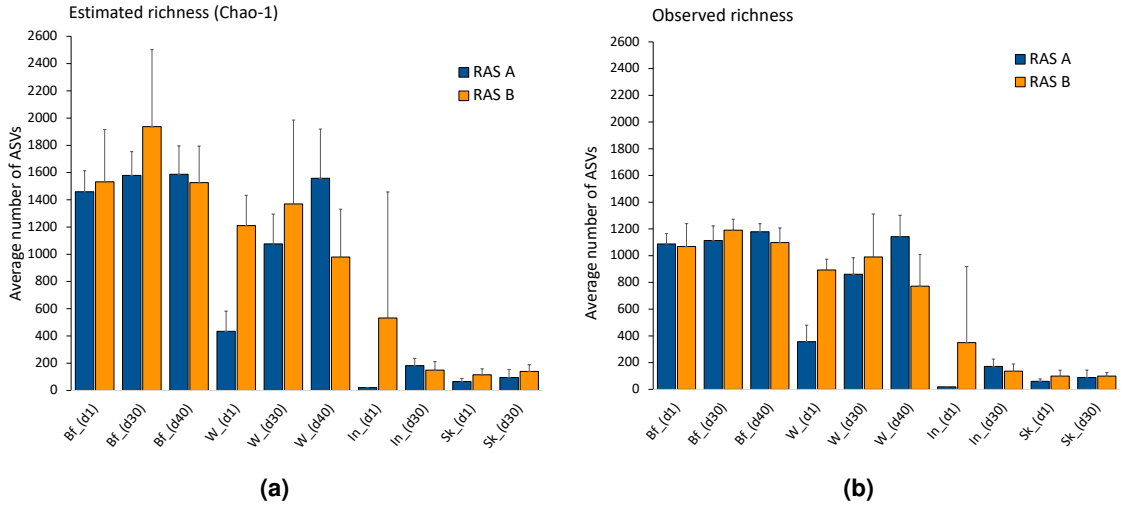


Figure 3.4.1: Average ASV richness indices for sample groups from lab-scale RAS_A and RAS_B. a) Estimated ASV richness (Chao1), and b) Observed ASV richness. The error bars indicate the standard deviations. The sample groups are designed as follows: Bf: biofilter samples; W: water samples; In: intestine samples, Sk: Skin samples; d1: sampling Day_1; d30: sampling Day_30; d40: sampling Day_40. The richness indices were based on the normalized ASV table (normalized to 16 400 reads per sample).

The average sequence coverage between the observed richness and the estimated richness (Chao-1) taking all sample groups into account was determined as 76.0 ± 14.1% (±SD). The biofilm carrier samples had the overall highest ASV richness, closely followed by the water samples, while the lowest ASV richness was found in the salmon fry samples. Further statistical tests revealed that the observed ASV richness was

significantly different between the different sample groups for one or multiple sample groups (biofilter, water, skin, and gut samples) (One-way ANOVA, $p<0.01$). A Post-hoc Tukey HSD Test showed a significant difference in observed ASV richness between all sample groups ($p<0.05$), with the exception of between skin and intestine samples ($p>0.05$). Comparing the observed ASV richness for RAS_A and RAS_B samples, no significant difference was found (one-way ANOVA $p>0.05$). Turning to the different biofilters, the observed ASV richness in sample groups where Kjærelva carriers were located in the RAS was not statistically different from the sample groups where Belsvik carriers were located in the RAS (t-test, $p>0.05$).

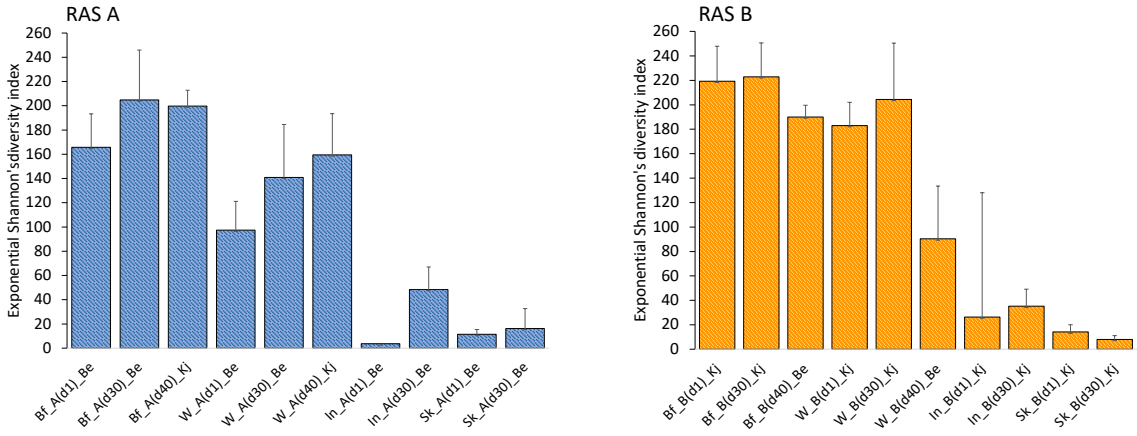


Figure 3.4.2: Average exponential Shannon's diversity for sample groups from RAS_A and B. The error bars indicate the standard deviations. The sample groups are designated as follows: Bf: biofilter samples; W: water samples; In: intestine samples; Sk: Skin samples; A: RAS_A; B: RAS_B; d1: sampling Day_1; d30: sampling Day_30; d40: sampling Day_40; Kj: biofilter with carriers from Kjærelva; Be: biofilter with carriers from Belsvik. The Shannon's diversity index was based on the normalized ASV table (normalized to 16 400 reads per sample).

The alpha diversity was also highest for the biofilter samples in terms of the exponential Shannon's diversity and lowest for the gut and skin samples (Figure 3.4.2). The exponential Shannon's diversity for the biofilter samples was significantly higher than for the water samples (t-test, $p<0.05$). There were no significant differences between the observed richness and Shannon's diversity in the intestine and skin samples (t-test, $p=0.28$ and $p=0.17$, observed richness and Shannon's diversity respectively).

3.4.2 Bacterial community compositions

The bacterial community composition for the biofilm carriers, rearing water, and salmon fry (skin and intestine) in RAS_A and B at the different time points were determined at order level (Figure 3.4.3).

The most dominant orders in skin and intestine samples were *Bacillales* in skin samples (up to 66.6%) and *Lactobacillales* in intestine samples (up to 60.9%). *Clostrodiales* were also abundant in the intestine samples (up to 41.7%). Further, in the bacterial

communities of the biofilter carriers, *Nitrospirales* was the most dominant order, with a maximum abundance as high as 40.9% in the Belsvik biofilm carrier samples and 15.1% in Kjærelva biofilm carrier samples. Other orders with similar high relative abundance in Belsvik- and Kjærelva biofilm carriers were *Rhodobacterales* and *Burkholderiales*. *Flavobacteriales* were more abundant in the Kjærelva carriers, especially on Day_1.

In the bacterial communities of the water samples, the most dominant order was *Burkholderiales* (up to 24.2%). In addition, *Sphingomonadales* (up to 20.0%) and *Rhodobacterales* (up to 14.6%) were abundant. In the water communities in RAS_B on Day_1 with Kjærelva biofilm carriers as the biofilter, a high abundance of *Flavobacteriales* was observed. Water samples from RAS_A on Day_1 with Belsvik carriers as the biofilter had high relative abundances of *Pseudomonadales* (up to 15.6%) and *Chlamydiales* (up to 5.44%). *Propionibacteriales* were also very abundant in one water sample from RAS_A on Day_1 (23.5%) and *Nitrospirales* were very abundant in one water sample from RAS_B on Day_1 (22.7%).

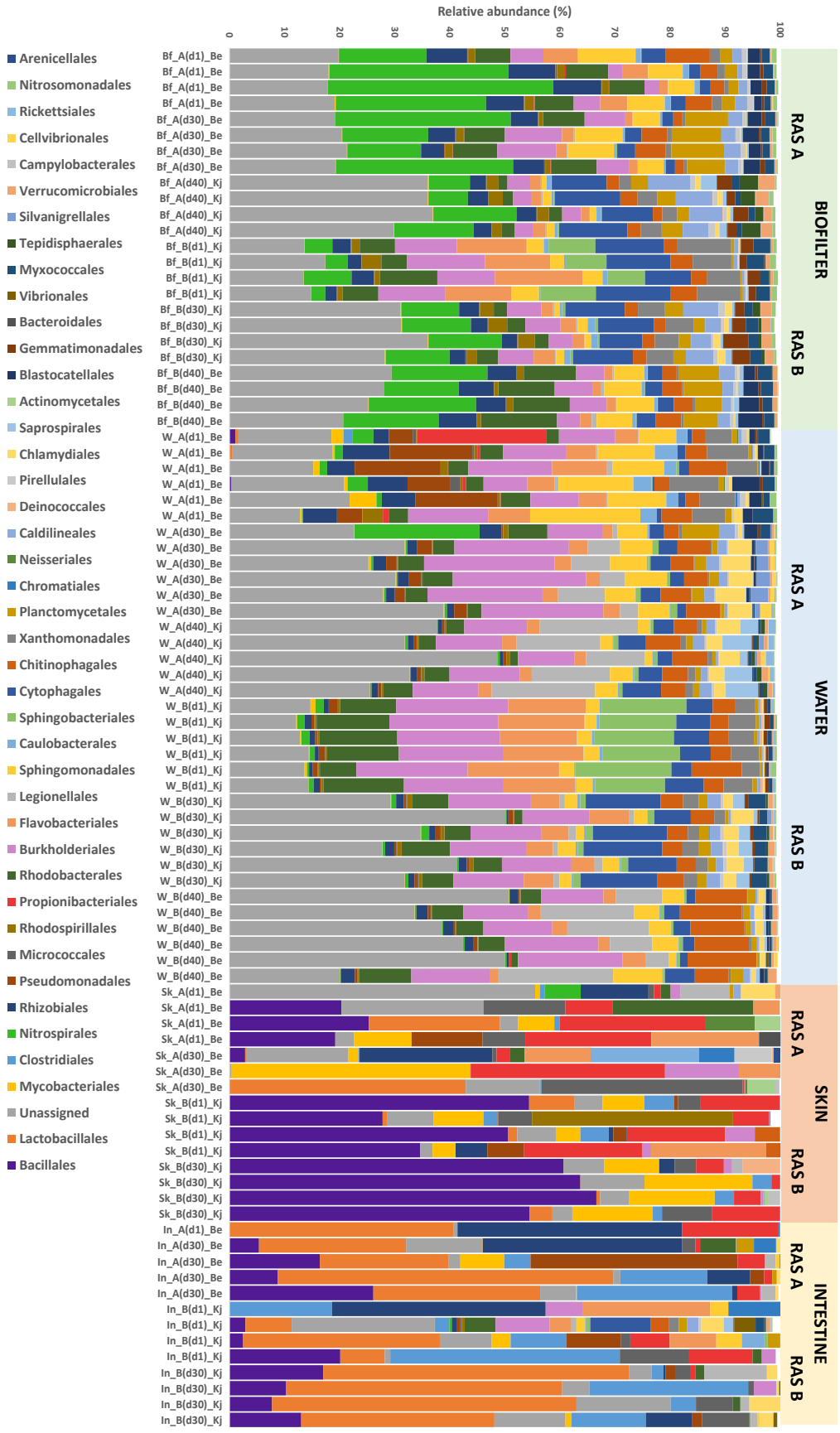


Figure 3.4.3: Bacterial community composition at order level for biofilter-, water- and salmon fry (skin and intestine) samples from RAS_A and RAS_B, at three different time points (d1, d30 and d40). Orders with a minimum relative abundance above 1% in at least one sample are included.

3.4.3 Beta diversity

To assess the similarities and dissimilarities in bacterial community between all samples, a PCoA ordination plot based on Bray-Curtis similarities was constructed (Figure 3.4.4). In the PCoA ordination, the Kjærelva biofilm carrier samples and the Belsvik biofilm carrier samples formed distinct clusters, regardless of which RAS they were located in. This observation suggests that the microbial compositions of the two types of biofilters, originating from hatcheries at different geographical locations, differed from each other and remained relatively stable over time.

Among the water, skin, and intestine samples, the water microbial community compositions exhibited a higher degree of similarity to the corresponding biofilter samples throughout the experiment (Figure 3.4.4). The water samples are clustered close to the belonging systems biofilter samples from the beginning of the experiment (Day_1) and are even closer to the belonging biofilter on Day_30. Then, on Day_40, ten days after the biofilter swap between RAS_A and RAS_B, the water samples shift towards the new belonging biofilter. This indicates that the bacterial community composition in the water samples was influenced by the biofilter's microbiota. Further, the PCoA ordination indicated that the microbiota of the salmon fry skin- and intestine differed from the microbiota of the water- and biofilter.

To visualize similarities and dissimilarities within each sample type, new PCoA ordination plots based on the Bray-Curtis similarities were composed for each sample type (Figure 3.4.5).

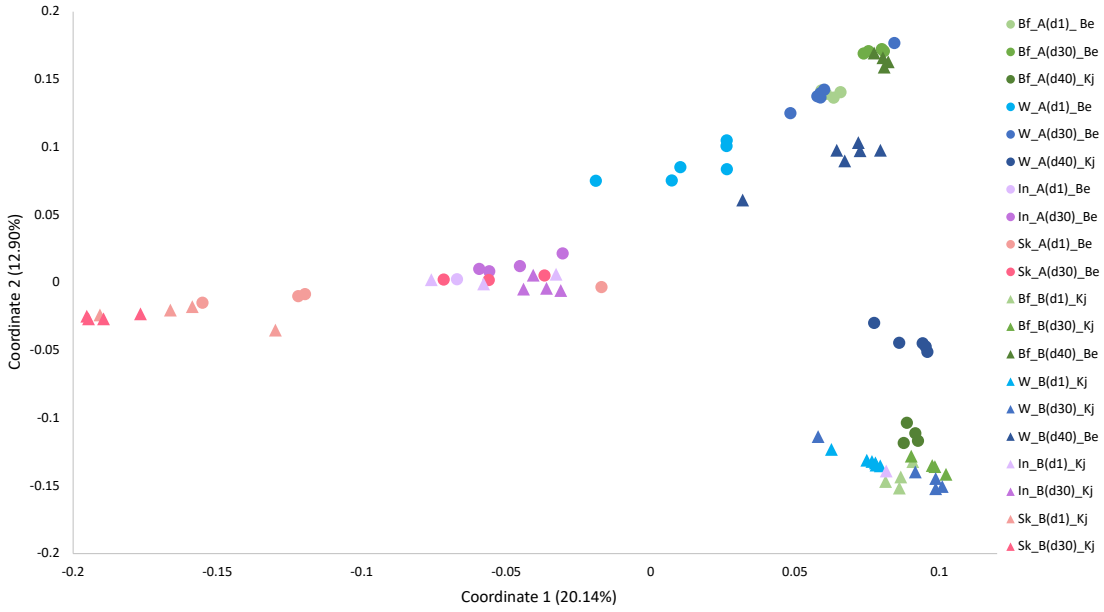


Figure 3.4.4: PCoA ordination based on Bray-Curtis similarities for microbial community profiles of all samples; Biofilter (Bf), Water (w), Skin (Sk), Intestine (In). A:RAS_A, B:RAS_B, d1:Day_1, d30:Day_30, d40:Day_40, Kj:biofilm carriers originating from Kjærelva located in the RAS, Be: biofilm carriers originating from Belsvik located in the RAS.

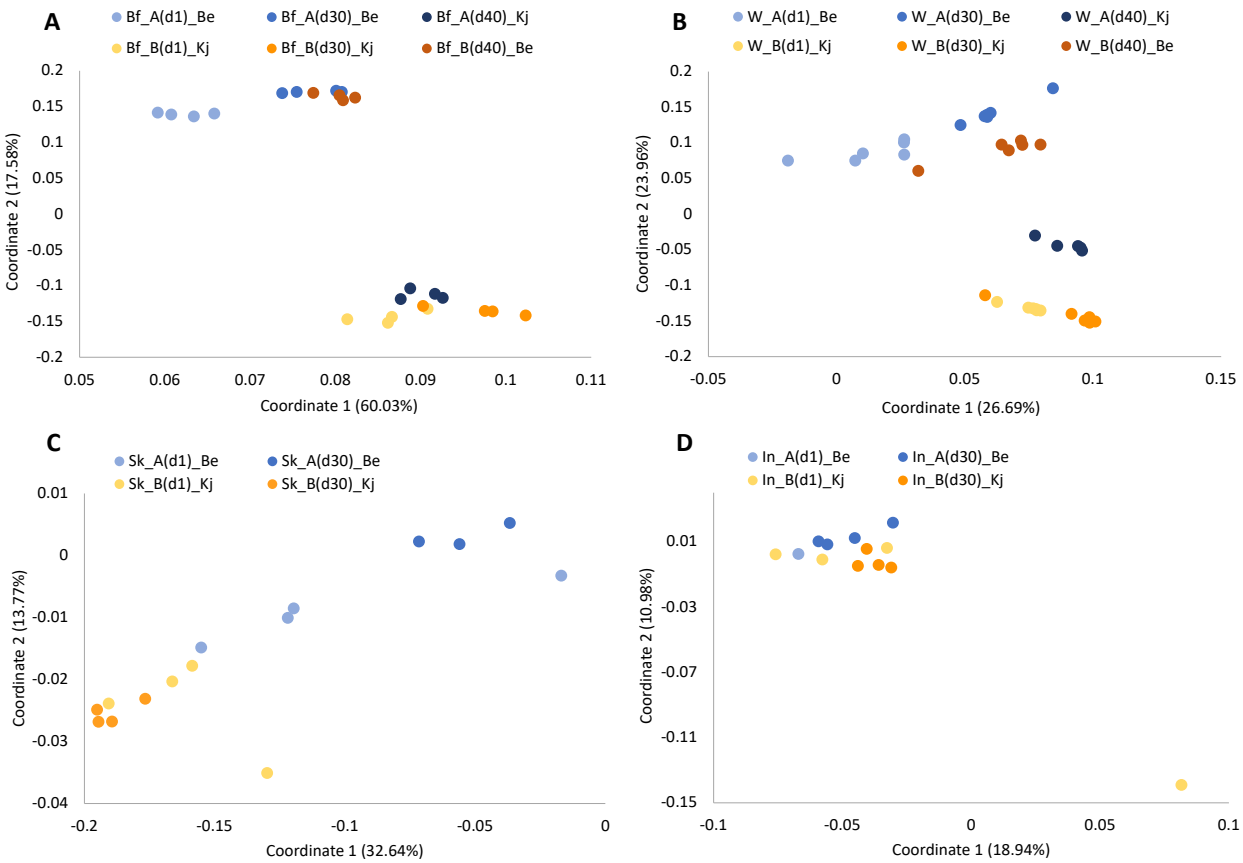


Figure 3.4.5: PCoA ordination plots based on Bray-Curtis similarities separated on sample type; A) Biofilter (Bf), B) Water (w), C) Skin (Sk), and D) Intestine. Sample groups are designed as follows: A:RAS_A; B:RAS_B; d1:Day_1; d30:Day_30; d40:Day_40; Kj:biofilm carriers originating from Kjærelva located in the RAS; Be: biofilm carriers originating from Belsvik located in the RAS.

Bacterial communities of the biofilter carrier samples

The bacterial communities on the biofilm carriers originating from the Kjærelva- and Belsvik biofilters were dissimilar throughout the experiment, as shown in Figure 3.4.5A. A one-way PERMANOVA confirmed that the bacterial communities on the two different types of biofilter-biofilm carriers were significantly different at all sampling times despite which RAS they were located in ($p=0.0001$). This is also observed when comparing the Bray-Curtis similarity between the biofilter-biofilm communities in RAS_A and RAS_B, which shows high dissimilarity throughout the experiment, with average Bray-Curtis similarities of 0.13 on Day_1 and 0.16 on both Day_30 and Day_40 (Figure 3.4.6c). Additionally, no significant differences were observed within the bacterial communities on the Kjærelva- and Belsvik biofilm carriers when comparing all three sampling times (one-way PERMANOVA with Bonferroni corrected p-values, $p>0.05$). This indicates that the biofilter-biofilm bacterial communities did not significantly change during the experiment.

The similarity in the biofilter biofilm communities within and between the sample times separated on RAS was investigated by determining the Bray-Curtis similarities (Figure 3.4.6). High similarities were observed among replicate carrier samples from the same sampling time, for RAS_A (Figure 3.4.6a) and RAS_B (Figure 3.4.6b). Replicate carriers sampled on the same day had highly similar bacterial communities within each of the RAS, with average Bray-Curtis similarities as high as 0.7 to 0.9. Furthermore, less variation was observed for the biofilm communities of the Kjærelva carriers within each sampling time.

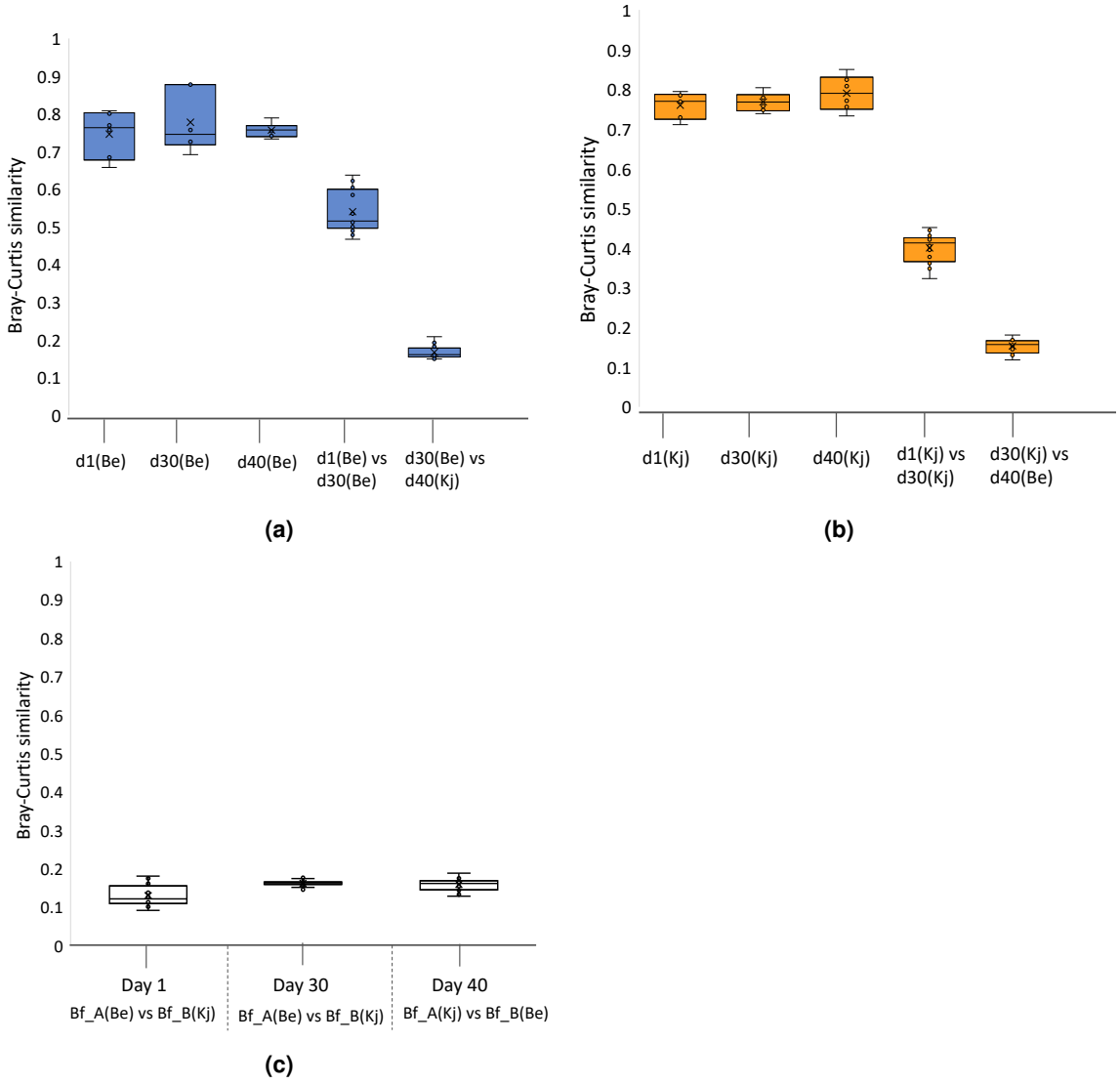


Figure 3.4.6: Box plot showing Bray-Curtis similarities for comparison of the biofilter-biofilm communities within and between the three sampling times in a) RAS_A and in b) RAS_B, and c) between RAS_A and RAS_B. There are four biofilm-carrier replicates from each biofilter from the three sampling days; d1:Day_1; d30:Day_30; d40:Day_40. Kj: Kjærelva biofilm carriers, Be: Belsvik biofilm carriers.

However, when comparing the bacterial communities between Day_1 and Day_30 in both RAS, less similarity was observed, implying that the biofilm communities of the carriers had changed during the first month of the experiment. The Kjærelva biofilm carriers in RAS_B underwent a larger change compared to the Belsvik biofilter in RAS_A between Day_1 and Day_30. Further, when comparing the bacterial communities of the Belsvik- and Kjærelva biofilm carriers from Day_30 and Day_40 of the experiment, the average Bray-Curtis similarities for RAS_A was 0.17 and for RAS_B 0.15, due to the swap of biofilm carriers between the systems.

In addition, when comparing the Bray-Curtis similarities between Day_30 and Day_40 across RAS, thus comparing the microbiota of the same biofilter type, high similarities were calculated (average Bray-Curtis similarities of 0.7 for both Kjærelva and Belsvik).

To further examine and compare the microbiota of the two different biofilters, the nitrifying communities were inspected (Figure 3.4.7). The ASVs representing nitrifying bacteria were identified manually by inspecting the normalized ASV table with the assigned taxonomy. A total of 40 ASVs were found to represent nitrifying bacteria, when including ASVs that had a maximum abundance of at least 0.1% in at least one sample. The majority of these ASVs were classified as the nitrite-oxidizing species *Nitrospira*, while a few were classified as the ammonia-oxidizing species *Nitrosomonas*. At the start of the experiment (Day_1), a much higher relative abundance of ASVs potentially representing nitrifying bacteria was observed in the Belsvik biofilm carrier communities. The most abundant ASV represented in the microbiota of all the biofilm carrier samples was ASV 7 (*Nitrospira*), with an average abundance of 12%. ASV 17 (*Nitrospira*) and ASV 32 (*Nitrospira*) were the other most abundant ASVs, with a total average abundance of 8% and 7% respectively. These ASVs were essentially only identified in the Belsvik biofilter samples. The Kjærelva biofilter samples had a high relative abundance of ASV 97 (*Nitrospira*), which is not highly represented in the Belsvik biofilter samples.

To identify the ASVs contributing to the most difference in the bacterial communities between Kjærelva and Belsvik biofilters a SIMPER analysis based on Bray-Curtis similarities was performed. The most contributing ASVs were ASV 17, ASV 16, ASV 32 and ASV 7. Only ASV 16 is not classified as Nitrospira but as *Bacteroidetes* and was most abundant in Belsvik carrier communities. These four ASVs contributed to 10% of the differences in bacterial community composition between the Belsvik- and Kjærelva carrier samples.

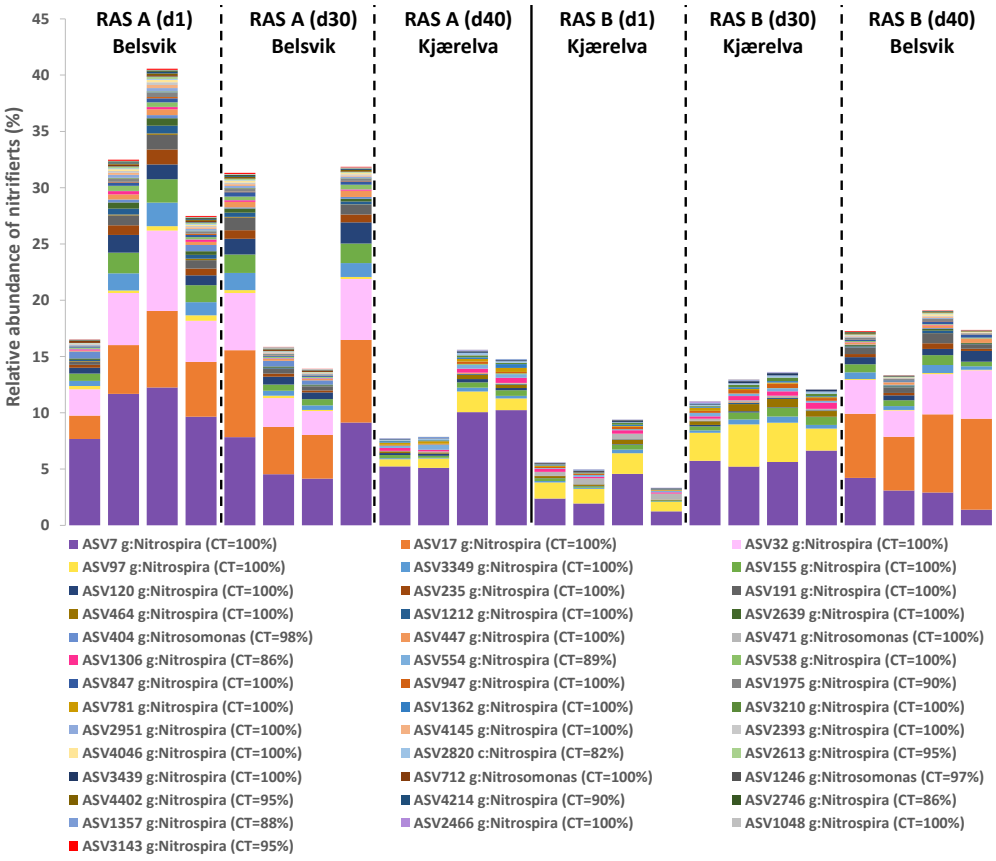


Figure 3.4.7: Relative abundance of ASVs classified as nitrifiers with an 80% confidence threshold (CT) in the biofilm carrier samples. Only ASVs with a maximum relative abundance over 0.1% in at least one sample are included. The taxonomy of the ASVs is given at the lowest level obtained, the majority at the genus (g) level, and one ASV at the class (c) level. The biofilter samples are separated into six groups based on RAS (A or B), biofilter (Belsvik or Kjærelva), and time points in the experiment (Day_1, Day_30, or Day_40). Each group consists of four samples.

The majority of ASVs representing the nitrifying communities in the biofilters were classified as Nitrospira. To investigate the relationship between these ASVs and previously described Nitrospira a phylogenetic tree was made (Figure 3.4.8).

The phylogenetic tree indicates that ASV 7, ASV 155, and ASV 3349 are related to N. defluvii, while ASV 97 is related to N. japonica. As previously mentioned, ASV 7 was the most abundant ASV in Belsvik carrier samples, while ASV 97 was very abundant in Kjærelva carrier samples. ASV 155 and ASV 3349 were not very abundant in any of the biofilter samples and were fairly evenly distributed, but slightly more abundant in the Belsvik carrier samples. Further, the phylogenetic tree indicates that multiple ASVs can be related to Nitrospira representing complete ammonia oxidizers (comammox), however, with relatively low bootstrap support.

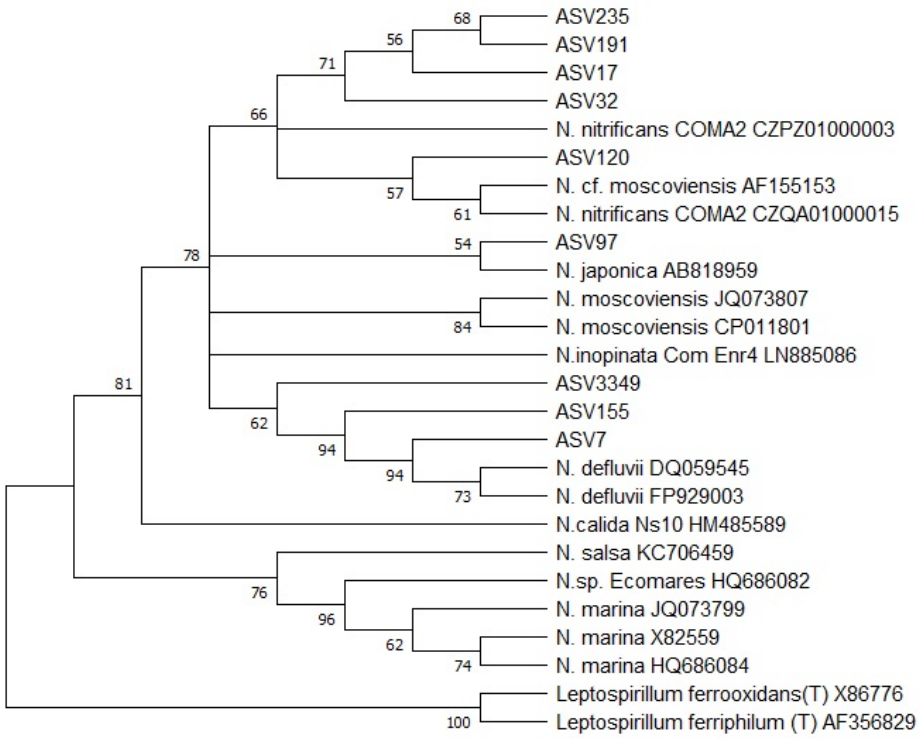


Figure 3.4.8: Phylogenetic tree showing the relationships between ASVs classified as Nitrospira and previously published Nitrospira 16S rDNA sequences. The tree was inferred in MEGA-X by using the Maximum Likelihood method with 1000 bootstrap replicates and the Tamura-Nei model for sequence evolution. The tree was rooted at the Leptospirillum node. Bootstrap support values are shown at the nodes. The tree was condensed with a 50% cutoff value. Only ASVs with a relative abundance >1% in at least one sample were included. Sequences were retrieved from RDP database or the NCBI Genbank. Accession numbers are specified for with the species names. Type strains are indicated by a (T).

Bacterial communities of the rearing water

The bacterial communities of the rearing water within RAS_A and RAS_B were significantly different at all three sampling times (one-way PERMANOVA with Bonferroni-corrected p-values, $p<0.05$ for both RAS). This indicates that the water microbiota changed over time within each RAS. Comparison of the water- and biofilter-biofilm microbiota, within RAS_A and RAS_B at each sampling time also resulted in a significant difference between the communities (One-way PERMANOVA, $p<0.05$ for all comparisons).

The PCoA ordination for water samples indicates a difference in the bacterial communities between RAS_A with Belsvik biofilter and RAS_B with Kjærelva biofilter on Day_1 and Day_30 of the experiment (Figure 3.4.4B). However, after the biofilter swap, on Day_40 of the experiment, the water communities were more similar to the new, swapped, biofilter carriers. This indicates that the bacterial community in the rearing water was affected by the bacterial community on the biofilm carriers.

The similarity in the water communities within and between the sampling times separated on RAS was investigated by determining the Bray-Curtis similarities (Figure 3.4.9). There was a higher similarity in the microbiota of the water samples collected on Day_1 of the experiment in RAS_B with the Kjærelva biofilter (Figure 3.4.9b, than in RAS_A with the Belsvik biofilter (Figure 3.4.9a). However, there was a comparable similarity and variation within samples collected on Day_30 of the experiment for both RAS. Comparison between the water microbiota on Day_1 and Day_30 in both RAS showed a high dissimilarity, indicating a change in the water microbiota during the first month of the experiment. A high dissimilarity was also observed between the water microbiota on Day_30 and Day_40 (before and after the biofilter swap) in both RAS, suggesting a change in the water microbiota in 10 days.

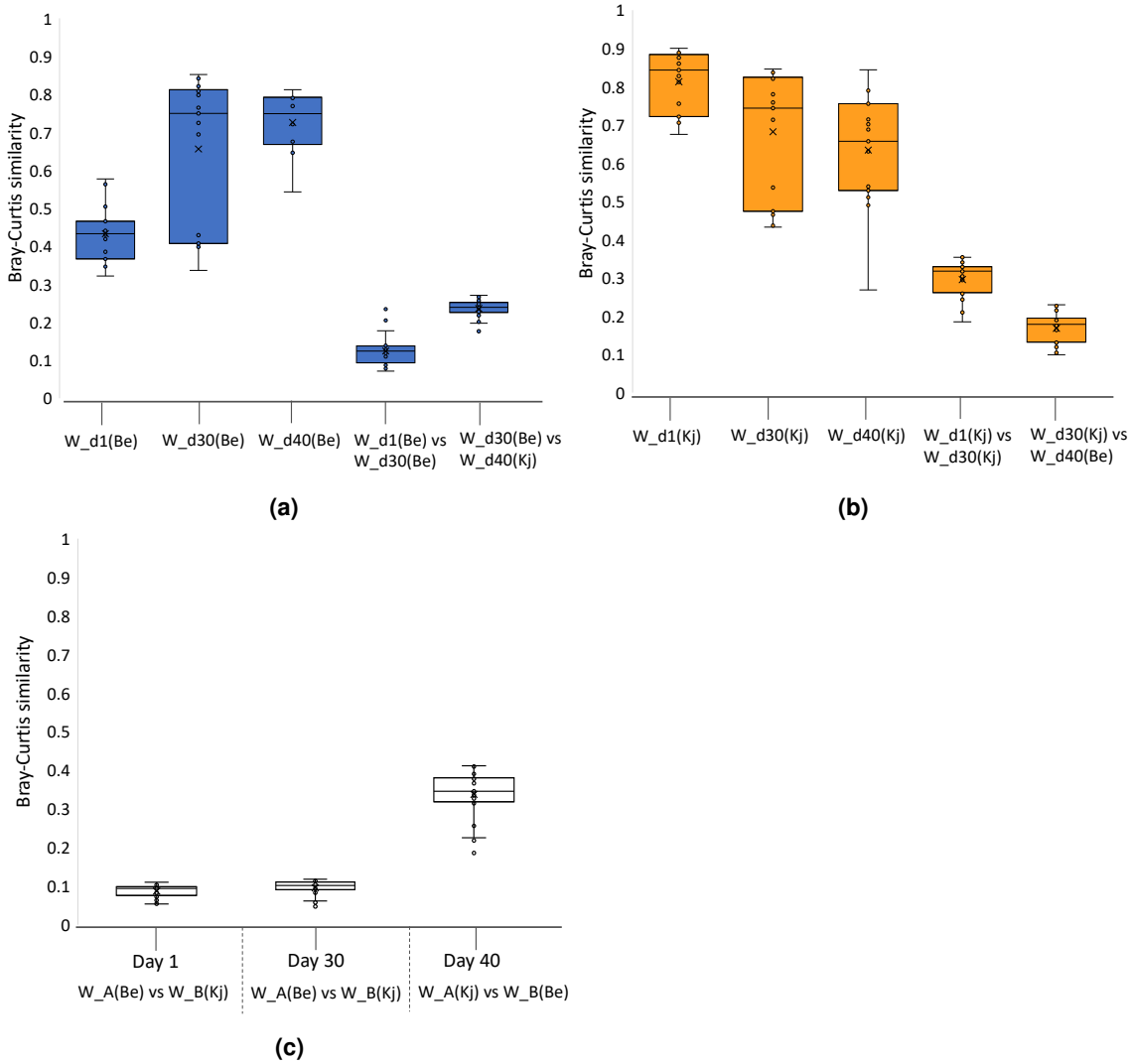


Figure 3.4.9: Box-plot showing Bray-Curtis similarities for comparison of the water microbiota for samples taken on the same dayand between days for a) RAS_A and b) RAS_B, and c) between RAS_A and RAS_B on the same day. W: water samples, d1:Day_1, d30:Day_30, d40:Day_40, Kj: Kjærelva biofilm carriers located in the RAS, Be: Belsvik biofilm carriers located in the RAS.

When comparing the water microbiota between RAS_A and RAS_B, very low Bray-Curtis similarities were found on Day_1 and Day_30 of the experiment (0.09 and 0.10 respectively) (Figure 3.4.9c). However, a higher similarity was observed when comparing the water communities in RAS_A and RAS_B on Day_40 of the experiment (average Bray-Curtis similarity of 0.34).

To compare the similarity between water- and biofilter-biofilm microbiota in RAS_A and RAS_B throughout the experiment, Bray-Curtis similarities were determined (Figure 3.4.10). Interestingly, the water microbiota in each RAS was more similar to the biofilm carriers located in the RAS, than the other biofilm carriers, at each sampling time. Even on Day_40, the water communities were more similar to the biofilm carriers located in the RAS for ten days, than the biofilm carriers previously located in the RAS for 30 days. The highest similarity between the microbiota of water-and biofilm carriers was observed in RAS_B with Kjærelva carriers on Day_1 of the experiment.

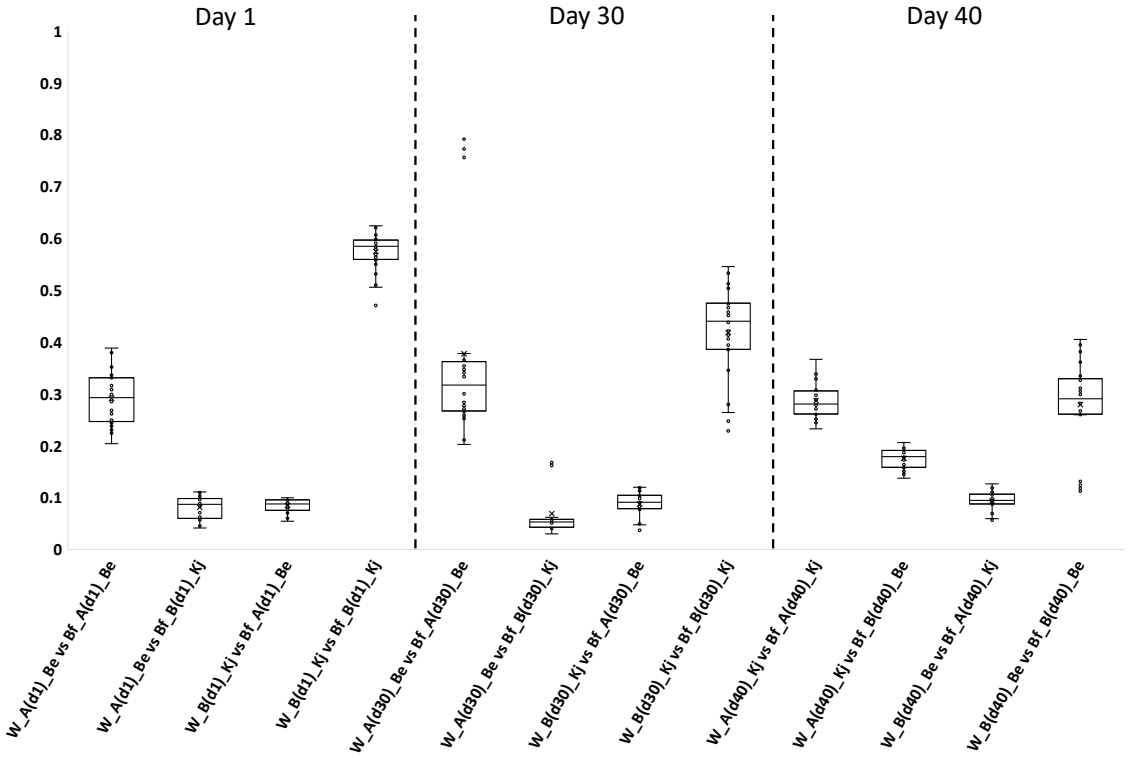


Figure 3.4.10: Box plot showing Bray-Curtis similarities for comparison between the microbiota of the biofilter-biofilm and water on the same sampling day within and between RAS. There are four biofilm-carrier replicates from each biofilter and six replicates of water. W: water samples, Bf: biofilter, d1:Day_1, d30:Day_30, d40:Day_40, Kj: Kjærelva biofilm carriers located in the RAS, Be: Belsvik biofilm carriers located in the RAS.

A SIMPER analysis found that the ASVs contributing to the most difference in the bacterial communities between biofilter and water samples were ASV_7 (*Nitrospira*), followed by ASV_8 (*p:Parcubacteria*) and ASV 17 (*Nitrospira*). ASV_8 is not classified at order level, and is the most abundant ASV in the water samples, indicating that it constitutes a high abundance of the unassigned orders in water samples. ASV_7 and ASV_17 were the two most abundant ASVs in the biofilm carrier communities.

Bacterial communities of the Atlantic salmon fry skin and intestine samples

For the salmon fry samples, only samples from Day_1 and Day_30 were analyzed. In the PCoA ordination of all samples, the microbial community composition of the intestine samples seemed to be similar across systems and remained stable over time, except for one outlier from RAS_B on Day_1 of the experiment (Figure 3.4.4). Further, the skin samples from RAS_A and RAS_B differed over time and the skin samples from RAS_A were more similar to the intestine samples on Day_30. In the PCoA, the skin samples from RAS_B were more clustered together, suggesting a more similar bacterial composition over time. One-way PERMANOVA confirmed that there was no significant difference between Day_1 and Day_30 of the skin microbiota in both RAS_A and RAS_B ($p>0.05$). The significant difference between Day_1 and Day_30 in RAS_A of the intestine microbiota was not investigated, since only one sample represented Day_1. However, for RAS_B the intestine microbiota was also not significantly different between Day_1 and Day_30 (One-way PERMANOVA, $p>0.05$).

The PCoA ordination of the salmon fry skin samples indicated that there was a greater similarity over time between samples from RAS_B compared to RAS_A (Figure 3.4.5). Additionally, in RAS_A, samples collected on Day_30 of the experiment were more different than samples collected on Day_1 of the experiment, which could be due to an indirect influence by the biofilter. However, in RAS_A on Day_30, the bacterial communities of the salmon fry skin were significantly different from both the Belsvik biofilm carriers and the rearing water (One-way PERMANOVA, $p=0.027$ and $p=0.011$ respectively).

The PCoA ordination of intestine samples identified a distinct outlier from RAS_B with Kjærelva biofilter on Day_1 of the experiment (Figure 3.4.5D). This sample is also represented in Figure 3.4.4 where it showed considerable similarity to the corresponding biofilter (Kjærelva). Without the outlier, the intestine bacterial compositions seemed to be highly heterogeneous throughout the experiment and across systems.

Box plots based on Bray-Curtis similarities were generated to further explore the similarity within and between the microbiota of the salmon fry skin and intestine (Figure 3.4.11). Only one intestine sample was analyzed from RAS_A on Day_1 of the experiment and is therefore not included in the box plot. In RAS_A on Day_1 and Day_30, a low similarity was observed between the microbiota of the skin samples and between the microbiota of the intestine samples (Figur 3.4.11a). Additionally in RAS_A, the average Bray-Curtis similarity between the skin microbiota on Day_1 and Day_30

was very low (0.08), indicating a change in the microbiota during the experiment. However, a one-way PERMANOVA states that the skin microbiota between Day_1 and Day_30 in RAS_A are not significantly different ($p>0.05$).

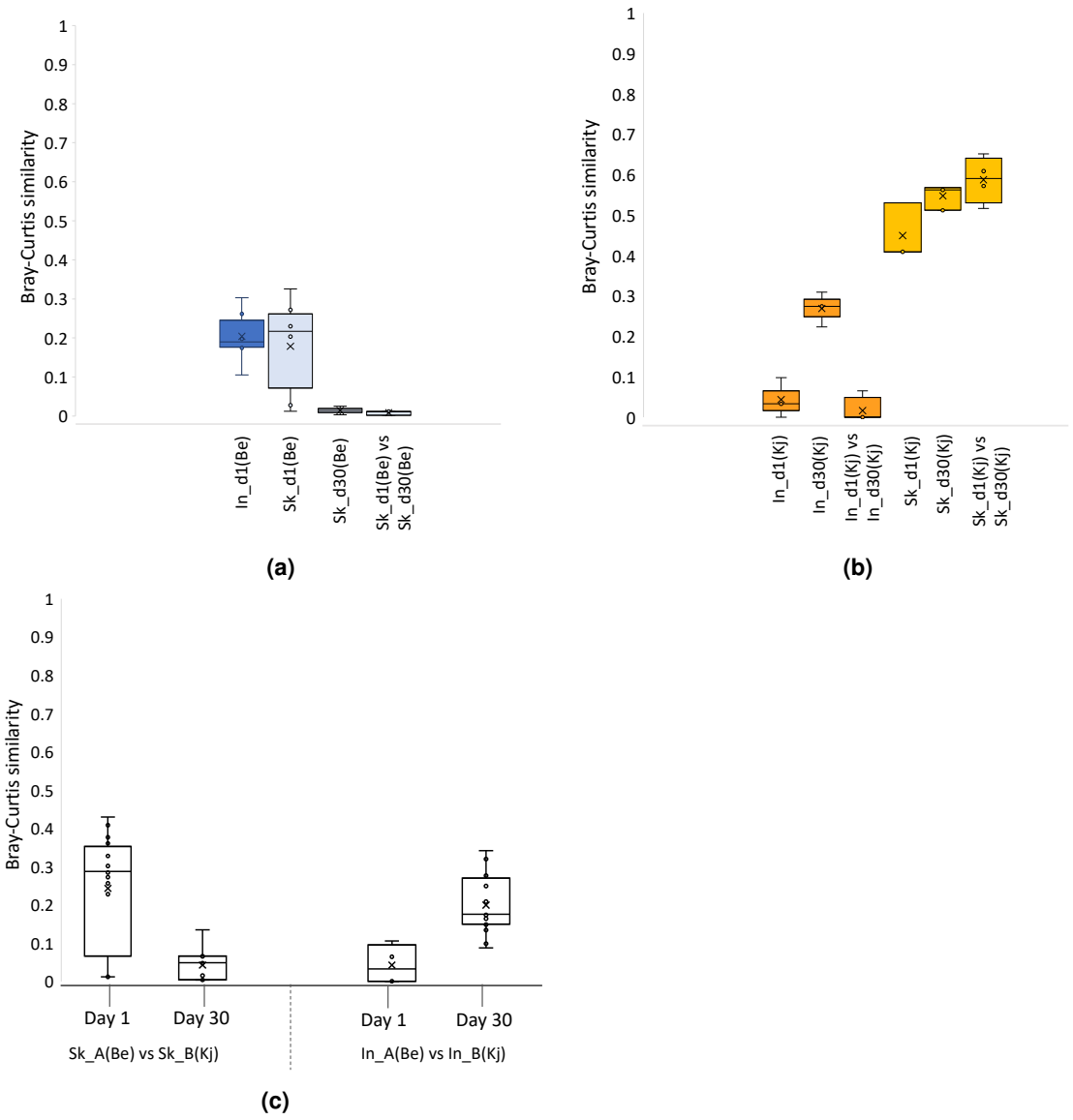
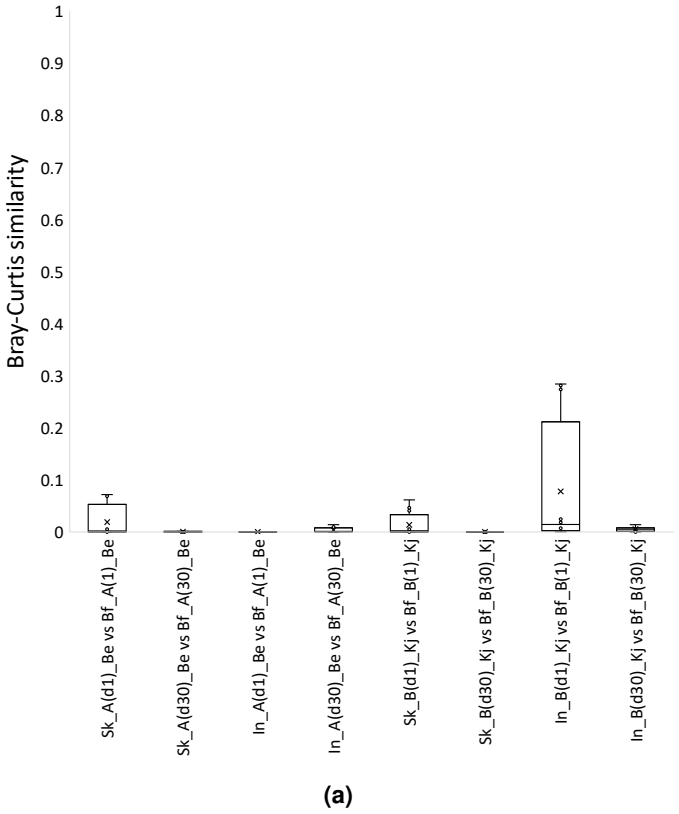


Figure 3.4.11: Box-plot showing Bray-Curtis similarities for comparison of salmon fry microbiota for samples taken on the same day and between days for a) RAS_A and b) RAS_B, and c) between RAS_A and RAS_B on the same day. In: intestine samples, Sk: skin samples, d1:Day_1, d30:Day_30, d40:Day_40, Kj: Kjærelva biofilm carriers located in the RAS, Be: Belsvik biofilm carriers located in the RAS.

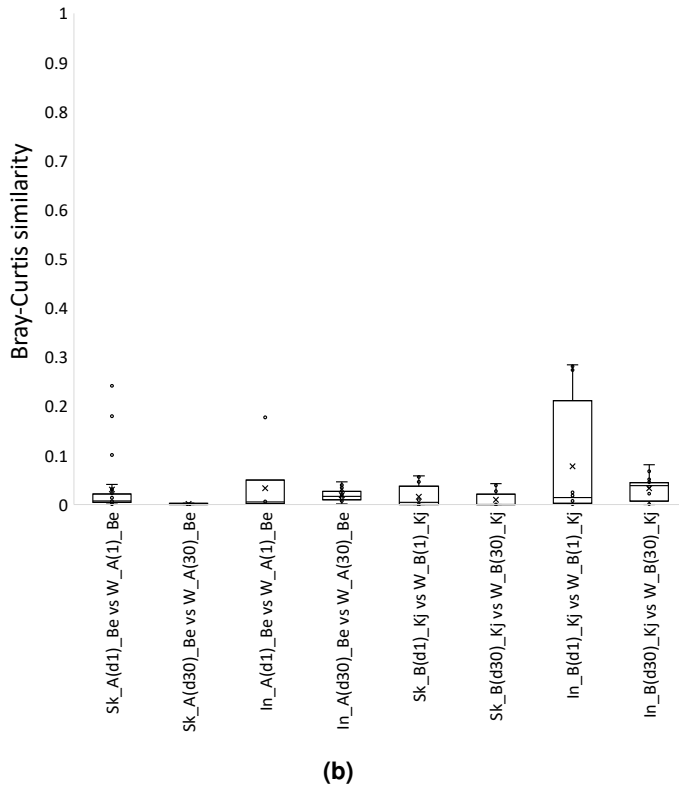
In RAS_B, higher Bray-Curtis similarities were observed within the skin microbiota compared to the intestine microbiota on each sample day (Figure 3.4.11b). When comparing the skin microbiota in RAS_B on Day_1 and Day_30 of the experiment, a higher similarity was observed, compared to the results in RAS_A. As for RAS_A, there was no significant difference between the bacterial communities in the skin samples from Day_1 and Day_30 of the experiment, suggesting that the biofilter may not have had a considerable influence on the skin microbiota (one-way PERMANOVA, $p>0.05$).

Comparison of the skin microbiota between RAS_A and RAS_B on Day_30 showed a very low average Bray-Curtis similarity of 0.04 (Figure 3.4.11c). Interestingly, there was no significant difference between the skin microbiota from RAS_A and RAS_B on Day_1, but on Day_30 there was a significant difference (one-way PERMANOVA, $p>0.05$ and $p=0.026$, respectively).

Bray-Curtis similarities between the salmon fry microbiota (skin and intestine) and the water- and biofilter-biofilm microbiota were determined to compare possible community similarities (Figure 3.4.12). When comparing the skin microbiota to the water and biofilter-biofilm microbiota, low Bray-Curtis similarities are observed in both RAS_A and RAS_B. The same is observed when comparing the intestine microbiota to the water and biofilter-biofilm microbiota, except for a bit higher Bray-Curtis similarity in RAS_B on Day_1 between intestine microbiota and both the water and biofilter-biofilm communities. This can be due to the outlier sample mentioned previously, which was located near the Kjærelva biofilm carriers in the PCoA with all samples (Figure 3.4.4).



(a)



(b)

Figure 3.4.12: Box-plot showing Bray-Curtis similarities for comparison between the microbiota of the salmon fry intestine and skin, with a) biofilm carriers and b) rearing water, for samples taken on the same day. In: intestine samples, Sk: skin samples, A: RAS_A, B: RAS_B, d1:Day_1, d30:Day_30, d40:Day_40, Kj: Kjærelva biofilm carriers located in the RAS, Be: Belsvik biofilm carriers located in the RAS.

4 Discussion

4.1 Nucleic acids extraction and PCR amplification

4.1.1 Evaluation of DNA/RNA-extraction kits

The research group ACMS has encountered challenges in previous studies when performing PCR amplification of bacterial 16S rDNA from fish samples. One of the reasons for this is believed to be the quality of the DNA and RNA extracts. The quality of the extracts can be affected by a variety of factors, such as DNA contamination in the extraction kits, contamination during the extraction process, or the presence of inhibiting factors in the final extracts. Avoiding DNA contamination in the DNA-extraction kit and PCR reagents is crucial, particularly when handling samples that present difficulties in obtaining a PCR product. Contaminating DNA has been found in commonly used DNA extraction kits and other laboratory reagents in previous studies[71][72]. When working with samples from Atlantic salmon, the yield of salmon DNA is significantly higher compared to bacterial DNA in the DNA extracts, which can potentially interfere with the amplification of bacterial DNA if co-amplification of salmon DNA occurs when using universal primers. An objective of this project was to evaluate four different DNA and/or RNA extraction kits with the use of KingFisher™ Flex Purification System to determine the most optimal kit for later PCR amplification of bacterial 16S rDNA.

The Atlantic salmon yolk-sac fry samples used for this project originated from a previous master project and were of the same age. Among the four kits evaluated, Kit A (MagAttract® PowerSoil® Pro DNA kit) provided the DNA extracts with the highest average DNA concentration and the purest DNA. However, none of the DNA extracts from Kit A, nor Kit C or D resulted in PCR products for the six yolk-sac fry samples. This could be due to PCR inhibitors in the extracts and low amounts of target DNA (bacterial DNA), compared to host DNA. Additionally, Kit A and Kit C were found to have contaminating bacterial DNA, as the PCR for the negative extraction kit controls resulted in PCR products of the expected length. PCR products for the negative extraction kit controls indicate bacterial DNA contamination from the extraction kits, which could possibly originate from previous use of the extraction kits or from the manufacturer. Only Kit B (96 MagBead DNA kit) resulted in PCR products of the DNA extracts for all six samples, despite not being the kit that yielded the highest concentration of DNA.

Since DNA extracts can contain PCR inhibitors[73], they were diluted 1:10 and 1:100 prior to PCR amplification. Diluting the DNA extracts 1:10, increased the number of PCR products from the salmon yolk-sac fry samples for kits A, C, and D, suggesting that PCR inhibitors were present in the DNA extracts. PCR of the DNA extracts from Kit B did not produce shorter, unspecific DNA fragments when the DNA extracts were diluted 1:10, potentially indicating increased primer specificity. However, residual kits resulted in PCR products at shorter nucleotide lengths for the skin samples. While a 1:100 dilution also helped against unwanted PCR products, it did not result in more PCR products. Additionally, a 1:100 dilution resulted in PCR products for the negative control for all the

DNA extraction kits. PCR product for the NTC was also observed and suggests PCR contamination. Thus, the 1:100 dilution test can't be trusted.

The PCR of the cDNA from the RNA extracts by Kit C and Kit D did not result in PCR products for all six yolk-sac fry samples. PCR products were obtained from four samples (one gut and three skin) from cDNA of RNA extracts by Kit D. There was little difference in the results obtained from using 4μL or 2μL RNA for cDNA synthesis, only a bit stronger products were observed when 4μL RNA was utilized. These results indicate that a PCR of cDNA produced by an RNA template volume between 4μL and 2μL would yield similar results. Diluting the cDNA samples (2μL RNA as template) was not optimal, only a weak PCR product for one skin sample was observed per kit, indicating that inhibitors in the cDNA samples were not a problem. The residual DNA present in the RNA extracts after DNase treatment did not amplify during PCR and was therefore not thought to be a problem in downstream cDNA synthesis and PCR amplification.

In conclusion, the most optimal DNA extraction kit of the four kits tested with the use of KingFisher™ Flex Purification System for later PCR amplification of 16S rDNA was Kit B (96 MagBead DNA kit, ZymoBIOMICS™). In addition, a 1:10 dilution of the DNA extracts helped against unspecific PCR amplification and primer dimer formation. The optimal extraction kit for isolating RNA for later amplification of regions of the 16S rRNA gene was Kit D (Quick-DNA/RNA™ MagBead, ZymoBIOMICS™), and the amount of RNA extract used as template for the cDNA synthesis did not affect the PCR result. Diluting the RNA extracts prior to cDNA synthesis did not yield any PCR products, and is not recommended.

4.1.2 PCR amplification of 16S rDNA from samples of Atlantic salmon

The amplification of the v3+v4 region of the 16S rRNA gene from the extracted DNA samples of the Atlantic salmon fry obtained from the lab-scale RAS proved to be challenging, despite the positive PCR results achieved using the extraction kit described in the section above. The difficulties of amplifying 16S rDNA from the DNA extracts of Atlantic salmon fry could be due to inhibitors in the DNA extract or low levels of bacterial DNA compared to host DNA, as previously discussed.

Prior to the DNA extraction of the salmon fry obtained from the lab-scale RAS experiment, the samples were homogenized. In this step whole tissue samples of the salmon were used, yielding a much higher presence of host DNA compared to bacterial DNA. The gill and skin tissue samples were larger than the intestine samples, and the DNA extracts of skin and gill samples yielded the highest DNA concentration and purity on NanoDrop™ One. Even so, the DNA extracts of intestine samples was the least troublesome to amplify, indicating that a lower DNA concentration and less host DNA, resulted in easier amplification of 16S rDNA. These results suggests an importance of using less sample material for nucleic acid extraction when the goal is to amplify bacterial 16S rDNA.

Different variables were tested to optimize the PCR, such as increased $MgCl_2$ concentration and cycling conditions. The cycling conditions included annealing temperature and the number of repeated cycles. The annealing temperature is the most important cycling condition to change since the smallest change in temperature could make the difference between specific and non-specific amplification[74].

In summary, 1:10 dilution of the DNA extracts prior to PCR amplification and using cycling conditions of 56 annealing temperature and 38 cycles resulted in the most optimal yield of PCR products. These conditions resulted in PCR products for the intestine samples, however for the skin and gill samples an initial PCR using primers that only represented the target sequences, were used to obtain PCR products.

4.2 Rearing of Atlantic salmon fry

4.2.1 Evaluation of water quality and system operation

The physiochemical water quality variables were relatively stable and satisfying during the experiment, indicating a well-dimensioned lab-scale RAS. The measured values were similar across RAS_A and RAS_B. Both biofilters (Belsvik and Kjærelva) were effective, and the total ammonia nitrogen (TAN) and nitrite (NO_2^-) concentrations did not exceed the threshold values of 2 mg/L and 0.1 mg/L respectively[35]. The measured TAN values in RAS_A were higher than in RAS_B during the first 17 days of the experiment, suggesting a greater nitrification efficiency and stability for the Kjærelva biofilter located in RAS_B during this period. The TAN and NO_2^- did not increase throughout the experiment, which could have been expected with increased input of feed and thus increased salmon fry biomass. A possible explanation for the decrease in TAN values and the low nitrite values could be the increased water exchange to compensate for water loss due to problems with the mechanical filtration in the period from Day_10 to Day 32. The low values also indicate that there was good nitrifying activity, and thus indicating that thenitrifying bacteria were not out-competed by the heterotrophic bacteria. Nevertheless, the TDS levels increased during the experiment, which is expected in a recirculating system. The amount of dissolved solids in the water increases due to feed, fecal solids, and biofilm shattered from the biofilter[34]. An increase in TDS could reduce water quality and decrease the nitrifying capacity of the biofilter[31]. Even so, the TDS values in this experiment did not seem to be high enough to affect the conversion of TAN and nitrite.

The concentration of nitrate NO_3^- increased during the experiment which could be expected when only a low amount of water is exchanged each day. The nitrate level exceeded recommended levels of nitrate (up to 400 mg/L)[34] in both RAS on Day_19 of the experiment and was then measured to be 640 mg/L. However, there was no observed increase in stressful behavior or mortality after this incident of elevated nitrate levels, suggesting that the concentrations were non-toxic for the reared salmon fry. After this incident, the nitrate levels decreased, seemingly due to the days with high water

exchange to compensate for the water loss.

Further, the water temperature in the rearing tanks was stable in the upper shift of 11^oC, which is within the optimal physiological thermal range (6-20^oC), but lower than the optimal temperature growth of Atlantic salmon (15-16^oC)[34]. This experiment did not intend for maximum growth of the salmon fry, so the intended temperature of 11^oC was satisfying.

An observation during the rearing of the salmon fry was that a lot more biofilm was detached from the Kjærelva biofilter carriers in the first few days of the experiment, making the water more turbid. This could be expected since the surface area of these carriers are flat and thus more exposed to external impact. Additionally, the mechanical filter sock in the system where the Kjærelva biofilter was located acquired a green color. This was observed in RAS_B the first month and quickly observed in RAS_A after the biofilter swap. A possible explanation for this could be the presence of bacteria or archaea in the biofilm carriers producing green pigments, such as chlorophyll. Two ASVs (ASV 2226 and ASV 4306) represented *Cyanobacteria*, which are aquatic and photosynthetic bacteria containing green pigment[75], but these were found in very low relative abundance. The presence of eukaryotic algae could be an alternative explanation.

Although the physiochemical water quality variables were mainly satisfying, the operation of the systems was challenging with consideration of the tank outlets and the mechanical filter unit. Feces and uneaten feed accumulated in the bottom of the tanks, indicating a sub-optimal design of the tank outlets. The accumulation of particles needed to be manually removed, increasing the stress level for the fish. Clogging of the mechanical filter sock led to water loss in the systems, resulting in a higher water exchange than 5% on the days this occurred. In retrospect, the systems should also have been better secured against such events as a power outage. During the experiment, an alarm was tried installed but without sucess. For the safety of the fish, an alarm and a backup source of power should have been installed for the most critical units of the systems, such as the oxygen supply.

4.2.2 Evaluation of fish performance

The Atlantic salmon fry reared in the lab-scale RAS was healthy throughout the experiment. Fish growth was normal (SGR 1.26-1.46%) and there was no mortality until the end of the experiment on Day_40, indicating good rearing conditions. There was no significant difference in the specific growth rate (SGR) of the salmon fry in RAS_A and RAS_B, which indicated similar rearing conditions. Further, there was no fish mortality in either of the systems during the experiment. This suggests good rearing conditions, up until the power outage on Day_40.

The reared fish encountered various stressful conditions, including handling and transportation of the fish into the lab-scale RAS. However, efforts were made to

minimize unnecessary stress during transportation such a quick transportation and minimizing disturbances. In addition, they encountered stress during the collection of fish for sampling, and when particles were removed from the tanks by siphoning. Since stress is known to decrease feed intake[76], the salmon fry was hand fed after their transport into the systems, and the first six days of the experiment. This way their feeding response could be observed, and excessive amounts of feed were avoided. During the rest of the experiment, they were fed according to a calculated feed plan with the automatized feeders. It was important to not feed them excessive amounts, since uneaten feed may affect gill respiration, predispose the fish to bacterial infections and promote growth of heterotrophic bacteria [77][29].

4.3 Bacterial community analysis

The understanding of microbial community dynamics in RAS is currently limited due to a lack of available research[78][79]. In this study, the results from the Illumina sequencing indicate differences and similarities between the bacterial communities of the biofilm carriers, rearing water, and salmon fry skin and intestine, from three different sampling times and two different RAS.

4.3.1 The biofilters bacterial community composition

In this experiment, the bacterial community composition was found to be significantly different for biofilters originating from hatcheries at different geographical locations (Kjærelva and Belsvik). This finding was also reported in a study conducted by Dahle et al.[58]. Nearly complete Bray-Curtis dissimilarity was found between the two different biofilters on Day_1, Day_30, and Day_40 of the experiment. The bacterial community composition within replicates of the biofilter biofilm carriers at each sampling time was very similar. Further, less similarity was observed between Day_1 and Day_30, indicating a change in the bacterial community composition of the biofilters. However, between Day_30 and Day_40, a higher similarity was observed for the biofilters, even though they were located in a new RAS from Day_30. PCoA ordinations also reflected this dissimilarity between the two different biofilter biofilm communities and the similarity within the communities throughout the experiment (Figure 3.4.5A). Here, the biofilm communities clustered according to the biofilter type, regardless of which RAS they were located in.

Nitrospirales were found to be abundant in both Belsvik and Kjærelva biofilm carriers, but more abundant in the Belsvik carriers (Figure 3.4.3). Interestingly the relative abundance of Nitrospirales decreased in the Belsvik biofilm carriers on Day_40, after the biofilter swap. Other orders with high maximum abundance in both biofilters were Rhodobacterales and Burkholderiales. These were also among the most abundant orders in the biofilter biofilm communities in a previous study by Dahle et al. 2023[80]. *Flavobacteriales* were abundant in Kjærelva biofilter samples on Day_1 of the

experiment.

A total of 40 ASVs representing nitrifying bacteria (including ASVs with a relative abundance higher than 0.1% in at least one sample) were found in the biofilter-biofilms (Figure 3.4.7). The majority of these were classified as the genus of nitrite-oxidizing bacteria (NOB) *Nitrospira*, while some were classified as the genus of ammonia-oxidizing bacteria (AOB) *Nitrosomonas*. Nitrospira-dominated nitrifying communities are commonly found in fresh and brackish RAS biofilters[81],[80],[82]. The low AOB:NOB ratio could indicate the presence of comammox *Nitrospira bacteria*, which are capable of complete oxidation of ammonia to nitrate[83]. Phylogenetic analysis identified multiple ASVs genetically related to comammox *Nitrospira*, however with low bootstrap values. Further research is needed to determine whether comammox plays an important role in nitrification in these biofilters. One possible strategy would be to attempt to amplify the amoA gene with comammox-specific PCR primers, and sequence the potential PCR product. Another possible reason for the low relative abundance of ammonia-oxidizing bacteria could be the presence of ammonia-oxidizing archaea. Primers utilized in this experiment did not target archaea, so this was not investigated in this study. Further investigation of the phylogenetic relationship with previously described *Nitrospira* indicates that ASV 7 was related to N.defluvii and represented one of the primary nitrite-oxidizing ASVs in both biofilters. ASV 97, related to N. japonica was most abundant in Kjærelva biofilter. N.defluvii and N. japonica are previously found in wastewater treatment plants, and N.defluvii is also found in the biofilters of brackish water RAS[84][85].

4.3.2 The bacterial communities in the biofilter's influence on the microbiota of the rearing water

The biofilter in a RAS is an essential water treatment component for the nitrification of toxic nitrogenous waste. Thus, being a biological filtration system, the unit contains a large amount of bacteria. In a RAS the biofilter is the largest reservoir for microorganisms, except for the culture animals[38]. The effect these bacteria has on the suspended water microbiota in RAS is not well known. Previous studies have found the biofilter-biofilm and suspended water communities to be significantly different in RAS[80][86]. This was no exception in this experiment. The bacterial communities in the biofilter biofilm and water was found to be significantly different at all sampling times within RAS_A and RAS_B. However, water and biofilter biofilm carriers shared common taxa. In addition, the water microbiota was more similar to the biofilm carriers microbiota located in the same RAS_At all sampling points than to the other type of biofilm carriers microbiota.

The biofilter biofilm had higher exponential Shannon's diversity than water, which is also found in previous studies[81][87]. In accordance with a study by Dahle et al.2023, *Rhodobacterales* and *Burkholderiales* were found in high abundances in the biofilter- and water samples. *Flavobacteriales* was abundant in the Kjærelva carriers communities from Day_1, as well as in the water communities in RAS_B on Day_1 when

Kjærelva carriers were located in the biofilter. This may be explained by the significant suspension of biofilm particles from the Kjærelva biofilter in the water in RAS_B during the initial days of the experiment. The suspended biofilm particles could have influenced the water microbiota, resulting in a higher similarity to the biofilm carrier communities. Another similarity between the communities in water and biofilm carriers from Kjærelva was the abundance of *Cytophagales* throughout the experiment. *Nitrospirales* and *Rhizobiales* were abundant in the bacterial communities on the Belsvik carriers at all three sampling times, and were observed abundant in the water communities in RAS_A on Day_1 when Belsvik carriers were located in the biofilter. This indicates that suspended biofilm particles from the Belsvik carriers in RAS_A, had an impact on the water communities as well, though mostly observed on Day_1.

Bray-Curtis similarities comparing water communities in RAS_A and RAS_ B suggests very dissimilar bacterial communities in the rearing water of the two RAS_At Day_1 and Day_30 of the experiment, indicating an influence by the biofilters. The bacterial communities in water from RAS_A and RAS_B were more similar on Day_40, which is natural since the water microbiota had been exposed to both types of biofilm carriers at this time point. High abundances of *Burkholderiales* and *Legionellales* were observed in both RAS on Day_40, explaining the higher similarity observed (Figure 3.4.9c).

As mentioned, previous studies have found the biofilter-biofilm and suspended water communities to be significantly different in RAS. However, in previous studies multiple factors could have affected this difference, not only the biofilter-biofilm microbiota. These factors include difference in system design and operational rutines, which was the case in the study by Dahle et al[58] where the difference in RAS microbiota between commercial facilities was inspected. Notably, since the only distinguishing factor between the two RAS in this study was the biofilm carriers, it provides knowledge about a direct influence by the biofilm carriers on the water microbiota.

4.3.3 The bacterial communities in the biofilter- and rearing water's influence on the microbiota of the Atlantic salmon fry

The potential impact of bacterial communities present in the biofilm carriers and rearing water on the microbiota of the salmon fry's skin and intestine was examined solely based on samples collected on Day_1 and Day_30 since the salmon fry died, and the samples of the dead fish at Day_40 were discarded.

A PCoA ordination indicated that the intestine and skin microbiota differed from the water and biofilter microbiota in RAS_A and RAS_B at both sampling times (Day_1 and Day_30) (Figure 3.4.4). This was confirmed by one-way PERMANOVA, where all comparisons of the water- and biofilm-carrier communities to the skin- and intestine communities resulted in significant differences ($p<0.05$ for all comparisons). Previous studies confirm the significant differences between the microbiota of salmon gut and water[88]. Nevertheless, the skin microbiota was observed to differ between RAS_A and RAS_B on Day_30 (Figure 3.4.4), and a significant difference was detected (one-way

PERMANOVA, p=0.026). This could suggest an influence by the biofilter mikrobiota on the skin mikrobiota. Moreover, the exponential Shannon's diversity was lowest for the bacterial communities of the salmon fry compared to the water and biofilm carriers. Higher exponential Shannon's diversity for water microbiota compared to salmon gut microbiota was also found in a study by Bugten et al.[88].

In the intestinal microbiota, the order *Lactobacillales* was found to be the most dominant order, with the average most abundant ASV (ASV 27) belonging to the genus *Secundilactobacillus*, which is closely related to the genus *Lactobacillus*. *Secundilactobacillus* was derived from the genus *Lactobacillus*, and were proposed in 2020 by Zheng et al.[89]. *Lactobacillus* is known to be a part of the normal intestinal microbiota of fish[90]. Many *Lactobacillus* species are used as probiotics and are in aquaculture utilized for protection of the fish against pathogen infection[91][92]. Considering this, the high abundance of ASV 27 in the intestine microbiota could potentially have a positive impact on the health of salmon fry. The second most average abundant ASV in intestine microbiota was ASV 15, representing the genus *Tepidimicrobium* within the order *Clostridiales*. This order also includes the genus *Clostridium*, which is anaerobic bacteria commonly found in the intestinal tract in animals[93]. In a previous study investigating the microbiomes of rearing water in RAS_And Atlantic parr hindgut, *Bacilli* and *Actionabacteria* were the highly dominant classes of the hindgut microbiome[88]. *Lactobacillales*, the dominating order in the intestine samples in this study belong to the class *Actionabacteria*, thereby supporting the previous findings by Bugten et al.[88].

Bacillales was found to be the most dominating order in skin microbiota, and was abundant in intestine microbiota. The genus *Bacillus*, belonging to the *Bacillales* order, is also used in probiotics for human and veterinary applications[91].

Further, the average most abundant ASV (ASV 1) in the skin microbiota represented the genus *Staphylococcus*, under the dominating order *Bacillales*. *Bacillales* was far more abundant in the skin microbiota from RAS_B with Kjærelva biofilm carriers, than the skin microbiota from RAS_A with Belsvik biofilm carriers (Figure 3.4.3). However, this order was hardly found in either type of biofilm carriers or in the water microbiota, making it difficult to explain the high abundance in RAS_A.

4.3.4 Future work and perspectives

This thesis has provided has demonstrated the influence the bacterial communities in the biofilter have on the microbiota of rearing water and salmon fry in RAS. To expand on these findings, future studies should be conducted on a larger scale and over a longer period of time to gather more comprehensive data, to evaluate the biofilters influence on the microbiota of the system also in commercial RAS. This can contribute to a better understanding of the bacterial communities' interactions within RAS.

The potential influence of the biofilter and water microbiota on the salmon fry microbiota should be further investigated. This could result in increased knowledge on factors that shape the salmon fry microbiota, which is advantageous for the fish health and for commercial production.

Comperative studies should be conducted to further understand the selection pressures resulting in different bacterial community compositions between biofilters originating from hatcheries at different geographical locations.

To further investigate the ratio of ammonia-oxidizing- and nitrite-oxidizing microbes in the biofilters, 16S rDNA amplicon sequencing could be performed with primers targeting both bacteria and archaea. The role of comammox in nitrification could also be investigated by using primers for the amoA gene and sequencing the potential PCR product.

These future research perspectives can help increase the understanding of the complex microbial dynamics in RAS, and thereby result in increased system control when rearing fish in RAS.

5 Conclusions

The evaluation of the four different DNA and RNA extraction kits showed that 96 MagBead DNA kit (Kit B; ZymoBIOMICS™) yielded the most optimal nucleic acid extracts from yolk-sac fry (gut and skin) for successful bacterial 16SrDNA amplification. Optimization of the PCR protocol for amplifying bacterial 16S rDNA from the lab-scale RAS salmon fry did not result in the successful amplification of all samples. As a consequence, fewer salmon fry skin and intestine samples were included in the amplicon library than planned.

The experiment of rearing the Atlantic salmon fry in the two lab-scale RAS was successful, with satisfying physiochemical water quality and high fish survival. However, the operation of the systems was not ideal in terms of particle removal which resulted in days with higher water exchange. Notably, the experiment ended tragically with the death of all the fish due to an unexpected power failure on Day_40 of the experiment.

Characterization of the bacterial communities associated with biofilter-biofilm, rearing water, and salmon fry from the experiment in the two lab-scale RAS showed a significant difference between the biofilter-biofilm communities and the microbiota of the rearing water and salmon fry. However, the water and biofilm carriers exhibited a high abundance of multiple shared taxa, such as *Rhodobacterales* and *Burkholderiales*.

A significant difference between the microbiota of biofilm carriers originating from different geographical locations was found throughout the experiment, suggesting different selection pressures in the two originating hatcheries resulting in dissimilar household bacterial populations. Furthermore, the high Bray-Curtis similarities within each biofilm-biofilter indicated stable bacterial compositions over time. Additionally, the highest alpha diversity was observed for the bacterial communities of the biofilter-biofilm carriers compared to the water-and salmon fry communities. The nitrifying communities in both types of biofilter carriers had a low AOB:NOB ratio, indicating the presence of comammox *Nitrospria* or ammonia-oxidizing archaea. The phylogenetic tree indicated the biofilter-biofilm communities consisted of many different *Nitrospira* species, and it indicated a difference in the *Nitrospira* population between the Kjærelva- and Belsvik biofilm carriers.

Further, it was observed that the water microbiota was more similar to the biofilm-biofilter microbiota within its own system. This was also observed after the biofilter swap and indicates a direct influence of the biofilter-biofilm microbiota on the water microbiota. The high abundance of *Flavobacteriales* and *Cytophagales* in the microbiota of biofilm carriers originating from Kjærelva was observed in the rearing water within the same system. For the microbiota of the Belsvik biofilm carriers, there was a high abundance of *Nitrospirales* and *Rhizobiales*, which was also found in the water microbiota in the same system. Comparing the microbiota of the salmon fry to the water and biofilter-biofilm microbiota, little similarities were observed. Nevertheless, the skin microbiota between the systems was found to not be significantly different on Day_1 of the experiment, but on Day_30 they were significantly different. Suggesting a possible influence by the biofilter-biofilm communities.

6 References

[1] “Salmon Farming Industry Handbook 2019”. en. In: *MOWI* (2020).

[2] United Nations. *World population projected to reach 9.8 billion in 2050, and 11.2 billion in 2100*. en. Publisher: United Nations. URL: https://www.un.org/en/desa/world-population-projected-reach-98-billion-2050-and-112-billion-2100.

[3] National Oceanic and Atmospheric Administration US Department of Commerce. *What is aquaculture?* EN-US. URL: https://oceanservice.noaa.gov/facts/aquaculture.html.

[4] Magdy Soltan. “Aquaculture systems”. In: Oct. 2016.

[5] C. I. M. Martins et al. “New developments in recirculating aquaculture systems in Europe: A perspective on environmental sustainability”. en. In: *Aquacultural Engineering* 43.3 (Nov. 2010), pp. 83–93. DOI: 10.1016/j.aquaeng.2010.09.002.

[6] Rosamond L. Naylor et al. “Effect of aquaculture on world fish supplies”. en. In: *Nature* 405.6790 (June 2000). Number: 6790 Publisher: Nature Publishing Group, pp. 1017–1024. DOI: 10.1038/35016500.

[7] *The Great (Farmed Fish) Escape*. en-GB. URL: https://www.oceanoculus.com/news-from-the-sea/aquaculture-escape.

[8] Biju Sam Kamalam and Pramod Kumar Pandey. “Nutrition and Environment Interactions in Aquaculture”. en. In: *Outlook of Climate Change and Fish Nutrition*. Ed. by Archana Sinha, Shivendra Kumar, and Kavita Kumari. Singapore: Springer Nature, 2022, pp. 407–422. ISBN: 978-981-19550-0-6.

[9] M. Badiola et al. “Energy use in Recirculating Aquaculture Systems (RAS): A review”. In: *Aquacultural Engineering* 81 (2018), pp. 57–70. DOI: https://doi.org/10.1016/j.aquaeng.2018.03.003.

[10] J. Lara and M. Lean. “Chapter 19 - Salmon and Vascular Risk in Young Healthy Subjects”. en. In: *Fish and Fish Oil in Health and Disease Prevention*. Ed. by Susan K. Raatz and Douglas M. Bibus. San Diego: Academic Press, Jan. 2016, pp. 207–215. DOI: 10.1016/B978-0-12-802844-5.00019-1.

[11] J. E. Williams et al. “Cold-Water Fishes and Climate Change in North America”. In: *Reference Module in Earth Systems and Environmental Sciences*. Elsevier, 2015. DOI: https://doi.org/10.1016/B978-0-12-409548-9.09505-1.

[12] Jeremiah J. Minich et al. “Microbial Ecology of Atlantic Salmon (Salmo salar) Hatcheries: Impacts of the Built Environment on Fish Mucosal Microbiota”. In: *Applied and Environmental Microbiology* 86.12 (2020), e00411–20. DOI: 10.1128/AEM.00411-20.

[13] Food and Agriculture Organization of the United Nations Fisheries Department. *The State of World Fisheries and Aquaculture, 2000*. en. Food & Agriculture Org., 2000. ISBN: 978-92-5-104492-6.

[14] *Farmed Salmon — Industries — WWF*. en. URL: https://www.worldwildlife.org/industries/farmed-salmon.

[15] Audun Iversen et al. “Production cost and competitiveness in major salmon farming countries 2003–2018”. en. In: *Aquaculture* 522 (May 2020), p. 735089. DOI: 10.1016/j.aquaculture.2020.735089.

[16] Federation of European, FEAP. *FEAP ANNUAL REPORT 2022*. Tech. rep. 2022. URL: https://feap.info/wp-content/uploads/2022/12/feap-annual-report-feap-2022.pdf.

[17] Vasco C. Mota et al. “The effects of carbon dioxide on growth performance, welfare, and health of Atlantic salmon post-smolt (Salmo salar) in recirculating aquaculture systems”. en. In: *Aquaculture* 498 (Jan. 2019), pp. 578–586. DOI: 10.1016/j.aquaculture.2018.08.075.

[18] NOAA Fisheries. *Atlantic Salmon (Protected) — NOAA Fisheries*. en. Archive Location: New England/Mid-Atlantic. Oct. 2022. URL: https://www.fisheries.noaa.gov/species/atlantic-salmon-protected.

[19] *Salmon Life Cycle — Marine Institute*. URL: https://www.marine.ie/site-area/areas-activity/fisheries-ecosystems/salmon-life-cycle.

[20] *Atlantic salmon (Salmo salar)*. en. June 2021. URL: https://www.fisheriesireland.ie/species/atlantic-salmon-salmo-salar.

[21] *Fisheries and Aquaculture - Cultured Aquatic Species - Salmo salar*. eng. Nov. 2004. URL: https://www.fao.org/fishery/en/culturedspecies/salmo_salar/en.

[22] “Salmon Farming Industry Handbook 2019”. en. In: *MOWI* (2022).

[23] Lena Geitung et al. “Cleaner fish growth, welfare and survival in Atlantic salmon sea cages during an autumn-winter production”. In: *Aquaculture* 528 (2020), p. 735623. DOI: https://doi.org/10.1016/j.aquaculture.2020.735623.

[24] Frances Dipper. “Chapter 8 - Human impacts 1: sea fisheries and aquaculture”. In: *Elements of Marine Ecology (Fifth Edition)*. Ed. by Frances Dipper. Fifth Edition. Butterworth-Heinemann, 2022, pp. 389–458. ISBN: 978-0-08-102826-1. DOI: https://doi.org/10.1016/B978-0-08-102826-1.00006-5.

[25] Odd-Ivar Lekang. *Aquaculture engineering*. eng. Third edition. Hoboken: Wiley-Blackwell, 2020. ISBN: 978-1-119-48901-6.

[26] James M. Ebeling and Michael B. Timmons. *Aquaculture Production Systems*. eng. 1st ed. John Wiley & Sons, Ltd, 2012. URL: https://onlinelibrary.wiley.com/doi/10.1002/9781118250105#page=259.

[27] Johanne Dalsgaard et al. “Farming different species in RAS in Nordic countries: Current status and future perspectives”. In: *Aquacultural Engineering* 53 (2013), pp. 2–13. DOI: https://doi.org/10.1016/j.aquaeng.2012.11.008.

[28] Y. Ulgenes, Kari Attramadal, and Bendik Terjesen. *The motivation for using recirculation in Norway - a shift from the need for restricted water consumption to better control of the culture water environment and higher product quality*. Journal Abbreviation: Aquacultural Engineering Society’s Fourth Issues Forum Pages: 93 Publication Title: Aquacultural Engineering Society’s Fourth Issues Forum. July 2008.

[29] Eugene Rurangwa and Marc C.J. Verdegem. “Microorganisms in recirculating aquaculture systems and their management”. en. In: *Reviews in Aquaculture* 7.2 (2015), pp. 117–130. DOI: 10.1111/raq.12057.

[30] *Recirculating Aquaculture System (RAS) Design*. en-US. URL: https://innovasea.com/land-based-aquaculture/ras-design/.

[31] Anders J. Fjellheim et al. *Resirkulering av vann i settefiskproduksjon*. Norsk. 2016. URL: https://folk.ntnu.no/skoge/diplom/prosjekt19/more-info-on-projects/RAS/7127-2017%20-%20RAS%20guide_NO_low.pdf.

[32] James M Ebeling. “Biofiltration-Nitrification Design Overview”. en. In: (), p. 62.

[33] Michael Ben Timmons and James M. Ebeling. *Recirculating aquaculture*. en. 3. ed. NRAC Publication 401-2013. Ithaca, NY: Ithaca Publ. [u.a.], 2013. ISBN: 978-0-9712646-5-6.

[34] Brit Hjeltnes. “Risk Assessment of Recirculation Systems in Salmonid Hatcheries”. en. In: 09 ().

[35] *Forskrift om drift av akvakulturanlegg (Akvakulturdriftsforskriften) — Mattilsynet*. URL: https://www.mattilsynet.no/om_mattilsynet/gjeldende_regelverk/forskrifter/forskrift_om_drift_av_akvakulturanlegg_akvakulturdriftsforskriften.616.

[36] Maria Teresa Gutierrez-Wing and Ronald F. Malone. “Biological filters in aquaculture: Trends and research directions for freshwater and marine applications”. In: *Aquacultural Engineering* 34.3 (2006), pp. 163–171. ISSN: 0144-8609. DOI: https://doi.org/10.1016/j.aquaeng.2005.08.003.

[37] Mark J. Sharrer et al. “Inactivation of bacteria using ultraviolet irradiation in a recirculating salmonid culture system”. en. In: *Aquacultural Engineering* 33.2 (Aug. 2005), pp. 135–149. DOI: 10.1016/j.aquaeng.2004.12.001.

[38] N Leonard, J. P Blancheton, and J. P Guiraud. “Populations of heterotrophic bacteria in an experimental recirculating aquaculture system”. en. In: *Aquacultural Engineering* 22.1 (May 2000), pp. 109–120. DOI: 10.1016/S0144-8609(00)00035-2.

[39] “Microbial Transformations”. en. In: *Environmental Biology for Engineers and Scientists*. Hoboken, NJ, USA: John Wiley & Sons, Inc., Oct. 2005, pp. 387–441. ISBN: 978-0-471-74179-4 978-0-471-72239-7. DOI: 10.1002/0471741795.ch13. URL: https://onlinelibrary.wiley.com/doi/10.1002/0471741795.ch13.

[40] F Fdz-Polanco et al. “Spatial distribution of heterotrophs and nitrifiers in a submerged biofilter for nitrification”. en. In: *Water Research* 34.16 (Nov. 2000), pp. 4081–4089. DOI: 10.1016/S0043-1354(00)00159-7.

[41] Michael Madigan et al. *Brock Biology of Microorganisms*. eng. 15th ed. Pearson Education Limited, 2019.

[42] Sean M Gibbons and Jack A Gilbert. “Microbial diversity — exploration of natural ecosystems and microbiomes”. In: *Current opinion in genetics & development* 35 (Dec. 2015), pp. 66–72. DOI: 10.1016/j.gde.2015.10.003.

[43] Alejandra Escobar-Zepeda, Arturo Vera-Ponce de León, and Alejandro Sanchez-Flores. “The Road to Metagenomics: From Microbiology to DNA Sequencing Technologies and Bioinformatics”. In: *Frontiers in Genetics* 6 (2015). URL: https://www.frontiersin.org/articles/10.3389/fgene.2015.00348.

[44] Mark Osborn and Cindy Smith, eds. *Molecular Microbial Ecology*. en. 1st ed. Taylor and Francis, 2005. URL: https://www.routledge.com/Molecular-Microbial-Ecology/Osborn-Smith/p/book/9781859962831.

[45] Torsten Thomas, Jack Gilbert, and Folker Meyer. “Metagenomics - a guide from sampling to data analysis”. In: *Microbial Informatics and Experimentation* 2 (Feb. 2012), p. 3. DOI: 10.1186/2042-5783-2-3.

[46] “Next-Generation Sequencing”. en. In: *Molecular Microbiology*. Ed. by Persing et al. American Society of Microbiology, Jan. 2016, pp. 68–79. ISBN: 978-1-55581-908-8 978-1-55581-907-1. DOI: 10.1128/9781555819071.ch6.

[47] Jared M. Churko et al. “Overview of High Throughput Sequencing Technologies to Elucidate Molecular Pathways in Cardiovascular Diseases”. In: *Circulation research* 112.12 (June 2013), 10.1161/CIRCRESAHA.113.300939. DOI: 10.1161/CIRCRESAHA.113.300939.

[48] Jason A. Reuter, Damek Spacek, and Michael P. Snyder. “High-Throughput Sequencing Technologies”. In: *Molecular cell* 58.4 (May 2015), pp. 586–597. DOI: 10.1016/j.molcel.2015.05.004.

[49] M. Begon, J. L. Harper, and C. R. Townsend. “Ecology. Individuals, populations and communities.” English. In: *Ecology. Individuals, populations and communities.* (1986). Publisher: Blackwell Scientific Publications. URL: https://www.cabdirect.org/cabdirect/abstract/19870540497.

[50] *Metagenomics*. en. Sept. 2022. URL: https://www.genome.gov/genetics-glossary/Metagenomics.

[51] Jan Torsten Jeske and Claudia Gallert. “Microbiome Analysis via OTU and ASV-Based Pipelines—A Comparative Interpretation of Ecological Data in WWTP Systems”. In: *Bioengineering* 9.4 (Mar. 2022), p. 146. DOI: 10.3390/bioengineering9040146.

[52] Jethro S. Johnson et al. “Evaluation of 16S rRNA gene sequencing for species and strain-level microbiome analysis”. en. In: *Nature Communications* 10.1 (Nov. 2019). Number: 1 Publisher: Nature Publishing Group, p. 5029. DOI: 10.1038/s41467-019-13036-1.

[53] Benjamin J. Callahan, Paul J. McMurdie, and Susan P. Holmes. “Exact sequence variants should replace operational taxonomic units in marker-gene data analysis”. en. In: *The ISME Journal* 11.12 (Dec. 2017). Number: 12 Publisher: Nature Publishing Group, pp. 2639–2643. DOI: 10.1038/ismej.2017.119.

[54] Tobias Andermann et al. “Estimating Alpha, Beta, and Gamma Diversity Through Deep Learning”. In: *Frontiers in Plant Science* 13 (Apr. 2022), p. 839407. DOI: 10.3389/fpls.2022.839407.

[55] Louise von Gersdorff Jørgensen. *RASOPTA*. en. Publisher: University of Copenhagen. May 2021. URL: https://rasopta.ku.dk/.

[56] *NanoDrop One User Guide*. English. 2022. URL: https://assets.thermofisher.com/TFS-Assets/MSD/manuals/nanodrop-one-user-guide-EN-309102-REV-A.pdf.pdf.

[57] *Qubit Assays Quick Reference*. 2014. URL: https://assets.fishersci.com/TFS-Assets/LSG/manuals/qubit_assays_qrc.pdf.

[58] Stine Wiborg Dahle et al. "Overvåkning av bakteriesamfunn i settefiskproduksjonen". Norsk. In: *Norsk fiskeoppdrett* (2020).

[59] *RK Bioelements - Bio-media in biological water purification - RK Bioelements*. en. URL: https://en.rkbioelements.dk/.

[60] Robert C. Edgar. "Search and clustering orders of magnitude faster than BLAST". In: *Bioinformatics* 26.19 (Oct. 2010), pp. 2460–2461. DOI: 10.1093/bioinformatics/btq461.

[61] Robert C. Edgar. *UNOISE2: improved error-correction for Illumina 16S and ITS amplicon sequencing*. en. Pages: 081257 Section: New Results. Oct. 2016. DOI: 10.1101/081257.

[62] Robert C. Edgar. *SINTAX: a simple non-Bayesian taxonomy classifier for 16S and ITS sequences*. en. Pages: 074161 Section: New Results. Sept. 2016. DOI: 10.1101/074161.

[63] Oyvind Hammer, David A T Harper, and Paul D Ryan. "PAST: Paleontological Statistics Software Package for Education and Data Analysis". en. In: ().

[64] Anne Chao et al. "A new statistical approach for assessing similarity of species composition with incidence and abundance data". en. In: *Ecology Letters* 8.2 (2005). _eprint: https://onlinelibrary.wiley.com/doi/pdf/10.1111/j.1461-0248.2004.00707.x, pp. 148–159. DOI: 10.1111/j.1461-0248.2004.00707.x.

[65] C. E. Shannon. "A mathematical theory of communication". In: *The Bell System Technical Journal* 27.3 (July 1948). Conference Name: The Bell System Technical Journal, pp. 379–423. DOI: 10.1002/j.1538-7305.1948.tb01338.x.

[66] J. Roger Bray and J. T. Curtis. "An Ordination of the Upland Forest Communities of Southern Wisconsin". en. In: *Ecological Monographs* 27.4 (1957), pp. 325–349.

[67] K. R. Clarke. "Non-parametric multivariate analyses of changes in community structure". en. In: *Australian Journal of Ecology* 18.1 (1993), pp. 117–143. DOI: 10.1111/j.1442-9993.1993.tb00438.x.

[68] James R. Cole et al. "Ribosomal Database Project: data and tools for high throughput rRNA analysis". eng. In: *Nucleic Acids Research* 42.Database issue (Jan. 2014), pp. D633–642. DOI: 10.1093/nar/gkt1244.

[69] Sudhir Kumar et al. "MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms". eng. In: *Molecular Biology and Evolution* 35.6 (June 2018), pp. 1547–1549. DOI: 10.1093/molbev/msy096.

[70] K. Tamura and M. Nei. “Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees”. eng. In: *Molecular Biology and Evolution* 10.3 (May 1993), pp. 512–526. DOI: 10.1093/oxfordjournals.molbev.a040023.

[71] Susannah J Salter et al. “Reagent and laboratory contamination can critically impact sequence-based microbiome analyses”. In: *BMC Biology* 12 (Nov. 2014), p. 87. DOI: 10.1186/s12915-014-0087-z.

[72] Angela Glassing et al. “Inherent bacterial DNA contamination of extraction and sequencing reagents may affect interpretation of microbiota in low bacterial biomass samples”. In: *Gut Pathogens* 8.1 (May 2016), p. 24. DOI: 10.1186/s13099-016-0103-7.

[73] C. Schrader et al. “PCR inhibitors – occurrence, properties and removal”. en. In: *Journal of Applied Microbiology* 113.5 (2012), pp. 1014–1026. DOI: 10.1111/j.1365-2672.2012.05384.x.

[74] Kenneth H. Roux. “Optimization and Troubleshooting in PCR”. en. In: *Cold Spring Harbor Protocols* 2009.4 (Apr. 2009). Publisher: Cold Spring Harbor Laboratory Press, pdb.ip66. DOI: 10.1101/pdb.ip66.

[75] Jay Shankar Singh et al. “Cyanobacteria: A Precious Bio-resource in Agriculture, Ecosystem, and Environmental Sustainability”. In: *Frontiers in Microbiology* 7 (2016). URL: https://www.frontiersin.org/articles/10.3389/fmicb.2016.00529.

[76] Ole Folkedal et al. “Food anticipatory behaviour as an indicator of stress response and recovery in Atlantic salmon post-smolt after exposure to acute temperature fluctuation”. en. In: *Physiology & Behavior* 105.2 (Jan. 2012), pp. 350–356. DOI: 10.1016/j.physbeh.2011.08.008.

[77] *FAO: Feed Production*. URL: https://www.fao.org/fishery/affris/species-profiles/atlantic-salmon/feed-production/en/.

[78] J. P. Blancheton et al. “Insight into bacterial population in aquaculture systems and its implication”. In: *Aquacultural Engineering* 53 (2013), pp. 30–39. DOI: https://doi.org/10.1016/j.aquaeng.2012.11.009.

[79] Olav Vadstein et al. “K-Selection as Microbial Community Management Strategy: A Method for Improved Viability of Larvae in Aquaculture”. In: *Frontiers in Microbiology* 9 (2018). URL: https://www.frontiersin.org/articles/10.3389/fmicb.2018.02730.

[80] Stine Wiborg Dahle et al. “Long-term microbial community structures and dynamics in a commercial RAS during seven production batches of Atlantic salmon fry (Salmo salar)”. In: *Aquaculture* 565 (2023), p. 739155. DOI: https://doi.org/10.1016/j.aquaculture.2022.739155.

[81] Sanni L. Aalto et al. “Microbiome response to foam fractionation and ozonation in RAS”. en. In: *Aquaculture* 550 (Mar. 2022), p. 737846. DOI: 10.1016/j.aquaculture.2021.737846.

[82] Ragnhild O. Fossmark et al. “A comparison of two seawater adaptation strategies for Atlantic salmon post-smolt (Salmo salar) grown in recirculating aquaculture systems (RAS): Nitrification, water and gut microbiota, and performance of fish”. en. In: *Aquaculture* 532 (Feb. 2021), p. 735973. DOI: 10.1016/j.aquaculture.2020.735973.

[83] Maartje A. H. J. van Kessel et al. “Complete nitrification by a single microorganism”. en. In: *Nature* 528.7583 (Dec. 2015). Number: 7583 Publisher: Nature Publishing Group, pp. 555–559. DOI: 10.1038/nature16459.

[84] Norisuke Ushiki et al. “Genomic Analysis of Two Phylogenetically Distinct Nitrospira Species Reveals Their Genomic Plasticity and Functional Diversity”. In: *Frontiers in Microbiology* 8 (2018). URL: https://www.frontiersin.org/articles/10.3389/fmicb.2017.02637.

[85] Myriam Kruse et al. “Relevance and Diversity of Nitrospira Populations in Biofilters of Brackish RAS”. In: *PLoS ONE* 8.5 (May 2013), e64737. DOI: 10.1371/journal.pone.0064737.

[86] Ingrid Bakke et al. “Microbial community dynamics in semi-commercial RAS for production of Atlantic salmon post-smolts at different salinities”. en. In: *Aquacultural Engineering*. Workshop on Recirculating Aquaculture Systems (III) 78 (Aug. 2017), pp. 42–49. DOI: 10.1016/j.aquaeng.2016.10.002.

[87] Ryan P. Bartelme et al. “Component Microenvironments and System Biogeography Structure Microorganism Distributions in Recirculating Aquaculture and Aquaponic Systems”. In: *mSphere* 4.4 (July 2019). Publisher: American Society for Microbiology, e00143–19. DOI: 10.1128/mSphere.00143-19.

[88] Anette Voll Bugten et al. “Changes in rearing water microbiomes in RAS induced by membrane filtration alters the hindgut microbiomes of Atlantic salmon (Salmo salar) parr”. en. In: *Aquaculture* 548 (Feb. 2022), p. 737661. DOI: 10.1016/j.aquaculture.2021.737661.

[89] Jinshui Zheng et al. “A taxonomic note on the genus Lactobacillus: Description of 23 novel genera, emended description of the genus Lactobacillus Beijerinck 1901, and union of Lactobacillaceae and Leuconostocaceae”. In: *International Journal of Systematic and Evolutionary Microbiology* 70.4 (2020). Publisher: Microbiology Society, pp. 2782–2858. DOI: 10.1099/ijsem.0.004107.

[90] Shruti Gupta et al. “Lactobacillus Dominate in the Intestine of Atlantic Salmon Fed Dietary Probiotics”. In: *Frontiers in Microbiology* 9 (Jan. 2019), p. 3247. DOI: 10.3389/fmicb.2018.03247.

[91] A. Panigrahi et al. “Immune modulation and expression of cytokine genes in rainbow trout Oncorhynchus mykiss upon probiotic feeding”. en. In: *Developmental & Comparative Immunology* 31.4 (Jan. 2007), pp. 372–382. DOI: 10.1016/j.dci.2006.07.004.

[92] Nopadon Pirarat et al. “Protective effects and mechanisms of a probiotic bacterium Lactobacillus rhamnosus against experimental Edwardsiella tarda infection in tilapia (Oreochromis niloticus)”. en. In: *Veterinary Immunology and Immunopathology* 113.3 (Oct. 2006), pp. 339–347. DOI: 10.1016/j.vetimm.2006.06.003.

[93] Pingting Guo et al. “Clostridium species as probiotics: potentials and challenges”. In: *Journal of Animal Science and Biotechnology* 11.1 (Feb. 2020), p. 24. DOI: 10.1186/s40104-019-0402-1.

7 Appendix

A Buffer and media solutions

A.1 Tris-acetate-EDTA (TAE) buffer

Table A.1.1: The composition of 50x TAE-buffer used for gel electrophoresis. Preparation of 1x TAE-buffer by diluting 40 ml 50x TAE in 1960 ml Milli-Q® water.

Component	Amount
Tris base	242g
Glacial acetic acid	57.1ml
0.5 M EDTA pH 8.0	100mL
Milli-Q® water	up to 1L

A.2 Tris-EDTA (TE) buffer

Table A.2.1: The composition of TE-buffer used during washing step for preparation of samples for Illumina sequencing.

Component	Amount (mg/l)	Final concentration (mM)
2M Tris-HCl pH 7.5	2.5	10.0
0.5M EDTA pH 8.0	1.0	1.0
dH_2O	496.5	-

A.3 Media used for the biofilm carriers before the start of the lab-scale RAS experiment

Table A.3.1: Composition of TAN-nutrient medium (10mg/L TAN) used to maintain the nitrifying community in the biofilters before the start of the lab-scale RAS experiment.

Component	Quantity (g/L)
$(NH_4)_2SO_4$	0.04717
$NaH_2PO_4 \cdot {}_2H_2O$	0.272
Na_2CO_3	0.2
$NaNO_2$	0.02463
Trace metal solution	10 ml of stock solution
HCl (pH adjustment)	Drops of HCl to pH 5 or pH 7

Table A.3.2: Composition of trace metal solution used in TAN-nutrient medium.

Chemicals	Quantity (g/L)
MgSO4·7H2O	2,5
CaCl2·2H2O	1,5
FeCl2·4H2O	0,2
MnCl2·4H2O	0,55
ZnCl2	0,07
CoCl2·6H2O	0,12
NiCl2·6H2O	0,12
EDTA; Titriplex III	2,8

B DNA/RNA-extraction kit protocols

B.1 MagAttract PowerSoil Pro DNA kit

October 2020

Quick-Start Protocol

MagAttract® PowerSoil® Pro DNA Kit with KingFisher®

This protocol describes the use of the MagAttract PowerSoil Pro DNA Kit (cat. no. 47109) with the KingFisher Flex instrument. For use with the epMotion® instrument, please refer to the *MagAttract PowerSoil Pro DNA Handbook*.

Solution CD2 should be stored at 2–8°C upon arrival. All other reagents and kit components should be stored at room temperature (15–25°C).

Further information

- *MagAttract PowerSoil Pro DNA Handbook*: **www.qiagen.com/HB-2816**
- Safety Data Sheets: **www.qiagen.com/safety**
- Technical assistance: **support.qiagen.com**

Notes before starting

- Use extra-long pipette tips (1000–1250 µl) for collection microtube racks (CMTRs).
- Add 400 µl RNase A Solution to 80 ml Solution CD1 for each 96-well plate to be processed.
- Prepare Buffer QSB1 and Buffer MW1 according to the instructions on the bottles.
- 80% ethanol is required in this protocol and needs to be supplied by the user.

Sample to Insight

Procedure

1. Spin the PowerBead Pro Plate (cat. no. 19311) or the PowerBead Pro Tube (cat. no. 19301) briefly to ensure that the beads have settled at the bottom of the wells or tube.
2. Add up to 250 mg of soil or 100 mg of stool into the plate/tube, and 800 µl Solution CD1/RNase A Solution. Seal the plate with sealing film or recap the tube.
3. Homogenize samples thoroughly using the TissueLyser II (cat. no. 85300). (For other homogenization methods, please refer to the *MagAttract PowerSoil Pro DNA Handbook*.)
   3a. If using a PowerBead Pro Plate, place a silicone compression mat on top of the sealing film, and then place the sealed plate and mat between 2 adapter plates (cat. no. 11990). Shake for 5 min at 25 Hz.
   Reorient the plates so that the sides that were closest to the machine body are now furthest from it. Shake again for 5 min at 25 Hz.
   3b. If using PowerBead Pro Tubes, place the tubes into a TissueLyser Adapter Set 2 x 24 (cat. no. 69982), or into a 2 ml Tube Holder (cat. no. 11993) and Plate Adapter Set (cat. no. 11990). Fasten the adapter into the TissueLyser II. Shake for 5 min at 25 Hz. Reorient the adapter so that the side that was closest to the machine body becomes furthest from it. Shake again for 5 min at 25 Hz.
4. Centrifuge the PowerBead Pro Plate at 4500 x *g* for 6 min, or the PowerBead Pro Tubes at 15,000 x *g* for 1 min.
5. Transfer the supernatant to the CMTRs.

   Note: Expect 500–600 µl. The supernatant may still contain some soil/stool particles.

6. Add 300 µl Solution CD2. Seal the CMTRs with the caps provided, and then vortex.
7. Centrifuge the CMTRs at 4500 x *g* for 6 min at room temperature.
8. Taking care to avoid any residual pellet, transfer no more than 450 µl supernatant from each well to a clean KingFisher deep-well 96 plate.
9. Resuspend MagAttract Suspension G Beads by vortexing. For each 96-well plate to be processed, add 3 ml of the resuspended MagAttract Suspension G Beads to 44 ml Buffer QSB1 and mix well. Immediately transfer to a multichannel pipette reservoir.

   Note: Maintain the MagAttract Suspension G Beads in suspension to ensure uniform distribution.
10. Add 470 µl of the MagAttract Suspension G beads/Buffer QSB1 mix to each well containing lysate in a KingFisher 96 deep-well plate.
11. Place the plate on the robotic deck at the specified location indicated in the program.
12. Add 500 µl Buffer MW1 to each well of one clean KingFisher 96 deep-well plate. Add 80% ethanol (provided by the user) to each well of 2 clean KingFisher 96 deep-well plates. Place the plates on the robotic deck at the specified locations indicated in the program.
13. Add 100 µl Solution C6 to each well of a clean KingFisher 96 microplate and place on the robotic deck at the specified location. Initiate the robotic program.
14. Upon completion of the robotic program, cover the wells of the KingFisher 96 microplate with an appropriate storage seal. DNA is now ready for downstream applications.

Document Revision History

Date	Changes
10/2020	Initial release

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit handbook or user manual.

B.2 ZymoBIOMICS 96 MagBead DNA kit (Cat.no: D4308)

For automated scripts and **Technical Assistance** regarding generation of scripts for automated platforms, contact **Zymo Research's Technical Department** at 1-888-882-9682 or E-mail to tech@zymoresearch.com.

[1]For water samples, filter using desired filter (not provided). Cut the filter into small pieces and place into ZR BashingBead™ Lysis Tubes (0.1 & 0.5 mm).

[2]Swabs can also be cut or broken and placed directly in bead beating tube. For more information on processing swab samples, see Appendix B.

[3] See Appendix A for additional information on sample collection in DNA/RNA Shield™.

[4] For validated bead beating devices and conditions, refer to the Optimized Lysis Protocols on the website product page under Documents.

[5] For optimal lysis efficiency and unbiased profiling all bead beater devices beyond those validated by Zymo Research should be calibrated using the ZymoBIOMICS™ Microbial Community Standard. See Appendix C.

Protocol

Sample Lysis

For all mixing steps: pipette mix or shake at max speed.

Note: Shaking speed will depend on sample volume and plate well depth. Use of shaker plates will require user optimization.

1. Add sample to the **ZR BashingBead™ Module** using the table below.
   a. If using **ZR BashingBead™ Lysis Tubes (0.1 & 0.5 mm)**, add 750 µl ***ZymoBIOMICS Lysis Solution*.**
   b. If using **ZR BashingBead™ Lysis Rack (0.1 & 0.5 mm)**, add 650 µl ***ZymoBIOMICS Lysis Solution*.**

   ***Note: DNA/RNA Shield** has been provided to optionally replace **ZymoBIOMICS Lysis Solution** to improve DNA integrity.*

Sample Type	Maximum Input
Feces	100 mg
Soil	200 mg
Liquid Samples[1] and Swab Collections[2]	250 µl
Cells (Suspended in DNA/RNA Shield™ or isotonic buffer, *e.g.* PBS)	5-20 mg (wet weight) (2×10^8 bacterial or 2×10^7 yeast cells)
Samples in DNA/RNA Shield™ (10% v/v Sample)[3]	250 µl

2. Secure in a bead beater fitted with the appropriate holder assembly for your bead beating module and process using optimized bead beating conditions (speed and time) for your device[4,5].
3. Centrifuge the ZR BashingBead™ Lysis Module:
   a. If using **ZR BashingBead™ Lysis Tubes (0.1 & 0.5 mm),** centrifuge at ≥10,000 x g for 1 minute.
   b. If using **ZR BashingBead™ Lysis Rack (0.1 & 0.5 mm),** centrifuge at ≥4,000 x g for 5 minutes.

Sample Purification

4. Transfer 200 µl supernatant to the deep-well block (not provided). Add 600 µl **ZymoBIOMICS™ MagBinding Buffer.**

   ***Note:** For samples with excessive amounts of solid particulate, centrifuge at 4,000 x g for 5 minutes to reduce clogging.*

5. Dispense 25 µl of **ZymoBIOMICS™ MagBinding Beads** to each well. Mix well by pipette or shaker plate for 10 minutes.

   ***Note:** ZymoBIOMICS MagBinding Beads settle quickly, ensure that beads are kept in suspension while dispensing.*

Sample Purification (Continued)

6. Transfer the 96-well block to a magnetic stand until beads pellet, then aspirate and discard the supernatant. Remove the 96-Well Block from the magnetic stand.
7. Dispense 500 µl of **ZymoBIOMICS™ MagBinding Buffer** and mix well by pipette or shaker plate for 1 minute.
8. Transfer the 96-well block to a magnetic stand until beads pellet, then aspirate and discard the supernatant. Remove the 96-Well Block from the magnetic stand.
9. Dispense 500 µl of **ZymoBIOMICS™ MagWash 1** and mix well by pipette or shaker plate for 1 minute.
10. Transfer the 96-well block to a magnetic stand until beads pellet, then aspirate and discard the supernatant. Remove the 96-Well Block from the magnetic stand.
11. Dispense 900 µl **ZymoBIOMICS™ MagWash 2** and mix well by pipette or shaker plate for 1 minute.

    Note: *If high speed shaker plates are used, dispense 500 µl ZymoBIOMICS™ MagWash 2.*
12. Transfer the deep-well block to a magnetic stand until beads pellet, then aspirate and discard the supernatant. Remove the 96-Well Block from the magnetic stand.
13. Repeat the wash (Steps 11-12) twice.
14. Transfer the 96-Well Block onto a heating element (55°C) until beads dry (approximately 10 minutes). If no heating element is available, air dry for approximately 20-30 minutes.
15. Dispense 50 µl of **ZymoBIOMICS™ DNase/RNase Free Water** to each well and re-suspend beads. Mix the beads well for 10 minutes and then transfer the plate onto the magnetic stand for 2-3 minutes until the beads pellet[6].
16. Transfer the supernatant (containing the eluted DNA) to a clean elution plate or tube[7].

    The eluted DNA can be used immediately for molecular based applications or stored ≤ -20ºC for future use.

[6] See Appendix D for additional elution information.

[7] For optimal spectrophotometric quantification, eluate may be centrifuged at max speed to pellet magbeads.

B.3 ZymoBIOMICS™ 96 MagBead DNA/RNA kit (Cat.no: R2135)

ZymoBIOMICS™ Magbead DNA/RNA – Paralell Extraction
Catalog Nos. R2135, R2136

Automation Reference Guide – Magnetic Bead Transfer Systems

ZYMO RESEARCH

Items needed for protocol (bold items are from Zymo Research):

- Reagent needed
  - **ZymoBIOMICS MagBead DNA/RNA (R2135, R2136)**
- Hardware / Labware
  - Magnetic Bead Transfer System
    - Thermo Fisher Scientific KingFisher Flex System
  - KingFisher 96 deep-well plate, v-bottom, polypropylene, 95040450
  - KingFisher 96 tip comb for deep-well magnets, 97002534
  - KingFisher 96 (200 µL) microplate, 97002540
- Script: R2135_ZymoBIOMICS DNARNA Magbead_DNA Purification_v1.bdz
  R2135_ZymoBIOMICS DNARNA Magbead_RNA Purification_v1.bdz

Assay Protocol:

Step 0: Reagent Preparation:

Materials:

- Proteinase K and Storage Buffer, D3001-2-20
- MagBead DNA/RNA Wash 1 (concentrate), R2130-1-30 or R2130-1-120
- MagBead DNA/RNA Wash 2 (concentrate), R2130-2-20 or R2130-2-80
- Ethanol (95-100%), user supplied
- DNase I Set, E1010

Protocol:

I. Preparation of Proteinase K
1. Add 1040 µl of **Proteinase K Storage Buffer** to the tube containing **Proteinase K**.
2. Dissolve completely then store at -20 °C.

II. Preparation of DNase I Digestion Mix
1. Reconstitute **DNase I** with **ZymoBIOMICS™ DNase/RNase-Free Water** (according to the table A below), transfer into an RNase-free tube and mix by inversion. Store frozen aliquots.
   Add **DNA Digestion Buffer** (according to table B below) to the reconstituted DNase I and mix by inversion, then place on ice until ready to use. Add 50 µl DNase I Reaction Mix per sample during RNA Purification.

A.

Prep Size	DNase I (lyophillized)	ZymoBIOMICS™ DNase/RNase-Free Water
96 preps	3 x 250 U	2.25 ml each
4 x 96 preps	2 x 1500 U	13.5 ml each

B.

DNA Digestion Buffer
0.25 ml per vial
1.5 ml per vial

III. Preparation of MagBead DNA/RNA Wash 1
1. Add isopropanol to **MagBead DNA/RNA Wash 1** concentrate to a final dilution of 40% (v/v)
Example: 20 ml isopropanol per 30 ml MagBead DNA/RNA Wash 1 concentrate (R2130-1-30) or 80 ml of Isopropanol per 120 ml MagBead DNA/RNA Wash 1 concentrate (R2130-1-120).

IV. Preparation of MagBead DNA/RNA Wash 2
1. Add isopropanol to **MagBead DNA/RNA Wash 2** concentrate to a final dilution of 60% (v/v).
Example: 30 ml isopropanol per 20 ml MagBead DNA/RNA Wash 2 concentrate (R2130-1-20) or 120 ml of Isopropanol per 80 ml MagBead DNA/RNA Wash 2 concentrate (R2130-2-80).

Step 1: Sample Preparation (Off-Deck)

Perform all steps at room temperature (20 - 30°C)

Materials:

- Sample Input
- Proteinase K, resuspended—from Step 0. I.
- DNA/RNA Shield, R1100-50 or R1100-250

I. Mechanical Homogenization

1. Add 750 µl **DNA/RNA Shield™** to a sample (see table below) and mix and/or homogenize[1]

Sample Type	Maximum Input
Feces	50 mg
Soil	50 mg
Liquid Samples[2] and Swab Collections	250 µl
Cells (resuspended in DNA/RNA Shield™ or isotonic buffer, e.g. PBS)	5-20 mg (wet weight; 2x108 bacterial, 2x107 yeast cells, 2x106 mammalian cells)
Samples in DNA/RNA Shield™ (10% v/v)	250 µ

2. To achieve unbiased lysis of different organisms, including hard-to-lyse microbes, perform mechanical homogenization[3] (Recommended e.g., lysis tubes S6012-50 or lysis rack S6002-96-3; each sold separately). Then centrifuge at 10,000 – 16,000 *x g* for 30 seconds to pellet debris.

[1]At this point, samples in DNA/RNA Shield can be stored at ambient temperature (4-25°C) for a month, 3 days at 37°C, or long-term (> 1 year) -20°C or below.
[2]For water samples, filter using desired filter (not provided). Cut the filter into small pieces and place into ZR BashingBead™ Lysis Tubes or into 96-well Lysis Rack. Swabs can also be cut or broken and placed directly in bead beating tube.
[3] Secure in a high-speed bead beater and process at maximum speed for ≤ 5 minutes (e.g., Bertin Precellys, FastPrep®) or for ≥ 10 minutes when using low speed homogenizers (e.g.,Disruptor Genie™).

II. Sample Digestion with Proteinase K

Materials:

- Sample Input (following mechanical homogenization)
- Proteinase K, resuspended – from Step 0. I.
- 1 x KingFisher 96 deep-well plate, v-bottom, polypropylene, 95040450

Protocol:

1. Prepare a KingFisher 96 deep-well plate for sample digestion as follows:

Sample Input A	200 µL
Proteinase K, resuspended	10 µL
Total Volume	210 µL

Note: Prevent cross contamination by dispensing the sample after the pipette tip is half-way into the well. DO NOT let liquid from the pipette tip touch another well while entering or removing the tip from the well.

2. Mix well and incubate the sample mixture at room temperature for 30 minutes.

Step 2: KingFisher Reagent Plates Preparation—DNA Extraction

Materials:

- DNA/RNA Lysis Buffer, R1060-1-50 or R1060-100
- MagBead DNA/RNA Wash 1, prepared in Step 0. III.
- MagBead DNA/RNA Wash 2, prepared in Step 0. IV.
- Ethanol (95-100%), user supplied
- RNA Prep Buffer, R1060-2-50 or R1060-2-100
- DNase I Digestion Mix, prepared in Step 0.II.
- ZymoBIOMICS DNase/RNase-Free Water, D4302-30 or D4302-5-50
- ZymoBIOMICS MagBinding Beads, D4302-6-6 or D4302-6-12
- KingFisher 96 deep-well plate prepared in Step 1. II.
  5 x KingFisher 96 deep-well plate, v-bottom, polypropylene, 95040450
- 1 x KingFisher 96 tip comb for deep-well magnets, 97002534
- 1 x KingFisher 96 (200 µL) microplate, 97002540
- KingFisher Flex Layout Program: R2135_ZymoBIOMICS DNARNA Magbead_DNA Purification_v1.bdz

Note: Use a different KingFisher 96 deep-well plate, v-bottom, polypropylene, 95040450, for each reagent unless specified. The number of wells used correspond to number of samples being processed. Orientation of plates should be noted.

Protocol:

I. Sample Plate Preparation:
1. Add 500 ul of **DNA/RNA Lysis Buffer** into the KingFisher 96-deep-well plate with the digested sample (prepared in Step 1. II.)
2. Vortex the container of **MagBinding Beads** for 10 seconds at maximum speed.
3. Aliquot 30 µL of MagBinding Beads into the same plate.

Note: Prevent cross contamination by dispensing the buffers after the pipette tip is half-way into the well. DO NOT let liquid from the pipette tip touch another well while entering or removing the tip from the well.

II. MagBead DNA/RNA Wash 1 Plate Preparation:
1. Aliquot 500 µL of **MagBead DNA/RNA Wash 1 Buffer** into a KingFisher 96 deep-well plate.

III. MagBead DNA/RNA Wash 2 Plate Preparation:
1. Aliquot 500 µL of **MagBead DNA/RNA Wash 2 Buffer** into a KingFisher 96 deep-well plate.

IV. Ethanol 1 Plate Preparation:
1. Aliquot 500 µL of Ethanol (95-100%) into a KingFisher 96 deep-well plate.

V. Ethanol 2 Plate Preparation:
1. Aliquot 500 µL of Ethanol (95-100%) into a KingFisher 96 deep-well plate.
2. Slowly load the KingFisher 96 tip comb into the plate. Do not cause the Ethanol to splash out of well.

VI. Elution Plate Preparation:
1. Aliquot 50 µL of **ZymoBIOMICS DNase/RNase-Free Water** into a KingFisher 96 (200 µL) microplate.
2. Ensure the elution buffer is at the bottom center of the plate

Step 3: Operating KingFisher and Loading Plates—DNA Extraction

Materials:

- Prepared Reagent Plates from Step 2.
  R2135_ZymoBIOMICS DNARNA Magbead_DNA Purification_v1.bdz

Protocol:

I. Thermo Fisher Scientific KingFisher Flex Initialization
   1. Power-on the KingFisher Flex.
   2. Load program (R2135_ZymoBIOMICS DNARNA Magbead_DNA Purification_v1.bdz) on the KingFisher software BindIt.
   3. Click on start to begin program.
   4. Follow program instructions on the KingFisher screen to load plates.
   5. Ensure orientation of plates is uniform.
   6. Close plastic shield and start script.
   7. After completion of the program, carefully remove KingFisher 96 (200 µL) microplate, which will contain the eluted DNA.
   8. Keep Sample Plate from DNA extraction. Supernatant will be used as sample input in Step 4.

Step 4: Supernatant Sample Transfer for RNA Extraction

Materials:

- Sample Plate from DNA extraction

Protocol:

1. Transfer 350 ul supernatant from the Sample Plate from the DNA Extraction into a new KingFisher 96 deep-well plate.

Note: Prevent cross contamination by dispensing the sample after the pipette tip is half-way into the well. DO NOT let liquid from the pipette tip touch another well while entering or removing the tip from the well.

Step 5: KingFisher Reagent Plates Preparation—RNA Extraction

Materials:

- RNA Sample Plate prepared in Step 4.
- MagBead DNA/RNA Wash 1, prepared in Step 0. III.
- MagBead DNA/RNA Wash 2, prepared in Step 0. IV.
- Ethanol (95-100%), user supplied
- DNA/RNA Prep Buffer, R1060-2-50 or R1060-2-100
- DNase I Digestion Mix, prepared in Step 0.II.
- ZymoBIOMICS DNase/RNase-Free Water, D4302-30 or D4302-5-50
- ZymoBIOMICS MagBinding Beads, D4302-6-6 or D4302-6-12
- KingFisher 96 deep-well plate prepared in Step 1. II.
- 7 x KingFisher 96 deep-well plate, v-bottom, polypropylene, 95040450
- 1 x KingFisher 96 tip comb for deep-well magnets, 97002534
- 1 x KingFisher 96 (200 µL) microplate, 97002540
- KingFisher Flex Layout Program: R2135_ZymoBIOMICS DNARNA Magbead_RNA Purification_v1.bdz

Note: Use a different KingFisher 96 deep-well plate, v-bottom, polypropylene, 95040450, for each reagent unless specified. The number of wells used correspond to number of samples being processed. Orientation of plates should be noted.

Protocol:

VII. Sample Plate Preparation:
1. Add 350 ul of ethanol (95 – 100%) into the KingFisher 96-deep-well plate with the supernatant (prepared in Step 4)
2. Vortex the container of **MagBinding Beads** for 10 seconds at maximum speed.
3. Aliquot 30 µL of MagBinding Beads into the same plate.

Note: Prevent cross contamination by dispensing the buffers after the pipette tip is half-way into the well. DO NOT let liquid from the pipette tip touch another well while entering or removing the tip from the well.

VIII. MagBead DNA/RNA Wash 1 Plate Preparation:
2. Aliquot 500 µL of **MagBead DNA/RNA Wash 1 Buffer** into a KingFisher 96 deep-well plate.

IX. MagBead DNA/RNA Wash 2 Plate Preparation:
2. Aliquot 500 µL of **MagBead DNA/RNA Wash 2 Buffer** into a KingFisher 96 deep-well plate.

X. Ethanol 1 Plate Preparation:
2. Aliquot 500 µL of Ethanol (95-100%) into a KingFisher 96 deep-well plate.

XI. Ethanol 2 Plate Preparation:
3. Aliquot 500 µL of Ethanol (95-100%) into a KingFisher 96 deep-well plate.
4. Slowly load the KingFisher 96 tip comb into the plate. Do not cause the Ethanol to splash out of well.

XII. Ethanol 3 Plate Preparation:
1. Aliquot 500 µL of Ethanol (95-100%) into a KingFisher 96 deep-well plate.

XIII. Ethanol 4 and Tip Plate Preparation:
1. Aliquot 500 µL of Ethanol (95-100%) into a KingFisher 96 deep-well plate.
2. Slowly load the KingFisher 96 tip comb into the plate. Do not cause the Ethanol to splash out of well.

XIV. Elution Plate Preparation:
3. Aliquot 50 µL of **ZymoBIOMICS DNase/RNase-Free Water** into a KingFisher 96 (200 µL) microplate.
4. Ensure the elution buffer is at the bottom center of the plate

XV. DNase I Treatment Plate Preparation:
1. Aliquot 50 ul of prepared DNase I Digestion Mix (prepared in Step 0. II.) into a KingFisher 96-deep-well plate.
2. Ensure the reaction mix is at the bottom center of the plate.

Note: During the KingFisher protocol, 500 ul of DNA/RNA Prep Buffer will be added to each sample. **DO NOT** add RNA Prep Buffer before prompted after starting the program.

Step 6: Operating KingFisher and Loading Plates—RNA Extraction

Materials:

- Prepared Reagent Plates from Step 5.
  R2135_ZymoBIOMICS DNARNA Magbead_RNA Purification_v1.bdz

Protocol:

II. Thermo Fisher Scientific KingFisher Flex Initialization

1. Power-on the KingFisher Flex.
2. Load program (R2135_ZymoBIOMICS DNARNA Magbead_RNA Purification_v1.bdz) on the KingFisher software BindIt.
3. Click on start to begin program.
4. Follow program instructions on the KingFisher screen to load plates.
5. Ensure orientation of plates is uniform.
6. Close plastic shield and start script.
7. After approximately 35 minutes, the script will pause.
8. Dispense 500 ul of **DNA/RNA Prep Buffer** to each well in the DNase Treatment deep-well plate.
9. Follow program instructions on the KingFisher screen to continue script.
10. After completion of the program, carefully remove KingFisher 96 (200 µL) microplate, which will contain the eluted RNA.

B.4 ZymoBIOMICS™ Quick-DNA/RNA™ MagBead (Cat. no: R2130)

***Quick*-DNA/RNA™ Magbead – CoPurification**
Catalog Nos. R2130, R2131

Automation Reference Guide – Magnetic Bead Transfer Systems

ZYMO RESEARCH

Items needed for protocol (bold items are from Zymo Research):

- Reagent needed
  - ***Quick*-DNA/RNA™ Magbead (R2130, R2131)**
- Hardware / Labware
  - Magnetic Bead Transfer System
    - Thermo Fisher Scientific KingFisher Flex System
  - KingFisher 96 deep-well plate, v-bottom, polypropylene, 95040450
  - KingFisher 96 tip comb for deep-well magnets, 97002534
  - KingFisher 96 (200 µL) microplate, 97002540
- Script: R2130_Quick-DNA RNA Magbead_KingFisherFlex_Copurification_v1.bdz

Assay Protocol:

Step 0: Reagent Preparation:

Materials:

- Proteinase K and Storage Buffer, D3001-2-20
- MagBead DNA/RNA Wash 1 (concentrate), R2130-1-30 or R2130-1-120
- MagBead DNA/RNA Wash 2 (concentrate), R2130-2-20 or R2130-2-80
- Isopropanol, user supplied

Protocol:

I. Preparation of Proteinase K
1. Add 1040 µl of **Proteinase K Storage Buffer** to the tube containing **Proteinase K**.
2. Dissolve completely then store at -20 °C.

II. Preparation of MagBead DNA/RNA Wash 1
1. Add isopropanol to **MagBead DNA/RNA Wash 1** concentrate to a final dilution of 40% (v/v)

Example: 20 ml isopropanol per 30 ml MagBead DNA/RNA Wash 1 concentrate (R2130-1-30) or 80 ml of Isopropanol per 120 ml MagBead DNA/RNA Wash 1 concentrate (R2130-1-120).

III. Preparation of MagBead DNA/RNA Wash 2
1. Add isopropanol to **MagBead DNA/RNA Wash 2** concentrate to a final dilution of 60% (v/v).

Example: 30 ml isopropanol per 20 ml MagBead DNA/RNA Wash 2 concentrate (R2130-1-20) or 120 ml of Isopropanol per 80 ml MagBead DNA/RNA Wash 2 concentrate (R2130-2-80).

Step 1: Sample Preparation (Off-Deck)

Materials:

- Sample Input
- Proteinase K, resuspended – from Step 0. I.
- RNA Lysis Buffer, R1060-1-50 or R1060-100
- DNA/RNA Shield™, R1200-25 or R1200-125
- DNase/RNase-Free Water, W1001-30 or W1001-100

a. Cells

1. Pellet up to 10^6 mammalian cells (≤500 x g for 1 minute), remove the supernatant and resuspend the cell pellet in 200 µl **DNA/RNA Shield™** (1X)[1].
2. Proceed to Step 2.

b. Solid Tissue & Blood Cells (PBMCs, WBCs)

1. Add ≥ 200 µl **DNA/RNA Shield™** (1X)[1] to a solid tissue sample (≤ 5 mg) and mechanically homogenize[2,3]. Centrifuge to pellet debris and transfer 200 µl supernatant to a new tube. For blood cells, buffy coat and pelleted PAXgene™ samples (≤1 ml blood) resuspend in 200 µl DNA/RNA Shield™ (1X)[1]
2. Add 10 µl **Proteinase K** for every 200 µl sample. Mix and incubate at room temperature (20-30ºC) for 30 minutes.
3. Proceed to Step 2.

c. Urine[4]

1. Generate pellet from up to 40 ml urine by adding 70 µl **Urine Conditioning Buffer** for every 1 ml of urine and mix by vortexing. Centrifuge at 3,000 x g for 15 minutes. Discard the supernatant and leave up to 50 µl pellet.
2. Add 150 µl **DNA/RNA Shield™** (1X)[1] and resuspend the pellet by pipetting.
3. Add 10 µl **Proteinase K**. Mix and incubate at room temperature (20-30ºC) for 30 minutes.
4. Proceed to Step 2.

d. Whole Blood (Mammalian)

1. DNA & RNA Purification (in two separate fractions) is not compatible.

e. FFPE Tissue

1. Remove (trim) excess paraffin wax from ≤5 mg FFPE tissue and transfer into a new tube.
2. Add 400 µl **Deparaffinization Solution**[5] to the sample. Incubate at 55ºC for 1 minute. Vortex briefly. Remove the Deparaffinization Solution.
3. Add 95 µl **DNase/RNase-Free Water**, 95 µl **2X Digestion Buffer**[1,5], and 10 µl **Proteinase K**.
4. Incubate at 55ºC for 1 hour. Incubate at 94ºC for 20 minutes to de-crosslink the sample.
5. Centrifuge to remove insoluble debris.
6. Proceed to Step 2.

f. Environmental (Plant/Seed, etc.)

1. Add up to 50 mg plant material and 750 µl **DNA/RNA Shield™** (1X)[1] to a lysis tube/rack[2].
2. Secure in a bead beater fitted with the appropriate holder assembly for your bead beating module and process at maximum speed[3] for 1 minute.
3. Add 10 µl **Proteinase K**. Mix and incubate at room temperature (20-30ºC) for 30 minutes.
4. Centrifuge to pellet debris.
5. Proceed to Step 2.

g. Swabs (Stool, Soil, Microbial samples, etc.)

1. Add 750 µl **DNA/RNA Shield™** (1X)[1] to a swab sample (or up to 50 mg stool or soil) and mix by vortexing. Centrifuge to pellet debris and transfer 200 µl supernatant to a new tube.

   Optional: To achieve unbiased lysis of different organisms (including hard-to-lyse microbes), add sample in DNA/RNA Shield™ to a lysis tube/rack[6]. Secure in a bead beater fitted with the appropriate holder assembly for your bead beating module and process at maximum speed[3] for 5 minutes.

2. Add 10 µl **Proteinase K**. Mix and incubate at room temperature (20-30ºC) for 30 minutes.
3. Proceed to Step 2.

[1] To prepare 1X solution, mix equal amounts of the supplied 2X concentrate with nuclease-free water (not provided).
[2] ZR-96 BashingBead™ Lysis Rack (2.0 mm) (S6002-96-2) and ZR BashingBead™ Lysis Tubes (2.0 mm) (S6003-50) are sold separately.
[3] Processing time will vary based on sample input and bead beater. For high-speed homogenizers (e.g. Precellys, FastPrep®), process for ≤ 5 minutes. For low-speed homogenizers (e.g., Disruptor Genie), process for ≥ 10 minutes.
[4] Warm up urine sample at 37˚C for 5-10 minutes if the urine is visually cloudy (salt precipitation). Samples containing bacterial contamination will not be clear.
After adding Urine Conditioning Buffer (sold separately; D3061-1-140), urine can be stored for up to 1 month at ambient temperature. Prior to processing, mix the sample thoroughly by vortexing.
[5] Deparaffinization Solution (D3067-1-20) and 2X Digestion Buffer (D3050-1-20) are sold separately.
[6] ZR-96 BashingBead™ Lysis Rack (0.1 & 0.5 mm) (S6002-96-3) and ZR BashingBead™ Lysis Tubes (0.1 & 0.5 mm) (S6012-50) are sold separately.

Step 2: KingFisher Reagent Plates Preparation for DNA Purification

Materials:

- DNA/RNA Lysis Buffer, D7001-1-50 or D7001-2-200
- MagBead DNA/RNA Wash 1, prepared in Step 0. II.
- MagBead DNA/RNA Wash 2, prepared in Step 0. III.
- Ethanol (95-100%), user supplied
- DNase/RNase-Free Water, W1001-30 or W1001-100
- MagBinding Beads, D4100-2-6 or D4100-2-24
- 5 x KingFisher 96 deep-well plate, v-bottom, polypropylene, 95040450
- 1 x KingFisher 96 tip comb for deep-well magnets, 97002534
- 1 x KingFisher 96 (200 µL) microplate, 97002540
- KingFisher Flex Layout Program: See appendix

Note: Use a different KingFisher 96 deep-well plate, v-bottom, polypropylene, 95040450, for each reagent unless specified. The number of wells used correspond to number of samples being processed. Orientation of plates should be noted.

Protocol:

I. Sample Plate Preparation:
   1. Add 200 µl of **DNA/RNA Lysis Buffer** into a KingFisher 96 deep-well plate.
   2. Add 400 µl of Ethanol (95-100%) into a KingFisher 96 deep-well plate.
   3. Vortex the container of MagBinding Beads for 10 seconds at maximum speed.
   4. Aliquot 30 µL of **MagBinding Beads** into the same plate.

II. MagBead DNA/RNA Wash 1 Plate Preparation:
   1. Aliquot 500 µL of **MagBead DNA/RNA Wash 1 Buffer** into a KingFisher 96 deep-well plate.

III. MagBead DNA/RNA Wash 2 Plate Preparation:
   1. Aliquot 500 µL of **MagBead DNA/RNA Wash 2 Buffer** into a KingFisher 96 deep-well plate.

IV. Ethanol 1 Plate Preparation:
   1. Aliquot 500 µL of Ethanol (95 – 100%) into a KingFisher 96 deep-well plate.

V. Ethanol 2 and Tip Plate Preparation:
1. Aliquot 500 µL of Ethanol (95 – 100%) into a KingFisher 96 deep-well plate.
2. Slowly load the KingFisher 96 tip comb into the plate. Do not cause the Ethanol to splash out of well.

VI. DNA Elution Plate Preparation:
1. Aliquot 50 µL of **DNase/RNase-Free Water** into a KingFisher 96 (200 µL) microplate.
2. Ensure the elution buffer is at the bottom center of the plate

Step 3: Transferring Samples into Sample Plate

Protocol:

1. Bring prepared Sample Plate from Step 2.I.
2. Transfer up to 200 µL of prepared sample from Step 1 into Sample Plate.
   Prevent cross contamination by dispensing the sample after the pipette tip is half-way into the well. DO NOT let liquid from the pipette tip touch another well while entering or removing the tip from the well.

Step 4: Operating KingFisher and Loading Plates

Materials:

- Prepared Reagent Plates from Step 2 and Step 3.
- R2130_ Quick-DNA RNA Magbead_KingFisherFlex_Copurification_v1.bdz

Protocol:

I. Thermo Fisher Scientific KingFisher Flex Initialization
1. Power-on the KingFisher Flex.
2. Load program (R2130_Quick-DNA RNA Magbead_KingFisherFlex_Copurification_v1.bdz) on the KingFisher software BindIt.
3. Click on start to begin program.
4. Follow program instructions on the KingFisher screen to load plates.
5. Ensure orientation of plates is uniform.
6. Close plastic shield and start script.
7. After completion of the program, carefully remove KingFisher 96 (200 µL) microplate, which will contain the eluted DNA and RNA.

Appendix:

Reagent info

Sample Plate		96 DW plate	
Name	**Well volume [µl]**	**Total reagent volume [µl]**	**Type**
Sample (after Digestion)	200	-	Reagent
DNA/RNA Lysis Buffer	200	-	Reagent
MagBinding Beads	30	-	Reagent
Ethanol (95 - 100%)	400	-	Reagent

DNA RNA Wash 1		96 DW plate	
Name	**Well volume [µl]**	**Total reagent volume [µl]**	**Type**
MagBead DNA/RNA Wash 1	500	-	Reagent

DNA RNA Wash 2		96 DW plate	
Name	**Well volume [µl]**	**Total reagent volume [µl]**	**Type**
MagBead DNA/RNA Wash 2	500	-	Reagent

Ethanol Wash 1		96 DW plate	
Name	**Well volume [µl]**	**Total reagent volume [µl]**	**Type**
95 - 100% Ethanol	500	-	Reagent

Ethanol Wash 2		96 DW plate	
Name	**Well volume [µl]**	**Total reagent volume [µl]**	**Type**
95 - 100% Ethanol	500	-	Reagent

Elution Plate		96 DW plate	
Name	**Well volume [µl]**	**Total reagent volume [µl]**	**Type**
DNAse/RNAse-Free Water	50	-	Reagent

Steps data

Tip		96 DW tip comb	
Pick-Up		Ethanol Wash 2	
Binding		Sample Plate	
	Beginning of step	Precollect	No
		Release beads	No
	Mixing / heating:	Mixing time, speed	00:10:00, Fast
		Heating during mixing	No
	End of step	Postmix	No
		Collect count	2
		Collect time [s]	5
DNA RNA Wash 1		DNA RNA Wash 1	
	Beginning of step	Precollect	No
		Release time, speed	00:00:10, Fast
	Mixing / heating:	Mixing time, speed	00:02:00, Fast
		Heating during mixing	No
	End of step	Postmix	No
		Collect count	2
		Collect time [s]	5
DNA RNA Wash 2		DNA RNA Wash 2	
	Beginning of step	Precollect	No
		Release time, speed	00:00:10, Fast
	Mixing / heating:	Mixing time, speed	00:02:00, Fast
		Heating during mixing	No
	End of step	Postmix	No
		Collect count	2
		Collect time [s]	5
Ethanol Wash 1		Ethanol Wash 1	
	Beginning of step	Precollect	No
		Release time, speed	00:00:10, Fast
	Mixing / heating:	Mixing time, speed	00:02:00, Fast
		Heating during mixing	No
	End of step	Postmix	No
		Collect count	2
		Collect time [s]	5
Ethanol Wash 2		Ethanol Wash 2	
	Beginning of step	Precollect	No
		Release time, speed	00:00:10, Fast
	Mixing / heating:	Mixing time, speed	00:02:00, Fast
		Heating during mixing	No
	End of step	Postmix	No
		Collect count	2
		Collect time [s]	5
Dry		Ethanol Wash 2	
		Dry time	00:10:00
		Tip position	Outside well / tube
Elution		Elution Plate	
	Beginning of step	Precollect	No
		Release time, speed	00:00:30, Bottom mix
	Mixing / heating:	Mixing time, speed	00:10:00, Slow
		Heating during mixing	No
	End of step	Postmix	No
		Collect count	5
		Collect time [s]	10
Leave		Ethanol Wash 2	

B.4.1 ZymoBIOMICS™ Quick-DNA/RNA™ MagBead RNA extraction

Reagent info

DNase Treatment		96 DW plate	
Name	**Well volume [µl]**	**Total reagent volume [µl]**	**Type**
DNase I Reaction Mix	50	-	Reagent
MagBinding Beads	30	-	Reagent

Ethanol Wash 1		96 DW plate	
Name	**Well volume [µl]**	**Total reagent volume [µl]**	**Type**
95 - 100% Ethanol	500	-	Reagent

Ethanol Wash 2		96 DW plate	
Name	**Well volume [µl]**	**Total reagent volume [µl]**	**Type**
95 - 100% Ethanol	500	-	Reagent

RNA Elution Plate		96 standard plate	
Name	**Well volume [µl]**	**Total reagent volume [µl]**	**Type**
RNA Elution Plate	50	-	Reagent

Dispensed reagents

DNase Treatment		96 DW plate	
Name	**Step**	**Well volume [µl]**	**Total reagent volume [µl]**
DNA/RNA Prep Buffer	Pause	500	-

Steps data

	Tip	96 DW tip comb	
	Pick-Up	Ethanol Wash 2	
	DNase Treatment	DNase Treatment	
	Beginning of step	Precollect	No
		Release beads	Yes
	Mixing / heating:	Mixing time, speed	00:10:00, Slow
		Heating during mixing	No
	End of step	Postmix	No
		Collect beads	No
	Pause	DNase Treatment	
		Message	Add 500 uL of DNA RNA Prep Buffer
		Dispensing volume [µl]	500
	Reagent(s)	Name	DNA/RNA Prep Buffer
		Volume [µl]	500
	RNA Prep Wash	DNase Treatment	
	Beginning of step	Precollect	No
		Release beads	No
	Mixing / heating:	Mixing time, speed	00:10:00, Medium
		Heating during mixing	No
	End of step	Postmix	No
		Collect count	2
		Collect time [s]	5
	Ethanol Wash 1	Ethanol Wash 1	
	Beginning of step	Precollect	No
		Release beads	Yes
	Mixing / heating:	Mixing time, speed	00:02:00, Fast
		Heating during mixing	No
	End of step	Postmix	No
		Collect count	2
		Collect time [s]	5
	Ethanol Wash 2	Ethanol Wash 2	
	Beginning of step	Precollect	No
		Release beads	Yes
	Mixing / heating:	Mixing time, speed	00:02:00, Fast
		Heating during mixing	No
	End of step	Postmix	No
		Collect count	2
		Collect time [s]	5
	Dry	Ethanol Wash 2	
		Dry time	00:10:00
		Tip position	Outside well / tube

	Elution	RNA Elution Plate	
	Beginning of step	Precollect	No
		Release time, speed	00:00:10, Fast
	Mixing / heating:	Mixing time, speed	00:10:00, Medium
		Heating during mixing	No
	End of step	Postmix	No
		Collect count	5
		Collect time [s]	15
	Leave	Ethanol Wash 2	

Lot info

No lot numbers have been defined.

C iScript™ cDNA Synthesis Kit

BIO-RAD

iScript™ cDNA Synthesis Kit

Catalog #	Description
1708890	**iScript cDNA Synthesis Kit**, 25 x 20 µl reactions
1708891	**iScript cDNA Synthesis Kit**, 100 x 20 µl reactions

For research purposes only.

Introduction

iScript cDNA Synthesis Kit provides a sensitive and easy-to-use solution for two-step reverse transcription quantitative PCR (RT-qPCR). This kit includes three tubes, which contain all the reagents required for successful reverse transcription.

The iScript Reverse Transcriptase is RNase H+, which provides greater sensitivity than RNase H– enzymes in qPCR. iScript is a modified Moloney murine leukemia virus (MMLV) reverse transcriptase, optimized for reliable cDNA synthesis over a wide dynamic range of input RNA. The enzyme is provided preblended with RNase inhibitor. The unique blend of oligo(dT) and random hexamer primers in the iScript Reaction Mix works exceptionally well with a wide variety of targets. This blend is optimized for the production of targets <1 kb in length. iScript cDNA Synthesis Kit produces excellent results in both real-time and standard RT-qPCR.

Storage and Stability

Store at –20°C. Guaranteed for 12 months at –20°C in a constant temperature freezer. Nuclease-free water can be stored at room temperature.

Note: Kits whose six-digit lot number begins with a 2 are not compatible with kits whose six-digit lot number begins with a 1. Please make note of this distinction if you have multiple lots of this kit in storage.

Kit Contents

Reagent	Volume for 25 Reactions	Volume for 100 Reactions
5x iScript Reaction Mix	100 µl	400 µl
iScript Reverse Transcriptase	25 µl	100 µl
Nuclease-free water	1.5 ml	1.5 ml

Reaction Setup

Note: The 5x iScript Reaction Mix may generate some precipitation upon thawing; this does not affect the quality of the mixture. If you do experience precipitation, please mix thoroughly to resuspend and use as directed in the following table.

Component	Volume per Reaction, µl
5x iScript Reaction Mix	4
iScript Reverse Transcriptase	1
Nuclease-free water	Variable
RNA template (100 fg–1 µg total RNA)*	Variable
Total volume	**20**

* When using larger amounts of input RNA (>1 µg), the reaction should be scaled up (for example, 40 µl reaction for 2 µg, or 100 µl reaction for 5 µg) to ensure optimum synthesis efficiency.

Reaction Protocol

Incubate the complete reaction mix in a thermal cycler using the following protocol:

Priming	5 min at 25ºC
Reverse transcription	20 min at 46ºC
RT inactivation	1 min at 95ºC
Optional step	Hold at 4ºC

Recommendation for Optimal Results Using the iScript cDNA Synthesis Kit

The maximum amount of the cDNA reaction that is recommended for downstream PCR is one-tenth of the reaction volume, typically 2 µl.

Related Products

Catalog #	Description
Reverse Transcription Reagents for Real-Time qPCR	
1708840	iScript Reverse Transcription Supermix for RT-qPCR
1725037	iScript Advanced cDNA Synthesis Kit for RT-qPCR
1708896	iScript Select cDNA Synthesis Kit
1725034	iScript gDNA Clear cDNA Synthesis Kit
Reagents for Real-Time qPCR	
1725270	SsoAdvanced™ Universal SYBR® Green Supermix
1725280	SsoAdvanced Universal Probes Supermix
1725120	iTaq™ Universal SYBR® Green Supermix
1725130	iTaq Universal Probes Supermix
1725160	SsoAdvanced PreAmp Supermix

Visit **bio-rad.com/web/iscriptcDNA** for more information.

Bio-Rad Laboratories, Inc.
2000 Alfred Nobel Drive, Hercules, CA 94547
510-741-1000

INST-653 Ver D (4106228) 15-1170 0915

D Amplicon library preparation protocol

Protocol for preparation of an amplicon library for Illumina sequencing

1 1ST PCR (TARGETED PCR)

Amplification with primers with Illumina adapters, e.g.

ill341F_KI (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGNNNNCCTACGGGNGGCWGCAG-3')

ill805R (5'- GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGNNNNGACTACNVGGGTATCTAAKCC-3')

IMPORTANT: Always include a negative control from the DNA extraction (particularly important for "difficult samples" and a non-template PCR control. Preferably, also add a positive control previously found to work well with these primers (e.g. DNA from a bacterial isolate).

Mastermix, per reaction

Reagent	**Final concentration**	**Amount x1**
DNA-free H_20		Up to final reaction volume
5x Phusion buffer HF (7,5 mM MgCl2)*	1x	5.0 µL
Ill341F_KI (10 µM)**	0.3 mM	0.75 µL**
Ill805R (10 µM)**	0.3 mM	0.75 µL**
dNTP (10mM each)	200 µM each	0.5 µL
$MgCl_2$ (50 mM; check concentration!)***	(add 0.5 mM)***	0.25 µL
Phusion Hot Start DNA polymerase (2 units/ µL)	0.02 units/µL	0.18 µL
Template		Usually 1.0 µL****
Total volume		25 µL

* For Phusion Hot Start High Fidelity polymerase (Molecular Biology, Thermo Fisher)

**Standard final primer concentration for PCR. In the case of primer dimer formation, try to reduce the primer concentration to the half.

*** Optional! Reduces stringency, not needed for all samples. Usually, the standard PCR reaction buffer gives a final $MgCl_2$ concentration of 1.5 mM. This additional MgCl2 will add 0.5 to the final concentration; i.e. 2 mM.

****Never let the amount of template exceed 10% of the total volume, due to high possibilities for inhibitory effects. Usually; if problems with achieving product, try to dilute the template rather than increasing the amount.

Typical cycling conditions (optimization may be needed for other primers)

98°C	2min	
98°C	15 sec	x33-38 cycles
55°C	20 sec	
72°C	20 sec	
72°C	5min	
4°C	1min	
10°C	∞	

Check PCR products on agarose gel. Expected product length: around 540 nt. A clearly visible band of the correct size, in combination with a negative result for both controls.

2 PURIFICATION AND NORMALIZING OF PCR PRODUCT

Use the SequalPrep Normalization Plate Kit for 96-well plates (supplied by Thermo Fisher) – follow the protocol.

https://www.thermofisher.com/order/catalog/product/A1051001

This kit both purifies and normalizes the PCR products.

NOTE: Save binding+PCR-product mix in a 96-well plate until you now that you get a product.

No need for a new agarose gel (too low DNA concentrations in the eluates), typical output is 1 - 2 ng/µL per well.

3 INDEXING PCR (SECOND PCR)

Here, a unique combination of a forward and a reverse sequence barcode/index is added to each of the PCR products generated in the first PCR in a new, "indexing" PCR.

Each kit contains 8x12 indices and can be used for indexing maximum 96 samples at the time. If more than 96 samples, you need a second indexing kit with another set of unique indexing sequences.

One indexing kit is originally meant for 2 times indexing of 96 samples; but we have scaled down; i.e. with the below protocol, one kit is enough for 4 time indexing of 96 samples. It works just fine!

https://www.illumina.com/products/by-type/sequencing-kits/library-prep-kits/nextera-xt-dna.html

Add 17.5 µL master-mix (see table below) to each well, then add indexing primers 1 and 2, and template.

Mastermix, per reaction

Reagent	**Amount**
DNA-free H_20	To total rx volume 25 µL
5x Phusion buffer HF (7,5 mM MgCl2)	5.0 µL
dNTP(10mM each)	0.5 µL
Phusion Hot Start DNA polymerase	0.19 µL
Index 1 (orange lid, N series, 8 unique)	2.5 µL
Index 2 (white lid, S series, 12 unique)	2.5 µL
Template (normalized from 1st stage PCR	2.5 µL

Cycling conditions

98°C 2 min

98°C 15 sec

55°C 20 sec

72°C 20 sec

} 8 – 12 cycles (if only week PCR products obtained in the first round, use 12 cycles)

72°C 5min

4°C 1min

10°C ∞

Agarose gel electrophoresis. If the PCR products are weak, run a new indexing PCR with more cycles.

4 NORMALIZATION OF 2.ST INDEXING PCR

Use the SequalPrep Normalization Plate Kit for 96-well plates (supplied by Thermo Fisher) – follow the protocol.

No need for a new agarose gel (too low DNA concentrations in the eluates), typical output is 1 - 2 ng/µL.

5 POOLING OF INDEXED, NORMALIZED PCR PRODUCTS

After the second normalization, pool all samples in one tube. Total volume: 20 µL x no. of samples.

Concentration of sample using an Amicon filter

Concentrate the sample using an AmiconUltra 0.5 centrifugal filter devices (30K membrane, Merck Millipore). Follow the protocol. Maximum volume per column is 500 µL. Normally you have close to 96 samples which gives a total volume of 1920 µL. If this is the case, you can devide the volume over two colums (to balance the centrifuge) by repeating step 4 once more to spin trough all your sample. Save the flow tough until you have confirmed the DNA concentratin.

Specific comments to the protocol:

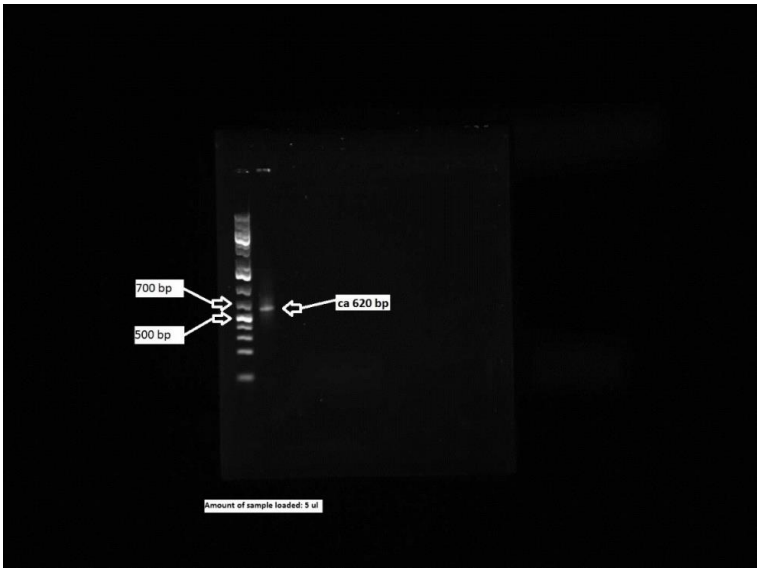
- For step 4 (page 9): spin at 14000 g for 10 minutes.
- For washing (according to table page 14): add 500 µL sterile filtrated 1xTE buffer. Spin at 14000 g for 10 minutes. Discard the eluate
- Repeat the washing step once more. The liquid remaining above the filter (typically 20 – 30 µL) is your concentrated sample.

https://www.fishersci.no/shop/products/emd-millipore-amicon-ultra-15-centrifugal-filter-units-15/10212584#?keyword=UFC500324

Determine the concentration and purity of the concentrated sample using nanodrop. The DNA concentration is typically around 10 – 20 ng/µL.

Tris in the elution buffer (e.g. TE buffer) will interfere with the 260/230 ratio, but the DNA is probably still OK!

The Norwegian sequencing centre (NSC) requires a picture of the final amplicon library sample on an agarose gel. On the picture, include info about the DNA ladder, the amount (in uL) of the sample applied on the gel; see the example below.



6 SENDING IN THE SAMPLES

While waiting for the gel, you can fill out the sample submission form and sample submission table. You find both forms following this link: https://www.sequencing.uio.no/illumina-submission/.

When you are done with the preparation of the samples, prepare a 1.5 mL tube containing 20 µL of your sample. NSC also require a sample of the elution buffer. Please prepare a 1.5 mL tube with 20 µL TE-buffer. Mark both tubes well and with the same name as you fill in the Sample submission form.

Store the tubes at -20°C until shipping.

E Sequalprep normalization plate kit

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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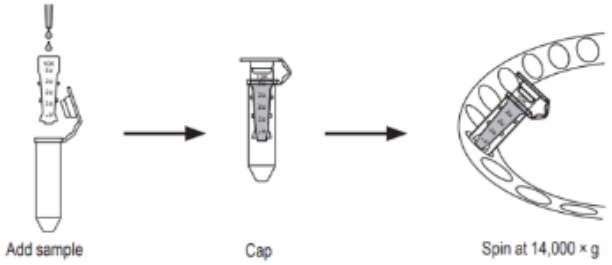
F Amicon® Ultra-0.5 Centrifugal Filter Devices

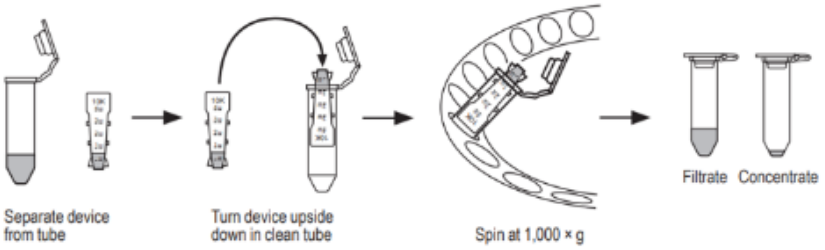
How to Use Amicon Ultra-0.5 Centrifugal Filter Devices

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



How to Use Amicon Ultra-0.5 Filter Devices, continued

5. Remove the assembled device from the centrifuge and separate the Amicon Ultra filter device from the microcentrifuge tube.
6. To recover the concentrated solute, place the Amicon Ultra filter device upside down in a clean micro centrifuge 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.



G QIAquick Gel Extraction Kit Protocol

QIAquick Gel Extraction Kit Protocol

using a microcentrifuge

This protocol is designed to extract and purify DNA of 70 bp to 10 kb from standard or low-melt agarose gels in TAE or TBE buffer. Up to 400 mg agarose can be processed per spin column. This kit can also be used for DNA cleanup from enzymatic reactions (see page 8). For DNA cleanup from enzymatic reactions using this protocol, add 3 volumes of Buffer QG and 1 volume of isopropanol to the reaction, mix, and proceed with step 6 of the protocol. Alternatively, use the new MinElute Reaction Cleanup Kit.

Notes:
- The yellow color of Buffer QG indicates a pH ≤7.5.
- Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).
- Isopropanol (100%) and a heating block or water bath at 50°C are required.
- All centrifugation steps are carried out at ≥10,000 x *g* (~13,000 rpm) in a conventional table-top microcentrifuge.
- 3 M sodium acetate, pH 5.0, may be necessary.

1. **Excise the DNA fragment from the agarose gel with a clean, sharp scalpel.**

   Minimize the size of the gel slice by removing extra agarose.

2. **Weigh the gel slice in a colorless tube. Add 3 volumes of Buffer QG to 1 volume of gel (100 mg ~ 100 µl).**

   For example, add 300 µl of Buffer QG to each 100 mg of gel. For >2% agarose gels, add 6 volumes of Buffer QG. The maximum amount of gel slice per QIAquick column is 400 mg; for gel slices >400 mg use more than one QIAquick column.

3. **Incubate at 50°C for 10 min (or until the gel slice has completely dissolved). To help dissolve gel, mix by vortexing the tube every 2–3 min during the incubation.**

   IMPORTANT: Solubilize agarose completely. For >2% gels, increase incubation time.

4. **After the gel slice has dissolved completely, check that the color of the mixture is yellow (similar to Buffer QG without dissolved agarose).**

   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 to yellow.

   The adsorption of DNA to the QIAquick membrane is efficient only at pH ≤7.5. Buffer QG contains a pH indicator which is yellow at pH ≤7.5 and orange or violet at higher pH, allowing easy determination of the optimal pH for DNA binding.

5. **Add 1 gel volume of isopropanol to the sample and mix.**

   For example, if the agarose gel slice is 100 mg, add 100 µl isopropanol. This step increases the yield of DNA fragments <500 bp and >4 kb. For DNA fragments between 500 bp and 4 kb, addition of isopropanol has no effect on yield. Do not centrifuge the sample at this stage.

6. **Place a QIAquick spin column in a provided 2 ml collection tube.**
7. **To bind DNA, apply the sample to the QIAquick column, and centrifuge for 1 min.**

   The maximum volume of the column reservoir is 800 µl. For sample volumes of more than 800 µl, simply load and spin again.
8. **Discard flow-through and place QIAquick column back in the same collection tube.**

   Collection tubes are re-used to reduce plastic waste.
9. **(Optional): Add 0.5 ml of Buffer QG to QIAquick column and centrifuge for 1 min.**

   This step will remove all traces of agarose. It is only required when the DNA will subsequently be used for direct sequencing, in vitro transcription or microinjection.
10. **To wash, add 0.75 ml of Buffer PE to QIAquick column and centrifuge for 1 min.**

    Note: If the DNA will be used for salt sensitive applications, such as blunt-end ligation and direct sequencing, let the column stand 2–5 min after addition of Buffer PE, before centrifuging.
11. **Discard the flow-through and centrifuge the QIAquick column for an additional 1 min at ≥10,000 x *g* (~13,000 rpm).**

    IMPORTANT: Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.
12. **Place QIAquick column into a clean 1.5 ml microcentrifuge tube.**
13. **To elute DNA, add 50 µl of Buffer EB (10 mM Tris·Cl, pH 8.5) or H_2O to the center of the QIAquick membrane and centrifuge the column for 1 min at maximum speed. 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 for 1 min.**

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

    Elution efficiency is dependent on pH. The 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 –20°C as DNA may degrade in the absence of a buffering agent. The purified DNA can also be eluted in TE (10 mM Tris·Cl, 1 mM EDTA, pH 8.0), but the EDTA may inhibit subsequent enzymatic reactions.

H Feeding table

Table H.0.1: The amount of feed given in each fish tank per day of the lab-scale RAS experiment. *The salmon fry was hand fed. **Installation of the automatic feeder. ***Amount of feed was decreased in order to decrease the organic loading in the RAS.

Feeding table	
Date	**Amount of feed (g) in each tank**
06/11/2023	5.00*
07/11/2023	5.00*
08/11/2023	5.00*
09/11/2023	5.00*
10/11/2023	5.00*
11/11/2023	5.00*
12/11/2023	5.00**
13/11/2023	5.08
14/11/2023	5.15
15/11/2023	5.23
16/11/2023	5.31
17/11/2023	5.39
18/11/2023	5.47
19/11/2023	5.55
20/11/2023	5.63
21/11/2023	5.72
22/11/2023	5.8
23/11/2023	5.89
24/11/2023	5.98
25/11/2023	6.07
26/11/2023	6.16
27/11/2023	4.00***
28/11/2023	4.04
29/11/2023	4.1
30/11/2023	4.16
01/12/2023	4.22
02/12/2023	4.29
03/12/2023	4.35
04/12/2023	4.42
05/12/2023	4.48
06/12/2023	4.55
07/12/2023	4.62
08/12/2023	4.69
09/12/2023	4.76
10/12/2023	4.83
11/12/2023	4.9
12/12/2023	4.98
13/12/2023	5.05
14/12/2023	5.13
15/12/2023	5.2
16/12/2023	5.28

I Water exchange lab-scale RAS experiment

Table I.0.1: The amount water exchanged during the lab-scale RAS experiment in both RAS A and RAS B.

Water exchange		
	RAS A and RAS B	
Date	Water added (L)	Water exhanged (L)
06/11/2023	10	
07/11/2023	0	
08/11/2023	4	
09/11/2023		
10/11/2023		3
11/11/2023		
12/11/2023		3
13/11/2023		
14/11/2023		
15/11/2023		
16/11/2023	15	
17/11/2023		
18/11/2023		5
19/11/2023		8
20/11/2023		8
21/11/2023		8
22/11/2023		
23/11/2023		38
24/11/2023		13
25/11/2023		8
26/11/2023		8
27/11/2023		8
28/11/2023		8
29/11/2023		8
30/11/2023		8
01/12/2023		8
02/12/2023		20
03/12/2023		8
04/12/2023		8
05/12/2023		8
06/12/2023		8
07/12/2023		8
08/12/2023		8
09/12/2023		18
10/12/2023		8
11/12/2023		8
12/12/2023		8
13/12/2023		8
14/12/2023		10
15/12/2023		8
16/12/2023		8

J Water quality control measurement protocols for the lab-scale RAS experiment

J.1 API freshwater kit protocol

ENGLISH

To remove childproof safety caps:
Push down on cap while turning.

pH TEST

Why Test pH?
pH is the measure of acidity of water. A pH reading of 7.0 is neutral. A pH higher than 7.0 is alkaline, and a pH lower than 7.0 is acidic. Maintaining the aquarium at the proper pH ensures optimal water quality. The pH should be tested weekly, since natural materials in the aquarium (such as fish waste and uneaten food) can cause pH changes.

Testing Tips
The minimum pH reading for this kit is 6.0 and the maximum is 7.6. Under extreme water conditions, readings below the minimum will read 6.0 and above the maximum will read 7.6. pH adjustments outside the range of this kit will not show any changes until the pH of the aquarium water is within the range of this kit. When keeping livebearers, goldfish, African Cichlids or marine fish & invertebrates use the API HIGH RANGE pH TEST KIT.

Directions
1. Fill a clean test tube with 5 ml of water to be tested (to the line on the tube).
2. **Add 3 drops of pH Test Solution**, holding dropper bottle upside down in a completely vertical position to assure uniformity of drops.
3. Cap the test tube & invert tube several times to mix solution.
4. Read the test results by comparing the color of the solution to the pH Color Chart. The tube should be viewed in a well-lit area against the white area of the chart. The closest match indicates the pH of the water sample.

Recommended pH Levels
A pH of 7.0 is ideal when keeping a community aquarium containing a variety of tropical fish. Goldfish and livebearers prefer a pH of 7.5. Many Amazonian fish, like angelfish and neon tetras, prefer a pH of 6.5 to 6.8. Mollies and swordtails thrive at pH 7.2 to 7.5. To raise or lower the pH of a freshwater aquarium, use API pH UP® or pH DOWN®. To automatically adjust pH to a preset level, use API PROPER pH® 6.5, 7.0, or 7.5.

HIGH RANGE pH TEST

Why Test pH?
pH is the measure of acidity of water. A pH reading of 7.0 is neutral. A pH higher than 7.0 is alkaline, and a pH lower than 7.0 is acidic. Maintaining the aquarium at the proper pH ensures optimal water quality. The pH should be tested weekly, since natural materials in the aquarium (such as fish waste and uneaten food) can cause pH changes.

Testing Tips
The minimum pH reading for this kit is 7.4 and the maximum is 8.8. Under extreme water conditions, readings below the minimum will read 7.4 and above the maximum will read 8.8. pH adjustments outside the range of this kit will not show any changes until the pH of the aquarium water is within the range of this kit.

Directions

1. Fill a clean test tube with 5 ml of water to be tested (to the line on the tube).
2. **Add 5 drops of High Range pH Test Solution**, holding dropper bottle upside down in a completely vertical position to assure uniformity of drops.
3. Cap the test tube & invert tube several times to mix solution.
4. Read the test results by comparing the color of the solution to the High Range pH Color Chart. The tube should be viewed in a well-lit area against the white area of the chart. The closest match indicates the pH of the water sample. Rinse the test tube with clean water after use.

Recommended pH Levels

A pH of 7.5 is ideal for most live-bearing fish, such as mollies & swordtails. Goldfish will also thrive at a pH of 7.5. African cichlids prefer a pH of 8.2. Marine fish & invertebrates require a pH between 8.2 – 8.4. To raise or lower the pH of a freshwater aquarium, use API pH UP or pH DOWN. Also, API PROPER pH 7.5 may be used to automatically adjust & hold pH at 7.5. PROPER pH 8.2 may be used in African cichlid and saltwater aquariums.

AMMONIA TEST

Why Test for Ammonia?

Fish continually release ammonia (NH_3) directly into the aquarium/pond through their gills, urine, and solid waste. Uneaten food and other decaying organic matter also add ammonia to the water. A natural mechanism exists that controls ammonia in the aquarium/pond – the biological filter. However, as with any natural process, imbalances can occur. So, testing for the presence of toxic ammonia is essential. Ammonia in the aquarium/pond may damage gill membranes, and prevent fish from carrying on normal respiration. High levels of ammonia quickly lead to fish death. Even trace amounts stress fish, suppressing their immune system and increasing the likelihood of disease. Using API QUICK START® will help accelerate the development of the biological filter.

Testing Tip: This salicylate-based ammonia test kit reads the total ammonia level in parts per million (ppm) [equivalent to milligrams per liter (mg/L)] from 0 - 8.0 ppm (mg/L).

Directions

1. Fill a clean test tube with 5 ml of water to be tested (to the line on the tube).
2. **Add 8 drops from Ammonia Test Solution #1**, holding the dropper bottle upside down in a completely vertical position to assure uniformity of drops.
3. **Add 8 drops from Ammonia Test Solution #2**, holding the dropper bottle upside down in a completely vertical position to assure uniformity of drops.
4. Cap the test tube & shake vigorously for 5 seconds.
5. **Wait 5 minutes for the color to develop.**
6. Read the test results by comparing the color of the solution to the Ammonia Color Chart. The tube should be viewed in a well-lit area against the white area of the chart. The closest match indicates the ppm (mg/L) of ammonia in the water sample. Rinse the test tube with clean water after use. Note: Do not pour test tube contents back into the aquarium.

Reducing Ammonia Levels

In a new aquarium or pond the ammonia level may rise and then fall rapidly as the biological filter becomes established. The

ammonia will be converted to nitrite (also toxic), then to nitrate. This process may take several weeks. It is recommended to use API QUICK START to help establish the biological filter, lower ammonia and nitrite, and reduce the risk of fish loss. In an established aquarium, the ammonia level should always remain at 0 ppm (mg/L); any level above 0 can harm fish.

To reduce risk of fish loss, if ammonia levels continue to test high in your aquarium or pond (4 ppm or mg/L), perform a water change of 25% or more, then add API AMMO LOCK® to quickly detoxify ammonia. AMMO LOCK will convert ammonia to a non-toxic form. The Ammonia test kit will still test positive for ammonia, even though treating with AMMO LOCK has made it non-toxic. A daily water change may be required over several days. Be sure to use a water conditioner, such as STRESS COAT®, when adding tap water back into the aquarium.

WARNING

AMMONIA TEST SOLUTION #1

Harmful if swallowed • Harmful in contact with skin • Harmful if inhaled • Causes serious eye irritation • Use only in outdoors or in a well-ventilated area • Avoid breathing dust /fume/ gas/mist/vapors/spray • Do not eat, drink or smoke when using this product • Wear protective gloves/protective clothes/eye protection/face protection • Specific treatment (see advice on this label) • IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. If eye irritation persists: Get medical advice/attention • IF SWALLOWED: Call a POISON CENTER / Doctor / Physician / first aider / if you feel unwell • IF ON SKIN: Wash with plenty of water and soap • IF INHALED: Remove person to fresh air and keep comfortable for breathing • Rinse mouth • Take off contaminated clothing and wash before reuse • Dispose of contents/container to authorized chemical landfill or if organic to high temperature incineration.

DANGER

AMMONIA TEST SOLUTION #2

May be corrosive to metals • Causes severe skin burns and eye damage • Causes serious eye damage • Harmful to aquatic life•Do not breathe dust / fume / gas / mist / vapors / spray • Wear protective gloves/ protective clothes/ eye protection/face protection • Keep only in original container • Avoid release to environment • IF SWALLOWED: Rinse mouth. DO NOT induce vomiting • IF ON SKIN (or hair): Take off immediately all contaminated clothing. Rinse skin with water/shower • IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing • Immediately call a POISON CENTER/Doctor/Physician/first aider • Specific treatment (see advice on this label) • Wash contaminated clothing before reuse • Absorb spillage to prevent material damage • IF INHALED: Remove person to fresh air and keep comfortable for breathing • Store locked up • Dispose of contents/container to authorized chemical landfill or if organic to high temperature incineration.

NITRITE TEST

Why Test For Nitrite?

Nitrite (NO_2^-) is produced in the aquarium/pond by the biological filter. Beneficial bacteria in the biological filter convert ammonia into nitrite. The biological filter then converts nitrite into nitrate (NO_3^-). Nitrite in the aquarium/pond is toxic; it will prevent fish from carrying on normal respiration, and high levels will quickly lead to fish death. Even trace amounts of nitrite stress fish, suppressing their immune system and increasing the likelihood of disease. Too many fish, as well as uneaten fish food and decomposing plants and other organic matter can cause excessive nitrite levels. Water should be tested for nitrite every

other day when the aquarium/pond is first set up, and once a week after the biological filter has been established (in about 4 - 6 weeks). Using API QUICK START will help accelerate the development of the biological filter.

Testing Tips

This test kit reads total nitrite (NO_2^-) level in parts per million (ppm) which are equivalent to milligrams per liter (mg/L) from 0 - 5.0 ppm (mg/L).

Directions

1. Fill a clean test tube with 5 ml of water to be tested (to the line on the tube).
2. **Add 5 drops of Nitrite Test Solution**, holding dropper bottle upside down in a completely vertical position to assure uniformity of drops.
3. Cap the test tube and shake for 5 seconds.
4. **Wait 5 minutes for the color to develop.**
5. Read the test results by comparing the color of the solution to the Nitrite Color Chart. The tube should be viewed in a well-lit area against the white area of the chart. The closest match indicates the ppm (mg/L) of nitrite in the water sample. Rinse the test tube with clean water after use.

What the Test Results Mean

In new aquariums/ponds the nitrite level will gradually climb to 5 ppm (mg/L) or more. As the biological filter becomes established, nitrite levels will drop to 0 ppm (mg/L). In an established aquarium, the nitrite level should always remain at 0; any level above 0 can harm fish. The presence of nitrite indicates possible over-feeding, too many fish, or inadequate biological filtration.

Reducing Aquarium Nitrite Levels

Add API NITRA-ZORB®/AQUA DETOX to the aquarium filter to remove nitrite from freshwater aquariums. Making partial water changes can also help reduce nitrite, especially if the initial level is very high. Use API QUICK START to help speed the development of the biological filter. Adding API AQUARIUM SALT will reduce nitrite toxicity to fish while the biological filter is removing the nitrite.

NITRATE TEST

Why Test for Nitrate?

Nitrate (NO_3^-) is produced in the aquarium by the biological filter. Beneficial bacteria in the biological filter convert toxic ammonia and nitrite into nitrate. A high nitrate level indicates a build-up of fish waste and organic compounds, resulting in poor water quality and contributing to the likelihood of fish disease. Maintaining a low nitrate level improves the health of fish & invertebrates. Excessive nitrate also provides a nitrogen source that can stimulate algal blooms. Aquarium water should be tested for nitrate once a week to make sure the nitrate does not reach an undesirable level.

Testing Tip: This test kit reads total nitrate (NO_3^-) level in parts per million (ppm) which are equivalent to milligrams per liter (mg/L) from 0 - 160 ppm.

Directions

1. Fill a clean test tube with 5 ml of water to be tested (to the line on the tube).
2. **Add 10 drops from Nitrate Test Solution #1**, holding dropper bottle upside down in a completely vertical position to assure uniformity of drops.

3. Cap the test tube & invert tube several times to mix solution.
4. **Vigorously shake the Nitrate Test Solution #2 for at least 30 seconds. This step is extremely important to insure accuracy of test results.**
5. **Now add 10 drops from Nitrate Test Solution #2**, holding dropper bottle upside down in a completely vertical position to assure uniformity of drops.
6. **Cap the test tube and shake vigorously for 1 minute. This step is extremely important to insure accuracy of test results.**
7. **Wait 5 minutes for the color to develop.**
8. Read the test results by comparing the color of the solution to the Nitrate Color Chart. The tube should be viewed in a well-lit area against the white area of the card. The closest match indicates the ppm (mg/L) of nitrate in the water sample. Rinse the test tube with clean water after use.

What the Test Results Mean

In new aquariums the nitrate level will gradually climb as the biological filter becomes established. A nitrate level of 40 ppm (mg/L) or less is recommended for freshwater aquariums. In marine aquariums, it is best to keep nitrate as low as possible, especially when keeping invertebrates.

Reducing Nitrate Levels

Add API NITRA-ZORB® / AQUA-DETOX to the filter to remove nitrate from freshwater aquariums. Making partial water changes can also help reduce nitrate, especially if the level is very high. However, because many tap water supplies contain nitrate, it can be difficult to lower nitrate levels by this method.

DANGER

NITRATE TEST SOLUTION #1

May be corrosive to metals • Harmful if inhaled • Causes severe skin and eye damage. Causes serious eye irritation • May cause respiratory irritation • Do not breathe dust / fume / gas / mist / vapors / spray • Use only in outdoors or in a well-ventilated area. Wear protective gloves / protective clothes / eye protection / face protection • Keep only in original container • IF SWALLOWED: Rinse mouth. DO NOT induce vomiting • IF ON SKIN (or hair): Take off immediately all contaminated clothing. Rinse skin with water/shower • IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing • Immediately call a POISON CENTER / Doctor / physician / first aider • Specific treatment (see advice on this label) • If eye irritation persists: Get medical advice/attention • Wash contaminated clothing before use • Absorb spillage to prevent material damage • IF INHALED: Remove person to fresh air and keep comfortable for breathing • Store locked up • Store in a well-ventilated place • Keep container tightly closed • Dispose of contents/container to authorized chemical landfill or if organic to high temperature incineration.

WARNING

NITRATE TEST SOLUTION #2

Wear protective gloves/protective clothes/eye protection / face protection • If exposed or concerned: Get medical advice / attention • Store locked up • Dispose of contents/container to authorized chemical landfill or if organic to high temperature incineration.

J.2 JBL PRO AQUATEST KH

Gebrauchsinformationen (de)

WICHTIG: Tropfflaschen beim Tropfen immer mit dem Tropfer **senkrecht** nach unten halten und **blasenfrei** tropfen. Tropfer **müssen außen trocken** sein.
Lagerung der Reagenzien: Trocken bei Raumtemperatur und in Originalverpackung.

JBL PRO AQUATEST KH (de)

Besonderheit: JBL PRO AQUATEST KH ist ein einfach zu handhabender Titrationstest zur Bestimmung der Karbonathärte (auch Säurebindungsvermögen oder Alkalinität genannt) im Süß- und Meerwasser sowie im Gartenteich.
Warum testen? Je nach Herkunft kann Wasser, z.B. bedingt durch die Beschaffenheit des Untergrundes, unterschiedlich hohe Mengen verschiedener Mineralsalze enthalten. Einen Großteil der gelösten Salze stellen Erdalkali- und Alkali-Hydrogenkarbonate dar. Hydrogenkarbonate bilden zusammen mit Karbonaten und Kohlendioxid (CO_2) ein wichtiges Puffersystem, das gefährlich hohe pH-Wertschwankungen im Wasser verhindert. Die gemessene Karbonathärte (KH) liefert die Gesamtkonzentration an Hydrogencarbonat im Wasser und kann daher in seltenen Fällen (wenn hauptsächlich Alkali-Hydrogenkarbonate vorhanden sind wie z.B. in ostafrikanischen Seen) höher sein als die Gesamthärte bei der nur Erdalkalisalze berücksichtigt werden. Die meisten Süßwasserfische und -pflanzen im Aquarium lassen sich bei einer Karbonathärte von etwa 5–16 °dH erfolgreich pflegen. Für eine optimale CO_2-Düngung sollte die Karbonathärte jedoch nicht unter 5 °dH liegen. Auch im Gartenteich sollte eine Karbonathärte von mindestens 5 °dH eingehalten werden. Bei CO_2-Mangel verbrauchen Wasserpflanzen bzw. vor allem Algen durch ihre schnelle Assimilation bei der Photosynthese Hydrogenkarbonat (biogene Entkalkung) und können dadurch den pH-Wert in für Fische gefährliche Höhen (über 10) treiben. Im Meerwasser sollte zur optimalen pH-Pufferung eine Karbonathärte um 7–13 °dH eingehalten werden.
Vorgehensweise:

1. Messgefäß mit dem zu untersuchenden Wasser mehrmals spülen.
2. Mit der beigefügten Spritze 5 ml Probenwasser in das Messgefäß füllen
3. Reagens tropfenweise zugeben. Nach jedem Tropfen schwenken und Tropfen zählen, bis ein Farbumschlag Blau nach Gelb-Orange erfolgt.
4. Ein Tropfen verbrauchter Reagenzlösung entspricht 1 Grad deutscher Karbonathärte (°dH), 1,78 Grad französischer Härte (°fH), einem Säurebindungsvermögen von 0,36 mmol/l und einem Hydrogencarbonatgehalt von 21,8 mg/l.

Korrektur abweichender Werte:
Zu gering: Anwendung von hydrogencarbonathaltigen JBL Wasseraufbereitern oder Mineralsalzmischungen.
Zu hoch: Wasserenthärtung z. B. durch Verwendung einer Umkehrosmoseanlage.

Information for use (en)

IMPORTANT: Always point the dropper **vertically** downwards when using the drop bottle and **avoid bubbles.** The exterior surface of the dropper should be **dry.**
Storage of reagents: Keep dry at room temperature and in original packaging.

JBL PRO AQUATEST KH (en)

Features: JBL PRO AQUATEST KH is an easy-to-use titration test to determine the carbonate hardness (also called acid-binding capacity or alkalinity) of fresh and marine water and of garden ponds.
Why test? Depending on its origin, and often due to the nature of the subsoil, water can contain different amounts of various mineral salts. Most of the salts dissolved in it are alkaline earth and alkaline hydrogen carbonates. Together with carbonates and carbon dioxide (CO_2), hydrogen carbonates form an important buffer system that prevents dangerously high pH fluctuations in water. The carbonate hardness (KH) measured provides the total concentration of hydrogen carbonate in the water and can therefore in rare cases (when alkali hydrogen carbonates are mainly present, e.g. in East African lakes) be higher than the general hardness, which only takes into account the alkaline earth salts. Most freshwater fish and plants in the aquarium can be kept successfully with a carbonate hardness of about 5–16 °dH. For an optimal CO_2 fertilisation, however, the carbonate hardness should not be below 5 °dH. A carbonate hardness of at least 5 °dH should also be maintained in the garden pond. If there is a CO_2 deficiency, aquatic plants and above all algae consume hydrogen carbonate (biogenic decalcification) through their rapid assimilation during photosynthesis and can thus drive the pH value to heights which are dangerous for fish (above 10). In marine water, a carbonate hardness of 7–13 °dH needs to be maintained for optimum pH buffering.
Procedure:

1. Rinse the measuring vessel several times with the water to be tested.
2. Put 5 ml sample water into the measuring vessel with the syringe provided.
3. Add the reagent drop by drop. After each drop, swirl and count the drops until the colour changes from blue to yellow-orange.

4. One drop of reagent solution used corresponds to 1 degree of German carbonate hardness (°dH), 1.78 degree of French hardness (°fH), an acid binding capacity of 0.36 mmol/l and a hydrogen carbonate content of 21.8 mg/l.

Correcting deviating values:
Too low: Use hydrogen carbonate-containing JBL water conditioners or mineral salt mixtures.
Too high: Soften the water by using a reverse osmosis system, or similar.

Notice d'emploi (fr)

IMPORTANT: toujours tenir les flacons compte-gouttes **verticalement** avec l'embout vers le bas et verser **sans bulles d'air.** Les compte-gouttes doivent être secs de l'extérieur.
Stockage des réactifs: Dans un endroit sec à température ambiante et dans l'emballage d'origine.

JBL PRO AQUATEST KH (fr)

Particularité: JBL PRO AQUATEST KH est un test de titrage facile à manipuler pour déterminer la dureté carbonatée (capacité de l'eau à neutraliser les acides, également appelée alcalinité) dans l'eau douce ou l'eau de mer et dans les bassins de jardin.
Pourquoi tester? En fonction de sa provenance, l'eau peut contenir des quantités plus ou moins importantes de différents sels minéraux, conditionnées par exemple par la nature du sous-sol. Une grande partie des sels dissous sont des carbonates alcalino-terreux et des hydrogénocarbonates alcalins. Avec les carbonates et le dioxyde de carbone (CO_2), les hydrogénocarbonates constituent un système tampon important qui empêche les fluctuations dangereusement élevées du pH dans l'eau. La dureté carbonatée (KH) mesurée donne la concentration totale d'hydrogénocarbonates dans l'eau et peut, dans certains cas rares (en cas de présence principale d'hydrogénocarbonates alcalins comme dans les lacs d'Afrique de l'Est), être plus élevée que la dureté totale où l'on ne tient compte que des sels alcalino-terreux. La plupart des poissons et des plantes en aquarium d'eau douce peuvent être parfaitement maintenus avec une dureté carbonatée de 5 à 16 °dH environ. Pour une fertilisation optimale au CO_2, la dureté carbonatée ne devrait pas être inférieure à 5 °dH. Dans le bassin de jardin, on devra également maintenir une dureté carbonatée d'au moins 5 °dH. En cas de pénurie de CO_2, les plantes aquatiques, et surtout les algues, qui l'assimilent rapidement au cours de la photosynthèse, vont consommer les hydrogénocarbonates (décalcification biogène) et peuvent, de ce fait, faire grimper le pH à des niveaux dangereux pour les poissons. Dans l'eau de mer, on devra maintenir une dureté carbonatée entre 7 et 13 °dH pour avoir un pouvoir tampon optimal du pH.
Mode d'emploi:
1. Rincer l'éprouvette plusieurs fois avec l'eau à tester.
2. Remplir l'éprouvette de 5 ml d'eau à tester à l'aide de la seringue fournie.
3. Ajouter du réactif au goutte à goutte. Agiter après chaque goutte et compter les gouttes jusqu'à ce que la couleur passe du bleu au jaune orangé.
4. Une goutte de solution réactive correspond à 1 degré de dureté carbonatée allemande (°dH), à 1,78 degré de dureté française (°fH), à une alcalinité de 0,36 mmole/L et à une teneur en hydrogénocarbonates de 21,8 mg/L.

Correction de paramètres divergents:
Trop faibles: recourir à des conditionneurs d'eau JBL contenant des hydrogénocarbonates ou à des mélanges de sels minéraux.
Trop élevés: adoucir l'eau p. ex. en utilisant un dispositif d'osmose inverse.

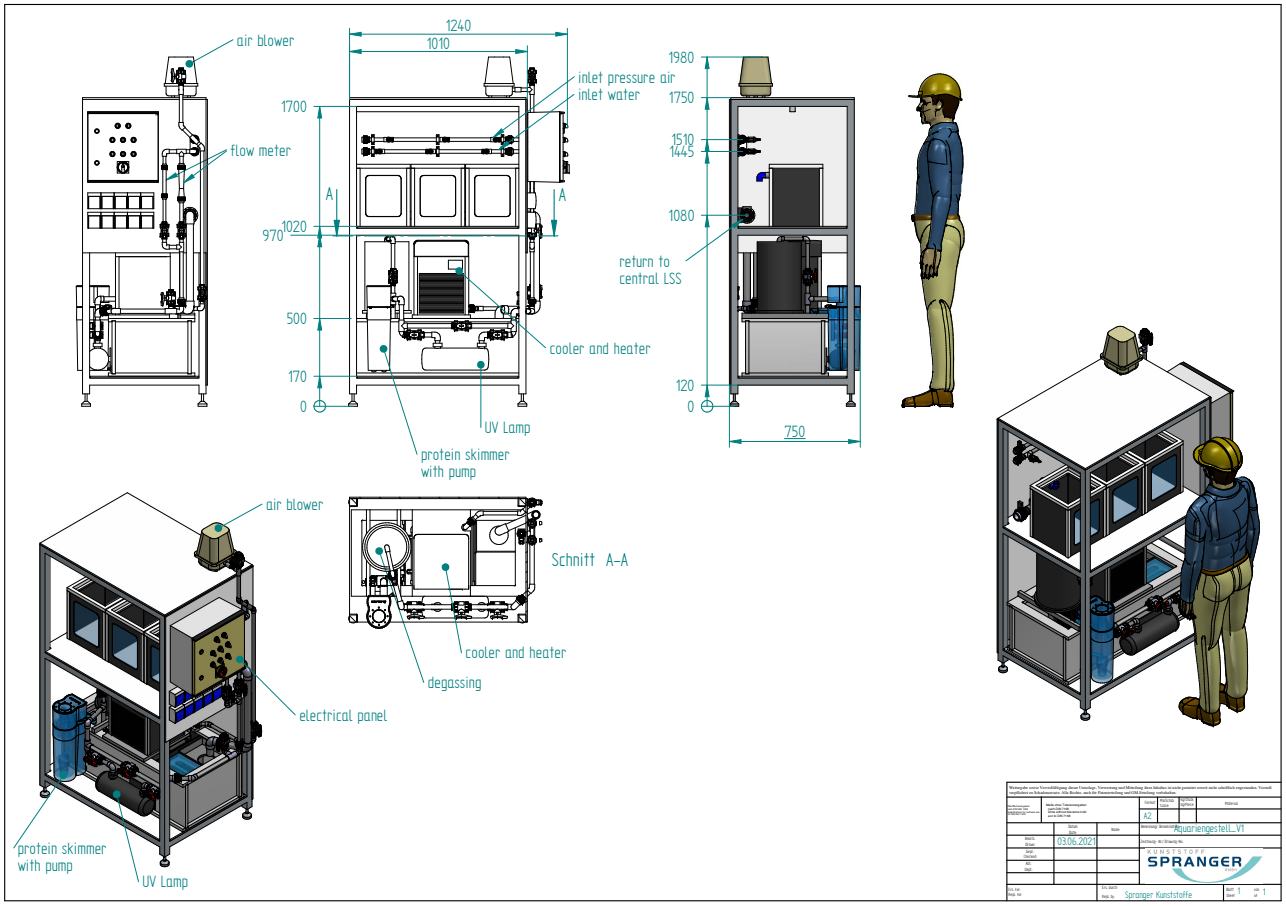
Informaciones para el uso (es)

IMPORTANTE: los frascos cuentagotas deben sujetarse siempre **en posición vertical** con el cuentagotas hacia abajo y gotear sin burbujas. El cuentagotas debe estar **seco** por fuera.
Cómo almacenar los reactivos: Guardar en un lugar seco a temperatura ambiente y en el envase original.

JBL PRO AQUATEST KH (es)

Características destacadas: JBL PRO AQUATEST KH es un análisis volumétrico fácil de usar para determinar la dureza de carbonatos (también denominada capacidad tampón o alcalinidad) en acuarios marinos y de agua dulce, así como en el estanque de jardín.
¿Por qué hacer la prueba? Dependiendo de su origen, p. ej., condicionada por las características del subsuelo, el agua puede contener diversas sales minerales en concentraciones distintas. Una gran parte de las sales disueltas está formada por hidrocarbonatos alcalinos y alcalino-térreos. Los hidrocarbonatos forman, junto con los carbonatos y el dióxido de carbono (CO_2), un importante tampón que impide que el valor del pH del agua oscile peligrosamente. La dureza de carbonatos (KH) medida indica la concentración total de hidrocarbonato en el agua y, por

K Manufacturers lab-scale RAS design



L Product specifications biofilm carriers

L.1 RK bioelements

RK BioElements
- for a cleaner world

Effective biological treatment for aquaculture

The injection moulded biofilter media, RK BioElements, from Dania Plast is a unique designed biomedia which can be used for both nitrification and denitrification in fixed-bed filters and moving-bed filters.

As RK BioElements combine a good flow with a large surface area of 750 m^2/m^3, the filters work with very high turnover rates, thus reducing the biological footprint. The unique design also means that RK BioElements cannot "wedge" together, which ensures an easy and simple cleaning of the filters

Since 2009 RK BioElements have successfully been used in biofilters around the world.

Characteristics

FORM STABLE

- Injection moulded – PP/PE material
- High breaking strength
- No compression/deformation

OPEN STRUCTURE

- Low hydraulic loss
- High water flow through the elements
- Easy to clean

DIFFERENT DENSITIES

- Light – density 0.93 g/m^3
- Medium – density 1.00 g/m^3
- Heavy – density 1.20 g/m^3

THE OBVIOUS CHOICE WHEN IT COMES TO BIOFILTER MEDIA

RK BioElements
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Specifications

	RK BioElements Light Density 0.93 g/cm³	RK BioElements Medium Density 1.00 g/cm³	RK BioElements Heavy Density 1.20 g/cm³
Applications	Primarily used for up-flow fixed-bed filters	Primarily used for moving-bed filter	Primarily used for down-flow fixed-bed filters
Surface area (m^2/m^3)	750	750	750
Number (pcs/m^3)	255,000	255,000	255,000
Volume weight (kg/m^3)	158	172	210
Bag size (m^3)	2.0 m^3, 2.5 m^3 & 3.0 m^3	2.0 m^3, 2.5 m^3 & 3.0 m^3	2.0 m^3, 2.5 m^3 & 3.0 m^3

RK BioElements are produced in Polypropylene (PP/PE), which contains no halogens and can be recycled or disposed of by incineration, where the end product is only water and carbon dioxide.
The filler used in RK BioElements Heavy and Medium is Barium Sulphate ($BaSO^4$). Barium Sulphate is environmentally neutral, ref. safety data sheet: *"No danger of toxity" the material is biological inactive.*

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L.2 AnoxK™Chip P

ANOXKALDNES

AnoxK™Chip P

Use

Carrier media for biofilm in biological wastewater treatment. For use in aerated reactors, with tank and reactor equipment designed for carrier media. This media is NOT to be used together with mixers.

Production

Injection molding. The carriers are produced in several injection molding machines and with several molds. Minor differences can be seen between individual pieces but this does not influence performance.

Form and size

Disk shape with 3.0 mm thickness and 45 mm diameter. Minor differences in the measures can be seen between individual pieces but this does not influence performance.

Protected surface area for biofilm growth:

Approx. 900 m^2/m^3 ± 1 %

Number per m^3: Approx. 132,000

Composition

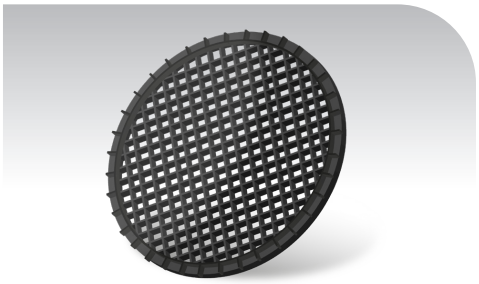
High-density polyethylene or polypropylene with lime addition to trim density. Virgin or recycled polymers are used.

Density

For most applications the density is 0.96kg/dm^3 (±0.02), which can be adjusted if required.

Weight per m^3: 145 kg at a density of 0.96 kg/dm^3

Displacement: 18 %, void 82 %.



AnoxK™Chip P

Handling

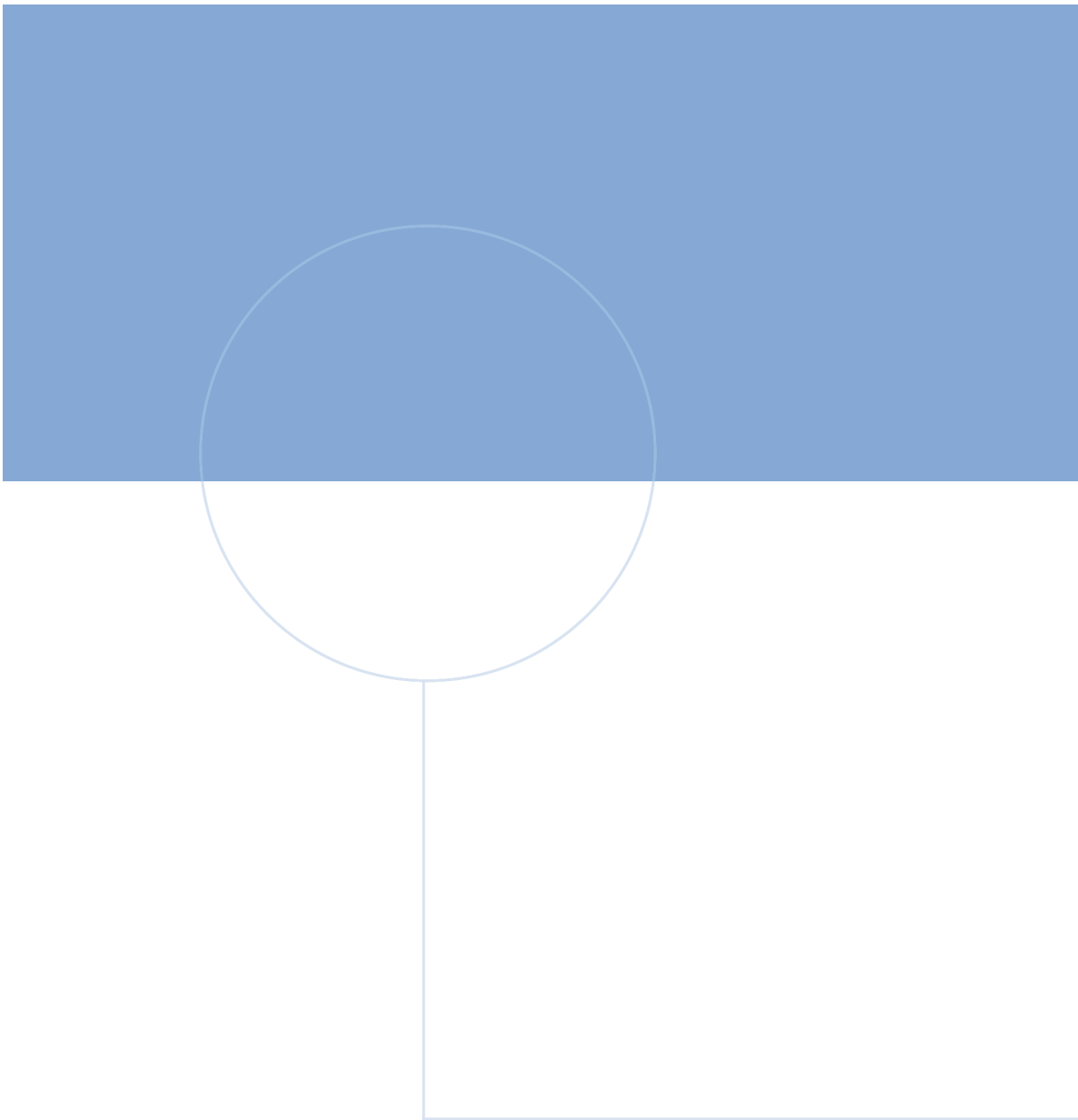
The carriers are packed and shipped in bags containing 3 m^3 carrier volume. When the carriers are emptied into the reactor, the reactor must already be filled with water and the aeration or mixing system must be running in order to avoid any impact damage to the carriers. Impact damages can be caused by a free fall of several meters and collisions with hard surfaces or other carriers. When filling, the suspended bags are usually simply cut open from the bottom with a knife.

The carriers should be stored in the bags. It is recommended no to stack more than 2 large bags (> 1.5 m^3) on one another for safety reasons as bag fall can occur and cause serious injuries. If the bags are stored, it is important that they are protected against sun light (UV light) and extreme temperatures.
The recommended range of temperatures for storage is 5 to 25°C.

If the carriers have to be moved from one reactor to another, centrifugal pumps or pneumatic conveyors are not suitable and will cause carrier breakage.
The carriers can be safely moved using an air lift pump or a belt conveyor. Refer to the 'Handling instructions' for more information.

Service life

The service life with normal use in a steel tank is more than 20 years and more than 15 years in a concrete tank with smooth walls. A rough concrete tank will wear the carriers and shorten the life expectancy. In concrete tanks, the rate of wear will depend on the aeration intensity and the roughness of the concrete. It is important that there are no sharp edges on the grid or other equipment in the reactor in order to avoid impact damage that can reduce service life.



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