Master's thesis

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

Kristine Hofstad

The Effects of Biofilter Disinfection on Nitrification Capacity and Bacterial Communities in RAS with Atlantic salmon (*Salmo salar L.*) fry

Master's thesis in Biotechnology (MBIOT5)
Supervisor: Ingrid Bakke
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Summary

Recirculating Aquaculture Systems (RAS) are increasingly popular as a technology for the production of Atlantic salmon (*Salmo salar L.*) smolts in Norway. In addition to lower water use than in traditional flow-through systems (FTS), advantages such as the high control of environmental conditions and physicochemical water quality increase fish welfare and production efficiency. The importance of microbial communities for fish welfare and chemical water quality in RAS is increasingly acknowledged. Biosecurity is generally high in RAS, but increasing concern about pathogenic outbreaks has provoked the need for disinfection procedures as a preventive measure. The biofilter in a RAS is a central component to ensure the removal of toxic nitrogenous compounds. However, there is concern about whether it may serve as a reservoir for pathogens. Therefore, chemical disinfection of the biofilter between fish batches is a proposed strategy in commercial production. Disinfection of the biofilter influences the dynamics of the resident communities of the RAS, but how and to what extent under the introduction of opportunistic bacteria is poorly understood. However, according to ecological theory, disinfection of the biofilter would increase the probability of pathogen invasion.

This thesis investigated whether the mature biofilter community in a RAS can counteract an invasion from opportunistic bacterial strains. To explore this question, two lab-scale RAS with Atlantic salmon fry were used, where one RAS had a biofilter with biofilm carriers that were partly disinfected, lowering the nitrification capacity to 20 % of the initial. The other RAS functioned as a control, having non-disinfected biofilm carriers in the biofilter. Further, pure cultures of four heterotrophic and presumably opportunistic bacterial isolates were introduced to both RAS. Characterization of the microbial communities of the biofilm carriers in the biofilter, rearing water and salmon gut was performed by Illumina sequencing of 16S rDNA amplicons. The opportunistic strains colonized the biofilm carriers and the rearing water of the RAS with disinfected biofilm carriers to a significant extent. In the RAS with non-disinfected biofilm carriers, i.e. mature, the opportunistic strains were not established either in the biofilm of the carriers or the rearing water. Moreover, the microbial communities in biofilm carriers and rearing water were more stable over time in the RAS with a mature biofilter. Regardless of the disinfection treatment, biofilm communities of the biofilter had higher alpha diversity than the rearing water communities and were significantly more stable over time. Also, the communities of the salmon gut were significantly less diverse than those of the biofilm carriers and rearing water, regardless of biofilter treatment.

This thesis has demonstrated the protective role of the mature biofilter during the invasion from four opportunistic bacterial strains during the rearing of Atlantic salmon fry in two lab-scale RAS. The work has provided new and vital knowledge that can be important for the management strategies for the biofilters of commercial RAS.

Sammendrag

Resirkulerende akvakultursystemer (RAS) har økt i popularitet for produksjon av atlantisk laksesmolt (*Salmo salar L.*) i Norge. I tillegg til lavere vannforbruk enn i tradisjonelle gjennomstrømningssystemer (FTS), bidrar fordeler som høy kontroll over miljøfaktorer og vannkvalitet til å øke fiskevelferden og produksjonseffektiviteten. Betydningen av de mikrobielle samfunnene for fiskevelferd og kjemisk vannkvalitet har fått økende anerkjennelse. Biosikkerheten er generelt høy i RAS, men en økende bekymring for utbrudd av patogener har aktualisert hvorvidt bruken av desinfeksjon bør utvides som et preventivt tiltak. Biofilteret i RAS er en sentral komponent for å sikre fjerning av nitrogenforbindelser som er toksiske for fisken, men det er bekymring for om det kan fungere som et reservoar for patogener. Derfor er kjemisk desinfeksjon av biofilteret mellom produksjonsbatcher en foreslått strategi i kommersiell produksjon. Desinfeksjon av biofilteret påviker dynamikken i de mikrobielle samfunnene i RAS, men hvordan og i hvilken grad, er ikke fullstendig kjent. I følge økologisk teori vil desinfeksjon av biofilteret føre til økt sannsynlighet for invasjon fra patogener.

Oppgaven har undersøkt hvorvidt det modne biofilteret kan motvirke invasjon fra opportunistiske bakteriestammer. To lab-skala RAS med lakseyngel ble brukt, der det ene systemet hadde et biofilter med biofilmbærere som var delvis desinfisert, slik at nitrifikasjonskapasiteten var redusert til 20 % av den initielle kapasiteten. Det andre systemet fungerte som en kontroll, der biofilmbærerne i biofilteret ikke var desinfisert. Videre ble rene kulturer av fire heterotrofe, antatt opportunistiske bakterieisolater tilsatt til begge RAS. Karakterisering av de mikrobielle samfunnene ble gjort ved Illumina-sekvensering av 16S rDNA amplikoner. De opportunistiske stammene koloniserte biofilmbærerne og vannet i det systemet som hadde desinfiserte bærerne på et signifikant nivå. I det systemet som hadde et modent biofilter, der bærerne ikke var desinfisert, etablerte ikke de opportunistiske stammene seg verken i biofilmbærerne eller i vannet. Videre var bakteriesamfunnene i systemet med modent biofilter mer stabile over tid. Samfunnene i biofilteret hadde høyere alfadiversitet enn samfunnene i vannet, og var mer stabile enn vannet over tid. De mikrobielle samfunnene i fisketarmen hadde signifikant lavere diversitet enn i biofilmbærerne og vannet, uavhengig av desinfeksjon av biofilteret.

Oppgaven har demonstrert den beskyttende rollen til det modne biofilteret under en invasjon fra fire opportunistiske bakteriestammer under oppdrett av lakseyngel i lab-skala RAS. Arbeidet har gitt ny og viktig kunnskap som kan være viktig for strategier for kontroll i biofilter i kommersielle RAS.

Preface

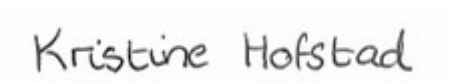
This thesis marks my completion of the five-year master´s program in Biotechnology (MBIOT5) at the Norwegian University of Science and Technology (NTNU) in Trondheim. The thesis was part of the RASOPTA project (956481) funded by the European Union. The work was conducted in the ”Analysis and Control of Microbial Systems” group at the Institute for Biotechnology and Food Science (IBT), and partly performed at the NTNU Centre for Fisheries and Aquaculture (SeaLab) at Brattørkaia.

I want to express my gratitude to my supervisor in the ACMS group, Professor Ingrid Bakke. Thank you for sharing your extensive knowledge of microbial ecology and aquaculture, always answering all my questions with a smile, and inviting me to exciting discussions. No one could ask for a better supervisor than you.

To my co-supervisor, Ph. D.-candidate Fernando Fernando, thank you for following me through this master’s project from start to finish. It has been a pleasure taking part in your work, especially the fish experiment in SeaLab, where you willingly shared your practical knowledge working with RAS. I also appreciate your passion for scientific communication and understanding of statistics, being invaluable during my writing. You truly deserve a gold medal for your patience. I also want to thank Ph.D.-candidate Sujan Khadka, for involving me in your work in such a pleasant manner, trusting my knowledge and always being helpful. And to my fellow master’s student Anna Aasen, thank you for the collaboration during the fish experiment. Working with such friendly people has taught me that we are better together. The staff at SeaLab, especially Arne and Iurgi, should also be acknowledged for their practical help and advice during the fish experiment.

My family and friends deserve the world for their patience and kindness during my work. For you, I am forever grateful. Lastly, I would like to thank my better half, Christopher, for always being there. I could never have done this without your endless support.

Trondheim, June 2023



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List of Abbreviations

AOA	Ammonia-oxidizing Archaea
AOB	Ammonia-oxidizing Bacteria
ASV	Amplicon Sequence Variant
CC	Carrying Capacity
CFU	Colony Forming Unit
COD	Chemical Oxygen Demand
COMAMMOX	Complete Ammonia Oxidation
dNTP	Deoxyribonucleotide triphosphates
DO	Dissolved Oxygen
EPS	Exopolymeric Substances
FTS	Flow Through System
HRT	Hydraulic Retention Time
LB medium	Lysogeny Broth medium
MBBR	Moving Bed Biofilm Reactor
NGS	Next Generation Sequencing
NOB	Nitrite-oxidizing bacteria
NTC	Non-template PCR Control
PBS	Phosphate Buffered Saline
PCoA	Principal Coordinates Analysis
PCR	Polymerase Chain Reaction
PERMANOVA	Permutational Analysis Of Variance
RAS	Recirculating Aquaculture Systems
RAS C	RAS Control (non-disinfected biofilm carriers in the biofilter)
RAS D	RAS Disinfected (disinfected biofilm carriers in the biofilter)
rDNA	Ribosomal Deoxyribonucleic acid
rpm	Revolutions per minute
SBS	Sequencing By Synthesis
SSR	Surface Specific Removal rate
TAN	Total Ammonia Nitrogen
TDS	Total Dissolved Solids
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
UV radiation	Ultraviolet radiation
V3	Variable region 3
V4	Variable region 4

1 Introduction

The demand for sustainable food production is increasing with the growing world population. Aquaculture is the fastest-growing food production sector in the world, and the production reached record numbers in 2020 at 214 million tonnes (FAO, 2022). The increasing demand requires further development of aquaculture for protein production. Norway is the world-leading producer of farmed Atlantic salmon (*Salmo salar L.*), with a total export of 1.67 million tonnes in 2020 (Norges Sjømatråd, 2023), accounting for more than half of the global export of the species (FAO, 2022). The global production of marine species had a six-fold increase from 1990 to 2016, and the main challenge will be maintaining the growth sustainably while lowering the environmental footprint (Thompson and Ahmed, 2019). To address this challenge, developing new technological solutions incorporating biology and fish welfare knowledge is necessary (Lekang, 2020). The same challenge affects the Norwegian salmon industry. Despite the record export numbers, several challenges affect the industry's growth. The ambition of a fivefold increase in Norwegian salmon production from 2012 to 2050 (Norsk Industri, 2017) is limited by problems with access to fresh water, eutrophication of the sea, escapes, sea lice infections, and pathogens (Thompson and Ahmed, 2019). There is a trend of moving production systems on land to solve some of these challenges sustainably (Lekang, 2020).

1.1 Landbased rearing of salmon smolts in Recirculating Aquaculture Systems (RAS)

The common practice in salmon production is to grow juveniles until smolt on land, then transfer and grow the smolt until harvest size in the open net pens in the sea. The grow-out period from smolt to slaughter typically lasts 16 to 24 months (Mobley et al., 2021). Recirculating Aquaculture Systems (RAS) is a widely used technology for producing smolts on land. It is estimated that around 70 % of the salmon smolts transferred to sea in Norway are grown in a RAS, and further advancement of the RAS technologies would even enable complete cycle production on land (Meriac, 2019).

Compared to production in open sea cages, the land-based systems can avoid problems with sea lice infections and escapes, which lowers production efficiency. Recirculating aquaculture systems (RAS) are an example of such a land-based system. Globally, most land-based systems are flow-through systems (FTS). Flow-through systems depend on continuously adding new water to ensure optimal water quality. In contrast, in RAS, the rearing water is treated and recirculated, thus limiting new water use (Balami, 2021). Figure 1.1 illustrates the differences in water flow between a conventional FTS and a RAS (Lekang, 2020).

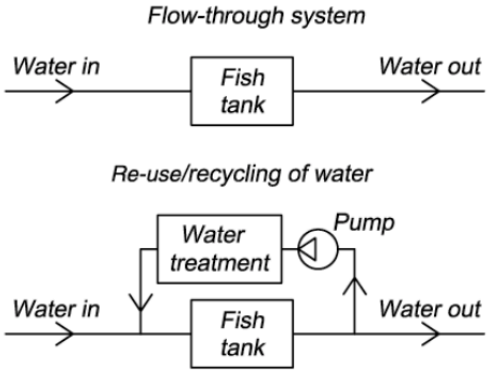

Figure 1.1: Illustration of RAS versus FTS, obtained from Lekang, 2020. In a flow-through system, the rearing water is continuously exchanged to ensure optimal water quality, while in a RAS, the water is recirculated, and a water treatment loop ensures optimal water quality.

Extensive control of the water quality and production parameters in RAS are the main advantages of the RAS technology (Holan et al., 2020 and Terjesen et al., 2013). The environmental conditions can be optimized to species and life stage, enhancing the production efficiency (Balami, 2021). The high degree of water reuse implies the demand for extensive water treatment systems to remove substances that cause stress, harm the fish, or limit growth (Bregnballe, 2015). Examples of factors that impact fish growth are shown in Figure 1.2.

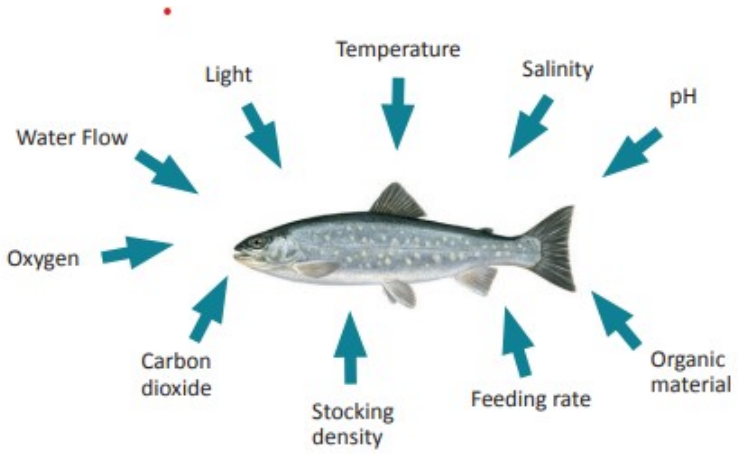

Figure 1.2: Factors that impact the growth and welfare of a farmed fish, adapted from Bregnballe, 2015.

RAS technology has advanced extensively in recent years for optimizing water quality and fish health. Water treatment in a RAS always includes 1) the removal of organic particles from faeces and uneaten feeds, 2) microbial conversion of toxic nitrogen compounds excreted by the fish, 3) removal of carbon dioxide from fish respiration and microbial decomposition, and 4) addition of oxygen (Bregnballe, 2015). The presence and order of each component in the water treatment loop may vary. An example from the RAS provider AKVAGroup is shown in Figure 1.3 (AKVAGroup, 2022). In the mechanical filter (4), larger particles ranging from 40-100 µm, depending on the technology, are removed. The water passes to a biological filter, or *biofilter* (5),

where bacteria convert toxic ammonia to less toxic nitrate. The moving bed bioreactor (MBBR) is a widely used biofilter technology in RAS, where bacterial biofilm grows on the surface of small plastic carriers in constant movement (Shitu et al., 2022). Some RAS include disinfection by ozonation or UV (6) and removal of smaller particles by a protein skimmer (not pictured). The *degasser* (7) strips the carbon dioxide from the water and has additional properties as a biofilter. Oxygenation cones (3) supply oxygen to the rearing tanks (2), and treatment of the sludge may be performed through the use of multiple technologies (9,10). (Lekang, 2020, Bregnballe, 2015).

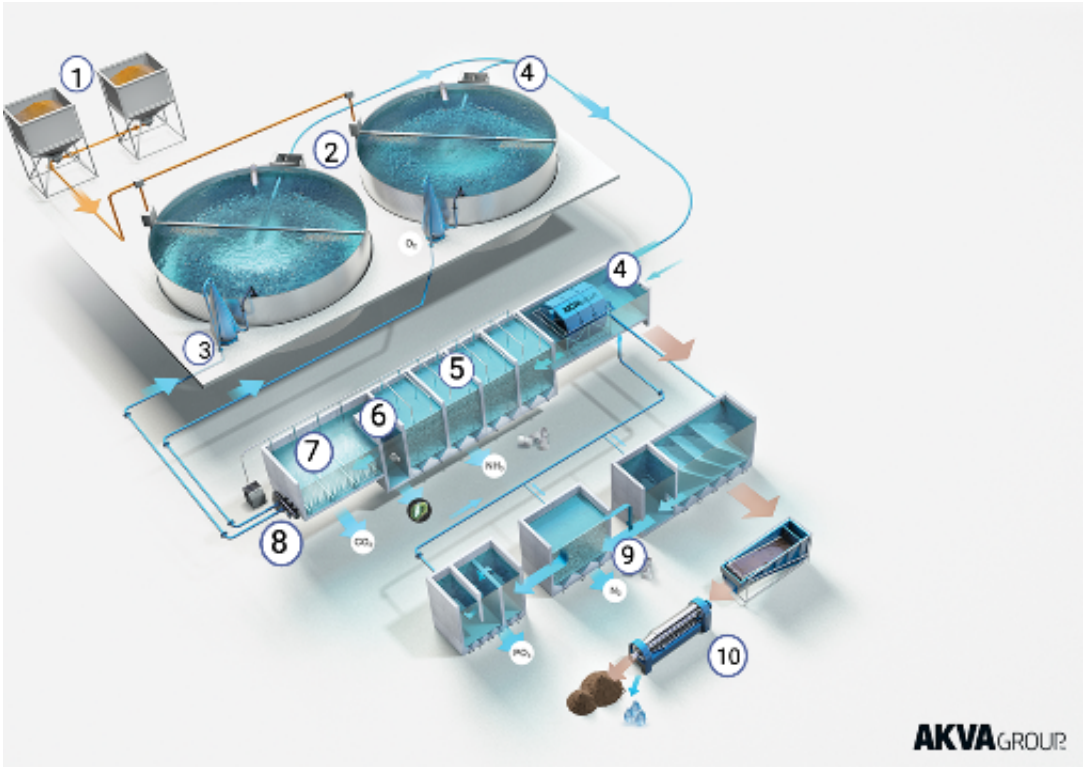

Figure 1.3: An example of a RAS, modified from AKVAGroup (AKVAGroup, 2022). Automatic feeders (1) supply feed to the rearing tanks (2). Oxygenation of recirculated water is performed through the oxygenation cones (3). The water enters the treatment loop: mechanical filtration of the rearing water removes particles larger than 40 µm, pictured is a *drum filter* (4). The water is treated in the biological filter (5), passing the ozonation chamber (6) to the degasser for the removal of carbon dioxide (7). Main pumps (8) circulate the treated water back to the rearing tanks. A side stream in the treatment can perform additional removal of phosphorus and nitrogen gas (9) while passing wastewater to the sludge treatment step (10).

Typically, some percentages of the RAS water are exchanged or added daily to account for the evaporation and dilute the nitrate produced by the nitrite-oxidizing bacteria. UV or ozone treatment of the inlet water is a common strategy for disinfecting the new water. pH and alkalinity adjustments are needed to account for the acid produced by the nitrifying bacteria as well as the disturbances in the carbonate equilibrium due to the production of carbon dioxide by the fish and bacteria (Bregnballe, 2015, Balami, 2021).

1.2 Microbial ecology in RAS

RAS hosts complex microbial communities. The bacteria are omnipresent in the system: in the rearing water, in the biofilms on every biotic or abiotic surface, and associated with host and organic matters (Rurangwa and Verdegem, 2015). The bacteria growing in RAS have positive and negative contributions to the water quality, and, therefore, to the efficiency of the production. While some bacteria can be pathogens and detrimental to fish, others are responsible for the conversion of harmful substances in the rearing water or support the normal development of the fish. Therefore, understanding the microbial ecology is vital in developing a well-functioning RAS from different aspects; for the fish's health and welfare and its interaction with the water quality (Dahle, 2022). Many factors, including the system design and management strategies, as well as external sources, interact with the microbiota of a RAS, as illustrated in Figure 1.4 (Dahle, 2022).

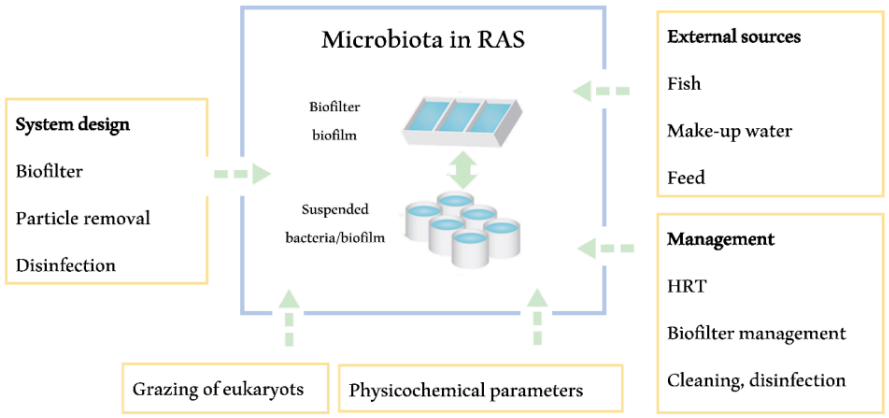

Figure 1.4: Factors interacting with the microbiota in RAS, from Dahle, 2022.

1.2.1 Bacterial growth in biofilm

The conversion of toxic ammonia in biofilters in RAS utilizes the unique behaviour of bacteria: the ability to grow in biofilm. Biofilm is the attachment and growth of a bacterial community on a surface. The bacteria are attached to each other and the surface with an exopolysaccharide matrix, allowing for growth on submerged surfaces such as the carriers in the biofilter (Bose and Ghosh, 2011). The matrix contains exopolymeric substances (EPS) produced by the bacteria, serving as a protective layer and aiding the bacterial attachment to each other and the surface.

Biofilm growth is favourable for bacteria because of the collective protection against the environment. Bacteria submerged in biofilm communities show distinct phenotypes compared to planktonic cells freely growing in water. The characteristic phenotypes, such as modified growth, intercellular communication, and reduced metabolism, allow the community to withstand environmental changes that can be detrimental to bacterial survival. Examples of such factors include antibiotics or disinfectants (Otter et al., 2015). Biofilm formation as a bacterial life strategy increases the probability of

survival through effective nutrient uptake and complex social cooperation, which are essential from an evolutionary perspective (Flemming et al., 2016).

Forming biofilm is a complex and dynamic process that can be divided into three main stages: early, intermediate, and mature. The early stage is characterized by the movement of planktonic cells along the surface, followed by surface recognition and adhesion to form a bacterial monolayer. During the intermediate stage, the bacteria irreversibly bind to the surface. The bacteria multiply and grow to form microcolonies that are now phenotypically different from the individual planktonic cells. The microcolonies are responsible for forming EPS, which expands during maturation (Malheiro and Simões, 2017).

Biofilms are heterogenous structures, and the heterogeneity is reflected in the layers of the biofilm, where a gradient of cells with different living strategies is found (Flemming et al., 2016). Nutrient and oxygen gradients are typically formed as the biofilm is established. Aerobe bacteria establish the higher layers of the biofilm. The fermenters and anaerobes thrive in the lower layers, as the oxygen is consumed faster than its diffusion. (Flemming et al., 2016).

1.2.2 Removal of nitrogenous compounds in the biofilter

The nitrogenous waste products from fish and bacteria are toxic to fish and result in reduced growth and welfare, with mortalities at high concentrations. In addition to the excretion due to the fish's metabolism of organic matter, bacterial decomposition, in addition to uneaten feed, adds to the nitrogenous compounds for removal (Ruiz et al., 2020). Due to the intensive recirculation of the RAS water, technologies to prevent their accumulation are a prerequisite for effective production and safeguarding of fish welfare (Nazar et al., 2013). The *biofilter* is a central part of the treatment loop in RAS, where a process called *aerobic nitrification* is performed by autotrophic bacteria growing in biofilm (Ruiz et al., 2020). Two phenotypically distinct groups of bacteria are well known for performing nitrification. Ammonia is converted to nitrite (Step 1) by ammonia-oxidizing bacteria (AOBs), such as *Nitrosomonas* and ammonia-oxidizing Archaea (AOAs), for example *Nitrosopumilus*. The nitrite is oxidized to nitrate (Step 2) by nitrite-oxidizing bacteria (NOBs), such as *Nitrobacter* (Ebeling and Timmons, 2012, Ruiz et al., 2020). In addition to the AOA/AOBs and NOBs, the presence of a newly discovered genus of *Nitrospira* performing complete ammonia-oxidization (COMAMMOX), was detected in RAS (Al-Ajeel et al., 2022). These simultaneously perform the job of the two groups mentioned above.

$$NH_4^+ + 1.5\,O_2 \rightarrow NO_2^- + 2\,H^+ + H_2O \qquad \textbf{(Step 1)}$$

$$NO_2^- + 1.5\,O_2 \rightarrow NO_3^- \qquad \textbf{(Step 2)}$$

Ammonia exists in equilibrium with the ionized form, ammonium. The toxicity to fish is highly pH-dependent: with a pH above 7, more of the total ammonia nitrogen (TAN) is present as ammonia gas dissolved in the water, being more toxic than the

ionized form due to the more efficient transport across the gills. Chronic exposure to sublethal doses of ammonia has been shown to cause gill damage in Atlantic salmon parr (Kolarevic et al., 2013); however, acute toxicity is the primary concern in RAS (Ruiz et al., 2020). A TAN <2 mg/L threshold is recommended in the rearing of Atlantic salmon in RAS in Norway (Norwegian Food Safety Authority, 2008). Monitoring other physicochemical water quality parameters influencing ammonia toxicity, such as pH, temperature, and salinity, is equally important to secure the welfare (Norwegian Veterinary Institute, 2018).

Nitrite may accumulate when the biofilter in a RAS operates in sub-optimal conditions. High nitrite concentration can be lethal to fish due to its affinity for the chloride gill transporter, affecting gas transport, ion regulation and excretion. Since nitrite is more toxic at lower salinity due to the lower chloride concentration, close monitoring is vital in the freshwater production of smolts in RAS (Gutiérrez et al., 2019). The recommendation for nitrite is a concentration below 0.1 mg/L in freshwater production of Atlantic salmon (Norwegian Food Safety Authority, 2008). Less research has been carried out to investigate the effects of nitrate on the welfare of Atlantic salmon juveniles and smolt. However, a study by Davidson et al. on Atlantic salmon post-smolt suggested that chronic exposure to nitrate levels lower than 100 mg/L had no significant effect on health and survival (Davidson et al., 2017).

1.3 Microbial selection theory and application in RAS

The r- and K-selection theory can describe some dynamic properties of microbial communities (Vadstein et al., 2018). The r-strategists have high growth rates when the availability of resources such as organic matter and oxygen per capita is high, i.e. the competition is low. On the other hand, the K-strategists are characterized by low maximum growth rates, and their adaptation to lower resource availability is high. The K-strategists are also called specialists since they are selected for when the availability of resources is close to the carrying capacity, and the competition is high (Vadstein et al., 2018).

The theory can apply to studies of microbial communities in RAS. K-selected communities are hypothesized to be more stable than the r-selected communities and are also termed *mature* (Skjermo et al., 1997). High biological stability, high diversity and stable biomass at carrying capacity also characterize the mature communities (Skjermo et al., 1997, Vadstein et al., 2018). Several studies demonstrated that RAS can promote K-selection, resulting in more stable microbial communities having a positive impact on fish production (Bakke et al., 2017, Attramadal et al., 2014, Vadstein et al., 2018). Several reasons are proposed, including the long hydraulic retention time in a RAS, which is favourable for the growth of the K-strategists as they are not washed out before they have had the opportunity to establish. The long hydraulic retention time (HRT) also ensures a stable carrying capacity in the RAS over time (Vadstein et al., 2018).

1.3.1 Heterotrophs

While some convert nitrogen species through nitrification or degrade organic matter accumulating in a RAS, the heterotrophic bacteria compete with the autotrophs for the available resources. They comprise most of the biofilter biofilm, and their competition with the nitrifiers for the available resources may lower the nitrification efficiency (Leonard et al., 2000). Generally, the heterotrophic bacteria are faster growing than the autotrophs, and the competition between these groups is, therefore, highly dependent on resource availability and the available space. The carbon-to-nitrogen (C/N) ratio and oxygen availability is critical since heterotrophic bacteria thrive with higher levels of organic matter and consume more oxygen (Navada et al., 2020). However, it has been shown that an equilibrium between the nitrifiers, characterized by growth in biofilms, is favourable. The protective properties of the thin outer layer of heterotrophs established in the biofilm-water surface prevent grazing and detachment of autotrophs. Furthermore, the coexistence of heterotrophs and autotrophs allows for the simultaneous conversion of organic matter and nitrogenous substances that limits fish growth (Blancheton et al., 2013). Importantly, heterotrophic bacteria may be pathogens causing infections that may harm the fish (King et al., 2004).

1.3.2 Disinfection as a microbial management strategy in RAS

To reduce the risk of pathogens entering and establishing in the RAS, ultraviolet radiation (UV), ozone and chemical methods are commonly used for disinfection. UV and ozone are commonly combined to inactivate heterotrophs, including fish pathogens, in the water treatment loop within the RAS (Martins et al., 2010). These methods kill neutral heterotrophs and obligate pathogens, reducing the bacterial load and thus bacterial competition for oxygen (Attramadal et al., 2014). Chemical disinfection, for example, with hydrogen peroxide, may be utilized for disinfection of facilities between fish batches. It is effective for inactivation of harmful bacteria. Consequently, the nitrifying communities of the biofilter biofilm are equally perturbed, and the nitrification capacity decreases (Møller et al., 2010). The toxicity of the disinfectants is also a concern when using chemical disinfectants (Gaikowski et al., 1999).

However, the disinfection procedures cause perturbations in the healthy microbial communities. It is hypothesized that these methods may be disadvantageous for the microbial water quality when used as a preventive measure to lower the probability of opportunistic pathogens entering the RAS (Gullian et al., 2012 and Attramadal et al., 2012). Disinfection in the RAS-loop by the use of ozone or UV has been shown to perturb the microbial communities and therefore cause a shift towards r-selection in RAS for marine larvae (Attramadal et al., 2012). This is due to the assumption that the r-selected species, which might be opportunistic pathogens causing infections in fish, are more effective re-colonizers when the resources per capita increase (Hess-Erga et al., 2010). The opportunistic heterotrophs, viewed as r-strategists, can quickly colonize the empty niches available after disinfection. Thus, it has been hypothesized that disinfection as a microbial management strategy can select the unwanted species since the carrying capacity is unaffected and the number of bacteria decreases

substantially. However, little research exists on the role of the biofilter biofilm communities after disinfection in RAS. Still, it is assumed that the K-selected microbial community is unfavourable for the opportunists (Blancheton et al., 2013). It is unlikely to completely eradicate the probability of pathogens entering a RAS (Attramadal et al., 2012). Thus, more research is needed investigating microbial management and what characterizes the ”healthy” communities in a RAS.

1.4 Biosecurity and the increased concern of infectious diseases in salmon farming

The 2021 Fish Health Report published by The Norwegian Veterinary Institute states the concern of increased incidence of some bacterial infectious diseases in farmed salmonids. In freshwater, these include typical fish infections such as flavobacteriosis (caused by *Flavobacterium psychrophilum*), yersiniosis (caused by *Yersinia ruckeri*) and mycobacteriosis (caused by species of Mycobacteria) (Sommerset et al., 2022). The disease caused by infectious pancreatic necrosis virus (IPNV) is among other pathogens posing a severe threat (Murray et al., 2014). The causes are complex and may relate to poor biosecurity measures allowing the spread of disease from farmed or wild reservoirs and more frequent handling, increasing the probability of spreading infections between individuals (Sommerset et al., 2022).

Although infection control is generally higher in RAS, this emerging concern of infectious disease also prevails for closed systems on land. More recently, there has been increasing interest and emphasis on the importance of biosecurity in these systems. The infection dynamics differ in RAS compared to production at sea (Mota et al., 2015). The higher stocking densities, increased organic load in the system, and long HRT increases the probability of pathogen proliferation (Mota et al., 2022). Preventive measures such as antibiotics, hygienic barriers, disinfection (chemical or with ozonation or UV), and management routines are utilized to limit the incidence of infections and increase biosecurity (Powell and Scolding, 2018 and Summerfelt, 2003).

The disinfection strategies’ consequences on the welfare of Atlantic salmon and the impact on the chemical and microbial water quality need more attention (Mota et al., 2022). In addition to the costs of the preventive disinfection procedures, the economic consequences of pathogen incidents in a RAS are significant. The affected fish batches cannot be sold, and high costs are linked to the time spent eliminating the pathogen from the system.

1.5 Methods for studying microbial communities

The complexity of microbial communities makes them challenging to study compared to pure bacterial strains. However, the development of culture-independent methods, such as next-generation sequencing (NGS) technologies, and bioinformatic methods, has started the revolution in characterizing such communities. Illumina sequencing of 16S ribosomal RNA is the most widely used method for studying microbial com-

munities (Fukuda et al., 2016). The high conservation of rRNA gene sequences over generations makes them suitable for identification and classification. Sequencing by synthesis (SBS) is the basis for Illumina sequencing, allowing for sequencing millions of fragments at a time. The workflow consists of four main steps: 1) library preparation, 2) cluster generation, 3) sequencing, and 4) data analysis (Illumina Inc., 2015). The region of interest is amplified during library preparation, and an adapter is added to both ends of all fragments. The amplicon library is loaded into a *flow cell*, where oligos bound to the surface of the cell have sequences complementary to the adapters, allowing for the capture of each amplicon. Bridge amplification is then utilized to generate clusters for each fragment. In the third step, sequencing, each template formed in the cluster generation is used in the incorporation of fluorescently labelled nucleotides that emit light. The light emitted by each cluster in the flow cell is recorded, and the strength of the signal is used to identify each base, and the process is repeated until the desired read length is achieved (Illumina Inc., 2015). The resulting sequences can be subjected to different data analyses to obtain the information of interest. The result of the 16S rDNA metabarcoding can be a table showing the presence and abundance of Amplicon Sequence Variants (ASVs) detected in a given sample, which can be further analyzed and used to characterize the microbial community composition (Zemb et al., 2020).

1.6 Study Aims and Hypotheses

Pathogenic bacteria, possibly causing detrimental disease in fish, is of increasing concern in RAS. Disinfection is a biosecurity measure that may eradicate pathogens while simultaneously perturbing the resident microbial communities. The importance of the resident microbial communities of the RAS during an invasion from opportunistic bacteria, specifically whether it can protect against such invasions, is a knowledge gap. Extended biosecurity measures, such as disinfection between fish batches, are considered. The role of the biofilter during opportunistic invasion in RAS is not understood. There is a concern about whether the biofilters in RAS may serve as reservoirs for pathogenic microorganisms. On the other hand, it is hypothesized that the high bacterial densities of the biofilter, secure high competition for organic matter, and, therefore, may protect the RAS against invasion from pathogens. Although some commercial RAS use disinfection of the biofilter as a biosecurity measure, there is a lack of knowledge on the significance of this procedure for preventing the growth of opportunistic bacteria. Altogether, this thesis aims to:

1. Develop more knowledge and understanding of lab-scale RAS as new experimental systems at NTNU.
2. Investigate whether the mature biofilter biofilm community can counteract an invasion by opportunistic bacteria.
3. Characterize the microbial communities associated with biofilter biofilm, rearing water and salmon, in two lab-scale RAS with either a 1) biofilter with disinfected biofilm carriers, or 2) biofilter with non-disinfected biofilm carriers after a challenge by opportunistic bacteria.

Hypotheses include

- The opportunistic bacteria will have higher colonization success in the RAS with a biofilter running with disinfected carriers compared to the RAS with a non-disinfected biofilter.
- The alpha diversity of the microbial communities in the RAS with a disinfected biofilter will be lower than in the system with a non-disinfected biofilter.
- The microbial communities in the RAS with a disinfected biofilter will be more unstable over time than the system with a non-disinfected biofilter.

2 Methods

The main experiment was the rearing of Atlantic salmon fry in two lab-scale RAS during a challenge from four opportunistic bacterial strains to determine how the microbial communities in fish, biofilm carriers, and rearing water changed over time; one system (control) had untreated biofilm carriers in the biofilter, and the other had partly disinfected biofilm carriers in the biofilter. Before the fish experiment, the nitrification capacity of the biofilm carriers was determined to aid the dimensioning of the biofilters of the lab-scale RAS. Secondly, a small-scale batch experiment was performed to develop a disinfection protocol for the biofilm carriers used in the fish experiment. Also, an experiment was conducted to investigate the opportunistic strain's ability to colonize the biofilm carriers prior to their use in the bacterial challenge in lab-scale RAS.

2.1 Nitrification capacity of biofilm communities on carriers from Lerøy

2.1.1 Experimental design

Biofilm carriers were obtained from a RAS operating at the Lerøy facility in Hemne, Trøndelag. Two similar batch reactors were set up according to Figure 2.1, one for determination of the TAN oxidation capacity, and one to test the nitrite oxidation capacity. They were incubated with medium (Appendix A, Table A1) for 48 hours before the experiment. The medium in the batch reactors was set to have an initial concentration of TAN of 10 mg/L (Appendix A, Table A1) or nitrite of 5 mg/L (Appendix A, table A2), prepared with MilliQ® water. The pH in both media was adjusted to 7-7.5, and a trace metal solution was added (Appendix A, Table A3).

The two bioreactors (1000 mL) with continuous cooling to 12 °C were filled with biofilm carriers (250 mL, RK BioElements) and medium containing ammonia or nitrite (650 mL, Appendix A1). Humidified and filtered air were continuously supplied to the reactors, and the temperature was controlled with a thermometer. For the reactor containing ammonia medium, the ammonia, nitrite, and nitrate concentrations were determined with the Hach-Lange™ method (Appendix B2). Samples were taken every 20-40 minutes over a period of 5 hours and 20 minutes. In the reactor containing nitrite medium, the concentration of nitrite and nitrate was measured every 10-20 minutes over a period of 70 minutes.

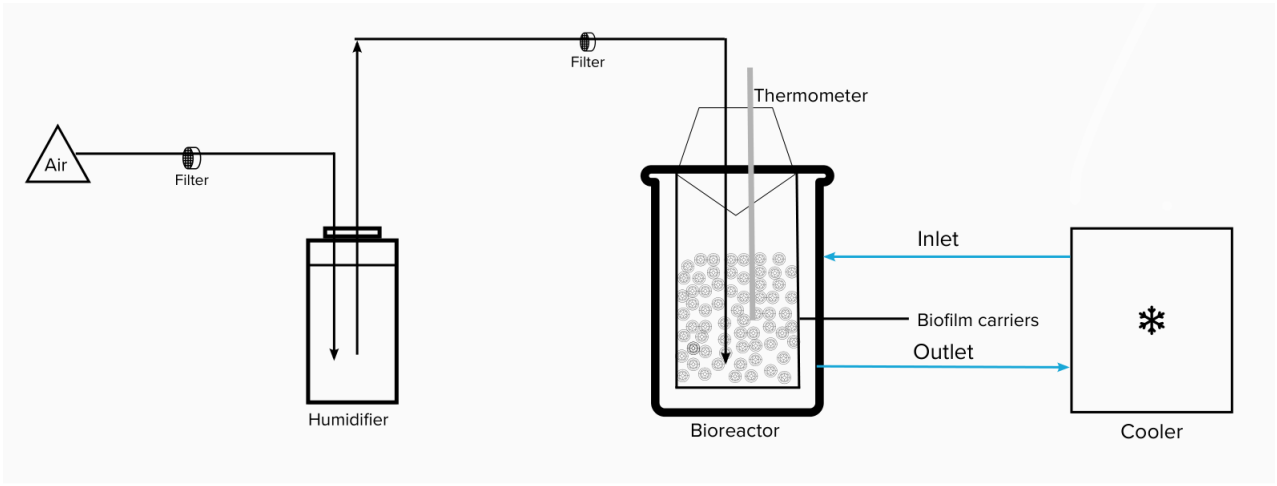

Figure 2.1: Experimental setup used in the batch experiment for determination of the ammonia- and nitrite-oxidation capacity of the microbial communities in the biofilm carriers from Lerøy. The moving bed bioreactor (MBBR) with biofilm carriers was supplied with ammonia- or nitrite medium, and humidified air was supplied continuously. The temperature was kept constant by the cooler.

2.1.2 Sampling procedure

Samples for the Hach-Lange tests were collected by withdrawing 4 mL of the bioreactor media with a syringe and passing it through a 0.2 µm filter before transfer to the respective test cuvette for ammonia, nitrite, or nitrate. In case of concentrations above the reference range of the kits, the media to be tested was diluted appropriately. The concentrations were determined by placing the cuvettes in the Hach-Lange UV-VIS spectrophotometer. By generating linear regression models using the measured concentration of each nitrogen substance over time in the two reactors, the surface-specific TAN- and nitrite removal rate (SSR) was calculated using Equation 1:

$$\text{SSR} = \frac{\text{slope of regression model x total volume of carriers in reactor}}{\text{total area of the biomedia}} \qquad \text{(Eq. 1)}$$

Hach-Lange™ protocols and their associated reference range are given in Appendix B.1. The raw data used to generate the regression models is in Appendix C.1,

2.2 Development of a disinfection protocol for the biofilm carriers

A batch experiment performed by Ph.D.-candidate Fernando Fernando tested the effect of different disinfection methods on nitrification capacity, developing a disinfection protocol for the biofilm carriers to be used in the experiment in RAS. Chemical disinfection with hydrogen peroxide was chosen as it is commonly used for disinfection purposes between fish batches in commercial RAS. However, the dosage relationships are not straightforward. A decrease in ammonia oxidation capacity to 20 % of the initial community was seen as a suitable level of disinfection (personal communication, Olav Vadstein, September 2022).

2.2.1 Experimental design

Four separate reactors with biofilm carriers were operated as described in Section 2.1 to test four disinfection treatments. Quantification of the nitrification capacity after four degrees of disinfection of the biofilm carriers was performed by using the Hach-Lange™ spectrophotometer to determine the ammonia concentration over time (Appendix B.1). Four batches of carriers (4 x 250 mL) were submerged in a bath of hydrogen peroxide for 3 hours prior to the batch experiment, containing one of four different treatments: 0 mg/L (control), 300 mg/L, 600 mg/L, and 1200 mg/L hydrogen peroxide. After the respective treatment, the carriers were distributed equally to the four reactors, and ammonia medium to a final concentration of 10 mg/L was added to each reactor (Appendix A, Table A1).

2.2.2 Sampling frequency and choice of protocol

The decrease in ammonia in each reactor was followed for the next 5 hours. Regression was used to generate a linear model of the decrease in ammonia over time for each treatment. The slope generated for each treatment (300 mg/L, 600 mg/L, and 1200 mg/L) compared to the control treatment (0 mg/L) was used to choose the treatment closest to 20 % of that of the initial community.

2.3 Determining the potential for the opportunistic bacterial strains *Flavobacterium, Proteus, Pseudomonas* and *Psychrobacter* to colonize the biofilm carriers

The main objective of the work with this thesis was to understand whether the mature biofilm community of a biofilter in RAS could protect against invasion from opportunistic bacteria. To answer the objective, four assumingly opportunistic bacteria were considered for introduction to the lab-scale RAS during the main experiment. Two aims were established: The determination of the unique nucleotide sequences of the four candidates, and the investigation of their colonization potential on the biofilm carriers to be used in the fish experiment. See also Section 2.4.1, where the rationale and method of introduction to the RAS are explained.

2.3.1 Sanger sequencing of the bacterial isolates

Culturing of the bacterial isolates and colony PCR was performed by Ph.D.-candidate Sujan Khadka. Freeze stocks of the four bacterial isolates in pure culture were used as the basis for the experiment. After culturing the isolates on tryptic soy agar (TSA), single colonies were subcultured multiple times, and the morphological characteristics were assessed. A polymerase chain reaction (PCR) amplified the bacterial DNA from single colonies representing each bacterial isolate before the unique nucleotide sequence of each genus was determined by sending the PCR products for Sanger sequencing. The primer 515F (Sigma-Aldrich) was used to amplify the 16S ribosomal

DNA of each isolate, and the primer sequence is given in Table 2.2. Contents of the PCR mix used are shown in Table 2.3, obtaining a final concentration of 0.3 mM of the primer, 0.25 mM of each dNTP (ThermoFisher™ Scientific), and 0.02 units/µŁ of Phusion Hot Start DNA polymerase (ThermoFisher™ Scientific). Temperature and cycling conditions for the PCR are given in Table 2.4. Examination of the yield and size of the PCR products was done by agarose gel electrophoresis. The PCR products were purified using the QIAquick® PCR Purification Kit (Qiagen, Appendix B.2), and 5 µŁ of each product was mixed with the sequencing primer (5 µŁ of 515F). Eurofins Genomics performed the Sanger sequencing of the 16S rDNA gene. A new glycerol stock of each bacteria was stored at -80°C for later use.

2.3.2 Colonization experiment with biofilm carriers

To investigate the potential of *Flavobacterium, Proteus, Pseudomonas* and *Psychrobacter* to colonize the biofilm carriers to be used in the main experiment, each strain was incubated in flasks containing new, sterile carriers. The biofilm on the carriers was detached, the bacteria were subcultured, and the growth potential was assessed before colony PCR was performed to confirm the 16S rDNA sequences with Sanger sequencing.

Cultivation of bacterial isolates and incubation with biofilm carriers

The culturing and incubation of the isolates with biofilm carriers were performed by Ph.D.-candidates Sujan Khadka and Fernando Fernando. In brief, each of the four strains was cultured in tryptic soy broth (TSB, 30 mg/L) for 12 hours. The optical density (OD_{600}) to colony forming units (CFU/mL) relationships had been established earlier and were used to introduce ”low” (10^3 CFU/mL) and ”high” (10^5 CFU/mL) concentrations to each flask later. The overnight cultures were centrifuged, and each culture’s OD-CFU relationship was used to adjust the volume of each culture representing the ”low” and ”high” treatment. The adjusted volume of each culture was transferred to a sterile flask and centrifuged (6000 rpm, 10 minutes, 4° C). The centrifuged cultures were washed twice with sterile PBS, and the pellets were resuspended in sterile flasks containing an organic medium (”feed medium”) and TSB (30 mg/L), with a final volume of 200 mL liquid altogether. The feed medium contained the salmon feed used in the fish experiment, based on a chemical oxygen demand (COD) of 100 mg O_2 per litre of medium. To achieve this COD value, 0.3 g/L of finely crushed fish feed was used. An equal volume of biofilm carriers (unused and sterile, RK BioElements) was added to each flask. Controls of the TSB and feed medium, without carriers, were also used, resulting in 10 flasks (Table 2.1). The flasks were incubated for 8 days at 12° C with a constant shaking speed of 155 rpm to allow colonization of the carriers.

Table 2.1: Experimental setup of the flasks used in the experiment to investigate the potential of the four opportunistic strains *Flavobacterium, Proteus, Pseudomonas* and *Psychrobacter* to colonize biofilm carriers. Eight flasks containing two different cell densities (CFUs) of each strain, were incubated for eight days with new (sterile, uncolonized) biofilm carriers, and a feed medium. Two controls without bacteria and carriers were included as a sterility check for the media used during the growth of the bacterial cultures and the feed medium.

Bacteria	**Treatment**	**Target CFU/mL media**
Proteus sp.	Feed medium + sterile carriers	10^3
Psychrobacter sp.	Feed medium + sterile carriers	10^3
Flavobacterium sp.	Feed medium + sterile carriers	10^3
Pseudomonas sp.	Feed medium + sterile carriers	10^3
Proteus sp.	Feed medium + sterile carriers	10^5
Psychrobacter sp.	Feed medium + sterile carriers	10^5
Flavobacterium sp.	Feed medium + sterile carriers	10^5
Pseudomonas sp.	Feed medium + sterile carriers	10^5
TSB control	No carriers	None
Feed medium control	No carriers	None

Cultivation of biofilm bacteria and colony PCR

After 8 days, the incubated carriers were washed twice with sterile MillQ® water, distributed in sterile Falcon tubes containing 1 mm glass beads and sterile MillQ® water, and shaken vigorously in a vortex shaker (3000 rpm, 2 minutes) to detach the biofilm from the carriers into the water. Serial dilutions of each were plated to tryptic soy agar (TSA) and incubated for 24 to 48 hours before plate counting to estimate the CFU/mL relationship after incubation with the carriers.

To confirm that the bacteria recovered from the biofilm carriers were the bacteria introduced, Sanger sequencing of the 16S rDNA from a few colonies from each treatment was performed. Amplification of the 16S rDNA gene performed by colony PCR as described in Section 2.3.1, using the Eub-8F primer (Sigma-Aldrich, sequence given in Table 2.2) instead of 515F.

Table 2.2: PCR primers 515F and Eub-8F (Sigma-Aldrich) used in the colony PCR for amplification of the 16S rDNA gene before Sanger sequencing.

Primer name	**Sequence**
515F	5‘-GTGCCAGCMGCCGCGGTAA-‘3
Eub-8F	5‘-AGAGTTTGATCCTGGCTCAG‘-3

The composition of the PCR mix is given in Table 2.3, obtaining a final concentration of 0.3 mM of the primer, 0.25 mM of each dNTP (ThermoFisher™ Scientific) and 0.02 units/µŁof Phusion Hot Start DNA polymerase (ThermoFisher™ Scientific). Temperature and cycling conditions for the colony PCR for amplification of the 16S rDNA gene are given in Table 2.4.

Table 2.3: PCR-mix used in the colony PCR to obtain a final reaction volume of 25 µL. The primer 515F (Sigma-Aldrich) was used in the colony PCR of the pure bacterial strains, and EuB8F (Sigma-Aldrich) was used in the colony PCR in the colonization experiment, to amplify the 16S rDNA gene.

Reagent	**Final concentration**	**Volume per reaction**
DNA-free H_2O		Up to final reaction volume of 25 µL
5x Phusion buffer HF (7,5 mM $MgCl_2$)	1x	5.0 µL
Primer 515F or EuB8F (10 µM)	0.3 mM	0.75 µL
dNTP (10 mM each)	250 µM each	0.625 µL
Phusion Hot Start DNA polymerase (2 units/µL)	0.02 units/µL	0.18 µL

Table 2.4: Temperature and cycling conditions used in the colony PCR for amplification of the 16S rDNA gene.

Step	**Temperature (°C)**	**Time (seconds)**	**Number of cycles**
Denaturation	98	120	1
Denaturation	98	15	35
Annealing	55	20	30
Elongation	72	20	30
Final elongation	72	300	1

Examination of the PCR products was done by agarose gel electrophoresis. The Sanger sequencing was performed as explained in Section 2.3.1, where the purified products were mixed with the PCR primer before sending, as required by Eurofins Genomics.

2.4 Rearing of Atlantic salmon in lab-scale RAS

During the autumn of 2022, Atlantic salmon fry from Lerøy Belsvik was reared in two identical lab-scale RAS established at NTNU SeaLab at Brattørkaia, Trondheim. The two RAS operated in the experiment in this study, are hereafter denoted RAS C (control) and RAS D (disinfected), where RAS C was running with non-disinfected biofilm carriers in the biofilter, and RAS D was running with partly disinfected biofilm carriers in the biofilter. Additionally, the impact of the biofilter community in seeding the rearing water and salmon microbiome was studied, to investigate how the microbiomes in different compartments of a RAS interrelate. This second objective was investigated by two other members of the ACMS group in two separate lab-scale RAS operated at the same time and is not addressed here. The two experiments were a collaboration between the Ph.D.-candidates Fernando Fernando and Sujan Khadka, master´s student Anna Aasen, and myself. The four lab-scale RAS were operated in parallel as two pairs for the two projects with their own objectives. However, the operation of the four RAS and the sampling events were shared, and all participants engaged equally.

2.4.1 Experimental design

Due to their small size, these RAS are suitable for experimental purposes, since a small research group can operate them. A timeline describing the planned workflow for the experiment is given in Figure 2.2, where sampling of the microbial communities of the biofilter carriers, salmon, and rearing water was planned for day 0 (baseline), day 7, day 14, and day 30. According to the experimental design, the planned duration was 30 days, with 4 sampling time points. On day 8, a power outage following the shutdown of the leading electricity to the room where the lab-scale RAS were situated, led to the death of all the fish, ending the experiment 22 days earlier than planned.

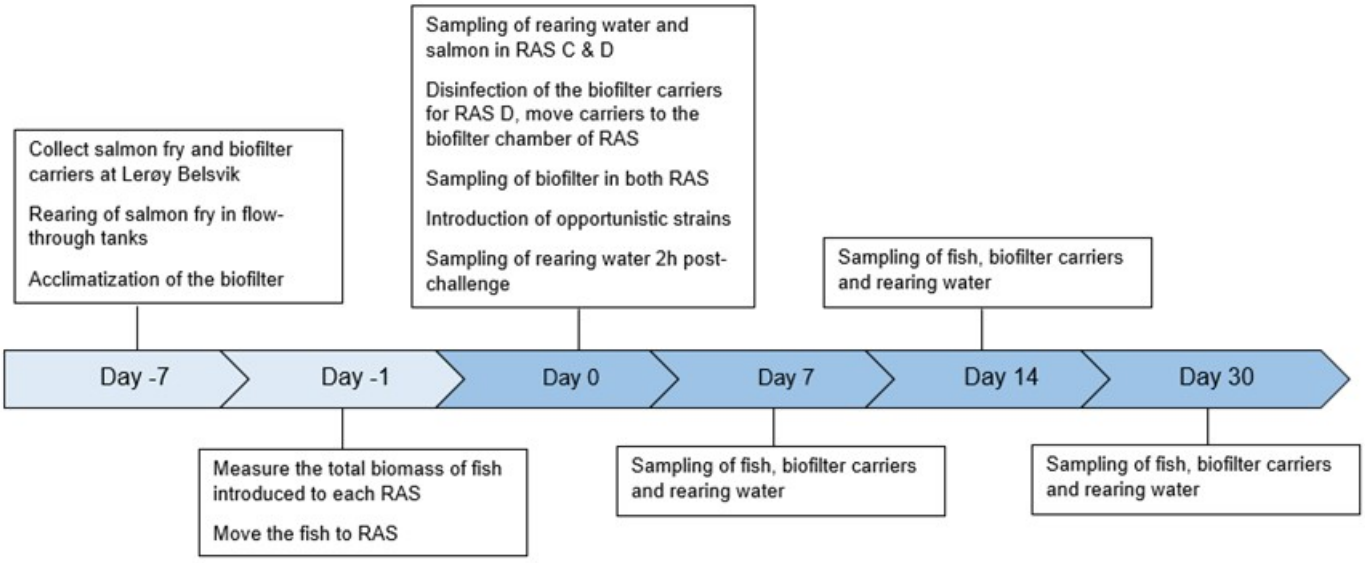

Figure 2.2: Planned timeline for rearing Atlantic salmon fry in lab-scale RAS. Four sampling time points (day 0, day 7, day 14 and day 30) were planned.

Figure 2.3 illustrates the design of the lab-scale RAS, manufactured by Spranger. The RAS, with a total volume of 165 L, included three rearing tanks with an individual volume of 35 L (Table 2.5). The water treatment loop consisted of mechanical filtration, biological filtration in an MBBR, a protein skimmer and a degasser. Outlet and aeration pipes, in addition to automatic feeders and tank lids of the rearing tanks not shown. The UV lamp was not in operation.

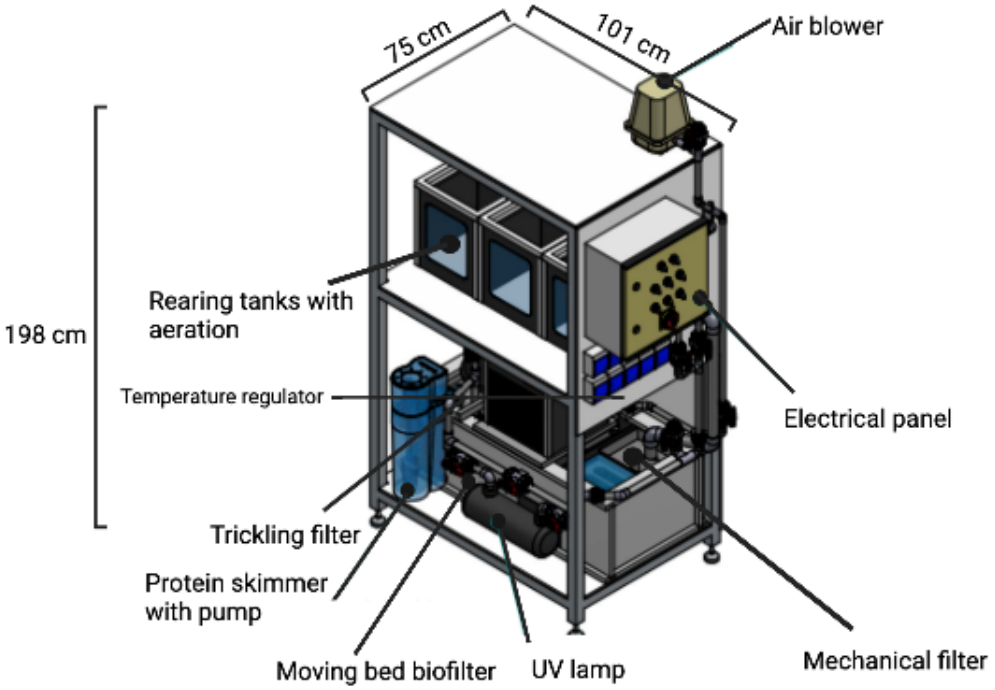

Figure 2.3: Illustration of the lab-scale RAS, adapted from Spranger. The rearing tanks were modified before the experiment, and the tank lids and automatic feeders are not pictured.

The direction of water flow and order of components in the RAS-loop is outlined in Figure 2.4. Flowing from each tank, the water was passed through the mechanical filter, before passing to biological filtration. The last treatment steps consisted of the protein skimmer removing larger particles, and the trickling filter, functioning as a de-gasser removing carbon dioxide. Before entering the trickling filter, the water passed the temperature regulator to ensure a temperature between 11 and 12 °C. The main pump, located in the sump upstream of the trickling filter, passed the treated water back to the rearing tanks. The flow rate was controlled by flow meters situated behind the tanks. Aeration was supplied to the biofilter to ensure continuous movement of the carriers, and additionally to the rearing tanks.

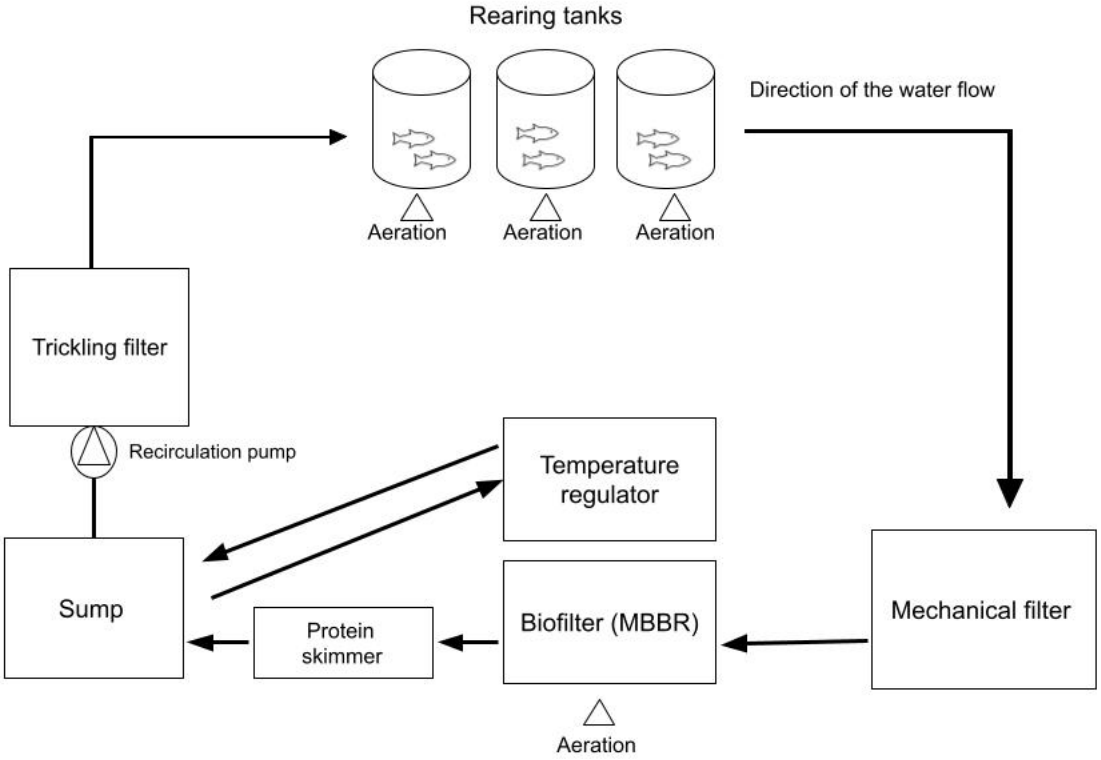

Figure 2.4: Schematic diagram of the lab-scale RAS for the rearing of Atlantic salmon at NTNU SeaLab showing the components and the direction of the water flow. The water from the three rearing tanks passed the mechanical filter to the MBBR with biofilm carriers, further to the protein skimmer. The trickling filter removed carbon dioxide, and the water was passed through the temperature regulator, before the recirculation pump passed the water back to the rearing tanks.

Properties of the RAS are shown in Table 2.5. The recirculation degree was planned to be 95%, with a planned exchange of 5% (8 L) of the total volume (165 L) per day. The biofilter chamber of 45 L was filled with 17.5 L biofilm carriers (RK BioElements), resulting in a biofilter filling degree of 38 % in both RAS. 35 fish were stocked in each tank.

Table 2.5: Properties of the lab-scale RAS used for the fish experiment performed at NTNU SeaLab.

Component	**Explanation**
Water volume of the lab-scale RAS	165 L
Recirculation degree	95 %
Rearing tanks	3 tanks with an individual water volume of 35 L
Number of fish per tank	35
Volume of biofilter chamber	45 L
Biofilter carrier type	RK BioElements
Biofilter surface area	750 m^2/m^3
Volume of biofilter carriers	17.5 L
Biofilter filling degree	38 %

Introduction of four opportunistic bacterial candidates to the lab-scale RAS
The cultivation and quantification of the four strains introduced to the lab-scale RAS were performed by Ph.D.-candidate Sujan Khadka, and is briefly described in this section. Four bacterial isolates from a strain collection previously established in the ACMS group were added to both RAS: *Flavobacterium sp., Proteus sp., Pseudomonas sp.* and *Psychrobacter sp.*, previously examined in the experiment described in Section 2.3. These candidates were used because they represent heterotrophic bacteria, assumingly rapid-growing and potentially opportunistic, chosen based on their taxonomy and earlier information about the characteristics of the genera. Due to the regulations of animal welfare, disease-causing pathogens could not be applied in this experiment for biosecurity reasons. The four opportunistic strains were not known as fish pathogens (personal communication, Ingrid Bakke, April 2023).

To ensure the introduction of a quantified number of cells from each the four strains, the Optical Density versus cell number was determined using flow cytometry. To ensure the opportunist's possibility to establish, the number of cells introduced was set to a total of $5*10^5$ cells per mL of the four strains combined.

The workflow is illustrated in Appendix C.2. In brief, frozen glycerol stocks of each candidate were plated individually to lysogeny broth (LB) agar, and incubated at room temperature for 24-48 hours. Single colonies were picked and incubated in liquid LB medium at 12° C for 24 hours. The optical density (OD_{600}) of each bacteria culture was measured, and the cell number versus optical density relationship was used to determine the amount of each culture to introduce. The culture was centrifuged (6000 rpm, 10 minutes), and the pellets were suspended in sterile phosphate-buffered saline (PBS) media. The cell suspension was divided into two sterile bottles before introduction to the two RAS on the first day of the experiment.

Acclimatization and disinfection of the biofilter before the experiment in RAS
Prior to the experiment, the biofilm carriers (around 30 L) were acclimatized in a batch reactor (100 L). The reactor was supplied with sufficient aeration to ensure the movement of the carriers, and the dissolved oxygen concentration was measured regularly. The reactor with biofilm carriers was fed 80 mL ammonia medium with a concentration of 2500 mg/L TAN according to the composition in Appendix A, Table A1, to obtain a final concentration of 10 mg/L TAN in the reactor. Every second day, the TAN, nitrite, and nitrate concentrations were monitored using the API Freshwater Test Kit (Appendix B.5). As ammonia and nitrite concentrations dropped, the biofilter carriers were supplied with more ammonia medium (80 mL, approximately every other day). The pH in the batch reactor was measured every second day (optimal range 7-8). Fluctuations in pH in the batch reactor were regulated by adjusting the pH in the ammonia stock solution.

2.4.2 Fish husbandry in the lab-scale RAS

The permission for the experiment was granted with FOTS ID 29715. The use of living animals in science is strictly regulated by the law to ensure good animal welfare, enforced by the Norwegian Food Safety Authority (Mattilsynet). The three R's in animal

research were the main ethical framework followed during the experiment: Replacement, Reduction, and Refinement. Good fish welfare was secured by establishing daily routines for feeding, cleaning, water exchange, and measurements of important water quality parameters. All daily measurements and observations were noted on a separate sheet, as shown in Appendix B.4.

Since the lab-scale RAS were new, and it was the first time they were used at NTNU, there was a need for extra time for remodelling and adjustments. The salmon fry was therefore reared in flow-through tanks for 54 days before the experiment's start in RAS ("acclimatization period"), instead of 7 days as outlined in the experimental design (Section 2.2.1).

Tank environment and observation of fish

The water current in the fish tanks was adjusted to the swimming of around 0.5 body lengths per second. The photoperiod was set to 24 hours of light, corresponding to the photoperiod of the RAS at the Lerøy Belsvik facility. Daily monitoring of the fish's behaviour and feeding habits was performed to ensure good welfare both in the acclimatization period and the rearing in RAS. This included recording the appetite (eagerness in the feeding situation), and the absence of obvious stress. Obvious stress was seen as extensive jumping and swimming in arbitrary patterns. Normal feeding behaviour was assessed as the immediate response when adding a small amount of feed to the individual tanks. Normal gill movement was assessed as the rhythmic opening and closing of the gill bow during respiration.

Feeding regime

The fish were fed the same feed as in the facility they were obtained from, developed, and manufactured by Cargill (EWOS) (Cargill, n.d.). The feed was the same throughout the acclimatization period in flow-through tanks, and the experiment in RAS, but the feeding routines were different. During the acclimatization period, the salmon were hand-fed morning and afternoon, while automatic feeders provided continuous feeding (24 h) in each tank in the lab-scale RAS. The feeding was adjusted as the fish were growing, maintaining a feeding rate of approximately 1.5 % of the total biomass in each tank. The automatic feeders (Fish Mate F14) (n.d.) were filled and controlled every morning.

Water quality, particle removal, and water exchange

The mechanical filter performed particle removal, a mesh sock with 200 pore size that was exchanged and cleaned by hand morning and afternoon. Excess large particles in the mechanical filter compartment (2.3 or the fish tanks were removed through siphoning. Water quality parameters in the rearing tanks were closely monitored during the experiment to secure good fish welfare, including the pH, alkalinity, dissolved oxygen (DO), nitrogen species (TAN, nitrite and nitrate), temperature, conductivity and total dissolved solids (TDS). The parameters, reference range used for Atlantic salmon, and measuring frequency are given in Appendix B.3. Nitrogen species were measured with the API®Freshwater Test Kit (Appendix B.5), and dissolved oxygen and temperature were measured with the ProfiLine Oxi 3310 IDS DO Meter (Catalog number 2BD350). The recirculation rate in the lab-scale RAS was set to 95 %. In the case of ammonia, nitrite or nitrate spikes, more water was exchanged, and a control

measurement was performed within the same day. Adjustments of water exchange were applied equally to both RAS, to ensure similar conditions.

2.4.3 Sampling of biofilm carriers, rearing water, and fish for characterization of microbial communities in the lab-scale RAS

Due to the accident on day 9, samples of the microbial communities in the two lab-scale RAS were taken on day 0 and day 7 of the experiment (D8 post-challenge). Five fish per RAS were sampled at each sampling point, three from one tank and two from the other, where skin, gill, and intestine from the same fish were individual samples. On day 0 (D0) of the experiment, three samples of the rearing water microbiome (one from each fish tank) were taken in each lab-scale RAS before the introduction of the opportunistic strains (D0 pre-challenge). Three samples (one from each fish tank) were taken 2 hours after the addition of the cultured bacteria (D0 post-challenge). Six samples of rearing water per RAS (two per tank) were taken on day 7 (day 8 post-challenge; D8). Five biofilm carriers per RAS were sampled before the introduction of the opportunistic strains at day 0. On day 7 (D8 post-challenge), five carriers and five of the uncolonized carriers that were autoclaved before the experiment were sampled. 104 samples were taken for both RAS across the two sampling days.

All fish, biofilter, and rearing water samples were stored in Precellys® 24 tubes (Bertin Instruments), filled with glass beads (0.1 mm) upon homogenization. A sampling of the salmon intestine, gill, and skin microbiome was done by dissection of randomly selected individuals from each system tank. Unnecessary suffering was avoided by following Norwegian regulations (Akvakulturdriftsforskriften), stating that fish to be euthanized is to be unconscious before the procedure (Norwegian Food Safety Authority, 2008). The individuals were put under anaesthesia in a solution of tricaine mesylate, MS-222 (50 mg/L MS-222 buffered with 50 mg/L Na_2CO_3) for 15 minutes, before being euthanized in an overdose of the same substance (150 mg/L MS-222 buffered with 150 mg/L Na_2CO_3). The fish was rinsed briefly with MilliQ® water prior to dissection to remove planktonic bacteria from the skin surface. Weight and length were recorded for each individual, before skin and gill samples were dissected and stored in individual tubes. The intestine was removed, and hindgut contents were emptied into separate tubes for the sampling of the gut microbiome, assuming that this part of the intestine contained the largest portion of digested gut content, serving as the best representation of the gut microbiome.

Representing one microbiome sample of the biofilter biofilm, one biofilm carrier from the biofilter compartment was taken out and rinsed briefly with MilliQ® water to remove planktonic bacteria. The biofilm carrier was sampled by cutting it into smaller pieces, before some of the pieces were transferred to the same Precellys® tube, representing one sample for DNA extraction. The carriers that were previously autoclaved were sampled similarly on D8.

The sampling of rearing water was performed by filtering 100 mL of the tank water through a 0.2 µm filter (Merck Millipore). The filter was transferred to a Precellys tube, representing one sample. All samples were transported on dry ice and stored

at -80 °C upon analysis.

2.5 Extraction and amplicon library preparation for Illumina sequencing of variable regions in 16S rDNA

2.5.1 DNA extraction and PCR

DNA extraction was performed using the ZymoBIOMICS™ 96 MagBead DNA Kit (ZymoResearch) (Appendix B.6) on the KingFisher™ Flex Purification System (ThermoFisher™ Scientific). Lysis was performed by bead-beating (5500 rpm x 2 cycles x 30 seconds x 15 seconds intervals between cycles), with the Lysis Buffer (550 µL) using the Precellys® 24 (Bertin Instruments) cell homogenizer. DNA extraction was performed according to the manufacturer's protocol, adapted to the KingFisher™ platform. The eluate volume (step 15) was increased to 100 µL.

Microbial composition and diversity in the two RAS were characterized by Illumina sequencing of 16S rDNA amplicons, using the Illumina primers Ill341F_Kl and Ill805R (Table 2.6), targeting the v3-v4 regions of the gene. A polymerase chain reaction amplifying the ribosomal 16S DNA of the bacterial domain was the first step of the amplicon library preparation. The PCR mixture contained a final concentration of 1x Phusion Buffer HF (ThermoFisher™ Scientific), 0.3 mM of each primer (Sigma-Aldrich), 0.2 mM of each dNTP (ThermoFisher™ Scientific), and 0.02 units/µL of Phusion Hot Start DNA polymerase (ThermoFisher™ Scientific) (Appendix B.9). The PCR mixture (25 µL) and the template (1 µL) were added for each reaction.

Table 2.6: Nucleotide sequences of primers (Sigma-Aldrich) used in the amplification of variable regions in the 16S rDNA for Illumina sequencing. The Illumina adapters are marked in bold, and the variable target region of each primer in 16S rDNA is given.

Primer name	Nucleotide sequence	Target region in 16S rDNA
Ill341F_Kl	5'-**TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG NNNN** CCT ACG GGN GGC WGC AG-3'	v3
Ill805R	5'- **GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G NNNN** GAC TAC NVG GGT ATC TAA KCC-3'	v4

The cycling conditions are given in Table 2.7. Protocol modification was considered for low or absent PCR products [reported in Results]. The PCR products were inspected with agarose gel electrophoresis, observing bands corresponding to an expected length of 520 bp.

Table 2.7: Cycling conditions for amplification of the variable regions in 16S rDNA for Illumina sequencing.

Step	Temperature (°C)	Time (seconds)	Number of cycles
Denaturation	98	120	
Denaturation	98	15	
Annealing	55	20	35-38
Elongation	72	20	
Final elongation	72	300	

2.5.2 Amplicon library preparation

Purification and normalization of the PCR products were performed using the SequalPrep Normalization Plate kit (Invitrogen, Appendix B.7), following the manufacturer´s protocol. Indexing is the process where unique pairs of Illumina sequence indexes are added to each amplicon in a second PCR. The Illumina Nextera Kit Set D (N7xx—Nextera XT Index Kit v2, and S5xx—Nextera XT Index Kit v2) was used. The PCR mixture contained a final concentration of 1X Phusion buffer HF (ThermoFisher™Scientific), 0.25 mM dNTP (ThermoFisher Scientific) and 0.015 units µL Phusion Hot Start DNA Polymerase (ThermoFisher™Scientific) (Appendix B.10). The mixture (17.5 µL per reaction) was transferred to a 96-well plate, before adding 2.5 µL of each unique index and 2.5 µL of the normalized PCR product. The PCR cycling conditions are given in Table 2.8. The indexed amplicons were investigated with

Table 2.8: Cycling conditions for the indexing PCR in amplicon library preparation. Unique pairs of indexing adapters (Illumina N7xx—Nextera XT Index Kit v2, and S5xx—Nextera XT Index Kit v2) were added to each normalized PCR product.

Step	Temperature (°C)	Time (seconds)	Number of cycles
Denaturation	98	120	
Denaturation	98	15	
Annealing	55	20	15
Elongation	72	20	
Final elongation	72	300	

agarose gel electrophoresis, observing bands corresponding to the expected length of 620 bp. The indexed PCR products were purified with the SequalPrep Normalization Plate kit (Invitrogen, Appendix B.7), following the manufacturer´s protocol.

Each indexed and normalized sample (20 µL) was pooled in a tube and concentrated using the AmiconUltra 0.5 centrifugal filter devices (30K membrane, Merck Millipore, User Guide given in Appendix B.8). The final pooled and concentrated sample was examined with agarose gel electrophoresis, observing a band corresponding to the expected length of 620 bp. Additionally, the DNA concentration and quality of the final sample were measured with a NanoDrop spectrophotometer (ThermoFisher™ Scientific) before sequencing, ensuring a DNA concentration above 10 ng/µL.

The pooled sample was sent for sequencing on the Illumina MiSeq platform at the Norwegian Sequencing Center. The total number of samples taken was 104, but

problems with the amplification of the microbial 16S rDNA in some samples limited the amplicon library sent to NSC to 60 samples.

2.6 Processing and statistical analysis of the sequencing data

2.6.1 Processing

The sequencing reads were processed using USEARCH (v.11; Edgar, 2010) following the pipeline recommended by the author. In brief, the pair reads were merged, and the primer binding sequences were stripped. Then, the merged reads were quality filtered and dereplicated to obtain unique sequences. Denoising, i.e., predicting the true biological sequence (ASVs) from these unique sequences, was performed. The chimeric and singletons (sequences that appear below 8 reads in all samples) were removed in the denoising process. Merged reads were mapped to ASVs to obtain the read counts for each sample. The taxonomic classification for the obtained ASVs was predicted using the SINTAX classifier (Edgar, 2016) with a bootstrap cutoff of 80 % and the RDP training set v18 (21 000 sequences) as the reference database. The ASVs representing non-bacterial taxa, e.g., chloroplasts, eukaryotes, and taxa of known kit contaminants, were removed. The ASV counts were normalized to 20 815 reads per sample. The ASVs of the four bacteria strains introduced to the RAS were identified by aligning the sequences obtained from Illumina sequencing with sequences obtained from Sanger sequencing of pure bacteria colonies. Only perfectly matched sequences, i.e., 100 % nucleotide similarity, were considered the true bacteria strains introduced to the RAS.

2.6.2 Statistical analyses

PAST (v4.12b; Hammer, Harper and Ryan, 2001) was used for the analysis of alpha- and beta-diversity in the sampled microbial communities of the two RAS. The alpha diversity indices for each sample (Observed ASV richness, Chao1, Shannon's diversity and Inverse Simpson) were retrieved with the 'Diversity indices' function, where the summary tables were imported to Excel for further analysis. The beta diversity (differences between sample groups) was investigated using the Principle Coordinates Ordination (PCoA) in PAST, based on the Bray-Curtis and Dice-Sørensen similarities. The Bray-Curtis index is a quantitative measure that takes both the presence-absence and the abundance into account. Conversely, the Dice-Sørensen index is a binary measure where abundance is abandoned, and the presence-absence of each ASV in each sample is the only parameter accounting for the differences. The PCoA is based on a three-dimensional distance matrix, where each sample is ordinated relative to its similarity to the other samples (Chao et al., 2006). One-way PERMANOVA (permutational analysis of variance) based on the Bray-Curtis similarities was used to determine if there were significant differences between pairs of sample groups, with a significance threshold of $p < 0.05$. The DESeq2 package (Love et al., 2014) using the R software (R Core Team, 2021) was used to identify the ASVs contributing most to the differences between the sample groups. Two-sample Student's T-test in Excel

was used to test if there were significant differences between pairs of sample groups, assuming equal variance. The significance threshold was set to $p < 0.05$.

3 Results

This section presents the following results: 1) the nitrification capacity of control versus disinfected biofilm carriers; 2) the colonization ability of four bacterial strains on the biofilm carriers; 3) the optimization of protocols for generating 16S rDNA PCR products before Illumina meta-barcoding; and 4) the fish experiment in lab-scale RAS and bacterial community analysis to investigate the role of the mature biofilm carrier community during the invasion from opportunistic bacterial strains.

3.1 Nitrification capacity and development of a disinfection protocol for the biofilm carriers from Lerøy

3.1.1 Nitrification capacity in the biofilm carriers from Lerøy

Quantifying the ammonia- and nitrite-oxidation capacity in the nitrifying biofilm carriers from Lerøy was necessary to aid the dimensioning of the biofilter for the fish experiment. Two separate experiments were therefore performed in lab-scale moving bed bioreactors (MBBRs). In one reactor, the biofilter carriers were submerged in a medium spiked with ammonia (10 mg/L), while the other reactor contained a media spiked with nitrite (5 mg/L). The concentrations of the nitrogen species were measured over time using the Hach-Lange™ method, and a linear regression analysis was performed (Figure 3.1).

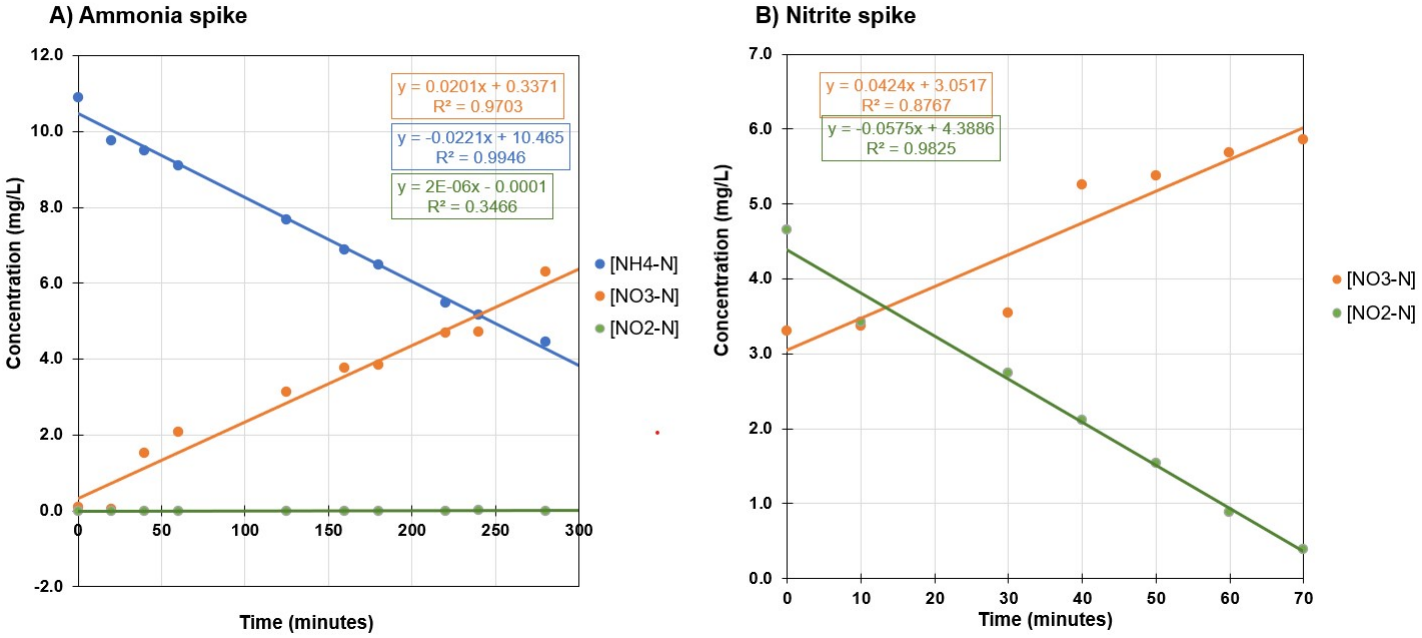

Figure 3.1: Linear regression models for TAN, NO2-N and NO3-N concentrations over time in two lab-scale batch reactors with biofilm carriers, spiked with A) 10 mg/L TAN, and B) 5 mg/L NO2-N. Concentrations were determined using spectrophotometry with the Hach Lange™method.

Subsequently, the surface-specific TAN removal rate (SSR) for the biofilter biofilm from Lerøy Belsvik is given by Equation 1, using the slope from the model in Figure 3.1A:

$$SSR_{TAN} = \frac{0.0221\ (\frac{mg-N}{L*min}) * 0.65\ L}{750\ (\frac{m^2}{m^3}) * \frac{56}{255000}\ (\frac{m^2}{m^3})} = 0.126\ g*m^{-2}*d^{-1} \qquad \text{(Eq. 1)}$$

The surface-specific nitrite removal rate for the biofilter biofilm from Lerøy is given by the same equation, using the slope from the model in Figure 3.1B:

$$SSR_{NO2-N} = \frac{0.0575\ (\frac{mg-N}{L*min}) * 0.65\ L}{750\ (\frac{m^2}{m^3}) * \frac{89}{255000}\ (\frac{m^2}{m^3})} = 0.206\ g*m^{-2}*d^{-1} \qquad \text{(Eq. 1)}$$

The surface-specific TAN removal rate of the biofilm carriers from Lerøy was 0.0399 g/m2*d, and the NO2-N removal rate was 0.143 g/m2*d.

3.1.2 Development of a disinfection protocol

Further, a protocol for disinfection of the biofilm carriers was developed for use in the fish experiment. An 80 % decrease in nitrification capacity was used as an indication of the loss of 80 % of the bacterial biomass. The decline in nitrification capacity in biofilm after disinfection at different concentrations was investigated using lab-scale MBBRs, as described in detail Section 2.2. Four concentrations of hydrogen peroxide (0 mg/L (control), 1200 mg/L, 600 mg/L, and 300 mg/L) were tested. Regression models assessing the decline in TAN in reactors containing carriers treated with different concentrations of hydrogen peroxide are shown in Figure 3.2.

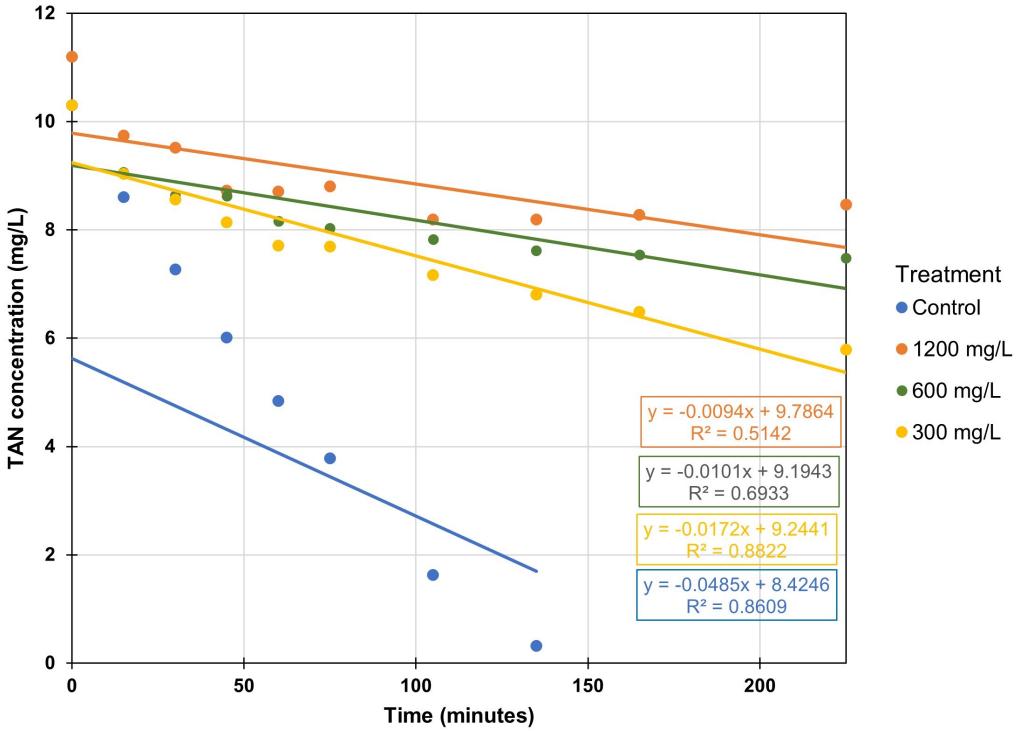

Figure 3.2: Linear regression models for the decrease in TAN over time in four lab-scale MBBRs with biofilm carriers subjected to different degrees of disinfection with hydrogen peroxide; control (0 mg/L), 300 mg/L, 600 mg/L and 1200 mg/L. The TAN concentration was determined using spectophotometry with the Hach-Lange™method.

With a decline in nitrification capacity to 21% of the control, the chosen strategy for disinfection was the incubation of the biofilm carriers in a solution of 600 mg/L hydrogen peroxide for 3 hours, as determined by the use of the regression models in Figure 3.2:

$$\text{Nitrification capacity} = \frac{\text{slope 600 mg/L}}{\text{slope control}} = \frac{0.0101}{0.0485} \approx 21\%$$

3.2 Colonization ability of four opportunistic bacteria candidates on the biofilm carriers from Lerøy

Colony PCR was followed by Sanger sequencing of the 16S rDNA gene of four bacterial isolates obtained from a strain collection maintained by the ACMS group. It was performed to confirm their taxonomy before use in a later fish experiment, hypothesized to represent opportunistic genera based on earlier research of bacterial communities in RAS. Prior to colony PCR, the morphological characteristics of each isolate were examined. TSA plates showing growth of the strains, representing isolates of *Proteus* sp., *Psychrobacter* sp., *Flavobacterium* sp., and *Pseudomonas* sp. are shown in Figure 3.3.

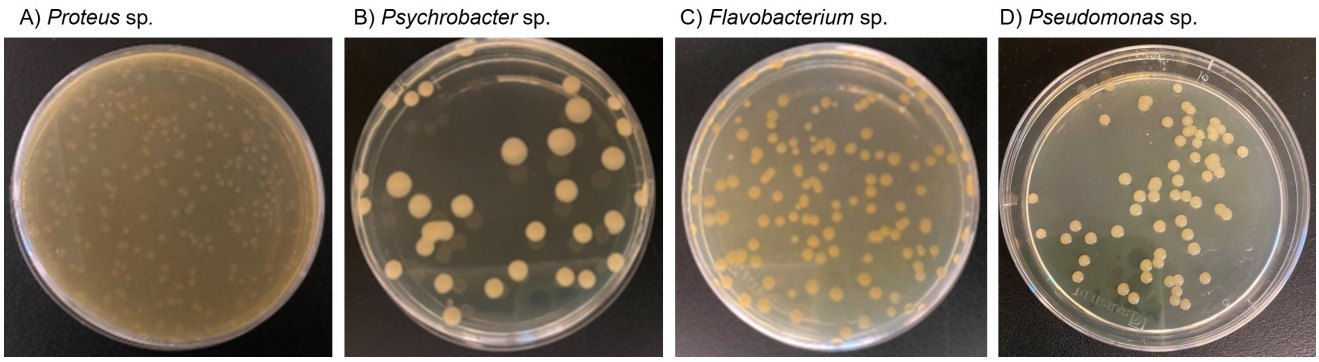

Figure 3.3: Tryptic soy agar (TSA) plates with a pure culture of four opportunistic strains, after incubation at 12°C for 2-5 days; A) *Proteus* sp., B) *Psychrobacter* sp., C) *Flavobacterium* sp, and D) *Pseudomonas* sp. Photos: Sujan Khadka.

PCR for amplification of the 16S rDNA gene from distinct colonies from each pure culture was followed by Sanger sequencing (Eurofins Genomics), and the obtained sequences are given in Appendix D.2.

An experiment was performed to determine the colonization potential on biofilm carriers of each of the presumably opportunistic strains to investigate their capability of growing in biofilm on the biofilter carriers later used in the fish experiment. As described in the Methods, incubation of the carriers in flasks with a known cell density of each isolate over 8 days, was followed by the determination of the colonization ability through CFU analysis. Table 3.1 reports the number of CFUs per mL determined after counting the CFUs on each plate (Appendix D1), as a measure of colonization success. Generally, the initial cell density ('Low' or 'High' treatment, as described in Methods) had little impact on the CFU/mL obtained from the carriers after incubation. The bacterial isolate of *Pseudomonas* had the highest growth on the carriers with the lower initial cell density. In comparison, *Psychrobacter* had the highest growth on the carriers with the higher initial cell density in the flasks. Sanger sequencing of 16S rDNA from single colonies in each treatment showed that the four strains had identical sequences to the first colony PCR, reported in Appendix D.2.

Table 3.1: Colony forming units (CFU) per mL of the four bacterial isolates recovered from the biofilm carriers after the colonization experiment, given for each treatment. The colonization success was determined by dividing the number of CFUs obtained from each carrier after 24-48 hours of incubation on TSA plates, with the initial cell density as determined by the CFU analysis.

Bacterial isolate	**Treatment**	**CFU/mL**
Proteus sp.	Low 1	43*10^4
Psychrobacter sp.	Low 2	58*10^3
Flavobacterium sp.	Low 3	12*10^3
Pseudomonas sp.	Low 4	20*10^4
Proteus sp.	High 1	25*10^4
Psychrobacter sp.	High 2	40*10^4
Flavobacterium sp.	High 3	49*10^3
Pseudomonas sp.	High 4	21*10^3

3.3 Optimization of the PCR protocol for amplification of 16S rDNA in fish samples

For the DNA extracts from the biofilm carriers and rearing water, the amplification of 16S rDNA before amplicon library preparation was mostly successful- Earlier research conducted in the ACMS group has proven the difficulties in obtaining 16S rDNA amplicons from fish samples, hereunder from the intestine, gills, and skin. The proportion of host DNA to bacterial DNA, in addition to the presence of PCR inhibitors in the DNA extracts, are assumed to be possible reasons. Therefore, participants in the research group have conducted experiments to test the efficiency of different DNA extraction kits for these sample types, leading to the choice of the kit used in the experiment in this thesis. Despite incorporating results from the earlier work conducted in the group, there were problems with the amplification of bacterial DNA from the fish samples collected in the fish experiment in lab-scale RAS. Therefore, further optimization of the PCR protocol was attempted, including

1. Testing dilutions of the DNA extracts used as templates
2. Testing of annealing temperatures through a gradient PCR
3. Testing a gradual increase in the number of PCR cycles (35-38X)
4. Testing dilutions of the primer concentration and increasing the magnesium chloride concentration.

Prior work performed in the group had shown that dilution of the DNA extracts obtained from the skin, gill, and intestine samples increases the yield of the expected product. Dilutions to 1:10 and 1:100 of the initial template concentration were tested, following the standard PCR protocol (Table 2.7). The PCR amplification was not successful for all samples [results not shown]. Investigation of the impact of annealing temperature on the primer specificity was therefore performed as a gradient PCR (Figure 3.4). Expecting more specific amplification with increasing annealing temperature due to higher stringency, the results were unclear. Unspecific amplification was observed to a greater extent with the lowest annealing temperatures (Figure 3.4A and C). Unexpectedly, the formation of primer dimers was a problem with the highest annealing temperature (Figure 3.4B). The results should be interpreted carefully due to the contaminations of the non-template control ('NTC') (Figure 3.4A and 3.4D), making it difficult to know if the observed results represent the specific PCR products or DNA contamination. Diluting the DNA extracts obtained from salmon skin, intestine, and gut ('S', 'I' and 'G', respectively) to 1:10 and 1:100 of the initial concentration (undiluted, 'UD') had different effects depending on the annealing temperature. Generally, diluting the extracts had a positive effect on the yield. The 16S rDNA in the extract from the salmon intestine ('I') was generally easier amplified than from skin ('S') and gill ('G').

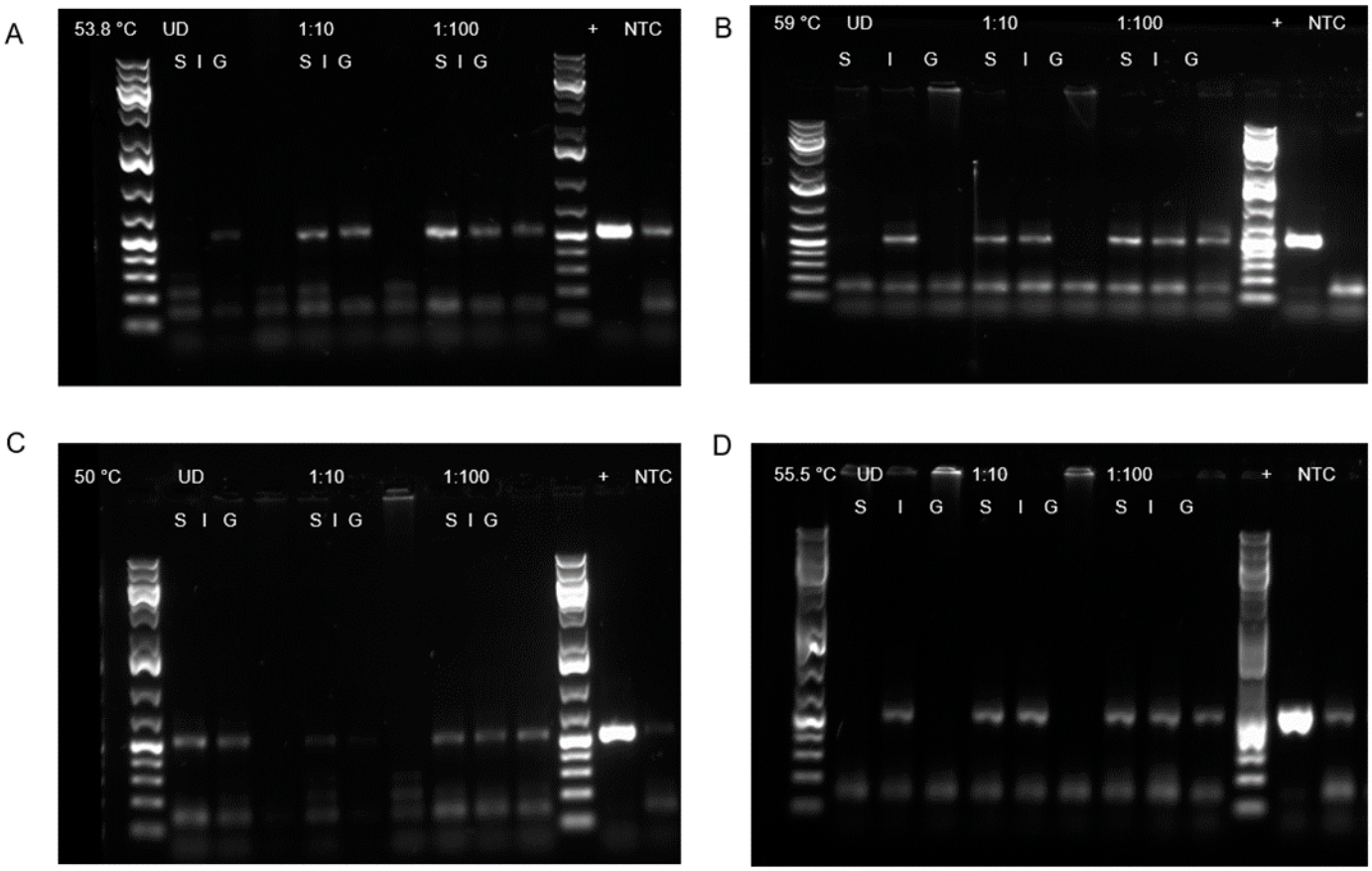

Figure 3.4: Agarose gels (1%) showing 16S rDNA v3-v4 region amplicons from the same 3 fish samples, testing a gradual increase of annealing temperature and dilutions of the extracts. The size of each amplicon was compared to a 1 kb Plus DNA ladder (Invitrogen). A) Annealing temperature of 53.8°C, B) Annealing temperature of 59 °C, C) Annealing temperature of 50.0°C, D) Annealing temperature of 55.5°C. UD=undiluted DNA extract, 1:10: DNA extract diluted to 1:10 of initial conc., 1:100: DNA extract diluted 1:100 of initial conc. S = skin, I = intestine, G = gill, NTC = non-template PCR control, + = positive PCR control.

The gradient PCR was repeated [results not shown]. Despite testing different annealing temperatures, primer concentrations, and additional magnesium chloride to enhance the polymerase efficiency, no clear conclusion could be made. The indication was that amplification of 16S rDNA for the skin and gill samples was unpredictable, while the undiluted DNA extracts from the salmon intestine had higher success. Increasing the number of PCR cycles from 35 to 38 increased the yield of the desired amplicons, but primer dimer formation became a more significant problem with this strategy [results not shown].

Due to the unambiguous results with unspecific and insufficient amplification, a two-step PCR was the final solution. Here, a first PCR with primers lacking the Illumina-adapter (341F_Kl and 805R) was followed by a PCR protocol with fewer cycles, where the Illumina tag was added. This strategy aimed to reduce the competition with the primer-dimer formation. The strategy was unsuccessful for the skin and gill samples [results not shown] while being partly successful for the intestine samples (undiluted

DNA extract, 38 cycles, 57°C annealing) (Figure 3.5). In the first round of PCR, the desired amplicons were obtained from 10 of 14 intestine samples, with varying amplification strengths.

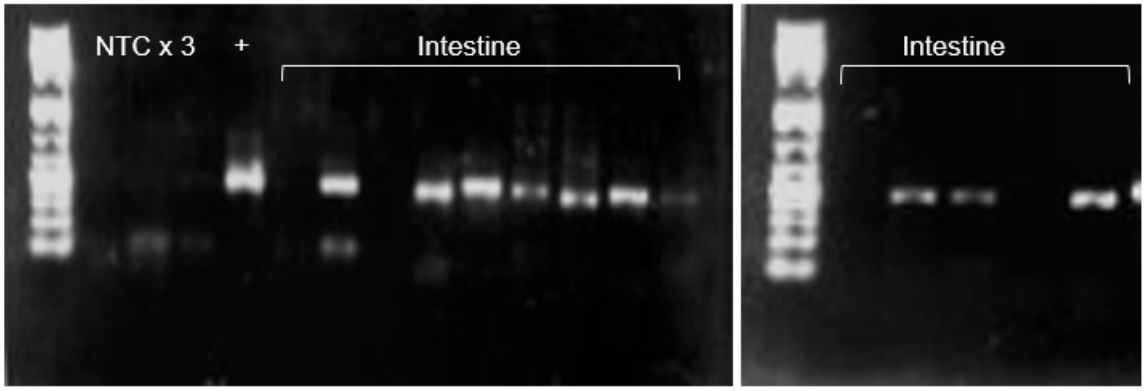

Figure 3.5: Agarose gel (1%) with 16S rDNA amplicons obtained from 14 salmon intestine samples with the 341F_Kl and 805R PCR primers (Sigma-Aldrich) targeting the v3-v4 regions of the gene. Samples are compared to a 1 kb Plus DNA ladder (Invitrogen). 'NTC' is a non-template PCR control, '+' is a positive PCR control.

A second PCR protocol with fewer cycles was used to add the Illumina adapter to the 10 PCR products resulting from the first PCR shown in Figure 3.5. First, these PCR products were normalized and purified using the SequalPrep Normalization Plate Kit before use in the second PCR protocol. Here, a similar PCR setup was followed, using the Ill341F_Kl and Ill805R primers and a protocol with fewer cycles (15X, 55°C annealing). This second PCR of 10 intestine samples to add the Illumina adapter was considered successful, with two samples having lower amplification observed as weaker bands on the agarose gel (Figure 3.6). After a second normalization of these samples, followed by the amplicon library preparation [results not shown], the final number of fish samples sent for Illumina sequencing was 10.

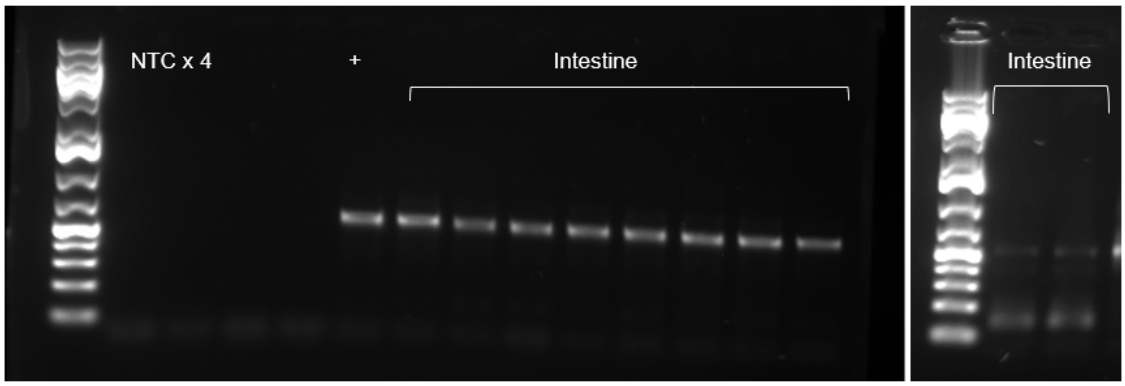

Figure 3.6: Agarose gel (1 %) with 10 salmon intestine 16S rDNA amplicons after short-protocol PCR (15 cycles) to add the Illumina adapter. DNA was extracted with the ZymoBIOMICS™ 96 MagBead DNA Kit (ZymoResearch) (Appendix B.6). The Ill341F_Kl and Ill805R primers (Sigma-Aldrich) were used, the annealing temperature was 55 °C with 15 cycles. 'NTC' represents a non-template control and '+' is a positive PCR control.

3.4 Rearing of Atlantic salmon in lab-scale RAS

The main experiment in the thesis was the rearing of Atlantic salmon fry in two identical lab-scale RAS, one having disinfected biofilm carriers (RAS D) and one with untreated carriers as a control (RAS C) during a challenge with four opportunistic bacterial strains. As described in the Methods, a power outage on day 8 ended the experiment 22 days too early; a short circuit in an electrical panel box of one of the lab-scale RAS triggered the fuse in the main circuit breaker (MCB) to shut down the electricity to the whole room. As a consequence, the water flow and oxygen supply to the rearing tanks, led to a fast decline in dissolved oxygen, being the cause of death within minutes.

3.4.1 Water quality

Table 3.2 summarizes the physicochemical water quality in RAS C and D during the experimental period, from day 0 to day 7 (D0; the day the opportunistic strains were introduced, or "the bacterial challenge", day 7 abbreviated as D8; day 8 post-challenge), shown as the range of the lowest and highest measurement of each parameter. Generally, there were variations in the water quality in both RAS. The total dissolved solids and conductivity increased steadily from D0-D8, indicating organic matter accumulation in both systems. The total dissolved solids increased more in RAS C than in RAS D, with a difference of 392 ppm to 153 ppm between the highest and lowest value, measured on day 0 and day 8. The temperature varied from 9.5-11.9 °C in RAS C, and 11.4-12.2 °C in RAS D, being more stable in RAS D.

Table 3.2: Physicochemical water quality during the experimental period (range) recorded in the two lab-scale RAS. All measurements were recorded two times daily (morning and afternoon, except the alkalinity and nitrogen species, measured every second day)

Parameter	RAS C	RAS D
Dissolved oxygen (mg/L)	5.9-9.2	5.8-9.5
pH	7.0-7.4	7.3-7.7
Temperature (°C)	9.5-11.9	11.4-12.2
Total dissolved solids (ppm)	170-562	179-323
Conductivity (mS/cm)	261-837	275-491
Alkalinity (mg $CaCO_3^-$/L)	54.5	54.5
TAN (mg/L)	0	0-0.5
NO_2^-(mg/L)	0	0
NO_3^-(mg/L)	0-10	0-5

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With pH ranging between 6.9 and 7.7. the pH in RAS C was continuously lower than in RAS D (Appendix D, Figure D3). The pH in both RAS decreased from day 0 to day 3. However, the decrease was more significant in RAS C. From day 3 to day 7. the pH increased in both RAS. Interestingly, the alkalinity was stable, neither varying with time nor type of RAS (Table 3.2).

In the case of additional water exchange, the same amount was exchanged in both RAS, to ensure similar conditions. The water exchange was generally higher than planned, due to unexpected events such as clogging of the mechanical filter. To obtain a recirculation degree of 95 %, the planned exchange was 8 L per day, making up 5 % of the total water volume in the RAS. The build-up of particles, causing clogging of the mechanical filter and subsequent water loss due to overflow in the mechanical filter sump, was a problem causing additional make-up water. On day 1, 38 L was added to RAS C after a leakage. A leakage in RAS C on day 7 led to the addition of 20 L of make-up water in C, to account for the loss of 12 L. The same amount was then exchanged in RAS D.

The concentrations of the nitrogen species (ammonia, nitrite, and nitrate) were generally stable during the experimental period. Ammonia spikes between 0.2 and 0.5 mg/L were recorded in RAS D at five sampling points, indicating incomplete ammonia oxidation while still being below the reference range of 0-2 mg/L tolerated by Atlantic salmon. No ammonia was detected in RAS C. The low concentrations of nitrogen species in both RAS can be linked to the low feeding rate due to the low biomass stocked in each tank, and the higher water exchange, where the maximum feeding per day was around 15 g feed per RAS.

3.4.2 Fish performance

The total biomass of fish was recorded for each RAS at the start (D0) and end (D9 post-challenge) of the experiment (Table 3.3). The total increase was 664 grams in RAS C and 593 grams in RAS D, corresponding to growth of 68.3 % and 60.3 %,

respectively, and the difference was insignificant (t-test, $p > 0.05$). The individual weight and length of individuals sampled for microbiome analysis were measured (averages are reported in Appendix D, Table D2). There were no mortalities during the experimental period (D0-D8).

Table 3.3: Average biomass per tank and total biomass for the three rearing tanks in the two lab-scale RAS, recorded at day 0 and day 9.

Day	RAS	Average biomass per tank (g)	Total biomass (g)
0	C (mature biofilter)	323	970
	D (disinfected biofilter)	328	983
9	C (mature biofilter)	545	1634
	D (disinfected biofilter)	525	1576

3.4.3 Microbial communities

Samples of biofilm carriers, rearing water and fish from both RAS, taken at day 0 and day 8 post-challenge, were subjected to microbial community analysis after Illumina sequencing of the 16S rDNA. Due to problems with amplifying the bacterial 16S rDNA in the fish gill and skin samples, these were not included in the analysis of microbial communities. The same problems limited the gut microbiome samples that were included to 10. In total, 60 samples from biofilm carriers, rearing water, and salmon hindgut were sent for Illumina sequencing of variable regions in the 16S rDNA gene for microbial community profiling.

Sequencing output and downstream processing

From the sequencing of 60 samples, 14.2 million pairs were obtained, i.e., the average sequencing depth was 236 000 pairs per sample. Of these 14.2 million pairs, only 9.1 million pairs were successfully merged, with the mean merged reads length being 463 bp. Most raw reads (4.6 million pairs) were lost because the merged reads were shorter than 360 bp, most likely the primer dimers from the PCR in the library preparation steps. Six samples were discarded due to having less than 20 815 reads (rarefaction threshold used), and only 54 samples were used in the downstream analysis. After data processing and manual inspection of the resulting ASV table as described in Section 2.6.1, 6 samples were removed due to possible DNA contaminations or a low number of reads. The further analyses are based on the ASV table normalized to 20 815 reads. In total, 3270 ASVs were included in the normalized ASV table.

Alpha diversity in biofilter biofilm, rearing water, and salmon hindgut

Alpha diversity metrics (observed ASV richness, Chao1, Exponential Shannon and Inverse Simpson) for the communities in biofilm carriers, rearing water, and salmon hindgut samples from the two lab-scale RAS varied with sampling day, but even more with sample type (Figure 3.7). Generally, the observed ASV richness was higher in the sampled biofilm carriers than in the rearing water and salmon hindgut (Figure 3.7A). The observed ASV richness in the rearing water increased after 8 days in both RAS but was more variable in water samples from RAS D (Figure 3.7A). The observed ASV

richness was considerably lower in the salmon hindgut samples than in the biofilm carrier and rearing water samples.

Comparing alpha diversity in the communities of biofilm carriers of RAS C and D
T-tests were performed to compare the sample groups to assess whether disinfection significantly impacted the alpha diversity of microbial communities on the biofilm carriers. At D0 pre-challenge, the observed ASV richness (Figure 3.7A) was not significantly different in biofilm carrier samples from C and D ($p > 0.05$). Nevertheless, the Exponential Shannon's diversity (Figure 3.7C) was significantly different with $p = 0.007$, indicating that differences in evenness may characterize the communities of the disinfected versus non-disinfected carriers. Interestingly, the comparison of the communities of the biofilm carrier sampled from the two RAS at D8, revealed that both the observed ASV richness (Figure 3.7A) and Exponential Shannon diversity (Figure 3.7C) differed significantly ($p = 0.003$ and $p = 0.03$, respectively).

Comparing alpha diversity in biofilm carriers with rearing water within the RAS
Similar tests were performed to assess whether the alpha diversity of the communities in the biofilm carriers was significantly different from the communities of the rearing water. At D8, both the observed ASV richness and the Exponential Shannon's diversity showed significant differences between the communities of biofilm carriers and water within RAS C and RAS D (Exponential Shannon; $p=0.008$ in C and $p=0.02$ in D, Observed ASV richness; $p=0.001$ in C and $p=0.002$ in D).

Temporal development of the alpha diversity within the RAS
It is also interesting to investigate the temporal effect of the bacterial challenge on the alpha diversity of disinfected and non-disinfected carrier samples, i.e. from D0 to D8 within RAS C and D. In the non-disinfected carriers of RAS C, neither the observed ASV richness nor the Exponential Shannon's diversity was significantly different from D0 pre-challenge to D8 post-challenge ($p > 0.05$). On the other hand, in the communities of the disinfected biofilm carriers of RAS D, a significant increase was observed for the observed ASV richness ($p = 0.04$) and Exponential Shannon's diversity ($p=0.00013$) from D0 pre-challenge to D0 post-challenge. A temporal decrease in alpha diversity was observed in rearing water samples within the same RAS taken at D0 (2h post-challenge) and D8. The observed ASV richness and the Exponential Shannon's diversity were significantly different from D0 to D8 in both RAS ($p=0.004$ and $p=0.0003$ in RAS C, $p=0.03$ and $p=0.004$ in RAS D, respectively).

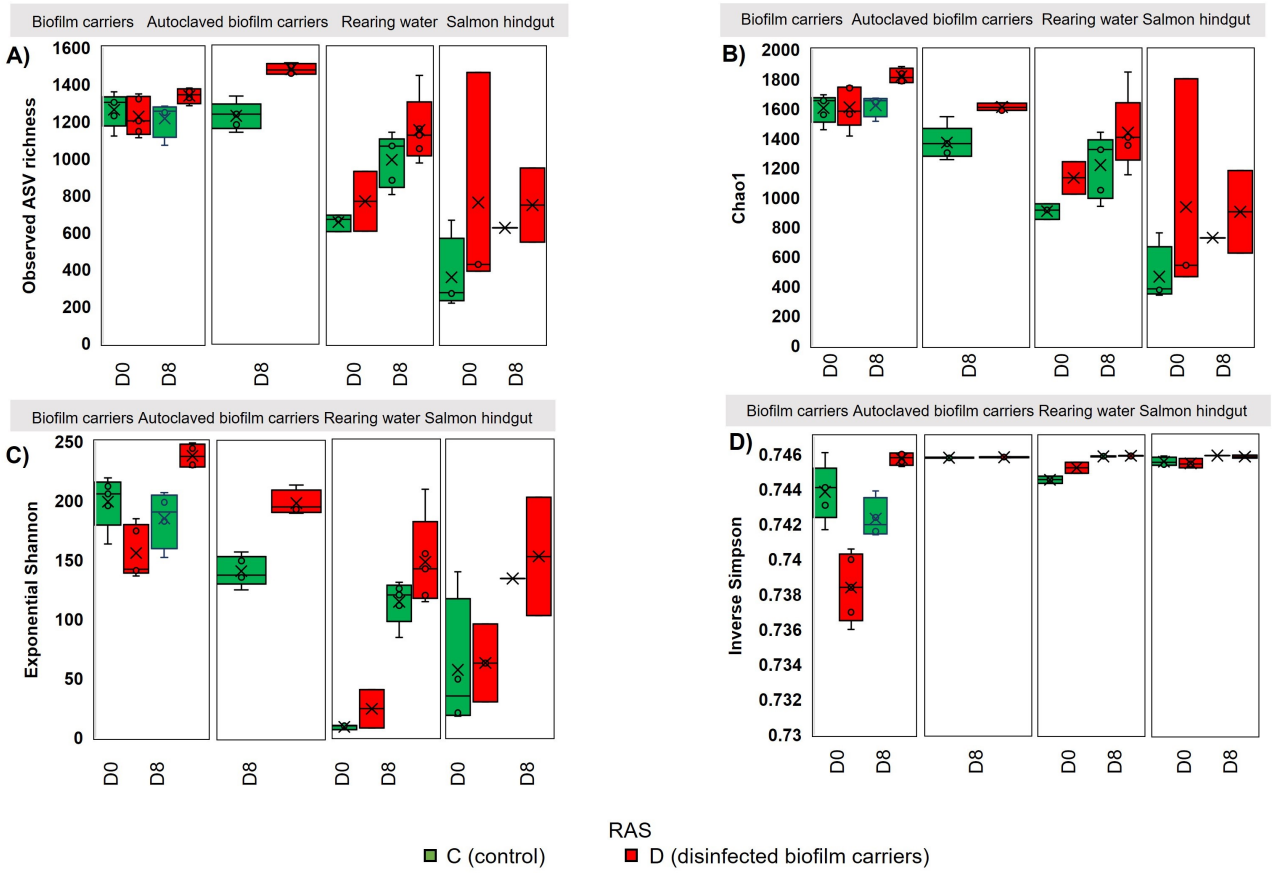

Figure 3.7: Alpha diversity indices for sampled microbial communities in the two RAS, where green = RAS C (control) and red is RAS D (disinfected biofilm carriers). The indices are shown for each sampling day; D0 pre-challenge for biofilm carriers and salmon gut, D0 2h-post-challenge for rearing water, and D8 post-challenge). A) Observed ASV richness; B) Chao1; C) Exponential Shannon; and D) Inverse Simpson.

Overview of the microbial community composition

A comparison of the observed ASV richness (Figure 3.7A) and the estimated ASV richness (Chao1) (Figure 3.7B) revealed an average sequencing coverage of 77 % across all samples. The microbial community composition at order level in biofilm carriers, rearing water, and salmon gut samples at D0 and D8 are presented in Figure 3.8). Pseudomonadales, Burkholderiales, and Flavobacteriales were the three most abundant orders in the microbial community profiles, after assessment of the average abundances across all samples, disregarding the unassigned ASVs. Pseudomonadales was the most abundant order in biofilm carrier and rearing water samples. Other orders dominating the microbial communities independent of sample type were Rhodobacterales, Sphingomonadales, and Chitinophagales. The overall community composition in the salmon hindgut appears different from the biofilm carrier and rearing water samples. The four orders with the highest average relative abundance were Clostridiales, Pseudomonadales, Bacilliales and Lactobacilliales (17.8, 12.0, 11.1 and 10.9 %, respectively). Interestingly, an ASV classified to the genus of *Janthinobacterium* had a high relative abundance in the gut samples, on average 3.7 %. This ASV was also present in some biofilm carrier and rearing water samples but in a lower relative abundance (0.3 % on average).

Overall, the community composition of the samples from the disinfected biofilm carriers of RAS D appeared similar to that of the non-disinfected carriers of RAS C before the introduction of the opportunistic strains on D0. Here, the most abundant orders in the samples of biofilm carriers, disregarding unassigned ASVs, were Nitrospirales, Sphingomonadales and Burkholderiales (on average 19.0, 15.4 and 4.2 %, respectively). Interestingly, a shift in composition was observed at D8. Here, the most abundant orders in the communities of the biofilm carriers in RAS D were Pseudomonadales, Flavobacteriales, and Nitrospirales (on average 12.2, 11.3 and 10.5 %, respectively). This was different to the communities of the biofilm carriers in RAS C, which still was dominated by Nitrospirales (on average 24.2 %) followed by Rhodobacterales and Sphingomonadales, which also dominated in the biofilm carriers of this RAS at day 0. The community composition in the rearing water samples appears different between RAS C and RAS D. However, the same three orders dominated the communities. Flavobacteriales was the most abundant order at D8 (average 12.1 % in C and 23.0 % in D), followed by Burkholderiales and Rhodobacteriales.

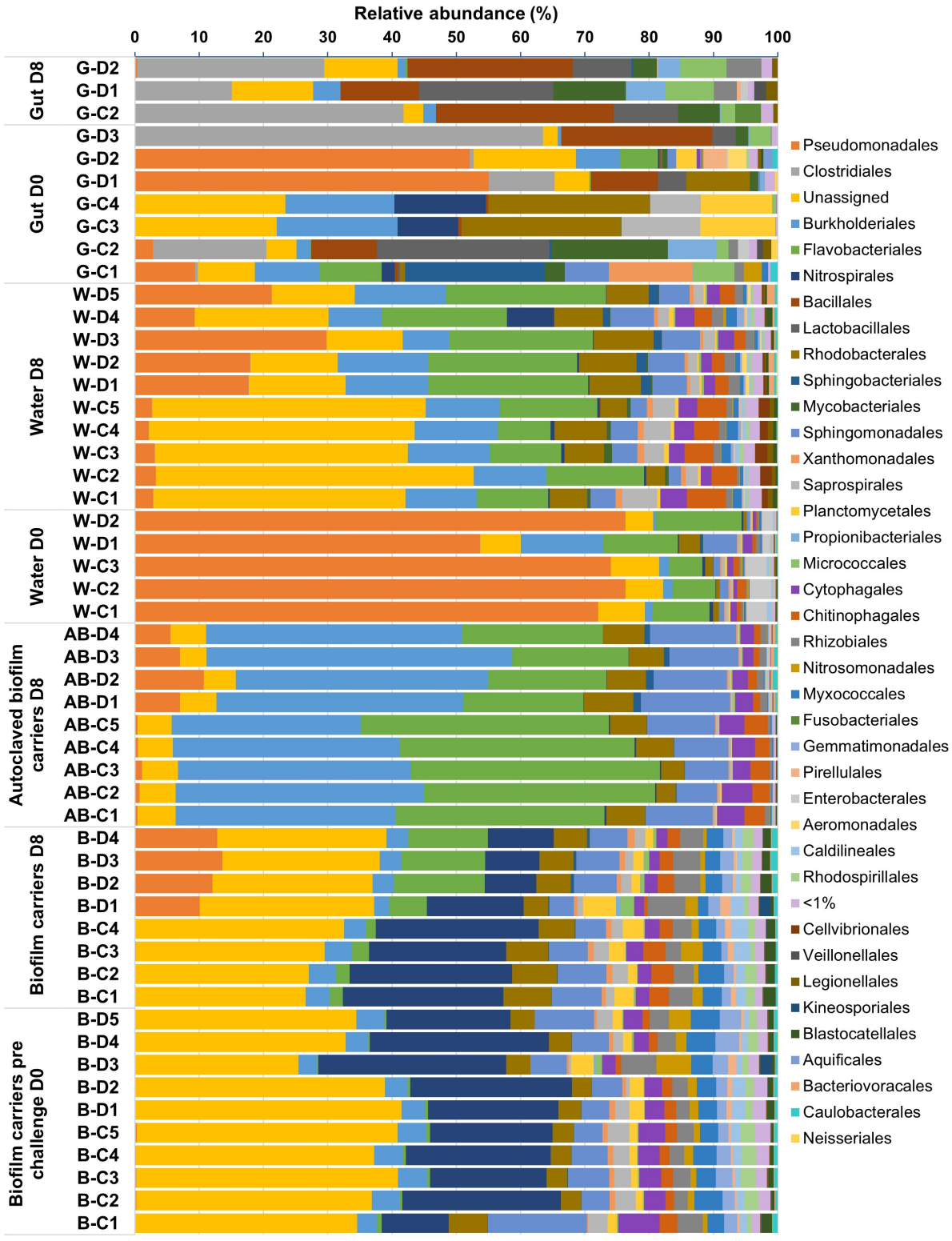

Figure 3.8: Community composition at the order level in rearing water, biofilm carriers, and fish gut samples before and after the challenge with four bacterial strains in RAS C (control) and D (disinfected biofilm carriers). B=biofilm carrier, W=rearing water, AB=autoclaved biofilm carrier. The samples of rearing water on D0 were taken 2 hours after the introduction of the opportunistic strains ('post-challenge), and biofilm carrier and gut samples were taken before the introduction ('pre-challenge). The ASVs are classified at the order level, whereas ASVs that could not be classified at the order level are displayed as 'Unassigned'. Orders with average abundance lower than 1 % in all samples are shown as '<1 %'.

Colonization success of the introduced bacterial strains

The relative abundance of the four ASVs identified as the introduced opportunistic strains in the biofilm carrier and rearing water samples were used when assessing their colonization success in the two RAS (Figure 3.9). The relative abundance of the opportunistic strains was higher in both the disinfected biofilter biofilm and autoclaved biofilter biofilm of RAS D at D8 compared to RAS C (t-test, p=1.04e-05 and p=0.0001, respectively). The total relative abundance in the samples from the biofilm carriers (average) was 6.25 % in RAS D and only 0.16 % in RAS C . No significant increase in the average relative abundance of the opportunistic strains was observed in RAS C from D0 to D8 (t-test, p>0.09 for all four strains). Interestingly, the proportion of opportunists was considerably higher also in the rearing water of RAS D than in C, where the total relative abundance of the introduced strains (average) on day 8 was 0.24 % in RAS C while being 9.5 % in RAS D. From an average relative abundance of 75.7 % in RAS C and 56.4 % in RAS D 2 h post-challenge, the decrease was significant. Notably, the sample size of rearing water at day 0 was small, and the variation between the samples from the same system was large, impacting the significance of this result. Nevertheless, the significantly higher relative abundance in RAS D indicates that the disinfection of the biofilm carriers led to a clear tendency of greater colonization success of the opportunistic strains.

The colonization success also varied with bacterial strain (Figure 3.9). *Proteus* (ASV51) had a low relative abundance among the four strains, and was close to absent in the biofilter biofilm and rearing water samples of RAS D at D8. *Psychrobacter* had a relative abundance above 20 % in all rearing water samples at D0 post-challenge, declining to around 3 % on day 8. Both *Flavobacterium* (ASV7) and *Pseudomonas* (ASV4) established more in the biofilter biofilm and rearing water communities in RAS D, where *Pseudomonas* had a relative abundance of 1.8 % in biofilm carrier samples and in rearing water, and *Flavobacterium* had an average relative abundance of 4.0 % in biofilm carriers and 5.9 % in rearing water.

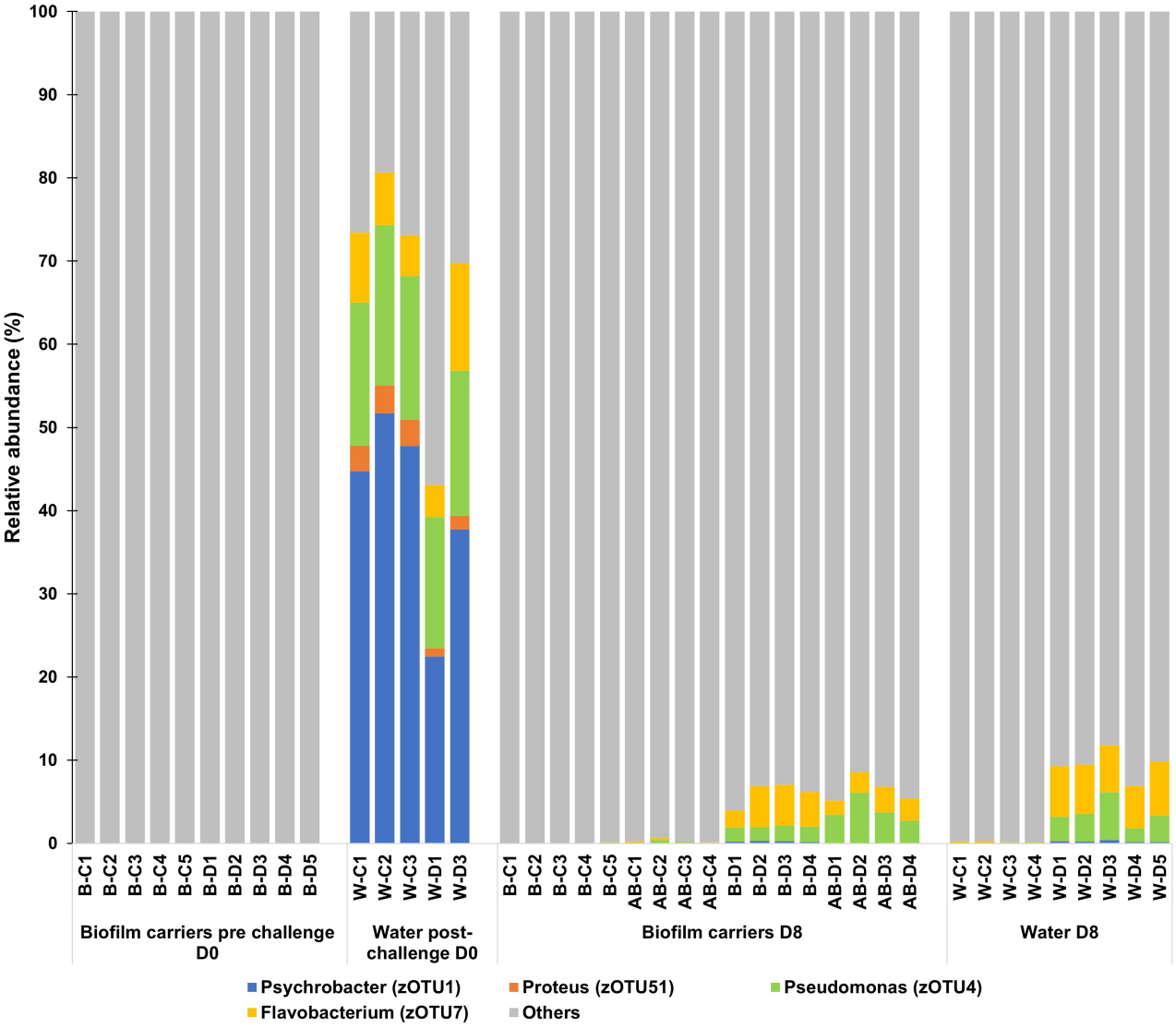

Figure 3.9: Relative abundance (%) of the ASVs corresponding to the four introduced strains *Psychrobacter, Pseudomonas, Proteus* and *Flavobacterium* in biofilter biofilm and rearing water samples. RAS; C (control) and D (disinfected biofilm carriers). Sample type; B=biofilm carrier, W=rearing water, AB=autoclaved biofilm carrier.

Beta diversity in the biofilm of the biofilter, rearing water and salmon hindgut

Differences in microbial communities between sample types

A Principle Coordinates Analysis (PCoA) was performed to investigate if the microbial community of biofilter biofilm, rearing water, and salmon hindgut were different (Figure 3.10). The PCoA-plots indicate that the biofilter biofilm, rearing water, and salmon hindgut samples have different microbial community compositions. However, there is some overlap between the samples of the same type (i.e. biofilter biofilm, rearing water, and salmon hindgut) between the systems. The samples of biofilm carriers and rearing water separate more along the first coordinate in the PCoA based on Bray-Curtis similarities (Figure 3.10A) than with the Dice-Sørensen coefficients (Figure 3.10B). This suggests that the biofilter biofilm and rearing water samples share ASVs, but that the relative abundances are different. As expected, the one-way PERMANOVA revealed significant differences between the microbial communities in biofilm carriers and rearing water samples. This was true for both RAS on both sampling days; for biofilm carriers and rearing water at DO ($p= 0.017$ in RAS C, $p=0.045$ in RAS D), and for biofilm carriers and rearing water at D8 ($p=0.006$ in RAS C, 0.0084 in RAS D). Interestingly, the salmon hindgut samples separate more from the other sample types in the PCoA based on the Dice-Sørensen coefficients (Figure 3.10B) along coordinate 1 than they do with Bray-Curtis similarities 3.10A). This indicates that the microbial communities in the gut samples share ASVs that are not present in the biofilm carrier and rearing water samples.

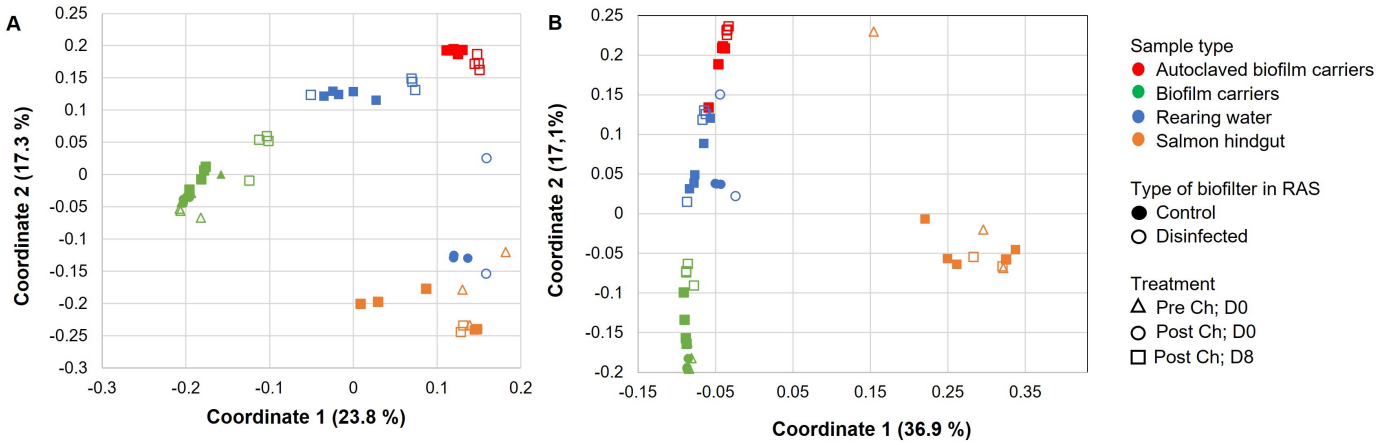

Figure 3.10: Principle coordinates analysis (PCoA) of microbial communities obtained by 16S rDNA sequencing from all samples analyzed.
A) PCOA based on Bray-Curtis similarities. B) PCoA based on the Dice-Sørensen coefficients.

Differences between RAS C and D

The microbial community composition appears different based on the biofilm carriers in the RAS (disinfected versus control). The PCoA based on Bray-Curtis similarities indicates that samples from the two RAS of the same type (biofilm carriers, rearing water and salmon hindgut) taken on the same day (D0 vs D8 post-challenge) have similar microbial communities (Figure 3.11). The rearing water samples at day 0, taken 2 hours after introducing the opportunistic strains, appear more similar than at D8 (Figure 3.11). As expected, the rearing water communities were not significantly

different (PERMANOVA, p=0.1) between RAS C and RAS D on D0 (2h post-challenge). Interestingly, the microbial communities in the rearing water were significantly different between RAS C and RAS D at D8 (p=0.0082). A one-way PERMANOVA revealed no significant differences between the gut samples from the two RAS (p >0.05 when comparing both systems and sampling days.

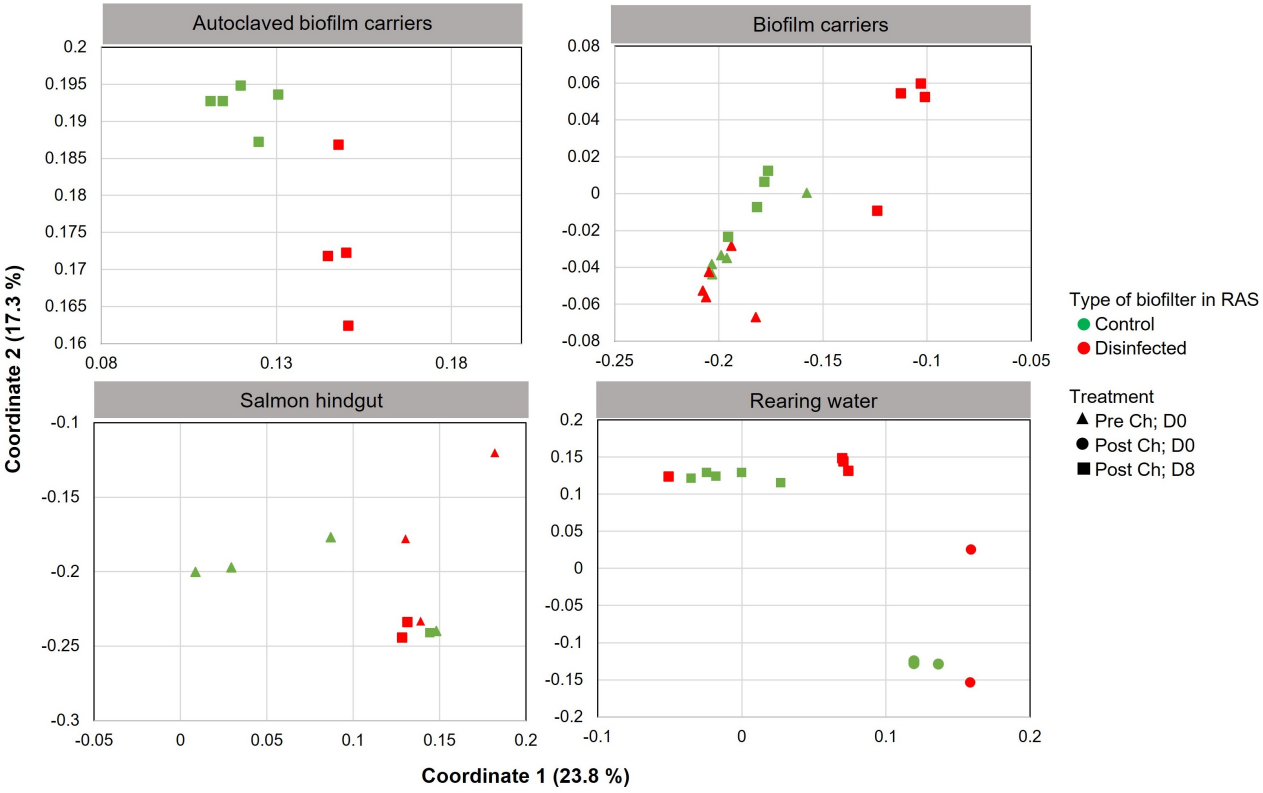

Figure 3.11: Principle coordinates analysis based on Bray-Curtis similarities, sorted on sample type (autoclaved biofilm carriers, biofilm carriers, salmon hindgut, and rearing water) and sampling timepoint in both RAS. Green = RAS C (control); red = RAS D (disinfected biofilm carriers).

Temporal developments within each RAS

The Bray Curtis dissimilarities (i.e. 1-Bray Curtis similarity) were used in the further assessment of the diversity in the microbial communities in the biofilm carriers over time (Figure 3.12). Overall, the samples of the biofilm carriers in RAS D were more dissimilar than the samples within RAS C on day 8 post-challenge. The comparison of the development of the communities within the two RAS over time (i.e. 'D0; Pre-Ch vs D8; Post-Ch'), shows significantly higher dissimilarity over time in RAS D than in C (t-test, p=6.4e-13). This suggests that the biofilter biofilm community of RAS D was more unstable over time (as observed on day 8 post-challenge). The average dissimilarity was not significantly different (t-test, p=0.28) in the two RAS at D8, indicating consistent sampling. However, the spread was more extensive in RAS D at D8.

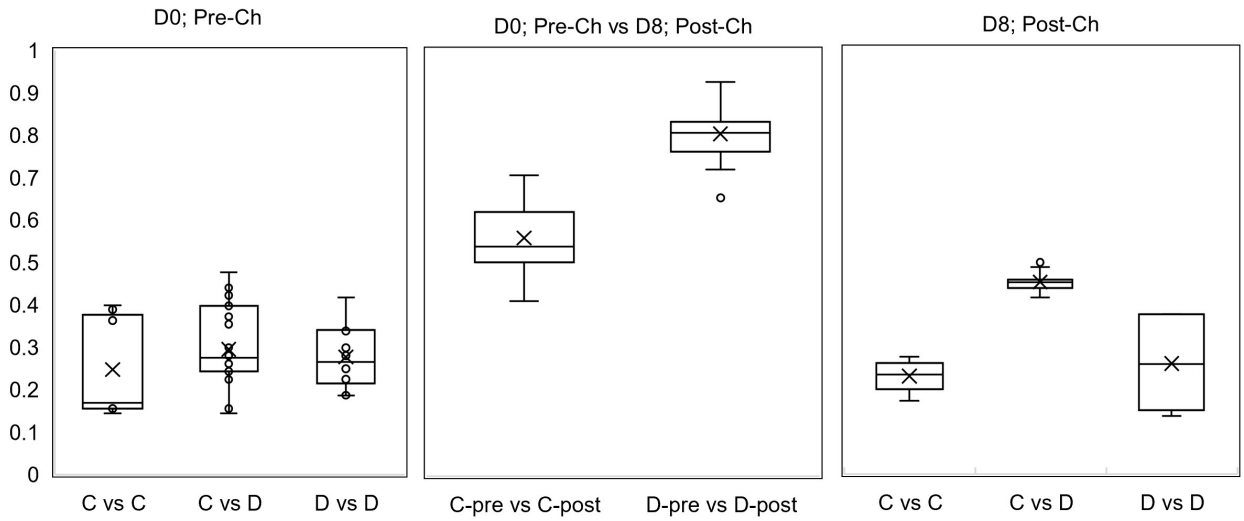

Figure 3.12: Box plots comparing the microbial communities based on Bray-Curtis dissimilarities in biofilm carriers from system C (control) and D (disinfected biofilter). x = Mean, o = Outlier data point, line=median.

Conversely, the Bray-Curtis dissimilarities of microbial community profiles in the rearing water samples were used to compare the temporal differences over time (Figure 3.13). Due to the low number of samples successfully sequenced at day 0 (2h post-challenge), these observed dissimilarities should be interpreted carefully. However, the comparison of the water samples from the two RAS shows a higher dissimilarity (i.e. larger spread) than the comparisons of samples within the same RAS on the same day. This can indicate a different influence from the bacterial challenge with non-disinfected versus disinfected biofilm carriers. When comparing the temporal changes within the same system (i.e. 'D0; Pre-Ch vs D8; Post-Ch'), the average dissimilarity is high in both RAS, suggesting high influence from the bacterial challenge. Nevertheless, the difference between the samples from RAS D was considerably higher than the samples from RAS C, indicating a stable community over time in RAS C compared to RAS D. As indicated, the PERMANOVA showed that rearing water communities were significantly different from D0 to D8 within the same RAS, with $p=0.018$ and $p=0.049$ in RAS C and D, respectively.

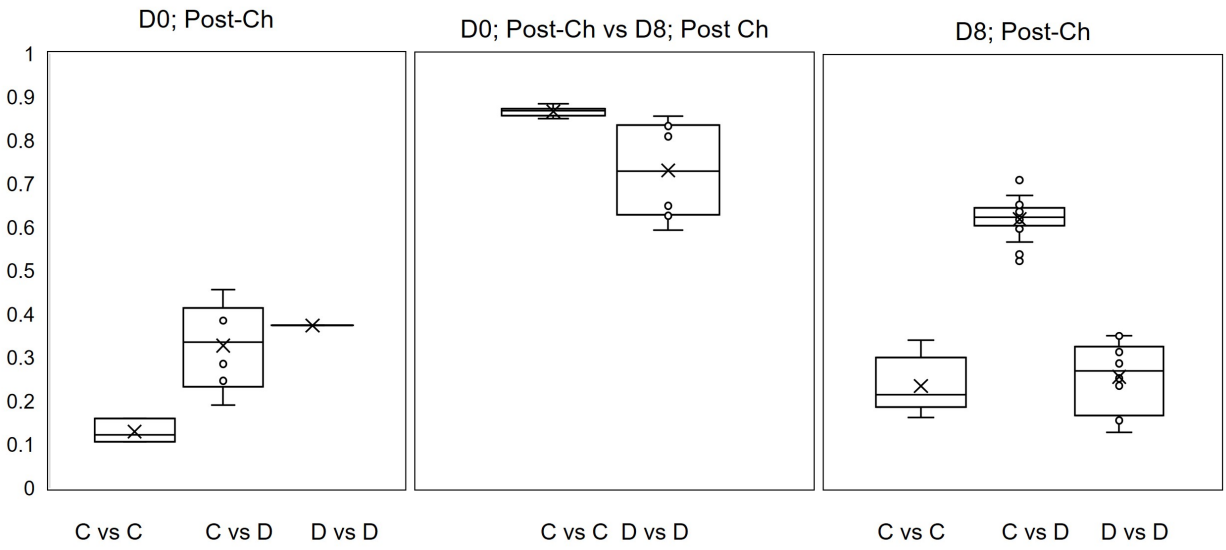

Figure 3.13: Box plots comparing the Bray-Curtis dissimilarities in rearing water from system C (control) and D (disinfected biofilter) over time. x=Mean, o=outlier datapoint, line = median.

Differential abundance test and top ASVs contributing to differences in microbial communities in the biofilm carriers

To identify the ASVs contributing most to the differences between the microbial communities in the biofilm carriers in RAS C and RAS D on day 7 of the experiment (D8 post-challenge), a DeSeq2 analysis was performed by Ph.D.- candidate Fernando Fernando. The top 10 ASVs contributing to the differences are listed in Table 3.4. All ASVs among the 10 were classified as Flavobacteriia or Gammaproteobacteria. 3 of the introduced opportunistic strains were among the 30 ASVs contributing most to the differences: ASV7 (*Flavobacterium*), ASV1 (*Psychrobacter*), and ASV4 (*Pseudomonas*).

Table 3.4: The ten ASVs contributing most to the differences between the microbial community profiles of the biofilm in the mature and immature biofilter at day 8 post-invasion. Based on the DeSeq2 analysis, the differential presence and abundance of each ASV in the samples are used in statistical analyses to assess their contribution to the differences observed between the sample groups. Each ASV is given at the genus (g) level, where c=class, obtained from the taxonomic assignment in the processed ASV table.

ASV ID	Taxonomy	log2FoldChange	Wald statistic	Adjusted p-value
ASV44	c: Gammaproteobacteria g: Pseudomonas	11.85747	11.10747	1.78E-25
ASV7	c: Flavobacteriia g: Flavobacterium	9.581912	18.41946	1.42E-72
ASV452	c: Gammaproteobacteria g: Acinetobacter	9.545061	8.509772	2.69E-14
ASV382	c: Gammaproteobacteria g: Pseudomonas	9.326113	8.392255	7.36E-14
ASV1261	c: Flavobacteriia g: Flavobacterium	9.313861	8.291881	1.72E-13
ASV2319	c: Gammaproteobacteria g: Acinetobacter	9.166886	8.293093	1.70E-13
ASV567	c: Flavobacteriia g: Flavobacterium	8.964896	8.106008	8.08E-13
ASV279	c: Gammaproteobacteria g: Pseudomonas	8.94808	8.367599	9.08E-14
ASV340	c: Flavobacteriia g: Flavobacterium	8.872472	8.169439	4.78E-13
ASV939	c: Flavobacteriia g: Flavobacterium	8.650587	7.379687	2.45E-10

Gammaproteobacteria and nitrifiers in the communities of the biofilm carriers

The total relative abundance of ASVs representing nitrifying bacteria in the communities of the biofilm carriers in RAS C and D on D0 and D8 were identified by manual inspection of the ASV-table and SINTAX file (Figure 3.14A). The proportion of ASVs hypothesized as nitrifiers were found by computing the average of ASVs assigned as *Nitrosomonas*, *Nitrosospira* or *Nitrospira* at the genus level. In total 51 ASVs the microbial samples from biofilm carriers across both RAS were assigned to these genera and incorporated in this analysis. Furthermore, it was hypothesized that a remarkable increase in the relative abundance of *Gammaproteobacteria* in the biofilm carriers could relate to a shift towards r-selection in the microbial communities. A manual inspection of the ASV-table showed that the relative abundance of *Gammaproteobacteria* was considerably higher ($p=0.0005$) in RAS D than RAS C, differing from 13.3 % to 3.2 % on sampling day 8 (Figure 3.14B). The increase was partly due to the opportunistic strains *Pseudomonas* and *Psychrobacter*. Interestingly, an ASV classified as *Acinetobacter* at the genus level, was the ASV having the highest contribution to the increase observed in RAS D, with an average increase of 4.8 % from D0 to D8.

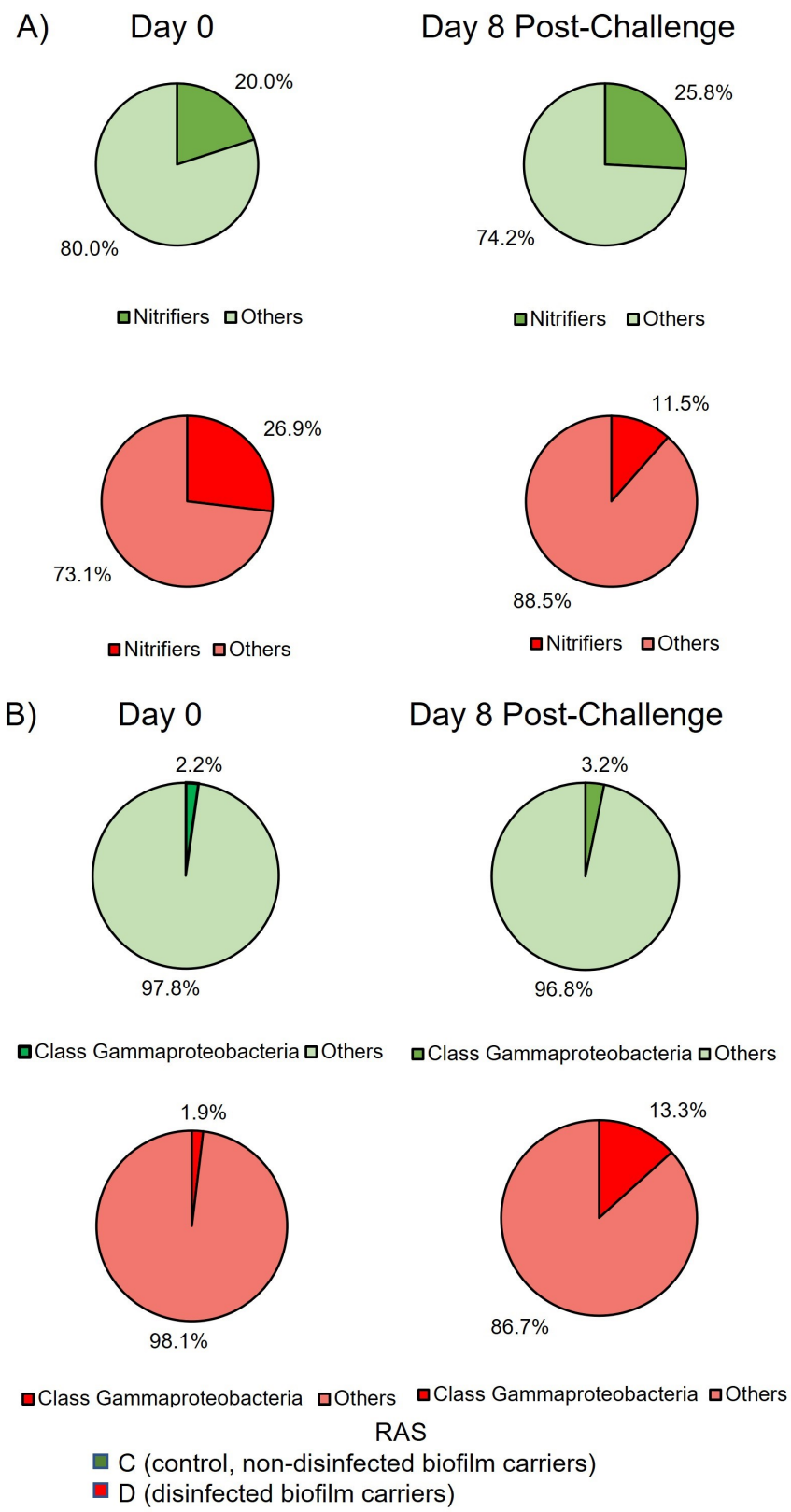

Figure 3.14: Pie charts representing A) the average relative abundance of nitrifying bacteria in the communities of the biofilm carriers, and B) the average relative abundance of Gammaproteobacteria, in the communities of the biofilm carriers, at D0 pre-challenge and D8 post-challenge in the two RAS. The proportions were calculated after manual inspection of the ASV-table and SINTAX file.

4 Discussion

4.1 Nitrification capacity and development of a disinfection protocol for the biofilm carriers from Lerøy

The decline in ammonia-N after the addition of 10 mg/L TAN to the reactor media (Figure 3.1A), in addition to the effective removal of 5 mg/L nitrite (Figure 3.1B), suggests a well-established community of nitrifying bacteria in the carriers obtained from the commercial RAS. Ammonia and nitrite were converted linearly. This corresponds to the reaction kinetics suggested in the literature with these initial concentrations of ammonia and nitrite (von Ahnen et al., 2015, Kinyage et al., 2019). A linear conversion of 0.33 g TAN/m^2*d and 0.31 g NO2-N/m^2*d was shown for communities established on polypropylene carriers in the form of saddle chips used in a commercial RAS for production of rainbow trout (*Oncorhynchus mykiss*) (Aalto et al., 2022). The somewhat higher conversion rates observed in this study could be due to the temperature difference (19 °C compared to 12 °C in our study), the different carrier shape, or the fact that the mentioned study was performed in an actual RAS where the environmental conditions will differ from the conditions in a lab-scale reactor. However, the rates were in the same order of magnitude, suggesting that our study correctly estimated the nitrification rate.

The development of a disinfection protocol for use on the biofilm carriers in the fish experiment was necessary to address the study's main objective. Chemical disinfection with hydrogen peroxide was chosen as the strategy, as it is routinely used to inactivate bacteria in the RAS loop between fish batches. Three concentrations of hydrogen peroxide were tested to establish the relationships between dose and nitrification efficiency. A decline to 20 % of the initial ammonia removal rate was achieved by treating the carriers with 600 mg/L hydrogen peroxide for 3 hours, indicating the inactivation of a substantial part of the nitrifying bacteria. Interestingly, a study by Linley et al. emphasized that remarkably little knowledge exists on hydrogen peroxide's exact mode of action as a biocide (Linley et al., 2012). However, the formation of oxidating hydroxyl radicals, leading to a combination of DNA damage and damage of proteins and lipids in the bacteria, was hypothesized as the main contributor to the inactivating properties in the previously mentioned study. A study by Christensen et al. assessing the effect of hydrogen peroxide on biofilms formed by two strains of *Pseudomonas* found some evidence of a breakdown of the extracellular matrix followed by increased dispersion of the biofilm in combination with cell lysis (Christensen et al., 1990). In our case, no measurements of biofilm thickness were performed, but visually, signs of increased turbidity in the hydrogen peroxide solution were observed. This could indicate detachment of the biofilm. Nevertheless, the mentioned study did not focus specifically on bacterial inactivation in biofilms, where other factors may apply to the mode of action. An interesting question to assess after the work in this thesis is the mode of action of hydrogen peroxide on the biofilm formed on the carriers and to which extent the biofilm detaches.

4.2 Colonization ability of four opportunistic bacterial strains on the biofilm carriers from Lerøy

To fulfil the study's main aim, it was necessary to investigate the possibility of four presumably opportunistic strains colonizing the biofilm carriers used in the fish experiment. Bacterial isolates of *Flavobacterium, Proteus, Pseudomonas* and *Psychrobacter* were chosen based on earlier research performed in the ACMS group, in addition to known taxonomy. In general, the four bacterial isolates grew fast and formed distinct colonies after 24-48h (Figure 3.3), and it was therefore assumed that these bacterial strains would be opportunists in the fish experiment as well. The assessment of the colony forming unit (CFU) in the biofilm after incubation of the carriers (Figure 3.1) showed variable growth with bacterial strain and initial cell density. Generally, the results could not be used to completely *quantify* the growth on the biofilm carriers but to investigate their *possibility* of growing in biofilm on the carriers. There was no clear tendency of higher growth on the biofilm carriers with higher initial cell densities. A possible explanation for this could be limiting resources for growth since the same media was supplied to the different initial cell densities. Nevertheless, the Sanger sequences of the 16S rDNA amplified with colony PCR from each treatment were similar to the Sanger sequences of the pure bacterial isolates. They showed their ability to grow in biofilm on the carriers since their unique 16S rDNA sequences were recovered from the carrier biofilm.

4.3 Optimization of the PCR protocol for the amplification of 16S rDNA

Although earlier work performed in the ACMS group to optimize PCR protocols for the amplification of 16S rDNA in fish samples (skin, gill and gut) was implemented in this study's laboratory work, obtaining sufficient amplicons of the target gene was still challenging. After testing a gradual increase in annealing temperature, in addition to diluting the DNA extracts, there was a slight tendency that diluting the skin and gill extracts increased the yield (Figure 3.4). Unexpectedly, the formation of primer dimers was a more significant problem with the higher annealing temperature in that PCR run, which was not corresponding to the hypothesis that increased annealing temperature gives more specific amplification due to higher stringency of the PCR-primer (Salter et al., 2014). However, the lowest annealing temperature obtained unspecific amplicons, in line with the hypothesis (Figure 3.4A and B). A two-step PCR, where 16S rDNA in undiluted DNA extracts from the salmon intestine was amplified with two separate primers with and without the Illumina adapter sequence, was partly successful (Figure 3.6). This was mainly due to the lower amplification of primer dimers, which had been a problem. One can speculate that a low amount of microbial DNA to host DNA in the extracts was the reason. However, the impact of this untraditional method on the Illumina sequencing results is unknown.

Since few DNA extracts from skin and gill samples resulted in sufficient amplification of the target gene, these samples were omitted in the following amplicon library

preparation. It has been shown that the skin and gill mucus contains a low amount of microbial DNA (Sandberg, 2021), which may explain the difficulties with amplifying the bacterial 16S rDNA. Furthermore, too much skin and gill tissue subjected to extraction could be a reason for the problems. This could lead to increased amounts of PCR inhibitors from host DNA that were not accounted for by diluting the extract. Most likely, a too high amount of skin and gill tissue in each sample tube for DNA extraction was the main reason, resulting in insufficient homogenization and a high proportion of inhibiting host DNA to microbial DNA in the extracts.

4.4 The effect of disinfection of biofilm carriers on the microbial communities in RAS after the invasion challenge from opportunistic strains

The study's main objective was to assess the role of disinfection of biofilm carriers in a RAS during an invasion from opportunistic bacteria, to increase the understanding of whether mature biofilm communities in the biofilter may protect against such invasions. To do this, Atlantic salmon fry was reared in two lab-scale RAS with identical conditions, where the only difference was the treatment of the biofilm carriers in the biofilter. One RAS had biofilm carriers subjected to partial disinfection with hydrogen peroxide, whereas the other RAS had untreated biofilm carriers (RAS C).

4.4.1 Evaluation of the lab-scale RAS as a model system in the fish experiment

A lab-scale RAS can be a good alternative for experimental purposes, where the aim is to understand more of the dynamics of this system in order to optimize the production of fish and other marine species on land. Although it must be thoroughly planned and dimensioned, its size makes it operable for a small research group. Several biological and technological challenges must be considered when dimensioning a RAS to secure good fish welfare and optimal production. The benefits and importance of optimizing the physicochemical water quality are well known, and the microbial water quality has been increasingly recognized in several studies. Before the start-up of the lab-scale RAS, the plan was to introduce salmon fry with an average weight of 0.5-1 gram per individual and around 0.5 kg biomass per RAS. It was discovered that the dimensioning relationship between the biofilter chamber and the rearing tanks was too large, meaning that for this biomass of salmon, the TAN production was insufficient to feed the nitrifiers in the biofilter. Therefore, it was decided to increase the rearing tanks' size to fit a larger biomass with the same stocking density, thus increasing the production of TAN. The time used for this adaptation meant that the RAS facility providing salmon fry was later in its production cycle, and the salmon had an average size of 3-3.5 grams on the day of delivery. The salmon was reared in flow-through tanks for 54 days due to the challenges connected to the dimensioning. Therefore, the average individual size was higher on day 0 of the experiment (Table 3.3).

Effective mechanical filtration for removing larger particles originating from faeces

and excess feed was a challenge in the lab-scale RAS, independent of biofilter maturation. The microbial communities are highly influenced by the increased organic load in the system, which will strengthen the r-selection and facilitate opportunistic growth. Although the mechanical filter sock was exchanged and rinsed every morning and afternoon, there were recurring problems with clogging and back-flow of the recirculated water from the rearing tanks. This suggests that the mechanical filter needed to be more extensive, considering the load of fish in the system, making maintenance difficult. Although siphoning in the mechanical sump and rearing tanks somewhat reduced the visible particles, an additional mechanical filter with a larger pore size would improve the performance of the mechanical filter sock used in this experiment.

Subsequently, the problems with clogging in the mechanical filter caused back-flow following leakages on day 1 and day 7, as described in the results, leading to the need for higher water exchange. The incident on day 1 of the experiment led to the exchange of 23 % of the total water volume, the highest exchange rate during the experimental period, and the higher dilution reduced the organic load in the RAS, further reducing the carrying capacity (CC). This could impact the microbial selection pressure, as suggested in the study by Vadstein et al. (Vadstein et al., 2018). Although this factor might have impacted the microbial communities observed, the exchange rate in the two RAS was equal, ensuring equal carrying capacity. Equalizing the feeding rate in both RAS was another important measure in controlling carrying capacity.

4.4.2 Fish performance and physicochemical water quality

No mortalities were observed in either RAS's first eight days of the experiment, suggesting that the dimensioning, physicochemical water quality and microbial water quality were tolerated. However, the consequences of the power outage were severe, causing the fish death and ending the experiment prematurely. Performance-wise, the biomass increase was slightly higher in the RAS with non-disinfected biofilm carriers, although insignificant. However, prolonging the experiment would be needed to assess the effect on the fish performance in the longer run.

As discussed earlier, the accumulation of particles was a problem in both RAS. Although only the total dissolved solids (TDS) were measured in the RAS, quantifying the number of dissolved particles below 2 µm, was in line with the observed increasing load of larger particles. As expected, the TDS increased from day 0 to day 7, justifying the increased carrying capacity. However, it is essential to note that the TDS also includes inorganic compounds when interpreting this. The maximum TDS observed in the rearing tank was higher in RAS C than in RAS D (562 ppm versus 323 ppm, on day 8 of the experiment), which could be connected to the clogging of the mechanical filter observed in RAS C. Additional quantification of the available organic matter over time, for example by analysis of chemical oxygen demand, would improve the understanding of this.

The concentration of the nitrogen species ammonia, nitrite and nitrate in the rearing

tanks is directly linked to the performance of the nitrifying communities of the biofilm carriers (Ruiz et al., 2020). Generally, the concentrations of nitrogen species (Table 3.2) were within the tolerance limits suggested for Atlantic salmon (<2 mg/L TAN, <0.1 mg/L nitrite, and <100 mg/L nitrate) (Davidson et al., 2017, Norwegian Food Safety Authority, 2008). However, spikes up to 0.15 mg/L TAN were observed for single measurements in the RAS with disinfected biofilm carriers. This supports the hypothesis of decreased ammonia oxidation capacity after disinfection due the partial inactivation of the nitrifying bacteria. Since the experiment's duration was shorter than planned, the TAN concentration in the rearing water would be expected to increase over time in this RAS, which could be detrimental to the fish. While incidents of ammonia spikes also may occur in commercial RAS with Atlantic salmon, especially during peak production, the ammonia is quickly consumed if the biofilter is correctly dimensioned and matured. Earlier studies have shown that incomplete ammonia removal can be linked to increased heterotrophic growth (Rojas-Tirado et al., 2018, Bugten et al., 2022). This correlates well with the hypothesis of the opportunistic growth in the RAS with disinfected biofilm carriers after the invasion challenge. The pH was constantly lower in the RAS with non-disinfected biofilm carriers (Figure D3 in Appendix D.3), suggesting the higher activity of the nitrifying community, producing more H^+. The aim was to ensure a nitrification capacity securing TAN <2 mg/L in the lab-scale RAS. Although this treatment maintained some nitrification in the MBBR, it was not sufficient to altogether avoid spikes of ammonia in the lab-scale RAS. A possible explanation for this is the difference between the bioreactors' controlled environment and the biofilm carriers' performance when operated in a RAS where a series of parameters may fluctuate. The high organic matter content in the two RAS enhances the competition with heterotrophs and may lower the nitrification efficiency. The role of the introduced opportunistic strains in the lab-scale RAS should also be considered, substantially increasing the bacterial load and impacting the competition for resources. Both nitrite and nitrate concentrations were low in both RAS during the experiment (Table 3.2). The short experimental duration and the higher water exchange are logical explanations for this. The lower maximum nitrate production in the RAS with disinfected biofilm carriers (5 mg/L, Table 3.2) aligns with the incomplete ammonia oxidation observed here, leading to less substrate available for the NOBs.

4.4.3 Microbial communities in RAS with disinfected biofilm carriers after the invasion from presumably opportunistic strains

The colonization success of the four bacterial strains was higher in the RAS with disinfected biofilm carriers

The verification of the opportunistic bacteria strains' ability to colonize the media of interest was a critical step since their ability to establish is a prerequisite for their ability to grow in biofilm (Flemming et al., 2016). A simple lab-scale batch experiment showed that all four strains of interest, *Flavobacterium, Proteus, Pseudomonas* and *Psychrobacter* were able to form biofilm on plastic carriers. The next step was an up-scaling of the experiment into lab-scale RAS systems with live fish and biofilm carriers with resident nitrifying communities from a commercial RAS. The main aim was to assess whether the opportunistic strains had a higher ability to colonize in

a RAS with partly disinfected biofilm carriers. As hypothesized, the four bacterial strains had a higher ability to colonize in the lab-scale RAS running with disinfected biofilm carriers compared to the control RAS with non-disinfected biofilm carriers. The colonization ability was higher in biofilm carriers, autoclaved biofilm carriers and rearing water of the mentioned RAS, as suggested by the higher average relative abundance of the ASVs corresponding to the four strains at D8 of the experiment (Figure 3.9). This implies that the four strains were able to establish themselves in the disinfected system to a greater extent over time, despite the shorter duration of the experiment than planned. In addition, the significantly lower abundance of the four opportunistic strains in the lab-scale RAS with non-disinfected biofilm carriers may imply that the resident communities of the carriers could counteract an invasion from opportunistic strains.

It is interesting to discuss this in relation to invasions in microbial ecology. Although little is known about the ecological processes explaining the mechanisms of invasion in residential communities, earlier ecological theories have hypothesized that high growth rates, resource efficiencies, and dispersal may characterize a good invader. Moreover, low diversity and resource availability fluctuations could make a resident community more prone to invasion (Acosta et al., 2015). In the context of our study in RAS, one may hypothesize the relationship between disinfection, leading to the inactivation of bacteria, and the empty niches that will become available to the invaders as a consequence (Li et al., 2019). Additionally, the opportunistic strains were characterized by fast growth during the colonization experiment, which could be part of the explanation, together with the empty niches. Despite the disinfection, the total availability of resources was the same as in the RAS with non-disinfected carriers, favouring the fast-growing opportunistic strains in this system. Moreover, it has been shown that disinfection in a RAS causes a perturbation in the resident community composition (Attramadal et al., 2012), which also in itself has been hypothesized to increase the probability of an invader successfully establishing in the resident communities (Abernethy, 2022). All over, the biofilm community of the non-disinfected carriers in RAS C may be viewed as mature, where the K-selected species dominated, and, thus, were more resistant to the perturbation (i.e. the bacterial challenge from r-strategists) (Vadstein et al., 2018). It is essential to note that the fast growth rate of a microbe is not negative *per se*, but the fact that most pathogens are r-strategists with a sum of traits making them unfavourable in the context of aquaculture and RAS (Vadstein et al., 2018). Furthermore, the introduced bacterial strains were established exclusively in the clean and uncolonized autoclaved carriers of RAS D (Figure 3.9), suggesting that the same mechanisms of selection for r-strategists ensured their establishment as a biofilm on these carriers. Regardless, it is essential to note that the autoclave-inactivated bacteria left on the carriers before use could have DNA of sufficient quality for PCR, possibly impacting the sequencing results. However, the increase of the opportunists was still significant.

Interestingly, the invasion success in the lab-scale RAS with disinfected carriers was not the same for the four strains, where *Flavobacterium* (ASV7) and *Pseudomonas* (ASV4) established more in both biofilm carriers and rearing water than *Proteus* and *Psychrobacter*. This implies that although the four strains had relatively similar growth

on agar plates assessed in the colonization experiment, high growth after a short time does not necessarily mean the strain will be a good invader. This is a good illustration of the complexity of the invader theories in microbial ecology, since a hierarchy of factors may affect the community dynamics.

A significant decrease of nitrifying bacteria and an increase of Gammaproteobacteria was observed in the disinfected biofilm carriers

We observed a significant increase in the relative abundance of Gammaproteobacteria in addition to a decrease in ASVs representing nitrifying bacteria in the biofilm carrier samples (Figure 3.14) during the experimental period, which may imply a shift in the microbial community composition. Interestingly, this trend was much more pronounced in the RAS with disinfected biofilm carriers, where the relative abundance increased by 11.4 % on average compared to a 1 % increase in the non-disinfected carriers. Relating this to microbial ecology, disinfection can be viewed as a non-selective strategy for eliminating bacteria (Vadstein et al., 2018). With this strategy, detrimental bacteria are killed after the general reduction in the number of bacteria. As suggested by Vadstein et al., this general reduction of bacterial biomass, in combination with the surplus of nutrients and space, leads to lower competition in the environment. This is in line with the theory of r-selection, where the lower competition supports the growth of opportunistic heterotrophic bacteria (Vadstein et al., 2018). In our case, disinfection of the biofilm carriers used in the lab-scale RAS led to inactivation and possibly detachment or thinning of the bacterial biofilm, which would free more space for the fast-growing heterotrophs. In addition, a surplus of nutrients was probably available, in the form of organic matter from the faeces, uneaten fish feed and dead bacteria circulating in the RAS loop. We assume, from the lab-scale MBBR test of nitrification as a degree of disinfection, that around 80 % of the bacteria in the biofilm was inactivated. Thus, we expected lower competition (r-selection) where more substrate is available per heterotrophic cell. The r-strategists have a high maximum growth rate, but not necessarily high substrate affinity. Thus, the environmental conditions after disinfection are better adapted to the added opportunistic strains.

Notably, the same study showed an increased presence of taxa belonging to the class Gammaproteobacteria in r-selected communities of natural seawater, obtained by nutrient pulsing to increase the availability of organic matter (Vadstein et al., 2018). Altered bacterial communities with an increase in Gammaproteobacteria taxa have also been observed after UV and ozonation disinfection of wastewater (Becerra-Castro et al., 2016). Among the Gammaproteobacteria genera in the r-selected communities mentioned in the study, multiple are known pathogens, for example, Vibrio, Yersinia, Pseudomonas, Aeromonas and others (Vadstein et al., 2018). Moreover, the study linked the bloom of r-strategists to detrimental host-microbe-interactions, and this may suggest a link between the theory of r-selection and pathogens, an interesting perspective in this study of disinfection in RAS (Vadstein et al., 2018). Notably, three of the four introduced strains belong to Gammaproteobacteria: *Proteus, Pseudomonas* and *Psychrobacter*, so the bloom in the RAS with disinfected carriers, could, to some extent, be explained by their establishment, which was also partly supported by the DeSeq2 analysis assessing the ASVs contributing most to the differences observed.

The ASVs of *Pseudomonas* and *Psychrobacter* were among the top 30 ASVs. But interestingly, other Gammaproteobacteria ASVs also contributed. Among them were other ASVs assigned to the genera of *Pseudomonas* and *Acinetobacter* (Table 3.4). The average decrease in ASVs representing nitrifying bacteria was 15.4 % in the communities of the disinfected carriers from D0 pre-challenge to D8 post-challenge while increasing from 20 % to 25.8 % in the non-disinfected carriers (Figure 3.14A), implying the stability of the nitrifying community in the non-disinfected carriers despite the bacterial challenge.

Higher similarity in community composition was observed between sample types than between the RAS

Overall, the PCoA-plot assessing the Bray-Curtis and Dice-Sørensen similarities at ASV level (Figure 3.10 and Figure 3.11) showed that the beta-diversity was more variable between sample types (autoclaved biofilm carriers, biofilm carriers, rearing water and salmon hindgut) than with RAS and day of the experiment (D0 or D8). The samples of autoclaved biofilm carriers, biofilm carriers and rearing water showed a more significant clustering along the first coordinate in the plot based on Dice-Sørensen similarities, which implies that the communities may share ASVs, but that they were present in different abundances in different sample types. A study by Bakke et al. showed different community dynamics in rearing water and biofilm samples in a RAS for post-smolt production, supporting the findings in our study (Bakke et al., 2017). The different environmental and competition conditions for bacteria growing in biofilm compared to suspended growth in the rearing water can be part of the explanation for the differences observed.

Furthermore, the microbial communities in samples of the rearing water 2 hours after the introduction of the opportunistic strains were not significantly different between the two RAS ($p > 0.05$). This may imply that both RAS were treated equally, i.e. that the same cell density of the opportunistic bacteria was introduced. There were some variations in the rearing water communities over time (i.e. from D0 to D8) (Figure 3.11). A part of the explanation could be the increase of total dissolved solids in the rearing water of both lab-scale RAS during the experiment (Table 3.2), which increases the carrying capacity and thereby support increased heterotrophic growth.

The communities were temporally more stable in the RAS with non-disinfected biofilm carriers

There were several implications that the microbial community composition generally was more stable in the RAS without disinfection of the biofilm carriers, which is in line with a previous study on the impact of disinfection with UV on the microbial composition and diversity in RAS (Dahle et al., 2023,Attramadal et al., 2021). The PCoA plot of Bray-Curtis similarities showed a higher clustering of the samples from the non-disinfected biofilm carriers from D0 to D8 3.11). This was supported by the comparisons of Bray-Curtis-dissimilarities in the biofilm carrier samples (Figure 3.12), showing a higher mean dissimilarity in C than D from D0 to D8.

In the rearing water communities, the PCoA-plot of Bray-Curtis similarities within each RAS (Figure 3.10A), showed a lower similarity over time than the communities of the

biofilm carriers for both RAS, despite the fact that the biofilm carriers were sampled before and after the introduction of the opportunistic strains. The comparison of Bray-Curtis dissimilarities in the rearing water from D0 2h post-challenge to D8 post-challenge (Figure 3.13), revealed high temporal dissimilarity in both RAS, possibly due to altered community dynamics after the introduction of the opportunistic strains, where the altered bacterial load in addition to the fast growth of the introduced strains caused perturbations in the water communities. However, the spread between the rearing water samples was higher in the RAS with disinfected biofilm carriers, suggesting that the introduced strains perturbed the communities of the rearing water more than in RAS C. The higher temporal dissimilarity in rearing water than biofilm communities observed may be due to the benefits of bacteria forming biofilm as a life strategy. Bacteria growing in a biofilm are more protected against the environment than the free-living bacteria in the rearing water (Flemming et al., 2016).

An increase in alpha diversity was observed in the disinfected carriers from D0 to D8

The experimental duration was shorter than planned, which should be taken into consideration when interpreting the development of alpha diversities. A significant reduction of exponential Shannon's diversity, but no significant reduction of observed ASV richness, was observed in the communities of the disinfected biofilm carriers when comparing their alpha diversity indices to the non-disinfected carriers at D0 before the introduction of the bacterial strains (Figure 3.7). This may imply that some bacterial populations are more resistant to disinfection than others since the evenness decreased slightly. At the same time, the richness was similar, meaning that the same ASVs were present but in different abundances. As discussed by Tong et al., bacteria may propose a variety of molecular mechanisms that reduce the membrane permeability to disinfectants as well as the production of reactive oxygen species and activation of enzymatic pathways to limit damage to the cell and degrade the disinfectant (Tong et al., 2021). Additionally, the biofilm serves as a stable and protective environment, and it has been shown that some bacteria can acquire resistance to a higher extent than others (Zhu et al., 2021).

Interestingly, the alpha diversity in the rearing water and biofilm carrier communities of the RAS running with disinfected biofilm carriers increased from D0 to D8. In contrast, no change in alpha diversity was observed in the non-disinfected carrier communities. At first, this finding seems counterintuitive. Multiple studies have proposed that high alpha diversity is a prominent characteristic of the K-selected communities (Attramadal et al., 2012, Vadstein et al., 2018, Dahle, 2022). However, one can speculate whether the "Intermediate Disturbance Hypothesis" may apply to the observation we did in the lab-scale RAS with disinfected biofilm carriers. The hypothesis was first launched by Grime (1973) and Horn (1975) and is a conceptual framework that implies a connection between species diversity and level of disturbance. (Moi et al., 2020). It proposes that the highest diversity in a community is reached with intermediate disturbance since the relationship between colonizers and competitors will be the most stable. With too intense or frequent perturbations, the community diversity will decrease since many species fail to survive and limited colonization is

observed (Moi et al., 2020). We can view our finding of higher alpha diversity in the context of this framework; our disinfection could be of a level of disturbance low enough to not eradicate too many species, and not too low, where a few species would dominate since they persisted in the disinfection. This would mean that the assumed 80 % disinfection, was an ”intermediate” level which eliminated a fraction of the most dominating species, allowing the rise and coexistence of the less competitive species. Regardless, this is only speculation since the duration of the experiment was very short, and we would need more data. Following the communities over a longer time would improve the understanding. Also, the hypothesis has been challenged by several researchers, implying that caution should be applied when using the framework (Moi et al., 2020).

Overall, independent of the disinfection of the biofilm carriers, the alpha diversity was lower in the rearing water communities than in the biofilm carrier communities. This has also been observed in commercial RAS for the production of Atlantic salmon (Dahle et al., 2023). It is also supported by other studies implicating that the rearing water has its own planktonic microbial communities while other communities establish in the biofilter (Blancheton et al., 2013, Bartelme et al., 2017).

The salmon gut samples had lower diversity than the biofilm carrier and rearing water samples

A limited number of salmon gut samples were successfully sent for 16S rDNA sequencing, lowering the significance of the observations done in these samples. However, they are interesting to discuss in the context of other studies. The diversity in the salmon gut communities was lower and more variable than in the biofilm carrier and rearing water (Figure 3.7), supported by the higher separation in the Dice-Sørensen PCoA (Figure 3.10B), suggesting that different ASVs were present). This correlates to findings in previous research (e.g. Bozzi et al., 2021) and may imply different selection pressures in the fish than in the surroundings. Furthermore, high inter-individual variation was observed in the communities, which corresponds to observations done in a study of gut communities of Atlantic salmon yolk-sac fry (Fiedler et al., n.d., submitted to Frontiers in Microbiology). It is suggested that selection pressure and drift are the major factors accounting for the low diversity and high variations (Vestrum et al., 2020).

Clostridiales (average 17.8 %), Pseudomonadales (average 12.0 %), Bacilliales (11.1 %), and Lactobacilliales (average 10.9 %) were the dominating orders in the salmon gut samples (Figure 3.8). This was expected since they have been observed in farmed Atlantic salmon in other studies (Egerton et al., 2018, Wang et al., 2021, Gajardo et al., 2016), suggesting that some orders make up the ”core-communities” that are observed even though the individual variation may be large. The feed composition has been shown to influence the microbial community composition in the gut of farmed fish (e.g. in Wang et al., 2021), which may be part of the explanation for the variations observed in the research.

Interestingly, an ASV classified to the genus of *Janthinobacterium* had the highest average relative abundance in the gut samples (3.7 %). The *Janthinobacterium lividum* is a known fish pathogen that may cause infection in salmonids (Oh et al., 2019),

but the genus has also been isolated from the gut of healthy fish (Egerton et al., 2018). However, strains of *Janthinobacterium* were grown and used in the ACMS lab in research simultaneously with this experiment. Since the ASV was present in some biofilm carrier- and rearing water samples in lower relative abundances, one could speculate if it could originate from contamination in reagents or the lab environment.

4.5 Future perspectives

This thesis emphasizes the role of the mature biofilter biofilm community in counteracting invasion from opportunistic and possibly pathogenic bacteria in RAS for the production of Atlantic salmon. With infections being an increasing concern in such systems in Norway, and disinfection suggested as a strategy, the study raises essential knowledge of the consequences of disinfection of the biofilter on the resident communities in a RAS.

As the duration of the experiment was limited to eight days, prolonging the study would be a natural continuation of this work. Studying the dynamics of the microbial communities in the two RAS having different degrees of maturation in the biofilter over a more extended period, would provide more knowledge on the effect of the opportunistic bacteria over time. Increasing the scale of the experiment and improving the lab-scale RAS as a model system would enhance the understanding and make it transferable to commercial RAS. Performing the same study with multiple fish batches and the same biofilter carriers would give insight into how the opportunistic strains behave when the rearing water is exchanged. Having different household communities by using biofilm carriers and fish from other localities, possibly other species, would also be interesting, in addition to testing other opportunistic strains. Investigating whether pathogenic bacteria can thrive in mature communities of the biofilm carriers, would also aid the understanding of the use of disinfectants.

5 Conclusions

This thesis has investigated the role of the mature biofilm carrier community in a lab-scale RAS for the rearing of Atlantic salmon fry, during the invasion from four opportunistic bacterial strains. The major findings of this thesis were:

- The lab-scale RAS functioned as intended, and the fish experiment was performed as planned until a power outage ended the experiment early.
- The opportunistic strains representing *Proteus*, *Psychrobacter*, *Pseudomonas*, and *Flavobacterium* established in high abundances in both water and biofilter in the RAS with a disinfected biofilter eight days after the bacterial challenge, but were hardly observed in the RAS with a mature biofilter.
- A slight increase in alpha diversity was observed for the communities of the biofilm carriers and rearing water from D0 to D8 in the RAS with a disinfected biofilter, which was not in line with the expectations or previous research.
- The microbial communities of the biofilm carriers and rearing water were more stable from D0 to D8 in the RAS running with non-disinfected carriers. The community compositions of the salmon gut samples were significantly different from the communities of the biofilm carriers and the rearing water. The gut communities were also less diverse than the communities of the biofilm carriers and rearing water.

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Appendix

A Media and solution compositions

Table A1: Composition of the nutrient medium used for determination of ammonia oxidation capacity in biofilm carriers from Lerøy, according to a start concentration of 10 mg/L TAN.

Chemical	Quantity (g/L)
$(NH_4)_2SO_4$	0.04717
NaH_2PO_4*2H2O	0.272
Na_2CO_3	0.2
$NaNO_2$	0.02463
Trace metal solution	10 mL of stock solution
pH adjustment	3-6 drops of HCl to pH 7.5

Table A2: Composition of the nutrient medium used for determination of nitrite oxidation capacity in carriers from Lerøy, according to a start concentration of 5 mg/L nitrite.

Chemical	Quantity (g/L)
$NaH_2PO_4 \cdot 2\,H_2O$	0.272
Na_2CO_3	1
$NaNO_2$	0.0242
Trace metal solution	10 mL of stock solution
pH adjustment	3-6 drops of HCl to pH 7.5

Table A3: Composition of trace metal solution used in media for the batch experiment and acclimatization of biofilm carriers in the fish experiment.

Chemical	Quantity (g/L)
$MgSO_4 \cdot 7\,H_2O$	2.5
$CaCl_2 \cdot 2\,H_2O$	1.5
$FeCl_2 \cdot 4\,H_2O$	0.2
$MnCl_2 \cdot 4\,H_2O$	0.55
$ZnCl_2$	0.07
$CoCl_2 \cdot 6\,H_2O$	0.12
$NiCl_2 \cdot 6\,H_2O$	0.12
EDTA; Tritriplex III	2.8

B Kits & Protocols

B.1 Hach-Lange protocols

LCK 303 Ammonium

DOC312.53.94008

2–47 mg/L NH_4-N or 2.5–60.0 mg/L NH_4 **LCK 303**

Scope and application: For surface water, wastewater, soil and substrates.

Test preparation

Test storage

Storage temperature: 2–8 °C (35–46 °F)

pH/Temperature

The pH of the water sample must be between pH 4–9.

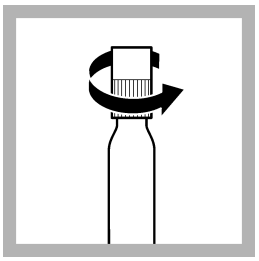
The temperature of the water sample and reagents must be 20 °C (68 °F).

Before starting

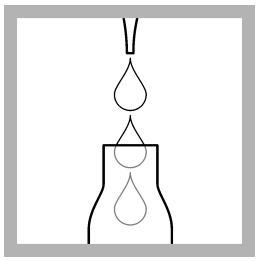
In case of not working at the correct recommended temperature an incorrect result may be obtained.
Analyze the samples as soon as possible for best results.
Time dependency: The final absorbance is reached after a reaction time of **15 minutes** and then remains **constant for a further 15 minutes**.
Review safety information and expiration date on the package.
Review the Safety Data Sheets (MSDS/SDS) for the chemicals that are used. Use the recommended personal protective equipment.
Dispose of reacted solutions according to local, state and federal regulations. Refer to the Safety Data Sheets for disposal information for unused reagents. Refer to the environmental, health and safety staff for your facility and/or local regulatory agencies for further disposal information.

Procedure

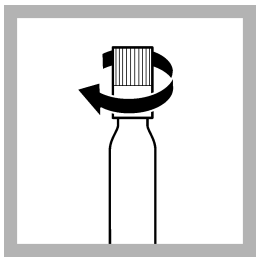
1. Carefully remove the foil from the screwed-on DosiCap Zip.



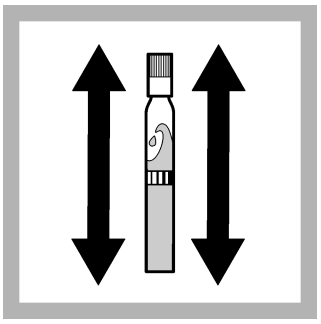
2. Unscrew the DosiCap Zip.



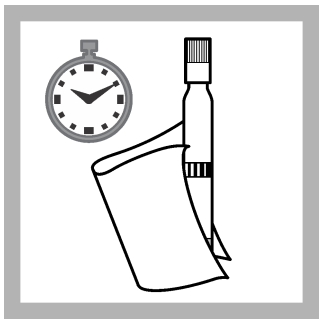
3. Carefully pipet **0.2 mL** of **sample**.



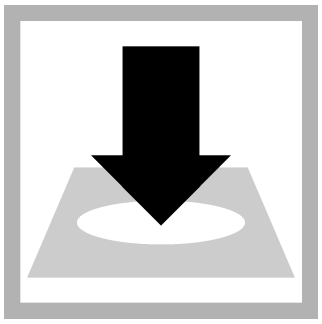
4. **Immediately** screw the DosiCap Zip back on; **fluting at the top**.



5. Shake **vigorously**.



6. After **15 minutes**, thoroughly clean the outside of the cuvette and evaluate.



7. Insert the cuvette into the cell holder.
DR 1900: Go to LCK/TNTplus methods.
Select the test, push **READ**.

Interferences

The ions listed in the table have been individually checked against the given concentrations and do not cause interference. The cumulative effects and the influence of other ions have not been determined.

Primary amines are also determined and cause high-bias results. A 10000-fold excess of urea does not interfere. All reducing agents interfere and cause low-bias results.

A large excess of ammonium can cause result displays within the measuring range. It is advisable to carry out a plausibility check by making dilutions.

The measurement results must be subjected to plausibility checks (dilute and/or spike the sample).

Interference level	Interfering substance
1000 mg/L	Cl^-, SO_4^{2-}
500 mg/L	K^+, Na^+, Ca^{2+}
50 mg/L	CO_3^{2-}, NO_3^-, Fe^{3+}, Cr^{3+}, Cr^{6+}, Zn^{2+}, Cu^{2+}, Co^{2+}, Ni^{2+}, Hg^{2+}
25 mg/L	Fe^{2+}
10 mg/L	Sn^{2+}
5 mg/L	Pb^{2+}
2 mg/L	Ag^+

Summary of method

Ammonium ions react at pH 12.6 with hypochlorite ions and salicylate ions in the presence of sodium nitroprusside as a catalyst to form indophenol blue.

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10/2019, Edition 1

LCK 342 Nitrite

DOC312.53.94019

0.6–6.0 mg/L NO_2-N or 2–20 mg/L NO_2 LCK 342

Scope and application: For wastewater, drinking water, table water, surface water, mineral water and process analysis.

Test preparation

Test storage

Storage temperature: 15–25 °C (59–77 °F)

pH/Temperature

The pH of the water sample must be between pH 3–10.

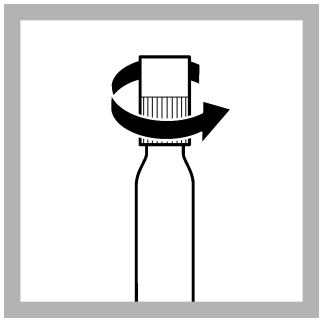
The temperature of the water sample and reagents must be between 15–25 °C (59–77 °F).

Before starting

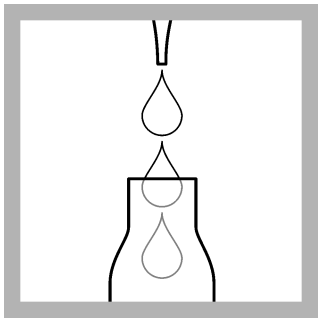
Not more than **3 hours** should elapse between sampling and analyzing the sample.
Review safety information and expiration date on the package.
Review the Safety Data Sheets (MSDS/SDS) for the chemicals that are used. Use the recommended personal protective equipment.
Dispose of reacted solutions according to local, state and federal regulations. Refer to the Safety Data Sheets for disposal information for unused reagents. Refer to the environmental, health and safety staff for your facility and/or local regulatory agencies for further disposal information.

Procedure

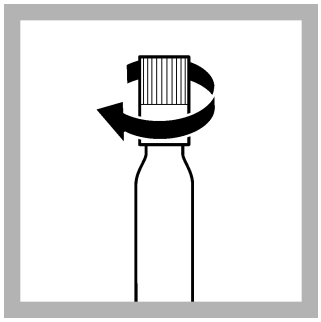
1. Carefully remove the foil from the screwed-on DosiCap Zip.



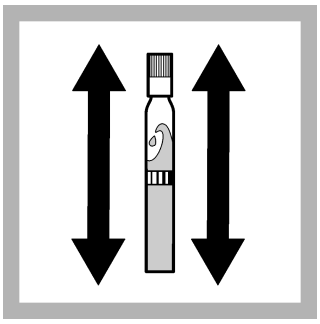
2. Unscrew the DosiCap Zip.



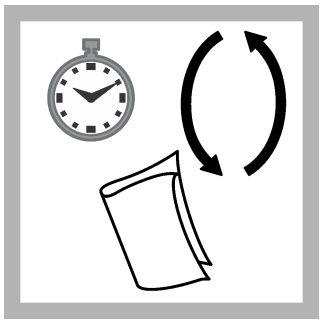
3. Carefully pipet **0.2 mL** of **sample**.



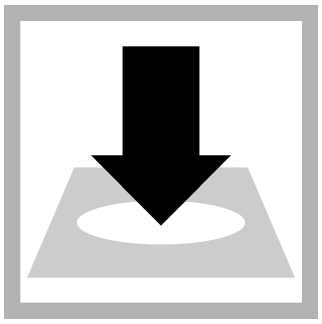
4. Immediately screw the DosiCap Zip back on; **fluting at the top**.



5. Shake **vigorously** until the freeze-dried contents are **completely dissolved**.



6. After **10 minutes**, invert a few more times, thoroughly clean the outside of the cuvette and evaluate.



7. Insert the cuvette into the cell holder.
DR 1900: Go to LCK/TNTplus methods.
Select the test, push **READ**.

Interferences

The ions listed in the table have been individually checked against the given concentrations and do not cause interference. The cumulative effects and the influence of other ions have not been determined. Chromium(VI) ions interfere with the determination. Copper(II) ions interfere with the determination even at concentrations below 1 mg/L.

The measurement results must be subjected to plausibility checks (dilute and/or spike the sample).

Interference level	Interfering substance
4000 mg/L	SO_4^{2-}
2000 mg/L	K^+, NO_3^-, Ca^{2+}, Cl^-
1000 mg/L	NH_4^+, PO_4^{3-}
200 mg/L	Mg^{2+}
100 mg/L	Cr^{3+}, Hg^{2+}
50 mg/L	Co^{2+}, Zn^{2+}, Cd^{2+}, Mn^{2+}
20 mg/L	Fe^{3+}, Ni^{2+}, Ag^+, Fe^{2+}
10 mg/L	Sn^{4+}

Summary of method

Nitrites react with primary aromatic amines in acidic solution to form diazonium salts. These combine with aromatic compounds that contain an amino group or a hydroxyl group to form intensively colored azo dyes.

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10/2019, Edition 1

LCK 339 Nitrate

DOC312.53.94016

0.23–13.50 mg/L NO_3-N or 1–60 mg/L NO_3 **LCK 339**

Scope and application: For wastewater (beware of interferences), drinking water, raw water, surface water, soils, substrates and nutrient solutions.

Test preparation

Test storage

Storage temperature: 15–25 °C (59–77 °F)

pH/Temperature

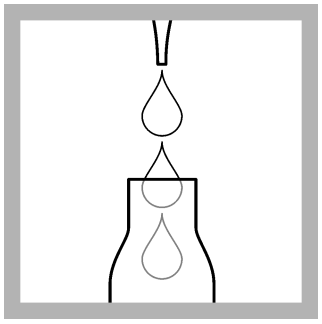
The pH of the water sample must be between pH 3–10.

The temperature of the water sample and reagents must be between 20–24 °C (68–75 °F).

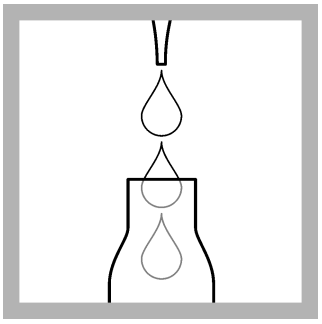
Before starting

In case of not working at the correct recommended temperature an incorrect result may be obtained.
Not more than **3 hours** should elapse between sampling and analysis. **Store in a cool place!**
Review safety information and expiration date on the package.
Review the Safety Data Sheets (MSDS/SDS) for the chemicals that are used. Use the recommended personal protective equipment.
Dispose of reacted solutions according to local, state and federal regulations. Refer to the Safety Data Sheets for disposal information for unused reagents. Refer to the environmental, health and safety staff for your facility and/or local regulatory agencies for further disposal information.

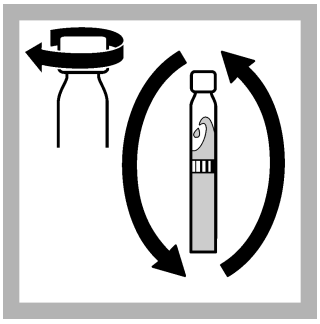
Procedure



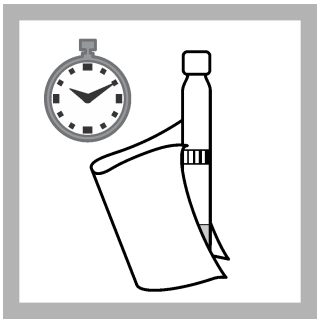
1. Carefully pipet **1.0 mL** of **sample**.



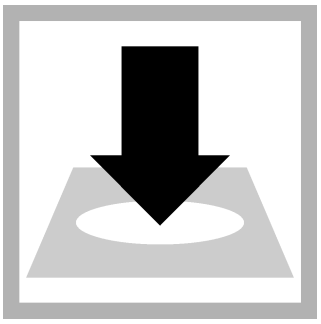
2. Carefully pipet **0.2 mL** of **solution A**.



3. Close the cuvette and invert a few times until **no more streaks** can be seen.



4. After **15 minutes**, thoroughly clean the outside of the cuvette and evaluate.



5. Insert the cuvette into the cell holder.
DR 1900: Go to LCK/TNTplus methods.
Select the test, push **READ**.

Interferences

The ions listed in the table have been individually checked against the given concentrations and do not cause interference. The cumulative effects and the influence of other ions have not been determined.

High loads of oxidizable organic substances (COD) cause the reagent to change color and to give high-bias results. The test can only be used for waste water analyses if the COD is less than 200 mg/L.

The measurement results must be subjected to plausibility checks (dilute and/or spike the sample).

Removal of Interferences

Nitrite concentrations of more than 2.0 mg/L interfere (high-bias results) and can be removed by the addition of a spatula-tip full of amidosulphonic acid. The chloride can be precipitated out as silver chloride by adding silver sulphate. High calcium concentrations cause turbidity. This interferes with the determination but can be prevented by adding a spatula-tip full of EDTA to the sample.

Interference level	Interfering substance
500 mg/L	K^+, Na^+, Cl^-
100 mg/L	Ag^+
50 mg/L	Pb^{2+}, Zn^{2+}, Ni^{2+}, Fe^{3+}, Cd^{2+}, Sn^{2+}, Ca^{2+}, Cu^{2+}
10 mg/L	Co^{2+}, Fe^{2+}
5 mg/L	Cr^{6+}

Summary of method

Nitrate ions in solutions containing sulphuric and phosphoric acids react with 2.6-dimethylphenol to form 4-nitro-2.6-dimethylphenol.

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10/2019, Edition 1

B.2 QIAquick ® PCR Purification Kit (Qiagen)

July 2018

Quick-Start Protocol

QIAquick® PCR Purification Kit
QIAquick® PCR & Gel Cleanup Kit

The QIAquick PCR Purification Kit and the QIAquick PCR & Gel Cleanup Kit (cat. nos. 28104, 28106, 28506 and 28115) can be stored at room temperature (15–25°C) for up to 12 months if not otherwise stated on label.

Further information

- *QIAquick Spin Handbook*: **www.qiagen.com/HB-1196**
- Safety Data Sheets: **www.qiagen.com/safety**
- Technical assistance: **support.qiagen.com**

Notes before starting

- This protocol is for the purification of up to 10 µg PCR products (100 bp to 10 kb in size).
- Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).
- All centrifugation steps are carried out at 17,900 x g (13,000 rpm) in a conventional table-top microcentrifuge at room temperature.
- Add 1:250 volume pH indicator I to Buffer PB. The yellow color of Buffer PB with pH indicator I indicates a pH ≤7.5. The adsorption of DNA to the membrane is only efficient at pH ≤7.5. If the purified PCR product is to be used in sensitive microarray applications, it may be beneficial to use Buffer PB without the addition of pH indicator I; do not add pH indicator I to buffer aliquots.
- Symbols: ● centrifuge processing; ▲ vacuum processing.

Sample to Insight

QIAGEN

1. Add 5 volumes Buffer PB to 1 volume of the PCR reaction and mix. If the color of the mixture is orange or violet, add 10 µl 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn yellow.
2. Place a QIAquick column in ● a provided 2 ml collection tube or into ▲ a vacuum manifold. For details on how to set up a vacuum manifold, refer to the *QIAquick Spin Handbook*.
3. To bind DNA, apply the sample to the QIAquick column and ● centrifuge for 30–60 s or ▲ apply vacuum to the manifold until all the samples have passed through the column. ● Discard flow-through and place the QIAquick column back in the same tube.
4. To wash, add 750 µl Buffer PE to the QIAquick column ● centrifuge for 30–60 s or ▲ apply vacuum. ● Discard flow-through and place the QIAquick column back into the same tube.
5. Centrifuge the QIAquick column once more in the provided 2 ml collection tube for 1 min to remove residual wash buffer.
6. Place each QIAquick column in a clean 1.5 ml microcentrifuge tube.
7. To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water (pH 7.0–8.5) to the center of the QIAquick membrane and centrifuge the column for 1 min. 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.
8. If the purified DNA is to be analyzed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel.

B.3 Water quality parameters and reference range for rearing of Atlantic salmon in lab-scale RAS

Table B1: Water quality parameters, measuring frequency and the reference range used during the rearing of Atlantic salmon in lab-scale RAS.

Parameter	**Frequency**	**Reference range**
Dissolved oxygen (DO)	Twice a day	>6 mg/L
Temperature	Twice a day	11-12 °C
pH	Twice a day	6.5-7.5
Conductivity	Once a day	-
Total dissolved solids (TDS)	Once a day	-
TAN	Every second day	<2 mg/L
Nitrite (NO_2^-)	Every second day	<1 mg/L
Nitrate (NO_3^-)	Every second day	<100 mg/L

B.4 Daily monitoring sheet used for the lab-scale RAS in the fish experiment

Daily fish and RAS monitoring sheet **RAS B**

Day of Experiment & Date/Day :
Personnel :

Fish welfare

Tank	Normal Feeding*		Normal gill movement*		Absence of obvious stress*		Absence of sores/lesions*	
1								
2								
3								

*Tick the box

Water quality parameters

Daily	Value		Every other day	Value
	AM	PM		
DO (mg/L)			TAN (mg L^{-1})	
pH			Nitrite (mg L^{-1})	
Temperature (°C)			Nitrate (mg L^{-1})	
TDS (ppm)			Alkalinity (mg $CaCO_3$ L^{-1})	
Conductivity (mS/cm)			COD (mg O_2 L^{-1})	

Tank and system condition

AM		PM	
Aeration		Aeration	
Water Flow		Water Flow	

Note:

B.5 API® Freshwater Test Kit

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.

B.6 ZymoBIOMICS™ 96 MagBead DNA Kit (D4308) (ZymoResearch)

ZYMO RESEARCH

Microbiomics
Made Simple™

ZymoBIOMICS™ 96 MagBead DNA Kit

DNA for microbiome or metagenome analyses

Highlights

- **Validated Unbiased for Microbiome Measurements:** Unbiased cellular lysis validated using the ZymoBIOMICS™ Microbial Community Standard.
- **Inhibitor-Free DNA from Any Sample:** Isolate ultra-pure DNA ready for any downstream application.
- **Certified Low Bioburden:** Boost your detection limit for low abundance microbes.
- **Simple Workflow:** Simply bead-beat sample, purify via spin-plate, and filter to remove PCR inhibitors. No precipitation or lengthy incubations!

Catalog Numbers:
D4302, D4306, D4308

Scan with your smart-phone camera to view the online protocol/video.

tech@zymoresearch.com www.zymoresearch.com Toll Free: (888) 882-9682

3. Centrifuge the **BashingBead™ Lysis Module**:
    a. If using **ZymoBIOMICS™ BashingBead™ Lysis Rack (0.1 & 0.5 mm)**, centrifuge at ≥ 4,000 x *g* for 5 minutes.
    b. If using **ZR BashingBead™ Lysis Tubes (0.1 & 0.5 mm)**, centrifuge at ≥ 10,000 x *g* for 1 minute.

4. Transfer up to 200 µl 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.*

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).

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[5].

16. Transfer the supernatant (containing the eluted DNA) to a clean elution plate or tube[6].

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

[5] In some cases a brown-colored pellet may form at the bottom of the tube after centrifugation. Avoid this pellet when collecting the eluted DNA.
[6] If fungi or bacterial cultures were processed; the DNA is now suitable for all downstream applications.

B.7 SequalPrep™ Normalization Plate (96) Kit (Invitrogen)

invitrogen™

SequalPrep™ Normalization Plate (96) Kit

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

Contents and Storage

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

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

Description

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

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

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

System Overview

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

System Specifications

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

Accessory Products

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

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

Part no: 100003531 Rev. date: 5 May 2008

General Guidelines

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

Generating PCR Amplicon

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

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

Sample Amount

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

Elution Options

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

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

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

Materials Needed

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

Binding Step

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

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

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

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

Washing Step

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

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

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

3. Completely aspirate the buffer from wells and discard.

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

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

Elution Step

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

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

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

2. Mix by pipetting up and down 5 times or seal the plate with PureLink™ Foil Tape (page 1), vortex to mix, and briefly centrifuge the plate. Ensure that the buffer contacts the entire plate coating (up to 20 µl level).

3. Incubate at room temperature for 5 minutes.

4. Transfer and pool the purified DNA as desired or store the eluted DNA at 4°C (short-term storage) or –20°C (long-term storage) until further use.

Expected Yield and Concentration

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

Optional: DNA Quantitation

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

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

Downstream Applications

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

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

Continued on next page

Troubleshooting

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

Quality Control

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

Limited Use Label License No. 5: Invitrogen Technology

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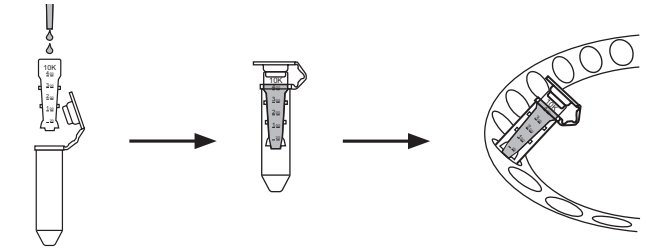
How to Use Amicon Ultra-0.5 Centrifugal Filter Devices

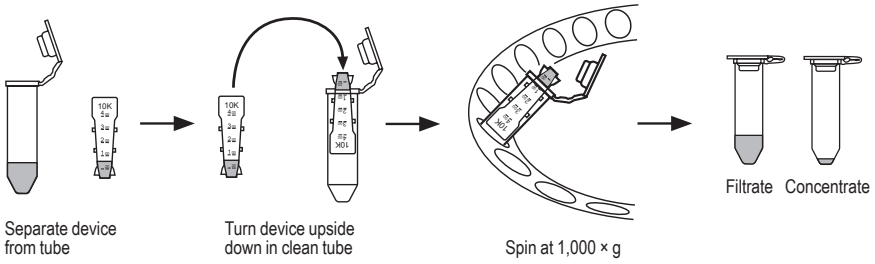
1. Insert the Amicon Ultra-0.5 device into one of the provided microcentrifuge tubes.
2. Add up to 500 µL of sample to the Amicon Ultra filter device and cap it.
3. Place capped filter device into the centrifuge rotor, aligning the cap strap toward the center of the rotor; counterbalance with a similar 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.



B.9 Targeted PCR Mastermix

Table B2: PCR Mastermix used in the amplification of the v3- and v4-regions of the 16S rDNA gene.

Reagent	**Final concentration**	**Volume per reaction**
DNA-free H_2O		Up to final reaction volume
5x Phusion buffer HF (7,5 mM $MgCl_2$)	1x	5.0 µŁ
Ill341F_KI (10 µM)	0.3 mM	0.75 µŁ
Ill805R (10 µM)	0.3 mM	0.75 µŁ
dNTP (10 mM each)	200 µM each	0.5 µŁ
Phusion Hot Start DNA polymerase (2 units/µŁ)	0.02 units/µŁ	0.18 µŁ
Template		1 µŁ

B.10 Indexing PCR Mastermix

Table B3: PCR Mastermix used in the indexing step in the amplicon library preparation for Illumina sequencing of the v3- and v4-regions in the 16S rDNA gene.

Reagent	**Volume per reaction**
DNA-free H_2O	To total reaction volume of 25 µŁ
5x Phusion buffer HF (7,5 mM $MgCl_2$)	5.0 µŁ
dNTP (10 mM each)	0.5 µŁ
Phusion Hot Start DNA polymerase (2 units/µŁ)	0.19 µŁ
Index 1	2.5 µŁ
Index 2	2.5 µŁ
Template (normalized from targeted PCR)	2.5 µŁ

C Raw data and workflow-diagrams

C.1 Raw data obtained during the batch experiment

Table C1: Raw data for TAN, nitrite- and nitrate-N concentrations measured with the Hach-Lange™ method during the batch experiment. NR=Negative range, UMR=Under measurement range

Time (min)	[NH_4-N] (mg/L)	[NO_2-N] (mg/L)	[NO_3-N] (mg/L)	Remarks
0	10.9	-0.052	0.083	NR-NO2, UMR-NO3
20	9.76	-0.038	0.037	NR-NO2, UMR-NO3
40	9.5	-0.037	1.53	NR-NO2
60	9.09	-0.029	2.06	NR-NO2
125	7.68	-0.021	3.14	NR-NO2
160	6.89	-0.017	3.77	NR-NO2
180	6.47	-0.019	3.85	NR-NO2
220	5.48	-0.009	4.68	NR-NO2
240	5.16	0.001	4.71	UMR-NO2
280	4.46	-0.009	6.31	NR-NO2
320	3.44	0.001	6.58	UMR-NO2

Table C2: Raw data for NO2-N and NO3-N concentrations in the batch reactor for testing nitrite oxidation capacity in biofilm carriers from Lerøy, measured with the Hach-Lange™ method. UMR=Under measurement range.

Time (min)	[NO_2-N] (mg/L)	[NO_3-N] (mg/L)	Remarks
0	4.66	3.3	
10	3.43	3.37	
30	2.74	3.55	
40	2.12	5.25	
50	1.54	5.37	
60	0.886	5.69	
70	0.393	5.86	UMR-NO2

C.2 Work-flow for the cultivation of the four opportunistic strains used in the fish experiment

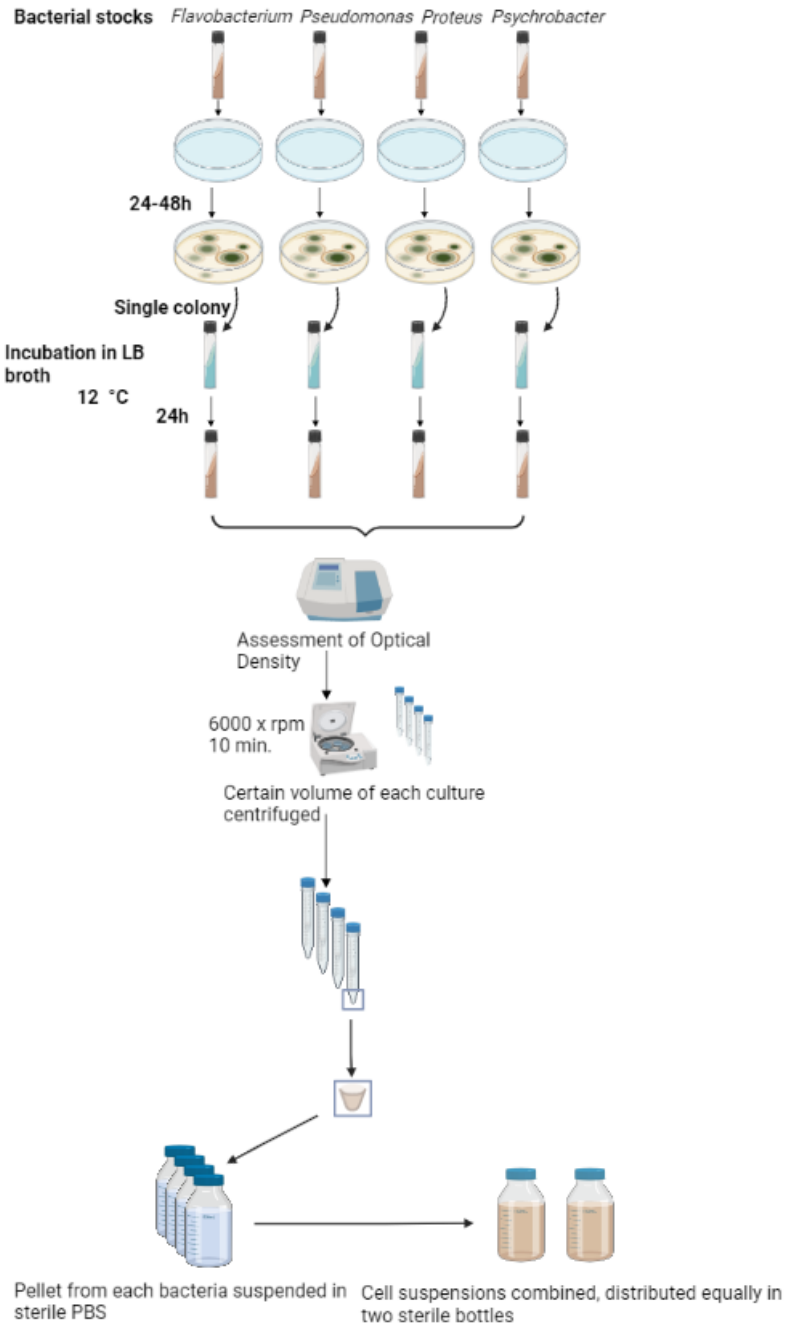


Figure C1: Illustration of the workflow for the cultivation of a defined cell density of the four opportunistic strains introduced to the two lab-scale RAS. LB = lysogeny broth, PBS = phosphate-buffered saline. The illustration was created with BioRender (biorender.com).

D Supplementary results

D.1 Colony-forming units (CFUs) counted during the colonization experiment

Table D1: Colony forming units (CFU) counted on the TSA plates of each strain, initial treatment (CFU/mL) introduced to each flask) and dilution of the cell extract detached from the biofilm carriers.

Bacterial isolate	**Initial CFU/mL**	**10^{-1}**	**10^{-2}**	**10^{-3}**	**10^{-4}**	**10^{-5}**
Proteus	10^3	> 300	> 300	240	53	0
Psychrobacter	10^3	> 300	121	58	3	0
Flavobacterium	10^3	> 300	> 300	12	3	0
Pseudomonas	10^3	> 300	> 300	204	10	0
Proteus	10^5	> 300	> 300	200	25	4
Psychrobacter	10^5	> 300	> 300	408	716	193
Flavobacterium	10^5	> 300	384	49	4	0
Pseudomonas	10^5	> 300	167	21	10	0
Feed medium only	-	0	0	0	0	0
TSB medium only	-	0	0	0	0	0

D.2 Sanger sequences of the 16s rDNA gene for the bacterial isolates used in the fish experiment

***>Proteus* sp.**

GGGAAAGCTTGCTTTCTTGCTGACGAGCGGCGGACGGGTGAGTAATGTATGGGGATCTGCCCGATAGAGGGGGATAACTACTGGAAACGGTGGCTAATACCGCATAAT
GTCTACGGACCAAAGCAGGGGCTCTTCGGACCTTGCACTATCGGATGAACCCATATGGGATTAGCTAGTAGGTGAGGTAATGGCTCACCTAGGCGACGATCTCTAGCTG
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GTTGTGGTCTTGAACCGTGGCTTCTGGAGCTAACGCGTTAAATCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGC
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GCGAGAGCAAGCGGAACTCATAAAGTCTGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTAGATCAGAATGCTACGGTGA
ATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCAAAAGAAGTAGGTAGCT++ACCTTCGGGAGGGCGCTTACCACTTTG

***>Psychrobacter* sp.**

GTAACAGGAGAAGCTTGCTTCTCGCTGACGAGCGGCGGACGGGTGAGTAATACTTAGGAATCTACCTAGTAGTGGGGGATAGCACGGGGAAACTCGTATTAATACCGC
ATACGACCTACGGGAGAAAGGGGGCAGTTTACTGCTCTCGCTATTAGATGAGCCTAAGTCGGATTAGCTAGATGGTGGGGTAAAGGCCTACCATGGCGACGATCTGTAG
CTGGTCTGAGAGGATGATCAGCCACACCGGGACTGAGACACGGCCCGGAYTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGGGAAACCCTGATCCAGCC
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GGCTTAACCTGGGAACTGCATCTGAAACTGTAGAGCTAGAGTATGTGAGAGGAAGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGAT
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***>Pseudomonas* sp.**

GAGCGGATGACAGGAGCTTGCTCCTGAATTCAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGACAACGTTTCGAAAGGAACGCTAATACC
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GCCAAGCCGCGAGGTGGAGCTAATCCCATAAAACCGATCGTAGTCCGGATCGCAGTCTGCAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGTGAATCAGAATGT
CACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCACCAGAAGTAGCTAGTCTAACCTTCGGGAGGACGGTTACCACGGTGT
GA

***>Flavobacterium* sp.**

AGGGGTATATGTCTTTCGGATATAGAGACCGGCGCACGGGTGCGTAACGCGTATGCAATCTACCTTTTACAGAGGGATAGCCCAGAGAAATTTGGATTAATACCTCATAG
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TCCGTGCCAGCAGCCGCGGTAATACGGAGGATCCAAGCGTTATCCGGAATCATTGGGTTTAAAGGGTCCGTAGGCGGTTTAATAAGTCAGTGGTGAAAGCCCATCGCTC
AACGGTGGAACGGCCATTGATACTGTTAAACTTGAATTATTAGGAAGTAACTAGAATATGTAGTGTAGCGGTGAAATGCTTAGAGATTACATGGAATACCAATTGCGAAGG
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AGGAAGGTGGGGATGACGTCAAATCATCACGGCCCTTACGCCTTGGGCTACACACGTGCTACAATGGCCGGTACAGAGAGCAGCCACTGGGCGACCAGGAGCGAATC

Figure D2: Sanger sequences of the 16S rDNA gene of four strains identified as *Proteus* sp., *Psychrobacter* sp, *Pseudomonas* sp., and *Flavobacterium* sp.

D.3 pH in the lab-scale RAS

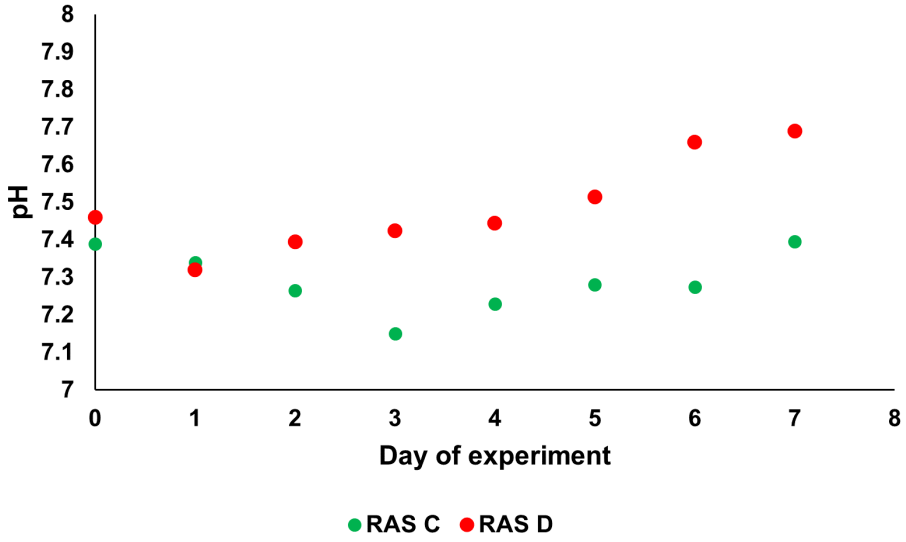
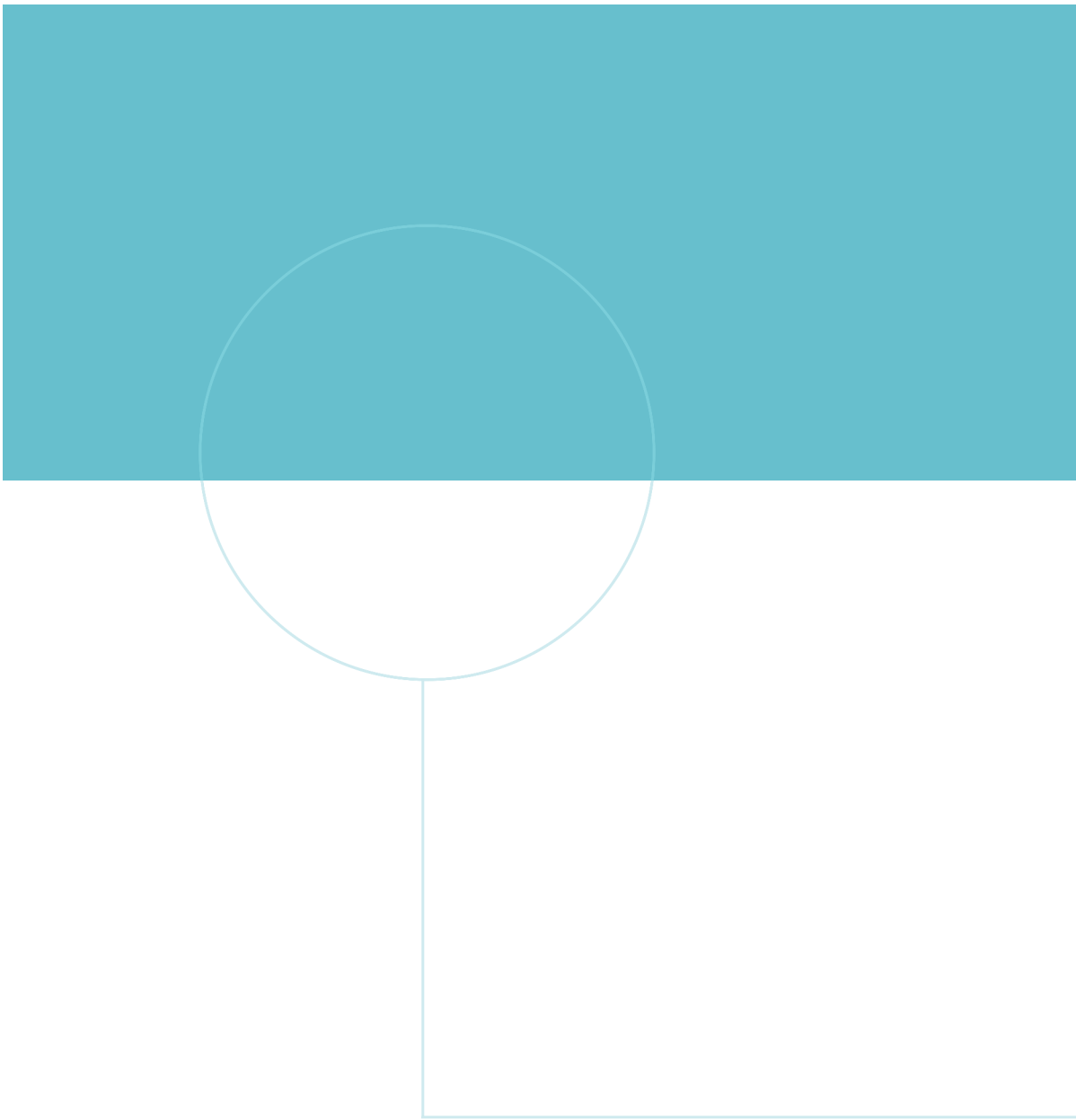


Figure D3: pH in the rearing tanks of the two lab-scale RAS during the experiment (D0-D7). On days where pH was recorded twice (morning and afternoon), the value is calculated as the average pH.

D.4 Individual weight and length of sampled fish

Table D2: Weight (g) and total length (cm) of sampled individuals, (mean ± SD) in the two lab-scale RAS at D0 and D8 of the experiment.

Day of experiment	RAS	Weight (g)	Total length (cm)
0	C (non-disinfected biofilter)	13.9 ±1.4	10.6 ± 0.5
	D (disinfected biofilter)	13.0 ± 3.6	11.2 ± 0.6
8	C (non-disinfected biofilter)	14.4 ± 3.7	10.9 ± 0.8
	D (disinfected biofilter)	16.1 ± 1.2	11.6 ± 0.3



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