Master's thesis

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Characterising temporal dynamics in microbial communities from biofilm and water during commercial production of Atlantic salmon smolts in RAS

Master's thesis in Ocean Resources

Supervisor: Ingrid Bakke

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Acknowledgements

This master thesis was part of the SINTEF Ocean research project "Monitoring microbiota in closed aquaculture systems (MonMic)" led by Stine Wiborg Dahle. The MonMic project was a collaboration between SINTEF Ocean and five commercial producers of Atlantic salmon smolts in recirculating aquaculture systems (RAS). The work of this thesis was mainly performed at NTNUs Centre of Fisheries and Aquaculture (SeaLab) at Brattørkaia and at the Department of Biotechnology and Food Science at NTNU Gløshaugen, Trondheim. In addition, some work was done at the studied RAS-facility.

First, I want to thank my main supervisor Ingrid Bakke at the Department of Biotechnology and Food Science, for always keeping the door open whenever I had questions. The feedback and knowledge you have provided me with throughout this process has been crucial for the result of my thesis.

I would also like to acknowledge my co-supervisor Stine Wiborg Dahle for valuable comments and for always being helpful and understanding during challenging times. A most sincere thank you to my other co-supervisor Kari Johanne Kihle Attramadal for introducing me to RAS and including me in the MonMic project. Your knowledge and passion for RAS is genuinely inspiring!

I wish to thank the staff at SINTEF Ocean for being welcoming and helpful, especially with details on methodology and laboratory work in the MonMic project. I also wish to thank the staff at the studied RAS-facility for always welcoming me and for providing vital information whenever I asked.

Most of all I want to thank my fiancé and my family for always believing in me and encouraging me. Lastly, I want to thank my friends and classmates for making my years as a student at NTNU memorable and fun.

Trondheim, June 2020

Sunniva Ingebrigtsen Gaarden

Abstract

Recirculating aquaculture systems (RAS) have become increasingly popular as a production system for Atlantic salmon smolts *(Salmo salar)* in Norway. Advantages such as the possibility of intensive and continuous production at optimal temperatures throughout the year, in addition to low freshwater consumption are some of the main reasons for the increasing popularity. Microorganisms can have great impacts on water quality and fish health in RAS. However, little is known about microbial communities in different units of commercial RAS during normal operation.

This study aimed to characterise the microbial communities in biofilms and water from a commercially operated RAS, during start-feeding of Atlantic salmon smolts. To study the microbial communities, samples for microbial community analysis were collected biweekly over 15 months from four different units inside a RAS-loop: water and biofilm samples from each of two rearing tanks, biofilm samples from a biofilter and water samples from a water sump downstream from the biofilter. A total of 245 samples were subjected to microbial community analysis by Illumina sequencing of 16S rDNA amplicons. To supplement the microbial community analyses, additional water samples were taken to quantify the number of bacteria, estimate the ratio of opportunistic bacteria, the bacterial growth potential and the bacterial cultivability.

The most abundant orders in both biofilm and water samples were Rhodobacterales, Burkholderiales and Sphingomonadales. Moreover, the microbial communities in biofilm and water were found to be significantly different from each other. Microbial communities in rearing tanks and the treated water in the water sump were similar, but varied over time. The similarity in water microbiota throughout the sampled RAS were believed to be caused by a low HRT in the rearing tanks. There were more slow-growing bacteria and a lower bacterial growth potential in the treated water in the water sump compared to the rearing tank water, suggesting a better microbial water quality in the treated water compared to the rearing tank water. The microbiota of rearing tank biofilm was significantly different from communities within biofilm from the biofilter. The biofilm communities in the biofilter were found to be more stable over time than biofilm communities from the rearing tanks. Large variations in relative abundance of nitrifiers were found in the biofilter biofilm. *Nitrospira* was the most abundant nitrifier, and one of the *Nitrospira* OTUs were found to be related to previously characterised COMAMMOX. There were in general low abundances of ammonia-oxidising bacteria.

This thesis has provided new knowledge on the complex microbial communities in start-feeding of salmon smolts in a commercial RAS. The results can be implemented in further research, to improve microbial management of fish production in RAS.

Sammendrag

Resirkulerende akvakultursystemer (RAS) har økt i popularitet i norsk oppdrett av atlantisk laksesmolt (*Salmo salar*). Muligheten til intensiv og kontinuerlig produksjon ved optimale temperaturer gjennom hele året, i tillegg til et lavt ferskvannsforbruk er noen av grunnene til at RAS har økt i popularitet i Norge. Selv om mikroorganismer kan ha en stor effekt på vannkvalitet og fiksehelse i RAS, finnes det lite kunnskap om de mikrobielle samfunnene under normal kommersiell drift.

Denne studien hadde som mål å karakterisere de mikrobielle samfunnene i biofilm og vann fra et kommersielt driftet RAS-anlegg, ved kommersiell startfôring av laksesmolt. For å studere disse mikrobielle samfunnene ble det tatt prøver annenhver uke, i over 15 måneder. Prøvene ble tatt fra fire ulike deler i RAS-loopen: Biofilm- og vannprøver fra to fisketanker, biofilm fra et biofilter og vann fra en sump etter biofilteret. Totalt ble 245 prøver sendt inn til Illumina-sekvensering av 16S rRNA genet og disse lå til grunn for den videre analysen av den mikrobielle sammensetningen. I tillegg til disse prøvene ble det tatt vannprøver for å estimere antall bakterier, andelen opportunistiske bakterier, det bakterielle vekstpotensialet og den bakterielle kultiverbarheten.

De mest tallrike ordenene i både biofilm- og vannprøver var Rhodobacterales, Burkholderiales og Sphingomonadales. De mikrobielle samfunnene var signifikant forskjellig fra hverandre i biofilm og vannprøver. De mikrobielle samfunnene i vannprøver var like i fisketankene og det behandlede vannet fra vannsumpen, men varierte over tid. Den gjennomgående likheten i vannmikrobiota var mest sannsynlig forårsaket av en lav HRT i fisketankene. Det var flere sakte-voksende bakterier og et lavere bakterielt vekstpotensial i det behandlede vannet i vannsumpen sammenlignet med vann fra fisketankene, dette foreslår at den mikrobielle vannkvaliteten var bedre i det behandlede vannet sammenlignet med vann fra fisketankene. Mikrobiotaen i biofilm fra fisketankene var signifikant forskjellig fra samfunnene i biofilm fra biofilteret. Biofilmsamfunnene i biofilteret var mer stabile i sammensetning enn biofilmsamfunn fra fisketankene. Det ble funnet store variasjoner i relativ forekomst av nitrifiserende i biofilm fra biofilteret. *Nitrospira* var den mest vanlige nitrifiserende i biofilteret og det ble oppdaget at en av *Nitrospira*-OTUene var i slekt med tidligere karakteriserte COMAMMOX bakterier. I tillegg var det lav relativ forekomst av ammoniakk-oksiderende bakterier.

Denne masteroppgaven har gitt ny kunnskap om de komplekse mikrobielle samfunnene i startfôring av laksesmolt i et kommersielt RAS. Resultatene kan implementeres i fremtidig forskning for å forbedre hvordan vi forvalter mikrober i fiskeproduksjon i RAS.

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1. Introduction

As the world population increases towards 9.7 billion in 2050, an ongoing issue is to meet the growing food demand sustainably. Aquaculture is currently the fastest growing food-producing sector in the world and has been responsible for the continuing growth in seafood-supply for human consumption since the peak in capture fisheries in the late 1980s (FAO 2018). Norway is the biggest producer of Atlantic salmon (*Salmo salar*) in the world (FAO 2018), producing over 1.28 million tonnes in 2018 (Norwegian Directorate of Fisheries 2020). Despite the ambitions of a fivefold increase in production by 2050 (DKNVS and NTVA 2012), the production-volume of Atlantic salmon in Norway has been relatively stable since 2012, varying between 1.2-1.3 million tonnes (Norwegian Directorate of Fisheries 2020). Several challenges have resulted in the stagnating growth in the Norwegian aquaculture sector, such as issues with sea-lice, pathogens, escapees (Forseth et al. 2017), limited access to freshwater (Kittelsen et al. 2006), and an increased focus on environmental sustainability (Tal et al. 2009). As a result, a current trend in Norwegian aquaculture is to move more of the production on land.

1.1 Production cycle and biology of Atlantic salmon smolts

Atlantic salmon (*S. salar*) is an anadromous fish species, spending its first life stages in freshwater before entering the sea after going through an adaptation process called smoltification (Røsvik 1997). The life cycle of Atlantic salmon can be divided into six stages: egg, alevin, fry (Figure 1.1), parr, smolt, and adult salmon. In Norwegian aquaculture, the earliest life stages, from egg to smolt, are traditionally produced in land-based systems, while the grow-out phase, from smolt to adult salmon, takes place in net cages at sea (Bergheim et al. 2009).

Figure 1.1. Atlantic salmon fry (Picture from Skretting)

The fertilised salmon eggs hatch after approximately 500 day-degrees (the number of days times the degrees Celcius in the water) and the alevins live off their yolk sac for around 300 additional day-degrees before they start eating formulated feed (Røsvik 1997). It takes around 8-18 months from the eggs hatch until smoltification at around 100 g, depending

on temperature, feeding, and light-regime (Havforskningsinstituttet 2019). In 2007, around 40 % of all smolts produced in Norway were under-yearlings, meaning that they were transferred to sea less than a year after hatching (Bergheim et al. 2009). Lately, following a lift in the ban of producing smolts over 250 g in 2012, the trend is to produce bigger fish on-land, called post-smolts, from 250-1000 grams (Dalsgaard et al. 2013), making even more of the production land-based. While the number of active licences for smolt production in Norway has decreased from 265 in 2000 to 184 in 2018, the production per license has increased considerably. In 2018 it was sold nearly 350 million Atlantic salmon smolts for further cultivation at sea. This was over 220 million more than in 2000 (Norwegian Directorate of Fisheries 2020), reflecting a substantially increased productivity in smolt production in Norway (Bergheim et al. 2009).

1.2 Land-based aquaculture in recirculating aquaculture systems (RAS)

Land-based aquaculture systems can be separated into two main types depending on how the water in the system is used; flow-through systems and re-use systems (Figure 1.2). In flow-through systems, the water is only used once, whereas, in re-use systems, the water is recycled inside the system and used several times (Lekang 2013). Aquaculture systems that re-use the water are also called recirculating aquaculture systems (RAS).

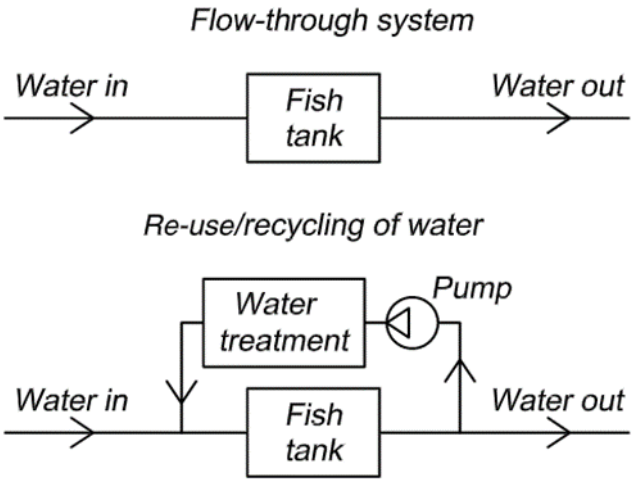

Figure 1.2. Schematic overview of a flow-through system (top) and a recirculating aquaculture system (RAS) (bottom) (Lekang 2013)

RAS have become increasingly popular as a production system for Atlantic salmon smolts in Norway, mainly because of the possibility of intensive and continuous production at optimal temperatures throughout the year, and low freshwater consumption. It is also easier to control the rearing conditions in RAS to create an optimal environment for the reared fish species. Controlling the rearing temperature gives the advantage of a highly predictable growth-rate and, thereby, a highly predictable production schedule (Ebeling and Timmons 2012). Other advantages, such as full control over more concentrated waste streams and thereby less environmental impacts, is also of increasing importance (Tal et al. 2009). As a result, the number of RAS in Norway are increasing rapidly, and almost

every new land-based system built in Norway today are RAS (Dalsgaard et al. 2013). Many farmers also plan to convert their flow-through systems to RAS (Bergheim et al. 2009). However, RAS also has some drawbacks, including high energy requirements, high initial investments cost, and increased complexity, which underlines the importance of qualified employees that can operate the systems (Badiola, Mendiola, and Bostock 2012).

In order to reuse the water in RAS, the water must be treated. The treatment loop consists of several water treatment steps in a more or less specific order. In a typical RAS, there is first a particle removal step, where particles from excess feed and fish faeces are removed. Subsequently, there is a biofilter where toxic ammonia from fish excretion is converted to less toxic nitrate by autotrophic nitrifying bacteria. Downstream from the biofilter is typically a degasser that airs out carbon dioxide (CO_2) from both bacteria- and fish metabolism. After the degasser, the water is oxygenated before re-entering the rearing tanks (Ebeling and Timmons 2012). In addition, pH- and alkalinity regulation is an important step, as a change in pH affects almost all other water-quality parameters. Some RAS also include disinfection in the RAS-loop, with ultraviolet irradiation or ozone-gas (O_3) as proactive measures to avoid diseases caused by bacteria, viruses, or fungi (Fjellheim et al. 2016). To account for evaporation and to dilute the end-metabolite from the biofilter, nitrate, new water must be added. This water must be thoroughly treated before entering the RAS-loop to ensure that unwanted substances do not enter.

1.2.1 Nitrifying biofilters in RAS

There are numerous different strategies and designs of nitrifying biofilters in RAS. Most RAS use fixed-film biofilters, as opposed to suspended growth systems, and these hold media such as plastic, sand, or rocks to support the growth of bacterial biofilms. These bacterial biofilms take up and degrade waste contaminants as the contaminated water flows through the biofilter (Vaccari, Strom, and Alleman 2006). The main goal of a nitrifying biofilter is to provide optimal conditions for nitrifying bacteria to grow. This includes providing a large surface area for microbial growth while simultaneously providing optimal conditions for the desired microorganisms (Fjellheim et al. 2016). Plastic pieces explicitly designed for biofilm-growth, with a large surface to volume ratio, are probably the most popular media in biofilters in Norwegian RAS.

Different types of submerged biofilters, including fixed bed (FBB)- and moving bed biofilters (MBB), are the most common in RAS for the production of salmon smolts (Fjellheim et al. 2016). As these biofilters are submerged in water and due to the oxygen consumption of nitrifying bacteria, they need additional supply of oxygen. The bacteria in the biofilter also produce carbon dioxide (CO_2), which makes it a logical step to have an aeration or degasser downstream from the biofilter. In a fixed bed biofilter (FBB), the media for biofilm-growth is fixed, while the water and air flow upwards (Figure 1.3A; Lekang 2013). FBBs trap small particles from the RAS-loop, and must be frequently washed to avoid clogging (Fjellheim et al. 2016). Washing of FBBs are often done by high pressure backflushing. In moving bed biofilters (MBB); Figure 1.3B), the biofilter-medium is suspended in water and kept in motion by the supplied oxygenation and the up-flowing water current. These biofilters are self-cleaning as the turbulence in the water knocks the media against each other, washing off excess biofilm growth. MBBs thereby release particles from biofilm growth into the RAS-loop (Fjellheim et al. 2016).

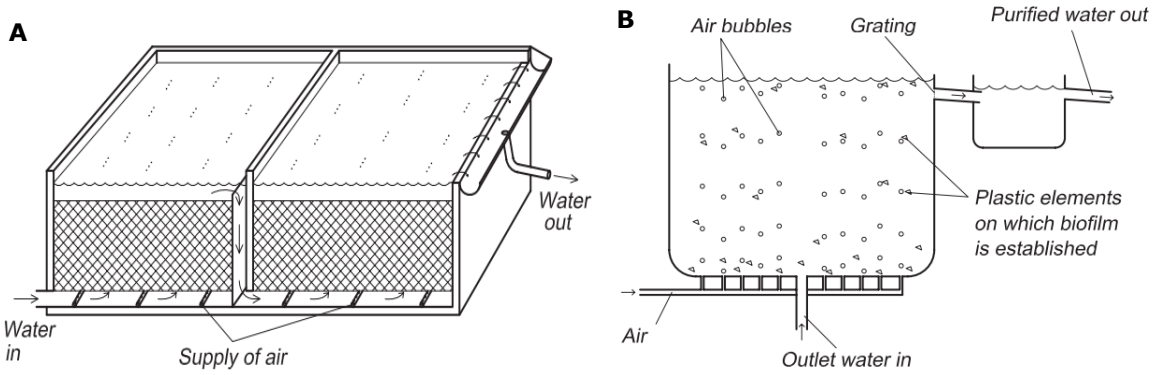

Figure 1.3. Examples of submerged biofilters. A) An up-flowing fixed bed biofilter. B) A moving bed biofilter. Both figures are from Lekang 2013.

1.3 Microbiology in RAS

Microorganisms are central for fish health and water quality in RAS and are found in large quantities in the water and biofilm that surrounds the cultivated fish. Microorganisms are introduced through several sources such as feed, make-up water, air, employees, and the fish itself (Rurangwa and Verdegem 2014), but also grow in the system. The microbial community composition is decided by a selection pressure in the system, and the selection pressure is affected by several different factors. Because the sources contribute with their unique microbial flora and the selection depends on system design and operational routines, the composition and diversity of microorganisms vary from RAS to RAS (Blancheton et al. 2013). Bacteria have both positive and negative effects on the water quality and the reared fish. Today, little is known about the interactions between different microbial communities in RAS, which include the microbiota associated with the fish, the suspended free-living bacteria in the water and biofilm communities on system surfaces and in the biofilter. Microorganisms are essential for maintaining good water quality, especially considering the biofilter, where autotrophic nitrifying bacteria convert toxic ammonia (NH_3) to nitrate (NO_3^-)(Ruiz et al. 2019). However, there are many other groups of bacteria in RAS that affect the physicochemical- and microbial water quality. When discussing bacteria in RAS it is practical to look at two main groups: the autotrophic nitrifying bacteria which mainly occur in biofilm in the biofilter, and the heterotrophic bacteria which are widely distributed throughout the whole RAS-system (Blancheton et al. 2013).

1.3.1 Autotrophic nitrifying bacteria converting ammonia

Ammonia is naturally excreted from the reared fish, but it is also a product from bacterial degradation of available organic material in the RAS. Removing ammonia is especially vital in the production of Atlantic salmon, as salmonids are known to be particularly sensitive to ammonia (Rosseland 1999). Ammonia (NH_3) is in equilibrium with the ammonium ion (NH_4^+) in the RAS-water (Eq. 1.1), and the sum of the two forms is called the total ammonia nitrogen (TAN). The suggested threshold for TAN in Norwegian aquaculture of Atlantic salmon is <2 mg/L (Hjeltnes et al. 2012). Ammonia gas (NH_3), which is dissolved in the RAS-water, is the most toxic of the two forms and the concentration of ammonia compared to the ionised ammonium, is primarily determined by the pH. A high pH, exceeding 7, shifts the equilibrium towards the left, resulting in more of the toxic ammonia

gas (Rosseland 1999). Exposure to toxic ammonia concentrations affects respiration, osmoregulation, stress-hormones, and the tissue structure of gills, liver, and kidney in fish (Fjellheim et al. 2016).

$$NH_3 + H^+ \leftrightarrow NH_4^+ \quad \text{(Eq. 1.1)}$$

The aerobic nitrification process in the biofilter performed in two steps by two main groups of bacteria: the first step by ammonia oxidising bacteria (AOB) and the second step by nitrite oxidising bacteria (NOB), as seen in equation 1.2. and 1.3, respectively (Lekang 2013). AOBs such as for example *Nitrosomonas*, *Nitrosospira* and *Nitrosovibrio* use two enzymes: ammonia monooxygenase and hydroxylamine dehydrogenase to oxidise ammonia to nitrite. While NOBs, such as *Nitrobacter*, *Nitrospira,* and *Nitrotoga* use the enzyme nitrite oxidoreductase to oxidise nitrite to nitrate (Ruiz et al. 2019).

Step 1 $$NH_4^+ + 1.5\,O_2 \rightarrow NO_2^- + H_2O + 2\,H^+ \quad \text{(Eq. 1.2)}$$

Step 2 $$NO_2^- + 0.5\,O_2 \rightarrow NO_3^- \quad \text{(Eq. 1.3)}$$

The intermediate in the nitrification process, nitrite, is very toxic to fish. The nitrite ions compete with chloride ions, which are actively taken up over the gills. If fish are exposed to high nitrite levels over a longer period, this can result in oxidation of haemoglobin to methaemoglobin, giving a reduced transport of oxygen in the blood (Kroupova, Machova, and Svobodova 2005). Increasing the chloride concentration in the rearing water could counteract the toxic effects (Kroupova, Machova, and Svobodova 2005). The Norwegian Food Safety Authority have suggested that nitrite concentrations should be kept under 0.1 mg/L in freshwater production of Atlantic salmon smolts (Hjeltnes et al. 2012).

In addition to AOBs and NOBs, other groups of bacteria and archaea have been found to oxidise ammonia and nitrite. One of them are ammonia oxidising archaea (AOA), which use the same enzymes and have the same functions as AOBs, oxidising ammonia to nitrite. AOAs have been found in RAS-biofilters, but their role in nitrification is still unclear (Ruiz et al. 2019; Schreier, Mirzoyan, and Saito 2010). AOAs are found in the phylum Thaumarchaeota, including genera such as *Nitrosoarchaeum*, *Nitrosopumilus* and *Nitrososphaera* (Ruiz et al. 2019).

Furthermore, a newly discovered group of bacteria in the genus *Nitrospira*, called complete ammonia oxidisers (COMAMMOX) have been found to singularly perform the complete oxidation of ammonia to nitrate (Daims et al. 2015; Van Kessel et al. 2015). Although COMAMMOX were only recently discovered, Costa, Pérez, and Kreft (2006) hypothesised the existence of complete ammonia oxidisers over a decade ago. The two COMAMMOX species characterised by Van Kessel et al. (2015), *Nitrospira nitrosa* and *Nitrospira nitrificans,* were found in a trickling filter connected to a RAS cultivating the freshwater species Common carp (Cyprinus carpio) (Van Kessel et al. 2015). Since then, still more COMAMMOX have been discovered and are continued to be discovered (Sobotka et al. 2018).

1.3.2 Heterotrophic bacteria

Heterotrophic bacteria use organic matter to grow, which is available in high doses due to the intensive feeding rates in RAS. Heterotrophic bacteria may be pathogens, and blooms of heterotrophic opportunistic bacteria can be detrimental to fish, especially if the fish already are under stress and live in suboptimal rearing conditions (Vadstein et al. 2018). As heterotrophic bacteria's doubling time is considerably faster than for autotrophic bacteria (hours versus days), they may outcompete the autotrophic bacteria in the competition for oxygen and space (Blancheton et al. 2013). High levels of heterotrophic bacteria are, thereby, considered a problem in RAS, and thus the reduction of available organic matter is a crucial issue (Blancheton et al. 2013). Besides, heterotrophs produce CO_2 and other metabolic by-products, thereby playing a part in the water quality degradation (Fjellheim et al. 2016).

In general, most heterotrophic bacteria are neutral and help to maintain a good microbial water quality by occupying available niches in the RAS, thus preventing colonisation of pathogenic bacteria. Moreover, certain types of heterotrophic bacteria have been shown to have positive effects on the development of morphology and digestive system in fish larvae (Attramadal et al. 2012b; Vadstein, Mo, and Bergh 2004). Heterotrophic bacteria dominate the microbial communities in RAS, even the nitrifying biofilters (Rurangwa and Verdegem 2014). In the biofilter an outer layer of heterotrophic bacteria can protect the autotrophic nitrifiers against detachment and grazing (Blancheton et al. 2013). However, as they quickly outgrow the autotrophic nitrifying bacteria, it is essential to keep this layer thin to sustain an adequate oxygen diffusion to the inner layers of the biofilm where the nitrifying bacteria are situated. A study by Michaud et al. (2006) showed that the nitrification efficiency of submerged biofilters decreased substantially with increasing carbon to nitrogen ratios, enhancing the importance of managing heterotrophic bacteria in RAS. Even though some studies have characterised the microbial communities in RAS-biofilters by deep-sequencing (Bartelme, McLellan, and Newton 2017; Schreier, Mirzoyan, and Saito 2010), there is limited knowledge about the factors that affect the composition of nitrifying communities in RAS-biofilters, as well as the temporal dynamics in community composition over a more extended period.

1.3.3 Microbial water quality and ecology

The bacteria that are associated with the RAS-water are those that mainly interact with the reared fish. These water-associated bacterial communities also contribute to the degradation of readily available organic matter in the water, and thereby affect the water quality. A good microbial water quality in an aquaculture system can be defined as the absence of pathogens and other bacteria that can be detrimental for the reared fish. The ecological theory of r- and K-strategists can be useful to describe and characterise the microbial water quality in aquaculture systems (Vadstein et al. 2018).

Bacteria that are r-selected are defined by their high maximum growth rate, and r-selection often occurs when the microbial density is far from the carrying capacity (the maximum bacterial biomass that can be sustained in the system over time). Most r-selected fast-growing bacteria are opportunistic, meaning that they could cause diseases in stressed and susceptible fish (Vadstein et al. 2018). On the other hand, K-selected bacteria, also called specialists, are adapted to a lower resource availability, making them more successful in crowded environments with high rates of competition. K-selection therefore occurs when the microbial communities approach the carrying capacity. As resources per bacterial cell become scarcer in an environment, the K-selected bacteria will eventually outcompete the

r-selected bacteria (Vadstein et al. 2018). K-selected microbial communities have also been termed microbially matured, and several studies have implied that K-selected microbial environments are more stable than r-selected ones and that this stability is favourable for the cultivated fish and the RAS (Attramadal et al. 2012b; Skjermo et al. 1997; Vadstein et al. 1993). In general, aquaculture systems often select for r-strategists, but studies have suggested that RAS could be a strategy for shifting towards a K-selection of bacteria (Attramadal et al. 2012b). The reason RAS selects for K-selection, more than conventional FTS, is that the water stays in the system for a longer time, creating a long hydraulic retention time (HRT) of the system. The long HRT keeps the slow-growing K-selected bacteria in the system. Moreover, due to the recirculation of the water, it keeps the carrying capacity relatively stable throughout the RAS-loop. However, if disinfection is included in the RAS-loop, the number of bacteria is immediately lowered, resulting in a more unstable carrying capacity and further on a more r-selected microbial community (Attramadal et al. 2012a).

1.4 Methods to study microbial communities

In general, microbial communities are challenging to study compared to communities consisting of perceptible organisms. For an extended period, microbes were only studied by culture-dependent methods, which are well-known to underestimate the diversity of microbes. Today, over 99 % of the microbial species in environmental samples are believed not to have been successfully grown in a laboratory (Madigan et al. 2014), however, as the methods to study microbial communities continuously develops, this number is expected to decrease considerably in the future.

Since the mid-80s, molecular methods have revolutionised the way microbial communities are studied, giving a better understanding of the composition of uncultivated communities (Head, Saunders, and Pickup 1998). The 16S rRNA gene, which encodes for the small subunit of ribosomal RNA in prokaryotes, has been especially significant for the study of microbial organisms (Head, Saunders, and Pickup 1998). This gene is found in all prokaryotes and consists of nine variable (V1-V9) and nine conserved regions. The variable regions in the 16S rRNA gene can be used to identify different species of bacteria and assess the diversity of microbial communities, as these regions are dissimilar for different bacteria. On the other hand, the conserved regions are used as targets for broad-coverage PCR primers (Yang, Wang, and Qian 2016). Following the complete human genome sequencing in 2001, the need for faster and more automated sequencing methods was emphasised (Rajesh and Jaya 2017). This led to the development of next-generation sequencing (NGS) technologies. These technologies have developed immensely in the last two decades and, currently, the clearly dominating NGS-technology is Illumina sequencing (Chiu and Miller 2016).

1.4.1 Illumina sequencing of 16S rRNA amplicons

Illumina sequencing is a so-called high-throughput sequencing (HTS) technology. HTS is a generic term for several sequencing technologies that can run parallel sequencing of many DNA-molecules at the same time with a high-throughput, compared to the traditional low-throughput Sanger sequencing (Churko et al. 2013). As HTS technologies has advanced

over the years, the sequencing costs has dramatically decreased, making these sequencing technologies easily accessible (Churko et al. 2013).

The workflow of Illumina sequencing can be divided into four steps: library preparation, cluster generation, sequencing, and data analysis. In the library preparation, the sample-DNA is fragmented, and specialised adapters are ligated to both ends of the DNA-fragments. In cluster generation, the DNA-fragments are loaded onto a flow cell (i.e. a small glass plate) that contains surface-bound oligonucleotides that are complementary to the specialised adapters that was added in the library preparation. The DNA-fragments then hybridise with the complementary oligonucleotide in the flow cell. Subsequently, the DNA-fragments are clonally amplified by bridge amplification into clusters of identical fragments. Following the amplification, the fragments are sequenced by a Sequencing by Synthesis method. Here, fluorescent-labelled nucleotides are included one at a time, emitting light as they are incorporated. These light-pulses are simultaneously analysed to find the sequence. In the last step, the data analysis, the sequences are quality filtered, aligned and/or assembled depending on the type of sequencing data. The data analysis step results in an OTU-table that is further basis for multivariate statistics (Illumina Inc. 2017). In microbial community analyses, regions of the 16s rRNA gene are targeted by universal broad-coverage PCR primers and subsequently amplified in all samples that are included in the study.

1.5 Study aims and hypotheses

Although microbial communities can have great effects on both fish health and water quality in RAS, little is known about microbial communities in different units of commercial RAS-facilities during normal operation. As stressed by Badiola, Mendiola, and Bostock (2012), most studies on microbiota in RAS are based on laboratory scale trials and the knowledge obtained in these studies are often not representative for commercial-scale RAS.

Thus, this study aimed to characterise the microbial communities in biofilms and water from a commercially operated RAS, during cultivation of Atlantic salmon smolts. By studying these microbial communities, one could potentially discover early-warning signals before disease outbreaks, or inexplicable mortality-events occurs. We aim at improving the understanding of microbial community dynamics in RAS, which might be necessary for further improvements and development in land-based aquaculture systems.

More specifically, the sub-aims of this study were to answer to the following hypotheses:

a) Microbial communities will differ between biofilm and water samples
b) Microbial communities in rearing tank water will be different from the water returning from the water treatment loop
c) Microbial communities in the biofilm from the rearing tank walls will differ from the biofilm in the biofilter
d) Microbial communities in the biofilter biofilm are expected to be more stable over time than that of the rearing tank biofilm
e) There will be more slow-growing, K-selected, bacteria in the water samples from the treated water compared to water samples from rearing tanks
f) Bacterial growth potential will be higher in the rearing tank water compared to the treated water

2. Methods

This thesis was primarily based on DNA-sequencing data from the SINTEF Ocean research project "Monitoring microbiota in closed aquaculture systems" (MonMic) led by Stine Wiborg Dahle. The overall aims of MonMic were to give fish farmers increased control of microbial water quality, to see if diseases may be detected at an early stage to ensure that preventive measures could be put into action, and to evaluate system- and operational design in RAS to optimize the microbial environment for the fish. The project was funded by FHF (Fishery- and aquaculture research funding) and five commercial producers of Atlantic Salmon smolts in recirculating aquaculture systems (RAS). The commercial producers provided microbial test-material and data on production, fish health and water quality that could be relevant for the evaluation of the results. Sampling for microbial community analyses in the MonMic project was conducted biweekly over a 15-months period, resulting in 33 sampling times (t0 – t32). This thesis will focus on analysing data from one of the commercial RAS-facilities in the MonMic project. To supplement the microbial community analyses, additional water samples were taken for this master thesis to quantify the number of bacteria, both by culture-dependent and culture-independent methods. In addition, these water samples were used to estimate the ratio of opportunistic bacteria, the bacterial growth potential and the bacterial cultivability.

2.1. Description of the RAS-unit

A schematic overview of the RAS studied in this master project is shown in Figure 2.1. The commercial RAS was used for start-feeding of Atlantic Salmon alevins/fry from around 0.2 g to around 3 g, after which they were moved to another department for further cultivation. The RAS consisted of 6 rearing tanks with fish (max biomass 22 kg/m^3), with an associated water treatment loop consisting of a mechanical drum filter (Hydrotech, mesh 60 µm) for particle removal, three fixed bed bioreactors (RK BioElements, 3 x 13.5 m^3, Denmark), a trickling filter (EXPO-NET BIO-BLOK®, 20 m^3, Denmark) for degassing, and ultraviolet filtration (MonoRay 10, UltraAqua, Denmark) for disinfection. In addition, the RAS included oxygenation from oxygen cones and pH regulation with calcium hydroxide slurry ($Ca(OH)_2$) added in the water sump before the biofilter (Figure 2.1). New water (Make-up water) was added in Water sump 1 (Figure 2.1). From t0 to t26 the rearing tanks had a volume of 22.6 m^3, with a hydraulic retention time (HRT) of 18 minutes. However, problems with removing all the faeces and wastes from the rearing tanks resulted in a period of reconstruction from t27 to t29 where all the rearing tank walls were raised around 30-50 cm. After the rebuild tanks had a volume of 35 m^3 and a HRT of 28 minutes. Total water flow in the start-feeding was 454 m^3/t at all sampling times.

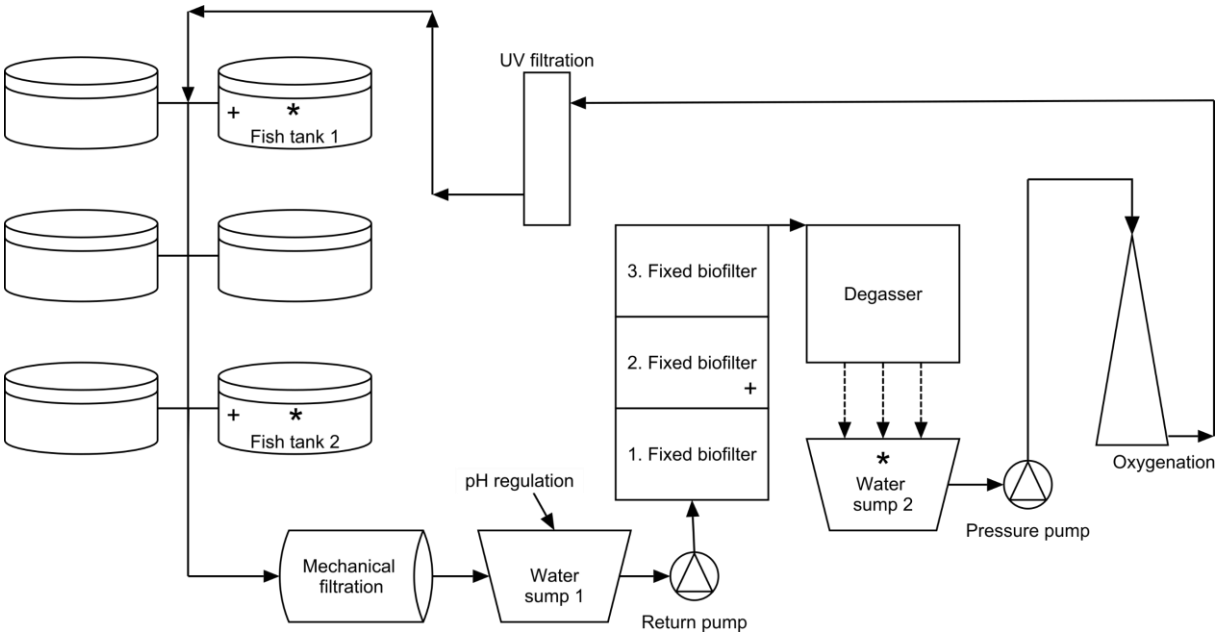

Figure 2.1. Schematic overview of the RAS-loop sampled. Units where water samples were taken are marked with a star (*), while units where biofilm samples were taken are marked with a plus sign (+).

2.2. Sampling

2.2.1. Sampling for microbial community analysis

Sampling for microbial community analyses in the MonMic project was conducted biweekly over a 15-months period, resulting in 33 sampling times (t0–t32). Production data was given for the whole period and divided into seven fish batches, and six fallowing periods between the fish batches (Figure 2.2). Sampling for microbial analyses was conducted from the 06th of November 2017 to the 28th of January 2019.

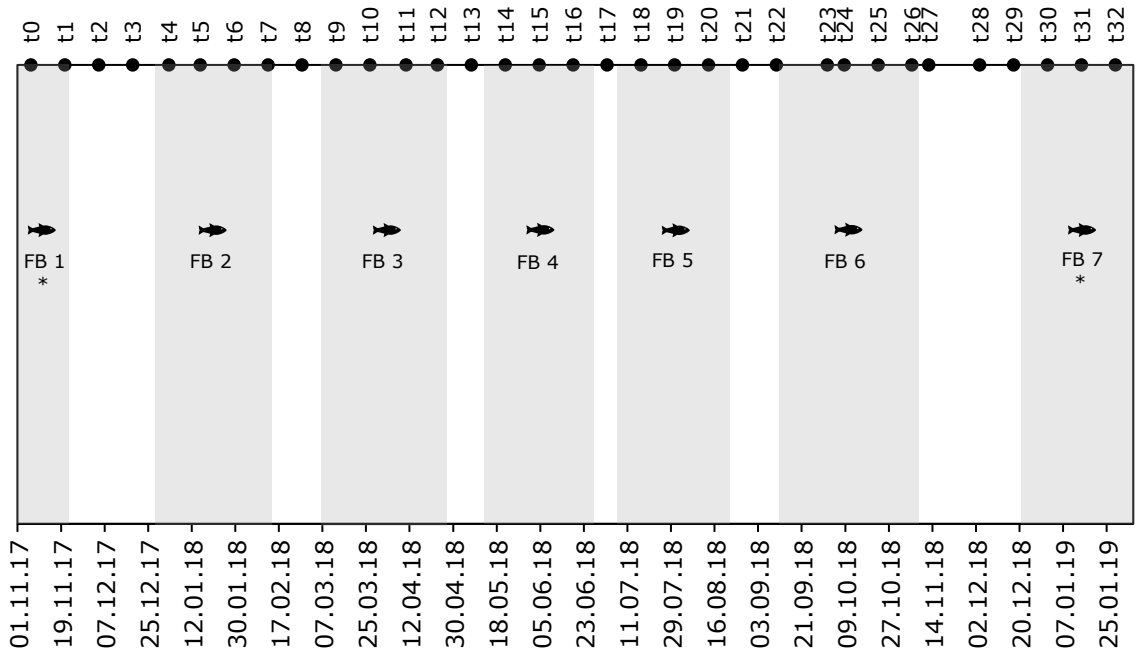

Figure 2.2. Timeline for sampling times (t0-t32) and the production periods consisting of seven fish batches showed in blue (FB = fish batch). White spaces in between fish batches represent the fallowing periods where there was no fish in the department. *FB 1 and FB 7 were not completely sampled fish batches.

Samples were collected by the staff at the RAS on Monday mornings, from four different sampling units inside the RAS-loop: water and biofilm samples from each of two rearing tanks, biofilm samples from one of the fixed bed biofilters and water samples from a water sump positioned downstream of the biofilters in the treatment loop (see Figure 2.1). S. I. Gaarden assisted with sampling for microbial community analyses two times. Primarily, from sample time t0 to t5, water samples were collected in triplicates from each sampled unit by filtering 150-200 mL water through a 0.22 µm Sterivex filter (Millipore) with Omnifix® syringes. Biofilm samples were taken by swabbing the tank walls of the two rearing tanks and the fixed bed biofilter. Swabs (Copan Diagnostics, USA) were rolled on a 10 x 5 cm area to ensure that enough material was collected. Samples of biofilm were also collected in triplicates from t0 to t5. However, a preliminary analysis of the results from t0 to t5 in both water and biofilm samples, indicated that replicates had similar microbial community compositions, and replicate samplings were therefore omitted from t6 to t32.

All collected samples were stored in a freezer (-20 °C) until they were sent by post to SINTEF. When samples arrived at SINTEF they were stored in a freezer (-80 °C) until further analyses were performed. A total of 245 samples was subjected to microbial community analysis by Illumina sequencing of 16S rDNA amplicons in this master project. Originally, there was in total 278 samples, but 34 of these were not successfully sequenced or not sent for sequencing due to reasons such as failed sequencing, shortage of extracted DNA and missing samples.

Extraction and sequencing of DNA

DNA-extraction from samples collected for microbial community analysis was done by laboratory personnel at SINTEF Ocean, with the assistance of S. I. Gaarden twice. For extraction of the DNA, two different kits were used: FastDNA® SPIN Kit for Soil (MP Biomedicals, Santa Ana, California) was used for samples taken from t0 to t17, while ZymoBIOMICS™ DNA Miniprep kit (Zymo Research, Irvine, California) was used for samples taken from t18 to t32. Extraction was done as described by the manufacturers (Appendix A.1 and A.2 respectively). To check if there was a difference between the two extraction kits, DNA from the same samples was extracted with each kit. The extracted DNA was sequenced, and the microbial community composition results were subsequently compared at different taxonomical levels. Only small differences were found in the microbial community composition between the two DNA-extraction kits.

After extraction, The Genomic DNA Clean & Concentrator™-10 kit (Zymo Research, Irvine, California) was used to purify the DNA (Appendix B). To determine the concentration and purity of the extracted DNA, a NanoDrop Spectrophotometer (Thermo Scientific Inc., England) was used. A Qubit 3.0 Fluorometer (Thermofisher Scientific Waltham, MA, USA) with the Qubit 1x dsDNA High Sensitivity Assay kit (Invitrogen, Thermofisher Scientific) was used to measure the concentration of double-stranded DNA (Appendix C).

The extracted DNA was sent to the Centre for Biotechnology (CeBiTec), Bielefeld University (Germany) for 16S rDNA amplicon library preparation and sequencing. Library preparation was conducted after standard Illumina instructions. The variable regions 3 and 4 (v3 + v4) of the 16S rRNA gene was amplified by two PCR rounds using the 2xHiFi HotStart ReadyMix (Kapa Biosystems, USA). To cover the domains of Bacteria and Archaea, the primers Pro341F (5'-CCTACGGGNBGCASCAG-3') and Pro805R (5'-GACTACNVGGGTATCTAATCC-3') were used for the first PCR round (Takahashi et al. 2014). Obtained amplicons were indexed, pooled and subsequently sequenced on an Illumina MiSeq platform (paired end sequencing; 2x300 bp).

2.2.2. Sampling for flow cytometry and CFU analyses

Water samples were collected in the RAS for flow-cytometry and CFU analyses at three sampling days in January 2019: the 17th-, the 21st- and the 28th of January 2019. Samples were taken from the same units as the water samples taken for microbial community analysis: the two rearing tanks and the water sump (See figure 2.1). Samples were collected by using a sampler consisting of a metal cup fixed onto a metal rod. The sampler was rinsed with sample-water before the final sample was collected and transferred onto sterilised 500 mL glass-bottles. The water samples were used to evaluate the number of bacteria by two methods, one culture-dependent; Colony forming units (CFU) on agar cultivation medium and one culture-independent; flow-cytometry.

CFU analysis for estimating bacterial densities and fraction of opportunistic bacteria

Plate count agar (PCA) was used for cultivation of bacteria from water samples. The medium was prepared by mixing 8.75 g PCA (Himedia), 1.50 g agar powder and 500 mL Milli-Q water. The agar medium was autoclaved at 120 °C for 20 minutes, before poured into sterile Petri dishes in a sterile cabinet. After solidifying, plates were put in the refrigerator in sterile plastic bags. Water samples were plated immediately after collection in the laboratory of the RAS-facility.

Serial dilutions were made using 2 mL sterile Eppendorf-tubes with tenfold dilutions ranging from $1:10^{-1}$ to $1:10^{-4}$. Samples were diluted with sterile physiologic saltwater (3 ppm) to resemble their natural habitat. All water samples were plated in triplicates. Plating was done by pipetting 70 µL sample onto each agar plate and spreading the sample using disposable plastic inoculation loops. After plating, the Petri dishes were incubated at 14 °C. Colony-forming-units (CFU) were registered each afternoon for 18 days of incubation. Plates containing 30-300 colonies were used to calculate the number of CFU mL^{-1} (Skjermo et al. 1997). CFU-analysis was used to estimate the fraction of opportunistic bacteria. Opportunistic bacteria were defined as the number of CFUs registered three days after incubation, divided by the total number of CFUs registered after 18 days of incubation (Skjermo et al. 1997).

Flow cytometry for enumeration, growth potential and cultivable bacteria

Flow cytometry is a laser-based technology that can be a useful tool in enumerating-, analysing-, and characterising cells. With a detection rate up to 10 000 cells per second, it is a very rapid method for examining and counting cells. With its large potential of applications, it can be used with a wide variety in the field of aquatic microbiology (Wang et al. 2010). Flow cytometry can be divided into three main systems: fluidics, optics and electronics. The fluidics system arranges the sample into a single stream so that cells can pass the laser one cell at a time. When cells pass the lasers, they scatter light, and if stained they can also emit fluorescent signals. These light signals are collected by the optics system by several different detectors. Subsequently, the electronic system converts the signals into numerical values that further can be analysed using a specially designed software (Macey, 2007).

The total number of bacterial cells in water samples was determined by flow cytometry using a BD Accuri™ C6 Flow Cytometer (BD Biosciences, San Jose). For each sampling day, Eppendorf 2 mL tubes were filled with 10 µL of the fixation-agent glutaraldehyde before 990 µL water sample was added. Subsequently, the Eppendoft tubes were turned six times, to thoroughly mix the samples. All water samples for flow cytometry had six replicates. Fixated samples were stored in the refrigerator for maximum 3 days before they were run at the flow cytometer.

Before flow cytometry analysis, samples were vortexed to ensure that they were homogeneous. To keep cell counts under 1000 events μL^{-1}, samples were diluted 1:10 with a filtrated (0.2µm) 1:10 TE buffer. Subsequently, diluted samples were stained with a 1:50 working solution of SYBR® Green II RNA Gel Stain (Life Technologies, Thermo Fisher Scientific Inc.). The stain was diluted with filtrated (0.2µm) 1:10 TE buffer, and 10 µL of stain was added to 1 mL diluted water sample. After staining, samples were incubated in the dark for 15 min. Only six samples were analysed each round to ensure that the stain stayed stable (Brandsegg, 2015). A medium flow rate (35 µL min^{-1}) and a 4 min collection time was used for all samples for enumeration of bacterial cells. The FL1 detector was set to a threshold value of 3000. The gating that was used for all flow cytometry samples excluded fluorescent intensity signals below approximately $10^{3.5}$ on the FL1 detector, as these events were considered to be noise and not bacterial cells (Figure 2.3).

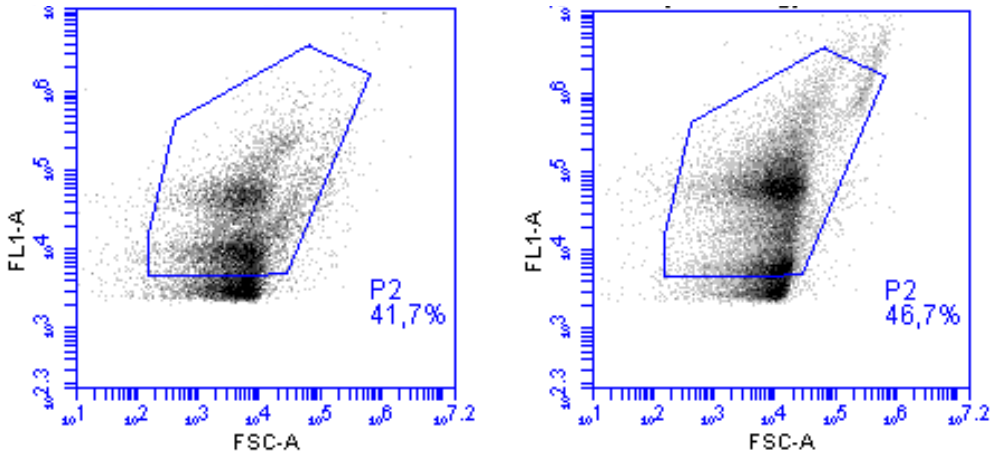

Figure 2.3. The gating option that was used for all flow cytometry samples, here showed for two of the samples collected for this master thesis. The software from BD Flow Cytometry, San Jose, 2008, was used for gating of all samples.

Triplicate water samples from the same sample units were also incubated at 14 °C for three days in open 50 mL plastic tubes to determine the bacterial growth potential. After three days samples were fixated and analysed as all other water samples for flow cytometry. The bacterial growth potential was calculated by dividing the difference in total bacteria after three days incubation by the original number of total bacterial cells (Attramadal et al. 2016). Bacterial counts from CFU analysis relative to the total number of bacterial cells determined by flow cytometry, was used to calculate the fraction of cultivable bacteria. Data was exported to Microsoft Excel 2016 for further analysis.

2.3. Processing of sequencing data for microbial community analysis

The Illumina sequencing data (one Fastq file for each sample) were processed with the USEARCH pipeline (version 9.2; https://www.drive5.com/usearch/). During merging of paired reads, primer sequences were removed and reads shorter than 380 base pairs were filtered out. The processing further included demultiplexing and quality trimming by the Fastq filter command (with an expected error threshold of 1). The UPARSE-OTU algorithm was applied for chimera removal and clustering at the 97% similarity level (Edgar, 2013). Taxonomy assignment was based on the SINTAX script (Edgar, 2016) with a confidence value threshold of 0.8 and the RDP reference data set (version 16). For identifying OTUs potentially representing nitrifiers, the OTUs were also classified using the MiDAS 3.2 reference data set based on 16S rRNA gene sequences obtained from activated sludge wastewater treatment systems (Nierychlo et al. 2019). The resulting OTU (Operational taxonomic units) table was normalised to 17 000 number of reads per sample by determining the fraction of the OTUs for each community profile, and subsequently multiplying by 17 000, and finally rounding off the read numbers to integers.

2.4. Statistical analyses

The USEARCH commands Alpha_div and Sintax_summary were used to calculate alpha diversity indices (observed OTU richness, Chao1 and Shannon's diversity) and generate taxa summary tables, respectively. OTU richness is the number of OTUs observed in each sample, while the Shannon's diversity index considers both the OTU richness and the evenness. If high values of Shannon's diversity are observed, this indicates high diversity of the community. Evenness is a measure that describes the equability of the OTU abundances. If the OTUs are present in similar abundances in a community, the evenness is high. The calculated alpha diversity indices were exported to Microsoft Excel for further analysis.

PAST (version 4.0; Hammer, Harper, and Ryan 2001) was used to calculate Bray-Curtis similarities. To compare the microbial community profiles within and between samples, a matrix of Bray-Curtis similarities was made and exported to Microsoft Excel for further analysis. Bray-Curtis similarities is a measure of beta diversity and are given in values between 0 and 1, where 0 represents completely dissimilar communities (no shared OTUs) and 1 represent identical communities.

Principal Coordinate Analysis (PCoA) ordinations based on Bray-Curtis similarities were made to illustrate the beta-diversity. PCoA plots are based on a distance matrix, where samples are ordinated into a three-dimensional space so that the distances between the samples represent the Bray-Curtis similarity. Subsequently, this three-dimensional space is projected into a two-dimensional plot where samples that have similar microbial communities are positioned closer to each other than samples that have less similar microbial communities (Hammer, Harper, and Ryan 2001).

One-way PERMANOVA (permutational multivariate analysis of variance) based on Bray-Curtis similarities was used to test if there was a statistically significant difference between sample-groups (Anderson 2001), with the significance threshold set to a p-value below 0.05. When more than two groups were compared, one-way PERMANOVAs with Bonferroni-corrected p-values were used.

SIMPER (Similarity Percentage) analysis based on Bray-Curtis values was performed to identify the OTUs which contributed the most to the difference in microbial community composition between selected groups (Clarke 1993).

When production data was divided into different fish groups and fallowing periods, the standard error of the mean (SE) was used to show the variation in the measured variables. Standard error was also used to show variation in microbial water quality measures, alpha diversity measures and in Bray-Curtis similarities. SE was calculated by dividing the standard deviation (SD) by the square root of the sample size (n) (Eq. 2.1). SD was calculated in Microsoft Excel using the function STDAV.S().

$$SE = \frac{SD}{\sqrt{n}} \qquad \text{(Eq. 2.1)}$$

To check if there were statistical differences between groups in the data, two-sample t-tests (assuming equal variances) were used with a significance threshold set to a p-value below 0.05. The data analysis tool in Microsoft Excel 2016 was used to perform the t-tests.

3. Results

3.1. Water quality and production data

3.1.1. Production data and physicochemical water quality

Production data and physicochemical water quality variables were provided by the RAS-facility for the 15-month period of the MonMic-project (Table 3.1 and 3.2). There were, in total, seven fish batches cultivated in the monitored RAS-unit during this period (Table 3.1). Of these batches, five were complete, meaning that production data and physicochemical water quality variables were given for the whole cultivation period from input of alevins until the fry were moved to another department for further cultivation. For the last two fish batches (Fish batch 1 and Fish batch 7), samples for microbial community analyses were not taken for the whole cultivation period (Table 3.1). Usually, fish were in the start-feeding department for 47-49 days until the fry reached an average weight of around 2.5 g. Nevertheless, fish batch 6 was in the RAS-unit for 58 days and reached an

Table 3.1. Production data from the RAS-site. The 15-month long production period in the MonMic project represented seven different fish batches and six fallowing periods. The average number of fish was calculated by adding together the number of fish on each production day and dividing it by the number of production days in the given fish batch and rearing tank. The average fish weights are the last measured fish weights before the fish were moved to another department. RT1 = Rearing tank 1, RT2 = Rearing tank 2.

				Average number of fish		Average fish weight	
	Day	Date	Sampling times(s)	RT1	RT2	RT1	RT2
Fish batch 1*	1-15	06.11.17 - 20.11.17	t0 – t1	269217	266522	2.8 g	3.0 g
Fallowing period 1	16-53	21.11.17 - 28.12.17	t2 – t3	-	-	-	-
Fish batch 2	54-100	29.12.17 - 13.02.18	t4 – t7	267348	266969	2.2 g	2.6 g
Fallowing period 2	101-121	14.02.18 - 06.03.18	t8	-	-	-	-
Fish batch 3	122-170	07.03.18 - 24.04.18	t9 – t12	267619	267552	2.5 g	2.5 g
Fallowing period 3	171-186	25.04.18 - 10.05.18	t13	-	-	-	-
Fish batch 4	187-234	11.05.18 - 27.06.18	t14 – t16	265666	261070	2.4 g	2.5 g
Fallowing period 4	235-240	28.06.18 - 03.07.18	t17	-	-	-	-
Fish batch 5	241-289	04.07.18 - 21.08.18	t18 – t20	163528	164549	2.4 g	2.6 g
Fallowing period 5	290-309	22.08.18 - 10.09.18	t21 – t22	-	-	-	-
Fish batch 6	310-367	11.09.18 - 07.11.18	t23 – t26	280233	276167	3.7 g	4.1 g
Fallowing period 6	368-408	08.11.18 - 18.12.18	t27 – t29	-	-	-	-
Fish batch 7*	409-456	19.12.18 - 04.02.19	t30 – t32	254594	254126	2.1 g [#]	2.2 g [#]

* Fish batch 1 and -7 were not followed for the whole cultivation period

[#] The average fish weights were the last reported fish weights but possibly not the final fish weight of this fish batch

average weight closer to 4 g. The average weight of the alevins when moved into the start-feeding department was 0.2 g for all fish batches. Between each fish batch there was a fallowing period for cleaning of rearing tanks and maintenance of the system before input of a new group of alevins (Table 3.1). Cleaning of rearing tanks between fish batches was done with a strong alkaline soap (HG Alkaskum 32, Norway) and high-pressure washing, before tanks were refilled with production water from the loop. The fallowing periods varied from 6 to 40 days. However, the 40-day long fallowing period was used for rebuilding the department.

Table 3.2 gives a summary of the physicochemical water quality in the different fish batches and fallowing periods. The mean temperature varied between 12°C - 14°C. When there was fish in the system, the temperature was higher than in periods of fallowing. Salinity varied from 0.1-5.2 ppt. The salinity was raised occasionally when the RAS-facility had problems with fungi. The oxygen saturation never fell below 83.5 % in either of the rearing tanks during fish cultivation and was observed to be on average higher in fallowing periods. The pH was usually very stable, varying between 6.9-7.0, but single measurements as low as 6.36 and as high as 7.21 was observed.

Table 3.2. Physicochemical water quality for different fish batches and fallowing periods (average ± SE). All variables were measured in Water sump 2 (see figure 2.1 in Methods), except from the oxygen saturation, which was measured in each rearing tank. Oxygen saturation was calculated as the average of both rearing tanks. Numbers reported without SE represent single measurements for the relevant production period. pH is given without SE because it was constantly <0.02.

	Temperature (°C)	Oxygen saturation (%)	pH	Salinity (ppt)	Total ammonia nitrogen (mg TAN/L)	Nitrite (mg NO_2^-/L)	Nitrate (mg NO_3^-/L)
Fish batch 1*	12.9±0.7	91.0±0.9	6.9	2.0±0.0	1.1±0.2	0.6±0.1	177.1±7.5
Fallowing period 1	12.1±0.1	100.2±0.4	6.9	1.1±0.0	0.2±0.0	<0.05	85.8±8.8
Fish batch 2	13.8±0.1	92.2±0.3	7.0	2.2±0.2	0.6±0.2	0.3±0.1	113.9±13.0
Fallowing period 2	12.0±0.2	104.0±0.4	7.0	1.0±0.1	0.5±0.1	<0.05	78.0±9.5
Fish batch 3	13.7±0.1	93.7±0.4	6.9	1.9±0.2	0.7±0.1	0.2±0.1	143.8±22.2
Fallowing period 3	12.3±0.1	101.5±0.9	6.9	1.0±0.0	0.6	<0.05	93.0
Fish batch 4	13.9±0.1	92.8±0.3	6.9	2.5±0.1	0.5±0.1	0.2±0.0	179.7±26.6
Fallowing period 4	13.5±0.3	99.4±0.4	6.9	0.3±0.1	0.2±0.1	0.1±0.1	97.0±33.0
Fish batch 5	14.0±0.0	93.2±0.2	6.9	0.9±0.0	0.5±0.1	0.1±0.0	120.9±17.9
Fallowing period 5	13.8±0.1	99.7±0.3	6.9	0.7±0.1	0.2±0.0	<0.05	140.0±10.0
Fish batch 6	13.7±0.1	95.2±0.5	7.0	1.1±0.0	0.8±0.1	0.3±0.1	194.6±21.5
Fallowing period 6	12.8±0.1	99.6±0.4	6.9	0.4±0.1	-	-	-
Fish batch 7*	13.5±0.2	92.3±0.2	6.9	1.6±0.1	0.7±0.0	0.2±0.1	135.7±14.0

*Fish batch 1 and 7 were not followed for the whole cultivation period.

Concentrations of nitrogenous substances in the RAS-unit during fish batches and fallowing periods was measured in Water sump 2 (see Figure 2.1). The general trend in concentrations of total ammonia nitrogen (TAN), nitrite (NO_2^-) and nitrate (NO_3^-) was that the concentrations increased throughout the fish batches followed by a decrease during fallowing periods (Figure 3.1). TAN varied between 0.1-2.0 mg/L, but was on average 0.6 mg/L. There were large variations in both nitrite (NO_2) and nitrate (NO_3) concentration, varying between <0.05-1.3 mg/L and 33-390 mg/L, respectively (Table 3.2).

In fallowing periods, the biofilters were fed 1 kg ammonium chloride (NH_4Cl) to a final concentration of 12.5 mg TAN /L, twice a day, to preserve the biofilter activity. If assuming that the addition of one kg fish feed produces 0.4 kg TAN (Personal communication, K. Attramadal, 24.04.20), the addition of 2 kg ammonium chloride each day equals 76.6 % of the TAN concentration that the fish produces each day at peak feeding and 1678.5 % of the concentration that the fish produces at minimum feeding.

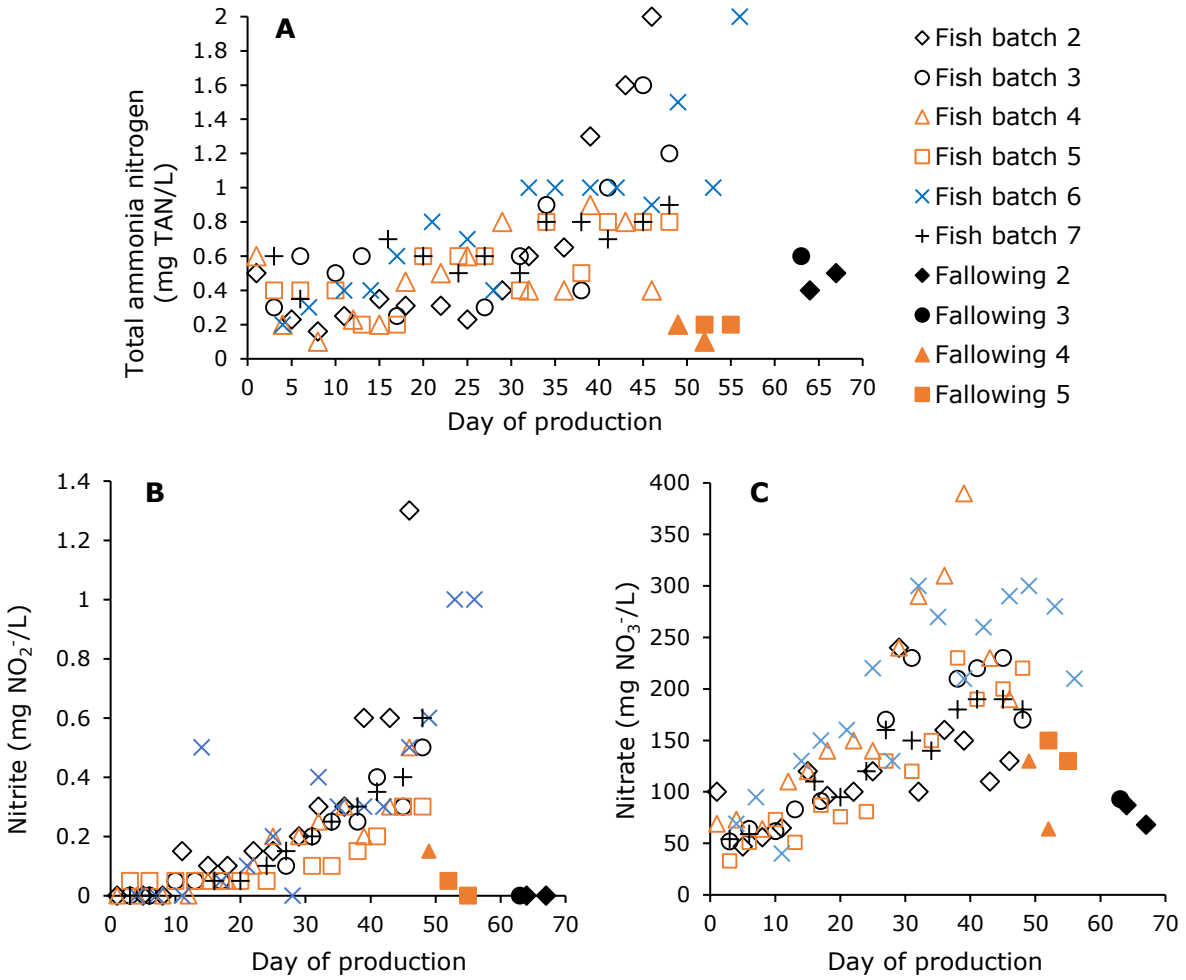

Figure 3.1. Concentration of nitrogenous substances in the RAS-unit throughout the fish batches and fallowing periods. A): Total ammonia nitrogen (TAN) in mg/L, B): Nitrite (NO_2^-) in mg/L and C): Nitrate (NO_3^-) in mg/L. Filled symbols are measurements taken in fallowing periods. Open symbols are measurements taken during fish batches. During fish batches, the nitrogenous substances was measured every Monday and Wednesday, while there was no set procedure for measuring nitrogenous substances in fallowing periods. Fallowing period 6 had no reported measurements. Fish batch 1 was not included due to no reported data for day 0. Fish batch 7 is included but data were not available for the complete production period.

The three biofilters were backflushed with high-pressure water every third week (one biofilter each week) to avoid clogging. The biofilters had never been disinfected throughout the seven years of operation.

Daily fish mortality (%) for the two sampled rearing tanks is shown in Figure 3.2. The rearing tanks had approximately the same pattern of mortality during the different fish batches and the mortality was in general relatively low. The average daily mortality for all fish batches and both rearing tanks was 0.12 %. The mortality was usually highest in the first days of production, except for fish batch 4 and 7 in rearing tank 1, and fish batch 7 in rearing tank 2. On the other hand, fish batch 7 had the highest single incident of mortality in both tanks, with a peak at day 16 with 1.7 % mortality in rearing tank 1 and a peak at day 15 with 1.7 % mortality in rearing tank 2 (Figure 3.2). Production day 16 in rearing tank 1 and production day 15 in rearing tank 2 both correspond to the same date, the 3rd of January 2019.

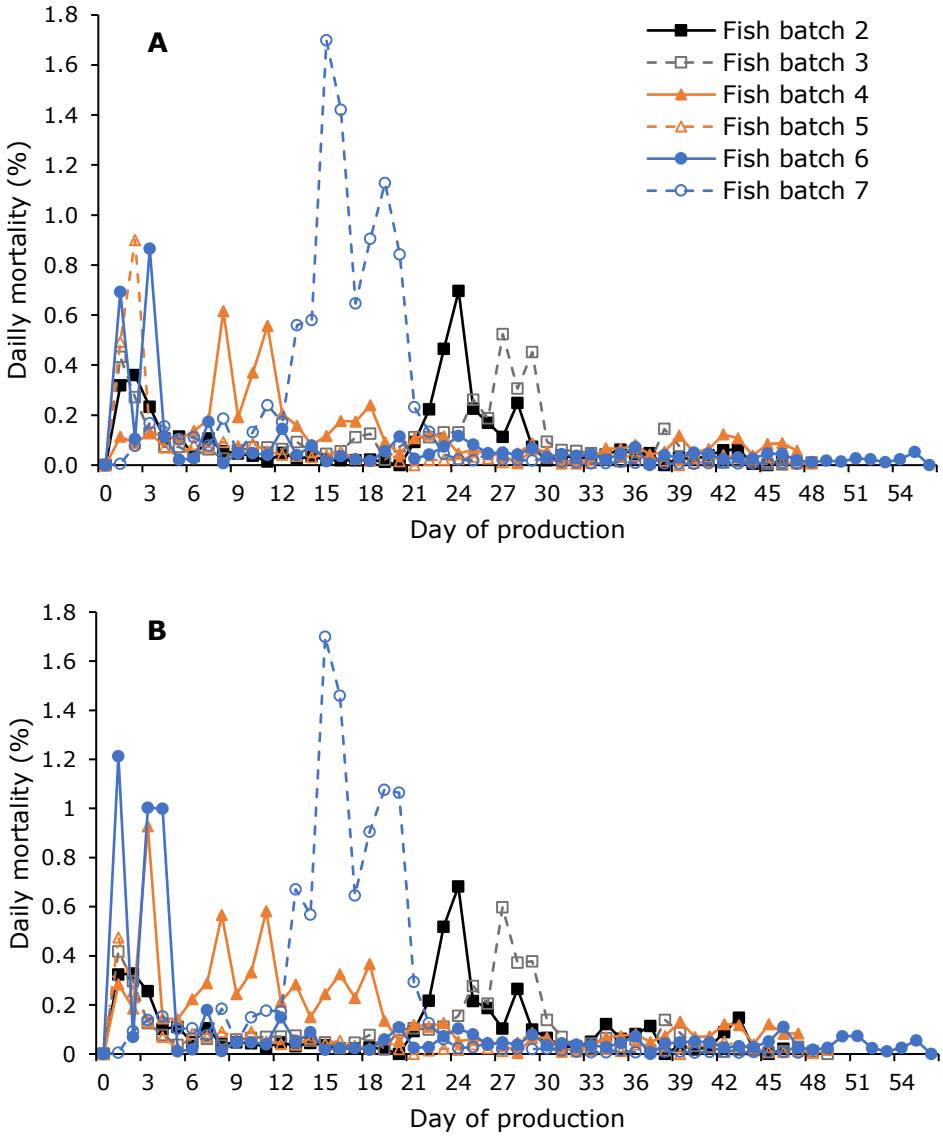

Figure 3.2. Daily mortality (%) during the production period for different fish batches in A): rearing tank 1 and B): rearing tank 2. Day 0 is when the fish were moved into the rearing tanks while the last days included are the days the fry were moved out of the rearing department.

3.1.2. Microbial water quality

The number of bacterial cells in the two rearing tanks and in the water sump was estimated at three sampling dates, using two different methods: colony forming units (CFU) and flow cytometry (Table 3.3). All microbial water quality samples were taken during fish batch 7 on cultivation day 30, 34 and 40, equivalent to the 17th, the 21st and the 28th of January 2019, respectively.

The total number of bacterial cells, as assessed by flow cytometry, varied between 3.2×10^5 and 5.7×10^5 cells mL^{-1}, while the number of CFU varied between 3.0×10^3 and 1.7×10^4 CFU mL^{-1}. On the 17th of January 2019 the number of bacteria was higher than in the two following sampling dates. The percentage of cultivable bacteria (CFU mL^{-1} relative to total bacterial cells as measured by flow cytometry) varied between 0.9-3.1 % and was on average highest on the 17th of January.

Table 3.3. Number of cultivable cells, total number of bacterial cells and percentage of cultivable bacteria in water samples from the RAS-loop (±SE). The average number of cultivable cells were estimated by colony forming units (average of three replicate water samples), while the total bacterial cell numbers were estimated by flow cytometry (average of six replicate water samples). Percentage cultivable bacteria is the portion of colony forming units compared to the total bacteria counts. RT1 = Rearing tank 1, RT2 = Rearing tank 2, WS = Water sump.

Sampling date	17.01.19			21.01.19			28.01.19		
Sampling site	RT1	RT2	WS	RT1	RT2	WS	RT1	RT2	WS
Colony forming units $\times 10^3$ mL^{-1}	13.2 ±2.1	16.8 ±0.5	14.4 ±2.9	3.7 ±0.5	5.3 ±0.3	6.1 ±0.9	7.8 ±0.9	6.5 ±0.7	8.4 ±1.4
Total bacteria cells $\times 10^5$ mL^{-1}	5.0 ±0.1	5.4 ±0.1	5.1 ±0.1	4.3 ±0.2	4.5 ±0.2	4.3 ±0.1	3.9 ±0.2	4.0 ±0.1	3.6 ±0.2
Cultivable bacteria (%)	2.6	3.1	2.8	0.9	1.2	1.4	2.0	1.6	2.3

Fraction of opportunistic bacteria in RAS-water

The fraction of presumptive opportunistic bacteria was measured at three water sampling sites and the three different sampling dates in January 2019, by calculating the fraction of visible CFUs after three days incubation (representing the fast-growing cells) compared to the total number of colonies after 18 days of incubation (Skjermo et al., 1997). There were large variations in the fraction of presumptive opportunistic bacteria between sampling days and sampling sites (Figure 3.3).

The fraction of opportunistic bacteria in the rearing tanks were significantly higher than in the water sump on January 17th (t-test, $p<0.001$). On January 21st there was no significant difference between the water sump and the rearing tanks while on January 28th there was a considerably higher fraction of opportunists in the rearing tanks, compared to the water sump, but this was not statistically significant (t-test, $p=0.06$).

On January 17th the fraction of opportunistic bacteria was significantly higher, compared to the two following dates (t-test, $p<0.001$). The percentage of opportunistic bacteria on the 17th of January varied between 55-86 %, while on January 21st and -28th, the percentages differed between 3-12 % and 11-43 %, respectively.

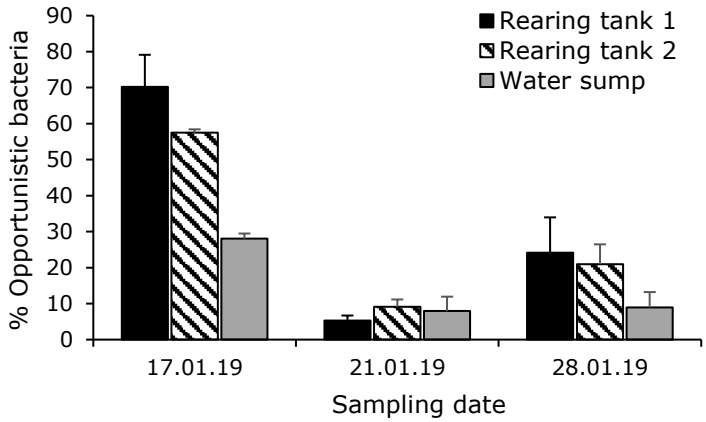

Figure 3.3. The average fraction of presumptive opportunistic bacteria (±SE) in water samples from three different sampling sites; Rearing tank 1, Rearing tank 2 and Water sump, in a RAS-loop on three different sampling dates in January 2019. The averages were calculated from three replicate water samples for each sample site on each sample date. The fraction of opportunistic bacteria was estimated by registering the number of CFUs after three days incubation (representing the fast-growing cells) and dividing it by the total number of CFUs registered after 18 days incubation.

Bacterial growth potential in RAS-water

A high bacterial growth potential indicates that there are high amounts of available resources and niches for bacterial growth (Attramadal et al. 2016). To estimate the bacterial growth potential in water from the RAS-unit, water sampled the 17th and 28th of January was incubated for three days at 14 °C after sampling, before they were fixated and analysed using flow cytometry. To calculate the growth potential the difference in total bacterial cells after three days incubation was divided by the original number of total bacterial cells (Attramadal et al. 2016).

On the 28th of January the bacterial growth potential was substantially higher for all sampled units compared to the growth potential on the 17th of January (Figure 3.4). Further, the analysis indicated that water from the water sump had a lower bacterial growth potential than water from the rearing tanks.

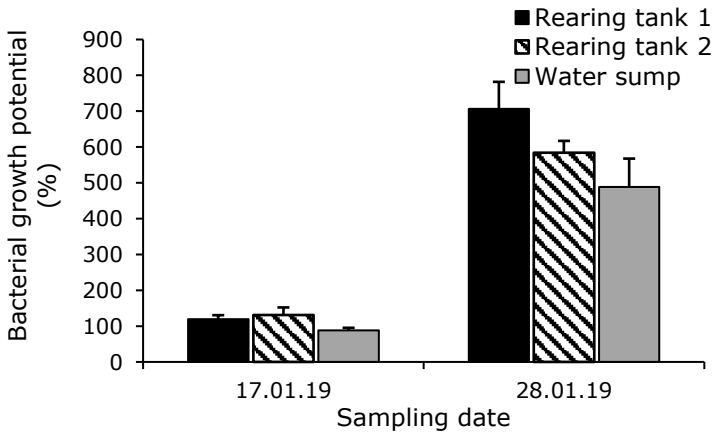

Figure 3.4. Average bacterial growth potential (±SE) at three different sampling sites and two different sampling dates in January 2019. The average bacterial growth potential was calculated by dividing the increase in number of bacterial cells after three days incubation by the total bacterial cell number for the respective sampling date. The averages were calculated from triplicate water samples for each sampling site on each sampling date.

3.2. Microbial community analysis

Sampling for microbial community analyses was conducted biweekly, over a 15-month period, resulting in 33 samplings (t0-t32). Samples were taken from four different units inside the RAS-loop, water samples from each of two rearing tanks and a water sump in the treatment loop, and biofilm samples from each of the same two rearing tanks and one of the fixed bed biofilters (see Figure 2.1). Water samples for microbial community analysis were taken from the same units as for microbial water quality samples (see chapter 3.1.2).

3.2.1. OTU richness and alpha diversity measures

In total, 1237 operational taxonomic units (OTUs) were identified from the OTU-table that was normalised to 17000 reads per sample. All further analyses of alpha- and beta diversity were based on this normalised OTU-table. There were five OTUs classified as chloroplasts that were removed, resulting in a final number of 1232 OTUs from the 245 samples subjected for 16S rDNA amplicon sequencing.

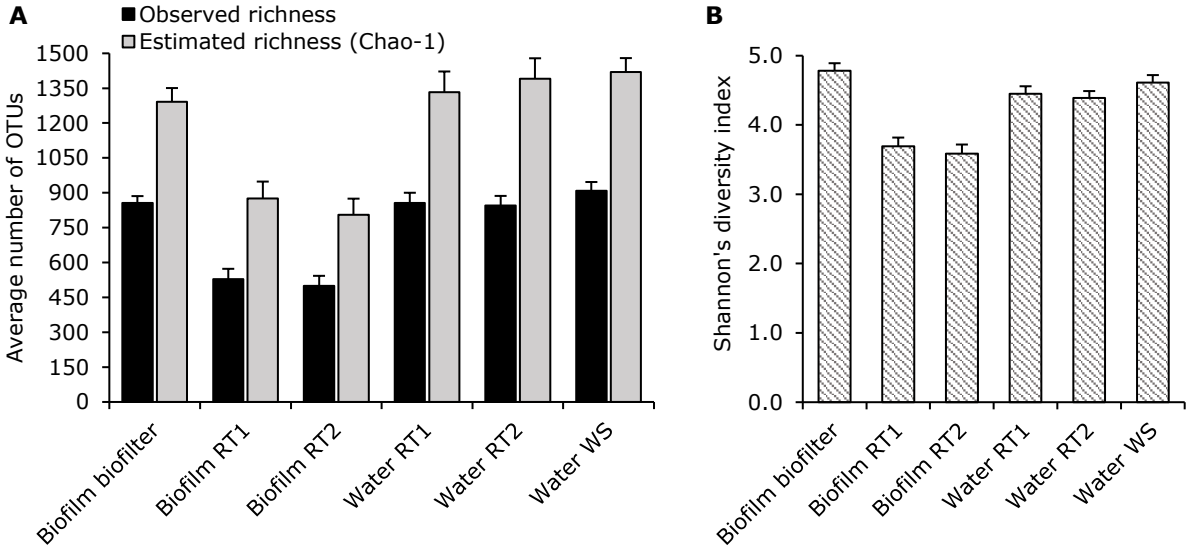

Figure 3.5. Alpha diversity indices for the four sampled units in the RAS-loop where microbial community samples were taken: Biofilm- and water samples from each of two rearing tanks, biofilm from one of the fixed bed biofilters and water from a water sump. The alpha diversity indices were based on the normalised OTU-table (normalised to 17000 reads per sample). All the alpha diversity indices were calculated as the mean (±SE) of all sampling times (t0 - t32). A) The average observed OTU richness and estimated richness (Chao-1). B) Shannon's diversity index. RT1 = Rearing tank 1, RT2 = Rearing tank 2, WS = Water sump.

The average of the alpha diversity measures was estimated by finding the mean of all sampling times (t0-t32) for the four sampled units in the RAS-loop; Biofilm and water samples from the two rearing tanks, biofilm from one of the fixed bed biofilters and water from a water sump in the RAS-loop (Figure 3.5). Comparison of the observed OTU richness to the estimated richness (Chao-1) in Figure 3.5A showed that the average sequence coverage was 63.0 ± 0.9 % (±SE). Both the lowest observed OTU richness and Shannon's diversity index were found for the biofilm samples in the rearing tanks (Figure 3.5) and

this was found to be significantly lower than in the biofilm samples from the biofilter (t-test, $p<0.001$ and $p<0.001$ for OTU richness and Shannon's diversity, respectively). Water samples from the rearing tanks had, in general, higher OTU-richness than biofilm samples from the same rearing tanks. The biofilm samples from the biofilter had a higher Shannon's diversity index than water from the water sump (Figure 3.5B) but this was not significant (t-test, $p=0.26$).

3.2.2. Comparing microbial communities in biofilm and water

To investigate if there was a difference in community profiles between sample types (water and biofilm) and RAS-units (rearing tanks, water sump and biofilter), a PCoA-ordination based on Bray-Curtis similarities was made (Figure 3.6). The PCoA-plot indicated that there was a difference between water and biofilm samples. However, it appears that there was some overlap within the biofilm and water samples. A one-way PERMANOVA test showed that there was a significant difference in the bacterial communities between the water samples and the biofilm samples ($p<0.001$).

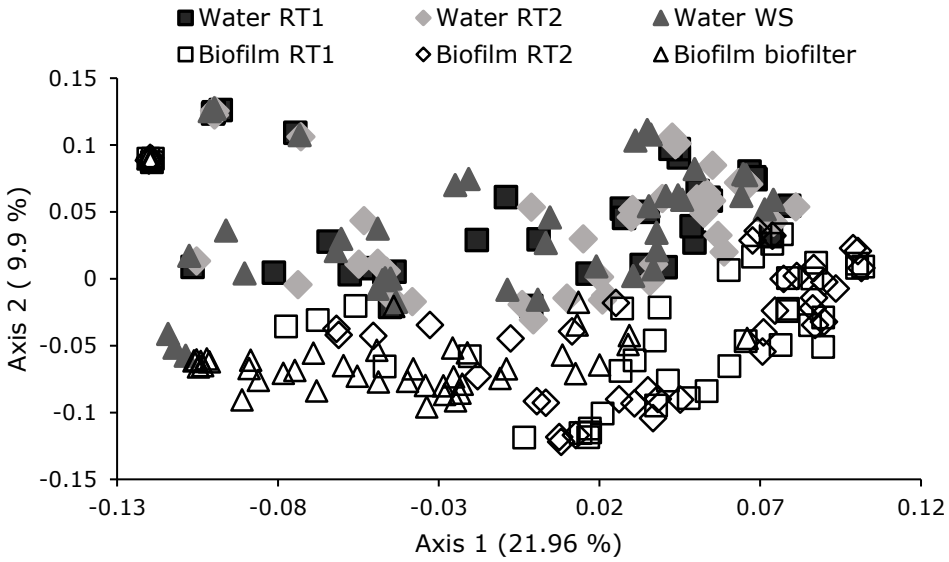

Figure 3.6. PCoA ordination based on Bray-Curtis similarities of microbial community profiles for water samples from rearing tank 1 (RT1), rearing tank 2 (RT2) and the water sump (WS), and biofilm samples from rearing tank 1 (RT1), rearing tank 2 (RT2) and the biofilter over a time period of 15 months.

When assessing the ten most common orders (sorted according to average relative abundance and disregarding unassigned bacteria) in the microbial community profiles from the sampled RAS-units, the microbial community composition was in general similar. Of the ten most common orders, all samples shared seven of them (Figure 3.8, Figure 3.11, and Figure 3.13). Rhodobacterales was the most common order in all samples (Figure 3.8, Figure 3.11, Figure 3.13). In all sampled units, except for biofilm samples from the biofilter, the second most common order was Burkholderiales. Burkholderiales was however, the fourth most common order in biofilm samples from the biofilter (Figure 3.13). The communities were otherwise dominated by the orders Sphingomonadales, Chlamydiales, Rhizobiales, Xanthomonadales and Actinomycetales.

To identify which OTUs contributed most to the difference between water and biofilm samples, a SIMPER analysis based on Bray-Curtis similarities was conducted. Collectively, ten OTUs contributed nearly 30 % to the difference between water- and biofilm samples (Table 3.4). OTU_1, representing the family Rhodobacteraceae, was the most contributing OTU, singularly explaining almost 10 % of the difference. However, the average relative abundances of OTU_1 in biofilm and water samples were approximately the same. OTU_4 (*Sphaerotilus*) was the second most contributing OTU, with a relative abundance of 0.05 in the biofilm samples but was hardly present in the water samples. OTU_2 (*Thiothrix*) was the third most contributing OTU, with a higher relative abundance in biofilm (0.04) compared to water samples (0.02). OTU_9 (*Rhodoferax*) and OTU_3 (*Mycobacterium*) were, on the other hand, more abundant in water samples (Table 3.4).

Table 3.4. The ten OTUs contributing most to the difference between the microbial communities in water samples (w) or biofilm samples (b), identified by SIMPER-analysis based on Bray-Curtis similarities. Relative mean abundances are calculated as the OTU mean abundance divided by the average number of normalised reads in each sample for biofilm and water respectively. The taxonomy for the OTUs are given at the lowest level obtained, either at order- (o), family- (f) or genus- (g) level.

OTU ID	Taxonomy	Cumulative contribution [%]	Relative abundance (b)	Relative abundance (w)
OTU_1	f: Rhodobacteraceae (Alphaproteobacteria)	9.74	0.14	0.15
OTU_4	g: *Sphaerotilus* (Betaproteobacteria)	13.00	0.05	0.00
OTU_2	g: *Thiothrix* * (Gammaproteobacteria)	16.10	0.04	0.02
OTU_9	g: *Rhodoferax* * (Betaproteobacteria)	18.91	0.02	0.04
OTU_3	g: *Mycobacterium* (Actinobacteria)	21.49	0.01	0.03
OTU_17	f: Sphingomonadaceae (Alphaproteobacteria)	23.03	0.02	0.02
OTU_5	o: Acidimicrobiales * (Actinobacteria)	24.49	0.02	0.02
OTU_11	f: Parachlamydiaceae (Chlamydiia)	25.89	0.01	0.02
OTU_12	g: *Chryseobacterium* (Flavobacteriia)	27.26	0.01	0.01
OTU_8	g: *Acinetobacter* (Gammaproteobacteria)	28.57	0.02	0.00

* OTU_2, OTU_5, and OTU_9 were classified subsequent to the Usearch data processing using the RDP Classifier tool.

Estimation of average Bray-Curtis similarities showed that microbial communities within water samples varied less in composition than communities within biofilm samples (Figure 3.7). Microbial communities in water samples were highly similar at each sampling time, with average Bray-Curtis similarities as high as 0.92. Microbial communities in biofilm samples however, varied more at each sampling time, indicating a relatively big difference between the units where biofilm was sampled. The similarity between water and biofilm samples also varied between sampling times, but less than similarities for biofilm comparisons, suggesting that there were considerable differences in community composition between biofilm and water samples.

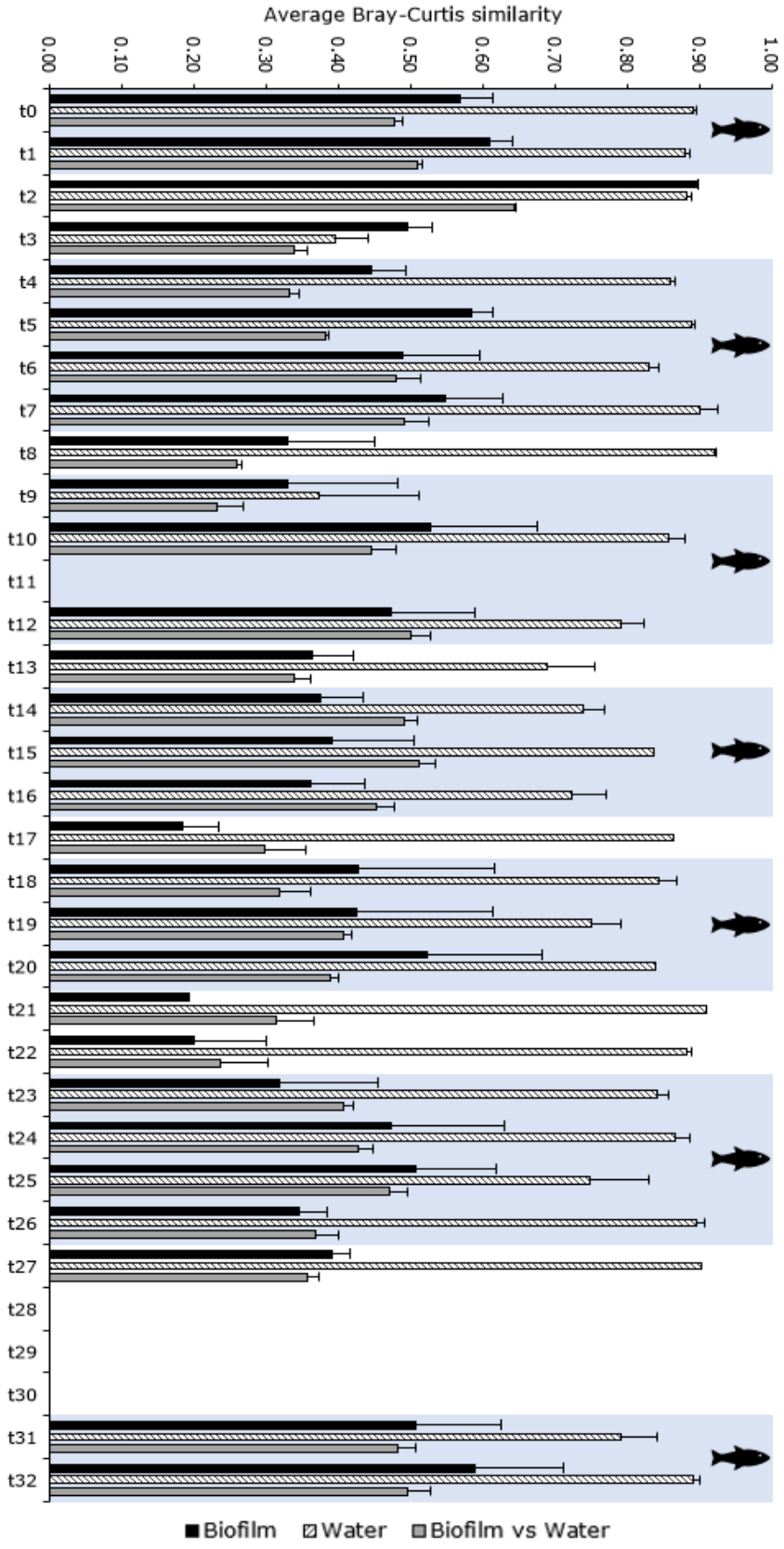

Figure 3.7. Average Bray-Curtis similarities (±SE) for comparisons within water and biofilm samples, and between water and biofilm samples at each sample time. At t11 and t28-t39 there was no data available. Bars without SE represent single comparisons for the relevant sampling time. Sampling times shown with blue background and a fish symbol represent the seven fish batches.

3.2.3. Microbial communities in RAS-water

The community compositions for water samples from the two rearing tanks and the water sump were determined at order level (Figure 3.8). The communities were, in general, similar for samples from the different RAS-units, however, relatively large variations in composition was observed over time. The most common orders in water samples were Rhodobacterials, Burkholderiales and Chlamydiales. Nitrospirales and Nitrosomonadales were also observed in the water samples, but at relatively low average abundances (0.2 %).

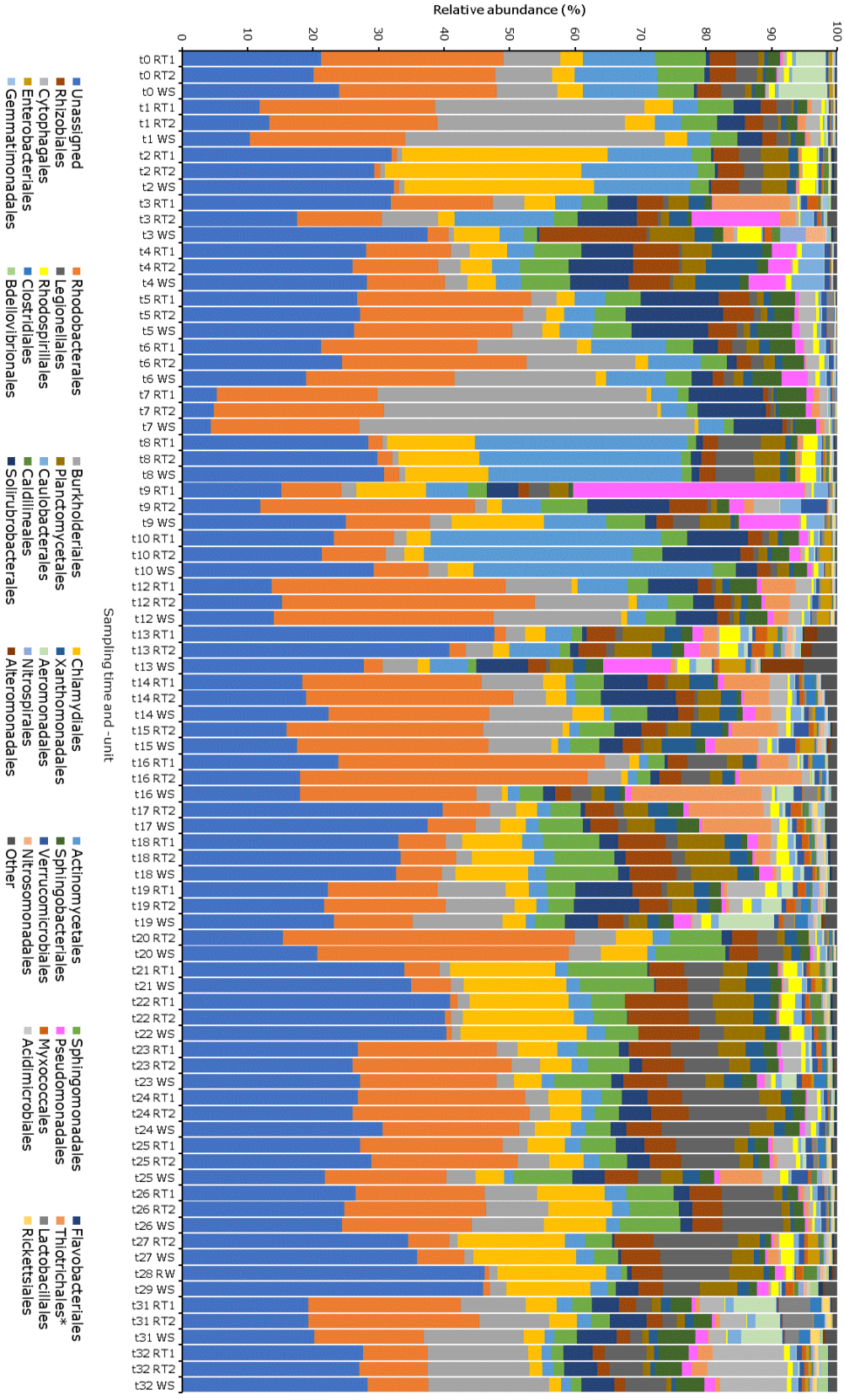

Figure 3.8. Microbial community composition at order level of water samples from rearing tank 1 (RT1), rearing tank 2 (RT2) and water sump (WS) from all sampling times. Orders with average relative abundance over 0.1 % are included in the plot. Orders with average relative abundance under 0.1 % are included in 'Other'. * Included in Thiothrichales is one OTU that was classified using the online RDP Classifier tool.

To examine if there was a difference in the microbial community composition between water samples from the two rearing tanks and the water sump, a PCoA ordination based on Bray-Curtis similarities was performed (Figure 3.9 A). The plot did not indicate differences between water samples, and a one-way PERMANOVA-test confirmed this (p=1.0). Furthermore, water samples clustered according to sample time in the ordination (Figure 3.9 B). This suggests that the microbiota in water samples are similar throughout the RAS-loop (Figure 3.7) and mainly changes over time. In addition, the microbial community composition seems to be affected by the phase in the production cycle (Figure 3.9 C).

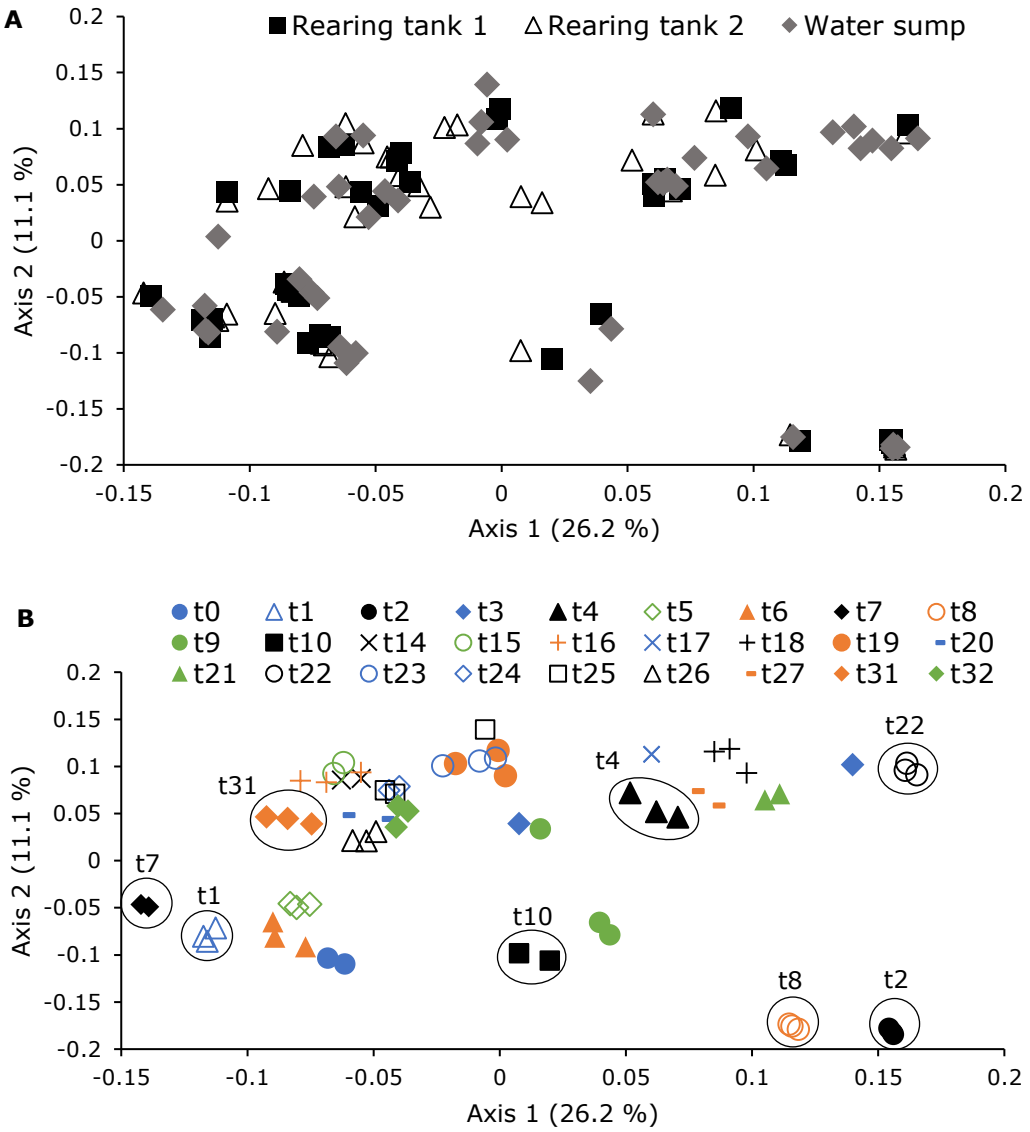

Figure 3.9. PCoA-ordination based on Bray-Curtis similarities from the three units microbial water samples were taken, rearing tank 1, rearing tank 2 and the water sump. A) Sorted by sample unit B) Sample units cluster together according to sample time. Matching symbols represent the three different sampled units at one sample time. Some random sampling times are encircled to emphasise the clustering. C) The ordination shows the development in the microbial communities of water samples during different steps of the production cycle. "No fish" are sample times when there were no fish in the system, "Early in production cycle" is the first sampling time after the fish is moved into the RAS-unit, "Late in production cycle" is the last sampling time before fish are moved out of the RAS-unit and "Middle of production cycle" are all sampling times in between early and late in the production cycle.

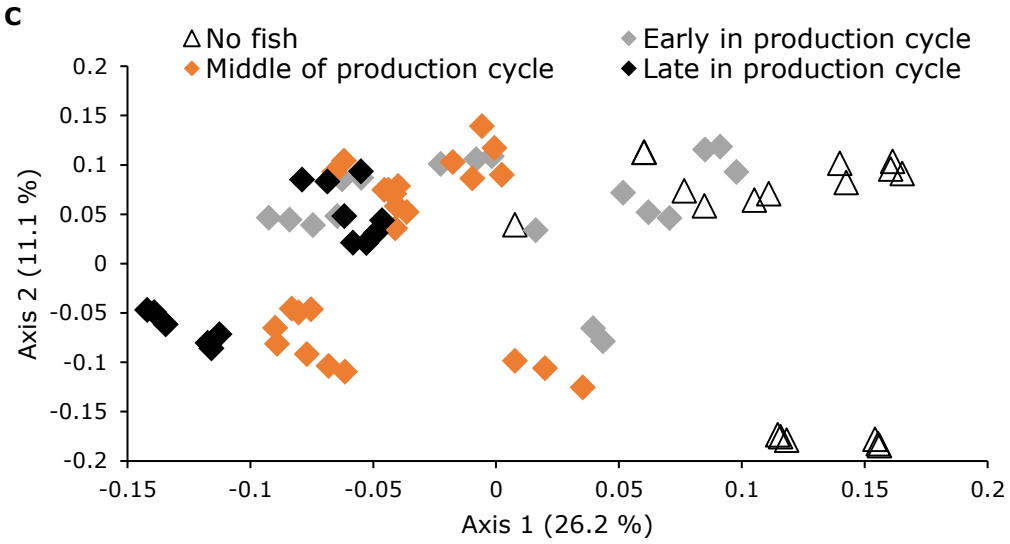

Figure 3.9 continued. PCoA-ordination based on Bray-Curtis similarities from the three units microbial water samples were taken, rearing tank 1, rearing tank 2 and the water sump. A) Sorted by sample unit B) Sample units cluster together according to sample time. Matching symbols represent the three different sampled units at one sample time. Some random sampling times are encircled to emphasise the clustering. C) The ordination shows the development in the microbial communities of water samples during different steps of the production cycle. "No fish" are sample times when there were no fish in the system, "Early in production cycle" is the first sampling time after the fish is moved into the RAS-unit, "Late in production cycle" is the last sampling time before fish are moved out of the RAS-unit and "Middle of production cycle" are all sampling times in between early and late in the production cycle.

3.2.4. Microbial communities in biofilm samples

A PCoA-plot for comparison of biofilm samples from all sampling times suggested that the biofilm communities in the two rearing tanks were different from that in the biofilter (Figure 3.10). This was confirmed to be statistically significant using a one-way PERMANOVA ($p<0.001$). Biofilm samples from the two rearing tanks had a high degree of overlapping in the PCoA-plot, suggesting that these two units had similar biofilm communities.

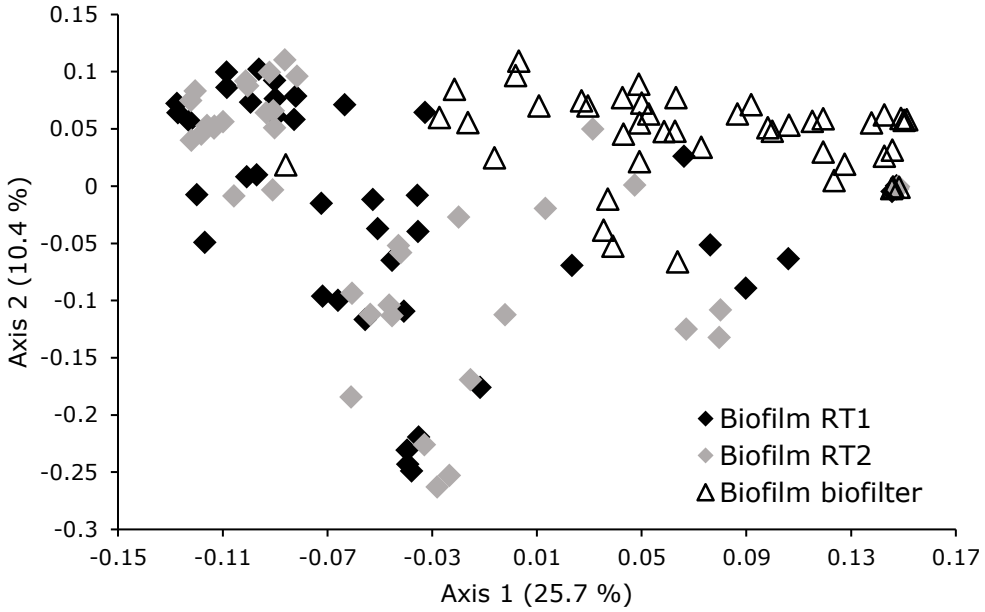

Figure 3.10. PCoA-ordination based on Bray-Curtis similarities for microbial community profiles of all biofilm samples. Biofilm samples were taken in two rearing tanks (biofilm rearing tank 1 (RT1) and biofilm rearing tank 2 (RT2) and from one of the fixed biofilters (biofilm biofilter).

In biofilm samples from the two rearing tanks OTU_1 (*Rhodobacteraceae*) was on average the most common in all samples, followed by OTU_4 (*Sphaerotilus*), OTU_9 (Comamonadaceae), and OTU_17 (*Sphingomonadaceae*). OTU_1 (*Rhodobacteraceae*) was, on average, the second most common in biofilm samples from the biofilter, only exceeded by OTU_2 (*Thiothrix*). OTU_6 (*Sphingomonadaceae*) and OTU 24 (*Nitrospira*) were the third and fourth most common OTUs in biofilm samples from the biofilter.

To examine which OTUs contributed most to the difference in microbial communities between biofilm in rearing tanks and biofilm in the biofilter, a SIMPER analysis based on Bray-Curtis similarities was conducted. The most contributing OTU was OTU_1 (*Rhodobacteraceae*), contributing nearly 10 % of the difference between the biofilms (Table 3.5). OTU_1, the most abundant OTU in the rearing tank biofilm communities (on average 18%) was over twice as abundant in rearing tank biofilm, compared to the biofilter biofilm (on average 7 %). OTU_2 (*Thiothrix*) was the second most contributing, clearly being most abundant in biofilm from the biofilter (around 7 % on average; Table 3.5). OTU_24, classified as the genus *Nitrospira* was found at an average abundance of 2 % in the biofilter biofilm. *Nitrospira* is a genus of nitrite oxidising bacteria commonly found in nitrifying biofilters. The *Nitrospira* OTU hardly occurred in the biofilm of rearing tanks (Table 3.5).

Table 3.5. The top 10 OTUs contributing to the difference between biofilm communities in rearing tanks (BT) and biofilter (BB) were found by conducting a SIMPER analysis based on Bray-Curtis similarities. Relative mean abundances are calculated as the OTU mean abundance divided by the average number of normalised reads in each sample for biofilm in rearing tanks and biofilm, respectively. The taxonomy for the OTUs are given at the lowest level obtained in the classification by the Usearch SINTAX command, either at family- (f) or genus- (g) level.

OTU ID	Taxonomy	Cumulative contribution (%)	Relative abundance (BT)	Relative Abundance (BB)
OTU_1	f: Rhodobacteraceae (Alphaproteobacteria)	9.85	0.18	0.07
OTU_2	g: *Thiothrix* * (Gammaproteobacteria)	15.00	0.02	0.07
OTU_4	g: *Sphaerotilus* (Betaproteobacteria)	18.88	0.07	0.02
OTU_8	g: *Acinetobacter* (Gammaproteobacteria)	20.82	0.02	0.01
OTU_17	f: Sphingomonadaceae (Alphaproteobacteria)	22.51	0.02	0.01
OTU_9	g: *Rhodoferax* * (Betaproteobacteria)	24.11	0.02	0.01
OTU_3280	g: *Pseudorhodobacter* (Alphaproteobacteria)	25.55	0.01	0.01
OTU_6	f: Sphingomonadaceae (Alphaproteobacteria)	26.95	0.02	0.02
OTU_24	g: Nitrospira (Nitrospira)	28.29	0.00	0.02
OTU_3	g: *Mycobacterium* (Actinobacteria)	29.61	0.02	0.01

*OTU_2 and OTU_9 were subsequently classified to a lower taxonomic level using the online RDP Classifier tool.

Biofilm in rearing tanks

The biofilm communities in the rearing tanks appeared to change considerably over time (Figure 3.11). However, the community profiles were observed to be relatively similar at the order level in the two rearing tanks at each sample time. The most common orders in the biofilm communities of the rearing tanks were Rhodobacterales, Burkholderiales, Flavobacteriales, Sphingomonadales and Pseudomonadales. There were also high abundances of OTUs that were not classified at the order level. In general, there were large variations in relative abundance in most orders, and large variations were particularly observed in Rhodobacterales, Sphingomonadales and Flavobacteriales.

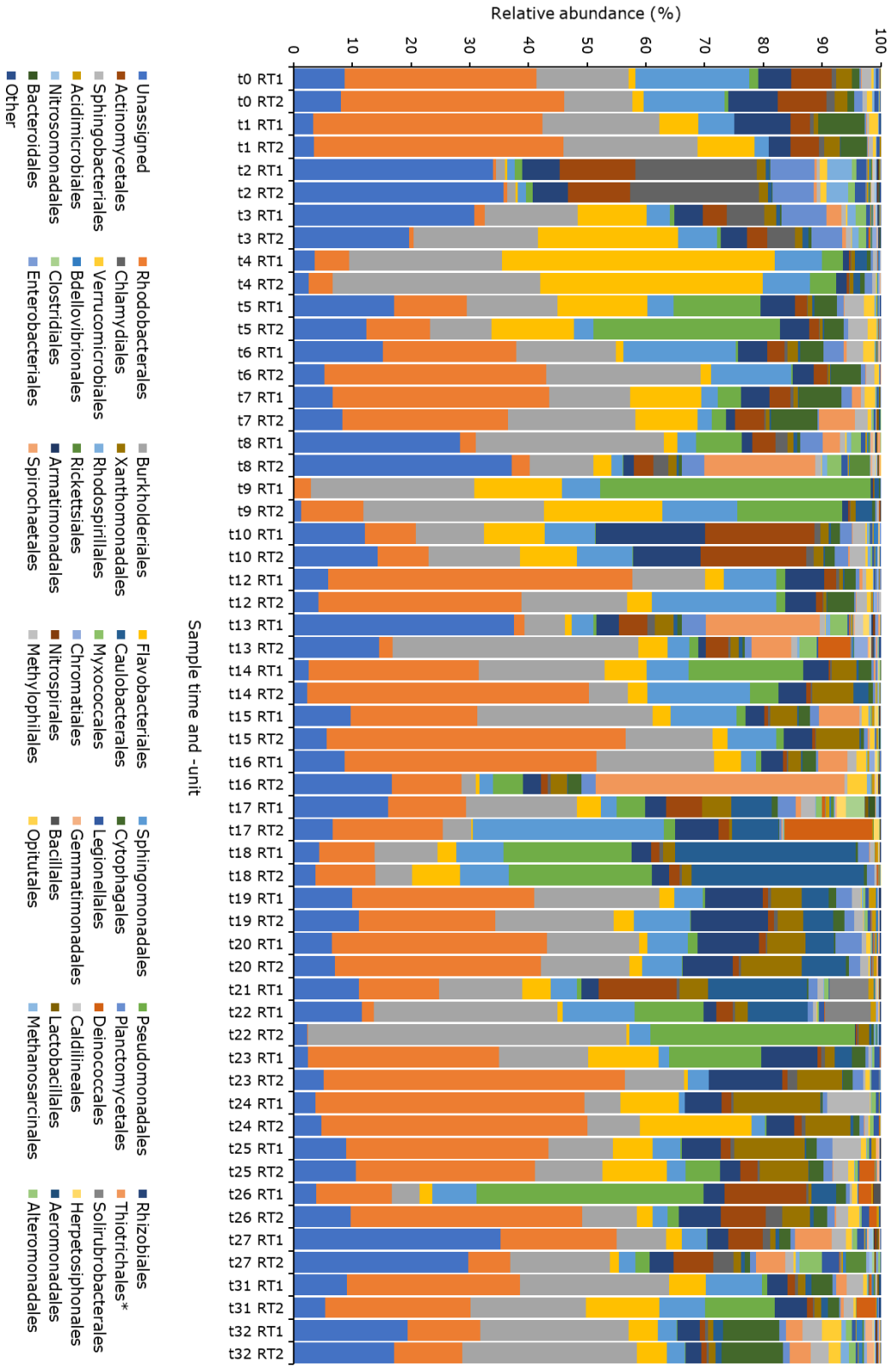

Figure 3.11. Community composition at the order level for biofilm samples from rearing tank 1 (RT1) and rearing tank 2 (RT2) at all sampling times. Orders with average relative abundance over 0.1 % are included in the plot. Orders with average relative abundance under 0.1 % are included in 'Other'. *Included in Thiothrichales was one OTU (OTU_2) that was classified using the RDP Classifier tool.

To study the development of the microbial communities in biofilm samples from the rearing tanks throughout the production period, a PCoA-plot based on Bray-Curtis similarities was made (Figure 3.12). The PCoA plot indicates that microbial communities in the rearing tank biofilm changes over time and become more similar to each other late in the production cycle compared to earlier in the production cycle.

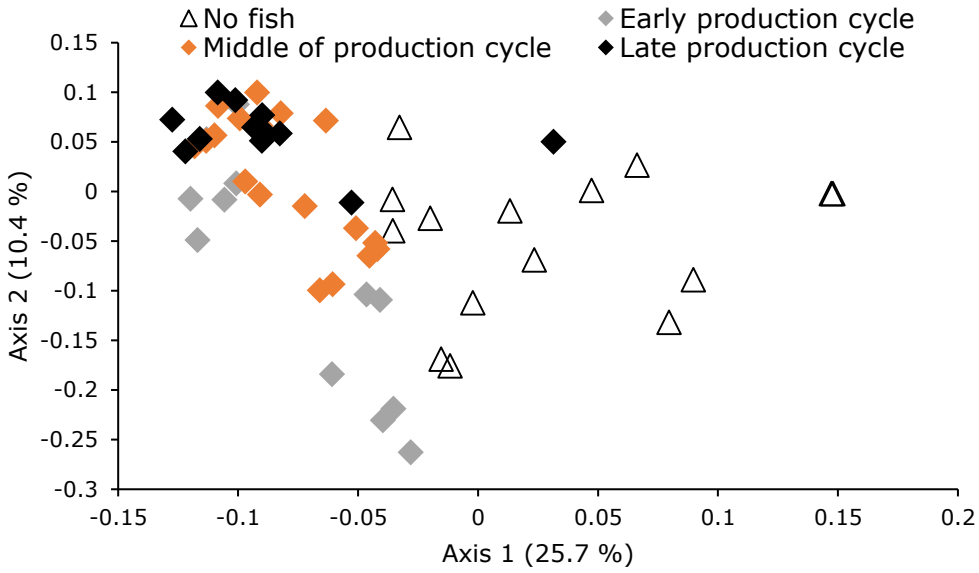

Figure 3.12. PCoA ordination based on Bray-Curtis similarities for microbial community profiles of biofilm samples from the rearing tanks. The ordination shows the development in the microbial communities during different steps of the production cycle. "No fish" are samples from periods without fish in the system, "Early production cycle" is the first sampling time after the fish is moved into the RAS-unit, "Late production cycle" is the last sampling time before fish are moved out of the RAS-unit and "Middle of production cycle" are all sampling times in between early and late in the production cycle.

Biofilm in the biofilter

The microbial communities in biofilm samples from the biofilter had relatively large variations in composition over time (Figure 3.13). When studying the community composition in biofilm samples from the biofilter on order level, Rhodobacterales, Rhizobiales, Thiothrichales and Burkholderiales were the most common (Figure 3.13). The order Thiothrichales had large variations in relative abundance in biofilm samples over time. Rhodobacterales also varied in relative abundance over time.

The nitrite-oxidising order Nitrospirales was the 8th most common order with a relative abundance of 3.7 %, when sorting after average relative abundance of all sampling times. The ammonia-oxidising Nitrosomonadales, on the other hand, had an average relative abundance of 0.9 % and was only the 18th most common order.

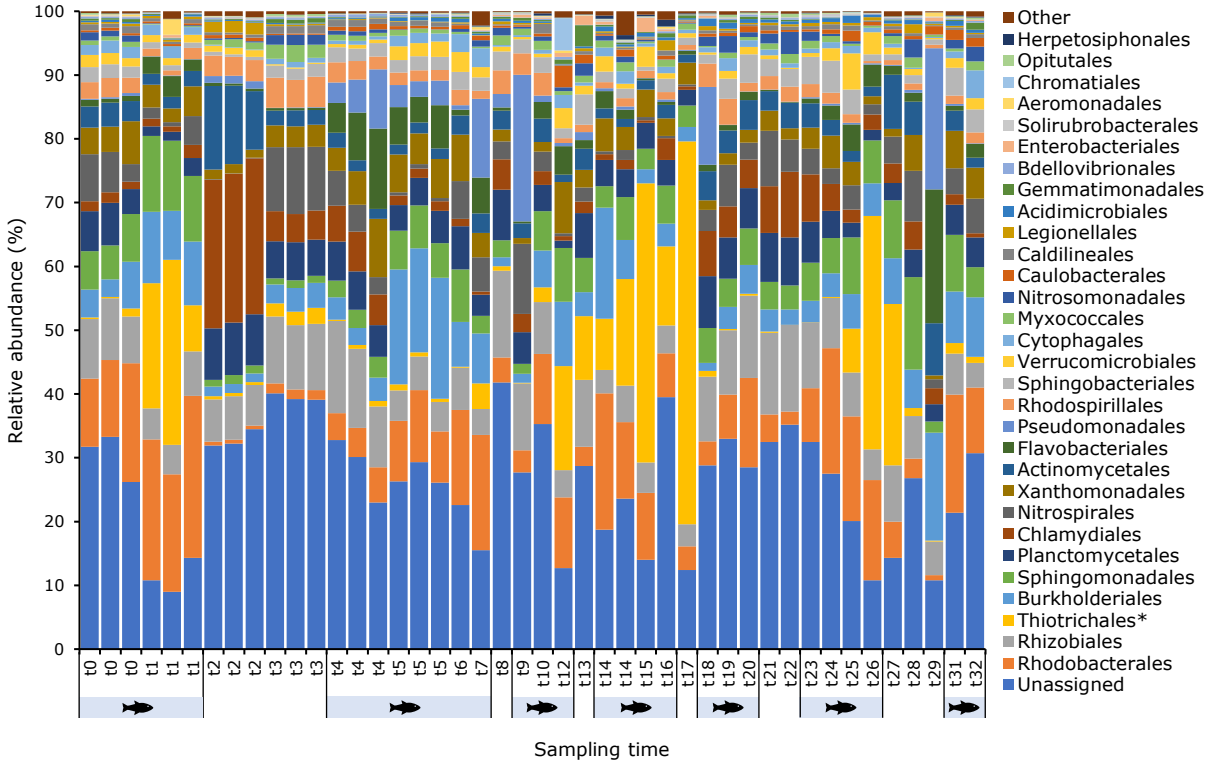

Figure 3.13. Microbial community composition at order level for biofilm samples from the biofilter. Sampling times marked with a fish symbol on a blue background represent the seven fish batches. Orders with a relative abundance over 0.01 % was included in the plot, while orders with a relative abundance under 0.01 % are included in "other". Replicates are included in sampling times t0-t5 and for t14. *Included in Thiotrichales is one OTU that was classified using the RDP Classifier tool.

A moving window analysis of the biofilter-biofilm samples, based on average Bray-Curtis similarities, where microbial community composition on one sample time are compared to the following sample time, showed large variations over time (Figure 3.14). The average Bray-Curtis values ranged from 0.19 when comparing t1 and t2, to 0.79 when comparing t21 and t22. On average, the Bray-Curtis similarity when comparing two subsequent sampling times was 0.47.

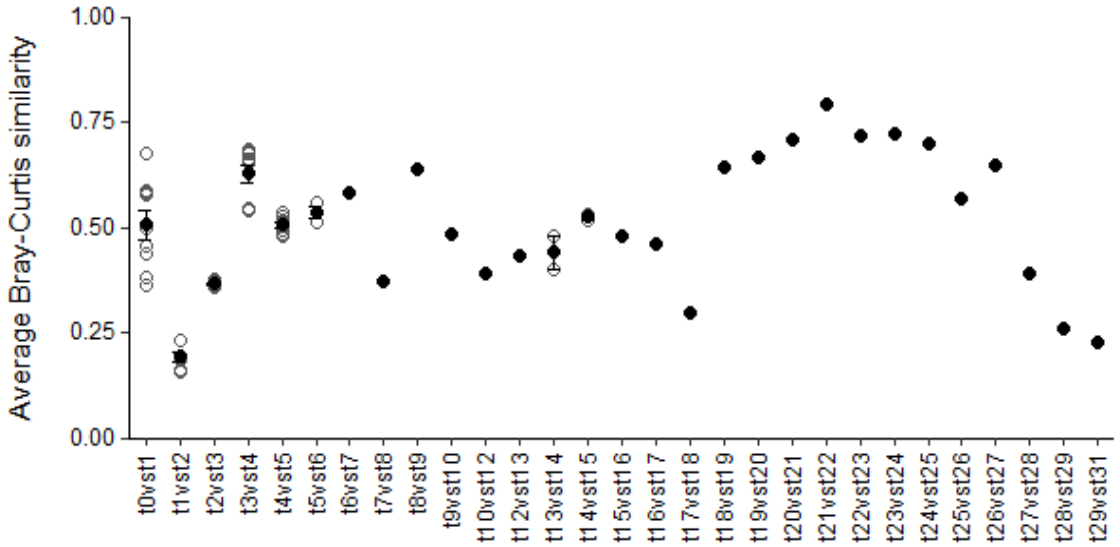

Figure 3.14. A moving window analysis (comparing microbial community composition of one sample time to the following sample time) based on average Bray-Curtis similarity in the biofilter biofilm. When there were replicate samples (t0-t5 and t14) the filled symbols represent the calculated mean.

A PCoA analysis indicated that the biofilm communities in the biofilter tended to change according to phase in the production cycle (Figure 3.15). The plot further indicated that the biggest differences in biofilm communities in the biofilter were between sample times without fish and sample times where the fish had been in the system for a longer period (late in production cycle).

A one-way PERMANOVA confirmed that there was a significant difference in microbial communities in the biofilm from the biofilter between samples representing periods when there were no fish in the system and samples representing the late phase in the production cycle ($p=0.012$). There was also a significant difference in the microbial communities in biofilm from the biofilter between the groups "No fish" and "Middle of production cycle" (PERMANOVA, $p=0.03$), also indicating that the phase of production affects the biofilter-biofilm communities.

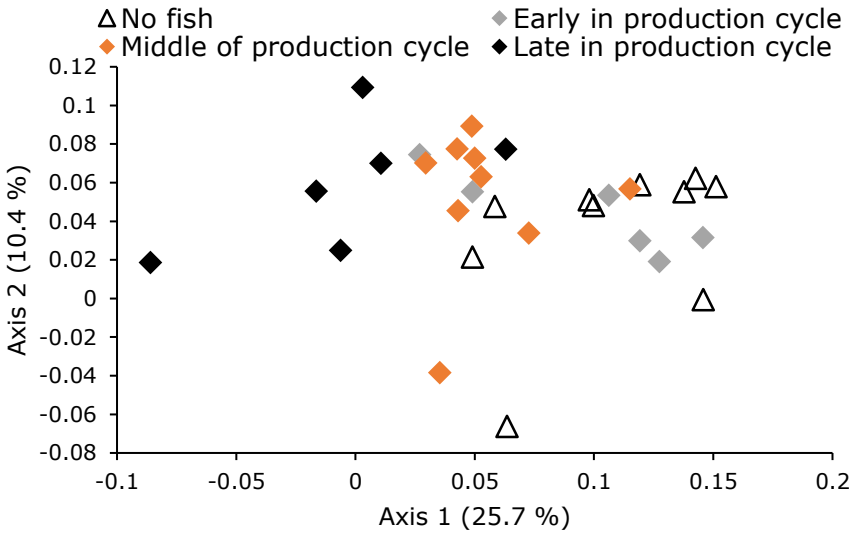

Figure 3.15. PCoA ordination based on Bray-Curtis similarities for microbial community profiles of biofilm-samples from the biofilter. The ordination shows the development in the microbial community in biofilm from the biofilter during different steps of the production cycle. "No fish" are sample times when there were no fish in the system, "Early in production cycle" are the first samplings time after the fish was moved into the RAS-unit, "Late in production cycle" are the last sampling time before fish were moved out of the RAS-unit and "Middle in production cycle" are all sampling times in between early and late in the production cycle.

To see which OTUs contributed the most to the difference in communities between the periods without fish and the last stage in the production cycles, a SIMPER-analysis based on Bray-Curtis similarities was conducted (Table 3.6). The top five contributing OTUs explained nearly 30 % of the difference. OTU_2 (*Thiothrix*) was the most contributing OTU, however it had the same relative abundance in both sampling periods. OTU_1, representing the family *Rhodobacteraceae,* was the second most contributing, with a considerable higher relative abundance late in the production cycle, compared to when there was no fish in the system. Of the top five contributing OTUs only one was more abundant in periods without fish, and this was OTU_11 (*Parachlamydiaceae*).

Table 3.6. A SIMPER-analysis was used to find the five OTUs contributing most to the difference between the microbial communities when there were no fish in the system (NF) and microbial communities late in the production cycle (LP). Relative mean abundances are calculated by dividing the OTU mean abundance by the average number of normalised reads in each sample for NF and LP, respectively. The taxonomy for the OTUs are given at the lowest level obtained, either at family- (f) or genus- (g) level.

OTU ID	Taxonomy	Cumulative contribution [%]	Relative abundance (NF)	Relative abundance (LP)
OTU_2	g: *Thiothrix* * (Gammaproteobacteria)	11.03	0.11	0.11
OTU_1	f: Rhodobacteraceae (Alphaproteobacteria)	20.03	0.01	0.13
OTU_3280	g: *Pseudorhodobacter* (Alphaproteobacteria)	24.93	0.00	0.07
OTU_76	f: Comamonadaceae (Betaproteobacteria)	26.74	0.00	0.03
OTU_11	f: Parachlamydiaceae (Chlamydiia)	28.51	0.03	0.01

*OTU_2 was classified using the RDP classifier tool.

Nitrifiers in the biofilter biofilm

OTUs representing nitrifying bacteria were identified by manual inspection of the normalised OTU-table. Nine OTUs were found to represent nitrifying bacteria. The relative abundances of the OTUs representing nitrifiers in the biofilter biofilm samples varied considerably over the 15 months period (Figure 3.16). The nitrite-oxidising genus *Nitrospira* was dominating among nitrifiers at most sampling times. *Nitrosomonas*, which is a genus of ammonia-oxidising bacteria, was the second most abundant genus among the nitrifiers.

The relative abundance of nitrifiers showed a slight tendency to decrease throughout the fish batches, followed by an increase during fallowing periods (Figure 3.16). The highest relative abundance of nitrifiers was observed late in the first fallowing period, at sampling time t3, with an average relative abundance of around 13 % of the total biofilm community. The lowest abundance was observed the preceding sampling time, t2, which was earlier in the same following period. The average relative abundance of nitrifiers in t2 was 0.6 % of the total biofilm community. There was exactly 14 days between these two sampling times.

Due to the high relative abundance of different *Nitrospira* OTUs and the low relative abundance of OTUs representing ammonia oxidising bacteria (AOBs), it was investigated if some of the *Nitrospira* OTUs could represent complete ammonia oxidisers (COMAMMOX). To examine this, the sequences of OTUs classified as *Nitrospira* were compared with previously published 16S rRNA sequences of COMAMMOX and other *Nitrospira* strains. A phylogenetic tree based on the neighbour-joining method was generated and interestingly indicated that one of the *Nitrospira* OTUs (OTU_1771) was related to two described COMAMMOX bacterial species, *Nitrospira nitrificans* and *Nitrospira nitrosa* (Figure 3.17). OTU_1771 was on average the third most abundant OTU in the biofilter biofilm with an average relative abundance of 0.85 %. OTU_24, which was the most abundant *Nitrospira* OTU, with an average relative abundance of 2.12 %, was found to be related to *Nitrospira defluvii*.

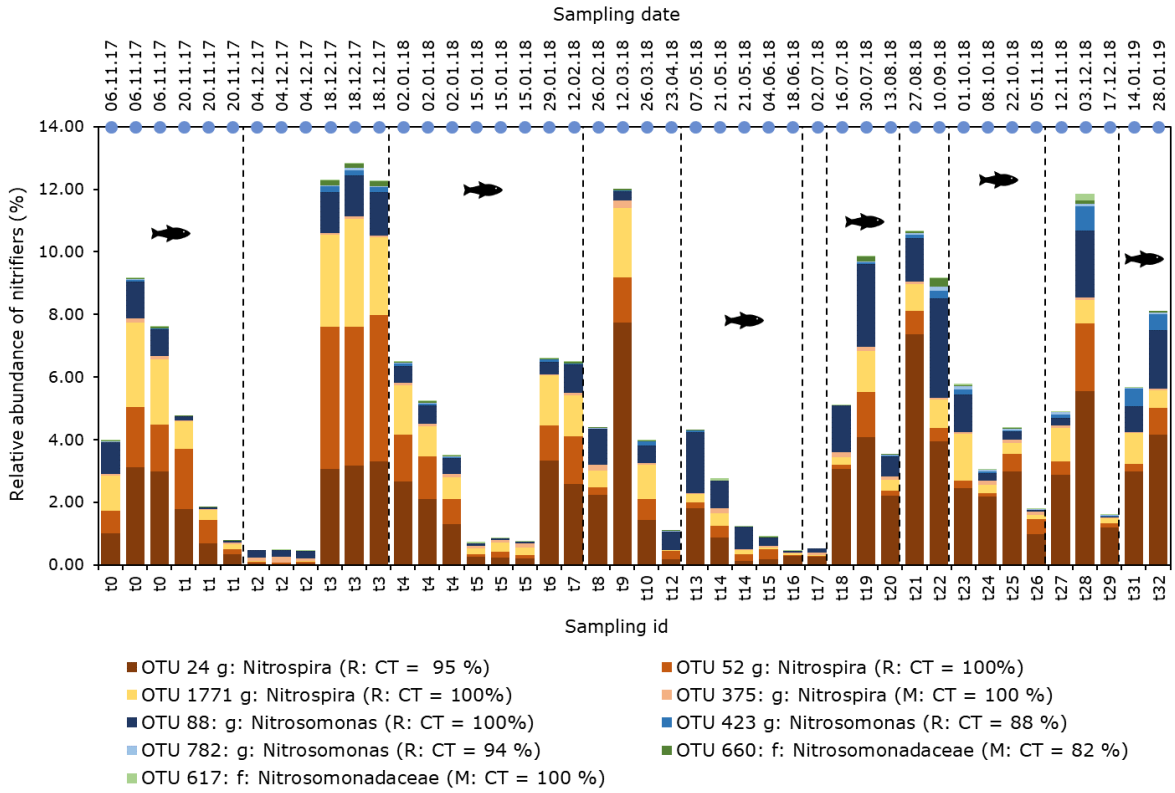

Figure 3.16. Relative abundance of OTUs classified as nitrifiers at different sampling times in the biofilter biofilm samples. The taxonomy for the OTUs are given at the lowest level obtained, either at family- (f) or genus- (g) level. Sample id t0-t5 and t14 are given with replicates to show replicate variation. R = classified with RDP, M = classified with MiDAS, CT = confidence threshold. Sampling times with fish in the system are symbolised with a fish ( ), representing the seven different fish batches. Sampling times without the fish symbol represent the fallowing periods between fish batches.

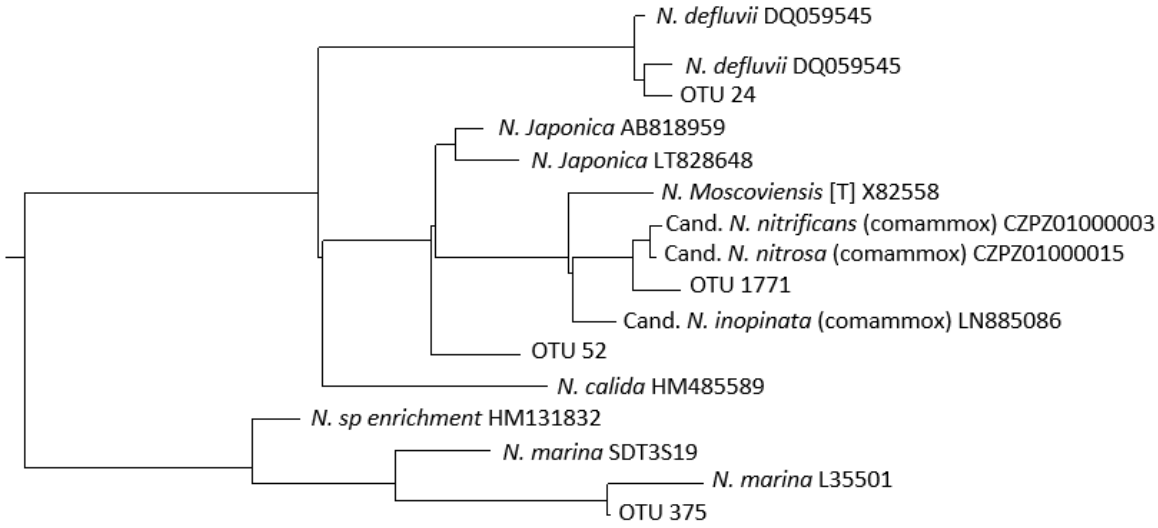

Figure 3.17. A Neighbour-joining tree for comparing OTUs classified as *Nitrospira* to previously published *Nitrospira* 16S rRNA gene sequences. Sequences were retrieved from the SILVA and RDP databases. Sequence accession numbers for these sequences are provided in the tree. The tree was generated using the Mulit-way alignment tool with default parameters in the program package Clone Manager (v9).

4. Discussion

4.1. Evaluation of production data and effects of water quality

4.1.1. Evaluation of growth, biomass and daily mortality

When comparing the seven fish batches that were cultivated in the studied RAS during the project period, there were some variations in duration of the production period, fish density, and fish weights. Fish batch 5 stands out with a considerably lower average number of fish in the two rearing tanks (Table 3.1). The lowered stocking density can positively affect the cultivated fish, and this was reflected in the daily mortality, as fish batch 5 had a considerably lower average mortality (on average 0.05 %) than the other fish batches (averages ranging from 0.10-0.21 %). The next fish batch, fish batch 6, also stands out, with a slightly higher average number of fish than the other fish batches. However, this did not seem to negatively affect the daily mortality. Fish batch 6 was also kept in the start-feeding department for a longer period than the other fish batches, and consequently reached a higher average fish weight. This is believed to have influenced the RAS-system as bigger fish needs more feed, and this subsequently leads to an increased loading on the system. This was supported by the relatively high concentrations of nitrogenous substances in fish batch 6 compared to the other fish batches (Figure 3.1).

When considering the two different rearing tanks, there is a tendency of bigger fish in rearing tank 2, for most fish batches. This could indicate a better cultivation environment in rearing tank 2, compared to rearing tank 1. One possible explanation can be that there were better tank hydraulics in rearing tank 2, such as better oxygen-dispersion or flow in the tank. However, one can only speculate on why this tendency was observed, as no obvious explanation comes to mind.

The daily mortality was on average 0.12 % for all fish batches, with a tendency of being slightly raised early in the production periods (Figure 3.2). However, no other known sources state what a normal daily mortality is in start-feeding of Atlantic salmon, which makes it difficult to evaluate if the mortality is high or low. On the other hand, the Norwegian Veterinary Institute uses a threshold of 1.5 % total mortality for start-feeding of Atlantic salmon alevins until the fish have reached 1 g (Personal communication, B. Tørud, 02.06.2020). The total mortality (from 0.2-1.0 g) in the studied start-feeding was on average 5 % for all fish batches, which is considerably higher than the suggested threshold. A heightened mortality rate is typical early in the production period in smolt production and is often associated with poor egg quality (Norwegian Veterinary Institute 2019). However, other factors such as the high stocking density in the studied start-feeding can be a contributing factor to the high mortality.

The pattern of mortality was similar for all fish batches in both rearing tanks. The highest single incident in mortality was found in both rearing tanks in Fish batch 7 on the 3rd of January 2019. The RAS-facility reported that they had problems with removing excess particles from the rearing tanks after rebuilding the department. High levels of suspended solids in the rearing water can have both direct and indirect effects on fish health. Firstly, suspended solids can have adverse effects on gills and fish health (Lu, Kania, and Buchmann 2018), and secondly, the particles can be substrate for bacterial growth, especially heterotrophic opportunistic bacteria (Michaud et al. 2006). This can further indicate that the aggregation of suspended solids occasioned the increased mortality.

4.1.2. Evaluating the physicochemical water quality

The physicochemical water quality variables were, in general, good and relatively stable for all fish batches and fallowing periods, indicating a well dimensioned RAS. The temperature was kept in a range between 12-14 °C during the whole production period (Table 3.2), which is slightly lower than the suggested optimal temperature, 15-16 °C, for optimal growth rate of Atlantic salmon (Hjeltnes et al. 2012). However, several studies have shown that freshwater rearing temperatures exceeding 12 °C can cause skeletal deformities in farmed salmon (Hjeltnes et al. 2012). This further indicates that optimal temperatures for growth rate of Atlantic salmon and biofilter function in RAS can, subsequently, cause losses in production. Nevertheless, no unusual mortality or occurrence of deformities were reported.

Concentrations of nitrogenous substances

As expected, the concentrations of nitrogenous substances (ammonia, nitrite and nitrate) increased throughout the fish production batches, followed by a decrease in fallowing periods (Figure 3.1). This is logical, as the increased input of fish feed as the cultivated fish grows, subsequently gives an increased production of wastes. The following reduction in nitrogenous substances is also logical as the feeding stops during fallowing periods. However, as a high dose of ammonia chloride was added upstream of the biofilter during fallowing periods, one would expect that the levels of nitrogenous substances would not decrease as much as observed in Figure 3.1. A possible explanation for the decline of nitrogenous substances could be that the biofilter achieved an enhanced nitrification efficiency during fallowing periods, possibly due to the sudden decrease of available organic matter, subsequently reducing the fraction of heterotrophic bacteria (Michaud et al. 2006).

The concentration of nitrite was, in general, low, however, there were single measurements as high as 1.3 mg NO_2^-/L (Figure 3.1 B). The suggested threshold for nitrite is <0.1 mg/L in freshwater (Hjeltnes et al. 2012); however, it is not stated if this threshold is based on nitrite (NO_2^-) or nitrite-nitrogen (NO_2^--N). There is a big difference in the molecular weights between these two molecules, where 1 mg/L NO_2^--N equals 3.3 mg/L NO_2^-. A nitrite concentration of 1.3 mg/L thus equals 0.39 mg/L nitrite-nitrogen, which is above the suggested threshold for nitrite in freshwater. However, as the toxicity of nitrite is considerably reduced with increasing chloride (Cl^-) levels, and the studied RAS had an average salinity between 1-2 ppt, it is not certain that the level of nitrite was toxic to the reared fish. However, this is not easy to prove as no known studies have researched effects of different nitrite levels in low-saline water. Nevertheless, there was no observed increase in daily mortality after the single incident of elevated nitrite levels, indicating that the levels were sub-lethal for the reared fish.

Concerning nitrate concentrations, there was one incident, in Fish batch 5, where the nitrate concentration reached 390 mg/L, corresponding to 88 mg/L NO_3^--N/L (Figure 3.1C). The Norwegian Food Safety Authority has no suggested threshold for nitrate levels in RAS cultivating Atlantic salmon, however, a study on juvenile rainbow trout (*Oncorhynchus mykiss*) concluded that chronic exposure to NO_3^--N concentrations of 80-100 mg/L affected the fish health and welfare (Davidson et al. 2014). On the other hand, no effects were found on Atlantic salmon post-smolts exposed to 100 mg/L NO_3^--N (Davidson et al. 2017). As there are no known studies on chronic nitrate exposure in juvenile Atlantic salmon, and as these are thought to be more sensitive than post-smolt, chronic exposures could possibly have adverse effects on juvenile Atlantic salmon. On the other hand, single

incidences of nitrate-nitrogen concentrations exceeding 100 mg/L are not believed to have major effects on fish health if other conditions are kept stable. Nevertheless, further research is needed on chronic nitrate exposures in juvenile Atlantic salmon.

4.1.3. Evaluation of the microbial water quality

The maximum number of bacterial cells in this study, as estimated by flow cytometry in fish batch 7, was 5.7 x 10^5/mL. Few known studies have estimated the total bacterial cells in freshwater RAS. However, a study investigating the effects of acetate on a freshwater RAS found total cell numbers to range between 5.0 x 10^6/mL – 72 x 10^6/mL (Rojas-Tirado et al. 2019). This could indicate that the bacterial density in the studied RAS was low. On the other hand, the estimated CFU/mL was also low, possibly confirming the low numbers of bacterial cells in the studied RAS. Besides, the fraction of cultivable bacteria (CB), calculated as the ratio between CFU/mL and total bacterial cells/mL, ranged between 0.9 - 3.1 %, which was as expected. As the CB was as expected, this can suggest that the bacterial numbers estimated by flow cytometry and colony forming units (CFU) were within normal range for a freshwater RAS. However, there are no known other studies estimating CB in a freshwater RAS, to compare the results to.

Low fractions of cultivable bacteria (CB) can indicate K-selected and stable microbial communities with low numbers of opportunistic bacteria (Skjermo et al. 1997). Both the percentage of cultivable bacteria and the fraction of opportunistic bacteria were on average highest on the 17th of January, compared to the two other sampled dates (Table 3.3 and Figure 3.3). At this point, the RAS still had some problems with removing particles from the rearing tanks after rebuilding the department, providing high levels of substrate for free-living heterotrophic opportunistic bacteria. This suggest that the microbial water quality was poorer on the 17th of January, compared to the two other sampled dates. In addition, the bacterial growth potential on the 17th of January was considerably lower than the 28th of January, suggesting that there was a lower amount of resources available for bacterial growth on the 17th compared to the 28th. A possible explanation for why the bacterial growth potential was lower on the 17th can therefore be that most of the resources available for growth already was utilised by the fast-growing opportunistic bacteria. The opposite can be seen on the 28th as there were low fractions of opportunistic bacteria, and a high bacterial growth potential.

A tendency of a higher fraction of opportunistic bacteria in the rearing tanks compared to the treated water in the water sump gave support to the proposed hypothesis, that there will be more slow-growing, K-selected, bacteria in the water samples from the treated water compared to water samples from the rearing tanks. However, this trend was only seen on two out of three sampling days in fish batch 7 (Figure 3.3). When considering the bacterial growth potential, we hypothesised that the bacterial growth potential would be higher in the rearing tank water compared to the treated water, and the results indicated that this might be true. A high bacterial growth potential can indicate that high amounts of resources and niches are available for bacterial growth (Attramadal et al. 2016). A possible explanation for the lower bacterial growth potential in the treated water can be that the biofilter reduces the amounts of available resources for bacterial growth. The biofilter houses large quantities of heterotrophic bacteria, and as the water goes through the biofilter, the heterotrophic bacteria consume the available organic matter, leading to a decrease in the bacterial growth potential. Together these results indicate that the

microbial water quality was better in the treated water in the water sump compared to the water in the rearing tanks.

The fraction of opportunists was considerably higher in rearing tank 1, compared to rearing tank 2, suggesting a poorer microbial water quality in rearing tank 1. This was supported by the reported average fish weights, which, on average, were higher in rearing tank 2, compared to rearing tank 1. However, as the fraction of opportunistic bacteria only was estimated on three sampling days, one should be careful with concluding, and data from more sampling days should have been collected to confirm the hypothesis.

4.2. Microbial community analysis

4.2.1. Comparing microbial communities in biofilm and water

As hypothesised, there was a significant difference between the microbial communities in water samples and biofilm samples. Moreover, Rhodobacterales was the most frequent order in all samples. Rhodobacterales was also found to be very common both in a brackish water (8 ppt) RAS, producing Atlantic salmon smolts (Rud et al. 2017), and a seawater (24 ppt) RAS producing Lumpsucker (*Cyclopterus lumpus*) (Dahle et al. 2020), suggesting that it is, in general, common in RAS in Norway.

Despite of similar abundances in water- and biofilm samples, OTU_1 (*Rhodobacteraceae*) was found to be the most contributing OTU to the difference between water and biofilm-samples. A possible explanation for the high contribution percentage, despite the similar abundances, can be that OTU_1 was common in both biofilm and water throughout the total sampling-period for microbial community analyses. Besides, the relative abundances in the SIMPER-analysis are given as an average of the abundance at all sampling times, meaning that the abundance could have varied a lot at different sampling times, resulting in a high contribution percentage in the SIMPER-analysis.

OTU_4 (*Sphaerotilus*) and OTU_2 (*Thiothrix*) was found (by SIMPER-analysis) to be more relatively abundant in biofilm samples (0.05) than in water samples (0.00). *Sphaerotilus* is a genus of heterotrophic bacteria commonly found in biofilm (Huang et al. 2019). In a study where Atlantic salmon parr were produced in a freshwater RAS, *Sphaerotilus* was found at relative abundances around 0.01-0.02 in water samples (Fossmark et al. 2020). Moreover, *Thiothrix* is a genus of filamentous sulphur-oxidising bacteria, previously found in wastewater treatment plants (Molina-Muñoz et al. 2007). *Thiothrix* was also found in microbial samples from a RAS-site producing Atlantic salmon post-smolts; however, this study did not state if it was more abundant in biofilm or water samples (Rud et al. 2017). These results suggest that both *Sphaerotilus* and *Thiothrix* are common genera found in RAS in Norway.

OTU_3 (*Mycobacterium*) was more abundant in water samples (0.03) than in biofilm samples (0.01). The genus *Mycobacterium* includes several highly contagious fish pathogens that can cause piscine mycobacteriosis. This disease causes characteristic nodules on internal organs; however, the external signs are often non-specific (Gauthier and Rhodes 2009). Fossmark et al. (2020) also found high relative abundances of a *Mycobacterium*-OTU and concluded that the high relative abundances were most likely due to a high availability of nutrients. Moreover, in the same study, the characterised *Mycobacterium*-OTU was found to be non-pathogenic. In the present study, regardless of the average relative abundance of 3 % in water samples and 1 % in the biofilm samples,

there were no reported disease outbreaks on the RAS-facility during the 15-months sampling period, suggesting that the found Mycobacterium OTU probably was non-pathogenic or that it was not present in high enough abundances to infect the fish. This could indicate that *Mycobacterium* is a generally common bacteria-genus in freshwater RAS under production of Atlantic salmon, and moreover, it underlines that many *Mycobacterium* species are not pathogenic.

4.2.2. Microbial communities in RAS-water

The microbial communities in the rearing tanks were hypothesised to be different from the communities in the water returning from the water loop (water sump 2), especially due to the UV-disinfection step before the treated water entered the rearing tanks. However, our results indicated that the microbial communities in the RAS-water were similar and rather stable throughout the RAS-loop (Figure 3.7, Figure 3.8 and Figure 3.9B). UV-disinfection has been previously found to destabilise the microbial communities in RAS by creating a gap in the carrying capacity, which creates favourable conditions for recolonization and proliferation of opportunistic bacteria (Attramadal et al. 2012a). However, the results in our study can possibly be explained by the short hydraulic retention time (HRT) in the rearing tanks, as the HRT in the rearing tanks was around 20-30 minutes. The time that the water was kept in the rearing tanks would probably not be sufficient for the microbiota to change enough for us to be able to see a change in the microbial community composition (Bakke et al. 2017).

On the other hand, the results regarding OTU richness and Shannon's diversity (Figure 3.5) showed a tendency of higher diversity in the treated water in the water sump, compared to the water in the rearing tanks. This tendency was seen in three of the total five commercial producers included in the MonMic project, indicating that the water treatment positively affected the alpha diversity of the rearing water. These seemingly contradictory results can possibly be explained by differences in methodology. The results indicating that the microbial communities in the RAS-water were similar throughout the RAS-loop were based on analyses using the Bray-Curtis similarity index. Rare OTUs does not have a big effect on the Bray-Curtis similarities, however, these rare OTUs influence the alpha-diversity measures. This indicates that if rare OTUs had been emphasised more, by for example using a different similarity index, there might have been a clearer difference between the microbial communities in rearing tank water compared to treated water. One such similarity index could have been the Sørensen-Dice index, which takes into account the presence of rare OTUs (Chao et al. 2006). Besides, as earlier mentioned, water samples from the treated water showed a lower number of opportunistic bacteria and bacterial growth potential than the water from the rearing tanks, suggesting that there was difference in the selection pressure there. Furthermore, this indicates that there might be a difference between the microbial water-communities in rearing tanks and the treated water, but that this difference was not big enough to be detected by the Bray-Curtis similarity index.

The results indicated that the microbiota in water samples was more affected by the sampling time, than the different sampled units (Figure 3.8 and Figure 3.9). The microbial communities in the water were also shown to change according to phase in the production cycle (Figure 3.9C). The microbiota in the water samples without fish was different from the microbiota in water samples with fish, and the microbial communities seemed to become more similar as the fish grew. This can indicate that the system selects for the

similar suspended microbiota in each fish batch possibly as a result of system design and operational routines. In addition, results from the MonMic-project indicate that the different RAS have their own distinct in-house water-microbiota, possibly laying the foundation for the microbiota-development during each fish batch (Personal communication, S. Dahle, 26.05.2020).

The microbial community composition in RAS-water was dominated by the orders Rhodobacterales, Burkholderiales and Chlamydiales. Chlamydiales is an order where all known species are obligately parasitic, which means that they cannot complete their life cycle without a host. Both fish and humans can be hosts for the Chlamydiales-parasites. In addition, many species within Chlamydiales can use amoebas as hosts for reproduction (Lienard et al. 2016). Amoebas are ubiquitous, particularly in water environments, and many can form resistant cysts that help them survive during unfavourable growth conditions (Lienard et al. 2016). This can make them resistant to the UV-disinfection inside the RAS-loop. Despite the relatively high abundance of Chlamydiales in water, there were no reported disease outbreaks in the studied RAS, indicating that the Chlamydiales-species were non-pathogenic or not present in high enough abundances to infect the fish.

4.2.3. Microbial communities in biofilm samples

As hypothesised, the microbiota in biofilm from the rearing tanks was significantly different from biofilm from the biofilter (Figure 3.10). This was expected as the two different biofilms are subjected to different selection pressures, resulting in dissimilar functions in the RAS. The alpha diversity was significantly lower in biofilm samples from the two rearing tanks compared to the other water- and biofilm samples (Figure 3.5). Moreover, there were large variations within biofilm communities over time, which was most likely caused by the less stable communities in the biofilm from the rearing tanks, compared to the more stable biofilm communities in the biofilter (Figure 3.11). As the rearing tanks was thoroughly cleaned with soap between each fish batch, they had to be frequently re-colonised with biofilm on the rearing tank walls. According to ecological theory on succession, biological communities become more diverse as the succession-process proceeds (Fierer et al. 2010). As opposed to the biofilm in the biofilter, which only was backflushed with water, the biofilm in the rearing tanks had to go through a primary succession process between each fish batch and were probably not able to develop an as mature biofilm as in the biofilter biofilm. This might explain the lower OTU richness, Shannon's diversity and stability over time in the biofilm in the rearing tanks.

Biofilm communities in the biofilter

There were some variations over time in the biofilm community composition in the biofilter (Figure 3.13). However, as hypothesised these variations were considerably smaller than those seen in the biofilm from rearing tanks (Figure 3.11). The biofilm communities in the biofilter tended to change according to phase in the production cycle (Figure 3.15). There were significant differences in Bray-Curtis similarities when comparing biofilm samples taken without fish in the system, and biofilm samples where the fish had been in the system for a more extended period. These results suggest that the fish had some impact on the community composition in the biofilter, where Rhodobacterales were found to be considerably more common during fish batches than in fallowing periods. These impacts were probably driven by the changing amounts of available organic matter and the fish

microbiota itself. Also, the additional feeding of the biofilter during fallowing periods, together with the lower amount of organic matter in these periods, are likely to have had an impact on the composition of microbial communities in the biofilm from the biofilter.

Nitrifiers in the biofilter biofilm

Nine nitrifying OTUs were found in the biofilter-biofilm, classified as *Nitrospira*, *Nitrosomonas*, and *Nitrosomonadaceae*. Several *Nitrospira*- and *Nitrosomonas* species are commonly found in RAS-biofilters (Ruiz et al. 2019), and these are also believed to be the primary nitrifiers in RAS-biofilters (Bartelme, McLellan, and Newton 2017). Moreover, there were large variations in the relative abundance of nitrifying bacteria in the biofilter biofilm (Figure 3.16). The relative abundances of nitrifiers showed a slight tendency to increase during fallowing periods and decrease throughout the fish batches. This is the opposite of what we initially expected; however, a possible explanation can be the intensive feeding of the biofilter during fallowing periods, which was calculated to be approximately 77 % of the TAN supplied at peak feeding. In addition, the observed trend in nitrifiers could be explained by the biofilter's changing selection pressure during fallowing periods. As the fish were moved out of the system, the organic matter influx subsequently stopped. This could possibly result in a disadvantage for the heterotrophic bacteria living in the biofilter. The increase in relative abundance could, therefore, possibly be explained by a decrease in the relative abundance of heterotrophs in the biofilter.

The high relative abundance of different *Nitrospira* OTUs and the low relative abundance of OTUs representing ammonia-oxidising bacteria, made it interesting to investigate if some of the *Nitrospira* OTUs could represent complete ammonia oxidisers (COMAMMOX). Comparisons of previously published 16S rRNA sequences of COMAMMOX and other *Nitrospira* strains indicated that one of the discovered *Nitrospira* OTUs (OTU_1771) was related to two described COMAMMOX bacterial species, *Nitrospira nitrificans* and *Nitrospira nitrosa* (Figure 3.17). Astonishingly, both of these COMAMMOX species were found in a trickling filter connected to a RAS (Van Kessel et al. 2015). OTU_1771 can therefore possibly represent a new COMAMMOX species.

Other possible reasons for the low relative abundance of ammonia-oxidising microbes can be that there were undiscovered ammonia-oxidising archaea (AOA) in the biofilm of the biofilter. It is possible that the broad-coverage primers, did not cover species of AOA. Bartelme, McLellan, and Newton (2017) also found high abundances of *Nitrospira* in a RAS-biofilter, together with high abundances of an AOA characterised as *Thaumarchaeota.* The same study concluded that RAS-biofilters can harbour complex ammonia oxidation communities, including a consortium of ammonia-oxidising archaea, *Nitrosomonas,* and COMAMMOX *Nitrospira*. This suggest that the ammonia oxidising communities in RAS can be very complex and that *Nitrosomonas* might not be as dominating as previously thought.

4.3. Future work and perspectives

This thesis has provided new knowledge on the dynamics of the complex microbial communities in start-feeding of salmon smolts in a commercial RAS. Future work on microbial ecology in RAS should focus on mapping the interactions between the free-living suspended bacteria, the reared fish, and the bacteria living in biofilms in RAS. This is important because the reared fish are in continuous contact with the free-living suspended bacteria in the water and these bacteria can have both positive and negative effect on the fish. In addition, all bacteria have an impact on the chemical water quality, potentially affecting the selection pressure in the system. Further research on this could contribute to understanding the dynamics and interactions of microbial communities in RAS.

Further research on the temporal dynamics of nitrifying communities in RAS and the potential COMAMMOX-species found in the biofilter would also be very interesting, as this can potentially change the way we describe the nitrifying bacteria. Ammonia-oxidising archaea are also a group of interest to look more into in further work on nitrifying communities.

Besides this, a study investigating chronic exposure of nitrate on juvenile Atlantic salmon would be useful to improve the current advices regarding cultivation of smolts in RAS. A threshold for nitrite concentrations in low-saline water should also be made, as a threshold could substantially increase the fish welfare of Atlantic salmon in RAS.

Bacterial abundances in freshwater RAS should also be further investigated, as the research on microorganisms in RAS today is mainly focusing on marine species, or the marine life stages of Atlantic salmon. This should be prioritised as the juvenile salmon might be more sensitive to fluctuations in bacterial abundances than the later life stages.

Also, how to achieve stable, healthy and beneficial microbial communities during production of salmon smolts in RAS should be investigated, to maintain good rearing conditions for optimised growth and high survival of fish.

5. Conclusions

To our knowledge, this is the first study investigating the microbial communities in a normally operated large-scale commercial RAS over a more extended period. New insights on microbial communities in a freshwater RAS were obtained in this thesis, and these results can be further implemented to improve microbial management of fish-cultivation in RAS.

The major findings in this thesis were:

- The microbial communities in biofilm and water were significantly different from each other and the most abundant orders in both biofilm and water communities were Rhodobacterales, Burkholderiales and Sphingomonadales.

- The water microbiota was similar in all the sampled units, probably because of the short hydraulic retention time.

- The microbial communities in biofilm from rearing tanks were significantly different from those in the biofilter and the microbial communities in the biofilter biofilm were more stable over time than that of the rearing tank biofilm.

- On two of three sampling days, there were more slow-growing bacteria in water samples from the treated water compared to water samples from the rearing tanks.

- The bacterial growth potential was higher in the rearing tank water compared to the treated water. However, this was only tested on two days in fish batch 7, and only one of these days had significantly higher growth potential in the rearing tank water.

- Large variations in relative abundance of nitrifiers were seen in the biofilter-biofilm communities throughout the sampling period and the variations were found to be connected to operational routines.

- The relative abundance of *Nitrospira* was high in the biofilm from the biofilter, together with a low abundance of ammonia-oxidising bacteria. Also, one of the *Nitrospira* OTUs was found to be related to two formerly characterised COMAMMOX.

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Appendix A – DNA-extraction protocols

A.1 – FastDNA™ Spin Kit for Soil (MP Biomedicals)

5.2 FastDNA™ SPIN Kit for Soil Detailed Protocol

1. Add up to 500 mg of soil sample to a Lysing Matrix E tube.

   NOTE: See section 3.2 for other important guidelines

2. Add 978 µL Sodium Phosphate Buffer to sample in Lysing Matrix E tube.
3. Add 122 µL MT Buffer.

   What's Happening: Begin to solubilize membrane proteins with detergents as well as extra-cellular proteins and contaminants in soil.

4. Homogenize in the FastPrep instrument for 40 seconds at a speed setting of 6.0.

   What's Happening: Mechanical disruption of cell walls of soil organisms and releasing nucleic acids into the protective buffer.

5. Centrifuge at 14,000 x g for 5-10 minutes to pellet debris.

   NOTE: Extending centrifugation to 15 minutes can enhance elimination of excessive debris from large samples or from cells with complex cell walls.

   What's Happening: Pellet insoluble cellular material and lysing matrix particles.

6. Transfer supernatant to a clean 2.0 mL microcentrifuge tube. Add 250 µL PPS (Protein Precipitation Solution) and mix by inverting the tube 10 times.

   What's Happening: Separate the solubilized nucleic acids from the cellular debris and lysing matrix. Flocculation of protein-containing micelles.

7. Centrifuge at 14,000 x g for 5 minutes to pellet precipitate. Transfer supernatant to a clean 15 mL microcentrifuge tube.

   NOTE: While a 2.0 mL microcentrifuge tube may be used at this step, more efficient mixing and DNA binding will occur in a larger tube.

   What's Happening: Removal of flocculated proteins.

8. Resuspend the Binding Matrix suspension and add 1.0 mL to the supernatant in the 15 mL tube.

9. Place on rotator or invert by hand for 2 minutes to allow binding of DNA. Place tube on a rack for 3 minutes to allow settling of silica matrix.

   What's Happening: Nucleic acids bind to the silica matrix in the presence of chaotropic salts.

10. Remove and discard 500 µL of supernatant being careful to avoid settled Binding Matrix.

11. Gently resuspend Binding Matrix in the remaining amount of supernatant. Transfer approximately 600 µL of the mixture to a SPIN™ filter and centrifuge at 14,000 x g for 1 minute. Empty the catch tube and add the remaining mixture to the SPIN Filter and centrifuge as before. Empty the catch tube again.

12. Add 500 µL prepared SEWS-M and gently resuspend the pellet using the force of the liquid from the pipet tip.

    NOTE: *Ensure that ethanol has been added to the Concentrated SEWS-M. See section 3.1.*

    What's Happening: Continuing to solubilize proteins.

13. Centrifuge at 14,000 x g for 1 minute. Empty the catch tube and replace.

    What's Happening: Desalting with ethanol and additional detergents remove impurities by centrifuging through the SPIN Filter bucket while the purified DNA is still bound to the silica.

14. Without addition of any liquid, centrifuge a second time at 14,000 x g for 2 minutes to "dry" the matrix of residual wash solution. Discard the catch tube with a new, clean catch tube.

15. Air dry the SPIN Filter for 5 minutes at room temperature.

    What's Happening: Removal of residual ethanol.

16. Gently resuspend Binding Matrix (above the SPIN Filter) in 50 - 100 µL of DES (DNase/Pyrogen-Free Water).

    NOTE: *To avoid over-dilution of the purified DNA, use smallest amount of DES required to resuspend Binding Matrix pellet*

    NOTE: *Yields may be increased by incubation for 5 minutes at 55°C in a heat block or water bath.*

    What's Happening: Purified nucleic acids elute from the silica with collapse of cation bridges because low salt elution solution rehydrates both the silica and the DNA.

17. Centrifuge at 14,000 x g for 1 minute to bring eluted DNA into the clean catch tube. Discard the SPIN Filter. DNA is now ready for PCR and other downstream applications. Store at -20°C for extended periods or 4°C until use.

DNA Analysis by Agarose Gel Electrophoresis

Load 5 µL of the eluted DNA per well on a 1% agarose gel in 0.5 x TBE electrophoresis buffer.

BBS loading dye, a 10X solution of bromophenol blue and sucrose solution, is supplied for this application.

A.2 – ZymoBIOMICS™ DNA Miniprep Kit (Zymo Research)

Protocol

1. Add sample to a **ZR BashingBead™ Lysis Tubes (0.1 & 0.5 mm)**. Add 750 µl **ZymoBIOMICS™ Lysis Solution** to the tube and cap tightly.

   Note: *For samples stored and lysed in* ***DNA/RNA Shield™ Lysis Tubes****, do not add ZymoBIOMICS™ Lysis Solution and proceed to Step 2.*

Sample Type	Maximum Input
Feces	200 mg
Soil	250 mg
Liquid Samples[1] and Swab Collections[2]	250 µl
Cells (isotonic buffer, *e.g.* PBS)	50-100 mg (wet weight) (10^9 bacterial and 10^8 yeast cells)
Samples in DNA/RNA Shield™[3]	≤ 1 ml

2. Secure in a bead beater fitted with a 2 ml tube holder assembly and process at maximum speed for ≥ 5 minutes.

   Note: *Processing time will vary based on sample input and bead beater. Times may be as little as 5 minutes when using high-speed cell disrupters (FastPrep® -24) or as long as 20 minutes when using lower speeds (e.g., Disruptor Genie®).*[4]

3. Centrifuge the **ZR BashingBead™ Lysis Tubes (0.1 & 0.5 mm)** in a microcentrifuge at ≥ 10,000 x *g* for 1 minute.

4. Transfer up to 400 µl supernatant to the **Zymo-Spin™ III-F Filter** in a **Collection Tube** and centrifuge at 8,000 x *g* for 1 minute. Discard the Zymo-Spin™ III-F Filter.

5. Binding preparation:

Feces and All Non-Soil Samples		Soil Samples
Add 1,200 µl of **ZymoBIOMICS™ DNA Binding Buffer** to the filtrate in the Collection Tube from Step 4. Mix well.	**OR**	Add 800 µl of **ZymoBIOMICS™ DNA Binding Buffer** and 400 µl of 95% ethanol to the filtrate in the Collection Tube from Step 4. Mix well.

6. Transfer 800 µl of the mixture from Step 5 to a **Zymo-Spin™ IICR Column** in a Collection Tube and centrifuge at 10,000 x *g* for 1 minute.

7. Discard the flow through from the Collection Tube and repeat Step 6.

8. Add 400 µl **ZymoBIOMICS™ DNA Wash Buffer 1** to the Zymo-Spin™ IICR Column in a new Collection Tube and centrifuge at 10,000 x *g* for 1 minute. Discard the flow-through.

9. Add 700 µl **ZymoBIOMICS™ DNA Wash Buffer 2** to the Zymo-Spin™ IICR Column in a Collection Tube and centrifuge at 10,000 x *g* for 1 minute. Discard the flow-through.

For **Technical Assistance**: 1-888-882-9682 or E-mail tech@zymoresearch.com

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

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

[3]Up to 1 ml of sample in DNA/RNA Shield can be processed directly in ZR BashingBead™ Lysis Tube. Adjust final volume to 1 ml with ZymoBIOMICS™ Lysis Solution or DNA/RNA Shield, if necessary.

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

For **Technical Assistance**:
1-888-882-9682 or E-mail
tech@zymoresearch.com

[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.

10. Add 200 µl **ZymoBIOMICS™ DNA Wash Buffer 2** to the Zymo-Spin™ IICR Column in a Collection Tube and centrifuge at 10,000 x *g* for 1 minute.

11. Transfer the Zymo-Spin™ IICR Column to a clean 1.5 ml microcentrifuge tube and add 100 µl (50 µl minimum) **ZymoBIOMICS™ DNase/RNase Free Water** directly to the column matrix and incubate for 1 minute. Centrifuge at 10,000 x *g* for 1 minute to elute the DNA[5, 6].

12. Place a **Zymo-Spin™ III-HRC Filter** in a new Collection Tube and add 600 µl **ZymoBIOMICS™ HRC Prep Solution.** Centrifuge at 8,000 x *g* for 3 minutes.

13. Transfer the eluted DNA (Step 11) to a prepared Zymo-Spin™ III-HRC Filter in a clean 1.5 ml microcentrifuge tube and centrifuge at exactly 16,000 x *g* for 3 minutes.

    The filtered DNA is now suitable for PCR and other downstream applications.

Appendix B – Genomic DNA Clean & Concentrator™-10

Buffer Preparation

✓ *Before starting:* Add 24 ml 100% ethanol (26 ml 95% ethanol) to the 6 ml **DNA Wash Buffer** concentrate. Add 96 ml 100% ethanol (104 ml 95% ethanol) to the 24 ml **DNA Wash Buffer** concentrate.

Protocol

Note: All centrifugation steps should be performed between 10,000 - 16,000 x g.

1. In a 1.5 ml microcentrifuge tube, add 2-5 volumes of **ChIP DNA Binding Buffer** to each volume of DNA sample[1] (see table below). Mix thoroughly.

Application	DNA Binding Buffer : Sample	Example
Plasmid, genomic DNA (>2 kb)	2 : 1	200 µl : 100 µl
PCR product, DNA fragment	5 : 1	500 µl : 100 µl

2. Transfer mixture to a provided **Zymo-Spin™ IC-XL Column**[2] in a **Collection Tube**.
3. Centrifuge for 30 seconds. Discard the flow-through.
4. Add 200 µl **DNA Wash Buffer** to the column. Centrifuge for 1 minute. Repeat the wash step.
5. Add ≥ 10 µl **DNA Elution Buffer**[3] or water[4] directly to the column matrix and incubate at room temperature for one minute. Transfer the column to a 1.5 ml microcentrifuge tube and centrifuge at for 30 seconds to elute the DNA.

   Ultra-pure DNA is now ready for use.

For **Assistance**, please contact Zymo Research Technical Support at 1-888-882-9682 or e-mail tech@zymoresearch.com.

Notes:

[1] It may be necessary to add RNase A to cell lysates prior to performing the procedure to ensure RNA-free DNA will be recovered in Step 5.

[2] The sample capacity of the column is 1 ml. It may be necessary to load and spin a column multiple times if a sample has a volume larger than 1 ml.

[3] **DNA Elution Buffer**: 10 mM Tris-HCl, pH 8.5, 0.1 mM EDTA

[4] Elution of DNA from the column is dependent on pH and temperature. If water is used, make sure the pH is >6.0. Tthe total yield may be improved by eluting the DNA with 60-70 °C **DNA Elution Buffer**.

Appendix C – Qubit 1x dsDNA High Sensitivity Assay kit

Read standards and samples

Qubit™ 3 and Qubit™ 4 Fluorometers

2.1 On the **Home** screen of the Qubit™ 3 or the Qubit™ 4 Fluorometer, press **DNA**, then select **1X dsDNA HS** as the assay type. The "Read standards" screen is displayed. Press **Read Standards** to proceed.

Note: If you have already performed a calibration for the selected assay, the instrument prompts you to choose between reading new standards and running samples using the previous calibration. If you want to use the previous calibration, skip to step 2.4. Otherwise, continue with step 2.2.

2.2 Insert the tube containing Standard #1 into the sample chamber, close the lid, then press **Read standard**. When the reading is complete (~3 seconds), remove Standard #1.

2.3 Insert the tube containing Standard #2 into the sample chamber, close the lid, then press **Read standard**. When the reading is complete, remove Standard #2.

The instrument displays the results on the Read standard screen. For information on interpreting the calibration results, refer to the *Qubit™ Fluorometer User Guide*, available for download at **thermofisher.com/qubit**.

2.4 Press **Run samples**.

2.5 On the assay screen, select the sample volume and units:

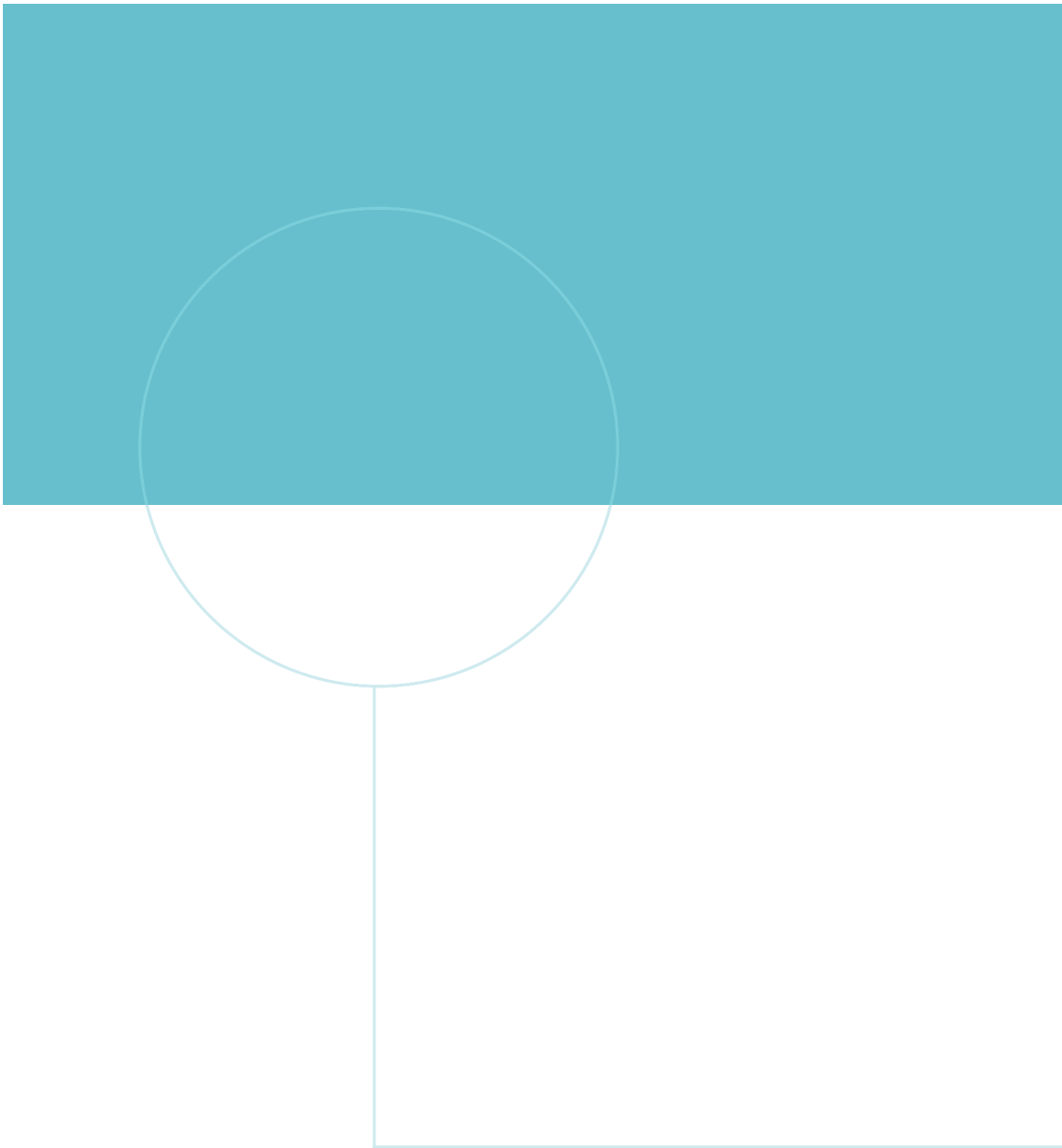
a. Press the **+** or **–** buttons on the wheel, or anywhere on the wheel itself, to select the **sample volume** added to the assay tube (from 1–20 µL).

b. From the **unit** dropdown menu, select the units for the output sample concentration.

2.6 Insert a sample tube into the sample chamber, close the lid, then press **Read tube**. When the reading is complete (~3 seconds), remove the sample tube.

The top value (in large font) is the concentration of the original sample and the bottom value is the dilution concentration. For information on interpreting the sample results, refer to the *Qubit™ Fluorometer User Guide*.

2.7 Repeat step 2.6 until all samples have been read.



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