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The Effect of Membrane Filtration on the Microbial Community Dynamics in RAS for Post-Smolt Production

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Biotechnology
Submission date: June 2016
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Acknowledgements

This thesis was part of the project “Improved rearing conditions for post-smolt production in RAS by membrane filtration: fish performances, welfare, and water quality” led by Dr. Astrid Buran Holan at Nofima Sunndalsøra. The work was performed at Nofima Centre for Recirculation in Aquaculture (NCRA) and NTNU Department of Biotechnology. My main supervisor was Ingrid Bakke at the Department of Biotechnology.

First of all, I want to thank Ingrid for your presence, guidance and support. It has not only meant a lot for the work of my master thesis, but also at a personal level. Your friendship is something I value highly.

I wish to express my gratitude to Astrid for letting me take part of such an interesting project, and for being so accommodating and helpful. I remember the road trips to Sunndalsøra being filled with interesting conversations and laughter. I want to thank Kari Attramadal, your enthusiasm for RAS and profound knowledge have really inspired me.

I want to thank the staff at Nofima Sunndalsøra, especially Jelena, Yuriy and Britt Kristin. Your help and friendliness made me really enjoy the time I spent at the research facility.

Professor Olav Vadstein and Jo Esten Hafsmo at the Institute of Biotechnology should be thanked, your advice on academics and kindness is highly appreciated.

My fellow classmates must be thanked for making the years at NTNU fun and memorable, my friends for always encouraging me, my parents for always showing their love and support no matter what, and my amazing sisters for always believing in me.

Trondheim June 2016 **Ragnhild Olsen Fossmark**

Abstract

An increased interest for recirculating aquaculture systems (RAS) the past years have led to more research on water treatment processes and how they affect water quality. Most studies have been focusing on the units governing the physicochemical parameters, however the microbial environment is also an important parameter in the cultivation of fish. Bacteria are highly abundant in the rearing water and thus in close contact with the fish. Knowledge on the potential health effects of the microbial environment in the cultivation of salmon fry, smolt and post-smolt are limited and remains to be studied.

Accumulation of solids is a known problem in RAS, and could affect the water quality and fish performance. The conventional particle removal systems in use today only manage to remove larger particles, leaving the fine solids in the system water for recirculation. Membrane technology has been proposed as a strategy for removal of the fine solids in the system and could potentially improve the water quality. The scope of this thesis was to evaluate the effect of membrane filtration on the bacterial concentrations and the microbial community structures in the rearing water of post-smolt production in two identical RASs, one control RAS (cRAS) and one RAS modified to include a membrane (mRAS). The community compositions of the gut microbiota from the post-smolts reared in the two systems were also investigated at the end of the experiment. To estimate the bacterial concentrations, flow cytometry analysis was used to count the bacteria in the rearing water. To investigate the microbial community compositions, 16S rRNA PCR/DGGE analysis was conducted.

Implementation of a membrane in the water treatment significantly reduced the bacterial concentrations in the rearing water. At the end of the experiment the bacterial concentrations of water in the fish tanks in mRAS were around 6 million/ml, and in cRAS 14 million/ml, respectively. The microbial community compositions in mRAS and cRAS were significantly different from each other, and the community compositions in mRAS were more diverse than those of cRAS. The water microbiota in both systems changed throughout the experiment, however it was observed a more stable microbiota over time in mRAS than cRAS. The microbial community compositions of the gut samples from mRAS and cRAS were significantly different from each other, thus the membrane filtration affected the gut microbiota of the salmon post-smolts.

Sammendrag

En økt etterspørsel etter resirkuleringsakvakultursystemer (RAS) de siste årene har ført til mer forskning på vannbehandlingsprosesser og hvordan de påvirker vannkvaliteten. De fleste studier har fokusert på enhetene som kontrollerer de fysiokjemiske parameterne, med det mikrobielle miljøet er også en viktig parameter i kultiveringen av fisk. Bakterier er tallrike i vann, og dermed i nær kontakt med fisken. Kunnskap om de potensielle helseeffektene av det mikrobielle miljøet i kultiveringen av lakseyngel, smolt og postsmolt er manglende og gjenstår å bli undersøkt.

Akkumulering av partikler og faste stoffer er et kjent problem i RAS og kan påvirke vannkvaliteten og fiskens ytelse. De konvensjonelle partikkelfjerningssystemene i bruk i dag fjerner bare de største partiklene, og de minste blir gjenværende i vannet og resirkulert. Membranteknologi har blitt foreslått som en strategi for å fjerne de fineste partiklene i systemet og dette kan potensielt forbedre vannkvaliteten. Formålet med denne masteroppgaven var å evaluere effekten av membranfiltrering på konsentrasjonen av bakterier og strukturen av de mikrobielle samfunnene i vannet brukt i postsmolt produksjon. To identiske RAS ble undersøkt, en kontroll RAS (cRAS) og en modifisert RAS (mRAS) med en membran. Sammensetningen av de mikrobielle samfunnene i tarmen til postsmolten fra de to systemene ble også undersøkt. For å estimere de bakterielle konsentrasjonene ble flowcytometri brukt for å telle bakteriene i vannet. For å undersøke strukturen av de mikrobielle samfunnene ble 16S rRNA PCR/DGGE brukt.

Inkluderingen av en membran i vannbehandlingen reduserte den bakterielle konsentrasjonen i vannet signifikant. På slutten av eksperimentet så var den bakterielle konsentrasjonen i fisketankene i mRAS henholdsvis 6 millioner/ml og i cRAS 14 millioner/ml. Sammensetningen av de mikrobielle samfunnene i mRAS og cRAS var signifikant forskjellige fra hverandre, og de mikrobielle samfunnene i mRAS var mer mangfoldig enn i cRAS. Vannmikrobiotaen i begge systemene endret seg gjennom hele forsøket, men det var observert en mer stabil mikrobiota i mRAS enn cRAS mot slutten av forsøket. Sammensetningen av de mikrobielle samfunnene i tarmprøvene fra mRAS og cRAS var signifikant forskjellige fra hverandre, dermed hadde membranfiltrering en effekt på tarmmikrobiotaen til postsmolten.

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1. Introduction

The Food and Agriculture Organization of the United Nations (FAO) reports that aquaculture is one of the fastest growing food-producing sectors today. Parallel with the increase in global population, the exploitation of our marine resources for food has never been higher. The interest for water reuse systems in fish production have considerably increased as a more sustainable way of aquaculture (Tal *et al*., 2009). Technology for rearing facilities with recirculating aquaculture systems (RAS) is improving and becoming more popular opposed to the traditionally single-pass flow through systems (FTS) (Dalsgaard *et al*., 2013, Terjesen *et al*., 2013). Today most Norwegian land based smolt production facilities are FTS, but the newly built facilities are usually reuse systems and many farmers plan to convert their single pass systems to RAS (Bergheim *et al*., 2009).

The Atlantic Salmon (*Salmo salar)* is the most farmed species in Europe in tonnes biomass produced (FEAP, 2015). As the rearing of salmon in RAS during the fresh water stages of their life cycle is accelerating, knowledge on optimal water quality and water treatment technology to meet these demands are continuously improving. Particle accumulation in RAS is detrimental on the fish, and several methods are used to maintain the concentration of particles in the rearing water at acceptable levels. Conventional particle removal units do not remove the fine suspended and colloidal fraction of particles, and suspended solids have shown a negative effect on gills and general fish health (Timmons and Ebeling, 2007, Chen *et al*., 1993). Furthermore, accumulation of these small particles cause an increase in organic matter and bacterial substrate in the water, inducing fluctuations in the microbial community structures of the rearing water (Wold *et al*., 2014). Water treatment that gives a stable microbial environment has shown to affect the survival and growth of marine fish larvae positively (Skjermo and Vadstein, 1999). However, knowledge on the potential health effects of microbial environments in the cultivation of salmon fry, smolt and post-smolt are lacking and remain to be studied. Knowledge on water treatments and its effect on the microbiota of rearing water are generally limited, and host-microbe interactions in fish are still poorly understood. A better understanding of the water treatment in RAS and how particles affect the water microbiota could lead to better rearing conditions yielding more robust cultivated species.

1.1 Cultivation of aquatic species and aquaculture technology

Water quality requirements depend upon the species cultivated along with different life stages of the individuals (Colt, 2006). The rearing conditions should be as optimal as possible to obtain healthy and fast-growing fish that utilize their feed at maximum efficiency. In their natural habitat, aquatic species are an integral part of a complex eco-system where multiple water quality parameters affect their welfare. Luckily, the most critical water quality parameters can be subjected to control in rearing facilities by man-made technology to ensure the species well being. The most important physical and chemical parameters are pH, temperature, alkalinity, suspended solids and concentrations of dissolved CO_2, oxygen, nitrogen, ammonia and nitrite (Timmons and Ebeling, 2007). Additionally, it is becoming more evident that microbial control is also an important water quality parameter in the cultivation of marine fish, especially at the early larvae stage after hatching (Vadstein *et al*., 2013).

In 2013 there were a total of 193 facilities for land based smolt and rainbow trout production in Norway, 168 of these were flow through systems and 25 were partially or fully using recirculation of water for cultivation (Mattilsynet, 2014). In 2015 the number of RAS for salmonid production on land was increased to 70, and more facilities are planned to be built (Veterinærinstituttet, 2015). In the traditional flow through systems (FTS) the water is usually treated with particle removal, disinfection and oxygenation before it enters the rearing thanks and is discharged after one lap through the facility (Figure 1.1). The accumulation of waste products within the systems is low as the water is constantly renewed. Fewer water quality parameters are subjected to monitoring and the technology needed to run a facility is less complex, which is why the majority of todays production facilities are of this character (Lekang, 2013). On the downside, the water consumption is huge and the systems are dependent on a continuous water flow. A lot of energy is required to obtain suitable rearing temperatures, especially during the winter months (Kolarevic *et al*., 2014), and there is usually less control over potential pollution of the discharges. In contrast, recirculating aquaculture system (RAS) facilities allows for lower water consumption due to recirculation (Figure 1.1), which is more environmentally friendly and economic in terms of energy for heating and water usage. Its closed-system characteristics allow for better control of environmental impacts, which is highly beneficial when controlling the surroundings receiving the waste load discharged in the effluent (Martins *et al*., 2010, Summerfelt *et al*.,

2001). Rearing conditions and temperatures are more easier to control, and of high significance to the aquaculturist as it correlates with net food conversion and growth of the cultivated species (Barton, 1996). The systems are not dependent on a continuous water flow, which is very advantageous as flow through systems require enormous amounts of water and can only be built by rivers where the water flow is high, stable and not exposed to seasonal water depletion (Timmons and Ebeling, 2007). The hydraulic retention time of the water in the system is long and provide more stable rearing environments in regard to abiotic physicochemical factors, but also the biotic part of the system; the bacteria. The microbial state of the water is more constant in RAS, which is favourable for cultivation (Attramadal *et al.*, 2012a, Skjermo *et al.*, 1997). RAS is considered as a more sustainable way of aquaculture, however the technology needed to assure the required water quality parameters are costly, high tech and demands thorough surveillance (Timmons and Ebeling, 2007).

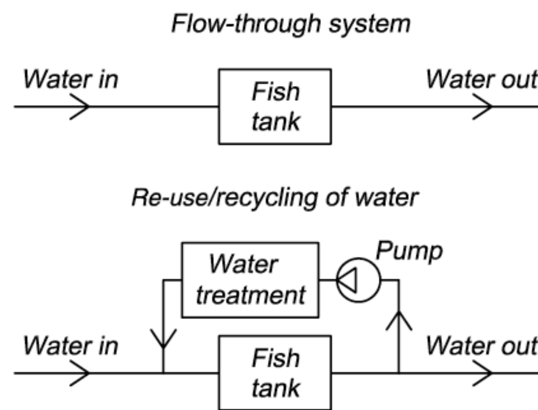

Figure 1.1: Illustration of a flow through system (FTS) and a recirculating aquaculture system (RAS), figure from Lekang, 2013.

Kolarevic *et al.* (2014) conducted a study rearing salmon smolts in two different systems of RAS and FTS in freshwater and then transferred the fish to sea cages. The study showed that 98 % more water was used in the production of salmon reared in FTS than in RAS. The salmon showed no significant difference in mortality or growth rate when reared in the two systems. However, there were some differences in levels regarding enzymes regulating the salinity tolerance at sea transfer between the smolts produced in the two systems, which should be further investigated. Nonetheless, studies have shown lower mortality rates of cod larvae reared in RAS as opposed to FTS (Attramadal *et al.*, 2014) and higher growth rates of trouts reared in RAS compared to FTS (Colson *et al.*, 2015). Thus, in addition to being more environmentally friendly and economic, RAS provides more stable microbial, physical and

chemical rearing conditions that may also yield healthier and bigger fish. Even though the investment cost for RAS is large, farming with recirculation of water may be very profitable if the system is well managed.

1.2 Rearing of the Atlantic Salmon (*Salmo salar)*

The high content of proteins and omega-3 fatty acids makes salmon a healthy food consumed all over the world (FAO, 2014). In 2014, 1.6 million metric tons were produced with 1,3 million metric tons coming from Norwegian production (FEAP, 2015). Farmed salmon is one of Norway's most important export industries (Larsen and Asche, 2011), and today the production has increased from less than 500 tons in the 1970's to 1.26 million tons in 2014 (Fiskeridirektoratet, 2014, Liu *et al*., 2011). The features of the salmonid life cycle and composition of needed feed makes the salmon a favourable reared species (Barton, 1996). At the rearing facilities, the fertilized eggs are hatched in fresh water to sac fry and they feed on their yolk sac the first month. After 4-6 weeks the fry is transferred to larger tanks and starts to eat dry feed. The fry swims and feed in freshwater and develops into a juvenile fish called parr, until it reaches the smoltification stage and is prepared for saltwater and sea transfer (Barton, 1996). Salmon requires a somewhat uncomplicated diet were the feed can be manufactured and the mortality rates after hatching is usually low, making it a species suitable for intense farming. Smolts are normally produced in land-based systems to a size of 70-140 g before stocking in sea cages (Bergheim *et al*., 2009). The sea transfer is tough on the smolts and the mortality rates are usually high. Stress due to the transport process, prior rearing conditions yielding low quality smolt and exposure to sea lice and other diseases at sea are distinct factors (Iversen *et al*., 2005, Finstad *et al*., 2003). This has led to more research the past years on farming post-smolt up to 1 kg on land that are more resilient towards the tough conditions at sea. In addition, by increasing the production time on land the fish will be exposed to the harsh environments in the ocean for a shorter period of the production cycle, which also increases the production efficiency at the farming facilities (Terjesen, 2016). The Atlantic salmon is considered a cold-water fish with optimal growth rate ranging 12.1 °C – 15.1 °C (Barton, 1996). The cold ocean waters and long coastlines of Norway and Chile are thus very suitable for salmon farming, which is why the highest production rates of salmonid worldwide are found there (FAO, 2014).

1.3 Water treatment in recirculating aquaculture systems

A typical RAS facility consists of rearing tanks and a water treatment section for mechanical removal, degradation and/or conversion of; organic matter, accumulated nutrients, gas and particles (Lekang, 2013, Timmons and Ebeling, 2007) and the degree of water recirculation is usually high (> 90 %) (Summerfelt *et al*., 2001). The different water treatment units of a RAS and the water quality parameters they are governing that are of particular relevance for this thesis are elaborated below

1.3.1 N-compounds and biofilters

Fish excrete ammonia as the end-product of the metabolism of protein catabolism, and especially salmon as its diet contain a lot of protein (Timmons and Ebeling, 2007). High levels of ammonia are lethal to the fish, and the primary purpose of a biofilter in RAS is the bacterial conversion of ammonia to the less toxic compound nitrate. The process is called nitrification and includes two steps; first an intermediate step where ammonia (NH_3) is oxidized to nitrite (NO_2^-) by ammonia oxidizing bacteria (AOB) and then a second step where nitrite is oxidized to the less harmful nitrate (NO_3^-) by nitrite oxidizing bacteria (NOB). These bacteria are autotrophs and grow on surfaces in the aerated biofilters (Schreier *et al*., 2010). Ammonia exists in an equilibrium between un-ionized NH_3 (ammonia) and ionized NH_4^+ (ammonium) which is a function of pH, temperature and salinity (Timmons and Ebeling, 2007). Unionized NH_3 is the most toxic form, due to its neutral charge it can diffuse through the gills and cell membranes and cause harm (Arillo *et al*., 1981). The sum of ammonium and ammonia is called total ammonia-nitrogen (TAN), a term often used when discussing concentrations of ammonia in RAS. At neutral pH the amount of NH_4^+ in fresh water systems at 10 °C is 99.2 %, and an increase in pH, salinity or temperature causes the proportion of NH_3 in TAN to increase leading to detrimental conditions for the fish (Timmons and Ebeling, 2007). Nitrate is the final product of the nitrification and the least toxic N-compound, and in reuse-systems the accumulation of nitrate is diluted by the daily water exchange (Lekang, 2013).

A second type of bacteria also inhabit the biofilter, they are the heterotrophic bacteria that remove particles from the water by utilizing organic matter and they are correlated to the organic loading in the system (Blancheton *et al*., 2013). They compete with the autotrophs for space and oxygen in the biofilter, and their maximum growth rate is a lot higher than the

autotrophs. They are generally more abundant in the biofilter and can reduce the nitrification efficiency of the AOBs and NOBs (Michaud *et al*., 2006, Blancheton, 2000). It is important that the concentration of the heterotrophic bacteria is kept at acceptable levels so that they do not interfere with the nitrifying bacteria. Some systems operate with a separate biofilter unit/chamber for heterotrophic growth prior to the nitrifying biofilter to remove particles and to maximize the nitrification efficiency (AKVAGroup, 2014). In an operating RAS the TAN-levels in the rearing tanks should be under 2 mg/l (Mattilsynet, 2012) otherwise the conditions are detrimental for the fish, hence a functioning biofilter is essential. Nitrification kinetics is very important in RAS as it is part of determining the water exchange rate (Chen *et al*., 2006). Having an optimal biofilter requires less water exchange and it correlates with the amount of water that needs to be heated before entering the RAS. A reduction in water heating is also a significant reduction in cost. One common type of biofilter is the moving-bed biofilter with plastic discs that provides a large surface area. The bacteria grow attached in biofilms on the surface of the discs and utilize the nitrogenous compounds and organic matter (Lekang, 2013).

1.3.2 Microbial water quality

Studies have emphasized the importance of microbial stability in aquaculture, particularly in the rearing of marine fish larvae and strategies to obtain microbial control have been proposed (Vadstein *et al*., 1993, Skjermo *et al*., 1997, Skjermo and Vadstein, 1999). The concept of microbial stabilization is based on the ecological r/*K*-selection theory (MacArthur and Wilson, 1967, Vadstein et al., 1993), and the basis for the theory is how organisms thrive in crowded or uncrowded environments. Low population densities (uncrowded) with surplus of resources selects for r-strategists and environments of high population density (crowded) and limited resources favours K-strategists (Andrews and Harris, 1986). r-strategists are considered opportunistic, they display rapid growth rates when resources are abundant and do not usually succeed when the competition for nutrients is high. r-strategists are unpredictable and sensitive to fluctuations in the environment, they are often members of pioneering communities and considered detrimental on the species cultivated (Vadstein *et al*., 1993, Vadstein *et al*., 2004). K-strategists have lower growth rates, and succeed in environments with high competition of nutrients as they can exploit limited resources better than r-strategists. Since K-strategists are not as dependent on high and balanced nutrient supplies, they are more stable and resilient to environmental fluctuations. They outcompete r-strategists

after the pioneering phase and become permanent members of the community. In aquaculture context, this is when the rearing water is microbial matured and stable, which can be obtained by creating a K-selective pressure with a reduction of the nutrient supply per bacteria (Skjermo *et al*., 1997).

RAS has been proposed as a possible strategy to obtain microbial control and has shown to increase the survival of marine larvae when compared to rearing in FTS (Attramadal *et al*., 2012a, Attramadal *et al*., 2014). Due to the high degree of water reuse, the water retention time is long, which provides good conditions for water maturation and the stable microbial matured water is retained in the system and not discharged (Attramadal *et al*., 2012a). The presence of a biofilter also functions as a maturation unit, by limiting the nutrient supply per bacteria and thus selecting for K-strategists that can outcompete the opportunists. The potential for microbial stabilisation is yet another advantage of RAS as opposed to FTS and the importance of microbial stability have not been studied in RAS and smolt production.

1.3.3 Particles in RAS and their effect on water and fish

Particles in aquaculture systems are produced from organic decomposing food, excreted waste, and dead and living bacteria (Chen *et al*., 1993). Several techniques are used to remove particles, e.g. mechanical filtration in a disk, belt or sand filter, and gravity separation (Lekang, 2013, Summerfelt *et al*., 2001). Accumulation of particles is a problem, lowering the quality of water, inducing stress on the fish by affecting gill tissue and leading to decreased performance and disease resistance (Cripps and Bergheim, 2000, Chen *et al*., 1993). Particles have also shown to reduce the disinfection of water by protecting the bacteria from UV-light and ozone disinfecting methods (Hess-Erga *et al*., 2008). In RAS, particles accumulate continuously and are very important to remove from the water before entering rearing tanks. However, the conventional particle removal systems in use today only manage to remove particles larger than 40-60 µm (Timmons and Ebeling, 2007), and the fine suspended solids (<35 µm) and colloidal particles (<1 µm) remains in the system and is recirculated. A study conducted by Chen *et al*., (1993) discovered that more than 95 % of the suspended solids had a diameter less than 20 µm when farming trouts in RAS, and that these small particles accounted for 40-70 % of the total suspended solids by weight. Accumulation and mineralization of particles will also lead to an increase in bacterial substrate that induce heterotrophic bacterial growth, causing competition for oxygen and space in the biofilter

which can affect the nitrification kinetics (Michaud *et al*., 2009, Chen *et al*., 2006). It can also cause increase in bacterial numbers and changes of the microbial community composition in the system, which is considered unfavourable for the cultivated species (Holan *et al*., 2014a, Wold *et al*., 2014, Attramadal *et al*., 2012a). There is undoubtedly a need for a more advanced particle removal system to remove the fine suspended solids and colloidal fraction of the particles from the system to enhance the water quality and fish performance.

1.4 Membrane technology

A membrane is a barrier that pure liquids and gas will flow through while pollutants are retained and discharged. The membrane can consist of a filter cloth or a porous media, it is semi-permeable and may separate impurities after size, shape, electrostatic charge, physicochemical interactions and polarity (Lekang, 2013, Chiam and Sarbatly, 2011). As membranes can remove bacteria, they have been used to treat drinking water, and are used in sewage and other waste water treatment (Figure 1.2) (Van der Bruggen *et al*., 2003).

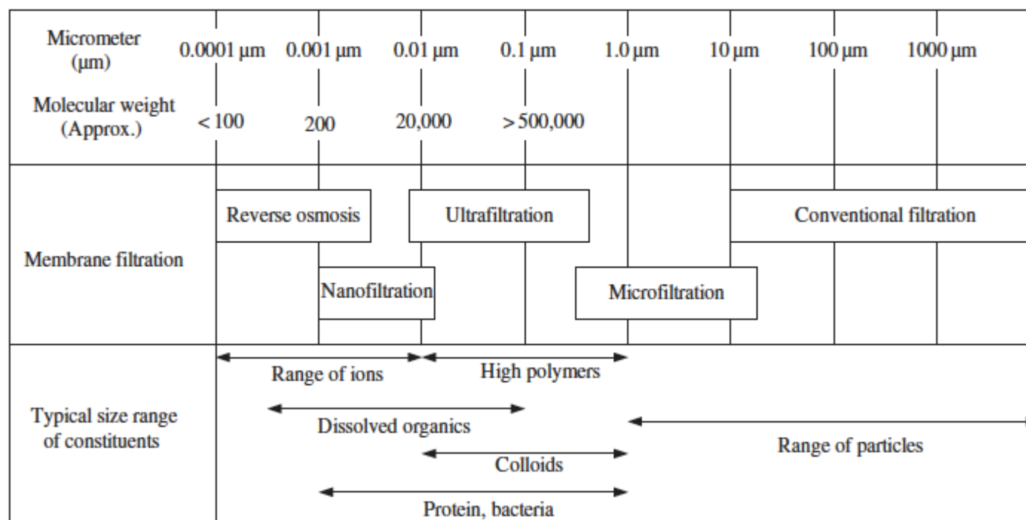

Figure **1.2**: Sizes, molecular weights and features of particles removed by different membrane filtration techniques. Figure from Chiam and Sarbatly, 2011.

The water passes through the separation unit due to the hydraulic pressure across the membrane, and this is called the transmembrane pressure (TMP). Operation of the membrane above recommended TMP might damage the membrane or the supplying system. The TMP needs to be monitored, and when reaching the acceptable pressure level, back flushing is required to remove the particles retained on the membrane surface (Lekang, 2013). Membrane

technology improves water quality by removing the smallest particles and suspended solids, however the technology is costly and requires continuous surveillance. Fouling problems and reduction in water flux are common shortcomings with this technology, which require frequent back flushing and sometimes chemical cleaning (Holan *et al*., 2014b, Le-Clech *et al*., 2006, Wu *et al*., 2008). Despite these drawbacks, membrane technology has achieved increased interest as a unit for removal of fine suspended and colloidal particles in aquaculture systems, as these particles are not removed by conventional particle removal systems. By integrating a membrane in RAS it can lower the concentration the smallest particles, which may reduce stress on the cultivated species, decrease organic matter and bacterial substrate in the system and thereby diminish heterotrophic blooms and improve nitrification efficiency (Holan *et al*., 2014a). It may stabilize the microbial state of the water by lowering bacterial fluctuations in the water and create a more favourable rearing environment (Wold *et al*., 2014). All of this emphasizes that membrane filtration of rearing water could improve the water quality and affect fish health.

1.5 Host-microbe interactions

Humans often associate bacteria with infection and disease, which is true for some pathogenic strains. Even though some bacteria can cause harm and in the worst-case death, a diversity of strains are essential for health and survival in higher organisms. Bacteria colonize the internal and external epidermal surfaces of humans and animals, creating a close symbiotic relationship between host and bacteria (Maynard *et al*., 2012). This relationship gives many metabolic and immunological advantages to the host and it is part of the mucosal immune system (Sekirov *et al*., 2010). In mammals, the intestinal microbiota is known to play a fundamental role in the development of the immune system and the digestive tract (Fraune and Bosch, 2010), harvesting nutrients from food (Nicholson *et al*., 2012, Maynard *et al*., 2012) and there are constantly new discoveries of correlations between disease/health and the gut microbiota (Round and Mazmanian, 2009). The importance of microbes to mammalian health and survival makes it reasonable to assume that the evolution of humans and metazoan organisms involved a coevolutional alliance with bacterial life (Gill *et al*., 2006).

Fish are surrounded with higher concentrations of microorganisms than terrestrial species as bacteria thrive more in water than air. Their main mucosal surfaces and immune barriers are gills, skin and the intestine (Gomez *et al*., 2013), which are in constant contact with the

microbes in the water surrounding them. Farmed fish are even more exposed to high bacterial concentrations, as the density of biomass is greater in rearing tanks/sea cages than in open waters. The microbial community compositions of rearing water is also different and unstable, which may stress the cultivated fish and pose additional challenges on their mucosal immune system (Gomez *et al*., 2013). The bacteria in rearing water may affect the microbiota of the skin and gills of the fishes in regard to competition of space, nutrients and oxygen (Llewellyn *et al*., 2014), and studies in tilapia and cod larvae indicate that the water microbes influence the composition of the larval microbiota (Giatsis *et al*., 2015, Bakke *et al*., 2015). After larvae hatch they are exposed to the bacteria present in the rearing water, thus these bacteria are probably colonizing the gut of the fish larvae (Hansen and Olafsen, 1999). Microbial balance is important for fish health, and to grasp the fish host and its microbiome as one unit may give a good foundation to develop better rearing regimes (Gómez and Balcázar, 2008, Sunyer, 2013). Different water treatment processes and rearing regimes, such as disinfection, RAS vs. FTS, membrane filtration and salinity have shown to affect the microbial communities of tank water (Attramadal *et al*., 2012a, Attramadal *et al*., 2012b, Wold *et al*., 2014, Bakke *et al*., 2016), however knowledge on the correlation between water microbiota and potential effects on fish health is limited and should be further investigated.

1.6 Analytical methods to study microbes, their community structures and diversity

Most eukaryotes can be recognized on the basis of their morphology, which makes it easy to study them and classify their function in an ecosystem. This is not the case for bacteria, as their simple and microscopic traits give limited clues for their identification and role in the environment (Theron and Cloete, 2000). To identify specific populations of microorganisms, methods including isolation of pure cultures by cultivation under conditions as close to their native habitat as possible have been used. However, only a minor fraction of bacteria can be cultivated in a laboratory thus posing a biased and incomplete description of the bacteria present in the sample (Theron and Cloete, 2000). Culture-dependent techniques are insufficient to study the bacterial diversity and community structures of natural or manipulated environments (Sanz and Köchling, 2007). The development of new molecular techniques for identification and characterization of bacterial communities without the need of cultivation have been essential for our increased understanding of microbial diversity and functionality in microbial ecology (Malik *et al*., 2008). PCR-based analyses of the 16S rRNA

genes of prokaryotes have in particular enabled microbial ecologists to attain better knowledge on the diversity of bacteria in different environments (Marchesi *et al*., 1998). The 16S rRNA is a component in the ribosome and thus a key element in the protein-synthesizing machinery, making the gene functionally conserved and present in all bacteria (Olsen *et al*., 1986). Universal bacteria primers that are complentary to the preserved regions are available, making it easy to amplify the genes with PCR for further analyses (Clarridge, 2004). Big databases of microbial rRNA gene sequences, like the Ribosomal Database Project (RDP), makes it possible to assign the taxonomy to the 16S rDNA sequences (Marchesi *et al*., 1998).

Denaturing gradient gel electrophoresis (DGGE) is a genetic fingerprinting technique well suited for comparing microbial community profiles between samples, e.g. dynamics of bacterial communities using 16S rDNA molecules (Theron and Cloete, 2000). After the extraction of DNA from the sample and amplification through PCR, the 16S rDNA molecules are separated in a poly acrylamide gel with a denaturing gradient according to sequence, creating a band pattern which displays the species in the sample (Sanz and Köchling, 2007). There are limitations to DGGE-analysis and this is not the method of choice if highly accurate taxonomically information of the sample is needed (Sanz and Köchling, 2007). The method display relatively low resolution, and the maximum numbers of bands that are expected to be separated are only around 70-80 (Personal communication, Ingrid Bakke). The use of DGGE in the investigation of microbial communities is becoming less common as technology for more accurate and quick methods of metagenomic studies are improving. The next-generation sequencing (NGS) approaches and technologies have opened up for opportunities of analysing microbial communities of very complex environmental samples (Shokralla *et al*., 2012). This can answer questions about microbial diversity at much lower costs and efforts, with much higher resolution, accuracy and throughput (Mardis, 2008). NGS for community profiling using the 16S rRNA gene is commonly used in microbial ecology since this gene is present in all domains of life (Bartram *et al*., 2011) as previously discussed. Roche 454 pyrosequencing and Solexa Illumina are examples of PCR-based next-generation sequencing methods often applied to microbial genetics (Shokralla *et al*., 2012). Roche 454 pyrosequencing is however, becoming out-competed compared to Illumina sequencing, which can do the same sequencing effort at much lower costs (Sinclair *et al*., 2015). Amplicon-sequencing through Illumina gives much more detailed taxonomic information and much better resolution than DGGE.

The first step in Illumina sequencing is clonal solid-phase bridge amplification to enhance the DNA template and resulting sequence signal. Adaptor sequences complementary to sequences anchored to the solid phase (e.g. glass plate) are ligated to single stranded template DNA and causes the DNA to attach to the solid phase and stand up. Furthermore, the template DNA strands fold as the adaptor sequence at the other end is attaching to the anchored complementary sequence forming a bridge. A DNA polymerase synthesizes dsDNA and clusters of identical DNA strands have formed. The resulting clusters are denatured leaving single strands of DNA as templates in the sequence reaction and fluorescent-labelled reversible terminator dNTP's are incorporated. As the dNTPs are incorporated they emit light that is detected and analysed to see the base sequence (Degnan and Ochman, 2012, Goodwin *et al*., 2016).

1.6.1 Measures of Microbial Diversity

The microbial diversity of a given environment can be measured in the amount of variation in the microbial populations present. These measures can include genetics and functional features of the bacteria (Gentry *et al*., 2015). Species richness and species evenness are common data to include in the measure of microbial diversity. Species richness is the number of different species present in a sample unit, and the species evenness describes the variability of species abundances in a sample unit (Gentry *et al*., 2015). If the species are present in equal amounts it is an extremely even community. The alpha (α) diversity refers to the diversity of a defined unit, sample or habitat (Rosenzweig, 1995), and is often represented by species richness, evenness and/or the Shannon's diversity index. The Shannon index (H') (Shannon and Weaver, 1949) is common to use in microbial ecology, and includes both species richness and their relative abundance in a sample. High values reflect communities with greater species richness and evenness, whereas lower values reflect communities with fewer species and/or low equality of abundances (Hollister *et al*., 2015). After acquiring diversity information within a community, the diversity between samples representing different microbial communities can be measured. This is known as beta (β) diversity, and the Bray-Curtis similarity index (Bray and Curtis, 1957) is a measure commonly used. This measure evaluates the degree of similarity between two communities using the number of shared species relative to the number of species held in both communities, and also evaluates the abundance data of each species (Hollister *et al*., 2015).

1.7 Objectives of the study

The main objective of this study was to evaluate the effect of membrane filtration as part of the water treatment in RAS on the rearing water microbiota and gut microbiota of post-smolt.

More specifically the goals were to evaluate the effects of membrane filtration on:

- Bacterial concentrations in rearing water
- Bacterial community composition in the rearing water
- Temporal dynamics of the rearing water microbiota
- The gut microbiota in salmon post-smolt

2. Materials and methods

An experiment with post-smolt (Atlantic salmon) production in a recirculating aquaculture system (RAS) was conducted at Nofima Centre for Recirculation in Aquaculture (NCRA), Sunndalsøra. The post-smolt were reared in two identical and separate RASs, and a membrane was included as part of the water treatment in one of the systems. Samples were collected during the study and analysed to investigate the effect of membrane filtration on number of bacteria and on the microbial community compositions of the water- and gut microbiota. All samples for this thesis were collected at Nofima and transported to NTNU, Department of Biotechnology for analyses.

2.1 Experimental design and system configuration

Post-smolt were cultivated (Atlantic salmon; start weight 250 g) in two separate RAS, one conventional RAS (cRAS) and one membrane modified RAS (mRAS) (Figure 2.1). Operational parameters are given in table 2.1. Each RAS consisted of 3 tanks (3.3 m^3) with post-smolt (biomass density 50 kg/m^3), and a separate water treatment system consisting of a microscreen belt filter (Salsnes Filter, mesh 90 μm, Norway) for particle removal, a biofilter (moving bed bioreactor, MBBR from Krüger Kaldnes, Norway), and a degasser (Aqua Optima). Total system volume was (47.5 m^3). The two systems (cRAS and mRAS) were identical except that a sidestream of 5.3 % of the total water flow (800 l/h) was treated by an ultrafiltration membrane unit (0.02 μm hallow fiber, polymeric) in mRAS for about 10 hours per day (Figure 2.1). Before the start of the experiment the fish tanks were thoroughly cleaned and the systems were totally disinfected, and the recirculating water was cross-run between the two systems to ensure equal conditions at start up. The duration of the experiment was 51 days (08.12.14 – 27.01.15)

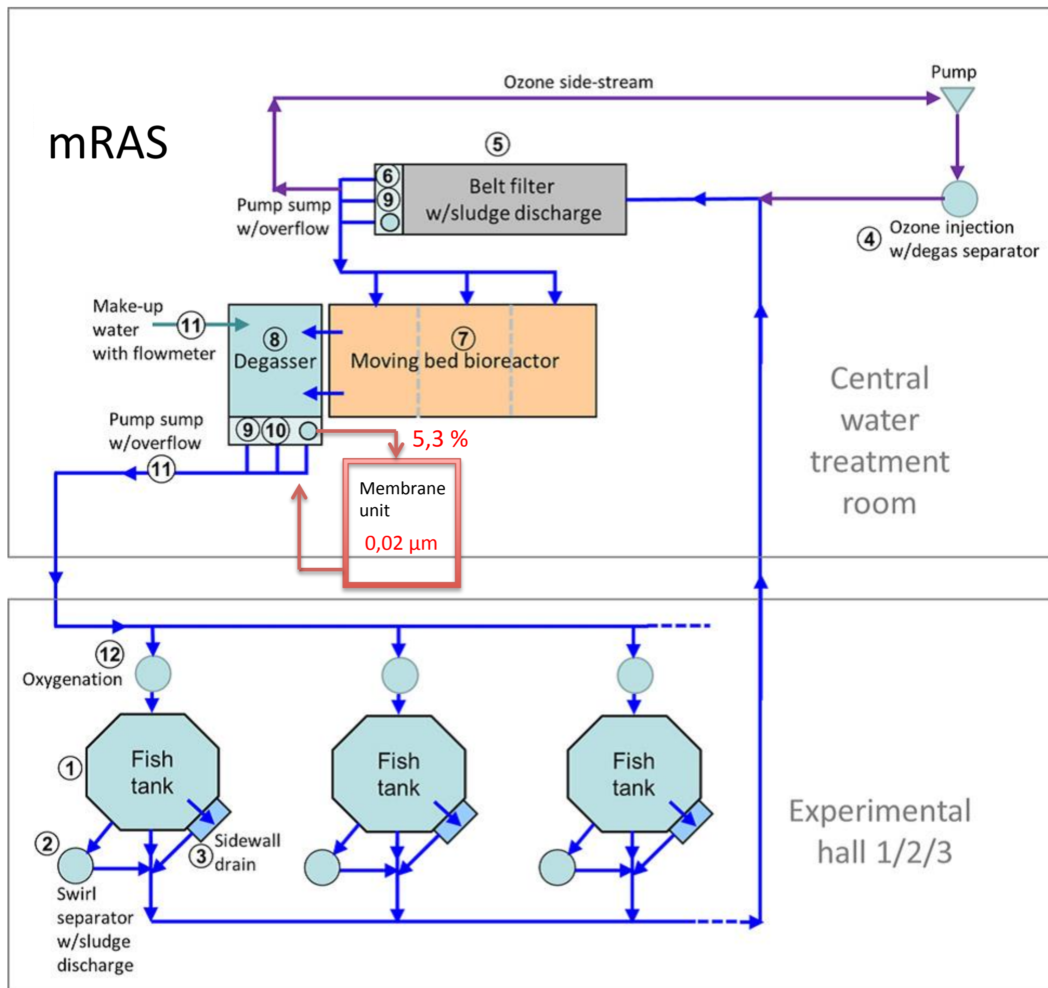

Figure 2.1: Illustration of mRAS at NCRA. Modified image from Terjesen *et al*., 2013.

Table 2.1: Average water quality parameters with standard deviations (± SD) throughout the experiment (Holan *et al*., 2016).

Parameter	mRAS	cRAS
pH	7.62 ± 0.16	7.57 ± 0.11
Temperature (°C)	12.9 ± 0.4	12.9 ± 0.4
Salinity (ppt)	12.3 ± 0.4	12.3 ± 0.6
O_2 (%)*	97.3 ± 4.5	96.5 ± 2.6

*Tank outlet

2.2 Sampling- times and procedures

For water microbiota analysis, water samples were collected at both inlet and outlet water from the fish tanks and the biofilters (Table 2.2). Fish tanks in mRAS were labelled 101, 102 and 104, fish tanks in cRAS were labelled 201, 202 and 204. For the bacterial count analysis, water was collected from the inlet and outlet water of the membrane in addition to fish tanks and biofilters. For gut microbiota analysis, 24 fish at day 51 of the experiment were euthanized and gut samples were collected from the colon.

Table 2.2: Sampling system

Sampling time	Day	Date	Water samples	Gut samples
1st (T1)	1	08.12.04	Membrane and biofilters	
1st (T1)	3	10.12.14	Fish tanks 101, 102, 104, 201, 202, 204	
2nd (T2)	8	15.12.14	Membrane and biofilters	
2nd (T2)	9	16.12.14	Fish tanks 101, 102, 104, 201, 202, 204	
3rd (T3)	36	12.01.15	Membrane and biofilters	
3rd (T3)	37	13.01.15	Fish tanks 101, 102, 104, 201, 202, 204	
4th (T4)	50	26.01.15	Membrane and biofilters	
4th (T4)	51	27.01.15	Fish tanks 101, 102, 104, 201, 202, 204	Fish f11, f12, f13, f14

2.2.1 Water sampling

For DGGE microbial community analysis, samples were collected from both inlet and outlet water in 50 mL Falcon tubes. The tubes were frozen and stored at -20 °C. At the last sampling time (T4), all tubes from previous sampling times were thawed in room temperature. The water was transferred to a 50 mL syringe and 30-50 mL of the water was pressed through a 0.22 µm Dynaguard filter tip. The filter tips were frozen and stored at -20 °C until DNA extraction.

For flow cytometry bacterial count analysis, samples were collected from both inlet and outlet water in 20 mL cryo tubes. Glutaraldehyde (50%) was added to a total concentration of 0,1 % to fixate the samples and avoid any further division and growth of bacteria. The samples were quickly snap freezed in liquid nitrogen and the tubes were stored at -80 °C.

2.2.2 Gut content sampling

The fish were euthanized and cut open to harvest the gut sample from the colon. The samples were put in 20 mL cryo tubes and snap freezed on liquid nitrogen. The tubes were stored at -80 °C.

2.3 Analytical methodology and principles of some methods used

To compare the number of bacteria between mRAS and cRAS, flow cytometry analysis was performed on the samples. To study the microbial community composition within and between cRAS and mRAS in both water and gut content of the fish, the 16S rDNA molecules were analysed using DGGE.

2.3.1 Flow Cytometry

Flow cytometry is a technology that allows for characterization of cells in a solution by means of identification through fluorescence- and light-scattering signal analysis. It can be used to identify cells in a sample and give information about number of cells, cell size, shape, density, and surface morphology (Díaz *et al*., 2010, Endo *et al*., 2000). Flow cytometry can be used in microbial ecology to identify bacterial cells in environmental samples. By labelling the cells with fluorescent dyes, they are illuminated by a laser beam in the flow cytometer, which create light-scattering and fluorescent signals. These signals are analysed and coupled to structural and/or functional cell features (Bressan *et al*., 2015, Díaz *et al*., 2010).

The samples were thawed in room temperature and prepared for analysis on a BD Accuri™ C6 Flow Cytometer (BD Biosciences, San Jose). The maximum cell counts per sample run for the flow cytometer is 1 million counts, therefore some samples were diluted 1:10 with sterile 0,22 µm filtered milliQ water depending on bacterial concentrations. SYBR® Green I nucleic acid gel stain (Life technologies) working solution (1:10) was made from the stock solution (10 000X conc.), and added to the water samples to a final 1:100 ratio of water sample and SYBR stain. The samples were incubated for 15 minutes in the dark before further analyses. A medium flow rate (34.5 µl/min) and 2 minute collections were conducted for all samples. As the samples are run through the flow cytometer they pass the blue laser (λ = 488 nm) light which is absorbed by the SYBR stain in the cells, and they emit green light (λ_{max} = 520 nm) that is collected by a detector (FL1) reading blue laser excited emissions (533±15 nm). The

number of emissions collected by the detector FL1 showed the amounts of bacteria in the samples. The results from the samples were further analysed with the BD Accuri™ C6 Software, where plots that displayed FSC-A (forward scattered light, correlates with size of cell) and FL1 for fluorescent signal detection were used. Fluorescent intensity signals below 10^4 on the FL1 detector was excluded from the results as these hits were considered noise, viruses and fluorescent algae present in the samples and not bacteria. The data was exported to Microsoft Excel, and bacterial concentrations were calculated.

2.3.1 DNA extraction

The PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc) was used to extract bacterial DNA from the gut and water samples according to the protocol supplied with the kit (Appendix C).

For water samples the hollow fibers of the Dynaguard filters were pulled out from the plastic tip using a needle and transferred to the PowerBead Lysis tubes, and the DNA was extracted as described by the protocol (Appendix C).
The gut samples were thawed in room temperature and a spatula was used mix the content of the tube. 1.0 – 1.5 ml of gut sample was added to Eppendorf tubes and centrifuged at 13 000 rpm for 10 minutes. 500 – 700 µl excess water/supernatant was removed and 0.25 g of gut sample was added to the PowerBead Lysis tubes. For following steps the protocol was followed.

2.3.2 PCR (Polymerase chain reaction)

To amplify the variable 3 region (V3) of the 16S rRNA gene, PCR was conducted. A mastermix was made from milliQ water (18.5 µl), 10 mM dNTPs (0.5 µl), 25 mM $MgCl_2$ (0.5 µl), 0.10 µM of each primer (table 2.3) (0.75 µl), 10x reaction buffer (2.5 µl), BSA (0.375 µl) and polymerase (0.125 µl) to a volume of 23 or 24 µl for each sample. The last step was adding 1 or 2 µl of approximately 5 ng/µl DNA extract as a template to the reaction mixture, giving a total reaction volume of 25 µl. A negative control without DNA template was always included to the PCR reactions.
The PCR reactions were run at 35-40 temperature cycles (Table 2.4) depending on the concentration of the DNA template (DNA extract). Several rounds of PCR with varying

numbers of temperature cycles, amount of DNA template added (1 or 2 μl) and different DNA polymerases were needed to optimize the PCR products for further DGGE-analysis. Gut-samples had generally very low DNA-concentrations. To gain a satisfactory PCR product for these samples, Phusion® High-Fidelity DNA Polymerase (Thermo Fisher Scientific), 2 μl added template and 38-40 reaction cycles were tested. This polymerase contains lower amounts of bacterial DNA in the polymerase and lowers the possibility of amplified bacterial enzyme DNA form the polymerase in the PCR product when conducting many reaction cycles (Quail et al., 2012). For water samples the concentration of DNA extract was higher, 1 μl template was added, Taq polymerase (Invitrogen) was used and 35 reaction cycles were enough to get acceptable PCR products.

Table 2.3: DNA sequences for primers used to amplify the (1) V3 region of the 16S rRNA gene and the (2) re-amplification and sequencing of excised DGGE-bands

	Primer	Sequence
1	338F-GC Forward	5' – CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGG GACTCCTACGGGAGGCAGCAG - 3'
	518R Reverse	5' – ATTACCGCGGCTGCTGG – 3'
2	338F-GC-M13R Forward	5' – CAGGAAACAGCTATGACCGCCCGCCGCGCGCGGCGGGCGG GGCGGGGGCACGGGGGACTCCTACGGGAGGCAGCAG – 3'
	518R Reverse	5' – ATTACCGCGGCTGCTGG – 3'
	M13 R Sequencing	5' – CAGGAAACAGCTATGACC – 3'

Table 2.4: PCR temperature cycling program for the amplification of the V3 region of the bacterial 16S rDNA. Steps 2-4 were repeated for 35-40 cycles.

Step	Reaction	Temperature (°C)		Time	
		Taq	Phusion	Taq	Phusion
1	Denaturation	95	98	3 min	2 min
2	Denaturation	95	98	30 sec	15 sec
3	Annealing	50	50	30 sec	20 sec
4	Elongation	72	72	60 sec	20 sec
5	Elongation	72	72	10 min	5 min
6		4-10	4-10	∞	∞

2.3.3 Agarose gel electrophoresis

To analyse the amplified PCR products in regards to quantity, quality and possible contamination, the PCR products were run through a 1 % agarose gel. The gel was prepared by mixing agarose powder (4 g) with 1 x TAE buffer (400 ml). The solution was heated in the microwave until all the agarose had dissolved and cooled in room temperature to 65 °C before GelRed (Biotium) (20 µl) was added. The agarose solution was poured into a gel mould, a well comb was applied to create wells and the gel was left to solidify for around 10 minutes. Then the well comb was carefully removed and 1 x TAE buffer was poured onto the gel and into the electrophoresis chamber. Loading dye (Thermo Fisher Scientific) (1 µl) was added to the PCR products (3-5 µl) and applied on the gel together with a DNA ladder (Thermo Fisher Scientific). The gel was run at 100-140 Volt (depending on the size of the gel) for 45 minutes, and then the gel was moved to the UV-cabinet for visualization of the DNA bands. The intensity of the bands gave an indication of quantity of DNA and bands in the negative control or at uncommon positions in the gel indicated contamination of the PCR products.

2.3.4 DGGE (Denaturing gradient gel electrophoresis)

Denaturing gradient gel electrophoresis (DGGE) is a quick method well suited for investigation of microbial community compositions, where DNA fragments of similar length but different sequences can be separated. The amplified rDNA molecules of a sample are separated due to the differences in electrophoretic mobility in the polyacrylamide gel with a

denaturing gradient (Muyzer and Smalla, 1998). As the rDNA molecules are migrating and denatured in the gel they create a band pattern that reflects the species present in the sample. The intensity of the bands reflect the abundance of the relevant strains in the sample (Sanz and Köchling, 2007). The number of bands reflects the species diversity of the sample, and more thorough taxonomic analysis can be done by sequencing particular bands of interest (Malik *et al*., 2008).

Denaturing gradient gel electrophoresis was performed using the INGENY phorU system. Two glass plates were washed and polished with ethanol (96 %) and Kimwipe paper, assembled on each side of the spacer and placed in the gel box. A comb to create wells was mounted and all the screws were tightened. Two 8% acrylamide solutions (0 % and 80 % denaturing) with urea and formamide as denaturing agents were made (Appendix D) and used to make two separate solutions representing 35 % and 55 % denaturing concentration, respectively (Table 2.5). Tetramethylenediamine (TEMED) and 10 % ammonium persulphate (APS) were added prior to casting the gel for polymerization. The gel was casted using a gradient mixer to create the denaturing gradient with the high denaturing concentration (55%) at the bottom and the lower denaturing concentration (35 %) towards the top, following a stocking solution (0 % denaturing) applied at the top of the gel. The comb was pressed down, and the gel was left to polymerize for two hours.

Table 2.5: Contents of solutions applied for casting an 8% polyacrylamide DGGE gel with 35 % - 55 % denaturing gradient

Denaturing	0 %	80 %	TEMED	APS	Tot.vol
35 %	13,5 ml	10,5 ml	16 µl	87 µl	24 ml
55 %	7,5 ml	16,5 ml	16 µl	87 µl	24 ml
Stack. gel 0%	8 ml	-	10 µl	40 µl	8 ml

The comb was then carefully removed and the spacer pushed down to open up the space below the gel for the electrical current to pass. The gel box was placed in a preheated (60 °C) buffer tank (17 L 0.5 x TEA). The water circulation was turned on and the voltage was set at 100 V, which should result in a current of 23-27 mA. Lower mA may imply air bubbles beneath the gel that should be removed or it may affect the migration of the samples. A too high buffer level in the tank could explain higher mA. The water circulation was switched off

and loading dye (Fermentas) (2-4 µl) was added to 5-15 µl of PCR product and applied to the wells. The amount of PCR product loaded on the gel was depending on the concentration of PCR product that was visualized by the agarose gel electrophoresis. The 7-8 outermost wells were left empty due to smiling effects in the gel after the electrophoresis. The water circulation was switched back on, and the gel was run for 20 hours at 100 V.

After the electrophoresis the gel was carefully removed from the glass plates, placed on a plastic sheet and stained with a solution containing milliQ water (30 ml), SYBR® Gold (Invitrogen) (3 µl) and 50 x TAE (600 µl) in a dark container for 1 hour. Then the gel was rinsed with milliQ water and placed in the UV-cabinet for visualization and photography. Selected bands were excised from the gel for sequence analysis. Eppendorf tubes containing sterile water (20 µl) were prepared, and pipette tips were used to excise the bands of interest. The pipette tips were then placed in the Eppendorf tubes and the gel-material containing DNA was pushed out of the pipettes and into the sterile water.

2.3.5 Reamplification of selected DGGE bands for sequence analysis

The excised DGGE-bands were prepared for reamplification by first vortexing the Eppendorf tubes with the gel-material was then spun down. A PCR mastermix was made from milliQ water (20 µl), 10 x reaction buffer (2.5 µl), 10 mM dNTPs (0.5 µl), 25 mM $MgCl_2$ (0.5 µl), 10 µM forward and reverse primers (table 2.3) (0.75 µl each), Taq polymerase (0.125 µl) and DNA template from the gel (1 µl) for each band. Then the PCR was carried out for 38 temperature cycles. The PCR-reaction cycles were as shown in table 2.4 for Taq polymerase with the exception of step 3 annealing, which was conducted at 53 °C in this case. The PCR products were analysed by agarose gel electrophoresis as described above. The QIAquick PCR purification kit was used to purify the PCR products according to the protocol supplied by the producer (Appendix E). Eppendorf tubes containing purified PCR product and the sequencing primer M13R (table 2.3) (5 µl of a 5 mM solution) were shipped to the commercial company GATC Biotech for sequencing.

2.3.6 Taxonomic classification of sequences from selected DGGE bands

The DNA sequence results received from GATC were presented as chromatograms, where the peaks in the chromatogram represent the bases in the sequences. The files were opened in

Chromas Lite (Technelysium Pty Ltd) and the sequence quality was examined by inspecting the chromatograms. The text files were exported and opened in word (or other text formatting programs). The forward and reverse primer sequences and noisy areas were removed and the remaining sequence was used for the taxonomical analysis. The sequences were analysed using the Ribosomal Database Project (RDP) Classifier tool. This tool can classify bacteria using the 16S rDNA-sequences in the hierarchy of biological classification. The bootstrap cut-off was set to 50 %, which was recommended by the Classifier tool for short sequences of less than 250 base pairs.

2.3.7 Analysis of DGGE gels and statistics

The DGGE images were analysed with Gel2k (Svein Norland, Department of Biology, University of Bergen, Norway). The software converts the bands profiles in the gel into histograms, where the area under of the peaks in the histogram represents the intensity of the bands in the gel. Bands that have migrated the same distance in the gel among the loaded samples are defined as one 16S rRNA gene sequence, representing the same species. The peak area values from the histograms were exported to Microsoft Excel, and the values were normalized for comparisons between samples by dividing each peak area value by the sum of all peak values of that specific lane, equation 2.1:

$$p_i = \frac{n_i}{N} \tag{2.1}$$

where, p_i = normalized peak area values, n_i = peak value of a single band and N = the sum of all the peak area values of all the bands in the lane. The statistical analyzes were based on the normalized values using the computer program package PAST (Hammer *et al*., 2001).

The alpha diversity indices calculated with PAST were band richness (S), Shannon's diversity index (H'), and the Busaz and Gibson's evenness ($e^{H'}/S$). Band richness reflects the number of species in the sample and thus the species richness, whereas Shannon's diversity index (Equation 2.2) reflects both band richness and evenness of bands in the community profile (cf. Section 1.8) expressed by equation

$$H' = -\sum_i \frac{n_i}{n} \ln \frac{n_i}{n} \tag{2.2}$$

where, n_i = number of individuals of species i and n = total number of individuals

Evenness (Equation 2.3) reflects the equality of abundances of species (cf. Section 1.8) and is a valued number between 0 and 1. Higher evenness numbers indicate more even communities, and low evenness may imply dominant species.

$$evenness = e^{H'}/S \tag{2.3}$$

where, H’ = Shannon’s diversity index and S = Band richness

For beta diversity measures to compare microbial community profiles, Bray-Curtis similarity matrices were calculated in PAST. The Bray-Curtis similarity values ranges from 0 – 1, where values of 0 indicate no common bands among the compared community profiles, and 1 imply identical community profiles (Bray and Curtis, 1957).

Based on the Bray-Curtis similarities, Non-metric multidimensional scaling (NM-MDS) was computed for a visual display of the similarities between samples. NM-MDS is based on a distance matrix, where the program attempts to plot data points of the samples in a coordinate system (Hammer *et al.*, 2001). Points (samples) that display larger distances are less similar, whereas points that are more clustered indicated more similarity.
Also based on the Bray-Curtis similarities, a Similarity Percentage (SIMPER) method was conducted to assess which bands contributed to most of the differences found between the groups of samples.

One-way PERMANOVA (Non-parametric MANOVA) was used to test whether there were significant differences in the microbial community profiles between groups of samples. This test is based on measures of distance or dissimilarity (e.g. Bray-Curtis) between samples. By comparing the distances among and within sample groups, it permutuate the comparisons to test whether there are significant differences in community composition (Anderson, 2001). If there are significant differences between samples, $p < 0.05$ (Hammer *et al.*, 2001).

3. Results

The samplings in mRAS and cRAS were conducted at four periods of time throughout the experiment. The samplings at the different units (biofilters, fish tanks and membrane) of mRAS and cRAS were carried out at different dates at the sampling times. Therefore, when discussing samples from several units of mRAS and cRAS, the abbreviations T1, T2, T3 and T4 are used for the first, second, third and fourth sampling time. When discussing samples from one unit, days are used to explain the time of the sampling. The sampling system from the units can be found in table 2.2 section 2.2.

3.1. Effect of membrane filtration in water treatment on the bacterial concentrations

3.1.1 Comparison of bacterial concentrations in mRAS and cRAS

To investigate the effect of membrane water filtration on the number of bacteria in mRAS, the bacterial concentrations were compared to those in cRAS. Flow cytometry analysis was conducted on samples from rearing tanks, biofilter and membrane at all sampling times. A sidestream of 5.3 % from the total water flow in mRAS was run through the membrane for 10 hours per day.

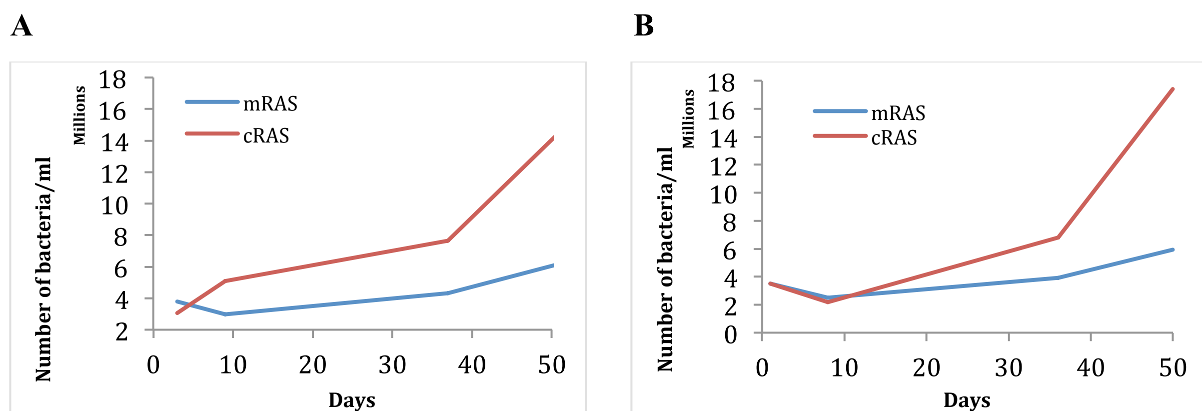

Figure 3.1: Number of bacteria in inlet and outlet* water of rearing tanks (A) and inlet and outlet* water of biofilter (B).

*The same bacterial concentrations in inlet and outlet water caused the graphs to completely overlap when plotted together, thus an average of inlet and outlet water concentrations were used as one graph instead of two per RAS (SD± were so low they were not visually present on the graph).

Table 3.1: Number of bacteria per millilitre from inlet and permeate water of membrane in mRAS.

Day	Water in (Number of bacteria/ml)	Permeate* (Number of bacteria/ml)	Amount of bacteria removed (%)
1	3 098 986	103 101	96.7
8	2 580 290	7 478	99.7
38	3 734 058	13 957	99.6
50	6 651 594	64 174	99.0

*Permeate water filtrated through pore sizes of 0.02 μm in the membrane

At the first sampling time at day 3, the bacterial concentration was somewhat higher in mRAS than cRAS in the inlet and outlet water of the fish tanks (Fig 3.1A**)**. By day 9, the bacterial concentration increased in both mRAS and cRAS until the fourth sampling time at day 51, however the increase was steeper and higher for cRAS. At day 9, 37 and 51 a t-test confirmed that the concentration of bacteria was significantly higher in cRAS than mRAS ($p = 0.0039$, 0.017 and 0.014, respectively). By day 51, the bacterial concentration in cRAS was found to be around 14 million/ml, while in mRAS it was around 6 million/ml. The bacterial concentrations in the biofilter (Fig 3.1B**)** indicated the same trends in mRAS and cRAS as the fish tanks. At the end of the experiment the bacterial concentration in the biofilter in cRAS was a lot higher (18 million/ml) than the concentration in fish tanks in cRAS (14 million/ml. The bacterial concentration in mRAS was about the same for both rearing tanks and biofilter (6 million/ml) at the end of the experiment.

The membrane removed high amounts of bacteria, and the reduction of bacterial numbers in the rearing water (sidestream of 5.3 % of total water flow) was more than 96% at all sampling times (Table 3.1), at the last three sampling times the removal of bacteria was 99 %.

3.2 Effect of membrane filtration in water treatment on the composition of water microbiota

3.2.1 Comparison of water microbiota in mRAS and cRAS

To study the effect of the membrane filtration as part of the water treatment in mRAS on the water microbiota, a DGGE gel was run to compare the microbial community profiles between the two systems mRAS and cRAS at the end of the experiment. Water samples from T3 and T4 were loaded on the gel (Fig. 3.2).

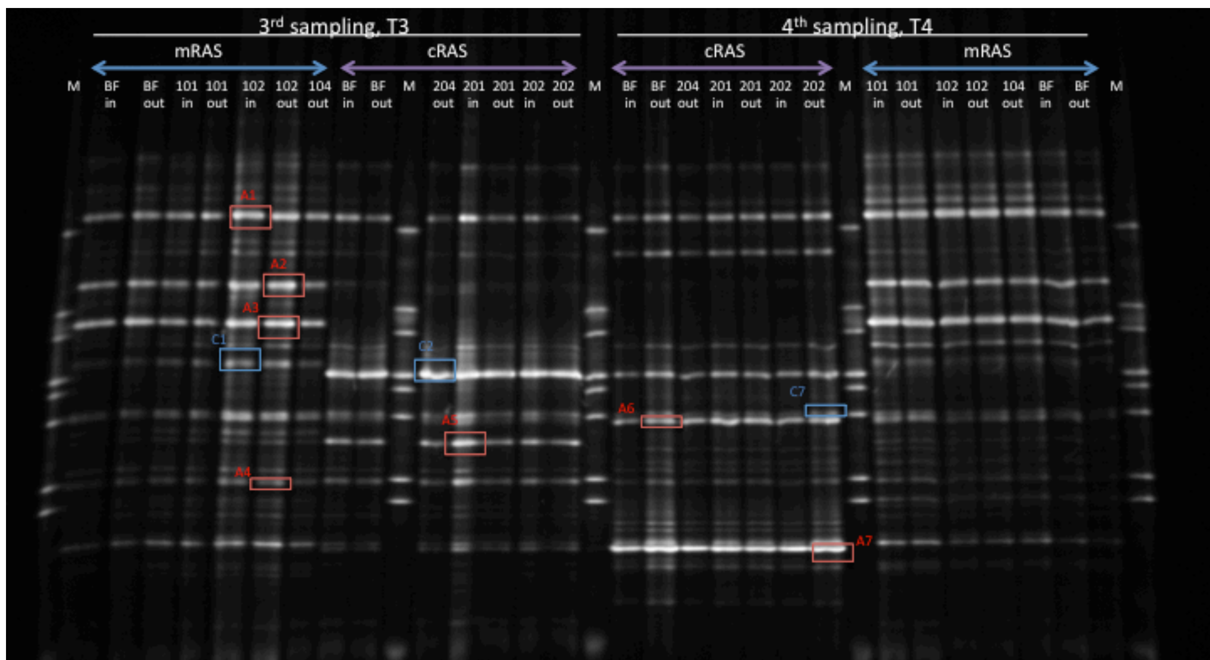

Figure 3.2: DGGE gel showing PCR-products of the 16S v3 region from water samples obtained from T3 and T4 in mRAS and cRAS. The samples were collected at the inlet and outlet water (in/out) of the biofilters (BF) and of different replicate rearing tanks (mRAS: 101, 102, 104, cRAS: 201, 202, 204). M = markers. Bands excised from the present gel and subjected to sequence analysis are marked with red squares. Blue squares show bands corresponding to bands that are excised from others gels. Overview of all bands excised is found in table 3.5.

The DGGE gel (Fig.3.2) indicated differences in the microbial community structures between mRAS and cRAS. All sample profiles in the gel were further analysed with NM-MDS ordination based on Bray-Curtis similarities to illustrate any variation between community profiles in a coordinate system (Fig.3.3). The NM-MDS plot showed that the community profiles of mRAS and cRAS were clearly separated in the plot. The samples of mRAS from T3 and T4 were more clustered together, indicating higher similarity and less fluctuations of water microbiota over time within this system than in the cRAS. The samples of cRAS from

T3 and T4 were separated according to sampling time, which could indicate a less stable water microbiota over time.

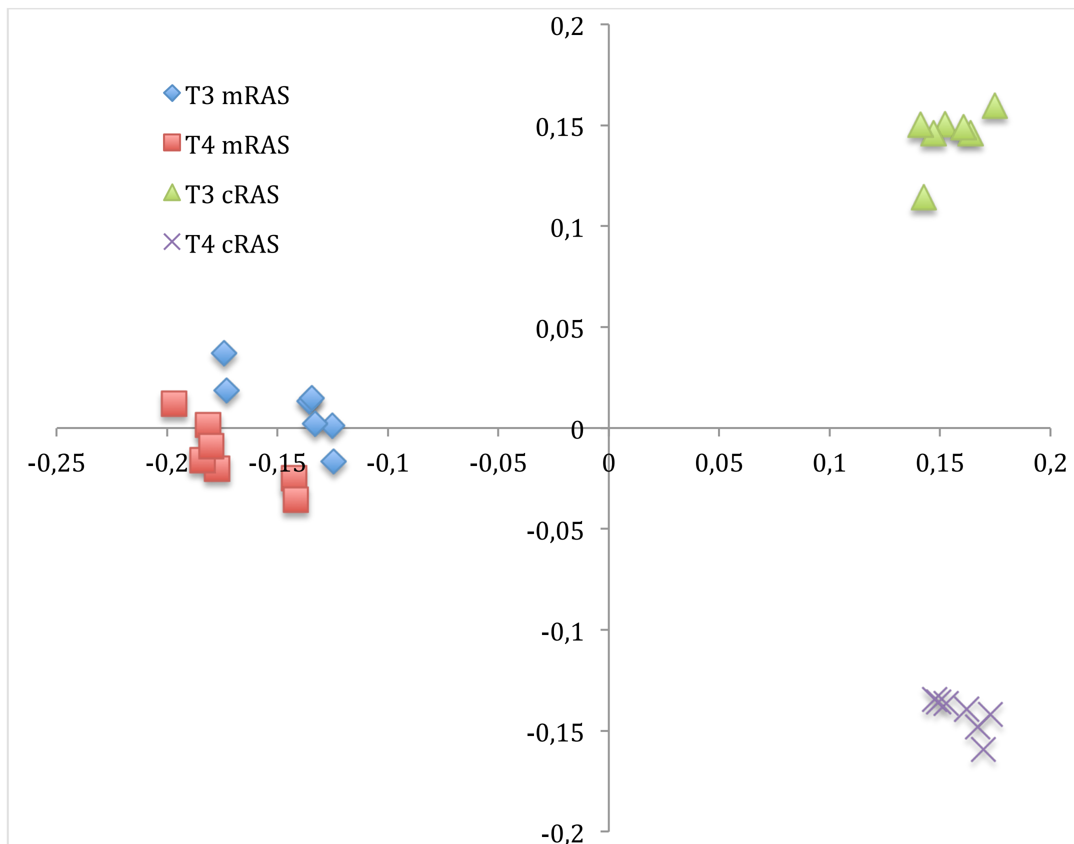

Figure 3.3: NM-MDS plot based on average Bray-Curtis similarities for mRAS and cRAS water community profiles from T3 and T4.

To further compare the microbial community profiles in mRAS and cRAS, average Bray-Curtis similarities were calculated within and between the samples of each system from T3 and T4. There were higher similarities within the samples of each system than between them (Fig. 3.4), which suggests that there are differences in community structures between the two systems. The similarity index between mRAS and cRAS were low at both sampling times, 0.4 at T3 and 0.35 at T4 that also indicated low similarity between systems. There were differences between T3 and T4 in mRAS and cRAS, and the samples from cRAS were less similar (0.5) than those in mRAS (0.72). This further implies lower stability of the water microbiota over time in cRAS than mRAS. A One-Way PERMANOVA test confirmed that the community profiles between all the samples were significantly different from each other ($p > 0.005$). The similarities within samples in cRAS (Fig. 3.4) were higher than mRAS, and a t-test confirmed that the similarities were significantly higher in cRAS ($p = 0.0001$).

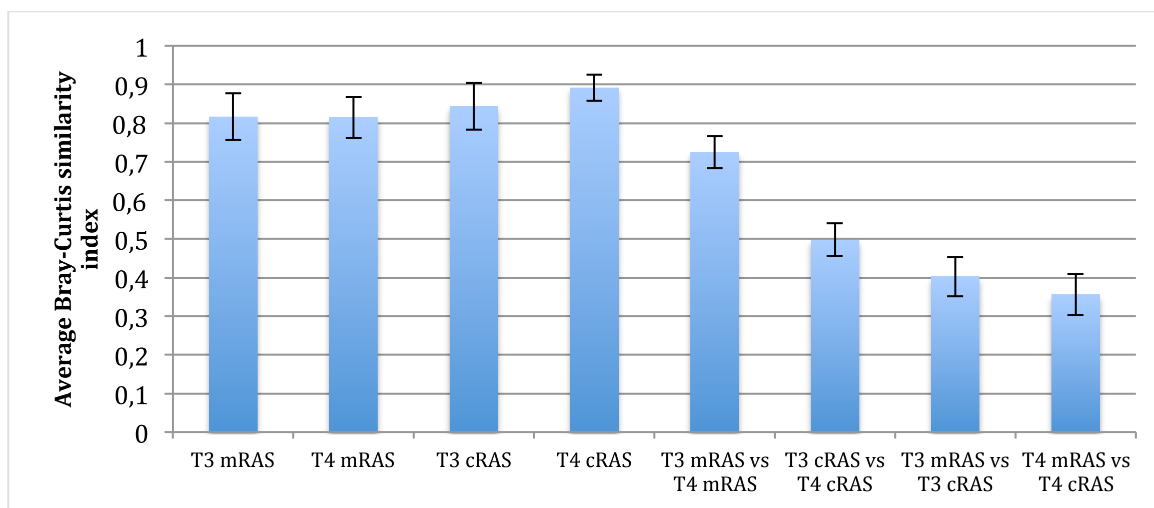

Figure 3.4: Average Bray-Curtis similarities with standard deviations (SD±) for comparison of community profiles within and between samples of mRAS and cRAS from T3 and T4.

To asses which bands that were primarily responsible for any differences in water community structure between mRAS and cRAS, a SIMPER analysis was performed. Eight bands explained 70 % of the dissimilarity between mRAS and cRAS (Table 3.2). Band C2 accounted for most of the differences between mRAS and cRAS; 15.6 %. This band was strong in cRAS (mean abundance 0.2) and weak in mRAS (0.01). Band A3, being strong in mRAS (0.2) and not present in cRAS, accounted for 15.1 %. The bands were excised, and sequence analysis revealed they both represented Flavobacteriia (Table 3.5). Even though the confidence threshold was low, the RDP classifier suggested that the bands represented two different Flavobacteriaceae genera (C2: *Persicivirga*, A3: *Joostella*, see table 3.5)

Table 3.2: Bands contributing to differences in mRAS and cRAS as identified by SIMPER analysis

Band ID	Contribution %	Cumulative %	Mean abun.* mRAS	Mean abun. cRAS
C2	15.60	15.60	0.01	0.20
A3	15.10	30.70	0.20	0.00
A2	10.50	41.20	0.14	0.01
A7	9.70	50.90	0.00	0.12
A5	5.70	56.60	0.01	0.07
A6	5.50	62.10	0.04	0.09
A1	4.30	66.40	0.17	0.12
C1	3.50	70.00	0.04	0.00

*Abundance calculated as the peak area of the band divided by the sum of all peak areas for the relevant DGGE community profile

To get insight on the microbial diversity for community profiles in mRAS and cRAS, average diversity indices were calculated from the DGGE profiles for each sample (Fig. 3.5). Both the band richness (Fig. 3.5A) and Shannon's diversity index (Fig. 3.5B) indicated more diverse microbial communities in mRAS than in cRAS. A t-test confirmed that the band richness ($p = 0.0001$) and Shannon's diversity index ($p = 0.0083$) were significantly higher in mRAS than cRAS. The richness and diversity increased in both systems from T3 to T4. The evenness index (Fig. 3.5C) was similar for mRAS and cRAS community profiles, and was lowest at T4 in mRAS that indicated lower equal abundance of bands here compared to the other samples. A t-test confirmed that the evenness indices were not significantly different between the two systems ($p = 0.252$).

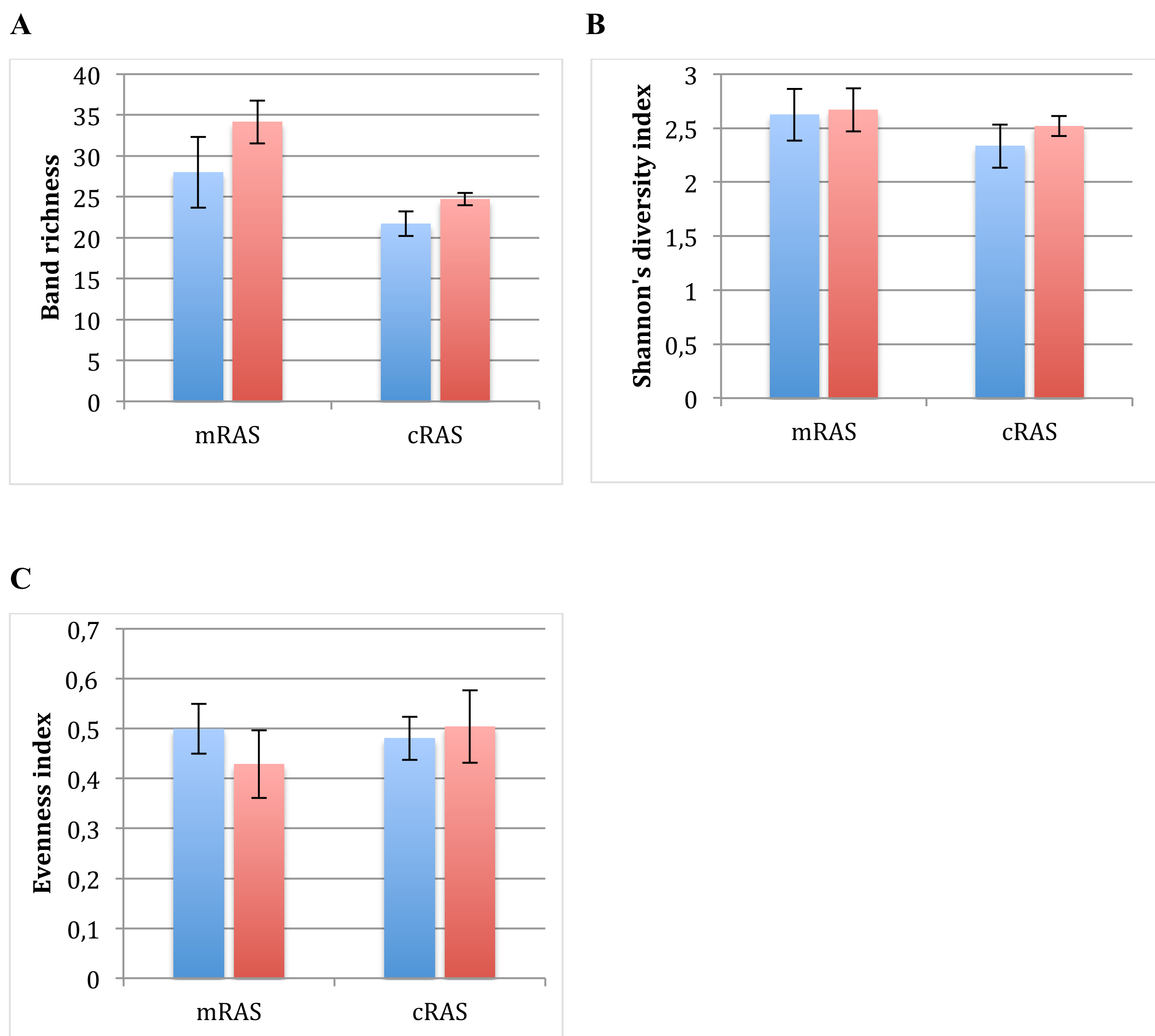

Figure 3.5: Average diversity indices with standard deviations (SD±) calculated from the microbial community DGGE profiles for each sample (Figure 3.2). **A**: Band richness, **B**: Shannon's diversity index, **C**: Evenness index. Samples taken from mRAS and cRAS at different sampling times,
■ = T3 ■ = T4

3.2.2 Temporal community dynamics of water microbiota in mRAS

To examine the microbial community dynamics over time in the system with membrane water treatment, a DGGE gel was run to compare the microbial community composition between all sampling times T1, T2, T3 and T4 within mRAS (Fig. 3.6).

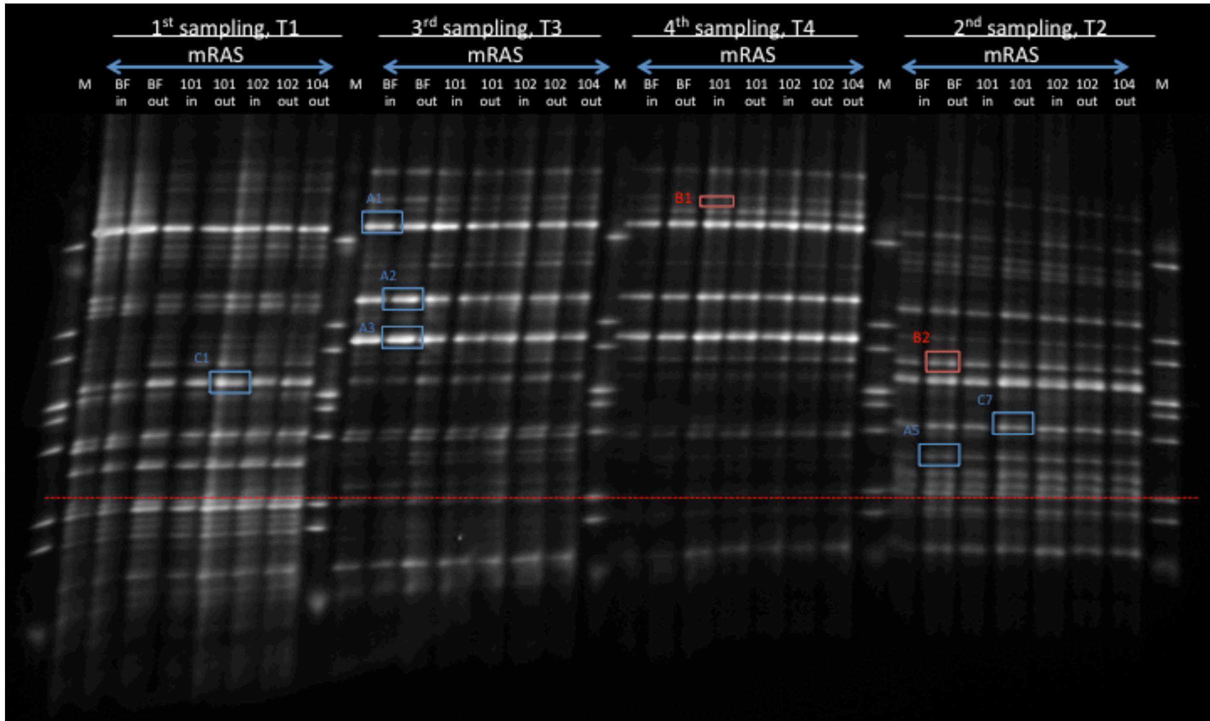

Figure 3.6: DGGE gel showing PCR-products of the 16S v3 region from water samples obtained from T1, T2, T3 and T4 in mRAS. The samples were collected at the inlet and outlet water (in/out) of the biofilters (BF) and of different replicate rearing tanks (mRAS: 101, 102, 104, cRAS: 201, 202, 204). M = markers. The gel was not analysed below the red line due to smiling effects and poor separation. Bands excised from the present gel and subjected to sequence analysis are marked with red squares. Blue squares show bands corresponding to bands that are excised from others gels. Overview of all bands excised is found in table 3.5.

Differences in the microbial community structure between the sampling times in mRAS were observed in the DGGE gel (Fig. 3.6). NM-MDS ordination based on Bray-Curtis similarities was conducted for further analysis to compare the community profiles and to illustrate differences/similarities between the samples in a coordinate system (Fig. 3.7). The NM-MDS plot suggested that the water microbiota in mRAS changed throughout the experiment given the different positions of the samples in the plot. The samples at T1 and T2 were far apart indicating higher variation and fluctuations in the microbial communities at the beginning of the experiment. The samples taken at T3 and T4 were clustered together indicating, more stability and less variation in the community structures during the last period of the experiment.

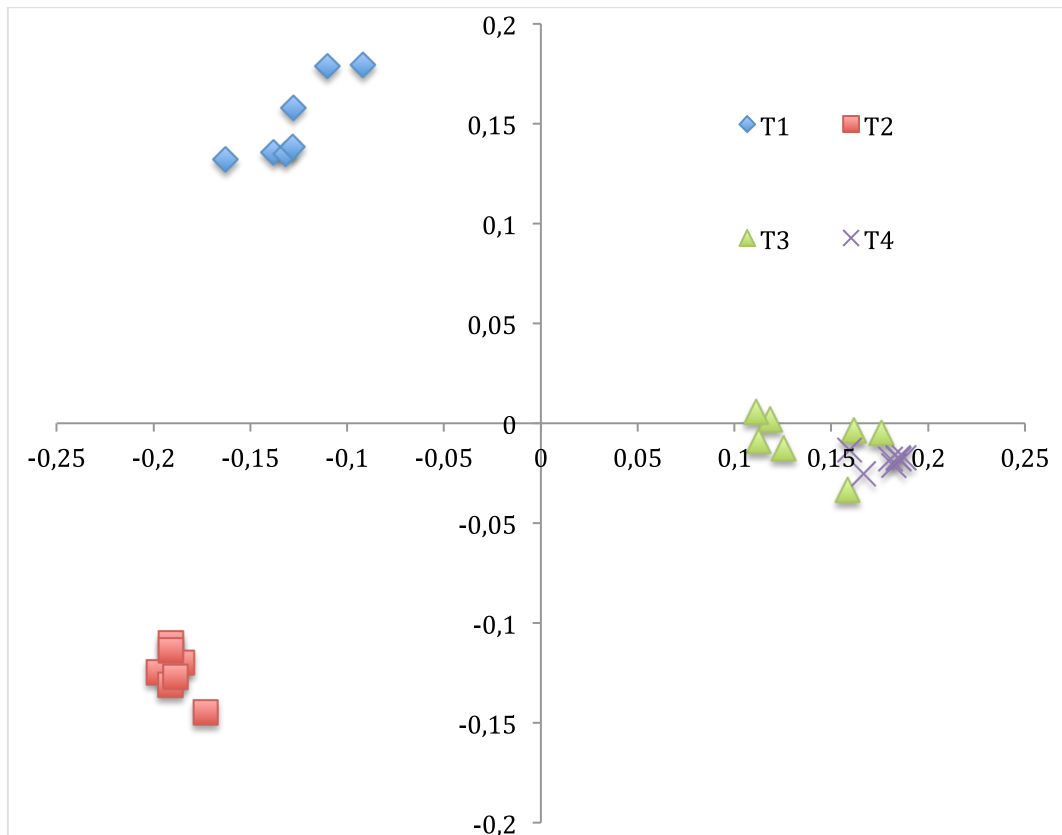

Figure 3.7: NM-MDS plot based on average Bray-Curtis similarities for mRAS water community profiles from T1, T2, T3 and T4.

Bray-Curtis similarities were computed to compare the water microbiota within and between sampling times. Comparisons of community profiles between sampling times for mRAS (Fig. 3.8) showed that the samples at T3 and T4 were more similar to each other (0.75) than the other sampling times. It also indicated early changes in the water microbiota due to the low similarity between T1 and T2 (0.5). The similarity within each sampling time was overall high (> 0.75) being lowest at T1 and highest at T4. The One-Way PERMANOVA test confirmed that all the community profiles between all the sample times were significantly different from each other ($p > 0.005$).

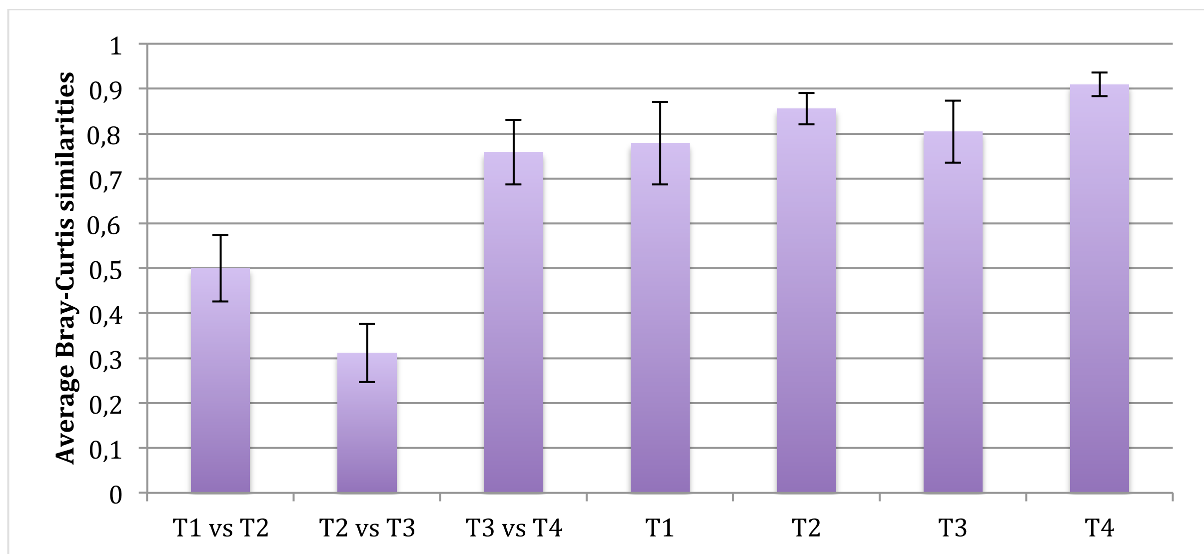

Figure 3.8: Average Bray-Curtis similarities with standard deviations (SD±) for comparison of community profiles in mRAS between and within sampling times T1, T2, T3 and T4.

A SIMPER analysis showed that eight bands explained 66.1 % of the dissimilarity between the community profiles (Table 3.3). The band that accounted for most of the differences (15.5 %) between the sampling times was A3. The band was strong in T3 (mean abundance 0.22) and T4 (0.3), and not present at the two first sampling times. Band A1 accounted for 14.7 % of the differences, it was barely present at the two last sampling times, but had mean abundances of 0.2 and 0.24 at sampling time T1 and T2, respectively. The bands were excised, and sequence analysis revealed that A3 represented Flavobacteriales and *Joostella* at the genus level even though the confidence threshold was very low (table 3.5). A1 represented *Francisella* **(**Thiotrichales, Gammaproteobacteria; table 3.5) with a fairly high confidence threshold. The mean abundances of band C1 and A2 increased over time, and both bands represented Flavobacteriaceae (C1: *Polaribacter,* A2: No genus data obtained). Band A3, C1 and A2 showed a general increase of Flavobacteriaceae over time in mRAS.

Diversity indices were calculated for mRAS (Appendix A), however due to unequal loading of samples on the gel and/or uneven staining, the changes in diversity reflected in the band richness, Shannon's diversity and evenness index may not display a real development of the microbial communities.

Table 3.3: Bands contributing to differences in mRAS at all sampling times as identified by SIMPER analysis

Band ID	Contribution %	Cumulative %	Mean abun.* T1	Mean abun. T2	Mean abun. T3	Mean abun.T4
A3	15.50	15.50	0.00	0.00	0.22	0.30
A1	14.70	30.20	0.11	0.24	0.06	0.02
C1	10.60	40.80	0.17	0.13	0.20	0.25
A2	9.90	50.70	0.00	0.04	0.16	0.19
A5	4.30	55.00	0.09	0.07	0.01	0.01
B2	4.00	59.00	0.03	0.10	0.02	0.04
-	3.60	62.60	0.06	0.06	0.02	0.00
C7	3.50	66.10	0.07	0.09	0.05	0.02

*Abundance calculated as the peak area of the band divided by the sum of all peak areas for the relevant DGGE community profile

3.2.3 Temporal community dynamics of water microbiota in cRAS

To investigate the microbial community dynamics over time in the system without membrane water treatment, a DGGE gel was run to compare the microbial community composition between all sampling times T1, T2, T3, and T4 within cRAS (Fig. 3.9).

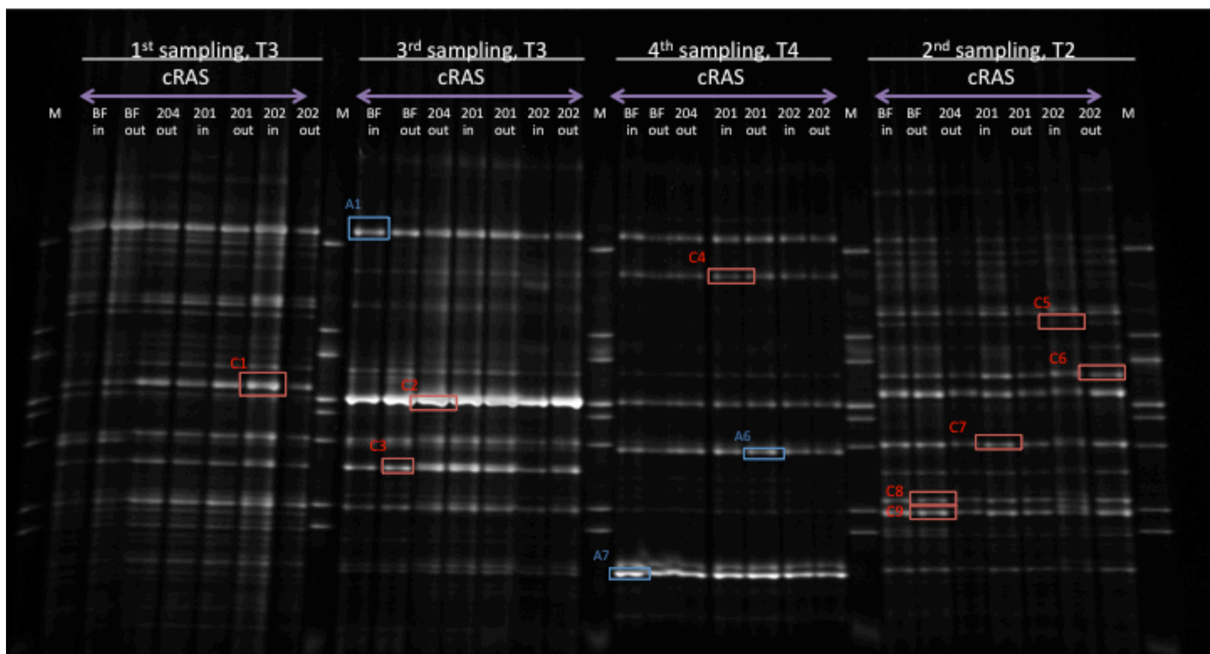

Figure 3.9: DGGE gel showing PCR-products of the 16S v3 region from water samples obtained from T1, T2, T3 and T4 in mRAS. The samples were collected at the inlet and outlet water (in/out) of the biofilters (BF) and of different replicate rearing tanks (mRAS: 101, 102, 104, cRAS: 201, 202, 204). M = markers. Bands excised from the present gel and subjected to sequence analysis are marked with red squares. Blue squares show bands corresponding to bands that are excised from others gels. Overview of all bands excised is found in table 3.5.

The gel (Fig. 3.9) showed variation in the microbial community structures between the sampling times in cRAS. All sample profiles in the gel were further analysed with NM-MDS ordination based on Bray-Curtis similarities to illustrate any differences/similarities between the community profiles in a coordinate system (Fig. 3.10). The samples from each sampling time were located at different positions in the plot, and suggested that there were changes in the community structures throughout the experiment. Samples taken at T1 and T2 were closer together which indicated smaller fluctuations in the community structures and a more stable water microbiota at the beginning of the experiment. The samples taken at T3 and T4 were further apart and gave reason to assume more variability in water microbiota over time in the system.

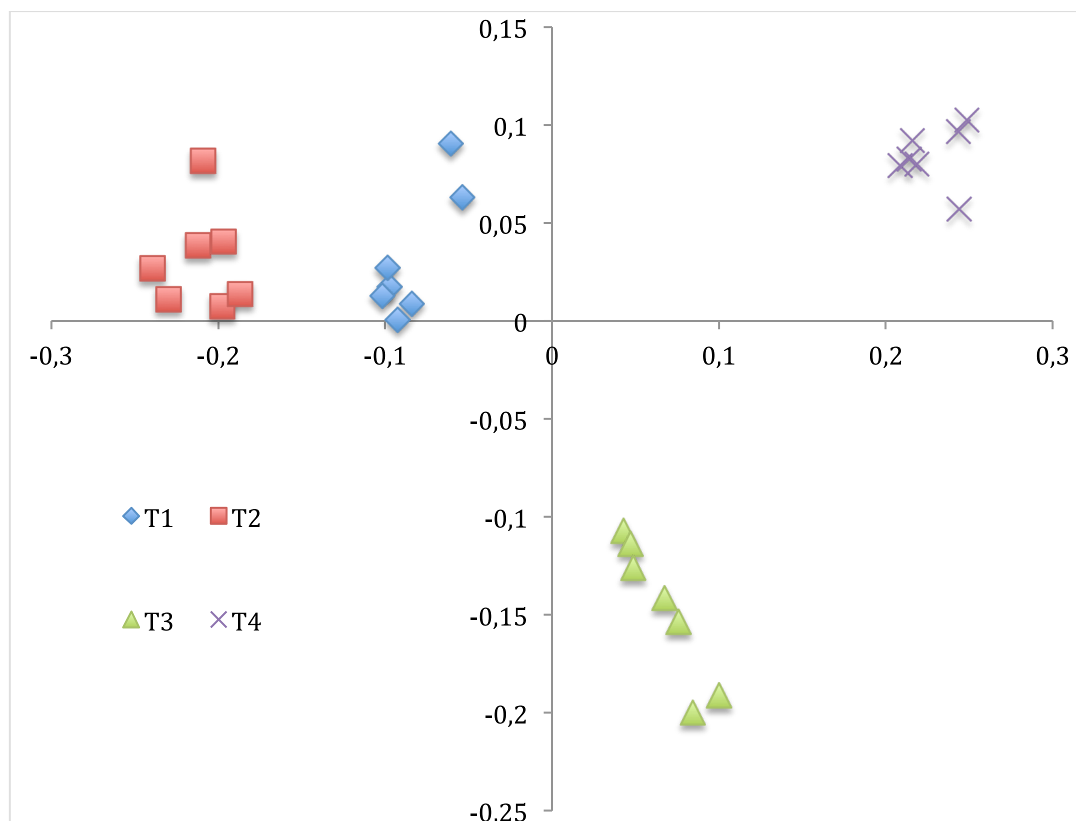

Figure 3.10: NM-MDS plot based on average Bray-Curtis similarities for cRAS water community profiles from T1, T2, T3 and T4.

To compare the microbial community profiles within and between all sampling times, average Bray-Curtis similarities were calculated. The calculations (Fig. 3.11) indicated that the community profiles at T1 and T2 were more similar to each other (0.58) than the other sampling times, which suggested more stable water microbiota at the start of the experiment.

The similarities between T3 and T4 were low (0.35), and could imply fluctuations in the water microbiota over time. The similarity within each sample time was overall high (> 0.75), lowest at the start (T1) and highest at the end (T4) of the experiment (Fig. 3.11). A One-Way PERMANOVA test showed that all the sample times were significantly different from each other ($p > 0.005$).

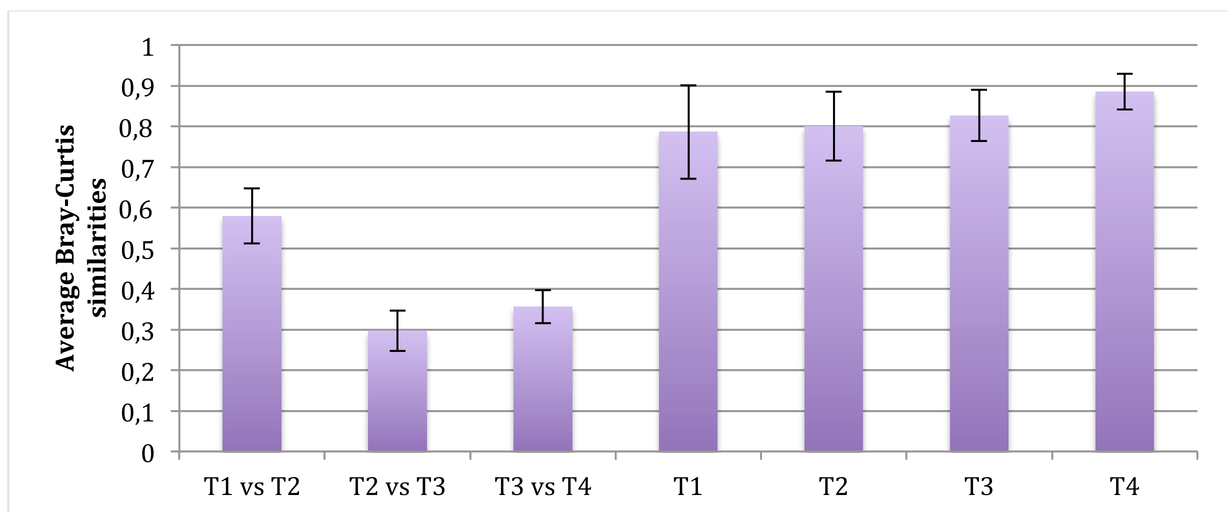

Figure 3.11: Average Bray-Curtis similarities with standard deviations (SD±) for comparison of community profiles in cRAS between and within sampling times T1, T2, T3, T4.

SIMPER analysis was performed to investigate which bands that were primarily accountable for the dissimilarity between the sampling times in cRAS (Table 3.4). It showed that eight bands were responsible for 62.7 % of the dissimilarity, and band C2 was responsible for most of the differences; 17.6 %. This band was very strong in T3 (mean abundance 0.44) with a steep decrease by T4 (0.09). In the beginning of the experiment at T1 and T2 the band was barely present with mean abundances of 0.02 and 0.01, respectively. Band A7 was responsible for 12.5 % of the differences between sampling times, and was only strong at the end of the experiment (T4: 0.32). The bands were excised and sequence analysis revealed that C2 represented Flavobacteriia and at genus level *Persicvirga*, however the confidence threshold was low (table 3.5). A7 represented *Marivita* with a very high confidence threshold (Rhodobacterales, Alphaproteobacteria; table 3.5).

Diversity indices were calculated for cRAS (Appendix B), but also here (as with mRAS) the loading of samples on the gel and/or staining was unequal. The observed changes in diversity showed in the band richness, Shannon's diversity and evenness index could thus not display a real development of the microbial communities.

Table 3.4: Contribution of bands to differences in microbial community structures within cRAS between T1, T2, T3 and T4

Band ID	Contribution %	Cumulative %	Mean abun.* T1	Mean abun. T2	Mean abun. T3	Mean abun. T4
C2	17.6	17.6	0.02	0.01	0.44	0.09
A7	12.5	30.1	0.01	0.00	0.01	0.32
C1	7.4	37.5	0.10	0.16	0.00	0.00
A1	5.7	43.2	0.16	0.02	0.11	0.10
-	5.6	48.8	0.03	0.05	0.03	0.16
C9	4.8	53.6	0.08	0.12	0.05	0.02
C3	4.6	58.2	0.04	0.03	0.11	0.00
A6	4.5	62.7	0.04	0.01	0.02	0.12
C7	3.4	66.1	0.07	0.12	0.05	0.04
C6	3.1	69.2	0.03	0.09	0.03	0.02
-	2.8	72.0	0.02	0.07	0.01	0.01
C5	2.7	74.7	0.05	0.06	0.01	0.00
C4	2.3	77.0	0.01	0.02	0.02	0.06

*Abundance calculated as the peak area of the band divided by the sum of all peak areas for the relevant DGGE community profile. Dash (-) indicates that band was either not excised or no taxonomic classification was obtained by the RDP classifier tool due to low quality sequence.

Table 3.5: Taxonomic classification of DGGE bands based on sequence analysis of excised bands. Confidence threshold given by the RDP Classifying tool. A bootstrap cut-off of 50 % was used due to the short sequencelenghts (> 250 bp).

ID*	Phylum	Class	Order	Family	Genus
A1	Proteobacteria 100 %	Gammaproteobacteria 100 %	Thiotrichales 90 %	Francisellaceae 68 %	Francisella 68 %
A2	Bacteriodetes 69 %	Flavobacteriia 54 %	Flavobacteriales 54 %	Flavobacteriaceae 47 %	-
A3	Bacteriodetes 67 %	Flavobacteriia 52 %	Flavobacteriales 52 %	Flavobacteriaceae 41 %	Joostella 3 %
A4	Proteobacteria 100 %	Alphaproteobacteria 100 %	Rhodobacterales 49 %	Rhodobacteracea 49 %	Pseudorhodobacter 36 %
A5	Proteobacteria 100 %	Alphaproteobacteria 100 %	Rhodobacterales 100 %	Rhodobacteracea 93 %	Roseovarius 49
A6	Proteobacteria 100 %	Alphaproteobacteria 100 %	Rhodobacterales 96 %	Rhodobacteracea 96 %	Sulfitobacter 56 %
A7	Proteobacteria 100 %	Alphaproteobacteria 100 %	Rhodobacterales 100 %	Rhodobacteracea 100 %	Marivita 87 %
B1	Proteobacteria 74 %	Alphaproteobacteria 41 %	Rhizobiales 27 %	Bradyrhizobiaceae 9 %	Blastobacter 6 %
B2	Bacteriodetes 61 %	Flavobacteriia 44 %	Flavobacteriales 44 %	Flavobacteriaceae 41 %	Jejuia 5 %
C1	Bacteriodetes 100 %	Flavobacteriia 100 %	Flavobacteriales 100 %	Flavobacteriaceae 100 %	Polaribacter 65 %
C2	Bacteriodetes 95 %	Flavobacteriia 84 %	Flavobacteriales 84 %	Flavobacteriaceae 84 %	Persicivirga 30 %
C3	Proteobacteria 100 %	Alphaproteobacteria 98 %	Rhodobacterales 100 %	Rhodobacteracea 100 %	Roseovarius 53 %
C4	Bacteriodetes 100 %	Flavobacteriia 100 %	Flavobacteriales 100 %	Flavobacteriaceae 100 %	Polaribacter 92 %
C5	Proteobacteria 78 %	Gammaproteobacteria 66 %	Thiotrichales 13 %	Piscirickettsiaceae 13 %	Piscirickettsia 13 %
C6	Proteobacteria 63 %	Betaproteobacteria 23 %	Rhodocyclales 6 %	Rhodocyclaceae 6 %	Sterolibacterium 4 %
C7	Proteobacteria 69 %	Alphaproteobacteria 30 %	Sneathiellales 2 %	Sneathiellaceae 2 %	Oceanibacterium 2 %
C8	Proteobacteria 98 %	Alphaproteobacteria 87 %	Sneathiellales 15 %	Sneathiellaceae 15 %	Oceanibacterium 14%
C9	Proteobacteria 99 %	Alphaproteobacteria 83 %	Sneathiellales 25 %	Sneathiellaceae 25 %	Oceanibacterium 15 %

*ID shows band numbers.

A = figure 3.2, section 3.2.1. **B** = figure 3.6, section 3.2.2, **C** = figure 3.9, section 3.2.3.

3.3 Effect of membrane in water treatment on the composition of gut microbiota in post-smolt

3.3.1 Comparison of microbial communities in water and gut microbiota within and between cRAS and mRAS

To study the effect of membrane filtration on the gut microbiota of the post-smolt reared in the system, a DGGE was run to compare the microbial community composition in gut- and water microbiota between mRAS and cRAS from T4.

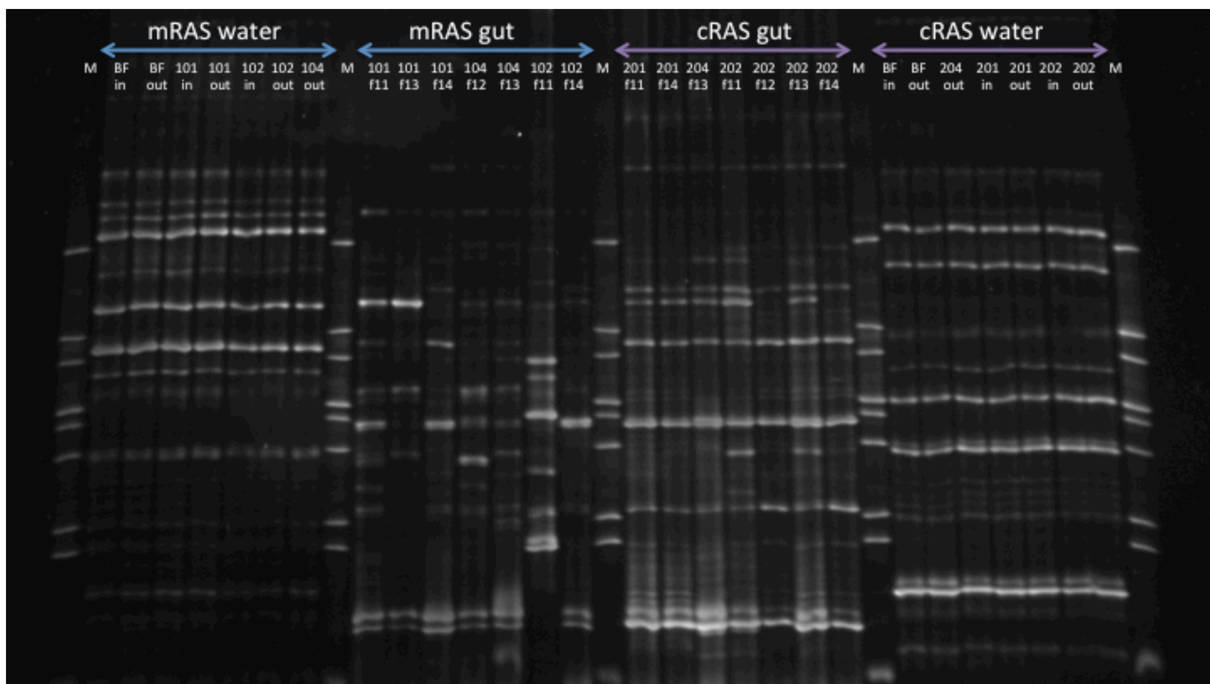

Figure 3.12: DGGE gel showing PCR-products of the 16S v3 region from water and gut samples obtained at T4 in mRAS and cRAS. The water samples were collected at the inlet and outlet water (in/out) of the biofilters (BF) and different replicate rearing tanks (mRAS: 101, 102, 104, cRAS: 201, 202, 204). The gut samples were collected from different fish (f11, f12, f13, f14) in the replicate rearing tanks. M = markers.

A DGGE gel (Fig. 3.12) with PCR products represented by water and gut microbial communities in both cRAS and mRAS at T4 showed clearly differences in community structures between water and gut in both systems. The band profiles that represented the gut samples in mRAS displayed especially high variation among individuals across and within rearing tanks. Ordination by NM-MDS based on Bray-Curtis similarities was performed on all profiles in the gel to illustrate any variation of the samples in a coordinate system (Fig. 3.13). The plot suggested that the microbiota was different between mRAS and cRAS due to

the clustering of the samples depending on system. Gut and water samples within both systems were clearly separated which suggested that the gut and water microbiota were different. The plot further indicated that water microbiota differed between mRAS and cRAS, thus corroborating the results describes above (c.f. section 3.2).

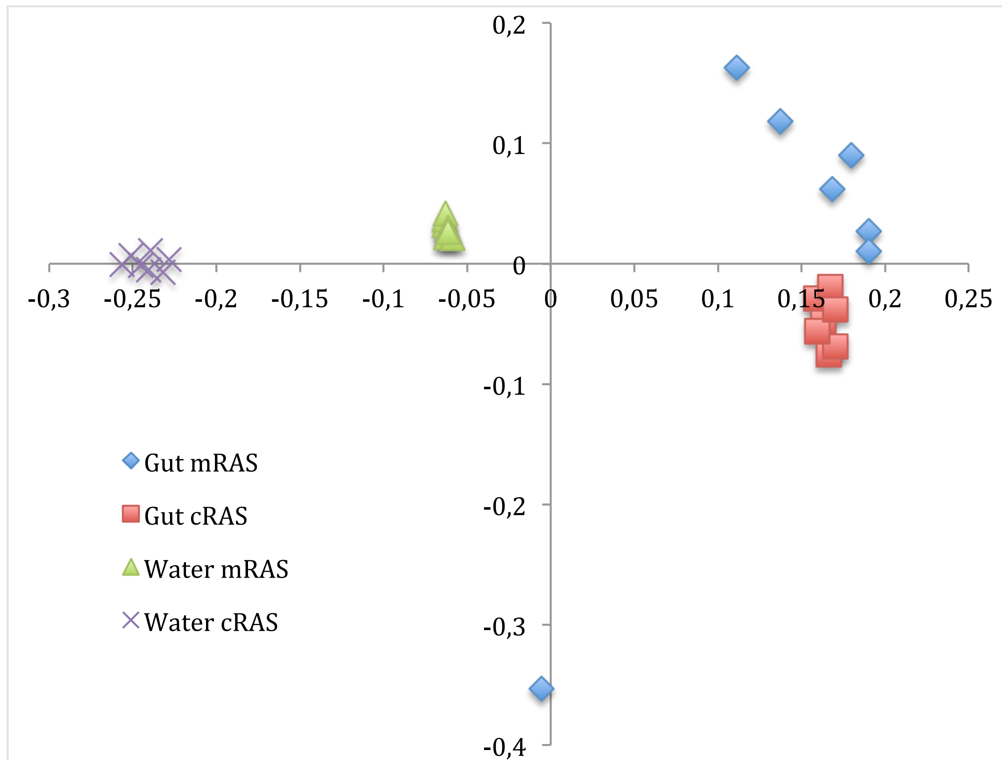

Figure 3.13: NM-MDS plot based on average Bray-Curtis similarities for water and gut samples taken from mRAS and cRAS at T4.

For further comparison of the microbial community profiles in the water and gut samples, average Bray-Curtis similarities were calculated within and between the samples of each system (Fig. 3.14). The similarities of community profiles from gut samples were low between mRAS and cRAS (> 0.4), which suggested that the gut microbiota of mRAS fish and cRAS fish were different. Gut and water microbiota within mRAS and cRAS were highly different when compared to each other, especially the community structures of gut and water in cRAS (0.05). A One-Way PERMANOVA test confirmed that the community profiles between all the samples were significantly different from each other (p > 0.0005)

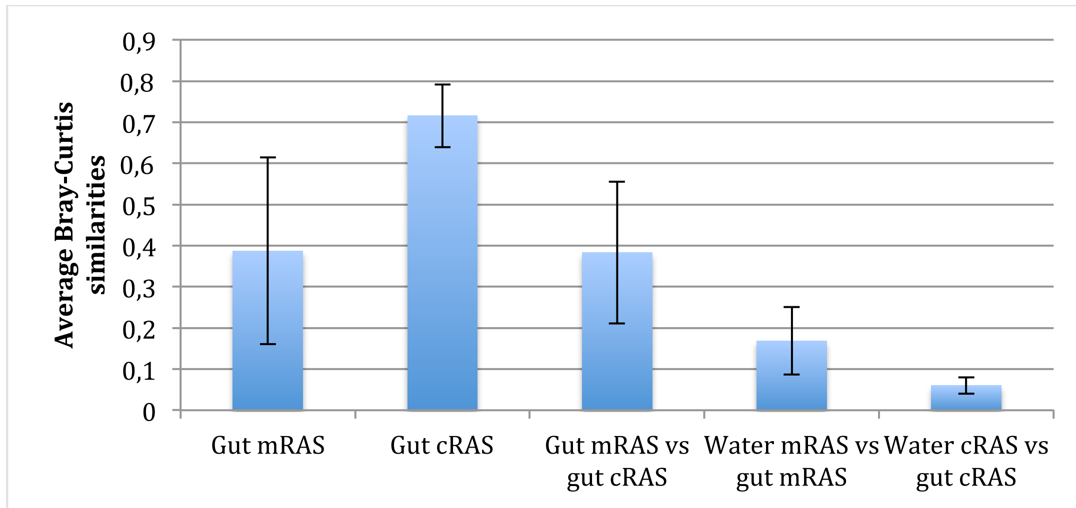

Figure 3.14: Average Bray-Curtis similarities with standard deviations (SD ±) for comparison of microbial community profiles in gut and water samples from mRAS and cRAS at T4.

4. Discussion

4.1 Evaluation of methods

The PCR/DGGE method was chosen to study the microbial community structures of water and gut samples from the two RASs. This method is well suited to get an overview of the diversity of the microbial communities present, and the method is relatively quick and inexpensive. As several PCR products can be compared simultaneously, one can investigate whether or not there are temporal variations or differences between samples from different systems. However, the method displays some limitations. The polymerase used in PCR may contain bacterial DNA that can be amplified together with the bacterial sample DNA, especially if many cycles of PCR are needed (Balázs *et al*., 2013). This may cause inaccurate results, and negative samples should always be included in PCR to exclude this event. It is not easy to create the same running conditions for each gel and the denaturing gradient may differ, thus the band patterns can vary and comparisons between gels should be avoided (Muyzer and Smalla, 1998). This also limits the amount of samples that can be compared to maximum 30. In addition, several 16S rDNA molecules can denature at the same locations in the gel. This creates an intensive band that can be interpreted as one band, which gives false impression of diversity. Some bacterial species may have multiple copies of the 16S rRNA-gene, which will result in more bands present in the gel than there are species and give an overestimation of the microbial diversity of the sample (Malik *et al*., 2008). However, this is also a possible source of error in all 16S rRNA-PCR based methods, also 16S rDNA amplicon sequencing and not just DGGE. The analyses of the DGGE gels with Gel2K involved a lot of manual processing, especially if the band richness of the samples were high. The program was not capable of accurately separate between the bands of each sample and identify all the bands of one species, and some bands were not detected. These mistakes were corrected manually, and could be a source of error. It was challenging to get adequate amounts of PCR product from the 16S rRNA genes, and several rounds of PCR were needed. Even so, after analysing some of the DGGE gels it became apparent that either the staining of the gel was unequal or it was loaded unequal amounts of PCR product on the DGGE gel. Thus, for samples where less PCR product was loaded on the gel, it was probable that the diversity was underestimated.

The DGGE method is not optimal for classifying microbial communities taxonomically. The sequences obtained from the excised bands can be of varying quality, making it harder to obtain a taxonomical classification with high confidence threshold. Furthermore, it is limited how many bands that are possible to obtain sequences from, and more easy to sequence and classify the strongest bands. High throughput sequencing like Illumina is better suited to obtain more accurate taxonomical data of the bacteria in the samples, however these methods are more expensive and require thorough bioinformatical analyses (MacLean *et al*., 2009). Despite all the drawbacks just discussed, the use of DGGE in this experiment answered the questions that were asked regarding the effect of membrane filtration on the microbial community structures of rearing water and was a suitable method. The results from the sequence analysis were not optimal, however the taxonomical classification that was obtained gave some information on the bacteria that could be present in mRAS and cRAS.

4.2 Effect of membrane filtration on particles and bacterial concentrations of system water

When comparing the bacterial concentrations in system water between mRAS and cRAS, the increase of bacterial concentrations throughout the experiment was clearly higher for the system without membrane filtration (Fig. 3.1). This shows that the membrane significantly lowered the bacterial concentration in the system water of mRAS.

Measurements of nitrification efficiency and total suspended solids (TSS) for samples taken in this experiment were performed by Holan *et al*., (2016). The nitrification efficiency was higher in mRAS than cRAS throughout the experiment, and the same trends could be observed for total suspended solids (TSS), where the solid concentration was significantly higher in cRAS compared to mRAS at all sampling times. In the work for this thesis, the microbial community structures of the nitrifying bacteria in the biofilter biofilm carriers were not studied. Removal of particles could result in reduced substrate for heterotrophic bacteria, and lower levels of organic material were observed for mRAS (Holan *et al*., 2016). This could pose for reduced heterotrophic growth in the biofilter, and could lead to increased share of nitrifying bacteria in the biofilter and increased nitrification efficiency (Michaud *et al*., 2006). It would be interesting to investigate if this could be another positive effect of membrane filtration, not only did the membrane remove bacteria, but also some of the substrate for the bacteria.

4.3 Effect of membrane filtration on water microbiota

Comparison of microbial community structures between mRAS and cRAS revealed that the water microbiota in the two systems were significantly different from each other (Fig. 3.3, 3.4). Furthermore, the band richness and Shannon's diversity index were significantly higher in mRAS than in cRAS (Fig 3.5 A & B), showing that membrane filtration had an effect on the alpha diversity of the water microbiota. Previous studies performed on marine larvae reared in RAS with membrane filtration have shown changes in microbial community structures and decreased levels of bacteria, organic matter in the rearing water and increased survival of the larvae (Holan *et al*., 2014a, Wold *et al*., 2014). Membrane filtration can influence the microbial community structures in to ways: Directly by removal of the bacteria, and indirectly by removal of substrate for the bacteria. Removal of organic matter present in the water thus might have decreased the potential of opportunistic blooms by lowering the bacterial substrate. Attramadal *et al*., (2012a) have shown an increase in survival of marine larvae using RAS as opposed to FTS, and suggests that it is due to the more K-selected microbial matured rearing water in RAS, which is considered more stable and favourable for cultivation of marine species (Skjermo *et al*., 1997). Membrane filtration may also pose for selection of K-strategists as the reduction of organic matter results in more limited resources for r-strategists. As the alpha diversity of the water microbiota in mRAS was higher than in cRAS, this implies that the water in mRAS could be more K-selected and matured, since high diversity correlates with matured water and low diversity with water containing more dominant r-strategists (Vadstein *et al*., 1993).

In this experiment, Holan *et al*., (2016) found that there were no differences in growth and survival between the post-smolt reared in mRAS and cRAS, and to the best of the knowledge of the author, there have been no other previous studies with post-smolt and membrane filtration. From previous studies (Attramadal *et al*., 2014, Holan *et al*., 2014a, Verner-Jeffreys *et al*., 2004) it seem like larvae are more susceptible to the microbial changes in the rearing environments than adult fish, however knowledge on the potential effect of microbial environments in the cultivation of salmon fry, smolt and post-smolt are limited and should be further studied. The fish in this experiment were not transferred to seawater, which is known to give high mortality rates of smolts. It cannot be excluded that fish reared in mRAS results in more robust smolt that are better fit for the sea transfer, however this was not tested in this

study. The smolts in this system were reared at a salinity around 12 ppt (table 2.1) and as salmon live their life after the juvenile stages in the sea (30-35 ppt), the microbes associated with the fish probably changes as different bacteria thrive in fresh, brackish and salty waters (Logares *et al*., 2009). How the present microbes interacting with the smolts are adapting to the new selection pressure in the salty water after sea transfer is unknown. The microbial community structures in the rearing water have shown to be different in systems where smolts have been reared at different salinities (Bakke *et al*., 2016), and it further implies that salinity is an important physicochemical parameter that influences the water microbiota.

DNA sequencing of the DGGE bands and SIMPER analysis comparing the community profiles of mRAS and cRAS samples (table 3.2) revealed that the bacteria that were shown to be most abundant in the systems at the two last sampling times belonged to the Flavobacteriales order. Even though the confidence threshold was low (table 3.5), the RDP classifier suggested that the systems selected for two different Flavobacteriaceae genera, *Persicivirga* in cRAS and *Joostella* in mRAS. The presence and abundance of this class of bacteria have been shown to be tightly correlated to resource availability (Eiler and Bertilsson, 2007). Both mRAS and cRAS contain organic material (feed, feces, etc.) and thus probably enough also in the system with membrane filtration for the Flavobacteriaceae to thrive. Flavobacteriaceae are of the Bacteriodetes phylum, and some bacteria of this phylum have been considered K-strategists (Fuchs et al., 2000, Sheik *et al*., 2014). When comparing the bacteria between the systems at the end of the experiment (table 3.2) it seemed like cRAS overall displayed a higher abundance of Rhodobacterales, which is an order of Alphaproteobacteria. mRAS displayed an overall higher abundance of Flavobacteriaceae. Alphaproteobacteria have been considered to be r-strategists (Fuchs et al., 2000, Sheik *et al*., 2014), thus it is interesting that these bacteria are more abundant in cRAS and that the Flavobacteriaceae (Bacteriodetes) were more abundant in mRAS. This could further support the assumption of more stable K-selected water in mRAS. However, these are general considerations. There are pathogenic opportunistic bacteria among the Flavobacteriaceae, and K-strategists among the Alphaproteobacteria.

4.4 Effect of membrane filtration on temporal community dynamics of rearing water

When comparing the microbial community structures from different sampling times within mRAS and cRAS, there were clearly temporal changes in the water microbiota between the sampling times in both systems (Fig. 3.7, 3.8, 3.9, 3.10). The similarities between samples in different units of the RASs within each system at each sampling time were very similar. This suggests that the microbial community structures were relatively stable throughout the system units, water from fish tanks and biofilters displayed similar water microbiota. Both systems were RAS and it is expected that these systems select for stable microbial matured water as HRT is long (4.1 days), and the water is not constantly exchanged as in FTS (Attramadal *et al*., 2014). Still, temporal changes are expected in RAS due to changes in operational conditions such as organic loading in the system. The mRAS seemed to have a more stable water microbiota over time than the cRAS, particularly at the last sampling times. Even though the microbial community structures were significantly different from each other at all sampling times, Bray-Curtis similarities and ordination by NM-MDS showed that the two last sampling times were more similar to each other than the two first in mRAS (Fig. 3.7, 3.8) and similarities between at the two last sampling times in cRAS were low (Fig 3.9, 3.10).

Sequence analysis of the DGGE bands showed the taxonomy for some abundant bacteria in mRAS and cRAS (table 3.3 and 3.4). In mRAS, *Joostella* (Flavobacteriales) was not observed the two first sampling times, but became highly abundant at the two last sampling times as just discussed. There are some bacteria of the Flavobacteriaceae family that are known fish pathogens, one known species is *Flavobacterium psychrophilum* that have caused a lot of damage in fish farming (Wu *et al*., 2015). Thus, some strains in the Bacteriodetes phylum could also be pathogenic and possible r-strategists. *Francisella* (Gammaproteobacteria) displayed high abundance at the beginning of the experiment, and the system selection pressure might have changed as the abundance decreased by the two last sampling times. Gammaproteobacteria are also considered to be r-strategists (Fuchs et al., 2000, Sheik *et al*., 2014), and the decreased abundance of this possible r-strategist at the end of the experiment in mRAS could imply that the water became more K-selected. *Francisella* is also a genus which includes pathogenic bacteria shown to cause disease in fish farming (Birkbeck *et al*., 2011). However, this does not mean that the Flavobacteriaceae and *Francisella* found in this experiment are pathogenic, and none of the classified bacteria in this experiment could be

identified on species level. As most fish-pathogenic bacteria belong to the category of opportunistic pathogens (Vadstein *et al*., 2004), many of them are not pathogenic until there are conditions weakening the fish allowing pathogenic thrive.

In cRAS the most abundant bacterial taxa at T3 appeared to be the *Persicvirga (*Flavobacteriaceae) but the abundance decreased drastically by T4. The only reported change in the rearing conditions at this time was the reduction of feeding in both systems at T3 (Holan *et al*., 2016). This could also be the cause of the pronounced decrease of *Persicvirga* at T4, thus this Flavobacteriaceae could be very affected by the availability of resources. The second most abundant band represented *Marivita (*Rhodobacteracea, Alphaproteobacteria), and this band was barely present at the three first sampling times, but very abundant at T4. Representatives form this bacterial genus have been isolated from marine waters and have also shown to utilize carbon sources (Hwang *et al*., 2009). *Francisella* (Gammaproteobacteria) was also relative abundant throughout the experiment (table 3.4), compared to mRAS where it was more abundant at the beginning of the experiment than the end (table 3.3).

To sum up, the higher diversity of the microbial communities in mRAS water at the end of the experiment could imply that the water was more K-selected than cRAS. In addition, since the microbial community structures at the two last sampling times were more similar to each other in mRAS than cRAS, it could further imply more stable water microbiota in mRAS. The sequence analysis showed that mRAS displayed higher abundance of Flavobacteriaceae than cRAS, even though this family of bacteria have been shown to be tightly correlated to resource availability, and there were more organic material in cRAS than mRAS (Holan *et al*., 2016). As discussed above, it is difficult to make assumptions about the degree of r-/K-selection based on the abundance of specific taxa. Notwithstanding, the fact that the abundance of Gammaproteobacteria, considered to be r-strategic, decreased over time in mRAS, might support the assumption of more K-selected water in mRAS over time.

4.5 Effect of membrane filtration on the gut microbiota of post-smolt

When comparing the gut microbiota of mRAS and cRAS reared post-smolt there were significant differences in their microbial community structures (Fig 3.13, 3.14). The gut samples were only collected at the end of the experiment. The microbiota of developing marine larvae changes with age, and can be explained by a selection pressure in the gut as the digestive systems develop (Bakke *et al.*, 2015, Olafsen, 2001). The fish used in this experiment were post-smolt, and the gut microbiota is believed to become more stable after first feeding at the juvenile stage (Romero and Navarrete, 2006, Hansen and Olafsen, 1999). Thus, the post-smolt would be expected to have developed a stable microbiota and would be less affected by external factors. The growth rate and survival of the post-smolt were the same for both systems, and the rearing conditions were identical for mRAS and cRAS throughout the experiment (HRT, feed etc.), therefore it seems likely that it was the effect of the membrane that changed the selective pressure on the microbes in the system inducing changes of the gut microbiota. There could be two possible mechanisms to cause changes in gut microbiota: 1) Different water microbiota that causes different gut microbiota, the bacteria in the water could affect the colonization of the gut. The bacterial communities in the water have been correlated to changes in the gut microbiota of tilapia larvae (Giatsis *et al.*, 2015) and the microbiota of developing cod larvae (Bakke *et al.*, 2015), suggesting that the microbes present in the environment does effect the microbiota associated with the reared species. 2) Stress have been known to cause changes in the gut microbiota of rats (O'Mahony *et al.*, 2009) and shown to alter the bacterial concentrations of the intestinal lining and feces in Atlantic salmon (Olsen *et al.*, 2002). Membrane filtration has reduced the concentrations of the colloidal fraction and fine suspended solids of the particles, and these particles have shown to cause stress on the cultivated species. Thus, the reduction of particles could have reduced the stress level of the smolts that explains the differences in gut microbiota. Also the assumption of more K-selected and microbial matured water in mRAS could be the reason for the differences in gut microbiota between the two systems.

It has not been previously shown for fully developed fish that rearing water affects the gut microbiota. Feed has been anticipated to be the one of the most important determinants for gut microbiota (Llewellyn *et al.*, 2014, Nayak, 2010), but the results of this experiment shows that water also affect the gut microbiota. The composition of gut microbiota is important for health, thus it is possible that water treatment could affect fish health through the gut microbiota. However, for this experiment there were few samples and only one sampling

time. In the PCR/DGGE-analysis it was very challenging to obtain good PCR-products from the gut samples. Samples of feces have shown to contain inhibitors for PCR (Lantz *et al*., 1997), which could be the situation here. Thus, only a limited number of samples were studied, and the number of samples and sampling times should be increased for further studies.

The first colonization of the larvae gut can be influenced by random processes, thus the bacterial species that settle in the gut can be by chance (De Schryver and Vadstein, 2014). Regarding the high variability among individuals in mRAS (Fig. 3.12 3.13, 3.14), if the water in this system was more K-selected, random events could be thought to have more impact on the colonization, thus explaining the differences of gut microbiota among the individuals. A microbial environment with higher abundances of r-strategist could be thought to more easily settle in the gut as these bacteria usually are pathogenic and dominant (Vadstein *et al*., 1993), thus the gut microbiota among the individuals in cRAS are more similar. However, it must be taken into consideration that the theories of colonization proposed by De Schryver and Vadstein (2014) were in larvae and the first colonization. The fish in this experiment were fully developed post-smolt with already colonized guts, and it is difficult to know if these ecological principles are applicable here.

4.6 Future work and perspectives

Most research on the effects of water treatment systems of fish health in aquaculture systems has been focused on marine larvae, but studies on salmon fry are lacking. More studies on salmon fry and smolt/post-smolt and how the microbes in the environment and water treatment processes affect the microbiota of the fish should be performed. Studies on smolt before and after sea transfer should also be conducted to investigate the dynamics of the microbial communities associated with the fish when transferred to seawater. The nitrification efficiency was lower in cRAS compared to mRAS and the microbial community compositions of the biofilter discs should have been studied. If there were more autotrophic bacteria present on the discs from mRAS than cRAS, it would further emphasize the benefit of membrane filtration on the nitrification efficiency. Gut samples from more sampling times should have been collected, and the temporal dynamics of gut microbiota should have been studied. Stress could be a possible explanation for the differences in gut microbiota between mRAS and cRAS. For future studies, stress responses in the fish could be measured with

qPCR on the gene expression level in the fish, and see if the levels are higher in fish reared in systems without membrane filtration. Illumina sequencing should be applied to obtain more high-resolution taxonomical information on the microbial communities and their temporal dynamics.

RAS could be a step towards making the aquacultural industry more sustainable, with reduction of water and decreased environmental influences. If more fish farming could be moved up on land, not just the first fresh water stages but perhaps the full life cycle, the environmental impacts would drastically decrease. More research on RAS and the microbial aspect of water treatment can make rearing conditions more favourable yielding lower mortality rates and fish of high quality. Today the technology is still developing and the enormous amounts of water and space needed for cultivation of adult fish on land is very challenging. Nonetheless, RAS is an excellent starting point and more research could lead to more robust cultivated species with lower water consumption and environmental impacts, leading to a more sustainable way of feeding the growing population.

5. Conclusions

- Membrane filtration significantly lowered the bacterial concentrations in the rearing water

- The microbial community compositions in mRAS and cRAS were significantly different from each other

- The water microbiota in both systems changed throughout the experiment, however the microbiota of mRAS was more stable at the last sampling times than that of cRAS

- The microbial community compositions of the gut from mRAS and cRAS post-smolt were significantly different from each other, thus the membrane filtration affected the gut microbiota

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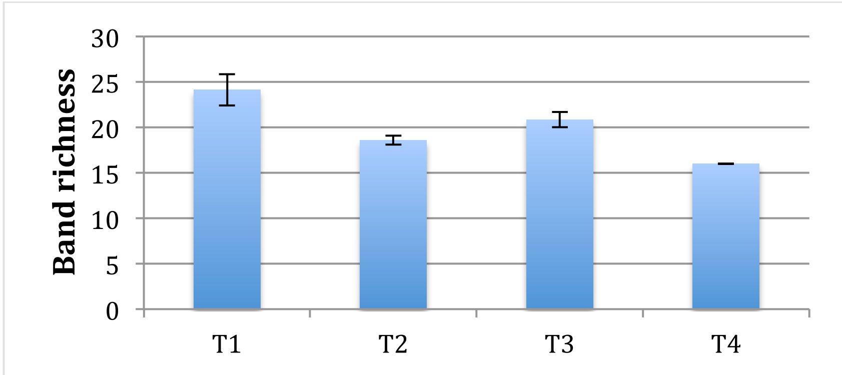
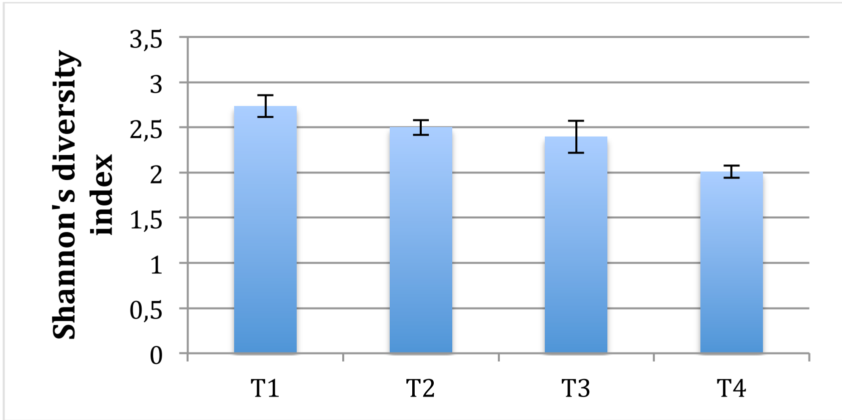
Appendix A Average diversity indices for mRAS

A



B



C

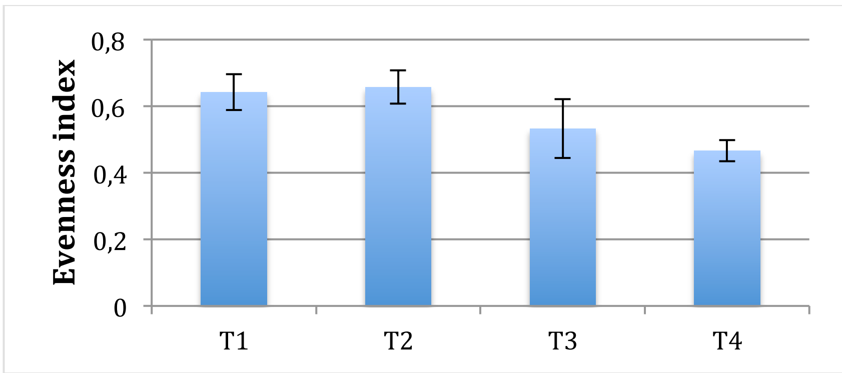
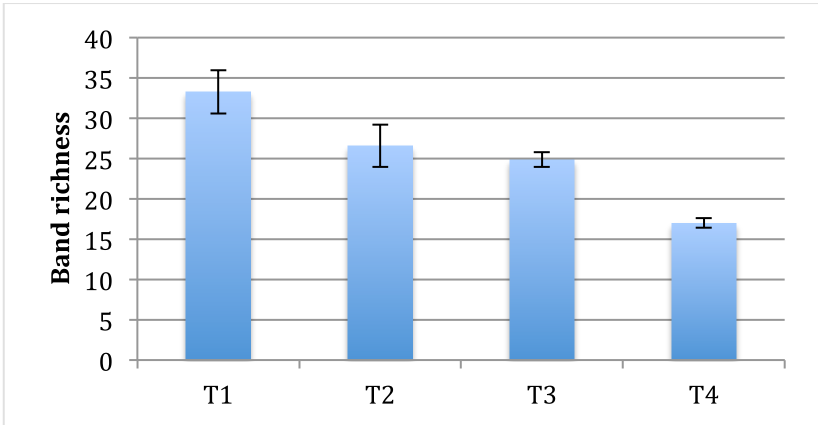


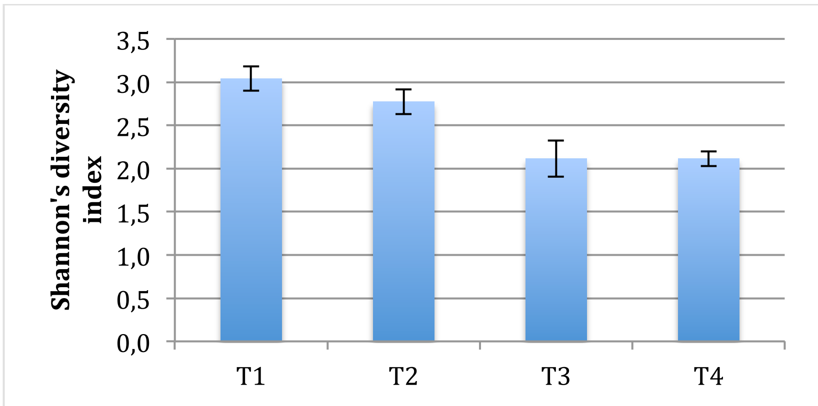
Figure A.1: Average diversity indices with standard deviations calculated from the microbial community DGGE profiles (Fig 3.6) for each sample of mRAS. **A**: Band richness, **B**: Shannon's diversity index, **C**: Evenness index

Appendix B Average diversity indices for cRAS

A



B



C

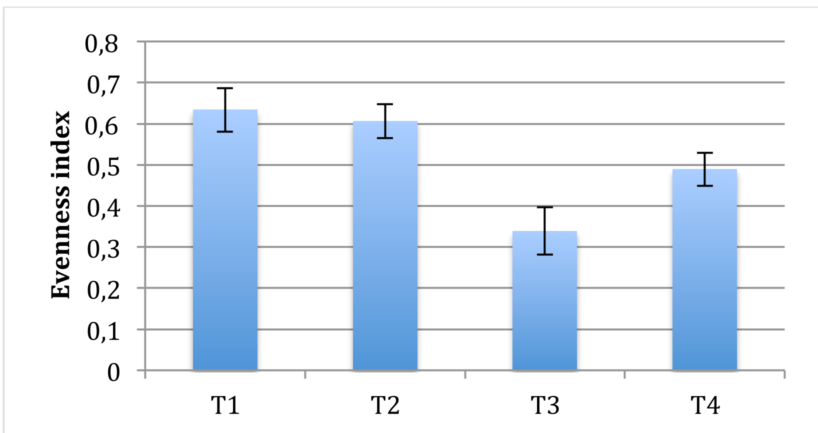


Figure B.1: Average diversity indices with standard deviations calculated from the microbial community DGGE profiles (Fig 3.9) for each sample of cRAS. **A**: Band richness, **B**: Shannon's diversity index, **C**: Evenness index

Appendix C DNA extraction protocol

Experienced User Protocol

Please wear gloves at all times

1. To the **PowerBead Tubes** provided, 0.25 grams of soil sample.
2. Gently vortex to mix.
3. **Check Solution C1.** If **Solution C1** is precipitated, heat solution to 60°C until dissolved before use.
4. Add 60 µl of **Solution C1** and invert several times or vortex briefly.
5. Secure **PowerBead Tubes** horizontally using the MO BIO Vortex Adapter tube holder for the vortex (MO BIO Catalog# 13000-V1) or secure tubes horizontally on a flat-bed vortex pad with tape. Vortex at maximum speed for 10 minutes.
   Note: If you are using the 24 place Vortex Adapter for more than 12 preps, increase the vortex time by 5-10 minutes.
6. Make sure the PowerBead Tubes rotate freely in your centrifuge without rubbing. Centrifuge tubes at 10,000 x *g* for 30 seconds at room temperature. **CAUTION:** Be sure not to exceed 10,000 x *g* or tubes may break.
7. Transfer the supernatant to a clean **2 ml Collection Tube** (provided).
   Note: Expect between 400 to 500 µl of supernatant. Supernatant may still contain some soil particles.
8. Add 250 µl of **Solution C2** and vortex for 5 seconds. Incubate at 4°C for 5 minutes.
9. Centrifuge the tubes at room temperature for 1 minute at 10,000 x *g.*
10. Avoiding the pellet, transfer up to, but no more than, 600 µl of supernatant to a clean **2 ml Collection Tube** (provided).
11. Add 200 µl of **Solution C3** and vortex briefly. Incubate at 4°C for 5 minutes.
12. Centrifuge the tubes at room temperature for 1 minute at 10,000 x *g.*
13. Avoiding the pellet, transfer up to, but no more than, 750 µl of supernatant into a clean **2 ml Collection Tube** (provided).
14. Shake to mix Solution C4 before use. Add 1200 µl of **Solution C4** to the supernatant and vortex for 5 seconds.
15. Load approximately 675 µl onto a **Spin Filter** and centrifuge at 10,000 x *g* for 1 minute at room temperature. Discard the flow through and add an additional 675 µl of supernatant to the **Spin Filter** and centrifuge at 10,000 x *g* for 1 minute at room temperature. Load the remaining supernatant onto the **Spin Filter** and centrifuge at 10,000 x *g* for 1 minute at room temperature.
    Note: A total of three loads for each sample processed are required.
16. Add 500 µl of **Solution C5** and centrifuge at room temperature for 30 seconds at 10,000 x *g.*
17. Discard the flow through.
18. Centrifuge again at room temperature for 1 minute at 10,000 x *g.*
19. Carefully place spin filter in a clean **2 ml Collection Tube** (provided). Avoid splashing any **Solution C5** onto the **Spin Filter**.
20. Add 100 µl of **Solution C6** to the center of the white filter membrane. Alternatively, sterile DNA-Free PCR Grade Water may be used for elution from the silica Spin Filter membrane at this step (MO BIO Catalog# 17000-10).
21. Centrifuge at room temperature for 30 seconds at 10,000 x *g.*
22. Discard the **Spin Filter**. The DNA in the tube is now ready for any downstream application. No further steps are required.

    We recommend storing DNA frozen (-20° to -80°C). **Solution C6** contains no EDTA. To concentrate the DNA see the Hints & Troubleshooting Guide.

Appendix D Recipes for DGGE solutions

0 % denaturing acrylamide solution:

8 % acrylamide in 0.5 X TAE (per 250 ml):

- 50 ml 40 % acrylamide solution (BioRad)
- 2.5 ml 50 x TAE
- Store at 4 °C, protect form light

80 % denaturing acrylamide solution:

8 % acrylamide, 5.6M urea, 32 % formamide in 0.5 X TAE (per 250 ml):

- 50 ml 40 % acrylamide solution (BioRad)
- 2.5 ml 50 X TAE
- 84 g urea
- 80 ml Deionized formamide
- Stir to dissolve urea
- Store at 4 °C, protect form light
- Solution must be filtered before use

50 x TAE –buffer

Per litre:

- 242 g tris base
- 57.1 ml glacial acetic acid
- 100 ml 0.5 M EDTA (pH 8.0)

Autoclave the buffer

10 % APS (ammonium persulphate)

- 10 g ammonium persulphate dissolved in 100 ml dH_2O
- Sterile filter the solution, distribute in eppendorf tubes (250 µl in each)
- Keep frozen

Appendix E QIA quick PCR purification protocol

Procedure

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

   For example, add 500 µl of Buffer PB to 100 µl PCR sample (not including oil).

2. **If pH indicator I has beein added to Buffer PB, check that the color of the mixture is yellow.**

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

3. **Place a QIAquick spin column in a provided 2 ml collection tube.**

4. **To bind DNA, apply the sample to the QIAquick column and centrifuge for 30–60 s.**

5. **Discard flow-through. Place the QIAquick column back into the same tube.**

   Collection tubes are re-used to reduce plastic waste.

6. **To wash, add 0.75 ml Buffer PE to the QIAquick column and centrifuge for 30–60 s.**

7. **Discard flow-through and place the QIAquick column back in the same tube. Centrifuge the column for an additional 1 min.**

   IMPORTANT: Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.

8. **Place QIAquick column in a clean 1.5 ml microcentrifuge tube.**

9. **To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water (pH 7.0–8.5) to the center of the QIAquick membrane and centrifuge the column for 1 min. Alternatively, for increased DNA concentration, add 30 µl elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge.**

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

   Elution efficiency is dependent on pH. The maximum elution efficiency is achieved between pH 7.0 and 8.5. When using water, make sure that the pH value is within this range, and store DNA at –20°C as DNA may degrade in the absence of a buffering agent. The purified DNA can also be eluted in TE buffer (10 mM Tris·Cl, 1 mM EDTA, pH 8.0), but the EDTA may inhibit subsequent enzymatic reactions.

10. **If the purified DNA is to be analyzed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel.**

    Loading dye contains 3 marker dyes (bromophenol blue, xylene cyanol, and orange G) that facilitate estimation of DNA migration distance and optimization of agarose gel run time. Refer to Table 2 (page 15) to identify the dyes according to migration distance and agarose gel percentage and type.