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Microbial community dynamics in three RAS with different salinities for production of Atlantic Salmon postsmolt

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Abstract

Bacteria in the water interacts with the fish and can affect their health and survival. A stable microbial community in the rearing water can promote a good rearing environment for the fish. The purpose of this study was to examine the microbial community dynamics in three recirculating aquaculture systems (RAS) that were run at different salinities. This was performed by comparing the microbial community structure of samples from biofilm carriers, biofilter water and rearing water that were sampled at different dates over a period of 5 months from the three RAS' that run salinities at 12‰, 22‰ and 32‰.

Total DNA were extracted from the samples and PCR (polymerase chain reaction) was used to amplify the variable region 3 of the highly conserved and universal bacterial gene 16S rRNA. The DGGE (denaturing gradient gel electrophoresis) method was used to analyze the resulting PCR products, and to characterize the microbial communities. The results were analyzed statistically to examine variations between communities of samples from the different systems and dates.

It was found that the different salinities gave different microbial communities, which indicate that the salinity had an impact on the microbial communities in RAS. Both the biofilter and rearing tank water communities showed significant differences when compared between systems and within systems at different dates. The results indicate that the microbial communities of the biofilm carriers were less affected by the salinity than the water communities. The microbial communities in biofilm carriers developed to become more similar to the communities of the biofilm carriers from the other RAS' over time, even though they were sampled from different salinities. The biofilm communities were also found to be more stable than water communities over time.

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Table of contents

1. Introduction

1.1 Aquaculture of Atlantic salmon

The high demand for fish has made aquaculture a necessity, due to overexploitation of the wild stocks at sea (FAO, 2014, Bostock et al., 2010). The Atlantic salmon (*Salmon salar*) is a highly commercial product worldwide and is among one of the biggest export products in Norway (Liu et al., 2011). The Atlantic salmon has a share of 90% of the aquaculture production in Norway, with rainbow trout at 7-8% and Atlantic cod with 2% of the remaining production (Bergheim, 2012). In 2012, aquaculture produced 42.2% of the total amount of fish consumed worldwide. However, the future world depends on aquaculture to provide enough products to feed the rapid growing population (FAO, 2014). An estimated 40% more protein from the sea is needed to meet the demands in year 2030 (Kaiser, 2011).

Atlantic salmon has always been an important food source for Norwegians and there has been a development from fishing wild salmon to high intensity farming. Norway has been a leading producer of Atlantic salmon throughout the history of the salmon industry, with an estimated share of 51% of the total production worldwide in 2010 and an estimated production of 1.2 million tons Atlantic salmon in 2012 (Asche et al., 2013). It has been necessary to increase the intensity of smolt farms to meet the increasing demands (Bergheim et al., 2009). The Norwegian hatcheries produced around 280 million smolt in 2010, compared to 57 million smolt in 1988 (Sandvold and Tveterås, 2014). Applied water treatments, e.g. oxygenation, CO_2 removal, ozonation and UV irradiation has made the intensification in smolt production possible (Bergheim et al., 2009).

The fertilized salmon eggs are transferred from specialized producers to the hatcheries, from either October to December or December to March, where they hatch in fresh water. The alevins get their nutrients from the yolk sac during the first weeks, and are transferred to larger freshwater tanks with daily feeding at 4-8 weeks. This usually takes place in either Recirculating Aquaculture systems (RAS) or traditional flow-through systems (FTS). The first spring after hatching is when the parr normally reach the smoltification stage. The

developmental process from parr to smolt is driven by hormones to enhance their fitness to the marine environment. It involves changes in e.g. osmoregulation, morphology, behavior and physiology (Björnsson et al., 2011). The parr changes color from a dark spotted body to a silver colored body with black fins and the body shape becomes more slender (Hoar, 1976). The Atlantic salmon becomes smolt at 60-100 g after 9 to 18 months in freshwater, and is ready for the transfer to sea water (Sandvold and Tveterås, 2014). However, the smolt size can vary. Due to the optimal conditions in RAS, the smolt grows to sizes of 140 to 170 g before delivery, compared to the farms using FTS, which usually deliver smolt at 50 to 70 g. The relocation of 1-year olds to cages takes place in the spring, while the out of season under-yearlings (9-10 months) is set out in the autumn (Bergheim et al., 2009, Joensen, 2008).

1.1.1 Production of postsmolt

During and after sea transfer, the Atlantic salmon smolt are exposed to new diseases, injuries and stress during the whole process from capture till unloading the stock in sea cages (Iversen et al., 2005). The reduced growth and mortalities mostly occur shortly after sea transfer due to these exposures. Recent change in regulations in Norway allow production of salmon up to 1 kg on land (Ytrestøl et al., 2013). The future trend seems to be to grow the postsmolt in closed containment systems on land, to sizes of 250 to 1000 g. The production of larger smolt can result in better tolerance to sea water transfer, which can reduce the losses at sea. With production of large postsmolt on land, there will be a reduction in production time in sea cages, because of optimal temperatures for growth on land. However, the optimal conditions and the effects of salinity for postsmolt is not yet clear (Ytrestøl et al., 2013, Dalsgaard et al., 2013).

1.2 Recirculating aquaculture system

In recent years, there has been an increase in investments in RAS in Norway, which is land-based systems that treats and reuses water from the fish production. In a future perspective of increasing intensification of smolt production and a growing limitation of water, RAS can be the solution for the Atlantic salmon production in Norway (Terjesen et al., 2013). RAS uses

mechanical treatments to remove solids and biological treatments to change or relocate harmful substances by nitrification, from the recirculated water. The dissolved components treated in RAS consists of nitrogen (N), phosphorus (P) and organic compounds, which originates from uneaten food, metabolic waste and defecation (Piedrahita, 2003).

The waste solids are highly organic, and will be broken down by bacteria that generate the toxic compound ammonia (NH_3) and consume dissolved oxygen if not removed. Drains, mechanical filters or sedimentation tanks can remove the solid waste that settle at the tank bottom. Mechanical filtration in the systems is used to remove the suspended solids in the water. However, the finer solids is harder to remove by filtration. Protein skimmers are efficient for removal of these particle sizes. By producing air bubbles at the bottom of a closed compartment of water, the solids attach to the surfaces of the bubbles and travel to the water surface, where the foam with dissolved solids is removed. Biological filtration in bioreactors have various design, but the principle is to optimize the nitrification process to remove NH_3. The biological filtration is based on adding substrates to biofilm carriers that have large surface areas, e.g. sand, beads and plastic plates, where the bacteria needed for the processes attach and proliferates (Losordo et al., 1998). The biofilters increases the water retention time in the system and allows microbial growth (Attramadal et al., 2012). Oxygenation is needed since the production intensity and oxygen demand in the system is high. Down-flow bubble contractors is one of the options, which can diffuse oxygen through the system. UV and ozonation (O_3) is two choices to disinfect the recirculating water, to prevent outbreak of diseases. The UV sterilization uses UV-lamps contained in pipes that are submerged, since it has to be in close proximity to the microorganisms to irradiate them. Usually, the O_3 is diffused in the water and is destroyed when in contact with microorganisms. The gas needs to be controlled due to its toxic effect in elevated amounts (Losordo et al., 1998).

RAS also makes it possible to control factors as temperature and oxygen levels that can give the fish optimal growth conditions (Sandvold and Tveterås, 2014). RAS has become the preferred system over the conventional FTS that consumes new water. Many Norwegian farmers have in recent years converted their flow through systems into RAS and new farms that are being built are of the RAS design (Kolarevic et al., 2014, Drenstig et al., 2011). It has

been shown that RAS can use 98% less water than FTS, and this large reduction in water is the driving force of the conversion from FTS to RAS. Additionally there are fewer cases of fin damage in the production of Atlantic salmon smolt in RAS (Kolarevic et al., 2014). RAS beholds a more stable and beneficial microbial community than FTS, due to the bacterial growth on the biofilm carriers' surfaces and the waters long retention time (Hess-Erga et al., 2010, Attramadal et al., 2012).

1.2.1 Interaction between fish and microbes in RAS

A high concentration of bacteria surrounds the fish constantly in RAS', and most of them are harmless (*K*-strategists). However, a small portion of the bacteria found in the water phase can be harmful and cause diseases. Opportunistic pathogens (*r*-strategists) are not dependent on a host like the obligated pathogens, but utilize opportunities like favorable physical and chemical environmental changes or weakened hosts, which happens on a regular basis in aquaculture due to stress and the varying organic loading. The harmless bacteria colonize the intestines, gills and mucus of the skin, while the *r*-strategist can infect these sites (Vadstein et al., 2007). The *r*- and *K*-selection term are related to growth rates and the number of individuals in the environment. The *r*-strategists grow quickly in environments with few individuals and high concentration of nutrients (Andrews and Harris, 1986) while the *K*-strategists grows slowly and is highly competitive in high densities of bacteria, close to the carrying capacity (CC) with low access to nutrients per cell (Skjermo and Vadstein, 1999). In RAS, it is possible to select for an environment suitable for the slow growing *K*-strategists, which will dominate and colonize, while outcompeting the *r*-strategists (Vadstein et al., 2004, Attramadal et al., 2012, 2014). By favoring neutral and space-consuming heterotrophs in a *K*-selected environment, the fish is exposed to a diverse community of harmless bacteria. The amount of heterotrophs can be controlled by the supply of organic matter (Attramadal et al., 2012). The RAS also promotes this stable environment, since it mostly consists of reused water that is already microbially matured (Attramadal et al., 2014). It is hypothesized that the mortality rate among larvae in *K*-selected water is low, since there are minor chances to encounter harmful pathogens that can interact with the fish (Attramadal et al., 2014, Salvesen et al., 1999). Bacteria-fish interaction may develop into a beneficial relationship for the host. However, the research on this topic is scarce and should get more attention since the bacteria interacts directly with the fish and can affect their health (Blancheton et al., 2013).

1.2.2 Biological water treatment in RAS

Biofilms main function are the nitrification process during which the dissolved toxic NH_3 is converted to less hazardous substances (Chen et al., 2006). The success and water quality of a RAS highly depends on the nitrification process, maintenance of homeostasis and structure of microbes that are present in the biofilm carriers (Schreier et al., 2010). The bacteria that take part in the nitrifying process are slow growing, but effectively oxidize NH_3 and nitrite (NO_2^-) to the non-toxic product nitrate (NO_3^-) (Rusten et al., 2006). Nitrification consists of a two-step process. The first step is oxidation of NH_3 to NO_2^- that is performed by ammonia-oxidizing bacteria (AOB). The second step is further oxidation of NO_2^- to NO_3^- by nitrite-oxidizing bacteria (NOB) (Sharrer et al., 2007, Sudarno et al., 2011). Further conversion of NO_3^- to nitrogen gas (N_2) is possible by denitrifying bacteria under anoxic conditions. However, to get an efficient denitrification, some RAS have anaerobic conditions with denitrification carriers (Sharrer et al., 2007). The rate of nitrification depends on the concentration of dissolved oxygen, the supply of organic load, salinity, temperature, alkalinity, pH, biomass composition and the concentration of total ammonium nitrogen (TAN) and nitrite (Rusten et al., 2006).

One type of bioreactors used in RAS are the Moving Bed Biofilm reactors (MBBR). The plastic biofilm carriers in the bioreactors move freely by mechanical stirring or aeration in a bioreactor with adjusted volume, depending on their aerobic or anaerobic application. The carriers have large surfaces to fix desired bacteria. The biomass growth takes place in the whole tank volume of the bioreactor (Rusten et al., 2006). The biofilm carriers are able to treat large amounts of water and protect the efficiency of processes by preventing wash-out of bacteria (Nogueira et al., 1998, Kumar et al., 2011). However, it is not only nitrifiers attached to the carriers. A large proportion of bacteria attached to the carriers are heterotrophs, which feed on organic compounds. The heterotrophs have high growth rates, which is an advantage when competing for space and oxygen with the nitrifiers (Leonard et al., 2002, Fdz-Polanco et al., 2000). The loading ratio of organic carbon and inorganic nitrogen (C/N) influences the percentage of heterotrophs in the biofilm carriers (Michaud et al., 2006).

1.2.3 The effects of salinity on microbial communities and fish

The efficiency of nitrification is lower in saltwater than in fresh water. The nitrifiers are sensitive and can be inhibited by the high salt concentrations, which can result in accumulation of nitrogen compounds (Sakairi et al., 1996). When exposed to salt stress there is a decrease in the diversity of bacteria (Moussa et al., 2006). However, the inhibition of nitrifiers can be avoided. By avoiding rapid changes and favoring a controlled adaption, the bacteria adapts to the new concentrations and the inhibition is limited (Dahl et al., 1997). There are varying results in studies of the effect of salinity on denitrifiers and nitrifiers (Sharrer et al., 2007) and the research on nitrification at high salinity is scarce (Aslan and Simsek, 2012). It is difficult to compare results, since there are many varying factors involved in the different experiments involving salt on nitrification (Moussa et al., 2006). There are clear differences in density and microbial communities in biofilters and rearing water between different RAS. Fish species, their microbiota, the amount and type of feed, and the rearing environment affect the microbial communities in the system (Sugita et al., 2005, Schreier et al., 2010).

Salinity can influence the growth and development of fish, from early stages and throughout its life. Different species have different tolerance limits and can have different optimal levels. Usually, marine fish prefer an intermediary concentration of salt, ranging from 8‰ to 20‰, depending on species. The salt influences the activation and inhibition on receptors and hormones. Low salinity lead to a lower energy demand in osmoregulation, compared to exposures to higher concentrations. Many species regulate their ingestion according to the external salinity. The achievement of better growth in intermediate salinity conditions can be due to lower metabolic rates and higher, controlled intake of food (Bœuf and Payan, 2001). The "Optimized Postsmolt Production" (OPP) experiment at Nofima Center for Recirculation in Aquaculture (NCRA) concluded that 12‰ salinity had positive effect on the postsmolt growth along with exercise, compared with the postsmolt exercised in 22‰ RAS and 32‰ RAS. The mortality of postsmolt increased according to salinity, and the salmon reared in 32‰ RAS did not handle the salinity conditions as good as postsmolt coming from the other systems with lower salinities. The removal efficiencies of CO_2 and total ammonia nitrogen (TAN) were higher in 12‰ RAS compared to the efficiencies in the two other systems (Ytrestøyl et al., 2013).

1.3 Analyzing microbial communities

Classifying and analyzing the composition of microbial communities were previously limited, due to old fashion techniques like microscopy and cultivation. These conventional techniques makes it challenging to differentiate bacteria, since only a small percentage of the bacteria can be cultivated. Additionally, the bacteria are small and do not always have external traits. Newer culture-independent methods makes it possible to analyze almost all bacteria by using PCR-based approaches. Short sequences of 16S rRNA have become the preferred gene marker for analyses of bacterial diversity and phylogeny, since it is universal in all bacteria and contains both functionally and highly conserved regions and variable regions (Clarridge, 2004, Nikolaki et al., 2013).

Denaturing Gradient Gel Electrophoresis (DGGE) and Temperature Gradient Gel Electrophoresis (TGGE) makes it possible to study the structure of microbial communities at a genetic level (Muyzer, 1999). Amplification of 16S rRNA creates a population of PCR products with variable sequences, which reflects the bacteria present in the original sample. The DGGE/TGGE methods can separate these sequences. The fragments of the different bacteria can be of varying length, but separates due to different DNA sequences and melting points. The separation of DNA sequences are due to the linear gradient in the gels, which is produced by a mixture of DNA denaturants in DGGE and by temperature in TGGE. Once a DNA fragment in a sample reaches its denaturing or melting position, it will partially melt and reduce the mobility to an eventual halt. The remaining bacterial DNA will continue the migration until they finally melt and stops (Muyzer and Smalla, 1998). In DGGE, a GC clamp is added to one of the PCR primers. The GC-clamp prevents complete denaturation of the double stranded PCR fragment, and the separation becomes more sensitive, to better separate DNA fragments with different DNA sequences (Sheffield et al., 1989). However, a drawback with these methods is the co-migration of DNA fragments that lowers the possibility to get clean sequences from the bands visualized on the gel. This makes the TGGE/DGGE methods poorly suited for obtaining taxonomic information, but appropriate for studying the dynamics and development of communities (Muyzer and Smalla, 1998).

High throughput sequencing techniques are replacing DGGE/TGGE by analyzing larger quantities of DNA sequences at low costs. The high coverage in sequencing makes it suitable

and preferred for taxonomic research (Di Bella et al., 2013). However, there is a large production of data from the high-throughput sequencing that needs to be handled (Di Bella et al., 2013). Tools have been designed to compare communities in multiple samples which can e.g. solve phylogenetic similarities or the community structure, by using diversity indices and total operational taxonomic unit (OUT) (Tringe and Hugenholtz, 2008). The assembly of data can be time-consuming and expensive due to the need of experts. The future trends will be to develop faster and specialized tools for handling all the data, to remove the need for assistance from experts (Di Bella et al., 2013).

Sequence technology apply 16 rRNA PCR products when studying the composition and structure of microbial communities. Fragments of the 16S gene is amplified by using universal PCR-primers. Each sample that is amplified receives a barcode of 10 base pairs. In this way, one can merge multiple samples and analyze the sequences using either pyro-sequencing or Illumina sequencing. Thereafter, one can sort the sequences by using the barcodes to find the samples the sequences comes from (Parameswaran et al., 2007).

Roche 454 sequencing uses the pyrosequencing technique and emulsion-based clonal PCR to amplify the sequences on beads. The beads are transferred to a picotiter plate where each well contains one bead. Each run is based on additions of specific dNTP to the plate and if attached to the sequence it will generate visible light that are detectable (Di Bella et al., 2013). Roche 454 do approximately 1 million readings each run with up to 1000 base pair long readings (eurofinsgenomics.eu). The Illumina technique uses bridge amplification to form clusters of each sequence on a solid glass plate. Presentations of nucleotides takes place in each round and each nucleotide has a specific dye that, when attached to a sequence, sends a corresponding wavelength. Blocking molecules removes the fluorescence to prepare for the next round of nucleotides (Di Bella et al. 2013). The Illumina can do reads from 25 million to 3 billion readings per flow cell, depending on the instrument used (illumina.com).

New high throughput sequencing techniques are already up and coming, giving competition to the sequencing techniques as those described. The prediction is that they will be less costly,

with better accuracy. It will not be necessary to amplify DNA and the samples will be run in real-time, which is less time-consuming (Di Bella et al., 2013, Liu et al., 2012).

2. Aim

The aim of this study was to examine the microbial communities in the rearing water, biofilter water and biofilm carriers of three RAS, which were run with different salinities of 12‰, 22‰ and 32‰, respectively. A PCR-DGGE strategy was used to characterize and compare the microbial communities in and between the different systems.

More specifically the objectives were to:

- Investigate if the different salinities resulted in differing water- and biofilm- communities

- Investigate if the biofilm carriers have different microbial community structure than its corresponding rearing water and biofilter water

- Investigate temporal dynamics of microbial communities within each RAS

3. Material and Methods

3.1 Experimental design

The samples analyzed in this study were collected during the OPP project that was carried out at NCRA, Sunndalsøra. Three semi-commercial RAS, each with 4 rearing tanks (3.3m^3) were used for production of postsmolt from average individual weights of 68.3g ±1.2g (±SD) to final average weights of 725.7g ±69.3g. Two of the systems were same in size, while one was larger in volume. However, the feed load and the rearing conditions were kept similar, as shown in Table 3.1. The system operation specification and main water quality parameters sign are shown in Figure 3.1. Each RAS had four octagonal rearing tanks that had triple outlets; the tank center had a particle trap, sludge collector outside the tank and drain at the sidewall. The water treatments consisted of the mechanical belt filters (Salsnes, Norway) and bioreactors consisting of three chambers (Kaldnes MBBR with Biofilm Chip P, KrügerKaldnes, Sandefjord, Norway) before CO_2 degassing column (AquaOptima). The ozone injection was not used. Thereafter, the water was oxygenized with down flow bubble contractors (AquaOptima) and returned back to the rearing tanks by one inlet to each tank (Kolarevic et al., 2014). Three separate RAS with different salinity were used in this experiment; RAS 1: 32‰ salinity; RAS 2: 22‰ salinity and RAS 3: 12‰ salinity.

Table 3.1: The rearing condition of the three systems throughout the experiment (Ytrestøl et al. 2013)

RAS	Temp °C	pH	CO_2 mg/l	Alkalinity $CaCO_3$ mg/l	% Reused flow	% Water exchange /day	Feedload , % of capacity	Feedload/ water exh (kg/m^3 /day)
12‰	12.4±0.9	7.5±0.2	6.1±1.5	72±20	98.7±0.8	25±7	11.0±3.4	0.44±0.30
22‰	12.1±0.8	7.6±0.1	7.0±0.2	110±26	98.8±0.7	24±6	10.6±3.9	0.49±0.28
32‰	12.6±0.9	7.8±0.1	6.9±0.4	137±33	98.9±0.4	25±2	9.1±3.9	0.46±0.20

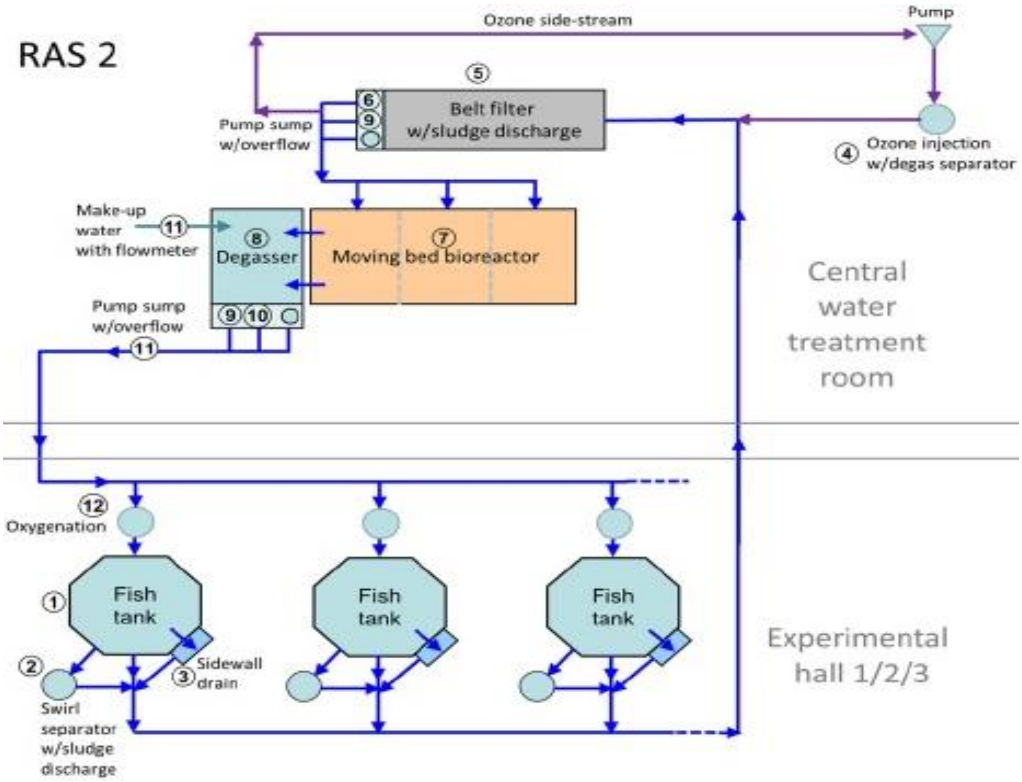


Figure 3.1: Water loop drawing of the RAS 2. Three out of four fish tanks are shown. The drawn components are not up to scale (Terjesen et al., 2013).

3.2 Sampling of biofilm carriers and water

The experiment lasted from 12 October 2012 to 19 May 2013 and the samples were taken from each system at several dates throughout the experiment and at different locations: water (50 ml) from two inlets and four outlets of the rearing tanks, inlet and outlet of the bioreactor, and biofilm carriers. The water samples from the rearing and bioreactor tanks were sampled on 3 January 2013, 26 February 2013 and 7 May 2013, while the biofilm carriers were sampled on 3 January 2013, 19 March 2013 and 14 May 2013. The water samples were stored at -20°C while the biofilm carriers were stored at -80°C. The water samples were thawed and filtrated through a 0.2 µm filter (Dynaguard) at Nofima's laboratory. The filters from the water samples, along with the biofilm carriers, were taken to the NTNU lab for further analyzes.

3.3 Analytical methods

3.3.1 DNA extraction

The biofilm carriers were cut into small fragments and the water samples hollow fibers from the Dynaguard filters were removed to be used. Both types of samples were transferred into PowerBead Tubes supplied with the PowerSoil®DNA Isolation Kit. Fragments of several biofilm carriers from the same system and date were added to the Powerbead Tubes to obtain a representative sample. The protocol for PowerSoil DNA extraction kit in Appendix 1 was followed. In a short description of the kit, the addition and mixing of disruption agents were needed to complete the cell lysis in the PowerBead Tubes containing the samples. The supernatant was transferred to a clean 2 ml collection tube. Thereafter, the solution was purified by several washes with different solutions to remove organic and inorganic matter. The DNA was eluted from the spin column into a clean collection tube with sterile Milli-Q water. Some of the DNA extract were selected to be tested on the Nanodrop ND1000 spectrophotometer (Thermo Fisher Scientific Inc) to quantify the amount of extracted DNA present in the samples. The DNA concentration for biofilm carriers were found to be between 7-14 ng/µl. For the water samples, the DNA concentration were between 1-5 ng/µl.

3.3.2 Polymerase chain reaction

The v3 region of the bacterial 16S rRNA gene was amplified with the 338F-GC and 518R primers. The 338F-GC primer had a GC clamp attached to it to prevent the PCR products from fully denature in the subsequent DGGE (Muyzer et al., 1993). The DNA sequence of the primers are given in Table 3.2.

Table 3.2: The primer sequences used for the amplification of the v3 region of the bacterial 16S rRNA gene and for reamplification/sequencing of DGGE bands.

338F-GC	5′- CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACG GGGGGACTCCTACGGGAGGCAGCAG-3′-
518R	5′-ATTACCGCGGCTGCTGG-3′
338F-GC-M13R	5’-CAGGAAACAGCTATGAC CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACG GGGGG ACTCCTACGGGAGGCAGCAG -3’
M13R	5’-CAGGAAACAGCTATGACC-3’

Several rounds of optimization with various amount of template (DNA extract) and PCR-cycles were necessary to improve the amplification and get sufficient amount of product. The adequate amount DNA template that were added to the reaction mix were 1 µl DNA template for biofilm carriers and 2 µl DNA template for water samples, which corresponded to approximately 9 ng/µl per reaction. The reaction mix made for each reaction also consisted of 2 mM $MgCl_2$, 0.2 mM dNTP, 0.3 µM for both forward and reverse primers and 0.125 µl Taq Polymerase (VWR) with reaction buffer and BSA (Appendix 2).

PCR cycling conditions with a 3 minute long hot start was used to lower the nonspecific annealing of primers to non-target DNA. PCR reactions were run for 31 cycles for samples of biofilm carriers and 40 cycles for water samples under the temperature cycling conditions given in Table 3.3.

Table 3.3: The PCR temperature-cycling program for the amplification of the v3 region of the bacterial 16S rRNA gene.

Cycling step	Temperature	Time
1 (denaturation)	95˚C	3 min
2 (denaturation)	95˚C	30 sec
3 (annealing)	50˚C	30 sec
4 (elongation)	72˚C	60 sec
5 (final step of elongation)	72˚C	10 min
6	10˚C	∞

For each PCR product, 5 µl with 1 µl loading dye were added and analyzed by 1% agarose gel electrophorese in 1 x TAE at 140V for 40 minutes to see if there was sufficient amount of PCR-product, if the product was of correct size and if unspecific products were present (Appendix 2).

3.3.3 Denaturing gradient gel electrophoresis

The DGGE was performed using the Ingeny PhorU system (Ingeny, Netherlands) and their protocol was followed. Two basic solutions were made: a 0% denaturing acrylamide solution and an 80% denaturing acrylamide solution, with urea and formamide as the denaturing agents. To create an 8% polyacrylamide gel with a 35-55% denaturing gradient, a 24 ml solution of both 35% and 55% denaturing were made. Additionally, a 0% "stacking gel" was made, to create a 0% denaturing gradient at the top of the gel. The 80% acrylamide solution had to be filtered to remove urea crystals and Dowex Resin beads that were used to make deionized formamide. To start the polymerization process, 16 µl TEMED and 87 µl APS were added to the solutions when ready to cast the gel. To the 8 ml 0% "stacking gel", 10 µl TEMED and 40 µl APS was added when the solution was ready for use. All the solutions were made after the recipes found in Appendix 2.

A gradient mixer was used when casting the gel to create an even denaturing gradient with the highest denaturing concentration (55%) at the bottom and lowest denaturing concentration

(35%) at the top of the gel. The “stacking gel” was added last, to fill up the remaining top space of the gel with no gradient. The comb was pressed down in the gel and the gel was left to polymerize for two hours before further work. The comb was then gently removed and the gel was transferred to a buffer tank, in a sharp angle to avoid air bubbles at the bottom of the gel, when setting down the gel in the tank. Cables and tubes from the buffer tank were attached to the gel box.

Loading dye (2-4 µl) was added to 5-15 µl of PCR product, depending on the concentration of the PCR products. The samples were applied to the wells along with a DGGE marker. The 5-6 outermost wells were avoided due to the “smiling effect”. The voltage was turned and the electrophoresis was run in 0.5 x TAE at 100V and 60˚C. A current of 23-27 mA at 100V in a startup was expected. Lower mA could be due to air bubbles beneath the gel, which could ruin the samples vertical migration if not removed. For a sufficient migration of the samples, the DGGE had to run at least 20 hours.

After 20 hours the instrument was turned off and the gel taken out of the buffer tank. The gel was stained with a staining solution consisting of 30 ml 1 x TAE and 3 µl SYBR gold (Invitrogen) for one hour. The gel was thereafter rinsed with Milli-Q water and transferred to a UV-plate and photographed under UV-light at different exposure times.

Some characteristic bands in the DGGE gels were chosen for sequencing. Mostly common bands from the DGGE profiles that had high intensity were sampled, to get higher chances of good quality sequencing results. Eppendorf tubes with 20 µl sterile Milli-Q water were prepared for each sample. Pipette-tips were used to stick out the gel samples and a pipette was used to blow the materials in the tip into the Eppendorf tubes with water.

3.3.4 Reamplification of DGGE bands

The DGGE bands that were picked out for sequencing had to be re-amplified. The PCR cycling conditions was performed as described in Table 3.3, with 53˚C in the annealing step and 38 cycles. The forward primer was 338F-GC-M13R (Table 3.2) and 518R as reverse

primer. The forward primers 5'-end includes a target sequence for the M13R sequencing primer and the rest is identical to the 338F-GC primer. The products were examined on 1% agarose gel electrophoresis to see if there were sufficient amount of PCR products without any contamination. To clean the PCR products, the QIAquick PCR Purification Kit protocol was used (Appendix 3) and the sequencing primer M13R (Table 3.2) were added to the samples. The sequencing was carried out at the commercial company GATC Biotech (Germany).

3.3.5 Classification of DGGE band sequences

The DNA sequencing results received from GATC Biotech included chromatograms and the DNA sequences on text format. Chromas Lite (Technelysium Pty Ltd, Australia; Windows) was applied for viewing the chromatograms. In the chromatograms, the sequences for each sample were illustrated through peaks, where the highest peak of a base were the result. However, DGGE samples may have a lot of background noise, which results in noisy DNA sequences, and only the regions of high quality were used in further analysis. The poor quality regions were often in the 5'- and 3'-ends of the sequences and these sequences were removed from the corresponding text format along with the sequences corresponding to the forward and reverse primers. The remaining sequence regions were analyzed using the Classifier tool (Wang, Garrity et al. 2007) at the Ribosomal Database Project. This tool can classify bacterial 16S rRNA according to taxonomy. The bootstrap cutoff for classification was set to 50%, as recommended by the Classifier tool for sequences shorter than 250 base pairs.

3.3.6 Statistics

The DGGE images were exported, converted and cropped in ImageJ (Rasband et al., 1997-2012). The images were further analyzed with the Gel2K software (Svein Norland, Department of Microbiology, University of Bergen, Norway). This software converts the DGGE profiles in the gel into histograms. The peaks of the histograms correspond to the bands in the profile and the peak area corresponds to the intensity of fluorescence of the bands. The software organizes the bands in a band pattern, where the bands that have migrated the same distance in the gel are defined into the same band in the band pattern. However, many of the bands that have similar migration pattern ended up being defined as

different bands and needed to be adjusted manually to fit into the correct band. Thereafter the peak area values were exported to Microsoft excel.

In excel, the peak area values for each sample were normalized by dividing each peak area value by the sum of all the peak areas in the relevant lane. It creates a fractional peak area from the total peak area of all the bands in one sample, as shown below, where n_i corresponds to peak area of one band and N is the sum of all the peaks in the sample.

- $p_i = \frac{n_i}{N}$

These normalized peak area values were imported into the program package PAST (Hammer et al., 2001) for the statistical analyzes.

The following diversity indices were calculated for all samples in PAST:

- Band richness (S), reflecting the species richness of a sample.
- Shannon index: a diversity index. Both band richness and evenness of species is taken into account. The diversity index increases when both richness and evenness increases (Hammer et al., 2001).

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

n: is the total number of individuals

n_i: is the number of individuals of taxon *i*

- Evenness of species were calculated as described by Buzas and Gibsons's evenness formula: e^H/S. The evenness reflects how equal the abundance of the different species is between the DGGE bands (Hammer et al., 2001).

The Bray-Curtis similarity is a similarity index. It was chosen as it puts no weight on absence-absence. Bray-Curtis similarities for comparisons between all pairs of DGGE profiles in a DGGE gel were calculated in PAST from the normalized peak area values. The similarities range from 0 (no common bands) to 1 (identical band profiles) (Bray and Curtis, 1957).

Principal coordinates analysis (PCoA) was performed based on the Bray-Curtis distances (=1-similarity). The ordination method visualizes the distance in the DGGE profiles between samples, and reflects the distance between the DGGE profiles by distances in the plot. Similar DGGE profiles are found in close proximity to each other in the plot and very dissimilar DGGE profiles far away from each other. The graph shows all the data points in relation to one another in a coordinate system (Hammer et al., 2001).

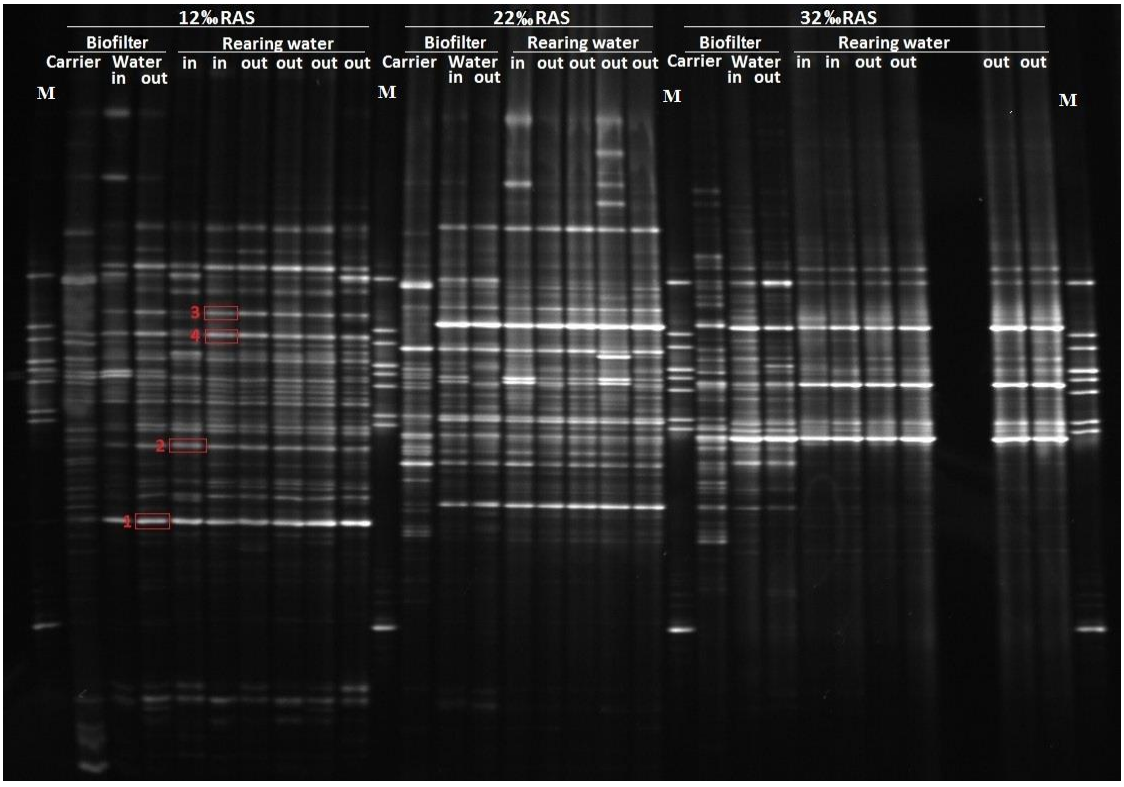
One-Way PERMANOVA (Non-Parametic MANOVA) based on Bray-Curtis similarities was used to test whether there were significant differences in bacterial community composition between groups of samples. If significant differences between groups are found, it is due to the differences in location. The test uses raw distances instead of ranking the results. The p-values are calculated using permutations instead of tabled P-values (Anderson, 2005). The Bonferroni correction of p-values was used when more than two comparisons were performed. In this correction the p-values are multiplied with the number of comparisons, which results in larger p-values. Differences between groups were considered significant if $p<0.05$ (Hammer et al., 2001). The One-Way PERMANOVA is relative robust and is a good tool to detect changes in structures of microbial communities in balanced experiments. If the H_0 hypothesis is true, there are no significant differences in the community profile between the selected groups (Anderson and Walsh, 2013).

4. Results

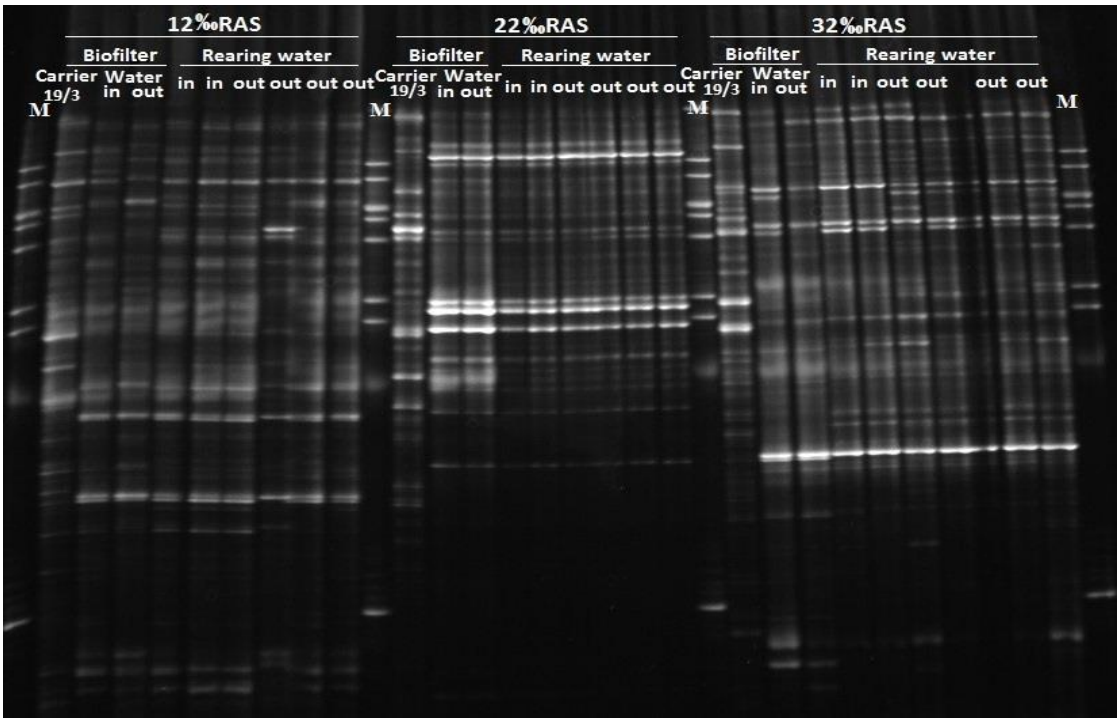
4.1 Comparisons of microbial communities between RAS with different salinities

To investigate if the salinity had an impact on the microbial communities, three DGGE gels were run (Figure 4.1). Each gel represents one sampling date with samples from the three systems for comparing the structure of the microbial communities at specific dates. The gel images indicate that there were large variations in the community structure between the salinities. The community profiles of biofilter water appear to be similar to those of the corresponding rearing water. The excised band taken from the 12‰ RAS (Figure 4.1A and C) showed a dominance of the class *Alphaproteobacteria* (band number 3-7). The listing of the all taxonomic assignments for the excised bands are found in Table 4.2.

A



B



C

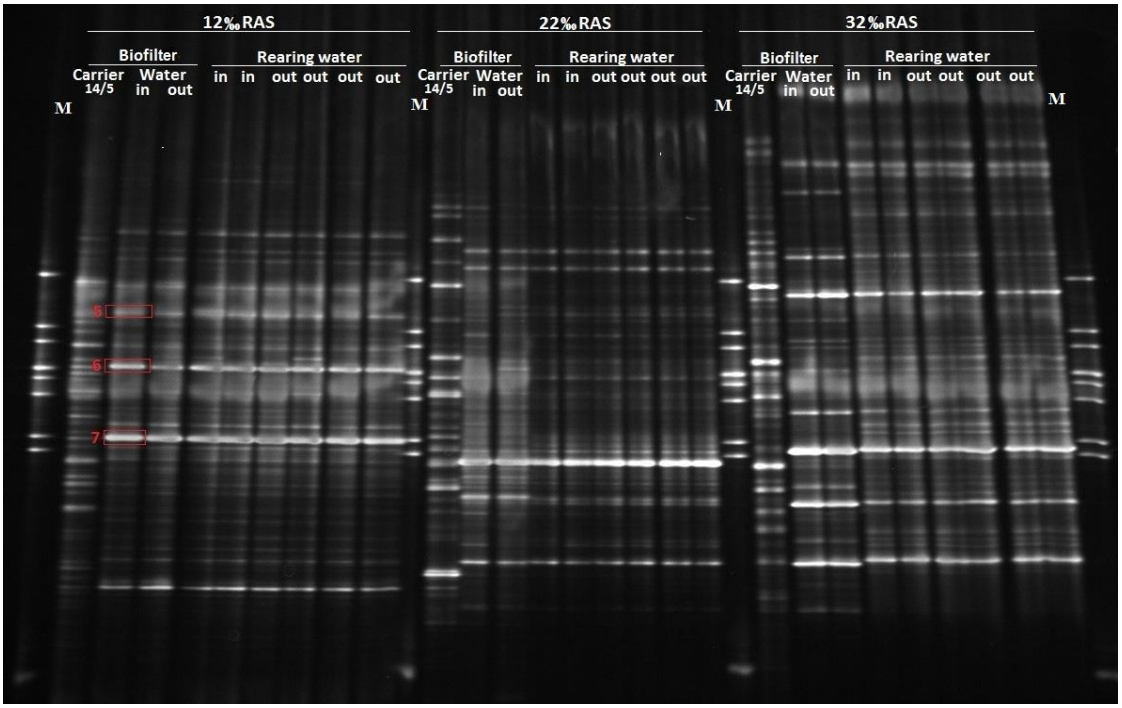
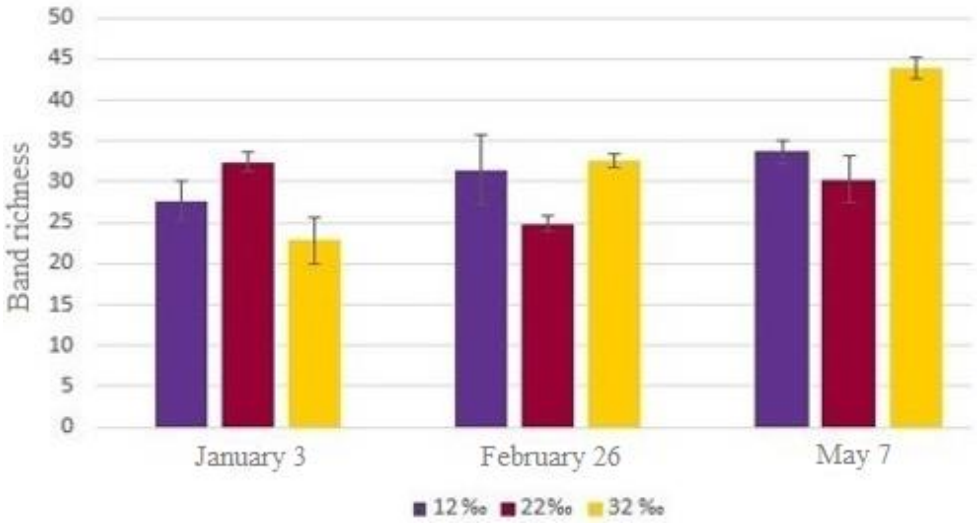


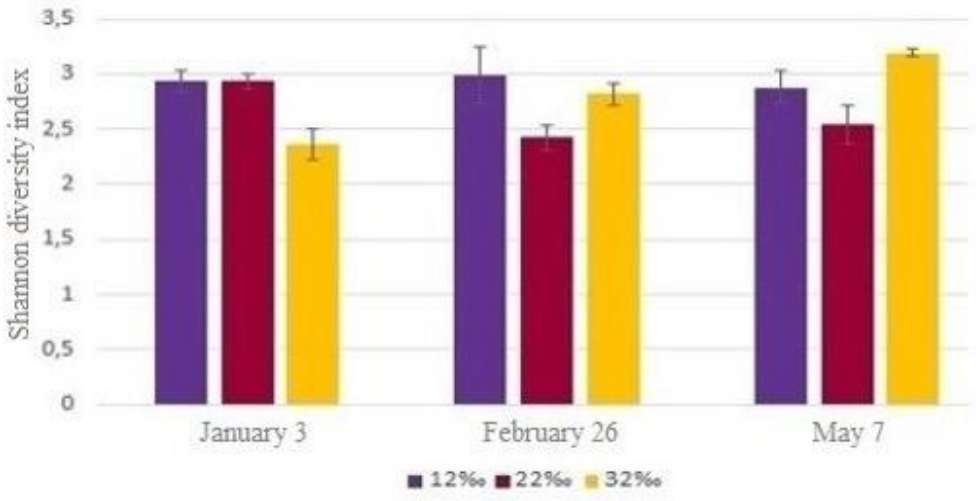
Figure 4.1: The DGGE gel images representing v3 16S rDNA PCR products of samples from the three RAS systems at January 3 (A), February 26 (B) and May 7 (C). "In" and "out" are the abbreviations for the samples taken of the incoming and outgoing water in biofilter and rearing tanks. The red rectangles display the bands excised for DNA sequencing (M = DGGE marker).

Average band richness, Shannon diversity index and evenness index were determined for all the DGGE profiles representing in- and outgoing rearing water for each system, at the various dates. At January 3, the microbial communities in 32‰ RAS were the least diverse, with low average results for all the diversity indices. At the same sampling date, the community profiles in 22‰ RAS had the highest average band richness, with an average Shannon's diversity index high and similar to the community profiles in 12‰ RAS. At the two last sampling dates, the DGGE profiles clearly indicated that the 22‰ RAS was the least diverse. The 32‰ RAS had the most diverse microbial communities at the last sampling date.

A



B



C

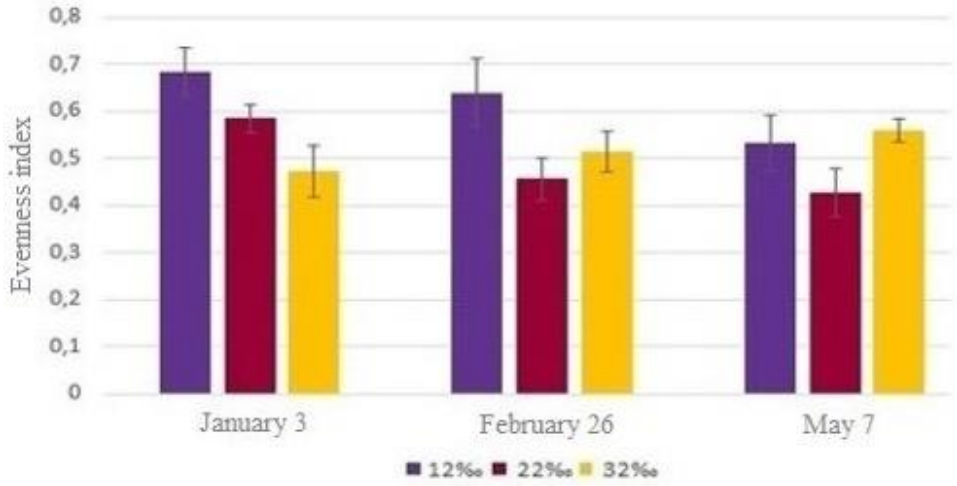
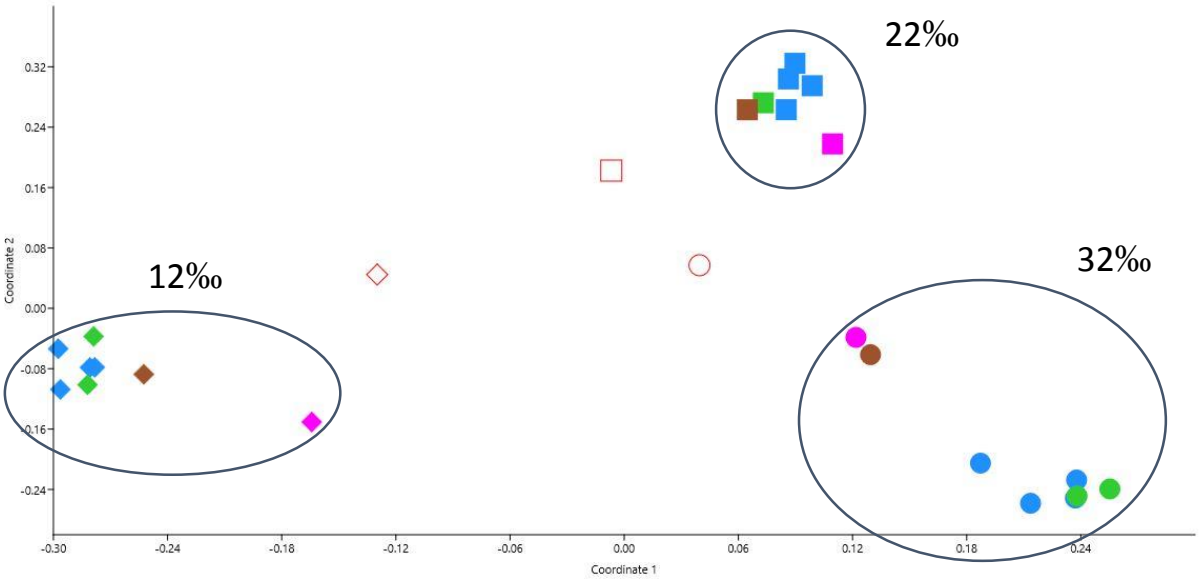


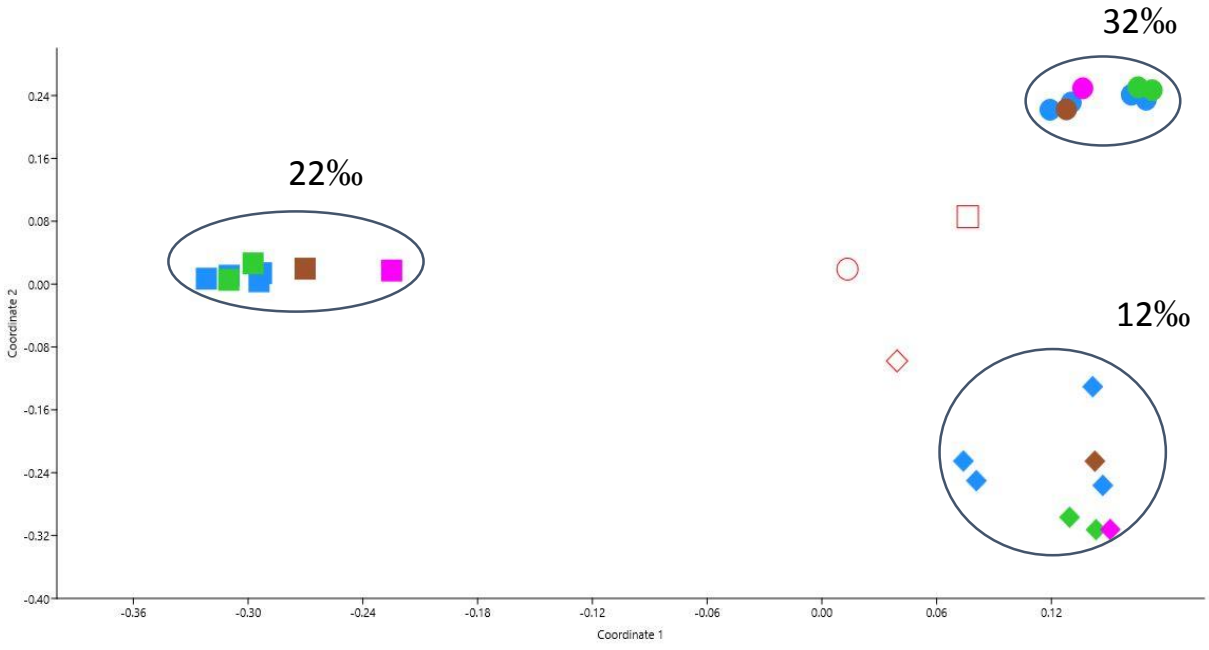
Figure 4.2: Average diversity indices with standard deviation for the DGGE profiles of rearing water in the three RAS systems at specific dates. A: Band richness, B: Shannon diversity index and C: evenness index.

Ordination by principal coordinates analysis based on Bray-Curtis similarities were performed for DGGE profiles representing all the samples in each gel (Figure 4.3). At all three sampling dates, the water samples grouped according to salinity. The one-way PERMANOVA test confirmed that there were significant differences in the rearing water between the three systems that represented distinct salinities, at all the sampling times ($p < 0.0001$). The PCoA plots indicate that the community profiles of biofilter water became more similar to its corresponding rearing water community profiles over time. The community profile of biofilm carriers seem to be more similar to its water samples community profiles at the first sampling (Figure 4.3A) than at later samplings. This was confirmed by average Bray-Curtis similarities for comparisons of microbial communities in the biofilm carriers and the rearing water, which was found to be considerably higher at the first sampling date (Figure 4.5A). However, at the last sampling, the biofilm communities were found quite similar to each other, even though they were taken from the different systems (Figure 4.3C). This imply that the samples of biofilm carriers became more similar to each other over time. This was confirmed by average Bray-Curtis similarities for comparisons of biofilm communities between the systems, which was found to increase over time (Figure 4.5B)

A


22‰
12‰
32‰
Coordinate 2
Coordinate 1
0.32
0.24
0.16
0.08
0.00
-0.08
-0.16
-0.24
-0.30
-0.24
-0.18
-0.12
-0.06
0.00
0.06
0.12
0.18
0.24

B


32‰
22‰
12‰
Coordinate 2
Coordinate 1
0.24
0.16
0.08
0.00
-0.08
-0.16
-0.24
-0.32
-0.40
-0.36
-0.30
-0.24
-0.18
-0.12
-0.06
0.00
0.06
0.12

C

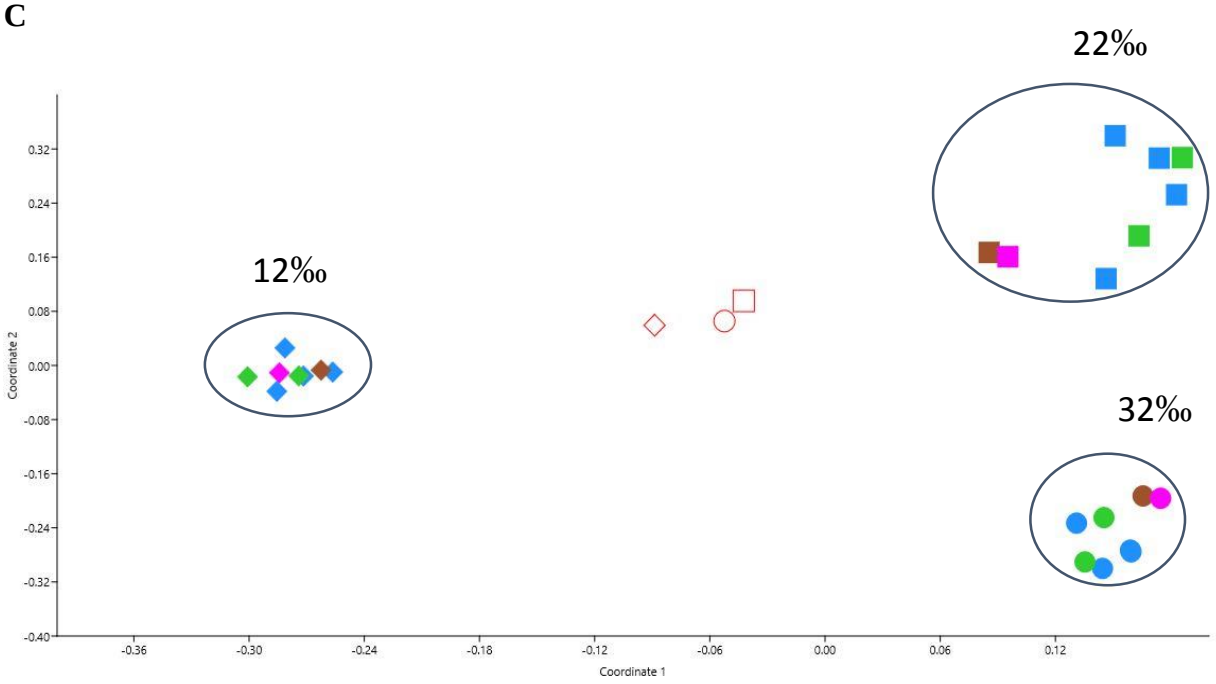
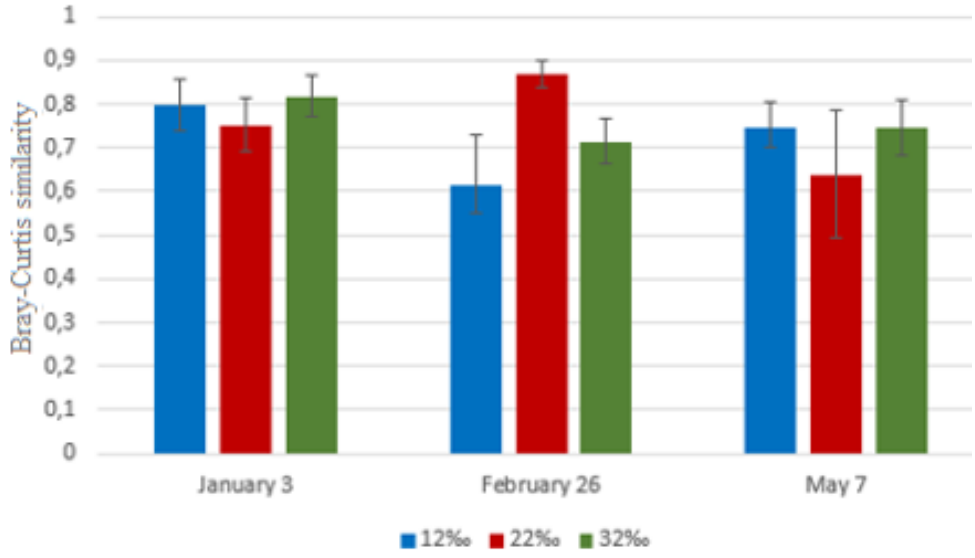


Figure 4.3: PCoA ordination plot based on Bray Curtis similarities for DGGE profiles of samples from the three systems at January 3 (A), February 26 (B) and May 7 (C). Circles represents the 32‰ RAS samples, squares represents 22‰ RAS samples and diamond 12‰ RAS samples. The samples representing biofilm carriers are represented by red, open symbols. Blue symbols represents outgoing rearing water samples, green represents incoming rearing water samples, brown represents outgoing biofilter water samples and purple represents incoming biofilter water samples.

To investigate the variability in microbial community structure in in- and out-going rearing water within each system between replicate tanks over time, average Bray-Curtis similarities were calculated for the community profiles of the rearing water for each system, at all the sampling times (Figure 4.4 A). The microbial communities of the rearing water within each system had high Bray-Curtis similarities, ranging from 0.6-0.88 at all dates. In the first and last sampling, both 12‰ RAS and 32‰ RAS had high average similarities. At February 26, the 22‰ RAS had the highest similarities between its rearing water microbial communities, while the 12‰ RAS had its lowest similarity between its communities at this date. Average Bray-Curtis similarities were also calculated to investigate the variability in microbial communities in rearing waters between the salinities over time (Figure 4.4 B). The comparisons of the rearing water microbial communities between the 22‰ vs 32‰ RAS' had the highest similarities in both the first and last sampling. The comparisons between the communities of 12‰ vs 22‰ had the lowest similarities at the two last sampling dates while the 12‰ vs 32‰ seem to be relatively stable in similarity over time.

A



B

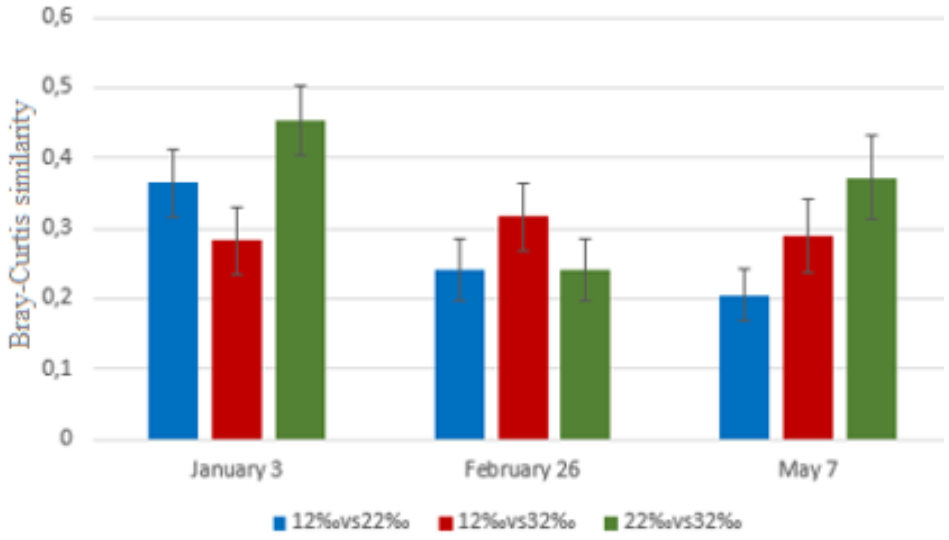
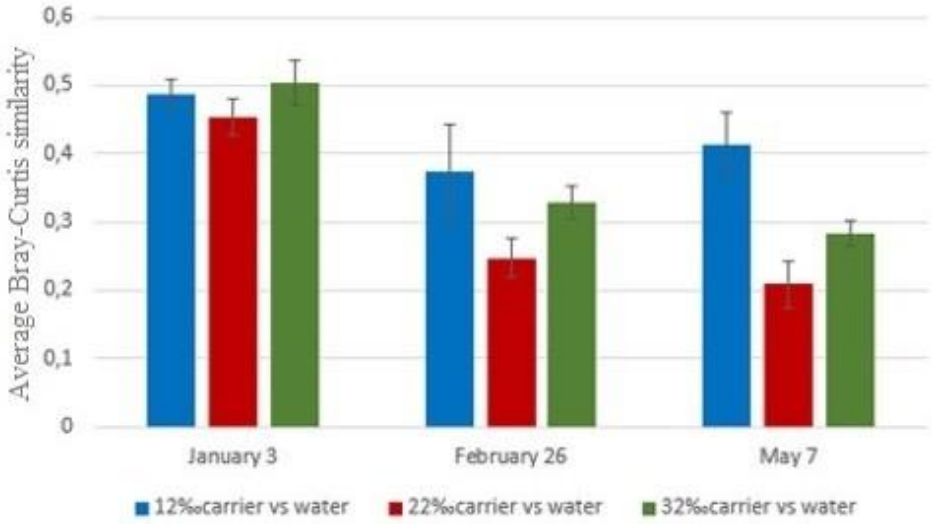


Figure 4.4: Average Bray-Curtis similarities with standard deviation for comparisons of A: microbial communities between replicate rearing tanks inlet and outlet water within each system at the three sampling dates, and B: microbial communities of rearing water between distinct salinities.

Average Bray-Curtis similarities were calculated for comparisons between the biofilm communities and its corresponding rearing water communities of the different RAS (Figure 4.5A) to investigate the development between the components in the systems. The average Bray-

Curtis similarities between the biofilm communities and the rearing water microbial communities were highest in the first sampling, but decreased over time. The 12‰ RAS' biofilm community and rearing water community had the highest similarities at the two last sampling dates compared to the other systems, while the 22‰ RAS microbial communities were the most dissimilar. Average Bray-Curtis similarities were calculated for comparisons of the biofilm communities between the systems at the specific dates to investigate the similarity in biofilm communities over time (Figure 4.5B). The Bray-Curtis similarities show a small increase in similarity between the biofilm communities of the three systems in the last sampling.

A



B

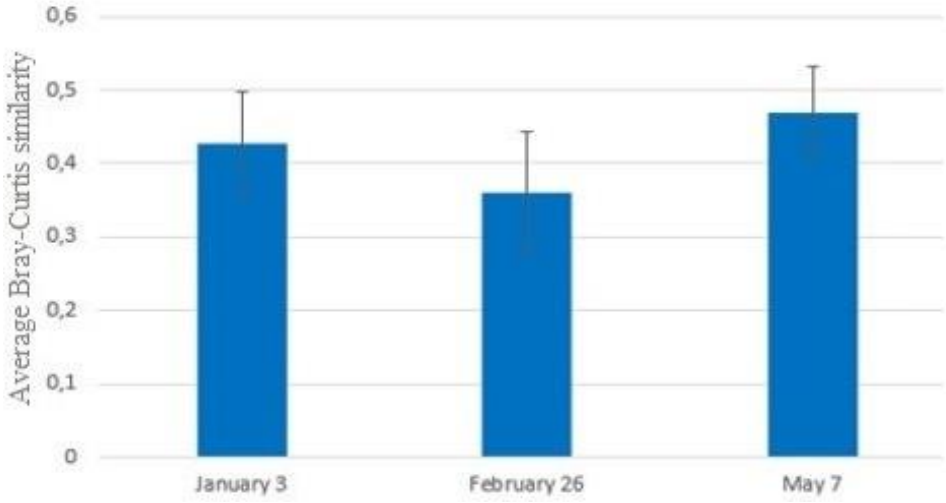
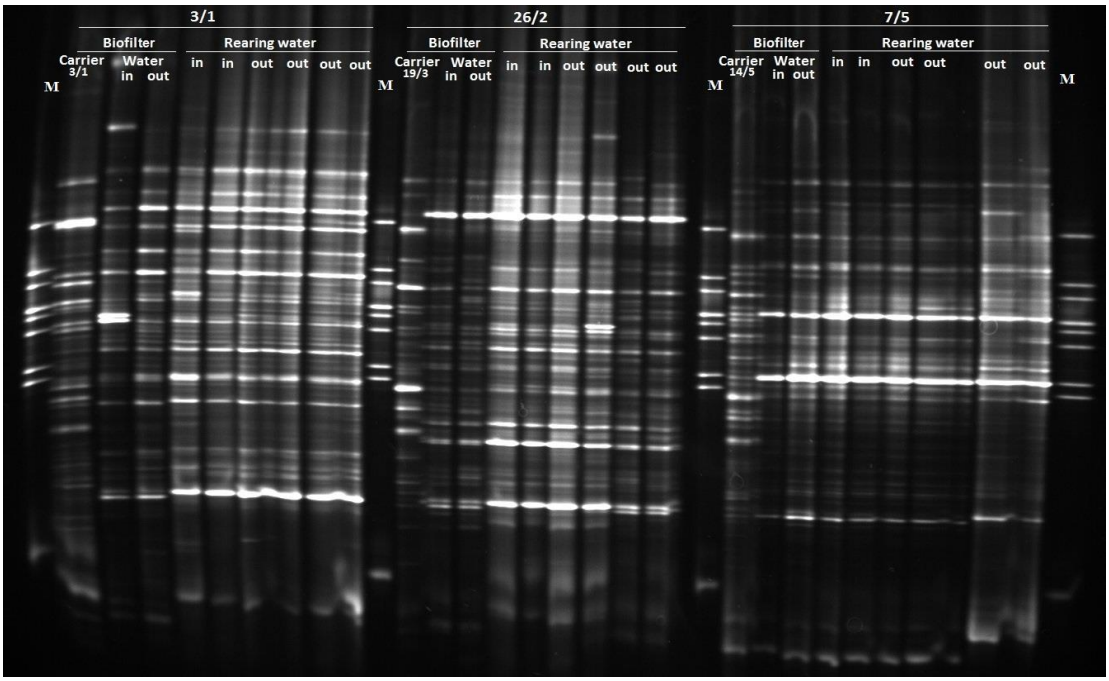


Figure 4.5: Average Bray-Curtis similarities with standard deviation for comparisons of A: community profiles between biofilm carriers and its corresponding rearing water, and B: biofilm communities between the systems at the specific sampling dates.

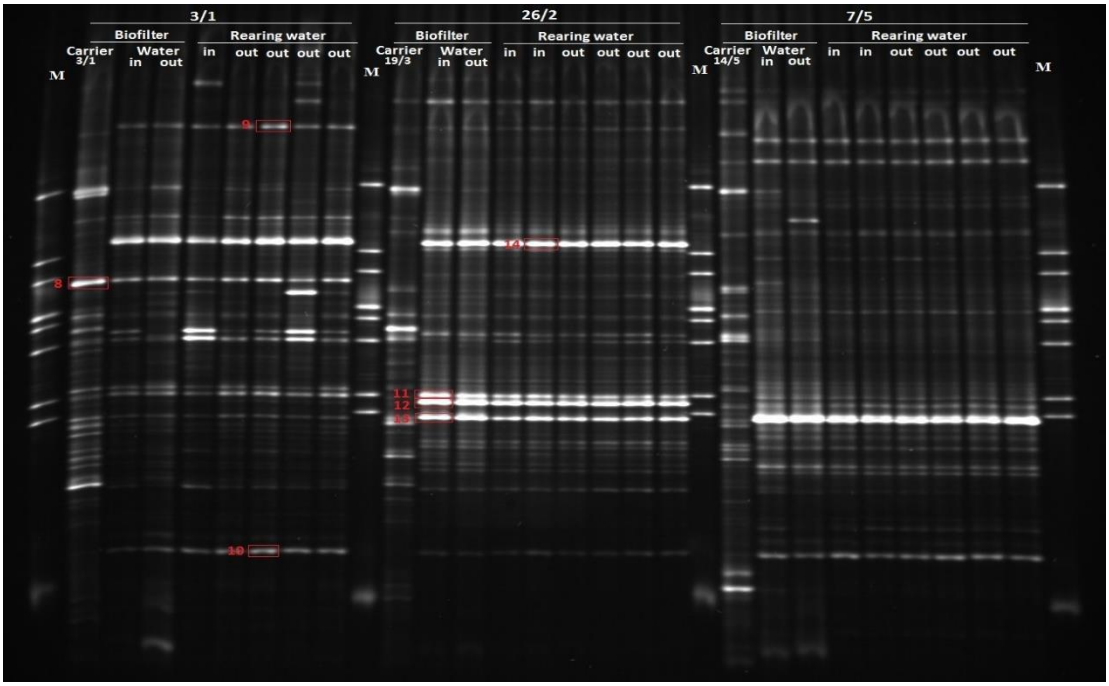
4.2 Temporal development of microbial communities in RAS with different salinities

To investigate the dynamics of the microbial communities in each system over time, three DGGE gels were run (Figure 4.6). Each gel represents one system with samples from the different sampling times for comparing the structure of the microbial communities at different dates within each system. The gel images indicate that there were large variations in community structure between dates. The community profile of biofilter water appear to be similar to those of the corresponding rearing water. The DGGE gels shows that there were bands that persisted over time within a system. Some of the bands appear to grow stronger in intensity while some disappeared over time. The excised band showed a majority of *Sulfitobacter* (band number 10, 12, 14) in the 22‰ RAS (Figure 4.6B). *Sulfitobacter* (band number 15, 20) was also found in the 32‰ RAS (Figure 4.6C) along with a majority of *Rhodobacteraceae* (band number 18, 19, 22).

A


3/1
Biofilter
Rearing water
Carrier 3/1
Water in out
in in out out out out
M
26/2
Biofilter
Rearing water
Carrier 19/3
Water in out
in in out out out out
M
7/5
Biofilter
Rearing water
Carrier 14/5
Water in out
in in out out out out
M
M

B


3/1
Biofilter
Rearing water
Carrier 3/1
Water in out
in out out out out
M
9
8
10
26/2
Biofilter
Rearing water
Carrier 19/3
Water in out
in in out out out out
M
14
11
12
13
7/5
Biofilter
Rearing water
Carrier 14/5
Water in out
in in out out out out
M
M

C

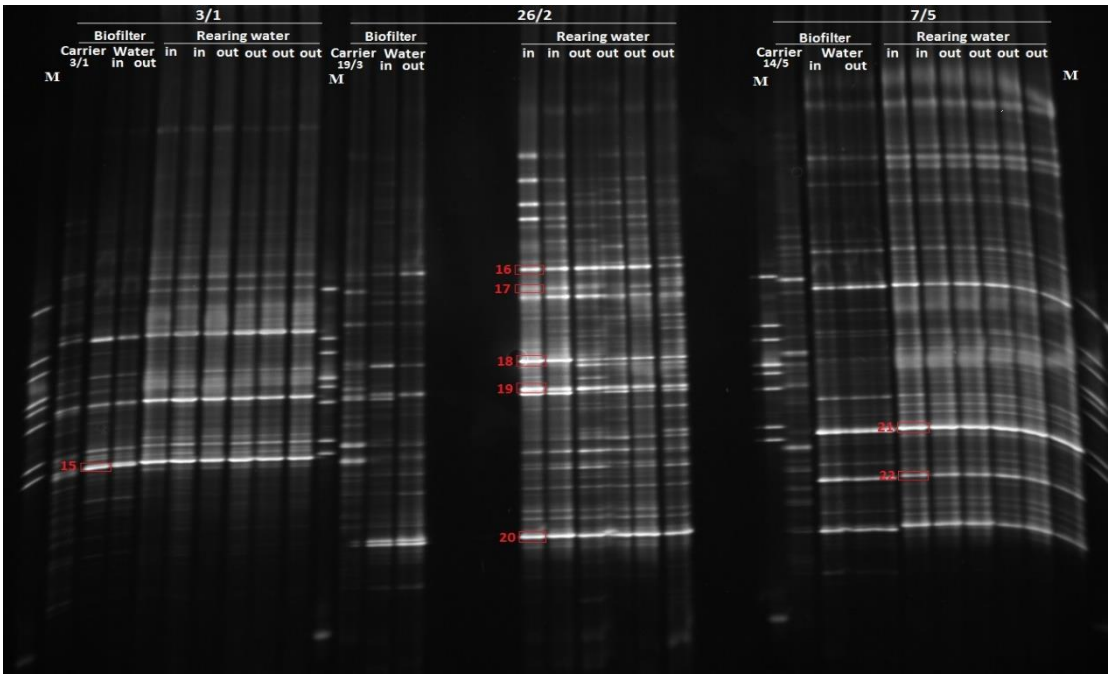
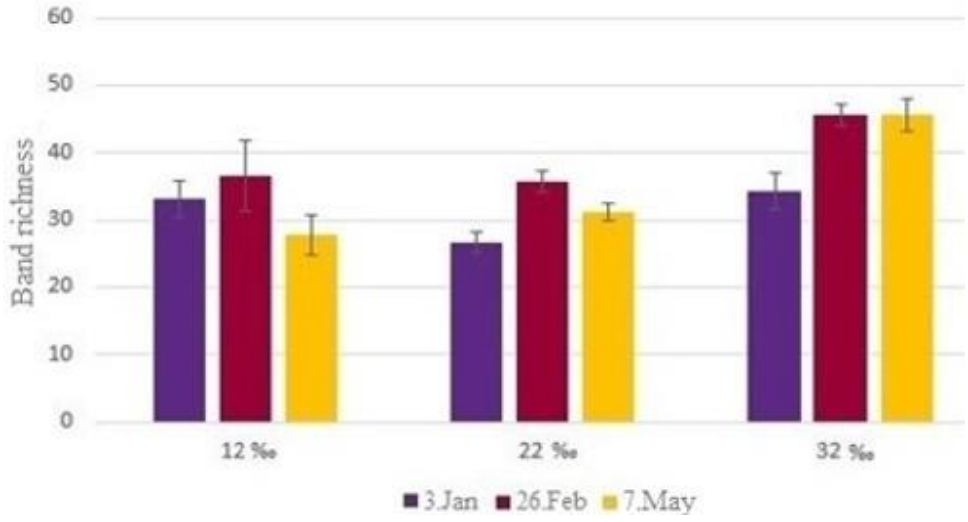


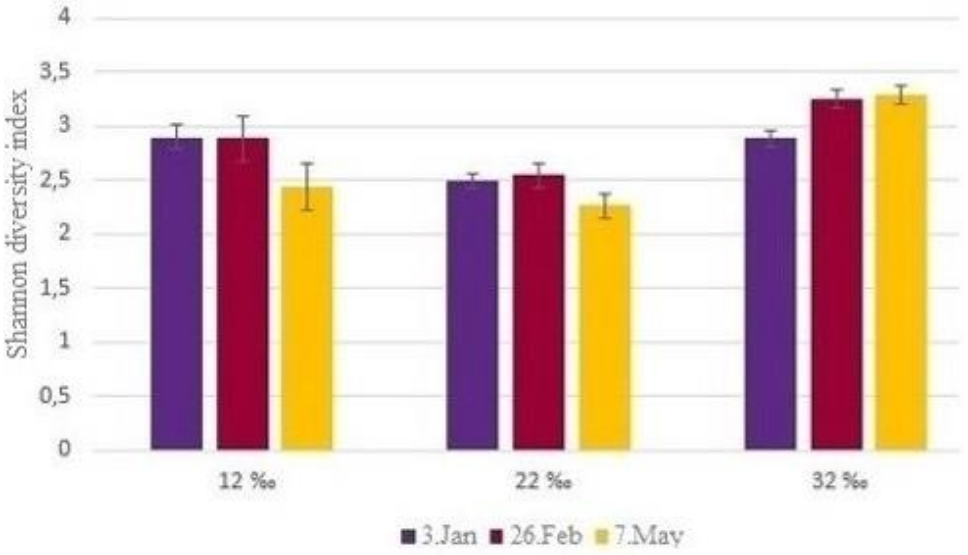
Figure 4.6: The DGGE gel images representing v3 16S rDNA PCR products of samples taken at different dates in 12‰ RAS (A), 22‰ RAS (B) and 32‰ RAS (C). "In" and "out" are the abbreviations for the samples taken of the incoming and outgoing water in biofilter and rearing tanks. The red rectangles display the bands excised for DNA sequencing (M = DGGE marker).

Average band richness, Shannon diversity index and evenness index were determined for all the DGGE profiles representing in- and outgoing rearing water in the systems (Figure 4.7). The 32‰ RAS had a clear increase in the diversity indices over time. At the two last samplings, the 32‰ RAS was highly diverse, with high average diversity indices. The 22‰ RAS had an increase in band richness over time, while the average results in Shannon diversity index and evenness index decreased. The average diversity indices in 12‰ RAS showed a tendency to decrease over time.

A


60
50
40
30
20
10
0
Band richness
12 ‰
22 ‰
32 ‰
3.Jan
26.Feb
7.May

B


4
3,5
3
2,5
2
1,5
1
0,5
0
Shannon diversity index
12 ‰
22 ‰
32 ‰
3.Jan
26.Feb
7.May

C

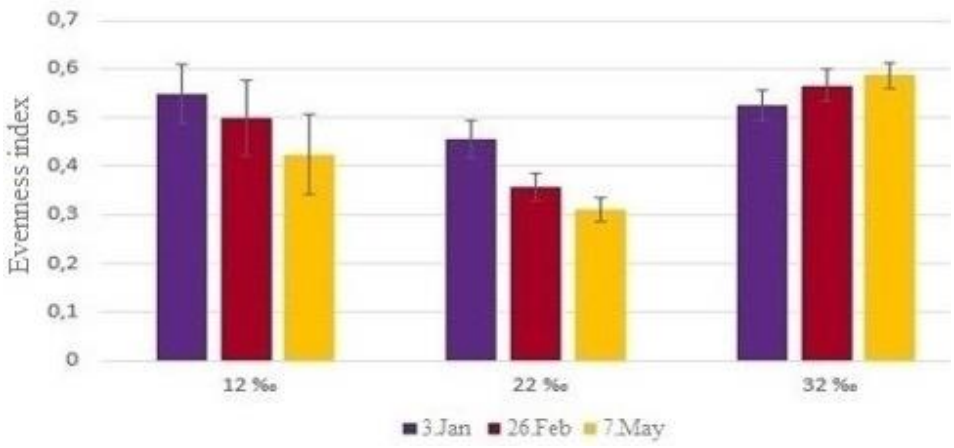
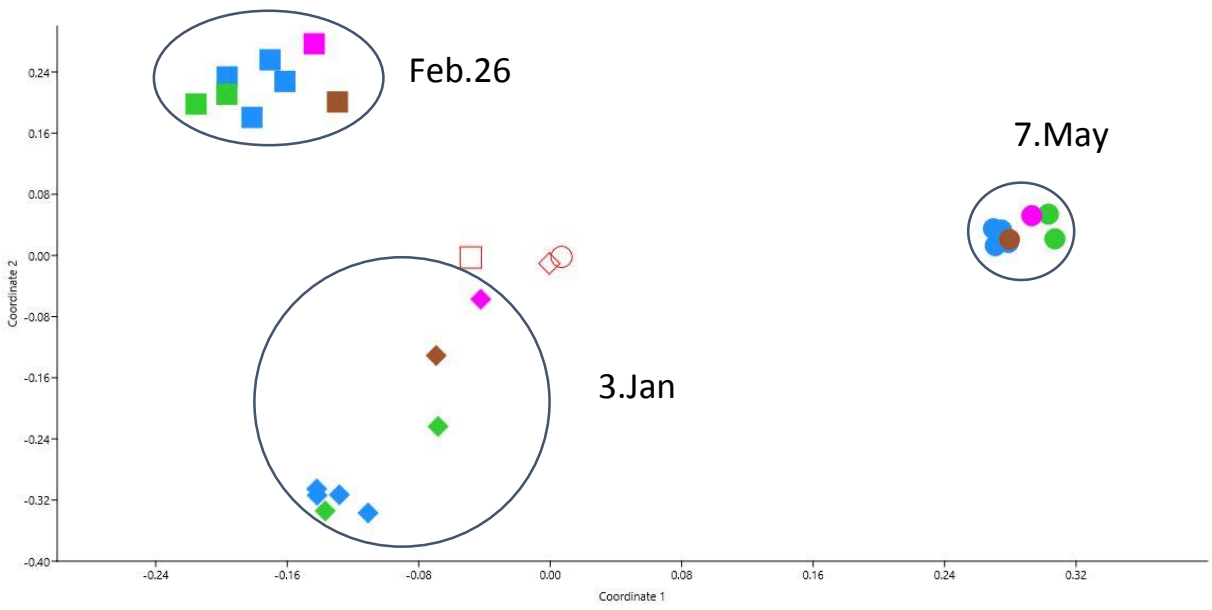


Figure 4.7: Average diversity indices with standard deviation for the DGGE profiles of rearing water at three specific dates in each RAS. A: Band richness, B: Shannon diversity index and C: evenness index.

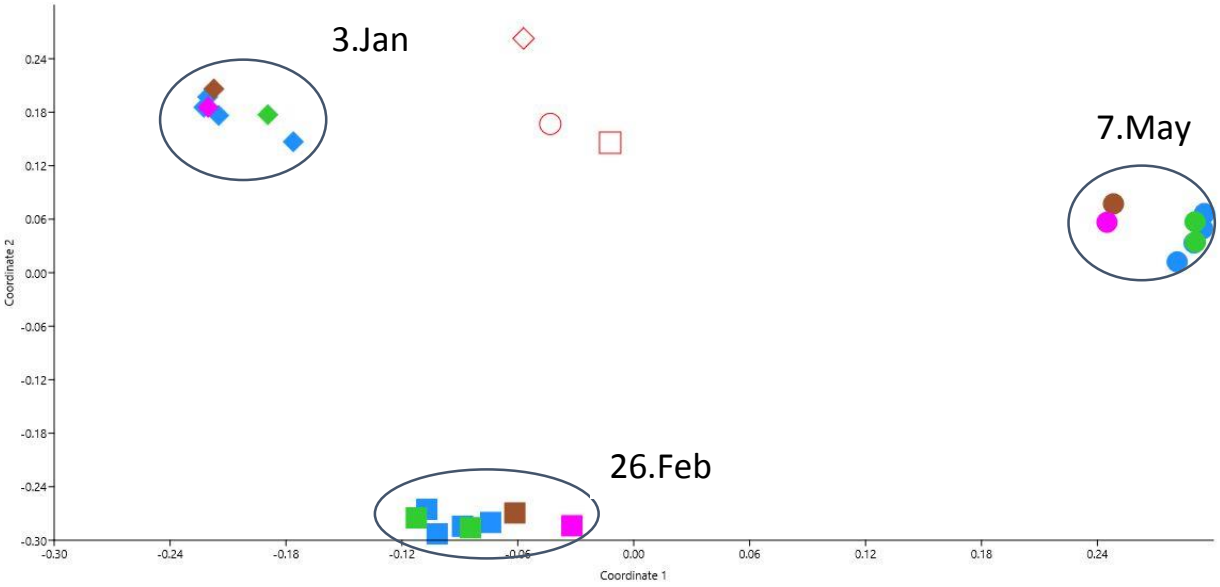
Ordination by principal coordinates analysis based on Bray-Curtis similarities were performed for DGGE profiles representing all the samples in each gel (Figure 4.8). In all the three systems, the rearing water samples grouped according to date in the plot. The one-way PERMANOVA test confirmed that there were significant differences in community profiles of the rearing water between the three dates in all systems ($p < 0.0001$). The one-way PERMANOVA test also confirmed that there were no significant differences in the microbial communities between the rearing tank inlet and outlet water at all dates for all the three systems (p-values ranged from $p<0.069$ to $p<0.66$). The PCoA plots further indicate that the community profiles of biofilter water appear to be similar to the community profiles of the rearing water. However, particularly at the first sampling date in 12‰ RAS (Figure 4.8A), the communities of biofilter water appear to be more similar to the biofilm community than rearing waters microbial communities. Additionally, at the last sampling in 22‰ RAS (Figure 4.8B) and 32‰ RAS (Figure 4.8C), the community profiles of the biofilter water appear to be somewhat dissimilar from the community profiles of the rearing water samples. One-way PERMANOVA tested if there were significant differences between the biofilter water communities and the rearing water communities (Table 4.1). The One-Way PERMANOVA test proved that there were significant difference in the microbial structure between the biofilter

water and the rearing water in most of the comparisons. The biofilter water and rearing waters microbial communities in 22‰ RAS showed similarities at the two first sampling dates, while in the 12‰ RAS the communities develop to become similar in the last sampling date. The PCoA plots also imply that the biofilm carriers in 12‰ RAS and 22‰ RAS had similar community profiles over time. This was confirmed by average Bray-Curtis similarities for comparisons of the biofilm communities within each system, which was found to be relatively high (Figure 4.9B). At the first sampling date in the 32‰ RAS, the biofilm community appear to be more similar to its corresponding rearing waters community profile. However, the biofilm carriers sampled at the two later dates in the 32‰ RAS appear to be more similar in community structures.

A



B



C

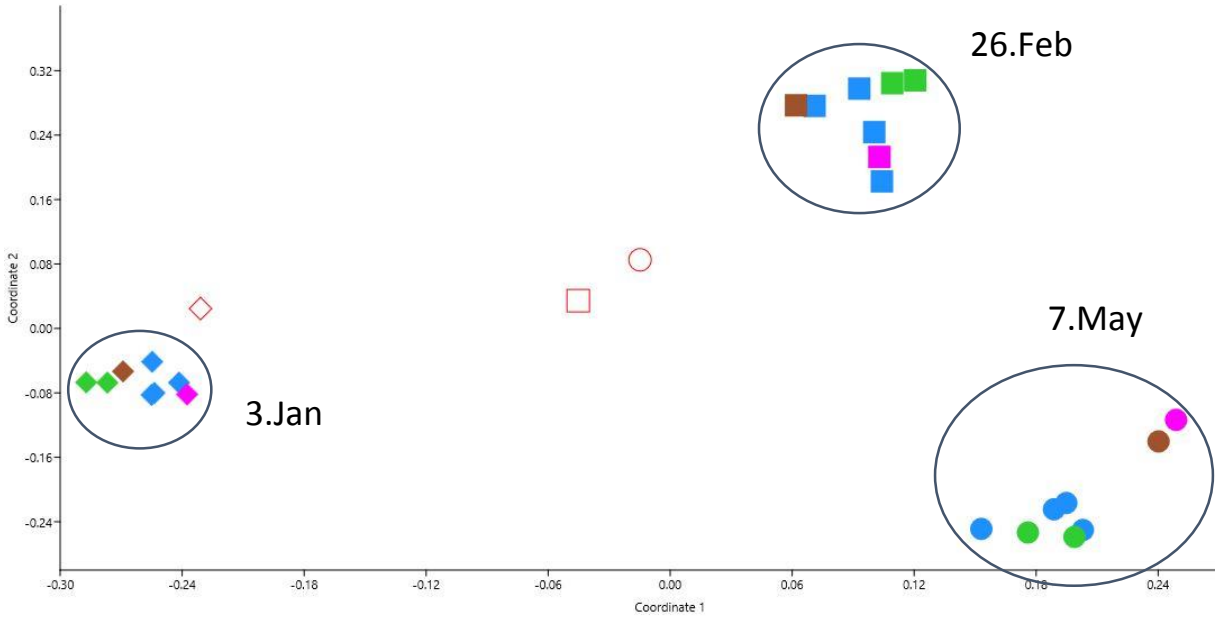


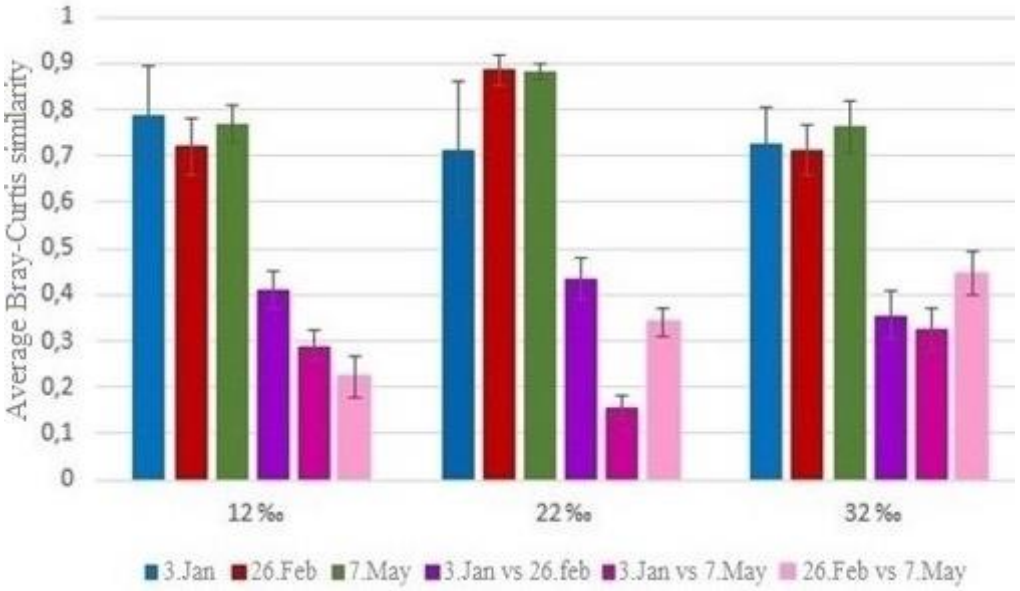
Figure 4.8: PCoA plot based on Bray Curtis similarities for DGGE profiles of samples that represents specific dates in 12‰ RAS (A), 22‰ RAS (B) and 32‰ RAS (C). Circles represents the May 7 samples, squares represents February 26 samples and diamond January 3 samples. The samples of biofilm carriers are represented by red, open symbols. Blue symbols represents outgoing rearing water samples, green represents incoming rearing water samples, brown represents outgoing biofilter water samples and purple represents incoming biofilter water samples.

Table 4.1: One-Way PERMANOVA results (Bonferroni-corrected p-values) for testing the hypothesis of no difference between the biofilter water and the rearing water communities for all the dates in each system. The grey squares visualize the significant differences.

System/Sampling date	January 3	February 26	May 7
12‰ RAS	0.0351	0.0359	0.3191
22‰ RAS	0.0987	0.0741	0.0341
32‰ RAS	0.0336	0.0376	0.0334

To investigate the variability in the microbial community structure over time within each system, average Bray-Curtis similarities were calculated for the rearing waters community profiles within each sampling date and between dates, in all the three systems (Figure 4.9A). There were 2-4 times higher similarities in the microbial communities within each date than between the different dates. The 22‰ RAS started out with a somewhat low similarity in microbial composition within the rearing water at first sampling, but at the two last samplings dates the rearing waters community profile became more similar to each other. In the 12‰ RAS and 22‰ RAS, the comparisons of microbial communities between the rearing water at 3.Jan vs 26.Feb showed the highest similarity compared to the other comparisons between dates in the systems. The 26.Feb vs 7.May comparison had the least similar microbial communities in 12‰ RAS, while the comparison had the highest similarity in the 32‰ RAS. Both 22‰ RAS and 32‰ RAS were the least similar in microbial communities in the 3.Jan vs 7.May comparison. Average Bray-Curtis similarities were also calculated for the biofilm communities within each system over time (Figure 4.9B). The Bray-Curtis similarities indicate that the biofilm communities in 12‰ were the most similar.

A



B

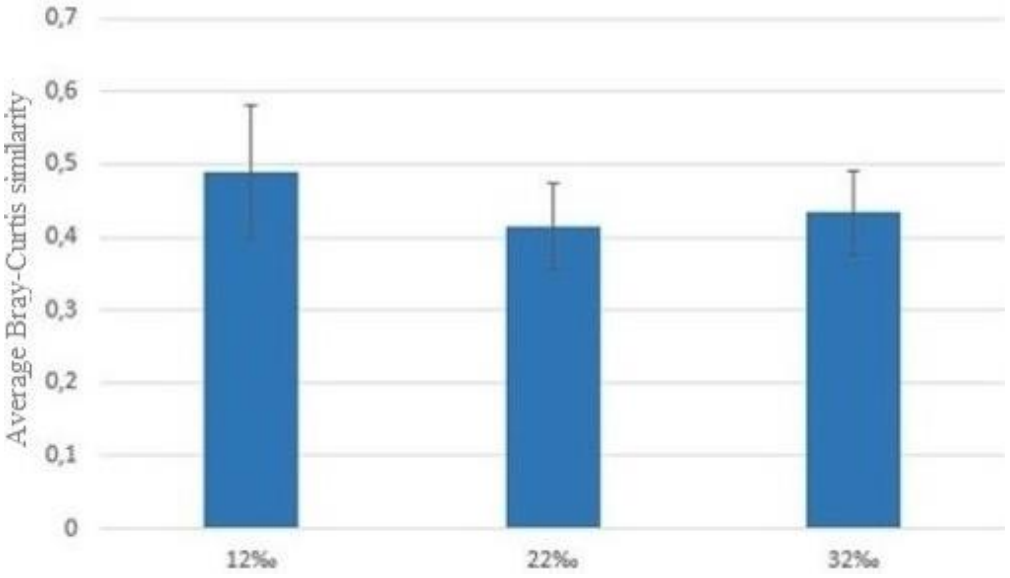


Figure 4.9: Average Bray-Curtis similarities with standard deviations for comparisons of A: microbial communities of water coming in and out of the replicate rearing tanks within each date and between dates in each system and B: biofilm communities over time within each system.

4.3 Comparisons of the microbial communities of biofilm and water in the biofilters of the three RAS

To investigate if the salinity had an impact on the biofilter communities, one gel was run with samples from biofilm carriers and water going in and out of the biofilter tanks from the three systems at the different dates (Figure 4.10). The gel image indicate that there were differences in community profiles for the biofilm carriers and the biofilter water in each system. The community structure of the biofilm carriers appear to be relatively similar over time in each system. The community profiles of water coming in seem to be similar to the water going out of the biofilter tanks. However, the community profiles of the biofilter water appear to change over time within each system. There are several high intensity bands in the DGGE profiles of biofilter water in the last sampling date compared to earlier samplings, indicating the dominance of a few taxa. The excised bands taken from the microbial profiles of biofilm carriers shows a broad spectrum of bacteria. One of them is *Pseudorhodobacter* (band number 27), which seems to be found as strong bands in the biofilter water in all the systems, at the last sampling date.

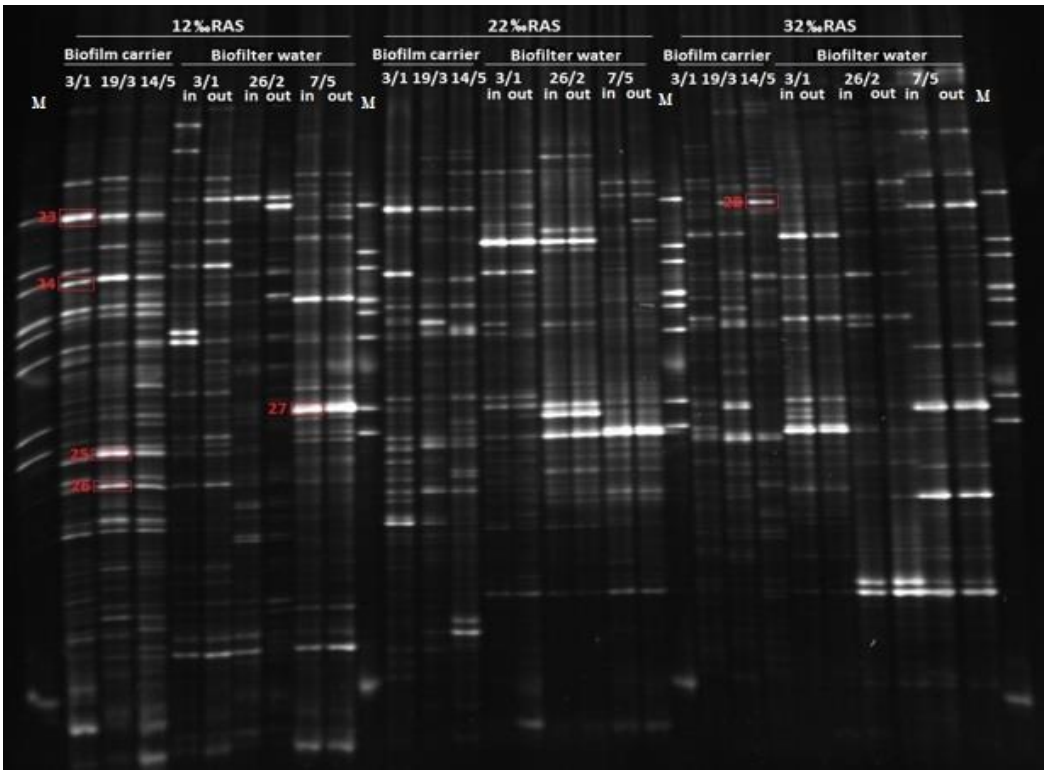
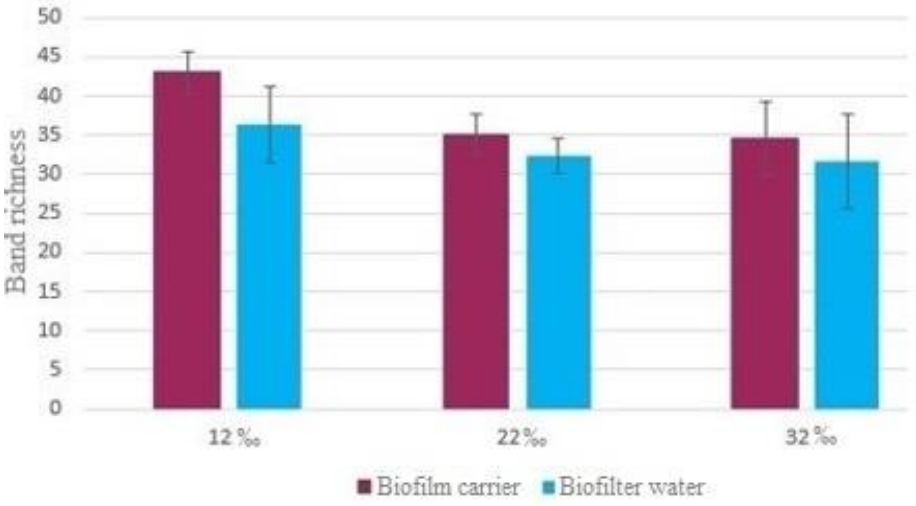


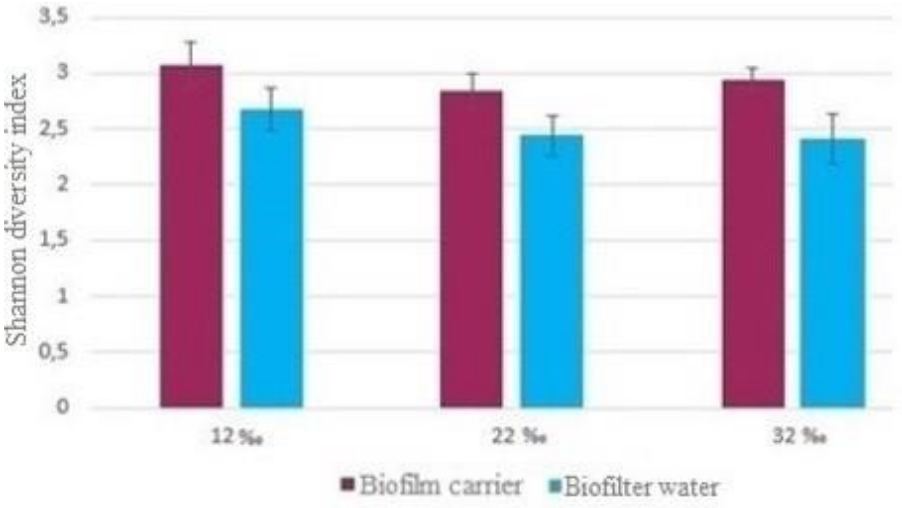
Figure 4.10: The DGGE gel image representing v3 16S rDNA PCR products for biofilter samples from the three RAS systems. "In" and "out" are the abbreviations for the samples taken of the incoming and outgoing water in the biofilter tanks. The red triangles display the bands excised for DNA sequencing (M = DGGE marker).

Average band richness, Shannon diversity index and evenness index were determined for all the DGGE profiles, compared between the biofilm communities and biofilter water communities (Figure 4.11). The figure shows that there was a tendency of higher average diversity indices for the biofilm communities than for the microbial communities of biofilter water, in all the systems. The microbial communities in 12‰ RAS' biofilm carriers and biofilter water had higher average diversity indices, compared to the other systems diversity indices. The average diversity indices for the biofilm carriers of 22‰ RAS and 32‰ RAS were relatively similar, and so were their corresponding biofilter water community profiles.

A



B



C

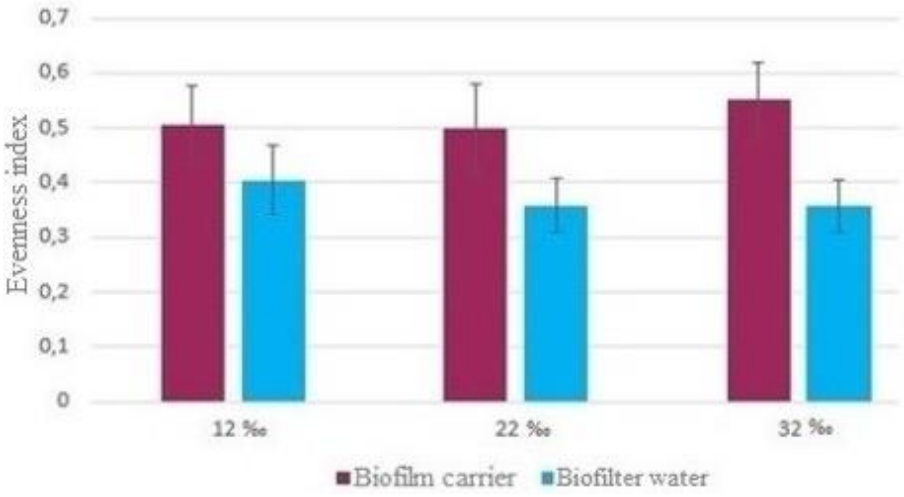


Figure 4.11: Average diversity indices with standard deviations for the DGGE profiles of biofilm discs and biofilter water in the three RAS systems. A: Band richness, B: Shannon diversity index and C: evenness index.

PCoA ordination based on Bray-Curtis similarities were performed for DGGE profiles representing all the samples in the gel (Figure 4.12). The biofilm communities appear to be relatively similar in microbial composition between salinities and time than the biofilter water. This was confirmed by average Bray-Curtis similarities for comparisons of biofilm communities and rearing waters communities between salinities, which found that the similarity in biofilm communities were higher (Figure 4.13A). The biofilm communities were generally not so similar to their corresponding biofilter waters microbial communities. The One-Way PERMANOVA confirmed that there were significant difference between the biofilm carriers and the biofilter water ($p<0.0001$). In both 12‰ RAS and 22‰ RAS, the biofilm carriers community profiles appear to group according to their system. The One-Way PERMANOVA confirmed that there were no significant difference between the biofilter inlet and outlet water at all dates in each system (32‰ $p>0.799$, 22‰ $p>0.69$ and 12‰ $p>0.90$). The communities in biofilter inlet and outlet water were more similar in both 12‰ RAS and 32‰ RAS' in the last sampling than at earlier dates, which can indicate that the biofilter water became even more similar in microbial community over time. The PCoA plot indicates the biofilter waters microbial communities were generally dissimilar to each other between the different sampling dates. This was confirmed by average Bray-Curtis similarities for comparisons of biofilter water communities within each system over time, which showed less similarity in the biofilter water over time than the biofilm communities (Figure 4.13B). The

plot also imply that community profiles of 12‰ RAS' biofilter water were somewhat similar over time.

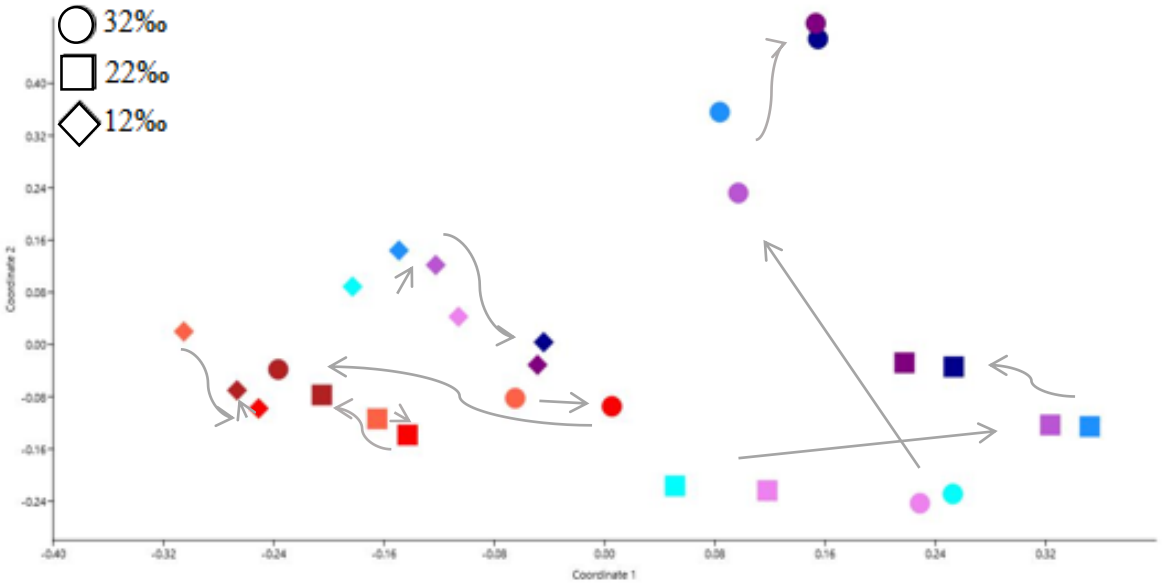
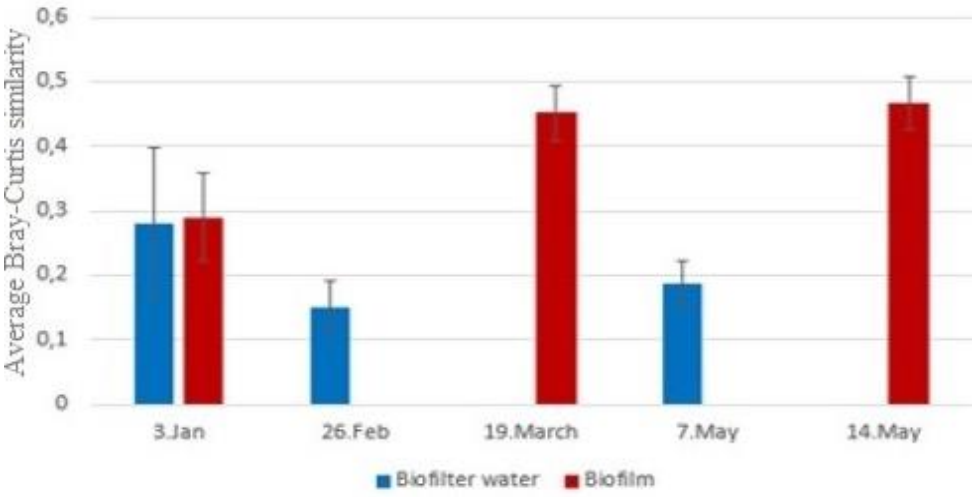


Figure 4.12: PCoA plot based on Bray Curtis similarities for DGGE profiles of samples representing different salinities and dates. Blue symbols represents outgoing biofilter water, purple represents incoming biofilter water and red represents biofilm carriers. Light shades represents January 3 samples, intermediate shades represents February 26 samples and dark shades represents May 7 samples.

Average Bray-Curtis similarities were calculated to investigate whether the microbial community structure of the samples from biofilm carriers were more similar to each other between salinities than the biofilter water communities between salinities (Figure 4.13). At the first sampling, the biofilter community comparisons and the water community comparisons both had average Bray-Curtis similarities close to 0.3. Comparing the water communities between salinities were considerably lower in similarity than those found in comparing biofilm communities between salinities, at the two latest sampling times. All the values were relatively low. Average Bray-Curtis similarities were also calculated for biofilm communities and biofilter water communities within each system over time. The biofilm communities were generally more similar within each system than the biofilter water and the biofilm communities similarity were highest in the 12‰ RAS. The biofilter water had low average Bray-Curtis similarities.

A



B

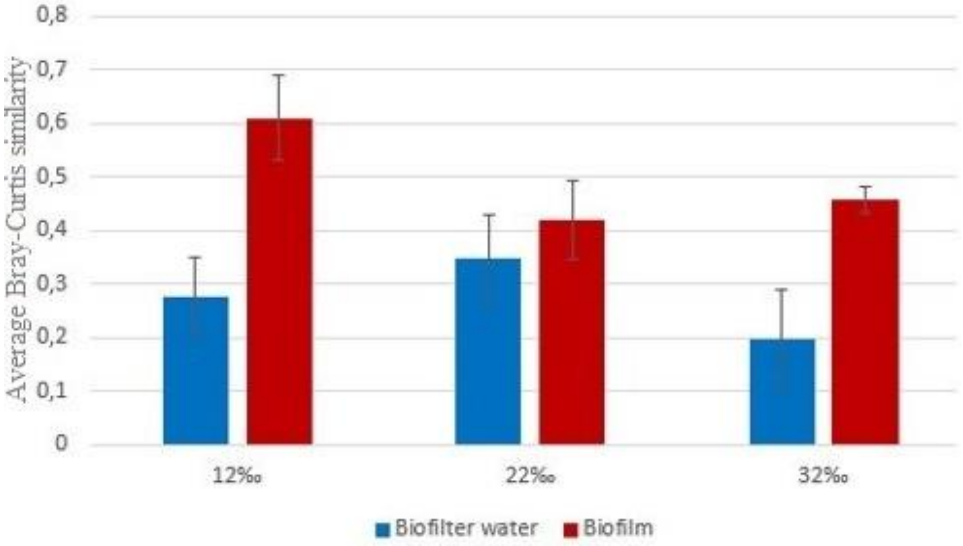


Figure 4.13: Average Bray-Curtis similarities with standard deviation for comparisons of A: biofilter water communities between salinities and biofilm communities between salinities and B: biofilter water within each system and biofilm communities within each system over time.

From the DGGE gels, 32 bands were excised for determining the DNA sequences for obtaining taxonomic assignments. Of these bands, sufficient sequence quality for classification was obtained for 28.

Table 4.2: The taxonomic assignments of the excised DNA sequences.

Band	Figure number	Salinity	Lowest taxonomic assignment (conf. Threshold >50%)
1	4.1A	12‰	Actinomycetales (96%)
2	4.1A	12‰	Gammaproteobacteria (58%)
3	4.1A	12‰	Alphaproteobacteria (78%)
4	4.1A	12‰	Alphaproteobacteria (77%)
5	4.1C	12‰	Alphaproteobacteria (61%)
6	4.1C	12‰	Alphaproteobacteria (61%)
7	4.1C	12‰	Alphaproteobacteria (70%)
8	4.6B	22‰	Flavobacteriaceae (97%)
9	4.6B	22‰	Alphaproteobacteria (58%)
10	4.6B	22‰	Sulfitobacter (67%)
11	4.6B	22‰	Phaeobacter (88%)
12	4.6B	22‰	Sulfitobacter (52%)
13	4.6B	22‰	Alphaproteobacteria (96%)
14	4.6B	22‰	Sulfitobacter (67%)
15	4.6C	32‰	Sulfitobacter (52%)
16	4.6C	32‰	Alphaproteobacteria (83%)
17	4.6C	32‰	Proteobacteria (77%)
18	4.6C	32‰	Rhodobacteraceae (99%)
19	4.6C	32‰	Rhodobaceraceae (100%)
20	4.6C	32‰	Sulfitobacter (67%)
21	4.6C	32‰	Phaeobacter (91%)
22	4.6C	32‰	Rhodobacteraceae (100%)
23	4.10	12‰	Betaproteobacteria (57%)
24	4.10	12‰	Proteobacteria (77%)
25	4.10	12‰	Rhodobacteraceae (66%)
26	4.10	12‰	Rhizobiales (54%)
27	4.10	12‰	Pseudorhodobacter (85%)
28	4.10	32‰	Rubritalea (86%)

5. Discussion

5.1 Evaluation of the methods

To investigate the impacts of salinity on the microbial communities in RAS, the rearing water, biofilter water and biofilm carriers had to be monitored. The PCR-DGGE method was used since it is well suitable for comparing the microbial community dynamics by running multiple samples simultaneously and separate the universal bacterial 16S rRNA gene. The method are also relatively cheap and fast. However, it is not beneficial to compare community profiles from different DGGE gels to each other since it is difficult to reproduce the exact same band pattern between gels due to the denaturing gradients and the conditions of electrophorese. Therefore, selecting samples to be compared had to be considered. The DGGE is not an optimal method for classifying microbial communities taxonomically. One can re-amplify bands in the DGGE to get some taxonomic assignments, but the quality of the sequencing results varies. High throughput sequencing of the 16S rDNA gene is more effective for taxonomical classification of microbial communities, but these methods are more expensive and are in need of bioinformatics (Clarridge, 2004, Nikolaki et al., 2013).

The Taq DNA polymerase (VWR) used in this universal PCR amplification contains some bacterial DNA. This bacterial DNA is amplified along with the samples and can give false-positive results (Tseng et al., 2003). For some samples, the non-template PCR control showed the same amounts of PCR products that was obtained for the samples. Several rounds with optimization of the amplification step had to be conducted, especially for the water samples. The samples were accepted for DGGE analysis only when they clearly had higher band-intensity than the product for the non-template control on the agarose gel.

The objective was to add equal amount of PCR-product for all the samples to the DGGE gel and the amount PCR product added to the DGGE gel were considered from the results in the gel electrophoresis, which showed varying quantities. Therefore, the intensity of the community profiles for some samples had higher or lower band-intensities than the other samples run in the DGGE. The intensity can affect the DGGE profiles of the samples, by leading to differences between the DGGE profiles that are not due to actual differences in

community structure between the samples. The DGGE method gave a good separation at 35-55% gradient. The DGGE profiles had to be analyzed in the Gel2k software to sort all the bands in a band pattern table, which was complicated due to the high band richness. Not all of the DGGE profiles were registered as a band and not all the bands were placed correctly. One band on the gel should theoretically correspond to a bacterial specie. However, several bands can originate from one bacterial specie due to the presences of many gene copies. In addition, several DNA molecules with varying sequences that can have the same denaturing point that makes them stop at the same location. Despite of this, band richness still reflects the species richness, and the similarities between the DGGE profiles reflects the similarity between the microbial communities (Ranjard et al., 2000).

5.2 Effect of salinity on the suspended microbial communities in rearing water

The community profiles of the rearing water from the three RAS were found to be significantly different from each other (Figure 4.3). The same freshwater and seawater sources were used to create the salinities in the three RAS. This indicates that the salinity affects the rearing water since they are significantly different, even though they have the same bacterial starting point and rearing conditions. The diversity indices of the rearing waters communities (figure 4.2) showed no clear differences between the different systems. The 12‰ RAS had high average diversity indices at the first sampling, which seem to decrease in the two last samplings dates. The diversity indices of the 32‰ RAS rearing water communities were highest at the last sampling date. The RAS' was started up with addition of freshwater containing bacteria adapted to that environment. The higher amount of salt added to the systems, the higher the selection pressure will be due to the harsh salinity condition. In the 12‰ RAS, it can be expected high diversity indices in the beginning, since the selection pressure is lower in this system. The decrease in diversity indices in the 12‰ RAS at the later sampling dates may be due to the ongoing low selection pressure, where only the bacteria adapted survives the conditions. In the systems with higher salinities, the selection pressure would be stronger, and when bacteria have had the time to adapt to their new environment there can be a development of high species richness of adapted bacteria, which is seen in the diversity indices of 32‰ RAS' last samples. Mostly freshwater and seawater, of approximately 32‰ are found in nature, which gives a high amount of bacteria that are highly

adapted to these environments (Zahran, 1997). However, the seawater is generally found to have less bacterial diversity than the freshwater due to its challenging salinity condition (Barberán and Casamayor, 2010). The systems had been running for three months already (October 2012) and it is possible that the diversity indices may have been even higher for 12‰ RAS and even lower for the 32‰ RAS before the first sampling due to selection pressure differences in the systems.

The rearing water from the replicate tanks showed generally high similarity in microbial communities within the systems over time (Figure 4.4A). Both 12‰ RAS and 32‰ RAS rearing water communities were found to have high similarities in the first and last sampling. The high similarity in communities within the replicate tanks of 32‰ RAS may be due to the high selection pressure, which can result in low species richness. A low species richness gives a less diverse microbial community (Moussa et al., 2006), which leads to higher similarities in communities within the different parts of the system. However, the different RAS' had already been running for three months before the first sampling date and the microbial communities may have overcome the selection pressure and adapted to the different conditions. The high average Bray-Curtis similarities within the three systems over time may be due to the possibility that the same specialized bacteria have adapted within the replicate rearing tanks within each systems. However, the development in similarity may also have been affected by the total RAS volume exchange rate. At the start of January, there was an increase in the total RAS volume exchange rate, where the systems had the highest total exchange rate in the end of February; the 12‰ RAS had a total volume exchange rate of 28.5%, 22‰ RAS had 26.7% and the 32‰ RAS had 24.6%. The total volume exchange rate thereafter decreased for both 12‰ RAS and 22‰ RAS, and all the RAS' had an exchange rate between 25.2-23.4% in May. The decrease in similarity between the replicate tanks in 12‰ RAS and 32‰ RAS at February 26 can be due to the higher total volume exchange rate. The decrease in total volume exchange rate can also be the reason why the similarity in 12‰ RAS and 32‰ RAS communities between the replicate tanks increases in the last sampling.

The 22‰ vs 32‰ communities had the highest similarity at the first and last sampling (Figure 4.4B). However, the comparison between the 12‰ vs 32‰ RAS communities was found higher in stability and similarity than the comparison of the two brackish waters 12‰ vs 22‰

communities, which was the comparison expected to be high in similarity. It was expected that the systems with closer salinities were more similar to each other than the lowest and highest salinity RAS. The dissimilarity in communities from the 22‰ RAS may be due its larger scale design. The 22‰ RAS had a 57 m^3 MBBR, compared to the other two systems 21 m^3 MBBR. This may have had an impact on the results, even though the media volume were filled up to 50% in all the systems (Terjesen et al., 2013).

5.3 Temporal development of the suspended microbial communities in rearing water in the three RAS with different salinities

The intensity of some DGGE bands increased while some bands disappeared over time (Figure 4.6) which indicate that some species disappear while some species become common in the systems over time. This is due to the many factors, like changing operational and environmental conditions, and random events in the systems. The selection pressure can also be an explanation. The rearing water communities within a system were found significantly different from each other (Figure 4.8) at different dates. The rearing waters diversity indices (Figure 4.7) showed that in both 12‰ RAS and 22‰ RAS the average values decreased over time, while the average values for 32‰ RAS' diversity indices increased. This may be due to a still ongoing selection pressure in the 32‰ RAS at first, which at later samplings may develop to be dominated by many bacteria adapted to the high salinities. The decreasing diversity indices of 12‰ RAS and 22‰ RAS may be due to lower amount of species that can adapt to these conditions. The similarity in microbial community composition in the different rearing tanks within each system was found highly similar (Figure 4.9A). The 3.Jan vs 7.May comparison within each system was expected to be highly dissimilar in microbial communities due to the development in this relative long time lapse, which was also the case. The comparisons between dates showed that the rearing water samples microbial communities clearly developed over time. It has been shown that microbial stability is important for the survival and growth of marine cod larvae (Attramadal et al., 2014). In most fish species, including Atlantic salmon, it is not until awhile after hatching that the juveniles have a fully matured specific immune system and are very sensitive to opportunistic bacteria. Therefore is a stable microbial community important for the juveniles' health (Vadstein et al., 2007, Attramadal et al., 2012). The accumulative survival of the postsmolt through the entire trial,

from the starting weight of 68.3g (±1.2g) to final average weights of 725.7g (±69.3g), showed that 12‰ and the 22‰ RAS had high survival percentages, with up to 95% survival. In the 32‰ RAS' cumulative survival decreases when the postsmolt reached 200g and was on average of 71% at the end of the trial. In this case it is not known how the unstable microbial communities in the rearing tanks affects the health of the fish over time. However, the rearing environment in 32‰ RAS were more unstable than the other systems, which can have contributed to the mortalities (Kolarevic et al., 2014). The unstable environment can have caused growths of *r*-strategists, which can have interacted with the postsmolt.

5.4 Effects of salinity on the microbial communities in the biofilters

The biofilm carriers from the different systems had similar microbial composition and developed to become highly similar across salinities at the last sampling (Figure 4.12). This development was supported in Figure 4.3, where the biofilm communities from the different salinities became more similar in microbial communities to each other over time (Figure 4.3). The biofilm communities between the systems had a stable and higher similarity over time (Figure 4.5B) than between the biofilm community and its rearing water community in each system over time (Figure 4.5A). The biofilm communities were found dissimilar from the microbial communities in its incoming and outgoing biofilter tank water (Figure 4.12). The biofilter waters communities changed over time and appeared different at different salinities. The communities of the biofilter water going in and out of the biofilter tanks were highly similar in all the systems at each date. This indicate that the water communities do not seem affected by passing through the biofilter tank. There was a general tendency that the communities in the biofilter inlet and biofilter outlet water became more similar to each other over time within the systems, which may be due to the dominance of some taxa. The biofilm communities between salinities were found to have higher average Bray-Curtis similarities than the microbial communities in biofilter water between salinities over time (Figure 4.13A). The biofilm carriers also had a higher similarity in its microbial communities than the rearing water communities within each system over time (Figure 4.13B). This was supported because biofilm communities were found to have a higher stability and similarity within each system between dates (Figure 4.8 and Figure 4.9B) than the rearing water communities between dates (Figure 4.9A). The biofilm communities were more stable and similar to each other between

salinities and between dates, than the biofilter water communities were. This may be due to the growth of similar bacteria, with different niches, that performs the same processes. Another reason can be that bacteria that are specialized in high salinity conditions also adapts to the systems with lower salinities. The findings suggest that the salinity had a higher impact on the microbial communities in the biofilter water and rearing water than on the biofilm communities.

The biofilm communities were found to be very different from the microbial communities from the rearing water and biofilter water. The dissimilarity in microbial communities between water and biofilm can be due to the rearing water being subjected to treatments in the system when recirculated, while the biofilm bacteria were less prone to the water treatment processes in the RAS. The treatments could be a factor in the dissimilarity between microbial communities in the water and biofilm carriers that develops over time (Blancheton et al., 2013). Bacteria from the rearing water where the ones to colonize the biofilms when starting the system. The biofilm bacteria, especially those that grow inwards in the biofilm, are more protected than the dissolved bacteria against changes in physical and chemical conditions. This can be the explanation for the smaller changes in biofilm communities than in the water communities. The diversity indices of the biofilter samples showed that the biofilm carriers had higher diversity indices than its biofilter water (Figure 4.11). The result was expected due to the proliferation of attached bacteria in the carriers and the accumulation of bacteria from the rearing water (Blancheton et al., 2013) that is protected against washout.

The removal of Total Ammonia Nitrogen (TAN) is varying between the salinities, which can be due to the varying microbial communities in the different systems. The high salt concentration affects the nitrification (Sakairi et al., 1996). The TAN removal efficiency was highest in the 12‰ RAS biofilter, and decreased according to salinity in the other systems. The removal efficiencies for 12‰ RAS were 38% at first sampling and 49% at last sampling, which can indicate an increase in adaption of the microbial communities specialized in nitrification to the 12‰ salinity. The 32‰ RAS had the lowest efficiencies at 12% TAN removal at the first sampling and 17% at the last sampling date, which indicate that the nitrifiers are heavily affected by the salinity (Ytrestøyl et al., 2013).

5.5 Future work

There is still many uncertainties about the optimal conditions for production of Atlantic salmon postsmolt in RAS. The chemical conditions and the optimal microbial composition of the water in RAS are important aspects of RAS production and can be used to improve the already great success of the land-based systems and optimize the fish welfare and production. One important approach would be to investigate the microbial communities found in RAS' and investigate if the bacteria are harmless to the fish, what processes the bacteria perform and the biofilter efficiencies. In this way, one can select for the most important bacteria needed in RAS', which can give excellent stability and growth conditions for the fish. In this case, it is also important to get more knowledge about the physical requirements the Atlantic salmon need in their different life stages in a RAS to secure their welfare, performance and health.

For further work on this experiment it would have been an idea to look at the microbial composition in the system over a longer period, to see the further development of the microbial communities and their function. It would also have been interesting to compare the microbial communities in the system to the incoming new water, to see if the systems microbial communities were somewhat similar and influenced by this water. Another suggestion would be to compare several carriers from the same system and date to each other, to see the variation between the carriers in the same bioreactor. To investigate further the microbial communities found in the experiment it would be an idea to sequence the bacteria using Illumina amplicon sequencing.

6. Conclusion

- The results of this master thesis found that the salinity affected the microbial communities since the RAS' with different salinities had different communities associated with rearing water, biofilter water and biofilm carriers.

- The biofilm communities developed to become more dissimilar from its corresponding water over time and more similar to the other biofilm communities across salinities. The water communities from the different RAS were significantly different from each other at all sampling dates. The microbial communities associated with the biofilm carriers and its corresponding water were generally dissimilar from each other.

- There were high similarities among the microbial communities associated with rearing water and biofilter water within each RAS at each date, but there were significant differences in water communities between the different sampling dates. The biofilm communities were found to be relatively similar to each other between dates, which indicates that the biofilm communities were more stable over time in each system than the water communities.

References

Anderson, M. J. (2005). "Permutational multivariate analysis of variance." Department of Statistics, University of Auckland, Auckland.

Anderson, M. J. and D. C. I. Walsh (2013). "PERMANOVA, ANOSIM, and the Mantel test in the face of heterogeneous dispersions: What null hypothesis are you testing?" Ecological Monographs **83**(4): 557-574.

Andrews, J. and R. Harris (1986). r- and K-Selection and Microbial Ecology. Advances in Microbial Ecology. K. C. Marshall, Springer US. **9:** 99-147.

Asche, F., et al. (2013). "SALMON AQUACULTURE: LARGER COMPANIES AND INCREASED PRODUCTION." Aquaculture Economics & Management **17**(3): 322-339.

Aslan, S. and E. Simsek (2012). "Influence of salinity on partial nitrification in a submerged biofilter." Bioresource Technology **118**(0): 24-29.

Attramadal, K. J. K., et al. (2012). "Recirculation as a possible microbial control strategy in the production of marine larvae." Aquacultural Engineering **46**(0): 27-39.

Attramadal, K. J. K., et al. (2014). "RAS and microbial maturation as tools for K-selection of microbial communities improve survival in cod larvae." Aquaculture **432**(0): 483-490.

Barberán, A. and E. O. Casamayor (2010). "Global phylogenetic community structure and ?-diversity patterns in surface bacterioplankton metacommunities." Aquatic Microbial Ecology **10**: 1-10.

Bergheim, A. (2012). "Recent growth trends and challenges in the Norwegian aquaculture industry." Latin American Journal of Aquatic Research **40**(3): 800-807.

Bergheim, A., et al. (2009). "Production of Atlantic salmon smolts in Europe—Current characteristics and future trends." Aquacultural Engineering **41**(2): 46-52.Beveridge, M. C. M. (2004). Cage aquaculture. Oxford, UK, Blackwell Pub.

Björnsson, B. T., et al. (2011). "Environmental endocrinology of salmon smoltification." General and Comparative Endocrinology **170**(2): 290-298

Blancheton, J. P., et al. (2013). "Insight into bacterial population in aquaculture systems and its implication." Aquacultural Engineering **53**(0): 30-39.

Bostock, J., et al. (2010). "Aquaculture: global status and trends." Philos Trans R Soc Lond B Biol Sci **365**(1554): 2897-2912.

Bray, J. R. and J. T. Curtis (1957). "An Ordination of the Upland Forest Communities of Southern Wisconsin." Ecological Monographs **27**(4): 326-349.

Bœuf, G. and P. Payan (2001). "How should salinity influence fish growth?" Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology **130**(4): 411-423.

Chen, S., et al. (2006). "Nitrification kinetics of biofilm as affected by water quality factors." Aquacultural Engineering **34**(3): 179-197.

Clarridge, J. E., 3rd (2004). "Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases." Clin Microbiol Rev **17**(4): 840-862, table of contents.

Dahl, C., et al. (1997). "Combined biological nitrification and denitrification of high-salinity wastewater." Water Science and Technology **36**(2–3): 345-352.

Dalsgaard, J., et al. (2013). "Farming different species in RAS in Nordic countries: Current status and future perspectives." Aquacultural Engineering **53**(0): 2-13.

Di Bella, J. M., et al. (2013). "High throughput sequencing methods and analysis for microbiome research." Journal of Microbiological Methods **95**(3): 401-414.

Drenstig, A. et al. (2011) "Norwegian salmon smolt farms embracing RAS to raise production". Global Aquaculture Advocate July/August: 67-69 .

FAO (2014). State of world fisheries and aquaculture. Rome, Food and Agriculture Org. of the United Nations.

Fdz-Polanco, F., et al. (2000). "Spatial distribution of heterotrophs and nitrifiers in a submerged biofilter for nitrification." Water Research **34**(16): 4081-4089.

Gatesoupe, F. J. (1999). "The use of probiotics in aquaculture." Aquaculture **180**(1–2): 147-165.

Hammer, Ø., et al. (2001). "PAST-Palaeontological statistics." www. uv. es/~ pardomv/pe/2001_1/past/pastprog/past. pdf, acessado em **25**(07): 2009.

Hess-Erga, O.-K., et al. (2010). "Recolonization by heterotrophic bacteria after UV irradiation or ozonation of seawater; a simulation of ballast water treatment." Water Research **44**(18): 5439-5449.

Hoar, W. S. (1976). "Smolt Transformation: Evolution, Behavior, and Physiology." Journal of the Fisheries Research Board of Canada **33**(5): 1233-1252.

Iversen, M., et al. (2005). "Stress responses in Atlantic salmon (Salmo salar L.) smolts during commercial well boat transports, and effects on survival after transfer to sea." Aquaculture **243**(1–4): 373-382.

Parameswaran, P., et al. (2007). "A pyrosequencing-tailored nucleotide barcode design unveils opportunities for large-scale sample multiplexing." Nucleic Acids Research **35**(19): e130.

Joensen, R., 2008. Resirkulering av vand i oppdrett. Presentation at Seminar of Recirculation of Water in Aquaculture, 27–28 February 2008, Sunndalsøra, Norway (in Danish)

Kaiser, M. J. (2011). Marine ecology: processes, systems, and impacts. Oxford, Oxford University Press.

Kolarevic, J., et al. (2014). "Performance and welfare of Atlantic salmon smolt reared in recirculating or flow through aquaculture systems." Aquaculture **432**(0): 15-25.

Kumar, V. J. R., et al. (2011). "Nitrification in a packed bed bioreactor integrated into a marine recirculating maturation system under different substrate concentrations and flow rates." Journal of Chemical Technology & Biotechnology **86**(6): 790-797.

Leonard, N., et al. (2002). "Bacteria and nutrients—nitrogen and carbon—in a recirculating system for sea bass production." Aquacultural Engineering **26**(2): 111-127.

Liu, L., et al. (2012). "Comparison of next-generation sequencing systems." J Biomed Biotechnol **2012**: 251364.

Liu, Y., et al. (2011). "Wild and farmed salmon in Norway—A review." Marine Policy **35**(3): 413-4

Losordo, et al (1998). "Recirculating Aquaculture Tank Production Systems – A overview of critical considerations". SRAC Publication No.451

Michaud, L., et al. (2006). "Effect of particulate organic carbon on heterotrophic bacterial populations and nitrification efficiency in biological filters." Aquacultural Engineering **34**(3): 224-233.

Moussa, M. S., et al. (2006). "Long term effects of salt on activity, population structure and floc characteristics in enriched bacterial cultures of nitrifiers." Water Research **40**(7): 1377-1388.

Muyzer, G., et al. (1993). "Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA." Appl Environ Microbiol **59**(3): 695-700.

Muyzer, G. (1999). "DGGE/TGGE a method for identifying genes from natural ecosystems." Current Opinion in Microbiology **2**(3): 317-322.

Muyzer, G. and K. Smalla (1998). "Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology." Antonie van Leeuwenhoek **73**(1): 127-141.

Nikolaki, S. and G. Tsiamis (2013). "Microbial Diversity in the Era of Omic Technologies." BioMed Research International **2013**: 15.

Nogueira, R., et al. (1998). "Influence of dissolved oxygen on the nitrification kinetics in a circulating bed biofilm reactor." Bioprocess Engineering **19**(6): 441-449.

Piedrahita, R. H. (2003). "Reducing the potential environmental impact of tank aquaculture effluents through intensification and recirculation." Aquaculture **226**(1–4): 35-44.

Ranjard, L., et al. (2000). "Monitoring complex bacterial communities using culture-independent molecular techniques: application to soil environment." Research in Microbiology **151**(3): 167-177.

Rasband WS. ImageJ, U.S. National Institutes of Health, Bethesda, Maryland, USA, imagej.nih.gov/ij/, 1997—2012

Rusten, B., et al. (2006). "Design and operations of the Kaldnes moving bed biofilm reactors." Aquacultural Engineering **34**(3): 322-331.

Sakairi, M. A. C., et al. (1996). "Nitrogen removal in seawater using nitrifying and denitrifying bacteria immobilized in porous cellulose carrier." Water Science and Technology **34**(7–8): 267-274.

Salvesen, I., et al. (1999). "Growth of turbot (Scophthalmus maximus L.) during first feeding in relation to the proportion of r/K-strategists in the bacterial community of the rearing water." Aquaculture **175**(3–4): 337-350.

Sandvold, H. N. and R. Tveterås (2014). "INNOVATION AND PRODUCTIVITY GROWTH IN NORWEGIAN PRODUCTION OF JUVENILE SALMONIDS." Aquaculture Economics & Management **18**(2): 149-168.

Schreier, H. J., et al. (2010). "Microbial diversity of biological filters in recirculating aquaculture systems." Current Opinion in Biotechnology **21**(3): 318-325.

Sharrer, M. J., et al. (2007). "Membrane biological reactor treatment of a saline backwash flow from a recirculating aquaculture system." Aquacultural Engineering **36**(2): 159-176.

Sheffield, V. C., et al. (1989). "Attachment of a 40-base-pair G + C-rich sequence (GC-clamp) to genomic DNA fragments by the polymerase chain reaction results in improved detection of single-base changes." Proceedings of the National Academy of Sciences **86**(1): 232-236.

Skjermo, J. and O. Vadstein (1999). "Techniques for microbial control in the intensive rearing of marine larvae." Aquaculture **177**(1–4): 333-343.

Southgate, P. C. and J. S. Lucas (2012). Aquaculture: farming aquatic animals and plants. Chichester, Wiley-Blackwell.

Sudarno, U., et al. (2011). "Effect of varying salinity, temperature, ammonia and nitrous acid concentrations on nitrification of saline wastewater in fixed-bed reactors." Bioresource Technology **102**(10): 5665-5673.

Sugita, H., et al. (2005). "Microbial communities associated with filter materials in recirculating aquaculture systems of freshwater fish." Aquaculture **243**(1–4): 403-409.

Terjesen, B. F., et al. (2013). "Design, dimensioning, and performance of a research facility for studies on the requirements of fish in RAS environments." Aquacultural Engineering **54**(0): 49-63.

Tringe, S. G. and P. Hugenholtz (2008). "A renaissance for the pioneering 16S rRNA gene." Current Opinion in Microbiology **11**(5): 442-446.

Tseng, C. P., et al (2003). "Broad-Range Ribosomal RNA Real-Time PCR after Removal of DNA from Reagents: Melting profiles for Clinically Important Bacteria." Clinical Chemistry **49**(0): 306-309.

Vadstein, O. (1997). "The use of immunostimulation in marine larviculture: possibilities and challenges." Aquaculture **155**(1–4): 401-417.

Vadstein, O., et al. (2007). Microbial Interactions, Prophylaxis and Diseases. Culture of Cold-Water Marine Fish, Blackwell Publishing Ltd**:** 28-72.

Wang, Q., et al. (2007). "Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy." Appl Environ Microbiol **73**(16): 5261-5267. The RDP Classifier and RDP Library Compare are available online at http://rdp.cme.msu.edu/.

Ytrestøyl et al. (2013) "Effekt av salinitet og trening på vekst, fysiologi og velferd hos stor postsmolt av Atlantisk laks i RAS".

Zahran, H. H. (1997). "Diversity, adaptation and activity of the bacterial flora in saline environments." Biology and Fertility of Soils **25**(3): 211-223.

Internet links:

www.eurofinsgenomics.eu

www.illumina.com

Appendix

Appendix 1: DNA isolation protocol

Experienced User Protocol

Please wear gloves at all times

1. To the **PowerBead Tubes** provided, add 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-V1-24) 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.

Thank you for choosing the PowerSoil® DNA Isolation Kit.

Technical Information: Toll free 1-800-606-6248, or 1-760-929-9811 Email: technical@mobio.com **Website:** www.mobio.com

Appendix 2: Recipes

Set-up for each PCR amplification and re-amplification of DGGE bands

- 10 x reaction buffer 2,5 µl
- dNTP (10mM of each) 0,5 µl
- $MgCl_2$ (25 (mM) 0,5 µl
- BSA 0,75 µl
- Primer fwd (10 µM) 0,75 µl
- Primer rev (10 µM) 0,75 µl
- Taq DNA polymerase 0,125 µl
- Filtered $_dH_2O$ To total volume of 25 µl

50 x TAE

- 242 g Tris base
- 57,1 ml Glacial acetic acid
- 100 ml 0,5 EDTA (pH 8,0)
- Add $_dH_2O$ until the total volume is 1000 ml

1 x TAE

- 1960 ml Milli-Q
- 40 ml 50 x TAE

1% Agarose gel with GelRed

- 4 g agarose
- 400 ml 1 x TAE
- Heat in a microwave until the agarose has dissolved. Add Gel red
- 20 µl GelRed when the solution has cooled to 65˚C.

Deionized formamide

- 200 ml formamide
- Add 7,5 g DOWEX RESIN AG 501X8 to deionize the solution
- Stir for 1 hour
- Separate the liquid to be used from the beads

0% denaturing acrylamid solution:

8% acrylamide in 0,5 x TAE (per 250 ml):

- 50 ml 40% acrylamide solution (BioRadLab Inc., Ca., USA)
- 2,5 ml 50 x TAE
- Store the solution at 4˚C, protected from light

80% denaturating acrylamide solution:

8% acrylamide, 5.6M urea, 32% formamide in 0,5 x TAE (per 250 ml):

- 50 ml 40% acrylamide solution (BioRadLab Inc., Ca., USA)
- 2,5 ml 50 x TAE
- 84 g Urea
- 80 ml Deionized formamide
- Store the solution at 4˚C, protected from light

10% APS (ammonium persulfate):

- 10 **g** Ammonium persulfate
- 100 ml $_dH_2O$

Composition of the low and high denaturing solution used:

Denaturing %	0%	80%	TEMED+10%APS	Total volume
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

0% "Stacking gel":

- 8 ml 0% acrylamide solution
- 10 µl TEMED
- 40 µl 10% APS.

Appendix 3: 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.