NTNU

Norwegian University of
Science and Technology

The effect of membrane filtration on the microbial communities associated with rearing water, gut and skin mucus of Atlantic salmon parr (*Salmo salar*) in recirculating aquaculture systems (RAS)

Anette Voll Bugten

Biotechnology (5 year)
Submission date: May 2018
Supervisor: Ingrid Bakke, IBT
Co-supervisor: Kari Attramadal, IBT

Norwegian University of Science and Technology
Department of Biotechnology and Food Science

Acknowledgements

This master's thesis was part of the research project "Developing water treatment technology for land-based closed containment systems (LBCC-RAS) to increase efficiency by reducing the negative effects of organic matter" (project acronym: RAS-ORGMAT, 2016 – 2018, ERA-Net COFASP). It was a collaboration project between SINTEF Ocean AS (Norway), the University of Copenhagen (Denmark), and CIIMAR and INESC TEC (Portugal). The work of this thesis was carried out at NTNU's Centre of Fisheries and Aquaculture (SeaLab) at Brattørkaia in Trondheim and at the Department of Biotechnology and Food Science at NTNU Gløshaugen. My main supervisor was Ingrid Bakke at the Department of Biotechnology and Food Science and my co-supervisor was Kari Johanne Kihle Attramadal at SINTEF Ocean.

I would like to thank Ingrid and Kari for extraordinary support and guidance throughout the work of this thesis. Your knowledge, constructive feedback and presence have been highly appreciated. Thank you for inspiring me and letting me take part in this really interesting project. I want to thank all the people in the "RAS-ORGMAT group" for both help, laughter and enthusiasm. We were such a team! Mia Tiller Mjøs should be thanked for all the help and advice, both in and outside the laboratory.

I also want to thank my fellow classmates for five fantastic years at NTNU. Last but not least I want to thank my family and friends for always believing in me, encouraging me and supporting me.

Trondheim May 2018 **Anette Voll Bugten**

Abstract

Fish are constantly in contact with microorganisms in the surrounding environment, and their mucosal surfaces; skin, gills and the gastrointestinal tract are colonized by microbiota that play vital functions for their health and welfare. Suboptimal rearing conditions may affect the stability and quality of the microbial communities associated with the rearing water and the fish host, allowing potentially harmful bacteria to proliferate.

Small accumulating organic particles are a problem in recirculating aquaculture systems (RAS) and affects both the water quality and the health of reared Atlantic salmon parr. The objective of this thesis was to investigate the effect of membrane filtration on the microbial communities of the rearing water, gut and skin mucus of Atlantic salmon parr. Water and fish microbiota from two RASs, one conventional RAS (cRAS) and one RAS with a membrane filtration step implemented (mRAS), was examined at two different sampling times representing different feed loadings and water exchange rates. The period prior to the first sampling time (t1) was characterized by high water exchange rate and normal feed loading, while moderate water exchange rate and intentional overfeeding characterized the period prior to the second sampling time (t2). The water, gut and skin mucus microbiota were characterized by Illumina sequencing of the V4-region of the bacterial 16S rRNA gene.

The salmon parr reared in mRAS grew larger compared to the fish in cRAS. The water microbiota was significantly different between cRAS and mRAS, with a more diverse microbial community in mRAS. Three OTUs representing *Mycobacterium, Spartobacteria_genera_incertae_sedis* and *Legionella* dominated the water microbiota in cRAS. The first two OTUs increased in abundance at t2. In mRAS, the water communities were dominated by a *Sphaerotilus*-OTU at t1 and a *Flavobacterium*-OTU at t2. Fish reared in mRAS had a significantly different gut microbiota and were more similar between the two sampling times compared to that of fish in cRAS. *Carnobacterium* was the dominant OTU in the gut microbiota of mRAS fish. A *Brochothrix*-OTU increased highly in abundance at t2, causing the largest difference between the two sampling times in mRAS. The OTUs *Mycobacterium, Spartobacteria_genera_incertae_*sedis and *Legionella* were more abundant also in the gut of cRAS fish compared to that of mRAS fish. The two former OTUs increased in abundance at t2, just as in the water microbiota. The skin microbial communities were significantly different at the two sampling times. Both *Mycobacterium* and *Spartobacteria_genera_incertae_sedis*

became more abundant after the feed overloading period (t2) in both systems, with a higher abundance in the skin microbiota of cRAS. The three presumed opportunists associated with the water also colonized the gut and skin mucus of the fish reared in cRAS. This strongly indicates that the water microbiota affected both the gut and skin mucus microbiota of Atlantic salmon parr.

Sammendrag

Fisk er i direkte kontakt med mikroorganismer som koloniserer vannet, og i tillegg, er fiskens mukosale overflater; skinn, gjeller og tarm, kolonisert av mikrober som spiller en vital rolle for vertens helse og velferd. Oppdrettsforhold som ikke er optimale har potensial til å påvirke stabiliteten og kvaliteten til de mikrobielle samfunnene i vannet samt mikrobene som koloniserer fisken. Dette kan føre til at potensielt patogene bakterier øker i antall.

Små organiske partikler som akkumuleres i vannsøylen er et problem i resirkulerende akvakultur systemer (RAS). De påvirker både den mikrobielle vannkvaliteten og lakseparrens helse. I denne masteroppgaven var det av interesse å undersøke hvilken effekt membranfiltrering har på det mikrobielle samfunnet som koloniserer vann, tarm og skinn hos Atlantisk lakseparr. Vann og fiskemikrobiota fra to RAS, ett konvensjonelt RAS (cRAS) og ett system med membranfiltrering (mRAS) ble undersøkt ved to prøvetidspunkt som representerte ulik fôringsbelastning og vannutvekslingsgrad. Perioden før prøvetidspunkt 1 (t1) var karakterisert med høy vannutveksling og normal fôringsbelastning, mens perioden før prøvetidspunkt 2 (t2) var karakterisert med moderat vannutveksling og høy fôringsbelastning av systemet. Vann, tarm og skinnmikrobiota ble karakterisert ved Illuminasekvensering av V4-regionen til det bakterielle 16S rRNA-genet.

Lakseparren i mRAS var større sammenliknet med parren i cRAS ved eksperimentslutt. Vannmikrobiotaen var signifikant forskjellig mellom cRAS og mRAS, med et mer mangfoldig mikrobielt samfunn i mRAS. *Mycobacterium, Spartobacteria_genera_incertae_sedis* og *Legionella* var de tre mest vanlige OTUene i vannet i cRAS. De to første ble mer vanlig ved t2. En *Sphaerotilus*-OTU dominerte i vannet i mRAS ved t1, mens en *Flavobacterium*-OTU ble mer vanlig ved t2. Det var signifikante forskjeller i tarmmikrobiota mellom fisk i cRAS og mRAS. Tarmmikrobiotaen for fisk i mRAS var mer lik ved de to prøvetidspunktene sammenliknet med fisk i cRAS. *Carnobacterium* var den dominerende OTU i tarmmikrobiotaen hos fisk i mRAS. Ved prøvetidspunkt 2 ble *Brochothrix* mye mer vanlig i mRAS, og skapte de største forskjellene mellom de to prøvetidspunktene. *Mycobacterium, Spartobacteria_genera_incertae_sedis* og *Legionella* var også de tre mest vanlige OTUene i tarmmikrobiotan hos fisk i cRAS sammenliknet med fisk i mRAS, hvor de to førstnevnte ble mer vanlig ved t2, slik som for vannmikrobiotaen. Det mikrobielle samfunnet tilknyttet fiskens skinn var signifikant forskjellig mellom de to prøvetidspunktene. Både *Mycobacterium* og *Spartobacteria_genera_incertae_sedis* ble mer vanlig ved t2 i begge system etter økt

fôringsbelastning. De var mer vanlig i skinnmikrobiotaen for fisk i cRAS. Siden de tre antatte opportunistene i vannet også var i stand til å kolonisere fiskens slimlag, er dette en sterk indikasjon på at vannmikrobiotaen påvirket den Atlantiske lakseparrens tarm- og skinnmikrobiota.

Abbrevations

AOB	Ammonia oxidizing bacteria
BF	Biofilter
cRAS	conventional RAS
DOM	Dissolved organic matter
DS	Drum screen
F	Fish
FT	Fish tank
FTS	Flow-through system
G	Gut
GI	Gastrointestinal (tract)
HRT	Hydraulic retention time
HTS	High-throughput sequencing
LBCC	Land-based closed containment
LBS	Land-based systems
MBB	Moving-bed biofilter
MF	Membrane filter
mRAS	membrane RAS
NOB	Nitrite oxidizing bacteria
OTU	Operational taxonomic unit
PCR	Polymerase chain reaction
POM	Particulate organic matter
RAS	Recirculating aquaculture system
S_x	Sump
SGR	Specific growth rate
SM	Skin mucus
TAN	Total ammonia nitrogen
TGC	Thermal-unit growth coefficient
TMP	Transmembrane pressure
W	Water

Table of contents

1. Introduction

1.1 The Aquaculture Industry

Aquaculture is the cultivation of marine or freshwater species, with some form of control over the population, like feeding and adjustment of water quality. It is thought to be among the fastest growing primary industries in the World, where China is one of the major contributors, producing over 60 % of the total and is the top exporter of aquaculture products (FAO, 2016). One of the reasons for the growth since 1990 is increased knowledge and improvements related to both biology, technology and nutrition. Due to the worlds' increasing population and limited food resources, aquaculture and fisheries is believed to have a great potential in securing enough food. The industry is also offering jobs, which have both positive economic and social impacts (FAO, 2016).

Norway is an important contributor to this growing industry. In 2016, the total production in the aquaculture sector was 1.3 million tons in total in Norway. The proportion of the two most produced species, Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*), was 1 233 619 tons and 87 853 tons respectively (SSB, 2017). With this biomass production of Atlantic salmon, the nation is the worlds' largest producer and also the top exporter of salmon (FAO, 2016). Salmon is an important source of nutritious food, containing high quality protein, omega-3 and important vitamins and minerals, making it a high-valued specie both nutritionally and economically (FAO, 2016).

1.1.1 The biology of Atlantic salmon (Salmo salar)

Atlantic salmon is a species in the Salmonidae family. They are wild caught and geographically distributed at both sides of the North Atlantic Ocean, in rivers located in North-America and Europe (Jobling et al., 2010). It is an anadromous fish, which means that the first years of its life cycle are spent as so-called parr in fresh water, before it smoltify and migrates to the sea. To "smoltify" means that the fish is adapting to higher salinities. Smoltification is a challenging process for the fish, and morphological, physiological and behavioral changes are necessary (Folmar and Dickhoff, 1980, Yamauchi et al., 1985). The smolt spends some years in sea water, where it grows, before the adult salmon return to its parental river again to spawn (Jobling et al., 2010).

1.1.2 The aquaculture production cycle and rearing conditions in land-based systems (LBS)

A normal production cycle from fertilized egg to harvestable salmon takes around three years. The initial fresh water period, carried out in hatcheries and juvenile production facilities, are typically land-based and takes 10 – 16 months. After smoltification, the smolt is transferred to sea cages for on-growing to market size (3 – 7 kg). This production stage takes approximately 14 – 24 months (Jobling et al., 2010). Transfer to sea cages is a challenging process for the smolt and is often characterized by high mortality rates. One of the main reason for this is thought to be suboptimal rearing conditions with low quality smolt outcome not able to handle stressful situations such as handling, transport and other critical situations related to sea transfer (Finstad et al., 2003, Iversen et al., 2005).

Several important factors must be fulfilled to obtain successful cultivation of salmon in land-based systems (LBS) (i.e. maximum growth and optimal health and welfare). To facilitate for optimal rearing conditions throughout the production cycle, it is important to have knowledge about their natural habitat, behavior, life cycle, developmental stages, nutrition, feeding regimes and water quality parameter limits. The increased demand for fish and the on-going intensification has increased the focus on fish health and welfare in order to optimize the rearing. Especially is conditions causing stress of interest (Ashley, 2007). Stress is the result of suboptimal rearing conditions (Lekang, 2013), and is defined by Barton and Iwama (1991, as sited by (Llewellyn et al., 2014) as "a physiological response to overcome a negative environmental stimulus or disturbance". Fish that experience stress over a longer period grow slower because they are forced to use their energy to compensate for suboptimal rearing conditions. In addition, stressed fish is less resistant to diseases caused by pathogenic microorganisms (Llewellyn et al., 2014). There is increased attention on microorganisms associated with the rearing water and the fish host, focusing on microbial water quality and how detrimental bacteria cause stressful situations for the reared fish. The symbiotic communities are thought to change in response to stress, so how to facilitate for stabile microbial communities, both in the rearing water and those associated with the host, is of interest. Homeostasis of the host-associated microbiota is thought to be a central key to obtain optimal fish health and welfare (Chiarello et al., 2015, Llewellyn et al., 2014).

1.2 Smolt production in recirculating aquaculture systems (RAS)

In 2013, there were 193 smolt production facilities for both salmon and rainbow trout in Norway. Of these, 165 were flow-through systems (FTS) and 25 were fully or partly recirculating aquaculture systems (RAS) (Mattilsynet, 2014). According to the Directorate of Fisheries (Fiskeridirektoratet), there was a total of 187 smolt production licences and 117 companies in Norway in 2016, both for salmon, trout and rainbow trout (Fiskeridirektoratet, 2017). How many RAS and FTS facilities that exist on a national basis today is unsure, but RAS is surely increasing in popularity (Badiola et al., 2017). Most of the newly build facilities are RAS and several companies rebuild their flow-through systems into recirculation systems (Personal communication, Fiskeridirektoratet).

RAS is a land-based cultivation system used worldwide in rearing of both marine and freshwater species at different developmental stages. In contrast to traditional flow-through systems, RAS is re-using the outlet water from the rearing tanks (Figure 1.1). The used water is treated in a water treatment loop that controls and adjusts water quality parameters before it enters the tanks again (Lekang, 2013). Water quality variables that need to be controlled is the amount of organic matter in the system, oxygen, pH, temperature, salinity, the concentration of total ammonia nitrogen (TAN), carbon dioxide (CO_2) concentration and the microbial stability, to mention some.

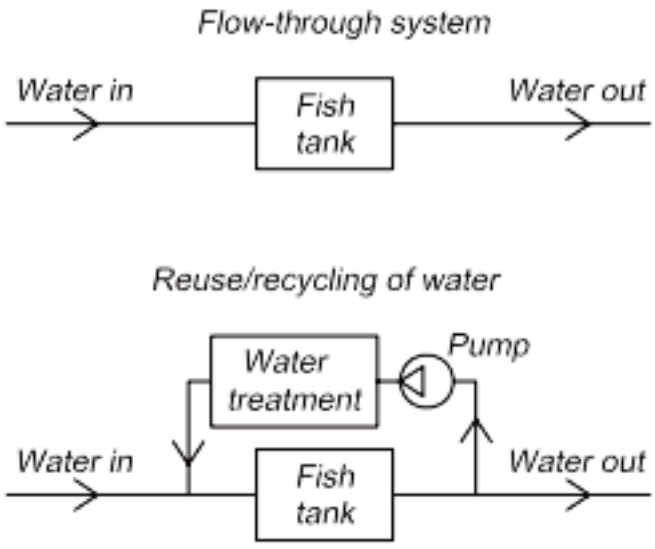


Figure 1.1 Flow-through system (FTS) (top) compared to a recirculation aquaculture system (RAS) (below). Figure from Lekang (2013).

To describe a recirculating aquaculture system, three parameters are important according to Lekang (2013); the degree of re-use, water exchange with respect of fish or feed and the degree of purification. New water is added continuously or in batches, but continuous adding of water is most common. RAS systems allows for 90 – 99 % water recirculation (Badiola et al., 2012).

Recirculating of rearing water has both positive and negative aspects. First of all, the volume of intake water to the system is reduced. This reduces the energy needed to heat and treat the inlet water (Lekang, 2013). With the reduced amount of water, it is easier to obtain a stable water temperature throughout the production year. There is limited access to good quality freshwater sources in the World, which is one of the reasons why many companies choose RAS rather than FTS. The reduction of water consumption is the main reason for why many companies in Norway choose RAS, as the authorities has set a limit for water use not to extend the volume used for salmon production in 2011. Another reason, is that the freshwater sources in Norway is often characterized with low pH, low buffering capacity and variable levels of metals like aluminum (Al) and iron (Fe) (Kristensen et al., 2009). RAS with treatment of the inlet water and treatment of the used water gives the farmer great control of both water quality and environmental parameters and may contribute to a healthier and more robust fish (Hambly et al., 2015, Badiola et al., 2012). Marine larvae reared in RAS has showed to onset growth earlier, have higher survival and higher tolerance to stressful conditions compared to larvae reared in FTS. This emphasizing the importance of the possibility to obtain stable, optimal rearing conditions, also for the microbiota (Attramadal et al., 2012, Verner-Jeffreys et al., 2004).

Investment and operating costs are the two major disadvantages of RAS (Lekang, 2013). Several steps in the water treatment loop are dependent on well-functioning technology and constant supply of electricity to be operated, and in case of failure, there is a need for back-up systems. However, these are not the only challenges. The intensification has brought up several challenges related to technology and biology that need to be solved in order to improve the rearing and outcome in RAS. Important issues suggested to improve the rearing conditions are more efficient removal of accumulated waste products, such as small particles and nitrogen compounds (Martins et al., 2010).

1.3 Water quality parameters and components of the treatment loop

The components of the water treatment loop controls and adjusts the water quality parameters, so that they are well within the acceptable range for the reared species. Optimal water quality parameters for Atlantic salmon are given in Table A.1 (Appendix A). Typically, the first step in the water treatment loop is particle removal by for example mechanical filtration, before nitrifying bacteria in the biofilter convert ammonia to less toxic nitrate. Stripping of the metabolic end product carbon dioxide takes place in a degasser. Oxygen could be supplied at several locations in the system. One opportunity is to add directly to the intake water of each rearing tank. Several aquaculture facilities also include disinfection as one of the last treatments of the re-used water, where ozone and ultraviolet light (UV) are used with the intention to inactivate microorganisms and reduce the concentration to an acceptable level. There is a lot of different technologies and several possibilities of set-up and design of a RAS and its treatment loop. The most optimal is related to the reared species, but also to what the farmers wishes and the functionality of each individual system.

1.3.1 Removal of particles and organic matter

Organic matter is the carbon-containing material in the system. Overfeeding, defecation, shedding of biofilm and dead organisms are all contributing to the organic loading of the rearing water. Organic matter can be classified into particulate organic matter (POM) (> 0.001 μm) and dissolved organic matter (DOM) (< 0.001 μm) based on size (Lekang, 2013). Further, particles may be classified as colloids (0.001 – 1 μm), supercolloids (1 – 100 μm) and suspended settleable particles (> 100 μm) (Lekang, 2013). Suspended non-settleable particles are smaller than 100 μm, and include algae and other living organisms (Holan et al., 2014).

The amount of organic matter in the system should be kept low for several reasons. Firstly, the suspended solids affect the fish health negatively by causing stressful conditions and gill irritation. This may cause reduced growth rates over time (Chapman et al., 1987, Fernandes et al., 2015). Secondly, the system itself could be negatively affected. Bacterial degradation of the particles contributes to oxygen consumption, as well as ammonia and CO_2 production in the system. Large particles have the potential to clog pipes and settle at dead zones where the water current is not optimal. These areas may turn into anoxic zones and release toxic gases such as hydrogen sulfide (H_2S). Moreover, high particle levels have the potential to reduce the effectiveness of ozone and UV disinfection, by shielding the pathogenic microorganisms

(Liltved and Cripps, 1999). Finally, POM and DOM are substrate for heterotrophic bacterial growth. High amounts of organic particles are followed by higher loads of bacteria in the system, which could cause rapid reduction of biofilter nitrification efficiency and additional unwanted problems described in more detail below section 1.3.4. Hence, removal of organic matter from the system is important to obtain optimal rearing conditions and good water quality.

Different removal technologies exist to eliminate both larger and smaller particles. Removal of free moving particles can be done by sedimentation of settleable particles or with hydrocyclones that utilizes the centrifugal forces (Lee, 2015). Hindered separation can be performed by filtration with sieves and grids or with different types of filters. The pore size determines which size of particles are removed. The most commonly used mechanical separation methods are screens and rotating microscreens with pore sizes from 60 to 200 μm available (Barrut et al., 2013, Cripps and Bergheim, 2000). It is difficult to remove particles smaller than 50 μm because they have slow sedimentation rates, and screen with small pore sizes results in reduced flow rate and finally clogging of the screen (Cripps and Bergheim, 2000). The consequence is accumulation of small particles, like colloids, and dissolved organic material in the RAS system's water. This is a problem and affects the water quality negatively (Martins et al., 2010). Protein skimmers and membrane filtration are two technologies capable of removing the accumulating particles (Lekang, 2013).

1.3.2 Membrane filtration technology

Membrane filtration is a physical separation method that has been used for decades in the process industry. It is a relatively new method in treatment of waste water and drinking water (Lekang, 2013). The method is not widespread in the aquaculture sector but considered a promising water treatment technology for removal of accumulating organic particles in land-based recirculating aquaculture systems.

The membrane has a semi-permeable function, meaning that some substances are rejected, while others are allowed to pass. Most of the membrane filters are made of synthetic or natural organic polymers (Sagle and Freeman, 2004, Li et al., 2008). A membrane filter has the potential to remove suspended and colloidal particles from the water. If the pore size is small enough, also microorganisms are removed, thereby functioning as a disinfection step (Li et al., 2008).

The main reason why membrane filtration is rarely used in aquaculture today is pore-blocking and fouling. These problems reduce the efficiency of the membrane and back-flushing is needed (Lekang, 2013). Membrane filters are expensive, so there are also some cost-related issues. Despite this, it is increasing in popularity due to its capability to improve the water quality by removing smaller organic particles that escape the traditional water treatment mechanisms (Wold et al., 2014).

1.3.3 Nitrogen compounds and the biofilter

The end product in the protein catabolism is inorganic nitrogen (ammonia). Most of the ammonia is excreted through the gills and some are released as urea in the urine. Ammonia is toxic for the fish (and other animals), even at low concentrations. The compound induces stress responses and reduce the growth. Longer exposure and high concentrations may have lethal effects (Randall and Tsui, 2002, Ruyet et al., 1997). Ammonia exists in two forms in water; as unionized ammonia (NH_3) and ionized ammonium (NH_4^+), where NH_3 is the most toxic form. The sum of NH_3 and NH_4^+ is called total ammonia nitrogen (TAN). This equilibrium depends on pH, temperature and salinity (Ip et al., 2001, Randall and Tsui, 2002).

The biofilter converts ammonia to less toxic nitrate in a two-step process called nitrification. In the first step, ammonia oxidizing bacteria (AOB) oxidize ammonia to nitrite (NO_2^-) before nitrite oxidizing bacteria (NOB) oxidize nitrite to nitrate (NO_3^-) in the last step. They grow in biofilm at surfaces (Chen et al., 2006). One possible setup commonly used in RAS is the moving-bed biofilter (MBB), where biofilm carriers with large surface area are suspended and mixed due to addition of air at the bottom (Rusten et al., 2006).

The nitrifying bacteria are not the only bacteria occupying space in the biofilter. Also, heterotrophic bacteria are found here, generally representing over 80 % of the biofilm bacterial communities (Michaud et al., 2006). Heterotrophs have higher growth rates than the nitrifiers and can outcompete the nitrifiers if there is enough substrate, oxygen and space available. This creates an unwanted situation since the availability of O_2 is lowered and the concentration of CO_2 increases. Hence, it is important to control the level of heterotrophic bacteria because they could lower the efficiency of the biofilter and have negative effects on the water quality (Fjellheim et al., 2016).

1.3.4 Microbial control in RAS systems

Microbial control is the control of the total number and types of microorganisms in the system. There is of interest to select for desirable water microbiota (i.e. microbial maturation) and to avoid proliferation of detrimental pathogens (Vadstein et al., 1993, Skjermo and Vadstein, 1999).

Microorganisms is a natural part of a recirculating aquaculture system. Some of them interfere with the fish and affect their health positively or negatively by causing diseases. The first barrier to avoid pathogenic entrance is disinfection of the inlet water. Furthermore, blooming of heterotrophic, opportunistic bacterial communities, due to high organic loading, is thought to affect the welfare of the fish negatively (Hambly et al., 2015). These bacteria may turn pathogenic and cause disease when the fish are weakened by stress (Vadstein et al., 1993). Therefore, several commercial recirculating facilities also include a disinfection step in the water treatment loop, intentionally to inactivate and reduce the microbial concentration in their system to an acceptable level.

In commercial facilities there has been little focus on microbial ecology and conditions that promotes microbial stability of the rearing water. Several studies focusing on marine larvae has shown that control of the microbial state of the water is essential to obtain optimal rearing conditions, health and welfare of the reared species (Vadstein et al., 1993, Skjermo et al., 1997, Attramadal et al., 2014). There has been proposed several different strategies to obtain microbial control and stability. The strategies are related to the r/K-selection theory (Vadstein et al., 1993, Skjermo et al., 1997). K-strategists dominates in stable mature environments where there is low supply of substrate per bacteria. They are characterized by their high substrate affinity and low growth rate. On the other side is the fast-growing r-strategists which are considered as opportunistic. They are bad competitors and thrive in unstable pioneer environments where the supply of nutrients per bacteria is high (Vadstein et al., 1993). Detrimental opportunists are mainly found among the opportunistic heterotrophs present in the system. Opportunistic heterotrophs are always present, but to obtain microbial control, the aim should be to keep the percentage down. Proliferation of heterotrophic bacteria is avoided by reducing the amount of organic matter available. In addition, disinfection should be avoided as it creates an unstable environment with high amounts of substrate available per bacteria, conditions that selects for unwanted r-strategists (Blancheton et al., 2013, Attramadal et al., 2012).

Attramadal et. al (2012) have suggested RAS as a possible strategy to obtain K-selection of the water microbiota. RAS operates with a high degree of water re-use, giving a long total system hydraulic retention time (HRT). The water remains in the system for a longer time period due to low water dilution. This keeps the slow-growing bacteria in the system. If a stable supply and removal of organic matter is obtained and disinfection of the re-used water is avoided, it is possible to maintain a low and stable carrying capacity throughout the system. This creates conditions that promotes K-selection, leading to more stable microbial communities over time and less variability between replicate tanks (Attramadal et al., 2012). Further, more efficient removal technologies for accumulating organic particles, such as membrane filtration, may improve the microbial water quality and the fish welfare. Atlantic cod (*Gadus morhua*) larvae reared in a RAS with membrane filtration showed higher survival and growth compared to the control group (Wold et al., 2014).

1.4 Fish – microbe interactions

The mucosal surfaces of vertebrates are colonized by bacteria, protozoa, virus, archaea and fungi, collectively called the microbiota. It is believed that the microorganisms outnumber their host cells by 10 to 1 (Turnbaugh et al., 2007). Several studies in this field has been performed on humans, where the "The Human Microbiome Project" launched in 2007 has been an important contributor in the study of the human microbiome and the relevance in normal physiology and disease (Turnbaugh et al., 2007). Through the studies of humans and model organisms like mice and zebrafish (*Danio rerio*), it has been revealed that commensal microbiota serves vital functions for their host. It is known that they, among other, facilitate digestion and nutrient absorption, synthetize vitamins, protect against pathogens and stimulates the immune system (Fraune and Bosch, 2010, Turnbaugh et al., 2007).

1.4.1 Teleost fish and commensal bacteria

Aquatic environments are highly colonized by microorganisms. Thus, fish and other aquatic animals are constantly in contact with water microbes, which includes both pathogenic and non-pathogenic species. The skin, gills and gastrointestinal (GI) tract are the primary mucosal barriers between the fish and its environment, and the site of colonization by commensal microbiota (Figure 1.2).

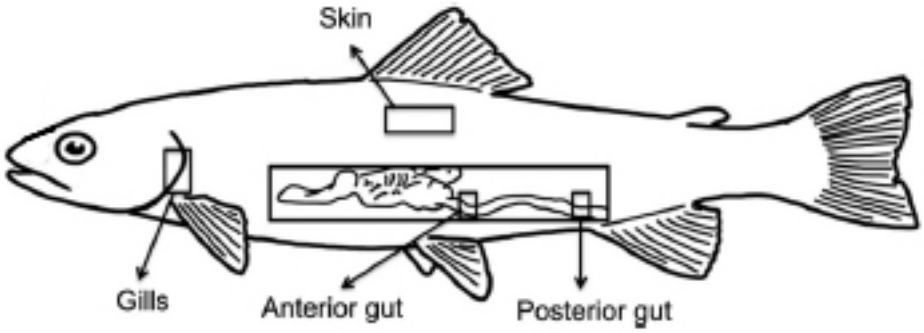


Figure 1.2 The commensal microbiota colonizes the primary mucosal barriers; skin, gills and gut (anterior and posterior gut) of the fish. Figure adapted from Lowrey et. al (2015).

The mucosal surfaces of teleost fish resemble the mammals' in several ways even though there exist some structural and functional differences (Gomez et al., 2013, Peterson, 2015). Mucus is a viscous secrete continually produced and secreted by goblet cells in the epithelial layer (Ángeles Esteban and Cerezuela, 2015). The main constituents are water, electrolytes, lipids and various proteins, making it a nutrient rich substance for adherence and growth of microbiota (Bansil and Turner, 2018, Gomez et al., 2013). It also contains lysozyme and different antimicrobial peptides, creating an antimicrobial barrier for invading pathogens (Bansil and Turner, 2018). A well-functioning mucus is characterized by balanced secretion of mucus and a stable number of commensal bacteria that outcompetes pathogens by competing for substrate or adhesion sites (Olsen et al., 2008).

Colonization of the mucus layers by commensal bacteria happens early in development. Teleost fish eggs becomes colonized possibly during oviposition and/or when it comes in contact with the surrounding water (Llewellyn et al., 2014). Water flow or chemotaxis mechanisms make the microbes come in contact with the egg (Merrifield and Rodiles, 2015). The skin of the newly hatched larvae become colonized by both the existing egg chorion bacteria and additional environmental bacteria (Llewellyn et al., 2014). Microbiota may colonize the gut after mouth opening and first-feeding, indicating that bacteria present at the fish surface, in the water and feed represent the initial gut communities (Llewellyn et al., 2014, Romero and Navarrete, 2006). Throughout life, the bacterial communities develop and matures due to different influencing factors described in the next two sections.

1.4.2 The Commensal Microbiota of the Gastrointestinal Tract

The gastrointestinal tract of salmonids is divided into esophagus, stomach, pyloric caeca, anterior intestine and posterior intestine, and is the site of food digestion (Bone, 2008, Løkka, 2013). Anatomical and physiological differences in each compartment favors different types and numbers of colonizing microbiota. Their main task is to facilitate nutrient absorption and protect against pathogenic invasion (Navarrete et al., 2008). The bacteria colonizing the GI tract are either autochthones or allochthones. Autochthones binds the mucus and allochthones do not. The transient allochthones lack the ability to colonize the mucus epithelial layer, or they are outcompeted, and will thus remain in the luminal content (Nayak, 2010b, Navarrete et al., 2008). Proteobacteria, Firmicutes, Bacteriodetes and Tenericutes has been found to be the dominating phyla in gut microbiota of teleost fish (Llewellyn et al., 2014, Dehler et al., 2017b, Sullam et al., 2012).

Homeostasis of the microbial community is important to avoid disease and maintain good health of the organism. The gut possesses a selective environment and the composition of microbiota is determined by developmental stage, host genetics, diet, stress and the surrounding water (Nayak, 2010b, Sullam et al., 2012, Merrifield and Rodiles, 2015). It is still under debate on which level host genetics determines the composition of commensal bacteria in the gut (and other mucosal surfaces), but it is thought that the innate immune system plays a major role (Llewellyn et al., 2014).

Feed and nutrition is also a factor that may shape the GI microbial communities. During first-feeding of rainbow trout the bacterial load of the gut increased, causing a change in the gut microbial communities (Ingerslev et al., 2014). Ringø et. al (2006) found that the gut microbiota of Atlantic cod fed with fish meal, standard soya bean meal and bioprocessed soya bean meal was affected by their different diets. However, Bakke et. al (2013) found that microbiota associated with the diet may not be the major determinant of shaping the gut microbiota in cod larvae, but rather the microbiota associated with the rearing water.

A third factor that affects the commensal communities is the environment. The physiology of the environment; pH, organic loading, temperature, salinity and so on, determines which microbes are present in the first place. Dehler et. al (2017b) studied the gut associated microbiota in Atlantic salmon parr reared in two different environments, one recirculating laboratory aquarium and one open freshwater loch, and found that the environment most likely

was the reason for different gut community composition representing the two groups. One of the reasons stated was that the fish in the natural open loch was exposed to higher bacterial loads and had access to additional food sources. In addition, the composition of bacterial communities in the intestine were found to differ between freshwater species and species living in seawater (Nayak, 2010b, Roeselers et al., 2011).

1.4.3 Teleost Skin and Associated Microbiota

The skin is the largest organ of the body and is often referred to as the integumentary system. It serves many functions, such as maintaining body shape, protection against physical damage, as well as invasion by pathogens. It consists of three layers, the mucus layer, epidermis and dermis, where the symbiotic microbiota colonize the outermost mucosal surface (Ángeles Esteban and Cerezuela, 2015).

The microbiota associated with the skin of teleost have been less studied than those associated with the gut. Still, little is known about the residing bacteria and their function and role in fish health (Lowrey et al., 2015). Studies so far, have found that the phyla Proteobacteria, Bacteriodetes, Firmicutes and Actinobacteria is abundant at teleost skin (Boutin et al., 2014, Lowrey et al., 2015, Chiarello et al., 2015). Lowrey et. al (2015) found that the diversity of bacteria associated with the skin of rainbow trout was higher than those associated with internal surfaces and may be due to the skins direct contact with the surrounding water.

The skin and its mucosal surface is the first barrier between the fish and its environment, and like the gut, influenced both by environmental factors and host-associated factors (Chiarello et al., 2015). These interacting factors create different physical and chemical surfaces colonized by microbiota that vary between teleost species, individuals and body parts (Larsen et al., 2013, Chiarello et al., 2015). Studies performed on Atlantic salmon migrating from freshwater to seawater, found that the phylogenetic diversity of skin mucus increased in the marine stage, indicating an environmental effect (Lokesh and Kiron, 2016). Host-associated factors that shapes the skin microbiota are genetics and excretion of metabolic waste and mucus. The different body parts of the fish are exposed to water flow and secrete in varying degree, creating different colonization surfaces for the microbiota (Chiarello et al., 2015).

To summarize, the gut and skin microbiota are influenced by several of the same complex shaping factors. Still the bacterial communities in these two mucosal habitats are dominated by

different genera, indicating that different selection pressures exist (Merrifield and Rodiles, 2015, Dehler et al., 2017b). Water and fish mucus surfaces has shown to be colonized by different bacterial communities (Minniti et al., 2017, Giatsis et al., 2015). As mentioned, different treatment of the rearing water, such as disinfection, microbial maturation and membrane filtration affects the microbial communities associated with the system water. But it is still unknown if and how the water microbiota affects the mucus associated microbiota which is determinant for fish health and welfare.

1.5 Investigation of microbial diversity – High-throughput sequencing (HTS) of 16S rRNA amplicons

The study of teleost symbiotic microbiota was previously based on culture-dependent methods. It is well-known that culture-dependent methods underestimate the bacterial diversity in samples from natural habitats (von Wintzingerode et al., 1997). Actually, more than 99 % of the species in environmental samples have never been successfully grown in the laboratory (Madigan et al., 2015). This is because laboratory conditions fail to mimic conditions that is necessary for the organism to proliferate (Muyzer et al., 1993). Thus, cultivation-dependent methods give no valid estimation of richness and abundance and does not represent the actual biodiversity (von Wintzingerode et al., 1997).

1.5.1 16S rRNA gene as marker gene

The 16S rRNA gene encodes the 16S rRNA component of the 30S subunit of the ribosome and is one of the most used marker genes in classification, phylogenetic analyses and microbial diversity analyses of microbial communities (Pepper, 2015). It holds a conserved secondary and tertiary structure (Gluick and Draper, 1992). The gene contains nine variable regions (V1 – V9) which can be amplified by polymerase chain reaction (PCR) with the use of universal bacterial primers that targets the conserved regions (Muyzer et al., 1993, Cole et al., 2014). Databases such as "The Ribosomal Database Project" (RDP, 2013) is offering tools for e.g. taxonomic assignment (Cole et al., 2014). PCR amplification of the 16S rRNA gene may not necessarily lead to results that represents the exact diversity of the microbial community because amplification may be affected by suboptimal hybridization and specification of the primers (von Wintzingerode et al., 1997). In addition, bacterial genomes have variable numbers of rRNA gene regions (rrn operons) which may cause over-representation of some bacteria (von Wintzingerode et al., 1997).

1.5.2 High-throughput sequencing (HTS)

An alternative method to study microbial diversity is by metagenomics and high-throughput sequencing (HTS). HTS is a collective name for a group of modern sequencing techniques, such as Illumina sequencing. These methods allow the researcher to sequence deoxyribonucleic acids (DNAs) and ribonucleic acids (RNAs) quicker and cheaper, in contrast to traditional Sanger sequencing, since the sequencing process runs in parallel (Clark, 2012, Reuter et al., 2015). Sequences representing the 16S rRNA gene is filtered out and used to describe the microbial community of interest (Pepper, 2015).

Illumina is one of the sequencing methods that dominate in HTS (Reuter et al., 2015). After generating an 16S rRNA amplicon library with primers containing Illumina adapters, the PCR amplicons is normalized to purify and obtain an even concentration of amplicons in each sample (Caporaso et al., 2012, de Muinck et al., 2017). In the next step, they are pooled together. This is possible because the adaptor contains a unique index that identifies every single amplicon. The double-stranded templates are cleaved into single-stranded sequences, and with the help of the adaptor, annealed to oligonucleotides bound to a flow cell surface (Illumina Inc, 2018). DNA polymerase and un-labeled nucleotides are added to initiate bridge amplification where double-stranded bridges are made, generating clusters of identical DNA strands. The following denaturation step leaves the sequencing templates single-stranded on the flow cell surface, before DNA polymerase, primers and reversible fluorescent deoxyribonucleotide triphosphates (dNTPs) are added. Each nucleotide is tagged with a fluorescent dye, where one color corresponds to each of the four dNTPs. The fluorescent tag ensures that only one nucleotide is added at each time by terminating further DNA-synthesis at the 3'-OH ends. A computer analyzes the fluorescent signal, telling which base is added, thereby sequencing the region of interest. With the help of the unique sequence indices each sequence read can be sorted according to belonging sample (Illumina Inc, 2018).

1.6 Objectives of the study

This master's thesis was part of the research project RAS-ORGMAT. The overall goal in the RAS-ORGMAT project was to examine the potential of membrane filtration to reduce the amount of accumulating organic particles in the rearing water of land-based closed containment recirculation aquaculture systems (LBCC-RAS) with Atlantic salmon parr to improve the water quality and smolt health and welfare.

The objective of this master project was to investigate the effect of membrane filtration on the microbiota associated with rearing water, gut and skin mucus of Atlantic salmon parr by Illumina sequencing of bacterial 16S rDNA amplicons. Furthermore, a sub-aim was to compare the microbial communities after a period with high water exchange rate and normal feed loading (day 90 – 118 of the experiment) (sampling time 1), and after a period with moderate water exchange rate and feed overloading of the system (day 119 – 140) (sampling time 2). To summarize, the aims of this thesis is to:

- Evaluate the effect of membrane filtration on;
    - Water associated microbiota
    - Gut associated microbiota
    - Skin mucus associated microbiota
- Compare the microbial communities associated with rearing water, gut and skin mucus within and between systems at the two sampling times (t1 and t2), representing different levels of feed loading and water exchange rates.

2. Materials and methods

2.1 Design and setup of the fish experiment

The fish experiment was carried out at NTNU's Centre of Fisheries and Aquaculture (SeaLab) at Brattørkaia in Trondheim. This experiment was part of the research project "Developing water treatment technology for land-based closed containment systems (LBCC-RAS) to increase efficiency by reducing the negative effects of organic matter" (project acronym: RAS-ORGMAT, 2016 – 2018, ERA-Net COFASP). SINTEF Ocean was the main responsible for the experiment, experiment setup, maintenance and water quality measurements. Gaute Helberg (master student, NTNU) was responsible for sampling and analyses related to fish health and performance. Ragnhild Fossmark (PhD student, NTNU) was responsible for sampling and analysis of water samples. Sampling and analyses of salmon gut and skin mucus microbiota were performed by the author of this thesis.

The experiment consisted of two recirculating aquaculture systems (RAS), one conventional RAS (cRAS) and one that included a water treatment step with membrane filtration (mRAS). Each system contained six rearing tanks (FT) (400 L/tank) (Nofitech, Norway) and a recirculation loop with water treatment. The rearing tanks were squared with rounded corners. A lid was installed on top of each tank to obtain photoperiodic light control. Each of the recirculating loops contained, a sump (S_1), a mechanical drum screen filter (DS) (HEX, CM Aqua Technologies, Denmark), a second sump (S_2), a moving bed biofilter (BF) (Nofitech, Norway) and a CO_2 degasser (CO_2). A membrane filtration step (MF) was installed between sump 2 and the biofilter in the mRAS system.

The two mechanical filters with a pore size of 63 μm and 26 μm, cRAS and mRAS respectively, were driven by pressure forces. To pump the water into the biofilter, a frequency-controlled pump (Grundfos, Denmark) was used. The membrane filter in the mRAS was a X-FLOW Compact 4.0G ultrafiltration membrane (PENTAIR, Netherlands) with 8 mm diameter and with a mean pore size of 30 nm. A 10 % side-stream of the total water flow was treated by the membrane. The transmembrane pressure (TMP) was set to approximately 0.2 bar. Each RAS contained a water volume of 3500 L in total. The total system hydraulic retention time throughout the experiment period is given in Table 2.1. For each system, the biofilter consisted of three connected chambers, each with a volume of 250 L and was prior to the experiment matured by addition of ammonium chloride (NH_4Cl) and fish feed for approximately two

months. Biofilm carriers with a total surface area of 100 m^2 were added to each chamber, giving a total carrier surface area of 300 m^2. The intake water was treated with UV before entering the systems.

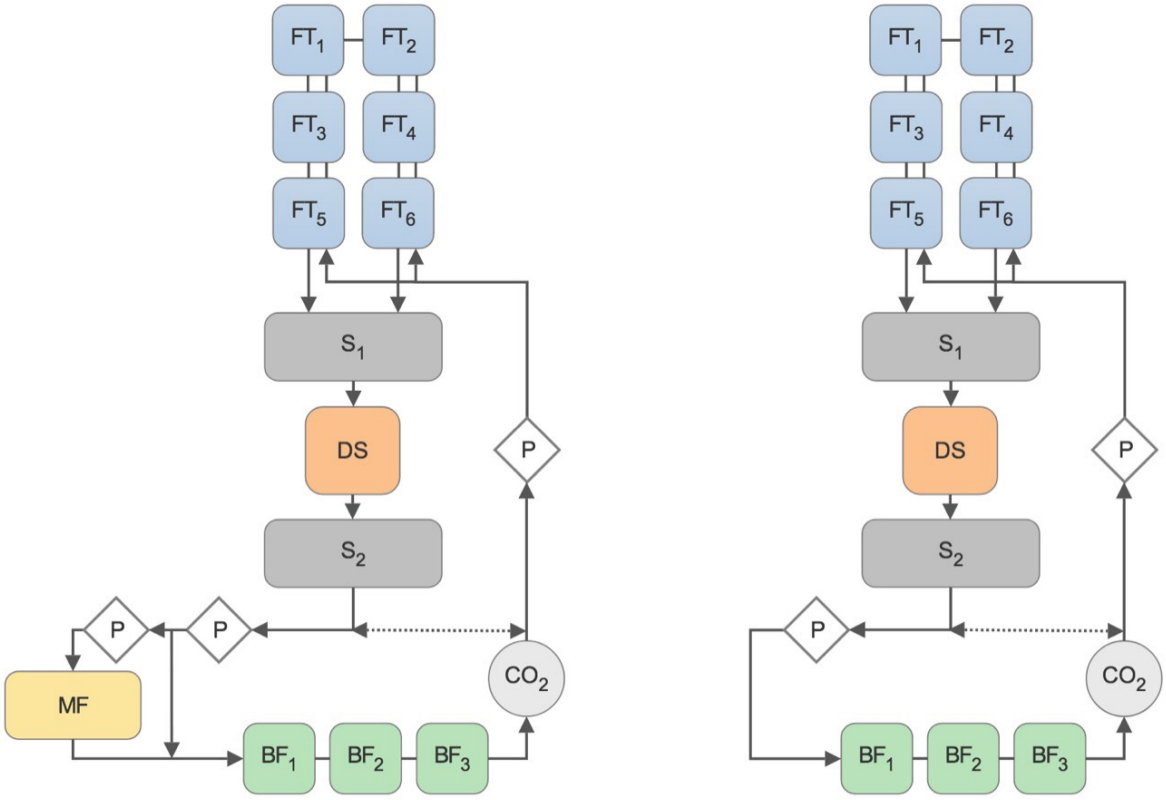


Figure 2.1. Illustration of the setup of mRAS (left) and cRAS (right). Each system consisted of six fish tanks (FT), two sumps (S1 and S2), a drum screen filter (DS), pumps (P), moving bed biofilter (BF) and a degasser (CO_2). In addition, mRAS had a membrane filter (MF) implemented before the biofilter, treating 10 % of the rearing water. Figure constructed by Jenny Nesje (master student, NTNU).

A total of 60 *S. salar* parr (40 ± 4 g) was stocked in each tank at day 0 (density: 6 kg/m^3). They came from the same sibling group and had been reared in a flow-through system before entering the experiment tanks. The experiment lasted for 140 days in total (24.01.17 – 13.06.17). Table 2.1 shows the rearing conditions for each of the experiment periods. Period 1 was an acclimatization period for the fish and the system, where the system was run as one unit to ensure equal start conditions. During period 2, the system was only semi-closed, giving the same water to the rearing tanks, but different water (membrane filtered or not) to the two biofilters. In period 3, the system was split in two separate systems (as cRAS and mRAS). Period 3 was characterized by low water exchange rate and moderate feed loading. Period 4

had high water exchange rate and normal feed loading. The last period, period 5, was characterized by moderate water exchange rate and high feed load to the systems. The first sampling (t1) was performed at day 118 (22.05.17), and reflects period 4, while the second sampling (t2) was performed at day 140 (13.06.17) and reflects period 5.

Table 2.1. Rearing conditions for the fish experiment. Each period was characterized by its respective average intake water volume (L/d), recirculation degree (%/d), total system hydraulic retention time (d) and degree of feeding (g/FT/d). In the last period (period 5) there was of interest to increase the load on the system by high overfeeding.

Day	Period	Intake water (L/d)	Recirculation degree (%/d)	HRT_{tot} (d)	Feed (g/FT/d)
0 – 13	1	190.1	94.6	18.4	31.7
14 – 52	2	190.1	94.6	18.4	43.5
53 – 89	3	273.1	92.2	12.8	73.3
90 – 118	4	2056.6	41.2	1.7	87.9
119 – 140	5	1478.0	57.8	2.4	192

From day 2 and throughout the experiment, the fish were fed dry feed (3.5 mm, Nutra Advance RC, Skretting, Norway) at every twenty minutes (3 – 4 times/hour) during the seven hours light period (8:00 – 15:00). Automatic feeders (Arvo-Tec Oy, Finland) were installed at every fish tank. Feed spill and feces were collected in a sieve located outside the tanks and were removed manually each day.

2.1.1 Sampling of rearing water, skin mucus and posterior gut content

Throughout the experimental period, water, skin mucus and posterior gut content were sampled. Water samples (approximately 200 mL) were collected in Erlenmeyer flasks from the outlet of fish tanks 1, 2 and 3 (cRAS) and 7, 8 and 9 (mRAS), before filtration through a sterile filter (0.22 μm) (Sterivex™) using a 60 mL syringe. The water volume filtered was approximately 200 mL for mRAS and 100-150 mL for cRAS. The filters were stored at -20 ℃ until further analysis.

Four fish from each of the tanks 1, 2 and 3 from cRAS and 7, 8 and 9 from mRAS were sampled. The fish were transferred with a dip net to a bucket and anesthetized in a 1:10 dilution of AQUI-S (16 mL) and 20 mL water from the recirculation system for approximately one minute. A

second bucket with 1 mL of the dilution and 10 L rearing water was prepared. The fish were transferred to the low concentration to maintain sedation and then euthanized before sampling.

Skin mucus was scraped off from each side of the fish (from right behind the pectoral fin, backwards and towards the end of the dorsal fin), transferred to a cryo tube (2 mL) and stored on ice. Posterior gut content was sampled by stroking the stomach from just behind the dorsal fin, and backwards following the intestine, and squeezed into a petri dish. The smallest fish were cut open, and gut content pushed out from the intestine with a tweezers. The gut samples were transferred into a cryo tube (2 mL) with a scalpel. Equipment used were rinsed with ethanol (70 %) between each fish. The samples were stored at -20 ℃ until further analysis.

2.2 DNA extraction

DNA was extracted from water (12 samples), skin (48 samples) and gut (48 samples) samples sampled at t1 and t2. The filter used during water filtration was cut into small pieces with a scalpel in a petri dish and transferred to a microcentrifuge tube (1.5 mL). A disposable inoculating loop was used to transfer approximately 25 mg feces and skin mucus to a microcentrifuge tube (1.5 mL). QIAamp® DNA Mini Kit (Qiagen, Germany) was used to extract total DNA from the samples according to manufacturer's protocol with minor alterations. An extra lysis step was added to ensure lysis of Gram-positive bacteria by using an enzymatic lysis buffer (180 μL) consisting of 2mM EDTA, 20 mM Tris-HCl (pH 8) and 1.2 % Triton and lysozyme (0.06 g), before a 1 hour incubation at 37 ℃. Proteinase K (40 μL) and ATL buffer (180 μL) were added in step 3 of the protocol (Appendix B) before the samples were vortexed and incubated at 56 ℃ for 2 hours. After adding AE buffer (200 μL) in step 11 and 12, the samples were incubated for 5 minutes in room temperature both times and centrifuged (Appendix B). The DNA concentration in each sample was determined using Nanodrop^{TM} One (Thermo Scientific).

2.3 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) was conducted to amplify the variable 4 region (V4) of the bacterial 16S rRNA gene from water, skin mucus and gut samples. The amplification was performed using Phusion Hot Start II DNA polymerase (Thermo Scientific). PCR reactions were performed with 0.2 mM of each dNTP (TaKaRa Clontech), 0.3 μM of each primer (Ill805R and Ill515F, SIGMA), 0.5 mM spermidine (Sigma-Aldrich), 2 mM $MgCl_2$ (Thermo

Scientific), 0.02 U/μL Phusion Hot Start II DNA polymerase and reaction buffer from Thermo Scientific in a total reaction volume of 25 μL, included ~1 ng/μL undiluted DNA extract as template. The primers contained Illumina adapters and their sequences are given in Table 2.2. A non-template control was always included, as well as a negative control for the DNA extraction kit using milliQ water instead of tissue sample with the DNA extraction kit. The PCR reactions were run with 36 temperature cycles using a T100^{TM} Thermal Cycler (BioRad) (Table 2.3).

Table 2.2. Primer sequence for forward and reverse primers used during PCR amplification of the V4 region of the bacterial 16S rRNA gene. Illumina adapter sequences are given in red.

Primer name	Primer sequence (5' – 3')
Ill805R	5'- GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G NNNN GAC TAC NVG GGT ATC TAA KCC-3'
Ill515F	5'- TCG TCG GCA GCG TCA GAT GTC TAT AAG AGA CAG NNNN GTG CCA GCM GCC GCG GTA A-3'

Table 2.3. Temperature steps for the PCR cycling. Step 2 – 4 were repeated for 36 cycles.

Step	Reaction	Temperature (°C)	Time
1	Denaturation	98	1 min
2	Denaturation	98	15 sec
3	Annealing	55	20 sec
4	Elongation	72	20 sec
5	Elongation	72	5 min
6	Storage	4	∞

2.4 Agarose gel electrophoresis

To examine quality and yield of the amplified PCR products, agarose gel electrophoresis was performed. A 1 % agarose gel was made by heating agarose (2 g) in 1 x TAE buffer (200 mL) in the microwave until the agarose was dissolved. For each gel, approximately 50 μL of 1 % agarose and a volume of 2.5 μL GelRed (Biotium) was mixed. The solution was poured into a gel tray and left for solidifying for 15 – 30 minutes. PCR products (5 μL) were mixed with DNA loading dye (1 μL, x6, Thermo Scientific) and applied to the wells. As size marker, GeneRuler 1 kb Plus ladder (Thermo Scientific) was used. The gel was run at 140 Volt for 45 – 75 minutes, depending on the size of the gel.

2.5 Illumina sequencing

The PCR products generated using primers with Illumina adapters were further used to make an amplicon library for Illumina sequencing. A SequalPrep Normalization Plate (96) kit (Invitrogen, USA) was used for normalization and purifying the PCR products. The protocol included in the kit was followed (Appendix C).

Nextera XT Index kit (Illumina) was used to add unique index sequences to each PCR product. The kit contains unique sequence tags attached during PCR, where 8 different sequence indexes are used with the forward primer and 12 different sequence indexes used with the reverse primer, which gives 96 unique index pairs in total, one unique index to each well. The indexes (2.5 μL each) were added just after the reagents (17.5 μL in total), followed by normalized template (2.5 μL), giving a total reaction volume of 25 μL. The reagents added were 0.25 mM of each dNTP (VWR), 2 mM $MgCl_2$ (Thermo Scientific), 0.015 U/μL Phusion Hot Start DNA polymerase and reaction buffer (Thermo Scientific). The PCR mixtures were distributed to a 96-well plate (BioRad) and run in a T100^{TM} Thermal Cycler machine (BioRad), with the program given in Table 2.4. To examine the PCR yields, gel electrophoresis was performed.

Table 2.4. Temperature during PCR used for indexing of 16S rDNA amplification. Step 2 – 4 was repeated for 10 cycles.

Step	Reaction	Temperature (℃)	Time
1	Denaturation	98	1 min
2	Denaturation	98	15 sec
3	Annealing	50	20 sec
4	Elongation	72	20 sec
5	Elongation	72	5 min
6	Cooling	4	1 min
7	Storage	10	∞

The index amplicons were normalized first time after indexing using a Sequal Prep Normalization plate (Invitrogen) following the same procedure as described above, before they were pooled to generate the amplicon library.

To concentrate the pooled sample, AmiconUltra 0.5 Centrifugal Filter (Merck Millipore, Ireland) was performed as described by the manufacturer (Appendix D). At step 4, TE buffer

(500 μL) was added before spinning down at 14 000 G in 10 minutes. This step was performed two times, and the eluate discarded. After the concentration, approximately 50 μL of concentrated DNA sample was obtained. The concentration and purity of the concentrated pooled sample was measured with NanoDrop. Gel electrophoresis was performed to examine the yield of the amplicon library.

The amplicon library was sequenced on a MiSeq lane (Illumina, San Diego, CA) with V4 reagents (Illumina) at the Norwegian Sequencing Centre (NCS).

2.6 Illumina sequencing data processing

The Illumina sequencing data were processed with the USEARCH pipeline (version 9.2; https://www.drive5.com/usearch/). During merging of paired reads, also primer sequences were trimmed and reads shorter than 230 base pairs were filtered out. The processing further included demultiplexing, quality trimming by the Fastq_filter command (with an expected error threshold of 1). Chimera removal and clustering at the 97% similarity level was performed using the UPARSE-OTU algorithm (Edgar, 2013). Taxonomy assignment was based on the Sintax script (Edgar, 2016) with a confidence value threshold of 0.8 and the RDP reference data set (version 15).

2.7 Statistical Analyses

Statistical analyses were performed using the program package PAST version 3.18 (Hammer, 2001). To examine the α-diversity for the microbial communities associated with individual rearing water, gut and skin samples, observed number of operational taxonomic units (OTUs), estimated richness (Chao1) and Shannon's diversity index (H') were calculated.

The observed numbers of OTUs reflects the numbers of species observed in a microbial community. But, there will always be some undetected species in a taxonomic survey and the observed richness do not represent the true species richness in a community. Chao1 is a non-parametric estimation of the total numbers of species present. It is calculated based on singletons and doubletons (sequence reads observed once and twice) (Chao, 1984, Chao, 2016).

Shannon's diversity index (Equation 2.1) includes both the species richness and species evenness, expressed by (Peet, 1974, as sited in (Beisel and Moreteau, 1997));

$$H' = -\sum_{i=1}^{S} \left(\frac{n_i}{n}\right) \left(ln \frac{n_i}{n}\right) \quad (2.1)$$

where, S corresponding to OTU richness, n_i to number of reads assigned to OTU i and n to the total number of sequence reads.

To investigate the β-diversity for comparing community profiles between samples, a Bray-Curtis similarity matrix were generated in PAST to compare microbial communities. The index describes the similarity between community profiles of two samples, ranging from 0 to 1, where the value 0 indicate no similarity between community profiles and 1 indicates fully identical community profiles (Bray and Curtis, 1957).

To visualize the β-diversity observed by the Bray-Curtis similarity matrix, a principal coordination analysis (PCoA) plot based on the Bray-Curtis similarities was computed. In PCoA, the Bray-Curtis similarity matrix is used to plot the samples in a multidimensional coordinate plot (Hammer, 2001). The distance between two samples in the plot reflects their similarity or dissimilarity; the closer positioned in the plot, the more similar the two microbial communities are to each other.

A one-way permutational multivariate analysis of variance (PERMANOVA) test based on Bray-Curtis similarities was conducted to examine whether there were significant differences in the microbial communities between two or more groups of samples (Anderson, 2001). If $p < 0.05$, they are significantly different (Hammer, 2001). Bonferroni-corrected p-values were used when more than two groups were compared.

To identify which OTUs contributed the most to the difference in community profiles between groups of samples, a Similarity Percentage (SIMPER) analysis was performed. The SIMPER analysis was based on the Bray-Curtis similarities.

Two-sample t-tests were used to examine whether diversity indices were significantly different among groups of samples, between samples and to examine individual variations within samples.

2.8 Fish growth and performance

Growth and performance of the salmon parr reared in cRAS and mRAS were examined and compared by calculating daily specific growth rate (SGR) according to Equation 2.2 (Cech et al., 1984):

$$SGR\ (\%/d) = \frac{\ln W_t - \ln W_0}{t - t_0} \times\ 100 \qquad (2.2)$$

where W_t is the average weight at time t (average weight of 4 fish per fish tank 1, 2 and 3 from cRAS and 7, 8 and 9 from mRAS at t2) and W_0 is the initial weight (of 4 fish per fish tank 1, 2 and 3 from cRAS and 7, 8 and 9 from mRAS at t1) at time t_0.

The thermal-unit growth coefficient (TGC) was calculated to correlate for the temperature differences in cRAS and mRAS (Appendix D, Table D.1 and D.2) according to Equation 2.3 (Jobling, 2003):

$$TGC = \frac{\sqrt[3]{W_t} - \sqrt[3]{W_0}}{T(t - t_0)} \times 1000 \qquad (2.3)$$

where $T(t - t_0)$ is degree-days based on the average temperature (℃) from period 4 and 5.

3. Results

The bacterial communities associated with rearing water (W), gut (G) and skin mucus (SM) of Atlantic salmon parr reared in two different recirculating aquaculture systems, one conventional RAS (cRAS) and one that included a membrane filtration step (mRAS) was examined by Illumina amplicon sequencing of the V4 region of the bacterial 16S rRNA gene. Water from three replicate fish tanks (FT) per system and gut and skin mucus samples from four fish (F) per tank were sampled at two different sampling times, t1 (day 118 of the experiment) and t2 (day 140 of the experiment) (Table 2.1). In the time before t1, the systems were characterized by high water exchange rate and normal feed loading. The time period before t2 was characterized by moderate water exchange rate and intentional feed overloading of the system (see Table 2.1). Raw data of water quality parameters and fish performance from the respective periods and statistical analyses was collected and performed by Gaute Helberg (master student, NTNU).

3.1 Richness and diversity of the microbiota associated with rearing water, gut and skin mucus of Atlantic salmon parr

A total of 6 502 926 sequence reads were obtained for samples of water (12 samples), gut (48 samples) and skin mucus (48 samples) after quality filtering and chimera removal, giving an average of 60 212 ± 41 590 reads per sample (Appendix F, Table F.1). The gut samples had the highest number of reads (average 94 933) followed by water samples (average 84 007) (Table 3.1). Number of reads for the skin mucus samples were highly variable and there were large variations between samples with an average of 19 543 reads per sample (Table 3.1).

Table 3.1. The average number of sequence reads (± standard deviation) achieved after quality filtering and chimera removal for water, gut and skin mucus samples.

Sample category	Average number of reads (± SD)
Water	84 007 ± 19 329
Gut	94 933 ± 22 965
Skin	19 543 ± 16 069

By comparing estimated OTU richness (Chao1) and the observed OTU richness, the sequencing effort was found to cover on average 86 % of the estimated richness for water samples, 91 % for the gut and 77 % for the skin mucus samples.

The bacterial communities associated with rearing water had both the highest OTU richness (Figure 3.1) and Shannon's diversity (Figure 3.2), followed by gut and skin microbiota. For the microbiota associated with the rearing water, mRAS showed both higher OTU richness and diversity compared to cRAS (t-test, observed OTUs: $p = 0.003$, Chao1: $p = 0.009$, H': $p = 0.02$). Thus, membrane filtration of the water appeared to result in a more diverse water microbiota. The gut microbial communities within mRAS were less diverse compared to cRAS at both sampling times (t-test, $p = 0.001$) (Figure 3.2), suggesting that membrane filtration may have lowered the diversity of the gut microbiota. The bacterial diversity decreased from t1 to t2 for the water in cRAS, whereas it increased for the rest (Figure 3.2). A t-test confirmed that the diversity was significantly different at t1 and t2 in both systems (W cRAS: $p = 0.02$, W mRAS: $p = 0.003$, G cRAS: $p = 4.22 \times 10^{-5}$, G mRAS: $p = 0.004$). No significant differences were observed in the diversity of the skin associated microbiota, neither between systems nor between the two sampling times (Figure 3.1 and 3.2).

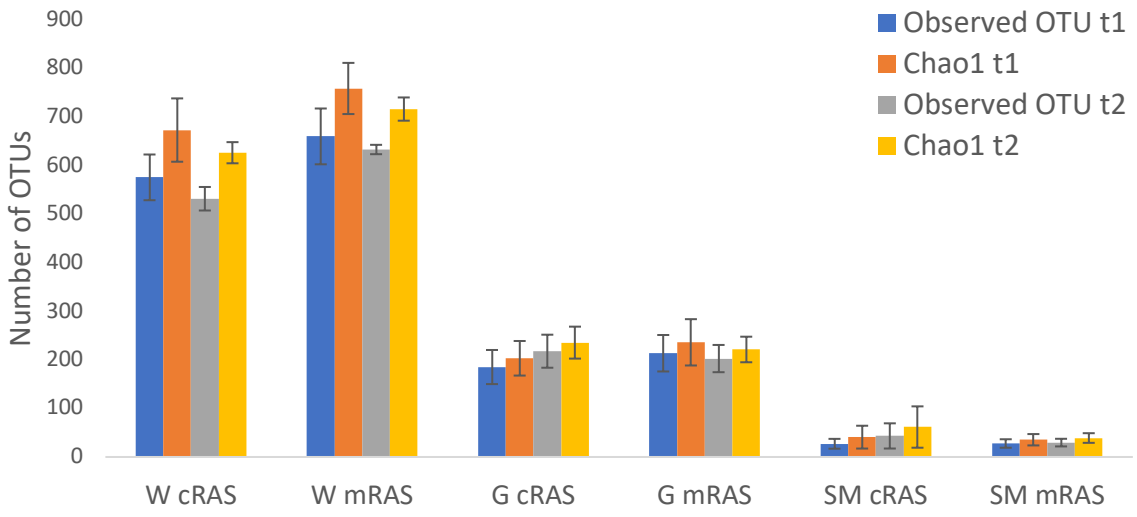


Figure 3.1. Average observed OTU richness and Chao1 richness index for water (W), gut (G) and skin mucus (SM) samples in cRAS and mRAS at sampling times t1 and t2. Error bars indicate standard deviation (±SD).

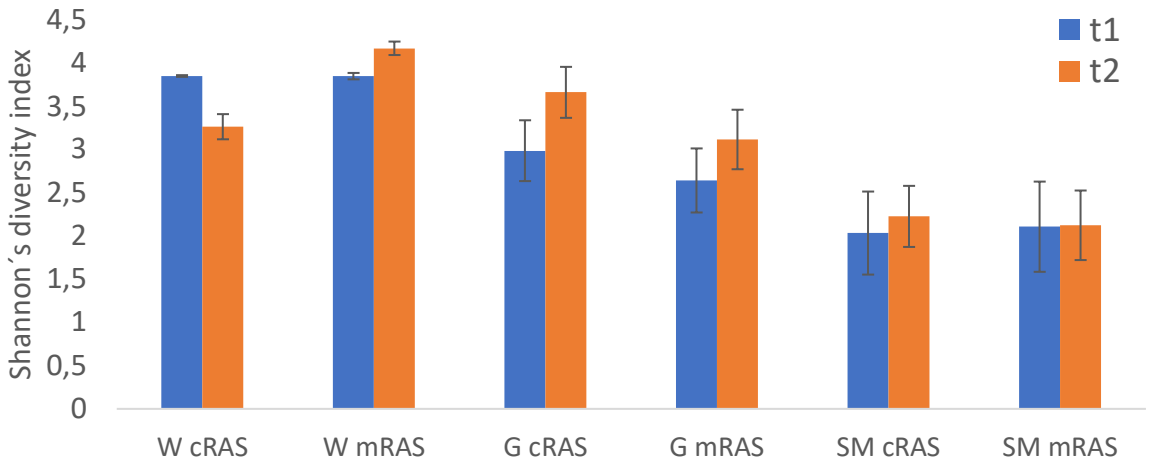


Figure 3.2. Shannon´s diversity index for water (W), gut (G) and skin mucus (SM) samples in cRAS and mRAS at sampling time t1 and t2. Error bars indicate standard deviation (±SD).

The relative abundances of the bacterial classes associated with the samples of rearing water, gut and skin mucus of salmon parr are represent in Figure 3.3. Water, gut and skin mucus are dominated by different classes of bacteria. The microbial communities associated with water were dominated by Betaproteobacteria and Alphaproteobacteria (16.09 ± 4.83 % and 15.48 ± 3.19 % respectively). Bacilli and Actinobacteria were the two most abundant classes in the gut microbiota (49.38 ± 13.25 % and 24.68 ± 9.13 % respectively). The increased feed loading affected the community composition of the skin microbiota, where Bacilli dominated at t1 and Actinobacteria and OTUs that could not be classified at the genus level dominated at t2 (25.54 ± 14.33 % and 17.60 ± 20.44 % respectively). Sequences representing Chloroplast originated most likely from the feed and were removed before further analyses.

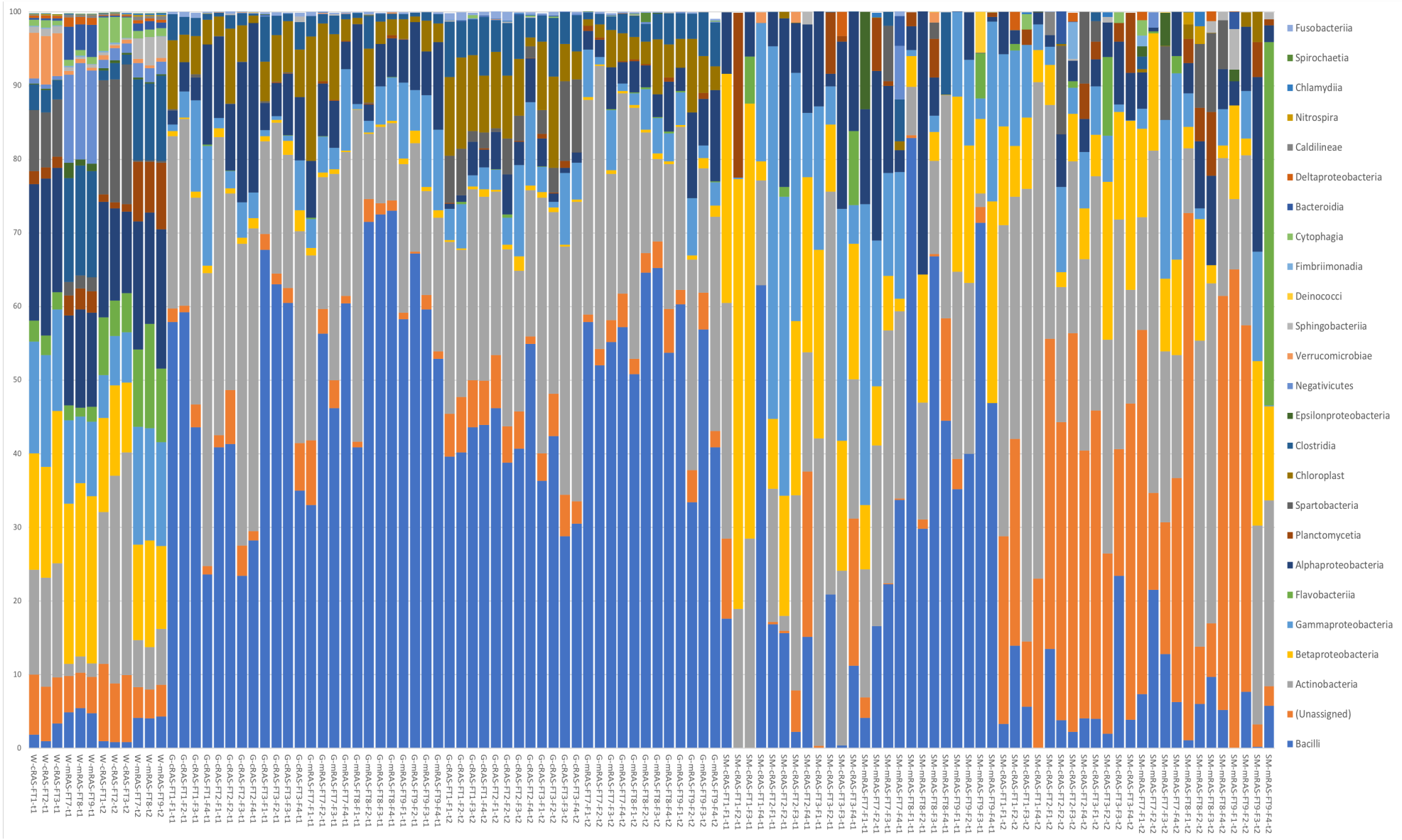


Figure 3.3. Relative abundances of classes represented in the bacterial V4 16S rDNA amplicons from water (W), gut (G) and skin mucus (SM) samples of each individual fish (F) in cRAS and mRAS and the respective fish tanks (FT) at the two sampling times t1 and t2. Only taxa represented by a proportion of ≥1% in at least one of the samples are shown.

3.2 Bacterial communities associated with the rearing water

A principal coordinate analysis (PCoA) suggested that the water microbiota was different between systems and sampling times (Figure 3.4). A One-Way PERMANOVA test confirmed that the microbial communities in cRAS and mRAS were significantly different ($p = 0.002$). There were larger differences in the water microbiota between systems at t1 and t2 (average Bray-Curtis similarities 0.32 – 0.35) than between sampling times within each system (average Bray-Curtis similarities 0.44 – 0.50) (Figure 3.5), indicating that membrane filtration affected the water microbiota composition. Average Bray-Curtis similarities showed large similarities between replicate tanks within each system at both sampling times (> 0.85) (Figure 3.5), which is in accordance with the PCoA plot (Figure 3.4).

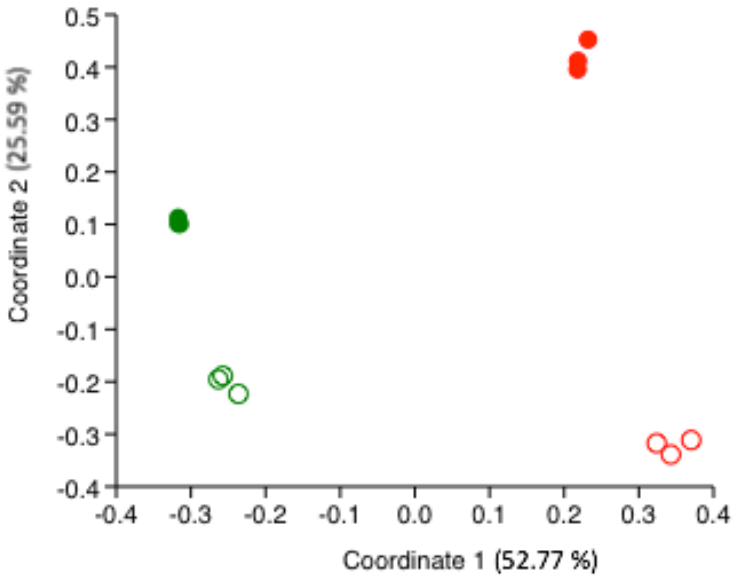


Figure 3.4. Principal coordinate analysis (PCoA) plot based on Bray-Curtis similarities for comparison of the microbiota associated with the rearing water of the mRAS (green) and cRAS (red) at sampling time t1 (filled circles) and t2 (open circles).

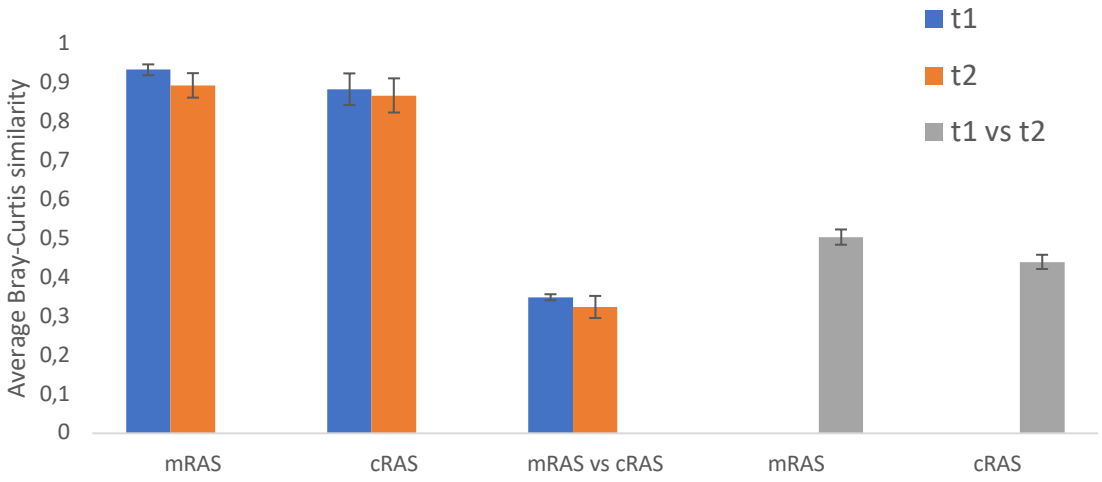


Figure 3.5. Average Bray-Curtis similarities for comparison of water microbiota witnin and between the two systems (mRAS and cRAS) and sampling time t1 and t2. Error bars indicate standard deviation (±SD).

The most common OTUs in the rearing water in cRAS were *Mycobacterium* (OTU_4), *Spartobacteria_genra_incerae_sedis* (OTU_6) and *Legionella* (OTU_16) (Table 3.2). For mRAS, the most abundant OTUs were *Sphaerotilus* (OTU_14), Rhodobacteraceae (OTU_34) and *Zymophilus* (OTU_28) (Table 3.3).

To examine which OTUs contributed the most to the differences observed between the water microbiota in cRAS and mRAS, a SIMPER analysis based on Bray-Curtis dissimilarities was performed (Appendix G, Table G.7). OTU_4 (*Mycobacterium*) contributed the most to the dissimilarity (11.47 %) and was most abundant in cRAS (17 %). Within cRAS, the OTU increased in abundance from t1 to t2 (Appendix G, Table G.2 and G.3). OTU_6, representing *Spartobacteria_genera_incertae_sedis*, was the second OTU contributing to the dissimilarities (8.37 %) and was also more abundant in cRAS (12 %). It increased in abundance from t1 to t2 (Appendix G, Table G.2 and G.3). *Legionella* (OTU_16) was the most abundant OTU at sampling time 1 in cRAS but decreased drastically in abundance at t2 (Appendix G, Table G.2 and G.3). It was the fourth OTU that contributed to the differences between cRAS and mRAS (4.66 %) (Appendix G, Table G.7).

Sphaerotilus (OTU_14) was the most abundant OTU in mRAS (14 %), and the OTU that contributed third most to the dissimilarities in the water microbiota between the two systems (7.04 %) (Appendix G, Table G.7). This OTU decreased in abundance from t1 to t2 in the

mRAS (Appendix G, Table G.5 and G.6). *Flavobacterium* (OTU_27) became the most abundant OTU in mRAS water microbiota at t2 (Appendix G, Table G.6) and was the fifth OTU contributing to the dissimilarities between cRAS and mRAS (3.91 %) (Appendix G, Table G.7). The results suggest that membrane filtration prevented proliferation of *Mycobacterium* (OTU_4), *Spartobacteria_genera_incertae_sedis* (OTU_6) and *Legionella* (OTU_16).

Table 3.2. The five most abundant OTUs in water samples of cRAS with average relative abundance.

OTU ID	Taxonomy	Average relative abundance
4	*Mycobacterium* (Actinobacteria)	0.17
6	*Spartobacteria_genera_incertae_sedis* (Spartobacteria)	0.12
16	*Legionella* (Gammaproteobacteria)	0.07
14	*Sphaerotilus* (Betaproteobacteria)	0.05
34	Rhodobacteraceae (Alphaproteobacteria)	0.04

Table 3.3. The five most abundant OTUs in water samples of mRAS with average relative abundance.

OTU ID	Taxonomy	Average relative abundance
14	*Sphaerotilus* (Betaproteobacteria)	0.14
34	Rhodobacteraceae (Alpharoteobacteria)	0.06
28	*Zymophilus* (Negativicutes)	0.06
27	*Flavobacterium* (Flavobacteria)	0.06
25	Lachnospiraceae (Clostridia)	0.05

3.3 Gut associated microbiota

Figure 3.6 show PCoA analysis of the microbial communities associated with the gut of salmon parr reared in cRAS and mRAS at different sampling times (t1 and t2). There were no large differences between the gut associated microbiota in cRAS and mRAS at t1 (PERMANOVA, Bonferroni-corrected $p = 0.05$). At sampling time 2, the gut microbial communities were different between the two systems (PERMANOVA, Bonferroni-corrected $p = 0.0001$). The gut microbiota in mRAS were relatively similar at t1 and t2 (PERMANOVA, Bonferroni-corrected $p = 0.10$) and may indicate that the gut community profiles in mRAS were more stable through time despite different feed loading. There was less variation in gut associated microbiota among individuals within each system (average Bray-Curtis similarities 0.5 – 0.6) (Figure 3.7). Additionally, the gut microbiota in mRAS was significantly more similar between individuals at both t1 and t2 compared to that in cRAS (t-test, $p < 0.01$) (Figure 3.7). Furthermore, the gut microbiota in cRAS and mRAS were significantly different (PERMANOVA, $p = 0.0001$).

Figure 3.8 show PCoA analysis comparing the gut microbial communities in tanks within each system. The gut samples grouped according to each fish tank at t2 (Figure 3.8B). The gut microbiota in the replicate tanks in mRAS was more similar to each other at t2 (Figure 3.8B). The replicate fish tanks of each system were not significantly different at t1 nor at t2 in either of the systems (PERMANOVA, $p > 0.05$) (Figure 3.8A and B).

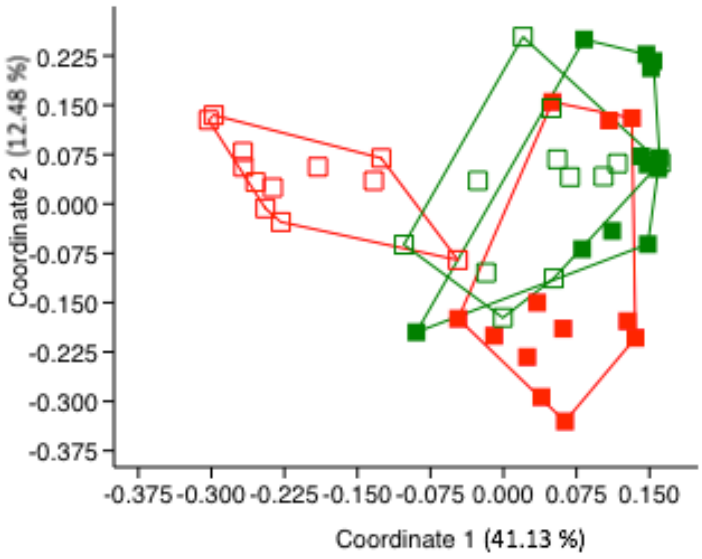


Figure 3.6. Principal coordinate analysis plot based on Bray-Curtis similarities for comparison of the gut microbiota of salmon parr in mRAS (green) and cRAS (red) at sampling time t1 (filled squares) and t2 (open squares).

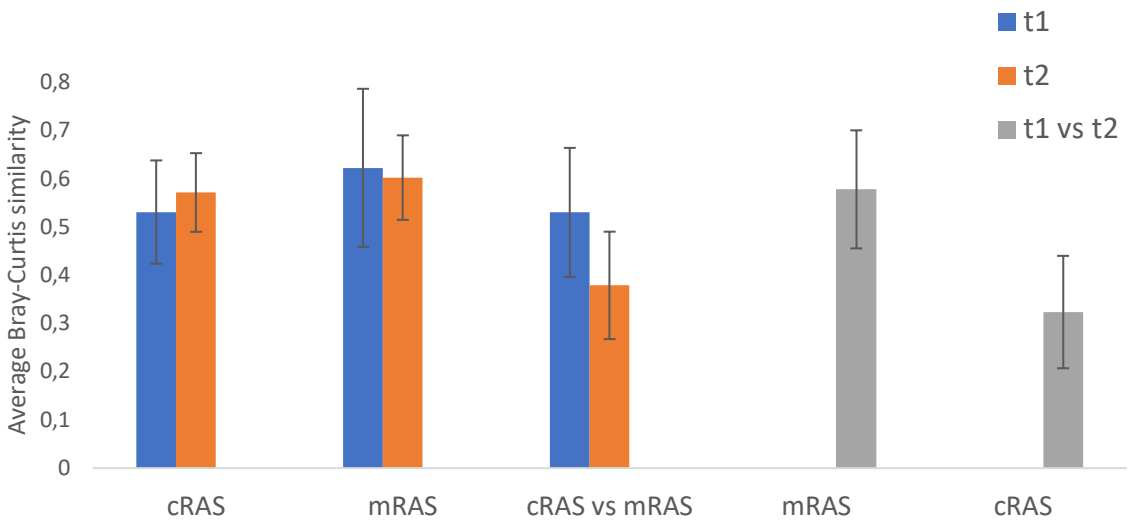


Figure 3.7. Average Bray-Curtis similarities for comparison of salmon parr gut microbiota within and between systems (mRAS and cRAS) at sampling time t1 and t2. Error bars indicate standard deviation (±SD).

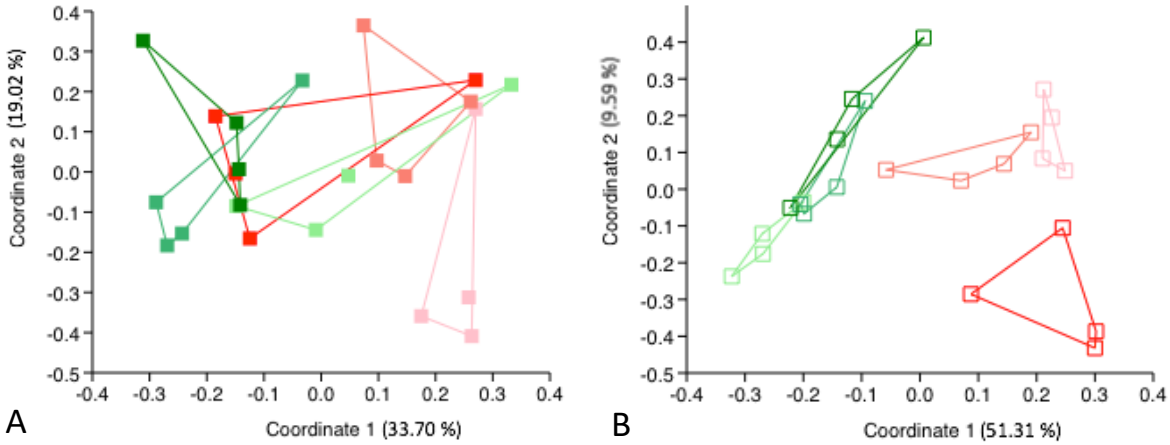


Figure 3.8. PCoA plot based on Bray-Curtis similarities for comparison of gut microbiota between replicate fish tanks in cRAS (FT1, 2 and 3, red color palette) and mRAS (FT7, 8 and 9, green color palette) at A) sampling time 1 (filled squares) and B) sampling time 2 (open squares).

Carnobacterium (OTU_2), *Brochothrix* (OTU_1) and *Rhodococcus* (OTU_3) were the most abundant genera in the gut microbial communities (Table 3.4). On average, OTU_1 representing the genus *Brochothrix* was the most abundant OTU in the gut microbial communities in cRAS, followed by *Carnobacterium* (OTU_2) and *Mycobacterium* (OTU_4) (Table 3.4). For mRAS, the most abundant OTU was OTU_2 *Carnobacterium* (Table 3.5). *Brochothrix* (OTU_1) and *Rhodococcus* (OTU_3) were the second and third most abundant OTUs in mRAS respectively (Table 3.5).

A SIMPER analysis showed that *Mycobacterium* (OTU_4) and *Spartobacteria_genera_incertae_sedis* (OTU_6) were the second and fifth OTUs contributing to the Bray-Curtis dissimilarities between cRAS and mRAS at sampling time 2 (contributing 13.55 % and 4.11 % respectively) and were most abundant in the gut of cRAS fish (17 % and 5 % respectively) (Appendix H, Table H.3). They were rarely not present in the gut microbiota of fish in mRAS at this sampling time. In addition, OTU_4 and OTU_6 were the two most abundant OTUs in the rearing water in cRAS (Table 3.2). *Legionella* (OTU_16), also abundant in the cRAS rearing water, was present as the 39th most abundant OTU in the gut microbial communities of fish in cRAS. The OTU that contributed the most to the dissimilarities between the two systems at t2 was *Carnobacterium* (OTU_2) (15.16 %) (Appendix H, Table H.3). This OTU was most abundant in the mRAS (23 %) (Table 3.5). *Rhodococcus* (OTU_3) and

Brochothrix (OTU_1) were also more abundant in mRAS at sampling time 2 (Appendix H, Table H.3).

Furthermore, OTU_4 representing *Mycobacterium*, increased highly in abundance from t1 to t2 in cRAS (Appendix H, Table H.4). This OTU contributed the most to the differences observed between the two sampling times (12.36 %). *Weisella* (OTU_8) increased in abundance at sampling time 2 and was the fifth OTU causing the differences between t1 and t2 (4.72 %). *Mycobacterium* (OTU_4)*, Rhodococcus* (OTU_3) and *Brochothrix* (OTU_1) were more abundant at sampling time 1 in cRAS (Appendix H, Table H.4). There were no large differences between the gut microbiota at t1 and t2 in mRAS (Appendix H, Table H.5). The main difference was caused by OTU_1, which increased considerably in abundance through the overfeeding period (Appendix H, Table H.5). Interestingly, also in the gut communities, the membrane filtration of the water seemed to prevent dominance of OTU_4 (*Mycobacterium*), OTU_6 (*Spartobacteria_genera_incertae_sedis*) and OTU_16 (*Legionella*). All three OTUs were not present among the 100th most abundant OTUs in mRAS gut microbiota.

Table 3.4. The five most abundant OTUs associated with the gut of salmon parr reared in cRAS and average relative abundance.

OTU ID	Taxonomy	Average relative abundance
1	*Brochothrix* (Bacilli)	0.12
2	*Carnobacterium* (Bacilli)	0.10
4	*Mycobacterium* (Actinobacteria)	0.08
3	*Rhodococcus* (Actinobacteria)	0.08
8	*Weissella* (Basilli)	0.05

Table 3.5. The five most abundant OTUs associated with the gut of salmon parr reared in mRAS and average relative abundance.

OTU ID	Taxonomy	Average relative abundance
2	*Carnobacterium* (Bacilli)	0.27
1	*Brochothrix* (Bacilli)	0.14
3	*Rhodococcus* (Actinobacteria)	0.13
11	*Pseudochrobactrum* (Alphaproteobacteria)	0.05
10	*Microbacterium* (Actinobacteria)	0.04

3.4 Skin mucus associated microbiota

Figure 3.9 show PCoA analysis of the microbial communities associated with the skin of salmon parr reared in cRAS and mRAS at different sampling times (t1 and t2). The PCoA plot indicated that sampling time, or the different loading of the system, influenced the changes in the skin microbiota more than water treatment. A PERMANOVA test confirmed that the skin microbiota within each system were significantly different between t1 and t2 (Bonferroni-corrected $p = 0.0006$) but not between cRAS and mRAS ($p = 0.19$). Average Bray-Curtis similarities further suggested that the skin associated microbiota within cRAS and mRAS were different between t1 and t2 (< 0.15) (Figure 3.10). The individuals at t1 showed a larger individual variation in the skin microbial communities both in cRAS and mRAS (average Bray-Curtis similarities < 0.20) (t-test, $p < 0.01$) (Figure 3.10).

The replicate fish tanks within each system were compared by a PCoA analysis to investigate dissimilarities between skin microbiota (Figure 3.11A and B). The skin samples in both mRAS and cRAS were more clustered and similar at sampling time 2 (Figure 3.11B), which was also supported by the average Bray-Curtis similarities (Figure 3.10). The skin microbiota in the replicate fish tanks of each system were not significantly different at t1 nor at t2 in either of the systems (PERMANOVA, $p > 0.05$).

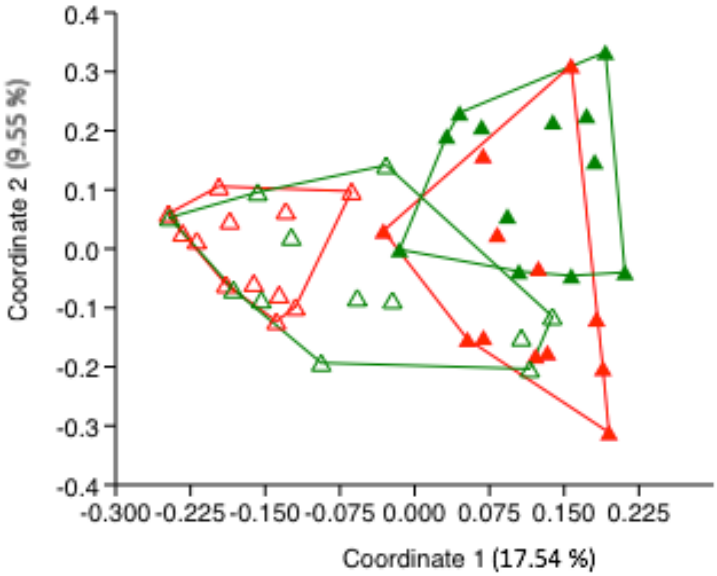


Figure 3.9. Principal coordinate analysis plot based on Bray-Curtis similarities for comparison of the skin microbiota of salmon parr in mRAS (green) and cRAS (red) at sampling time t1 (filled triangles) and t2 (open triangles).

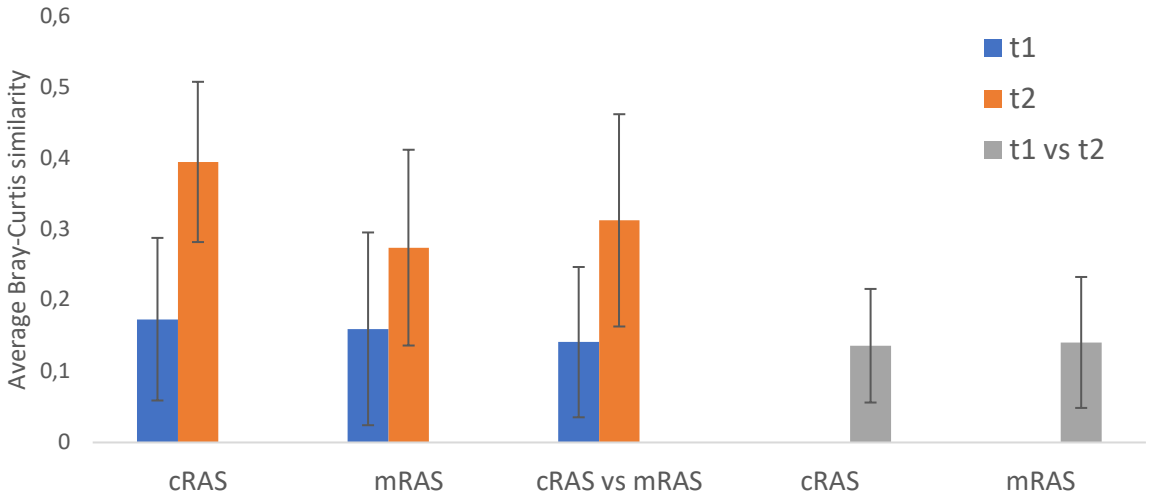


Figure 3.10. Average Bray-Curtis similarities for comparison of salmon parr skin microbiota within and between systems (mRAS and cRAS) at sampling time t1 and t2. Error bars indicate standard deviation (±SD).

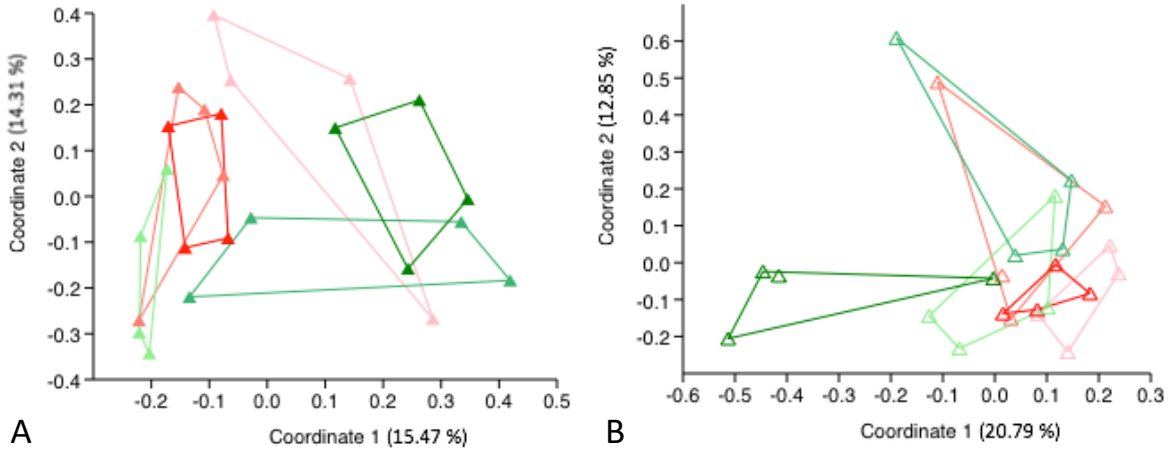


Figure 3.11. PCoA plot based on Bray-Curtis similarities for comparison of skin microbiota between replicate fish tanks in cRAS (FT1, 2 and 3, red color palette) and mRAS (FT7, 8 and 9, green color palette) at A) sampling time 1 (filled triangles) and B) sampling time 2 (open triangles).

The three most abundant OTUs in the skin microbiota in cRAS were *Mycobacterium* (OTU_4), *Pelomonas* (OTU_15) and *Propionibacterium* (OTU_13) (Table 3.6), whereas for mRAS it was *Mycobacterium* (OTU_4), *Carnobacterium* (OTU_2) and *Pelomonas* (OTU_15) (Table 3.7). *Mycobacterium* (OTU_4) contributed to the largest dissimilarity between t1 and t2 in both

systems (SIMPER, 19.31 % for cRAS and 11.23 % for mRAS) (Appendix I, Table I.2 and I.3. It was more abundant in cRAS compared to mRAS and increased highly in abundance at t2 in cRAS (37 %). *Pelomonas* (OTU_15) was the OTU that contributed the second most to the differences in cRAS (SIMPER, 8.27%) and decreased in abundance through time. *Propionibacterium* (OTU_13) decreased in abundance in cRAS at t2 and was the OTU that caused the third most differences observed between t1 and t2 in cRAS (Appendix I, Table I.2).

For mRAS, the OTU causing the second largest dissimilarity between t1 and t2 was *Carnobacterium* (OTU_2) (11.07 %) (Appendix I, Table I.3). The OTU decreased in abundance at sampling time 2. *Pelomonas* (OTU_15) was also present in mRAS and increased in abundance at t2. OTU_1 representing *Brochothrix* decreased drastically at t2 in mRAS and was the OTU that contributed fifth most to the dissimilarity in skin microbiota between sampling times. *Mycobacterium* (OTU_4)*, Spartobacteria_genera_incertae_sedis* (OTU_6) and *Legionella* (OTU_16) were all among the ten most abundant OTUs in both systems. They followed the same developmental pattern as for the rearing water, indicating that the skin associated microbiota was affected by the surrounding water.

Table 3.6. The five most abundant OTUs associated with the skin of salmon parr reared in cRAS and average relative abundance.

OTU ID	Taxonomy	Average relative abundance
4	*Mycobacterium* (Actinobacteria)	0.20
15	*Pelomonas* (Betaproteobacteria)	0.12
13	*Propionibacterium* (Actinobacteria)	0.07
26	*Staphylococcus* (Bacilli)	0.03
45	*Sphingomonas* (Alphaproteobacteria)	0.03

Table 3.7. The five most abundant OTUs associated with the skin of salmon parr reared in mRAS and average relative abundance.

OTU ID	Taxonomy	Average relative abundance
4	*Mycobacterium* (Actinobacteria)	0.11
2	*Carnobacterium* (Bacilli)	0.10
15	*Pelomonas* (Betaproteobacteria)	0.09
13	*Propionibacterium* (Actinobacteria)	0.08
26	*Staphylococcus* (Bacilli)	0.06

3.5 Comparison of rearing water, gut and skin samples

The microbial communities in water, gut and skin mucus were significantly different (PERMANOVA, Bonferroni-corrected p = 0.0003, average Bray-Curtis similarities, 0.07 – 0.10) (Figure 3.12). Water and skin microbiota were more equal to each other than to the gut microbiota (PCoA analysis of Bray-Curtis similarities, Figure 3.12). The skin microbial communities showed the largest individual variation (average Bray-Curtis similarities, 0.18) (Figure 3.13).

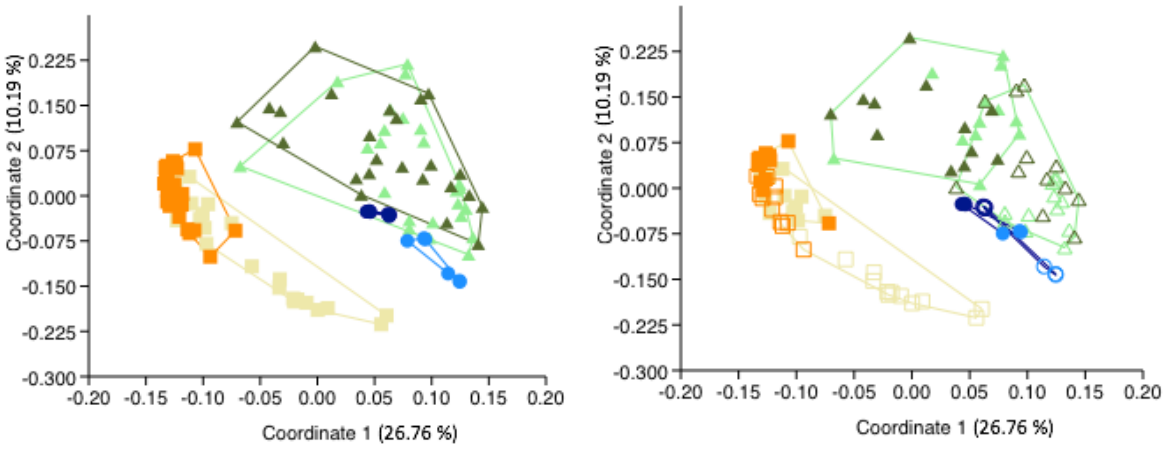


Figure 3.12. PCoA plot based on Bray-Curtis similarities for comparison of water (blue), gut (orange) and skin (green) associated microbiota based on A) mRAS (light colors) and cRAS (dark colors) and B) sampling time t1 (filled symbols) and t2 (open symbols).

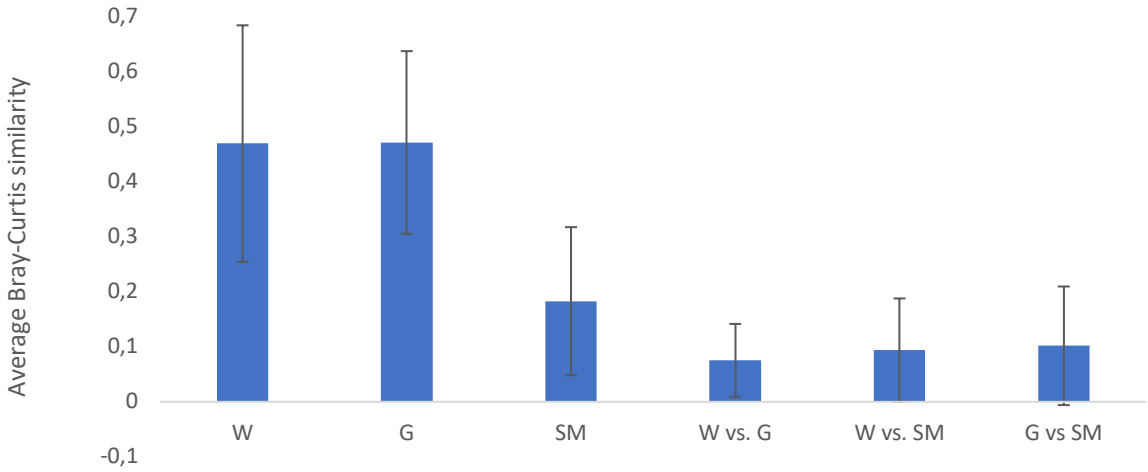


Figure 3.13. Average Bray-Curtis similarities for comparison of the bacterial communities associated with rearing water (W), gut (G) and skin (SM). Error bars indicate standard deviation (±SD).

The OTU *Carnobacterium* (OTU_2) was found to account for the largest difference between water, gut and skin samples (SIMPER, 9.20 %) (Table 3.8). The highest abundance of OTU_2 was found within the gut samples (19 %). *Mycobacterium* (OTU_4) contributed with 8.25 % to the dissimilarity between the three sample groups, with highest abundance in the skin samples (16 %). Also, OTU_1 and OTU_3, *Brochothrix* and *Rhodococcus* respectively, were more abundant in the gut samples. *Pelomonas* (OTU_15) was rarely not present in the water samples and was more abundant in skin samples (9.60 %). None of the five OTUs accounting for the majority of differences between the sampling groups were more abundant in water, which indicated that different microbial community profiles dominated within the fish host compared to water.

Table 3.8. The five OTUs contributing the most to the dissimilarity observed between rearing water (W), gut (G) and skin (SM) microbiota.

OTU	Taxonomy	Contribution %	Mean abundance W	Mean abundance G	Mean abundance SM
2	*Carnobacterium* (Bacilli)	9.20	0.01	0.19	0.06
4	*Mycobacterium* (Actinobacteria)	8.25	0.09	0.04	0.16
1	*Brochothrix* (Bacilli)	6.14	0.006	0.12	0.03
3	*Rhodococcus* (Actinobacteria)	4.71	0.0008	0.10	0.02
15	*Pelomonas* (Betaproteobacteria)	4.38	7.63×10^{-5}	0.002	0.10

3.6 Fish growth and performance

The salmon parr reared in mRAS were significantly longer, grew larger and had a significantly higher specific growth rate (SGR) and thermal-unit growth coefficient (TGC) compared to the fish in cRAS (Table 3.9). This indicate that the fish in mRAS grew and thrived better.

Table 3.9. Specific growth rate (SGR) and thermal-unit growth coefficient (TGC) from sampling time 1 to sampling time 2. Weight (g) and length (cm) measurements represent t2. All parameters are given in average ± SD.

Parameter	cRAS	mRAS	p-value*
SGR	0.64 ± 0.83	0.93 ± 0.92	0.001
TGC	0.15 ± 0.19	0.20 ± 0.20	0.008
Weight (g)	124.22 ± 22.90	140.63 ± 28.80	2.22×10^{-10}
Length (cm)	21.96 ± 1.43	23.02 ± 1.67	1.17×10^{-11}

*statistical analyses (SGR and TGC: Welch Two Sample t-test, length and weight: Mann-Whitney-Wilconxon test) were performed by Gaute Helberg (master student, NTNU)

The mortality through the experiment period (day 0 – 140) was three fish per system, resulting in a survival rate of 99 %.

4. Discussion

4.1 Evaluation of Illumina sequencing method and data quality

4.1.1 Illumina sequencing of 16S rDNA amplicons

To study the bacterial communities associated with rearing water, gut and skin mucus of Atlantic salmon parr reared in two RAS systems with different water treatment, Illumina sequencing of bacterial 16S rDNA amplicons was used. Illumina sequencing provides high number of sequence reads, high resolution and detailed taxonomic information (Illumina Inc, 2018). There exist several databases with tools that allows for processing and analyses of 16S rDNA sequence data (Bacci et al., 2015). There are, however, also drawbacks and biases related to deep sequencing of 16S rDNA amplicons that may affect the resulting community profiles. Bacterial genomes contain variable numbers of the rRNA gene regions (rrn operon), which cause over-representation of certain bacteria in the community. PCR bias may also give a wrong representation of the bacterial community in the sample. Metagenomics is an alternative method to study microbial communities. The total DNA from a defined habitat is isolated and cloned, before the microbial sequences of interest in the DNA library is analyzed (Sleator et al., 2008, Langer et al., 2006). Thus, PCR bias is avoided. However, the method requires more complex bioinformatic analyses, and if the microbial community is diverse, metagenomics may give low resolution (Jünemann et al., 2017, Cibrián-Jaramillo and Barona-Gómez, 2016). HTS of bacterial 16S rRNA amplicons is preferred to study the microbial community diversity.

The number of sequence reads varied among both water, gut and skin mucus samples. Especially the sequence reads for the skin samples were highly variable, indicated by their high standard deviation (Table 3.1). This may have given a wrong representation of the actual bacterial richness and diversity.

4.1.2 The skin mucus samples and co-amplification of salmon gene sequences

The skin samples had the lowest average numbers of reads per sample (~20 000 on average), more than five times lower than water and gut samples (Table 3.1). There were several problems related to analyses and processing of the skin mucus samples. First of all, they had lower DNA concentration during the extraction of DNA. Secondly, PCR amplification of the bacterial V4-region of the 16S rRNA gene turned out to be more difficult than for the other samples, indicating that the fish skin mucus may have contained some sort of inhibitors. Lastly, the

Illumina sequencing results for skin samples showed to be highly dominated by a mitochondrial (mt) 12S rRNA sequence from *S. salar*.

The samples were processed two times using the USEARCH pipeline. First, a threshold of minimum 150 bp was set for the paired sequences. However, the resulting community profiles were highly dominated by an OTU representing the salmon mt 12S rRNA gene. An "OTU_1" was found to represent the mitochondrial 12S rRNA gene of *S. salar*, where especially the skin samples were highly represented by this OTU (average abundance in all skin samples was 0.88, and up to 0.97 average abundance at the most in one sample). The co-amplified mt 12S rRNA PCR product was expected to be 214 bp (including primers). To compare with the gut samples, the average abundance of OTU_1 in the gut samples was 0.04. Thus, at the second processing, the minimum threshold for paired sequences was increased to 230 bp to eliminate the mt 12S rRNA gene reads from the data set. The reason for this may be that the skin samples contained low numbers of bacteria and a larger proportion of salmon-DNA (Personal communication, Ingrid Bakke).

A possible strategy to avoid co-amplification of the *S. salar* mt 12S rRNA gene is to use other broad-range 16S rDNA primer sets. The primers used in this project were 515F and 805R (amplifies the V4 region), which have large homology to the regions in the salmon 12S rRNA gene. The forward primer 338F (amplifies the V3 region) is an alternative which has less homology to the salmon rRNA genes. The primers, however, gives a longer PCR product in combination with 805R (V3-V4 region), which in turn may result in poorer amplification efficiency. When using the primer combination 338F/805R for amplification of the V3-V4 regions of the 16S rRNA gene of problematic samples, such as fish skin samples, there is often problems with primer dimer formation (Personal communication, Ingrid Bakke).

After removal of the salmon mt 12S rDNA reads, ten skin mucus samples had lower than 10 000 sequence reads (ranging from 4000 to 10 000 reads). With this low number of sequence reads, compared to that for the other skin samples, the observed number of OTUs in these samples may be less than the actual number of OTUs in the skin bacterial community profiles. The samples with low sequence reads were compared to the other samples in a PCoA analysis (Appendix J, Figure J.1) which showed that they had no deviant community profile. No large effect in the PCoA plot was observed if they were excluded (Appendix J, Figure J.2). This was the reason for why they were included in further statistical analyses.

4.1.3 Propionibacterium and Sphingomonas, possible contaminants?

DNA extraction kits have shown to be contaminated by DNA, probably during the production process (Mollerup et al., 2016, Peters et al., 2004, Evans et al., 2003). The genera *Propionibacterium* and *Sphingomonas* has been detected in non-template controls for DNA extraction kit (milliQ water instead of tissue sample during DNA extraction) during PCR and affected the sequencing results in studies of microbial community diversity (Salter et al., 2014). In this study, two OTUs representing these genera were detected in the QIAamp DNA mini kit negative control ("kit-blank") at low abundances (average relative abundance 1.80×10^{-5} and 0.03, respectively). The negative control for the DNA extraction kit was amplified and sequenced on another sequencing lane. Therefore, it cannot be excluded that other contaminants can be represented in the data set in this study. Furthermore, the data set in this study showed that *Propionibacterium* (average relative abundance 0.03) and *Sphingomonas* (average relative abundance 0.01) OTUs were represented in 37 of 48 gut samples and were the third and sixth most abundant OTUs associated with the skin mucus of Atlantic salmon parr respectively (Appendix E, Table E.1). The ten skin mucus samples with the lowest numbers of reads (4000 – 10 000) had higher abundances of these two OTUs that the skin samples with higher number of reads. Moreover, since the most abundant OTU in the control was an *Azomonas*-OTU (average relative abundance 0.50), which was not represented in the fish microbiota at all, and the two OTUs were relatively little represented in the "kit-blank", is it highly likely that the *Propionibacterium* and *Sphingomonas* OTUs were not contaminants. Boutin et. al (2013) investigated the effect of stress on skin associated microbiota of brook charr (*Salvelinus fontinalis*) and detected *Propionibacterium* and *Sphingomonas* as two of the most abundant genera of the skin microbial communities of unstressed healthy fish. Both genera have also been isolated from the GI tract of Atlantic salmon (Dehler et al., 2017b, Zarkasi et al., 2017).

4.2 The effect of membrane filtration on fish growth and performance

The fish reared in mRAS was significantly larger and grew faster compared to the fish in cRAS in period 4 and 5 (Table 3.9). The water in mRAS had a higher temperature compared to cRAS, but the temperature corrected growth (TGC) was still significantly higher for the fish in mRAS. Studies performed on marine larvae has shown that larvae reared in a RAS with an implemented membrane filter grew better compared to the control (Wold et al., 2014). This result may indicate that membrane filtration and removal of accumulating organic particles from the water facilitated for more optimal water quality and microbial state of the rearing water, and thus, increased growth of the fish. The fish reared in cRAS may have been more stressed due to

higher particle levels and poorer microbial water quality. This may have had an impact on the microbiota colonizing the salmon parr, causing the lower observed growth rate.

Furthermore, the observed growth rates of the fish in both cRAS and mRAS were lower than the potential growth rates previously found at the respective water temperatures (Austreng et al., 1987). This may indicate suboptimal rearing conditions within both systems. Throughout the experiment period there were shorter and longer periods with unstable and suboptimal water quality variables, such as very high particle levels in cRAS, increased water temperature due to heat production by the membrane and sudden activation of the emergency oxygen. Such variations in water quality may have triggered stress responses in the exposed fish. Chronic stress exposure is known to reduce the growth rate of Atlantic salmon parr (Madaro et al., 2015) and also lower the resistance against opportunistic bacteria (Llewellyn et al., 2014). Potential opportunistic pathogens were detected, however, there were no signs to disease in either of the systems and the survival rate was equal in both systems.

4.3 The effect of membrane filtration on the water microbial communities

The bacterial communities associated with the rearing water had the highest richness and Shannon's diversity index of all three sample groups (Figure 3.1 and 3.2). Furthermore, the water microbiota of mRAS had a significantly higher diversity compared to cRAS ($p < 0.05$) (Figure 3.2). This indicates that the membrane filter may have affected the alpha diversity related to the bacterial communities, both by removal of bacteria directly and by removal of their substrate, organic matter. Removal of organic matter reduces the carrying capacity of heterotrophic bacteria and may promote formation of a more stable environment with high competition for substrate, favoring proliferation of K-strategists. On the other hand, large perturbances (e.g. addition of organic matter), unstable conditions and a high and unrealized carrying capacity promotes r-selection and opportunistic proliferation. K-strategists dominates in mature communities which are characterized by high diversity (Vadstein et al., 1993). The significantly higher diversity in mRAS suggest that the system may have gained a more K-selected water community, or at least a less r-selected community. These findings coincide with a study performed on post-smolt reared under the similar conditions as in this experiment, with one conventional RAS and a RAS with membrane filtration (Fossmark, 2016). At the second sampling time, the diversity of the water microbiota was reduced in cRAS and increased in mRAS, compared to that of the first sampling time. The feed overloading period prior to the second sampling time increased the bacterial carrying capacity and probably facilitated for a

pioneer community with lower diversity in cRAS compared to mRAS (Figure 3.2), where a few species (opportunists) dominate.

The most noticeable difference between the water microbiota communities at OTU and class level was the presence of *Mycobacteria* (Actinobacteria) and *Spartobacteria_genera_incertae_sedis* (Spartobacteria). At the OTU level, also *Legionella* (Gammaproteobactera) contributed to the differences. Interestingly, they were all more abundant in cRAS, the system with a higher bacterial carrying capacity. A *Sphaerotilus*-OTU (Betaproteobacteria) dominated in mRAS at t1 and *Flavobacterium* (Actinobacteria) became the most abundant OTU at t2. The microbiota present in the water is a reflection of the water quality variables such as temperature, pH, salinity and substrate availability. *Mycobacterium* (Actinobacteria) are rod-shaped but may even undergo filamentous growth. They thrive in different natural water environments and several opportunistic species of the genus have been isolated from municipal water distribution systems (Madigan et al., 2015, Percival and Williams, 2014). Spartobacteria is a class in the phylum Verrucomicrobiota, ubiquitous in freshwater sources, but little studied (He et al., 2017). This genus is found to co-occur with cyanobacterial blooms in brackish water of the Baltic sea during the summer period (Herlemann et al., 2013) and increase in abundance during periods with more labile dissolved organic matter (DOC) (Arnds et al., 2010). *Legionella* is a well-known genus consisting of several waterborne opportunistic pathogens (Madigan et al., 2015).

Furthermore, the feed overloading period (day 119 – 140) affected the water microbial community profiles in both systems. Both OTU_4 and OTU_6 increased in abundance after the feed overloading period in cRAS, whereas *Sphaerotilus* (Betaproteobacteria) decreased in abundance in mRAS. There was not observed large concentration differences of DOC between the systems during the last two periods (day 95 – 128), but cRAS had a higher total organic carbon (TOC) concentration (Appendix B, Table B.2). The supply of DOC and TOC increased during the feed overloading period in both systems, but more extreme in cRAS (since a lot of the organic matter was removed by the membrane in the mRAS). This may explain why *Mycobacterium* and *Spartobacteria* increased even more in abundance in cRAS (Figure 3.3). These findings indicate that membrane filtration facilitated for a mature community with higher competition for substrate that prevented proliferation of these presumed opportunists. A microbial environmental balance is an important factor to prevent opportunistic heterotrophic blooms and thought to facilitate for good health and well-being of the reared fish (Llewellyn et

al., 2014). These results indicate that membrane filtration may be a strategy to obtain a more preferable water microbial community in recirculating aquaculture systems.

4.4 The effect of membrane filtration on the gut associated microbiota

The bacterial communities associated with the gut of Atlantic salmon parr reared in cRAS and mRAS were found to be significantly different from each other ($p < 0.05$). The gut microbiota of the fish in mRAS were less diverse compared to that of fish in cRAS (Figure 3.2). Factors that are known affect and shape the gut microbial composition are genetics, developmental stage, diet, environmental conditions and stress (Nayak, 2010b, Sullam et al., 2012, Merrifield and Rodiles, 2015). The salmon parr in this experiment had the same genetic background, were in the same developmental stage and fed the same diet. Because of this, the observed differences in the microbial communities between cRAS and mRAS were most likely were caused by different exposure to stress and/or the two rearing environments colonized by different microbiota.

Membrane filtration reduces the number of organic particles in the water column. A reduction in particle levels is related to both stress reduction and improved water quality. Colloids and fine suspended solids that accumulate during operation may adhere to the gill tissue and further affect respiration and osmoregulation, causing consecutive stressful situations for the fish (Chapman et al., 1987, Fernandes et al., 2015). A study performed on Atlantic salmon showed that stress induced structural changes in the GI tract; with increased shedding of mucus and changes the content of colonizing microbiota in the gut (Olsen et al., 2002). Thus, the fish in cRAS may have been more stressed due to higher particle levels in the water column, causing the significant differences in fish gut microbiota between cRAS and mRAS. Furthermore, the environment and associated microbiota may affect the intestinal microbial communities. The water microbiota compositions in cRAS and mRAS were found to be significantly different from each other and may be the reason for the different gut microbiota in the two systems. Giastis et. al (2015) found that changes in the gut microbiota of tilapia larvae most likely correlated with changes in the microbial communities of the surrounding water. Another study performed on Atlantic salmon parr reared under two different rearing conditions, one RAS and one open loch facility, found that the dissimilarities in the gut microbial communities between the two groups probably were a reflection of the different water microbiota (Dehler et al., 2017b). In addition, seawater transfer affected the intestinal community profiles of Atlantic salmon (Dehler et al., 2017a). The results in this study indicates that the bacterial taxa common

in the rearing water, are also more common in the fish gut. Especially for cRAS was this the case. *Spartobacteria_genera_incertae_sedis* (Spartobacteria), *Mycobacterium* (Actinobacteria) and *Legionella* (Gammaproteobacteria) (OTU_16) were highly abundant in the gut microbiota of fish in cRAS. All of these OTUs were abundant in the rearing water of both systems, but interestingly, OTU_4, OTU_6 and OTU_16 were nearly not present in the gut of the fish reared in mRAS. These findings strongly indicate that the gut microbiota was affected by the water microbiota and that the three presumed opportunists were able to colonize the gut of fish reared in cRAS. Even though the water microbiota clearly had an influence on the communities associated with the gut, there were low similarities between the microbiota associated with water and gut due to other selection pressures existing in these environments.

The diversity of the gut microbiota in both systems increased from t1 to t2, which may be due to the feed overloading period. It also created noticeable different gut microbiota in cRAS compared to the more similar gut microbiota in mRAS (Figure 3.6 and 3.7). The two OTUs that increased in abundance in the water at t2, OTU_4 and OTU_6, also increased in abundance in the gut microbiota, further suggesting that the microbial state of the gut became altered after the feed overloading period. *Carnobacterium* (Bacilli) and *Brochothrix* (Bacilli) were the two most abundant OTUs in the gut microbiota in both systems, but interestingly, mRAS fish had a higher abundance of these OTUs in their gut at both sampling times (Table 3.4 and 3.5). *Carnobacterium* is thought to be a part of the normal gut flora in healthy fish (Ringø and Gatesoupe, 1998). It is a common probiotic used in aquaculture due to its potential to inhibit growth of fish pathogens such as *Aeromonas salmonicida* and different *Vibrio* spp. (Nayak, 2010a, Ringø, 2008), but some species could also be pathogenic (Leisner et al., 2007). *Brochothrix* has been isolated from the intestine of Atlantic cod and used as probiotic against *Aeromonas bestiarum* in rainbow trout (Ringø et al., 2006, Pieters et al., 2008). These findings further indicate that membrane filtration may prevent large changes in the gut microbiota in periods with different feed loading due to more stable concentration of organic particles in the water column.

Thus, different exposure to stress and/or the different water microbiota may be the reason for the different gut microbiota between the cRAS and mRAS. There is, however, no guarantee that the microbes associated with the water actually affects gut microbiota of fish. Studies performed on teleost fish in early life stages (tilapia and Atlantic cod larvae) showed that the water microbiota affects the colonizing of the gut (Giatsis et al., 2015, Bakke et al., 2015).

However, research on how water microbiota affects the gut microbiota of fish in later developmental stages are scarce.

4.5 The effect of membrane filtration on the skin associated microbiota

The bacterial communities associated with the skin were significantly different between the two sampling times ($p < 0.05$), but not between cRAS and mRAS (Figure 3.9). The largest differences between the skin microbiota between the two systems was the higher dominance of *Mycobacterium* (Actinobacteria) in cRAS compared to mRAS. Furthermore, *Carnobacterium* (Bacilli) and several other related genera was more abundant in mRAS (Figure 3.3). The microbiota associated with fish skin is little studied, but species of the class Bacilli are known to belong to the normal microbiota in fish gut (Ringø and Gatesoupe, 1998). In the present study, Bacilli was the most abundant class in the gut microbiota of fish reared in mRAS. The skin is directly and constantly in contact with the surrounding water, and the environment and associated microbiota are one of the factors thought to affect the skin communities (Lokesh and Kiron, 2016, Chiarello et al., 2015). The skin and its mucus are colonized by several of the same bacterial strain as found in the environment (Minniti et al., 2017, Boutin et al., 2013) and a change in the environment reshape the skin associated microbiota (Lokesh and Kiron, 2016). Lokesh and Kiron (2016) showed that transition of Atlantic salmon from freshwater to seawater increased the diversity of the bacterial skin community. Interestingly, in the present study, OTUs representing *Mycobacterium* (Actinobacteria), *Spartobacteria_genera_incertae_sedis* (Spartobacteria) and *Legionella* (Gammaproteobacteria) were highly abundant in the skin microbiota of fish in both systems. This indicate that the skin associated microbiota was strongly affected by the surrounding water microbiota. Like for the gut microbiota, there was still differences in the microbial community compositions in the rearing water and fish skin due to different selection pressures.

Furthermore, stress may induce changes in the skin microbial communities. A study performed on brook charr found that stress may affect the skin microbiota by lowering the abundance of commensal microbiota, allowing potential pathogenic bacteria to colonize the skin. The skin microbiota of stressed brook charr was dominated by Actinobacteria strains (Boutin et al., 2013, Llewellyn et al., 2014). Thus, the higher particle levels in cRAS may have induced stress responses, allowing the three presumed opportunists (OTU_4, OTU_6 and OTU_16) found in the rearing water to colonize the skin of fish in cRAS to a higher extent than in mRAS.

The microbial communities associated with the skin of the salmon parr were different between sampling time t1 and t2 (Figure 3.3, Appendix I, Table I.2 and I.3). This was most likely due to different feed loading prior to t1 and t2. As for the water and gut microbiota, both *Mycobacterium* (Actinobacteria), *Spartobacteria_genera_incertae_sedis* (Spartobacteria) and OTUs that could not be classified at the class level, became more abundant in the skin-associated communities in both systems. Feed overloading of the systems thus increased the abundance of the presumed opportunists on the skin of the fish. Two OTUs representing *Propionibacterium* and *Sphingomonas* were found to be highly abundant in the skin microbiota of fish in both systems. They decreased in abundance in cRAS and increased in abundance in mRAS after the overloading period. Interestingly, Boutin et. al (2013) found that *Propionibacterium* and *Sphingomonas* were the two most abundant genera of the skin microbial communities of unstressed healthy fish, further indicating increased stress exposure in cRAS due to higher levels of organic matter.

These results further indicate that optimal and stable water quality variables (including microbial quality) are even more important for the skin associated microbiota, since the skin is constantly in contact and probably shaped by the rearing water and associated microbiota.

4.6 Comparison of the water, gut and skin microbiota

The skin samples had the lowest richness and diversity among all three sample groups (Figure 3.1 and 3.2). Lowrey et. al (2015) found, in contrast to this study, higher microbial diversity compared to the internal colonization sites. They suggested that the intestine has a more stable community "that shape specialized microbial communities" (Lowrey et al., 2015). The reason for the low diversity in this study, might have been problems related to PCR amplification and the low number of reads obtained for the skin samples.

The bacterial communities associated with rearing water, gut and skin mucus were significantly different from each other ($p < 0.05$). The fish host possess a selective environment, offering a different environment compared to the surrounding water. Several studies have shown that the host microbiota and water microbiota are different from each other (Minniti et al., 2017, Bakke et al., 2013). Interestingly in this study is that several OTUs in the rearing water colonized both the gut and skin environment of salmon parr in both cRAS and mRAS.

4.7 Future work and perspectives

Research on microbial stability in land-based aquaculture systems has mainly focused on marine larvae. There has been little focus on rearing of Atlantic salmon parr toward sea transfer. The most hazardous phase of the salmon production cycle is the transfer of smolt to sea cages and the following weeks at sea. A larger smolt with good health and welfare are thought to handle this stressful situation better. How to facilitate for optimal and stable rearing conditions and microbial state of the rearing water is valuable information that can be used to facilitate for better health and welfare of salmon parr in RAS.

The results in this study showed that the salmon parr reared in mRAS were larger compared to the fish in cRAS. An explanation for the differences in growth may be that the fish in cRAS were stressed due to high amounts of accumulating organic particles in the water column. Stress and a poorer microbial water quality may have affected the commensal microbial communities, causing the observed differences. More knowledge related to the effect of stress and the effect of water microbiota on the commensal microbial communities associated with the fish is needed. The experimental period investigated here was characterized by periods with suboptimal rearing conditions that varied over time within and between the systems. This may have affected both the fish growth and the microbial communities associated with water and fish in the two systems differently. It would be more optimal to compare two systems with equal and stable water quality variables, only differing by the membrane filtration step. In future studies, water, gut and skin samples from a larger number of sampling times should be analyzed to investigate the effect of water microbiota on the microbial communities associated with the host. It could be interesting to study the growth, welfare and the total production output of smolt reared in membrane filtered water also after transfer to on-growing in sea cages. Microbial water quality is expected to be even more important for salmon in early developmental stages because they are less robust. A “first-feeding experiment” with salmon fry and the importance of optimal and stable microbial water quality would be interesting to study. Better health and welfare in early developmental stages is fundamental for further development. Furthermore, gene expression analyses (for example; transcriptomics, qPCR or microarray analyses) could be applied to study the effect of different microbiota on fish gene expression, for example, how expression of genes in the fish immune system is affected by bacterial loads or different pathogens, giving a more detailed information beyond growth responses.

5. Conclusions

The objectives of this thesis were to evaluate the effect on membrane filtration on the microbial communities associated with rearing water, gut and skin mucus of Atlantic salmon parr reared in cRAS and mRAS at two sampling times (t1 and t2) with different levels of feed loading and water exchange. The major findings were as follows:

The microbial communities associated with the rearing water in cRAS and mRAS were significantly different. The microbial communities in mRAS were more diverse compared to that of cRAS. In cRAS, three OTUs representing *Mycobacteria*, *Spartobacteria_genera_incertae_sedis* and *Legionella* were more abundant compared to mRAS. Except from *Legionella*, these OTUs increased in abundance in cRAS after the feed overloading period. A *Sphaerotilus*-OTU dominated in the water community of mRAS at t1, before an OTU representing *Flavobacterium* became more abundant at t2.

The gut microbiota of fish reared in cRAS and mRAS were significantly different from each other. Fish reared in mRAS had a more similar gut microbial composition between the sampling times, despite different feed loading. This may indicate that the gut microbiota was more stable over time. The OTUs *Mycobacterium, Spartobacteria_genera_incertae_sedis* and *Legionella* were more abundant also in the fish gut microbiota in cRAS. After the overfeeding period, the two former OTUs became even more abundant. This indicated that membrane filtration reduced these few opportunists associated with the rearing water from colonizing the gut.

The microbial communities associated with the skin of salmon parr were significantly different between t1 and t2, but not between cRAS and mRAS, suggesting that the skin microbiota was more affected by the change in feed loading than water treatment. *Mycobacteria*, *Spartobacteria_genera_insterae_sedis* and *Legionella* colonized the skin mucus of salmon parr in both systems, but with a higher dominance in the fish of cRAS. Also, in the skin communities, both *Mycobacterium* and *Spartobacteria_genera_incertae_sedis* increased after the feed overloading period.

The three presumed opportunists associated with the rearing water were able to colonize both the gut and skin mucus of the salmon parr. These results indicate that the water microbiota affected the microbial communities associated with the gut and skin mucus.

The Atlantic salmon parr reared in mRAS grew larger compared to the fish reared in cRAS. Higher particle levels and suboptimal microbial water quality may have induced stress responses and affected the microbiota associated with the fish, causing the lower growth rate of fish in cRAS.

6. References

ANDERSON, M. J. 2001. A new method for non-parametric multivariate analysis of variance. *Austral Ecology,* 26**,** 32-46.

ÁNGELES ESTEBAN, M. & CEREZUELA, R. 2015. 4 - Fish mucosal immunity: skin A2 - Beck, Benjamin H. *In:* PEATMAN, E. (ed.) *Mucosal Health in Aquaculture.* San Diego: Academic Press.

ARNDS, J., KNITTEL, K., BUCK, U., WINKEL, M. & AMANN, R. 2010. Development of a 16S rRNA-targeted probe set for Verrucomicrobia and its application for fluorescence in situ hybridization in a humic lake. *Systematic and Applied Microbiology,* 33**,** 139-148.

ASHLEY, P. J. 2007. Fish welfare: Current issues in aquaculture. *Applied Animal Behaviour Science,* 104**,** 199-235.

ATTRAMADAL, K. J. K., SALVESEN, I., XUE, R., ØIE, G., STØRSETH, T. R., VADSTEIN, O. & OLSEN, Y. 2012. Recirculation as a possible microbial control strategy in the production of marine larvae. *Aquacultural Engineering,* 46**,** 27-39.

ATTRAMADAL, K. J. K., TRUONG, T. M. H., BAKKE, I., SKJERMO, J., OLSEN, Y. & VADSTEIN, O. 2014. RAS and microbial maturation as tools for K-selection of microbial communities improve survival in cod larvae. *Aquaculture,* 432**,** 483-490.

AUSTRENG, E., STOREBAKKEN, T. & ÅSGÅRD, T. 1987. Growth rate estimates for cultured Atlantic salmon and rainbow trout. *Aquaculture,* 60**,** 157-160.

BACCI, G., BANI, A., BAZZICALUPO, M., CECCHERINI, M. T., GALARDINI, M., NANNIPIERI, P., PIETRAMELLARA, G. & MENGONI, A. 2015. Evaluation of the Performances of Ribosomal Database Project (RDP) Classifier for Taxonomic Assignment of 16S rRNA Metabarcoding Sequences Generated from Illumina-Solexa NGS. *Journal of Genomics,* 3**,** 36-39.

BADIOLA, M., GARTZIA, I., BASURKO, O. C. & MENDIOLA, D. 2017. Land-based growth of Atlantic salmon (Salmo salar) and consumers' acceptance. *Aquaculture Research,* 48**,** 4666-4683.

BADIOLA, M., MENDIOLA, D. & BOSTOCK, J. 2012. Recirculating Aquaculture Systems (RAS) analysis: Main issues on management and future challenges. *Aquacultural Engineering,* 51**,** 26-35.

BAKKE, I., COWARD, E., ANDERSEN, T. & VADSTEIN, O. 2015. Selection in the host structures the microbiota associated with developing cod larvae (Gadus morhua). *Environmental Microbiology,* 17**,** 3914-3924.

BAKKE, I., SKJERMO, J., VO, T. A. & VADSTEIN, O. 2013. Live feed is not a major determinant of the microbiota associated with cod larvae (Gadus morhua). *Environmental Microbiology Reports,* 5**,** 537-548.

BANSIL, R. & TURNER, B. S. 2018. The biology of mucus: Composition, synthesis and organization. *Advanced Drug Delivery Reviews,* 124**,** 3-15.

BARRUT, B., BLANCHETON, J.-P., CALLIER, M., CHAMPAGNE, J.-Y. & GRASMICK, A. 2013. Foam fractionation efficiency of a vacuum airlift—Application to particulate matter removal in recirculating systems. *Aquacultural Engineering,* 54**,** 16-21.

BEISEL, J.-N. & MORETEAU, J.-C. 1997. A simple formula for calculating the lower limit of Shannon's diversity index. *Ecological Modelling,* 99**,** 289-292.

BLANCHETON, J. P., ATTRAMADAL, K. J. K., MICHAUD, L., D'ORBCASTEL, E. R. & VADSTEIN, O. 2013. Insight into bacterial population in aquaculture systems and its implication. *Aquacultural Engineering,* 53**,** 30-39.

BONE, Q. 2008. *Biology of fishes,* New York, Taylor & Francis.

BOUTIN, S., BERNATCHEZ, L., AUDET, C. & DERÔME, N. 2013. Network Analysis Highlights Complex Interactions between Pathogen, Host and Commensal Microbiota. *PLoS One,* 8**,** e84772.

BOUTIN, S., SAUVAGE, C., BERNATCHEZ, L., AUDET, C. & DEROME, N. 2014. Inter Individual Variations of the Fish Skin Microbiota: Host Genetics Basis of Mutualism?(Research Article). *PLoS ONE,* 9.

BRAY, J. R. & CURTIS, J. T. 1957. An Ordination of the Upland Forest Communities of Southern Wisconsin. *Ecological Monographs,* 27**,** 326-349.

BREGNBALLE, J. 2015. A Guide to Recirculating Aquaculture. *An introduction to the new environmentally friendly and highly productive*

closed fish farming systems. Food and Agriculture Organization of the United Nations (FAO)

EUROFISH International Organisation.

CAPORASO, J. G., LAUBER, C. L., WALTERS, W. A., BERG-LYONS, D., HUNTLEY, J., FIERER, N., OWENS, S. M., BETLEY, J., FRASER, L., BAUER, M., GORMLEY, N., GILBERT, J. A., SMITH, G. & KNIGHT, R. 2012. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *The Isme Journal,* 6**,** 1621.

CECH, J. J., MITCHELL, S. J. & WRAGG, T. E. 1984. Comparative growth of juvenile white sturgeon and striped bass: Effects of temperature and hypoxia. *Estuaries,* 7**,** 12-18.

CHAO, A. 1984. Nonparametric Estimation of the Number of Classes in a Population. *Scandinavian Journal of Statistics,* 11**,** 265-270.

CHAO, A., CHIU, C. H. 2016. Species Richness: Estimation and Comparison. *Wiley StatsRef: Statistics Reference Online.,* 1-26.

CHAPMAN, P. M., POPHAM, J. D., GRIFFIN, J., LESLIE, D. & MICHAELSON, J. 1987. Differentiation of physical from chemical toxicity in solid waste fish bioassays. *Water, Air, and Soil Pollution,* 33**,** 295-308.

CHEN, S., LING, J. & BLANCHETON, J.-P. 2006. Nitrification kinetics of biofilm as affected by water quality factors. *Aquacultural Engineering,* 34**,** 179-197.

CHIARELLO, M., VILLÉGER, S., BOUVIER, C., BETTAREL, Y., BOUVIER, T. & MARCHESI, J. 2015. High diversity of skin-associated bacterial communities of marine fishes is promoted by their high variability among body parts, individuals and species. *FEMS Microbiology Ecology,* 91.

CIBRIÁN-JARAMILLO, A. & BARONA-GÓMEZ, F. 2016. Increasing Metagenomic Resolution of Microbiome Interactions Through Functional Phylogenomics and Bacterial Sub-Communities. *Frontiers in Genetics,* 7**,** 4.

CLARK, D. D. P. 2012. *Molecular biology,* Oxford, Elsevier Academic Press.

COLE, J. R., WANG, Q., FISH, J. A., CHAI, B., MCGARRELL, D. M., SUN, Y., BROWN, C. T., PORRAS-ALFARO, A., KUSKE, C. R. & TIEDJE, J. M. 2014. Ribosomal Database Project: data and tools for high throughput rRNA analysis. *Nucleic acids research,* 42**,** D633.

CRIPPS, S. J. & BERGHEIM, A. 2000. Solids management and removal for intensive land-based aquaculture production systems. *Aquacultural Engineering,* 22**,** 33-56.

DE MUINCK, E. J., TROSVIK, P., GILFILLAN, G. D., HOV, J. R. & SUNDARAM, A. Y. M. 2017. A novel ultra high-throughput 16S rRNA gene amplicon sequencing library preparation method for the Illumina HiSeq platform. *Microbiome,* 5**,** 68.

DEHLER, C., SECOMBES, C. & MARTIN, S. 2017a. Seawater transfer alters the intestinal microbiota profiles of Atlantic salmon (Salmo salar L.). *Sci Rep,* 7**,** 13877-13877.

DEHLER, C. E., SECOMBES, C. J. & MARTIN, S. A. M. 2017b. Environmental and physiological factors shape the gut microbiota of Atlantic salmon parr (Salmo salar L.). *Aquaculture,* 467**,** 149-157.

EDGAR, R. 2016. SINTAX: a simple non-Bayesian taxonomy classifier for 16S and ITS sequences. *bioRxiv*.

EDGAR, R. C. 2013. UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nat Methods,* 10**,** 996-8.

EFSA 2008. Scientific Opinion of the Panel on Animal Health and Welfare on a request from the European Commission on Animal welfare aspects of husbandry systems for farmed Atlantic salmon. *The EFSA Journal.*

ELLIOTT, J. M. 1991. Tolerance and resistance to thermal stress in juvenile Atlantic salmon, Salmo salar. *Freshwater Biology,* 25**,** 61-70.

EVANS, G. E., MURDOCH, D. R., ANDERSON, T. P., POTTER, H. C., GEORGE, P. M. & CHAMBERS, S. T. 2003. Contamination of Qiagen DNA Extraction Kits with Legionella DNA. *Journal of Clinical Microbiology,* 41**,** 3452.

FAO 2016. The State of World Fisheries and Aquaculture 2016. *The State of World Fisheries and Aquaculture.* Food and Agriculture Organization of the United Nations.

FERNANDES, P., PEDERSEN, L.-F. & PEDERSEN, P. B. 2015. Microscreen effects on water quality in replicated recirculating aquaculture systems. *Aquacultural Engineering,* 65**,** 17-26.

FINSTAD, B., IVERSEN, M. & SANDODDEN, R. 2003. Stress-reducing methods for releases of Atlantic salmon (Salmo salar) smolts in Norway. *Aquaculture,* 222**,** 203-214.

FISKERIDIREKTORATET 2017. Key figures from Norwegian aquaculture industry.: Norwegian Directorate of Fisheries.

FJELLHEIM, A. J., HESS-ERGA, O.-K., ATTRAMADAL, K. & VADSTEIN, O. 2016. Recycling of water in hatchery production. *Background booklet for courses in recycling technology for hatchery production.*

FOLMAR, L. C. & DICKHOFF, W. W. 1980. The parr—Smolt transformation (smoltification) and seawater adaptation in salmonids: A review of selected literature. *Aquaculture,* 21**,** 1-37.

FOSSMARK, O. R. 2016. *The effect of membrane filtration on the microbial community dynamics in RAS for post-smolt production.* Master's thesis, Norwegian University of Science and Technology.

FRAUNE, S. & BOSCH, T. C. G. 2010. Why bacteria matter in animal development and evolution. *BioEssays,* 32**,** 571-580.

GIATSIS, C., SIPKEMA, D., SMIDT, H., HEILIG, H., BENVENUTI, G., VERRETH, J. & VERDEGEM, M. 2015. The impact of rearing environment on the development of gut microbiota in tilapia larvae. *Scientific Reports (Nature Publisher Group),* 5**,** 18206.

GLUICK, T. C. & DRAPER, D. E. 1992. Tertiary structure of ribosomal RNA. *Current Opinion in Structural Biology,* 2**,** 338-344.

GOMEZ, D., SUNYER, J. O. & SALINAS, I. 2013. The mucosal immune system of fish: The evolution of tolerating commensals while fighting pathogens. *Fish and Shellfish Immunology,* 35**,** 1729-1739.

HAMBLY, A. C., ARVIN, E., PEDERSEN, L. F., PEDERSEN, P. B., SEREDYŃSKA-SOBECKA, B. & STEDMON, C. A. 2015. Characterising organic matter in recirculating aquaculture systems with fluorescence EEM spectroscopy. *Water Research,* 83**,** 112-120.

HAMMER, Ø., HARPER, D.A.T., RYAN, P.D. 2001. PAST: Paleontological Statistics software package for education.

HE, S., STEVENS, S. L. R., CHAN, L.-K., BERTILSSON, S., GLAVINA DEL RIO, T., TRINGE, S. G., MALMSTROM, R. R. & MCMAHON, K. D. 2017. Ecophysiology of Freshwater Verrucomicrobia Inferred from Metagenome-Assembled Genomes. *mSphere,* 2**,** e00277-17.

HERLEMANN, D. P. R., LUNDIN, D., LABRENZ, M., JÜRGENS, K., ZHENG, Z., ASPEBORG, H., ANDERSSON, A. F. & JURGENS, K. 2013. Metagenomic De Novo Assembly of an Aquatic Representative of the Verrucomicrobial Class Spartobacteria. *Mbio,* 4**,** e00569-12.

HOLAN, A. B., WOLD, P. A. & LEIKNES, T. O. 2014. Membrane performance and fouling behavior of membrane bioreactors installed in marine recirculating aquaculture systems. *Aquacultural Engineering,* 58**,** 45-51.

ILLUMINA INC. 2018. *An Introduction to Next-Generation Sequencing Technology* [Online]. Illumina Inc. [Accessed].

INGERSLEV, H. C., VON GERSDORFF JØRGENSEN, L., LENZ STRUBE, M., LARSEN, N., DALSGAARD, I., BOYE, M. & MADSEN, L. 2014. The development of the gut microbiota in rainbow trout (Oncorhynchus mykiss) is affected by first feeding and diet type. *Aquaculture,* 424-425**,** 24-34.

IP, Y. K., CHEW, S. F. & RANDALL, D. J. 2001. Ammonia toxicity, tolerance, and excretion. *Fish Physiology.* Academic Press.

IVERSEN, M., FINSTAD, B., MCKINLEY, R. S., ELIASSEN, R. A., CARLSEN, K. T. & EVJEN, T. 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**,** 373-382.

JOBLING, M. 2003. The thermal growth coefficient (TGC) model of fish growth: a cautionary note. *Aquaculture Research,* 34**,** 581-584.

JOBLING, M., ARNESEN, M.-A., BENFEY, T., CARTER, C., HARDY, R., LE FRANCOIS, R. N., O'KEEFE, R., KOSKELA, J. & LAMAARE, G. S. 2010. The Salmonids (Family: Salmonidae). *In:* LE FRANCOIS, N., JOBLING, M., CARTER, C. & BLIER, P. (eds.) *Finfish Aquaculture Diversification.*

JÜNEMANN, S., KLEINBÖLTING, N., JAENICKE, S., HENKE, C., HASSA, J., NELKNER, J., STOLZE, Y., ALBAUM, S. P., SCHLÜTER, A., GOESMANN, A., SCZYRBA, A. & STOYE, J. 2017. Bioinformatics for NGS-based metagenomics and the application to biogas research. *Journal of Biotechnology,* 261**,** 10-23.

KRISTENSEN, T., ÅTLAND, Å., ROSTEN, T., URKE, H. A. & ROSSELAND, B. O. 2009. Important influent-water quality parameters at freshwater production sites in two salmon producing countries. *Aquacultural Engineering,* 41**,** 53-59.

LANGER, M., GABOR, E. M., LIEBETON, K., MEURER, G., NIEHAUS, F., SCHULZE, R., ECK, J. & LORENZ, P. 2006. Metagenomics: an inexhaustible access to nature's diversity. *Biotechnol J,* 1**,** 815-21.

LARSEN, A., TAO, Z., BULLARD, S. A. & ARIAS, C. R. 2013. Diversity of the skin microbiota of fishes: evidence for host species specificity. *FEMS Microbiology Ecology,* 85**,** 483-494.

LEE, J. 2015. Practical applications of low-pressure hydrocyclone (LPH) for feed waste and fecal solid removal in a recirculating aquaculture system. *Aquacultural Engineering,* 69**,** 37-42.

LEISNER, J. J., LAURSEN, B. G., PRÉVOST, H., DRIDER, D. & DALGAARD, P. 2007. Carnobacterium: positive and negative effects in the environment and in foods. Oxford, UK.

LEKANG, O.-I. 2013. *Aquaculture engineering,* Oxford, Wiley-Blackwell.
LI, N. N., FANE, A. G., HO, W. S. W. & MATSUURA, T. 2008. Advanced Membrane Technology and Applications. John Wiley & Sons.
LILTVED, H. & CRIPPS, S. J. 1999. Removal of particle-associated bacteria by prefiltration and ultraviolet irradiation. *Aquaculture Research,* 30**,** 445-450.
LLEWELLYN, M. S., BOUTIN, S., HOSEINIFAR, S. H. & DEROME, N. 2014. Teleost microbiomes: The state of the art in their characterization, manipulation and importance in aquaculture and fisheries. *Frontiers in Microbiology,* 5**,** 1-1.
LOKESH, J. & KIRON, V. 2016. Transition from freshwater to seawater reshapes the skin-associated microbiota of Atlantic salmon. *Sci. Rep.,* 6**,** 19707.
LOWREY, L., WOODHAMS, D. C., TACCHI, L. & SALINAS, I. 2015. Topographical Mapping of the Rainbow Trout (Oncorhynchus mykiss) Microbiome Reveals a Diverse Bacterial Community with Antifungal Properties in the Skin. *Applied and environmental microbiology,* 81**,** 6915.
LØKKA, G., AUSTBØ, L., FALK, K., BJERKÅS, I., KOPPANG, E. O. 2013. Intestinal morphology of the wild atlantic salmon (Salmo salar). *Journal of Morphology,* 274**,** 859-876.
MADARO, A., OLSEN, R. E., KRISTIANSEN, T. S., EBBESSON, L. O. E., NILSEN, T. O., FLIK, G. & GORISSEN, M. 2015. Stress in Atlantic salmon: response to unpredictable chronic stress. *Journal of Experimental Biology,* 218**,** 2538-2550.
MADIGAN, M. T., MARTINKO, J. M., BROCK, T. D., BENDER, K. S., BUCKLEY, D. H. & STAHL, D. A. 2015. *Brock biology of microorganisms,* Harlow, Pearson.
MARTINS, C. I. M., EDING, E. H., VERDEGEM, M. C. J., HEINSBROEK, L. T. N., SCHNEIDER, O., BLANCHETON, J. P., D'ORBCASTEL, E. R. & VERRETH, J. A. J. 2010. New developments in recirculating aquaculture systems in Europe: A perspective on environmental sustainability. *Aquacultural Engineering,* 43**,** 83-93.
MATTILSYNET 2014. Vannkvalitet og vannbehandling i settefiskanlegg. *Nasjonalt tilsynsprosjekt 2012-2014.* Mattilsynet.
MERRIFIELD, D. L. & RODILES, A. 2015. 10 - The fish microbiome and its interactions with mucosal tissues A2 - Beck, Benjamin H. *In:* PEATMAN, E. (ed.) *Mucosal Health in Aquaculture.* San Diego: Academic Press.
MICHAUD, L., BLANCHETON, J. P., BRUNI, V. & PIEDRAHITA, R. 2006. Effect of particulate organic carbon on heterotrophic bacterial populations and nitrification efficiency in biological filters. *Aquacultural Engineering,* 34**,** 224-233.
MINNITI, G., HAGEN, L. H., PORCELLATO, D., JØRGENSEN, S. M., POPE, P. B. & VAAJE-KOLSTAD, G. 2017. The Skin-Mucus Microbial Community of Farmed Atlantic Salmon (Salmo salar). *Frontiers in Microbiology,* 8.
MOLLERUP, S., FRIIS-NIELSEN, J., VINNER, L., HANSEN, T. A., RICHTER, S. R., FRIDHOLM, H., HERRERA, J. A., LUND, O., BRUNAK, S., IZARZUGAZA, J. M., MOURIER, T., NIELSEN, L. P. & HANSEN, A. J. 2016. Propionibacterium acnes: Disease-Causing Agent or Common Contaminant? Detection in Diverse Patient Samples by Next-Generation Sequencing. *J Clin Microbiol,* 54**,** 980-7.
MUYZER, G., DE WAAL, E. C. & UITTERLINDEN, A. G. 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**,** 695-700.
NAVARRETE, P., ESPEJO, R. T. & ROMERO, J. 2008. Molecular Analysis of Microbiota Along the Digestive Tract of Juvenile Atlantic Salmon (Salmo salar L.). *Microbial Ecology,* 57**,** 550.

NAYAK, S. K. 2010a. Probiotics and immunity: A fish perspective. *Fish & Shellfish Immunology,* 29**,** 2-14.

NAYAK, S. K. 2010b. Role of gastrointestinal microbiota in fish. Oxford, UK.

OLSEN, R. E., SUNDELL, K., HANSEN, T., HEMRE, G.-I., MYKLEBUST, R., MAYHEW, T. M. & RINGØ, E. 2002. Acute stress alters the intestinal lining of Atlantic salmon, Salmo salar L.: An electron microscopical study. *Fish Physiology and Biochemistry,* 26**,** 211-221.

OLSEN, R. E., SUNDELL, K., RINGØ, E., MYKLEBUST, R., HEMRE, G.-I., HANSEN, T. & KARLSEN, Ø. 2008. The acute stress response in fed and food deprived Atlantic cod, Gadus morhua L. *Aquaculture,* 280**,** 232-241.

PEAKE, S., MCKINLEY, R. S. & SCRUTON, D. A. 1997. Swimming performance of various freshwater Newfoundland salmonids relative to habitat selection and fishway design. *Journal of Fish Biology,* 51**,** 710-723.

PEPPER, I. L. 2015. *Environmental microbiology,* San Diego, Calif, Academic Press.

PERCIVAL, S. L. & WILLIAMS, D. W. 2014. Chapter Nine - Mycobacterium. *Microbiology of Waterborne Diseases (Second Edition).* London: Academic Press.

PETERS, R. P. H., MOHAMMADI, T., VANDENBROUCKE-GRAULS, C. M. J. E., DANNER, S. A., VAN AGTMAEL, M. A. & SAVELKOUL, P. H. M. 2004. Detection of bacterial DNA in blood samples from febrile patients: underestimated infection or emerging contamination? *FEMS Immunology & Medical Microbiology,* 42**,** 249-253.

PETERSON, T. S. 2015. 3 - Overview of mucosal structure and function in teleost fishes A2 - Beck, Benjamin H. *In:* PEATMAN, E. (ed.) *Mucosal Health in Aquaculture.* San Diego: Academic Press.

PIETERS, N., BRUNT, J., AUSTIN, B. & LYNDON, A. R. 2008. Efficacy of in-feed probiotics against Aeromonas bestiarum and Ichthyophthirius multifiliis skin infections in rainbow trout (Oncorhynchus mykiss , Walbaum. *Journal of Applied Microbiology,* 105**,** 723-732.

RANDALL, D. J. & TSUI, T. K. N. 2002. Ammonia toxicity in fish. *Marine Pollution Bulletin,* 45**,** 17-23.

REUTER, J. A., SPACEK, D. & SNYDER, M. P. 2015. High-Throughput Sequencing Technologies. *Molecular cell,* 58**,** 586-597.

RINGØ, E. 2008. The ability of carnobacteria isolated from fish intestine to inhibit growth of fish pathogenic bacteria: a screening study. *Aquaculture Research,* 39**,** 171-180.

RINGØ, E. & GATESOUPE, F.-J. 1998. Lactic acid bacteria in fish: a review. *Aquaculture,* 160**,** 177-203.

RINGØ, E., SPERSTAD, S., MYKLEBUST, R., REFSTIE, S. & KROGDAHL, Å. 2006. Characterisation of the microbiota associated with intestine of Atlantic cod (Gadus morhua L.): The effect of fish meal, standard soybean meal and a bioprocessed soybean meal. *Aquaculture,* 261**,** 829-841.

ROESELERS, G., MITTGE, E. K., STEPHENS, W. Z., PARICHY, D. M., CAVANAUGH, C. M., GUILLEMIN, K. & RAWLS, J. F. 2011. Evidence for a core gut microbiota in the zebrafish. *The Isme Journal,* 5**,** 1595.

ROMERO, J. & NAVARRETE, P. 2006. 16S rDNA-Based Analysis of Dominant Bacterial Populations Associated with Early Life Stages of Coho Salmon (Oncorhynchus kisutch). *Microbial Ecology,* 51**,** 422-430.

RUSTEN, B., EIKEBROKK, B., ULGENES, Y. & LYGREN, E. 2006. Design and operations of the Kaldnes moving bed biofilm reactors. *Aquacultural Engineering,* 34**,** 322-331.

RUYET, J. P.-L., GALLAND, R., LE ROUX, A. & CHARTOIS, H. 1997. Chronic ammonia toxicity in juvenile turbot (Scophthalmus maximus). *Aquaculture,* 154**,** 155-171.

SAGLE, A. & FREEMAN, B. 2004. *Fundamentals of Membranes for Water Treatment*.

SALTER, S., COX, M., TUREK, E., CALUS, S., COOKSON, W., MOFFATT, M., TURNER, P., PARKHILL, J., LOMAN, N. & WALKER, A. 2014. Reagent and laboratory contamination can critically impact sequence-based microbiome analyses. *BMC Biology,* 12**,** 87.

SKJERMO, J., SALVESEN, I., ØIE, G., OLSEN, Y. & VADSTEIN, O. 1997. Microbially matured water: a technique for selection of a non-opportunistic bacterial flora in water that may improve performance of marine larvae. *Aquaculture International,* 5**,** 13-28.

SKJERMO, J. & VADSTEIN, O. 1999. Techniques for microbial control in the intensive rearing of marine larvae. *Aquaculture,* 177**,** 333-343.

SLEATOR, R. D., SHORTALL, C. & HILL, C. 2008. Metagenomics. *Letters in Applied Microbiology,* 47**,** 361-366.

SSB. 2017. *Akvakultur* [Online]. Statistisk sentralbyrå. Available: https://www.ssb.no/jord-skog-jakt-og-fiskeri/statistikker/fiskeoppdrett [Accessed].

SULLAM, K. E., ESSINGER, S. D., LOZUPONE, C. A., O'CONNOR, M. P., ROSEN, G. L., KNIGHT, R., KILHAM, S. S. & RUSSELL, J. A. 2012. Environmental and ecological factors that shape the gut bacterial communities of fish: a meta-analysis. *Molecular Ecology,* 21**,** 3363-3378.

THORARENSEN, H. & FARRELL, A. P. 2011. The biological requirements for post-smolt Atlantic salmon in closed-containment systems. *Aquaculture,* 312**,** 1-14.

TURNBAUGH, P. J., LEY, R. E., HAMADY, M., FRASER-LIGGETT, C. M., KNIGHT, R. & GORDON, J. I. 2007. The Human Microbiome Project. *Nature,* 449**,** 804.

VADSTEIN, O., ØIE, G., OLSEN, Y., SALVESEN, I., SKJERMO, J. & SKJÅK-BRÆK, G. 1993. *A strategy to obtain microbial control during larval development of marine fish*.

VERNER-JEFFREYS, D. W., SHIELDS, R. J., BRICKNELL, I. R. & BIRKBECK, T. H. 2004. Effects of different water treatment methods and antibiotic addition on larval survival and gut microflora development in Atlantic halibut (Hippoglossus hippoglossus L.) yolk-sac larvae. *Aquaculture,* 232**,** 129-143.

VON WINTZINGERODE, F., GOBEL, U. B. & STACKEBRANDT, E. 1997. Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. *FEMS Microbiol Rev,* 21**,** 213-29.

WOLD, P.-A., HOLAN, A. B., ØIE, G., ATTRAMADAL, K., BAKKE, I., VADSTEIN, O. & LEIKNES, T. O. 2014. Effects of membrane filtration on bacterial number and microbial diversity in marine recirculating aquaculture system (RAS) for Atlantic cod (Gadus morhua L.) production. *Aquaculture,* 422-423**,** 69-77.

YAMAUCHI, K., BAN, M., KASAHARA, N., IZUMI, T., KOJIMA, H. & HARAKO, T. 1985. Physiological and behavioral changes occurring during smoltification in the masu salmon, Oncorhynchus masou. *Aquaculture,* 45**,** 227-235.

ZARKASI, K. Z., TAYLOR, R. S., GLENCROSS, B. D., ABELL, G. C. J., TAMPLIN, M. L. & BOWMAN, J. P. 2017. In vitro characteristics of an Atlantic salmon (Salmo salar L.) hind gut microbial community in relation to different dietary treatments. *Research in Microbiology,* 168**,** 751-759.

Appendix A Optimal water quality variables for rearing of Atlantic salmon parr at commercial scale

Table A.1 Optimal water quality variables for rearing of Atlantic salmon parr.

Parameter	Value	Reference
Temperature (°C)	12 – 14	Elliot (1991)
Oxygen (%)	> 60	EFSA (2008)
CO_2 (mg/L)	< 15	Bregnballe (2015)
pH	6.5 – 7.5 (Nitrification: 7.0 – 8.0)	Bregnballe (2015)
TAN (mg/L)	< 0.02	Bregnballe (2015)
Nitrite (mg/L)	< 0.5	Bregnballe (2015)
Nitrate (mg/L)	< 100	Bregnballe (2015)
Salinity (ppt)	< 10	EFSA (2008)
Alkalinity (mg/L)		
TSS (mg/L)	< 15	Thorarensen and Farell (2011)
Water flow (cm/s)	< 60	Peak et. al (1997)

Appendix B QIAamp® DNA Mini Kit extraction protocol (Qiagen)

Protocol: DNA Purification from Tissues (QIAamp DNA Mini Kit)

This protocol is for purification of total (genomic, mitochondrial, and viral) DNA from tissues using the QIAamp DNA Mini Kit.

Tissues

Important points before starting

- All centrifugation steps are carried out at room temperature (15–25°C).
- Use carrier DNA if the sample contains <10,000 genome equivalents (see page 17).
- Avoid repeated freezing and thawing of stored samples, since this leads to reduced DNA size.
- Transcriptionally active tissues, such as liver and kidney, contain high levels of RNA which will copurify with genomic DNA. RNA may inhibit some downstream enzymatic reactions, but will not inhibit PCR. If RNA-free genomic DNA is required, include the RNase A digest, as described in step 5a of the protocol.

Things to do before starting

- Equilibrate the sample to room temperature (15–25°C).
- Heat 2 water baths or heating blocks: one to 56°C for use in step 3, and one to 70°C for use in step 5.
- Equilibrate Buffer AE or distilled water to room temperature for elution in step 11.
- Ensure that Buffers AW1 and AW2 have been prepared according to the instructions on page 16.
- If a precipitate has formed in Buffer ATL or Buffer AL, dissolve by incubating at 56°C.

Procedure

1. **Excise the tissue sample or remove it from storage. Determine the amount of tissue. Do not use more than 25 mg (10 mg spleen).**

   Weighing tissue is the most accurate way to determine the amount.

   If DNA is prepared from spleen tissue, no more than 10 mg should be used.

   The yield of DNA will depend on both the amount and the type of tissue processed.

   1 mg of tissue will yield approximately 0.2–1.2 µg of DNA.

2. **Cut up (step 2a), grind (step 2b), or mechanically disrupt (step 2c) the tissue sample.**

   The QIAamp procedure requires no mechanical disruption of the tissue sample, but lysis time will be reduced if the sample is ground in liquid nitrogen (step 2b) or mechanically homogenized (step 2c) in advance.

2a. Cut up to 25 mg of tissue (up to 10 mg spleen) into small pieces. Place in a 1.5 ml microcentrifuge tube, and add 180 µl of Buffer ATL. Proceed with step 3.

It is important to cut the tissue into small pieces to decrease lysis time.

2 ml microcentrifuge tubes may be better suited for lysis.

2b. Place up to 25 mg of tissue (10 mg spleen) in liquid nitrogen, and grind thoroughly with a mortar and pestle. Decant tissue powder and liquid nitrogen into 1.5 ml microcentrifuge tube. Allow the liquid nitrogen to evaporate, but do not allow the tissue to thaw, and add 180 µl of Buffer ATL. Proceed with step 3.

2c. Add up to 25 mg of tissue (10 mg spleen) to a 1.5 ml microcentrifuge tube containing no more than 80 µl PBS. Homogenize the sample using the TissueRuptor or equivalent rotor–stator homogenizer. Add 100 µl Buffer ATL, and proceed with step 3.

Some tissues require undiluted Buffer ATL for complete lysis. In this case, grinding in liquid nitrogen is recommended. Samples cannot be homogenized directly in Buffer ATL, which contains detergent.

3. **Add 20 µl proteinase K, mix by vortexing, and incubate at 56°C until the tissue is completely lysed. Vortex occasionally during incubation to disperse the sample, or place in a shaking water bath or on a rocking platform.**

   Note: Proteinase K must be used. QIAGEN Protease has reduced activity in the presence of Buffer ATL.

   Lysis time varies depending on the type of tissue processed. Lysis is usually complete in 1–3 h. Lysis overnight is possible and does not influence the preparation. To ensure efficient lysis, a shaking water bath or a rocking platform should be used. If not available, vortexing 2–3 times per hour during incubation is recommended.

4. **Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid.**

5. **If RNA-free genomic DNA is required, follow step 5a. Otherwise, follow step 5b.**

   Transcriptionally active tissues, such as liver and kidney, contain high levels of RNA which will copurify with genomic DNA. RNA may inhibit some downstream enzymatic reactions, but will not inhibit PCR.

5a. First add 4 µl RNase A (100 mg/ml), mix by pulse-vortexing for 15 s, and incubate for 2 min at room temperature (15–25°C). Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from inside the lid before adding 200 µl Buffer AL to the sample. Mix again by pulse-vortexing for 15 s, and incubate at 70°C for 10 min. Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from inside the lid.

It is essential that the sample and Buffer AL are mixed thoroughly to yield a homogeneous solution.

A white precipitate may form on addition of Buffer AL. In most cases it will dissolve during incubation at 70°C. The precipitate does not interfere with the QIAamp procedure or with any subsequent application.

5b. Add 200 µl Buffer AL to the sample, mix by pulse-vortexing for 15 s, and incubate at 70°C for 10 min. Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from inside the lid.

It is essential that the sample and Buffer AL are mixed thoroughly to yield a homogeneous solution.

A white precipitate may form on addition of Buffer AL, which in most cases will dissolve during incubation at 70°C. The precipitate does not interfere with the QIAamp procedure or with any subsequent application.

6. Add 200 µl ethanol (96–100%) to the sample, and mix by pulse-vortexing for 15 s. After mixing, briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from inside the lid.

It is essential that the sample, Buffer AL, and the ethanol are mixed thoroughly to yield a homogeneous solution.

A white precipitate may form on addition of ethanol. It is essential to apply all of the precipitate to the QIAamp Mini spin column. This precipitate does not interfere with the QIAamp procedure or with any subsequent application.

Do not use alcohols other than ethanol since this may result in reduced yields.

7. Carefully apply the mixture from step 6 (including the precipitate) to the QIAamp Mini spin column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 x *g* (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate.*

Close each spin column to avoid aerosol formation during centrifugation.

It is essential to apply all of the precipitate to the QIAamp Mini spin column.

Centrifugation is performed at 6000 x *g* (8000 rpm) to reduce noise. Centrifugation at full speed will not affect the yield or purity of the DNA. If the solution has not completely passed through the membrane, centrifuge again at a higher speed until all the solution has passed through.

* Flow-through contains Buffer AL or Buffer AW1 and is therefore not compatible with bleach. See page 6 for safety information.

8. **Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW1 without wetting the rim. Close the cap, and centrifuge at 6000 x *g* (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the collection tube containing the filtrate.***

9. **Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed (20,000 x *g*; 14,000 rpm) for 3 min.**

10. **Recommended: Place the QIAamp Mini spin column in a new 2 ml collection tube (not provided) and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.**

    This step helps to eliminate the chance of possible Buffer AW2 carryover.

11. **Place the QIAamp Mini spin column in a clean 1.5 ml microcentrifuge tube (not provided), and discard the collection tube containing the filtrate. Carefully open the QIAamp Mini spin column and add 200 µl Buffer AE or distilled water. Incubate at room temperature for 1 min, and then centrifuge at 6000 x *g* (8000 rpm) for 1 min.**

12. **Repeat step 11.**

    A 5 min incubation of the QIAamp Mini spin column loaded with Buffer AE or water, before centrifugation, generally increases DNA yield.

    A third elution step with a further 200 µl Buffer AE will increase yields by up to 15%.

    Volumes of more than 200 µl should not be eluted into a 1.5 ml microcentrifuge tube because the spin column will come into contact with the eluate, leading to possible aerosol formation during centrifugation.

    Elution with volumes of less than 200 µl increases the final DNA concentration in the eluate significantly, but slightly reduces the overall DNA yield (see Table 5, page 25). Eluting with 4 x 100 µl instead of 2 x 200 µl does not increase elution efficiency.

    For long-term storage of DNA, eluting in Buffer AE and placing at –30 to –15°C is recommended, since DNA stored in water is subject to acid hydrolysis.

    Yields of DNA will depend both on the amount and the type of tissue processed. 25 mg of tissue will yield approximately 10–30 µg of DNA in 400 µl of water (25–75 ng/µl), with an A_{260}/A_{280} ratio of 1.7–1.9.

    For more information about elution and how to determine DNA yield, length, and purity, refer to pages 24–25 and Appendix A, page 50.

* Flow-through contains Buffer AL or Buffer AW1 and is therefore not compatible with bleach. See page 6 for safety information.

Appendix C SequalPrep ™ Normalization Plate (96) Kit

invitrogen

SequalPrep™ Normalization Plate (96) Kit

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

Contents and Storage

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

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

Description

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

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

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

System Overview

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

System Specifications

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

Accessory Products

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

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

Part no: 100003531 Rev. date: 5 May 2008

For technical support, email tech_support@invitrogen.com. For country-specific contact information, visit ***www.invitrogen.com***.

General Guidelines

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

Generating PCR Amplicon

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

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

Sample Amount

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

Elution Options

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

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

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

Materials Needed

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

Binding Step

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

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

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

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

Washing Step

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

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

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

3. Completely aspirate the buffer from wells and discard.

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

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

Elution Step

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

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

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

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

3. Incubate at room temperature for 5 minutes.

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

Expected Yield and Concentration

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

Optional: DNA Quantitation

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

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

Downstream Applications

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

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

Continued on next page

Troubleshooting

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

Quality Control

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

Limited Use Label License No. 5: Invitrogen Technology

The purchase of this product conveys to the buyer the non-transferable right to use the purchased amount of the product and components of the product in research conducted by the buyer (whether the buyer is an academic or for-profit entity). The buyer cannot sell or otherwise transfer (a) this product (b) its components or (c) materials made using this product or its components to a third party or otherwise use this product or its components or materials made using this product or its components for Commercial Purposes. The buyer may transfer information or materials made through the use of this product to a scientific collaborator, provided that such transfer is not for any Commercial Purpose, and that such collaborator agrees in writing (a) not to transfer such materials to any third party, and (b) to use such transferred materials and/or information solely for research and not for Commercial Purposes. Commercial Purposes means any activity by a party for consideration and may include, but is not limited to: (1) use of the product or its components in manufacturing; (2) use of the product or its components to provide a service, information, or data; (3) use of the product or its components for therapeutic, diagnostic or prophylactic purposes; or (4) resale of the product or its components, whether or not such product or its components are resold for use in research. For products that are subject to multiple limited use label licenses, the most restrictive terms apply. Invitrogen Corporation will not assert a claim against the buyer of infringement of patents owned or controlled by Invitrogen Corporation which cover this product based upon the manufacture, use or sale of a therapeutic, clinical diagnostic, vaccine or prophylactic product developed in research by the buyer in which this product or its components was employed, provided that neither this product nor any of its components was used in the manufacture of such product. If the purchaser is not willing to accept the limitations of this limited use statement, Invitrogen is willing to accept return of the product with a full refund. For information on purchasing a license to this product for purposes other than research, contact Licensing Department, Invitrogen Corporation, 5791 Van Allen Way, Carlsbad, CA 92008. Phone (760) 603-7200. Fax (760) 602-6500. Email: outlicensing@invitrogen.com

For research use only. Not intended for any animal or human therapeutic or diagnostic use.

SOLiD™ is a trademark of Applera Corporation.

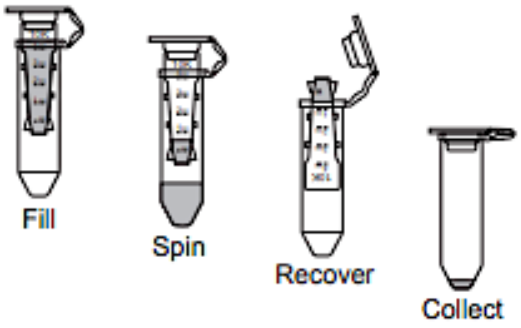
Appendix D Amicon® Ultra-0.5 Centrifugal Filter Devices User Guide

Amicon® Ultra-0.5

Centrifugal Filter Devices

for volumes up to 500 µL

User Guide

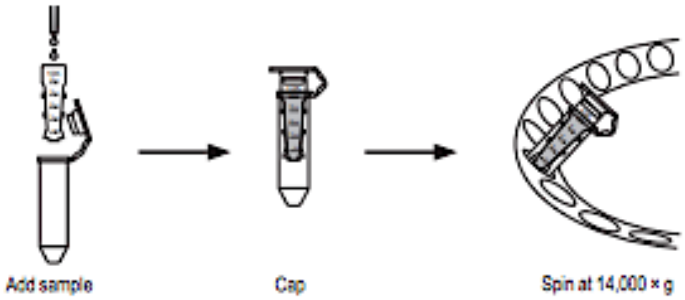


For research use only;
not for use in diagnostic procedures

MILLIPORE

How to Use Amicon Ultra-0.5 Centrifugal Filter Devices

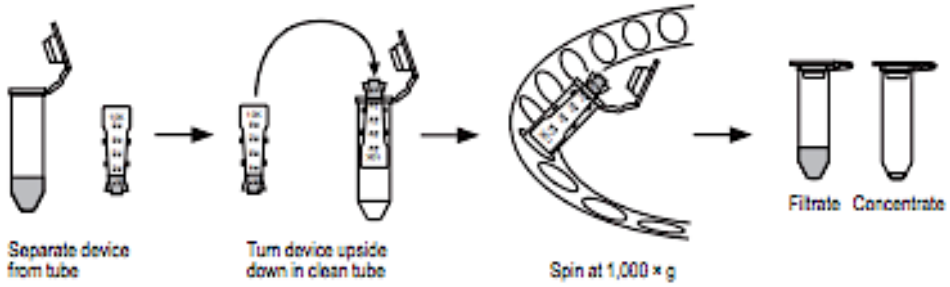
1. Insert the Amicon Ultra-0.5 device into one of the provided microcentrifuge tubes.
2. Add up to 500 µL of sample to the Amicon Ultra filter device and cap it.
3. Place capped filter device into the centrifuge rotor, aligning the cap strap toward the center of the rotor; counterbalance with a similar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



How to Use Amicon Ultra-0.5 Filter Devices, continued

5. Remove the assembled device from the centrifuge and separate the Amicon Ultra filter device from the microcentrifuge tube.
6. To recover the concentrated solute, place the Amicon Ultra filter device upside down in a clean micro centrifuge tube. Place in centrifuge, aligning open cap towards the center of the rotor; counterbalance with a similar device. Spin for 2 minutes at 1,000 × g to transfer the concentrated sample from the device to the tube. The ultrafiltrate can be stored in the centrifuge tube.

   NOTE: For optimal recovery, perform the reverse spin immediately.



Performance - DNA Concentration

The Amicon Ultra-0.5 30K device provides the best balance between recovery and spin time for double-stranded DNA for base pairs ranging from 137 to 1159. To achieve maximum PCR product recovery and primer removal with primers greater than 20 bases, one or two additional spins with Tris-EDTA (TE) buffer are recommended.

Table 2. Typical recovery of nucleotides from Amicon Ultra-0.5 30K device

PCR Product (base pairs)	PCR Primer (bases)	PCR Recovery (%)	PCR Primer Removal (%)	TE Washes (number)
137	10	≥ 95	≥ 90	0
	20	≥ 90	≥ 85	1
	48	≥ 90	≥ 75	2
301	10	≥ 90	≥ 90	0
	20	≥ 85	≥ 90	1
	48	≥ 90	≥ 80	2
648	10	≥ 95	≥ 90	0
	20	≥ 90	≥ 90	1
	48	≥ 95	≥ 90	2
1159	10	≥ 90	≥ 90	0
	20	≥ 90	≥ 95	1
	48	≥ 95	≥ 95	2

Spin conditions: 40° fixed angle rotor, 14,000 × g, room temperature, 500 µL starting volume, 20–30 µL final volume, 10 minute spin, n=12.

Appendix E Water quality variables at period 4 and period 5 in cRAS and mRAS

Water quality variables measured throughout period 4 (day 90 – 118) and period 5 (day 119 – 140) were temperature, oxygen saturation, CO_2 concentration, pH, total ammonia nitrogen (TAN), nitrite ($NO_2 - N$), nitrate ($NO_3 - N$), salinity, alkalinity, DOC, TOC and turbidity. Average variables (± SD) are given in Table E.1 for period 4 and Table E.2 for period 5.

Table E.1 Average water quality parameters (± SD) for cRAS and mRAS at period 4 (day 90 – 118).

Parameter	cRAS	mRAS
Temperature (℃)	12.28 ± 0.25	13.79 ± 0.06
Oxygen (%)	104.82 ± 11.46	98.98 ± 27.43
CO_2 (mg/L)	1.62 ± 0.68	1.6 ± 0.59
pH	7.76 ± 0.08	7.79 ± 0.09
TAN (mg/L)	0.06 ± 0.02	0.07 ± 0.07
Nitrite (mg/L)	0.02 ± 0.01	0.02 ± 0.01
Nitrate (mg/L)	13.44 ± 4.98	17.15 ± 6.89
Salinity (ppt)	1.78 ± 0.43	1.2 ± 0.3
Alkalinity (mg/L as $CaCO_3$)	48.92 ± 2.22	47.76 ± 2.59
DOC (mg/L)	-	-
TOC (mg/L)	-	-
Turbidity (NTU)	3.9 ± 1.63	0.68 ± 0.39

Table E.2 Average water quality variables (± SD) for cRAS and mRAS at period 5 (day 119 – 140).

Parameter	cRAS	mRAS
Temperature (°C)	12.71 ± 0.48	14.01 ± 0.46
Oxygen (%)	94.38 ± 6.00	86.18 ± 6.14
CO_2 (mg/L)	1.62 ± 1.08	2 ± 1.09
pH	7.64 ± 0.25	7.61 ± 0.26
TAN (mg/L)	0.08 ± 0.03	0.08 ± 0.05
Nitrite (mg/L)	0 ± 0	0.01 ± 0
Nitrate (mg/L)	5.4 ± 1.07	8.60 ± 2.81
Salinity (ppt)	1.65 ± 0.2	1.62 ± 0.31
Alkalinity (mg/L as $CaCO_3$)	48.53 ± 5.63	49.63 ± 8.27
DOC (mg/L) *	5.64 ± 0.68	5.47 ± 0.61
TOC (mg/L) *	8.58 ± 1.20	6.39 ± 0.88
Turbidity (NTU)	6.45 ± 3.49	2.55 ± 1.75

*Average DOC and TOC from day 95 – 128

The temperature was higher in mRAS overall due to heat production by the membrane and uneven distribution of temperature in the fish hall. cRAS had a slightly higher oxygen saturation during period 4 and 5 than mRAS due to differences in oxygenation.

Appendix F Total number of reads per sample

Table B.1 Number of sequence reads for water (blue), gut (orange) and skin (green) samples. Sample ID explanation for gut and skin samples: R2 = cRAS, R1 = mRAS, T# = fish tank, F# = fish, T = gut, S = skin mucus, 22 = t1, 13 = t2. For water samples: 14-71 = mRAS t1 FT9, 14-72 = mRAS t1 FT8, 14-73 = mRAS t1 FT7, 24-71 = cRAS t1 FT3, 24-72 = cRAS t1 FT2, 24-73 = cRAS t1 FT1, 16-91 = mRAS t2 FT9, 16-92 = mRAS t2 FT8, 16-93 = mRAS t2 FT7, 26-91 = cRAS t1 FT3, 26-92 = cRAS t2 FT2, 26-93 = cRAS t2 FT1.

Sample ID	Number of reads
R2T2F3T-22	70963
R1T7F1T-22	59933
R2T1F1S-22	50183
R1T8F3T-22	83762
R2T1F1T-13	66746
R2T2F3T-13	67054
R1T7F1T-13	115323
R1T8F3T-13	96712
R2T1F2S-22	22756
R2T2F4S-22	11209
R1T7F2S-22	3983
R1T8F4S-22	10766
R2T2F3S-22	11043
R2T1F2S-13	7987
R2T2F4S-13	12458
R1T7F2S-13	7494
R1T8F4S-13	13907
R2T1F2T-22	158060
R2T2F4T-22	92821
R1T7F2T-22	89992
R1T8F4T-22	97761
R2T1F2T-13	92456
R2T2F4T-13	20354
R1T7F2T-13	133332
R1T7F1S-22	26436
R1T8F4T-13	127560
R2T1F3S-22	11596
R2T3F1S-22	28086
R1T7F3S-22	11876
R1T9F1S-22	29482
14-71w	118351
R2T1F3S-13	11973
14-72w	62467
R2T3F1S-13	11648
14-73w	104311
R1T7F3S-13	10824

Sample ID	Number of reads
R1T9F1S-13	6785
R2T1F3T-22	104691
R2T3F1T-22	74453
24-71w	63555
R1T7F3T-22	68429
R1T8F3S-22	33771
24-72w	64723
R1T9F1T-22	112971
24-73w	102462
R2T1F3T-13	73553
R2T3F1T-13	90849
R1T7F3T-13	83423
R1T9F1T-13	104121
R2T1F4S-22	103548
R2T3F2S-22	33147
R1T7F4S-22	23093
R1T9F2S-22	29379
R2T1F4S-13	7740
R2T3F2S-13	26367
R2T1F1S-13	24417
R1T7F4S-13	16694
R1T9F2S-13	12652
R2T1F4T-22	77217
R2T3F2T-22	100161
R1T7F4T-22	77979
R1T9F2T-22	99926
16-91w	78133
R2T1F4T-13	81815
16-92w	83969
R2T3F2T-13	94959
16-93w	70192
R1T7F4T-13	124546
R1T9F2T-13	103572
R2T2F1S-22	42036
R2T2F3S-13	7352
R2T3F3S-22	28107

Sample ID	Number of reads
26-91w	81852
R1T8F1S-22	15226
26-92w	71110
R1T9F3S-22	26665
26-93w	106961
R2T2F1S-13	14995
R2T3F3S-13	15722
R1T8F1S-13	10407
R1T9F3S-13	4118
R2T2F1T-22	91687
R2T3F3T-22	90164
R1T8F1T-22	91383
R1T9F3T-22	127534
R1T7F1S-13	8836
R2T2F1T-13	85437
R2T3F3T-13	94385
R1T8F1T-13	110954
R1T9F3T-13	108929
R2T2F2S-22	21689
R2T3F4S-22	16848
R1T8F2S-22	7377
R1T9F4S-22	23682
R2T2F2S-13	12984
R2T3F4S-13	4036
R1T8F2S-13	30213
R1T8F3S-13	17622
R1T9F4S-13	18848
R2T2F2T-22	83529
R2T3F4T-22	71847
R1T8F2T-22	88573
R1T9F4T-22	102015
R2T1F1T-22	132170
R2T2F2T-13	98193
R2T3F4T-13	105847
R1T8F2T-13	119744
R1T9F4T-13	108892

Appendix G The most abundant OTUs in the water samples and SIMPER analyses

Table G.1 The five most abundant OTUs in the rearing water in cRAS with average relative abundances.

OTU ID	Taxonomy	Average relative abundance
4	*Mycobacterium* (Actinobacteria)	0.17
6	*Spartobacteria_genera_incertae_sedis* (Spartobacteria)	0.12
16	*Legionella* (Gammaproteobacteria)	0.07
14	*Sphaerotilus* (Betaproteobacteria)	0.05
34	Rhodobacteraceae (Alphaproteobacteria)	0.04

Table G.2 The five most abundant OTUs in the rearing water in cRAS at sampling time 1 with average relative abundances.

OTU ID	Taxonomy	Average relative abundance
16	*Legionella* (Gammaproteobacteria)	0.13
4	*Mycobacterium* (Actinobacteria)	0.09
6	*Spartobacteria_genera_incertae_sedis* (Spartobacteria)	0.07
14	*Sphaerotilus* (Betaproteobacteria)	0.07
29	Actinomycetales (Actinobacteria)	0.04

Table G.3 The ten most abundant OTUs in the rearing water in cRAS at sampling time 2 with average relative abundances.

OTU ID	Taxonomy	Average relative abundance
4	*Mycobacterium* (Actinobacteria)	0.25
6	*Spartobacteria_genera_incertae_sedis* (Spartobacteria)	0.17
34	Rhodobacteraceae (Alphaproteobacteria)	0.06
51	Bacteria	0.05
47	*Polaribacter* (Flavobacteria)	0.04
50	Cytophagaceae (Cytophagia)	0.04
1315	*Albidiferax* (Betaproteobacteria)	0.03
64	Comamonadaceae (Betaproteobacteria)	0.03
43	Bacteroidetes	0.03
14	*Sphaerotilus* (Betaproteobacteria)	0.02

Table G.4 The ten most abundant OTUs in the rearing water in mRAS with average relative abundances.

OTU ID	Taxonomy	Average relative abundance
14	*Sphaerotilus* (Betaproteobacteria)	0.14
34	Rhodobacteraceae (Alphaproteobacteria)	0.06
28	*Zymophilus* (Negativicutes)	0.06
27	*Flavobacterium* (Flavobacteria)	0.06
25	Lachnospiraceae (Clostridia)	0.05
31	Xanthomonadaceae (Gammaproteobacteria)	0.04
30	*Blastopirellula* (Planctomycetia)	0.04
49	Lachnospiraceae (Clostridia)	0.02
2	*Carnobacterium* (Bacilli)	0.02
16	*Legionella* (Gammaproteobacteria)	0.02

Table G.5 The ten most abundant OTUs in the rearing water in mRAS at sampling time 1 with average relative abundances.

OTU ID	Taxonomy	Average relative abundance
14	*Sphaerotilus* (Betaproteobacteria)	0.20
28	*Zymophilus* (Negativicutes)	0.10
25	Lachnospiraceae (Clostridia)	0.06
31	Xanthomonadaceae (Gammaproteobacteria)	0.05
34	Rhodobacteraceae (Alphaproteobacteria)	0.05
49	Lachnospiraceae (Clostridia)	0.03
53	*Prevotella* (Bacteroidia)	0.03
2	*Carnobacterium* (Bacilli)	0.03
30	*Blastopirellula* (Planctomycetia)	0.02
6	*Spartobacteria_genera_incertae_sedis* (Spartobacteria)	0.02

Table G.6 The ten most abundant OTUs in the rearing water in mRAS at sampling time 2 with average relative abundances.

OTU ID	Taxonomy	Average relative abundance
27	*Flavobacterium* (Flavobacteria)	0.11
14	*Sphaerotilus* (Betaproteobacteria)	0.08
34	Rhodobacteraceae (Alphaproteobacteria)	0.08
30	*Blastopirellula* Planctomycetia)	0.06
25	Lachnospiraceae (Clostridia)	0.05
31	Xanthomonadaceae (Gammaproteobacteria)	0.04
16	*Legionella* (Gammaproteobacteria)	0.03
766	Microbacteriaceae (Actinobacteria)	0.02
94	*Pedobacter* (Sphingobacteria)	0.02
133	*Devosia* (Alphaproteobacteria)	0.02

Table G.7 The five OTUs that contributed most to the difference (as measured by the Bray-Curtis dissimilarity) between microbial communities in cRAS and mRAS as determined by SIMPER analysis. The mean abundance in each system is included.

OTU	Taxonomy	Contribution %	Mean abundance cRAS	Mean abundance mRAS
4	*Mycobacterium* (Actinobacteria)	11.47	0.17	0.01
6	*Spartobacteria_genera_incertae_sedis* (Spartobacteria)	8.37	0.12	0.01
14	*Sphaerotilus* (Betaproteobacteria)	7.04	0.05	0.14
16	*Legionella* (Gammaproteobacteria)	4.66	0.07	0.02
27	*Flavobacterium* (Flavobacteria)	3.91	0.01	0.06

Appendix H The most abundant OTUs in the gut samples and SIMPER analyses

Table H.1 The ten most abundant OTUs associated with the gut of salmon parr. OTU ID, related taxonomy, average normalized and maximum numbers of reads are shown.

OTU	Taxonomy	Average relative abundance
2	*Carnobacterium* (Bacilli)	0.19
1	*Brochothrix* (Bacilli)	0.13
3	*Rhodococcus* (Actinobacteria)	0.10
11	*Pseudochrobactrum* (Alphaproteobacteria)	0.04
4	*Mycobacterium* (Actinobacteria)	0.04
8	*Weissella* (Bacilli)	0.04
10	*Microbacterium* (Actinobacteria)	0.03
17	*Leuconostoc* (Bacilli)	0.02
12	*Psychrobacter* (Gammaproteobacteria)	0.02
18	*Arthrobacter* (Actinobacteria)	0.02

Table H.2 The five OTUs that contributed most to the difference (as measured by the Bray-Curtis dissimilarity) between gut microbial communities in cRAS and mRAS as determined by SIMPER analysis. The mean abundance in each system is included.

OTU	Taxonomy	Contribution %	Mean abundance cRAS	Mean abundance mRAS
2	*Carnobacterium* (Bacilli)	18.44	0.10	0.28
1	*Brochothrix* (Bacilli)	9.77	0.12	0.14
4	*Mycobacterium* (Actinobacteria)	7.46	0.09	0.0002
3	*Rhodococcus* (Actinobacteria)	5.77	0.08	0.13
8	*Weisella* (Bacilli)	3.66	0.05	0.030

Table H.2 The five OTUs that contributed most to the difference (as measured by the Bray-Curtis dissimilarity) between gut microbial communities in cRAS and mRAS at sampling time 1 as determined by SIMPER analysis. The mean abundance in each system is included.

OTU	Taxonomy	Contribution %	Mean abundance cRAS t1	Mean abundance mRAS t1
2	*Carnobacterium* (Bacilli)	22.80	0.15	0.32
1	*Brochothrix* (Bacilli)	12.40	0.19	0.16
3	*Rhodococcus* (Actinobacteria)	5.73	0.12	0.12
12	*Psychrobacter* (Gammaproteobacteria)	4.70	0.03	0.03
11	*Pseudochrobactrum* (Alpharoteobacteria)	4.07	0.06	0.06

Table H.3 The five OTUs that contributed most to the difference (as measured by the Bray-Curtis dissimilarity) between gut microbial communities in cRAS and mRAS at sampling time 2 as determined by SIMPER analysis. The mean abundance in each system is included.

OTU	Taxonomy	Contribution %	Mean abundance cRAS t2	Mean abundance mRAS t2
2	*Carnobacterium* (Bacilli)	15.16	0.04	0.23
4	*Mycobacterium* (Actinobacteria)	13.55	0.17	0.0004
3	*Rhodococcus* (Actinobacteria)	7.29	0.05	0.14
1	*Brochothrix* (Bacilli)	7.05	0.05	0.12
6	*Spartobacteria_genera_incertae_sedis* (Spartobacteria)	4.11	0.05	4.34E-05

Table H.4 The five OTUs that contributed most to the difference (as measured by the Bray-Curtis dissimilarity) between gut microbial communities in cRAS at t1 and t2 as determined by SIMPER analysis. The mean abundance in each system is included.

OTU	Taxonomy	Contribution %	Mean abundance cRAS t1	Mean abundance cRAS t2
4	*Mycobacterium* (Actinobacteria)	12.36	0.001	0.17
1	*Brochothrix* (Bacilli)	11.02	0.19	0.05
2	*Carnobacterium* (Bacilli)	9.23	0.15	0.04
3	*Rhodococcus* (Actinobacteria)	5.19	0.12	0.05
8	*Weisella* (Bacilli)	4.72	0.02	0.08

Table H.5 The five OTUs that contributed most to the difference (as measured by the Bray-Curtis dissimilarity) between gut microbial communities in mRAS at t1 and t2 as determined by SIMPER analysis. The mean abundance in each system is included.

OTU	Taxonomy	Contribution %	Mean abundance mRAS t1	Mean abundance mRAS t2
2	*Carnobacterium* (Bacilli)	18.49	0.32	0.23
1	*Brochothrix* (Bacilli)	10.90	1.61E-01	0.12
3	*Rhodococcus* (Actinobacteria)	7.06	0.12	0.14
11	*Pseudochrobactrum* (Alphaproteobacteria)	4.65	0.06	0.05
8	*Weisella* (Bacilli)	4.36	0.01	0.05

Appendix I The most abundant OTUs in the skin mucus samples and SIMPER analyses

Table I.1 The ten most abundant OTUs associated with skin of Atlantic salmon parr.

OTU	Taxonomy	Average relative abundance
4	*Mycobacterium* (Actinobacteria)	0.16
15	*Pelomonas* (Betaproteobacteria)	0.10
13	*Propionibacterium* (Actinobacteria)	0.07
2	*Carnobacterium* (Bacilli)	0.06
26	*Staphylococcus* (Bacilli)	0.04
45	*Sphingomonas* (Alphaproteobacteria)	0.03
1	*Brochothrix* (Bacilli)	0.03
16	*Legionella* (Gammaproteobacteria)	0.02
6	*Spartobacteria_genera_incertae_sedis* (Spartobacteria)	0.02
41	Vibrionaceae (Gammaproteobacteria)	0.02

Table I.2 SIMPER analysis generating the top five genera contributing to the dissimilarities observed between cRAS at sample time 1 (t1) and 2 (t2).

OTU	Taxonomy	Contribution %	Mean abundance cRAS t1	Mean abundance cRAS t2
4	*Mycobacterium* (Actinobacteria)	19.31	0.04	0.37
15	*Pelomonas* (Betaproteobacteria)	8.27	0.18	0.07
13	*Propionibacterium* (Actinobacteria)	6.28	0.11	0.03
26	*Staphylococcus* (Bacilli)	2.91	0.005	0.05
1	*Brochothrix* (Bacilli)	2.54	0.04	0.003

Table I.3 SIMPER analysis generating the top five genera contributing to the dissimilarities observed between mRAS at sample time 1 (t1) and 2 (t2).

OTU	Taxonomy	Contribution %	Mean abundance mRAS t1	Mean abundance mRAS t2
4	*Mycobacterium* (Actinobacteria)	11.23	0.02	0.20
2	*Carnobacterium* (Bacilli)	11.07	0.19	0.02
13	*Propionibacterium* (Actinobacteria)	5.97	0.06	0.10
15	*Pelomonas* (Betaproteobacteria)	5.23	0.07	0.11
1	*Brochothrix* (Bacilli)	4.31	0.07	0.0001

Appendix J Evaluation of Illumina sequencing data quality

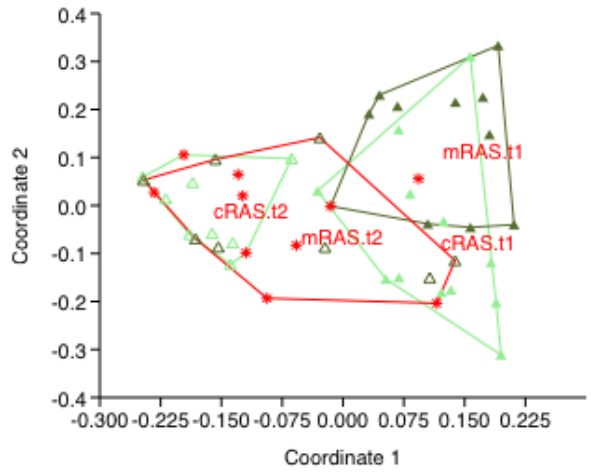


Figure J.1. Principal coordinate analysis (PCoA) plot based on Bray-Curtis similarities for the bacterial communities associated with skin of salmon parr. Red stars represent the ten samples which obtained 4000 – 1000 reads. Light green represents cRAS and dark green represents mRAS. Filled triangles represent sampling time 1 (t1) and triangles represent sampling time 2 (t2).

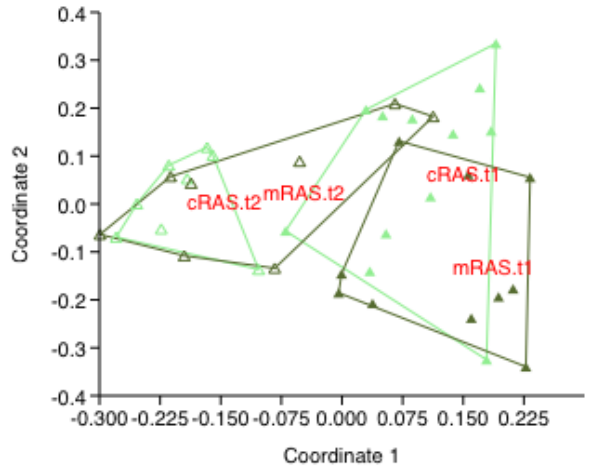


Figure J.2. Principal coordinate analysis (PCoA) plot based on Bray-Curtis similarities for the bacterial communities associated with skin of salmon parr. The ten samples that obtained 4000 – 1000 reads are removed. Light green represents cRAS and dark green represents mRAS. Filled triangles represent sampling time 1 (t1) and triangles represent sampling time 2 (t2).