Master's thesis

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

Amalie Johanne Horn Mathisen

Effect of Host Strain and Microbial Water Quality on the Colonization of Salmon Fry

Master's thesis in Biotechnology
Supervisor: Ingrid Bakke
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Trondheim May 2018 **Amalie Johanne Horn Mathisen**

Abstract

The salmon fry hatches germ-free, but the gut is rapidly colonized by bacteria in the surrounding environment as soon as the mouth opens. Both host-genetics and microbial water quality have previously been found to affect the microbiota of the fish gut. However, the relative importance of each of these factors is poorly understood. The purpose of this study was to investigate the influence of host-genetics and microbial water quality on the colonization of Atlantic salmon yolk-sac fry. An experiment was conducted with two strains of Atlantic salmon (a wild strain and an aquaculture strain). The salmon fry was hatched in a germ-free environment and later exposed to two distinct microbial water qualities, namely r- and K-selected water. Illumina sequencing of the V3-V4 region of the 16S rRNA gene was applied to characterize and compare the microbiota associated with the water and salmon gut. The most abundant phyla in the fish gut for all rearing groups were Proteobacteria, Actinobacteria, Firmicutes, and Bacteroidetes. The salmon gut microbiota was found to be significantly different from the water microbiota. No differences were observed between the wild and aquaculture strain of Atlantic salmon. However, a significant difference was found in the composition of the gut microbiota for fish reared in K-selected water and the fish reared in r-selected water. This study indicates that the water microbiota is a more significant factor than host genetics for the composition of the gut microbiota of Atlantic salmon yolk-sac fry.

Sammendrag

Lakseyngel klekkes bakteriefritt, men man antar at tarmen koloniseres raskt av bakterier i omgivelsene når munnen åpnes. Både vertsgenetikk og mikrobiell vannkvalitet er vist å påvirke tarmmikrobiotaen til fisk, men man kjenner ikke den relative betydningen for den enkelte av disse faktorene. Formålet med denne masteroppgaven var å undersøke betydningen av vertsgenetikk og mikrobiell vannkvalitet på sammensetningen av tarmmikrobiotaen hos plommesekkyngel av laks. Et eksperiment ble utført med to stammer av laks (en villstamme og en akvakulturstamme). Lakseyngelen ble klekt i et bakteriefritt miljø og senere eksponert for to ulike mikrobielle vannkvaliteter, nemlig r- og K-selektert vann. Illuminasekvensering av V3-V4 regionen av 16S-rRNA genet ble anvendt for å karakterisere og sammenligne mikrobiotaen assosiert med vann og laksetarm. Proteobacteria, Actinobacteria, Firmicutes og Bacteroidetes var de vanligste fylaene i laksetarmene for alle fiskegruppene. Tarmmikrobiotan til laks var signifikant forskjellig fra vann mikrobiotan. Ingen forskjell ble observert i tarmmikrobiota for vill- og oppdrettslaks. Derimot var det en signifikant forskjell i sammensetningen av tarmmikrobiotaen for fisk som hadde fått K-selektert vann og fisk i r-selektert vann. Denne studien indikerer at vannmikrobiotaen er en mer signifikant faktor enn vertsgenetikk for sammensetningen av tarmmikrobiotaen hos plommesekkyngel av laks.

Abbreviations

Aq	Aquaculture
Aw	Added water
Ax	Axenic
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphates
DOM	Dissolved organic matter
dpe	Days post-exposure
dph	Days post-hatching
F	Flask
GI	Gastrointestinal (tract)
HTP	High-throughput sequencing
K	K-selected
KB	"Kit blanc"
KC	Kit control
L	Larvae
NGS	Next-generation sequencing
NTC	Non-template control
OTU	Operational taxonomic unit
PC	Positive control
PCoA	Principal coordinate analysis
PCR	Polymerase Chain Reaction
PERMANOVA	Non-parametric multivariate analysis of variance
r	r-selected
RAS	Recirculating aquaculture system
rDNA	Ribosomal deoxyribonucleic acid
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
Rw	Rearing Water

SBS	Sequence by Synthesis
SGM	Salmon Gnotobiotic Media
SIMPER	Similarity percentage
t-RFLP	Terminal restriction fragment length polymorphism
V3	Variable region 3
V4	Variable region 4
Wi	Wild
wpe	Weeks post-exposure

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1 Introduction

The population of the world is expected to reach 9.3 billion in 2050. During the last decades, the aquaculture industry has been the fastest growing food-producing sector. Fish is a good source of essential fatty acids and proteins, which will contribute to the increased food demand for the growing population, especially in low-income countries. In European aquaculture, Atlantic salmon is one of the main species, with Norway as the primary producer (Jensen et al., 2012, Merino et al., 2012).

Newly-hatched fish larvae are highly susceptible to diseases due to an immature immune system. The larvae are surrounded by the water, which makes it essential to control the opportunistic pathogens in the water. In recent years, it has been an increased focus on the selection of a more healthy microbiota in the rearing water. The manipulation of the natural water microbiota has a high potential of preventing pathogenic infections and has also been shown to increase survival (Vadstein et al., 2018b). The development of culture-independent methods has made it possible to examine complex microbial communities, and contributed to a new understanding of the importance of the microbes colonizing the host (Fraune and Bosch, 2010). As the most important aquaculture species in Norway, several studies on the intestinal microbiota have been conducted on Atlantic salmon (Gajardo et al., 2016). The microbial communities of the fish gut vary between species and are assumed to be affected by host genetics, rearing water, diet, habitat, season, and stage of development (Sullam et al., 2012). However, host-microbe interactions and the factors affecting the colonization of fish fry are still poorly understood. A better understanding of factors influencing the colonization of Atlantic salmon yolk-sac fry could potentially provide more positive host-microbe interactions and thereby contribute to a more sustainable aquaculture.

1.1 Host-Microbe Interactions

Culture-independent methods have increased our knowledge of bacterial colonizing and their interactions with the host, especially in mammals. Several studies over the last years show that the intestinal microbiota has a role in the development of the immune system, regulations of genes, brain function and behavior, among others (Fraune and Bosch, 2010, Heijtz et al., 2011). Dysbiosis in the gut is linked to several diseases, including obesity and irritable bowel disease

(Rosenbaum et al., 2015, Ott et al., 2004), which show the importance of a healthy gut microbiota.

Microbial populations associated with teleost fish have also received increased attention in the last years. The gastrointestinal tract (GI-tract), and other outer mucosal surfaces like the fish skin and gills, are colonized by microorganisms that live in a symbiotic relationship with the host (Romero et al., 2014). This consortium of microbes consists of viruses, protozoa, yeast, archaea, and bacteria (Merrifield and Rodiles, 2015). Microorganisms in the fish intestine play an essential role in the development of the host immune system. Colonization by commensal bacteria in the gut is assumed to act as a barrier against pathogens, by stimulating the host immune response, producing bacteriocins, competing for adhesion sites and nutrients, and by altering the physiochemical environment of the gut (Wang et al., 2018, De Schryver and Vadstein, 2014). The mucosal surfaces of the fish gut, skin, and gills are the main site for interactions between the environmental microorganisms and the host (Pérez et al., 2010).

1.1.1 Fish Mucus

The mucosal surface is the first barrier microorganisms face in contact with a fish host. The fish skin, gills, and intestine are covered by a layer of mucus, secreted by goblet cells in the epidermal surfaces. The main component of the mucosal surface is mucins (highly *O*-glycosylated long peptides), in addition to a complex mixture of proteins, ions, and lipids which creates a niche for microorganism. The innate and adaptive components of the mucosal immune system create a selective barrier where the commensal bacteria are able to adhere to the surface but at the same time protect the host against pathogens. Thus, precise homeostatic regulatory mechanisms from the host are required for colonization of commensals. (Salinas and Parra, 2015, Gomez et al., 2013).

1.1.2 Factors Influencing the Colonization of Fish Larvae Gut

The fish larva hatches germ-free, but the mucosal surface is rapidly colonized by bacteria (Romero et al., 2014). The fish live in close contact with the surrounding water and its bacteria, and the establishment of gut microbiota of fish larvae and fry is complex. The first bacteria colonizing of the fish intestine probably originates from the egg surface, feed, and rearing water (Ringø and Birkbeck, 1999). In traditional intensive hatcheries, the rearing water has a high bacterial load and are different in composition and quantity compared to what found in natural environments (Hansen and Olafsen, 1999). In the yolk-sac stage, the fish does not feed (Le

Francois et al., 2010). Thus, the fish is primarily exposed to the bacteria in the rearing water before the fish larvae actively start feeding. The fish larvae will probably also ingest egg debris in the water, and the microbial community found on the egg will therefore also potentially influence the establishment of a bacterial community in the GI tract. The first community colonizing the gut at yolk sac stage remain until after first feeding, and a more stable bacterial community in the gut establishes weeks to months after first feeding (Hansen and Olafsen, 1999).

The environment of the GI-tract creates an ecological niche for microorganisms, and several factors influence the composition and the diversity of the gut microbiota. Gut morphology, host genetics, trophic level, rearing environment, and diet are all factors shown to influence the microbiota associated with the fish intestine (Romero et al., 2014, Nayak, 2010). Several studies have reported changes in the gut microbiota with age and variation between seasons and regions of the gut (Merrifield and Rodiles, 2015).

However, most of the studies have focused on adult fish, and the importance of each of these factors on the early colonization of fish fry is still not well understood. Several studies have observed high inter-individual variation in the composition of the microbial community of the gut, even between fish reared from the same tank, explained partly by stochastic processes (Fjellheim et al., 2007, Giatsis et al., 2014). A study conducted by Bakke et al. (2015) on the microbiota of developing cod larvae (*Gadus morhua*), showed a changing community structure in the gut with age. The larval microbiota was also found to be dissimilar from the water and live feed microbiota, and several OTUs were found unique to the fish larva. Stochastic processes could not only explain these findings, but rather a selection process by the host (Bakke et al., 2015).

Despite the numerous of factors shaping the complex community of the fish GI-tract, there is some evidence of a core microbiota. Roeselers et al. (2011) compared the gut microbiota from fish collected from their natural habitat and fish collected from different rearing lab facilities. Microbial analysis of the 16S rRNA gene showed variations between the wild and the domesticated zebrafish, as well as between the different lab facilities. However, the dominant bacterial taxa of both wild and domesticated fish were similar, indicating a common bacterial community selected by the host (Roeselers et al., 2011). Species from the phyla Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes, Fusobacteria, and Tenericutes are commonly reported to be the most dominant members of the fish GI-tract (Merrifield and Rodiles, 2015,

Romero et al., 2014). However, the microbiota associated with the fish larvae at early development stages are not well understood. Lokesh et al. (2019) reported stage-specific microbial communities of Atlantic salmon, where Proteobacteria is most abundant in the early life stages.

1.1.3 Germ-free Models to Study Host-Microbe Interactions

Germ-free animal models have been crucial to understand the host-microbe interactions. In such studies, the animals are raised in the absence of microbes and later colonized by bacteria in a controlled manner. Thus, it is possible to study the effect of a single microbe or more complex communities on the host response (Rawls et al., 2006, Wang et al., 2018). The later years, protocols to rear germ-free zebrafish (*Danio rerio*) and Atlantic cod larvae (*Gadus morhua L.*) has been established (Rawls et al., 2006, Forberg et al., 2011). Gnotobiotic studies on zebrafish have revealed the role of the microbes in the development of the fish immune system and regulations of genes, and also conservation of host response between mammals and fish (Kanther and Rawls, 2010, Rawls et al., 2006). A protocol to rear germ-free Atlantic salmon yolk-sac fry are now developed at NTNU, Department of Biotechnology and Food Science (Gomez de la Torre Canny et al, in preparation). The salmon yolk-sac fry is large and robust upon hatching and easy to handle. The yolk-sac stage of the fry is relatively long and to external feeding is required (Le Francois et al., 2010). Thus making it a suitable candidate as a germ-free model.

1.2 Aquaculture of Atlantic Salmon

Atlantic salmon is an anadromous species, that spends part of their life cycle in the freshwater and another part in the sea. In the wild, salmon hatches and live its first phase in freshwater (from hatching to parr). After morphological and physiological adaptations from freshwater to seawater (smoltification), the fish migrates to the sea (smolt), where it continues to develop until sexual maturation. The salmon migrates back to the rivers to spawn (Figure 1-1) (Le Francois et al., 2010).

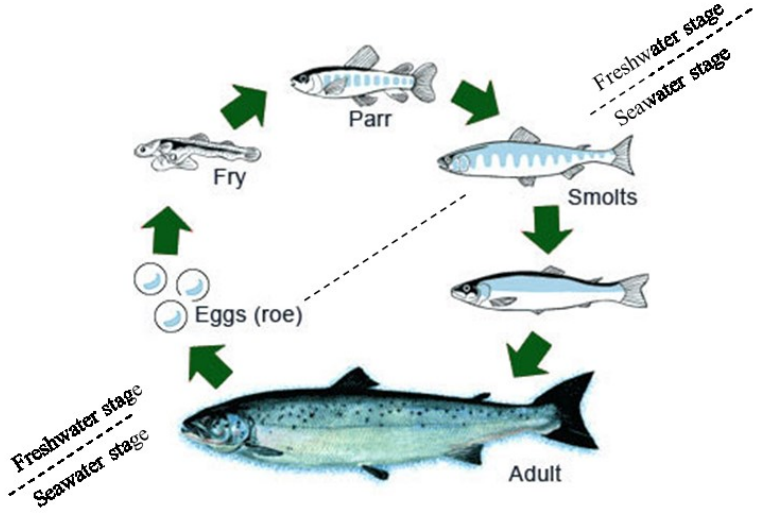

Figure 1-1: Life cycle of Atlantic salmon. Picture is modified from MESA (2014).

In aquaculture of Atlantic salmon, the freshwater stage is conducted in land-based facilities. Atlantic salmon is a suitable species for intensive farming. The eggs are easily assessable from the broodstock and spawning can be controlled with photomanipulation. The eggs are large (5-6 mm in diameter), and the larva is usually 15-25 cm and well developed at hatching. This makes the incubation of eggs and rearing of the larvae relatively easy. At the first stage of its life, the salmon receives nutrition from the yolk sac approximately 300 day-degrees after hatching. When 90% of the yolk sac is consumed, the feeding is initiated in rearing facilities. After the fish has completed the parr-smolt transformation (i.e. smoltification), the salmon is transferred to sea cages to grow to the market size (3-6 kg) (Le Francois et al., 2010).

1.3 Microbial Water Quality

1.3.1 Microbial Community Dynamics

Microorganisms exist in complex communities which varies in both composition and relative abundance of their members (Degnan and Ochman, 2011). Within a community, the populations are in competition to occupy the available niches. To survive and maintain their existents in the communities, microorganisms have developed different strategies to adapt to their environment. r- and K-strategists are one way to classify the organisms and originates from the equation for population growth (1-1),

$$\frac{dX}{dt} \cdot \frac{1}{X} \equiv r - \frac{r}{K} X \qquad (1\text{-}1)$$

where $\frac{dX}{dt} \cdot \frac{1}{X}$ is the specific rate of population increase, r is the population growth rate, X is the population density, and K is the carrying capacity (Andrews and Harris, 1986).

The carrying capacity is defined as the maximum biomass a system can sustain with the given resources, and is the carrying capacity in the relevant environment is the limiting factor when the population density is high (Andrews and Harris, 1986, Hui, 2006). Maximum growth rate occurs when the population density is near zero and substrate concentration is high. The theory of r- and K-selection is based on the assumption that evolution has favored adaptation to either high reproduction rate (r-strategists) or optimal utilization of recourses (K-strategists). r-strategists are characterized by a high growth rate and tend to dominate in uncrowded environments where the nutrient supply is high. These communities are often unstable, with unpredictable and transitory environmental changes. Opportunistic pathogens are typically r-strategists. In contrast, non-opportunistic bacteria, or K-strategists, is characterized by a low maximum specific growth rate and have a high affinity for substrate at low concentrations. They dominate in mature communities with high species diversity, narrow niche specialization and high stability on nutrient changes. These bacteria are more stable members of the community (Andrews and Harris, 1986, Skjermo et al., 1997).

1.3.2 Microbial Control of Rearing Water Microbiota

The microbiota associated with the rearing water in traditional hatcheries differs both in quantity and quality compared to the natural environments of fish. The inlet water in rearing systems is often disinfected to reduce the microbial content in the water, while the organic load is high in the fish tanks. These conditions promote the growth of fast-growing, opportunistic bacteria that generally is found at low densities in the sea (Skjermo et al., 1997, De Schryver and Vadstein, 2014). These bacteria can be opportunistic pathogens that may cause disease, especially in young and stressed fish (De Schryver and Vadstein, 2014).

In theory, a microbial community consisting of non-opportunistic bacteria should be more beneficial for the fish by selecting against opportunists (Skjermo et al., 1997). If the water is dominated by K-strategists (microbially matured water), it may inhibit the growth of opportunistic bacteria in the water and their ability to colonize the skin and gut surfaces of the larvae because the r-strategists are bad at competing when the resources are low (Skjermo et al., 1997). Selection of more beneficial water microbes (i.e., K-strategists) is possible to achieve in aquaculture systems. Recirculating aquaculture systems (RAS) is compatible with

K-selection if properly designed and managed (Attramadal et al., 2014, Vadstein et al., 2018b). RAS has shown to affect fish health positively by increased survival and growth (Attramadal et al., 2014). However, the importance of the microbial water quality at the early life stages of Atlantic salmon is poorly studied.

1.4 Approaches to Study Microbial Communities

To study the diversity of microbial communities are not as straightforward as for animal and plant species. Most microorganisms are indistinguishable by studying them under a microscope. Thus, it is not possible to rely on morphological and functional traits. Also, equivalent ecological niches can be filled by distantly related microorganisms (Gibbons and Gilbert, 2015). Traditionally, the study of the microbiota associated with the fish gut was conducted with culture-dependent methods. Several disadvantages are reported with these methods, by being time-consuming, and only a small portion (~0.1%) of the bacteria associated with the fish gut are assumed to be culturable (Romero et al., 2014). Since the mid-80s, the diversity and ecology of microorganisms in natural environments have been studied by application of molecular methods. The use of molecular techniques has revolutionized the field of microbial ecology, by giving a better understanding of microbial diversity and functionality in the environment (Head et al., 1998). During the last decades, the development of next-generation sequencing (NGS) methods has made it possible to simultaneously read thousand of sequences, and the ability to detect low-abundant phylotype and to examine complex microbial communities. This has contributed to a new understanding of the importance of the microbes colonizing the host (Madigan et al., 2015, Fraune and Bosch, 2010).

To study the microbial diversity one often rely on the phylogenetic information obtained from highly conserved genes (e.g., 16S rDNA) (Gibbons and Gilbert, 2015). The most commonly used gene marker in microbial diversity studies is the 16S ribosomal RNA (rRNA) gene (Stoddard et al., 2015). Even before the development of NGS methods, the 16S rRNA gene was well represented gene in GenBank (Ibarbalz et al., 2014). The gene is ubiquity distributed and conserved between species. The 16S rRNA gene has nine variable regions in addition to highly conserved regions, making it a useful phylogenetic marker in culture-independent methods (Stoddard et al., 2015). In amplicon-based bacterial community studies, universal PCR primers are used to target one or more of the nine variable regions of the 16S rRNA gene (Ibarbalz et al., 2014). However, the bacteria have multiple copies of the rRNA operon and can cause a misrepresentation of the community (Stoddard et al., 2015). Bias is also related to in general

multi-template PCR-based methods where the amplification can be affected by insufficient coverage of primers, primer-template mismatches, unequal amplification, and a differential efficiency of annealing (Ibarbalz et al., 2014).

Characterization of the sequence diversity of PCR amplicons has previously been studied by gel-based fingerprinting methods like denaturing gradient gel electrophoresis (DGGE) and terminal-restriction fragment length polymorphism (T-RFLP) (Liu et al., 1997, Muyzer et al., 1993). High-throughput sequencing (HTS) platforms, like Illumina, is now the most commonly used method with the 16S rRNA as a target gene (Pepper et al., 2015). The NGS techniques have the ability to process millions of sequence reads simultaneously. The sequences derived from the samples are clustered together based on nucleotide similarity (often 97%) called "operational taxonomic units" (OTUs). This is the most commonly used diversity unit in microbial ecology, and are used as an estimator of the number of species in a sample (Gibbons and Gilbert, 2015). The species richness (i.e., number of OTUs) and species evenness or indices that combine both, are often used to describe the variation within a community (alpha(α)-diversity). Comparison of the communities between samples is known as the beta(β)-diversity. To characterize the β-diversity, the number of shared species between communities are often used (Pepper et al., 2015, Lozupone and Knight, 2008).

1.5 Aims of the Study

The overall aim of this study was to assess the relative importance of water microbiota and host genetics on the early gut microbiota in Atlantic salmon yolk-sac fry. The concept of r- and K-selection was used to study the effect of microbial water quality on the colonization of salmon fry by exposing two salmon strains to the two distinct microbial water qualities. The main hypothesis is that the fry microbiota will differ between the two salmon strains, but exposure to opportunistic bacteria in the water may obscure theses strain-specific differences. A sub-aim was to evaluate the effect of the water selection regime on the rearing water microbiota.

PCR amplification of regions of the bacterial 16S rRNA gene has in previous studies been challenging for samples from yolk-sac salmon fry. It is assumed that the main reason for this problem is due to the presence of unknown inhibitors in the DNA extract in addition to the low concentration of bacterial DNA compared to host DNA in the samples. For this reason, another sub-aim of this study was to establish a method for DNA extraction and successful amplification of bacterial 16S rDNA amplicons for Atlantic salmon yolk-sac fry.

2 Materials and Methods

2.1 Experiment on Atlantic Salmon

An experiment was conducted to study the effect of host strain and microbial water quality on the colonization of Atlantic salmon yolk-sac fry. Two strains of Atlantic salmon, a wild strain and an aquaculture strain, were hatched under germ-free conditions. One week post-hatching (wph), bacteria were introduced to the fish by exposing them to either r- or K-selected water. The bacterial density of the rearing water was analyzed by flow cytometry throughout the experiment. To study the microbial community composition of the salmon fry gut and rearing water by Illumina sequencing of the 16S rDNA amplicons, samples were collected two weeks post-exposure to bacteria (wpe).

The experiment was conducted in the time period 03.04-08.05.2018, and all samples in this study were collected and analyzed at NTNU, Department of Biotechnology and Food Science. The wild and aquaculture eggs were delivered from Haukvik (Vinjeøra) and AquaGen AS (Hemne), respectively.

2.1.1 Experimental Design

Two strains of Atlantic salmon, a wild strain and an aquaculture strain, was reared under two different water quality treatments. By arrival, the salmon eggs were surface sterilized with antibiotics and buffodine (see Section 2.1.2). The salmon fry was reared in 500 mL tissue flasks with a density of 15-18 fish in each flask. Before hatching and until one wph, all groups were reared under germ-free conditions with sterile fresh water. The experimental hatching day was determined to when at least 75 % of the fish was hatched in all fish flasks. One wph the flasks were randomly divided into two groups according to rearing water quality. Four replicate flasks from each salmon strain were then exposed to either with r- or K-selected water. For the experimental timeline, see Figure 2-1. Samples for microbial community analysis were collected 8 and 15 days post exposure (dpe) to r- and K-selected bacteria. Seven flasks for each salmon strain were kept germ-free as a control group for survival and growth. The experiment was conducted in a dark room at ~7°C.

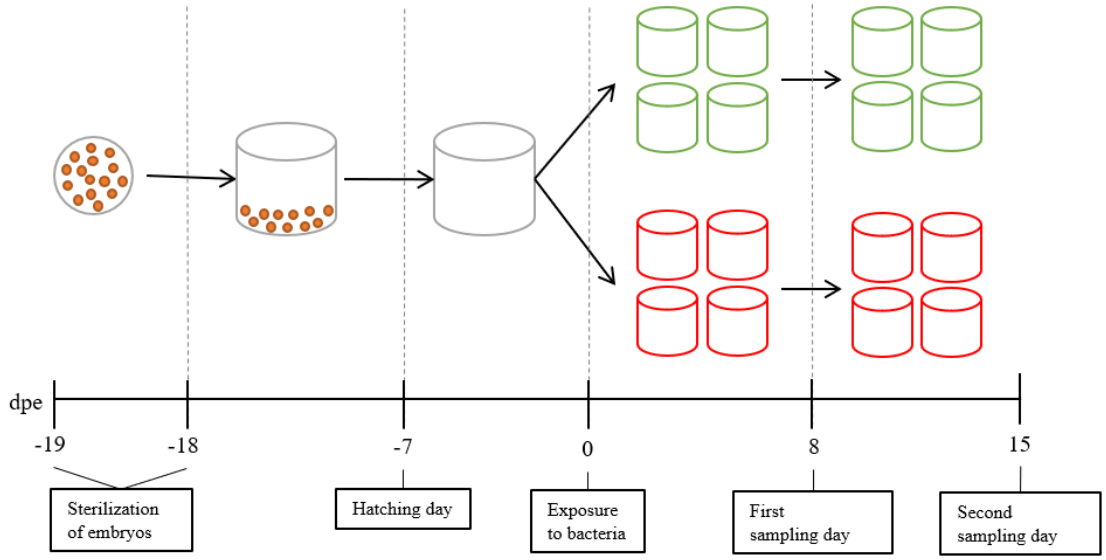

Figure 2-1: Illustration of the experimental timeline for an experiment conducted on Atlantic salmon (*Salmo salar*), representing one strain. The Atlantic salmon was reared in sterile fresh water (gray) until 7 days post-hatching. The flasks were then randomly divided into two groups according to the water treatment and received either r-selected (red) or K-selected (green) water. Four replicate flask of each water quality treatment was reared with ~15 fish in each flask before the first sampling point and ~9 fish in each flask after the first sampling point. The experiment ended 15 days post exposure to r- or K-selected bacteria (dpe).

2.1.2 Sterilization of Atlantic Salmon Eggs and Fish Rearing

To control the microbial community colonizing the fish, the eggs were surface sterilized to produce germ-free salmon yolk-sac fry. Upon arrival, the eggs were distributed into petri dishes (~100 embryos in each petri dish) and covered with sterile salmon gnotobiotic media (SGM, Appendix A). The embryos were incubated in the dark at 7°C for 24 hours to acclimatize.

The SGM was poured of the embryos, and approximately 100 mL of an antibiotic cocktail (Appendix B) was added to the eggs, and the plates were gently swirled to mix. The eggs were incubated in the dark for another 24 hours at 7°C.

Sterilization of the embryos with buffodine was performed in a laminar flow cabinet, and all equipment was UV-radiated before use. A buffodine solution (final concentration of 100 mgL^{-1}) was prepared in 50 mL conical vials with SGM. Approximately 15 embryos were transferred to an empty 50 mL conical vial by using plastic forceps. The embryos were inspected, and only healthy ones were used. The buffodine solution was added to the embryos and incubated for 30 minutes. After incubation, the embryos were rinsed four times in SGM and transferred to 500

mL tissue flasks containing 100 mL SGM. This was repeated for in total of 30 fish rearing flasks.

After the eggs were aliquoted into fish rearing flasks, water exchange was performed in a sterile laminar flow cabinet three times a week (Mondays, Wednesdays, and Fridays). Around 60 % of the water was exchanged by removing 60 mL of the rearing water with a 50 mL serological pipette. New water was poured in the fish flasks up to 100 mL. The experiment were conducted in the dark at 7°C.

2.1.3 Water Selection Regime

The freshwater used in this experiment was supplied by Vikelvdalen drinking water plant, and represented untreated water collected from Jonsvatnet at 70 meters depth (Trondheim (63.3655°N 10.5820°E)). The water was pre-filtered (1 µm) to remove larger particles and organisms, and stored in 20 L containers in the fish room at 7°C in the dark. Sterile fresh water was prepared by sterile filtering (0.2 µM) and autoclaving the water. This water was used for the germ-free control group and to dilute the r-selected water.

r- and K-selected water was made to every day of water exchange. Based on the theory of r- and K-selection (see Section 1.3.1), selection regimes were set up to have water dominated by either r- or K-strategic bacteria (Figure 2-2). In theory, K-strategist will dominate in water with a low nutrient supply, close to its carrying capacity. Based on this, the selection of K-strategists was done by "starving" the bacteria by keeping it untreated in the dark at room temperature (~22°C). The day before water exchange, the water was incubated at the fish room (~7°C). In contrast, r-strategists is good at competing when the nutrient supply is high, and will, in theory, dominate in communities where nutrients are in surplus. K-selected water (300 mL) was given a nutrient pulse of 15 mL M65-nutrient solution (Appendix C) one day before addition to the fish flasks. The bacterial density of samples from both r- and K-selected water was measured with flow cytometry. r-selected water was diluted in sterile freshwater to have approximately the same bacterial density as the K-selected water.

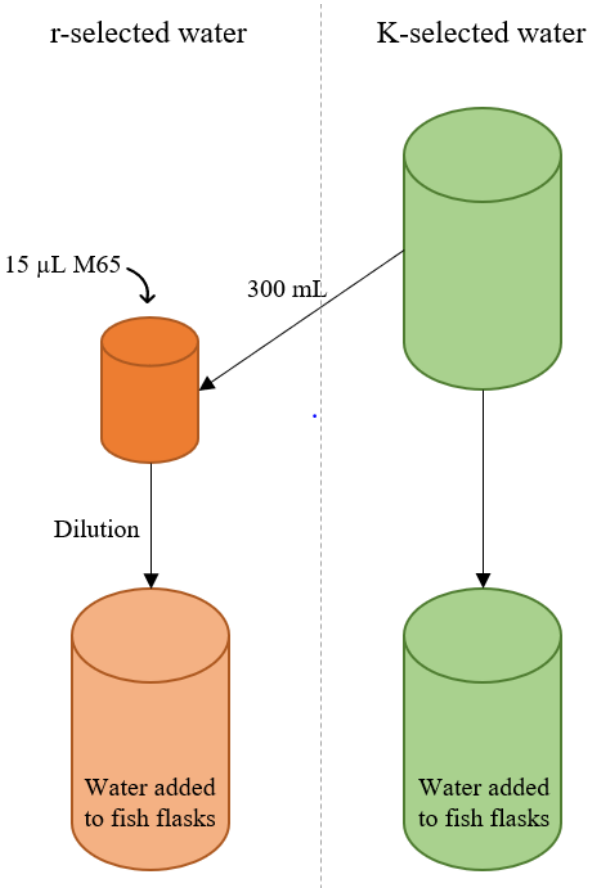

Figure 2-2: Illustration of the water selection regime of r- and K-strategists. 300 mL of the K-selected (green) water was used to make r-selected (red) water by adding 15 µL M65. The r-selected water was diluted in sterile fresh water to the same bacterial density as the K-selected water, before added to the fish flasks.

2.2 Collection of Samples

To determine the bacterial density of the water, samples of the rearing water and added water were collected for the days of water exchange for flow cytometry analysis. The exchanged rearing water and excess added water were collected in sterile 50 mL conical vials and stored at 4°C until further use for flow cytometry analysis the same day. Samples for microbial community analysis of the rearing water were collected 15 dpe to r- and K-selected bacteria. Rearing water samples for microbial community analysis were obtained by filtrating the rearing and added water through a 0.2 µm syringe tip filter (DynaGard®) using a 20 mL syringe. The filters were stored at -20°C until further analysis. The added r- and K-selected water was sampled for microbial community analysis every day of water exchange, with the same procedure as described above.

Samples for microbial community analysis of the salmon yolk-sac fry gut were collected 8 and 15 dpe to r- and K-selected water. The gut from six individuals was dissected out from each rearing flask. However, only four samples from 15 dpe were sequenced. The fish was transferred into petri dishes with sterile fresh water by using a serological pipette. The fish was euthanized in sterile ethyl 3-aminobenzoate methane sulfonate ($5.2gL^{-1}$) (Sigma), and then transferred to an empty petri dish. The dissection was performed under a microscope. The yolk sac was sacrified, and the gut was gently pulled out and transferred into a cryo tube. All equipment was cleaned in 70% ethanol between individuals. The samples were put on ice and stored at -80°C until further analysis.

2.3 Analytical Methods

2.3.1 Flow Cytometry Analysis

Flow cytometry is a method which uses light-scattering and fluorescence to rapidly measure single cells in a suspension. The analysis can give information about cell size, shape, density, and surface morphology. Fluorescence labeling of cells can be used to detect and quantify the number of bacteria in complex environments, such as water (Bressan et al., 2015).

The samples were diluted in 0.1x TE-buffer (Appendix D) accordingly to the recommended detection limit (<1 000 cells/µL). A working solution of SYBR® Green I nucleic acid gel stain (Life Technologies, Thermo Fisher Scientific Inc.) (1:50) was prepared by diluting the stock solution (10 000x in DMSO) in sterile filtrated 0.1x TE-buffer. SYBR Green I was added to 1 mL of the diluted samples and incubated in the dark for 15 minutes. The samples were analyzed on a BD Accuri™ C6 Flow Cytometer (BD Bioscience, San Jose) with medium flow rate (34.5 µL/min) for 2 minutes or until 10 000 counts were reached. The emitted light was detected by the FL1 detector (533±15 nm). The number of bacteria in the sample is the number of emissions detected by the FL1 detector. The results were processed using BD Accuri™ C6 Software. FL1 versus FSC-A (forward scatter, correspond with the size of the cell) was plotted to filter out the noise and larger particles. The data were imported to Microsoft Excel for further analysis to estimate the bacterial density of the added and rearing water.

2.3.2 Microbial Community Analysis

2.3.2.1 DNA Extraction

Four DNA extraction kits were tested for Atlantic salmon gut samples to find the kit giving the highest yield of bacterial DNA (see Section 3.1). The DNA extraction kits tested were DNeasy Powersoil DNA Isolation Kit (Qiagen), PureLink™ Microbiome DNA Purification Kit (Thermo Scientific), Ultra-Deep Microbiome Prep (Molzym), and ZymoBIOMICS™ DNA Miniprep Kit (Zymo research), and are hereby referred to as PowerSoil, PureLink, Ultra-Deep, and Zymo, respectively. The DNA extractions were performed according to the manufacturer's protocols (Appendix E) with a few deviations. A deviation common to all kits was that the samples were transferred by adding the first lysis solution buffer to the sample tube and dissolving the gut in the solution before transferring it back. This was done to ensure the whole gut was transferred because of the small size. In sample pre-treatment and step 6 and 7 in the protocol from Ultra-Deep, the samples were going to be incubated in a thermomixer. In these steps, the samples were incubated at the given temperature in a heating block, and regularly vortex. In step 2 in the extraction with Zymo, a vortex adapter for 2 mL tubes was used at maximum speed for 30 minutes. Elution volume for all extractions was 100 µL.

Several measures were done to reduce DNA contamination through the DNA extraction protocols. All collection tubed were UV-radiated in at least 20 minutes before use. Spin columns used in PureLink and PowersSoil was washed with preheated DNase-, protease- and RNase-free water. This was done by adding water to the columns and incubating them at 55°C in 20 minutes. The columns were centrifuged at 10 000 G for one minute. The two other kits claim to contain no or little amount of DNA, and columns used in these kits were not washed. The DNA extractions performed with the Zymo kit was conducted under a laminar flow cabinet and all equipment was UV-radiated. With each DNA extraction round, a positive control (a cell pellet from a pure bacterial strain) and a negative control was included.

The DNA extraction kit from Zymo was found to be the kit giving the highest yield of bacterial DNA with the given PCR conditions (see Section 3.1.1). This kit was therefore used to extract DNA from salmon gut and rearing water samples from the last sampling day (15 dpe), in addition to samples of added r- and K-selected water from water exchange day 7, 9, 11 and 14. The samples were thawed in room temperature, and DNA extraction was performed as described above.

2.3.2.2 Polymerase Chain Reaction (PCR)

Bacterial 16S rDNA was amplified by polymerase chain reaction (PCR). The variable region 3 and/or 4 (V3 and V4) were amplified by various primer pairs (Table 2-1). To establish a PCR protocol yielding sufficient concentration of bacterial DNA, several components of the reaction mixture and cycling conditions were tested, both in terms of reaction mixture composition and temperature cycling conditions (Table 2-2).

The amplification of the 16S rDNA was conducted on a T100^{TM} Thermal Cycler (BioRad) with a reaction volume of 25 µL. When testing the effect of increased magnesium chloride concentration ($MgCl_2$, final concentration 2 Mm) (Thermo Scientific) or addition of spermidine (0.5 mM) (Sigma-Aldrich), the volume of water was adjusted to give a total volume of 25 µL. The variable region V3, V4, and V3-V4 were amplified using primers specified in Table 2-1. Various PCR reaction and cycling conditions were tested, and the results are reported in Section 3.1.

After optimization of the PCR amplification protocol (see Section 3.1), the following conditions were used to amplify the V3-V4 region of the 16S rDNA from water and gut samples for Illumina amplicon sequencing. Atlantic salmon fry gut samples were amplified using 1:10 diluted DNA extract as a template for the PCR reaction. The reaction mixture consisted of 1x Phusion buffer HF (Thermo Scientific), 0.3 µM of each primer (Sigma-Aldrich), 0.2 µM each dNTP (Thermo Scientific), 0.025 UμL^{-1} Phusion Hot Start DNA Polymerase (Thermo Scientific), and 2 µL template. The reaction was run with an annealing temperature of 55°C and 38 thermal cycles. Water samples were amplified using the same reaction mixture but with 1 µL undiluted DNA extract as a template. The reaction was run with an annealing temperature of 55°C and 35 cycles.

Table 2-1: PCR primers (Sigma-Aldrich) name and sequence used for amplification of the 16S rDNA for Illumina and Sanger sequencing. Illumina adapters are marked in bold.

Primer name	Nucleotide sequence	Target region
Ill338F	5'-**TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG NNNN** CCT ACG GGW GGC AGC AG-3'	V3
Ill805R	5'- **GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G NNNN** GAC TAC NVG GGT ATC TAA KCC-3'	V4
Ill515F	5'- **TCG TCG GCA GCG TCA GAT GTC TAT AAG AGA CAG NNNN** GTG CCA GCM GCC GCG GTA A-3'	V4
Ill532R	5'- **GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G NNNN** TTA CCG CGG CKG CTG GCA C -3′	V3
518R	5'- ATT ACC GCG GCT GCT GG -3'	V3
338F	5'- CCT ACG GGW GGC AGC AG -3'	V3
805R	5'- GAC TAC NVG GGT ATC TAA KCC -3'	V4

Table 2-2: Temperature cycling conditions in the PCR reaction used to amplify variable regions of the 16S rDNA.

Step	Temperature [°C]	Time	
Denaturation	98	1 min	
Denaturation	98	15 sec.	
Annealing	55-57	20 sec.	x 35-39
Elongation	72	20 sec.	
Final elongation	72	5 min	

2.3.2.3 Agarose Gel Electrophoresis

The quantity and possible contamination of PCR products were analyzed using agarose gel electrophorese.

An agarose solution (1 or 1.5 %) was prepared by dissolving agarose in 1% TEA-buffer (Appendix D - Buffer Solutions) by heating the solution. The solution was poured into a gel chamber with gel comb and settled to a gel (20-30 minutes depending on thickness). Either a 1 or 1.5 % agarose gel was used depending on the desired separation resolution.

Samples (1-4 µL) mixed with 1 µL 6x DNA Loading dye (Thermo Scientific) were applied to the gel wells and ran at 100-120 V until sufficient separation of products was achieved (normally 1-1.5 h). GeneRuler™ 1 kb Plus DNA Ladder (Thermo Scientific) or GeneRuler™ 1 kb DNA Ladder (Thermo Scientific) was used as a size marker. The samples were stained by adding GelRed® (Biotium) (final concentration of 50 mM) to either the loading dye or the agarose gel and visualized by using a UV-chamber.

2.3.2.4 Preparation of Samples for Sanger Sequencing

Some samples were sequenced by Sanger sequencing to evaluate the quality of the PCR amplification product of the 16S rDNA. The PCR products were purified using the QIAquick® PCR Purification Kit (Qiagen) by following the manufacturer's protocol (Appendix F). The purified PCR product (5 µL) and a sequencing primer (5 µL of 5 mM 338F) were mixed and sent to Eurofins Genomics for Sanger sequencing.

The sequence results were analyzed by manual inspection of the chromatograms.

2.3.2.5 Preparations of Amplicon Library for Illumina Sequencing

The microbial diversity in environmental samples can be characterized by Illumina sequencing of 16S rDNA amplicons. After PCR amplification and normalization, the amplicon library is prepared for sequencing by tagging both ends of the sequence with unique oligomers and adapter sequences that allows for pooling of PCR products representing multiple samples. The amplicon library is loaded into a flow cell and the DNA molecules are attached to surface-bound oligomers that are complementary to the adapters. Illumina sequencing technology is based on sequence by synthesis (SBS) chemistry. Fluorescent-labeled deoxyribonucleotide triphosphates (dNTPs) with a reversible 3´ block are added to the flow cell. These modified

dNTPs ensure that only a single base is added each cycle. Between each base cycle, an imaging step identifies the attached dNTP and a chemical step remove the 3´block and the fluorescent group. This cycle is repeated until the DNA molecule is sequenced (Mardis, 2008, Illumina, 2017).

After optimizing the DNA extraction and PCR protocols, PCR products from Atlantis salmon fry gut and water samples including Illumina adapters were successfully generated. The products were further used to make an amplicon library for Illumina sequencing. To prepare the samples for Illumina sequencing, the samples were first normalized and purified by using a Sequal Prep Normalization plate Kit (Invitrogen). The manufacturer's protocol (Appendix G) were followed, by using 15 µL of the amplicons representing gut samples, and 7.5 µL of amplicons representing water samples due to more products for the water samples.

Further, eight forward sequence indexes and twelve reverse sequence indexes were used to give each sample a unique index combination by using the Nextera XT Index Kit Set D (Illumina). Each primer sequence index (2.5 µL each) were added to a PCR reaction mixture consisting of 1x Phusion buffer HF (Thermo Scientific), 0.25 mM dNTP (Thermo Scientific), and 0.015 $U\mu L^{-1}$ Phusion Hot Start DNA polymerase (Thermo Scientific), before the normalized PCR products were added as template. The samples were prepared in a 96-well plate. Samples were run for 10 cycles on a thermocycler with the temperature and cycling conditions listed in Table 2-3. The yield of PCR products was analyzed using agarose gel electrophoresis. For the samples with low yield, the procedure was repeated with 12 cycles.

Table 2-3: Temperature and cycling condition used in the PCR reaction when indexing PCR amplification products.

Step	Temperature [°C]	Time	
Denaturation	98	1 min	
Denaturation	98	15 sec.	
Annealing	50	20 sec.	x 10-12
Elongation	72	20 sec.	
Final elongation	72	5 min	

The indexed PCR products were normalized and purified using the Sequal Prep Normalization plate Kit (Invitrogen) as described above, by using 10 µL of each of the PCR products. All 95

samples were then pooled together and concentrated by using Amicon Ultra 0.5 Centrifugal Filter units (Merck Millipore, Ireland). The manufacturer's protocol was followed (Appendix H). After step four, an additional washing step was performed. TE-buffer (500 µL, Appendix D) was added, and the sample was centrifuged in 10 minutes at 14 000 G. The washing step was repeated once more. The concentration and purity of the concentrated product were measured with NanoDrop™ One (Thermo Scientific), and the size of the product was determined by running an agarose gel.

The concentration of the pooled samples was not satisfying, and the sample was concentrated once more following the same procedure.

The amplicon library was sent to the Norwegian Sequencing Centre (NCS) for sequencing on one MiSeq lane (Illumina, San Diego, CA) with V3 reagents (Illumina).

2.3.2.6 Processing of Illumina Sequencing Data

The Illumina sequencing data were processed using the USEARCH pipeline (version 10; https://www.drive5.com/usearch/). The command Fastq_mergepairs was used for merging of paired reads, trimming off primer sequences, and filtering out reads shorter than 400 base pairs. The processing further included demultiplexing and quality trimming (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. Taxonomy assignment was performed applying the Sintax script (Edgar, 2016) with a confidence value threshold of 0.8 and the RDP reference data set (version 16).

The resulting OTU table was manually inspected, and OTUs that represented salmon genes were excluded from the table. Furthermore, OTUs that were dominating in non-template controls and negative controls for the DNA extraction protocol, were excluded. The resulting OTU table was normalized to 20 000 number of reads per sample by first determining the fraction of the OTUs for each sample, and then multiply with the relevant number of reads, and finally rounding off the read numbers to integers. The Usearch commands Alpha_div and Sintax_summary was used to calculate alpha diversity indices and generate taxa summary tables (at various taxonomic levels as specified with the results), respectively.

2.3.2.7 Statistical Analysis

The alpha(α)-diversity measurements OTU richness (*S*), Chao1, and Shannon diversity index (*H*) were calculated in the USEARCH pipeline and exported to Microsoft Excel for further analysis. Number of OTUs in a sample reflects the species richness. Chao1 is an estimation of the true number of species in a sample, where singletons and doubletons are taken into consideration when calculating the number of OUTs (Chao et al., 2005). Both the number of unique species and their relative abundance in a sample is considered in the Shannon diversity index (Shannon, 1948). In general, a large value Shannon value indicates a higher diversity (Pepper et al., 2015). As recommended by Lucas et al. (2016), the exponential Shannon index (e^H) were used. Busaz and Gibson's evenness (e^H/S) were calculated based on the OTU richness and Shannon diversity index (Buzas and Hayek, 1996). The species evenness is a term which describes the variability of the species abundance, where a perfectly even community consist of species in equal abundance (Pepper et al., 2015).

The beta(β)-diversity statistics were calculated in PAST. Bray-Curtis similarities and Dice index were used for comparison of the β-diversity within and between samples. The Dice index is based on the presence/absence of species compared between samples and is calculated according to the number of species shared and unique between samples. The Bray-Curtis similarity is a modified version of Dice, and are based on the abundance of species (Chao et al., 2005, Bray and Curtis, 1957, Dice, 1945). To compare the microbial community profiles within and between the samples a Bray-Curtis similarity matrix was calculated. The output is a number between 0 (dissimilar) and 1 (similar), quantifying the similarities between the samples (Bray and Curtis, 1957). Based on the matrix, average Bray-Curtis similarities were calculated within and between sample groups using Microsoft Excel. A principal coordination analysis (PCoA) plot was computed based on Bray-Curtis similarities and Dice indices. PCoA plot a graph in low-dimension based on a distance matrix. The data is arranged in a way that the distance between the points reflects the similarity/dissimilarity as good as possible. The closer the samples are arranged in the plot, the more similar they are to each other (Harper, 1999, Davis and Sampson, 1986).

To examine whether there was a significant difference or not between the microbial communities between sample groups, one-way PERMANOVA (permutational multivariate analysis of variance) based on Bray-Curtis or Dice similarities was conducted (Anderson,

2001). The result indicates a significant difference if the p-value is below 0.05 (Hammer et al., 2001).

SIMPER (Similarity Percentage) analysis based on Bray-Curtis similarities were conducted to identify the OTUs contributing to the difference in the microbial community composition between groups (Clarke, 1993).

A two-sample T-test was used to examine potential differences between two groups. If the variance between samples were significantly different (F-test, $p<0.05$), however, the p-value of the unequal variance t-test (Welch t-test) were used (Hammer et al., 2001).

3 Results

3.1 Optimization of DNA Extraction and Amplification of 16S rDNA from Atlantic Salmon Fry Gut

Based on previous studies and master projects in the research group, PCR amplification of regions of the 16S rRNA gene from DNA samples from Atlantic salmon has proven to be difficult. This is believed to be mainly because of inhibitors in the DNA extract and/or low amount of bacterial DNA compared to host DNA. Most of the DNA extraction kits contain contaminating DNA (Glassing et al., 2016, Salter et al., 2014), and might be a problem when the concentration of target DNA is low. Another challenge is co-amplification of host DNA because of primer homology to regions of the 18S rDNA and the mt 12S rDNA of Atlantic salmon. When the fraction of bacterial DNA is low, it can be outcompeted during amplification. Thus, an essential part of this study was to optimize the DNA extraction and PCR protocol.

In total four different DNA extraction kits were tested in combination with various PCR protocols to achieve a sufficient product yield. Several reagents in the PCR reaction mixture and PCR cycling conditions were tested in an attempt to optimize the product yield. Gut samples used in the optimization process was dissected from Atlantic salmon yolk-sac fry.

The first DNA extraction kit tested was DNeasy Powersoil DNA Isolation Kit (Qiagen). PCR amplification of the V3-V4 region of the 16S rDNA was conducted by adding extra magnesium chloride ($MgCl_2$) to a final concentration of 2 mM in the PCR reaction mixture (Figure 3-1). A weak band of the expected size was observed for all samples except DNA extract representing six pooled individual guts. A product of a larger size was observed in two of the samples, probably due to co-amplification of host DNA. The product yield was similar for samples representing a single and two pooled guts, indicating that it is sufficient to extract DNA from one gut to obtain an amplification product. A weak PCR product was obtained in DNA extraction “kit blanc” (KB; negative control for the DNA extraction kit), which indicates contaminating DNA in the DNA extraction kit. The product yield was still low, and samples were further amplified by increasing the number of PCR cycles (to 38). To improve the specificity, and thus reduce co-amplification of host DNA, the $MgCl_2$ concentration was reduced 1.5 mM.

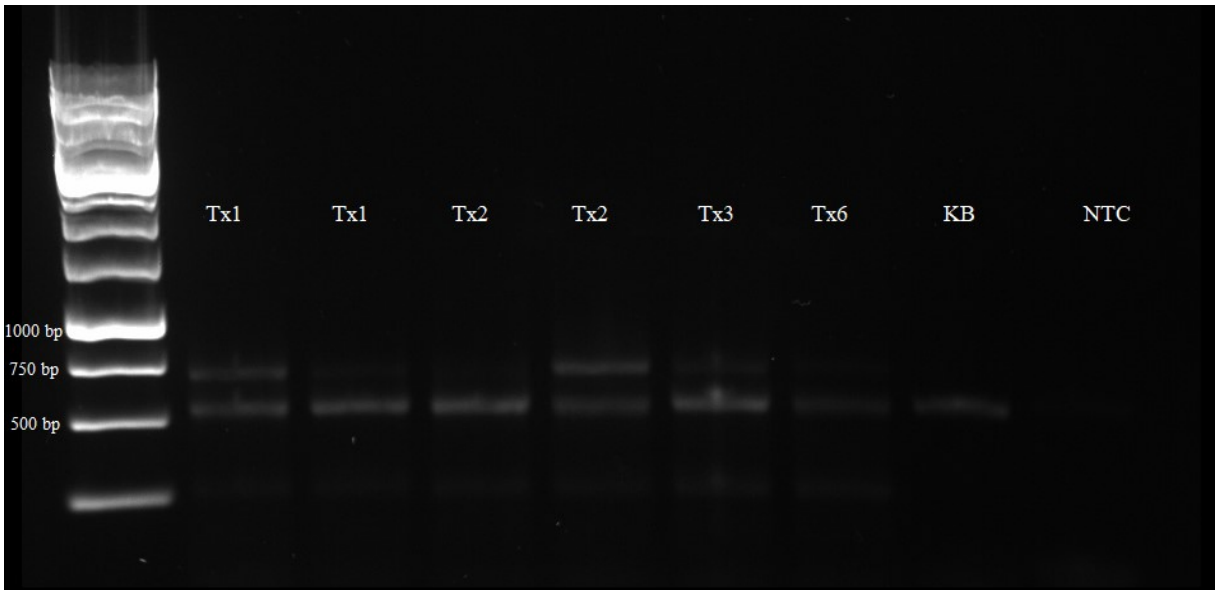

Figure 3-1: Agarose gel (1%) showing V3-V4 16S rDNA amplicons obtained with the primers Ill-338F and Ill-805R (Table 2-1). Samples represent DNA extracted with DNeasy Powersoil DNA Isolation Kit (Qiagen) from a single gut (Tx1) and pooled guts from two (Tx2), three (Tx3), and six (Tx6) individuals from Atlantic salmon yolk-sac fry. PCR reactions had a final concentration of 2 mM $MgCl_2$ and were run with an annealing temperature of 55 °C and 36 temperature cycles. KB and NTC represent a negative DNA extraction control ("kit blanc") and a PCR non-template control, respectively.

Amplification of the V3-V4 region of the 16S rDNA gene with 38 cycles and a final $MgCl_2$ concentration of 1.5 mM was conducted. Reduction of the $MgCl_2$ concentration resulted in improved specificity, and only one band of the expected size was obtained for all gut samples (Figure 3-2). Also, no effect on the product yield of the desired product was observed, and it was concluded to further run PCR with no extra $MgCl_2$ in the reaction mixture. However, the relative concentration of the expected product compared to the KB was not satisfying, and further testing was conducted to increase the product yield.

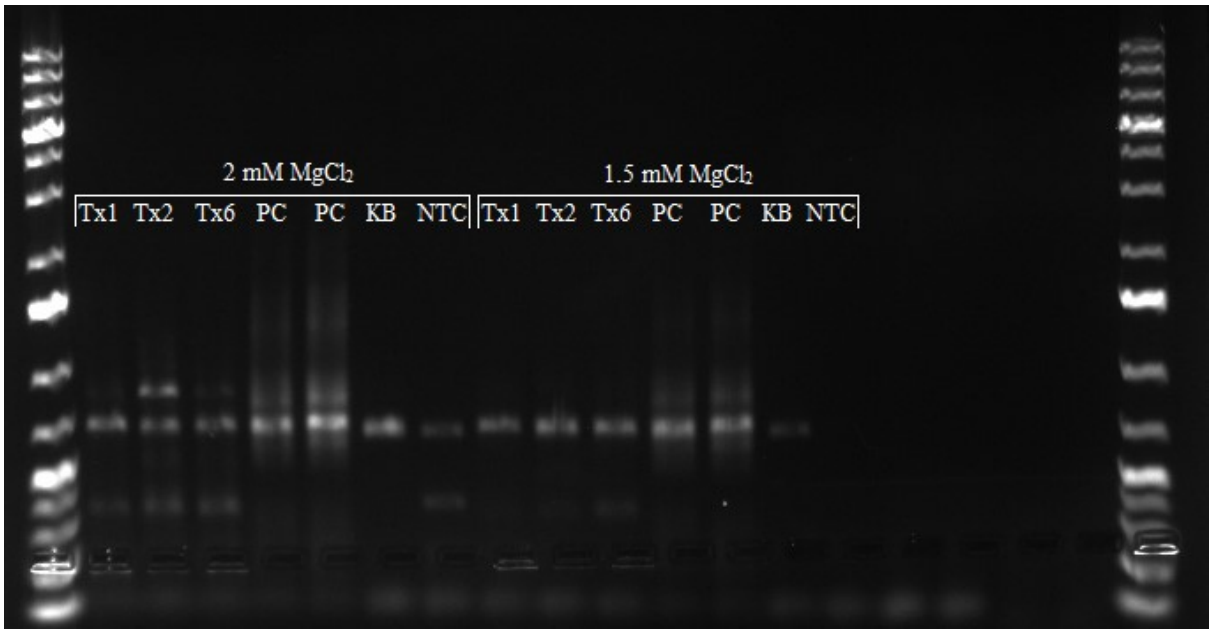

Figure 3-2: Agarose gel (1%) showing V3-V4 16S rDNA amplicons obtained with the primers Ill-338F and Ill-805R (Table 2-1). Samples represent DNA extracted with DNeasy Powersoil DNA Isolation Kit (Qiagen) from a single gut (Tx1) and pooled guts from two (Tx2), three (Tx3), and six (Tx6) individuals from Atlantic salmon yolk-sac fry. PCR reactions were run with and without extra $MgCl_2$ (final concentration of 2 mM or 1.5 mM) at an annealing temperature of 55 °C and with 38 temperature cycles. KB, PC, and NTC represent a negative DNA extraction control ("kit blanc"), a positive PCR control and a PCR non-template control, respectively. The ladder used as a size marker was not diluted correctly, and the size of the bands are not marked on the gel to prevent confusion.

Due to a low product yield of the expected product, the effect of the facilitator spermidine in the PCR reaction mixture was tested. Spermidine was added to the PCR reaction mixture to a final concentration of 0.5 µM. Adding spermidine had no significant effect on the product yield of the desired product (Figure 3-3). Also, spermidine resulted in an increased product yield for the KB, and thus it was concluded not to use the facilitator in the reaction mixture.

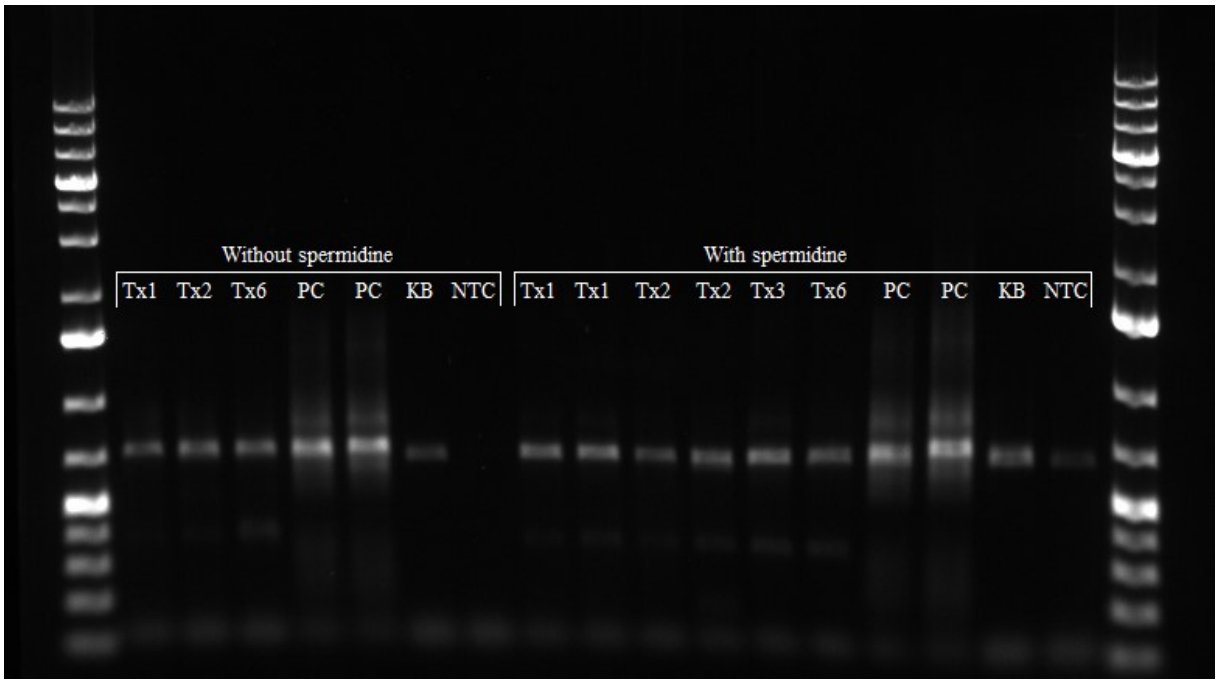

Figure 3-3: Agarose gel (1%) showing V3-V4 16S rDNA amplicons obtained with the primers Ill-338F and Ill-805R (Table 2-1). Samples represent DNA extracted DNeasy Powersoil DNA Isolation Kit (Qiagen) from a single gut (Tx1) and pooled guts from two (Tx2), three (Tx3), and six (Tx6) individuals from Atlantic salmon yolk-sac fry. PCR reactions were run with and without spermidine (final concentration of 0.5 mM) at an annealing temperature of 55 °C and with 38 temperature cycles. KB, PC, and NTC represent a negative DNA extraction control ("kit blanc"), a positive PCR control and a PCR non-template control, respectively. The ladder used as a size marker was not diluted correctly, and the size of the bands are not marked on the gel to prevent confusion.

In an attempt to reduce the amount of contaminating bacterial DNA obtained from the DNA extraction kit, the spin columns were washed with DNA-free water and collection tubes were UV-radiated (see 2.3.2.1). The V3-V4 region of the 16S rDNA was amplified using DNA extracted with PowerSoil and PureLink as a template. A low yield of the expected product was obtained, as well as PCR product for the KB (gel picture not included) and indicate contamination of bacterial DNA in these DNA extraction kits.

A third kit was tested to try to solve the problem with contamination of bacterial DNA in DNA extraction kits and low product yield of the desired product. Ultra-Deep Microbiome Prep (Molzym) claims to be DNA-free and include steps to remove host DNA. Amplification of the V4-V3 and V4 region of the 16S rDNA resulted in no product for KB nor non-template control (NTC) (Figure 3-4). However, only a weak band of the expected length were observed in one of the samples for both regions amplified. Co-amplification of host DNA was also observed for amplification of the V4 region as shown in Figure 3-4. Due to the poor yield of the desired product, this kit was found non-optimal for the salmon gut samples.

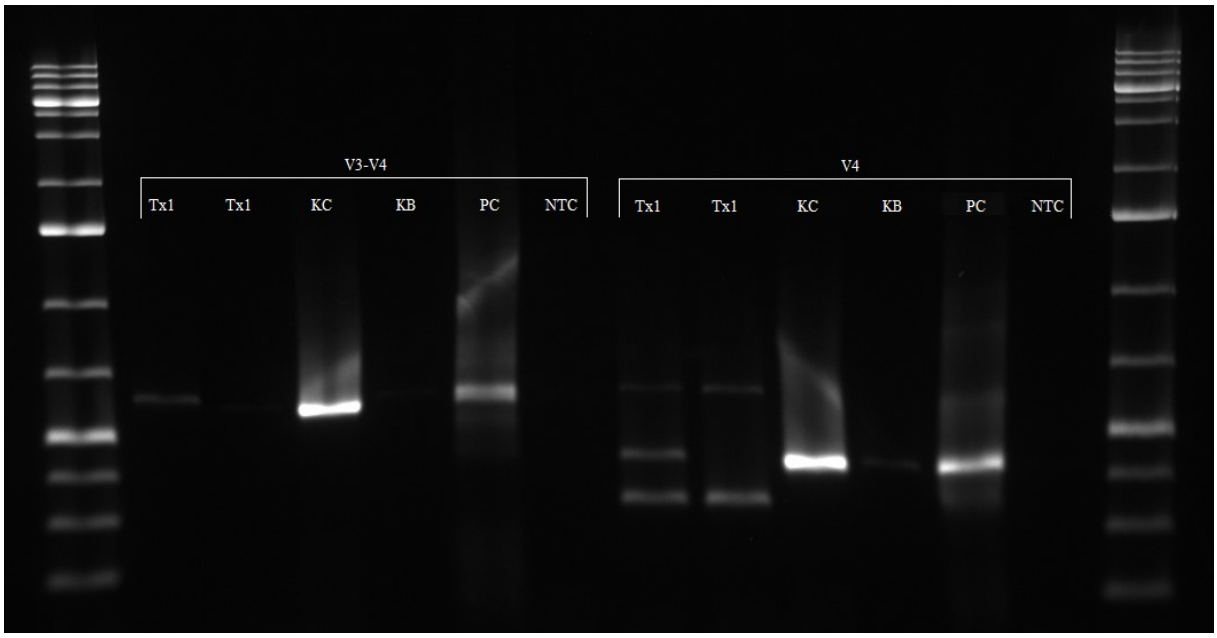

Figure 3-4: Agarose gel (1.5%) showing V3-V4 and V4 16S rDNA amplicons obtained with the primers Ill-338F and Ill-805R and Ill-515F and Ill-805R, respectively (Table 2-1). Samples represent DNA extracted with Ultra-Deep Microbiome Prep (Molzym) from a single gut (Tx1) from Atlantic salmon yolk-sac fry. PCR reactions were run with an annealing temperature of 55 °C and with 36 temperature cycles. KB, KC, PC, and NTC represent a negative DNA extraction control ("kit blanc"), an DNA extraction positive control, a PCR positive control (water bacterial community) and a PCR non-template control, respectively. The ladder used as a size marker was not diluted correctly, and the size of the bands are not marked on the gel to prevent confusion.

Further, DNA extracts obtained form gut samples with PureLink, PowerSoil, and Ultra-Deep were used to amplify the V3 region of the 16S rDNA using primers both with and without Illumina sequences in an attempt to achieve a higher yield of the desired product. Amplification with Illumina adapters was not successful and resulted in primer dimers (gel picture not included). However, by using primers without Illumina adapters, great product yield was obtained from gut samples extracted with PureLink and PowerSoil (Figure 3-5). By comparing primer sequences with the sequence of the 18S rRNA gene of Atlantic salmon, homologous regions were found both for the forward and the reverse primer indicating this could be amplified host DNA. The expected length of the salmon and bacterial amplification product is similar, and it was not possible to distinguish them from each other using agarose gel electrophoresis. PCR products were sequenced using Sanger sequencing to evaluate the samples. The results showed that the amplified product originated from the 18S rRNA gene of Atlantic salmon.

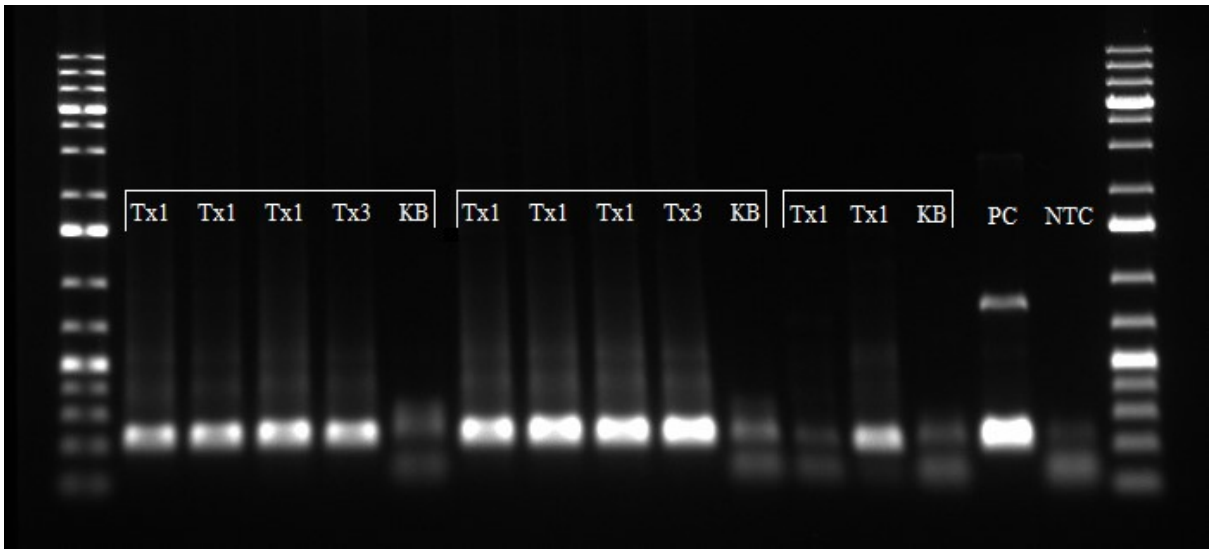

Figure 3-5: Agarose gel (1.5%) showing V3 16S rDNA amplicons obtained with the primers 338F and 518R (Table 2-1). Samples represent DNA extracted with DNeasy Powersoil DNA Isolation Kit (Qiagen), PureLink™ Microbiome DNA Purification Kit (Thermo Scientific), and Ultra-Deep Microbiome Prep (Molzym) from a single gut (Tx1) and pooled guts from three (Tx3) individuals from Atlantic salmon yolk-sac fry. PCR reactions were run with an annealing temperature of 55 °C and with 38 temperature cycles. KB, PC, and NTC represent a negative DNA extraction control ("kit blanc"), a positive PCR control and a PCR non-template control, respectively. The ladder used as a size marker was not diluted correctly, and the size of the bands are not marked on the gel to prevent confusion.

A last DNA extraction kit was tested in the hope of achieving a higher product yield and lower levels of contaminating bacterial DNA. ZymoBIOMICS™ DNA Miniprep Kit (Zymo research) claims to contain a low amount of bacterial DNA and also has a purifying step at the end. Amplification of the V3-V4 region of the 16S rDNA was conducted for samples representing single guts extracted with Zymo and Ultra-deep. The result showed a greater product yield for samples extracted with the Zymo kit compared to samples extracted with Ultra-Deep (gel picture not included). One PCR product form Zymo was sequenced using Sanger sequencing. The results revealed a mix of sequences, indicated that the amplified product most likely originated from bacteria in the salmon gut. Based on the results, the Zymo kit was used to extract DNA from Atlantic salmon fry guts. Further optimization was conducted to increase the product yield.

In the presence of inhibitors or high concentration of host DNA, diluting the DNA extract may affect the amplification yield of the desired product. Dilution of the template was conducted to investigate the effect on amplification using DNA extracts from Zymo as template. The product yield in the KB decreased significantly when diluted (Figure 3-6). However, no reduction in

band intensity was obtained in the gut samples, indicating the presence of inhibitors. It was decided to further use 2 µL of 1:10 diluted samples as a template in the PCR reactions.

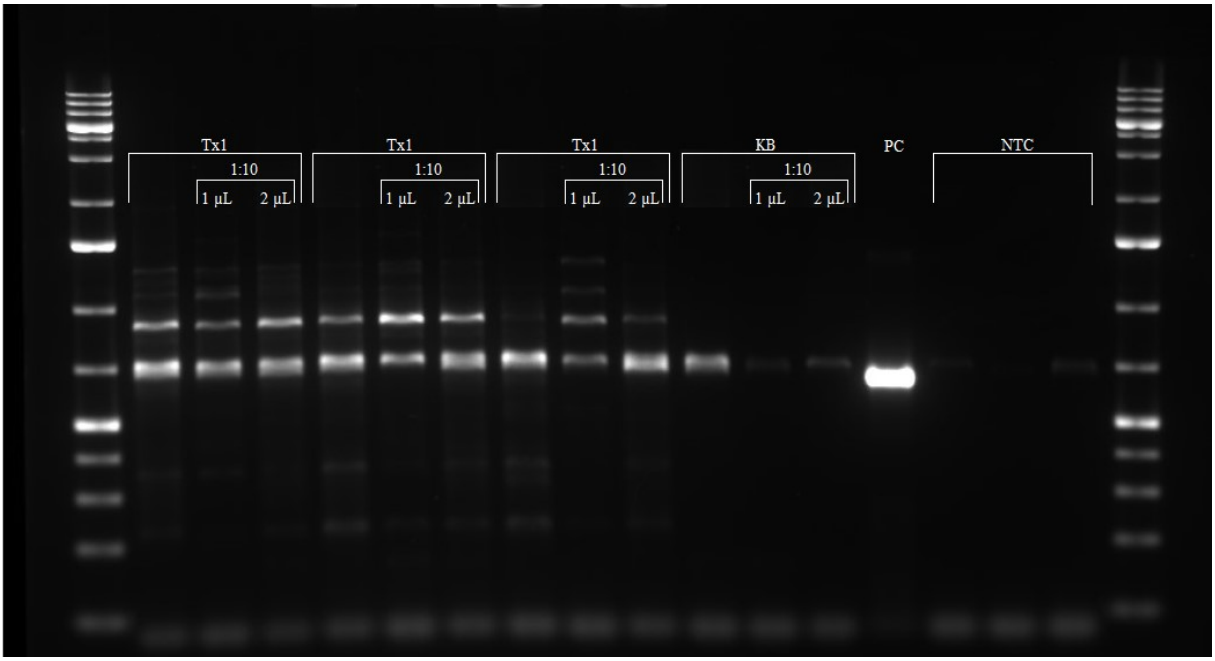

Figure 3-6: Agarose gel (1.5%) showing V3-V4 16S rDNA amplicons obtained with the primers Ill-338F and Ill-805R (Table 2-1). Samples represent DNA extracted with ZymoBIOMICS™ DNA Miniprep Kit (Zymo research) from a single gut (Tx1) from Atlantic salmon yolk-sac fry amplified using 1 µL undiluted and 1 and 2 µL of 1:10 diluted DNA extract as a template. PCR reactions were run with an annealing temperature of 55 °C and with 38 temperature cycles. KB, PC, and NTC represent a negative DNA extraction control ("kit blanc"), a PCR positive control and a PCR non-template control, respectively. The ladder used as a size marker was not diluted correctly, and the size of the bands are not marked on the gel to prevent confusion.

In several of the samples extracted with Zymo, it was obtained an extra band expected to be co-amplified host DNA. In an attempt of reducing the amount of unspecific product, the annealing temperature was increased. By increasing the annealing temperature, the stringency of the primers will increase, making the amplification more specific. A PCR was conducted by increasing the annealing temperature to 56 °C but showed little effect on unspecific amplification. Also, a negative effect on the yield of the expected product was observed (Figure 3-7). Using an annealing temperature of 55°C was found to be preferable for amplification of the V3-V4 region of the 16S rDNA for Atlantic salmon gut samples.

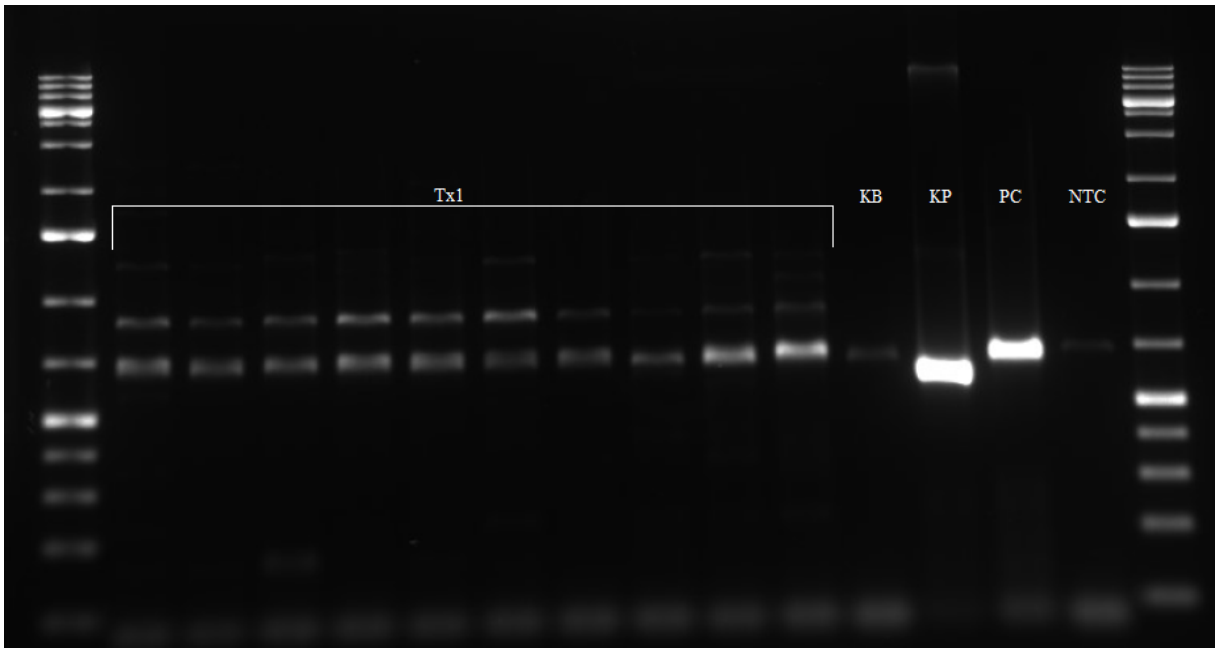

Figure 3-7: Agarose gel (1.5%) showing V3-V4 16S rDNA amplicons obtained with the primers Ill-338F and Ill-805R (Table 2-1). Samples represent DNA extracted with ZymoBIOMICS™ DNA Miniprep Kit (Zymo research) from a single gut (Tx1) from Atlantic salmon yolk-sac fry amplified using 2 µL of 1:10 diluted extract as a template. PCR reactions were run with an annealing temperature of 56 °C and with 38 temperature cycles. KB, KC, PC, and NTC represent a negative DNA extraction control ("kit blanc"), an DNA extraction positive control, a PCR positive control and a PCR non-template control, respectively. The ladder used as a size marker was not diluted correctly, and the size of the bands are not marked on the gel to prevent confusion.

3.1.1 Conclusion for the PCR Optimization

The greatest amplification yield for the expected PCR product was obtained for the DNA extracted with the kit from Zymo. This kit was used to extract the DNA from Atlantic salmon gut samples. The final PCR protocol was conducted for the V3-V4 region of the 16S rDNA using 2 µL of 1:10 diluted sample as a template. PCR was run with 38 temperature cycles with an annealing temperature of 55°C. Amplicons were successfully made for gut samples with a low product yield for the KB control using these conditions (Figure 3-8). Co-amplification, probably of salmon 18S rRNA gene, was observed for some of the samples, but the yield was less compared to the bacterial 16S rDNA product. Low yield of amplicons was observed in a few of the samples. A satisfying yield was finally obtained for these samples by increasing PCR cycles to 39.

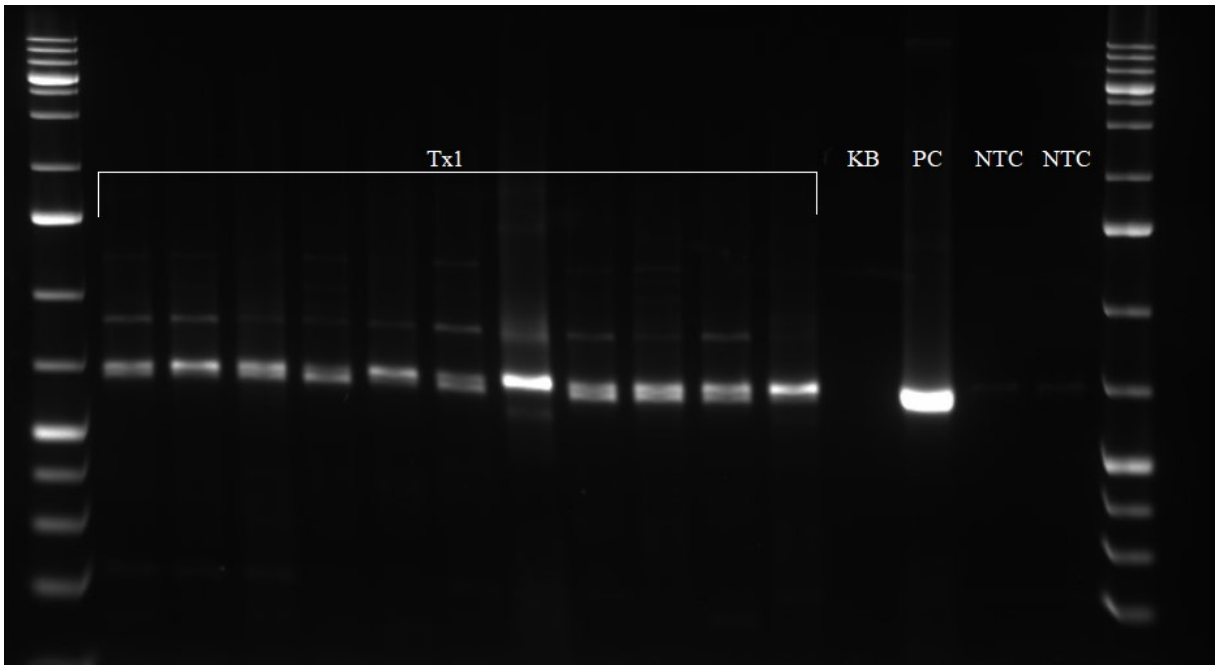

Figure 3-8: Agarose gel (1.5%) showing V3-V4 16S rDNA amplicons obtained with the primers Ill-338F and Ill-805R (Table 2-1). Samples represent DNA extracted with ZymoBIOMICS™ DNA Miniprep Kit (Zymo research) from a single gut (Tx1) from Atlantic salmon yolk-sac fry amplified using 2 µL of 1:10 diluted extract as a template. PCR reactions were run with an annealing temperature of 55 °C and with 38 temperature cycles. KB, KC, PC, and NTC represent a negative DNA extraction control ("kit blanc"), an DNA extraction positive control, a PCR positive control and a PCR non-template control, respectively. The ladder used as a size marker was not diluted correctly, and the size of the bands are not marked on the gel to prevent confusion.

3.2 Microbial Community Analysis

The microbial communities of the Atlantic salmon fry guts and water samples were analyzed using Illumina sequencing of the V3-V4 region of the bacterial 16S rDNA to study the effects of host strain and microbial water quality on the colonization of the salmon fry gut. Two strains of Atlantic salmon, a wild strain and an aquaculture strain, were raised under germ-free conditions and exposed to either r- or K-selected water one week after hatching (wph) (see Figure 2-1). Four replicate flasks were reared in each experimental group. The experimental groups were wild salmon strain reared in r-selected water (Wi-r), wild salmon strain reared in K-selected water (Wi-K), aquaculture strain reared in r-selected water (Aq-r), and aquaculture strain reared in K-selected water (Aq-K).

The whole gut from four individuals was sampled from each rearing group at 15 days post exposure (dpe) to either r- or K-selected bacteria, and the microbial communities from a total 64 gut samples were characterized. Also, 16 rearing water samples from the last sampling day (15 dpe) and eight samples representing r- and K-selected water added to the fish flasks at the last four water exchanges (7, 9, 11, and 14 dpe) were characterized.

3.2.1 Bacterial Density of Added and Rearing Water

The bacterial density of the rearing water was analyzed by flow cytometry to investigate the effect the water treatment regime on the bacterial density in the rearing water. Flow cytometry analysis was conducted on samples from the added water and rearing water each day of water exchange.

The added r- and K-selected water contained approximately the same bacterial density at each day of water exchange except for 9 dpe (Figure 3-9A). This indicates that the bacterial load added to the fish flasks were similar each day of water exchange. However, from day 9, the bacterial density of the added water had large day-to-day fluctuations. These fluctuations were also observed in the bacterial density of the rearing water (Figure 3-9B). An increase in the bacterial density was observed between 9 and 14 dpe, while the bacterial density decreased between 14 to 15 dpe. This reflected the trends for the added water. Large variations were observed between rearing water in the same rearing group, but in general, the bacterial density was higher in the K-selected rearing water compared to the r-selected rearing water.

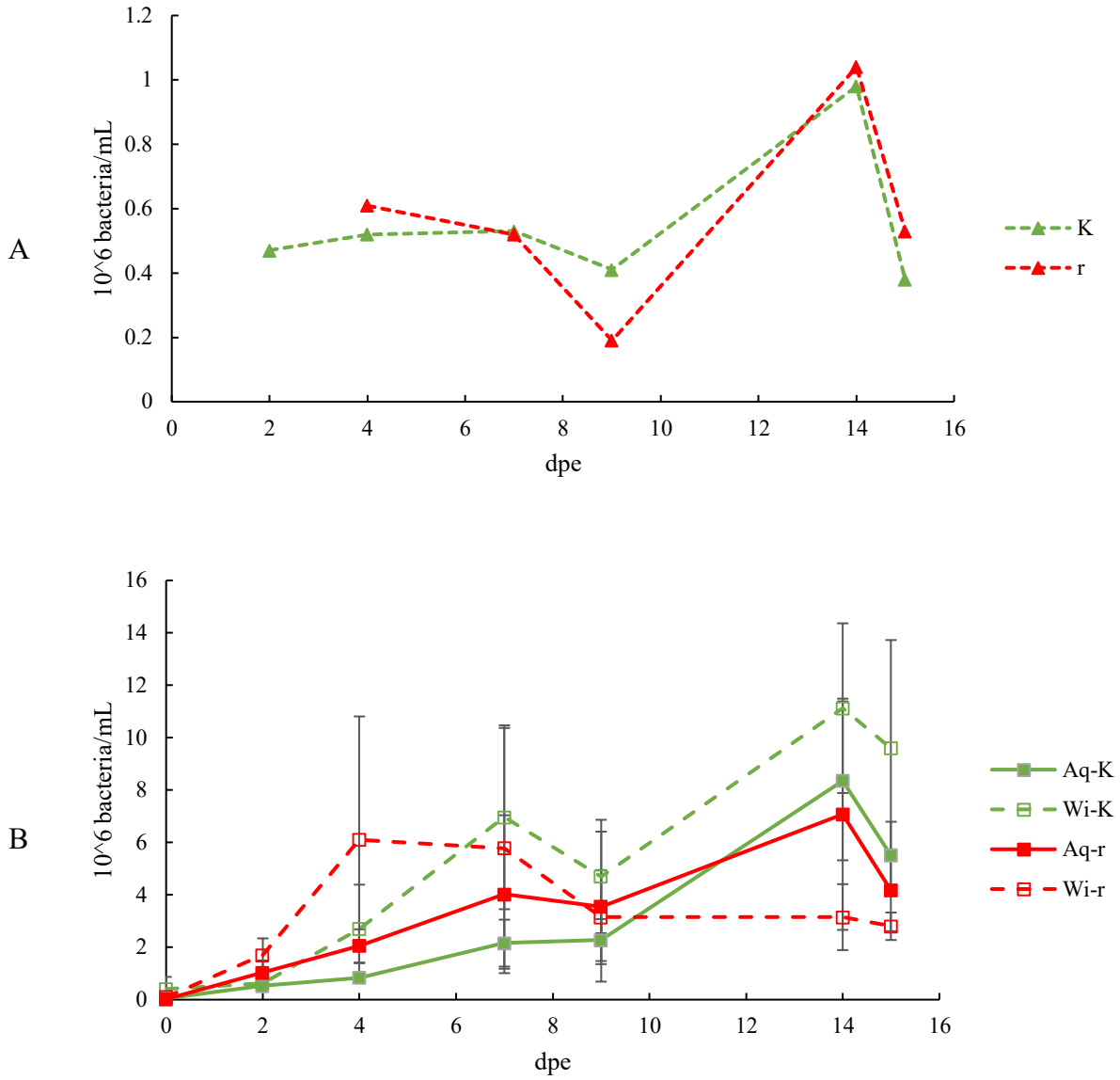

Figure 3-9: Bacterial density as determined by flow cytometry of the added water (A) and rearing water (B) with standard deviation (±SD). The rearing water samples represent water collected from four replicate fish flasks with either aquaculture (Aq) or wild (Wi) Atlantic salmon reared in r-selected (r) and K-selected (K) water. Only one sample were analyzed each day for the added r- and K-selected water. The time scale on the x-axis for the added water is adjusted so the added water correspond to the water in the fish flask.

3.2.2 Alpha Diversity of the Microbiota Associated with Water and Atlantic Salmon Gut

In total 5 517 512 reads were obtained for the samples after quality filtering, chimera removal and removal of reads representing salmon DNA or contaminating DNA (see Section 2.3.2.6). The highest number of reads were obtained from the rearing water samples, followed by the

samples of the added water (Table 3-1). The fewest number of reads were obtained in the gut samples. Clustering the sequence reads into operational taxonomic units (OTUs) based on 97% similarity resulted in a total of 970 OTUs.

Table 3-1: Average number of reads with standard deviations (±SD) for samples from Atlantic salmon fry gut, rearing water and added water after quality filtrating and chimera removal.

Sample:	Average number of reads (±SD)
Gut	53848 ± 17783
Rearing water	90356 ± 9769
Added water	78194 ± 14106

Diversity indices were calculated based on the OTU table normalized to 20 000 reads. Comparison of the observed number of OTUs and estimated richness (Chao1) show on average an 81±15% sequencing coverage. The added K-selected water had almost four times higher OTU richness compared to the added r-selected water (Figure 3-10), and the OTU richness was significantly different confirmed with a two-sample t-test ($p<0.05$). The OTU richness of the added K-selected water decreased significantly (Welch t-test, $p<0.05$) after addition to the rearing flasks, indicating a reduction in the number of species. No change was observed in the OTU richness of the rearing water in the flasks added r-selected water, confirmed with a two-sample t-test ($p<0.05$).

The OTU richness indicates a general trend where the richness was higher for the microbial communities for both gut and rearing water samples in the flasks added K-selected water compared to samples from the flasks added r-selected water (Figure 3-10). Welch t-test showed that these differences were significant, both for the gut and rearing water microbiota ($p<0.05$).

The OTU richness was higher for the rearing water microbiota compared to the fish gut microbiota (t-test, $p<0.05$). However, the Shannon diversity index (Figure 3-11A) showed more diverse communities for the gut samples. This indicated more evenly distributed communities for the gut samples, whereas the microbial communities associated with the rearing water was more dominated by fewer OTUs (Figure 3-11B).

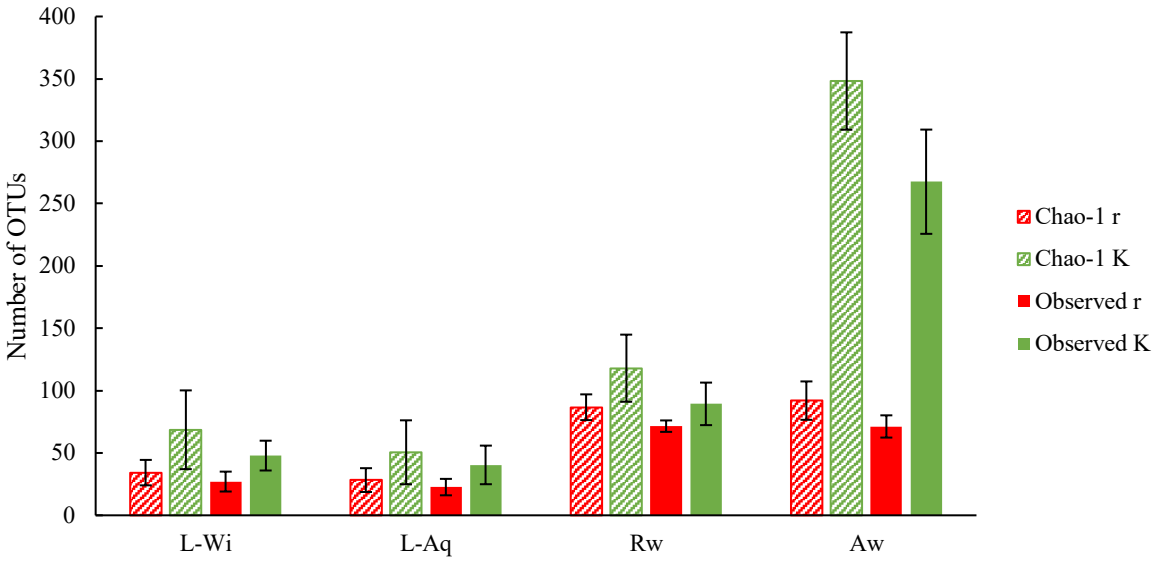

Figure 3-10: Average OTU richness with standard deviations (±SD) for gut samples (L), rearing water (Rw), and added water (Aw) for aquaculture (Aq) and wild (Wi) strain of Atlantic salmon reared in r-selected (r) and K-selected (K) water. Samples represent single gut dissected from four individuals reared in four replicate fish rearing flasks. Gut and rearing water samples were collected 15 days post-exposure to r- or K-selected bacteria (dpe), and samples of the added water were collected 7, 9, 11, and 14 dpe.

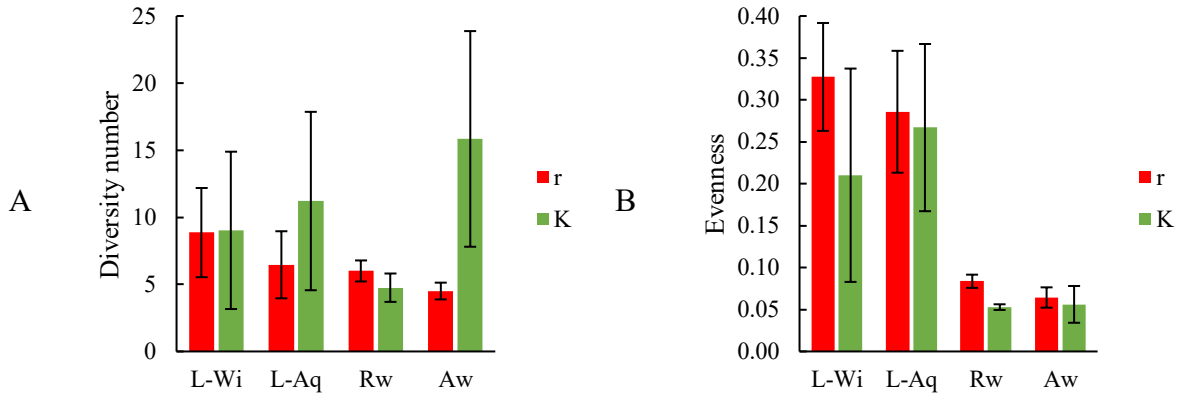

Figure 3-11: Average alpha diversity indices with standard deviations (±SD) for gut samples (L), rearing water (Rw), and added water (Aw) for aquaculture (Aq) and wild (Wi) strain of Atlantic salmon reared in r-selected (r) and K-selected (K) water. A; Shannon diversity index, and B; Evenness. Samples represent single gut dissected from four individuals reared in four replicate fish rearing flasks. Gut and rearing water samples were collected 15 days post-exposure to r- or K-selected bacteria (dpe), and samples of the added water were collected 7, 9, 11, and 14 dpe.

3.2.3 Effect of the Water Treatment on the Microbial Community of the Water

The community composition at the genus level showed that only seven families were dominant in the water microbiota. The community structure of the rearing water appears to be relatively similar to the r-selected water (Figure 3-12). *Pseudomonadaceae* (Gammaproteobacteria) and *Oxalobacteraceae* (Betaproteobacteria) were the most abundant families observed in all rearing water samples at 15 dpe and the added r-selected water samples from 1, 9, and 11 dpe. Observations of the OTU table showed that *Pseudomonadaceae* (OTU_1, identified at genus level as *Pseudomonas*) and *Oxalobacteraceae* (OTU_3, not identified at genus level) were mainly represented by one OTU each. The microbial community of the added K-selected water deviates from the other water samples, and large day-to-day variations were observed. *Pseudomonadaceae* is the dominating family on day 9 but was nearly not present in the K-selected water the other days. The family composition on 7 and 11 dpe were similar, with the highest abundance of *Moraxellaceae*.

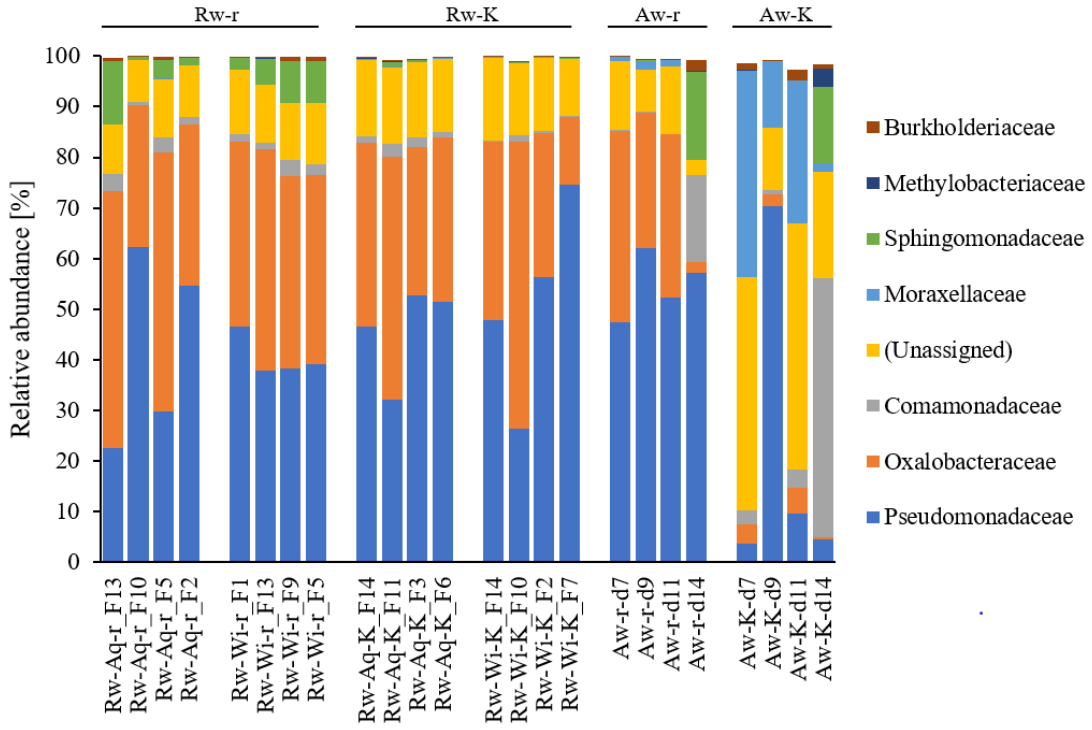

Figure 3-12: Composition of the microbial communities at the family level for added water (Aw) and rearing water (Rw) collected from fish flasks with two strains, aquaculture (Aq) and wild (Wi), reared in r-selected (r) and K-selected (K) water. Rearing water samples were all collected 15 days post-exposure to r- or K-selected bacteria (dpe), while the added water samples were collected 7, 9, 11, and 14 dpe. Families with an abundance of less than 1% in all samples are not included.

A selection regime was applied to select for either r- or K-strategic bacteria in the water used to cultivate the two strains of Atlantic salmon (see Section 2.1.3). To evaluate the effect of the selection regimes, a PCoA ordination based on Bray-Curtis similarity was performed (Figure 3-13). The added r-selected water from day 7, 9, and 11 dpe are clustered together, indicating a similar microbial composition. The added K-selected water appeared to be less similar indicating that there was more variation among the samples, who also was observed in the taxa summary at the genus level. However, the PCoA plot showed a low similarity between the r- and K-selected water, suggesting a successful selection of two distinct microbial water communities. A one-way PERMANOVA based on Bray-Curtis similarity confirms that the community profiles for the two groups were significantly different from each other ($p<0.05$).

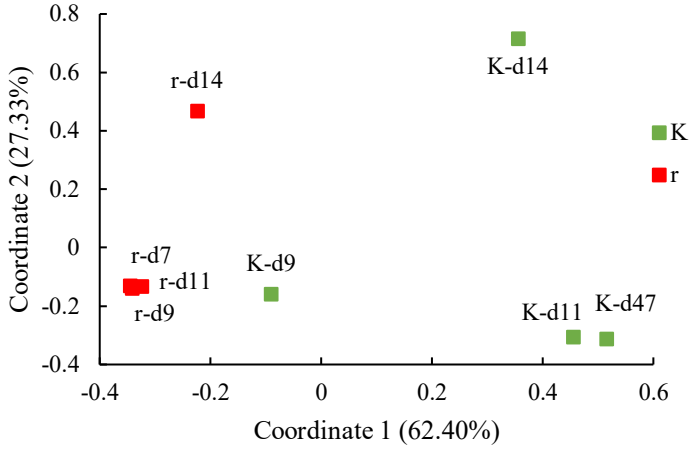

Figure 3-13: A PCoA plot based on Bray-Curtis similarities for community profiles of the added r-selected (r) and K-selected (K) water. The samples were collected from the added water from 7, 9, 11, and 14 days post-exposure to r- and K-selected water.

To further analyze the microbial community of the water, the average Bray-Curtis similarity was calculated within and between the groups (Figure 3-14). The results showed a greater similarity among the r-selected samples compared to the K-selected samples, confirming the result from the PCoA plot. A low average Bray-Curtis similarity (0.24) was observed between the two microbial water qualities, indicating a different community structure.

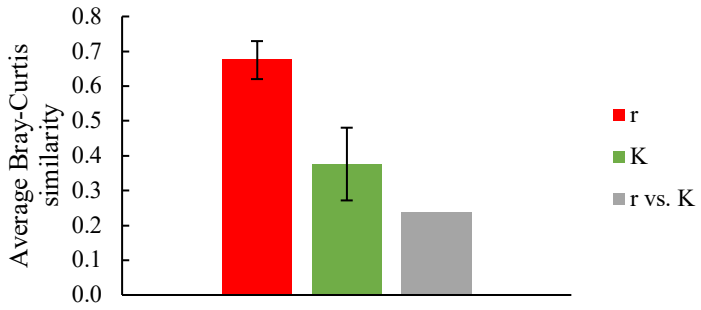

Figure 3-14: Average Bray-Curtis similarities with standard deviation (±SD) within and between groups calculated for community profiles for samples of the added r-selected (r) and K-selected (K) water. The samples were collected from the added water from 7, 9, 11, and 14 days post-exposure to r- and K-selected water.

To identify the OTUs contributing most to the difference between the added r- and K-selected water samples, a SIMPER analysis based on Bray-Curtis dissimilarity was conducted. A *Pseudomonas* OTU (OTU_1) was found to explain approximately 26% of the difference. This was the most abundant OTU in both the added r- and K-selected water (Appendix I). However, the relative abundance was over twice as high in the r-selected water samples (0.53) compared to the K-selected water (0.21). Further, OTU_3 and OTU_12, representing *Oxalobacteraceae* and *Moraxellaceae*, respectively, explained together around 25% of the dissimilarity. *Oxalobacteraceae* were more common in the added r-selected water, while *Moraxellaceae* were more common in the added K-selected water. The OTUs contributing most to the differences are summarized in Table 3-2.

Table 3-2: The five OTUs contributing most the difference between the microbial community of the added r- and K-selected water, identified by SIMPER analysis based on Bray-Curtis dissimilarity. The OTUs contribution and relative mean abundance are given with the taxonomy specified at the lowest taxonomic level obtained.

OTU ID	**Taxonomy**	**Cumulative [%]**	**Rel. abundance K**	**Rel. abundance r**
1	*Pseudomonas* (Gammaproteobacteria)	25.83	0.21	0.53
12	*Moraxellaceae* (Gammaproteobacteria)	38.82	0.21	0.01
3	*Oxalobacteraceae* (Betaproteobacteria)	51.29	0.01	0.20
26	*Comamonadaceae* (Betaproteobacteria)	56.54	0.07	0.02
202	Proteobacteria	61.00	0.00	0.07

To study how the added water affected the microbial composition of the rearing water, a PCoA ordination based on Bray-Curtis similarity was plotted to visualize the variation between the communities (Figure 3-15). The PCoA plot showed a clustering of the community profiles associated with all rearing water and the added r-selected water, thus indicating an effect of r-selection on the rearing water. It appears to be a change of the community structure of the rearing water added K-selected water. A one-way PERMANOVA test showed that the microbial communities of the K-selected water were significantly different from all the other water groups ($p<0.05$), suggesting that the water microbiota of the K-selected water was subjected to a different selection pressure in the fish rearing flasks.

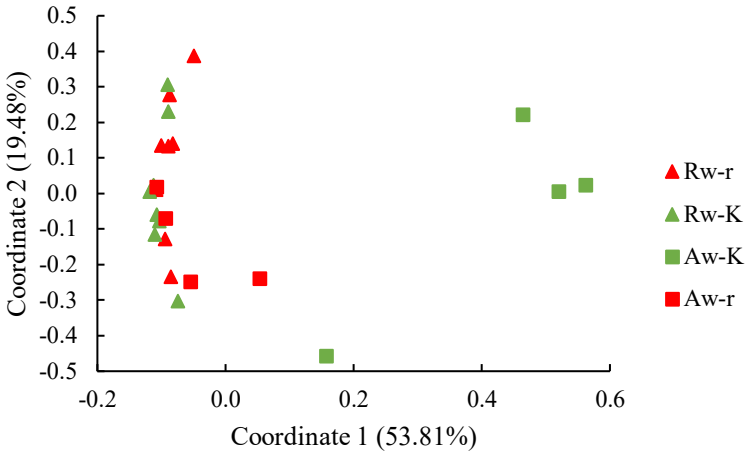

Figure 3-15: A PCoA plot based on Bray-Curtis similarities for community profiles of added water (Aw) and rearing water (Rw) from fish flasks with Atlantic salmon reared in r-selected (r) or K-selected (K) water. The rearing water samples represent the water microbiota from four replicate flask in each rearing group collected 15 days post-exposure (dpe) to r- and K-selected bacteria. The added water samples were collected 7, 9, 11, and 14 dpe.

Average Bray-Curtis similarities were calculated for comparison of the community profiles between the rearing water and the relevant added water (Figure 3-16). A high similarity was observed between the r-selected rearing water and its associated added water, indicating similar microbial compositions of the water. The low similarity between the community profiles of the K-selected rearing water and its associated added water is consistent with the observation of the PCoA plot, and indicate a change in the community structure of the K-selected water microbiota after addition to the fish rearing flasks.

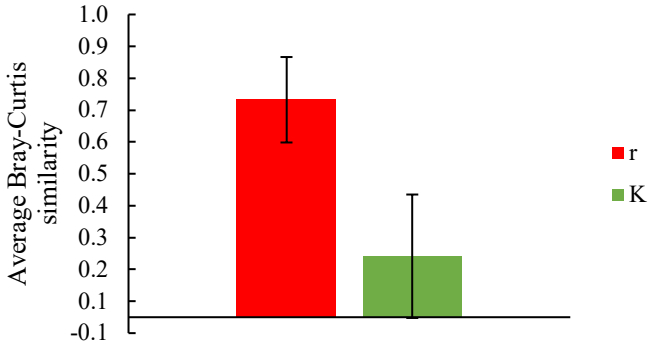

Figure 3-16: Average Bray-Curtis similarities with standard deviation (±SD) between rearing water and its relevant added water from fish flasks with Atlantic salmon reared in r-selected (r) or K-selected (K) water. The rearing water samples represent the water microbiota from four replicate flask in each rearing group collected 15 days post-exposure (dpe) to r- and K-selected bacteria. The added water samples were collected 7, 9, 11, and 14 dpe.

To compare the community profiles of the rearing water samples, a PCoA ordination based on Bray-Curtis similarity was performed (Figure 3-17). A clustering of the rearing water samples according to selection regime of the added water was observed. A one-way PERMANOVA test confirmed a significant difference between the microbial communities of the r- and K-selected rearing water ($p<0.05$), indicating different community structures.

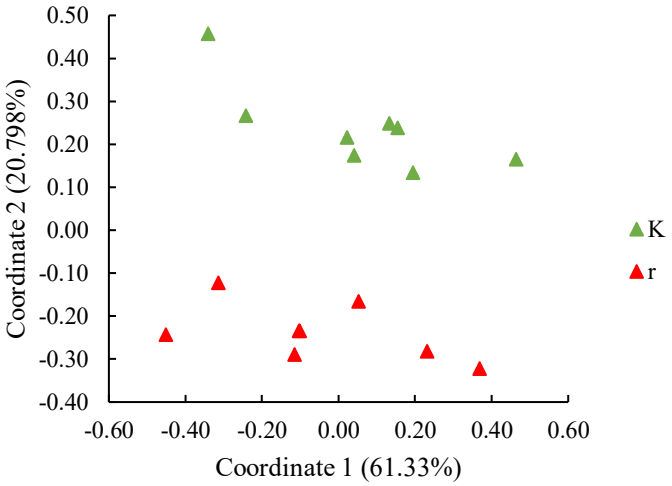

Figure 3-17: PCoA plot based on Bray-Curtis similarities for the community profiles of the rearing water from fish flasks with Atlantic salmon reared in r-selected (r) or K-selected (K) water. The rearing water samples represent the water microbiota from four replicate flask in each rearing group collected 15 days post-exposure (dpe) to r- and K-selected bacteria.

The average Bray-Curtis similarity was calculated for comparison of community profiles within and between the r- and K-selected rearing water (Figure 3-18). The results showed an overall high similarity within groups (>0.79), both for the r- and K-selected rearing water. A high

similarity was also observed between the two rearing water groups. However, the similarities of the community profiles were higher within the groups than between them, indicating an effect of the added water microbiota.

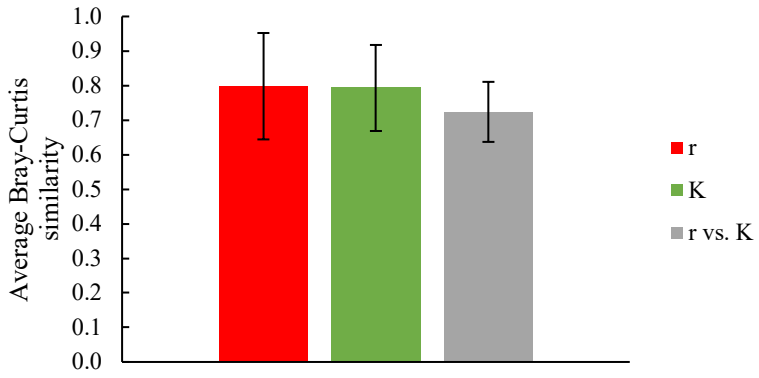

Figure 3-18: Average Bray-Curtis similarities with standard deviation (±SD) within and between rearing water from fish flasks with Atlantic salmon reared in r-selected (r) and K-selected (K) water. The rearing water samples represent the water microbiota from four replicate flask in each rearing group collected 15 days post-exposure (dpe) to r- and K-selected bacteria.

OTU_1 (*Pseudomonas*), OTU_3 (*Oxalobacteraceae*), and OTU_202 (Proteobacteria) were found to be the three most abundant OTUs of both the r- and K-selected rearing water (Table 3-3 and Table 3-4, respectively). Further were OTU_130 (*Oxalobacteraceae*) and OTU_886 (Proteobacteria) among the most abundant OTUs of the rearing water associated with the added K-selected water, but were only found in very low abundance in the r-selected rearing water. Reversely, OTU_16 (*Duganella*) and OTU_18 (*Sphingomonas*) were among the most abundant OTUs associated with the r-selected rearing water and were found in very low abundance in the K-selected rearing water. The most abundant OTUs of the microbial communities of both the r- and K-selected rearing water was similar. However, a difference in the microbial profiles was observed between OTUs of lower abundance, indicating a difference in the rearing water microbiota between flasks supplied with either r- or K-selected water.

Table 3-3: The five most abundant OTUs associated with the r-selected rearing water. The OTUs relative abundance is given with the taxonomy specified at the lowest taxonomic level obtained.

OTU ID	Taxonomy	Average relative abundance
1	*Pseudomonas* (Gammaproteobacteria)	0.40
3	*Oxalobacteraceae* (Betaproteobacteria)	0.30
202	Proteobacteria	0.07
16	*Duganella* (Betaproteobacteria)	0.06
18	*Sphingomonas* (Alphaproteobacteria)	0.05

Table 3-4: The five most abundant OTUs associated with the K-selected rearing water. The OTUs relative abundance is given with the taxonomy specified at the lowest taxonomic level obtained.

OTU ID	Taxonomy	Average relative abundance
1	Pseudomonas (Gammaproteobacteria)	0.48
3	Oxalobacteraceae (Betaproteobacteria)	0.29
202	Proteobacteria	0.11
130	Oxalobacteraceae (Betaproteobacteria)	0.03
886	Proteobacteria	0.02

3.2.4 Comparison of Rearing Water and Gut Microbiota

To compare the microbial communities associated with the rearing water and the Atlantic salmon gut, PCoA ordinations based on Bray-Curtis similarity and Dice were performed. The PCoA plot based on Bray-Curtis similarity (Figure 3-19A) might indicate that the fish reared in K-water have a more similar gut microbiota to its associated rearing water. However, no clear clustering was observed. A one-way PERMANOVA test based on Bray-Curtis similarities showed a significant difference in the microbial profiles of the fish reared in r-selected water and its relevant rearing water, and the fish reared in K-selected water and its relevant rearing water ($p<0.05$). In the PCoA plot based on Dice (Figure 3-19B), there was a stronger indication

of clustering of the gut microbiota of the fish reared in the same microbial water quality treatment. This indicates that the groups are more dissimilar when only considering the present/absent of species in the communities and that there are some rare OTUs that only occurs in one of the groups. A one-way PERMANOVA based on Dice showed that the microbiota of all fish groups is significantly different from its associated rearing water ($p<0.05$).

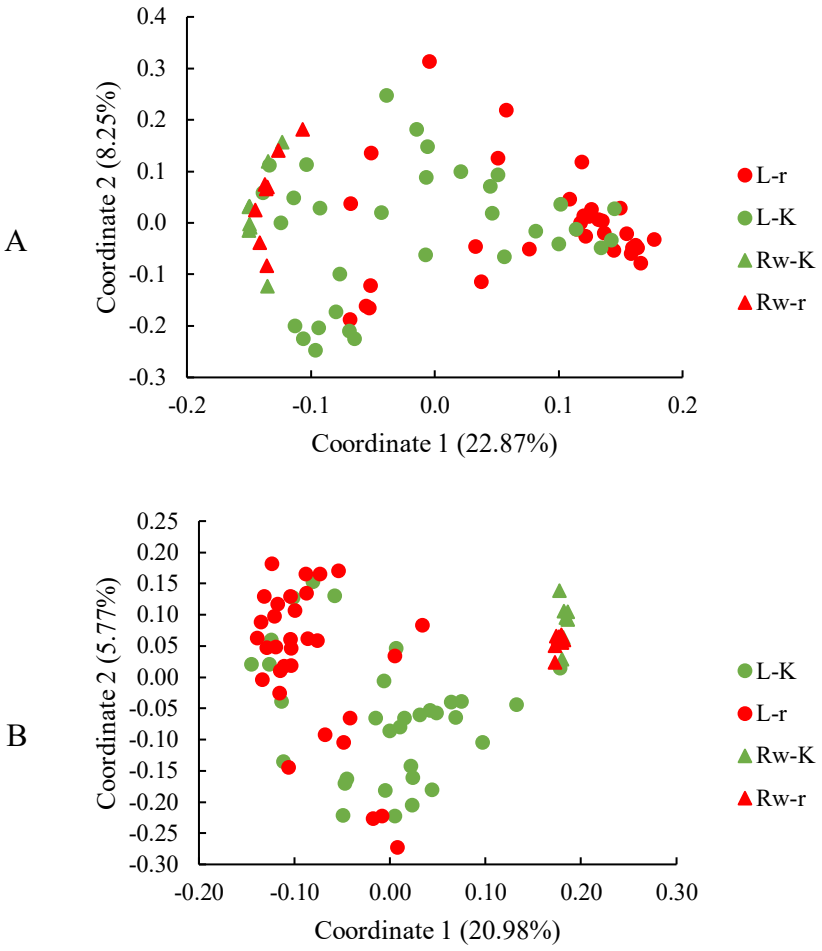

Figure 3-19: PCoA plots based on Bray-Curtis (A) and Dice (B) similarities for comparison of the microbiota associated with the rearing water (Rw) and gut microbiota (L) of Atlantic salmon fry reared in either r-selected (r) or K-selected (K) water. Samples represent single gut dissected from four individuals reared in four replicate fish rearing flasks. Gut and rearing water samples were collected 15 days post-exposure to r- or K-selected bacteria.

The Bray-Curtis similarities were calculated between the microbial communities for the gut and its relevant rearing water (Figure 3-20). In general, a low similarity was observed between the communities, indicating a gut microbiota different from the microbiota in the surrounding water. However, the fish reared in K-selected water had significantly greater similarity to its associated rearing water compared to the fish reared in r-selected water (Welch t-test, $p<0.05$).

This suggesting that the K-selected rearing water affected the gut microbiota more than the r-selected water.

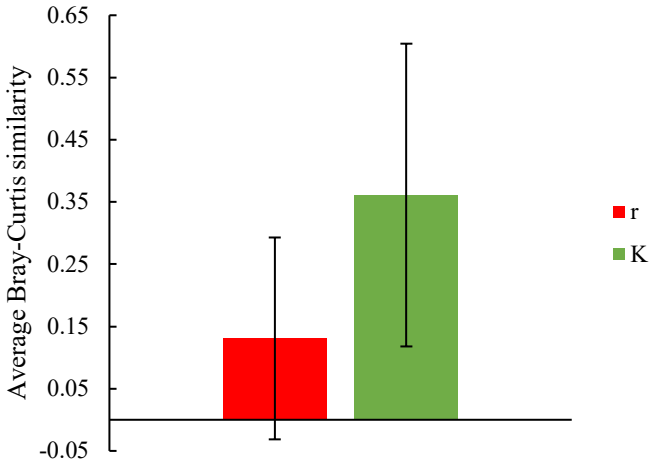

Figure 3-20: Average Bray-Curtis similarities with standard deviation (±SD) calculated between groups for comparison of the microbial community associated with rearing water (Rw) and Atlantic salmon reared in r-selected (r) and K-selected (K) water. Samples represent single gut dissected from four individuals reared in four replicate fish rearing flasks. Gut and rearing water samples were collected 15 days post-exposure to r- or K-selected bacteria.

By comparison of the OTU table, OTU_1 (*Pseudomonas*) and OTU_3 (*Oxalobacteraceae*) were the most abundant OTUs in all rearing water samples (see Table 3-3 and Table 3-4). These OTUs were found in more abundant in the fish reared in K-selected water than the fish reared in r-selected water (Appendix I). For the fish reared in r-selected water OTU_11 (*Staphylococcus*) and OTU_8 (*Acinetobacter*) were among the most abundant OTUs for the gut microbiota and were found unique for the fish gut.

3.2.5 Effect of Host Strain and Rearing Water Microbiota on the Colonization of Atlantic Salmon Yolk-Sac Fry Gut

Large inter-individual variation was observed for the composition of the gut microbiota, even between individuals belonging to the same groups (Figure 3-21). However, the gut microbiota of fish exposed to r- or K-selected water appears to different. *Pseudomonadaceae* (Gammaproteobacteria) and *Oxalobacteraceae* (Betaproteobacteria) were more abundant in the gut microbiota of fish reared in K-selected water than those reared in r-selected water. No family appears to be dominant in the gut microbiota of the fish reared in r-selected water. However, the most abundant orders were common to all fish groups and identified as

Actinobacteria, Gammaproteobacteria, Betaproteobacteria, and Bacilli. These orders were present in most of the individuals.

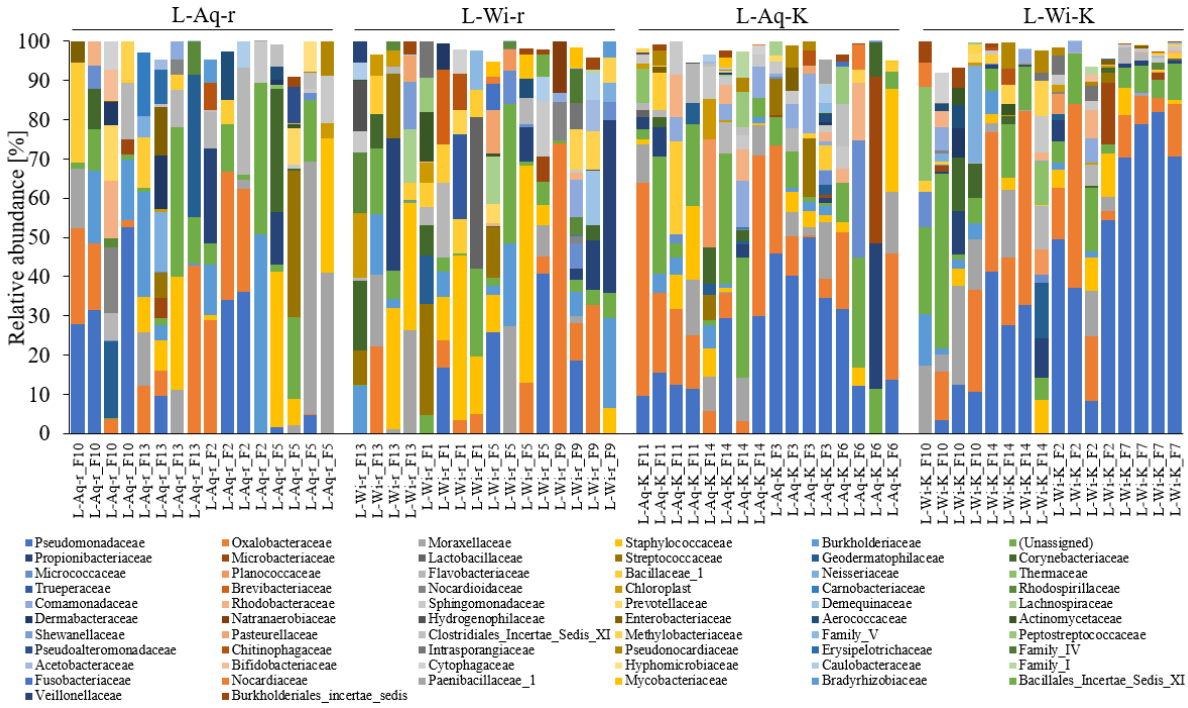

Figure 3-21: Composition of the microbial communities at the family level associated with larvae gut (L) from two strains of Atlantic salmon, aquaculture (Aq) and wild (Wi) strain, reared in either r-selected (r) or K-selected (K) water. One bar represents a single gut collected 15 days post-exposure to r- or K-selected bacteria. Four samples were collected from each replicate rearing flasks (F). Families with an abundance of less than 5 % in all samples are not included.

To compare potential differences in the microbiota associated with wild or aquaculture strain of Atlantic salmon receiving the same microbial water quality, PCoA plots based on Bray-Curtis similarities were performed (Figure 3-22A and B). No clustering of genetic groups was observed, indicating no differences in the community profiles between wild and aquaculture strain. A One-way PERMANOVA test based on Bray-Curtis similarities confirmed that it was not a significant difference between the gut microbiota for fish belonging to different strains ($p>0.05$).

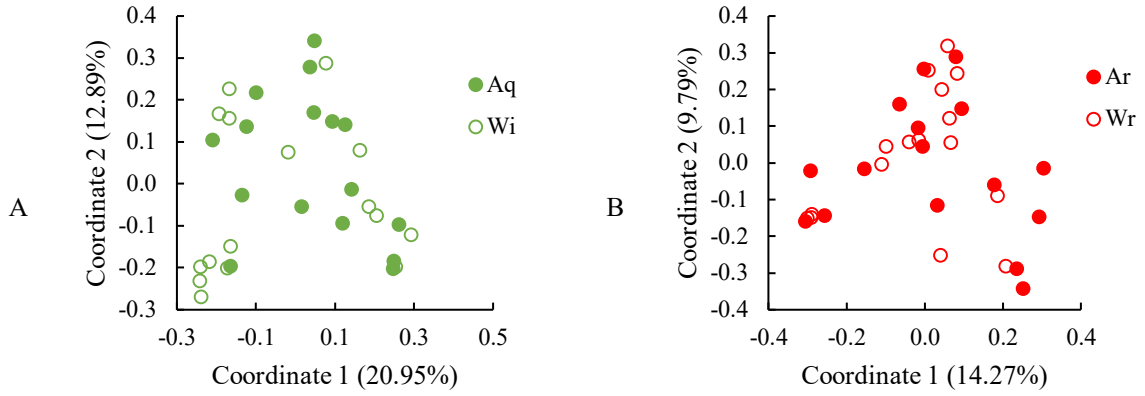

Figure 3-22: PCoA plot based on Bray Curtis similarities for two strains of Atlantic salmon, wild strain (Wi) and aquaculture strain (Aq), reared in r-selected (r) (plot B) and K-selected (K) water (plot A). Samples represent single gut dissected from four individuals reared in four replicate fish rearing flasks. Gut was collected 15 days post-exposure to r- or K-selected bacteria.

The average Bray-Curtis similarities were calculated within and between the rearing groups (Figure 3-23) and confirms a low overall similarity within the groups (≤0.26). This correlates with the observations of the PCoA plot based on Bray-Curtis similarity (Figure 3-22). However, a greater similarity was observed within individuals and between salmon strains reared in K-selected water compared to the fish reared in r-selected water, suggesting an effect of the water microbiota on the colonization of the fish gut. The average Bray-Curtis similarity was similar within and between the wild and aquaculture strain reared in the K-selected water, suggesting a similar variation within and between the two rearing groups (Figure 3-23). This was also observed for wild and aquaculture strain reared in r-selected water. The result may indicate that the water microbiota is a more significant factor than host strain on the microbial composition of the gut of Atlantic salmon fry. Further analyses were conducted to examine the potential effect of the rearing water microbiota on the colonization of the fish gut.

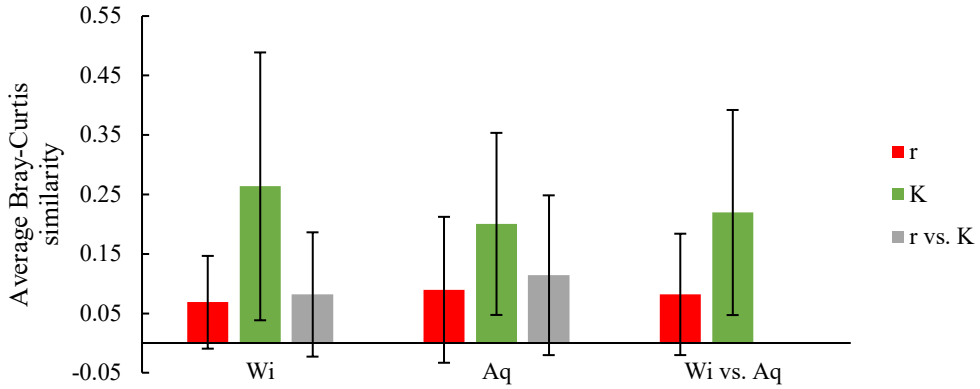

Figure 3-23: Average Bray-Curtis similarities with standard deviations (±SD) calculated within and between groups for comparison of the microbial community associated with the gut of two strains of Atlantic salmon, wild (Wi) and aquaculture (Aq) strain, reared in r-selected (r) and K-selected (K) water. Samples represent single gut dissected from four individuals reared in four replicate fish rearing flasks. Gut was collected 15 days post-exposure to r- or K-selected bacteria.

To evaluate the effect of the water microbiota on the colonization of the fish gut, a PCoA ordination based on Bray-Curtis similarity was performed (Figure 3-24). A large variation was observed between individuals associated with the same microbial water quality, correlating with the inter-individual differences in gut microbiota within the groups. However, there is a tendency of clustering of the community profiles for fish reared in the same microbial water quality. A one-way PERMANOVA confirms a significant difference in the gut microbiota between fish reared in different microbial water qualities ($p<0.05$).

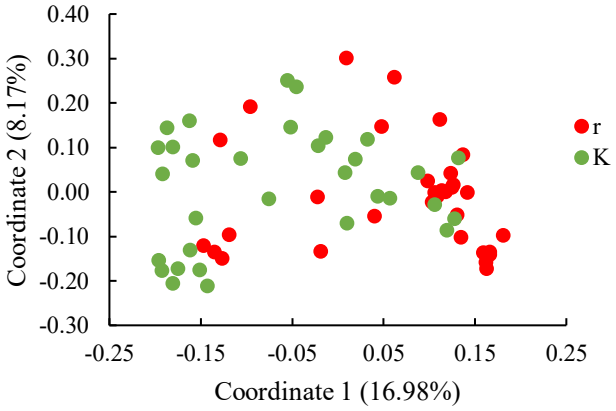

Figure 3-24: PCoA plot based on Bray Curtis similarities for community profiles of the gut microbiota of Atlantic salmon reared in r-selected (r) and K-selected (K) water. Samples represent single gut dissected from four individuals reared in four replicate fish rearing flasks. Gut was collected 15 days post-exposure to r- or K-selected bacteria.

Average Bray-Curtis similarities were calculated within and between the community profiles of the fish reared in either r- or K-selected water (Figure 3-25). The community profiles of fish reared in K-selected water are greater compared to the fish reared in r-selected water (Welch t-test, $p<0.05$). This indicates a higher variation in the gut microbiota between individuals reared in r-selected water compared to fish reared in K-selected water.

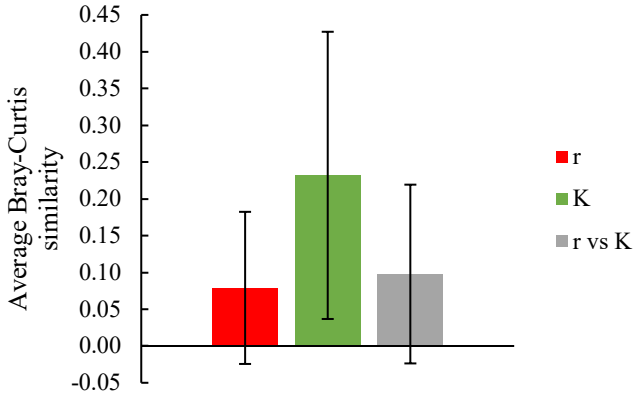

Figure 3-25: Average Bray-Curtis similarities with standard deviation (±SD) calculated within and between groups for comparison of the microbial community associated with Atlantic salmon reared in r-selected (r) or K-selected (K) water. Samples represent single gut dissected from four individuals reared in four replicate fish rearing flasks. Gut samples were collected 15 days post-exposure to r- or K-selected bacteria.

A SIMPER analysis based on Bray-Curtis dissimilarities was conducted for further comparison of the gut microbiota for all fish reared in K-selected water and for all fish reared in r-selected water (Table 3-5). OTU_1 (*Pseudomonas*) and OTU_3 (*Oxalobacteraceae*) contributed most to the difference between the groups. These OTUs were abundant in the gut of fish reared in K-selected water, but less abundant in the gut of fish reared in r-selected water. OTU_3 was absent from many of the gut samples (14 of 32) associated with r-selected water, but present in nearly all samples originating from Atlantic salmon reared in K-water (31 of 32). OTU_6 and OTU_11 have a greater average abundance in the gut of fish reared in r-selected water but was less frequent (15 of 32 and 17 of 32 samples, respectively) compared to the gut microbiota associated with K-selected water (19 of 32 and 22 of 32 samples, respectively).

Table 3-5: The five OTUs contributing most the difference between the microbial community of the gut microbiota of Atlantic salmon fry reared in r- or K-selected water, identified by SIMPER analysis based on Bray-Curtis dissimilarity. The OTUs contribution and relative mean abundance are given with the taxonomy specified at the lowest taxonomic level obtained.

OTU ID	Taxonomy	Cumulative [%]	Rel. abundance r	Rel. abundance K
1	*Pseudomonas* (Gammaproteobacteria)	14.39	0.08	0.27
3	*Oxalobacteraceae* (Betaproteobacteria)	21.78	0.04	0.13
11	*Staphylococcus* (Bacilli)	27	0.08	0.04
6	*Ralstonia* (Betaproteobacteria)	30.87	0.07	0.02
8	*Acinetobacter* (Gammaproteobacteria)	34.51	0.05	0.03

3.3 Fish Performance

The performance of the fish exposed to the different water qualities was measured by observing the survival after exposure to bacteria and by measuring the length of the fish. After exposure to bacteria, no mortality was observed in any of the groups. The length of the Atlantic salmon fry was measured at two time points, 8 and 15 dpe to r- and K-selected water (Figure 3-26A and B). There was no significant difference between the groups 8 dpe, neither between the fish strains nor between the fish reared in different microbial water qualities. However, at 15 dpe, the wild salmon strain reared in r-selected water were significantly longer than the germ-free wild strain and the wild strain reared in K-selected water (t-test, $p<0.05$).

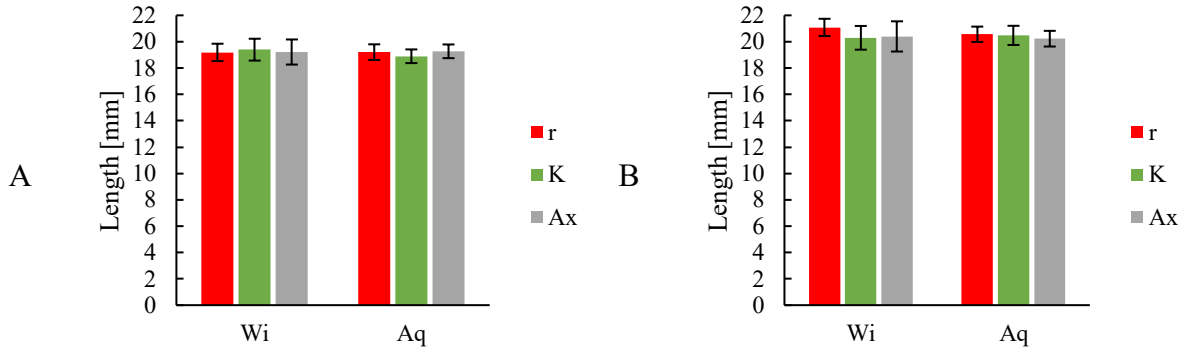

Figure 3-26: Average standard length with standard deviations (±SD) for two strains of Atlantic salmon yolk-sac fry (a wild strain (Wi) and an aquaculture strain (Aq)) measured 8 (A) and 15 (B) days post-exposure to r-selected (r) and K-selected (K) water and a germ-free control group (Ax). Each bar represent in total 24 samples, where six individuals were measured form four replicate flask in each rearing group.

4 Discussion

4.1 Evaluation of Model System and Experimental Design

Germ-free models have been fundamental for the present knowledge about host-microbe interactions (Wang et al., 2018, Kanther and Rawls, 2010). Previous studies conducted on fish larvae have often based their conclusion of the influence of a single factor on the gut microbiota on observations that coincide with changes of environmental factors (e.g., change in feeding regime). To assess the relative influence of several factors, a well-design experiment is needed. In the present study, a two-factor design was used to study the effect of host-genetic and microbial water quality on the colonization of Atlantic salmon yolk-sac fry. A two-factor model system made it possible to compare the effect of host-genetics a microbial water quality. The salmon fry is a suitable candidate as a germ-free model due to its relatively long yolk-sac stage (Le Francois et al., 2010). This means that the fish does not require external feeding and the complications related to generating germ-free feed. By the use of this model system, it was possible to control the water microbiota and create two different microbial water communities. Flow cytometry analysis of the rearing water was used to evaluate whether the Atlantic salmon fry was germ-free before exposure to bacteria. The number of counts in the water before exposure to bacteria was low and assumed not to be bacteria. Thus it was concluded that the fish were germ-free before exposure to bacteria.

4.2 Evaluation of Methods

4.2.1 Amplification of 16S rDNA from Atlantic Salmon Gut

PCR amplification of regions of the 16S rRNA gene for Atlantic salmon samples has in previous studies been challenging in the research group "Analysis an Control of Microbial Systems". It is assumed that the main reasons for the problems are due to the presence of unknown inhibitors in the DNA extracts, low concentration of bacterial DNA compared to host DNA in the samples, DNA contaminations in the DNA extraction kits and PCR reagents, and co-amplification of salmon rRNA genes with the use of universal primers. In total, four different DNA extraction kits were tested in combination with various PCR protocols in order to achieve a sufficient product yield from single gut samples dissected from Atlantic salmon yolk-sac fry.

The presence of bacterial contamination in the DNA extraction kit is especially a challenge when working with samples which contain a low concentration of bacterial DNA. This could

lead to misleading results. The presence of bacterial DNA in DNA extraction kits are reported in several studies (Salter et al., 2014, Glassing et al., 2016). Thus it is essential to choose the DNA extraction kit carefully and include negative controls for the DNA extraction kit. During the optimization process, two DNA extraction kits which claim to contain low or little contaminating DNA were tested (Ultra-Deep Microbiome Prep (Molzym), and ZymoBIOMICS™ DNA Miniprep Kit (Zymo research). DNA extracted from the Ultra-deep kit showed low levels of contaminating DNA in the negative extraction control ("kit blanc" (KB)). However, only a low yield for the 16S rDNA amplicon for the gut samples was obtained. PCR amplification products for DNA template extracted with Zymo resulted in a greater product yield, and also low levels of product in the KB relative to the gut samples. The kit from Zymo has a purifying step at the end, which will reduce the concentration of inhibitors. The DNA extracts from this kit were also diluted. This will also dilute the concentration of potential inhibitors and also potentially contaminating DNA from the DNA extraction kit. might explain the increased product yield obtained from the samples. The PCR products with DNA extracted with from the two other DNA extraction kits (DNeasy Powersoil DNA Isolation Kit (Qiagen) and PureLink™ Microbiome DNA Purification Kit (Thermo Scientific)) were weak, in addition to high levels of contaminating DNA obtained for the kit (KB). This emphasizes the importance to choose the right DNA extraction kit for difficult samples.

Contaminating DNA can be a challenge in PCR when using universal bacterial primers, and can cause a misleading result of the microbial community due to amplification of bacterial DNA originally not present in the samples (Salter et al., 2014). Precautionary measures were conducted to reduce the bacterial contamination to the minimum, and only a weak band were occasionally observed in the non-template control for PCR. Thus the main source of contaminating DNA was identified to originate from the DNA extraction kit. However, both negative controls from the DNA extraction kit and PCR were sequenced with the samples to identify OTUs potentially representing contaminating DNA.

During the PCR optimization process, primers targeting the V3, V4, and V3-V4 regions of the 16S rDNA were tested. PCR amplification of the V4 region of the 16S rDNA yielded low amount of bacterial product compared to co-amplified host rDNA, which probably originated from salmon 18S rDNA and mt 12S rDNA (personal communication, Ingrid Bakke). Thus, the primers used to amplify the V4 region of the 16S rDNA were found to not be optimal for the Atlantic salmon gut samples. PCR amplification of the V3 region of the 16S rDNA did not result in the expected bacterial product. Sanger sequencing of the PCR product showed that the

sequence represented salmon 18S rRNA. PCR amplification of the V3-V4 region of the 16S rDNA, however, resulted in a product of the expected size and a reasonable yield. Some co-amplification of host DNA also occurred with these primers, however, the band intensity was much greater for the bacterial product. Several OTUs representing 18S and mt 12S rDNA were found in the Illumina sequencing data set, both for the rearing water and gut samples. Still, these OTUs did not dominate the community profiles for the samples and were removed.

4.2.2 Diversity Analysis Based on Amplification of the 16S rRNA gene

To characterize the microbial communities for water and salmon gut samples, Illumina sequencing of the 16S rDNA amplicons were conducted. There are several drawbacks related to PCR-based methods in general, and to deep sequencing of 16S rDNA amplicons for microbial diversity analysis. This may cause an under- or over-estimation of species in the bacterial communities. Insufficient coverage of primers, primer-template mismatches, unequal amplification, and a differential efficiency of annealing are sources for bias during PCR amplification (Ibarbalz et al., 2014). The bacteria also have multiple copies of the rRNA operons, varying from a single copy up to 15 copies of the operon in the genome. A high copy number is correlated with a high maximum growth rate of the bacteria (Stoddard et al., 2015). The growth rate of r-strategists are higher compared to K-strategists, and it is likely that these have a higher copy number of the rRNA operon. Thus, this variation in copies of the rRNA operon might introduce bias in the 16S rDNA amplicon library, with an over-estimation of the r-strategic bacteria. However, next-generation sequencing platforms provide an enormous number of reads at high speed and enables in-depth and accurate sequencing data. This makes it possible to detect members of the microbial community who are rare or in low abundance (Ghanbari et al., 2015). Thus, Illumina sequencing of the 16S rRNA gene was a preferable method to characterize the microbial communities of Atlantic salmon gut and water samples.

4.2.3 Quality of the DNA Sequencing Data set

To detect bacterial contamination originating from the DNA extraction kit and PCR reagents, samples were sequenced together with the gut and water samples. *Curvibacter* (OTU_5), *Propionibacterium* (OTU_2), *Corynebacterium* (OTU_9), *Aeromonas* (OTU_14), *Micrococcus* (OTU_10), and *Pelomonas* (OTU_4) were abundant in the KB control and NTC, and these OTUS were excluded from the OTU table. *Corynebacterium*, *Micrococcus*, *Pelomonas*, and *Aeromonas* were not observed in any of the water or gut samples. However,

Curvibacter and *Propionibacterium* were observed in some samples, mostly from those originating from the gut. All OTUs mentioned above have previously been detected as contaminants in DNA extraction kits and PCR reagents (Salter et al., 2014, Glassing et al., 2016), and were assumed not to represent bacterial DNA originally in the samples.

After quality filtering, chimera removal and removal of reads representing salmon DNA or contaminating DNA, the OTU table were normalized to 20 000 reads. Only one gut sample was less than 20 000 reads (16 690) and was treated with caution in further analysis. The sample did not deviate from other gut samples in PCoA analysis and was not excluded in statistical analysis.

4.3 Fish Performance

Several opportunistic pathogens are known to be r-strategists (Vadstein et al., 2018a). However, no mortality was observed after exposure to bacteria for neither the fish receiving r- or K-selected water. This may be because the Atlantic salmon is robust and well developed by hatching. Besides no specific pathogens were observed in the rearing water or fish microbiota.

The wild Atlantic salmon strain reared in r-selected water was significantly longer compared to the same strain reared in K-selected water and the germ-free salmon 15 dpe to bacteria. However, only small variations were observed between the length of the salmon fry both at 8 and 15 dpe (Figure 3-26). No obvious explanation was found for the observed difference.

4.1 Microbial Community Analysis

4.1.1 Effect of Water Treatment on the Microbial Composition of the Water

A water selection regime was applied to select for r- and K-strategist to achieve a rearing environment of two different water microbiotas. A K-selected community is characterized by a high species richness and diversity (Vadstein et al., 2018b). The species richness and diversity were both over three times higher in the added K-selected water compared to the added r-selected water. This indicates that the K-selection was successful and that K-strategists dominated the added water. A low Average Bray-Curtis similarity was observed between the r- and K-selected water. A low similarity was also observed for the community composition of the added water at the genus level (Figure 3-12) and indicated a selection of two distinct microbial water qualities.

Vadstein et al. (2018b) found that r-selected seawater communities were dominated mainly by Gammaproteobacteria. This order includes many genera with known pathogens. The K-selected communities were more diverse and were composed of Verrucomicrobia, Alpha-, Beta-, and Deltaproteobacteria. This is only partly consistent with the findings of the present study. Gamma- and Betaproteobacteria were the dominating orders of the r-selected water. The microbiota of the K-selected water was more diverse and composed of a varying abundance of Actinobacteria, Alpha-, Beta-, and Gammaproteobacteria. However, different microbiota may thrive in seawater compared to freshwater. It was surprising to observe a high abundance of Gammaproteobacteria since many of the species associated with this order are known pathogens and represent typical r-strategists.

The OTU richness and diversity decreased significantly in the rearing water after the K-selected water had been added to the fish flasks. This reduction in diversity indicates a change in the community structure of the K-selected water after addition to the rearing flasks, and this was also observed at the genus level (Figure 3-12). The composition of the microbiota of the added K-selected water and the corresponding rearing water were significantly different (PERMANOVA, $p<0.05$), confirming the assumption of a change in the community structure. On the other hand, the communities of the r-selected rearing water were very similar to the added r-selected water and indicated a similar selection pressure in the added water and the fish rearing flasks.

A high similarity was observed for the rearing microbiota for the fish flasks receiving both r- and K-selected water. This might suggest a similar selection regime inside the fish rearing flasks, independent of the type of water added. The salmon larvae and bacteria will release dissolved organic matter (DOM) which is a resource and basis for the growth of heterotrophic bacteria. The maximum sustainable bacterial biomass of the system (i.e., the carrying capacity) determines the growth of bacteria within the fish rearing flasks (Vadstein et al., 2018b). The concentration of DOM for the rearing water was not measured. However, the flow cytometry analysis showed a low bacterial load added to the system compared to what observed within the rearing water. Approximately 60 % of the water was exchanged for every day of water exchange, which will promote the growth of fast-growing, opportunistic bacteria until the carrying capacity inside the fish flasks is reached. Thus it is likely that the conditions in the fish rearing flasks give rise to a selection of r-strategists.

Regardless of a great observed similarity for the rearing water microbiota, the microbial communities of the rearing water receiving either r- or K-selected water were found significantly different (PERMANOVA, $p<0.05$). Thus, the salmon fry was successfully reared in two different microbial water qualities.

4.1.2 Effect of Host Genetic and Water Quality on the Colonization of Atlantic Salmon Yolk-Sac Fry Gut

Comparison of the OTU richness and Shannon's diversity associated with the rearing water and salmon gut showed a higher OTU richness in the rearing water, but Shannon's diversities was greater for the salmon gut. This was surprising since the water microbiota has previously been found to have both a higher OTU richness and diversity compared to fish microbiota (Uren Webster et al., 2018). The alpha-diversity indices for the gut samples had a relatively large standard deviation (Figure 3-21), and the average diversity may reflect the samples with the highest diversity. However, the water microbiota was dominated by a few OTUs, who can explain a lower Shannon diversity compared to the gut samples.

The community profiles of the gut samples and rearing water samples were significantly different (PERMANOVA; $p<0.05$), and indicates a selection process inside the host. However, the microbial community associated with the salmon gut showed large variations between individuals (Figure 3-21), and also between the fish within the same rearing group. The high variation observed between individuals may partly be explained by scholastic processes during the early colonization, where the bacteria that happened to be "in the right place at the right time" can establish in the intestine and thereby outcompete other bacteria. Verschuere et al. (1997) found that both deterministic and stochastic factors influenced the microbial community colonizing *Artemia*. The high variation observed between individuals indicates that the environment of the gut microbiota can harbor many different species.

The most abundant phyla in the salmon gut samples were Proteobacteria, Actinobacteria, Firmicutes, and Bacteroidetes. Lokesh et al. (2019) found life stage specific microbial compositions of the Atlantic salmon gut. Acinetobacter, Firmicutes, Tenericutes, Spirochaetes, and Deinococcus-Thermus was the most abundant phyla in Atlantic salmon hatchlings (whole larvae), whereas Proteobacteria was the most dominant phyla in the gut of Atlantic salmon at seven weeks post-hatching. This phylum is also shown to be a part of the microbial community associated with older fish at the freshwater stage. Dehler et al. (2017) found that Proteobacteria,

Bacteroidetes, and Firmicutes were members of the core microbiota of Atlantic salmon parr reared in two different water environments. The finding in this study correlates with previous observations on Atlantic salmon at different life stages, and suggest that these phyla are a part of the commensal microbiota of Atlantic salmon in the freshwater stage.

Comparison of the gut microbiota associated with the wild and aquaculture strain of Atlantic salmon indicated no strain-specific differences (PERMANOVA; $p<0.05$). Several studies have shown a different microbiota associated with the host genotype of fish reared under the same conditions. Li et al. (2012) investigated the gut microbiota of four freshwater fish larvae species (silver carp, grass carp, bighead carp, and blunt snout bream). They found a distinct microbiota associated to each of the species when reared in the same environment. Navarrete et al. (2012) analyzed the 16S rRNA and rDNA community profiles of rainbow trout originating from four unrelated broodstock families reared in the same tank under the same environmental conditions. The results revealed a significant difference in the gut microbiota associated with the families (Navarrete et al., 2012). However, in the present study, the fish were only three weeks old when sampled, and the gut was not fully developed at this stage. The microbial communities of the Atlantic salmon fry are shown to change with age and at first-feeding (Lokesh et al., 2019). One can not exclude that strain-specific differences would emerge at a later point in the development. A strain-specific variation could also have been masked by the high inter-individual variation observed in the same rearing group. On the other hand, recent studies on the gut microbiota of healthy humans indicate that the microbial composition of the gut is determined by environmental factors, and not by the host genetics (Jackson et al., 2018, Rothschild et al., 2018). It is possible that the host factors are less important in the colonization of the fish gut than previously assumed, but this needs to be further studied in well-designed experiments.

Comparison of the gut microbiota of the fish reared in r- and K-selected water showed a significant difference between the two groups (PERMANOVA; $p<0.05$). The only bacterial source for colonization of the fish intestine was the rearing water microbiota. Regardless of a high similarity observed in the microbial composition of the rearing water in the flasks supplied r- and K-selected water, they were significantly different (PERMANOVA; $p<0.05$). Thus, the colonization of the fish intestine is affected by microbial water quality. Dehler et al. (2017) found a dissimilar gut microbiota of Atlantic salmon parr with reared in a RAS and an open loch facility. The difference observed in the gut microtia was most likely due to different rearing environment. Another study by Giatsis et al. (2015), found a correlation between changes in the

water and gut microbiota of Nile tilapia larvae over time. However, in these studies, the water microbiota was not the only water condition affecting the gut microbiota. Diet, salinity, and stage of development, among others, have also shown to affect the microbial composition of the fish intestine (Romero et al., 2014, Nayak, 2010). It can not be exclude that other factors may have shaped the observed difference in gut microbiota for the fish. In the present study, the fish were not fed, and were of the same age, and the rearing conditions were identical between the groups except for the water microbiota. The differences observed between the gut microbiota of the fish reared in r- and K-selected water were therefore highly probably caused by different microbial rearing environments.

Surprisingly, the fish reared in the K-selected water showed a greater similarity to the microbiota associated with the rearing water when compared to the fish reared in r-selected water. Giatsis et al. (2015) found a correlation between changes in the water and gut microbiota over time for Nile tilapia larvae reared in two different microbial water environments. In contrast to what found in the present study, Giatsis et al. (2015) found no evidence that the water in one of the systems affected the gut microbiota more than the other. *Pseudomonas* and *Oxalobacteraceae* were abundant in the microbiota of both the r- and K-selected rearing water, but their relative abundance was greater for the fish reared in flasks with K-selected water compared to those reared with r-selected water (Table 3-5). Both *Pseudomonas* and *Oxalobacteraceae* have previously been found in the gut microbiota of Atlantic salmon (Gajardo et al., 2016, Lokesh et al., 2019). The OTUs associated with the fish reared in r-selected water varied among individuals, and no OTU was found to dominate the microbial communities of all fish larvae. By comparing the water and gut microbiota between the fish reared in r- and K-selected water, no obvious explanation is found for the observed differences between the gut microbiota of the fish reared in r- and K-selected water. However, the microbial communities of the rearing water and salmon gut were only characterized at one time point. The microbiota of the added water varied from day-to-day (Figure 3-12) and it is reasonable to think that this could also be the case for the microbial community of the rearing water. Thus, further studies are needed to understand the complex interactions between the water microbiota and fish microbiota.

4.1.3 Future Work and Perspectives

The present study showed that the colonization of the Atlantic salmon yolk-sac fry is a complex process, which needs to be further studied. Rearing water and gut samples for microbial

community analysis were only characterized at one time point. To investigate the effect of microbial water quality and host genetics over time (e.g., from the moment of mouth opening to the end of the yolk-sac stage) could provide more information on the colonization dynamics.

Based on the result obtained from the microbial water profiles, there was most likely an r-selection pressure in the fish flasks independent of the microbial quality of the water added to the flasks. In future studies, a stricter microbial water selection regime should be conducted. This could be achieved by adding a higher load of bacteria to the fish flasks (closer to the systems carrying capacity) or adjust the water exchanging regime to promote r- or K-selection pressure inside the fish flasks.

The germ-free model system is a fundamental method to study host-microbe interactions and should be applied in further studies on Atlantic salmon yolk-sac fry. The findings in the present study could serve as a base for further studies on the colonization of the salmon fry. More knowledge on the colonization and development of the gut microbiota in the salmon fry may potentially provide more positive host-microbe interactions, and thereby contribute to a more sustainable aquaculture.

5 Conclusion

Illumina sequencing of the 16S rDNA amplicons was used to study the effect of microbial water quality and host genetics on the colonization of Atlantic salmon yolk-sac fry. A successful PCR amplification protocol for the V3-V4 region of the 16S rDNA of salmon fry samples was established.

The microbial water selection regime was successful, and two distinctly different microbial water qualities were added to the fish rearing flasks. However, the microbial communities for the fish rearing water were more similar, indicating an r-selection pressure inside the fish flasks. Despite this, the microbial communities in the r- and K-selected rearing flasks were significantly different. Thus, the Atlantic salmon fry was exposed to two different microbial water communities.

The water and gut microbiota were significantly different from each other. The most abundant phyla in all fish, independent of rearing groups, were Proteobacteria, Actinobacteria, Firmicutes, and Bacteroidetes. These findings coincide with previous studies on Atlantic salmon.

The major finding of the present study was that the water microbiota was a more significant factor than host genetics on the composition of the gut microbiota of Atlantic salmon yolk-sac fry. The gut microbiota of the Atlantic salmon yolk-sac fry did not differ between the wild and aquaculture strain. However, the colonization was affected by the rearing water microbiota. The microbial communities of fish reared in K-selected water were more similar to the water microbiota compared to the fish reared in r-selected water. Thus, the main hypothesis for this study, namely that the salmon fry microbiota would differ between the two salmon stains, but exposure to opportunistic bacteria in the water could obscure theses strain-specific differences, was not supported by the findings in this study.

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Appendix A - Salmon Gnotobiotic Media

Salmon Gnotobiotic media (SGM)

Sol Gómez de la Torre Canny, modified from on US EPA/600/4-90/027F artificial water recipe

Salt Stocks

$MgSO_4 \bullet 7H_2O$ 100X
Dissolve 12.3 g in 1 l. Autoclave.
KCl 100X
Dissolve 0.4 g in 1 l. Autoclave.
NaHCO3 100X
Dissolve 9.6 g in 1 l. Autoclave.
$CaSO_4 \bullet 2H_2O$ 5X
Dissolve 0.3 g in 1 L. Filter sterilize.

SGM prep

$MgSO_4 \bullet 7H_2O$ 100X 10 ml
KCl 100X 10 ml
$NaHCO_3$ 100X 10 ml
$CaSO_4 \bullet 2H_2O$ 5X 200 ml
Miiq H_2O 700 ml

1000 ml
Prepare in pre-autoclaved 1 L glass bottles.

Autoclave and store in fish room.

Appendix B - Antibiotic Cocktail

AB-GSM

Sol Gómez de la Torre Canny

Antibiotic Cocktail Preparation

Rifampicin (Rif)

(557303-1, VWR)
Stock: 50 mg/ml in DMSO
Dissolve 1000mg of powder in 20 ml of DMSO.
Aliquot in sterile Eppendorff tubes, date, and store at -20 C.
NOTE—To facilitate dissolving, I recommend splitting powder from original vial, shake at room temperature, and make sure to diffuse any clumps of powder at the bottom of the vial before adding DMSO. Shaking at RT for about an hour helped getting powder into solution.

Kanamycin (Kan)

(420311-5, VWR)
Stock: 50 mg/ml in H_2O
Dissolve 1000mg of powder in 20 ml of filtered/autoclaved mqH2O.
Filter sterilize using a 0,22 µm syringe filter.
Aliquot in sterile Eppendorff tubes, date, and store at -20 C.

PenicillinG (PenG)

(A1837.0025, VWR)
Stock: 100 mg/ml in H_2O
Dissolve 5000mg of powder in 50 ml of filtered/autoclaved mqH_2O.
Filter sterilize using a 0,22 µm syringe filter.
Aliquot in sterile Eppendorff tubes, date, and store at -20 C.

Ampicillin (Amp)

(171254-5, VWR)
Stock: 100 mg/ml in H_2O
Dissolve 5000mg of powder in 50 ml of filtered/autoclaved mqH2O.
Filter sterilize using a 0,22 µm syringe filter.
Aliquot in sterile Eppendorff tubes, date, and store at -20 C.

Oxolinic acid (Ox)

(J66637.06, VWR)
Stock: 12,5 mg/ml in 0,05N NaOH
Dissolve 1000mg of powder in 80ml of 0,05 N NaOH.
NOTE—0,05 N NaOH was prepared by diluting filter-sterilized 1N NaOH with filtered/autoclaved mqH_2O.
Filter sterilize using a 0,22 µm syringe filter.
Aliquot in sterile Eppendorff tubes, date, and store at -20 C.

Amphotericin B (Fun)

Stock: 250 µg/ml pre-made solution
Aliquot in sterile Eppendorff tubes, date, and store at -20 C.

Erythromycin (Ery)

(329815-5, VWR)
Stock: 50 mg/ml in 90% EtOH
Dissolve 1000mg of 20ml of 96% OH.
NOTE—96% EtOH was prepared by diluting absolute EtOH in filtered/autoclaved mqH_2O (19,2 ml of EtOH + qs 20 ml mqH_2O=
Aliquot in sterile Eppendorff tubes, date, and store at -20 C.

Antibiotic working concentrations

Rifampicin 10 mg/l
Erythromicin 10 mg/l
Kanamycin 10 mg/l
Ampicillin 100 mg/l
Amphotericin B 250 ug/l
Penicillin 150 mg/l
Oxolinic acid 75 mg/l
Rifampicin 0,2 ml
Kanamycin 0,2 ml
Ampicillin 1 ml
Amphotericin B 1 ml
Penicillin 1,5 ml
Oxolinic acid 6 ml
qs 1 L GSM

Preparation

1. Thaw the Abx stocks in advance.
2. Prepare solution in a pre-autoclaved GSM bottle, by the addition of the Abx stocks as described above inside of the laminar flow cabinet.

NOTE: Do not irradiate Abx with UV light.

3. Filter sterilize the solution Abx cocktail and aliquot 100 ml in the polycarbonate bottles (qs for a large petri Dish of ~150 salmon embryos.
4. Frozen aliquots or freshly made Abx work well for derivations.

NOTE: Upon thawing, there will be a white precipitate in the ABx

Appendix C - M65-Nutrient Solution

Table C-1: Recipe for M65 concentrated stock solution. Yielding a final concentration of 150 gL^{-1}

Component	Amount	Final concentration
Yeast extract	5 g	50 gL^{-1}
Bacteriological peptone	5 g	50 gL^{-1}
Tryptone	5 g	50 gL^{-1}
Distilled water (MilliQ)	100 mL	

Appendix D - Buffer Solutions

Recipe for 50x TAE-buffer are presented in Table D-1. 1x TAE-buffer was prepared by diluting 40 mL 50x TAE-buffer in 1960 mL MQ-water.

Table D-1: Recipe for 50x TAE-buffer.

Component	**Amounts**
Tris base	242 g
Glacial acetic acid	57.1 mL
0.5M EDTA pH 8.0	100 ml
dH2O	Up to 1L

Table D-2: Recipe for 1x Tris-EDTA buffer (TE-buffer)

Component	**Amounts**	**Final concentration**
2M Tris-HCl pH 7.5	2.5 ml	10.0 mM
0.5M EDTA pH 8.0	1.0 ml	1.0 mM
dH2O	496.5 ml	-

Appendix E – DNA Extraction Protocols

PowerSoil ® DNA Isolation Kit (Mo Bio)

EXPERIENCED USER PROTOCOL
PowerSoil® DNA Isolation Kit
Catalog No. 12888-50 & 12888-100

Please wear gloves at all times

1. To the **PowerBead Tubes** provided, add 0.25 grams of soil sample.
2. Gently vortex to mix.
3. **Check Solution C1**. If **Solution C1** is precipitated, heat solution to 60°C until dissolved before use.
4. Add 60 µl of **Solution C1** and invert several times or vortex briefly.
5. Secure **PowerBead Tubes** horizontally using the MO BIO Vortex Adapter tube holder for the vortex (MO BIO Catalog# 13000-V1-24) or secure tubes horizontally on a flat-bed vortex pad with tape. Vortex at maximum speed for 10 minutes.

Note If you are using the 24 place Vortex Adapter for more than 12 preps, increase the vortex time by 5-10 minutes.

6. Make sure the **PowerBead Tubes** rotate freely in your centrifuge without rubbing. Centrifuge tubes at 10,000 x g for 30 seconds at room temperature. **CAUTION**: Be sure not to exceed 10,000 x g or tubes may break.
7. Transfer the supernatant to a clean **2 ml Collection Tube** (provided).

Note Expect between 400 to 500 µl of supernatant. Supernatant may still contain some soil particles.

8. Add 250 µl of **Solution C2** and vortex for 5 seconds. Incubate at 4°C for 5 minutes.
9. Centrifuge the tubes at room temperature for 1 minute at 10,000 x g.
10. Avoiding the pellet, transfer up to, but no more than, 600 µl of supernatant to a clean **2 ml Collection Tube** (provided).
11. Add 200 µl of **Solution C3** and vortex briefly. Incubate at 4°C for 5 minutes.
12. Centrifuge the tubes at room temperature for 1 minute at 10,000 x g.
13. Avoiding the pellet, transfer up to, but no more than, 750 µl of supernatant into a clean **2 ml Collection Tube** (provided).
14. Shake to mix **Solution C4** before use. Add 1200 µl of **Solution C4** to the supernatant and vortex for 5 seconds.

15. Load approximately 675 µl onto a **Spin Filter** and centrifuge at 10,000 x g for 1 minute at room temperature. Discard the flow through and add an additional 675 µl of supernatant to the **Spin Filter** and centrifuge at 10,000 x g for 1 minute at room temperature. Load the remaining supernatant onto the **Spin Filter** and centrifuge at 10,000 x g for 1 minute at room temperature.

Note

A total of three loads for each sample processed are required.

16. Add 500 µl of **Solution C5** and centrifuge at room temperature for 30 seconds at 10,000 x g.

17. Discard the flow through.

18. Centrifuge again at room temperature for 1 minute at 10,000 x g.

19. Carefully place spin filter in a clean **2 ml Collection Tube** (provided). Avoid splashing any **Solution C5** onto the **Spin Filter**.

20. Add 100 µl of Solution C6 to the center of the white filter membrane. Alternatively, sterile DNA-Free PCR Grade Water may be used for elution from the silica Spin Filter membrane at this step (MO BIO Catalog# 17000-10).

21. Centrifuge at room temperature for 30 seconds at 10,000 x g.

22. Discard the **Spin Filter**. The DNA in the tube is now ready for any downstream application. No further steps are required.

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

DNeasy® PowerSoil® Kit protocol (Qiagen)

Quick-Start Protocol June 2016

DNeasy® PowerSoil® Kit

The DNeasy PowerSoil Kit can be stored at room temperature (15–25°C) until the expiry date printed on the box label.

Further information

- Safety Data Sheets: **www.qiagen.com/safety**
- Technical assistance: **support.qiagen.com**

Notes before starting

- Perform all centrifugation steps at room temperature (15–25°C).
- If Solution C1 has precipitated, heat at 60°C until precipitate dissolves.
- 2 ml collection tubes are provided.

1. Add 0.25 g of soil sample to the PowerBead Tube provided. Gently vortex to mix.
2. Add 60 µl of Solution C1 and invert several times or vortex briefly.
   Note: Solution C1 may be added to the PowerBead tube before adding soil sample
3. Secure PowerBead Tubes horizontally using a Vortex Adapter tube holder (cat. no. 13000-V1-24).
4. Vortex at maximum speed for 10 min.
   Note: If using the 24-place Vortex Adapter for more than 12 preps, increase the vortex time by 5–10 min.
5. Centrifuge tubes at 10,000 x *g* for 30 s.
6. Transfer the supernatant to a clean 2 ml collection tube.
   Note: Expect between 400–500 µl of supernatant. Supernatant may still contain some soil particles.
7. Add 250 µl of Solution C2 and vortex for 5 s. Incubate at 2–8°C for 5 min.

Sample to Insight

Note: You can skip the 5 min incubation. However, if you have already validated the DNeasy PowerSoil extractions with this incubation we recommend you retain the step.

8. Centrifuge the tubes for 1 min at 10,000 x *g*.
9. Avoiding the pellet, transfer up to 600 µl of supernatant to a clean 2 ml collection tube.
10. Add 200 µl of Solution C3 and vortex briefly. Incubate at 2–8°C for 5 min.

    Note: You can skip the 5 min incubation. However, if you have already validated the PowerSoil extractions with this incubation we recommend you retain the step.
11. Centrifuge the tubes for 1 min at 10,000 x *g*.
12. Avoiding the pellet, transfer up to 750 µl of supernatant to a clean 2 ml collection tube.
13. Shake to mix Solution C4 and add 1200 µl to the supernatant. Vortex for 5 s.
14. Load 675 µl onto an MB Spin Column and centrifuge at 10,000 x *g* for 1 min. Discard flow through.
15. Repeat step 14 twice, until all of the sample has been processed.
16. Add 500 µl of Solution C5. Centrifuge for 30 s at 10,000 x *g*.
17. Discard the flow through. Centrifuge again for 1 min at 10,000 x *g*.
18. Carefully place the MB Spin Column into a clean 2 ml collection tube. Avoid splashing any Solution C5 onto the column.
19. Add 100 µl of Solution C6 to the center of the white filter membrane. Alternatively, you can use sterile DNA-Free PCR Grade Water for this step (cat. no. 17000–10).
20. Centrifuge at room temperature for 30 s at 10,000 x *g*. Discard the MB Spin Column. The DNA is now ready for downstream applications.

    Note: Solution C6 is 10 mM Tris-HCl, pH 8.5. We recommend storing DNA frozen (–20° to –80°C) as Solution C6 does not contain EDTA. To concentrate DNA see the Hints & Troubleshooting Guide.

Ultra-Deep Microbiome Prep (Molzym)

Ultra-Deep Microbiome Prep
Sample Pre-Treatment*

Molzym

Protocol 2, part A: Tissue Sample Preparation

Kit 1: Buffers & Consumables (+18 to +25°C)
Kit 2: Enzymes & Reagents (-15 to -25°C)

- Transport the specimen under conditions avoiding contamination to the laboratory for analysis.
- Per specimen, place a *Sample tube* (*ST*, Kit 1,) in a rack and mark. Pipette 180µl of buffer *PKB* (Kit 1) into the *Sample tube* (*ST*).
- Transfer the specimen to a sterile support (e.g., Petri dish). Cut the specimen (~0.5x0.5cm) into small pieces by using a sterile scalpel.
- Transfer the cut specimen to the *Sample tube* (*ST*) filled with buffer *PKB*. **Add 20µl of *Proteinase K*** (Kit 2), **vortex for 15s.**
  Incubate at 56°C, 10min, 1,000rpm (thermomixer).
  → 400rpm + 3min 1000 rpm
- Fill up to 1ml with the transport solution, if available, or with buffer *TSB* (use the measure line of the tube).

Continue with the instructions of the scheme Ultra-Deep Microbiome Prep / Protocol 2, part B: DNA Isolation (page 5, short manual).

***Please note:** **Before using this scheme inform yourself of the details of the procedure. Please consult the manual.** Special care is required for working under DNA-free conditions and secure working conditions, please consult the manual for more information.

Molzym GmbH & Co.KG; Mary-Astell-Str. 10; 28359 Bremen; Germany; www.molzym.com; info@molzym.com

x

Ultra-Deep Microbiome Prep
Protocol 2, part B: DNA Isolation*

Molzym

Kit 1: Buffers & Consumables (+18 to +25°C)
Kit 2: Enzymes & Reagents (-15 to -25°C)

Arrange bottles according to the sequence of steps as below:
CM – DB1 – RS – RL – RP – CS – AB – WB –70% Ethanol – Deionized Water

Continued from Ultra-Deep Microbiome Prep / Tissue Sample Preparation, part A (page 2, short manual).

Per sample:

Removal of Human DNA

1. Add **250µl buffer *CM***, vortex for 15s.
   Let stand at room temperature (+18 to +25°C) for 5min.
2. Briefly centrifuge.
   Add **250µl buffer *DB1***.
   Add **10µl *MolDNase B*** (Kit 2), vortex for 15s.
   Incubate at room temperature (+18 to +25°C) for 15min.
3. Centrifuge at ≥12,000x*g*, 10min.
   Remove supernatant by pipetting and discard.
4. Resuspend pellet in **1ml buffer *RS*** by pipetting.
5. Centrifuge at ≥12,000x*g*, 5min.
   Remove supernatant by pipetting.
   (Optional: freeze pellet at -15 to -25°C for storage).

Lysis of Pathogens

6. Resuspend pellet in **80µl buffer *RL***, briefly centrifuge tube.
   Add **20µl *BugLysis*** (Kit 2).
   Add **1.4µl *ß-mercaptoethanol*** (Kit 2), vortex for 15s.
   Take care not to inhale.
   Incubate at 37°C, 30min, 1,000rpm (thermomixer).
7. Briefly centrifuge.
   Add **150µl buffer *RP***.
   Add **20µl *Proteinase K*** (Kit 2), vortex for 15s.
   Incubate at 56°C, 10 min, 1,000 rpm (thermomixer).

Continue on page 6

***Please note:** **Before using this scheme inform yourself of the details of the procedure. Please consult the manual.** Special care is required for working under DNA-free conditions and secure working conditions, please consult the manual for more information.*

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Ultra-Deep Microbiome Prep
Protocol 2, part B: DNA Isolation*

Molzym

During 10 min incubation:

Kit 1: Buffers & Consumables

Unpack *Spin columns* (*SC*), 2 ml *Collection tubes* (*CT*) and 1.5 ml *Elution tubes* (*ET*), label; heat ***Deionized Water*** (100µl each sample) vial to **70°C** (thermomixer).

Lysis of Pathogens

8. Briefly centrifuge.
   Add **250µl buffer *CS***, vortex for 15s.

9. Briefly centrifuge.
   Add **250µl buffer *AB***, vortex for 15s.

DNA Purification

10. Briefly centrifuge to clear lid.
    Pipette lysate into a *Spin column.*
    Pipette the fluid phase in the column.
    Avoid transfer of any unresolved particles!
    Centrifuge: ≥12,000x*g*, 30 to 60s.

11. Remove column and place in a new 2 ml *Collection tube.*
    Add **400µl buffer *WB***.
    Centrifuge: ≥12,000x*g*, 30 to 60s.

12. Remove column and place in a new 2 ml *Collection tube.*
    Add **400µl *70% Ethanol***.
    Centrifuge: ≥12,000x*g*, 3min.

DNA Elution

13. Carefully remove column and place in a 1.5 ml *Elution tube*.

14. Add **100µl *Deionized Water*** heated to 70°C.
    Incubate at room temperature (+18 to +25°C) for 1min .
    Centrifuge: ≥12,000x*g*, 1min.
    Discard column, close lid of *Elution tube*.

15. Store eluted DNA (1.5 ml *Elution tube*) at -15 to -25°C.

***Please note:** **Before using this scheme inform yourself of the details of the procedure. Please consult the manual.** Special care is required for working under DNA-free conditions and secure working conditions, please consult the manual for more information.*

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Version 2.1

ZymoBIOMICS™ DNA Miniprep Kit (Zymo Research)

Protocol

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

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

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

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

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

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

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

5. Binding preparation:

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

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

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

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

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

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

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

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

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

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

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

[5] In some cases a brown-colored pellet may form at the bottom of the tube after centrifugation. Avoid this pellet when collecting the eluted DNA.

[6] If fungi or bacterial cultures were processed; the DNA is now suitable for all downstream applications.

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

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

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

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

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

Appendix F – QIAquick ® PCR Purification Kit (Qiagen)

QIAquick PCR Purification Kit Protocol

using a microcentrifuge

PCR Purification Spin Protocol

This protocol is designed to purify single- or double-stranded DNA fragments from PCR and other enzymatic reactions (see page 8). For cleanup of other enzymatic reactions, follow the protocol as described for PCR samples or use the MinElute Reaction Cleanup Kit. Fragments ranging from 100 bp to 10 kb are purified from primers, nucleotides, polymerases, and salts using QIAquick spin columns in a microcentrifuge.

Important points before starting

- Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).
- All centrifugation steps are carried out at 17,900 x *g* (13,000 rpm) in a conventional tabletop microcentrifuge at room temperature.
- Add 1:250 volume pH indicator I to Buffer PB (i.e., add 120 µl pH indicator I to 30 ml Buffer PB or add 600 µl pH indicator I to 150 ml Buffer PB). The yellow color of Buffer PB with pH indicator I indicates a pH of ≤7.5.
- Add pH indicator I to entire buffer contents. Do not add pH indicator I to buffer aliquots.
- If the purified PCR product is to be used in sensitive microarray applications, it may be beneficial to use Buffer PB without the addition of pH indicator I.

Procedure

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

   For example, add 500 µl of Buffer PB to 100 µl PCR sample (not including oil).
2. **If pH indicator I has beein added to Buffer PB, check that the color of the mixture is yellow.**

   If the color of the mixture is orange or violet, add 10 µl of 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn to yellow.
3. **Place a QIAquick spin column in a provided 2 ml collection tube.**
4. **To bind DNA, apply the sample to the QIAquick column and centrifuge for 30–60 s.**
5. **Discard flow-through. Place the QIAquick column back into the same tube.**

   Collection tubes are re-used to reduce plastic waste.
6. **To wash, add 0.75 ml Buffer PE to the QIAquick column and centrifuge for 30–60 s.**
7. **Discard flow-through and place the QIAquick column back in the same tube. Centrifuge the column for an additional 1 min.**

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

8. **Place QIAquick column in a clean 1.5 ml microcentrifuge tube.**
9. **To elute DNA, add 50 µl Buffer EB (10 mM Tris-Cl, pH 8.5) or water (pH 7.0–8.5) to the center of the QIAquick membrane and centrifuge the column for 1 min. Alternatively, for increased DNA concentration, add 30 µl elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge.**

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

   Elution efficiency is dependent on pH. The maximum elution efficiency is achieved between pH 7.0 and 8.5. When using water, make sure that the pH value is within this range, and store DNA at –20°C as DNA may degrade in the absence of a buffering agent. The purified DNA can also be eluted in TE buffer (10 mM Tris·Cl, 1 mM EDTA, pH 8.0), but the EDTA may inhibit subsequent enzymatic reactions.
10. **If the purified DNA is to be analyzed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel.**

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

Appendix G - SequalPrep™ Normalization Plate (96) Kit (Invitrogen)

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 Tape	50 tapes	12261-012
E-Gel® *96* gels 1% (or 2%)	8 gels	G7008-01 (G7008-02)

Part no: 100003531 Rev. date: 5 May 2008

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.

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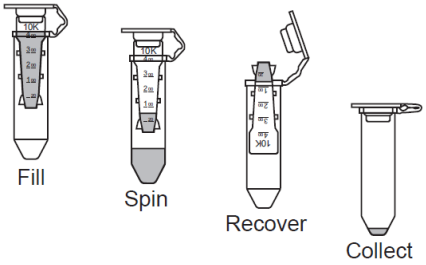
Appendix H - Amicon® Ultra-0.5 Centrifugal Filter Devices User Guide

Amicon® Ultra-0.5
Centrifugal Filter Devices
for volumes up to 500 µL

User Guide



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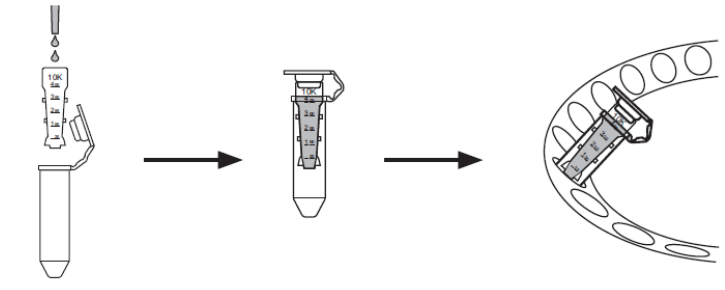
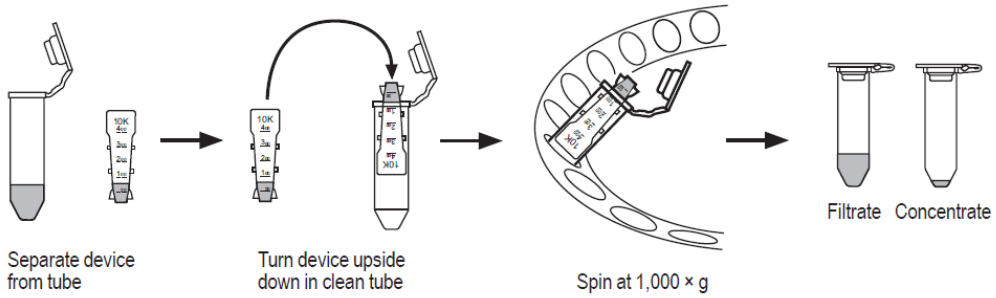
How to Use Amicon Ultra-0.5 Centrifugal Filter Devices

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



How to Use Amicon Ultra-0.5 Filter Devices, continued

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



Appendix I – Most abundant OUTs in Water and Salmon Gut Samples

Table I-1: The five most abundant OTUs associated with an aquaculture strain of Atlantic salmon yolk-sac fry reared in r-selected water. The OTUs relative abundance is given with the taxonomy specified at the lowest taxonomic level obtained.

OTU ID:	Taxonomy:	Average abundance L-AK:
1	Pseudomonas	0.19
3	Oxalobacteraceae	0.15
11	Staphylococcus	0.05
55	Agromyces	0.03
8	Acinetobacter	0.02

Table I-2 The five most abundant OTUs associated with a wild strain of Atlantic salmon yolk-sac fry reared in r-selected rearing water. The OTUs relative abundance is given with the taxonomy specified at the lowest taxonomic level obtained.

OTU ID:	Taxonomy:	Average abundance
1	Pseudomonas	0.12
8	Acinetobacter	0.08
6	Ralstonia	0.08
11	Staphylococcus	0.08
15	Chryseobacterium	0.04

Table I-3 The five most abundant OTUs associated with an aquaculture strain of Atlantic salmon yolk-sac fry reared in K-selected water. The OTUs relative abundance is given with the taxonomy specified at the lowest taxonomic level obtained.

OTU ID	Taxonomy:	Average abundance:
1	Pseudomonas	0.35
3	Oxalobacteraceae	0.11
8	Acinetobacter	0.03
11	Staphylococcus	0.03
6	Ralstonia	0.02

Table I-4: The five most abundant OTUs associated a wild strain of Atlantic salmon yolk-sac fry reared in K-selected rearing water. The OTUs relative abundance is given with the taxonomy specified at the lowest taxonomic level obtained.

OTU ID:	Taxonomy:	Average abundance :
11	Staphylococcus	0.09
3	Oxalobacteraceae	0.05
6	Ralstonia	0.05
1	Pseudomonas	0.05
17	Streptococcus	0.04

Table I-5: The five most abundant OTUs associated with the added K-selected rearing water. The OTUs relative abundance is given with the taxonomy specified at the lowest taxonomic level obtained.

OTU ID	Taxonomy:	Average abundance Aw-K
1	Pseudomonas	0.21
12	Moraxellaceae	0.21
26	Comamonadaceae	0.07
34	Alphaproteobacteria	0.06
25	Acidovorax	0.05

Table I-0-6: The five most abundant OTUs associated with the added r-selected rearing water. The OTUs relative abundance is given with the taxonomy specified at the lowest taxonomic level obtained.

OTU ID	Taxonomy:	Average abundance
1	Pseudomonas	0.53
3	Oxalobacteraceae	0.20
202	Proteobacteria	0.07
18	Sphingomonas	0.04
25	Acidovorax	0.02

Amalie Johanne Horn Mathisen

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NTNU
Norwegian University of
Science and Technology