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Effects of Salmon Lice Treatment on Bacterial Density and Community Composition of the Atlantic Salmon Skin Mucus Microbiota

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Trondheim, May 2018 **Charlotte Nilsen**

Abstract

The salmon lice (*Lepeophtheirus salmonis*) infesting Atlantic salmon (*Salmo salar*) in the on-growing sea water phase is one of the major issues in the Norwegian aquaculture industry. To obtain health and welfare of the farmed Atlantic salmon, medicinal and non-medicinal treatment methods are used for control of the salmon lice. The effect of salmon lice treatment on the bacterial density and the community composition of the skin mucus barrier is not known. In this project, the effect of various salmon lice treatments on the bacterial colonization were investigated using PCR, DGGE and Illumina sequencing of 16S rDNA variable regions. The bacterial load on fish skin mucus was investigated by real-time PCR. The samples were taken from fish that had been treated with freshwater and H_2O_2 bath, and the oral treatment SLICE, in addition a group of fish had ulcer. Proteobacteria was the dominant phylum in Atlantic salmon skin mucus from all samples investigated, and the genus *Pseudomonas* was prevailing in almost all samples. There was no significant difference in the skin mucus microbiota between fish treated with the different salmon lice treatments and untreated fish. The bacterial load seemed to be lower in skin mucus for some of the fish treated with freshwater and H_2O_2 bath. However, the most noticeable difference was found between the ulcerated fish and all the other fish samples, where *Psychrobacter* was most abundant in the ulcerated fish. In addition to a distinct community composition, the skin of ulcerated fish had the highest bacterial load.

Sammendrag

I oppdrettsnæringen i Norge i dag er et av hovedproblemene lakselus som infiserer Atlanterhavslaks i påvekstfasen i havet. For å opprettholde god fiskehelse hos oppdrettslaks må medisinske og ikke-medisinske behandlingsmetoder tas i bruk for å kontrollere nivåene av lakselus i merdene. Effekten lakselusbehandlingen har på bakteriemengden og bakteriesammensetningen i slimlaget på skinn er ikke kjent. I dette prosjektet ble det undersøkt om lakselusbehandlingen hadde en effekt på koloniseringen av skinn og slimlaget til Atlanterhavslaksens ved hjelp av PCR, DGGE og Illuminasekvensering av variable regioner i 16S rDNA. Mengden bakterier i slimlaget ble også undersøkt ved "Real-time" PCR. Fisken som ble undersøkt hadde gjennomgått ferskvann og hydrogenperoksid badebehandling og fôrbehandlingen SLICE. I tillegg var en det en gruppe med sårfisk som hadde blitt behandlet med ferskvann. Rekken Proteobakterier dominerte slimlaget til all fisken som ble undersøkt, og slekten *Pseudomonas* dominerte i nesten alle slimlagprøvene. Det var ingen signifikant forskjell i slimmikrobiotaen i prøver som var behandlet med ulike lakselus behandlinger og ubehandlet fisk. Mengden med bakterier så ut til å være lavere i slimlaget til fisk som var badebehandlet i ferskvann eller hydrogenperoksid. Den mest slående resultatet var forskjellen i slimmikrobiotaen mellom sårfisk og resten av fisken, der *Psychrobacter* dominerte i sårfiken. I tillegg til en annerledes bakteriesammensetning i slimlaget hadde sårfisk størst bakteriemengde.

Abbreviations

AGD	Amoebic gill disease
ANOVA	Analysis of variance
APS	Ammonium persulfate
C	Control sample (positive)
CN	Copy number
Cq	Quantification cycle
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphates
EB	Emamectin benzoate
HTS	High throughput sequencing
KB	"Kit blank"
M	Skin mucus sample
MQ	MilliQ water
NC	non-template control in PCR reaction
NTC	Non-template control in qPCR reaction
OTU	Operational taxonomic unit
PCoA	Principal coordinate analysis
PCR	Polymerase chain reaction
PERMANOVA	Non-parametric multivariate analysis of variance
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
rDNA	Ribosomal deoxyribonucleic acid
rRNA	Ribosomal ribonucleic acid
RDP	Ribosomal Database Project
S	Skin sample
SBS	Sequencing by synthesis
SC	Sea cage
SIMPER	Similarity percentage
SM	Salmon muscle sample
TEMED	Tetramethylethylenediamine
V3	Variable region 3
V4	Variable region 4

Table of Contents

1 Introduction

1.1 Aquaculture of the Atlantic Salmon (*Salmo salar*)

Aquaculture of the Atlantic salmon (*Salmo salar*) is one of Norway's major industries that started in the early 1970s (Asche and Bjørndal, 2010, Jobling et al., 2010). The production of Atlantic salmon in Norway has increased dramatically, from less than 500 tons in the 1970s, to 1.23 million tons in 2016 (SSB, 2017). The industry is economically important in Norway, and in some other countries like Chile, Scotland and Canada. The total production of Atlantic salmon in the world exceeded 2 million tons in 2016 (Marine Harvest, 2017).

Despite the phenomenal growth of Atlantic salmon production in Norway over the last decades, the production has recently stabilized. The salmon lice (*Lepeophtheirus salmonis* (Krøyer, 1838)) infesting Atlantic salmon in the on-growing sea water phase is one of the major issues in the industry. Salmon lice feed on skin, mucus and blood from the Atlantic salmon. If the number of lice per fish is too high, this can harm the fish. Skin lesion and anemia usually leads to secondary infections and problems with the osmoregulation. Higher lice numbers have been observed on wild salmonids in the surrounding areas of the Atlantic salmon farms, suggesting transfer of lice from farmed to wild salmon (Serra-Llinares et al., 2014). The salmon lice infection is regarded as the most expensive health issue for the Atlantic salmon industry (Torrissen et al., 2013). The economic loss is related to treatment costs, negative impacts on the growth rate, product downgrading, and in the worst case, early slaughter and a lower biomass output.

The world's population is expected to reach 9 billion people in 2050 (FAO, 2016). To meet the global demand for fish protein, sustainable approaches to reduce the cost and increase the yield of aquaculture is necessary (FAO, 2016). To obtain health and welfare for the farmed Atlantic salmon, effective control of the salmon lice is necessary. When the salmon lice number reaches 0.5 adult female lice per fish in a sea cage, it is treated against salmon lice (Heuch and Mo, 2001). A wide range of medicinal and non-medicinal treatment methods are used. Throughout the treatment process the Atlantic salmon is exposed to stressors, both chemical and mechanical factors. The treatments may affect the skin and mucus barrier properties, and the microbial community associated with it.

1.2 Atlantic Salmon

The Atlantic salmon is an anadromous species populating the northern regions of the Atlantic Ocean (Jobling et al., 2010). With optimum growth at 12.1-15.1 °C, it is considered a cold-water species (Pennell and Barton, 1996). Adult Atlantic salmon spawns in freshwater during autumn or early winter, where eggs are laid in gravel nests. The life cycle starts when the alevins emerge from the hatched eggs (Fig. 1.1). In the alevin stage, the fish have yolk sacs and stays in the gravel. When the yolk sac is consumed, fry emerge from the gravel and start to feed on insect larvae, as the Atlantic salmon is carnivorous (Jobling et al., 2010). The fry stage is followed by the parr stage, and the fish develops vertical stripes and spots on the skin as camouflage. The freshwater phase is terminated when the parr undergoes morphological, physiological and behavioural changes and becomes smolt. During smoltification the fish adapts to the marine environment and the osmoregularity of the fish changes from hyper- to hypo-osmoregulation. Smoltification usually happens within one to eight years (Jobling et al., 2010). The fish migrates to feeding grounds in the sea, where the majority of the growth happens. After one to four years, the mature Atlantic salmon may return to its native river and spawn (Jobling et al., 2010).

In aquaculture, Atlantic salmon is reared in land-based freshwater facilities, and transferred to sea cages to reach market size. The Atlantic salmon is an easily cultivable specie due to features of the salmonid life cycle and the composition of needed feed (Jobling et al., 2010). The eggs are obtained from captive broodfish, where the spawning is controlled by manipulation of the temperature and photoperiod. The Atlantic salmon produce large demersal eggs, resulting in well-developed offsprings. Moreover, the fish grow fast on formulated dry feed. When the 50-100 grams Atlantic salmon smolt are transferred to the sea, it can reach four to five kilograms in 18 months. Crowding and handling during transfer from land-based facilities to sea cages, is tolerated to a certain extent. The full-grown adult Atlantic salmon produced in the sea cages give a high yield of fish fillet, which is easily sold to an accepting market (Jobling et al., 2010).

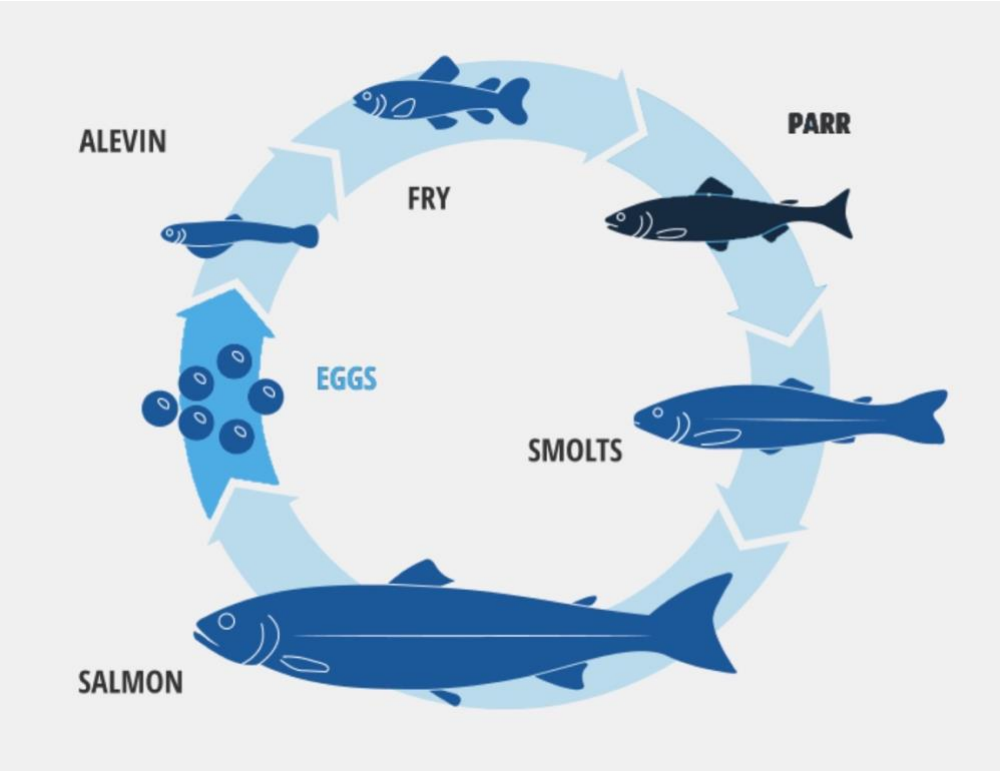


Figure 1.1: Life stages, from the eggs to alevin, fry, parr, smolt and finally adult Atlantic salmon. Figure from Scottich Sea Farms (2018).

1.3 Salmon Lice

The salmon louse is a parasitic copepod in the family Caligidae that naturally affects Atlantic salmon in the marine environment on the northern hemisphere (Llewellyn et al., 2017). The salmon louse has eight life stages (Fig. 1.2), separated by ecdysis. The planktonic nauplius 1 is hatched from an egg, and further developed into the nauplius 2 stage (Hamre et al., 2013). Infection of the Atlantic salmon occurs in the subsequent copepodid stage. The following stages, chalimus 1 and 2 are the non-motile stages where the salmon louse is attached to the Atlantic salmon. In the final motile pre-adult sages, 1 and 2, the lice can move and spread in the water column. During the fully adult female stage the louse is able to produce ten to eleven pairs of egg strings, and the mean number of eggs produced per string has been recorded to 152 eggs at 7.2°C (Heuch et al., 2000). The generation time is six weeks at 9 °C (Hayward et al., 2011). Along the Norwegian coast, about 300 million smolts are transferred to sea cages every year. Ultimately, the amount of eggs produced and the short generation time, results in the release of more than a billion salmon lice larvae daily (Taranger et al., 2015).

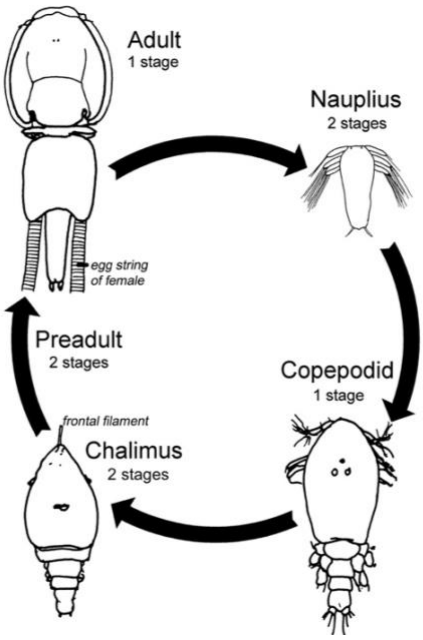


Figure 1.2: The lifecycle of *L. salmonis* showing the eight life stages from nauplius to adult female lice with egg strings. Figure from Hayward et al. (2011).

1.4 Medicinal Treatments Combatting Salmon Lice

To reduce the number of lice in the sea cage a variety of methods are used. Pharmaceuticals, applied through feed or as bath treatment, are used during medicinal treatment. Bath treatments are conducted in tarpaulin enclosed cages or in wellboats. Examples of pharmaceuticals used in bath treatments are azamenthiphos, pyrethroids and hydrogen peroxide (H_2O_2). Furthermore, emamectin benzoate (EB) and flubenzurones are substances used in feed as oral salmon lice treatment. An overview of the different medicinal treatments and non-medicinal treatment used in Norway from 2011 to 2017 are presented in Table 1.1. From 2015 to 2016 the use of the medicinal treatments azamenthiphos and pyrethroids was reduced by 60 percent, and the use of H_2O_2 was reduced by 50 percent (Mattilsynet, 2017). The reason for the reduced use of medicinal treatment is the increased resistance developed against the chemicals (Aaen et al., 2015). The delousing agent H_2O_2 was used between 1993 and 1997, but the use was terminated, due to the discovery of more efficient chemicals (Aaen et al., 2015). But, H_2O_2 was reintroduced in 2009 as a result of increased resistance against the other chemicals (Aaen et al., 2015). Another concern using the medicinal treatments is the potential environmental risk to negatively impact non-target organisms and sediments in the surroundings of the farm, affecting the species composition (Burridge et al., 2010).

Table 1.1: Overview over active substances and non-medicinal treatments used to combat salmon lice from 2011 to 2017. The numbers of medicinal treatments are the numbers of requisitions given on each active substance registered VetReg 16.01.18. The number of non-medicinal treatment is the number of reported mechanical treatments to Mattilsynet 18.01.18. Table from Hjeltnes et al. (2018).

Active Substance	**2011**	**2012**	**2013**	**2014**	**2015**	**2016**	**2017**
Azamenthipos	409	691	480	794	616	257	58
Pyrethrioids	456	1155	1123	1043	662	276	80
Emamectin benzoate	288	164	162	481	523	608	319
Flubenzurones	23	129	170	195	201	173	79
Hydrogen peroxide	172	110	250	1009	1279	629	214
Sum	1348	2249	2185	3477	3284	1943	750
Non-medicinal treatments		136	110	177	202	1178	1669

To fight pre-adult and adult lice attached to the fish, H_2O_2 was the bath treatments applied the most in 2017 (Tab. 1.1). The salmon lice exposed to H_2O_2 is filled with oxygen gas bubbles in the gut and the haemolymph. It floats up to the water surface and stays lifeless for several hours (Thomassen, 1993). Furthermore, H_2O_2 is toxic to Atlantic salmon and may cause gill damage at the wrong treatment criteria. The lethal toxicity increases with temperature, which results in restricted use during the summer (Thomassen, 1993).

The most used oral treatment in 2017 was the active substances EB (4"-deoxy-4" epi-methylamino-avermectin B_1) (Tab. 1.1). EB is the active ingredient in SLICE® feed, which is fed to reduce the salmon lice number in the sea cage. EB is semi-synthetic and belongs to the group of avermectins, a family of compounds isolated from the microorganism *Streptomyces avermitilis* (Burg and Stapley, 1990). The active substance is absorbed and distributed to tissues in the Atlantic salmon. The concentration of the compound is maintained in the tissue due to the limited metabolism of EB in the fish (Kim-Kang et al., 2004). EB has been demonstrated as effective against all life stages of the salmon lice (Lees et al., 2008). It affects the muscle cells and synapses in the peripheral nervous system, causing paralysis and death of the parasite. Atlantic salmon tolerates EB three and a half times higher than the therapeutic dose used to kill the salmon lice (Roy et al., 2000).

The main technique fighting salmon lice have been medicinal treatments, but due to increasing resistance against the pharmaceuticals, the use of medicinal treatments has decreased. Since 2012, there has been a dramatic increase in the use of non-medicinal treatment methods (Tab. 1.1).

1.5 Non-medicinal Treatments Combatting Salmon Lice

Non-medicinal treatments are treatments without the use of pharmaceuticals and can be thermic, mechanical or non-mechanical. An example of thermic treatment is the Thermolicer, which use a flow-through system where the fish are pumped through pipes with heated seawater. The method is effective against mobile lice in the water, but not on lice attached to the fish (Grøntvedt et al., 2015). In mechanical treatment, such as the SkaMik delicer, the fish is flushed with water and brushed with rotating brushes to remove the lice (Holan et al., 2017). Other mechanical treatment methods use flushing with seawater. The thermic and the mechanical treatment require fish handling, that may stress the fish. A method that does not require handling is the use of cleaner fish, such as ballan wrasse (*Labrus bergylta*) and lumpsucker (*Cyclopterus lumpus*). The method is an effective, preventive biological control against salmon lice (Leclercq et al., 2014), as the cleaner fish eats the salmon lice directly from the skin of Atlantic salmon. The use of farmed cleaner fish is considered both environmentally friendly and sustainable (Holan et al., 2017). However, the welfare of the cleaner fish is compromised, as many fish die when they are transferred to sea cages (Holan et al., 2017).

Treatment of Atlantic salmon in freshwater wellboats is a common bath treatment, where the low salinity seems to reduce the levels of salmon lice (Tucker et al., 2000, Bricknell et al., 2006). The change from seawater to freshwater disturbs the osmotic balance, and the salmon lice is paralysed and will eventually die. Freshwater seems to have an effect on the copepodid stage of the salmon lice, but not on the attached adult lice survival (Wright et al., 2016). The disadvantage of freshwater treatment is the development of freshwater tolerant salmon lice, because there are genotypes of salmon lice that are tolerant towards lower salinities (Ljungfeldt et al., 2017). Freshwater treatments are also used to treat amoebic gill disease (AGD), caused by *Neoparamoeba perurans* (Powell et al., 2015).

Wellboats are used during non-medicinal and medicinal treatment of the fish. These treatments includes transfer by netting and pumping, which is stressful to the fish (Erikson et al., 1997). There has also been reported loss of fish scales and skin haemorrhage in this process (Holan et al., 2017). These treatments may affect the mucus layer colonized by bacteria on the skin of the fish.

1.6 Fish Skin Mucus and Microbiota

The fish skin is covered by a mucus layer colonized by bacteria, creating a physical, chemical and biological barrier towards the external environment. The skin integument of fish consists of the hypodermis and dermis, covered by the epidermis and the goblet cells (mucous glands) (Fig. 1.3). The goblet cells supply the outermost mucus layer. The mucosal barrier both protect the internal milieu towards entry of pathogens and prevent leakage of water, solutes and nutrients. The mucus covering the epidermis is a thin barrier with a complex composition of protective compounds, and is an important part of the first line immune defence against infectious agents (Esteban, 2012).

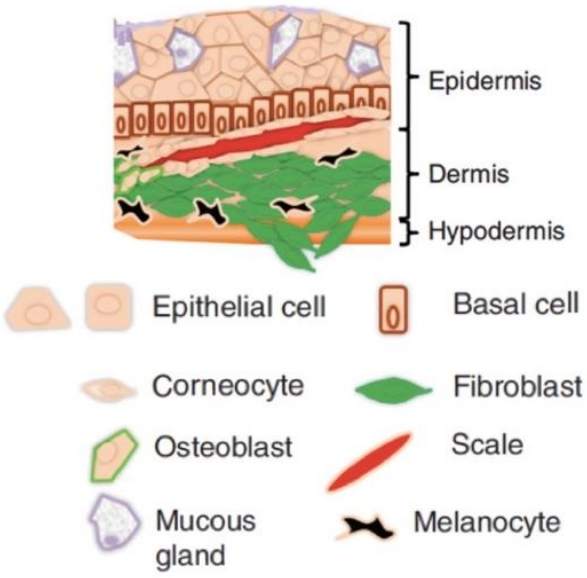


Figure 1.3: The skin integument of fish showing the hypodermis, dermis and epidermis with the mucus producing goblet cells (Mucous glands). Figure from Rakers et al. (2013)

The protective mucus layer contains a variety of biologically active substances. Mucins are highly glycosylated glycoproteins and constitute the main component of mucus (Esteban, 2012). Mucins form a matrix that contributes to the protection against chemical, enzymatic,

microbial and mechanical impact on the underlying epidermis. Certain cells in the epidermis excrete antimicrobial compounds after injury, or when in contact with pathogens. Antimicrobial compounds, such as lysozyme, immunoglobulins, complement proteins, lectins, C-reactive proteins and proteolytic enzymes are found in the matrix of the mucins (Rakers et al., 2013, Subramanian et al., 2007). Pathogens, virus and particles are captured and removed due to continuous exchange of the viscoelastic mucus.

The fish skin and mucus colonized by bacteria are continuously in contact with the aqueous environment, which is rich in microbes. However, the microbes in the water are not necessarily found on the fish skin (Chiarello et al., 2015). Alongside the immune system, mucus helps to maintain a healthy fish. The commensal microbiota is also assumed to protect against pathogens (Kelly and Salinas, 2017). Bacteria adhere to the nutrient rich Atlantic salmon mucus, and the balance between the mutualistic, commensal and pathogenic bacteria in the skin mucus is an important factor to preserve fish health (Gomez et al., 2013). The fish skin microbiota is diverse and a high variety of phyla are found. Moreover, the most abundant phyla found in Atlantic salmon skin are Proteobacteria, Actinobacteria, Bacteroidetes and Firmicutes (Lokesh and Kiron, 2016, Minniti et al., 2017, Reid et al., 2017).

Pathogenic bacteria can be categorized as obligate or opportunistic bacteria (Vadstein et al., 2004). Obligates cannot survive without infecting and causing disease in a host. The opportunistic bacteria have a variety of survival strategies and is not dependent on infecting a host in order to survive. Most fish pathogens are opportunistic bacteria, as they often are naturally present in the water column and in the sediments. The opportunists can infect weakened fish reared in unfavourable environments. Pathogens such as *Vibrio* species causing Hitra disease (Enger et al., 1989), *Yersinia ruckeri* causing enteric redmouth, *Flexibacter* species causing columnaris disease and *Moritella viscosa* causing ulcer (Bakke and Harris, 1998), have been a problem in the salmon farming industry. However, in Norway the problems with bacterial infections in farmed salmon is generally under control, due to vaccines preventing these diseases (Hjeltnes et al., 2018).

The healthy balance between commensals and pathogenic bacteria can be disturbed by a variety of environmental factors, such as stress and fish handling (Karlsen et al., 2017, Boutin et al.,

2013, Minniti et al., 2017). The host genotype may also influence the fish skin microbiota. One study indicates that the host genotype may regulate the abundance of specific genera among its surface microbiota (Boutin et al., 2014). It has also been shown that bacterial communities are variable between different individuals and body parts (Chiarello et al., 2015). Atlantic salmon transferred from freshwater to seawater had a higher phylogenetic diversity after the transfer (Lokesh and Kiron, 2016). Transfer of fish between two different environments can be a stressful situation for the fish, which have shown to affect the microbiota associated with the fish skin. The homeostasis of the fish skin microbial community was extensively disturbed on brook charr (*Salvelinus fontinalis*) after physiological stress was introduced. The abundance of probiotic-like bacteria decreased after stress exposure, and pathogenic bacteria increased following the stress exposure (Boutin et al., 2013). When the fish is exposed to stress over a longer period, it may affect the fish health (Llewellyn et al., 2014). Another stressful situation for the Atlantic salmon is salmon lice infection and salmon lice treatment. Among Atlantic salmon infected with salmon lice, a significant reduction in microbial richness, increased diversity and destabilisation of the microbial community have been observed (Llewellyn et al., 2017). However, the effect of salmon lice treatment on the microbial community on fish skin mucus is yet to be explored.

1.7 Studying Microbial Communities and Diversity

Traditionally culturing was the best way to study and characterize bacteria (Amann et al., 1995). Bacteria were isolated in pure cultures and biochemical and physiological traits were tested. However, many prokaryotic organisms are uncultivable. Studying microbial communities by culturing is time-consuming, and the lack of growth does not prove the absence of the bacteria in a sample. Slower growing bacteria may be outcompeted by faster growing bacteria, and the culturing environment may not favour growth at all. The number of cultured bacteria is very small compared to the actual number of bacteria, and it is difficult to get an overview of the diversity in an ecosystem using traditional culturing methods. Molecular methods have made it possible to do taxonomic assignment and study the phylogenetic relationships and the diversity in microbial communities.

A current approach of studying microbial communities is by polymerase chain reaction (PCR)-based analysis of sequence variation in the small subunit 16S ribosomal RNA (16S rRNA) genes encoded by the rDNA (Acinas et al., 2004). The small subunit of 16S rDNA molecules

is a component of the ribosome and a vital part of the protein-synthesizing machinery. The molecule and its nucleotide sequences is conserved and universally found in all bacteria (Olsen et al., 1986). The 16S rDNA of approximately 1500 base pairs (bp) is composed of highly conserved regions and regions with sequence variations. The nine variable regions (V) can be amplified by PCR using universal bacterial primers binding to the conserved regions of the DNA. Microorganisms can be characterized based on the sequence of one variable region. However, it is optimal to combine more variable and conserved regions, to obtain more sequence information (Hamady and Knight, 2009).

The sequences can be analysed by denaturing gradient gel electrophoresis (DGGE), a genetic fingerprinting method applied to compare microbial communities in different samples. After amplification of the PCR product, the 16S rDNA can be separated on a polyacrylamide gel with a denaturing gradient according to sequence variation. The different species are separated based on the differences in the variable regions of the 16S rDNA, and one band theoretically represents a single species. The pattern displays the community profile for each PCR product, and represents the microbial diversity in the sample (Muyzer et al., 1993). DGGE is a simple and cheap method for examining population dynamics. The bands in the gel can be manually excised, reamplified and sequenced to obtain taxonomic information of the bacteria represented. The sequences are aligned with known sequences in databases, such as the Ribosomal Database Project (RDP) (Cole et al., 2013), and taxonomic assignment can be conducted. However, the taxonomic information obtained is of limited resolution. The information obtained from Sanger sequencing (Sanger et al., 1977) of the 16S rDNA bands from DGGE is insufficient to adequately describe and compare microbial communities (Bartram et al., 2011). The use of DGGE has decreased and more accurate technologies, such as high throughput sequencing (HTS), are becoming more prominent. HTS has been a paradigm shift for molecular ecology and especially microbial diversity analysis using marker genes, such as the 16S rRNA gene. Information of the species present in an environmental sample can rapidly be obtained and the microbial diversity may be examined at a higher resolution (Mardis, 2008). By indexing different samples, thousands of sequences from several samples can be pooled together and sequenced. HTS also makes it possible to detect rare microorganisms that exist at low relative abundance (Bartram et al., 2011). However, HTS of 16S rDNA amplicons is not a quantitative method.

1.8 Quantification of Bacteria

Culture dependent methods have been used for enumeration of bacteria in environmental samples. However, the method is tedious and uncultivable bacteria are not detected. Fluorescent methods, such as flow cytometry using universal probes to measure total rRNA abundance in aquatic environmental samples, are well established (Amann et al., 1990). However, aggregation of bacteria and the presence of contaminating matrices can make counting difficult. Alternatively, molecular methods, such as real-time PCR, also called quantitative PCR (qPCR), can be used to quantify bacterial DNA in any environmental sample. Broad-range primers designed to amplify 16S rDNA by qPCR have been applied to quantify bacteria in environmental samples (Malinen et al., 2003, Nadkarni et al., 2002). Formation of DNA product is rapidly detected as the fluorescent dye binds specifically to double stranded DNA in each round of amplification. The need for post-PCR processing is unnecessary and the 96-well format allows large amounts of samples to be analysed simultaneously (Nadkarni et al., 2002). An alternative method to qPCR are droplet digital PCR (ddPCR). ddPCR is less affected by inhibitors in the samples and standard curve generation is not needed. However, the method is twice as expensive as qPCR and the sensitivity of the methods are comparable (Yang et al., 2014).

1.9 Hypothesis and Aims

The main hypothesis of this project is that Atlantic salmon exposed to salmon lice treatments have reduced skin mucus barrier properties, and that their skin therefore will be colonized by more and other bacteria than healthy, untreated fish.

The aim of this study was to investigate whether the salmon lice treatments affected the quantity of bacteria and composition of bacterial communities colonizing the fish skin mucus. A sub-aim was to investigate whether ulcerated fish had a different bacterial load and bacterial community composition in their skin mucus than the other fish. The methods used to investigate this were qPCR, and DGGE analysis and Illumina sequencing of 16S rDNA amplicons.

Previous experiments in the research group "Analysis and Control of Microbial Systems (NTNU, Norway)" have shown that PCR amplification of microbial 16S rDNA from Atlantic salmon skin samples is challenging, probably due to the presence of inhibitors and low fraction of bacterial DNA in such samples. A second sub-aim was therefore to optimize a protocol for isolating DNA and subsequent PCR amplification of microbial 16S rDNA from Atlantic salmon skin mucus using broad-range bacterial PCR primers.

2 Materials and Methods

2.1 Sampling

2.1.1 Sampling from Farmed Atlantic Salmon in Sea Cages

Samples from the skin of farmed Atlantic salmon exposed to various salmon lice treatment in six sea cages representing three aquaculture sites were collected by Åkerblå during winter 2016-2017. The fish from sea cage 1 (SC1), collected 8.12.2016, had ulcers, where the shells and the subcutaneous layer of the fish had lesions, and parts of the salmon muscle were visible. The fish from this sea cage had undergone freshwater bath treatment, conducted as an AGD treatment, in week 43. Samples from untreated fish were collected from sea cage 2 (SC2) 11.01.2017. The samples collected 20.12.2016 were from four different sea cages (SC3-6) at the same aquaculture site. Fish in all sea cages had been treated with SLICE (Emamectin benzoate 5 mg/kg, 1.4% of the feed for seven days) in week 30/31. The fish in SC4 were in addition to SLICE, treated in a freshwater bath for three hours in week 44/45. The fish in SC6 were, in addition to SLICE, treated with H_2O_2 (1100ppm) for 32 minutes in week 44/45. At sampling, squares of skin were cut and stored in plastic bags. The samples were frozen and stored at -20°C until further use. An overview of the samples used and the number of individuals sampled in each sea cage is presented in Table 2.1.

Table 2.1: Samples collected from Atlantic salmon in six different sea cages. Five of the sea cages were exposed to various salmon lice treatments. Sampling dates, number of individuals and samples used are presented.

Treatment (Week)	**Sampling Date (Week)**	**Sea Cage**	**Number of Individuals**	**Number of Samples**
Freshwater* (43)	08.12.16 (49)	SC1	4	5
No treatment	11.01.17 (2)	SC2	4	5
SLICE (30/31)	20.12.16 (51)	SC3	3	4
SLICE + Freshwater (30/31 + 44/45)	20.12.16 (51)	SC4	2	4
SLICE (30/31)	20.12.16 (51)	SC5	4	4
SLICE + H_2O_2 (30/31 + 44/45)	20.12.16 (51)	SC6	2	4
Total:				26

*The fish had ulcer.

2.1.2 Samples for Optimizing the PCR Protocol

Previous experiments in the research group "Analysis and Control of Microbial Systems (NTNU, Norway)" have shown that PCR amplification of microbial 16S rDNA from Atlantic salmon skin samples has been challenging, probably due to the presence of inhibitors and low fraction of bacterial DNA (personal communication, Ingrid Bakke). Samples of Atlantic salmon fry (40 g ± 4.4 g) collected at SINTEF SeaLab in Trondheim 24.1.17 was therefore used to optimize DNA extraction and a PCR amplification protocol. Prior to DNA extraction, the skin samples (S) were collected by separating the skin from the salmon muscle using a sterile scalpel

(Swann-Morton). These samples included both skin and skin mucus. Skin mucus samples (M) were collected by scraping the mucus off the Atlantic salmon skin using a sterile scalpel (Swann-Morton). Bacterial samples were collected from biofilm material, and was later used for generating positive controls (C) for PCR reactions.

2.1.3 DNA Extraction Kits

Total DNA was extracted from the Atlantic salmon fry samples from SINTEF SeaLab using three different DNA extraction kits. DNeasy Powersoil DNA isolation Kit (Qiagen), PureLink™ Microbiome DNA Purification Kit (Thermo Scientific) and QIAamp DNA Mini Kit from Tissues (Qiagen) were used to extract DNA from skin, skin mucus and biofilm samples. DNA extraction was performed using 0.2 g of salmon skin and all the mucus available scraped of the skin (not more than 0.2 g). The extractions were performed according to the manufacturer`s protocol (Appendix A-C), and an overview of the DNA extracts are showed in Table 2.2.

The volume of the DNA eluated from each sample were 100-200 μL. For extraction with DNeasy Powersoil DNA isolation Kit (Qiagen) (Appendix A) minor alterations were performed: in step 5 the vortex time of samples were extended from 10 to 15 minutes. Twice the amount of the solutions C1 and C2 were used compared to the instructions in the protocol, because the skin sample was hard to dissolve. For extraction from PureLink™ Microbiome DNA Purification Kit (Thermo Scientific) (Appendix B) alteration were performed in step 1e., where the vortex time of the samples were extended from 10 to 15 minutes.

Table 2.2: Overview of the DNA extracts used for PCR protocol optimization. DNA was extracted from skin and skin mucus from Atlantic salmon fry from SINTEF SeaLab (24.1.17, Trondheim). DNA used in positive control samples was extracted from biofilm material. Three DNA extraction kits were used.

	Sample Names		
Extraction Kits	Skin	Skin mucus	Biofilm/control
DNeasy Powersoil DNA isolation Kit (Qiagen),	S1.1-S1.3	M.1.1-M1.3	C1.1-C1.3
PureLink™ Microbiome DNA Purification Kit (Thermo Scientific)	S2.1-S2.3	M2.1-M2.3	C2.1-C2.3
QIAamp DNA Mini Kit from Tissues (Qiagen)	S3.1-S3.3	M3.1-M3.3	C3.1-C3.3

2.2 Optimizing PCR Amplification of Bacterial 16S rDNA Variable Regions from Atlantic Salmon Skin and Skin Mucus

PCR was performed to amplify variable regions 3 and 4 (V3 and V4) of bacterial 16S rDNA. Different polymerases, reaction components and cycling conditions were used to optimize a PCR protocol for bacterial DNA retrieved from Atlantic salmon skin and skin mucus samples.

A nested PCR protocol for the V3 region of the bacterial 16S rRNA gene, was applied using the Phusion Hot Start polymerase II (Thermo Scientific). The nested PCR protocol can be used to avoid co-amplification of eukaryotic small sub unit rDNA, in two separate rounds of external and internal PCR amplification (Bakke et al., 2011). The primers EUB8F and 984yR were used for external PCR, and the primers 338F-GC and 518R were used for internal PCR. The sequences of the primers used are presented in Table 2.3. The PCR reaction was performed in a total volume of 25 µL on a T100™ Thermal Cycler (BioRad). The template (0.1 µL of the DNA extracts (~1 ng μL^{-1})) was amplified in a reaction with 2 mM $MgCl_2$ (Thermo Scientific), 0.2 mM of each dNTP (Thermo Scientific), 0.3 µM of each primer (Sigma-Aldrich), 0.025 U μL^{-1} of the Phusion Hot Start polymerase II (Thermo Scientific) and 1x Phusion HF

Buffer™ (Thermo Scientific). Amplification product (1 µL) from the external PCR was used as template in the internal PCR, and amplification was performed with the same reaction components as in the external PCR. External and internal PCR were carried out for 20 cycles (95°C 30 sec. (denaturation), 53°C 30 sec. (annealing), 72°C 60 sec. (elongation) and 72 °C 10 min. (final elongation)).

Four different polymerases; Phusion Hot Start polymerase II (Thermo Scientific), KAPA 2G Robust PCR kit (Sigma-Aldrich), PrimeStar DNA polymerase (TaKaRa Bio USA, Inc.) and ExTaq polymerase (TaKaRa Bio USA, Inc.) were used in attempt to amplify the variable regions of the bacterial 16S rDNA. PCR reactions were performed in a total volume of 25 µL. The template (1 µL of the DNA extracts (~1 ng μL^{-1}) was amplified in a reaction with various primers (Tab. 2.3). Exact reagents concentrations used for each polymerase are listed in Table 2.4. The cycling conditions for the PCR reaction are given in Table 2.5. The amplification of the variable region of the bacterial 16S rDNA was performed on a T100™ Thermal Cycler (BioRad). Furthermore, the PCR facilitators glycerol (VWR; final concentration 10%) or spermidine (Sigma-Aldrich; final concentration 0.5 mM) were applied to investigate potential positive effects on the amplification.

Table 2.3: PCR primers (Sigma-Aldrich) used to amplify bacterial 16S rDNA regions for qPCR, DGGE and Illumina amplicon sequencing. Primer names, primer sequence and the application are presented. Illumina adapter sequences are marked in red.

Primer Name	Sequence (5'-3')	Application
Ill515F	5′- TCG TCG GCA GCG TCA GAT GTC TAT AAG AGA CAG NNNN GTG CCA GCM GCC GCG GTA A-3′	V4 region
Ill338F	5′-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG NNNN CCT ACG GGW GGC AGC AG-3′	V3 region
Ill805R	5′- GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G NNNN GAC TAC NVG GGT ATC TAA KCC-3′	V4 region
Ill532R	5′- GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G NNNN TTA CCG CGG CKG CTG GCA C -3´	V3 region
EUB8F	5′- AGA GTT TGA TCM TGG CTC AG -3′	V3 region
984yR	5′- GTA AGG TTC YTC CGC GT -3′	V3 region
338F-gc	5′-cgc ccg ccg cgc gcg gcg ggc ggg gcg ggg gca cgg gggg ACT CCT ACG GGA GGC AGC AG -3′	V3 region
518R	5′- ATT ACC GCG GCT GCT GG -3′	V3 region
338F-gc-M13R	5′ – CAG GAA ACA GCT ATG ACC GCC CGC CGC GCG CGG CGG GCG GGG CGG GGG CAC GGG GGG ACT CCT ACG GGA GGC AGC AG – 3′	Sanger seq.
M13R seq.	5´ - CAG GAA ACA GCT ATG ACC – 3´	Sanger seq.
RT-996F	5´- GCA ACG GCM RGA ACC TTA CCT A - 3´	qPCR
RT-1089R	5´- CSG GAC TTA ACC SAA CAT YTC A - 3´	qPCR

Table 2.4: PCR reaction components for four different polymerases used to amplify variable regions of the bacterial 16S rDNA.

	Phusion Hot Start polymerase II (Thermo Scientific)	**KAPA 2G Robust PCR kit (Sigma-Aldrich)**	**The PrimeStar DNA polymerase (TaKaRa Bio USA, Inc.)**	**ExTaq polymerase (TaKaRa Bio USA, Inc.)**
Reagents				
Reaction buffer	1X (1.5 mM $MgCl_2$)	1X (1.5 mM $MgCl_2$)	1 X (1 mM $MgCl_2$)	1X (2 mM $MgCl_2$)
dNTP (Thermo Scientific)	0.2 mM each	0.2 mM each	0.2 mM each	0.2 mM each
$MgCl_2$ (Thermo Scientific)	0.5 mM	0.5 mM	-	-
Primers (F&R)	0.3 μM each	0.5 μM each	0.5 μM each	0.4 μM each
Polymerase	0.02 U μL^{-1}	0.02 U μL^{-1}	0.015 U μL^{-1}	0.025 U μL^{-1}
Glycerol (VWR)/Spermidine (Sigma-Aldich)*	10%/0.5mM	-	-	10%/0.5mM

* Were applied in some of the reactions as PCR facilitators

Table 2.5: Cycling conditions for the four polymerases used to amplify the variable regions of the bacterial 16S rDNA.

Step	**Phusion Hot Start polymerase II (Thermo Scientific)**		**KAPA 2G Robust PCR kit (Sigma-Aldrich)**		**The PrimeStar DNA polymerase (TaKaRa Bio USA, Inc.)**		**ExTaq polymerase (TaKaRa Bio USA, Inc.)**	
	Temp	Time	Temp	Time	Temp	Time	Temp	Time
Denaturation	98°C	15 sec.	98°C	15 sec.	98°C	10 sec.	94°C	10-30 sec.
Annealing	55°C	15 sec.	54-58°C	15 sec.	55°C	15 sec.	53-55°C	30-60 sec.
Elongation	72°C	15 sec.	72°C	15/30 sec.	68°C	60 sec.	72°C	45-60 sec.
Final elongation	72°C	5 min.	72°C	5 min.	68°C	5 min.	72°C	2 min.
Number of cycles	36		35		30-35		30	

2.3 Denaturing Gradient Gel Electrophoresis (DGGE)

DGGE can be used to investigate the microbial community composition, where DNA fragments are separated according to sequence variation. The polyacrylamide gel consists of a linearly increasing gradient of the denaturants formamide and urea. The DNA molecule migrates in the gel until denaturation, and the denaturation depends on the molecules melting point. A GC-clamp is added to one of the primers to prevent complete denaturation of the PCR product, resulting in a more sensitive separation of the sequences (Sheffield et al., 1989). As the rDNA molecules migrates and denatures, a pattern of bands are created in the gel. This pattern reflects the variety of species present in the samples, where different bands indicate different bacterial species (Muyzer and Smalla, 1998).

To characterize the microbial diversity on the Atlantic salmon skin mucus, the 26 samples in Table 2.1 were subjected to DGGE analysis of the V3 16S rDNA region. The Atlantic salmon skin samples were cut out in equal sizes (25 mm in diameter) using the Beef Steaker (Bürkle) and mucus were scraped off using a sterile scalpel (Swann-Morton). DNA was extracted from

the skin mucus using the DNeasy PowerSoil Kit (Qiagen) following the manufacturer's protocol (Appendix A).

The V3 region of the 16S rDNA was amplified using 338F-gc and 518R primers (Tab. 2.3), spermidine and the reagents and cycling conditions for Phusion polymerase (Tab. 2.4 and Tab. 2.5). DGGE was performed on the PCR amplicons encompassing the V3 region of the 16S rRNA gene on the INGENY phorU system (Ingeny), for a rapid fingerprint analysis of microbial community composition. Two glass plates, the spacer and the comb were washed using Deconex soap and hot tap water. One side of each glass plate was polished using 70% ethanol and Kimwipe paper. The glass plates and the spacer were placed in the gel box and the comb was put on top. An 8% acrylamide gel with 35% to 55% denaturing gradient (Tab. 2.6), where 100% denaturation equals 7M urea and 40% formamide, was used. Tetramethylenediamine (TEMED) and 10% ammonium persulphate (APS) were added prior to casting the gel for polymerization. The gel was casted using a gradient mixer to create the denaturing gradient with the high denaturing concentration (55%) at the bottom and the lower denaturing concentration (35%) towards the top, following a stocking solution (0% denaturing) applied at the top of the gel. The comb was pressed down, and the gel was left to polymerize in 20L electrophoresis buffer (0.5 TAE) at 60°C for two hours.

The gel cassette was placed in the buffer tank and prepared for loading of samples. A mixture of loading dye (3 µL) and PCR product (15 µL) were loaded on the gel. The gel was run for approximately 22 hours at 100V.

After electrophoresis, the gel was transferred to a plastic foil sheet and stained with a mixture of 3 uL SYBR Gold (Invitrogen) and 30 mL 1 x TAE (Appendix D) in the dark for one hour, at room temperature. After staining, the gel was visualized under UV-light (G:BOX GelDoc, Syngene) and photographed using GeneSnap software (SynGene). Selected bands were excised from the gel for sequence analysis.

Table 2.6: Reagents used in the gradient gel with 0%, 35% and 55% denaturing acrylamide used to separate the V3 regions of bacterial 16S rDNA from skin mucus samples collected from six sea cages.

Denaturing acrylamide (%)	0%	80%	TEMED + 10% APS	Total Volume
0	8 mL		10 + 40 uL	8 mL
35	13.5 mL	10.5 mL	16 + 87 uL	24 mL
55	7.5 mL	16.5 mL	16 + 87 uL	24 mL

2.3.1 Reamplification, Purification and Sanger Sequencing of DGGE Products

The 19 excised DGGE bands were reamplified using the primers 338F-GC-M13R and 518R (Tab. 2.3), spermidine and the reagents and cycling conditions presented in Table 2.4 and Table 2.5 for Phusion Hot Start Polymerase II. QIAquick purification kit (Qiagen) was used to purify the PCR products. As described by the manufacturer, 20 µL PCR product and 100 µL PB buffer were used (Appendix E). The purified PCR products (5 µL) were sequenced by Sanger sequencing at GATC Biotech (Germany) using 2.5 µM of M13R as primer (Tab. 2.3).

Taxonomic assignment was performed for the DNA sequences received from GATC Biotech. The results were presented as chromatograms, where the peaks in the chromatogram represent the bases in the sequences. The files were opened in SnapGene Viewer (version 3.2.1) and the sequence quality was examined by inspecting the chromatograms. The text files were exported as fasta files. Primer sequences and noisy areas were removed and the remaining sequence was used for the taxonomic analysis. The sequences were analysed using the Ribosomal Database Project (RDP) Classifier tool (Wang et al., 2007). The confidence threshold was set to 50%, as recommended by the Classifier tool, for sequences shorter than 250 base pairs.

2.4 Preparation of Amplicon Library for Illumina Sequencing

HTS methods, such as Illumina sequencing of 16S rDNA amplicons, can be used to characterize microbial diversity in environmental samples. An amplicon library can be created by amplifying 16S rDNA regions using broad-range bacterial primers. After the PCR amplification, normalization is conducted to adjust amplicon concentration. The normalized PCR products in all samples are then “barcoded” by introducing unique indices to separate sample groups. The indices are introduced as a part of the primers in a second round of PCR.

This is necessary, due to eventual pooling of all samples. The DNA is denatured into single strands and attached to a flow cell where bridge amplification occurs. Clusters of DNA fragments with identical sequences are formed. The DNA is made single-stranded, and further sequenced by synthesis (SBS). Fluorescently labelled deoxyribonucleotide triphosphates (dNTPs) are incorporated into the DNA template strand, and the incorporation is identified by excitation of the fluorophore (Illumina Inc, 2016).

To characterize the microbial diversity on the Atlantic salmon skin mucus, the 26 samples in Table 2.1 were subjected to Illumina sequencing of the V4 16S rDNA amplicons. The DNA extracts used, were the same as the DNA extracts prepared for DGGE analysis (see 2.3). The amplicons were prepared as described in Table 2.4 and Table 2.5 for Phusion Hot Start Polymerase II with the ill515F and ill805R primers (Tab. 2.3) and spermidine. To examine the yield and band size of the PCR product, an 1% agarose gel with GelRed™ (VWR) in 1 x TAE buffer (Appendix D) was run. The PCR products were normalized and purified using the SequalPrep™ Normalization Plate kit (Invitrogen) following the manufacturer's protocol (Appendix F), to achieve similar concentration of DNA in all samples.

Nextera XT Index Kit (Illumina) was used to add unique index sequences to each PCR product. The PCR reactions were performed in a total volume of 25 µL, using 0.25 mM dNTP (Thermo Scientific), 2 mM $MgCl_2$ (Thermo Scientific), 0.015 U μL^{-1} Phusion Hot Start Polymerase II (Thermo Scientific) and 1x Phusion HF Buffer™ (Thermo Scientific). The reagents were mixed in an Eppendorf tube and distributed in a 96 well plate (BioRad). The 8 different index I and 12 different index II (2.5 µL each) were distributed in the 96 wells creating 96 unique index pairs, which are incorporated as a part of the primers in the PCR reaction. The normalized, purified PCR products (2.5 µL) were used as templates, and amplification was performed at 98°C 15 sec. (denaturation), 50°C 20 sec. (annealing) and 72°C 20 sec. (elongation) for 8 cycles in the T100™ Thermal Cycler (BioRad).

The indexed PCR products were run on 1% agarose gel electrophoresis to examine PCR yield. After indexing, the products were normalized and purified a second time using the SequalPrep™ Normalization Plate kit (Invitrogen) following the manufacturer's protocol

(Appendix F), before all the 26 samples were pooled together with 70 samples not related to this project.

The DNA in the pooled sample was concentrated to 12.9 ng μL^{-1} using Amicon Ultra Centrifugal Filter Units (Merck Millipore, Ireland) according to the manufacturer's protocol (Appendix G). Concentration and purity of the sample were determined by NanoDrop™ One (Thermo Scientific). The size of the final product was determined by running a 1% agarose gel.

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

2.4.1 Processing of Illumina Sequencing Data

The sequencing data were processed using the USEARCH pipeline (version 9.2; https://www.drive5.com/usearch/). At the step of merging paired reads, primer sequences were trimmed, and reads shorter than 230 base pairs were excluded. The processing further included demultiplexing, quality trimming by the Fastq_filter command (with an expected error threshold of 1). Chimera removal and clustering at the 97% similarity level was performed using the UPARSE-OTU algorithm (Edgar, 2013). Taxonomy assignment was based on the Sintax script (Edgar, 2013) with a confidence value threshold of 0.8 and the RDP reference data set (version 15). The RDP tools Classifier and sequence Match (Wang et al., 2007) were used to analyse OTUs of interest.

2.4.2 Statistical Analysis

The diversity in a bacterial community can be measured using genetic data retrieved from DNA sequencing of 16S rDNA amplicons. The microbial diversity includes both the species richness and the species evenness. Species richness is the number of species in a community and species evenness is the variability of species abundances in a community. Microbial "species" are often defined by a limit of percent sequence similarity and are usually presented as operational taxonomic units (OTUs) at 97% similarity level, due to sequencing and PCR errors (Bartram et al., 2011). The OTUs with 97% similarity level are presented as the observed OTU richness.

Statistical analyses were performed using the program package PAST version 3.18 (Hammer et al., 2001). To estimate the theoretical OTU richness, the Chao1 (Chao, 1984) index was calculated. The Chao1 index is a correction factor for the observed OTU richness and is an alpha (α) diversity measurement (Whittaker, 1960), that represents the diversity in one habitat or one sample unit. The α-diversity can also be represented by Shannon´s diversity index (Shannon, 1948), which reflects both the relative abundance and the species richness. In this representation, higher values reflect communities with greater spices richness and evenness (Hollister et al., 2015). Both Chao1 and Shannon's diversity indices were calculated for each sample from the absolute OTU table.

A two-sample t-tests was performed to investigate statistical significance between two selected sample groups. However, if the variance were statistically different from each other (p- value < 0.05) revealed by the F-test, an unequal variance t-test was performed. When several sample groups were compared, a one-way analysis of variance (ANOVA) was used. The ANOVA test assumes that the data is normally distributed and that the groups have similar variance. If the variance was similar a Tukey´s pairwise post-hoc test was performed. However, if the data used in the test violated this assumption the Kruskal-Wallis test was used.

To investigate the difference in microbial diversity between samples the beta (β) diversity indices (Whittaker, 1960), Bray-Curtis similarity (Bray and Curtis, 1957) and Jaccard index (Jaccard, 1901) were determined. Bray-Curtis similarity evaluates the degree of similarity between two communities using the number of shared species OTUs to the number of OTUs in both communities, as well as the abundance data of each OTU. The Jaccard index evaluates the degree of similarity between two communities by quantifying the number of OTUs uniquely held by each community, and is a presence/absence index. The β-diversity can be visualised by ordination, such as principal coordinate analysis (PCoA). The PCoA plot is multidimensional scaling which assign each sample to a location in a multidimensional space. The distance between the samples on the plot indicate the similarity/dissimilarity, where similar samples are more closely positioned in a two dimensional plot (Hammer et al., 2008). Bray-Curtis similarity was calculated for community profiles within the sample groups. The similarity/distance measures between all pairs of rows were computed. The average and the standard error of the mean within the groups were calculated in Microsoft Excel. The similarity indices are between

0 and 1, where 1 imply identical community profiles.

A normalized OTU table, where the proportion of each OTU was calculated as the number of reads divided by the total number of reads for each sample, was used to calculate the multivariate statistics. Principal coordinate analysis (PCoA) plots based on Bray-Curtis and Jaccard similarity indices was generated. To investigate if there were any statistical significance between the community profiles between groups of samples, a one-way non-parametric multivariate analysis of variance (PERMANOVA) test was performed for both Bray-Curtis and Jaccard similarity indices. The Bonferroni corrected p-values were applied when more than two sample groups were compared. A similarity percentage (SIMPER) test using Bray-Curtis similarity measures was performed to determine which OTUs that were primarily responsible for the observed difference in community profiles between the sample groups.

2.5 Real-time PCR

Real-time PCR (qPCR) can be used to quantify bacterial DNA in environmental samples. In qPCR experiments, the formation of PCR product is monitored as the amount of DNA synthetized throughout the PCR is amplified. This amplification can be monitored in real time by using a fluorescent signal, such as SYBR® Green that binds double stranded DNA. The fluorescent signal increases dramatically when SYBR® Green binds to the minor groove of double stranded DNA and form a DNA-dye complex during amplification. A quantification cycle (Cq) value is determined at the point where the samples fluorescence signal is larger than the background fluorescence. The Cq value is directly proportionate to the amount of starting DNA template, and can be used as a basis to quantify a specific gene in microorganisms (Cakilci and Gunduz, 2007).

qPCR was conducted to quantify 16S rDNA copies in the skin mucus samples using the broad-range bacterial primers RT996F and RT1089R (Tab. 2.3). To calculate the copy number (CN) of 16S rDNA in the samples, a standard curve had to be created. DNA extracted from a *Vibrio* strain (RD5-30) with a known sequence was amplified using the RT996F and RT1089R primers (Tab. 2.3). The PCR reaction was performed with Phusion Hot Start Polymerase II and spermidine as described in section Table 2.4 and Table 2.5, with the exception of the annealing step, which was conducted at 60 °C. The PCR product was purified using the QIAquick PCR

purification (Qiagen) kit according to the manufacturers protocol (Appendix E). The DNA concentration was measured using the iQuant™ HS dsDNA quantitation assay and Qubit 3 Fluorometer (Invitrogen, Thermo Scientific) (Appendix H). The sample was diluted to 1 ng μL^{-1}.

The qPCR was performed in triplicate reactions in a total volume of 20 µL per reaction in a LightCycler® 96 (Roche). The samples were prepared in a 96 well plate (Thero Scientific) with 0.25 µM of each primer (RT996F and RT1089R), LightCycler 480 Probes Master (1x) (Roche) and 5 µL template (~1 ng μL^{-1}). The samples were pre-incubated at 95 °C (600 sec.), before the denaturing step at 95°C (10 sec.), annealing at 60 °C (10 sec.) and elongation step at 72 °C (10 sec.) were repeated 45 times. After amplification, melting analysis was performed at 95 °C (5 sec.), 65 °C (60 sec.), 97 °C (1 sec.) and the plate was finally cooled at 37 °C (30 sec.). Four individual salmon skin mucus DNA extracts from each of the sample groups (SC1-SC6), a salmon muscle control (SM) and a non-template control (NTC) were used as templates. The PCR product generated from DNA extracts from an isolated *Vibrio* (RD5-30) (~1 ng μL^{-1}) and its ten-fold dilution series, were used as templates in the qPCR reaction to create a standard curve.

2.5.1 Processing of qPCR Data

The data obtained in this project were processed using LightCycler® 96 software (Roche). Amplification products within a triplicate with a Cq value differentiating strongly from the other samples were removed (higher or lower than Cq 3). The average of each triplicate sample was calculated by the LightCycler 96 software (Roche) and exported to Microsoft Excel. The CN in the *Vibrio* (RD5-30) sample was calculated using equation 2.1, with the DNA length of 123 bp and the DNA concentration of the diluted sample (1 ng μL^{-1}).

$$CN_{stock}\left(\frac{molecules}{\mu L}\right) = \frac{DNA_{conc}(g/\mu L)\times 6.022\times 10^{23}(molecules/mol)}{DNA_{length}\times 660(g/mol)} \qquad (2.1)$$

A standard curve was made using the values from the ten-fold dilution series of the DNA extracted from *Vibrio* (RD5-30), excluding the sample with the highest DNA concentration. An

ANOVA test was conducted (see 2.4.2), to examine whether the 16S rDNA copy number determined for the samples differed between the sample groups.

3 Results

3.1 Optimization of PCR Amplification of the Bacterial 16S rDNA from Atlantic Salmon Skin and Skin Mucus

Previous attempts to amplify bacterial 16S rDNA from Atlantic salmon skin have shown to be complicated, probably due to the presence of inhibitors and low fraction of bacterial DNA (personal communication, Ingrid Bakke). A sub-aim of this master thesis was to optimize DNA isolation and a PCR protocol targeting the variable regions V3 and/or V4 of the bacterial 16S rDNA from Atlantic salmon skin and skin mucus. The samples presented in Table 2.2 were used in the DNA extraction and in PCR optimization.

The following processes were tested:

- Nested and non-nested protocols
- The effect of different DNA extraction kits
- Different polymerases
- Different PCR facilitators
- Annealing temperatures
- Amount of template

3.1.1 The Effect of Different DNA Extraction Kits on PCR Amplification Efficiency

The effect of the different DNA extraction kits on PCR success was tested on skin, skin mucus and biofilm control samples, using a nested PCR protocol (see 2.2). The protocol is effective for amplification of the V3 16S rDNA from samples with high fractions of eukaryotic DNA without co-amplification of eukaryotic 18S rDNA (Bakke et al., 2011). However, the nested protocol gave no amplification product for the selected skin and skin mucus samples (Fig. 3.1). Positive control biofilm samples yielded PCR product of the expected length (~230 base pairs) from all DNA extraction kits (Fig. 3.1). The amplification product obtained from DNeasy Powersoil DNA extracts resulted in higher PCR yield than those for the Purelink and QIAamp DNA extracts. The result indicated that better amplification efficiency was obtained by using the DNeasy Powersoil DNA extracts.

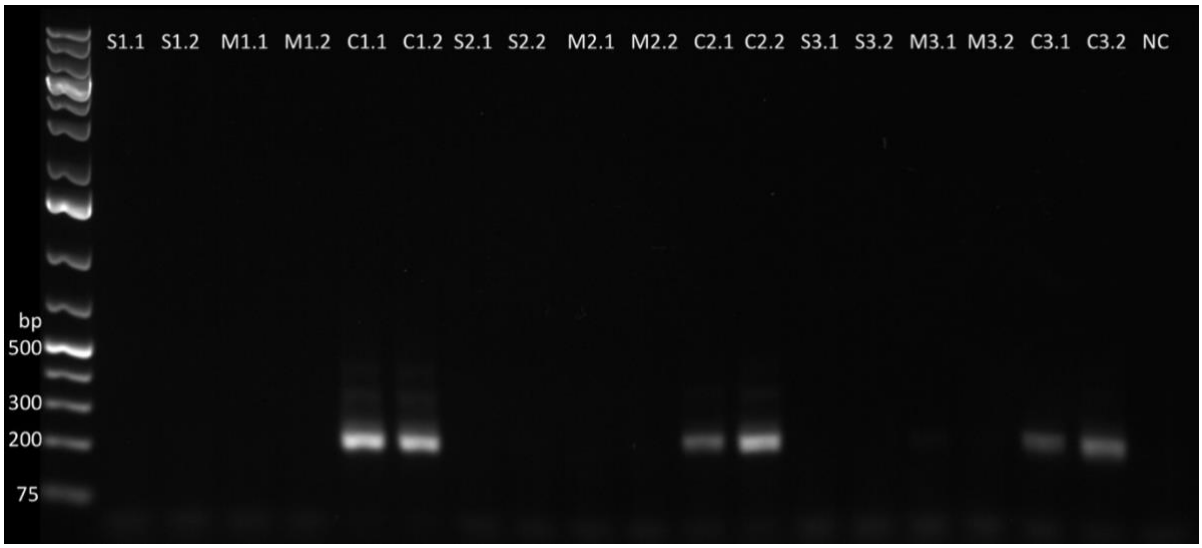


Figure 3.1: Agarose gel of PCR products representing the V3 region of the bacterial 16S rDNA amplified from DNA extracted from skin (S) skin mucus (M) and control biofilm samples (C) using three different DNA extraction kits (DNeasy Powersoil (1), Purelink (2) and QIAamp (3)). The V3 region was amplified using the nested PCR protocol and Phusion polymerase. NC represents the non-template control.

The four PCR polymerases KAPA 2G Robust PCR kit (Sigma-Aldrich), PrimeStar DNA polymerase (TaKaRa Bio USA, Inc.), ExTaq polymerase (TaKaRa Bio USA, Inc.) and Phusion Hot Start polymerase II (Thermo Scientific) were used to amplify the V4 region of the bacterial 16S rDNA (see Tab. 2.4 and 2.5). Selected samples from all DNA extraction kits listed in Table 2.2 were used. KAPA 2G Robust PCR kit (Sigma-Aldrich) and PrimeStar DNA polymerase (TaKaRa Bio USA, Inc.) did not succeed in producing PCR product of the expected length (~360 bp) for the skin and skin mucus samples. However, PCR product of the expected length was produced for the biofilm control samples (gel not shown). This indicated no efficient amplification of the V4 region of skin and skin mucus samples using these two polymerases.

PCR products of the expected length were produced when using ExTaq polymerase (TaKaRa Bio USA, Inc.) and Phusion Hot Start polymerase II (Thermo Scientific) for skin and skin mucus samples. Generally, higher yields of PCR products were obtained for skin samples than skin mucus samples with the Phusion polymerase, independent of DNA extraction kit (Fig. 3.2). However, the length of the PCR products obtained for the skin samples were shorter than those obtained from control samples of the expected length (~360 bp). For the skin mucus samples, the PCR products yielded double bands. One band was a shorter PCR product and the other band had the expected length. However, the bands were very weak and could be a

contamination. The PCR product in the non-template control was weak, but of the expected length, and indicated contaminating DNA. (The weak bands are representing low PCR product yield are not very well visualized on the gel pictures.)

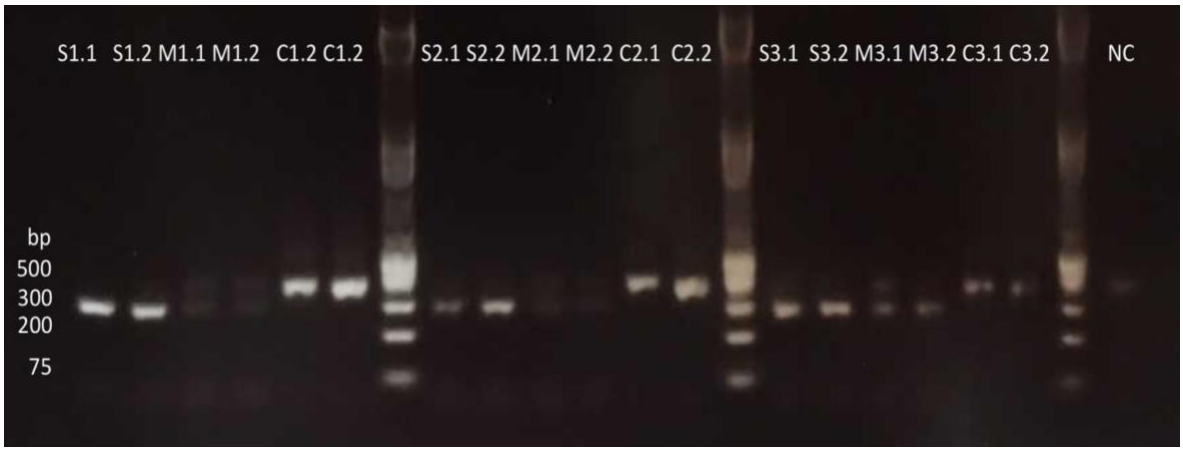


Figure 3.2: Agarose gel of PCR products representing the V4 region of the bacterial 16S rDNA from skin (S), skin mucus (M) and a control biofilm samples (C). Samples were amplified using Phusion polymerase. NC represents the non-template control. Three different DNA extraction kits (DNeasy Powersoil (1), Purelink (2) and QIAamp (3)) were used to extract DNA from the samples.

The PCR product obtained from skin and skin mucus samples from the amplified V4 region using ExTaq polymerase (Fig. 3.3) were similar to the results using the Phusion polymerase. Higher yield of PCR product was obtained from the skin samples than the skin mucus samples. Both skin and skin mucus PCR products were shorter than the biofilm control samples of expected length. Although, in the non-template control showed no contaminating DNA, sample M3.1 had a double band. The band of the unexpected length in both skin and skin mucus samples may represent a non-specific PCR product, for instance salmon rDNA (personal communication, Ingrid Bakke).

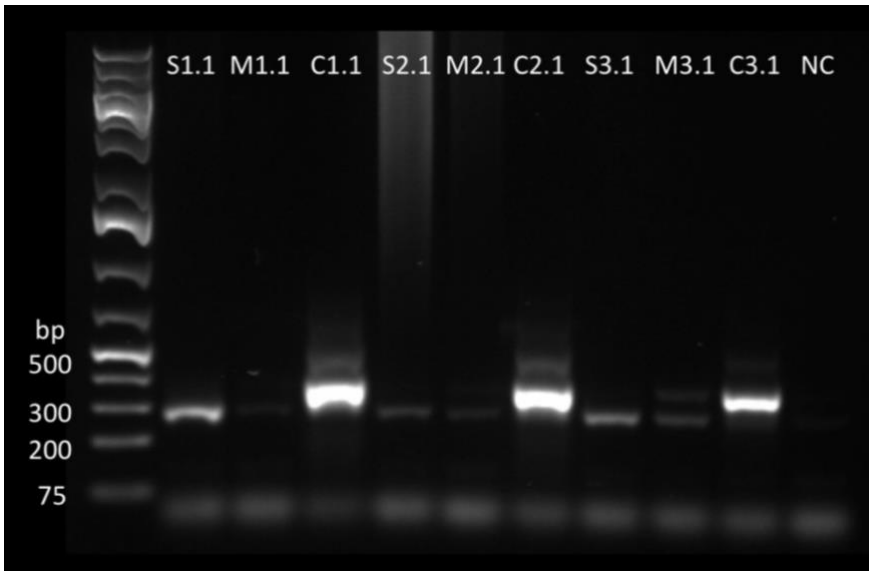


Figure 3.3: Agarose gel of PCR products representing the V4 region of the bacterial 16S rDNA from skin (S), skin mucus (M) and a control biofilm samples (C). Samples were amplified using ExTaq polymerase. NC represents the non-template control. Three different DNA extraction kits (DNeasy Powersoil (1), Purelink (2) and QIAamp (3)) were used to extract DNA from the samples.

Amplification with both Phusion polymerase and ExTaq polymerase resulted in high PCR yield for DNeasy Powersoil DNA extracts (S1.1-C1.2). The DNeasy Powersoil DNA extracts were further used to optimize the PCR protocol.

3.1.2 Comparison of Reaction Components Using ExTaq and Phusion Polymerase

Amplification of the V4 16S rDNA from the skin and skin mucus DNA extracts (Tab. 2.4 and 2.5) yielded amplification products of unexpected length (< 360 bp). Thus, various reverse PCR primers, PCR reaction buffers and PCR facilitators were tested to obtain the expected PCR product. Comparison of ExTaq and Phusion polymerase was conducted by using the DNeasy Powersoil DNA extracts.

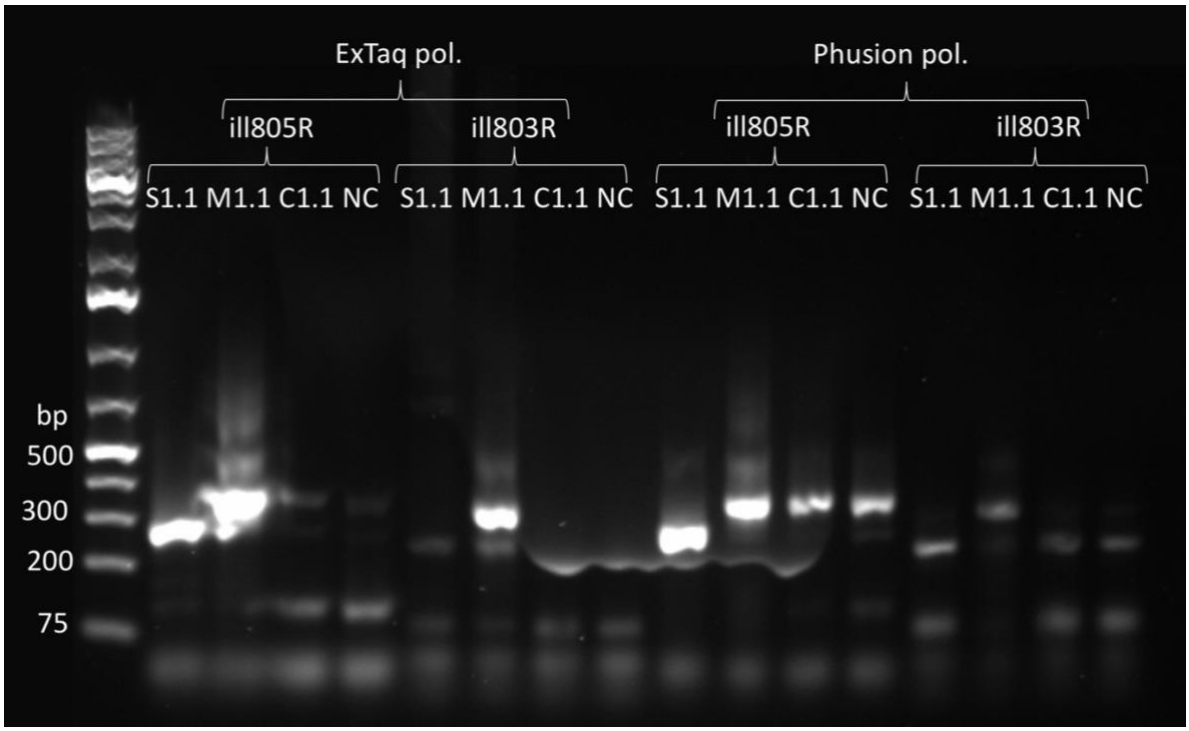


Figure 3.4: Agarose gel of PCR products representing the V4 region of the bacterial 16S rDNA extracted by the DNeasy Powersoil kit from skin (S), skin mucus (M) and a control biofilm samples (C). Samples were amplified using ExTaq polymerase and Phusion polymerase. NC represents the non-template control. The reverse primers ill805R and ill803R were used with for both polymerases.

First, the reverse primer ill805R was compared to ill803R, by using both ExTaq and Phusion polymerase. Both ExTaq and Phusion polymerase had high PCR product yield using the ill805R primer, indicating high amplification efficiency (Fig. 3.4). Although PCR products was generated for the non-template controls, higher PCR product yields were obtained for the skin and skin mucus samples. The amplification products for skin mucus and control samples were of the expected length, indicating amplification of the bacterial V4 16S rDNA region. The skin sample amplification products were shorter than expected, and may represent salmon rDNA. The reverse primer ill805R was used for further optimization of the PCR protocol.

Due to unspecific amplification of the skin samples (Fig. 3.4), the PCR facilitators spermidine and glycerol were tested with both ExTaq and Phusion polymerase. Phusion Hot Start polymerase II (Thermo Scientific) was tested with two different reaction buffers, the standard HF-buffer and GC-buffer (for GC-rich templates). Four reactions were performed with the Phusion polymerase. The first reaction was performed with the HF-buffer, as previously. The

second reaction was performed with GC-buffer, and the two last reactions were performed with HF-buffer. The third reaction had additional glycerol and the fourth reaction had additional spermidine. Three reactions were performed with the ExTaq polymerase. A reaction with no PCR facilitators was compared to two separate reactions with either additional glycerol or spermidine.

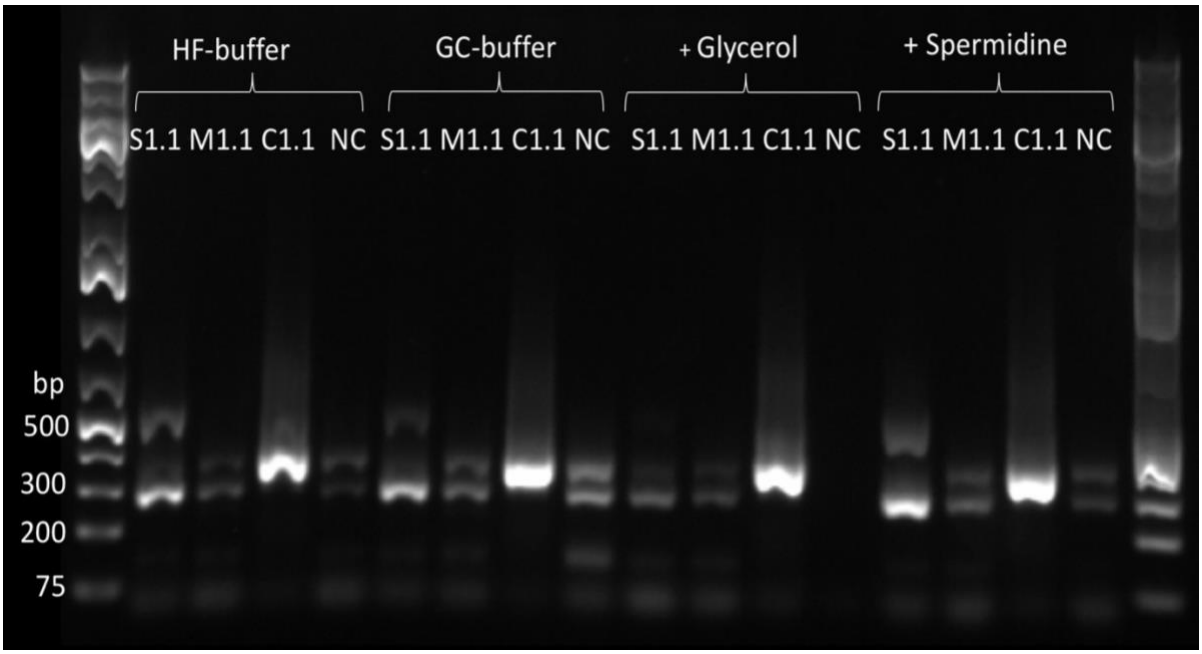


Figure 3.5: Agarose gel of PCR products representing the V4 region of the bacterial 16S rDNA extracted by the DNeasy Powersoil kit from skin (S), skin mucus (M) and a control biofilm samples (C). Samples were amplified using Phusion polymerase. NC represents the non-template control. Four different reactions were performed. First reaction was performed with HF-buffer, second reaction with GC-buffer, third and fourth reaction with HF-buffer. The third reaction had additional glycerol and fourth reaction had additional spermidine.

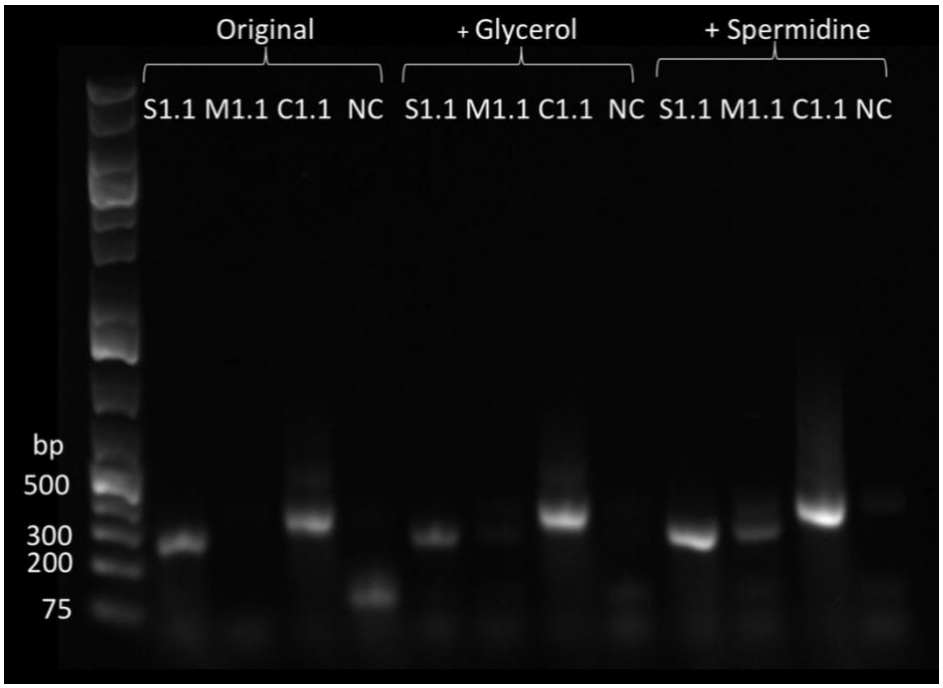


Figure 3.6: Agarose gel of PCR products representing the V4 region of the bacterial 16S rDNA extracted by the DNeasy Powersoil kit from skin (S), skin mucus (M) and a control biofilm samples (C). Samples were amplified using ExTaq polymerase. NC represents the non-template control. Three different reactions were performed. First reaction had no PCR facilitators, the second reaction had additional glycerol and the third reaction had additional spermidine.

Spermidine had a positive effect on the amplification using the ExTaq polymerase (Fig. 3.6). Both spermidine and glycerol had a positive effect on the amplification using the Phusion polymerase (Fig. 3.5). However, PCR product was generated for the non-template controls in the spermidine reaction using Phusion polymerase (Fig 3.5), indicating contaminating DNA. Since addition of spermidine improved the PCR product yield for both polymerases it was used for further optimization. Phusion polymerase amplified the expected length of the PCR product from the skin mucus samples and was further used in PCR optimization.

PCR product in the non-template control showed that contamination was a problem. A DNA sample, representing a "kit blank" control (KB) was included to investigate bacterial contamination associated with the DNA extraction kit. This DNA sample was produced by extracting DNA from a MQ-water sample (expected to be bacteria free) by the DNeasy powersoil kit. The KB sample was used in subsequent PCR optimization experiments.

To investigate the effect of annealing temperature on PCR specificity, a gradient PCR was conducted. PCR products were generated by using the Phusion polymerase at three different annealing temperatures; 57 °C, 55 °C and 53°C.

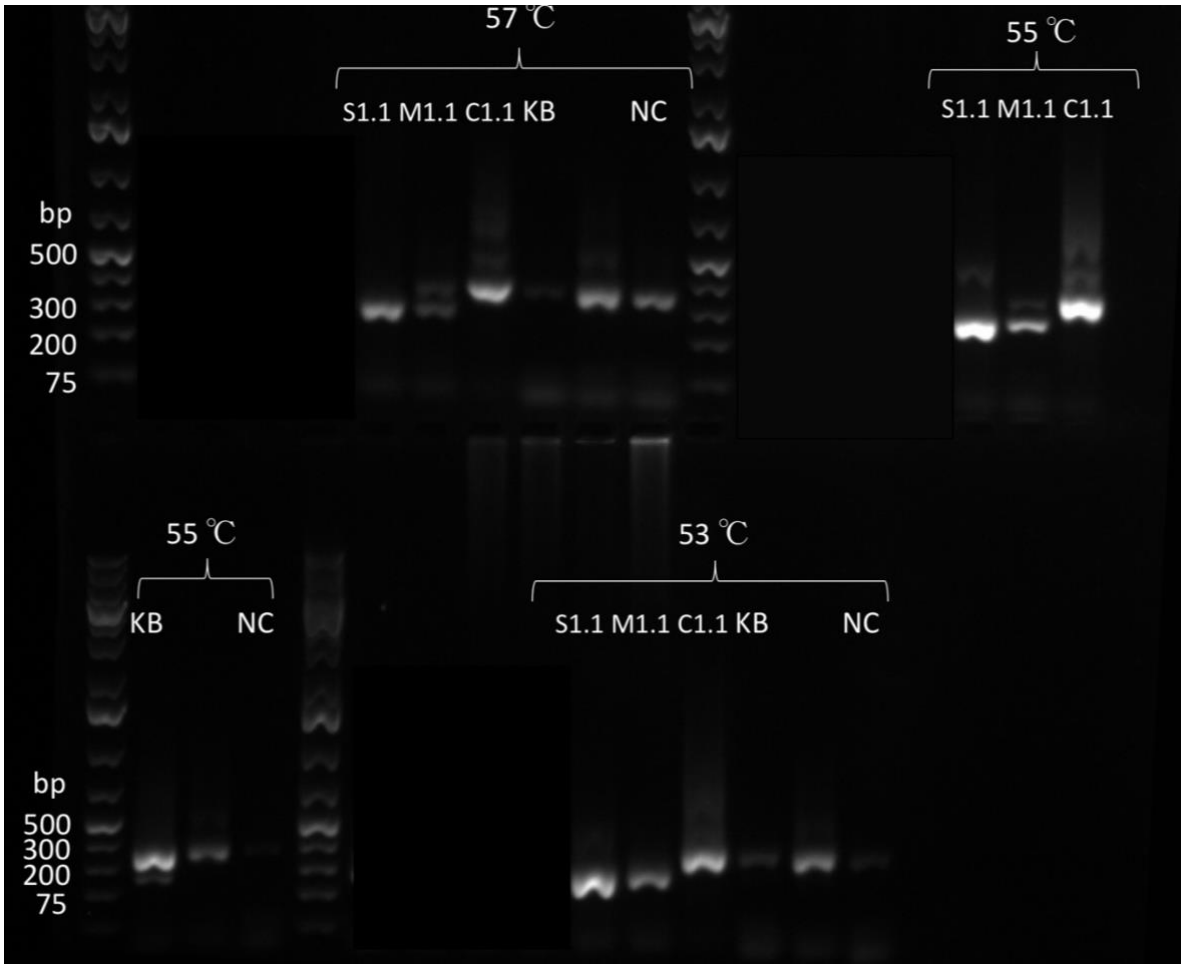


Figure 3.7: Agarose gel of PCR products representing the V4 region of the bacterial 16S rDNA extracted by the DNeasy Powersoil kit from skin (S), skin mucus (M) and a control biofilm samples (C). Samples were amplified using Phusion polymerase. KB represents the "kit blank" control and NC represents the non-template control. The same reaction was performed at three different annealing temperatures 57 °C, 55 °C and 53°C.

An increased annealing temperature had no effect on the amplification specificity of bacterial V4 16S rDNA from both the skin and skin mucus samples (Fig. 3.7). However, more amplification products were obtained at 55 °C annealing temperature, compared to the other temperatures. Annealing at 55 °C was used in the optimized PCR protocol.

The amplification efficiency may be affected by inhibitors in the DNA extract. Throughout the optimization of the PCR protocol, 1:10 diluted DNA extracts (~1 ng μL^{-1}) were used as templates. However, low amounts of bacterial DNA template in the DNA extract may lower the amplification efficiency. As a last step in the PCR protocol optimization, the effect of template amounts was investigated. Undiluted DNA samples, 1:5 diluted and 1:10 diluted samples were used as templates. Undiluted samples (~10 ng μL^{-1} DNA) resulted in higher PCR product yield and were therefore used in the optimized PCR protocol.

To avoid amplification of eukaryotic DNA from the Atlantic salmon skin, skin mucus was used to amplify the 16S rDNA variable regions of the bacteria associated with the fish skin. DNA from the samples collected from Åkerblå presented in Table 2.1 were extracted using DNeasy Powersoil kit, and further amplified using the optimized PCR protocol. The optimized PCR protocol was performed using Phusion polymerase, HF-buffer and the PCR facilitator spermidine, together with necessary reaction components described in section 2.2 for Phusion polymerase (Tab 2.4 and 2.5). The annealing temperature used were 55 °C and the number of temperature cycles were 36.

3.2 PCR Amplification of the Bacterial 16S rDNA from Atlantic Salmon Skin Mucus

The optimized PCR protocol was used with two primer pairs to amplify the V3 region (ill338F and 518R) and the V4 region (ill515F and ill805R). PCR products of the expected length were obtained for both primer pairs, which indicated successful amplification of bacterial DNA from the skin mucus samples (Fig. 3.8). A salmon muscle sample (SM), expected to contain only eukaryotic DNA, was included to identify potential amplification of product representing Atlantic salmon genes. The SM sample amplification product was shorter than the control biofilm sample and the skin mucus samples of the expected length. This indicated that the eukaryotic DNA is targeted by the broad-range primers as well. However, bacterial DNA was amplified from the skin mucus DNA extracts.

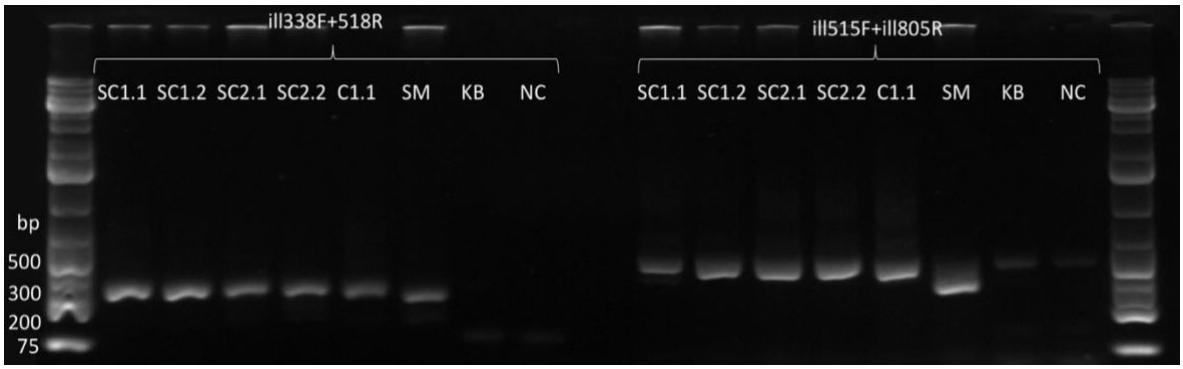


Figure 3.8: Agarose gel of PCR products representing the V3 (ill338F+518R) and V4 (ill515F+ill805R) region of the bacterial 16S rDNA and eukaryotic DNA (for SM) extracted by the DNeasy Powersoil kit from skin mucus samples and control biofilm samples (C). Samples were amplified using Phusion polymerase. SM represents DNA extracted from salmon muscle, KB represents the "kit blank" and NC represents the non-template control.

There were little or no PCR product for the KB and NC samples. This indicated that there no longer was a problem with contaminating DNA. The primer pair ill515F+ill805R was further used to amplify the bacterial V4 16S rDNA region from all skin mucus samples obtained by Åkerblå. These amplification products were subsequently sequenced by Illumina MiSeq sequencing.

3.3 DGGE Analysis of Skin Mucus Microbiota

DGGE was performed to study the microbial community profiles of skin mucus samples from six sea cages with Atlantic salmon treated with various salmon lice treatment (SC1-6). The primer pair 338F-gc and 518R was used to amplify the bacterial V3 16S rDNA used in the DGGE analysis of skin mucus microbiota (see 2.3).

The gel indicated some variation in the microbial community structures between the different salmon lice treatments (Fig. 3.9). DGGE profiles of samples from salmon treated with SLICE, SLICE + freshwater and SLICE + H_2O_2, in SC3, SC4 and SC6 were similar to each other. The skin mucus from ulcerated fish treated with freshwater in SC1 had a different microbial community profile compared all the other individuals sampled. The untreated samples from SC2 and the SLICE treated samples from SC5, also appeared to have a different microbial community profile. The SM sample differed from the other samples and showed only one strong

band on the gel. This indicated that the sample represented eukaryotic DNA. No strong bands appeared at the eukaryotic DNA denaturing point in the samples from the six sea cages, which indicated specific amplification of bacterial DNA from skin mucus. The bands subjected to reamplification and DNA sequencing (1 to 19; Fig. 3.9) (see 2.3.1) were taxonomically assigned (Tab. 3.1).

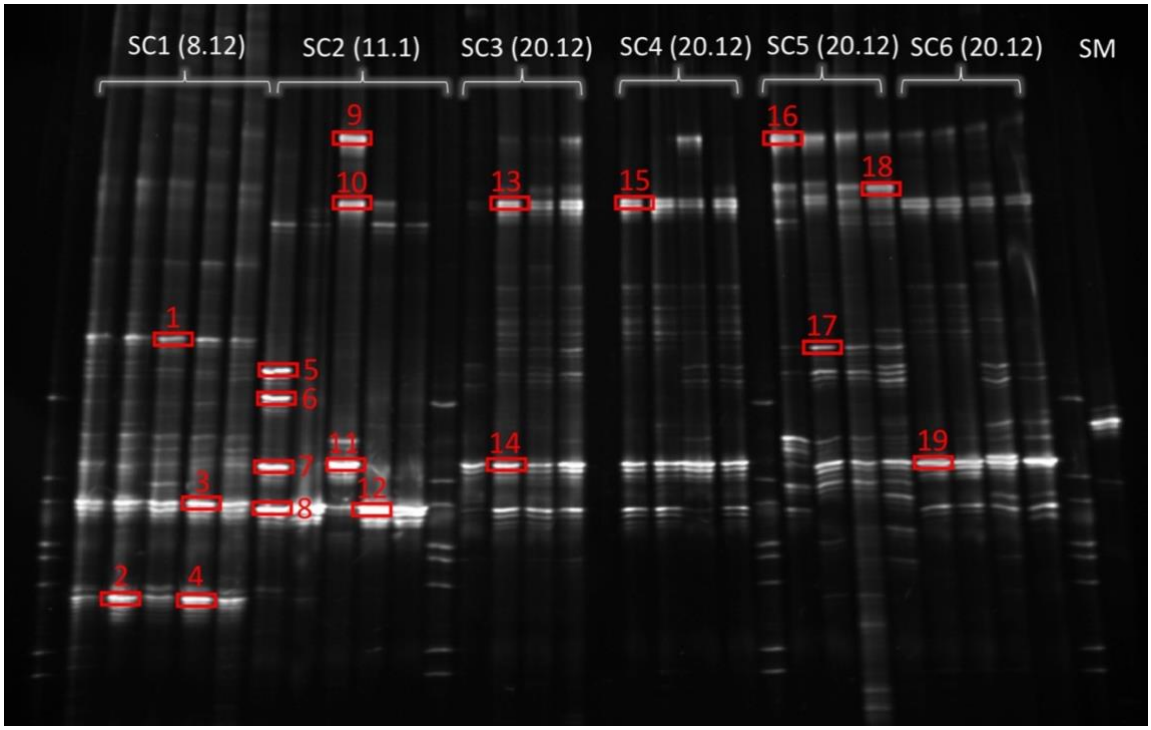


Figure 3.9: DGGE profiles for V3 region of bacterial 16S rDNA amplicons obtained from DNA extracted from skin mucus of Atlantic salmon from six sea cages (SC1-6). The bands marked 1-19 (in red) were isolated and sequenced. SM represents an Atlantic salmon muscle sample.

Table 3.1: Taxonomic assignment based on sequence analysis of excised bands from the DGGE gel in Figure 3.9. The Phylum, class, family and genus are given for each excised band and the confidence threshold (CT) is given by the RDP classifying tool.

Band ID	Sea cage	Phylum	Class	Family	Genus
1	SC1	Proteobacteria 100%	Gammaproteobacteria 93%	Moraxellacea 86%	*Psychrobacter* 65%
2	SC1	Firmicutes 100%	Bacilli 100%	Listeriaceae 99%	*Brochothrix* 99%
3	SC1	Firmicutes 34%	Bacilli 27%	Carnobacteriaceae 23%	*Dolosigranulum* 11%
4	SC1	Firmicutes 100%	Bacilli 100%	Listeriaceae 99%	*Brochothrix* 99%
5	SC2	Proteobacteria 100%	Gammaproteobacteria 100%	Pseudomonadaceae 100%	*Pseudomonas* 100%
6	SC2	Proteobacteria 100%	Gammaproteobacteria 100%	Pseudomonadaceae 100%	*Pseudomonas* 100%
7	SC2	Proteobacteria 100%	Gammaproteobacteria 100%	Pseudomonadaceae 99%	*Pseudomonas* 98%
8	SC2	Proteobacteria 100%	Gammaproteobacteria 100%	Pseudomonadaceae 100%	*Pseudomonas* 100%
9	SC2	Proteobacteria 100%	Gammaproteobacteria 100%	Pseudomonadaceae 100%	*Pseudomonas* 98%
10	SC2	Proteobacteria 100%	Gammaproteobacteria 100%	Pseudomonadaceae 100%	*Pseudomonas* 100%
11	SC2	Proteobacteria 100%	Gammaproteobacteria 100%	Pseudomonadaceae 100%	*Pseudomonas* 100%
12	SC2	Proteobacteria 100%	Gammaproteobacteria 100%	Pseudomonadaceae 99%	*Pseudomonas* 70%
13	SC3	Proteobacteria 100%	Gammaproteobacteria 100%	Pseudomonadaceae 100%	*Pseudomonas* 100%
14	SC3	Proteobacteria 100%	Gammaproteobacteria 100%	Pseudomonadaceae 100%	*Pseudomonas* 100%
15	SC4	Proteobacteria 100%	Gammaproteobacteria 100%	Pseudomonadaceae 100%	*Pseudomonas* 100%
16	SC5	Proteobacteria 100%	Gammaproteobacteria 100%	Pseudomonadaceae 100%	*Pseudomonas* 100%
17	SC5	Proteobacteria 100%	Gammaproteobacteria 100%	Pseudomonadaceae 100%	*Pseudomonas* 99%
18	SC5	Proteobacteria 100%	Gammaproteobacteria 100%	Pseudomonadaceae 100%	*Pseudomonas* 100%
19	SC6	Proteobacteria 100%	Gammaproteobacteria 100%	Pseudomonadaceae 100%	*Pseudomonas* 100%

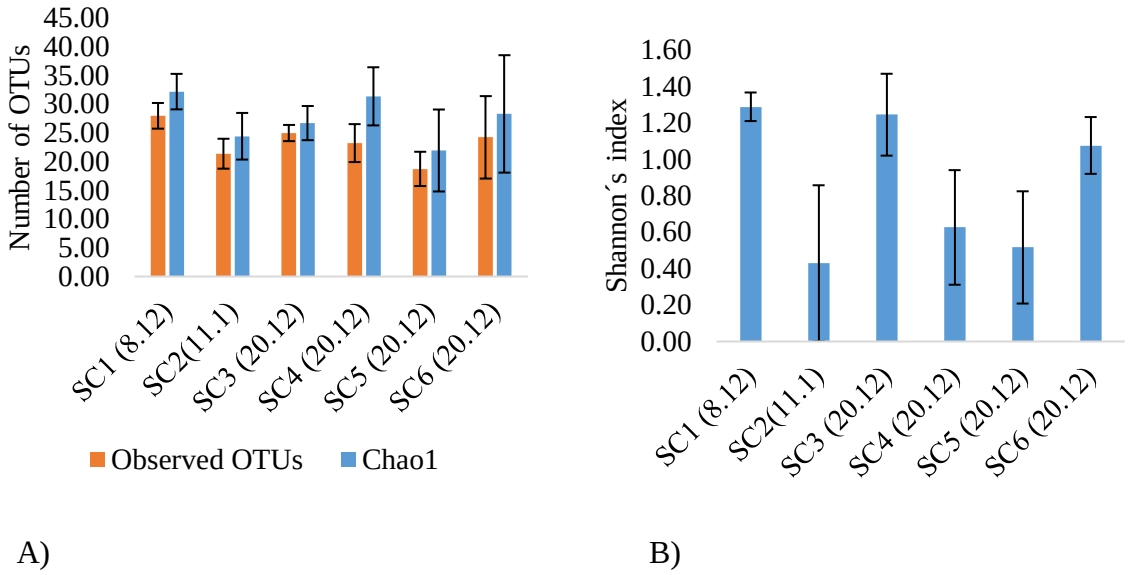
The strong bands 1-4 (Fig. 3.9) in the DGGE profile seemed to be unique for skin mucus from ulcerated fish in SC1 treated with freshwater. The most abundant bacteria in the community profiles were found to represent *Psychrobacter*, *Brochothrix* and *Dolosigranulum* (Tab. 3.1). The strong bands 5-19 (Fig. 3.9) in the DGGE profile were all found to represent *Pseudomonas* (Tab. 3.1). The bands representing *Pseudomonas* were common in samples treated with various salmon lice treatments from SC2-6, but did not appear in the skin mucus samples from ulcerated fish in SC1 treated in a freshwater bath. The bands had different melting points, and alignment studies revealed sequence variation indicating presence of several *Pseudomonas* strains.

3.4 Characterization of Skin Mucus Communities by Illumina Sequencing of 16S rDNA Amplicons

Bacterial communities in skin mucus samples from the Atlantic salmon treated with various salmon lice treatments were examined after Illumina MiSeq sequencing of the V4 16S rDNA amplicons (~230 bp) (see 2.4). The total number of reads after USEARCH quality filtering and chimera removal were 164 686 for the 26 samples. The average number of reads for each sample were 63 341 ± 11 816. The total number of reads for each individual sample is given in Appendix I (Tab. AI.1). Clustering of sequence reads into operational taxonomic units (OTUs) at the 97% similarity level (see 2.4.1) resulted in a total of 68 OTUs.

3.4.1 Alpha Diversity

The α-diversity of the OTU community profiles was investigated, by estimating OTU richness (Chao1) and the observed number of OTUs (Fig. 3.10A). Comparison of the Chao1 and the observed OTU richness showed that the sequencing effort covered 86.7 ± 9.6% on average. The Shannon´s diversity index, representing both the richness and evenness, was determined for all community profiles associated with the skin mucus samples (Fig. 3.10B).



A) B)

Figure 3.10: Average diversity indices for skin microbiota of Atlantic salmon individuals from six sea cages, treated with different salmon lice treatments. A) Observed OTU richness and estimated Chao1 OTU richness. B) Shannon´s diversity index. Error bars represent standard error of the mean.

The OTU richness appeared to be relatively similar for samples from skin mucus from different sea cages (Fig. 3.10A). No significant difference in OTU richness between the individuals in the different sea cages were found (ANOVA p-value > 0.05). Average Shannon´s diversity indicated that the skin mucus microbiota had greater OTU richness and evenness for individuals in SC1, SC3 and SC6, which had undergone freshwater, SLICE and SLICE + H_2O_2 treatment, respectively (Fig. 3.10B). The samples from SC2, SC4 and SC5, which were untreated, treated with SLICE + freshwater and SLICE, respectively, reflected communities with fewer OTUs and/or uneven distribution among them.

The relative microbial community composition at genus level showed that *Pseudomonas* was the most abundant genera in almost all samples (Fig. 3.11). An exception were samples retrieved from ulcerated fish treated with freshwater treatment in SC1, where *Psychrobacter* was the most abundant genus.

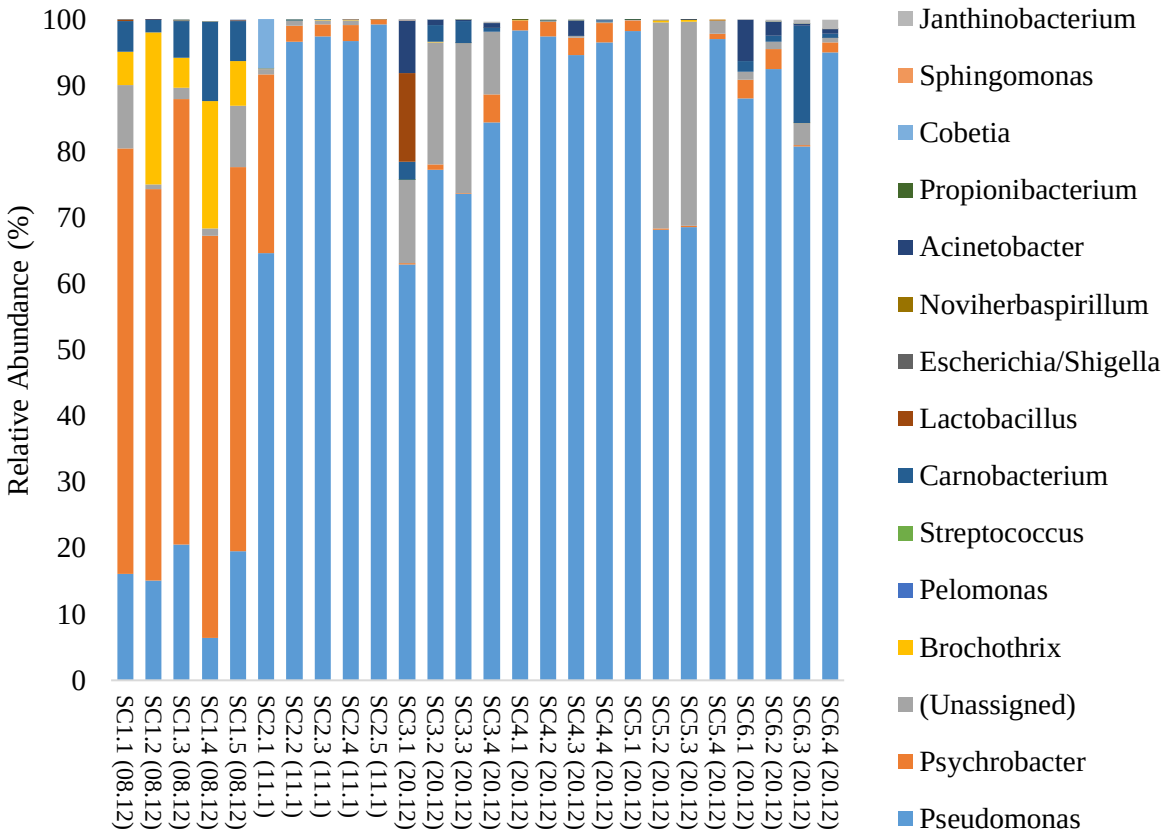


Figure 3.11: Relative microbial community composition at genus level in individual samples. Only genera more abundant than 1% in at least one sample is included.

To get an overview of the most abundant OTUs in the samples from each sea cage, the five most abundant OTUs for each group of were determined (Tab. 3.2). OTU_1, *Pseudomonas* was most abundant in the samples from SC2-6, and highly abundant in the samples from SC1. OTU_24, also representing *Pseudomonas*, was highly abundant in samples from all sea cages, except SC1. *Pseudomonas* was representing at least 70% of the bacteria in samples from SC2-SC6. For SC1 samples, OTU_2, *Psychrobacter* was the most abundant, and represented 61% of the number of reads. Sequences form OTU_1 and OTU_24 were aligned and showed a difference of 3% (Appendix J).

The ulcerated fish treated with freshwater (SC1) had a different microbial community distribution in the skin mucus compared to the samples from the other sea cages (Fig 3.11 and Tab. 3.2). Samples from SC3 and SC5, both treated with SLICE, differed from the other sample groups, where the relative abundance of *Pseudomonas* was somewhat lower. OTU_3 and OTU_6, the family Enterobacteriaceae, were present in 14-16% of the number of reads in these

samples (Tab. 3.2). The genus level was not assigned for Enterobacteriaceae and these OTUs were one of the five most abundant OTUs in samples from SC1, SC3 and SC5 (Tab. 3.2). Thus, OTU_3 and OTU_6 (Enterobacteriaceae) were most likely responsible for the majority of the unassigned genera in Figure 3.11.

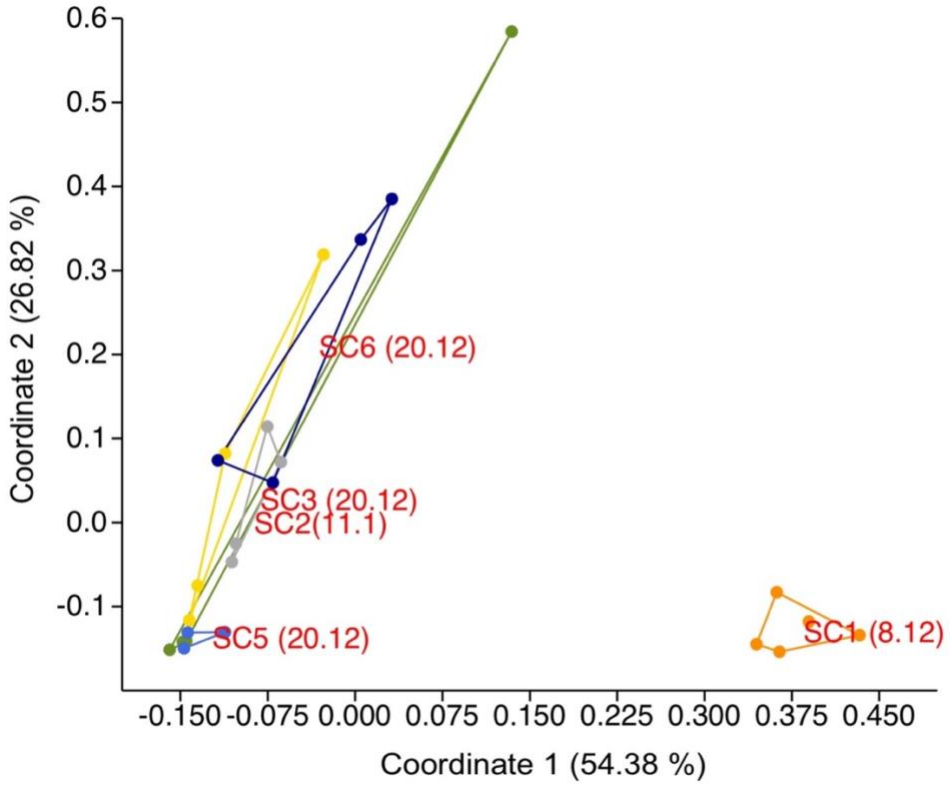
Table 3.2: Overview over the five most abundant OTUs in the six different sea cages. OTU_ID, Taxonomy (Family or *Genus*) and relative average number of reads are listed.

SC1			**SC2**		
OTU ID	Taxonomy	Average	OTU ID	Taxonomy	Average
OTU_2	*Psychrobacter*	0.61	OTU_1	*Pseudomonas*	0.78
OTU_1	*Pseudomonas*	0.12	OTU_24	*Pseudomonas*	0.12
OTU_4	*Brochothrix*	0.12	OTU_9	*Psychrobacter*	0.06
OTU_5	*Carnobacterium*	0.06	OTU_17	*Cobetia*	0.02
OTU_3	Enterobacteriaceae	0.03	OTU_2	*Psychrobacter*	0.01
SC3			**SC 4**		
OTU ID	Taxonomy	Average	OTU ID	Taxonomy	Average
OTU_1	*Pseudomonas*	0.60	OTU_1	*Pseudomonas*	0.74
OTU_24	*Pseudomonas*	0.14	OTU_24	*Pseudomonas*	0.22
OTU_6	Enterobacteriaceae	0.14	OTU_2	*Psychrobacter*	0.02
OTU_11	*Lactobacillus*	0.03	OTU_18	*Acinetobacter*	0.01
OTU_5	*Carnobacterium*	0.02	OTU_22	*Pseudomonas*	4.92E-03
SC5			**SC6**		
OTU ID	Taxonomy	Average	OTU ID	Taxonomy	Average
OTU_1	*Pseudomonas*	0.81	OTU_1	*Pseudomonas*	0.50
OTU_3	Enterobacteriaceae	0.16	OTU_24	*Pseudomonas*	0.38
OTU_24	*Pseudomonas*	0.02	OTU_5	*Carnobacterium*	0.04
OTU_2	*Psychrobacter*	0.01	OTU_13	*Acinetobacter*	0.02
OTU_20	*Pseudomonas*	3.15E-03	OTU_2	*Psychrobacter*	0.01

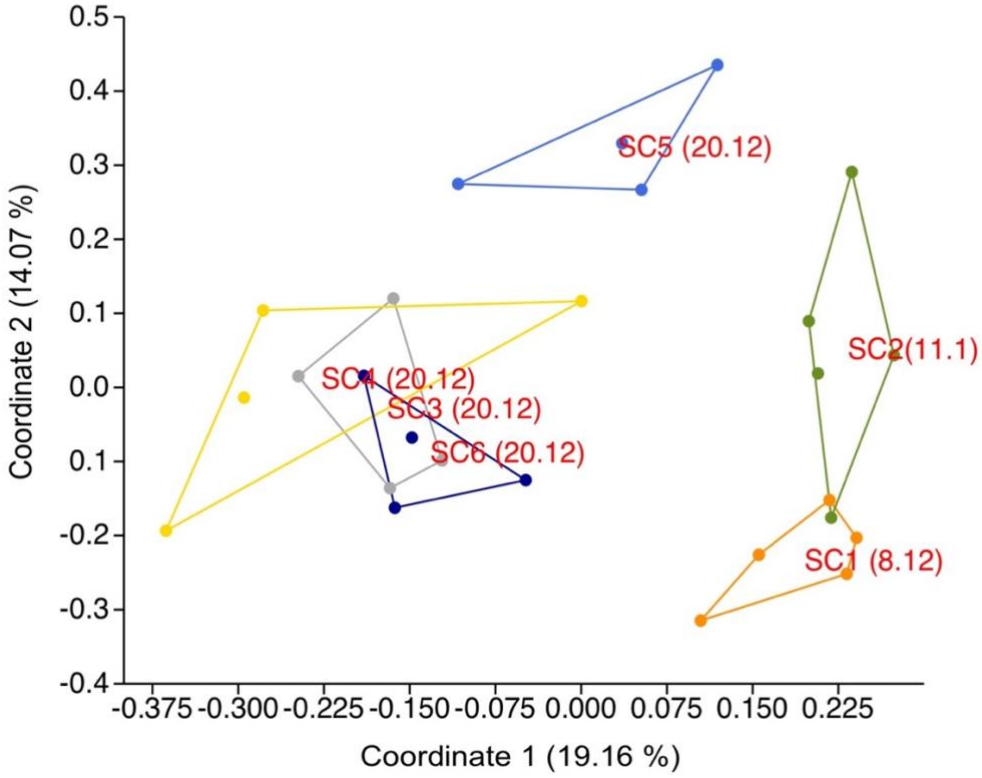
3.4.2 Beta Diversity, Comparing Microbial Community Profiles Between Samples

The normalized OTU table was used to calculate the β-diversity for the skin mucus microbial communities. Principal coordinates analysis (PCoA) based on Bray-Curtis similarity (Fig. 3.12A) and Jaccard indices (Fig. 3.12B) was used to compare community profiles between skin mucus samples from the different sea cages.

The PCoA plot based on Bray-Curtis similarities (Fig 3.12A) showed that the samples from SC1, representing ulcerated fish treated with freshwater, were different from the other samples. The skin mucus microbiota from the other sea cages showed great similarity. This indicated that the microbiota in skin mucus samples treated with various salmon lice treatment were similar. The PCoA plot based on the Jaccard index (Fig. 3.12B) indicated that untreated skin mucus samples from SC2 and samples from SC5 treated with SLICE had a slightly different microbiota than SC3, SC4 and SC6 treated with SLICE, SLICE + freshwater and SLICE + H_2O_2, respectively. However, there were no significant differences in the skin mucus microbiota of individuals from the different sea cages (One-way PERMANOVA: p-value > 0.05, Bonferroni-corrected p-values) for either Bray-Curtis or Jaccard similarity indices.



A)



B)

Figure 3.12: PCoA plot based on A) Bray-Curtis and B) Jaccard indices for comparison of microbiota from Atlantic salmon mucus from six different sea cages (SC1-6). Orange: SC1, Green: SC2, Grey: SC3, Yellow: SC4, Light blue: SC5, Dark blue: SC6.

Average Bray-Curtis similarities were calculated for the microbial communities within the skin mucus samples from the six sea cages and between skin mucus samples from selected sea cages (Figure 3.13). The average Bray-Curtis similarity showed relative high similarity in the skin mucus microbiota among individuals within each sea cage (~0.7-0.8). SC2 had the least similarity between individuals within the samples group (~0.6). Average Bray-Curtis similarity between samples from SC1 and SC2 as well as between SC1 and all samples from 20.12, confirmed that the skin microbiota of the ulcerated fish treated with freshwater (SC1) were different from the other sea cages.

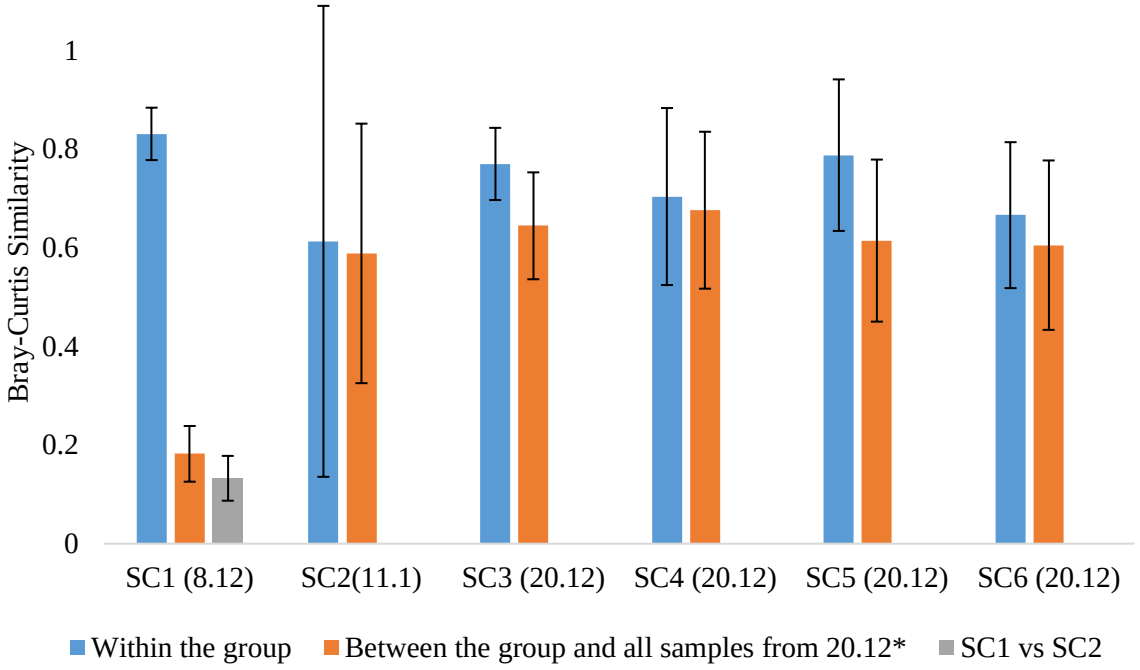


Figure 3.13: Average Bray-Curtis similarity indices for comparison of the microbial communities within and between sea cages. Error bars indicate standard error of the mean. * For samples from SC3-6: the sample group compared to the remaining sample groups from 20.12.

A SIMPER analysis showed that five OTUs explained 85.8% of the Bray-Curtis dissimilarity between the skin mucus sample groups (Tab. 3.3). OTU_1 representing *Pseudomonas* was primarily responsible for the difference between the groups of samples, and accounted for 36.76% of the difference. OTU_1 was highly abundant in samples treated with various salmon lice treatments in SC2-6, where it accounted for ~70% of the number of reads. OTU_1 was also abundant (12% of the number of reads) in skin mucus microbiota of ulcerated Atlantic salmon treated with freshwater in SC1. OTU_24, also representing *Pseudomonas*, was more abundant in samples from SC2-4 and SC6, accounting for ~21% of the reads. OTU_24 were less abundant in fish with ulcer (SC1) and fish treated with SLICE (SC5) and accounted for ~2-3% of the number of reads. OTU_2 representing *Psychrobacter* contributed to 20.9% the dissimilarity, and was highly abundant in microbiota of ulcerated fish in SC1, and accounted for 61% of the number of reads.

Table 3.3: SIMPER analysis based on Bray-Curtis dissimilarities used to identify the OTUs that contribute to the difference between the sample groups SC1-SC6. The OTUs cumulative contribution to dissimilarity and mean abundance of the five OTUs in the samples from each of the six sea cages (SC1-6). The taxonomy is presented as Family or *Genus*.

OTU ID	Taxonomy	Cumulative (%)	Mean SC1 (8.12)	Mean SC2 (11.1)	Mean SC3 (20.12)	Mean SC4 (20.12)	Mean SC5 (20.12)	Mean SC6 (20.12)
OTU_1	*Pseudomonas*	36.76	0.118	0.783	0.60	0.739	0.807	0.496
OTU_2	*Psychrobacter*	57.66	0.611	0.010	0.008	0.016	0.005	0.012
OTU_24	*Pseudomonas*	76.11	0.028	0.124	0.141	0.223	0.019	0.384
OTU_3	Enterobacteriaceae	81.80	0.032	1.13E-04	0.018	5.99E-05	0.160	7.55E-04
OTU_4	*Brochothrix*	85.80	0.117	2.72E-04	5.38E-05	1.21E-04	0.002	7.03E-06

The skin microbiota for fish treated with various salmon lice treatments obtained from six sea cages at three different aquaculture sites was investigated by DGGE and Illumina sequencing. The ulcerated fish treated with freshwater from SC1 had a different skin mucus microbiota compared to the fish in the remaining sea cages (Fig. 3.9 and 3.12A), where *Psychrobacter* (OTU_2) was highly abundant (Tab. 3.2). The most abundant OTUs in all the other samples treated with various salmon lice treatments in SC2-SC6 were represented by *Pseudomonas* (OTU_1 and OTU_24).

3.5 Real-time PCR for Quantification of Bacteria in the Salmon Skin Mucus Samples

To quantify the 16S rDNA copies in the different skin mucus samples, real-time PCR (qPCR) was conducted (see 2.5). It was used to estimate the bacterial load on skin mucus of Atlantic salmon treated with different salmon lice treatments.

3.5.1 Determination of Amplification Efficiency and Standard Curve for *Vibrio* DNA

To be able to calculate the 16S rDNA copy numbers (CN) in the skin mucus samples a standard curve was prepared by using DNA extracted from an isolated *Vibrio* strain (RD5-30; (Skjermo et al., 2015)) with known sequence. The primer pair RT996F and RT189R (Tab. 2.3) was used to amplify a DNA fragment of 123 bp from the 16S rRNA gene of the isolated *Vibrio* strain (RD5-30) (see 2.5). The measured amount of DNA was 9.7 ng μL^{-1} in the PCR product, and the sample was diluted to obtain 1 ng μL^{-1}. A standard curve was made by ten-fold dilution from 1 ng μL^{-1} to $1.0 \cdot 10^{-5}$ ng μL^{-1}. According to equation 2.1 (see 2.5.1), 1 ng μL^{-1} of this specific PCR product corresponds to $7.4 \cdot 10^{9}$ copies. A slope of -3.32 in the standard curve would indicate 100% amplification efficiency. When the first sample in the dilution series (DNA concentration: 1 ng μL^{-1}) was excluded from the data, the slope was -3.49, which indicated an amplification efficiency of 93% (Fig. 3.14).

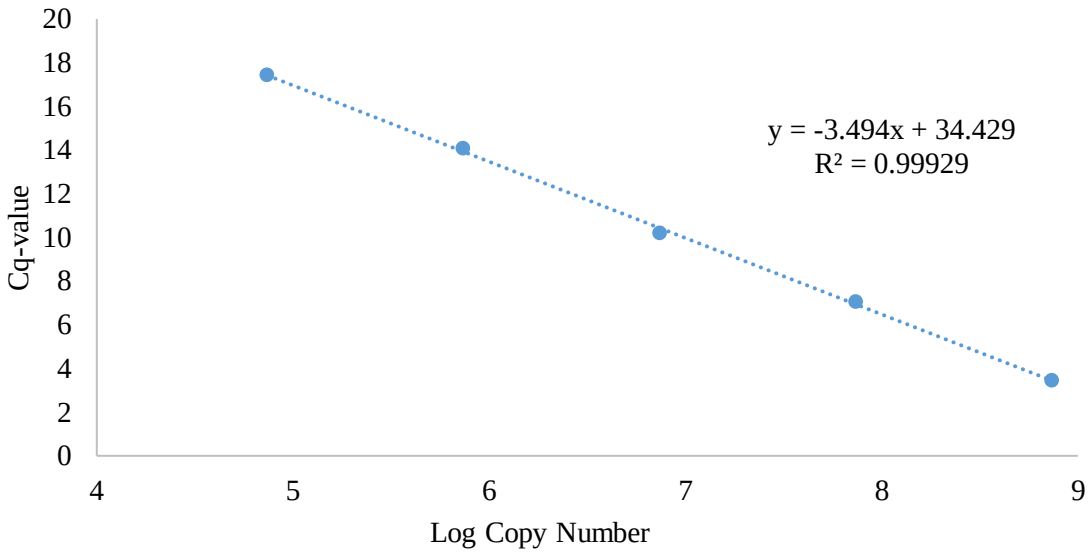


Figure 3.14: Standard curve obtained using a 123 bp long PCR product of 16S rDNA from *Vibrio* (RD5-30) as template in qPCR in a Light Cycler 96 (Roche).

3.5.2 Quantification of the 16S rDNA Copies in Skin Mucus Samples

qPCR was conducted with the RT996F and RT189R (Tab. 2.3) primers on DNA extracts form the skin mucus samples from the six sea cages. The amplification curves for the skin mucus samples indicated that the amplification efficiency was similar to what obtained for the standard curve samples (Fig. 3.15). To estimate the quantity of 16S rDNA copies in the samples amplified by qPCR, the average CN of the triplicate samples based on the standard curve were retrieved from the LightCycler 96 software (Roche). The area of the skin from which the DNA was extracted was 4.9 cm^2, and the CN cm^{-2} of skin was calculated for each triplicate. The CN ranged from 20-1.6·10^4 copies cm^{-2} of skin.

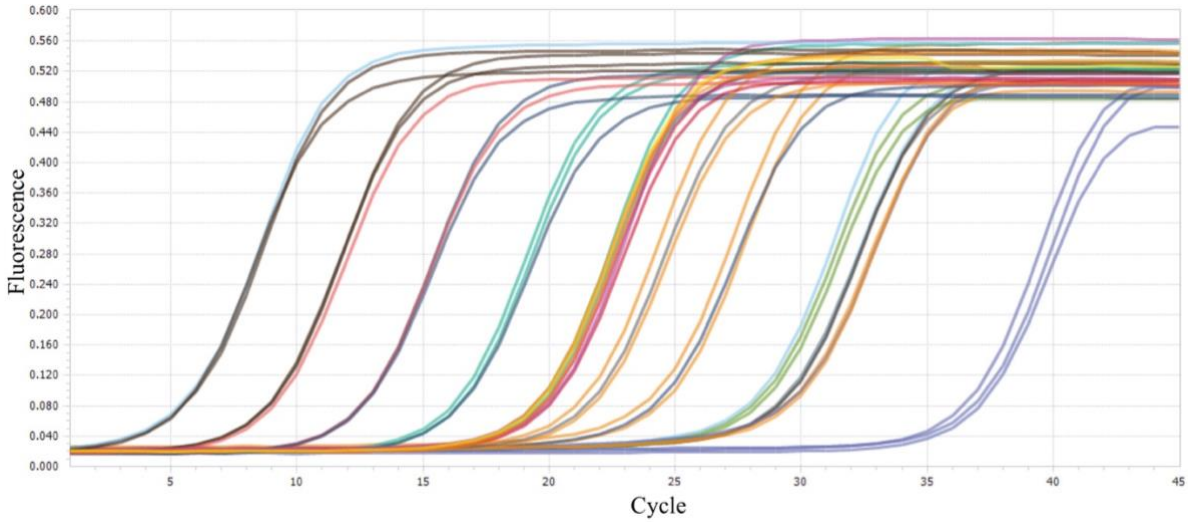


Figure 3.15: Amplification curve from the qPCR, where the first four amplification curves to the left represents the samples used as standard curve and the remaining amplification curves are the amplified samples from 16S rDNA form skin mucus microbiota DNA extracts. The amplification curve to the right (purple) represented the salmon muscle sample.

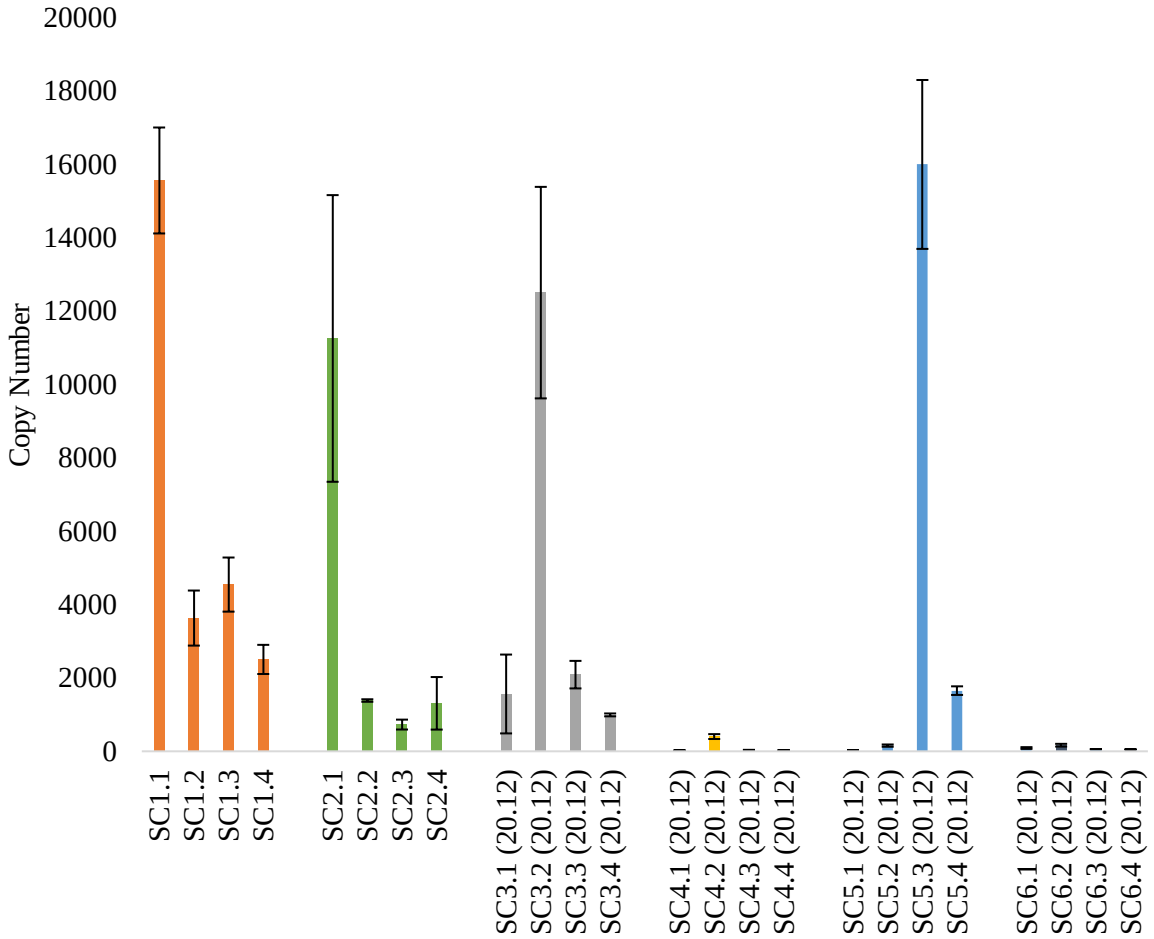


Figure 3.16: The copy number of 16S rDNA cm^{-2} skin of four skin mucus samples from each sea cage (SC1-6) treated with different salmon lice treatments. The error bars represent standard error of the mean.

The CN of individuals from the same sea cage showed great variation (Fig. 3.16), and the outliers (SC1.1, SC2.1, SC3.2, SC4.2 and SC5.3) were removed. The average CN of the skin mucus samples from each sea cage were calculated (Fig. 3.17). The CN was significantly higher in skin mucus samples from ulcerated fish in SC1, compared to the skin mucus samples in remaining sea cages (Tukey´s pairwise post-hoc, p-value < 0.05). The CN in samples exposed to bath treatment with freshwater (SC4) and H_2O_2 (SC6) were very low compared to the samples not exposed to bath treatment in SC2, SC3 and SC5. Results from qPCR indicated that the bacterial load was higher in ulcerated fish and lower in the freshwater treated samples (SC4), as well as the H_2O_2 treated samples (SC6).

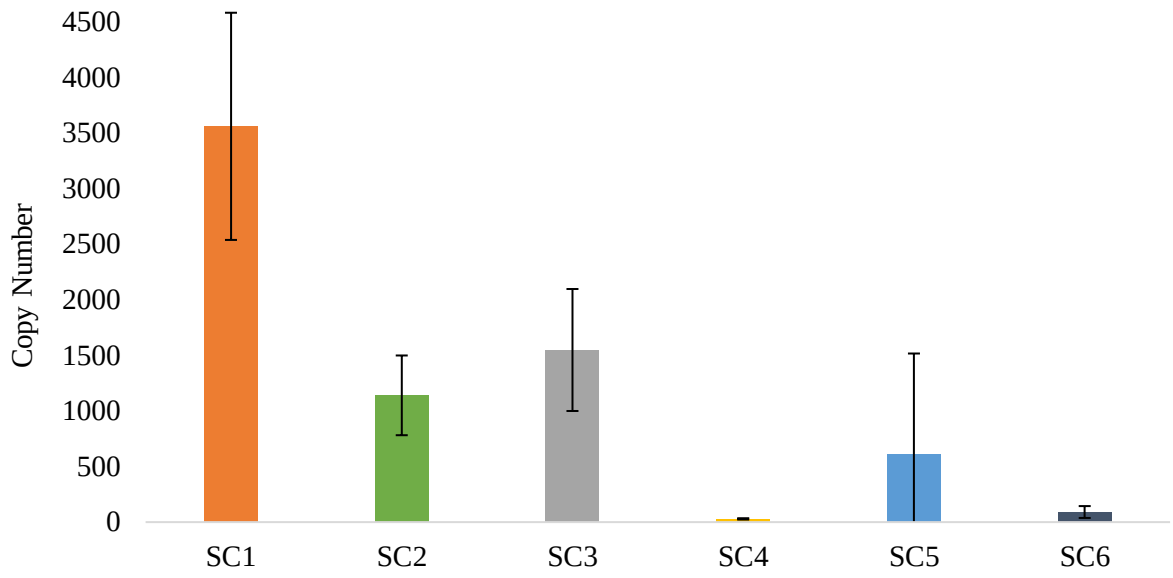


Figure 3.17: The average copy number of 16S rDNA cm^{-2} skin from each sea cage (SC1-6) treated with different salmon lice treatments. The error bars represent standard error of the mean.

4 Discussion

4.1 Evaluation of Methods

4.1.1 DNA Extraction and Amplification of Bacterial 16S rDNA from Skin Mucus Samples

Previous experiments in the research group "Analysis and Control of Microbial Systems" have shown that PCR amplification of microbial 16S rDNA from Atlantic salmon skin samples has been challenging, probably due to the presence of inhibitors and low fraction of bacterial DNA. The effect of different DNA extraction kits and PCR polymerases were tested with objective to improve amplification efficiency of variable regions of 16S rDNA. DNeasy Powersoil kit DNA extracts gave a higher PCR product yield during PCR amplification, compared to DNA extracts from the DNA extraction kits PureLink and QIAamp. Other DNA extraction kits have been compared in similar research investigating 16S rDNA amplicon sequencing, showed higher amplification efficiency of DNA isolated from insects using Phenol-chloroform (Sigma-Aldrich) and DNeasy Blood & Tissue Kit (Qiagen), than DNeasy Powersoil kit (Rubin Benjamin et al., 2014). This indicates that DNA extraction kits should be carefully chosen, when extracting DNA from various samples.

Further, the amplification efficiencies of four PCR polymerases were investigated. The polymerases KAPA 2G Robust PCR kit (Sigma-Aldrich) and PrimeStar DNA polymerase (TaKaRa Bio USA, Inc.) did not produce a significant amount of PCR product for the skin and skin mucus samples. However, ExTaq polymerase (TaKaRa Bio USA, Inc.) and Phusion Hot Start polymerase II (Thermo Scientific) both succeeded in producing PCR products with the expected length (~360 bp; V4 region of the bacterial 16S rRNA gene including Illumina adapter sequences). This indicates that choice of DNA extraction kit and polymerase could have an impact on amplification of the target DNA.

Among the two successful polymerases, Phusion Hot Start polymerase II gave the best yield, and was therefore chosen for amplification of skin mucus bacterial DNA during the rest of the project.

Contamination issues appeared during PCR amplification, with PCR product in the non-template control. However, the PCR product yields from the skin mucus samples were always considerably higher than the yield for the non-template control. This indicates that the bacterial DNA from the templates had been amplified in a larger degree than what the contamination contributed to. Contamination of PCR is a known challenge when using universal bacterial primers, and the contamination probably originated from the polymerase. In an earlier paper by Iulia et al. (2013) six of the seven polymerases tested contained traces of bacterial DNA. The contaminating DNA may also have derived from the lab, carry-over contamination or other reagents. This may cause inaccurate results due to detection of bacteria not present in the original samples. However, in the final amplification of the V4 16S rDNA used for Illumina sequencing it was managed to reduce the contamination to a minimum (weak or no bands in the non-template control). This was done by aliquoting all the reagents before use, UV-irradiate the Eppendorf tubes used for the master mix and setting up PCR by using a sterile bench. This showed that the polymerase might not have been the major cause to the contamination.

Illumina primers encompassing the V4 16S rDNA region yielded PCR products that formed a shorter PCR product than expected from skin samples, with a size of >300 bp. In addition to this, it was observed double bands on the agarose gel after amplification of the DNA extracted from skin mucus samples. The shorter PCR products only occurred in the fish samples and not in bacterial biofilm samples used as positive controls, and may therefore be due to co-amplification of eukaryotic DNA. This co-amplification might be a consequence of primer homology with the mitochondrial (mt) 12S rRNA gene in Atlantic salmon (personal communication, Ingrid Bakke). Thus, the final DNA extractions were performed from skin mucus samples scraped off the skin to avoid profusion of eukaryotic DNA in the DNA extract. The bacteria free DNA amplicon from salmon muscle showed that the eukaryotic DNA sample generated a shorter PCR product, which corresponded to the expected mt 12S rRNA gene (Fig. 3.8). This result confirmed that the broad-rage bacterial primers had homology with this gene.

4.1.2 Analysis of Microbial Community Diversity

PCR amplification of the bacterial 16S rDNA variable region was performed prior to DGGE and Illumina amplicon sequencing, and limitations are related to this method. Bacterial species

have intragenomic variations in 16S rDNA copy numbers, and species with high copy numbers will be overrepresented in the samples (Kembel et al., 2012). Thus, community profile will not reflect the actual abundance of the species in the sample. In addition, some taxa may not be detected because DNA from every specie may not be targeted by the primers (Mao et al., 2012). Moreover, the amplification efficiency can vary due to the length of the DNA amplification fragments and the GC-content in the sequences (Suzuki and Giovannoni, 1996). Sequence analysis of the amplified 16S rDNA variable region can also be difficult, due to the limited resolution of 16S rDNA among closely related species (Poretsky et al., 2014) An alternative approach to avoid PCR bias is metagenomics, where HTS is performed on total DNA extracted from the environment (Madigan et al., 2015). However, metagenomics requires thorough bioinformatic analysis, and the sequence assembly of high species diversity samples (for instance soil samples) has shown to be complicated (Tringe et al., 2005).

DGGE

The DGGE method was chosen to study the microbial community profiles of Atlantic salmon skin mucus samples from six sea cages treated with various salmon lice treatments. This method was used to get an overview of the diversity of the microbial communities in a relatively quick and inexpensive manner. When running a DGGE-gel several PCR products can be compared simultaneously, and variations in community profiles between skin mucus samples from different sea cages can be investigated. In DGGE, PCR bias may cause overestimation of the microbial diversity in a sample. One organism may potentially contribute to several bands on the DGGE-gel and thus give a false impression of the species diversity (Malik et al., 2008). In addition, there are limitations related to the method itself. The number of samples compared are limited, due to slightly different running conditions for each gel, as the denaturing gradient may differ. Thus, the band patterns can vary and comparisons between gels should be avoided (Muyzer and Smalla, 1998). In addition, some 16S rDNA molecules may denature at the same position in the gel giving an underestimation of the diversity, because several bands are interpreted as one band. Further, the taxonomic information obtained from Sanger sequencing (Sanger et al., 1977) of the bands from DGGE is insufficient to adequately describe and compare microbial communities (Bartram et al., 2011). Nevertheless, the results from the sequence analysis based on the V3 region of the 16S rDNA and taxonomic assignment gave some information on the most abundant bacteria present in skin mucus samples from the different sea cages.

Illumina Sequencing

Illumina sequencing was performed on the bacterial V4 16S rDNA amplicons. The sequence information was further used to analyse the microbial community in the sample groups. In this project a greater extent of taxonomic information was retrieved from HTS of 16S rDNA amplicons, compared to Sanger sequencing of DGGE bands. The amplified V4 region is a good choice for regional sequencing, as it is well represented in the RDP database and capable of detecting most Bacteria (Caporaso et al., 2011). However, it is preferred to use a combination of more variable and conserved regions of the 16S rDNA for higher resolution sequence analysis (Hamady and Knight, 2009). Compared to Sanger sequencing of DGGE bands, Illumina amplicon sequencing is more costly and require thorough bioinformatic analysis (Mardis, 2008). Yet, the problems related to DGGE, PCR bias and intragenomic copy number variation, also applies for Illumina sequencing of the 16S rDNA.

Identical genera and families were detected by sequencing of the bands indicating the highest yield on the DGGE-gel and Illumina sequencing of V4 16S rDNA amplicons. The sequence analysis showed that the DGGE bands represented *Psychrobacter*, *Brochothrix* and the family Carnobacteriaceae for the microbial community in ulcerated fish skin mucus (Tab. 3.1). The same taxa were detected by Illumina sequencing of 16S rDNA extracted from the same skin mucus samples (Tab. 3.2). For all other DNA extracts, strong DGGE bands represented *Pseudomonas* species. Sequences classified by Illumina amplicon sequencing of 16S rDNA revealed that *Pseudomonas* was highly abundant in these skin mucus samples, represented by two different OTUs; OTU_1 and OTU_24 (Tab. 3.2). The reason why *Pseudomonas* created several bands at different positions in the denaturing gradient gel may be due to heterogeneity between intragenomic 16S rRNA operons (Coenye and Vandamme, 2003). The DGGE results complied with the Illumina amplicon results when identifying the most abundant community members, but the Illumina amplicon sequencing gave a more detailed taxonomic assignment of all sequences.

4.1.3 Quantification of Bacterial 16S rDNA copies by Real-time PCR

Real-time PCR (qPCR) was applied to quantify the 16S rDNA copies and estimate the load of bacteria in skin mucus samples from Atlantic salmon treated with various salmon lice

treatments. This method has been used to quantify specific strains of bacteria and the total load of bacteria (Skjermo et al., 2015, Castillo et al., 2006, Nadkarni et al., 2002). The DNA was quantified by using the double stranded DNA binding dye, SYBR® Green. The SYBR® Green binding dye is very simple and inexpensive to use. However, the problem with this assay is that both specific and nonspecific PCR products are detected. Therefore, careful optimization of PCR conditions is required. Broad-range PCR primers intended for amplification of all bacterial DNA were used in the quantification of 16S rDNA copies. To maintain fluorescent detection of bacterial DNA as broad coverage as possible, TaqMan probes were not used. Using qPCR in quantification of bacterial 16S rDNA copies allows for detection of both live and dead bacteria, as all intact DNA encompassed by the primers are amplified (Rinttilä et al., 2004). This may lead to an overestimation of the number of 16S rDNA copies in the skin mucus samples. Furthermore, the presence of PCR inhibitors in the DNA extract can have an effect on the qPCR amplification efficiency. The amplification efficiency of all samples and the DNA template used to make the standard curve should be equivalent (Smith and Osborn, 2009). The amplification curves in this project indicated similar efficiency, and it was most likely no problem with inhibitors in the DNA extracts from the samples. Finally, the 16S rDNA copy numbers for the environmental samples cannot be converted to cell numbers due to variation in the 16S rDNA copy numbers in different bacterial species. However, the copy number detected gave an indication of the bacterial load in the samples.

4.2 Effect of Salmon Lice Treatment on the Skin Mucus Microbiota

Variations in the microbial community composition associated with the fish skin mucus were observed among individuals from the same sea cage (Fig. 3.11). The bacterial colonization of fish starts in the egg phase, and as the fish is continuously in contact with the external environment, the individuals microbiota can potentially be affected over time (Austin, 2006). Similar research focusing on characterizing skin microbiota in marine fish have found variation in the skin microbiota between individuals complying with the results in this project. In a previous paper by Chiarello et al. (2015) the skin associated bacterial communities of marine fish varied between individuals, but also between different body parts.

For samples from some sea cages, the average skin mucus microbiota appeared to have higher Shannon's diversity, compared to that of other groups (Fig. 3.10B). There were low Shannon´s diversity in untreated samples, samples treated with SLICE + Freshwater and SLICE (SC2,

SC4 and SC5), where OTU_1 representing *Pseudomonas* prevailed. This OTU was responsible for over 74% of the reads across all samples, and the high abundance of one OTU lowers the Shannon´s diversity. The Shannon´s diversity and the observed OTU richness found for the salmon skin microbiota in this project were low compared to results from similar research focusing on characterizing Atlantic salmon skin microbiota (Minniti et al., 2017, Llewellyn et al., 2017, Karlsen et al., 2017). In the study conducted by Karlsen et al., (2017) the number of OTUs in the skin mucus were 178, in comparison to the total of 68 OTUs found in this project.

The most abundant phyla in the salmon skin mucus samples were Proteobacteria, Firmicutes, Bacteriodetes and Actinobacteria, which corresponds to similar research focusing on characterizing Atlantic salmon skin microbiota. In an earlier paper by Minniti et al. (2017) Proteobacteria was the most abundant phyla, followed by Firmicutes and Acidobacteria. Interestingly, the Proteobacteria prevailed and accounted for an average of 94% of the reads across all skin mucus samples in this project. This correlates with the findings by Lokesh and Kiron (2016), where Proteobacteria was the dominant phylum in salmon from both freshwater (45%) and seawater (> 89%). It is suggested that Proteobacteria are part of the residential skin associated microbiota of Atlantic salmon because psychrophiles, which thrive in cold environments, are mostly Gram-negative Proteobacteria (Lokesh and Kiron, 2016).

Pseudomonas (Proteobacteria) was abundant in all samples, where two OTUs (OTU_1 and OTU_24) prevailed in skin mucus samples from SC2-6. *Pseudomonas* are Gram-negative, chemoorganotrophic bacteria and all species of this genus thrive in aerobic environments (Madigan et al., 2015). This genus is ubiquitous in soil and aquatic systems, and many species cause diseases in plants and animals. Several *Pseudomonas* species are considered opportunistic pathogens in salmonids (Boutin et al., 2013, Austin et al., 2012). Opportunistic pathogens may cause an infection if the fish are stressed when exposed to unfavourable conditions. *Pseudomonas* have also been associated with high louse burdens on Atlantic salmon, together with *Vibrio, Flavobacterium* and *Tenacibaculum* (Llewellyn et al., 2017). A majority of the Atlantic salmon in this project have most likely been infected with salmon lice, because many fish have undergone salmon lice treatment. This could be the reason why *Pseudomonas* prevailed in the bacterial communities of most skin mucus samples. However, *Pseudomonas* also prevailed in the untreated samples in SC2 with 90% of the reads, samples that should not be associated with high louse burdens. In addition to pathogenic *Pseudomonas*,

the range of non-pathogenic *Pseudomonas* is large. *Pseudomonas* is identified as one of the main genera of skin microbiota of various fish species, such as cod (*Gadus morhua*), herring (*Clupea* spp.), and Atlantic salmon, among others (Wilson et al., 2008, Horsley, 1973, Horsley, 1977). This indicates that *Pseudomonas* might be part of the commensal microbiota of Atlantic salmon skin.

Psychrobacter, Pseudomonas and Acinetobacter (Proteobacteria) associated with many of the skin mucus samples in this project (Tab. 3.2 and Fig. 3.11) have been associated with stressed brook charr (Boutin et al., 2013). Bacteria associated with unstressed and healthy control individuals was species from the genera *Sphingomonas, Methylobacterium, Propionibacterium,* and *Thiobacter* (Boutin et al., 2013). They were not detected in this project. The reason for this could be that the fish were stressed during the salmon lice treatment. Stress has been reported to cause a compositional shift in the mucus proteins. This shift in mucus composition may further change the composition of microbial communities associated with the mucus (Llewellyn et al., 2014). The resident microbiota and inhibitory compounds in the fish skin mucus may also affect the colonization of other bacteria from the environment (Austin, 2006).

Individuals from SC3 and SC5, treated with SLICE and no bath treatment, had a slightly different skin mucus community composition, where Enterobacteriaceae was among the most abundant OTUs (Tab. 3.2). Both OTU_3 and OTU_6 represented Enterobacteriaceae and were unassigned at the genus level. The Enterobacteriaceae is a family of Gram-negative bacteria that includes many harmless symbionts, but also many pathogens, such as *Salmonella, Escherichia coli* and *Yersinia* in this family (Ghanem et al., 2014). However, the difference in the microbial composition between the treated and untreated samples was not significant, and the slight difference observed may be due to individual differences as previously discussed (Chiarello et al., 2015).

The salmon lice treatment had no significant effect on the Atlantic salmon skin mucus microbiota composition, with an exception of the skin mucus microbiota of the fish in SC1 which was significantly different from the remaining skin mucus samples. All individuals sampled from SC2-6 had a community composition where *Pseudomonas* was prevailing.

However, the SLICE treated individuals in SC3 and SC5 had a slightly different microbiota than the bath treated and the untreated samples, where Enterobacteriaceae were present in the Atlantic salmon skin mucus microbiota.

4.2.1 Ulcerated Atlantic Salmon with a Different Skin Mucus Microbiota

The microbial community structure of skin microbiota for fish with ulcers differed from the community structure seen in the other fish. These findings were confirmed by DGGE analysis and Illumina amplicon sequencing (Fig. 3.9, Fig. 3.12A). When comparing the average Bray-Curtis similarity between ulcerated fish and the other samples, the similarity was low (Fig. 3.13). The ulcerated fish skin mucus was mostly colonized by *Psychrobacter,* with an average abundance of 61%. Compared to a previous paper by Boutin et. al (2013), *Psychrobacter* had the relative abundance of 7.3% in stressed brook charr. In addition to *Psychrobacter*, *Brochothrix*, *Carnobacterium* and *Pseudomonas* were also represented in these individuals (Tab. 3.2).

Psychrobacter (Proteobacteria) was the most abundant genera associated with ulcerated fish in SC1. *Psychrobacter* includes Gram-negative, aerobic, cold-adapted and osmotolerant bacteria, which is widespread, and found in slightly to highly saline environments with large variations in temperature (Bowman, 2006). A species of *Psychrobacter* (*P. immobilis*) is an opportunistic pathogen on farmed salmonids fish (Hisar et al., 2002). *Psychrobacter* might be present in the skin mucus because the fish were stressed as a result of the freshwater bath treatment, as this species is previously detected in stressed brook charr (Boutin et al., 2013). Moreover, the ulcerated fish could be more susceptible towards opportunistic pathogens due to its health conditions.

Another abundant genus in the ulcerated fish analysed in this project, was *Brochothrix* (Firmicutes). *Brochothrix* is Gram-positive and facultative anaerobe bacteria (Stackebrandt and Jones, 2006). The natural habitat of *Brochothrix* has not been determined, and the species are detected in a wide variety of environments. The genus has been isolated from soil and grass, but also from fish and frozen food products (Stackebrandt and Jones, 2006). *B. thermosphacta* is one of the most common spoilage bacteria reported in fish and fish products (Rudi et al., 2004), and contamination almost always occurs during slaughter and post slaughter

(Stackebrandt and Jones, 2006). *Carnobacterium* (Firmicutes) was also present in the ulcerated fish. This Gram-positive bacteria has been isolated from cold and temperate environments (Leisner et al., 2007), and they frequently predominate a range of food, including fish. *Carnobacterium* species (*C. inhibens*) have also been isolated from Atlantic salmon intestine (Joborn et al., 1999). Some species thrive in permafrost and are capable of growth under low temperatures (0 °C), low pressure and anoxic conditions (Nicholson et al., 2013). However, none of these genera has previously been associated with Atlantic salmon ulcer.

Bacteria related to ulcerated fish are *Moritella viscosa* shown to cause the ulcer (Karlsen et al., 2017). *Tenacibaculum* and *Alivibri* species are among other bacteria associated with Atlantic salmon ulcer (Karlsen et al., 2017). Their role in Atlantic salmon ulcer is still to be defined. However, these bacteria were not detected in the microbial community analysis in this project, indicating that the ulcerated samples may have been exposed to secondary infection. The freshwater treatment applied to the ulcerated fish was an Amoebic gill disease (AGD) treatment, indicating that AGD was a problem for these fish, in addition to ulcer. The bacterial colonization could also be different from the other sample groups due to AGD, as *Psychrobacter* have previously been associated with gill mucus samples in Atlantic salmon with AGD (Bowman and Nowak, 2004). Since the community composition was significantly different in the ulcerated fish compared to the individuals from the other sea cages, it is likely that the health status of the fish can affect the susceptibility of bacterial colonization. However, it is not known whether the microbial populations inhabiting the skin mucus are actively selected by the host or if the community is structured by the rearing environment. In addition, bacterial growth on the fish skin mucus may be limited by inhibitors (Karlsen et al., 2017).

In this project the mucus was scraped of the skin, and the determination of the microbial diversity and the load of bacteria on the fish skin mucus can be challenging. For example, there is no appropriate way to sample skin mucus without collecting water associated with it (Minniti et al., 2017). The fish skin samples were stored at -20°C post slaughter, and the skin microbiota could potentially be affected after fish death. The fish with ulcer were likely susceptible to post slaughter colonization of other bacteria due to open wounds and a possible harmed mucus barrier. It is not known whether the bacterial genera related to spoilage bacteria found in stored fish and fish products (*Brochothrix* and *Carnobacterium*) are actually present on the live fish in the sea cage. To avoid colonization of other bacteria during slaughter and storage, the skin

mucus sampling should be performed directly from the fish in the sea cage at the aquaculture site.

4.2.2 Effect of Salmon Lice Treatment on Bacterial Density

The copy number of 16S rDNA detected by qPCR analysis ranged from 20-$1.6 \cdot 10^4$ copies cm^{-2} of skin, and low values indicate low bacterial numbers in the skin mucus. It is difficult to compare quantitative data across studies, as several different approaches based on culturing and molecular methods are used (Minniti et al., 2017). The quantity detected is dependent on sampling technique and body regions sampled from, as bacterial communities varies among body parts (Chiarello et al., 2015). In general, the bacterial load on skin is lower than in the surrounding water and in the gastrointestinal tract (Minniti et al., 2017, Merrifield and Rodiles, 2015). Moreover, previously reported quantity of bacteria in fish skin mucus is within the range of ~10^2 to ~10^4 cm^{-2} (Austin, 2006), but may vary between species. The quantity of 16S rDNA copies detected in this project differed between individuals in the same sea cage (Fig. 3.16). Interestingly, the DNA extracted from ulcerated fish had significantly higher copy number of 16S rDNA, indicating a higher bacterial load. Furthermore, qPCR analysis in this project indicated that the fish subjected to the bath treatment (Freshwater; SC4, and H_2O_2; SC6), which is considered stressful, had a considerably lower bacterial load than the salmon not treated by bath treatments (Fig. 3.17). The reason for this may be the high activity of antibacterial agents produced by the fish in response to the stress connected to the treatment. However, ulcerated fish were also treated in freshwater bath and had higher bacterial loads than all other samples. It is known that the production of mucus is altered when the fish is subjected to stressful situations, such as chemical aggressions, which induce higher expression and activity of antibacterial agents (Tort et al., 2003). It has also been found an increase in mucus production after a Thermolicer treatment for Atlantic salmon (Pittman and Merkin, 2016). It might seem reasonable that the salmon lice treatment and the handling related to the treatment would harm the skin mucus barrier and make it more vulnerable to bacterial colonization. The results from qPCR suggests the opposite, where the bacterial colonization was lower in the bath treated samples. Increased production of mucus and antimicrobial compounds in respond to the stressful situations have been reported (Tort et al., 2003). The salmon lice bath treatment may have been stressful for the fish and led to an increased production of mucus and shredding of bacteria. The loss of mucus may only be a problem in mechanical treatment using brushing and flushing, and not during bath treatments. However, this must be investigated further.

4.3 Future Perspectives

It is important to continue research on aquacultured fish, due to the increasing demand for fish protein as the human population is increasing. Analysis of the fish in its natural environment as well as at aquaculture sites, might contribute to find the optimum conditions to maintain fish health and high product yield. As the issue with salmon lice currently is controlled by various salmon lice treatments, the investigation on health and effect of the treatments is important because it may affect the fish welfare. The effect of salmon lice treatments on the salmon skin microbiota and the mucus production has not been studied previously, and there are no published articles related to this topic, to our knowledge.

The effect of the different salmon lice treatments on the skin mucus production should be investigated in more detail, to increase the knowledge on how it affects the skin mucus barrier. The skin mucus can potentially be scraped off during handling, flushing and brushing in mechanical treatments. However, stress and handling also lead to an increased production of mucus and antimicrobial compounds, and result in a stronger skin mucus barrier. It would be interesting to investigate the amount, composition, and mechanical barrier properties of the skin mucus and its components after various salmon lice treatments. Controlled experiments where different treatment parallels with fish reared in the same environment should be compared at several time points after treatment to examine temporal dynamics. The sampling of fish skin mucus should be conducted at the aquaculture site, preferably pre slaughter, to avoid contamination of bacteria associated with the slaughter process. Moreover, the fish should not be affected by any diseases, as the ulcerated fish seemed to have had a major impact on the fish skin microbiota.

The project showed that qPCR and the use of Illumina amplicon sequencing of bacterial 16S rDNA were efficient methods to investigate the bacterial load and characterize the microbial communities on fish skin mucus.

5 Conclusions

In this project, DGGE and Illumina sequencing of 16S rDNA amplicons were used to investigate the potential influence of salmon lice treatment on the composition of the skin mucus microbiota of farmed Atlantic salmon. A PCR protocol for successful amplification of the V4 region of bacterial 16S rDNA from salmon skin mucus DNA extracts was developed.

Proteobacteria was the dominant phylum in all skin mucus samples of Atlantic salmon. The genus *Pseudomonas* was prevailing in almost all samples. There was no significant difference in the community composition between samples from treated and untreated Atlantic salmon. However, qPCR analysis indicated that the bacterial load was lower for samples originating from fish that had been exposed to freshwater and H_2O_2 in bath treatments. Thus, the main hypothesis for this project, namely that salmon lice treatments would reduce skin mucus barrier properties, and that the fish skin therefore would be colonized by more and other bacteria than healthy, untreated fish, did not conclude with the results obtained. The most noticeable difference in the salmon skin microbiota was the difference between ulcerated fish and all the other individuals sampled. Ulcerated fish had distinct community composition, with *Psychrobacter* as the most abundant genus, and the highest bacterial load among all fish samples.

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Appendix A

DNeasy Powersoil DNA isolation Kit (Qiagen, former Mo BIO Laboratories, Inc.) used to extract DNA from fish skin, skin mucus, salmon muscle, *Vibrio* (RD5-30) and biofilm for positive controls.

Experienced User Protocol
Please wear gloves at all times

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

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

Thank you for choosing the PowerSoil® DNA Isolation Kit.

Technical Information: Toll free 1-800-606-6246, or 1-760-929-9911 Email: technical@mobio.com **Website:** www.mobio.com
7

Appendix B

PureLink™ Microbiome DNA Purification Kit (Thermo Scientific) used to extract DNA from fish skin, skin mucus and biofilm for positive controls.

Methods

Perform the procedure at room temperature (20–25°C), unless otherwise indicated.

1 Prepare the lysate

a. Add 600 µL of S1—Lysis Buffer to the Bead Tube.

b. Add 0.2±0.05 g of soil, cap securely, then vortex.

c. Add 100 µL of S2—Lysis Enhancer, cap securely, and vortex briefly.

d. Incubate at 65°C for 10 minutes.

e. Homogenize by bead beating for 10 minutes at maximum speed on the vortex mixer.
Use the hands-free adapter and horizontal agitation.

f. Centrifuge at 14,000 × *g* for 5 minutes.

g. Transfer up to 400 µL of the supernatant to a clean microcentrifuge tube.

IMPORTANT! A layer of debris may be present on top of the bead pellet. Avoid transfer of this debris with the supernatant.

h. Add 250 µL of S3—Cleanup Buffer, and vortex immediately.
Vortex immediately to ensure even dispersion of S3—Cleanup Buffer and uniform precipitation of inhibitors.

i. Incubate on ice for 10 minutes.

j. Centrifuge at 14,000 × *g* for 1 minute.

k. Transfer up to 500 µL of the supernatant to a clean microcentrifuge tube, avoiding the pellet.

2 Bind the DNA to the column

a. Add 900 µL of S4—Binding Buffer, and vortex briefly.

b. Load 700 µL of the sample mixture onto a spin column-tube assembly, and centrifuge at 14,000 × *g* for 1 minute.

c. Discard the flow-through, and repeat step 2b with the remaining sample mixture.
Ensure that the entire sample mixture has passed into the collection tube by inspecting the column. If sample remains in the column, centrifuge again at 14,000 × *g* for 1 minute.

3 Wash and elute the DNA

a. Place the spin column in a clean collection tube, add 500 µL of S5—Wash Buffer, then centrifuge the spin column-tube assembly at 14,000 × *g* for 1 minute.

b. Discard the flow-through, then centrifuge the spin column-tube assembly at 14,000 × *g* for 30 seconds.
The second centrifugation optimizes removal of S5—Wash Buffer, which could interfere with downstream applications.

c. Place the spin column in a clean tube, add 100 µL of S6—Elution Buffer, then incubate at room temperature for 1 minute.

d. Centrifuge the spin column-tube assembly at 14,000 × *g* for 1 minute, then discard the column.
The purified DNA is in the tube.

The DNA is ready for immediate use. Alternatively, store the purified DNA:

- At 4°C for up to 1 week.
- At –20°C for long-term storage.

Appendix C

QIAamp DNA Mini Kit from Tissues (Qiagen) used to extract DNA from fish skin, skin mucus and biofilm for positive controls.

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

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

Important points before starting

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

Things to do before starting

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

Procedure

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

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

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

   The yield of DNA will depend on both the amount and the type of tissue processed. 1 mg of tissue will yield approximately 0.2–1.2 µg of DNA.

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

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

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

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

2 ml microcentrifuge tubes may be better suited for lysis.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

   Close each spin column to avoid aerosol formation during centrifugation.

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

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

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

8. **Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW1 without wetting the rim. Close the cap, and centrifuge at 6000 x *g* (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the collection tube containing the filtrate.***
9. **Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed (20,000 x *g*; 14,000 rpm) for 3 min.**
10. **Recommended: Place the QIAamp Mini spin column in a new 2 ml collection tube (not provided) and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.**

    This step helps to eliminate the chance of possible Buffer AW2 carryover.
11. **Place the QIAamp Mini spin column in a clean 1.5 ml microcentrifuge tube (not provided), and discard the collection tube containing the filtrate. Carefully open the QIAamp Mini spin column and add 200 µl Buffer AE or distilled water. Incubate at room temperature for 1 min, and then centrifuge at 6000 x *g* (8000 rpm) for 1 min.**
12. **Repeat step 11.**

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

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

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

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

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

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

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

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

Appendix D

Buffers and acrylamide solutions used for gel electrophoresis and/or DGGE.

50 x TAE-buffer

Per litre	
Tris base	242 g
Glacial acetic acid	57,1 mL
0.5 M EDTA (pH 8.0)	100 mL

Add distilled water to obtain final volume.

Autoclave the buffer

1 x TAE-buffer

1960 mL Milli-Q water + 40 mL 50 x TAE-buffer

Acrylamide solution (0% denaturing)

8% acrylamide in 0.5 x TAE (per 250 mL)

40% acrylamide solution (BioRadLab Inc.)	50 mL
50 x TAE	2.5 mL

Store the solution at 4 °C, protected from light

Denaturing acrylamide solution (80%denaturing):

8% acrylamide, 5,6M urea, 32% formamide i 0,5 x TAE (per 250 ml):

40% acrylamide solution (BioRadLab Inc.)	50 mL
50 x TAE	2.5 mL
Urea	84 g
Deionized formamide	80 mL

TE-buffer: 10 mM Tris-HCl, 1 mM EDTA

1 M Tris-HCl (pH 8.0)	1 mL
EDTA (0.5 M)	0.2 mL
Distilled water up to 100 mL	

Store the solution at 4 °C, protected from light. The solution must be sterile filtered before pouring the gel.

10% APS (ammonium persulfate)

10 g APS dissolved in 100 mL distilled H_2O, sterile filtered and distributed in Eppendorf tubes in each)

Appendix E

QIAquick purification kit (Qiagen) was used to purify PCR products from *Vibrio* (RD5-30) and DGGE bands.

QIAquick® PCR Purification Kit

Notes 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 table-top microcentrifuge at room temperature.
- Add 1:250 volume pH indicator I to Buffer PB. The yellow color of Buffer PB with pH indicator I indicates a pH of ≤7.5. 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. Do not add pH indicator I to buffer aliquots.

1. Add 5 volumes Buffer PB to 1 volume of the PCR reaction and mix. If the color of the mixture is orange or violet, add 10 µl 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn yellow.
2. Place a QIAquick column in ▲ a provided 2 ml collection tube or into ● a vacuum manifold. For details on how to set up a vacuum manifold, refer to the *QIAquick Spin Handbook*.
3. To bind DNA, apply the sample to the QIAquick column and ▲ centrifuge for 30–60 s or ● apply vacuum to the manifold until all the samples have passed through the column. ▲ Discard flow-through and place the QIAquick column back in the same tube.
4. To wash, add 0.75 ml Buffer PE to the QIAquick column ▲ centrifuge for 30–60 s or ● apply vacuum. ▲ Discard flow-through and place the QIAquick column back in the same tube.
5. Centrifuge the QIAquick column once more in the provided 2 ml collection tube for 1 min to remove residual wash buffer.
6. Place each QIAquick column in a clean 1.5 ml microcentrifuge tube.
7. 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. 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.
8. 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.

Appendix F

SequalPrep™ Normalization Plate (96) kit (Invitrogen) used to normalize and purify the 16S rDNA variable region 4 (V4) that were further sequenced by MiSeq Illumina sequencing.

invitrogen™

SequalPrep™ Normalization Plate (96) Kit

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

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.

Appendix G

The protocol for the Amicon® Ultra-0.5 Centrifugal Filter Devices (Merck Millipore Ltd.) was performed on the pooled, indexed samples that were going to be sequenced by MiSeq Illumina sequencing. In step 4, the device was spun for 10 minutes. After of step 4, 500 µL of TE-buffer was added and the device spun at 14 000 x g for 10 minutes and eluate was discarded. This process was repeated once more before continuing on to step 5.

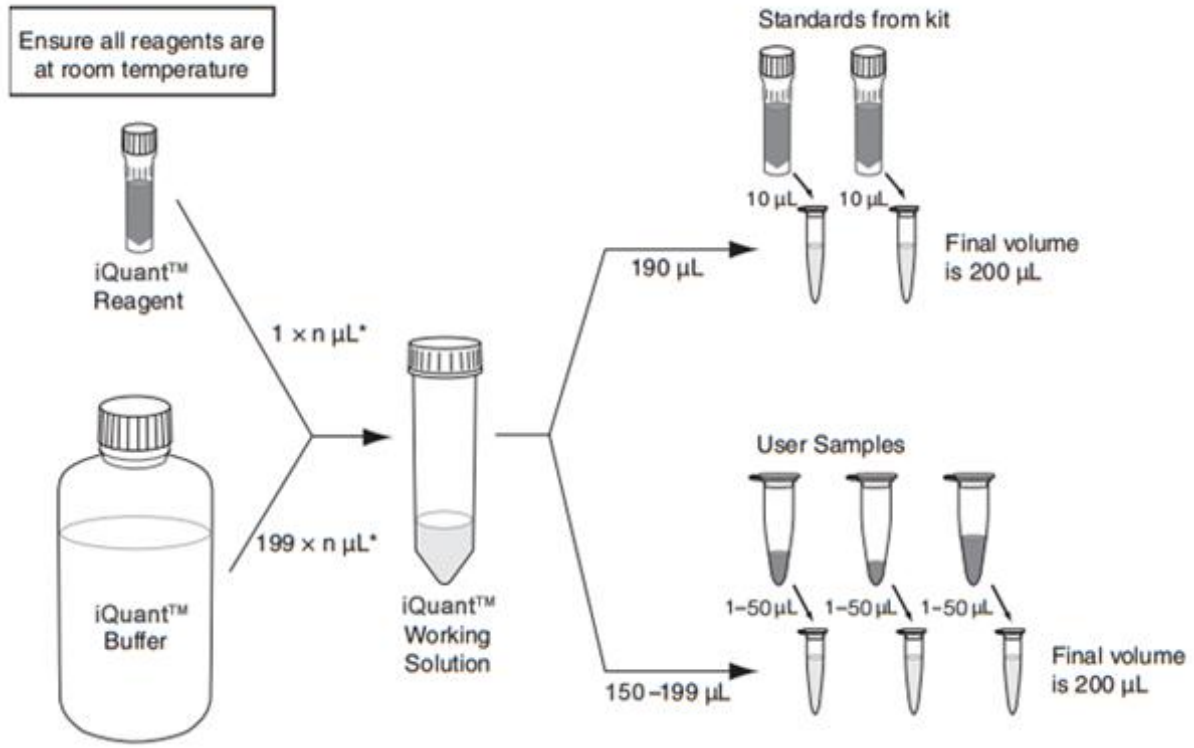
How to Use Amicon® Ultra-0.5 Centrifugal Filter Devices

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 2 for typical spin times.
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 microcentrifuge 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 H

The concentration of the purified *Vibrio* RD5-30 amplicon product was measured using the iQuant™ HS dsDNA quantitation assay and Qubit3 Fluorometer (Invitrogen, Thermo Scientific). 199 µL of the iQuant Working Solution were mixed with 1 µL amplicon product to get the right dilution.



Appendix I

The total number of reads in each individual skin mucus sample after USEARCH filtering showed in Table AI.1.

Table AI.1 Number of reads of the V4 region of the bacterial 16S rRNA in all samples sequenced by Illumina MiSeq.

Sample Name	Number of Reads	Sample Name	Number of Reads
SC1.1 (08.12)	50534	SC4.1 (20.12)	54344
SC1.2 (08.12)	55267	SC4.2 (20.12)	53864
SC1.3 (08.12)	49532	SC4.3 (20.12)	64468
SC1.4 (08.12)	83502	SC4.4 (20.12)	53275
SC1.5 (08.12)	57534	SC5.1 (20.12)	73521
SC2.1 (11.1)	52787	SC5.2 (20.12)	61583
SC2.2 (11.1)	80550	SC5.3 (20.12)	65677
SC2.3 (11.1)	71031	SC5.4 (20.12)	50895
SC2.4 (11.1)	91441	SC6.1 (20.12)	61846
SC2.5 (11.1)	82813	SC6.2 (20.12)	54044
SC3.1 (20.12)	67678	SC6.3 (20.12)	69970
SC3.2 (20.12)	59032	SC6.4 (20.12)	72235
SC3.3 (20.12)	60049		
SC3.4 (20.12)	49395		

Appendix J

Sequences form OTU_1 and OTU_24 from Illumina sequencing results were aligned and showed a difference of 3%. OTU_1 and OTU_24 both represented the genera *Pseudomonas*.

```
OTU_1                1 tacagagggtgcaagcgttaatcggaattactgggcgtaaagcgcgcgta
OTU_24               1 ..................................................

OTU_1               51 ggtggtttgttaagttggatgtgaaatccccgggctcaacctgggaactg
OTU_24              51 .....c...a.....ca..............................

OTU_1              101 cattcaaaactgacaagctagagtatggtagagggtggtggaatttcctg
OTU_24             101 ..............tga..............................

OTU_1              151 tgtagcggtgaaatgcgtagatataggaaggaacaccagtggcgaaggcg
OTU_24             151 ..................................................

OTU_1              201 accacctggactgatactgacactgaggtgcgaaagcgtggggagcaaac
OTU_24             201 ............a.....................................

OTU_1              251 a
OTU_24             251 .
```