Microbiological monitoring of an Atlantic salmon recirculating aquaculture system (RAS) – Health and quality assessment

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Master Thesis

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Abstract

Aquaculture is an important industry in Norway, particularly the production of Atlantic salmon (*Salmo salar* L.). Health problems and environmental impacts during the seawater stage are limiting factors for the industry. To combat these issues, closed and land-based aquaculture facilities for post-smolt production have become more popular. Although, vaccinations control several infectious diseases in Norwegian aquaculture, bacterial and viral infections and salmon lice cause losses in aquaculture and pose a threat to fish health and welfare. Also, off-flavor compounds, such as geosmin, have the potential to be important quality-limiting factors in the production of Atlantic. The change to land-based production increases the risk of accumulation of pathogenic and geosmin-producing bacteria, and little is known how such a production method affects the overall health and skin microbiota of fish. In the present study, microbiological and molecular methods were used to monitor fish health and product quality in connection to off-flavor in a land-based saltwater RAS facility. *Brevundimonas vesicularis* was amongst the most dominating bacteria in all samplings, whilst bacteria such as *Chryseobacterium indologenes* were only present in freshwater fish mucus samples. Overall, less bacterial diversity was observed in the second saltwater RAS sampling. Geosmin-producing bacteria were identified in most of the samples, with an increase of target DNA in the saltwater RAS samplings, particularly in RAS biofilm. The results presented in this study indicate that monitoring of bacterial microbiota of fish skin, RAS biofilm, and biofilters by traditional bacteriology methods could be used to reveal wound problems in an aquaculture facility. *Aliivibrio wodanis* was isolated from fish and environment during a period with ulcer problems in the RAS facility and could thus potentially be used as a biomarker for wound development. Quantitative PCR was established and used as a method for early detection of pathogens important in Norwegian aquaculture and geosmin-producing bacteria. This approach can be used to implement changes in production quickly if pathogens or geosmin-producing bacteria are detected.

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1 – Introduction

1.1 - Aquaculture in Norway

Norway has great conditions for aquaculture, partly due to its long coastline of approximately 102,900 kilometers (Kartverket, 2019), as well as good water quality, optimum water temperature ranges, and an abundance of wild fish which gave Norway a start to the industry (Bjerkestrand et al., 2013). Since the industry was introduced in 1960 seafood has become Norway's third most important export product (Statistisk sentralbyrå, 2019b). Many species are farmed in Norway, such as Rainbow trout (*Onchorhynchus mykiss* L.), cod (*Gadus morhua* L.), and halibut (*Hippoglossus hippoglossus* L.), in addition to cleaner fish like lumpfish (*Cyclopterus lumpus* L.) and wrasse (*Labrus bergylta* L.) (Misund, 2009). Since 2012 no cod was produced in Norway due to unprofitable production (Fauske, 2019), though recent successful breeding research gives positive outlooks on future production of cod (Johansen, 2019). Production of Atlantic salmon (*Salmo salar* L.) is the largest contributor to the growth in Norwegian aquaculture, with an export value of 72.5 billion NOK in 2019 (Norges Sjømatråd, 2020a). Export prices for Norwegian aquaculture products have risen considerably in the last few years (Richardsen et al., 2018). In May of 2020, the value of Norwegian salmon decreased for the first time since 2018, attributed to a low product demand because of the SARS-CoV-2 pandemic (Pettersen & Aandahl, 2020). Even still, the value of exported salmon corresponds to 64.5% of all Norwegian seafood export value in 2020 (Norges Sjømatråd, 2020b).

1.2 - Fish health in Norwegian aquaculture

In the 1980's bacterial diseases such as furunculosis, cold-water vibriosis, vibriosis, and yersiniosis caused large losses (Håstein, 2009a; Misund, 2009) and a major increase in the use of antibacterial drugs in Norwegian aquaculture. Especially classic furunculosis caused by *Aeromonas salmonicida* subspecies *salmonicida* infected thousands of farmed fish and was hard to treat before an effective oil based vaccine was introduced (Midtlyng et al., 2011). In the late 1980s vaccines against vibriosis caused by *Vibrio anguillarum* and

cold-water vibriosis caused by *Aliivibrio salmonicida* were introduced, an oil-based vaccine against furunculosis was introduced in 1993 (Sommerset et al., 2020), and in addition to stricter laws, and better farm operation, the use of antibiotics decreased with 99% between 1987 and 2013 (Misund, 2009). The introduction of effective vaccines also allowed for larger production volumes as fishing of wild stocks stagnated (Hoel, 2009). Thus, vaccination is one of the main reasons for the good health status of Norwegian farmed salmon (World Health Organization, 2015). Fish can be vaccinated in different ways, such as a dip or bath method, or orally through feed. The most prominent method in Norway is intraperitoneal injection of an oil-based vaccine (Sommerset et al., 2020; Statens legemiddelverk, 2020). Atlantic salmon are vaccinated against furunculosis (*A. salmonicida* subsp. *salmonicida*), cold water vibriosis (*Al. salmonicida*), vibriosis (*V. anguillarum*), winter wounds (*Moritella viscosa*), IPN (infectious pancreatic necrosis-virus), and yersiniosis (*Yersinia ruckeri*), though other vaccines may also be chosen depending on the geographic location of the farm (Sommerset et al., 2020).

According to the Fish Health Report (Fiskehelserapporten, Norwegian) published by the Norwegian Veterinary Institute, illness and injury accounted for 88% of Atlantic salmon losses in 2017 (Hjeltnes et al., 2019). Overall, bacterial infections are under control in Norwegian aquaculture and illnesses such as furunculosis and vibriosis do no longer cause many losses due to comprehensive vaccination efforts (Hjeltnes et al., 2019). Several illnesses do not yet have effective vaccines and still have negative impacts on the aquaculture industry. Bacteria such as *Flavobacterium psychrophilum*, *Tenacibaculum finnmarkense,* and *Tenacibaculum dicentrarchi* cause severe wounds in fish. Other bacteria of importance are *Renibacterium salmoninarum* and different mycobacteria. Though bacteria are not the only challenge in fish health and viral diseases also have a detrimental effect on fish health. The most important viruses in aquaculture are infectious salmon anemia (ISA), pancreas disease (PD), cardiomyopathy syndrome (CMS), heart- and skeletal muscle inflammation (HSMI), and infectious pancreas necrosis (IPN). All of these diseases have been reported at least ten times in 2019 (Sommerset et al., 2020). What poses one of the largest threats to the health of Norwegian farmed fish today is salmon lice (*Lepeophtheirus salmonis* L.). Salmon lice are natural parasites of salmonid fish in a marine environment and live off fish skin, mucus, and blood (Sommerset et al., 2020). The amount of salmon lice present in an aquaculture facility is under constant surveillance. A combination of preventative measures, medication, and continuous

delousing, either by cleaner fish or mechanical measures, are used to fight the spread of salmon lice (Veterinærinstituttet). Previously antiparasitic drugs were used, though widespread resistance led to the use of alternative, non-medicinal methods (Aaen et al., 2015). Sea lice larvae also pose a threat to wild salmon stocks, as they will spread from farmed fish to wild salmon in the spring (Sommerset et al., 2020). The rise in salmon lice treatment has considerably raised production cost of salmon and added strain on fish during delousing (Sommerset et al., 2020).

The aquaculture industry in Norway is subject to a large number of laws and regulations which ensure correct and optimum operation of farms (Akvakulturloven, 2006). These laws also constrict the use of antibiotics in Norwegian aquaculture. In 2019, a total of 213 kilograms of active-substance antibiotics were used in Norwegian aquaculture (Sommerset et al., 2020). This is a very low amount considering that 1,35 million tons of consumable fish and shellfish was produced in Norwegian aquaculture the same year (Statistisk sentralbyrå, 2019a). Spikes in antibiotics use seen in 2017 and 2018 can be attributed to outbreaks of yersiniosis in large post-smolt in sea cages. As salmon production is such an essential part of Norwegian aquaculture industry, research that will give better animal welfare, health, and profit, is sought after.

1.3 - Biology of Atlantic salmon

1.3.1 - Wild Atlantic salmon

Atlantic salmon is a fish species naturally found in the northern parts of the Atlantic Ocean and in its surrounding rivers. Atlantic salmon are anadromous, meaning they hatch in freshwater, juvenile fish migrate to the sea and stay there until they return to freshwater to spawn (Vøllestad, 2009a). The appearance of Atlantic salmon depends on the life stage. The first life-stages occur in freshwater, often in cold rivers. After hatching, the young fish are called fry, and become parr once they have started eating live feed and are roughly 2-2.5 cm long. Parr are recognizable by their brown color and their distinct markings, which camouflage them from predators. After approximately two to five years in the river, salmon will go through smoltification. Smoltification is a biochemical process that allows the fish to move from freshwater to saltwater (Jørgensen, 2014). The salmon, now called smolt, has a silver belly and an almost black back. Smolt will grow in the ocean

for one to four years, before they return to freshwater for spawning. Spawning salmon will again have a more brown color, to easily blend in with the river environment, and have a size of around 5-20 kg (males) (Vøllestad, 2009a). Atlantic salmon can spawn several times in their life, unlike other salmon species, such as the Pacific salmon (*Oncorhynchus spp.* L.), that die after spawning once (Vøllestad, 2009b).

1.3.2 - Life cycle of farmed Atlantic salmon

The life cycle of Atlantic salmon produced in aquaculture differs from the wild. Atlantic salmon will be hatched in hatching trays in a freshwater facility, typically in the fall. Then they will be moved to startfeeding tanks, where artificial feeding starts once fry have used up most of their yolk sac. Salmon will usually stay in the same freshwater facility until they go through smoltification. Two types of production models are used, depending on water temperature and the overall production plan. 0-year smolt will be in freshwater for 11-13 months before being moved to seawater in the fall. 1-year smolt will stay in the freshwater facility an additional winter, with setout in early summer. In total, salmon are in a freshwater facility between 11-22 months from eyed-egg stage until smoltification (Bjerkestrand et al., 2013). Once salmon are ready for seawater, they will be moved, either by truck or well boat, to a saltwater facility. Here, they have a grow-out period in the sea cages until they reach 4-6 kilograms, approximately 12-18 months from setout (Bjerkestrand et al., 2013) followed by slaughter and processing.

1.3.2 - Biology of fish skin

Fish skin is a dynamic tissue comprised of an epidermal layer, which also contains scattered goblet cells, allowing the secretion of mucus and together with keratocytes these form the outermost layer of fish skin (Karlsen et al., 2018; Shephard, 1994). The dermis lies underneath and contains connective tissue, blood vessels, scales, and collagen-rich tissue for flexibility. This innermost layer connects to the adipose and muscle tissues (Karlsen et al., 2018). Mucus found on the outer layer is mostly made up of glycoproteins and water, and shows many similarities to mucus found in mammalian species (Shephard, 1994). It serves a protective function mechanically, chemically, and immunologically (Fast et al., 2002). Different types of stress can affect mucus composition, such as handling and

changes in pH or temperature. Changes in mucus composition will make fish more susceptible to bacterial and viral infections (Reverter et al., 2018).

1.4 - Aquaculture systems

Intensive aquaculture is defined by artificial feed and higher fish density, whilst extensive aquaculture has very low density of fish with very little human intervention and fish live off natural feed from the environment (Misund, 2009). Norwegian aquaculture consists of only intensive aquaculture, though extensive aquaculture is normal in production of, for example, carp (*Cyprinus carpio* L.) in China (Misund, 2009). Two types of intensive aquaculture are represented in Norway; traditional flow-through systems with a grow-out period in sea cages, and recirculating aquaculture systems, which may or may not have a grow-out stage in sea cages (Skoglund, 2017).

1.4.1 - Flow-through aquaculture

A flow-through system is an aquaculture facility where water is continuously supplied from a nearby freshwater source, normally a lake or a river. Atlantic salmon will be hatched in this type of facility and stay there until it goes through smoltification, when it will be moved to a sea cage until slaughter. When water enters the farm, it passes through a mechanical filter to remove solids from the water. Some facilities also have a UV-filter for disinfection before the water moves to the tanks. In the colder months, water will be heated to optimum growth temperature, increasing electricity costs. Water is also aerated and added oxygen is used to keep the oxygen concentration constant in the tank. Water flows through the tank and is then filtrated to remove uneaten feed and feces, before it is released into a recipient water body (Lekang, 2013).

Flow-through systems have many advantages. One of them is the very little water treatment that is needed. The general layout of an intensive flow-through farm is relatively simple. Additionally, the fact that clean water is continuously supplied minimizes the risk of accumulation of pathogens, and also ensures that uneaten feed and feces are easily removed. A big disadvantage to a conventional flow-through system is the amount of water that is consumed. The average flow-through farm uses 0.3L of water per

kilogram biomass per minute (Agnalt et al., 2004). Also, a freshwater source has to be nearby. The water source needs to be large and clean to ensure optimum growth conditions for the biomass. Another disadvantage is the additional grow-out period in sea cages, with that come the challenges associated with sea lice, escaping fish, and the environmental impact.

1.1.3 - Recirculating aquaculture systems

Recirculating aquaculture systems (RAS) are fish farming facilities were outlet water is re-used instead of being released to the recipient water body (Lekang, 2013). Water enters the tanks in the same way, but will go through several cleaning steps, before being used again. Water treatment consists of a mechanical filtration to remove solids and a biological filtration to denitrify the water, followed by a degasser, oxygen supplementation, and a UV-disinfection step (Bregnballe, 2015). An important technical fixture in a RAS-facility is the biofilter used for denitrification after mechanical filtration. The biofilter consists of either a moving bed or a fixed bed. Both contain plastic chips with a large surface area on which microbes colonize, many of which oxidize ammonia to nitrite in a multi-step process (Könneke et al., 2005). Recirculation of water is not fully closed, as water that is lost through evaporation and filtration has to be replaced. However, many RAS-facilities have a 90-99% degree of recirculation (Bregnballe, 2015).

RAS-facilities have many advantages, such as significant reduction of water use. Where the conventional flow-through system uses 0.3 L of water per kilogram biomass per minute, a RAS facility can reduce that amount to 0.15 L (Agnalt et al., 2004). As freshwater is a limited resource, interest in RAS-facilities has increased (Lekang, 2013). Another advantage is that wastewater treatment costs will be drastically reduced, as very little water is released from the facility (Lekang, 2013). A RAS-facility will also give total control over production, as it ensures good water quality through thorough water treatment and all other water parameters, such as temperature and oxygenation are under tight control (Bregnballe, 2015). Potential diseases are controlled, due to the fact that very little new water enters the facility and the risk for pathogens entering through water is lowered (Bregnballe, 2015).

Recirculating systems have several disadvantages. Production cost is one of the biggest, as all water treatment steps and pumps give very high energy costs, overall consuming more electricity than a flow-through system (Lekang, 2013). Hydrogen sulfide (H_2S) is a big issue in RAS-facilities, though it has also been observed in some flow-through facilities recently (Sommerset et al., 2020). Hydrogen sulfide is produced as a bacterial byproduct when they metabolize organic material. Problems arise when there is a build up of H_2S, which occurs when a lot of uneaten feed is present, or when seawater is used, as seawater has naturally larger amounts of H_2S than freshwater (Åtland et al., 2020). Another important factor is the potential accumulation of pathogens. As mentioned, the chance of pathogens entering the facility is lowered, though if pathogens do enter they get the opportunity to form biofilms. RAS-facilities have many areas where removal of biofilm can be difficult, allowing an accumulation of biofilm and potentially pathogens. Additionally, the problems of sea lice, escaping fish, and environmental impact are not necessarily eliminated in a RAS-facility, because many of them still use a grow-out period in sea cages (Hjeltnes et al., 2019).

A way to avoid some of the issues associated with sea cages is to use a completely closed-off farm. Closed farms can choose to use seawater or brackish water for production after smoltification. This type of farming is emerging in Norway, and the first such facility in Norway is Fredrikstad Seafood AS. Here the entire production from the smolt life-stage is in a closed RAS facility, with slaughter and processing on-site (Nordic Aquafarms, 2020; Olsen, 2017). Fredrikstad Seafood AS is a salt-water farm with a moving-bed biofilter and an average salt concentration of 34 parts per thousand (ppt). The average water temperature is 12°C, with O2 levels at 100% and a CO2 concentration of 4.5 mg/L. The potential for pathogens entering the facility is lowered too, but contamination may occur through already infected smolt entering the farm or through the additional seawater that is taken into the facility.

1.1.4 - Sea cages

Sea cages are used to grow smolt and salmon will stay in a sea cage from smoltification until slaughter. One large disadvantage to sea cages is the natural presence of salmon lice in the marine environment. Escaping fish and environmental impact on surrounding wild

life are also drawbacks to sea cages. Farmed salmon have been bred for many generations to make them grow faster and larger, giving them a different genetic make-up compared to wild salmon (Glover et al., 2017). If farmed salmon mix with the wild populations, they may have a genetic (Hindar et al., 1991) and ecological (Thorstad et al., 2008) impact on wild salmon populations. Escaped farmed salmon also potentially introduce diseases and parasites to wild fish stocks, such as sea lice mentioned above. In the 1980s, farmed salmon caused a large outbreak of furunculosis in wild fish (Bjerkestrand et al., 2013). Farmed salmon can escape through breaks in the net due to poorly constructed nets, old nets, wear-and-tear due to weather or equipment, or boats too close to the nets (Bjerkestrand et al., 2013).

The environmental impact imposed by sea cages has been a challenge for Norwegian aquaculture. Sea cages used to be located in shallow waters that were sheltered from harsh weather and strong currents. These localities polluted the surrounding seafloor, with aggregations of uneaten feed, feces, and bacterial deposits below the sea cages (Bjerkestrand et al., 2013). Sea cages were moved to more suited localities, where the distance between the net and the seafloor is larger, and currents are present to diffuse waste (Bjerkestrand et al., 2013). Due to legislations in the aquaculture law and better technology, the environmental impact is under control and most production regions in Norway are considered low risk for environmental impact on seafloor (Hansen & Husa, 2019). As per April 2020, there are 3,489 active sea cages in Norway for farming Atlantic salmon and Rainbow trout, and this number has been relatively stable the last few years (Fiskeridirektoratet, 2005-2020). More aquaculture facilities may be built, as aquaculture is an important factor in future food production to feed a growing world population (Misund, 2009). Norwegian aquaculture is said to have a potential for six-doubling in value by 2050 (Almås & Ratvik, 2017). To do that better technology is needed to solve issues associated with the industry today and produce salmon in a more environmentally sustainable way. A way to remove some of the issues associated with aquaculture is to remove the grow-out period in sea cages, such as in a closed recirculating aquaculture system.

1.5 - Bacteria in aquaculture

1.5.1 - Normal microbiota of Atlantic salmon

Normal microbiota is defined as the microorganisms that naturally live on skin and mucosal membranes. Normal microbiota consist for the most part of bacteria, but may also contain fungi and protozoa (Tønjum, 2009). These microorganisms serve a protective role and are, in humans, extremely complex with more than 200 species of bacteria (Todar, 2020). Composition of skin flora in humans depends on the location, where moist areas usually have a higher density of bacteria than dry areas (Todar, 2020). Less densely colonized areas are typically comprised of bacteria such as staphylococci and micrococci (David, 1996).

Normal microbiota of fish is variable and depends on the area of the fish. In the eyed egg stage, *Pseudomonas* and *Cytophaga* species dominate (Cahill, 1990), though the microbial community changes once fry hatch. Many bacterial species grow on fish gills, and the most abundant are *Moraxella*, *Pseudomonas*, *Flavobacterium*, *Acinetobacter*, *Aeromonas*, *Bacillus*, and *Vibrio* species (Horsley, 1973). Additionally, the gastrointestinal tract of Atlantic salmon has a wide range of bacterial growth. Several bacterial taxa have been identified such as *Aeromonas, Acinetobacter, Enterobacter, Proteus, Moraxella,* and *Pseudomonas,* among others. Typical bacteria found in the gastrointestinal tract in fish in a marine environment are species from *Alcaligenes, Flavobacterium,* and *Vibrio* (Austin, 2002).

Normal skin microbiota in fish is still being discussed. Typically, normal microbiota is identified using a cultivation approach and/or 16S rRNA gene sequencing in a metagenomics approach (Minniti et al., 2017). Many argue that skin microbiota is transient and dependent on the microbes in the surrounding environment (Cahill, 1990; Horsley, 1973). More recently scientists suggested that not only does the skin microbiota change with the environment, but it also changes with stress (Minniti et al., 2017). Minniti and colleagues (2019) also suggest that the protein composition of Atlantic salmon mucus changes over time. They found that, when reared in disinfected salt water, mucus contained mostly proteins from Atlantic salmon during the first two days of the experiment, but soon evolved to contain mostly bacterial proteins, almost diminishing the

salmon proteins previously found (Minniti et al., 2019). The bacterial community found in mucus does not change significantly between fish that are fed a pellet diet, as is the norm in aquaculture, and fish that are fed a diet comprised of macroinvertebrates, which is similar to the natural diet of wild salmon (Landeira-Dabarca et al., 2013). Though, the composition of bacteria changes when the fish is not fed and the amount of epidermal mucus cells decreases during starvation, making it easier for bacteria to establish themselves on the skin (Landeira-Dabarca et al., 2013).

Other studies have found that the microbial community on salmon skin is distinct from the environment (Karlsen et al., 2017), further strengthening the theory that, though the environment may affect the composition of microbes, the overall flora is not identical to that of the environment. Karlsen et al (2017) also suggest that salmon mucus has a stable normal microbiota comprised of species of *Tenacibaculum* and *Acrobacter.* Other bacteria commonly found on the skin mucus of Atlantic salmon are *Pseudomonas, Lysobacter, Burkholderia, Methylobacterium, Sphingomonas, Vibrio, Rubritalea,* and *Pseudoalteromonas* (Minniti et al., 2017) (Minniti et al., 2019). What has not been explored much, is the composition of skin microbiota when salmon are farmed in environments different from sea cages, such as saltwater RAS or closed-off systems. Though it has been suggested that overall, RAS-farmed salmon are healthier with a higher number of skin mucosal cells than fish reared in a flow-through system (Minich et al., 2019).

Biofilm formation

In most natural environments, the prevailing microbial lifestyle is in association with a surface in a structure known as a biofilm. Biofilms are aggregations of bacteria connected to each other and the surface through a matrix. This matrix is produced by the bacteria and consists of extracellular material, lipids, polysaccharides, DNA, and proteins (Lönn-Stensrud, 2009). Biofilm formation typically occurs in four stages. The first stage is bacterial adhesion to surfaces and the second is the formation of micro-colonies, followed by stage three maturation of the biofilm. The last stage is the detachment of cells to colonize new surfaces (Armbruster & Parsek, 2018).

Biofilms are a problem in many industries, such as hospitals, food production, and water systems (Lönn-Stensrud, 2009). Aquaculture is no exception and in RAS facilities biofilms present a large problem. A RAS facility has many components and pumps for water treatment and transportation, many of which are difficult to clean and disinfect, giving a perfect environment for biofilm formation (Sommerset et al., 2020). Additionally, RAS facilities that produce post-smolts have to use brackish or seawater due to smolt biology. This can increase the risk of bacterial diseases where the infectious agent requires higher salt concentration to live (Sommerset et al., 2020). It has been suggested that there is a species-specific effect on bacterial communities in RAS-systems, meaning that microbial communities differ, depending on the fish species that live in the system (Martins et al., 2013). Additionally, studies have shown that the bacterial community found in biofilm will, over time, differ from that of the surrounding water environment (Bakke et al., 2017; Blancheton et al., 2013).

1.5.2 - Bacterial diseases in Norwegian aquaculture

Diseases caused by bacteria are a concern for aquaculture, and many bacterial fish infections exist worldwide. Norwegian aquaculture is mainly intensive aquaculture and the spread of disease poses a great threat, as large amounts of fish are held in small environments and the risk of infection is increased. The spread of infectious diseases in Norway is kept low by preventative measures such as vaccination. Even though the situation in Norwegian aquaculture is relatively stable, bacterial infections still occur. Several bacterial infections have mandatory notification to the Norwegian Food Safety Authority that they do not go undetected. Bacteria that have mandatory notification in Norway are *Renibacterium salmoninarum*, *Francisella sp.* (in cod), *Aeromonas salmonicida* spp. *salmonicida*, and *Flavobacterium psychrophilum* (in Rainbow trout) (Mattilsynet, 2014; Sommerset et al., 2020). Other bacteria are also of importance in Norway, such as *Yersinia ruckeri, Tenacibaculum dicentrarchi, Vibrio anguillarum, Moritella viscosa,* and *Aliivibrio wodanis.* These bacteria are widespread in Norway and cause losses in aquaculture despite vaccination efforts (Sommerset et al., 2020).

Detection and identification of these pathogens is done by traditional diagnosis of sick fish using clinical, histopathological and/or microbiological methods. Additionally, molecular diagnosis is playing an increasingly important role for rapid detection and

identification of pathogens. PCR followed by 16S rRNA gene sequencing offers an alternative to culturing due to the genetic variation of ribosomal genes in bacteria. PCR using species-specific primers is another method to confirm the presence of pathogens in diseased fish, and is a faster and easier method of identification than DNA sequencing.

Yersinia ruckeri

Yersinia ruckeri is a facultative aerobic, Gram-negative, motile, rod-shaped bacterium (Kumar et al., 2015). The bacterium grows easily on blood agar with creamy-white, smooth, and round colonies (Garrity, 2004). While *Y. ruckeri* can grow at a wide range of temperatures, its optimum growth temperature is between 20-28°C. The bacterium causes yersiniosis, or enteric redmouth disease (ERM), a serious septicemic disease of salmonid fish including Rainbow trout and Atlantic salmon (Sommerset et al., 2020). The bacterium has been isolated from other non-fish species, like humans, sea gulls, and turtles (Kumar et al., 2015). In Norway ERM is exclusively a problem in the farming of Atlantic salmon (Gulla et al., 2018). Infection temperature for *Y. ruckeri* is 18°C, lower than the bacterium's optimal growth temperature (Tobback et al., 2007). The disease can occur both before and after fish are introduced to seawater, but it is often first introduced in the freshwater stage. *Y. ruckeri* is transferred horizontally through direct contact between infected and healthy fish. The bacterium is found in the lower intestine of healthy fish and shedding of the bacterium in feces when carrier fish become stressed is likely to play an important role in disease transmission (Kumar et al., 2015). It has been suggested that ERM occurring late in the seawater grow-out stage is due to subclinical or latent infections activated by stress caused by delousing (Sommerset et al., 2020). *Y. ruckeri* can survive at least four months outside the host mostly due to the formation of biofilms that promote survival of bacteria on surfaces and in sediments in aquatic environments (Coquet et al., 2002). These biofilms could be a source of recurring infections in aquaculture facilities (Kumar et al., 2015). ERM has a broad geographical distribution and causes significant losses in aquaculture industry worldwide. *Y. ruckeri* has several identified serotypes, and serotype O1 is the most widespread in Norway, all large ERM outbreaks in Norwegian aquaculture can be attributed to this serotype (Gulla et al., 2018). Antibiotics are still in use to combat ERM. A vaccine is available, though with varying degree of effect and some farms feel the need to vaccinate all fish with auto-vaccines to be able to minimize outbreaks (Bornø & Lie, 2015; Kumar et al., 2015).

Aeromonas salmonicida

Aeromonas salmonicida ssp. *salmonicida* is a facultative anaerobic, Gram-negative, non-motile, rod-shaped bacterium (Veterinærinstituttet). It is the causative agent of furunculosis in farmed and wild salmonid fish, an important bacterial disease that causes large economic losses in aquaculture worldwide (Reith et al., 2008). *A. salmonicida* ssp. *salmonicida* grows with grey-white translucent colonies with a creamy consistency that shows hemolysis on ox blood agar (Garrity, 2004), colonies produce a brown, water-soluble pigment on agar containing tyrosine and/or phenylalanine (Sommerset et al., 2020). The optimum temperature for bacterial growth is 25°C, though the bacterium loses its virulence at temperatures above 22°C (Ishiguro et al., 1981). The infective dose of *A. salmonicida* ssp. *salmonicida* is low and bacteria are transmitted horizontally from diseased fish and latent carrier fish to healthy fish (Sommerset et al., 2020). Other subspecies of *A. salmonicida* exist and disease caused by subspecies other than *salmonicida* is called atypical furunculosis. Atypical furunculosis is especially problematic in cleaner fish (Sommerset et al., 2020). Furunculosis occurs at temperatures above 10°C, whilst at temperatures below 7-8°C the disease will only appear in latent form (Håstein, 2009b). *A. salmonicida* ssp. *salmonicida* has the ability to form biofilms, allowing for bacterial establishment in various environments, such as aquaculture facilities (Sommerset et al., 2020). Vaccines against furunculosis exist and are a part of the vaccine regimen in all Norwegian aquaculture facilities. No furunculosis was diagnosed in farmed fish in Norway in 2019, though *A. salmonicida* subsp. *salmonicida* was isolated from diseased wild salmon found in Trøndelag (Sommerset et al., 2020).

Vibrio anguillarum

Vibrio anguillarum is a facultative anaerobic, Gram-negative, motile and rod-shaped bacterium. It is a ubiquitous bacterium widely distributed in marine and estuarine environments, with an ability to survive for long periods during starvation (Garrity, 2004). Colonies grow rapidly on nutrient rich agar, such as blood agar, though bacteria can grow on media without added salt, a sodium chloride concentration of 1.5-2% is typically used to stimulate growth (Frans et al., 2011). They appear as hemolytic, round,

and cream in color (Buller, 2004). *V. anguillarum* is the causative agent of vibriosis, a deadly hemorrhagic septicemia affecting various marine, fresh- and brackish water fish (Garrity, 2004). Several different serotypes of *V. anguillarum* exist, though serotype O1 is the one causing most disease in aquaculture. Outbreaks usually occur at water temperatures above 15°C (Austin & Austin, 2016). *V. anguillarum* causes disease in many species such as Atlantic salmon, Rainbow trout, Sea bass (*Lateolabrax japonicus* L.), cod, and eel (*Anguilla anguilla* L.) (Frans et al., 2011). Additionally, it is known to cause disease in cleaner fish, surprisingly also at temperatures as low as 6°C (Sommerset et al., 2020). In both aquaculture and larviculture, this disease is responsible for severe economic losses worldwide. In Norway *V. anguillarum* serotype O1 was diagnosed once in Rainbow trout in 2019. Two non-serotyped infections occurred as well, both in fish with additional infections (Sommerset et al., 2020).

Aliivibrio salmonicida

Aliivibrio salmonicida is a facultative anaerobic, Gram-negative, curved, motile, rod-shaped bacterium that is widely distributed in marine habitats (Veterinærinstituttet). The bacterium is halophilic, it requires 0.5 – 4.0% sodium chloride for growth and makes small and grayish, non-hemolytic colonies when cultivated on ox blood agar with 1.5% NaCl. The optimum temperature for growth is 10-15°C. *Al. salmonicida* is the causative agent of cold-water vibriosis, a bacterial septicemia of farmed salmonid fish and Atlantic cod. The disease occurs when the water temperature is below 10°C (Colquhoun & Sørum, 2001). The bacterium is able to form biofilms, allowing the bacteria to establish themselves in the environment (Bjelland et al., 2012). Today more or less all farmed Atlantic salmon in Norway are vaccinated against this pathogen, which makes *Al. salmonicida* no longer a threat to the fish farming industries, and no infections were diagnosed in 2019 (Sommerset et al., 2020). However, disease is sporadically identified and in 2012-2013 outbreaks were reported in the Northern part of Norway (Veterinærinstituttet).

Moritella viscosa

Moritella viscosa is a facultative anaerobic, Gram-negative, motile and rod-shaped bacterium that is found in marine water and sediments. The bacterium grows well on

blood agar supplemented with 1% to 4% sodium chloride at temperatures between 4°C and 25°C (Garrity, 2004). Colonies are hemolytic, white and viscous, and form long strands when picked off agar (Benediktsdóttir & Heidarsdóttir, 2007). *M. viscosa* is considered the main etiological agent of winter ulcer disease in farmed salmonid fish, typically in cold regions with sea water temperature below 8°C (Einarsdottir et al., 2018). Infections occur in saltwater once temperature drops, though fish will recover in spring, when water temperatures increase (Lunder et al., 1995). Usually, mortalities are low during a *M. viscosa* outbreak, but deep ulcers will significantly lower fish welfare and product quality. Little is known about the transmission of infection though it has been suggested that horizontal disease transfer through water is not enough to cause disease (Lunder et al., 1995; MacKinnon et al., 2020). In Norway, all farmed fish are vaccinated against *M. viscosa*, reducing the amount of disease outbreaks (Sommerset et al., 2020), though the vaccine does not give optimum protection and even vaccinated fish may get infected (Lunder et al., 1995). In the period between 2008-2011 a total of 211 *M. viscosa* outbreaks were registered in Norway (Karlsen et al., 2014). In 2019 antibiotics were used to treat four outbreaks of *M. viscosa* in Atlantic salmon in sea cages (Sommerset et al., 2020).

Aliivibrio wodanis

Aliivibrio wodanis is a facultative anaerobic, Gram-negative, rod-shaped, motile bacterium. Bacterial growth is observed at temperatures below 20°C with an optimum growth temperature of 10°C (Lunder et al., 2000). Colonies appear yellow-orange in color and exhibit hemolysis when grown on blood agar infused with 2% sodium chloride (Takle et al., 2015). The bacterium is often isolated from winter ulcers alongside *M. viscosa,* though it has not been shown that *Al. wodanis* can cause winter ulcers without the presence of the latter*. Al. wodanis* causes disease in salmonid fish at low water temperatures, and grows faster than *M. viscosa* at 7°C (Hjerde et al., 2015). It can be found in the wounds of fish, but can also deposit in fish kidney (Takle et al., 2015). The bacterium is naturally found in seawater and it has been suggested that stress due to transportation, handling or high fish density could increase the risk of infection for this opportunistic pathogen (Sommerset et al., 2020). *Al. wodanis* transmits disease horizontally, from sick and carrier fish to healthy fish (Lunder et al., 2000). In Norway, winter ulcer disease does not have mandatory

notification, so it is hard to estimate the amount of infections that occur (Sommerset et al., 2020).

Tenacibaculum finnmarkense and *Tenacibaculum dicentrarchi*

Tenacibaculosis is an ulcerative skin disease of many economically important farmed marine fish species worldwide caused by members of the genus *Tenacibaculum*. *Tenacibaculum* are aerobic and motile, Gram-negative, rod-shaped, and thread-like cells, that are found in various marine environments (Klakegg et al., 2019). These bacteria produce yellow pigment and require specific growth media typically marine agar is used. On marine agar colonies appear as pale to bright yellow, round, convex, and are somewhat viscous, though they do not stick to the agar (Småge et al., 2016).

Signs of tenacibaculosis are similar to those seen in *M. viscosa* infections with deep wounds, and also occur in colder water temperatures (Sommerset et al., 2020). Contrary to *M. viscosa* infections, in infections with *Tenacibaculum*, the bacterium is solely isolated from the wound and not in any internal tissue (Sommerset et al., 2020). Particularly two *Tenacibaculum* species are of interest in Norwegian aquaculture, *Tenacibaculum finnmarkense* and *Tenacibaculum dicentrarchi*. *T. finnmarkense* was first identified in 2015 from winter ulcer affected fish in sea cages in Finnmark, Norway (Småge et al., 2016). It is strictly aerobic and has an optimum growth temperature of 2-20°C. *T. finnmarkense* is typically isolated with *M. viscosa* at sea temperatures between 3-6°C (Småge et al., 2016). *Tenacibaculum dicentrarchi* is also associated with winter ulcers, though *T. dicentrarchi* has a slightly higher growth temperature at 2-25°C (Klakegg et al., 2019). Norway has not had many *T. dicentrarchi* outbreaks, but the bacterium has been associated with tail and fin rot in Atlantic salmon in Chile (Avendaño-Herrera et al., 2016). An outbreak of winter ulcers was observed in Norway recently and isolates were similar to *T. dicentrarchi* strains found in Chile (Klakegg et al., 2019). Here, the water temperature during infection was 12°C, significantly higher than what is typically observed in infections with *T. finnmarkense*. *T. dicentrarchi* seems to transfer disease horizontally, but *T. finnmarkense* does not spread easily between fish (Småge et al., 2018). No vaccine is available against tenacibaculosis, and antibiotics have little effect, so diseased fish should be culled to avoid further spread (Sommerset et al., 2020).

Flavobacterium psychrophilum

Flavobacterium psychrophilum is another yellow-pigmented bacterium naturally found in soil, freshwater, and marine habitats (Garrity, 2004). The bacterium is Gram-negative, long and rod-shaped, motile, and strictly aerobe (Avendaño-Herrera et al., 2016). It is relatively difficult to grow in the laboratory, requires specific growth media, such as Ordal agar, and takes 3-6 days to grow with an optimum temperature between 15-20°C (Garrity, 2004; Veterinærinstituttet). *F. psychrophilum* causes Rainbow trout fry syndrome (RTFS) in young Rainbow trout and bacterial cold-water disease (BCWD) in adult Atlantic salmon, though it is also known as flavobacteriosis (Sommerset et al., 2020). BCWD will give wounds, large boils, fin rot, and may spread to internal organs leading to death (Sommerset et al., 2020). The disease is typically found in freshwater or brackish waters and outbreaks most often occur at water temperatures below 16°C, with more severe cases below 10°C (Starliper, 2011). *F. psychrophilum* spreads both horizontally (Jari et al., 2000) and vertically (Kumagai & Nawata, 2011). Autogenous vaccines against *F. psychrophilum* exist, but few choose to vaccinate their fish in Norway (Sommerset et al., 2020). If infection occurs in non-vaccinated fish, antibiotics are usually used to prevent further spread, as the disease spreads horizontally. Troubling is that *F. psychrophilum* seems to be gaining resistance to the antibiotic oxolinic acid (Sommerset et al., 2020), but the bacterium is sensitive to common disinfection methods, so thorough cleaning and disinfection is necessary (Veterinærinstituttet). In both 2018 and 2019 *F. psychrophilum* was diagnosed in Rainbow trout at four localities in Norway, though no infections occurred in Atlantic salmon (Sommerset et al., 2020).

Renibacterium salmoninarum

Renibacterium salmoninarum is an aerobic, Gram-positive, non-motile bacterium causing bacterial kidney disease (BKD) in wild and farmed salmonids. The bacterium grows very slowly with an optimum growth temperature of 15-28°C (Invasive Species Compendium, 2019; Sanders & Fryer, 1980). *R. salmoninarum* requires serum and cysteine for growth on agar, both of which are components of the most used medium, KDM-2. On this agar, colonies appear after more than six weeks of incubation as creamy-white, round, and

convex (Sanders & Fryer, 1980). When observed under a microscope, the rod-shaped bacteria usually appear in pairs. BDK is a serious, chronic infection in salmonid species and the disease transmits vertically from parents to offspring through infected roe (eggs) (Sommerset et al., 2020). The disease typically occurs in hatcheries and will be diagnosed post smoltification some time after fish are set out in sea cages when high mortality is observed (Veterinærinstituttet). No treatment or vaccine is available, so infection has to be avoided by constant surveillance. If infection occurs in spawning fish, they are slaughtered and replaced by disease-free fish to prevent further spread (Sommerset et al., 2020). BKD has been diagnosed one to three times on average per year. It was not at all diagnosed in Atlantic salmon in 2019, though, it was found once in Rainbow trout in a sea cage facility on the west coast of Norway (Sommerset et al., 2020).

Pasteurella sp.

Bacteria of the *Pasteurella* genus are typically Gram-negative, non-motile coccobacilli or rods. They are mostly aerobic, though facultative anaerobic species exist. Bacteria will grow on blood agar, though for some species, specific growth media may be required (Garrity, 2004). Symptoms of pasteurellosis are boils in muscle tissue and inner organs. The disease has been reported to cause high mortalities in different fish species such as wild white perch (*Morone americanus* L.), striped bass (*M. saxatilis* L.), and farmed yellowtail (*Seriola quinqueradiata* L.) (Bullock, 1978). *Pasteurella* bacteria have recently been causing disease in farmed Atlantic salmon and lumpfish (Sommerset et al., 2020). Pasteurellosis was first detected in Norway in 1989 and a significant increase in outbreaks in salmon was observed in 2019 with 14 affected saltwater localities, compared to only 1 in 2012 (Sommerset et al., 2020).

1.5.3 - Geosmin and geosmin-producing bacteria

Geosmin is a simple chemical compound (**Figure 1**) that causes a salmon fillet to have an earthy off-flavor. The compound has no known biological or toxic effects however the human detection limit is very low (400-500 ng kg^{-1}) (Burr et al., 2012) and due to this, geosmin should be avoided for best possible fillet quality. Geosmin is a by-product of bacterial metabolism, specifically it is produced by a group of bacteria known as actinomycetes and myxobacteria (Lindholm-Lehto & Vielma, 2019). These bacteria are

soil dwelling and can be found in hummus-rich waters or in biofilms (Dworkin, 1996). Important known geosmin-producing bacteria are *Nocardia cummidelens, N. fluminea, N. salmonicida, Streptomyces luridiscabiei, S. albidoflavus,* and *Saccharopolyspora erythraea* (Auffret et al., 2011; Burr et al., 2012; Schrader & Summerfelt, 2010).

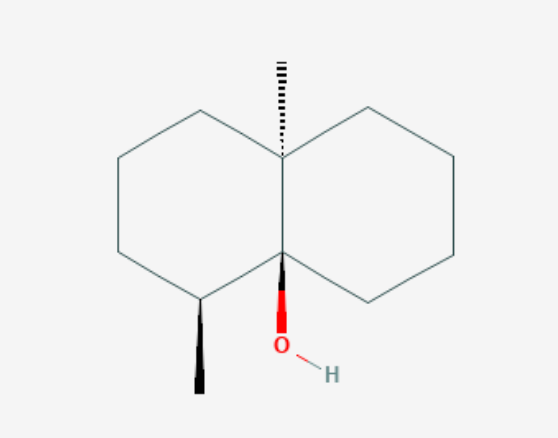

Figure 1: Chemical structure of off-flavor geosmin; a lipophilic, bicyclic alcohol made up of two carbon-rings with a single hydroxyl-group and two methyl-groups (National Center for Biotechnology Information, [online]).

Geosmin-producers are introduced into aquaculture systems through water, and whether the bacteria get to stay in the facility, and thereby impact fillet quality, depends on the type of aquaculture. In a flow-through system, water will move through the facility, and there will be less build-up of geosmin-producers, as all water is continuously exchanged with fresh water. In a RAS-facility biofilm will accumulate, which gives growth opportunities to more bacteria, including geosmin-producing bacteria (Davidson et al., 2015). Though, Schrader et al (2005) and Gonçalves & Gagnon (2011) suggest that the RAS biofilters have the highest amount of geosmin-producers, whilst Lukassen et al (2017) argue that the water itself contains just as many geosmin-producers as the biofilter.

Geosmin uptake occurs primarily through the gills of salmonid fish and will deposit in lipid-rich tissues. Consequently, the higher the fat-content of the fish, the more geosmin is deposited (Howgate, 2004). Uptake of geosmin is also dependent on other factors, such as water temperature, geosmin concentration, and exposure time. Overall, excretion of geosmin is significantly slower than uptake of the compound (Tucker, 2000). Removal of geosmin occurs through the gills by simple diffusion once the geosmin concentration is lower in the environment (Davidson et al., 2014). Water temperature will affect the

elimination of geosmin from the fish, where lower temperatures will extend the elimination time (Tucker, 2000).

In aquaculture, geosmin is removed from the fish through a depuration process. Here, fish are moved to clean seawater to excrete geosmin naturally. During this period, fish are not fed and the longer the depuration period, the more weight loss occurs (Burr et al., 2012). Additionally, more fat and water deviations were observed after a longer depuration period. Consequently, the depuration should be kept as short as possible to achieve best fillet quality without losing too much biomass. Many different methods have been tested for geosmin-removal, such as low-dose ozonation (Schrader et al., 2010), use of activated carbon, chlorine, UV-light, and combinations of those (Lindholm-Lehto & Vielma, 2019). Most effective geosmin-removal was shown to be pre-disinfecting the tank with hydrogen peroxide (H_2O_2) and using a water system devoid of aeration media (Davidson et al., 2014). Here, fillet geosmin concentration was considerably below the human detection limit after a 6-day depuration period (Davidson et al., 2014). Considering that high doses of harsh chemicals are needed for the desired effect makes these methods harder to implement in commercial RAS farming. Accordingly, depuration in clean saltwater is still the easiest and most accessible solution to off-flavor problems.

It has been shown that two essential water treatment compartments in RAS facilities harbor geosmin-producing bacteria: the biofilter and the denitrification chamber (Podduturi et al., 2020). Geosmin levels fluctuate over time, and were particularly high during and immediately after cleaning of both compartments, indicating the need to limit cleaning of biofilters and denitrification chambers to avoid a surge in geosmin (Podduturi et al., 2020). By monitoring the geosmin dynamics in RAS operations, farmers could prevent the compound from proliferating and negatively impacting the marked value of produced fish. Measuring geosmin can be done directly through gas-chromatography mass-spectrometry (GC-MS), or indirectly through measuring geosmin-producing bacteria by direct microscopy or PCR. GC-MS is an effective instrument for specific detection of geosmin, though it is a complicated process that requires expensive equipment and technical understanding (Broad Institute, [online]). Microscopy methods can be imprecise and will also not necessarily give a valid identification of the bacteria (John et al., 2018). Quantitative PCR is a simple detection method for geosmin-producing

bacteria to monitor the level of geosmin in a production facility without the need for GC-MS equipment, or the potential inaccuracy of microscopy.

The *geoA* gene encodes germacradienol synthase, which is an important enzyme in geosmin synthesis (Auffret et al., 2011). The gene is found in both *Myxobacteria* and *Actinomycetes* and may therefore be used as a molecular marker for detecting geosmin-producing bacteria in water and biofilm samples (Lukassen et al., 2017). *Saccharopolyspora erythraea* (previously identified as *Streptomyces erythraeus*) is a Gram-positive actinomycete that is typically found in soil (Liu et al., 2013). It produces antibiotics and geosmin as a metabolic byproduct (Auffret et al., 2011). As described by Auffret et al (2011), *S. erythraea* also contains the *geoA* gene, and its sequence matches standard *geoA* sequences 72-78%. For this study, *S. erythraea* was chosen to be a representative of geosmin producers.

2 – Aims

It is still unknown how production of Atlantic salmon in land-based saltwater facilities affects the skin microbiota of fish. Therefore, the overall focus of this study was to investigate the bacterial microbiota in a land-based saltwater RAS system for production of Atlantic salmon, from smoltification until slaughter. Additionally, the potential occurrence and accumulation of pathogenic as well as geosmin-producing bacteria is a challenge in RAS systems. An early detection method for changes in bacterial composition, as well as for pathogens and geosmin-producers could be used to take action and minimize the impact on fish health and product quality. Thus, this study aimed to develop and investigate screening methods for fast and reliable identification of bacteria as biomarkers to implement preventive measures against bacterial fish diseases and off-flavor fish products.

The aims of this study were:

> To describe the development of the bacterial microbiota in an Atlantic salmon saltwater RAS system and evaluate the usability of such microbiological monitoring for health and quality assessment.

To achieve these aims, the following sub-aims were established:

1. Establishing and optimizing a qPCR protocol as method for early detection of pathogenic and geosmin-producing bacteria found in a saltwater RAS facility
2. Describe the development of the cultivable microbiota in Atlantic salmon skin as well as in RAS biofilm and biofilters throughout part of a production cycle
3. Describe the development of geosmin-producing bacteria in a seawater RAS facility throughout part of a production cycle
4. Investigate and describe the occurrence of pathogenic bacteria in a seawater RAS facility throughout part of a production cycle

3 - Methods

3.1 - Establishment and optimization of qPCR protocol

3.1.1 - Verification of positive controls and optimizing template concentrations

Table 1: Bacterial isolates used as positive control DNA for all qPCRs.

Table 1 – Bacterial isolates for positive DNA controls

Bacterium	Strain	Source	Supplier	Growth medium
Aeromonas salmonicida subsp. *salmonicida*	ATCC® 14174™	Diseased Brook trout	ATCC, Manassas, USA	Ox blood agar base no. 2, 2.5% NaCl
Tenacibaculum dicentrarchi	In-house strain	Diseased fish	Bacteriology lab, NMBU	Marine agar
Renibacterium salmoninarum	In-house strain	Diseased fish	Norwegian veterinary institute, Oslo, Norway	Kidney disease medium (KDM)
Flavobacterium psychrophilum	NCIMB 2282	Diseased Coho salmon	NCIMB, Aberdeen, Scotland	Ordal medium
Yersinia ruckeri	In-house strain	Diseased fish	Bacteriology lab, NMBU	Ox blood agar base no. 2, 0.5% NaCl
Vibrio anguillarum	ATCC® 14181™	Diseased Sea trout	ATCC, Manassas, USA	Ox blood agar base no. 2, 2.5% NaCl
Saccharopolyspora erythraea	ATCC® 11635™	Soil	ATCC, Manassas, USA	N/A
Moritella viscosa	NCIMB 2263	Kidney of diseased Atlantic salmon	NCIMB, Aberdeen, Scotland	Ox blood agar base no. 2, 2.5% NaCl
Aliivibrio wodanis	In-house strain	Diseased fish	Bacteriology lab, NMBU	Ox blood agar base no. 2, 2.5% NaCl

Bacterial isolates chosen for positive DNA controls are shown in **Table 1**. *A. salmonicida* ssp. *salmonicida*, *V. anguillarum*, *M. viscosa* and *Al. wodanis* were grown on ox blood agar (Blood agar base no. 2, Thermo Fisher Scientific™, Waltham, USA) supplemented with 2.5% sodium chloride (NaCl). *Y. ruckeri* was grown on blood agar and *R. salmoninarum* was grown on kidney disease medium (KDM). Ordal medium was used to grow *F. psychrophilum*, and marine agar was used for *T. dicentrarchi*. DNA was isolated according to the QIAamp "Isolation of genomic DNA from bacterial plate cultures" protocol (DNA blood and mini handbook, QIAGEN, Hilden, Germany). *S. erythraea* DNA was re-hydrated from freeze-dried state as recommended by the producer. DNA was stored at -20°C.

To verify the identity of positive controls and to find the optimal template concentrations for future qPCRs, DNA from all bacteria were run through a qPCR at different dilutions; 1:10, 1:100, and 1:1000. All DNA dilutions as well as negative controls were run in duplicates. qPCRs were performed in a 20μL reaction using TaqMan™ Universal Master Mix (Applied Biosciences™, Waltham, USA) with 300nM of each primer and 100nM of probe in addition to 2μL of total DNA. *S. erythraea* qPCRs were performed using the Maxima SYBR green qPCR Master Mix (Thermo Fisher Scientific™), also in a 20μL reaction, with 250nM of each primer, and 2μL of DNA (1ng/μL).

The qPCR (Mx3000P, Agilent Technologies, Santa Clara, USA) cycles were pre-heating at 95°C for 10 minutes followed by 40 cycles of heating at 95°C for 15 seconds and annealing at 60°C (62°C for *A. salmonicida*) for 60 seconds. qPCR cycles for *S. erythraea* were as follows: pre-heating at 95°C for 10 minutes, then 40 cycles of 95°C for 15 seconds, annealing at 66°C for 60 seconds, and 72°C for 15 seconds, followed by a standard melting curve with heating to 95°C for one minute, 55°C for 30 seconds, and a final heating to 95°C for 30 seconds. To analyze qPCR results, the MxPro ET qPCR software (Agilent Technologies) was used.

3.1.2 - Testing of primers and probes and design of new primers

Table 2 shows all primers and probes used in this study. New primers were designed for two of the pathogens: *F. psychrophilum* and *T. dicentrarchi*. Primers for *F. psychrophilum* and *T. dicentrarchi* were designed based on the *gyrB* gene. The basis for the choice of *gyrB* is described by Habib et al. (2014) for *T. dicentrarchi*, and by Suzuki et al. (2001) for *F. psychrophilum*. FASTA sequences were aligned using Jalview. All primers were manufactured by Invitrogen (Thermo Fisher Scientific™) and tested using a SYBR green qPCR protocol. The qPCR was run as a 20μL reaction with 10μL of Maxima SYBR green PCR master mix (Thermo Fisher Scientific™), 0.6μL of each primer, and 2μL of total DNA. qPCR conditions were as follows: pre-heating at 50°C for 2 minutes, then heating at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and annealing at 58°C for 60 seconds. 58°C, 60°C, and 62°C were tested as annealing temperatures. An Mx3000P qPCR system (Agilent Technologies) was used for all qPCR analyses.

Newly designed primers and probes were test-run together. Here, TaqMan™ Universal PCR Master Mix (Thermo Fisher Scientific™) was used in a 20μL reaction with 300nM of each primer, and 100nM of probe, in addition to 2μL of template DNA. The qPCR temperatures were adjusted to probe detection as suggested by the producer of the master mix. The qPCR conditions were denaturation at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60 seconds at annealing temperature. Three different annealing temperatures were tested, 58°C, 60°C, and 62°C. FAM was the detection dye, and ROX was the reference dye. DNA from *F. psychrophilum* and *T. dicentrarchi* was added as template at four different dilutions, 1:1, 1:10, 1:100, and 1:1000. All were run in triplicates, with an addition of three negative controls per bacteria, where the template was DEPC water.

Table 2: Primers and probes sequences, including target genes and expected product length. *Two sets of primers were tested for *R. salmoninarum*.

Table 2 – Primers and probe sequences with references

Bacterium	Target gene	Forward/ Reverse primer	Nucleotide sequence (5'-3')	Expected amplicon length	Reference
Aeromonas salmonicida	*vapA*	Asal-vap-A-F Asal-vap-A-R Asal_probe	CGTTGGTGCTTCTATCACTGCTA AACAGCTACTTCACCCTGATTGG CCGTCAGGCTCGC	79 bp	(Fløgum, 2016)
Tenacibaculum dicentrarchi (set A)	*GyrB*	TeGyrB F TeGyrB R TeGyrB probe	TCGTATGCGTGAGTTGGCGTATTTAA GGTAAACCTTCTTTACTATGAAATGTTTCAG ATCATCTGTATTACGTTTATCTG		This paper
Tenacibaculum dicentrarchi (set B)	*GyrB*	T.dicenF T.dicenR T.dicenP	TAGCCTTTAGAAATGAAGATTA CCGTTACCTTACCATCTA ATTCGGCATCGGAACACTATTAA	119 bp	This paper
Renibacterium salmoninarum (set B)*	16S rRNA gene	Reni F Reni R Reni probe	TGGATACGACCTATCACCGCAT TCGCCTTGGTTAGCTATTACC TTTTTGCGGTTTTGGATGGACTCG	107 bp	(Jansson et al., 2008)
Flavobacterium psychrophilum (set A)	*GyrB*	FlGyrB F FlGyrB R FlGyrB probe	GTTGCTGAACCTCAATTTGAAGGTCAAA ATCGCCAACCGCTTGAGAAACTG TACTTCTCTATTTCCTAATTTGGT		This paper
Flavobacterium psychrophilum (set B)	hypo-thetical protein	FpSig-F FpSig-R FpSig-P	GGTAGCGGAACCGGAAATG TTTCTGCCACCTAGCGAATACC CGCTTCCTGAGCCAGA	220 bp	(Marancik & Wiens, 2013)
Yersinia ruckeri	*recA*	YRA-F1 YRA-R1	TCTGGACATCGCTCTGG AGTTTTTTTGCGTAGATAGGA	188 bp	(Bastardo et al., 2012)

		YRA-P	TATCGCCTCTGCACAGC		
Vibrio anguillarum	*toxR*	ToxR F ToxR R ToxR probe	ACACTGCAAAGCAAATTGATG TGATGGGCGTATTCACAACT TGGCTCTTCTATTGACTAGCCCTGCA	133 bp	(Kim & Lee, 2014)
Saccharopolyspora erythraea	*geoA*	AMgeo F AMgeo R	GAGTACATCGAGATGCGCCGCAA GAGAAGAGGTCGTTGCGCAGGTG	167 bp	(Auffret et al., 2011)
Universal 16S rRNA primers	16S rDNA gene	B27F U1492R	AGAGTTTGATCATGGCTCAGA GGTTACCTTGTTACGACTTC		(Weisburg et al., 1991) (Dojka et al., 1998)

3.1.3 - Specificity testing of primers and probes

Cross-reactivity to DNA from bacterial species other than the target bacterium was evaluated by performing qPCR reactions containing primers and probe for the target bacterium and template DNA from a panel of relevant bacteria. Relevant bacteria are listed in **Table 3**. A qPCR master mix was made, containing primers and probes for one of the selected bacteria. DNA from bacteria as described in **Table 1** was used as templates. **Figure 2** illustrates qPCR plate set-up.

Table 3: Illustration of cross-reactivity testing of primers and probes within selected bacteria.

Table 3 – Cross-reactivity testing

Bacterium	**Cross-reactivity tested against:**							
	A. salmonicida	*V. anguillarum*	*Y. ruckeri*	*R. salmoninarum*	*T. dicentrarchi*	*F. psychrophilum*	*M. viscosa*	*Al. wodanis*
A. salmonicida								
V. anguillarum								
Y. ruckeri								
R. salmoninarum								
T. dicentrarchi								
F. psychrophilum								

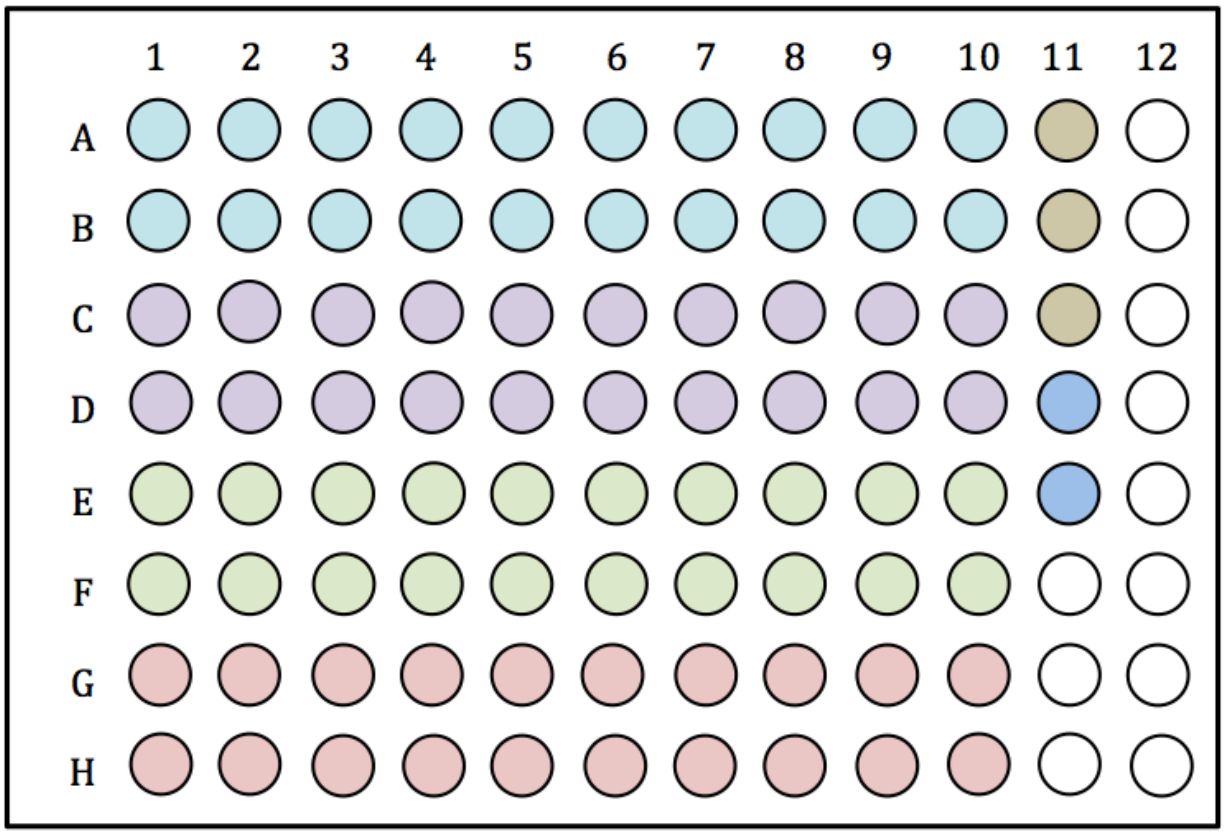

Figure 2: Example of a qPCR plate for a cross-reactivity test. Here, the master mix contains primers and probe for *F. psychrophilum*. The templates for the wells were as follows: light blue = DNA from *V. anguillarum*, purple = DNA from *Y. ruckeri*, green = DNA from *R. salmoninarum*, red = DNA from *T. dicentrarchi*, brown= DNA from *F. psychrophilum* (positive control), dark blue = no DNA, DEPC water as template (negative control). On a new plate, with the same master mix, the remaining bacteria were tested (*A. salmonicida, Al. wodanis* and *M. viscosa*). Cross tests were performed for all bacteria, except for *Al. wodanis* and *M. viscosa*, as there were no primers and probes available for these.

3.2 - Sampling

3.2.1 - Location and time of sampling

The sampling for this study was conducted at two salmon production facilities. Nesfossen smolt AS, located in Alver municipality (Vestland County, Norway), is a land-based freshwater smolt production farm. Fredrikstad Seafood AS (Nordic Aquafarms, Viken County, Norway) is a newly established saltwater, land-based grow-out facility that uses advanced recirculating aquaculture systems for salmon production and on-site fish processing. The fish had an average weight of 700g at the first RAS-sampling, and 1000g at the second RAS-sampling. No deviations from normal water parameters were

observed. The facility has two large production modules, fish holding tanks, with separate biofilters and water treatment systems.

Samples were collected on three occasions: once at the freshwater farm in Vestland, and twice at the RAS-facility in Fredrikstad. The sampling dates were 27th of September 2019, 31st of January 2020, and 09th of March 2020, denoted as sampling 1, sampling 2, and sampling 3. A sampling was also planned to take place in November 2019, though it was cancelled due to wounds and increased fish mortality. The sampling group remained the same over the course of these experiments, as fish that were sampled in the freshwater facility were moved to the saltwater RAS-farm on October 9th 2019. The sampling group was the second group of fish in the facility, the first group entered the farm in May 2019.

3.2.2 - Sampling procedures

Bacterial samples were obtained from fish mucus, and in the RAS-facility samples were also taken from tank biofilms, and the biofilter. Biofilm and biofilter samples were taken from two set locations in both production modules. At all three samplings, ten fish were randomly selected. Fish were anesthetized with MS-222 (Tricaine methane-sulfonate, PHARMAQ AS, Overhalla, Norway) at the Nesfossen facility, and with Benzoak vet (ACD Pharmaceuticals AS, Leknes, Norway) at the Fredrikstad facility. Dosages of anesthetics were calculated as recommended by the producers, 80mg MS-222 per liter water, and 20mL Benzoak vet per liter water.

After anesthetizing, mucus samples were taken from the left pectoral fin to the dorsal fin, using a sterile inoculation loop. Mucus samples were plated on blood agar plates (Blood agar base no. 2, Thermo Fisher Scientific™). For sampling 2 and sampling 3, blood agar was supplied with 2.5% sodium chloride due to environmental salt concentration of the RAS rearing water. Bacterial samples were also plated on bromo-thymol blue lactose agar (BTB agar, Thermo Fisher Scientific) at all samplings. Agar plates were incubated at 12°C for 2-5 days. Additionally, a Copan ESwab® was used across the same area of the fish and stored at 4°C until DNA extraction.

Tank biofilm was sampled on four different occasions over the course of eight months, all at the Fredrikstad Seafood facility. Biofilm samples were taken using a sterile cotton

swab, which was scraped along the tank wall in two different locations per fish tank module. Using an inoculation loop, the biofilm collected was scraped off the cotton swab and into an Eppendorf tube containing 350μL of DNeasy PowerBiofilm kit (QIAGEN, Hilden, Germany) lysis buffer. Additionally, the same cotton swab was streaked on a 2.5% NaCl blood agar plate for bacterial culturing. Samples were kept at 4°C during transportation. Once the samples arrived in the lab, the Eppendorf tubes containing the DNA samples were kept at 4°C for short-term storage, and the blood agar plates were incubated at 12°C for 2-5 days.

Biofilter chips were sampled twice. To sample bacteria from the biofilter, three to four biofilter chips were removed from the tank and transported in biofilter water. When the biofilter chips arrived in the lab they were immediately removed from the transportation water. Chips were cut into smaller pieces of approximately 5-8mm in diameter, and then placed in 350μL DNeasy PowerBiofilm (QIAGEN) lysis buffer. Biofilter samples were stored in lysis buffer at 4°C until DNA extraction.

3.2.3 - DNA extraction

DNA was extracted no later than two days after sampling. DNA from biofilter chips and from biofilm was extracted using the DNeasy PowerBiofilm kit (QIAGEN) with slight modifications. Briefly, the samples were placed in 350μL of kit-buffer on-site, and then heated at 55°C for 5 minutes in the lab to initiate cell lysis, followed by an additional heating step at 65°C for 5 minutes with an added 100μL of FB buffer. Lysis was completed by bead beating at full speed for 10 minutes. IRS solution was added, and then the samples were incubated at 4°C for 5 minutes for inhibitor removal. After DNA binding to a spin column, a series of washing and centrifugation steps followed, before DNA was eluted in 100μL of EB solution.

DNA was extracted from mucus using the QIAamp DNA blood and mini kit (QIAGEN). The protocol used was “Isolation of genomic DNA from Gram-positive bacteria” with some modification. The sample was obtained using an ESwab® (Copan Group, Brescia, Italy), and to extract DNA the ESwab® was centrifuged, to elute bacteria into the transportation liquid. Further, as much liquid as possible was pressed from the swab by pressing the

swab against the holding tube. Liquid was homogenized by vortex. All samples were centrifuged at full speed (13,000 rpm) for 10 minutes to form a pellet, and the supernatant was discarded. The bacterial pellet was then suspended in 180µL of 20mg/mL lysozyme, followed by a 30-minute incubation at 37°C. 20µL of proteinase K and 200µL of buffer AL was added, mixed by vortex, and incubated at 56°C for 30 minutes, followed by a denaturation period of 15 minutes at 95°C. After, the DNA purification from tissues protocol was followed. DNA was eluted in 200µL of elution buffer. Extracted DNA was stored at -20°C until further use.

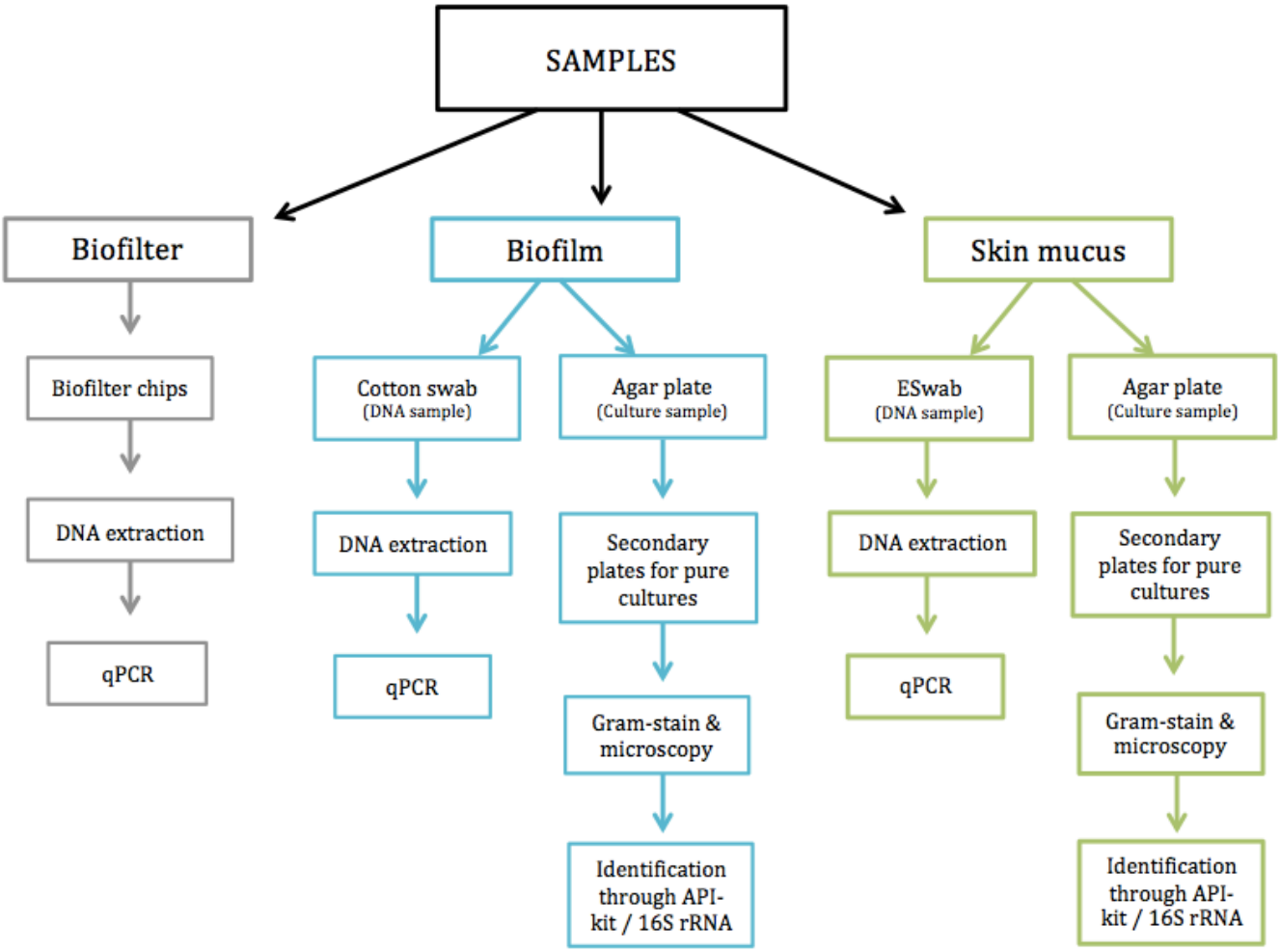

Figure 3: Schematic illustrations of all samples collected for this study and their processing.

3.3 - Bacterial identification

3.3.1 - Culture conditions

Bacterial isolates were obtained from salmon skin mucus and from biofilm. Growth media are described in section 2.2.2. Two agar plates were used for each sample and samples were diluted in a three-phase streaking pattern. All bacterial cultures were incubated at 12°C for 2-5 days before analysis.

3.3.2 - Identification of bacterial isolates from biological samples

The most prevalent colonies found on the plates were re-streaked on new plates to attain a pure culture for further analysis. Pure cultured bacteria were Gram-stained and then examined under a light microscope (Leica DM1000, Leica Microsystems, Wetzlar, Germany) at 100X magnification with oil. Colonies that appeared similar, but came from different types of agar, were tested on the same agar side by side, to confirm identical bacteria.

Isolates were further identified using the oxidase test and the API 20NE system (bioMérieux, Marcy I'Etoile, France). Inoculation of the API 20NE test strips was done according to the producer, and the strips were incubated at 30°C for 48 hours. The first two reactions, nitrate reduction (NO_3) and indole reaction (TRP) were interpreted after 24 hours, the rest after a total incubation period of 48h. Identification of isolates was done using the bioMérieux' Apiweb™ software.

Some isolates were unable to be identified due to delays in diagnostics because of COVID-19.

3.3.3 - 16S rRNA gene sequencing

Bacterial isolates that were not possible to identify by microscopy, traditional bacteriological methods and/or through the API 20NE kit were identified using 16S rRNA gene sequencing. Such isolates were for example isolates that gave negative results on all tests, with the exception of the oxidase test (API 20NE ID: 0000004/0000000).

3.3.3.1 - DNA isolation

DNA was isolated from a pure culture using a half-automated DNA extraction manual (QIAGEN). First, lysozyme buffer was made using frozen lysozyme and a lysis buffer. Bacteria were mixed into the lysis buffer by vortex, and then incubated at 37°C for 30 minutes. After the first incubation AL-buffer and proteinase K (DNA Blood and Tissue kit) were added and mixed. The solution was then incubated a further 30 minutes at 56°C. The DNA isolation was completed using the QIACube (Classic model, QIAGEN) program QIAamp DNA Blood mini – Blood and body fluid – manual lysis protocol.

3.3.3.2 - PCR

The PCR reaction was prepared using 2.5μL of 10X buffer, 1.0μL of magnesium chloride ($MgCl_2$), 0.5μL of deoxyribose nucleoside triphosphate (dNTP), 1.0μL of each primer (forward and reverse), and 0.2μL of Taq polymerase (Agilent Technologies). 2.0μL of total DNA was used in the reaction, and 16.8μL of DEPC water was used to raise the total reaction volume to 25μL. The PCR conditions were initial denaturation at 94°C for three minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 56°C for 30 seconds, and a 2-minute extension stage at 72°C. The final step was an extension at 72°C for a further five minutes and an infinite hold at 4°C (Weisburg et al., 1991).

3.3.3.3 - Agarose gel electrophoresis and gel extraction

A 1% agarose gel was prepared using 1g of agarose powder (Sigma Aldrich, St. Louis, USA) in 100mL of 1X TAE buffer and 10μL of SYBR safe gel stain (Invitrogen™, Thermo Fisher Scientific) was added to be able to visualize bands of DNA. The gel was casted and set in a plastic tray before loading samples. Two μL of loading dye was used. In addition to the sample DNA, a 1kb DNA ladder (Thermo Fisher, Waltham, USA) was run on the gel. The agarose gel was run at 90V for 60 minutes on a plastic box filled with 1X TAE buffer. To confirm the presence of correct PCR product, a picture of the gel was taken under UV-light (ChemiDoc™ XRS+ system with Image Lab™ Software, Bio Rad, Hercules, USA).

PCR product was extracted from the gel using the QIAquick gel extraction kit (QIAGEN). The producers' protocol was followed. The samples were sent for 16S rRNA gene sequencing to GATC-biotech (Ebersberg, Germany). Sequences were viewed using JalView and blasted using the Basic Local Alignment Search Tool (BLAST) at the National Center for Biotechnology Information (NCBI).

3.4 - Screening for pathogenic and geosmin-producing bacteria

3.4.1 - Screening for pathogenic bacteria

DNA that was extracted from biofilm, biofilter, and swab samples from fish skin mucus (see section 3.2.3), were screened for presence of pathogenic bacteria. The qPCR protocols established in section 3.1 were used to screen for the pathogenic bacteria *A. salmonicida, T. dicentrarchi, V. anguillarum, Y. ruckeri, R. salmoninarum,* and *F. psychrophilum*.

Samples from all sampling dates were run in one qPCR, with the master mix corresponding to one bacterium, as illustrated in **Table 4**. This was done specifically as some of the bacteria have different qPCR cycles, as established in section 3.1.

Table 4: DNA that was extracted from fish skin swabs are named after the fish they came from and which sampling date, e.g. F204 is DNA extracted from fish number 4 from the second sampling. F101-F310 = DNA from fish skin swabs, number and sampling date, BF = DNA extracted from biofilm, first number corresponds to sampling date, second number corresponds to location, BC = DNA extracted from biofilter chips, first number corresponds to sampling date, second number corresponds to location, PosC = positive control, DNA from bacteria to which the primers and probe belong, NegC = no template, control

Table 4 – qPCR plate set-up for screening for pathogens

	1	2	3	4	5	6	7	8	9	10	11
A	F101	F105	F109	F202	F206	F210	BF24	F303	F307	BF31	BC31
B	F101	F105	F109	F203	F207	BF21	BC21	F303	F307	BF31	BC31
C	F102	F106	F110	F203	F207	BF21	BC21	F304	F308	BF32	BC32
D	F102	F106	F110	F204	F208	BF22		F304	F308	BF32	BC32
E	F103	F107		F204	F208	BF22	F301	F305	F309	BF33	PosC

F	F103	F107	F201	F205	F209	BF23	F301	F305	F309	BF33	PosC
G	F104	F108	F201	F205	F209	BF23	F302	F306	F310	BF34	NegC
H	F104	F108	F202	F206	F210	BF24	F302	F306	F310	BF34	NegC

All samples including the positive control were run in duplicates. This process was performed for all six selected bacteria (see Appendix 1 for detailed qPCR cycles for all bacteria). For relative quantification of the target DNA, DNA concentration of all positive samples was measured using the Nanodrop™ ND1000 Spectrophotometer (Thermo Fisher Scientific). Samples were then adjusted to a DNA concentration of 2ng/µL, and a new qPCR was performed.

3.4.2 - Screening for geosmin producing bacteria

The qPCR protocol for *Saccharopolyspora erythraea* differs from previous protocols, as no probe was used. All samples from all sampling dates were processed in parallel in the laboratory. The qPCR reaction was performed in a 20µL reaction, with 250nM of each primer, and 10µL of Maxima SYBR green PCR master mix (Thermo Scientific™) (with added ROX, Agilent Technologies). 2µL of template was used. The plate set-up is shown in **Table 5**.

The qPCR cycle for *S. erythraea* included pre-heating at 95°C for 10 minutes, followed by 40 cycles of heating at 95°C for 15 seconds, annealing at 66°C for 60 seconds, and extension at 72°C for 15 seconds. Finally, a standard melting curve was performed to confirm dissociation of the double stranded DNA. The melting curve cycle was heating at 95°C for 1 minute, then 55°C for 30 seconds, and a final heating to 95°C for 30 seconds.

Table 5: qPCR plate set up for screening for geosmin-producer *S. erythraea*.

Table 5 – qPCR plate set up for screening for *S. erythraea*

	1	2	3	4	5	6	7	8	9	10	11
A	F101	F105	F109	F202	F206	F210	BF24	F303	F307	BF31	BC31
B	F101	F105	F109	F203	F207	BF21	BC21	F303	F307	BF31	BC31

C	F102	F106	F110	F203	F207	BF21	BC21	F304	F308	BF32	BC32
D	F102	F106	F110	F204	F208	BF22		F304	F308	BF32	BC32
E	F103	F107		F204	F208	BF22	F301	F305	F309	BF33	
F	F103	F107	F201	F205	F209	BF23	F301	F305	F309	BF33	PosC
G	F104	F108	F201	F205	F209	BF23	F302	F306	F310	BF34	PosC
H	F104	F108	F202	F206	F210	BF24	F302	F306	F310	BF34	NegC

For relative quantification of the target DNA, DNA concentration of all positive samples was measured using the Nanodrop™ ND1000 Spectrophotometer (Thermo Fisher Scientific) and adjusted to a DNA concentration of 2ng/µL before a new qPCR was performed. Standard deviation of positive Ct-values was calculated in Microsoft Excel.

4 - Results

4.1 - Establishment and optimization of qPCR protocol

The identification of bacterial strains used as positive controls in this study were confirmed by qPCR (data not shown). Additionally optimal template concentrations for future qPCRs were found. The preferred DNA concentrations were 5 ng/μL for *A. salmonicida* and *T. dicentrarchi*, 2 ng/μL for *Y. ruckeri, F. psychrophilum,* and *V. anguillarum,* and 8 ng/μL for *R. salmoninarum*. *S. erythraea* had the lowest DNA concentration of the positive controls with 1 ng/μL.

All primers and probes were found to bind specifically to their respective target DNA with Ct-values varying between 17.61 for the higher DNA concentrations and 34.55 for low DNA concentrations (detailed data shown in **Table 6**), except for set A of *R. salmoninarum* that gave positive Ct-values in 14 of 26 negative controls. Therefore, primers and probe set B was introduced and found to bind *R. salmoninarum* DNA satisfactorily (see **Table 6**). Here, Ct-values varied from 15.70 to 23.09, depending on DNA concentration. Due to this, *R. salmoninarum* set B was used for further testing and screening for pathogens. Preferable annealing temperature was found to be 60°C for all primer sets except for *A. salmonicida* (62°C) and *S. erythraea* (66°C). Dissociation curves for all primer sets were performed using a SYBR green qPCR protocol, and all sets had satisfying melting curves.

Table 6: Ct values of positive controls. *Dilution was not run.

Table 6 – Ct-values for positive controls

	Ct-values, $\bar{x}$					
Template dilution	*A. salmonicida* ssp. *salmonicida*	*F. psychrophilum*	*R. salmoninarum* (set B)	*T. dicentrarchi*	*V. anguillarum*	*Y. ruckeri*
1:1	28.07	-*	-*	-*	-*	-*
1:10	30.17	17.61	15.70	26.32	25.50	18.63
1:100	34.55	20.78	19.38	29.76	28.04	21.82
1:1000	no Ct	24.39	23.09	33.79	31.42	25.92

Cross-reactivity of the tested qPCR primers and probes is illustrated in **Table 7**. No cross-reactivity was found for primers and probes of *A. salmonicida* ssp. *salmonicida*, *T. dicentrarchi* (set A), and *F. psychrophilum* (set A). Cross-reactivity between primers and probes of *V. anguillarum*, *Y. ruckeri, R. salmoninarum* and *M. viscosa* and DNA from other bacterial species was observed for a limited number of qPCR reactions and with high Ct-values (>37.00). For these reactions, cut-off values were defined to avoid misinterpretation of results for later analyses.

V. anguillarum showed eight cross-reactions out of a total of 133 cross-reactivity reactions. Five out of 20 positive cross-reactions occurred between *V. anguillarum* primers and *M. viscosa* template DNA (Ct-values: 37.44, 37.62, 37.77, 38.29, 38.29), and three out of 20 between *V. anguillarum* primers and *F. psychrophilum* DNA (Ct-values: 37.41, 37.53, 37.81). A cut-off value of Ct≥37.00 was set for *V. anguillarum*, to avoid possible false positives due to cross-reaction between the primers and *F. psychrophilum* and/or *M. viscosa* DNA. *Y. ruckeri* primers showed cross-reactivity to DNA from *M. viscosa* (Ct-value: 39.87), *R. salmoninarum* (Ct-value: 39.91) and *F. psychrophilum* (Ct-value: 39.93). Thus, a cut-off value of Ct≥39.00 was defined. *R. salmoninarum* primers cross-reacted with DNA from *Y. ruckeri* (Ct-values: 37.15, 37.36, 37.44), *F. psychrophilum* (Ct-value: 39.25) and *Al. wodanis* (Ct-value: 39.31). A cut-off value of Ct≥37 was defined for *R. salmoninarum* primer set B.

Primers and probe set B for *T. dicentrarchi* and *F. psychrophilum* demonstrated cross-reactivity to DNA from other bacteria in several reactions. *T. dicentrarchi* set B cross-reacted with *R. salmoninarum* DNA (Ct-values: 36.50, 37.14, 37.19, 37.19, 37.29, 37.33, 37.36, 37.42, 37.45, 37.72, 37.72, 38.07, 38.31, 38.38, 38.42, 38.88, 39.13, 39.40). The same was seen for set B of primers and probe for *F. psychrophilum* that reacted to DNA from both *R. salmoninarum* (Ct-values: 33.71, 34.98, 35.22) and *Y. ruckeri* (Ct-values: 34.90, 34.99, 35.03, 35.06, 35.06, 35.74). Due to the low specificity for set B of both *T. dicentrarchi* and *F. psychrophilum* these primers and probes were replaced by set A and not further used in this study.

Table 7: Cross-reactivity testing of primers and probes. Color-coded, where black represents cross-reactivity not applicable, and green means all reactions were negative. Orange indicates a positive cross-reaction in the

indicated reaction only, not in the opposing reaction. Red color indicates a positive reaction both ways. All orange and red boxed are denoted with the number of positive reactions that occurred.

Table 7 – Cross-reactivity testing results

Primers and probes from:	Cross-reactivity tested against template DNA:							
	A. salmonicida	*V. anguillarum*	*Y. ruckeri*	*R. salmoninarum*	*T. dicentrarchi*	*F. psychrophilum*	*M. viscosa*	*Al. wodanis*
A. salmonicida								
V. anguillarum						3/20 positive	5/20 positive	
Y. ruckeri				1/20 positive		1/20 positive	1/20 positive	
R. salmoninarum (Set B)			3/20 positive			1/20 positive		1/20 positive
T. dicentrarchi (Set B)			16/20 positive	18/20 positive				
F. psychrophilum (Set B)			6/20 positive	3/20 positive				
T. dicentrarchi (Set A)								
F. psychrophilum (Set A)								

4.2 - Bacteriological results

4.2.1 - Bacteria isolated from fish mucus

Bacterial isolates grown from fish mucus samples were identified using API-20NE kits and 16S rRNA sequencing. The most dominating bacterial genera were identified from fish mucus over the course of all three samplings and are illustrated in **Figures 4-6**. The number of times each genus was identified includes isolates of the same species but may also include different species in that genus.

Samples from the freshwater facility generally had more diverse bacterial growth on blood agar plates, compared to the samples from the RAS facility. Overall, the freshwater fish had many apparently different bacteria, though few colonies of each. All bacteria that were found on plates were Gram-negative, and most were rod shaped, though some were coccobacilli, such as *Acinetobacter junii*. Plates did not differ much from one another in bacterial growth. On average, four different bacterial species were identified on agar plates from freshwater samples.

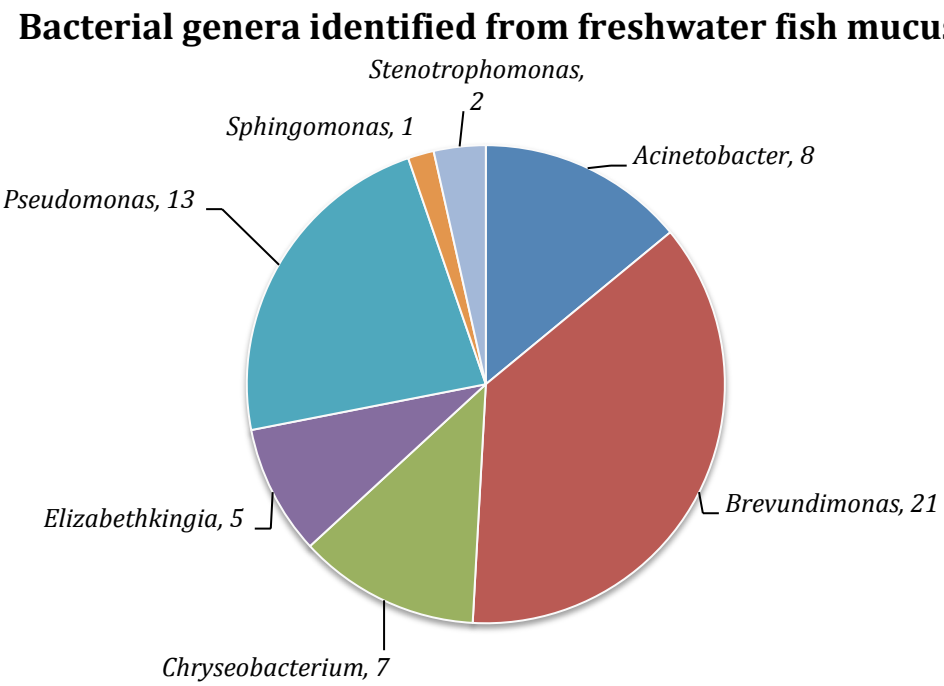

Figure 4: Overview over bacterial genera identified from fish mucus during freshwater sampling, pre-smoltification. Number of times a genus was identified may include more than one species.

Brevundimonas vesicularis and *Pseudomonas fluorescens* had the most abundant growth out of all bacteria in freshwater fish mucus samples. Furthermore, *Acinetobacter* species were identified eight times and included *A. junii/johnsonii, A. lwoffii,* and *A. baumanii/calcoaceticus.* These bacterial species were however not identified in the RAS samples. Presence of *Pseudomonas fluorescens* remained relatively stable over the sampling period. The bacterium was identified 13 times in freshwater fish mucus, nine and ten times in mucus from RAS-fish. *B. vesicularis* also remained among the most dominating bacteria in RAS samples.

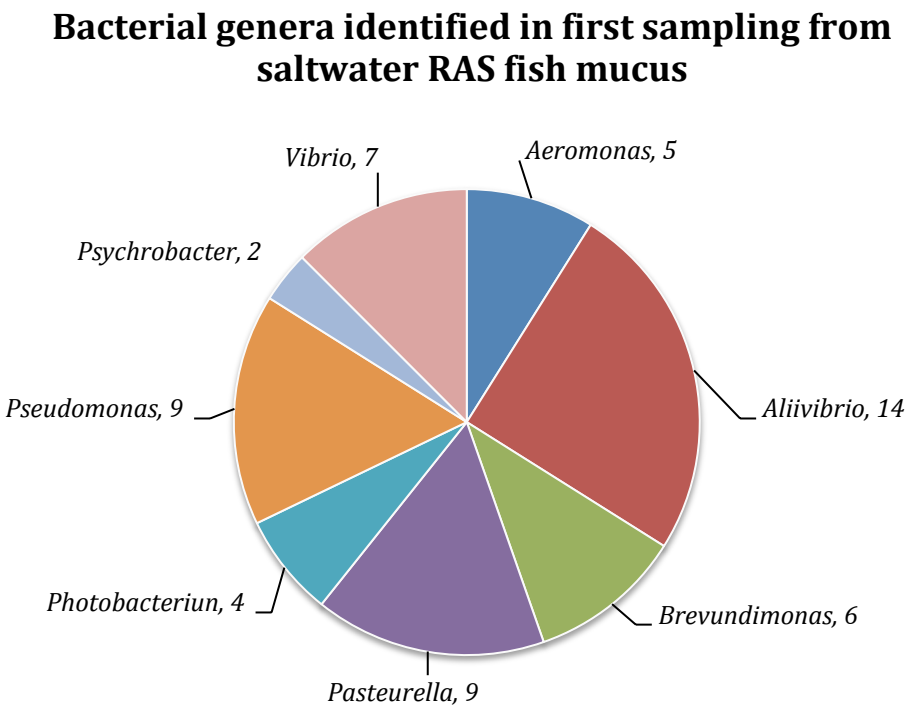

Figure 5: Overview over bacterial genera identified from fish mucus using traditional bacteriological methods from the first saltwater RAS sampling, post-smoltification. Number of times a genus was identified may include more than one species.

C. indologenes was found in fish mucus from freshwater, though it was not found in mucus in RAS, but in biofilm samples. On average, three different colony types were identified on each agar plate from the first saltwater RAS sampling. Overall, saltwater RAS fish skin mucus samples were dominated by *Aliivibrio, Pseudomonas, Pasteurella,* and *Vibrio* species. At the first saltwater RAS sampling, the most frequently identified bacterium was *Aliivibrio wodanis*. Isolates from wound samples were identified as *Aliivibrio wodanis* and *Pasteurella multocida*.

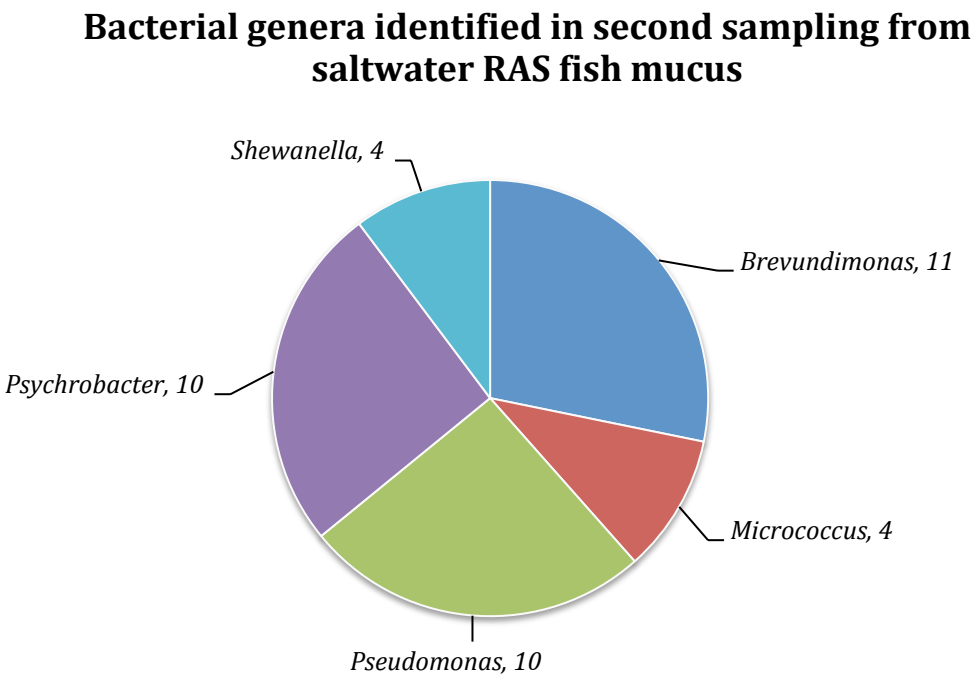

Figure 6: Overview over bacterial genera identified using traditional bacteriological methods from fish mucus during the second saltwater RAS sampling, post-smoltification. Number of times a genus was identified may include more than one species.

The second saltwater RAS sampling differed from the first in that there was less bacterial diversity. Further, less bacterial growth was observed in samples, and on average two different colonies were observed per blood agar plate and on BTB agar the average was less than one colony per plate. The most frequently identified species was *Brevundimonas vesicularis*, similar as in the first RAS sampling. More *Psychrobacter* species were identified compared to the previous RAS-sampling. No *Aliivibrio* species were identified at the second RAS sampling.

4.2.2 - Bacteria isolated from biofilm samples

Bacterial isolates grown from biofilm samples were identified using API-20NE and 16S rRNA sequencing. The bacterial genera that were identified in RAS-biofilm over the course of 8 months are shown in **Figure 7**.

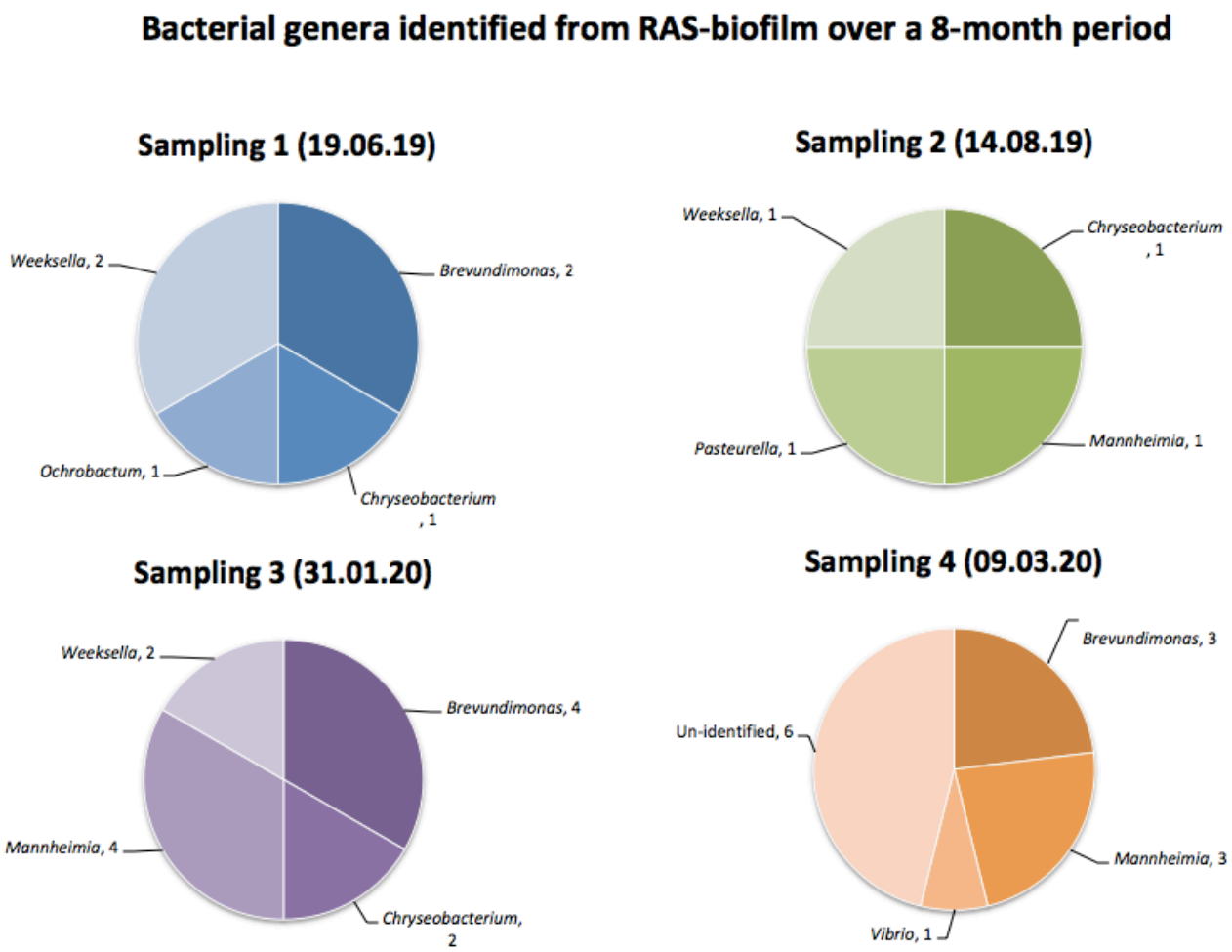

Figure 7: Overview over all bacterial genera identified in RAS-biofilm samples using traditional microbiological methods. Un-identified bacteria from sampling 4 were not identified due to delays in diagnostics during COVID-19.

Overall, the bacterial growth and diversity from biofilm samples were lower compared to fish mucus samples with an average of three isolates per blood agar plate. Four different locations were sampled for biofilm at each sampling, and did not differ from one another, suggesting that biofilm microbiota is similar across the RAS facility. Although, there was a decrease in *Weeksella* species and an increase in *Brevundimonas* and *Mannheimia* species identified, the isolated bacteria from biofilm did not change significantly over the sampling period. Several bacterial species that were found in biofilm were not identified in mucus samples, such as *Weeksella virosa, Ochrobactum anthropi, Mannheimia haemolytica,* and *Vibrio splendidus/atlanticus.*

4.3 - Presence of geosmin producing bacteria

qPCR for detection of the geosmin-producing bacterium *S. erythraea* were performed for freshwater skin mucus samples, saltwater RAS skin mucus samples, as well as biofilm and biofilter locations in the RAS facility. *S. erythraea* was found in all samples, except for fish skin mucus from the second saltwater RAS sampling in a qPCR using stock DNA. In the original DNA stock total DNA concentrations varied between 2.98 ng/µL to 85.66 ng/µL. After standardizing the total DNA to 2 ng/µL, one of the freshwater samples did no longer have detectable amounts of target DNA. **Figure 8** shows the relative quantification of *S. erythraea* found in fish skin mucus, biofilm, and biofilter samples.

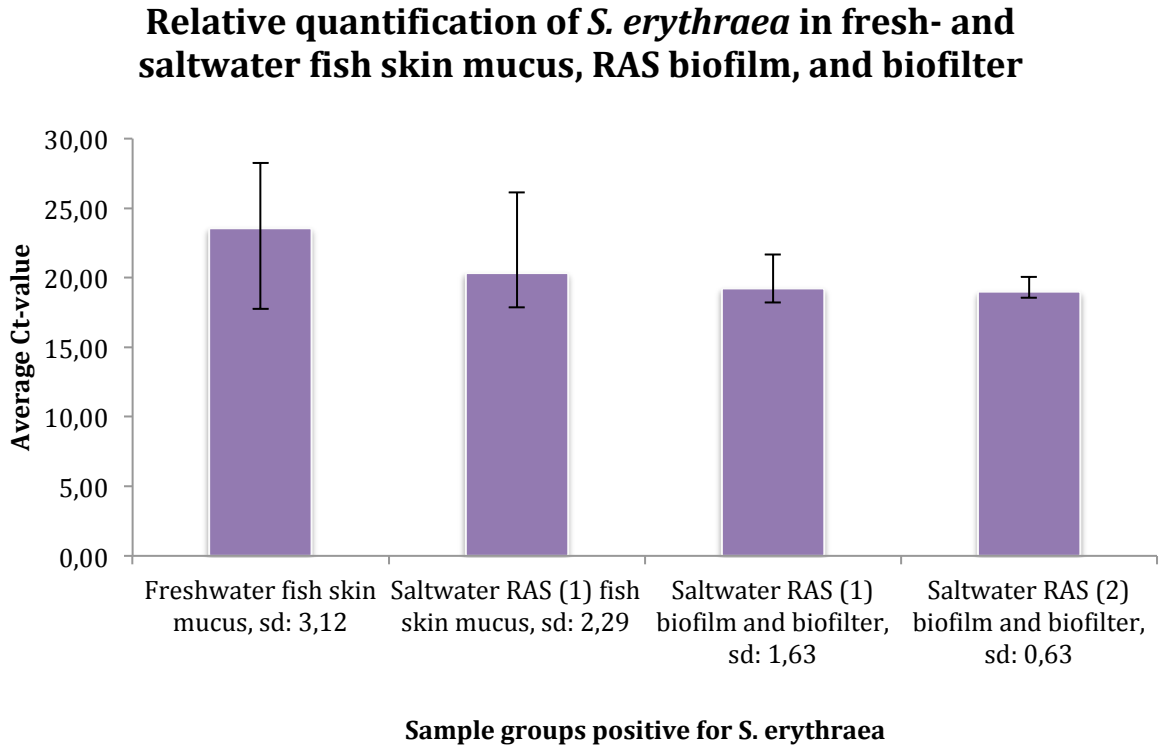

Figure 8: Relative quantification of representative geosmin-producer, *S. erythraea* in fish skin mucus, RAS-biofilm, and biofilter with standard deviation. Error bars represent maximum and minimum Ct-values measured for that group.

The concentration of *S. erythraea* DNA was higher in samples from biofilm and biofilter compared to fish mucus, indicating that the geosmin-producing bacterium is more abundant in environmental surfaces than in fish mucus. However, the relative concentration of *S. erythraea* DNA in fish mucus varied greatly. No *S. erythraea* was detected in fish mucus sampled in the second RAS-sampling, though it was still present in

both biofilters and biofilm samples. Fish mucus samples sampled from freshwater demonstrated a lower relative concentration of geosmin producing bacteria compared to fish mucus sampled from the seawater facility.

4.4 - Screening for pathogens using qPCR

The established qPCR protocol was used to screen for fish pathogens. Fish mucus, biofilm, and biofilter samples were screened for *A. salmonicida* subsp. *salmonicida*, *T. dicentrarchi, F. psychrophilum, R. salmoninarum, Y. ruckeri,* and *V. anguillarum*. No qPCRs showed Ct-values for *A. salmonicida* subsp. *salmonicida*, *R. salmoninarum, Y. ruckeri,* or *V. anguillarum*. DNA from *T. dicentrarchi* and *F. psychrophilum* were identified in some samples with total DNA concentrations ranging from 2.62ng/μL to 199.59ng/μL in sample stocks. Before standardization to 2ng/μL, 21 samples gave Ct-values ranging from 24.84 to 39.97 when screening for *T. dicentrarchi*. The relative quantification of *T. dicentrarchi* is shown in **Figure 9**. All standardized samples had Ct-values higher than 35.9, indicating low concentrations of target DNA. Very little target DNA was detected in two of the sampling locations, one in the first RAS sampling and one in the second RAS sampling. Here, Ct-values are above 39 and could be cut off, though they are included here to illustrate the difference in biofilm sampling locations. Generally more target DNA was detected in samples from the first RAS sampling, with more *T. dicentrarchi* found in fish tank module 2 (biofilm1.1 and 1.2). In fish tank module 1, *T. dicentrarchi* was only detected in one of two locations (biofilm1.4).

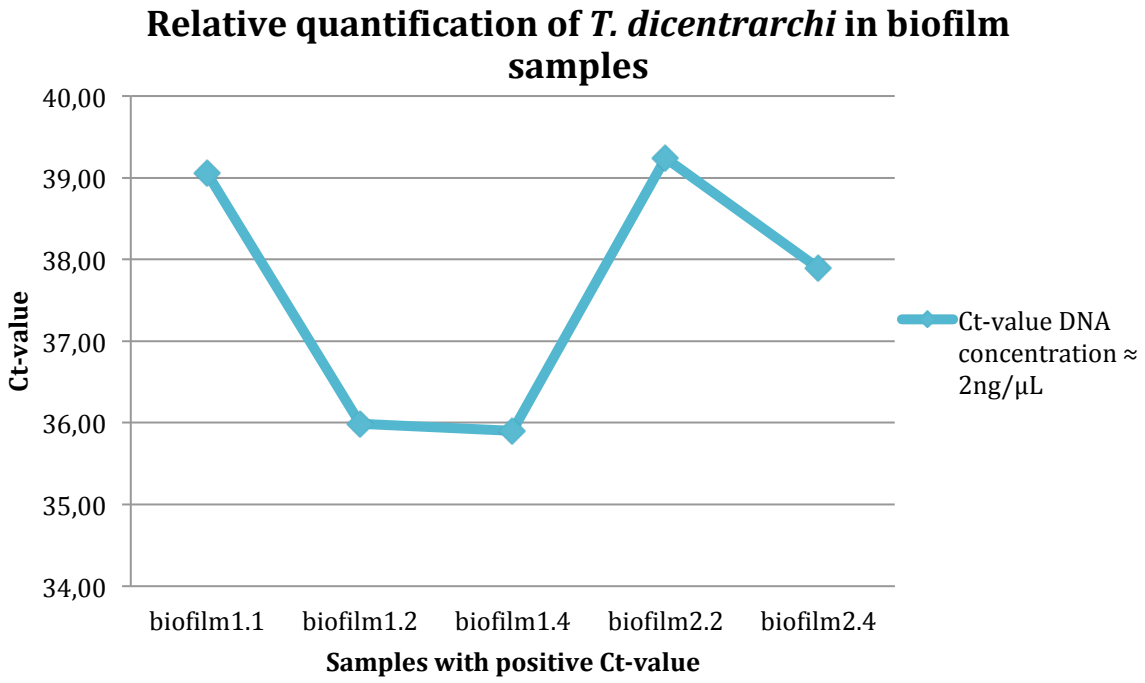

Figure 9: Relative quantification of *T. dicentrarchi* in RAS-biofilm. Biofilm1.1 and 1.2 are two sampling locations in fish tank module 2, and 1.3 (not shown) and 1.4 are from fish tank module 1 in the first RAS sampling. Biofilm2.2 (module 2) and biofilm2.4 (module 1) are the two locations from the second RAS sampling.

During qualitative screening for *F. psychrophilum*, 10 out of 41 samples had Ct-values ranging from 34.92 to 39.09. Results for the qualitative screening can be seen in **Figure 10**. As shown, higher DNA concentration corresponded with lower Ct-values. Once DNA was standardized to 2ng/µL for relative quantification, no samples had detectable amounts of *F. psychrophilum* DNA. This could indicate that the DNA was too diluted, though the qualitative screening also had very high Ct-values, which shows that very little DNA was detected even in higher DNA concentrations. Less *F. psychrophilum* DNA was observed in the biofilter samples compared to biofilm samples, though biofilter samples also had less total DNA. Amount of *F. psychrophilum* detected did not vary much between sampling dates, but most DNA was detected in biofilm samples from fish tank module 1 in both the first and second RAS sampling.

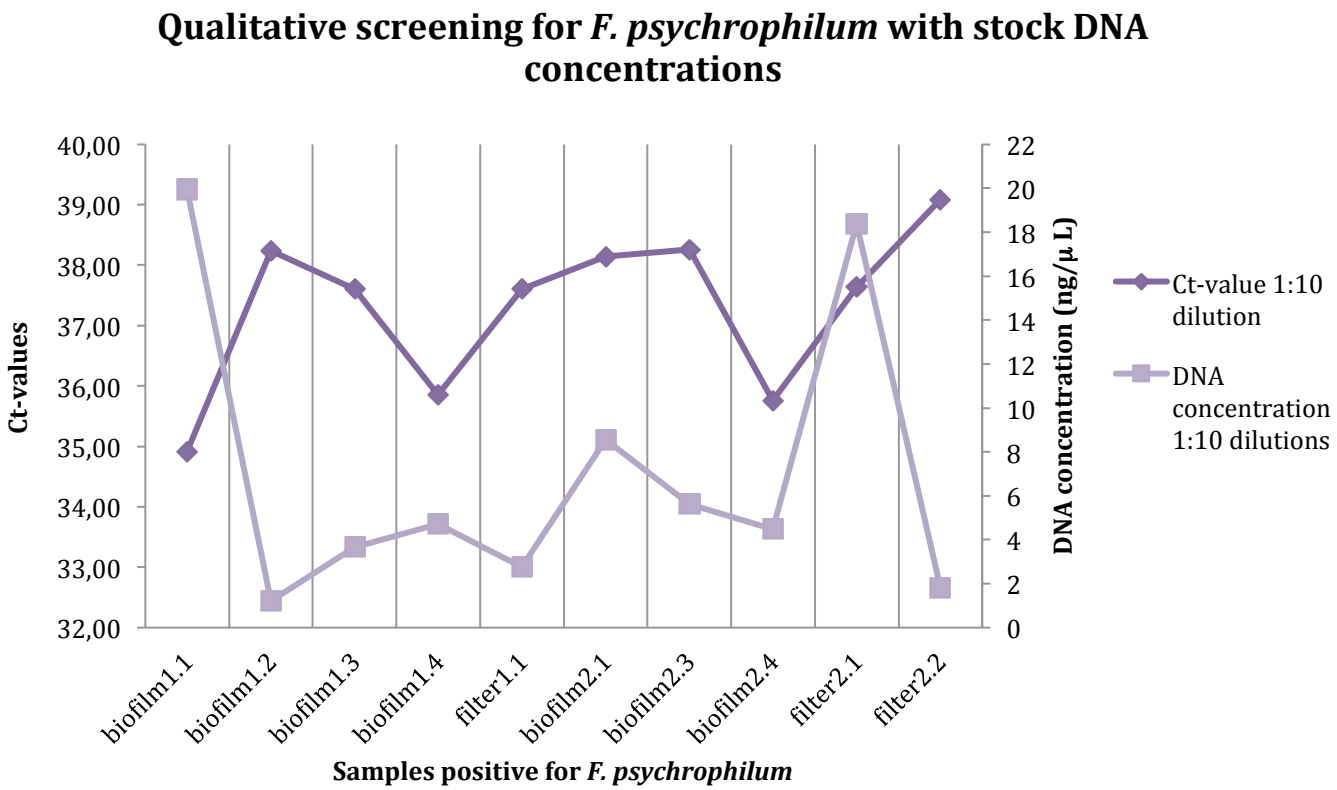

Figure 10: Qualitative qPCR screening for *F. psychrophilum* in RAS-biofilm and biofilter. Biofilm1.1-1.4 are biofilm samples from four sampling locations, filter1.1 is a biofilter sample from fish tank module 2, both are from the first saltwater RAS sampling. Biofilm2.1-2.4 and filter2.1-2.2 represent the same locations from the second saltwater RAS sampling.

5 - Discussion

5.1 - Bacteria isolated from fish skin mucus and RAS biofilm

In this study the goal was to describe the development of the bacterial flora in an Atlantic salmon saltwater RAS system and evaluate the applicability of such microbiological monitoring for health and quality assessment. The most prevalent isolates were identified through traditional bacteriology, API-kits, and/or 16S rRNA sequencing. Normal skin microbiota in farmed fish has been explored using many different approaches, such as 16S rRNA gene sequencing (Minniti et al., 2017), microarray analyses (Karlsen et al., 2018), traditional bacteriology (Horsley, 1973), or a combination of those (Karlsen et al., 2017). Many of these approaches are costly and time consuming, and may require high technical knowledge. The advantage of using a cultivation approach is that very little equipment is needed. In the present study, both cultivation and 16S rRNA sequencing was used to identify the most dominating bacteria. Most dominating isolates were chosen for identification to be able to see if different bacteria dominate over time and if that change could be indicative of the overall health status. A metagenomics approach would of course give a more detailed look at the overall microbiota, though it is more costly and requires more bioinformatics understanding. It would be advantageous to be able to identify changes in the dominating flora quickly, so that changes in production can be implemented to minimize any negative health effects.

In the present study different bacteria were isolated from fish skin mucus in freshwater. The most dominating bacterial genera in freshwater samples were *Brevundimonas, Pseudomonas, Acinetobacter,* and *Chryseobacterium*. All of these are naturally found in water and soil (Garrity, 2004; Hsueh et al., 1996). *Pseudomonas* species have been suggested as part of the normal microbiota of fish skin (Minniti et al., 2017), which corresponds to the findings of this study. Once fish were introduced to the saltwater RAS system, the composition of isolated bacteria changed, which is not surprising as the non-halophilic bacteria isolated from freshwater will no longer grow in the salt concentration in the RAS facility. In the first saltwater RAS sampling, some of the previously dominating species were identified, such as *Pseudomonas fluorescens* and *Brevundimonas vesicularis*.

The dominating bacterium isolated from fish skin mucus in the first RAS sampling was *Aliivibrio wodanis*. This bacterium is ubiquitous in marine environments, but is also known to be isolated from wounds together with *M. viscosa* (Karlsen et al., 2014). *Al. wodanis* was isolated both from direct sampling of wounds, but also from mucus samples from unaffected areas. Other genera dominating in the first RAS sampling were *Pseudomonas, Vibrio,* and *Pasteurella*. All three can be found in marine environments, and *Vibrio* species have been assumed to be part of the Atlantic salmon normal microflora (Minniti et al., 2017). *Pasteurella* species have recently been associated with several disease outbreaks in post-smolt Atlantic salmon (Sommerset et al., 2020). This could be cause for concern, though the bacterium was not identified in any samples from the second saltwater RAS sampling. The RAS facility had problems with wounds and high mortality before the first sampling was conducted, and wounds were still observed in the first RAS sampling, though fewer wounds were observed in the second RAS sampling. It could be speculated that the decrease in wounds is due to the decrease of *Pasteurella sp.* and *Aliivibrio wodanis* in the second RAS sampling. Although the bacteria could be secondary infections in fish that have acquired mechanical injury.

The second RAS sampling had overall less bacterial growth and diversity. Genera that dominated were *Brevundimonas, Psychrobacter*, and *Pseudomonas*. *Brevundimonas* species have been isolated from fish (Ibrahim et al., 2016), though they are also found in different water sources (Ryan & Pembroke, 2018). *Psychrobacter* are also known to be part of the normal skin microbiota of Atlantic salmon (Minniti et al., 2017), so it is expected to also find these in this study. Important to note is that no *A. wodanis* or *Pasteurella sp.* were isolated during the second RAS sampling, though some wounds were still present in the facility at this point. It appears as though the microflora in fish skin stabilizes over time, especially after fish have been transferred to seawater. Wounds occurred right after fish were moved to saltwater and lessened after time. This is mirrored in the bacteria isolated, in that bacteria associated with wounds were isolated in the first RAS sampling, but not in the second sampling. This is supported by literature that suggests that Atlantic salmon is particularly susceptible to bacterial infections directly after transfer to seawater, and that the skin barrier will evolve after transfer to the sea (Karlsen et al., 2018). In this study, simple microbiological methods could be used to reveal health issues in the facility. Preferably an additional saltwater sampling should have been performed before the wounds started occurring, to see if bacteria such as *Al.*

wodanis were already present and to get an indication on the skin microflora directly after sea transfer.

Many bacteria found in fish skin mucus were also isolated from biofilm. *Brevundimonas* was the dominating species across all biofilm samplings. Additionally, less bacterial growth and diversity was observed at later biofilm samplings. This could be due to the fact that the most prevalent bacteria in biofilm will relocate to form new biofilms during the last stage of biofilm formation (Armbruster & Parsek, 2018), thereby allowing the bacteria to establish themselves in the facility over long periods of time. However, this does not correspond to literature, which states that bacterial diversity in RAS biofilm increases over time (Gao et al., 2012). Weaknesses in the cultivation approach are that relatively few bacteria are identified at a time. Additionally, bacteria that are difficult to cultivate will be lost on blood agar plates, either due to insufficient growth medium or by overgrowth from other bacteria. This is especially true for some of the pathogens chosen for this study, which require specific growth media, e.g. *T. dicentrarchi*. Specific growth media could have been used to strengthen the method, and a metagenomics approach would give a more detailed look at the composition of biofilm. Additionally, metagenomics are becoming simpler and less expensive each year, which could make it a more applicable method in the future. The method presented in this study is more useful when the technologies and resources needed for metagenomics are not available.

5.1 - Establishment and optimization of qPCR protocol

In this study, qPCR assays were used to detect bacterial DNA from freshwater and saltwater fish skin mucus, and saltwater RAS biofilm and biofilter. The aim was to establish a fast and reliable detection method, so as to implement measures early if a pathogen is detected. Real-time PCR has been used as a detection method for bacterial pathogens in several other studies (Bastardo et al., 2012; Fløgum, 2016; Jansson et al., 2008; Kim & Lee, 2014; Marancik & Wiens, 2013). Based on these publications, qPCR protocols for detection of pathogenic bacteria known to cause disease in aquaculture, specifically bacteria that could be found in the environment of saltwater RAS, were implemented. In addition, qPCR protocols for the detection of *T. dicentrarchi* and *F. psychrophilum* were established in this study.

Primers and probes used in this study, detected target DNA specifically, though cross-reactivity was observed in some of the primer sets. Introducing new primers, where no cross-reactivity occurred or setting Ct cut-off values resolved this issue. Very high Ct-values are typically interpreted as amplification or fluorescens artifacts, contaminations, or amplification of background nucleotides (Caraguel et al., 2011). In this study, Ct-values that are very close to the maximum amplification were considered false-positives.

The qPCR protocols developed in this study were successful in detecting DNA from the selected bacteria, additionally, relative quantification of the detected bacterial DNA in each sample allowed for comparison of bacterial abundance between sample types and sampling times. Though, the detection limit of bacterial DNA was not defined, and it is possible that bacteria were present that were under the detection limit of the qPCR. Relative quantification of target DNA was performed by standardizing the input of total DNA (template) in the qPCR reactions, by diluting the isolated total DNA to 2ng/μL. A disadvantage to this protocol is that there is a possibility for diluting the DNA too much, thereby no longer detecting DNA that was originally present in the sample. A way to avoid this is by standardizing the sampling procedure, by always extracting DNA from the same amount of sample material at all samplings, be it skin mucus, biofilm, or biofilter.

A protocol for qualitative detection of *S. erythraea* was established as well. It was used as an indicator for the presence of geosmin, so that measures can be taken to minimize the amount of geosmin in the facility. The target gene was *geoA* that is found in cyanobacteria and other bacteria that produce geosmin as a metabolic byproduct (Auffret et al., 2011). All bacteria with the *geoA* gene produced geosmin, making it a great target for qPCR detection of the component (Lukassen et al., 2017). Though variations of the *geoA* gene exist, it has been shown that the AMgeo-primers detect a wide range of *geoA*. The primer set used in the present study detected DNA from *S. erythraea*, making it possible to detect *geoA* in fish mucus, biofilm, and biofilter samples.

Other potential primers exist for bacteria that produce geosmin, such as primers that detect cyanobacterial 16S rDNA (Shaw et al., 1999; Tsao et al., 2014). This could be a potential way to further strengthen this detection method, by screening not only for *geoA* but also for other genes found in geosmin-producing bacteria. GC-MS could be used to measure the amount of geosmin that is present in a facility once the qPCR has confirmed

the presence of geosmin-producing bacteria, which could be especially useful during the depuration period to determine when geosmin-levels are below (human) detection limit. Although the addition of GC-MS would make this detection method less accessible for the industry.

5.3 - Screening for pathogens

In the present study, a simple and reliable detection method for pathogenic and geosmin-producing bacteria in aquaculture was explored. *Y. ruckeri, A. salmonicida* ssp. *salmonicida*, *V. anguillarum, T. dicentrarchi, F. psychrophilum,* and *R. salmoninarum* were chosen as the most important pathogens in this study.

Bacteria to screen for were chosen based on their potential, or previous, impact on the welfare of farmed Atlantic salmon in Norway. Pathogens that have mandatory notification to the Norwegian Food Safety Authority, such as *A. salmonicida* ssp. *salmonicida* and *R. salmoninarum*, were chosen to monitor the presence of pathogens and health status. Additionally, temperature and salinity of the sampling facility were taken into consideration. *T. finnmarkense* was not screened for in the present study, even though it is an important bacterium in Norwegian aquaculture, due to the relatively high water temperature in the saltwater RAS facility. As *T. finnmarkense* is typically isolated from water with temperatures between 3-6°C (Småge et al., 2016) and the water temperature in the saltwater RAS is 12°C the bacterium was unlikely to cause disease in this environment. Because of this, *T. dicentrarchi* was chosen, as this bacterium has a generally higher growth temperature. In similar, *M. viscosa* and *Al. salmonicida* were not included in the screening due to their psychrophilic nature. *F. psychrophilum* was included, even though it is typically a problem in freshwater and brackish water, as Fredrikstad Seafood originally planned on using brackish water instead of seawater. All bacteria were chosen to be able to implement preventative measures if pathogens are detected in the facility.

T. dicentrarchi was detected in RAS biofilm and biofilter from both RAS samplings, but not in samples taken from fish mucus, and during both samplings several fish had wounds. *T. dicentrarchi* will cause wounds in salmonids, though it is also naturally found in marine

environments (Klakegg et al., 2019). *T. dicentrarchi* was not cultivated from fish mucus samples either, though that could be due to the fact that the bacterium requires seawater for growth, and fish skin mucus was only plated on blood agar and BTB agar. To overcome this, samples could have been plated on marine agar additionally. This issue was overcome, as the qPCR was able to detect the bacterium. The results indicate that the skin wounds observed were not caused by *T. dicentrarchi*, and that the bacterium is not common in the natural microbiota of the RAS fish. The DNA detected probably came from *T. dicentrarchi* found in the environment, particularly from deposits in the biofilm. The bacterium is able to form biofilms after a 24-hour incubation period, with cell-detachment phase starting after 92 hours (Levipan et al., 2019). During the first sampling *T. dicentrarchi* was found in all four biofilm sampling sites, showing that the bacterium had built up biofilms in both RAS production modules. No *T. dicentrarchi* was detected in the biofilter during the first sampling, though it was found during the second sampling, indicating that *T. dicentrarchi* was able to establish itself in the biofilter in the time between sampling one and two. Wounds in the facility had lessened at the second RAS-sampling and less *T. dicentrarchi* DNA was detected in biofilm and biofilter samples. It is impossible to say if there was any *T. dicentrarchi* in the wounds, as wound samples only were grown on blood agar, but the bacterium is typically isolated from gills or wounds (Klakegg et al., 2019). To overcome this an additional swab could have been used to sample wounds, thereby giving a DNA sample for qPCR analysis.

During qualitative qPCR very low amounts of *F. psychrophilum* were detected in biofilm and biofilter samples from the first and second RAS sampling. Using standardized DNA as qPCR template (concentration of 2ng/μL) gave no Ct-values in any samples. This is probably because the target DNA was diluted too much thereby falling below the detection limit. *F. psychrophilum* is typically found in freshwater or brackish waters, and in the present study, the positive samples all came from saltwater RAS samples. This could be why the amount of *F. psychrophilum* DNA detected was so low, and why no DNA was detected when using the standardized DNA.

5.3 – Screening for geosmin-producer

qPCR was used to screen for geosmin-producer *S. erythraea*. Total DNA was standardized to 2ng/μL. *S. erythraea* was identified in virtually all samples, except for fish skin mucus

from the second saltwater RAS sampling. *S. erythraea* DNA concentrations seemed to increase over time, with the least amount of geosmin detected in the freshwater sampling, and most in the second RAS sampling. Additionally, less variation in DNA concentration was observed in RAS biofilm samples, whilst skin mucus samples showed high variation. In previous studies it has been suggested that geosmin concentrations will vary greatly at different sites in the facility, and that cleaning measures greatly impact the amount of geosmin deposited in the facility (Podduturi et al., 2020). This is not reflected in the findings of this study, as all biofilm sampling locations had similar *S. erythraea* DNA concentrations. It could be that few sampling locations and sampling times affect the overall variation of geosmin-producing bacteria in the facility, and that a large-scope experiment would reveal the same variations observed in previous studies. Though no direct variation between sampling sites was observed, more *S. erythraea* was found in environmental samples compared to fish skin mucus. A shortcoming of this approach is that the actual geosmin concentrations remain unknown, and this method does not give an indication to how much geosmin has entered the fish. An advantage to this method is that fish can be sampled without having to euthanize fish, and since geosmin enters the fish through the gills (Howgate, 2004) it can be assumed that geosmin has affected fillet quality.

6 - Conclusion

Aquaculture is an important industry in Norway, though diseases and environmental impact have negative effects on the growth of the industry. Saltwater RAS facilities have become more popular as a new production method to avoid some of these challenges. Pathogenic bacteria and geosmin greatly reduce product quality and fish welfare. The goal was to describe the development of the bacterial flora in an Atlantic salmon saltwater RAS system and evaluate the applicability of such microbiological monitoring for health and quality assessment. In this study traditional cultivation and qPCR methods were used as an overall indicator of fish health, the presence of geosmin and pathogenic bacteria. Bacteria that typically cause wounds in fish, such as *A. wodanis* and *T. dicentrarchi* were detected using both methods, though the microflora did no longer contain these at later sampling dates, when wound occurrence was reduced. The results indicate that changes in the RAS microflora related to health issues are possible to detect

by traditional microbiological cultivation. Collecting more samples would strengthen the method overall, and using routine samplings with a set time between samplings would give a better overview of bacterial development in the facility. It was possible to use the qPCR as a fast and reliable detection method and could be used to implement preventative measures to prevent accumulation of unwanted bacteria. A way to reduce geosmin in the fillet could be moving fish to a clean and pre-disinfected tank without the use of a biofilter, as suggested by (Davidson et al., 2014) before slaughter. To minimize the risk of spread of pathogenic bacteria throughout a RAS facility, it is important to keep strict hygiene routines, and, if possible, cull sick fish to prevent horizontal disease spread.

Appendices

Appendix 1 – Primers and probes for qPCR: names, abbreviations and concentrations

Overview over primers, probes, and cycles used for qPCR							
Bacteria	Forward primer	Reverse primer	Probe	Dye	qPCR master mix	qPCR cycles	Concentration primers/probe
Aeromonas salmonicida (Furunculosis)	Asal-vapA-F	Asal-vapA-R	Asal-vapA_probe	FAM	PerfeCTa qPCR ToughMix	95°C 10min – (95°C 15sec - 62°C 60sec)x40	Primers: 300nM Probe: 100nM
Yersinia ruckeri (Enteric redmouth disease)	YRA-F1	YRA-R1	YRA probe	FAM	PerfeCTa qPCR ToughMix	95°C 10min – (95°C 15sec - 60°C 60sec)x40	Primers: 300nM Probe: 100nM
Renibacterium salmoninarum (Bacterial kidney disease) (Set A)	16S F	16S R	16S probe	FAM	PerfeCTa qPCR ToughMix	95°C 10min – (95°C 15sec - 60°C 60sec)x40	Primers: 300nM Probe: 100nM
Renibacterium salmoninarum (Bacterial kidney disease) (Set B)	Reni F	Reni R	Reni probe	FAM	PerfeCTa qPCR ToughMix	95°C 10min – (95°C 15sec - 60°C 60sec)x40	Primers: 300nM Probe: 100nM
Flavobacterium psychrophilum (Bacterial cold water disease) (Set A)	FlGyrB F	FlGyrB R	FlGyrB probe	FAM	PerfeCTa qPCR ToughMix	95°C 10min – (95°C 15sec - 60°C 60sec)x40	Primers: 300nM Probe: 100nM
Flavobacterium psychrophilum (Bacterial cold water disease) (Set B)	FpSig F	FpSig R	FpSig probe	FAM	PerfeCTa qPCR ToughMix	95°C 10min – (95°C 15sec - 60°C 60sec)x40	Primers: 300nM Probe: 100nM
Vibrio anguillarum (Vibriosis)	ToxR F	ToxR R	ToxR P	FAM	PerfeCTa qPCR ToughMix	95°C 10min – (95°C 15sec - 60°C 60sec)x40	Primers: 300nM Probe: 100nM
Tenacibaculum dicentrarchi (Tenacibaculosis) (Set A)	TeGyrB F	TeGyrB R	TeGyrB probe	FAM	PerfeCTa qPCR ToughMix	95°C 10min – (95°C 15sec - 60°C 60sec)x40	Primers: 300nM Probe: 100nM
Tenacibaculum dicentrarchi (Tenacibaculosis) (Set B)	T.dicenF	T.dicenR	T.dicen NY (new nov.2018)	FAM	PerfeCTa qPCR ToughMix	95°C 10min – (95°C 15sec - 56°C 60sec)x40	Primers: 300nM Probe: 100nM

Saccharopolyspora erythraea (geosmin producer)	AMgeo-F	AMgeo-R	N/A	ROX	Maxima SYBR green qPCR Master Mix	95°C 10 min – (95°C 15 sec - 66°C 60 sec - 72°C 15 sec) x40 + standard melting curve ((95°C 1 min – 55°C 30 sec - 95°C 30 sec)	Primers: 250nM Probe: N/A

Appendix 2 – Sequences of primers and probes

Sequences of primers and probes 5'-3'			
Bacteria	Forward primer	Reverse primer	Probe
Aeromonas salmonicida subsp. *salmonicida*	CGTTGGTGCTTCTATCACTGCCTA	AACAGCTACTTCACCCTGATTGG	CCGTCAGGCTCGC
Yersinia ruckeri	TCTGGACATCGCTCTGG	AGTTTTTTTGCGTAGATAGGA	TATCGCCTCTGCACAGC
Renibacterium salmoninarum (Set A)	ACGTTATGGTGGGGACTCATAGG	GGGATTAGCTCCACCTCACAGTA	CTTATGTCTTGGGCTTCA
Renibacterium salmoninarum (Set B)	TGGATACGACCTATCACCGCAT	TCGCCTTGGTTAGCTATTACC	TTTTTGCGGTTTTGGATGGACTCG
Flavobacterium psychrophilum (Set A)	GTTGCTGAACCTCAATTTGAAGGTCAAA	ATCGCCAACCGCTTGAGAAACTG	TACTTCTCTATTTCCTAATTTGGT
Flavobacterium psychrophilum (Set B)	GGTAGCGGAACCGGAAATG	TTTCTGCCACCTAGCGAATACC	CGCTTCCTGAGCCAGA
Tenacibaculum dicentrarchi (Set A)	TCGTATGCGTGAGTTGGCGTATTTAA	GGTAAACCTTCTTTACTATGAAATGTT-TCAG	ATCATCTGTATTACGTTTATCTG
Tenacibaculum dicentrarchi (Set B)	TAGCCTTTAGAAATGAAGA	CCGTTACCTTACCATCTA	ATTCGGCATCGGAACACTATTAA
Vibrio anguillarum	ACACTGCAAAGCAAATTGATG	TGATGGGCGTATTCACAACT	TGGCTCTTCTATTGACTAGCCCTGCA

Moritella viscosa			N/A
Aliivibrio wodanis			N/A
Saccharopolyspora erythraea	GAGTACATCGAGATGCGCCGCAA	GAGAAGAGGTCGTTGCGCAGGTG	N/A

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