Master's thesis

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

Renate Bringsli

Antimicrobial Resistance and Biofilm Formation among *Pseudomonas* spp., *Serratia* spp. and *Aeromonas* spp. isolated from Farmed Salmon in a Norwegian Processing Plant

Master's thesis in Chemical Engineering and Biotechnology
Supervisor: Lisbeth Mehli
Co-supervisor: Gunn Merethe B. Thomassen
June 2021

NTNU
Norwegian University of
Science and Technology

Renate Bringsli

Antimicrobial Resistance and Biofilm Formation among *Pseudomonas* spp., *Serratia* spp. and *Aeromonas* spp. isolated from Farmed Salmon in a Norwegian Processing Plant

Master's thesis in Chemical Engineering and Biotechnology
Supervisor: Lisbeth Mehli
Co-supervisor: Gunn Merethe B. Thomassen
June 2021

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

NTNU
Norwegian University of
Science and Technology

Abstract

As Norway has become the world´s largest producer of farmed salmon, the Norwegian salmon industry has a large responsibility to provide microbially safe and healthy products to consumers worldwide. Now that antimicrobial resistant bacteria are recognized as an emerging threat to food safety and public health, a comprehensive mapping of the occurrence, evolution and diversity of antimicrobial resistance in bacteria associated with Norwegian seafood is needed. This master study contributes to this work, as the resistance status of *Pseudomonas* spp., *Serratia* spp., and *Aeromonas* spp. isolated from farmed salmon in a Norwegian processing plant is illuminated. As bacterial biofilms pose a serious challenge to the seafood industry, the biofilm formation among these bacteria was tested at the operating temperature of salmon processing plants (12°C). The efficiency of Aqua Des Foam PAA was also tested, as it is commonly applied to disinfect surfaces and equipment in salmon processing plants.

A disk diffusion assay revealed that 36.2% of the bacteria were resistant to ampicillin, 6.4% to florfenicol and 4.3% to tetracycline, and a microtiter susceptibility assay showed that florfenicol MIC- and MBC values ranged from 19 – 300 µg/mL and 75 – 2400 µg/mL, respectively. This demonstrate a low occurrence of antibiotic resistance compared to other main seafood producing countries such as Chile. The observed incidences of resistance in this master study are likely caused by intrinsic mechanism rather than acquisition of resistance genes caused by antibiotic usage in Norwegian salmon farming. The PCR-based detection of antimicrobial resistance genes revealed that none the bacteria possessed either the *ampC*, *bcrABC, floR*, *sul1*, *tetG, qacΔE1* or the *qacH* gene. Thus, the observed incidences of resistance are caused by mediators other than these resistance genes.

A microtiter biofilm assay illuminated that *Pseudomonas* spp. and particularly *P. fluorescens* were efficient biofilm producers at 12°C, and are therefore of particular concern to the processing plants. The commonly applied Aqua Des Foam PAA disinfection procedure was sufficient to eradicate all planktonic cells, while more than 10% of biofilm cells survived the disinfection. To avoid surviving bacteria potentially acquiring an increased tolerance to the disinfectant caused by repeated exposure, the usage concentration should be increased.

Sammendrag

Norge er en verdensledende produsent av oppdrettslaks, noe som innebærer at norsk lakseindustri har et stort ansvar når det kommer til levering av sunne og trygge produkter som eksporteres verden over. Nå som antibiotikaresistente bakterier har blitt en anerkjent trussel mot matsikkerhet og folkehelse, er det behov for en grundig kartlegging av status, utvikling og diversitet av resistens hos bakterier i norsk sjømat. Denne masteroppgaven er et bidrag til dette arbeidet, ettersom antibiotikaresistens undersøkes hos *Pseudomonas*-, *Serratia*- og *Aeromonas*-slekten isolert fra oppdrettslaks i et norsk lakseslakteri. Siden bakteriell biofilm er en stor utfordring for sjømatindustrien, ble biofilmdannelsen hos disse bakteriene undersøkt ved driftstemperaturen hos norske lakseslakteri (12°C). Effektiviteten av Aqua Des Foam PAA ble også testet, ettersom dette desinfeksjonsmiddelet brukes til desinfeksjon av utstyr og overflater i lakseslakteri.

En diskdiffusjonstest viste at 36.2% av bakteriene var resistent mot ampicillin, 6.4% mot florfenicol og 4.3% mot tetrasyklin, og et brønneplateforsøk for testing av følsomhet illustrerte at MIC og MBC verdiene til florfenicol var henholdsvis 19 – 300 µg/mL og 75 – 2400 µg/mL. Disse resultatene viser et lavt nivå av antibiotikaresistens sammenlignet med andre lakseindustri-nasjoner slik som Chile. De observerte tilfellene av antibiotikaresistens i denne masteroppgaven ser heller ut til å skyldes iboende mekanismer enn tilegnelsen av resistensgener grunnet antibiotikabruk i norsk lakseoppdrett. En PCR-basert påvisning av gener knyttet til antimikrobiell resistens viste at ingen av bakteriene hadde verken *ampC*, *bcrABC, floR*, *sul1*, *tetG, qac*Δ*E1* eller *qacH* genet. Den påviste resistensen skyldes derfor andre gener og assosierte mekanismer enn disse genene.

Et biofilm brønneplateforsøk viste at *Pseudomonas* spp., og spesielt *P. fluorescens*, var en effektiv biofilm-produsent ved 12°C, og disse bakteriene er derfor av ekstra stor bekymring hos lakseslakteri. Desinfeksjonsprosedyren med Aqua Des Foam PAA som anvendes av lakselakteriet var tilstrekkelig for å drepe planktoniske celler, mens over 10% av biofilm-cellene overlevde desinfeksjonen. For å unngå at overlevende bakterier potensielt tilegner seg en økt toleranse mot desinfeksjonsmiddelet grunnet gjentatt eksponering, bør brukskonsentrasjonen av Aqua Des Foam PAA økes.

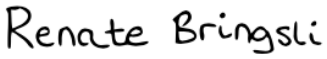
Preface

This master´s thesis was conducted at The Norwegian University of Science and Technology (NTNU) at the Department of Biotechnology and Food Science (IBT). It accounts for the 30 final credits of the study program “Chemical Engineering and Biotechnology” and is a continuation of the specialization project carried out Autumn 2020. The practical work was performed in the microbiology and analytical chemistry laboratories at campus Akrinn located in Trondheim, in the time period from January to May 2021. The master project is supported by a larger research project executed by PhD candidate Gunn Merethe Bjørge Thomassen and it is financed by the NTNU research program OPTiMAT (Optimal Utilization of Marine Food Resources).

First, I would like to thank my supervisor Lisbeth Mehli for giving me the opportunity to do my master project within a field I find very interesting: antibiotic resistance among bacteria. She also gave me guidance and support, especially during the writing period of my specialization project report and master´s thesis. Also, a special thanks to my co-supervisor Gunn Merethe Bjørge Thomassen, for all her advice and everything she taught me in the lab. I would also like to thank fellow students and employees working in the lab for their helpfulness. Finally, I would like to thank my family, friends, and flatmates for giving me support and inspiration throughout the years of being a civil engineering student, and especially Olaf van der Veen who also put some serious effort in correcting the English language of my thesis. Lastly, working on this project has given me a lot of valuable experience I am sure will become useful in my future career.

Trondheim, 17.06.2021

Renate Bringsli



List of abbreviations

ANOVA – analysis of variance

ARB – antimicrobial resistant bacteria

ARGs – antimicrobial resistance genes

EPS - extracellular polymeric substances

MBC – minimum biocidal concentration

MBEC – minimum biofilm eradication concentration

MHA – Mueller-Hinton agar

MIC – minimum inhibitory concentration

OD_{650} – optical density measured at 650 nm

PAA – peracetic acid

TSA – tryptone soya agar

TSB – tryptone soya broth

QACs – quaternary ammonium compounds

QS – quorum sensing

Table of contents

1. Introduction

Salmon farming is the fastest growing food production system in the world, and Norway has become the world´s largest producer of farmed fish (ISFA, 2018; Jensen, 2021). As salmon is exported to more than 100 countries, the Norwegian salmon industry has a large responsibility to provide healthy and microbially safe products to consumers worldwide (NSC, 2021). Now that antimicrobial resistance is recognized as an emerging threat to food safety and public health, there is an urgent need for a comprehensive mapping of occurrence, diversity and evolution of antimicrobial resistance among bacteria associated with Norwegian farmed salmon (WHO, 2011). More investigation of biofilm formation in salmon industry is also required, as it is a phenomenon tightly linked to bacteria´s acquisition of antimicrobial resistance, as well as intrinsically being a threat towards food safety. As this master´s thesis will be a contribution to this work, the introduction is focused on antimicrobial resistance and biofilm formation among bacteria.

1.1 Antibiotic resistant bacteria

1.1.1 The consequences of antibiotic resistance

Bacteria can over time become resistant to antibiotics by slowly developing the ability to survive (and also possibly grow) despite being exposed to antibiotics designed to kill them (CDC, 2020). However, the misuse and overuse of antibiotics which have been ongoing for decades have led to the acceleration of this process. In aquaculture as well as agriculture, antibiotics are applied to treat infections of food animals, prevent disease and promote growth. Nevertheless, antibiotic resistance genes (ARGs) can be transferred from non-pathogens to pathogens, and antibiotic resistant bacteria (ARB) can also spread from food production animals to humans through the food chain (WHO, 2011). As food products of animal origin are contaminated with bacteria, this is often the main way of transmitting ARB from food animals to humans. Aquaculture systems are genetic hotspots for gene transfer and spreading of ARGs, as they naturally contain high numbers of diverse bacteria (Watts *et al.*, 2017).

When ARB infect humans, it is challenging to treat the infection as the antibiotics traditionally used to treat the infection are less efficient, which further leads to higher medical costs and increased mortality rate (WHO, 2020). Besides, many modern medical practices such as surgery, organ transplantation and chemotherapy in cancer treatment would be high risk procedures without having effective antibiotics available (WHO, 2011). Spreading of ARGs and ARB are therefore an emerging threat towards public health and global food security.

1.1.2 Bacterial disinfectant resistance – a food safety problem

In addition to bacteria developing resistance to antibiotics, another problem especially relevant in food industry is the emergence of disinfectant-resistant bacteria. Due to the increased focus on hygiene in the food industry during recent years, the use of chemical disinfectants has also increased. This is expected to contribute to the emergence of disinfectant-resistant bacteria, which is of high concern as the antibacterial effect of disinfectants is crucial for controlling foodborne pathogens in food processing environments (Langsrud *et al.*, 2003). Low numbers of bacteria will remain after ineffective disinfection routines as well as cleaning routines which do not aim to completely sterilize the surfaces. Remaining bacteria can then possibly contribute to the selective pressure for acquisition of resistance genes among bacteria (Møretrø *et al.*, 2017).

1.2 Antimicrobials used in salmon farming and processing plants

1.2.1 Antibiotics used in Norwegian and global salmon farming

The usage of antibiotics in the Norwegian food production is strictly regulated, and Norway is at the global top level when it comes to low usage of antibiotics in salmon production (Figure 1.1). The low need for antibiotics in Norway compared to other main producing countries such as the UK, Ireland, Canada and Chile is explained by effective vaccination of the salmon (Olsvik, 2019; SeaBOS, 2019). Also, in Norway, antibiotics are in addition not being used to promote growth or prevent diseases in healthy fish, and there is also a high focus on fish welfare (Larsen, 2017). Antibiotics are however much more extensively used in other countries, and especially in developing countries (Vivekanandhan *et al.*, 2002). Globally, the

most commonly used classes of antimicrobials in aquaculture are quinolones (such as oxalinic acid), tetracyclines (such as oxytetracycline), amphenicols (such as florfenicol) and sulphonamides (such as ampicillin) (Schar *et al.*, 2020).

Norwegian aquaculture uses 201 – 1591 kg of antibiotics per year, at least when considering the time period from 2010 – 2019 (NORM-VET, 2019). The total amount prescribed in 2019 was 222 kg, where approximately 70% were florfenicol and 30% oxalinic acid. These are the two main antibiotics used by the Norwegian aquaculture, followed by oxytetracycline. The maximum annual usage of oxytetracycline in the time period from 2010 – 2019 was 20 kg in 2019, however, most years it was not being used at all. Oxalinic acid has to a large extent replaced oxytetracycline in recent years, and ampicillin and other sulphonamides have not been used in the Norwegian aquaculture since 1993 (Lunestad & Samuelsen, 2008; Grave, 2006). The use of antibiotics in Norwegian aquaculture is extremely low compared to Chile, which is the world´s second largest salmon producer. The Chilean aquaculture had an annual usage of 143200 – 563200 kg during the time period from 2010 – 2019 (Soto, 2020). Florfenicol is also the most commonly used antibiotic in Chile, followed by oxytetracycline (Miranda *et al.*, 2018).

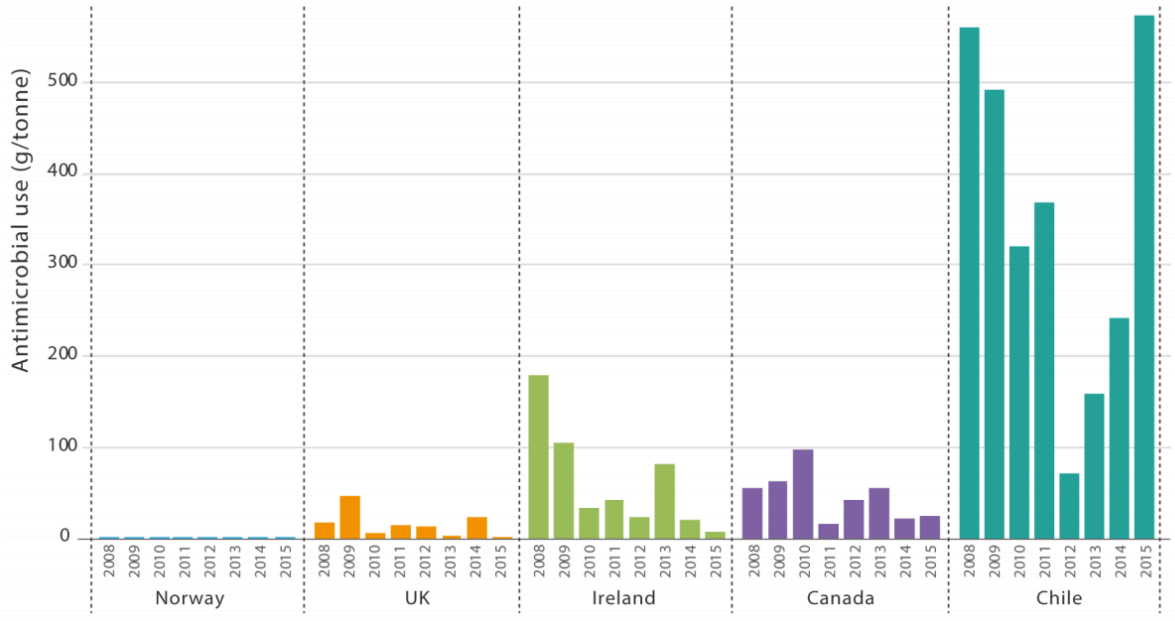


Figure 1.1: Comparison of the annual antimicrobial usage for salmon production in Norway and the four other main producing countries; UK, Ireland, Canada and Chile, in the time period from 2008 – 2015 (SeaBOS, 2019).

Investigations of the antibiotic resistance in bacteria associated with Norwegian agricultural animals are mainly performed by NORM-VET, which has stated that the probability of developing antimicrobial resistance in farmed fish and thus their transmission to humans is very low, due to the minimal use of antibiotics (NORM-VET, 2019). However, the residue of antibiotics even at low concentrations in the environment is likely to impose selective pressures on environmental microbes, inducing the emergence of diverse ARGs and promoting the evolution of novel genes conferring antibiotic resistance mechanisms (Zhao *et al.*, 2016).

It seems like very little effort has been made to investigate the occurrence of antibiotic resistance in Norwegian seafood, as NORM-VET is mostly focusing on the occurrence of resistance among clinical bacteria isolates. Recent studies concerning antibiotic resistance of seafood products available on the Norwegian market did however reveal that 98% of tested isolates were highly resistant to several antibiotics (Lee *et al.*, 2021). No recent studies concerning the antimicrobial resistance status of Norwegian farmed salmon have been found. Hence, more investigations are needed, understanding the resistance diversity of aquaculture-associated bacteria will help to improve the understanding of human health risk related to the usage of antibiotics (Fernández-Alarcón *et al.*, 2010).

1.2.2 Disinfectants used in salmon processing plants

Disinfectants are commonly being used in food processing plants to sanitize food contact surfaces and equipment. Major classes of disinfectants applied by the food industry are quaternary ammonium compounds (QACs), peroxides, iodophors, phenols, chlorine and glutaraldehyde (Chapman, 2003). QACs, such as benzalkonium chloride, seem to be among the most frequently used disinfectants in Norwegian salmon processing plants (Møretrø *et al.*, 2016). Disinfectants based on peracetic acid (PAA) are also commonly being used, such as the disinfectant Aqua Des Foam PAA. This chemical is a mixture of hydrogen peroxide, acetic acid and benzenesulfonic acid, and is widely used for surface hygiene in Norwegian salmon processing plants as it is effective on most microorganisms (Zoellner *et al.*, 2018; AquaticChemistry, 2018).

1.2.3 Antimicrobials´ mechanism of action

Florfenicol is extensively used to treat infections in aquacultural species such as salmon. By binding the 50S ribosome, this antibiotic prevents the transfer of amino acids from tRNA to the growing peptide chain, thus inhibiting the protein synthesis (Schwarz *et al.*, 2004). It has a broad spectrum of antibacterial activity as it is effective against most gram-negative, gram-positive, aerobic and anaerobic bacteria (Fernández-Alarcón, 2010). Oxalinic acid acts by inhibiting bacterial DNA synthesis, and has bactericidal activity as it kills the bacteria rather than simply inhibiting growth (Rubinstein & Lagacé-Wiens, 2017). It is effective against gram-negative bacteria including many fish pathogens.

Tetracyclines, such as oxytetracycline, have a bacteriostatic mode of action as they inhibit bacterial growth. This is accomplished by inhibiting protein synthesis by being transported into the cell and further preventing the attachment of aminoacyl-tRNA to the RNA-ribosome complex (Chopra & Roberts, 2001). Tetracyclines exhibit activity to a wide range of gram-positive and gram-negative bacteria (CLSI, 2012). Ampicillin, which is a ß-lactam and a sulphonamide, functions by inactivating penicillin-binding proteins located in the inner membrane of the cell wall. The cross-linkages of peptidoglycan chains which are necessary for bacterial cell wall strength then gets disrupted, leading to further cell lysis (NCBI, 2021).

Peracetic acid is a potent biocide as it leads to oxidation of cellular constituents by disrupting the chemiosmotic and transport functions of the bacterial cell membrane (PeroxyChem, 2017). QACs are cationic surfactants which adsorb to and penetrate bacterial cell walls, causing degradation of proteins and nucleic acids, followed by cell death (Falk, 2019).

1.2.4 Mechanisms of antimicrobial resistance in bacteria

There are two main ways bacteria can acquire antimicrobial resistance: by horizontal gene transfer that allows genetic exchange within microbial populations, or by naturally occurring mutations in the DNA during cell replication (Watts *et al.*, 2017). The former implies that ARGs from an antibiotic resistant bacteria is transferred to another bacterial cell, making both cells resistant. As ARGs often are carried on mobile genetic elements such as plasmids and transposons, this facilitates horizontal transfer among bacteria and thus increased spreading of these genes in the environment (Wang *et al.*, 2013).

There are four main categories of antimicrobial resistance mechanisms which all can exist in gram-negative bacteria (Figure 1.2). These are: (1) limiting the uptake of the antibiotic, (2) modification of the antibiotic target, (3) inactivation of the antimicrobial and (4) possession of an active drug efflux (Reygaert, 2018). Gram-negative bacteria possess a cell wall which is naturally impermeable to certain types of antibiotics (first mechanism). As certain antibiotics target components in the bacterial cell, some bacteria exhibit resistance by escaping this binding as they have modified the target (second mechanism). By drug inactivation, the bacteria degrade the antibiotic or transfer a chemical group to it, leading to its inactivation (third mechanism). Most bacteria possess chromosomally encoded genes for efflux proteins, which are membrane proteins that export toxic molecules from within cells into the external environment (fourth mechanism). In addition to these four mechanisms, the formation of biofilms can also be regarded as a mechanism of antimicrobial resistance, as the biofilm serves as a diffusion barrier limiting the antibiotic exposure to the bacterial cell (Pang *et al.*, 2019).

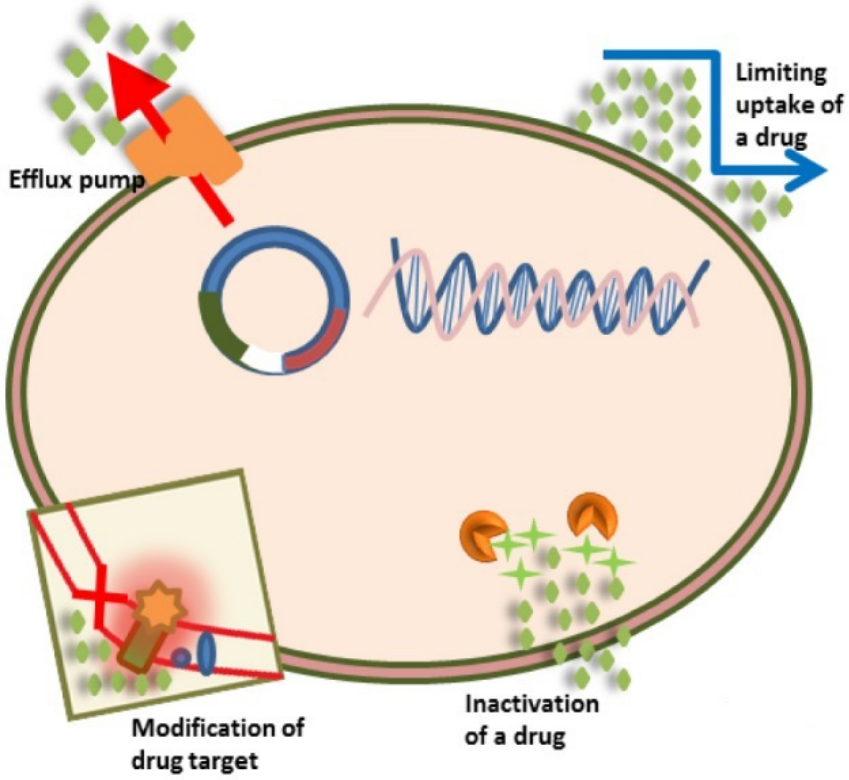


Figure 1.2: Illustration of the four mechanisms of antimicrobial resistance which bacteria can possess: limiting the antibiotic uptake, modification of the antibiotic target, inactivation of the antibiotic, and the possession of an active drug efflux (Reygaert, 2018). In addition, the formation of biofilm can be regarded as a mechanism of antimicrobial resistance.

Most of these efflux pumps are multidrug efflux pumps, as they are capable of transporting a large variety of toxic compounds out of the cell (Wassenaar *et al.*, 2015). In bacteria, there are five major families of efflux pumps: (1) major facilitator, (2) multidrug and toxic efflux, (3) resistance-nodulation-division, (4) small multidrug resistance and (5) the ATP binding cassette ABC (Webber & Piddock, 2003). All these efflux pumps utilizes the proton driving force as an energy source, except the ABC family which utilize ATP hydrolysis to drive the export of the toxic molecules.

1.3 Seafood-associated bacteria and their antimicrobial resistance status

1.3.1 The genus of *Pseudomonas* spp.

Pseudomonas spp. is a bacterial genus belonging to the order of Pseudomonadales, and consists of gram-negative, flagellated, aerobic rods that are able to utilize diverse carbon sources and carry out respiratory metabolisms (Madigan *et al.*, 2015). This genus is a highly phylogenetically diverse group of bacteria which are broadly distributed in different ecological niches. *Pseudomonas* spp. is highly abundant in natural environments such as soil and aquatic systems, and many species are known to cause diseases in plants, animals and humans (Agaras & Valverde, 2018). The best known member of this genus is *P. aeruginosa*, as it is an opportunistic pathogen being the principal cause of mortality in cystic fibrosis patients, and it has been reported as a contaminator in seafood (Drenkard & Ausubel, 2002; Boss *et al.*, 2016). Treatment of *P. aeruginosa* infections is challenging, as it is resistant towards a variety of antibiotics including quinolones and ß-lactams, due to both innate and acquired resistance mechanisms (Pang *et al.*, 2019).

Pseudomonas spp. have been reported as major spoilage bacteria in aerobically stored chilled fish and processing equipment, as *P. fluorescens*, *P. lundensis*, *P. libanensis*, *P. gessardii* and *P. veronii,* have been detected in salmon filet and processing equipment (Møretrø *et al.*, 2016). In the specialization project, nine different species of *Pseudomonas* spp. were detected in the skin or fillet of farmed salmon in a processing plant (Bringsli, 2020).

P. fluorescens was the most abundant one, and as it is known to form biofilms this species poses a serious threat to food industries (Kumar *et al.*, 2019). Due to the high metabolic diversity and abundancy of *Pseudomonas* spp., new species of this genus are constantly being discovered in various environments, such as the highly stress-resistant *P. extremaustralis* isolated from Antarctic ponds (López *et al.*, 2009). This species (or a highly similar unidentified species) was also detected in in a salmon processing plant, and this species was the second most abundant of *Pseudomonas* spp. (Bringsli, 2020).

A relatively high frequency of low-level resistance to QACs has been reported in *Pseudomonas* spp. (Langsrud *et al.*, 2003). This genus has an intrinsic resistance to many antimicrobials, resulting from the natural possession of broadly specific multi-drug efflux systems (Poole, 2001). *Pseudomonas* spp. being resistant to antibiotics used in aquaculture is therefore not necessarily a consequence of farming activity, but could be explained by its innate resistance (Kerry *et al.*, 1994).

1.3.2 The genus of *Aeromonas* spp.

Aeromonas spp. is a genus of rod-shaped, gram-negative, facultative anaerobic bacteria belonging to the order of Enterobacteriales (Percival & Williams, 2014). This genus is commonly found in freshwater reservoirs, marine environments, soil and agricultural products, as well as inhabiting fish and other aquatic animals (Joseph, 2005). *Aeromonas* spp. can be divided into two groups: the non-motile psychrophilic aeromonads such as *A. salmoncida*, and the motile mesophilic aeromonads such as *A. hydrophila*. The former is a fish pathogen, while the latter is associated with infectious diarrhoea in humans (Percival & Williams, 2014).

Several other species of this genus are also responsible for human disease and are considered potential foodborne pathogens (Parker & Shaw, 2011). Genes encoding virulence factors were detected in *Aeromonas* spp. isolated from ready-to-eat seafood available on the Norwegian market (Lee *et al.*, 2021). Virulence factors are components evoking disease, such as toxins, surface coats inhibiting the host´s immune response and surface receptors that bind to host cells (Cross, 2008). Both *A. hydrophila* and *A. salmoncida* have been detected inside Norwegian salmon processing plants (Møretrø *et al.*, 2016; Bringsli, 2020). *Aeromonas* spp.

readily form biofilms as they are proficient at adhering to surfaces, and they have been reported as frequent pioneer colonizers in water systems (Doğruöz *et al.*, 2009).

Aeromonas spp. are generally ampicillin resistant, while being susceptible to tetracycline, chloramphenicol and several other antibiotics (Burgos *et al.*, 1990; Koehler & Ashdown, 1993). However, there has been an increasing amount of incidents of multidrug resistance among *Aeromonas* spp. isolates observed worldwide (Jacobs & Chenia, 2007). A study concerning mesophilic *Aeromonas* spp. isolated from ready-to-eat seafood available on the Norwegian market, revealed that 98% were multidrug-resistant (Lee *et al.*, 2021).

1.3.3 The genus of *Serratia* spp.

Serratia spp. is a genus belonging to the order of Enterobacteriales, and consists of gram-negative, facultatively anaerobic bacteria. They have the morphology of straight rods with rounded ends, and are generally motile (Grimont & Grimont, 2015). This genus is widespread in natural environments and easily detected in soil and water, as well as being associated with plants and animals (Mahlen, 2011). The most studied is *S. marcescens* as it is a human pathogen. Nevertheless, several members of this genus are opportunistic pathogens, including *S. fonticola* and *S. liquefaciens* which are found in aquatic environments (Kurz *et al.*, 2003). The latter produces proteolytic extracellular products which can cause death in Atlantic salmon (McIntosh, 1990). Both these species have been detected in the skin and fillet of salmon in a processing facility, where *S. liquefaciens* was the most abundant species of all isolated bacterial species (Bringsli, 2020).

Serratia spp. is naturally resistant to several antibiotics and is capable of readily acquiring resistance (Remuzgo-Martínez *et al.*, 2015). *S. liquefaciens* isolated from human clinical specimen has been reported as naturally resistant to a long line of antibiotics including florfenicol, ampicillin and tetracycline, and this bacterium should be considered a probable agent of human disease (Stock *et al.*, 2003). Studies concerning seafood-associated *Serratia* spp., did however report this bacteria as susceptible to tetracycline and ampicillin (Pongsilp & Nimnoi, 2018).

1.4 Bacterial genes associated with antibiotic- and disinfectant resistance

The genes encoding efflux pumps seem to be the most relevant ones when it comes to spreading of ARGs. Many gram-negative bacteria possess an intrinsic resistance towards antibiotics and disinfectants as their genomes naturally contain genes encoding these efflux pumps, as mutations have led to overexpression of these genes in bacterial chromosomes (Munita & Arias, 2016). However, when resistance genes are present on transferable genetic elements such as plasmids and transposons, this can lead to the epidemic spread of resistance between species (Wang *et al.*, 2007).

1.4.1 Florfenicol resistance genes

There are several specific resistance genes related to florfenicol resistance, including the phenicol-specific exporter genes *fexA*, *fexB* and *floR*, as well as the multidrug resistance gene *cfr*. The *floR* gene, which encodes a specific phenicol drug exporter, was first identified in fish pathogens and has since been frequently reported in several florfenicol-resistant bacteria of animal origin (Zhao *et al.*, 2016). In gram-negative bacteria associated with agriculture, florfenicol resistance seems to be primarily mediated by the *floR* gene (Schwarz *et al.*, 2004). Most of florfenicol-resistant *Pseudomonas* spp. isolated from Chilean salmon farms possessed this gene (Fernández-Alarcón, 2010). Those that did not, had instead their resistance explained by the possession of a non-specific multi-drug efflux pump. As the *floR* gene coexists with bacterial mobile genetic elements such as plasmids, florfenicol resistance genes can easily spread to numerous bacterial species and genera (Poole, 2002).

1.4.2 Tetracycline resistance genes

Tetracycline resistance is often caused by acquisition of new genes encoding energy-dependent efflux of tetracyclines, and the family of *tet* genes are commonly reported mediators (Roberts & Schwarz, 2016). These genes encode tetracycline resistance efflux proteins, which exchange a protein for a tetracycline-cation complex against a concentration gradient (Yamaguchi *et al.*, 1990). The *tet* genes have been identified in gram-negative, environmental and aquaculture-associated bacteria (Hedayatianfard *et al.*, 2014). Studies of marine bacteria in Chilean salmon aquaculture found that resistance to tetracycline was most

commonly encoded by the *tetA* and *tetG* genes (Shah *et al*., 2014). However, a limited number of bacteria acquire tetracycline resistance by mutations which alter the permeability of the outer membrane porins or change regulation of innate efflux systems (Chopra & Roberts, 2001).

1.4.3 Ampicillin resistance genes

Resistance to ampicillin can often be caused by acquisition of the *ampC* gene, which encodes the enzyme β-lactamase (Li *et al.*, 2012). This enzyme hydrolyses cephalosporins such as ampicillin, and the bacteria thus confer resistance by inactivation of this antibiotic. Studies of chlorination effects on microbial antibiotic resistance in drinking water found that chlorination caused enrichment of *ampC* (Shi *et al.*, 2013). This gene has also been detected in plasmids of bacteria isolated from Turkish fish farms, where it was also the most commonly reported resistance gene in all tested bacteria (Capkin & Altinok, 2015).

1.4.4 Disinfectant resistance genes

During the past years, resistance to QACs has been detected in many gram-negative bacteria associated with food, such as the pathogen *L. monocytogenes* and *Pseudomonas* spp. (Langsrud *et al.*, 2003). Genes conferring reduced susceptibility to QACs, such as benzalkonium chloride, are called *qac* genes (Wassenaar *et al.*, 2015). These genes encode efflux pumps capable of expelling many QAC structures from the bacterial cell. The *qac*Δ*E1,* which is an attenuated version of the *qacE* gene, has been widely detected in gram-negative bacteria, including the Enterobacteriaceae family and *Pseudomonas* spp. (Vijayakumar & Sandle, 2019). The *qacH* gene has also been found in gram-negative bacteria.

Resistance to disinfectants can also be achieved by gene cassettes such as *bcrABC,* giving an increased tolerance to benzalkonium chloride by encoding an efflux pump, and this gene cassette have been reported in *L. monocytogenes* isolated from food (Jiang *et al.*, 2016). Many disinfectants contain sulphonamide, and bacteria can achieve resistance to this component by the *sul1* plasmid-borne resistance gene encoding a sulphonamide resistance protein (Rådström & Sköld, 1991). A wide range of bacteria can harbour this gene, which is located in transposons and plasmids (Wang *et al.*, 2014).

1.5 Bacterial biofilms

1.5.1 Introduction to biofilms

Bacteria can exist in two different states: (1) planktonic cells and (2) biofilm cells. In the planktonic state, the bacteria live as an individual entity, occurring as a free-floating cell in a suspension. In the biofilm state, the cell exists in a complex community with other microbial cells. This community can either consist of bacterial cells from the same species, a mixture of several bacterial species, or even a mixture containing additional microorganisms such as fungi and algae (Elias & Banin, 2012). A biofilm is a cohesive matrix of microorganisms, mucopolysaccharides (slime), DNA, lipids and other extracellular constituents being produced by the microorganism (Madigan *et al.*, 2015). The microbes occupy about 10-30% of the biofilm volume, while the major part consists of water providing the necessary flow of nutrients.

Bacteria have a tendency to spend most of their lives in the biofilm state, and approximately 99% of all microbial species on earth have been estimated to exist in biofilms (Hall-Stoodley *et al.*, 2004). Biofilms are extremely widespread in natural environments, especially in aqueous environments such as lakes and ponds. These bacterial communities are also commonly found in living tissues, teeth, and medical devices inserted into the body, as well as industrial surfaces and pipes (Donlan, 2002). The structure of the biofilm largely depends on the environment, implying that biofilms are adapting to the surrounding conditions (Toyofuku *et al.*, 2016).

Bacteria form biofilms as a response to environmental cues with the aim of self-defence, protection and increased survival (Karatan & Watnick, 2009). Bacterial cells living in a biofilm community achieve an increased resistance towards physical forces, UV exposure, dehydration, salinity, destruction by the immune system, toxic metals and antimicrobials. Biofilms can also aid the bacteria in remaining in a favourable niche, such as the surface of nutrient rich animal tissue. In addition, biofilms bring bacterial cells in close proximity of each other, and thus facilitate the transfer of favourable genes such as ARGs. In mixed species biofilms, the by-product of one organism might serve to support the growth of another, while the adhesion of one species might provide ligands allowing attachment of other species (Dunne, 2002).

The increased tolerance to antibiotics among cells in the biofilm state have several causes, including physical, physiological and genetic factors (Ciofu & Tolker-Nielsen, 2019). As the cells in a biofilm are embedded in extracellular components, these may retard the penetration of the antibiotics. Also, as cells living in the interior of a biofilm have low physiological activity or no growth at all compared to planktonic cells, their tolerance to antibiotics can be increased a 1000-fold as antibiotics often target bacterial metabolism. Biofilm-induced expression of genes can as well increase the tolerance to stress. Mixed biofilms can also show higher resistance towards disinfectants such as QACs compared to single species biofilms (Rodríguez-López *et al.*, 2018).

1.5.2 Mechanisms of biofilm formation and development

A biofilm typically forms as bacteria attach to a solid surface such as steel, glass or animal tissue, but can also form by bacteria aggregating with each other without the presence of a surface (Harrison *et al.*, 2010). Biofilm formation occurs as cells switch from the planktonic state to the biofilm state, as they respond to changing environmental conditions by altering gene expression (Toyofuku *et al.*, 2016).

Cell-to-cell communication is a crucial factor in biofilm formation, and especially a communication mechanism known as quorum sensing, which enables bacteria to collectively modify behaviour in response to changes in cell density (Papenfort & Bassier, 2016). This mechanism involves the production, release, and group-wide detection of extracellular signalling molecules called autoinducers. In *P. aeruginosa*, the autoinducers responsible for biofilm formation are acyl homoserine lactones (De Kievit, 2009). Bacteria continuously monitor the concentration of autoinducers to receive information about the local cell number of their own species, and they will respond accordingly by altering their gene expressions collectively. Cyclic diguanosine-5′-monophosphate is a secondary messenger and a key regulator of biofilm formation in many bacterial species, including *P. aeruginosa*. This compound is involved in the intracellular signalling leading to production of extracellular components forming the biofilm (Ciofu & Tolker-Nielsen, 2019).

Biofilm development can be divided into five stages (Figure 1.3). In the first stage, planktonic cells typically adhere to a surface using cellular structures like pili or flagella (Madigan *et al.*, 2015). The attachment of these cellular structures promotes the gene expression related to

matrix assembly, further leading to production of extracellular polymeric substances (EPS). In the second stage, a microcolony is developed by simultaneous bacterial aggregation and growth (Preda & Săndulescu, 2019). Lectins, adhesins and additional EPS formation is important for proper building of the matrix and localization of its components in the early biofilm (third stage) (Passos da Silva *et al.*, 2017). In the fourth stage, the biofilm becomes mature, as internal cages and channels are created in the biofilm structure to provide the gradient-based entry of nutrients and bacterial signalling molecules. As the biofilm grows thicker and develops an anaerobic environment of the interior, there is a detachment and dispersal of cells and cells clusters ready to colonize new sites (fifth stage) (Preda & Săndulescu, 2019).

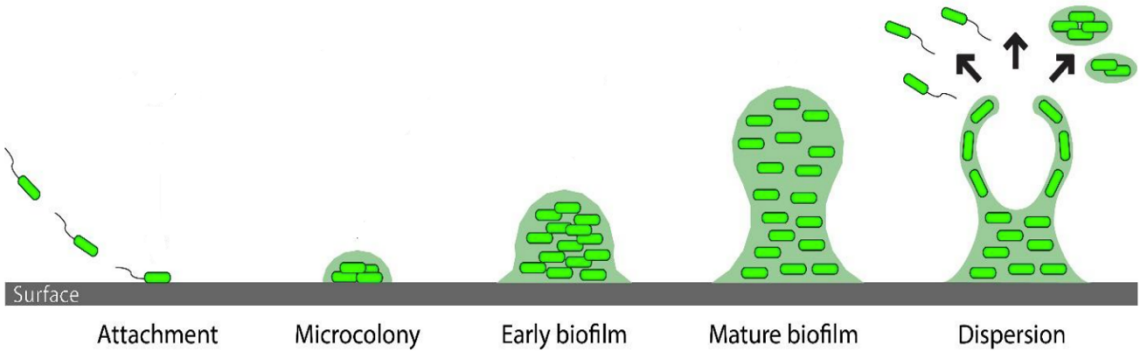


Figure 1.3: The five stages of biofilm development; (1) attachment of planktonic cells to a surface, (2) microcolony formation, (3) early biofilm development, (4) biofilm maturation and (5) dispersion of cells ready to colonize new sites. The figure does however not illustrate the formation of internal cages and channels during maturation of the biofilm, which takes place during the fourth stage (Passos da Silva *et al.*, 2017).

1.5.3 Implications of biofilm formation in seafood industry

Biofilms are especially of concern in the food industry, as diverse bacteria are able to grow on the surface of foods and along processing infrastructures such as surfaces and equipment (Cunault *et al.*, 2018; Galié *et al.*, 2018). As the biofilm matrix is fixed to solid surfaces, bacteria in biofilms commonly attach to food industry equipment of stainless steel, glass, and polyethylene, as well as biological structures such as fish skin. Attachment of potential spoilage and pathogenic bacteria leading to further biofilm formation represent a serious challenge to all food industries (Giaourisa *et al.*, 2014). Biofilm formation is also associated with alteration of organoleptic properties of the food due to bacterial secretion of lipases and

proteases, causing reduced shelf life of the product and corrosion of metal surfaces in the food processing plant.

Of particular concern are human pathogens capable of forming biofilms, leading to their persistence in the environment of the food processing plant. Common seafood pathogens that readily form biofilms are *Vibrio* spp., *A. hydrophila*, *Salmonella* spp., and *L. monocytogenes* (Mizan *et al.*, 2015). In Norway, there are 5000-7000 reported annual cases of food- and waterborne infections, but these are expected to only be a small fraction of the actual cases (NIPH, 2017). About 100 cases of listeriosis are reported annually in Norway, making it the most dominant pathogen responsible for hospitalization, as it poses a serious health risk to elderly humans, pregnant women and people having reduced immunity.

As the biofilm-associated bacteria are more tolerant to disinfectants than planktonic cells, it is extremely challenging to eradicate biofilms. Current biofilm control strategies applied by the food industry are simply based on the prevention of bacterial contamination, using chemical and mechanical cleaning, disinfection, and surface preconditioning (Giaourisa *et al.*, 2014; Preda & Săndulescu, 2019). These strategies have some efficiency, but do however not provide the desired level of effect and control. Approaches specifically targeting biofilms seem to not have been developed so far, as the detailed molecular mechanisms behind bacterial biofilm formation are still poorly understood (Rabin *et al.*, 2015).

1.6 The aim of the project

1.6.1 Previous work in the specialization project

In the specialization project, 90 bacterial isolates collected from farmed salmon in a Norwegian processing plant were identified (Bringsli, 2020). The DNA of these was isolated prior to a PCR amplification of the *rpoD* and 16S rRNA genes, followed by gel visualization and Sanger sequencing performed by an external company. BLASTN analysis of the obtained gene sequences was used for identity prediction. By this approach, 37% of isolates were identified as *Pseudomonas* spp., 34% as *Serratia* spp., and 7% as *Aeromonas* spp. The remaining bacteria were identified as either *Hafnia* spp., *Shewanella* spp., *Acinetobacter* spp., *Strenotrophomonas* spp. or *Enterobacter* spp.

1.6.2 Aim of master´s thesis

The specialization project is the starting point of this master´s thesis, as the properties of the previously identified *Pseudomonas* spp., *Serratia* spp. and *Aeromonas* spp. will now be investigated. The master project has four aims: (1) achieve a more certain identification of the intrageneric identity of *Pseudomonas spp*., (2) investigate the occurrence of antimicrobial resistance, (3) determine the biofilm formation capacity and (4) validate the efficiency of the disinfection routine applied by the salmon processing plant. The second aim will however be the main focus of the thesis.

1. A more certain prediction of the intrageneric identity of the bacteria isolates already being predicted to be *Pseudomonas* spp. will be attempted. A PCR amplification of the *gyrB* gene will be performed, and further BLASTN analysis of gene sequences will hopefully ensure a high-resolution identification at the *Pseudomonas* spp. intrageneric level. Morphology observations are also performed for all bacteria isolates, to see if this can be an approach for identification of these bacterial species.

2. The microbial resistance status of the isolates will be illuminated, and the focus will be on resistance to florfenicol, tetracycline, ampicillin, and QACs. A PCR-based detection of relevant ARGs will be performed to reveal the resistance at the genotypic level, and a disk diffusion assay and microtiter susceptibility assay will be applied to cover the resistance at the phenotypic level.

3. By using a microtiter biofilm assay, the biofilm formation capacity of the isolates at 12°C will be investigated, as this is the operating temperature of the salmon processing plant.

4. The efficiency of the disinfectant Aqua Des Foam PAA will be tested, as it is commonly applied to disinfect surfaces and equipment of the salmon processing plant. A microtiter susceptibility assay will be utilized for this purpose.

2. Materials and methods

2.1 Description of the bacteria isolates

To investigate the antimicrobial resistance and biofilm formation capacity among bacteria associated with Norwegian salmon industry, the isolates previously identified in the specialization project were the starting point (Bringsli, 2020). These were isolated from a salmon processing facility in the time period 2018-2019, being collected from the skin (87%), fillet (12%) or gills (1%) of gutted salmon prior to final packaging. A full list of sample ID, sampling point and predicted identity is presented in Appendix A. The isolates belonging to the genera of *Pseudomonas* spp., *Serratia* spp. and *Aeromonas* spp. were selected for this further investigation, as the former two were the most abundant genera and the latter represents potential pathogens (Table 2.1).

Table 2.1: Overview of the bacteria isolates selected for further investigation of antimicrobial resistance and biofilm formation capacity. These were isolated from gutted salmon in a Norwegian processing plant. The *rpoD* & 16S rRNA-based identifications are indicated both at genus and species level, and the sample ID and collection point (gills, skin or fillet) are presented in Appendix A.

Genus	Species	Quantity of isolates
***Pseudomonas* spp.** **(23 isolates in total)**	*P. anguilliseptica*	1
	P. azotoformans	1
	P. extremaustralis	5
	P. fluorescens	9
	P. gessardii	1
	P. libanesis	2
	P. lundensis	2
	P. marginalis	1
	P. veronii	1
***Serratia* spp.** **(20 isolates in total)**	*S. liquefaciens*	18
	S. fonticola	2
***Aeromonas* spp.** **(4 isolates in total)**	*A. hydrophila*	1
	A. salmoncida	3

2.2 Morphology observations

The morphological appearance of the visible bacteria isolates growing on agar plates was observed, and since morphology can be influenced by environmental conditions, all observations were executed at the same conditionings: after a 72 h incubation at 25°C. The colour and consistency were then observed as they were propagated from one Tryptone Soya Agar plate (TSA) (Oxoid, CM0131) to another TSA plate. One way analysis of variance

(ANOVA) was performed to detect significant differences ($P < 0.05$) in morphology among and within the bacteria genera, as well as detecting morphological differences in the isolates collected from fillet- and skin (Appendix E). When differences were statistically significant, Tukey or LSD post hoc tests were performed for comparison. All statistical analysis was conducted using the computer program IBM SPSS Statistics 27.

2.3 Intrageneric identification of *Pseudomonas* spp. based on the *gyrB* gene

To achieve a more certain identification of the isolates being already predicted to be species within the *Pseudomonas* spp. genera using *rpoD* or 16S rRNA genes, the identification was further extended to the *gyrB* gene. This is a housekeeping gene in *Pseudomonas* spp., and should provide a high-resolution identification. A specific primer pair designed to target highly conserved regions within this gene was used, having the following sequences: AGCATYAARGTGCTGAARGG (primer *gyrB*-F) and GGTCATGATGATGATGATGTTGTG (primer *gyrB*-R), and the expected amplicon size is 1461-1467 bp (Agaras & Valverde, 2018).

2.3.1 PCR amplification and gel visualization

A 25 µL reaction mixture was prepared and added to the wells of a sterile PCR plate. This mixture contained 2.50 µL of 10x PCR buffer (203203, QIAGEN), 0.50 µL of 100 mM dNTP mixture (201901, QIAGEN), 0.50 µL of 25 mM $MgCl_2$ (203203, QIAGEN), 1.00 µL of each primer (at 10 µM concentration), 0.13 µL of 5 units/µL HotStarTaq® DNA Polymerase (203203, QIAGEN), 19.37 µL nuclease-free water (129114, QIAGEN) and 1.00 µL template DNA. Also, a negative control using nuclease-free water and a positive control being *P. aeruginosa* NCTC 13717 was applied, and a thermocycler (Doppio Gradient, 732-2551, VWR) was used for the PCR amplifications. To achieve an optimal amplification, the PCR cycle consisted of an initial denaturation step of 15 min at 95°C, followed by 35 cycles of 30 s at 95°C, 30 s at 56°C, and 90 s at 72°C, and a final extension step at 72°C for 5 min.

Gel electrophoresis was used to verify if the gene had been successfully amplified. A 1.2% agarose gel was prepared by mixing 1.8 g agarose powder (SeaKem®, 50004), 150 mL 1x EDTA buffer (Thermo Fisher Scientific, B49) and 6 µL GelRed (Biotium, 41003). A mixture

of 12 µL PCR product and 5 µL DNA loading dye (Thermo Fisher Scientific, R0611) was applied to the gel. Also, 6 µL of Gene Ruler 1 kb DNA ladder (SM1331, Thermo Fisher Scientific) was added to the first and last well of the gel. The gel was run at 85 V for 75 min, followed by a visualization of the genes as bands appearing in the gel, using a gel doc system and Genesys software (G:BOX Chemi XRQ, Syngene™). PCR products achieving a successful amplification were further purified. For this purpose, a mixture of 2 µL ExoSAP-IT™ PCR Product Cleanup (Thermo Fisher Scientific, 78250.40.UL) and 5 µL PCR product was added to wells of a PCR plate, before being incubated the thermocycler. The incubation was first set to 37°C for 15 min to degrade remaining primers and nucleotides, followed by This was followed by an incubation at 80°C for 15 min to inactivate the EXOSAP-IT reagens.

2.3.2 Preparation for sequencing and BLASTN bioinformatic analysis

All PCR products were quantified using a spectrophotometer (BioTekTM PowerWaveTM Microplate Spectrophotometer, 04, Thermo Fisher Scientific) and Gen5 2.0 data analysis software. The PCR products were then individually diluted using nuclease-free water (129114, QIAGEN), to achieve a DNA concentration of 25 ng/µL as this is a requirement for DNA Sanger sequencing at Eurofins Genomics. Each diluted PCR product was added to two different 1.5 mL tubes, using a volume of 5 µL PCR product per tube. The primers were diluted to 5 mM, and 5 µL of primer *gyrB*-F was added to the first tube while 5 µL of primer *gyrB-R* was added to the second tube, using a volume of 5 µL primer per tube. Each tube was given a sequence identify before being manually sent to Eurofins Genomics GATC services in Cologne, Germany.

BLASTN bioinformatic analysis using the NCBI web was used to predict the identity of the obtained gene sequences. The chosen database was set to ”Nucleotide collection” standard database, excluding “Uncultured/environmental sample sequences” and the program was optimized for highly similar sequences (Megablast). In the list generated by the similarity search, the output sequence obtaining the highest parameter scores (query coverage, percentage identity and max score) was chosen as the best prediction. Only the top hits being *gyrB* gene sequences of officially identified species (not simply representing isolates) were considered as candidates in the identity prediction.

2.4 PCR-based detection of antimicrobial resistance genes

A PCR-based approach was performed to detect the presence of ARGs in all of the 47 bacteria isolates collected from Norwegian gutted salmon in a processing plant. Several genes related to a variety of antimicrobial resistances were attempted to be amplified, by using different PCR assays with selected primers and appropriate positive controls (Table 2.2).

2.4.1 PCR amplification and gel visualization

The PCR reaction mixtures were prepared with slight variations among the eight different PCR assays, and a thermocycler was used for the amplification (Table 2.3). To make each individual resistance gene amplification optimal, different PCR cycles was applied in the various assays (Table 2.4). To detect the presence of genes associated with florfenicol resistance, two different primer pairs were used in each individual PCR assay to ensure that both of the two existing gene versions (*floR* & *floR2*) were covered (Reiche, 2021). Visualization of the potentially amplified resistance genes was performed using a gel electrophoresis procedure (section 2.3.1).

Table 2.2: Overview of the antimicrobial resistance genes attempted amplified using PCR. Primer sequences, positive controls and expected amplicon sizes are presented.

Target gene	Primer sequence (5'- 3')	Resistance	Amplicon size	Positive control	Reference
ampC	**F:** CCTCTTGCTCCACATTTGCT	Ampicillin	189 bp	*E. coli* CCUG 17620	(Shi *et al.*, 2013)
	R: ACAACGTTTGCTGTGTGACG				
bcrABC	**F:** GGAGGGTAATCATGTCAG	Benzalkonium chloride/ QACs	1312 bp	*L. monocytogenes* MF 4624	(Elhanafi *et al.*, 2010)
	R: GTATAATCCGGATGCTGCCC				
floR/floR2	**F1**: GCTTTAGCGCCGGTATGG	Florfenicol (phenicol specific exporter genes)	120 bp	*A. baumannii* NCTC 13305, E. coli CCUG 17620	(Zhao *et al.*, 2016)
	R1: GACAGTGGCGAAGGCAAAG				
	F2: TCGCCCGGTATTCCTTAATCG		963 bp	*A. baumannii* NCTC13305	(Reiche, 2021)
	R2: TGAAGGTGAGGAATGACGGC				
sul1	**F:** CGGCGTGGGCTACCTGAACG	Sulphonamide	433 bp	*P. aeruginosa* CCUG59347	(Shi *et al.*, 2013)
	R: GCCGATCGCGTGAAGTTCCG-				
tetG	**F:** GCTCGGTGGTATCTCTGCTC	Tetracycline	468 bp	*P. aeruginosa* CCUG 59347	(Shi *et al.*, 2013)
	R: AGCAACAGAATCGGGAACAC				
qacH	**F:** ATGTCATATCTATATTTAGC	QACs	366 bp	*L. monocytogenes* MF5634	(Müller *et al.*, 2013)
	R: TCACTCTTCATTAATTGTAATAG				
qacΔE1	**F:** TAGCGAGGGCTTTACTAAGC	QACs	300 bp	*P. aeruginosa* CCUG59347	(Wang *et al.*, 2007)
	R: ATTCAGAATGCCGAACACCG				

Table 2.3: PCR reaction mixtures used in the amplification of selected antimicrobial resistance genes. If not otherwise specified, the given volume of a specific reactant was used irrespective of gene type being amplified.

PCR Reaction mixture	Volume per reaction
10x PCR buffer (203203, QIAGEN)	2.50 µL
dNTP mixture, 10 mM of each dNTP (201901, QIAGEN)	0.50 µL
25 mM $MgCl_2$ (203203, QIAGEN)	0.50 µL (*bcrABC, tetG, qacH, qacΔE1*) 0.00 µL (*ampC, floR, sul1*)
10 µM Primer F	0.50 µL
10 µM Primer R	0.50 µL
5 units/µL HotStarTaq® DNA Polymerase (203203, QIAGEN)	0.13 µL
Nuclease-free water (129114, QIAGEN)	18.88 µL (*bcrABC, tetG, qacH, qacΔE1*) 19.38 µL (*ampC, floR, sul1*)
Template (DNA sample)	1.5 µL
Total volume	**25 µL**

Table 2.4: PCR cycles used in the various PCR assays for the amplification of the ARGs.

PCR cycles for amplification of resistance genes	
Initial denaturation	94°C, 15 min (*ampC, sul1, tetG, qacΔE1*) 95°C, 15 min (*bcrABC, floR, qacH*)
Denaturation	93°C, 30 s (*qacΔE1*) 94°C, 30 s (*ampC, tetG, sul1, qacH*) 94°C, 40 s (*bcrABC*) 95°C, 30 s (*floR*)
Hybridization	55°C, 30 s (*bcrABC, sul1, tetG, qacΔE1*) 56°C, 90 s (*qacH*) 58°C, 30 s (*ampC*) 60°C, 30 s (*floR*)
Elongation	72 °C, 30 s (*floR, qacH*) 72 °C, 60 s (*ampC, sul1, tetG, qacΔE1*) 72 °C, 90 s (*bcrABC*)
Reaction cycles	30 (*bcrABC, qacH*) 35 (*ampC, sull, tetG, qacΔE1*) 40 (*floR*)
Final elongation	72 °C, 5 min (*bcrABC, floR, qacΔE1*) 72 °C, 7 min (*ampC, sul1, tetG*) 72 °C, 10 min (*qacH*)

2.5 Investigating phenotypic resistance patterns by disk diffusion assay

2.5.1 Introduction to disk diffusion assay

The prevalence of antibiotic resistance was phenotypically investigated by applying a disk diffusion assay. The assay was conducted for all *Pseudomonas* spp., *Serratia* spp. and *Aeromonas* spp. isolated from gutted salmon in a processing plant, and their susceptibility to florfenicol, tetracycline and ampicillin was tested. Florfenicol and oxytetracycline are used in Norwegian aquaculture at present time, while ampicillin has not been used for almost three decades. As oxytetracycline was not available as antimicrobial disks, tetracycline had to be

applied instead, and their effect should be the same. The disk diffusion assay was performed according to the standards and recommendations of the Clinical and Laboratory Standards Institute (CLSI, 2012).

Explaining the principles behind this method in brief: an inoculum is spread onto the surface of a Mueller-Hinton agar plate (MHA) and antimicrobial disks are applied. The antimicrobial susceptibility is revealed by the growth pattern after an incubation period, and the bacteria are classified as either resistant, intermediate or susceptible. In case of susceptibility, a circular uniform inhibition zone surrounding the antimicrobial disk will be present, while the rest of the agar surface consists of a continuous lawn of growth. To gain reliable results, individual colonies should not appear on the MHA plate. The diameter of the inhibition zone determines the classification, as a large zone corresponds to a sensitive isolate while a small zone corresponds to a resistant one. Reference tables provide the actual inhibition zone interpretive criteria necessary for this classification.

2.5.2 Inoculum preparation

The bacteria isolates were propagated twice in advance using TSA plates, to ensure fresh colonies. Between each propagation, the plates were incubated at 25°C for 24 h. Fresh colonies from the agar plate were transferred to a sterile glass tube containing 10 mL 0.9% saline solution, by using a disposable plastic loop, and the bacteria suspension was thoroughly mixed. To standardize all of the bacteria suspensions to an equal cell number, McFarland Barium Sulphate turbidity standards were prepared (Table 2.5). The bacteria suspensions were standardized to 0.5 McFarland, being equivalent to 67 ± 3% transmittance and a cell number of $1.5 * 10^8$ CFU/mL. A turbidimeter (21907, Biolog) was used to determine the turbidity, and additional colonies or sterile saline solution was added to the glass tube to adjust the turbidity closer to the desired transmittance.

Table 2.5: Preparation of McFarland turbidity standards. The indicated volume of reagents was mixed to achieve the corresponding McFarland standards. In the disk diffusion assay, only McFarland standard 0.5 is relevant for the standardization of bacteria suspensions. However, McFarland standard 1.0 and corresponding cell number of inoculum used for biofilm cultivation are also presented as they become relevant in further experiments.

McFarland standard	Volume of 1% $BaCl_2$ (mL)	Volume of 1% H_2SO_4 (mL)	Approximate cell number of corresponding bacteria suspension (CFU/mL)	Approximate cell number of inoculum used for biofilm cultivation (CFU/mL)
0.5	0.05	9.95	$1.5 * 10^8$	$5.0 * 10^6$
1.0	0.10	9.90	$3.0 * 10^8$	$1.0 * 10^7$

2.5.3 Inoculation of MHA plates and disk dispension

Prior to inoculation, the MHA plates (CM0337, Oxoid) were placed in a ventilated sterile cabinet at 37°C for approximately 15 min to ensure a dry agar surface. A sterile cotton swab (TX705W, Texwipe) was dipped into the bacteria suspension and rotated several times, before inoculating the plates by streaking out the cotton swab on the entire surface several times, rotating approximately 60° each time. This streaking pattern was performed a total of three times, and the cotton swab was dipped into new inoculum between each round. The lid was then left partially off the MHA plate for approximately 5 min to allow excess surface moisture to be absorbed. The antimicrobial disks were dispensed evenly onto the surface of the inoculated plate using sterile tweezers, and the disks were pressed down to ensure complete contact with the agar surface. Antimicrobial susceptibility disks of ampicillin (10 µg, CT0003B, Oxoid^{TM}), florfenicol (30 µg, CT1754B, Oxoid^{TM}) and tetracycline (30 µg, CT0053B, Oxoid^{TM}) were applied. Lastly, the MHA plates were inverted and incubated at 37°C for 16–18 h.

2.5.4 Classification and statistical analysis

After incubation, the growth pattern appearing on the MHA plates was observed, and inhibition zone diameters were measured to the nearest whole millimetre using a ruler. The isolates were classified as susceptible, intermediate or resistant based on inhibition zone diameter interpretive criteria (Table 2.6). Statistical analysis was performed to see if the resistance patterns varied significantly ($P < 0.05$) among the bacteria genera, as well as between the two collection points (skin and fillet), using the previously explained procedure from section 2.2 (Appendix E.2).

Table 2.6: Inhibition zone diameter interpretive criteria applied to classify the bacteria isolates as resistant (R), intermediate (I) or susceptible (S) in the disk diffusion assay, and references are also presented (Ref.).

Bacteria genera	**Inhibition zone diameter interpretive criteria**											
	Florfenicol, 30 µg (mm)				**Ampicillin, 10 µg (mm)**				**Tetracycline, 30 µg (mm)**			
	(R)	(I)	(S)	Ref.	(R)	(I)	(S)	Ref.	(R)	(I)	(S)	Ref.
Pseudomonas spp.	≤ 14	-	>14	(Miranda & Rojas, 2007)	13 ≤	-	>13	(Miranda & Rojas, 2007)	≤ 14	-	>14	(Miranda & Rojas, 2007)
Serratia spp.	≤ 14	15-18	≥ 19	This study	13 ≤	14-16	≥ 17	(CLSI, 2014)	≤ 11	12-14	≥ 15	(CLSI, 2014)
Aeromonas spp.	≤ 14	15-18	≥ 19	This study	13 ≤	-	>13	This study	≤ 11	12-14	≥ 15	CLSI, 2014)

2.6 Biofilm capacity testing by microtiter biofilm assay

2.6.1 Introduction to microtiter biofilm assay

A microtiter biofilm assay was applied to test the biofilm formation capacity of the bacteria isolates collected from farmed salmon in a processing facility. The temperature for biofilm cultivation was set to 12°C as this is the operating temperature of the salmon processing plant. The working procedure was based on the MBEC^{TM} assay procedural manual (Innovotech, 2015) and a microtiter susceptibility testing protocol (Harrison *et al.*, 2010). This assay is a small-volume, high-throughput experimental approach, allowing biofilm formation capacity of several bacteria to be tested simultaneously. By applying a 96-well microtiter plate, biofilms of different species can be cultured separately without cross-contamination between wells. By representing each bacteria isolate by four wells each, 23 isolates can be tested per microtiter plate (four last wells serves as growth media sterility control). A ped lid is placed onto the plate, containing pegs which function as biofilm inoculators as they provide a contact surface which planktonic cells can attach to and develop biofilm. The experiment involves several steps expanding over three days (Figure 2.1). All equipment, media and solutions were prepared and sterilized in advance.

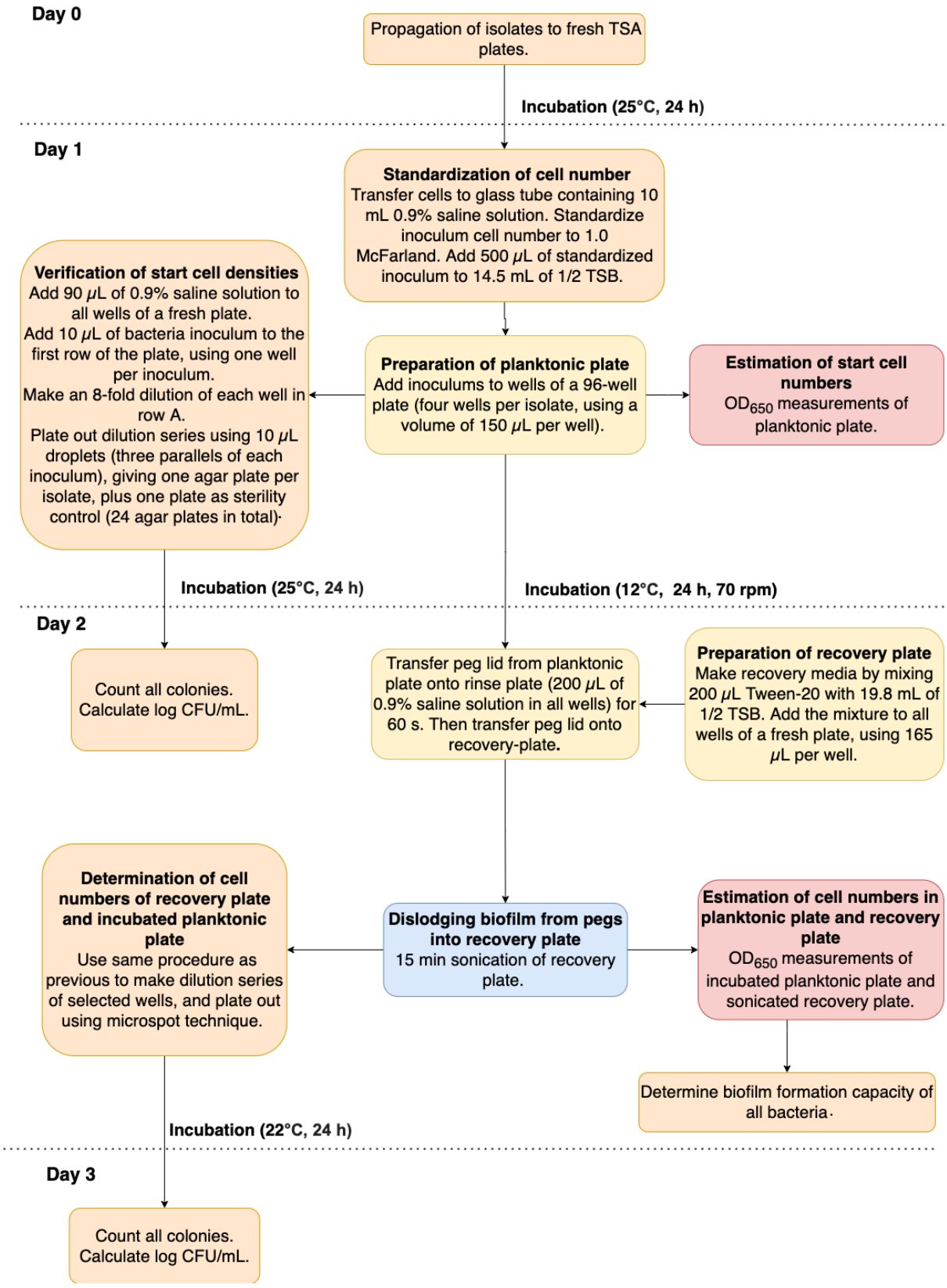


Figure 2.1: Working procedure executed to investigate the biofilm formation capacity at 12°C, among the *Pseudomonas* spp., *Serratia* spp. and *Aeromonas* spp. isolated from farmed salmon in a processing plant.

2.6.2 Inoculum preparation and cell number standardization

An inoculum preparation was then prepared and standardized using the previously explained procedure (section 2.5.2). This time, the bacteria suspensions were standardized to 1.0 McFarland standard, being equivalent to 50 ± 3% transmittance. This would ensure a cell number corresponding to approximately $3.0 * 10^8$ CFU/mL in the bacteria suspension, and further $5.0 * 10^6$ CFU/mL in the inoculum used for biofilm cultivation. After the standardization, 500 µL of bacteria suspension was transferred to a sterile centrifuge tube (10025-698, VWR) prefilled with 14.5 mL of half-concentrated tryptone soya broth ($^1/_2$TSB) (CM0129, Oxoid).

2.6.3 Preparation of planktonic plate

The centrifuge tube containing the inoculum was vortexed and poured into a sterile reagent reservoir (89094-662, VWR). Each bacteria inoculum was added to four wells of a 96-well microtiter plate (732-2719, VWR), using a volume of 150 µL per well. The first inoculum was added to the four first wells of column one (A1, B1, C1, D1), the second inoculum was added to the four last wells of column one (E1, F1, G1, H1), the third inoculum to the four first wells of column two (A2, B2, C2, D2), and so on (Figure 2.2a). A multichannel-pipette was utilized to transfer the inoculums into the wells, and the pipette tips were changed between each step. After this procedure, the microtiter plate (hence forth referred to as planktonic plate) contained 23 isolates, plus one growth media sterility control consisting of 150 µL of $^1/_2$ TSB in the last four wells.

2.6.4 OD_{650} measurements and incubation of planktonic plate

To assure the cell number of all wells in the planktonic plate were approximately equal, measurements of the optical density at 650 nm (OD_{650}) were performed using a spectrophotometer and data analysis software. The lid was left off the microtiter plate during measurements to achieve an accurate reading. Afterwards, a peg lid (445497, Thermo Fisher Scientific) was placed carefully onto the planktonic plate (Figure 2.2b & c). The plate with the peg lid was placed onto a rocking table in a platform shaker (MaxQ 6000 Digital Incubating and Refrigerating Stackable Orbital Shakers, 89032-320, VWR). The temperature was set to 12°C, and low stirring (70 rpm) was applied. A biofilm incubation period of 24 h was used.

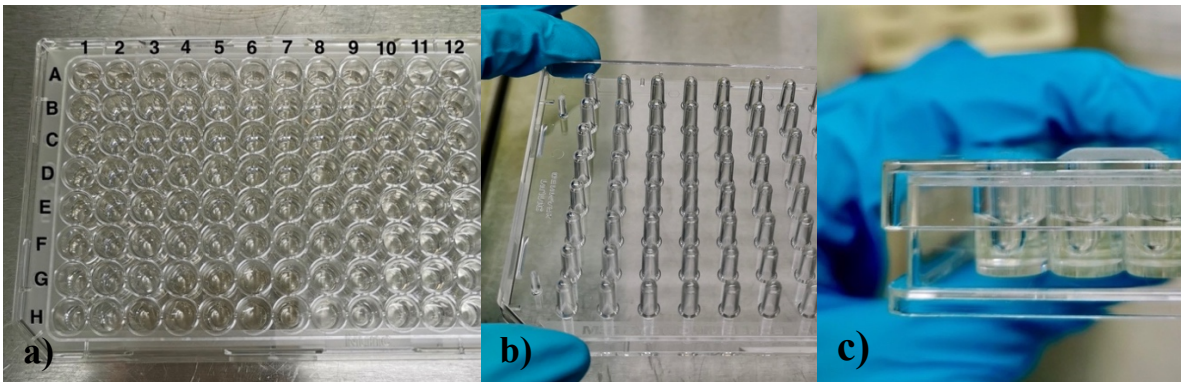


Figure 2.2 a) 96-well microtiter plate. **b)** peg lid. **c)** peg lid placed onto the 96-well plate.

2.6.5 Determining cell number of inoculum used for biofilm cultivation

As OD_{650} does not provide actual cell numbers, the "Miles and Misra" method was applied to determine CFU/mL of all inoculums used for the biofilm cultivation, as they should be approximately equal to $3.0 * 10^8$ CFU/mL. This is a more time efficient method compared to conventional plate count methods (Miles *et al.*, 1938; Munsch-Alatossava *et al.*, 2007). First, two new microtiter plates were prefilled with 90 µL of 0.9% saline solution in all wells. Then, 10 µL of the first inoculum was transferred from the plastic reservoir to the first well of column one in the new plate. The second inoculum was added to the first well of column two, and so on, until the last inoculum was added to column 11 of the second dilution plate. To achieve a serial dilution of each bacteria inoculum, 10 µL from each well of row A was transferred to corresponding wells of row B. Next, 10 µL from the wells of row B was transferred to the corresponding wells of row C, and so on, until 10 µL had been transferred to the last row (row H). Each well was thoroughly mixed between each step using the pipette.

By following this procedure, a serial dilution with dilution factors ranging from -1 in row A to -8 in row H was formed (Figure 2.3a). To allow quicker drying when plating out the dilution series, the TSA plates were in advance stored in a sterile incubator at 36°C for 30 min. Each dilution series, represented by one column each, was dropped onto the surface of an agar plate using a volume of 10 µL. Three parallels per dilution series were plated out onto the same agar plate (Figure 2.3b). One agar plate was used per series, giving 23 agar plates in total plus one plate of growth media sterility control. The droplets were allowed to dry before an incubation at 22°C for 24 h. In cases where growth was insufficient, the incubation period was prolonged for another 24 h at 15°C. All viable cells appearing in the microspots were

counted, and log CFU/mL were calculated (Appendix C.1). Acceptable log CFU/mL values of the inoculums were 6.7 ± 1.5.

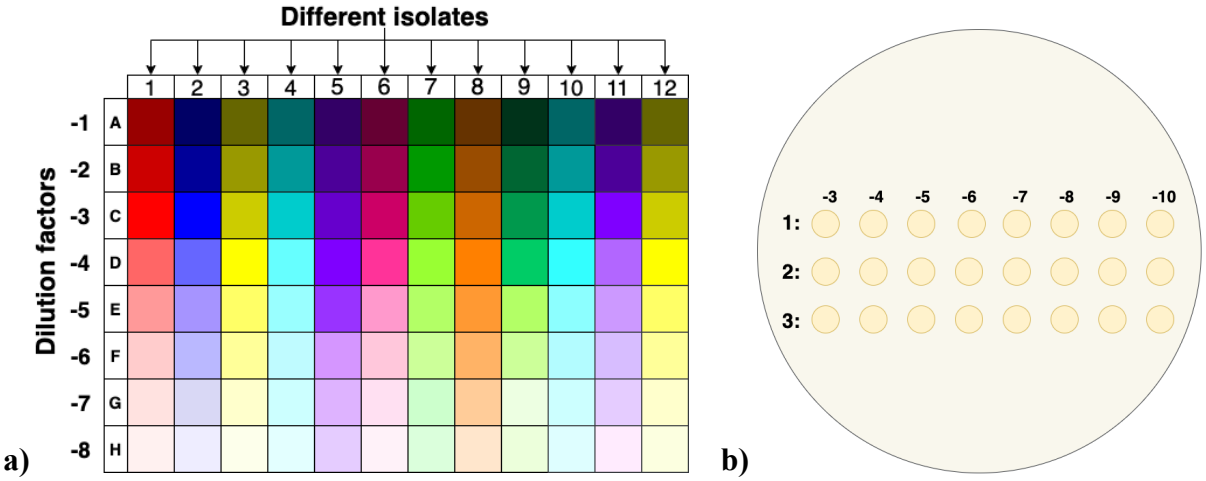


Figure 2.3: Dilution procedure. **a)** Illustration of dilution plate. Each bacteria inoculum is represented by one column, with dilution factors ranging from -1 to -8. **b)** Dilution microspot arrangement on agar plate. As a volume of 10 µm was plated out per well, the final dilution factors were ranging from -3 to -10. For each bacteria inoculum, three parallels were plated onto the same agar plate.

2.6.6 Removal of planktonic cells and dislodging biofilms

A rinse plate was prepared by adding 200 µL of 0.9% saline solution to all wells of a new 96-well plate. A recovery plate was prepared as well, by adding 165 µL of recovery medium to all wells of another new plate. This recovery medium was made by mixing 200 µl of Tween-20 (VWR, 0777-1L) with 19.8 mL of $^1/_2$ TSB. After the incubation period, the peg lid was carefully transferred from the planktonic plate onto the rinse plate for approximately 60 s, to remove loosely associated planktonic cells from the pegs. Next, the peg lid was further transferred onto the recovery plate, and the plate was placed in a stainless steel insert tray before being placed in a water bath sonicator (Branson 5800 Cleaner). The water level was adjusted to touch the bottom of the plate. A 15 min sonication was then performed to dislodge the biofilm from the pegs into the recovery media using low-frequency (60 Hz) vibrations.

2.6.7 OD_{650} measurements of incubated planktonic- and recovery plate

After sonication, OD_{650} measurements were performed for the incubated planktonic plate and recovery plate. This was done to determine the growth of planktonic cells (in the planktonic

plate) and growth of biofilm cells (being dislodged from pegs into the recovery plate). Also, to see if the OD_{650} measurements actually reflected the log CFU/mL values, these were determined for selected wells of the planktonic- and recovery plate using the "Miles and Misra" procedure (section 2.6.5). For each of the plates, two wells receiving a low OD_{650}, two wells receiving a medium OD_{650} and two wells receiving a high OD_{650} were selected. The wells containing the growth media sterility controls were plated out.

2.6.8 Determination of biofilm formation capacity and statistical analysis

The percentage difference in OD_{650} between incubated planktonic plate and sonicated recovery plate ($OD_{650,planktonic}$ minus $OD_{650,recovery}$) was used to classify the bacteria as good or poor biofilm producers. A large difference would imply that the growth of planktonic cells is large compared to growth of biofilm cells. To be classified as a good biofilm producer, the following criteria had to be fulfilled: $10\% \leq OD_{650,planktonic} - OD_{650,recovery} \leq 20\%$. Those bacteria achieving a difference higher than 20% were classified as poor biofilm producers, while those achieving a difference lower than 10% had insufficient growth. Statistical analysis was performed to investigate if the biofilm formation capacity varied significantly ($P < 0.05$) among and within the bacteria genera, using the previously explained procedure (section 2.5.4).

2.7 Testing antimicrobial efficiency by microtiter susceptibility assay

2.7.1 Introduction to MIC, MBC, MBEC and log reduction analysis

The efficiency of the disinfectant Aqua Des Foam PAA and the antibiotic florfenicol towards the bacteria isolates was tested. This disinfectant is commonly used to disinfect surfaces in salmon processing plants, while florfenicol is the most commonly used antibiotic in aquacultural farming. The test was conducted using a microtiter susceptibility assay, and the working procedure was also based on the MBEC™ assay procedural manual (Innovotech, 2015) and the microtiter susceptibility testing protocol (Harrison *et al.*, 2010). Isolates representing a variety of bacterial species, biofilm formation properties and antimicrobial resistance patterns were selected for the testing.

In this method, biofilms growing on pegs are introduced to the antimicrobial in a wide range of concentrations. Four parameters related to the efficiency of the antimicrobial are determined: MIC, MBC, MBEC and log reductions. MIC is defined as the minimum antimicrobial concentration preventing growth of planktonic cells in the recovery media after dislodging the biofilm. MBC is the minimum concentration necessary to kill the planktonic cells that have been shed from the biofilm during the antimicrobial challenge. MBEC is the minimal concentration necessary to eradicate the biofilm cells which have survived the antimicrobial challenge. Log reductions of biofilm cells are also determined for each antimicrobial concentration, by first using the "Miles and Misra" method to determine CFU/mL.

The overall working procedure extended over four or five days, depending on the antimicrobial being tested (Figure 2.4). As the commonly applied exposure time of Aqua Des Foam PAA in the processing plant is 15-20 min, an exposure time of 17 min was chosen. A 24 h incubation period was thought to be appropriate for florfenicol.

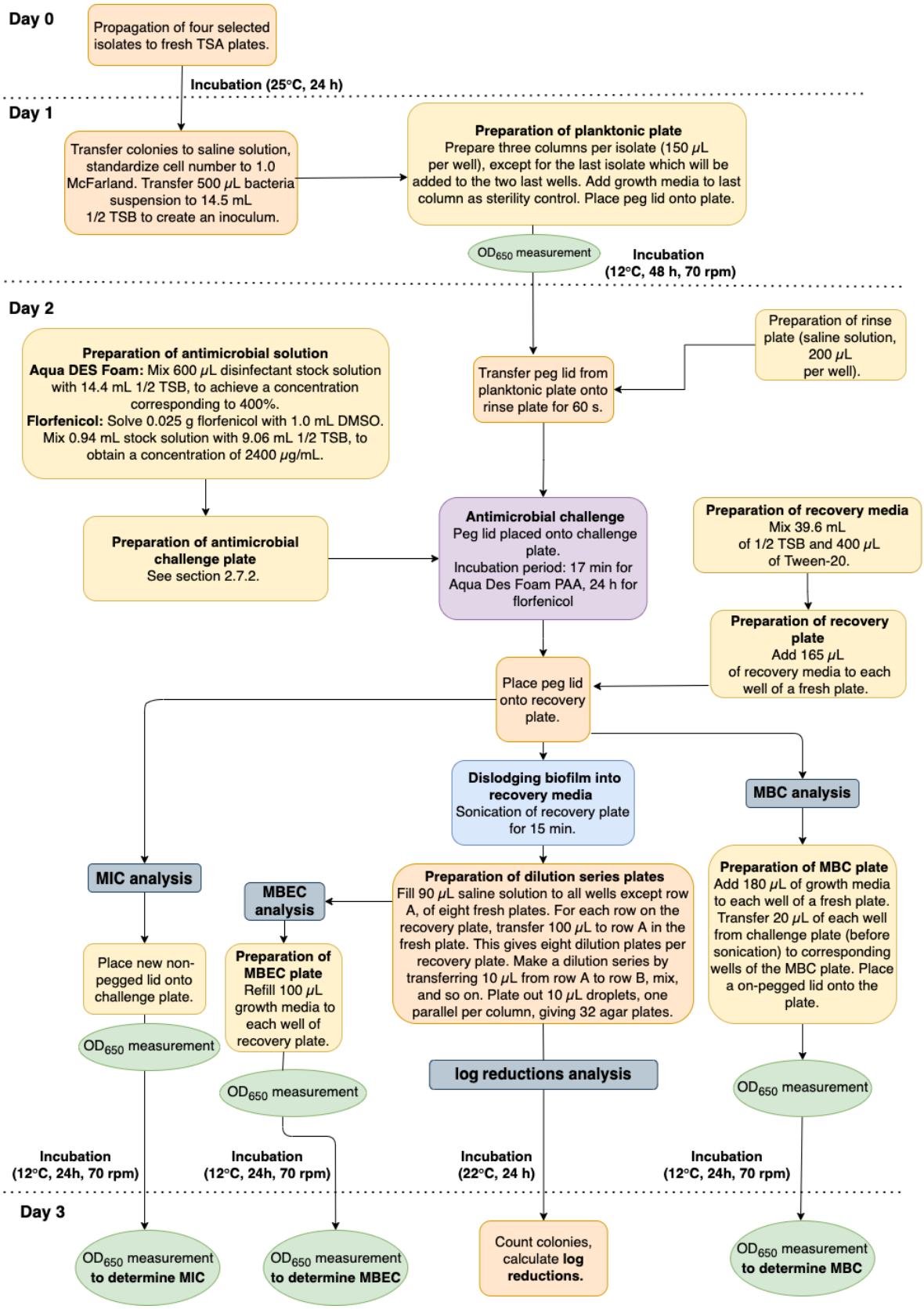


Figure 2.4: Overall working procedure to determine MBEC, MBC, MIC and log reductions of selected bacteria isolates, to test the efficiency of Aqua Des Foam PAA and florfenicol.

2.7.1 Inoculum preparation and incubation for biofilm formation

The bacteria inoculums were made following the procedure explained in section 2.6.2. Due to the comprehensiveness of the experiment, it was divided into several runs where four isolates were tested per run. The three first isolates were represented by three columns of the planktonic plate each, while the fourth was represented by two columns (plus two more columns in the next run). The planktonic plate was prepared by adding 150 µL of each inoculum to the wells of a microtiter plate. Then, 150 µL of $^1/_2$ TSB was added as a growth media sterility control in the last column, and OD_{650} measurements were performed. A peg lid was placed onto the plate before incubation at 12°C for 48 h, using low stirring (70 rpm).

2.7.2 Preparation of antimicrobial challenge plates

Aqua Des Foam PAA challenge plate

As a 1% disinfectant concentration is used by the salmon processing plant, a concentration gradient approximately representing the following concentrations was applied: 4.00%, 2.00%, 1.00%, 0.50%, 0.25%, 0.13% and 0.06%. First, a 4.00% disinfectant solution was prepared by mixing 1.2 mL of Aqua Des Foam PAA (H608B, AquatiqChemistry) with 28.8 mL of $^1/_2$ TSB. As the chemicals in the disinfectant can react with organic compounds in the growth media over time, the disinfectant solution was made immediately prior to usage.

To prepare the challenge plate, 200 µL of $^1/_2$ TSB was first added to the wells of column 12 of a new microtiter plate as growth media sterility control, and also 100 µL was added to all wells from B1→B11 to H1→H11. Then, 200 µL of the 4.00% disinfectant solution was added in A1→ A11, and also 100 µl in B1→B11 and C1→C11. Afterwards, 100 µL from the wells of C1→C11 was transferred to the corresponding wells of row D. The same transfer procedure was done from row D to E, and so on, until 100 µL was added to row G. After each transfer, the new solution was mixed thoroughly using the pipette. After this transfer, 100 µL was discarded from G1→G11 after mixing. At last, the wells from C1→C11 to H1→H11 were filled with 100 µL $^1/_2$ TSB. All wells in the challenge plate would now have an equal volume of 200 µL. Row H, containing only $^1/_2$ TSB, represented growth control without disinfectant. A disinfectant concentration gradient was now achieved, ranging from 4.00% in A1→A11 to 0.06% in G1→G11. The challenge plate was allowed to stand at room temperature for 30 min to equilibrate prior to the next step.

	1	2	3	4	5	6	7	8	9	10	11	12
A	4	4	4	4	4	4	4	4	4	4	SC+	SC-
B	2	2	2	2	2	2	2	2	2	2	SC+	SC-
C	1	1	1	1	1	1	1	1	1	1	SC+	SC-
D	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	SC+	SC-
E	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	SC+	SC-
F	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.13	SC+	SC-
G	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	SC+	SC-
H	GC-	GC-	GC-	GC-	GC-	GC-	GC-	GC-	GC-	GC-	GC-	SC-

Figure 2.5: Illustration of the Aqua Des Foam PAA challenge plate, representing concentrations ranging from 4.00% to approximately 0.06%. SC+/SC- corresponds to sterility control of growth media with and without added disinfectant, and GC-corresponds to a growth control without the disinfectant.

Florfenicol challenge plate

Firstly, 0.025 g of solid florfenicol (F1427, SigmaAldrich) was solved in 1.0 mL of DMSO (1.16743.1000, EMPLURA®). Then, 0.94 mL of this solution was mixed with 9.06 mL of $^1/_2$ TSB to achieve a florfenicol stock solution with a concentration of approximately 2400 µg/mL. The florfenicol challenge plate would be prepared in a specific manner to approximately represent the following concentrations: 2400, 600, 300, 150, 75, 19 and 5 µg/mL (Figure 2.6). The florfenicol challenge plate was prepared by first adding 200 µL of $^1/_2$ TSB to all wells of column 12 of a new microtiter plate as a growth media sterility control. Next, 100 µL of $^1/_2$ TSB was added to all wells from B1→B11 to H1→H11 of the challenge plate, and also to the three first rows of a new plate (dilution plate). Also, 100 µL of the florfenicol stock solution was added to the challenge plate in A1→ A11, representing a concentration of 2400 µg/mL.

First, 100 µl from A1→A11 in the challenge plate was transferred to row A of the dilution plate. Then, 100 µL from this dilution plate was further transferred to B1→B11 of the challenge plate (now representing a concentration of 600 µg/mL). The same transfer procedure was done from row B to C of the challenge plate, and further from C to D, and D to E. Row C, D and E would now represent concentrations of 300, 150 and 75 µg/mL

respectively. Next, 100 µL was transferred from row E of the challenge plate to row B of the dilution plate, and 100 µL from row B of the dilution plate further to row F of the challenge plate (now representing approximately 19 µg/mL). From row F, 100 µL was transferred to row C of the dilution plate, and another 100 µL from row C back to the challenge plate in row G, representing the final concentration step of approximately 5 µg/mL. At last, 100 µL was discarded from G1→G11 in the dilution plate, and wells from C1→C11 to H1→H11 were filled with 100 µL ½ TSB to achieve an equal volume of 200 µL in all wells. Row H, containing only growth media, would now represent growth control without disinfectant.

	1	2	3	4	5	6	7	8	9	10	11	12
A	2400	2400	2400	2400	2400	2400	2400	2400	2400	2400	SC+	SC-
B	600	600	600	600	600	600	600	600	600	600	SC+	SC-
C	300	300	300	300	300	300	300	300	300	300	SC+	SC-
D	150	150	150	150	150	150	150	150	150	150	SC+	SC-
E	75	75	75	75	75	75	75	75	75	75	SC+	SC-
F	18.75	18.75	18.75	18.75	18.75	18.75	18.75	18.75	18.75	18.75	SC+	SC-
G	4.68	4.68	4.68	4.68	4.68	4.68	4.68	4.68	4.68	4.68	SC+	SC-
H	GC-	GC-	GC-	GC-	GC-	GC-	GC-	GC-	GC-	GC-	GC-	SC-

Figure 2.6: Illustration of the florfenicol challenge plate, representing concentrations from 2400 to approximately 5 µg/ml. SC+/SC- corresponds to sterility control of growth media with and without added florfenicol, and GC- corresponds to a growth control without florfenicol.

2.7.3 Antimicrobial challenge and recovery of biofilm

Planktonic cells being loosely attached to the biofilms were removed following the previously described rinsing procedure (section 2.6.6). The peg lid was transferred further to the challenge plate. A 17 min exposure time at room temperature was used for Aqua Des Foam PAA, and a 24 h exposure period at 12°C was applied for florfenicol. After incubation, the peg lid was transferred from the challenge plate to a recovery plate prepared as previously explained (section 2.6.6). The recovery media helps reduce toxicity from the carry-over of biologically active compounds from the challenge plate to the recovery plate, and it also aids

in recovering any remaining biofilm after the challenge. A 15 min sonication was applied to dislodge the biofilms.

2.7.4 Determining log reductions of biofilm cells

Following sonication of the recovery plate, 100 µL of each well in row A was transferred to corresponding wells of row A in a new microtiter plate. From the wells of row B on the recovery plate, 100 µL was transferred to corresponding wells of row A in another fresh plate. This procedure was done for all eight rows of the recovery plate, resulting in eight dilution plates. Then, 180 µL of 0.9% saline solution was added in the remaining empty wells of all the dilution plates. The "Miles and Misra" method was again applied as previously explained (section 2.6.5). One parallel per column was plated out, and three columns were spotted on the same TSA agar plate. Plating out these eight dilution plates would then give 32 agar plates, which were further incubated at 22°C for 24 – 48 h. Colonies were counted to determine the quantity of viable biofilm cells surviving the antimicrobial challenge. Log CFU/peg (the log density for each peg) was determined, and further log reductions were calculated (Appendix D.2).

2.7.5 Determination of MIC, MBC and MBEC values

The MBEC plate was simply prepared by refilling 100 µL of $^1/_2$ TSB to each well of the recovery plate to replace the volume transferred to the dilution plates. An MBC plate was also prepared, by first filling the wells of a new microtiter plate with 180 µL $^1/_2$ TSB. Then, 20 µL was transferred from the challenge plate into the corresponding wells of the MBC plate. The MIC plate was prepared by placing a new non-pegged lid into the challenge plate. Then, OD_{650} measurements were performed for the three plates, and they were incubated at 12°C for 24 h using 70 rpm stirring. After incubation, OD_{650} measurements were again performed. Plots of the OD_{650} measurements were constructed for each bacteria isolate and antimicrobial concentration level. A criterium for adequate biofilm growth of growth control wells was defined: $(OD_{650,24\ h} - OD_{650,\ 0\ h}) \geq 0.05$. Thus, the difference in OD_{650} between start and stop times had to be larger than 0.05, and bacteria isolates achieving a smaller difference were excluded from further analysis. To determine the MIC, MBC and MBEC values, the cut-off value for growth was set to $OD_{650} \geq 0.09$. The lowest concentration level having OD_{650} higher or equal to 0.09 would thus represent the MIC, MBC or MBEC value, depending on the plate being considered.

3. Results

3.1 Morphology observations

The colour of the bacteria isolates was observed when growing on TSA agar, after an incubation at 25°C for 72 h. The consistency was also noticed during the propagations as colonies were transferred from one agar plate to another. Three different colour shades were observed: beige, brown and yellow (Figure 3.1). Also, two different consistency patterns were observed: soft and firm/gel. Most isolates were classified as light beige with a soft consistency. Full list of morphology observations are presented in Appendix A.

Figure 3.1: Illustration of the appearance of bacteria isolates on TSA plates after an incubation at 25°C for 72 h. The first isolate (LPJ882: *P. libanesis*) was classified as yellow, the second isolate (LPJ863: *P. extremaustralis*) as beige, and the third isolate (LPJ823: *P. anguilliseptica*) as brown.

Statistical analysis was used to detected morphological variation among the genera, which revealed that neither the colour nor consistency had a significant difference ($P < 0.05$) among *Pseudomonas* spp., *Serratia* spp., and *Aeromonas* spp. isolates at the level of genera (Appendix E). To detect differences at the species level, the bacteria species being represented by less than two isolates were excluded (P. *anguilliseptica*, *P. azotoformans*, *P. gessardii*, *P. marginalis*, *P. veronii* and *A. hydrophila*). In this case, both colour and consistency had a highly significant difference among the species. The largest variation was between *P. fluorescens* and *P. libanesis*, as all nine members of the former were light beige with a soft consistency, while the two members of the latter were yellow with a firm/gel consistency. Whether the isolates had been collected from salmon skin or fillet did not significantly affect ($P < 0.05$) the observed morphology.

3.2 Intrageneric identification of *Pseudomonas* spp. based on the *gyrB* gene

The identity prediction of *Pseudomonas* spp. (23 isolates) was extended to the *gyrB* gene, as only the *rpoD* and 16S rRNA genes had been considered in the previous specialization project. This was achieved by applying PCR amplification and gene sequence analysis. The gel visualization of the *gyrB* gene revealed that the amplicons had an approximately size of 2000 bp, despite the expected size was 1500 bp (Figure 3.2). No band was achieved for positive control (*P. aeurgionos*a NCTC 13717), as well as no band appeared in four of the 23 isolates.

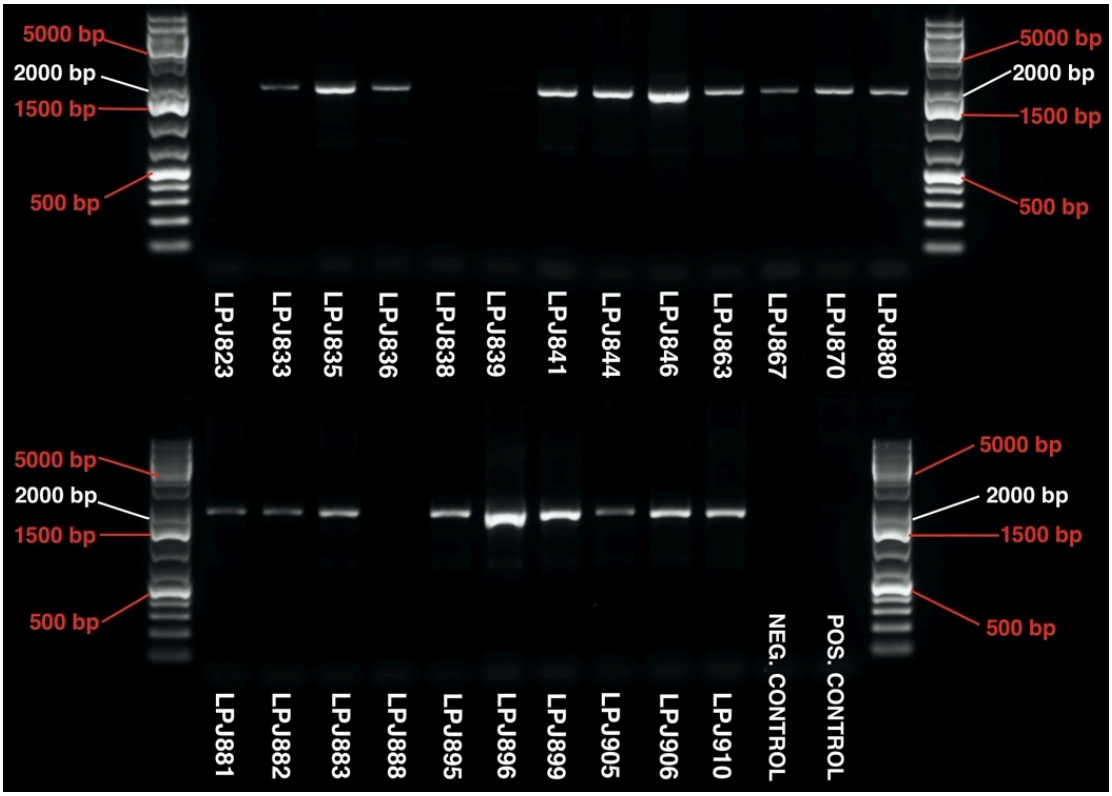


Figure 3.2: Visualization of the *gyrB* gene amplicons of all *Pseudomonas* spp. isolates on a gel, these are represented by the sample IDs. No band was achieved for positive control (*P. aeurgionos*a NCTC 13717) as well as in four of the isolates. The applied ladder was Gene Ruler 1 kb DNA ladder (SM1331, Thermo Fischer Scientific).

The amplicons of five isolates were sent for external Sanger sequencing to test if they actually represented the *gyrB* gene despite not having the expected length, using two parallels per isolate: one parallel with primer R and one with primer F. Only four of the in total 10 parallels

being sent for sequencing actually achieved gene sequence of appropriate length (being 522-938 bp) for identification, as the remaining were shorter than 100 bp. Of the four isolates (LPJ835, LPJ863, LPJ896 and LPJ899) achieving a gene sequence of sufficient length, only LPJ899 had a successful sequencing for both primers. Three gene sequences were based on the R primer, and two on the F primer, thus, the primer type did not affect the successfulness of the sequencing. BLASTN analysis of the gene sequences were performed and their identity predicted using the explained criteria (section 2.3.4). The *gyrB* identity and score parameters was compared to those obtained from the previous 16S rRNA & *rpoD* analysis (Table 3.1). When comparing the novel *gyrB* identity with the previous *rpoD*/16S rRNA identity, the max score is smaller for *gyrB* and the top hit identity was equal in most of the cases.
Since less than half of the prepared amplicons did not obtain a successful sequencing, as well as the parameter scores not being larger this time, it was decided to not continue the *gyrB*-based prediction of the remaining isolates and keep their *rpoD*/16S rRNA identities.

Table 3.1: Comparison of *gyrB*, *rpoD* and 16S rRNA identification of four *Pseudomonas* spp. isolates. The E value is not presented as it was equal 0.0 in all cases. Identity predictions based on different genes are coloured differently.

Isolate ID	BLASTN-identification	Sequence ID	Query Cover	Percentage identity	Max score
LPJ835	16S rRNA-based: *P. gessardii*	MN069032.1	99%	99.91%	2023
	gyrB-based: P. fluorescence	AB039381.1	100%	97.12%	1583
LPJ863	*rpoD*-based: *P. extremaustralis*	LT629689.1	99%	96.21%	1162
	gyrB-based: *P. extremaustralis*	HE800479.1	98%	95.35%	821
LPJ896	*rpoD*-based: *P. extremaustralis*	LT629689.1	99%	96.88%	588
	gyrB-based: *P. extremaustralis*	HE800479.1	99%	96.34%	1480
LPJ899	*rpoD*-based: *P. fluorescens*	KY950266.1	99%	99.58%	1288
	gyrB-based: *P. iridis*	LR797690.1	100%	98.08%	905
	gyrB-based: *P. fluorescens*	AB039384.1	99%	98.88%	1279

3.3 PCR-based detection of antimicrobial resistance genes

Several PCR assays were applied to potentially detect ARGs among the 47 isolates collected from farmed salmon in a processing plant. Several genes were attempted to be amplified, being associated with florfenicol, ampicillin, tetracycline, sulphonamide or QACs/benzalkonium chloride resistance. All of the positive controls appeared as bands on the gel, implying all PCR runs had been successful. However, no bands appeared for any of the isolates in any of the runs, with one exception: a single band of approximately 2000 bp appeared for LPJ895 (only isolate being *P. veronii*) in the *qac*Δ*E1* run. The expected

amplicon size of this gene was however only 300 bp, which was obtained for the positive control. The amplicon was prepared and sent for external sequencing to see if further BLASTN analysis would identify it as a *qacΔE1* gene, however, no gene sequence was obtained. It was therefore concluded that none of the isolates possessed any of the tested ARGs.

3.4 Investigating phenotypic resistance patterns by disk diffusion assay

A disk diffusion assay was applied to test the phenotypic resistances of the 47 isolates, towards florfenicol, ampicillin and tetracycline (Figure 3.3). The isolates were classified as resistant or susceptible based on the inhibition zone diameter interpretive criteria presented in section 2.5.4. A list of all obtained inhibition zones and corresponding resistance patterns are presented in Appendix B.

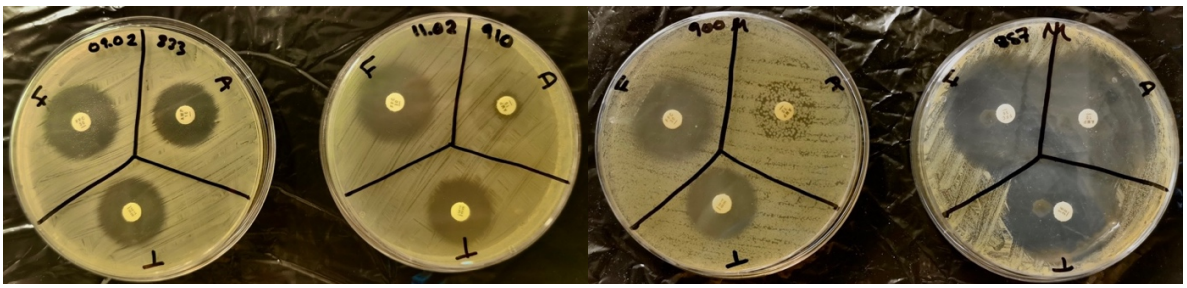


Figure 3.3: Illustration of different inhibition zone scenarios occurring on MHA plates during the disk diffusion test. On the agar plates, F, A and T corresponds to florfenicol, ampicillin and tetracycline, respectively. The first plate shows an isolate being susceptible to all tested antibiotics, while the second and the third plate shows ampicillin resistant isolates, and the fourth plate illustrates a highly susceptible isolate.

Of all tested bacteria, 42.7% were resistant to one or more of the tested antibiotics. The highest observed resistance was among *Aeromonas* spp. (represented by four isolates) as 100% were resistant to ampicillin. Among *Pseudomonas* spp. isolates, 43.5% exhibited resistance, while 30.0% of *Serratia* spp. were resistant (Figure 3.4). Among *Pseudomonas* spp., 30.0% were resistant to ampicillin, 10.0% to florfenicol and none were resistant to tetracycline. Among *Serratia* spp., 20.0% were resistant to ampicillin, 10.0% to tetracycline and 5.0% to florfenicol. Among all tested isolates, three were resistant to more than one

antibiotic tested (LPJ863: *P. extremaustralis,* LPJ882: *P.libanesis* and LPJ847: *S. liquefaciens*) as they exhibited resistance to both ampicillin and florfenicol. For the few cases of florfenicol resistance being observed, it was always accompanied by ampicillin resistance.

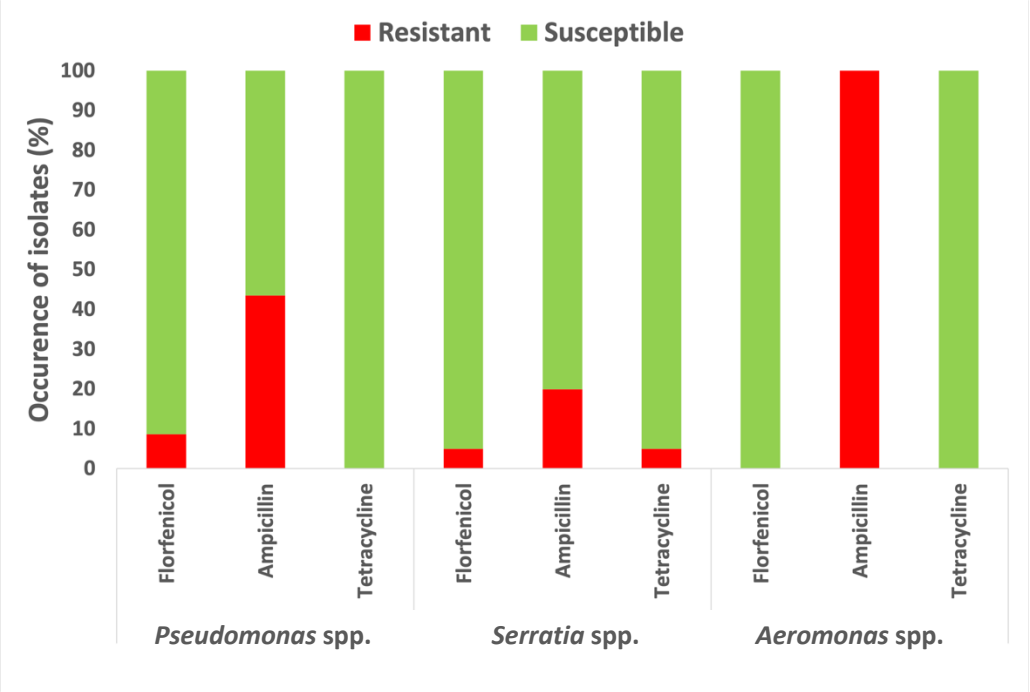


Figure 3.4: Antibiotic resistance patterns of florfenicol, ampicillin and tetracycline of the bacteria isolates at the level of genera. These were obtained by applying a disk diffusion assay performed for *Pseudomonas* spp., *Serratia* spp. and *Aeromonas* spp. isolated from Norwegian farmed salmon in a processing plant.

When observing the resistance patterns at the bacteria species level of the nine different species of *Pseudomonas* spp., the isolates exhibiting resistance were *P. veronii*, *P. lundensis*, *P. libanesis*, *P. extremaustralis* and *P. fluorescens* (Figure 3.5). All of these exhibited resistance towards ampicillin, while *P. libanesis* and *P. extremaustralis* were in addition resistant to florfenicol. Tetracycline resistance was only detected in one isolate of *S. liquefaciens* (LPJ847) and one isolate of *S. fonticola* (LPJ874). One-way ANOVA detected a significant difference ($P < 0.05$) in resistance at the generic level, and further Tukey post hoc test revealed that the ampicillin resistance pattern of *Aeromonas* spp. was significantly different from the patterns to *Pseudomonas* spp. and *Serratia* spp. isolates (Appendix E). In addition, the abundancy of resistance varied significantly ($P = 0.095$) between isolates being collected from either salmon skin and fillet.

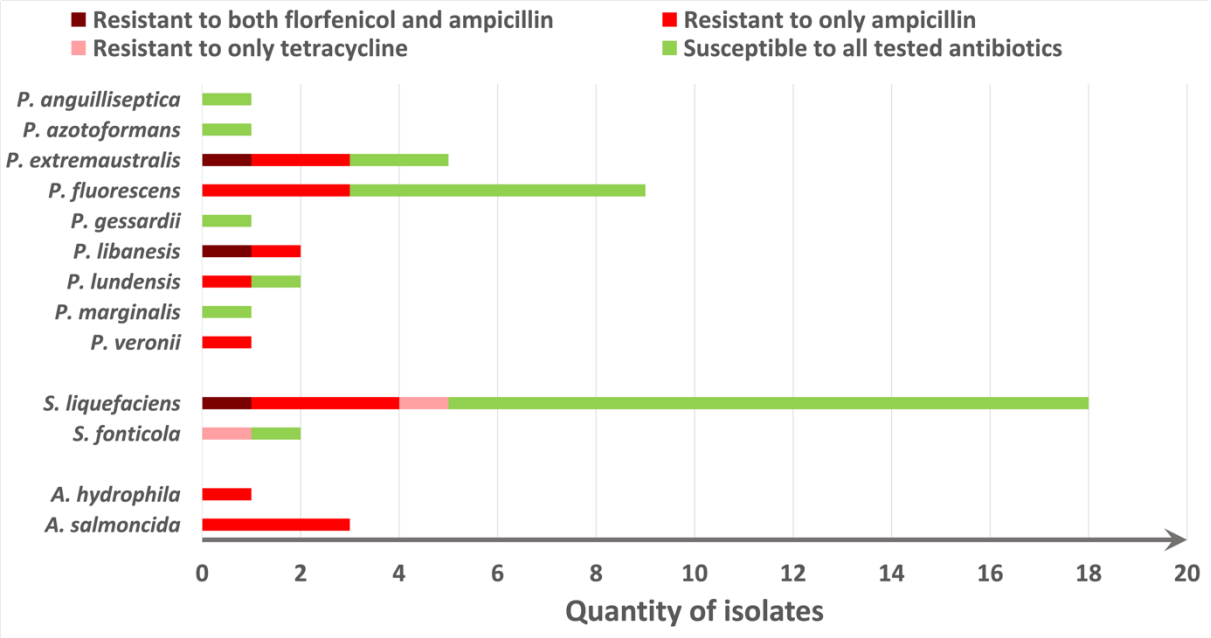


Figure 3.5: Overview of the different antibiotic resistance patterns at species level, achieved by a disk diffusion assay performed on bacteria isolates collected from Norwegian farmed salmon in a processing facility.

3.5 Biofilm capacity testing by microtiter biofilm assay

A microtiter biofilm assay was applied to test the biofilm formation capacity of the bacteria isolates at 12°C, using a 24 h biofilm incubation period. All *Pseudomonas* spp. and *Aeromonas* spp. were tested, while one of the 20 isolates of *Serratia* spp. was excluded for practical reasons (LPJ825: *S. liquefaciens*). All isolates had an acceptable cell number in the inoculum used for biofilm cultivation, as their log CFU/mL value was within 6.7 ± 1.5 (Appendix C.1). The isolates were classified as good or poor biofilm producers based on the selected criterium: $10\% \leq OD_{650,planktonic} - OD_{650,recovery} \leq 20\%$ (Appendix C.2). Two isolates did not fulfil the criterium and were thus excluded from the classification (LPJ823: *P. anguilliseptica* and LPJ895: *P. veronii*).

Eight of the 44 isolates being included in the analysis were classified as good biofilm producers, and all of these were *Pseudomonas* spp., except for one *Serratia* spp. isolate (Figure 3.6). Among the good biofilm producers of *Pseudomonas* spp., 42.9% were *P. fluorescens*, 28.6% were *P. libanesis*, 14.3% were *P. extremaustralis* and 14.3% were *P.*

marginalis (Figure 3.7). Among the *Serratia* spp. being good biofilm producers, only a single isolate of *S. fonticola* was represented.

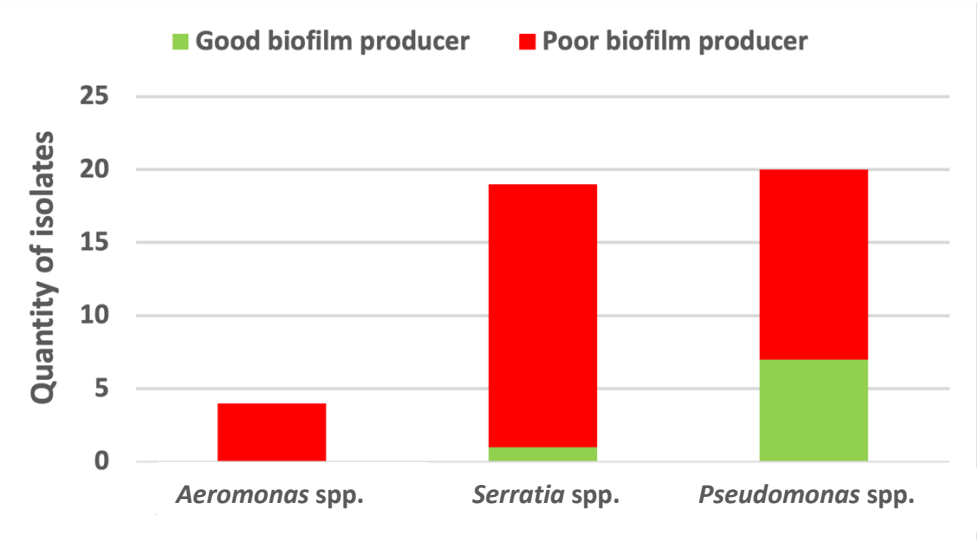


Figure 3.6: Biofilm formation capacity among the genera, when tested at 12 °C using a biofilm incubation period of 24 h. Isolates were classified as good or poor biofilm producer based on the difference in OD_{650} between planktonic- and recovery plate. Two isolates (*P. veronii and P. anguillispetica*) did not have sufficient growth, and one isolate (*S. liquefaciens*) was excluded from the testing.

Statistical analysis is SPSS detected a significant difference ($P < 0.05$) in the biofilm producing capacity between *Pseudomonas* spp., *Serratia* spp. and *Aeromonas* spp. at the generic level. *Pseudomonas* spp. had the highest abundance of good biofilm producers. Tukey post hoc analysis (excluding species being represented by less than two isolates) revealed that several isolates were significantly different from each other. Among these, *P. libanesis*, *S. fonticola* and *P. fluorescens* were highly different from *P. lundensis*, *S. liquefaciens* and *A. salmoncida*. Among the bacteria species, *P. fluorescens* had the highest abundance of good biofilm producers.

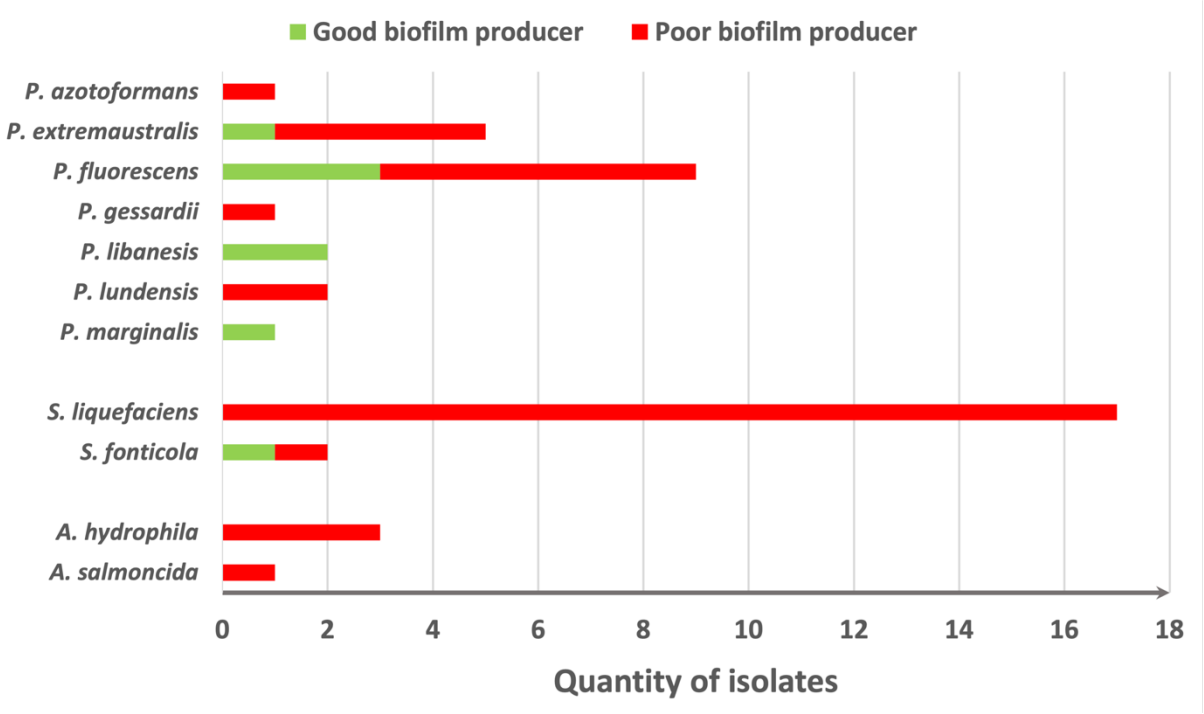


Figure 3.7: Biofilm formation capacity of the bacteria isolates at 12 °C, using a 24 h biofilm incubation period. Two isolates (*P. veronii and P. anguillispetica*) did not have sufficient growth and were thus excluded from the classification, and one isolate (*S. liquefaciens*) was excluded from the testing for practical reasons.

3.6 Testing antimicrobial efficiency by microtiter susceptibility assay

The antimicrobial efficiency of Aqua Des Foam PAA and florfenicol was tested towards the bacteria isolates using a microtiter susceptibility assay. Seven isolates were selected for the analysis, and these represented a variety of morphological appearances, antimicrobial resistance patterns and biofilm formation capacities (Table 3.2). A criterium for adequate growth of the growth controls was set to $OD_{650,24\ h}$ - $OD_{650,\ 0\ h} \geq 0.05$. Thus, a difference in OD_{650} larger or equal to 0.05 between the measurements performed before and after the 24 h incubation period would imply a sufficient growth. This led to one measurement point being excluded from MIC analysis and three excluded from MBC analysis. All OD_{650} differences of growth controls are presented in Appendix D.1.

Table 3.2: The seven isolates selected for antimicrobial susceptibility testing to Aqua Des Foam PAA and florfenicol. The indicated letters F, A and T, corresponds to florfenicol, ampicillin and tetracycline resistance, respectively. The three genera, as well as the different morphologies, are coloured differently.

Bacteria genus	Sample ID	Species ID	Morphology observations (when grown on TSA plates after 72 h incubation at 25°C)	Antibiotic resistance pattern	Ability to form biofilm (when using an incubation at 12°C for 24 h)
Pseudomonas spp.	LPJ841	*P. marginalis*	Dark beige. Firm/gel consistency.	-	Good
	LPJ863	*P. extremaustralis*	Light beige. Soft consistency.	F & A	Poor
	LPJ882	*P. libanesis*	Strong yellow. Firm/gel consistency.	F & A	Good
	LPJ895	*P. veronii*	Dark beige. Firm/gel consistency.	A	Good
	LPJ906	*P. fluorescens*	Light beige. Soft consistency.	A	Good
Serratia spp.	LPJ847	*S. liquefaciens*	Dark beige. Firm/gel consistency.	F & A	Poor
Aeromonas spp.	LPJ900	*A. hydrophila*	Light beige. Firm/gel consistency.	A	Poor

The OD_{650} measurements obtained from the MIC, MBC and MBEC analysis of Aqua Des Foam PAA and florfenicol was plotted to observe the differences among the tested isolates at the various antimicrobial concentrations (Figure 3.8 & 3.9). Sample standard deviations of each measurement point was also calculated. The actual MIC, MBC and MBEC values were determined as the lowest antimicrobial concentration achieving growth, using the set criteria for bacterial growth corresponding to $OD_{650} \geq 0.09$ (Table 3.3).

Log reductions of biofilm cells and corresponding standard deviations were calculated at each antimicrobial concentration level (Appendix D.2). The log reductions were then plotted to illustrate the variation in antimicrobial tolerance among the seven tested isolates (Figure 3.10).

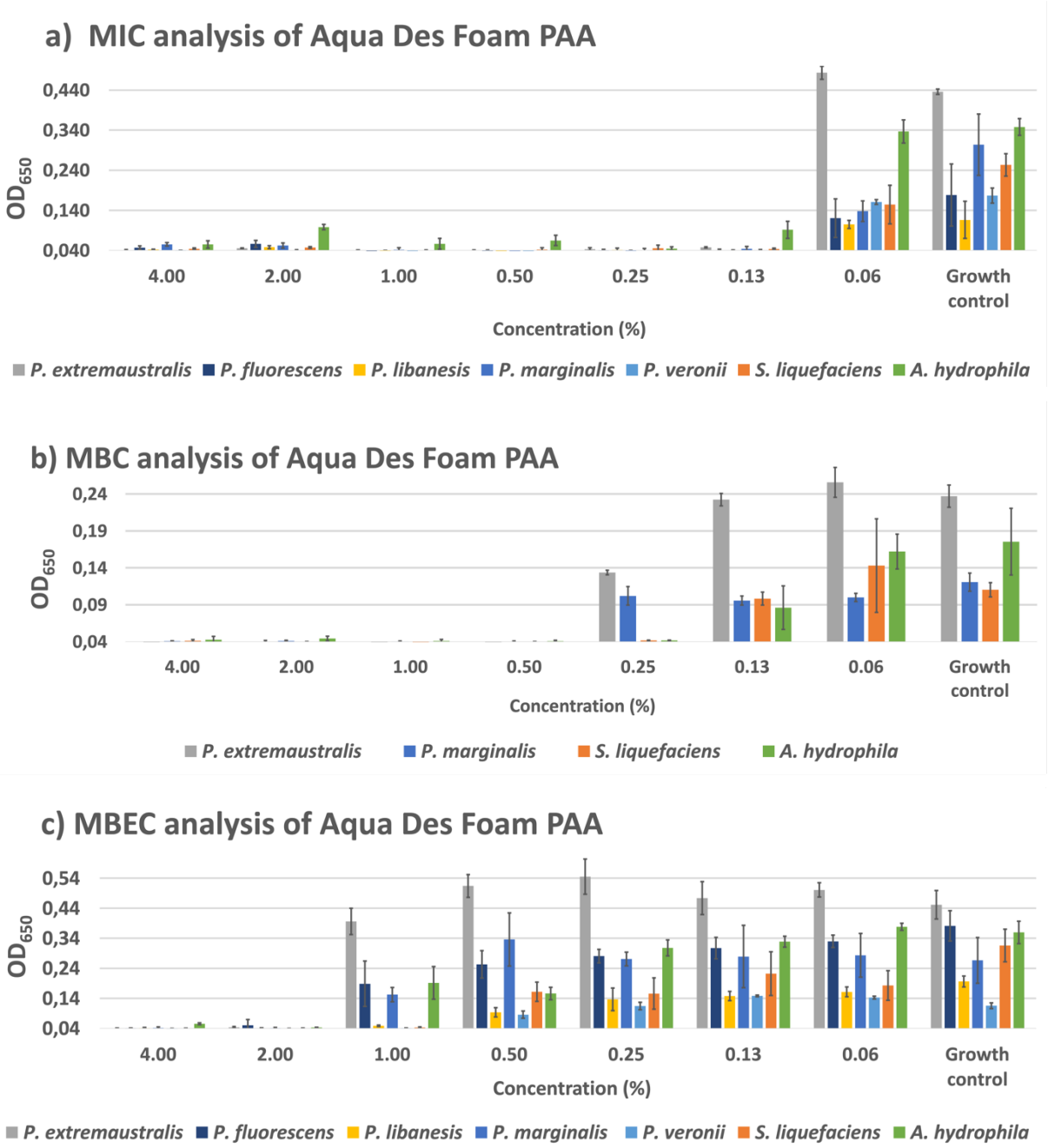


Figure 3.8: a) MIC, **b)** MBC and **c)** MBEC analysis of the disinfectant Aqua Des Foam PAA. A 17 min exposure time at room temperature was used. In the MBC analysis, *P. fluorecens*, *P. libanesis* and *P. veronii* were excluded from the MBC analysis, as the growth controls of these isolates did not have sufficient growth (OD_{650} < 0.09). The plots start at 0.04 as this was the OD_{650} value of sterility controls. Seven isolates are included: *P. extremaustralis* (LPJ863), *P. fluorescens* (LPJ906), *P. libanesis* (LPJ882), *P. marginalis* (LPJ841), *P. veronii* (LPJ895), *S. liquefaciens* (LPJ847) and *A. hydrophila* (LPJ900).

The Aqua Des Foam PAA susceptibility test was conducted using a 17 min exposure time at room temperature. At these conditions, the achieved MIC values ranged from 0.06-0.13%, MBC values from 0.06-0.25% and MBEC values from 0.25-1.00%. Among the seven tested isolates, the lowest tolerance was observed in *S. liquefaciens and P. veronii.*

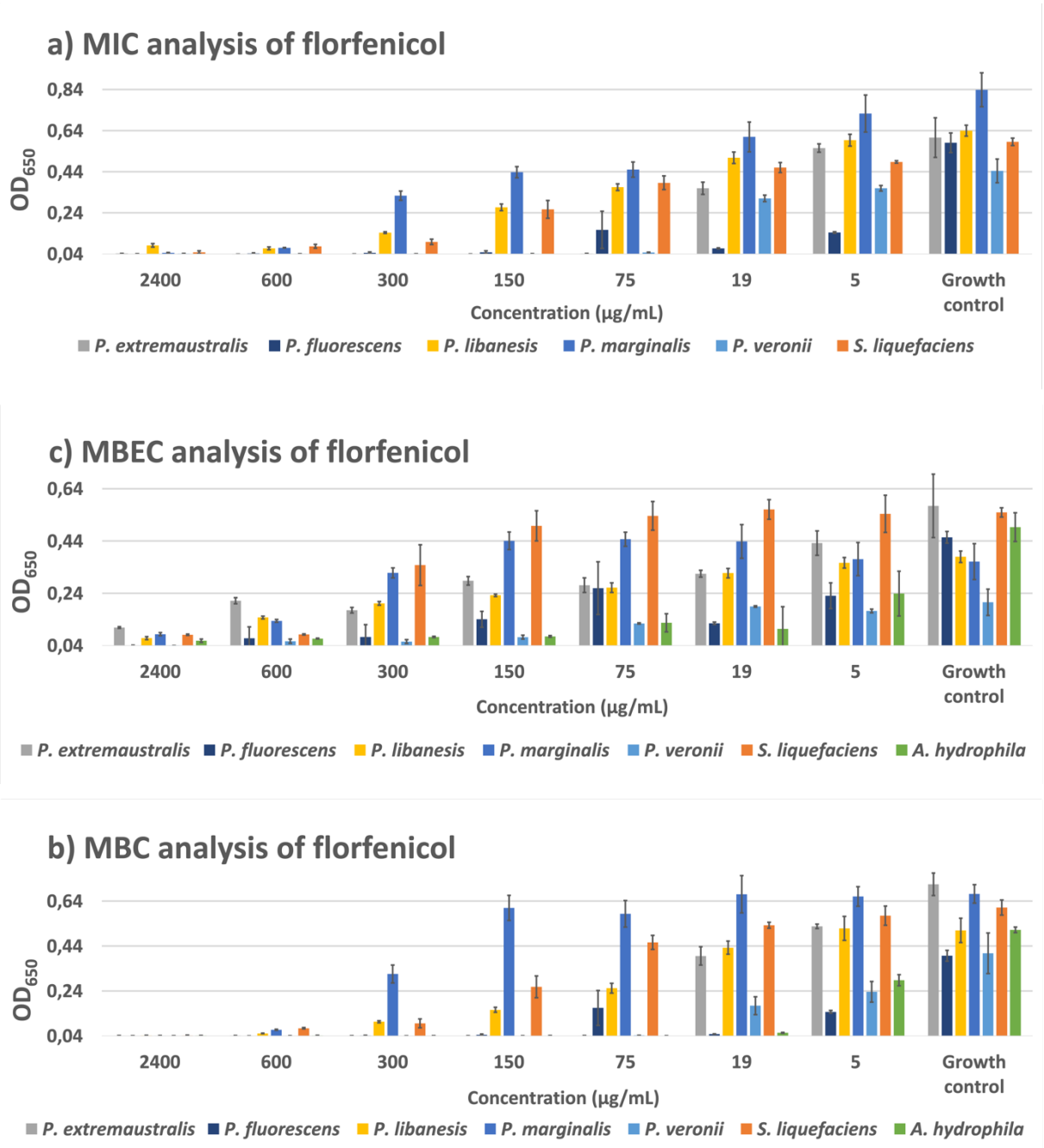


Figure 3.9: a) MIC, **b)** MBC and **c)** MBEC analysis of florfenicol, when applying an incubation period of 24 h at 12 °C. In the MIC analysis, *A. hydrophila* was excluded as the growth control of this isolate did not have sufficient growth (OD_{650} < 0.09). The plots start at 0.04 as this was the OD_{650} value of sterility controls. Seven isolates are included: *P. extremaustralis* (LPJ863), *P. fluorescens* (LPJ906), *P. libanesis* (LPJ882), *P. marginalis* (LPJ841), *P. veronii* (LPJ895), *S. liquefaciens* (LPJ847) and *A. hydrophila* (LPJ900).

The florfenicol susceptibility test was conducted using a 24 h exposure period at 12 °C. At these conditions, the achieved MIC values ranged from 19-300 mg/µL, MBC values from 19-600 mg/µL and MBEC values from 75 to >2400 mg/µL. Among the seven tested isolates, the highest tolerance was observed in *P. libanesis*, *P. marginalis* and *S. liquefaciens*.

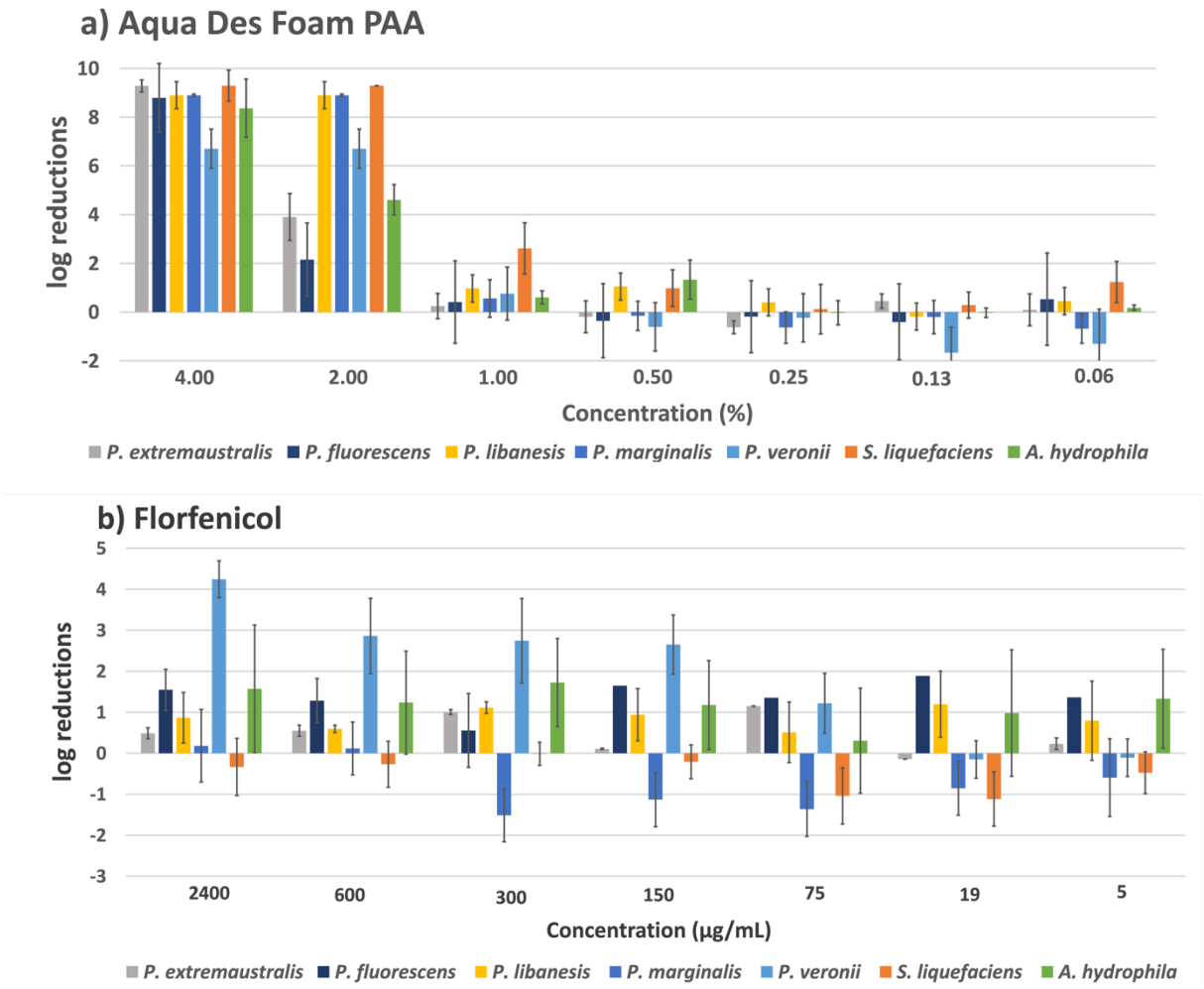


Figure 3.10: Log reduction plots for **a)** Aqua Des Foam PAA and **b**) florfenicol, illustrating the log reductions achieved by applying the disinfectant for 17 min at room temperature or applying florfenicol for 24 h at 12 °C, respectively. Seven isolates are included: *P. extremaustralis* (LPJ863), *P. fluorescens* (LPJ906), *P. libanesis* (LPJ882), *P. marginalis* (LPJ841), *P. veronii* (LPJ895), *S. liquefaciens* (LPJ847) and *A. hydrophila* (LPJ900).

When using an exposure time of 17 min at room temperature for the disinfectant Aqua Des Foam PAA at the 1% level of concentration, less than one log reduction was achieved for all isolates except *S. liquefaciens* having a 2.5-log reduction. When using a 2% concentration, the log reductions ranged from 2-9, while more than a 6-log reduction is achieved at 4% concentration. The plot of florfenicol log reductions reveals that less than a 1-log reduction is achieved for four isolates at the highest tested concentration (2400 mg/µL). Among the seven tested isolates, *P. marginalis* was the most susceptible to florfenicol, as more than a 1-log reduction was already achieved at 75 mg/µL. The lowest florfenicol concentration necessary to achieve a 1-log reduction and the lowest Aqua Des Foam PAA concentration achieving a 5-log reduction was determined (Table 3.3).

Table 3.3: Overview of the achieved MIC, MBC and MBEC values for the seven isolates, after being exposed to Aqua Des Foam PAA for 17 min at room temperature or florfenicol for 24 h at 12 °C. For the Aqua Des Foam PAA analysis, the disinfectant concentration necessary to achieve a 5-log reductions are presented, as well as the florfenicol concentration necessary to achieve a 1-log reduction. Four values are excluded due to insufficient growth of growth controls at these measurement points, these are denoted by (-). The three genera are coloured differently.

Bacteria	**Sample ID**	**Aqua Des Foam PAA (%)** **1.00% represents the usage concentration for disinfection in the salmon processing plant**				**Florfenicol (µg/mL)**			
		MIC	**MBC**	**MBEC**	**5-log reduction of biofilm cells**	**MIC**	**MBC**	**MBEC**	**1-log reduction of biofilm cells**
P. extremaustralis	LPJ863	**0.06**	**0.25**	**1.00**	**4.00**	**19**	**75**	**>2400**	**>2400**
P. fluorescens	LPJ906	**0.06**	**-**	**1.00**	**4.00**	**75**	**19**	**150**	**75**
P. libanesis	LPJ882	**0.06**	**-**	**0.50**	**2.00**	**300**	**600**	**600**	**300**
P. marginalis	LPJ841	**0.06**	**0.25**	**1.00**	**2.00**	**300**	**600**	**600**	**>2400**
P. veronii	LPJ895	**0.06**	**-**	**0.25**	**2.00**	**19**	**75**	**75**	**75**
S. liquefaciens	LPJ847	**0.06**	**0.13**	**0.50**	**2.00**	**300**	**600**	**300**	**>2400**
A. hydrophila	LPJ900	**0.13**	**0.13**	**1.00**	**4.00**	**-**	**19**	**75**	**75**

4. Discussion

4.1 Morphology and detection of ARGs

The appearance and consistency of the bacteria isolates collected from farmed salmon in a processing plant was observed on TSA plates, after an incubation at 25ºC for 72 h. No significant difference ($P < 0.05$) in morphology was detected among the genera, and morphological variation was also abundant within a species, as three different colour shades were observed in *S. liquefaciens* isolates. This is however not an uncommon observation, and this phenomenon is referred to as phenotypic variation among isogenic bacteria (Smits *et al.*, 2006).

All *P. fluorescens* isolates appeared light beige with a soft consistency, which corresponds to the characterizations by others as it has been stated that *Pseudomonas* spp. colonies have a white, cream or yellow colour (NHS, 2015). The most significant difference ($P < 0.05$) in morphology among the species irrespective of genera, was however observed between *P. fluorescens* and *P. libanesis.* The former had a light beige colour and soft consistency, while the latter appeared yellow with a firm/gel consistency. As the genus of *Pseudomonas* spp. is a large and complex heterogeneous group, the morphology can differ among the species (Madigan *et al.*, 2015). Most *Serratia* spp. appeared light beige, however, other studies have reported this genus as either white, pink or red in colour (Grimont & Grimont, 2015).

Whether the bacteria had been collected from salmon skin or fillet did not affect the observed morphology as no significant difference ($P < 0.05$) was detected. Thus, the morphology of the bacteria associated with farmed salmon seems to be more dependent on intrageneric species rather than genera and collection point. Morphology observations are however not appropriate for the identification of these bacterial species, at least when using this type of agar and these incubation conditions.

The presence of several ARGs was investigated: *floR*, *ampC*, *sul1*, *bcrABC, qacΔE1, qacH* and *tetG,* being associated with resistance to florfenicol, ampicillin, sulphonamide, benzalkonium chloride, QACs and tetracycline, respectively. The gel visualization did however reveal that no such genes were amplified by the PCR, which demonstrates that none of the isolates possessed any of these resistance genes. The tested genes have however been frequently detected in other studies concerning water- and aquaculture-associated bacteria.

The *sul1*, *tetG, qacΔE1* and *ampC* genes were detected in bacteria isolated from a drinking water treatment plant in China, and the high abundancy of the two former genes was explained by the long-term exposure to tetracycline and sulphonamide (Shi *et al.*, 2013). In studies concerning bacteria associated with Chilean salmon farms and nearby marine sediments, the *tetG, sul1* and *floR* genes were detected (Buschmann *et al.*, 2012). As the annual florfenicol usage in Chilean aquaculture is more than 90 times larger than in Norwegian aquaculture, the selective pressure to acquire resistance will be much smaller in bacteria associated with Norwegian salmon farming, which could explain the lack of these resistance genes in this master study (NORM-VET, 2019; Soto, 2020).

4.2 Antibiotic resistance patterns obtained by disk diffusion assay

Florfenicol is the dominating antibiotic in Norwegian aquaculture to treat salmon infections (NORM-VET, 2019). Therefore, if any of the isolates in this master study have acquired resistance caused by a long-term exposure to an antibiotic, it is likely going to be the acquisition of florfenicol resistance. However, florfenicol resistance was only observed in three of the 47 tested isolates: *P. extremaustralis* (LPJ863), *P. libanesis* (LPJ882) and *S. liquefaciens* (LPJ847). Florfenicol resistance was also only demonstrated in 10% of *Pseudomonas* spp., which is a very low percentage compared to observations in Chilean salmon farms where 100% of *Pseudomonas* spp. was resistant to florfenicol (Miranda & Rojas, 2007). The extensive usage of florfenicol in Chilean aquaculture compared to Norwegian aquaculture is likely the reason for these highly different observations.

The observed incidences of florfenicol resistance in this master study is clearly not caused by possession of the *floR* gene, despite this gene being a commonly reported mediator of florfenicol resistance in gram-negative bacteria associated with salmon aquaculture (Buschmann *et al.*, 2012; Miranda *et al.*, 2003). However, some studies have found the majority of florfenicol resistance among seafood-associated bacteria to be caused by non-specific efflux pump systems and undescribed florfenicol resistance genes (Fernández-Alarcón *et al.*, 2010; Tomova *et al.*, 2015).

Ampicillin resistance was the most abundant antibiotic resistance in all genera, as 36.2% of total isolates were ampicillin resistant. The highest occurrence was demonstrated in

Aeromonas spp., as all these four isolates exhibited ampicillin resistance. *Aeromonas* spp. isolated from seafood available on the Norwegian market, as well as from South African wastewater treatment plants, also had a 100% occurrence of ampicillin resistance (Lee *et al.*, 2021; Igbinosa & Okoh, 2012). Despite the usage of ampicillin in Norwegian aquaculture during past years, the resistance is more likely caused by intrinsic mechanisms rather than farming activity. It has been demonstrated that *Aeromonas* spp. are intrinsically resistant to ampicillin due to a natural possession of chromosomally-located genes encoding β-lactamases (Grave, 2006; Piotrowska & Popowska; 2014). *Aeromonas* spp. in natural aquatic environments with no ampicillin exposure have also been reported as resistant (Zdanowicz *et al*., 2020). Nevertheless, the demonstrated resistance in this master study is not caused by possession of the *ampC* gene.

The occurrence of ampicillin resistance in *Pseudomonas* spp. was 30%, which is low compared to *Pseudomonas* spp. associated with Chilean salmon industry as 90% were ampicillin resistant (Miranda & Rojas, 2007). Among *Serratia* spp. isolates in this master study, 20% were ampicillin resistant, which is also considerably low compared to *Serratia* spp. collected from seafood in Thailand as all tested *S. fonticola* isolates were reported as ampicillin resistant (Pongsilp & Nimnoi, 2018).

Among the tested antibiotics, tetracycline is the only one presently being used in both salmon aquaculture as well as the treatment of human infections. Tetracycline resistant bacteria among aquaculture-associated bacteria therefore pose a higher risk than those simply being resistant to antibiotics extensively used in aquaculture. Pathogens being resistant to healthcare antibiotics can, in the worst case scenario, lead to untreatable infections. Two of the 47 isolates were resistant to tetracycline: one isolate being *S. liquefaciens* and one being *S. fonticola*, where the former can act as an agent of human disease (Stock *et al*., 2003). No incidences of tetracycline resistance was observed among *Aeromonas* spp. and *Pseudomonas* spp. in this master study, although tetracycline resistance has been detected in these genera isolated from Indian fish as well as from Chilean salmon farms (Vivekanandhan *et al.*, 2002; Miranda & Rojas, 2007).

While the observed tetracycline resistance is not caused by possession of the *tetG* gene, it could possibly be caused by other genes in the *tet* family, as tetracycline resistance among bacteria in fish farms is mainly mediated by these genes (Roberts, 2005). In studies

concerning Chilean salmon farms, the majority of the tetracycline resistant bacteria possessed at least one *tet* gene: *tetA*, *tetB*, *tetE*, *tetH*, *tet34* or *tet35* (Miranda *et al.*, 2003). This Chilean study also reported cases of tetracycline resistance in gram-negative bacteria in fish farms with no recent antibiotic use, and this resistance was also not caused by the *tetG* gene. The detected presence of tetracycline resistance in this master study seems therefore more likely to be caused by intrinsic mechanisms than exposure to oxytetracycline by farming activity.

There was a significant difference ($P = 0.095$) in the occurrence of resistance among the two collection points, as 48.7% of isolates collected from salmon skin were resistant compared to only 14.3% of those collected from fillet. However, as only seven isolates were collected from fillet compared to 39 collected from skin, more isolates from fillet should be included in the study. The bacteria collected from fillet are likely to only originate from the interior of the processing plant, while the bacteria isolated from skin also originate from the external environment. A comparison of resistance occurrence among these could therefore be interesting, as slight residues of antibiotics are only present in the external farming environment.

Among the resistance patterns, only the ampicillin resistance pattern varied significantly among the genera ($P < 0.05$), as the pattern of *Aeromonas* spp. was significantly different from the other genera. However, the resistance pattern of florfenicol and tetracycline did not vary significantly ($P < 0.05$) among the genera. Nonetheless, most studies seem to conclude that the antibiotic resistance patterns among aquaculture-associated bacteria are independent of species type and taxonomic groups (Miranda & Zemelman, 2002a; Lee *et al.*, 2021).

4.3 Biofilm formation capacity and efficiency of antimicrobials

Pseudomonas spp. was clearly the most efficient biofilm producer at 12°C, as 33.0% were classified as good biofilm producers compared to only 5.3% of *Serratia* spp. and 0.0% of *Aeromonas* spp. The occurrence of good biofilm producers among *Pseudomonas* spp. was significantly higher ($P < 0.05$) compared to *Serratia* spp., and *P. fluorescens* was the dominating species as it accounted for 37.5% of total species being good biofilm producers. Studies concerning dairy processing plants also reported *P. fluorescens* as a good biofilm producer, and they also discovered that this is especially the case at low temperatures (10°C) (Rossi *et al.*, 2016). *Pseudomonas* spp. have also been recognized as good biofilm producers

in seafood industry (Rajkowski, 2009). However, the latter study also reported *Aeromonas* spp. as good biofilm producers, while none of *Aeromonas* spp. in this master study had good biofilm formation capacities, at least when studying biofilm formation at 12°C.

Among the *Serratia* spp. isolates being good biofilm producers, only a single isolate of *S. fonticola* was represented, thus, 95% of *Serratia* spp. were poor biofilm producers at 12°C. This finding is shared by another master study by Boyko, who investigated bacteria from the same salmon processing facility as this master study, and also reported this genus as a poor biofilm producer (Boyko, 2020). In general, these findings demonstrate that *Pseudomonas* spp., and particularly *P. fluorescens*, is of concern to the salmon industry, as they are good biofilm producers at the operating temperature in the salmon processing facility.

A microtiter susceptibility assay was performed to test the antibacterial efficiency of the disinfectant Aqua Des Foam PAA. For the disinfection routine in the processing plant (1% disinfectant concentration applied for 17 min), the MIC values of tested isolates ranged from 0.06-0.13%, MBC values from 0.06-0.25% and MBEC values from 0.25-1.00%. Accordingly, this disinfection procedure is very efficient at both inhibiting planktonic growth as well as killing planktonic cells of all tested bacteria, as well as eradicating biofilm cells. However, as the cut-off value for growth was set to $OD_{650} \geq 0.09$ (despite OD_{650} of sterility control wells were approximately 0.04), a certain level of living bacteria is likely present below this cut-off value. This was however a necessary criterium to account for the large uncertainty in OD_{650} measurements at low cell densities. The MBEC values were 4-17 times higher than the corresponding MBC values, demonstrating the fact that cells in biofilms possess an increased tolerance to antimicrobials compared to planktonic cells (Ciofu & Tolker-Nielsen, 2019).

The log reductions of biofilm cells achieved by this disinfection procedure are however less than one for all tested bacteria except one. When less than a 1-log reduction is achieved, this implies that more than 10% of biofilm cells will remain after disinfection. However, the producer of Aqua Des Foam PAA recommends using a 1.5-3% concentration for 5-15 min (AquaticChemistry, 2018). At 2% concentration, the log reductions of biofilm cells are increased to 2-9, while for 4% concentration they are further increased to 6-8. At high concentrations, *P. extremaustralis* and *P. fluorescens* are the ones exhibiting the strongest tolerance to the disinfectant. When it comes to the emergence of disinfectant resistant bacteria, it is mostly ineffective disinfection routines which cause a selective pressure for

acquisition of resistance genes among the surviving bacteria, as these are repeatedly exposed to the disinfectant (Møretrø *et al.*, 2017). Thus, to eradicate a higher percentage of biofilm cells using a 17 min exposure time, a higher disinfectant concentration should be applied.

In the florfenicol susceptibility testing, an exposure period of 24 h at 12°C was used. At these conditions, the achieved MIC values of tested bacteria ranged from 19 – 300 µg/mL, and MBEC values from 75 – 2400 µg/mL. Florfenicol resistant bacteria isolated from Chilean salmon farms achieved MIC values from 64 – 2048 µg/mL, and they classified the bacteria as resistant when MIC ≥ 512 µg/mL (Miranda & Zemelman, 2002b). When using this classification criterium, none of the tested isolates in this master study were florfenicol resistant in their planktonic state. Also, four of the seven isolates achieved MBEC values below this limit, suggesting that these are still more susceptible to florfenicol than the planktonic bacteria associated with Chilean salmon farming.

There was however some disagreement between the florfenicol resistant patterns obtained from the disk diffusion assay and the results from the microtiter susceptibility assay. Among the tested isolates, only *P. marginalis* was classified as susceptible to florfenicol in the disk diffusion test. Despite this observation, this isolate received MIC, MBC and MBEC values just as large as *S. liquefaciens* and *P. libanesis* which had been classified as resistant. Disagreement between results obtained from disk diffusion assay and microtiter susceptibility testing have been reported by others as well, and they concluded that disk diffusion testing had limitations (Edelmann *et al.*, 2007).

4.4 Noteworthy comments on applied experimental procedures

A prerequisite for using inhibition zone interpretive criteria during the disk diffusion assay is an incubation of MHA plates at 35 ± 2°C for 16 –18 h. However, as some of the tested species were psychrophiles and could not grow at high temperatures, the assay for these was conducted at 25°C instead. Thus, it is clearly necessary to develop interpretive criteria allowing lower incubation temperatures to meet the need for resistance testing of aquatic isolates.

In addition, 80% of isolates classified as resistant had a firm consistency, which was a significant difference ($P < 0.05$) from the occurrence of resistance among isolates having soft

consistency. It may be possible that bacteria having a firm consistency are in the biofilm state, as a characteristic of this state is the production of slimes, and this would surely affect the diameter of the inhibition zone. Studies investigating this topic have said that bacterial colonies growing on agar plates behave like planktonic cells "stranded" on a surface, while other studies have described the colonies of *P. aeruginosa* as air-exposed biofilms (Mikkelsen *et al*., 2007; Kolter & Greenberg; 2006). It is therefore not clear if the obtained resistance patterns of the isolates are reflecting the resistance of cells in the planktonic- or biofilm state.

The basis for classifying the bacteria as good/poor biofilm producers, as well as determining MIC, MBC and MBEC values, was the difference in cell density measured as OD_{650}. These measurements are however more uncertain at low absorbances. As a 24 h biofilm cultivation period was first applied, the incubation was prolonged to 48 h to achieve higher absorbance values and thus reduce the uncertainty. Another factor leading to unreliable OD_{650} values was the agglomeration of cells, as it seemed like the position of these particles within a well affected the read absorbance. Furthermore, morphological differences among the species in this master study exist, such as *P. fluorescens* being 0.5 µm longer than *S. liquefaciens*, and these differences will likely affect the read absorbance (Rafii *et al.*, 2014; Martínez-García *et al*., 2015). The MIC, MBC and MBEC plots certainly revealed that standard deviations were quite large at certain measurement points, and these would be reduced if more parallels per bacteria had been applied in the microtiter plates.

In addition, the obtained log CFU/mL values did not always correspond to their respective OD_{650} values. As the correlation coefficient of log CFU/mL versus OD_{650} data was 0.87, there was clearly no perfect correlation (Appendix C.2). This is however not just being caused by the uncertainty of OD_{650} measurements, but could also be explained by shortcomings in applying viable cell count in determinations of CFU/mL. Several bacteria, including *Pseudomonas* spp., will sometimes grow as individual units while other times they occur in pairs of two bacteria or even as short chains of several bacteria (Bennik, 1999). In cases of the latter, the clustered bacteria will appear as a single colony on the agar surface, leading to miscounting and a further CFU/mL value not reflecting the actual cell number.

Sonication was applied to dislodge the biofilms into the recovery media, which generated strong vibrations leading to cross-contamination between wells. Placing a plastic plate between the steel tray and the microtiter plate did however partially reduce the risk of cross-

contamination. The intensity of vibrations did also vary among the wells, and it is not certain if this affected the amount of biofilm being dislodged into the recovery media. Nevertheless, other studies have reported that sonication is producing an unexpectedly high variation in the ability to dislodge biofilms (Sandbakken *et al.*, 2020).

4.5 Suggestions for further research

Despite there having been a low occurrence of antimicrobial resistance in this study, it should not be taken for granted that it will remain low in the future, as high levels of multidrug resistance among bacteria isolated from seafood available on the Norwegian market have already been demonstrated (Lee *et al.*, 2021). A regular surveillance of the resistance patterns in bacteria associated with Norwegian seafood is needed to understand how the occurrence of resistance will evolve with time. To receive a more complete depiction of the abundancy of antibiotic resistance among the bacteria, several more antibiotics should be tested, including oxalinic acid as it is the second most dominating antibiotic in Norwegian aquaculture. Also, as bacteria being multidrug resistant to several antibiotics applied for human infections is a threat to public health, more antibiotics important in healthcare should be covered.

As the molecular mechanisms behind the observed cases of resistance were not illuminated, more ARGs should be tested. In addition, as some of the bacteria isolates can be potential human pathogens, genes encoding virulence factors could also be investigated to validate the potential health risk of consuming Norwegian farmed salmon. As the Aqua Des Foam PAA disinfection procedure used in salmon processing has a 10% survival rate of biofilm cells, the resistance to this commonly applied disinfectant should be monitored on a regular basis. This will reveal if there is an evolving resistance among the bacteria due to repeated exposure to this disinfectant.

5. Conclusion

The obtained antibiotic resistance patterns from disk diffusion assay, as well as the MIC- and MBC values, did generally demonstrate a low level of antibiotic resistance among the bacteria isolates collected from farmed salmon in a Norwegian processing plant. This becomes clear when comparing to other main seafood producer countries such as Chile, where the antibiotic usage in aquacultural farming is extensive. Incidences of all tested antibiotics (florfenicol, ampicillin, and tetracycline) were however detected, and ampicillin resistance had the highest abundance among isolates. It was especially present in *Aeromonas* spp. as all four isolates exhibited ampicillin resistance. The observed incidences of resistance are likely caused by intrinsic mechanisms rather than the exposure to antibiotics by Norwegian aquacultural farming. However, the resistance status should be monitored regularly, as other Norwegian studies have detected multidrug resistant bacteria in seafood.

The attempted detection of antimicrobial resistance genes revealed that none of the bacteria isolates possessed any of the tested genes: *floR*, *ampC*, *sul1*, *bcrABC, qacΔE1, qacH* and *tetG.* Thus, the phenotypically demonstrated cases of florfenicol, ampicillin and tetracycline resistance were not caused by the possession of the *floR*, *ampC* and *tetG* gene, respectively. Other undetected genes and associated mechanisms must be mediating these resistances. When it comes to biofilm producing capacity of the isolates at 12°C, the genus of *Pseudomonas* spp., and particularly *P. fluorescens*, was the most efficient biofilm producer. This genus is therefore of particular concern, as these species are good biofilm producers at the operating temperature of the salmon processing plant.

The Aqua Des Foam PAA disinfection procedure applied by the processing facility was sufficient at eradicating planktonic cells. However, the log reduction analysis revealed that more than 10% of biofilm cells would survive the disinfection. To avoid surviving bacteria potentially acquiring an increased tolerance to the disinfectant caused by the repeated exposure, the concentration should be increased. Morphology observations was not appropriate to distinguish between *Pseudomonas* spp., *Serratia* spp., and *Aeromonas* spp., as morphological differences were even observed between some isolates being the same bacterial species.

Literature and references

Agaras, B. C., & Valverde, C. (2018). A Novel Oligonucleotide Pair for Genotyping Members of the Pseudomonas Genus by Single-Round PCR Amplification of the gyrB Gene. *Methods and Protocols, 1*(3). doi:10.3390/mps1030024

AquaticChemistry. (2018). *Aqua Des Foam PAA*. Retrieved from: https://chemistry.aquatiq.com/n%C3%A6ringsmiddel/desinfeksjon/aqua-des-foam-paa-21-kg-ah-des-pe/H608B [Downloaded 05.06.21]

Bennik, M. H. J. (1999). Pseudomonas aeruginosa. *Encyclopedia of Food Microbiology,*1867-1871. doi:10.1006/rwfm.1999.1295.

Boss, R., Overesch, G., & Baumgartner, A. (2016). Antimicrobial Resistance of Escherichia coli, Enterococci, Pseudomonas aeruginosa, and Staphylococcus aureus from Raw Fish and Seafood Imported into Switzerland. *Journal of Food Protection, 79*(7), 1240-1246. doi:10.4315/0362-028X.JFP-15-463

Boyko, V. (2020). *Identifisering av, og biofilmegenskaper blant, Pseudomonas spp. fra et lakseslakteri - påvirkning if overlevelse av Listeria innocua.* (Master´s thesis). Norwegian University of Science and Technology (NTNU), Trondheim.

Bringsli, R. (2020). *rpoD & 16S rRNA-based identification of Pseudomonas spp. and other bacterial species in Norwegian Farmed Salmon.* (Specialization project). Norwegian University of Science and Technology (NTNU), Trondheim.

Burgos, A., Quindós, G., Martínez, R., Rojo, P., & Cisterna, R. (1990). In vitro susceptibility of Aeromonas caviae, Aeromonas hydrophila and Aeromonas sobria to fifteen antibacterial agents. *European Journal of Clinical Microbiology and Infectious Diseases, 9*(6), 413-417. doi:10.1007/BF01979472

Buschmann, A. H., Tomova, A., López, A., Maldonado, M. A., Henríquez, L. A., Ivanova, L., Moy, F., Godfrey, H. P., & Cabello, F. C. (2012). Salmon Aquaculture and Antimicrobial Resistance in the Marine Environment. *PloS one, 7*(8). doi:10.1371/journal.pone.0042724

Capkin, E., Terzi, E., & Altinok, I. (2015). Occurrence of antibiotic resistance genes in culturable bacteria isolated from Turkish trout farms and their local aquatic environment. *Diseases of Aquatiq Organsisms* 114(2), 127-137. doi:10.3354/dao02852

CDC. (2020). *Antibiotic Resistance, Food and Food Animals*. Centers for Disease Control and Prevention. Retrieved from: https://www.cdc.gov/foodsafety/challenges/antibiotic-resistance.html [Downloaded 05.06.21]

Chapman, J. S. (2003). Disinfectant resistance mechanisms, cross-resistance, and co-resistance. *International Biodeterioration & Biodegradation, 51*(4), 271-276. doi:10.1016/S0964-8305(03)00044-1

Chopra, I., & Roberts, M. (2001). Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiology and molecular biology reviews, 65*(2), 232-260. doi:10.1128/MMBR.65.2.232-260.2001

Ciofu, O., & Tolker-Nielsen, T. (2019). Tolerance and Resistance of Pseudomonas aeruginosa Biofilms to Antimicrobial Agents - How P. aeruginosa Can Escape Antibiotics. *Frontiers in Microbiology, 10*, 913. doi:10.3389/fmicb.2019.00913

CLSI. (2012). *Performance Standards for Antimicrobial Susceptibility Tests; Approved Standard* (11th ed.). *M02-A11* (32). Clinical and Laboratory Standards Institute.

CLSI. (2014). *Performance Standards for Antimicrobial Susceptibility; Testing; 24th Informational Supplement*, *M100-S24* (34). Clinical and Laboratory Standards Institute.

Cross, A. S. (2008). What is a virulence factor? *Critical care, 12*(6), 196-196. doi:10.1186/cc7127

Cunault, C., Faille, C., Briandet, R., Postollec, F., Desriac, N., & Benezech, T. (2018). Pseudomonas sp. biofilm development on fresh-cut food equipment surfaces – a growth curve – fitting approach to building a comprehensive tool for studying surface contamination dynamics. *Food and Bioproducts Processing, 107*, 70-87. doi:10.1016/j.fbp.2017.11.001

De Kievit, T. R. (2009). Quorum sensing in Pseudomonas aeruginosa biofilms. *Environmental Microbiology, 11*(2), 279-288. doi:10.1111/j.1462-2920.2008.01792.x

Doğruöz N., Göksay, D., Ilhan-Sungur, E., & Cotuk, A. (2009). Pioneer colonizer microorganisms in biofilm formation on galvanized steel in a simulated recirculating cooling-water system. *Journal of Basic Microbiology*. doi:10.1002/jobm.200800250

Donlan, R. M. (2002). Biofilms: Microbial Life on Surfaces. *Emerging Infectious Diseases Journal, 8*(9)*,* 881–890*.* doi:10.3201/eid0809.020063

Drenkard, E., & Ausubel, F. M. (2002). Pseudomonas biofilm formation and antibiotic resistance are linked to phenotypic variation. *Nature, 416*(6882), 740-743. doi:10.1038/416740a

Dunne, W. M., Jr. (2002). Bacterial adhesion: seen any good biofilms lately? *Clinical microbiology reviews, 15*(2), 155-166. doi:10.1128/cmr.15.2.155-166.2002

Edelmann, A., Pietzcker, T., & Wellinghausen, N. (2007). Comparison of direct disk diffusion and standard microtitre broth dilution susceptibility testing of blood culture isolates. *Journal of Medical Microbiology, 56*(2), 202-207. doi:10.1099/jmm.0.46937-0

Elhanafi, D., Dutta, V., & Kathariou, S. (2010). Genetic Characterization of Plasmid-Associated Benzalkonium Chloride Resistance Determinants in a Listeria monocytogenes Strain from the 1998-1999 Outbreak. *Applied and Environmental Microbiology, 76*(24), 8231. doi:10.1128/AEM.02056-10

Elias, S., & Banin, E. (2012). Multi-species biofilms: living with friendly neighbors. *FEMS Microbiology Reviews, 36*(5), 990-1004. doi:10.1111/j.1574-6976.2012.00325.x

Falk, N. A. (2019). Surfactants as Antimicrobials: A Brief Overview of Microbial Interfacial Chemistry and Surfactant Antimicrobial Activity. *Journal of surfactants and detergents, 22*(5), 1119-1127. doi:10.1002/jsde.12293

Fernández-Alarcón, C., Miranda, C. D., Singer, R. S., López, Y., Rojas, R., Bello, H., Domínguez, M., & González-Rocha, G. (2010). Detection of the *floR* Gene in a Diversity of Florfenicol Resistant Gram-Negative Bacilli from Freshwater Salmon Farms in Chile. *Zoonoses and Public Health*, 57,181-188. doi:10.1111/j.1863-2378.2009.01243.x

Galié, S., García-Gutiérrez, C., Miguélez, E. M., Villar, C. J., & Lombó, F. (2018). Biofilms in the Food Industry: Health Aspects and Control Methods. *Frontiers in Microbiology, 9*(898). doi:10.3389/fmicb.2018.00898

Giaourisa, E., Heir, E., Hébraudc, M., Chorianopoulos, N., Langsrud, S., & Møretrø, T. (2014). Attachment and biofilm formation by foodborne bacteria in meat processing environments: Causes, implications, role of bacterial interactions and control by alternative novel methods. *Meat Science, 97*(3), 298-309. doi:10.1016/j.meatsci.2013.05.023

Grave, K. (2006). Previous and current trends in the usage of antimicrobial drugs in Norwegian aquaculture. In: National Veterinary Institute*,* Norway.

Retriwed from: https://www.fhi.no/globalassets/dokumenterfiler/rapporter/arkivert-rapporter/previous-and-current-trends-in-the-usage-of-antimicrobial-drugs-in-norwegian-aquaculture.pdf [Downloaded 10.05.21]
Grimont, F., & Grimont, P. A. D. (2015). Serratia. *Bergey's Manual of Systematics of Archaea and Bacteria*, 1-22. doi:10.1002/9781118960608.gbm01167
Hall-Stoodley, L., Costerton, J. & Stoodley, P. (2004). Bacterial biofilms: from the Natural environment to infectious diseases, *Nature Reviews Microbiology, 2,* 95–108. doi: https://doi.org/10.1038/nrmicro821
Harrison, J. J., Stremick, C. A., Turner, R. J., Allan, N. D., Olson, M. E., & Ceri, H. (2010). Microtiter susceptibility testing of microbes growing on peg lids: a miniaturized biofilm model for high-throughput screening. *Nature Protocols, 5*, 1236–1254. doi:10.1038/nprot.2010.71
Hedayatianfard, K., Akhlaghi, M., & Sharifiyazdi, H. (2014). Detection of tetracycline resistance genes in bacteria isolated from fish farms using polymerase chain reaction. *Veterinary research forum: an international quarterly journal, 5*(4), 269-275. Retrieved from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4299992/ [Downloaded 01.06.21]
Igbinosa, I. H., & Okoh, A. I. (2012). Antibiotic susceptibility profile of Aeromonas species isolated from wastewater treatment plant. *The Scientific World Journal, 2012*, 764563-764563. doi:10.1100/2012/764563
Innovotech. (2015). MBEC ASSAY - For High-Throughput Antimicrobial Susceptibility Testing of Biofilms, Procedural Manual, Version 1.1.
ISFA. (2018). *Salmon Farming*. International Salmon Farmers Association. Retrieved from: https://sjomatnorge.no/wp-content/uploads/2018/06/ISFA-Report-2018-FINAL-FOR-WEB.pdf [Downloaded 25.05.21]
Jacobs, L., & Chenia, H. Y. (2007). Characterization of integrons and tetracycline resistance determinants in Aeromonas spp. isolated from South African aquaculture systems. *International Journal of Food Microbiology, 114*(3), 295-306. doi:10.1016/j.ijfoodmicro.2006.09.030
Jensen, P. M. (2021). *Norway growing more salmon for 2021*. In: Fishfarming expert. Retrieved from: https://www.fishfarmingexpert.com/article/norway-december-salmon-biomass-8-higher-year-on-year/ [Downloaded 01.06.21]
Jiang, X., Yu, T., Liang, Y., Ji, S., Guo, X., Ma, J., & Zhou, L. (2016). Efflux pump-mediated benzalkonium chloride resistance in Listeria monocytogenes isolated from retail food. *International Journal of Food Microbiology, 217*, 141-145. doi:10.1016/j.ijfoodmicro.2015.10.022
Joseph, M. C. A. (2005). Aeromonadalesord. nov. In :Bergey's Manual® of Systematic Bacteriology: Volume Two The Proteobacteria Part B The Gammaproteobacteria ed.. Boston, MA. doi:10.1007/0-387-28022-7_12
Karatan, E., & Watnick, P. (2009). Signals, Regulatory Networks, and Materials That Build and Break Bacterial Biofilms. *Microbiology and Molecular Biology Reviews, 73*(2), 310. doi:10.1128/MMBR.00041-08
Kerry, J., Hiney, M., Coyne, R., Cazabon, D., NicGabhainn, S., & Smith, P. (1994). Frequency and distribution of resistance to oxytetracycline in micro-organisms isolated from marine fish farm sediments following therapeutic use of oxytetracycline. *Aquaculture, 123*(1994)*,* 43-54. doi:10.1016/0044-8486(94)90118-X
Koehler, J. M., & Ashdown, L. R. (1993). In vitro susceptibilities of tropical strains of Aeromonas species from Queensland, Australia, to 22 antimicrobial agents. *Antimicrobial Agents and Chemotherapy, 37*(4), 905-907. doi:10.1128/aac.37.4.905

Kolter, R., Greenberg, E. (2006). The superficial life of microbes. *Nature, 441,* 300-302. doi:10.1038/441300a

Kumar, H., Franzetti, L., Kaushal, A., & Kumar, D. (2019). Pseudomonas fluorescens: a potential food spoiler and challenges and advances in its detection. *Annals of Microbiology, 69*, 873-883. doi:10.1007/s13213-019-01501-7

Kurz, C. L., Chauvet, S., Andrès, E., Aurouze, M., Vallet, I., Michel, G. P. F., Mitch, U., Celli, J., Filloux, A., de Bentzmann, S., Steinmetz, I., Hoffmann, J. A., Finlay, B. B., Gorvel, J.-P., Ferrandon, D., & Ewbank, J. J. (2003). Virulence factors of the human opportunistic pathogen Serratia marcescens identified by in vivo screening. *The EMBO journal, 22*(7), 1451-1460. doi:10.1093/emboj/cdg159

Langsrud, S., Sidhu, M. S., Heir, E., & Holck, A. L. (2003). Bacterial disinfectant resistance-a challenge for the food industry. *International Biodeterioration & Biodegradation, 51*(4), 283-290. doi:10.1016/S0964-8305(03)00039-8

Larsen, A. K. (2017). *Forebyggende arbeid for fremtidens fiskehelse*. University of Tromsø (UiT). Retrieved from: https://uit.no/nyheter/artikkel?p_document_id=512952&p_dim=88163 [Downloaded: 10.05.21]

Lee, H. J., Hoel, S., Lunestad, B. T., Lerfall, J., & Jakobsen, A. N. (2021). Aeromonas spp. isolated from ready-to-eat seafood on the Norwegian market: prevalence, putative virulence factors and antimicrobial resistance. *Applied Microbiology, 130*(4). doi:10.1111/jam.14865

Li, G.-L., Duo, L.-B., Luan, Y., Wang, C.-Y., Wang, W.-P., Zhang, H.-G., Sun, Q., & Qi, G.-Y. (2012). Identification of genotypes of plasmid-encoded AmpC beta-lactamases from clinical isolates and characterization of mutations in their promoter and attenuator regions. *Gene expression, 15*(5-6), 215-223. doi:10.3727/105221613x13571653093204

López, N. I., Pettinari, M. J., Stackebrandt, E., Tribelli, P. M., Põtter, M., Steinbüchel, A., & Méndez, B. S. (2009). Pseudomonas extremaustralis sp. nov., a Poly(3-hydroxybutyrate) producer isolated from an antarctic environment. *Current Microbiology, 59*(5), 514-519. doi:10.1007/s00284-009-9469-9

Lunestad, B. T., & Samuelsen, O. (2008). Veterinary drug use in aquaculture. *Improving Farmed Fish Quality and Safety*, 97-127. doi:10.1533/9781845694920.1.97

Madigan, M. T., Martinko, J. M., Bender, K., Buckley, D. H., & Stahl, D. A. (2015). *Brock Biology of Microorganisms* (14th ed.), 820-850. Upper Saddle River, Prentice Hall/Pearson Education.

Mahlen, S. D. (2011). Serratia Infections: from Military Experiments to Current Practice. *Clinical Microbiology Reviews, 24*(4), 755-791. doi:10.1128/CMR.00017-11

Martínez-García, P. M., Ruano-Rosa, D., Schilirò, E., Prieto, P., Ramos, C., Rodríguez-Palenzuela, P., & Mercado-Blanco, J. (2015). Complete genome sequence of Pseudomonas fluorescens strain PICF7, an indigenous root endophyte from olive (Olea europaea L.) and effective biocontrol agent against Verticillium dahliae. *Standards in genomic sciences*, *10*, 10. doi:10.1186/1944-3277-10-10

McIntosh, D. A., B. (1990). Recovery of an extremely proteolytic form of Serratia liquefaciens as a pathogen of Atlantic salmon, Salmo solar, in Scotland. *Journal of Fish Microbiology, 36*(5), 765-772. doi:10.1111/j.1095-8649.1990.tb04330.x

Mikkelsen, H., Duck, Z., Lilley, K. S., & Welch, M. (2007). Interrelationships between colonies, biofilms, and planktonic cells of Pseudomonas aeurginosa. *Journal of bacteriology*, *189*(6), 2411-2416. doi:10.1128/JB.01687-06

Miles, A. A., Misra, S. S., & Orwin, I. J. (1938). The estimation of bactericidal powder of the blood *Epidemiology & Infection, 38*(6), 732-749. doi:10.1017/S002217240001158x

Miranda C. D., Godoy, F. A., & Lee, M. R. (2018). Current Status of the Use of Antibiotics and the Antimicrobial Resistance in the Chilean Salmon Farms. *Frontiers in Microbiology, 9*(1284). doi:10.3389/fmicb.2018.01284

Miranda, C. D., Kehrenberg, C., Ulep, C., Schwarz, S., & Roberts, M. C. (2003). Diversity of tetracycline resistance genes in bacteria from Chilean salmon farms. *Antimicrobial agents and chemotherapy, 47*(3), 883-888. doi:10.1128/AAC.47.3.883-888.2003

Miranda, C. D., & Rojas, R. (2007). Occurence of florfenicol resistance in bacteria associated with two Chilean salmon farms with different history of antibacterial usage. *Aquaculture, 266*(1-4), 39-46. doi:10.1016/j.aquaculture.2007.02.007

Miranda, C. D., & Zemelman, R. (2002a). Antimicrobial multiresistance in bacteria isolated from freshwater Chilean salmon farms. *Science of The Total Environment, 293*(1), 207-218. doi:10.1016/S0048-9697(02)00022-0

Miranda, C. D., & Zemelman, R. (2002b). Bacterial resistance to oxytetracycline in Chilean salmon farming. *Aquaculture, 212*(1), 31-47. doi:10.1016/S0044-8486(02)00124-2

Mizan, M. F. R., Jahid, I. K., & Ha, S.-D. (2015). Microbial biofilms in seafood: A food-hygiene challenge. *Food Microbiology, 49*, 41-55. doi:10.1016/j.fm.2015.01.009

Munita, J. M., & Arias, C. A. (2016). Mechanisms of Antibiotic Resistance. *Microbiology spectrum, 4*(2). doi:10.1128/microbiolspec.VMBF-0016-2015

Munsch-Alatossava, P., Rita, H., & Alatossava, T. (2007). A faster and more economical alternative to the standard plate count (SPC) method for microbiological analyses of raw milks. *Communicating Current Research and Educational Topics and Trends in Applied Microbiology, 1, 395-499.*

Müller, A., Rychli, K., Muhterem-Uyar, M., Zaiser, A., Stessl, B., Guinane, C. M., Cotter, P. D., Wagner, M., & Schmitz-Esser, S. (2013). Tn6188 - A Novel Transposon in Listeria monocytogenes Responsible for Tolerance to Benzalkonium Chloride. *PloS one, 8*(10). doi:10.1371/journal.pone.0076835

Møretrø, T., Moen, B., Heir, E., Hansen, A. Å., & Langsrud, S. (2016). Contamination of salmon fillets and processing plants with spoilage bacteria. *International Journal of Food Microbiology, 237*, 98-108. doi: 10.1016/j.ijfoodmicro.2016.08.016

Møretrø, T., Schirmer, B. C. T., Heir, E., Fagerlund, A., Hjemli, P., & Langsrud, S. (2017). Tolerance to quaternary ammonium compound disinfectants may enhance growth of Listeria monocytogenes in the food industry. *International Journal of Food Microbiology, 241*, 215-224. doi:10.1016/j.ijfoodmicro.2016.10.025

NCBI. (2021). *PubChem Compound Summary for CID 6249, Ampicillin.* National Center for Biotechnology Information. Retrieved from: https://pubchem.ncbi.nlm.nih.gov/compound/Ampicillin [Downloaded 15.05.21]

NHS. (2015). Identification of Pseudomonas species and other Non-Glucose fermenters. *UK Standards for Microbiology Investigations*, *3.* National Health Service. Retrieved from:https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/422699/ID_17i3.pdf [Downloaded 15.06.21]

NIPH. (2017). *Food and waterborne diseases*. Norwegian Institute of Public Health. Retrieved from: https://www.fhi.no/en/op/hin/infectious-diseases/food-and-waterborne/ [Downladed 15.05.21]

NORM-VET. (2019). *Usage of Antimicrobial Agents and Occurence of Antimicrobial Resistance in Norway*. Norwegian Veterinary Institute. Retrieved from: https://www.vetinst.no/overvaking/antibiotikaresistens-norm-vet [Downladed 15.05.21]

NSC. (2021). *Norwegian salmon - the world's most popular fish.* Norwegian Seafood Council. Retrieved from:

https://www.mynewsdesk.com/seafood/pressreleases/norwegian-salmon-the-worlds-most-popular-fish-3102344 [Downladed 01.05.21]
Olsvik, Ø. (2019). *Norge fortsatt best i verden på lavt antibiotikabruk i oppdrett.* Forskersonen.no. Retrieved from:https://forskersonen.no/fisk-hav-og-fiske-kronikk/norge-fortsatt-best-i-verden-pa-lavt-antibiotikabruk-i-oppdrett/1575729 [Downladed 01.05.21]
Pang, Z., Raudonis, R., Glick, B. R., Lin, T.-J., & Cheng, Z. (2019). Antibiotic resistance in Pseudomonas aeruginosa: mechanisms and alternative therapeutic strategies. *Biotechnology Advances, 37*(1), 177-192. doi:10.1016/j.biotechadv.2018.11.013
Papenfort, K., & Bassier, B. L. (2016). Quorum sensing signal-response systems in Gram-negative bacteria. *Nature Reviews Microbiology, 14*. doi:10.1038/nrmicro.2016.89
Parker, J. L., & Shaw, J. G. (2011). Aeromonas spp. clinical microbiology and disease. *Journal of Infection, 62*(2), 109-118. doi:10.1016/j.jinf.2010.12.003
Passos da Silva, D., Schofield, M. C., Parsek, M. R., & Tseng, B. S. (2017). An Update on the Sociomicrobiology of Quorum Sensing in Gram-Negative Biofilm Development. *Pathogens, 6*(4), 51. doi:10.3390/pathogens6040051
Percival, S. L., & Williams, D. W. (2014). Aeromonas. *Microbiology of Waterborne Diseases (*2th ed.*)*, 49-64. London: Academic Press.
PeroxyChem. (2017). *Mechanisms of Peracetic Acid Inactivation of Microbes*. Disinfection forum. Retrived from: https://www.peroxychem.com/media/227023/DisinfectionForum_Feb2017Microbial-Inactivation.pdf [Downloaded 28.05.21]
Piotrowska, M., & Popowska, M. (2014). The prevalence of antibiotic resistance genes among *Aeromonas* species in aquatic environments. *Annals of Microbiology* 64**,** 921–934. doi:10.1007/s13213-014-0911-2
Pongsilp, N., & Nimnoi, P. (2018). Diversity and antibiotic resistance patterns of enterobacteria isolated from seafood in Thailand. *CyTA - Journal of Food, 16*(1), 793-800. doi:10.1080/19476337.2018.1479453
Poole, K. (2001). Multidrug efflux pumps and antimicrobial resistance in Pseudomonas aeruginosa and related organisms. *Journay of Molecular Microbiology and Biotechnology, 3*(2001), 255-264. PMID: 11321581.
Poole, K. (2002). Mechanisms of bacterial biocide and antibiotic resistance. *Journal of Applied Microbiology, 92*(1), 55-64. doi:10.1046/j.1365-2672.92.5s1.8.x
Preda, V. G., & Săndulescu, O. (2019). Communication is the key: biofilms, quorum sensing, formation and prevention. *Discoveries, 7.* doi:10.15190/d.2019.13
Rabin, N., Zheng, Y., Opoku-Temeng, C., Du, Y., Bonsu, E., & Sintim, H. O. (2015). Biofilm formation mechanisms and targets for developing antibiofilm agents. *Future Medicinal Chemistry, 7*(4), 493-512. doi:10.4155/fmc.15.6
Rafii, F. (2014). Serratia. *Encyclopedia of Food Microbiology,* 371-375. doi: 10.1016/B978-0-12-384730-0.00304-9
Rajkowski, K. T. (2009). Biofilms in fish processing. *Biofilms in the Food and Beverage Industries,* 499-516. doi:10.1533/9781845697167.4.499
Remuzgo-Martínez, S., Lázaro-Díez, M., Mayer, C., Aranzamendi-Zaldumbide, M., Padilla, D., Calvo, J., Marco, F., Martínez-Martínez, L., Icardo, J. M., Otero, A., & Ramos-Vivas, J. (2015). Biofilm Formation and Quorum-Sensing-Molecule Production by Clinical Isolates of Serratia liquefaciens. *Applied and environmental microbiology, 81*(10), 3306-3315. doi:10.1128/AEM.00088-15
Reiche, T. (2021). Phenotypic and Genotypic Characterization of Antimicrobial Resistance in Pseudomonas spp. isolated from the Salmon Industry. (Master´s thesis). Norwegian University of Science and Technology (NTNU), Trondheim.

Reygaert, W. C. (2018). An overview of the antimicrobial resistance mechanisms of bacteria. *AIMS microbiology, 4*(3), 482-501. doi:10.3934/microbiol.2018.3.482
Roberts, M. C. (2005). Update on acquired tetracycline resistance genes. *FEMS Microbiology Letters, 245*(2), 195-203. doi:10.1016/j.femsle.2005.02.034
Roberts, M. C., & Schwarz, S. (2016). Tetracycline and Phenicol Resistance Genes and Mechanisms: Importance for Agriculture, the Environment, and Humans. *Journal of Environmental Quality, 45*(2), 576-592. doi:10.2134/jeq2015.04.0207
Rodríguez-López, P., Rodríguez-Herrera, J. J., Vázquez-Sánchez, D., & López Cabo, M. (2018). Current Knowledge on Listeria monocytogenes Biofilms in Food-Related Environments: Incidence, Resistance to Biocides, Ecology and Biocontrol. *Foods, 7*(6), 85. doi:10.3390/foods7060085
Rossi, C., Chaves-López, C., Serio, A., Goffredo, E., Goga, B. T. C., & Paparella, A. (2016). Influence of Incubation Conditions on Biofilm Formation by Pseudomonas Fluorescens Isolated from Dairy Products and Dairy Manufacturing Plants. *Italian journal of food safety, 5*(3), 5793-5793. doi:10.4081/ijfs.2016.5793
Rubinstein, E., & Lagacé-Wiens, P. (2017). Quinolones. *Infectious Diseases (4th ed.),* 1239-1248. doi: 10.1016/B978-0-7020-6285-8.00144-1
Rådström, P., Swedberg, G., & Sköld, O. (1991). Genetic analyses of sulfonamide resistance and its dissemination in gram-negative bacteria illustrate new aspects of R plasmid evolution. *Antimicrobial agents and chemotherapy, 35*(9), 1840-1848. doi:10.1128/aac.35.9.1840
Sandbakken, E. T., Witsø, E., Sporsheim, B., Egeberg, K. W., Foss, O. A., Hoang, L., Bjerkan, G., Løseth, K., & Bergh, K. (2020). Highly variable effect of sonication to dislodge biofilm-embedded Staphylococcus epidermidis directly quantified by epifluorescence microscopy: an in vitro model study. *Journal of Orthopaedic Surgery and Research, 15*(1), 522. doi:10.1186/s13018-020-02052-3
Schar, D., Klein, E. Y., Laxminarayan, R., Gilbert, M., & Van Boeckel, T. P. (2020). Global trends in antimicrobial use in aquaculture. *Scientific Reports, 10*(21878). doi:10.1038/s41598-020-78849-3
Schwarz, S., Kehrenberg, C., Doublet, B., & Cloeckaert, A. (2004). Molecular basis of bacterial resistance to chloramphenicol and florfenicol. *FEMS Microbiology Reviews, 28*(5), 519-542. doi:10.1016/j.femsre.2004.04.001
SeaBOS. (2019). *Antibiotics in Aquaculture.* Retrieved from: https://seabos.org/wp-content/uploads/2019/12/Brief4-Antibiotics-in-Aquaculture.pdf [Downloaded 15.05.21]
Shah, S. Q. A., Cabello, F. C., L'Abée-Lund, T. M., Tomova, A., Godfrey, H. P., Buschmann, A. H., & Sørum, H. (2014). Antimicrobial resistance and antimicrobial resistance genes in marine bacteria from salmon aquaculture and non-aquaculture sites. *Environmental Microbiology, 16*(5), 1310-1320. doi:10.1111/1462-2920.12421
Shi, P., Jia, S., Zhang, X., Zhang, T., Cheng, S., & Li, A. (2013). Metagenomic insights into chlorination effects on microbial antibiotic resistance in drinking water. *Water Research, 47*(1), 111-120. doi:10.1016/j.watres.2012.09.046
Smits, W., Kuioers, O., & Veening, J. W. (2006). Phenotypic variation in bacteria: the role of feedback regulation. *Nature Reviews Microbiology*, *4*, 259-271. doi:10.1038/nrmicro1381
Soto, F. (2020). Chilean farmers cut antibiotic use in 2019. *Fishfarmingexpert*. Retrieved from: https://www.fishfarmingexpert.com/article/chilean-farmers-cut-antibiotic-use-in-2019/ [Downloaded 15.05.21]
Stock, I., Grueger, T., & Wiedemann, B. (2003). Natural antibiotic susceptibility of strains of Serratia marcescens and the S. liquefaciens complex: S. liquefaciens sensu stricto, S.

proteamaculans and S. grimesii. *International Journal of Antimicrobial Agents, 22*(1), 35-47. doi:10.1016/S0924-8579(02)00163-2
Tomova, A., Ivanova, L., Buschmann, A. H., Godfrey, H. P., & Cabello, F. C. (2018). Plasmid-mediated quinolone resistance (PMQR) genes and class 1 integrons in quinolone-resistant marine bacteria and clinical isolates of *Escherichia coli* from an aquacultural area. *Microbial Ecology,* 75, 104–112. doi:10.1007/s00248-017-1016-9
Toyofuku, M., Inaba, T., Kiyokawa , T., Obana, N., Yawata, Y., & Nomura, N. (2016). Environmental factors that shape biofilm formation. *Bioscience, Biotechnology, and Biochemistry, 80*(1), 7–12. doi:10.1080/09168451.2015.1058701
Vijayakumar, R., & Sandle, T. (2019). A review on biocide reduced susceptibility due to plasmid-borne antiseptic-resistant genes- special notes on pharmaceutical environmental isolates. *Journal of Applied Microbiology, 126*(4), 1011-1022. doi:10.1111/jam.14118
Vivekanandhan, G., Savithamani, K., Hatha, A. A. M., & Lakshmanaperumalsamy, P. (2002). Antibiotic resistance of Aeromonas hydrophila isolated from marketed fish and prawn of South India. *International Journal of Food Microbiology, 76*(1), 165-168. doi:10.1016/S0168-1605(02)00009-0
Walpole, R. E., Myers, R. H., Myers, S. H., & Ye, K. (2012). *Probability & Statistics for Engineers & Scientists* (9th ed), 507-540. Prentice Hall.
Wang, C., Cai, P., Guo, Y., & Mi, Z. (2007). Distribution of the antiseptic-resistance genes qacEΔ1 in 331 clinical isolates of Pseudomonas aeruginosa in China. *Journal of Hospital Infection, 66*(1), 93-95. doi:10.1016/j.jhin.2007.01.012
Wang, N., Yang, X., Jiao, S., Zhang, J., Ye, B., & Gao, S. (2014). Sulfonamide-resistant bacteria and their resistance genes in soils fertilized with manures from Jiangsu Province, Southeastern China. *PloS one, 9*(11). doi:10.1371/journal.pone.0112626
Wang, Z., Zhang, X., Huang, K., Miao, Y., Shi, P., Liu, B., Long, C., & Li, A. (2013). Metagenomic Profiling of Antibiotic Resistance Genes and Mobile Genetic Elements in a Tannery Wastewater Treatment Plant. *PloS one, 8*(10), doi:10.1371/journal.pone.0076079
Wassenaar, T., Ussery, D., Nielsen, L., & Ingmer, H. (2015). Review and phylogenetic analysis of qac genes that reduce susceptibility to quaternary ammonium compounds in Staphylococcus species. *European Journal of Microbiology and Immunology, 5*(1), 44-61. doi:10.1556/eujmi-d-14-00038
Watts, J. E. M., Schreier, H. J., Lanska, L., & Hale, M. S. (2017). The Rising Tide of Antimicrobial Resistance in Aquaculture: Sources, Sinks and Solutions. *Marine Drugs, 15*(6), 158. doi:10.3390/md15060158
Webber, M. A., & Piddock, L. J. V. (2003). The importance of efflux pumps in bacterial antibiotic resistance. *Journal of Antimicrobial Chemotherapy, 51*(1), 9-11. doi:10.1093/jac/dkg050
WHO. (2011). *Tackling antibiotic resistance from a food safety perspective in Europe*. World Health Organization. Retrieved from: https://www.euro.who.int/__data/assets/pdf_file/0005/136454/e94889.pdf [Downloaded 18.05.21]
WHO. (2020). *Antibiotic resistance*. World Health Organization. Retrieved from: https://www.who.int/news-room/fact-sheets/detail/antibiotic-resistance [Downloaded 18.05.21]
Wilson, C., Lukowicz, R., Merchant, S., Valquier-Flynn, H., Caballero, J., Sandoval, J., Okuom, M., Huber, C., Brooks, T. D., Wilson, E., Clement, B., Wentworth, C. D., & Holmes, A. E. (2017). Quantitative and Qualitative Assessment Methods for Biofilm

Growth: A Mini-review. *Journal of engineering and technology, 6*(4), Retrieved from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6133255/ [Downloaded 24.05.21]

Yamaguchi, A., Ono, N., Akasaka, T., Noumi, T., & Sawai, T. (1990). Metal-tetracycline/H+ antiporter of Escherichia coli encoded by a transposon, Tn10. *The Journal of biological chemistry, 265*, 15525-15530. PMID: 2168416.

Zdanowicz, M., Mudryk, Z. J. & Perliński, P. (2020). Abundance and antibiotic resistance of *Aeromonas* isolated from the water of three carp ponds. *Veterinary Research Communications,* 44**,** 9-18. doi:10.1007/s11259-020-09768-x

Zhao, Q., Wang, Y., Wang, S., Wang, Z., Du, X.-d., Jiang, H., Xia, X., Shen, Z., Ding, S., Wu., C., Zhou, B., Wu, Y., & Shen, J. (2016). Prevalence and Abundance of Florfenicol and Linezolid Resistance Genes in Soils Adjacent to Swine Feedlots. *Scientific Reports, 6*(1), 32192. doi:10.1038/srep32192

Zoellner, C., Aguayo-Acosta, A., Siddiqui, M. W., & Dávila-Aviña, J. E. (2018). Peracetic Acid in Disinfection of Fruits and Vegetables. Postharvest Disinfection of Fruits and Vegetables, 53-66. doi:10.1016/B978-0-12-812698-1.00002-9

Appendix

Appendix A: Overview of morphology and characteristics

The overall results from morphology observations, disk diffusion assay and microtiter biofilm assay are presented in Table 6.1, as well as their BLASTN-predicted identity (*rpoD*/16S rRNA-based). As the bacteria had been isolated from either skin, fillet or gills of farmed salmon in a processing plant, their collection points are also presented.

Table 6.1: Overall results from morphology observations, disk diffusion- and microtiter biofilm assay. The collection point of the isolates is given by letters and numbers: "S" corresponds to isolates collected from salmon skin, "F" to fillet, and "G" to gills. The indicated number corresponds to which of the five distinct salmon the isolates were collected from. For example, F3 corresponds to an isolate collected from the filet of salmon nr.3. Species ID refers to the species identity of the isolates, predicted from BLASTN-analysis of *16S rRNA* or *rpoD* gene sequences. The colour and consistency, as well as resistance patterns are presented. The indicated letter (F, A & T) corresponds to resistance toward florfenicol, ampicillin and tetracycline, respectively. The three different genera are coloured differently.

Genus ID	Species ID	Sample ID	Collection point	Observations of appearance and consistency when grown on TSA plates after 72 h incubation at 25°C	Resistance pattern	Ability to form biofilm at 12°C, using 24 h incubation period
Pseudomonas spp.	*P. anguilliseptica*	LPJ823	G2	Light brown. Soft consistency.	-	-
	P. azotoformans	LPJ844	S3	Light beige. Soft consistency.	-	Poor
	P. extremaustralis	LPJ846	F2	Light beige. Soft consistency.	-	Poor
	P. extremaustralis	LPJ863	F2	Light beige. Soft consistency.	F & A	Poor
	P. extremaustralis	LPJ870	S1	Light beige. Soft consistency.	-	Poor
	P. extremaustralis	LPJ896	S2	Light brown. Firm/gel consistency.	A	Good
	P. extremaustralis	LPJ910	S2	Light beige. Firm/gel consistency.	A	Poor
	P. fluorescens	LPJ836	S1	Light beige. Soft consistency.	-	Poor
	P. fluorescens	LPJ838	F2	Light beige. Soft consistency.	-	Poor
	P. fluorescens	LPJ839	F2	Light beige. Soft consistency.	-	Poor
	P. fluorescens	LPJ867	F5	Light beige. Soft consistency.	-	Poor
	P. fluorescens	LPJ880	S5	Light beige. Soft consistency.	A	Good
	P. fluorescens	LPJ881	S2	Light beige. Soft consistency.	A	Good
	P. fluorescens	LPJ883	S2	Light beige. Soft consistency.	-	Poor
	P. fluorescens	LPJ899	S2	Light beige. Soft consistency.	-	Poor
	P. fluorescens	LPJ906	S2	Light beige. Soft consistency.	A	Good
	P. gessardii	LPJ835	S1	Light beige. Soft consistency.	-	Poor
	P. libanesis	LPJ882	S5	Strong yellow. Firm/gel consistency.	F & A	Good
	P. libanesis	LPJ888	S5	Pale yellow. Firm/gel consistency.	A	Good
	P. lundensis	LPJ833	F2	Dark beige. Soft consistency.	-	Poor
	P. lundensis	LPJ905	S2	Pale yellow. Firm/gel consistency.	A	Poor
	P. marginalis	LPJ841	F2	Dark beige. Firm/gel consistency.	-	Good
	P. veronii	LPJ895	S2	Dark beige. Firm/gel consistency.	A	-
Serratia spp.	*S. fonticola*	LPJ874	S4	Light beige. Soft consistency.	T	Poor
	S. fonticola	LPJ892	S5	Yellow beige. Firm/gel consistency.	-	Good
	S. liquefaciens	LPJ827	S2	Light beige. Soft consistency.	-	Poor
	S. liquefaciens	LPJ828	S2	Light beige. Soft consistency.	-	Poor
	S. liquefaciens	LPJ829	S2	Light beige. Soft consistency.	-	Poor
	S. liquefaciens	LPJ832	S3	Light beige. Soft consistency.	-	Poor
	S. liquefaciens	LPJ847	S3	Dark beige. Firm/gel consistency.	F & A	Poor
	S. liquefaciens	LPJ849	S3	Light beige. Soft consistency.	A	Poor
	S. liquefaciens	LPJ851	S3	Dark beige. Soft consistency.	A	Poor
	S. liquefaciens	LPJ856	S3	Light beige. Soft consistency.	A	Poor
	S. liquefaciens	LPJ857	S4	Light beige. Soft consistency.	-	Poor
	S. liquefaciens	LPJ861	S4	Dark beige. Soft consistency.	-	Poor
	S. liquefaciens	LPJ862	S4	Light beige. Soft consistency.	-	Poor
	S. liquefaciens	LPJ869	S4	Dark beige. Soft consistency.	-	Poor
	S. liquefaciens	LPJ873	S4	Yellow beige. Soft consistency.	-	Poor

	S. liquefaciens	LPJ826	S2	Light beige. Soft consistency.	-	Poor
	S. liquefaciens	LPJ876	S4	Light beige. Soft consistency.	T	Poor
	S. liquefaciens	LPJ878	S5	Light beige. Soft consistency.	-	Poor
	S. liquefaciens	LPJ891	S5	Light beige. Soft consistency.	-	Poor
	S. liquefaciens	LPJ825	S2	Light beige. Soft consistency.	-	Not tested
Aeromonas spp.	*A. hydrophila*	LPJ868	S1	Light beige. Firm consistency.	A	Poor
	A. salmoncida	LPJ886	S2	Dark beige. Soft consistency.	A	Poor
	A. salmoncida	LPJ897	S5	Light beige. Soft consistency.	A	Poor
	A. salmoncida	LPJ900	S5	Light beige. Soft consistency.	A	Poor

Appendix B: Inhibition zone diameters

A disk diffusion assay was applied to investigate the antibiotic resistance pattern of the isolates towards florfenicol, ampicillin and tetracycline. The measured inhibition zone diameters are presented in Table 6.2.

Table 6.2: Inhibition zone diameters of all isolates achieved by disk diffusion assay. The antibiotics tested were disks of 30 µg florfenicol, 10 µg ampicillin and 30 µg tetracycline. Resistant isolates are marked in red, while green represents isolates being susceptible. The classification of isolates as resistant or susceptible were done in accordance with inhibition zone diameter interpretive criteria given in Table 2.6. The three different genera are coloured differently.

Genus ID	Sample ID	Origin	Species ID	Inhibition zone diameter (mm)		
				Florfenicol	Ampicillin	Tetracycline
Pseudomonas spp.	LPJ823	G2	*P. anguilliseptica*	29	24	20
	LPJ844	S3	*P. azotoformans*	23	17	22
	LPJ846	F2	*P. extremaustralis*	26	22	23
	LPJ863	F2	*P. extremaustralis*	10	5	41
	LPJ870	S1	*P. extremaustralis*	22	20	21
	LPJ896	S2	*P. extremaustralis*	51	5	41
	LPJ910	S2	*P. extremaustralis*	25	10	20
	LPJ838	F2	*P. fluorescens*	25	19	23
	LPJ839	F2	*P. fluorescens*	26	21	23
	LPJ867	F5	*P. fluorescens*	28	21	24
	LPJ883	S2	*P. fluorescens*	25	20	21
	LPJ899	S2	*P. fluorescens*	27	21	23
	LPJ880	S5	*P. fluorescens*	26	11	23
	LPJ881	S2	*P. fluorescens*	27	10	22
	LPJ906	S2	*P. fluorescens*	21	5	27
	LPJ836	S1	*P. fluorescens*	25	21	23
	LPJ835	S1	*P. gessardii*	26	21	24
	LPJ888	S5	*P. libanesis*	22	5	49
	LPJ882	S5	*P. libanesis*	6	5	35
	LPJ905	S2	*P. lundensis*	27	10	22
	LPJ833	F2	*P. lundensis*	25	21	22
	LPJ841	F2	*P. marginalis*	25	21	21
	LPJ895	S2	*P. veronii*	27	5	28
Serratia spp.	LPJ874	S4	*S. fonticola*	28	25	10
	LPJ892	S5	*S. fonticola*	28	21	18
	LPJ825	S2	*S. liquefaciens*	24	23	19
	LPJ891	S5	*S. liquefaciens*	28	21	18
	LPJ826	S2	*S. liquefaciens*	21	21	17
	LPJ827	S2	*S. liquefaciens*	21	21	17
	LPJ828	S2	*S. liquefaciens*	24	23	20
	LPJ829	S2	*S. liquefaciens*	27	21	21
	LPJ832	S3	*S. liquefaciens*	24	21	18
	LPJ847	S3	*S. liquefaciens*	14	5	28
	LPJ849	S3	*S. liquefaciens*	19	5	28
	LPJ851	S3	*S. liquefaciens*	19	5	28
	LPJ856	S3	*S. liquefaciens*	18	5	31
	LPJ857	S4	*S. liquefaciens*	18	46	31
	LPJ861	S4	*S. liquefaciens*	29	24	21
	LPJ862	S4	*S. liquefaciens*	29	24	21
	LPJ869	S4	*S. liquefaciens*	28	23	22
	LPJ873	S4	*S. liquefaciens*	24	23	20
	LPJ878	S5	*S. liquefaciens*	27	22	21
	LPJ876	S4	*S. liquefaciens*	26	22	10
Aeromonas spp.	LPJ900	S5	*A. hydrophila*	29	5	22
	LPJ886	S2	*A. salmoncida*	35	5	36
	LPJ868	S1	*A. salmoncida*	37	5	36
	LPJ897	S5	*A. salmoncida*	38	5	32

Appendix C: Biofilm capacity testing

C.1 Calculations of CFU/mL in inoculums used for biofilm cultivation

C.1.1 Relevant formulas to calculate CFU/mL

The biofilm formation capacity of the bacteria isolates was tested using a microtiter biofilm assay, using a biofilm incubation at 12ºC for 24 h. Standardization of the bacteria suspensions was performed to achieve a cell number of inoculum used for biofilm cultivation of approximately $5.0 * 10^6$, corresponding to a log CFU/mL value of 6.70. Three parallels per dilution of each isolate inoculum were plated out and counted after the incubation, to further calculate log CFU/mL by using relevant formulas (Innovotech, 2015; Walpole *et al.*, 2012). This presented calculation procedure was performed for all bacteria isolates.

The log CFU/mL value of each bacteria inoculum parallel was first calculated as following:

$$\log\left(\frac{\text{CFU}}{\text{mL}}\right)_{\substack{\text{ISOLATE i}\\ \text{PARALLEL i}}} = \log\left(\frac{\text{sum of counted colonies in all microspots}}{\text{sum of dilution factors}}\right)$$

Once log CFU/mL values were calculated for all three parallels, the mean CFU/mL value was calculated:

$$\log\left(\frac{\text{CFU}}{\text{mL}}\right)_{\substack{\text{ISOLATE i}\\ \text{MEAN}}} = \frac{\log\left(\frac{\text{CFU}}{\text{mL}}\right)_{\substack{\text{ISOLATE i}\\ \text{PARALELL 1}}} + \log\left(\frac{\text{CFU}}{\text{mL}}\right)_{\substack{\text{ISOLATE i}\\ \text{PARALELL 2}}} + \log\left(\frac{\text{CFU}}{\text{mL}}\right)_{\substack{\text{ISOLATE i}\\ \text{PARALELL 3}}}}{3}$$

And the sample standard deviation, σ, of the log CFU/mL mean value:

$$\sigma = \sqrt{\frac{\sum\left(\log\left(\frac{\text{CFU}}{\text{mL}}\right)_{\substack{\text{ISOLATE i}\\ \text{PARALLEL i}}} - \log\left(\frac{\text{CFU}}{\text{mL}}\right)_{\substack{\text{ISOLATE i}\\ \text{MEAN}}}\right)^2}{N-1}}$$

Where N is the number of parallels, thus N = 3.

C.1.2 Example calculation

An example calculation is provided, using the viable cell counts of isolate LPJ823. In the first well of the dilution plate, 10 uL of inoculum was added to 90 uL of saline solution, and further 10 uL of diluted inoculum was plated out on the agar. Thus, the final dilution factors ranged from -3 to -10.

Table 6.3: Counted colonies for isolate LPJ823. Spots having an uncountable colony number are denoted UC.

LPJ823	Dilution factors							
Parallel	-3	-4	-5	-6	-7	-8	-9	-10
1	UC	UC	UC	27	2	0	0	0
2	UC	UC	UC	19	0	0	0	0
3	UC	UC	UC	23	1	0	0	0

First, the log CFU/mL values was calculated for each parallel:

$$\log\left(\frac{\text{CFU}}{\text{mL}}\right)_{\substack{\text{LPJ823}\\ \text{PARALLEL1}}} = \log\left(\frac{\text{sum of all counted colonies}}{\text{dilution factors}}\right) = \log\left(\frac{27+2}{10^{-6}+10^{-7}}\right) = 7.46$$

This gave a log CFU/mL value of 7.46 for the first parallel, 7.28 for the second parallel and 7.38 for the third parallel. Thus, the mean log CFU/mL value for this isolate was:

$$\log\left(\frac{\text{CFU}}{\text{mL}}\right)_{\substack{\text{LPJ823}\\ \text{MEAN}}} = \frac{7.46+7.28+7.38}{3} = 7.37$$

And σ of the mean log CFU/mL value:

$$\sigma_{LPJ823} = \sqrt{\frac{\sum\left(\log\left(\frac{\text{CFU}}{\text{mL}}\right)_{\substack{\text{LPJ823}\\ \text{paralell i}}} - \log\left(\frac{\text{CFU}}{\text{mL}}\right)_{\substack{\text{LPJ823}\\ \text{MEAN}}}\right)}{N-1}} = 0.09$$

Where *i* represents individual parallels and N is the number of parallels, thus N = 3.

C.1.3 Calculated log CFU/mL values of all bacteria

The standardization of the inoculums used for biofilm cultivation would give a cell number of approximately $5.0 * 10^6$, which corresponds to a log CFU/mL value of 6.70. All isolates

achieving a log CFU/mL value of 6.7 ± 1.5 were considered acceptable, thus, all calculated values were acceptable (Figure 6.1).

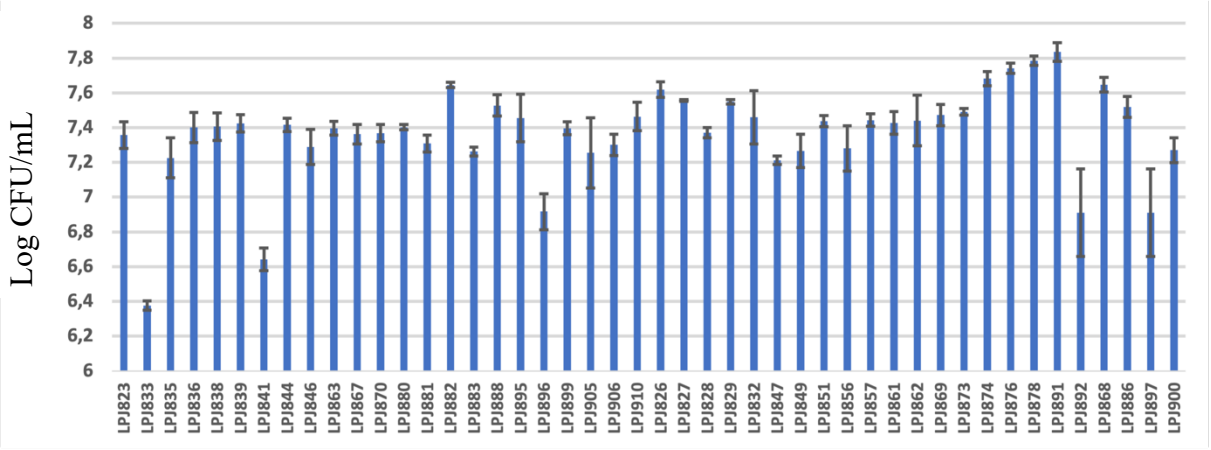


Figure 6.1: Cell numbers of all bacteria inoculums used for biofilm cultivation, during the experiment conducted to test biofilm formation capacity of the isolates.

C.2 Procedure to classify isolates as good or poor biofilm producers

The difference in OD_{650} between the planktonic plate and recovery plate was used to determine if the bacteria were good or poor biofilm producers. As each isolate was represented by four wells in the plates, the mean OD_{650} value of parallels was applied in further calculations. Isolates achieving a difference between 10-20% were classified as good biofilm producers, while those achieving a difference higher than 20% were classified as poor biofilm producers. Those under 10% were considered to have insufficient growth for the classification. The mean OD_{650} values of the recovery plate and planktonic plate, as well as their percentage difference, was determined (Table 6.4).

In addition to determining bacteria cell number in the inoculum for biofilm cultivation, cell number of selected wells in the planktonic plate and recovery plate was also determined. This was done to check the correspondence between the OD_{650} values and CFU/mL values. Five or six wells from both the planktonic and biofilm plate were selected after reading the OD_{650}, for further determination of CFU/mL. Values of log CFU/mL were calculated using the procedure in Appendix C.1.1. For the selected wells, the calculated CFU/mL are also presented (Table 6.4). A correlation coefficient of 0.87 was achieved by plotting the log CFU/mL values in Table 6.4. versus the corresponding OD_{650} data.

Table 6.4: Mean OD_{650} values of the recovery and planktonic plates, and their percentage difference. For selected wells, the calculated log CFU/mL values are indicated, as well as the corresponding OD_{650} value of that well (abs:). The three different genera are coloured differently.

Sample ID	Species ID	Recovery plate		Planktonic plate		Percentage difference
		Absorbance	Log CFU/mL	Absorbance	Log CFU/mL	
LPJ823	*P. anguilliseptica*	0.042		0.043		0.1%
LPJ833	*P. lundensis*	0.055		0.304		24.9 %
LPJ835	*P. gessardii*	0.062		0.323		26.1 %
LPJ836	*P. fluorescens*	0.058		0.325		26.7 %
LPJ838	*P. fluorescens*	0.061		0.324		26.3 %
LPJ839	*P. fluorescens*	0.060		0.331	9.538 (abs: 0.339)	27.1 %
LPJ841	*P. marginalis*	0.063	7.747 (abs: 0.064)	0.219	8.702 (abs: 0.226)	15.6 %
LPJ844	*P. azotoformans*	0.062		0.318		25.6%
LPJ846	*P. extremaustralis*	0.051		0.303		25.3%
LPJ863	*P. extremaustralis*	0.052		0.323		27.1%
LPJ867	*P. fluorescens*	0.037		0.315		27.8%
LPJ870	*P. extremaustralis*	0.060		0.302		24.2%
LPJ880	*P. fluorescens*	0.047		0.216	8.798 (abs: 0.209)	16.9%
LPJ881	*P. fluorescens*	0.048		0.209		16.1%
LPJ882	*P. libanesis*	0.046		0.210		16.4%
LPJ883	*P. fluorescens*	0.058		0.307		24.9%
LPJ888	*P. libanesis*	0.045		0.151		10.5%
LPJ895	*P. veronii*	0.058	7.577 (abs: 0.070)	0.130	8.532 (abs: 0.138)	7.2%
LPJ896	*P. extremaustralis*	0.101	6.836 (abs: 0.104)	0.263		16.2%
LPJ899	*P. fluorescens*	0.057		0.287		23.0%
LPJ905	*P. lundensis*	0.050		0.312		26.2%
LPJ906	*P. fluorescens*	0.045		0.215		17.0%
LPJ910	*P. extremaustralis*	0.039	6.314 (abs: 0.038)	0.300	8.808 (abs: 0.362)	26.1%
LPJ825	*S. liquefaciens*	Not tested.				
LPJ826	*S. liquefaciens*	0.059		0.396	9.505 (abs: 0.407)	33.7%
LPJ827	*S. liquefaciens*	0.061		0.379		31.8%
LPJ828	*S. liquefaciens*	0.060		0.335		27.6%
LPJ829	*S. liquefaciens*	0.062		0.362	9.656 (abs: 0.410)	30.0%
LPJ832	*S. liquefaciens*	0.061		0.332		27.1%
LPJ847	*S. liquefaciens*	0.044	6.566 (abs: 0.041)	0.324		28.0%
LPJ849	*S. liquefaciens*	0.056		0.368		31.2%
LPJ851	*S. liquefaciens*	0.054		0.365		31.1%
LPJ856	*S. liquefaciens*	0.057		0.330		27.3%
LPJ857	*S. liquefaciens*	0.055	7.784 (abs: 0.054)	0.348	9.136 (abs: 0.330)	29.2%
LPJ861	*S. liquefaciens*	0.057		0.336		28.0%
LPJ862	*S. liquefaciens*	0.059		0.351		29.2%
LPJ869	*S. liquefaciens*	0.058		0.330		27.2%
LPJ873	*S. liquefaciens*	0.060		0.329	9.143 (abs: 0.332)	26.9%
LPJ874	*S. fonticola*	0.061		0.340		27.8%
LPJ876	*S. liquefaciens*	0.058		0.300		24.2%
LPJ878	*S. liquefaciens*	0.060		0.334		27.4%
LPJ891	*S. liquefaciens*	0.050	8.038 (abs: 0.067)	0.369		31.9%
LPJ892	*S. fonticola*	0.045		0.239	9.005 (abs: 0.205)	19.4%
LPJ868	*A. salmoncida*	0.081	7.897 (abs: 0.074)	0.361		28.0%
LPJ886	*A. salmoncida*	0.081		0.302		22.1%
LPJ897	*A. salmoncida*	0.078	7.872 (abs: 0.092)	0.297	8.747 (abs: 0.258)	21.9%
LPJ900	*A. hydrophila*	0.074		0.324		25.0%

Appendix D: Testing antimicrobial efficiency

D.1 Determination of MIC, MBC and MBEC values

The criteria for adequate biofilm growth of growth control wells were as follows: ($OD_{650,48\ h}$ minus $OD_{650,0h}$) ≥ 0.05. Thus, the difference in OD_{650} mean values for the control wells before and after the 48 h incubation period had to be larger than or equal to 0.05. Growth controls having insufficient growth were excluded from the analysis (Table 6.5).

Table 6.5: Differences in OD_{650} between pre-incubated and post-incubated plates ($OD_{650,24\ h}$ minus $OD_{650,\ 0\ h}$). As the criteria were set to ($OD_{650,24\ h}$ minus $OD_{650,\ 0h}$) ≥ 0.05, one measurement point was excluded from the MIC analysis and three from the MBC analysis. These are coloured in red, while the valid differences are coloured in green. The three different genera are coloured differently.

MBEC analysis	Florfenicol			Aqua Des Foam PAA		
	Growth controls (mean values)					
	OD_{650} (0 h)	OD_{650} (24 h)	Difference	OD_{650} (0 h)	OD_{650} (24 h)	Difference
P. extremaustralis	0.068	0.575	0.507	0.047	0.451	0.404
P. fluorescens	0.067	0.454	0.387	0.045	0.381	0.336
P. libanesis	0.056	0.380	0.324	0.045	0.196	0.151
P. marginalis	0.047	0.361	0.314	0.046	0.266	0.220
P. veronii	0.053	0.206	0.153	0,040	0.115	0.075
S. liquefaciens	0.072	0.550	0.478	0.047	0.316	0.269
A. hydrophila	0.081	0.493	0.412	0.045	0.359	0.314

MIC analysis	Florfenicol			Aqua Des Foam PAA		
	Growth controls (mean values)					
	OD_{650} (0 h)	OD_{650} (24 h)	Difference	OD_{650} (0 h)	OD_{650} (24 h)	Difference
P. extremaustralis	0.388	0.606	0.218	0.044	0.436	0.392
P. fluorescens	0.310	0.581	0.271	0.040	0.178	0.138
P. libanesis	0.333	0.640	0.307	0.039	0.116	0.077
P. marginalis	0.622	0.839	0.217	0.043	0.304	0.261
P. veronii	0.156	0.444	0.288	0.039	0.177	0.138
S. liquefaciens	0.386	0.585	0.199	0.041	0.253	0.212
A. hydrophila	0.396	0.376	-0.02	0.056	0.348	0.292

MBC analysis	Florfenicol			Aqua Des Foam PAA		
	Growth controls (mean values)					
	OD_{650} (0 h)	OD_{650} (24 h)	Difference	OD_{650} (0 h)	OD_{650} (24 h)	Difference
P. extremaustralis	0.109	0.715	0.606	0.040	0.237	0.197
P. fluorescens	0.085	0.397	0.312	0.040	0.089	0.049
P. libanesis	0.066	0.510	0.444	0.043	0.062	0.019
P. marginalis	0.066	0.673	0.607	0.040	0.121	0.081
P. veronii	0.054	0.408	0.354	0.039	0.051	0.012
S. liquefaciens	0.126	0.611	0.485	0.041	0.110	0.069
A. hydrophila	0.075	0.512	0.437	0.040	0.175	0.135

D.2 Calculation of log reductions

D.2.1 Relevant formulas

For each well of the recovery plate, a dilution series was spotted out and incubated, to further determine the CFU/mL at each concentration step of the antimicrobial. To see how effective the antimicrobial was to reduce the microbial population of biofilm cells, log reductions were calculated. Instead of calculating CFU/mL like previously, CFU/peg was instead calculated, which corresponds to the cell number of each peg. As each isolate was represented by three columns of the recovery plate, the log CFU/peg values were first for each parallel. Then, the mean CFU/peg value of each concentration was calculated (Innovotech, 2015).

Log CFU/peg for each parallel was calculated as follows:

$$log\left(\frac{CFU}{peg}\right) = log\left[\left(\frac{X}{B}\right)(D) + 1\right]$$

Where:

X = Number of counted colonies of all spots in one parallel

B = Volume plated: 10 µL spots corresponds to 0.01 mL

D = Dilution

And the sample standard deviation, σ, of the mean log CFU/peg was calculated:

$$\sigma = \sqrt{\frac{\sum\left(\log\left(\frac{\text{CFU}}{\text{peg}}\right)_{\substack{\text{ISOLATE i}\\\text{PARALLEL i}}} - \log\left(\frac{\text{CFU}}{\text{peg}}\right)_{\substack{\text{ISOLATE i}\\\text{MEAN}}}\right)^2}{N-1}}$$

Where N is the number of parallels, thus 3.

Log reductions for each dilution were calculated as follows:

$$\log\ \text{reduction} = \text{mean log}\ \ \text{growth control pegs}\ - \text{mean log treated pegs}$$

The uncertainty of the log reduction, δlog reduction, was also calculated:

$$\delta\text{log reduction} = \sqrt{\left(\sigma_{Mean\,\log CFU/peg}\right)^2 + \left(\sigma_{CFU/peg\ growth\ control}\right)^2}$$

Where:

$\sigma_{Mean\,\log CFU/peg}$ = standard deviation of the mean log CFU/peg of relevant antimicrobial concentration

$\sigma_{CFU/peg\ growth\ control}$ = standard deviation of the mean log CFU/peg of corresponding growth control

D.2.2 Example calculation

The log reductions of the isolate LPJ863 after the Aqua Des Foam PAA challenge at 2% concentration are used as an example (Table 6.6).

Table 6.6: Counted colonies for isolate LPJ863 being exposed to Aqua Des Foam PAA at 2% concentration.

LPJ863, 2% concentration of Aqua Des Foam PAA	**Dilution factors**							
Parallel	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**
1	88	4	0	0	0	0	0	0
2	16	3	0	0	0	0	0	0
3	52	5	1	0	0	0	0	0

First, log CFU/peg was calculated for each parallel:

$$log\left(\frac{CFU}{peg}\right)_{\substack{LPJ863,\\ parallel1}} = log\left[\left(\frac{88+4}{0.01}\right)\left((10^0)+(10^1)\right)\right] = 6.01$$

This gave a log CFU/peg value of 6.01 of the first parallel, 4.32 of the second parallel and 5.81 of the third parallel. The mean value of log CFU/peg were thus calculated to 5.38.

The sample standard deviation of the parallels was further calculated:

$$\sigma_{LPJ863} = \sqrt{\frac{\sum\left(\log\left(\frac{\text{CFU}}{\text{peg}}\right)_{\substack{\text{ISOLATE i}\\ \text{PARALLEL i}}} - \log\left(\frac{\text{CFU}}{\text{peg}}\right)_{\substack{\text{ISOLATE i}\\ \text{MEAN}}}\right)^2}{N-1}} = 0.92$$

The mean log CFU/peg of corresponding growth control was 9.28 ± 0.57, thus, the log reduction was:

$$\log \text{reduction}_{\substack{\text{LPJ863}\\ \text{1\% Aqua Des Foam PAA}}} = 9.28 - 6.01 = 3.90$$

The uncertainty of this log reduction was calculated as following:

$$\delta log\ reduction = \sqrt{(0.57)^2 + (0.92)^2} = 0.96$$

Thus, the resulting log reduction of isolate LPJ863 was 3.90 ± 0.96, when using a concentration of the disinfectant corresponding to 2%.

Appendix E: Statistical analysis in SPSS

E.1 Morphology observations

First, one-way ANOVA was performed to see if the observed colour and consistency varied significantly ($P < 0.05$) among the three genera. This revealed that neither colour nor consistency varied significantly among *Pseudomonas* spp., *Serratia* spp., and *Aeromonas* spp. (Table 6.7).

Table 6.7: ANOVA table for testing colour and consistency variance among the genera.

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Colour	Between Groups	.254	2	.127	.260	.772
	Within Groups	21.050	43	.490		
	Total	21.304	45			
Consistency	Between Groups	.503	2	.252	1.478	.239
	Within Groups	7.323	43	.170		
	Total	7.826	45			

Further, one-way ANOVA was performed to investigate if there was a significant difference ($P < 0.05$) at the species level. The species being represented by less than two members were excluded from the analysis. Among the tested species, there was a significant difference ($P < 0.05$) in both colour and consistency (Table 6.8).

Table 6.8: ANOVA table for testing colour and consistency variance among the species.

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Colour	Between Groups	9.985	6	1.664	5.677	.000
	Within Groups	9.967	34	.293		
	Total	19.951	40			
Consistency	Between Groups	2.660	6	.443	4.794	.001
	Within Groups	3.144	34	.092		
	Total	5.805	40			

Further Tukey post hoc analysis was applied for comparison, to see which of the species had a significant difference between their morphologies (Table 6.9).

Table 6.9: Tukey post hoc analysis of colour and consistency differences among the species

Colour

	Species	N	Subset for alpha = 0.05		
			1	2	3
Tukey HSD[a,b]	P. fluorescens	9	1.0000		
	P. extremaustralis	5	1.2000	1.2000	
	S. liquefaciens	18	1.3333	1.3333	
	A. salmoncida	3	1.3333	1.3333	
	S. fonticola	2	2.0000	2.0000	2.0000
	P. lundensis	2		2.5000	2.5000
	P. libanesis	2			3.0000
	Sig.		.260	.063	.260

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.182.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

Consistency

	Species	N	Subset for alpha = 0.05	
			1	2
Tukey HSD[a,b]	P. fluorescens	9	1.0000	
	A. salmoncida	3	1.0000	
	S. liquefaciens	18	1.0556	
	P. extremaustralis	5	1.4000	1.4000
	P. lundensis	2	1.5000	1.5000
	S. fonticola	2	1.5000	1.5000
	P. libanesis	2		2.0000
	Sig.		.390	.195

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.182.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

ANOVA was also used to see if the morphology varied significantly ($P < 0.05$) between isolates collected from salmon skin and isolates collected from filet. The analysis did not detect any significant difference (Table 6.10).

Table 6.10: ANOVA performed to see if there was a significant variance in morphology between the two collection points.

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Consistency	Between Groups	.044	1	.044	.252	.618
	Within Groups	7.829	45	.174		
	Total	7.872	46			
Colour	Between Groups	.175	1	.175	.370	.546
	Within Groups	21.314	45	.474		
	Total	21.489	46			

E.2 Antibiotic resistance patterns

One-way ANOVA analysis was performed to see if the antibiotic resistance patterns of florfenicol, ampicillin and tetracycline varied significantly ($P < 0.05$) among the genera. The analysis revealed that this was the case for ampicillin resistance patterns, but not for the others (Table 6.11). Further Tukey post hoc analysis revealed that the ampicillin resistance pattern of *Aeromonas* spp. differed significantly from those of the two other genera (Table 6.12).

Table 6.11: ANOVA performed on **a)** florfenicol resistance pattern **b)** ampicillin resistance pattern, and **c)** tetracycline resistance pattern among the genera.

ANOVA

Flor_pattern

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.032	2	.016	.257	.775
Within Groups	2.776	44	.063		
Total	2.809	46			

a)

ANOVA

Amp_pattern

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2.254	2	1.127	5.602	.007
Within Groups	8.852	44	.201		
Total	11.106	46			

b)

ANOVA

Tet_pattern

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.115	2	.057	1.404	.256
Within Groups	1.800	44	.041		
Total	1.915	46			

c)

Table 6.12: Tukey post hoc analysis of ampicillin resistance pattern differences among the genera.

Amp_pattern

Tukey HSD[a,b]

		Subset for alpha = 0.05	
Genera	N	1	2
Serratia	20	1.2000	
Pseudomonas	23	1.4348	
Aeromonas	4		2.0000
Sig.		.523	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 8.734.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

To see if the collection point of the isolates had any influence on the general abundancy of resistance, one-way ANOVA was used. This revealed that the abundancy of resistance among bacteria collected from salmon skin and the abundancy of those collected from fillet was significantly different ($P < 0.10$) at the 10% level of significance (Table 6.13)

Table 6.13: ANOVA revealed that there was a significant difference in resistance abundancy between bacteria collected from skin and those collected from fillet, at the 10% level of significance.

ANOVA

Resistance_pattern

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.704	1	.704	2.920	.095
Within Groups	10.601	44	.241		
Total	11.304	45			

E.3 Biofilm formation capacity

ANOVA revealed that the biofilm formation capacity was significantly different ($P < 0.05$) at the level of genera (Table 6.14). Further LSD post hoc analysis showed that *Pseudomonas* spp. and *Serratia* spp. had a significant difference ($P = 0.021$) in their biofilm formation capacity (Table 6.15).

Table 6.14: ANOVA revealed that there was a significant difference in biofilm formation capacity among the genera, at the 5% level of significance.

ANOVA

Biofilm

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.931	2	.466	3.401	.043
Within Groups	5.614	41	.137		
Total	6.545	43			

Table 6.15: LSD post hoc analysis revealed that there was a significant difference in biofilm formation capacity between *Pseudomonas* spp. and *Serratia* spp.

Multiple Comparisons

Dependent Variable: Biofilm
LSD

(I) Genus	(J) Genus	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Pseudomonas	Serratia	-.28070*	.11716	.021	-.5173	-.0441
	Aeromonas	-.33333	.20187	.106	-.7410	.0744
Serratia	Pseudomonas	.28070*	.11716	.021	.0441	.5173
	Aeromonas	-.05263	.20356	.797	-.4637	.3585
Aeromonas	Pseudomonas	.33333	.20187	.106	-.0744	.7410
	Serratia	.05263	.20356	.797	-.3585	.4637

*. The mean difference is significant at the 0.05 level.

The differences in biofilm formation capacity at species level were also investigated, excluding those species being represented by less than one member. This revealed that there was a significant difference ($P < 0.05$) in biofilm formation capacity (Table 6.16). Tukey post hoc analysis revealed that the largest difference was between *P. libanesis* and *A. salmoncida*.

Table 6.16: ANOVA performed to detect significant differences in biofilm formation capacity at the species level.

ANOVA

Biofilm

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2.475	6	.412	4.125	.003
Within Groups	3.300	33	.100		
Total	5.775	39			

Table 6.17: Tukey post hoc analysis performed on biofilm formation capacity at species level.

Biofilm

	Species	N	Subset for alpha = 0.05	
			1	2
Tukey HSD[a,b]	P. libanesis	2	1.0000	
	S. fonticola	2	1.5000	1.5000
	P. fluorescens	9	1.6667	1.6667
	P. extremaustralis	5		1.8000
	P. lundensis	2		2.0000
	S. liquefaciens	17		2.0000
	A. salmoncida	3		2.0000
	Sig.		.142	.438

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.177.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

NTNU

Norwegian University of
Science and Technology