Norwegian University of Life Sciences

Master's Thesis 2024 60 ECTS
Faculty of Chemistry, Biotechnology and Food Science

Mapping the presence of antibiotic resistant bacteria in water habitats in Gjesdal, Moss, and Våler municipality.

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Acknowledgement

First of all, I would like to express my gratitude to my supervisor, Professor Bjørn-Arne Lindstedt. Thank you for letting me take part in this meaningful research on environmental antibiotic resistant bacteria. You have given me invaluable feedback on my thesis, and always been there to answer whatever question that has popped up in my mind. You have also been very positive and reassuring during the whole process, which has helped me to keep calm and avoid stressing too much.

Secondly, I would like to thank Ahmed Abdelghani for all your help in the laboratory, and for showing such an interest in the progress and the results. Additionally, I must thank my fellow student Hanne Brænd Rabbås for making the days in the laboratory more enjoyable, although it's been a challenging not to contaminate the isolates by singing, when groovy music has been playing on the radio!

I also want to thank everyone who has made my time in Ås as good as it has been over the past five years. Thank you to my study buddies, all my good friends from my time in Skogården, and all of you whom I got to meet while on exchange to Vienna. I am really grateful for you all.

Lastly, I must thank my family for always supporting me and my decisions, and for making it easy for me to stand by my choices. I am also extremely grateful for all the support and motivation I have received from my boyfriend, and an extra thank you is in order for all the dinners you have cooked for me this past year! This thesis would have been a lot harder to finish without all the support I have received from you all.

Eirunn Ø. Søyland

Ås, May 2024

Abstract

The last few decades have seen a massive use of antibiotics worldwide, in all from human health care and veterinary use to agriculture and aquaculture. This has led to a rise in emergence of antibiotic resistant bacteria (ARB), where bacteria harbouring genes for extended-spectrum β-lactamases (ESBL) and carbapenem resistance are of particular concern. Infectious diseases caused by these bacteria can be very challenging to treat, and a staggering number of deaths every year result directly or indirectly from antibiotic resistance. With no measurements taken to stop the ARB spread, this problem will only keep on growing.

This study aimed to investigate the existence of ARBs in six different water habitats from the southwestern and southeastern part of Norway. By the help of ESBL and CRE agar screening plates, ARB were isolated from all water sites. The DNA from a total of 20 bacterial isolate was extracted, and identified through Sanger sequencing. Multiplex and singleplex PCR were performed, using primer pairs of known ESBLs and carbapenemases, to identify the presence of these genes. Antibiotic susceptibility tests were performed on 13 of the isolates, out of which three were chosen for whole genome sequencing.

Eight different bacterial genera were identified, namely *Caulobacter, Chonella, Chromobacterium, Herbaspirillum, Novosphingobium, Pseudomonas, Serratia,* and *Stenotrophomonas*. The WGS results revealed two certain, and three putative β-lactamase genes present in the three bacteria that were sequenced. These were a class A β-lactamase gene in *Caulobacter, bla*FONA-8 and *bla*SFDC in *Serratia*, and *bla*L1 and *bla*L2 in *Stenotrophomonas*. This revealed that β-lactam resistant bacteria can be found in various water habitats in the southern part of Norway.

Samandrag

Dei siste tiåra har det vore ein massiv antibiotikabruk på verdsbasis, både innanfor menneskeleg helse, veterinærmedisin, jordbruk og akvakultur. Dette har ført til ein auke i førekomsten av antibiotikaresistente bakteriar (ARB), og bakteriar med gen for β-laktamasar med utvida spektrum og karbapenemresistens er særleg urovekkande. Infeksjonssjukdommar som kjem av slike bakteriane kan vere veldig vanskelege å behandle, og kvar år er eit overveldane tal av dødsfall direkte eller indirekte knytt til antibiotikaresistens. Utan nokon tiltak for å stoppe spreiinga av ARB, vil dette problemet berre fortsette å auke.

Denne studia hadde som føremål å undersøke om ARB var til stade i seks ulike vassmiljø frå sørvest og søraust Noreg. Ved hjelp av ESBL og CRE agar undersøkingsplater blei det isolert ARB frå alle prøvestadane. DNAet frå totalt 20 bakterieisolat blei ekstrahert og identifisert ved hjelp av Sanger sekvensering. For å undersøke om bestemte gen var til stade i prøvane blei multiplex og singleplex PCR utført, med primer-par for kjente ESBL- og karbapenemasegen. Sensitivitetstest for antibiotika blei utført på 13 av isolata, og til slutt blei tre av isolata valt til heilgenomsekvensering.

Åtte ulike bakterieslekter blei identifisert, og desse var *Caulobacter, Chonella, Chromobacterium, Herbaspirillum, Novosphingobium, Pseudomonas, Serratia,* or *Stenotrophomonas*. Resultata frå heilgenomsekvenseringa avslørte to sikre og tre trulege β-laktamasegen blant dei tre prøvane som blei sekvensert. Desse var eit klasse A β-laktamasegen i *Caulobacter*, *bla*FONA-8 og *bla*SFDC i *Serratia,* og *bla*L1 og *bla*L2 i *Stenotrophomonas*. Dette viste dermed at β-laktamresistente bakteriar kan bli funne i ulike vassmiljø i det sørlege Noreg.

Abbreviations

ARB	Antibiotic resistant bacteria
ARG	Antibiotic resistance gene
bla	β-lactamase gene
BLAST	Basic Logical Alignment Search Tool
BLASTn	Nucleotide Basic Logical Alignment Search Tool
BLASTp	Protein Basic Logical Alignment Search Tool
Bp	Base pairs
BSA	Broad-spectrum antibiotic
CARD	The Comprehensive Antibiotic Resistant Database
CF	Cystic fibrosis
CLSI	Clinical & Laboratory Standards Institute
CRE	Carbapenem resistant *Enterobacteriaceae*
DHFR	Dihydrofolate reductase enzyme
DNA	Deoxyribonucleic acid
EMBL-EBI	European Molecular Biology Laboratory, European Bioinformatics Institute
ESBL	Extended-spectrum β-lactamase
EUCAST	The European Committee on Antimicrobial Susceptibility Testing
IS	Insertion sequence
MDR	Multi-drug resistance
MGE	Mobile genetic elements
MIC	Minimum Inhibitory Concentration
NAG	*N*-acetylglucosamine
NAM	*N*-acetylmuramic acid
NCBI	National Centre for Biotechnology Information
NMBU	Norges miljø- og biovitenskapelige universitet
NSA	Narrow-spectrum antibiotic

OMP	Outer membrane protein
PBP	Penicillin-binding protein
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
STP	Sewage treatment plant
VFDB	The Virulence Factor Database
WGS	Whole genome sequencing
WWTP	Wastewater treatment plant

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1 Introduction

In 1928, a Petri dish unintentionally contaminated by a *Penicillin* mould, sparked what is considered the greatest discovery in modern medicine (Hutchings et al., 2019). Alexander Flemings finding of this antibiotic, known today as penicillin G, was the starting point of an era filled with great optimism, regarding tackling infectious diseases. The utilisation of antibiotics gave modern medicine a new weapon to combat minor and major bacterial infections, many of which were previously associated with high morbidity and mortality rates (Aminov, 2010; Haque et al., 2016).

Soon after the discovery of antibiotics, even before their introduction to the drug-market, resistance to antibiotics was discovered. Resistance genes to antibiotics occur naturally in many bacteria, but the exaggerated use of antibiotics in the last century has led to an acceleration of resistance development (Lobanovska & Pilla, 2017). In turn, emergence of bacteria resistant to one compound from three or more antibiotic classes, so called multidrug resistant (MDR) bacteria, are the cause of great concern (Magiorakos et al., 2012). Most worrisome are carbapenem-resistant *Enterobacteriaceae* (CRE) and bacteria producing extended-spectrum β-lactamases (ESBLs).

Traditionally, the primary research on antibiotic resistant bacteria have centred on clinical samples, as hospitals are hot spots for acquiring infections (Mills & Lee, 2019). However, the dissemination of antibiotics into the environment has been a major factor for the spread of ARB and antibiotic resistance genes (ARG) due to selection pressure. Nevertheless, knowledge regarding antibiotic resistance in environmental bacteria is scarce and needs greater attention (Rizzo et al., 2013).

In this thesis, water samples from three sites in Western Norway, and three sites in Eastern Norway were collected. All sampling sites are located near human civilisation, and the existence of problematic bacteria in these habitats can therefore have an impact on human life. As part of a broader study lead by professor Bjørn-Arne Lindstedt, the aim of this thesis is to investigate the occurrence of antibiotic resistant bacteria in the chosen habitats, with an emphasis on bacteria resistant to β-lactam antibiotics.

2 Theory

2.1 Antibiotics

Antibiotics are chemical agents produced and secreted in low concentration by microbes, such as bacteria and fungi, to inhibit or kill other microbes in their vicinity (Lima et al., 2020). This strategy gives the antibiotic producer an advantage in the constant competition for crucial resources, such as nutrition and water. Actinomycetes are the most productive antibiotic producers, with more than 70 % of the medically used antibiotics deriving from their secondary metabolites (Mak et al., 2014; Sitotaw et al., 2022). Although many microorganisms produce antibiotic compounds, the antibiotics can be toxic to humans or have minimal effect on the pathogen, thus, only a few of them are medically significant (Tronsmo, 2019).

The utilisation of antibiotic compounds for medicinal purposes is not new. Historical evidence shows that ancient civilisations in Greece, Rome, and China, among others, used antibiotics to treat serious infections, for example through topical application of mouldy bread (Gould, 2016). In human skeletal remains from Sudanese Nubia, traces of the antibiotic tetracycline have been detected (Aminov, 2010; Gould, 2016). However, it was only after Fleming discovered the potential of penicillin in 1928, that more extensive research on this topic was conducted. By the end of the Second World War, a vast distribution of antibiotics helped save the life of thousands of soldiers wounded in the battles (Haque et al., 2016). The sharp rise in life expectancy since then can partly be contributed to the commercialisation of antibiotics, which became possible due to the antibiotics' high efficiency, wide availability, and relatively low costs (Abadi et al., 2019; Smith & Bradshaw, 2008; Sutterlin et al., 2017).

2.1.1 Antibiotics mechanism of action

Bacteria are dependent on functional cell growth and cell division, to replicate a large enough quantity needed to cause an infection. Antibiotics work by interfering with one or several of these processes and can be either bactericidal, killing the target bacteria, or bacteriostatic, inhibiting their cell growth (Neu & Gootz, 1996). An important property of antibiotics as a treatment option for human infections, is their selective toxicity. This means that the antibiotic effectively targets the invading microorganism, without being

harmful to the host. Selective toxicity is possible when the antibiotic interacts with targets unique to prokaryotes, or has a higher affinity for the prokaryotic rather than eukaryotic pathway in similar mechanisms (Dalhoff, 2021). The mechanisms of action can be divided into five groups, based on their antibiotic target sites (figure 1): (1) Inhibition of cell wall synthesis, (2) affecting the cytoplasmic membrane, (3) inhibition of replication and transcription of DNA, (4) inhibition of protein synthesis, and (5) affecting other biochemical targets (Etebu & Arikekpar, 2016; Neu & Gootz, 1996; Tronsmo, 2019).

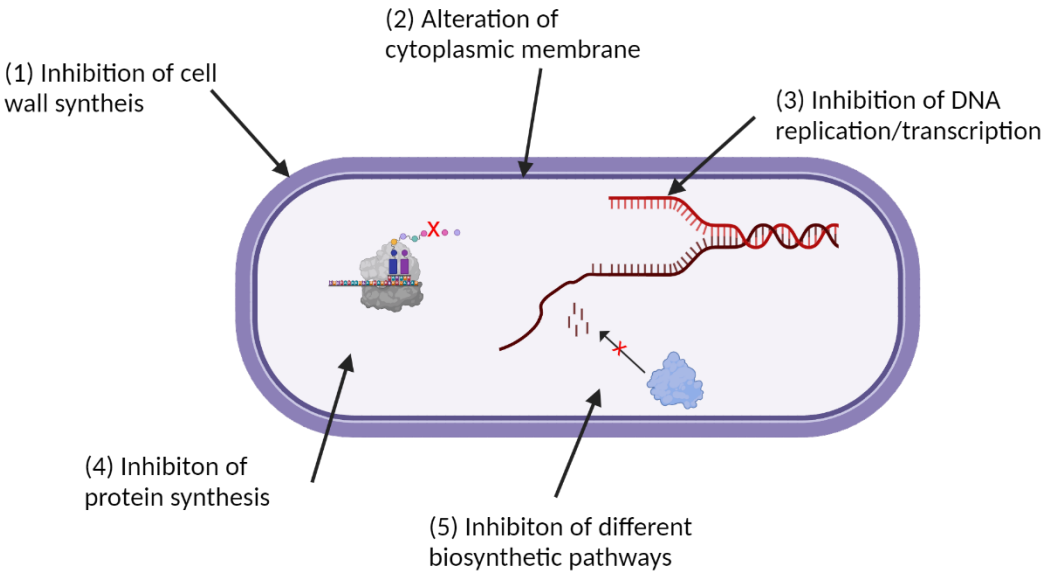


Figure 1. Depiction of the different antibiotic target sites. Created by the author with BioRender.com.

2.2 Impact of human antibiotic use

While the utilization of antibiotics proved an efficient tool in battling infectious diseases, the bacteria's constant adaptability to their surroundings soon became evident. Already during his Nobel prize lecture in 1945, Fleming predicted the development of antibiotic resistant bacteria when exposed to non-lethal doses of the antibiotic (Abadi et al., 2019; Fleming, 1945).

Antibiotics have a variety of applications, and are used both therapeutically and preventative in human and veterinary medical settings (O'Neill, 2016). Additionally, they are used in animal husbandry, aquaculture, and crop cultivation to hinder diseases, stimulate growth, and improve the overall yield (Lobanovska & Pilla, 2017; Taylor &

Reeder, 2020). Waste from these facilities and industries is released into the environment through urine and faeces excretion. Between 25-90 % of the antibiotics used in human and animal therapy are not absorbed by the body, but released as bioactive substances (Le et al., 2023; Polianciuc et al., 2020). Waste water treatment plants (WWTP) and sewage treatment plants (STP) treat the contaminated water and sewage before final release into the environment, but a 100 % removal of antibiotics is not possible. In addition, ARBs and ARGs have been seen to escape the WWTP processes (Gao et al., 2018; Polianciuc et al., 2020; Rizzo et al., 2013). In effect, the high density of bacteria in treatment plants along with antibiotic residues, can lead to increased development and genetic exchange of ARGs due to selection pressure (Zhang et al., 2009).

The increasing number of resistant bacteria is of massive concern, and the issue is listed as one of the top ten global health threats by the World Health Organization (2019). The aquatic environment is among the habitats with the highest abundance of bacteria and bacterial diversity. The water, including rivers, lakes, and oceans, serves as a link connecting all life on Earth, and is an important contributor in disseminating bacteria between different environments (Vaz-Moreira et al., 2014). Most environmental bacteria are not pathogens, but exposed to sub-inhibitory concentrations of antibiotics, they may develop ARGs. Thus, they serve as reservoirs for these genes, and can later transfer them to pathogenic bacteria (Finley et al., 2013; Gao et al., 2018).

Human exposure to ARBs through the environment and the food chain, emphasizes the need to see the spread of ARB and MDR in light of a bigger whole (Djordjevic et al., 2020). One Health is a concept seeking to better the collaboration between human, animal, and the environment in order to improve the collective health, and the problem surrounding ARBs is considered a critical One Health issue (One Health Initiative, 2024). A report from 2016 suggests that, if no measures are taken, by 2050 there will be a staggering 10 million deaths per year linked to antimicrobial resistance. This is a substantial increase from the 2016 figure of 700 000 deaths per year, and is higher than the number of people currently dying from cancer (O'Neill, 2016).

2.3 Antibiotic resistance

Antibiotic resistance (AR) is not a novel phenomenon, and ARBs have been isolated for 30,000-year-old ice layers, long predating the modern use of antibiotics (Finley et al., 2013; Le et al., 2023). Bacteria can quickly adapt to external factors, due to their ability to incorporate external DNA into their genome, as well as their short generation time, ranging from approximately 20 minutes and up to a few hours. Antibiotic resistance can either be an intrinsic part of the bacteria, or acquired from the surrounding environment (Sandner-Miranda et al., 2018; Tronsmo, 2019).

2.3.1 Intrinsic antibiotic resistance

Many of the antibiotic producers encode ARGs. These genes protect the microbes from succumbing to their own defence mechanisms when secreting the antibiotics (D'Costa et al., 2006; Mak et al., 2014; Zhang & Cheng, 2022). ARGs are present on the chromosome and are part of the bacteria's intrinsic resistance (Sandner-Miranda et al., 2018). The antibiotics are produced by enzymatic pathways that are encoded in adjacent gene clusters. For each gene cluster, there is usually at least one resistance gene for its associated antibiotic. Some are expressed constitutively, while others are highly regulated and are only switched on in the presence of the antibiotic compound (Mak et al., 2014). Intrinsic antibiotic resistance is often the result of the interaction of multiple genes, and is therefore not easily transferred through horizontal gene transfer (HGT) (Vaz-Moreira et al., 2014).

2.3.2 Acquired antibiotic resistance.

Acquired antibiotic resistance occurs when a bacterium, previously susceptible to an antibiotic, develops resistance to it. This can happen through genetic mutations, or by acquisition of new, resistant DNA from the environment (Hawkey, 1998; Le et al., 2023). Mutations can take place spontaneously, at any time, and without any involvement of antibiotics. If the mutation results in an advantage to the bacterium, such as resistance to a particular antibiotic, this mutation is favourable and will be kept through evolution. The favourable mutations can be transferred by insertion sequences or transposons to the plasmid, and from here, they can be transferred from one organism to another through HGT. (Hawkey, 1998).

2.3.2.1 Horizontal gene transfer

There are three ways HGT occurs, namely conjugation, transformation, and transduction. Conjugation is the event in which a donor bacterium and a recipient bacterium are in physical contact via a sex pilus. DNA is exchanged between them with the help of mobile genetic elements, such as conjugative plasmids. The plasmid is replicated, and the copied plasmid is transferred to the recipient cell (Michaelis & Grohmann, 2023; Tronsmo, 2019).

Transformation is the process where a bacterium takes up free DNA from the environment, and incorporates it into its own genome. For this to happen, the bacteria must be in a competent state. The regulation of competence is dependent on quorum-sensing and several conserved competence-inducing genes (Michaelis & Grohmann, 2023).

During transduction, novel DNA is introduced into a bacterial cell via bacteriophages. Bacteriophages are viruses capable of infecting bacteria. When the bacteriophages introduce their DNA into the bacteria, they take control over the bacterial cell, and force it to produce copies of the bacteriophage. When the copied DNA is packed in the protein capsid of new bacteriophages, some bacterial DNA might follow. This can, in turn, be introduced into the genome of the next bacteria these bacteriophages infect (Michaelis & Grohmann, 2023; Tronsmo, 2019).

2.4 Development of antibiotics

Antibiotics are roughly divided into broad- and narrow-spectrum antibiotics. Broad-spectrum antibiotics (BSAs) target a wide range of bacteria and often work against both Gram-positive and Gram-negative bacteria. On the other hand, narrow-spectrum antibiotics (NSAs) target more specifically, and aim to affect particular or a few similar types of bacteria. Usually, these include Gram-positive bacteria, as they are easier to target due to their lack of outer membrane (Saxena et al., 2023; Tronsmo, 2019).

Using BSAs is advantageous if multiple bacteria are causing the infection, or in situations where an immediate initiation of treatment is necessary, but the cause of infection is unknown. A drawback of using BSAs is that they affect bacteria important for

the host's microbiota in addition to the target bacteria. The time it takes to restore the gut microbiota back to its normal form can take years, depending on the extent of the antibiotic treatment (Lozupone et al., 2012; Melander et al., 2018). Additionally, the use of BSAs can lead to an increase in ARBs due to selection pressure, and the host's microbiota can serve as a reservoir for resistance genes. These drawbacks are minimized when using narrow-spectrum antibiotics (Alm & Lahiri, 2020). One strategy to stagger the spread of ARBs is therefore to expand the search for finding new NSAs. This faces economic difficulties, as NSA development is expensive due to their limited application. A shift to wider use of NSAs also calls for rapid diagnostic tools able to quickly identify the bacterial agent (Melander et al., 2018; O'Neill, 2016).

In general, the development of new antibiotics is slow. Between January 2013 and December 2022, nineteen new antibacterial drugs were launched. However, none of these were first-in-class antibiotics, but were instead modified from already existing agents (Butler et al., 2023; Karvouniaris et al., 2023; Zhang & Cheng, 2022). The most common antibiotic agents today, are the β-lactam antibiotics.

2.5 Types of antibiotics

2.5.1 β-lactam antibiotics

The first ever marketed antibiotic, penicillin G, belongs to the β-lactam antibiotic group, which is a large group harbouring antibiotics with both broad- and narrow-spectrum target modes (Carcione et al., 2021). The β-lactams all exhibit a distinctive, highly reactive, four-member ring (figure 2), which contributes to the inhibition of cell wall synthesis and results in bactericidal effects (Babic et al., 2006; Lima et al., 2020). Their activity corresponds to the antibacterial group 1 mode of action.

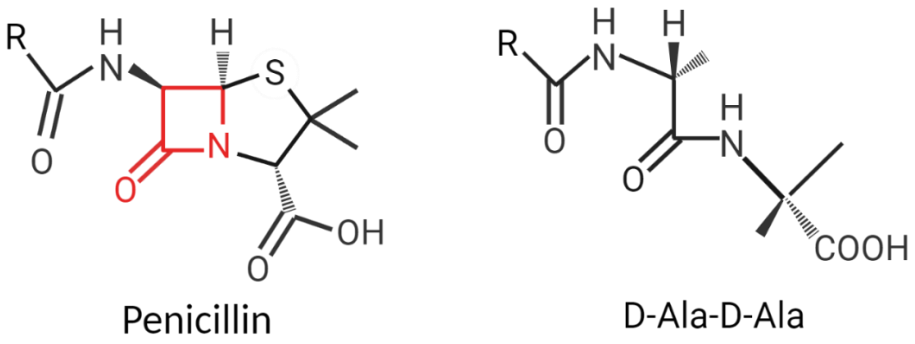


Figure 2. The similarity of Penicillin and D-Ala-D-Ala. The β-lactam ring is highlighted in red. Created by the author with BioRender.com, adapted from (Zeng & Lin, 2013).

The bacterial cell wall consists of a rigid, complex macromolecule, called peptidoglycan (PG). The PG is made up of glycan chains of alternating *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM) units. These are cross-linked to adjacent glycan chains with the help of a pentapeptide attached to the NAM units (figure 3).

The PG functions as the bacteria's "skeleton" and is crucial for maintaining the cell's shape and withstanding the osmotic pressure (Babic et al., 2006). One enzyme involved in synthesis of PG is the transpeptidase called Penicillin-binding protein (PBP). PBPs bind to the D-Ala-D-Ala (figure 2) of the pentapeptide connected to the NAM units, and assist in the cross-linking of the glycan chains. This cross-linking is crucial in the formation of the characteristic net structure of PG. β-lactams are sterically similar to D-Ala-D-Ala and can therefore target the transpeptidase domain of PBP. This hinders PBPs from binding to D-Ala-D-Ala, and subsequently blocks the formation of the cross-links. This causes the bacterial cell well to become permeable to water. As a result of fluid uptake, the bacteria can no longer withstand the osmotic pressure and eventually lyse (Babic et al., 2006). The PBP enzyme has proven to be a great antibiotic target due to its importance in PG synthesis. Because there are no homologues pathways in eukaryotes, it also reduces the chance of off-target effects due to toxic selectivity (Cochrane & Lohans, 2020; Lima et al., 2020). Multiple β-lactam antibiotic classes have been developed through the years, with slight alterations to their mode of action.

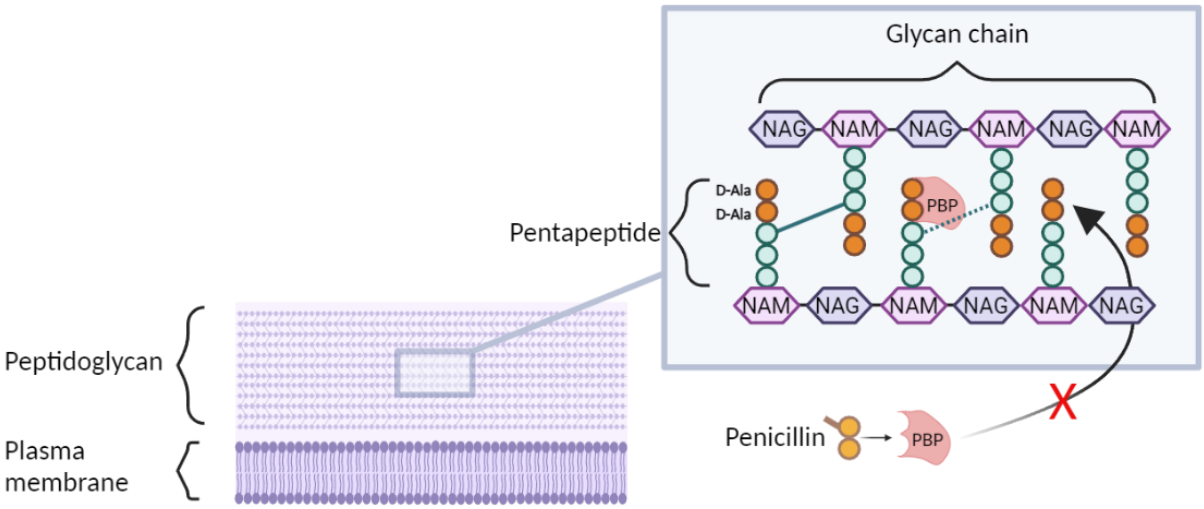


Figure 3. Depiction of the cell wall of a gram-positive bacterium. PBP is involved in the cross-linking of the NAG-NAM network of the peptidoglycan. When a β-lactam antibiotic, e.g. penicillin, binds to PBP, the PBP can no longer bind to the D-Ala-D-Ala unit. Thus, penicillin inhibits the PBPs transpeptidase activity. Created by the author with BioRender.com.

2.5.1.1 Penicillins

Two examples of antibiotics in the penicillin class are ampicillin and the already mentioned penicillin G. Penicillin G has a narrow antibacterial spectrum, and works specifically against Gram-positive bacteria, e.g. *Staphylococcus, Streptococcus* and *Pneumococci* (Lima et al., 2020). Its low activity towards gram-negative bacteria is due to the antibiotic's hydrophobic nature, which stops it from passing through the hydrophilic outer membrane porins (OMPs) of these bacteria, and thus, hindering its binding to PBPs. With the addition of a single amino group, ampicillin is more hydrophilic than penicillin G. This enables it to enter through the OMPs, subsequently attacking the PG layer in the periplasmic space and rendering it more effective against gram-negative bacteria (Lehtinen & Lilius, 2007; Sharma et al., 2013). Ampicillin was one of the first broad-spectrum penicillins introduced on the drug-market (Lima et al., 2020). By manipulating the side chains and basic ring structure of these antibiotics, a multitude of penicillins with improved activity against different bacteria have been developed (Lehtinen & Lilius, 2007).

2.5.1.2 Cephalosporins

Another class of β-lactam antibiotics are the cephalosporins, first isolated from *Cephalosporium acremonium* cultures. They are divided into different generations, based on their spectrum of coverage (Lima et al., 2020). Cefotaxime and cefepime are regarded as 3rd and 4th generation cephalosporins, respectively. Both have good coverage against gram-negative bacteria, but cefepime and other 4th generation cephalosporins, have a better coverage against gram-positive bacteria compared to the 3rd generation agents. So far, five generations of cephalosporin antibiotics have been developed, where the 5th generation antibiotic ceftaroline has coverage against methicillin-resistant *Staphylococcus aureus* (MRSA), a serious, nosocomial, MDR bacterium. Just as penicillins, cephalosporins are bactericidal agents, using their β-lactam ring to bind to and inactivate the PBP. They differ mainly from penicillins in their core structure (figure 4), and amongst themselves by different R_1 and R_2 groups (Chaudhry et al., 2019; Lima et al., 2020).

2.5.1.3 Carbapenems

The carbapenem antibiotic class has a broader antibacterial spectrum than both penicillins and cephalosporins. Carbapenems bind to PBPs, but distinguishes itself from the other β-lactams by their ability to bind to multiple different PBPs (Dewi et al., 2021). This makes carbapenem highly effective against MDR infections, and it is considered a “last resort” antibiotic. The core structure of carbapenem is very similar to that of other β-lactams, but a few alterations increase its antibiotic spectrum and stability. The alterations include a carbon at the C-1 position instead of a sulfur, a *trans* instead of *cis* configuration at position C-5 and C-6, and a methyl group attached to the C-1 carbon (figure 4). Examples of carbapenem antibiotics are imipenem and meropenem, where meropenem is slightly more efficient against gram-negative bacteria (Papp-Wallace et al., 2011).

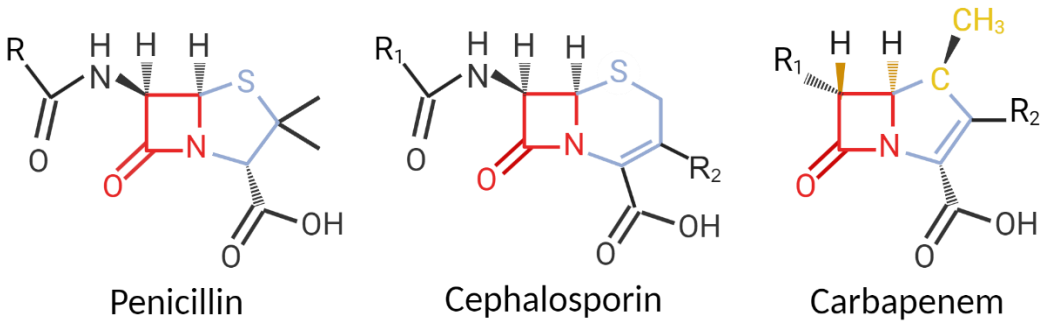


Figure 4. The basic structure of penicillin, cephalosporin and carbapenem. The common β-lactam ring is marked in red, and the differentiating core structure is marked in blue. The alterations increasing carbapenems potency is highlighted in yellow. Created by the author with BioRender.com, adapted from (Papp-Wallace et al., 2011)

2.5.2 Quinolone antibiotics

A different antibiotic group is the quinolones. This is a group of synthetic antibiotics and have a basic structure consisting of a bicyclic core. The class fluoroquinolone achieves broad-spectrum activity by the introduction of a fluorine atom into its chemical structure (Tang & Zhao, 2023). The antibiotic interacts with the bacterial enzymes topoisomerase II and IV, which are crucial for opening the helical DNA during replication and transcription. When fluoroquinolones bind to the topoisomerases, they distort these processes and lead to inhibition of cell growth, and eventually to bacterial cell death (Hernández et al., 2011; Tang & Zhao, 2023). Thus, fluoroquinolones antibacterial mode of action fits in the 3rd group. Ciprofloxacin is a 2nd generation fluoroquinolone, and one of the most used antibiotics from this class. Derivates of ciprofloxacin have shown biological properties, such as being anti-TB, anti-tumor, anti-HIV, and anti-malarial, among others (Zhang et al., 2018).

2.5.3 Antifolate antibiotics

An antibiotic group exhibiting group 5 mode of action, are the antifolates. Antifolate agents are compounds inhibiting the dihydrofolate reductase enzyme (DHFR) in bacteria. This enzyme synthesizes essential cofactors that bacteria need to create the nucleobases adenine and guanine. When antifolates inhibit DHFR, the RNA and DNA production is halted, and subsequently stop the cell from growing. Trimethoprim is a well-known antifolate agent. It is highly species-specific, binding primarily to bacterial

DHFR, but the inhibition is weak. Therefore, it is often used in combination with the sulphonamide drug sulfamethoxazole, creating a synergistic antifolate effect which increases its antibiotic properties (Kompis et al., 2005).

2.6 Resistance mechanisms

There is a constant race between development of new antibiotics, and the bacteria's ability to evolve new strategies to overcome these effects. In their article, Zhang & Cheng (2022) mentions nine different resistance mechanisms of bacteria. Three of them are (1) target modification or mutation, (2) efflux pumps, and (3) hydrolase or inactivating enzymes. Target modification or mutation alters the antibiotic target site so that the antibiotic no longer can bind to them. Such modifications can occur in PBPs, causing the β-lactams to lose their affinity to them. Efflux pumps is an effective system for bacteria to get rid of toxic compounds, by pumping them out of the cell. Many efflux systems are non-specific, potentially leading to multidrug resistance. However, of greatest concern are enzymes capable of hydrolysing or inactivating the antibiotic, which is the case for β-lactamases (Zhang & Cheng, 2022).

2.6.1 β-lactamases

β-lactamases hydrolyse the β-lactam ring in β-lactam antibiotics by breaking the amide bond, and render the antibiotic ineffective. This prevents it from binding to PBP, and subsequently to hinder cell wall synthesis. β-lactamase (*bla*) genes can be present either chromosomally, on plasmids, or on transposons. *bla* genes located on mobile genetic elements (MGEs), such as plasmids and transposons, facilitates a possible rapid spread of the resistance gene (Babic et al., 2006; Bradford, 2001).

To combat β-lactamase-mediated resistance, β-lactamase inhibitors have been put into use. β-lactamase inhibitors are ineffective in themselves, but used in combination with other β-lactam antibiotics, they have proven efficient. Clavulanic acid is a common β-lactamase inhibitor. Clavulanic acid has a low antibacterial effect, but can bind irreversibly to β-lactamase enzymes, thereby effectively inhibiting their hydrolysis of β-lactams. The combination of clavulanic acid and e.g. amoxicillin has been proven effective against both gram-positive and gram-negative bacteria (Arer & Kar, 2023).

There are two classification systems used for β-lactamases: the Ambler classification and the Bush-Jacoby-Medeiros classification. Ambler classification groups the β-lactamases into groups A-D, based on their similarity in amino acid sequence. In Bush-Jacoby-Medeiros classification, they are grouped into classes 1-4, based on their substrate and inhibition profile. In this thesis, the Ambler classification will be used as a reference. Ambler classes A, C, and D contain a serine residue in the active site, and are called serine β-lactamases. Class B contains a metal ion (Zn^{2+}), and is named metallo-β-lactamases (Babic et al., 2006). The metallo-β-lactamases are further subdivided into groups B1, B2, and B3, where B3 is most distinguishable among them (Hall & Barlow, 2005). Extended-spectrum β-lactamases (ESBL), AmpC β-lactamases, and carbapenemases are the β-lactamases of primary concern (Babic et al., 2006; Zhang & Cheng, 2022).

2.6.1.1 Extended-Spectrum β-Lactamases

ESBLs are enzymes containing resistance mechanisms capable of inhibiting the effect of the most common antibiotic groups, including penicillin and cephalosporine (Folkehelseinstituttet, 2023). So far, ESBLs have mainly been found in gram-negative bacteria, and *Escherichia coli* and *Klebsiella pneumoniae* are the most common ESBL-producing species in the *Enterobacteriacea*e family (Husna et al., 2023; Tanimoto et al., 2021). In the Ambler classification, ESBLs belong to classes A and D, where class A includes mostly penicillinases, while class D is oxacillinases able to hydrolyse oxacillin (Babic et al., 2006). The ESBL-encoding genes are often located on plasmids or transposons, aiding in their rapid spread (Husna et al., 2023). Some of the most common ESBL gene families are the class A *bla*CTX-M, *bla*TEM and *bla*SHV genes, and the class D *bla*OXA gene (Rahman et al., 2018).

ESBLs are thought to have evolved Gram-negative bacteria either by mutations in already existing genes, or through the uptake of β-lactamases in the metagenome. For example, *bla*TEM and *bla*SHV are believed to have evolved from mutations in already existing TEM and SHV enzymes, while the *bla*CTX-M gene type is believed to have been acquired through HGT from *Kluyvera sp.* (Rahman et al., 2018). CTX-Ms are normally carried on conjugative plasmids, and the capture and mobilization of *bla*CTX-M genes are attributed to their association with insertion sequences (ISs) (D'Andrea et al., 2013).

2.6.1.2 AmpC β-lactamases

AmpC β-lactamases are found among the Ambler class C enzymes, and can be located both chromosomally and on the plasmid. These β-lactamases have shown resistance to many antibiotics, including penicillins, 3rd generation cephalosporins, and sometimes to carbapenems. Additionally, they have shown resistance to β-lactamase inhibitors, such as clavulanic acid (Dong et al., 2021). The plasmid mediated AmpCs are a source for spread in β-lactamases, and have become a problem in hospitals and intensive care units around the world (Babic et al., 2006).

2.6.1.3 Carbapenemases

As mentioned, carbapenems are regarded as last resort antibiotics as they have a broad antibacterial spectrum. This is due to their high resilience towards common class A, C and D β-lactamases (Babic et al., 2006). Carbapenem-resistant Enterobacteriaceae (CRE) are therefore of great concern, and some of the most transferrable genes resulting in carbapenem resistance, are those producing carbapenemases (Mills & Lee, 2019). Carbapenemases enable the hydrolysis of almost all β-lactam antibiotics, and many carbapenemase genes are located on MGEs (Dewi et al., 2021). Carbapenemases are found in both class A, B, and D. The chromosomally encoded *bla*SFC-1 and the plasmid encoded *bla*KPC can be found among class A β-lactamases. The most important class B Metallo-β-lactamases include *bla*NDM, *bla*VIM, and *bla*IMP, and are often found on plasmids or other MGEs. Class D encompasses the *bla*OXA genes (Caliskan-Aydogan & Alocilja, 2023). The carbapenemase genes, along with ESBLs, have been detected in aqueous environments around the world (Mills & Lee, 2019). This further emphasises the water habitats important role in the circulation and spreading of antibiotic resistance genes.

2.7 Methodological theory

2.7.1 Phenotypic and genotypic determination of resistant bacteria

Oxoid Brilliance™ CRE and Oxoid Brilliance™ ESBL agar plates were used for selective isolation and simple screening of CRE and ESBL-producing bacteria from the selected water habitats. To select for the specific bacteria, the CRE and ESBL agar plates contained in combination with other antimicrobial agents, a mediated carbapenem and

the cephalosporin cefpodoxime, respectively. The two-chromogenic system distinguished primarily between *E. coli* isolates and members of the KESC-group (*Klebsiella, Enterobacter, Serratia* and *Citrobacter*), while other isolates appeared brown, beige, or colourless (Oxoid, 2010, 2011). This is because the plates specifically screen against bacteria in the *Enterobacteriaceae* family, resistant to carbapenems (CRE) and ESBLs, as these are considered a great threat to clinical patient care and the public health (Oxoid, 2011). To identify the bacterial isolates, genotypic determination was performed through Sanger sequencing of the 16S rRNA genes of the extracted bacterial DNA.

2.7.2 Resistance gene detection

To detect potential resistance genes, multiplex and singleplex PCR were performed. Multiplex PCR is a technique enabling the amplification of multiple DNA products in the same PCR reaction. This is achieved by adding two or more primer pairs in the reaction mix. The different products in the mix should be of distinct sizes, to easily distinguish them from one another. The technique is an important and cost-effective high-throughput analysis (Marmiroli & Maestri, 2007; Zhang et al., 2009). Singleplex PCR, with one primer pair present in the reaction mix, was subsequently performed on relevant isolates. Possibly relevant fragments were finally sequenced by Sanger sequencing, for a genotypic verification of the gene.

2.7.3 Antibiotic susceptibility testing

For each bacterial isolate, its susceptibility against seven different antibiotics were tested. Knowing if a bacterium is susceptible to specific antibiotics is important to provide the best healthcare, and to know which antibiotic should be used, to treat patients with bacterial infections. One method to assess this is the minimum inhibitory concentration (MIC) test. This is a method that uses thin plastic strips impregnated with a dried antibiotic concentration gradient on the underside. On the upper side, a concentration scale is marked (Jorgensen & Ferraro, 2009). The strips were placed on agar plates inoculated with the bacterium of interest, and incubated overnight. Bacteria with susceptible to the antibiotic displayed a visible inhibition zone around the strip. The MIC value was then determined based on the point where the inhibition zone intersected the concentration scale. This value was compared with the breakpoint

values given by The European Committee on Antimicrobial Susceptibility Testing (EUCAST) or Clinical & Laboratory Standards Institute (CLSI), to determine whether the bacteria were resistant or susceptible to the antibiotic.

2.7.4 Whole genome sequencing

The bacteria with highest interest and relevance were sent to whole genome sequencing (WGS). WGS is a next-generation sequencing (NGS) method that quickly and relatively cheap generate high-throughput sequence data (Park & Kim, 2016). The method allows for parallel sequencing of many DNA strands, compared to traditional Sanger sequencing where only one strand can be sequenced at a time. Sanger sequencing is the gold standard for sequencing small regions of DNA and for sequencing a limited number of samples. NGS is, however, more suitable for sequencing multiple samples simultaneously, and to sequence entire genomes, as is the case for WGS (Illumina, 2024).

3 Method

3.1 Sample collection

The water samples were collected from three sites in two different regions in Norway at separate time points. From the first three collection sites, both summer and winter samples were taken, while only summer samples were collected from the three sites in the second region.

3.1.1 Sampling from Gjesdal municipality

The first round of sample collection took place on the west coast of Norway in Gjesdal municipality on August 22,2023. Prior to the sampling, three 0.5 l jam jars had been cleaned and sterilized by heating the jars and their lids in the oven at 100 °C for ca. 15 minutes, until they were completely dry. Sample 1 was taken from the lake Galtatjørna on Søyland. The sample was taken from the edge of the water, right next to where a small stream runs out in the water and brings with it sand and soil from a newly cultivated field a couple of hundred meters further up. The second sample was taken from the stream Oppsalåna, at its starting site in Kydland. The stream is part of the bigger Figgjovassdraget river system. The third sample was taken from a private well, supplying the addresses Søyland 58, Søyland 62 and Søyland 64 with drinking water. The samples were stored at room temperature for 6 days before further use.

The winter samples were collected on January 6, 2024. The samples were taken from the same sites as for the summer samples, but pre-autoclaved 1 litre flasks were used. In Galtatjørna lake, there was a ca. 15 cm thick layer of ice. Prior to the sampling an axe was used to cut a hole in the ice. The second sample, taken from the well, also had a thin layer of ice on top which was cracked in order to take the sample. Oppsalåna river had not frozen completely, and the third sample could be taken directly. The samples were then stored between 2-8 degrees for 2 days before further use.

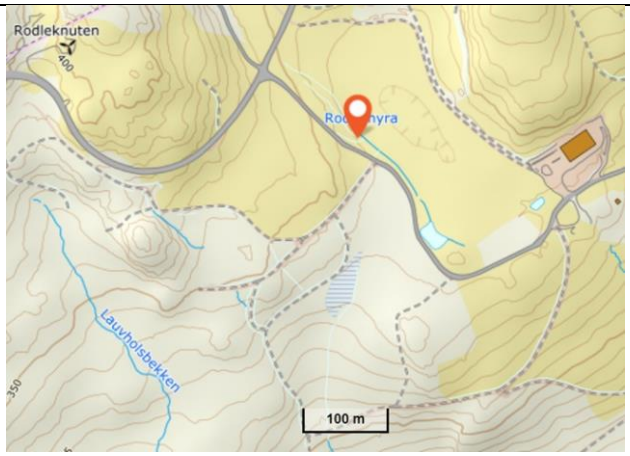
3.1.2 Sampling from Moss- and Våler municipality

The second round of sample collection took place in eastern Norway, in Moss and in Våler municipality on September 9, 23. All samples were collected in 1 l glass flasks that had been autoclaved in advance. The first sample was collected from Oslofjorden, from

Fiskestranda on Jeløya in Moss municipality. The second sample was taken from Vestre Vansjø Lake, right next to a popular recreational site called Vaskeberget. The final sample was collected in Våler Municipality from the river Veidalselva/Kirkeelva, right next to Våler Church. The samples were stored at 4 °C for 1 day before further use.

Table 1. Overview of the different collection sites. All illustrations are screenshots from norgeskart.no, and the marker is placed where the samples were taken.

Water ID	Name of water	Type of water	Location
W1	Galtatjørna	Lake	
W2	Oppsalåna	River	

W3	Brønn	Well	
W4	Oslofjorden	Fjord	
W5	Vestre Vansjø	Lake	
W6	Veidalselva/Kirkeelva	River	

3.2 Sample inoculation

Samples that contained mud and soil particles underwent a primary filtration, by pouring the water through a previously autoclaved funnel containing a filter, into a new, sterile flask. The flasks were inverted two times, before 1 ml was pipetted directly onto one Oxoid *Brilliance*™ ESBL and one Oxoid *Brilliance*™ CRE agar screening plate, for each sample. Sterile plastic Drigalski spatulas were used to even out the sample on the plates. They were then left to dry on a sterile bench (Thermo Scientific, HeraGuard Eco) with the lid partially on.

Filtered water samples were prepared using a Microfil Support Stainless Steel Frit (MISP00002 Millipore, Sigma-Aldrich) system connected to a water tap. The steel filters and the stations where they were placed were all sterilized with 96 % ethanol and burned off using a FLAMEBOY (Integra Bioscience) portable Bunsen burner. With a sterile tweezer, a 0.45 µl EZ Pak-membrane filter (Merck KgaA) was placed on top of each steel filter. Over the filters, one 100 ml Microfil® Filtration Funnel (Millipore®, Sigma-Aldrich) was placed.

To secure a homogenized sample, the flasks were inverted a couple of times, before 100 ml was poured into the filtration funnels. The water tap was turned on to create a vacuum in the filtration system. The taps on the individual stations were turned on until all the water was sucked through the filter. Two filters were prepared from each sample and placed on both ESBL and CRE agar plates. The plates were stored at 37 °C for 1-2 days, until bacterial colonies had grown.

3.3 Purification of bacterial colonies

The first round of samples yielded low bacterial growth of potentially interesting bacteria, and a second collection and inoculation process was therefore conducted. After approximately 2 days of inoculation, the new samples revealed sufficient bacterial growth. By using sterile plastic inoculation needles (Sarstedt AG & Co. KG), single colonies were picked and plated onto new plates of the same agar medium. The newly inoculated plates were then incubated at 37 °C, until new colonies grew. This process was repeated until all bacteria had formed pure colonies.

3.4 DNA extraction

To extract the DNA, the DNeasy® PowerFood® Microbial DNA extraction protocol from Qiagen was followed, with some diversions. First, a 10 µl inoculation loop (Sarstedt AG & Co. KG) was used to scoop up some bacteria. This was resuspended in 1 ml of Ringer's solution and centrifuged at 13 000 g for 1 minute. The supernatant was discarded, and the pellet was used further, following the protocol from step 3 and onward. The only diversions from the protocol from this point on were that 50 µl of the Solution EB elution buffer were used to elute the DNA, instead of 100 µl. After the elution buffer was added, the tube was left for 2 minutes on the counter before centrifugation.

3.4.1 Quantification and purity determination

After DNA extraction, the DNA was quantified, and the purity was determined using Nanodrop 2000 Spectrophotometer (Thermo Scientific). First, the Nanodrop was blanked by adding 2 µl of the EB Buffer used to elute the DNA to the lens. The lens was wiped clean before 2 µl of each isolate was added and measured. Between each measurement, the lens was wiped clean with a tissue. The concentration value, as well as the 260/280 and 260/230 ratio were noted.

3.5 Glycerol stock preparation

A glycerol stock was prepared by mixing an 86 % glycerol solution with distilled water to a desired concentration of 17 %. The bacteria that had their DNA extracted were plated once more and incubated at 37 °C for 1-2 days. 1 ml of the 17 % glycerol solution were added to 2 ml cryotubes (Sarstedt Inc. Screw Cap Micro tube), and inoculation loops (Sarstedt AG & Co. KG) were used to pick up some bacteria and mix it in the glycerol solution. The bacteria were properly dissolved by pipetting up and down and the cryotubes were stored at -80 °C for long-term storage.

3.6 Sanger sequencing of 16S rRNA

To identify the bacterial strains, the 16S rRNA was amplified by PCR and sent to Eurofins Genomics (Germany) for sequencing.

3.6.1 PCR of 16S rRNA

A reaction mix was prepared in a sterile bench following the protocol for Q5® HotStart High-Fidelity 2x Master Mix (New England BioLabs inc.). The primer pair 1F (5'-GAGTTTGATCCTGGCTCAG-3') and 5R (5'-GGTTACCTTGTTACGACTT-3') were used. 23 µl of the mix, and 2 µl DNA templates was added to respective wells in the PCR-strips. A negative control was included, using Ambion™ Nuclease-Free Water (Thermo Fisher Scientific) as template. The PCR-strips were placed in a BioRad C1000™ Thermal Cycler and followed the program settings noted in the Q5® HotStart Polymerase protocol, as described in table 2.

Table 2: PCR settings for Q5® HotStart Polymerase.

Step	**Temperature**	**Time**	
Initial denaturation	98°C	30 sec.	
Denaturation of templates	98°C	10 sec.	} 32 x
Annealing	55°C	30 sec.	
Elongation	72°C	42 sec	
Final extension	72°C	2 min.	
Hold	4°C	∞	

3.6.2 Gel electroporation and visualization of the 16S amplicons

A gel tray was assembled, and a 2 % agarose solution was prepared by mixing 1x Tris-acetate-EDTA (TAE) buffer solution with SeaKem® LE Agarose (BioNordika). The amount needed varied depending on the size of the gel tray. The solution was heated in a microwave until it had completely cleared. After cooling down to ca 60 °C, 0.5 µl SYBRSafe DNA Gel Stain was added to every 10 ml of TEA buffer. When it had properly mixed, the solution was poured into the gel tray. Bubbles in the agarose solution around the wells were removed with the help of a pipette tip, and aluminium foil was used to cover the tray while solidifying.

In new PCR-strips a mix of 7 µl Ambion™ Nuclease-Free Water, 2 µl Gel Loading Dye, Purple 6X (New England BioLabs inc.) and 2 µl of the respective PCR products was added. The mixes were applied to the wells, along with a Quick Load Purple 100 bp DNA

Ladder (New England BioLabs inc.). The trays were placed in a Sub-Cell® GT Agarose Gel Electrophoresis System (BioRad). To let the samples settle properly, a low voltage (80-100 V) was set initially. After a few minutes, the volt was turned up to 120-150 V, and run for 1.5-2 hours.

Finally, the gel was visualised under UV-light to indicate bands corresponding with the expected size of the 16S gene. For a clearer band visualization, the gel was placed in a Molecular Imager® Gel Doc™ XR+ (BioRad). The image was viewed on the computer using the BioRad Image Lab™ Software.

3.6.3 PCR-product clean-up

Before sending the 16S sequences to Sanger sequencing, the amplicons had to be cleaned. GeneElute™ PCR Clean-Up Kit (Sigma-Aldrich) was used. The protocol was followed as described, and all centrifugations were at 16 000 x g. The product was eluted with a non-diluted Elution Solution. The concentration of the cleaned product was measured by Nanodrop 2000 Spectrophotometer (Thermo Scientific).

3.6.4 Preparation for Sanger-sequencing

The cleaned PCR product was made ready for Sanger sequencing by allocating one tube for the forward primer and one tube for the reverse primer, for each isolate. Each tube contained 5 µl of a 5 µM primer solution, and 5 µl of the isolate with a concentration of 10 ng/µl. The volume needed from each isolate to reach the desired concentration was calculated based on the concentration measured by Nanodrop. The volume varied from 1.4-5 µl, and Ambion™ Nuclease-Free H_2O was used to dilute the isolates requiring adjustments. The samples were then put in a plastic bag and sent to Eurofins Genomics, Germany for sequencing.

3.6.5 Analysis of the sequence results

The sequence results were analysed using BioEdit Sequence Alignment Editor version 7.7.1 The corresponding forward and reverse sequence of each sample were selected, and a consensus sequence was created. The resulting FASTA format of the consensus sequence was pasted into the nucleotide BLAST tool (BLASTn) from the National Centre for Biotechnological Information (NCBI) web page, and a presumptive bacterial genus was given.

3.7 Detection of resistance genes

The bacterial strains were screened for specific resistance genes, by running a Multiplex PCR followed by Singleplex PCR. Bands of interest were cut from the gel, cleaned, and sent to sequencing.

A 100 µM stock solution of the primers was prepared by adding Ambion™ Nuclease-Free H_2O to the tube. The volume of water required was determined by multiplying the specified number of nanomole of the primer by 10. To prepare the multiplex mixes, 4 µl of three primer pairs were added to 176 µl nuclease-free H_2O. For the singleplex, 50 µl of 10 µM working solution for each primer was prepared.

3.7.1 Multiplex PCR

For the multiplex PCR, the QIAGEN® Multiplex PCR Kit protocol was used. Protocol 1 was chosen as this is the standard multiplex protocol. The Q-solution was not added, as complex secondary structures of the DNA extract was not expected. Five different multiplex primer mixes were prepared as seen in table 3, and reaction mixes were prepared as described in the protocol. The sequence of all primers are found appendix A.

Table 3: Multiplex mixes 1-4 contained three different primer pairs each, while multiplex 5 served as a control containing 16S rRNA primers and primers for the rpoB gene.

Primer mix	Primers	Base pairs
Multiplex 1	• *bla*CTX-M (gr. 2): Forward + Reverse • *bla*OXA: Forward + Reverse • *bla*SHV: Forward + Reverse	404 564 713
Multiplex 2	• *bla*CTX-M (gr. 9): Forward + Reverse • *bla*CTX-M (gr. 1): Forward + Reverse • *bla*TEM: Forward + Reverse	561 688 800
Multiplex 3	• *bla*NDM: Forward + Reverse • *bla*VIM: Forward + Reverse • *bla*KPC: Forward + Reverse	157 564 460
Multiplex 4	• *bla*CMY: Forward + Reverse • *bla*OXA-48: Forward + Reverse • *bla*IMP: Forward + Reverse	188 281 393

Multiplex 5 (Control)	• *rpoB*: Forward + Reverse • 16s rRNA: Forward + Reverse	512 1505

In the PCR-strips, 24 µl reaction mix and 1 µl DNA template were added. A positive and a negative control were prepared using DNA from *Klebsiella pneumoniae* and Ambion™ Nuclease-Free H_2O as template, respectively. The *K. pneumoniae* DNA was known to be positive for *bla*CTX-M (gr. 1), *bla*TEM, *bla*SHV and *bla*OXA, and therefore only used in multiplex 1 and 2. Subsequently, the PCR strips were placed in a BioRad C1000™ Thermal Cycler and followed the program settings noted in the QIAGEN® Multiplex PCR Kit protocol, shown in table 4.

Table 4. The PCR program settings for QIAGEN® Multiplex PCR.

Step	**Temperature**	**Time**	
Initial denaturation	95°C	15 min.	
Denaturation of templates	94°C	30 sec.	} 35 x
Annealing	60°C	90 sec.	
Elongation	72°C	90 sec.	
Final extension	72°C	10 min.	
Hold	4°C	∞	

While the PCR was running, an agarose gel was prepared as previously described. After amplification, 2 µl of the PCR-product was mixed with 7 µl Ambion™ Nuclease-Free Water and 2 µl purple 6x Loading dye. 10 µl of this mixture was loaded on the gel and run for ca 1.5-2 hours at 150 V. Finally, the bands were visualised using Molecular Imager® Gel Doc™ XR+ (BioRad).

3.7.2 Singleplex PCR

All isolates displaying bands around the desired lengths from multiplex PCR, were carried on to singleplex PCR. A reaction mix was prepared by following the Q5 Polymerase Protocol, and the primer pair of the desired genes was used. The PCR-strips were prepared in the same fashion as for the multiplex PCR, but the PCR program followed the settings presented in table 5.

Table 5. The PCR settings for Q5® HotStart DNA Polymerase (New England BioLabs inc.).

Step	**Temperature**	**Time**	
Initial denaturation	98°C	30 sec.	
Denaturation of templates	98°C	10 sec.	} 32 x
Annealing	55°C	30 sec.	
Elongation	72°C	42 sec.	
Final extension	72°C	2 min.	
Hold	4°C	∞	

The finished Singleplex PCR-product was loaded onto a 2% Agarose gel as previously described. Once the gel run was completed, the bands were visualised in Molecular Imager® Gel Doc™ XR+ (BioRad).

3.7.3 Troubleshooting

Troubleshooting was performed in attempts to improve the singleplex gel results, and minimize the occurrence of unspecific bands. To rule out degradation of primers and DNA, new primers, and new DNA, extracted from freshly grown bacteria from the glycerol stock, was separately tested. Both multiplex and singleplex PCRs were run with higher annealing temperatures, to optimise the binding of the primers to the template. A multiplex PCR were run at 62 °C, while singleplex PCRs were run at both 63 °C and 66 °C. In addition to the original Q5 polymerase, Hemo KlenTaq and iProof polymerases were tested.

3.7.4 Gel clean-up for Sanger sequencing

Isolates that continued to display a band around the desired length were loaded onto new gels, in every other well. This was done to secure clear separation of the isolates when cut from the gel. A higher volume of the PCR-product was also applied, to increase the product amount.

The gel was visualised under UV light in the Gel Doc Imager, to cut out bands of desired lengths. A sterile scalpel was used to cut directly over and under the desired bands. The UV light was turned off to protect the DNA from degrading before vertical cuts on the sides were made. The gel slices were placed in pre-labelled Eppendorf tubes.

To clean the gel, NucleoSpin® Gel and PCR clean-up protocol (Takara Bio Inc.) was used. The steps from § 5.2 DNA Extraction from Agarose Gel, were followed as described in the protocol, except for the following notations. In step 1, 500 µl NTI buffer was used to dissolve the agarose gel, regardless of the weight of the gel lump. To completely remove Buffer NT3 in step 4, a 3-minute centrifugation was performed instead of a 1-minute centrifugation. When eluting the flow-through, 15 µl Buffer NE was added to the column and incubated in room temperature for 5 minutes, instead of 1. After centrifugation, the eluate was placed onto the column and centrifuged once more.

The concentration of the gel clean-up product was measured by Nanodrop 2000 Spectrophotometer (Thermo Scientific). The isolates were then prepared for Sanger sequencing by mixing 8 µl of the cleaned gel product with 2 µl of the corresponding 10 µM forward primer. The tubes were placed in Falcon tubes for secure shipment to Eurofins Genomic (Germany) where they were sequenced.

3.8 Antibiotic susceptibility testing

The bacteria were once again grown on corresponding ESBL and CRE agar plates, to test their susceptability to different antibiotics. The Glycerol stocks of the bacteria of interest were thawed, and an inoculation loop was used to spread the bacterial solution on the plates. The plates were incubated at 37 ºC for 1-2 days, until bacterial colonies had formed. Some bacterial strains took longer than others to grow, so the following procedure was performed at different time points for the bacteria, depending on their growth efficiency.

When colonies had formed on the agar plates, an inoculation loop was used to scoop up some of the bacteria and mix it in a tube containing 9.9 ml Ringer's solution. The bacteria were suspended completely by vortexing and pipetting. A Bürker counting chamber (Marienfeld Superior, Germany) was prepared by streaking water with a glove-covered finger along each side of the counting chamber. A cover glass was placed on top, covering the counting grid, before 10 µl of the bacteria solution was applied to the edge of the cover glass with a pipette. The counting chamber was placed in a microscope (Leitz Laborlux K, Wetzlar Germany), and the 40x objective and the PH2

condenser annulus were applied. The bacteria found in the B-square was counted. The desired turbidity of the inoculum suspension was a 0.5 McFarland standard, which corresponds to approximately 1.5 x 10^8 CFU/ml (Aryal, 2021). Mueller-Hinton (MH) agar plates were marked and the inoculum suspension with the appropriate turbidity was applied with a cotton swab. The suspension was thoroughly spread out on the whole plate by swabbing the entire plate while rotating it. After application, the plates were placed in a sterile bench for ca. 10 minutes to dry.

Finally, a tweezer was sterilised with 96% ethanol and burned off using a Bunsen burner. The Liofilchem® MIC Test Strips intended for testing were kept on the bench until they reached room temperature, to avoid condensation during application. Table 6 displays the antibiotics tested for in this thesis. When the plates had completely dried, one strip was taken out and placed on the MH agar plates. The plates were then invertedly stored at 37 °C for 1-2 days, until a confluent lawn of growth was present.

Isolate 5 showed no signs of growth on the MH-agar after both 1 and 2 days. A new inoculation was done on MH-agar, blood agar and plate count agar (PCA), and only PCA revealed growth after 1 day. Isolate 5 was therefore plated on PCA instead of MH agar.

After incubation, their susceptibility to the antibiotics were determined. The value where the inhibitory zone of bacterial growth started was read from the E-strip, and this value indicated the minimum inhibitory concentration (MIC) (Tang & Zhao, 2023).

Table 6. The antibiotics tested for in this thesis and their mechanism of action.

Antibiotic	**Antibiotic class**	**Antibiotic group**	**Mechanism of action**
Cefotaxime	3rd generation Cephalosporin	β-lactam	Inhibits cell wall synthesis
Cefepime	4th generation Cephalosporin	β-lactam	Inhibits cell wall synthesis
Meropenem	Carbapenem	β-lactam	Inhibits cell wall synthesis
Penicillin G	Penicillin	β-lactam	Inhibits cell wall synthesis
Ampicillin	Penicillin	β-lactam	Inhibits cell wall synthesis
Ciprofloxacin	2nd generation Fluroquinolone	Quinolone	Prevents replication and translation of bacterial DNA
Trimethoprim	Trimethoprim	Antifolate	Inhibits folate synthesis

3.9 Whole genome sequencing

Based on the antibiotic susceptibility testing, isolates 4, 7, and 19 were chosen to undergo whole genome sequencing. New DNA extractions were performed from freshly grown bacteria, and their concentration and purity were measured using both Nanodrop and Qubit. The Qubit™ 1X dsDNA HS Assay Kit (Thermo Fisher Scientific) was used. A standard 1 (0 ng/µl) and a standard 2 (10 ng/µl) solution were prepared by mixing 190 µl of the Qubit Working Solution with 10 µl of the respective Qubit Standard solution, and subsequently used to calibrate a standard curve. A 10-fold dilution of the DNA extracts were made, and 3 µl of this was mixed with 197 µl of Qubit Working Solution in Qubit tubes. The tubes were placed in the Qubit® 2.0 Fluorometer (Invitrogen, Thermo Fisher) and the concentrations were measured. 30 µl of each sample were placed in new Eppendorf tubes, sealed with Parafilm, and sent to Novogene (UK) for whole genome sequencing.

3.9.1 Analysis of WGS results

The results were analysed using the Galaxy database (www.usegalaxy.eu). For each sequenced bacteria, Novogene provided two files, which were selected and uploaded to Galaxy. The forward and reverse reads of each bacterium were uploaded in the "Shovill" assembly tool. Trimmomatic was performed by choosing "yes" on the Trim reads-button before running Shovill. Shovill assembled the reads provided by Novogene into a consensus sequence, and the generated contig-file was further used to scan for resistance and virulence genes. This was done using the "ABRicate" tool, which was run three times, scanning against CARD (The Comprenensive Antibiotic Resistance Database), NCBI Bacterial Antimicrobial Resistance Reference Gene Database and VFDB (Virulence Factor Database). "Prokka" annotation tool was used to annotate genes and genomic features in the bacteria based on the consensus sequences. Additionally, a quality assessment of the genome assembly was performed by using the "Quast" tool.

On the web platform Centre for Genomic Epidemiology (www.genomicepidemiology.org), the ResFinder service was used to search for antibiotic resistance genes in the isolates. Isolate 19 was also typed against MLST (Multi Locus Sequence Typing). The gbk. (GenBank) dataset provided by "Prokka" was opened

in Excel, and a search for lactamases and resistance genes were conducted. The resulting lactamase sequences were blasted in NCBI's protein BLAST (BLASTp), to determine the specific lactamases.

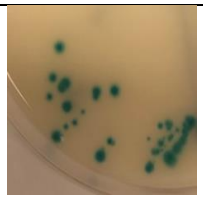
4 Results

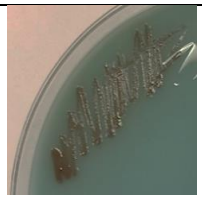
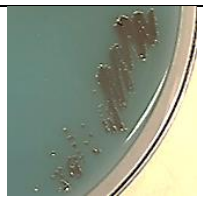
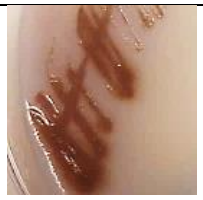
4.1 Phenotypic bacterial determination

The Brilliance CRE- and ESBL Agar plates inoculated with water samples displayed a variation of bacterial growth. For all samplings, the 1 ml direct inoculation resulted in lower bacterial diversity and growth, than the corresponding filter inoculations. Sampling site W3 (the well) showed the lowest amount of growth out of all sampling sites.

Based on their colour, the bacterial colonies considered most interesting were selected for further investigation. A phenotypic determination of them was conducted, using the datasheet provided by the producers (Oxoid, 2010, 2011). In total, the DNA of 11 summer samples and 9 winter samples were extracted. Table 7 shows the growth and phenotypic determination of the 13 isolates that were later carried on to MIC-testing.

Table 7. Phenotypic determination of bacterial isolates grown on ESBL - and CRE-agar.

Isolate	**Water ID (collection date)**	**Agar growth**	**Agar type**	**Colour**	**Colour screening**
1	W5 (03.09.23)		ESBL	Dark Purple	Colour not described by the producer
3	W5 (03.09.23)		CRE	Yellow	Colour not described by the producer
4	W6 (03.09.23)		ESBL	Green	*Klebsiella, Enterobacter, Serratia* or *Citrobacter* (KESC)

5	W5 (03.09.23)		CRE	Blue	*Klebsiella, Enterobacter, Serratia* or *Citrobacter* (KESC)
7	W5 (03.09.23)		CRE	Blue	*Klebsiella, Enterobacter, Serratia* or *Citrobacter* (KESC)
8	W5 (03.09.2023)		CRE	Pink	*E. coli*
11	W2 (22.08.23)		CRE	Pink	*E. coli*
12	W1 (06.01.2024)		ESBL	Brown	Colour not described by the producer
13	W2 (06.01.2024)		ESBL	Green	*Klebsiella, Enterobacter, Serratia* or *Citrobacter* (KESC)
16	W1 (06.01.2024)		CRE	Blue	*Klebsiella, Enterobacter, Serratia* or *Citrobacter* (KESC)
17	W3 (06.01.2024)		ESBL	Beige /colourless	*Salmonella, Acinetobacter* or other

18	W2 (06.01.2024		CRE	Pink	*E. coli*
19	W1 (06.01.2024)		CRE	Yellow	Colour not described by the producer

The colour of isolates 1, 3, 12, and 19 was not described by the manufacturer, thus, not giving any indication of their bacterial identity. Isolates 8, 11, and 18 all displayed a pink colour, which according to the Oxoid datasheet suggested *E. coli* bacteria. However, only isolate 18 showed an actual likeness to the picture presented in the datasheet, while isolate 8 and 11 showed a darker shade of pink. Isolates 4, 5, 7, 13, and 16 were all suspected to belong to the KESC-group, based on the phenotypic determination.

4.2 Quantification and purity determination

To verify the success of the DNA extraction, the concentration and purity of the bacterial isolates was measured using Nanodrop. The results are presented in table 8.

Table 8. The measured concentration and purity of the bacteria isolates. Summer isolates are presented with a light green background, and winter isolates with a light blue background.

Isolate ID	Concentration (µl)	260/280	260/230
1	9.0	2.01	1.56
2	31.1	1.85	1.50
3	37.1	1.81	1.45
4	37.1	1.76	1.48
5	29.4	1.77	1.37
6	34.5	1.79	1.48
7	30.7	1.78	1.51
8	32.0	1.80	1.45
9	27.9	1.78	1.28
10	35.9	1.79	1.41
11	23.6	1.84	1.37
12	105.2	2.28	0.63

13	96.8	2.26	0.41
14	205.4	2.11	1.31
15	145.2	2.19	0.90
16	105.8	2.29	0.67
17	36.0	2.49	0.65
18	4.8	-0.77	0.03
19	80.5	2.50	0.68
20	56.2	2.86	0.31

The summer samples (ID 1-11) had a low variation in concentration, and the 260/280 value for all isolates hovered around 1.8, indicating pure DNA extracts. The 260/230 values were somewhat low, and could be due to some residual phenol from the DNA extraction (Matlock, 2015). The winter isolates (ID 12-20) had a high concentration variation, varying from 4.8 - 205 µl. The 260/280 ratio was generally higher compared to the summer isolates, but did not indicate any issues. On the other hand, the 260/230 ratio revealed very low values in the winter isolates, suggesting some contamination. Isolate 18 had the lowest concentration out of all isolates and exhibited a negative 260/280 value. This suggested an abnormal isolate.

4.3 Genotypic bacterial determination

The 16s rRNA sequences, with an expected size of 1505 bp, were amplified by PCR. The amplicons were visualized on the agarose gel to verify their presence, and is illustrated by the winter samples in figure 5.

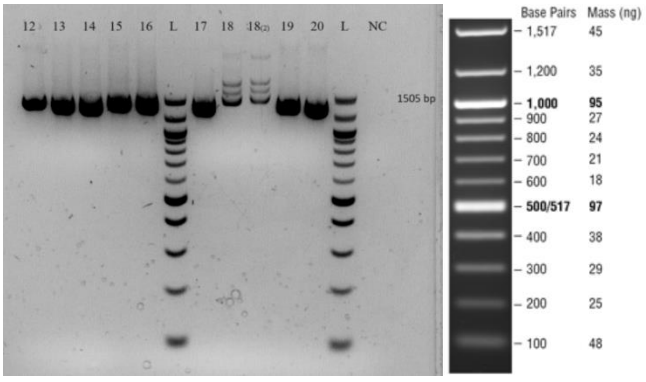


Figure 5. The agarose gel visualization of the winter isolates (ID 12-20). The figure shows presence of a band around 1300-1500 bp for all isolates. The ladder used is a Quick Load Purple 100 bp DNA Ladder.

There were bands present in all isolates, corresponding to the expected length of the 16S rRNA sequence. Isolate 18 was extracted twice, due to its low DNA concentrations. Both extracts showed a band, albeit weaker, around the same length as the other isolates.

The 16S rRNA PCR-products were prepared and sent to Sanger sequencing. The resulting FASTA sequences were uploaded in NCBI's BLASTn tool, and the bacteria were identified. The results are presented in table 9.

Table 9. The bacterial identification of all DNA extracts, based on Sanger sequencing of the 16S rRNA and identified by BLASTn search.

Isolate ID	16S rRNA BLASTn results	Bacterial family
1	*Chromobacterium vaccinii*	*Chromobacteriaceae*
2	*Chromobacterium vaccinii*	*Chromobacteriaceae*
3	*Novosphingobium*	*Sphingomonadaceae*
4	*Serratia fonticola*	*Enterobacteriaceae*
5	*Caulobacter* sp.	*Caulobacteraceae*
6	*Caulobacter* sp.	*Caulobacteraceae*
7	*Caulobacter* sp.	*Caulobacteraceae*
8	*Herbaspirillum huttiense*	*Oxalobacteraceae*
9	*Herbaspirillum huttiense*	*Oxalobacteraceae*
10	*Herbaspirillum huttiense*	*Oxalobacteraceae*
11	*Herbaspirillum huttiense*	*Oxalobacteraceae*
12	*Pseudomonas aeruginosa*	*Pseudomonadaceae*
13	*Serratia fonticola*	*Enterobacteriaceae*
14	*Pseudomonas* sp.	*Pseudomonadaceae*
15	*Serratia fonticola*	*Enterobacteriaceae*
16	*Cohnella xylanilytica*	*Paenibacillaceae*
17	*Pseudomonas* sp.	*Pseudomonadaceae*
18	Uncultured sp.	
19	*Stenotrophomonas maltophilia*	*Xanthomonadaceae*
20	*Caulobacter* sp.	*Caulobacteraceae*

The results from the BLAST search revealed that only isolates 4, 13, and 15 corresponded to their phenotypic identification, as they all came back as *Serratia*, one of the species in the KESC-group. Isolate 18 yielded low-quality Sanger sequencing results. When aligning the forward and reverse sequence in BioEdit, two potential consensus sequences were given. Both were of poor quality according to their provided

chromatograms, and with lengths of only around 500 bp instead of 1505 bp. The BLASTn search came back as uncultured for both sequences, further confirming an abnormal isolate. *Herbaspirillum huttiense* and *Caulobacter sp.* were the most isolated bacteria, with four isolates each. *H. huttiense* were isolated from four different sampling sites (W1, W2, W5, and W6), suggesting a ubiquitous occurrence of this species.

4.4 Detection of resistance genes

Five multiplex PCRs were run, with each multiplex mix containing three primer pairs. The PCR products were loaded to a 2 % agarose gel to visualize possible resistance genes. Multiplex 2 from the winter isolates are used as an illustration (figure 6). Additional multiplex images can be found in appendix B.

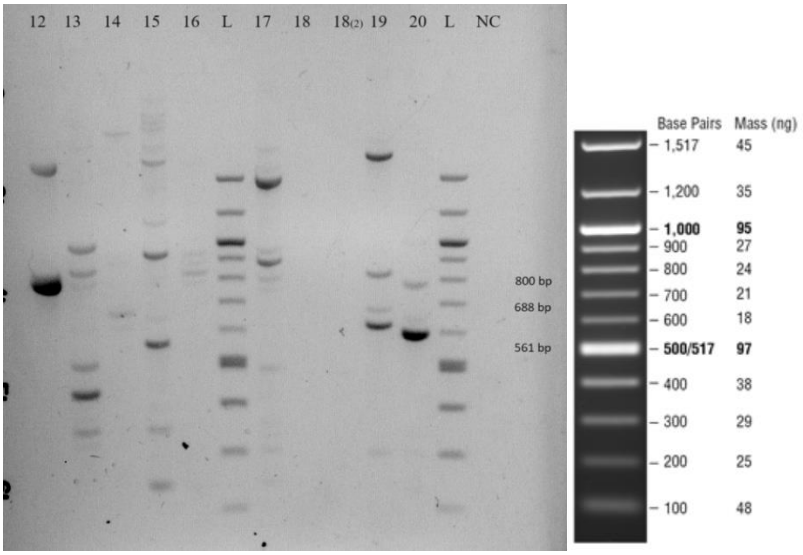


Figure 6. PCR-product of isolates E12-E20 and negative control (NC) of multiplex 2. Primer pairs in the mix was CTX-M (gr. 9), CTX-M (gr. 1) and TEM with expected band lengths of 561 bp, 688 bp and 800 bp, respectively. The ladder is Quick Load Purple 100 bp DNA Ladder. The 100 bp band has been cropped out from the gel picture.

Isolates 12, 13, 16, 17, 19, and 20 all displayed bands at approximately 800 bp. No bands perfectly aligned at 688 bp, and only isolate 15 had a band overlapping at 561 bp.

A new, singleplex PCR was run for the isolates showing bands at the expected lengths for one of the primer pairs. Additional bands closely aligning one of the desired band lengths were also included to make sure nothing was omitted. The weak bands barely

visible at ca. 688 bp in isolates 14 and 15 were also further tested, by running singleplex PCR for CTX-M (gr. 1). The singleplex agarose gel for the relevant isolates from multiplex 2, winter isolates, are shown in figure 7. A complete list of genes tested for each isolate by singleplex can be seen in appendix C.

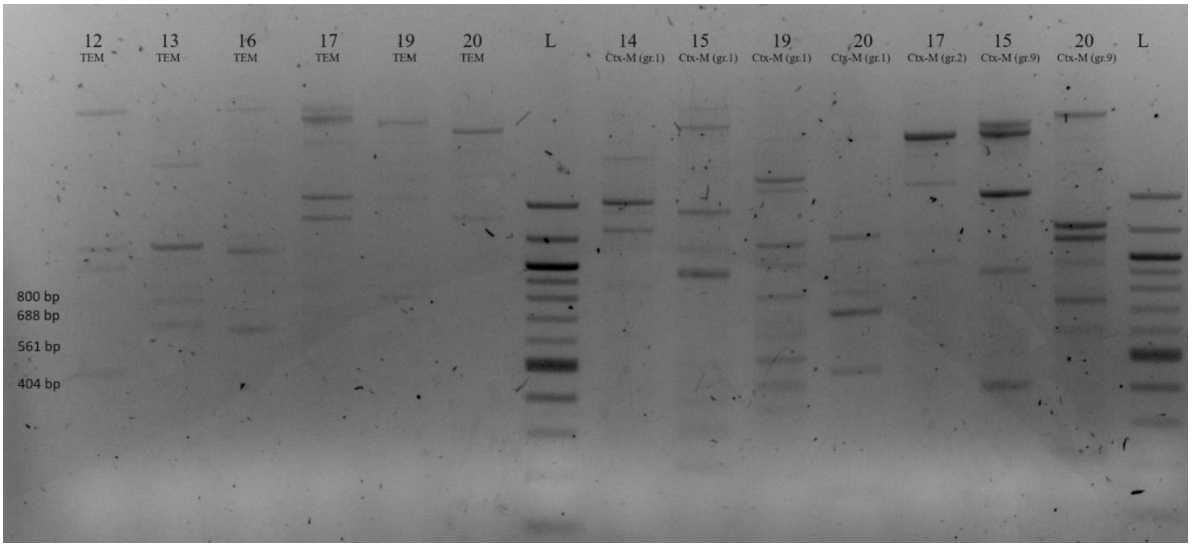


Figure 7. Visualisation of the singleplex bands of the isolates possibly displaying the gene for TEM (800 bp), CTX-M (gr. 1) (688 bp), CTX-M (gr. 2) (404 bp) and CTX-M (gr. 9) (561 bp). The ladder is Quick Load Purple 100 bp DNA Ladder.

Instead of displaying one single band at the desired lengths, multiple bands are seen in all isolates. This is a deviation from the expected singleplex result. Nonetheless, bands closely aligning the desired length were Sanger sequenced, but none revealed results corresponding to the gene of interest when BLASTn in NCBI was performed.

4.5 Antibiotic susceptibility test results

A total of 13 isolates were selected to assess their susceptibility towards seven different antibiotics. Two replicates of each antibiotic were performed for each isolate to better verify the results, and the mean value was calculated. The MIC values were interpreted following the examples presented in figure 8.

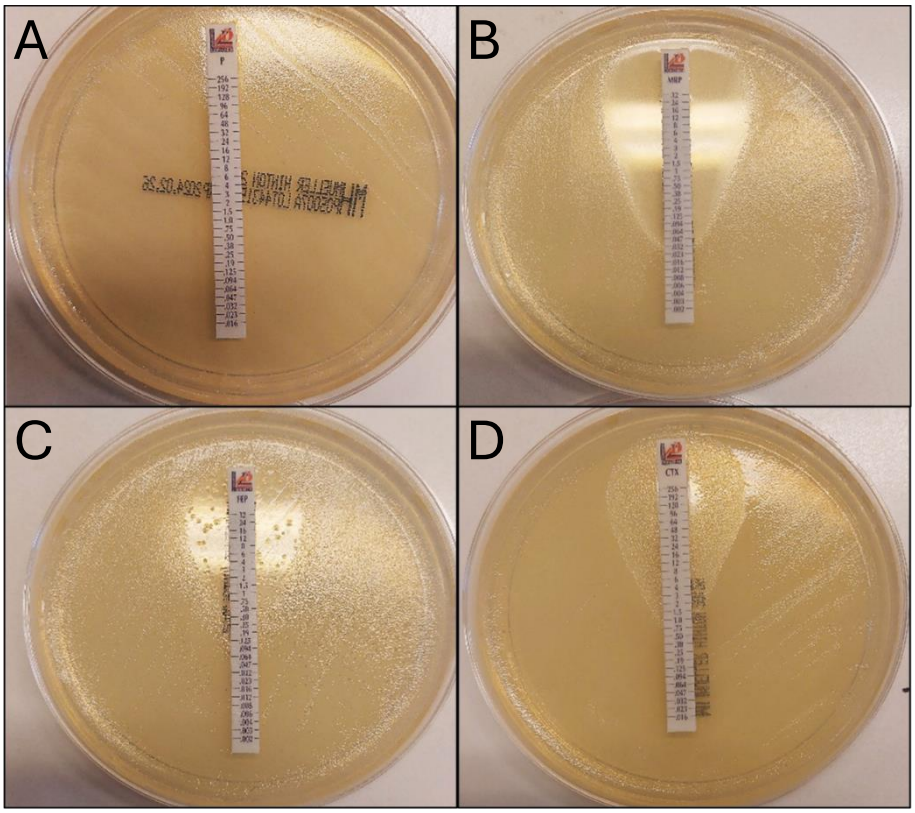


*Figure 8. (**A**) With no signs of bacterial inhibition, the MIC value is given as >256 or >32, depending on the max value of the E-strip. (**B**) The MIC-value of plates with clear inhibition zones are read as the lowest value inside the inhibition zone, in this example 0.032. (**C**) Plates with a clear inhibition zone but with bacterial colonies inside this zone are market with two stars (**) in front of the MIC-value. (**D**) Plates where a halo are seen but no clear zones, are given the maximum MIC-value and marked with one star (*).*

In **A**, there is no indication of bacterial inhibition by the antibiotic. Thus, the MIC-value is higher than the maximum of the E-strip, which is illustrated with the “>” sign. For plates where the inhibition zone exceeds the minimum value of the E-strip, a “<” symbol is used to indicate this. As there are no clear inhibition zone in **D**, this is also noted with the maximum value. To distinguish these plates showing some sign of inhibition, from the plates showing no signs of inhibition, an “*” is included in the table. **D** reveals a small, clear zone to the right of the E-strip, but as there are no such zone on the left, this has not been accepted as a clear zone. **C** also have colonies growing at maximum MIC-value, but they are distinctly different from the ones in **D** as they are single colonies inside a clear inhibition zone. Therefore, the value of the inhibition zone is given, but

marked with two stars. All values are presented in table 10, and pictures of the plates for isolate 4, 7, and 19 can be found in appendix D, figure D.1.

Table 10. The MIC values are given in µg/ml for each antibiotic for each isolate. The mean value is highlighted in a colour, where dark green represents bacterial susceptibility, yellow represents intermediate susceptibility, and dark red represents resilience towards the antibiotic. The lighter green and red colours are based on the EUCAST guidelines for when there are no breakpoint values, and represent if therapy with the agent should be discouraged (light red) or not (light green). The grey boxes indicate that no valid reference point has been found to determine bacterial susceptibility. The max/min column states the range of the MIC scale in µg/ml for each antibiotic.

Antibiotic	Isolate													Max/min value
	1	3	4	5	7	8	11	12	13	16	17	18	19	
Cefotaxime	0.69[1]	<0.016[1]	*256[2]	>256[1]	20[1]	0.625[1]	0.285[1]	**56[4]	**20[2]	4[3]	32[4]	>256	20[1]	256/0.016
Cefepime	0.75[1]	0.127[1]	3.5[2]	>32[1]	>32[1]	0.407[1]	0.094[1]	1[1,2]	0.38[2]	>32	1.5[1,2]	>32	*32[1]	32/0.002
Meropenem	0.142[1]	0.11[1]	0.064[2]	0.285[1]	1.5[1]	**0,5[1]	0.565[1]	0.125[1,2]	0.04[2]	0.44[3]	0.158[1,2]	>32	**5[1]	32/0.002
Penicillin G	>256[3]	0.094[3]	>256[3]	>256[3]	>256[3]	**256[3]	**144[3]	>256[3]	>256[3]	0.5[3]	>256[3]	>256	*256[3]	256/0.016
Ampicillin	140[3]	<0.016[3]	>256[3]	56[3]	22[3]	**256[3]	20[3]	>256[3]	>256[3]	1.25[3]	>256[3]	>256	*256[3]	256/0.016
Ciprofloxacin	0.003[1]	32[1]	0.023[2]	6[1]	*32[1]	12[1]	6.75[1]	0.125[1,2]	0.032[2]	0.22[3]	0.032[1,2]	>32	0.88[5]	32/0.002
Trimethoprim	20	*32	0.5	>32	>32	>32	32	>32	0.19	2.25	>32	>32	>32	32/0.002

Susceptibility references: [1] (CLSI, 2023), [2] (EUCAST, 2024a), [3] (EUCAST, 2024b), [4] (Thornsberry et al., 1982), [5] (Ba et al., 2004).

Table 10 shows that isolate 3, *Novosphingobium*, was susceptible to the most antibiotics tested, whereas isolate 19, *Stenotrophomonas,* and isolates 5 and 7, *Caulobacter,* showed the highest tendencies toward resilience. When preparing isolate 18 for antibiotic susceptibility testing, viewing in the microscopy revealed yeast-like cells (Appendix E). Its identity was ultimately confirmed as the yeast *Candida gleabosa* by Sanger sequencing, using the eukaryotic primers ITS, N4 and 18S. Therefore, the isolate was not further considered although it showed no growth inhibition to any antibiotic. Meropenem was the antibiotic exhibiting the highest bacterial inhibition effectivity, with only isolate 19 showing elevated resistance levels towards it. Penicillin G, followed by ampicillin, displayed the lowest inhibition effectivity. No breakpoint values for these specific antibiotics were given by neither EUCAST nor CLSI, hence, the susceptibility or resistance to these antibiotics could not be determined. However, the

values were checked against EUCASTs guidance on when there are no breakpoint tables. According to this guidance, the use of penicillin G or ampicillin is only advised for treatment of isolate 3. Breakpoint values for trimethoprim were only given in combination with sulfamethoxazole in both the CLSI and EUCAST tables, and the susceptibility or resistance to the bacteria could therefore not be evaluated for trimethoprim alone.

4.5 Analysis of whole genome sequencing

The assembled contigs created by Shovill in Galaxy from the WGS reads, were uploaded in PubMLST (https://pubmlst.org/bigsdb?db=pubmlst_rmlst_seqdef_kiosk) to verify the bacterium ID identified by NCBI's BLASTn, and to determine the bacterial strain. The PubMLST results are presented in table 11.

Table 11. The bacterial ID suggested by BLASTn based on the Sanger sequence, and suggested by PubMLST based on WGS sequence is listed, with the percentage support from PubMLST.

Isolate ID	BLASTn results	PubMLST conformation	Support (%)
4	*Serratia fonticola*	*Serratia fonticola*	96
7	*Caulobacter sp.*	*Caulobacter segnis*	36
19	*Stenotrophomonas maltophilia*	*Stenotrophomonas maltophilia*	100

PubMLST confirmed species 19 as *Stenotrophomonas maltophilia* with a 100 % support, and isolate 4 was strongly indicated as *Serratia fonticola*, with a support of 96 %. Isolate 7 was classified as a Caulobacter species via BLAST, and PubMLST suggested the species *Caulobacter segnis*, however, only with a 36 % support.

The consensus sequences were further used for typing and phenotyping in different services provided by Centre for Genomic Epidemiology (https://www.genomicepidemiology.org/services/). The results are presented in table 12.

Table 12. All isolates were typed against ResFinder and MGE in Centre for Genomic Epidemiology. Isolate 19 was additionally typed against MLST.

Bacterial ID	Service	Results	Note
4	ResFinder	blaFONA-6	96,62 % sequence identity
4	MGE	None	
7	ResFinder	None	
7	MGE	None	
19	ResFinder	blaL1	90,03 % sequence identity
19	MLST	Unknown	Nearest ST: 837
19	MGE	None	

Only two resistance genes among the three isolates, were found using ResFinder. The threshold was set to 90 %, meaning all possible resistance genes below this percentage were not displayed. Isolate 19 was the only one typed against MLST, as *Stenotrophomonas* was the only organism out of these three that could be selected for. The strain came back as unknown, with the nearest strain type being ST 837. The difference from this strain type can be seen in appendix F.

A mass screening of antibacterial genes was performed using the ABRicate tool, and screening against the NCBI Bacterial Antimicrobial Resistance Reference Gene Database and CARD. This provided the results presented in table 13. Virulence genes detected by screening against VFDB are found in appendix G, table G.2.

Table 13. Antibacterial genes found by the databases NCBI and CARD.

Isolate	Gene	Database(s)	Gene product	% Coverage	% Identity
4	FONA-6	CARD, NCBI	FONA-6 class A B-lactamase	100	96,62
4	H-NS	CARD	Histone-like protein repressing the membrane fusion protein genes acrE mdtE and emrK	97	81,58
4	CRP	CARD	Global regulator repressing MdtEF multidrug efflux pump expression	100	84,83
7	CAU-1	CARD, NCBI	CAU-1 class B3 metallo-B-lactamase	96,9	82,69
19	APH(3')-IIc	CARD, NCBI	Chromosomal-encoded aminoglycoside phosphotransferase	99,88	84,08
19	APH(6)-Smalt	NCBI	Putative aminoglycoside O-phosphotransferase	100	89,42

19	L1	NCBI	L1 family subclass B3 metallo-beta-lactamase	99,89	90,24
19	L1	CARD	blaL1 Class B3 metallo-B-lactamase	95,38	84,54
19	smeF	CARD	Outer membrane multidrug efflux protein of the smeDEF complex	96,79	93,36
19	smeE	CARD	RND protein of the efflux complex smeDEF	99,55	97,11
19	smeD	CARD	Membrane fusion protein of the smeDEF multidrug efflux complex	100	96,62

The mass screening detected more possible resistance genes than ResFinder, also finding a possible resistance gene in isolate 7. The presumptive FONA-6 found in isolate 4 was given the same sequence identity as in ResFinder, while the L1 found in isolate 19 had a lower sequence identity when screened against CARD, and slightly higher when screened against NCBI, compared to ResFinder. CARD additionally detected the presence of the *sme*DEF efflux pump protein in isolate 19.

The GenBank file (gbk.) provided from Prokka was opened in Excel, and a search for lactamases and resistance genes was conducted. A BLASTp of the sequence of all suggested lactamases was performed and the results are listed in table 14. The resistance genes found are listed in appendix G, table G.1.

Table 14. The NCBI BLASTp result off all predicted lactamases, along with their query coverage, identity, and accession number.

Isolate	**Gene**	**Product**	**Query coverage (%)**	**Identity (%)**	**Accession number**
4	*bla*FONA-8	Class A B-lactamase FONA-8	100	100	WP_024530279.1
4	*bla*SFDC	SFDC family class C beta-lactamase	100	99,21	WP_074031142.1
7		TIGR01244 family sulphur transferase	100	94,51	WP_309999812.1
7	*bla*	Class A B-lactamase	100	92,19	WP_013080480.1
7		Serine hydrolase	100	89,9	WP_099442791.1
19	*bla*L1	L1 family subclass B3 metallo-beta-lactamase	100	98,78	WP_332329658.1
19	*bla*L2	L2 family extended-spectrum class A B-lactamase	100	100	WP_329848933.1

The BLASTp results revealed that two of the three presumptive lactamases found in isolate 7 were not lactamases, but rather a transferase and a hydrolase. The remaining

five BLAST searches came back positive for lactamases. *bla*FONA-8 and *bla*L2 had a 100 % identity with their subject strains, which for isolate 19 was a *Pseudomonas hibiscicola (*appendix H, figure H.2). The β-lactamase found in isolate 7 was suggested as a class A β-lactamase by BLASTp in NCBI, in contrast to the suggestion from the antimicrobial mass screening in NCBI and CARD, presented in table 13, which suggested a class B metallo-β-lactamase.

To further investigate the genetics of the FONA-8 gene, an alignment and a phylogenetic tree were made between this gene, three other FONA-genes found in previous theses from NMBU, and four FONA-genes found in the NCBI database. The alignment, and construction of phylogenetic tree, was performed by Professor Bjørn-Arne Lindstedt, using UniProt Align (The UniProt Consortium, 2023) and the multiple sequence alignment tool Clustal Omega from EMBL-EBI, respectively. The phylogenetic tree is presented in figure 9, while the multiple sequence alignment are found in appendix H, figure H.3.

Figure 9. The phylogenetic tree depicting the evolutionary distance between the FONA-8 gene found in this study (E4), and seven other FONA-genes.

The FONA-8 found in this study (E4) was identical with a FONA-8 found in the NCBI database. The gene had highest similarity to A16_Jonsvatnet, followed by A15_Fossbekken, to which it differed by only one amino acid. Furthermore, it differed from FONA-6 by 2 amino acids, FONA-5 and As5_NMBU by 6 amino acids, and FONA-1 by 8 amino acids.

The quality assessment of isolate 4, 7, and 19 yielded good results, each revealing an average number of 0.0 N's per 100 kbp (appendix I).

5 Discussion

5.1 Phenotypic and genotypic bacterial determination

The Brilliance™ CRE and ESBL screening plates can quickly and easily help indicate if samples contain carbapenemase or ESBL producing bacteria. As all the collected water samples yielded some bacterial growth when grown on the plates, an indication of the presence of bacteria harbouring these genes was given. Sample W3 grew fewer colonies compared to the other water samplings, hinting at a lower occurrence of CRE and ESBL producers at this sample site. As this water was collected from a private well supplying three households with drinking water, this discovery must be considered preferable. The well has its source from natural groundwater, which is a more protected water source than surface water, and is generally considered to be of higher quality. Although runoff, erosion, and leakage of ARG to the groundwater do occur, a lower abundance of microbes, therein ARB, is expected in groundwater compared to surface water (Tollan, 2023; Zhang et al., 2009). However, the low growth on the screening plates cannot rule out the presence of non-resistant bacteria, or resistant bacteria with other resistance mechanisms.

Only a few of the isolated bacteria displayed a colour resembling the ones described by the producer, suggesting most of the isolates to be bacteria other than *E. coli* and bacteria in the KESC-group. The BLASTn result of the 16s rRNA Sanger sequence revealed isolate 4, 13, and 15, all displaying green colonies on the ESBL plates, to correspond with the bacterial group predicted by the manufacturer, as all were identified as *Serratia sp*. The genotypic results revealed that none of the remaining isolates belonged to the *Enterobacteriaceae* family (table 7), explaining why these isolates could not easily be phenotypically identified by the Brilliance™ CRE and ESBL plates, as they focuses mainly on detecting bacteria in the *Enterobacteriaceae* family.

The phenotyping is thus not a reliable method for determining bacterial species other than the ones specified by the producer. However, having some knowledge surrounding bacteria phenotypic traits, can give an indication of their identity. For example, isolates 1 and 2 displayed a dark, violet colour, and BLASTn identified them as *Chromobacterium*. Most isolates from this genus carry the pigment Violacein, causing them to appear violet

(Benomar et al., 2019). Thus, the phenotypic and genotypic results for these isolates corresponded well.

5.2 DNA purity and identification of isolate 18

The purity of the extracted DNA revealed a mostly successful DNA extraction, as a 260/280 ratio around 1.8 is accepted as pure DNA (Matlock, 2015). However, isolate 18 displayed a negative 260/280 ratio, giving a clear indication of an abnormal isolate. The isolate was eventually identified as a yeast, which explained the low purity, as the kits used in this thesis are meant for bacteria. Additionally, this explains why this isolate was resistant to all antibiotics tested against, as these antibiotics are not designed to affect eukaryotes.

5.3 Screening for β-lactamases

When looking for β-lactamase genes, the multiplex gels revealed bands possibly matching one of the desired target sequences. The subsequent singleplex however, resulted in multiple bands for most isolates. As singleplex PCR only amplifies one target sequence, one band per isolate is expected. To improve the singleplex results, some troubleshooting was performed, but none were deemed effective. One explanation as to why the singleplex yielded multiple bands, is that the primers have been developed to fit clinical isolates of specific bacteria. The bacteria investigated in this thesis are all environmental isolates, and many of them are not well studied clinically (Rizzo et al., 2013). Therefore, the primers possibly did not fit the bacteria in question, and bound unspecifically. One evidence to support this assumption was that the positive control, a clinically well-studied *K. pneumoniae* bacterium, only revealed one, clear band on the gel. This demonstrated a successful protocol and expected results, when working with bacteria known to fit the primer pairs.

Following BLASTn of the sequences closely aligning the desired sequence lengths, the results showed that none of them matched the presumptive gene. As there were a poor match between the bands and the expected band-length, this was as expected.

5.4 Evaluation of antibiotic susceptibility

The antibiotic susceptibility results (table 10) revealed the highest tendency to resistance towards penicillin G and ampicillin. This can be explained by the fact that all bacteria, except isolate 16, are gram-negative, while penicillins have a better coverage against gram-positive bacteria (Benomar et al., 2019; Kämpfer et al., 2006; Takeuchi et al., 2001). This is further supported by the fact that isolate 16 displayed a noticeably lower MIC-value to these antibiotics. Many β-lactam antibiotics struggle to get through the gram-negative bacteria's first layer of protection, the outer membrane, due to their hydrophobicity. With the addition of an amino group, ampicillin is better suited than penicillin G to tackle gram-negative bacteria. This was true for four of the isolates tested, who displayed a lower MIC-value for ampicillin compared to penicillin G. EUCAST and CLSI had not given any breakpoints to determine susceptibility or resistance towards penicillin G and ampicillin, for the bacteria identified in this thesis. However, the EUCAST guidance for when no breakpoints are given in the tables, considered these MIC-value as so high that they discouraged the use of these antibiotics as a treatment option for all bacteria, except for isolate 3 (EUCAST, 2024b). Isolate 3 was identified as the bacterium *Novosphingobium*, and despite being gram-negative, displayed the highest susceptibility to both penicillin G and ampicillin, with MIC values as low as 0.094 and <0.016, respectively. To understand why this is so, further investigation of the bacterium would have to be performed.

Of the antibiotics tested, meropenem was deemed the most effective overall, with MIC-values below the susceptible breakpoints published by EUCAST and CLSI for all isolates, except isolate 19. As meropenem is a carbapenem-antibiotic, often used as a last-resort antibiotic, it was as expected to find most of the bacteria susceptible to this antibiotic. Isolate 19 exhibited an elevated MIC value of 5, which was above the CLSI susceptibility breakpoint for non-*Enterobacteriales,* set at 4, but below the resistance breakpoint set at 16, and was therefore considered intermediately resistant.

Isolate 3 showed the broadest susceptibility, being susceptible to 5 out of the 7 antibiotics tested for. In contrast, isolate 19 was only susceptible to one antibiotic, namely ciprofloxacin. If isolates showing no inhibition towards an antibiotic (marked with >x) are accepted as resistant to this antibiotic, isolates 5, 7, and 12 are considered

MDR bacteria (Magiorakos et al., 2012). Isolates 5 and 7 displayed resistance to antibiotics in the classes penicillin, cephalosporin, fluoroquinolone and antifolate, and isolate 12 to the classes penicillin, cephalosporin, and antifolate.

Isolate 12, *Pseudomonas aeruginosa*, is an opportunistic human pathogen. The organism is extremely resilient to different environmental conditions, able to survive temperatures from 4-42°C, and to live on dry, abiotic surfaces for up to six months. These factors contribute to the bacterium's influence as a nosocomial pathogen (Liao et al., 2022). Carbapenem-resistant *P. aeruginosa* was categorized as priority 1: critical, in WHO's priority pathogens list for research and development of new antibiotics, published in 2017 (World Health Organization, 2017). Although the *Pseudomonas* species observed in this thesis showed high susceptibility to meropenem, it is important to monitor its susceptibility profile, so that quick action can be taken if carbapenem-resistant *P. aeruginosa* were to be detected in the environment.

Isolate 8, *Herbaspirillum*, showed high susceptibility towards meropenem, with a mean value of 0.5, but in both parallels some colonies were observed growing inside the inhibition zone (appendix D, figure D.2). This could be due to spontaneous mutations leading to acquired resistance, or due to heteroresistance (Gutiérrez et al., 2021). Heteroresistance is a phenotype of a bacterial isolate, where a subpopulation have an increased level of resistance compared to the main population. This can cause the subpopulation to grow at a higher antibiotic level than the rest, leading to the appearance of colonies growing inside the inhibition zone (Andersson et al., 2019). *Herbaspirillum* is a rare human pathogen, but *H. huttiens* has shown to cause serious infections, even in immunocompetent patients (Bloise et al., 2021; Ruiz de Villa et al., 2023). Publications on clinical findings, susceptibility profiling and epidemiology for this organism is limited, but as a possibly emerging human pathogen, increased research and knowledge surrounding this bacterium is important (Bloise et al., 2021).

5.5 Whole genome sequencing

WGS was performed on isolates 4, 7, and 19. Isolate 19 was chosen, as it displayed high levels of resistance to most antibiotics during the antibiotic susceptibility test,

particularly towards meropenem. Isolate 7 was a possible MDR bacterium based on the MIC results, and had the highest MIC value for meropenem, other than isolate 19. Isolate 4 was chosen because Serratia is a relevant clinical isolate, and among the bacteria known to produce ESBLs resistant to different antibiotics. PubMLST identified isolate 19 as *S. maltophilia* with 100 % support, and strongly suggested isolate 4 as *S. fonticola* with 96 % support (table 11). Isolate 7 was proposed as the species *C. segnis*, but had only 36 % support. This suggests that the *Caulobacter* species isolated in this thesis might still be uncultured, but has the highest sequence identity with *C. segnis* out of the yet cultured species.

5.5.1 WGA analysis of isolate 4, *Serratia fonticola*

Serratia is a genus in the *Enterobacteriaceae* family. The species are free-living, gram-negative, and ubiquitously distributed in the environment (Williams et al., 2022). Most *Serratia* species are considered rare human pathogens, and the majority of infections caused by this bacterium are attributed to *S. marcescens,* which can cause a range of infections (Sandner-Miranda et al., 2018). However, recent reports have described cases of MDR *S. fonticola* causing serious, difficult-to-treat infections, sometimes leading to lethal outcomes (Hai et al., 2020; Kunjalwar et al., 2024). This rise in MDR bacteria is a cause for concern surrounding the pathogens in the *Serratia* genus (Williams et al., 2022).

The results presented in table 14 reveal the identification of two lactamases in this isolate. One had the highest sequence identity to an SFDC family class C β-lactamase. SFDC-1 was first identified in *S. fonticola* in 2021, as a novel chromosomal-encoded AmpC β-lactamase (Dong et al.). The *bla*SFDC-1 gene was suggested to be conserved in *S. fonticola*, as all genes found in the NCBI database, with sequence similarity >92.72 % to the *bla*SFDC-1 investigated in the paper, came from this species. Thus, there is currently no evidence suggesting that the gene has been transferred to other species, which corresponds with the gene being located on the chromosome. The complete ORF *bla*SFCD-1, along with its promoter region, was cloned into a pUCP24 vector, to determine the resistance effectivity of the gene. The recombinant strain revealed elevated resistance activity against different β-lactam antibiotics, including penicillin G, ampicillin, and all cephalosporins, except cefoselis. However, the recombinant strain

showed no elevated MIC values for the two carbapenems tested, meropenem and imipenem (Dong et al., 2021). These findings coincide with the MIC values seen in table 10. Isolate 4 showed susceptibility towards meropenem, and resistance or intermediate resistance towards the two penicillins and cephalosporins. These findings further support that the presumptive *bla*SFCD found in isolate 4 is closely related to the *bla*SFDC-1 characterized by Dong et al.

The other β-lactamase found by in the GenBank-file was identified as class A β-lactamase FONA-8 by the NCBI GenBank, having 100 % sequence identity to the subject sequence (Appendix H, figure H.1). ResFinder, NCBI's antibacterial resistance database, and CARD all suggested the lactamase FONA-6 with 96.62 % support, which corresponds to FONA-6 being the gene available in these databases, who resembles FONA-8 the most (figure 9).

*bla*FONAs are minor ESBL genes encoded in the *S. fonticola* chromosome. FONAs are species specific for *S. fonticola*, but another minor, plasmid-encoded ESBL gene, called *bla*SFO-1, is believed to have derived from *bla*FONA. This gene has been found in *Enterobacter cloacae* and could mean that FONA derivatives can be transferred between members of the *Enterobacteriaceae* family (Dong et al., 2021; Tanimoto et al., 2021). Whether or not it can be transferred outside of this family is not yet known.

In their study, Tanimoto et al. tested the antibiotic susceptibility of five FONA-producing *S. fonticola* strains (2021). Their findings correspond well with the susceptibility profile of the two *S. fonticola* strains isolated in this thesis, revealing high susceptibility towards meropenem and ciprofloxacin, and high resistance towards ampicillin. Moreover, the resistance towards cefotaxime varied greatly among the five strains, with MIC values ranging from 4 to >128 (Tanimoto et al., 2021). Interestingly, a great variation in resistance towards this antibiotic was also seen in this study, with the MIC values for isolates 4 and 13 being >256 and 20, respectively. This could mean that factors other than the FONA genes are responsible for these bacteria's resistance to cefotaxime, but to answer this, further investigation would have to be conducted.

As presented in the phylogenetic tree, FONA-8 resembled isolate A16_Jonsvatnet, followed by isolate A15_Fossbekken, the most, differing only by one amino acid.

Interestingly, isolate A15_Fossbekken, differs from FONA-8 by an Ala→Val substitution at position 24, while A16_Jonsvatnet, has an Asn→Asp substitution at position 106 (appendix H, figure H.3). Alanine (Ala) and valine (Val) are amino acids with similar chemical properties, as both have hydrophobic side chains and are of approximately the same size. Thus, a substitution between them would not have a great impact on the property of the protein. Asparagine (Asn) is a polar amino acid, with uncharged side chains, while aspartic acid (Asp) has a negative side chain. This substitution would therefore have a greater impact, making isolate A16_Jonsvatnet, genetically farther apart from FONA-8 than A15. However, this is not the case.

5.5.2 WGS analysis of isolate 7, *Caulobacter sp.*

The *Caulobacter* genus consists of gram-negative, rod-like shaped bacteria. *Caulobacter* have been observed in the rhizosphere, soil and in aqueous environments and was for a long time considered a non-pathogenic bacterium. However, the last couple of decades have seen reports of infections associated with *Caulobacter* species. The reported cases of *Caulobacter* infections are hospital-acquired by immunocompromised patients, emphasizing the species' role as an opportunistic pathogen (Moore & Gitai, 2020). *Caulobacter crescentus* has properties making it suitable for bioengineering applications, such as bioremediation for heavy metal and anti-tumour immunization (Bhatnagar et al., 2006; Patel et al., 2010). Therefore, the establishment of its pathogenicity is important.

ResFinder did not detect any lactamases in isolate 7, while NCBI identified the presence of a presumptive class A β-lactamase. Class A β-lactamases are recognized by three conserved motifs, namely S-X-X-K, S-D-N, and K-T-G at position 70, 130, and 234, respectively (Singh et al., 2009). All motifs are present in the lactamase sequence of isolate 7, but at positions 76, 136 and 244. Although not present at the exact position as described by Singh et al., this gives a clear indication of its class A identity. Previous literature on *Caulobacter sp*. has only reported findings of class B metallo-β-lactamases in the *C. crescentus* species (Docquier et al., 2002; Simm et al., 2001). This makes the finding of a class A-lactamase interesting, although the lack of this reporting in previous studies could be due to scarce research on *Caulobacter* in general, as it is considered a rare human pathogen (Penner et al., 2016).

5.5.3 WGS analysis of isolate 19, *Stenotrophomonas maltophilia*

Stenotrophomonas is a genus of gram-negative bacteria found in different environmental niches. *S. maltophilia* is a ubiquitous bacterium and is as of 2021 the only validated human pathogenic species in the *Stenotrophomonas* genus (Patil et al., 2021). *S. maltophilia* is an opportunistic pathogen and causes a range of infections, where lung infection in individuals with cystic fibrosis (CF) is of particularly concern (Bhaumik et al., 2024). Mortality rates of *S. maltophilia* bacteraemia are high, having been reported between 21-69 % (Kim et al., 2019). A concerning aspect of *S. maltophilia* is its intrinsic antibiotic resistance, making it resistant to a multitude of common antibiotics, including carbapenems (Bhaumik et al., 2024; Brooke, 2014; Urase et al., 2022).

Two lactamases were identified in the *S. maltophilia* isolate: namely *bla*L1 and *bla*L2. The BLASTp result revealed the L2-lactamase to be identical with an L2 β-lactamase previously found in *Pseudomonas hibiscola*. According to Van den Mooter & Swings (1990), *P. hibiscola* is a synonym of *Xanthomonas maltophilia*, which has later been reclassified as *S. maltophilia* (Denton & Kerr, 1998).

L1, a class B metallo-β-lactamase, and L2, a class A β-lactamase, are known to be intrinsic in *S. maltophilia*, and the cause for its β-lactam resistance (Okazaki & Avison, 2008). The metallo-β-lactamases, in particular, are known to exhibit carbapenem resistance (Liu et al., 2012). The presence of this gene can explain the elevated resistance of *S. maltophilia* towards meropenem, compared to the other isolates tested in this study. However, the MIC value is not high enough to be considered resistant. This could be due to the presence of inhibitors, hindering its abilities to hydrolyse β-lactam antibiotics, but this cannot be confirmed without further research. L1 and L2 are inducible, and are produced at high levels during β-lactam challenge due to the *ampR* gene found immediately upstream of *bla*L2 (Okazaki & Avison, 2008). Blocking the expression of the *ampR* gene or the function of the ampR protein has therefore been suggested as possible targets to tackle the β-lactam resistance in *S. maltophilia* (Lin et al., 2009).

Trimethoprim displayed no inhibition of the bacteria in this study, but the combination of trimethoprim/sulfamethoxazole (TMP/SMX) has been the treatment of choice for

infections caused by *S. maltophilia*. However, the TMP/SMX treatment option is challenged by the overexpression of the multidrug efflux pump smeDEF (Sánchez & Martínez, 2018). The presence of this efflux pump was detected in sample 19 by CARD (table 13), but to determine its expression level and subsequently its role in *S. maltophilia* resistance profile, further research is needed.

A comparative study revealed similar mortality between groups treated with TMP/SMX and those treated with fluoroquinolones, when infected by *S. maltophilia* (Junco et al., 2021). Of the antibiotics tested in this study, only the fluoroquinolone ciprofloxacin revealed susceptibility towards the bacteria, suggesting its potential as a useful drug to tackle infections caused by *S. maltophilia*.

6 Conclusion

The aim of this study was to investigate the occurrence of ARBs in Norwegian water habitats, with a focus on β-lactamase producers. All sample sites were selected based on their proximity to human activity, meaning that possible pathogenic bacteria in these habitats could potentially be transferred to humans, and cause infectious diseases.

The water samples taken in this study, harboured mainly non-pathogenic and treatable bacteria, given by the fact that all revealed high susceptibility to at least one antibiotic. This does not, however, exclude the possibility of problematic bacteria being present in these water habitats, as only one sample was taken from each site. A more comprehensive study would need to be conducted at each site, to properly conclude whether or not pathogenic ARBs can be found here.

The β-lactamase genes identified in this study belonged to Ambler classes A, B, and C. Out of these, the L1 lactamase found in *S. maltophilia* could be considered the most problematic yet, as it is intrinsic in the *S. maltophilia* genome and known to exhibit resistance to carbapenems. However, the growing issue of serious infections caused by *S. fonticola* calls for a greater awareness surrounding this bacterium. More research is needed to determine whether the FONA genes are the main contributor to the bacterium's pathogenicity.

Even though the bacteria detected in this study are not considered the most critical pathogens, it is worth noting that serious infectious diseases caused by these bacteria, have been reported. Mapping the occurrence of ARBs at different sites is therefore important, as a way to monitor the spread and development of them. This study revealed antibiotic resistant bacteria at all sample sites, and three of the bacteria could be considered MDR, as they were resistant to antibiotics in three or more antibiotic classes. This emphasise the ubiquity of antibiotic resistance, and shows that ARBs can be found in many water environments. However, to fully understand the extent of this issue, further comprehensive studies on this subject must be conducted.

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8 Appendices

Appendix A – Primer sequences

Table A.1. The primer sequence of the forward and reverse primer of each resistance gene tested for in this thesis, along with the length of the gene product and their references.

Gene	Primer sequence (5'-3')	Sequence length (bp)	Reference
*bla*CTX-M (gr. 2)	F- CGTTAACGGCACGATGAC R- CGATATCGTTGGTGGTTCCAT	404	(Dallenne et al., 2010)
*bla*OXA	F- GGCACCAGATTCAACTTTCAAG R- GACCCCAAGTTTCCTGTAAGTG	564	(Dallenne et al., 2010)
*bla*SHV	F- AGCCGCTTGAGCAAATTAAAC R- ATCCCGCAGATAAATCACCAC	713	(Dallenne et al., 2010)
*bla*CTX-M (gr. 9)	F- TCAAGCCTGCCGATCTGGT R- TGATTCTCGCCGCTGAAG	561	(Dallenne et al., 2010)
*bla*CTX-M (gr. 1)	F- TTAGGAARTGTGCCGCTGYA R- CGATATCGTTGGTGGTRCCAT	688	(Dallenne et al., 2010)
*bla*TEM	F- CATTTCCGTGTCGCCCTTATTC R- CGTTCATCCATAGTTGCCTGAC	800	(Dallenne et al., 2010)
*bla*NDM	F- TGGCCCGCTCAAGGTATTTT R- GTAGTGCTCAGTGTCGGCAT	157	(Finton et al., 2020)
*bla*VIM	F- ATAGAGCTCAGTGTGTCGGCAT R- TTATTGGTCTATTTGACCGCGT	564	(Finton et al., 2020)
*bla*KPC	F- TCCGTTACGGCAAAAATGCG R- GCATAGTCATTTGCCGTGCC	460	(Finton et al., 2020)
*bla*CMY	F- GCATCTCCCAGCCTAATCCC R- TTCTCCGGGACAACTTGACG	188	(Finton et al., 2020)
*bla*OXA-48	F- GCTTGATCGCCCTCGATT R- GATTTGCTCCGTGGCCGAAA	281	(Dallenne et al., 2010)
*bla*IMP	F- ACAGGGGGAATAGAGTGGCT R- AGCCTGTTCCCATGTACGTT	393	(Finton et al., 2020)
rpoB	F- CAGGTCGTCACACGGTAACAAG R- GTGGTTCAGTTTCAGCATGTAC	512	Universal primers
16S rRNA	F- GAGTTTGATCCTGGCTCAG R- GGTTACCTTGTTACGACTT	1505	Universal primers

Appendix B – Multiplex PCR, agarose gel pictures

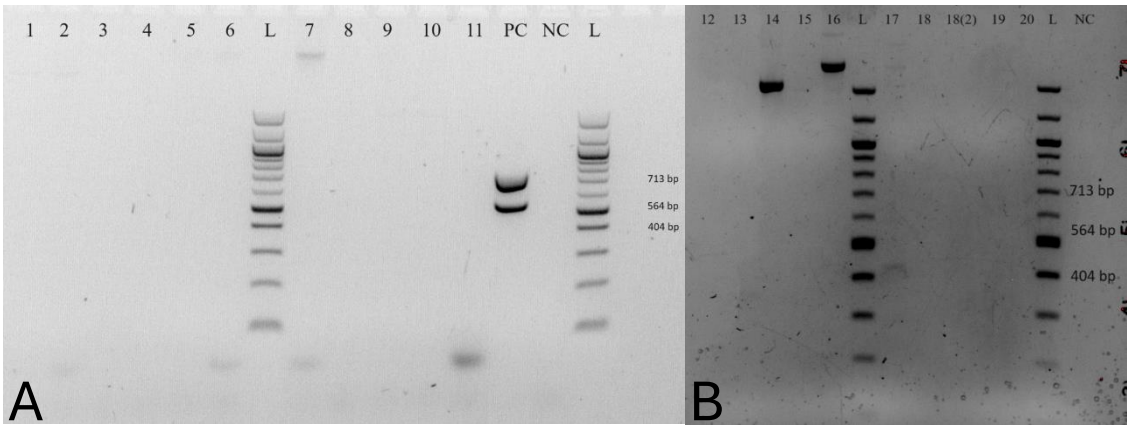

*Figure B.1. Multiplex 1 from (**A**) summer isolates and (**B**) winter isolates. Containing primers for CTX-M (gr. 2) (404 bp), OXA (564 bp) and SHV (713 bp). Positive control (K. pneumoniae) for OXA and SHV. Ladder is Quick Load Purple 100 bp DNA Ladder.*

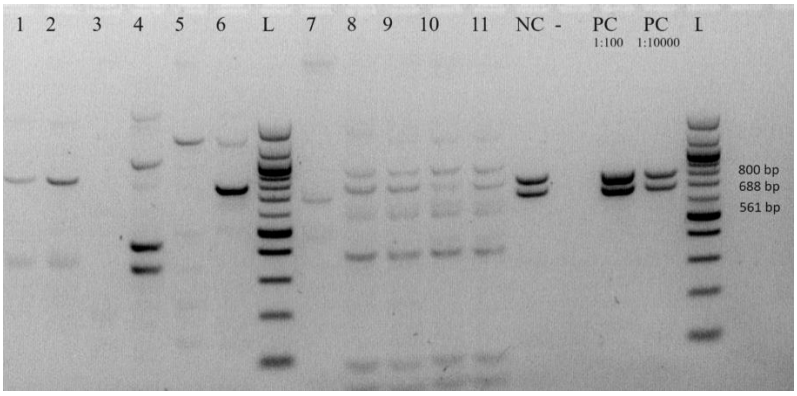

Figure B.2. Multiplex 2 from summer isolates. Containing primers for CTX-M (gr. 9) (561 bp), CTX-M (gr. 1) (688 bp) and TEM (800 bp). Positive control (K. pneumoniae) for CTX-M (gr. 1) and TEM. Ladder is Quick Load Purple 100 bp DNA Ladder. Negative control is contaminated.

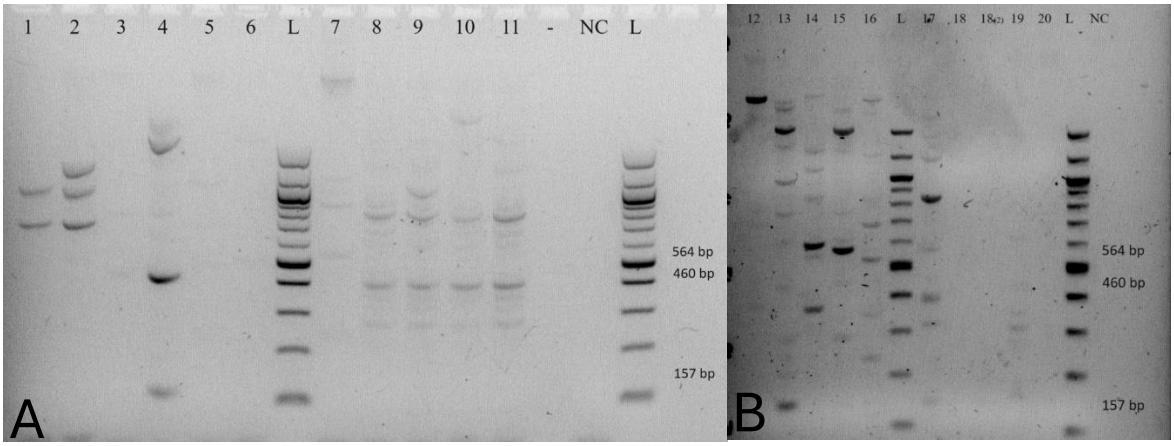

*Figure B.3. Multiplex 3 from (**A**) summer isolates and (**B**) winter isolates. Containing primers for NDM (157 bp), VIM (564 bp) and KPC (460 bp). Ladder is Quick Load Purple 100 bp DNA Ladder.*

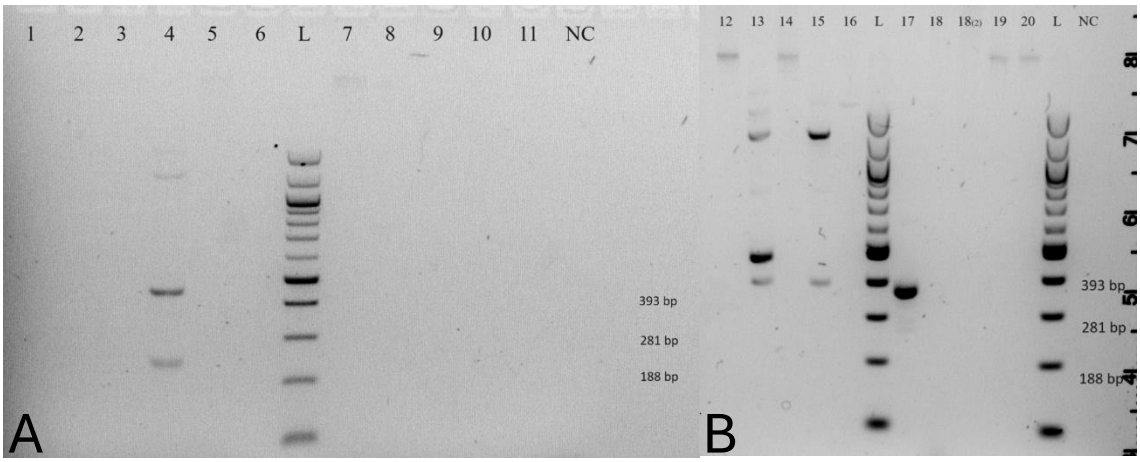

*Figure B.4. Multiplex 4 from (**A**) summer isolates and (**B**) winter isolates. Containing primers for CMY (188 bp), OXA-48 (281 bp) and IMP (393 bp). Ladder is Quick Load Purple 100 bp DNA Ladder.*

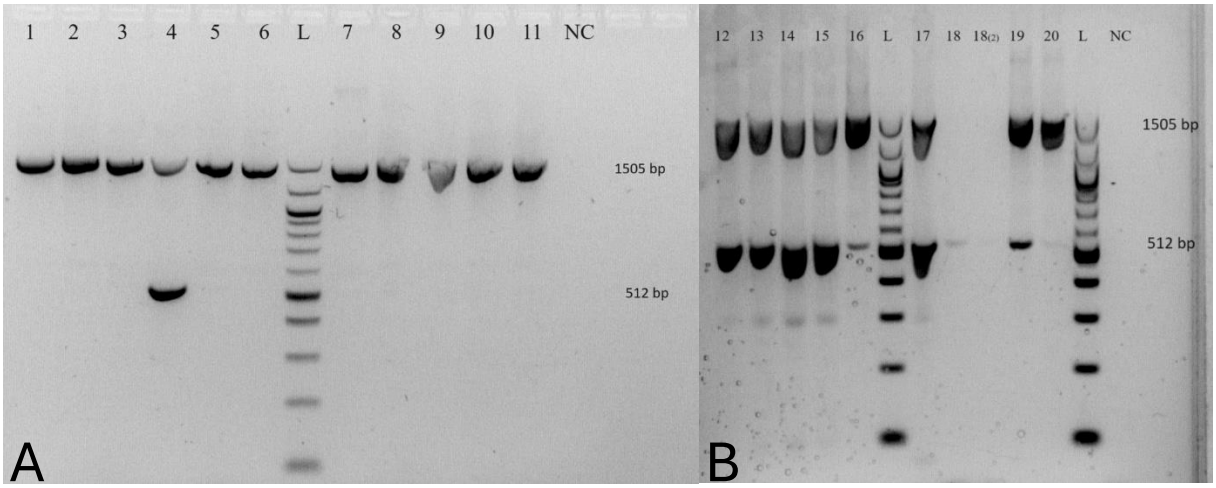

*Figure B.5. Multiplex 5 (control) from (**A**) summer isolates and (**B**) winter isolates. Containing primers for 16S rRNA (1505 bp) and rpoB (512 bp). Ladder is Quick Load Purple 100 bp DNA Ladder.*

Appendix C – Genes tested for by singleplex PCR

Table C.1. Full list of which resistance genes were tested for by singleplex PCR for each isolate.

Gene \ Isolate	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
CTX-M 1	x	x	x				x	x	x	x	x			x	x				x	x
CTX-M 2																	x			
CTX-M 9	x	x		x	x	x	x								x					x
TEM	x	x	x	x	x	x		x	x	x	x	x	x			x	x		x	x
NDM				x									x				x		x	
VIM							x							x	x	x	x			
KPC			x	x				x	x	x	x		x		x				x	
CMY				x																
OXA-48				x																
IMP				x									x		x					

Appendix D – Antibiotic susceptibility

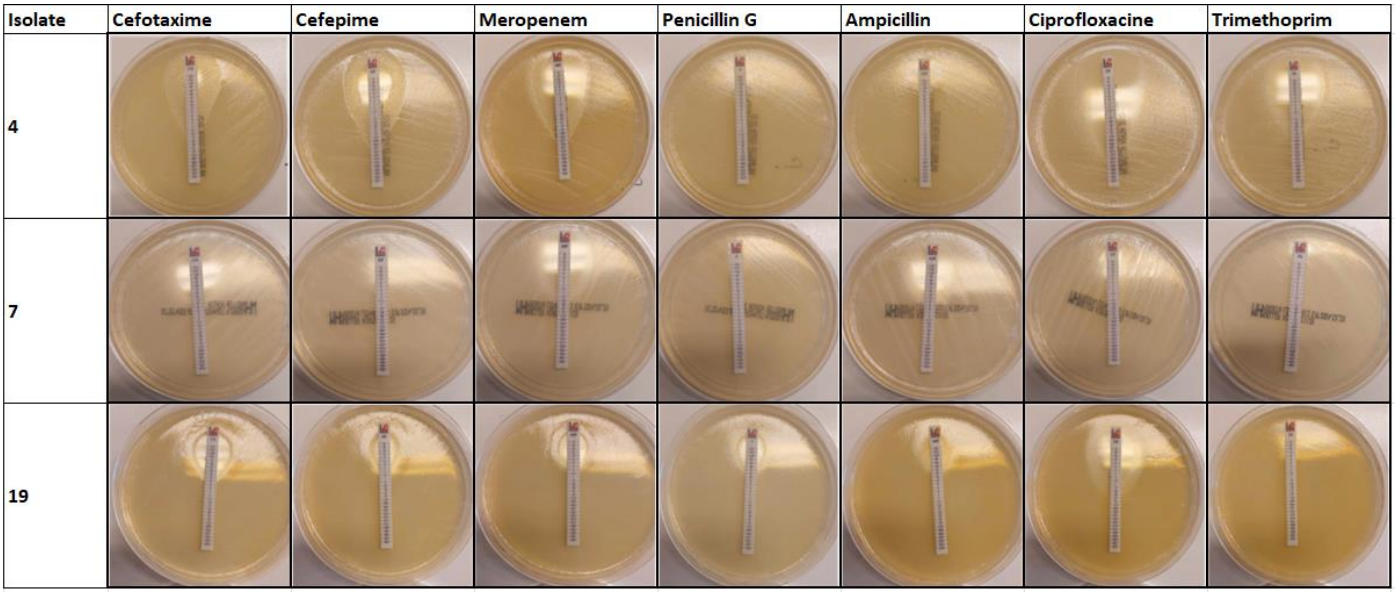

Figure D.1. One replicate of isolates 4, 7, and 19, displaying their antibiotic susceptibility.

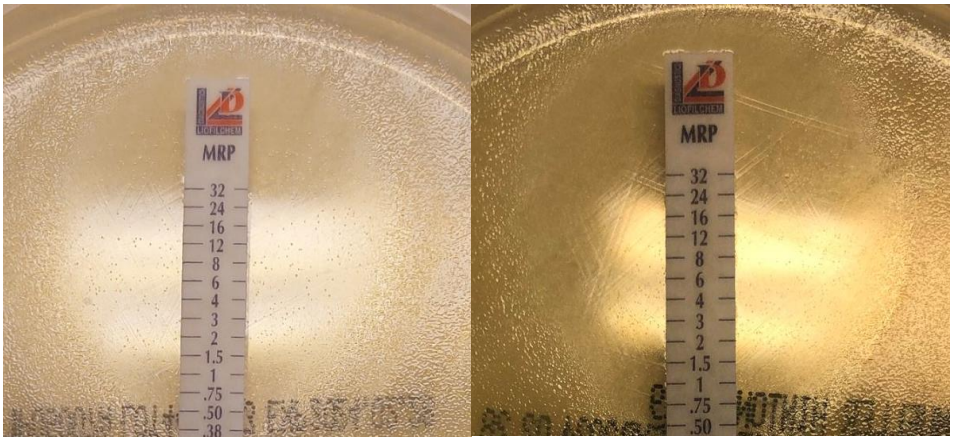

Figure D.2. The MIC sensibility testing of meropenem on isolate 8 displays some minor colonies inside the inhibition zone in both parallels.

Appendix E – Microscopy picture of isolate 18

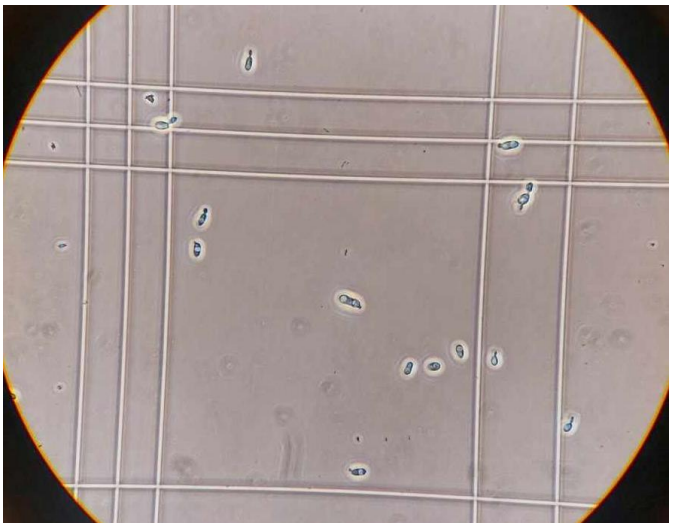

Figure E.1. Isolate 18 under microscop, revealing yeast cell structure.

Appendix F – MLST result of isolate 19

MLST-2.0 Server - Results

mlst Profile: *smaltophilia*

Organism: *Stenotrophomonas maltophilia*

Sequence Type: *Unknown*

Nearest ST: *837*

Locus	Identity	Coverage	Alignment Length	Allele Length	Gaps	Allele
atpD	100	100	531	531	0	atpD_78
gapA	100	100	558	558	0	gapA_145
guaA	99.8188	100	552	552	0	guaA_410*
mutM	100	100	465	465	0	mutM_239
nuoD	100	100	444	444	0	nuoD_72
ppsA	100	100	495	495	0	ppsA_177
recA	100	100	546	546	0	recA_135

Notes: * alleles with less than 100% identity found

*** guaA:** *Novel allele, ST may indicate nearest ST.*

Figure F.1. Isolate 19 shows highest similarity to Stenotrophomonas maltophilia strain 837, differing only slightly from it in the guaA locus.

Appendix G – Detection of resistance and virulence genes

Table G.1. Resistance genes in isolates 4, 7, and 19, found in the GenBank-file provided by Prokka.

Isolate	Resistance gene
4	Bicyclomycin resistance protein
	Bifunctional polymyxin resistance protein ArnA
	Cobalt zinc-cadmium resistance protein CzcA
	Fosfomycin resistance protein AbaF
	Fosmidomycin resistance protein
	Inhibitor of hydrogen peroxide resistance
	Linearmycin resistance permease protein LnrN
	Methyl viologen resistance protein SmvA and YddG
	Multidrug resistance protein D, MdtA, MdtB, MdtC, MdtH, MdtK, MdtL, MdtN, MdtO, and Stp
	Multidrug resistance-like ATP-binding protein
	Multiple resistance protein 3
	Multiple stress resistance protein BhsA
	Organic hydroperoxide resistance protein OhrB
	Peroxide stress resistance protein YaaA
	Persistence and stress-resistance antitoxin
	Phenazine antibiotic resistance protein EhpR
	Putative multidrug resistance ABC transporter
	Putative multidrug resistance protein MdtD, EmrK and EmrY
	Quinolone resistance transporter
	Vancomycin B-type resistance protein VanW
7	Multidrug resistance protein MdtA
	Bicyclomycin resistance protein
	Bleomycin resistance protein
	Cobalt zinc-cadmium resistance protein CzcA and CzcC
	Colistin resistance protein EmrA and EmrB
	Copper resistance protein A and B
	Daunorubicin/doxorubicin resistance ATP-binding
	Fatty acid resistance protein FarA
	Multidrug resistance protein 3, MdtA, MdtB, MdtC, MdtE, MdtG, MdtL, MexA, NorM and Stp
	Nickel and cobalt resistance protein CnrA, CnrB and CnrC
	Non-motile and phage-resistance protein
	Organic hydroperoxide resistance protein OhrB
	Organic hydroperoxide resistance transcriptional
	Peroxide stress resistance protein YaaA
	Putative multidrug resistance protein MdtD
	Tetracycline resistance protein, class C
19	Antiseptic resistance protein
	Arsenical-resistance protein Acr3

	Bicyclomycin resistance protein
	Cobolt-zinc-cadmium resistance protein CzcA, CxcB and CzcC
	Colistin resistance protein EmrA
	Copper resistence protein A and B
	Fosmidomycin resistance protein
	Mercuric resistance operon regulatory protein
	Multidrug resistance ABC transporter
	Multidrug resistance protein MdtA, MdtB, MdtC, MdtG, MdtL, MexA, MexB and NorM
	Mutiple antibitoc resistance protein MarA
	Nickel and Cobalt reistance protein CnrA
	Organic hydroperoxide resistance protein OhrB
	Persistence and stress-resistance antitoxin
	Persistence and stress-resistance toxin PasT
	Phenaine antibiotic resistance protein EhpR
	Putative multidrug resistance protein EmrY and MdtD

Table G.2. Virulence genes detected by mass screening in VFDB.

Isolate	Gene	Database(s)	Gene product	% Coverage	% Identity
4	fliG	VFDB	Flagellar motor switch protein	100	81,47
4	fliM	VFDB	Flagellar motor switch protein	99,9	81,21
4	fliP	VFDB	Flagellar biosynthetic protein	98,84	80,56
4	flgG	VFDB	Flagellar basal-body rod protein	99,08	80,25
4	cheW	VFDB	Purine-binding chemotaxis protein	94,38	80,21
19	pilT	VFDB	Twitching motility protein	96,62	80,57

Appendix H – Sequence alignments

MULTISPECIES: class A beta-lactamase FONA-8 [Serratia]

Sequence ID: WP_024530279.1 Length: 295 Number of Matches: 1

See 5 more title(s) See all Identical Proteins(IPG)

Range 1: 45 to 295 GenPept Graphics ▼ Next Match ▲ Pre

Score	Expect	Method	Identities	Positives	Gaps
518 bits(1334)	0.0	Compositional matrix adjust.	251/251(100%)	251/251(100%)	0/251(0%)

```
Query  1    EKNSGGRLGVALIDTADNSQILYRADERFPMCSTSKVMAVSALLKQSETDKNLLAKRMEI  60
            EKNSGGRLGVALIDTADNSQILYRADERFPMCSTSKVMAVSALLKQSETDKNLLAKRMEI
Sbjct  45   EKNSGGRLGVALIDTADNSQILYRADERFPMCSTSKVMAVSALLKQSETDKNLLAKRMEI  104

Query  61   KQSDLVNYNPIAEKHLDTGMTLAEFSAATIQYSDNTAMNKILEHLGGPAKVTEFARTIGD  120
            KQSDLVNYNPIAEKHLDTGMTLAEFSAATIQYSDNTAMNKILEHLGGPAKVTEFARTIGD
Sbjct  105  KQSDLVNYNPIAEKHLDTGMTLAEFSAATIQYSDNTAMNKILEHLGGPAKVTEFARTIGD  164

Query  121  KTFRLDRTEPTLNTAIPGDKRDTTSPLAMAKSLQNLTLGKALGEPQRAQLVEWMKGNTTG  180
            KTFRLDRTEPTLNTAIPGDKRDTTSPLAMAKSLQNLTLGKALGEPQRAQLVEWMKGNTTG
Sbjct  165  KTFRLDRTEPTLNTAIPGDKRDTTSPLAMAKSLQNLTLGKALGEPQRAQLVEWMKGNTTG  224

Query  181  GASIRAGLPTTWVVGDKTGSGDYGTTNDIAVIWPANHAPLVLVTYFTQPQQNAEARKDVL  240
            GASIRAGLPTTWVVGDKTGSGDYGTTNDIAVIWPANHAPLVLVTYFTQPQQNAEARKDVL
Sbjct  225  GASIRAGLPTTWVVGDKTGSGDYGTTNDIAVIWPANHAPLVLVTYFTQPQQNAEARKDVL  284

Query  241  AAAAKIVTEGL  251
            AAAAKIVTEGL
Sbjct  285  AAAAKIVTEGL  295
```

Figure H.1. Sequence alignment showing 100 % pair identity between blaFONA gene found in isolate 4 in this study, and blaFONA-8 found in a previous study.

L2 family extended-spectrum class A beta-lactamase [[Pseudomonas] hibiscicola]

Sequence ID: WP_329848933.1 Length: 303 Number of Matches: 1

Range 1: 45 to 303 GenPept Graphics ▼ Next Match ▲ Pre

Score	Expect	Method	Identities	Positives	Gaps
522 bits(1344)	0.0	Compositional matrix adjust.	259/259(100%)	259/259(100%)	0/259(0%)

```
Query  1    SDFAALEKACAGRLGVTLLDTASGRRVGHRQDERFPMCSTFKSMLAATVLSQAERMPALL  60
            SDFAALEKACAGRLGVTLLDTASGRRVGHRQDERFPMCSTFKSMLAATVLSQAERMPALL
Sbjct  45   SDFAALEKACAGRLGVTLLDTASGRRVGHRQDERFPMCSTFKSMLAATVLSQAERMPALL  104

Query  61   DRRVPVRDADLLSHAPVTRRHAGKDMTVRDLCRATIITSDNTAANLLFDVVGGPPAVTAF  120
            DRRVPVRDADLLSHAPVTRRHAGKDMTVRDLCRATIITSDNTAANLLFDVVGGPPAVTAF
Sbjct  105  DRRVPVRDADLLSHAPVTRRHAGKDMTVRDLCRATIITSDNTAANLLFDVVGGPPAVTAF  164

Query  121  LRASGDAVSRSDRLEPELNSFAEGDPRDTTTPAAMAGSLQRVVLGKVLQPASRQQLADWL  180
            LRASGDAVSRSDRLEPELNSFAEGDPRDTTTPAAMAGSLQRVVLGKVLQPASRQQLADWL
Sbjct  165  LRASGDAVSRSDRLEPELNSFAEGDPRDTTTPAAMAGSLQRVVLGKVLQPASRQQLADWL  224

Query  181  IDNETGDACLRAGLGKRWRVGDKTGSNGEDARNDIAVLWPRAGGAPWVLTAYLQASAISN  240
            IDNETGDACLRAGLGKRWRVGDKTGSNGEDARNDIAVLWPRAGGAPWVLTAYLQASAISN
Sbjct  225  IDNETGDACLRAGLGKRWRVGDKTGSNGEDARNDIAVLWPRAGGAPWVLTAYLQASAISN  284

Query  241  EQRAQVLAQVGRIADRLIG  259
            EQRAQVLAQVGRIADRLIG
Sbjct  285  EQRAQVLAQVGRIADRLIG  303
```

Figure H.2. Sequence alignment showing 100 % pair identity between blaL2 gene found in isolate 19 in this study, and blaL2 found in a previous study.

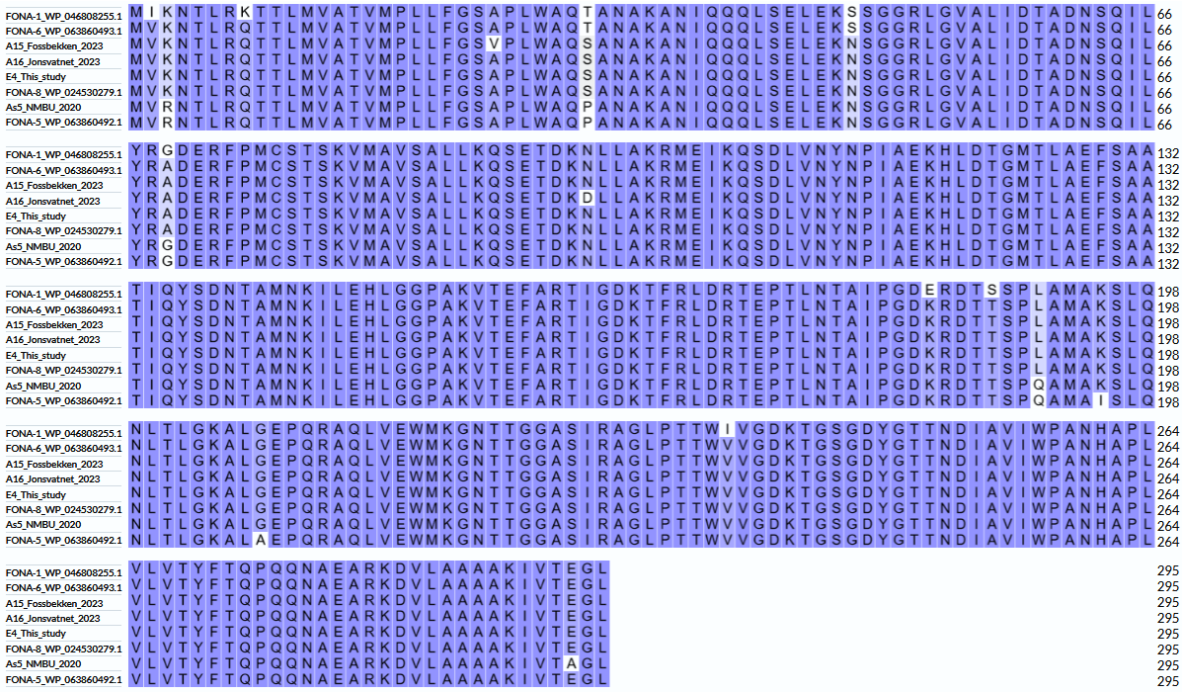

Figure H.3. Multiple sequence alignment of FONA-1,5,6 and 8 from NCBI, and four FONA genes found by students at NMBU, including the FONA found in this study (E4). The alignment visualizes the distance seen in the phylogenetic tree.

Appendix I – Quality assessment of isolates 4, 7, and 19

	Shovill_on_data_2_and_data_1__Contigs
# contigs (>= 0 bp)	73
# contigs (>= 1000 bp)	22
Total length (>= 0 bp)	6001098
Total length (>= 1000 bp)	5989753
# contigs	25
Largest contig	1339672
Total length	5991501
GC (%)	53.72
N50	670171
N90	186661
auN	691228.0
L50	3
L90	12
# N's per 100 kbp	0.00

Figure I.1. Quality assessment by Quast of isolate 4, S. fonticola.

	Shovill_on_data_22_and_data_21__Contigs
# contigs (>= 0 bp)	76
# contigs (>= 1000 bp)	28
Total length (>= 0 bp)	4419815
Total length (>= 1000 bp)	4411627
# contigs	30
Largest contig	1241637
Total length	4412715
GC (%)	67.77
N50	257883
N90	98919
auN	505893.5
L50	4
L90	14
# N's per 100 kbp	0.00

Figure I.2. Quality assessment by Quast of isolate 7, Caulobacter sp.

	Shovill_on_data_42_and_data_41__Contigs
# contigs (>= 0 bp)	123
# contigs (>= 1000 bp)	75
Total length (>= 0 bp)	4495740
Total length (>= 1000 bp)	4487029
# contigs	77
Largest contig	303604
Total length	4488069
GC (%)	66.43
N50	105393
N90	30444
auN	129206.8
L50	13
L90	42
# N's per 100 kbp	0.00

Figure I.3. Quality assessment by Quast of isolate 19, S. maltophilia.

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