UiT The Arctic University of Norway

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Antibiotic resistance profiles and polystyrene biofilm formation capabilities of bacteria isolated from wastewater in Tromsø

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Abstract

Pollution of plastic in itself has become a growing focus in the last 15 years. In addition, the impact of plastic as a reservoir for pathogenic bacteria and transfer of antibiotic resistance genes has become a global concern, for individuals, organizations, policymakers and health organizations. It is important to document the relationship of microbiota associated to different types of plastic material. This project is part of a larger project (MICROPLASTRESIST) and aims to document the connection between antibiotic resistance patterns in bacteria, identify potential pathogens in wastewater, and further document biofilm formation capabilities on polystyrene (PS) plastic.

Antibiotic resistant bacteria were isolated from two wastewater treatment plants in Tromsø, Norway, using ampicillin agar. The identities of the pure isolated strains of bacteria were confirmed using mass spectrometric analysis (MALDI-TOF) that is mainly based on species specific ribosomal proteins. The result showed an abundance of bacteria from genus *Pseudomonas,* followed by *Aeromonas, Klebsiella, Raoultella* and *Acinetobacter*, which are all Gram-negative bacteria. Some of the species were closely related to known pathogens like *A. salmonicida.* Others were characterized within the same genus, but being non-pathogens, like *A. media.* This implies that the bacteria are able to exchange genetic material, both resistance and pathogenicity from pathogens to non-pathogenic bacteria. Biofilms in particular are believed to create opportunities for this exchange of genetic material and thus form resistance reservoirs.
Antibiotic resistance patterns were mapped out using 8 of the most prescribed antibiotics used at the University Hospital of North Norway (UNN). In total, 13/55 of the identified bacterial species contained antibiotic resistant genes to ≥ 5 of these 8 antibiotics. Most of the identified strains were able to form biofilm on polystyrene microtiter plates at 30°C and 35°C. Most biofilm mass was formed using TSB and BHI broth for the raw wastewater, whereas bacteria in wastewater without any added nutrients form biofilm after 24 h at 30°C.

This pilot study confirms existence of multi-resistant bacteria, both human and fish pathogens, at both wastewater stations in Tromsø. Most of them are also biofilm forming on polystyrene at 30°C and 35°C.

List of abbreviations

ABR	antibiotic resistance
ABRG	antibiotic resistant genes
AMR	antimicrobial resistance
BLAST	Basic Local Alignment Search Tool
CHCA	α-cyano-4-hydroxycinnamic acid
DHB	2,5-dihydroxybenzoic acid
EMA	European Medical Agency
EPM	extracellular polymeric matrix
EPS	extracellular polymeric substances
EU	European Union
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FA	ferulic acid
HGT	horizontal gene transfer
LB	Luria Bertani medium
MP	microplastic
MRG	metal resistent genes
PE	polyetylen
PP	polypropylen
SA	sinapinic acid
SW	sewage water
WW	wastewater
WWTP	wastewater treatment plant

1 INTRODUCTION

Plastic has become a huge pollution problem, posing a threat in both their physical presence and their potential to harbor toxins and microorganisms. The ability to house microorganisms on plastics pose a threat, particularly in connection to wastewater. Because of the pathogens in said water and its distribution potential, there is a need for more information on the actual composition of different bacteria in wastewater. Important questions to find the answers to are; i) what kind of resistance do they carry and thus might transfer to other bacterial strains ii) can the microorganism form biofilms?

This master thesis project is done in collaboration with Genøk- center for biosafety, and is connected to a project, MICROPLASTRESIST, co-funded by National Research Foundation (South Africa) and the Norwegian Research Council. This project aims to investigate the dispersal of microplastics in wastewater and characterize their association with biofilm forming bacteria as a potential facilitator in the spread of antibiotic resistant genes or bacteria.

1.1 Aim

The main goal of the thesis is to characterize the microbial composition and antibiotic resistance patterns of bacteria and their biofilm formation capabilities on plastic from wastewater.

Research questions related to this aim are:

- Can the presence of (micro) plastics in wastewater (WW) influence the growth and exchange of genetic material between different strains of bacteria that otherwise might not have been in in close contact?
- Are there multi-resistant strains of bacteria (particularly human pathogens) present in the WW samples from both Breivika and Langnes wastewater treatment plants in Tromsø, Norway?
- Are some of these bacteria opportunistic pathogens?
  Are there more pathogens in WW from Breivika than Langnes, because of its proximity and connection to the University Hospital in North Norway (UNN)?

From this, the following **subgoals** are:

- Identify genus and strains of antibiotic resistant bacteria from the WW.
- Evaluate the ability of the antibiotic resistant bacteria to grow on plastics, like polystyrene (PS), polypropylene (PP).
- Evaluate the growth and biofilm formed at different temperatures.
- Evaluate the growth and biofilm formed in different media.

1.2 Background

The dispersal of plastics in marine and freshwater systems is an ever-growing problem on a global scale. Plastics ranging in sizes from the micro to macro have been found in all the world's oceans, along beaches, in the sediment, in the deepest crevice known to man – in the Marina trench there is a plastic bag at 10 898 m (Chiba et al., 2018; Jamieson et al., 2019). As well as on large floating trash patches mainly made up of plastic (Law, 2017; Solomon & Palanisami, 2016) along with other floating debris. Ever since plastic production reached an all-time high for that era of production in the 1970s, it has only steadily increased, due to its versatile applicability and cheap manufacture – in 2016 production reached a staggering 355 million tons (Alimba & Faggio, 2019).

Over time, plastic has become an incorporated feature in most of the world's environments and ecosystems. Research has also indicated that their presence, has become a vector for new toxicological development, as well as being an ecological niche for bacterial communities (Alimba & Faggio, 2019). This is due to the favourable conditions (more than being suspended in seawater) for development of biofilms, which creates a potential for an even more interactive surface in terms of genetic exchanges between strains and species (Arciola, Campoccia, Speziale, Montanaro, & Costerton, 2012).

Wastewater treatment plants receive wastewater containing antibiotic residues from anthropogenic sources (Kumar & Pal, 2018). As the current treatments are not specialized enough to filter them out and non–existent in some facilities. Increased concentrations of antibiotics in wastewater over times, makes the treatment plants reservoirs for antibiotic

resistance, due to the selective pressure caused by the presence of antibiotics (Kelly et al., 2021; Kumar & Pal, 2018). This can in turn, increase the amount of resistance genes present in the environmental microbiome as resistance genes are exchange through horizontal gene transfer, both in the plant and after expulsion into nature (Ester M. Eckert et al., 2018; Kelly et al., 2021; Kumar & Pal, 2018; Moura, Pereira, Henriques, & Correia, 2012)

There have also been observations of non-random co-occurrence of antibiotic resistance genes and metal resistance genes on plastics in marine environments, and an even higher prevalence (likely due to higher anthropogenic influence) in sewage sludge and leachate from landfills (Yang et al., 2019). This, along with emerging zoonotic diseases - any kind of infection that is transmittable from animal to humans, has caught the attention of researchers worldwide.

Inspiring a multi-disciplinary approach with the umbrella term “One Health” (supported by WHO, FAO, OIE, UNICEF, World bank and more) “One health” aims to highlight consequences, responses and actions in relation to the animal-human and environmental interactions. Focusing on (1) emerging and endemic zoonoses – the latter being impertinent in regard to diseases in the developing world, with a detrimental societal impact in resource poor settings (2) Antimicrobial resistance (AMR). Resistance may arise in animals, humans or the environment and has the potential to spread from one to the other, even across borders (Mackenzie & Jeggo, 2019). The initiative is comprised of different scientific disciplines such as social science, ecology, ecosystem and environmental health, wild-life, land use, veterinary and human medicine. The term as well as the disciplines are clearly illustrated in Figure 1, it

all emphasises the need to work together towards a global problem that will affect all of mankind eventually, if left unchecked.

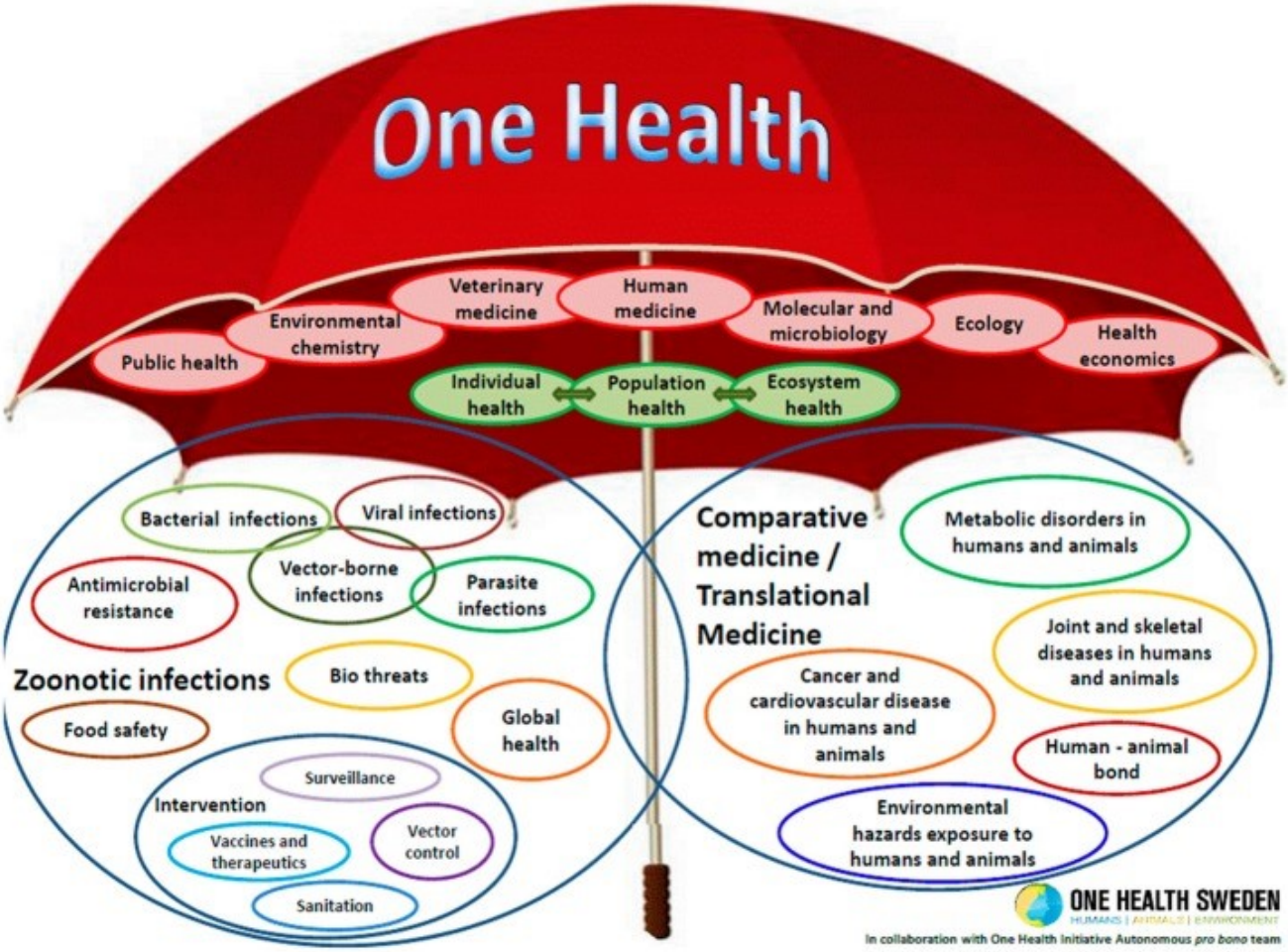

Figure 1.One Health concept summed up. Umbrella designed by One health Sweden and One Health initiative autonomous pro bono team.

1.3 Microplastics – Composition, origins and dispersal

This leads us to one of the major consequences of anthropogenic activity, plastic. Microplastics are polymeric particles ≤ 5 mm and nano plastic (<100nm) (Koelmans, Besseling, & Shim, 2015; Solomon & Palanisami, 2016). These particles are either primary plastics from the production of microbeads, used to manufacture other plastic products or secondary plastics formed by degradation of plastics over time (Ester M. Eckert et al., 2018).

Samples from marine sediment have shown densities as high as 100 000 items per m^3 (Kershaw & Rochman, 2015).

There are seven classes of plastics that dominate in the marine environment: Polyvinyl Chloride (PVC), Polypropylene (PP), Poly-urethan (PUR), High/low density Polyethylene (HDPE/ LDPE), Polystyrene (PS) and Polyethylene Terephthalate (PET) (Figure 2). They all have a wide area of use and a varying degree of recyclability (Kershaw & Rochman, 2015).

Figure 2. ***PLASgran recycling guideline and grading system of different plastics****. The number inside the Möbius loop indicates recyclability from high (1) to low (7) (PLASgranLtd, 2021)*

The grade of recyclability is most often demarcated by a loop of three triangular green arrows called the Möbius loop. Though most plastic products have this loop on the packaging, not all are recycled. Often because the facilities available in the area are unsuitable for certain

degradation methods as well as local legislation regulating certain plastics (PLASgranLtd, 2021). Without proper legislations and implementation, plastic is left to degrade in the environment where it spreads, be it from human causation or at the mercy of nature (Kershaw & Rochman, 2015).

Evidence implies that the mode of transportation of plastic particles for both fresh and marine water systems is mainly through surface currents. Before being deposited in the sediments, with some particles remaining afloat freely in the pelagic layer. Most of the particles ultimately end up in the sediments, where they accumulate for centuries or unfortunately enter the food webs of the marine ecosystems, when marine organisms mistake them for food (Solomon & Palanisami, 2016)

1.3.1 Degradation – by natural forces and human interaction

Microplastics are created when external forces such as UV-light, wave action, ocean currents, microbial degradation and even chemical processes affect larger pieces and fragments them into micro particles that are easily dispersed (Solomon & Palanisami, 2016).
In addition to the forces listed above, there is a continuous combustion of plastic in order to disintegrate them. This process releases some of the smallest fragments (on a nanoscale) into the atmosphere where they are dispersed, as well as releasing toxic chemical (Liu et al., 2019; Verma, Vinoda, Papireddy, & Gowda, 2016).
Table 1. **Overview of different modes of polymeric degradation, both natural and man-made**. Content borrowed from (Muthukumar & Veerappapillai, 2015)

Factors (requirement/activity)	Photo-degradation	Thermooxidative degradation	Biodegradation
Active agent	UV-light or high-energy radiation	Heat and oxygen	Microbial agents
Requirement of heat	Not required	Higher than ambient required temperature	Not required
Rate of degradation	Initiation is slow. But propagation is fast	Fast	Moderate
Other consideration	Environment friendly if high-energy radiation is not used	Environmentally not acceptable	Environment friendly
Overall acceptance	Acceptable but costly	Not acceptable	Cheap and very much acceptable

Free plastic particles act as pollutants and are a threat to the biodiversity by their physical presence, as they may be mistaken as food by marine life. In addition to the nanoparticles that have proven to be adept at harbouring toxic chemicals due to their large surface area (Koelmans et al., 2015).

1.3.2 Plastispheres and genetic exchange

While the plastic particles drift about, they facilitate a hydrophobic perch for different strains of microorganisms, creating a suitable habitat for biofilm formation as the microbes colonise them. This creates a plastisphere - a community of autotrophs, heterotrophs, predators, and symbionts (Radisic, Nimje, Bienfait, & Marathe, 2020).

Additionally, these spheres enable horizontal gene transfer of antibiotic resistant genes (ABRG) amongst the microbes and its previously been shown to facilitate multidrug resistance in microbial communities found on marine plastics (Yang et al., 2019)

A study done in China links Metal resistant genes (MRGs) to ABRG on plastics as they were often found together. This is likely due to a co-selection caused by the same factors that regulate efflux pump gene expression. These may also regulate resistance gene expression (Eckert, Di Cesare, Coci, & Corno, 2018; Perron et al., 2004). MRGs and ABRG can be coupled on the same mobile genetic element, such as a plasmid. Making it possible for them to be transferred on the same mobile gene, this has been observed in different environments, such as in soil (Johnson et al., 2016) and sediments (Rosewarne, Pettigrove, Stokes, & Parsons, 2010). The class 1 integron - integrase gene (*intl1)* is a key mobile genetic component linked to these occurrences (Gillings et al., 2015). This rout of gene transference and specific transference in general will not be focused on in this thesis, this is simply to highlight how a close proximity via a biofilm can contribute to ABRG transfer thru MRG.

1.4 Biofilm

In short, a biofilm is a consortium of bacteria in a structured self-produced matrix on any kind of surface. Each film has a unique composition based on, environmental conditions, strain type and bacterial species. As well as containing an array of other substances such as proteins, exopolysaccharides, extracellular DNA/RNA (eDNA/eRNA) and teichoic acids, collectively referred to as extracellular polymeric substances (EPS) (Nazir, Zaffar, & Amin, 2019). Particularly, wastewater biofilms have been found to contain large amounts of eDNA, but also this varies from biofilm to biofilm based on species composition (Flemming & Wingender, 2010).
When structured like this, bacterial biofilms are quite resilient and resistant to antibiotics, disinfectants, phagocytosis, and other defense mechanism of both the adaptive and innate immune system of a potential host (Arciola et al., 2012; Costerton, Montanaro, & Arciola, 2005; Høiby et al., 2011; Stewart & Costerton, 2001).

Known biofilm forming bacteria are *Staphylococcus epidermidis, Shewanella oneidensis* and *Staphylococcus aureus,* hence a lot of models for biofilm formation are based on staphylococcal biofilm formation.
This is a four-part process commencing with the initial adhesion of bacterial cells, that followingly accumulate and aggregate in several cell layers. Lastly, it settles in a stage of maturation that ends in detachment where the biofilm converts into a planktonic state and drifts of in search of new perches to start the cycle anew(Arciola et al., 2012).

During the early stages of formation, the interactions are random and purely driven by physical forces; electrostatic, Lifshitz-Van der Waals and hydrophobic forces. (Legeay, Poncin-Epaillard, & Arciola, 2006). At this stage different bacteria are passively adsorbed onto the surface of nearby material. Most of the existing adhesion models are based on *Staphylococcal* behavior and these indicate hydrophobicity plays a central role to initial attachment, along with proteins like autolysin which helps mediate adhesion to abiotic surfaces (Heilmann, Hussain, Peters, & Götz, 1997; Legeay et al., 2006). Herein, there are hydrophobic and ionic interactions with a dual role as peptidoglycan hydrolases and adhesives. For example, in *S. epidermidis* the adhesion protein AtlE mediates adhesion to polystyrene, similar properties have been observed in homologs due to their glycine-tryptophane dipeptide repeats (important for biofilm production and surface association)

(Legeay et al., 2006).

The second occurrence is accumulation in several bacterial layers. This process is facilitated by Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMM) and intracellular adhesion (Legeay et al., 2006; Patti, Allen, McGavin, & Höök, 1994).

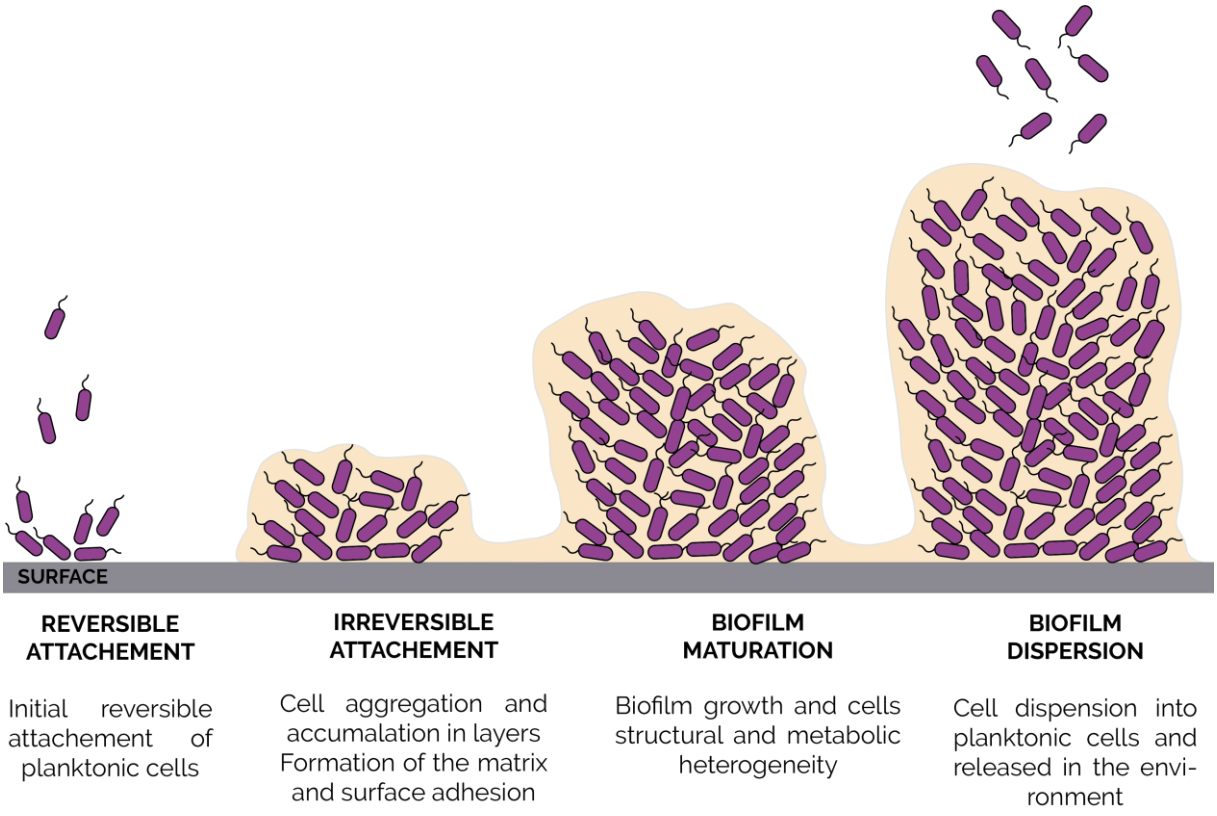

Figure 3. ***The biofilm life cycle from initial attachment to re-dispersion into planktonic stage*** *(E. Maunders 2017)*

Through these steps the biofilm is gradually colonizing the surface where its attached and encased. Followed by the last and final steps of maturation and re-entry to a planktonic state. It's also at this stages the biofilm develops the structural characteristics which identifies it as a biofilm, specific characteristics are dependent on present species (Legeay et al., 2006). Which varies in biofilms as some species die off, stop producing EPS or detach (Flemming & Wingender, 2010).

1.4.1 Genetic exchange in biofilms

A biofilm is a protected environment for the microbes within, making it difficult for antibiotics to penetrate. The extracellular polymeric matrix controls which molecules and the amount that is allowed to penetrate the film to the inner layers, where interaction with antibiotic targets may occur. Thus, creating a physical barrier, on the other hand there are a number of cationic and anionic like glycolipids, glycoproteins and proteins that are capable of binding charged antimicrobial agents, yet again creating an obstacle (Nadell, Drescher, Wingreen, & Bassler, 2015). There are even implications that the decreased diffusion of antibiotic agents elicits a delay big enough to allow time for an adaptive phenotypic response (Tseng et al., 2013). So, in order for antibiotics to penetrate a biofilm there are several obstacles to be cleared, such as high cell density, substance delivery, resistant mutants, persistent cells and efflux pumps (Nadell et al., 2015; Tseng et al., 2013). The matrix also acts as a storage for lysed cells, keeping the different components for recycling, including DNA, making it a potential vault for genes to transferred in HGT (Flemming & Wingender, 2010).

Genetic transfer can happen through horizontal or lateral transference by conjugation, transduction or transformation. All of these mechanisms involve the transfer of genetic material from one bacterium to another, this can be interspecies or same-species transference. Causing a swift transmission of new phenotypic traits by either operons or complete genes. Conjugation and transformation are most common for biofilm (Cvitkovitch, 2004).

1.5 Antibiotic resistance - a global challenge

Antibiotics or antimicrobials are therapeutic agents used to inhibit growth and impede survival of bacteria. They are produced as secondary metabolites by some microorganisms, like fungi to defend themselves against other microorganisms, like bacteria. In today's production they may be semi synthesized analogous, chemically produced compounds or natural products (Ben et al., 2019).

Since Fleming's accidental discovery of Penicillin in the early 1900s the use of antibiotics has steadily been increased and the utility broadened from treating human diseases, to livestock and aquaculture. As new drugs are discovered and used over time there is a developing resistance within the microbial community as an adaptation to the drugs, largely due to

overuse and residues that are present in the environment driving a selective pressure in favor of resistance (Gupta, Shin, Han, Hur, & Unno, 2018; Kumar & Pal, 2018; Moura et al., 2012). Antimicrobial resistance (AMR) is when a strain of bacteria no longer responds to an antibiotic drug. This may be due to phenotypic resistance – resistance acquired without genetic alteration (Corona & Martinez, 2013) which is not inheritable. AMR evolves either through genetic variations caused by genetic mutation or through horizontal gene transfer of resistance determinants on e.g. plasmids (Ben et al., 2019)

There are several feedstock producers that have included antibiotics as an additive to their food as a prophylactic and growth promotor (Gelband et al., 2015). This creates another outlet for antibiotics into the environment, together with an array of routs for dispersal including municipal sewage, animal husbandry, agricultural runoff from livestock manure, aquaculture ponds. The biggest source of them all being the pharmaceutical industry. Albeit the life of most antibiotics spans from hours to a couple of months, they are still considered persistent contaminants, as they are found frequently over vast areas (Ben et al., 2019).

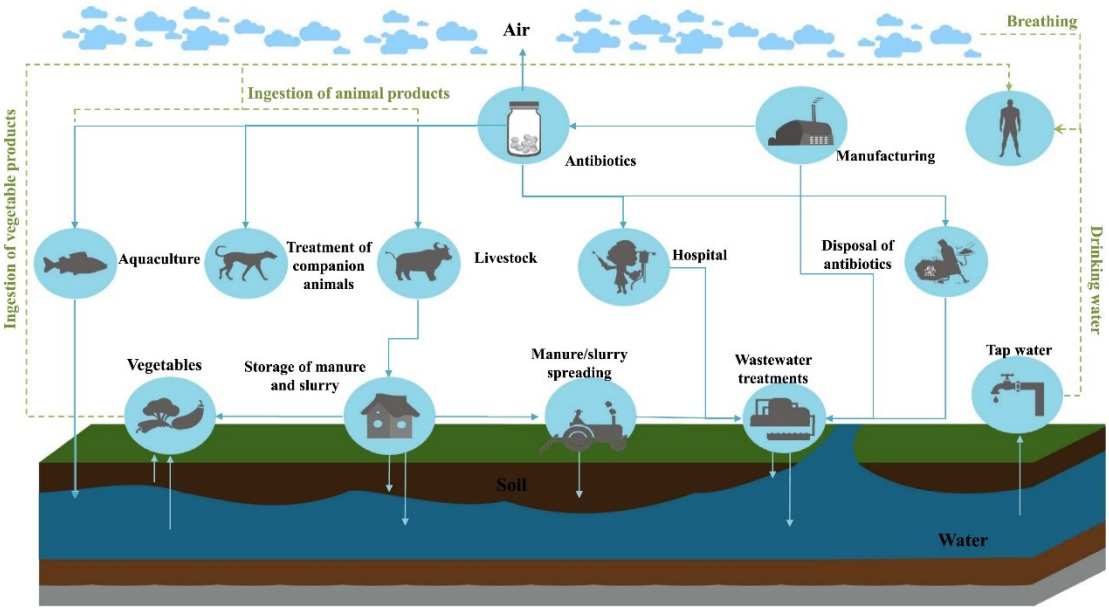

Figure 4. ***Illustration of the different outlets for antibiotics found in the environment*** *from (Ben et al., 2019)*

According to WHO there are several priority pathogens on the rise for which new antibiotics need to be focused on in the perspective of human health and mortality rates in the fight against antibiotic resistant infections. The list is divided into critical, high and medium, with critical being the number one priority.

The list from WHO of bacterial strains with clinically relevant antibiotic resistance:

- **Priority 1: CRITICAL**
- *Acinetobacter baumannii*, carbapenem-resistant
- *Pseudomonas aeruginosa*, carbapenem-resistant
- *Enterobacteriaceae*, carbapenem-resistant, ESBL-producing
- **Priority 2: HIGH**
- *Enterococcus faecium*, vancomycin-resistant
- *Staphylococcus aureus*, methicillin-resistant, vancomycin-intermediate and resistant
- *Helicobacter pylori*, clarithromycin-resistant
- *Campylobacter* spp., fluoroquinolone-resistant
- *Salmonellae*, fluoroquinolone-resistant
- *Neisseria gonorrhoeae*, cephalosporin-resistant, fluoroquinolone-resistant
- **Priority 3: MEDIUM**
- *Streptococcus pneumoniae*, penicillin-non-susceptible
- *Haemophilus influenzae*, ampicillin-resistant
- *Shigella* spp., fluoroquinolone-resistant

‘

1.5.1 Antibiotic resistance in the environment

Resistance in the environment is a fickle thing, as non-pathogenic bacteria can acquire resistance genes through HGT. Environmental bacteria can also pass resistance on to pathogenic bacteria. A study by (Forsberg et al., 2012) has found evidence that shows how antibiotics are excreted naturally by soil bacteria. This is used to explain how resistance has

developed in other environmental organisms, that do not inherently produce antibiotics themselves. In a large scale sampling/screening of soil bacteria, it turned out that all of the strains tested were multi drug resistant (Costa, McGrann, Hughes, & Wright, 2006). Several antibiotics were used in the screening, including synthetic, semi-synthetics and natural products, with at least one targeting the major bacterial pathways (Costa et al., 2006). The study uncovered intrinsic resistance in all the antibiotic classes, even for relatively recent antibiotics such as daptomycin, had a reduced susceptibility to numerous isolates (Costa et al., 2006). This just goes to show the already existing natural reservoir residing in the soil-dwelling microbiome is a potential threat to human and animal health (Forsberg et al., 2012). Furthermore, how detrimental a mix of these determinants along with the forming WW reservoirs could be if they proliferate and pass on their pathogenicity.

1.5.2 Antibiotics: mechanisms of action

There are several different types of antibiotics worldwide developed to combat microbial infections. Different classes of antibiotics have specialized mechanisms of action devoted to disrupting a specific part of the microbial machinery/anatomy. Bacteria are divided into Gram-positive and Gram-negative based on the composition of their cell wall, in particular the presence of an outer membrane. An outer membrane consisting of lipopolysaccharides and proteins coats the outer bounds of gram-negative bacteria, along with the inner layers of peptidoglycan, periplasmic space and a plasma membrane. It functions as an extra protective layer as well as a stiffener which provides protection against mechanical and osmotic stress while maintaining the characteristic shape (Kapoor, Saigal, & Elongavan, 2017). Gram-positive bacteria on the other hand do not have the outer membrane, but a thick layer of peptidoglycan, a periplasmic space and the plasma membrane (Kapoor et al., 2017). These traits may be observed on figure 5.

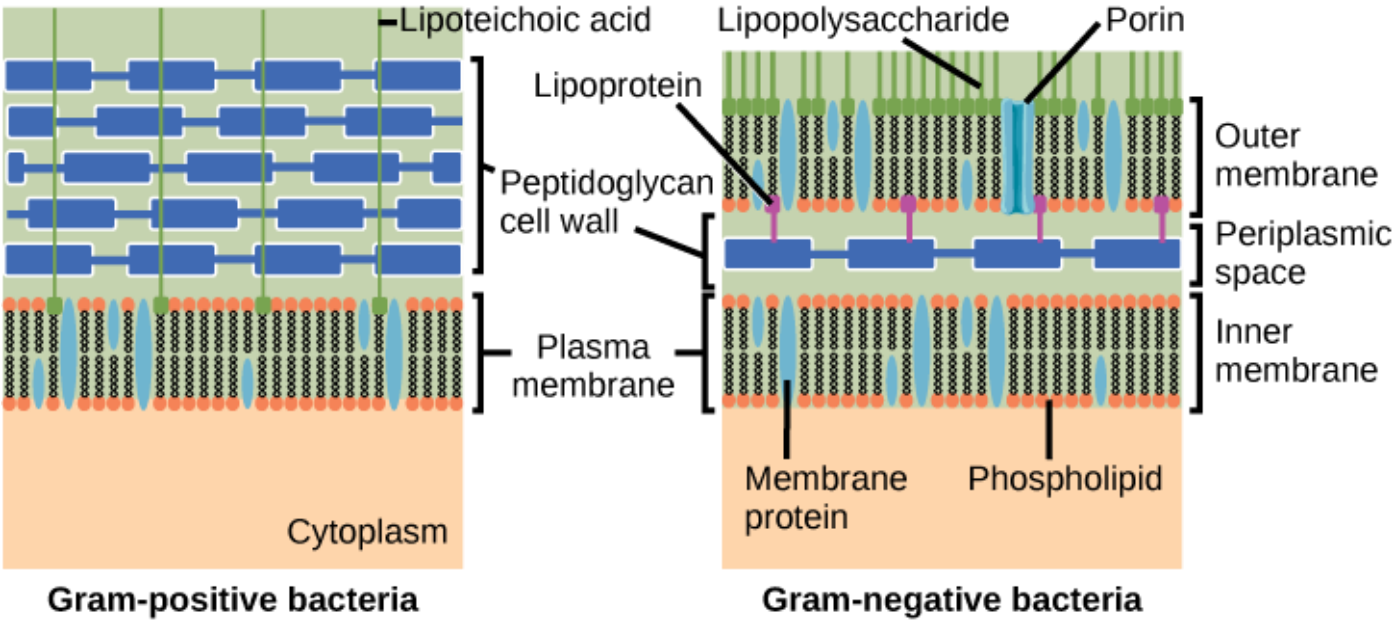

Figure 5. ***Gram- Positive vs. Gram-Negative Cell wall anatomy*** *(Samanthi, 2018)*

Antibiotics may target several biosynthetic pathways such as nucleic synthesis, protein synthesis and cell wall synthesis. By disrupting these processes, the cell growth becomes static or lysis incurs. The classification of antibiotics is based on the mechanisms illustrated in figure 6.

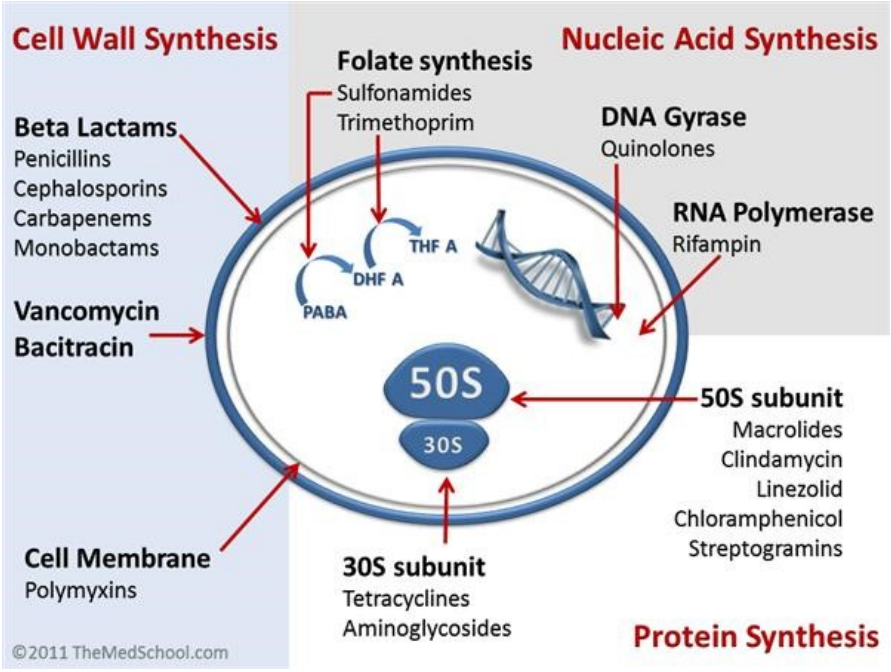

Figure 6. **Mechanism of action by antibiotics** (TheMedSchool, 2011)

There are 6 different classes of antibiotics grouped based on their blocking properties – cell wall synthesis, protein synthesis inhibitors, DNA/RNA synthesis inhibitors, Mycolic acid synthesis inhibitor and folic acid inhibitors.

Table 2. Antibiotics grouped by mechanism.

Antibiotic grouping by mechanism		**Antibiotics for disk diffusion test in this thesis**
Cell Wall Synthesis	Penicillin's Cephalosporins Vancomycin Beta-lactamase Inhibitors Carbapenems Aztreonam Polymyxin Bacitracin	Cefotaxime Mecilinam Ampicillin Penicillin
Protein Synthesis Inhibitors	Inhibit 30s Subunit Aminoglycosides (gentamicin) Tetracyclines Inhibit 50s Subunit Macrolides Chloramphenicol Clindamycin Linezolid Streptogramins	Gentamicin Tetracycline
DNA Synthesis Inhibitors	Fluoroquinolones Metronidazole	Ciprofloxacin
RNA synthesis Inhibitors	Rifampin	
Mycolic Acid synthesis inhibitors	Isoniazid	
Folic Acid synthesis inhibitors	Sulphonamides Trimethoprim	Trim-sulfa

In December 2019, the European medicine agency (EMA) published an updated list of categorized antibiotics within the European union (EU) based on their usage and risk to public health in regard to antimicrobial resistance. This report was based on the use of antimicrobials in veterinary medicine and focused on the list of critically important antibiotics from the WHO list. The criteria and weight for each category is based on the need for that specific class/subclass of antibiotic in human medicine in addition to the danger and likeliness of resistance spreading from animals to humans (EMA, 2019). The categorization is only based on antibiotics authorized for human and or veterinary application in the EU, also taking into consideration possible consequences of antimicrobial resistance (AMR) transfer from animal to human in regard to instances where a single gene confers multi-resistance or resistance to several classes. Finally, the availability of an alternative (sub)class of antibiotics in veterinary medicine that has a lower risk with AMR to animals and public health is also weighted (EMA, 2019).

1.5.3 Intrinsic antibiotic resistance in Gram negative bacteria

As can be seen in the section above, Gram negative bacteria have an outer membrane with lipopolysaccharides that makes them distinguishable from Gram-positive. This outer membrane acts as a structural component and protects against large molecules entering the cell, only allowing molecules up to 30-57kDa due to its mesh like structure (Brock, 2009; Cox & Wright, 2013). This is structure also protects against entry from a lot of antimicrobials, by acting as a semipermeable membrane to molecules of the right size and making them intrinsically insusceptible to an array of antibiotics (Cox & Wright, 2013; Vaara, 1992). This size selection is also present in the membrane porins, including hydrophobicity and charge repulsion (Cox & Wright, 2013). Some species like *P. aeruginosa* has ß-lactamase present innately within the periplasmic space, so when some ß-lactam antibiotics are able to permeate the membrane they are immediately inactivated by the enzyme (Nakae, Nakajima, Ono, Saito, & Yoneyama, 1999).

The outer membrane also has active efflux pumps enabling them to expel any antibiotics that have permeated the cell, allowing them to slow down the rate of intrusion. This can cause a synergistic effect when acting alongside f.ex the ß-lactamase or other resistance mechanisms (Cox & Wright, 2013). Efflux pumps are present in almost all organisms, also Gram- positive bacteria. In addition to these protection mechanisms, they also have chromosomally encoded protection through dubbed the “intrinsic resistome” which refers to an array of different

genetic loci that encode different cellular functions contributing to Gram-negative resistance(Cox & Wright, 2013)

1.6 Wastewater microbiota

Wastewater contains a multitude of bacteria from animals (husbandry/pets) and humans, a great deal of these contain acquired antibiotic resistance genes (Célia M. Manaia et al., 2018; Pruden, 2014). They run a risk of carrying ARG into the natural environment, increasing the chances of enriching the environmental resistome through horizontal gene transfer or selection. Which in the end might influence the rise of pathogenic bacteria, that threaten animal and human health (Célia M Manaia, 2017). The nutritious water allows for increase in biomass and room to create biofilms for biofilm forming bacteria. This in turn gives rise to cell-cell communication and coordination

Bacterial taxa/genus associated with wastewater in samples from the US are *Acinetobacter, Campylobacteraceae, Klebsiella, Pseudomonas* and *Sphingomonas (Kelly et al., 2021)* In Norway, bacteria like *Morganella morganii, Aeromonas salmonicida, Acinetobacter beijerinckii* and *Aeromonas popoffii* were all found with multiple resistance genes when isolated from WW plastic (Radisic et al., 2020).

Removing undesirable microorganism, organic matter and chemical pollutants from sewage is an important step in safe-guarding the environment where the effluent flows out (Célia M. Manaia et al., 2018). Not all WWTP have incorporated such cleaning steps, the WWTPs in Tromsø are some of them.

1.6.1 Wastewater treatment in Tromsø

Wastewater treatment plants are cleaning systems that collect water from drainage and sewers in order to filter and purify it to such an extent that it is safe to redistribute into nature without causing foreseen harm, through criteria of local legislation (Lucas et al., 2014).

In Tromsø there are six WW stations processing water for its some 76 973 inhabitants (SSB, 2020) As this is a small study, only two stations were selected sampling;

- Breivika located close to the hospital, university and industrial area.
- Langnes primarily receives water from Kvaløyas residential area and the local airport.

The selection is also influenced by practicality of collection but the main point being a definite source of varying antibiotics in the hospital and some potential lower concentration from prescription drugs in the residential area.

The process of WW management is divided into several steps that are physical, biological and chemical in order to neutralize all potential toxins, obstructions and chemical before the water is re-released and may be processed into new drinking water. In essence they are a critical part in sustaining modern life infrastructure (Figure 7).

Figure 7. Breivika wastewater treatment station (private photo)

At Breivika and Langnes there are only mechanical cleaning steps

Step one captures lager object like wipes, wood, toys etc. the larger items collected (Figure 8)

are sent off to a landfill or disposal (Bentzen, 2020).

Figure 8. Filter mat at Breivik WWTP (private photo)

The flow is then pumped through a “grit chamber” where smaller fragments like sand and rock are removed by pumping the viscous mass in a specialized chamber at a speed that allows the sediments to be filtered out in a sand bed.

The next steps differ for the two stations:
At Breivika the flow is divided onto four different sifts-sheets with a mask size of 350 um meant to capture more sludge which is scraped off and sent to a landfill while the filtered water is pumped out to Tromsøysundet (Bentzen, 2020).
While at Langnes the flow continues onto a coarse sift with a mask size of 1 mm, followingly a polymer is added called MacoZoll which binds the smaller particles remaining. This makes the next filtering step more efficient; the flow is filtered through 300 um masks, then decanted into a final sift. Additionally, sand is removed into its own container before the filtered water is released into the fjord (Bentzen, 2020).

1.7 Analyses and principles

1.7.1 Culturing bacteria - importance of nutrients in media

Agar plates are sterile petri dishes filled with a nutrient rich substance. There are several different types available depending on which kind of traits one is selecting for and what kind of results are expected. If it is low selective media or a more restrictive type. All-purpose medias such as tryptic soy broth allows a wide range of growth in contrast to specialized medias enriched with antibiotics, specific vitamins and growth factors that promote the well-being of a select organism (Julianne Zedalis, 2018).

In this study only a handful were selected as there were quite a few unknown factors involved.

The first and most widely used is a classic Luria- Bertani (LB) media, it consists of distilled water, amino acids like tryptone or peptone, yeast extract, NaCl and agarose unless a liquid media is desired. As with most plates, they may be added antibiotics if resistant strains are suspected and make pure cultures or to exclude certain resistant strains (Aryal, 2019). LB medium is considered as one of the least selective medias and is often used to simply establish growth.

CLED also known as Cystine-Lactose-Electrolyte Deficient Agar; Bromothymol-blue Lactose Cystine Agar acts as a source of nutrients like carbon, nitrogen and amino acids. It consists of L-cystine, Lactose, Peptic digest of animal tissues, Casein enzymatic hydrolysate, Beef extract, Agar and Bromothymol blue. Each component has different modes of indication for activity on the plate such as Bromothymol changes color from blue to yellow when acid is produced in a fermentation process whilst it changes to a deep blue in alkalinization. Lactose provides a fermentable carbohydrate and lactose -positive bacteria appear as yellow colonies. Whilst bacteria that decarboxylates L-Cystine which causes an alkaline reaction that appears as deep blue colonies (KGaA, 2018). It is typically used to grow aerobically growing microorganism like *Pseudomonas, Enterobacteriaceae* as well as other non-fermenting gram negative rods (Aryal, 2019).

Mueller-Hinton was developed primarily for meningococci and gonococci, it consists of meat infusion, casein hydrolysate, starch paste and water (Mueller & Hinton, 1941).

Blood agars are made with blood cells from animals like sheep or cow, these are not selective Chocolate agar is made of lysed blood cells and is particularly good for growing bacteria the inhabit the respiratory system.

Brain Heart infusion is a common liquid media, it consists of sodium chloride sodium citrate, sodium phosphate, dextrose peptone, polyanethol sulfonate (SPS) and brain heart infusion broth (normally from an ox) (Parija, 2009).

Table 3.Types of culture media and their purposes(Pearson® & education, 2012)

Type	Purpose
Chemically defined	Growth of chemoautotrophs and photoautotrophs, and microbiological assays.
Complex	Growth of most chemoheterotrophic organisms.
Reducing	Growth of obligate anaerobes.
Selective	Suppression of unwanted microbes; encouraging desired microbes.
Differential	Differentiation of colonies of desired microbes from others.
Enrichment	Similar to selective media but designed to increase numbers of desired microbes to detectable levels.

There are several types of media categorized by the desired growth and needs of intended target organism(s). This can be observed in table 3 which explains the different categories of culture media (Pearson® & education, 2012).

1.7.2 MALDI-TOF

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry also known as MALDI-TOF MS. It is an accurate, rapid and cost- effective method utilized for microbial characterization and identification, each microorganism has a unique spectral fingerprint. This unique fingerprint makes for an accurate identification at genus and species level with potential for strain typing and identification. It was first registered in 1996 that this method of spectral fingerprinting was an efficient method for microbial identification and has since been reproduced by several research teams(Biswas & Rolain, 2013; Giebel et al., 2010)
The instrument works by a spectrometer which consists of three functional units

(1.) An ion source to ionize and transfer sample molecules ions into a gas phase. This is followed by (2) A mass analyzer which separates ions based on their mass to charge ratio (m/z) (3) lastly a detection device to monitor separated ions (Croxatto, Prod'hom, & Greub, 2012). MALDI incorporates a soft ionization technique which allows ionization and vaporization of large nonvolatile biomolecules like intact proteins, moreover MADLI mostly generates single charged ions and thus providing a spectrum that may include a large number of proteins. TOF mass analyzers are well suited for interacting with pulse laser ionization and provides rapid analysis and miniaturization making it well suited for intact microorganisms (Croxatto et al., 2012).

In order to analyze the samples, they have to be mixed with a matrix which leads to a crystallization. Within the matrix there are small acid molecules that have a strong optical absorption with wavelength in the range of the laser being used (Fenselau & Demirev, 2001). Common matrices are 2,5-dihydroxybenzoic acid (DHB), α-cyano-4-hydroxycinnamic acid (CHCA), sinapinic acid (SA), ferulic acid (FA), and 2,4-hydroxy-phenyl benzoic acid. The different matrices have different properties – SA, FA and CHCA have proven effective for detecting proteins biomarkers (Fenselau & Demirev, 2001; Vaidyanathan & Azar, 2015) while DHB is the better choice for detecting glycopeptides and glycoproteins (Giebel et al., 2010). The selected matrix affects the size and intensities of the peaks of the detected molecules. Studies have shown that CHCA and DHB are usually optimal for detection of lower mass ions, all the way down to 10kDa in combination with a proper solvent (Croxatto et al., 2012; Sagen et al., 2004). For higher mass ions (>15kDa), both SA and FA have proven more adept at detection (Conway, Smole, Sarracino, Arbeit, & Leopold, 2001; Madonna et al., 2000; Vargha, Takáts, Konopka, & Nakatsu, 2006) although they have a lower sensitivity than CHCA (Clark, Egan, Frazier, & Wang, 2013; Ruelle, Moualij, Zorzi, Ledent, & Pauw, 2004).

Whole microorganisms may be directly processed by MALDI-TOF without any pretreatment, generally vegetative bacteria get lysed after exposure to organic solvents, water and/or strong acids in MALDI matrix. Microorganisms that are resistant like viruses, yeast cells, bacterial spores often go through pre-treatment where they are added strong organic acids or alcohols. Some species of bacteria such as Actinomyces also require protein extraction or specific pretreatment (Croxatto et al., 2012). The process is done by mixing the microbial sample on a conductive metal plate or alternatively deposited and dried out on a metal support before adding the matrix (Figure 9). After crystallization of the mixture, the metal plate carrying the target is placed in the spectrometer where it is flushed with brief laser pulses. In most cases a nitrogen laser is utilized.

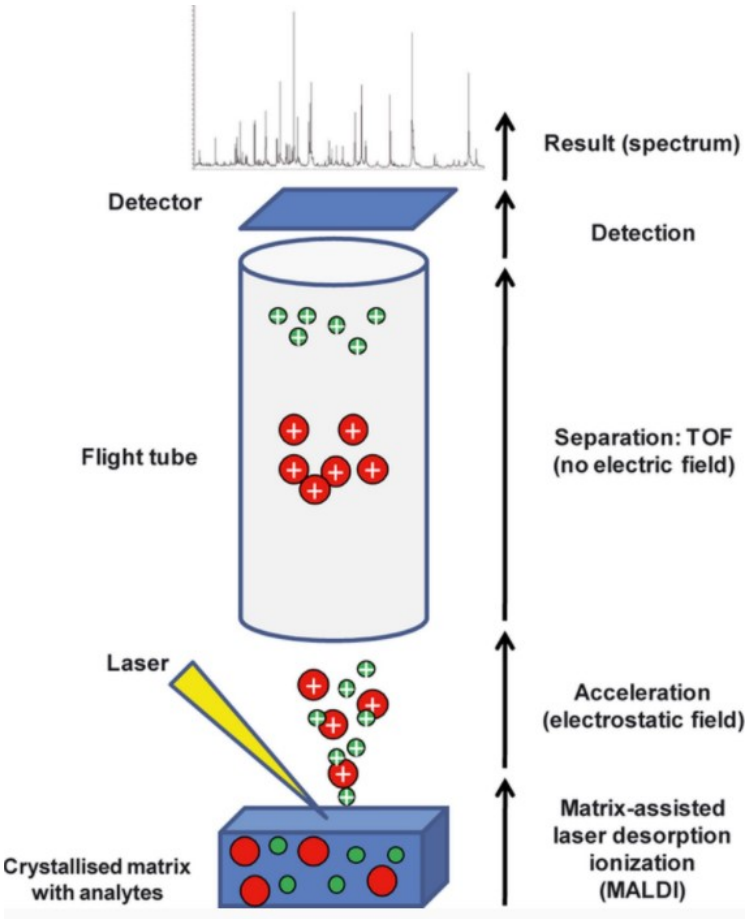

Figure 9. ***Illustration of MALDI TOF mechanism from start to finish****.*

The high energy from the laser is absorbed in matrix causing to desorption of the analytes which then are vaporized and ionized in the gas phase. Ionization of the analytes causes formation of primarily single charged ions from the sample. Followingly the now desorbed and ionized molecules are accelerated through an electrostatic field and then ejected through a

metal flight tube which is in vacuum. The molecules travel through the vacuum until they reach a detector, smaller ions will travel faster and reach the detector ahead of lager ions. Time of flight (TOF) is linked to the mass (m) and charge (z) of the bio analyte and is also proportional to the square root of m/z. Hence, each component of the complex sample is separated based on their TOF, this created a mass spectrum characterized by the ions intensity as well as m/z, defined by the number of an ion with a specific m/z that hit the detector (Croxatto et al., 2012).

Finally, the bombardment of ions results in a diagram with spectral signature spikes which ordinarily range from 1000 – 20 000 m/z. MALDI usually makes single charged ions (z =1) making m/z of an analyte match the value of its mass (Croxatto et al., 2012). The MALDI score values are classified like this: ≥2 species identification, ≤ 1.9 ≥1.7 genus identification < 1.7 no identification(Ferreira et al., 2011)

1.7.3 Horizontal submerged electrophoresis

Gel electrophoresis may be used to check if the PCR product is the right size and has an insert. This is a technique that separates DNA fragments (or other macromolecules) by their size. It's done by running a current of electricity through a porous agarose gel. The molecules of interest are loaded into wells at the top and move through the gel until they lodge themselves and create bands, the gel itself is submerged in a running buffer. Distance and patterns are decided by molecular charge and size, this gives a clear count of fragments. Size is determined by comparing the columns to a ladder with DNA fragments of a known size. (Reece, 2012)

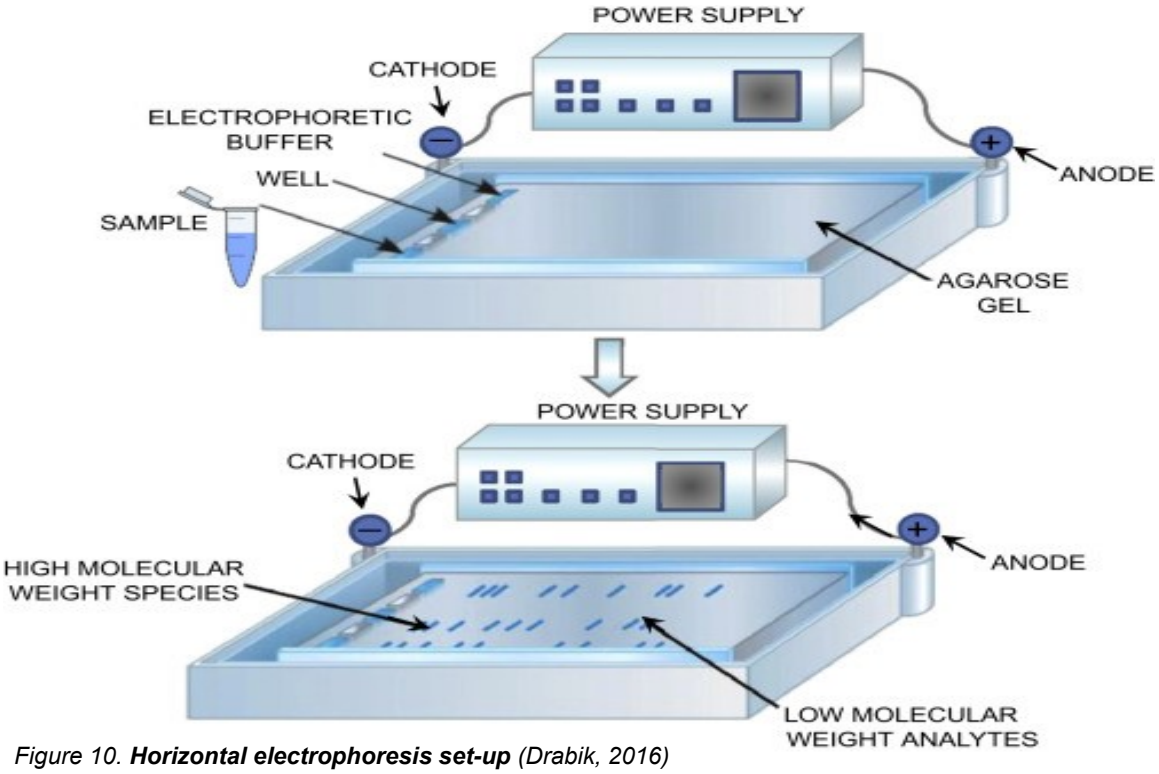

Figure 10. ***Horizontal electrophoresis set-up*** *(Drabik, 2016)*

1.7.4 Biofilm forming assay – Christensen method

This standard method was established as a quantitative model for adherence of *staphylococci* to medical devices. It was originally published in American Society from Microbiology in 1985. The isolates were from catalase producing Gram-positive *Staphylococci* isolated from intravascular catheters of hospitalized patients. Samples were cultivated on tryptic soy agar and supplemented with 5% sheep blood every 2-3 months.

The samples were diluted in TSB without glucose in 1:100 dilutions. The aliquots were

inoculated on sterile PS tissue culture plates (Micro Test III (Falcon no. 3072; Becton Dickinson, Oxnard, Calif.) and Cell Wells (no. 25860; Corning Glass Works, Corning, N.Y.), with 200 µl pr. well.

Plates were placed for stationary incubation for 18 h at 37°C. After incubation, plates were emptied using a low vacuum 100 µl Pasteur hand pipette. The wells were washed 4 times with 200 µl PBS (ph7.2).

The film that adhered was fixed with a Bouin fixative and stained with Hucker crystal violet; excess was rinsed under running water.

Reading were made on an Micro ELISA auto reader at 670 nm with single wavelength mode (lambda test). Measurements were done in quadruplicates and repeated three times. Isolates that were the same strain were merged and averaged together (Christensen et al., 1985).

2 Material & methods

2.1 Study design

As this is a pilot study, most of the workflow has been based on previous results (Figure 11).

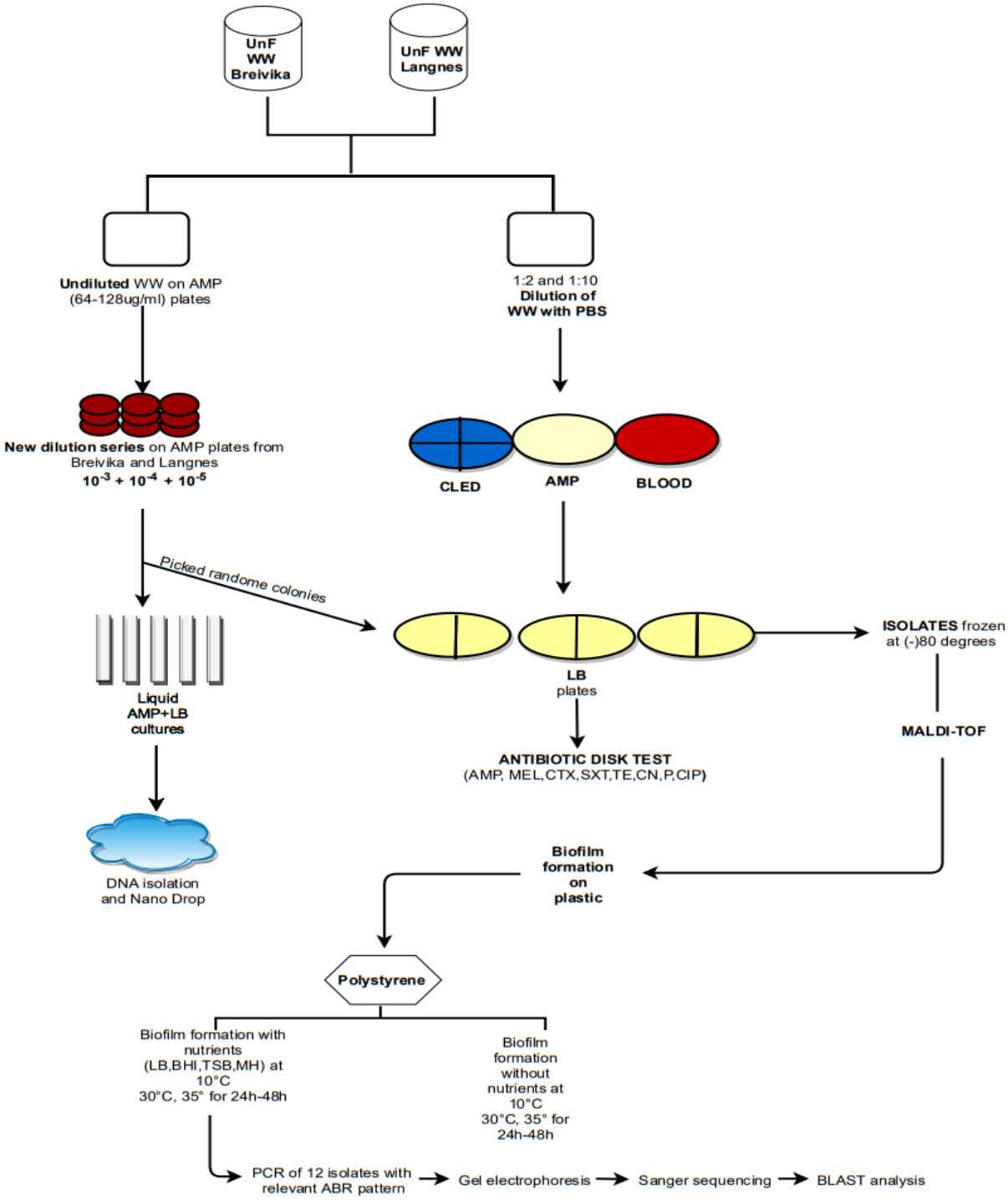

Figure 11. ***Thesis workflow****, starting at sampling to the final sequencing and BLAST analysis.*

Sampling was done at Breivika and Langnes WWTP in 1L bottles. Small amounts were diluted and streaked onto different agars that were available at the time in order to isolate individual bacterial colonies from the WW. Random colonies (n= 57) from the AMP, CLED and blood agar were re-streaked onto LB plates. Identification with MALDI-TOF and Kirby-Bauer disk diffusion was performed on the isolates and frozen stocks were made.
Eight bactericidal antibiotics representing four different modes of action were selected based on a list of the most utilized antibiotics from UNN (limited by availability).
Frozen stocks were used to test the biofilm formation ability of each isolate on PS plastic at 30°C with the Christensen method. The same procedure was performed with WW added LB, BHI, MH, TSB and a set of raw sewage water without nutrients.
Additionally, PCR was performed on 12 isolates which were biofilm forming on PS and were resistant to ≥5 of the eight antibiotics. And 8 of these were amplified with degenerated primers Bak11w Bak 2 and sent to external lab for 16S sequencing for identification.

2.2 Material

2.2.1 Biological material

Sewage water, 24h samples

Collected in September 2020:

1 L of mechanically filtered effluent Breivika

5 L unfiltered influent from Breivika and Langnes

Collected in January 2021:

1 L Unfiltered influent from Breivika

Positive controls

Staphylococcus epidermidis ATCC 35984 (RP62A 42-77)

Shewanella oneidensis, strain ID: LMG 19005 (MR-1)

2.2.2 Kits, Chemical, Medias

QIAamp® DNA Stool Mini Kit (50), Qiagen

BigDye® Direct Sanger Sequencing Kit

Chemicals

1 X Phosphate – buffered Saline (PBS) solution, Sigma Aldrich

NaCl: 137 mM

KCl: 2.7 mM
Na2HPO4: 10 mM
KH2PO4: 1.8 mM

Running buffer Tris -borat- EDTA (TBE) x1, Sigma Aldrich
0.13 M tris (pH 7.6)

45 mM boric acid

2.5 mM EDTA

Adjust volume to 1 L with MiliQ dH2O

phiX174 DNA/BsuRI (HaeIII) Marker, Sigma Aldrich
ΦX174 DNA was completely digested with BsuRI, purified and dissolved in a storage buffer.

The Marker contains the following 11 discrete fragments (in base pairs): 1353, 1078, 872, 603, 310, 281, 271, 234,194, 118, 72.

1kb DNA ladder, Sigma Aldrich
10 mM Tris-HCl (pH 8.0), with 1.0 mM EDTA.

DNA Loading Dye x6, Thermo Fisher
10 mM Tris-HCl (pH 7.6), 0.03 % bromophenol blue, 0.03 % xylene cyanol FF, 60 % glycerol and 60 mM EDTA.

GelRed™, Sigma Aldrich

Crystal violet 0,1%, Sigma Aldrich
10 mg Crystal violet

100ml MiliQ H_2O

Autoclaved

NaCl (85%)

80% glycerol, Sigma Aldrich

Diluted in dH_2O

ExoSAP-IT PCR Product Cleanup Reagent (Thermo Fischer Scientific)

Broth and Agar

LB Broth (Miller), Sigma Aldrich (Merck™ KGaA, Darmstadt, Germany)
NaCl, 10 g/L

Tryptone, 10 g/L

Yeast Extract, 5 g/L

(pH range 6.8 - 7.2 (2.5% solution))
1. Suspend 25 g in 1 L of distilled water.

2. Autoclave for 15 minutes at 121 °C.

MH Broth, Sigma Aldrich (Merck™ KGaA, Darmstadt, Germany)
beef infusion solids, 2.0 g/L

casein hydrolysate, 17.5 g/L

starch, 1.5 g/L
Dissolve 21 g in 1L of distilled water. Sterilize by autoclaving at 121°C for 15 minutes.

TSB Broth, Sigma Aldrich (Merck™ KGaA, Darmstadt, Germany)
casein peptone (pancreatic), 17 g/L

dipotassium hydrogen phosphate, 2.5 g/L

glucose, 2.5 g/L

sodium chloride, 5 g/L

soya peptone (papain digest.), 3 g/L

Suspend 30 g of dehydrated media in 1L of purified filtered water. Heat with frequent agitation and boil for one minute. Sterilize at 121°C for 15 minutes.

BHI Broth, Sigma Aldrich (Merck™ KGaA, Darmstadt, Germany)
beef heart (infusion from 250g), 5 g/L

calf brains (infusion from 200g), 12.5 g/L

disodium hydrogen phosphate, 2.5 g/L

D (+)-glucose, 2 g/L

peptone, 10 g/L

sodium chloride, 5 g/L
Dissolve 37 g in 1 L distilled water. Sterilize by autoclaving at 121°C for 15 minutes

AGAR

Muller-Hinton agar, Sigma Aldrich (Merck™ KGaA, Darmstadt, Germany)
agar, 17.0 g/L

beef infusion solids, 2.0 g/L

casein hydrolysate, 17.5 g/L

starch, 1.5 g/L
Suspend 38 g in 1 L of distilled water, bring to the boil to dissolve the medium completely and sterilize by autoclaving at 121°C for 15 minutes.

LB agar Sigma, Aldrich (Merck™ KGaA, Darmstadt, Germany)
Agar, 15 g/L

NaCl, 10 g/L

Tryptone, 10 g/L
1. Suspend 40 g in 1L of distilled water.

2. Heat to boiling while stirring to dissolve.

3. Autoclave for 15 minutes at 121°C.

4. Cool to 50°C prior to dispensing into sterile petri dishes.

Clade agar Sigma Aldrich (Merck™ KGaA, Darmstadt, Germany)
agar, 15 g/L

Beef extract, 3 g/L

Bromo thymol blue, 0.02 g/L

Casein enzyme hydrolysate, 4 g/L

L-cysteine, 0.128 g/L

lactose, 10 g/L

peptic digest of animal tissue, 4 g/L
Suspend 36.15g in 1000ml distilled water. Boil to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

Chocolate agar Sigma Aldrich (Merck™ KGaA, Darmstadt, Germany)
agar, 15 g/L
meat extract, 10 g/L
peptone, 10 g/L

sodium chloride, 5 g/L
Suspend 40 g in 1L of distilled water. Bring to the boil to dissolve completely. Sterilize by autoclaving at 121°C for 15 minutes.

Blood agar (horse blood) Sigma Aldrich (Merck™ KGaA, Darmstadt, Germany)
agar, 15 g/L

meat extract, 10 g/L

peptone, 10 g/L

sodium chloride, 5 g/L
Suspend 40 g in 1L of distilled water. Bring to the boil to dissolve completely. Sterilize by autoclaving at 121°C for 15 minutes. For blood agar, cool to 45-50°C and add aseptically 6% of sterile defibrinated blood.

Ampicillin infused agar plates (64 µl/ml -128 µl/ml)

Agar, 15 g/L

NaCl, 10 g/L

Tryptone, 10 g/L

Ampicillin 64 µl/ml/128 µl/ml
1. Suspend 40 g in 1L of distilled water.

2. Heat to boiling while stirring to dissolve.

3. Autoclave for 15 minutes at 121°C.

4. Cool to 50°C prior to dispensing into sterile petri dishes.

2.3 Sample preparation and harvesting

Samples were collected from the wastewater treatment plants in the morning (16/9/20) the samples, both filtered (1 L from Breivika) and unfiltered (5 L from Langnes and Breivika) were brought back and processed (Figure 12).

Figure 12.Sampling sites at Breivika and Langnes on the northern point of Tromsø peninsula (Rambøll, 2020).

The wastewater being treated at Langnes is a collection of WW from the residential area on the western end of Kvaløya and the airport. While WW at the Breivika plant water is collected from UNN, The Arctic University of Tromsø and the closest residential houses and the industrial nearby.

Two liters of the unfiltered samples from each station were filtered in the sink using a sift with 200 µm masks size, the sludge was collected in marked tubes and frozen at -80 °C for further investigation at a later point. About 1 L from inlet + outlet from each station was also collected for analysis at an external lab (Eurofins Environment testing Norway AS) to establish the distribution and composition of microplastics present.

2.3.1 Culturing wastewater on agar plates

Two liters of unfiltered sample, from each station and one filtered from Breivika was utilized. They were incubated on Muller-Hinton, LB agar, CLED agar, Chocolate agar, Blood agar and LB with ampicillin (AMP, 64-128 µg/mL). Each of the three samples were divided onto three plates, making 9 plates in total.

One undiluted

One 1:2 dilution – 1 mL sample to 1 mL PBS

One 1:10 dilution – 200 µL sample to 1.8mL PBS

A 10 µL loop was used to evenly distribute 100 µL of sample on each plate. The same loop was used on samples with the same degree of filtration and origins, starting from the highest dilution to the undiluted sample. Loop was changed once it had touched the ampicillin infused plate. Plates were incubated at 30°C for 24 h.

Streaking for purification of single isolates

After 24 h of incubation the plates were inspected. Three new AMP plates with dilutions 10^{-3}, 10^{-4}, 10^{-5} were set for 24 h incubation using T-streak/three phase streak technique. Using 100µl diluted sample from Breivika filtered + unfiltered.

Liquid LB media infused with AMP were made in 15mL tubes, five tubes with 1mL media in each – four with one individual colony from the AMP infused plate (Unfiltered, undiluted from Langnes) and one with a scoop of colonies from the same plate. After 24 h incubation, made same dilution series and T-streaks on LB+AMP plates.

The remaining colonies were placed in a fridge to suspend further growth. All the steps involving agar plates were done next to a lit Bunsen burner with sterile equipment.

2.3.2 Colony isolation on LB

Single colonies were picked from diluted AMP plates and set on LB+AMP, blood and CLED agar.

Colonies from Langnes/Breivika Unfiltered on AMP and CLED plates were picked. Each original plate was divided onto 3-4 new plates (of identical make), these were again divided in four sections, allowing four single colonies to be streaked with decreasing concentration within its designated area. One blood agar plate was split in two, one side for Breivika

(unfiltered) and one for Langnes (unfiltered). Incubated at 30°C - 37°C for~24 h.
Plates were stored in the fridge after 24 h incubation and later transferred to LB plates.

2.4 Antimicrobial susceptibility testing by disk diffusion test

2.4.1 Disk diffusion test prep on isolates

Colonies from LB + AMP, CLED and one blood agar plate was transferred to new LB plates divided in two parts rather than four. Each plate was marked with, date, name and the type of agar the colonies had been transferred from. Random swabs with an inoculation loop were made in a selected quadrant before being T- streaked onto the new two-part plate. The plates from CLED Breivika colony were named Q1-Q4 and AMP Breivika were called P1-P4. Plates were incubated at 30°C - 37°C for~24 h. The same procedure was done with plates from Langnes, these were named A1-A5 for AMP Langnes and B1-B4 for CLED Langnes.

Disk diffusion test

Overnight LB plates prepped for the disk test. And new fresh Muller-Hinton plates were marked with numbers, as the antibiotic disks would be placed on these and each single culture would be on separate plates. New plates were made in duplicates as there were eight antibiotics – placing four on each plate.
A cotton swab was used to remove a small portion of colonies and dipped into a glass tube with NaCl (85%) in a McFarland device (Grant McFarland Densitometer DEN-1B) which measures the optical density. The solutions were made to be 0.5 McF, followingly the swab was discarded, and a new swab was used to smear the solution evenly across the plate in a grid pattern, this was repeated 3 times or until the plate was dry.
Each duplicate had four disks of different antibiotics with varying concentration on them as seen in table 4. These plates were incubated over night at 30°C for 24 h.
After incubation the plates with antibiotic disks were looked over, and each plate was logged with the diameter (measured with a ruler) of inhibition for the different antibiotics, deviations and general appearance. Plates with contamination, implying more than one obvious strain were discarded. Degree of resistance is determined by studies done on the different genera/species by EUCAST – European Committee on antimicrobial Susceptibility testing (EUCAST, 2021).

Table 4. Antibiotics utilized in Kirby-Bauer disk diffusion test and the amount of antibiotics in each disk(µg)

Antibiotics	**Total amount on the patch (µg)**
Penicillin (P)	10
Gentamycin (CN)	10
Ampicillin (AMP)	10
Tetracycline (TE)	30
Trim-sulfa (SXT)	25
Cefotaxime (CTX)	5
Ciprofloxacin (CIP)	5
Mecillinam (MEL)	10

2.5 Freeze stock preparation

Freeze stocks (-80°C) were prepared for preservation of isolates, 64 tubes with 250 µL 80% glycerol and 750 µL LB medium were made in a Laminar air flow cabinet, each vile and piece of equipment brought into the cabinet was wiped down with 80% ethanol. The tubes were stored at room temp. Freeze tubes with glycerol and LB medium were inoculated in Laminar air flow cabinet. A scoop from each isolate was transferred to the tubes and placed in -80°C freezer for further study.

2.5.1 Re-vitalizing freeze stocks

Frozen stocks were taken out 5-6 at a time in a specialized cooling box (to keep them from thawing) into a laminar flow cabinet. A small piece of sample was scraped out from each isolate and T-streaked out onto new LB plates with a plastic inoculating loop. The plates were incubated at 30°C degrees for 24 h.

After the incubation, plates that were clean isolates, were transferred to a 4°C fridge awaiting further treatment. Some plates did not yet have any visible growth and were therefore re-

incubated. Impure isolates were sorted onto new separate plates, making new pure isolates, which were incubated at 30°C degrees for 24 h.

Plates that did not exhibit any growth were discarded, the cultures they originated from were thawed and new liquid cultures were made. This was done by transferring 700 µl LB+AMP broth and incubating it on a shaker at 37°C degrees for 24 h, meant to be frozen with 80% glycerol. However, the cultures were not viable.

2.6 Biofilm formation on PS pegs

Cultures meant for peg biofilm assay were made by adding 5 mL LB media and one colony to a falcon tube, this was done with sterile media and inoculation happened in a laminar air flow cabinet. There were 18 cultures in triplicates on a 96F well plate (Thermo Fisher Scientific) in addition to a positive control, a lid with polystyrene pegs Nunc TSP (Thermo Fisher Scientific, Transferable Solid Phase Screening System, 96 simultaneous assays, Denmark). Isolates were incubated with 5 ml LB and positive control S. epidermidis with 5 ml TSB. After 24 h incubation at the plate was treated as stated in the modified Christensen biofilm method (Appendix E) for semi quantitative determination of biofilms.
Absorbance was measured with a Synergy H1 Hybrid Multi-Mode Microplate reader (BioTek). There were some additional adaptations regarding the incubation temperature, as some of the samples had optimal growth at 30°C, they were incubated at this temperature for the overnight cultures and at 35 °C for biofilm formation in the wells.

2.6.1 Biofilm formation on flat-bottom and convex microtiter plates

The same procedure was followed as for biofilm formation on pegs, except plates with standard lids were used. Incubation was at 30°C for both overnight shake cultures and static incubation in the wells. Positive control was *S. oneidensis* was incubated with 5 ml LB.

2.6.2 Biofilm formation with different media

A new 1 L sample was collected from the inlet at Breivika WWTP for the raw sample biofilm formation.
Four different medias - **LB, TSB, MH, BHI**
Were divided onto two 96 well plates with lids and incubated at 30°C for 24 h and 150 µl samples per well. There were two medias per plate in addition to a positive control (*S.*

oneidensis) and negative controls which was whatever media being used on that plate. Samples of the raw sewage water were diluted with media and placed in a dilution gradient from undiluted, 10^2, 10^4, 10^6, 10^8, 10^{10} and 10^{12} in triplicates. One series with raw undiluted sewage water and a series diluted with distilled water was also made.

2.7 DNA isolation bacteria

A selection of the samples identified with MALDI-TOF were chosen for further sequencing, subsequently the DNA from said samples were isolated using QIAamp® DNA Stool Mini kit (50). The protocol provided by the manufacturer was followed (Appendix E). The purity was determined using Nano Drop™ 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Extracted DNA was stored at -20°C to be used for PCR and sequencing.

2.8 PCR & 16S sequencing

Eluted DNA from isolated bacterial samples was thawed and used as template for the 16S rRNA sequencing. There was a total of 12 samples in duplicates making 24 PCR tubes in addition to a duplicated negative control (dH2O) with a total volume of 20µL and a total of 26 PCR tubes. The tubes were filled with 3 µl template and 17 µl Master Mix.

PCR Master Mix composition

Sample size	**x1**	**x30**	
BAK 11W	1	30	µl
BAK 2	1	30	µl
DreamTaq (x2)	10	300	µl
dH2O	5	150	µl
Templat (max. 3 µl, 50-100 ng)	3	3	µl
Tot.	**20**		
Negativ ctrl	3		

PCR temperature cycle

16S	*Degrees(°C)*	*Time(t)*
Initial	95	**2min**
Denaturation		
Denaturation	95	**30sec**
Annealing	60	**30sec**
Elongation	72	**40sec**
Fin.elongation	72	**5min**
Cooldown	10	**∞**

2.8.1 Sequencing: Big Dye Terminator V3.1

The PCR product of 8 isolates were cleaned using ExoSAP-IT™ Express PCR product cleanup from Thermo Fisher (©2017 Thermo Fisher Scientific Inc).
A 96-well microtiter plate for PCR was filled with 2 µl SAP and 5 µl PCR product from the 8 samples in duplicates.

After cleaning a new plate with 18 µl Master mix and 1 µl Bak11w primer (forward) was added. Template was added last in a sterilized fume hood. Visuals were provided via Gel Doc with Quantity One software.

Big Dye v3.1 Master Mix composition

Sample size	**x1**	**x20**	
BAK 11W	1	20	µl
SeqBuffer	4	80	µl
Big Dye	1	20	µl
dH2O	13	230	µl
Clean PCR product	1	1	µl
Tot.	**20**		

Big Dye v3.1 temperature cycle

BigDye v3.1	*Degrees(°C)*	*Time(t)*
Initial	96	**1 min**
Denaturation		
Denaturation	96	**10 sec**
Annealing	50	**5 sec**
Fin.elongation	60	**4 min**
Cooldown	4	**∞**

Purified PCR product was sent for 16S rRNA sequencing with Applied Biosystems 3130xl Genetic Analyzers for Sanger sequencing at UNN – University hospital in Northern-Norway.

3 Results

The following results are from samples collected at Langnes and Breivika wastewater stations in September (Figure 13). In order to see a difference in microbial composition and ABR patterns in the WW, and biofilm formation capabilities on plastic. After collection, the wastewater was cultivated on MH agar plates with 64-128 µg/mL ampicillin as well as LB, CLED, Chocolate and blood agar. There was a lot of growth on all the plates, in the initial undiluted set. The different plates had distinguishable colonies – dominated by large round white ones, some slimy and some with a dry look. As well as small white ones (~1mm diameter) were observed. Later, after isolates had been made and frozen and re-streaked, some large redish colonies were also observed.

A decision was made to only move forward with colonies from the AMP plates. A new set of AMP plates were streaked with higher dilutions from the same sample, in order to grow single colonies. About 4 L from at Breivika and Langnes was sent to Eurofins for microplastic analysis, 1L from the inlet and outlet at both stations. These analysis reports are added in appendix A.

Water samples collected from Breivika and Langnes

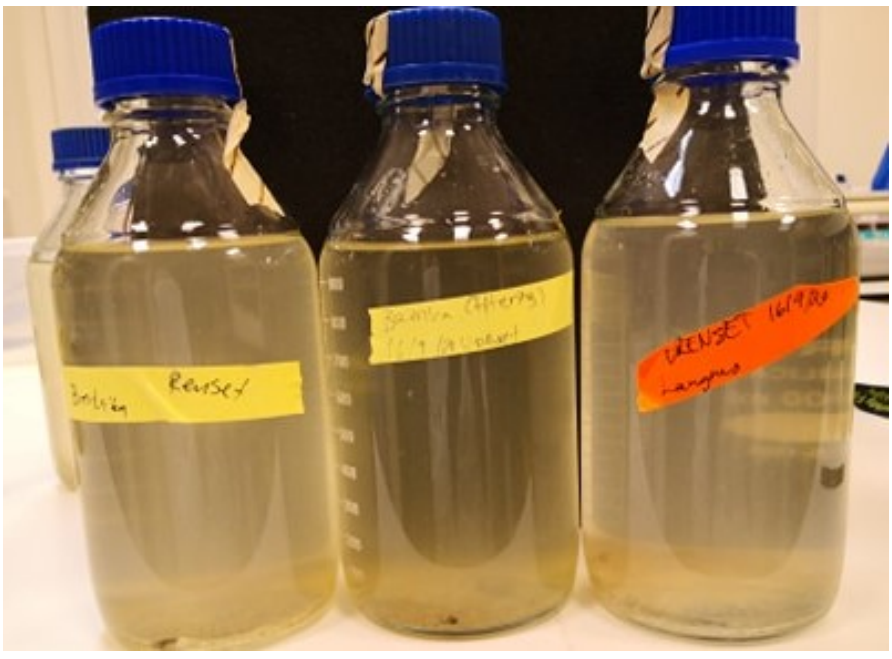

Figure 13.Water samples from wastewater stations Breivika and Langnes in 1L bottles. Seen from left to right; Breivika filtered, Breivika unfiltered and Langnes unfiltered.

After 24 h of incubations all the plates had substantial growth, even 1:10 dilution and plates infused with AMP. Therefor a new dilution series of 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} in order to inspect the individual colonies at a higher dilution. New AMP plates and liquid LB media infused with AMP, were set with dilutions 10^{-3}, 10^{-4}, 10^{-5} to test the strains for antibiotic resistance, phenotypic or inherited genetic resistance.

DNA concentration was measured with Nano Drop to determine purity in each isolate in relation to the presence RNA and other proteins. However, too much of the DNA was left in the filter as the sample size was 2 mL there was simply too much for the elution buffer. There was a large mass of slime in the sample after the lysate was incubated with ethanol which would not be eluted. During Nano Drop, the concentrations in K1-K3 were low indicating that there was an insufficient flowthrough of DNA in the filter, indicating that much of it was still tied up in the slime. This prompted a new round of elution of the slime with a longer incubation period. The two filters with the most visible slime were selected for further elution to prove the theory, one of 200 µL and one of 400 µL elution buffer were prepped and processed. The new concentrations measured with Nano Drop were higher for both volumes, however the 200 µL had a higher concentration.

These results indicated that a sample volume of 1 mL is more suitable to the kit when isolating DNA, also in regard to acquiring enough DNA that may be used for the sequencing.

3.1 Genera and species identified with MALDI-TOF

Using MALDI-TOF this is the genus distribution of the samples that were analyzed with MS. There is a clear abundance of Pseudomonas in the selection of colonies that thrived within the set conditions (Figure 14). Closely followed by *Aeromonas, Klebsiella, Raoultella* and *Acinetobacter.*

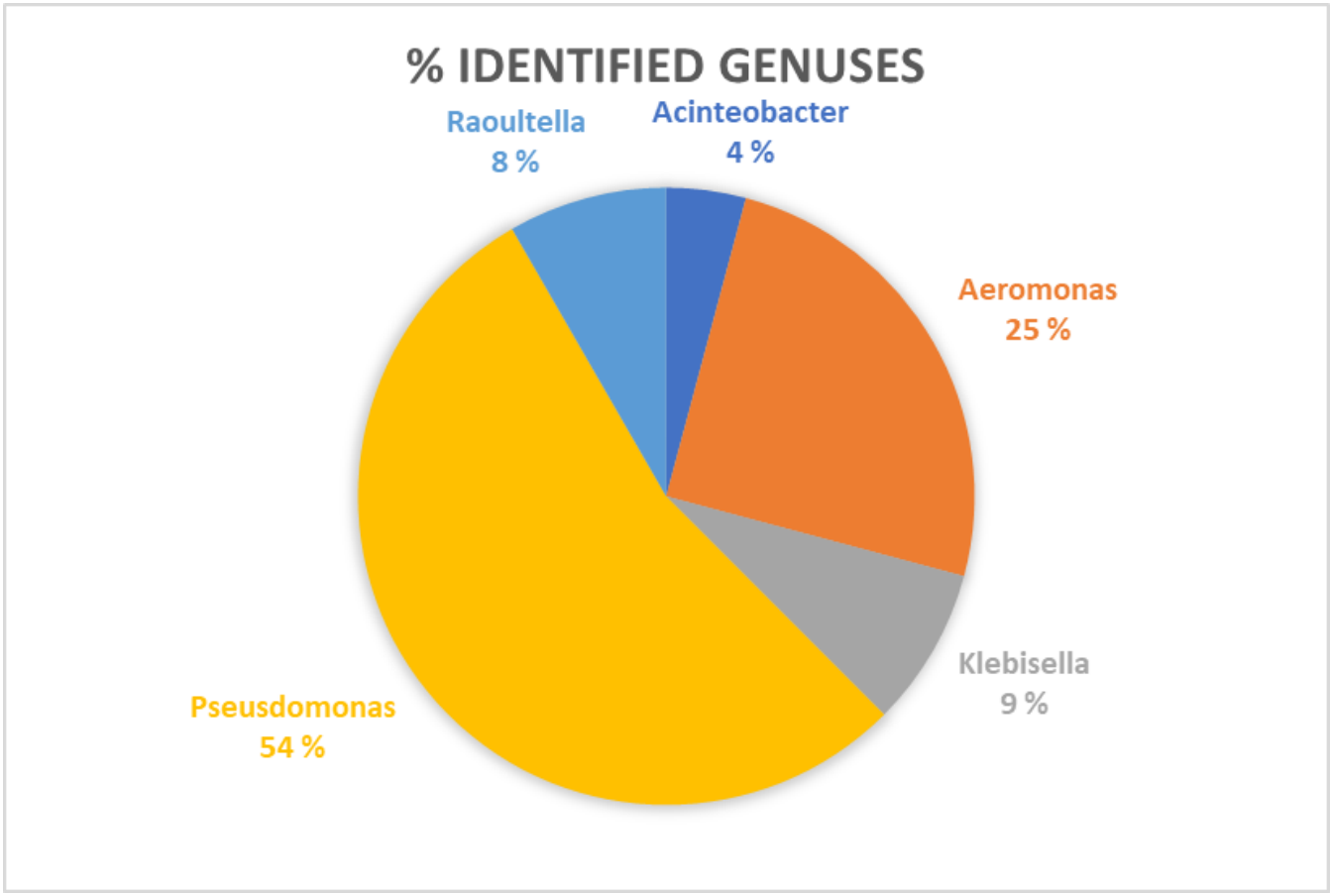

Figure 14.Out of the 57 samples collected and screened these were the genera present and their distribution. In total there were 24 unique species identified by MALDI-TOF. There is a clear abundance of Pseudomonas and Aeromonas in the WW at the time of sampling,

Out of the 57 isolates analyzed there were 24 unique species identified as seen in table 5. The full table with the individual identification for each isolate can be found in appendix C.

Table 5.Species identified by MALDI -TOF. Names in bold are species that have been described as human, fish or potential plant pathogens (Daskalov, 2006; Ghatak et al., 2016; Haruki et al., 2014; Martínez-Murcia et al., 2005; Pitout, Nordmann, & Poirel, 2015; Sa

Bacteria identified
1.ACINETOBACTER JOHNSONII
2. AEROMONAS BESTIARUM
3. *AEROMONAS EUCRENOPHILA*
4.AEROMONAS HYDROPHILA
5.*AEROMONAS MEDIA*
5.AEROMONAS SALMONICIDA
7.AEROMONAS VERONII
8.KLEBSIELLA OXYTOCA
9.KLEBISELLA PNEUMONIAE
10.PSEUDOMONAS LIBANESIS
11.PSEUDOMONAS ANTARCTICA
12.PSEUDOMONAS AZOTOFORMANS
13.PSEUDOMONAS BRENNERII
14.PSEUDOMONAS CHLORORAPHIS
15.PSEUDOMONAS EXTEMORIENTALIS
16.PSEUDOMONAS FLUORESCENS
17.PSEUDOMONAS FREDRKSBERGENSIS
18.PSEUDOMONAS GESSARDII
19.PSEUDOMONAS KILONESIS
*20.****PSEUDOMONAS PUTIDA***
21.PSEUDOMONAS VERONII
22.PSEUDOMONAS FRAGI
23.RAOULTELLA ORNITHINOLYTICA
24.RAOULTELLA TERRIGENA

The 9 species in bold are ones found clinically relevant in literature as being the disease-causing pathogen, associated with one. There are four *Aeromonads,* followed by two *Klebsiella,* two *Pseudomonas* and one *Raoultella.*

Overall, there are thirteen *Pseudomonas*, six *Aeromonads*, two *Klebsiella*, two *Raoultella* and one *Acinetobacter*. (Daskalov, 2006; Ghatak et al., 2016; Haruki et al., 2014; Martínez-Murcia et al., 2005; Pitout, Nordmann, & Poirel, 2015; Radisic et al., 2020; Sawada, Fujikawa, Tsuji, & Satou, 2021; Singh, Cariappa, & Kaur, 2016). These will be divulged further in the discussion.

3.1.1 BLAST identification of 16S rRNA sequence

The table 6 displays 16S rRNA Sanger sequencing results after being run through the nucleotide BLAST database at NCBI as well as the species identified with MALDI-TOF. There are some (2/8) species that differed between the two identification methods, however 6 out 8 were identified at least within the same genus for both identification methods. BLAST had several hits with identical ID- percentage and E-values, this was the case for all 8 samples.

Table 6. MALDI-TOF identification and 16S rRNA sequence BLAST results

ISOLATE #	MALDI SPECIES ID.	SCORE	BLAST ID	% IDENTITY
B2L4	*ACINETOBACTER JOHNSONII*	*2,06*	*Pseudomonas ludensis*	98
A1L4	*PSEDOMONAS KILONESIS*	*2,29*	*Pseudomonas migulae*	100
P2B2	*PSEDOMONAS FREDRIKBERGENSIS*	*1,99*	*Pseudomonas sp. strain 25-A10 16S ribosomal RNA gene, partial sequence*	99,17
P3B3	*PSEDOMONAS LIBANESIS*	*2,13*	*Pseudomonas lactis*	100
Q3B4 LHK	*ACINETOBACTER JOHNSONII*	*2,13*	*Acinetobacter johnsonii*	100
P4B2	*AEROMONAS MEDIA*	*2,23*	*Pseudomonas sp. strain TP34 16S ribosomal RNA gene*	100
P2B3	*AEROMONAS MEDIA*	*2,41*	*Aeromonas media*	99,59
Q3B4	*RAOULTELLA ORNITHINOLYTICA*	*2,18*	*Raoultella ornithinolytica*	99,73

The MALDI score values are classified like this: ≥2 species identification, ≤ 1.9 ≥1.7 genus identification, < 1.7 no identification (Ferreira et al., 2011) meaning only one (P2B2) has been identified to genus level by MALDI. The rest are according to the scores identified to species level.

3.2 Antibiotic susceptibility of single isolates from wastewater

Samples isolated from WWTP Breivika and Langnes were diluted to 0.5 McFarland and incubated 24 h at 30°C with eight different types of antibiotic disks on each isolate (Figure 15). Following, are the resistance patterns, visualized by an inhibition zone where the diffused antibiotic is effective, indicating the isolate is susceptible. Or there is a small to non-existent circle where the isolate is intermediate to resistant.

Degree of resistance is determined by studies done on the different genera/species by EUCAST – European Committee on antimicrobial Susceptibility testing (EUCAST, 2021).

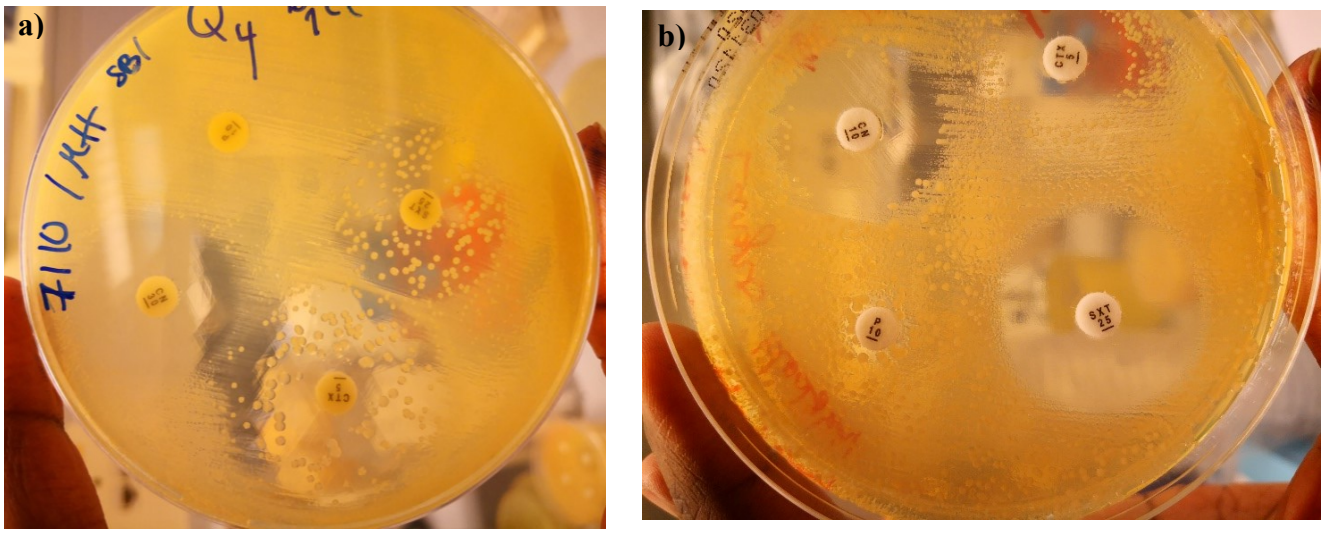

Figure 15. Kirby Bauer disk diffusion test picture a) to the left and b) to the right.
a) LB plate with four different discs of antibiotic. ***Top left*** *corns is Penicillin (P)10 µg, the growth is all the way to the disc.* ***Lower left*** *is Gentamycin 10 µg (CN); has a clear ring of inhibition around the disc.* ***Top right*** *is Trim sulfa 25 µg (SXT); it has a ring of inhibition but there are still some that have adapted and managed to grow past the line.*
Low right *is Cefotaxime 5 µg (CT); there is a large ring of incomplete inhibition a considerable amount of colonies have been established*

b) LB plate with four different discs of antibiotic. ***Top left*** *is Gentamycin 10 µg (CN); has a clear ring of inhibition around the disc* ***Lower left*** *corner is Penicillin, the growth is all the way to the disc.* ***Top right*** *is Cefotaxime 5 µg (CT); there is a small ring of incomplete inhibition, with a few colonies inching towards the center.*
Low right *is trim sulfa 25 µg (SXT); it has a clear ring of inhibition with no growth past the line.*

Most of the isolates are resistant to β-lactam antibiotics from both sample sites, likely due to intrinsic resistance (Cox & Wright, 2013; EUCAST, 2021).

Langnes 28/30 were resistant to all β-lactam antibiotics, and 2 susceptible to intermediate.

Breivika 27/27 were resistant to AMP and P. And 17/27 were intermediate to resistant to MEL and CXT.

There was a lot of variation from isolate to isolate for Trim-sulfa and cefotaxime, as can be seen in Figure 15 a) and b), some colonies grew past the line, while others had a clear distinction. This might indicate that the ones with a ring of inhibition and some colonies growing within the inhibition zone were impure or the bacteria adapted. These are likely resistant mutants able to grow beyond the inhibition zone. Based on the name code, these colonies stem from isolates grown on CLED agar (presumably due to is low selective nature), these often had small, glazed colonies accompanied by large white slimy ones.

The result of antibiotic resistance test by using disk test, is shown in Figure 16 and Figure 17.

The shortest bars indicate are either multi-resistant or it had fewer antibiotics tested for that isolate. There was a shortage on CIP, MEL, TE, P and AMP discs, the ones affected were; A3L3, B2L4.
The tallest bars uniformly have a wide range of susceptibility to the antibiotic discs at given concentration. Height is determined by the resistance diameter in millimeter (mm) which is visible within each bracket. Most of the strains seem to be resistant to penicillin's (AMP, P, MEL) and the cephalosporin (CTX).
However, there are some genus/species that are intrinsically resistant to certain antibiotics (EUCAST, 2021; Radisic et al., 2020):
* Klebsiella pneumoniae (complex)[1] – Ampicillin/Amoxicillin
*Raoultella spp. – Ampicillin/Amoxicillin
*Aeromonas hydrophila - Ampicillin/Amoxicillin
*Aeromonas veronii - Ampicillin/Amoxicillin
* Acinetobacter spp. - Ampicillin/Amoxicillin, Tetracyclines
* Pseudomonas spp. - Ampicillin/Amoxicillin, Tetracyclines

[1] Also *K.pneumoniae* sensu stricto*, K. quasipneumoniae* and *K.variicola (Rodrigues, Passet, Rakotondrasoa, & Brisse, 2018)*

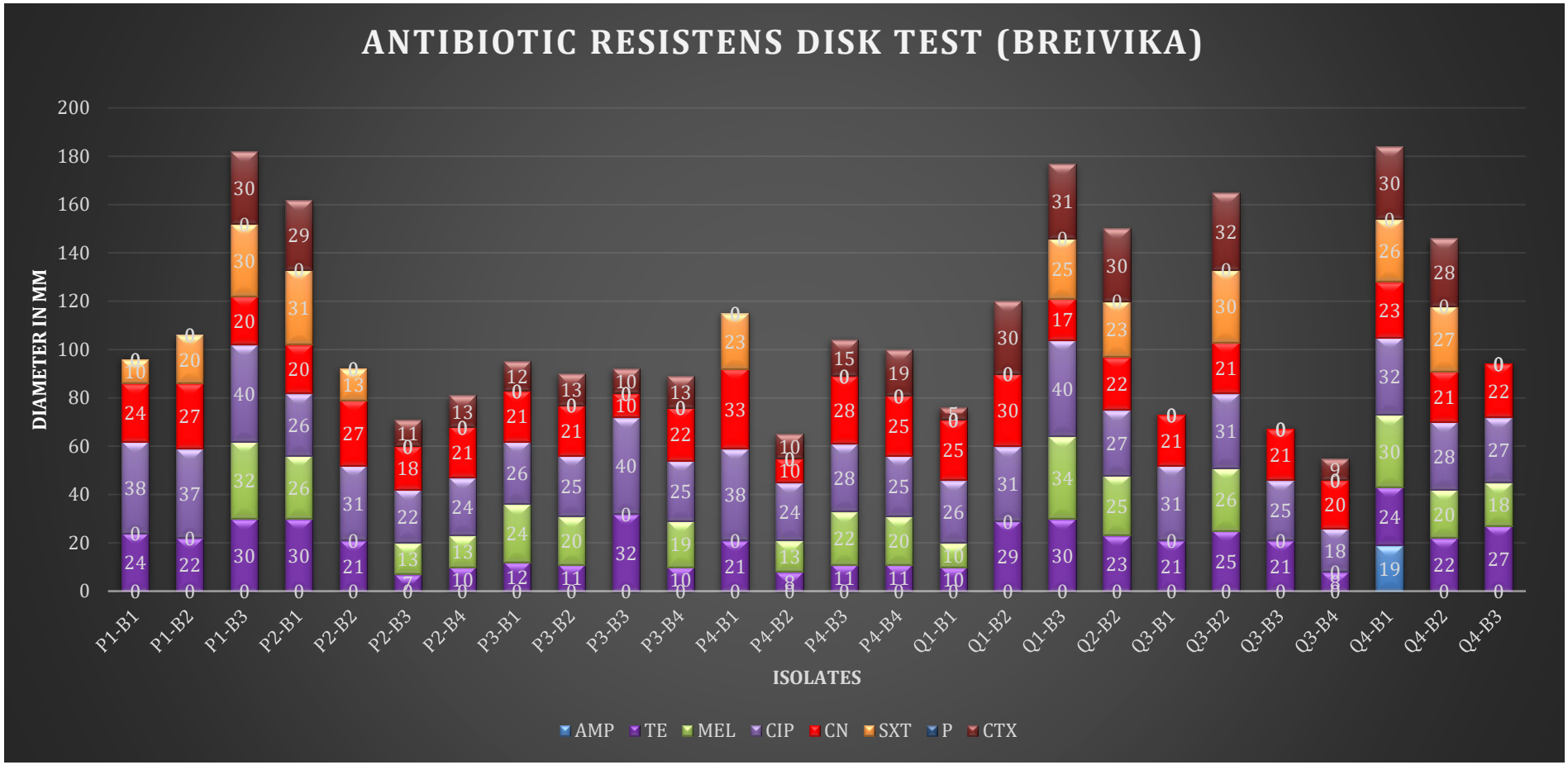

Figure 16. ***Antibiotic resistance detected in strains, Breivika****. After testing each isolate (n= 27) with disks with Penicillin (P) 5ug, Gentamycin (CN) 30 µg, Ampicillin (AMP) 10 µg, Tetracycline (TE) 30 µg, Trim-sulfa (SXT) 25 µg, Cefotaxime (CTX) 5 µg, Mecillinam (MEL) 10 µg these are the diameters of the inhibition rings created by the antibiotics. These isolates were collected at Breivika, the shortest posts have either highly efficient antibiotics for chosen isolates or/and the isolate is multi-resistant. The number on each block is the diameter in mm for that specific antibiotic according to the color codes.*

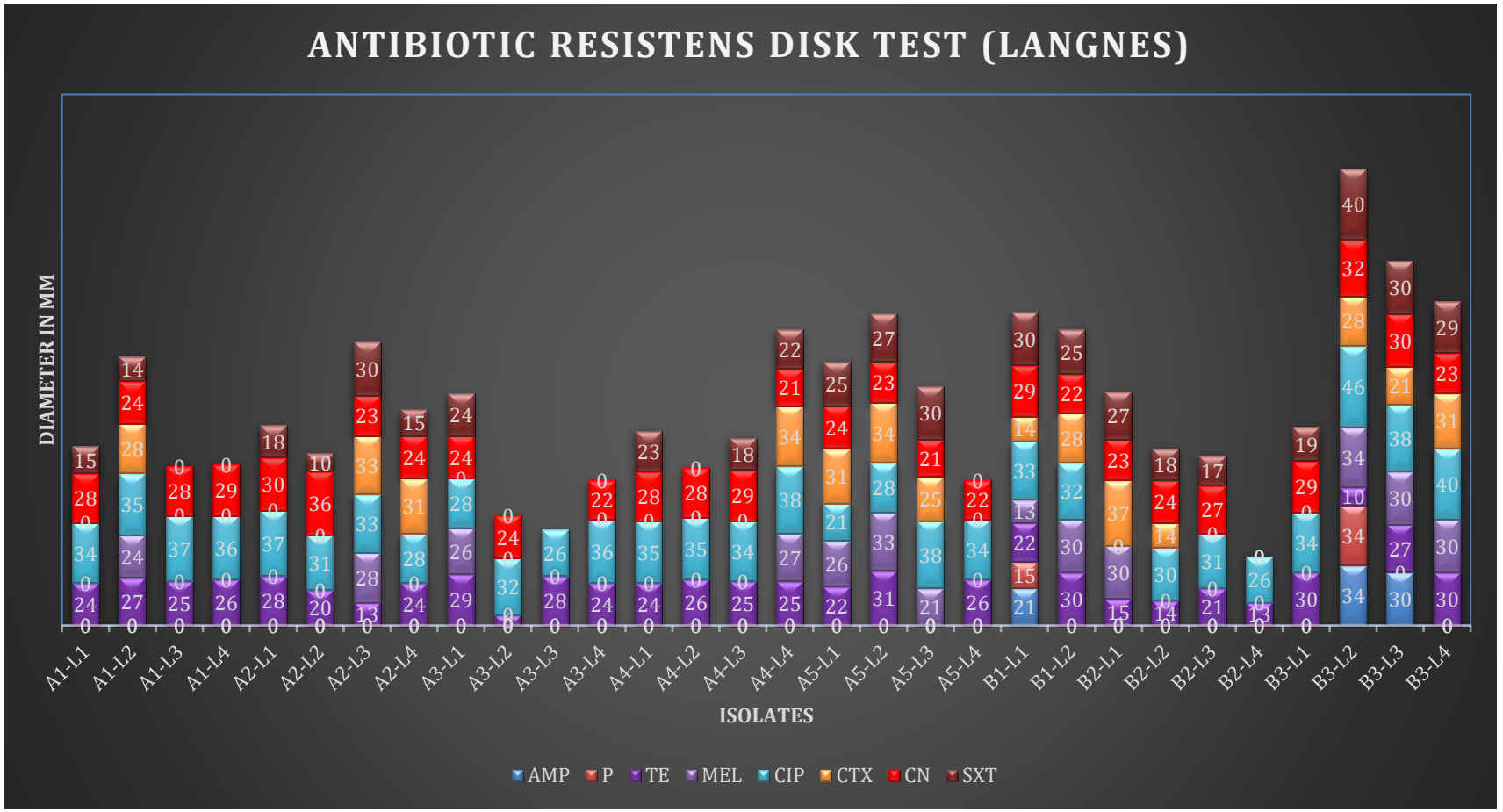

Figure 17. ***Antibiotic resistance detected in strains, Langnes.*** *After testing each isolate (n= 30) with disks with Penicillin (P) 5 µg, Gentamycin (CN) 30 µg, Ampicillin (AMP) 10 µg, Tetracycline (TE) 30 µg, Trim-sulfa (SXT) 25 µg, Cefotaxime (CTX) 5 µg, Mecillinam (MEL) 10 µg and Ciprofloxacin (CIP) 5 µg. These are the diameters of the inhibition rings created by the antibiotics. These isolates were collected at Langnes, the shortest posts have either highly efficient antibiotics for chosen isolates or/and the isolate is multi-resistant. The number on each block is the diameter in mm for that specific antibiotic according to the colour code*

3.2.1 Resistance profiles of isolates excluding βlactam antibiotics

Figure 18 and Figure 19 display the resistance patterns of the isolates without the β-lactam antibiotics as this likely is an intrinsic resistance in most of the samples. Caused by only picking colonies growing on AMP plates. In this figure there are antibiotics representing aminoglycosides, tetracyclines, fluroquinolones, Trimethoprim/Sulfonamides and gentamicin. These target 30S subunit by binding and inhibiting protein synthesis, DNA synthesis and folic acid metabolism through dihydrofolate reductase and dihydropteroate synthase. CIP is also considered a second line antibiotic, meant for more serious cases. CN, SXT and TE are used in a varying degree based on the type infection (Shorvon, 1989; WHO, 2017)

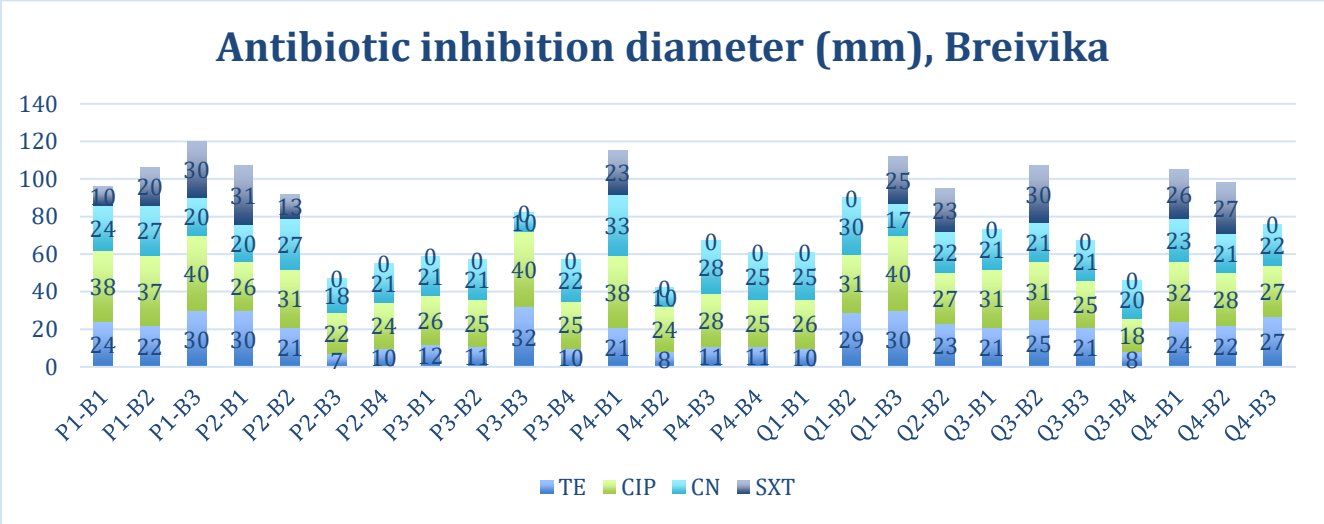

Figure 18 Resistance profiles of isolates (n=26) from Breivika without β-lactam antibiotics AMP, MEL, P & CTX

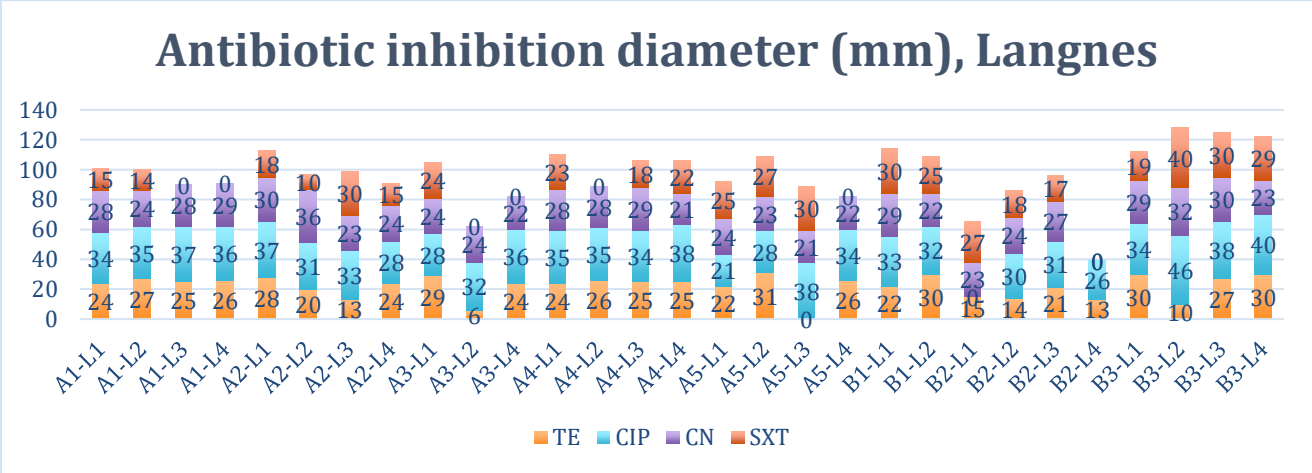

Figure 19 Resistance profiles of isolates (n=29) from Langnes without β-lactam antibiotics AMP, MEL, P& CTX.

Based on the overall accumulated inhibition diameter from each station on each antibiotic, Langnes samples has more susceptible colonies than the ones from Breivika. However, Langnes also has more resistant isolates to the non-β-lactams conveyed by the flat dot-plot line in Figure 20 for minimum values. This indicates a largely varying resistance composition in the WW samples from Langnes.

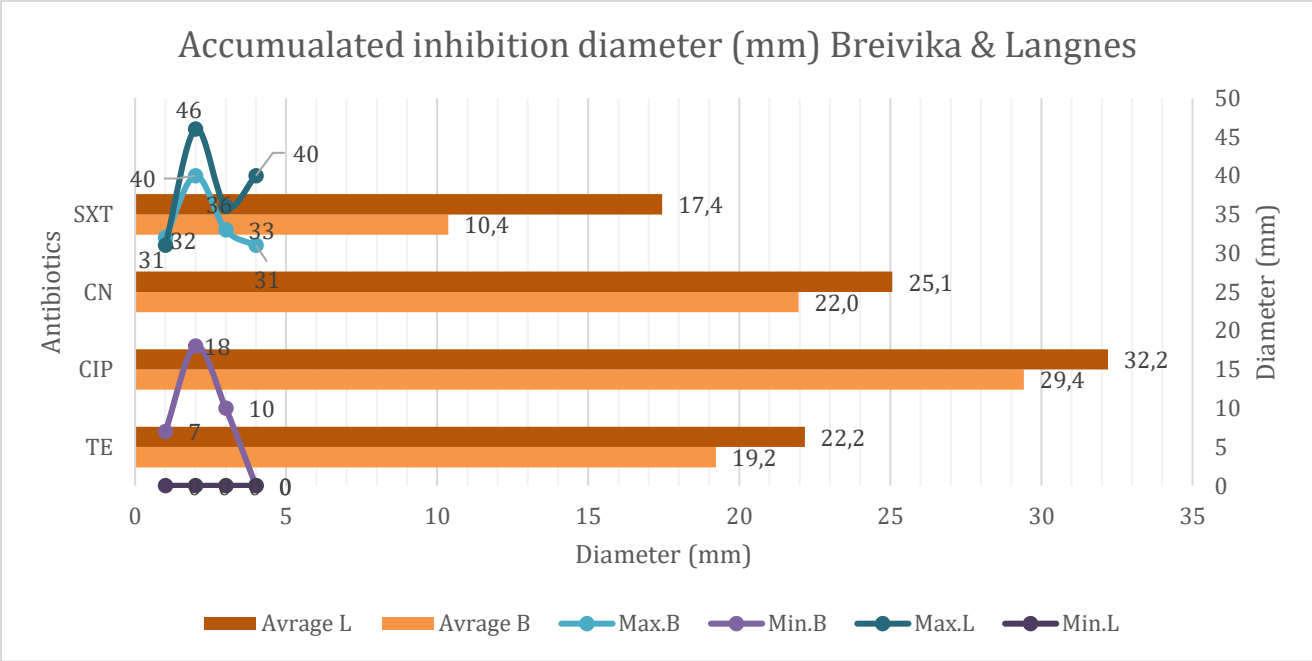

*Figure 20. Graph displaying the average inhibition diameter of all isolates form each sampling sites to the four non-beta-lactam antibiotics tested. The **lined dot-plot represents the maximum and** minimum values of each antibiotic, the **bars represent the average** values.*

The average accumulated inhibition of Breivika samples is lower than in the Langnes samples. However, the minimum values from Breivika are higher than Langnes and the maximum values are below the max values at Langnes. This suggests a more uniform resistance pattern in the samples from Breivika, but no clear conclusion can be drawn based on the low amounts of samples.

Overall, the isolates have a low susceptibility to SXT which inhibits folate synthesis which is a vital step in replication. CN and TE also seems intermediately effective against the isolates relative to the other antibiotics. Both CN and TE target the 30S ribosomal unit and inhibit protein synthesis. The one with the most effect, based on the large diameters is CIP, in both Breivika and Langnes samples. CIP is a fluroquinolone, which targets GyrA subunit in DNA gyrase and topoisomerase IV, essentially inhibiting DNA synthesis (Bjørsvik et al., 2017; Kapoor et al., 2017) .

3.3 Biofilm formation on PS peg lids

Biofilm formation on pegs is a standard method used to quantify biomass of biofilms and was therefore chosen as the starting point for these experiments.

The OD/absorbance was measured in order to quantify biofilm formation on both PS peg lids and on the bottom of polystyrene wells. Crystal violet is absorbed by cells and the extracellular matrix in the biofilm, making it possible to measure biomass. This was done at 30°C and 35°C for 24h with either MH, LB, BHI or TSB.

Note that not all the experiments had a positive control as there was difficulty finding available biofilm forming species able to thrive in said conditions. On the PS peg lids *S. epidermidis* was used as the positive control. However, in the remaining biofilm experiments *S. oneidensis.* was utilized as the control.

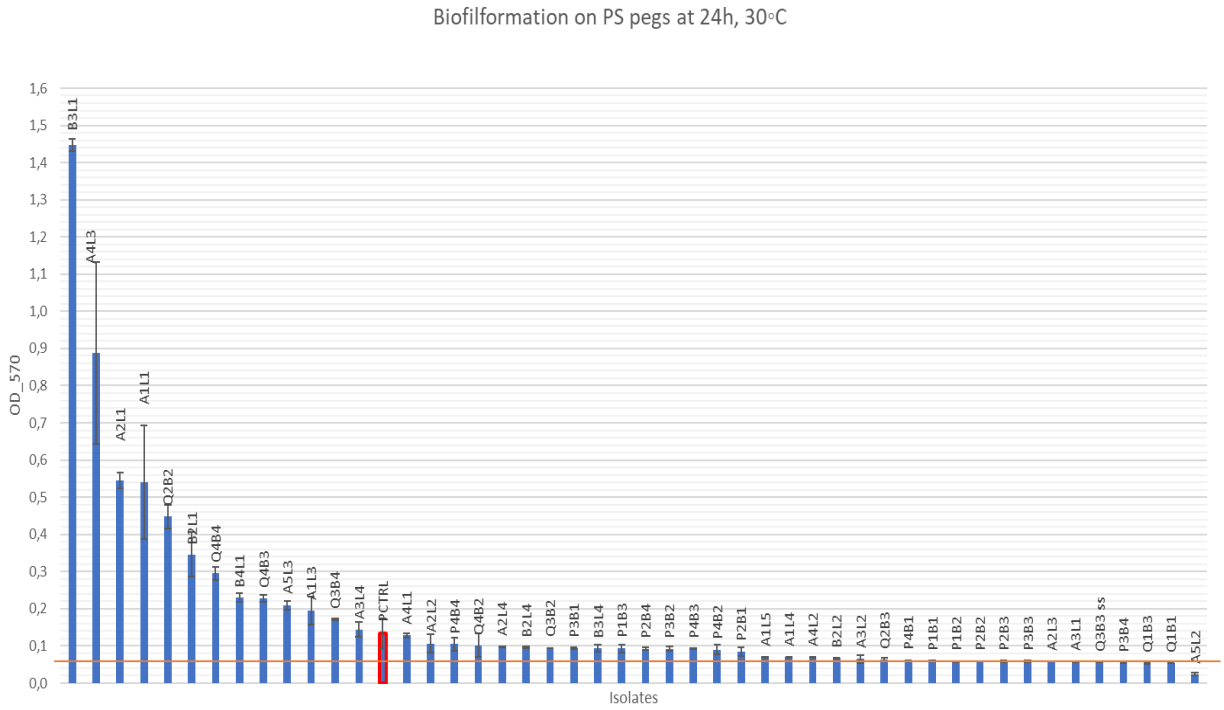

Figure 21.Biofilm formation on polystyrene peg lids n= 50. In red is the positive control S. epidermidis, the red line is the negative controls, where TSB and LB were utilized. The average absorbance = 0.17

Based on results from this study, a biofilm forming bacteria need an absorbance from ≥0.1, based on the results from these experiments, any values above 0.2 OD are deemed a decent biofilm forming bacteria. The average growth of isolates on PS pegs is 0.17.

3.3.1 Biofilm on flat bottom polystyrene 96-well plate

These results show how different surface allow varying adherence of biofilm forming bacteria to surfaces. Due to a mix up with the plates being used in these experiments, one set of isolates was incubated and fixated on plates with a convex surface rather than a flat one.

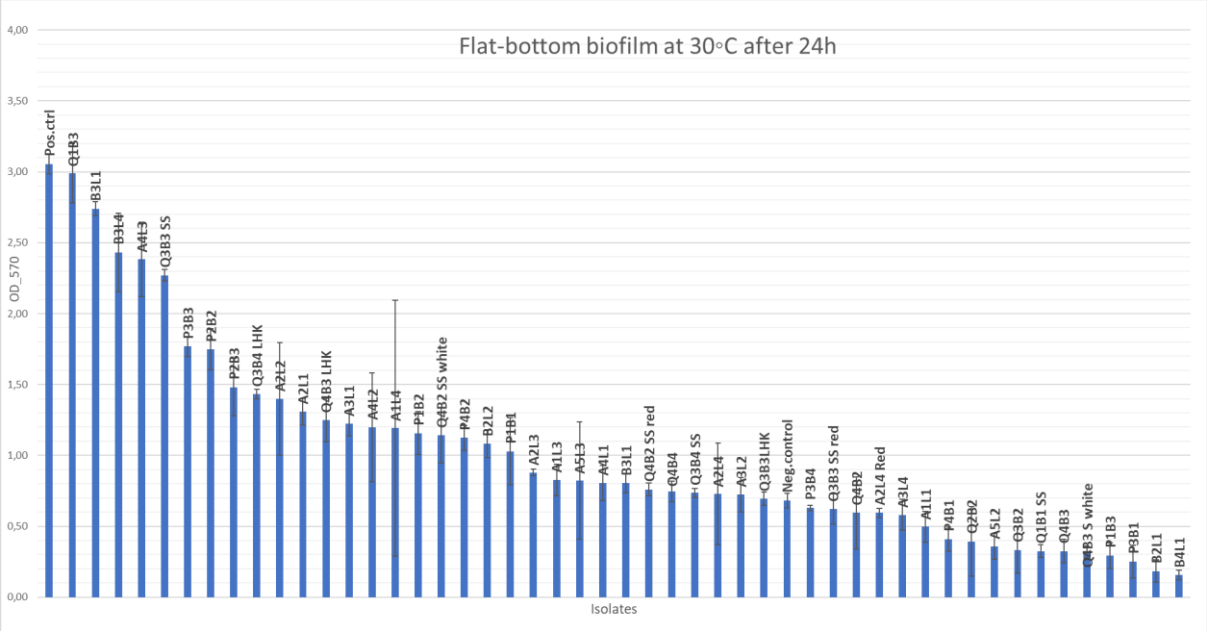

Figure 22. Biofilm formation on Nunc treated polystyrene plates after 24 h incubation at 30 degrees Celsius with 49 isolates. The different abbreviations after the number id are to explain the colonies physical appearance. The average growth for all the isolates is Avg. absorbance = 1.03

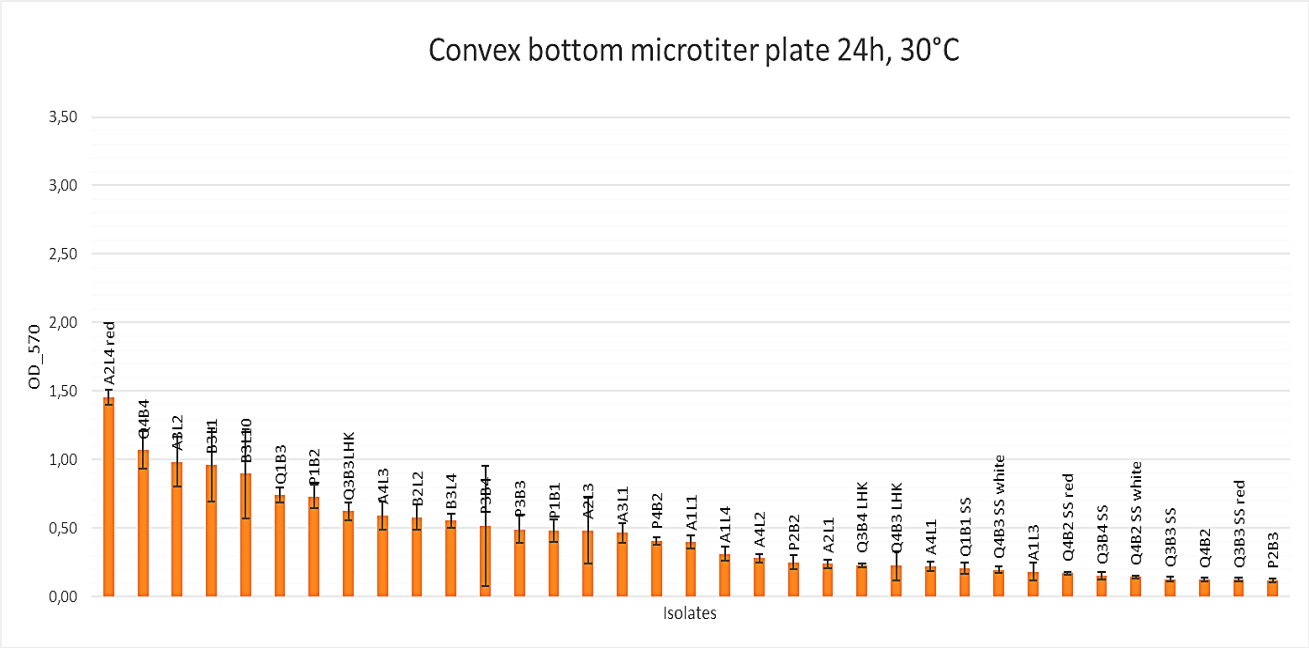

Figure 23. Biofilm formation on Nunc treated polystyrene plates with convex bottom after 24 h at 30 degrees Celsius. The different abbreviations after the number id are to explain the colonies physical appearance. The average growth for all the isolates is Avg. absorbance = 0.45.

Overall, the isolates that grew better on the flat bottom microtiter plates after 24 h, than in the convex shaped one under the same conditions. The average absorbance on flat bottom is 1.03 (Figure 22), while the average of the convex plate is 0.45 (Figure 23), which is a low number but still indicative of growth. However, there were fewer isolates tested on the concave plates as these plates were not part of the original plan.

This indicates that surface shape influences adhesion/biofilm formation on polystyrene plastic.

3.3.2 Suitable biofilm growth assays from wastewater on PS and PP

A hundred-fold dilution series was made with WW diluted in different medias, in order to find the nutrients which promoted the most growth in 24 h at 30°C.

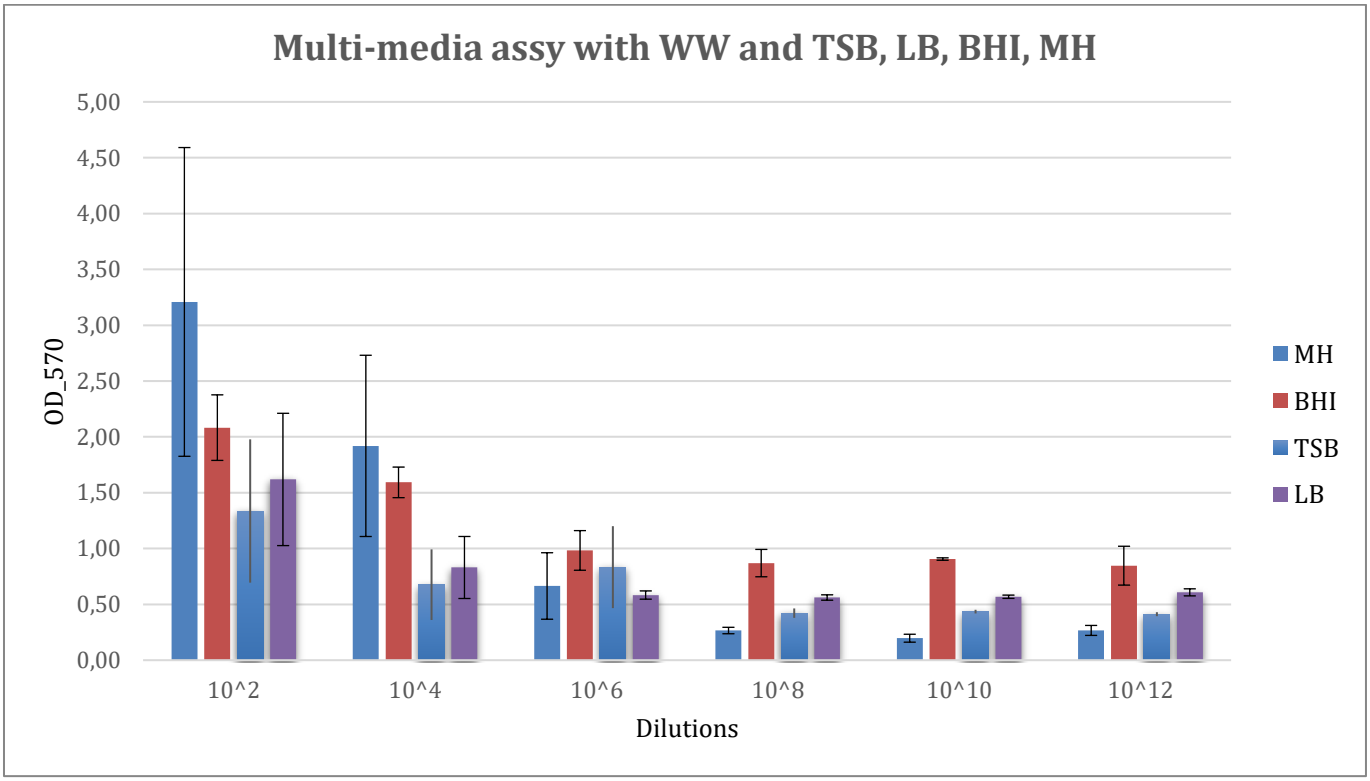

Figure 24.Wastewater with MH, TSB, LB and BHI broth at different dilutions

Based on figure 24, MH media seems to be a good growth promotor for biofilm formation on PS in lower dilutions. However, looking at the standard deviation BHI is more stable throughout all the dilutions. This indicates that the microbes in the WW may be better able to sequester nutrients from BHI based on this one parallel.

3.3.3 Biofilm formation with pure wastewater in dilution series

The higher the dilutions, the more biomass is measured based on Figure 25. These readings are only from two replicates, therefor it is not possible to calculate the standard deviation. There is biofilm formation in all dilutions, and the average growth is OD = 0.52. The highest values are 0.69 and the lowest 0.28.

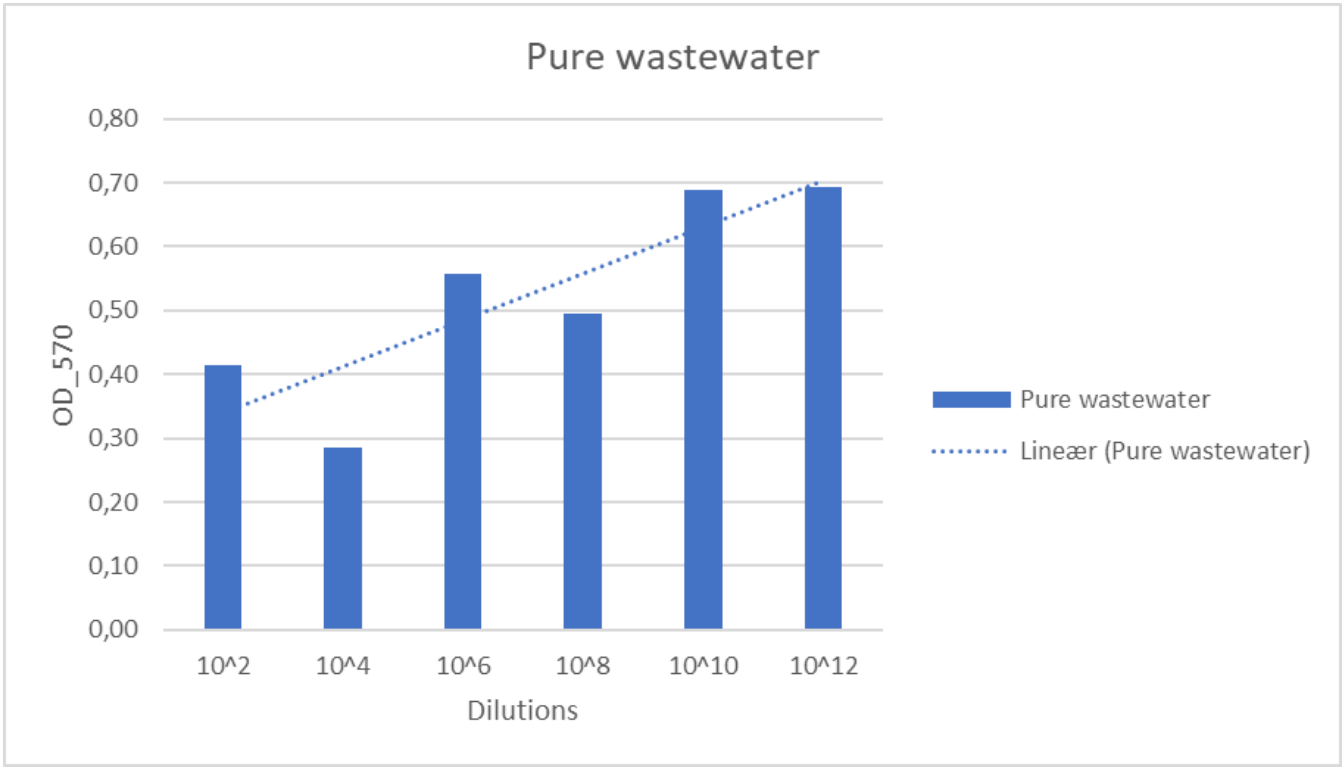

Figure 25.Biofilm formation of pure wastewater diluted in dH_2O.

3.4 16S PCR gel product

PCR is a standard procedure performed in order to amplify a specific region of genetic material before sequencing. Here, primers Bak 11w and Bak 2 were utilized, these are specific for the 16S rRNA sequence when identifying bacterial strains. The gel is used as a confirmation method for the PCR and reveals bands of RNA between 1000 bp and 630 bp, as observed on Figure 26 a) and Figure 27 b). Four samples did however not display any bands with 3µl template. These were run again with double the amount of template.

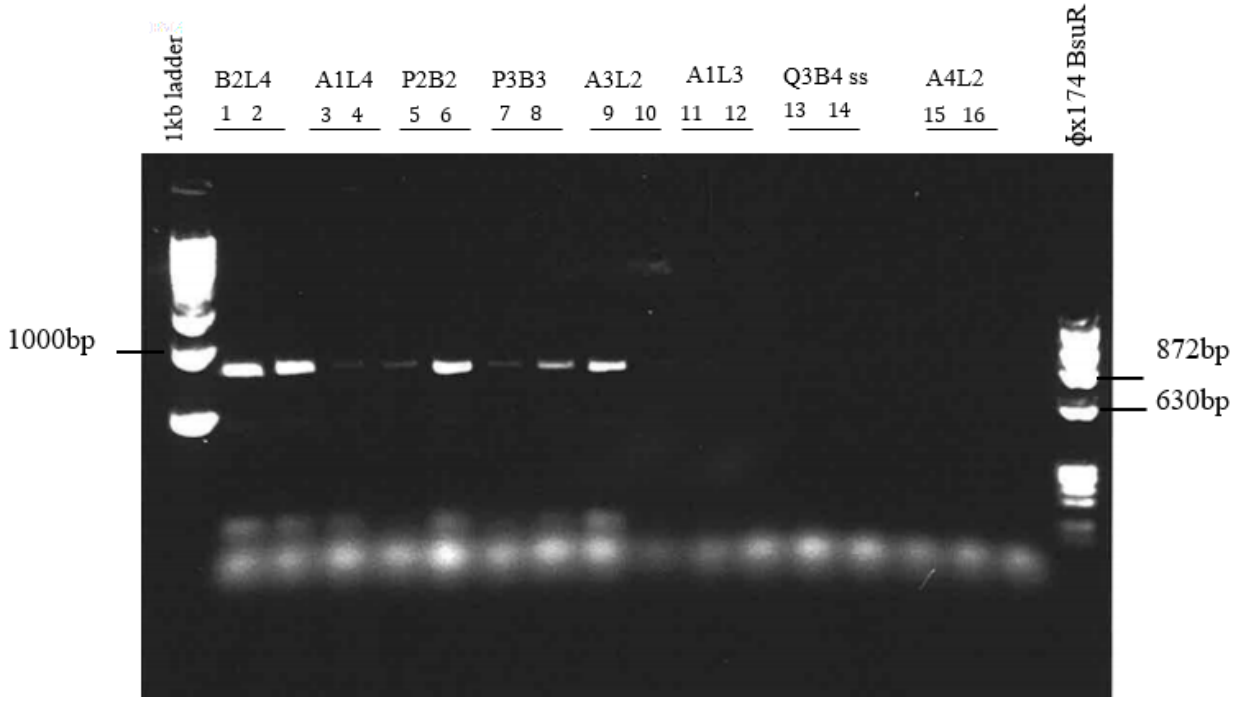

Figure 26. a) These are 8 of the 12 isolates selected for sequencing based on their multi resistant pattern after the Disc test with 8 different types of antibiotics. The isolates have bands between 1000bp and 630 bp

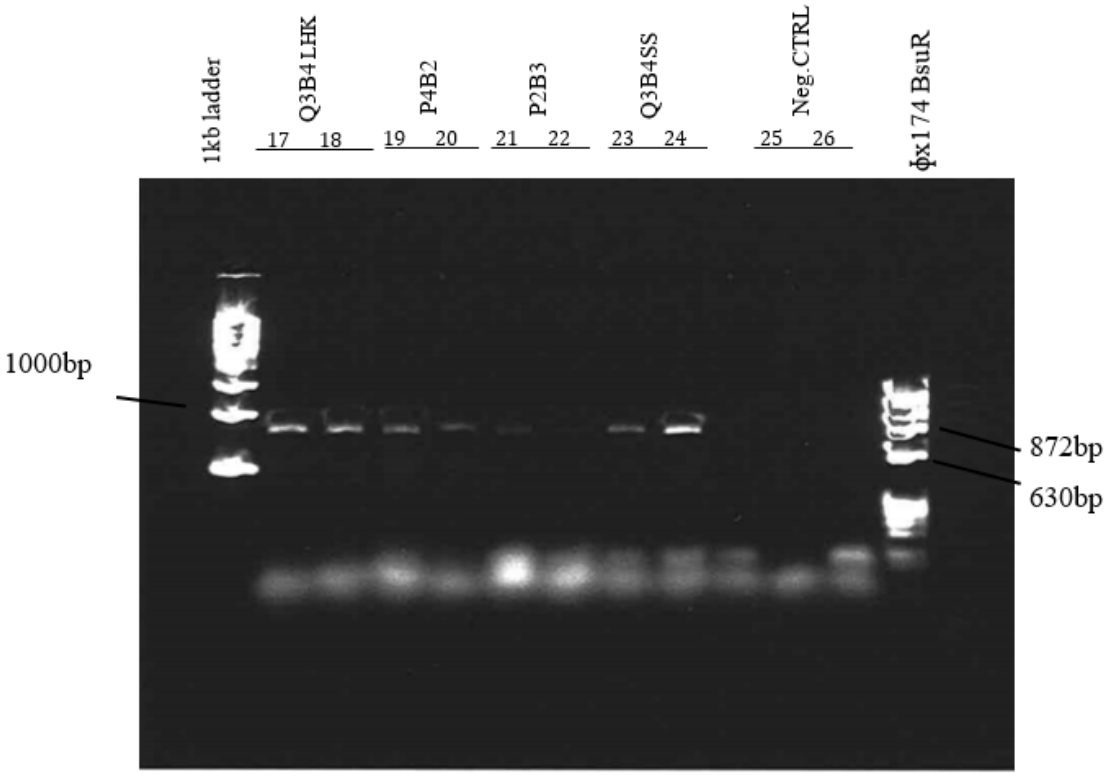

Figure 27. b) These are 4 of the 12 isolates selected for sequencing based on their multi-resistant pattern after the Disc test with 8 different types of antibiotics. The isolates have bands between 1000 bp and 630 bp

A selection was made based in whether the isolates were biofilm forming and resistant to more than 5 of the 8 antibiotics tested or 2 of the 4 non-beta-lactam antibiotics. This left 12 isolates that had these attributes to be sequenced based on their affinity to the selected primers in the PCR. Based on the PCR products, only 8 isolates were left for sequencing.

4 Discussion

There is an increasing interest and focus on plastics from wastewater and how the nutritious fluid is contributing to ABR/ABRG and the number of studies/literature linking plastics and ABR/ABRG increase every year. Wastewater is aiding transfer of genetic material and resistance between microbes by creating a favorable environment for proliferation and biofilm formation on plastics (Alimba & Faggio, 2019; Ester M. Eckert et al., 2018; Eerkes-Medrano, Thompson, & Aldridge, 2015; Radisic et al., 2020; Solomon & Palanisami, 2016). This pilot study is one of the first to document ABR bacteria that also form biofilms on PS in Tromsø. The main goal of the thesis was to characterize the microbial composition and antibiotic resistance patterns of bacteria and their biofilm formation capabilities on plastic in wastewater. Focusing on their ability to form biofilms on plastics like polystyrene (PS), polypropylene (PP) or other plastics that were abundant in the wastewater (APPENDIX A – Plastic analysis, influent and effluent). Different assays were evaluated to identify a good method for microbial biofilm formation with samples from wastewater. We were able to identify several bacterial species with ABRG. In addition to documenting biofilm formation abilities of different bacteria from wastewater on different polystyrene material. Hence, this study is laying the foundation for further investigation of wastewater microbiota associated with plastic.

4.1 Approach

The choice in plastic was based on the availability and amount of PS found in the water samples collected at Breivik/Langns. PS is also one of the most utilized plastic polymers in the laboratory and therefor easily available. This also applies to the choice in antibiotics. The most prescribed antibiotics at the University hospital of Northern Norway (UNN) from 2019 were chosen (7.2). These results are determined by the selection process early on in the experiment. By using AMP agar (64-128 µg/ml) a selection was made favoring bacteria with an intrinsic resistance to penicillin's and other β-lactams like cephalosporin (Radisic et al., 2020). Secondly, all isolates were incubated at 30°C throughout the biofilm formation, disk diffusion experiments and MALDI-TOF preparation. A series of bacterial suspensions were made through out the isolation stage, the suspensions were initially incubated at 35-37°C.However, few of the isolates thrived at this temperature and grew slowly. Since this project was established within the frame of the "One health commission" (1.2) it relates back

to the kind of impact, potential pathogens cultivated can affect or be a threat to human health. So, it was still important to keep the temperature as close to 37°C as possible, while still keeping the isolates alive. The suspensions made at 30°C proved to be in the goldilocks zone, as most isolates from Breivika and Langnes grew reasonably (visible opaque liquid) within 24 h.

Media chosen for culturing could not be selective at the initial phase, as the project needs to encompass a wide a range of potentially resistant bacteria to grow a representative set of isolates. Figure 14 displays a majority of *Pseudomonas* followed by *Aeromonas, Klebsiella, Raoultella* and *Acinetobacter* respectively, from the WW. All of which have an intrinsic resistance to β-lactams and are Gram-negative (Cox & Wright, 2013; Nakae et al., 1999; Nikaido, 1994). These are all genera with known pathogen.

As a side note: throughout the study, some plates were discarded due contamination. Some we managed to separate and others not. Hence, there are some variations in the number of isolates tested in the different experiments.

Below I will discuss in more detail the different findings and steps for the process of investigation. Starting with the ABR pattern of influent from Breivika and Langnes from the first sampling, which took place in October 2020.

4.2 ABR pattern in wastewater treatment plant

The resistance patterns from the disk test reveals several multi-resistant strains present in the WW at both Breivika and Langnes. There are 13 resistant strains, to ≥ 5 of the 8 antibiotics, including the β-lactam antibiotics. As previously stated, a majority of the samples have an intrinsic resistance (3.2) to β-lactams, rendering them less efficient. They either lack the antibiotic target, are presenting with a modified version of said target or by producing enzymes which degrades the antibiotic (Cox & Wright, 2013). Especially Gram-negative bacteria have a low susceptibility to a plethora of antibiotics. This is due to its outer membrane which is impermeable to several molecules and to multidrug resistant efflux pumps, which are adept at lowering the intracellular concentration of several drugs (Nikaido, 1994). As mentioned in the introduction, Gram negative bacteria are wrinkle makers in the intrinsic resistome, with the many ways of resisting antibiotics (Cox & Wright, 2013).
The one third generation cephalosporine, was the only β-lactam with any kind of effect overall in the samples, as can be seen in Figure 16 and Figure 17. Cefotaxime has a modified β-lactam, making it difficult to degrade by the β-lactamase. Based on this, a decision was made to focus on the non-β-lactam antibiotics; ciprofloxacin, tetracycline, trim-sulfa and gentamycin. Also, removing isolates that were not tested with all the antibiotics leaves us with n = 55 isolates.

After the β-lactams, Trim-sulfa had the most resistance in the sample pool with 25/55 samples within the resistance range (figure 16 and 17). Trimethoprim-sulfamethoxazole is a combination of two bacteriostatic drugs, that make a bactericidal mix. They have been utilized liberally, because of availability and cheap production cost (Huovinen, Sundström, Swedberg, & Sköld, 1995; Sköld, 2001). The main resistance mechanism against these antibiotics, has been spread through HGT, causing expression of drug-insensitive versions of the target enzymes dihydrofolate reductase and dihydropteroate (Sköld, 2001). The resistance is most frequently mediated by plasmids and transposons for sulfonamide, particularly by genes sul1 and sul2. As for Trimethoprim, by 2001, there were about 20 phylogenetically different resistance genes characterized as expressing drug-insensitive dihydrofolate reductase (Sköld, 2001). A study by ((Kadlec et al., 2011) documents several species of *Aeromonads* as resistant to Trim-sulfa through sul1 and gene cassettes found on class 1 integrons (mentioned earlier as being associated with transposons and HGT). *Aeromonads* are often pathogens

responsible for furunculosis and hemorrhages in fish and intestinal/extraintestinal diseases in humans (Janda & Abbott, 2010; Kadlec et al., 2011; Parker & Shaw, 2011), amongst the fish pathogens are *A. salmonicida* , *A. hydrophila, A. veronii, A. bestiarium, A. caviae* and *A. sobria* (Kadlec et al., 2011). The first three are amongst the identified species from both Breivika and Langnes.

The next antibiotic is tetracycline. It's mechanism blocks translation by inhibiting aminoacyl-t-RNA binding (Chopra & Roberts, 2001).Of the 55 isolates tested on the non-β-lactams, only 5/55 were considered resistant, with 9/55 in the range of intermediate to susceptible. This indicates a moderate distribution of TE resistance within this samples. 3/55 of the resistant isolates were from Breivika and 2/55 from Langnes. Amongst the resistant isolates are *A. media, A. putida, R. ornithinolytica* and *K. oxytoca* which will be discussed in 4.3.1 as pathogens.

Resistance to TE is often associated with acquired resistance through transposons or mobile plasmids. The resistance mechanism is normally through protection of the ribosome, blocking the antibiotic or active efflux pumps (Roberts, 1996). A study by (Roberts, 1996) found more resistance genes linked to TE resistance in Gram negative bacteria. As stated previously (1.5.3), active efflux pumps are important in Gram-negative resistance (Cox & Wright, 2013; Nikaido, 1994).

The las two antibiotics are ciprofloxacin and gentamycin. The first inhibits DNA gyrase, while the other binds the 30S subunit. These two were overall the most efficient antibiotics in this study.

For CIP only 4/55 isolates were considered resistant or intermediately resistant, the rest where well within the susceptible range with diameters >25 mm. Amongst these were two documented pathogens: *R. ornithinolytica* and *A. salmonicida* (Haruki et al., 2014; Martínez-Murcia et al., 2005). In addition to *A. media,* which for the time being has not been flagged as pathogen and has only found in environmental samples. But it is closely related to *A. salmonicida* (Allen, Austin, & Colwell, 1983). Indicating that it might have the potential to become pathogenic through HGT when in close proximity to or as part of a biofilm. The last isolate was not identified because its freeze stock was poorly made and it could not be revived.

Gentamycin also had 4/55 isolates considered resistant with diameter <17 mm. *A. media* was also resistant to gentamycin, along with *P. libanesis.* This species is normally found in spring

water and has shown cytotoxic effect towards HEP-2 sells in humans, but has no known pathogenic outbreaks (Meghdas, Hamze, Dabboussi, Baida, & Izard, 2004). Though its closely related to *P. fluorescens,* which is a known pathogen, giving it the potential to become one in circumstances that allow genetic exchange. Or it could act as a reservoir for pathogenic genes (ELOMARI et al., 1996; Flemming & Wingender, 2010; Kumar & Pal, 2018; Savage, Chopra, & O'Neill, 2013).

Overall, the interpretation of inhibition zones was mostly based on *Pseudomonas, Aeromonas* and *Acinetobacter* in EUCAST. The ones with the closest relation were used to assess the inhibition zones, when the genus or species was missing.
Though, there were not many pathogens in the sample. The non-pathogenic bacteria are key-players, acting as reservoirs for ABRG (Roberts, 1996) in the wastewater systems with a big distribution potential, if/when they grow on plastic. There are already examples of plastic found downstream from WWTP with a bacterial taxa that is associates with human gastrointestinal infections (Kelly et al., 2021). Showing how colonization and biofilm formation on plastic serves as protection for the bacteria. Allowing them to travel great distances while keeping their genetic material intact, with potentially new acquired ABRG as a result from the time spent in the WWTP, where the selective pressure potentially is higher than in nature.

4.3 Microbiota in wastewater - Breivika vs Langnes

The initial hypothesis was that a majority of the potential pathogens in the sample would originate from WW at Breivika. Considering one of its primary waste sources being a hospital, a place where patients are administered antibiotics daily. Presumably, said patients relive themselves into sewer systems that end at Breivika WWTP. Not only is the water from the hospital, but UiT- university of Tromsø, the industrial wastewater from the northern part of Tromsø peninsula, as well as the residential area close to the treatment plant. Based on the results from the disk test and MALDI identification - out of the 58 isolates sent in for MS analysis, 24 different species were discovered. From these there are 9 previously documented pathogens, with *Aeromonas* being the dominant genus (5/9), followed by *Klebsiella* (2/9), *Pseudomonas* (2/9) and *Raoultella* (1/9). Around 8/9 of the pathogens were found at Langnes with two found at both stations and 4/9 found at Breivika. Most of the documented human pathogens*, K. oxytoca, P. fluorescens, R. ornithinolytica* were found at Langnes, while *A.*

hydrophila and *K. pneumonia* were found at Breivika (Daskalov, 2006; Ghatak et al., 2016; Haruki et al., 2014; Iglewski, 1996; Janda & Abbott, 2010; Martínez-Murcia et al., 2005; Parker & Shaw, 2011; Pitout et al., 2015; Sawada et al., 2021; Singh et al., 2016).
There is no clear reason for this distribution and as the sample pool is small, it is barely representative of the actual biota as this might change based on the amount of precipitate or drought (Lucas et al., 2014). But it gives an indication of the bacteria present and their characteristics.

4.3.1 Pathogens identified at WWTP Breivika and Langnes

There was an abundance of *Pseudomonas sp.* in the samples. Another study looking at microplastics in WW from the west of Norway WW also found *Pseudomonas sp.* in a majority of their samples, making up a total of 70% (Radisic et al., 2020). Both studies did a selection early on by only continuing forward with colonies grown on agar with AMP, which favors bacteria with an intrinsic resistance to penicillin's (Radisic et al., 2020).
Pseudomonas is a large genus with a variety of species with several opportunistic pathogens and some that are directly tied to human infection (ELOMARI et al., 1996). They are commonly found in soil, water, vegetation and on various surfaces of healthy humans as well as the intestines (Iglewski, 1996). Amongst these are *P. putida* and *P. fluorescens* that were both found only in WW from Langnes. This station receives water from mostly residential areas in addition to the local airport "Tromsø lufthavn – Avinor".
Unfortunately, the *P. putida* and *P. fluorescens* MS identification score was 1.8, indicating identification to genus level, not species. The PCR was unsuccessful in producing a product for sequencing. Likely due to degradation on the template or absence of primer sequence.
P. putida did present resistance toward the β-lactams, Trim-sulfa and tetracycline, in addition to being biofilm forming on PS. *P. fluorescens,* showed intermediate resistance to Trim-sulfa, bordering towards resistance with a diameter of 18mm when the resistance limit is <17mm (EUCAST, 2021) in addition to β-lactam resistance. *P. azotoformans is* another pseudomonad associated with cereal grain infections (Iizuka & Komagata, 1963) But never directly implicated as a pathogen itself. All these potential pathogens were biofilm forming.

A majority of the remaining pathogens belong to *Aeromonas spp.* they are common in the marine environment and are mostly opportunistic pathogens. They are attributed several infections in both fish and humans (Parker & Shaw, 2011; Radisic et al., 2020). Especially *A.*

salmonicida is an important fish pathogen responsible for making cultured and wild salmon sick (Radisic et al., 2020). In this study isolates identified as *A. salmonicida* had a varying degree of resistance. However, all four were resistant to β-lactams, one to TE, one to CIP and the last to SXT. This indicates potential genetic variations within the species, sequencing would have to be carried out to confirm, or it could be contamination. β-lactam resistance was also confirmed in *A .salmonicida* in isolates grown on plastic from Bergen (Radisic et al., 2020)

Other fish pathogens discovered were *A. hydrophila, A bestiarum* and *A. veronii,* these were resistant to β-lactams except for A. *bestiarium and A. veronii* also had resistance towards SXT and intermediate resistance to CIP. *A. bestiarium, A. salmonicida, A. media, A. hydrophila* were biofilm forming and *A. veronii* was a weak biofilm former.

Now, over to some well-established opportunistic human pathogens, *Klebsiella oxytoca* and *Klebsiella pneumoniae.* These two are often associated with hospital infections, primarily of immunosuppressed individuals in hospitals (Singh et al., 2016). But given the right circumstance and virulence factors, *K. pneumoniae* has been known to infect healthy individuals as well (Li, Zhao, Liu, Chen, & Zhou, 2014), causing infections such as bronchopneumonia, septicemia, and urinary tract infections. Previously, *K. Pneumonia* was the main cause of concern in term of hospital infections from the genus, but *K. oxytoca* is currently making itself known as an up-and-coming pathogen in the hospital environment (Singh et al., 2016) *Klebsiella* are normally found in vegetation, surface waters and soil (Struve & Krogfelt, 2004). They have documented resistance towards β-lactams, carbapenemase and lowered sensitivity to aminoglycosides and quinolones (Singh et al., 2016).

In this study, both Klebsiellas had resistance towards AMP, P. While *K. Pneumonia* also had an intermediate resistance to CIP, curiously it was one of the few susceptible to MEL and CTX. *K. oxytoca,* was resistant to TE in addition to the AMP, P. Here we also observed an intermediate resistance to MEL and CTX. This might indicate that these isolates do not stem from a hospital infection, but the environment. Based on the modest resistances found. *Klebsiellas* are known for easily exchanging genetic material through plasmids, especially with other Gram negative bacteria (Singh et al., 2016). Both species produced biofilms on PS at 30°C after 24h.

The last pathogen is *R. ornithinolytica* formerly classified as *Klebsiella.* They are normally found in soil and water. Similar to *Klebsiella*, *R. ornithinolytica* have now been found in association with virulence infection in co-morbid at-risk patients that are hospitalized. Additionally, they have the ability to convert histidine to histamine causing histamine poisoning in humans (Hajjar, Ambaraghassi, Sebajang, Schwenter, & Su, 2020). There are only a few clinical cases linked to the species, since it is difficult to correctly identify, so there is some uncertainty around the actual numbers. A majority of the species in the genus are sensitive to most antibiotics except ampicillin due to their β-lactamase production. Though, there are clinical specimens of *R. ornithinolytica* exhibiting acquired ABRG like the ones for carbapenemase and metallo- β-lactamase production (Hajjar et al., 2020; Haruki et al., 2014). In total there were three R. ornithinolytica isolates with a varying degree of resistance. All were resistant to AMP and P, one also showed resistance to TE, MEL, CIP, SXT and intermediate to CTX– making it one of the more resistant isolates in this samples with 7/8 antibiotics.
The other isolate was also resistant to SXT and intermediate to CIP and CTX. While the last one only had four antibiotics tested and was not a pure culture. Therefor its results are disregarded. Both pure cultures were biofilm forming on PS at 30°C after 24 h.

All of these pathogens have a varied resistance pattern with the β-lactams as a common feature, as expected. And at least on species from each genus is biofilm forming which makes HGT easier and potentially more frequent. As was the case on a study with *S. aureus,* where the frequency of plasmid transfer increased and indirectly increased the spread of resistance determinants. Likely caused by close proximity and the stabilizing effect between close bacteria of the matrix, however there are more complex interactions also at work (Savage et al., 2013; Stalder & Top, 2016).

4.3.2 16S rRNA – Genus or species identification

The 16S gene is a conserved region in all bacterial DNA and is a key area when sequencing for confirmation of bacterial DNA. This study utilized degenerate primers Bak2 and Bak w11. These are a mix of primers with small variations in the last oligonucleotide, which allows them to amplify different sequences (Shamir, 2005). This was considered an advantage when sequencing different genera and trying to identify them by the small unique sequence variations. The method has been used successfully on fastidious Gram negative bacteria

according to (de Melo Oliveira, Abels, Zbinden, Bloemberg, & Zbinden, 2013). However, out of the 12 isolates only 8 were amplified, there could be several reasons. Moste likely, this is linked to the template rather than the primers, as it worked for some of the samples. This was an anticipated problem, since some isolates had a low DNA conc. With some impurities based on the Nano Drop chromatogram, where the peak at 260nm were misaligned, indicating the presence of salts and proteins.

During the DNA isolation we struggled with eluating the DNA from the spin column, there was often visible lumps of supposed DNA left on the filter, paradoxically caused by too much DNA in the initial volume. The isolation kits and buffers were also expired, which might have affected efficiency (Wilfinger, Mackey, & Chomczynski, 1997). A slightly modified protocol should be used next time, adjusting the initial sample volume and elute buffer volume. And a fresh kit, as this eliminates some of the uncertainty around the quality/integrity of the buffers.

After PCR and clean-up of the eight remaining isolates, there were 16S rRNA genes detected in all the samples. Using BLAST for nucleotide sequencing, there were numerus hits for several different strains, with 100% identity. As should be expected when sequencing a conserved region. Most of them were within the same genus as the MALDI identification except two (table 6). Only two samples coincided to species level between the identification methods – *R. ornithinolytica* and *A. johnsonii.*

Overall, this method is decent for genus identification based on our samples where 6/8 were accurate to genus level.

4.4 Biofilm growth on PS plastic

4.4.1 Growth on PS pegs

After the preliminary analysis of the wastewater from Eurofins (7.1), PS was one of the more abundant plastics in the influent at 11.2 µg/l after PET at 12.5 µg/l. The initial plan was to add microplastic beads from different polymers to the cultures, but because of the covid-19 situation and delayed shipment from South Africa. Therefor we changed plans and worked with PS pegs and PS 96 well microtiter plates. These are part of several standard methods for biofilm formation (Stephanovic et al., 2007).

As previously mentioned(4.1), small growth experiments were conducted to decide that 30°C was an ideal temperature, to maintain growth in most of the isolates while also filtering out potential human pathogens. However, all experiments on pegs were incubated at 35°C.

This could be part of the explanation to why the biomass is lower on pegs, than the flat-bottom microtiter plates.
The next step was to find a suitable surface, where most of the cells would adhere. The general approach in this study is based on a standard method, where the biofilm adheres to pegs. But a point of this thesis was also to look into an efficient way to cultivate biomass on plastic. Trials with PS peg lids, PS microtiter plates with a flat-bottom and convex bottom were made with varying result. Both the flat-bottomed and convex series were grown at 30°C for 24 h with LB medium.
As can be seen in Figure 22, pegs were the least suitable surface for the isolates to grow on, with an average absorbance of 0.17. The bacterial growth using convex bottom microtiter plates showed a density of growth with an average of OD = 0.50. The flat bottom microtiter plate were clearly the more suitable option, with an average growth of OD = 1.03. However, the flat bottom series had more variations in det standard deviation, in comparison to the peg and convex series.
And it is also important to keep in mind that the pegs were incubated at 35°C, which was not an ideal temperature for the isolates to grow.

The positive control S. epidermidis, was not a good fit for the temperature intervals, and did not always grow/form biofilm in the peg series. Based on Christensen method protocol, it grows at 37°C, which suggests that 30°C is an unsuitable temperature and might slow its growth. A switch was made to *S.* oneidensis, a genus often found in cold environments and that is known to form biofilms (Venkateswaran et al., 1999). This one however, proved to be difficult to keep alive on agar and when inoculated, would not always proliferate in cultures or form biofilms in the wells. Therefore, these positive controls were removed from some of the figures, but the numbers are available in the 7.4.
All the plates were Nunc treated, meaning the surface is made to promote cell adhesion, this would definitely have affected the results. For further study, untreated plates should also be tested.

4.4.2 Optimal biofilm assay on PS and PP

Raw WW samples from the influent were incubated with TSB, BHI, LB and MH medium for 24h at 30°C on PS microtiter plates, to find a suitable assay. They were placed in a hundred-fold dilution series from 10^{-2} to 10^{-12}, in order to properly discern the difference in growth provided by the nutrients in each broth. This was also based on a failed attempt with a ten-fold dilution, where the growth was indiscernible.

As seen in Figure 24, MH medium has the higher absorbance, followed by BHI, LB then TSB. But it also has the biggest variations in the triplicate values, based on the standard deviation in comparison to the wells grown with BHI. Though, is difficult to draw certain conclusions based on the low number of replicates.

A theory as to why some wells with MH grew better than the other is competition in the well. Since these samples are a mix of microbes, it stands to reason that there might be some bacteria that are more adept at sequestering the nutrients from the broth than others. Giving them an advantage and more rapid growth. And said bacteria, might not be present in all the wells, as the pipetting is done by mixing up and down a few times before filling each one.

The two broths also have different nutrient composition – BHI contains beef heart and calf infusion, with Na_2HPO_4, D (+)-glucose, peptone and NaCl. While MH contains beef infusion, casein hydrolysate and starch. The different ingredients might benefit different bacteria that are able to use them as substrate.

So, for a continuous and stable growth the Brain heart infusion might be a better option to avoid these irregularities.

Though, the general set up might also be part of the problem. Due to the time constraint, a bachelor student was tasked with running parallel experiments with the same medias and some additional temperatures with more replicates. Testing the WW on both PS and PP. The student still faced the same issues with irregularities when using n = 12 replicates pr. dilution at room temp and with n = 3 at 30°C. Suggesting a high sensitivity with this set-up. They used a tenfold dilution from 10^{-1} to 10^{-4} at 30°C the microbes grew best on PS and had OD = 4.0 as the highest absorbance value measured using BHI and TSB at 10^{-1} and 10^{-2} dilutions. The OD values from PP plate were very similar to PS but at the highest dilution, PS remained the favored polymer. As for the preferred media, both BHI and TSB had less fluctuations (based on low STD) and gave decent growth to the microbes present.

A small section of the plates was used for side experiment with just WW diluted in dH_2O. These were incubated at 30°C to observe biofilm formation. As can be seen in Figure 25, the mix of different bacteria without nutrients are able to form biofilm in 24h with an average OD = 0.52. Albeit, not as efficiently as with additional nutrient. Proving that the microbes in WW are fully capable of creating biofilms without additional nutrients, though it might be at a slower pace.

4.4.3 Further studies

Interesting future studies to perform, would have been to sequence and look for specific genes related to antibiotic resistance using relevant primers and plasmid purification to identify where the resistance might be located.

It would further be interesting to look closer at the conditions and include other plastic polymers in the experiment to further investigate the differences between them. And whether the treatment of the plastic surface versus untreated surfaces influence the biofilm formation and growth.

These experiments should be done with enough parallels to perform sturdy statistical calculations on the data.

In addition, several other optimizations could be done, - like increasing the sample size considerably, performing more sampling over time to follow the variation of microbial composition.

These studies can also be followed up in a way that document the origins of bacteria in the wastewater before they end up at the plant. Another interesting question is if the hospital has additional cleaning steps to filter their wastewater and if these steps could reduce the presence of ABRG in the wastewater before it is flushed out into sea.

5 Summary

There is still much to be done in this area of research and plastic pollution is not going away anytime soon. This study documents some of the microbial composition and antibiotic resistance patterns of bacteria and biofilm formation capabilities at the two wastewater treatment plants in Tromsø.

There are multi-resistant bacteria at both Langnes and Breivika in Tromsø. In total, 13 of the 55 isolates and identified bacterial species showed resistance towards ≥ 5 of the 8 antibiotics that were tested, based on them being the most prescribed antibiotics at UNN.

There was an abundance of *Pseudomonas,* followed by *Aeromonas, Klebsiella, Raoultella* and *Acinetobacter* which all are Gram-negative bacteria.
Some of the species were closely related, like *A. salmonicida* and *A. media* which implies that they might be able to exchange genetic material since this is a way of spreading both resistance and pathogenicity to non-pathogenic bacteria, particularly through biofilm formation.

Most of the pathogens isolated originated from Langnes, where the water is collected from a residential area at Kvaløya, in addition the local airport.

The bacterial strains isolated from WW do grow and form biofilms on PS and PP plastic at 30°C and 35°C.

The bacteria from wastewater form the highest biofilm mass when BHI and TSB are used as media, but biofilms were also formed after 24 h at 30°C without any additional nutrients to the wastewater, likely because the wastewater already contains nutrients.
Interactions like this in the environment pose a threat to human and animal health. Re-enforcing the need for initiatives like One health, that work towards mapping out and enlightening these interactions.
Using the modified Christensen method is a sensitive way of measuring the biofilm formation of WW samples.

6 Referances

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7 Appendix

7.1 APPENDIX A – Plastic analysis, influent and effluent

Enclosed below in Table a and Table b, is the result from Eurofins on the type of plastic identified in Breivika wastewater plant from influent and effluent.

Table a. Plastic analysis from Eurofins. Displays composition and qunatity of plastic from influent

Eurofins Environment Testing Norway AS (Bergen)
F. reg. NO9 651 416 18
Sandviksveien 110
5035 Bergen

Tlf: +47 94 50 42 42
bergen@eurofins.no

Genøk-senter for biosikkerhet
Forskningsparken i Breivika
9291 TROMSØ
Attn: Odd-Gunnar Wikmark

AR-20-MX-006775-01

EUNOBE-00039934

Prøvemottak: 23.04.2020
Temperatur:
Analyseperiode: 23.04.2020-05.06.2020

Referanse: MikroplastResist 1. runde

ANALYSERAPPORT

Prøvenr.:	**441-2020-0427-097**	Prøvetakingsdato:	15.04.2020
Prøvetype:	Avløpsvann	Prøvetaker:	OGW
Prøvemerking:	S2000002 Inntak Breivika Døgn 1/2	Analysestartdato:	23.04.2020

	Analyse	Resultat	Enhet	LOQ	MU	Metode
*	**Mikroplast i avløpsvann >27µm (8 polymere)**					
*	Polyetylen (PE)	<3	µg/l	3		Py-GC-MS
*	Polypropylene (PP)	<1	µg/l	1		Py-GC-MS
*	Polystyren (PS)	11.2	µg/l	1		Py-GC-MS
*	Polyvinylklorid (PVC)	<1	µg/l	1		Py-GC-MS
*	Polyetylentereftalat (PET)	12.5	µg/l	1		Py-GC-MS
*	Polyamid 6 (PA6)	<1	µg/l	1		Py-GC-MS
*	Polymetylmetakrylat (PMMA)	<1	µg/l	1		Py-GC-MS
*	Polykarbonat (PC)	<1	µg/l	1		Py-GC-MS
*	Sum kvantifiserte polymere	23.6	µg/l	10		Py-GC-MS
*	**Gummipartikkel-komponenter >27µm**					
*	Polyisoprene	43.1	µg/l	1		Py-GC-MS
*	Polybutadiene	<1	µg/l	1		Py-GC-MS
*	Innhold av gummi	Se kommentar				Py-GC-MS
*	**Volum filtrert/analysert for mikroplast**					
*	Volum	969	ml			Volumetri

Merknader:
Ingen signal for polybutadiene, tydelige signaler for polyisoprene.

Bergen 05.06.2020

Tommie Christensen
ASM - Analytical Service Manager

Table b. Plastic analysis from Eurofins. Displays composition and quantity of plastic from effluent.

Eurofins Environment Testing Norway AS (Bergen)
F. reg. NO9 651 416 18
Sandviksveien 110
5035 Bergen

Tlf: +47 94 50 42 42
bergen@eurofins.no

Genøk-senter for biosikkerhet
Forskningsparken i Breivika
9291 TROMSØ
Attn: Odd-Gunnar Wikmark

AR-20-MX-006773-01

EUNOBE-00039934

Prøvemottak: 23.04.2020
Temperatur:
Analyseperiode: 23.04.2020-05.06.2020

Referanse: MikroplastResist 1. runde

ANALYSERAPPORT

Prøvenr.: **441-2020-0427-095**
Prøvetype: Avløpsvann
Prøvemerking: S2000010
Utløp Breivika Døgn 1/2

Prøvetakingsdato: 15.04.2020
Prøvetaker: OGW
Analysestartdato: 23.04.2020

	Analyse	Resultat	Enhet	LOQ	MU	Metode
*	**Mikroplast i avløpsvann >27µm (8 polymere)**					
*	Polyetylen (PE)	170	µg/l	3		Py-GC-MS
*	Polypropylene (PP)	<1	µg/l	1		Py-GC-MS
*	Polystyren (PS)	4.4	µg/l	1		Py-GC-MS
*	Polyvinylklorid (PVC)	<1	µg/l	1		Py-GC-MS
*	Polyetylentereftalat (PET)	7.0	µg/l	1		Py-GC-MS
*	Polyamid 6 (PA6)	<1	µg/l	1		Py-GC-MS
*	Polymetylmetakrylat (PMMA)	<1	µg/l	1		Py-GC-MS
*	Polykarbonat (PC)	<1	µg/l	1		Py-GC-MS
*	Sum kvantifiserte polymere	181	µg/l	10		Py-GC-MS
*	**Gummipartikkel-komponenter >27µm**					
*	Polyisoprene	14.32	µg/l	1		Py-GC-MS
*	Polybutadiene	11.59	µg/l	1		Py-GC-MS
*	Innhold av gummi	Se kommentar				Py-GC-MS
*	**Volum filtrert/analysert for mikroplast**					
*	Volum	915	ml			Volumetri

Merknader:
Tydelige signaler for både polyisoprene og polybutadiene.

Bergen 05.06.2020

Tommie Christensen
ASM - Analytical Service Manager

7.2 APPENDIX B – List of prescribed antibiotics from UNN

The most prescribes antibiotics at the UNN- University hospital of Northern Norway, retrieved from the hospital pharmacist Nord HF via Sykehusapotekenes legemiddelstatestikk (SLS).

Table c. Displays the distribution of antibiotics prescribes from the hospital pharmacist at UNN. Numbers are retrieved from SLS, hospital pharmacists statistics.

HUMAN ATC		
J - ANTIINFECTIVES FOR SYSTEMIC USE		
J01 - ANTIBACTERIALS FOR SYSTEMIC USE		
J01A - TETRACYCLINES		
J01AA - Tetracyclines		
J01AA02 - doxycycline	**652,40**	**6 597,00**
J01AA04 - lymecycline	**8,00**	**123,50**
J01AA07 - tetracycline	**22,00**	**210,00**
J01AA12 - tigecycline	**2,00**	**10,00**
J01B - AMPHENICOLS		
J01BA - Amphenicols		
J01BA01 - chloramphenicol		
Chloramphenicol ess inj sub 1g 1 MLHGL --349888	84,00	28,01
J01C - BETA-LACTAM ANTIBACTERIALS, PENICILLINS		
J01CA - Penicillins with extended spectrum		
J01CA01 - ampicillin	**1 514,50**	**4 717,21**
J01CA04 - amoxicillin	**439,00**	**4 710,95**
J01CA08 - pivmecillinam	**342,33**	**3 960,03**
J01CA11 - mecillinam	**151,00**	**125,83**
J01CE - Beta-lactamase sensitive penicillins		
J01CE01 - benzylpenicillin	**2 954,70**	**18 247,60**
J01CE02 - phenoxymethylpenicillin	**263,33**	**4 439,22**
J01CE08 - benzathine benzylpenicillin	**62,00**	**12,40**
J01CF - Beta-lactamase resistant penicillins		
J01CF01 - dicloxacillin	**459,00**	**3 461,25**
J01CF02 - cloxacillin	**1 806,50**	**16 987,50**
J01CR - Combinations of penicillins, incl. beta-lactamase		
J01CR02 - amoxicillin and beta-lactamase inhibitor	**84,33**	**414,06**
J01CR05 - piperacillin and beta-lactamase inhibitor	**2 229,70**	**6 329,18**
J01D - OTHER BETA-LACTAM ANTIBACTERIALS		
J01DB - First-generation cephalosporins		
J01DB01 - cefalexin	**82,25**	**380,00**
J01DB03 - cefalotin	**169,20**	**749,25**
J01DB04 - cefazolin	**1 295,70**	**8 638,28**
J01DC - Second-generation cephalosporins		
J01DC02 - cefuroxime	**460,60**	**2 093,86**
J01DD - Third generation cephalosporins		
J01DD01 - cefotaxime	**2 133,20**	**8 436,50**

J01DD02 - ceftazidime	**101,00**	**447,50**
J01DD04 - ceftriaxone	**82,40**	**735,00**
J01DD52 - ceftazidime and beta-lactamase inhibitor	**1,00**	**3,33**
J01DF - Monobactams		
J01DF01 - aztreonam	**44,00**	**22,00**
J01DH - Carbapenems		
J01DH02 - meropenem	**284,00**	**864,89**
J01DH03 - ertapenem	**32,00**	**32,00**
J01E - SULFONAMIDES AND TRIMETHOPRIM		
J01EA - Trimethoprim and derivatives		
J01EA01 - trimethoprim	**95,30**	**876,80**
J01EE - Combinations of sulphonamides and trimethoprim, inc		
J01EE01 - sulfamethoxazole and trimethoprim	**1 765,00**	**7 122,50**
J01F - MACROLIDES, LINCOSAMIDES AND STREPTOGRAMINS		
J01FA - Macrolides		
J01FA01 - erythromycin	**166,00**	**1 598,00**
J01FA09 - clarithromycin	**25,33**	**314,02**
J01FA10 - azithromycin	**94,00**	**805,58**
J01FF - Lincosamides		
J01FF01 - clindamycin	**1 091,57**	**3 204,08**
J01G - AMINOGLYCOSIDE ANTIBACTERIALS		
J01GB - Other aminoglycosides		
J01GB01 - tobramycin	**89,00**	**349,25**
J01GB03 - gentamicin	**2 561,30**	**6 613,21**
J01GB06 - amikacin	**1,00**	**5,00**
J01M - QUINOLONE ANTIBACTERIALS		
J01MA - Fluoroquinolones		
J01MA02 - ciprofloxacin	**360,40**	**2 633,25**
J01MA12 - levofloxacin	**15,00**	**150,00**
J01MA14 - moxifloxacin	**6,00**	**42,00**
J01X - OTHER ANTIBACTERIALS		
J01XA - Glycopeptide antibacterials		
J01XA01 - vancomycin	**1 273,40**	**1 307,00**
J01XB - Polymyxins		
J01XB01 - colistin	**1,00**	**1,11**
J01XC - Steroid antibacterials		
J01XC01 - fusidic acid	**63,50**	**211,62**
J01XD - Imidazole derivatives		
J01XD01 - metronidazole	**409,65**	**3 143,50**
J01XE - Nitrofuran derivatives		
J01XE01 - nitrofurantoin	**41,16**	**241,75**
J01XX - Other antibacterials		
J01XX05 - methenamine	**116,67**	**1 512,50**
J01XX08 - linezolid	**42,10**	**240,51**
J01XX09 - daptomycin	**1,00**	**1,79**
J02 - ANTIMYCOTICS FOR SYSTEMIC USE		
J02A - ANTIMYCOTICS FOR SYSTEMIC USE		

J02AC - Triazole derivatives	399,71	4 046,75
J02AX - Other antimycotics for systemic use		
J02AX04 - caspofungin	**90,00**	**99,20**
J02AX05 - micafungin	**18,00**	**18,00**
J02AX06 - anidulafungin	**358,00**	**358,00**
J02AA - Antibiotics		
J02AA01 - amphotericin B	**27,50**	**315,75**
J04 - ANTIMYCOBACTERIALS		
J04A - DRUGS FOR TREATMENT OF TUBERCULOSIS		
J04AB - Antibiotics		
J04AB02 - rifampicin	**47,86**	**502,00**
J04AB04 - rifabutin	**3,00**	**90,00**
J04AC - Hydrazides		
J04AC01 - isoniazid	**2,00**	**200,00**
J04AD - Thiocarbamide derivatives		
J04AD01 - protionamide	**1,00**	**33,33**
J04AK - Other drugs for treatment of tuberculosis		
J04AK01 - pyrazinamide	**3,00**	**99,99**
J04AK02 - ethambutol	**4,00**	**108,32**
J04AM - Combinations of drugs for treatment of tuberculosis		
J04AM02 - rifampicin and isoniazid	**3,00**	**84,00**
J04AM05 - rifampicin, pyrazinamide and isoniazid	**2,00**	**33,34**
J04AM06 - rifampicin, pyrazinamide, ethambutol and isoniazid	**7,00**	**105,00**
J04B - DRUGS FOR TREATMENT OF LEPRA		
J04BA - Drugs for treatment of lepra		
J04BA01 - clofazimine	**1,00**	**50,00**
J04BA02 - dapsone	**6,00**	**600,00**
L - ANTINEOPLASTIC AND IMMUNOMODULATING AGENTS		
L01 - ANTINEOPLASTIC AGENTS		
L01B - ANTIMETABOLITES		
L01BA - Folic acid analogues		
L01BA01 - methotrexate	**383,80**	**531,24**
L01D - CYTOTOXIC ANTIBIOTICS AND RELATED SUBSTANCES		
L01DB - Anthracyclines and related substances		
L01DB01 - doxorubicin	**415,00**	**25,70**
	25	**130**
Totalsum	**750,41**	**450,44**

7.3 APPENDIX C – Raw data: MALDI-TOF

The details of the results of the MALDI-TOF analysis to identify the isolated strains of bacteria in wastewater.

Table d. Species identified with MALDI MS analysis for each pure isolate.

Sample ID	Best match organsism	Score
A1L1	*PSEUDOMONAS GESSARDII*	2,03
A1L3	*PSEDOMONAS KILONESIS*	1,95
A1L4	*PSEDOMONAS KILONESIS*	2,06
A2L1	*PSEUDOMONAS FLUORESCENS*	1,86
A2L2	*PSEUDOMONAS EXTEMORIENTALIS*	1,92
A2L3	*AEROMONAS SALMONICIDA*	2,15
A2L4	*PSEUDOMONAS GESSARDII*	2,14
A2L4 red	*AEROMONAS MEDIA*	2,12
A3L1	*AEROMONAS BESTIARUM*	2,19
A3L2	*PSEUDOMONAS PUTIDA*	1,88
A3L4	*AEROMONAS SALMONICIDA*	2,24
A4L1	*PSEDOMONAS GESSARDII*	2
A4L2	*PSEUDOMONAS KILONESIS*	2,07
A5L2	*AEROMONAS SALMOCIDA*	2,15
A5L3	*KLEBISELLA OXYTOCA*	2,44
B2L1	*AEROMONAS SALMONICIDA*	2,2
B2L2	*PSUDOMONAS FRAGI*	2,09
B2L4	*ACINETOBACTER JOHNSONII*	2,29
B3L1	*PSEUDOMONAS AZOTOFORMANS*	1,82
B3L1	*PSEUDOMONAS ANTARCTICA*	1,92
B3L4	*AEROMONAS BESTIARUM*	2,13
B4L1	*AEROMONAS VERONII*	2,13
P1B2	*PSEUDOMONAS VERONII*	2,28
P1B3	*AEROMONAS EUCRENOPHILA*	2,19
P2B1	*AEROMONAS HYDROPHILA*	2,11
P2B2	*PSEDOMONAS FREDRIKBERGENSIS*	1,99
P2B3	*AEROMONAS MEDIA*	2,13
P2B4	*AEROMONAS MEDIA*	2,19
P3B1	*AEROMONAS VERONII*	2,19
P3B2	*AEROMONAS MEDIA*	2,22
P3B3	*PSEDOMONAS LIBANESIS*	2,13
P3B4	*AEROMONAS MEDIA*	2,22
P4B1	*PSEUDOMONAS VERONII*	2,38
P4B2	*AEROMONAS MEDIA*	2,23
P4B3	*AEROMONAS MEDIA*	2,09
P4B4	*AEROMONAS MEDIA*	2,24
Q1B1SS	*AEROMONAS MEDIA*	2,22

Q2B2*	*PSEUDOMONAS VERONII*	2,12
Q2B2	*KLEBISELLA PNEUMONIAE*	2,44
Q3B2	*RAOULTELLA TERRIGENA*	2,3
Q3B4	*RAOULTELLA ORNITHINOLYTICA*	2,41
Q3B4 LHK	*ACINETOBACTER JOHNSONII*	2,18
Q4B2	*RAOULTELLA ORNITHINOLYTICA*	2,53
Q4B3	*RAOULTELLA ORNITHINOLYTICA*	2,31
Q4B3 LHK	*ACINETOBACTER JOHNSONII*	2,32
Q4B4*	*AEROMONAS MEDIA*	2,21
Q4B4	*ACINETOBACTER JOHNSONII*	2,41
A1L1	*PSEUDOMONAS GESSARDII*	2,04
A1L1*	*PSEUDOMONAS BRENNERII*	2,04
A1L3	*PSEUDOMONAS CHLORORAPHIS*	1,96
A3L2	*PSEUDOMONAS PUTIDA*	1,79
A4L1	*PSEUDOMONAS BRENNERII*	2
B3L4	*AEROMONAS BESTIARUM*	2,15
P1B1	*PSEUDOMONAS ANTARCTICA*	2,05
P2B2	*PSEUDOMONAS FREDRKSBERGENSIS*	1,86
Q1B1SS	*AEROMONAS MEDIA*	2,09
Q3B3SS	*AEROMONAS EUCRENOPHILA*	2,05

7.4 APPENDIX D – Raw data: Biofilm formation

Data from ELISA plate reader with the average, standard deviation and standard error for the isolate in triplicates. Collected in order to determine biofilm biomass.

Table e. Raw biofilm data with the triplicates of each isolates on pegs

PEGS	35°C					
ISOLATES	**OD_absorbance**			**AVG.**	**STD**	**SE**
A5L2	0,084	0,076	0,08	0,024	0,004	0,00
A3L1	0,057	0,059	0,058	0,058	0,001	0,00
A3L2	0,06	0,057	0,077	0,065	0,011	0,01
Q2B3	0,057	0,065	0,067	0,063	0,005	0,00
Q4B2	0,069	0,105	0,131	0,102	0,031	0,02
Q3B3 ss	0,058	0,057	0,059	0,058	0,001	0,00
Q1B3	0,052	0,056	0,056	0,055	0,002	0,00
Q4B3	0,227	0,219	0,239	0,228	0,010	0,01
P2B3	0,06	0,059	0,057	0,059	0,002	0,00
A2L3	0,058	0,058	0,058	0,058	0,000	0,00
Q1B1	0,055	0,054	0,055	0,055	0,001	0,00
Q3B4	0,175	0,17	0,171	0,172	0,003	0,00
A5L3	0,2	0,223	0,207	0,210	0,012	0,01
P3B4	0,055	0,056	0,056	0,056	0,001	0,00
LB	0,056	0,057	0,058	0,056	0,001	0,00
TSB	0,057	0,057	0,058	0,057	0,001	0,00
Pos.control	0,125	0,175	0,212	0,171	0,044	0,03
B4L1	0,317	0,33	0,342	0,230	0,013	0,01
Q3B2	0,095	0,095	0,095	0,095	0,000	0,00
P3B1	0,096	0,094	0,091	0,094	0,003	0,00
A2L4	0,099	0,099	0,096	0,098	0,002	0,00
Q2B2	0,43	0,429	0,485	0,448	0,032	0,02
P4B3	0,093	0,092	0,092	0,092	0,001	0,00
P2B1	0,089	0,071	0,093	0,084	0,012	0,01
P2B4	0,092	0,09	0,096	0,093	0,003	0,00
P4B4	0,095	0,095	0,124	0,105	0,017	0,01
B3L4	0,097	0,083	0,101	0,094	0,009	0,01
A2L2	0,104	0,083	0,133	0,107	0,025	0,01
A3L4	0,156	0,155	0,121	0,144	0,020	0,01
P3B2	0,098	0,094	0,086	0,093	0,006	0,00
P4B2	0,075	0,097	0,099	0,090	0,013	0,01
P1B3	0,105	0,092	0,084	0,094	0,011	0,01
Q4B4	0,315	0,279	0,291	0,295	0,018	0,01
B2L4	0,096	0,094	0,098	0,096	0,002	0,00
A1L4	0,118	0,128	0,149	0,082	0,016	0,01
A1L3	0,171	0,205	0,208	0,195	0,021	0,01
P4B1	0,1	0,107	0,106	0,104	0,004	0,00

P2B2	0,053	0,056	0,124	0,078	0,040	0,02
P3B3	0,051	0,054	0,069	0,058	0,010	0,01
A2L1	0,508	0,582	0,546	0,545	0,037	0,02
A4L2	0,149	0,166	0,168	0,161	0,010	0,01
B2L2	0,114	0,097	0,094	0,102	0,011	0,01
A4L1	0,165	0,178	0,186	0,176	0,011	0,01
P1B2	0,072	0,063	0,059	0,065	0,007	0,00
B3L1	1,177	1,657	1,506	1,447	0,245	0,14
A4L3	0,863	0,898	0,903	0,888	0,022	0,01
P1B1	0,067	0,062	0,056	0,062	0,006	0,00
B2L1	0,054	0,057	0,049	0,053	0,004	0,00

Table f. Raw biofilm data with the triplicates of each isolates on convex microtiter plates

Convex wells	30°					
ISOLATES	**OD_absorbance**			**AVG.**	**STD**	**SE**
Q3B4 LHK	0,227	0,219	0,245	0,23	0,0	0,01
Q1B1 SS	0,198	0,187	0,233	0,21	0,0	0,01
P3B4	1,021	0,26	0,263	0,51	0,4	0,25
Q3B3LHK	0,696	0,61	0,57	0,63	0,1	0,04
B2L2	0,472	0,641	0,626	0,58	0,1	0,05
B3L10	1,26	0,801	0,63	0,90	0,3	0,19
P1B1	0,576	0,462	0,416	0,48	0,1	0,05
A1L4	0,302	0,408	0,375	0,36	0,1	0,03
Q4B4	1,01	1,226	0,972	1,07	0,1	0,08
A4L3	0,486	0,694	0,597	0,59	0,1	0,06
A3L1	0,549	0,416	0,438	0,47	0,1	0,04
A3L2	1,144	1,024	0,789	0,99	0,2	0,10
A2L3	0,324	0,363	0,762	0,48	0,2	0,14
P1B2	0,823	0,658	0,71	0,73	0,1	0,05
A2L4 Red	1,502	1,467	1,399	1,46	0,1	0,03
P4B2	0,401	0,381	0,436	0,41	0,0	0,02
P3B3	0,412	0,603	0,461	0,49	0,1	0,06
B3L1	1,145	1,085	0,652	0,96	0,3	0,16
Q4B3 LHK	0,349	0,175	0,158	0,227	0,11	0,06
Q3B4 SS	0,161	0,149	0,146	0,152	0,01	0,00
P2B3	0,116	0,122	0,121	0,120	0,00	0,00
Q4B2	0,114	0,128	0,136	0,126	0,01	0,01
Q3B3 SS	0,115	0,133	0,136	0,128	0,01	0,01
A4L1	0,213	0,197	0,262	0,224	0,03	0,02
A4L2	0,243	0,293	0,305	0,280	0,03	0,02
A1L3	0,167	0,231	0,244	0,214	0,04	0,02
P2B2	0,274	0,288	0,191	0,251	0,05	0,03
Q1B3	0,734	0,799	0,691	0,741	0,05	0,03
A2L1	0,27	0,209	0,241	0,240	0,03	0,02

B3L4	0,512	0,543	0,611	0,555	0,05	0,03
A1L1	0,459	0,372	0,372	0,401	0,05	0,03
Q3B3LHK	0,181	0,156	0,13	0,156	0,03	0,01
Q4B3 SS Ligth	0,193	0,262	0,133	0,196	0,06	0,04
Q4B2 SS Red	0,18	0,175	0,162	0,172	0,01	0,01
Q4B2 SS Ligth	0,164	0,128	0,138	0,143	0,02	0,01
Q3B3 SS Red	0,142	0,121	0,114	0,126	0,01	0,01

Table g raw biofilm data with the triplicates of each isolates on flat- bottom microtiter plates

Flat -bottom 30°C

ISOLATES	**OD_absorbance**			**AVG.**	**STD**	**SE**
Q3B4 LHK	1,471	1,418	1,405	1,43	0,03	0,02
Q1B1 SS	0,305	0,377	0,292	0,32	0,05	0,03
P3B4	0,64	0,642	0,608	0,63	0,02	0,01
Q3B3LHK	1,029	0,778	0,357	0,72	0,34	0,20
B2L2	1,037	1,197	1,014	1,08	0,10	0,06
B3L10	2,7	2,794	2,723	2,74	0,05	0,03
P1B1	1,298	0,93	0,859	1,03	0,24	0,14
A1L4	2,224	0,785	0,565	1,19	0,90	0,52
Q4B4	0,694	0,717	0,829	0,75	0,07	0,04
A4L3	2,675	2,167	2,305	2,38	0,26	0,15
A3L1	1,262	1,286	1,125	1,22	0,09	0,05
A3L2	0,596	0,737	0,838	0,72	0,12	0,07
A2L3	0,855	0,9	0,883	0,88	0,02	0,01
P1B2	1,274	1,212	0,984	1,16	0,15	0,09
A2L4 Red	0,622	0,559	0,603	0,59	0,03	0,02
P4B2	1,19	1,156	1,024	1,12	0,09	0,05
P3B3	1,844	1,703	1,757	1,77	0,07	0,04
B3L1	0,746	0,877	0,788	0,80	0,07	0,04
Pos.control	2,973	3,088	3,103	3,05	0,07	0,04
Neg.control	0,735	0,679	0,634	0,68	0,05	0,03
Q4B3 LHK	1,268	1,39	1,086	1,25	0,15	0,09
Q3B4 SS	0,719	0,77	0,716	0,74	0,03	0,02
Q3B3 SS	2,254	2,319	2,239	2,27	0,04	0,02
A4L1	0,95	0,729	0,738	0,81	0,13	0,07
A4L2	1,011	0,94	1,639	1,20	0,38	0,22
A1L3	0,904	0,878	0,698	0,83	0,11	0,06
P2B2	1,744	1,893	1,604	1,75	0,14	0,08
Q1B3	2,806	3,216	2,941	2,99	0,21	0,12
A2L1	1,361	1,363	1,202	1,31	0,09	0,05
B3L4	2,536	2,117	2,643	2,43	0,28	0,16

A1L1	0,482	0,611	0,397	0,50	0,11	0,06
Q4B3 SS white	0,269	0,353	0,335	0,32	0,04	0,03
Q4B2 SS red	0,75	0,81	0,721	0,76	0,05	0,03
Q4B2 SS white	0,957	1,342	1,123	1,14	0,19	0,11
Q3B3 SS red	0,542	0,745	0,578	0,62	0,11	0,06
B4L1	0,196	0,13	0,142	0,16	0,0	0,02
B2L1	0,273	0,127	0,152	0,18	0,1	0,05
A2L4	0,343	0,786	1,052	0,73	0,4	0,21
A5L2	0,289	0,325	0,464	0,36	0,1	0,05
P1B3	0,193	0,333	0,356	0,29	0,1	0,05
Q3B2	0,201	0,515	0,285	0,33	0,2	0,09
Q2B2	0,131	0,427	0,621	0,39	0,2	0,14
P3B1	0,122	0,295	0,34	0,25	0,1	0,07
A2L2	0,975	1,456	1,761	1,40	0,4	0,23
A5L3	0,358	0,95	1,154	0,82	0,4	0,24
Q4B3	0,356	0,231	0,387	0,32	0,1	0,05
A3L4	0,488	0,554	0,7	0,58	0,1	0,06
P4B1	0,336	0,503	0,389	0,41	0,1	0,05
P2B3	1,502	1,671	1,268	1,48	0,2	0,12
Q4B2	0,888	0,449	0,448	0,60	0,3	0,15

7.5 APPENDIX E – Protocols

Semiquantitative determination of biofilm formation (modified Christensen method)

Media:

Appropriate solid and liquid growth medium (LB for environmental samples)

0,1% Crystal violet

Other equipment:

96well microtiter plates

Peg-lid for biofilm cultivation

ELISA reader

Procedure:

1. Streak out isolates under investigation from freeze stock.
2. Inoculate a single colony from agar into 5 ml liquid growth medium and let grow overnight in a shaker (approx. 220 rpm) at 37°C.
3. Dilute the overnight culture 1:100 with appropriate liquid growth medium.
4. Pipette 150µl of the bacterial suspension in one column of a 96-well polystyrene tissue culture plates included positive control and negative control. Each isolate should be used to inoculate at least four wells.

Incubate for 24 hours at 37 °C without shaking.

5. Wash pegs by inserting into clean microtiter plate with 200 µl PBS. Repeat 3 times.
6. Put the plates in an incubator for 1 hour at 55 °C. The heat will fix the biofilm-forming bacteria.
7. 200µl crystal violet (0.4%) is added in each well of a microtiter plate and the lid inserted to stain the biofilm for 5 minutes.
8. Wash the pegs gently with tap water.
9. Add 200µl 70% EtOH in each well and insert the peg-lid. Incubate on the bench for 5 minutes.
10. Remove peg lid and measure OD in an ELISA reader at 570nm and single wavelength.
11. Three biological replicates are advised.
12. Calculate the average value and standard deviation:
    - To avoid the effect extreme low or extreme high observations will have on the average, we have to remove these values from the data.
    - Calculate the average value of each parallel.

$$\text{Average} = \frac{X_1 + X_2 + X_3 + \ldots\ldots X_n}{N}$$

- Calculate the average values of the parallels
- Calculate the standard deviation (STD) for the average value

13. Calculate the average of the controls. Add three standard deviations to find the OD-limit between biofilm and non-biofilm forming strains.
14. Investigate reliability of each strain as to their biofilm or non-biofilm forming phenotype by adding or subtracting one standard deviation from the average of the 3 parallels.

Reference List

1. **Christensen, G. D., Simpson, W. A., and Younger, J. J.** Adherence of coagulase-negative staphylococci to plastic tissue culture plates: a quantitative model for the adherence of staphylococci to medical devices. Journal of Clinical Microbiology 22(6), 996-1006. 1985.

2. **Fredheim, E. G. A., C. Klingenberg, H. Rohde, S. Frankenberger, P. Gaustad, T. Flaegstad, and J. E. Sollid**. 2009. Biofilm Formation by Staphylococcus haemolyticus. J.Clin.Microbiol. **47**:1172-1180.

3. **Klingenberg, C., E. Aarag, A. Ronnestad, J. E. Sollid, G. Kjeldsen, and T. Flaegstad**. 2005. Coagulase-Negative Staphylococcal Sepsis in Neonates: Association Between Antibiotic Resistance, Biofilm formation and the Host Inflammatory Response. Pediatric Infectious Disease Journal **24**:817-822.

DNA isolation Protocol

QIAamp® Fast DNA Stool Mini Kit

The QIAamp Fast DNA Stool Mini Kit (cat. no. 51604) can be stored at room temperature (15–25°C) for up to 12 months.

Further information

- QIAamp Fast DNA Stool Mini Kit Handbook: www.qiagen.com/HB-1764
- Safety Data Sheets: www.qiagen.com/safety
- Technical assistance: support.qiagen.com

Notes before starting

Prepare a thermomixer with 2 ml inlays or a water bath at 70°C for use in steps 3 and 8.

Perform all centrifugation steps at room temperature (15–25°C) at 20,000 x g (~14,000 rpm).

Redissolve any precipitates in Buffer AL and InhibitEX® Buffer by heating and mixing.

Add ethanol to Buffer AW1 and Buffer AW2 concentrates.

Mix all buffers before use.

Symbols: ● pathogen detection; ▲ human DNA analysis

1. Weigh 180–220 mg stool in a 2 ml microcentrifuge tube (not provided) and place tube on ice.

2. Add 1 ml InhibitEX Buffer to each stool sample. Vortex continuously for 1 min or until the stool sample is thoroughly homogenized.

3. ▲ Skip this step and continue with step 4. ● Heat the suspension for 5 min at 70°C. The lysis temperature can be increased to 95°C for cells that are difficult to lyse. Vortex for 15 s.

4. Centrifuge sample for 1 min to pellet stool particles.

5. Pipet ● 15 µl or ▲ 25 µl Proteinase K into a new ● 1.5 ml or ▲ 2 ml microcentrifuge tube (not provided).

6. Pipet ● 200 µl or ▲ 600 µl supernatant from step 4 into the ● 1.5 ml or ▲ 2 ml

microcentrifuge tube containing Proteinase K.

7. Add □ 200 µl or ▲ 600 µl Buffer AL and vortex for 15 s. Note: Do not add Proteinase K directly to Buffer AL. It is essential that the sample and Buffer AL are thoroughly mixed to form a homogeneous solution.

8. Incubate at 70°C for 10 min.

9. Add □ 200 µl or ▲ 600 µl of ethanol (96–100%) to the lysate, and mix by vortexing.

10.Carefully apply 600 µl lysate from step 9 to the QIAamp spin column. Close the cap and centrifuge for 1 min. Place the QIAamp spin column in a new 2 ml collection tube, and discard the tube containing the filtrate. □ Repeat step 10 until all lysate is loaded.

11.Carefully open the QIAamp spin column and add 500 µl Buffer AW1. Centrifuge for 1 min.

Place the QIAamp spin column in a new 2 ml collection tube, and discard the collection tube containing the filtrate.

12.Carefully open the QIAamp spin column and add 500 µl Buffer AW2. Centrifuge for 3 min.

Discard the collection tube containing the filtrate.

13.Place the QIAamp spin column in a new 2 ml collection tube (not provided) and discard the old collection tube with the filtrate. Centrifuge for 3 min.

14.Transfer the QIAamp spin column into a new, labeled 1.5 ml microcentrifuge tube (not provided) and pipet 200 µl Buffer ATE directly onto the QIAamp membrane. Incubate for 1 min at room temperature, then centrifuge for 1 min to elute DNA. If yield will be quantified by UV absorbance, blank the measuring device using Buffer ATE to avoid false results.

Changes between Revision 2 and Revision 3

Step 5. Addition of 2 ml microcentrifuge tube for the human DNA analysis protocol.

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit handbook or user manual.

Disk diffusion test protocol

Method 3.2.1: Antimicrobial Gradient Strip Testing

Version No. 1.0

Author: Elizabeth G.A. Fredheim & Nicole L. Podnecky

Approved by: Nicole L. Podnecky

Date: 19 February 2018

1. Purpose/ brief description

Determination of the minimal inhibitory concentration (MIC) of Escherichia coli isolates to various antimicrobials using gradient strip testing. This is a quick an easy procedure to determine MIC and clinical interpretation using reference cut-off values (e.g EUCAST - R, I, S).

2. Scope This protocol was written specific for Enterobacteriaceae according to Liofilchem and BioMerieux guidelines. Check the manufacturer guidelines and EUCAST or CLSI guidelines when working with other bacterial species.

3. Safety precautions Biosafety precautions based on bacterial strain.

Toxic compounds and antimicrobials in the testing strips.

4. Risk waste Standard waste treatment appropriate for the organism. Autoclave.

5. References NS-EN ISO 20776-1:2006

http://www.eucast.org/clinical_breakpoints/

CLSI M100-S20

6. Attachments BioMerieux – Product Insert

BioMerieux – MIC reading guide

Liofilchem – MIC reading guide

7. Notes The control strain should be tested regularly and compared to the expected range of MIC values given by EUCAST or CLSI.

Material and Equipment:

- Inoculation loops
- MIC test strips (BioMerieux® or Liofilchem®)
- Sterile cotton swabs
- Sterile forceps
- Sterile toothpicks
- Rotator
- Calibrated densitometer

Enterobacteriaceae specific:

- Mueller Hinton II (cation-adjusted) agar plates (BD – 211438/ 211441/ 212257)
- Sterile saline, 0.85% NaCl
- Incubation at 37°C for 18 +/- 2 hours
- Control strain: Escherichia coli ATCC 25922

Procedure:

Before you start: Use standard microbiological and aseptic techniques to ensure sterility of the culture. It is recommended to work in a biological safety cabinet to provide an aseptic workspace, protect the sample(s) and provide ample light for interpretation.

1. Streak for isolation a pure culture of the strain of interest on appropriate agar medium and incubate overnight.

a. Alternatively an overnight liquid culture may be used.

b. Subculture and growth to mid-log phase can further reduce inter-assay variability.

2. Allow MIC testing strips to equilibrate to room temperature before testing according to the manufacturer.

a. Typically 15 minutes for those at 4°C and 1 hour for those at -20°C.

3. Suspend several fresh colonies in 3-5 mL of 0.85% NaCl with a sterile cotton swab to the optical density of a 0.5 McFarland.

a. 0.5 McFarland(s) should not be prepared more than 15 min in advance of step 4.

b. 0.5 McFarland has an OD600nm ≈ 0.08 – 0.1 if using a spectrophotometer or can be compared by eye to a reference standard looking through the culture to something dark in colour.

4. Place a sterile cotton swab in the inoculum to saturate it. Then press it against the inside of the tube to remove excess liquid.

5. Streak a Mueller Hinton agar plate for confluency.

a. Use a rotator, hold the swab gently to the surface and count to 10 while spiralling the swab to the just past the centre. Rotate the swab slightly to use a different surface and then count to 10 spiralling out again.

b. Or streak the plate for confluence 3 times, rotating the plate 60° each time and including the outer edge of the plate and using different sides/surfaces of the swab.

6. Allow the plate to dry for a few minutes (must be completely dry). Maximum 15 mins.

7. Use sterile forceps or strip application device to place gradient strips with scale facing up.

a. Do not move the strip once it has touched the agar, drug diffusion occurs rapidly.

b. When using forceps only handle the strip from the top edge - above the highest concentration.

8. Use a sterile toothpick to gently tap the strip and remove any air bubbles from under the strip. Do this in the direction of lowest to highest concentration.

a. If using forceps for this step, forceps must be re-sterilized before further use.

9. Incubate at 37°C for 18 +/- 2 hours.

a. Place the plate in the incubator no more than 15 minutes after the strip was applied.

b. Do not stack more than 5 plates, ideally no more than 3.

10. Analysis: Read the MIC value according to the manufacturer's guidelines.

a. Typically where the edge of the growth ellipse cuts the strip.

b. For most bactericidal drugs include any form of growth in the interpretation.

c. Single colonies within the inhibition zone should be evaluated.

d. Always increase half-values to the next 2-fold dilution before interpretation of resistance category according to EUCAST or CLSI guidelines.

e. For bacteriostatic antimicrobials the MIC should be read at ≥ 80% inhibition, the first point of significant inhibition as judged by the unaided eye.

Variables that might affect the results:

Low risk:

- The bacteria: colonies should not be older than 24-48h, to ensure a correct relationship between bacterial growth and antimicrobial diffusion. It is ideal to work with pure cultures.

- Growth conditions: Temperature, incubation time, atmosphere (plate stacking)

- MIC strips: diffusion speed, drug solubility, charge, molecular weight, durability, controls, application.

Medium risk:

- Analysis/interpretation: bactericidal antimicrobials should be read at total inhibition, bacteriostatic are read at 80% inhibition, which can be difficult. Single colonies within the inhibition zone have to be evaluated.

- Storage: The strips are very sensitive to moisture, it's very important to store them dry with a desiccant and bring them to ambient temperature before opening packaging and use.

- Agar: thickness, viscosity, pH, cation concentration, thymine/thymidine content.

- Inoculum: too thin gives poor growth, which can result in too low MIC. Too dense can give too high MIC. Inoculum has to be used within 15 min.