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THE EFFECT OF TETRACYCLINE EXPOSURE ON THE MINIMUM INHIBITORY CONCENTRATION, SPREAD TO ANTIBIOTIC RESISTANT GENES IN A LABORATORY SCALE ACTIVATED SLUDGE REACTOR

MASTER´S THESIS

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Abstract

Antibiotics, which are used to treat bacterial infections have saved countless lives and their development has been a huge step in modern medicine. Unfortunately, the overuse of antibiotics has led to the development of antibiotic resistance. An increasing number of bacteria strains are becoming resistant to antibiotics, where horizontal gene transfer (HGT) plays a crucial role in the spread of resistance. Wastewater treatment plants (WWTPs) are hotspots for HGT of antibiotic resistant genes (ARGs), as they contain large numbers of bacteria, which are exposed to low levels of antibiotics thus exerting selective pressure for the selection of antibiotic resistance genes.

This study looks at the spread of ARGs by horizontal gene transfer in artificial wastewater, because WWTPs constitute the final barrier for ARGs and antibiotic resistant bacteria (ARB) before being released to the environment. Sludge from the local WWTP which was maintained in an aerobic sludge bioreactor was used to inoculate two aerobic AS reactors, one with the concentration of 1,4 µg/L tetracycline, and a control reactor without tetracycline. After addition of tetracycline to Reactor 2, the reactors were maintained for four weeks. Samples were taken from both reactors twice a week, where MIC values were determined for the different bacterial isolates at each sampling time. In addition, qPCR was used to quantify the total number of bacteria.

Both the MIC values and qPCR suggests that subinhibitory levels of tetracycline led to a tendency for the selection of higher antibiotic resistance in the reactor where tetracycline was added.

Keywords: *Tetracycline, Wastewater treatment, antibiotic resistant genes, HGT, MIC, qPCR, aerobic AS reactor*

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TABLE OF CONTENT

List of tables

List of figures

List of abbreviations

WWTPs - wastewater treatment plants

ARB - antibiotic resistant bacteria

ARGs - antibiotic resistant genes

HGT - horizontal gene transfer

MGE - mobile genetic elements

AS - activated sludge

ADS - anaerobic digestion sludge

MIC - minimum inhibitory concentration

CFU - colony forming units

PCR - polymerase chain reaction

qPCR - quantitative polymerase chain reaction

CT value - cycle threshold value

OD - optical density

AS - activated sludge

1.0 INTRODUCTION

Antibiotic resistance is one of the greatest threats to global health, food security and development in the world today. Antibiotic resistance occurs naturally. However, misuse of antibiotics in humans and animals is accelerating the process (World Health Organization, 2020). Large quantities of antibiotics, antibiotic resistant bacteria (ARB) and antibiotic resistant genes (ARGs) are ending up in wastewater. Therefore, wastewater treatment plants are hotspots for antibiotics, ARB and ARGs, and the wastewater treatment plants (WWTPs) also serve as the last barrier to remove ARBs and ARGs before being released to the environment. To avoid further spreading to the environment, proper mechanisms for removal of ARB, ARGs and antibiotics are critical.

1.1 Objectives

The main objective of this study was to look at the effect an antibiotic subinhibitory concentration had on the spread of antibiotic resistance, and the minimum inhibitory concentration (MIC) to the antibiotic in a wastewater treatment plant. As tetracycline is commonly found in wastewater treatment plants, a realistic wastewater concentration of tetracycline was used to study the effects on the spread of antibiotic resistance in wastewater treatment plants. Two model aerobic activated sludge reactors were used to study this effect, one containing tetracycline and one control reactor without tetracycline. This was further divided into two subobjectives:

i) The effect of tetracycline on the minimum inhibitory tetracycline concentration
ii) The effect of tetracycline on the abundance to the tetracycline resistance gene (*tet*M) over time by using qPCR.

2.0 BACKGROUND

2.1 Antibiotics

Antibiotics are antimicrobial agents. These can be natural or synthetic chemicals that either kill or inhibit growth of microorganisms. Antibiotics can be either bactericidal or bacteriostatic.

Antibiotics are agents killing or inhibiting bacteria. Different types of antibiotics have different targets in the bacteria cell as shown in table 1-1 below. Examples of targets can be the bacteria cell wall, cell membrane, protein synthesis (30S or 50S), folate synthesis, DNA synthesis or RNA synthesis (Madigan, 2015, p. 836).

Table 1-1: Antibiotics mode of action.

Antibiotic class	**Mode of action**	**Reference**
Glycopeptides	Inhibiting cell wall synthesis by binding to the D-Ala-D-Ala terminal of the growing peptide chain and prevents further elongation	(Reynolds, 1989)
β-lactams	Inhibiting synthesis of peptidoglycan	(Lin et al., 2015)
Chloramphenicol	Binds to ribosome, blocking peptidyl transferase, and inhibit protein synthesis	(DrugBank Online)
Aminoglycosides	Binds to 30S ribosomal subunit and inhibit protein synthesis	(Kotra et al., 2000)
Oxazolidinone	Inhibits protein synthesis by binding to the P site at the ribosomal 50S subunit	(Shinabarger, 1999)
Macrolides	Inhibits protein synthesis by binding to 50S ribosomal subunit, preventing the addition of amino acids.	(Patel & Hashmi, 2021)
Quinolones	Block the ligase domain of DNA gyrase to prevent bacterial DNA replication	(Doble, 2007)
Tetracyclines	Reversible binding to bacterial 30S ribosomal subunits inhibits protein synthesis by preventing binding of new incoming amino acids	(Kester et al., 2012)
Sulfa drugs	Binds to and inhibit dihydropteroate synthase (DHPS), an enzyme required for folate production, an important vitamin necessary for cell survival	(Lesley Earl, 2015)

2.2 Antibiotics mode of action

2.2.1 Inhibition of cell wall synthesis

Human cells do not have a cell wall nor possesses peptidoglycan which is found in prokaryotic cells. Since the antibiotic will not destroy the human cell, only the bacterial cell, the bacterial cell wall becomes an optimal target for the antibiotic (Kaufman, 2011). Bacterial cells primary functions are to provide rigidity to the cell and therefore avoid cell lysis. The peptidoglycan backbone is what is causing the cell's rigidity (Sarkar et al., 2017). The mode of action for inhibition of cell wall synthesis is therefore to interrupt the peptidoglycan synthesis (Madigan, 2015, p. 838).

β-lactam antibiotics are an example of cell wall synthesis inhibitors and include the medically important penicillin and cephalosporins (Madigan, 2015, p. 838) These two antibiotics enter the bacterial cell and bind to penicillin-binding proteins (Kaufman, 2011). This results in a weak cross-linking of two peptidoglycans which makes the cell wall deformed and forces the cell wall to swell and then burst (Kaufman, 2011; Madigan, 2015, p. 836). Another antibiotics that inhibit cell wall synthesis are the glycopeptide vancomycin, which is often used to treat MRSA (Madigan, 2015, p. 836).

2.2.2 Cell membrane inhibition

Polymyxins and daptomycin are antibiotics whose target is the cell membrane (Madigan, 2015, p. 836). Polymyxins are cationic agents that bind to the anionic bacterial outer membrane, which lead to a detergent effect and leakage of membrane integrity (Landman et al., 2008). Daptomycin binds specifically to bacterial cytoplasmic membranes, forms a pore, and provides rapid depolarization of the membrane. The depolarized cell quickly loses its ability to synthesize macromolecules such as nucleic acids and proteins, resulting in cell death (Madigan, 2015, p. 840).

2.2.3 Nucleic acid synthesis inhibition

Deoxyribonucleic acid (DNA) is every organism's hereditary material in every living organism. DNA synthesis and mRNA transcription require modulation of chromosomal supercoiling (through strand breakage and rejoining reactions catalyzed by topoisomerase). Quinolone is a class of antibiotics that targets the topoisomerase (II and IV) in prokaryotes and traps these enzymes at the DNA cleavage stage and prevents strand rejoining. DNA

synthesis is therefore inhibited, which immediately leads to bacteriostasis and eventually cell death (Kohanski et al., 2010).
Ribonucleic acid (RNA) converts the DNA into proteins. Rifamycin is a class of bactericidal antibiotics that is able to inhibit RNA synthesis by binding to the β-subunit of RNA polymerase (Kohanski et al., 2010; Madigan, 2015, p. 838).

2.2.4 Protein synthesis inhibition

The mRNA translation process occurs in three stages; initiation, elongation and termination which involve the ribosome and other cytoplasmic factors. The ribosome consists of two subunits: 30S and 50S. 50S ribosome inhibitors include macrolides, lincosamides, streptogramins, amphenicols and oxazolidinones, and work by physically block initiation of protein translation or translocation of peptidyl tRNAs, which serves to inhibit the reaction that elongates the peptide chain (Kohanski et al., 2010).
30S inhibitors include tetracyclines and aminocyclitols (includes spectinomycin and aminoglycosides) (Kohanski et al., 2010). Tetracyclines block the access for aminoacyl tRNAs to the ribosome by binding to the 30S subunit and cause inhibition of bacterial protein synthesis (Anderson et al., 2012, p. 210). Aminocyclitols bind to the 16S rRNA component of the 30S ribosome subunit which means it occupies the space where tRNA are supposed to bind, and inhibits the protein synthesis (Anderson et al., 2012, p. 158; Madigan, 2015, p. 839).

2.2.5 Folic acid synthesis inhibition

Folic acid is a nutrient that is required for the production of proteins and nucleic acids (DNA and RNA). Bacteria produce folic acid from the substrate para-amino-benzoic acid (PABA), catalyzed by the enzyme dihydropteroate synthase (DHPS), and all cells require folic acid for survival (Constable et al., 2017). DHPS is not found in eucaryotic cells, and mammals must obtain folic acid from their diet as it cannot be synthesized by mammalian cells. Folic acid is indirectly involved in DNA synthesis as the enzyme cofactors which are needed for synthesizing purine and pyrimidine bases of DNA (Anderson et al., 2012, p. 111).
Sulfonamides and Trimethoprim are antibiotics targeting the folic acid metabolism (Madigan, 2015, p. 836). Sulfonamides are highly selective as they interfere with a key process in bacterial cells which does not take place in eukaryotic (mammalian) cells. By binding to the PABA binding domain, the active site of DHPS , and therefore inhibiting the DHPS enzyme, will stop the production of folic acid (Anderson et al., 2012, p. 112).

Trimethoprim is inhibiting the dihydrofolate reductase (DHFR) which is resulting in a bacteriostatic effect due to the disruption of the folate cycle and reduction of tetrahydrofolate (Anderson et al., 2012, p. 131). The DHFR enzyme in bacteria and mammalian cells shows a lot of simulatities, and there are two possible binding sites for trimethoprim. However, the binding sites in non-bacterial DHFR the active binding site clefts are wider than that of the bacterial enzyme, where bindings of trimethoprim to the mammalian DHFR would lead to conformational changes that are less favorable than the bindings if trimethoprim to the bacterial DHFR. Therefore, trimethoprim is highly selective and will bind to the bacterial DHFR and stop cell growth and proliferation. (Anderson et al., 2012, p. 134).

2.3 Antibiotic resistance in bacteria

According to Luby et al. (2016) antibiotic resistance is defined as "the ability of a bacterial cell to survive and grow in the presence of an antibiotic concentration that is inhibitory to susceptible cells." The efficiency of antibiotics has been compromised over the last century by a growing number of antibiotic resistant pathogens (Lin et al., 2015). Misuse and overuse of antibiotics provides selective pressure on bacteria which accelerate the resistant process of the selection of resistance mechanisms towards antibiotics (World Health Organization, 2020; Wright, 2010). The ability bacteria have to develop these mechanisms are expressed by ARGs in the bacteria (Lin et al., 2015).There are several ways in which bacteria can obtain ARGs; natural resistance, spontaneous mutations, vertical gene transfer from parent to offspring or by horizontal gene transfer (Lin et al., 2015; Pazda et al., 2019).

2.3.1 Horizontal gene transfer

Horizontal gene transfer (HGT) can occur through three different mechanisms: phage-mediated transduction, transformation of "naked" DNA, and conjugation (Graf et al., 2019), (see Figure 1). HGT has been considered as the most efficient method of spreading ARGs between bacteria, as it can even spread to those of different genera (Colomer Lluch et al., 2011; Wright, 2010). Some of the HGT mechanisms are mediated by mobile genetic elements (MGE) such as plasmids, transposons and integrons which can carry one or more resistance genes (Rizzo et al., 2013; Wright, 2010). MGE refers to segments of DNA that move as units from one location to another within other DNA molecules (Madigan, 2015, p. 334). This can occur either between distinct cells or within the same cell.

Plasmids are small, often circular, double stranded segments of DNA that are found in almost all prokaryotic cells in addition to the chromosome. Plasmids rely on the chromosomally encoded enzymes for their replication through cell division, even though they possess their own origin of replication. Plasmids do not contain essential, but they encode functions that can be beneficial for the host and increase fitness, such as ARGs. Plasmids encoding antibiotics resistance are called R-plasmids (Madigan, 2015, p. 138).
Transposons are a type of genetic element that "jumps" to new places within a genome (Britannica, T. Editors of Encyclopedia, 2018). Some transposons, called conjugative, are able to move from different bacterial species by conjugation in addition to jumping from location to location within the bacterial genome, thus transferring resistant genes between different bacterial cells (Madigan, 2015, p. 334).
Integrons are genetic elements with a site-specific recombination system capable of integrating, expressing, and exchanging certain DNA elements known as gene cassettes (Domingues et al., 2012). Gene cassettes are not always part of the integron. Resistance integrons, found on the chromosome or on plasmids, carry gene cassettes that encode resistance to antibiotics and disinfectants (Fluit & Schmitz, 2004).

Conjugation is a mechanism where two bacteria which are in direct contact can transfer gene material. In conjugation ´the donor bacterium transfers some of its DNA, often a plasmid, to the donor bacterium through thin "tunnels" called pilus (Madigan, 2015, pp. 73, 323). A conjugative apparatus, which is expressed by genes on autonomously replicating plasmids or on integrative conjugative regions on the chromosome. The *tra*-genes encode for proteins needed for its own transfer to other cells (Graf et al., 2019; von Wintersdorff et al., 2017).

Conjugation is thought to have the largest impact on the spread of ARGs compared with the other three conventional HGT processes. This is because it offers better protection from the environment and a more efficient means of accessing the host cell than transformation, as well as a wider host range than bacteriophage transduction. While transformation and transduction are regarded to be less important, recent findings reveal that they may play a larger role than previously thought (von Wintersdorff et al., 2017).

Transduction refers to the mechanism where a bacteriophage (bacterial virus) has taken up bacterial DNA due to incorrect packing and then transferring bacterial DNA into a new host (Madigan, 2015, p. 327). Bacteriophages might use specialized or generalized transduction to mobilize genes. Generalized transduction mobilizes any DNA fragment of the bacterial genome, whereas specialized transduction mobilizes just a few specific genes (Brown-Jaque et al., 2015; von Wintersdorff et al., 2017). Transductions is regarded as less important for spreading ARGs because the packaging of bacterial DNA into viruses is inefficient and bacteriophages are usually limited in the bacterial species they are able to infect (Rogers & Kadner, 2020). qPCR has been used in several studies to detect ARGs in bacteriophages from wastewater samples, river water and animal fecal samples confirming their contribution to the mobilization of resistance genes (Colomer-Lluch, Calero-Cáceres, et al., 2014; Colomer-Lluch, Jofre, et al., 2014).

In transformation a competent bacteria can take up free DNA through its cell membrane from the environment and incorporate it, resulting in a genetic alteration (Madigan, 2015, p. 325). A competent bacterium is defined as having the ability to bind free fragments of DNA. Competent bacteria occur naturally only in a small number of bacteria such as *Haemophilus*, *Neisseria*, *Streptococcus*, and *Bacillus* (Rogers & Kadner, 2020). Other species capable of natural transformation may only establish competence under certain conditions, such as presence of peptides or autoinducers, nutritional status or other stressful conditions (von Wintersdorff et al., 2017). WWTPs have a large reservoir of naked DNA fragments, making them an ideal habitat for transformation, especially since antibiotics have shown to select for ARGs (von Wintersdorff et al., 2017).

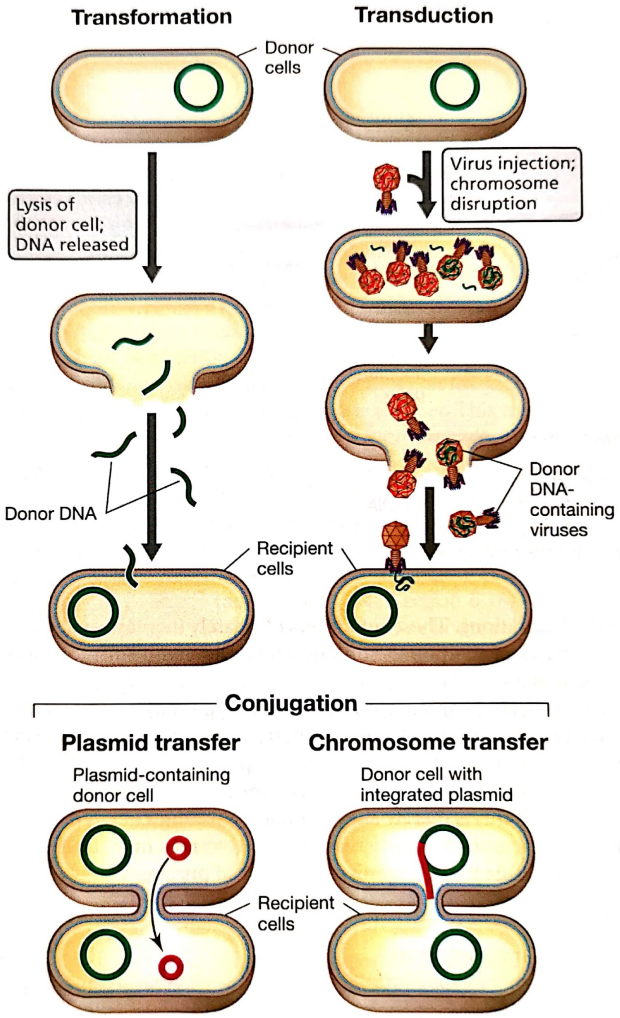

Figure 1: Horizontal gene transfer. Gathered from (Madigan, 2015, p. 324).

2.3.2 Resistance mechanisms

ARGs encode mechanisms that make the bacteria overcome the antibiotics (Luby et al., 2016). These resistance genes, in general, encode proteins that either inactivate the antibiotic or protect the cell in some way. A single R plasmid can encode numerous antibiotic resistance genes; conversely, a cell with multiple resistance may include many sperate R plasmids (Madigan, 2015, p. 138). These different resistance mechanisms will now be further discussed.

2.3.2.1 Reduced permeability

Porins are channels through the outer membrane of the cell that regulate influx and efflux of components. Expression of porins is regulated in response to the environment. Temperature, pH, nutrients, and chemicals in the environment affect the expression of porin genes, and

therefore the size and characteristics of the porins. The characteristics of constitutively expressed porins have a significant impact on the intrinsic level of antibiotic resistance in gram-negative bacteria. *Pseudomonas aeruginosa* shows a well-known intrinsic resistance to a wide range of antibiotics, mainly due to the use of a slow porin as the main channel. The low-permeability outer membrane becomes even more effective in preventing the inflow of antibiotics (Nikaido, 2003).

2.3.2.2 Inactivation of antibiotics

The R plasmid genes can encode enzymes that phosphorylate, acetylate, or adenylate the drug to alter and inactivate it. As a result, the antibiotic activity of the modified drug is lost (Madigan, 2015, p. 843).

Antibiotics known as β-lactams inhibit the development of bacterial cell wall and are frequently used to treat a variety of bacterial infections (Lin et al., 2015). β-lactams antibiotics includes among other penicillins and chloramphenicol. For penicillin, the bacteria's' R plasmid encode β-lactamase, which is an enzyme that break the β-lactam ring, and therefore inactivating the antibiotic. Resistance to chloramphenicol is caused by an enzyme encoded by the R plasmid that acylates the antibiotic (Madigan, 2015, p. 843).

Another important type of antibiotics treating various infections are the aminoglycosides (e.g., kanamycin, streptomycin), where the effectiveness has been compromised lately due to resistance. The synthesis of aminoglycoside-modifying enzymes is a major mechanism of aminoglycoside resistance (Lin et al., 2015).

2.3.2.3 Alteration of target

In bacterial pathogens, altering the target site is one of the most common methods of antibiotic resistance. Point mutations in the gene encoding the target site, enzymatic alterations of the binding site (e.g., addition of methyl group), and/or replacement or bypass of the original target are examples of target alterations (Munita & Arias, 2016).

Macrolides (e.g., erythromycin and rifamycin) and quinolones (and their derivatives - fluoroquinolones, e.g., norfloxacin) have resistance mechanisms that involve alteration of target (Madigan, 2015, p. 844; Pazda et al., 2019). The most well-known mechanism of bacterial resistance to macrolide antibiotics is based on the production of methylase, which has the function of methylating 23S rRNA – the antibiotic's target site (Pazda et al., 2019). The methylation of antibiotics decreases their affinity for their target, most likely by blocking

direct access to the target or altering the conformation of the binding site (Leclercq & Courvalin, 2002).

Quinolones and fluoroquinolones inhibit two enzymes which are essential for bacterial DNA replication: DNA gyrase and topoisomerase IV. Quinolone resistance have been discovered to be mediated by the *qnr* genes, which encodes proteins that protect DNA gyrase against quinolone compounds, and are found on MGEs such as plasmids, which might potentially transfer genes by HGT (Pazda et al., 2019; Ruiz, 2003). However, the *qnr* genes effect on topoisomerase VI is unclear (Ruiz, 2003).

2.3.2.4 Development of resistant biochemical pathway

Bacteria can create an alternative metabolic pathway that performs similar or identical functions to the drug-inactivated pathway (Pazda et al., 2019).

Sulfonamides is an example of antibiotics that develop resistant biochemical pathways as a resistance mechanism (Madigan, 2015, p. 844). Resistance to sulfonamides is caused by a variety of mechanisms, including metabolic alterations (overproduction of target enzyme or physiological metabolite), mutational changes in the target enzyme that reduce its affinity for the inhibitor, changes in the cell permeability that impact the drug uptake and the synthesis of drug-resistant bypass enzymes (Pazda et al., 2019).

2.3.2.5 Efflux pump

Efflux pumps are transport proteins involved in the extrusion of hazardous substrates from the inside of cells to the external environment (including nearly all types of therapeutically relevant antibiotics) Pups can be specific to a single substrate or may transport a variety of structurally different compounds (Webber & Piddock, 2003).

Transcriptional regulators that either repress or activate the transcription of multidrug efflux genes usually control the expression of the bacterial multidrug efflux system (Lin et al., 2015). One of the two main resistance mechanisms for tetracycline antibiotics is the reduction of intracellular drug concentration due to membrane associated proteins coded by genes that export drugs out of the cell (Pazda et al., 2019). Some bacteria species (such as *Klebsiella pneumoniae*) are able to operate with multidrug efflux pumps, which can remove a wide range of pharmaceuticals from the cell (Pazda et al., 2019).

2.3.3 Resistance mechanisms for Tetracycline

Tetracycline inhibits protein synthesis by attaching to the 30S subunit of the bacterial ribosome and distorting the A site, making it impossible for charged tRNA to bind (Salyers et al., 1990, p. 151). Three distinct bacterial resistance mechanisms have been discovered (see figure 2). They interfere with three elements of tetracycline's antibacterial activity: (1) tetracycline intracellular concentration decrease by efflux pumps, (2) ribosome protection as as an antibiotic target, where the ribosome has been modified so that tetracycline no longer can bind productively, and (3) antibiotic inactivation via modifying enzymes, where tetracycline (T) is being transported into the cytoplasm and altered to an inactive form (t) which then diffuses out of the cell (Salyers et al., 1990, p. 151; Schnappinger & Hillen, 1996, p. 361).

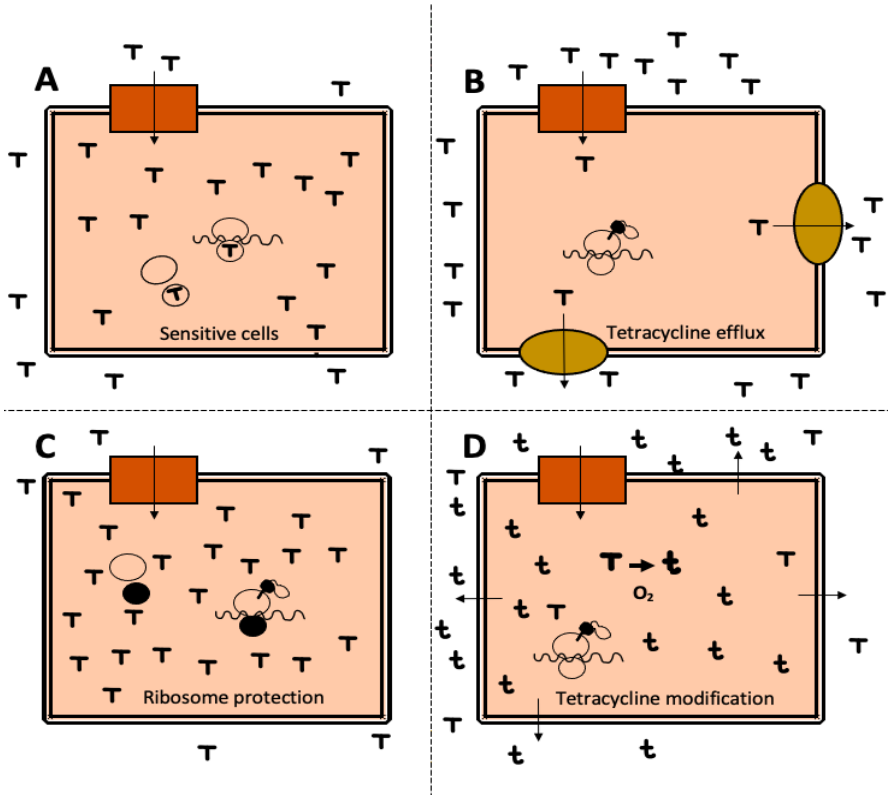

Figure 2: Different mechanisms of tetracycline resistance adapted by Salyers et. Al. (1990, p. 152) (B,C and D).
A: In susceptible bacteria tetracycline (T) accumulates to the point where it binds to ribosomes and stops protein synthesis (Speer et al., 1992, p. 389). B: Bacteria with an efflux resistance gene generate a cytoplasmic membrane protein (yellow oval) that pumps tetracycline out of the cell at the same rate as it is pumped in keeping the intracellular concentration of tetracycline too low for efficient ribosome binding (Salyers et al., 1990, p. 152; Speer et al., 1992, p. 389). C: The cell accumulates tetracycline similarly to the sensitive cell, but the ribosome has been changed so the tetracycline can no longer bind effectively to the ribosome (Salyers et al., 1990, p. 152). D: Bacteria that have a tetracycline modification resistance gene generate an enzyme that chemically converts tetracycline (T) to an inactive form (t) that may readily exit the cell. Oxygen and NADPH are needed for the enzymatic process (Speer et al., 1992, p. 389).

2.4 Wastewater treatment

Wastewater is essentially the community's water supply after it has been used for a number of purposes and has been contaminated with elements that make it unfit for most usage without treatment (Tchobanoglous et al., 2014, p. 3). A variety of unit processes are put together to provide primary, secondary, tertiary, and advanced treatment in order to remove constituents. *Primary* refers to implementations of physical unit processes; *secondary* refers to chemical and biological treatment; and *tertiary* refers to the combination of all three (Tchobanoglous et al., 2014, p. 10,12). A more detailed description of the wastewater treatment stage is shown in table 2-1 below.

Table 2-1: Levels of wastewater treatment. Adapted from Tchobanoglous et al. (2014, p. 13) .

Treatment level	**Description**
Preliminary	Removal of wastewater elements such as rags, sticks, floatables, grit and grease that might create maintenance or operational issues with the treatment operations, processes, and ancillary systems.
Primary	A fraction of the suspended particles and organic debris in the wastewater is removed.
Advanced Primary	Enhanced wastewater removal of suspended solids and organic materials. Chemical addition or filtration are usually used to accomplish this.
Secondary	Suspended solids and biodegradable organic materials (in solution of suspension) are removed. In most cases, disinfection is included in the criteria of traditional secondary therapy.
Secondary with nutrient removal	Biodegradable organics, suspended solids, and nutrients are removed (nitrogen, phosphorous, or both nitrogen and phosphorous).
Tertiary	After secondary treatment, remaining suspended solids are removed using granular medium filters, cloth filters, or microscreens. Disinfection is usually included in tertiary therapy. This term frequently includes nutrient removal.
Advanced	When necessary for certain water reuse applications, removal of dissolved and suspended materials remaining after conventional biological treatment.

2.5 Antibiotics and antibiotic resistance in WWTP

Intensive use of antibiotics in humans, veterinary and agricultural purposes is causing a continuous release into the environment which contributes to the spread of antibiotic resistance. Wastewater treatment plants are one of the main sources of ARGs in the natural environment (Yang et al., 2014). ARB and ARGs are present together with antibiotics present in the wastewater. Genes encoding for resistance have been detected in all stages of wastewater treatment plants (Pazda et al., 2019). A study using high-throughput sequencing-based metagenomic approach was done by Yang et al. (2014) to investigate the broad-spectrum profiles and fate of ARGs in a full-scale treatment plant. They identified 271 ARGs subtypes that belonged to 18 ARGs types using the broad scanning of metagenomic analysis. 78 subtypes of ARGs were persevered through the biological wastewater and sludge treatment process. This study showed that the highest ARGs abundance was found in the influent, followed by the effluent, anaerobic digestion sludge and activated sludge respectively. The influent also had the highest diversity of ARGs and abundance as the influent contained various ARB from municipal wastewater and hospital wastewater. The ARGs found in the influent were the source of ARGs in other samples (effluent, activated sludge (AS) and anaerobic digestion sludge (ADS)). The average abundance was measured in ppm. The influent had an average ARGs subtype abundance at 595,26 ppm. In the effluent the average abundance dropped to 82,62 ppm, whereas in the AS and ADS the average abundance were 29,94 ppm and 47,41 ppm respectively.

It was not just the abundance of ARGs in the influent that were reduced in the effluent, AS and ADS - the diversity of ARGs were also reduced. 263 ARGs subtypes were identified in the influent, whereas 155 in the effluent, 119 in the AS and 102 in the ADS.

By using metagenomic sequencing both Yang et al. (2014) and Pazda et al. (2019) found that >98% of ARGs were removed from the influent to the effluent wastewater after wastewater treatment processes. This suggests that the sewage treatment process is an effective method in reducing ARGs (Yang et al., 2014).

2.6 Methods for enumerating ARB and ARGs

2.6.1 Agar dilution method

To evaluate MIC in bacterial isolates, antibiotic susceptibility testing by agar dilution can be used. The MIC is evaluated as the concentration of the first plate showing no growth of the relevant microorganism. Different concentrations of the antimicrobial agent are added to the media before solidification. Bacteria that are to be tested are grown and diluted into a standardized concentration suspensions which are spread on the agar plates.
The agar dilution method has several advantages, including the ability to test multiple strains at once, quick contamination identification, and the ability to test non-transparent materials (López-Carballo et al., 2012).

2.6.2 Broth dilution method

Broth dilution involves inoculating a specific number of bacterial cells (0.5 McFarland standard, of 1-5 x 10^5 CFU/mL) into a liquid growth medium containing increasing concentrations of the antimicrobial agent (usually a twofold dilution series) (Do et al., 2017, p. 335). Broth dilution can be prepared in two ways, either by macro dilution or micro dilution. The final volume of the test determines whether the approach is referred to as macrodilution which uses a volume of 2 ml, or microdilution which uses ≤500 µl per well (Wiegand et al., 2008).
For both macro and micro dilution, the MIC-value is determined as the first test tube/well not showing growth. Turbidity or cell deposit at the bottom of the well indicates growth. Depending on the microorganism and antimicrobial treatment used, the appearance of growth varies (EUCAST, 2021).
The dilution series for macrodilution is carried out in test tubes, which has the advantage of reduced costs. However, there are a few drawbacks to this approach, including the requirement for a significant number of reagents for each test, the need for a large laboratory area to conduct the tests, the time it takes to prepare the antibiotic solutions for each test, and it can be hard to reproduce (subject to human errors) (Do et al., 2017, p. 335).
Microdilution is a miniature version of macrodilution, performed on a 96 well plate, which allows for various antibiotics to be tested in a range of twofold dilutions. In addition, the microdilution method is less time consuming, makes better use of reagents and space, and the convenience of using prepared plates, which contribute to high reproducibility. Using

prepared plates, on the other hand, might be a disadvantage because standard commercial panels do not include a wide range of antibiotics (Do et al., 2017, pp. 335–336).

For all anaerobic organisms, agar dilution is the recommended reference method (Carpenter et al., 2018). For rapidly growing aerobic bacteria however, broth microdilution is the reference method for antimicrobial susceptibility testing (except for mecillinam and fosfomycin, where agar dilution is the reference method) (EUCAST, 2021).

The broth microdilution method is a more user-friendly method compared to agar dilution, that allows several antimicrobial agents to be tested on one microdilution tray for one isolate (Carpenter et al., 2018).

2.6.3 Kirby Bauer disk diffusion method

In the Kirby Bauer disk diffusion method, a standardized suspension of bacterial cells of approximately 1-2 x10^8 CFU/mL is spread on an agar plate. The surface is covered with filter paper disks with known concentrations of antibiotics. The plates are incubated at a temperature specific to the bacterial species being tested. The antibiotics will diffuse into the media and form a gradient with decreasing concentration going outwards from the filter paper disks. If the microbe shows growth all the way to a certain filter paper patch, the microbe is resistant to this antimicrobial agent. On the other hand, if the microbe is sensitive to the agent, there will be a circular clear area around the filter paper disk where the bacteria were unable to grow, which is called the zone of inhibition (Do et al., 2017, p. 336).

The diameter of the inhibition zone can be correlated to the MIC values from tables. By using breakpoint tables, the diameter of the inhibition zone can interpret susceptibility categorization of the bacteria (S-sensitive, I-intermediate, R-resistant) (EUCAST, 2021).

2.6.4 Agar gradient diffusion method (Etest)

Agar gradient diffusion method is similar to the Kirby Bauer disk diffusion method. Instead of using filter paper disks, a paper strip to which contains a gradient of the antimicrobial agent is laid on the agar plate. The concentration of the agent is halved for each “step” downwards on the strip. These steps are called dilution steps. The strip is placed on the agar disk after applying the microbe to be examined. After incubation an elliptical inhibition zone is formed. In this case the size of the inhibition zone is not of significance to the MIC value. The MIC

value is read where the edge of the growth zone “crosses” the strip (Kahlmeter & Brown, 2010).

2.6.5 Conventional Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) is an enzyme-based method for amplifying target genes or gene fragments using oligonucleotide primers that are complementary to the flanking areas of the target (Luby et al., 2016). Natural DNA replication is mimicked in PCR, by using a mix of chemical reagents and the DNA template. Each cycle is divided into three steps as seen in figure 3: denaturation, annealing and elongation. In denaturation the temperature is raised to separate the two strands of DNA, followed by annealing where the temperature is lowered so the primers can attach to the DNA strands. Finally, elongation, where DNA polymerase binds to the primers and fills in the missing strands. At the end of many cycles, the target sequence of interest is now greatly enriched, and the size of the PCR product can be identified by using agarose gel electrophoresis. Further analysis of the amplified genetic information is also possible, for example the PCR products may be sequenced (Pestana et al., 2010, pp. 10–11). For identifying the presence ARGs of interest in environmental samples, polymerase chain reaction has become a common method (Luby et al., 2016). The reason for this is that PCR is a easy, adaptable, sensitive, specific and repeatable technique (Rodríguez-Làzaro & Hernàndez, 2019).

The PCR method however has some disadvantages. Since PCR is such a sensitive method, even the tiniest amount of DNA contamination in the sample might generate false results. Furthermore, some prior sequence data is required in order to design primers for PCR. As a result, PCR can only be used to determine if a pathogen or gene is present or absent (Garibyan & Avashia, 2013).

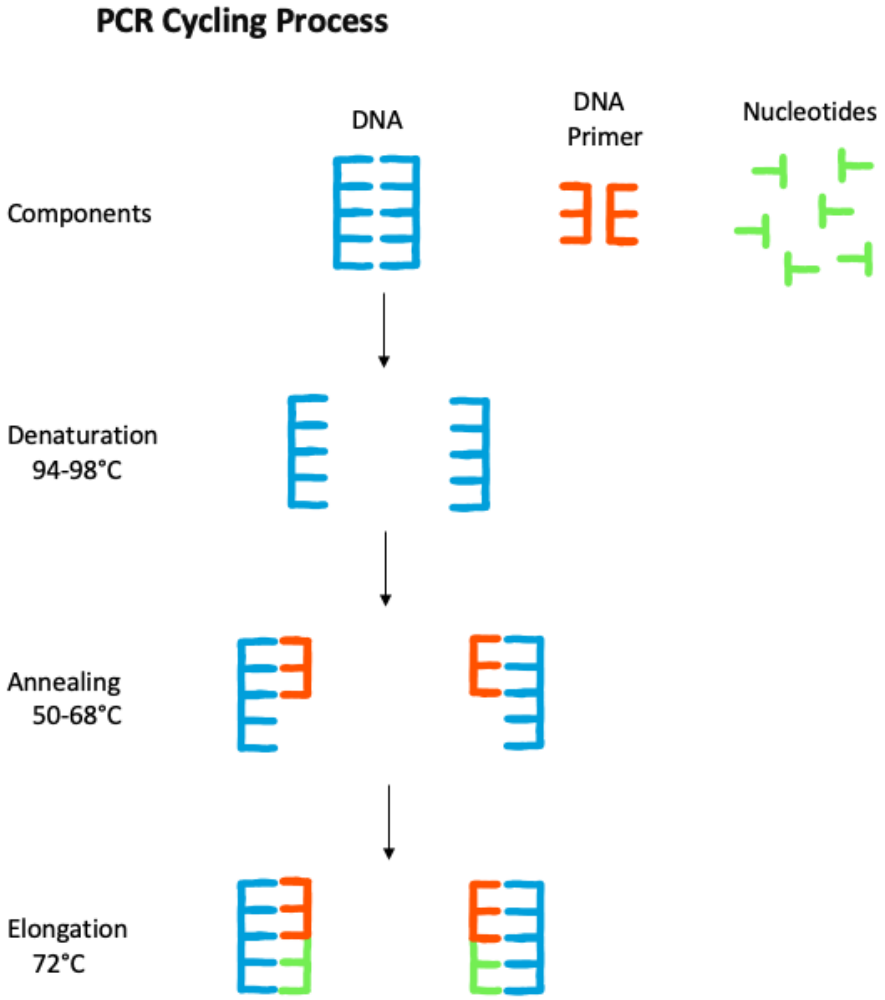

Figure 3: Principles of traditional PCR.

2.6.6 Quantitative PCR (qPCR)

qPCR enables real-time monitoring of amplicon molecule production during PCR. Therefore, data is collected throughout the PCR process to monitor the amplification process in real time, rather than only at the end of the reaction, as in conventional PCR (Rodríguez-Làzaro & Hernàndez, 2019, p. 3). Different fluorescent chemicals, such as SYBR Green I, TaqMan, Molecular Beacons, and Scorpions are used to measure the PCR product accumulation. The experimental design and objectives will determine which chemical is used (Pestana et al., 2010, p. 32). qPCR has the same three thermal steps as conventional PCR, as shown in figure 4, and the cycle is repeated several times. The CT value, or threshold cycle, is the cycle at which the signal crosses the baseline. This CT value may be compared to a standard curve which contains known numbers of the amplicon of interest and then used to estimate the gene copy number of an ARG (Luby et al., 2016).

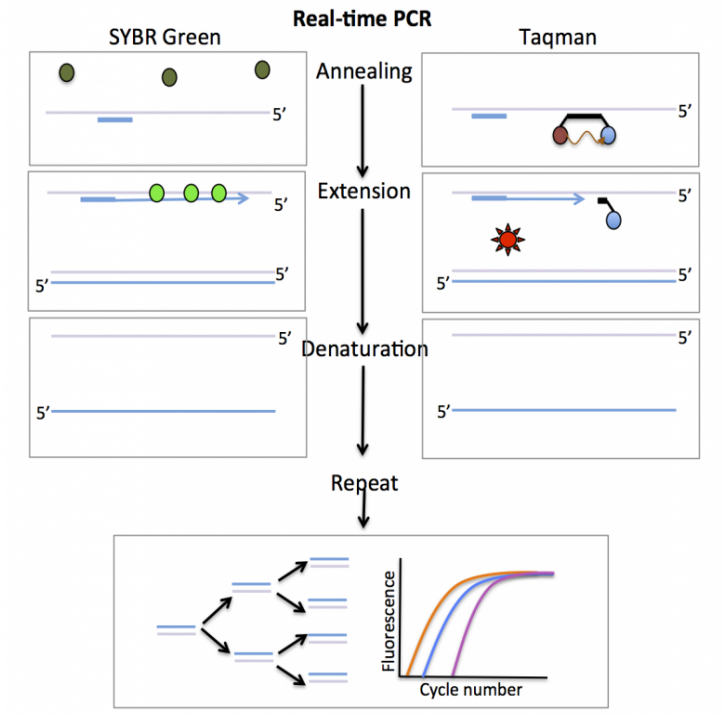

Figure 4: Real-time PCR entails reverse transcription of RNA to cDNA, followed by several rounds of PCR to amplify and detect the genes of interest. SYBR-green or TaqMan probes can be used to detect the products in real time (Huerta & Burke, 2020).

SYBR Green I, which binds nonspecifically to double-stranded DNA (dsDNA) is the most used DNA-binding dye for real-time PCR. When SYBR Green is free in solution, it has very little fluorescence, but when it attaches to dsDNA, its fluorescence increases up to 1,000-fold,

as shown in figure 5. As a result, the total fluorescence signal from a reaction is proportional to the quantity of dsDNA present, and as the target is amplified, the overall fluorescent signal will grow (Bio-Rad Laboratories, 2006).

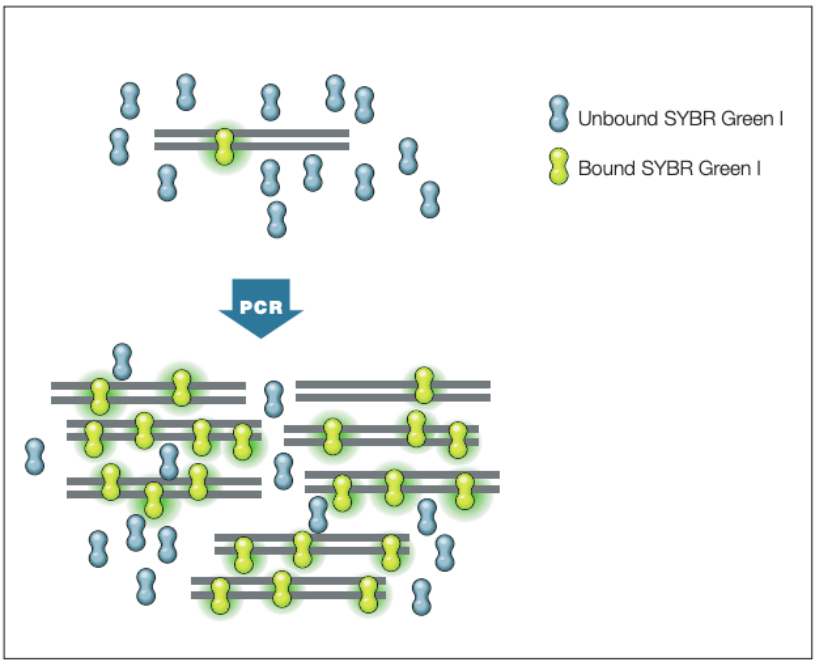

Figure 5: SYBR Green I DNA binding probes in real-time PCR. When the dye binds to dsDNA the fluorescence increases drastically. Gathered from (Bio-Rad Laboratories, 2006).

The closed-tubes format (which eliminates the risk of contamination), the quick and simple analysis, the extremely wide dynamic range of quantification (more than eight orders of magnitude), and significantly higher reliability and sensitivity of the results especially for quantifying nucleic acids and genotyping, are all major advantages of qPCR compared to conventional PCR (Rodríguez-Làzaro & Hernàndez, 2019, p. 4).
Because SYBR green I is a non-specific dye that may bind to any dsDNA, a disadvantage is that it might cause false positive results due to primer-dimer formation or non-specific products. To check for potential primer-dimer or non-specific product formation, the melting curve must be analyzed at the end of the amplification reaction (Pestana et al., 2010, p. 33).
Another significant drawback of both PCR and qPCR is that each study/reaction can only target a small number of genes (Luby et al., 2016).
To avoid the binding to dsDNA, TaqMan probes which are much more specific could be used. By using TaqMan probes it is also not necessary to check the melting curves. However, TaqMan probes are much more expensive than the SYBR green I probes which is why SYBR Green I probes are more commonly used (Tajadini et al., 2014).

2.6.7 Metagenomics methods

Antibiotic resistance genes have been discovered in DNA libraries from environmental samples using metagenomic methods in recent years (Torres-Cortés et al., 2011). Studies employing a metagenomic method directly recover DNA from all micro-organisms in a biological sample, therefore removing the bias that is generated by picking particular species, and allowing for the exploration of the resistome of microbial ecosystems (von Wintersdorff et al., 2017).

The main benefit of these methods is that they do not require a priori genetic target selection, such as specific ARGs and genetic elements, because they do not use PCR. By using next generation sequencing, collective genomes in a given sample can be sequenced in a single step. Antibiotic genes can then be detected and quantified by searching and comparing against online genome databases (Luby et al., 2016).

Recent studies performed by Torres-Cortés et al. (2011) and Yang et al. (2014) have used metagenomic methods for detecting ARGs and MGEs in various environmental samples, such as WWTPs and soil, where the first mentioned were able to identify 11 new ARGs.

One major issue of metagenomics is that existing databases are still limited to few isolates, focusing mostly on clinical ARGs, compromising the quality of ARGs profile comparisons across samples, particularly environmental samples (Luby et al., 2016; Pazda et al., 2019). Searches for undiscovered bioactive compounds may be missed if related genes aren't found in the database. However, the most significant limitation to widespread use of metagenomics techniques for detecting antibiotic resistance in the environment is currently the cost (Luby et al., 2016).

3.0 METHODS

3.1 Study area

The original sludge sample was taken in November 2020 from the WWTP IVAR Merkjavik Sentralrenseanlegg Nord-Jæren (SNJ), which is in Rogaland County, the southwestern part of Norway. The plant is receiving wastewater from more than 300 000 inhabitants daily and is the biggest and most advanced treatment plant in Norway. It is also the first plant in Europe to produce fertilizer from the sludge (IVAR, 2018).

3.2 Setting up reactors

This original sample of sludge was used to inoculate a 10 L aerobic activate sludge reactor in November 2020 and has been maintained since by being fed daily by first wasting 1 L of the sludge and removing 7 L of the supernant after the reactor was allowed to settle for 30 min. The removed supernant was replaced with 1 X artificial wastewater. This allowed the microbial population in the reactor to stabilize before being used for experimental work. 200 ml sludge from this reactor was used to inoculate two different reactors, the control reactor without tetracycline and the experimental reactor containing 1,4 µg/L named "1" and "2" respectively, as shown in figure 6.

The reactors were set up as described below. Fresh E.*faecalis* was grown for 24 hours in 2x100 ml Mueller Hinton broth (+100 µl of 10 mg/ml tetracycline stock solution = 10 µg/ml tetracycline in the 10% *E.faecalis* inoculum) and 100 ml were added to each reactor. E.*faecalis* contains a plasmid with *tet*M, which is a gene causing tetracycline resistance mechanisms. 200 ml sludge was added to each reactor. 200 ml sludge were added to each reactor. A 100-fold strength OECD synthetic sewage, referred to as artificial wastewater stock solution (AF WW stock solution), was prepared according to (OECD, 2010). 700 ml of **1X** AF WW stock solution was prepared as shown in appendix 9.1 (formula 1) and added to each reactor giving the reactors a final volume of 1 L (200 ml + 100 ml + 700 ml).

10 µl of tetracycline stock solution (140 mg/L) was added to reactor 2, prepared as described in appendix 9.1 (formula 2) giving a final concentration of 1,4 µg/L of tetracycline in reactor 2.

Both reactors were stirred and aerating continuously, except for the "feeding time" every weekday.

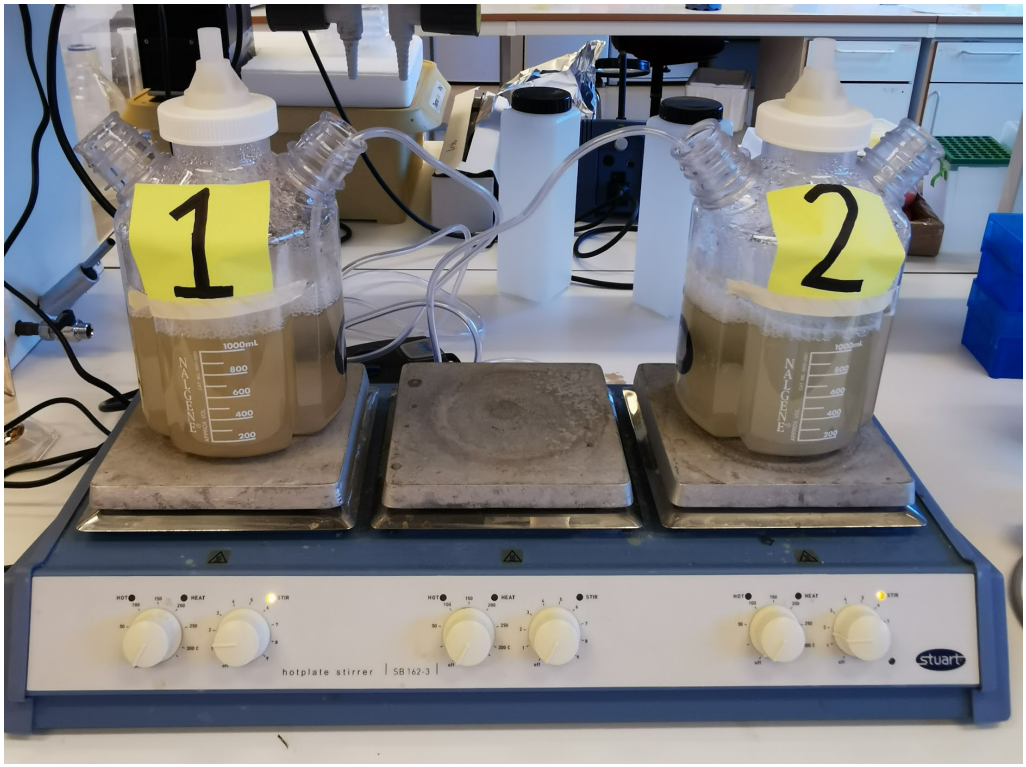

Figure 6: Reactor 1 without tetracycline and reactor 2 containing of 1,4 µg/L tetracycline.

3.3 Reactor maintenance

Both reactors were fed everyday Monday through Friday as described in table 3-1.

Table 3-1: Reactor maintenance.

Monday	Tuesday, Wednesday, and Thursday	Friday
10 am, Turn off air and stirring and let settle for 30 min	09:50 am, waste 100 ml (sludge and wastewater mixed)	09:50 am, waste 100 ml (sludge and wastewater mixed) -
Pump out 800 ml of the liquid phase (i.e., not the sludge)	10 am, turn off air and stirring and let settle for 30 min	10 am, turn off air and stirring and let settle for 30 min
Add 800 ml **1X** AF WW as described in appendix 9.1 (formula 3)	Pump out 700 ml of the liquid phase	Pump out 700 ml of the liquid phase
Add 8 µl Tetracycline stock solution (140 mg/L) to reactor 2 as described in appendix 9.1 (formula 4)	Add 800 ml **1X** AF WW as described in appendix 9.1 (formula 3)	Add **2X** AF WW as described in appendix 9.1 (formula 5)
	Add 8 µl Tetracycline stock solution (140 mg/L) to reactor 2 as described in appendix 9.1 (formula 4)	Add 8 µl Tetracycline stock solution (140 mg/L) to reactor 2 as described in appendix 9.1 (formula 4)

40-50 ml of the 100 ml waste (sludge and wastewater mixed) from each reactor are frozen down in sterile falcon tubes at -20°C every Tuesday and Friday so DNA could be extracted later for qPCR analysis.

3.4 Collecting sample and growing pure colonies

Samples were taken every Tuesday and Friday from the 100 ml waste (sludge and wastewater mixed). 100 µl were used for growing colonies.

The first samples were taken from both reactors immediately after setting up the reactors (Day 0).

The sample was serially diluted in peptone and spread on Mueller Hinton agar plates. The plates were then incubated at room temperature for 72 hours. A sterile inoculation loop was used to pick the isolate colonies from the plates and transfer them one by one to and nutrient broth, and a Mueller Hinton agar plate (see figure 7) to control that the colonies were pure. The tubes and plates were incubated at room temperature for 48-72 hours. The tubes were shaken constantly during incubation to ensure the cultures were well aerated.

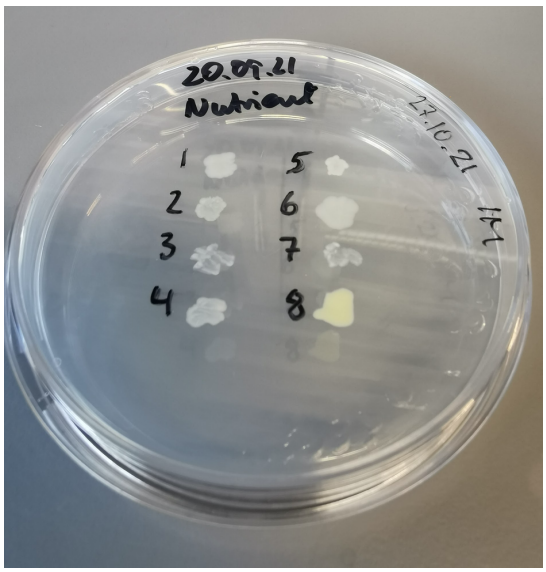

Figure 7: The different colonies from the original sample were spread on a new agar plate to control that the colony spread to broth was pure.

3.5 MIC testing

3.5.1 Preparation of 0.5 McFarland standard

The Mc Farland standard was made according to (Batra, 2018). 1% Barium chloride ($BaCl_2$) solution was prepared by mixing 1g of anhydrous barium chloride into 100 ml distilled water. 1% sulfuric acid (H_2SO_4) solution was also prepared by mixing 1 ml of concentrated sulfuric acid into 100 ml distilled water.

0.5 ml of 1% barium chloride solution and 99.5 ml of 1% sulfuric acid were mixed well and kept stored in a tightly sealed glass flask at room temperature.

3.5.2 Preparation of culture for MIC testing

The wavelength of the spectrophotometer was set to 600 nm, and distilled water was used as "blank". The ABS of the McFarland standard was measured for each time, and was ~0.120, and this was used as the reference for the bacteria cultures. The OD for the bacteria cultures grew in nutrient broth was checked by pipetting 1000 µl into a cuvette. Each culture was diluted in a cuvette with Mueller Hinton bro by trying different amounts of the culture and broth (with a total volume of 1000 µl) until the desirable OD (~0.120) was obtained.

3.5.3 Preparation of microtiter plates with tetracycline stock solution

The microtiter plates were prepared and set up as described in (ISO, 2019). Tetracycline stock solution was prepared with the desired concentration, in this case 5120 mg/l, by weighing 512 mg and adding to a 100 ml volumetric flask and filling with sterile water and mixed well. By using a syringe and sterile syringe filter w/ 0,2 µm Cellulose Acetate Membrane (VWR) the solution was filter-sterilized into sterile falcon tubes. The tetracycline stock solution was stored at -80°C and thawed/refrozen each time it was used.

Tetracycline stock solution were diluted with Mueller Hinton broth as shown in table 3-2.

Table 3-2: Preparation of working dilution of antimicrobial agents for use in broth dilution susceptibility tests. Gathered from (ISO, 2019).

Antimicrobial agent concentration in stock solution	**Volume Stock solution**	**Volume broth**	**Antimicrobial agent concentration obtained**
mg/l	ml	ml	mg/l
5120	1	9	512
512	1	1	256
512	1	3	128
512	1	7	64
64	1	1	32
64	1	3	16
64	1	7	8
8	1	1	4
8	1	3	2
8	1	7	1
1	1	1	0.5
1	1	3	0.25
1	1	7	0.125

100 µl of each tetracycline concentation was transferred to a microtiter plate in increasing order. Clean Mueller Hinton broth was added to two wells for positive and negative controls. The microtiter plates were stored at -80°C until use and were thawed for 2 hours prior to use. In addition, if the plates were not used 4 hours they were discarded for a new freshly thawed plate.

3.5.4 Transfer of bacteria cultures to microtiter plates

Each bacteria culture was diluted with Mueller Hinton broth in Eppendorf tubes to the OD of ~0.120 by using the ratio found by using the spectrophotometer. 10 µl of the bacteria cultures were added to teach well of the series except for the negative control.

The 96 well plates were set up as shown in figure 8 where each of the colored rows (blue, yellow, orange, and pink) represents the dilution series used for each colony tested. Light green and dark green wells are the positive (no tetracycline) and negative control wells (Mueller Hinton broth only). The white wells were unused.

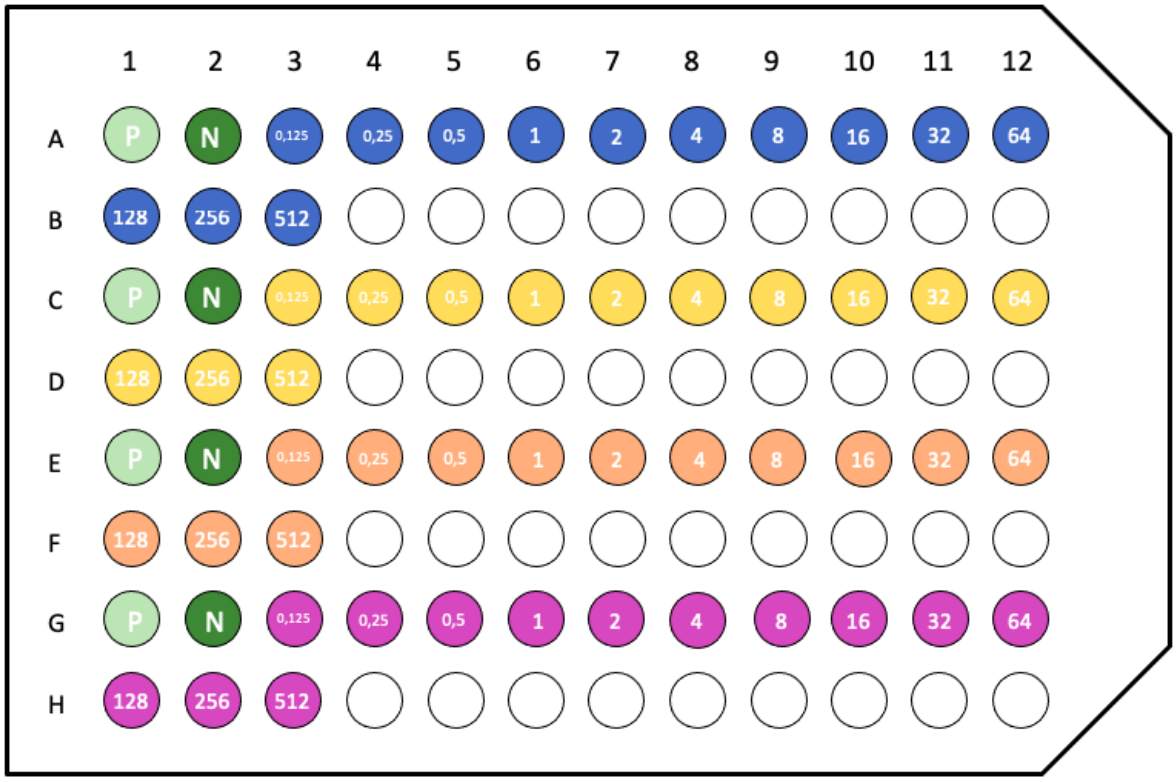

Figure 8: Microtiter plate with positive (P) control, negative (N) control and two-fold increasing concentrations of tetracycline.

3.5.5 Reading of results from microtiter plates

The microtiter plates were interpreted visually, and the MIC-value for the relevant strain was evaluated as soon as there was growth in the positive control well. Wells showing turbidity were scored as positive. The MIC-value was determined using the first well without growth.

3.6 Sample preparations for DNA extraction and qPCR

The frozen samples taken every Tuesday and Friday were thawed. The falcon tubes were centrifuged at 5000 rpm for 20 min, and supernatant removed so only the sludge pellets remained in the tube. 0,3 g of the sludge from each sample were transferred to powerbead tubes.

3.7 DNA extraction

The DNeasy® PowerSoil® isolation kit manufactured by Quiagen was used for DNA extraction. The powerbead tubes were filled with solution C1 (200 µl) from the package. For appropriate mixing, the tubes were inverted and vortexed. The powerbead tubes were vortexed for 60 seconds in a bead beater at 5 m/s. The rest of the DNA extraction carried out in accordance with the manufacturer's instructions with the exception of the final step. In the

final elution step, 40 µl of ddH_2O was used instead of the C6 solution, as this have tended to interfere with other components. The extracted DNA samples were frozen and kept stored at -20°C.

3.8 Verification of DNA templates

The DNA products from the extraction were verified using agarose gel electrophoresis on a 1% gel at 100 volts for 60 minutes. A GelDoc (Bio-Rad) was used for visualization of the DNA products.

3.9 qPCR

qPCR was running using *rpo*B gene to quantify the total number of bacteria and the *tet*M gene. First 10 µM of each primer were prepared by mixing 80 µl ddH_2O, 10 µl of forward primer and 10 µl of backward primer. The primer sequences along with the PCR reaction conditions can be found in table 3-3. A Master mix for each primer were made by mixing 770 µl SsoAdvanced Universal SYBR® Green Supermix (Bio-Rad), 77 µl of the primer mix, and 616 µl molecular grade H_2O.

The reactions were set up in 96 wells plates with a final volume of 20 µl, which consisted of 19 µl Master mix and 1 µl template. 1 µl of the standard DNA were added to the control well for the standard and molecular grade water was used for the negative control. All samples were run in triplicate.

Table 3-3: Tetracycline resistant genes, primers, and thermal conditions.

Gene	Primer	Sequence	Amplicon size	Thermal cycle conditions
*rpo*B	*rpo*B - For	CGAACATCGGTCTGATCAACTC	~366 bp	Pre-denaturation: 10 min at 95°C, 30 cycles: 30 sec at 95 °C, 60 sec at 62°C, 30 sec at 72°C.
	*rpo*B - Rev	GTTGCATGTTCGCACCCAT		
*tet*M	*tet*M - For	ACCTGAGCAATGGGATGTGG	176 bp	Pre-denaturation: 2 min at 95°C, 30 cycles: 30 sec at 95 °C, 20 sec at 60°C.
	*tet*M - Rev	GCTGCTCAATCCCTATGTTGC		

4.0 RESULTS

4.1 Method optimization

Different steps of the method were tested and adjusted before a whole set up could be performed and MIC value determined. From table 9-1 in appendix only isolates from the sample taken 29.10.21 were performed according to the optimized method. The names of the isolates (1-8) are random, and the numbers are not corresponding to the same type of colony.

4.2 MIC values

Results of MIC-values from samples taken over 23 days for Reactor 1 is presented in figure 9, and Reactor 2 presented in figure 10 below. The isolates MIC values were divided into categories low (<0,125 - 2 mg/L), medium (4 - 16 mg/L), and high (32 - >512 mg/L). The results are varying a lot, but the low and medium categories are dominating (except for the sample at day 13) for Reactor 1, with a decreasing percentage of high isolates the last 10 days, but an increasing percentage of medium isolates. At day 6 and 23 no high isolates were detected. The dominating resistance categories in Reactor 2 are also varying, but more stable than Reactor 1, where no high isolates are present at day 0 or 23, but a fair amount are medium and high for the remaining days.
MIC values for Reactor 1 are given in Table 9-2, and table 9-3 for Reactor 2 in appendix.

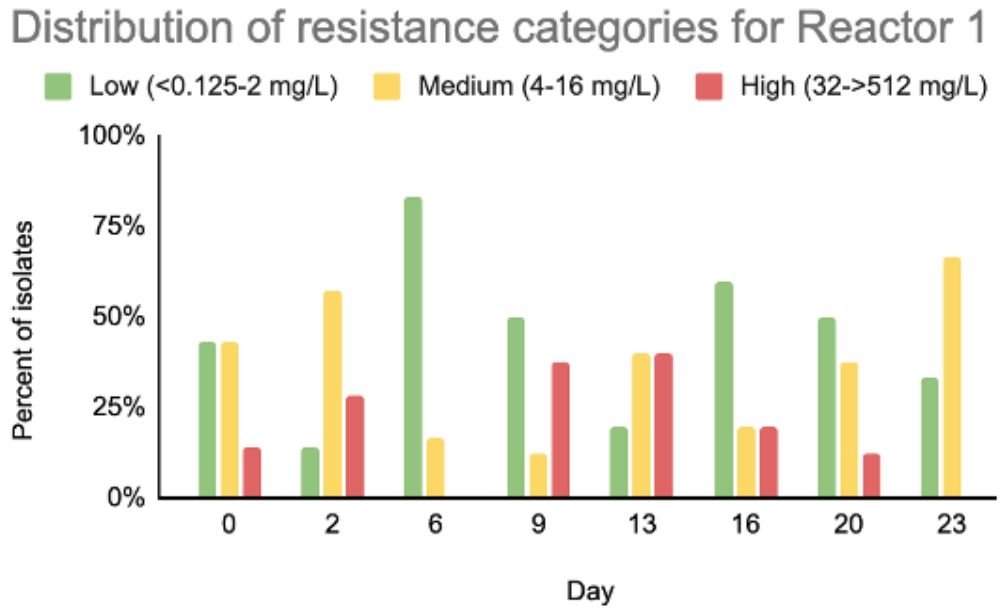

Figure 9: Percent of isolates distribution of resistance categories (low, medium, and high) from each sampling day.

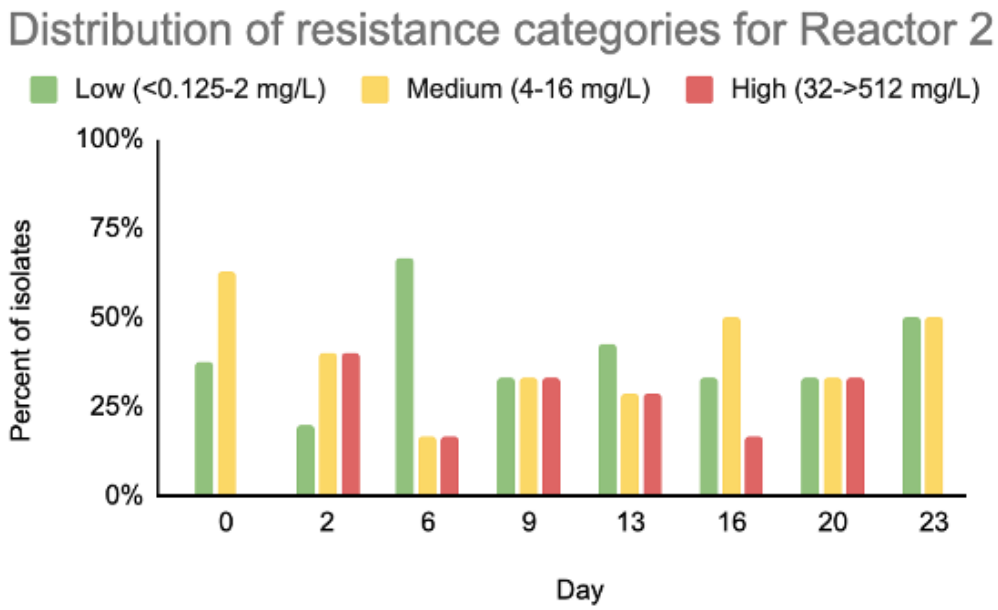

Figure 10: Percent of isolates distribution of resistance categories (low, medium, and high) from each sampling day.

4.3 Verification of DNA products

Figure 11 and 12 shows that the DNA extraction was performed successfully as there are DNA product in all lanes after performing gel electrophoresis.

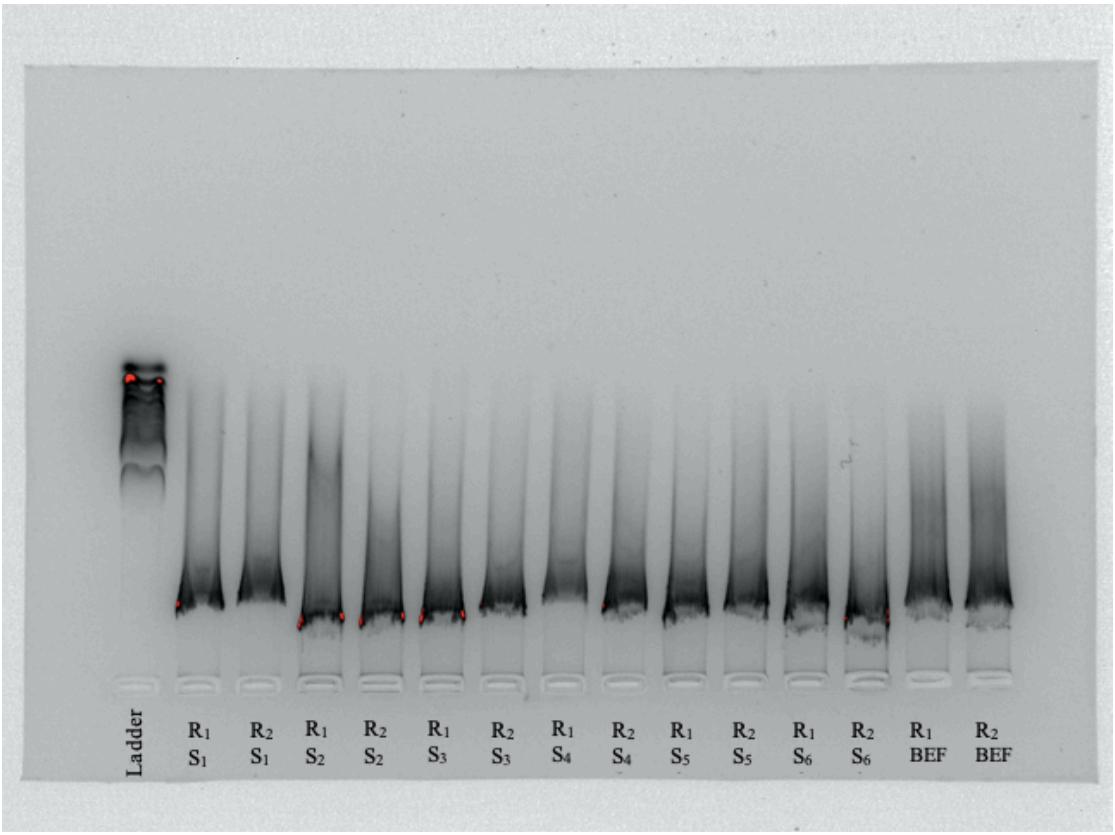

Figure 11: DNA extraction control. All lanes contain DNA product. R_1= Reactor 1, R_2= Reactor 2, S_i= sample I, BEF= before adding *E. faecalis*.

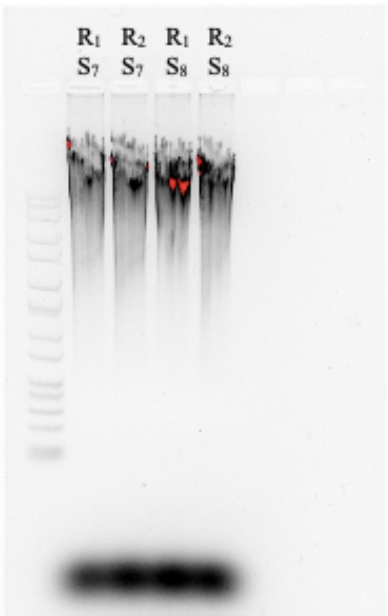

Figure 12: DNA extraction control. All lanes contain DNA product. From left to right: R_1S_7, R_2S_7, R_1S_8, R_2S_8.

4.4 qPCR

The mean Cq value were calculated for the duplicates of each standard, and a standard curve were made for both *tet*M and *rpo*B, see figure 14 and 15 in appendix. Mean value of the Cq values for each sample were calculated, followed by the calculation of the quantity of genes present in each sample, (see formula 6 in appendix). The ratio between *tet*M genes and *rpo*B genes present in each sample is shown in figure 13 below, where *tet*M genes are no longer present in Reactor 1 after 13 days, and all *tet*M genes are gone in Reactor 2 after 20 days.

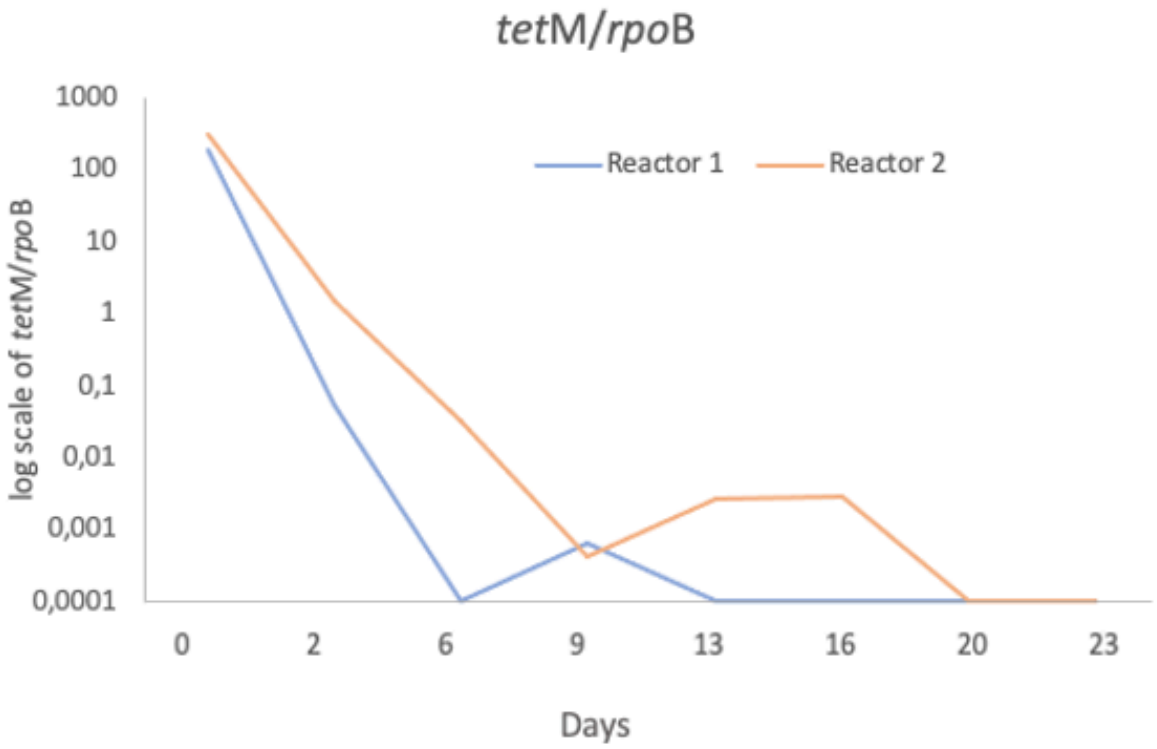

Figure 13: The ratio of *tet*M/*rpo*B in a logarithmic form. 8 samples from each reactor have been taken over 23 days, where the blue line is Reactor 1 and orange line is Reactor 2.

5.0 DISCUSSION

5.1 Method optimization

The method used for analyzing the MIC values was based on ISO 20776-1:2019 (E), but as that method is used for medical isolates several adjustments were required so that the method worked for the reactor samples used in this study.

Samples spread on agar plates were first incubated at 37°C as this was the temperature in the ISO, designed for human pathogens. This resulted in growth of only one type of colony. As the reactor was maintained in room temperature (25°C) it was decided to incubate the agar plates at room temperature where bacteria grew well, although many of them were slow growers. Due to the time it took for the colonies to grow they were not transferred to broth until after 72 hours.

Originally, 48 hours or longer were used to incubate the microtiter plates at room temperature, or the plates were incubated more than 48 hours if there was no growth in the positive control. However, some of the colonies were found to grow after 24 hours incubation time. For these bacteria it was observed that if the bacteria were allowed to grow longer, the number of positive wells increased, which resulted in an overestimation of the MIC values. Therefore, it was decided to read off the MIC values when the positive control well contained growth.

After attempting to grow the bacteria in round bottom and flat bottom microtiter wells, it was decided that the flat-bottomed microtiter plates were easier to read. The samples inoculated in the round bottom microtiter plates tended to look like there was growth when there was not, because the inoculum sunk to the bottom of the wells. Shaking of plates before reading was also used as that made the readings easier. The optimized method was then used to analyze the MIC values from the reactor samples.

5.2 MIC-values

The reactors seem to have relatively similar results for MIC values in all the samples taken from day 0 to day 23. MIC values from day 0 are overestimated as these MIC values was read off as first well with no growth at all. From day 2 of it was determined that it was better to set the MIC value as the first well that had a lower turbidity than the positive control well, even though it still had some growth in it. Therefore, some of the bacteria cultures from day 2 of have a slightly lower MIC value compared to day 0. The rest of the bacteria cultures had very clear distinction between negative and positive wells and the MIC-value was easy to determine for these.

Both reactors had at least one isolate with a MIC-value in the low category for each day sampling. For Reactor 1 the percentage of isolates in the low category varied from 14-83%, but it was mostly between 30-60%. The percentage of isolates in the low category in Reactor 2 were more stable, ranging from 20-67%. The medium category also had a varying percentage of isolates, ranging from 13-67% in Reactor 1, but usually between 13-43%. In Reactor 2 the percentage of isolates in the medium category was between 17-63%, but between 29-63% for all samples except day 6. Percentage of isolates in the high category were between 0-40% for both reactors. The percentages can be misleading as the number of isolates tested were very low (5-8 isolates per sample), and they also varied from sampling day to sampling day, where usually more isolates were tested from Reactor 1 which could affect the distribution of the isolates, where it is a bit random if the “extra” isolates included each time are sensitive or resistance. This could give skewed distribution in the diagram as so few isolates were included in the first place. The number of bacterial isolates chosen on each sampling day was based on colony morphology and many of the colonies looked the same. Samples from day 0 to day 9 showed an overall higher percentage in the high and medium category in Reactor 2 compared to Reactor 1, with an increasing tendency of resistance in Reactor 2 these first nine days. There are no clear trends in Reactor 1 the first nine days. At day 13 Reactor 1 showed a higher percentage of medium and high isolates than Reactor 2. However, there are the same amount of medium and high isolates in Reactor 1 and 2, with two isolates in each category for both reactors. In this sample, Reactor 1 only have one isolate in the low category, whereas Reactor 2 had three isolates in this category. Samples from day 16 and 20 also show a higher resistance tendency in Reactor 2, and the percentage of isolates in the high category in Reactor 1 were clearly starting to decrease between day 16 to 20, whereas the medium category is increasing, indicating that less ARGs that gave high antibiotic resistance were present. The last sampling day, day 23, show a higher resistance in

Reactor 1. Here, the margins were small, with 2 and 3 isolates in the low category, and 4 and 3 isolates in the medium category, for Reactor 1 and 2 respectively. No isolates were present in the high category at sample day 23, which could mean that there were less of the highly resistance bacteria, but there were still some resistance as some isolates were in the medium category.

Lundström et al (2016) looked at MICs of tetracycline in complex aquatic bacterial biofilms, using the Etest to determine MIC values. Six aquaria with different tetracycline concentrations (0, 0.1, 1, 10, 100 and 1000 µg/L) were set up, establishing biofilms. MIC values were determined from each TC exposure, and their results were presented as percent of isolates within three different MIC ranges, grouped as low (0.016 - 2 µg/mL), medium (3 - 24 µg/mL) and high (32 - 256 µg/mL). The distribution of their isolates was very similar in the aquaria with TC concentrations of 0, 0,1 and 1 µg/L, i.e., these concentrations did not influence the resistance of significance. Their isolates' resistance patterns appeared to vary at 10-100 µg/L in the MIC-assay. Fewer isolates showed a low MIC at these tetracycline exposure levels, while an increasing number of isolates showed a medium or high MIC, indicating selectivity for tetracycline resistance when the bacteria were exposed to tetracycline at these concentrations.

By comparing figure 9 (Reactor 1) and figure 10 (Reactor 2), Reactor 2 tended to have an overall higher percentage of isolates in the medium and high tetracycline resistance category, than Reactor 1, which corresponds to the results of the study performed by Lundström et al. (2016). Their sensitivity pattern also did not vary much for tetracycline concentrations under 10 µg/L, in the study by Lundström et al. (2016) and in this study the concentration in Reactor 2 was only 1,4 µg/L. Therefore, a greater impact would probably been seen if the tetracycline concentration were even higher. However, the concentrations used in this study was based on realistic concentrations found in wastewater treatment plants. As the sensitivity did not vary much for the concentrations used in this study, which is a high concentration for tetracycline in WWTPs, it is possible that WWTPs are not as important reservoirs for the spread of antibiotic resistance as previously thought. However, much more work needs to be done to come to any conclusion about this.

Due to time limitations the reactors could not be set up again to compare the results, but in future studies more colonies should be checked for resistance from each sample. In addition, it would be important to sequence the colonies to check which bacteria species that are present, and if the resistance bacteria have clinical significance.

Other antibiotics, such as ampicillin which is one of the most prescribed antibiotics in Europe (ECDC, 2015), and is most likely present in the local WWTP, could be studied to compare the results with the MIC values of tetracycline.

5.3 qPCR

The *rpo*B gene, which codes for the β subunit of the RNA polymerase and is present in all bacteria. Therefore, *rpo*B were used as a reference for the total abundance of bacteria. A plasmid in the *E. faecalis* strain used in this study contains the *tet*M gene, and by adding the *E. faecalis* culture to the reactors, the quantity of *tet*M should increase. The number of *et*M genes could also increase via HGT. By quantifying the *rpo*B and *tet*M genes, the abundance of the genes in the two reactors could be compared.

*tet*M/*rpo*B ratio is higher in Reactor 2 from the beginning at time 0, as seen in figure 13. A way to avoid this in further studies is to prepare one larger culture of the *E. faecalis* and divide equal volumes of the same culture in the 2 reactors, instead of growing it in two different cultures. Regardless of this, the slope for Reactor 1 is steeper than for Reactor 2, which means that *tet*M genes were removed faster in Reactor 1 compared to Reactor 2. Figure 13 is presented in a logarithmic scale as the quantity of genes present had a very wide range of values.

The *tet*M genes were removed from both reactors quickly; for Reactor 1 the *tet*M gene was no longer present in the qPCR results after 13 days, while for Reactor 2 *tet*M was present until the very last sample taken after 23 days where *tet*M was no longer present in the day 23 sample. This indicates a selective pressure for tetracycline resistance in Reactor 2 compared to Reactor 1, and that vertical gene transfer has occurred. Similarly Chen et al. (2019) determined a strong positive correlation between abundance of *tet*M genes and tetracycline concentration.

Even though the *tet*M genes were declining (figure 13), there are still some isolates having high and medium MIC value (figure 9 and 10). This could indicate that another or several *tet*-resistant gene(s) were already present in the reactors in the natural population. If this is the case *E.faecalis* might not be as fit in this environment as the established bacteria.

Another study performed by Zhang et al. (2021) were they looked at changes of antibiotic resistance genes and bacterial communities in advanced biological wastewater treatment system under low selective pressure of tetracycline. They found that in biological wastewater treatment systems, long-term sub-MIC tetracycline exposure was linked to an increase in the relative number and concentration of tetracycline resistance genes. Antibiotics' low-

concentration selection pressure leads to the vertical transfer of ARGs in single bacterial systems and complex aquatic bacterial communities, highlighting the importance of ARGs propagation in wastewater treatment processes.

Among others Pazda et al. (2019) can confirm that wastewater and WWTPs are hotspots for the spread of ARGs via HGT. In my study, all colonies transferred to broth have been frozen. To check if HGT have occurred in my study, qPCR could be performed to see if the colonies have picked up the *tet*M gene.

5.4 Other observations

After just a few days the density of the flocs in Reactor 2 changed. While removing the liquid phase the sludge started mixing, and the suction strength from the peristaltic pump had to be reduced drastically. This did not happen with Reactor 1.

Also, after a few days the sludge started to stick to the edges of Reactor 2, i.e., formation of biofilms. When unicellular organisms get together to create a community that is adhered to a solid surface and enclosed in an exopolysaccharide matrix, a biofilm is produced. Biofilms can be composed of a single bacterial species or several species (Mah & O'Toole, 2001). Biofilms have been found to have higher antibiotic resistance than planktonic cells, and in fact, according to Mah & O'Toole (2001) cells in biofilms can become 10-1000 times more resistant to antimicrobial agents. There are several mechanisms that can cause an increased antibiotic resistance of biofilms. These can be (i) limited diffusion of antimicrobial agents through the biofilm matrix, (ii) communication of the antimicrobial agents with the biofilm matrix (polymer and cells), (ii) enzyme-mediated resistance, (iv) levels of metabolic activity inside the biofilm, (v) genetic adaption, (vi) efflux pumps and (vii) outer membrane structure (Singh et al., 2017).

Antibiotics at lower concentrations, such as chloramphenicol and tetracycline, stimulate the development of multi-drug resistance operons and efflux pumps in the bacterial biofilm (Singh et al., 2017).

6.0 CONCLUSION

MIC values for both reactors were varied, but there was still a tendency towards higher resistance in Reactor 2 where tetracycline was added. qPCR results corresponded to the tendency seen in the MIC results, as the *tet*M gene remained present for 23 days in the reactor containing tetracycline, whereas it only remained for 13 days in the reactor without tetracycline. Biofilm formation was also observed on the reactor walls containing tetracycline, which also strengthen the theory that even a subinhibitory concentration of tetracycline increase the presence of antibiotic resistant genes as biofilms are known to protect against the addition of antibiotics.

Because of time limitation further studies could not be done. However, a new set up of the reactors should be conducted to confirm the results if this study and strengthen the theory that even subinhibitory levels of tetracycline can lead to the selection for resistance.

7.0 CHALLENGES AND IMPROVEMENT

One of the main challenges in preventing antibiotic resistance is that in many countries without standard treatment guidelines, antibiotics are often over-prescribed by health workers and veterinarians and then overused by the public. To meet this challenge individuals can among other things use antibiotics only when it is prescribed by certified health professionals, never share, or use leftover antibiotics, prevent infections by washing hands regularly and have good hygiene, and prepare food hygienically (World Health Organization, 2020).

There are challenges in the methods for detecting ARB and ARGs in agricultural ecosystems. Limitations for PCR and qPCR are the limited availability of primers and false-negative results being caused by an enzyme inhibitor in the environmental samples. The metagenomic analysis can overcome these drawbacks of amplification methods (Yang et al., 2014). Extended genome data from environmental reference bacteria should be applied in data bank entries to make metagenomics (next generation sequencing) even better (Rizzo et al., 2013).

Challenges with the culture dependent methods are the determination of the MIC-value. Sometimes the inhibition zone may have some colonies in it. For the broth dilution method, it can be hard to determine which tube/well has no growth, and the read may vary from person to person.

WWTP has microbial communities that are very complex and most of the microorganisms cannot be cultivated. To further sort the relation between ARGs and bacteria communities and provide more information on the control of ARGs by sewage/sludge treatment process it is necessary to learn more about ARGs profiles as well as microbial community composition (Yang et al., 2014).

In future studies samples should be taken from the biofilm to study both MIC values and for qPCR analysis to determine if the bacteria in the biofilm are more resistant and if *tet*M persists longer in the biofilm.

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9.0 APPENDIX

9.1 Media compositions

Formula 1: Diluting artificial wastewater stock solution when setting up the reactor.

1X (1%) of 100-fold dilution to V_{tot} = 1000ml; 1X=$\frac{1000\ ml}{100\ ml}$= 10 ml

When adding only 700 ml, how much of AF WW stock solution is needed (**1X**)?

1X (700 ml) AF WW = $\frac{10ml*1000ml}{700ml}$ = 14,3 ml of AF WW stock solution

V_{tot} = 700 ml

$V_{tap\ water}$ = 700 ml - 14,3 ml = 685,7 ml tap water

Formula 2: Tetracycline added to reactor 2 when setting up the reactor.

V_1=?

C_1= 140*10^{-3}g/L

V_2=1,0 L

C_2= 1,4*10^{-6}g/L (desirable concentration in reactor 2)

V_1=$\frac{1{,}4*10-6g/L*1{,}0L}{140*10-3g/L}$ = 1*10^{-5}L = 10*10^{-6} = 10 µl

Formula 3: Diluting artificial wastewater stock solution when feeding the reactor Monday-Thursday.

1X (800 ml) AF WW = $\frac{10ml*1000ml}{700ml}$ = 12,5 ml of AF WW stock solution

V_{tot} = 800 ml

$V_{tap\ water}$ = 800 ml - 12,5 ml = 787,5 ml tap water

Formula 4: Tetracycline added to reactor 2 when feeding reactor 2 Monday-Friday with 800 ml AF WW.

V_1=?

C_1= 140*10^{-3}g/L

V_2= 0,8 L

C_2= 1,4*10^{-6}g/L (desirable concentration in reactor 2)

$V_1 = \frac{1{,}4*10-4g/L*0{,}8L}{140*10-3g/L}$= 8µl

Formula 5: Diluting artificial wastewater stock solution when feeding the reactor Fridays (double the concentration).

2X (800 ml) AF WW = $2 * 12{,}5\ ml$ = 25 ml of AF WW stock solution

V_{tot} = 800 ml

$V_{tap\ water}$ = 800 ml - 25 ml = 775 ml tap water

9.2 Additional data for MIC-values

The MIC values from the method optimization is presented in table 9-1. Tables over MIC-values for Reactor 1 in table 9-2 and Reactor 2 in table 9-3.
NA is stated if the MIC value could not be read, if e.g., does not grow in positive well or skipping of wells. The names of the isolates (1-8) are random, and the numbers are not corresponding to the same type of colony.

Table 9-1: MIC-values for method optimization.

Date of sampling	01.10.2021	08.10.2021	15.10.2002	29.10.2021
Isolate				
1	2		0.25	>512
2	8		0.25	<0.125
3	0.5		4	0.25
4	<0.125		<0.125	<0.125
5	64		4	<0.125
6	>512		>512	<0.125
7				<0.125
8				4
E.faecalis		>512		

Table 9-2: MIC-values for Reactor 1.

Day sampling	0	2	6	9	13	16	20	23
Isolate								
1	4	4	4	4	8	64	2	4
2	128	32	<0.125	128	>512	<0,125	2	8
3	<0.125	128	2	32	32	0,5	2	2
4	4	4	2	2	NA	4	4	4
5	<0.125	2	<0.125	2	0.25	0,125	2	0.5
6	4	4	1	2	16		128	8
7	NA	8		<0.125			8	
8	<0.125			128			8	

Table 9-3: MIC-values for Reactor 2.

Day sampling	0	2	6	9	13	16	20	23
Isolate								
1	16	4	4	8	8	8	8	16
2	8	128	>512	>512	128	>512	64	2
3	<0.125	8	2	4	2	2	2	8
4	8	2	2	2	4	4	2	4
5	<0.125	32	<0.125	0.5	NA	4	16	2
6	8		2	128	2	2	64	0.5
7	16				0.5	64		
8	<0.125				32			

9.3 Additional information for qPCR

Formula 6: Calculating the quantity of genes present in each sample:

Quantity = $10^{\frac{C_q - b}{a}}$. See "a" and "b" from the standard curves in figure 14 and 15.

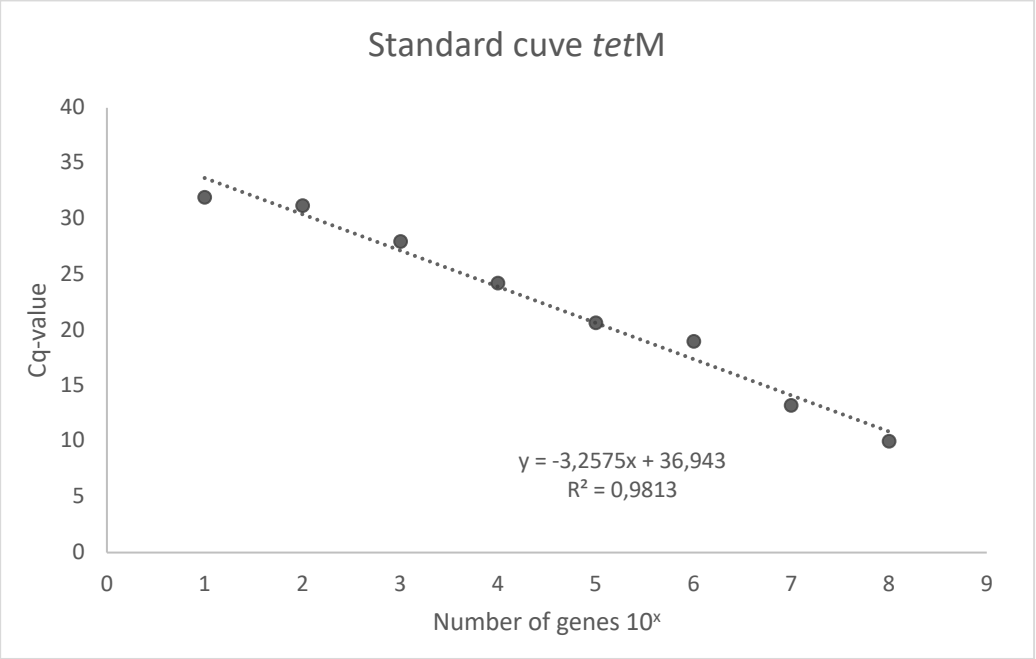

Figure 14: Standard curve for *tet*M. a=-3,2575, b= 36,943, efficiency=102,7%.

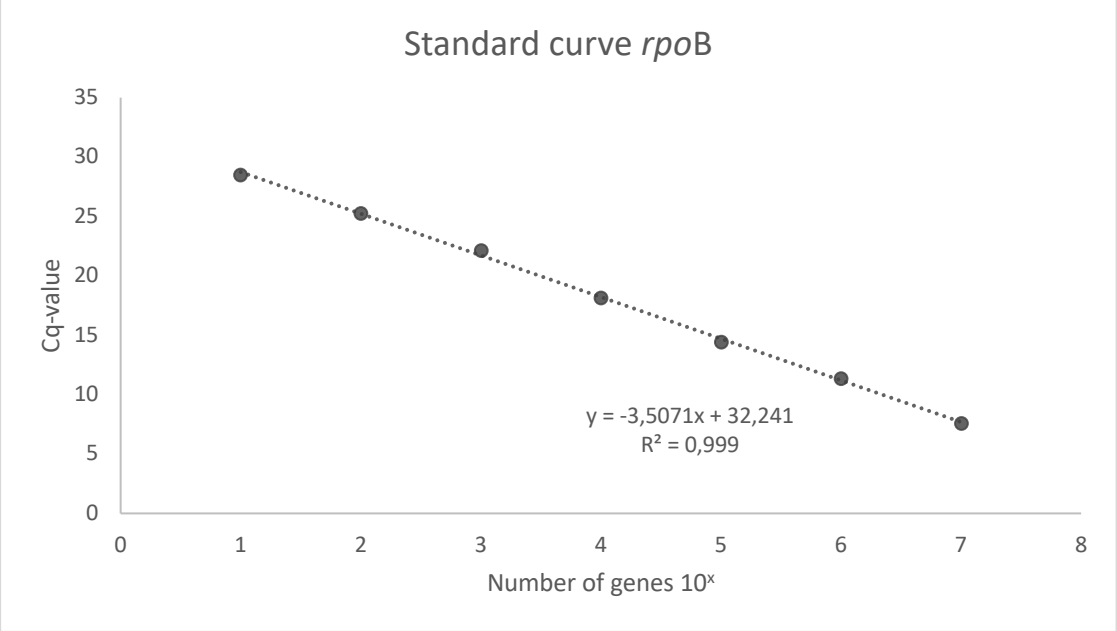

Figure 15: Standard curve for *rpo*B. a=-3,5071, b= 32,241, efficiency=92,7%.