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The effect of benzalkonium chloride exposure on the minimum inhibitory concentration, and the spread of resistance to tetracycline in a laboratory-scale sludge reactor

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Abstract

With the increased use of disinfectants due to the Covid-19 pandemic an increased understanding of their effect on antimicrobial resistance is of paramount importance. This study looks at the effect low concentrations of the disinfectant benzalkonium chloride (BAC) have on the minimum inhibitory concentration (MIC) of antibiotic tetracycline (TEC). A laboratory-scale sludge reactor was used to investigate if there is a correlation between low concentrations of benzalkonium chloride and the minimum inhibitory concentration of both tetracycline and benzalkonium chloride. During two experiments, two aerobic sludge reactors were maintained for 4 weeks, one containing low levels of BAC and the second one functioning as a control reactor. During the first experiment, a concentration of 2,0 mg/L BAC was added to the test reactor. In the second experiment, this concentration got raised to 10 mg/L. The effects of BAC on the MIC and microbial populations were examined. The effect on the microbial community was determined using total suspended solids (TSS) and volatile suspended solids (VSS). Both the MIC values and the results from the TSS and VSS suggest that subinhibitory levels of benzalkonium chloride led to a higher resistance to benzalkonium chloride. The MIC value for tetracycline resistance however indicate that benzalkonium chloride did not affect the resistance of the microbial community. The MIC values for tetracycline seemed to be stable and didn't get influenced through the expose to benzalkonium chloride. These results indicate that overuse of a disinfectant can lead to resistance to these products leading to a decrease in their effectiveness. Despite the fact that in this study disinfectant use did not affect the resistance to tetracycline, more research needs to be conducted to obtain a better understanding of the effect of disinfectants on antibiotic resistance.

Keywords: *Wastewater treatment, Tetracycline, Benzalkonium chloride, MIC, TSS, VSS, Laboratory-scale sludge reactor*

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TABLE OF CONTENT

List of tables

List of figures

List of abbreviations

WWTPs – wastewater treatment plants

MIC – minimum inhibitory concentration

ARB – antibiotic resistant bacteria

MDR – multi drug resistant

QACs – Quaternary ammonium compounds

BAC – benzalkonium chloride

TEC – tetracycline

OD – optical density

CMC – critical micelle concentration

TSS – total suspended solids

VSS – volatile suspended solids

CFU – colony forming units

HGT – horizontal gene transfer

SNJ – Sentralrenseanlegget Nord-Jæren

1.0 INTRODUCTION

The increase in antibiotic resistance in the world today is of growing global concern. Antibiotic resistance is a naturally occurring event where microorganisms such as bacteria, viruses and fungi develop resistance to the drugs designed to kill them (Centers for Disease Control and Prevention, 2021). This process is being accelerated due to the misuse and overuse of antibiotics, therefore, antibiotic resistance is rapidly growing to be a public health crisis (World Health Organization, 2020). In addition to this crisis, another form for resistance is increasing rapidly in all types of microbes. The resistance against disinfectants and biocides has been a silent crisis that has been going on for decades. Only now, during the last few years and during the pandemic of Covid-19, has this underreported crisis received more attention (Mahoney et. al, 2021). Disinfectants are widely used to help prevent the spread of infectious microorganisms. During the Covid-19 pandemic, disinfectant use and availability has increased significantly. This overuse of disinfectants in the world today comes with little considered risk. The overuse is leading to resistance in all type of microorganisms. Remnants of disinfectants are found everywhere disinfectants are used. In most cases, those remnants end up in Wastewater treatment plants (WWTPs), which are hotspots for the development of new multi-drug resistant bacteria.

Large amounts of remnants from antibiotic drug therapy are ending up in wastewater, which makes it a potential hotspot for interactions between microorganisms and antimicrobial agents leading to antibiotic resistant bacteria (ARB). Disinfectants are also extensively used in all type of institutions all over the world to help prevent the spread of harmful microorganisms on all type of surfaces and objects. Hospitals are probably the biggest consumers of disinfectants and antimicrobial solvents and are therefore regarded as being an important source for antimicrobial drug and disinfectant residues which released in wastewater are ending up in wastewater treatment plants (WWTPs). This is leading to a new type of resistant bacteria, multi drug resistant bacteria (MDR). Proper methods for removing antibiotic, disinfectants, ARBs and MDRs from wastewater in WWTPs are therefore crucial to prevent their spread to the environment.

While the misuse of antibiotics clearly has contributed to the emergence of resistance in bacterial pathogens with major health consequences, it still remains less clear if the widespread use of disinfectants such as benzalkonium chlorides have contributed to this

problem (Kim et. al, 2018). In this study, the effect of benzalkonium chloride exposure on the minimum inhibitory concentration, and the spread of resistance to the antibiotic tetracycline was tested.

1.1 Objectives

The main objectives of this study were to look at the effect a disinfectant at subinhibitory concentrations had on the minimum inhibitory concentration (MIC) to the disinfectant in a wastewater treatment plant, and if the MIC for the antibiotic tetracycline (TEC) would be affected as well by the presence of disinfectants.

As Quaternary ammonium compounds (QACs) are frequently found in WWTPs their effect on MIC was investigated, two different concentrations of benzalkonium chloride (BAC) which is a QAC were used to study the effects they had on the MIC for both BAC and TEC. This study also looked at how the exposure to the disinfectant affected both the total suspended solids and the volatile suspended solids in the reactor over time.

2.0 BACKGROUND

2.1 Biocides

Biocide is a general term that is used to describe a variety of chemicals and biological agents that are used to fight against all types of microorganisms, with the function of either inhibiting the growth, inactivating their mode of action or killing them (SCENIHR, 2009). Biocides can be further classified into different categories which include: disinfectants, preservatives, pest control and other biocidal products (Liu et. al., 2017). In this study, the focus was on disinfectants, more specifically Benzalkonium chloride (BAC), and how it can lead to resistance in microbes found in WWTPs.

2.2 Covid-19 and the use of disinfectants during the pandemic

During the Covid-19 pandemic that started early in 2020, a huge number of disinfectants and antibiotics have been utilized in public health sector leading to the misuse and overuse of disinfectants. Several studies have found out that the concentration of disinfectants and antibiotics is increasing rapidly in different environments such as wastewater, surface waters, solids and sediments (Chen et. al., 2021). Traditional WWTPs can only remove between 20-80% of pharmaceuticals, and the remaining pharmaceuticals in the effluent end up in the environment. During the pandemic, huge amounts of pharmaceutical remnants ended up in the environment. Levels of up to 1 mg disinfectants per liter have frequently been found in different environments such as ground waters, wetlands, wastewaters and soils. (Chen et. al., 2021). In comparison, Quaternary ammonium compounds (QACs) such as benzalkonium chlorides (BACs) are extremely toxic to the environment at levels as low as 1 mg disinfectant per liter. (Elersek et. al., 2018)

2.3 Quaternary ammonium compounds

Benzalkonium chlorides (BACs) are a variety of quaternary ammonium compounds (QACs), which are positively charged derivatives of ammonium compounds. They are widely used as antiseptic agents due to their cationic amphiphilic property. The hydrophilic part in BAC is a nucleophilic substitution of alkyl dimethylamine and benzyl chloride. (Microbewiki, 2022). The hydrophobic alkyl residues are paraffinic chains containing everything between 8-18 carbon atoms (Aronson J.K, 2016).

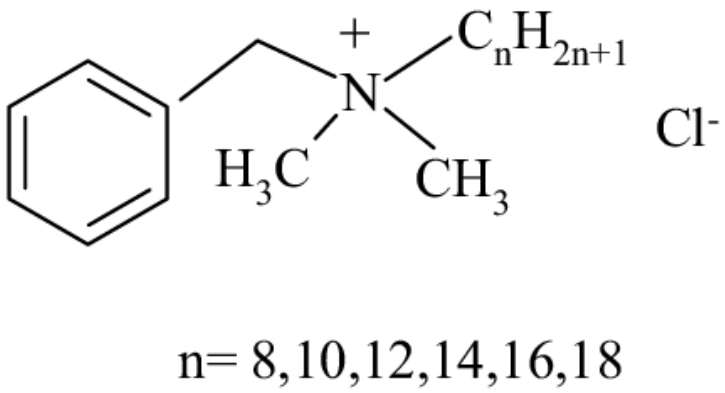

Figure 1. Benzalkonium chloride derivatives. Gathered from: https://www.researchgate.net/figure/Structure-of-Benzalkonium-chloride-C-12-C-14-and-C-16-homologues-are-the-most-common_fig1_314488478

All QACs are classified as surfactants, which are surface-active agents (McDonnell & Russell, 1999). Surfactants are amphiphilic molecules that have both hydrophilic and hydrophobic parts. Due to their amphiphilic nature, they are greatly absorbed at interfaces between two different substances, where each of the different ends of the surfactant molecule aligns in a way so that the different parts are in each its substance, for example water (hydrophilic part) and oil (hydrophobic part) (Laurén, 2018).

A surfactants purpose is to reduce the interfacial tension between two surfaces to stabilize the interface. If the concentration of surfactant molecules in a solution is high enough to reach the critical micelle concentration (CMC), they start to form structures called micelles. In micelles, the molecules of the surfactant are arranged in a special way, usually building a ring-type structure with the hydrophobic tails pointing towards the center, and the hydrophilic heads pointing outwards into the solution (Independent Chemical, 2019). Forming micelles assist the surfactant to inhibit or kill the bacteria at lower disinfectant concentrations than without micelle forming. Therefore, the lower the CMC for a surfactant type disinfectant, the better its ability to inhibit growth of microorganisms.

2.3.1 Quaternary ammonium compounds as disinfectants

QACs as disinfectants are membrane-active agents. That means they target the membranes in living organisms. In bacteria for example, the inner cytoplasmic membrane is the active target site (McDonnell & Russell, 1999). The way in how QACs attack their target was described in a proposal from Salton in the 1960s, with a series of events to study the mechanisms behind how QACs work as disinfectants. This series of events are the following five steps as shown

in figure 2: (i) adsorption and penetration; (ii) reaction with the cytoplasmic membrane followed by membrane disorganization; (iii) leakage of intracellular lower-weight material; (iv) degradation of proteins and nucleic acids; and (v) cell wall lysis caused by autolytic enzymes. (Garcia & Cabo, 2018).

Due to their amphiphilic composition, QACs are effective against most pathogen organisms. Their hydrophobic activity for example, makes them effective against lipid-containing viruses and bacteria, whereas non-lipid viruses and organisms are attacked by their hydrophilic end. Due to their targeting bacterial membranes bacteria, gram negative bacteria are much more resistant and likely to survive treatment of QACs since they have both an outer membrane layer and inner membrane layer. QACs must overcome both layers to neutralize the organism (Mandell, 2010).

QACs may be both bacteriostatic, and bactericidal, depending on their concentrations. At low concentrations (usually between 0,5 – 5,0 mg/l) QACs are bacteriostatic, whereas at higher concentrations (usually between 10 – 50 mg/l) QACs are bactericidal for these same groups. The MIC varies widely among different microorganisms (Gerba, 2015).

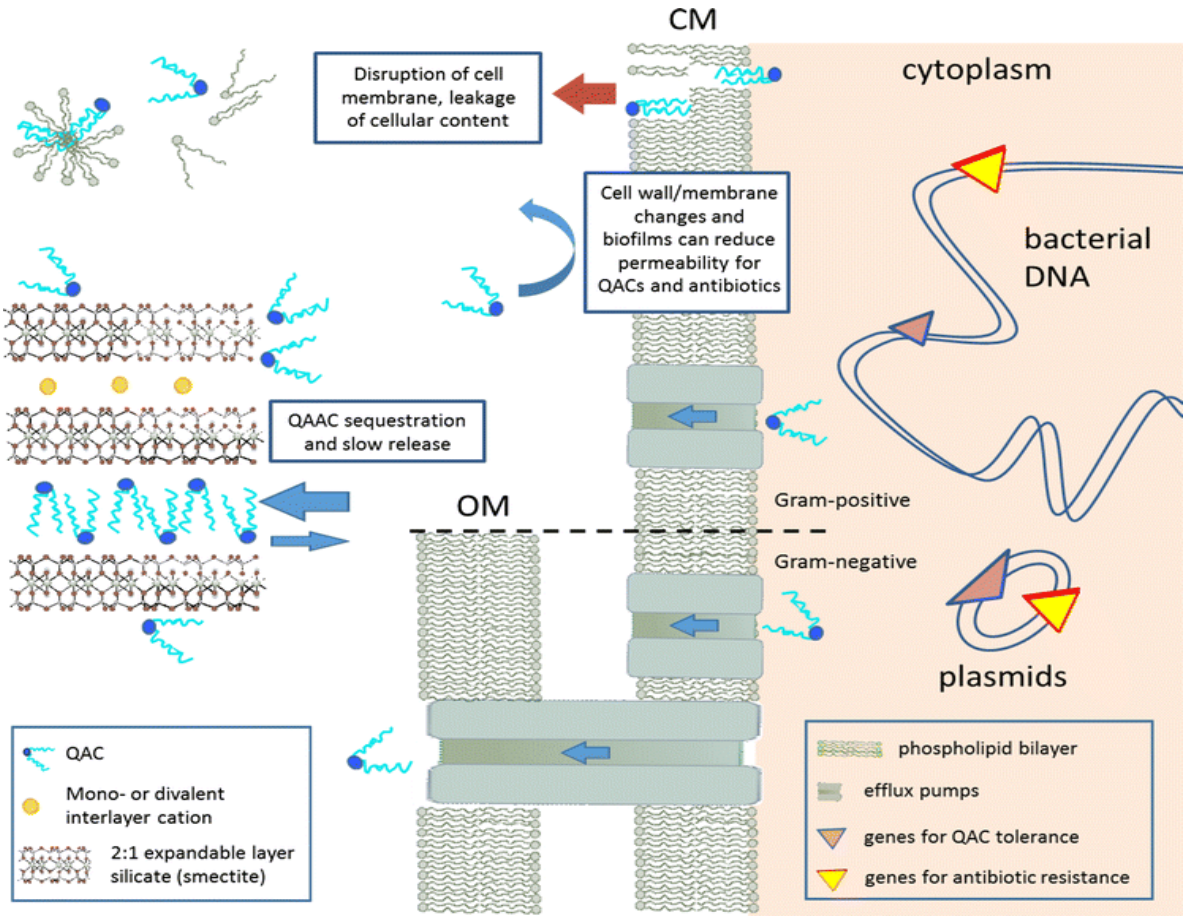

Figure 2. Mode of action of QACs targeting the membranes in bacterial cells, ending up in cell lysis. Gathered from: https://www.researchgate.net/figure/Schematic-view-of-QAC-mode-of-action-and-QAC-tolerance-mechanisms-in-the-presence-of-soil_fig3_321581279

2.4 Disinfectant resistance mechanisms in bacteria

Bacteria are using a lot of different mechanism to resist attacks by disinfectants. The first and most common interaction is producing biofilms. Biofilm producing means that the bacteria attach to a surface and excrete exopolysaccharides to help with attachment resulting in layers of bacteria protecting the bacteria by reducing the concentration of the disinfectant on the surface of the bacteria membranes (Tong et. al, 2021). This effect could clearly be seen during this study in reactor 2 where all the sludge started to bind to the edge of the reactor. See also figure 14. If the disinfectant manages to penetrate the cell wall of the bacteria, and enters the cell, the bacteria trigger an oxidative stress response and produce high levels of reactive oxygen species. Those help the bacteria to withstand the disinfectants for a while by inactivating the bactericidal effect of the disinfectant. The bacteria also react with a general SOS response when in contact with disinfectants to repair damage to the DNA through error-prone DNA-repair. In addition, the efflux system of the bacteria is activated to discharge the

disinfectants from the body of the bacterial cell. Bacteria can also use enzymatic activity to reduce the bactericidal efficiency of the disinfectants (Tong et. al, 2021).

2.4.1 Disinfectants diffusion is limited by biofilm

A biofilm is a group of bacteria as well as possibly other microorganisms that attach to a surface surrounded by extracellular polymeric substances (EPS) that were excreted by the bacteria. Biofilm formation leads to a reduce in the concentration of the disinfectant on the bacterial cell wall and membranes particularly for the bacteria in the inner most part of the biofilm. Biofilm formation happens everywhere bacteria are present, often on surfaces and in new environments for the bacteria. Biofilms act as a barrier and create a stable internal environment for microbial life activities (Tong et. al, 2021)

Some studies have shown that biofilms of *Salmonella spp*. and *Staphylococcus aureus* are more resistant to BAC than planktonic bacteria. The disinfectant resistance of the biofilms is found to be related to the age of the biofilm and the nutrient content in the environment. That means, mature biofilms are more resistant to disinfectants than newly build biofilms (Tong et. al, 2021).

2.4.2 Change in cell surface permeability

If the bacteria decrease the membrane permeability, a lower volume of disinfectant will be able to enter the bacterial cell. Hydrophobic drugs enter the bacterial cell via diffusion directly through the membrane. Outer membrane proteins can prevent them from diffusing through the membrane. Smaller hydrophilic drugs and substances can diffuse through small porins in the membrane and are hard to stop for the bacteria (Delcour, 2009). Studies have found out that gram-negative bacteria show a higher resistance against hydrophilic drugs due to their composition of two cell membrane layers, that make it more difficult for the drugs to enter the cell (Tong et. al. 2021). This outer hydrophobic membrane allows hydrophilic molecules to pass only through its aqueous pores. Many bacteria can change the absolute numbers of pores in function to reduce the diffusion of the antimicrobial agents entering the cell. This mechanism of reduced permeability can also lead to cross-resistance to several families of antibiotics (J C Nguyen Van and L Gutmann, 1994).

2.4.3 Efflux pump systems

Bacterial efflux pumps are proteins that are found imbedded in the plasma membrane of bacterial cells that play a major role in drug resistance in bacteria. The function of the efflux pumps is to recognize toxic agents that have penetrated the protective cell wall or plasma membrane of the bacterial cell and entered the cytoplasm and extrude them before they manage to damage the inner structure of the bacterial cell. The pumps can be both specific for a single substrate or may also handle structurally different compounds (Amaral et. al, 2014). Efflux pumps can be divided into five different membrane protein families which are; resistance-nodulation-division (RND), main promoter superfamily (MFS), multidrug and toxic compound extrusion (MATE), small multidrug resistance family (SMR) and the ATP binding cassette (ABC). All those different efflux pumps are working together, also with other resistance mechanisms in order to resist damage of the disinfectants on the cell. For gram-negative bacteria the RND is the most common efflux pump, while for gram-positive bacteria the MFS plays the most important role. Benzalkonium chloride is mainly extruded by pumps belonging to the families of MFS, SMR and MATE. (Tong et. al. 2021)

2.4.4 Enzyme inactivation

The interaction between bacteria and different biocides leads to the production of enzymes specific for their targeted disinfectant. The difference between the bacterial resistance to disinfectants and antibiotics lies in the production of different enzymes regarding to what kind of substances the bacteria is exposed to. It is not uncommon for bacteria to develop resistance against multiple biocides and antibiotics resulting in bacteria that are called multi-drug resistant bacteria (MDR). There have been studies which have shown that bacteria are capable of deactivating different disinfectants such as hypochlorous acid (HOCl), H_2O_2, QACs and formaldehyde through enzyme catalysis (McDonnell & Russell, 1999). The exposure to QACs may result in increased drug resistance and decreased microbial diversity for some species. That happens because different strains of bacteria in the same species may have variable tolerances against different disinfectants, what means the strains with the highest tolerance will out compete the other strains. (Tong et. al. 2021)

2.4.5 Target alterations

Target alteration is another mechanism bacteria may use to develop resistance against antibiotics and disinfectants. Some bacteria evade antimicrobials by changing and

camouflaging their target sites for the specific antimicrobials. The antimicrobial compounds will therefore not recognize the bacteria and no binding and inhibition can take place (Lambert, 2005).

2.5 Antibiotics

Antibiotics are drugs that are used to fight bacterial infections in people and animals (MedlinePlus, 2022). They can be classified in two major groups, bacteriostatic and bactericidal. The difference between those two groups is in how they work. Bacteriostatic antibiotics only inhibit the growth of the bacteria, whereas bactericidal antibiotics kill the microbe. An important consideration to make when antibiotics are used is that many of them are specific and only work against certain types of bacteria. When using an antibiotic therapy, it is important to know what type of microbe is causing the disease, so the right type of antibiotic can be chosen for the treatment (Stephens E. M. D., 2021). Different types of antibiotics have different ways of killing or inhibiting the microbe. There are five major modes for how antibiotics function, which are: Inhibition of Cell Wall Synthesis, Inhibition of Protein Synthesis, Alteration of Cell Membranes, Inhibition of Nucleic Acid Synthesis and Antimetabolite Activity (Rollins M. David, 2000)

2.5.1 Overview over different antibiotics

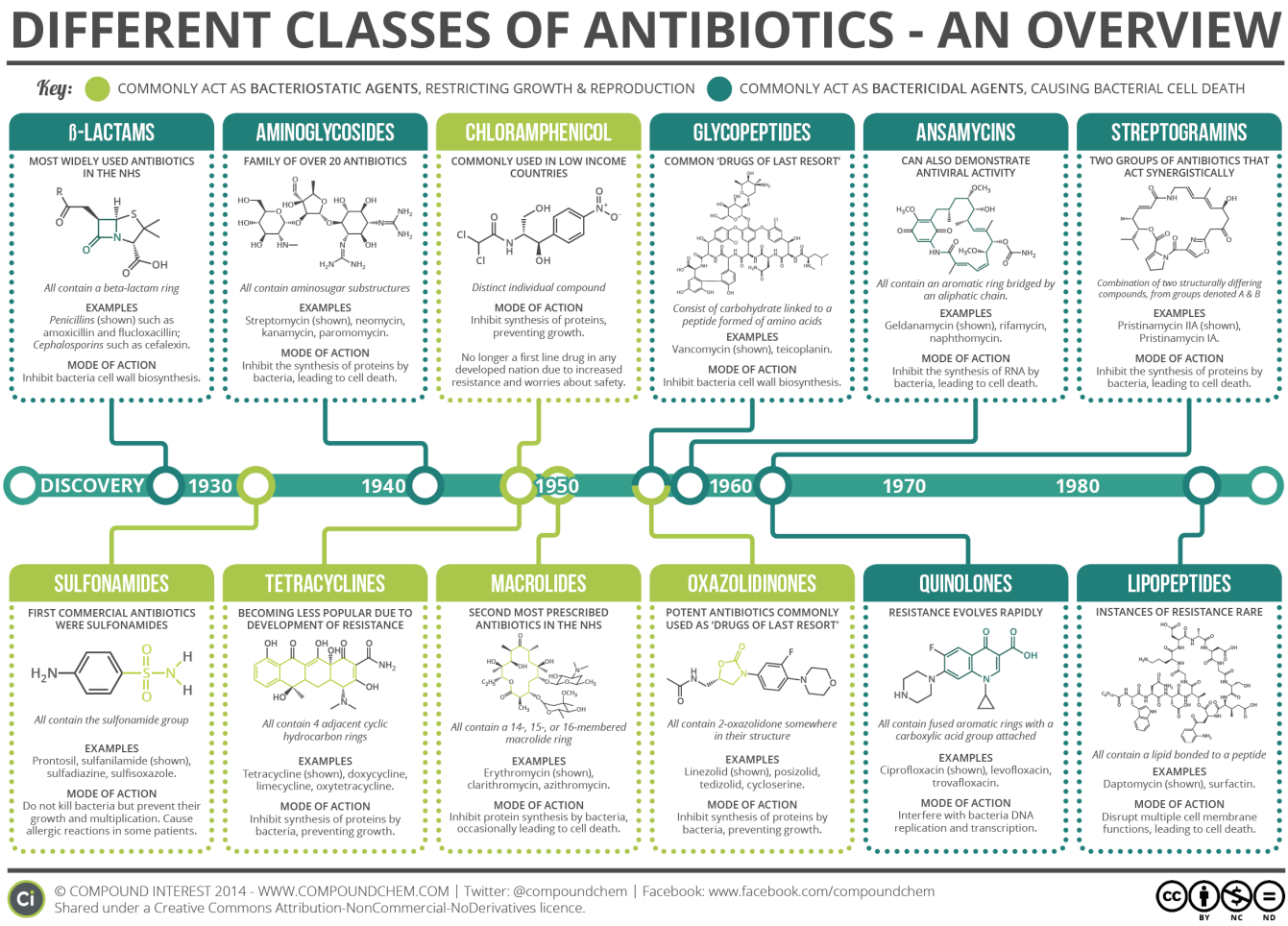

Figure 3: Overview over different classes of antibiotics and their mode of action. Gathered from: http://www.compoundchem.com/wp-content/uploads/2014/09/Major-Classes-of-Antibiotics-Summary-v2.png

2.5.2 Antibiotics and their mode of action

2.5.2.1 Inhibition of Cell Wall Synthesis

Bacterial cells are surrounded by cell walls made of peptidoglycan. Peptidoglycan biosynthesis is essential for the integrity of the cell wall structure, and it is the outermost layer and the main component of the cell wall. Some antibiotics, like Vancomycin, interfere with the biosynthesis of peptidoglycans, thereby destroying the integrity of the cell wall. Since animal cells do not have peptidoglycan in their cell walls, this mode of action has no negative affect on animal host cells. This mode is therefore optimized to destroy only the bacteria invading the host cell (Trevor et. al., 2015)

2.5.2.2 Inhibition of Protein Synthesis

Protein Synthesis is a complex, multi-step process involving many enzymes as well as conformational alignment that is mainly going on in the ribosomes. Those consist of 2 subunits, called the 30S subunit and 50S subunit. Most antibiotics in this category interfere with the processes at the 30S or 50S subunit of the 70S bacterial ribosome. Since antibiotics target different stages of mRNA translation, they can be swapped if resistance develops. (Merck, 2022)

Tetracyclines for example, prevent the binding of aminoacyl-tRNA by blocking the A site of the 30S ribosome. Tetracyclines inhibit the process in the 30S subunit in prokaryotic cells due to structural differences in the 30S subunits in eukaryotic and prokaryotic cells. In addition, tetracycline uses the bacterial cell transport system to its advantage to get inside the cell causing a higher concentration of tetracycline inside the bacterial cell than in the outside environment, and therefore do not harm the animal host cells. (Merck, 2022)

2.5.2.3 Alteration of Cell Membranes

The plasma membrane in both prokaryotic and eukaryotic cells play an important role in the creation of a border between the outside environment and the inside of the cell by constantly maintaining a membrane potential. Some antibiotics like daptomycin, a cyclic lipopeptide, disrupt the plasma membrane, which causes rapid depolarization of the cell, leading to a loss of membrane potential and the inhibition of protein, DNA and RNA synthesis, which automatically leads to cell death. (Földesi, 2021)

2.5.2.4 Inhibition of Nucleic Acid Synthesis

A group of antibiotics, called Quinolones, interfere with the DNA synthesis by inhibiting topoisomerase, most frequently topoisomerase 2 (DNA gyrase), an enzyme that is involved in DNA replication. The purpose of the enzyme topoisomerase 2 is to relax supercoiled DNA molecules and prepare them for replication by DNA and RNA polymerases. Since topoisomerases are present in both prokaryotic and eukaryotic cells, the quinolones therefore specific to only the bacterial topoisomerases do not harm mammalian topoisomerases. Most of the antibiotics that use that mode of action are effective against both gram-negative and gram-positive bacteria (Merck, 2022)

2.5.2.5 Antimetabolite Activity

Some antibiotics use the pathway of interfering with DNA and RNA synthesis by acting as false metabolites, often as purines or pyrimidine nucleotides. They get incorporated into the DNA or RNA, but do not interact in the same way. This leads to inhibition of important steps in the cells metabolite and function of the cell (Britannica, 2017).

2.6 Antimicrobial resistance in bacteria

2.6.1 Horizontal gene transfer

Horizontal gene transfer (HGT) allows bacteria to exchange their genetic materials among diverse species, greatly fostering collaboration among bacterial population in MDR development (Sun et. al, 2019). Multiple mechanisms of HGT liberate genes from normal vertical inheritance. Conjugation by plasmids, transduction by bacteriophages, and natural transformation by extracellular DNA each allow genetic material to jump between strains and species (Lerminiaux & Cameron, 2018). HGT can help antibiotic resistant genes establish at a low frequency in a population, and even in the absence of the antibiotic (Woods et. al, 2020).

2.6.2 Cross-resistance & Co-resistance

Cross-resistance corresponds to resistance to all the antibiotics belonging to the same class due to a single mechanism. Drugs chemical similar to each other, are often assigned to the same class of drugs and thus have the same target of action in the cell, and therefore subject to cross-resistance: bacteria that are resistant to one member of the class are generally also resistant to the other members in the same class. (Périchon et. al, 2019). Cross-resistance can also apply to antibiotics of different classes and with different mechanisms of action. For example, mutations affecting drug efflux can confer drug resistance to multiple classes of antibiotics. That means that exposure to a single antibiotic can lead to resistance or reduced sensitivity in multiple antibiotics and thus multidrug resistance. (Global Antibiotic Research & Development Partnership, 2022)

Co-resistance involves transfer of several genes into the same bacteria or the mutation in different genetic loci affecting different antimicrobials (Cantón & Ruiz-Garbajosa, 2011). There is experimental and observational evidence that exposure to biocides used as disinfectants can induce or select for bacterial adaptations that result in decreased susceptibility to one or more antibiotics (Wales and Davies, 2015).

2.7 Wastewater treatment

Wastewater is all water that has been affected by domestic, industrial and commercial use (Tuser, 2020). Wastewater contains of 99,9% water, but the remaining 0,1% is matter that contaminates the water and needs to be removed from the wastewater in order to release it back into the environment. That 0,1% of the wastewater consists of organic matter, microorganisms, and inorganic compounds. However, the composition of wastewater varies a lot due to different usages and origins.

2.7.1 The different stages in wastewater treatment

There are different stages in wastewater treatment in order to convert the wastewater into water of sufficient quality that it can be released back into the environment. The first stage is where larger elements such as sticks, leaves, rags, cans and bottles and other garbage, also called the “easy pickings”, are removed. This stage is called the pretreatment phase. After pretreatment, the wastewater is pumped into large basins and sedimentation tanks in a phase called primary treatment. There, gravity allows smaller particles to settle out. Also grease and fats are often removed in this stage before the wastewater is sent to the next stage. In the next stage, called secondary treatment, in most cases the wastewater is aerated by plants and microorganisms are added to break down organic matter into sludge. However, in some cases, this process may also be anaerobic depending on the type of secondary treatment used. During breakdown into sludge, carbon, nitrogen and phosphor are removed. (Grosfield, 2018) The final stage of wastewater treatment is often referred to as sludge treatment. The remaining water and biosolids (sludge) are removed by different methods, for example centrifuging or filtering. The last to happen before returning the water to the supply is disinfecting with chlorine, ozone or ultraviolet light. (Bank 2017).

2.7.2 Antimicrobial resistance in WWTPs

Due to the increasing use of antibiotics in the world and the following consequences of antibiotic resistant bacteria, scientists and doctors are now looking for new drugs to help prevent the spread of infectious microbes. The overuse of all type of biocides ending up in WWTPs makes them perfect hotspots for gene transfer and the emergence of multi-drug resistant bacteria (Rodriguez-Molina et. al., 2019). According to several studies, isolates in wastewater have shown resistance against several antibiotics including tetracycline, methicillin and sulphonamide, with tetracycline as one of the most commonly occurring

ARGs in wastewater treatment plants. Some organic compounds such as QACs can increase the selective pressure for ARGs through the mechanisms of cross-resistance and co-resistance (Uluseker et. al., 2021).

2.8 Methods for MIC-testing

The minimum inhibitory concentration of an antimicrobial agent is the lowest possible concentration that inhibits the visible growth of the bacteria tested (Wiegand et. al. 2008). To determine the minimum inhibitory concentrations (MICs) of antimicrobial agents, several different methods can be used, depending on what agent and bacteria are used for testing (Rodriguez-Tudela et. al., 2003). Here at three of the most common methods used for MIC-testing will be discussed, these include the agar dilution method, broth dilution method and agar disk diffusion method. The most appropriate methods to determine MIC-values are dilution methods, as they make it possible to exactly determine the concentration of the antimicrobial agent used for testing (Balouri et. al. 2015).

2.8.1 Agar dilution method

In the agar dilution method, the antimicrobial agent is incorporated with different concentrations into the agar medium, usually with a serial two-fold dilution. Testing the MIC is quite simple by inoculating the desired microorganism on the surface of the agar plates and look for growth on the plates after incubation. The plate containing the lowest concentration of the antimicrobial agent and with no growth of colonies on the surface is the MIC-plate. This method makes is possible to test several strains of colonies on the same plate, making it the preferred method of MIC-testing (Balouri et. al. 2015).

2.8.2 Broth dilution method

This method is one of the most basic methods used for MIC-testing, and the method that was used during this study. There are two possible ways, to perform this method, one known as macrodilution, and the other known as microdilution. The principle is the same, the only difference is the volumes used to determine the MIC.

In this method, bacterial colonies are grown in a liquid growth medium, also called broth. The concentration of these cultures is adjusted to a 0,5 McFarland standard solution to contain a specific number of bacterial cells (usually 1-5 x 10^5 CFU/mL). The diluted bacterial suspensions are then inoculated into a liquid growth media containing the antimicrobial agent

usually in a two-fold dilution series (Balouri et. al. 2015). In the case of microdilution, microtiter plates are prepared and used for inoculating the bacteria suspensions. The plates are containing 96 wells, organized as a field of 8 rows containing 12 wells each. Each of the rows of wells are containing the antimicrobial agent dilution series. The MIC-value is determined by reading the plates by looking at the plates and determining, the first well in each row where growth is not evident. Growth is indicated by turbidity or visible bacterial colonies in the wells (Tenover, 2009).

2.8.3 Agar disk diffusion method

This agar disk diffusion method was developed during the 1940s and is still an official method used in many clinical microbiology laboratories for routine antimicrobial susceptibility testing. In this method, agar plates are inoculated with a standardized inoculum of the microorganism that is tested. Then, small filter papers that are containing different but known concentrations of the antimicrobial agent are placed on the agar surface. The agar plates are then incubated at the temperature suited for the microorganism, and normally the antimicrobial agent diffuses into the agar and inhibits the growth of the tested microorganism. After incubation, the diameter of the inhibited growth zone can be measured and be compared to standardized values. However, this method is not appropriate to measure the MIC as it is impossible to quantify the amount of the antimicrobial agent diffused into the agar medium. (Balouri et. al. 2015).

2.9 Total Suspended Solids & Volatile Suspended Solids

2.9.1 Total suspended solids

Total Suspended solids (TSS) are defined as solids in water that can be trapped by a glass-fiber filter. TSS can be all kind of particles that are found in the aquatic system, both inorganic particles such as minerals, salts and metals, as well as organic particles such as algae, silt and bacteria. The TSS data is critical in determining the operational behavior of a wastewater treatment system.

To test the total suspended solids in a solution, a sample with an exact volume is well mixed and filtered through a dried and weighed standard glass-fiber filter. The residue left on the filter is then dried in an oven at a temperature between 103°C and 105°C for around 1 hour, or until the weight of the filter is constant. The increased in the weight of the filter is calculated as the total suspended solids of the sample (Environmental Business Specialists, 2022).

2.9.2 Volatile Suspended Solids

After determination of the TSS, another analysis can be performed, called volatile suspended solids (VSS). The VSS data is critical for determining the biological concentration throughout the system. In this method, the filter from the TSS analysis is used. The filter is ignited in a special oven for VSS at a temperature of 550°C for 30 minutes. The VSS data can be read as the weight loss of the filter during the heating phase (Environmental Business Specialists, 2022).

Volatile solids are substances that easily can transform from their solid phase into their vapor phase, without going through their liquid phase. Most organic matter is considered volatile solids, such as bacteria. VSS is a method normally applied to sludge to measure and to achieve balance between the food amounts present in or entering the water, and the bacteria amounts available to eat that food. The ratio between TSS/VSS is used to determine how much of the TSS is organic matter. (Theobald, 2014)

Both the TSS and VSS data are measured and expressed in milligrams per liter (mg/L).

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 (Malmberg Ingrid, 2021). 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

For the first run, the reactors were set up at the same time in the morning of Wednesday 16th of February 2022, using 250 ml of maintained sludge, that originated from the original sludge sample taken in November 2020, in each reactor. A 100-fold strength OECD synthetic sewage, referred to as synthetic wastewater was so prepared according to (OECD, 2010). This 100**X** stock solution was used to prepare 750 ml of 1**X** synthetic wastewater as shown in formula 1, and got added to each of the reactors. The final volume in each reactor was 1L. The second run was started on Friday 1st of April 2022, with the same set up as in the first run.

Reactor 1 was used as control reactor, to make sure that all the procedures and solutions added to the reactors worked properly. To reactor 2 an additional volume of 7,81 µl of BAC stock solution (256 g/L) was added to the reactor in the first run as shown in formula 4. The addition of 7,81 µl gave a final concentration of ≈ 2 mg/L of BAC in reactor 2 during the first run. In the second run, the BAC concentration in reactor 2 was increased fivefold compared to the first run. 39,1 µl of BAC stock solution was added during the second run when setting up the reactor, giving a BAC concentration of ≈ 10 mg/l in reactor 2 as shown in formula 5. During the feeding days in the first run, a volume of 6,25 µl BAC got added to reactor 2 according to formula 6 in appendix section 9.1. During the second run, a volume of 31,4 µl BAC got added according to formula 7 in appendix 9.1. Both reactors were stirred and aerated continuously, except for the feeding time (≈ 30 minutes) every weekday.

3.3 Maintenance of reactors

Both reactors were fed everyday Monday through Friday following the same schedule as described in table 3-1. Table 3-1 however follows the exact procedures for the first run. For the second run, a higher volume of BAC-stock solution got added to reactor 2 to obtain a

higher concentration of BAC. During the second run, 31,4 µl of BAC-stock solution got added instead of 6,25 µl, giving the higher concentration of ≈ 10,0 mg/l BAC.

Table 3-1. Maintenance of reactors.

Monday	Tuesday, Wednesday, and Thursday	Friday
07:00 AM, Turn off air and stirring and let settle for 30 min	06:50 AM, waste 100 ml (sludge and wastewater mixed)	06:50 AM, waste 100 ml (sludge and wastewater mixed)
Pump out 800 ml of the liquid phase (i.e., not the sludge)	07:00 AM, turn off air and stirring and let settle for 30 min	07:00 AM, turn off air and stirring and let settle for 30 min
Add 800 ml **1X** S WW as described in appendix 9.1 (formula 2)	Pump out 700 ml of the liquid phase	Pump out 700 ml of the liquid phase
Add 6,25 µl BAC stock solution (256 g/L) to reactor 2 as described in appendix 9.1 (formula 6)	Add 800 ml **1X** S WW as described in appendix 9.1 (formula 2)	Add **2X** S WW as described in appendix 9.1 (formula 3)
	Add 6,25 µl BAC stock solution (256 g/L) to reactor 2 as described in appendix 9.1 (formula 6)	Add 6,25 µl BAC stock solution (256 g/L) to reactor 2 as described in appendix 9.1 (formula 6)

3.4 Collecting samples and growing pure colonies

Samples were taken every Monday, Wednesday and Friday from both reactors. For the samples, 100 µl (sludge and wastewater mixed) was pipetted out from each reactor around 10 minutes after the feeding procedure. The feeding procedure helped to stabilize the sludge in the reactors before collecting the samples. The samples were serially diluted in Eppendorf tubes with Mueller Hinton broth and spread on Mueller Hinton agar plates, with the dilution

series ranging from 10^{-3} to 10^{-6}, to ensure the growth of isolate colonies were obtained from both reactors. The plates were incubated at room temperature for 48 to 72 hours. After incubation, the best dilution agar plate was used to pick 8 different colonies and transfer them to reagent tubes with Mueller Hinton broth. Sterile pipette-tips were used to pick one and one colony on a new Mueller Hinton agar plate to control that the colonies were pure.
The tubes and new agar plates were again incubated at room temperature, this time for around 24 hours. During incubation, the tubes were constantly shaken to ensure that the cultures were well aerated.

3.5 MIC testing

3.5.1 Preparation of 0.5 McFarland standard

The McFarland standard was made according to (Wiegand, 2008). First, 1% Barium chloride ($BaCl_2$) solution was prepared by mixing 1 g of anhydrous barium chloride into 100 ml distilled water. Then, 1% sulfuric acid (H_2SO_4) solution was prepared by mixing 1 ml of concentrated sulfuric acid into 99 ml distilled water.
To get a 0.5 McFarland standard solution, 0.5 ml of the 1% barium chloride solution and 99.5 ml of the 1% sulfuric acid solution were mixed together. The McFarland standard was stored in a tightly sealed, foil-covered glass flask at room temperature.

3.5.2 Preparation of cultures for MIC testing

After 24 hours of incubation, the reagent tubes with the pure colonies in nutrient broth got diluted till they matched the turbidity of the 0.5 McFarland standard, to ensure the nutrient broth contained the right amount of CFU/ml. A spectrophotometer was set to a wavelength of 600 nm, and distilled water was used as a blank to reset the spectrophotometer. The absorbance of the 0.5 McFarland standard was measured to ≈ 0,096. This functioned as the reference value for the bacteria cultures. To check the value of the bacteria cultures, 600 µl of the bacteria culture was pipetted into a microcuvette. The bacteria cultures had to be diluted considerably to obtain an OD value that match the reference value. This was done by diluting the culture suspensions with Mueller-Hinton broth until the absorbance value of the bacteria culture was approximate the same as the reference value ≈ 0,096.

3.5.3 Preparing microtiter plates

Two different types of microtiter plates were prepared as described in (ISO, 2019). One batch of plates containing benzalkonium chloride (BAC), and the other batch of plates containing tetracycline (TEC). The concentrations of the antimicrobial solutions in the valves of the plates ranged from 0.5 mg/l to 256 mg/l. The step of making a stock solution was omitted, instead the highest concentration solution used to make the dilutions for the microtiter plates (256 mg/l) . The 256 mg/l solution got so was serially giving a total of the 10 different concentrations shown in table 3-2.

The microtiter plates consisted of rows with 12 wells each, where two of the wells were used as positive and negative controls only containing clean Mueller Hinton broth. The remaining 10 wells were used for the dilution series of the antibacterial solutions with concentrations ranging from 0,5 mg/l to 256 mg/l. A total volume of 50 µl was added to each well. The prepared batches of microtiter plates were stored at -80°C until use. The plates were than thawed for 2 hours prior to use.

Table 3-2. Preparation of dilution series of antimicrobial agents for use in broth dilution MIC-testing. Inspired by (ISO, 2019)

Antimicrobial agent concentration in stock solution	**Volume Stock solution**	**Volume broth**	**Antimicrobial agent concentration obtained**
mg/L	ml	ml	mg/L
256	11	0	256
256	5,5	5,5	128
128	5,5	5,5	64
64	5,5	5,5	32
32	5,5	5,5	16
16	5,5	5,5	8
8	5,5	5,5	4
4	5,5	5,5	2
2	5,5	5,5	1
1	5,5	5,5	0,5

3.5.4 Transfer of bacteria cultures to microtiter plates

Each pure grown bacteria culture got diluted to the OD of the 0.5 McFarland standard that was around 0,096. 50 µl of the bacteria cultures were added to each of the wells, except for the negative control. Due to adding the 50 µl of bacteria culture to each well, the final concentration in the wells were halved, now giving a final concentration ranging from 0,25 mg/l to 128 mg/l. The microtiter plates were set up as shown in figure XX. Each row (A-H) was used for one bacteria colony, giving space for a total of eight colonies in each plate. The first and the second column were used as positive (only containing Mueller Hinton broth and bacteria culture) and negative control wells (only containing Mueller Hinton broth). The remaining 10 columns contained the antibacterial solutions in increasing concentrations from left to right.

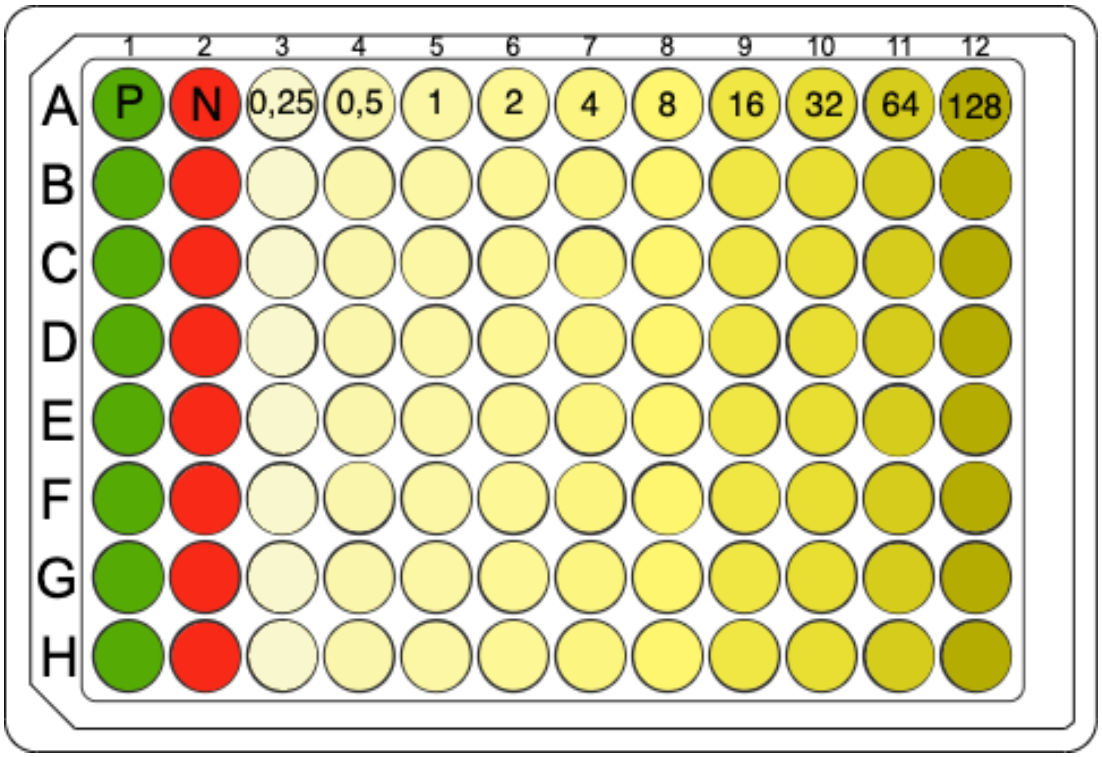

Figure 4. Microtiter plate with positive control (P), negative control (N), and two-fold increasing concentrations of either benzalkonium chloride or tetracycline measured in mg/L.

3.5.5 Reading of results from microtiter plates

The microtiter plates were incubated at room temperature and shaken continuously to ensure the bacteria got aerated during their growth period. The results from the plates were read after around 18 - 24 hours of incubation, by looking at the wells from underneath the plate. Turbid

wells showing growth and were read as positive. The MIC-value was determined by using the first well without any growth. The MIC-value for both BAC and TEC of a total of eight different bacteria colonies got read three times every week.

3.6 TSS and VSS testing

3.6.1 Preparing the filters

For the TSS and VSS, glass fiber membranes filters with a pore size of 1,5 µm were used. The filters had to be washed and dried prior to use to prevent weight differences between the weighing phases due to lose particles that could have affected the weight. To wash the filters, deionized water was filtered through the filter several times under vacuum. After washing, the filters were dried overnight in an oven at a temperature of 105°C, and then placed into a desiccator for storage until use to prevent the filters from absorbing water from humidity.

3.6.2 Filtering the samples

Each day bacteria samples were taken from the reactors, a certain volume of 25 ml of the water and sludge mixture was pipetted out from each reactor while the reactors were still stirred and aerated. The samples were pipetted into clean falcon tubes, one containing the sample reactor 1, and the other containing the sample from reactor 2. A washed and dried filter was prepared for filtration by weighing the weight of the filter prior to filtration of the sample. Then, the filter got put on the filtration setup and the sample with the known volume of 25 ml got filtered through the filter. To ensure that the entire sample with all its particles got filtered, both the falcon tube containing the sample and the filtration setup got cleaned with deionized water that also was filtered through the filter after the sample.

3.6.3 Drying the samples

After filtration, the filters were dried at a temperature of 105°C for around 60 minutes. After drying, the filters were again put into the desiccator for cooling down to room temperature before weighing again.

3.6.4 Volatile suspended solids

For the analysis of VSS, the dried filters from the TSS were used. The filters were put into a special oven made for VSS that can withstand extremely high temperatures. The filters were heat up to a temperature of 550°C for around 30 minutes, before the oven started to cool

down. Due to the time it took the oven to reach a lower temperature, the samples were not weighed before the next day. Before weighing, the samples were again put into the desiccator for around half an hour to ensure the filters were completely dry before weighing. After weighing, the filters were thrown away into the normal trash.

4.0 RESULTS

4.1 Method optimization

In order to determine the MIC, the method had to be tried and optimized before the real test run could be carried out. The first run was used to determine the MIC for BAC and TEC, in order to find out what concentration of BAC needed to be used in order to influence the MIC values over time.

4.2 MIC values

During the first run, a total of 12 samples were taken over a time period of 25 days from both reactors and tested for MIC-values for both BAC and TEC. Both reactors were run in room temperature (22°C). A concentration of 2,0 mg/L benzalkonium chloride was added to reactor 2. Reactor 1 functioned as control reactor and did not contain benzalkonium chloride. Each sampling day, a total of 8 colonies were tested for their MIC value. Each bacteria colony is represented as a bar in the graph. The MIC values were measured using 96 well microtiter plates. The results for the first run can be seen in figure 5 to figure 8.

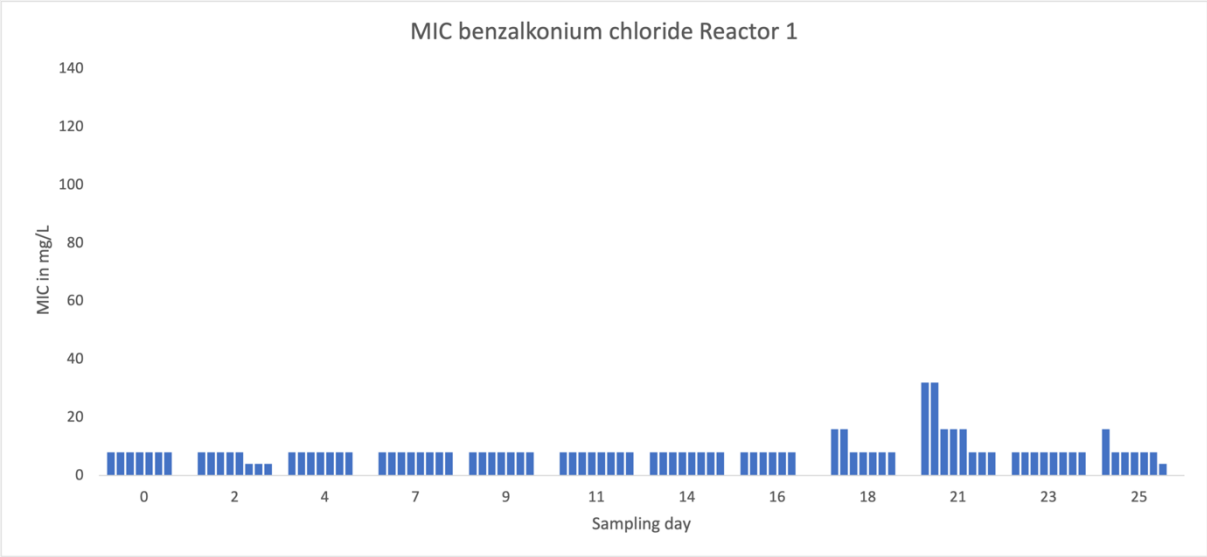

Figure 5. MIC values for BAC in the control reactor (reactor 1) during run 1. The reactor was run in room temperature, no BAC was added.

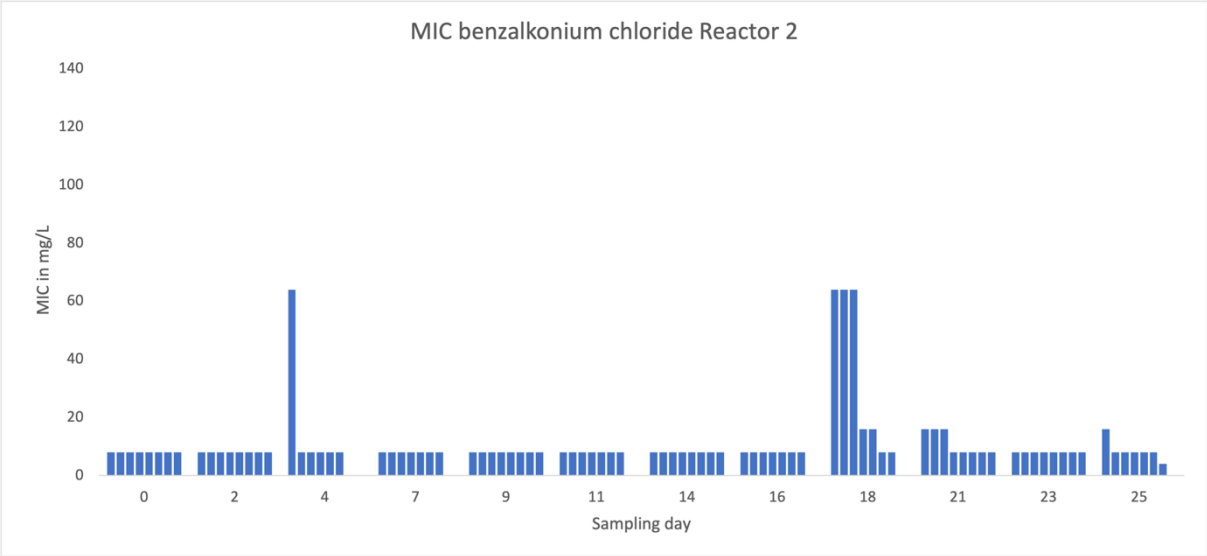

Figure 6. MIC values for BAC in the test reactor (reactor 2) during run 1. The reactor was run in room temperature with a concentration of 2,0 mg/L benzalkonium chloride.

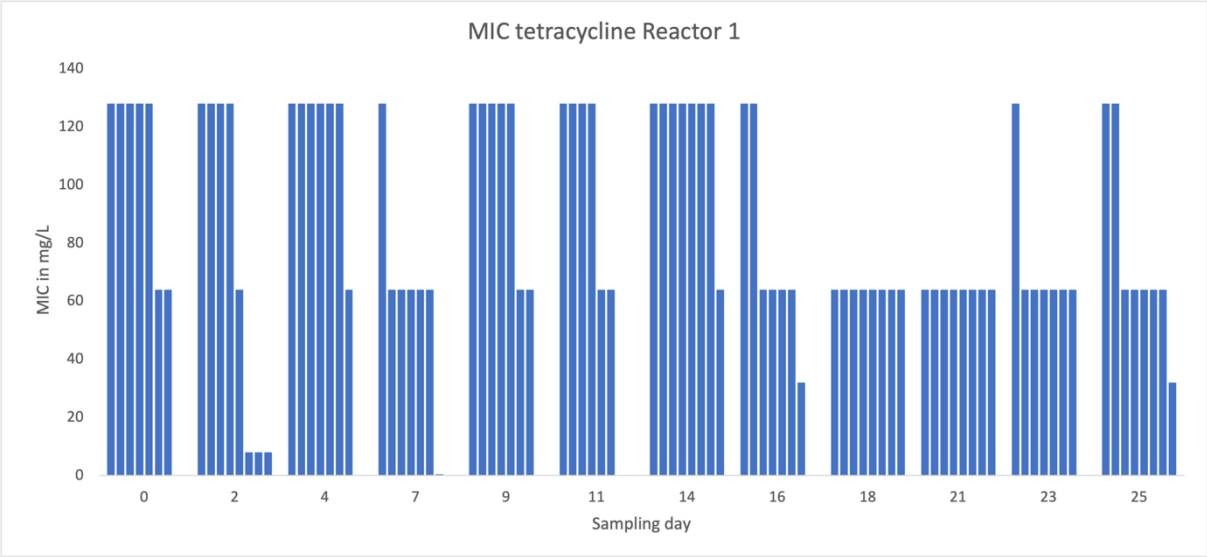

Figure 7. MIC values for TEC in the control reactor (reactor 1) during run 1. The reactor was run in room temperature, no BAC was added.

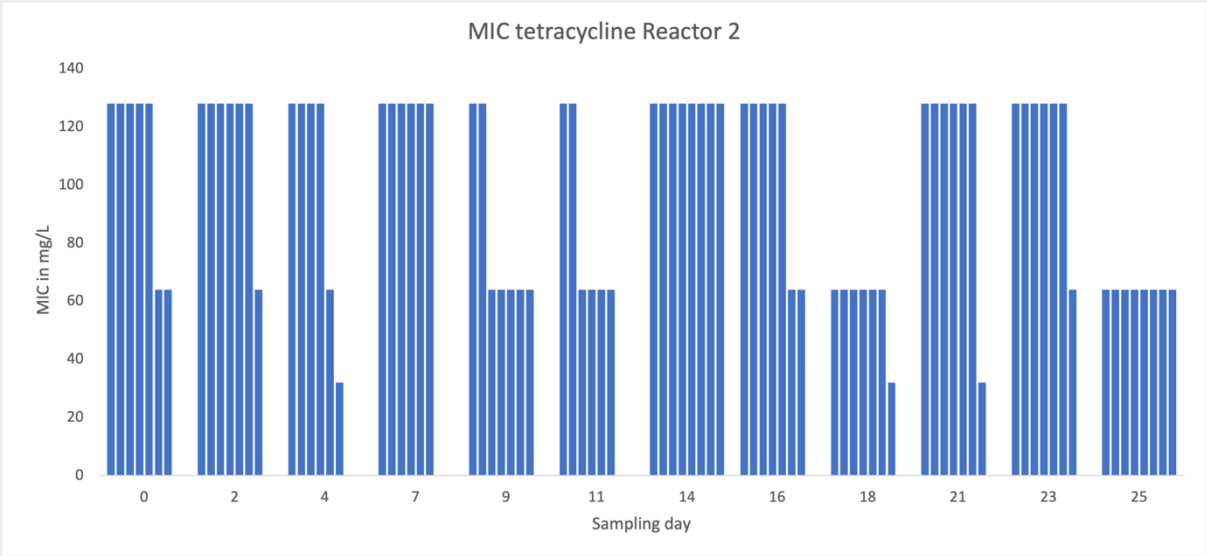

Figure 8. MIC values for TEC in the test reactor (reactor 2) during run 1. The reactor was run in room temperature with a concentration of 2,0 mg/L benzalkonium chloride.

During the second run, a total of 10 samples were taken over the time period of 25 days, and tested for MIC values for both BAC and TEC. Both reactors were again run in room temperature (22°C). This time, a concentration of 10 mg/L benzalkonium chloride was added to the test reactor (reactor 2). Reactor 1 functioned as control reactor with no benzalkonium chloride added. Each bacteria colony is represented as a bar in the graph. The MIC values were measured using 96 well microtiter plates. The results for the MIC values for both TEC and BAC during the second run can be seen in figure 9 to figure 12.

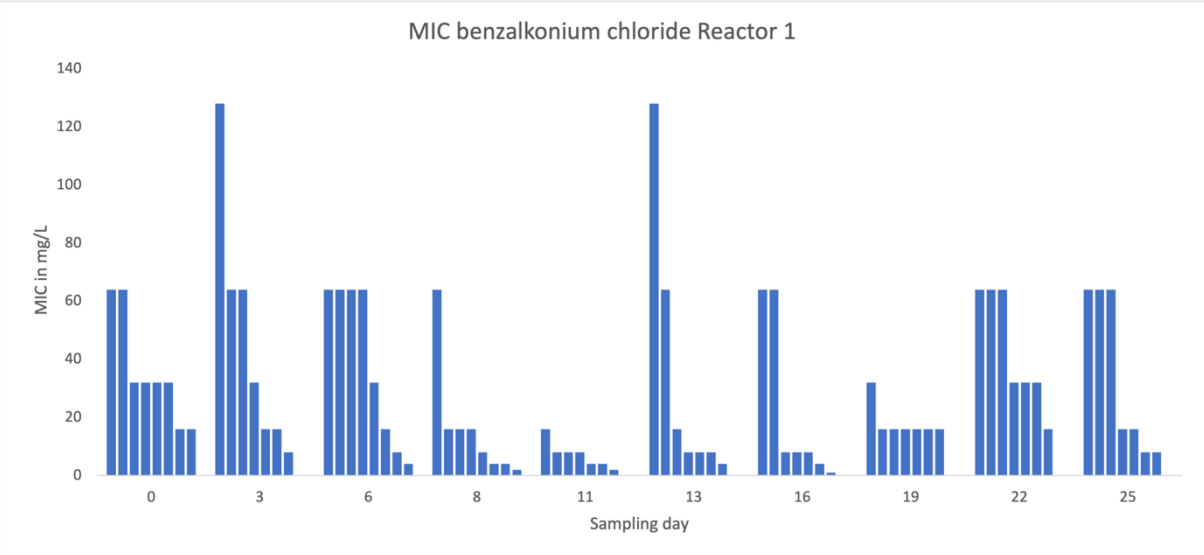

Figure 9. MIC values for BAC in the control reactor (reactor 1) during run 2. The reactor was run in room temperature, no BAC was added.

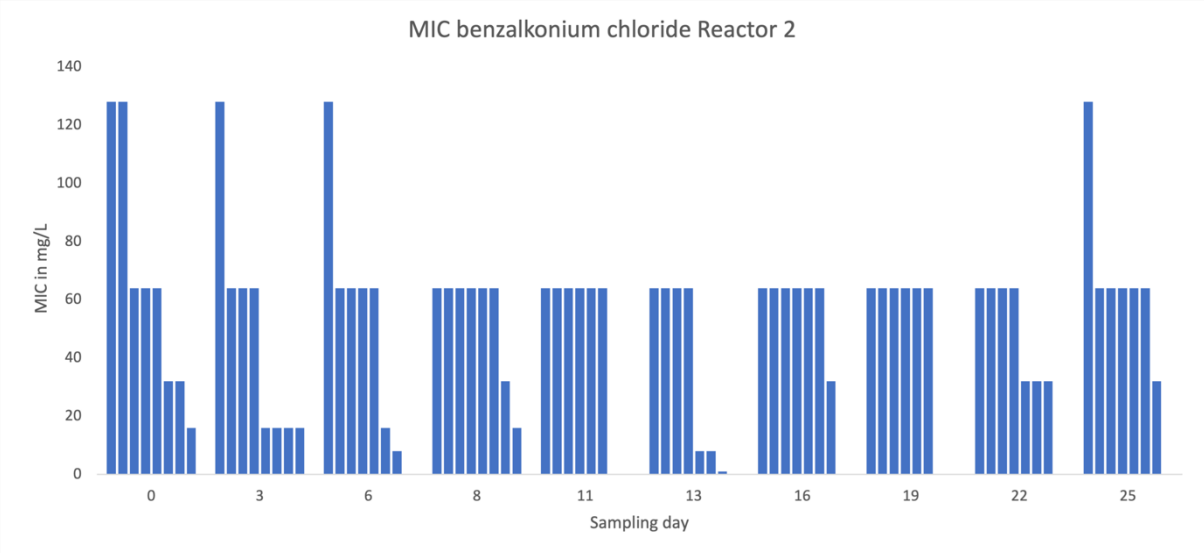

Figure 10. MIC values for BAC in the test reactor (reactor 2) during run 2. The reactor was run in room temperature with a concentration of 10 mg/L benzalkonium chloride.

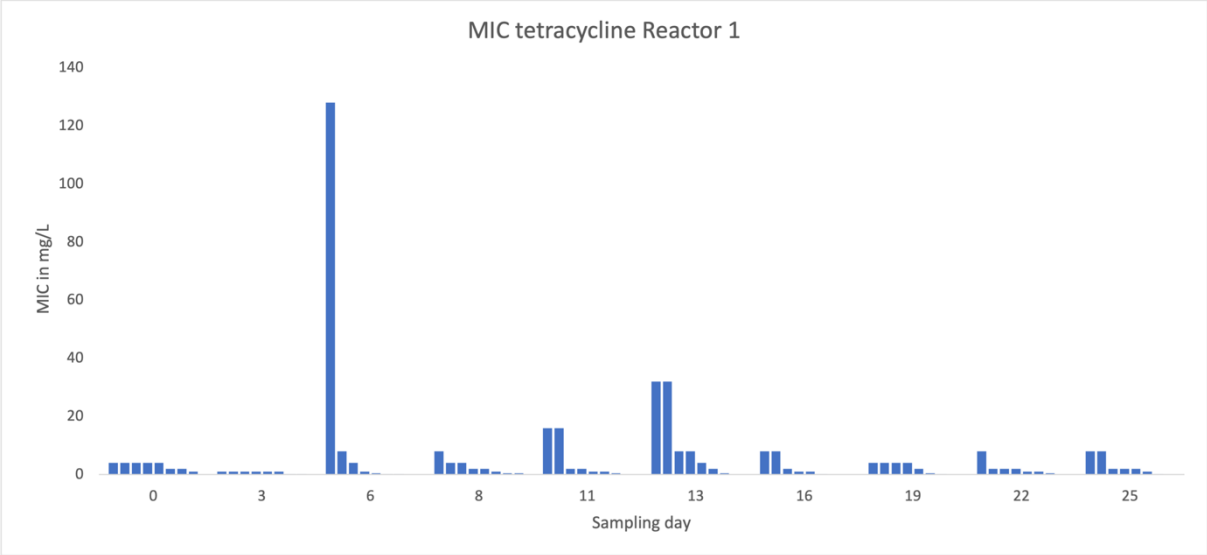

Figure 11. MIC values for TEC in the control reactor (reactor 1) during run 2. The reactor was run in room temperature, no BAC was added.

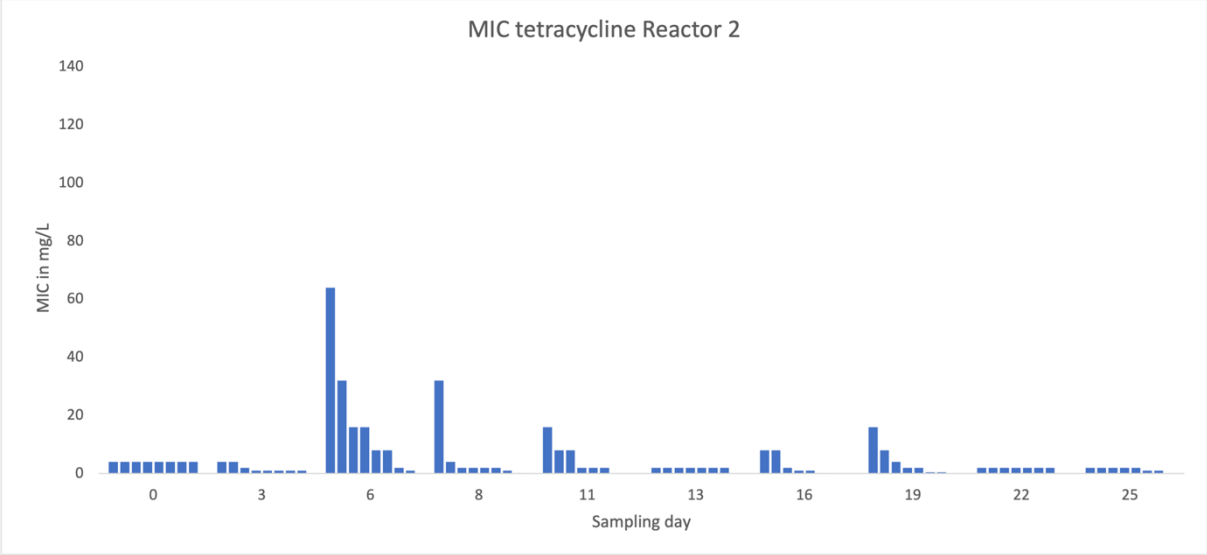

Figure 12. MIC values for TEC in the test reactor (reactor 2) during run 1. The reactor was run in room temperature with a concentration of 10 mg/L benzalkonium chloride.

4.3 TSS/VSS

The results for the TSS and VSS are shown in figure 13. This analysis was only performed during the second run. Reactor 1 functioned as control reactor where no BAC was added. Reactor 2 contained a concentration of 10 mg/L BAC during the whole period of 25 days. It shows clearly the increase of TSS in reactor 1, whereas the TSS in reactor 2 decreases significantly.

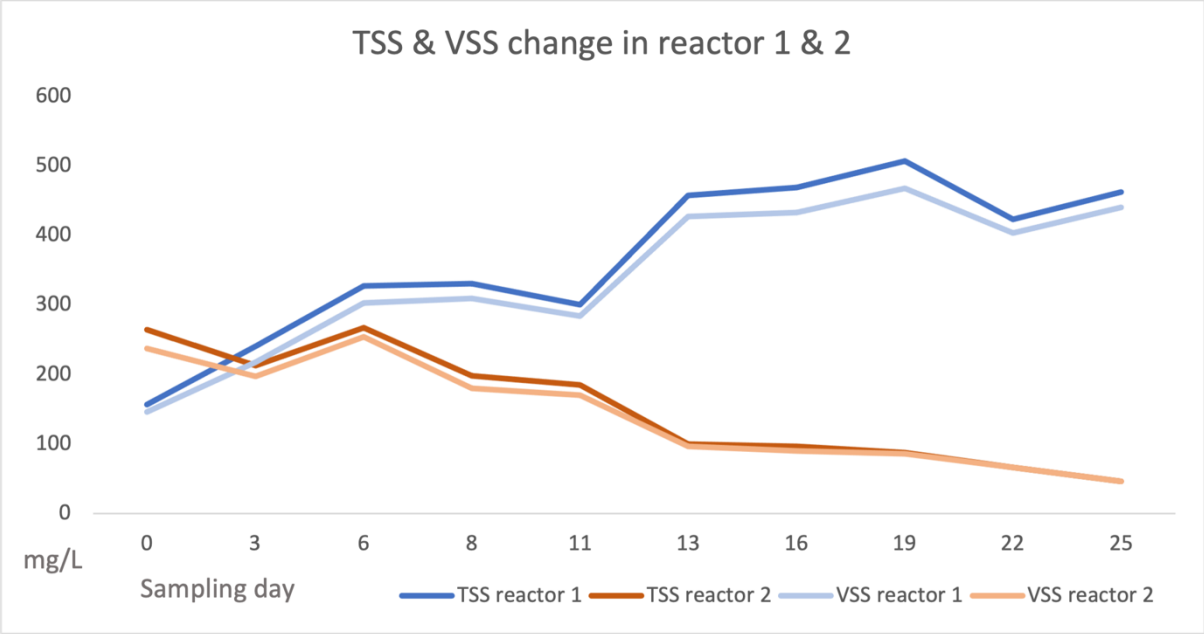

Figure 13. TSS and VSS data for reactor 1 (R1) and reactor 2 (R2) over the period of 25 days. Both reactors were run in room temperature. Reactor 1 did not contain BAC, whereas reactor 2 contained a concentration of 10 mg/L BAC.

5.0 DISCUSSION

5.1 Method optimization

The method used for analyzing the MIC-values was based on ISO 20776-1:2019 (E). However, this method is designed for medical isolates, meaning several adjustments needed to be done in order to perform this method on the sludge reactors. This method had previous been adapted for this use by Ingrid Malmberg in her thesis (Malmberg, 2021).
Samples spread on agar plates were incubated at room temperature (22°C), instead of 37°C shown in the ISO. This resulted in the growth more different types of bacteria since the reactor was maintained at room temperature and thus selected for organisms that grew optimally at this temperature. Also, some of the organisms grew slowly, as it took 48 hours or more before growth was visible on the plates, resulting in the delayed colony to broth transfer compared to the ISO which was designed for medically isolate organisms .

5.2 MIC values

All MIC-values range from a maximum of 128 mg/L to a minimum of 0,25 mg/L.
During the first run, the reactors when compared to each other seem to have relatively similar MIC results for both BAC and TEC during the whole experiment. Both reactors show some tendency of decreasing MIC-values for TEC on days 22 and 25 (figures 7, 8, 11 and 12), whereas the MIC-values for BAC seem to increase during the last five days of testing. The MIC for TEC is quite constant throughout the entire experiment with values between 64 – 128 mg/L in both reactors, whereas the MIC for BAC was around 8 mg/L during the whole period except for sampling day 18 and 21, where the MIC seems to be a little bit higher in both reactors, with values of up to 64 mg/L. However, the differences in MIC-values between reactor 1 and reactor 2 are too small to state any significant increase of resistance due to the addition of BAC to reactor 2 during the first run. Also, the concentration of 2,0 mg/L BAC in reactor 2 during the first run seemed to be a subinhibitory concentration for BAC. This concentration might have been too low to select for the development of resistance against BAC.
During the first run, two batches of microtiter plates were made. The first batch of plates ended on 28th of February (day 6 of testing), meaning a new batch of plates was used from 2nd of March on (day 7 of testing). Slight differences in the concentration of the different solutions may have occurred in making those batches due to weighing errors or pipetting

errors. Those slight differences in concentration that might have occurred may be high enough for some strains to get inhibited or by allowing some strains to grow at higher concentrations. However, no such differences in the results could be seen in this study.

During the second run the MIC-values seem to be quite different compared to the MIC values from the first run. The MIC values for TEC were now a lot lower, whereas the MIC values for BAC seemed to have increased a little as seen in figure 9 to figure 12 in the results section, During the first run, the MIC values for TEC were constantly high, mostly lying between 64 and 128 mg/L, whereas that changed in the second run with the values lying around in the lower section mostly ranging from 1 – 8 mg/L. The MIC values for BAC increased from an average of 8 mg/L in the first run to mostly higher values in the second run ranging from 32 – 128 mg/L.
The MIC values for TEC are also varied a lot more than the MIC values for BAC as shown in figure 9 to figure 12 in the results section. The MIC values for TEC are quite unstable with a lot more variation between the different colonies tested. In one sample, it was possible to find colonies with totally different MIC values. This may be due to developing resistance in some strains, whereas other strains remained unaffected. However, the MIC values for TEC were similar for both reactors with no significant change in MIC during the experiment. The MIC values for TEC are constantly swinging in the lower section with values between 1 and 4 mg/L. Only a few colonies were found to have quite high MIC values for TEC, therefore it appears that BAC had little impact on TEC resistance. The MIC values for BAC on the other hand seem to be somewhat higher on average in reactor 2. As shown in figure 10, there are higher MIC values for BAC in reactor 2. MIC values of 64 mg/L and higher were common in reactor 2, indicating the presence of BAC at this concentration may lead to an increase in resistance when compared to the control reactor.

The study by Khan et. al (2016), looked at the effect chlorination has on the development of resistance to different antibiotics. As chlorination is a commonly used method to disinfect the water in WWTPs, they used normal tap water and tested the bacteria in the water to obtain their MIC values for different antibiotics including tetracycline. The study of Sadia Khan et. al (2016) used standardized maximum MIC values for organisms described by CLSI (Clinical and Laboratory Standards Institute) and concluded that 13,5% of the 145 tested colonies in tap water were resistant to tetracycline. Also, a recently published study by van Dijk H. F. G. et. al. (2022) proved that most disinfectants can lead to antimicrobial resistance and may

increase the development of resistance to antibiotics. In this study, they looked at the MIC values for different antimicrobial agents in different strains at random locations. The MIC values in strains at locations where disinfectants are commonly used appeared to be significantly higher than for locations where disinfectants hardly are used.

5.3 TSS/VSS

The data for the TSS and VSS are presented in figure 13 in the results section. This analysis was only performed during the second run. There is a significant difference in the two reactors for the TSS and VSS values. The TSS in reactor 1 is constantly increasing during the first 3 weeks of testing, from around 150 mg/L at day 0, to around 500 mg/L at day 19. From there, the TSS drops a little, but stabilizes just below 500 mg/L and remains around this value between days 13 to 25.

In reactor 2, the TSS decreased during the entire experiment except for day 6 were it raised a little. TSS value of reactor 2 started at 250 mg/L at day 0 but decreased to 46 mg/L during this experiment. This confirms that the BAC added to reactor 2 impacted the sludge. This could also be seen visually, as the water in reactor 2 became clearer over time, containing less and less sludge (Figure 14). The decreasing TSS values tells us that the total amount of bacteria in the reactor decreased, meaning only the bacteria with a higher MIC value than 10 mg/L BAC in the reactor survived. That may also be the reason to why no isolates with MIC values below 16 mg/L BAC in reactor 2 were found as shown in figure 10. MIC values under 16 mg/L BAC could still be found in reactor 1 at that time as seen in figure 9.

The VSS values are also quite different for the 2 reactors. Both reactors start with a quite similar ratio between VSS/TSS, with the VSS values being around 90% of the TSS. Reactor 1 maintained this ratio between TSS/VSS over the whole period of 25 days. These result mean that the sludge in reactor 1 was able to process the added food, and the bacteria were able to reproduce until they reached a level where the population stabilized. Reactor 2 on the other hand ended up with a ratio of 1:1 between VSS/TSS after 25 days, meaning the sludge is lacking minerals and other sediments, and only contains of organic matter. This is a clear sign of imbalance in the sludge, most likely due to the addition of BAC.

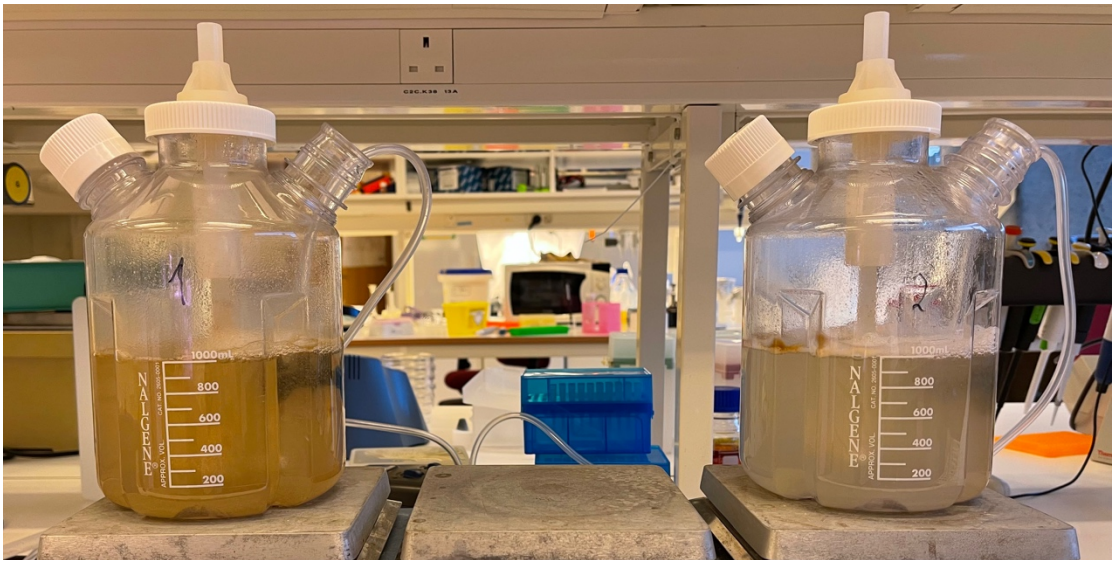

Figure 14. Reactor 1 on the left, and reactor 2 on the right side. Flocs and forming of biofilms in reactor 2 can be seen. The water in reactor 2 became clearer over time.

5.4 Other observations

A few days after adding BAC to reactor 2, the behavior of the sludge in reactor 2 changed. The sludge started to come together in flocs and attached to the edges of the reactor in order to form biofilms, which is one of the first responses of bacteria to withstand attacks from antimicrobial agent. Bacteria associated with biofilm are more resistant to antimicrobials, because the complex structure of biofilm prevents the access of antimicrobials to bacteria. Due to changes in environmental conditions the bacteria in biofilms grow more slowly than planktonic bacteria (Tasneem et. al. 2018). This could also explain the drastic decrease in TSS/VSS values in reactor 2.

During the second run, changes in the colonies collected from the reactors could be observed. The colonies grown on agar plates from reactor 1 didn't change their morphology, whereas the collected colonies from reactor 2 changed in color (Figure 15). This indicates a change in the microbial community. The colonies became greener in color and started to grow more slowly than before. Many of the colonies didn't grow before the third day after sampling. That means a new type of bacteria species took over in reactor 2 as the concentration of BAC increased.

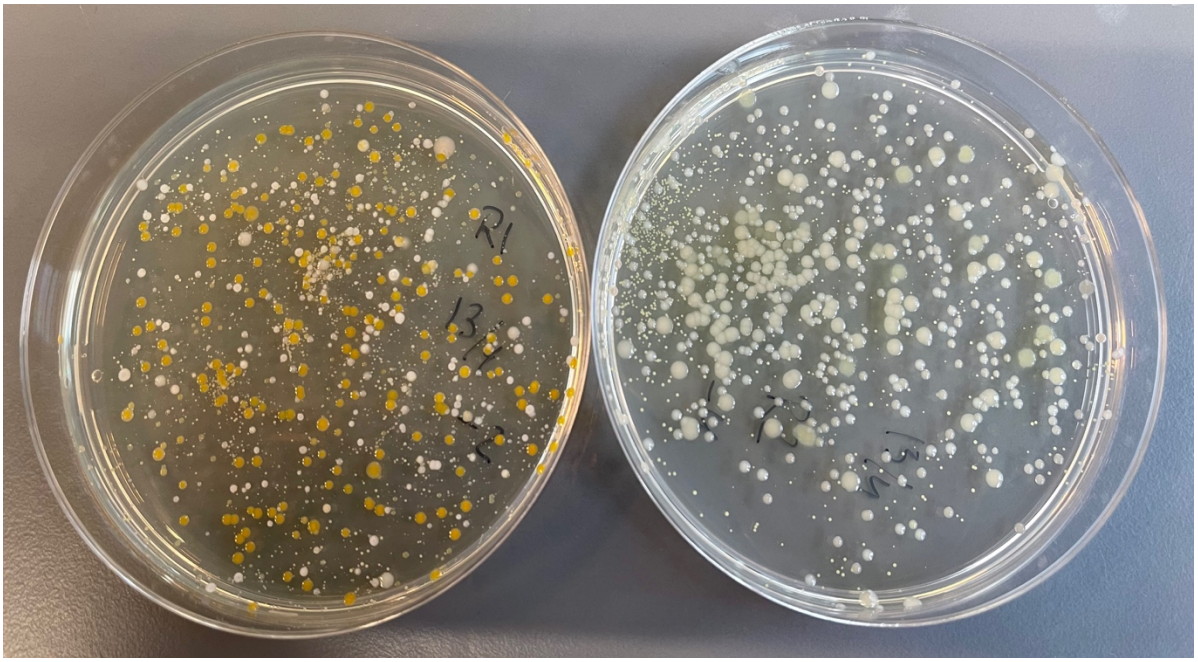

Figure 15. Significant change in microbial community in reactor 2 compared to reactor 1. Colony samples from reactor 1 on the left, colony samples from reactor 2 on the right. Both samples were taken on sampling day 13 during the second experiment.

6.0 CONCLUSION

The MIC values for both reactors varied for TEC quite a bit, with values ranging between 0,25 mg/L to 128 mg/L. As those values could be found in both reactors, it seems like there is some general variation of tetracycline resistance in the sludge from the SNJ. The MIC values for BAC seemed to be more stable. During the second run, a higher resistance against BAC in reactor 2 could be seen compared with the control reactor. The addition of BAC in reactor 2 did not impact the MIC values for TEC in both experiments at either of the concentrations used in the two experiments. Therefore, it can be concluded that in this study the presence of BAC did not contribute to tetracycline resistance. The TSS/VSS data also support the findings of the MIC values for BAC, as BAC addition clearly had an impact on reactor 2, with the decrease of sludge over time. Biofilm formation was also observed during both runs in reactor 2, meaning the bacteria in the sludge in reactor 2 were affected and started to protect against the addition of the antimicrobial agent. From this it can be concluded that not only can the addition of BAC decrease its effectiveness by increased resistance to the disinfectant, if BAC is present in high enough concentration such as the concentration used in the second experiment in this study, they may affect the sludge microbial community. Finally, these results indicate the effect of disinfectant in WWTPs warrants more research.

7.0 FUTURE PERSPECTIVES

Due to time limitations further studies could not be done. A new setup of the reactors should be conducted to confirm the results of this study and strengthen the theory that subinhibitory levels of BAC do not have any impact on the MIC for tetracycline. Also, a setup with the use of different antimicrobial agents should be performed to determine if BAC addition effects the MIC values and thus resistance of other antibiotics.

In future experiments it is important to operate the reactors for a longer time period to see if this could eventually result in a clear effect on antibiotic resistance using MIC values. 25 days appear to be a too short time period for the development of antimicrobial resistance.
The number of colonies checked for resistance should be higher in future studies as in this study there was a great spread of resistance on some of the sampling days potentially giving an unclear representation of the bacteria in the sample.

Due to time restrictions the bacteria in this work were not sequenced to obtain a better understanding of the effects BAC addition has on the microbial community. This should have been done. The bacteria isolated for the MIC tests should be sequenced to determine what bacteria species are present in the samples, and if the exposure to BAC changes the bacteria communities in the reactor and if the bacteria developing increased resistance are of medical significance.

As the TSS/VSS analysis only was performed during the second run, it is important to repeat the experiment with using the same conditions as in the first run not just to confirm the results but to perform TSS/VSS analysis using these conditions as well to check what impact the lower BAC concentration had on the TSS/VSS values.

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9.0 APPENDIX

9.1 Media compositions

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

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

When adding only 750 ml, how much of AF WW stock solution is needed (**1X**)?

1X (750 ml) AF WW = $\frac{10ml \times 1000ml}{750ml}$ = 13,3 ml of AF WW stock solution

V_{tot} = 750 ml
$V_{tap\ water}$ = 750 ml – 13,3 ml = 786,7 ml tap water

Formula 2: Diluting artificial wastewater stock solution when feeding the reactors Monday-Thursday.

1X (800 ml) AF WW = $\frac{10ml \times 1000ml}{800ml}$ = 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 3: Diluting artificial wastewater stock solution when feeding the reactors on Fridays

2X (800 ml) AF WW = $2 \times 12{,}5\ ml$ = 25,0 ml of AF WW stock solution

V_{tot} = 800 ml
$V_{tap\ water}$ = 800 ml – 25,0 ml = 775,0 ml tap water

Formula 4: Benzalkonium chloride added to reactor 2 when setting up the reactors (first run).

V_1 = ?
C_1 = 256g/L
V_2 =1,0 L

C_2 = 2,0 × 10^{-3}g/L (desirable concentration of BAC in reactor 2)

$V_1 = \frac{2{,}0 \times 10^{-3}\ g/L \times 1{,}0\ L}{256\ g/L} = 7{,}81 \times 10^{-6}\ L$ = 7,81 µl

Formula 5: Benzalkonium chloride added to reactor 2 when setting up the reactors (second run).

V_1 = ?
C_1 = 256g/L
V_2 =1,0 L

C_2 = 10,0 × 10^{-3}g/L (desirable concentration of BAC in reactor 2 during run 2)

$$V_1 = \frac{10{,}0 \times 10^{-3}\ g/L \times 1{,}0\ L}{256\ g/L} = 3{,}91 \times 10^{-5}\ L = \underline{39{,}1\ \mu l}$$

Formula 6: Benzalkonium chloride added to reactor 2 when feeding the reactor Monday – Thursday (first run)

V_1 = ?
C_1 = 256g/L
V_2 =0,8 L

C_2 = 2,0 × 10^{-3}g/L (desirable concentration of BAC in reactor 2 during run 1)

$$V_1 = \frac{2{,}0 \times 10^{-3}\ g/L \times 0{,}8\ L}{256\ g/L} = 6{,}25 \times 10^{-6}\ L = \underline{6{,}25\ \mu l}$$

Formula 7: Benzalkonium chloride added to reactor 2 when feeding the reactor Monday – Thursday (second run)

V_1 = ?
C_1 = 256g/L
V_2 =0,8 L

C_2 = 10,0 × 10^{-3}g/L (desirable concentration of BAC in reactor 2 during run 2)

$$V_1 = \frac{10{,}0 \times 10^{-3}\ g/L \times 0{,}8\ L}{256\ g/L} = 3{,}13 \times 10^{-5}\ L = \underline{31{,}3\ \mu l}$$

Formula 8: Total suspended solids expressed in mg/L.

$$\frac{Weight_{final}\ (g) - Weight_{initial}\ (g)}{Volume\ (ml)} \times 10^6 = TSS\ in\ mg/L$$

Weight initial: Weight of filter before use in grams.

Weight final: Weight of filter + residue in grams.

Volume: Volume of sample filtered through the filter in ml.

Formula 9: Volatile suspended solids expressed in mg/L.

$$\frac{Weight\ of\ Material\ lost\ by\ burning\ (g)}{Volume\ (ml)} \times 10^6 = VSS\ in\ mg/L$$

Weight of material lost by burning: $Weight_{initial} - Weight_{final}$

Weight initial: Weight of filter + residue in grams.

Weight final: Weight of filter after burning.

Volume: Volume of sample filtered through the filter in ml.

9.2 Additional data MIC values

The data for the MIC values for both BAC and TEC in reactors 1 and 2 are shown in the tables 9-1 to 9-8 The data for the MIC values are presented in a descending order to make the reading of the tables easier. Isolate 1 does therefore not necessary belong to isolate 1 on the microtiter plates. Also, the isolates are completely random for every day they got tested, meaning the number of the isolate is not corresponding to the same colony during the 4 weeks of testing. If the MIC value could not be read due to different reasons as growth in the negative control, no growth in positive well or if wells got skipped, NA is being stated instead of a value. During both runs, the MIC values got tested over a time period of 25 days. However, the frequency of testing is lower during the second run due to closed labs during the easter holidays.

Table 9-1: MIC values for BAC reactor 1 (first run)

Day sampling	0	2	4	7	9	11	14	16	18	21	23	25
Isolate												
1	8	8	8	8	8	8	8	8	16	32	8	16
2	8	8	8	8	8	8	8	8	16	32	8	8
3	8	8	8	8	8	8	8	8	8	16	8	8
4	8	8	8	8	8	8	8	8	8	16	8	8
5	8	8	8	8	8	8	8	8	8	16	8	8
6	8	4	8	8	8	8	8	8	8	8	8	8
7	8	4	8	8	8	8	8	NA	8	8	8	4
8	NA	4	NA	8	NA	8	8	NA	NA	8	8	NA

Table 9-2: MIC values for TEC reactor 1 (first run)

Day sampling	0	2	4	7	9	11	14	16	18	21	23	25
Isolate												
1	128	128	128	128	128	128	128	128	64	64	128	128
2	128	128	128	64	128	128	128	128	64	64	64	128
3	128	128	128	64	128	128	128	64	64	64	64	64
4	128	128	128	64	128	128	128	64	64	64	64	64
5	128	64	128	64	128	64	128	64	64	64	64	64
6	64	8	128	64	64	64	128	64	64	64	64	64
7	64	8	64	0,5	64	NA	128	32	64	64	64	64
8	NA	8	NA	NA	NA	NA	64	NA	64	64	NA	32

Table 9-3: MIC values for BAC reactor 2 (first run)

Day sampling	0	2	4	7	9	11	14	16	18	21	23	25
Isolate												
1	8	8	64	8	8	8	8	8	64	16	8	16
2	8	8	8	8	8	8	8	8	64	16	8	8
3	8	8	8	8	8	8	8	8	64	16	8	8
4	8	8	8	8	8	8	8	8	16	8	8	8
5	8	8	8	8	8	8	8	8	16	8	8	8
6	8	8	8	8	8	8	8	8	8	8	8	8
7	8	8	NA	8	8	8	8	8	8	8	8	4
8	8	8	NA	NA	8	NA	8	NA	NA	8	8	NA

Table 9-4: MIC values for TEC reactor 2 (first run)

Day sampling	0	2	4	7	9	11	14	16	18	21	23	25
Isolate												
1	128	128	128	128	128	128	128	128	64	>128	128	64
2	128	128	128	128	128	128	128	128	64	128	128	64
3	128	128	128	128	64	64	128	128	64	128	128	64
4	128	128	128	128	64	64	128	128	64	128	128	64
5	128	128	64	128	64	64	128	128	64	128	128	64
6	64	128	32	128	64	64	128	64	64	128	128	64
7	64	64	NA	NA	64	NA	128	64	32	32	64	64
8	NA	NA	NA	NA	NA	NA	128	NA	NA	NA	NA	64

Table 9-5: MIC values for BAC reactor 1 (second run)

Day sampling / **Isolate**	0	3	6	8	11	13	16	19	22	25
1	64	128	64	64	16	128	64	32	64	64
2	64	64	64	16	8	64	64	16	64	64
3	32	64	64	16	8	16	8	16	64	64
4	32	32	64	16	8	8	8	16	32	16
5	32	16	32	8	4	8	8	16	32	16
6	32	16	16	4	4	8	4	16	32	8
7	16	8	8	4	2	4	1	16	16	8
8	16	NA	4	2	NA	NA	NA	NA	NA	NA

Table 9-6: MIC values for TEC reactor 1 (second run)

Day sampling / **Isolate**	0	3	6	8	11	13	16	19	22	25
1	4	1	128	8	16	32	8	4	8	8
2	4	1	8	4	16	32	8	4	2	8
3	4	1	4	4	2	8	2	4	2	2
4	4	1	1	2	2	8	1	4	2	2
5	4	1	0,5	2	1	4	1	2	1	2
6	2	1	0,25	1	1	2	0,25	0,5	1	1
7	2	0,25	0,25	0,5	0,5	0,5	NA	0,25	0,5	0,25
8	1	0,25	NA	0,5	NA	NA	NA	NA	NA	NA

Table 9-7: MIC values for BAC reactor 2 (second run)

Day sampling / **Isolate**	0	3	6	8	11	13	16	19	22	25
1	128	128	128	64	64	64	64	64	64	128
2	128	64	64	64	64	64	64	64	64	64
3	64	64	64	64	64	64	64	64	64	64
4	64	64	64	64	64	64	64	64	64	64
5	64	16	64	64	64	8	64	64	32	64
6	32	16	16	64	64	8	64	64	32	64
7	32	16	8	32	NA	1	32	NA	32	32
8	16	16	NA	16	NA	NA	NA	NA	NA	NA

Table 9-8: MIC values for TEC reactor 2 (second run)

Day sampling / **Isolate**	0	3	6	8	11	13	16	19	22	25
1	4	4	64	32	16	2	8	16	2	2
2	4	4	32	4	8	2	8	8	2	2
3	4	2	16	2	8	2	2	4	2	2
4	4	1	16	2	2	2	1	2	2	2
5	4	1	8	2	2	2	1	2	2	2
6	4	1	8	2	2	2	0,25	0,5	2	1
7	4	1	2	1	NA	NA	NA	0,5	2	1
8	4	1	1	0,25	NA	NA	NA	NA	NA	NA

9.3 Additional data TSS and VSS

The data for TSS and VSS are shown in table 9-9 and 9-10. All values are expressed in mg/L. TSS and VSS was only performed during the second run.

Table 9-9: TSS values expressed in mg/L

Day sampling	0	3	6	8	11	13	16	19	22	25
Reactor 1	156,4	240,8	327,2	330,4	300,4	457,2	468,8	507,0	423,5	462,5
Reactor 2	264,4	212,8	267,2	198,0	184,8	100,0	96,4	87,2	66,4	46,0

Table 9-10: VSS values expressed in mg/L

Day sampling	0	3	6	8	11	13	16	19	22	25
Reactor 1	146,0	217,2	302,8	309,6	284,2	427,6	432,8	468,0	403,5	440,5
Reactor 2	237,6	197,2	254,0	180,0	169,8	96,0	89,4	85,4	66,0	46,0