Removal efficiency of antibiotic resistance using the O3-STEP filter

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Removal efficiency of antibiotic resistance using the O3-STEP filter

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TU Delft

Preface

This document presents the final report for the TU Delft master thesis of Civil Engineering, Watermanagement, specialization Sanitary Engineering. This is the culmination of work I have been doing since November 2020.

First of all, I would like to thank Bruno Bicudo Pérez for teaching me so much about antibiotic resistance. His enthusiasm and knowledge on waste water and the microorganisms living in it, have been a real inspiration to me. I would also not know if I could have done it without the coffee breaks and occasional pep-talks he gave me. Furthermore, I would like to thank the rest of my graduation committee from the TU Delft: Jan Peter van der Hoek, Doris van Halem and Merle de Kreuk. My research has become much more indepth because of their input during the meetings. Every meeting I started by thinking I knew a lot, only to find out there was so much more to learn. These meetings were certainly a real motivation boost. Lastly, I would like to thank all my supervisors for the extensive feedback I received on my report; the work is now much improved.

I would further like to thank Witteveen+Bos and more specifically Coen de Jong for the opportunity to write my master thesis at Witteveen+Bos. Coen had the inspiration of conducting research about the impact of the O3STEP$^{(R)}$ on antibiotic resistance. From the beginning, Coen has been very helpful, supportive and a source of real positivity. I am looking forward to working for Witteveen+Bos in September. I am also grateful to the other employees and operators of the O3STEP$^{(R)}$ for their interest in my research, for their help with sample taking and for the time they took to answer my questions. It was fascinating to join the meetings of a project in which so many different parties are involved.

I would like to thank ozone expert Max Fu for all the hours he spend in the laboratory helping me with making spike solutions. As well as the rest of the staff and students in the laboratory who have been very approachable and willing to help with my research but also to share a coffee and vent some of our struggles to each other.

Finally, I would like to thank my family and friends for their support and interest during the past months. More specifically, Ross Williams for his patience in helping me with the plots and graphs in R and for proofreading my thesis (twice). And last but not least, my parents, Jan Spit and Angelien Karsten for their love and support during this journey all the way from Kenya. I would also like to thank Jan for proofreading but more importantly for being a real source of inspiration to change my career path to the wonderful world of waste water.

T. P. M. Spit
Rotterdam, July 2021

Executive summary

Waste Water Treatment Plants (WWTPs) are broadening their focus to substances of emerging concern such as Organic Micro Pollutants (OMPs). At the same time, a new concern is already emerging; Antibiotic Resistance (AR). As antibiotics now save millions of human and animal lives anually, the World Health Organisation (WHO, 2019) declared AR as the number five most dangerous risk to global health. Several researchers mention WWTPs to be an important source for AR as the growth of bacteria is stimulated in an environment with a selective pressure facilitated by a relatively high concentration of antibiotics.

Tertiary treatment steps are implemented primary to polish the effluent and reduce the OMP load of the effluent. The O3-STEP$^{(R)}$ filter is an innovative example of such a tertiary treatment combining the oxidative effects of ozone (O_3) with the adsorption effects of a Granular Activated Carbon (GAC) filter. In addition to these effects, a coagulant is dosed for the removal of Phosphorus (P). As the focus of the O3-STEP$^{(R)}$ filter is on the removal of OMPs, the implications for AR are still unknown. In literature varying results are found on the disinfecting effects of ozone, GAC and coagulation and nothing is found on the combination of these treatment steps yet. The purpose of this document is to present how effective the treatment with ozone and coagulation is in the removal of ARB in comparison with antibiotic sensitive bacteria (ASB). The hypothesis is that ozonation disinfects ARB as good as ASB and thus does not promote resistance. For the coagulation it is expected that a higher coagulant dosage is needed for attenuation of both the ARB and ASB than is needed for the removal of P. ARB might grow in the biofilm during the GAC filter step and although these bacteria are not directly harmful, they can pass down their resistance to pathogenic bacteria.

In this research Agar growth media are used to test four different microorganisms: two faecal indicators (*E. coli* and Enterococci) and a resistant strain of each of these bacteria (ESBL-*E. coli* and Vancomycin Resistant Enterococci (VRE)). Several experiments are conducted in the laboratory to test disinfection on these microorganisms. Firstly, an ozone spike solution is dosed to two types of effluent to find if this removed resistant bacteria differently than ASB and if the effectiveness increases when the ozone dosage is increased. Secondly, jar tests are conducted with three different kinds of coagulants ($FeCl_3$, Fe-EC and poly-aluminium chloride (PAC)). Lastly, the performance of the pilot is tested at several stages in the filter: after ozonation, after filtration and coagulation and in the backwash water. Samples of a full-scale GAC filter (1-STEP$^{(R)}$) are also taken into account to give an indication of the effects of the biofilm which is not yet grown in the O3-STEP$^{(R)}$ filter.

In confirmation of the hypothesis, ozonation and coagulation disinfect ARB as well as ASB. This means that ASB are indicators for the removal of ARB. However, as both treatment steps are not aiming at the removal of microorganisms but at the removal of OMPs and phosphorus respectively, the microorganism removal is limited (with 0.7 log on average). Increasing the coagulant and ozone dosage showed promising results in the laboratory. Dosing the coagulant above the optimum sweep coagulation dosage is not favourable because the increase in removal stagnated above this concentration whilst the residual coagulant concentration in the supernatant water increased.

As the GAC filter located after the ozonation, stimulates bacterial growth in an environment with an increased OMP and potentially increased ARG content, risk of ARB enhancement occurs. In the GAC of the O3-STEP$^{(R)}$ filter and its backwash water, an increase of VRE is found relative to Enterococci in comparison with the ozonated water but not over the complete O3-STEP$^{(R)}$ filter. This was different for the 1-STEP$^{(R)}$ filter which showed the worrying result that VRE increased in the same amounts as Enterococci decreased. As the 1-STEP$^{(R)}$ filter is a filter with a matured biology, where O3-STEP$^{(R)}$ was recently started, the O3-STEP$^{(R)}$ filter might increase the absolute and relative amount of ARB in long term as well.

In case the aim for the O3-STEP$^{(R)}$ filter is to remove AR as well, several suggestions are made. As ozone and coagulation both potentially remove ARB, an increase in these concentrations is a no-regret possibility. Furthermore, ultrafiltration (UF) and the combination of ultraviolet (UV) and O_3 are proposed as possible alternatives. Using a multicriteria analysis (MCA), increasing the ozone dosage with the existing bypass is recommended.

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List of Abbreviations

- AMR - Antimicrobial Resistance
- ANOVA - Analysis of Variance
- ARB - Antibiotic Resistant Bacteria
- ARG - Antibiotic Resistance Genes
- ASB - Antibiotic Sensitive Bacteria (bacteria not resistant to the described drug)
- BAC- Biological Activated Carbon
- BOD - Biochemical Oxygen Demand
- CDC - Centers for Disease Control and Prevention
- COD - Chemical Oxygen Demand
- *C. perfringens* - *Clostridium perfringens*
- DBP - Disinfection byproduct
- DNA - Deoxyribonucleic Acid
- DOC - Dissolved Organic Carbon
- EC - Electronic Conductivity
- *E. coli* - *Escherichia coli*
- eARB - environmental ARB
- ESBL - ExtendedSpectrum Betalactamase *E. coli*
- Fe-EC - Iron Electrocoagulation
- GAC - Granular Activated Carbon
- HGT - Horizontal Gene Transfer
- HM - Horstermeer
- HP - Harnaschpolder
- KRW - Kader Richtlijn Water (Dutch water regulations)
- MCA - Multi Criteria Analysis
- MGE - Mobile Genetic Elements
- MIC - Minimal Inhibitory Concentration
- N- Nitrogen
- NOM - Natural Organic Matter
- OMP - Organic Micro-Pollutant
- O_3 - Ozone

- O3-STEP(R) filter - pilot project combining ozone treatment with a pilot scale GAC filter (including methanol dosage for biological growth and PAC dosage for P-removal)
- P - Phosphorus
- PAC - Poly-Aluminium Chloride
- PACa - Powdered Activated Carbon
- pARB - pathogenic ARB
- RedOx - Reduction Oxidation
- RNA - Ribonucleic Acid
- ROS - Reactive Oxygen Species
- SS - Suspended Solids
- TN - Total Nitrogen
- TOC - Total Organic Carbon
- UF - Ultrafilteration
- UV - Ultraviolet
- VGT - Vertical Gene Transfer
- VRE - Vancomycin Resistant Enterococci
- WHO - World Health Organisation
- WWTP - Wastewater Treatment Plant
- 1-STEP(R) filter - full scale GAC filter (including methanol dosage for biological growth and PAC dosage for P-removal)

1 Introduction

1.1. Background

In the 19th century, due to rapid industrialisation and urbanisation, pandemics such as cholera and typhoid demonstrated the need for more sophisticated water management as dilution was no longer sufficient. In the late 19th century, sanitary conditions improved with the introduction of sewer systems and the first WWTPs. Originally, these WWTPs were started as a measure solely to reduce the level of Biochemical Oxygen Demand (BOD) and Chemical Oxygen Demand (COD) and suspended solids (SS) in the water to prevent considerable pollution of the rivers. Later also Nitrogen (N) and Phosphorus (P) attracted attention due to the eutrophication of the water (Seeger, 1999). This shows that the focus of WWTPs continues to broaden to adapt to new issues affecting the protection of downstream environments.

Developments in the field of wastewater management have led to renewed water standards for the effluent. The European Water Framework Directive (WFD) came into effect in 2000 and required, among other things, that surface water had to reach an ecologically and chemically sufficient quality by 2015. The WFD lists ”focus substances” whose load should be reduced in surface waters, including the effects of WWTP effluent (WFD, 2000). These water standards are asking for a more broadened focus of WWTPs. Research is being directed to the removal of Organic Micro Pollutants (OMPs), such as pesticides and pharmaceuticals, that in very small concentrations can have a relatively large impact on the downstream environment (Schwarzenbach et al., 2006). For example, antibiotics are released into municipal wastewater due to incomplete metabolism in humans or due to incorrect disposal of unused antibiotics (Bouki et al., 2013). One of the potential risks of these OMPs is that the antibiotic residues can give a selection pressure that leads to an increase in antibiotic resistance amongst pathogenic bacteria, resulting in a decreased effectiveness of antibiotic treatments (Goldstein et al., 2014). As antibiotics now save millions of human and animal lives every year, the World Health Organisation (WHO, 2017) declared antibiotic resistance as the number five most dangerous risk to global health. To give an example of the scope of the issue, the WHO estimates that in 2018, half a million new cases of (multi-drug) resistant tuberculosis (TB) were identified globally (WHO, 2018). Furthermore, the United Nations (UN) stated that yearly 700,000 deaths are caused by AR diseases, which could increase to 10 million yearly by 2050 if no action is taken (UN, 2019). Antibiotic resistance (AR) puts pressure on the success of modern medicine in treating infections, including during major surgery and chemotherapy. AR leads to longer hospital stays, higher medical costs and increased mortality (WHO, 2020).

For AR specifically, there are currently no regulations for WWTPs. The conventional treatment techniques in WWTPs are sub-optimal to remove AR (Andersen, 1993, Karkman et al., 2018). Unfortunately, the large concentration and growth of the bacteria (for sludge and biofilms) together with the relatively high antibiotic concentrations, are suspected to even create a hotspot for antibiotic resistant bacteria and genes (ARB/ARG) in the WWTPs (Bréchet et al., 2014; Dropa et al., 2016). This is particularly important for the reuse of the reclaimed water in for example agriculture, for which a higher treatment standard is needed.

A new feature of WWTPs is the introduction of tertiary treatment steps to aid in the removal of OMPs

and nutrients. This prevents these contaminants from reaching the environment and thus means the plant complies with the WFD. Examples of these tertiary treatment steps can be divided into three types. Firstly, adsorptions processes such as activated carbon which can be for instance applied in a powdered (PACAS) or filter form (1-STEP$^{(R)}$ filter) (STOWA, 2009). Secondly, oxidation processes as ozone, applied in for example the in Section 1.1.1 explained O3-STEP$^{(R)}$ filter or in combination with ultrasonic sounds (Usoniq, STOWA, 2020). And thirdly, membrane processes such as ultrafiltration (UF) and nanofiltration (STOWA, 2020). About the impact of these tertiary treatment steps on AR a lot is still unknown.

1.1.1. O3-STEP$^{(R)}$ filter

At the WWTP in Horstermeer, in The Netherlands, a pilot of a new tertiary treatment method, the O3-STEP$^{(R)}$ filter, is being tested. This method combines ozone dosage with a Granular Activated Carbon (GAC) filter (Figure 1.1). The filter concept is focused on the removal of OMPs and nutrients.

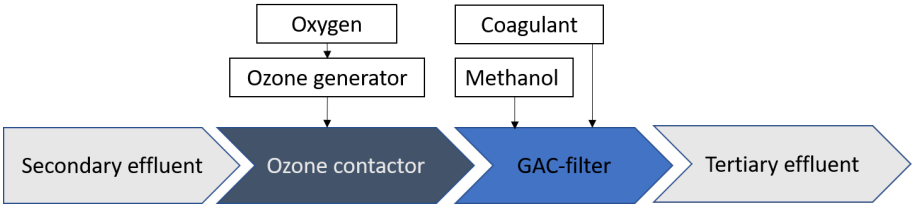

Figure 1.1: Schematic overview of the O3-STEP$^{(R)}$ filter

Currently, a full-scale GAC-filter is located at Horstermeer called the 1-STEP$^{(R)}$ filter which has the same concept as the O3-STEP$^{(R)}$ but without ozone dosage. In the 1-STEP$^{(R)}$ filter, Nitrogen (N) is removed by denitrification (with methanol, CH_4O), phosphorus (P) by coagulation and OMPs via adsorption in the micropores of the GAC and broken down by the biofilm. In addition, suspended solids (SS) are removed by filtration. Over the years, it appeared that the drawback of this 1-STEP$^{(R)}$ filter is that the micropores in the carbon are exhausted and clogged after 4 to 6 months, meaning the GAC should be replaced or regenerated for the OMP removal (Menkveld et al., 2009).

Regeneration needs to be done offsite and is costly. The O3-STEP$^{(R)}$ filter is designed to test if the use of ozone treatment can increase the lifetime of the GAC filter by breaking down the OMPs beforehand (STOWA, 2018). After the ozone treatment, residues, transformation products and ozone-resistant OMPs are filtered out in the GAC-filter. Whether the lifetime is increased, is outside of the scope of this research. Apart from OMPs and nutrients, other contaminants, such as microorganisms, will likely also be removed by the addition of the ozone. Ozone (O_3) is a strong oxidizer that reacts instantaneously (within seconds or a few minutes) with the organic substances in the water. It is therefore a fast and effective method for the removal of OMPs and pathogens leaving oxygen as end-product. Its disinfectant ability was discovered in the 1880's after which The Netherlands was the first country to apply ozone in a full-scale drinking water treatment plant (Diamant, 1980). After this success, the use of ozone spread throughout Europe and America and is now applied in wastewater treatment as well.

The combination of ozone treatment and the 1-STEP$^{(R)}$ filter might result in tertiary effluent with a high water quality that is a suitable starting-point for most reuse applications. This research will focus on the impact on AR in the effluent when using the O3-STEP$^{(R)}$ filter.

1.2. Antibiotic resistance (AR)

Antibiotic resistance (AR), is the resistance of a bacterium against antibiotics, threatening the effective prevention and treatment of infections. There are many definitions of AR provided throughout literature which shows the importance of defining the meaning for the scope within this research. In the clinical definition, resistance means that the infectious disease treatment will fail as there is a reduced susceptibility for the antibiotic (Martinez, 2014, Walsh, 2013). This definition is inadequate for non-pathogenic bacteria that have obtained antibiotic resistance as they do not require treatment. The more technical definition of AR uses the study of genetic material (metagenomics) to compare different gene sets to find antibiotic resistance. This definition is applicable for functional metagenomics as well as experimental studies on the spread of the antibiotic resistance (Martínez et al., 2015). The technical definition

does include non-pathogenic bacteria, but requires the analysis of large amounts of genetic data which is out of the scope of this research. Another definition including the non-pathogenic bacteria is the epidemiological definition. This definition is based on the screening of a large number of isolates of a given bacterial population. It defines resistant bacteria as presenting a higher minimal inhibitory concentration (MIC) than the bulk of the population. The MIC is the lowest concentration of a drug that prevents the growth of a bacterium (Martínez et al., 2015; Kahlmeter, 2014; Kronvall et al., 2011). The epidemiological definition is used in this research as the focus will be on resistant bacteria rather than genes. This section will define Antibiotic resistance genes (ARG) in Section 1.2.1, Antibiotic resistant bacteria (ARB) in Section 1.2.2 and describe several mechanisms of antibiotic resistance in Section 1.2.3. When talking about bacteria that are not resistant to antibiotics, the term Antibiotic Sensitive Bacteria (ASB) is used.

1.2.1. Antibiotic resistance genes (ARGs)

Antibiotic resistance genes (ARG), expressed through DNA or RNA, encode for the synthesis of proteins that protect a bacterium from inhibitory effects of antibacterial agents (Martínez et al., 2015). Normally, this DNA or RNA is present in a host, such as a microorganism. However, ARGs can also survive as extracellular DNA from lysed microbial cells, causing them to be spread widely throughout different environments (Paulus et al., 2019). Depending on the time in which the ARG occurs, two types can be distinguished. Firstly, the environmental ARGs, that have acquired their non-clinical resistance before the emergence of antibiotics. Secondly, the type evolved to counteract the activity of antibiotics (Martínez et al., 2015). The environmental ARGs obtain their resistance by inherent characteristics, by random mutation without the presence of an antibiotic or by Horizontal Gene Transfer (HGT, Section 1.2.2). As clinical antibiotics largely originate from (soil) microorganisms, it becomes clear that environmental ARGs are omnipresent in nature (Paulus et al., 2019). For instance, even before the introduction of the clinical antibiotic Penicillin in 1945, there were publications on the occurrence of antibiotic resistance of it (Abraham and Chain, 1940). This resistance was however not a problem before the emergence of clinical antibiotics. As its risk is indirect, ARGs only become a health risk in combination with a pathogen of which the disease is treated with an antibiotic. However, after years of antibiotic (over- and mis)use, inadequate disposal and non-essential use in agriculture, the risk increases for the second type of resistance, the one counteracting the antibiotics (Ventola, 2015).

1.2.2. Antibiotic resistant bacteria (ARB)

Antibiotic resistant bacteria (ARB) are bacteria that carry ARGs and are thus resistant to antibiotics. Depending on the host bacteria, the ARG can generate either environmental ARB (eARB) or pathogenic ARB (pARB)(Ashbolt et al., 2013). The direct risk for human health comes through pARB. However, eARB can also be resistant to antibiotics as antibiotics do not discriminate for pathogens in their attack of a bacterium. The risk from these bacteria is due to the potential of their resistant genes being passed on to pathogenic bacteria (Baquero et al., 2008).

Cells can obtain antibiotic resistance through horizontal (HGT) or vertical gene transfer (VGT). VGT is also called mutation, and is a spontaneous change in the DNA that can be selected due to the presence of an antibiotic. HGT is a (faster) significant platform for acclimatization to environmental stress by horizontally transferring across bacterial phyla. As shown in Figure 1.2, 1.3 and 1.4 the three mechanisms for HGT are transformation, transduction and conjugation (Furuya and Lowy, 2006).

The first mechanism for HGT is transformation in which free DNA, released by cell lysis, is taken up by the cell from its environment (Figure 1.2). For this mechanism, there is no need for protein-mediated mechanisms to occur as it uses the natural capability of prokaryotes to uptake plasmids or fragments (Keen and Fugère, 2017).

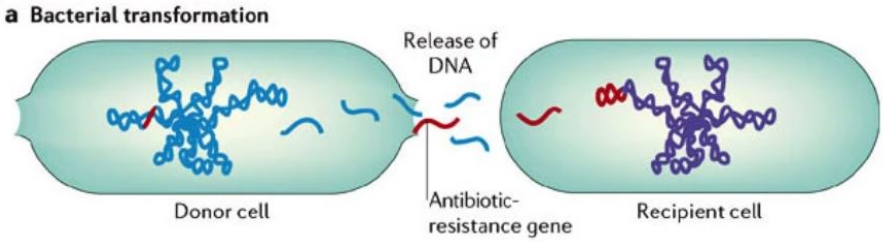

Figure 1.2: Horizontal gene transfer: transformation (Furuya and Lowy, 2006)

The second mechanism of HGT is transduction for which bacteriophage capsules are used to transfer genetic material (Figure 1.3). Host genome segments can be randomly included in the bacteriophage capsule in some circumstances. The capsule can directly be transferred to the host by infection (Doulatov et al., 2004).

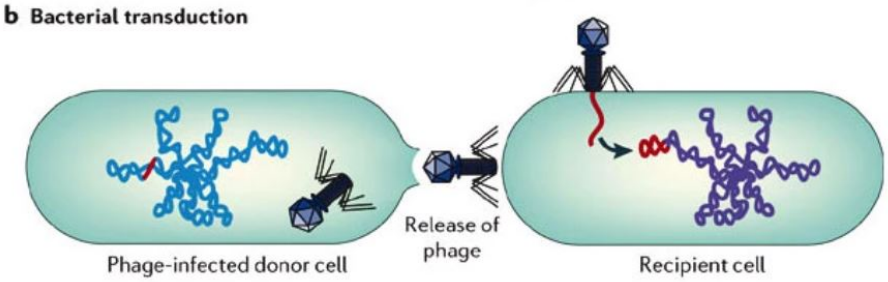

Figure 1.3: Horizontal gene transfer: transduction (Furuya and Lowy, 2006)

Lastly, for conjugation (Figure 1.4) a conjugative bridge is constructed between the donor and the recipient bacterium through which mobile genetic elements (MGEs), such as plasmids or transposons, carrying genetic information are transferred (Smillie et al., 2011). Genes encoding antibiotic resistant materials can be located on these MGEs. Consequently, these MGEs have the ability to facilitate the mobilization or rearrangement of ARGs within a cell or between different bacteria. Part of the genes can be transposed from the MGE into the genome of the cell, making the cell, including its offspring resistant. The presence of a MGE in a cell itself can also lead to temporary resistance for as long as the MGE is present in the cell and the genes are expressed. The ARB can thus become ASB again after losing the plasmids and in that case the offspring does not necessarily become resistant as well (Furuya and Lowy, 2006).

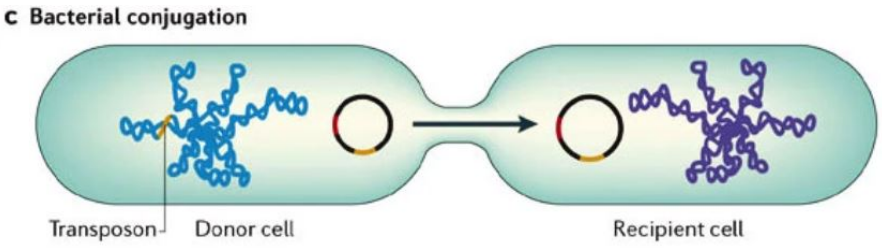

Figure 1.4: Horizontal gene transfer:conjugation (Furuya and Lowy, 2006)

1.2.3. Mechanisms of antibiotic resistance

There are several modes of action for antibiotics to either destroy (bactericidal) or slow down the growth (bacteriostatic) of the bacteria. Consequently, there are several mechanisms of antibiotic resistance to stop or reduce the effects of these modes of action. It is a complex issue as the existence of over 20,000 potential ARGs of nearly 400 different types is predicted from available bacterial genome sequences and listed in a database (Liu and Pop, 2009). Only a selection of the most common or worrisome mechanisms is given.

Some bacteria are already intrinsically resistant to certain antibiotics, not because they carry ARGs, but because they lack the antibiotic specific target. For instance, a separation can be made between gram positive and gram negative bacteria. Gram positive bacteria have a thicker peptidoglycan layer whilst gram negative bacteria have an extra membrane layer. Gram negative bacteria already have resistance to antibiotics such as penicillin as these antibiotics are not able to get through the extra membrane layer. As human cells are eukariotes, they don't have a cell wall (peptidoglycan) and are therefore also not affected by such treatments (Vellai et al., 1998).

Next to intrinsic antibiotic resistance, other mechanisms of resistance are encoded on the ARGs. The Beta-lactam antibiotics for example inhibit the synthesis of the peptidoglycan layer on the cell wall, which provokes the death of the cell by rupture. One mechanism of antibiotic resistance encoded by ARGs produces enzymes, called beta-lactamases, that break the so-called beta-lactam ring and this way inactivate the antibiotic before it is able to attack the cell wall (Fernandes et al., 2013). ARGs encoding for beta-lactamases are generally noted as $bla_{specific\ enzyme}$ (Munita and Arias, 2016).

Another group of antibiotics is the Polimixins. Polimixins disrupt the cell membrane, affecting its permeability and ultimately causing massive leakage of the cells contents. It is a group of antibiotics that mostly affects Gram-negative bacteria. Some ARGs, such as *mcr-1*, can modify target proteins so the antibiotic cannot attach itself to this protein anymore (Baron et al., 2016).

More examples of antibiotic groups are the ones target the synthesis of genetic material (such as macrolides or quinolones), or the production of proteins by blocking the ribosomes (aminoglycosides). For each group, again several mechanisms of resistance against the modes of action exist.

1.3. Indicators of fecal contamination and ARB

For this research municipal wastewater is used, thus the risk of fecal contamination and their AR is of great importance. Bacteria can act as indicators of fecal contamination in water and the health risks that accompany such a contamination. The indicators do not necessarily have to be pathogenic themselves, but indicate the presence of a larger group of bacteria, viruses and protozoa originating from the guts of humans and other warm-blooded animals. The wastewater can contain a wide variety of pathogens that are often in too low concentrations to detect in a reliable way, but are still hazardous to human health. Although originating as a tool in drinking water management to indicate contamination with sewage, recreational waters and foodborne exposure routes are nowadays also typically obligated to assess fecal contamination. *Escherichia coli* (*E. coli*) and enterococci are the the most typically used bacterial indicators of fecal contamination, used to indicate hazard and to test regulatory compliance (Holcomb and Stewart, 2020). For this research these two are measured including one resistant strain of each; Extended-Spectrum Beta-lactamase (ESBL) producing *E. coli* and Vancomycin Resistant Enterococci (VRE) respectively.

1.3.1. E. coli

E. coli is a fecal coliform bacterium used as an indicator organism to monitor the microbial quality of water and thus as hazard identification and for regulatory compliance. *E. coli* is, just as all coliforms, gram-negative (Berg, 2008). As mentioned before, most strains of *E. coli* are non-pathogenic. However, some can cause human illnesses such as diarrhoea and stomach cramps. The non-pathogenic strains are part of the normal microbiota of human and warm blooded animal intestines, producing vitamins and preventing colonisation of the guts with pathogenic bacteria. As their origin is almost exclusively fecal and they appear in much higher concentrations than other fecal bacteria, the monitoring can provide valuable insights into the routes of fecal contamination (Keen and Fugère, 2017).

1.3.2. Extended-Spectrum Beta-lactamase (ESBL)-Producing *E. coli*

In WWTPs high resistance rates of *E. coli* for, among others, amniopenicillins, sulfonamides and tetracyclines are found. ESBL-producing *E. coli* is listed as a "serious threat" due to its ability to transfer resistance within the Enterobacteriaceae family, which includes germs that can cause common infections such as urinary tract infections (Solomon and Oliver, 2014). Conventional WWTP are unsuccessful in the removal of these ARB and ARGs and even a slight increase was found by Ferreira da Silva et al., 2007 using culture methods. ESBL-producing *E. coli* are reported to be released in large numbers into the environment and is therefore used as an indicator for the assessment of environmental antimicrobial resistance(Bréchet et al., 2014, Holcomb and Stewart, 2020).

1.3.3. Enterococci

One of the other most studied bacteria in WWTP are enterococci. Enterococci are gram-positive cocci of the lactic acid bacteria. To date, 12 pathogenic species of Enteroccoci are identified of which two species are common commensal organisms, *E. faecalis* (90–95%) and *E. faecium* (5–10%). Both originating from the intestines of humans (B. E. Murray, 1990). Pathogenic enterococci cause between 5 and 15% of cases of endocarditis, which is best treated by the combination of a cell wall-active agent (such as penicillin or vancomycin) and an aminoglycoside as together they form a synergistic bactericidal effect. High inherent and acquired resistance traits were found for enterococci in WWTP including aminopenicillins and sulfonamides (Keen and Fugère, 2017). Penicillin resistance and vancomycin resistance are the most recent and worrying traits reported of them as both can be transferred to other enterococci, resulting in an increase in enterococci for which no treatments is adequate (B. E. Murray, 1990).

1.3.4. Vancomycin Resistant Enterococci (VRE)

As stated earlier, Vancomycin is an important antibiotic for the treatment of enterococci related infections as it is a last resort antibiotic against a broad range of gram-positive bacteria (such as *Staphilococcus aureus*, *Clostridium dificile*, *S. pneumoniae*). The organism itself is not the largest threat, however they are very capable of transferring resistance genes to other gram-positive bacteria. VRE is listed as a serious threat by the Centers for Disease Control and prevention (CDC) as it is responsible for a large part of the nonsocomial infections (CDC, 2019). In the USA enterococci is responsible for 9% of the nosocomial blood infections of which 60% of the isolates for *E. faecium* is Vancomycin-resistant (Tacconelli and Cataldo, 2008). As a percentage of the total enterococci detected, in secondary effluent samples taken in Europe 2-3 % VRE was found. This number is 52% for WWTPs that treat the water with Chlorination as a tertiary treatment step (Keen and Fugère, 2017).

1.4. Other microbial (non AR-related) fecal indicators

For some of the experiments, using the water samples from the O3-STEP$^{(R)}$ and the 1-STEP$^{(R)}$ filter, some extra indicators are included. Next to (antibiotic resistant) bacteria; viruses and protozoa are affected by the treatment with ozone and coagulants. As indicators, *Clostridium perfringens* (*C. perfringens*) and bacteriophages are included in this research. The focus of this research will not be on these indicators, however they are are useful tools for the assessment of the general disinfection performance.

1.4.1. Protozoa

Protozoa are unicellular eukaryotes, feeding on organic matter including other microorganisms. Well-known waterborne pathogenic protozoa are Giardia and Cryptosporidia. Protozoa have two life phases, the vegetative and resistant (cysts) phase. During the cysts, protozoa are much more resistant to treatment techniques than viruses and bacteria vegetative forms. This results in the survival and growth of protozoa after exposure to levels of disinfection (such as chlorine) that would have killed free-living bacteria. Several bacteria even show a higher resistance to disinfection treatment as they are digested by and surviving within protozoan cells (King et al., 1988). Previous research has shown that ozone at an appropriate concentration inactivates certain pathogenic protozoa that show resistance to methods as chlorine. This is explained by ozone destroying the protozoa cell membrane (Khalifa et al., 2001).

As protozoa are difficult and expensive to measure and this research is executed in a level 1 lab, *Clostridium perfringens* is used as an indicator. According to Payment and Franco, 1993, *Clostridium perfringens* (*C. perfringens*) appear to have a relationship to cysts and oocysts and could be used as indicators of their inactivation and removal. The cysts of protozoa can live under harsh conditions, hence following the *C. perfringens* gives valuable information on the removal of protozoa in the filter.

1.4.2. Viruses

Viruses are small (between 20 and 400 nm) particles identified between live beings and inert matter. Contrary to bacteria, viruses are always harmful and can cause several diseases as they can only live and reproduce parasiting cells and by doing so causing their destruction. Ozone has proven to be an effective treatment technique as it acts on viruses by oxidizing the proteins of their envelope and modifying their three-dimensional structure. This way, the virus cannot anchor itself onto the host

cell and thus cannot reproduce resulting in its death. Some types of viruses are less resistant and are completely destroyed by ozone treatment (Rojas-Valencia, 2011). For this research somatic coliphages are used as an indicator for the virus removal.

1.5. WWTP as ARB hot spot

Wastewater treatment plants do not aim for effective removal of ARB and pathogens from the water but to remove solids, degradable organic substances and nutrients (Ravasi et al., 2019). However, as a side effect conventional WWTPs normally obtain a 2-3 log removal of microorganisms in conventional biological treatment (Karkman et al., 2018). Similar results were obtained for the AR strains of these microorganisms (Bicudo et al., 2021). It is to be determined if the ARGs are removed equally or contain a preferential advantage for resistance determinants. In the WWTP the antibiotic resistance is driven by two factors: first of all the relatively high concentrations of antibiotic residues that can select for antibiotic determinants whilst a relatively low concentration is needed to select for AR. Even at a non-lethal concentration several hundred-folds below MIC (which for VRE is between 2-8 μg/l(Taučer-Kapteijn et al., 2016)) an antibiotic can already select for resistant bacteria (Karkman et al., 2018, Andersson and Hughes, 2014). Secondly the abundance and diversity of bacteria that are either suspended in the influent or grown in biofilms and flocs (Rizzo et al., 2013). In fact, in biological tanks, characterized by the continuous mixing between a large number of microorganisms and pollutants, bacteria can easily mutate and exchange resistance (Ravasi et al., 2019).

On top of these factors, the so called co-selecting residues, such as biocides and metals, positively correlate to the amount of ARB in the WWTP in both water and sludge phases (Ashbolt et al., 2013). Co-selection entails the localization of multiple resistance mechanisms on a single MGE. As mentioned in Section 1.2.2, MGEs constitute the driving force in HGT as they help by arranging genes within a bacterium or between bacteria. As metal resistance genes are often localized together with ARGs on the MGE the presence of a metal can select for ARGs without the occurrence of an antibiotic (Keen and Fugère, 2017). For example, the presence of metals such as arsenic (As) can select for a reduced permeability for the As itself whilst at the same time reducing the permeability for antibiotics such as beta-lactams. The same is found for the alteration and efflux of As as well as of beta-lactams (Baker-Austin et al., 2006). Cross-selection is a similar mechanism in which the resistance against a single antibiotic confers resistance to a larger group of antibiotics belonging to the same class. This cross-resistance occurs when different antibiotics attack the same target, initiate a common pathway to cell death, or share a common route of access to their respective targets (Keen and Fugère, 2017, Chapman, 2003).

1.6. Effects of antibiotic resistance in downstream environments

If a WWTP can create a hotspot for ARB and ARGs, it is important to look into the consequences of effluent and WWTP byproducts on the downstream environments. The effects of AR in the environment have been controversial in literature. As ARGs can be found everywhere, stating that they form a direct risk would lead to unworkable interventions. The ARGs become a risk for humans when they are acquired by pathogenic bacteria and they rarely exist in the same microbial ecosystem. On top of that, humans need to be exposed to these bacteria to get infected (Bengtsson-Palme and Larsson, 2015). Nonetheless, the effects for public health can be disastrous when antibiotic treatment loses its effectiveness. The WHO therefore declared AR as one of the five biggest global public health issues (WHO, 2019, O'neill, 2016). This is why the so-called relative risk, should be based on the chance of the acquirement of resistance and the public health criteria. Figure 1.5 shows how ARGs or antibiotics in the environment could become a risk for human health (Ashbolt et al., 2013).

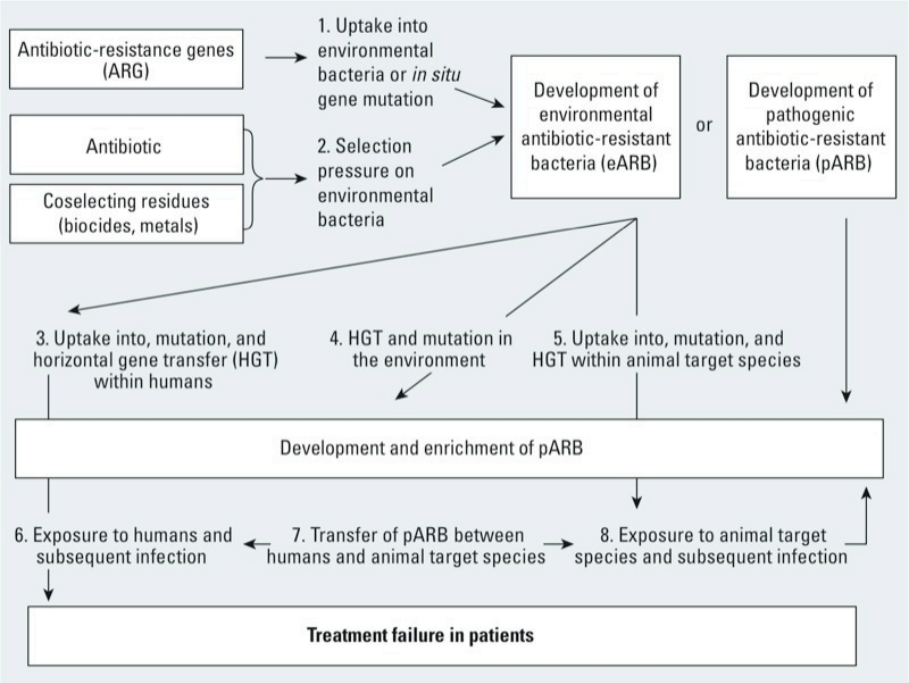

Figure 1.5: Risk assessment path for antibiotic resistance (Ashbolt et al., 2013)

An *E. coli* O104:H4 outbreak in Germany in 2011 demonstrates the risk to human health when humans get in touch with pathogens that have acquired AR. During this event there were 3,816 infections, most likely caused by the consumption of sprouts, which lead to 54 deaths in total. During the outbreak a resistance to beta-lactam antibiotics, third-generation cephalosporins and a partial resistance to fluorquinolones was classified. What caused the sprouts to get infected is unknown (Frank et al., 2011, Burger, 2012).

Due to the controversy about the risks of ARB and ARGs from WWTPs on the environment, regulations concerning AR are absent in most countries (Paulus et al., 2019). Only regulations on the level of *E. coli* and Enterococci exist in certain types of water such as swimming water (Table 5.1). Recently there is an increasing focus on adding a tertiary treatment step for the removal of Organic Micropollutants (OMPs) as well. If ARB an ARG treatment does become regulated, the usage of the water will be important to consider in these regulations. Specifically, if the water is intended to be used for swimming or reuse, the likelihood of human exposure is high and thus the regulations hold a greater significance than in other settings.

1.7. Effects of treatment methods on ARB and ARGs

In WWTPs 2-3 log of the indicator ARB can be removed, which is similar to the ASB removal (Ravasi et al., 2019). Although not aiming for the removal of ARB and ARGs, tertiary treatment methods may add to this removal as well (Novo et al., 2013, Keen and Fugère, 2017). Advanced treatment methods include physical processes (such as Reverse Osmosis, ultrafiltration (UF), membrane bioreactors and micro-filtration), biological processes (like wetlands), physical-chemical processes (coagulation, flocculation and sedimentation) and lastly chemical (oxidation) processes to degrade the bacterial load (Keen and Fugère, 2017). In this research two different oxidation processes will be compared: ozone and chlorine. Furthermore three different coagulants are compared. Lastly, the effects of the GAC of the O3-STEP$^{(R)}$ filter on the antibiotic resistance will be discussed briefly.

1.7.1. Ozonation

Ozone (O_3) is a strong oxidant that has shown to be effective for the inactivation of microorganisms and oxidation of inorganic ions (such as iron and ammonium) and organic pollutants. With a boiling point of -111.9 ℃, O_3 exists at room temperature as a bluish coloured gas (Rodríguez et al., 2008). When this gas is dissolved into water, it is highly unstable due to its self-decomposition as shown in Equations

1.1a and 1.1b.

$$O_3 + OH^- \rightarrow HO_2^- + O_2 \quad (1.1a)$$

$$O_3 + HO_2^- \rightarrow OH^- + O_2 + O_2^{\cdot-} \quad (1.1b)$$

As ozone has an oxidation potential of 2.07 V, it is a one of the strongest oxidation agents in water treatment. On top of that, ozone oxidation is an advanced oxidation process (AOP), which means it treats the water not only directly but also by generating powerful radicals such as $HO^{\cdot}$ with an oxidation potential of 2.8 V, $O_3^{\cdot-}$ and $HO_2^{\cdot}$ as is shown in Equations 1.2a, 1.2b and 1.2c (Glaze et al., 1987).

$$O_3 + O_2^{\cdot-} \rightarrow O_2 + O_3^{\cdot-} \quad (1.2a)$$

$$HO_3^{\cdot} \rightarrow HO^{\cdot} + O_2 \quad (1.2b)$$

$$HO^{\cdot} + O_3 \rightarrow HO_2^{\cdot} + O_2 \quad (1.2c)$$

The mechanisms of direct O_3 oxidation competes with the indirect oxidation with the oxidation agents. Where O_3 is in abundance, it is also slower than the oxidation agents. The molecular ozone reactions select for organic molecules with nucleosphilic moieties whilst the reactions with $HO^{\cdot}$ are non-selective and thus attack organic as well as inorganic compounds (Keen and Fugère, 2017). At a higher pH (above 8), more $HO^{\cdot}$ are generated and a higher oxidation capacity is achieved than with an acidic pH (Benner and Ternes, 2009). However, when ozone is used as disinfectant, indirect ozone reactions only play a minor role (Von Gunten, 2003).

Ozone is an excellent disinfectant as it has has shown to deactivate pathogenic microorganisms resistant to conventional disinfectants such as chlorine (Rodríguez et al., 2008, Von Gunten, 2003). Ozone obtains its effectiveness against bacteria, and thus potentially ARB, by reacting with organic functional groups within the cellular wall/membrane of Gram positive bacteria and in the wall of Gram-negative bacteria. Firstly, the ozone reacts with the unsaturated bonds within the membrane-bound phospholipids and lipopolysaccharides on the surface of the bacterial cell. Then, when the membranes permeability and structural integrity is disrupted, the interior gets exposed to the external conditions, resulting in leakage of the cellular compounds. The ozone oxidation is less effective in the removal of the inner molecular components, such as DNA, so they might remain present in the water after the ARB cell lysis (Dodd, 2012).

In wastewater treatment, the ozone concentration is often reported in grams O_3 per gram DOC (g O_3/g DOC) to compare treatment efficiency in different source waters with the same DOC concentrations. According to the research of Lüddeke et al., 2015, dosing 0.73 mg O_3/mg DOC in treated sewage led to a removal of 3.8 log for *E. coli* and Enterococci. For the antibiotic resistant strains of these bacteria a removal of 3.5 and 4.2 log was found respectively. For the inactivation of ARGs ozone showed a lot less effective results. According to Zhuang et al., 2015 ozone is less effective in the reduction of genes than chlorine and UV are.

Ozone solubility in water

The efficiency of the ozone treatment depends on the rate of oxidation and thus the concentration of dissolved ozone in the solution, C_{O3}. Equation 1.3 shows the mass balance of dissolved ozone.

$$\frac{dC_{O_3}}{dt} = K_L a(C^*_{O_3} - C_{O_3}) - k_{O3} C_{react} C_{O3} - k_d C_{O3} \quad (1.3)$$

In this equation the term on the right is the rate of oxidation in which the k_d value is the ozone decomposition kinetic constant. The second term is the part of the ozone that reacts with compounds in the water where k_{O3} is the reaction rate of this compound with ozone and C_{react} the concentration of this compound. The left term gives the rate in which the ozone dissolves in the solution in which $k_L a$ is the volumetric transfer coefficient, C^*_{O3} is the maximum ozone solubility in water and C_{O3} the concentration of dissolved ozone. When assuming ideal gas behaviour and no ozone transfer resistance, Henry's law can be applied for the solubility of ozone in water.

$$C^*_{O_3} = \frac{H_e}{P_{O3}} \quad (1.4)$$

In Equation 1.4 the P_{O3} is the partial pressure of ozone and H_e the Henry's law constant that will be larger at lower temperatures. Thus for lower temperatures, a higher solubility can be obtained (Rodríguez et al., 2008).

Disinfection byproducts (DBPs)

The formation of several organic as well as inorganic disinfection byproducts (DBPs) are identified during the ozonation/disinfection. Especially when removing the microorganisms resistant to other disinfectants, high concentrations of ozone are needed and thus high concentrations of the DBPs are found. The by-product of main concern is bromate, which is formed in bromide-containing waters. It is of concern as it can cause cancer and is not biodegraded in biological filters (Von Gunten, 2003). In certain cases, when bromide concentrations are above about 50 μg/l, it may be necessary to use control measures to lower bromate formation by for example lowering the pH or by the addition of ammonia. Iodate is the main by-product formed during ozonation of iodide containing waters. The reactions involved are direct ozone oxidations. However, Iodate is considered non-problematic because it is transformed back to iodide endogenically thus the main point of concern should be on bromide (Von Gunten, 2003).

1.7.2. Chlorination

Chlorination is chosen as comparator for the ozone in the O3-STEP$^{(R)}$ filter as it has been successfully used for the control of waterborne infectious disease for over a century (Dodd, 2012). In essence the mechanism of chlorine is quite similar to ozone. Both are oxidation methods which induce the leakage of macromolecules from the cells indicating the permeability changes of the membrane. The Reduction-Oxidation (RedOx) potential of chlorine is lower than the RedOx potential of ozone and $HO^{\cdot}$ and thus chlorine is a less reactive element (Lee and Von Gunten, 2012). Most researchers agree that a large part of the ARB are effectively inactivated by chlorination. However, it is considered not effective in controlling antimicrobial resistance as after chlorination a large part of the ARGs in the wastewater remain present (Yuan et al., 2015). After applying chlorination to the treated wastewater, a higher proportion of the surviving bacteria was resistant to ampicillin and cephalothin (G. Murray et al., 1984).

In small facilities chlorine is often dosed as sodium hypochlorite, while at a larger scale chlorine is dosed as a gas. One of the problems with the dosage of chlorine is that in the 1970's the formation of undesirable DBPs were discovered. DBPs, such as trihalomethanes (THM), occur through their reaction with natural organic matter (NOM). These DBPs are undesirable because they carry health risks such as cancer (Bull et al., 1995). This is mostly a problem for drinking water treatment where people directly consume the water. However, avoiding these compounds to end up in the environment is favourable, especially as wastewater typically has a high NOM content.

1.7.3. Coagulation

In the O3-STEP$^{(R)}$ filter, after ozonation, a coagulant is dosed in the supernatant water above the GAC filter (Figure 1.1). Coagulation is the process by which solutions are destabilized in order to form particles (Bratby, 2016). For coagulation, two mechanisms are commonly described to add to the removal: adsorption (or charge neutralization) and sweep coagulation (Dennett et al., 1996). For the O3-STEP$^{(R)}$ filter, the focus is on the first mechanism of coagulation where dissolved contaminants are adsorbed to the coagulant. This is because the filters main aim for the coagulation dosage is the removal of phosphorus (P). A reaction occurs between the contaminant (P) and the coagulant, leading to the removal of the P (Figure 1.6). The second mechanism refers to contaminant removal through the formation of solid precipitates (Dennett et al., 1996). On top of the P-removal, the coagulation may lead to the removal of more contaminants, such as (antibiotic resistant) bacteria that are removed by enmeshment or entrapment within a mass of the solid precipitate. The latter is of importance for this research. Figure 1.6 shows the schematics of these different mechanisms of removal.

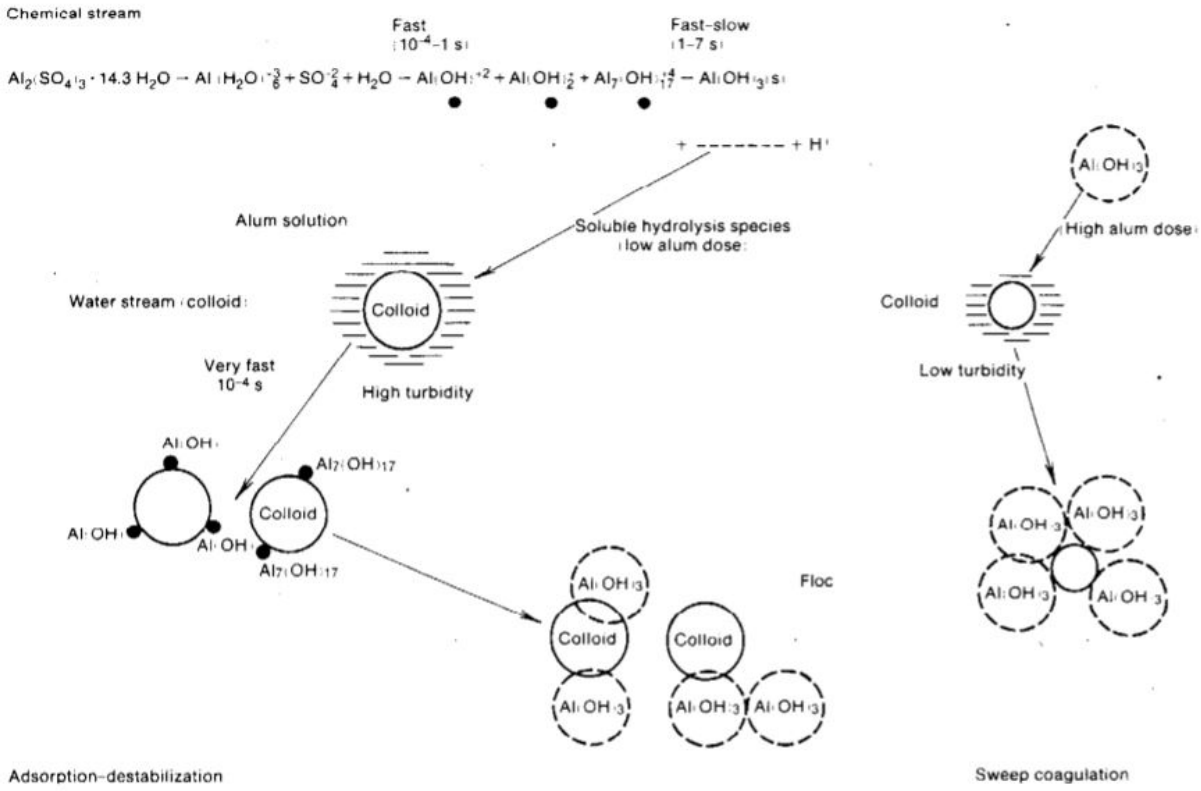

Figure 1.6: Mechanisms schematics of aluminium coagulation (Amirtharajah and Mills, 1982)

The focus of this research is on two different types of metal coagulants, iron (Fe^{2+}) on the one side and aluminium (Al^{3+}) on the other side. By using a Fe^{2+} coagulant, either dosed as a chemical with chloride or by using electrocoagulation (EC) when chloride is undesirable, oxidation takes place to create Fe^{3+}. Resulting in an extra treatment mechanism; disinfection by the oxidation of iron with the use of intermediate oxidants. Unlike the previously discussed ozone, this coagulant does not work as a common “primary” oxidizer. This means that its action is due to an intermediate oxidant that is formed when the iron coagulant is fully oxidized (Heffron et al., 2019). Examples of intermediate oxidants include the Reactive Oxygen Species (ROS) that potentially help with the treatment through disinfection and breaking down OMPs. ROS are the partially reduced molecules containing oxygen and are often highly reactive. Examples of ROS associated with the reaction from Fe^{2+} to Fe_{3+} are hydroxyl ($^{\cdot}OH$), superoxide ($^{\cdot}O_2-$) and peroxide ($ROO^{\cdot}$) radicals. The $^{\cdot}OH$ shows largest impact on the *E. coli* inactivation. The mechanism of disinfection is therefore comparable with the mechanism as described with ozone. However, in natural waters, due to the high NOM content bacteria attenuation by Fe-EC is primarily due to physical removal with flocs (Delaire et al., 2016).

The other considered coagulant is aluminium which is widely used as coagulant in water treatment plants in the form of Aluminum sulfate or polyaluminum chloride (PAC) (Boaventura et al., 2000). When using the aluminium coagulant, no oxidation takes place and thus only removal by the adsorption and sweep coagulation is expected. As the coagulation of the sample traps and removes bacteria, viruses and protozoa, the lack of disinfection is hypothesised to have no practical effect on the O3-STEP$^{(R)}$ filter. Furthermore, no selectivity for ASB compared to resistant bacteria is expected. The difference between the coagulants is expected to be found only in the sludge production. Not only because the activity of the microorganisms in the sludge might be lower after disinfection, but also by the upcoming knowledge on the potential harmful effects of aluminium contamination in the sludge.

For the effective removal of pollutants, it is essential that the right amount of coagulant is dosed. By using the Amirtharajah diagram, this amount can be decided based upon the pH and the type of removal that is desired. The diagram is split into regions of sweep coagulation and charge neutralization for turbidity removal (Figure 1.6). Whilst for the removal of dissolved contaminants such as P the adsorption is of importance, for the removal of microorganisms the sweep coagulation is more impor-

tant. For sweep coagulation, at an equal pH a higher Aluminium dose should be added to the water. Thus the goal is to find an area where the combination of sweep coagulation and adsorption can be obtained. Figures A.1 and A.2 in Appendix A show the Amirtharajah diagrams for Aluminium and Iron.

The expectation for the coagulant is that the choice between aluminium or iron does not have meaningful effects on the removal of resistant bacteria as for the entrapment in the flocs, it does not matter if the bacteria are first oxidised or not. Furthermore, there are no expected differences between the ASB and resistant bacteria. The dosage of coagulant will however have meaningful impacts as the adsorption and removal of P requires much lower concentrations than sweep coagulation and thus than is needed for the removal of (antibiotic resistant) bacteria.

1.7.4. Effectiveness of the GAC filter

After ozonation and coagulation, the O3-STEP$^{(R)}$ filter treats the water with granular activated carbon (GAC) filtration. The GAC filter is designed to adsorb the antibiotics and other OMPs from the water. No proof is found yet to confirm the contribution of the adsorption process to the disinfection of urban wastewater and the fate of ARGs during the combined process (Michael et al., 2019). According to a pilot research in Germany (Lüddeke et al., 2015), the removal of AR Enterococci and *E. coli* shows the same order of magnitude for the combination of ozone and GAC as for the removal of ozone only (3.6-3.9 log-units). Research about the removal of ARB with powdered activated carbon (PACa) treatment showed a removal for both susceptible and resistant microorganisms in the treated water of 99.70% (Ravasi et al., 2019). This is not directly comparable to a GAC filter as PACa is directly mixed with the wastewater and therefore PACa has no filter on which a biofilm grows.

Eventually a biofilm will grow on the GAC granuals. Thus a risk occurs that these bacteria become antibiotic resistant due to the presence of ARGs, that are not as effectively removed by ozone, and antibiotics in the water. In drinking water treatment, Wan et al., 2021 described that in biological activated carbon (BAC) filters, an accumulation of ARGs could exist as a result of HGT. According to this research, backwashing did not remove these ARGs, but even caused a relative abundance of 1.5 to 3.8-folds compared to ASB. During filter ripening the absolute ARGs abundance even increased by 0.9 to 1.12 log, meaning they accumulate in this period. This can lead to the pollution with ARGs of the effluent of these filters as well. Most of these bacteria are not harmful for humans. However, when released into the water, HGT can potentially add to the spread of resistance to pathogens.

2

Research approach

2.1. Problem statement and aim

As the O3-STEP(R) filter is originally designed to remove micro-pollutants, the impact on the Antibiotic resistant bacteria and genes (ARB/ARG) is still unknown. Literature does not give a clear picture of ARB and ARGs in these purification steps, and certainly not in the combination of these processes. The aim of this research is to measure the effectiveness of primarily ozone and coagulation for the removal of antibiotic resistance in comparison to conventional disinfection methods such as chlorine. The research will also investigate using two different water matrices to test the generalisability of the effectiveness of the filter.

2.2. Research questions

The research question is: How effective is the treatment with ozone and coagulation in the removal of antibiotic resistant bacteria compared to ASB. How does this compare to chlorine disinfection?

Five sub-questions are posed to provide an answer to the main research question:

1. Is there a difference between the removal of ARB and ASB when using ozone treatment?
2. Is there a difference between the removal of ARB and ASB when using coagulation?
3. What is the composition of bacteria in the GAC filter and does the GAC filter enhance the growth of resistant bacteria?
4. What is the influence of the water matrix on the performance of ozone and coagulation?
5. What are the potential changes to the design of the O3-STEP(R) filter when aiming to remove ARB as well?

2.3. Hypothesis

Based upon the literature, the hypothesis is that ozonation will disinfect ARB as good as ASB. For the coagulation it is expected that a higher coagulant dose is needed for attenuation of both the ARB and ASB. Some ARB might grow in the biofilm during the GAC filter step and although these bacteria are not directly harmful, they can pass down their resistance to harmful bacteria.

3

Materials and methods

3.1. Waste water samples

Different types of samples were collected from different sampling sites. For the ozone and chlorine efficiency tests secondary effluent of WWTP Horstermeer (every other week morning grab samples in January and February 2021 at location A of Figure 3.1) as well as of WWTP Harnaschpolder (every other week morning grab samples in January and February 2021) were taken. For the coagulation experiments, only grab of location Horstermeer after ozonation (Location B) are taken (March 2021). 10 liter of effluent per plant was stored in jerrycans in the refrigerator below 5 °C to prevent degradation and microbial activity (Keen and Fugère, 2017).

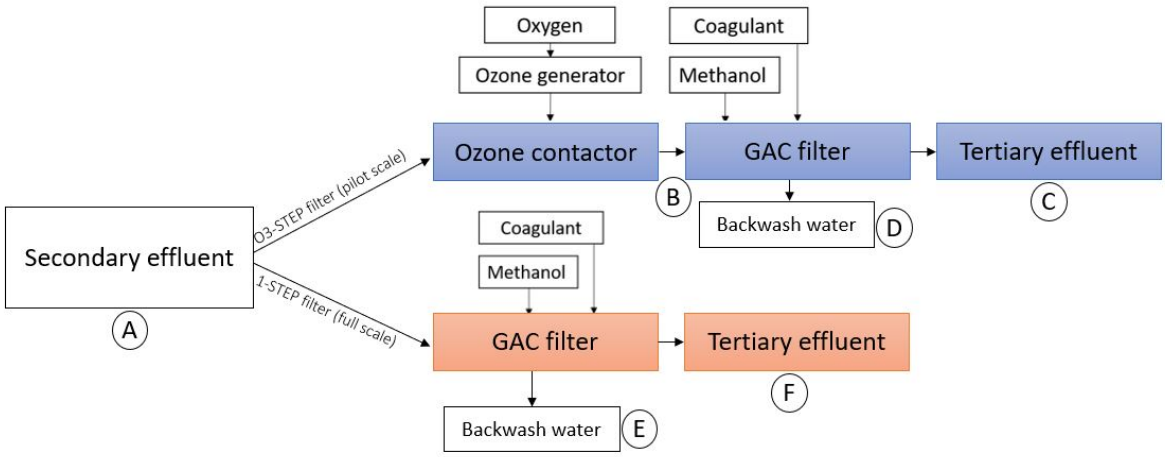

Figure 3.1: Sample locations (letters) of the pilot O3-STEP$^{(R)}$ at top and the fullscale 1-STEP$^{(R)}$ below

In April 2021, another set of grab samples was taken from WWTP Horstermeer from the secondary clarifier, after the ozone step (location B), after the GAC filter (location C) so directly before the water is discharged and from the backwash water of the O3-STEP$^{(R)}$ filter (location D). Furthermore, samples were taken from the effluent (location F) and backwash water (location E) of the fullscale 1-STEP$^{(R)}$ filter. The latter was done because the biological filter of the O3-STEP$^{(R)}$ filter is not yet fully grown. The samples were analyzed preferably immediately, or within the first 24 hours to prevent decay of the bacteria (Keen and Fugère, 2017).

WWTP Horstermeer has a influent capacity of 180,000 population equivalents (PE) and is a conventional treatment plant with nitrification and denitrification processes in two anoxic tanks and an aerated tank for nitrogen removal (vereniging nederlandse watersector, 2020). Furthermore, physical-chemical removal of phosphorus is obtained by coagulation. After conventional treatments, part of the water (1550 m^3/h) is treated by the 1-STEP$^{(R)}$ filter to tertiary effluent. This is a GAC filter that has not

been regenerated for eight years and thus likely working as a regular filter with biofilm and coagulant dosage. Next to the full-scale 1-STEP$^{(R)}$ filter, the O3-STEP$^{(R)}$ pilot filter is located through which part of the effluent is filtered (Figure 3.1, designed for 5 m^3/h ozonation and 3 m^3/h GAC filtration). The effluent of Horstermeer is discharged at the river De Vecht. WWTP Harnaschpolder is the largest WWTP of the Netherlands with a capacity of 1.3 million PE. The sewage of Harnaschpolder is treated by using activated sludge without any disinfection. It is pumped over 2.5 km into the North sea to be discharged.

3.2. Wastewater quality parameters

The samples were tested for several water quality parameters:

- The pH of the sample is of importance for several factors such as the RedOx potential (Lee and von Gunten, 2012). During ozone treatment, more radicals are formed at a higher pH (Benner and Ternes, 2009). For the coagulation keeping the pH stable is important to be able to distinguish the effects of the coagulant from the effects of the pH. Furthermore, the pH indicates reactions taking place in the water. The pH was measured by using the inoLab_IDS multimeter with the WTW pH-Electrode Sentix 940 probe.
- The electronic conductivity (EC) was measured by using the inoLab_IDS measurement as well but with a WTW TetrCon 324 probe. The conductivity is the ability of water to conduct an electrical current. As dissolved ions are conductors, a large EC will mean there are more dissolved ions in the water.
- The dissolved organic carbon (DOC) was measured by using a HACH kit (LCK380 range 2-65 mg/L) for the total organic carbon (TOC) kit after the sample was filtrated with a 0.45 micron filter. This way the suspended solids were filtered out and only the DOC was present in the sample. After the samples were prepared, they were measured in the HACH Lange DR3900 spectrophotometer. The DOC is important to know as the ozone concentration is dosed upon this. To improve the accuracy, the results of the HACH kit were compared with the results by using a TOC analyser (Shimadzu TOC-V CPH combined with the ASI-V).
- The turbidity, thus the cloudiness caused by suspended, non-settleable particles in a sample, was measured by using a turbidimeter (HACH 2100N).
- The water colour was measured with absorbance using a spectrophotometer (GENESYS 10S UV-Vis) set at a wavelength of 254 nm. This data gives insights on the organic carbon content in the water.
- The Phosphate (PO_4) and chemical oxygen demand (COD) were also measured by using HACH kits (LCK349 range 0.15-4.5 mg/L and LCK314 range 15-150 mg/L respectively), analysed in the HACH Lange DR3900 spectrophotometer.
- The total nitrogen (TN) was measured by using Merck photometric kits (114537, range 0.5-15 mg/L N), analysed in the Merck NOVA 60 Spectroquant.

All measurements were done in the laboratoryin triplicates of which an average with standard deviation is taken.

3.3. Target microorganisms

The focus of this research is on antibiotic resistant bacteria (ARB). As Enterococci and *E. coli* are often used as water quality indicators in regulations and monitoring activities (Keen and Fugère, 2017), these are chosen as relevant bacteria's for this research as well. From each of these bacteria one resistant strain is measured. This is for Enterococci the VRE (Section 1.3.4) and for ESBL-producing *E.coli* (Section 1.3.2). As discussed in Section 1.3.1, *E. coli*, and therefore also ESBL-producing *E.coli*, is a gram-negative bacteria and *Enterococcus spp.* and VRE gram-postive bacteria. As such, by using both bacteria, information on the flow of both Gram-negative and Gram-positive resistance traits in the water chain can be provided including their different reactions to disinfection. Furthermore, these bacteria can serve as reservoirs of resistance genes that can be transferred to human pathogens transiting the intestinal tract (WHO, 2017). The removal of somatic coliphages and *C. perfringens* is taken into account as well to asses the performance of the O3-STEP$^{(R)}$ filter.

3.3.1. Agar growth media

For the assessment of antibiotic resistant bacteria, culture-based analysis was done. On a selective media, bacteria were grown including a resistant strain of it. This method only gives a selection of usually anthropological associated culturable bacteria, such as *Enteroccocus* and Enterobacteriaceae (Keen and Fugère, 2017). By using a different growth medium, the ASB was distinguished from the resistant bacteria. Table 3.1 gives an overview of the tested microorganisms and the correlated culture media.

Table 3.1: Parameters and standard deviations of Horstermeer and Harnaschpolder

Microorganism	Culture media	Identification	Incubation
E. coli	Chromocult coliform agar	Violet colonies	19-23 h at 34-38 °C
ESBL *E. coli*	Biomerieux Chromid ESBL	Pink & violet colonies	18-24 h at 35-37 °C
Enterococci	Slanetz-Bartley	Red & pink colonies	40-48 h at 34-38 °C
VRE	Biomerieux Chromid VRE	Violet: E. faecium & green: E. faecalis	40-48 h at 34-38 °C
Somatic coliphages	Scholtens agar	Colourless plagues	24 h at 34-38 °C
C. perfringens	Chromacar C. perfringens	Orange colonies	24 h at 37 °C anaerobic

3.3.2. Preparation of the culture plates

To count the colony-forming units (cfu) using the agar media method, the aim was to produce between 30 and 300 cfu per plate. Below 30 there is an effect of randomness and above 300 the plates become uncountable. Different volumes were filtered over a 0.45 micron filter to obtain the suitable number of colonies. No serial dilutions were needed.

When doing filtrations a positive and negative control was done to check for systematic errors. For the negative control, a sample without any bacteria was tested in the same way as was done for the filtration itself. This way, there was a check for contamination in the experiment. The positive control was done with a sample of which it was certain that it contained the relevant bacteria. The positive control is not possible for the ARB and Enterococci as the research is done in a Biosafety Level 1 laboratorywhere it is not allowed to grow or keep dangerous stock bacteria. Therefore only *E. coli* was checked on positive systematic errors by using a pure culture of *E. coli* WR1.

3.3.3. Viruses and protozoa

To assay the somatic coliphages, the ISO 10705-2 protocol was followed. A growth media was made of a broth called ”modified Scholten’s Broth”, mixed with host bacteria (*E. coli* WG5) and the sample. After a day in the incubator (Table 3.1), the viruses showed perforations of the agar. Counting these perforations indicated the amount of viruses in plague-forming units per milliliter (pfu/ml). This was based on the assumption that each plaque formed is representative of one infective virus particle.

For protozoa an indicator bacteria was used (*C. Perfringens*) (Payment and Franco, 1993). To measure the *C. perfringens*, the samples were preheated to 70 degrees for 15 minutes to kill all non-cysts *C. perfringens*. After this, the samples were filtered over membranes and placed on an agar plate, like the other bacteria. However, because *C. perfringens* are strict anaerobes the bacteria needed to grow in an anaerobic environment (Table 3.1).

3.4. Ozone set-up

Figure 3.2 is showing the laboratoryset-up for dosing the ozone gas into the sample (bubbling tank). The ozone was generated after which the ozone gas was continually measured. Then the ozone gas bubbles were sparged through the sample during which it was stirred with a magnetic stirrer. The part of the ozone that was not dissolved nor reacted was measured again before being destructed. The residual concentration of ozone measured at the outflow is the inflow concentration minus the ozone reacted or transferred to the water phase (Equation 1.3).

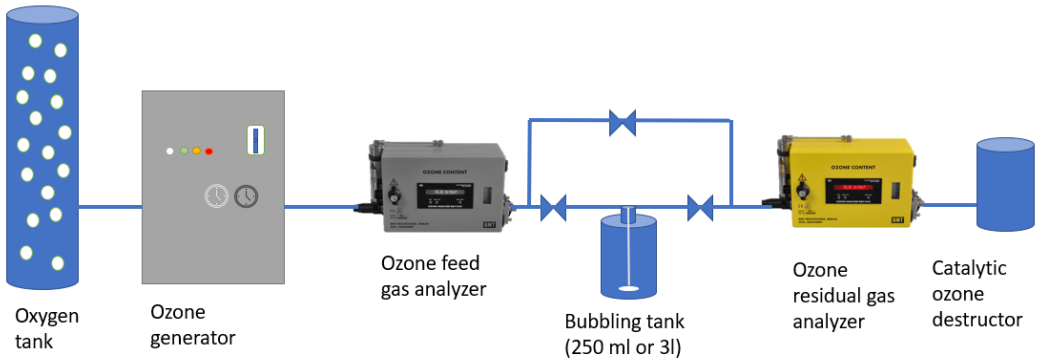

Figure 3.2: Schematic diagram of the experimental ozone set-up

By using this set-up, there are two possible ways of dosing the ozone into the effluent;

- By bubbling the ozone gas directly through the effluent sample (in a 3 liter bubbling tank) or;
- Indirectly, by bubbling ozone up to the maximum solubility in a spike solution (of 250 ml) which can then be dosed in different volumes to an effluent sample.

As the pilot project in Horstermeer is using a side stream in which the ozone is dosed before going to the mainstream and the stock solution allows the ozone concentration to be determined very precise, the ozone spike solution method was used.

The aqueous ozone stock solution was prepared by sparging ozone obtaining oxygen through effluent samples cooled in an ice-bath to increase the maximum solubility of ozone in the water (Equation 1.4). By using the indigo colorimetric method, the ozone concentration in the stock solution was measured (Equation 3.1).

$$mgO_3/L = \frac{\Delta A * 100}{f * b * V} \tag{3.1}$$

This method compares the difference in absorbance of a blank solution with the sample solution (ΔA) at 600 nm (Bader and Hoigné, 1981). The V is the volume of the measured sample and b the path length of the sample cell. The f is conversion factor correcting for the constant loss on the unstable ozone, that is taken as 0.42 as no commercial lot is found to deviate from this number (American Public Health Association, 1992).

After the concentration of ozone in the stock solution and the DOC concentration in the sample were measured, the stock solution was added to the WWTP effluent sample in different proportions to the DOC concentrations. Of the 250 ml spike solution, different volumes were added to a 2 liter jar filled with effluent. The ozone dosage was given in gram O_3/gram DOC so for different water samples a comparable effectiveness can be found. Due to the fast degradation of ozone, it was key to spike the ozone solution within seconds after preparing the ozone solution. Furthermore, the samples were directly covered after dosing so most of the stock solution reacted with the sample rather than degassifying into the atmosphere (Equation 1.3). Quenching is not needed as all ozone can be assumed to have reacted within a small amount of time.

To maintain in a countable range for the agar growth media plates (Section 3.3.2), the aim is to remove between 1 and 2 log-units of *E. coli* and Enterococci. By trying multiple concentrations between 0.2 and 1 mg O_3/mg DOC and keeping the concentration dosed at the O3-STEP$^{(R)}$ pilot plant into account (0.4 mg O_3/mg DOC) concentrations of 0.38, 0.57 and 0.68 mg O_3/mg DOC are dosed which is obtained by dosing 150, 200 and 250 ml of the spike solution per liter effluent sample respectively.

3.5. Chlorination

As mentioned previously, this research aims at the maximum removal whilst preventing the microbial concentration to drop below the measurable range which in practise showed to be between 1 and 2 log of the microorganisms. Different concentrations of Sodium Hypochlorite (NaOCl [1.25%]) in different

reaction times are examined to obtain this removal. For insights on the chlorine concentration in the samples, the free chlorine is measured by using dipropyl-p-phenylenediamine (DPD) measurements (Merck chlor-test range 0.010-6.00 mg/l Cl_2). The free chlorine is measured at three different moments: before treatment, in the concentration after 30 seconds of reacting and the chlorine concentration after quenching. First, the reaction time is calculated based upon the desired concentration*time (Ct) and the concentration of chlorine after 30 seconds of reacting in a 100 ml beaker. After the desired Ct is received, the Free Chlorine is quenched using Sodium Thiosulfate ([1.4% - 0.1M]) to stop the disinfection. After quenching DPD is used to verify that all chlorine is removed from the sample.

3.6. Coagulation set-up

For the coagulation three different coagulants are compared. The coagulant used in the O3-STEP$^{(R)}$ filter, Polyaluminium chloride (PAC), a chemical iron coagulant based on Fe^{3+}, $FeCl_3$ and an iron coagulant based on Fe^{2+} dosed with iron electrocoagulation (Fe-EC). To obtain enough water for the culture-based analysis, all experiments were conducted in 2 liters beaker glasses of ozonated effluent from the pilot. The dosed concentrations are based on the minimum and optimal floc concentration according to Figure A.1 and A.2 (Table 3.2).

Table 3.2: Coagulant concentrations. Fe-Ec at I=0.058 A. Al dosage based on PAX-214 (1.3 g/ml and 7% Al with a 1:10 dilution). $FeCl_3$ dosage based on 195 mg Fe/ml with a 1:10 dilution.

	Fe (mmol/L)	Al (mmol/L)	Fe (mg/L)	Al (mg/L)	Fe-EC (s)	Al-dosage (ml)	$FeCl_3$ (ml)
Based on P-removal	0.004	0.004	0.22	0.11	13.67	0.012	0.011
Minimum sweep for Al	0.03	0.03	1.67	0.81	102.41	0.089	0.086
Optimal sweep coagulation Al	0.2	0.2	11.17	5.40	682.76	0.60	0.57
Minimum sweep for Fe	0.01	0.01	0.56	0.27	34.14	0.03	0.029
Optimum sweep Fe	1	1	55.85	26.98	3413.80	2.96	2.86

3.6.1. Chemical coagulation

Both PAC and $FeCl_3$ were dosed as a chemical, therefore the same procedure is followed. The dosed concentrations are based on the minimum and optimal floc concentration according to Figure A.1 and A.2. These figures also show that pH has a large influence on the floc formation. Therefore the pH is measured and adjusted by dosing NaOH so the pH stays the same as in the original concentration. Together with the coagulant, the NaOH concentrations are dosed in 2 liter beakers filled with ozonated effluent sample.

3.6.2. Electrocoagulation

For the Fe-EC, first two electrodes are prepared, one for an anode and one for a cathode (3.3). By using a 30V-3A TENMA 72-10500 bench DC power supply, a constant current of I=0.058 A is applied for a time specified in Table 3.2. During this period, a magnetic stirrer is used to continuously mix the 2 liter beakers filled with ozonated effluent sample. Furthermore a constant oxygen supply was placed in this beaker. Throughout the experiment, pH, DO and EC are constantly measured.

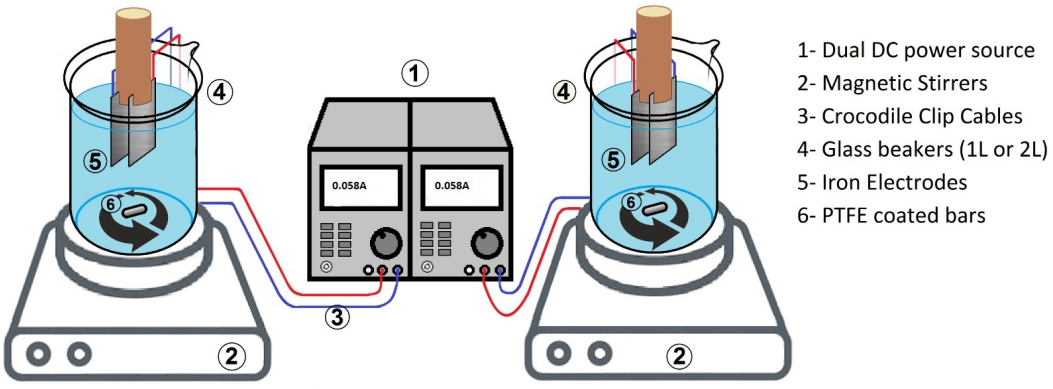

Figure 3.3: Schematic diagram of the experimental Fe-EC set-up (based on Bicudo et al., 2021)

3.6.3. Jar tests

After chemical coagulation or electrocoagulation, the beakers are placed into a jar set-up. For the jar tests, first rapid mixing is applied for 30 seconds (G=150 s^{-1}), then the first slow mixing for 10 minutes at G= 80 s^{-1} followed by the second slow mixing for 10 min at G = 30 s^{-1}. Lastly, the beakers were left to settle for another 1 hour and 40 minutes. The supernatant water is tested as is described in Section 3.2 and 3.3.

3.7. Modeling and documentation

The ”Ct” approach, as commonly used for disinfection in drinking water, is the product of the residual disinfection concentration (C) and the corresponding ’disinfection contact time’(t). It is an approach used to compensate for the use of indicator microorganisms. When the inactivation of an indicator organisms is more effective than for the undesired (resistant) microorganisms, this can give an overestimation of the effectiveness. Thus, to overcome this problem, the Chick-Watson equation found that the logarithmic relative decrease is proportional to $c^n t$ (Von Gunten, 2003). A given value can be obtained by increasing or decreasing either the time or the concentration and thus changing the surface under the Ct curve.

$$log(\frac{N}{N_0}) = -kc^n t \tag{3.2}$$

In this equation N_0 are the colony forming units of the microorganisms before exposure to ozone or chlorine and N the colony forming units of the microorganisms after a ”t” time of exposure to ozone or chlorine. Furthermore, k is the rate constant for the inactivation of a particular microorganism, C the concentration of a disinfectant (in mg/l), t the contact time (in minutes) and n the fitting parameter for non-first order behavior (although this is often 1 and thus first order). For ozone treatment of E. coli this k-value is for instance 130 $mg^{-1}min^{-1}$ whilst this value is much lower for microorganisms that are more difficult to inactivate (Von Gunten, 2003).

According to previous research, the best fitting model is Hom’s model for chlorination and ozonation (Mezzanotte et al., 2007). Looking at the R^2 value, the model is more suitable for ozonation than it is for chlorination.

$$log10(\frac{N_0}{N}) = -k \times C^n \times t^m \tag{3.3}$$

The parameters for this Equation are explained in Table 3.4.

	Chlorination			Ozonation		
Parameters	**Total coliforms**	**Fecal coliforms**	***E. coli***	**Total coliforms**	**Fecal coliforms**	***E. coli***
k	1.39	1.47	2.01	0.38	0.36	0.36
n	0.41	0.38	0.34	0.81	0.82	1.02
m	0.10	0.10	0.01	0.57	0.58	0.47
R^2	0.53	0.39	0.45	0.74	0.83	0.79

Figure 3.4: Typical values for the Homs equation for k, n, m and the R^2 (Mezzanotte et al., 2007).

4

Results

4.1. Results of the water quality tests

4.1.1. Different Matrices

As shown in Table 4.1, the secondary effluent of Horstermeer and Harnaschpolder gave fairly similar water quality results. As previously mentioned, these parameters are of importance as the ozone is dosed on the dissolved organic carbon (DOC) in the water and ozone reacts with the organics in the water. Harnaschpolder would need a slightly higher ozone concentration to obtain the same treatment capacity due to its higher DOC as it is dosed in mg O_3/mg DOC.

Table 4.1: Parameters and standard deviations of Horstermeer and Harnaschpolder, based on four samples measured in triplicates

	Mean values HM	+/- st. dev. HM	Mean values HP	+/- st. dev. HP	Unit
EC	809.0	145.3	958.2	64.5	uS/cm
pH	7.2	0.1	7.5	0.1	(-)
Turbidity	1.6	0.7	2.6	0.8	NTU
DOC	12.4	1.3	14.1	0.4	mg/l
COD	78.0	55.7	48.5	5.7	mg/l
UV254	0.05	0.01	0.08	0.01	A

4.1.2. Effect of ozone and chlorine on water quality parameters

The effluent parameters all decreased after the treatment with ozone. After treatment, visual differences were shown as the water lost colour. This is explained by the decrease in organic compounds by the ozone. Figure C.1 confirms this with the UV254 measurements, showing that the UV absorbing organic compounds, at different ozone concentrations decrease.

The parameters after chlorination changed less than after ozonation. The colour of the sample did not change as much as with the ozone treatment, which was confirmed by the UV254 and COD measurements that stayed similar after treatment. Furthermore, no significant changes were found in the pH, EC and turbidity of the chlorinated samples compared to the untreated effluent samples. This difference in treatment can be explained by the selectivity of ozone for organic matter compared to the non-selective chlorine (Mehta et al., 1989).

4.1.3. Removal of Natural organic matter

As oxidisers, such as chlorine and ozone, react with organics, the Natural Organic Matter (NOM) is measured before and after each treatment (Equation 1.3). NOM is typically measured as DOC (Sadrnourmohamadi and Gorczyca, 2015). No change in DOC was found after the water is treated with ozone. Therefore COD measurements and the UV254 are used to track the aromatic compounds.

After ozonation, a wavelength of 254 nm an absorbance decrease of about 60-70% was found in the ozone stock solution compared to the raw effluent. This means the organic matter decomposed to smaller substances resulting in a similar DOC with a decrease in aromatic compounds. Ozone can not

completely mineralize the NOM to CO_2. Therefore, the DOC measured remains unchanged whilst the aromatic compounds are decomposed to smaller ones. The ring of the compound is opened and thus a lower 254 nm value is found without a decrease in DOC (Siddiqui et al., 1997). The ozone reacts first with the NOM before the ozone stock solution can start saturating (Equation 1.3), after which it can be dosed to the sample.

Ozone spike solution

The spike solution is prepared in demi-water as well as in effluent. In the case of reacting with effluent, the ozone first reacts with the background concentration (COD and microorganisms) of the sample before it increases to the maximum solubility. After a few minutes the effluent looked completely clear and although the ozone concentration in the effluent sample increased slower than in demi-water, it finally increased to almost the same concentration as the demi-water did. Thus, a similar final (maximum solubility) ozone concentrations was found. This is seen in Figure 4.1 measured in a bubbling tank of 3 l and confirmed by using the indigo method. Based on these results, for the rest of the research effluent is used for the ozone spike solution. This way, the samples are not diluted by demi-water.

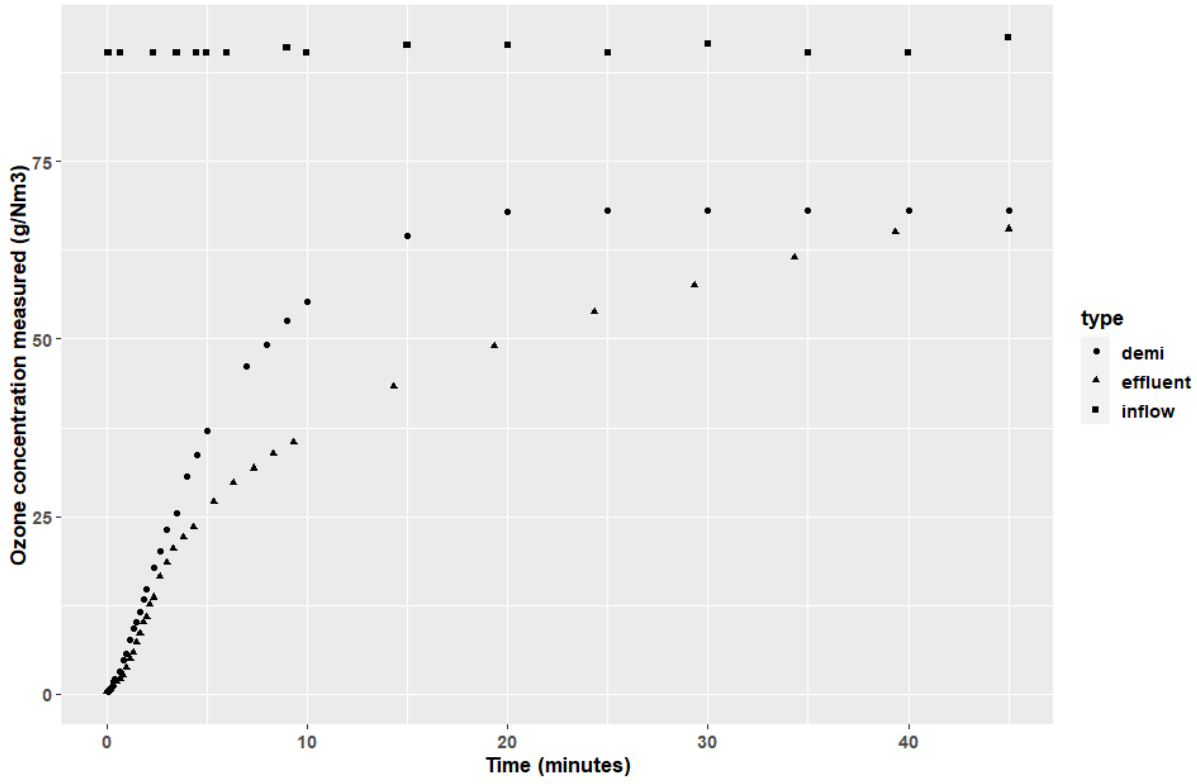

Figure 4.1: In- and outflow ozone gas in 3 l bubbling tank

When applying the indigo method on the treated samples after spiking, no ozone was found. Consequently, quenching of the treated samples was not needed as it can be assumed that all ozone has reacted. Based upon the 1-2 log removal of *E. coli* an ozone dosage was found between 0.4-0.6 mg ozone/mg DOC.

4.1.4. Effect of the O3-STEP and 1-STEP filter on the water quality parameters

Figure 4.2 shows the effects of the O3-STEP$^{(R)}$ and the 1-STEP$^{(R)}$ (in % change) on the UV254, COD, PO4 and TN.

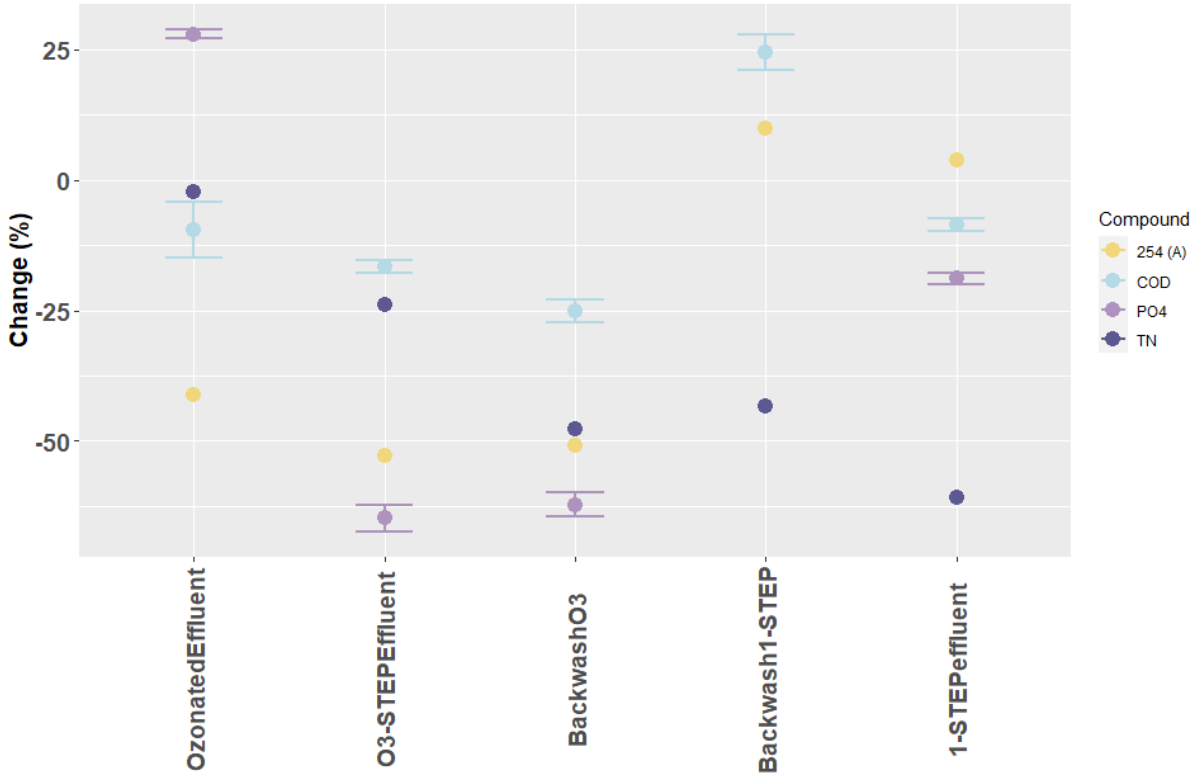

Figure 4.2: Scatter plot of the change (%) of different compounds in different treatment steps (respectively locations B, C, D, E and F at Figure 3.1). Note: Each point with error bars represents the average of three values. Error bars indicate standard deviation.

By measuring the UV254, organic compounds, specifically those that contain aromatic rings or unsaturated carbon bonds, absorb a lower amount of UV after ozonation (reduced by 40%). The rest of the compounds are more removed by the GAC-filtration. For instance, PO_4 is not removed after ozonation but showed a removal of 60% after the GAC-filter and thus in the effluent of the O3-STEP$^{(R)}$ step. Remarkable is that the changes of the compounds in the backwash water of the O3-STEP$^{(R)}$ filter were similar to the O3-STEP$^{(R)}$ filter effluent, even a higher TN removal is obtained in this water.

In the 1-STEP$^{(R)}$ backwash water an increased COD and UV254 is found and a decreased TN in comparison with the secondary effluent. After the filter some removal is found for TN, COD and PO_4 but not as much as for the O3-STEP$^{(R)}$ filter, again showing the impact of the ozone.

4.2. Lab results of ARB removal during ozonation

Figure 4.3 shows a bar plot of the log removal for chlorine and for the three different concentrations of ozone (0.38, 0.47 and 0.58 mg O_3/mg DOC) in Harnaschpolder as well as in Horstermeer. No significant patterns are found between the removal of ARB and ASB. Although the concentrations when applying ozone and chlorine do seem to give a different result for the resistant and ASB, by applying an ANOVA-test no significant differences were found between the removal of ARB and ASB. Even when adding the data for the different ozone concentrations up, no significant differences were found in the removal of ARB and ASB. Therefore, no proof was found that for either disinfection method one group is better or worse removed than the other for a given dose of the disinfectant. This result suggests that treatment with ozone and chlorine has the same effect on both types of bacteria and the ASB can thus be used as in indicator for the removal of resistant bacteria. As resistant bacteria often appear in much lower concentrations that are more difficult to measure, the use of an ASB as an indicator for ARB can provide a significant advantage. There is a large spread between the different removals resulting in large error bars. The current sample size is not large enough to compensate for this differentiation. Therefore more experiments to validate this result are recommended.

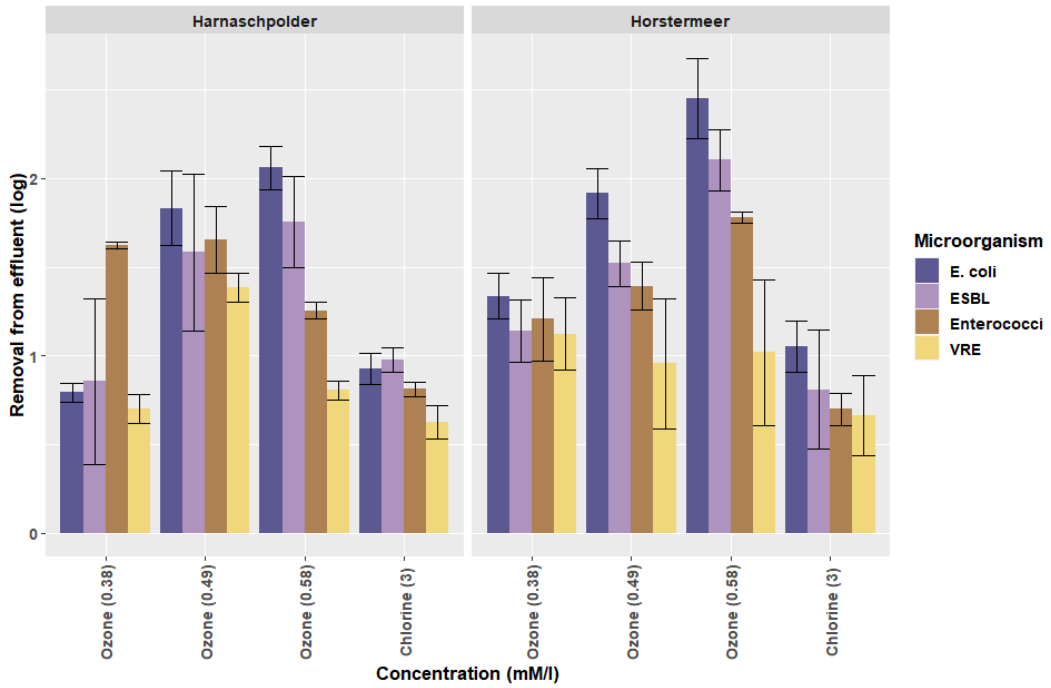

Figure 4.3: Bar graph of the log removal of ARB and ASB in Harnaschpolder and Horstermeer water. Based upon four weekly grab samples per water type, measured in triplicates. The concentration of ozone is given in mg O_3/mg DOC and for chlorination this number is mg/l/min.

Figure 4.3 shows a few trends in the removal capacity with the increase of the ozone. A significant link was found between the amount of ozone dosed and the effectiveness of the treatment with ozone as a disinfectant in the removal of *E. coli*. By applying an One-way ANOVA test on the removal of *E. coli*, a p-value lower than 0.05 is found (p=0.015), suggesting that the treatments are significantly different and thus increasing. For the resistant ESBL, the same pattern is shown in the graph, however with a p-value of 0.115, this increase was not found to be significant.

By applying the same method for Enterococci, no significant difference was found (p=0.52), although an increasing trend is visable in Horstermeer effluent. The lack of significance can either be explained by a limitation in data points and the large standard deviation in these groups or by an absence of differences between the groups. No increasing pattern was shown for VRE. The absence of a pattern with the removal of VRE is explained by a correction in the VRE measurement (Further explained in Section 5.2.3).

Lastly, no significant difference was found in the treatment in effluent of Horstermeer and effluent of Harnaschpolder (p=0.66).

As Figure 4.3 shows, the treatment with chlorine showed no significant differences between the removal of ASB and the removal of resistant bacteria. The resistant bacteria show a slightly lower median removal but this difference is not significant. Therefore, as mentioned earlier, the removal of ASB can be used as an indicator for the removal of resistant bacteria for chlorine as well.

4.3. Lab results of the ARB removal by coagulation

Figure 4.4 shows the results of the removal of bacteria by using three different kinds of coagulants in four different concentrations, based on the minimum and optimum coagulant dosage. For Aluminium one extra concentration is added based on a 3.1 mole ratio of the coagulant and P for the P-removal. Due to the low P concentration at the moment of the grab sampling, resulting in a negligible low coagulant dosages, this dosage is left out for the iron experiments.

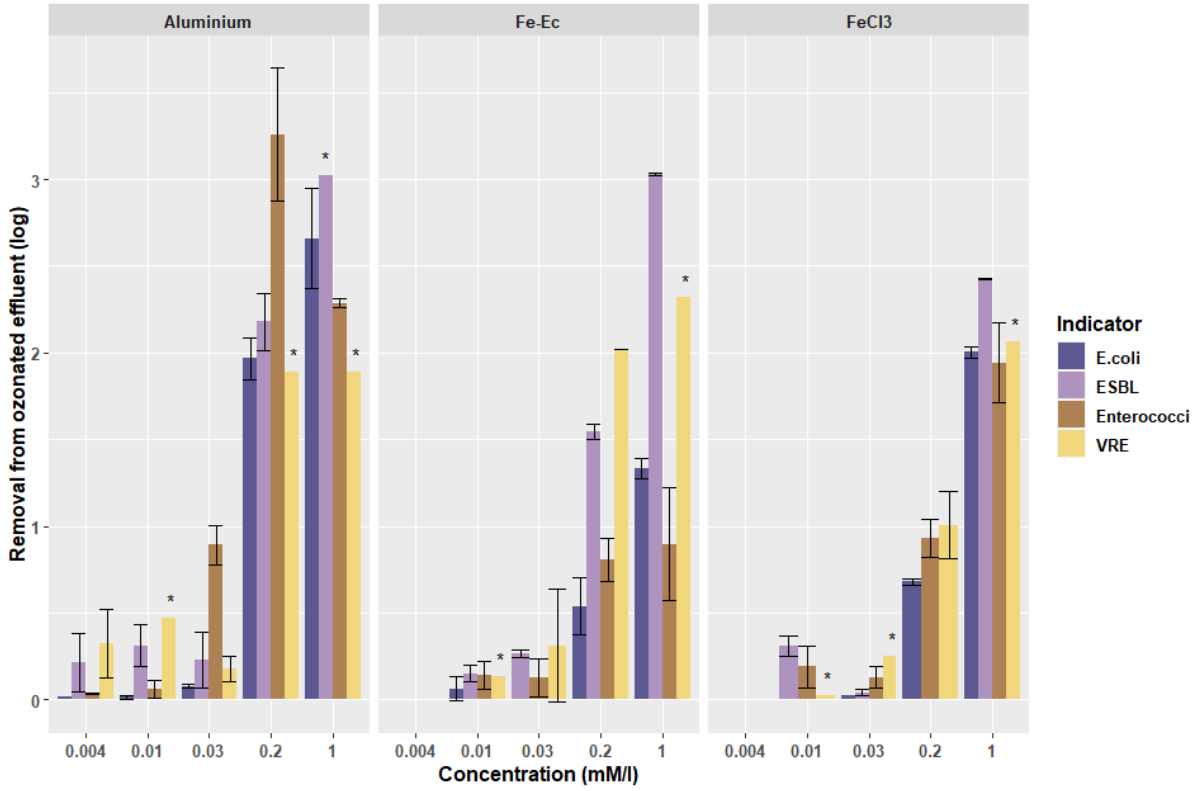

Figure 4.4: Bar graph of the log removal with different coagulants at different concentrations in ozonated (location B in Figure 3.1 of 0.4 mg O_3/mg COD) effluent. Note: Bars marked with an asterisk (*) indicate a minimum estimated removal, due to concentrations below the detection limit. Each bar represents the average of three values (triplicate microbial screening, single assay). Error bars indicate standard deviation.

Figure 4.4 shows a larger removal by using PAC than with either of the iron treatments. By looking at the Amirtharajah diagram, the minimum and optimum dosage were chosen for as well Aluminium as for Iron. The optimum concentration for aluminium is lower than the one for iron (0.2 mM/l vs 1 mM/l), explaining part of the higher performance for aluminium at 0.2 mM/l. However, when comparing the optimal concentration of aluminium with the optimal concentration of iron, aluminium still performs better than iron.

By using the Fe-EC, some removal of OMPs is also found. As shown in the Appendix in Graph B.1, up to 30% extra OMP removal can be obtained by applying Fe-EC.

4.4. Results of the influence of the pilot on ARB

After finding the lab-results for the ozonation as well as for coagulation in a lab environment, the results of the concentrations in the pilot are shown in Figure 4.5. As discussed in Section 1.4, *C. Perfringens* and Coliphages are also added to the experiments to give an idea of the general disinfection performance in the filters.

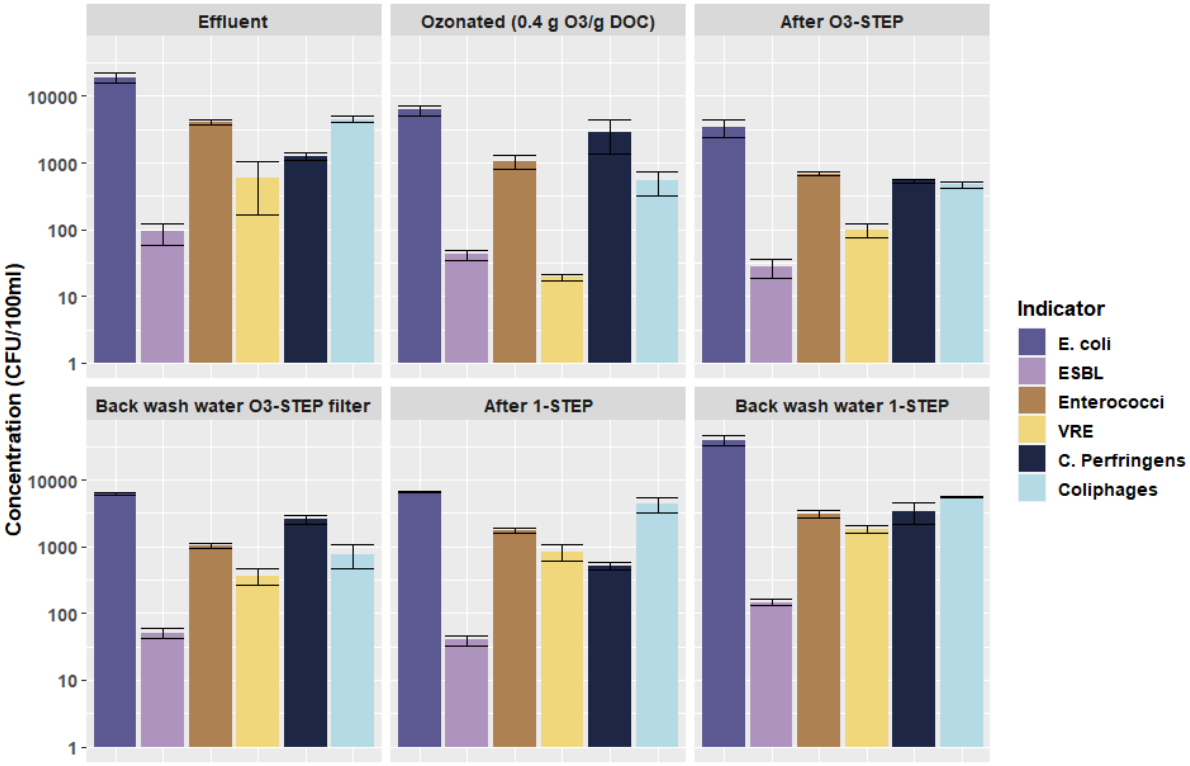

Figure 4.5: Bar graph of the concentrations (cfu or pfu/100 ml) of different microorganisms in different treatment steps (from left to right first row location A, B and C and second row D, F and E of Figure 3.1). Note: Each bar represents the average of three values (triplicate microbial screening, single assay). Error bars indicate standard deviation.

In Figure 4.5, the concentrations of several microorganisms are shown before the O3-STEP$^{(R)}$ filter (effluent, location A of Figure 3.1), after ozonation of 0.4 mg O_3/mg DOC (location B) and after the GAC-filter, thus of the tertiary effluent (location C). Furthermore, also the microorganisms in the backwash water of the filter are measured (location D). As the biology in the O3-STEP$^{(R)}$ filter is not fully set-up yet, the results of the microorganisms of the full-scale 1-STEP$^{(R)}$ filter are added to this figure as well to obtain information on the impact of the bio-film on the antibiotic resistance (location E and F).

In total after the O3-STEP$^{(R)}$ filter (location C of Figure 3.1) an average of 0.7 log removal was found against the secondary effluent without a significant difference between the removal of ARB and the ASB. This is significantly (with $p = 0.0035$) smaller than the 1 log found by dosing 0.38 mg O_3/mg DOC ozonation in the lab.

In the results of the 1-STEP$^{(R)}$ filter, a significant difference between the change of VRE and Enterococci was found ($p=0.0026$). Where after the 1-STEP$^{(R)}$ filter a removal in Enterococci of 0.5 log was found, an increase in VRE was noted. The same difference can be seen in the backwash water of the 1-STEP$^{(R)}$ filter with a significance of $p=0.0002$ (Figure D.1).

By presenting the numbers as a log removal compared to the effluent, we obtain Figure 4.6. Some of these bars obtain negative results, a negative log removal microorganisms an increase in the concentration.

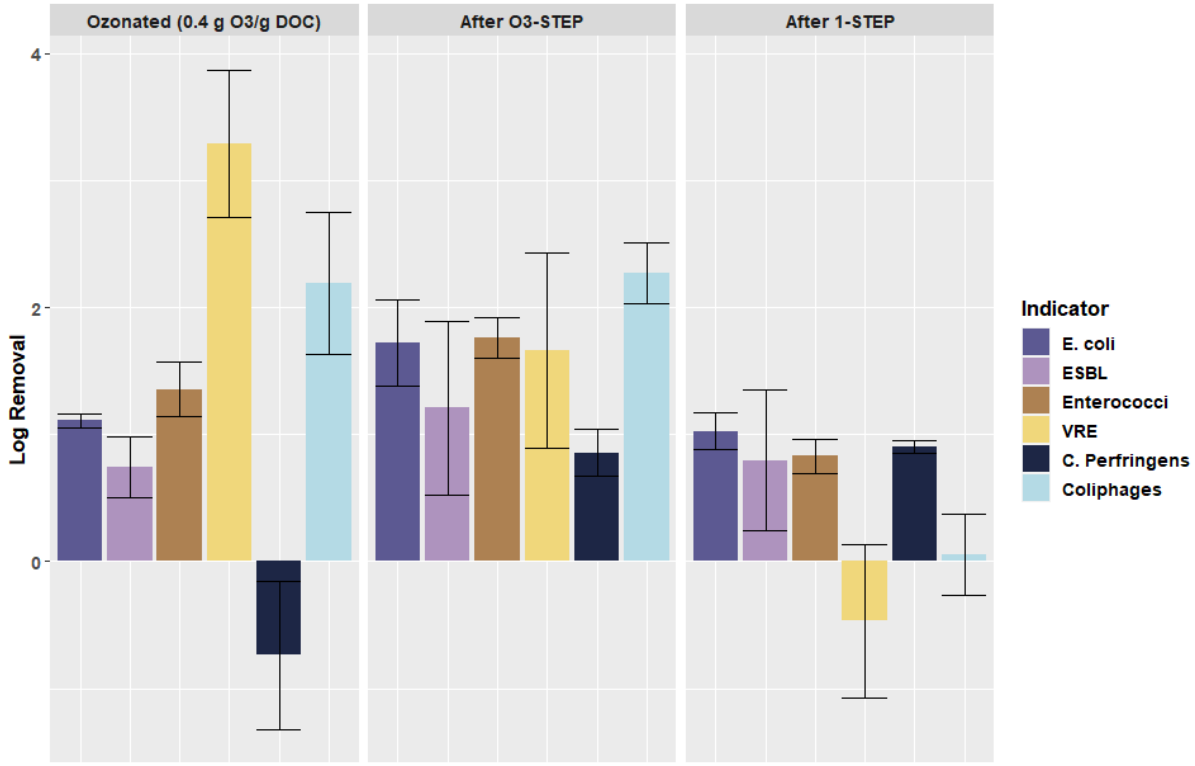

Figure 4.6: Bar graph of the log removal of different microorganisms in different treatment steps (Left to right; location B, C and F of Figure 3.1). Note: Each bar represents the average of three values (triplicate microbial screening, single assay). Error bars indicate standard deviation. Negative removal should be read as an increase in comparison with the secondary effluent.

5

Discussion

5.1. Interpretation of the results

In this section, the results found in Chapter 4 are interpreted and compared with literature. Looking at the results concerning the two different types of secondary effluent (Section 4.1.1), a few points are worth noticing. Keen and Fugère (2017) described that as a percentage of the total Enterococci detected, in secondary effluent samples taken in Europe 2-3% VRE are found. Although fluctuating per sample, in the secondary effluent of this research an average percentage of VRE in Enterococci was found of 2.8%, thus in line with the expectation in the literature. The effects on this effluent found after ozonation (Section 5.1.1), (Section 5.1.2) and after the GAC-filter (Section 5.1.3) are presented in this section.

5.1.1. Ozonation

Regarding the ozonation, in this research no significant difference was found between the removal of ASB and ARB. In the O3-STEP filter$^{(R)}$ the VRE percentage dropped to 1.8% after ozonation (location B of Figure 3.1). A significant removal of the (AR) indicators was found in the O3-STEP$^{(R)}$ filter. This log removal was not high (0.7 log) compared to effective disinfectant, such as ultra-filtration (UF) that can remove up to 6 log removal (Hembach et al., 2019). This is explained by the removal of OMPs requiring much lower ozone concentrations (around 0.4 mg O_3/mg DOC) than disinfection does (between 1 and 1.5 mg O_3/mg DOC) and is in line with the literature (Hembach et al., 2019). Applying the Hom's model for ozonation in wastewater by using the parameters as shown in Table 3.4, the best performances were found by Mezzanotte et al., 2007 at an ozone concentration of 3.6 mg/L (at 12.8 minutes contact time). This results in a removal of 4.4 log. With a design contact time of 15 minutes and average DOC concentration of 12.4 mg/l at Horstermeer, this would be equal to a concentration of 0.35 mg O_3/mg DOC for the same removal. As in this research a much lower log removal is found than 4.4 log, the Hom's model is not applied.

The ARGs are not taken into account in this research. Alexander et al., 2016 revealed accumulations of some ARGs after ozone treatment due to the mechanism of ozonation in which the cell membrane is attacked and the ARGs might remain after cell lysis (Section 1.7.1). Therefore in future research, these ARGs are recommended to be included.

Different microorganisms tend to cope differently with the bactericidal effects of ozone. This is also shown by *C. Perfringens* that was not removed by the ozone treatment of the water, whilst the GAC-filter was able to remove these bacteria. The opposite is true for coliphages which are removed mostly by the application of the ozonation and not much extra removal is found by the GAC-filter.

Chlorine was used as a comparison method for the ozone. The percentage of VRE in Enterococci increases according to Keen and Fugère (2017) more than by using ozone. They found a percentage of VRE of 52% for WWTPs that treat the water with Chlorination as a tertiary treatment step. This is significantly higher than this research found where this percentage increased to 6.28% after chlorination.

5.1.2. Coagulation

By looking at the Amirtharajah diagram (Figure A.1) the dosage used for the removal of phosphorus (3.1 mole Al/mole P), is suitable for the adsorption and destabilization of the compounds in the water. Although microorganisms are negatively charged and can thus be partly removed by destabilization, the removal is mostly dependent on the mechanism of sweep coagulation. This is confirmed by the results. Although removal took place with the minimum dosages of Iron and Aluminium, much higher results are obtained with the optimal dosages (Figure 4.4). To entrap (antibiotic resistant) bacteria in the flocs, higher coagulant concentrations should thus be dosed. Furthermore, the required phosphorus removal for the O3-STEP$^{(R)}$ filter is very low. When the phosphorus concentration is low enough (below 0,15 mg P), which is often the case in Horstemeer, the coagulant dosage is not performed.

Figure 3.2 shows that when aluminium is dosed five times higher than the optimal dosage (1 mM/l instead of 0.2 mM/l), the relative increase in removal was low (3 log instead of 2.6 log removal on average). Furthermore, more dissolved aluminium was found in the water after the higher dosage (0.82 mg/l in the supernatant). The dosage of aluminium can present aluminium phytotoxicity in case it is still in the effluent which could be the case if more aluminium is dosed for the sweep coagulation. This toxicity can also become a problem for the sludge or backwash water of the filter. Overdosing might ask for extra treatment considerations, such as lime dosage to remove this compound. This means there is a limit to the coagulant dosed to still make it an effective measure.

Aluminium performed better in the lab tests than iron coagulation did. This result is aligned with the findings at the pilot for the removal of P. Their explanation for the higher susceptibility for aluminium is the usage of iron in the secondary treatment. Why this is the case is outside the scope of this research but would be interesting to test in future research.

Even though iron obtained lower log removal than aluminium, iron should not be taken out of consideration. Iron is often cheaper than the dosage of aluminium as $FeCl_3$ is a byproduct of blast furnaces. Furthermore, iron does not have the same issues with toxicity as aluminium does. When looking into Fe-EC specifically, the lowest effectiveness for microorganism removal was obtained (Figure 4.4). However, benefits are that no extra electrolyte (Cl^-) is needed, such as is the case for $FeCl_3$. Extra benefits are the additional OMP removal by applying Fe-EC (Figure B.1) and the usage of oxygen, an element in abundance after ozonation, because of the oxidation process from Fe^{2+} to Fe^{3+}.

5.1.3. Granular activated carbon (GAC) - filter

There was no relative increase of ARB found after either the total O3-STEP$^{(R)}$ filter or in its backwash water compared with the secondary effluent, implying there is no enhancement of AR in the filter. However, after the GAC-filter of the O3-STEP$^{(R)}$ (location C) the percentage of VRE in Enterococci increased to 14.3% whilst after ozonation this was only 1.8%. This means that the ozone prevents the enhancement of antibiotic resistance in the filter, but the GAC has a negative impact on the relative ARB concentration in the water. For *E. coli* and ESBL-*E. coli*, this relative increase is not found. The difference between the bacteria is that *E. coli* is a gram-negative bacteria whilst Enterococci is gram-positive.

Increased AR in activated carbon filters is a known problem which is recently getting more attention (Section 1.6.4). This difference is possibly explained by the increase of ARGs in the activated carbon filter as described by Wan et al., 2021. Although the growth of VRE in the filter is unlikely as Enterococci is a faecal bacterium and the filter will therefore not give the desired growth conditions, it is possible that the existing Enterococci take up the ARGs by using HGT. This is a hypothesis that can be tested in future research involving gene transfer.

The 1-STEP$^{(R)}$ filter

The 1-STEP$^{(R)}$ filter is looked into because the biofilm in the O3-STEP$^{(R)}$ filter GAC filter is not yet ripe, and it can be assumed that similar populations will be developed in time. After the 1-STEP$^{(R)}$ filter (location F), the percentage of VRE in comparison with Enterococci went even up to 48%. In the 1-STEP$^{(R)}$ filter, where VRE showed an increase of 0.5 log after the filter whilst the antibiotic sensitive Enterococci was removed with 0.5 log (Figure 4.6).

Over time, when a biofilm has grown over the filter, a similar relative and absolute increase in AR may occur at the O3-STEP$^{(R)}$ as well. The possible abundance of ARGs, due to cell lysis by ozonation could potentially add to the selective pressure in this filter. It is therefore recommended to look keep track of these changes in AR cause by the GAC-filter.

5.2. Limitations

5.2.1. Sample taking

During this research grab samples were taken from the treatment plants. By using grab samples, one deals with the situation that applies at the moment that the sample is taken. For instance, a storm peak would dilute the effluent but also other factors might temporarily affect the water quality. This limitation can be overcome by taking flow proportional 24 hour composite samples.

Another limitation about the sample taking can be found in the absence of coagulation and biological growth at the moment the samples were taken. On the day the grab samples were taken of the entire filter, the P concentrations were low, for which the operators were not dosing coagulants. This meant that the removal by applying coagulation was not measured on the pilot. As mentioned, the O3-STEP$^{(R)}$ filter was not ripened at the time of the sample taking, meaning no biology had grown on the filter yet. Based upon previous research, this growth is the main risk for the selection for AR in the filter. This limitation is the reason measurements of the full-scale 1-STEP$^{(R)}$ filter were implemented in this research. The 1-STEP$^{(R)}$ has a fully grown biology but is not identical to the O3-STEP$^{(R)}$ filter: First of all, this filter does not have the inflow of ARGs potentially enhanced by the ozonation step. Secondly, due to the long period the 1-STEP$^{(R)}$ filter is in use, the micropores of the activated carbon in the filter are likely to be saturated thus taking the effect of these micropored not into account. Extra measurements in the O3-STEP$^{(R)}$ filter after the biology in the filter is completely started, could confirm or reject these results found in the 1-STEP$^{(R)}$ filter for the O3-STEP$^{(R)}$ filter.

5.2.2. Culture based methods

There are several limitations in the use of culture-based methods. First of all, the method is limited to a bacteria which are culturable, and these are a small fraction of the total (<1%). Secondly, only the impact on bacteria is measured whereas a gene analysis can give additional information on the ARGs in the system. As the mechanisms of oxidisers is to damage the cell wall of the bacteria, leakage of the (antibiotic resistance) genes can take place. These genes can potentially cause problems in a later stage. Although no significant increase of the tested ARB was found in the filter after ozonation, it does not necessarily mean there is no increase in ARGs after ozonation. Especially as the filter is designed to remove antibiotics, resulting in a higher antibiotic level in the filter, this could induce the antibiotic resistance by increased selective pressure. For future research, it would be interesting to measure these genes to consider the implications for the water quality (Figure 5.1). A possible method for this is quantitative Polymerase Chain Reaction (qPCR) which measures the ARG and MGE abundance in WWTPs. It determines the concentration of a specific gene from an extracted DNA sample, using gene-specific probes, without the need for cultivation. Due to the complexity of the data analysis of genes, it is left outside the scope of this research.

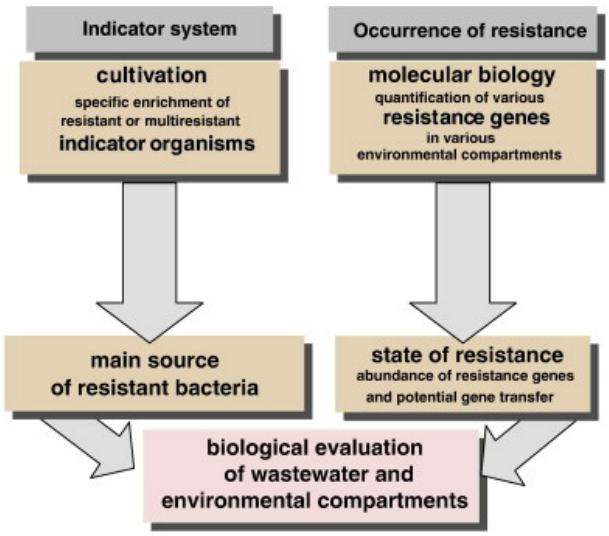

Figure 5.1: Combining cultivation and qPCR (Rizzo et al., 2013)

Another limitation of plate methods is the inability to check for higher removals due to the detection limit. At 500 ml, membranes usually clog when using effluent samples. When concentrations drop below 30 cfu per 500 ml, the results become unreliable or even not measurable. Although not measured, this does not mean the bacteria are not present in the water. When testing for higher concentrations of ozone, this limitation was that the ARB fell outside of the measurement range. This means it became difficult to test the significant difference between the removal of antibiotic sensitive and resistant bacteria at higher concentrations of ozone. The use of an additional gene-centric method, such as qPCR, would resolve this limitation. The result that ASB can be used as an indicator for ARB could also help overcome this limitation as ASB are often present in much higher concentrations.

5.2.3. Plate counting

As mentioned in Section 4.2, the VRE concentration was corrected during the research resulting in inconsistent measurement outcomes and therefore larger error bars. When VRE plates are overloaded, only few VRE get their colour at the edge of the plate (Figure 5.2). During the first weeks the plates were counted in this overloaded way, resulting in a lower count of VRE than in the later weeks where less water was filtered over the membranes (Figure 5.3). In general, the culture plates of VRE show less clear results due to the growth of other bacteria than VRE. This makes VRE more difficult to count than *E. coli* or ESBL. However, when using VRE, using smaller volumes and leaving the samples for 48 hours instead of the recommended 24 hours already gave less ambiguous results. The manufacturer of the agar plates (Biomerieux) confirmed that the 24 hours is based on clinical samples (such as blood) rather than effluent treatment.

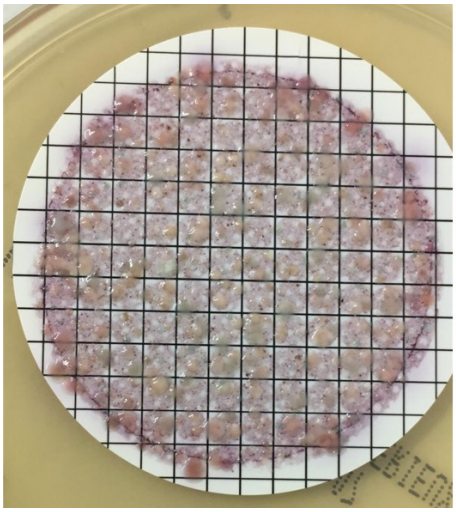

Figure 5.2: Overloaded VRE membrane

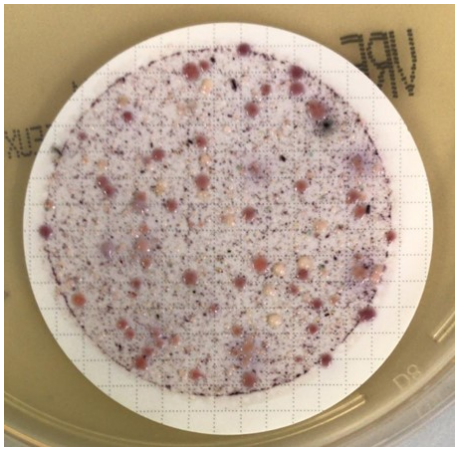

Figure 5.3: Less water over membrane on VRE plate

Another way of combating this issue is by using different plates. Different brands, such as CHROMagar could give more clear results on the VRE concentration. Instead of using VRE, also different gram-positive resistant bacteria could be used to test for antibiotic resistance. Examples are the gram positive *Staphylococcus* and *Enterococcus* resistant to linezolid.

5.2.4. Limitations in the results

As bacteria are living organisms, a relatively high randomness can be found in the measurements. As such, large standard deviations occur. The current amount of measurements might not be enough for calculating reliable significance as the impact of measurement errors is much larger than when implementing more results. This is outside the scope and period of this research. Consequently, a recommendation is to increase the length of this research to improve the reliability of the results.

5.3. Water quality implications

With only 0.7 log removal on average, improvement in the microbial quality of the O3-STEP$^{(R)}$ effluent was very limited, and could even result in a negative impact once the biofilm in the filter is fully started.

On the other hand, the O3-STEP$^{(R)}$ is very effective (>90%) in the removal of OMPs. The removal of OMPs reduces the selective pressure on the bacteria (as described in Section 1.5), potentially resulting in a decrease in ARB in the surface waters and an improved water quality (Hembach et al., 2019). The question is therefore if this effluent needs additional treatment to combat AR. There are no national or European regulations for ARB or ARGs yet, although the awareness over its severity of the issue is increasing (Zwemwaterrichtlijn, 2016). Released ARB and ARGs can persist in an aquatic environment for a considerable amount of time. Furthermore, as mentioned previously, ARGs can distribute regardless of their initial origin species (Courvalin, 1994). Once released in the environment they are able to transfer to autochthonous bacteria as well as other pathogenic bacteria, leading to an overall increase of resistance in the discharge areas. The WHO and UN assembly therefore recommend to interrupt dissemination of ARB to the environment (WHO, 2020). To make the O3-STEP$^{(R)}$ filter future-proof by removing not only the selective pressure of OMPs, but also the ARB from the effluent, it is recommended to consider changing the design of the O3-STEP$^{(R)}$ filter to be ahead of regulations for antibiotic resistance. Concluding from the results, with an increased ozone dosage and coagulation, more removal could already be obtained.

In comparison with existing regulations, the European Directive uses an ”acceptable quality” for *E. coli* and Enterococci in bathing water as described in Table 5.1. This is implemented to promote the public health and national prosperity (Zwemwaterrichtlijn, 2016). The levels for these regulations are not yet reached by using the O3-STEP$^{(R)}$ filter. To comply with the acceptable quality for coast and transitional water ways for *E. coli* and Enterococci in Horstermeer, an additional log removal of 2.3 log should be added to the O3-STEP$^{(R)}$ filter effluent on a 95%-percentile. For the implications for the design (Section 5.4), these levels are adopted as indicator guidelines. If the removal of ASB can indeed be used to indicate a sufficient removal of ARB, thus if 2.3 log removal of the ARB in Horstermeer is enough to promote public health, would be an interesting question for a follow-up research.

Table 5.1: Swimming water regulations for Enterococci and E. coli in The Netherlands, based on a 95-percentile (Zwemwaterrichtlijn, 2016)

Water Type	Parameter	Excellent quality	Good quality	Acceptable
Inland water ways (cfu/100ml)	E. coli	200	400	330
Inland water ways (cfu/100ml)	Enterococci	500	1000	900
Coast and transitional water ways (cfu/100ml)	E. coli	100	200	185
Coast and transitional water ways (cfu/100ml)	Enterococci	250	500	500

5.4. Engineering implications

To comply with the indicator guidelines as described in Section 5.3 and thus to remove pathogens and antibiotic resistance from the effluent, the design of the O3-STEP$^{(R)}$ filter should be adjusted. The current removal of 0.7 log is considered insufficient for disinfection and therefore extra measures need to be taken. Several implementations could be considered to obtain this additional removal. This Section describes the considerations between keeping the current design whilst increasing the ozone and coagulant concentration (Subsection 5.4.1) and implementing an additional treatment step that is proven effective against AR: Ultra-filtration (UF, Section 5.4.2) or UV/O_3 (Section 5.4.3). Furthermore, the effects of a bypass are implemented (Section 5.4.4). Lastly, in Section 5.4.5, a multi-criteria analysis (MCA) is done to compare these different implementations.

5.4.1. Enhancing the current design by dosage adjustment

Both ozonation and coagulation could remove microorganisms when dosed in higher dosages to obtain effective removal. In this case the investment costs would remain similar to the current O3-STEP$^{(R)}$ filter, as ozonation and coagulation are already applied in the system.

As described in Section 5.1.1, ozone is an effective disinfection method. In the correct dosages (between 1 and 1.5 mg O_3/mg DOC) ozone removes between 2-6 log for bacteria, 3-6 log for viruses and over 0.5 log for *Clostridium Perfringens* (Collivignarelli et al., 2018, Xu et al., 2002). When the ozone dosage is increased to a concentration between 1 and 1.5 mg O_3/mg DOC, this will have several implications on the O3-STEP$^{(R)}$ filter. First of all, more liquid oxygen (0.20 euro/kg) and energy is needed to produce the ozone, which increases the cost of the filter. Furthermore, the oxygen level in the GAC- filter might increase as a consequence of the ozonation (Equation 1.2c), although the current

concentration is already super saturated with peaks of 40 mg/L. An increased oxygen level would be disadvantageous for the removal of nitrates as denitrification requires anoxic conditions to take place. On top of this, different bacteria will grown in an aerobic environment. In practice this means a higher amount of methanol (0.35 euro/kg) needs to be dosed, which increases the operational costs (methanol is currently 5% of the operational costs)(STOWA, 2018). For considerably higher ozone concentrations, ecotoxicological effects could occur such as the formation of bromate (Section 1.7.1). Therefore the bromide concentration should be constantly measured to prevent the bromate concentration.

Coagulation showed also promising results in Section 4.3. The effects of increasing the three different coagulants are described in Section 5.1.2. Increasing the coagulation amount for microorganism removal is a low-cost measure as next to the absent extra investment-costs, the operational costs are low as well: the chemical costs for $FeCl_3$ is around 0.23 euro per kg and for PAC this is 0.26 euro (STOWA, 2018). This results in only a small fraction (1-3%) of the operational costs of the total filer which makes it a ”no regret” measure to increase the coagulant concentration. In terms of ARB removal, aluminium is most effective and with an optimal dosage for sweep coagulation of 0.2 mM/l an additional 2.6 log could be obtained (0.02 euro/m^3 on aluminium dosage in effluent). When using Fe-EC, there would be investment costs for the installation of the iron plates. However, lower operational costs could be obtained for the favourable microbial removal set of conditions (Bicudo et al., 2021). As Fe-EC uses oxygen for the oxidation of the Fe, this might positively impact the dosage of methanol as bacteria then consume more nitrate instead of oxygen.

5.4.2. Ultra-filtration (UF)

According to Hembach et al., 2019, the combination between ozonation and UF, shows most potential for the removal of AR from waste water. Ozonation removes the selective pressure for ARB as it effectively removes the OMPs. After which UF, as a physical technology, removes an average of 5 log units of both ARB and ARGs. Protozoa and viruses are effectively filtered out by the treatment with UF as well. As both GAC and UF are filtration techniques and the combination of ozone, GAC and UF would give an extremely high removal, UF can be considered to replace the GAC filter. As a new treatment step is needed for this design, the investment costs would be higher than for increasing the concentration of ozone or of the coagulant. According to Gómez et al., 2007, the variable costs for UF are 0.033 euro/m^3 and the fixed costs 0.081 euro/m^3. Furthermore, next to the clean permeate, UF produces a concentrate which asks for additional treatment. This can be returned to the beginning of the treatment plant. The spatial impact of the UF skids can be kept to a minimum as they are stackable.

5.4.3. UV/O_3

Ultraviolet in combination with ozone (UV/O_3) combines the action of the UV radiation with the enhance production of radicals (ROS) by ozone (Dodd, 2012). UV radiation interacts with target moieties in bacterial cells by physical processes, leading to photochemical reactions contributing to cellular inactivation. The ozone works as an oxidiser as explained in Section 1.6.1. Several studies found complete inactivation (<LOD, 4 log decrease) of ARB by using UV/O_3 (Sousa et al., 2017, Dodd, 2012). The ozone can be dosed before, during or after the application UV. According to Fang et al., 2014 0.5–0.9 log extra can be obtained when the UV and ozone are applied simultaneously. As with the UF, the new treatment step will increase the investment costs. The variable costs for a conventional UV are 0.033 euro/m^3 and the fixed costs 0.042 euro/m^3 (Gómez et al., 2007).

For the UV, in certain countries radiation of sunlight can be applied by using a Solar photo-reactor, resulting in lower operational and electricity costs (Ferro et al., 2015). To remove possible byproducts and use the dosed ozone, it is advised to place the UV before the GAC-filter. The downside of this order is the possible growth of ARBs in the biofilm as described in Section 5.1.3 (Sousa et al., 2017).

5.4.4. By-passing

As the water inflow in a WWTP can fluctuate and high peak flow rates can occur due to heavy rainfall, it is common practise that part of the water by-passes the treatment. This part of the water is not treated and as the removal of bacteria is calculated in logs, the impact of such a by-pass can be significant. In Appendix E, a flow rate analysis is done for location Horstermeer for an imaginary treatment step removing 3 log of the bacteria (Figure E.3). All water above 1500 m^3/h by-passes the 1-STEP$^{(R)}$ filter, which is taken as a starting point for this analysis. When the by-pass starts, the log-removal of the total inflow drops rapidly below the 2.1 log decided in Section 5.3. As the bacterial load after an event

of heavy rainfall is diluted by storm water and thus less (antibiotic resistant) bacteria by-pass the filter than during dry water flow. Depending on the requirements of the water body the effluent is discharged into, this exceedance can be seen as acceptable as in only 13% of the cases the water is by-passed.

In case the requirements of the receiving water do not allow water to bypass, several options can be considered. Designing a treatment step for the entire flow is undesirable as the size of the construction is then almost twice the size whilst only necessary in 13% of the hours. One option could be to build a buffer in the system that collects the water flow above 1500 m^3/h and treats this water once the flow rate drops below this limit again (second option in Figure 5.4). Based on the same dataset as used for Figure E.3, to treat the complete inflow, a buffer of 45,000 m^3 would need to be designed. This requires a large surface area and brings the risk of bad odour and growth of bacteria. Another option, in case the location does not have the surface area available for such a buffer, would be to start the by-pass after ozonation and before the GAC-filter. During peak water flows, shorter contact-times could be applied with a higher ozone dosage to obtain the same log removals. This way the complete flow can be treated with ozonation resulting in a lower bacterial load without doubling the size of the GAC-filter.

5.4.5. Multi-criteria analysis (MCA)

Based on the engineering implications for each of the design options, a multi-criteria analysis (MCA) is done in which the design options are compared on several criteria (definitions in Table E.1). As mentioned in Section 5.3, no regulations are applicable on antibiotic resistance meaning the requirements for the process additions are still open. The weight factors are dependent on how important the decision-maker considers the criteria. An example MCA without weight factors, is done based on the findings and results in this research (5.4 and Appendix F). Scoring the criteria as done in Figure E.2, with red = very bad, light red = bad, yellow = acceptable, light green = good and dark green = very good. increasing the ozone concentration with a bypass is considered the best addition to the design.

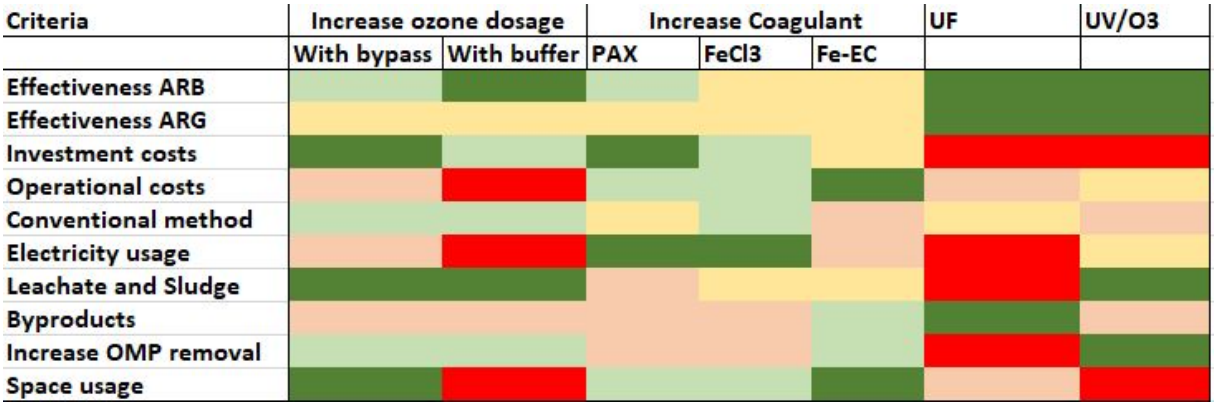

Figure 5.4: Multi-criteria analysis for seven different designs. The criteria definitions and colours for the score on the requirement are explained in Appendix E.

6

Conclusions and recommendations

6.1. Conclusions

The purpose of this research was to test the following hypothesis: *Ozonation will disinfect ARB as good as ASB. For the coagulation it is expected that a higher coagulant dose is needed for attenuation of both the ARB and ASB. Finally, ARB might grow in the biofilm during the GAC filtration*. The research confirms this hypothesis.

In answering this general hypothesis, several intermediate research questions were posed in chapter 2. The answers to those specific questions are as follows:

1. Is there a difference between the removal of ARB and ASB when using ozone treatment? The research showed no significant differences were found between the removal of ARB and ASB. This means that ASB can be used as an indicator for the removal of ARB for ozonation and coagulation. The extent to which ARB are removed by ozone from the water is low due to the relatively low ozone concentrations used for OMPs compared with concentrations required for disinfection. As the main mechanism of removal by ozone is by reacting with the cell membrane causing cell lysis, leakage of the antibiotic resistance genes (ARGs) can occur. There is a risk that through horizontal gene transfer (HGT) these genes could be taken up by other bacteria.

2. Is there a difference between the removal of ARB and ASB when using coagulation? Again, no significant difference were found between ARB and ASB removal. For the coagulation it was observed that a higher coagulant dose is needed for attenuation of both the ARB and ASB. The aluminium showed a higher removal than the iron coagulants. Above the optimum sweep coagulation concentration, the increase in removal stagnated, implying higher concentrations are not necessarily favourable. Especially as the superfluous coagulant should then be removed from the water.

3. What is the composition of bacteria in the GAC filter and does the GAC filter enhance the growth of resistant bacteria? The GAC was observed to impact Enterococci and VRE differently in the O3-STEP$^{(R)}$ filter. There is an increase observed in the VRE to Enterococci ratio for the GAC filter in comparison with the ozonated water. This effect was also seen in the 1-STEP$^{(R)}$ filter which showed the worrying result that VRE increased in the same amounts as Enterococci decreased. As the 1-STEP$^{(R)}$ filter is a filter with a matured biology, whereas the O3-STEP$^{(R)}$ filter does not yet have this, this filter might increase the ARB in the longer term as well. As the GAC filter stimulates bacterial growth in an environment with an increased OMP and potentially increased ARG content, risk of ARB enhancement occurs and thus during the maturing of the O3-STEP$^{(R)}$ filter the ARB should be monitored. Some ARB might grow in the biofilm during the GAC filter step and although these bacteria are not necessarily directly harmful, they can pass down their resistance to harmful bacteria using HGT.

4. What is the influence of the water matrix on the performance of ozone and coagulation? The matrix has a significant impact on the performance of ozone and coagulation. Where literature showed large removals for certain techniques in drinking water or artificial effluent, this was not observed in real effluent. This might be explained by the sensitivity to natural organic matter (NOM) of both ozone and coagulation, which is of importance as this is one characteristic of effluent. When considering the point estimates from the two sites, Horstermeer showed a slightly larger removal capacity than Harnaschpolder which might be explained by the higher NOM in Harnaschpolder. However, this difference was

insignificant. In order to fully understand the impact of the new filter, this observation should be fully investigated.

5. What are the potential changes to the design of the O3-STEP$^{(R)}$ filter when aiming to remove ARB as well? Several design solution were proposed to optimise the O3-STEP$^{(R)}$ filter for ARB removal. As ozone and coagulation both potentially remove ARB, an increase in these concentrations is a potential solution. Furthermore, ultrafiltration (UF) and the combination of ultraviolet (UV) and O_3 were proposed as possible alternatives. Using a multi-criteria analysis (MCA), increasing the ozone dosage with the existing bypass is recommended as a ”no regret” measure.

6.2. Recommendations

During this research, several general recommendations and recommendations for further research are made.

The UN and WHO recommend to interrupt dissemination of ARB to the environment. As WWTPs are an important source for this AR, the implementation of additional measures to counteract this is highly recommended. The recommendations for the design are dependent upon the needs of the WWTP. The ”no regret” measure that scores highest at the MCA is to increase the ozone dosage whilst keeping the rest of the O3-STEP$^{(R)}$ filter the same. This is only possible when the bromide concentration in the effluent is sufficiently low. In case a higher standard of ARB and ARG removal is required, an additional UF or UV/O_3 step can be considered. Lastly, the recommendation is to monitor the AR in the GAC-filter to prevent an increase in ARB in the effluent. As the results for the 1-STEP$^{(R)}$ filter show a worrying increase of AR, the AR in the O3-STEP$^{(R)}$ filter should be closely monitored.

For further research, it would be interesting to look into the ARGs in the different filter steps. As mentioned previously, the mechanism of ozone disinfection can cause leakage of the DNA resulting in free ARGs. Monitoring these ARGs adds valuable information on the spread of antibiotic resistance to the environment. When combining the plate methods with quantitative Polymerase Chain Reaction (qPCR) the ARGs can be included whilst at the same time overcoming the limitations related to plate methods. Secondly, instead of taking grab samples, day (24 hour) composite samples could be used. By doing this, fluctuation in concentrations can be avoided. Thirdly, during the sample taking, the biofilm on the GAC filter was not yet ripe in the O3-STEP$^{(R)}$ filter. As there were worrying observations produced by measurements taken from the mature 1-STEP$^{(R)}$, it is recommended to conduct further research after the biology in the O3-STEP$^{(R)}$ is fully matured. Furthermore, when the samples were taken, the phosphorus concentration was too low to dose a coagulant. Therefore, extra experiments are recommended at the moment the coagulant is dosed. The last recommendation for future research is to increase the length of this research to improve the reliability of the results.

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A

Amirtharajah diagrams for Aluminium and Iron

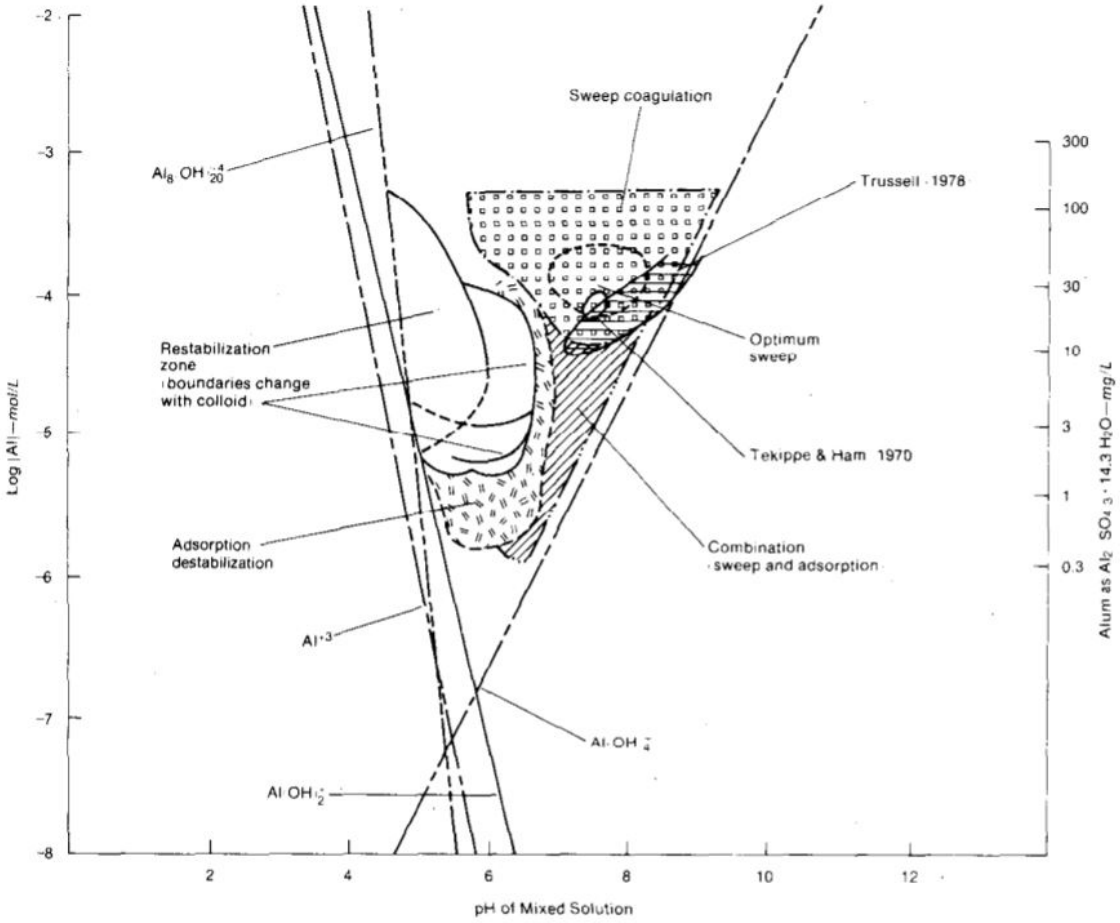

Figure A.1: Amirtharajah diagram Al (Amirtharajah and Mills, 1982)

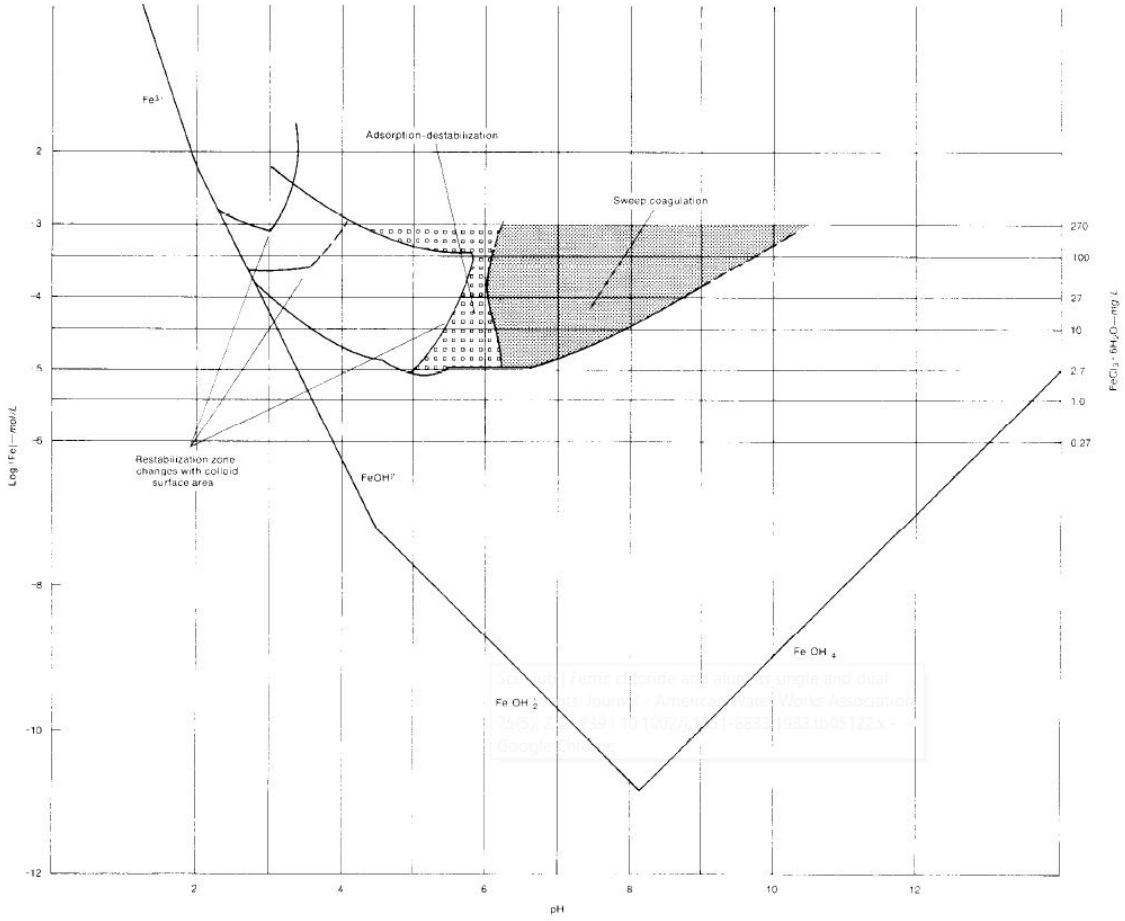

Figure A.2: Amirtharajah diagram Fe (Johnson and Amirtharajah, 1983)

B

OMP removal by Iron electrocoagulation

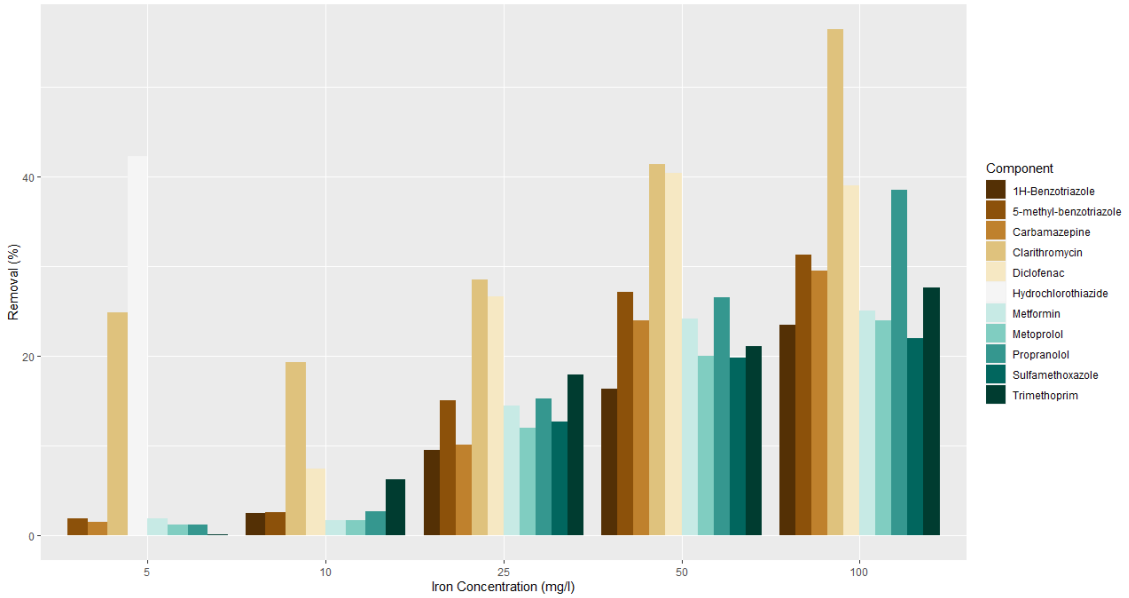

Figure B.1: Bar graph of the removal (%) of OMPS by Fe-EC in different concentrations in ozonated (0.4 mg O3/mg COD) effluent

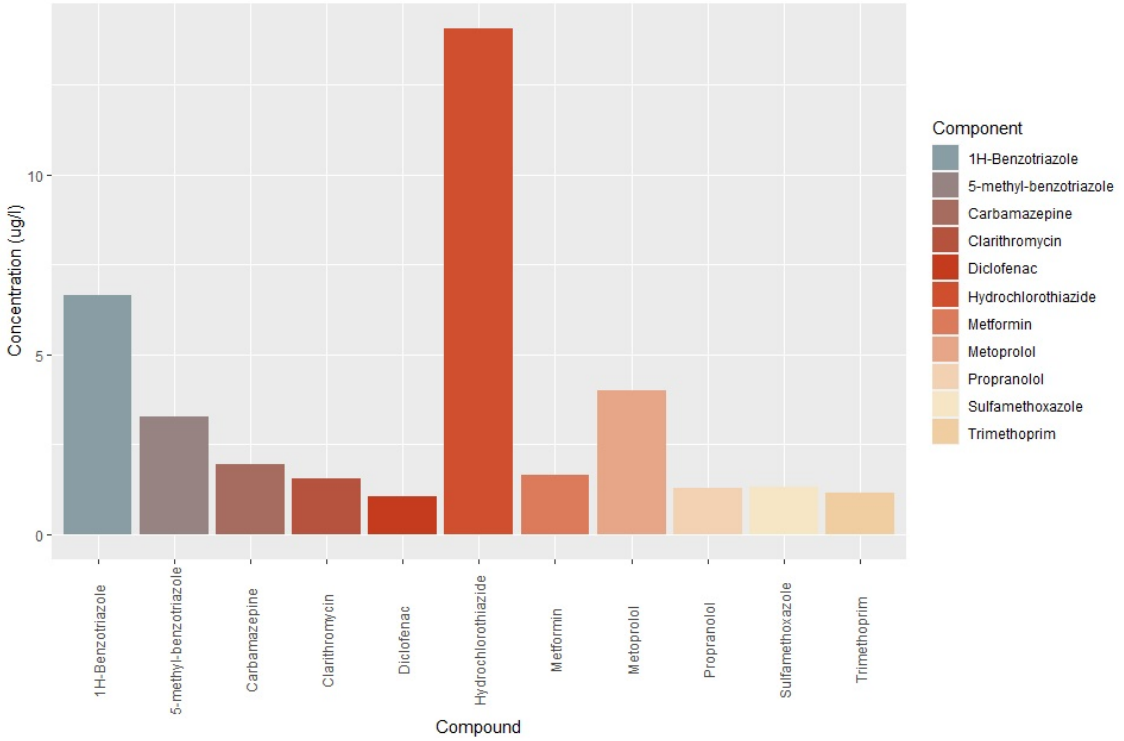

Figure B.2: Bar graph of the concentration OMPS (µg/l) in untreated effluent

C

Change in parameters by using ozonation

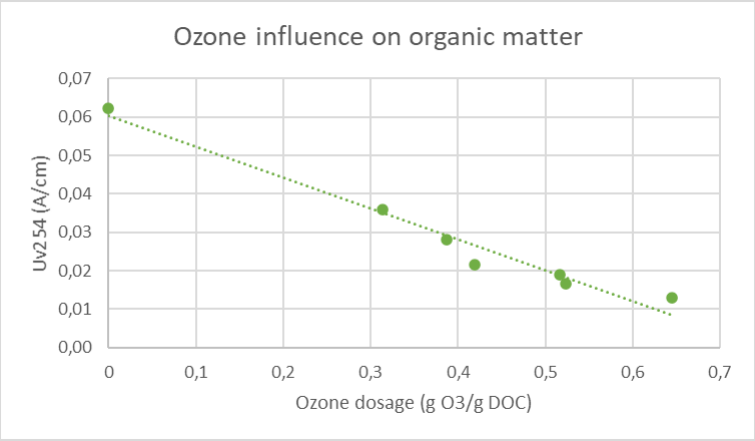

Figure C.1: UV254 with different ozone concentrations

D

Concentration of indicators in the O_3-STEP filter

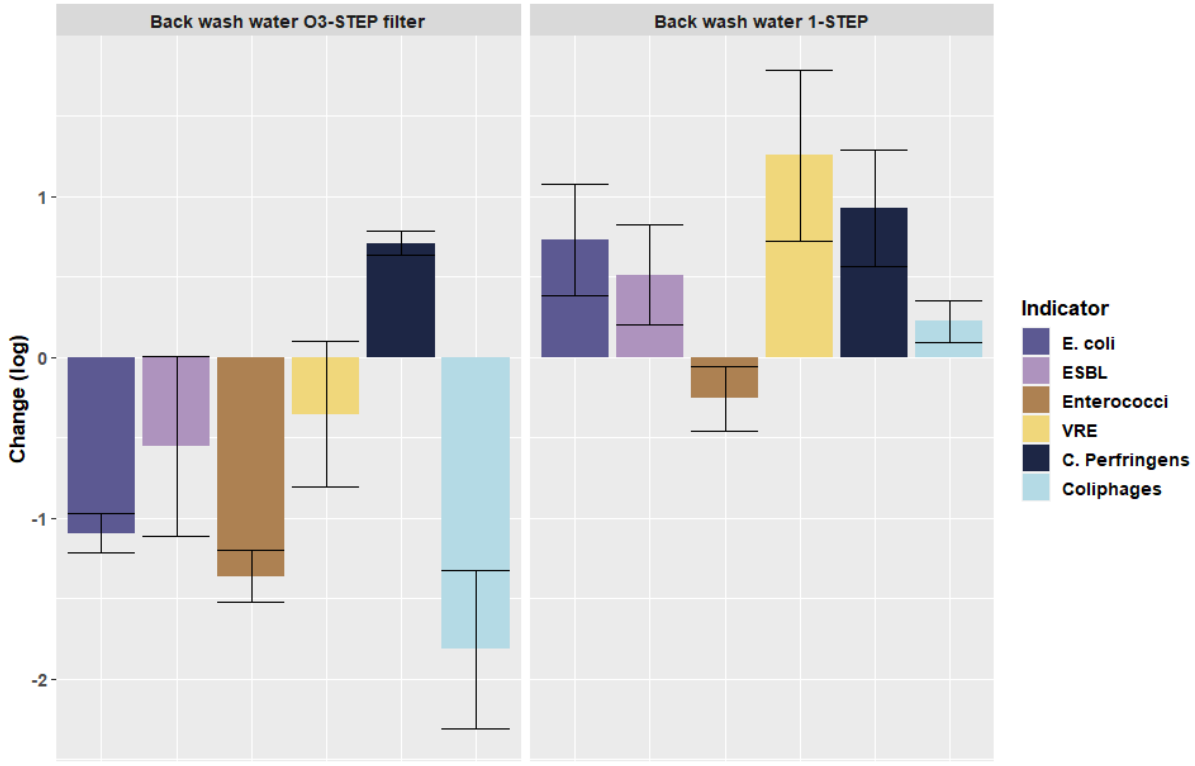

Figure D.1: Bar graph of the change (log) of different microbes in the backwash water in comparison with the secondary effluent. Note: Each bar represents the average of three values (triplicate microbial screening, single assay). Error bars indicate standard deviation.

E

Criteria scoring and definitions of the multi-criteria analysis (MCA)

	Criteria definitions
Effectiveness ARB	The treatment is effective in removing ARB (mediocre = current design)
Effectiveness ARG	The treatment is effective in removing ARGs (mediocre = current design)
Investment costs	What are the initial costs of the investment (Very good: no increase from the current design)
Operational costs	What are the operational costs of the investment (Very good: no increase from the current design)
Conventional method	It is a used application in WWTPs, thus less risks than with new innovations
Electricity usage	Additional electricity usage on top of current O3-STEP design (none: very good)
Leachate and Sludge	No extra sludge treatment is necessary
By-products	Risk of the formation of additional (disinfection) by-products
Increase OMP removal	Can increase the removal of OMPs
Space usage	There is no additional space needed next to the O3-STEP (= Very good)

Figure E.1: Criteria Definitions

Figure E.2: Criteria Scores

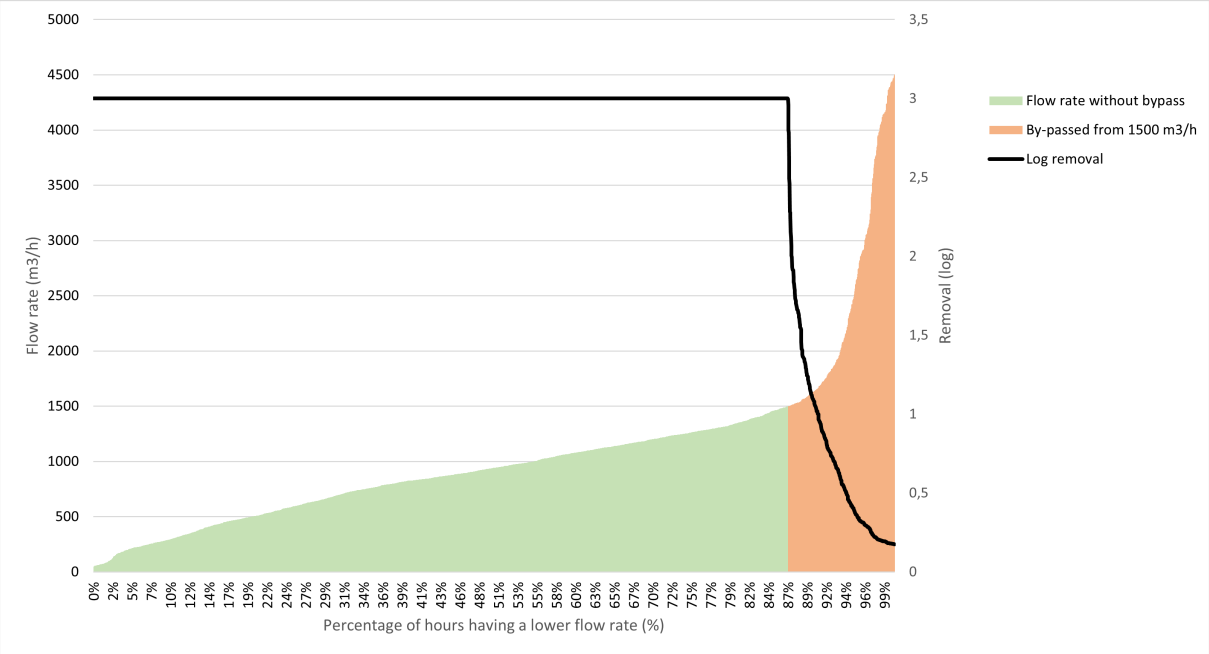

Figure E.3: Flow rate analysis. Based on 2870 hours in 2021 at location Horstermeer. All water over 1500 m^3/h is by-passed, such as is the case for the 1-STEP filter. When the log removal drops below 2.1 log, the removal is noted as ”insufficient” (orange part of the graph) with the false assumption that the incoming bacteria stay the same. This is the case in 13% of the hours and immediately at the point where the by-passing starts.