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Reduction of Antibiotic-Resistance Genes during Advanced Water Treatment in the New Goreangab Water Reclamation Plant for Direct Potable Reuse

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Abstract

This study analysed the fate and removal efficiency of antibiotic-resistant genes (ARGs) throughout the water reclamation process of the New Goreangab Water Reclamation Plant (NGWRP) in Windhoek, Namibia. The focus was on quantifying ARGs via quantitative PCR (qPCR) across different influent sources and throughout the entire treatment train, as well as on examining the interplay between absolute and relative abundance (ARGs normalized to *16S rRNA* gene). Physicochemical parameters and pharmaceutical concentrations were measured throughout the system to supplement molecular data and assess their potential influence on microbial resistance mechanisms.

The results from qPCR, supplemented by high-throughput qPCR, showed that all ARG classes entering the system mostly originated from effluent from Gammams Water Care Works. The treatment train of the NGWRP reduced the total microbial load to background levels, as confirmed by *16S rRNA* gene quantification and heterotrophic plate count analysis. All targeted ARGs: *sul1*, *tetW*, *ermB*, $bla_{CTX\text{-}M\text{-}1}$, and class 1 integron *intl1*, were reduced to below amplifiable levels in the final product.

The genes *sul1* and *intl1* both serve as proxies for horizontal gene transfer, and their absolute and relative abundances declined throughout the treatment process. This implies that the genes themselves were removed, and that their contribution to the total microbial load decreased simultaneously. The treatment steps Dissolved Air Flotation (DAF) and Biological Activated Carbon (BAC) increased the absolute abundance of all measured genes; however, their relative abundance did not increase. This indicates no selective enrichment of ARGs during these processes. Contrastingly, Dual Media Filtration (DMF) decreased absolute gene abundances but increased the relative abundance of detectable genes *(intl1, sul1*, and *tetW*). This result suggests that the DMF removal mechanism, based on particle size separation, is insufficient to remove smaller-particle-associated ARGs or extracellular ARGs at the same rate as the total microbial load (which consists of a larger proportion of inDNA). Taken together, the results offer insights into the presence, fate, and ultimate reduction of ARGs in the NGWRP system. They might support further efforts to reduce the risk of antimicrobial resistance in water reclamation for direct potable reuse.

Kurzfassung

Diese Studie analysiert den Verlauf und die Entfernungseffizienz von Antibiotikaresistenzgenen (ARGs) im Verlauf des Wasseraufbereitungsprozesses der New Goreangab Water Reclamation Plant (NGWRP) in Windhoek, Namibia. Der Schwerpunkt liegt auf der Erfassung von ARGs mittels quantitativer PCR (qPCR) aus verschiedenen Zulaufquellen entlang des gesamten Behandlungsprozesses sowie auf der Untersuchung des Zusammenspiels zwischen absoluter und relativer Häufigkeit (ARGs normalisiert auf das *16S-rRNA*-Gen). Physikochemische Parameter und Konzentrationen von Pharmaka wurden im gesamten System gemessen, um die molekularen Daten zu ergänzen und den potenziellen Einfluss der Pharmaka auf mikrobielle Resistenzmechanismen zu bewerten.

Die Ergebnisse der qPCR, ergänzt durch Hochdurchsatz-qPCR, zeigten, dass alle in das System eingehenden ARG-Klassen größtenteils aus dem Ablauf von Gammams Water Care Works stammten. Der Behandlungsprozess des NGWRP reduzierte die gesamte mikrobielle Belastung auf Hintergrundwerte, wie durch die Quantifizierung des *16S-rRNA*-Gens und die Analyse der heterotrophen Plattenzählung bestätigt wurde. Alle untersuchten ARGs: *sul1*, *tetW*, *ermB*, $bla_{CTX\text{-}M\text{-}1}$ und das Klassen-1-Integron *intI1* wurden im Endprodukt auf nicht nachweisbare Werte reduziert.

Die Gene *sul1* und *intI1* dienen beide als Indikatoren für horizontalen Gentransfer, und ihre absolute sowie relative Häufigkeit nahmen während des Behandlungsprozesses ab. Dies deutet darauf hin, dass die Gene selbst entfernt wurden und gleichzeitig ihr Anteil an der Gesamtmikrobenpopulation abnahm. Die Behandlungsschritte „Dissolved Air Flotation“ (DAF) und „Biological Activated Carbon“ (BAC) führten zu einem Anstieg der absoluten Häufigkeit aller gemessenen Gene; ihre relative Häufigkeit stieg jedoch nicht. Dies zeigt, dass während dieser Prozesse keine selektive Anreicherung von ARGs stattfand. Im Gegensatz dazu reduzierte die „Dual Media Filtration“ (DMF) die absoluten Genhäufigkeiten, erhöhte jedoch die relative Häufigkeit der nachweisbaren Gene (*intI1*, *sul1* und *tetW*). Dieses Ergebnis deutet darauf hin, dass der DMF-Entfernungsmechanismus, der auf Partikelgrößenseparation basiert, nicht in der Lage ist, ARGs, die mit kleineren Partikeln assoziiert oder extrazellulär vorliegen, im gleichen Maße wie die Gesamtmikrobenpopulation (die einen größeren Anteil an inDNA enthält) zu entfernen. Zusammengefasst liefern die Ergebnisse Einblicke in das Vorkommen und die Reduktion von ARGs in der NGWRP. Sie könnten weitere Maßnahmen zur Reduzierung des Risikos antimikrobieller Resistenz bei der Wasseraufbereitung zur direkten Trinkwasserwiederverwendung unterstützen.

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List of Abbreviations

AR	Antibiotic Resistance
ARB	Antibiotic-Resistant Bacteria
ARG	Antibiotic Resistance Gene
DNA	Deoxyribonucleic Acid
DOC	Dissolved Organic Carbon
DPR	Direct Potable Reuse
EEA	European Environmental Agency
exDNA	Extracellular Deoxyribonucleic Acid
GWCW	Gammams Water Care Works
HDPE	High-Density Polyethylene
HGT	Horizontal Gene Transfer
HPLC-MS/MS	High-Performance Liquid Chromatography-Tandem Mass Spectrometry
HTS-qPCR	High-throughput Screening qPCR
inDNA	Intracellular Deoxyribonucleic Acid
IC	Ion Chromatography
IPR	Indirect Potable Reuse
MCE	Mixed Cellulose Ester
MGE	Mobile Genetic Element
MSC	Minimum Selective Concentration
NGWRP	New Goreangab Water Reclamation Plant
pbDNA	Particle-bound Deoxyribonucleic Acid
PMA	Propidium Monoazide
qPCR	Quantitative Polymerase Chain Reaction
SMX	Sulfamethoxazole
SOP	Standard Operating Procedure
SPE	Solid Phase Extraction
TOC	Total Organic Carbon
VGT	Vertical Gene Transfer
WHO	World Health Organization
WMO	World Meteorological Organization
WWTP	Wastewater Treatment Plant

1. Introduction

Clean water is vital to life and therefore, one of the most essential natural resources on earth. Population growth, urbanization, and climate change increase the pressure on freshwater resources, affecting almost half of the global population (Leijnse et al., 2024; Shemer et al., 2023). This is exacerbated by climatic events, such as prolonged droughts, and by challenges in efficient water management (Dos Santos et al., 2017). As water demands are expected to increase, especially in regions prone to drought, climate-resilient water systems are essential to ensure a consistent, high-quality freshwater supply (WMO, 2024).

In urban areas lacking a consistent source of freshwater, water reclamation may be a solution (Jimenez & Asano, 2008; Rygaard et al., 2011). Water reclamation refers to the use of treated wastewater for agricultural, industrial, or potable purposes. Potable reuse is the practice of producing drinking water from treated wastewater. A distinction can be made between Direct Potable Reuse (DPR) and Indirect Potable Reuse (IPR). During IRP, treated water is returned to an environmental buffer before being reused, for example, injected into a groundwater aquifer or surface water reservoir. If treated wastewater is directly introduced into the drinking water distribution system without an environmental buffer, this is referred to as DPR (Jimenez & Asano, 2008; WHO, 2017). Both forms of potable reuse can reduce reliance on unstable or insufficient fresh water sources. However, water reclamation, particularly for potable reuse, may raise public concerns about the potential presence of microbial pathogens and other contaminants of concern (Moesker et al., 2024). Commonly detected contaminants in wastewater and water reclamation systems include antibiotics and antibiotic resistance bacteria (ARB) and genes (ARG) (Garner et al., 2018; Uluseker et al., 2021). Indicating the relevance of assessing their potential presence and removal during water reclamation for potable reuse.

As antibiotics are essential in modern medicine for preventing and treating bacterial infections (Hutchings et al., 2019; Larsson, 2014), their worldwide consumption continues to rise due to population growth and improved global access to health care. Antibiotics are also widely used in agriculture, often incorporated into animal feed to prevent disease and enhance livestock growth and productivity (Gustafson, 1991; Odey et al., 2023). Antibiotic Resistance (AR) is the ability of bacteria to evolve and counteract the effects of antibiotics, despite exposure to concentrations that would typically cause growth inhibition or cell death. The mechanisms for AR are encoded in the bacterial DNA by ARGs. However, the widespread and prophylactic use of antibiotics can exert selective pressure on microbes, leading to the development of AR. AR can significantly reduce or

eliminate the efficacy of antibiotics against pathogenic and non-pathogenic bacterial strains (Davies & Davies, 2010). AR makes bacterial infections more difficult to treat (Naghavi et al., 2024; Ventola, 2015). As a result, AR is noted as one of the most pressing health challenges in low- and middle-income regions globally (WHO, 2022; Murray et al., 2022)

Antibiotics target cellular bacterial components or functions, such as cell walls, membrane integrity, protein synthesis, or metabolic pathways. Bacteria can be intrinsically resistant, for example, when they lack the drug's target structure. In the context of AR, acquired resistance is particularly relevant. Acquired resistance arises from chromosomal mutations that confer resistance to antimicrobial compounds (Davies & Davies, 2010). The most common resistance mechanisms are enzymatic degradation or modification of the antibiotic, modification of antibiotic target sites, which reduces the binding affinity, reduced permeability of the cell membrane, which limits the uptake of the antibiotic in the cell, and active efflux of antibiotics through membrane-efflux pumps (Naghavi et al., 2024; Reygaert, 2018). In the presence of an antibiotic, selective pressure is exerted on the microbial community, even at sub-inhibitory concentrations, thereby eliminating susceptible bacteria and allowing the bacteria with resistance mutations to survive and multiply. Thus, when bacterial communities are continuously exposed to antibiotics, resistant strains are promoted. These resistance mechanisms are then further spread along the bacterial community via two main mechanisms. Firstly, vertical gene transfer (VGT) occurs when genes are passed from the mother cell to the daughter cell during replication. Secondly, horizontal gene transfer (HGT) occurs when genes are exchanged between unrelated bacterial strains or species. This is mainly done via transformation or conjugation. During transformation, bacteria take up DNA fragments released by bacterial cells. This DNA may be dissolved in aquatic environments or adsorbed to organic matter, thereby preventing degradation and enhancing persistence. Conjugation involves the formation of extracellular follicles, which can form a physical bridge between bacteria to transport genes (Lawrence & Hendrickson, 2008). These processes all contribute to the emergence and propagation of antibiotic resistance among bacterial communities.

Antibiotics and their metabolites are excreted by humans and animals and enter wastewater systems from domestic, hospital, or agricultural sources. Consequently, wastewater treatment plants (WWTPs) serve as collection points for antibiotic residues, antibiotic-resistant bacteria (ARB), and other contaminants that can influence the development of ARGs (La Rosa et al., 2025; Uluseker et al., 2021). The presence of co-contaminants, such as heavy metals and biocides, can further promote ARG formation and persistence (Drane et al., 2024). This is because co-contaminants exert selective pressures, and the frequent co-occurrence of ARGs and other resistance genes within bacteria

enhances their survival (See paragraph Mobile Genetic Elements and Co-selection). Conventional wastewater treatment processes are not designed to specifically remove antibiotics or ARBs, and thus these are discharged into the environment after treatment (Uluseker et al., 2021; J. Wang et al., 2022). As a result, antibiotics, ARBs, and ARGs are frequently detected in the aquatic environment. For example, in rivers (Wilkinson et al., 2022), groundwater (Böckelmann et al., 2009), and drinking water sources (Alawi et al., 2022; Hu et al., 2018). As analytical and molecular methods improve, antibiotics and ARGs can be detected at increasingly lower concentrations and, therefore, better monitored. This helps in recommended monitoring and assessment of removal efficiencies of water treatment plants, which is especially relevant for the quality and safety of water treatment for DPR (Keller et al., 2022; Mosher et al., 2016). DPR is more likely to be employed in regions with scarce freshwater resources (Keller et al., 2022), such as in Sub-Saharan Africa.

The World Resources Institute (2023) predicted that Sub-Saharan Africa will experience the highest increase in water demand worldwide in the coming decades. This arid region's historic water scarcity makes efficient, sustainable water use and management essential. Namibia, located in South-Western Africa, is one of the driest countries in sub-Saharan Africa, receiving only 300-400 mm of precipitation per year. Due to evaporation rates up to eight times the annual rainfall, long periods of drought, and a growing population, the country's water shortages are exacerbated by a limited water supply (Mapani et al., 2023; Murray et al., 2018). The capital, Windhoek, where the population and infrastructure are concentrated, relies on groundwater from the Windhoek aquifer and from the von Bach Dam (Murray et al., 2018). These water sources are augmented with reclaimed water from the New Goreangab Water Reclamation Plant (NGWRP), which was investigated in this thesis. The NGWRP was built in 2001 as an updated version of the world's first DPR plant, built in 1969 (Du Pisani, 2006; Lahnsteiner et al., 2024). The NGWRP produces a maximum of 21.000 m^3 of drinking water per day (Du Pisani, 2006; Law et al., 2015).

Besides, predictions state that the African continent will experience the largest increase in antibiotic usage by 2030 (Tiseo et al., 2020). Global trends show that antibiotic use increases, positively correlating with increases in AR (Mendelsohn et al., 2023). A study even estimated the "all-age death rate attributable to resistance to be highest in western Sub-Saharan Africa" (Murray et al., 2022). Comparable to African trends, antibiotic use (Pereko et al., 2016) and the occurrence of AR are increasing in Namibia. The country relies heavily on livestock for domestic consumption and export, making antibiotic use in agriculture a major contributor to the increase in AR (Odey et al., 2023). To combat this, the Namibian government has set up a national action plan to stop the overuse of antibiotics (Namibian Ministry of Health and Social Services, 2017). However, implementing these

plans may face challenges due to the required expertise and training on AR (Emberger et al., 2018). Thus, these trends make continuous monitoring and adequate treatment essential within DPR installations (Keller et al., 2022; Soller et al., 2017), especially since limited research has been conducted on the prevalence of ARBs and ARGs in Namibia's aquatic environments or water treatment systems.

To understand ARGs and their potential spread and persistence in the environment, it is necessary to consider how and where they occur within bacteria and in the environment. Bacteria are prokaryotic, and therefore the genes that make up their DNA are not contained in a nucleus but rather present in the cytoplasm, either as chromosomal DNA or as smaller circular plasmid DNA. However, although ARGs originate from bacterial cells, they are not exclusively present within them. ARGs may be released from the bacterial cell in various ways. In the context of this thesis, the main release mechanisms are cell death, autolysis, and the formation of extracellular vesicles. In this case, the ARGs are present extracellularly (exDNA), where some degrade rapidly, and others can persist before also ultimately being degraded, depending on the environment. This exDNA can be found free-floating, within vesicles, or particle-bound (pbDNA) due to the preferential binding of negatively charged DNA to positively charged minerals or organic matter. When assessing the fate of ARGs, it is crucial to distinguish between intracellular DNA (inDNA) and exDNA+pbDNA, as they can contribute to AR in distinct ways. InDNA is considered actively functional DNA in living bacteria and can express resistance mechanisms, whereas exDNA and pbDNA can persist and propagate in environmental systems and later be taken up by competent bacteria through horizontal gene transfer (Bairoliya et al., 2025; Nagler et al., 2018; S. Wang et al., 2024).

Quantitative Polymerase Chain Reaction (qPCR) amplifies and quantifies specific genes, yielding a measure of target gene abundance by comparing the target to a known DNA standard. Despite its higher costs, PCR is frequently favored over culture-dependent methods for monitoring AR in water because it is more time-efficient, can also detect non-viable organisms, and is often considered more sensitive (Alfahl et al., 2024). However, qPCR amplifies the target DNA sequence, irrespective of whether the DNA originates from intact cells or membrane-compromised (non-viable) cells or is present extracellularly. A method to distinguish between inDNA and exDNA in qPCR is to use viability dyes such as propidium monoazide (PMA) (Nocker et al., 2007; Slimani et al., 2012). PMA binds to all double-stranded DNA present outside the cell and prevents its amplification during PCR. PMA can’t penetrate bacterial cells, meaning that the measured gene abundance represents the inDNA fraction. Thus, the difference between PMA-treated and untreated samples theoretically represents the exDNA fraction.

2. State of Knowledge

2.1 Selective Media Cultivation

One of the oldest, and in clinical settings, a standard way to measure AR is to cultivate bacteria on agar plates containing selective media. These media can include specific antibiotics, allowing resistant organisms to grow while inhibiting susceptible microorganisms. In the present study, the presence and occurrence of antibiotic-resistant bacteria was measured by culturing bacteria on agar enriched with or without sulfamethoxazole (SMX) (Figure 1). Sulfamethoxazole ($C_{10}H_{11}N_3O_3S$) is part of the antibiotic class of sulfonamides. Sulfonamides prevent bacterial growth by inhibiting folic acid synthesis. Folic acids are necessary for the synthesis of DNA and proteins. Due to the similar chemical structure to para-aminobenzoic acid (PABA), sulfonamides take their place by binding to the enzyme dihydropteroate synthase. In this way, they act as competitive inhibitors of the enzyme, preventing the production of folic acid. Therefore, sulfonamides are bacteriostatic, as they inhibit bacterial growth but do not kill the bacteria (Hitchings, 1973).

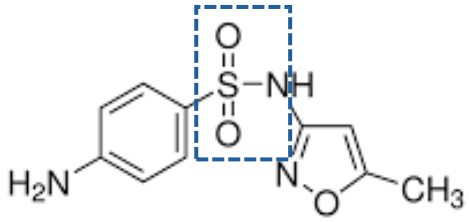


Figure 1: Chemical structure of sulfamethoxazole; blue dashed line indicates sulfonamide functional group.

SMX is effective against a broad range of bacterial infections caused by both gram-negative and gram-positive bacteria. To increase its effectiveness, it is commonly administered with trimethoprim (SMX-TMP), a bactericidal that interferes with the final step of folic acid synthesis (Hitchings, 1973). Together, TMP-SMX has been on the market since 1960 and is on the WHO Model List of Essential Medicines as a first-choice treatment for urinary tract infections. As urinary tract infections are among the most common bacterial infections worldwide, TMP-SMX is widely used (Mancuso et al., 2023). Bacteria can develop resistance to sulfonamides by altering the structure of dihydropteroate synthase, so that sulfonamide antibiotics like sulfamethoxazole cannot bind to the enzyme. This is mainly caused by chromosomal mutation or through the acquisition of plasmid genes such as *sul1*, *sul2*, and *sul3* (Sköld, 2001).

Due to its long history of use globally in human and veterinary medicine, sulfamethoxazole is often detected in wastewater (Zhuang et al., 2021) and surface waters (Wilkinson et al., 2022). This frequent detection made sulfamethoxazole a common indicator compound for studying

anthropogenic influences, antibiotic occurrence, and the potential for microbial resistance. By using sulfamethoxazole during bacterial cultivation in this thesis, insight was gained into the resistant cultivable bacteria present in the NGWRP and their removal rates. Correspondingly, *sul1* is also often used as a genetic indicator of anthropogenic influences and of AR spread via horizontal gene transfer; see Mobile Genetic Elements and Co-selection.

Indicator organisms commonly used to assess drinking water quality include heterotrophic microorganisms and faecal coliform bacteria. Heterotrophs encompass all bacteria, yeasts, and moulds that require an organic carbon source for their growth. Quantifying heterotrophs on suitable media provides insights into the efficacy of water treatment processes and serves as an indicator of drinking water safety (Bartram, 2013). Faecal coliforms are naturally occurring gut bacteria used as general indicators of microbial contamination, as their presence often correlates with that of other pathogens. They are frequently used as a microbial quality parameter of drinking water, and most regulations require a limit of zero coliforms per 100 ml (World Health Organization, 2022).

Selective media cultivation works well for clinically relevant pathogens as they are adapted to laboratory conditions and grow readily on standard media (Carroll et al., 2019). However, many environmental bacteria are difficult or impossible to cultivate on agar plates. As a result, a large part of the bacteria present in a sample do not form countable colonies on agar plates under laboratory conditions. This may be because some bacteria are in a viable but non-culturable state or depend on specific microbial interactions that are absent in isolated cultures. Additionally, many bacteria have specific growth requirements for temperature, pH, or nutrient composition to thrive, which can be challenging to replicate in laboratory settings (Li et al., 2014). As a result, culture-based methods may give a biased view and underestimate the microbial abundance in environmental samples. Nonetheless, in clinical settings, culturing is considered an essential first step in detecting resistance and can be complemented by microscopy or DNA-based techniques, such as qPCR, which allow detection and quantification of known resistance genes without culturing (Evans & Riley, 2021; Kaprou et al., 2021).

2.2 Mobile Genetic Elements and Co-selection

A bacterial cell contains the following components (Figure 2). Within the bacterial cell, DNA can exist in two states: chromosomal or on a plasmid. The chromosome contains most essential genes that mediate processes such as replication, metabolism, and cell maintenance. Chromosomal DNA is mostly inherited via VGT. The second type of DNA within the cell is plasmid DNA. A plasmid is a small, non-chromosomal, circular DNA. Plasmids often harbour genes that are not essential for the normal

functioning of bacteria but encode adaptive mechanisms when exposed to external stressors. These mechanisms can involve the degradation of toxic compounds and the development of resistance to heavy metals and antibiotics. Therefore, ARGs are frequently found on plasmids.

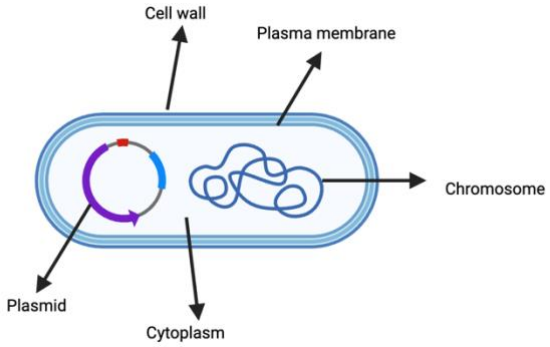


Figure 2: Components of a bacterial cell. Intracellular DNA is defined as being present within the plasma membrane/cell wall, either on a plasmid or on the chromosome. Both are in the cytoplasm. Chromosomal DNA can be transferred between bacterial cells via vertical gene transfer from mother to daughter cell during replication. Plasmids are mobile genetic elements that can be exchanged between (un)related bacterial species via horizontal gene transfer (figure created with BioRender.com).

Plasmids are mobile genetic elements (MGEs). MGEs can move and disperse within or transfer between bacterial cells. Main MGEs include plasmids, transposons, and integrons, which can all contribute to the spread of resistance within populations (Figure 3). Transposons are small pieces of DNA that can take up a gene and transport it via transposase enzymes, which lets the gene 'jump' from plasmid to chromosome within one cell, or vice versa. In this way, a resistant bacterium might be able to change its resistance traits from cell-bound to mobile and spread further to other bacteria. Integrons are other MGEs that can add, organize, and express gene-cassettes, which move as a unit. They often contain multiple resistance genes. They are not mobile themselves but are frequently carried on transposons or plasmids, on which they depend for transport (Frost et al., 2005; Johansson et al., 2025; Vale et al., 2022).

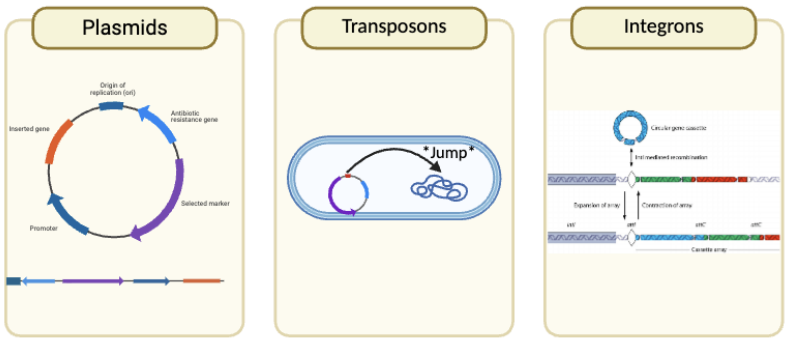


Figure 3: Main mobile genetic elements (MGEs) that facilitate horizontal gene transfer (HGT). ARGs are often contained on plasmids. As a result, ARGs can be transferred between (un)related bacterial species via HGT. Transposons facilitate transfer between the plasmid and the chromosome. ARGs can thus be transferred from chromosome to plasmid and out of the cell or introduced into a new cell via plasmids and subsequently incorporated into chromosomal DNA via transposons. Integrons are cassettes that can incorporate new genes. These MGEs facilitate ARG spread (figure created with BioRender.com).

Genes should be mobile to be transferred between bacterial species via HGT. The most relevant forms of HGT in the context of AR are conjugation and transformation (Figure 4). Conjugation is the main cause for the spread of plasmid-based ARGs, including associated integrons or transposons, via a pilus from the donor cell to the recipient cell. Transformation occurs when a competent bacterial cell takes up exDNA from its surrounding environment (Frost et al., 2005; Johansson et al., 2025). Recipient bacteria will be able to incorporate new genetic traits, such as AR, into their DNA. This exDNA is released from bacteria following cell lysis due to environmental stressors, antimicrobial or heavy-metal exposure, or natural cell death. Additionally, exDNA might persist for days to weeks before it is taken up through transformation. This persistence is dependent on environmental factors such as temperature, pH, and sorption to organic matter or clay particles in sediments (Joseph et al., 2022; Nielsen et al., 2007). This may lead to the persistence and spread of resistance genes (Mao et al., 2014).

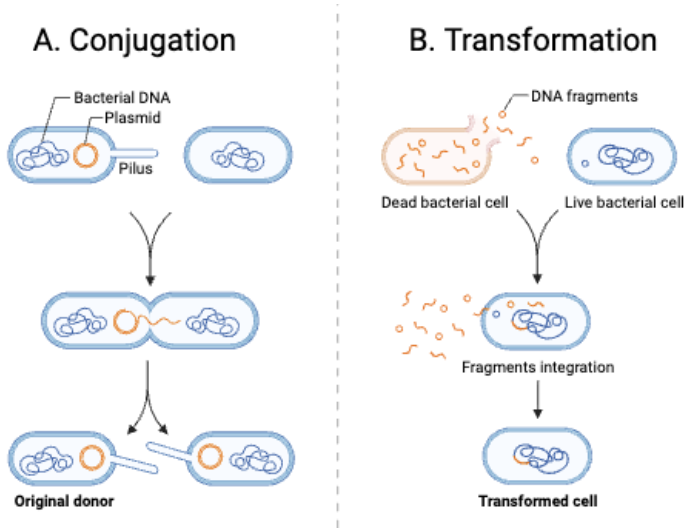


Figure 4: Main mechanisms of HGT in the context of this thesis. Conjugation involves the formation of a pilus, and transformation is the uptake of exDNA fragments by competent bacteria (Figure source: BioRender.com).

When antibiotics exert selective pressure on microbes, bacteria carrying MGEs with ARGs will have an advantage, persisting and spreading more readily. Integrons and plasmids often carry multiple resistance genes. This means that the genetic elements most prone to mobility are also those that confer multiple resistances. When they are exposed to selective pressure from an environmental stressor, co-selection occurs. Where not only the specific gene to that respective stressor will survive, but also the other resistance genes that were contained on the same plasmid or integron. This can be the case for genes conferring resistance to a broad range of environmental stressors, such as different antibiotics, heavy metal(s), or disinfectants. Co-occurrence thus exacerbates multi-resistance within microbial communities, without requiring direct exposure to specific stressors (Engin et al., 2023; Partridge et al., 2018).

This causes a problem for numerous reasons. Firstly, even if antibiotic input decreases due to new regulations, other stressors, such as heavy metal exposure, might still exert selective pressure on bacterial communities, favouring heavy metal resistance genes combined with ARGs. Similarly, the spread of multi-resistant bacteria poses risks. Aquatic environments, such as WWTPs and surface waters, often contain mixtures of antibiotics, heavy metals, and other organic pollutants. Additionally, these systems contain diverse microbial communities that can develop resistance to selective pressure from constant exposure to stressors. The combination of chronic exposure and a densely populated microbial community made researchers point to WWTPs as hotspots for ARG reservoirs (Guo et al., 2017; Pärnänen et al., 2019) and for HGE between environmental and pathogenic bacteria due to high bacterial densities (Brown et al., 2024).

2.3 Distinction between Intracellular and Extracellular DNA

A limitation of PCR is the inability to determine whether the detected genes originate from live or dead cells or are present extracellularly. In the context of resistance genes and their spread in the environment, it is relevant to determine whether the genes are present in living cells and can therefore be expressed, or are present extracellularly, where they play an important role in HGT and might persist longer. Different distinctions are found in the literature regarding exDNA, with no universal definition of exDNA and its subcategories existing yet. In this thesis, exDNA refers to genes that are either free-floating, particle-bound (pbDNA), or associated with cells without an intact cell membrane, see Figure 5 (Zhang et al., 2018).

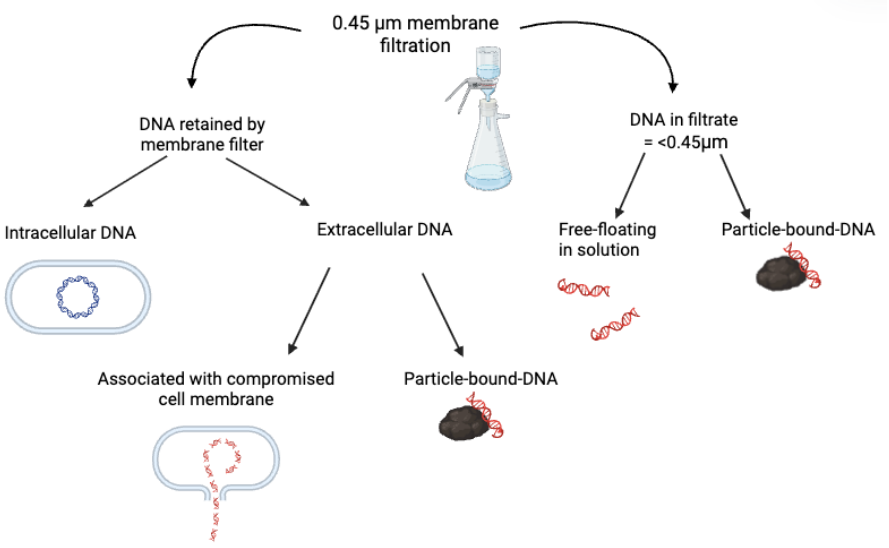


Figure 5: Most relevant forms of intracellular and extracellular DNA retained on the membrane filter and present in the filtrate as defined by this study. DNA retained by the filter may include intracellular DNA within intact bacterial cells or extracellular DNA, either bound to particles of organic matter or present in a bacterial cell with a compromised cell membrane, which leaves the bacterial cell and becomes extracellular DNA. The filter is expected to retain most intact bacterial cells, but free-floating extracellular DNA and DNA bound to particles smaller than 0.45 µm are supposed to largely pass through the filter and into the filtrate (figure created with BioRender.com).

Intracellular genes can be expressed and spread to other bacteria mainly through VGT and HGT via MGEs, whereas exDNA spreads and persists in the environment and can be taken up via transformation. The distinction between intracellular and extracellular resistance genes plays an essential role in the spread of resistance between bacteria.

PMA is a viability dye that complements qPCR by distinguishing between inDNA and exDNA. The dye binds covalently to double-stranded DNA, preventing amplification during qPCR (Figure 6). However, PMA cannot pass through intact cell membranes, making intracellular DNA inaccessible. Consequently, the dye binds only to extracellular DNA in the sample. After measuring the quantity of genes in the same sample, both treated with and without PMA, the difference in these samples would correspond to the amount of eDNA.

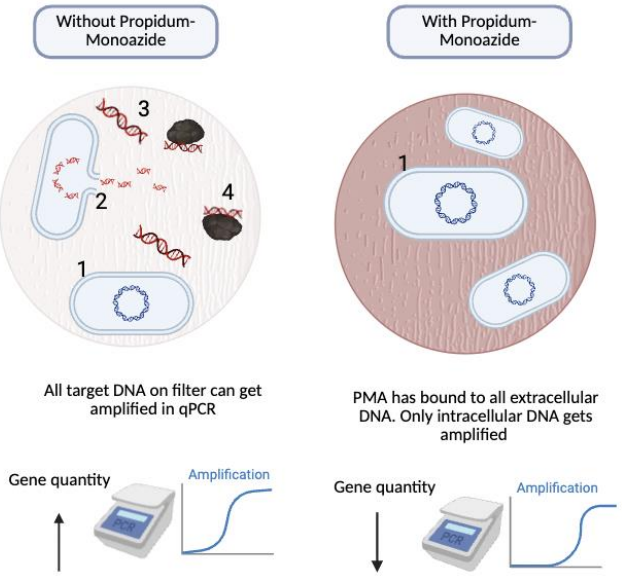


Figure 6: Left; untreated filter after membrane filtration, contains all possible forms of intracellular and extracellular DNA. Right: PMA-treated membrane filter; PMA has intercalated with extracellular DNA, so only intracellular DNA will be amplified and is 'visible'. (1) Intracellular DNA; (2) extracellular DNA stemming from compromised cell membranes; (3) free-floating extracellular DNA; (4) particle-bound extracellular DNA.

2.4 Gammams Water Care Works Wastewater Treatment Plant

The Namibian Water Resources Management Act (2013) specifies the main quality parameters that should be met after wastewater treatment. These include aesthetic and physical parameters, such as colour, turbidity, and inorganic chemicals, as well as bacteriological parameters, such as total coliforms and *E. coli*. Generally, the main objective is to achieve water quality that can be responsibly discharged into the receiving water body. At the Gammams Water Care Works (GWCW) WWTP, effluent is not discharged into surface water but is instead directly transferred to the NGWRP as influent for the water reclamation process. The GWCW WWTP process consists of primary settling tanks to remove organic and inorganic solids, followed by the activated sludge process to remove

further dissolved organic matter, nitrogen, and phosphorus. Finally, the water is directed into eight consecutive maturation ponds, with a total retention time of three days. In this last step, mainly pathogens and leftover nutrients are removed, and Biological Oxygen Demand (BOD) and Total Suspended Solids (TSS) are also lowered. When the resulting water meets the quality criteria, it is pumped to the NGWRP and serves as influent to the advanced water treatment process, producing reclaimed water for DPR. The GWCW is expected to reduce the general bacterial and pathogenic load. This is confirmed through biweekly faecal coliform cultivation of the settling tank and the maturation ponds, which Scientific Services analyse. However, conventional water treatment is known to be less effective at removing exDNA and can even increase ARGs during biological processes with high microbial densities, such as activated sludge (Wang & Chen, 2022). Therefore, the effluent from GWCW likely contains measurable amounts of ARGs, which will go into the NGWRP. Due to the high proportion of WWTP Effluent in the NGWRP Influent, this stream is expected to contribute significantly to the inflow of ARB and ARG into the system

2.5 New Goreangab Water Reclamation Plant

To ensure sufficient drinking water for Windhoek's population despite its climatic vulnerability, the Goreangab Water Reclamation Plant was put into use in 1968, making Windhoek the first city in the world to employ direct potable reuse (DPR). In 2002, the New Goreangab Water Reclamation Plant (NGWRP) replaced the original installation. However, the original installation is still in use and now provides irrigation water (Du Pisani, 2006; Lahnsteiner et al., 2024).

The water reclamation process is based on a multi-barrier advanced water treatment process. Multi-barrier refers to independent barriers designed to maintain control over the production process for safe, potable water. The first barrier is the exclusive use of wastewater effluent from a domestic source, which is then mixed with recycled water from within the system. The second barrier refers to the actual water reclamation process, which employs an eleven-step advanced treatment train, as shown in Figure 7. The third and final barrier consists of ultimately blending the final product with the groundwater and dam water in the water distribution system. Reclaimed water from the NGWRP provides about a third of the distributed water. Additionally, the entire process is live monitored at various stages, and action is taken when needed to ensure the desired water quality.

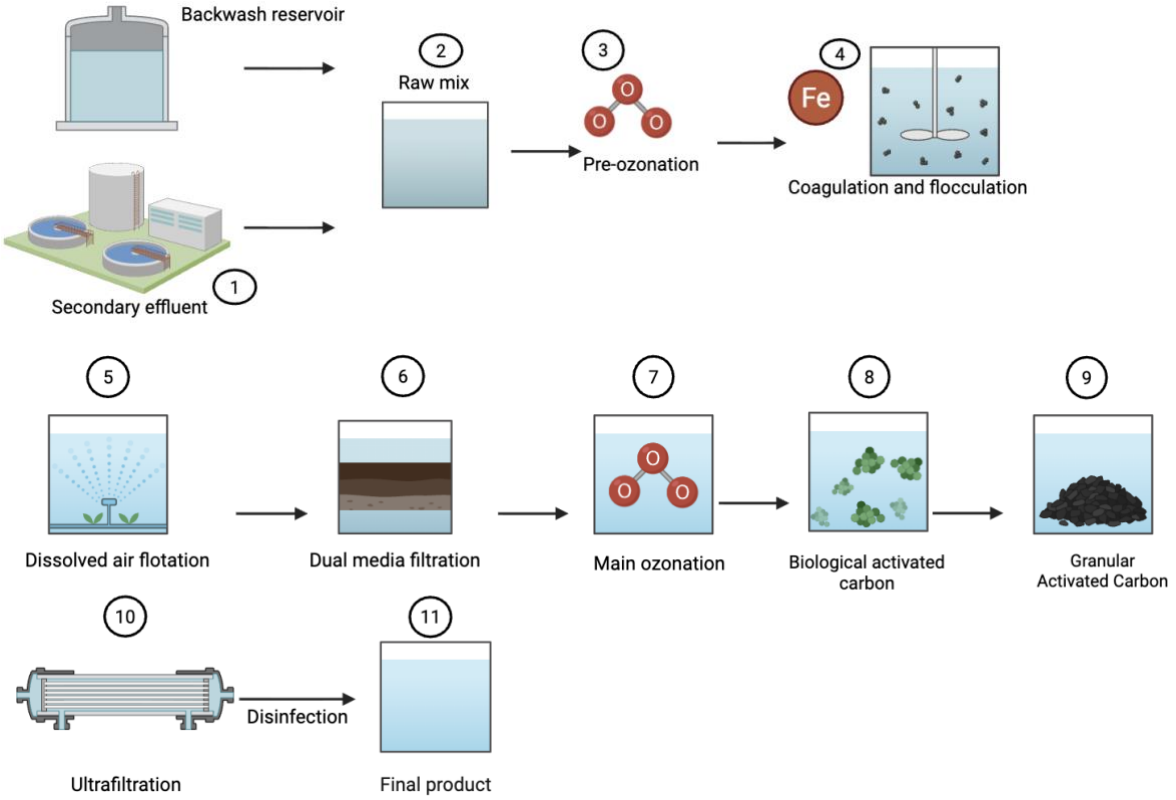


Figure 7: Treatment process of the water reclamation process of the New Goreangab Water Reclamation Plant. The main influent comes from treated wastewater from Gammams Water Care Works WWTP, supplemented with recycled water from within the system after membrane and filter backwashing. This is combined into the Raw Mix going into the system. Pre-ozonation and main-ozonation break up larger particles and improve the efficiency of the following steps. Coagulation, flocculation, and dissolved air flotation are based on the removal of larger particles that float to the surface. Dual media filtration is based on size separation. Biological activated carbon and granular activated carbon are based on biodegradation and adsorption of pollutants, respectively. Ultrafiltration removes the tiniest particles, and disinfection maintains quality during distribution (figure created with BioRender.com).

During the first step, the effluent from the GWCW maturation ponds is blended with water from the internal backwash reservoir and combined into the raw mix (2). The Raw Mix (2) consists of effluent from GWCW maturation ponds combined with internal backwash from filters (steps 6, 8, and 9; Figure 8 and Figure 8), as they need to be backwashed periodically to prevent clogging and maintain efficiency. The NGWRP has an inflow of approximately 20,400 m^3/day (about 15% backwash water and 85% secondary effluent).

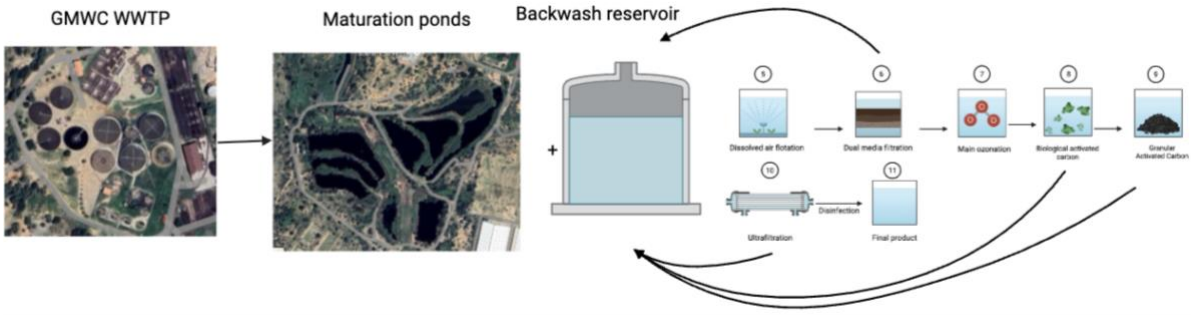


Figure 8: Raw Mix (Influent) of the NGWRP consists of wastewater treated by Gammams Water Care Works with consecutive maturation ponds serving as the final step. From the maturation ponds, the water is pumped to the NGWRP, where it accounts for 85% of the inflow and is combined with reservoir water recycled from within the NGWRP during membrane backwashing. Providing the remaining 15% of the total inflow (figure created with BioRender.com).

The second step (3) is pre-ozonation (3 – 5 mg O_3/L). Ozone is a strongly oxidizing compound that breaks down larger organic compounds, thereby lowering the organic load, which is beneficial for the efficiency of later steps. Ozone is produced on-site via pressure swing adsorption with zeolite filters, separating oxygen and nitrogen from ambient air and generating ozone gas. Potassium permanganate ($KMnO_4$) is dosed to precipitate manganese (Mn^{2+}) in solution. Then, in the following step (4), ferric chloride (± 90 mg $FeCl_3$/L) is added, which is a coagulant that binds to suspended solids and dissolved organic matter. The water is slowly stirred to improve floc formation. During dissolved air flotation (5), water saturated with air is sprayed upward from the bottom of the basin, which forms bubbles. These bubbles attach to the flocs formed during the previous step, causing them to float to the surface. This floc layer on the water's surface is scraped off. Sodium hydroxide (NaOH) is added for pH stabilisation and potassium permanganate ($KMnO_4$) for residual iron and manganese precipitation. This prevents iron and manganese from causing colour or taste in water or membrane fouling. Dual media filtration (6) consists of an anthracite filter to remove larger particles and a sand filter to remove smaller particles. These filters are backwashed at least every 60 hours, or earlier if the membrane pressure reaches its maximum. Main-ozonation (7) (8 – 11 mg O_3/L) further breaks down dissolved organic compounds and natural organic matter, and it also inactivates bacteria by oxidation of cell membranes and DNA. The ozone concentration applied is adjusted based on the microbial load entering the system. Hydrogen peroxide (H_2O_2) is added to break down residual ozone. Biological activated carbon (BAC) (8) consists of granular activated carbon onto which micro-organisms have grown, forming a biofilm layer. These microorganisms consume and remove the previously fragmented organic matter as nutrients. The BAC is replaced every 8 – 10 years. The subsequent granular activated carbon (GAC) is based on the activated carbon's absorption of remaining dissolved organic compounds, such as pharmaceuticals (9). The adsorption process is aided by the high surface area of the activated carbon granules' pores. The GAC is replaced every 4–6 months to prevent microbial growth, which would interfere with the activated carbon's adsorption capacity. BAC and GAC are periodically backwashed. Finally, the water is filtered through an ultrafiltration (UF) system (10) consisting of membranes with a 20 nm pore size. Lastly, chlorine (Cl_2) and sodium hydroxide (NaOH) are added for disinfection and pH adjustment, respectively (11). Multiple steps in the process contain membranes or filters that require periodic backwashing to prevent clogging. The water that is released after backwash is stored in the 'backwash reservoir' and used to supplement the incoming influent from GWCW works.

Literature indicates that ARGs can build up in drinking water treatment systems, potentially increasing through biofilm formation (Abe et al., 2020) and during backwashing (Spit et al., 2022; Wan et al., 2021). Biological water treatment processes depend on an active microbial community

and are susceptible to biofilm development. A biofilm is a complex matrix of microorganisms embedded in an extracellular polymeric substance excreted by bacteria that grows on surfaces. It is commonly found in industrial, hospital, or environmental systems. Biofilm provides bacteria with a growth advantage; it harbours and preserves exDNA and contributes to HGT due to the high bacterial population density (Abe et al., 2020; Balcázar et al., 2015).

Spit et al. (2022) analysed the levels of microbial indicators in backwash water from a new small-scale GAC filter compared with those from a mature GAC filter. It was found that backwash water from the mature biomass filter showed increased concentrations of microbial indicators. This can be caused by the fact that some activated carbon methods rely on biofilm growth, which is known to concentrate microbes and genetic elements. However, other sources report a reduction in exDNA after BAC treatment because microbes can consume it as a nutrient source (Ibáñez De Aldecoa et al., 2017).

2.6 Aim and Research Questions

This study aims to understand ARG removal at different treatment steps in the NGWRP, with a special focus on differentiating intracellular and extracellular ARGs. Additionally, reductions in culturable ARB will be assessed to complement these analyses. In this way, the NGWRP will serve as a case study for the fate of AR through advanced water treatment for DPR. This leads to the following research questions:

1. How do the two influent sources entering the NGWRP (WWTP Effluent and internal backwash water) differ in the ARG composition?
2. To what extent do the different treatment steps within the NGWRP reduce absolute and relative ARGs?
3. How do intracellular (inARG) and extracellular + particle-bound ARG (exARG + pbARG) fractions change across treatment steps?
4. Can ARG concentrations be influenced by pharmaceutical concentrations and selected physicochemical parameters in the NGWRP?

Based on the state of knowledge and research questions, the following hypothesis is formulated:
It is expected that advanced water treatment steps, as performed during the water reclamation process of the New Goreangab Water Reclamation Plant for direct potable reuse, will reduce the absolute and relative abundance of ARBs and ARGs and will be more efficient at removing inARGs compared to exARG/pbARGs.

3. Materials and Methods

Before the experiments at the von der Emde Technikum and the NGWRP could be planned, several preliminary experiments were conducted at the TU Wien. The materials and methods of these preliminary experiments are described in Appendix II: Method Preliminary Experiments TU Wien.

3.1 Experiments von der Emde Technikum

Before the sampling period in Windhoek, the same procedures were carried out at the Van der Emde Technikum at the TU Wien to confirm the method. The Technikum consists of a small-scale wastewater treatment plant with a maximum capacity of 300 L/day. It employs a three-step conventional treatment process, including a tank for nitrification, denitrification, and gravitational settling. Additionally, the Technikum has an ultrafiltration unit (polyether sulfone, 20 nm pore size) that is not directly connected to the small-scale WWTP. During the pre-experiments, treated wastewater was sampled as 'influent', mimicking the water reclamation process of the NGWRP. Additional samples were collected after the ultrafiltration unit, which was run in crossflow mode due to the expected high suspended solid content of the treated wastewater. Lastly, a disinfection step was performed to mimic the NGWRP, which employs similar treatment steps (Figure 9).

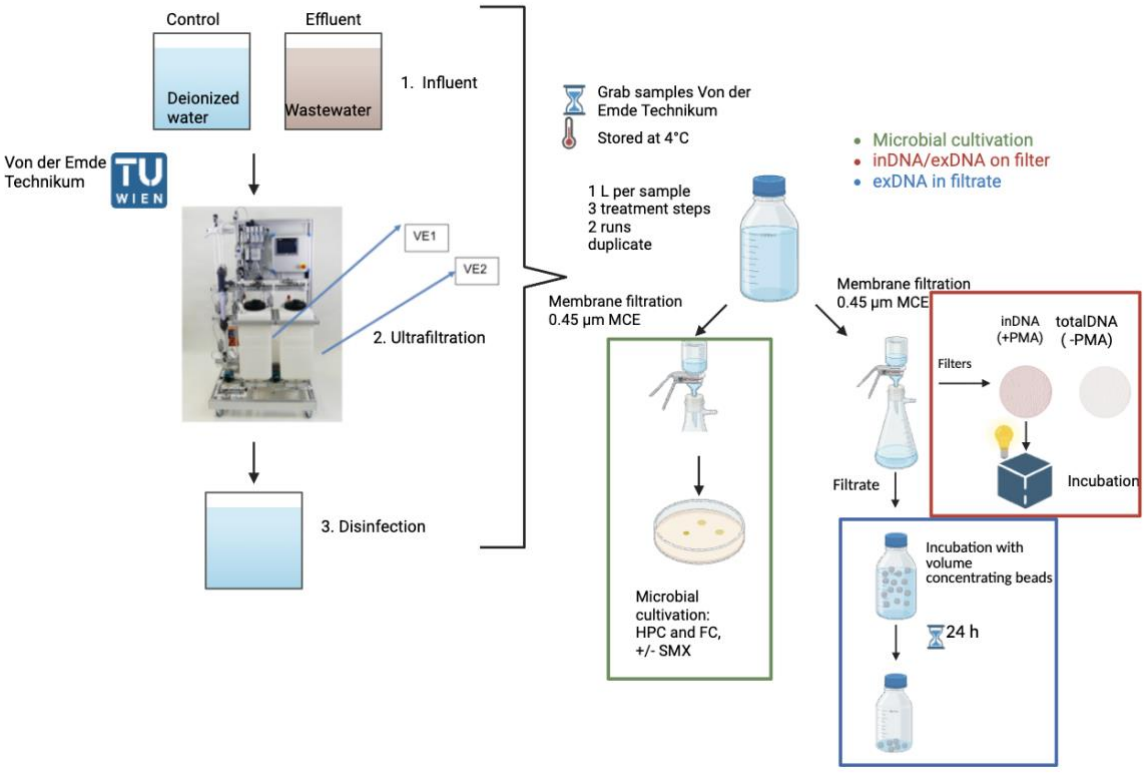


Figure 9: Sampling points and subsequent analyses during pre-experiments in Von der Emde Technikum at the TU Wien (figure created with BioRender.com)

After each step, 1 L samples were collected in duplicate for both the control and treated wastewater runs. Samples were taken in glass Schott bottles, which were first dishwasher-cleaned, then swirled with deionized water to remove residual detergent, rinsed with 1.4% NaOCl, and the lids were submerged in the same solution. Lastly, they were rinsed again with deionized water and either baked at 170 ± 10 °C for at least 1 hour or autoclaved at 121 ± 3 °C for at least 15 minutes (*ISO 19458*, 2006).

The feed (VE1) and permeate (VE2) tanks were rinsed with deionized, autoclaved water. First, a run was done with deionized water as a control. A run included sampling influent (either deionized water or treated wastewater effluent), loading 25 L of influent into VE1, and sampling from VE2 after ultrafiltration. Lastly, 0.4 ml/L of 0.5% sodium hypochlorite (NaOCl) was added to VE2 for 30 minutes to achieve a free chlorine concentration of 0.2-2 mg/L at the end of the exposure time (WHO, 2022).

After the exposure time, sodium thiosulfate was added to quench residual chlorine. The theoretical mass of sodium thiosulfate (pentahydrate) necessary to inactivate 1 mg of chlorine is 7.1 mg. This will inactivate 2-5 mg/L of free chlorine residual, depending on inactivation dynamics (*ISO 19458*, 2006). A sodium thiosulfate pentahydrate solution (18 mg/L) was made by dissolving 90 mg in 5 mL of autoclaved deionized water. Of the solution, 1 mL was added to each 1 L chlorinated sample.

3.2 New Goreangab Water Reclamation Plant

Figure 10 shows the experimental plan for the two main sampling days at the NGWRP in Windhoek, Namibia (days 1 and 2). On day 1, duplicate samples were collected at all eleven steps of the treatment process and analysed by microbial cultivation and qPCR for inDNA and exDNA on filters. On day 2, another set of duplicate samples was collected at five of the eleven steps for inDNA + exDNA analysis on filters, as well as for exDNA analysis of the retained filtrate. All experimental steps depicted were executed on the respective day of sampling. Afterwards, all resulting filters were immediately stored at -20°C, and the filtrate samples on the next day after the 24-hour incubation, before being transported to TU Wien on dry ice for DNA extraction and subsequent molecular biological analyses. Additionally, approximately 250 mL of untreated liquid samples were transported at 4°C for chemical analyses of selected pharmaceuticals.

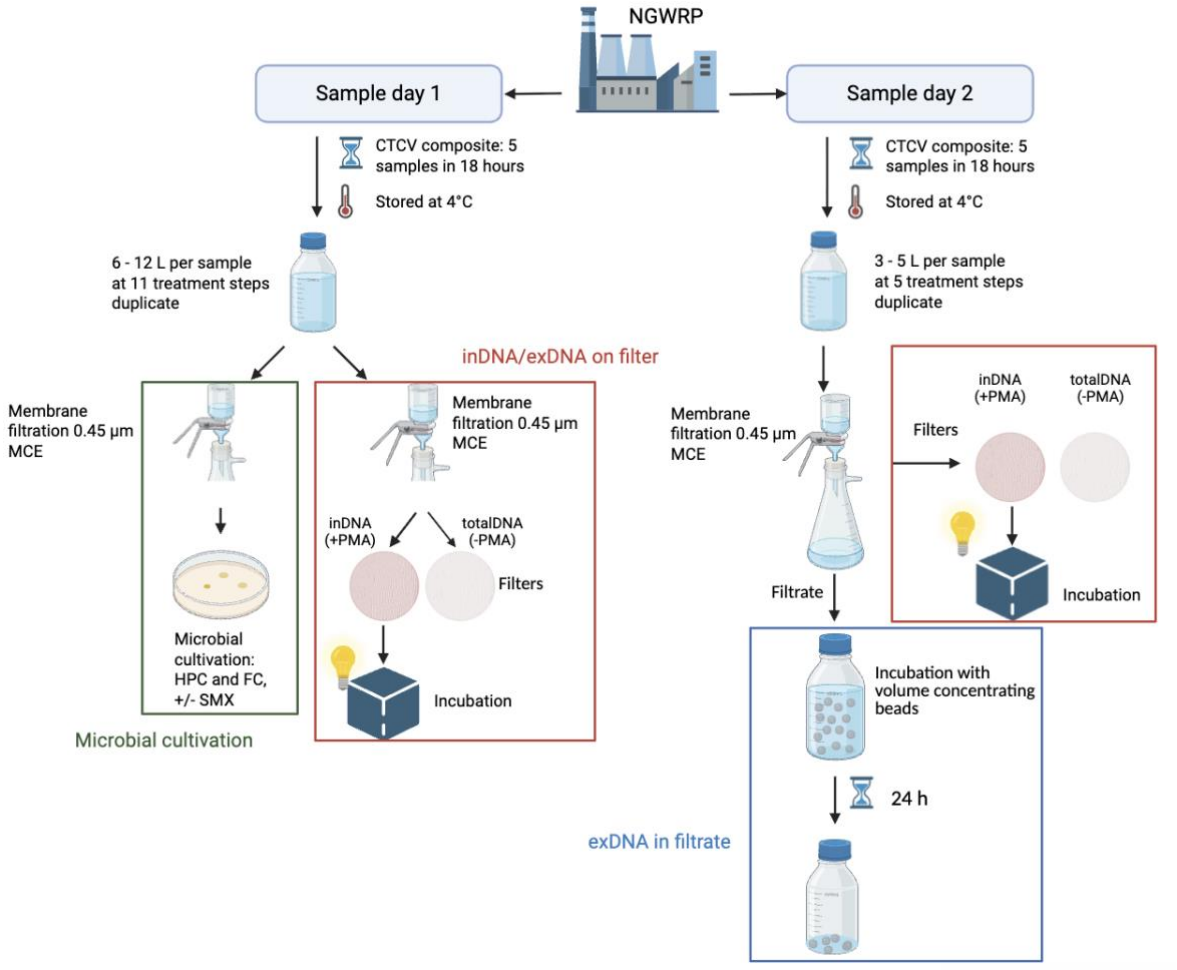


Figure 10: Overview of the experimental steps that have been conducted at NGWRP in Winhoek (Day 1, Day 2). On Sampling Day 1, part of the sample was used to cultivate heterotrophs (HPC) and faecal coliforms (FC) with and without sulfamethoxazole (+/- SMX). The other part was used for membrane filtration on mixed cellulose ester (MCE) filters to measure DNA retained on the filter. Half of these membranes were then treated with the chemical dye propidium monoazide (PMA) to distinguish between intracellular DNA (inDNA) and extracellular DNA (exDNA). The PMA-treated filters were then incubated under a light source to activate the dye. Furthermore, the direct filtrate was retained in a cleaned flask. This filtrate was incubated for 24 hours with the TCT Target Concentration kit. These beads concentrated the DNA concentration by taking up liquid. After the incubation, samples of the filtrate were taken. Membrane filters and filtrate after incubation were stored frozen and transported to Austria via air cargo (figure created with BioRender.com).

3.2.1 Sampling

Sampling was conducted on July 29th and July 31st, 2025, by WINGOC's staff at the NGWRP. To minimize the effects of diurnal variation, all samples were time-proportional composite samples taken every 4 hours, either auto-sampled or manually sampled, and subsequently pooled, since not all treatment steps were equipped with an autosampler. Time-proportional sampling refers to a constant-volume sampling at a constant time (CTCV). Sampling began the day before at 12.00 p.m. It continued until 6.00 the following morning, with samples taken every 4 hours and the last at 6.00. The final sample was taken at 6.00 to improve representativeness, resulting in 5 samples per composite. The samples arrived at the Scientific Services Laboratory in the morning after composite sample completion. In both sample methods, samples were stored at 4 °C until completion. Samples were taken and stored in new High-Density Polyethylene (HDPE) bottles; the bottles were rinsed

with sample three times before sampling. To the final product samples, 1 mL of sodium thiosulfate (100 mg/L) was added as a quenching agent to stop further reaction of residual free chlorine. The total volume sampled per treatment step depended on the expected filtration volumes and varied across experiments; see Table 1 for an overview.

Table 1: Sample volumes per treatment step for each sampling day at the NGWRP. X, not sampled.

Treatment	***Volume sampled (mL)***		
	Pre-test microbial cultivation	*Day 1: Microbial cultivation + inDNA/exDNA*	*Day 2: inDNA/exDNA + exDNA in filtrate*
WWTP Effluent	250	6000	X
NGWRP Influent	250	6000	3000
Pre-ozonation	X	6000	X
Coagulation and Flocculation	X	X	X
Dissolved Air Flotation	4000	8000	X
Dual Media Filtration	X	12000	5000
Main Ozonation	X	6000	X
Biological Activated Carbon	1000	8000	5000
Granular Activated Carbon	X	8000	5000
Ultrafiltration	X	8000	X
Final Product	80000	8000	5000

3.2.2 Filtration

Samples were filtered through a 0.45 µm (Ø 47 mm) mixed cellulose ester membrane filter (Pall Corporation, United States) using a three-fold filtration manifold for cultivation of faecal coliforms (FC) and molecular biological analyses. The manifold was rinsed with autoclaved water and sterilized with a gas burner in between samples. Initially, it was planned to use polycarbonate (PC) filters (Ø 47 mm; 0.2 µm) for molecular biological analyses, similar as during preliminary tests. This was not possible during the sampling period due to logistical challenges; therefore, MCE filters were used as the best available option. Filtration volumes for FC cultivation are given in Table 2 and for microbiological analyses in Table 3.

3.2.3 Bacterial cultivation

Heterotrophic Plate Count

Tryptone Glucose Extract Agar (TGEA) (Sigma-Aldrich, Germany) was used for the enumeration of heterotrophs. The media was freshly prepared by Scientific Services staff according to the manufacturer's instructions on the morning of the experiment. The standard operating procedure from Scientific Services was followed: the respective volume of each sample (Table 2) was added to an empty petri dish, then warm TGEA media (kept in a water bath at 46–48°C) was added to the dish, and the agar was swirled. The plates were incubated at 35±2°C for 48 hours.

Faecal Coliforms

Difco FC agar (BD, USA) was prepared by Scientific Services staff according to the manufacturer's instructions, and the poured plates were allowed to solidify and then stored at 4°C for up to 4 days before use. To test for the presence of faecal coliforms, the volumes described in Table 2 were filtered through a 0.45 µm (Ø 47 mm) mixed cellulose ester membrane filter and plated onto Difco FC agar. Petri dishes were incubated at 44.5 ± 1°C for 24 hours.

Sulfamethoxazole selective media

The TGEA and FC media were prepared in duplicates. Each replicate of the media was prepared at a volume of one litre. For each media type, 0.5 mL of a 1 mg /mL SMX stock solution was added to one replicate, resulting in a final concentration of 0.5 mg/L in the enriched media. The TGEA media was autoclaved after the addition of sulfamethoxazole according to the manufacturer's instructions. This was possible because sulfamethoxazole is shown to be stable well above the autoclave temperature of 121°C (Svahn & Björklund, 2015).

Counting

Colony counting was done according to the Scientific Services standard operating procedure. Plates were counted until 300 colonies per plate. If plates contained more than 300 colonies, the block-counting method was used as per routine. Plates with ten colonies or fewer are included in the results but marked (▽), also known as the LOQ. The LOD is marked as X and set to 1 colony per plate. The CFUs associated with the LOD and LOQ are dependent on the effective filtration or plate volume. The HPC plates were counted by Staff from Scientific Services.

Microbial Cultivation Volumes

A pre-test was conducted earlier to determine suitable sample volumes that yielded countable colonies for a selection of treatment steps (Table 1). Based on these results and recent sample volumes provided by Scientific Services, the volumes shown in Table 2 were used for microbial cultivation during the main experiment. A challenge also faced by the microbiological section of scientific services is the variability in the system's microbiological quality. This can lead to differences of two orders of magnitude in the volumes needed for HPC and FC cultivation. The samples were grown on agar plates with (+) or without SMX (-) in duplicates. Dilutions were made with autoclaved deionized water, and negative controls were conducted in parallel with the samples to confirm the sterility of the autoclaved water, the membrane filters, and the media.

Table 2: Volumes used for microbial cultivations per treatment step.

Step	**Treatment**	**Sample name**	**Volume added to agar (mL)**		**Volume filtered on membrane (mL)**	
			TGEA + SMX	*TGEA - SMX*	*FC + SMX*	*FC - SMX*
1	**WWTP Effluent**	MP	0.01	0.01	0.1	0.1
2	**NGWRP Influent**	RM	0.01	0.01	0.1	0.1
3	**Pre-ozonation**	PO	1	1	100	100
4	**Coagulation and Flocculation**	CO	1	1	100	100
5	**Dissolved Air Flotation**	DA	1	1	100	100
6	**Dual Media Filtration**	DM	1	1	100	100
7	**Main Ozonation**	MO	1	1	100	100
8	**Biological Activated Carbon**	BA	1	1	100	100
9	**Granular Activated Carbon**	GA	1	1	100	100
10	**Ultrafiltration**	UF	1	1	100	100
11	**Final Product**	FP	10	10	100	100
12	**Autoclaved water control**	W	10	10	100	100
13	**Empty filter control**	E	0	0	0	0

3.2.4 Molecular Biological Analyses

Filter Volumes

On day 1, volumes filtered are based on the amount of sample remaining after filtration for microbiological cultivation. Filters were stored at 4°C until all samples were processed. This resulted in 4 membrane filters per treatment step per day. Half of these were folded and stored in Eppendorf tubes at -20°C; these will later account for the total gene abundance in the sample (inDNA + exDNA). The remaining duplicates were further treated with PMA, see Propidium Monoazide Treatment.

Table 3: Sample volumes filtered per treatment steps for molecular biological analyses in duplicates (A, B).

Step	Treatment	Sample name	Volume filtered (mL)			
			With PMA		*Without PMA*	
			A	*B*	*A*	*B*
1	**WWTP Effluent**	MP	1500	1200	1200	1000
2	**NGWRP Influent**	RM	1200	1200	1200	1200
3	**Pre-ozonation**	PO	1200	1200	1200	1500
4	**Dissolved Air Flotation**	DA	1500	1000	1200	1200
5	**Dual Media Filtration**	DM	2000	2000	2000	2000
6	**Main Ozonation**	MO	1500	1200	1200	1200
7	**Biological Activated Carbon**	BA	1700	1700	1700	1700
8	**Granular Activated Carbon**	GA	1700	1700	1700	1700
9	**Ultrafiltration**	UF	1700	1700	1700	1700
10	**Final Product**	FP	1750	1700	1700	2000
12	**Autoclaved water control**	W	1500	1500	1500	1500

For each of the five treatment steps sampled on day 2, 2000 mL was filtered in duplicates, except for the NGWRP Influent, which was filtered in duplicate at 1500 mL (see Table 4). Each filter was cut in half with sterilised scalpels, and PMA was added to one half. After each filtration, the filtrate was retained, split into duplicates, and concentrated as described in Filtrate Processing.

Table 4: Sample volumes filtered per treatment steps for molecular biological analyses on filters and in filtrate.

Step	Treatment	Sample name	Volume filtered (mL)	
			A	*B*
1	**NGWRP Influent**	RM	1500	1500
2	**Dual Media Filtration**	DM	2000	2000
3	**Biological Activated Carbon**	BA	2000	2000
4	**Granular Activated Carbon**	GA	2000	2000
5	**Final Product**	FP	2000	2000
6	**Autoclaved water control**	W	2000	2000

Propidium Monoazide Treatment

Based on pre-test results at TU Wien and the literature, the method by Leifels et al. (2015) was adapted, and a concentration of 7.5 µM and a dye volume of 650 µl were chosen for the experiments at the NGWRP (see Method Preliminary Experiments TU Wien and Results Preliminary Experiments TU Wien). All handling with PMA was done in a dimly lit room due to the dye's light sensitivity. A stock solution of 7.5 µM was prepared by adding 20 µL of 20 mM PMA (Biotium USA) to 53.3 mL of autoclaved deionized water. The membrane filters through which the samples were filtered were placed in sterile petri dishes with the sample facing upward. From the stock solution, 650 µL was added to each filter. An incubation box (Figure 11) was built on-site that could be completely closed, containing aluminium foil and LED light strips (Amazon, USA) at 460-465 nm, the range in which PMA is activated. After adding PMA to the filters, they were incubated in the dark for 10 minutes in the incubation box to allow PMA to bind to the DNA. Afterwards, the light source was turned on (Figure 11), and the samples were further incubated for 30 minutes for photoactivation. The manufacturer recommends incubating complex samples for >15 minutes under the light source. After this, the filters were rolled into Eppendorf tubes and stored at -20°C until transportation to Vienna, Austria.

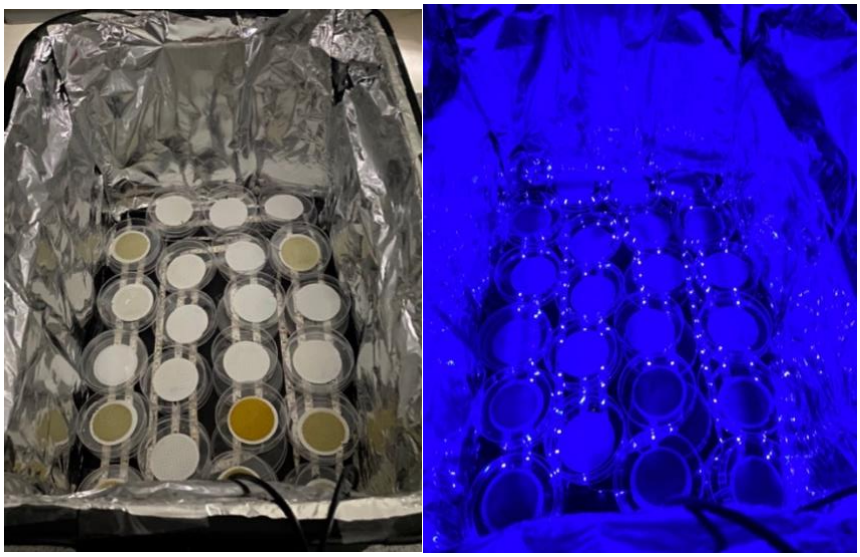


Figure 11: Left, membrane filters after filtration of samples and treatment with PMA before incubation; Right: The same samples incubated with the light source turned on.

Filtrate Processing

To measure a substantial number of antibiotic resistance genes in the filtrate, the volume should be maximized. However, volumes >1.5 L were not feasible to transport to Vienna for molecular biological analyses. To reach a transportable volume containing the highest concentration of ARGs, the filtrate was concentrated in Windhoek. The volume concentration step was performed in 2 L glass Schott bottles from the Scientific Services laboratory. They were cleaned as thoroughly as possible: starting with rinsing with tap water, rinsing with 5% bleach concentrate (World of Hygiene,

Namibia) diluted to 1% with autoclaved deionized water. Afterwards, they were rinsed again with Extran® MA02 (Sigma-Aldrich, Germany), diluted by adding 2 mL to 1 L of autoclaved deionized water, and then incubated overnight with Extran® at the same concentration. On the day of the incubation, the bottles were again rinsed with autoclaved deionized water. These extensive cleaning steps were performed because the bottles are generally used for non-molecular research purposes, and cleaned with a high volume of common detergent, which is not beneficial for molecular DNA analysis.

The filtrate was concentrated using the InnuPREP TCT Target Concentration Kit Water (IST Innuscreen, Germany). This was done by adding 12 grams of beads to 1 L of filtrate, and the mixture was incubated overnight. The principle is shown in Figure 12: the liquid in the control is almost entirely taken up, whereas the Dual Media Filtration sample, which most likely contains more DNA, retains more liquid after incubation. However, the volume is still concentrated compared to the start volume of 1 L. From the leftover concentrated filtrate of each sample, approximately 5 mL was taken, divided into 2 mL Eppendorf tubes, and stored at -20 °C in Windhoek and Vienna, and transported on dry ice to the TU Wien for molecular biological analyses.

Figure 12: Retained filtrate after 24-hour incubation with TCT Target Beads for two different treatment steps (Control and Dual Media Filtration). These treatment steps are expected to differ greatly in DNA concentration, resulting in different final volumes.

Storage and transport

All samples (filter papers and concentrated filtrates) were immediately stored at -20°C in Eppendorf tubes after their respective treatments. The samples were shipped in a Styrofoam box with ice packs from Windhoek, Namibia, to Johannesburg, South Africa. From Johannesburg, the parcel was supplemented with dry ice and transported to Vienna via air cargo. Upon arrival at TU Wien, the samples were stored again at -20°C until subsequent analysis. Figure 13 shows which samples were transported from Windhoek to the TU Wien.

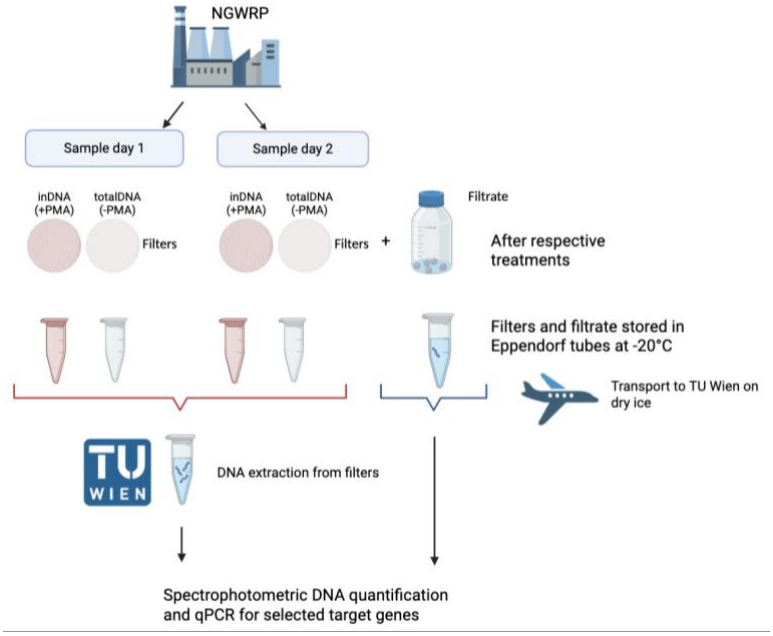


Figure 13: Schematic depiction of sample transport to the TU Wien and subsequent molecular biological analysis.

DNA Extraction

DNA from the filters was extracted with the DNeasy PowerWater Kit (Qiagen, Germany). The manufacturer's instructions were followed, with the following adjustments: the filter was cut into small pieces with a sterilized scalpel before the lysis step, and the bead-beating step was performed with a Fastprep-24™-5 (MP Biomedicals, USA) instead of a horizontal vortex mixer, using the pre-settings for environmental wastewater samples. All DNA extracts were stored at -20°C until further analysis. Filtrate samples have not been subjected to DNA extraction because pre-tests showed that DNA extraction from such samples did not result in a significant increase in DNA concentration, see DNA Extraction Filtrate in the Appendix.

DNA Quantification

The samples were analysed photometrically using the BioSpectrometer® (Eppendorf, Germany) to determine total DNA concentration. DNA concentration was measured by absorbance at 260 nm using a sample volume of 1.5 µl, as DNA absorbs strongly at this wavelength. Additionally, the A260/A280 absorbance ratio indicates purity: a value of 1.8 is considered pure, and deviations from this value may indicate contamination. Values below 1.8 may indicate contaminants such as EDTA, carbohydrates, or phenols, as they absorb at lower wavelengths (e.g., 230 nm).

Target Genes

For this study, several target genes were selected:

- The *16S rRNA* gene is a marker of overall bacterial abundance and will be used to normalize the other genes.

- *Intl1* is the integrase gene of class 1 integrons that encodes the integrase enzyme, which can insert genes into the gene cassette. Class 1 integrons are the most observed integrons. They are often used as a proxy marker of ARG abundance (EEA, 2025). Because they can incorporate multiple genes into their gene cassette, they are also strongly associated with multidrug resistance and HGE.

In addition, several specific ARGs were selected, each encoding different antibiotic classes and thus distinct resistance mechanisms:

- *Sul1* encodes for resistance to sulphonamides through an altered version of the enzyme dihydropteroate synthase (DHPS), which is targeted by sulphonamides. Usually, sulphonamides act as competitive inhibitors of DHPS. However, this altered DHPS enzyme has a lower affinity for sulphonamide, and thus the bacteria with altered DHPS can continue their essential folate biosynthesis (Sköld, 2001). *Sul1* is often found within class 1 integrons and is highly abundant in both clinical and environmental samples; it is usually used as a marker for anthropogenic influences and ARGs (EEA, 2025).
- *ErmB* encodes an rRNA methyltransferase that alters the 23S rRNA of the 50S ribosomal subunit. This mechanism alters the macrolide-binding site, allowing protein synthesis to continue despite exposure to a macrolide.
- *TetW* encodes for resistance to tetracyclines through ribosomal protection. It dislocates tetracycline molecules from the 30S ribosomal subunit, thereby preventing their inhibitory binding. Macrolides are widely used in human medicine worldwide, and resistance genes are detected in wastewater effluents in South Africa (Ramessar et al., 2025) and in rivers (Ekwanzala et al., 2018).
- $Bla_{CTX\text{-}M\text{-}1}$ produces an extended-spectrum β-lactamase (ESBL) which can hydrolyse the β-lactam ring of the respective group of antibiotics. This inactivates the antibiotic; therefore, cell wall synthesis is not inhibited (Mcmanus, 1997).

qPCR quantification

The abundance of selected target genes in the extracted samples was analysed and quantified using the QuantStudio™ 6 Pro Real-Time PCR System (Thermo Fisher Scientific, USA). The target genes were amplified using a primer pair specific to each gene (Table 5). Primers are short synthetic DNA sequences that are specific to a region of the target gene. Templates were amplified on 96-well microtiter plates in a total volume of 10 µL. For the *16S rRNA* gene, quantification was based on SYBR® Green, which intercalates all double-stranded DNA and emits a fluorescent signal proportional to the quantity of DNA. SYBR Green may thus also form non-specific qPCR products, such as primer dimers. For the other genes, sequence-specific, fluorescently labelled TaqMan probes were used.

They are designed to bind exclusively to the target gene's sequence and emit a fluorescent signal only when bound, thereby reducing the risk of non-specific binding. Each reaction well contained 2 µL of sample and 5 µL of SYBR™ Green Universal Master Mix (Thermo Fisher Scientific, USA) for the *16S rRNA* gene or Luna® Universal Probe qPCR Master Mix (New England Biolabs, USA) for all other genes. The concentrations of forward- and reverse primers and probe used differed per target and are specified in Table 5. Non-template controls were performed on every plate, in which 2 µL of PCR-grade water (Sigma-Aldrich, USA) was added instead of the sample. All samples were analysed in duplicates, and a Cq difference of <0.5 between duplicates was accepted.

The amplification efficiency of each target gene depends on primer/probe design, amplicon length, and qPCR conditions, including annealing temperature, mastermix composition, and sample characteristics. The qPCR temperature profiles are shown in qPCR Protocols. For all *16S rRNA* gene measurements, a melt curve was drawn during each qPCR run to assess nonspecific binding and confirm that amplification occurred at the correct temperature.

Table 5: Target genes and their primer and probe specificities.

Gene	**Primer and probe sequences**	**Amplicon length [bp]**	**Reference**
16S rRNA gene	F: AGA GTT TGA TCC TGG CTC AG R: TGC TGC CTC CCG TAG GAG T	351	Savio et al. (2015)
sul1	F: CCG TTG GCC TTC CTG TAA AG R: TTG CCG ATC GCG TGA AGT P: FAM-CAG CGA GCC TTG CGG CGG-BHQ-1	66	Heuer & Smalla (2006)
intl1	F: GCC TTG ATG TTA CCC GAG AG R: GAT CGG TCG AAT GCG TGT P: FAM-ATT CCT GGC CGT GGT TCT GGG TTT T-BHQ-1	196	Barraud et al. (2010)
ermB	F: GGA TTC TAC AAG CGT ACC TTG GA R: GCT GGC AGC TTA AGC AAT TGC T P: FAM-CAC TAG GGT TGC TCT TGC ACA CTC AAG TC-BHQ-1	92	Böckelmann et al. (2009)
tetW	F: CGG CAG CGC AAA GAG AAC R: CGG GTC AGT ATC CGC AAG TT P: FAM-CTG GAC GCT CTT ACG-MQ530	58	Walsh et al. (2011)
$bla_{CTX\text{-}M\text{-}1}$	F: ACC AAC GAT ATC GCG GTG AT R: ACA TCG CGA CGG CTT TCT P: FAM-TCG TGC GCC GCT G-MQ530	101	Colomer-Lluch et al. (2011)

F, forward primer; R, reverse primer; P, probe. Sequences of primers and probes noted are in the 5'-3' direction.

The cycle threshold (Cq) is the PCR cycle at which the fluorescent signal first surpasses the baseline threshold, as calculated by the software. This Cq value is inversely proportional to the quantity of

the target gene in the sample. For quantification, the Cq value of an unknown sample is compared to the standard curve generated from serial dilutions of plasmid standards containing the cloned target sequence. Multiple target genes can be cloned onto the same plasmid standard. The plasmid standards were diluted in PCR-grade water and ranged from $5*10^{-2}$ copies/µL to $5*10^{8}$ copies/µL; dilutions ≤ 5 copies/µL were included only for quality control and not used in the standard curve cultivation. A new standard curve was generated for each qPCR run.

By comparing the Cq value of a sample with the standard curve, the initial amount of target nucleic acid in the sample can be quantified. The amplification efficiency (Equation 1) reflects the quality of the qPCR run. If the efficiency is 1 (100%), the amplified product doubles with each cycle. A slope of -3.3 corresponds to an efficiency of 100% and the dilution steps are 3.3 cycles apart.

$$E = 10^{-\frac{1}{slope}} - 1$$

Equation 1: qPCR efficiency

HTS-qPCR

High-throughput Screening qPCR (HTS-qPCR) is an automated technique that uses very small sample volumes to measure and quantify up to 100 target genes in many samples simultaneously. Resistomap is a company based in Helsinki, Finland, that specializes in HTS-qPCR. Two samples from the NGWRP were analysed at Resistomap: the WWTP Effluent and the NGWRP Influent. Through Resistomap, insight was gained into the Resistome profile and whether the WWTP Effluent or the backwash reservoir contributed to a higher ARG load.

3.2.5 Chemical Analyses Windhoek

Physical-chemical parameters

The conductivity, pH, and turbidity were measured for all samples using the devices in Table 6. All devices were calibrated daily, and probes were rinsed with deionized water in between measurements. If samples could not be measured within 2 hours of sampling, they were stored at 4°C and measured within 24 hours.

Table 6: Measurement devices for physicochemical parameters.

Parameter	**Device**	**Manufacturer**
Conductivity	Seven Multi	Mettler Toledo
pH	Seven Multi	Mettler Toledo
Turbidity	TL2350 Tungsten Lamp Turbidimeter 0-10000 NTU	Hach

Dissolved Organic Carbon

Dissolved organic carbon (DOC) was analysed with a Teledyne Tekmar TOC Fusion (Teledyne Labs, United States) using a non-purgeable organic method for measuring dissolved organic compounds in water samples after removing all volatile and inorganic carbons. Samples were first filtered through 0.45 µm syringe filters into glass vials to separate the dissolved and particulate carbon fractions. After placing the vials into the autosampler, the subsample is acidified with phosphoric acid (H_3PO_4) to convert the inorganic carbon to purgeable CO_2, which is then stripped with high-purity nitrogen gas. After this, only the organic carbon remains and is oxidized by persulfate ($S_2O_8^{2-}$) in the presence of UV light into CO_2. Water vapor can absorb infrared light, which can interfere with quantification. Therefore, the CO_2 fraction is purged and dried before measurement by a non-dispersive infrared (NDIR) sensor. Samples were measured in duplicates and, if necessary, diluted with deionized water before analysis.

Cations and anions

Cations and anions were analysed using a Thermo Scientific Dionex ICS-5000 DC Ion Chromatographer (IC) coupled with a Dionex Variable Wavelength Detector (VWD). Samples are filtered through a 0.24 µm membrane filter (Whatman, United Kingdom) and degassed before analysis. Samples were diluted with deionized water if necessary. Anion separation is performed on an anion-exchange column at 40°C using potassium hydroxide as the eluent at a flow rate of 4 ml/min. Cation separation was performed on a cation-exchange column at 40°C using methanesulfonic acid as the eluent at a flow rate of 4 ml/min. The cations analysed were sodium (Na^+), potassium (K^+), magnesium (Mg^{2+}), and calcium (Ca2+). Anions analysed were fluoride (F^-), chloride (Cl^-), bromide (Br^-), nitrate-nitrogen (NO_3-N), phosphate-phosphorus (PO_4-P), and sulphate (SO_4^-).

3.2.6 Chemical Analyses Vienna

Pharmaceuticals

The concentrations of 11 commonly monitored pharmaceuticals in aquatic environments were measured; see Table 7. As organic micropollutants, they can persist in the environment and may contribute to mixtures that can alter their chemical properties and toxicological effects. The tendency of each compound to remain in solution, interact with organic matter or other compounds, and its affinity for different removal mechanisms can be partly explained by its molar mass and hydrophobicity, as expressed by its Log Octanol-Water Coefficient (Log K_{OW}).

Table 7: Pharmaceuticals analysed with their properties. Data obtained from EPA Comptox dashboard.

Compound	Therapeutic purpose	Formula	CAS	Supplier	Molar mass	Log K_{ow}
					g/mol	
Sulfamethoxazole	Antibiotic	$C_{10}H_{11}N_3O_3S$	723-46-6	Sigma-Aldrich	253.3	0.8
Carbamazepine	Psychiatric/ Neurological drug	$C_{15}H_{12}N_2O$	298-46-4	Sigma-Aldrich	236.3	2.4
Metoprolol	Cardiovascular drug	$C_{15}H_{25}NO_3 \cdot C_4H_6O_6$	37350-58-6	RTC, Sigma-Aldrich	267.4	1.8
Trimethoprim	Antibiotic	$C_{14}H_{18}N_4O_3$	738-70-5	Alfa Aeser	290.3	0.9
Diclofenac	Pain/Inflammation drug (NSAIDs)	$C_{14}H_{10}Cl_2NO_2Na$	15307-79-6	Sigma-Aldrich	318.1	4.4
Citalopram	Psychiatric/ Neurological drug	$C_{20}H_{21}FN_2O \cdot HCl$	85118-27-0	LGC	360.9	3.2
Venlafaxine	Psychiatric/ Neurological drug	$C_{17}H_{28}ClNO$	99300-78-4	Supelco	313.9	3.2
Bezafibrate	Cardiovascular drug	$C_{19}H_{20}ClNO_4$	41859-67-0	Sigma-Aldrich	361.8	3.8
Ibuprofen	Pain/Inflammation drug (NSAIDs)	$C_{13}H_{17}O_2Na$	31121-93-4	Sigma-Aldrich	228.3	3.7
Hydrochlorothiazide	Cardiovascular drug	$C_7H_8ClN_3O_4S_2$	58-83-5	Sigma-Aldrich	297.7	-0.1

Sample preparation

Samples for pharmaceutical analysis were collected in ethanol-rinsed HDPE bottles, transported at ambient temperature, and stored at 4 °C upon arrival in Vienna and until analysis. At the TU Wien, the samples were filtered through VWR glass fibre filters with a pore size of 0.45 µm (Ø 45 mm). The analytical standard was prepared at 1 mg/mL in ethanol. For automated online solid-phase extraction (online SPE), 10 mL of sample was used with a 25 µm (20 x 2.0 mm) Phenomenex Strata X On-Line extraction cartridge. The gradient program for online SPE and HPLC can be found in Appendix 0.

HPLC-MS/MS

Chromatographic separation of the samples was performed on an Agilent System with two binary pumps, a degasser, a CTC PAL autosampler with a Peltier-cooler, and a Rheodyne 2-position, 6-port switching valve. The eluents were 0.1% Acetic acid solution in deionized water (A) and 0.1% Acetic acid in Acetonitrile solution (B). HPLC-grade organic solvents were purchased from Sigma-Aldrich: Ethanol (CAS 64-17-5), Acetonitrile (CAS 75-05-8), and Acetic acid (CAS 64-19-7). Mass spectrometric detection was done via a Hybrid Triple Quadrupole Linear Ion Trap Tandem Mass Spectrometer Q

Trap 6500 from Applied Biosystems. Quantification was performed using MRM Analysis with Electrospray Ionisation (ESI) in positive and negative modes at a source temperature of 500 °C, with nitrogen as the collision gas. The LOQ/LOD values from the measurements are provided in Appendix 0.

3.3 Data analysis

3.3.1 Limits and standards

Bacterial Cultivation

LOD and LOQ values for the bacterial cultivation were determined as 1 colony per plate and 10 colonies per plate, respectively. Results <LOQ are marked in the results with '▽', all samples <LOQ are considered not robust. Bacterial growth is reported as CFU/mL, the sample LOD, and sample LOQ expressed as CFU/mL varied depending on the filtered volume, and were calculated according to Equation 2 and Equation 3:

$$\text{Sample LOD (CFU/mL)} = \frac{1\ colony/plate}{Volume\ filtered/plated\ (mL)}$$

Equation 2: Bacterial cultivation

$$\text{Sample LOQ (CFU/mL)} = \frac{10\ colonies/plate}{Volume\ filtered/plated\ (mL)}$$

Equation 3: Bacterial cultivation LOQ

Molecular Biological Analysis

Accepted qPCR efficiency ranged from 95 – 115%. For each run, the LOQ was defined as 10 copies/µL, the lowest reliably quantifiable point on the standard curve. The LOD is defined as 1 copy/µL, and samples that did not amplify were labelled 'not amplified' (NA).

The qPCR quantities for the filters and the filtrate were corrected using Equation 4 and Equation 5Equation 5, respectively. This leads to the absolute gene quantity (copies/mL) per sample.

$$\text{Copies/mL} = \frac{Copies/\mu L_{on\ filter} \times elution\ volume\ (\mu L) \times qPCR\ dilution\ factor}{filtered\ volume\ (mL)}$$

Equation 4: Absolute gene quantity on filter

$$\text{Copies/mL} = \frac{Copies/\mu L_{in\ filtrate} \times concentration\ factor \times qPCR\ dilution\ factor}{filtered\ volume\ (mL)}$$

Equation 5: Absolute gene quantity in filtrate

The relative gene abundance was calculated by normalization of the target gene to the total microbial load (Equation 6)

Relative gene quantity = $\frac{Copies\ target\ gene/mL}{Copies\ 16S\ rRNA/mL}$

Equation 6: Relative gene quantity

Due to the low number of replicates (2 or 4), the error may be amplified after log transformation, especially when values from multiple days are combined. To account for this, the Log Removal Values (LRV) were calculated for each absolute and relative sample by calculating the effective log removal and propagated error using the equations in Table 8

Table 8: Equations used to find the effective log reduction and its propagated error.

Number	Equation	Result
Equation 7	$log_{10}\left(\frac{C_{in}}{C_{out}}\right)$	Log reduction (LRi)
Equation 8	$\frac{1}{ln(10)} \times \sqrt{\left(\frac{\sigma_{in}}{C_{in}}\right)^2 + \left(\frac{\sigma_{out}}{C_{out}}\right)^2}$	Log reduction error (LRi error)
Equation 9	10^{-LR_i}	Reduction rate (ri)
Equation 10	$r_i \times\ ln(10) \times \sigma_{LRi}$	Error in ri (σri)
Equation 11	$\frac{1}{n}\Sigma\, r_i$	Mean reduction rate ($\bar{r}$)
Equation 12	$\frac{1}{n}\sqrt{\Sigma\sigma^2{}_{r_i}}$	Error in mean reduction rate ($\sigma_{\bar{r}}$)
Equation 13	$-log_{10}(\bar{r})$	Effective log reduction
Equation 14	$\frac{1}{\bar{r}\ \times\ ln(10)} \times \sigma_{\bar{r}}$	Error in effective LR

The LRV, propagated error, and reduction rate were calculated for each replicate separately. From the mean reduction rate, the effective log reduction was calculated. If a treatment step reduced the gene quantity below LOQ or LOD, no meaningful quantities could be attributed to these values with the desired certainty. Therefore, no LRVs could be calculated for treatment steps that reduced gene copies to levels below the LOQ or LOD, or for those that were not amplified (NA).

3.3.2 Statistical analysis

The gene abundance on the two sampling days was compared statistically to determine whether they differed significantly. The absolute abundance was log-transformed, and t-tests were performed for *16S rRNA* gene*, ermB, and tetW*, as the data were normal after log transformation. For *intI1* and *sul1* (non-normal distribution before and after transformation), the Wilcoxon test was done. Data normality was assessed using the Shapiro-Wilk test.

3.3.3 Software

qPCR plate design and analysis were done with Design & Analysis, version 2.8.0. ChatGPT (version GPT-5) was used to improve the code for data mutation, analysis, and visualisation. Data mutation, (statistical)analysis, and data visualization were done in RStudio Version 2024.12.1+563. PerplexityAI was used to find relevant sources. Biorender was used to create figures. Grammarly was used for grammatical improvement of the written text. DeepL was used as a helping tool to translate the written ‘Abstract’ into German for the ‘Kurzfassung’.

4. Results and Discussion

4.1 Bacterial Cultivation

Comparing the WWTP Effluent and the NGWRP Influent (Raw Mix) shows that the NGWRP Influent contains more heterotrophic organisms than the WWTP Effluent (Figure 14). No binding legal limit exists for heterotrophs in drinking water. HPC is typically used to monitor the overall efficiency of water treatment systems, not as a health indicator. The NGWRP removes heterotrophs to below 100 CFU/mL after the GAC step. This value is used as an operational parameter by Scientific Services. Historically, the limit for faecal coliforms in drinking water standards used to be 0 CFU/100 mL (EU, 1998) while in the current WHO guideline and EU directive, this indicator has been replaced by *E.coli* with the identical numerical limit (EU, 2020; WHO, 2022). This value was achieved after the UF step (Figure 14). Three controls were conducted in duplicate: empty plates, plates with a dry membrane filter, and the filtration of autoclaved distilled water to confirm the sterility of the plates, the membrane filter, and the autoclaved water, respectively. All cultivation controls showed no colony growth on either TGEA or FC media, both ± SMX, and are thus indicated as <LOD. See Table S 2 for all respective LOD and LOQ values (CFU/mL). Raw plate counts are provided in Table S 3.

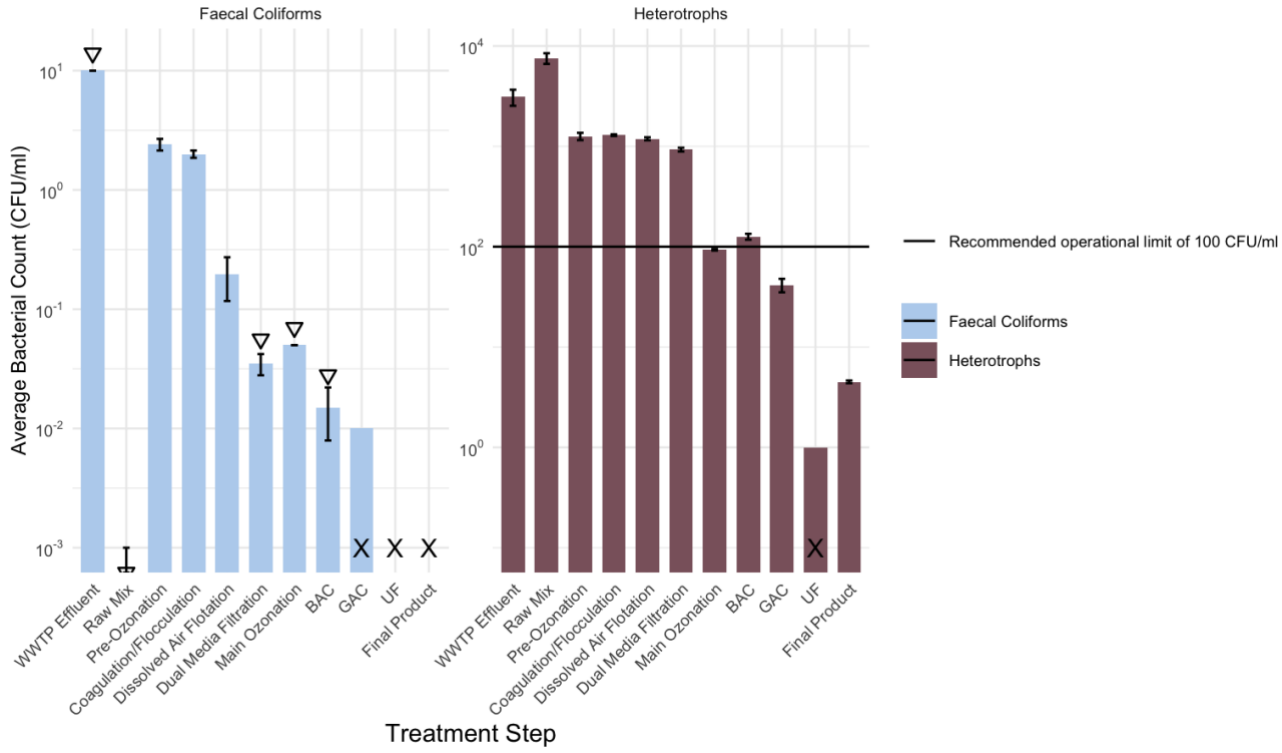


Figure 14: Total heterotrophs and faecal coliforms across treatment steps. LOD and LOQ values are calculated from the effective volume filtered or plated, which differs per treatment step (See Raw Data and Limits of Detection Bacterial Cultivation). Symbols indicate values below LOQ or LOD. X = <LOD, ▽= <LOQ. Values <LOQ are considered not robust. When one replicate was <LOQ, averages and SD are shown. If one replicate was <LOD and the other <LOQ, only the <LOQ value is plotted without SD. Error bars indicate standard deviation between replicate plates (n=2).

The treatment train reduced heterotrophs to the recommended monitoring limit of 100 CFU/mL and faecal coliforms to 0 CFU/mL. Contrary to expectations, plates containing sulfamethoxazole showed equal or, in some cases, greater growth than plates without the antibiotic. This occurred for heterotrophs as well as faecal coliforms. The same happened during preliminary experiments at the TU Wien when the method was tested at the Von der Emde Technikum. The cause of this unexpected growth is unclear, as the addition of sulfamethoxazole should have inhibited bacteria sensitive to this antibiotic (Masters et al., 2003). Wallmann et al. (2021) reported suppressed growth due to the addition of sulfamethoxazole by three orders of magnitude in the influent of the NGWRP. However, the calculation described in the method section of that study does not appear to yield the mentioned desired concentration. Therefore, it is unclear at what concentration of SMX the observed counts occurred. In the present study, a concentration of 0.5 mg/L was used, the same concentration reported to have been used by Wallmann et al. (2021), to compare their results with the present study. However, this concentration led to unanticipated bacterial growth. Due to this unexpected outcome, the results of the plates enriched with SMX are not described in detail, as they cannot be reliably interpreted. For completeness, they are given in Table S 3.

This concentration of 0.5 mg SMX/L as applied by Wallmann et al. (2021) was based on the minimum inhibitory concentration (MIC) from the Clinical and Laboratory Standards Institute (CLSI, 2020). However, based on standard methods, no uniform MIC for SMX alone is available. SMX is rarely administered alone, as it is generally combined with trimethoprim at a ratio of 1 TMP:19 (CLSI, 2020) SMX Therefore, the MIC is also based on the combined effect of TMP-SMX. Additionally, the MIC differs per target organism, often being specific clinical pathogens, which might respond very differently from environmental indicators such as heterotrophs or faecal coliforms (Kneis et al., 2025).

Al-Ahmad et al. (1999) reported sulfamethoxazole MIC_{50} values differing 5 orders of magnitude, depending on the organism, confirming the difficulty of applying a single concentration to estimate resistance in complex bacterial communities. Environmental studies have reported the use of SMX incorporated into agar at 56 mg/L (Piaggio et al., 2023) and of sulfamethazine, a closely related compound, at 350 mg/L (Ou et al., 2015). This implies that a higher SMX concentration is often used in agar than the 0.5 mg/L used here. Rauseo et al. (2021) tested the exposure of the digestate microbial community in liquid cultures and found no inhibition of microbial abundance, as measured by *16S rRNA* gene concentrations, at SMX concentrations below 4.75 mg/L. Lastly, Müller et al. (2013) found that activated sludge communities can utilize SMX as carbon and/or nitrogen source for

growth after an adaptation phase. This could be relevant because the influent from the NGWRP is also undergoing activated sludge treatment before entering the maturation pond.

Therefore, possible reasons for the lack of difference in bacterial growth between -SMX and +SMX plate counts could include a low concentration in the +SMX plates, prior adaptation of the organisms during wastewater treatment, or an unknown error in preparing the media containing the antibiotic. If resistance determination via cultivation is desired, it is recommended to conduct preliminary tests to compare CFUs after adding a range of antibiotic concentrations, based on concentrations reported in studies of relevant environmental systems with corresponding microbial communities. In this thesis, a range of volume samples was tested preliminarily, but not a range of antibiotic concentrations. Additionally, it would be useful to cultivate a positive control with a susceptible-SMX strain.

4.2 Absolute Gene Removal during Water Reclamation

4.2.1 Marker Genes

Figure 15 shows the absolute abundance and reductions in marker genes throughout the NGWRP treatment system. The NGWRP Influent contained *16S rRNA* gene copies at 5.9×10^5 copies/mL, a measure of total bacterial abundance. The treatment train of the NGWRP reduced this to 1.9×10^2 copies of *16S rRNA*/mL in the final product, indicating a reduction in bacterial load. Wallmann et al. (2021) measured 16S rRNA at <LOD (SLOD = 16 copies/µL) in the final product and 5.07×10^2 copies/mL at the point of use, which is comparable to the value observed in the final product in the present study.

The most effective removal was achieved by the DMF and UF units with effective log removals of 1.22 (±0.10) and 2.52 (±0.12), respectively. The *intl1 gene*, a marker for class 1 integrons, was detected at 1.6×10^3 copies/mL in the influent. Integrons can integrate multiple resistance genes into their gene cassettes and are therefore seen as a proxy for MGE-mediated HGT potential. After the treatment, *intl1* could not be detected by qPCR and was thus reduced to undetectable levels. This indicates that the treatment train, particularly the ultrafiltration unit, is effective at lowering the risk of HGT via integrons (Figure 15).

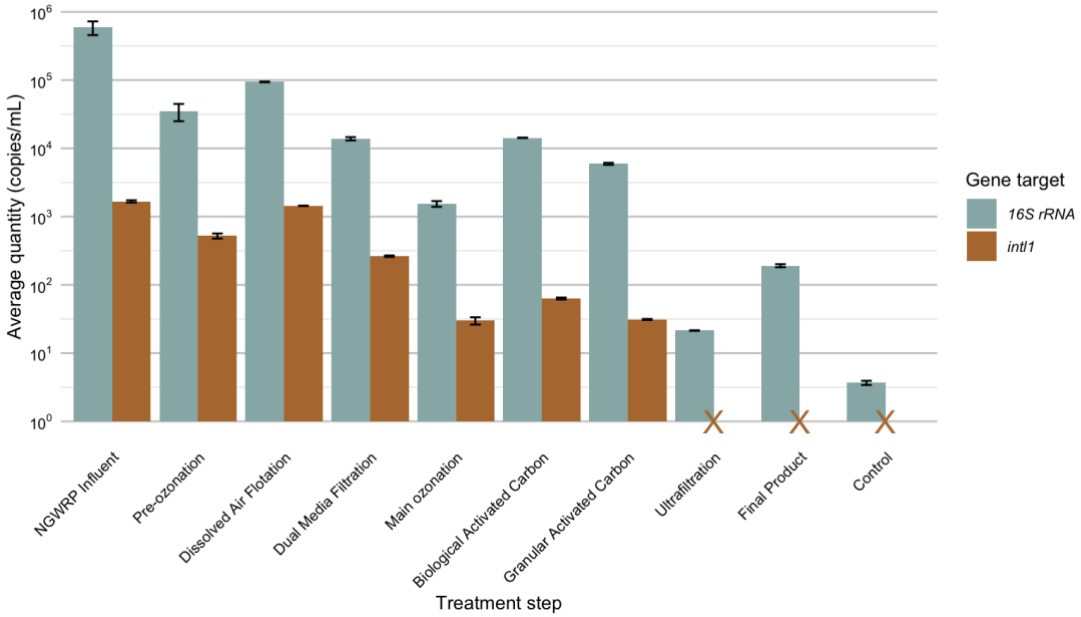


Figure 15: Average gene quantities for 16S rRNA gene (marker for total bacterial load) and intI1 (marker for horizontal gene transfer) throughout the treatment train. X indicates not amplified by qPCR.

4.2.2 Antibiotic Resistance Genes

Four antibiotic resistance genes were analysed, each conferring resistance to different classes of antibiotics: *sul1* (sulfonamide resistance), *tetW* (tetracycline resistance), *ermB* (macrolide resistance), and $bla_{CTX\text{-}M\text{-}1}$ (beta-lactam resistance). Figure 16 shows the absolute abundance of these resistance genes along the treatment train.

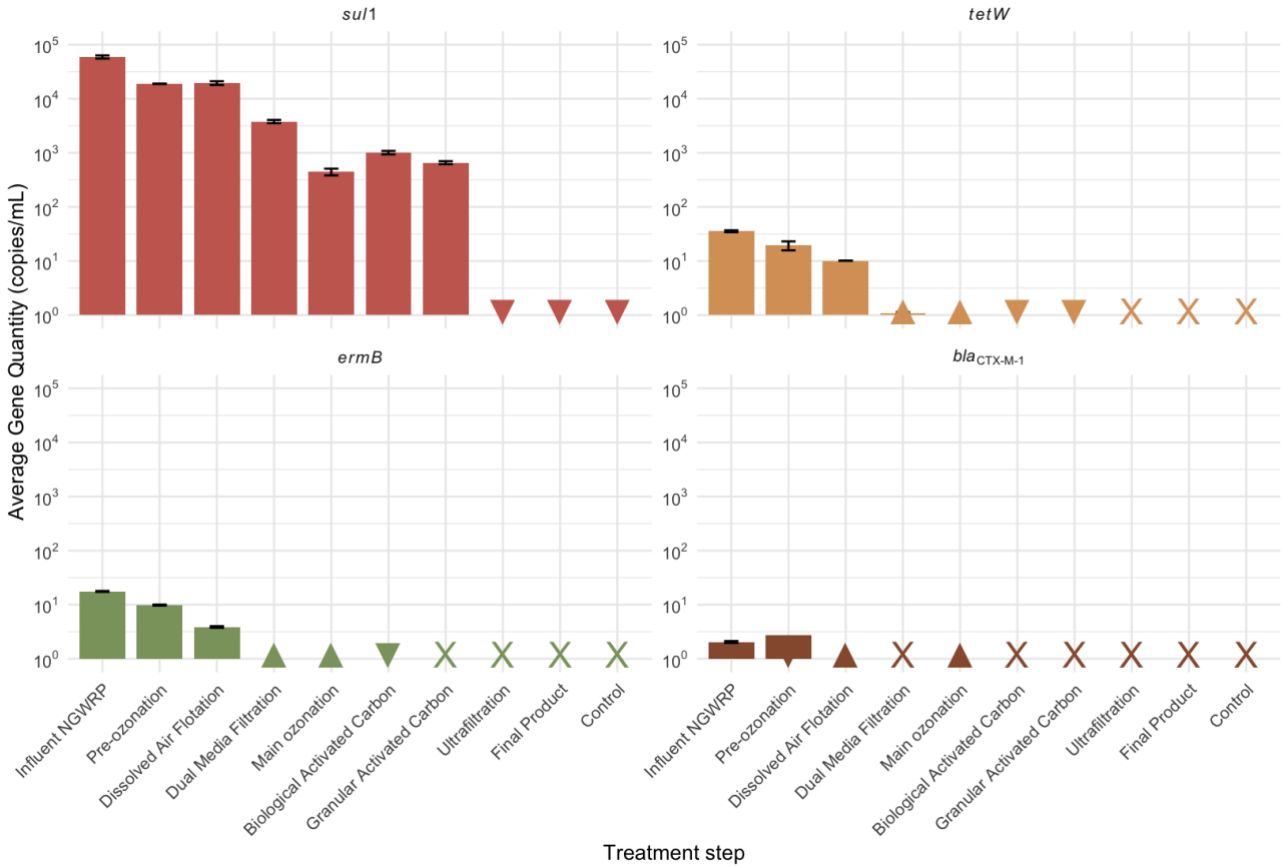


Figure 16: Average quantities of antibiotic resistance genes (sul1, tetW, ermB, and bla$_{CTX-M-1}$) throughout the treatment system, with the standard deviation (n=2) indicated with an error bar. X indicates not detectable in qPCR, ▼ indicates reduction to < LOD (1 copy/µL), ▲ indicates reduction to <LOQ (10 copies/µL), For the gene tetW during dual media filtration, one replicate was measurable, and one replicate was <LOQ. Similarly, for bla$_{CTX-M-1}$, during pre-ozonation, one replicate was measurable and one was <LOD.

Except for the *16S rRNA gene* and *sul1*, none of the targets yielded an amplification signal by qPCR in the control, the final product, or after UF. The *sul1* gene was detected below the detection threshold (LOD = 1 copy/µL) in the control, after UF and in the final product. If a target gene is not amplified by qPCR, the gene may still be present at unknown concentrations below the defined LOD threshold, but at levels no longer detectable by qPCR (Not Amplified).

Among these genes, *sul1* was the most abundant. This is consistent with previous studies reporting *sul1* among the most abundant ARGs in wastewater globally (Zhuang et al., 2021) and in South Africa (Smith et al., 2024). The ARG *tetW* was slightly more abundant than *ermB*; they were detected up until DMF and DAF, respectively. Both *tetW* and *ermB* often co-occur with *sul1* and other mobile resistance genes, as they are frequently plasmid-based. Their detection indicates the presence of mobile and environmentally relevant resistance genes. However, their removal to below detection limits means that the treatment train effectively reduces their abundance and the potential for their spread via HGT. All analysed resistance genes were removed to below detectable levels by qPCR in the final product.

For the *16S rRNA* gene, *intl1*, and *sul1*, a decrease in average quantity was observed until the main ozonation. This indicates effective removal of bacterial abundance and resistance marker genes. However, all genes that remained quantifiable during BAC showed increased abundance. This corresponds to the findings by Wallmann et al. (2021). However, in the present study, this increase is approximately one order of magnitude for the *16S rRNA* gene and even smaller for *sul1* and *intl1*. This makes the increase during BAC less pronounced than observed in 2021, when increases of 4 and 2 orders of magnitude for the *16S rRNA* gene and *sul1*, respectively, were observed between main ozonation and BAC.

4.2.3 Absolute Log Removal Values per Treatment

Table 9 presents the absolute log removal values (LRVs) for each treatment step. All treatment steps except DAF and BAC resulted in an absolute positive effective LRV for the *16S rRNA* gene, *sul1*, and *intl1*. With the exception that disinfection before the final product increased the abundance of the *16S rRNA* gene and led to a negative LRV. This might be due to an increase in eDNA that can occur during chlorination (Liu & Kasuga, 2024) or to contamination during any of the experimental steps, as *16S rRNA* gene contamination is very easily introduced in non-molecular laboratories (Table 9). Similarly, an increase in HPC after disinfection was observed. No LRV was calculated for the ultrafiltration unit because it removed all ARGs and *intl1* to below a detectable level. However, it showed the highest effective LRV for the *16S rRNA* gene, a proxy for total microbial load. Wallman et al. (2021) also found that the ultrafiltration unit achieved the highest log removal rate for the *16S rRNA* gene in the NGWRP.

Table 9: Absolute effective log removal values of individual treatment units. If a treatment step reduced the gene quantity below LOQ or LOD, no meaningful quantities could be attributed to these values with the desired certainty. Therefore, no LRVs were calculated for treatment steps that reduced gene copies to levels below the LOQ or LOD; thus, their LRVs are indicated as > or >>, respectively. Not amplified is indicated with >>>.

Treatment step	*16S rRNA*	*intl1*	*sul1*	*tetW*	*ermB*	$bla_{CTX\text{-}M\text{-}1}$
Pre-ozonation	0.28 (±0.48)	0.29 (±0.20)	0.28 (±0.32)	0.13 (±0.35)	0.14 (±0.16)	>>0.05 (±1.12)
Dissolved air flotation	-1.36 (±0.16)	-1.33 (±0.08)	-0.90 (±0.40)	-0.40 (±0.05)	-0.33 (±0.19)	>>
Dual Media filtration	1.22 (±0.10)	0.73 (±0.12)	0.35 (±0.12)	>0.98 (±0.10)	>	>>>
Main Ozonation	0.74 (±0.18)	1.51 (±1.42)	0.98 (±0.24)	>	>	>>
Biological Activated Carbon	-1.41 (±0.27)	-0.37 (±1.35)	-0.35 (±0.22)	>>	>>	>>>
Granular Activated Carbon	0.32 (±0.27)	0.27 (±0.06)	0.19 (±0.34)	>>	>>>	>>>

Ultrafiltration	2.52 (±0.12)	>>>	>	>>>	>>>	>>>
Disinfection	-0.94 (±0.08)	>>>	>	>>>	>>>	>>>
Total	1.37	>>>1.1	>0.55	>>>0.71	>>>-0.19	>>>0.05

DAF shows a negative removal for all quantifiable genes, indicating an increase. DAF is based on the removal of larger particles that floc together and float upwards. This upper scum layer is then scraped off. Dual media filtration and main ozonation effectively remove all analysed genes, consistent with findings from Wang et al. (2022) that sand filtration is a practical removal step for ARGs.

Main ozonation causes a positive absolute removal for all genes. However, main ozonation followed by activated carbon might amplify the increase of ARGs, as found by Spit et al. (2022). Main ozonation uses a high concentration of the oxidizing agent O_3 to fragment organic matter. The resulting smaller, easier biodegradable compounds can serve as a nutrient source for biofilm growth in the subsequent BAC. Additionally, ozonation may increase exDNA levels through cell lysis; however, this effect varies by ARG and ozone concentration (Alexander et al., 2016).

Similar to Wallmann et al. (2021), a negative removal was observed after BAC, indicating an increase for all analysed genes. BAC filters harbour biofilms with active microbial communities, thereby increasing total bacterial abundance. BAC filters have more often been pointed to as a possible reservoir of ARB and ARG, and are known to increase the concentration of ARGs in water treatment systems. Proia et al. (2016) found that inflow from WWTP Effluents favoured the proliferation and spread of ARGs in biofilms, suggesting that the quality of the inflow influences the role of biofilms in ARG proliferation. This is relevant because the NGWRP inflow also includes WWTP Effluent. Additionally, the bacterial communities in BAC filters may also include competent bacteria that could take up exDNA via transformation (Abe et al., 2020). Despite the risk of ARG spread associated with BAC filters, subsequent GAC and, mainly, UF reduce all analysed resistance genes to non-detectable levels in the final product.

4.3 Relative Gene Removal during Water Reclamation

To assess the selective removal or enrichment of ARGs by the treatment steps, the relative abundance (gene copies/*16S rRNA gene copies*) was calculated by normalizing gene abundance to total microbial abundance (*16S rRNA gene*). Additionally, lack of standardization across scientific studies on sampling methods, DNA extraction, and qPCR workflows limits direct comparisons with the literature. Therefore, it is advised that the molecular results presented are interpreted based on

their course within the NGWRP system, and, if compared with other studies, only qualitatively rather than quantitatively.

4.3.1 Molecular Resistome Influent Sources

The influent to the water reclamation process is treated wastewater from GWCW. It is expected that this still contains a high number of ARGs, as conventional wastewater treatment systems might reduce ARBs and ARGs but cannot completely remove them (Drane et al., 2024; Guo et al., 2017). The two sources of influent in the NGWRP are WWTP Effluent and recycled backwash water from within the system during membrane backwashing. Figure 17 shows the relative abundance of target genes between the WWTP Effluent alone and the Influent NGWRP (WWTP Effluent + backwash water). The figure shows minimal differences in the absolute average quantity of all target genes between the WWTP Effluent and the NGWRP Influent on sampling day 1.

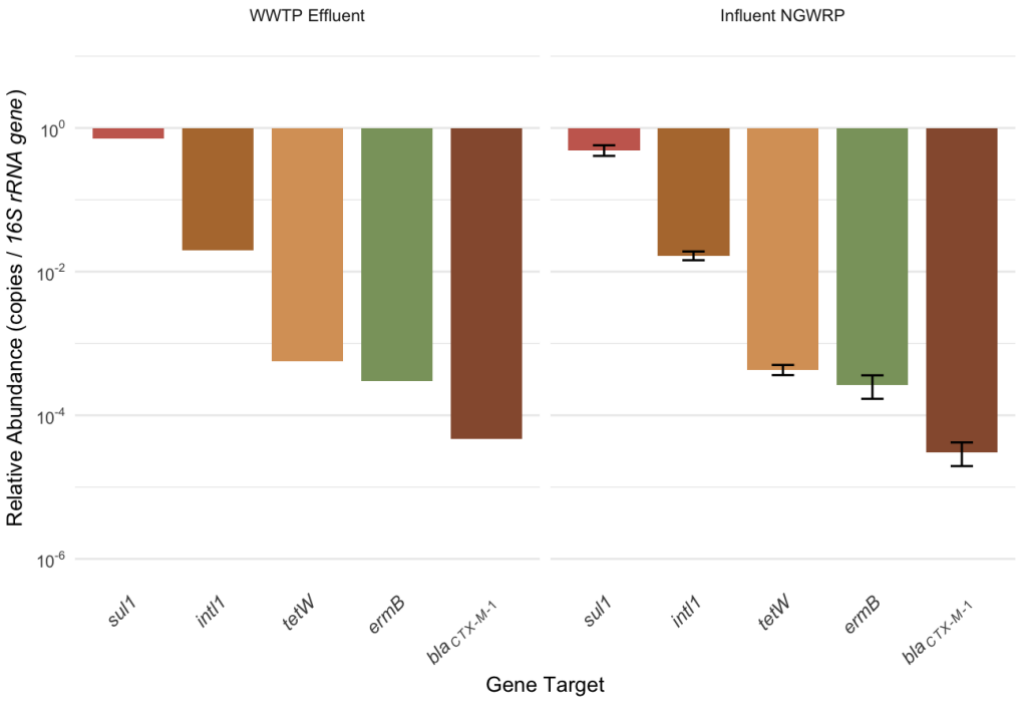


Figure 17: Absolute gene abundance in influent sources on sampling day 1. Error bars display the standard deviation between two replicate filters. Only one WWTP effluent replicate could be analysed due to a DNA extraction error; therefore, no standard deviation is available.

When comparing the absolute abundances of marker and resistance genes between the WWTP Effluent and the NGWRP Influent on day 1, the relative gene abundances were comparable. Thus, there is no indication of potential severe ARG accumulation in backwash water, as reported for pathogen and ARG accumulation in water from backwashing sand filter beds and biological activated carbon filters (Huang et al., 2024) or in distribution systems (Fahrenfeld et al., 2013).

4.3.2 High Throughput Screening - qPCR

The same samples from the NGWRP were analysed at Resistomap: the WWTP Effluent and the NGWRP Influent. Through HTS-qPCR, insight was gained into the resistome profile and whether the WWTP effluent or the NGWRP Influent contained a higher ARG load. Of the 120 genes analysed, 72 and 73 ARGs were detected in the WWTP Effluent and the NGWRP Influent, respectively. The differences in the presence of gene classes observed were minor Table 10. However, the NGWRP Influent contained additional genes from the quinolone and tetracycline classes but did not exhibit altered overall ARG diversity.

Table 10: Number of genes detected from all antibiotic resistance classes as measured by HTS-qPCR in the WWTP Effluent and NGWRP influent.

Antibiotic class	**Number of genes detected within class**		
	WWTP Effluent		NGWRP Influent
Mobile Genetic Elements	**9**	**=**	**9**
Beta-lactam	**15**	**=**	**15**
Vancomycin	**2**	**=**	**2**
Multi-drug resistance	**6**	**=**	**6**
Thrimethoprim	**2**	**=**	**2**
Phenicol	**2**	**=**	**2**
Quinolone	**3**	**<**	**5**
Sulfonamide	**2**	**=**	**2**
Tetracycline	**6**	**<**	**7**
Aminoglycoside	**8**	**=**	**8**
Macrolide, Lincosamide and Streptogramin B	**7**	**>**	**5**

The relative abundance of specific genes that serve as proxies for HGT, such as integrons and transposons, was within the same order of magnitude for both influent sources, see Figure 18.

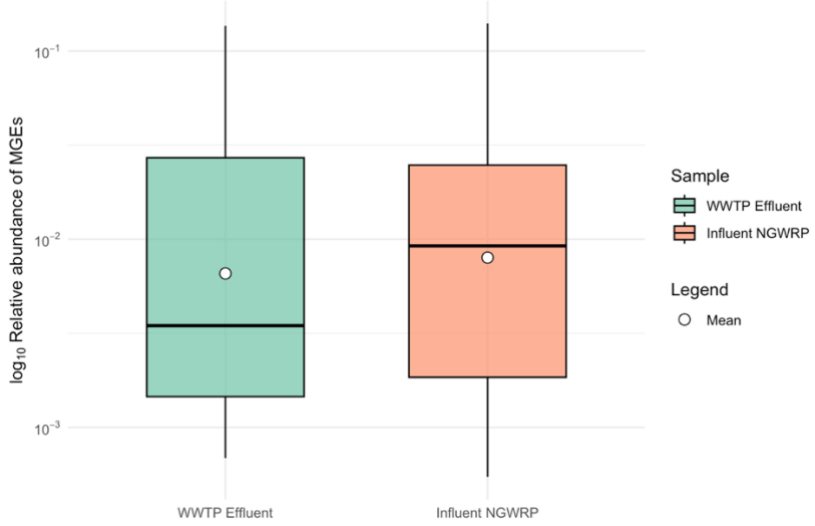


Figure 18: Relative abundance of mobile genetic elements as analysed by Resistomap. In both samples, a total of 9 mobile genetic elements were detected. In both cases, the 9 MGEs comprised four insertion sequence genes, one plasmid incompatibility gene, and four transposase genes (Data obtained from Resistomap).

Notable differences, defined as order-of-magnitude differences, were observed for several specific resistance genes. These had a higher relative abundance in the NGWRP Influent: *merA* (mercury resistance), *vanA* (vancomycin resistance), bla_{OXA-48} (beta-lactamase/carbapenemase). Even though combining WWTP Effluent with backwash into the NGWRP Influent had a minimal effect on the absolute number of target genes analysed in this study, Resistomap data show that mixing with backwash water might increase the relative abundance of *merA, vanA*, and bla_{OXA-48}. Especially *vanA* and bla_{OXA-48} are clinically relevant, as they confer resistance to the more advanced/near-last-resort class of antibiotics, vancomycin and carbapenems, respectively. Both fall within the WHO's WATCH group, indicating a high potential for antimicrobial resistance, and should be monitored (WHO, 2023). Interestingly, Manna et al. (2025) found that 58–75% of the ARGs in WWTP Effluent were more strongly influenced by the resistome profile of the conventional activated sludge process than that of the WWTP influent, indicating that results for ARGs in WWTP Effluent should not be automatically correlated with the WWTP influent.

The $bla_{CTX-M-1}$ gene was not specifically included in the analysed genes by Resistomap. However, the bla_{CTX-M} gene class was present in both Influent sources. Notably, some clinically relevant genes showed higher relative abundance after mixing with backwash water. Hereby, analysis with Resistomap provides supplementary information in addition to the qPCR assays from the present study. Given these results, the relative abundance of ARG markers measured by Resistomap and in the present study, *intl1, sul1, tetW*, and *ermB*, are comparable and indicate a low risk of accumulation in the system's backwash water.

4.3.3 Relative Log Removal Values

To assess ARG removal by the treatment steps, the relative abundance (gene copies/*16S rRNA gene* copies) was calculated. Figure 19 shows the difference in the contribution of each gene before and after treatment (each gene was reduced to <LOD after different treatment steps). It shows that *sul1* and *intl1* clearly make up a smaller proportion of the total microbial load in the GAC effluent than in the influent. This is also the case for *ermB* after its last detectable treatment step, DAF, compared to the influent. The higher relative abundance at the beginning of the treatment process, compared to the end, indicates better removal of *intl1*, *sul1,* and *ermB* than of the total microbial load (*16S rRNA* gene). No distinct increase or decrease in the proportion of *tetW* relative to the total microbial load can be seen between influent and DMF in the figure, due to the high standard deviation. <LOQ/<LOD/not amplified samples were not used to calculate the relative abundance. Which steps yielded <LOD/<LOD/not amplified values can be found in Figure 16, as the relative abundance is calculated based on the steps for which absolute values were available.

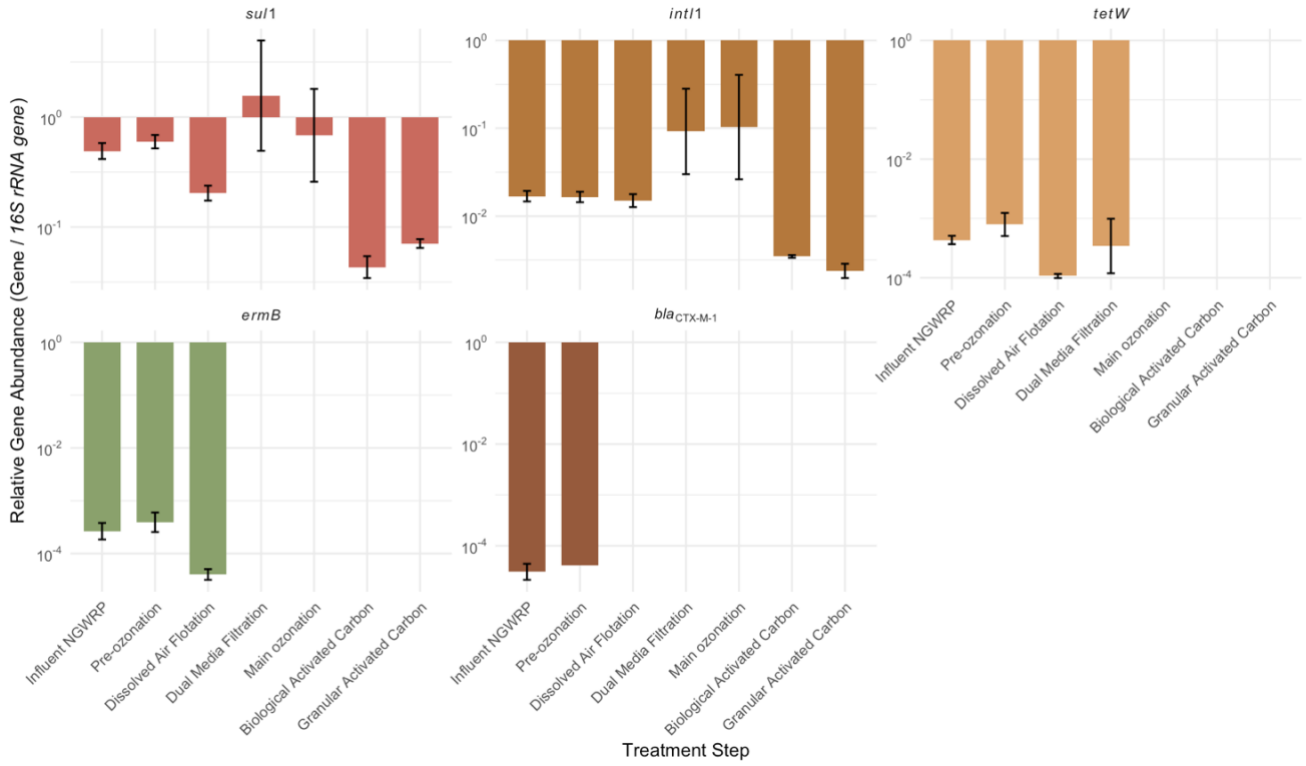


Figure 19: Log relative abundance of target genes/16S rRNA along the treatment train. This graph shows if the proportion of the target gene relative to the total microbial load increased or decreased throughout the treatment process. The lower the relative abundance (y-axis), the smaller the fraction of the target gene is compared to the total microbial load (16S rRNA). No relative abundances are calculated for any abundances <LOQ, <LOD or not amplified.

Table 11 shows the relative effective LRVs of individual treatment units analysed. When comparing the absolute and relative values, the LRVs relative to *16S rRNA* are generally smaller than the absolute LRVs. If ARGs and *16S rRNA* are removed similarly, the removal ratio will appear more consistent. The lower relative removal values might indicate that the total bacterial load is removed

more effectively than *sul1* and *intl1*, suggesting stronger ARG persistence. Moreover, this shows that, especially the earlier treatment units, might be more effective at decreasing total bacterial load than at removing specific ARGs.

Table 11: Relative effective log removal values of individual treatment units.

Treatment step	*intl1*	*sul1*	*tetW*	*ermB*
Pre-ozonation	0.01 (±0.02)	-0.10 (±0.01)	-0.25 (±0.06)	-0.16 (±0.05)
Dissolved Air Flotation	0.04 (±0.02)	0.47 (±0.01)	0.83 (±0.04)	0.97 (±0.04)
Dual Media filtration	-1.14 (±0.05)	-0.71 (±0.24)	-0.53 (±0.20)	>
Main Ozonation	0.15 (±0.84)	0.24 (±0.41)	>	>
Biological Activated Carbon	0.26 (±0.02)	0.87 (±0.14)	>>	>>
Granular Activated Carbon	-0.02 (±0.10)	-0.15 (0.01)	>>	>>>
Ultrafiltration	>>>	>	>>>	>>>
Disinfection	>>>	>	>>>	>>>
Total	>>>1.1	>0.62	>>>0.05	>>>0.81

Similarly to Wallmann et al. (2021), GAC was less effective at removing relative ARG abundance than BAC. This can be explained by the main GAC mechanism, namely, adsorption onto activated carbon surfaces. Adsorption mostly removes organic micropollutants rather than mediating biological degradation, which is needed for ARG removal (Hu et al., 2018; Zhang et al., 2025). Interestingly, the relative removal is negative for DMF and GAC, but positive for BAC.

4.4 Comparison of Absolute and Relative Removal

Figure 20 compares the absolute and relative removal values by combining the LRVs for each target gene. It indicates, for each treatment step, whether it yielded a positive (+) or negative (-) absolute and relative removal. See Illustration of absolute and relative LRV interpretation for an explanation of how to interpret positive and negative absolute or relative LRVs.

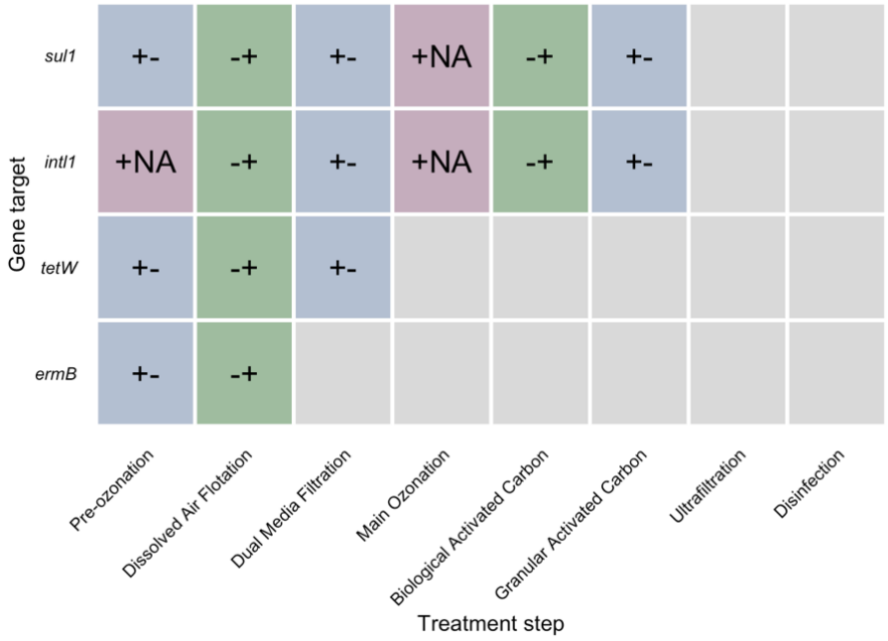


Figure 20: Comparison of combinations of absolute and relative removal per treatment step for all measured target genes. +- positive absolute removal and negative relative removal, -+ negative absolute removal and positive relative removal, +NA is positive absolute removal. All steps showed similar patterns in absolute and relative LRV of target genes.

Figure 20 clearly shows that all treatment steps followed the same pattern in their effects on absolute and relative removal for almost all targets. PO, DMF, MO, and GAC all show positive absolute removal of the target gene; however, they do not preferentially remove ARGs relative to the total microbial load (+-). This was expected, as none of the treatment steps was explicitly designed to achieve greater ARG removal than total microbial load removal. DAF and BAC show a contrasting picture compared to the other steps, as the absolute target gene amount increases, but the relative target gene concentration decreases (-+). As discussed earlier, both the total microbial load and the target genes increase in absolute quantity, leading to a negative absolute removal. However, both treatment steps still achieve a positive relative removal for all target genes.

This implies that the biofilm on BAC increases total biomass, but that resistant genes are not selectively enriched or grow at rates similar to those of non-ARG strains. Two possible causes could be: Firstly, for biofilm to serve as an ARG hotspot and stimulate HGT, it needs to receive an ARG, ARB, or antibiotic-rich stream (Balcázar et al., 2015; Haenelt et al., 2023). Since the water has already been severely pre-treated before it reaches the BAC, the concentration of ARGs entering the BAC may not be high enough to cause this proliferation effect on the biofilm. This is consistent with Zhang et al. (2025), who found that antibiotic resistance decreased in BAC after pre-treatment with ozone (>2 mg/L). In comparison, the NGWRP main-ozonation step before BAC applies 5 – 8 mg O_3/L. Secondly, ARG promotion is also dependent on the biofilm maturity in BAC. Throughout biofilm development, the primary mechanisms for pollutant removal also shift. In young biofilm, bacterial

density remains low, and adsorption to activated carbon is the primary removal mechanism. As the biofilm progresses, biodegradation becomes more important (Saini et al., 2023). The BAC at the NGWRP is replaced every 8 years, which is considered sufficient time for the biofilm to mature. The increase in absolute ARG concentrations during BAC, combined with the decrease in relative ARG concentrations, indicates that the biofilm contains a high amount of non-ARG DNA. This general microbial load increases more strongly, diluting the absolute ARG increase and achieving a positive effective relative removal.

Overall, BAC is a complex process with contradicting literature. For example, studies reach different conclusions about whether older or younger biofilms cause more HGT. Some argue that increased HGT of resistance plasmids at the air-liquid interface correlates with a younger biofilm (Król et al., 2011). In contrast, others found that a mature biofilm may increase HGT due to high bacterial density, exDNA preservation in EPS matrix, and, depending on the composition of inflow water, a higher microbial diversity. These factors could lead to more ARGs and antibiotics, thereby exerting selective pressure (Abe et al., 2020; Engin et al., 2023; Michaelis & Grohmann, 2023). The risk of HGT is therefore dependent on biofilm maturity, nutrient availability, and backwash frequency (Saini et al., 2023). However, in the context of the genes analysed in this study, BAC effectively removed the relative ARG concentrations.

4.5 Extracellular DNA distinction

The results described above represent the total DNA (inDNA + exDNA). However, the effects of water treatment steps on ARG levels might depend on whether genes are located on a chromosome or a plasmid, and on whether they are intracellular, extracellular, or particle-associated (Drane et al., 2024; Liu et al., 2022). Two methods were used to distinguish inDNA from exDNA; the results are described below.

4.5.1 Propidium Monoazide Viability Dye

On both sampling days of the main experiment, the viability dye PMA was used to distinguish between inDNA and exDNA retained on the membrane filter. Preliminary tests have been conducted to determine a suitable method for PMA. These preliminary tests are discussed in more detail in the Appendix: Propidium Monoazide Treatment and in Results Preliminary Experiments TU Wien.

Testing different dye volumes (500 µL, 1000 µL, 1500 µL) showed that 650 µL was the minimal volume needed to completely cover a membrane filter (Ø 47 mm) and ensure that all parts of the filter received equal amounts of the PMA solution. Figure 21 shows the *sul1* abundance after

filtration of wastewater from the Technikum and PMA treatment at a range of PMA concentrations (0.5–50 µM). Lower concentrations (0–0.5 µM) were hypothesized to contain insufficient PMA to bind all exDNA in the sample, as shown by a higher measurable *sul1* abundance. Whereas higher concentrations increased the risk of non-specific binding beyond that of exDNA alone, or of interfering with amplification, both will lead to overestimation of the amount of exDNA (15 – 50 µM). These effects were likely to be minimized at 5 – 10 µM; therefore, 7.5 µM was chosen for further experiments.

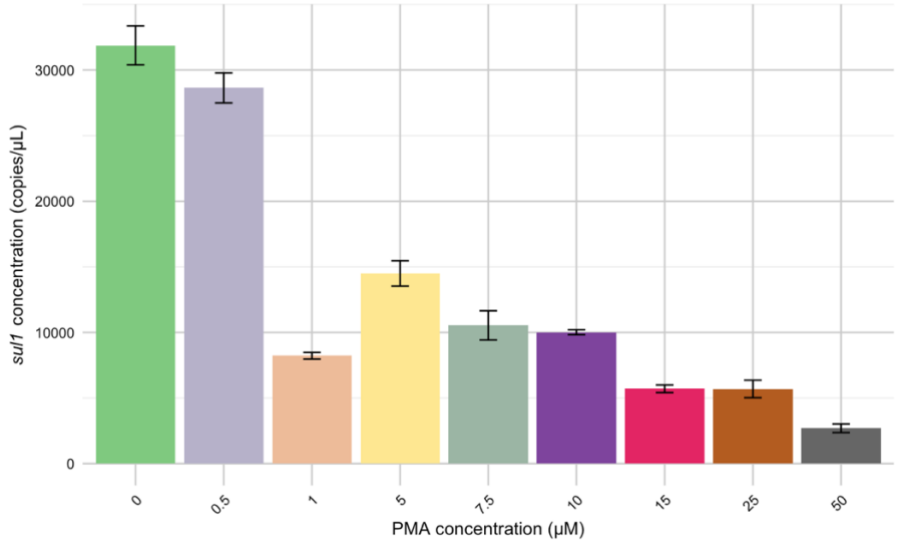


Figure 21: Result from preliminary test to determine suitable PMA concentration.

On both sampling days of the main experiment, the viability dye PMA was used to distinguish between inDNA and exDNA retained on the membrane filter. Theoretically, PMA binds to all exDNA, so that this DNA fraction can no longer be amplified in qPCR. When analysing a replicate with and without PMA in qPCR, the amount of DNA was expected to be lower for PMA-treated samples. The results were first analysed to determine whether the PMA-treated samples indeed yielded lower DNA levels in qPCR than the untreated samples. Figure 22 shows a heat map of which target genes and which treatment steps showed reduced gene abundance for treated samples compared to untreated samples.

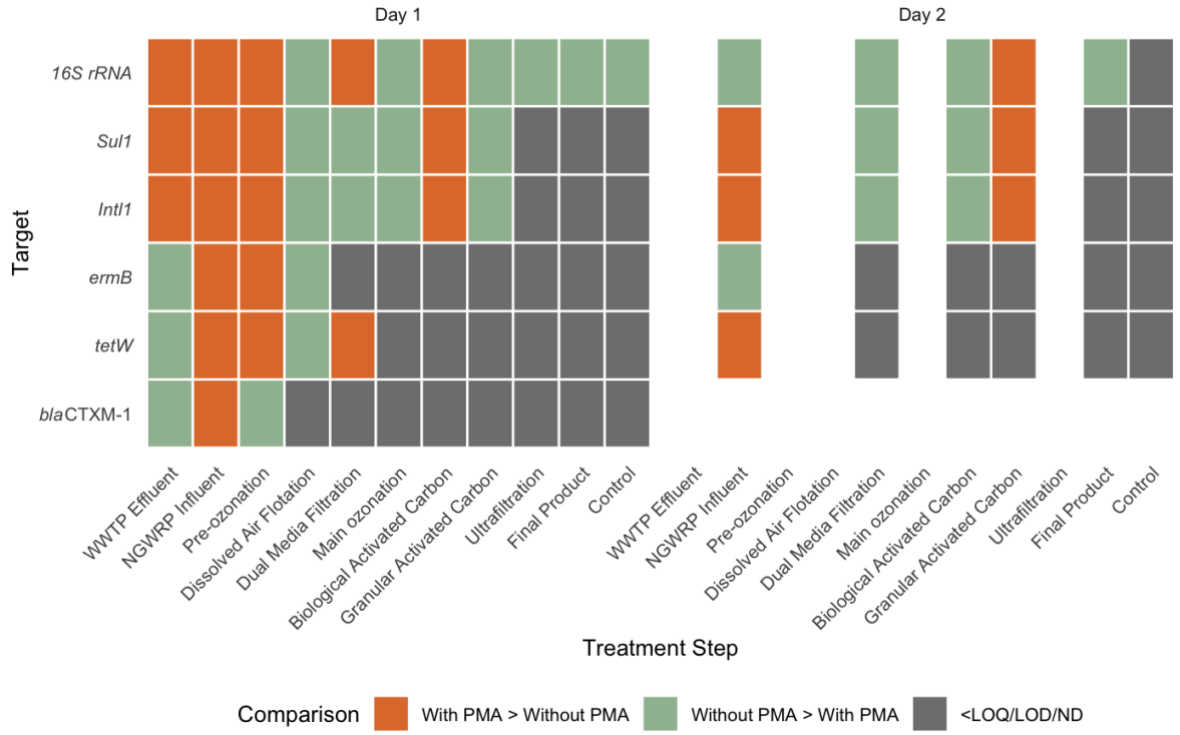


Figure 22: Heat map of target genes and treatment steps showing reduced gene abundance for PMA-treated samples compared to untreated samples. Orange indicates the unexpected pattern: In this sample, the PMA-treated replicate showed higher gene abundance than the untreated sample. Green means the expected pattern: the PMA-treated sample showed lower gene abundance than the untreated sample. Grey samples could not be analysed as they were <LOQ/LOD/Not amplified.

For the more abundant genes (*16S rRNA* gene*, sul1, and intl1*), the unexpected result (With PMA > Without PMA) occurs more frequently at the beginning of the treatment train than later in the treatment train and during BAC. The first treatment steps, as well as BAC, are characterized by a relatively high turbidity, cell density, and DOC. High turbidity and suspended solids may scatter or absorb light, thereby compromising the dye's penetration and preventing PMA from binding to exDNA (Biotium, 2024). Similar effects of complex matrices have been described for anaerobic fermentor sludge (Wagner et al., 2008), wastewater (Varma et al., 2009), aerated sludge, and marine sediment (Nocker et al., 2007). Slimani et al. (2012) also reported that high-biomass samples may be less suitable for PMA treatment.

Several factors could have led to the method's ineffectiveness, e.g., matrix interferences and too low dye concentrations. Studies often optimize the PMA method for each sample type and amplicon length (>100 bp) of the target gene by adjusting the concentration, incubation time, and light source. Unsurprisingly, using a single method for all treatment steps and targets yielded inconsistent results. It is likely that a combination of factors, as described before, caused the limited effectiveness. An attempt was made to optimize the method in Vienna (see Propidium Monoazide Treatment and Results Preliminary Experiments TU Wien). Still, due to time constraints, this could not be sufficiently

developed and adapted to the samples in Windhoek. This leads to the conclusion that, in the present study, the viability dye PMA could not effectively distinguish between inDNA and exDNA. Too many samples were unsuccessful, and no pattern could be found between successful and unsuccessful samples.

4.5.2 Extracellular DNA in filtrate

On day 2 of sampling, the filtrate was retained to analyse the amount of (assumed) exDNA that passed through the 0.45 µm membrane filter. exDNA can be smaller than 0.45 µm or associated with particulate matter or compromised membranes and therefore pass through the filter. However, it still represents a fraction that needs to be accounted for. Only the *16s rRNA* gene, *sul1*, and *intl1* were detected in the filtrate. They were measured at concentrations above the limit of detection (LOD: 1 copy/µL, SLOD: 2 copies/reaction) but below the limit of quantification (LOQ: 10 copies/µL, SLOD: 20 copies/reaction) and therefore not quantifiable. The genes *sul1* and *intl1* had a higher abundance in the autoclaved water control than in the influent. However, their respective dry filter and extraction controls were all below the limit of detection.

The *16S rRNA* gene could not be measured in the autoclaved water control due to qPCR inhibition, and dilutions up to 1:100 could not mitigate it. Additional dilution series or additional clean-up steps could have improved quantification. These observations suggest contamination or residual inhibitors, such as cleaning detergents. This is likely due to difficulties in working as sterilely as required for molecular biology standards at the on-site laboratory, despite thorough cleaning of the glass bottles as described in the Filtrate Processing section.

No DNA extraction has been performed on the filtrate samples. This was decided after preliminary tests at TU Wien on filtrate samples containing similar DNA concentrations (< 10 ng/µl); see Appendices I and III. This might have influenced the results, as the DNA can still be bound to very small particles (<0.45 µm), preventing amplification by qPCR. The use of MCE filters (0.45 µm) due to logistical challenges, rather than the intended PC filters (0.2 µm), might also have influenced the results, as the risk is higher that smaller particles were not fully retained. The two filters also differ in structure, with the uneven pore structure of mixed cellulose ester filters potentially leading to lower or more variable DNA yields compared to the smooth, uniform surface of PC filters. When logistically possible, PC filters are recommended (Liu et al., 2024; Putri et al., 2024).

4.6 Pharmaceuticals

Lastly, several pharmaceuticals were measured using HPLC-MS/MS. The results might provide a first indication of the order of magnitude of pharmaceuticals in the system and their removal. The pharmaceuticals were grouped based on their known effects on microbial communities and, therefore, how they could potentially influence the spread or occurrence of AR (selective pressure, stress responses, unknown effects). Two out of the analysed pharmaceuticals were antibiotics: sulfamethoxazole and trimethoprim. The presence of antibiotics can exert selective pressure, favouring the proliferation of SMX- or TMP-resistant bacteria, as well as other resistant genes that might co-occur with SMX or TMP resistance genes (Gualerzi et al., 2014; Sköld, 2001). Carbamazepine, Citalopram, diclofenac, ibuprofen, and metoprolol are non-antibiotic pharmaceuticals that, although they cannot directly cause AR because they lack antimicrobial properties, can still contribute to AR. They might induce physiological stress responses in microbes and may indirectly affect the spread or induction of AR. Ibuprofen, diclofenac, and metoprolol have been reported to increase reactive oxygen species production, which may compromise cell membrane stability, leading to exDNA release and increased HGT via transformation (Wang et al., 2020) and conjugation (Wang et al., 2021). Carbamazepine has been reported to increase ARGs during anaerobic digestion, promote bacterial aggregation, and thereby stimulate conjugation (Xiang et al., 2024). The effects of hydrochlorothiazide, bezafibrate, and venlafaxine on AR are still largely unknown. Bezafibrate and venlafaxine were among the most frequently found pharmaceuticals in wastewater in Spain (Gracia-Lor et al., 2012). The results of the HPLC-MS/MS analysis are summarized in Table 12. The LOQ and LOD values can be found in the HPLC-MS/MS section of the Appendix.

Table 12: Concentrations of pharmaceuticals (ng/L) throughout the treatment system grouped by their potential effect on antibiotic resistance.

Effect on AR	Treatment step	Influent NGWRP	Dual Media Filtration	Biological Activated Carbon	Granular Activated Carbon	Final Product
				(ng/L)		
Selective pressure	Sulfamethoxazole	724	888	36	<LOQ	<LOQ
	Trimethoprim	5388	1997	101	<LOD	<LOD
Stressors	Carbamazepine	279	229	36	<LOD	<LOD
	Metoprolol	461	130	56	130	58
	Diclofenac	340	184	6	15	8
	Citalopram	219	152	<LOQ	<LOQ	<LOQ
	Ibuprofen	102	<LOD	<LOD	<LOD	12
Unknown	Hydrochlorothiazide	1432	667	<LOD	<LOD	<LOD

Bezafibrate	15	15	<LOD	<LOD	<LOD
Venlafaxin	657	60	10	<LOQ	4

SMX was detected in the NGWRP Influent at 724 ng/L and increased to 888 ng/L during DMF; subsequent treatment steps reduced it to <LOQ after GAC. Studies assessing the selective pressure of sulfamethoxazole exposure range from 0.07 mg/L to 76 mg/L (Rauseo et al., 2021), where the lowest concentration tested is still 10 times higher than the concentration in the NGWRP Influent. Trimethoprim was present at 5388 ng/L in the Influent NGWRP, reduced to 101 ng/L after ozonation/BAC, and to <LOD after subsequent GAC, indicating overall efficient removal by the treatment train. BAC achieved similar removal for both antibiotics, after which GAC complemented BAC, achieving higher TMP removal than SMX. This can be explained by TMP's higher K_{OW} compared to SMX's. The higher K_{OW} indicates that TMP is more hydrophobic and that GAC sorption is a more preferred removal mechanism for TMP.

Among the non-antibiotic pharmaceuticals capable of inducing bacterial stress responses, citalopram was removed to <LOQ, and only carbamazepine was removed to <LOD. This is surprising, as carbamazepine is usually considered highly stable and resistant to biodegradation and oxidation during water treatment (Feijoo et al., 2023; Kosjek et al., 2009). Ibuprofen shows the most variable pattern as it reappears in the final product after being removed to <LOD earlier. Ibuprofen is sensitive to biodegradation (Rastogi et al., 2024) and ozonation (Quero-Pastor et al., 2014), and its sorption to activated carbon is pH-dependent. However, in the NGWRP system, this is not expected to cause variability in the observed ibuprofen concentration, as the pH is kept remarkably constant throughout the system, as shown by the physical-chemical measurement results (see Physical-Chemical Parameter Results.

Metoprolol and diclofenac were still detectable in the final product at 58 ng/L and 8 ng/L, respectively. Both are reduced until GAC but then increase. Diclofenac has a higher log K_{OW} than metoprolol, which can explain its lower concentration in the final product. The composition of the inflow, including TOC and other organic pollutants, and the regeneration frequency of the activated carbon can lead to competitive sorption and desorption. As activated carbon approaches saturation and other compounds are introduced, it will preferentially sorb hydrophobic compounds with strong binding energies due to their high electron densities (Schwarzenbach et al., 2003). The difference in sorption affinity arising from the compounds' hydrophobicity can also make metoprolol more prone to desorption (Kibbey et al., 2007). The DOC concentration in the NGWRP was measured after BAC and ranged from 2.35 to 2.50 mg/L. This might already be within the range, which could cause a compound with a low K_{OW}, such as metoprolol, to desorb and increase in concentration after GAC

treatment. However, the GAC of the NGWRP is replaced every three months, which is quite frequent, and the changes could also be less pronounced if more replicates were analysed. With regard to antimicrobial resistance, metoprolol has been shown to significantly increase conjugative gene transfer within bacterial species at a concentration of 0.005 mg/L (Wu et al., 2024), which is 10^3 times higher than the concentrations found in the Influent NGWRP.

In conclusion, most compounds were reduced to below quantifiable or detectable levels. Studies that found increased antimicrobial resistance mechanisms associated with these compounds involved higher concentrations than observed within the NGWRP. The specific effects of the concentrations reported in the present study are unknown, but the NGWRP reduced all the analysed pharmaceuticals. However, more research is needed to confirm the results, as they are based on only two sampling days.

4.7 Outlook

Seasonal variability is often mentioned as an important factor when assessing the removal efficiency of ARGs by different water treatment processes (Hu et al., 2018; Ramessar et al., 2025). These variances were not accounted for in the present study, as sampling occurred over one week during winter in Windhoek. The source of reclaimed water is WWTP Effluent from GWCW; seasonal variability and extreme events are expected to influence ARG load into the system. For example, during high-rainfall events, when the WWTP receives stormwater or during peak discharge events from hospitals. Ramessar et al. (2025) analysed ARB and ARG prevalence in wastewater effluent in South Africa and the influence of temporal variability. Strong correlations were found between shifts in physicochemical parameters (influenced by seasonal variability), such as temperature and pH, and prevalence of ARGs. Temperature was also identified previously as an important parameter affecting the removal efficiency of a water reclamation plant (Tram Vo et al., 2014). Higher temperatures lead to greater microbial growth in the system. This could either negatively impact ARG prevalence by promoting biofilm growth, thereby enhancing horizontal gene transfer of ARG (Abe et al., 2020) or have a positive impact by supporting exDNA degradation, as this was found to be correlated with higher microbial growth (Strickler et al., 2015). Taking this into account, the effects of increased temperature can be either positive or negative for ARGs. Results from the present study can thus not be extrapolated to summer, highlighting the need for further research across different seasonal regimes.

Furthermore, it is important to obtain an overview of the presence of selective pressures, such as antibiotics and non-antibiotic pharmaceuticals, as they are known to affect the prevalence of ARGs and HGT. Mass spectrometric analysis might provide insight into whether compounds are present at concentrations capable of exerting selective pressure and into the extent of reduction by the system. Still, robust conclusions would require more replicates. Additionally, it might provide insight into concentrations of organic compounds entering the BAC directly.

Even though outside the scope of this thesis, from an environmental and health perspective, formation of ozonation and disinfection byproducts can be considered relevant, especially when producing water for potable purposes. This poses a risk, as hospitals with expected pharmaceutical outflows are connected to the GWCW WWTP and, indirectly, to the NGWRP. Ozonation generates reactive intermediates from organic compounds, which are prone to further oxidation or subsequent halogenation during disinfection. Possible formation products are strongly dependent on factors such as the composition of the inflow, dissolved organic matter content, precursor concentrations, and oxidizing agent concentration. Formed products may be more toxic or persistent than their precursors; they may have significant health and ecotoxicological effects, providing grounds for future research.

5. Conclusion

This study analysed the New Goreangab Water Reclamation Plant's (NGWRP's) ability to effectively remove microbial, genetic, and antibiotic-resistance markers from treated wastewater effluent for potable reuse. Microbial water quality was analysed through the cultivation of heterotrophs and faecal coliforms. Results showed that the treatment train effectively reduced total heterotrophic bacteria and faecal coliform bacteria to below their respective operating limits for water treatment systems, set by the WHO and the EU after the Granular Activated Carbon (GAC) step. Cultivation plates enriched with sulfamethoxazole (0.5 mg SMX/L) to test for SMX-resistant bacteria showed equal or even greater bacterial growth than plates without SMX. This might be due to a concentration that was too low, microbial adaptation from prior exposure during the WWTP process before water reclamation, or improperly prepared media containing SMX. Therefore, results from SMX-enriched plates were not considered in further interpretations.

The genes measured: *16S rRNA* gene (total bacterial load), *intl1* (integron class 1; proxy for horizontal gene transfer (HGT)), *sul1* (sulfonamide resistance), *tetW* (tetracycline resistance), *ermB* (macrolide resistance), $bla_{CTX\text{-}M\text{-}1}$ (beta-lactam resistance) most likely originated from the wastewater treatment plant (WWTP) effluent from Gammams Water Care Works (GWCW), treating domestic wastewater. Mixing of WWTP Effluent with recycled water from membrane backwashing within the NGWRP system has no effect or a diluting effect on the quantity of analysed AR-related target genes. Indicating a low likelihood that the accumulation of DNA within the treatment train substantially influences their ingoing amount. HTS-qPCR analyzed over 100 target genes simultaneously and found that the compositions of WWTP Effluent and backwash reservoir effluent, which form the influx to the NGWRP, were comparable. Although the ARGs analysed in this study were chosen to represent a broad palette of commonly occurring environmental ARGs, the occurrence of other ARGs might differ from the results observed here.

All target genes, except the *16S rRNA* gene, were reduced to below the level of detection (LOD) in the final product. The remaining *16S rRNA* gene quantity is more likely due to background levels, as expected for this bacterial load marker. The highest absolute effective log removal (LRV) values were achieved by Dual Media Filtration (DMF) and Ultrafiltration (UF). An increase in absolute quantities of all target genes that were still quantifiable (*16S rRNA* gene*, intl1, sul1*) was observed during Dissolved Air Flotation (DAF) and Biological Activated Carbon (BAC). This does align with a broader observed trend of biofilm-associated enrichment in total microbial load and ARGs during BAC. Relative removal values normalized to total bacterial load (target gene/*16S rRNA* gene) indicate that

during BAC, the total microbial load increased, as did the absolute abundances of ARGs. Nevertheless, relative removal of *intI1* and *sul1* was positive. This indicates that, even though microbial load and ARGs increased, non-ARG DNA increased more than ARGs, diluting ARGs and resulting in a positive relative removal. This also suggests that no specific enrichment of HGT-associated genes *intI1* and *sul1* occurred. This might be caused by the multi-step pre-treatment, which limits the amount of ARGs entering the BAC. The same is observed for DAF, indicating that even though DAF causes an absolute increase of microbial load and target gene concentration, potentially due to mixing or incomplete removal of the scum layer, no specific enrichment of ARGs is observed. In contrast, Dual Media Filtration (DMF) effectively reduced the total bacterial load but increased the relative abundance of ARGs.

The results above are based on total DNA (inDNA + exDNA) measured after membrane filtration and DNA extraction. Due to differences in proliferation mechanisms of inDNA and exDNA, their roles in HGT, and the inability of qPCR to distinguish between them, two methods were used aiming to distinguish inDNA from exDNA. Firstly, a set of replicates was treated with the viability dye propidium monoazide (PMA) after filtration. Treated samples showed gene abundances equal to, or even higher than, those in untreated samples. This unexpected pattern was most often seen during early treatment steps and during BAC. This might be due to high turbidity and organic matter during these steps, as suspended solids can interfere with the dye's light-activation. However, no patterns could be observed, and thus PMA treatment did not reliably distinguish between inDNA and exDNA during this study. Secondly, the filtrate was retained after membrane filtration to determine the quantity of exDNA smaller than 0.45 µm. In the filtrate, *16S rRNA* gene, *sul1,* and *intI1* were detected. However, higher abundances were observed in all controls, which possibly indicates contamination in the filtrate samples.

In conclusion, the NGWRP treatment train reduced total heterotrophs and faecal coliforms to levels below their recommended operational limits for drinking water treatment systems. Even though no successful distinction could be made between inDNA and exDNA, the NGWRP reduced the class 1 integron gene *intI1* and ARGs conferring resistance to four antibiotic classes to levels below the level of detection in the final effluent, as measured by qPCR and representing the inDNA + exDNA fraction in the samples. Comparisons of absolute and relative gene abundances provide insight into the mechanisms behind gene reduction by advanced water treatment technologies. The results offer insights into the presence, fate, and ultimate reduction of ARGs in the NGWRP system. They can support further efforts to reduce the risk of antimicrobial resistance in water reclamation for direct potable reuse.

References

Abe, K., Nomura, N., & Suzuki, S. (2020). Biofilms: Hot spots of horizontal gene transfer (HGT) in aquatic environments, with a focus on a new HGT mechanism. *FEMS Microbiology Ecology*, *96*(5), fiaa031. https://doi.org/10.1093/femsec/fiaa031

Al-Ahmad, A., Daschner, F. D., & Kümmerer, K. (1999). Biodegradability of Cefotiam, Ciprofloxacin, Meropenem, Penicillin G, and Sulfamethoxazole and Inhibition of Waste Water Bacteria. *Archives of Environmental Contamination and Toxicology*, *37*(2), 158–163. https://doi.org/10.1007/s002449900501

Alawi, M., Torrijos, T. V., & Walsh, F. (2022). Plasmid-mediated antimicrobial resistance in drinking water. *Environmental Advances*, *8*, 100191. https://doi.org/10.1016/j.envadv.2022.100191

Alexander, J., Knopp, G., Dötsch, A., Wieland, A., & Schwartz, T. (2016). Ozone treatment of conditioned wastewater selects antibiotic resistance genes, opportunistic bacteria, and induce strong population shifts. *Science of The Total Environment*, *559*, 103–112. https://doi.org/10.1016/j.scitotenv.2016.03.154

Alfahl, Z., Chueiri, A., Carolan, S., Darcy, G., Hussain, N., Cahill, N., & O'Connor, L. (2024). Antimicrobial resistance detection methods in water environments: A scoping review. *Sustainable Microbiology*, *1*(1), qvae034. https://doi.org/10.1093/sumbio/qvae034

Bairoliya, S., Koh Zhi Xiang, J., & Cao, B. (2025). Extracellular DNA in Environmental Samples: Occurrence, Extraction, Quantification, and Impact on Microbial Biodiversity Assessment. *Applied and Environmental Microbiology*, *88*(3), Article 3. https://doi.org/10.1128/aem.01845-21

Balcázar, J. L., Subirats, J., & Borrego, C. M. (2015). The role of biofilms as environmental reservoirs of antibiotic resistance. *Frontiers in Microbiology*, *6*. https://doi.org/10.3389/fmicb.2015.01216

Barraud, O., Baclet, M. C., Denis, F., & Ploy, M. C. (2010). Quantitative multiplex real-time PCR for detecting class 1, 2 and 3 integrons. *Journal of Antimicrobial Chemotherapy*, *65*(8), 1642–1645. https://doi.org/10.1093/jac/dkq167

Bartram, J. (with Cotruvo, J. A., Fricker, C., Glasmacher, A., & Exner, M.). (2013). *Heterotrophic Plate Counts and Drinking-water Safety: The Significance of HPCs for Water Quality and Human Health*. IWA Publishing.

Böckelmann, U., Dörries, H.-H., Ayuso-Gabella, M. N., Salgot De Marçay, M., Tandoi, V., Levantesi, C., Masciopinto, C., Van Houtte, E., Szewzyk, U., Wintgens, T., & Grohmann, E. (2009). Quantitative PCR Monitoring of Antibiotic Resistance Genes and Bacterial Pathogens in

Three European Artificial Groundwater Recharge Systems. *Applied and Environmental Microbiology*, *75*(1), 154–163. https://doi.org/10.1128/AEM.01649-08

Borka, S. (2024). Removal of antibiotic resistance in a multi-barrier water treatment system. *Bachelor Thesis*.

Brown, C. L., Maile-Moskowitz, A., Lopatkin, A. J., Xia, K., Logan, L. K., Davis, B. C., Zhang, L., Vikesland, P. J., & Pruden, A. (2024). Selection and horizontal gene transfer underlie microdiversity-level heterogeneity in resistance gene fate during wastewater treatment. *Nature Communications*, *15*(1), 5412. https://doi.org/10.1038/s41467-024-49742-8

Carroll, K. C., Pfaller, M. A., Landry, M. L., McAdam, A. J., Patel, R., Richter, S. S., & Warnock, D. W. (Eds.). (2019). *Manual of clinical microbiology. Volume 2* (12th edition). ASM Press.

Colomer-Lluch, M., Jofre, J., & Muniesa, M. (2011). Antibiotic Resistance Genes in the Bacteriophage DNA Fraction of Environmental Samples. *PLoS ONE*, *6*(3), e17549. https://doi.org/10.1371/journal.pone.0017549

Council Directive 98/83/EC on the Quality of Water Intended for Human Consumption, No. CD 98/83/EC, Council of the European Union (1998).

Davies, J., & Davies, D. (2010). Origins and Evolution of Antibiotic Resistance. *Microbiology and Molecular Biology Reviews*, *74*(3), 417–433. https://doi.org/10.1128/MMBR.00016-10

DIRECTIVE (EU) 2020/2184 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 16 December 2020 on the quality of water intended for human consumption. (2020). *Official Journal of the European Union*, L 435/1-62.

Dos Santos, S., Adams, E. A., Neville, G., Wada, Y., De Sherbinin, A., Mullin Bernhardt, E., & Adamo, S. B. (2017). Urban growth and water access in sub-Saharan Africa: Progress, challenges, and emerging research directions. *Science of The Total Environment*, *607–608*, 497–508. https://doi.org/10.1016/j.scitotenv.2017.06.157

Drane, K., Sheehan, M., Whelan, A., Ariel, E., & Kinobe, R. (2024). The Role of Wastewater Treatment Plants in Dissemination of Antibiotic Resistance: Source, Measurement, Removal and Risk Assessment. *Antibiotics*, *13*(7), 668. https://doi.org/10.3390/antibiotics13070668

Du Pisani, P. L. (2006). Direct reclamation of potable water at Windhoek's Goreangab reclamation plant. *Desalination*, *188*(1–3), 79–88. https://doi.org/10.1016/j.desal.2005.04.104

EEA. (2025, November 18). *Antimicrobial resistance in European surface waters – a developing area*. https://www.eea.europa.eu/en/analysis/publications/antimicrobial-resistance-in-european-surface-waters-a-developing-area?activeTab=def89e87-9459-4a15-a9e0-dc6030d0c497

Ekwanzala, M. D., Dewar, J. B., Kamika, I., & Momba, M. N. B. (2018). Systematic review in South Africa reveals antibiotic resistance genes shared between clinical and environmental settings. *Infection and Drug Resistance*, *Volume 11*, 1907–1920. https://doi.org/10.2147/IDR.S170715

Emberger, J., Tassone, D., Stevens, M. P., & Markley, J. D. (2018). The Current State of Antimicrobial Stewardship: Challenges, Successes, and Future Directions. *Current Infectious Disease Reports*, *20*(9), 31. https://doi.org/10.1007/s11908-018-0637-6

Engin, A. B., Engin, E. D., & Engin, A. (2023). Effects of co-selection of antibiotic-resistance and metal-resistance genes on antibiotic-resistance potency of environmental bacteria and related ecological risk factors. *Environmental Toxicology and Pharmacology*, *98*, 104081. https://doi.org/10.1016/j.etap.2023.104081

Evans, T. J., & Riley, P. A. (2021). Principles of microscopy, culture and serology-based diagnostics. *Medicine*, *49*(10), 648–653. https://doi.org/10.1016/j.mpmed.2021.07.009

Fahrenfeld, N., Ma, Y., O'Brien, M., & Pruden, A. (2013). Reclaimed water as a reservoir of antibiotic resistance genes: Distribution system and irrigation implications. *Frontiers in Microbiology*, *4*. https://doi.org/10.3389/fmicb.2013.00130

Feijoo, S., Kamali, M., & Dewil, R. (2023). A review of wastewater treatment technologies for the degradation of pharmaceutically active compounds: Carbamazepine as a case study. *Chemical Engineering Journal*, *455*, 140589. https://doi.org/10.1016/j.cej.2022.140589

Frost, L. S., Leplae, R., Summers, A. O., & Toussaint, A. (2005). Mobile genetic elements: The agents of open source evolution. *Nature Reviews Microbiology*, *3*(9), 722–732. https://doi.org/10.1038/nrmicro1235

Garner, E., Chen, C., Xia, K., Bowers, J., Engelthaler, D. M., McLain, J., Edwards, M. A., & Pruden, A. (2018). Metagenomic Characterization of Antibiotic Resistance Genes in Full-Scale Reclaimed Water Distribution Systems and Corresponding Potable Systems. *Environmental Science & Technology*, *52*(11), 6113–6125. https://doi.org/10.1021/acs.est.7b05419

Global Antimicrobial Resistance and Use Surveillance System (GLASS) Report 2022 (1st ed). (2022). World Health Organization.

Gracia-Lor, E., Sancho, J. V., Serrano, R., & Hernández, F. (2012). Occurrence and removal of pharmaceuticals in wastewater treatment plants at the Spanish Mediterranean area of Valencia. *Chemosphere*, *87*(5), 453–462. https://doi.org/10.1016/j.chemosphere.2011.12.025

Gualerzi, C. O., Brandi, L., Fabretti, A., & Pon, C. L. (2014). *Antibiotics: Targets, Mechanisms and Resistance*. Wiley-VCH Verlag GmbH & Co.

Guo, J., Li, J., Chen, H., Bond, P. L., & Yuan, Z. (2017). Metagenomic analysis reveals wastewater treatment plants as hotspots of antibiotic resistance genes and mobile genetic elements. *Water Research*, *123*, 468–478. https://doi.org/10.1016/j.watres.2017.07.002

Gustafson, R. H. (1991). Use of Antibiotics in Livestock and Human Health Concerns. *Journal of Dairy Science*, *74*(4), 1428–1432. https://doi.org/10.3168/jds.S0022-0302(91)78299-4

Haenelt, S., Richnow, H.-H., Müller, J. A., & Musat, N. (2023). Antibiotic resistance indicator genes in biofilm and planktonic microbial communities after wastewater discharge. *Frontiers in Microbiology*, *14*, 1252870. https://doi.org/10.3389/fmicb.2023.1252870

Heuer, H., & Smalla, K. (2006). Manure and sulfadiazine synergistically increased bacterial antibiotic resistance in soil over at least two months. *Environmental Microbiology*, *9*(3), 657–666. https://doi.org/10.1111/j.1462-2920.2006.01185.x

Hitchings, G. H. (1973). Mechanism of Action of Trimethoprim-Sulfamethoxazole—I. *Journal of Infectious Diseases*, *128*(Supplement 3), S433–S436. https://doi.org/10.1093/infdis/128.Supplement_3.S433

Hu, Y., Jiang, L., Zhang, T., Jin, L., Han, Q., Zhang, D., Lin, K., & Cui, C. (2018). Occurrence and removal of sulfonamide antibiotics and antibiotic resistance genes in conventional and advanced drinking water treatment processes. *Journal of Hazardous Materials*, *360*, 364–372. https://doi.org/10.1016/j.jhazmat.2018.08.012

Huang, Y., Weng, X., Qu, X., Sun, T., Zhang, W., Yang, S., He, X., Xiao, Y., Zhang, T., & Tang, Y. (2024). Identification of microbiological genomes carrying antibiotic resistance genes and virulence factor genes for microbiological risk assessment in filter backwash water. *Journal of Environmental Chemical Engineering*, *12*(5), 113980. https://doi.org/10.1016/j.jece.2024.113980

Hutchings, M. I., Truman, A. W., & Wilkinson, B. (2019). Antibiotics: Past, present and future. *Current Opinion in Microbiology*, *51*, 72–80. https://doi.org/10.1016/j.mib.2019.10.008

Ibáñez De Aldecoa, A. L., Zafra, O., & González-Pastor, J. E. (2017). Mechanisms and Regulation of Extracellular DNA Release and Its Biological Roles in Microbial Communities. *Frontiers in Microbiology*, *8*, 1390. https://doi.org/10.3389/fmicb.2017.01390

Jimenez, B., & Asano, T. (2008). Water Reuse: An International Survey of current practice, issues and needs. *Water Intelligence Online*, *7*(0), 9781780401881–9781780401881. https://doi.org/10.2166/9781780401881

Johansson, M. H. K., Petersen, T. N., Nag, S., Lagermann, T. M. R., Birkedahl, L. E. K., Tafaj, S., Bradbury, S., Collignon, P., Daley, D., Dougnon, V., Fabiyi, K., Coulibaly, B., Dembélé, R., Magloire, N., Ouindgueta, I. J., Hossain, Z. Z., Begoum, A., Donchev, D., Diggle, M., … Aarestrup, F. M. (2025). Investigation of mobile genetic elements and their association with

antibiotic resistance genes in clinical pathogens worldwide. *PLOS One*, *20*(8), e0330304. https://doi.org/10.1371/journal.pone.0330304

Joseph, C., Faiq, M. E., Li, Z., & Chen, G. (2022). Persistence and degradation dynamics of eDNA affected by environmental factors in aquatic ecosystems. *Hydrobiologia*, *849*(19), 4119–4133. https://doi.org/10.1007/s10750-022-04959-w

Kaprou, G. D., Bergšpica, I., Alexa, E. A., Alvarez-Ordóñez, A., & Prieto, M. (2021). Rapid Methods for Antimicrobial Resistance Diagnostics. *Antibiotics*, *10*(2), 209. https://doi.org/10.3390/antibiotics10020209

Keller, A. A., Su, Y., & Jassby, D. (2022). Direct Potable Reuse: Are We Ready? A Review of Technological, Economic, and Environmental Considerations. *ACS ES&T Engineering*, *2*(3), 273–291. https://doi.org/10.1021/acsestengg.1c00258

Kibbey, T. C. G., Paruchuri, R., Sabatini, D. A., & Chen, L. (2007). Adsorption of Beta Blockers to Environmental Surfaces. *Environmental Science & Technology*, *41*(15), 5349–5356. https://doi.org/10.1021/es070152v

Kneis, D., De La Cruz Barron, M., Konyali, D., Westphal, V., Schröder, P., Westphal-Settele, K., Schönfeld, J., Jungmann, D., Berendonk, T. U., & Klümper, U. (2025). Ecology-based approach to predict no-effect antibiotic concentrations for minimizing environmental selection of resistance. *The ISME Journal*, *19*(1), wraf172. https://doi.org/10.1093/ismejo/wraf172

Kosjek, T., Andersen, H. R., Kompare, B., Ledin, A., & Heath, E. (2009). Fate of Carbamazepine during Water Treatment. *Environmental Science & Technology*, *43*(16), 6256–6261. https://doi.org/10.1021/es900070h

La Rosa, M. C., Maugeri, A., Favara, G., La Mastra, C., Magnano San Lio, R., Barchitta, M., & Agodi, A. (2025). The Impact of Wastewater on Antimicrobial Resistance: A Scoping Review of Transmission Pathways and Contributing Factors. *Antibiotics*, *14*(2), 131. https://doi.org/10.3390/antibiotics14020131

Lahnsteiner, J., Honer, T., Ashipala, L., Nikodemus, K., Poussade, Y., & Van Rensburg, P. (2024). Windhoek/Goreangab Direct Potable Water Reuse, Case Study Namibia. In J. Lahnsteiner (Ed.), *Handbook of Water and Used Water Purification* (pp. 1301–1318). Springer International Publishing. https://doi.org/10.1007/978-3-319-78000-9_170

Larsson, D. G. J. (2014). Antibiotics in the environment. *Upsala Journal of Medical Sciences*, *119*(2), 108–112. https://doi.org/10.3109/03009734.2014.896438

Law, I. B., Menge, J., & Cunliffe, D. (2015). Validation of the Goreangab Reclamation Plant in Windhoek, Namibia against the 2008 Australian Guidelines for Water Recycling. *Journal of Water Reuse and Desalination*, *5*(1), 64–71. https://doi.org/10.2166/wrd.2014.138

Lawrence, J. G., & Hendrickson, H. (2008). Genomes in Motion: Gene Transfer as a Catalyst for Genome Change. In M. Hensel & H. Schmidt (Eds.), *Horizontal Gene Transfer in the Evolution of Pathogenesis* (1st ed., pp. 3–22). Cambridge University Press. https://doi.org/10.1017/CBO9780511541520.002

Leifels, M., Jurzik, L., Wilhelm, M., & Hamza, I. A. (2015). Use of ethidium monoazide and propidium monoazide to determine viral infectivity upon inactivation by heat, UV-exposure and chlorine. *International Journal of Hygiene and Environmental Health*, *218*(8), 686–693. https://doi.org/10.1016/j.ijheh.2015.02.003

Leijnse, M., F P Bierkens, M., H M Gommans, K., Lin, D., Tait, A., & Wanders, N. (2024). Key drivers and pressures of global water scarcity hotspots. *Environmental Research Letters*, *19*(5), 054035. https://doi.org/10.1088/1748-9326/ad3c54

Li, L., Mendis, N., Trigui, H., Oliver, J. D., & Faucher, S. P. (2014). The importance of the viable but non-culturable state in human bacterial pathogens. *Frontiers in Microbiology*, *5*. https://doi.org/10.3389/fmicb.2014.00258

Liu, H., Li, Z., Liu, C., Qiang, Z., Karanfil, T., & Yang, M. (2022). Elimination and Redistribution of Intracellular and Extracellular Antibiotic Resistance Genes in Water and Wastewater Disinfection Processes: A Review. *ACS ES&T Water*, *2*(12), 2273–2288. https://doi.org/10.1021/acsestwater.2c00286

Liu, M., & Kasuga, I. (2024). Impact of chlorine disinfection on intracellular and extracellular antimicrobial resistance genes in wastewater treatment and water reclamation. *Science of The Total Environment*, *949*, 175046. https://doi.org/10.1016/j.scitotenv.2024.175046

Liu, Q., Tan, J., Wang, M., Xin, N., Qi, R., & Wang, H. (2024). Optimization of pore size and filter material for better enrichment of environmental DNA. *Frontiers in Environmental Science*, *12*, 1422269. https://doi.org/10.3389/fenvs.2024.1422269

Mancuso, G., Midiri, A., Gerace, E., Marra, M., Zummo, S., & Biondo, C. (2023). Urinary Tract Infections: The Current Scenario and Future Prospects. *Pathogens*, *12*(4), 623. https://doi.org/10.3390/pathogens12040623

Manna, B., Zhou, X., & Singhal, N. (2025). ROS-induced stress promotes enrichment and emergence of antibiotic resistance in conventional activated sludge processes. *Water Research*, *277*, 123366. https://doi.org/10.1016/j.watres.2025.123366

Mao, D., Luo, Y., Mathieu, J., Wang, Q., Feng, L., Mu, Q., Feng, C., & Alvarez, P. J. J. (2014). Persistence of Extracellular DNA in River Sediment Facilitates Antibiotic Resistance Gene Propagation. *Environmental Science & Technology*, *48*(1), Article 1. https://doi.org/10.1021/es404280v

Mapani, B. S., Shikangalah, R. N., & Mwetulundila, A. L. (2023). A review on water security and management under climate change conditions, Windhoek, Namibia. *Journal of African Earth Sciences*, *197*, 104749. https://doi.org/10.1016/j.jafrearsci.2022.104749

Masters, O'Bryan, T., Zurlo, J., Miller, D., & Joshi, N. (2003). *Trimethoprim-Sulfamethoxazole Revisited*. https://doi.org/doi:10.1001/archinte.163.4.402

Mcmanus, M. C. (1997). *Mechanisms of bacterial resistance to antimicrobial agents*.

Mendelsohn, E., Ross, N., Zambrana-Torrelio, C., Boeckel, T. P. V., Laxminarayan, R., & Daszak, P. (2023). *Global patterns and correlates in the emergence of antimicrobial resistance in humans*.

Michaelis, C., & Grohmann, E. (2023). Horizontal Gene Transfer of Antibiotic Resistance Genes in Biofilms. *Antibiotics*, *12*(2), 328. https://doi.org/10.3390/antibiotics12020328

Moesker, K., Pesch, U., & Doorn, N. (2024). Public acceptance in direct potable water reuse: A call for incorporating responsible research and innovation. *Journal of Responsible Innovation*, *11*(1), 2304382. https://doi.org/10.1080/23299460.2024.2304382

Mosher, J. J., Vartanian, G. M., & Tchobanoglous, G. (2016). *Potable Reuse Research Compilation: Synthesis of Findings*.

Murray, C. J. L., Ikuta, K. S., Sharara, F., Swetschinski, L., Robles Aguilar, G., Gray, A., Han, C., Bisignano, C., Rao, P., Wool, E., Johnson, S. C., Browne, A. J., Chipeta, M. G., Fell, F., Hackett, S., Haines-Woodhouse, G., Kashef Hamadani, B. H., Kumaran, E. A. P., McManigal, B., … Naghavi, M. (2022). Global burden of bacterial antimicrobial resistance in 2019: A systematic analysis. *The Lancet*, *399*(10325), 629–655. https://doi.org/10.1016/S0140-6736(21)02724-0

Murray, Louw, D., Van Der Merwe, B., & Peters, I. (2018). Windhoek, Namibia: From conceptualising to operating and expanding a MAR scheme in a fractured quartzite aquifer for the city's water security. *Sustainable Water Resources Management*, *4*(2), 217–223. https://doi.org/10.1007/s40899-018-0213-0

Naghavi, M., Vollset, S. E., Ikuta, K. S., Swetschinski, L. R., Gray, A. P., Wool, E. E., Robles Aguilar, G., Mestrovic, T., Smith, G., Han, C., Hsu, R. L., Chalek, J., Araki, D. T., Chung, E., Raggi, C., Gershberg Hayoon, A., Davis Weaver, N., Lindstedt, P. A., Smith, A. E., … Murray, C. J. L. (2024). Global burden of bacterial antimicrobial resistance 1990–2021: A systematic analysis with forecasts to 2050. *The Lancet*, *404*(10459), 1199–1226. https://doi.org/10.1016/S0140-6736(24)01867-1

Nagler, M., Insam, H., Pietramellara, G., & Ascher-Jenull, J. (2018). Extracellular DNA in natural environments: Features, relevance and applications. *Applied Microbiology and Biotechnology*, *102*(15), 6343–6356. https://doi.org/10.1007/s00253-018-9120-4

Namibian Antimicrobial Resistance National Action Plan. (2017). Namibian Ministry of Health and Social Services. https://cdn.who.int/media/docs/default-source/antimicrobial-resistance/amr-spc-npm/nap-library/amr-nap_final--namibia-2017.pdf?sfvrsn=b6629668_5

Nielsen, K. M., Johnsen, P. J., Bensasson, D., & Daffonchio, D. (2007). Release and persistence of extracellular DNA in the environment. *Environmental Biosafety Research*, *6*(1–2), 37–53. https://doi.org/10.1051/ebr:2007031

Nocker, A., Sossa-Fernandez, P., Burr, M. D., & Camper, A. K. (2007). Use of Propidium Monoazide for Live/Dead Distinction in Microbial Ecology. *Applied and Environmental Microbiology*, *73*(16), 5111–5117. https://doi.org/10.1128/AEM.02987-06

Odey, T. O. J., Tanimowo, W. O., Afolabi, K. O., Jahid, I. K., & Reuben, R. C. (2023). Antimicrobial use and resistance in food animal production: Food safety and associated concerns in Sub-Saharan Africa. *International Microbiology*, *27*(1), 1–23. https://doi.org/10.1007/s10123-023-00462-x

Ou, D., Chen, B., Bai, R., Song, P., & Lin, H. (2015). Contamination of sulfonamide antibiotics and sulfamethazine-resistant bacteria in the downstream and estuarine areas of Jiulong River in Southeast China. *Environmental Science and Pollution Research*, *22*(16), 12104–12113. https://doi.org/10.1007/s11356-015-4473-z

Pärnänen, K. M. M., Narciso-da-Rocha, C., Kneis, D., Berendonk, T. U., Cacace, D., Do, T. T., Elpers, C., Fatta-Kassinos, D., Henriques, I., Jaeger, T., Karkman, A., Martinez, J. L., Michael, S. G., Michael-Kordatou, I., O'Sullivan, K., Rodriguez-Mozaz, S., Schwartz, T., Sheng, H., Sørum, H., … Manaia, C. M. (2019). Antibiotic resistance in European wastewater treatment plants mirrors the pattern of clinical antibiotic resistance prevalence. *Science Advances*, *5*(3), eaau9124. https://doi.org/10.1126/sciadv.aau9124

Partridge, S. R., Kwong, S. M., Firth, N., & Jensen, S. O. (2018). Mobile Genetic Elements Associated with Antimicrobial Resistance. *Clinical Microbiology Reviews*, *31*(4), e00088-17. https://doi.org/10.1128/CMR.00088-17

Pereko, D. D., Lubbe, M. S., & Essack, S. Y. (2016). Surveillance of antibiotic use in the private sector in Namibia using sales and claims data. *The Journal of Infection in Developing Countries*, *10*(11), 1243–1249. https://doi.org/10.3855/jidc.7329

Performance Standard for Antimicrobial Susceptibility Testing. (2020). Clinical and Laboratory Standards Institute.

Piaggio, A., Mittapalli, S., Calderón-Franco, D., Weissbrodt, D., Van Lier, J., De Kreuk, M., & Lindeboom, R. (2023). The fate of sulfamethoxazole and trimethoprim in a micro-aerated anaerobic membrane bioreactor and the occurrence of antibiotic resistance in the

permeate. *Water Science & Technology*, *88*(9), 2344–2363. https://doi.org/10.2166/wst.2023.324

Proia, L., Von Schiller, D., Sànchez-Melsió, A., Sabater, S., Borrego, C. M., Rodríguez-Mozaz, S., & Balcázar, J. L. (2016). Occurrence and persistence of antibiotic resistance genes in river biofilms after wastewater inputs in small rivers. *Environmental Pollution*, *210*, 121–128. https://doi.org/10.1016/j.envpol.2015.11.035

Putri, R. E., Vrouwenvelder, J. S., & Farhat, N. (2024). Enhancing the DNA yield intended for microbial sequencing from a low-biomass chlorinated drinking water. *Frontiers in Microbiology*, *15*, 1339844. https://doi.org/10.3389/fmicb.2024.1339844

Quero-Pastor, M. J., Garrido-Perez, M. C., Acevedo, A., & Quiroga, J. M. (2014). Ozonation of ibuprofen: A degradation and toxicity study. *Science of The Total Environment*, *466–467*, 957–964. https://doi.org/10.1016/j.scitotenv.2013.07.067

Ramessar, K., Egbewale, O. S., Kumar, A., & Olaniran, A. O. (2025). Prevalence and removal of antibiotic-resistant bacteria and genes in treated effluents of four wastewater treatment plants and receiving surface waters. *Journal of Water Process Engineering*, *77*, 108432. https://doi.org/10.1016/j.jwpe.2025.108432

Rastogi, A., Chaudhary, S., Tiwari, M. K., & Ghangrekar, M. M. (2024). Ibuprofen degradation by mixed bacterial consortia: Metabolic pathway and microbial community analysis. *Chemosphere*, *359*, 142354. https://doi.org/10.1016/j.chemosphere.2024.142354

Rauseo, J., Barra Caracciolo, A., Spataro, F., Visca, A., Ademollo, N., Pescatore, T., Grenni, P., & Patrolecco, L. (2021). Effects of Sulfamethoxazole on Growth and Antibiotic Resistance of A Natural Microbial Community. *Water*, *13*(9), 1262. https://doi.org/10.3390/w13091262

Reygaert, W., C. (2018). An overview of the antimicrobial resistance mechanisms of bacteria. *AIMS Microbiology*, *4*(3), 482–501. https://doi.org/10.3934/microbiol.2018.3.482

Rygaard, M., Arvin, E., Bath, A., & Binning, P. J. (2011). Designing water supplies: Optimizing drinking water composition for maximum economic benefit. *Water Research*, *45*(12), 3712–3722. https://doi.org/10.1016/j.watres.2011.04.025

Saini, S., Tewari, S., Dwivedi, J., & Sharma, V. (2023). Biofilm-mediated wastewater treatment: A comprehensive review. *Materials Advances*, *4*(6), 1415–1443. https://doi.org/10.1039/D2MA00945E

Savio, D., Sinclair, L., Ijaz, U. Z., Parajka, J., Reischer, G. H., Stadler, P., Blaschke, A. P., Blöschl, G., Mach, R. L., Kirschner, A. K. T., Farnleitner, A. H., & Eiler, A. (2015). Bacterial diversity along a 2600 km river continuum. *Environmental Microbiology*, *17*(12), 4994–5007. https://doi.org/10.1111/1462-2920.12886

Schwarzenbach, R. P., Imboden, D. M., & Gschwend, P. M. (2003). *Environmental Organic Chemistry* (2nd ed). Wiley. https://doi.org/10.1002/0471649643

Shemer, H., Wald, S., & Semiat, R. (2023). Challenges and Solutions for Global Water Scarcity. *Membranes*, *13*(6), 612. https://doi.org/10.3390/membranes13060612

Sköld, O. (2001). *Resistance to trimethoprim and sulfonamides*.

Slimani, S., Robyns, A., Jarraud, S., Molmeret, M., Dusserre, E., Mazure, C., Facon, J. P., Lina, G., Etienne, J., & Ginevra, C. (2012). Evaluation of propidium monoazide (PMA) treatment directly on membrane filter for the enumeration of viable but non cultivable Legionella by qPCR. *Journal of Microbiological Methods*, *88*(2), 319–321. https://doi.org/10.1016/j.mimet.2011.12.010

Smith, A. M., Ramudzulu, M., Munk, P., Avot, B. J. P., Esterhuyse, K. C. M., Van Blerk, N., Kwenda, S., & Sekwadi, P. (2024). Metagenomics analysis of sewage for surveillance of antimicrobial resistance in South Africa. *PLOS ONE*, *19*(8), e0309409. https://doi.org/10.1371/journal.pone.0309409

Soller, J. A., Eftim, S. E., Warren, I., & Nappier, S. P. (2017). Evaluation of microbiological risks associated with direct potable reuse. *Microbial Risk Analysis*, *5*, 3–14. https://doi.org/10.1016/j.mran.2016.08.003

Spit, T., Van Der Hoek, J. P., De Jong, C., Van Halem, D., De Kreuk, M., & Perez, B. B. (2022). Removal of Antibiotic Resistance From Municipal Secondary Effluents by Ozone-Activated Carbon Filtration. *Frontiers in Environmental Science*, *10*, 834577. https://doi.org/10.3389/fenvs.2022.834577

Strickler, K. M., Fremier, A. K., & Goldberg, C. S. (2015). Quantifying effects of UV-B, temperature, and pH on eDNA degradation in aquatic microcosms. *Biological Conservation*, *183*, 85–92. https://doi.org/10.1016/j.biocon.2014.11.038

Svahn, O., & Björklund, E. (2015). *Thermal stability assessment of antibiotics in moderate temperature and subcritical water using a pressurized dynamic flow-through system*.

Taylor, M. J., Bentham, R. H., & Ross, K. E. (2014). Limitations of Using Propidium Monoazide with qPCR to Discriminate between Live and Dead *Legionella* in Biofilm Samples. *Microbiology Insights*, *7*, MBI.S17723. https://doi.org/10.4137/MBI.S17723

Tiseo, K., Huber, L., Gilbert, M., Robinson, T. P., & Van Boeckel, T. P. (2020). Global Trends in Antimicrobial Use in Food Animals from 2017 to 2030. *Antibiotics*, *9*(12), 918. https://doi.org/10.3390/antibiotics9120918

Tram Vo, P., Ngo, H. H., Guo, W., Zhou, J. L., Nguyen, P. D., Listowski, A., & Wang, X. C. (2014). A mini-review on the impacts of climate change on wastewater reclamation and reuse.

Science of The Total Environment, *494–495*, 9–17. https://doi.org/10.1016/j.scitotenv.2014.06.090

Uluseker, C., Kaster, K. M., Thorsen, K., Basiry, D., Shobana, S., Jain, M., Kumar, G., Kommedal, R., & Pala-Ozkok, I. (2021). A Review on Occurrence and Spread of Antibiotic Resistance in Wastewaters and in Wastewater Treatment Plants: Mechanisms and Perspectives. *Frontiers in Microbiology*, *12*, 717809. https://doi.org/10.3389/fmicb.2021.717809

Vale, F. F., Lehours, P., & Yamaoka, Y. (2022). Editorial: The Role of Mobile Genetic Elements in Bacterial Evolution and Their Adaptability. *Frontiers in Microbiology*, *13*, 849667. https://doi.org/10.3389/fmicb.2022.849667

Varma, M., Field, R., Stinson, M., Rukovets, B., Wymer, L., & Haugland, R. (2009). Quantitative real-time PCR analysis of total and propidium monoazide-resistant fecal indicator bacteria in wastewater. *Water Research*, *43*(19), 4790–4801. https://doi.org/10.1016/j.watres.2009.05.031

Ventola, C. L. (2015). *The Antibiotic Resistance Crisis*.

Wagner, A. O., Malin, C., Knapp, B. A., & Illmer, P. (2008). Removal of Free Extracellular DNA from Environmental Samples by Ethidium Monoazide and Propidium Monoazide. *Applied and Environmental Microbiology*, *74*(8), 2537–2539. https://doi.org/10.1128/AEM.02288-07

Wallmann, L., Krampe, J., Lahnsteiner, J., Radu, E., Van Rensburg, P., Slipko, K., Wögerbauer, M., & Kreuzinger, N. (2021). Fate and persistence of antibiotic-resistant bacteria and genes through a multi-barrier treatment facility for direct potable reuse. *Journal of Water Reuse and Desalination*, *11*(3), 373–390. https://doi.org/10.2166/wrd.2021.097

Walsh, F., Ingenfeld, A., Zampicolli, M., Hilber-Bodmer, M., Frey, J. E., & Duffy, B. (2011). Real-time PCR methods for quantitative monitoring of streptomycin and tetracycline resistance genes in agricultural ecosystems. *Journal of Microbiological Methods*, *86*(2), 150–155. https://doi.org/10.1016/j.mimet.2011.04.011

Wan, K., Guo, L., Ye, C., Zhu, J., Zhang, M., & Yu, X. (2021). Accumulation of antibiotic resistance genes in full-scale drinking water biological activated carbon (BAC) filters during backwash cycles. *Water Research*, *190*, 116744. https://doi.org/10.1016/j.watres.2020.116744

Wang, J., & Chen, X. (2022). Removal of antibiotic resistance genes (ARGs) in various wastewater treatment processes: An overview. *Critical Reviews in Environmental Science and Technology*, *52*(4), 571–630. https://doi.org/10.1080/10643389.2020.1835124

Wang, J., Sha, X., Chen, X., Zhuo, H., Xie, W., Zhou, Z., He, X., Wu, L., & Li, B. (2022). Removal and distribution of antibiotics and resistance genes in conventional and advanced drinking water treatment processes. *Journal of Water Process Engineering*, *50*, 103217. https://doi.org/10.1016/j.jwpe.2022.103217

Wang, S., Tian, R., Bi, Y., Meng, F., Zhang, R., Wang, C., Wang, D., Liu, L., & Zhang, B. (2024). A review of distribution and functions of extracellular DNA in the environment and wastewater treatment systems. *Chemosphere*, *359*, 142264. https://doi.org/10.1016/j.chemosphere.2024.142264

Wang, Y., Lu, J., Engelstädter, J., Zhang, S., Ding, P., Mao, L., Yuan, Z., Bond, P. L., & Guo, J. (2020). Non-antibiotic pharmaceuticals enhance the transmission of exogenous antibiotic resistance genes through bacterial transformation. *The ISME Journal*, *14*(8), 2179–2196. https://doi.org/10.1038/s41396-020-0679-2

Wang, Y., Lu, J., Zhang, S., Li, J., Mao, L., Yuan, Z., Bond, P. L., & Guo, J. (2021). Non-antibiotic pharmaceuticals promote the transmission of multidrug resistance plasmids through intra- and intergenera conjugation. *The ISME Journal*, *15*(9), 2493–2508. https://doi.org/10.1038/s41396-021-00945-7

Water quality—Sampling for microbiological analysis. (2006, January 8).

Water Resources Management Act 11 of 2013 - Republic of Namibia, Pub. L. No. GN 268/2023 (GG 8187) (2013). https://www.lac.org.na/laws/annoSTAT/Water%20Resources%20Management%20Act%2011%20of%202013.pdf

Wilkinson, J. L., Boxall, A. B. A., Kolpin, D. W., Leung, K. M. Y., Lai, R. W. S., Galbán-Malagón, C., Adell, A. D., Mondon, J., Metian, M., Marchant, R. A., Bouzas-Monroy, A., Cuni-Sanchez, A., Coors, A., Carriquiriborde, P., Rojo, M., Gordon, C., Cara, M., Moermond, M., Luarte, T., … Teta, C. (2022). Pharmaceutical pollution of the world's rivers. *Proceedings of the National Academy of Sciences*, *119*(8), e2113947119. https://doi.org/10.1073/pnas.2113947119

World Health Organization. (2017). *Potable reuse: Guidance for producing safe drinking-water*. World Health Organization. https://iris.who.int/handle/10665/258715

World Health Organization (Ed.). (2022). *Guidelines for drinking-water quality* (Fourth edition incorporating the first and second addenda). World Health Organization.

World Health Organization. (2023). *AWaRe classification of antibiotics for evaluation and monitoring of use*. https://www.who.int/publications/i/item/WHO-MHP-HPS-EML-2023.04

World Meteorological Organization. (2024). *State of Global Water Resources 2023*. United Nations.

World Resources Institute. (2023, August 16). *Highest Water Stressed Countries*. https://www.wri.org/insights/highest-water-stressed-countries

Wu, Q., Wu, G.-G., Pan, K.-N., Wang, X.-P., Li, H.-Y., Tian, Z., Jin, R.-C., & Fan, N.-S. (2024). Beta-blocker drives the conjugative transfer of multidrug resistance genes in pure and complex biological systems. *Journal of Hazardous Materials*, *477*, 135403. https://doi.org/10.1016/j.jhazmat.2024.135403

Xiang, Y., Jia, M., Xu, R., Xu, J., He, L., Peng, H., Sun, W., Wang, D., Xiong, W., & Yang, Z. (2024). Carbamazepine facilitated horizontal transfer of antibiotic resistance genes by enhancing microbial communication and aggregation. *Bioresource Technology*, *391*, 129983. https://doi.org/10.1016/j.biortech.2023.129983

Zhang, Xue, X., & Hu, J. (2025). Combined ozonation-biological activated carbon process for antibiotic resistance control in treated effluent from wastewater treatment plant. *Water Research*, *268*, 122610. https://doi.org/10.1016/j.watres.2024.122610

Zhang, Y., Li, A., Dai, T., Li, F., Xie, H., Chen, L., & Wen, D. (2018). Cell-free DNA: A Neglected Source for Antibiotic Resistance Genes Spreading from WWTPs. *Environmental Science & Technology*, *52*(1), 248–257. https://doi.org/10.1021/acs.est.7b04283

Zhuang, M., Achmon, Y., Cao, Y., Liang, X., Chen, L., Wang, H., Siame, B. A., & Leung, K. Y. (2021). Distribution of antibiotic resistance genes in the environment. *Environmental Pollution*, *285*, 117402. https://doi.org/10.1016/j.envpol.2021.117402

Appendix

I. Method Preliminary Experiments TU Wien

a. Propidium Monoazide Treatment

PMA method development for the discrimination between inDNA and total DNA (and indirectly exDNA via calculation of total DNA - inDNA) strongly depends on the sample matrix and the type of microorganisms. Several pre-tests have been conducted at the TU Wien to determine a suitable concentration at which PMA effectively prevents the amplification of exDNA. Important parameters were incubation time, dye concentration, dye volume, and the method of addition.

Several preliminary tests were executed to determine a suitable PMA concentration. For all filtrations, polycarbonate (PC) filters were used. A PMA range of 0, 10, 15, and 50 µM was first tested on filters containing wastewater. To quantitatively assess the effect of different PMA concentrations on the amplifiable DNA concentration in qPCR, filtrations were conducted using a plasmid standard at 10^6 cells/filter and a bacterial solution (Zymobiomics, USA) containing <0.02% exDNA at a range of cell volumes (10^7, 10^5, 10^3 cells/filter). It was hypothesized that extracting the DNA from these combined additions after PMA treatment would yield lower DNA abundance, corresponding to the amount of plasmid standard used, as the plasmid standard would be mainly exDNA. In contrast, the microbial solution should contain only intact DNA, see Figure S .

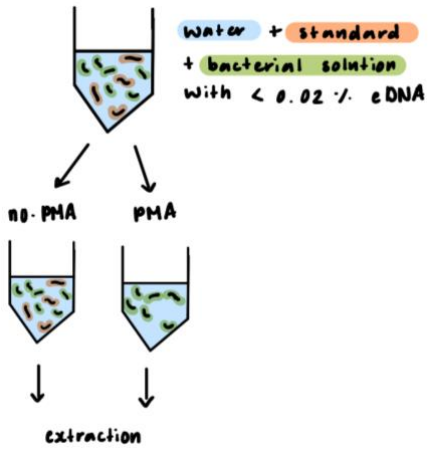


Figure S 1: Principle of propidium monoazide experiment using plasmid standard and bacterial solution containing expected intact DNA.

This experiment was repeated due to an extraction mistake. For all PMA concentrations, duplicates were made to assess the effect of freezing the samples after PMA addition. Finally, a broader range (0, 0.5, 1, 5, 7.5, 10, 15, 25, 50 µM) was tested, using wastewater to find an optimal amplification range during which there is enough PMA present to bind most exDNA, but avoid toxic effects to intact cells, which might happen at higher concentrations (Taylor et al., 2014).

b. Filtrate Processing

Two main methods for concentrating DNA from large volumes of filtrate were investigated. Firstly, the InnuPREP TCT Target Concentration Kit Water (IST Innuscreen, Germany). The beads function by absorbing liquid but repulsing biomolecules. In this way, the volume will decrease while the DNA concentration increases. The less DNA is present in the sample, the more liquid will be absorbed by the beads, thereby concentrating the DNA in the remaining liquid. Additionally, Amicon ultra centrifugal filters with a 10 kDa MWCO (Sigma-Aldrich, Germany) were used to further concentrate and purify the filtrate. They were tested both as an additional concentration step to complement the TCT Target Concentration Kit and independently.

Three experiments were conducted with the TCT Target Concentration Beads:

1. 100 mL of tap water was spiked with a high *sul1* concentration ($5*10^4$ copies/µL), a low *sul1* concentration (2.5 copies/µL), and 100 mL without a spiked standard (blank) and incubated with 2 grams of TCT beads for 2.5 hours. The leftover volume was measured afterward, and the theoretical DNA increase was calculated from the volume decrease and compared to the actual DNA increase.
2. 500 mL of tap water containing 2.5 *sul1* copies/µL, and 500 mL without a spiked standard (blank), were incubated with 5 grams of TCT beads for 24 hours. After 24 hours, the remaining liquid from the low-spike and blank samples was further concentrated by spinning down with Amicon ultra centrifugal filters. If all the liquid was taken up, the beads were washed with 10 mL of phosphate-buffered saline (PBS), briefly swirled, and then sampled according to the manufacturer's instructions.
3. 500 mL of tap water was spiked with 2.5 *sul1* copies/µL and incubated with 5 grams of TCT beads for 24 hours. The remaining 15 mL was supplemented with 10 mL PBS, swirled with the TCT beads, and brought to a final volume of 25 mL This volume was subsequently concentrated further using Amicon ultra centrifugal filters.

c. DNA Extraction of Filtrate

Borka., (2024) similarly investigated AR in an advanced water treatment plant, yielding filtrate samples that were further concentrated locally in India using Amicon ultracentrifugal filters (10 kDa). The present study measured the DNA quantity of these samples with and without additional DNA extraction of the filtrate. The aim was to test the efficiency Amicon ultra centrifugal filters in further concentrating the filtrate using 'real-life' samples. As well as investigating whether the extraction of filtrate, which is assumed to contain a low amount of inDNA, will increase the DNA yield.

II. Protocol von der Emde Technikum

Simulation of an advanced water treatment plant with three sampling points: Influent, Ultrafiltration (UF), and Disinfection. Two runs of this system will be done: One with Control water and one with effluent from the Von der Emde wastewater treatment plant.

Agar

Difco m FC agar: Feacal Coliforms and Tryptone Glucose Yeast Extract Agar for HPC was prepared according to the manufacturer's instructions. Petri dishes were cooled overnight and stored at 4°C.

Cleaning of Ultrafiltration installation

1. Organic Fouling: Use NaOCl Concentrations and procedures are specified by the membrane supplier. High concentrations (0.5% NaOCl) should be used for a maximum of 30 seconds to 1 minute. Rinse thoroughly between applications.
2. Inorganic and Organic Fouling: Use NaOH and NaOCl sequentially, as per supplier instructions.
3. Pilot Plant Cleaning: Use NaOH in very low concentrations (similar membrane cleaning), circulated in the system for 5-10 minutes. Rinse thoroughly with water for at least 10 minutes.

Sampling

1. Rinse 50 L feed tank with distilled water
2. Fill up feed tank with 25L of distilled water (control)
3. Mix well and take 2 times 1L sample in cleaned 2L glass bottles, store in fridge.
4. Turn on Ultrafiltration and let system run until all water is processed
5. Mix well and take 2 times 1L sample from retentate tank in cleaned 2L glass bottles, store in fridge.
6. Add 0.4 ml/L of sodium hypochlorite (NaOCl) with a concentration of 0.5 % to retentate tank with an exposure time of 30 mins to achieve a concentration between 0.2 mg/l and 2 mg/l of free chlorine after the exposure time (World Health Organization (Ed.). (2022). *Guidelines for drinking-water quality* (Fourth edition incorporating the first and second addenda). Geneva: World Health Organization.)
7. Stop the disinfection reaction of chlorine by adding 1 ml to 1 L of sample of sodium thiosulphate solution of 18 mg/l ($S_2O_3^{2-}$) (Borka, 2023)
8. Mix well and take 2 times 1L sample from retentate tank in cleaned 2L glass bottles, store in fridge.

Repeat above with effluent from the Von der Emde wastewater treatment plant. See Table S 1 for an overview of all samples after sampling.

Table S 1: Overview of all samples and replicates from Von der Emde Technikum preliminary tests.

Number	*Name*	*Definition*	*Volume*
1	C-IF-A	Control Influent A	1L
2	C-IF-B	Control Influent B	1L
3	C-UF-A	Control Ultrafiltration A	1L
4	C-UF-B	Control Ultrafiltration B	1L
5	C-DI-A	Control Disinfected A	1L
6	C-DI-A	Control Disinfected B	1L
7	E-IF-A	Effluent Influent A	1L
8	E-IF-B	Effluent Influent B	1L
9	E-UF-A	Effluent Ultrafiltration A	1L
10	E-UF-B	Effluent Ultrafiltration B	1L
11	E-DI-A	Effluent Disinfected A	1L
12	E-DI-B	Effluent Disinfected B	1L

Microbiological analysis

1. Take the determined volumes from the dilution experiment out of glass bottle four times (In duplicates so filter volume e.g. 40 mL * 8 = 320 mL) and filter each through a separate polycarbonate (PC) filter (Ø 47 mm, 0.2 µm).
2. Depending on turbidity and expected cell count a higher volume could be filtered. However, only 1 L of filtrate will be used for the later concentration step.
3. Add filters to agar plates and incubate for 48 hours at 36°C.
4. Count grown colonies after incubation.

Molecular biological analysis

1. Take the remaining +- 1L of sample and filter the following amounts per water source and sampling points:
    a. Controls: 1L at all sampling points
    b. Effluent: 40 mL for Influent, 1000 mL after Ultrafiltration and Disinfection
2. Incubate the filtrate with beads from the TCT InnuPrep concentration kit overnight. Determine the volume of beads like the following:
    a. E.g. 1000 mL filtrate/100 = 10g beads to be added

3. Prepare a Propidium Monoazide (PMA) solution at 7.5 µM by adding 2.925 µL to 7797.075 µL. Which is 7.5 µM for 12 samples with 650 µL per sample.
4. Make solution only shortly before use. Store in a tube covered in aluminum foil and work in a darkened environment.
5. Place the filters in their respective petri dishes and add 650 µL of Propidium Monoazide solution to each filter. Make sure the whole filter is covered with PMA-dye solution. Incubate petri-dishes for 10 minutes in the dark. Thereafter, incubate the petri dishes for 30 minutes in the UV-box.
6. Fold filters, store in Eppendorf tubes, and freeze.

Extraction

1. Add 2g of magnetic beads to Fastprep-compatible tubes.
2. Cut up stored filters into smaller pieces.
3. Fill filter into tube.
4. Extract filters according to TCT Innuprep DNA extraction kit
5. Store extracted samples in Eppendorf tube and freeze

III. Results Preliminary Experiments TU Wien

a. Propidium Monoazide Treatment

The results of the preliminary tests to determine a concentration for the Propidium Monoazide treatment are shown in the main text (see Propidium Monoazide Viability Dye)

b. Filtrate Processing

1. The first experiment showed an increase in concentration for both the high-spiked and low-spiked samples; however, it was not as large as the increase proportional to the volume decrease.
2. The main takeaway was that the concentration increased with longer incubation time, as indicated by a higher *sul1* quantity measured when only 15 mL remained compared to when 100 mL remained, with a lower volume remaining after a more prolonged incubation. Strikingly, after subsequent Amicon ultrafiltration of this exact 15 mL volume, no *sul1* could be measured anymore (Figure S 1)

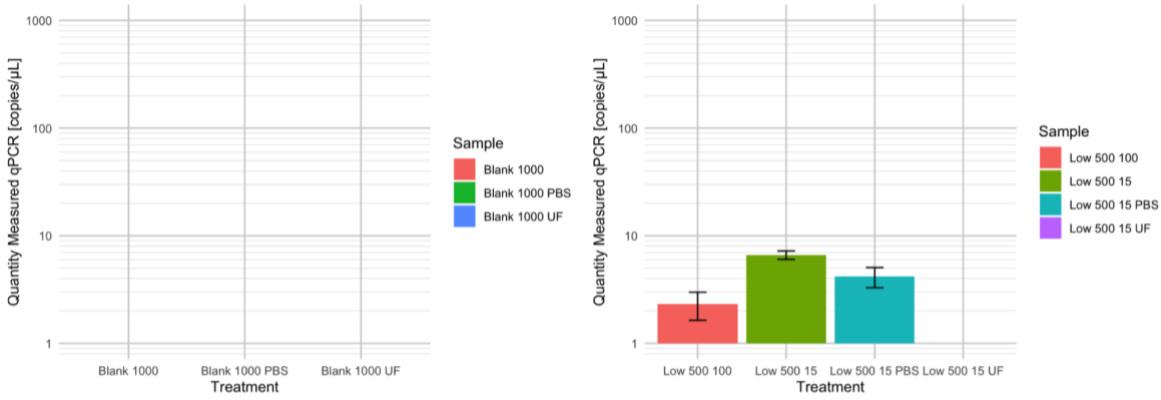


Figure S 1: The quantity of the sul1 gene measured for a low spike sample, after 100 mL volume is left over from incubation, and later, when only 15 mL is left. Additionally, the sul1 quantity is shown after spinning down 15 mL with Amicon Ultra centrifugal columns.

3. During the third experiment, the same was observed. To concentrate 25 mL of the sample using Amicon Ultra centrifugal filters, multiple rounds of spinning down should be performed. To learn why no signal could be detected after Amicon ultra centrifugal filter in the second experiment, a small aliquot of 20 µL was sampled after each subsequent round of spinning down. Figure S 2 shows a clear decrease in *sul1* quantity each time the sample is spun down. Due to this unexpected result, the challenges in obtaining a suitable centrifuge in Windhoek and the high cost of the Amicon filters, it was decided not to use the Amicon ultra centrifugal filters during the sampling period at the NGWRP and to concentrate the filtrate only using the TCT target beads.

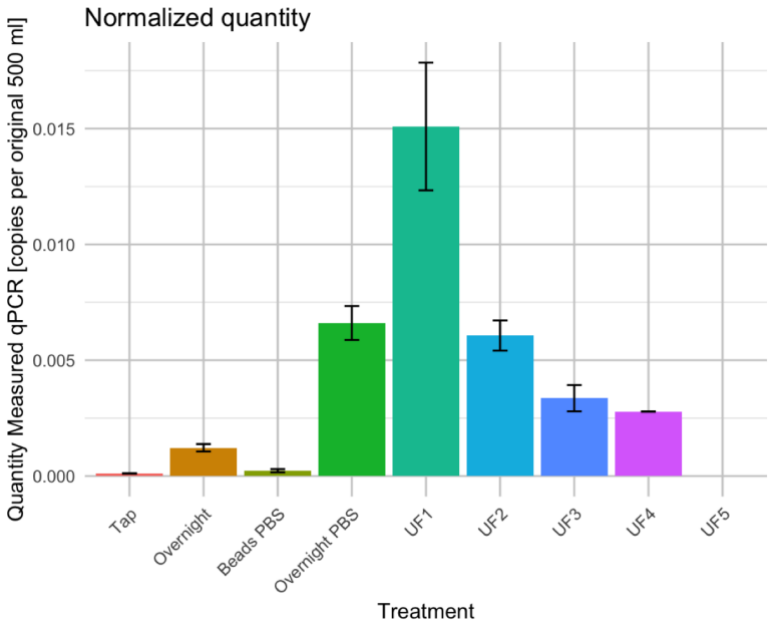


Figure S 2: Normalized sul1 quantity after each round of spinning down with Amicon Ultra centrifugal.

c. DNA Extraction Filtrate

Borka. (2024) executed a study in India into AR in a tertiary treatment reverse osmosis plant in Koyambedu (Chennai, India). Samples were filtered through a 0.2 µm polycarbonate filter. Most bacterial cells are 0.5 – 5 µm in diameter and will therefore be efficiently separated from extracellular DNA (eDNA), as even long eDNA strands are only about 0.2 nm long. Therefore, it is assumed that the filtrate will mainly contain eDNA. As DNA extraction is based on cell lysis, the release of DNA, and subsequent purification to prepare the samples for the following analysis. The filtrates from India, which were assumed to contain mainly extracellular DNA, were shipped to Vienna. These samples were extracted during the present study using the Power Water Kit to test the effectiveness of filtrate extraction (Figure S 3).

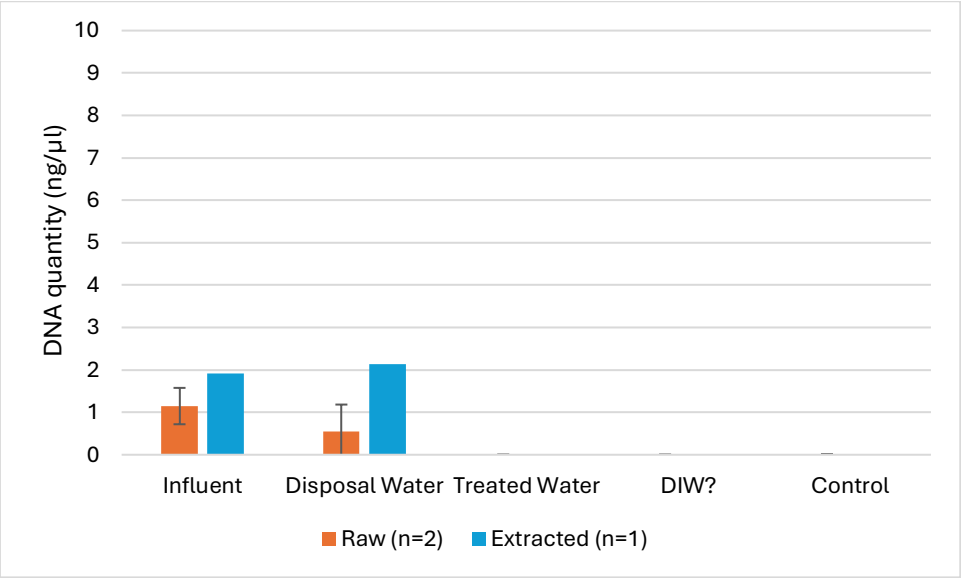


Figure S 3: DNA quantity measured in filtrate samples from India that were previously concentrated with Amicon Ultracentrifugal filters before and after DNA extraction.

The graph shows the measured DNA concentrations (ng/µl) for the four filtrate samples. Known measured sampling points from left to right are Influent, Disposal Water, Treated Water, and Distilled Water. For the DIW samples, the thesis does not specify which exact sampling points they are from. H2O is a distilled water extraction control. The not-extracted samples were measured fluorometrically in duplicates. DNA concentrations of 1.15 ng/µl (±0.4) and 0.54 ng/µl (±0.64) were measured for Influent and Disposal Water, respectively. The DNA concentration for Treated Water and "DIW" was below the limit of detection, while a distilled water control (made in Vienna) measured 0.0041 ng/µl (±0.00021). These samples are concentrated with Amicon centrifugal filters (10 kDa). After DNA extraction from the sample, the DNA concentration was measured at 1.91 ng/µl in the Influent and 2.13 ng/µl in the Disposal Water. However, at concentrations <10 ng/µl, interpretations of results should be done cautiously. Given uncertainties about the exact sampling points and procedures, the concentration methods used by staff (UF Amicon), and the low DNA concentration measured, the samples were not considered reliable.

Therefore, for the present study, it was decided to conduct additional tests using the UF Amicon (10 kDa) with new samples and not to extract eDNA from filtrate samples, as the minimal increase in quantity observed in these samples does not greatly improve the measurable DNA concentration

IV. Raw Data and Limits of Detection Bacterial Cultivation

Table S 2: LOD and LOQ for bacterial cultivation per cultivation and treatment steps.

Organisms	Treatment step	LOD (CFU/mL)	LOQ (CFU/mL)
Faecal Coliforms	WWTP Effluent Raw Mix	10	100
	All others	0.01	0.1
	Autoclaved water control	0.01	0.1
Heterotrophic Plate Count	WWTP Effluents Raw Mix	1000	100
	All Others	10	1
	Final Product	1	0.1
	Autoclaved water control	1	0.1

Table S 3: Raw data Bacterial Cultivation

Sample	Treatment step	Replicate	Plate	CFU (counts)	Flag	Sulfamethoxazole (Yes/No)	Filter volume (mL)	Bacterial count (CFU/ml)	LOD (CFU/mL)	LOQ (CFU/mL)
MP-A-FC+	WWTP Effluent/ Maturation Pond	A	FC	5	<LOQ	Y	0,1	50	10	100
MP-B-FC+	WWTP Effluent/ Maturation Pond	B	FC	4	<LOQ	Y	0,1	40	10	100
MP-A-FC-	WWTP Effluent/ Maturation Pond	A	FC	1	<LOQ	N	0,1	10	10	100
MP-B-FC-	WWTP Effluent/ Maturation Pond	B	FC	1	<LOQ	N	0,1	10	10	100
IN-A-FC+	Raw Mix	A	FC	1	<LOQ	Y	0,1	10	10	100

IN-B-FC+	Raw Mix	B	FC	1	<LOQ	Y	0,1	10	10	100
IN-A-FC-	Raw Mix	A	FC	0	<LOQ	N	0,1	0	10	100
IN-B-FC-	Raw Mix	B	FC	0	<LOQ	N	0,1	0	10	100
PO-A-FC+	Pre-Ozonation	A	FC	230		Y	100	2,3	0,01	0,1
PO-B-FC+	Pre-Ozonation	B	FC	170		Y	100	1,7	0,01	0,1
PO-A-FC-	Pre-Ozonation	A	FC	222		N	100	2,22	0,01	0,1
PO-B-FC-	Pre-Ozonation	B	FC	260		N	100	2,6	0,01	0,1
CO-A-FC+	Coagulation/ Flocculation	A	FC	143		Y	100	1,43	0,01	0,1
CO-B-FC+	Coagulation/ Flocculation	B	FC	170		Y	100	1,7	0,01	0,1
CO-A-FC-	Coagulation/ Flocculation	A	FC	190		N	100	1,9	0,01	0,1
CO-B-FC-	Coagulation/ Flocculation	B	FC	210		N	100	2,1	0,01	0,1
DA-A-FC+	Dissolved Air Flotation	A	FC	15		Y	100	0,15	0,01	0,1
DA-B-FC+	Dissolved Air Flotation	B	FC	19		Y	100	0,19	0,01	0,1
DA-A-FC-	Dissolved Air Flotation	A	FC	14		N	100	0,14	0,01	0,1
DA-B-FC-	Dissolved Air Flotation	B	FC	25		N	100	0,25	0,01	0,1
DM-A-FC+	Dual Media Filtration	A	FC	1	<LOQ	Y	100	0,01	0,01	0,1
DM-B-FC+	Dual Media Filtration	B	FC	5	<LOQ	Y	100	0,05	0,01	0,1
DM-A-FC-	Dual Media Filtration	A	FC	3	<LOQ	N	100	0,03	0,01	0,1
DM-B-FC-	Dual Media Filtration	B	FC	4	<LOQ	N	100	0,04	0,01	0,1
MO-A-FC+	Main Ozonation	A	FC	0	<LOD	Y	100	0	0,01	0,1
MO-B-FC+	Main Ozonation	B	FC	1	<LOQ	Y	100	0,01	0,01	0,1
MO-A-FC-	Main Ozonation	A	FC	5	<LOQ	N	100	0,05	0,01	0,1

MO-B-FC-	Main Ozonation	B	FC	5	<LOQ	N	100	0,05	0,01	0,1
BA-A-FC+	BAC	A	FC	3	<LOQ	Y	100	0,03	0,01	0,1
BA-B-FC+	BAC	B	FC	3	<LOQ	Y	100	0,03	0,01	0,1
BA-A-FC-	BAC	A	FC	2	<LOQ	N	100	0,02	0,01	0,1
BA-B-FC-	BAC	B	FC	1	<LOQ	N	100	0,01	0,01	0,1
GA-A-FC+	GAC	A	FC	1	<LOQ	Y	100	0,01	0,01	0,1
GA-B-FC+	GAC	B	FC	1	<LOQ	Y	100	0,01	0,01	0,1
GA-A-FC-	GAC	A	FC	0	<LOD	N	100	0	0,01	0,1
GA-B-FC-	GAC	B	FC	1	<LOQ	N	100	0,01	0,01	0,1
UF-A-FC+	UF	A	FC	0	<LOD	Y	100	0	0,01	0,1
UF-B-FC+	UF	B	FC	0	<LOD	Y	100	0	0,01	0,1
UF-A-FC-	UF	A	FC	0	<LOD	N	100	0	0,01	0,1
UF-B-FC-	UF	B	FC	0	<LOD	N	100	0	0,01	0,1
FP-A-FC+	Final Product	A	FC	0	<LOD	Y	100	0	0,01	0,1
FP-B-FC+	Final Product	B	FC	0	<LOD	Y	100	0	0,01	0,1
FP-A-FC-	Final Product	A	FC	0	<LOD	N	100	0	0,01	0,1
FP-B-FC-	Final Product	B	FC	0	<LOD	N	100	0	0,01	0,1
E-A-FC+	Empty filter control	A	FC	0	<LOD	Y	0			
E-B-FC+	Empty filter control	B	FC	0	<LOD	Y	0			
E-A-FC-	Empty filter control	A	FC	0	<LOD	N	0			
E-B-FC-	Empty filter control	B	FC	0	<LOD	N	0			
W-A-FC+	Autoclaved water control	A	FC	0	<LOD	Y	100	0	0,01	0,1
W-B-FC+	Autoclaved water control	B	FC	0	<LOD	Y	100	0	0,01	0,1
W-A-FC-	Autoclaved water control	A	FC	0	<LOD	N	100	0	0,01	0,1

W-B-FC-	Autoclaved water control	B	FC	0	<LOD	N	100	0	0,01	0,1
MP-A-TGEA+	Maturation Pond	A	TGEA	18		Y	0,01	1800	100	1000
MP-B-TGEA+	Maturation Pond	B	TGEA	19		Y	0,01	1900	100	1000
MP-A-TGEA-	Maturation Pond	A	TGEA	35		N	0,01	3500	100	1000
MP-B-TGEA-	Maturation Pond	B	TGEA	27		N	0,01	2700	100	1000
RM-A-TGEA+	Raw Mix	A	TGEA	61		Y	0,01	6100	100	1000
RM-B-TGEA+	Raw Mix	B	TGEA	80		Y	0,01	8000	100	1000
RM-A-TGEA-	Raw Mix	A	TGEA	82		N	0,01	8200	100	1000
RM-B-TGEA-	Raw Mix	B	TGEA	69		N	0,01	6900	100	1000
PO-A-TGEA+	Pre-Ozonation	A	TGEA	1014		Y	1	1014	1	10
PO-B-TGEA+	Pre-Ozonation	B	TGEA	1162		Y	1	1162	1	10
PO-A-TGEA-	Pre-Ozonation	A	TGEA	1333		N	1	1333	1	10
PO-B-TGEA-	Pre-Ozonation	B	TGEA	1185		N	1	1185	1	10
CO-A-TGEA+	Coagulation/Flocculation	A	TGEA	1311		Y	1	1311	1	10
CO-B-TGEA+	Coagulation/Flocculation	B	TGEA	1231		Y	1	1231	1	10
CO-A-TGEA-	Coagulation/Flocculation	A	TGEA	1276		N	1	1276	1	10
CO-B-TGEA-	Coagulation/Flocculation	B	TGEA	1311		N	1	1311	1	10

DA-A-TGEA+	Dissolved Air Flotation	A	TGEA	386		Y	1	386	1	10
DA-B-TGEA+	Dissolved Air Flotation	B	TGEA	392		Y	1	392	1	10
DA-A-TGEA-	Dissolved Air Flotation	A	TGEA	1162		N	1	1162	1	10
DA-B-TGEA-	Dissolved Air Flotation	B	TGEA	1219		N	1	1219	1	10
DM-A-TGEA+	Dual Media Filtration	A	TGEA	741		Y	1	741	1	10
DM-B-TGEA+	Dual Media Filtration	B	TGEA	946		Y	1	946	1	10
DM-A-TGEA-	Dual Media Filtration	A	TGEA	900		N	1	900	1	10
DM-B-TGEA-	Dual Media Filtration	B	TGEA	957		N	1	957	1	10
MO-A-TGEA+	Main Ozonation	A	TGEA	78		Y	1	78	1	10
MO-B-TGEA+	Main Ozonation	B	TGEA	85		Y	1	85	1	10
MO-A-TGEA-	Main Ozonation	A	TGEA	92		N	1	92	1	10
MO-B-TGEA-	Main Ozonation	B	TGEA	94		N	1	94	1	10
BA-A-TGEA+	BAC	A	TGEA	92		Y	1	92	1	10
BA-B-TGEA+	BAC	B	TGEA	96		Y	1	96	1	10
BA-A-TGEA-	BAC	A	TGEA	120		N	1	120	1	10
BA-B-TGEA-	BAC	B	TGEA	132		N	1	132	1	10
GA-A-TGEA+	GAC	A	TGEA	59		Y	1	59	1	10

GA-B-TGEA+	GAC	B	TGEA	52		Y	1	52	1	10
GA-A-TGEA-	GAC	A	TGEA	37		N	1	37	1	10
GA-B-TGEA-	GAC	B	TGEA	46		N	1	46	1	10
UF-A-TGEA+	UF	A	TGEA	0	<LOD	Y	1	0	1	10
UF-B-TGEA+	UF	B	TGEA	0	<LOD	Y	1	0	1	10
UF-A-TGEA-	UF	A	TGEA	0	<LOD	N	1	0	1	10
UF-B-TGEA-	UF	B	TGEA	1	<LOQ	N	1	1	1	10
FP-A-TGEA+	Final Product	A	TGEA	46		Y	10	4,6	0,1	1
FP-B-TGEA+	Final Product	B	TGEA	44		Y	10	4,4	0,1	1
FP-A-TGEA-	Final Product	A	TGEA			N	10	0	0,1	1
FP-B-TGEA-	Final Product	B	TGEA			N	10	0	0,1	1
E-A-TGEA+	Empty filter control	A	TGEA	0	<LOD	Y	10	0	0,1	1
E-B-TGEA+	Empty filter control	B	TGEA	0	<LOD	Y	10	0	0,1	1
E-A-TGEA-	Empty filter control	A	TGEA	0	<LOD	N	10	0	0,1	1
E-B-TGEA-	Empty filter control	B	TGEA	0	<LOD	N	10	0	0,1	1
W-A-TGEA+	Autoclaved water control	A	TGEA	0	<LOD	Y	0	#DIV/0!	#DIV/0!	#DIV/0!
W-B-TGEA+	Autoclaved water control	B	TGEA	0	<LOD	Y	0	#DIV/0!	#DIV/0!	#DIV/0!

W-A-TGEA-	Autoclaved water control	A	TGEA	0	<LOD	N	0	#DIV/0!	#DIV/0!	#DIV/0!
W-B-TGEA-	Autoclaved water control	B	TGEA	0	<LOD	N	0	#DIV/0!	#DIV/0!	#DIV/0!

V. qPCR Protocols

Table S 4: qPCR temperature and cycling protocols

Gene target		qPCR step			
		Denaturation	Annealing	Elongation	Cycles
16S rRNA gene	Temperature (°C)	95	95	60	45x
	Time	00:10:00	00:00:15	00:01:00	
sul1	Temperature (°C)	95	96	60	40x
	Time	00:10:00	00:00:15	00:01:00	
intl1	Temperature (°C)	95	95	60	45x
	Time	00:10:00	00:00:30	00:01:00	
ermB	Temperature (°C)	95	95	60	40x
	Time	00:10:00	00:00:15	00:01:00	
tetW	Temperature (°C)	96	96	60	40x
	Time	00:10:00	00:00:15	00:01:00	
$bla_{CTX\text{-}M\text{-}1}$	Temperature (°C)	95	95	60	45x
	Time	00:02:00	00:00:15	00:00:30	

VI. HPLC-MS/MS

Table S 5: Online SPE and HPLC elution gradient overview (E. Saracevic, TU Wien).

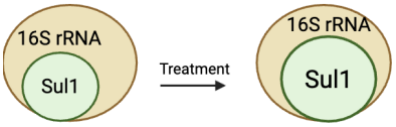
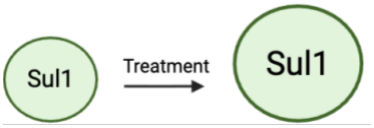
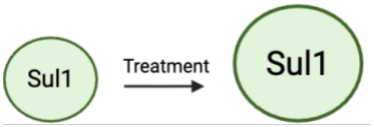
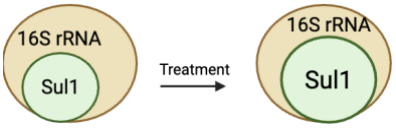
Time	Online SPE			HPLC		
	Flow	Gradient		Flow	Gradient	
min	Ml/min	%A	%B	Ml/min	%A	%B
0	1,0	100	0	0,8	100	0
5,6	1,0	100	0	0,8	100	0
0	1,0	100	0	0,8	80	20
5	1,0	100	0	0,8	90	10
8	0,5	0	100	0,8	90	10
8,2	1,0	0	100	0,8	0	100
8,5	1,0	0	100	0,8	60	40
17	1,0	0	100	0,8	60	40
19	1,0	100	0	0,8	5	95
25	1,0	100	0	0,8	80	20

Table S 6: LOQ and LOD values of pharmaceutical analysis.

Compound	LOQ	LOD
	ng/L	
Sulfamethoxazole	2.2	0.7
Trimethroprim	0.7	0.2
Carbamazepine	22.2	0.7
Metoprolol	10.1	3.1
Diclofenac	5.1	1.6
Citalopram	14.5	4.4
Ibuprofen	2.5	0.8
Hydrochlorothiazid	0.9	0.3
Bezafibrat	9.5	2.9
Venlafaxin	1.7	0.5

VII. Illustration of absolute and relative LRV interpretation

Table S 7: Illustrative explanation of absolute and removal mechanisms and how these relate to each other in the context of ARGs/16S rRNA.

Absolute removal of gene	Relative removal of gene
+ 	+ 
+ 	- 
- 	+ 
- 	- 

VIII. Physical-Chemical Parameter Results

Table S 8: Physical-Chemical parameters measured in the NGWRP on several sampling days.

Date	Treatment step	pH	Turbidity	Conductivity	Na+	K+	Mg^{2+}	Ca^{2+}	F^-	Cl^-	Br^-	NO_3^--N	PO_4-P	SO_4^-	TOC	TOC stdev
			(NTU)	(µS/cm)	(mg/l)										(ppmC)	
24/7/2025	Raw Mix	7,8	1,23	1227	195,44	30,65	15,02	43,59	0,28	120,79	NA	2,10	0,42	85,74		
	Raw Mix	7,8	1,23	1227	195,69	30,58	14,97	43,42	0,32	139,03	NA	2,44	0,46	99,49		
	Biological Activated Carbon	7,8	0,25	1380	218,51	30,70	14,89	43,63	0,32	193,94	NA	2,82	0,01	103,52		
	Biological Activated Carbon	7,8	0,25	1380	218,72	30,64	14,83	43,45	0,30	187,15	NA	2,79	NA	99,17		
	Final Product	7,7	0,12	1316	226,64	30,78	14,91	44,52	0,24	210,23	NA	3,08	0.2201	110,46		
	Final Product	7,7	0,12	1316	225,63	30,70	14,81	44,16	0,23	206,30	NA	2,99	0.0494	108,53	1,76	0,33
28/7/2025	Raw Mix	7,8	1,89	1026	192,72	31,28	14,92	43,81	0,28	133,57	0,04	2,58	0,41	82,57	5,53	0,26
	Raw Mix	7,8	1,89	1026	193,02	31,30	14,92	43,73	0,31	148,46	0,05	2,31	0,50	86,78	5,53	0,26
	Dual media filtration	7,8	0,40	1102	219,38	31,87	15,37	44,85	0,29	187,19	NA	2,68	0,04	91,74	2,75	0,34
	Dual media filtration	7,8	0,40	1102	219,20	31,76	15,34	44,68	0,28	185,70	NA	2,67	NA	86,24	2,75	0,34
	Final Product	7,7	0,21	649	267,12	32,18	14,39	39,99	0,20	190,17	NA	2,87	NA	88,88	1,93	0,24
	Final Product	7,7	0,21	649	266,41	32,09	14,39	39,78	0,20	190,59	NA	2,88	NA	89,94	1,93	0,24

29/7/2025	Dual media filtration	7,8		1137					0,39	243,56	0,06	3,43	NA	116,62	2,84	0,29
	Dual media filtration	7,8		1137					0,30	189,83	NA	2,65	NA	90,64	2,84	0,29
	Biological Activated Carbon	7,8							0,41	245,02	0,06	3,56	NA	117,84	2,35	0,26
	Biological Activated Carbon	7,8							0,32	197,87	NA	2,90	NA	94,42	2,35	0,26
31/7/2025	Raw Mix				206,53	31,17	15,60	45,02	0,37	184,15	0,08	3,25	0,44	126,88	5,65	0,15
	Dual Media Filtration				229,31	31,36	15,47	44,28	0,40	247,74	0,07	3,69	NA	131,13	2,95	0,25
	Biological Activated Carbon				225,24	30,90	15,26	43,92	0,39	240,48	0,07	3,64	NA	128,40	2,50	0,29
	Granular Activated Carbon				226,18	31,06	15,35	44,28	0,39	246,60	0,08	3,69	NA	129,91	1,66	0,30
	Final Product				245,49	31,55	15,67	44,69	0,38	250,04	NA	3,61	NA	126,48	1,56	0,16

IX. List of Figures

X. List of Tables