Assessing treatment technologies for reducing antibiotic resistant gene abundance and diversity in domestic wastewater treatment effluents

Panagiota Adamou

A thesis submitted to Newcastle University for the degree of Doctor of Philosophy within the Faculty of Science, Agriculture and Engineering

School of Engineering
Newcastle University
Newcastle upon Tyne
NE1 7RU

August 2020

Statement of declaration

I hereby certify that the work presented in this thesis is my original research work. Due reference is given to literature and any research collaborations where appropriate. No part of this thesis has been submitted previously for a degree at this or any other university.

Panagiota Adamou

Abstract

Antibiotic resistance is natural, but the wide use of antibiotics in anthropogenic activities has accelerated the rate of evolution and dissemination of antimicrobial resistance (AMR) strains around the world. One location of interest relative to AMR is domestic wastewater treatment plants (WWTPs). WWTPs are very effective at reducing the discharge of water contaminants and pathogens to the environment, and AMR levels are generally lower in places where wastewater is treated. However, understanding the relative value of different treatment options is less clear, which is central to this work. Here, four full-scale biological wastewater treatment technologies (trickling filter, granular activated sludge, activated sludge, and membrane bioreactor) were compared in their relative ability to reduce antibiotic resistance genes (ARGs) in treated effluents. Further, two advanced oxidation processes (AOPs; i.e., ozonation and hydrogen peroxide coupled with UV) and different combinations, and also a Fenton-like system using Fe-bearing clay minerals were assessed as additional steps to biological treatment. Data based on quantitative polymerase chain reaction (qPCR) and high-throughput qPCR showed that all biological options effectively reduced ARG abundance and diversity in final effluents, and enrichment of ARGs was not evident. The efficacy of each WWTP at reducing ARGs and overall bacterial loads was highly related to the treatment technology applied, with secondary treatment contributing > 0.73 to the total ARG removal in all WWTPs, while, membrane bioreactor was the most effective technology achieving up to a log removal of 6.1. Ozonation (3 g/m^3) as an additional step to an activated sludge system showed potential to reduce further abundances and diversities of ARGs by 1.25 log unit, achieving a total removal of 3.61. Using propidium monoazide (PMA)-qPCR to differentiate viable vs non-viable cell carriage of ARGs, we showed that ARGs were often carried in non-viable cells, which has implications to downstream gene exchange related to AMR spread. Finally, removal rates up to log 2.94 of target ARGs achieved by using iron-bearing clay minerals, which shows promise as an alternate to AOPs for ARG reduction in WWTPs. With a few AOP exceptions, all treatment technologies removed ARGs from domestic wastewater, although the membrane bioreactor was the most effective overall.

Keep Ithaka always in your mind.
Arriving there is what you're destined for.
But don't hurry the journey at all.
Better if it lasts for years,
so you're old by the time you reach the island,
wealthy with all you've gained on the way,
not expecting Ithaka to make you rich.
Ithaka gave you the marvelous journey.
Without her you wouldn't have set out.
...
Wise as you will have become, so full of experience,
you'll have understood by then what these Ithakas mean.

Constantine P. Cavafy

To all these people who never left my hand to this long journey to Ithaka.

Acknowledgments

I would sincerely like to thank my supervisors Professor David Graham and Dr. Anke Neumann for the opportunity they gave me to accomplish a PhD and all their support during my PhD. Working with them has always been a great experience that helped me broaden my knowledge and most importantly my way of thinking.

I would also like to thank the Engineering and Physical Sciences Research Council (EPSRC) and Thames Water (TW) for funding this work. I specifically would like to thank Dr. Eve Germain-Cripps and Dr. Ben Martin from TW for their guidance and help during my fieldwork; working with them has improved my understanding of the different demands and priorities of industrial research and given me the opportunity to incorporate this to an academic context. I also would like to thank all employees of TW for their help during my fieldwork; they made me feel really welcome.

I am grateful to Professor Simon Andrews from the University of Reading for providing his laboratories and assistance during my PhD, which allowed me to conduct my experimental work during my placement. He and his fantastic group of scientists made me feel really welcome during my placement away from Newcastle. I would also like to thank Dr. Yong-Guan Zhu and Dr. Jian Qiang Su from the Chinese Academy of Science for analysing my samples using high throughput quantitative polymerase chain reaction (HT-qPCR).

I would like to thank the Environmental Engineering group and my research group in Newcastle University for all their advice, support, chats and great summer barbeques! Special thanks to Marcos and Kishor for their lab guidance which helped me gain knowledge in critical techniques for my PhD tasks. Thanks also to Amelie who was always there for me and not only in the good moments!

But mostly, a huge thanks to Roberto for teaching me how to be a better scientist and person! Without his patience and constant support this PhD would not have been as complete as it is.

Lastly many thanks to my grandfather Panos, my parents Eleni and Kostas, my sister Efi and my cousin Xenia for their patience and unconditional support. I am really grateful that they never lost their faith in me.

Table of Content

List of Figures

List of Tables

List of Equations

Abbreviations

A^2O: Anaerobic-anoxic-aerobic
Al: Aluminium
AMR: Antimicrobial resistance
ANOVA: Analysis of variance
AO: Advanced oxidation
AOPs: Advanced oxidation processes
APHA: American Public Health Association
AR: Antibiotic resistance
ARB: Antibiotic resistant bacteria
ARDB: Antibiotic Resistance Genes Database
ARGs: Antibiotic resistance genes
AS: Activated sludge
COD: Chemical oxygen demand
Cq: Quantification cycles
Ct: Threshold cycle
CW: Constructed wetlands
DGGE: Denaturing gradient gel electrophoresis
DO: Dissolved oxygen
DOC: Dissolved organic carbon
DOM: Dissolved organic matter
EPA: Environmental Protection Agency
ESBL: Extended-spectrum β-lactamase
FCA: Fluoroquinolone/quinolone/florfenicol/chloramphenicol/ amphenicol
Fe: Iron
FISH: Fluorescence in situ hybridization
GAC: Granular activated carbon
GAS: Granular activated sludge
H_2O_2: Hydrogen peroxide
HCl: Hydrochloric acid
HGT: Horizontal gene transfer
HNO_3: Nitric acid
HRT: Hydraulic retention time
HT-qPCR: High-throughput quantitative polymerase chain reaction
LEDs: Light emitting diodes
LoD: Limit of detection
LoQ: Limits of quantification
LP: Low pressure
MBR: Membrane bioreactors
Mg: Magnesium
MGEs: Mobile genetic elements
MIC: Minimal inhibitory concentration
MLSB: Macrolide/lincosamide/streptogramin B

MRSA: Methicillin-resistant *S. aureus*
MRSE: Methicillin-resistant *S. epidermidis*
NAu-1: Nontronite-1
NCBI: National Center for Biotechnology Information
NGS: Next generation sequencing
NOM: Natural organic matter
$^{\bullet}$OH: Hydroxyl radicals
O_2: Oxygen
PBS: Phosphate-buffered saline
PCR: Polymerase chain reaction
PMA: Propidium monoazide
qPCR: Quantitative polymerase chain reaction
RAS: Return activated sludge
rNAu-1: Reduced NAu-1
ROS: Reactive oxidative species
rrndb: Ribosomal RNA Database
RT-qPCR: Reverse transcription PCR
RWW: Reclaimed wastewater
SBR: Sequential batch reactors
Si: Silicon
SRT: Sludge retention times
SS: Suspended solids
TF: Trickling filter
TiO_2: Titanium dioxide
TN: Total nitrogen
TOC: Total organic carbon
TON: Total oxidised nitrogen
TP: Total phosphorus
TSS: Total suspended solids
UV: Ultraviolet
UVT: UV transmittance
VBNC: Viable but not culturable bacteria
WHO: World Health Organization
WWTPs: Wastewater treatment plants

Chapter 1 Introduction

1.1 Overview and Context

Compounds with antibiotic properties are natural and have been produced by bacteria and fungi for millions of years (Dcosta *et al.*, 2011; Kahn, 2017). In 1928, Alexander Fleming discovered antibacterial properties from a mould of the genus *Penicillium* against bacteria causing hard-to-treat diseases (Tan and Tatsumura, 2015). Since first mass use during the World War II (Nesme *et al.*, 2014), antibiotics have been widely used in both medicine and agriculture to treat infections in humans, animals, and plants (Andersson and Hughes, 2014). However, the extensive use of antibiotics, as a result of human and other use due to releases of non-metabolised antibiotics into human and animal wastes, antibiotics and their impact have increased across nature.

The spread of antibiotics into natural environments has influenced ecosystem functions (Ding and He, 2010), human health, accumulation in water sources and edible crops (Kemper, 2008), but also greater selection and exposure to antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) (Berendonk *et al.*, 2015). ARB and ARGs have been detected in a wide range of environmental matrices, including lakes, rivers, sediments and soils, as well as wastewater samples (Martinez, 2009). Although the current knowledge on types and prevalence of antibiotic resistance phenotypes in the environment is not clear, ARGs, which drive antibiotic resistance, seem to be able to spread among bacteria and distribute from humans and animals to natural environments, food, and drinking water (Dodd, 2012; Berendonk *et al.*, 2015). Transfer of ARGs can be vertical (e.g., cell division) or horizontal (e.g., exchange of genetic material through conjugation or transduction, and transformation) (Dodd, 2012). As a result, the therapeutic potential against human and animal pathogens might be compromised by the dispersal of ARGs, which reflect resistance potential, across the environment (Kemper, 2008).

Antimicrobial resistance (AMR) has now been recognized by the World Health Organization (WHO) as a major public health problem, as a wide range of microorganisms (i.e. bacteria, viruses, parasites, and fungi) have increased their resistance to antimicrobial agents (WHO, 2018). In fact, the increasing emergence of antibiotic resistance in human, animal, and plant pathogens is a matter of concern for treating infectious diseases, because previously effective

medical procedures using antibiotics now are harder to treat (Martinez, 2009). This has resulted in prolonged and expensive treatments at hospitals, as well as elevated death rates (WHO, 2018).

AMR is a global concern and its spread widely across different environments imposes a great threat. Since ARB are discharged in human and animal wastes, wastewater treatment plants (WWTPs) can play an important role in mitigating their dissemination across the environment. In fact, high-income countries, with adequate waste management and sanitation, exhibit lower levels of antibiotic resistance rates compared to middle- and low-income countries with poor or absent sanitation (Collignon *et al.*, 2018). Therefore, improving personal hygiene and waste management worldwide, introducing where possible basic treatment processes, is considered critical (Graham *et al.*, 2018).

Several attempts have been made to provide insight into the relative role of current wastewater treatment technologies on mitigating AMR. Although WWTPs have significantly improved the quality of water discharged after treatment, inconsistent findings exist regarding ARB and ARG removal in final effluents. Several studies, for example, have suggested that biological treatment might possibly be an environment for the selection of resistance among bacteria since they are in abundance and continuously mixed with nutrients and antibiotics at sub-inhibitory concentrations (Davies *et al.*, 2006; Rizzo *et al.*, 2013b). Assessment of different treatment technologies, including activated sludge, anaerobic-anoxic-aerobic (A^2O) systems, biofilters, aerated lagoons, sequential batch reactors (SBR), and combination of these, some with additional treatment steps (e.g., UV, chlorination), showed variable removal rates, resulting in increases in relative and often absolute abundances of target ARB and ARGs after treatment (Al-Jassim *et al.*, 2015; Mao *et al.*, 2015; Naquin *et al.*, 2015; Rafraf *et al.*, 2016; Korzeniewska and Harnisz, 2018; Proia *et al.*, 2018). Therefore, according to these studies, dogma has suggested WWTPs are a principal pathway of ARB and related ARGs into the environment.

On the other hand, decreases in absolute and in some cases relative abundances of specific ARB and ARGs are more often reported from WWTPs using biological treatment (Chen and Zhang, 2013a; Laht *et al.*, 2014; Rodriguez-Mozaz *et al.*, 2015). Among treatment strategies constructed wetlands (CW) have shown good performance (Cardinal *et al.*, 2014; Chen *et al.*, 2016a; Fang *et al.*, 2017); while, membrane bioreactors (MBR) proved to be the most effective technology, achieving the highest removal rates, and decreasing abundances often below the detection limits (Du *et al.*, 2015; Zhang *et al.*, 2015a; Le *et al.*, 2018; Zhang *et al.*,

2018). It is also worth noting that studies based on resistomes, assessing a wide range of ARGs, in contrast to those focusing on few ARGs, showed that WWTPs decrease effectively both abundance and diversity (Yang *et al.*, 2014; Christgen *et al.*, 2015; Bengtsson-Palme *et al.*, 2016; Karkman *et al.*, 2016; Jiao *et al.*, 2017). Also, combination of resistomics and metagenomics revealed that ARB and ARG characteristics in both final effluent and downstream river were not similar to these of the return activated sludge of a WWTP (Quintela-Baluja *et al.*, 2019), challenging the wide view that WWTPs are reactors for propagation and dissemination of antibiotic resistance in natural environments (Rizzo *et al.*, 2013b; Krzeminski *et al.*, 2019).

Current evidence suggests that tertiary treatment contributes to further decreases in antibiotic resistance levels even up to 3 log removals of ARGs (Graham *et al.*, 2018). Although an additional tertiary treatment step can highly increase operating costs, it may be considered critical under some circumstances, such as reuse purposes or discharge into sensitive receiving environments (Christou *et al.*, 2017). Hence, considerable literature exists on conventional tertiary processes (e.g., chlorination, UV irradiation) or investigating new advanced technologies (e.g., ozonation, Fenton processes, hydrogen peroxide driven oxidation, and catalyst activation). Many studies have revealed the potential of a wide range of different treatment strategies to improve removal rates; however, several factors affecting treatment still need to be elucidated (Michael-Kordatou *et al.*, 2018).

Chlorine is widely used in WWTPs as it shows good performance on removing bacteria, including many pathogenic microorganisms (Sharma *et al.*, 2016; Barancheshme and Munir, 2018). Although chlorination is effective against ARB, ARGs are less readily reduced using conventional concentrations (Rizzo *et al.*, 2013a; Calero-Cáceres and Muniesa, 2016; Yoon *et al.*, 2017). This may be due to the fact that chlorine's principal mechanism relies on reaction with cell membrane and cell wall constituents (Dodd, 2012). Therefore, in order to deactivate genes, high contact times and non-practical doses are required (Zhuang *et al.*, 2015); while, regrowth of ARB after treatment has also been reported (Fiorentino *et al.*, 2015). UV irradiation, another widely applied treatment system, has also been tested as an alternative to chlorination, mainly due to its high potential on penetrating the membrane and reacting directly with the genetic material (Eischeid *et al.*, 2009; Dodd, 2012). Although UV irradiation reduce ARGs more effectively than chlorination, several factors can limit its performance increasing the need for higher doses which also implies higher costs (Rizzo *et*

al., 2013a; Guo *et al.*, 2015; Calero-Cáceres and Muniesa, 2016). Further, regrowth of bacteria through dark repair or photoreactivation also have been seen (Guo and Kong, 2019).

Advanced treatment systems, such as ozonation, hydrogen peroxide (H_2O_2)/UV, Fenton, and photocatalytic oxidation have been used less on controlling antibiotic resistance in wastewater. However, they seem to have value on ARB and ARG removal, as hydroxyl radicals ($^{\bullet}OH$), the main oxidant in these processes, reacts rapidly and non-selectively with most organic compounds including DNA and RNA fractions (Lee and von Gunten, 2010). Several studies have shown that advanced treatment options can be very effective against both ARB and ARGs, however, the effectiveness is often highly associated to doses, contact times, and wastewater matrix (Dunlop *et al.*, 2015; Moreira *et al.*, 2016; Zhang *et al.*, 2016; Sousa *et al.*, 2017; Yoon *et al.*, 2017). Moreover, advanced treatment can be very expensive and often requires elevated chemical doses and energy. In this regard, new natural antibacterial materials, such as clay minerals, are under investigation as alternative options to minimize the need for chemicals and energy (Williams *et al.*, 2011; Morrison *et al.*, 2016; Wang *et al.*, 2017; Venieri *et al.*, 2020).

The last two decades, the effectiveness of a variety of treatment technologies, both conventional and quaternary on reducing target ARB and ARGs have been assessed. However, few studies have monitored a broader spectrum of ARGs, which can provide a more comprehensive understanding on the performance of a treatment, monitoring not only abundances but also diversities of ARGs. Further, although considerable research has been devoted to examining ARGs between inlet and outlet of a WWTP, rather less attention has been paid to the contribution of each treatment step to the overall ARG removal in a WWTP. As a WWTP is a combination of treatment technologies, considering each unit can give important insight into the overall treatment. Similarly, advanced tertiary treatment, although exhibiting some potential, has been primarily evaluated at lab-scale; while, when tested in pilot- or full-scale, they have been examined as individual processes rather as part of the whole system. Therefore, optimization of advanced oxidation processes (AOPs) under real treatment conditions is an identified gap in the literature.

Finally, research to date has not yet assessed the physiological ('health') state of bacterial cells carrying ARGs in wastewater systems. Differentiating gene carriage in viable cells versus membrane-compromised cells can be key for understanding better the fate of ARGs during or after treatment and their contribution to resistance dissemination, nevertheless, very little is yet known. There also is an increasing interest on testing new natural materials as

cost- and energy-effective alternatives to typical tertiary advanced technologies. Clay minerals have shown good antibacterial potential against ARB, however, so far most of the studies have focused on skin infection applications and there are no data on studying its effectiveness on wastewater.

1.2 Thesis Summary

The thesis evaluates and compares a variety of treatment strategies for reducing ARG abundance and diversity in domestic wastewater treatment effluents. This work specifically aims to fill knowledge gaps, especially related to the fate of ARGs in conventional processes, and contribute to AMR mitigation solutions through wastewater management. For this reason, four wastewater treatment technologies, trickling filter (TF), granular activated sludge (GAS), activated sludge (AS), and a membrane bioreactor (MBR) were evaluated and compared at full-scale in their relative ability to decrease abundance and diversity from final effluents. Samples from primary, secondary, and tertiary (where applied) treatment were collected to evaluate the contribution of each step to the overall removal rates of the WWTP; while, the flow of a broad-spectrum of ARGs within treatment was monitored using resistomics. The 'health' state of bacteria carrying ARGs was also one of the main objectives of Chapter 3.

The work then proceeds to evaluate the added value of quaternary treatment on the overall performance of a WWTP (Chapter 4). Considering both a wide range of ARGs and the physiological state of bacteria, two advanced technologies, i.e., ozonation and H_2O_2/UV, and their combination, i.e., O_3/H_2O_2/UV were examined in pilot-scale. Optimization of the processes were evaluated by examining different experimental conditions; while, one of the principal goals was the additional benefit of advanced treatment to a conventional WWTP.

Additional lab-scale experiments were conducted with main objective to elucidate key factors affecting AOPs and examine alternate technologies intending to minimize cost, energy and use of chemicals. In this regard, Fe-bearing clay minerals were tested in combination with either H_2O_2 or in a reduced state to treat real wastewater (Chapter 5). Although an increasing body of the literature has shown the antibacterial potential of clay minerals, to the best of our knowledge, this is the first attempt to test its efficacy against ARGs in real wastewater. Therefore, nontronite-1 (NAu-1), which is an Fe rich clay mineral, was tested under various experimental conditions.

The UK in compliance with global initiatives (WHO, 2015a) is among the first countries to establish a national action plan on AMR (GOV.UK, 2019), highlighting that research and development of new treatments and technologies are key areas. Hence, evaluation of the effectiveness of existing wastewater technologies and/or development of new complementary alternative treatment options are considered essential. Therefore, the ultimate goal of this work is to determine best treatment options for reducing AMR releases from WWTPs, which will assist water companies in the future.

Chapter 2 Literature Review

2.1 Antibiotics: Occurrence and their Impact on Bacteria

"Antibiotics are medicines used to prevent and treat bacterial infections", as the World Health Organisation (WHO) states (WHO, 2018). The production of antibiotics by bacteria and fungi originated 2 billion to 40 million years ago, suggesting that antibiotics are natural (Dcosta *et al.*, 2011; Khan *et al.*, 2013). Alexander Fleming, a Scottish physician, accidentally discovered a mild antibacterial enzyme, the lysozyme, in 1922, which was the basis of his next great discovery (Tan and Tatsumura, 2015). In 1928, Fleming subsequently discovered a mould of the genus *Penicillium* was able to deactivate Gram-positive pathogens associated with difficult to treat diseases, such as pneumonia, diphtheria, meningitis, and gonorrhoea. For this discovery he was awarded the Nobel Prize in Physiology/Medicine in 1945.

Antibiotics were first used to treat bacterial infections in soldiers during World War II, and most production took place between 1940 and 1990 (Nesme and Simonet, 2015). Since being introduced to the market, they have been widely applied in both medicine and agriculture (Andersson and Hughes, 2014). It was after World War II that the first antibiotics were also used to promote growth and reduce mortality of animals in agriculture in the USA (Kahn, 2017). Antibiotics are classified as broad- or narrow-spectrum antibiotics; broad-spectrum antibiotics are effective against a wide range of infections, while, narrow-spectrum antibiotics are used to treat a limited number of infections both in human and veterinary treatment. Macrolides, β-lactams, sulfonamides, penicillin and tetracyclines are among the most widely used antibiotics (Hiller *et al.*, 2019). Last resort antibiotics (e.g. vancomycin, colistin, cefotaxime, ceftriaxone, ceftazidime) are less common and reserved for use when other antibiotics are not effective.

Andersson and Hughes (2014) stress that 20-80 % of antibiotics consumed by humans or animals are not metabolised and consequently pass into wastewater, sludge, and manure through urine and faeces. Hence, a fraction of residual antibiotics associated with human treatment, agriculture and aquaculture may end up in environmental ecosystems, such as soil, rivers and lakes, through the wastewater route, or via accidental releases by pharmaceutical plants. Consequently, bacteria in environmental compartments are exposed to antibiotic concentrations that are higher or lower than the minimal inhibitory concentration (MIC), which is the lowest concentration to inhibit visual growth of bacteria *in vitro*. They also point

out that lower than MIC (sub-MIC) concentrations allows growth of susceptible bacterial strains and can promote antibiotic resistance in bacteria (altering their susceptibility to antibiotics). Although more study is needed to clarify mechanistically the effect of low levels of antibiotics on inducing antibiotic resistance on bacteria, elevated mutagenesis and recombination between identical and near-identical intrachromosomal sequences have been suggested to accelerate microorganism evolution promoted by sub-MIC concentrations.

Antibiotic resistance spread among bacteria was traditionally associated to antibiotic usage in clinical environments; however, it is now known to be also connected to the wider environment (Berendonk *et al.*, 2015; Graham *et al.*, 2019). Both antibiotics and antibiotic resistance bacteria (ARB) can disseminate among different environments, in a cycle connecting hospitals and human activities to natural ecosystems (figure 2.1). Antibiotic residuals in environmental compartments both naturally produced, by bacteria and fungi, and released by human sources can equally select for ARB (Khan *et al.*, 2013). According to Hughes and Andersson (2012), given that bacterial exposures to sub-MIC concentrations cannot inactivate them, genes conferring resistance to antibiotics can be easily amplified and spread among bacteria. Further, mutations are more frequent in such environments, enhancing evolutive phenomena that increase selective pressure among bacteria. They also suggested that bacteria with elevated mutation rates are easily adapted and enriched in environments with sub-MIC concentrations, while, horizontal gene transfer (HGT) as well as recombination, and mutagenesis can also be affected by non-lethal antibiotic concentrations.

As Kahn (2017) observes, disagreements exist between medicine and agriculture about who is most responsible for antibiotic releases into the environment. In fact, levels of antibiotics often used as growth promoters in agriculture are normally at low concentrations, but food (e.g. meat or milk) that contain low levels of antibiotics can affect natural microbiota in human bodies selecting for antibiotic resistant strains (Andersson and Hughes, 2014). This, as WHO states, “leads to longer hospital stays, higher medical costs and increased mortality” (WHO, 2018).

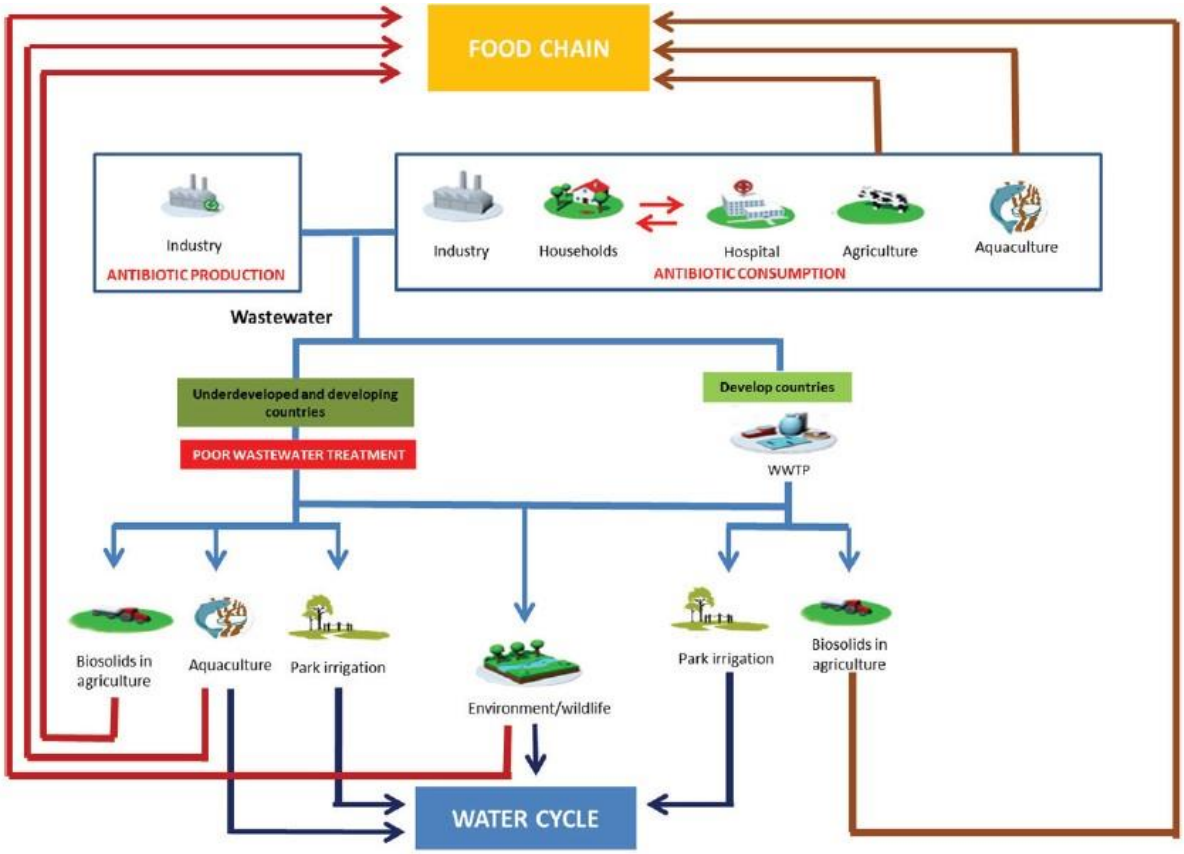

Figure 2.1 Antibiotics and antibiotic resistant bacteria cycle spreading through different pathways, adapted from Quintela-Baluja *et al.* (2015).

2.2 Antimicrobial Resistance Mechanisms and Dissemination

There are three major mechanisms that make bacteria resistant to antibiotics, including efflux pumps, antibiotic deactivation, and cellular protection, and they are summarised below as discussed in Walsh (2000).

Efflux pumps. Antibiotics need to penetrate cell membranes and walls, and be present in adequate concentrations inside the cytoplasm, where proteins are synthesised, to be effective against bacteria. However, as Walsh (2000) reports, staphylococci resistant to erythromycin, or other bacteria resistant to tetracycline (Levy, 1992), have developed efflux pumps resistance mechanisms, producing membrane proteins that can remove drugs from the cell. In this way, protein synthesis is not inhibited, as the ability to removing antibiotics prevents their accumulation inside the cell.

Antibiotic deactivation. Destroying the chemical structure of antibiotics is another important mechanism of resistance. For example, bacteria resistant to β-lactams (e.g., cephalosporins and penicillins) use a hydrolytic enzyme, called β-lactamase, to deactivate the β-lactam ring, destroying the functionality of the antibiotic. β-lactamases have been described to deactivate

100 million penicillin molecules inside cytoplasm each second (Philippon *et al.*, 1989). Similarly, aminoglycoside molecules are deactivated by enzymes that add chemical atoms in the periphery of the drug, inhibiting attachment to RNA targets; hence protein synthesis is not interrupted (Shaw *et al.*, 1993).

Cellular protection. Some ARB have developed mechanisms to protect themselves by cell reprogramming or disguising the antibiotic target. For instance, *S. aureus* resistant to erythromycin can activate an Erm mechanism, in which the enzyme Erm modifies the affinity of RNA to erythromycin (Bussiere *et al.*, 1998). Vancomycin-resistant enterococci have also evolved similar strategies, where resistant-specific enzymes modify the cell wall intermediates, reducing vancomycin's capacity to bind in the cell by 1,000-fold (Bugg *et al.*, 1991). Penicillin resistant strains, such as methicillin-resistant *S. aureus* (MRSA), have developed mutations on the proteins which binds to penicillin or have expressed new proteins with no affinity for the drug (Song *et al.*, 1987; Spratt, 1994).

Resistance to antibiotics can be inherent or the result of mutations; and can be transferred vertically, by cell replication, or may be acquired through HGT, including conjugation, transduction and natural transformation (Dodd, 2012).

Conjugation. Transfer by conjugation is very common. Specifically, the genetic information conferring resistance to antibiotics can be transferred between two viable bacterial strains through plasmids or other mobile genetic elements (MGEs). Plasmids are defined as molecules inside bacteria containing extrachromosomal DNA with the ability to auto-replicate, integrate in the chromosomal DNA, and move to a new bacterial cell through conjugation contributing to HGT (Sherratt, 1974). The donor cell needs to be in physical contact with the recipient cell for sufficient time in order to allow the transfer or exchange of genetic material (Salyers *et al.*, 1995; Sørensen *et al.*, 2005). It has been seen that conjugation is common in environments where the bacterial density and the antibiotic levels (e.g., gut) are high (Shoemaker *et al.*, 2001).

Transduction. Transfer of genetic material containing antibiotic resistance information also can occur through bacteriophages. Bacteriophages, which are viruses that infect and replicate inside bacteria, can disseminate ARGs by infecting various bacterial cells following cell lysis (Lorenz and Wackernagel, 1994). For example, bacteriophages that infect ARB can erroneously reproduce not only viral genome but also part of bacterial DNA (Miller, 2001). Consequently, bacteriophages carrying information that confer resistance to antibiotics, after inducing cell lysis, may proliferate inside the next infected bacterial cell. Hence, it is likely

that viral DNA, integrated into the bacterial genome during lysogenic growth, will be vertically spread during cell division.

Natural transformation. Some bacteria are suggested to have the ability to receive and integrate in their genome extracellular plasmids or chromosomal DNA derived from lysed cells (Lorenz and Wackernagel, 1994). Although not all bacteria can take up DNA from the exterior environment, some strains, including meningococci and streptococci, have been found to be able to acquire antibiotic resistance through natural transformation.

2.3 Analytical Methods for the Detection and Quantification of ARB and ARGs

Collecting and preserving samples in a reliable manner is one of the first steps for a robust analysis. As Ju and Zhang (2015) suggest, statistical accuracy depends on numbers of biological and technical replicates. Biological replicates refer to number of samples collected per experimental condition and should be high enough (at least three) to account for variation inside the group, making comparisons among different experimental conditions more accurate. Technical replicates refer to the number of replicates evaluated per sample which is a measure of reproducibility of the data produced under a specific analysis.

After collection, preservation of samples (to maintain the functionality and structure of the microbial community) is crucial and depends on the type of sample (e.g. sludge, wastewater, biofilms). Storage at -80 ^{o}C or -20 ^{o}C is typical; while, fixing of samples in 50% ethanol before storage also is used (Ju and Zhang, 2015). Collection of the bacterial fraction is achieved with centrifugation or membrane filtration depending on sample characteristics. For membrane filtration, 0.2 or 0.45 μm pore size filters are commonly used to capture bacteria (EPA, 2002). It is noteworthy that although 0.2 μm filters can capture all known bacteria types, as bacterial sizes range between 0.2 – 2.0 μm (Young, 2006), many studies claim to also capture free DNA using membrane filtration without further processing (Ferro *et al.*, 2016; Yoon *et al.*, 2017; Wang *et al.*, 2020), which leads to misinterpretation of results. Antibiotic resistance in environmental samples are mainly assessed using culture- or molecular-based methods. Methodological approaches and basic principles are summarised in Rizzo *et al.* (2013b).

2.3.1 *Culture-based techniques*

Culture-based methods, commonly used to identify clinically related strains, also have been used to monitor bacterial strains in environmental samples. Guidelines for these methods are

provided by standard procedures (EUCAST, 2016; CLSI, 2017). Selective culture media allow the isolation and quantification of bacteria, such as enterococci and coliforms, which are common indicators for water quality. Disc diffusion and micro-dilution are among techniques often used to distinguish between susceptible and resistant to antibiotics bacterial strains by using selective culture media containing antibiotics in MIC levels. Enrichment of bacteria with resistance genetic elements to identify the resistance potential among strains can be achieved by adapting standard methods (Novo and Manaia, 2010).

Culture-based methods have both advantages and disadvantages (Bouki *et al.*, 2013; Rizzo *et al.*, 2013b; Graham *et al.*, 2019; Hiller *et al.*, 2019). For instance, they are cost-effective, robust and easy to analyse, producing reliable data that can be compared among different laboratories, as universal standard methods exist. They are also very useful at determining multidrug resistance mechanisms. Although these methods are used as a direct indicator of viable bacteria in a sample, injured or viable but not culturable bacteria (VBNC) cannot be identified. Moreover, they are laborious and time-consuming methods. As these approaches were primarily developed for clinical and veterinary use, targeting specific microorganisms, one major drawback relies on their ineffectiveness in detecting environmental bacteria or viruses that have been shown to play a key role in antimicrobial resistance dissemination.

2.3.2 *Molecular-based techniques for identification and quantification of genes*

Molecular-based methods are used for both quantifying and identifying ARB and ARGs. These methods require extraction of DNA from the bacterial community, where polymerase chain reactions (PCR) can be applied to identify the target gene, and quantitative PCR (qPCR) can be used to quantify specific genes in the sample. For molecular assessments, the quality of the extracted DNA is essential. Molecular methods are very sensitive; therefore, the matrix of environmental samples, which contain humic substances, detergents and salts can inhibit the analysis resulting in poor performance (Gallup and Ackermann, 2006; Lloyd *et al.*, 2010). Thus, extraction methods and purification techniques are important.

Although qPCR is a quantitative technique with fairly standard protocols (Green and Sambrook, 2012), exact methods vary among research groups (Michael-Kordatou *et al.*, 2018). Some of the advantages of qPCR, as summarised in the reviews by Rizzo *et al.* (2013b) and Hiller *et al.* (2019), are high performance in gene specificity and fast identification. In contrast to culture-based methods, qPCR is sensitive and can detect ARGs in whole microbial communities, as well as in plasmids; while, MGEs, such as integrons and

transposons, also can be monitored. Therefore, identification and quantification of microorganisms that are not cultivable or which grow slowly is possible. Using adequate extraction techniques, 'free' DNA or DNA present in bacteriophages can be identified and quantified. The fact that there is no need for cultivation of bacterial strains prior to using this analysis has extended its use to samples from different environmental sources, such as rivers, lakes, drinking water and wastewater.

qPCR is mainly useful for monitoring ARG levels and antibiotic resistance (AR) potential. Plasmids, bacteriophages, transposons, and integrons can facilitate the spread of ARGs among bacterial cells and qPCR can identify the presence of such elements. Transposons are determined as genetic sequences that can move inside a genome, modifying both size and characteristics, leading often to mutations (Salyers *et al.*, 1995). In bacteria, transposons carrying ARGs can be exchanged between chromosomal and plasmid DNA, contributing to ARG dissemination often through conjugation. However, integrons are the most studied MGEs across literature. They are genetic structures that can incorporate sequences, including ARGs (Mazel, 2006), that can only be easily identified by molecular-based techniques, such as qPCR. Integrons can be immobile inside genomes, and/or located in mobile elements, such as transposons, insertion sequences, and conjugative plasmids; therefore, they are classified as superintegrons or mobile integrons, respectively. Five mobile integrons have been identified, with class I (*int*1) being the most studied, as this group is extensively found in clinical isolates and in gene cassettes conferring resistance to different antibiotic classes, such as aminoglycosides, β-lactams, chloramphenicol, erythromycin and others (Mazel, 2006). Detection of integrons inside a cell or community does not mean *de facto* expression of ARGs, but their presence suggests possible ARG transmission.

Some drawbacks associated with qPCR have also been reported in relevant review papers (Bouki *et al.*, 2013; Rizzo *et al.*, 2013b; Michael-Kordatou *et al.*, 2018; Graham *et al.*, 2019; Hiller *et al.*, 2019). For example, genes are detected in both viable and non-viable cells, therefore the physiological ('health') state of the microorganism carrying the genes cannot be differentiated using conventional qPCR. Similarly, although target genes in the whole microbial community can be monitored, host bacteria cannot be easily identified. Further, the accuracy of this method strongly depends on extracted DNA quality, influencing the sensitivity of the method. The use of this technique often can be limited due to high reagent and enzyme costs, as well as heterogeneity in protocols used among laboratories, also mentioned before. The need for a large amount of DNA can be problematic, as it increases the time and complexity of sample collection and preparation. Moreover, conventional qPCR

only targets one specific gene per assessment, limiting the capacity of this method to analyse a wider spectrum of genes which is essential for the robust evaluation of a study case.

qPCR data interpretation issues and bias are other limitations of this method. Reporting qPCR data is not coherent among literature. For instance, the fact that qPCR protocols are not standardized among laboratories and different primer sets are used makes comparisons among studies difficult (Michael-Kordatou *et al.*, 2018). Further, as Hiller *et al.* (2019) identifies, there are differences in reporting qPCR data. There is a major inconsistency on the presentation of relative abundances, which have been presented either as absolute abundances normalised to 16S rRNA ('all' bacteria) or to total amount of DNA. This can make comparisons among different studies challenging and implies errors on the interpretation of data when not correctly defined. For example, 16S rRNA copies per cell are different among bacterial types, ranging from 1 to 15; therefore, 4.1 as an estimation of the average number of 16S rRNA that encodes genes per bacterial genome, as reported in the Ribosomal RNA Database, may be considered when determining relative abundances (Klappenbach *et al.*, 2001).

2.3.3 *Complementary qPCR-associated techniques*

qPCR is effective at quantifying target genes carried in bacterial cells, however it cannot distinguish between genes carried in cells with integrated (viable) or broken membranes (presumably non-viable). DNA sample treatment with propidium monoazide (PMA) has been described as an appropriate method to quantify genes carried only in viable cells. This dye passes through fragmented bacteria cell membranes and binds to DNA, inhibiting the amplification of DNA during qPCR (Nocker *et al.*, 2007).

Using PMA-qPCR assessments have proved to be useful when processing environmental samples, therefore many researchers have focused on optimising this method. For example, validation techniques have been based on statistical comparisons between qPCR and PMA-qPCR absolute concentrations of samples spiked with bacteria previously exposed to thermal inactivation (Li *et al.*, 2014; Truchado *et al.*, 2016; Eramo *et al.*, 2019). In other studies, culture-based techniques were compared to both qPCR and PMA-qPCR methods, showing that bacteria can often be in viable but not cultivable state (Li *et al.*, 2014; Lin *et al.*, 2016; Truchado *et al.*, 2016; Tombini Decol *et al.*, 2019); while, other validation methods consisted of using either an exogenous control (Sketa DNA) to confirm reduction of known concentrations after PMA treatment (Kibbee and Örmeci, 2017), or comparing PMA-qPCR

against reverse transcription PCR (RT-qPCR), which is a method used to quantify active cells (Lin *et al.*, 2016).

There are other methods to quantify viable cells, however high decay rates, resulting in cell viability loss have been reported in RNA-based approaches. Whereas, other techniques, such as flow cytometry, have limitations based on cell clumping and non-specific gene identification of viable cells (Nocker *et al.*, 2007). Although factors, such as presence of suspended solids and humic acids can limit the effectiveness of PMA (Nocker *et al.*, 2007; Bae and Wuertz, 2009; Li *et al.*, 2014; Kibbee and Örmeci, 2017), recent studies have shown that qPCR in combination with PMA pre-treatment works well in environmental samples and can be performed quickly (Lin *et al.*, 2016; Pang *et al.*, 2016).

qPCR is effective at quantifying only one specific gene per assessment; however, in order to have better understanding on AMR spread, quantifying a wider range of ARGs is better because one often does not know key genes *a priori*. To overcome this limitation, high-throughput qPCR (HT-qPCR), with the capacity to simultaneously quantify hundreds ARGs has been developed. This method uses multiple fluorogenic probes to differentiate different amplicons within a sample (Arya *et al.*, 2005). The technique has been very advantageous, decreasing reagent costs and preserving samples (Arya *et al.*, 2005), and has been used successfully in monitoring a broad-spectrum of genes in arctic soil (McCann *et al.*, 2019), rivers (Zhou *et al.*, 2017), Chinese swine farms (Zhu *et al.*, 2013) and wastewater treatment plants (WWTPs) (Christgen *et al.*, 2015; Bengtsson-Palme *et al.*, 2016; Karkman *et al.*, 2016; Quintela-Baluja *et al.*, 2019). On the other hand, limitation of available fluorophores and application in qPCR machines of a monochromatic light source can reduce the efficacy of this analysis and increase detection limits (Arya *et al.*, 2005).

2.3.4 *Complementary molecular-based techniques*

Denaturing gradient gel electrophoresis (DGGE), fluorescence in situ hybridization (FISH) and next generation sequencing (NGS) are useful molecular tools. All of them are applied to identify microbial communities from various environments, including wastewater (Ju and Zhang, 2015). NGS is a powerful tool because due to cost decreases, metagenomics (identification of the microbial community based on DNA from environmental samples) has enriched our knowledge on microbial diversity in several environments, both engineered and natural (van Dijk, 2014). Examples of NGS platforms include Ion Torrent, SOLiD, 454, and Illumina; the latter, due to its high performance and cost-effectiveness, leads nowadays.

Metagenomics is very useful to explore new bacterial units originated from environmental samples, and when combined with metatranscriptomes (gene expression of microorganisms) can reveal important information about active bacteria including their gene expression patterns (Yu and Zhang, 2012). On the other hand, metagenomics is still an expensive method and high expertise in bioinformatics is required (Hiller *et al.*, 2019). Further, metagenomics cannot give any information regarding functional ARGs, or whether they are expressed in bacteria; even if detected, they cannot be associated to specific bacteria types, and no information is revealed whether ARGs are found in 'viable' cells, or if they form part of the intra-chromosomal or extra-chromosomal genome (Graham *et al.*, 2019).

Although a variety of useful tools exist to assess environmental samples, a systematic approach on analysing such samples there has not been determined yet. Each technique has advantages and disadvantages, and provides a unique piece of information, as Graham *et al.* (2019) comment; therefore, culturing, qPCR/HT-qPCR, and sequencing, should be ideally combined in order to provide a better understanding of complex microbial systems.

2.4 Occurrence and Fate of Anthropogenically Derived AMR

Systematic effort is needed to monitor AMR across environment. ARB and ARGs have been found in high concentrations in several environmental samples, including hospital and domestic wastewater. For example, high abundances of carbapenem-resistant Enterobacteriaceae and other clinically important antibiotic resistant strains were detected in hospitals in India (Lamba *et al.*, 2017). Although one would expect higher concentrations of ARB in clinical waste, bacterial load in domestic wastewater was found to be higher in some cases (Graham *et al.*, 2019). In fact, several studies have reported high presence of ARB and ARGs not only in hospital raw wastewater but also in domestic sewage, and both influent and effluents of municipal WWTPs (Sigala and Unc, 2012; Li *et al.*, 2015).

Although WWTPs decrease bacterial loads, including ARB, between 10 and 1000 times or more, ARB are not completely removed (Hong *et al.*, 2018). Municipal WWTPs, for example, have been erroneously described as hotspots for the dissemination of ARGs, mainly due to abundancy of microbes in a matrix that nutrient levels, antibiotic residuals, and ARB are high (Dröge *et al.*, 2000; Schlüter *et al.*, 2008; Rizzo *et al.*, 2013b); whereas recent data imply this may not be true (Quintela-Baluja *et al.*, 2019). Regardless, WWTPs are pathway of ARB and ARG into environment, although their relative contribution is not fully known (Graham *et al.*, 2019).

The impact of wastewater discharges and other human activities (e.g. agriculture) on surface waters and their ARB/bacterial communities has been the focus of many studies. For example, Proia *et al.* (2016) studied biofilm samples, collected from upstream and downstream rivers from four WWTPs, and they showed that WWTPs discharges affected both physicochemical and biological parameters of the receiving water, contributing to elevated ARG relative concentrations (gene copies/16S rRNA) as far as 1 km downstream of a WWTP. Similar findings were observed on sediments collected from both pristine and human-impacted locations in a river in Colorado (USA) (Pei *et al.*, 2006). According to this study, both ARG relative concentrations (gene copies/16S rRNA) and ARB abundances were higher in streams close to urban and agricultural areas than in pristine areas.

Other studies, targeting a broad-spectrum of ARGs using HT-qPCR, also have shown a similar trend. Quintela-Baluja *et al.* (2019) showed that ARB not treated or retained within WWTPs pass into the liquid phase of the treated water and subsequently end up in receiving waters; while, ARG diversity and abundances in the sources of the Jiulongjiang River in China were lower compared to sampling locations in the centre of the town (Ouyang *et al.*, 2015). Combined sewer overflows can contribute elevated ARG loads to rivers, as absolute concentrations of specific genes were higher in such effluents compared to treated effluents (Alexander *et al.*, 2015). While, transport of tetracycline-resistance genes was also seen in a river near Vancouver (Keen *et al.*, 2018). This study showed that seasonality affected ARG abundances and transport rate, with both exhibiting higher values during wet than dry season, possibly due to elevated presence of particulate matter in the water body as result of increased rainfalls following an agricultural intensive period. Dairy and poultry industries' activities as well as intensive manure application in agricultural fields along the river may have influenced seasonality in ARG presence.

Reusing treated wastewater for agricultural purposes, or using biosolids or manure as fertilizers are common practices worldwide; however, they can be a direct source of ARB into environments, affecting plant surfaces with an indirect impact on humans and livestock through consumption (Kahn, 2017; Graham *et al.*, 2019; Hiller *et al.*, 2019). Application of treated sludge from WWTPs and manure in agriculture, can have a direct effect on crops and soil, as they often contain ARB in high concentrations (Davis *et al.*, 2011; Yang *et al.*, 2014). Further, persistency of ARGs in soil after manure use has also been seen for up to a year (Luby *et al.*, 2016) or extended periods of time. For example, persistency of ARGs in soil between 1940 and 2010, as a human impact result, was shown by a study of Knapp *et al.* (2010), where increases in abundances of 18 ARGs in soil samples were observed throughout

these years with tetracycline resistant genes being 15 times higher in 2008 than in 1970; while, Graham *et al.* (2016) showed that changes on the use of antibiotics in agriculture are also reflected in the presence of ARGs in the soil throughout years. Despite the evidences that ARGs persist in soil, it is not clear yet what impact this may have on human health (Graham *et al.*, 2019), and further investigation is needed.

Faeces produced by both humans and animals are an important source of ARB into environment, especially in places where open defecation is practiced due to poor sanitation systems (Graham *et al.*, 2019). Many people in Sub-Saharan Africa and South Asia practice open defecation (UNICEF, 2017) and fecal matter releases are estimated to increase over 4.6 trillion kilograms annually by 2030 (Berendes *et al.*, 2018). As Graham *et al.* (2019) observe, the risk of enteric-related ARB and ARG dissemination increases in places with poor or absent sanitation systems, as people are close to contaminated faeces. Studies have shown elevated concentrations of ARGs in samples from Ganges River during pilgrimages compared to other seasons (Ahammad *et al.*, 2014). Similarly, comparisons between locations along the Ganges River with and without latrines showed that carbapenem-resistant Enterobacteriaceae were 5 orders of magnitude lower in the former (Graham *et al.*, 2018).

Other transmission routes of AMR include companion animals and their close relationship to humans (Pomba *et al.*, 2017). Wildlife also has seen to affect AMR spread globally (Arnold *et al.*, 2016); birds, for example, seem to be able to transfer ARB among different locations. Interestingly, clinically related ARGs, such as $bla_{\text{NDM-1}}$, were found in Arctic soil in places where birds, fox and reindeer live, indicating a possible route via wildlife movements (McCann *et al.*, 2019). Human international travel is also suggested to affect antibiotic resistance spread, however, the mechanisms of spread through that route are not yet clear (Kennedy and Collignon, 2010; Graham *et al.*, 2014; Petersen *et al.*, 2015).

2.5 Conventional Biological Wastewater Treatment

WWTPs, when correctly operated, play an important role in reducing spread of ARB and ARGs in receiving environments. Since AMR spread is closely associated to human and animal wastes, improvements in sanitation and hygiene levels is essential (Kahn, 2017; Graham *et al.*, 2018). Even if developed countries have effective management of wastes, 73% of the world has no access to waste treatment (Graham *et al.*, 2019). Antibiotic resistance rates are elevated in low- and middle-income countries relative to high-income countries; possibly due to poor personal hygiene, high numbers of people leaving in the same settlement,

and inadequate water system and sanitation (Collignon *et al.*, 2018). Wastewater treatment is expensive, and cost depends on several parameters such as technology, population to serve, climatic and geographical factors, and water use (Graham *et al.*, 2018). Secondary treatment is 2-3 times more costly than primary (Tchobanoglous *et al.*, 2003), while tertiary or quaternary options are three times more expensive than secondary (figure 2.2), and they are mostly applied for water reuse purposes, or when the treated water is discharged to sensitive environments (EPA, 1983). Although the overall cost of a WWTP closely depends on treatment technologies, constructing a sewer is the most expensive part of a complete sanitation system, being at least five to six times more costly than a secondary WWTP itself (Tchobanoglous *et al.*, 2003; Eggimann *et al.*, 2015).

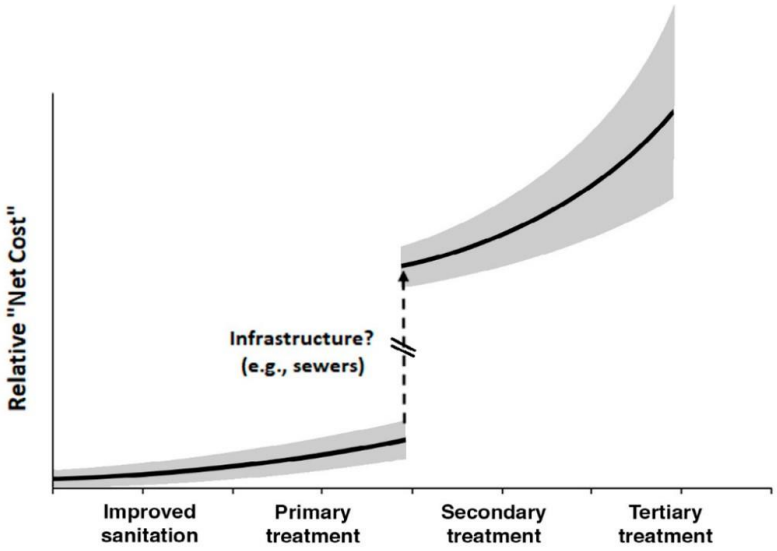

Figure 2.2 Estimated increases in relative costs per treatment step associated with controlling AMR pressure on environment adapted from Graham *et al.* (2018).

Graham *et al.* (2018) observed that passing from open defecation to a basic sanitation system by physically separating faeces from people and water can be a first step for antibiotic resistance improvement. Moving from basic sanitation to primary treatment will increase the overall cost but it will provide reductions in antibiotic resistance pressure, while the addition of secondary treatment will result in reductions of ARG concentrations and infection rates, as well as water quality improvements (figure 2.3). Although, secondary treatment can elevate the overall cost, cheaper solutions exist, as for example, the combination of septic tanks or condominium sewers with decentralized secondary treatment. Tertiary treatment will improve antibiotic resistance levels in final effluents, by up to 3 log removals of ARG, however, the cost will sharply increase, and its additional value may be much lower compared to previous

treatment steps and raw sewage. Therefore, tertiary treatment is considered essential mainly in places with water scarcity.

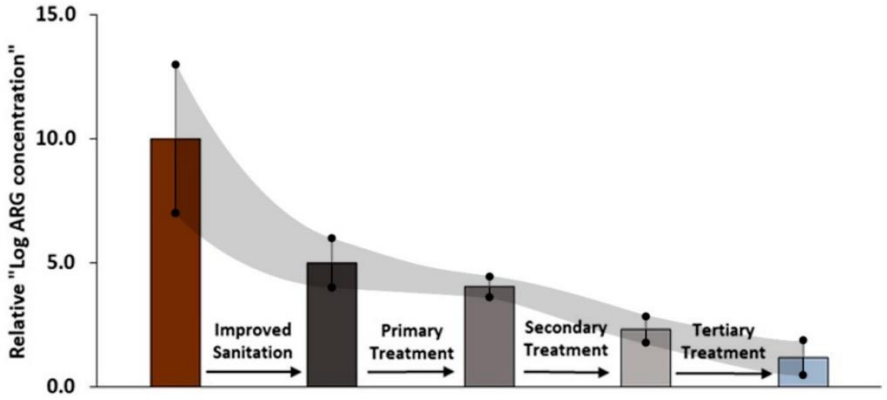

Figure 2.3 Decreases in relative ARG concentrations as a result of wastewater treatment step adapted from Graham *et al.* (2018).

A typical WWTP, in Europe and other places worldwide, consist of a primary step, with primary clarifiers to separate heavy solids (e.g., grit, sand) from fat or grease; secondary treatment, usually activated sludge or modifications for removing organic and suspended solids; and optional tertiary treatment, including a variety of biological, chemical or mechanical technologies (e.g. biofilters, chlorination, advanced processes) (EEC, 1991). There is a large volume of published studies on WWTPs, worldwide, dealing with ARB and ARGs removal from wastewater. The main objectives of these studies concern identification and removal efficiencies of ARB and ARGs in wastewater; factors affecting the treatment performance; and the fate of ARB and ARGs in receiving environments (e.g. rivers, sea, crops) (Xue *et al.*, 2019). Although there are several investigations into a variety of wastewater treatment technologies, inconsistent findings have been reported. Herein, representative examples of studies dealing with a wide range of conventional treatment technologies worldwide are reviewed.

2.5.1 *Activated sludge WWTPs*

Several biological treatment technologies are available, including activated sludge (AS), membrane bioreactors (MBR), and constructed wetlands. Among them, activated sludge is one of the most used, with different configurations (e.g. aerobic, anaerobic, anaerobic/anoxic/aerobic) being effective at treating wastewater (Xue *et al.*, 2019); as aerobes (oxygen-using bateria) degrade organic contaminants producing biomass, water and carbon dioxide, while anaerobes, with no need for oxygen, can produce carbon dioxide gas and

methane (Tchobanoglous *et al.*, 2003). Biological processes are generally effective at reducing pathogens and organic contaminants.

Some studies have suggested that WWTPs facilitate the propagation of ARB and the spreading of ARGs among bacteria within treatment and concluded that WWTPs may be contributors to AMR in receiving environments (Al-Jassim *et al.*, 2015; Mao *et al.*, 2015; Naquin *et al.*, 2015; Rafraf *et al.*, 2016; Korzeniewska and Harnisz, 2018; Proia *et al.*, 2018). However, more studies argue that WWTPs are very effective barrier to control ARB and ARG releases into the environment (Chen and Zhang, 2013b; Laht *et al.*, 2014; Rodriguez-Mozaz *et al.*, 2015; Zhang *et al.*, 2015a; Le *et al.*, 2018). Twelve wastewater treatment technologies, including anaerobic digesters in combination with either constructed wetlands or other technologies, anaerobic biofilters, oxidation ditches and anaerobic/anoxic/aerobic (A^2O) systems were assessed both in rural and urban locations in China (Chen and Zhang, 2013b). Comparison among technologies showed that urban WWTPs were more effective at reducing absolute concentrations of the target ARG compared to WWTPs in rural areas; while, among rural WWTPs, constructed wetlands improved ARG removals. Although ARG loads released into environment decreased significantly in all treatment systems, differences in relative abundances (ARGs copies/16S rRNA copies) were seen among WWTPs, and strong correlations were found between ARGs and the receiving capacity of the treatment plant, as well as ARGs with the 16S rRNA and the *int*1.

Studies in Europe also support the view that WWTPs can effectively reduce ARG loads in final effluents. For example, monitoring of influents and effluents of three AS followed by bio-filters in Finland and Estonia (Laht *et al.*, 2014) revealed that among all target ARGs, only *tet*M, *sul*1, and *sul*2 were always present in the samples, and absolute abundances decreased significantly after treatment. Even though complete removal of target ARGs were not achieved, enrichment was not evident after treatment and a weak correlation between relative abundances and physicochemical parameters suggest a possible relationship between performance and antibiotic resistance. Effective ARG absolute and relative abundance reduction in treated effluent were also achieved in a WWTP in Spain, receiving domestic and untreated wastewater from a hospital (Rodriguez-Mozaz *et al.*, 2015). In this research, although ARG abundances were reduced, different removals for each gene, and increased relative abundances of some ARGs after treatment were observed implying gene specificity; while, elevated concentrations of ARG and antibiotics were also found downstream of the WWTP.

2.5.2 *Membrane bioreactor WWTPs and other treatment technologies*

Among all treatment technologies, MBRs appear particularly effective at reducing ARGs from the liquid phase of wastewater. Three WWTPs in China, one operating with AS followed by a secondary clarifier, and the other two by MBR were assessed and compared revealing that all WWTPs were effective against total heterotrophs and coliforms as well as multidrug resistant strains (Zhang *et al.*, 2015a), with the highest removal rates in an MBR system. From all identified genera, only six persisted in the effluent of one WWTP, and among all target ARGs, only *bla*$_{\text{CTX-M}}$ and *sul*A were more frequent in effluent than influent. Likewise, Le *et al.* (2018) assessing an AS and an AS followed by MBR in Southeast Asia, reported that antibiotic levels were reduced by 70 % in both treatment systems. ARB concentrations were lower by 2 to 3 orders of magnitude in the AS secondary effluent, and no ARB were present in the MBR permeate. Similar to previous study, higher ARG removals were observed in the MBR (7.1 ARG log) than the AS system (4.2 ARG log).

Du *et al.* (2015), monitoring ARGs throughout a year in a WWTP with A^2O compartments followed by an MBR in China, showed that seasonality did not affect the treatment process, and that ARG levels were reduced significantly in MBR effluents. They also observed positive correlations between ARGs and 16S rRNA, as well as ARGs and physicochemical parameters. However, they postulated a possible risk from the sludge, as both absolute and relative abundances increased progressively from the anaerobic and anoxic tanks to aerobic compartment and MBR sludge, similar trends are also seen elsewhere (Zhang *et al.*, 2015a). On the other hand, a recent study from China found that MBR were less effective than an anoxic/anaerobic/aerobic process (inversed A^2O) (Zhang *et al.*, 2018). Three different configurations of a WWTP, including an inversed A^2O, a traditional A^2O, and an A^2O followed by an MBR were tested for their efficacy against ARGs; while, additional lab experiments examined an A^2O-MBR system with different sludge retention times (SRT). Their results suggested that the inversed A^2O exhibited the best performance at reducing ARGs and was the only one to prevent ARG relative abundances increases in the effluent. They also showed correlations between specific bacterial species and ARGs, indicating potential microbial hosts; while, longer SRTs resulted in higher decreases in ARG abundances in the effluent, at the cost of increasing relative abundances in the effluent, and both relative and absolute abundances in the sludge.

During the past decades, much attention has been drawn in constructed wetlands (CW), as they have shown good performance on reducing nutrients and organic contaminants from

wastewater, in a simple and cost-effective way (Sharma *et al.*, 2016; Barancheshme and Munir, 2018; Xue *et al.*, 2019). To better understand the effect of CWs on controlling AMR in natural ecosystems, recent studies have been conducted in full- and mesocosm-scale projects. Six different configurations of CWs in a mesocosm were investigated, quantifying levels of physicochemical parameters, eight antibiotics and twelve ARGs in wastewater (Chen *et al.*, 2016a). All configurations were effective at reducing both ARGs and antibiotics, and they improved the quality of the treated wastewater, with aqueous removal efficiencies ranging between 75.8 and 98.6 %. Among all CWs, the horizontal subsurface flow and the vertical subsurface flow constructed wetlands had the best performance, and removals were improved by the presence of plants. Adsorption in the substrate, plant uptake, and biodegradation were the main antibiotics and ARGs reduction mechanisms. Similar findings were also reported by Cardinal *et al.* (2014), where ARGs were effectively removed from the liquid phase, here subsurface flow was discussed to play a more important role than macrophytes at reducing ARGs.

ARG removals of 77.8 % and 59.5 % were achieved in dry and wet season, respectively, in a full-scale CW operated over ten years in China (Fang *et al.*, 2017), showing that treatment was affected by seasonality, with both absolute and relative concentrations being higher during the wet season. Meanwhile, ARG abundances in the CW sediment were always higher than these in the liquid phase. The authors concluded that CWs are effective at reducing ARGs in wastewater, however, data suggested enrichment of the sediment and positive correlations between *int*1 and some ARGs. Overall, previous studies indicated that CWs are effective on reducing ARG loads and they are in agreement with Chen and Zhang (2013a) who showed that CWs improved ARG removal in rural areas.

Controlling AMR in sewage has been the focus of lab-scale projects testing granular activated sludge (GAS). For example, Zou *et al.* (2016) tested the effectiveness of a granular sludge system against ARG dissemination through conjugation. Sludge was classified in four groups according to the size, from small flocculent sludge to bigger granules, and PCR-DGGE was performed to identify dominant species. Results revealed that plasmids invaded flocculent sludge and small aggregates more easily than granules of bigger size, while showing a lower transfer capacity in granular sludge compared to activated sludge. Although granular sludge systems are effective against antibiotic resistance by decreasing ARG transmission, the presence of antibiotics may affect the treatment process. The effect of sulfadiazine in four sequencing batch reactors (SBRs) with granular activated sludge was examined, and data

revealed that *sul*1 and *sul*2 levels in the sludge increased, and the microbial community structure changed, suggesting that sulfadiazine imposed selective pressure on dominant species (Wan *et al.*, 2018). In a similar study, SBRs systems were tested after the addition of tetracycline (Zhang *et al.*, 2019a), where tetracycline changed the structure of the microbial community in both sludge and effluent and had a negative impact on increasing ARG levels. For instance, presence of tetracycline resulted in increases in absolute and relative abundances of *tet*A, *sul*2, and $bla_{\text{TEM-1}}$; while, a positive correlation between *tet*A, and both *sul*2 and $bla_{\text{TEM-1}}$ was shown indicating a possible co-selection.

2.5.3 *WWTPs studies based on resistomes*

As defined by Wright (2007), resistomes include a wide range of ARGs found in both pathogenic and non-pathogenic bacterial strains, including environmental microorganisms. Recent studies using resistomics have revealed a decreasing trend in both abundances and diversities of ARGs after treatment. A recent study of Quintela-Baluja *et al.* (2019) has provided new insights on the view that WWTPs are hotspots for the spread of ARB and ARGs across environment. In this investigation a WWTP in Spain that operates with AS was studied. Samples were collected from hospital and domestic raw sewage, influents to the WWTP, return activated sludge (RAS), and secondary clarifier effluent, while liquid and sediment river samples were collected upstream and downstream of the treatment plant discharge point. This study, based on metagenomic sequencing and resistomics (i.e., HT-qPCR), challenges the dogma that WWTPs enhance ARGs exchange in a microbially dense environment where HGT may occur. In fact, they showed that ARG characteristics of the secondary effluent and downstream of the WWTP were more similar to raw sewage than to the RAS. Meanwhile the RAS was less diverse compared to both secondary effluent and downstream river. They concluded that WWTPs are effective barrier on mitigating AMR spread, and they stress that effort should be put on optimising biosolids separation in WWTPs, as they observed that ARB that cannot flocculate can disperse in the receiving water.

Similar observations were seen by Karkman *et al.* (2016) in a WWTP using mechanical, chemical and biological processes followed by biofilters for enhanced removal of nitrogen in Helsinki. Analysis monitoring up to 300 ARGs using HT-qPCR, showed significant decreases of ARG abundances and diversities in both effluent and dried sludge compared to influent, and relative abundances were also lower during all four seasons. Although nine ARGs were enriched in the final effluent, only three of them increased their richness downstream in the

river. This study suggested that WWTPs are effective on reducing ARG loads in discharged water; however, they stressed the risk of using the dried sludge due to ARGs enrichment. In contrast, Bengtsson-Palme *et al.* (2016) found decreases in relative abundances of ARGs in sludge, with $bla_{\text{OXA-48}}$ being the only enriched gene. Using metagenomic and resistomic analyses, they assessed samples collected from influent, secondary effluents, sand filtration effluents, and sludge of three WWTPs in Sweden. Although concentrations of ciprofloxacin and tetracycline were high in influent, enrichment for these or other antibiotics was not seen in effluents. Additionally, both absolute and relative abundances, and ARGs diversities decreased significantly in treated effluent. Although a shift in microbial community from obligate to facultative anaerobes were observed, this took place in the sewerage system and not in the WWTP. A similar study, using metagenomic sequencing and analysing 271 different ARG-like subtypes in a WWTP in Hong Kong, also showed that ARG abundances decreased in effluent by 99.8%, with lower removal rates (20.70%) being observed in anaerobic digestion sludge (Yang *et al.*, 2014). Although 78 ARGs, some positively correlated with five pathogenic genera, persisted during treatment, removal of these ARGs were >94 % in the effluent.

2.5.4 *General observations*

There is an increasing number of studies worldwide on different treatment technologies at reducing ARB and ARGs in wastewater, however, consistent conclusions are hard to draw. This is due to a variety of different factors, from operating and climatic parameters to sampling campaigns and methodological assessments, which make comparisons among studies particularly challenging (Barancheshme and Munir, 2018; Michael-Kordatou *et al.*, 2018; Hiller *et al.*, 2019). However, some general observations are presented below.

It is evident from the literature that process operational parameters play a key role in ARG and ARB fate. For example, Zhang *et al.* (2018) found longer SRT decreased ARGs in effluent, at a cost of increasing enrichment in sludge, and long hydraulic retention time (HRT) had a positive correlation in increasing both ARB and ARGs in effluents (Korzeniewska and Harnisz, 2018). Several studies also showed the importance of the influent characteristics. Raw sewage containing untreated hospital effluent can influence treatment performance, sometimes resulting in elevated relative or absolute ARG abundances in final effluents (Rafraf *et al.*, 2016; Korzeniewska and Harnisz, 2018). Also a recent study, based on resistomics, showed that ARG relative concentrations in a mixture of domestic and industrial

wastewater were higher compared to domestic raw sewage, with enhanced transfer frequency for plasmids carrying ARGs (Jiao *et al.*, 2017).

Heavy metals and antibiotics also found to correlate positively with ARG and ARB abundances, as well as HGT during treatment (Mao *et al.*, 2015; Wan *et al.*, 2018; Zhang *et al.*, 2018; Zhang *et al.*, 2019a). Season is another factor that may affect the performance of WWTPs with ARG removal being higher the dry season (Fang *et al.* (2017); however, no seasonal differences were seen elsewhere (Du *et al.*, 2015; Karkman *et al.*, 2016). Although a relationship between these factors and ARB/ARG removal has observed, very few studies include this information, raising the need for more comprehensive investigation in future research.

The treatment technology is a key factor to the removal of ARGs and ARB. For example, MBR seem to be the most effective treatment (Zhang *et al.*, 2015a; Le *et al.*, 2018), which may be attributed to MBR's capacity to separate biosolids. In fact, several studies showed positive correlations between ARGs with microorganisms (Chen and Zhang, 2013a; Du *et al.*, 2015; Fang *et al.*, 2017; Jiao *et al.*, 2017; Zhang *et al.*, 2018; Quintela-Baluja *et al.*, 2019), suggesting that decreasing bacteria loads leads to ARG elimination. AS systems have also showed good performance. Anaerobic/aerobic treatment proved to be particularly effective on reducing both abundances and diversities of ARGs (Christgen *et al.*, 2015), and inversed A^2O exhibited higher ARG removal compared to MBR in a study of Zhang *et al.* (2018). It is worth mentioning that most studies consider only influents and effluents without differentiating treatment steps in a WWTP (Laht *et al.*, 2014; Naquin *et al.*, 2015; Rodriguez-Mozaz *et al.*, 2015; Rafraf *et al.*, 2016; Korzeniewska and Harnisz, 2018; Proia *et al.*, 2018). As a result, important information, such as the contribution of each step in the total treatment, is not presented. Monitoring each treatment step of a WWTP is essential as this will allow for a better understanding of the whole process which can influence future decisions on applying the most effective treatment strategy.

Inconsistent findings are often result of different methodological approaches. Most studies have been based on qPCR data targeting specific genes, often using different primers which make comparisons, even between the same genes, challenging. Besides, monitoring of a broader spectrum of ARGs that can lead to more comprehensive results is rare. For example, a high amount of studies focusing on activated sludge using qPCR have revealed poor performance on ARG removal (Al-Jassim *et al.*, 2015; Mao *et al.*, 2015; Rafraf *et al.*, 2016; Korzeniewska and Harnisz, 2018; Proia *et al.*, 2018), mainly because target ARGs show

specificity to each technology, making general conclusions difficult. On the other hand, studies based on resistomics, considering a wider range of ARGs, showed that activated sludge decreases both abundances and diversities (Yang *et al.*, 2014; Bengtsson-Palme *et al.*, 2016; Karkman *et al.*, 2016; Quintela-Baluja *et al.*, 2019). It is suggested, therefore, that although qPCR is an accurate method for the quantification of genes of interest, resistomics is a complementary method that should be considered, as it can provide insight into a wider range of ARGs. Furthermore, resistomics when combined with metagenomic sequencing can provide useful information on potential pathogenic hosts correlated with ARGs (Zhang *et al.*, 2018; Quintela-Baluja *et al.*, 2019). Therefore, combining methods can lead to more comprehensive results, while methods standardisation should be a priority.

Several studies revealed that treatment changes the microbial community (Zhang *et al.*, 2015a; Wan *et al.*, 2018; Zhang *et al.*, 2019a), however, little is known about the physiological state of these bacteria. For example, Eramo *et al.* (2019) showed that ARGs, discharged with treated water, are carried both in viable and non-viable bacteria. Using conventional qPCR (total cells) and PMA-qPCR (viable cells), to differentiate ARGs carriage in viable cells from total cells, they showed that ARGs in viable cells were much lower compared to total ARGs after treatment with chlorination, suggesting that most of ARGs are carried in non-viable cells. In contrast, non-significant differences between total and viable cells were observed in downstream river samples, leading them to speculate that ARB with fragmented membranes contribute to ARG spread through natural transformation. Hence, identifying ARGs in viable and non-viable cells may be essential to understand the impact of different technologies on the 'health' state of ARB discharged with final effluents and their further fate.

2.6 Tertiary Treatment

Tertiary treatment is suggested to improve ARB and ARG removal from final effluents, which may be essential for reuse purposes or when wastewater needs to be discharged in sensitive natural environments (Christou *et al.*, 2017). Therefore, optimizing conventional tertiary processes (i.e., chlorination, UV irradiation, ozonation) or developing new advanced technologies is the focus of many recent studies (Fiorentino *et al.*, 2015; Zhuang *et al.*, 2015; Zhang *et al.*, 2016; Ferro *et al.*, 2017; Guo *et al.*, 2017; Yoon *et al.*, 2017).

2.6.1 *Conventional tertiary treatment*

Chlorination is a widely applied disinfection process in wastewater treatment (Rizzo *et al.*, 2013b; Sharma *et al.*, 2016; Barancheshme and Munir, 2018). Chlorine's germicidal effect is mainly based on bacteria cell oxidation, cell permeability modification, and inhibition of enzyme activity (Rizzo *et al.*, 2013b), while, when high doses and contact times are applied chlorine may pass into the cytoplasm and react with genetic material (Dodd, 2012). As an alternative to chlorination, the application of ultraviolet (UV) irradiation for wastewater disinfection has grown significantly, and many WWTPs have converted their treatment strategy from chemical to UV-based in recent years (Das, 2001). UV irradiation can have direct effect on genetic material, such as DNA and RNA, affecting the reproducibility of bacteria, altering or breaking the DNA, and leading to cell lysis (Michod *et al.*, 2008; Eischeid *et al.*, 2009; Dodd, 2012).

Various studies, most of them at lab-scale, have assessed the efficacy of chlorination in comparison with UV irradiation to reduce ARB and ARGs from wastewater. ARG transfer through conjugation between Gram-negative strains of *E. coli* during UV and chlorination treatment was examined by Guo *et al.* (2015). In this study, UV and chlorine doses higher than 10 mJ/cm^2 and 80 mg Cl_2 min/L, respectively, resulted in the complete inhibition of conjugative transfer. They also observed that bacteria concentration, nutrient, and mating time significantly affected the conjugative transfer and they suggested that UV irradiation does not affect the cell membrane significantly but directly damages the plasmid containing ARGs, whereas, chlorine affects cell permeability. They concluded that UV irradiation performed better than chlorination, however, higher concentrations than those normally applied on WWTPs, were needed to repress conjugation. A similar trend was seen by Rizzo *et al.* (2013a), where comparison between UV irradiation and chlorination showed that ARB were inactivated in shorter contact time under UV than chlorination. Multidrug resistant (resistant to > 2 antibiotics) *E. coli* strains, were completely removed after 60 min of UV irradiation (0-2.5 µW s/cm); whereas, total inactivation was observed after 120 min when chlorination applied (0.2 mg/L, conventional dose according to Italian standards for disinfection). The authors also found that despite the decreases in bacterial counts, bacteria were still resistant to all three antibiotics after 120 min treatment with chlorination, while, resistant to ciprofloxacin was affected after 60 min of UV irradiation, with no effect seen for the other two antibiotics. This research stresses that ARB removal depends on parameters such as treatment method, contact time, and target antibiotic.

Comparison of various UV irradiation (UV doses of 5.94, 29.7, 59.4 & 178.2 mJ/cm^2 for 1, 5, 10 & 30 min, respectively) and chlorination doses (10 ppm, for 1, 3, 5, 10 and 30 min) with thermal treatment (60^{o}C and 80^{o}C for 30 and 60 min), and an air-open mesocosm (samples collected on 1, 3, 7, 14, 21 and 28 days) showed that UV performed better than chlorination (Calero-Cáceres and Muniesa, 2016); however, the open-air mesocosm had the best performance on eliminating *E. coli*, somatic coliphages and ARGs in the bacterial fraction. This study concluded that, although significant decreases were achieved under experimental conditions based on high doses and contact times, ARGs persisted under all treatments, especially in the bacteriophage fraction, indicating that bacteriophages possibly play a vital role on ARG spread through the environment. It has also been suggested that combination between chlorination and UV can be more effective on reducing ARB and ARGs, with benefits such as decreases in doses, energy, and total costs (Umar *et al.*, 2019). While few studies have been conducted on UV/chlorination combinations, they have shown good performances against both ARB and ARGs (Destiani and Templeton, 2019; Zhang *et al.*, 2019b). The sequential UV/chlorination effect on ARG removal was assessed and compared with chlorination and UV irradiation alone in bench scale experiments using secondary effluent by Zhang *et al.* (2015b). Different chlorination doses (5-30 mg/L) and contact times (5-1020 min), as well as various UV doses (62.4, 124.8, 249.5 mJ/cm^2) were tested at reducing ARGs and *int*1 abundances. In this study, higher concentrations and contact times resulted in higher gene removals, while high ammonia concentrations negatively affected ARG reduction during treatment with chlorination. Chlorine alone was more effective than UV irradiation; however, UV irradiation following chlorination was more effective than both treatments alone, suggesting that combinations between different technologies may decrease the need for high doses and eliminate the formation of disinfection by-products while producing better results.

A few studies conducted at full-scale suggest that neither chlorination nor UV irradiation improve removal of ARB or ARGs (Al-Jassim *et al.*, 2015; Mao *et al.*, 2015; Naquin *et al.*, 2015). On the other hand, assessment of UV irradiation and chlorination on *E. coli* phenotypes and resistance to 21 antibiotics in two WWTPs in Australia suggested that both treatments were effective in preventing pathogenic *E. coli* from entering receiving environments (Anastasi *et al.*, 2013). Specifically, influent, secondary effluent and effluents treated by either UV (35 mJ/cm2) or chlorination (7 mg/L), were collected during dry season. Main findings suggested that *E. coli* phenotypes in effluent post-treated by UV irradiation were more diverse than post-chlorination, and in contrast to chlorination, bacterial strains that

survived UV irradiation carried virulence genes. Nonetheless, the amount of bacterial strains that survived was low in both treatments. Dominant phenotypes were resistant to 11-12 antibiotics, with strains resistant to sulfamethoxazole being more prominent; however, most of them did not carry virulence genes.

2.6.2 *Quaternary treatment*

Ozonation has been used at full-scale applications mainly due to its high oxidative potential of 2.08 V under acidic conditions and 1.24 V under basic conditions (Sharma *et al.*, 2016). Ozone is very effective at attacking phospholipids or lipopolysaccharides from external bacterial membranes (Dodd, 2012), carbon double bonds, functional groups or aromatic rings from organic molecules (Ikehata *et al.*, 2006), leading to cell permeability and facilitating nucleic acid oxidation (Von Sonntag, 2001; Cho *et al.*, 2010). The effect of ozonation on disintegrating ARGs and *int*1 genes was compared to chlorination and UV irradiation in bench scale experiments (Zhuang *et al.*, 2015). Among all treatment strategies, ozonation, with doses up to 177.6 mg/L, achieved 1.68–2.55 log removals, whereas, chlorination (at 120 min contact time and 160 mg/L dose), and UV irradiation (at a dose of 12,477 mJ/cm^2) achieved 2.98–3.24 log and 2.48–2.74 log removals, respectively. The authors postulate that the lower ozonation removals may be attributed to ozone consumption by organic matter in wastewater and they concluded that although chlorination and UV irradiation adequately reduced ARGs, the doses and contact times applied were higher than those normally used in WWTPs. Data also showed that ozonation, in contrast to other two treatments, highly increased relative abundances (log change of ARGs/16S rRNA) under all doses, which is in agreement with other studies (Luczkiewicz *et al.*, 2011; Lüddeke *et al.*, 2015), possibly indicating selection for specific genes.

Although more studies are needed to optimise the performance of ozonation, it has been found that this technology has achieved > 90 % removals of ARB and ARG abundances (Oh *et al.*, 2014; Zhuang *et al.*, 2015; Alexander *et al.*, 2016). In fact, ozonation was as effective as UV in a recent study by Sousa *et al.* (2017), where lab scale experiments testing ozonation (150 cm^3/min effluent at 15, 30, and 60 min) and UV irradiation (150 cm^3/min effluent at 30 min) were conducted treating real and synthetic secondary effluent. Both treatment methods resulted in similar removal efficiencies after 30 min; while, log removals between 3 and 4 were achieved for cultivable microorganisms, while, ARGs were detected close to the quantification limit. Although both ozonation and UV irradiation were effective at reducing absolute bacterial and gene abundances, regrowth in abundances were seen after three days

storage, while UV selected for ARGs and *int*1 by increasing the levels of relative concentrations.

Advanced oxidation processes (AOPs), such as Fenton and photo-Fenton, H_2O_2/UV, and heterogeneous photocatalytic oxidation, although used less in wastewater treatment practices, have shown a great potential on ARG removal, since hydroxyl radical ($^{\bullet}OH$), which is one of the main oxidants in these processes, has an oxidative potential ranging between 1.89 and 2.80 V (Sharma *et al.*, 2016), reacts rapidly and non-selectively with most compounds including fatty acids in biomembranes, enzymes, and nucleotides (Park *et al.*, 2005; Giannakis *et al.*, 2018).

UV/H_2O_2 oxidation, is an advanced technology with great potential on dealing with pollutants such as contaminants of emerging concern (Oturan and Aaron, 2014). Therefore, in recent years, it has been subject of investigation for its usefulness in controlling antibiotic resistance transmitted through wastewater. Its effect on the inactivation of susceptible and antibiotic resistant strains, as well as the removal of the bla_{TEM}, *qnr*S, and *tet*W ARGs were examined in a study by Ferro *et al.* (2016). In bench-scale experiments, hydrogen peroxide (20 mg/L) was combined with UV irradiation in a range of 0-2.5 x 10^5 µW s/cm^2 by altering the exposure time, and both intracellular DNA from resistant *E. coli* cells and total DNA present in water suspension were analysed using PCR and qPCR assays. Main results showed complete inactivation of antibiotic resistant *E. coli* after 90 min contact time, and ARGs in intracellular DNA also entirely reduced after 60 min. On the other hand, ARG removal in water suspension was less affected. The authors postulated that low H_2O_2 dose resulted in a mild hydroxyl radical effect on DNA, concluding that UV/H_2O_2 process performed under realistic conditions may not be effective against ARG dissemination. Similar findings were also observed in a recent study of the same group, showing persistence of bla_{TEM} abundances in wastewater treated by H_2O_2/UV (20 mg/L H_2O_2 dose and 240 min contact time), despite the complete inactivation of resistant *E. coli* (Ferro *et al.*, 2017).

In contrast to both previous studies, Yoon *et al.* (2017) showed that H_2O_2/UV reduced extracellular ARGs more readily than intracellular ARGs. H_2O_2/UV (10 mg/L H_2O_2) has been compared with UV (60-130 mJ/cm^2) and chlorination (37-376 mg min/L dose) at reducing amp_R and kan_R plasmid-associated ARGs in both extracellular and *E. coli* intracellular DNA. Analyses showed that ARG disintegration was slower than bacterial inactivation in all three treatment options, whereas, membrane damage was faster than ARG removal under chlorination and slower under UV and H_2O_2/UV. They also found that addition of H_2O_2 did

not enhance UV; while, the matrix (i.e. clean water vs wastewater) significantly influenced the treatment performance. Further, pH played an important role on chlorination, with no effect on UV or H_2O_2/UV treatment. Similar findings were seen in a full-scale study in a WWTP in Spain, where H_2O_2/UV was compared with UV alone (Rodríguez-Chueca *et al.*, 2019). In this study, although H_2O_2/UV enhanced the removal of antibiotics, UV alone had better performance on reducing the target ARGs, indicating that in real applications, the matrix plays a key role on the overall performance of a WWTP.

The efficacy of H_2O_2/UV at reducing target ARGs (*sul*1, *tet*X, *tet*G) and *int*1 from secondary effluent was compared with Fenton, which is an AOP with great potential (Zhang *et al.*, 2016). Both technologies were very effective on decreasing ARG and *int*1 abundances, with Fenton process being slightly better. In each disinfection method, various pH, Fe^{2+}/H_2O_2 molar ratios or H_2O_2 concentrations, and reaction times were examined. Removals in a range of 2.58–3.79 logs were observed under the optimum condition for the Fenton oxidation, at pH equal to 3.0, however, lower reductions (~2.26–3.35 logs) were achieved at natural pH (7.0). UV/H_2O_2 at pH 3.5 reduced ARGs by 2.8–3.5 logs, while, 1.55–2.32 log removal was observed at a natural pH of 7.0. According to this research, ARG decrease is attributed to the generation of $^{\bullet}OH$ by the decomposition of either H_2O_2 catalyzed by Fe^{2+} or the reaction of H_2O_2 with UV. Nonetheless, the low pH required during process, especially during Fenton oxidation, or the scavenging effect of $^{\bullet}OH$ as a result of high H_2O_2 and the presence of other organic contaminants in the matrix were identified as limitations of these technologies.

In order to overcome the low pH values required for the Fenton oxidation, and reduce the need for chemicals, Fenton-like processes based on natural clay minerals showed good performance on reducing emerging contaminants. Clay minerals, which are hydrous aluminium phyllosilicates, with variable amounts of iron (Fe), aluminium (Al), magnesium (Mg), silicon (Si) and other elements (Brigatti *et al.*, 2006), are known to produce hydroxyl radicals, superoxide and/or other reactive oxidative species (ROS) upon contacting with H_2O_2 through a Fenton-like process (Remucal and Sedlak, 2011). This process can be an effective alternative to the traditional Fenton oxidation, as it performs well in neutral pH and does not produce an iron sludge; hence, its application for *in situ* remediation of organic contaminants in soil, groundwater and wastewater is highly recommended (Pham *et al.*, 2009; Krembs *et al.*, 2010; Pham *et al.*, 2012; Liu *et al.*, 2014).

Over the past decade, clay minerals have been studied for their antimicrobial potential against bacteria causing skin or other nosocomial infections, and first findings show promise at

reducing antimicrobial resistant strains (Haydel *et al.*, 2008; Williams *et al.*, 2011; Morrison *et al.*, 2014; Morrison *et al.*, 2016; Otto *et al.*, 2016). For instance, the bactericidal effect of the French clay mineral CsAg02 has been reported against a wide range of both susceptible and antibiotic resistant pathogenic bacteria, e.g., extended-spectrum β-lactamase (ESBL) *E. coli*, *P. aeruginosa*, and *M. marinum* (Haydel *et al.*, 2008), while, illite, montmorillonite, and kaolinite clays reduced the abundances of methicillin-resistant *S. aureus* (MRSA) (Otto *et al.*, 2016). Furthermore, Oregon Blue clay from different hydrothermal deposit zones also showed an antibacterial effect on pathogens including methicillin-resistant *S. epidermidis* (MRSE), and MRSA (Morrison *et al.*, 2014; Morrison *et al.*, 2016); while, a recent study showed the antibacterial properties of ancient medicinal earths and their potential use in epidemics, indicating that treatment with clays is not something new (Venieri *et al.*, 2020). Also, modifications to the redox state of clays (i.e., Fe^{3+} to Fe^{2+}) was shown to enhance the production of ROS upon contact with oxygen, attacking membrane components of *E.coli*, such as cardiolipin, accelerating cell lysis (Wang *et al.*, 2017). Therefore, clay minerals, either alone or combined with H_2O_2 have shown great potential against ARB and may be a good Fenton-like alternative option to be considered in wastewater treatment strategies.

Using an external light source, mainly sunlight, is another way to enhance Fenton oxidation under neutral pH and, thus, the focus of many studies. The effect of photo-Fenton using solar light on several antibiotic resistant bacterial strains was assessed and compared with solar light alone (Giannakis *et al.*, 2018). Lab-based experiments examined photo-Fenton, at a 10 mg/L H_2O_2 dose and 1 mg/L Fe^{2+} to a 1:10 Fe^{2+}:H_2O_2 ratio, with no adjustments of the initial pH (6.5). The main results showed that both treatments completely reduced ARB concentrations with solar photo-Fenton inducing bacterial inactivation faster (90-120 min) than solar light (180-240 min) alone, this trend also was seen for the target ARGs. They also showed that the treatment was not affected by the bacterial type (i.e., Gram+ or Gram-), and multidrug-resistant strains were not more difficult to treat than bacteria resistant to one antibiotic. This study concluded that both technologies were effective against ARB, with photo-Fenton accelerating removal, which is in agreement with other research conducted under natural light (Karaolia *et al.*, 2014; Ferro *et al.*, 2015; Fiorentino *et al.*, 2015). Although an increase in antibiotic resistance among survived ARB was not evident, regrowth was observed in contact times shorter than 180 min, which also reported by Karaolia *et al.* (2017).

Decrease of ARB in secondary effluent by solar photo-Fenton and other solar-driven AOPs, such as H_2O_2/sunlight, TiO_2/sunlight, and H_2O_2/TiO_2/sunlight, was also seen in lab- and pilot-

scale projects (Ferro *et al.*, 2015; Fiorentino *et al.*, 2015). Both studies showed complete inactivation of ARB under all photo-AOPs, with photo-Fenton being one of the most effective options. In the study of Fiorentino *et al.* (2015) six log units reduction, and total inactivation of ARB were achieved after 4 hrs solar exposure when solar irradiance reached the highest levels. On the other hand, solar-driven AOPs achieved ARB inactivation faster than solar irradiation alone, requiring significantly lower cumulative energy. Although the TiO_2/H_2O_2/sunlight process was the most effective in terms of energy needs (total inactivation achieved in the range 3–5 kJ/L), the authors concluded that solar photo-Fenton (5:10 Fe^{2+}:H_2O_2 ratio at 15 kJ/L) and H_2O_2/sunlight (50 mg/L at 8 kJ/L) are more feasible and cost-effective processes. Further, the comparison between H_2O_2/sunlight and conventional chlorination (1.0 mg Cl_2/L) showed that the later was faster to inactivate multidrug *E. coli* than H_2O_2/sunlight, but less effective in controlling regrowth.

Titanium dioxide (TiO_2) photocatalysis has also seen to be effective against ARB, with complete inactivation being achieved by increasing TiO_2 concentration and UV dose (Tsai *et al.*, 2010; Xiong and Hu, 2013). The effect of photocatalysis on both sensitive and antibiotic (rifampicin, chloramphenicol) resistant bacterial strains, as well as on bacterial regrowth was tested in an immobilised TiO_2 reactor (Dunlop *et al.*, 2015). Experiments, conducted with both distilled water and sterile secondary effluent, showed that although both antibiotic resistant strains were completely inactivated after 180 min treatment, ARB abundances of both strains increased to initial levels after 24 hrs. Conjugation experiments also revealed that the number of conjugants increased four times after photocatalysis compared to control, whereas, increases were lower in secondary effluent, leading the authors to speculate that scavenging effect of ROS during treatment in real wastewater decreases the oxidative pressure on the bacteria. They concluded that photocatalysis is effective against ARB, and that longer contact times could reduce regrowth after treatment.

Higher bacterial inactivation and decreased contact times were observed by using metal-modified TiO_2 (Rizzo *et al.*, 2014; Venieri *et al.*, 2017) or combinations between oxidation processes. Photocatalysis and ozonation has been studied by Moreira *et al.* (2016) in a pilot-scale project, where photocatalytic ozonation was tested in continuous mode with TiO_2-coated glass rings and light emitting diodes (LEDs) against different types of contaminants, including chemical priority substances and contaminants of emerging concern, as well as ARB and ARGs (*bla*$_{TEM}$, *qnr*S, *sul*1) in domestic wastewater. Photocatalytic ozonation was much more effective than ozonation or photocatalysis alone at decreasing abundances of

micropollutants, ARB and ARGs. On the other hand, although photocatalytic ozonation effectively removed microorganisms and ARG abundances, bacteria resistant to meropenem, ciprofloxacin and gentamicin, as well as heterotrophs, fungi, and the genes 16S rRNA and *int1* increased after 3-days of storage, indicating a potential risk of microbial regrowth also seen elsewhere (Dunlop *et al.*, 2015; Biancullo *et al.*, 2019).

Other tertiary methods recently tested for the removal of ARGs and ARB from treated secondary effluents are coagulation and filtration. Coagulation has been traditionally used to improve water quality by attaching positively charged coagulants with negatively charged colloidal particles (Zainal-Abideen *et al.*, 2012). This method, used to treat colour, turbidity, heavy metals and organic contaminants, has recently been tested for ARGs removal. For example, the coagulants polyferric chloride and $FeCl_3$ were tested in terms of reducing ARGs and *int*1 abundances, as well as physicochemical parameters, such as NH_3-N, dissolved organic carbon (DOC), and total phosphorus (TP) (Li *et al.*, 2017). Strong correlations between physicochemical parameters and ARGs, and removal rates between 0.5 and 3.1 logs were seen at doses between 6 and 30 mg/L. Similarly, the presence of colloids was proven to enhance ARG removal in an ultrafiltration membrane system (Riquelme Breazeal *et al.*, 2013).

Filtration is often used as the last treatment step in WWTPs, therefore its efficacy on reducing ARGs have been also investigated. Sand filtration increased the abundances of multidrug-resistant bacteria and ARGs in a WWTP in Switzerland, however, it is speculated that this may be due to selection pressure on bacteria which occurred as a result of the use of the same filtration system over 18 years in this WWTP (Czekalski *et al.*, 2016). On the other hand, in a pilot-plant study performed in a WWTP in Germany, sand filtration following an ozonation step was very effective at reducing antibiotic resistant *E.coli*, staphylococci, and enterococci (Lüddeke *et al.*, 2015). In this study, examination of various treatment options, including flocculation/filtration, granular activated carbon (GAC), O_3/sand filtration, O_3/GAC, and O_3/sand filtration/GAC following a secondary settler, showed that both O_3/sand filtration and O_3/GAC achieved the best performance (0.8-1.1 logs), while the combination O_3/sand filtration/GAC did not improve ARB removals. The authors also observed that ozonation was selective against antibiotic resistant strains, nevertheless, post-treatment with O_3/sand filtration or O_3/GAC improved ARB removal from the discharged water. High removal of ARGs in treated wastewater by subsurface filters following a system of two lagoons were also observed in Anderson *et al.* (2015); however, the authors observed that ARGs were retained

on the filters therefore they stressed that filters should be carefully handled at the end of their use. Although filtration can further eliminate releases of ARB and ARGs, several factors such as operating parameters, filter material, pore size, backwash procedures, as well as levels of antibiotics, ARB and ARGs in the inlet can greatly affect their performance against AMR (Hiller *et al.*, 2019).

2.6.3 *General observations*

A large and growing body of the literature has investigated the effect of tertiary treatment on ARB and ARG removal. Previous studies and reviews (Rizzo *et al.*, 2013b; Barancheshme and Munir, 2018; Michael-Kordatou *et al.*, 2018; Hiller *et al.*, 2019) compare the effectiveness of both conventional (UV irradiation, chlorination) and quaternary treatment processes, and several factors are critical, such as operating parameters, doses, contact times, target microorganisms, and experimental conditions. For example, UV and chlorination seem to be effective against ARB and ARGs only when high doses and contact times are applied, which in full-scale application is impractical and costly (Rizzo *et al.*, 2013a; Guo *et al.*, 2015; Zhang *et al.*, 2015b). The same trend is seen with advanced treatment, such as ozonation and H_2O_2/UV, where ARB and ARGs are not hindered at low doses (Zhuang *et al.*, 2015; Ferro *et al.*, 2016; Ferro *et al.*, 2017). The wastewater matrix or the generation of by-products such as chloramines have suggested to be among principal reasons for limiting the performance of conventional and advanced technologies, due to their scavenging effect on disinfectants and oxidants, delaying the treatment process against ARB and ARGs (Rizzo *et al.*, 2013a; Guo *et al.*, 2015; Zhang *et al.*, 2015b; Zhuang *et al.*, 2015; Ferro *et al.*, 2016). Therefore, the characteristics of the wastewater are important in the optimisation of the treatment process.

Operating parameters are also crucial for the treatment process. Regardless of doses and contact times, Fe^{2+}/H_2O_2 molar ratios (e.g., Fenton oxidation), pH, light intensity or light absence, and types of catalysts (e.g., TiO_2, clay minerals) play key role for the treatment (Zhang *et al.*, 2016; Wang *et al.*, 2017; Giannakis *et al.*, 2018). For example, Fenton oxidation is enhanced at low pH (Zhang *et al.*, 2016) or by an external light source, such as solar light (Fiorentino *et al.*, 2015; Giannakis *et al.*, 2018). Combination of treatment processes also seem to increase performance at decreased doses and with less need for chemicals (Zhang *et al.*, 2015b; Moreira *et al.*, 2016). Further, filtration material, type of coagulants, and general operating parameters were also seen to be important for physical tertiary technologies based on filtration or coagulation (Riquelme Breazeal *et al.*, 2013; Lüddeke *et al.*, 2015; Czekalski *et al.*, 2016; Li *et al.*, 2017). Therefore, improving the quality

of water in pre-treated water, especially removing particles, and optimising operating parameters could be of value and further investigation is warranted.

Although AOPs or other UV- or ozone-based options have been described as non-selective, mainly due to the formation of •OH (Sharma *et al.*, 2016) or the ability to attack DNA directly (Dodd, 2012), some gene-to-gene and ARB specificity have been shown (Anastasi *et al.*, 2013; Zhuang *et al.*, 2015; Calero-Cáceres and Muniesa, 2016; Zhang *et al.*, 2016; Sousa *et al.*, 2017). Further, both AOPs and conventional tertiary treatment generally decrease ARG absolute abundances; however, increases in relative abundances have been occasionally reported for specific genes (Zhuang *et al.*, 2015; Sousa *et al.*, 2017), suggesting that ARG selection may be possible under certain experimental conditions. Although some advanced technologies seem to enhance selection, a better understanding requires examination of a wider range of ARB and ARGs. Also intracellular DNA has been described to be more recalcitrant than extracellular, mainly due to scavenging effects (Yoon *et al.*, 2017), but the opposite also has been reported (Ferro *et al.*, 2016). Additionally, comparison among several quaternary treatments (UV, chlorination, thermal treatment etc.) showed that they all appear to be ineffective at reducing ARGs carried in bacteriophages, possibly influencing ARG transfer potential via HGT (Calero-Cáceres and Muniesa, 2016). Regrowth of bacterial populations also has been shown as a result of photoreactivation or dark repair, sometimes reaching pre-treatment levels and promoting ARB in storage tanks (Dunlop *et al.*, 2015; Moreira *et al.*, 2016). Therefore, regrowth of bacteria especially in reclaimed water should be taken under consideration in future work.

Although there are many AOP studies performed at the lab-scale, less is known about how these technologies perform under real conditions at the pilot- or full-scale (Anastasi *et al.*, 2013; Rodríguez-Chueca *et al.*, 2019). Some full-scale studies have assessed tertiary treatment steps, such as chlorination, UV, or ozonation (Al-Jassim *et al.*, 2015; Naquin *et al.*, 2015; Rafraf *et al.*, 2016), but differentiation among treatment steps have not been considered or they have focused only on pre- and post-tertiary treated effluent. As a result, our knowledge regarding the added value of tertiary treatment in a conventional WWTP is limited; this is a key new contribution from this Thesis. Further, more comprehensive analysis is included herein on a broader spectrum of ARGs as well as the physiological state of exposed ARB. Finally, alternate, natural materials, such as clay minerals, are considered as possible lower cost AOP options, decreasing reliance on extraneous chemicals or elevated levels of energy.

2.7 Aim and Objectives

AMR worldwide has reached alarming levels. There now is evidence that AMR is associated not only with clinical environments, but also with the wider environment. Both ARB and ARGs, as well as antibiotics, reach WWTPs through sewage and WWTPs. Although WWTPs are a known pathway of AMR into environment, it also can be an effective barrier. Hence, over the last decade, a large and growing body of literature has examined many treatment technologies on their relative ability to control ARB and ARG releases in receiving environments. Our knowledge on AMR has increased considerably the last decades, but inconsistent findings make comparisons among studies challenging and many questions remain unanswered. Therefore, this research aims to fill some of the gaps previously identified and elucidate important factors influencing wastewater treatment.

In the thesis, I aim to assess and compare the performance of different conventional and quaternary technologies at full-, pilot-, and bench-scale at controlling the presence of ARGs in final effluents. Trickling filter, granular activated sludge, activated sludge, and a membrane bioreactor were evaluated at full-scale. The main objectives were to assess the effectiveness of each WWTP to reduce both abundance and diversity of a broad spectrum of ARGs relying on molecular-based techniques (qPCR and HT-qPCR); while, also assessing the relative carriage of ARGs in viable and non-viable cells within wastewater systems. Moreover, as the four WWTPs included different secondary and tertiary steps (in most WWTPs), the contribution of each treatment step to the total removal of ARGs was estimated.

The added value of ozonation, H_2O_2/UV, and O_3/H_2O_2/UV to a main WWTP was also evaluated in pilot testing, as AOPs have shown added value for controlling releases of ARB and ARGs in discharged water. Aiming to produce as much comparable data as possible among all treatment technologies tested, resistomes were also used here to examine abundances and diversities of a wide range of ARGs; while, ARG carriage in both viable and total cells were also evaluated to clarify any effect of advanced oxidation on the ‘health’ state of bacteria entering receiving waters. Furthermore, as most studies have focused on oxidation systems as individual processes, here we considered AOPs within the context of whole treatment systems, determining their added value to the main WWTP.

Lab-scale experiments were also conducted, aiming to give insight into key factors influencing AOP performance previously tested in pilot-scale, and develop alternative treatments based on natural materials which will presumably decrease operational costs and needs in energy and chemicals. Fe-rich clay minerals, reduced or combined with H_2O_2, can

produce ROS through Fenton-like processes or other oxidative pathways. Therefore, the oxidative potential of nontronite-1 (NAu-1), with a high content on Fe, either coupled with H_2O_2 or in its reduced state combined with air oxygen, were examined against target ARGs in real wastewater effluent. A variety of experimental conditions (e.g., concentrations and contact times) were assessed. To the best of our knowledge this is the first attempt to evaluate the antibacterial properties of clay minerals for ARG removal in real wastewater.

The Chapters 3, 4 and 5 are written in a style for publication. All Chapters include a small introduction on the topic, methodology, results, discussion and conclusions. The Chapter 3, focusing on conventional treatment at reducing ARG abundance and diversity of both viable and total cells in domestic wastewater, is intended to be published in the *Water Research*, which is a leading journal in the field of water science and technology for improving water quality and management. The *Environmental Science & Technology* is the target journal for the Chapter 4 on AOP options evaluated in a pilot plant. This is a high impact journal covering topics on environmental technology and science where studies on advanced treatment in wastewater have been published before. Chapter 5 on clay minerals tested as an alternative AOP is intended to be published also in the *Environmental Science & Technology* as a high rank journal for novel technologies. For Chapters 3 and 4, I would like to acknowledge the contribution of Dr. Yong-Guan Zhu and Dr. Jian Qiang Su from the Chinese Academy of Science for analysing the samples using HT-qPCR, Professor Simon Andrews from the University of Reading for providing his laboratories which allowed me to conduct my experimental work, and also the employees of Thames Water for their assistance during my fieldwork.

Chapter 3 Fate of Antibiotic Resistance Genes in Wastewater Treatment Plants

3.1 Introduction

Antibiotics have been widely used since the 1940s to prevent and treat bacterial infections in human, animals, and plants. However, their effectiveness has been compromised due to increasing antibiotic resistance in clinically important strains. Antibiotic resistance is natural, but the wide use of antibiotics in anthropogenic activities, such as medicine, agriculture, and aquaculture, has accelerated the rate of evolution and dissemination of antibiotic resistant strains on global scales (Andersson and Hughes, 2014). Antibiotic resistance, which comprises ARB and ARGs, can find their way into the water cycle, either as sewage discharged into aquatic environments or land irrigation with treated wastewater (Hiller *et al.*, 2019). Therefore, WWTPs are a pathway of resistance spread to the environment. For this reason, a comparison of how different treatment processes handle resistance is of value in understanding better the role of WWTPs in combating the spread of antibiotic resistance and, more widely, AMR.

WWTPs are generally effective at reducing the discharge of many water contaminants and pathogens to the environment. As a result, considerable interest now exists in understanding the diversity and abundance of ARGs in and through WWTPs. Many wastewater treatment technologies are used, but differences among options are not fully defined (Barancheshme and Munir, 2018). Therefore, a comparison among different treatment processes is needed to quantify the potential of each treatment option to deal with resistance dissemination.

Over the last decade, many WWTPs have been assessed and compared in their relative ability to reduce ARB and ARGs from the discharged effluents. Activated sludge, including tertiary treatment or not (Laht *et al.*, 2014; Yang *et al.*, 2014; Al-Jassim *et al.*, 2015; Mao *et al.*, 2015; Naquin *et al.*, 2015; Rodriguez-Mozaz *et al.*, 2015; Bengtsson-Palme *et al.*, 2016; Karkman *et al.*, 2016; Rafraf *et al.*, 2016), anaerobic-anoxic-aerobic treatment processes (Li *et al.*, 2016; Wen *et al.*, 2016), constructed wetlands (Chen *et al.*, 2016a; Fang *et al.*, 2017) and membrane bioreactors (Chen and Zhang, 2013a; Du *et al.*, 2015; Sun *et al.*, 2016) are treatment technologies that they have been assessed at full-scale.

Among them, activated sludge is one of the most studied technologies in terms of reducing antibiotic resistance from final effluents. Although a high number of studies have been

conducted, consistent conclusions are hard to draw. Many studies, based on qPCR data, have shown increases in relative abundances or even absolute abundances of genes after treatment, suggesting that biological reactors enhance the dissemination of ARGs among bacterial strains due to horizontal gene transfer (HGT) or mutations within the WWTP (Al-Jassim *et al.*, 2015; Rafraf *et al.*, 2016). In reality, most studies show that conventional activated sludge reduces both absolute and relative ARG abundances in final effluents (Chen and Zhang, 2013a; Mao *et al.*, 2015), whereas other studies indicate an increase or no change in ARG relative concentrations despite decreases in absolute abundances (Laht *et al.*, 2014; Rodriguez-Mozaz *et al.*, 2015; Le *et al.*, 2018). It is noteworthy, however, most studies using metagenomic analysis, which assess a wider range of ARGs, showed a decreasing trend in both absolute and relative abundances, as well as diversity of ARGs after treatment (Bengtsson-Palme *et al.*, 2016; Karkman *et al.*, 2016; Jiao *et al.*, 2017; Quintela-Baluja *et al.*, 2019).

A number of studies have also focused on membrane bioreactors (MBR), suggesting that this technology is a viable solution to improve antibiotic resistance removal from final effluents. For example, recent studies showed that MBRs significantly reduce relative and absolute levels of target genes (Zhang *et al.*, 2015a; Le *et al.*, 2018). Conversely, a few studies found increases in relative concentrations of genes in MBR permeate despite large reductions in absolute abundances (Du *et al.*, 2015; Zhang *et al.*, 2018). Recently, investigators have also examined the effectiveness of aerobic granular sludge at reducing ARGs. For example, lab-scale testing demonstrated that this technology effectively reduced transfer of plasmids, presumably carrying ARGs, compared to flocculent sludge (Zou *et al.*, 2016). On the other hand, it has also been found that the presence of antibiotics in the matrix may influence resistance selection (Wan *et al.*, 2018; Zhang *et al.*, 2019a), although this may require elevated antibiotic concentrations.

Although there is rising interest in assessing conventional treatment technologies, direct comparisons among studies is challenging. Different operational and climatic parameters, as well as differences in sampling campaigns and variation in methodological approaches have made comparisons across studies hard (Krzeminski *et al.*, 2019). Further, most studies assessed the overall efficiency of a WWTP without considering the impact of different treatment steps; hence, conclusions cannot be drawn on contribution of each step to the total removal efficiencies. In addition, most studies use qPCR data, which is biased towards specific genes, lacking data on a wider spectrum of ARGs in the wastewater. Besides, all these methods focused on ARGs present in all bacteria without identifying if these were

carried by cells with intact membranes or fragmented membranes. This is important to understand the impact of different technologies on viability of bacteria carrying ARGs (Michael-Kordatou *et al.*, 2018; Hiller *et al.*, 2019), which is critical to understanding if HGT exists or not.

In this study, the intent was to fill some of the knowledge gaps and clarify key factors that influence the effectiveness of a treatment technology to deal with antibiotic resistance. For this reason, four wastewater treatment technologies were assessed at full-scale, including a trickling filter (TF), granular activated sludge (GAS), activated sludge (AS), and an MBR. They were evaluated in their relative ability to reduce ARG diversity and abundances in final effluents using common methods and sampling regimes. Samples included primary, secondary and tertiary (where applied) treated discharges, and a broad-spectrum of ARGs were measured using qPCR and HT-qPCR Therefore, the contribution of each treatment step to the total efficiency of each WWTP was one of the main objectives in this Chapter. Finally, carriage of ARGs in viable and total bacterial cells was assessed using propidium monoazide (PMA).

3.2 Materials and Methods

3.2.1 *Sampling campaign*

The aim of this project was to compare the fate of ARGs and MGEs within different wastewater treatment technologies. Sampling was performed at four different full-scale municipal treatment at SE England, including TF, AS, GAS and MBR systems.

Sampling campaign was performed in January and February 2019. On each sampling day, samples were collected for physicochemical analysis and molecular analysis, differentiating samples for assessing total and viable bacteria (see Section 3.2.5). Volumes collected per sampling location varied according to WWTP and analytical purpose. For example, 1.5 L per location were distributed in appropriate vials and sent to analytical laboratories for physicochemical analyses, within 24 hours of collection (tables A.2, A.3). The volumes for the molecular analysis were determined in previous sampling campaigns taking into consideration characteristics of each WWTP, such as the type of treatment technology and the density of biomass in each location. To avoid variations in sample dilution due to precipitation events, sampling was primarily conducted during dry days. However, due to winter conditions and limited time, occasional samples were collected in rainy or snowy days.

For this reason, larger volumes, between 10 to 50 L from final effluents, were taken to ensure enough biomass was collected for molecular analysis purposes.

Samples were collected after primary, secondary and tertiary (where applied) treatment in all four plants, although sampling locations and volumes varied according to location and WWTP. The samples collected from the TF (figure 3.1a) were primary settled sewage (called influent), TF discharge (secondary effluent) and disc filter discharge (tertiary/final effluent); whereas primary settled sewage (influent), GAS discharge (secondary effluent) and pile cloth filter discharge (tertiary/final effluent) were sampled from the GAS WWTP (figure 3.1c). Sample locations of the AS system (figure 3.1b), included primary settled sewage (influent), AS discharge (mixed liquor), and secondary clarifier effluent (secondary/final effluent). Additionally, return activated sludge (RAS) was also collected from this WWTP. Four locations were sampled in the MBR (figure 3.1d), including screened sewage (influent), biological reaction tank effluent (mixed liquor), MBR permeate (secondary effluent), and final effluent after treatment with granular activated carbon (GAC) followed by chlorination.

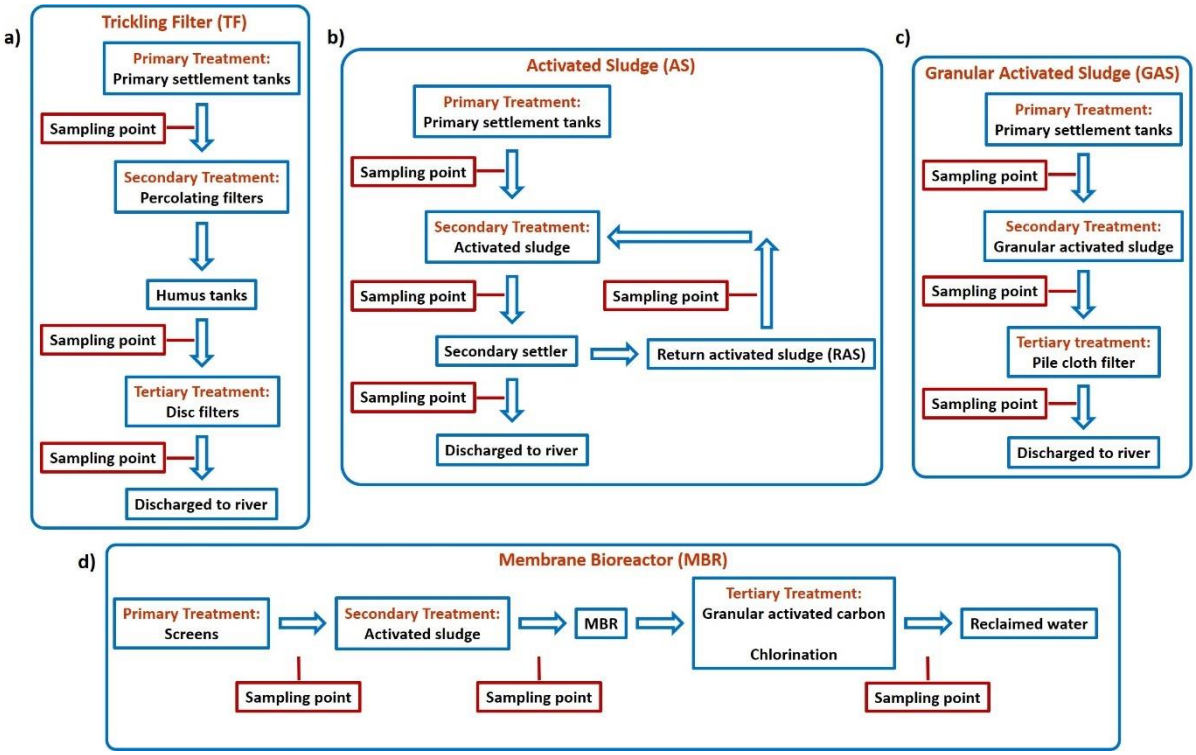

Figure 3.1 Schematics of the four WWTPs assessed in this work. The treatment technologies were **a)** TF, **b)** AS, **c)** GAS, and **d)** MBR. Blue boxes show the main treatment stages while red boxes indicate the sampling locations in each plant.

All samples for molecular biological analysis were collected in sterile polypropylene containers (Fisher Scientific, UK) and subsequently transferred to the laboratory where they stored at 4 ^{o}C until further processing. All samples were processed within 24 hours of

collection. The only exceptions were the MBR permeate and MBR final effluent. Due to large volumes, these samples were filtered on site (see Section 3.2.2) and filter units were subsequently transferred to laboratory for further processing.

Three independent samples (one per sampling day per treatment location) were collected for each type of analysis (i.e., molecular biological analysis for either total or viable bacteria and physicochemical analysis). Grab samples were taken from the TF, AS and GAS (table 3.1). On the other hand, MBR permeate and GAC & chlorination discharge from the MBR were continuously collected from approximately 10 am to 2 pm. As it is shown on table 3.1, large volumes were needed from these two sampling locations to collect enough biomass for analysis (100 L per location). Since effluents were collected in four hour intervals, screened sewage, and mixed liquor were taken every hour and combined to obtain one parallel sample per treatment location.

Table 3.1 Principal information about the sampling campaign containing sampling dates, WWTPs, sampling locations per plant and volumes collected per treatment stage. The volumes refer to samples for molecular analysis; the same amount was collected twice (x2), one half for total and the other half for viable bacteria analysis.

Sampling Dates	WWTP	Sampling locations	Volume (L)
Sampling day 1: 4/2/2019	**Trickling Filter (TF)**	Primary settled sewage	0.1 (x2)
Sampling day 2: 6/2/2019		TF discharge	5 (x2)
Sampling day 3: 13/2/2019		Disc filter discharge	10 (x2)
Sampling day 1: 31/1/2019	**Granular Activated Sludge (GAS)**	Primary settled sewage	0.1 (x2)
Sampling day 2: 14/2/2019		GAS discharge	5 (x2)
Sampling day 3: 27/2/2019		Pile cloth filter discharge	10 (x2)
Sampling day 1: 11/2/2019	**Activated Sludge (AS)**	Primary settled sewage	0.1 (x2)
Sampling day 2: 18/2/2019		Mixed liquor	0.01 (x2)
Sampling day 3: 21/2/2019		Secondary clarifier effluent	10 (x2)
		RAS	0.005 (x2)
Sampling day 1: 21/1/2019	**Membrane Bioreactor (MBR)**	Screened sewage	0.1 (x2)
Sampling day 2: 25/1/2019		Mixed liquor	0.005 (x2)
		MBR permeate	53 (x2)
Sampling day 3: 28/1/2019		GAC & chlorination discharge	51 (x2)

3.2.2 *Sample processing*

At the end of each sampling day, samples for biological molecular analysis were transferred to the laboratories. Influents, mixed liquors and RAS were centrifuged (Eppendorf 5810R) at 4,000 rpm for 30 min. Subsequently, centrates were removed and 20% of glycerol (Sigma-

Aldrich, UK) was added to the pellets until levelled to 10 mL (see Section A.1). The pellets were re-suspended and retained 30 minutes in room temperature before storage at -20 ^{o}C.

Secondary and tertiary effluents were filtered using hollow-fibre ultrafiltration with 30 kDa pore size. This method was used because large volumes of water can be filtered in relative short time. REXEED 25A hollow-fibre ultrafilters (TPS, UK) were chosen due to their high hydraulic performance (Smith and Hill, 2009) and their wide use in similar wastewater studies (Brinkman *et al.*, 2018; Quach-Cu *et al.*, 2018). The set-up consisted of hollow-fibre ultrafilters, a peristaltic pump (Watson-Marlow 520S) and silicone tubing. Filtration of each sample included three different set-ups (figure 3.2). First, the ultrafilters were established with the input port on the top, connected with the tubing (extended to the peristaltic pump), and subsequently emerged in 1 L of sterile reagent grade water. The ports on the side were closed and the bottom output port was attached to a small piece of tubing. After the filtration apparatus was established, the filter was flushed with 1 L of sterile water to flush out storage liquid of the filter units. Filtered water exited the ultrafilter through the bottom output port and collected in a waste-tank.

A second set-up was adjusted for filtering the samples. In this setup, the same tubing was emerged into the tank with the sample. In this case, the output port and the side port at the bottom were both closed, while new tubing was connected to the side port at the top (figure 3.2a). After the new set-up was established, 5 to maximum 100 litters per sample were filtered with a single pass through hollow-fibre ultrafilter with the peristaltic pump.

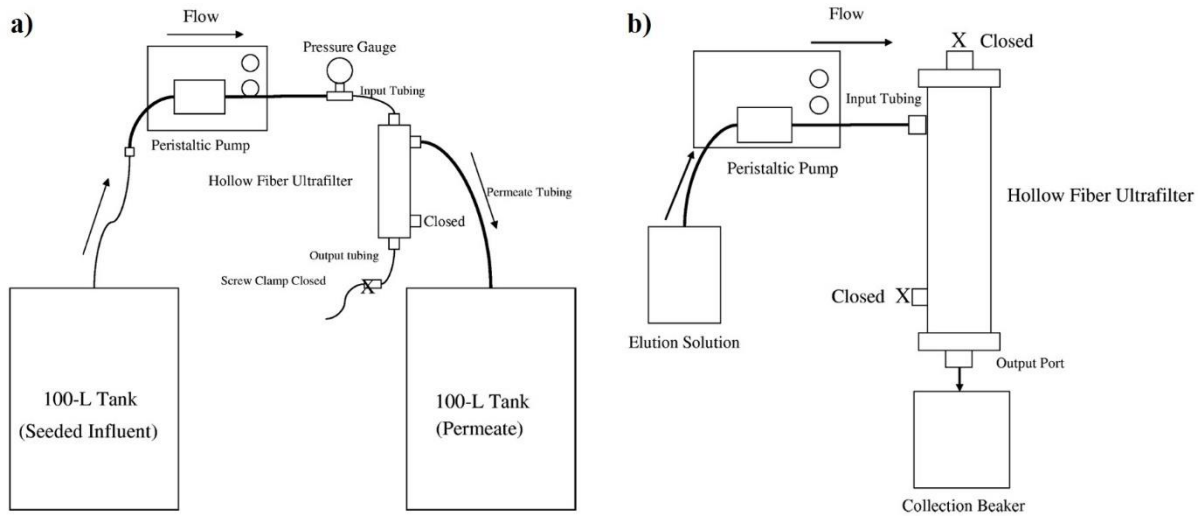

Figure 3.2 Ultrafiltration system set-ups for **a)** filtering the secondary or tertiary effluents, and **b)** backflushing the concentrated biomass. Scheme adapted by Smith and Hill (2009).

Microbes trapped in the hollow fibres of the ultrafilter were recovered by backflushing, using a surfactant solution. This solution was consisted of 1% Antifoam 204 (Sigma-Aldrich, UK), 10% Tween® 80 (Sigma-Aldrich, UK) and 1 % sodium pyrophosphate tetrabasic (Sigma-Aldrich, UK). The filtration set-up was adjusted for backflushing. The filter was inverted, and a new sterile tubing was connected to the new input port at the top, which was extended to the peristaltic pump and emerged into the elution buffer (figure 3.2b). In this configuration, the side ports were closed, and a sterile collection vessel was placed under the output port at the bottom so that there was no liquid loss from the filter. Five hundred millilitres of backflush solution were pumped through the input port at the top and the permeate was collected in the collection vessel.

The recovered backflush samples were then distributed into 50 mL falcon tubes, which were centrifuged. The content was preserved in glycerol, as described before. The same process was followed for tertiary and secondary effluents of TF, GAS and AS. The resulting eluate from the MBR plant was additionally filtered using membrane filtration to ensure all microbes were collected. Briefly, the eluate was filtered through 0.22 µm pore size hydrophilic polyethersulfone membrane filters (Merck Millipore, UK). The filtration system consisted of autoclavable polyphenylsulfone magnetic membrane filter funnels (VWR, UK) fitted in PYREX® borosilicate glass vacuum filtering flasks (VWR, UK), which were connected to a pump through tubing. Sterile membrane filters were applied on the filter funnels where the biomass was retained, whereas filtrates were collected in the filtering flask. Finally, membrane filters were also preserved in 10 mL of 20 % glycerol.
As it was previously mentioned, samples were processed for total bacteria and viable cells. One sample per sampling location per day was collected, providing three biological replicates in each category. Frozen samples were thawed at room temperature and then centrifuged at 4,000 rpm for 20 min. The glycerol centrate was decanted and the pellet or the membrane filters were prepared for DNA extraction. DNA was extracted from the samples for total bacteria analysis using the Fast DNA™ Spin Kit for Soil (MP Biomedicals, USA), according to the manufacturer's instructions. While samples for viable bacteria analysis were processed using PMA (see Section 3.2.5) before DNA was extracted. After DNA extraction, the quality was assessed spectrophotometrically in a NanoDrop apparatus (NanoDrop 2000C, NanoDrop Technologies, Willmington, DE), and DNA was quantified using the Qubit™ dsDNA HS Assay Kits (Fisher Scientific, UK) for use with the Qubit® 2.0 Fluorometer (Thermo Fisher Scientific, UK). Subsequently samples were stored at −20 °C until further use. Purification of DNA was performed using the QIAquick Nucleotide Removal Kit (QIAGEN, UK), according

to the manufacturer's instructions. After DNA was purified, the quality and quantity were re-assessed and an appropriate amount of DNA per sample was freeze-dried and used for HT-qPCR analysis (Section 3.2.3).

HT-qPCR was used to quantify ARGs and MGEs in all samples, except for the MBR permeate and GAC & chlorination discharge. Membrane bioreactor is a hollow-fibre technology which each fibre contains pores that are 0.04 μm in size. Therefore, solids and bacteria larger than 0.04 microns in size are retained on the surface of the fibre. Approximately 50 L per sample were filtered in order to collect enough biomass for HT-qPCR analysis, which requires high amounts of DNA, however the amount of DNA obtained was considerably low. Therefore, qPCR was performed for all four samples collected from this plant, whereas HT-qPCR was used for influents and mixed liquors. The target genes for qPCR were 16S rRNA, *tetM, tetQ, blaOXA-10* and *int1* and the process followed is described in Section 3.2.4.

3.2.3 *High-throughput qPCR analysis*

The dried DNA was sent to the Chinese Academy of Sciences in Xiamen, China for HT-qPCR analysis. Details on the analysis have been reported before (Wang *et al.*, 2014b; Ouyang *et al.*, 2015; Su *et al.*, 2015). The Wafergen SmartChip Real-time PCR system was used to assess the HT-qPCR reactions. The advantage of this system is that many gene quantifications can be performed in parallel by processing 5,184 nanowell reactions per run. The HT-qPCR amplification protocol included 100 nL reaction containing 1× LightCycler 480 SYBR® Green I Master Mix (Roche Inc., USA), nuclease-free PCR-grade water, 1 ng/μL bovine serum albumin, 20 ng/μL DNA template, and 1 μM of each forward and reverse primer. The thermal cycle was set up at initial enzyme activation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 30 s, and annealing at 60 °C for 30 s; finally a melting curve analysis was auto-generated by the programme.

Up to 296 primer sets (table A.4) were included in the assessment, targeting 283 ARGs, 12 MGEs and one eubacterial 16S rRNA gene. ARGs were grouped in nine classes of antibiotics to which they encode resistance to and BLAST on the Antibiotic Resistance Genes Database (ARDB) or the National Center for Biotechnology Information (NCBI) databases were initially used to identify the target genes (Zhu *et al.*, 2013; Wang *et al.*, 2014a; Zhou *et al.*, 2019).

Conventional qPCR was used to quantify absolute copy numbers of 16S rRNA targeting bacteria (table A.4). Roche 480 (Roche Inc., USA) was used to amplify the target gene. The reaction (20 μL total volume) used for the amplification included 10 μL 2× LightCycler 480 SYBR® Green I Master Mix (Roche Inc., USA), 7 μL DNase/RNase-free water, 1 μL DNA template and 1 μM of the same primer used in the HT-qPCR assessment. The amplification cycle was consisted of pre-incubation at 95 °C for 5 min, 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 1 min and 72 °C for 15 s. Since the data produced from the SmartChip Real-time PCR system and the Roche 480 were significantly correlated, relative abundances were transformed to absolute concentrations by normalized to 16S rRNA absolute copies numbers (Ouyang *et al.*, 2015).

3.2.4 *qPCR assays*

Quantification of genes for the MBR was performed using qPCR. In this study, target genes were the 16S rRNA, *int*1, $bla_{\text{OXA-10}}$, *tet*M and *tet*Q. These genes were chosen as they were known to be very abundant in the secondary effluent mainly assessed in the thesis, according to previous work from our group (Jong *et al.*, 2018),. Specific primers, published elsewhere, were used to amplify the target genes and they are shown in table 3.2.

Table 3.2 Primers used in this study.

Target	Primer	Primer sequence (5'-3')	*Base-pairs	Annealing temperature (ºC)	Reference
16s rDNA	1055 F	ATGGCTGTCGTCAGCT	337	60	(Harms *et al.*, 2003)
	1392 R	ACGGGCGGTGTGTAC			
*tet*Q	*tet*Q F	AGAATCTGCTGTTTGCCAGTG	167	60	(Wang *et al.*, 2015)
	*tet*Q R	CGGAGTGTCAATGATATTGCA			
$bla_{\text{OXA-10}}$	$bla_{\text{OXA-10}}$ F	AGAGGCTTTGGTAACGGAGG	191	60	(Wang *et al.*, 2015)
	$bla_{\text{OXA-10}}$ R	TGGATTTTCTTAGCGGCAAC			
*tet*M	*tet*M F	GGTTTCTCTTGGATACTTAAATCAATCR	88	60	(Peak *et al.*, 2007)
	*tet*M R	CCAACCATAYAATCCTTGTTCRC			
*int*1	*int*1 F	GCCTTGATGTTACCCGAGAG	196	60	(Barraud *et al.*, 2010)
	*int*1 R	GATCGGTCGAATGCGTGT			

*Amplicon size

qPCR was performed on a BioRad CFX C1000 System (BioRad, Hercules, CA USA). For quantification of all genes, 2 μL template DNA was used in a reaction mixture containing 7.5 μL SsoFast™ EvaGreen® Supermix (Bio-Rad, USA), 300 nmol/L of each forward and reverse primer (Thermo Fisher Scientific, UK), and H_2O to a final volume of 15 μL. Reaction

conditions for gene quantification included an initial denaturing step at 98 °C for 2 min, followed by 40 cycles with each cycle consisting of denaturation at 98 °C for 5 sec and annealing temperature at 60 °C for 30 sec. Each sample was amplified in triplicate and H_2O replaced template in control reactions to check any contamination. In order to avoid amplification inhibition due to the potential presence of humic acids or other impurities in the samples (Gallup and Ackermann, 2006; Lloyd *et al.*, 2010), DNA template were diluted to a working solution of 5 ng/µL.

Standards for each specific gene were produced from sequenced genes from environmental samples. Initially, PCR was performed, and gel electrophoresis on 1 % agarose gels was assessed for each gene to verify the specificity of the PCR product. The PCR cycling program included 10 sec of initial denaturation at 98 ^{o}C, followed by 30 cycles at 98 ^{o}C for 1 sec (denaturation), 60 ^{o}C for 5 sec (annealing) and 72 ^{o}C for 15 sec (extension), then the final extension was set at 72 ^{o}C for 1 min. The PCR product was then sequenced and the software SnapGene (https://www.snapgene.com/) was used to verify gene sequences. The PCR product was further purified with GenElute^{TM} PCR Clean-Up Kit (Merck Millipore, UK) and Quant-iT^{TM} PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific, UK) was used for quantification. Afterward 10-fold serial dilutions were prepared in a range of 10^8 to 10^1 gene copies/mL. The standards were further validated by performing qPCR. Standards were considered accurate based on the melt curve specificity, efficiency approximately between 90 and 110 %, and $R^2 \geq 0.99$ (figure A.1) (Green and Sambrook, 2012).

Standards exponentially increase between 10 and 35 quantification cycles (Cq), thus, any standard with Cq > 35 were considered unreliable (Green and Sambrook, 2012). Based on the lowest accurate standard on the curves, which was gene-specific, the limits of quantification (LoQ) were 2.53 copies/mL (Cq = 34.4), 22.45 copies/mL (Cq = 34.5), 16.86 copies/mL (Cq = 35), 218.8 copies/mL (Cq = 32.4) and 2.48 copies/mL (Cq = 34.1) for the 16S rRNA, *int*1, $bla_{\text{OXA-10}}$, *tet*M and *tet*Q, respectively.

3.2.5 *Determination of ARGs in viable cells*

Propidium monoazide processing was used to segregate ARGs in cells with intact membranes (here defined as “viable”) from ARGs carried by cells with compromised membranes (“non-viable”). PMA passes through cells with fragmented membranes, therefore only binds to DNA in cells with damaged membranes. Due to this, PCR amplification does not occur since the cell’s DNA is intercalated by covalent bonds with PMA (Nocker *et al.*, 2007). Previous

studies showed this dye distinguishes genes in viable cells, as they showed that DNA extracted from samples with no PMA treatment resulted in genes amplification in both viable and non-viable cells (Li *et al.*, 2014; Truchado *et al.*, 2016; Kibbee and Örmeci, 2017; Eramo *et al.*, 2019).

Here, two protocols were compared to find the most appropriate assay for my experiments. These protocols were considered because they were tested in similar samples to this work. The first protocol was based on the work of Kibbee and Örmeci (2017) and the second on the work of Pang *et al.* (2016). Both PMA protocols were pre-tested on secondary effluents from a WWTP. PMA- and no PMA-treated samples were amplified by qPCR targeting *tet*M (as a "typical" ARG) which are known to be abundant in this effluent, according to previous work from our group (Jong *et al.*, 2018). As it is shown in table 3.3, all data produced from the first protocol were close to the LoQ (see Section 3.2.4), whereas no signal was observed in more dilute samples. This showed that this protocol was probably not going to work because its sensitivity was too close to the LoQ. The probable reason for such a high Cq was because the amount of DNA from only 10 mL of secondary effluent was too low to give an accurate amplification, resulting in false positive signal. On the other hand, when samples were amplified using the second protocol, lower Cq values were found, indicating a stronger signal. Additionally, the samples amplified with PMA-qPCR were significantly lower (Tukey; $p < 0.01$) compared to qPCR confirming that the genes were located in viable cells (figure 3.3). Interestingly, statistical differences were also observed between samples with different dilutions amplified by both qPCR and PMA-qPCR, suggesting, similarly to previous studies (Gallup and Ackermann, 2006), that presence of humic acids or other impurities can be inhibitors for the amplification of genes.

Table 3.3 Quantification cycle (Cq) values of the two protocols tested plus/minus the standard deviation of three replicates per sample. 'Dilution' refers to the dilution factor tested in qPCR per sample and 'Sample' to secondary effluent treated by each protocol.

Sample	Dilution	Cq Mean
1st Protocol PMA - qPCR	x 1	33.19 ± 0.06
	x 10	NA
1st Protocol qPCR	x 1	31.12 ± 0.06
	x 10	NA
2nd Protocol PMA - qPCR	x 1	27.66 ± 0.07
	x 10	29.63 ± 0.51
2nd Protocol qPCR	x 1	23.43 ± 0.07
	x 10	25.75 ± 0.07

Based on pre-testing, the (Pang *et al.*, 2016) method was used here, but with a few modifications. Briefly, 500 mL of fresh collected effluent were filtered using 0.22-μm polyethersulfone membrane filters (see Section 3.2.2). The filters (cut into pieces) were transferred to sterile 15 mL centrifuge tubes, 5 mL of sterile phosphate-buffered saline (PBS) solution added to the tube, and the mixture vortexed for 3 min to release the bacteria into the eluent solution. The pieces of filter were removed, using sterile forceps, and the samples were centrifuged at 4,000 rpm for 20 min. The resultant supernatant was removed, and the pellets were re-suspended in the 500 μL of fresh PBS in sterile ultra-clear polypropylene 2 mL tubes (VWR, UK). The next step consisted of adding PMA to the concentrate, where 2.5 μL of PMA solution (20 mM) was added to obtain a final PMA concentration of 100 μM. The sample was incubated in the dark with occasional mixing for 5 min, followed by light exposure using a 500 W halogen light source (500w Halogen Lamp, TLC Electrical Supplies, UK) for 10 min. In order to avoid excessive heating during light exposure and to maximize light effect, the tubes containing the concentrated samples with PMA were placed horizontally on ice at a distance of approximately 20 cm from the light source and were periodically mixed. After PMA treatment, the DNA was immediately extracted using the Fast DNA™ Spin Kit for Soil (MP Biomedicals, USA), according to manufacturer's instructions, and stored at −20 °C to subsequent analysis.

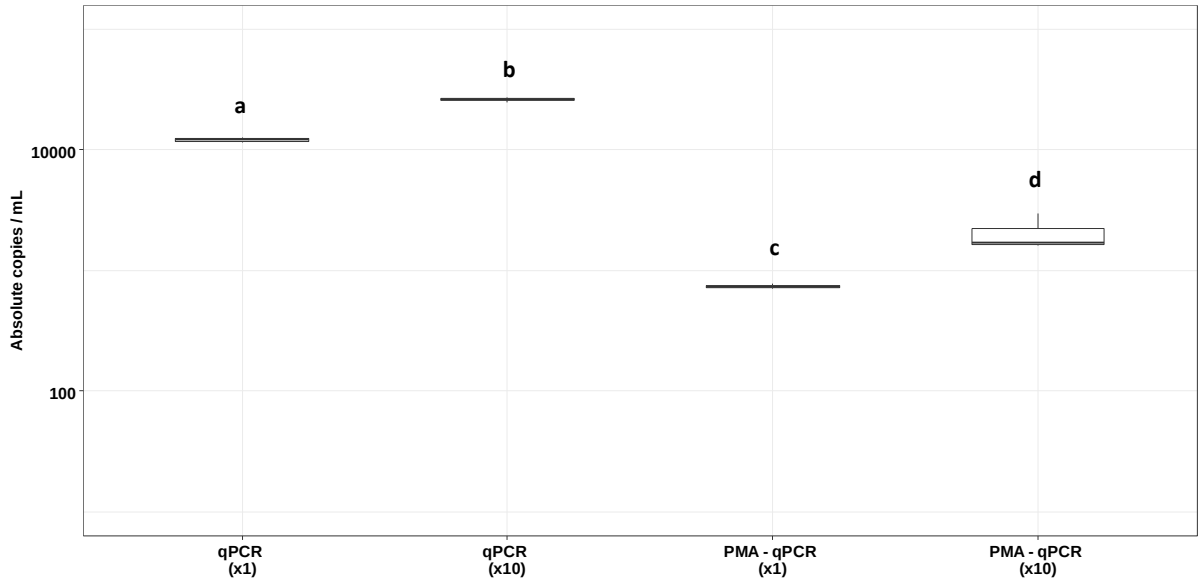

Figure 3.3 Boxplots shows absolute abundances of *tet*M gene in samples treated by the protocol of Pang *et al.* (2016). Boxplots depict each sample in triplicate, tested in different dilution factors ('x1' – no dilution & 'x10' – 1/10 dilution) with or without PMA treatment. The letters (a, b, c, d) indicate significantly different groups ($p < 0.05$).

3.2.6 *HT-qPCR and qPCR data processing and statistical analysis*

Both HT-qPCR and qPCR data were processed and statistically analysed with R studio (version 3.5.2, http://www.r-project.org/). Analysis of the qPCR data is previously described in Section 3.2.4. Regarding HT-qPCR, a threshold cycle (Ct) of 31 was used as the detection limit and any value more than 31 was discarded before subsequent analysis (Ouyang *et al.*, 2015; Xie *et al.*, 2016). Further, as all samples were assessed in triplicate, only samples with amplification in two replicates or more were considered positive and analysed further (Quintela-Baluja *et al.*, 2019). The equation 3.1 was used to calculate relative copy numbers (Chen *et al.*, 2016b). Relative data were transformed to absolute abundances by normalizing to 16S rRNA gene copy numbers quantified with conventional qPCR as described in Section 3.2.3.

$$\text{Relative gene copy number} = 10^{(31-\text{CT})/(10/3)} \qquad \textbf{Equation 3.1}$$

In the Ribosomal RNA Database (rrndb), an estimation of the average number of 16S rRNA that encodes genes per bacterial genome is 4.1 (Klappenbach *et al.*, 2001; Su *et al.*, 2015; Quintela-Baluja *et al.*, 2019). Therefore, 16S rRNA copy numbers were divided by this value to estimate the number of bacterial genomes, and ARG or MGE abundances where then normalised into copies per bacterial cell.

Both HT-qPCR and qPCR data were statistically tested with a significant cutoff of $\alpha = 0.05$. The Bartlett and Sapiro-Wilk tests were used to validate the assumption that sample populations tested were homogeneous and normally distributed, respectively. If both assumptions were met, the data were assumed homogeneous and normally distributed, and significant differences were assessed using one-way analysis of variance (ANOVA) and Tukey post-hoc test for pairwise comparisons. Non-parametric tests, such as Kruskall-Wallis and Games-Howell post-hoc tests, were performed to determine significant differences and assess pairwise comparisons, respectively, when the assumptions for normality and homoscedasticity were violated even in either log or square root transformed data. Additionally, correlation between the genetic and physicochemical data were analysed by the non-parametric Spearman's rank correlation since the assumptions for normality and homoscedasticity were not met. Spearman correlation analysis was performed between physicochemical and molecular data, including influents and final effluents of all WWTPs, excluding MBR final effluent, which data were not available.

3.2.7 *Calculation of ARG and MGE removal rates*

All four WWTs were compared by determining removal rates for ARGs, MGEs and 16S rRNA in each WWTP. The equation used was:

$$\text{Log removal} = \text{Log}_{10}\left(\frac{C_{\text{influent}}}{C_{\text{final effluent}}}\right) \qquad \textbf{Equation 3.2}$$

where, $C_{influent}$ refers to ARG, MGE or 16S rRNA absolute concentrations of the primary settled sewage of the TF, GAS or AS, and screened sewage of the MBR; while $C_{final\ effluent}$ represents ARG, MGE or 16S rRNA absolute concentrations of disc filter discharge (TF system), pile cloth filter discharge (GAS system), secondary clarifier effluent (AS system), and GAC & chlorination discharge (MBR system) depending on treatment technology.

3.3 Results and Discussion

3.3.1 *Diversities of ARGs and MGEs within WWTPs*

A total of 283 ARGs, 12 MGEs and the bacterial 16S rRNA gene were analysed using HT-qPCR. This method quantifies ARGs and clusters them in nine groups including aminoglycosides, fluoroquinolone/quinolone/florfenicol/chloramphenicol/ amphenicol (FCA), β-lactams, macrolide/lincosamide/streptogramin B (MLSB), non-specific (mostly multidrug-efflux pumps), sulfonamides, tetracyclines, vancomycin and 'other' for any gene that does not

clearly classify within the other groups. In addition, MGEs were classified in two groups; transposases and integrases.

Quantifying ARG diversity within treatment plants was one of the principal goals of this work. Therefore, the number of unique genes was totalled across treatment steps in the four WWTPs. Almost one third of the total ARG types were detected in the influent of the TF (figure 3.4a), which was 176 ± 5.5 for total cells and 168 ± 11.2 for viable bacteria. ARG numbers declined in biological unit and disc filter discharges (table A.5), although reductions were not statistically significant (Games-Howell; $p > 0.05$) (table A.6). No significant differences in ARG numbers were found between total and viable cells (Wilcoxon; $p > 0.05$) (table A.7), whereas MGE numbers did not change across treatment steps in TF system.

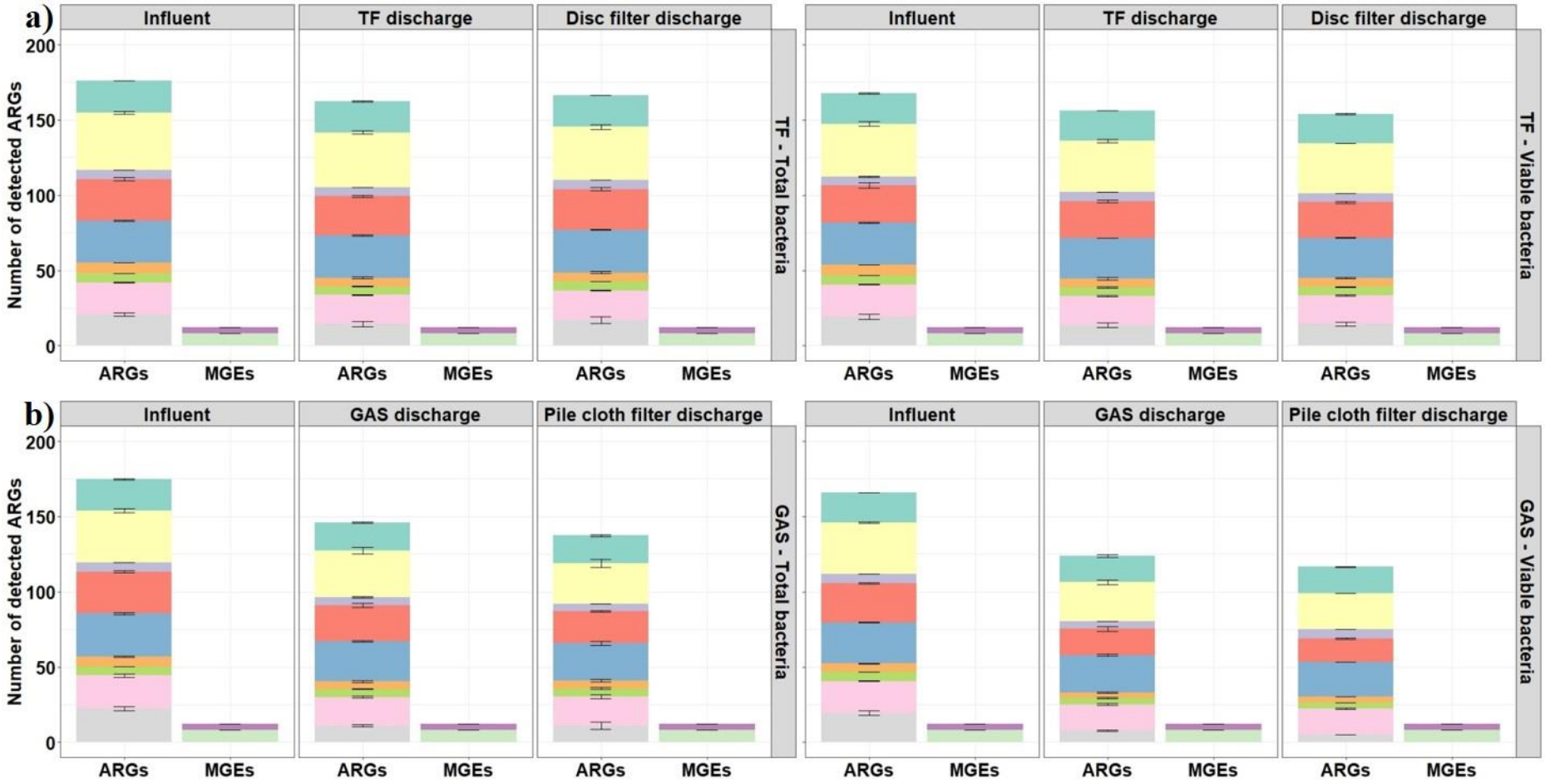

Figure 3.4 Number of unique ARGs and MGEs, classified by antibiotics to which they confer resistance, detected in total and viable bacteria of each treatment stage in **a)** TF, **b)** GAS, error bars represent the mean of triplicate samples collected at each site.

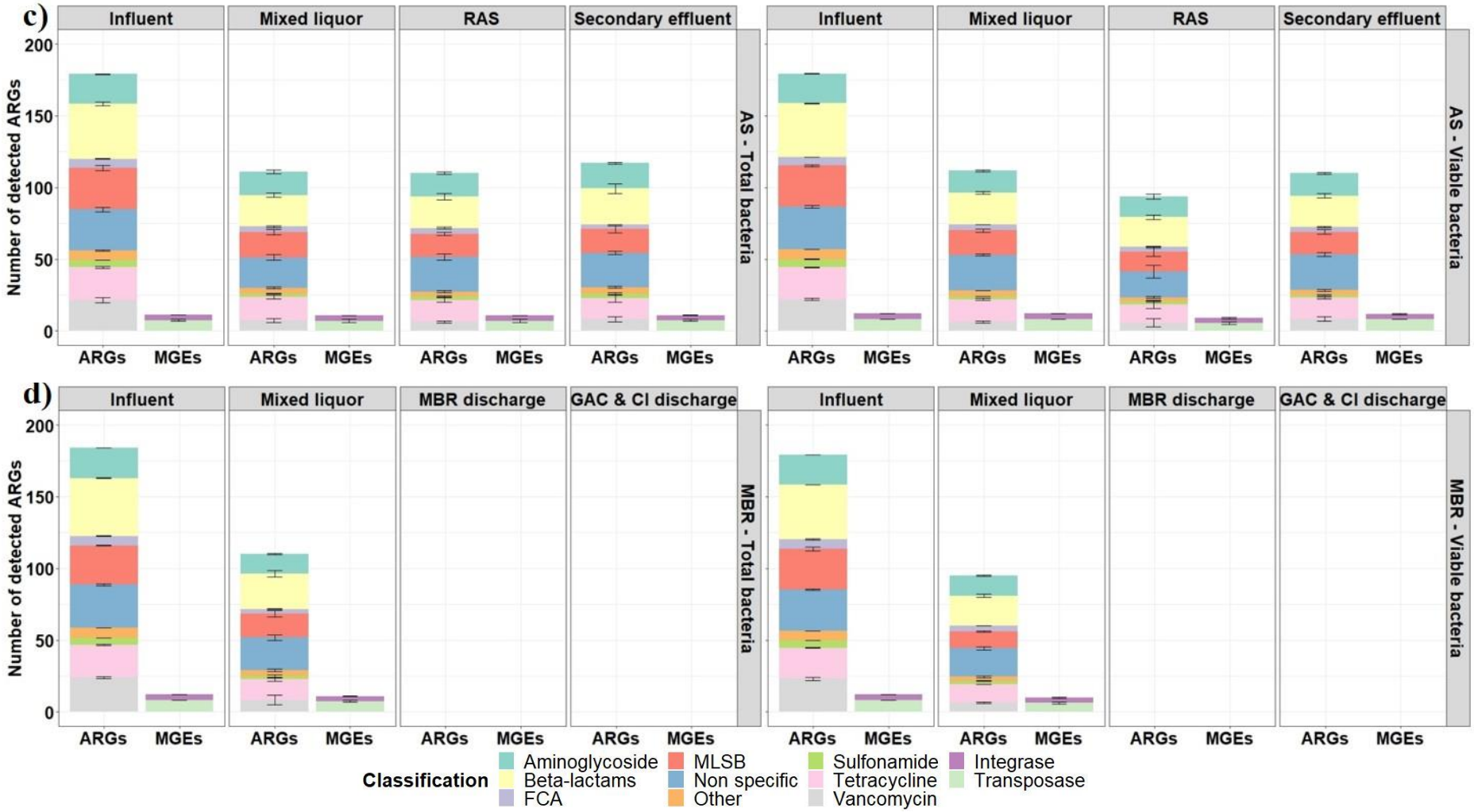

Figure 3. 4(cont.) As above for **c)** AS and **d)** MBR. Empty spaces in the graphs represent the absence of HT-qPCR data for the corresponding samples and error bars represent the mean of triplicate samples collected at each site.

In the GAS system (figure 3.4b), 175 ± 7.7 and 166 ± 1.0 unique ARGs were detected in total and viable bacteria in the influent, respectively (table A.5), which are similar to parallel location in the TF system. Decreases in gene numbers were seen in both GAS discharge (146 ± 6.2 in total and 124 ± 8.7 in viable cells) and the pile cloth filter discharge (137 ± 16.5 and 77.7 ± 67.2 in total and viable cells, respectively); however, these decreases were not statistically significant (table A.6). Although overall ARG number in viable cells was lower than total, no statistical differences were observed (table A.7). ARG numbers of individual classes such as MLSB and vancomycin in total bacteria significantly decreased in GAS and pile cloth filter discharges. These treatment steps also reduced significantly, apart from MLSB and vancomycin, ARG numbers of β-lactams, non-specific, 'other' and tetracycline in viable cells.

In the influent of the AS system (figure 3.4c), 179 ± 12.12 ARGs, were detected, which number was the same for total and viable bacteria (table A.5). Numbers decreased to 111 ± 17.58 (same number for total and viable cells) in the mixed liquor, and to 117 ± 21 and 110 ± 10.8 in the secondary clarifier effluent for total and viable bacteria, respectively. The total number of genes in RAS were 110 ± 17 for total and 93.7 ± 30.3 for viable cells. A comparison between total and viable bacteria per treatment step, revealed no statistically significant differences, as overall numbers of unique ARGs and MGEs were similar (table A.7). Relative to total bacteria, ARG numbers in both mixed liquor and RAS were lower (Games-Howell; $p = 0.03$) compared to influent (table A.6), while significant lower ARG viable cells were observed in mixed liquor, RAS and, secondary clarifier effluent compared to influent (Games-Howell; $p \leq 0.03$).

Activated sludge also was particularly effective at reducing gene diversity in important classes of both broad-spectrum and last resort antibiotics. For instance, FCA and vancomycin classes of total bacteria were lower (Games-Howell; $p < 0.05$) in secondary clarifier effluent compared to influent. In addition, MLSB, β-lactams, sulfonamide, tetracycline and vancomycin ARG numbers were decreased significantly in mixed liquor; whereas all except tetracycline had significant lower numbers in RAS. Regarding viable bacteria, most ARG classes, except non-specific and 'other', were reduced significantly in secondary clarifier effluent; whereas aminoglycoside, β-lactams, MLSB, non-specific, sulfonamide, vancomycin and tetracycline ARG numbers declined significantly in mixed liquor. Finally, β-lactam ARG numbers in viable cells was the only group with significantly reduced numbers in RAS.

Similar to TF and GAS, this WWTP did not significantly reduce MGE numbers in any treatment step (Games-Howell; $p > 0.05$).

These findings are consistent with data obtained in studies of (Yang *et al.*, 2014) and Jiao *et al.* (2017), where they showed that activated sludge reduced ARG diversity in final effluent of an activated sludge process; while others showed that diversity was less affected after treatment (Bengtsson-Palme *et al.*, 2016; Quintela-Baluja *et al.*, 2019). It is noteworthy that ARG diversity in RAS of the AS was significantly lower compared to influent which is in accordance with the study of Quintela-Baluja *et al.* (2019). This suggests that sludge did not enrich wastewater during treatment, since RAS was less diverse than the incoming wastewater. In addition, AS secondary clarifier effluent was more diverse than RAS, although non-significant difference was observed between these two samples. This also shows that ARB that do not flocculate, cannot settle, and will eventually be released in the discharged effluent, as also observed by Quintela-Baluja *et al.* (2019). The present data and other studies (Rafraf *et al.*, 2016; Korzeniewska and Harnisz, 2018) indicated that influent often determines effluent characteristics.

As previously mentioned in Section 3.2.2, due to low DNA amount collected from the MBR permeate and final effluent, HT-qPCR was performed only for the screened sewage and mixed liquor samples (figure 3.4d). Similar ARG numbers as other WWTPs detected in influent from the MBR, which were 184 ± 3 for total and 179 ± 5.5 for viable cells (table A.5). ARG numbers were reduced significantly (Games-Howell; $p < 0.01$) in mixed liquor to 110 ± 21.6 and 95 ± 4.3 for total and viable cells, respectively (table A.6). Relative to individual classes, significant reductions in numbers were achieved for aminoglycoside, β-lactams, FCA, MLSB, vancomycin and tetracycline in total cells, whereas all ARG classes of viable cells in mixed liquor were lower in number (Games-Howell; $p < 0.01$) compared to influent.

Gene diversity of influents was similar among all four plants, however, the effectiveness of each treatment technology at reducing ARG diversity varied. TF had no effect on diversity after treatment, while GAS affected only individual classes. Diversity was significantly decreased in secondary clarifier effluent of the AS. Likewise, a decreasing trend on unique ARG numbers was detected in mixed liquor of the MBR. Although data are not available for MBR permeate and GAC & chlorination discharge, increases in ARG numbers are considered unlikely. MGE diversities were not reduced by any technology.

Even though each treatment technology had a different impact on reducing actual ARG numbers, the overall distribution of genes did not change after treatment. As is shown in figure 3.5a, similar ARG and MGE diversities were observed among the influent of all WWTPs and the distribution of genes of total and viable bacteria was also similar (figure 3.5c). β-lactams had the highest presence in all four plants, with 21.78, 19.85, 21.42 and 21.92 % presence in total cells, and 20.87, 20.68, 20.82 and 21.19 % in viable cells for TF, GAS, AS and MBR, respectively. Aminoglycoside, MLSB, non-specific, tetracycline and vancomycin exhibited similar percentages (15 %), whereas, FCA, 'other', and sulphonamide were the least present (table A.9). Treatment had no impact on the distribution of genes in the final effluents, for both total (figure 3.5b) and viable cells (figure 3.5d). No changes were observed along treatment (figure A.2). This observation as well as the fact that final effluents were less diverse than influents indicate that none of the technologies selected for ARGs.

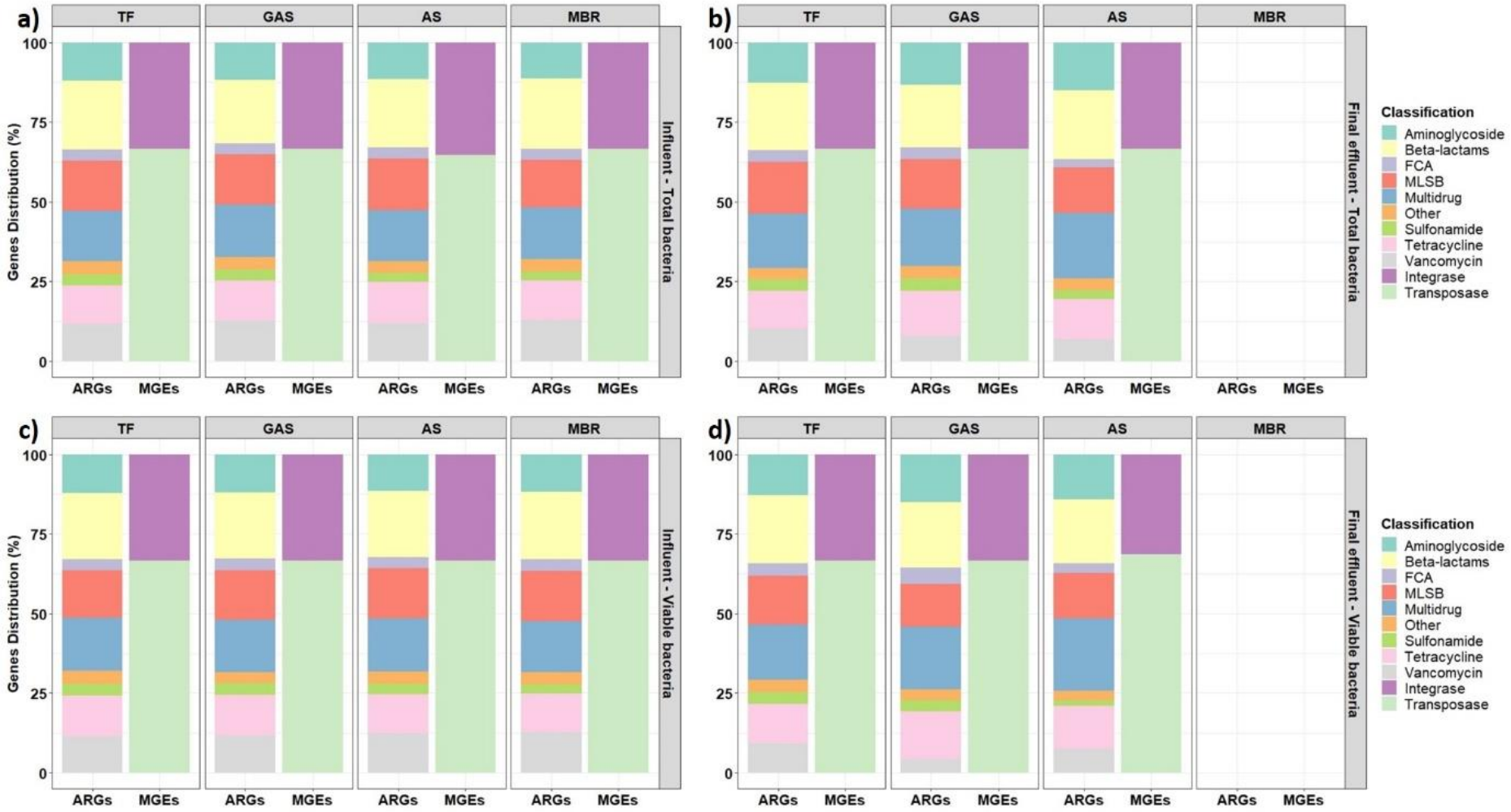

Figure 3.5 Gene distribution in influents and final effluents of **a-b)** total bacteria, and **c-d)** viable bacteria. Influent refers to primary settled sewage of the TF, GAS and AS and screened sewage of the MBR, while final effluents present the disc filter discharge, pile cloth filter discharge, secondary clarifier effluent and GAC & chlorination discharge of the TF, GAS, AS and MBR, respectively.

The three major resistance mechanisms encompassed by ARGs include antibiotic deactivation, efflux pump and cellular protection systems. In the influent of the TF, antibiotic deactivation (44.0 %) was the principal resistance mechanism, followed by efflux pumps (30.6 %) and cellular protection (23.1 %). A similar trend was observed in trickling filter (45.71, 31.43, and 21.43 %) and disc filter discharges (44.39, 31.31, and 22.90); both total and viable bacteria followed the same pattern (table A.8). Regarding influent in GAS, 41.5 % of total bacteria encompassed antibiotic deactivation mechanism, whereas 30.3 and 25.4 % were efflux pumps and cellular protection, respectively. Decreases in cellular protection were observed in GAS discharge (19.7 %) and pile cloth filter discharge (19.4 %). In contrast, percentages of antibiotic deactivation (43.8 %) and efflux pump ARGs (35 %) increased in pile cloth filter discharge. Despite small changes in the percentages, differences were not significant and the overall trends were similar for total and viable cells.

In influent of the AS, similar to TF and GAS, antibiotic deactivation (43 %) was the principal resistance mechanism among total bacteria, followed by efflux pump (30.3 %) and cellular protection (23.6 %). Antibiotic deactivation numbers did not change in percentages between treatment steps, although small decreases in cellular protection ARGs (19 %) were observed in parallel to slight increases in efflux pump ARGs (34.2 %) in secondary clarifier effluent. The same pattern was also detected for viable cells; however, changes in efflux pumps were larger compared to total bacteria. It is also worth mentioning that resistance mechanisms in RAS followed the same pattern observed for mixed liquor and secondary effluent, where antibiotic deactivation was the main mechanism in both total and viable bacteria (table A.8). Similar patterns were also observed for ARG mechanisms in the MBR plant. Specifically, 42.5, 30.2 and 24.2 % were the percentages for antibiotic deactivation, efflux pumps and cellular protection, respectively, for total bacteria in influent. Increases (38.9 %) in efflux pumps and deceases (16.7 %) in cellular protection mechanisms were detected in mixed liquor, while antibiotic deactivation remained the principal resistance mechanism. Similar trends were observed for viable cells.

Overall, results across WWTPs show that antibiotic deactivation was the principal resistance mechanism among ARGs in influents, followed by efflux pump and cellular protection mechanisms. However, decreases in cellular protection and increases in efflux pump ARGs were evident along the process train in GAS, AS and MBR WWTPs.

3.3.2 *Persistent genes in final effluents*

Venn diagrams overlaying ARGs and MGEs present in all samples show the number of persistent genes in the final effluent of each treatment plant, that is, the types of genes that are always present after all treatment steps. Furthermore, only ARGs detected on all three sampling days were taken into consideration for this analysis.

The number of persistent genes in TF samples for both total and viable cells are shown in figure 3.6a. Persistent gene numbers in total bacteria (170) were higher compared to those in viable cells (157). Interestingly, all persistent genes in viable cells also were present in total bacteria (table A.10). It is also noteworthy that all ARG types were introduced to the treatment system from the influent; nevertheless, few ARGs were uniquely present in TF discharge and disc filter discharge (table A.11). Persistent genes in total bacteria were higher (146) than the viable cells (113) in the pile cloth filter discharge of the GAS. Similar to TF, all persistent genes in viable cells were also present in total bacteria (table A.10); whereas in contrast to TF, no unique ARGs were only found in pile cloth filter discharge which is the final effluent in this WWTP (figure 3.6b).

In the AS system, persistent genes in total bacteria (115) were higher than those in viable cells (87) (figure 3.6c), and all genes in viable cells were also present in the total fraction (table A.10). Like GAS, all ARGs present in secondary clarifier effluent (final effluent) were also present in previous treatment steps; whereas one gene in viable cells was seen only in the secondary clarifier effluent and not in other treatment locations. RAS included no gene previously present in either influent or mixed liquor. Persistent genes in final effluent of MBR were not determined as HT-qPCR analysis was not conducted for these samples (figure 3.6d). However, most genes in mixed liquor also were present in influent, both in viable (129) and total cells (105). Among genes present solely in mixed liquor (table A.11), *bl2a_iii* was the one detected in both total and viable fractions, whereas the rest were not associated with a specific class or resistance mechanism.

In summary, AS system had the lowest number of persistent genes in final effluent, both total and viable bacteria, whereas the TF had the most. However, despite the lack of data, the MBR probably was lower than AS, but this cannot be proved due to lack of DNA. A few ARGs were detected only in final effluents (e.g., in the TF), but this is most likely anomalous due to small differences in abundances of ARGs near the HT-qPCR detection limit.

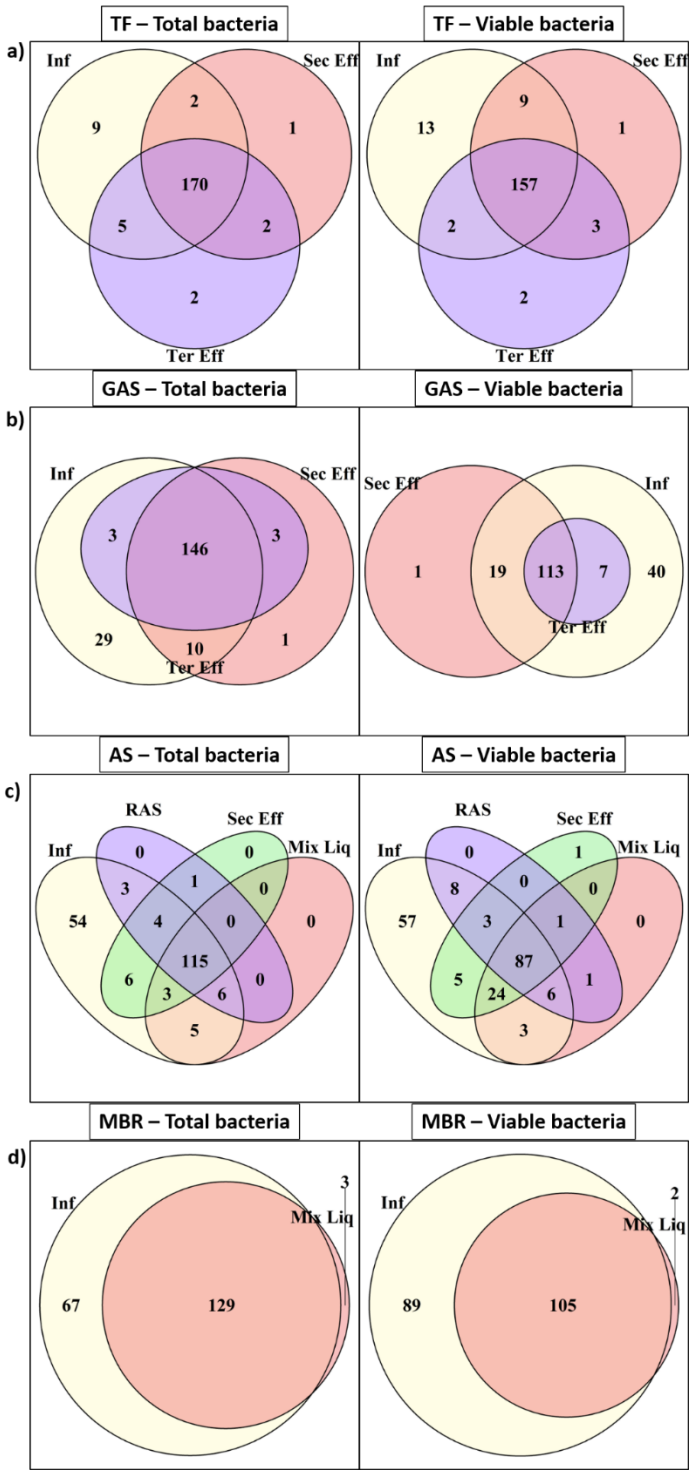

Figure 3.6 Venn diagrams showing common genes among all treatment stages in **a)** TF, **b)** GAS, **c)** AS and **d)** MBR for both total (right) and viable (left) cells. 'Inf' stands for influent, 'Sec eff' for secondary effluent, 'Ter eff' for tertiary effluent, and 'Mix liq' for mixed liquor (see Section 3.2.1 for determination of samples). The number located in the intersection of all treatment stages shows genes that entered the treatment system and were not removed in any treatment step (persistent genes).

3.3.3 *ARG and MGE abundances within the WWTPs*

One of the principal objectives of this work was to determine the efficacy of each WWTP technology to reduce absolute ARG concentrations. Thus, absolute abundances of ARGs and MGEs in each treatment step were determined as described previously in Section 3.2.7. Table A.12 summarises summed ARG or MGE absolute concentrations per treatment step in total and viable cells. Standard deviations represent variation across three sampling days (biological replicates) of which each day had three technical replicates.

ARG and MGE absolute concentrations of both total and viable cells in influent, TF and disc filter discharges of the TF system are depicted in figure 3.7a. Absolute abundances in influent were $8.96 \times 10^7 \pm 3.32 \times 10^7$ and $5.89 \times 10^7 \pm 2.25 \times 10^7$ copies/mL, for ARGs and MGEs, respectively, in total bacteria. Absolute concentrations for both ARGs and MGEs in total cells significantly decreased (Games-Howell; $p < 0.01$) in the following treatment steps (table A.13). Similar trends were observed for ARG and MGE absolute abundances in viable cells. Although gene means in viable cells were lower than genes in total cells, these differences were not statistically significant (table A.14). However, ARG absolute concentrations in viable cells of the disc filter discharge were significantly lower compared to these in total bacteria; although the same trend was not seen for MGEs.

GAS also was effective at reducing absolute abundances (figure 3.7b). ARGs in influent were $1.32 \times 10^8 \pm 9.77 \times 10^7$ copies/mL in total bacteria and $4.70 \times 10^7 \pm 2.34 \times 10^7$ copies/mL in viable (table A.12). All treatment steps significantly reduced (Games-Howell; $p < 0.01$) ARG absolute concentrations of both total and viable cells with similar decreasing patterns also observed for MGEs (table A.13). ARG and MGE viable cell abundances were lower compared to total bacteria (table A.14), which was significant (Wilcoxon; $p < 0.05$) in most steps, including the pile cloth filter discharge.

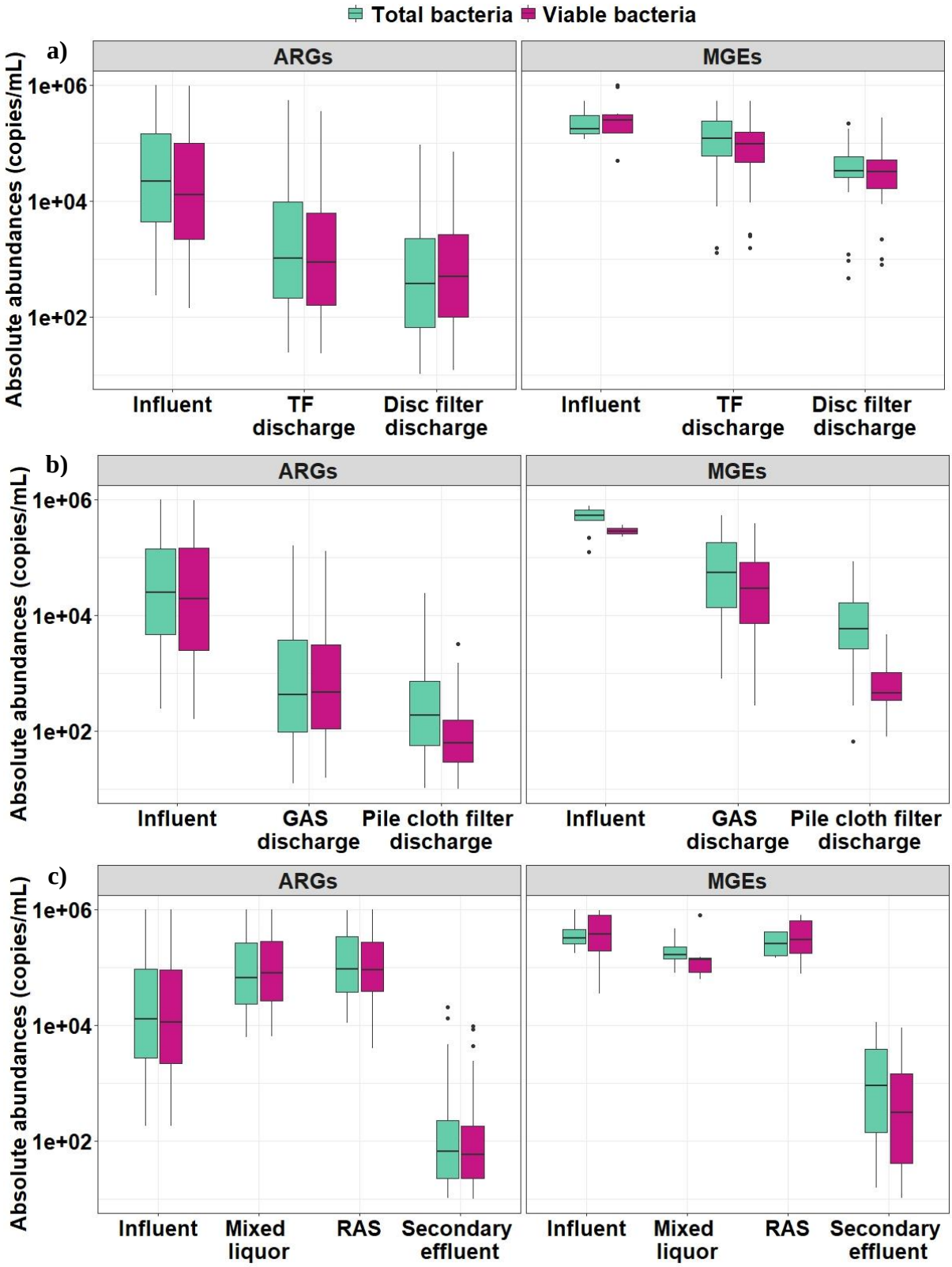

Figure 3.7 Absolute abundances grouped by ARGs and MGEs and treatment stage in **a)** TF, **b)** GAS, and **c)** AS WWTPs. Boxplots contain absolute concentrations of all ARGs and MGEs per treatment stage in both total and viable cells. Boxes represent the first quartile and third quartile of the data, the vertical line shows the median, whiskers extend from each quartile to minimum and maximum data points and outliers are depicted with dots.

Regarding AS, absolute abundances of ARGs and MGEs in total bacteria were $7.84 \times 10^7 \pm 3.50 \times 10^7$ and $5.69 \times 10^7 \pm 2.48 \times 10^7$ copies/mL, respectively (table A.12); whereas higher absolute concentrations (Games-Howell; $p < 0.01$) were observed in samples from the mixed liquor (figure 3.7c). This was expected since mixed liquor contains bacteria from both wastewater and activated sludge. Despite these increases during treatment, significant decreases were observed in the secondary clarifier effluent of this WWTP (table A.13). Additional to the main treatment locations, samples also were collected from the RAS. Absolute concentrations of both ARGs and MGEs were higher (Games-Howell; $p < 0.01$) compared to influent; nevertheless, non-significant differences were observed between RAS and mixed liquor.

As it is shown in figure 3.7c, viable cells followed similar pattern with total bacteria for both ARGs and MGEs absolute concentrations in AS system. Comparing viable versus total cells, gene means in viable cells were lower than those in total cells in most samples. However, in contrast to TF and GAS, in this WWTP non-significant differences were seen between total and viable cells in the final effluent (table A.14). This is interesting as it suggests that TF and GAS, which include tertiary filtration, had significantly lower ARG and MGE abundances in viable cells compared to total cells. This might suggest that tertiary filtration was particularly effective at reducing the viable fraction; nonetheless, it might also indicate that bacteria with compromised membranes (non-viable bacteria) were produced as a result of the mechanical process. In contrast, secondary sedimentation, with no mechanical stress applied to bacteria, reduced both viable and non-viable samples at the same rate.

In the MBR system, HT-qPCR was performed only for influent and mixed liquor (figure A.3). Data showed that both ARG and MGE absolute concentrations in total cells increased significantly (Games-Howell; $p < 0.01$) in mixed liquor (table A.13), whereas non-significant increases were observed for viable cells. Even though similar absolute concentrations were identified between total and viable cells in influent (table A.14), ARG and MGE absolute concentrations in viable cells were lower than total cells (Wilcoxon for total and t-test for viable cells; $p < 0.01$) in mixed liquor, indicating that many genes were carried in non-viable cells.

Since HT-qPCR was not performed for the final treatment steps in the MBR system, qPCR was used to estimate gene reduction in this WWTP (figure 3.8). Absolute concentrations of 16S rRNA in total bacteria were $1.66 \times 10^8 \pm 5.09 \times 10^7$ copies/mL and they were increased significantly (Games-Howell; $p < 0.01$) to $2.07 \times 10^9 \pm 2.47 \times 10^8$ copies/mL in mixed liquor.

Such increases were followed by sharp decreases in MBR permeate and GAC & chlorination discharge (tables A.15 and A.16). Although absolute concentrations in MBR permeate and GAC & chlorination discharge were both lower (Games-Howell; $p < 0.01$) compared to previous treatment steps, no significant differences were observed between them (Tukey; $p = 0.56$). *Int*1 followed similar trend to the 16S rRNA for influent and mixed liquor; whereas absolute abundances in both later steps were below the LoQ. In contrast to 16S rRNA and *int*1, $bla_{\text{OXA-10}}$, *tet*M and *tet*Q absolute abundances were significantly (Games-Howell; $p < 0.01$) lower in mixed liquor compared to influent, whereas they were below the LoQ in MBR permeate and GAC & chlorination discharge samples. Genes in viable cells followed similar pattern to total bacteria (table A.15), always being significantly lower (t-test; $p < 0.01$) than total cells (table A.17).

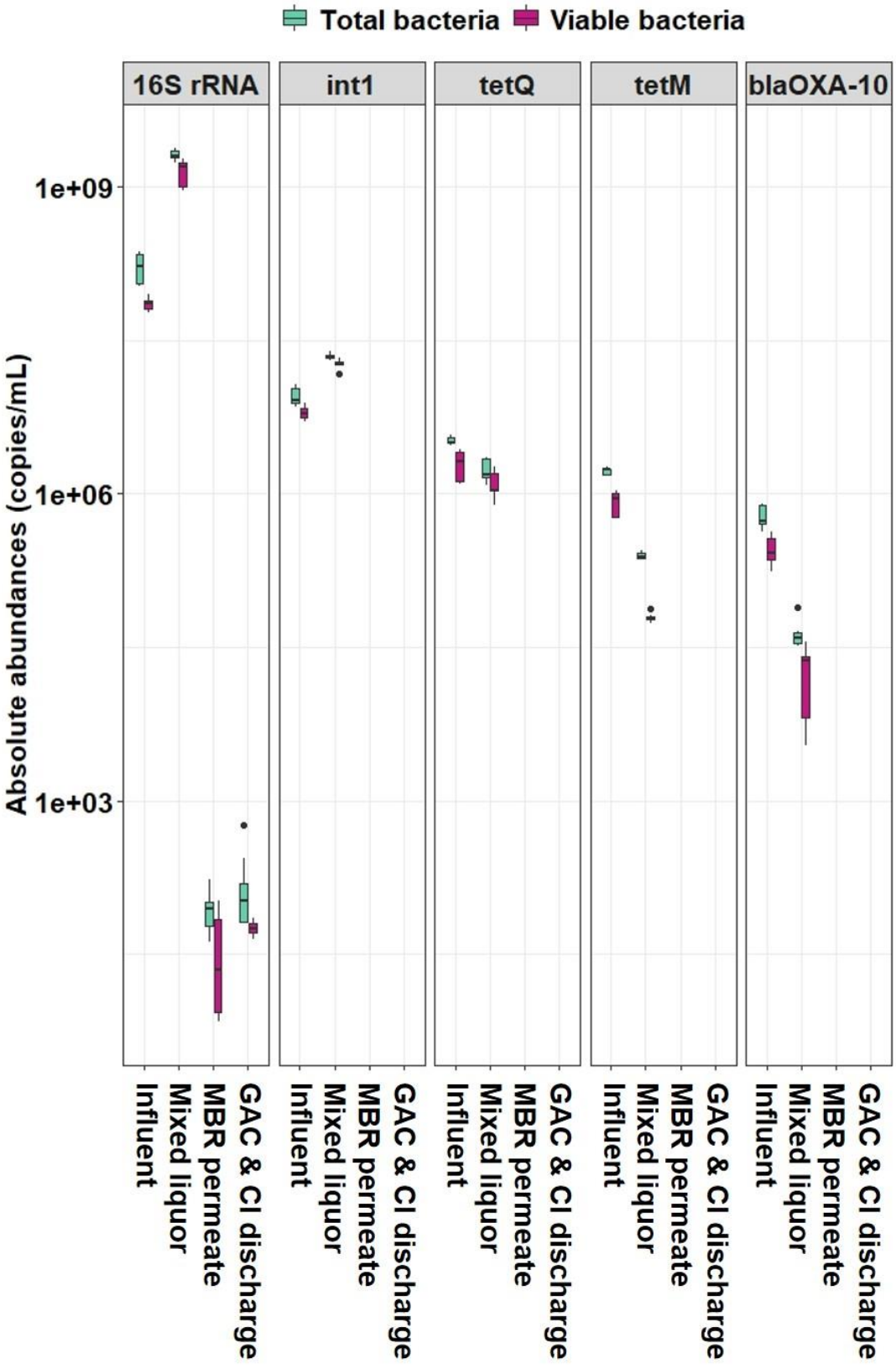

Figure 3.8 Absolute abundances of 16S rRNA, *int*1, *tet*Q, *tet*M and $bla_{\text{OXA-10}}$ in both total and viable cells in MBR WWTP. Boxplots represent absolute concentrations of genes per treatment step.

3.3.4 *Relative ARG and MGE removal rates across the different WWTPs*

The removal rates of ARGs, MGEs and 16S rRNA for both total and viable bacteria are provided in figure 3.9. The TF was the least effective at gene removal compared to other treatment technologies across the whole WWTP. Specifically, log removals of ARGs, MGEs and 16S rRNA genes in total bacteria were 1.98 ± 0.01, 2.04 ± 0.06 and 1.57 ± 0.04, respectively, and 1.62 ± 0.02, 1.71 ± 0.11 and 1.27 ± 0.07 in viable cells (table A.18). Significantly higher log removals (Games-Howell; $p < 0.05$) were achieved by GAS compared to TF for all three groups (table A.19). GAS achieved 2.80 ± 0.02, 2.90 ± 0.10 and 2.77 ± 0.13 log removals for ARGs, MGEs and 16S rRNA in total cells, respectively, and 3.20 ± 0.02, 3.43 ± 0.08 and 2.90 ± 0.14 in viable cells. Similarly, higher removal rates were observed in the AS WWTP, where log removals were 3.18 ± 0.04, 3.76 ± 0.18 and 2.80 ± 0.07, and 3.18 ± 0.04, 3.91 ± 0.18 and 2.74 ± 0.07 in total and viable cells for ARGs, MGEs and 16S rRNA genes, respectively. Although AS was significantly (Games-Howell; $p < 0.01$) better at gene removal than TF, AS and GAS were not significantly different. ARG and MGE removals in AS and GAS were significantly different among total bacteria (Games-Howell; $p < 0.05$), but differences were not significant for viable cells.

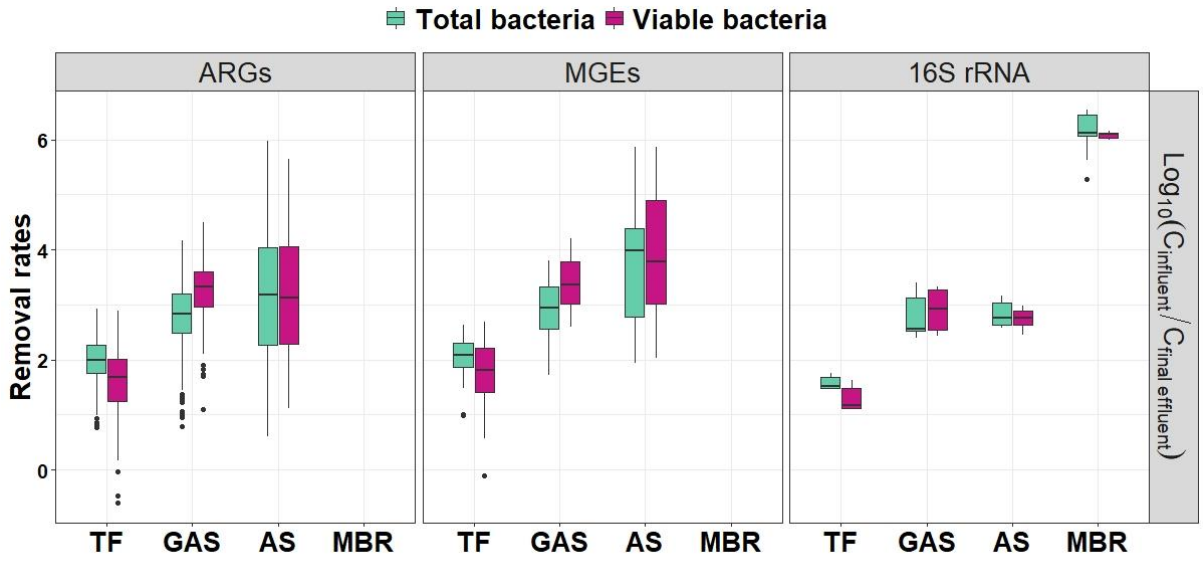

Figure 3.9 Removal rates per treatment technology grouped in ARGs, MGEs and 16S rRNA. Boxes represent the first quartile and third quartile of the data, the vertical line shows the median, whiskers extend from each quartile to minimum and maximum data points and outliers are depicted with dots.

Removal rates of ARGs and MGEs in MBR plant were not determined, as neither HT-qPCR nor qPCR data were available for the final effluent. Therefore, comparisons among all four plants were based on removal rates from 16S rRNA data. Very high removal rates were achieved in the MBR plant; i.e., 6.10 ± 0.14 and 6.08 ± 0.02 log removals for total and viable bacteria, respectively. These were significantly higher than the other three WWTPs (Games-Howell; $p < 0.01$).

One interesting finding was a strong positive correlation between 16S rRNA, ARGs and MGEs (figure 3.10). This suggests that decreases of 16S rRNA genes (i.e., "all" bacteria) mirror reductions in bacteria carrying ARGs and MGEs, including both total and viable cells; removing bacteria in general reduces AMR levels. This is in agreement with other studies that have suggested ARG reductions are driven by "all" bacteria removal (Chen and Zhang, 2013b; Yang *et al.*, 2014; Al-Jassim *et al.*, 2015; Bengtsson-Palme *et al.*, 2016; Karkman *et al.*, 2016). Based on this, MBR would be expected to be the most effective technology at reducing ARBs, which data here hint. This could not be 100% confirmed due to lack of HT-qPCR data, nonetheless, the log removal of over six of 16S rRNA genes implies that is the case. Quintela-Baluja *et al.* (2019) recently showed that improving biosolids separation after previous treatment was critical to reducing ARG releases, and MBRs might be a good option. In fact, our data revealed that the technology with the greater capacity to remove bacterial loads from the liquid phase effluent was the most effective at reducing ARB.

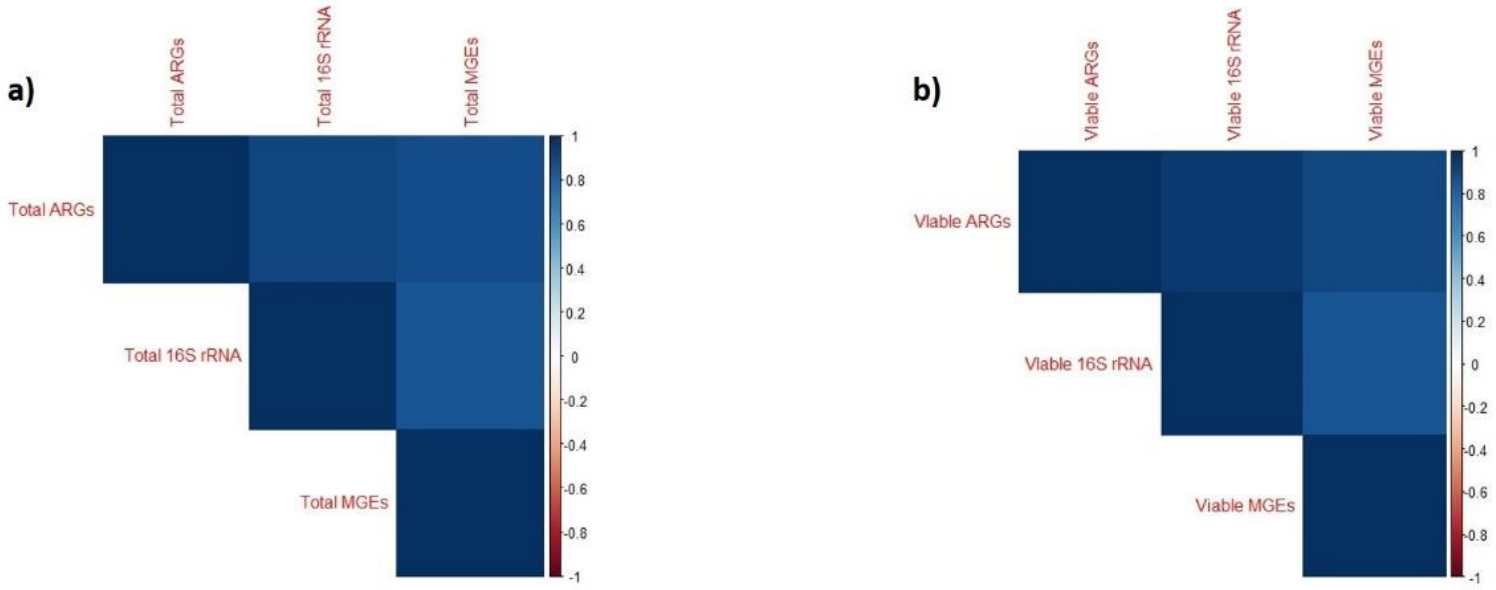

Figure 3.10 Correlograms, based on Spearman rank correlation, present the correlations among ARG, MGE and 16S rRNA removal rates. Data includes removal rates of TF, GAS and AS plants for **a)** total bacteria and **b)** viable bacteria. Blue and red present positive and negative correlations, respectively.

Among WWTP options, AS is the most studied technology in literature. As identified before (Krzeminski *et al.*, 2019), direct comparisons among technologies is particularly challenging due to different methodological approaches (cultivation or culture-independent methods), different operational or water characteristics, as well as climate conditions and sampling schemes; nevertheless, general observations are possible. Although most studies performed culture- or qPCR-dependent assays, there is a rising interest in resistomic analysis. Hence, many studies monitoring a wide range of ARGs in conventional activated sludge WWTPs, some combined with additional treatment steps (Karkman *et al.*, 2016), and others not (Bengtsson-Palme *et al.*, 2016; Jiao *et al.*, 2017; Jiao *et al.*, 2018; Quintela-Baluja *et al.*, 2019), revealed significant decreases in ARG absolute abundances in the discharged water, reporting up to 99.82 % total removal (Yang *et al.*, 2014). Our data agree with these findings, showing that AS systems can significantly reduce a broad array of ARGs from wastewater. Other studies show similar decreases in targeted ARGs using qPCR (Laht *et al.*, 2014; Mao *et al.*, 2015; Rodriguez-Mozaz *et al.*, 2015; Zhang *et al.*, 2015a), including log removals of 1.2 to 1.8 (Wen *et al.*, 2016), 1.0 to 3.0 (Chen and Zhang, 2013b) and 4.2 (Le *et al.*, 2018). However, AS also has shown poor performance in reducing ARG (Al-Jassim *et al.*, 2015; Rafraf *et al.*, 2016). Although this difference is concerning, we speculate the reason for such poor performance is related to operational problems in those cases, although it is hard to prove because inadequate operating data were provided.

A number of studies have examined the relative effectiveness of MBRs to reduce AMR from wastewater. Data from qPCR analysis showed large decreases in ARGs, even below the LoQ, of targeted genes in MBR effluents (Du *et al.*, 2015; Zhang *et al.*, 2015a; Zhang *et al.*, 2018), and log removals up to 7.1 have also been reported (Le *et al.*, 2018). These findings agree with our results where 6.10 log removals were achieved for 16S rRNA genes. Furthermore, some studies also showed increases in specific ARG concentrations in mixed liquor of aerobic compartments (Du *et al.*, 2015; Zhang *et al.*, 2015a; Zhang *et al.*, 2018), while Le *et al.* (2018) showed that increases or decreases closely depended on gene type. The later observation is in accordance with the results of this work, where qPCR data revealed decreases in target ARG absolute concentrations (qPCR data), whereas an increasing trend was seen for a wider range of genes (HT-qPCR data) in mixed liquor. It can thus be suggested that aerobic reactors affect differently ARGs, which may be due to the fact that sludge retention time (SRT) may affect in a different extent the microbial community, as discussed before (Zhang *et al.*, 2018).

The effectiveness of GAS at reducing ARGs in full-scale WWTPs has not been conducted before, although previous lab-scale testing showed this technology has potential for reducing ARGs by decreasing plasmid transfer among bacteria (Zou *et al.*, 2016), which agrees with our data. On the other hand, possible risk of ARB selection, in the presence of high levels of antibiotics in the matrix, has been shown as well (Wan *et al.*, 2018; Zhang *et al.*, 2019a). Relative to trickling filter, biofilters either as the principal treatment compartment or mainly in association with other treatment technologies such as activated sludge or constructed wetlands have shown good performance (Chen and Zhang, 2013b; Karkman *et al.*, 2016) but they are less studied.

3.3.5 *Relative abundances of ARGs and MGEs in the different WWTPs*

Relative abundances, expressed as gene absolute abundances normalized per bacterial genome (see Section 3.2.6), were quantified to determine the relative selection of ARGs and MGEs carried by cells within treatment steps in each WWTP. Summed relative abundances of total ARGs decreased significantly (Games-Howell; $p < 0.01$) (table A.21) from 0.875 ± 0.215 gene copies/cell in influent to 0.427 ± 0.075 and 0.286 ± 0.072 gene copies/cell in TF discharge and disc filter discharge, respectively. As is shown in figure 3.11a, tetracycline (0.273 ± 0.042 gene copies/cell) was the most abundant class per cell in influent, followed by MLSB (0.241 ± 0.055 gene copies/cell), non-specific (0.152 ± 0.012 gene copies/cell), aminoglycoside (0.097 ± 0.009 gene copies/cell), and β-lactams (0.082 ± 0.010 gene copies/cell) (table A.20). Tetracycline had significant lower (Games-Howell; $p < 0.05$) relative abundances after later treatment steps. Furthermore, relative abundances of aminoglycosides, β-lactams and transposons also were significantly reduced in the disc filter discharge. Interestingly, a non-significant increase in non-specific class was detected from influent (0.152 ± 0.012 gene copies/cell) to TF discharge (0.202 ± 0.011 gene copies/cell), followed by a significant decrease (Games-Howell; $p = 0.04$) in disc filter discharge (0.092 ± 0.008 gene copies/cell). This class is primarily non-specific efflux pumps, which have previously been observed to increase in WWTPs, probably due to the multiple minor stressors in biological treatment units.

Similar patterns were seen for total and viable bacteria cells in TF samples. Although the overall relative abundances in viable cells were lower than in total bacteria (table A.20), no significant differences were observed (table A.22). Significant decreases were achieved in TF and disc filter discharges compared to influent (table A.21). Specifically, tetracycline (0.222 ± 0.031 gene copies/cell), non-specific (0.142 ± 0.004 gene copies/cell), MLSB (0.132 ± 0.020

gene copies/cell), β-lactams (0.073 ± 0.008 gene copies/cell) and aminoglycoside (0.068 ± 0.008 gene copies/cell), were significantly decreased after disc filters. This WWTP was effective at reducing (Games-Howell; $p < 0.01$) relative abundances of transposases in both trickling filter and disc filter discharges; nevertheless, integrons did not consequently change.

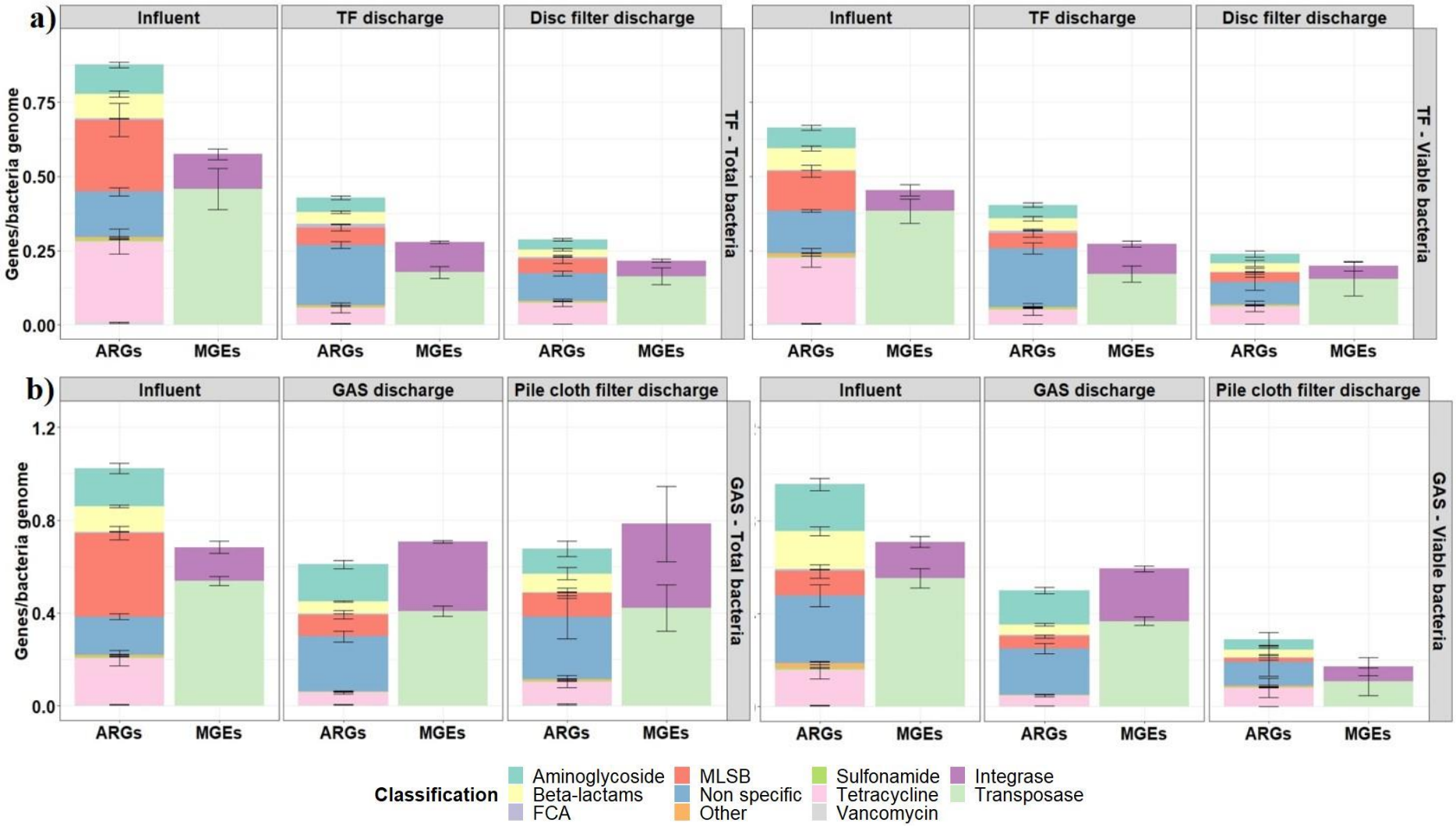

Figure 3.11 ARGs and MGEs relative abundances normalised per bacteria genome in total and viable bacteria of each treatment stage in **a)** TF, **b)** GAS, error bars represent the mean of triplicate samples collected at each site.

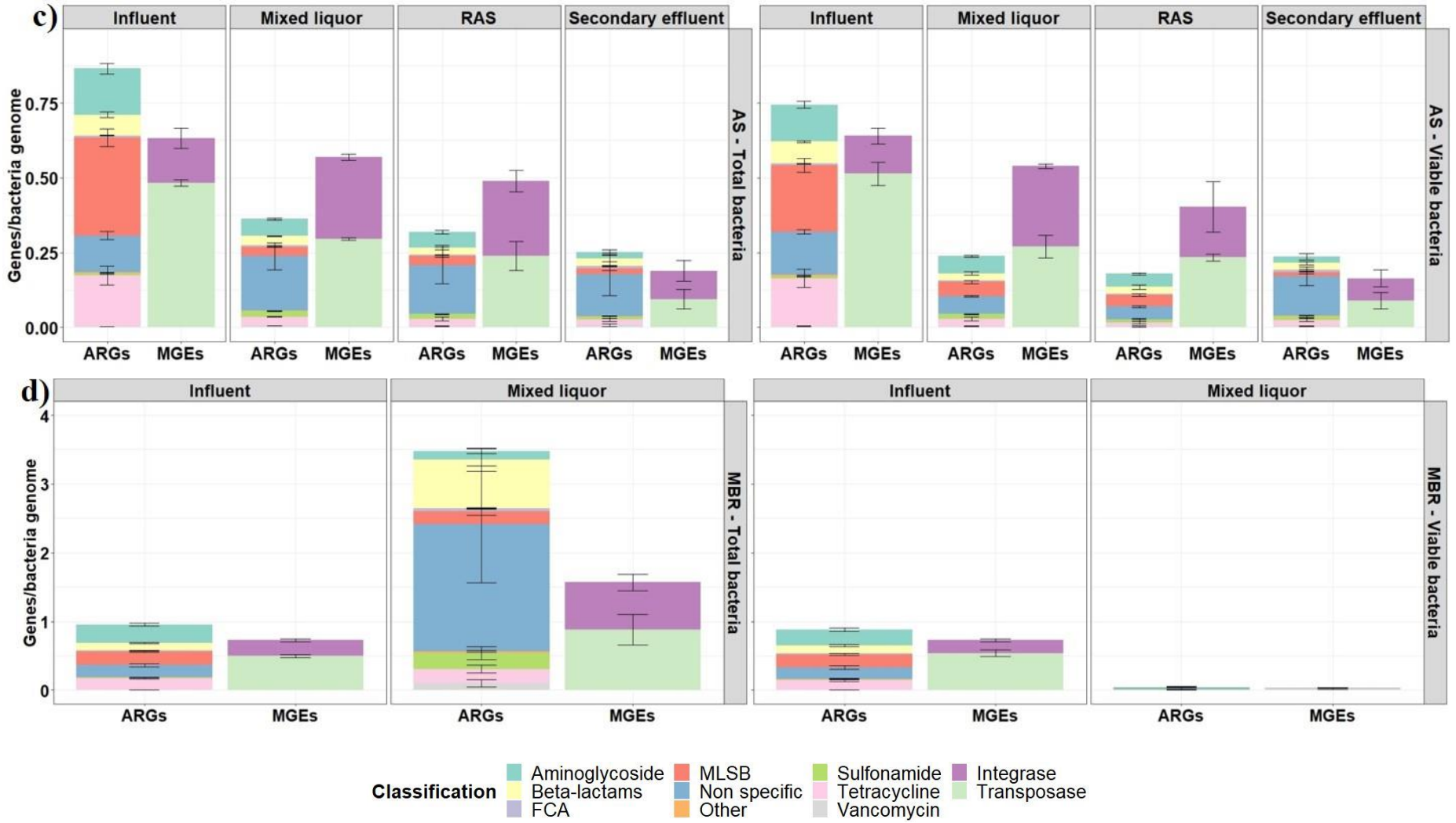

Figure 3. 11(cont.) ARGs and MGEs relative abundances normalised per bacteria genome in total and viable bacteria of each treatment stage in **c)** AS, **d)** MBR, error bars represent the mean of triplicate samples collected at each site.

In the GAS system, relative abundances of total bacteria (figure 3.11b) in influent (1.022 ± 0.055 gene copies/cell) declined in the following treatment steps, including GAS discharge (0.609 ± 0.077 gene copies/cell) and pile cloth filter discharge (0.677 ± 0.318 gene copies/cell) (table A.20). However, these decreases were not statistically significant (table A.21). The most abundant classes in influent were MLSB (0.359 ± 0.029 gene copies/cell), tetracycline (0.200 ± 0.033 gene copies/cell), non-specific (0.163 ± 0.011 gene copies/cell), aminoglycoside (0.163 ± 0.022 gene copies/cell), and β-lactams (0.109 ± 0.006 gene copies/cell). Although relative abundances of most classes in GAS and pile cloth filter discharges were lower compared to influent, only tetracycline and β-lactams significantly decreased in GAS discharge.

A different pattern was observed in viable versus total bacterial cells (figure 3.11b). The overall relative abundances decreased from 0.955 ± 0.146 gene copies/cell in influent to 0.500 ± 0.07 and 0.290 ± 0.270 gene copies/cell in GAS and pile cloth filter discharges, respectively (table A.20). Significantly lower values were seen between influent and pile cloth filter discharge (table A.21). Among individual classes, β-lactams' and FCA relative abundances reduced significantly. Similarly, transposases were significantly lower in the pile cloth filter discharge (Games-Howell; $p = 0.01$), whereas integrases were not significantly different (Games-Howell; $p > 0.05$). The relative abundances between total and viable bacteria in pile cloth filter discharge were significantly different for ARGs and MGEs (Wilcoxon; $p < 0.01$) (table A.22), suggesting that differentiating total from viable fraction may be important in understanding how each technology affects total versus viable cells.

In the AS system (figure 3.11c), summed relative abundances per genome in total bacteria decreased significantly (Games-Howell; $p < 0.05$) from 0.865 ± 0.067 gene copies/cell in influent to 0.238 ± 0.04 and 0.251 ± 0.192 gene copies/cell in mixed liquor and secondary clarifier effluent, respectively (table A.20). Likewise, overall ARG relative abundances in RAS (0.179 ± 0.043 gene copies/cell) were significantly lower (Games-Howell; $p < 0.01$) compared to influent. The most abundant class in influent was MLSB (0.327 ± 0.029 gene copies/cell), followed by tetracycline (0.170 ± 0.031 gene copies/cell), aminoglycoside (0.154 ± 0.017 gene copies/cell), non-specific (0.123 ± 0.014 gene copies/cell), and β-lactams (0.069 ± 0.009 gene copies/cell). Among these, aminoglycoside, MLSB, tetracycline and transposons were significantly reduced in secondary clarifier effluent (table A.21) compared to relative influent levels. Relative abundances of the aminoglycoside, 'other' and tetracycline classes in RAS also were significantly lower (Games-Howell; $p < 0.05$) than influent. An increase in

non-specific relative presence per genome was also observed during treatment in this WWTP; however, this increase was not significant.

Regarding overall relative abundances in viable cells of the AS, decreases were detected across treatment steps (figure 3.11c); however, these decreases were not statistically significant (table A.21). This shows that significant decreases in the total bacteria, we saw previously, were mostly related to non-viable cells. Regarding individual classes, secondary clarifier resulted in significant reductions of relative abundances in dominant classes, including aminoglycosides, MLSB, tetracyclines, and transposases; whereas RAS reduced tetracycline relative abundances significantly compared to influent. What is surprising is that relative abundances of the non-specific class exhibited a decreasing pattern in both mixed liquor and RAS compared to influent; whereas similar relative abundances were observed between secondary clarifier effluent and influent. This is in contrast with the total bacteria results, indicating a different pattern between total and viable cells, which is statistically significant for mixed liquor (table A.22).

Relative abundances from MBR influent and mixed liquor are shown in figure 3.11d. Large increases (Games-Howell; $p < 0.01$) were seen in ARG relative abundances of total bacteria in mixed liquor (table A.21). Interestingly, this trend was different compared to the AS WWTP where a decrease was seen. The most abundant classes in screened sewage were aminoglycoside (0.271 ± 0.023 gene copies/cell), MLSB (0.202 ± 0.007 gene copies/cell), non-specific (0.177 ± 0.018 gene copies/cell), tetracycline (0.167 ± 0.009 gene copies/cell) and β-lactams (0.104 ± 0.01 gene copies/cell). Although increasing trends were observed, only β-lactams, non-specific and vancomycin increased significantly.

A different pattern was observed for viable cells (figure 3.11d) in mixed liquor. Summed relative abundances significantly declined (table A.21), from 0.879 ± 0.178 copies/cell in influent to 0.051 ± 0.057 gene copies/cell in mixed liquor (table A.20). All ARG classes, but sulfonamide and vancomycin, decreased significantly (Games-Howell; $p < 0.01$) in mixed liquor compared to influent. Similarly, MGE's relative concentrations were lower (Games-Howell; $p < 0.01$) in mixed liquor. Although similar relative abundances were observed for both ARGs and MGEs in influent between total and viable cells, significant differences (Wilcoxon; $p < 0.01$) were seen for both fractions in mixed liquor (table A.22). Relative concentrations were not determined for MBR permeate and final effluent, due to lack of HT-qPCR data, however, all target genes in qPCR analysis were detected below the LoQ.

Overall, relative abundances normalised per genome were decreased across treatment by the four WWTPs. All treatment systems reduced significantly relative concentrations of their most abundant AGR classes, such as tetracycline, aminoglycoside, MLSB and β-lactams, which have been suggested to be monitored in WWTPs (Hiller *et al.*, 2019); while, selection for ARGs was no evident. An increase in the non-specific relative abundances, which is related to efflux pumps mechanism, also seen previously in Section 3.3.1, was observed during biological treatment; although increases were not statistically significant and reduced in later treatment steps. A possible increasing trend in biological processes may be explained by the fact that genes encoding for efflux pumps are often carried in MGEs (Webber and Piddock, 2003; Piddock, 2006; Baquero *et al.*, 2008), which can be easily transferred among bacteria. Wastewater treatment matrixes consist of various chemical stressors, such as heavy metals, disinfectants, antibiotics and other selectors, that may drive microbial communities to acquire multiple efflux pump mechanisms to defend themselves against them (Christgen *et al.*, 2015). It has also been suggested that selection from more specific to less specific resistance mechanisms, including efflux-pumps, might be enhanced during treatment process (Davies, 1994; Lambert, 2005).

Different trends between total and viable cells in some WWTPs, e.g. GAS and MBR, suggest that differentiating total from viable fraction may be important. This observation, for example, can possibly challenge previous studies based only in total bacteria showing that WWTPs increase relative abundances of ARGs (Al-Jassim *et al.*, 2015; Rafraf *et al.*, 2016), implying selection. Our data showed that this might not be the case, as ARGs in viable cell decreased despite the increasing trend on the total bacteria. It is evident that ARGs are carried in viable and non-viable cells. Although bacteria have repairing mechanisms, and ARG dissemination is possible through natural transformation (Dodd, 2012), no clear evidence exists, so far, regarding how non-viable cells can contribute to the spread of ARGs in receiving water. On the other hand, decreasing viable ARB prevent ARG dissemination through HGT. Therefore, evaluating the 'health' status of bacteria is important.

3.3.6 *Proportional contribution of each treatment step on removing ARG abundances*

Although numerous studies have dealt with the effectiveness of different types of full-scale technologies at removing ARGs, most of these reported results from combined treatment systems, often including tertiary or disinfection steps, without differentiating the contribution of each step in overall reductions. A typical WWTP system includes primary, secondary and tertiary treatment steps, based on mechanical, biological and a combination of chemical,

mechanical and biological processes, respectively (Laht *et al.*, 2014). In this study, all three main steps were included in TF, GAS and MBR, while no tertiary technology was present in the AS system. The contribution of each treatment step, based on the proportion of each step (secondary or tertiary) to the total removal rate, is shown in figure 3.12. Secondary treatment was the main contributor to the removal of ARG, MGE and 16S rRNA total cells in all four WWTPs, resulting in $\geq 0.64 \pm 0.11$ proportional contribution to the total removal occurring within the WWTPs (table A.23). In contrast, the tertiary step was proportionately less important (e.g., $\leq 0.36 \pm 0.11$ proportional contribution), although this presumes effective solids separation after secondary treatment. Similar patterns were observed for the viable cells in the TF, AS and MBR, where secondary treatment contributed a proportional contribution $\geq 0.73 \pm 0.19$ to the total removal. Interestingly, different pattern was observed for the GAS, where secondary and tertiary treatment exhibited similar efficiencies at reducing ARG, MGE and 16S rRNA viable cells.

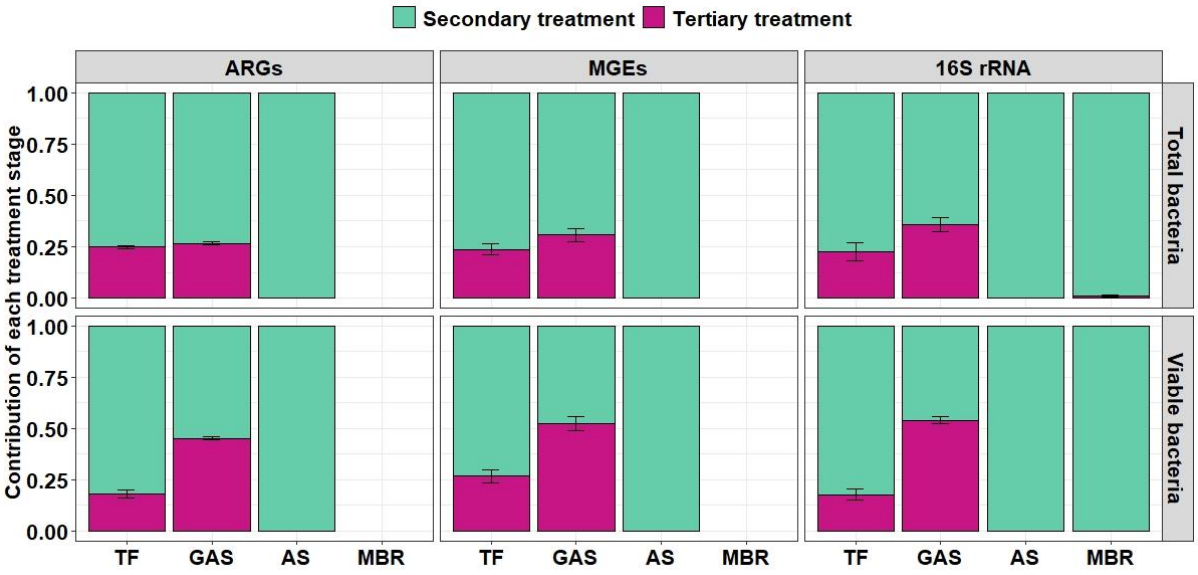

Figure 3.12 Contribution of each treatment step at reducing ARGs, MGEs and 16S rRNA in TF, GAS, AS and MBR systems of both total and viable bacteria. Secondary treatment refers to trickling filter, granular activated sludge, activated sludge and membrane bioreactor compartments, and tertiary treatment represents the disc filter, pile cloth filter, and GAC & chlorination tank for the TF, GAS, and MBR, respectively. No tertiary treatment was applied in the AS and empty spaces show absence of data.

General observations on the proportional contributions suggest that secondary treatment plays a key role at reducing both antibiotic and non-antibiotic resistance bacteria in all four treatment plants, also discussed by Graham *et al.* (2018). Similar observations were seen by Li *et al.* (2016), where significantly higher ARG reductions were achieved after secondary treatment while almost negligible was the added value of a UV disinfection system. The

present study additionally showed that removal efficiencies possibly depend on the 'health' state of bacterial cell, as for instance, in GAS, secondary treatment was far superior than tertiary treatment at reducing ARG total cells but they were equal at removing ARG viable cells.

3.3.7 *Viable versus total cells ratio*

The efficacy of each WWTP to affect viable to total cell ratio was evaluated dividing gene concentrations (copies/mL) between the viable and total cells. The viable/total ratios, determined in each treatment step, revealed different patterns among technologies (figure 3.13). For instance, mean viable to total cell ratios increased along the TF treatment chain (figure 3.13a). This increasing trend was seen for all ARGs, MGEs and 16S rRNA groups (table A.24); however, it was statistically significant only for ARG data, as well as MGEs between influent and disc filter discharge (table A.25). Surprisingly, increases higher than one were observed in some data, mostly related to the third day of sampling. A possible explanation for this might be that different filters were used to identify genes in total versus viable bacteria that led on slight variation on the biomass captured in each filter. Different susceptibility to the dye of the microbial community is also possible; however, PMA has shown good performance at targeting a wide range of environmental bacteria (Lin *et al.*, 2016; Pang *et al.*, 2016). Although experimental bias cannot be ruled out, these results indicate that the viable/total ratio increased during treatment in the TF system.

A different pattern was observed in the GAS (figure 3.13b), where decreases in the viable/total ratio were seen along treatment. Statistically significant reductions (Games-Howell; $p < 0.01$) were observed for ARGs and MGEs in pile cloth filter discharges compared to influent. This is consistent with the absolute abundances data that showed high reduction in ARG and MGE viable cells compared to total. Regarding AS (figure 3.13c), non-significant differences were observed on the viable/total ratios among treatment steps which also agrees with the absolute abundances data that showed no significant differences between total and viable cells. In contrast, significant lower viable to total ratios (Games-Howell; $p < 0.01$) were detected in MBR for both ARGs and MGEs in mixed liquor compared to influent (figure 3.13d). This different trend in mixed liquors between AS and MBR, may be due to different operating parameters, such as hydraulic retention time (HRT) or SRT, applied in the biological reactor of each WWTP (data not provided), which resulted in a lower viable to total cell ratio in MBR.

Different patterns were observed for ARGs and MGEs among the four WWTPs. For instance, TF increased the viable to total cells ratios, suggesting that most bacteria in the discharged water are viable. On the other hand, GAS decreased the viable/total ratio. Although both treatment systems use tertiary filtration, pile cloth filter (GAS) was more effective than disc filters (TF) at reducing viable cells. This also indicate that non-viable cells carrying ARGs and MGEs are also a result of mechanical pressure in a pile cloth filter. No change was observed in AS among treatment locations suggesting that this technology removed equally total and viable cells. Although information is not available for ARGs and MGEs in MBR, data hint a decreasing trend in the viable/total ratios along treatment train. Viable to total cells ratio in final effluents is, therefore, associated to treatment steps within each WWTP. For example, filtration is key to reduce viable cells; however, characteristics of the filtration system can be significant. On the other hand, settlers reduce equally both viable and non-viable bacteria. A general observation from our data suggests that ARG and MGE viable/total ratios followed similar pattern to 16S rRNA, which shows that reducing bacterial load drives ARB reduction in discharged water. Different pattern was observed in MBR's mixed liquor between ARGs and MGEs, and 16S rRNA, nevertheless, this might be a methodological bias between HT-qPCR and qPCR data

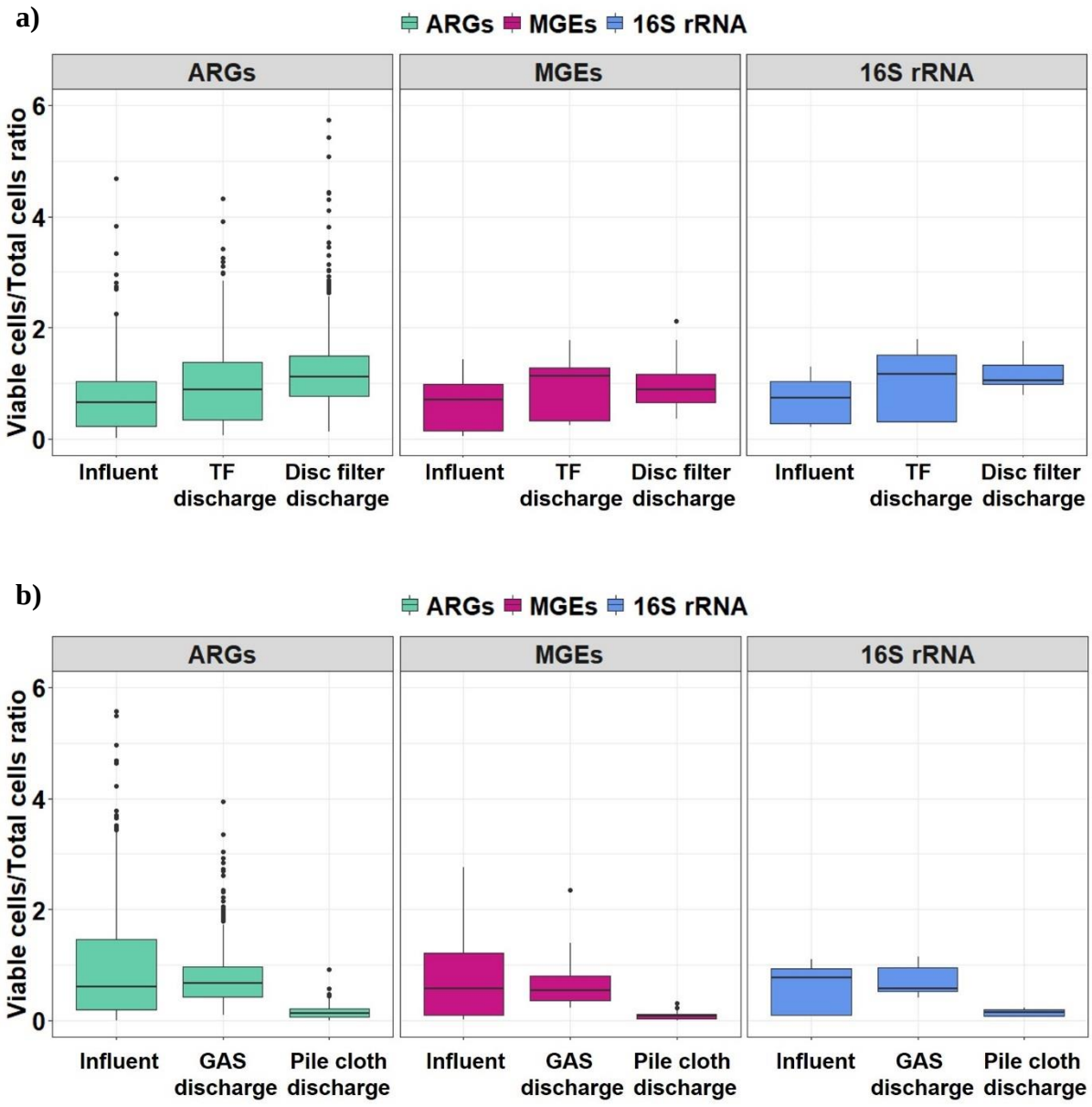

Figure 3.13 Ratios of gene absolute concentrations in viable cells to gene absolute concentrations in total cells in each treatment stage of the **a)** TF, and **b)** GAS systems.

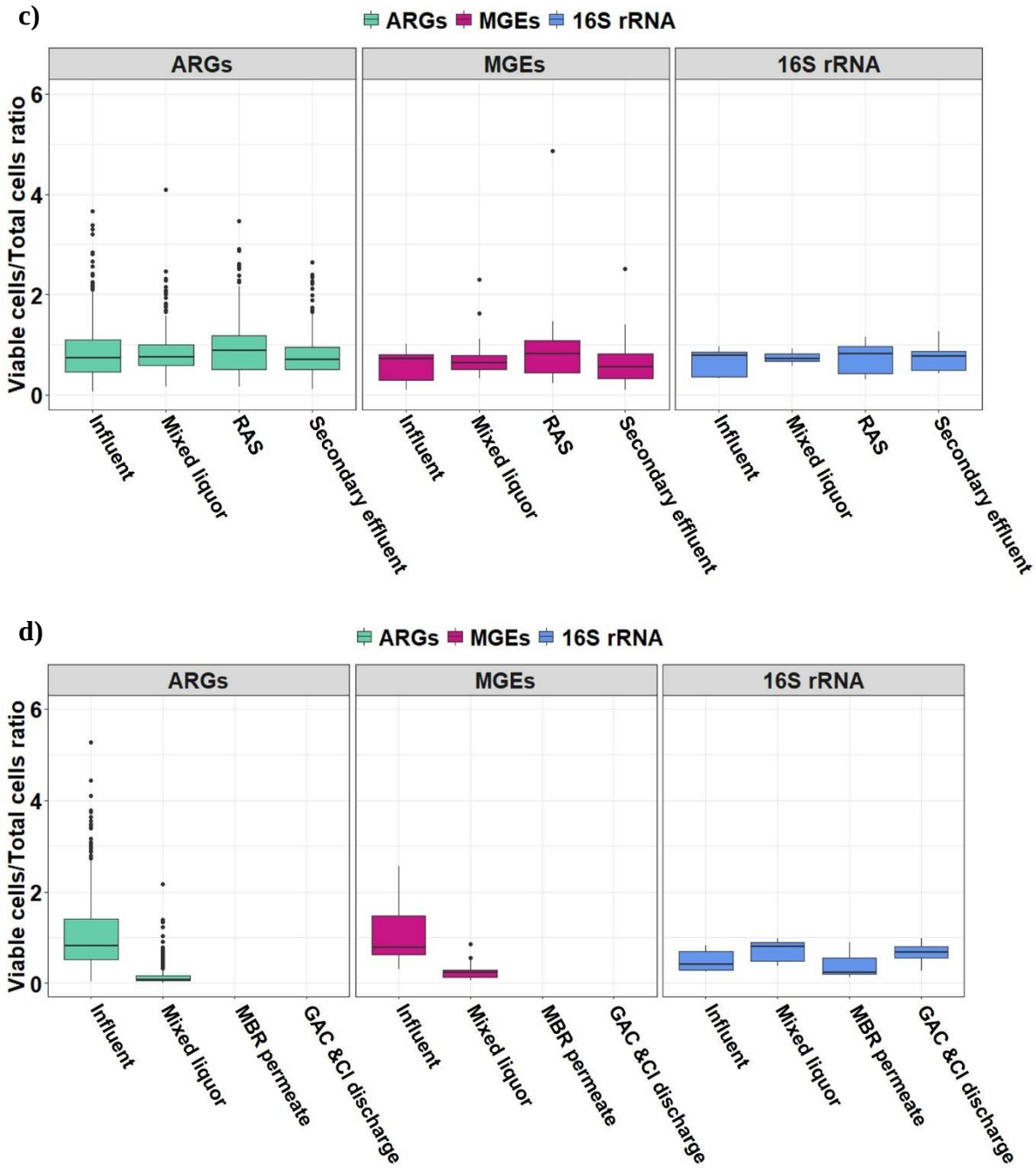

Figure 3.13(cont.) As above for **c)** AS and **d)** MBR. Empty spaces in the graphs represent the absence of HT-qPCR data for the corresponding samples.

3.3.8 *Physicochemical parameters affecting ARG and MGE removal*

Spearman's rank correlation was assessed between physicochemical and molecular data (Section 3.2.6) to explore any significant ($p < 0.05$) positive or negative correlations (figure 3.14). Regarding influent, only dissolved organic carbon (DOC) was strongly correlated with ARG and MGE total cell absolute abundances, while a positive correlation was observed between MGE viable cell absolute abundances and ammoniacal nitrogen. Physicochemical parameters were not related to gene diversities in influent.

In contrast, a greater number of significant correlations between physicochemical parameters and absolute concentrations was identified for final effluent samples. Both ARG and MGE absolute concentrations in total bacteria were positively correlated with ammoniacal nitrogen, total organic carbon (TOC) and pH; while, viable ARG and MGE absolute concentrations were significantly correlated with total oxidised nitrogen (TON). DOC, TOC and pH were also positively correlated with ARG diversity in total cells, while viable ARG diversities were only correlated with ammoniacal nitrogen.

Although no consistent pattern was observed between absolute abundances and diversities, organic carbon, either as TOC or DOC, and pH are always correlated with both, while viable cells are more related to nitrogen. This work indicated that ARG reduction was strongly correlated with physicochemical factors. Physicochemical parameters, such as pH, nitrogen and COD, have been suggested to influence the capacity of treatment plants to deal with ARB and ARGs (Krzeminski *et al.*, 2019); therefore, many studies have explored such correlations. Although some works have shown a strong correlation between ARGs and physicochemical parameters (Chen and Zhang, 2013a; Du *et al.*, 2015; Bengtsson-Palme *et al.*, 2016; Sun *et al.*, 2016), others show weaker correlations (Laht *et al.*, 2014). ARG reduction seems to be related with the capacity of each WWTP to treat wastewater; therefore, optimising operational processes could be key for increasing the effectiveness of a WWTP to deal with ARB and ARG dissemination.

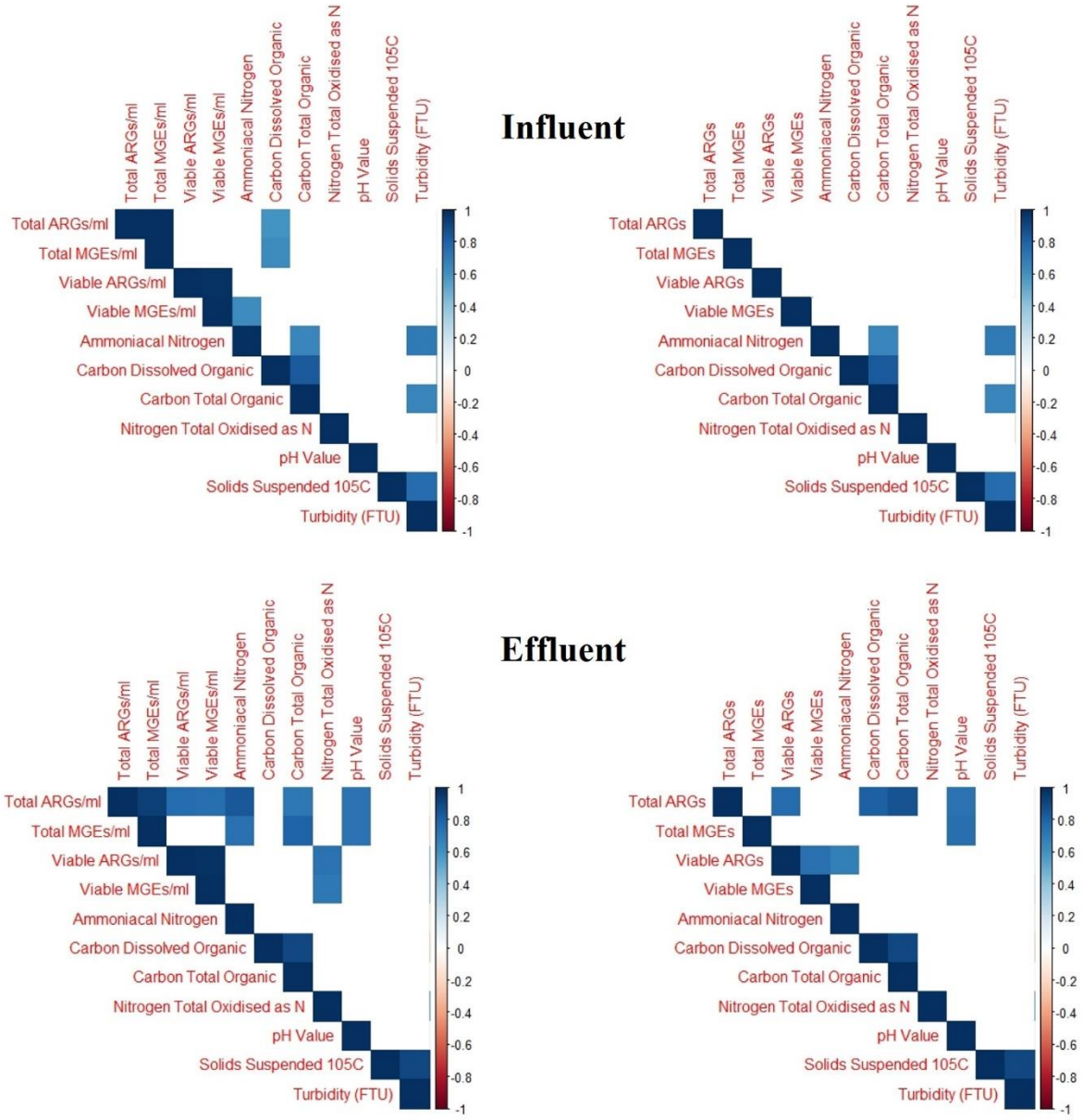

Figure 3.14 Correlations, based on Spearman rank correlation, among physicochemical parameters and ARG, MGE absolute concentration on the left, and diversities on the right. Influent includes data from all four WWTPs and effluent from TF, GAS and AS.

3.4 Practical Implication

Differences among WWTPs in their effectiveness at dealing with ARG abundances and diversities were observed. All treatment technologies were effective at reducing ARG absolute abundances at final effluents. The MBR was the most effective technology, followed by AS and GAS (figure 3.9). AS and GAS exhibited similar efficiencies; however, AS was more effective at reducing MGEs. The lowest removal rates were observed in the TF system. Among all treatment steps, secondary treatment had the highest contribution to the total effectiveness of each plant in reducing ARGs and MGEs (figure 3.12). This pattern was

observed for both total and viable cells; however, some differences were seen in GAS system, where secondary and tertiary treatment were equally effective at reducing ARG viable cells.

ARG diversities were less affected by TF and GAS, while significant decreases were seen in AS and MBR (figure 3.4). On the other hand, MGE diversities were not changed during treatment by any treatment system. Selection of ARGs is not evident in this study. All four WWTPs decreased ARG relative concentrations per genome along treatment, and final effluents as well as other compartments, such as mixed liquors and RAS, were less diverse than influents (figure 3.11). Furthermore, data showed that diversities in final effluents are determined by the influent characteristics rather than internal compartments (figure 3.5).

Together these results provide important insight into key factors that can affect each treatment technology at reducing ARB from final effluents. Technologies that achieved the highest removals of 'all' bacteria (16S rRNA) were also the most effective at reducing ARGs and MGEs, demonstrating a strong correlation between bacterial removal and these genes (figure 10). Filtration is very effective against bacteria but our data showed that the filter type is important. A difference also was seen between filtration and settling at reducing viable cells, as the former can decrease more effectively the viable to total cells ratio (figure 3.13). Furthermore, a strong positive correlation between ARGs and physicochemical parameters suggest that optimising operational parameters can enhance the effectiveness of WWTPs against ARB spread (figure 3.14). The data here hint that operating parameters can greatly affect the whole performance of a WWTP, e.g. ARG relative abundances per genome increases (total cells) in mixed liquor in the MBR system versus decreases (total cells) in mixed liquor of the AS system (figure 3.11). This indicates that different patterns in mixed liquor of the two WWTPs can be a result of different operating parameters; however, operational details were not provided from the industrial sponsor to explore further this finding as they considered sensitive information.

The PMA viability method also showed the importance of differentiating between total and viable cells in future studies. Although this method is new, and more studies will be warranted for the optimisation of this analysis, interesting findings were seen. For example, using this method, we showed that viable cells are not affected in the same way by different treatment technologies (figures 3.7 and 3.8). Furthermore, different patterns observed between total and viable cells, e.g., in relative concentrations per genome, showed that considering only total bacteria data may misinterpret the performance of WWTPs to reduce ARG viable cell (figure 3.11). Furthermore, it shows that ARGs are carried in non-viable cells suggesting

further investigation in the potential impact of non-viable cells carrying ARGs in discharged water.

3.5 Conclusions

In this study four different WWTPs (TF, GAS, AS and MBR) were assessed and compared in their relative ability to reduce ARG abundance and diversity in domestic wastewater. All four WWTPs reduced ARG and MGE absolute abundances of both total and viable cells in final effluents. However, the efficacy of each WWTP to reduce ARG abundance was related to treatment technology, with removal rates ranging from 1.27 logs (TF system) to 6.10 logs (MBR system). Significant positive correlations between ARGs, MGEs, and 16S rRNA were observed indicating that technologies that were more effective at eliminating 'all' bacteria, they also achieved higher removal rates at reducing ARGs and MGEs. Also, positive correlations between ARGs and physicochemical parameters, such as pH, TOC, DOC and TON, hint that operating parameters can greatly affect the performance of WWTPs at removing ARB.

In this study, the performance of each treatment step was examined. The contribution of the secondary treatment at reducing ARGs and MGEs was higher than the tertiary treatment (where applied), achieving > 0.73 proportional contribution to the total removal in all WWTPs. Although the same pattern was seen for ARGs and MGEs of both total and viable cells in most systems, secondary (GAS) and tertiary (pile cloth filtration) treatments were equally effective at removing ARGs and MGEs of viable cells in the GAS system. This suggests each treatment step can affect differently viable and total cells patterns and its effect on the whole system may be underestimated focusing only on total bacteria.

Although decreasing patterns were observed in diversities in all WWTPs after treatment, MBR and AS systems reduced significantly ARG diversity, while no treatment technology affected MGEs. Interestingly, no change in overall gene distribution within all treatment systems was observed, and all WWTPs reduced ARG relative abundances per genome of all major classes. Less ARG diversity and deceasing patterns in relative concentrations imply no evident ARG enrichment during treatment, suggesting WWTPs did not contribute to ARG selection during treatment.

Comparing PMA-qPCR data with conventional qPCR data revealed differences in total versus viable ARG patterns. For example, abundances and diversities of ARGs and MGEs carried in viable cells were always lower compared to those detected in total cells; while decreasing patterns of viable ARG relative abundances per genome versus increasing patterns of total ARG relative abundances (either overall relative abundances or in specific classes) were seen

in the GAS and MBR systems. Also, filtration significantly affected the viable to total cells ratio compared to secondary settlers which had no effect, indicating that technologies affect differently the viable fraction. Differentiating between total and viable cells can give insights into the real fate of ARGs within a treatment system mitigating overestimations of ARG abundances available for HGT, hence, it should be considered in future studies.

Chapter 4 Pilot-Scale Testing of Advanced Oxidation Processes for Reducing Abundances and Diversities of ARGs from Secondary Effluent

4.1 Introduction

Reuse of wastewater has become essential in the 21st century. Water is getting scarcer and the need for reclaimed wastewater (RWW) is rising, especially with increasing urbanisation, and in arid or semi-arid regions. In many places around world, reclaimed wastewater is used for many purposes, such as agricultural and municipal irrigation, direct and indirect potable use by aquifer discharges, recreational and other uses (Negreanu *et al.*, 2012; Gatica and Cytryn, 2013; Han *et al.*, 2016; Hong *et al.*, 2018). Although reclaimed wastewater is particularly beneficial, it can also be a pathway of biological and chemical contaminants into natural environment (Deng *et al.*, 2019). Among those, ARB and ARGs in such wastewater can be released into the environment through that route (Christou *et al.*, 2017).

Many studies have focused on possible impacts of RWW released via irrigation on different ecosystems, suggesting natural environments receiving RWW with elevated ARB abundances can be antagonistic to native bacterial species, potentially selecting AMR bacteria and genes in soils (Nesme *et al.*, 2014; Nesme and Simonet, 2015). Recent studies have identified elevated ARGs abundances in urban parks irrigated with RWW compared to fresh water in China and Australia (Wang *et al.*, 2014a; Han *et al.*, 2016). In contrast, other studies from Israel have found limited impact on ARB and ARGs abundances and diversities in regions irrigated with RWW, showing that AMR releases minimally affected natural microbiomes (Negreanu *et al.*, 2012; Gatica and Cytryn, 2013). These contradicting results, according to Christou *et al.* (2017), could be a result of different natural factors of each study area, and-or practical and methodological differences, such as quantification protocols, heterogeneity of samples, or different methods targeting often relic DNA. However, the lack of sufficient and consistent findings does not imply absence of risk, and minimisation of ARB and ARGs presence into RWW should be considered as high priority.

The importance of mitigating the presence of AMR in discharged wastewater is reflected on initiatives globally (WHO, 2015b; EC, 2017; GOV.UK, 2019). On May 2018, the European Commission updated the regulations on water reuse (EC, 2018) with additional and stricter requirements for water quality, listing AMR metrics among the targets. Since stricter water regulations implies quality improvement of the produced water, additional treatment steps,

including tertiary treatment and disinfection/oxidation, may be needed to enhance conventional biological treatment processes (Rizzo *et al.*, 2013b; Sharma *et al.*, 2016; Michael-Kordatou *et al.*, 2018).

Several tertiary and quaternary treatment options exist, including physical, disinfection, and advanced oxidation technologies. Granular activated carbon, sand filtration, and other membrane processes (Lüddeke *et al.*, 2015; Alexander *et al.*, 2016; Czekalski *et al.*, 2016) are examples of physical treatment options. Research suggests investigation on suitable operational conditions might be valuable because they have shown promise in reducing ARB and ARG from wastes (Hiller *et al.*, 2019). The successful implementation of disinfection processes, including chlorination, ozonation and UV irradiation, at improving water quality drove many scientists to study their effectiveness at reducing ARB and ARG abundances (Sharma *et al.*, 2016). Findings suggest that disinfection can be quite effective, nevertheless higher doses than these applied conventionally were often needed (Michael-Kordatou *et al.*, 2018; Hiller *et al.*, 2019). The formation of hydroxyl radicals ($^{\bullet}OH$) and their capacity to react non-selectively with a wide range of organic and non-organic compounds is the main advantage of advanced oxygen processes (AOPs) (Ruppert *et al.*, 1994), such as H_2O_2/ UV, solar-H_2O_2, homogenenous (Fe^{2+}, Fe^{3+}/H_2O_2) or heterogeneous photocatalysis (TiO_2). However, a more comprehensive examination of the oxidation mechanisms against both ARB and ARGs and also testing beyond bench-scale is crucial (Michael-Kordatou *et al.*, 2018).

Much effort has been made in trying to identify the most suitable and reliable technologies that might be an effective barrier of AMR spread through wastewater discharges or reuse. However, many questions still need to be answered. Work herein aimed to fill some of these knowledge gaps. For example, many previous studies focused on cultivable ARB or target ARGs, but less is known in ARG diversity in tertiary treated/disinfected effluents, especially differences between viable and non-viable bacterial hosts. Hence, one of the main goals of this work was to identify ARG diversities before and after different treatment options, using HT-qPCR that can simultaneously monitor most major classes of ARGs. Furthermore, very few studies have considered the physiological state of the bacterial cells (viable, non-viable, disintegrated), as most previous methods do not distinguish the state of host cells. This is huge knowledge gap because knowing the "health" status of cells is essential for understanding the fate of ARB, particularly horizontal gene transfer (HGT), because only viable cells will theoretically be able to actively contribute to HGT. Here the PMA method was used to differentiate ARGs in putative viable cells and all cells present (Nocker *et al.*, 2007; Pang *et al.*, 2016; Kibbee and Örmeci, 2017). It also worth noting that most previous

studies only compared physical and disinfection/oxidation systems as individual processes, without assessing their function within the whole treatment system. For this reason, one of the principal objectives of this study was to evaluate the additional benefit of different treatment options to the main WWTP. In summary, objectives of work in this Chapter was to assess and compare different quaternary technologies in terms of ARG removal and changes in diversity in pilot-scale systems; assess the distribution and fate of ARGs in the viable cell fraction; and then determine the relative value of such technologies compared with other technological options for reducing AMR in wastewater effluents.

4.2 Materials and Methods

4.2.1 *Advanced oxidation processes pilot plant*

This study took place in a WWTP in SE England. The treatment process in this plant is activated sludge with unit operations including screening, primary and secondary treatment, with the final effluent being discharged to a river. Pilot-scale assessment of the AOPs were performed at this WWTP. The AOP pilot plant received effluent from the secondary clarification tanks of the main plant after passing through pile cloth filters (Eliquo Hydrok, UK) that reduce total solids levels to less than 5 mg/L. As it is shown in figure 4.1, the feedwater to the AOP pilot plant passes a hydrogen peroxide dosing point, an ozone dosing point, and then enters four ozone and-or hydrogen peroxide contactors, which operate in parallel (figure 4.2, left-up). The flow ultimately ends up in two UV exposure reactors. Sampling access points are at the inlet and outlet of the pilot plant (figure 4.1), upstream and downstream of each ozone and hydrogen peroxide contactor, and downstream of the UV system (figure 4.2).

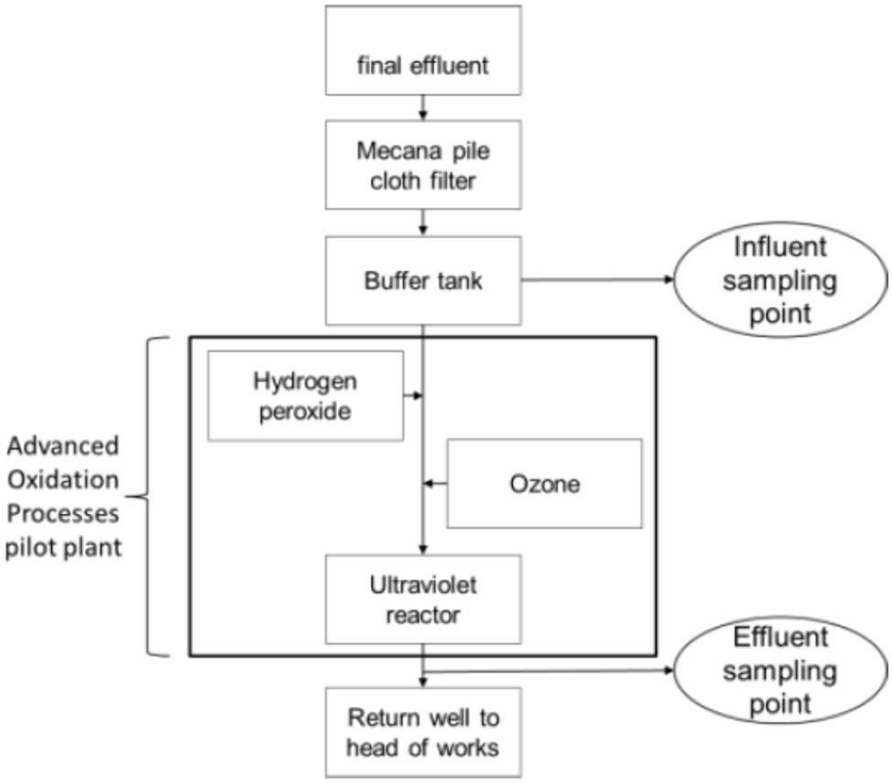

Figure 4.1 Overview schematic of the AOP pilot plant displaying the sampling points used in this study.

For the purpose of pilot testing, hydrogen peroxide was supplied by Airedale Chemical Company Ltd. (UK). It was stored in a container next to the AOP plant and transferred into the plant by pumps. Ozone was provided to the system by an ozone generator (figure 4.2), which produces ozone from atmospheric air inside the pilot plant. The ozone gas was introduced to the feedwater at the dosing point and was regulated by a flow valve. The UV system was equipped with two low pressure (LP) reactors obtained from Xylem WEDECO (Herford, Germany) with an automatic wiping system and two low pressure/high output lamps.

Figure 4.2 Sampling points highlighted upstream and downstream of the ozone and hydrogen peroxide contactors (left up) and UV system (left down). Ozone generator (right).

4.2.2 *Experimental set-up*

Experiments were performed from October 2017 to December 2017. For the testing, the AOP plant was monitored two days per week, which allowed the testing of two different experimental conditions per day. The specific advanced oxidation (AO) options tested were ozonation (O_3) alone, H_2O_2 coupled with UV (H_2O_2/UV), and combining O_3/H_2O_2/UV (table 4.1). The tested doses of H_2O_2, O_3 and UV were based on doses determined in previous projects at the AOP pilot plant (information not presented), which were modified according to data from previous pilot- and full-scale assessments performed elsewhere (Luczkiewicz *et al.*, 2011; Lekkerkerker-Teunissen *et al.*, 2012; Bourgin *et al.*, 2017; Bourgin *et al.*, 2018). Each condition was tested in triplicate.

Typically, the liquid feed from pile cloth filters was pumped into the pilot plant at 5.00 L/s with hydraulic retention time (HRT) at 140 seconds. A control panel was used to set automatically O_3, H_2O_2 and UV doses. Hydrogen peroxide and UV were adjusted to '0' when the ozonation condition was operated, whereas O_3 was set at '0' when the H_2O_2/UV condition was tested. Other test conditions were controlled according each specification, including

combined additions of oxidants. Previous testing at the pilot plant showed stable (with time) operating conditions were achieved at the last sampling point after 10 min. Hence, samples were collected after 15 to 20 minutes after each setting adjustment. Control samples also were taken without oxidant additions to quantify any impact of "other" factors on the overall pilot plant performance.

Table 4.1 Experimental conditions tested per day. 'Influent' refers to primary settled sewage from the main plant and 'feedwater' is the pile cloth filter discharge which enters the AOP pilot plant. The condition 'Control' (column 'AOP 1') refers to samples collected from the final sampling location of the pilot plant with no AOP in operation. UV dose, when applied, was always constant at 650 mJ/cm^2.

Dates	Influent	Feedwater	AOP 1	AOP 2
31/10/2017	Primary settled sewage	Pile cloth filter discharge	O_3 (1g/m^3)	H_2O_2/UV (1 g/m^3)
02/11/2017	Primary settled sewage	Pile cloth filter discharge	O_3 (1g/m^3)	H_2O_2/UV (1 g/m^3)
07/11/2017	Primary settled sewage	Pile cloth filter discharge	O_3 (1g/m^3)	H_2O_2/UV (1 g/m^3)
09/11/2017	Primary settled sewage	Pile cloth filter discharge	O_3 (3 g/m^3)	H_2O_2/UV (3 g/m^3)
14/11/2017	Primary settled sewage	Pile cloth filter discharge	O_3 (3 g/m^3)	H_2O_2/UV (3 g/m^3)
16/11/2017	Primary settled sewage	Pile cloth filter discharge	O_3 (3 g/m^3)	H_2O_2/UV (3 g/m^3)
21/11/2017	Primary settled sewage	Pile cloth filter discharge	O_3 (9 g/m^3)	H_2O_2/UV (9 g/m^3)
23/11/2017	Primary settled sewage	Pile cloth filter discharge	O_3 (9 g/m^3)	H_2O_2/UV (9 g/m^3)
28/11/2017	Primary settled sewage	Pile cloth filter discharge	O_3 (9 g/m^3)	H_2O_2/UV (9 g/m^3)
30/11/2017	Primary settled sewage	Pile cloth filter discharge	Control	O_3/H_2O_2/UV (3 g/m^3 / 3 g/m^3)
05/12/2017	Primary settled sewage	Pile cloth filter discharge	Control	O_3/H_2O_2/UV (3 g/m^3 / 3 g/m^3)
07/12/2017	Primary settled sewage	Pile cloth filter discharge	Control	O_3/H_2O_2/UV (3 g/m^3 / 3 g/m^3)

4.2.3 *Sample collection and processing*

Each sampling day consisted of collecting influent, feedwater and advanced treated effluent samples, in this order. Samples were sub-divided for molecular analyses and physicochemical analyses, respectively, with volumes taken according to sampling location and analyses' purposes. Typically, 90 mL was divided from influent samples, and between 1.5 and 2.5 L for feedwater and AO treated effluent for molecular analyses purposes. All efforts were made to conduct experiments on dry weather days (to avoid influent dilution), but due to time limitations, AOP testing was occasionally performed on rainy days. In such days, larger volumes of feedwater and advanced treated effluent samples were taken to assure that enough DNA was available for extraction and molecular analyses. Molecular samples were collected in sterile polypropylene containers (Fisher Scientific, UK) after letting the water to flow for 5 minutes to remove any stagnate water in the sampling lines. Additionally, 1.5 L of feedwater and treated effluents were distributed in appropriate vials and sent to analytical laboratories for physicochemical analyses, within 24 hours of collection.

At the end of each sampling day, samples for molecular analyses were transferred to the laboratories, being stored at 4 ^{o}C in the refrigerator. They were processed within 24 hours to prevent changes in the microbiota due to long storage (see figure B.1). Influents were centrifuged (Eppendorf 5810R) at 4,000 rpm for 30 min and the pellet was immediately stored at -20 ^{o}C after removing centrates. Both feedwater and treated effluents were filtered (process described in the Section 3.2.2) and the membrane filters with the concentrated biomass were also stored at -20 ^{o}C. Total bacteria (viable and non-viable cells) captured in these samples were preserved frozen until DNA extraction and HT-qPCR analysis.

At a later stage, the frozen samples were thawed at room temperature and DNA was extracted from the concentrated biomass (either pellets or membrane filters), using the Fast DNA™ Spin Kit for Soil (MP Biomedicals, USA), according to the manufacturer's instructions. Routine analysis included the quality and quantity determination of the extracted DNA with the methods described in Section 3.2.2 and subsequently storage at −20 °C until further use. Purification of DNA was performed to improve the quality, using the QIAquick Nucleotide Removal Kit (QIAGEN, UK), according to the manufacturer's instructions. After sample "cleaning", the quality and quantity were re-assessed and appropriate amounts of DNA per sample were freeze-dried and sent to the Chinese Academy of Sciences in Xiamen, China for HT-qPCR analysis. Details on the analysis have been described in Section 3.2.3.

Feedwater and AO treated samples were also processed for total bacteria and viable cells. Here fresh samples were processed with the method described in Section 3.2.5 and DNA was immediately extracted and stored at -20 °C for further use. These samples were analysed with qPCR for the genes 16S rRNA, *int*1, $bla_{\text{OXA-10}}$, *tet*M and *tet*Q with the procedure described in Section 3.2.4.

4.2.4 *HT-qPCR and qPCR data processing and statistical analysis*

HT-qPCR and qPCR data were processed and statistically analysed as previously described in Section 3.2.6. Pairwise comparisons for normal and homogeneous data were assessed with one-way analysis of variance (ANOVA) and Tukey post-hoc test, and samples where normality and homoscedasticity were violated were analyzed with non-parametric tests (Kruskal-Wallis and Games-Howell post-hoc). Significant differences among samples are denoted by $p < 0.05$.

4.3 Results

4.3.1 *Water quality conditions surrounding AO process testing*

The water quality of the feedwater was quantified to check any water characteristics that might affected AOP effectiveness. The physicochemical parameters are presented in tables B.1 and B.2 (Appendix B). Nitrate, nitrite and suspended solids (SS) were very low or below detection limit, and the pH was neutral. According to the United States Environmental Protection Agency (EPA), nitrate, nitrite, pH and suspended solids are important parameters affecting the treatment process where UV is involved (EPA, 2003) as the former in large amounts can absorb UV light decreasing the disinfection capacity and pH can influence the solubility of metals and carbohydrates, affecting also the treatment process. Total organic carbon (TOC) and presence of particles (SS/turbidity) in the matrix also affect ozonation's effectiveness (Alexander *et al.*, 2016; Czekalski *et al.*, 2016; Michael-Kordatou *et al.*, 2018), hence low levels of both in the feedwater suggest limited effects on AO treatment. Moreover, pre-filtration of inlet water in systems using ozonation has been suggested as essential to reduce high molecular weight compounds in the processes (Wojtenko, 2001); this is important because the presence of small particles can increase the risk of regrowth in post-treatment effluents.

It is worth noting that ozonation here improved the quality of water in various ways. For example, UV transmittance (UVT) increased with increases in ozone doses, whereas turbidity

levels decreased (table 4.2). More specifically, increases of 43.7, 58.9 and 90.6 % in UVT and decreases of 12.8, 21.9 and 41.2 % in turbidity were detected at 1, 3 and 9 g/m^3 O_3, respectively. On the other hand, an increasing pattern in turbidity was observed with increasing dose of H_2O_2 in the H_2O_2/UV system, which may be a result of organic carbon disintegration caused by UV irradiation (Shaw *et al.*, 2000; Liu *et al.*, 2012); while, no significant effect was observed in UV transmittance. Interestingly, an increase of 65.8 % in UVT and decrease of 23.6 % in turbidity were achieved in effluents treated by the combination (O_3/H_2O_2/UV), showing synergistic effect at improving the quality of water. Increases of UV transmittance during processes combining H_2O_2, ozone and UV were reported before where ozone was suggested to react with dissolved organic carbon to decrease its levels, increasing UV transmittance in the water matrix (Lekkerkerker-Teunissen *et al.*, 2012).

Table 4.2 Turbidity decreases and UV transmission (UVT) increases (%) after control (no oxidation), H_2O_2 coupled with UV (UV constant at 650 mJ/cm^2), ozonation and the combination O_3/H_2O_2/UV (3 g/m^3/3 g/m^3/650 mJ/cm^2).

Treatment stage	**Turbidity Decreases (%)**	**UVT Increases (%)**
Control	-0.51	0.26
1 g/m^3 H_2O_2/UV	9.90	8.37
3 g/m^3 H_2O_2/UV	0.00	8.81
9 g/m^3 H_2O_2/UV	-11.17	9.08
1 g/m^3 O_3	12.85	43.69
3 g/m^3 O_3	21.94	58.93
9 g/m^3 O_3	41.21	90.61
Combination	23.59	65.79

4.3.2 *HT-qPCR results and statistical analysis*

Since sampling was conducted across different weeks, statistical comparisons among all influent and all feedwater samples were performed to test for any significant differences in the wastewater across the whole program. Three categories of data, including ARGs and MGEs unique gene numbers, absolute and relative abundances, were compared to test normality and homogeneity, as described in Section 4.2.4, and nonparametric tests were performed to determine significance levels. Multiple comparisons of means (Games-Howell test) were performed among all influent and feedwater samples for each group, and subsequent p-values are presented in tables B.3 and B.4.

The non-parametric Games-Howell test was performed and a p-value greater than 0.05 for all comparisons between influent data for all three categories showed that the null hypothesis was not rejected, which implies WWTP influent conditions were not significantly different across all sampling weeks. Similarly, feedwater to the AOP pilot plant across was not significantly different for relative ARG abundances and gene numbers per sample (i.e. HT-qPCR diversities); however, absolute ARG abundances were significantly different for some sample-week pairs (i.e., p-values < 0.05). These significant differences were mainly attributed to decreases in absolute ARG concentrations over the last three weeks of sampling, possibly due to operational changes in the main WWTP. That shows that from a statistical point of view these data belong to different populations and were treated as separate groups. This implies that direct comparisons among absolute ARG concentrations across AOP were not possible, which is discussed below.

4.3.3 *Diversities of ARGs and MGEs within treatment steps*

High-throughput qPCR is a very useful, complementary analysis to traditional PCR and qPCR that provides much broader spectrum of ARGs present in environmental samples (Wang *et al.*, 2018), although detection limits are higher than conventional qPCR. As it has been mentioned before (Section 3.2.3), the HT-qPCR assessment used has the capacity to track up to 296 primer sets, from which 283 ARGs, 12 MGEs and one eubacterial 16S rRNA gene. ARGs were classified in nine groups according to their antibiotic class, including aminoglycosides, β-lactams, non-specific (mostly multidrug efflux pumps), tetracyclines, vancomycin, fluoroquinolone/quinolones/florfenicol/chloramphenicol/amphenicol (FCA), macrolide/lincosamide/streptogramin B (MLSB), sulfonamides, and ‘other’ for any gene not

conferring resistance to previous or specific antibiotic classes. Furthermore, MGEs were classified in two groups, including transposases and integrases.

ARGs and MGEs totals per class (table B.5) are means from twelve sampling days for both influent and AOP feedwater and means from three sampling days for each AOP option tested (see Section 4.3.2). Figure 4.3 shows the numbers of unique ARGs and MGEs detected under each AOP operating condition. Total ARG numbers in feedwater (tertiary filtered discharge) were significantly lower (Games-Howell; $p < 0.01$) than the WWTP influent (table B.6), showing reduction of ARGs numbers from 130 ± 2.6 to 78.2 ± 1.5. This implies that tertiary filtration following secondary treatment processes significantly reduced ARG diversity. For comparison, under control conditions when no AO processes were operating, non-significant differences were observed in both ARG and MGE numbers compared to feedwater. This verifies the pilot plant itself did not have any actual effect on ARG and MGE diversity.

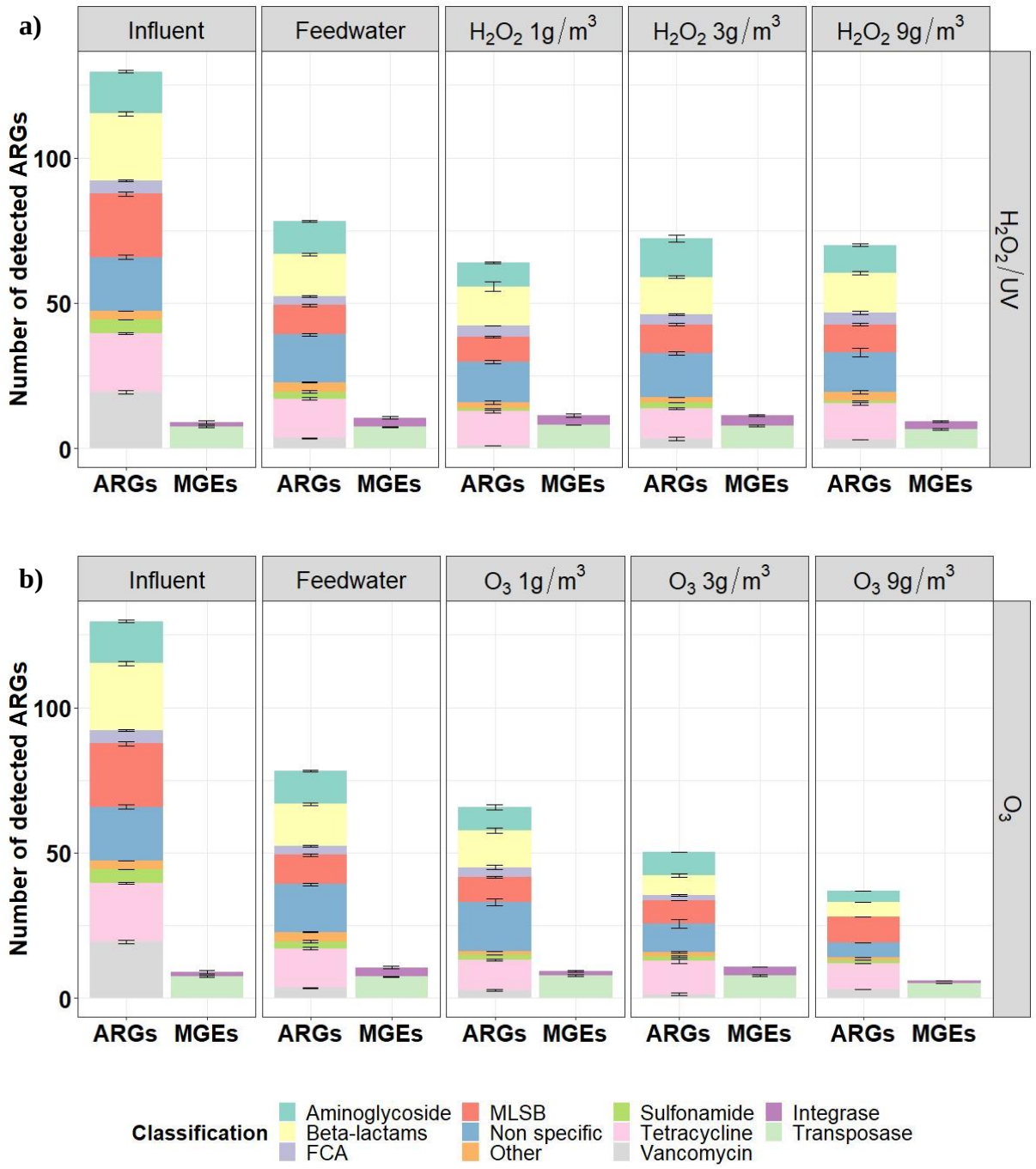

Figure 4.3 Number of unique ARGs and MGEs detected in each treatment step, classified in antibiotics to which they confer resistance. Treatment stages are grouped according to treatment type in **a)** H_2O_2/UV (UV constant at 650 mJ/cm^2), and **b)** ozonation. Error bars represent the variability of data from twelve sampling days for both influent and feedwater and from three sampling days for AOPs.

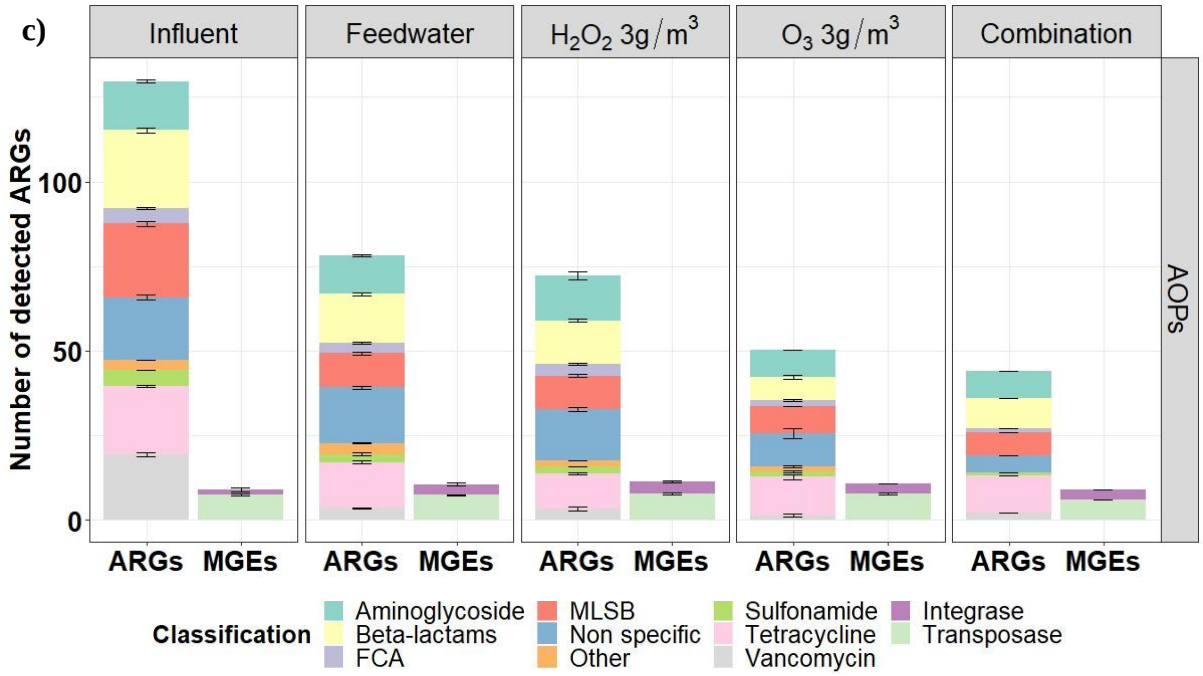

Figure 4.3(cont.) As above for **c)** all different types of AOPs including the "combination", i.e. O_3/H_2O_2/UV (3 g/m^3/3 g/m^3/650 mJ/cm^2).

The added value of each AOP option for further reducing ARG numbers was tested. In effluents treated by H_2O_2/UV (figure 4.3a), effluent aminoglycoside and vancomycin classes were significantly less diverse (based on unique ARG numbers, Games-Howell; $p < 0.05$) compared to feedwater after applying 1 g/m^3 H_2O_2, and tetracycline ARG numbers decreased significantly (Games-Howell; $p = 0.02$) at 3 g/m^3 (table B.6). Despite these observations, no significant decreases of the total ARGs and MGEs numbers were achieved after treatment with any H_2O_2 dose. In contrast, increases in ozone doses from 1 to 3 and 9 g/m^3, resulted in progressively decreases in ARG number; i.e., 65.7 ± 3.0, 50.3 ± 5.7 and 37.0 ± 0.0, respectively. Among these, effluent ARG numbers in the 3 and 9 g/m^3 O_3 effluents were significantly lower than the feedwater (Games-Howell; $p < 0.02$). On the other hand, MGE numbers were less influenced by O_3 dose, with only the highest dose (9 g/m^3) achieving significant reductions (Games-Howell; $p < 0.01$). Although a decreasing trend in total ARG numbers were seen with increasing O_3 dose (figure 4.3b), significant differences among doses were confirmed only for aminoglycoside, MLSB, non-specific, and sulfonamide ARG, and for integrases.

The combined effect of multiple AOP options at reducing ARGs and MGEs numbers also was examined. Combining AOPs (figure 4.3c) achieved lower (Games-Howell; $p < 0.01$) total ARG (44.0± 0.0) and transposases (6.0 ± 0.0) numbers compared to feedwater. Although a clear decreasing trend was observed after treatment with the combination, this reduction was

not statistically significant for the overall ARGs and MGEs compared to ozonation and H_2O_2/UV operating separately. Nevertheless, individual MLSB, non-specific and sulfonamide groups were significantly lower (Games-Howell; $p < 0.01$) in effluents treated by the combination, compared to the individual AO options.

The three major resistance mechanisms encompassed by ARGs span antibiotic deactivation processes, efflux pumps, and cellular protection systems. In influent, antibiotic deactivation was the principal resistance mechanism (39.8 %), followed by cellular protection (28.8 %), and then efflux pump systems (29.4 %). Similar trends were seen in the feedwater, with slight increases in antibiotic deactivation processes (46.3 %) and efflux pumps (32.3 %), but slight decreases in cellular protection (19.2 %) resistance mechanisms. Regarding each AOP, no major differences in resistance mechanism patterns were detected after treatment with H_2O_2/UV and ozonation at 1 and 3 g/m^3 (figure B.2). However, the proportion of efflux pump ARGs (24.5 %) declined and cellular protection mechanisms (28.6 %) increased after applying 9 g/m^3 O_3. These data indicated that tertiary filtration nor the AOPs changed the ARB resistance mechanisms patterns, except where O_3 addition at 9 g/m^3 tended to select away from ARGs conferring efflux pumps mechanisms.

4.3.4 *Removal of persistent genes after advanced oxidation*

Venn diagrams overlaying ARGs and MGEs present in all samples help one to identify the number and types of persistent genes in processes. Figure 4.4 shows all treatment options grouped by AOP (horizontal), and corresponding influent and feedwaters (vertical). Persistent ARG numbers in the feedwater were higher than AOP effluents Treatment with H_2O_2/UV decreased numbers persistent ARGs, nevertheless higher H_2O_2 concentration did not have an added effect. In contrast, increasing ozone doses decreased the number of persistent ARGs, resulting in 62, 49 and 36 ARGs by applying 1, 3 and 9 g/m^3 O_3, respectively. Interestingly, combining AO processes did not enhance removal of persistent ARGs compared to ozonation alone at 3 g/m^3, suggesting that ozonation probably drove the reduction in persistent ARGs.

Occasionally, some ARGs were only present in AOP effluents (figure 4.4). These genes were explored further (table B.7) to explain why ARGs might appear as a result of an AOP. Previous studies have suggested that AOPs can select for antibiotic resistance strains (Luczkiewicz *et al.*, 2011; Rizzo *et al.*, 2013b; Lüddeke *et al.*, 2015). In contrast, we found no evidence here suggesting patterned ARG selection, despite appearance of ARGs after AOPs. Some ARGs appeared only in tertiary filtered effluent (i.e., the feedwater), but seemed to be

bare not relation with previous treatment steps. This suggests that these ARGs are more likely randomly or arbitrarily detected in the AOP effluents, possibly due to levels being slightly above versus slightly below HT-qPCR detection limits.

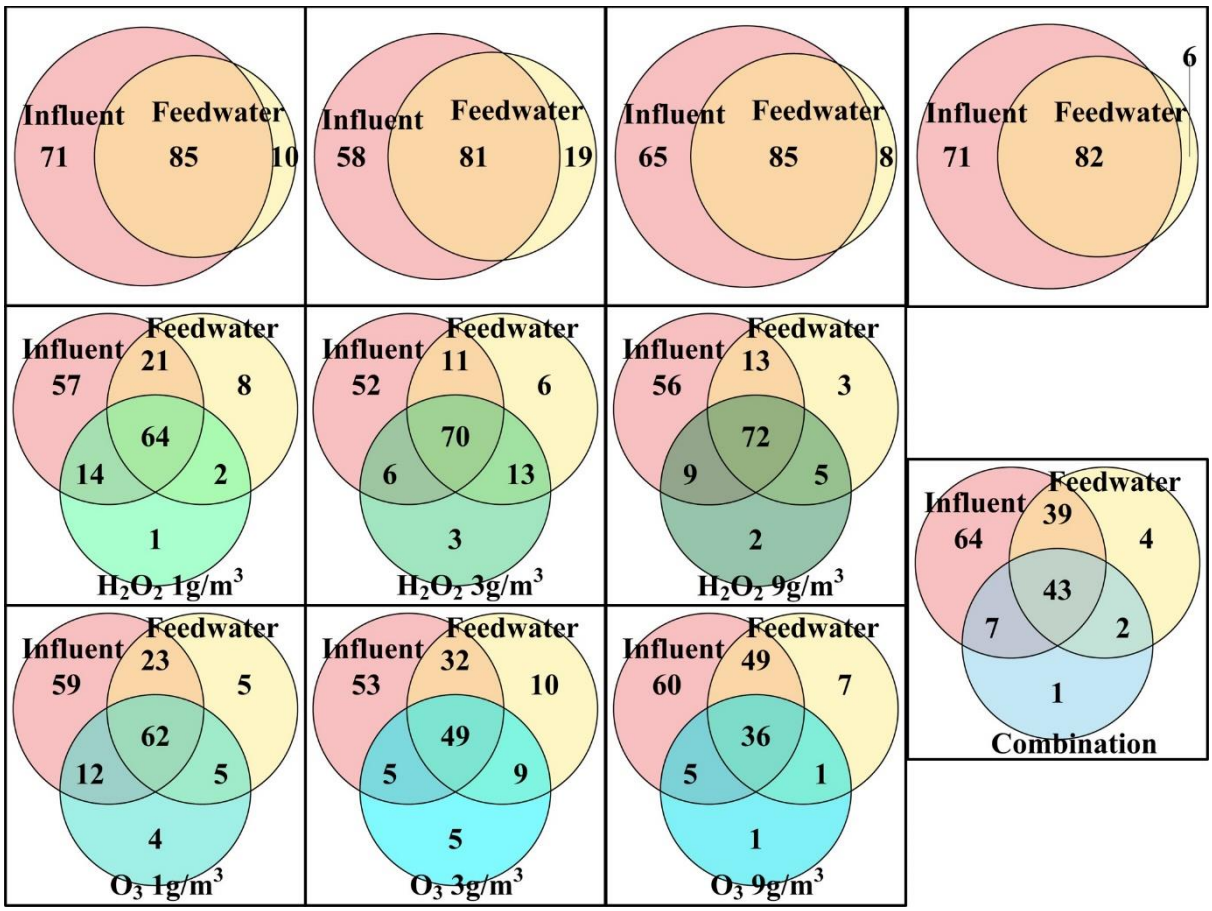

Figure 4.4 Venn diagrams showing common genes among influent, feedwater and the corresponding AOPs effluent. Each box represents detected genes during the whole treatment process from the influent to final effluent. The number located in the intersection of all treatment stages shows genes that entered the main WWTP and were not removed in any treatment stage (persistent genes).

Common genes in AOP final effluents were identified using Venn diagrams (figure B.3). This information let us explore ARGs that were not removed by any dose or type of advanced oxidation system. H_2O_2/UV was the least effective at removing ARGs (52 persistent genes), especially compared to ozonation (25), while combining AO options did not provide any added value (25) relative to ozonation alone (table B.8). Persistent genes crossed all major ARGs classes without being associated with any particular resistance mechanism. Therefore, based on systems and methods here, surviving ARG types after AOP treatment indicated no clear selection of bacteria carrying specific groups of ARGs.

4.3.5 *Absolute abundances and removal rates of ARGs and MGEs*

ARG and MGE absolute abundances (copies/mL) of influent, feedwater and AOP effluents were calculated as described in Section 3.2.3. Table 4.3 shows summed ARGs and MGEs per treatment. Absolute gene concentrations associated with each AOP are means of three sampling days compared with feedwater genes from the same days. Due to statistical variance among feedwater samples (see Section 4.3.2), each AOP treatment could only be statistically compared with their own feedwater.

Table 4.3 Comparisons of ARG and MGE absolute concentrations in AOP effluents and the corresponding feedwater. Standard deviation (SD) represent data from three sampling days, and significant differences between feedwater and effluent were examined in pairwise comparisons using the Games-Howell post hoc test. The 'Trend' column shows whether the concentration in the AOP effluent decreased significantly (↓) or it was not significantly different (-) compared to feedwater.

		Feedwater		Effluent			
AOPs	**Type**	**Average**	**SD**	**Average**	**SD**	**p-value**	**Trend**
1g/m^3 *H_2O_2	ARGs	**$6.06 \cdot 10^5$**	$1.17 \cdot 10^5$	**$2.06 \cdot 10^5$**	$2.65 \cdot 10^4$	**<0.01**	↓
	MGEs	**$3.91 \cdot 10^5$**	$7.18 \cdot 10^4$	**$1.27 \cdot 10^5$**	$4.05 \cdot 10^3$	**<0.01**	↓
3g/m^3 H_2O_2	ARGs	**$4.18 \cdot 10^5$**	$1.85 \cdot 10^5$	**$2.93 \cdot 10^5$**	$2.22 \cdot 10^4$	**0.24**	-
	MGEs	**$3.08 \cdot 10^5$**	$1.20 \cdot 10^5$	**$1.76 \cdot 10^5$**	$3.91 \cdot 10^4$	**0.02**	↓
9g/m^3 H_2O_2	ARGs	**$1.16 \cdot 10^5$**	$9.79 \cdot 10^4$	**$8.29 \cdot 10^4$**	$1.04 \cdot 10^5$	**0.59**	-
	MGEs	**$7.71 \cdot 10^4$**	$5.02 \cdot 10^4$	**$5.28 \cdot 10^4$**	$6.03 \cdot 10^4$	**0.84**	-
1g/m^3 O_3	ARGs	**$6.06 \cdot 10^5$**	$1.17 \cdot 10^5$	**$2.82 \cdot 10^5$**	$8.82 \cdot 10^4$	**0.02**	↓
	MGEs	**$3.91 \cdot 10^5$**	$7.18 \cdot 10^4$	**$1.13 \cdot 10^5$**	$2.68 \cdot 10^4$	**<0.01**	↓
3g/m^3 O_3	ARGs	**$4.18 \cdot 10^5$**	$1.85 \cdot 10^5$	**$5.35 \cdot 10^4$**	$1.34 \cdot 10^4$	**<0.01**	↓
	MGEs	**$3.08 \cdot 10^5$**	$1.20 \cdot 10^5$	**$4.17 \cdot 10^4$**	$5.45 \cdot 10^3$	**<0.01**	↓
9g/m^3 O_3	ARGs	**$1.16 \cdot 10^5$**	$9.79 \cdot 10^4$	**$5.94 \cdot 10^3$**	$6.17 \cdot 10^3$	**<0.01**	↓
	MGEs	**$7.71 \cdot 10^4$**	$5.02 \cdot 10^4$	**$6.51 \cdot 10^3$**	$6.12 \cdot 10^3$	**<0.01**	↓
Combination	ARGs	**$7.98 \cdot 10^4$**	$7.25 \cdot 10^4$	**$3.95 \cdot 10^3$**	$3.50 \cdot 10^3$	**<0.01**	↓
	MGEs	**$6.22 \cdot 10^4$**	$4.72 \cdot 10^4$	**$6.78 \cdot 10^3$**	$5.89 \cdot 10^3$	**<0.01**	↓
Control	ARGs	**$7.98 \cdot 10^4$**	$7.25 \cdot 10^4$	**$3.90 \cdot 10^4$**	$6.74 \cdot 10^3$	**0.06**	-
	MGEs	**$6.22 \cdot 10^4$**	$4.72 \cdot 10^4$	**$3.66 \cdot 10^4$**	$1.23 \cdot 10^4$	**0.3**	-

*H_2O_2 refers to the different concentrations tested in the system H_2O_2/UV (UV constant at 650 mJ/cm^2).

As discussed before (Section 4.3.2), direct comparisons of the absolute concentrations of the AOPs were not possible due to significant variance of feedwater ARG data among sampling days. For this reason, removal rates for feedwater and AOPs were calculated using the equations 4.1 & 4.2;

$$Log\ removal\ (feedwater) = Log_{10}\left(\frac{C_{primary\ settled\ sewage}}{C_{pile\ cloth\ filter\ discharge}}\right) \qquad \textbf{Equation 4.1}$$

$$Log\ removal\ (AOPs) = Log_{10}(\frac{C_{pile\ cloth\ filter\ discharge}}{C_{AOPs}})$$ **Equation 4.2**

Where *C* stands for absolute ARG or MGE concentrations, and AOPs for AO options tested.

Absolute influent ARG and MGE concentrations varied between 1.19 x 10^8 - 1.43 x 10^8 and 7.57 x 10^7 - 8.57 x 10^7 copies/mL, respectively. A significant decrease (Games-Howell; $p \leq 0.1$) in absolute abundances in both ARGs and MGEs were observed between the influent and feedwater (tertiary filtered effluent) (table B.9), achieving log removal rate of 2.50 and 2.64 (table B.10), respectively. Small decreases in absolute concentrations of both ARGs and MGEs from feedwater were observed after treatment with H_2O_2/UV (figure 4.5a). Interestingly, among all doses tested, statistically significant changes in ARG abundances were seen in final effluents with a dose of 1 g/m^3 H_2O_2, and for MGEs, with 1 and 3 g/m^3 H_2O_2 (table 4.3), although only log removals of < 0.63 were seen for H_2O_2 doses.

Ozonation was more effective at reducing absolute abundances of both ARGs and MGEs compared to H_2O_2/UV (figure 4.5b). Increases in ozone doses resulted in significant decreases (Games-Howell; $p < 0.01$) of absolute abundances, with removals up to 1.25 for ARGs and 1.47 for MGEs (table B.10). ARG log removals with the 3 and 9 g/m^3 O_3 doses were greater than for the 1 g/m^3 O_3 dose, although differences were not statistically significant (table B.11). Ozone doses at 3 and 9 g/m^3 also reduced MGEs concentrations, which was statistically significant for 3 g/m^3 O_3 between them (table B.11). Absolute concentration reductions (Games-Howell; $p < 0.01$) were also achieved by combing H_2O_2/UV with O_3. Similar to O_3 (3 g/m^3), about a 1.0 log reduction was observed with the AO combination (figure 4.5c), achieving significantly higher removal rates (Games-Howell; $p \leq 0.01$) than H_2O_2/UV (3 g/m^3) for both ARGs and MGEs.

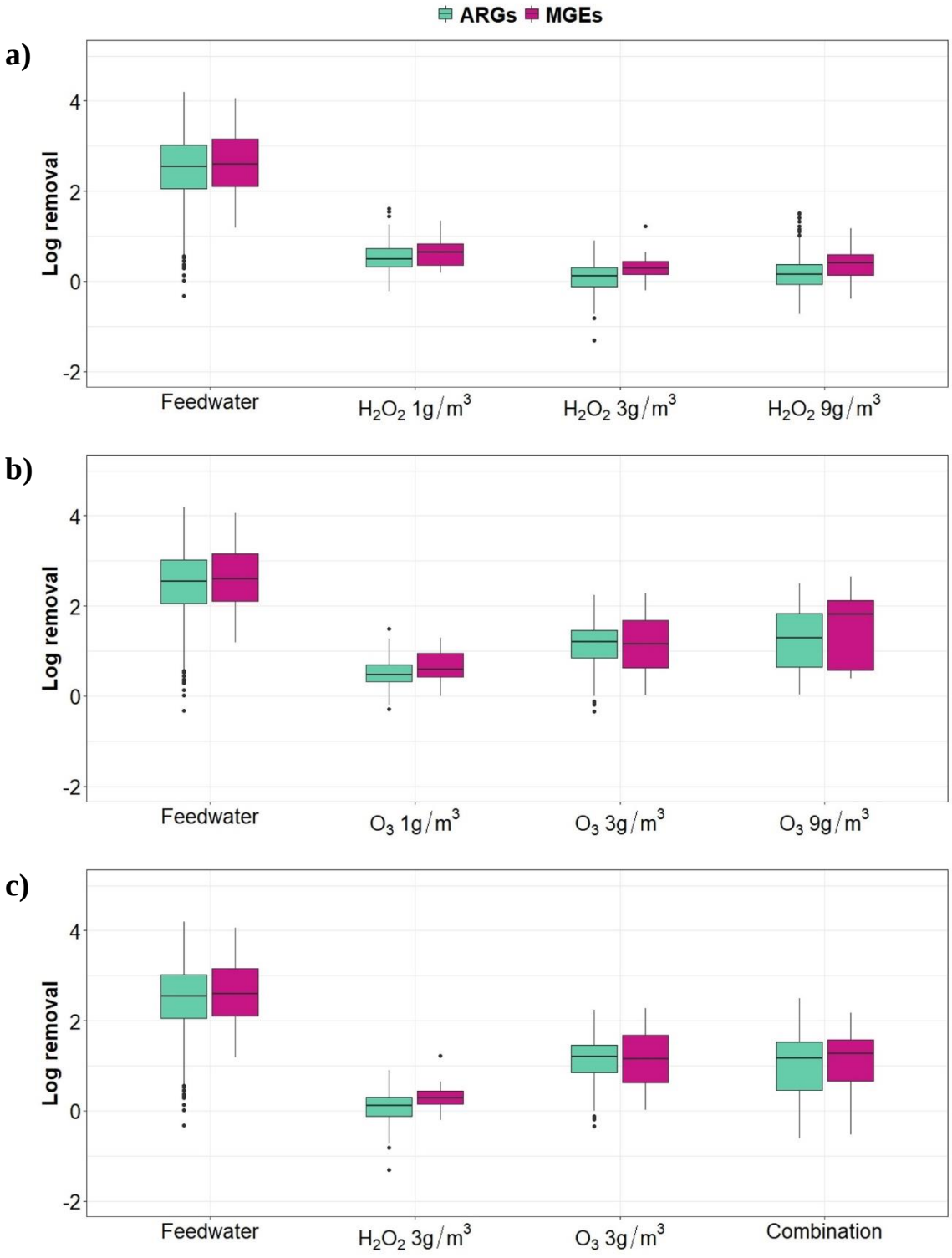

Figure 4.5 Removal rates per treatment condition, grouped in **a)** different H_2O_2 doses in the H_2O_2/UV system (UV constant at 650 mJ/cm^2), **b)** different O_3 doses and **c)** different AOPs (combination: O_3/H_2O_2/UV at 3 g/m^3/3 g/m^3/650 mJ/cm^2). Boxplots represent removal rates achieved for all ARGs and MGEs per treatment condition. Boxes represent the first quartile and third quartile of the data, the vertical line shows the median, whiskers extend from each quartile to minimum and maximum data points and outliers are depicted with dots.

4.3.6 *Relative abundances of ARGs and MGEs*

Relative abundance, expressed as absolute abundance normalized per bacterial genome (see Section 3.2.6), was determined for each treatment step to monitor the relative selection of ARGs and MGEs carried per bacterial cell among processes. As it shown in figure 4.6, MLSB was the most abundant ARG class per genome in influent (0.54 ± 0.04 gene copies/cell), followed by tetracycline (0.231 ± 0.013 gene copies/cell), non-specific ARGs (0.092 ± 0.012 gene copies/cell), aminoglycoside (0.077 ± 0.008 gene copies/cell) and β-lactams (0.05 ± 0.009 gene copies/cell), whereas sulfonamide and vancomycin (0.003 ± 0.000 gene copies/cell) were the least selected (table B.12). Relative ARG abundances were lower in feedwater compared with the WWTP influent; however, this decrease was not statistically significant for all classes (table B.13). It is worth noting that MLSB and tetracycline, the two most abundant classes in the influent, were significantly reduced (Games-Howell; $p < 0.01$) after tertiary filtration (feedwater) to 0.060 ± 0.012 and 0.043 ± 0.005 gene copies/cell, respectively. Likewise, a significant decrease from 0.552 ± 0.004 to 0.126 ± 0.012 in transposase genes was observed, whereas relative integrase genes abundances significantly increased (Games-Howell; $p = 0.017$). This increase might be attributed to interactions among bacteria within previous treatment steps in the main plant, or possible interactions of bacterial cells within the filter structure.

The AOPs reduced further the overall relative abundances of both ARGs and MGEs. Among them, relative MLSB ARGs decreased significantly in most AO conditions, except at the 1 g/m^3 O_3 dose (figure 4.6b). Transposase genes were significantly reduced (Games-Howell; $p \leq 0.01$) by all H_2O_2/UV conditions (figure 4.6a), and relative FCA ARG level was below detection at a dose of 9 g/m^3 O_3. Similar to H_2O_2/UV and ozonation, combining AOPs reduced further relative abundances per genome (by 0.006 ± 0.005) (figure 4.6c), and significant decreases were seen for MLSB and tetracycline ARG classes (Games-Howell; $p \leq 0.03$). However, comparisons between AOP types revealed non-significant differences among most classes, although relative MLSB ARG abundances significantly reduced (Games-Howell; $p < 0.01$) as O_3 dose was increased. Comparing AOPs, 3 g/m^3 O_3 and the AO combination decreased further (Games-Howell; $p < 0.05$) the most abundant class (MLSB), nevertheless similar relative abundances were observed for both treatment options. It is worth noting that MLSB, which was the most abundant class per genome in both influent and feedwater, was reduced significantly in most AOPs. In contrast, transposase genes were

reduced more effectively using H_2O_2/UV. Dissimilarities between AOP and relative ARGs may indicate different ARB may respond differently to different AOPs.

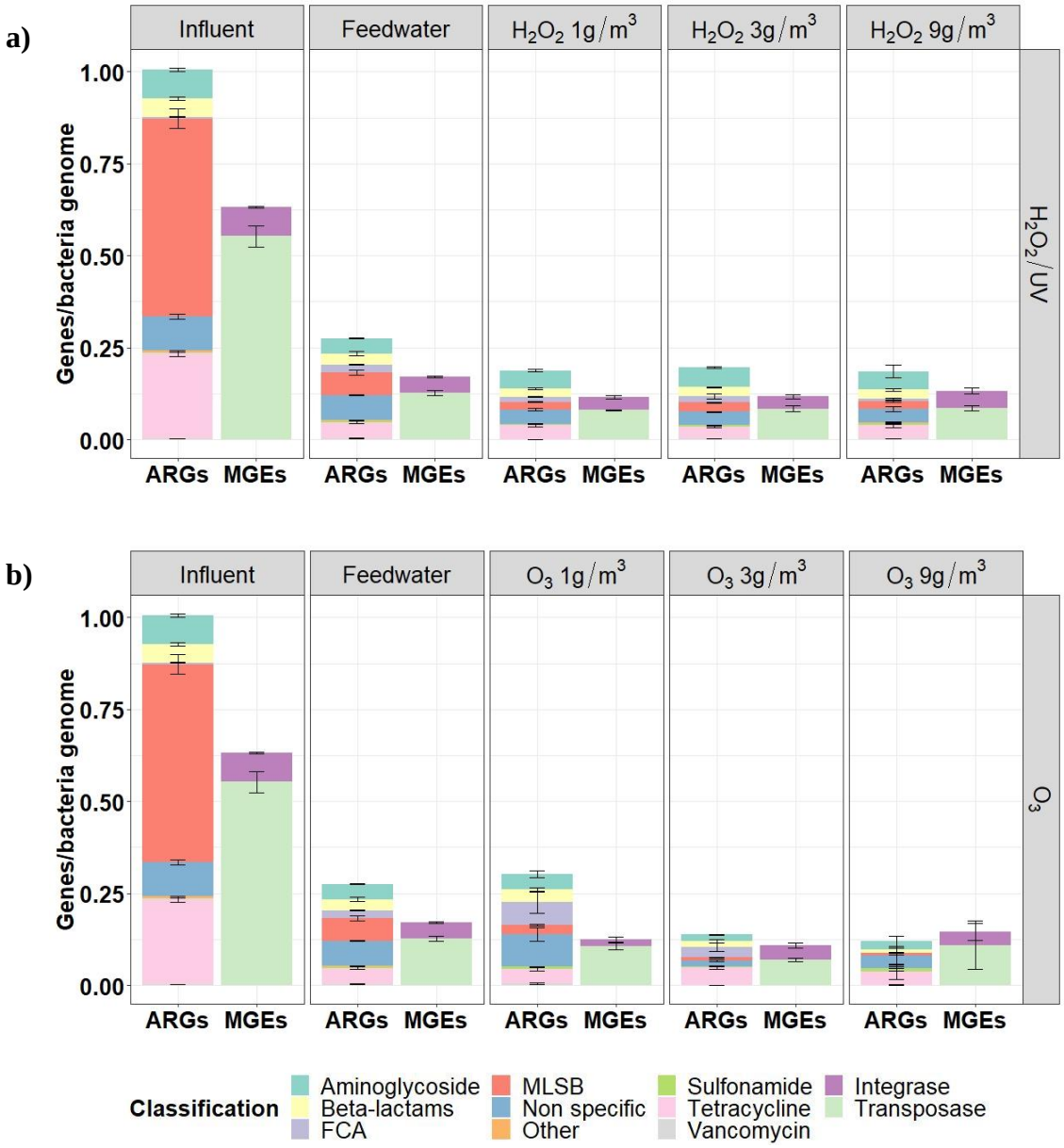

Figure 4.6 Relative abundances of ARGs and MGEs normalised per bacteria genome in each treatment step. Treatment conditions are grouped according to treatment type in **a)** H_2O_2/UV (UV constant at 650 mJ/cm^2), **b)** ozonation. Error bars represent the variability of data from 12 sampling days for both influent and feedwater and from 3 sampling days for AOPs.

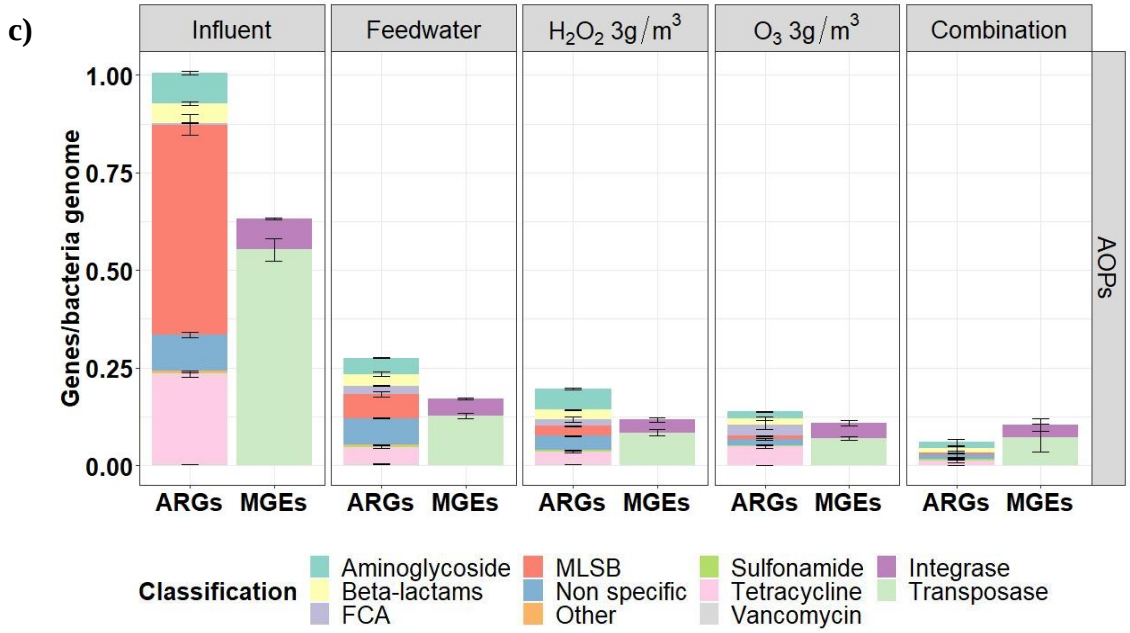

Figure 4.6(cont.) As above for **c)** all different types of AOPs including the combination O_3/H_2O_2/UV (3 g/m^3/3 g/m^3/650 mJ/cm^2).

4.3.7 *Total and viable-cell quantification of ARGs, int1 and 16S rRNA in feedwater and AOPs effluents*

ARGs in the viable bacterial fraction were quantified to assess the impact of AO processes on the destruction of ARGs in viable versus all cells (viable versus non-viable). Gene abundances in both total and viable cells were quantified using qPCR with the methods described in Section 3.2.5. Three ARGs (*tet*M, *tet*Q, bla_{OXA-10}), one MGE (*int*1) and one eubacterial 16S rRNA were the target genes. The first step of analysis consisted of statistically comparing absolute gene abundances in viable cells to those all total cells per treatment. The t-test was applied in pair samples with normal distribution and equal variances, while the non-parametric Wilcox-test was used when the assumptions for normality and homoscedasticity were not met even in transformed data.

Figures 4.7 depict the absolute concentrations of each target gene per condition in both total and viable cells. Absolute abundances of target genes in viable cells were always significantly lower (Wilcoxon; $p < 0.01$) compared with total cells in feedwater samples, although results were less consistent for AOP effluents (table B.14). For example, for H_2O_2/UV at 3 g/m^3 H_2O_2 (figure 4.7a), gene concentrations in viable cells were lower than in the total fraction, but only *tet*Q gene levels were significantly lower (Wilcoxon; $p < 0.01$). On the other hand, H_2O_2 at 9 g/m^3 achieved significant lower gene concentrations in viable cells compared to

total cells for most genes, except *int*1. Relative to ozonation (figure 4.7b), genes in viable cells in effluents treated with ozone at 3 g/m^3 were always significantly lower (t-test; $p < 0.01$) compared to the total fraction. Similarly, ozonation at 9 g/m^3 reduced significantly ARGs carried in viable cells, but 16S rRNA and *int*1 genes levels in viable cells were lower, but not significantly. However, it is worth mentioning that *tet*M genes in viable cells were below the limit of detection (LoD) for both ozone concentrations. Likewise, significantly lower concentrations of genes were found in the viable versus total cell fractions for combined AO treatment (figure 4.7c), with *tet*M and *bla*$_{\text{OXA-10}}$ being below the LoD.

4.3.8 *Comparisons between tertiary-treated versus AOP-treated effluents*

Gene concentrations of viable and total cells were statistically compared between feedwater and AOP effluents. Control samples, including samples from the final sampling location in the pilot plant before oxidation, showed gene concentrations in control samples identical to levels in feedwater (t-test; $p > 0.99$) for both viable and total cells (table B.15). This agreed with the HT-qPCR data, confirming the pilot plant itself did not have any effect on absolute cell concentrations.

When comparing AOP effluents with feedwater, ARGs in total cells were always significantly lower (Games-Howell; $p < 0.05$), with the exception being *bla*$_{\text{OXA-10}}$ genes in combined effluents, although this might be attributed to the wide distribution of the data (figure 4.7c). Similar trends were seen with ARGs detected in viable cells, where all AOP achieved either significantly lower ARG concentrations or genes were under the LoD. Integron genes in the total cells fraction also were reduced significantly (Games-Howell; $p < 0.05$) by AOP treatment, except H_2O_2/UV at 3 g/m^3. On the other hand, absolute concentrations in viable cells were lower (Games-Howell; $p < 0.05$) after applying combined AOPs or the highest doses of both H_2O_2/UV and ozonation (9 g/m^3) (table B.15). This suggested that AOPs had additional value at further reducing absolute concentrations of the target ARGs, however integron genes in viable cells were more readily reduced at higher oxidant doses. Different patterns between viable and total cells also were observed for relative levels to 16S rRNA. Total cells in effluents treated at the highest doses of both H_2O_2/UV and ozone (9 g/m^3) were significantly lower (Games-Howell; $p < 0.01$) compared to feedwater. In contrast, statistically significant decreases of viable cells were observed only after applying ozone at 3 and 9 g/m^3, while the combined AOPs was effective at reducing (Games-Howell; $p < 0.05$) both viable and total fractions.

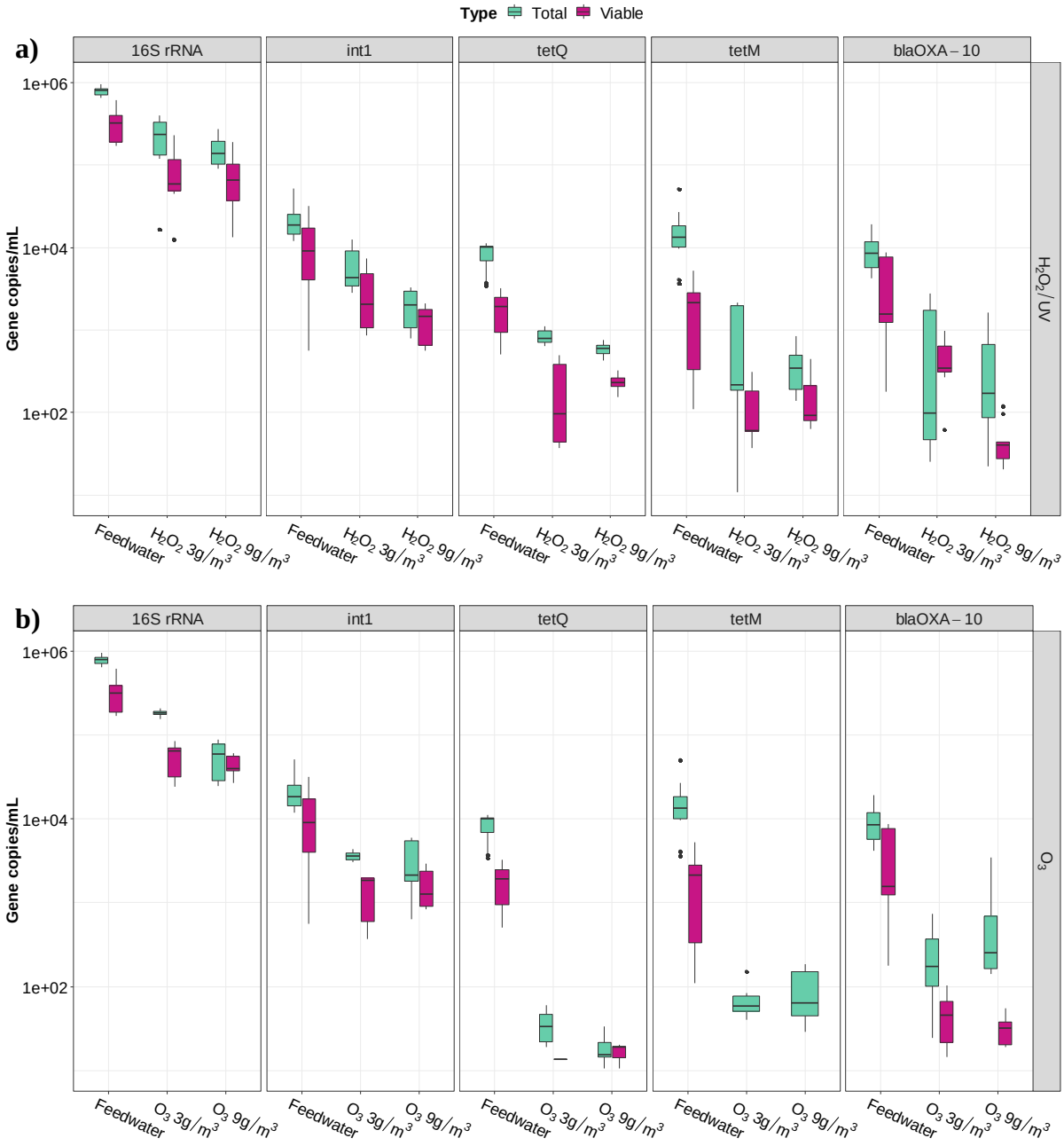

Figure 4.7 Genes absolute concentrations of 16S rRNA (both ARB & non-resistant bacteria), *int*1 (MGEs), *tet*Q, *tet*M and *bla*$_{OXA-10}$ (ARGs) are presented in total (viable & non-viable) and in viable cells measured upstream and downstream of the AOP pilot plant. Plot **a)** shows the different H_2O_2 doses applied with UV always constant at 650 mJ/cm^2 and feedwater include the corresponding samples to the oxidation conditions presented. Plot **b)** depicts the different ozone doses tested and the feedwater includes the corresponding samples to these oxidation conditions. Boxes represent the first quartile and third quartile of the data, the vertical line shows the median, whiskers extend from each quartile to minimum and maximum data points and outliers are depicted with dots.

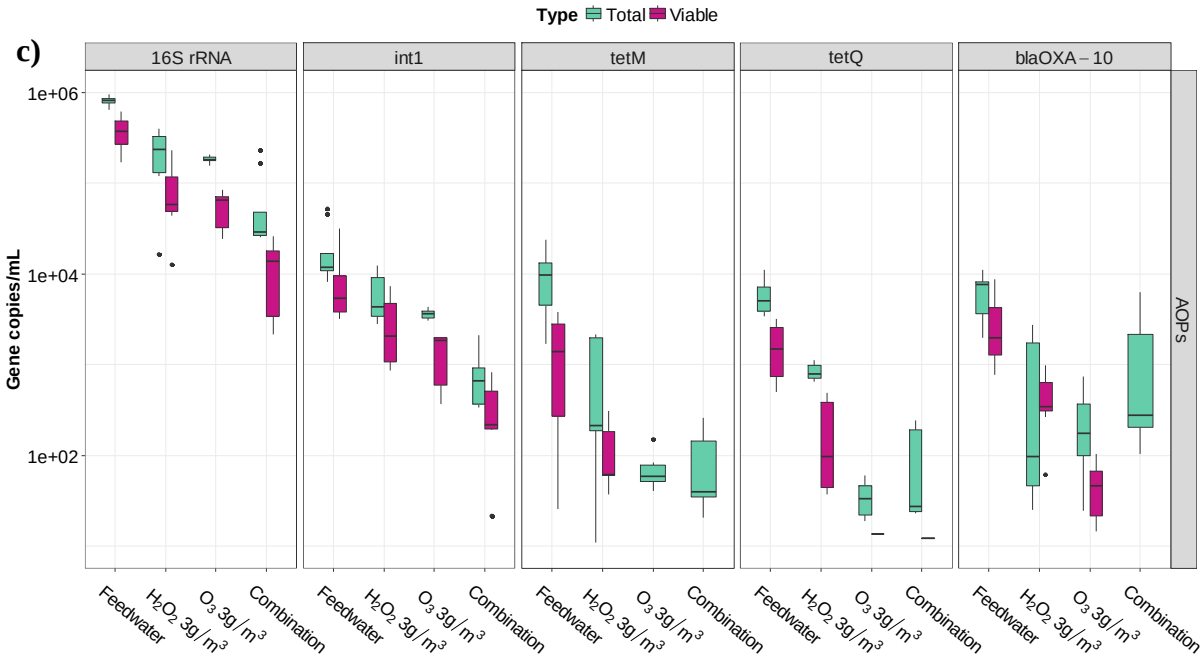

Figure 4.7(cont.) As above plot **c)** shows the different AO treatments; H_2O_2 was combined with UV (always constant at 650 mJ/cm^2), and the 'Combination' include the combination of H_2O_2 and O_3 both at 3 g/m^3 with 650 mJ/cm^2 UV.The feedwater include the corresponding samples to these oxidation conditions.

Absolute ARG concentrations also were compared among AOPs to further understand differences among oxidation options. Statistical analyses demonstrated no significant differences (Games-Howell; $p > 0.05$) among feedwater samples collected on different sampling days (tables B.16 & B.17), therefore direct statistical comparisons among different AOPs data were permitted since the inlet water throughout sampling campaigns came from the same data population. Regarding total bacteria, increases in H_2O_2 dose resulted in lower concentrations of all ARGs and also *int*1 (figure 4.7a), nevertheless only *tet*M genes were decreased significantly (Games-Howell; $p < 0.05$). Similarly, increasing ozonation dose from 3 to 9 g/m^3 did not result in further reductions in ARGs or *int*1 (figure 4.7b), which was statistically confirmed. Comparing AOP types (figure 4.7c), the combined treatment and ozonation alone at 3 g/m^3 significantly reduced *tet*Q concentrations compared to H_2O_2/UV at 3 g/m^3 (Games-Howell; $p < 0.05$), whereas the combined treatment reduced *int*1 the most relative to other AO options (Games-Howell; $p < 0.05$). Moreover, the combination achieved lower 16S rRNA absolute concentrations compared the other AOPs, although this was not statistically significant.

Interestingly, a different pattern was observed for ARGs and MGEs carried by viable cells. An increase in H_2O_2 dose increased $bla_{OXA\text{-}10}$ reduction (Games-Howell; $p = 0.03$) and gene

levels were often below the LoQ (figure 4.7a). It is also worth mentioning that data close to LoQ might be considered false positive as they are amplified close to the accuracy limit of the machine (Green and Sambrook, 2012). Similarly, some effluent *tet*M gene data were close to the LoQ treated with H_2O_2/UV, indicating that bacteria carrying *tet*M genes might be especially susceptible to this treatment. Likewise, both ozone doses resulted in effluent *tet*M levels under the LoD, while no significant differences were detected regarding other ARGs and *int*1. Comparing AOP, AOPs tested effectively reduced *tet*M and $bla_{\text{OXA-10}}$ in viable cells, although 3 g/m^3 O_3 and the combined treatment achieved levels below the LoD (figure 4.7c). Alternately, no significant statistical differences were seen for *tet*Q, *int*1 and 16S rRNA in absolute concentrations among the different AOPs.

4.3.9 *Viable cells versus total cells ratios*

The viable to total cell ratio was compared among different treatment conditions (figure 4.8). The mean ratio of all ARGs in feedwater was always ≤ 0.5 viable/total, indicating that ARGs in WWTP bacterial cells are more often in non-viable organisms, including in the effluents of all processes tested (table B.18). Mean ratios for some genes did increase after H_2O_2/UV treatment, but this is possibly due to small sample sizes and variability among samples amplified with qPCR and PMA-qPCR; differences were not significant compared to feedwater (Games-Howell; table B.19). Likewise, ozonation effluents retained viable/total ratios of ≤ 0.5. Nevertheless, *tet*M genes in viable cells were effectively removed under both conditions examined. However, the combined AO treatment did not alter the ratio (Games-Howell; $p > 0.05$) compared to neither feedwater nor other advanced treatment technology. Similar to ozonation, $bla_{\text{OXA-10}}$ and *tet*M in viable cells were below detection, suggesting these genes were more likely present in non-viable cells.

These data suggested that the different AOPs were not able to further reduce the viable/total cell ratio, except under a few conditions. On the other hand, the ratios across the WWTP and AOPs were almost always ≤ 0.5, implying most ARGs are in non-viable cells. Furthermore, despite the variance of data due to dividing into samples amplified with qPCR and others amplified with PMA-qPCR, all treatments resulted in bacteria with membranes sufficiently damaged to allow PMA enter, implying a preponderance of ARGs in un-viable cells.

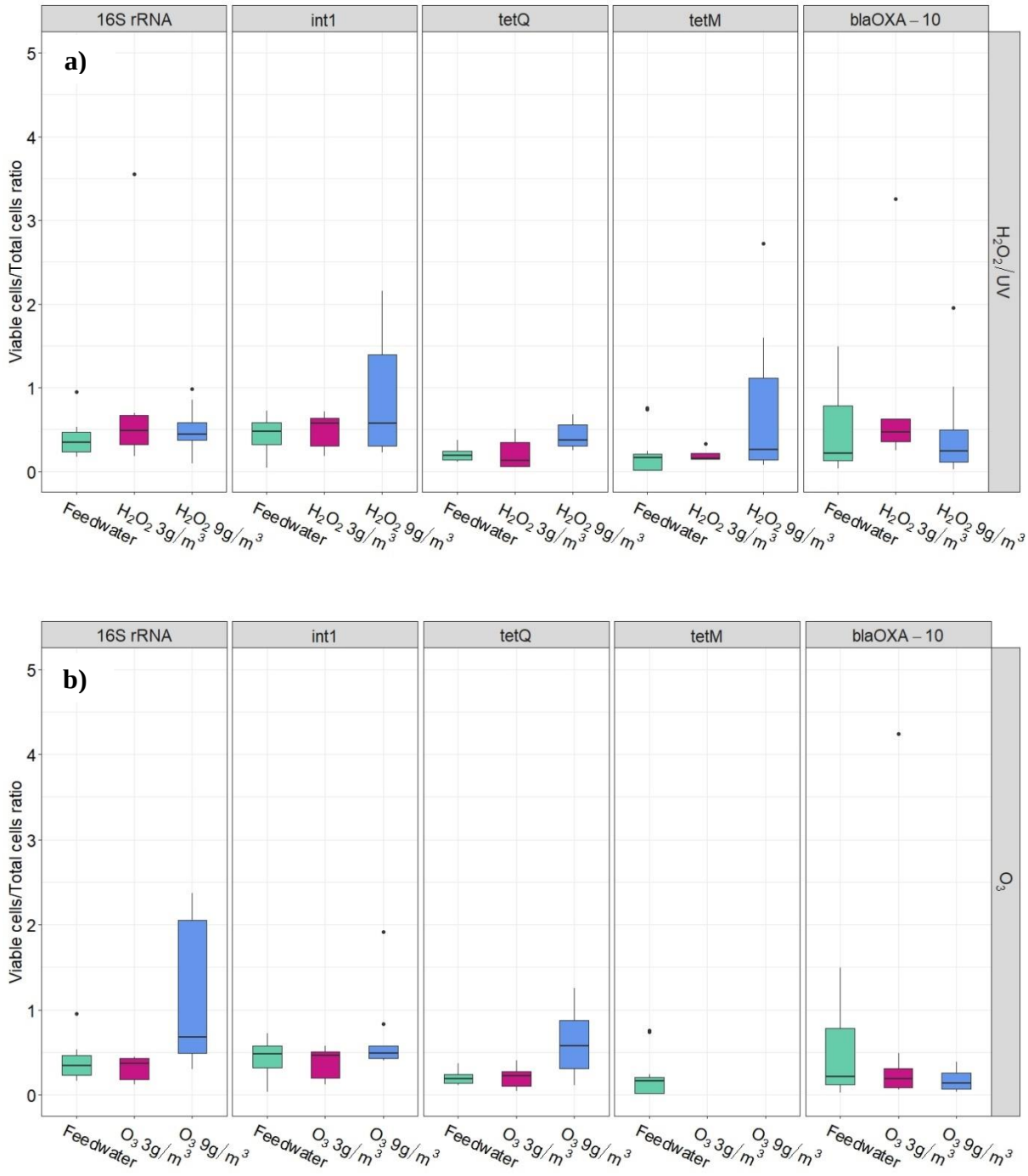

Figure 4.8 Ratios of gene concentrations in viable cells to gene concentrations in total cells in feedwater and AOP technologies. Plot **a)** shows the different H_2O_2 doses applied with UV always constant at 650 mJ/cm^2 and feedwater include the corresponding samples to the oxidation conditions presented. Plot **b)** depicts the different ozone doses tested and the feedwater includes the corresponding samples to these oxidation conditions.

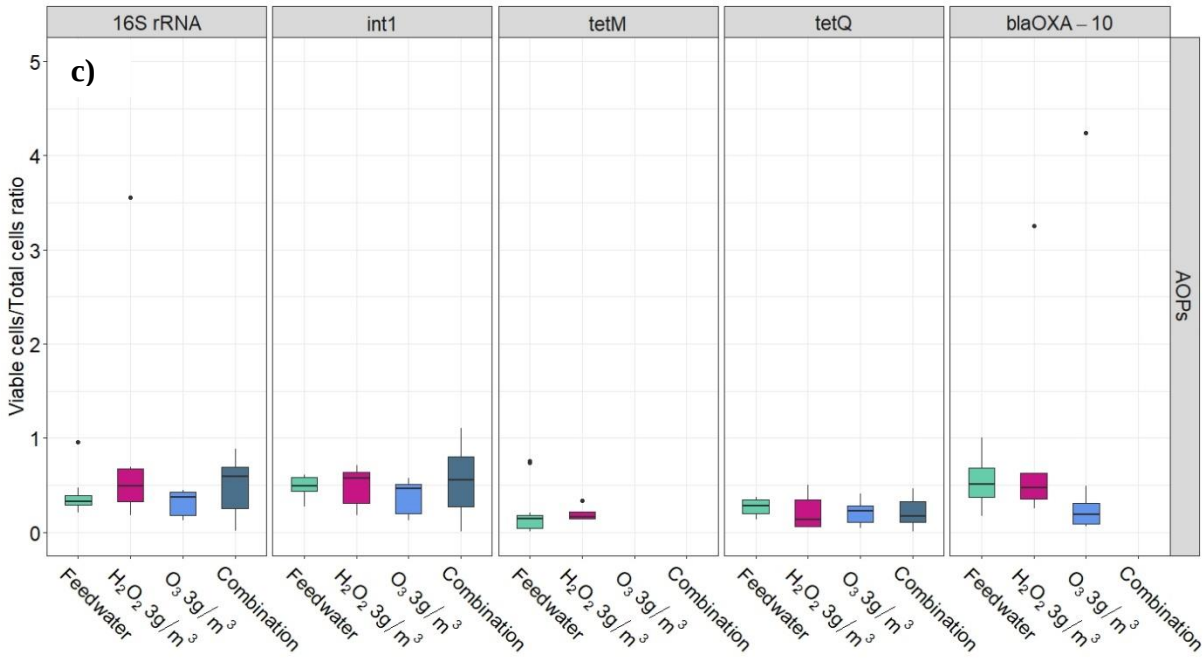

Figure 4.8(cont.) As above plot **c)** shows the different AO treatments; H_2O_2 was combined with UV (always constant at 650 mJ/cm^2), and the 'Combination' include the combination of H_2O_2 and O_3 both at 3 g/m^3 with 650 mJ/cm^2 UV.The feedwater include the corresponding samples to these oxidation conditions.

4.4 Discussion

Many studies have tested the relative effectiveness of AOPs to mitigate AMR spread into the environment from wastewater systems, but most have been lab-based and did not assess a wide spectrum of ARB or ARGs (Zhang *et al.*, 2016; Karaolia *et al.*, 2017; Sousa *et al.*, 2017). Furthermore, variability among methods and qPCR primer sets often made direct comparisons between genes and bacterial strains difficult (Michael-Kordatou *et al.*, 2018). The importance of consistently monitoring a wide spectrum of AMR agents in pilot- to full-scale systems has been highlighted before (Hiller *et al.*, 2019). Therefore, work in Chapter 4 aimed to fill the gap between bench and pilot assessments by comparing three different AOPs in terms of reducing abundances and diversities of a wide range of ARGs in larger scale systems.

β-lactam, MLSB, non-specific (e.g., multidrug-efflux pumps), tetracycline and vancomycin were ARG classes with the greatest presence in the influent to the main WWTP (figure 4.3). Numbers of unique ARGs of all classes were significantly lower after tertiary filtration (feedwater) in almost all groups. However, unique MGE numbers did not change across the main WWTP, although this might simply be due to small number of MGEs quantified.

Regarding the pilot plant AOPs, HT-qPCR data showed that ozonation at 3 and 9 g/m^3 were most effective at reducing unique ARG numbers, whereas MGEs only were reduced significantly at the highest ozone dose (9 g/m^3) (figure 4.3). Although H_2O_2/UV was generally not effective at reducing ARG numbers, combining it with a dose of O_3 3 g/m^3 reduced significantly (Games-Howell; $p < 0.05$) the overall number of ARGs (figure 4.3). It is also noteworthy that ozonation at 9 g/m^3 and H_2O_2/UV/O_3 both reduced FCA and 'Other' to below the LoD, indicating these conditions may be particularly effective at reducing ARG diversity in wastewater effluents. This may be because the effectiveness of ozonation is often close related to bacterial type (Wojtenko, 2001; Alexander *et al.*, 2016; Czekalski *et al.*, 2016). Although ozonation and the combined treatment reduced the total number of unique ARGs, there was evidence of specific ARG classes and mechanisms being more or less effectively reduced in these systems. Furthermore, unique persistent genes in AOP effluents also did not follow either distinct pattern according to class or mechanism, suggesting no obvious selective removals.

HT-qPCR data also indicated significant decreases in absolute abundances of ARGs in tertiary filtered discharge (feedwater) compared to primary settled sewage (influent) (table 4.3). Here, we additionally show the AOPs further reduced ARGs from final effluents. As identified before (Michael-Kordatou *et al.*, 2018; Hiller *et al.*, 2019; Xue *et al.*, 2019), absolute abundance ARG decreases observed here depended on technology type and AO dose. For instance, H_2O_2/UV was not as effective as other AO options at reducing absolute ARG abundances, while increasing H_2O_2 dose did not enhance ARG removal (figure 4.5). Interestingly, higher removal rates achieved with lower H_2O_2 doses which have been reported before. Zhang *et al.* (2016) showed that higher concentrations of H_2O_2 combined with UV resulted in reduced removal rates, suggesting the scavenging effect of higher $^{\bullet}OH$ radicals may become significant with high H_2O_2 concentrations. Furthermore, Michael-Kordatou *et al.* (2018) reported that reactive oxidative species (ROS) scavenging can be activated by bacterial cells, including b-carotene which is a singlet oxygen scavenger, and superoxide dismutase that protects bacteria from radiation and oxidative stress.

In contrast, ozonation significantly and increasingly reduced ARG abundance as O_3 was increased (figure 4.5). However, although higher ARG removal rates were achieved by increasing O_3 dose from 1 to 3 and 9 g/m^3, no significant differences were observed between the two highest doses, which contradicts a previous study that showed higher doses increased reductions (Zhuang *et al.*, 2015). Our data is promising because the middle dose, 3 g/m^3 O_3, is conventionally recommended for continuous full-scale applications at the WWTP Neugut in

Switzerland (Bourgin *et al.*, 2018), implying this dose may be adequate for efficient ARG reduction via ozonation. With respect to the combined AOP treatment, significantly higher ARG reductions were observed compared with H_2O_2/UV alone (3 g/m^3) (figure 4.5), but removals were not significantly greater than ozone alone at 3 g/m^3, suggesting that H_2O_2/UV inclusion was not needed for ARG removal. Combining H_2O_2/UV (3 g/m^3) and O_3 (3 g/m^3) did increase UV transmittance and reduce effluent turbidity, however the combination provided no added value for reducing ARG diversities and abundances in treated effluent.

In this study, we also assessed the fate of ARGs carried in both viable and total cells. Using the PMA viability test, ARGs in viable cells in feedwater were always less than in total cells in the same samples, confirming that most of the ARGs found in feedwater (tertiary filtered discharge) are in non-viable cells (figure 4.7). This was expected given that bacterial cells were exposed to physical and biological stress within tertiary filtration and previous treatment steps of the main plant. This has been also shown in the GAS system including pile cloth filtration as the last treatment step (figure 3.7), and it is in agreement with previous studies, using culture-based techniques, that showed that conventional WWTPs have the capacity to reduce both viable and non-viable cells within a conventional WWTP (Lavender and Kinzelman, 2009; Li *et al.*, 2014; Eramo *et al.*, 2019). Our data additionally suggests that the AOPs also result in lower ARGs in viable compared with non-viable cells. This is an invaluable new observation. Previous studies have highlighted the importance of distinguishing between ARGs carried in viable versus non-viable cells because it has implications to HGT and other active gene exchange AMR transmission pathways (Michael-Kordatou *et al.*, 2018; Eramo *et al.*, 2019; Hiller *et al.*, 2019).

Therefore, using PMA and qPCR in tandem shows that detected ARGs are not always present in viable cells and conventional qPCR and other previous metagenomic methods almost certainly overestimate “viable” ARG levels, and not accurately reflect the hazard associated with ARB and ARGs in treated effluents. More information is needed about the fate of genes in viable and non-viable host cells after the treatment process, as bacteria with broken cells can activate reparation mechanisms and re-produce in post-treated effluents (Alexander *et al.*, 2016; Deng *et al.*, 2019). However, Hiller *et al.* (2019) and Hong *et al.* (2018) discussed that more accurate investigation is required on the fate of ARB and ARGs released into the environment, while it is quite reasonable that any technology that can enhance the die-off of viable cells carrying ARGs will almost certainly reduce HGT of ARGs released in wastewaters.

Similar to HT-qPCR, qPCR showed a decreasing trend on the target ARGs and MGEs concentrations after applying advanced oxidation that in many conditions was also statistically confirmed (table B.15). It is noteworthy that significant reductions of some ARGs were achieved using lower oxidant levels, which is promising from an economic perspective, where other ARGs require higher oxidant doses. These differences agree with differences in observed ARG prevalence and diversity patterns, confirming gene-to-gene differences (implicitly cell-to-cell differences) are key critical in how ARB respond to each type of oxidative stress.

To understand context, a quick review of oxidation processes is of value. The main advantage of AOPs lie on the formation of ROS, which can damage and-or modify cell walls and membranes (Huang *et al.*, 2000; Alrousan *et al.*, 2009). However, oxidative pathways and mechanisms vary (Michael-Kordatou *et al.*, 2018; Hiller *et al.*, 2019; Xue *et al.*, 2019). The principle oxidative mechanisms in the system of UV/H_2O_2 are UV, H_2O_2, and $^{\bullet}OH$ formed by the reaction between UV and H_2O_2 (Ruppert *et al.*, 1994). UV irradiation typically attacks DNA, resulting in ruptures, single strand breaks, nucleic acid modifications and replication disruption, often leading to cell inactivation or death (Michod *et al.*, 2008; Eischeid *et al.*, 2009; Dodd, 2012). Additionally, H_2O_2 itself can cause mutations, and in combination with UV, can form $^{\bullet}OH$ that can be highly damaging to external cell structures as well as intracellular components, accelerating cell death (Park *et al.*, 2005; Giannakis *et al.*, 2018). Hydroxyl radicals are expected to target first the extracellular structure of the bacterial cell, as described previously, resulting in ruptures in membranes, producing non-viable cells after treatment. As we discussed previously, no significant differences were observed for H_2O_2 at 3 g/m^3 between viable and total cells, suggesting that this condition was effective at eliminating non-viable cells by oxidation.

Our data suggested that ozonation was far more efficient at reducing ARG diversity and abundances in both viable and total cells than H_2O_2/UV. A recent study suggested that the superiority of ozonation over other options is probably based on the presence of two powerful oxidants, O_3 (2.07 V) and $^{\bullet}OH$ (2.8 V), present in the process (Ikehata *et al.*, 2006). Many oxidative mechanisms are reported, with the most common including reactions of O_3 with external membrane structural components, such as phospholipids or lipopolysaccharides (Dodd, 2012), and with organic molecules, attacking carbon double bonds, functional groups or aromatic rings (Ikehata *et al.*, 2006). Furthermore, $^{\bullet}OH$ promote radical-radical and electron transfer reactions, mineralization of organic compounds, compromising cells by increasing cell permeability, and release of intracellular components to exterior environments,

thus facilitating nucleic acid oxidation (Von Sonntag, 2001; Cho *et al.*, 2010). Hence, the main pathway in ozonation include attacks on membrane walls and cells, and subsequent reactions with internal components and structures exposing intracellular content to the exterior environment. Both HT-qPCR and qPCR data showed that ozonation at 3 and 9 g/m^3 doses were effective at reducing absolute concentrations achieving similar removals (Games-Howell; $p > 0.05$), whereas ozonation at 9 g/m^3 was slightly more effective on both viable and non-viable cells, although not significantly. This suggests 3 g/m^3 might be an optimal dose, at least for contact times used in this pilot plant. By combining H_2O_2/UV with ozonation, we expected greater impact on bacterial cell destruction, given the collective of oxidative species (Ruppert *et al.*, 1994). However, an amplified oxidant effect was not seen. This suggests that ozonation was the principle oxidative driver factor in pilot plant removals.

It should be noted that relative abundances per genome of ARGs in feedwater were significantly lower compared to influent (figure 4.6); suggesting that relative ARG levels were effectively reduced prior to the AOP processes. This implies the AOPs may not be needed, except under conditions with sensitive receiving waters or for reuse. The same decreasing pattern was also detected in AOP effluents, confirmed statistically in some classes such as MLSB (table B.13), which was the most abundant class per genome in both influent and tertiary filtered discharge. This contrasts with previous studies that showed a positive selection of target antibiotic resistance strains after advanced oxidation treatment such as ozonation (Luczkiewicz *et al.*, 2011; Lüddeke *et al.*, 2015).

Overall, tertiary filtration was effective at reducing both absolute and relative abundances as well as diversities of ARGs and MGEs. AOPs did enhance ARG reductions, nevertheless effectiveness closely depended upon technology type and dose, as well as oxidative mechanism and ARG class. H_2O_2/UV was the least effective technology; nevertheless, this could be a result of the insufficient contact time in pilot tests here. In fact, lab-scale studies have shown that longer reaction times can increase effectiveness (Fiorentino *et al.*, 2015; Ferro *et al.*, 2016; Zhang *et al.*, 2016; Ferro *et al.*, 2017). On the other hand, increases in ozonation dose, as demonstrated before (Zhuang *et al.*, 2015), affected significantly reductions in both diversities and gene concentrations, while its combination with H_2O_2/UV did not enhance further its effectiveness.

4.5 Conclusions

This study assessed the added value of quaternary treatment to the overall performance of an AS WWTP. H_2O_2/UV, ozonation and the combination O_3/H_2O_2/UV were tested in a pilot plant, studying the effect of different H_2O_2 and O_3 doses against ARG and MGE abundances and diversities in the treated effluent. HT-qPCR analysis of total bacteria showed that although pile cloth filtration (feedwater) significantly reduced ARG abundances and diversities compared to primary settled sewage (influent) of the main WWTP, AOPs enhanced the overall treatment.

ARG and MGE removal depended on technology type and AO dose, as well as oxidative mechanism and ARG class. For instance, H_2O_2/UV had no added value at reducing ARG and MGE absolute concentrations (up to 0.63 log removal) and diversity; however, this may be result of the short treatment contact time. On the other hand, increasing doses of O_3 resulted in higher removal rates (up to 1.47 log removal, considering also MGEs); while 3 and 9 g/m^3 O_3 reduced significantly ARG diversity. Although no statistical differences at reducing ARG abundances were observed between 3 and 9 g/m^3 at reducing abundances, 9 g/m^3 O_3 had a significant effect on reducing MGE diversity. Interestingly, the combination did not enhance ARG and MGE reduction compared to ozonation, achieving similar removal rates (approximately 1 log removal). This implies that ozonation was the main oxidative driver in ARG removal, these findings are promising because they show that there is no need to include additional chemicals or UV irradiation in the process.

Differentiating gene carriage in viable and total cells revealed that target ARG abundances in viable cells of pile cloth filter discharge (feedwater) were always lower than in total cells, which was also seen in the GAS system (Chapter 3). In addition, AOPs treatment resulted in lower viable ARG abundances compared to total and it is worth noting that the viable to total cell ratio observed in effluents treated with ozonation and the combination were close to 0.5, implying that these technologies can enhance the die-off of viable cells carrying ARGs.

The PMA-qPCR data confirmed the absolute abundances decreasing patterns after quaternary treatment, also seen in HT-qPCR data. Both these data also shows gene-to-gene differences across AOPs, including genes below the LoD, for example, PMA-qPCR data shows *bla*$_{OXA10}$ and *tet*M below the LoD after ozone treatment, while HT-qPCR data shows FCA and 'Other' classes below the LoD after the same treatment. Different ARG prevalence and diversity patterns suggest ARB respond differently to different type of oxidative stress. Regardless of

the specificity of oxidants against bacterial types, selection for ARGs were not evident as both diversity and relative abundances per genome were significantly decreased after treatment with advanced processes.

Although AOPs enhanced the performance of the main WWTP, application of quaternary treatment is justified depending on the use of water or sensitivity of receiving environments, as well the water quality standards to be met in future legislation.

Chapter 5 Using Fe-bearing clay minerals to remove antibiotic resistance genes from domestic wastewater

5.1 Introduction

Disinfection technologies, typically used for treating wastewater for reuse purposes, are an alternate option for eliminating ARB and ARGs from WWTP final effluents (Sharma *et al.*, 2016; Barancheshme and Munir, 2018; Hiller *et al.*, 2019). Conventional disinfection processes, including chlorination, ozonation and UV irradiation, have been successfully applied as the final treatment step in WWTPs, improving further the quality of water before reuse or discharge. Recent findings suggest that disinfection processes also can be quite effective at reducing ARB and ARGs from final effluents, although higher doses than conventional use are often needed, elevating energy use and operational costs, as well as the need in chemicals (McKinney and Pruden, 2012; Zhang *et al.*, 2015b; Zhuang *et al.*, 2015; Calero-Cáceres and Muniesa, 2016).

Regardless, AOPs are an option for reducing AMR dissemination via wastewater pathways, especially when used in combination. Combined AOPs have value due to their intended formation of $^{\bullet}OH$, which have the most positive reduction potential among all oxidants involved in such processes (Sharma *et al.*, 2016), and react non-selectively with a wide range of organic and inorganic compounds (Oturan and Aaron, 2014). AOPs, such as UV/H_2O_2 or solar/H_2O_2, homogenenous (Fe^{2+}, Fe^{3+}/H_2O_2) or heterogeneous (photo)catalysis (UV/TiO_2), have been studied for the treatment of emerging contaminants from wastewater (Michael-Kordatou *et al.*, 2018). However, an increasing number of studies also have been conducted on ARB and ARG reduction from wastewater (Ferro *et al.*, 2015; Fiorentino *et al.*, 2015; Zhang *et al.*, 2016; Ferro *et al.*, 2017; Yoon *et al.*, 2017; Rodríguez-Chueca *et al.*, 2019), some of which have shown promise.

Although AOPs can be very effective reducing ARGs and ARB from wastewater effluents under optimum conditions, their utility can be limited when UV or ozonation are involved in the process due to elevated operating and energy costs. Therefore, alternate technologies based on natural materials may be more sustainable, such as ones that promote reactive oxygen species (ROS) formation with less energy. For instance, there is growing interest in clay minerals due to their antimicrobial effect on treating skin and other nosocomial infections

(Morrison *et al.*, 2014; Behroozian *et al.*, 2016; Morrison *et al.*, 2016; Otto *et al.*, 2016; Zhanel and Karlowsky, 2016; Svensson *et al.*, 2017).

Approximately 5% of clays consist of mineral assemblages with antibacterial properties (Williams, 2017). Clay is a term that refers to assemblage of different types of minerals with fraction size < 2 μm (Guggenheim *et al.*, 2006) . Clay minerals, in contrast, are a defined class of minerals, hydrous aluminium phyllosilicates, with variable amounts of iron (Fe), aluminium (Al), magnesium (Mg), silicon (Si) and other elements in their structure (Guggenheim *et al.*, 2006) . The use of clays and clay minerals as antibacterial agents is an example of 'geomimicry'; a term often used to describe materials and processes linked to geology (Williams, 2017). Several mechanisms have been linked to clays and clay minerals being antibacterial, as summarised in Williams (2017). The processes include: physical interactions between clay minerals and bacteria that can prevent bacteria from essential nutrients by adsorbing them; release of Fe^{2+} and other transition metals that attack bacterial membranes; and formation of ROS initiated by reduced Fe ions, which then can enter and interact with intracellular material such as DNA.

Investigating clays in remediation is a new field of interest and early work is sufficiently promising to consider their use against AMR genes and strains. For instance, the antibacterial potential of illites, montmorillonites, kaolinites and other Fe-rich clay minerals have been shown on both antibiotic-susceptible and antibiotic-resistant pathogenic strains (Haydel *et al.*, 2008; Otto *et al.*, 2016); while, other studies have provided insights into antibacterial mechanisms of hydrated clay minerals against bacterial cells. Oregon Blue clay, which has shown broad-spectrum antibacterial activity, was tested against a wide range of pathogens, including *E. coli*, ESBL *E. coli*, *S. enterica* serovar Typhimurium, *P. aeruginosa*, *S. epidermidis*, MRSE, *S. aureus*, and MRSA (Morrison *et al.*, 2016). Study on the antimicrobial mechanisms showed that Oregon Blue clays absorb cations and release metals which can be bactericidal, and the production of hydroxyl radicals also can contribute antimicrobial properties. Furthermore, a synergistic effect of Fe^{2+} and Al^{3+} was shown to attack various bacterial cellular systems, suggesting that Al^{3+} and Fe^{2+} may misfold membrane proteins and, after entering the cytoplasm, react with intracellular proteins and DNA, inducing hydroxyl radicals' formation. The same group also studied the effect of antibacterial clays from different hydrothermal deposit zones near Crater Lake (Morrison *et al.*, 2014), where they found antibacterial effects varied according to levels of oxidation. They showed that the uptake of Fe^{2+} by bacteria compromises bacterial metabolism by saturating cell with excess of Fe^{2+} and also by impacting iron storage proteins; whereas the formation of ROS, induced by

intracellular Fe^{2+}, can react with biomolecules and precipitate Fe-oxides , which is consistent with Williams *et al.* (2011).

The potential of clay minerals to form ROS can be enhanced by combining Fe-bearing clay minerals with H_2O_2. Specifically, H_2O_2 can be converted to hydroxyl radicals, superoxide and/or other ROS upon contacting with clay minerals, in a Fenton-like process (Remucal and Sedlak, 2011). As Pham *et al.* (2012) suggested, this process has advantages compared to Fenton reactions because it can be effective under non-acidic conditions and does not produce an iron sludge. Although longer contact times are needed, it has been effectively applied *in situ* for remediation of organic contaminants in soil, groundwater and wastewater (Pignatello *et al.*, 2006; Krembs *et al.*, 2010), and with more knowledge of the process kinetics, process performance can be enhanced (Pham *et al.*, 2009; Pham *et al.*, 2012; Liu *et al.*, 2014).

Reduced clay minerals, i.e. clay minerals with their structural Fe partly or completely reduced to ferrous Fe (Fe^{2+}), have also been examined for their oxidising potential. Structural Fe^{2+} in reduced clays can be oxidised by oxygen (O_2), initiating a series of reactions involving the production of ROS with high oxidative potential (Tong *et al.*, 2016; Liu *et al.*, 2017). Recently, the antibacterial properties of a reduced Fe-rich clay mineral (nontronite NAu-2) has been tested in *E.coli*, where oxidative mechanisms targeting a membrane lipid, cardiolipin, has been suggested as a potential path of oxidising agents accumulation inside cytoplasm inducing cell lysis (Wang *et al.*, 2017).

Given the unexpectedly poor performance of H_2O_2/UV in the AOP pilot plant (see Chapter 4), it was decided to perform follow-up laboratory scale experiments to elucidate important parameters that affect H_2O_2/UV performance, potentially developing alternate options that are more effective and also might have lower operating and energy costs (i.e. by replacing UV and if possible H_2O_2). Within this context, we assessed the antimicrobial activity of clay minerals targeting antibiotic resistance strains, and to the best of our knowledge, this is the first attempt to study the potential of clay minerals for reducing ARGs from real wastewater. We used an Fe-rich clay mineral, nontronite NAu-1 (~20 wt% Fe) as a) a natural mineral catalyst for H_2O_2 activation and, b) after reduction of the structural Fe to Fe^{2+}, to produce reactive oxidising species upon Fe^{2+}oxygenation without the need to add H_2O_2. We evaluated the ability of NAu-1 to reduce ARGs from domestic wastewater across a range of H_2O_2 concentration and contact times and compared it with the efficiencies of conventional UV and H_2O_2/UV technologies.

5.2 Materials and Methods

5.2.1 *Wastewater samples collection*

The value of UV irradiation and AOP treatment for reducing ARG abundances from wastewater was tested on secondary clarifier effluents from an activated sludge WWTP. Domestic wastewater was collected from the secondary clarifier tank from a WWTP in NE England. Typically, on the day of each experiment, grab samples were collected from the WWTP in sterile autoclavable polypropylene containers (Fisher Scientific, UK) and transferred in cool boxes to Newcastle University for further analysis and use within 24 hrs from collection. Basic water quality parameters, such as pH, dissolved oxygen (DO) and conductivity, were always measured on site with a digital HQ40D Portable Multi Meter (Hach Lange, UK), pre-calibrated before sample collection (table C.1).

5.2.2 *Wastewater quality determination of pre-treated samples*

Chemical oxygen demand (COD) was analysed in triplicate for each sample, using the LCK 314 COD cuvette test (Hach Lange, UK), according to manufacturer's instructions. The range used was 15-150 mg/L, employing an automatic recognition system in a DR 6000 Benchtop spectrophotometer (Hach Lange, UK). Total suspended solids (TSS) also were analysed in triplicate according to American Public Health Association (APHA) standard method (APHA, 2005). Glass fibre filters (VWR, UK) were dried in an oven at 104 ^{o}C for 15 minutes. The papers then were heated to constant weight at 550 ^{o}C in a muffle furnace for 10 minutes. Afterwards, they were allowed to cool in a desiccator and weighed to the nearest 0.1 mg using an analytical balance (PG503 DeltaRange). Typically, 20 mL per sample of secondary clarifier effluent were filtered, using a vacuum filtration unit connected to a pump through tubing. For TSS, the filters were dried at 104 ^{o}C for one hour. After drying, filters allowed to cool in a desiccator and weighed to nearest 0.1 mg. TSS are reported in mg/L using the equation 5.1:

$$mg\ \text{TSS}/L = \frac{(A-B)\ x\ 1000}{sample\ volume,mL} \qquad \textbf{Equation 5.1}$$

where, A = weight of dried residue + filter (mg), and, B = weight of filter (mg).

5.2.3 *Preliminary experiments*

The clay mineral-related AOP experiments were conducted in two phases. A preliminary set of experiments were performed to benchmark UV, H_2O_2/UV, and H_2O_2/NAu-1 performance

relative to gene reduction. Further tests then were performed to assess the influence of selected operating factors on NAu-1 gene removal performance. A summary of the preliminary experiments is shown in table 5.1.

Table 5.1 Preliminary experiments.

Experiment	Treatment	Contact/exposure times	Concentrations	Irradiance
AOPs	UV	5, 15, 30 min	-	320 µW/cm^2
	H_2O_2 UV	5, 15, 30 min	680 g/m^3 (20 mM) -	- 320 µW/cm^2
	H_2O_2 NAu-1	8 hrs	680 g/m^3 (20 mM) 1 g/L	-
	NAu-1 Control	8 hrs	1 g/L	-

The first batch of reactor experiments consisted of testing different UV doses in their relative ability to reduce ARGs from domestic wastewater. The main objective was to examine the effect of the UV dose in the treatment, which could not be evaluated in the AOP pilot plant due to time constraints. A 15-W, germicidal UV lamp (254-nm wavelength, model SC8D; Eurodyne, UK) apparatus inside a sterile microbiological hood was used. A UVP radiometer (VWR, UK) determined the irradiance of the UV lamp.

Evaluation of the lamp's UV intensity showed stabilised performance within 5 min, although the lamp was warmed up for 15 min before starting the experiments, as suggested elsewhere (Macauley *et al.*, 2006; Calero-Cáceres and Muniesa, 2016). The UV dose was determined using the equation $D = I \times T$, where D is the dose, T is the exposure time and I the intensity of the lamp (Calero-Cáceres and Muniesa, 2016).

According to Dodd (2012) and Hijnen *et al.* (2006), the doses commonly applied in water treatment practice are from 10 to several hundred mJ/cm^2. Therefore, in this initial experiment, UV doses of 96, 288, and 576 mJ/cm^2 were used to test a broad UV range, including a conventional UV dose (100 mJ/cm^2) (Michael-Kordatou *et al.*, 2018) and higher doses close to those used in the AOP pilot plant (650 mJ/cm^2). The irradiance was fixed throughout the experiment at 320 µW/cm^2, and the UV doses were controlled by changing the exposure time. Therefore, the contact times were 5, 15 and 30 min which correspond to 96, 288, and 576 mJ/cm^2, respectively. UV experiments were carried out in 10 cm diameter 500 mL cylindrical borosilicate glass beakers (VWR, UK) (herein called "reactors"), containing

250 mL of secondary clarifier effluent with a ~ 4.0 cm water height. Each UV dose was performed in triplicate, including three separate reactors per condition. The reactors were placed 47 cm from the UV lamp and magnetically stirred during the UV exposure period at a room temperature (22 ± 2 ^{o}C).

The second reactor experiments assessed combinations of UV and H_2O_2. The experimental set-up was similar to the previous UV experiments. However, hydrogen peroxide (30% w/v stock; Fisher Scientific, UK) was diluted into the reactors and the resulting solution was stirred during treatment. The initial concentration of the hydrogen peroxide was 680 g/m^3 (20 mM), and each condition was examined in triplicate in three reactors run in parallel. This H_2O_2 concentration was chosen because it has been tested before in studies including the combination of H_2O_2 with either UV or clay minerals (Liu *et al.*, 2014; Zhang *et al.*, 2016). At the end of the H_2O_2/UV treatment, 20 µL of 2300 units/mg catalase of bovine liver (Sigma-Aldrich, UK) at 0.1 g/L was added to 1.0 mL samples to eliminate residual H_2O_2, terminating the reactions (Ferro *et al.*, 2015; Fiorentino *et al.*, 2015). This dose has been shown to not affect bacterial viability (García-Fernández *et al.*, 2012).

In parallel with the H_2O_2/UV experiment, H_2O_2 doses in combination with clay minerals (NAu-1) also were tested. The NAu-1 used in this study was purchased form the Source Clays Repository of The Clay Minerals Society (www.clays.org) and had an Fe content of 19.8 wt%. To create the clay mineral powder, as-received NAu-1 was dried, crushed in a ball mill, and size-fractionated to ≤ 2 µm particles (Entwistle *et al.*, 2019). The NAu-1 powder was then sterilised in an autoclave cycle at 121 ^{o}C for 15 min by using saturated steam under at least 15 psi of pressure. Experiments on the effect of autoclaving on the structure of NAu-1 were not conducted; however, according to a study, divalent- and trivalent-cation smectites, such as NAu-1, lost up to 15 and 18 % elements weight (Cs, Rb, K, Na, and Li) at 175 ^{o}C and 1 atm (14.7 psi). (OSTI.GOV, 1988). Therefore, weight losses are expected to be lower, if any, at the autoclave conditions mentioned above. Similar to experiments previously described, reactions were performed in 500 mL cylindrical glass reactors, which contained 250 mL of secondary clarifier effluent. H_2O_2 and NAu-1 at initial concentrations of 680 g/m^3 (20 mM) and 1 g/L, respectively, were added to each reactor. The concentration of NAu-1 was the lowest concentration tested in the study of Pham *et al.* (2012), whose experimental set-up was similar to the one used here. After the addition of both H_2O_2 and NAu-1, secondary effluent was stirred for 8 hrs (Pham *et al.*, 2009). The reactors were open to the atmosphere, but covered with parafilm to minimize evaporation. All experiments were carried out at room temperature (22 ± 2 ^{o}C) in dark conditions to prevent reactions between H_2O_2 and

light. Control reactors were run under the same experimental conditions and included NAu-1 only and H_2O_2 only, to examine for any effect on gene removal. At the end of treatment, catalase from bovine liver was added to the reactors to eliminate residual H_2O_2 and terminate the reactions, as described above.

5.2.4 *H_2O_2/NAu-1 treatment system*

The effect of oxidative species formation by combining NAu-1 and H_2O_2 for removing ARGs from secondary effluent was examined further, including variation of H_2O_2 concentration and contact time. The experimental set-up was the same as described in Section 5.2.3 for both experiments. In the first series of experiments, NAu-1 was provided at a constant concentration of 0.5 g/L, combined with different H_2O_2 doses. The concentrations of H_2O_2 were 3.4 g/m^3 (0.1 mM), 9 g/m^3 (0.265 mM) and 340 g/m^3 (10 mM). The first two H_2O_2 concentrations were chosen because they were used in the H_2O_2/UV system of the AOP pilot plant. The 340 g/m^3 H_2O_2 dose was chosen because it was presumed to be high enough to cause large changes in gene abundances. Further, production of $^{\bullet}OH$ had been examined before in a H_2O_2/UV and H_2O_2/Fe^{2+} study at this H_2O_2 dose displaying elevated reduction of target ARGs (Zhang *et al.*, 2016). Controls were also tested to verify that oxidation resulted from reactions between clay minerals and H_2O_2 and all conditions are shown in table 5.2. All reactions were conducted in triplicate (in three parallel reactors) with an 8 hrs reaction time.

The second series of experiments used the same experimental set-up, but used constant H_2O_2 and NAu-1 concentrations at 9 g/m^3 and 0.5 g/L, respectively, and compared three different contact times: 30 min, 4 hrs and 24 hrs. A dose of 9 g/m^3 was chosen for the contact time experiments because this concentration exhibited similar removal rates with the highest dose tested (340 g/m^3) in the experiments where H_2O_2 was varied. Further, this dose was considered more practical as it was also tested in the AOP pilot plant. The control reactors are shown in table 5.2.

Table 5.2 H_2O_2/NAu-1 **e**xperimental conditions.

Experiment	Condition	NAu-1	H_2O_2	Contact times
H_2O_2/NAu-1	H_2O_2/NAu-1	0.5 g/L	9 g/m^3 (0.265 mM)	30 min 4 hrs 8 hrs 24 hrs
			3.4 g/m^3 (0.1 mM) 340 g/m^3 (10 mM)	8 hrs
	NAu-1 control	0.5 g/L	-	8 hrs 24 hrs
	H_2O_2 control	-	9 g/m^3 (0.265 mM)	30 min 4 hrs 8 hrs 24 hrs
			3.4 g/m^3 (0.1 mM) 340 g/m^3 (10 mM)	8 hrs
	'No treatment' control	-	-	8 hrs 24 hrs

5.2.5 *Reduced NAu-1 treatment system*

Experiments also were conducted using pre-reduced clay minerals with a high Fe^{2+} content that can produce reactive oxidising species once reacted with oxygen. Thus, Fe in the NAu-1 was chemically reduced by the citrate-bicarbonate-dithionite method, using an adapted version of Stucki *et al.* (1984) described elsewhere (Neumann *et al.*, 2011; Entwistle *et al.*, 2019). Similar to the previous experiments, reactions took place in 500 mL cylindrical borosilicate glass reactors containing 250 mL of secondary clarifier effluent. An initial concentration of 0.5 g/L of reduced NAu-1 (rNAu-1) was used. Each condition was tested in triplicate in three parallel reactors, under magnetic stirring at room temperature (22 ± 2 ^oC).

In this experimental set-up, controlled oxygenation of rNAu-1 was intended to lead to oxidising species formation; therefore, secondary clarifier effluent was deoxygenated by bubbling with N_2 for 60 minutes per litre before the beginning of the treatment. To prevent accidental reaction of rNAu-1 with the DO of the water, the preparation of all reactors took place inside a glovebox (100% N_2, GS Glovebox Systemtechnik GmbH) containing an

atmosphere with oxygen levels lower than 2 ppm ('low oxygen' in table 5.3). rNAu-1 was added to the reactors within the glovebox and these were subsequently placed in a dark room with ambient oxygen levels (~21%, 'high oxygen'). Controls were tested under both low and high oxygen conditions as shown in table 5.3, to examine the effect of O_2 level for gene removal. The contact times tested were 30 min, 4, 8 and 24 hrs. These reaction times were chosen in order to be comparable with the H_2O_2/NAu-1 systems. DO was measured at the end of each reaction, using a digital HQ40D Portable Multi Meter.

Table 5.3 rNAu-1 **e**xperimental conditions.

Experiment	Condition	rNAu-1	Condition	Contact times
rNAu-1	rNAu-1	0.5 g/L	*High oxygen	30 min 4 hrs 8 hrs 24 hrs
	rNAu-1 control	0.5 g/L	**Low oxygen	8 hrs 24 hrs
	'No treatment' control	-	High oxygen	8 hrs 24 hrs
			Low oxygen	8 hrs 24 hrs

*Low oxygen: reaction in atmosphere with oxygen < 2 ppm
**High oxygen: reaction in ambient atmosphere with oxygen 21%

5.2.6 *Effect of wastewater deoxygenation on microbial viability*

To ensure that the effects observed in the experiments in Section 5.2.5 were caused by the treatment rather than the absence of oxygen (e.g., changes in ARG absolute abundances), changes in cell viability in response to deoxygenation were assessed. Fresh secondary clarifier effluent was collected and divided into two aliquots. One aliquot was stored at 4 ^{o}C, whereas the other was deoxygenated by nitrogen bubbling and degassing for 60 min per litre (figure 5.1).

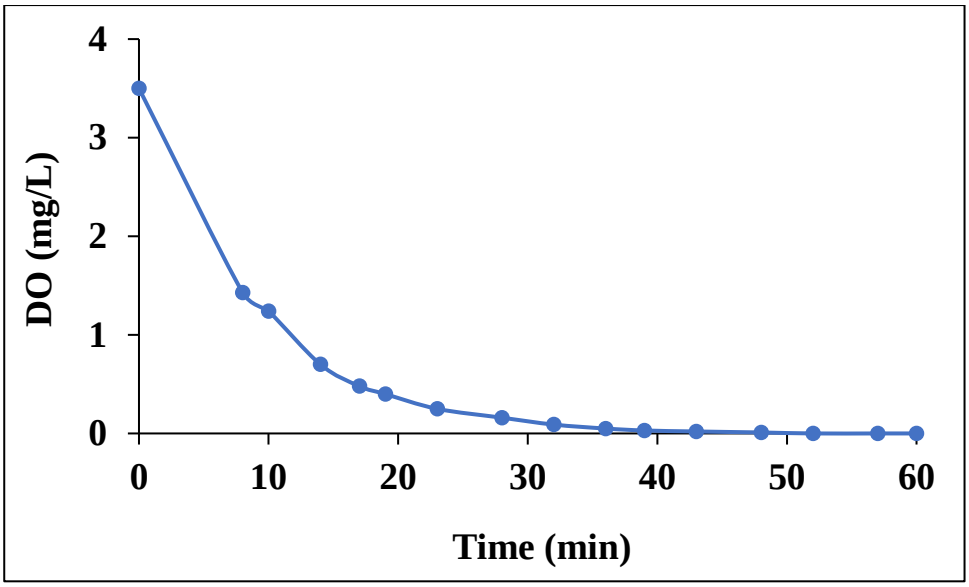

Figure 5.1 Time needed to deoxygenate one litre of secondary clarifier effluent.

The deoxygenated and aerobic samples were treated with PMA, similar to Section 3.2.5. DNA was subsequently extracted and qPCR was performed for the 16S rRNA gene. As the data revealed, 16s rRNA absolute concentrations in the deoxygenated samples ($9.28 \times 10^5 \pm 3.49 \times 10^5$ copies/mL) were not significantly different than in the aerobic samples ($7.37 \times 10^5 \pm 8.67 \times 10^4$ copies/mL) (Tukey; $p = 0.2$). Therefore, cell viability did not appear to be impacted by deoxygenation (see figure C.1).

5.2.7 *Preparation of samples for H_2O_2 and metal analysis*

Samples from all experimental set-ups were pre-processed with the same procedure for H_2O_2 and metal determination. After experimental treatment and before the addition of catalase (Section 5.2.4, Section 5.2.5), 50 mL of treated effluent was withdrawn from each reactor and centrifuged (Sigma 3-16P bench centrifuge) at 4000 rpm for 10 min to separate the solids from the aqueous phase. Supernatant was then filtered with Supor® hydrophilic polyethersulfone membrane 0.22 µm syringe filters (VWR, UK) to separate any remaining mineral after centrifugation from the aqueous phase. Analyses for metals and H_2O_2were carried out with the filtered aqueous phase. Each sample was then divided into three aliquots; the first aliquot (5 mL) was mixed with ethanol in a ratio 1:1 to quench any radicals present and stop oxidation reactions, and subsequently stored at 4 ^oC until H_2O_2 determination was conducted (Section 5.2.8). The other two fractions were both acidified to preserve the dissolved metals; i.e., 1 mL was mixed with 40 µL of hydrochloric acid (HCl) and stored at 4 ^oC until Fe analysis (Section 5.2.8) and the third aliquot (20 mL) was mixed with nitric acid

(HNO_3) to a ratio of 1 % and stored at 4 ^oC until trace metals were analysed by ICP-OES (Section 5.2.8).

5.2.8 *Post-treated water physicochemical analysis*

The residual concentration of H_2O_2 at the end of each treatment was quantified by a colorimetric method based on the use of titanium (IV) oxysulfate in an adapted version of the method suggested by Eisenberg (1943), which forms a stable yellow complex with H_2O_2 with maximum absorbance at 407 nm (Ferro *et al.*, 2015; Fiorentino *et al.*, 2015). Absorbance was measured on a UV/vis spectrophotometer (Hach Lange, UK) using disposable optical polystyrene cuvettes (VWR, UK). Specifically, 2 mL of sample were mixed with 1 mL of titanium oxysulfate reagent in a cuvette, prior to measurement. When solution turned to yellow, absorbance at 407 nm was measured on the UV/vis spectrophotometer and was linearly correlated with a H_2O_2 standard curve. A blank contained 1 mL of deionised water and 1 mL of ethanol was tested in parallel to quantifying absorbance in samples.

Fe^{2+} and total Fe (Fe^{2+} + Fe^{3+}) were determined by the 1,10-phenanthroline method (Tamura *et al.*, 1974). The process for the determination of the Fe^{2+} included addition of 200 µL of 1,10-phenanthroline and 200 µL of acetate buffer in 1 mL of sample previously acidified with HCl (Section 5.2.7). In the samples for total Fe, prior to adding 200 µL of 1,10-phenanthroline and 200 µL of acetate buffer in HCl, 30 µL of hydroxylamine was added and the mixture was allowed to react for 5 min. Both Fe^{2+} and total Fe samples were allowed to react in the dark for 30 min before absorbance measurements at 510 nm on the UV/vis spectrophotometer.

Trace metals were also analysed in samples before and after treatment to quantify metal released after oxidation in the treated water. The metals tested were Fe, Al, Si and Mg, selected because of their elevated presence in the NAu-1 structure (Keeling *et al.*, 2000). Standards for these metals were purchased from Sigma-Aldrich (UK) preserved in HNO_3, and dilutions were prepared in a range of 1, 2, and 3 mg/L for Al and Fe, and 5, 10, and 15 mg/L for Si and Mg. An ICP-OES instrument (Varian Vista-MPX CCD Simultaneous ICP-OES) was used and samples previously acidified in HNO_3 (Section 5.2.7) were analysed with no further dilution.

The pH was not adjusted during treatment and was measured at the end of the treatment using a Jenway 3010 pH-meter (Jenway, UK) with double junction electrode (VWR, UK), calibrated with commercial certified standards of pH 4 and pH 7 (VWR, UK).

The effect of H_2O_2/NAu-1 treatment on water quality was additionally assessed for conditions with H_2O_2 and NAu-1 concentrations constant at 9 g/m^3 and 0.5 g/L, respectively, and for contact times of 4 and 8 hrs, using tests described in Section 5.2.2 and the following additional assays. All analyses of secondary clarifier effluent were assessed using samples directly from the WWTP, whereas samples from laboratory experiments were first centrifuged (Sigma 3-16P bench centrifuge) at 4,000 rpm for 10 min in order to separate clay minerals and aqueous phase before further evaluation.

Total phosphorus (TP) was measured using Phosphate Ortho/Total cuvette test (HACK, UK), according to manufacturer's instructions, with pre-dosed reagents in a 0.5-5.0 mg/L PO_4-P range using a DR 6000 Benchtop spectrophotometer (Hach Lange, UK). Additionally, UV absorbance of the samples at 254 nm was measured in the same spectrophotometer. For total organic carbon (TOC) analysis, all samples were filtered using Supor® hydrophilic polyethersulfone 0.45 membrane syringe filters (VWR, UK), and the filtrate was then analysed by an Elementar vario TOC select TOC/TNb Analyzer.

5.2.9 *Gene quantification*

A volume of 200 mL of untreated and treated samples were filtered through 0.22 µm pore size hydrophilic polyethersulfone membrane filters (Merck Millipore, UK) to capture bacterial cells in the samples (for the filtration system see Section 3.2.2). Membrane filters with the sample were stored at -20 ^oC in 2 mL sterile ultra-clear polypropylene tubes (VWR, UK) until further processing. DNA was subsequently extracted using the Fast DNA™ Spin Kit for Soil (MP Biomedicals, USA), according to the manufacturer's instructions and the quality and quantity were assessed as described in Section 3.2.2. Quantification of genes was performed using qPCR for the 16S rRNA, *int*1, bla_{OXA-10}, *tet*M and *tet*Q. For more details on the qPCR assessments of this study see Section 3.2.4.

5.2.10 *Statistical analysis*

The statistical analysis of data in Chapter 5 was the same as Section 3.2.6. One-way analysis of variance (ANOVA) and Tukey post-hoc test for pairwise comparisons for normal and homogeneous data, and non-parametric tests (Krustall-Wallis and Games-Howell post-hoc) for samples where normality and homoscedasticity were violated, were performed to determine significant differences ($p < 0.05$).

5.3 Results

5.3.1 *Preliminary experiments*

The relative removal rates under each preliminary condition was determined using the equation 5.2 below:

$$Log\ removal\ (AOPs) = Log_{10}(\frac{C_{feedwater}}{C_{AOPs}}) \qquad \textbf{Equation 5.2}$$

Where, $C_{feedwater}$ and C_{AOPs} refer to the absolute gene concentrations in secondary clarifier effluent ('feedwater') and each AOP condition, including UV alone, H_2O_2/UV and H_2O_2/NAu-1.

Increases in UV dose resulted in gene removal rate increases (figure 5.2). For instance, log removal rates of 16S rRNA and *tet*M ranged from 0.69 ± 0.08 to 1.56 ± 0.50, and 0.58 ± 0.08 to 1.13 ± 0.12, respectively (table C.2). On the other hand, *int*1 levels reduced less, achieving only up to 0.52 ± 0.47 log removal. Among UV doses tested, 576 mJ/cm^2 increased 16S rRNA and *tet*M removal rates significantly compared to 96 mJ/cm^2, whereas no significant differences for *int*1 were seen among UV doses (table C.3).

Since UV dose alone increased removal rates, the effect of UV dose was tested in combined H_2O_2/UV treatments with H_2O_2 constant at 680 g/m^3 and UV dose varied in the same range previously tested (figure 5.2). Interestingly, with combined treatments, UV dose had no effect on gene removal (table C.2). The variations of log removal rates observed for different UV doses (0.89-1.05, 0.99-1.10, and 0.79-0.92 for 16S rRNA, *int*1 and *tet*M genes, respectively) were small and not significant (Table C.3).

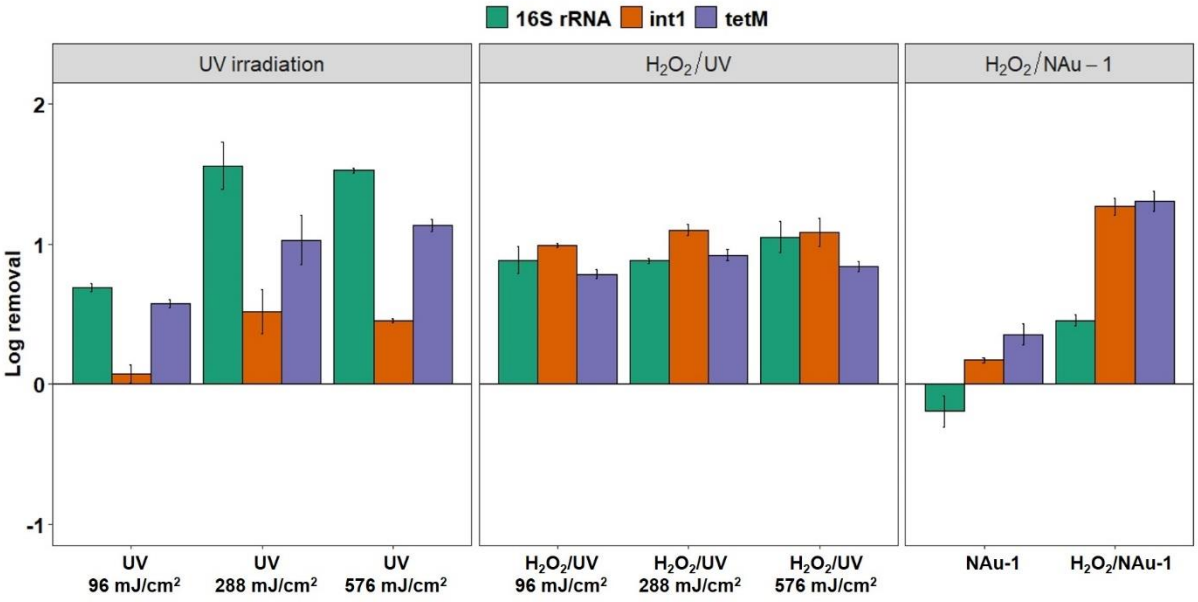

Figure 5.2 Removal rates achieved by UV, H_2O_2/UV, and H_2O_2/NAu-1. H_2O_2 dose in both H_2O_2/UV and H_2O_2/NAu-1 were constant at 680 g/m^3, and NAu-1 at 1 g/L (8 hrs contact time).

Comparison between UV and H_2O_2/UV shows that combining 680 g/m^3 H_2O_2 with 96 mJ/cm^2 UV dose enhanced removal of *tet*M and *int*1 genes significantly, while H_2O_2 addition had little effect on reducing 16S rRNA genes. In contrast, non-significant differences were observed between UV and H_2O_2/UV at a 288 mJ/cm^2 UV dose. Likewise, H_2O_2 did not enhance gene removal at a UV dose of 576 mJ/cm^2 against 16S rRNA and *tet*M genes. A significant decrease (Games-Howell; $p < 0.01$) was achieved only for *int*1 when combining 576 mJ/cm^2 UV with 680 g/m^3 H_2O_2.

Combined treatment of H_2O_2 with NAu-1 was examined in parallel, and compared with UV alone and H_2O_2/UV (figure 5.2). H_2O_2/NAu-1 achieved log removals of 0.46 ± 0.13, 1.27 ± 0.19, and 1.31 ± 0.21 for 16S rRNA, *int*1 and *tet*M genes, respectively, which were significantly higher removals (Games-Howell; $p < 0.01$) than NAu-1 alone (Table C.3). Although H_2O_2/NAu-1 exhibited the lowest removal rate at reducing 16S rRNA compared to both H_2O_2/UV and UV alone, it was more effective at removing *int*1 and *tet*M, achieving the highest removal rates. Statistically, H_2O_2/NAu-1 significantly reduced *tet*M genes compared to all H_2O_2/UV conditions, and UV alone at 96 mJ/cm^2. Significant differences were also observed between H_2O_2/NAu-1 and 96 and 576 mJ/ cm^2 UV alone, and between H_2O_2/NAu-1 and 96 mJ/ cm^2 H_2O_2/UV for *int*1.

5.3.2 *H_2O_2/NAu-1 treatment: effect of H_2O_2 concentration*

Because preliminary experiments combining H_2O_2 and NAu-1 showed promising results of significant gene abundance reduction (figure 5.2), further experiments were conducted using this AOP. In these experiments, different doses of H_2O_2 (3.4, 9, 340 g/m^3) were assessed with a constant concentration of NAu-1 (0.5 g/L) to evaluate how H_2O_2 dose influenced 16S rRNA, *int*1 and the three ARGs (*bla*$_{OXA-10}$, *tet*M, *tet*Q) in secondary clarifier effluent. Similar trends were seen for all target genes, which are typified in figure 5.3 for 16S rRNA genes. Results for all genes (absolute abundances ± standard deviation from triplicate parallel reactors per treatment, including controls) are presented in table 5.4.

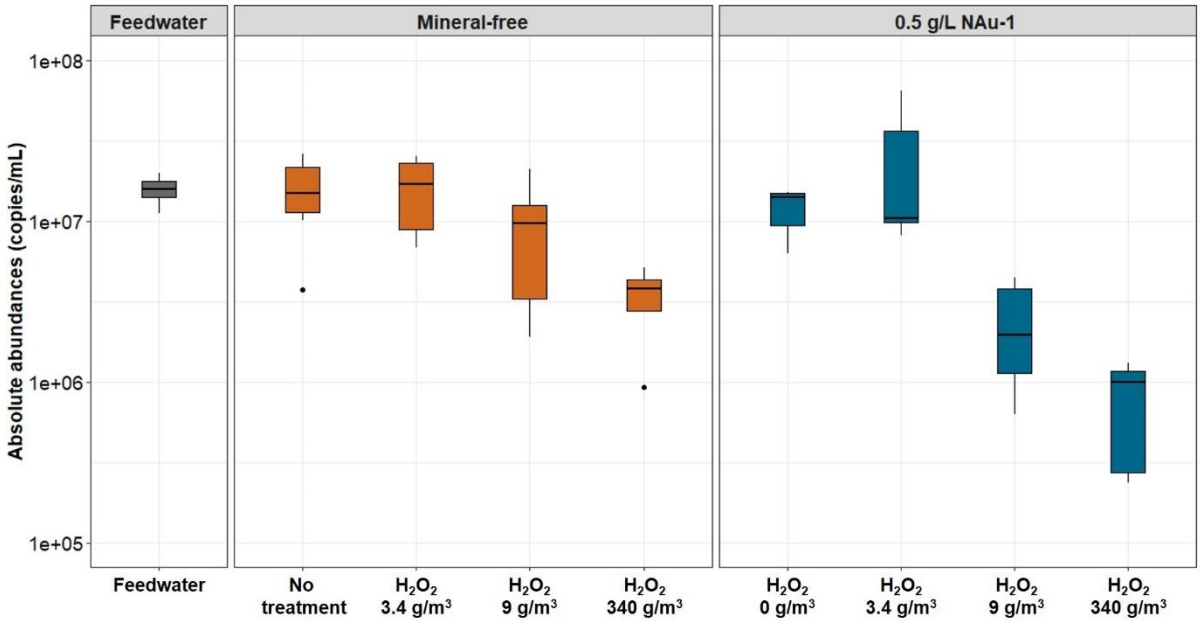

Figure 5.3 Absolute concentrations in copy genes per mL of 16S rRNA in each experimental condition. 'Feedwater' refers to secondary clarifier effluent and 'no treatment' to secondary clarifier effluent with no addition of H_2O_2 or NAu-1. Contact time: 8 hrs.

Control reactors with no addition of H_2O_2 or NAu-1 were tested in parallel to treatments. 'No treatment' showed no statistically significant differences in gene absolute abundances compared to feedwater (table C.4). Similarly, addition of 3.4 g/m^3 H_2O_2 alone had no significant effect on the absolute concentrations of all genes. However, increases in H_2O_2 dose were significant to gene abundance reduction. For example, *int*1 and *tet*M absolute abundances were reduced significantly after treatment with 9 g/m^3 H_2O_2, and all genes were significantly less abundant (Games-Howell; $p < 0.01$) when treated with a dose of 340 g/m^3 H_2O_2 and compared to feedwater. Additionally, *tet*M and *tet*Q gene abundances were

significantly lower in reactors with NAu-1 alone compared to feedwater, whereas *blaOXA-10* concentrations increased (Games-Howell; p = 0.02).

Table 5.4 Absolute concentrations ± standard deviation from three separate reactors per condition of all target genes at each condition tested. 'Feedwater' refers to secondary clarifier effluent and 'no treatment' to secondary clarifier effluent with no addition of H_2O_2 or NAu-1. Conditions: 0.5 g/L NAu-1, 8 hrs contact time.

Conditions	16S rRNA	*int1*	*blaOXA-10*	*tetM*	*tetQ*
Feedwater	**1.57E+07** ± 3.05E+06	**1.43E+05** ± 3.83E+04	**8.44E+02** ± 1.07E+02	**1.94E+04** ± 2.37E+03	**1.04E+03** ± 3.56E+02
No treatment	**1.57E+07** ± 7.01E+06	**1.67E+05** ± 6.19E+04	**2.55E+03** ± 1.46E+03	**1.68E+04** ± 4.24E+03	**1.45E+03** ± 5.69E+02
NAu-1	**1.23E+07** ± 3.35E+06	**1.48E+05** ± 8.04E+04	**2.09E+03** ± 7.89E+02	**1.06E+04** ± 3.05E+03	**2.80E+02** ± 7.52E+01
3.4 g/m^3 H_2O_2	**1.67E+07** ± 7.45E+06	**1.36E+05** ± 1.29E+05	**1.16E+03** ± 7.65E+02	**1.19E+04** ± 3.90E+03	**5.44E+02** ± 4.17E+02
9 g/m^3 H_2O_2	**9.49E+06** ± 6.22E+06	**3.75E+04** ± 4.89E+03	**8.14E+02** ± 9.34E+01	**9.32E+03** ± 4.32E+03	**5.14E+02** ± 7.08E+01
340 g/m^3 H_2O_2	**3.43E+06** ± 1.40E+06	**2.29E+04** ± 1.55E+04	**3.76E+02** ± 2.20E+02	**6.49E+03** ± 2.14E+03	**3.50E+02** ± 2.46E+02
3.4 g/m^3 H_2O_2/NAu-1	**2.21E+07** ± 2.00E+07	**3.04E+05** ± 3.76E+05	**1.91E+03** ± 1.73E+03	**1.31E+04** ± 4.85E+03	**4.58E+02** ± 1.20E+02
9 g/m^3 H_2O_2/NAu-1	**2.24E+06** ± 1.43E+06	**2.41E+03** ± 8.94E+02	**1.62E+02** ± 1.18E+02	**1.80E+03** ± 1.39E+03	**9.45E+01** ± 8.27E+01
340 g/m^3 H_2O_2/NAu-1	**7.50E+05** ± 4.76E+05	**7.77E+02** ± 3.64E+02	**5.53E+01** ± 3.01E+01	**8.36E+02** ± 4.88E+02	**2.59E+01** ± 1.15E+01

After 8 hrs of treatment, the combination of 3.4 g/m^3 H_2O_2 with 0.5 g/L NAu-1 showed no effect on reducing target gene abundances compared to feedwater, 3.4 g/m^3 H_2O_2 alone and NAu-1 alone (table 5.4). On the other hand, 9 g/m^3 H_2O_2 combined with NAu-1 resulted in decreases in absolute abundances of all genes, which were significantly lower compared to both feedwater, 9 g/m^3 H_2O_2 alone and NAu-1 alone for all genes. Similarly, 340 g/m^3 H_2O_2/NAu-1 resulted in significantly lower concentrations for all genes compared to feedwater and the corresponding controls. Comparisons among all H_2O_2/NAu-1 conditions revealed that both concentrations of 9 and 340 g/m^3 H_2O_2 resulted in significantly lower 16S rRNA, *int*1, and ARG absolute abundances, compared to the 3.4 g/m^3 H_2O_2 dose (figure 5.4). In contrast, no significant differences were observed between 9 and 340 g/m^3 H_2O_2 doses,

with the only exception being *int*1 gene, where the highest H_2O_2 dose (340 g/m^3) resulted in significant decreases in gene abundance (Games-Howell; $p < 0.01$, Table C.4).

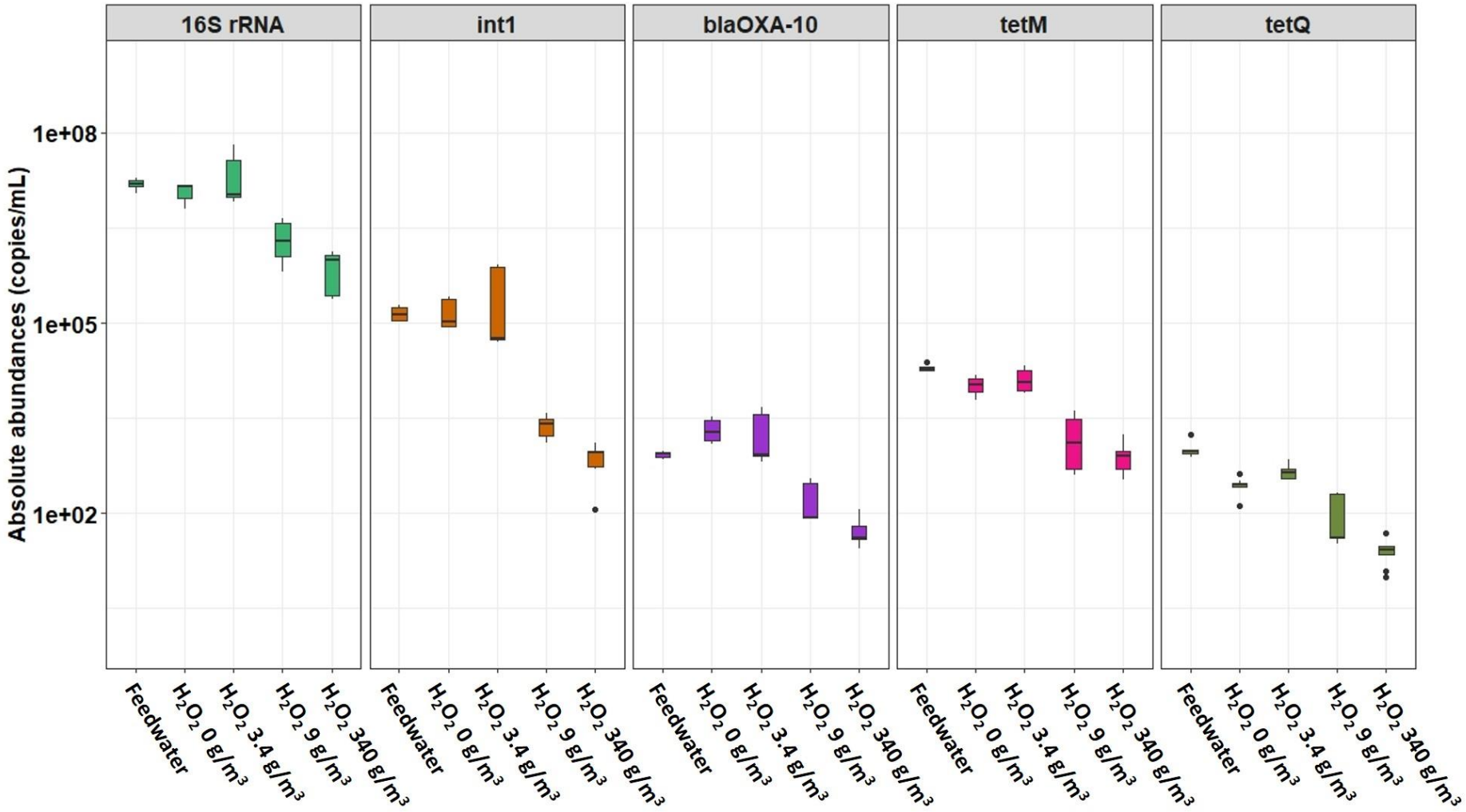

Figure 5.4 Absolute concentrations in copy genes/mL per target gene for the combined H_2O_2/NAu-1 treatments. 'Feedwater' refers to secondary clarifier effluent. Conditions: 0.5 g/L NAu-1, 8 hrs contact time.

How different H_2O_2/NAu-1 combinations reduced absolute gene abundances is summarised in figure 5.5. The lowest removal rates were observed with 3.4 g/m^3 H_2O_2/NAu-1, where log removals were less than 0.37, which is not significantly different than in the presence of NAu-1 alone. The only exception was *tet*Q, which showed slightly higher removal rates than with NAu-1 alone (Table C.5). On the other hand, 9 and 340 g/m^3 H_2O_2/NAu-1 both resulted in significantly higher removal rates compared to NAu-1 alone and 3.4 g/m^3 H_2O_2/NAu-1, with log removals of 0.81 ± 0.29 - 1.80 ± 0.17 for the 9 g/m^3 H_2O_2 dose and 1.23 ± 0.21 - 2.34 ± 0.32 for the 340 g/m^3 H_2O_2 dose (Table C.6). Although not significant differences in log removal were evident for 16S rRNA, *tet*M and *tet*Q between H_2O_2/NAu-1 at H_2O_2 doses of 9 and 340 g/m^3; the higher H_2O_2 dose was more effective at reducing *int*1 and *bla*$_{OXA-10}$ genes.

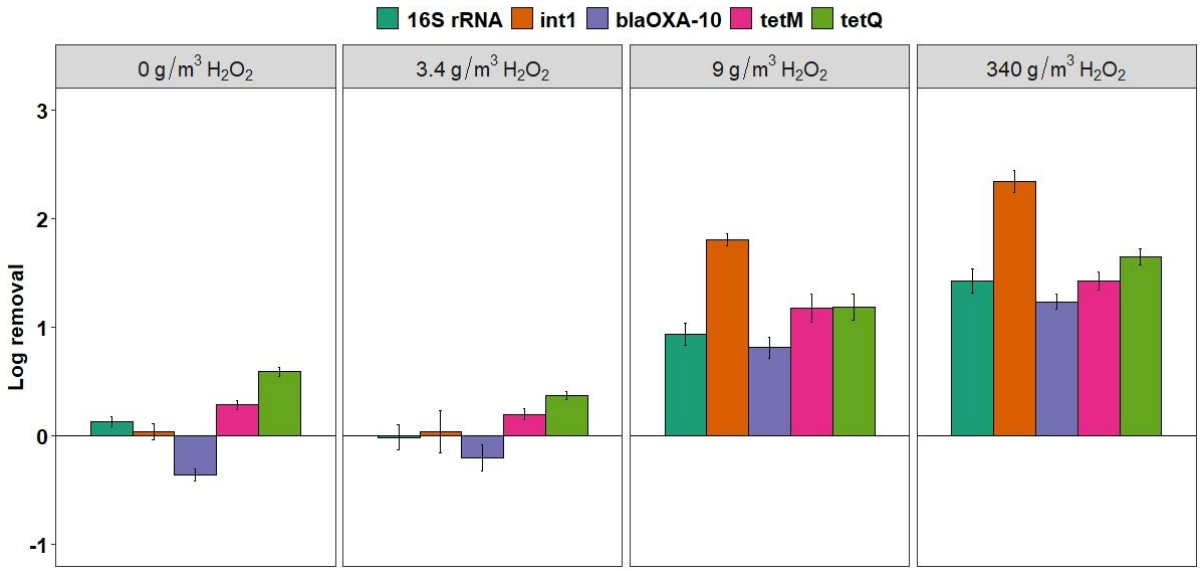

Figure 5.5 Effect of H_2O_2 concentrations on the removal rates of all target genes for the combined H_2O_2/NAu-1 treatment. Conditions: 0.5 g/L NAu-1, 8 hrs contact time.

Relative abundance, expressed as absolute abundance normalized per bacterial genome (see Section 3.2.6), was quantified for each treatment condition to monitor the relative selection of ARGs and *int*1 carried per bacterial cell during the AOPs. 'No treatment', as expected, had no effect on the relative abundance of target ARGs and *int*1 (figure C.2). On the other hand, addition of NAu-1 in the treatment process influenced relative removals (table C.7). For instance, NAu-1 alone reduced *tet*M and *tet*Q relative abundances compared to feedwater and increased *bla*$_{OXA-10}$ significantly (table C.8). Combining NAu-1 with 3.4 g/m^3 H_2O_2 did not have any effect on relative abundances compared to feedwater, whereas *int*1 and ARG relative abundances declined significantly at both 9 and 340 g/m^3 H_2O_2 doses.

5.3.3 *H_2O_2/NAu-1 treatment: Effect of contact time*

The effect of contact time in the H_2O_2/NAu-1 treatment was evaluated for constant NAu-1 and H_2O_2 concentrations of 0.5 g/L and 9 g/m^3, respectively, and contact times of 30 min, 4 and 24 hrs were applied. The 9 g/m^3 H_2O_2 dose was chosen because our experiments on the effect of H_2O_2 concentration (Section 5.3.2) showed elevated gene removal rates and this dose is within the range used in full-scale WWTP systems (Wagner *et al.*, 2002; Rodríguez-Chueca *et al.*, 2019). Furthermore, the 'no treatment' and NAu-1 alone controls were tested only for 24 hrs contact time because our preliminary experiments with 8 hrs contact time showed no consequential changes in ARG concentrations with or without NAu-1 (figure 5.2), and therefore a similar outcome was assumed for the shorter contact times of 30 min and 4 hrs.

Overall, similar patterns relative to variations in contact time were seen among all target genes. The 16S rRNA data are provided in figure 5.6, which is representative of the observed patterns and table 5.5 provides the means (± standard deviation) of all target genes.

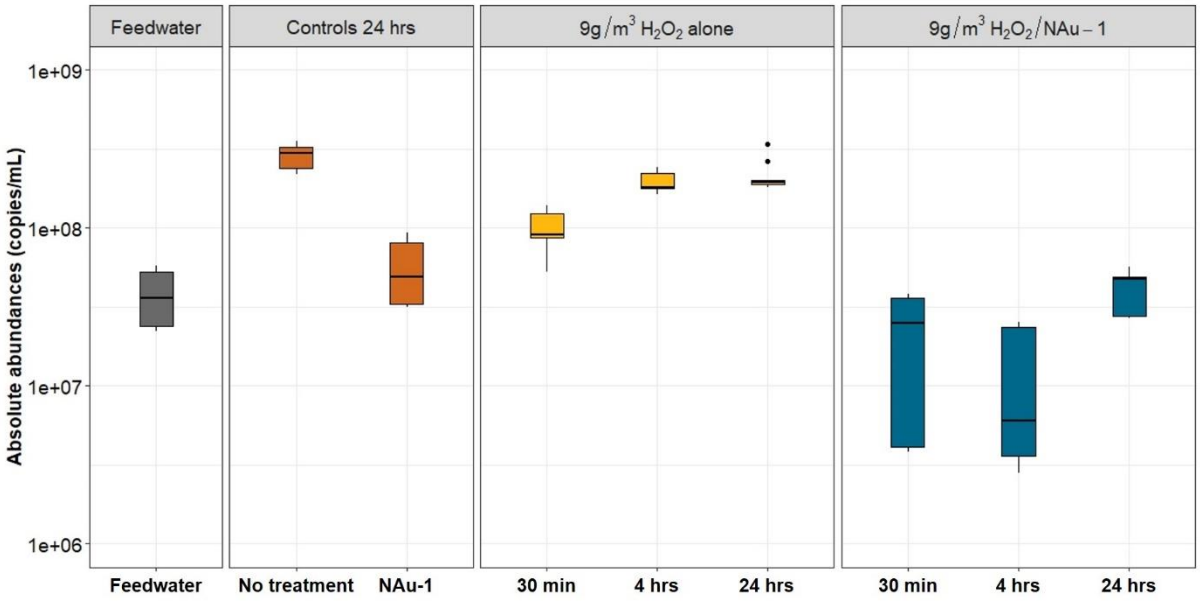

Figure 5.6 Effect of contact time on absolute gene concentrations (copy genes per mL) of 16S rRNA for the combined H_2O_2/NAu-1 treatment. 'Feedwater' refers to secondary clarifier effluent and 'no treatment' to secondary clarifier effluent with no addition of H_2O_2 or NAu-1. Conditions: 9 g/m^3 H_2O_2, 0.5 g/L NAu-1.

Using 24 hours as the control, significant increases in all target gene abundances were seen for the 'no treatment' condition (Games-Howell; $p < 0.01$) compared to feedwater (table C.9). In contrast, non-significant differences were seen between feedwater and NAu-1 alone for all genes except *tet*Q, where a significant decrease was observed after treatment. The effect of 9 g/m^3 H_2O_2 alone at 30 min, 4 and 24 hrs contact times also was tested because this H_2O_2

concentration affected gene abundances in our experiments with varying H_2O_2 concentrations (figure 5.3). In this experiment, higher absolute gene concentrations (Games-Howell; $p < 0.05$) were detected at all three contact times compared to feedwater, showing that 9 g/m^3 H_2O_2 alone had no effect at reducing target gene abundances.

Table 5.5 Absolute concentrations ± standard deviation from three separate reactors per condition of all target genes for the combined H_2O_2/NAu-1 treatment. 'Feedwater' refers to secondary clarifier effluent and 'no treatment' to secondary clarifier effluent with no addition of H_2O_2 or NAu-1. Conditions: 9 g/m^3 H_2O_2, 0.5 g/L NAu-1.

Conditions	**16S rRNA**	***int*1**	***bla*$_{OXA-10}$**	***tet*M**	***tet*Q**
Feedwater	**3.90E+07 ±** 1.69E+07	**1.28E+05 ±** 1.71E+04	**1.84E+02 ±** 8.96E+01	**1.44E+04 ±** 7.17E+03	**7.18E+02 ±** 2.28E+02
No treatment*	**2.87E+08 ±** 4.75E+07	**5.94E+05 ±** 3.73E+04	**1.01E+03 ±** 1.75E+02	**2.88E+04 ±** 6.18E+03	**2.30E+03 ±** 4.87E+02
NAu-1	**5.67E+07 ±** 2.55E+07	**1.92E+05 ±** 9.62E+04	**1.94E+02 ±** 1.11E+02	**4.13E+03 ±** 5.21E+02	**1.84E+02 ±** 8.06E+01
H_2O_2 alone					
30 min	**9.83E+07 ±** 2.88E+07	**2.37E+05 ±** 5.24E+04	**4.49E+02 ±** 1.02E+02	**2.52E+04 ±** 6.50E+03	**1.62E+03 ±** 4.07E+02
4 hrs	**1.94E+08 ±** 2.88E+07	**3.70E+05 ±** 3.58E+04	**7.60E+02 ±** 1.50E+02	**3.44E+04 ±** 4.67E+03	**2.57E+03 ±** 3.74E+02
24 hrs	**2.16E+08 ±** 5.37E+07	**1.87E+05 ±** 1.76E+04	**5.74E+02 ±** 6.94E+01	**2.83E+04 ±** 1.20E+04	**3.86E+03 ±** 1.33E+03
H_2O_2/NAu-1					
30 min	**2.20E+07 ±** 1.46E+07	**4.62E+04 ±** 3.84E+04	**7.44E+01 ±** 5.32E+01	**3.70E+03 ±** 2.59E+03	**2.67E+02 ±** 2.23E+02
4 hrs	**1.11E+07 ±** 1.01E+07	**1.57E+04 ±** 2.03E+04	**2.98E+01 ±** 3.09E+01	**1.60E+03 ±** 2.12E+03	**1.21E+02 ±** 1.09E+02
24 hrs	**4.28E+07 ±** 1.21E+07	**4.76E+04 ±** 8.36E+03	**1.20E+02 ±** 3.58E+01	**7.63E+03 ±** 2.97E+03	**4.73E+02 ±** 1.45E+02

* contact time 24 hrs

Combining H_2O_2 with NAu-1 had a significant effect on reducing gene absolute abundances compared to feedwater, but not all genes were affected in the same manner over the contacts times (figure 5.7). For instance, contact time had no effect on changes in 16S rRNA absolute concentrations (Games-Howell; $p > 0.05$), whereas *int*1 abundances declined significantly under all contact times (Table C.9). Likewise, tetracycline gene concentrations decreased significantly after 30 min and 4 hrs treatment, whereas declines were not statistically significant for 24 hrs. Relative to *bla*$_{OXA-10}$, all treatment conditions resulted in lower abundances that were very close to the LoQ, however, decreases were statistically significant

only between feedwater and 4 hrs contact time. Overall, all H_2O_2/NAu-1 conditions significantly reduced absolute gene abundances compared to control reactors containing H_2O_2 alone (e.g. figure 5.6), whereas significant differences among different H_2O_2/NAu-1 conditions were observed only between 4 and 24 hrs (Table C.9). Also, absolute abundances of all genes in both H_2O_2/NAu-1 and NAu-1 alone at 24 hrs were significantly lower compared to 'no treatment' (table 5.5), while no significant differences ($p>0.05$) were detected for most genes between H_2O_2/NAu-1 and NAu-1 alone (table C.9). Finally, H_2O_2/NAu-1 was more effective at reducing *int*1 (Games-Howell; $p = 0.02$) than NAu-1 alone, whereas NAu-1 alone more readily reduced *tet*Q genes (Games-Howell; $p < 0.01$) compared to H_2O_2/NAu-1.

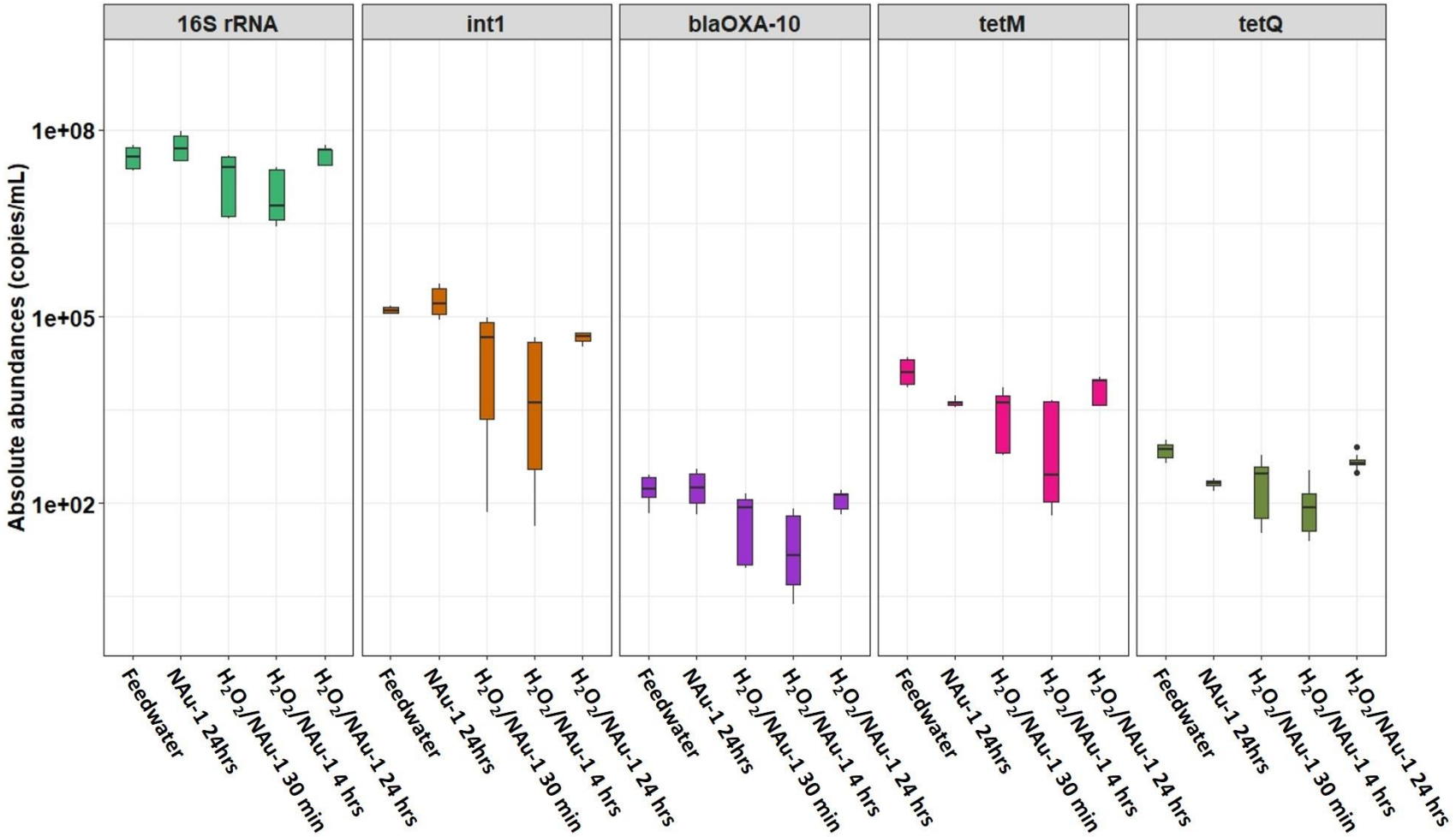

Figure 5.7 Effect of contact time on absolute concentrations (copy genes/mL per target gene) for the combined H_2O_2/NAu-1 treatment. 'Feedwater' refers to secondary clarifier effluent. Conditions: 9 g/m^3 H_2O_2, 0.5 g/L NAu-1.

Log removal rates varied between 0.40 ± 0.45 and 0.96 ± 1.09 after 30 min treatment with H_2O_2/NAu-1 (Table C.10), increased to 0.71 ± 0.40-1.65 ± 1.12 after 4 hrs and decreased to 0.44 ± 0.08 and lower after 24 hrs contact time, including negative log removal values for 16S rRNA genes (figure 5.8). Among all contact times tested, including the 8 hrs examined in the experiment with varying H_2O_2 concentrations (Section 5.3.2), 4 and 8 hrs resulted in the highest removal rates (figure 5.8), and non-significant differences ($p>0.92$) were observed between these two contact times for all target genes (table C.11). Both 4 and 8 hr contact times resulted in greater log removals than 30 minutes, but due to large standard deviations, these differences were not statistically significant ($p>0.17$). Although both 4 and 8 hrs had significantly higher log removal rates than after 24 hrs contact time, differences were not significant between 30 min and 24 hrs contact time (Table C.11).

Compared to NAu-1 alone, the combined H_2O_2/NAu-1 treatment achieved significantly higher removal rates after 8 hrs treatment. In contrast, non-significant differences were observed between H_2O_2/NAu-1 and NAu-1 alone after 24 hrs (for most genes), with the combined treatment resulting in significantly higher removal rates only for *int*1.

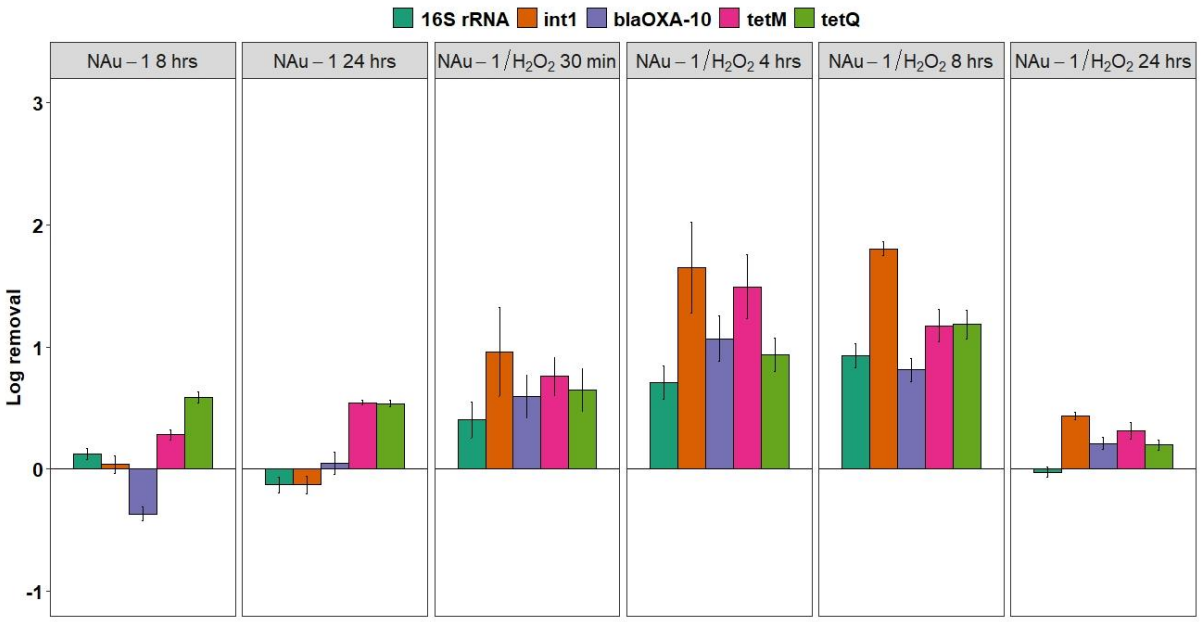

Figure 5.8 Removal rates of all target genes after adding NAu-1 in the treatment with both NAu-1 and H_2O_2 constant at 0.5 g/L and 9 g/m^3, respectively.

Relative gene abundances under the 'no treatment' condition increased significantly after 24 hrs compared to feedwater (figure C.3, tables C.12 and C.13). In contrast, 24 hrs contact with NAu-1 alone resulted in relative gene abundances similar to those observed in the feedwater, with the exception of *tet*Q, which relative abundances decreased significantly in reactors

containing NAu-1 (p=0.02, table C.13). The combined H_2O_2/NAu-1 treatment decreased target genes' relative concentrations after 30 min, 4 and 24 hrs, however significant differences between H_2O_2/NAu-1 conditions and feedwater were only evident for *int*1 at all contact times and *tet*Q at 30 min and 4 hrs. Among the all contact times applied to the H_2O_2/NAu-1 experiments, significant differences in relative gene concentrations were only observed between 4 and 24 hrs, which is consistent with the absolute abundance data (table C.9).

5.3.4 *Reduced NAu-1 treatment: Effect of contact time and oxygen levels*

The effect of rNAu-1 level on removing specific ARGs from secondary effluent was assessed. All genes followed a similar trend to 16S rRNA genes, which is exemplified in Figure 5.9 (see table 5.6 for all data). As described in Section 5.2.5, experimental conditions included reactors with and without rNAu-1 under low oxygen (glovebox with <2 ppm O_2) versus aerobic conditions (dark room with ambient O_2 levels). No significant differences in absolute gene concentrations between feedwater (deoxygenated secondary clarifier effluent) and control reactors were observed in the absence of rNAu-1 at both 8 and 24 hrs contact time and for both low and high oxygen conditions (table C.14). The only exception was *int*1, where a significant increase was observed after 8 hrs under high oxygen relative to feedwater levels.

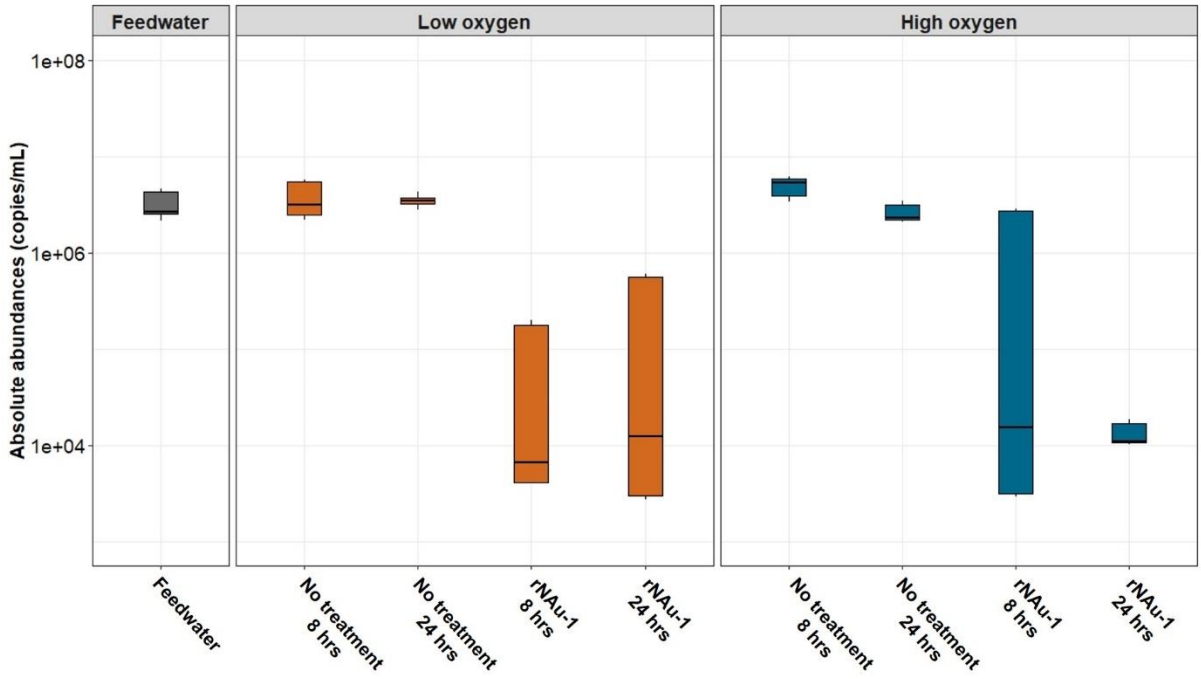

Figure 5.9 Effect of contact time and oxygen content on absolute concentrations (copy genes per mL of 16S rRNA) for the rNAu-1 treatment. 'Feedwater' refers to deoxygenated secondary clarifier effluent and 'no treatment' to deoxygenated secondary clarifier effluent in absence of rNAu-1. 'Low oxygen' refers to experiments conducted in the anaerobic glovebox (<2 ppm O_2), and 'high oxygen' to those carried out in a dark room with ambient O_2 levels. rNAu-1 concentration: 0.5 g/L.

On the other hand, addition of rNAu-1 under high oxygen conditions had a significant effect on gene absolute abundances compared to both feedwater and the 'no treatment' controls at 8 and 24 hrs. As data in table 5.6 show, all target genes declined significantly with $bla_{\text{OXA-10}}$ being below the LoQ after 24 hrs. Surprisingly, all gene absolute concentrations also declined after addition of rNAu-1 under low oxygen conditions, with $bla_{\text{OXA-10}}$ abundances close to the LoQ after 8 hrs. Decreases were significant compared to both feedwater and reactors in absence of rNAu-1. It is also interesting that no statistical differences were observed in absolute abundances of samples treated by rNAu-1 under low and high oxygen conditions (table C.14).

Table 5.6 Absolute concentrations ± standard deviation from three separate reactors per condition of all target genes in the rNAu-1 treatment. 'Feedwater' refers to deoxygenated secondary clarifier effluent and 'no treatment' to secondary clarifier effluent in absence of rNAu-1. Conditions: 0.5 g/L rNAu-1, 'low oxygen' refers to <2 ppm O_2, 'high oxygen' are ambient O_2 levels.

Conditions	**16S rRNA**	***int*1**	***bla*$_{OXA-10}$**	***tet*M**	***tet*Q**
Secondary clarifier effluent	**2.07E+06 ±** 2.48E+05	**8.66E+05 ±** 5.80E+05	**9.23E+02 ±** 6.88E+01	**6.86E+04 ±** 2.35E+04	**1.42E+04 ±** 5.63E+03
Feedwater	**3.14E+06 ±** 9.53E+05	**9.72E+05 ±** 3.03E+05	**1.14E+03 ±** 2.80E+02	**5.67E+04 ±** 2.93E+04	**2.01E+04 ±** 6.49E+03
No treatment 8 hrs (high O_2)	**4.91E+06 ±** 1.09E+06	**1.57E+06 ±** 2.71E+05	**1.49E+03 ±** 2.32E+02	**9.32E+04 ±** 1.70E+04	**1.87E+04 ±** 2.82E+03
No treatment 24 hrs (high O_2)	**2.59E+06 ±** 5.45E+05	**9.99E+05 ±** 1.59E+05	**1.05E+03 ±** 2.11E+02	**4.43E+04 ±** 1.00E+04	**4.82E+03 ±** 7.99E+02
No treatment 8 hrs (low O_2)	**3.72E+06 ±** 1.47E+06	**8.62E+05 ±** 1.85E+05	**1.23E+03 ±** 3.59E+02	**6.29E+04 ±** 1.79E+04	**1.36E+04 ±** 3.09E+03
No treatment 24 hrs (low O_2)	**3.51E+06 ±** 5.07E+05	**1.28E+06 ±** 2.31E+05	**1.58E+03 ±** 2.09E+02	**4.42E+04 ±** 4.00E+03	**1.38E+04 ±** 1.84E+03
rNAu1 30 min (high O_2)	**2.76E+05 ±** 3.98E+05	**1.57E+05 ±** 2.30E+05	**8.49E+01 ±** 1.18E+02	**3.40E+03 ±** 4.34E+03	**1.08E+03 ±** 1.53E+03
rNAu1 4 hrs (high O_2)	**6.86E+03 ±** 1.68E+03	**2.75E+03 ±** 8.39E+02	**8.58E+00 ±** 2.80E+00	**2.92E+02 ±** 1.54E+02	**4.65E+01 ±** 2.15E+01
rNAu-1 8 hrs (high O_2)	**9.22E+05 ±** 1.37E+06	**4.04E+05 ±** 6.00E+05	**3.54E+02 ±** 4.74E+02	**9.44E+03 ±** 1.49E+04	**2.67E+03 ±** 3.91E+03
rNAu1 24 hrs (high O_2)	**1.31E+04 ±** 3.55E+03	**4.67E+03 ±** 1.91E+03	**1.28E+01 ±** 5.59E+00	**3.35E+02 ±** 1.31E+02	**2.63E+01 ±** 1.49E+01
rNAu1 8 hrs (low O_2)	**6.49E+04 ±** 8.95E+04	**4.30E+03 ±** 2.62E+03	**1.67E+01 ±** 1.94E+01	**3.75E+02 ±** 4.20E+02	**1.74E+02 ±** 1.76E+02
rNAu1 24 hrs (low O_2)	**2.01E+05 ±** 2.90E+05	**7.80E+04 ±** 1.14E+05	**1.07E+02 ±** 1.41E+02	**1.67E+03 ±** 2.43E+03	**4.83E+02 ±** 6.64E+02

rNAu-1 was also tested at 30 min and 4 hrs contact times under high oxygen conditions. Similar to 8 and 24 hrs, contact for both time durations significantly reduced absolute concentrations of all genes compared to feedwater. In fact, high removal rates were achieved under all contact times for all target genes (table C.15). For example, 30 min resulted in log removals between 1.61 ± 0.62 and 1.98 ± 0.88, and log removals of > 2 were achieved after 4 hrs, varying between 2.15 ± 0.16 and 2.67 ± 0.10 (figure 5.10). Eight and 24 hr contact times also exhibited high log removal rates, 1.31 ± 1.01 to 1.82 ± 1.17 and 1.98 ±0.17 to 2.94 ± 0.24, respectively. Although 4 and 24 hrs had the highest removals, non-significant differences were seen among all rNAu-1 conditions under high oxygen conditions (table C.16). All treatments containing rNAu-1 under low oxygen conditions also had elevated gene removal rates, which varied between 2.15 ± 0.56 and 2.46 ± 0.53, and 1.53 ± 0.73 and 2.51 ± 1.16 for 8 and 24 hrs contact, respectively. In summary, treatments containing rNAu-1 under low and high conditions effectively reduced all targeted genes with removal rates not being statistically different among both conditions and all contact times.

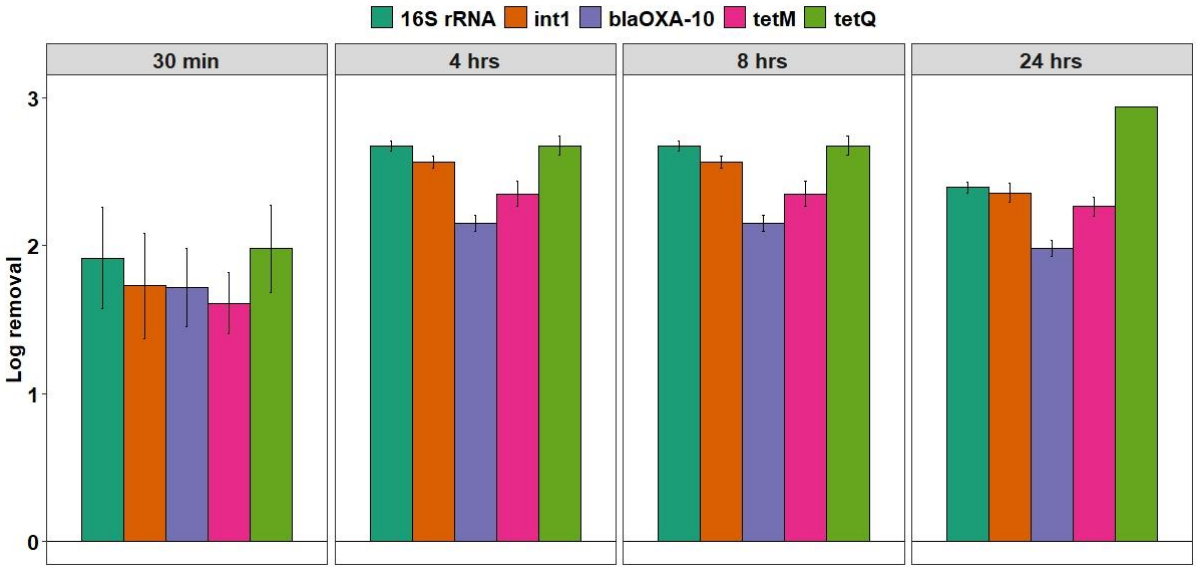

Figure 5.10 Removal rates of all target genes after adding rNAu-1 (0.5 g/L) under high oxygen conditions.

Relative abundances of target genes with rNAu-1 under high oxygen conditions and for all contact times were significantly lower compared to feedwater (figure C.4 and table C.17), whereas differences existed, but were not significant among different contact times (table C.18). The only exception was *int*1, which relative abundances after 8 hrs treatment were statistically

similar to the feedwater. Likewise, significantly lower relative abundances were observed in treatment containing rNAu-1 under low oxygen conditions, which reflects the elevated absolute abundances removal (table 5.6).

5.3.5 *Physicochemical characterisation of pre- and post-treated effluent*

As described previously, pH was not adjusted during treatment, but was monitored at the end of each experiment. As table C.19 shows, an increase in pH was observed at the end of each experimental condition. In general, increases were lower in 'no treatment' controls and higher in reactors containing H_2O_2 and/or NAu-1 in either native or reduced state. For instance, in the H_2O_2/NAu-1 experiment that varied H_2O_2 dose, initial pH in the feedwater was 6.25, whereas it increased to between 7.92 and 8.14 in reactors containing H_2O_2, NAu-1 or H_2O_2/NAu-1, which is an additional increase of 1.25-1.5 pH units. Although different H_2O_2 concentrations had no significant impact on the pH, different contact time did. For example, over the range of contact times from 30 min to 24 hrs in the H_2O_2/NAu-1 experiments, a trend of increasing pH with time occurred from 6.76 to between 7.84 and 8.24. A similar temporal trend also was seen in control reactors containing either H_2O_2 (pH of 7.62 to 8.29) or NAu-1 alone (8.26), with all reactors reaching approximately the same final pH value. Higher pH values, compared to H_2O_2/NAu-1, were observed in reactors containing rNAu-1, where pH up to 8.86 was observed in samples under low oxygen conditions, while a range of 8.05 to 8.41 was seen in reactors with high oxygen conditions. However, these reactors had higher initial pH values (7.72 vs 6.25-6.76; table C.19), resulting in an increase of up to 0.7 pH units, which is smaller than that observed in the H_2O_2, NAu-1 or H_2O_2/NAu-1 treatments, and an additional increase due to the presence of rNAu-1 of 0 and 0.27 pH units for low and high oxygen conditions, respectively. In contrast to the H_2O_2/NAu-1 treatment, pH did not follow an increasing trend over time. Despite the differences, the combined evidence demonstrates that treatment with either H_2O_2 or clay mineral shifted pH to more alkaline conditions.

Residual H_2O_2 was monitored at the end of each treatment in reactors containing H_2O_2, H_2O_2/NAu-1 and rNAu-1. In the experiments with variation in H_2O_2 dose, H_2O_2 concentrations were generally lower after reaction compared to initial concentrations. As can be seen in table C.20, H_2O_2 in H_2O_2/NAu-1 reactors containing initial 3.4 g/m^3 H_2O_2 decreased to 0.91 ± 0.14 g/m^3 H_2O_2 after 8 hrs treatment. Similarly, H_2O_2 levels in reactors with initial concentrations of 9

and 340 g/m^3 H_2O_2 declined to 2.53 ± 0.14 and 263 ± 33.8 g/m^3, respectively. Reductions in H_2O_2 concentrations were also observed in reactors containing H_2O_2 alone, although decreases were smaller compared to combined H_2O_2/NAu-1 treatments. It is also noteworthy that residual H_2O_2 was high (263 g/m^3) in reactors with 340 g/m^3 H_2O_2 initial concentration. Considering contact time in the H_2O_2/NAu-1 experiment with H_2O_2 concentration constant at 9 g/m^3, residual H_2O_2 declined gradually with time (figure 5.11) to residual H_2O_2 levels as low as 2.53 ± 0.14 g/m^3 at 8 hrs reaction time and 1.68 ± 1.19 g/m^3 after 24 hrs treatment, with decreasing H_2O_2 concentration differences between experiments with H_2O_2 alone and with combined H_2O_2/NAu-1 (table C.21). H_2O_2 was also measured at the end of the rNAu-1experiments, but absorbance at 407 nm was negative for all measurements, suggesting H_2O_2 levels were below detection.

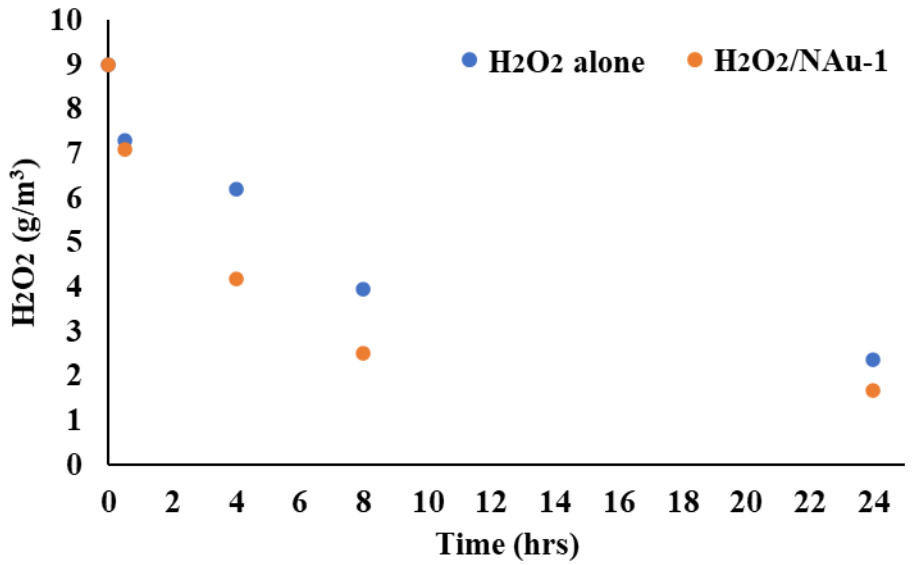

Figure 5.11 H_2O_2 concentrations over contact time in reactors with an added 9 g/m^3 H_2O_2 dose. H_2O_2 alone: blue; combined H_2O_2/NAu-1 (0.5 g/L NAu-1): orange.

NAu-1 mainly consists of Fe, Al, Si and Mg (Neumann *et al.*, 2013), therefore the presence of such metals in the matrix was analysed by ICP-OES (Section 5.2.8). Analysis revealed that metal concentrations in samples with H_2O_2/NAu-1 were very low, close to levels in pre-treated samples (table 5.7) and a similar pattern was observed in the results from the 1,10-phenanthroline method (Section 5.2.8) for the soluble Fe^{2+} and total Fe. As table C.22 shows, in all H_2O_2/NAu-1 experiments, $Fe^{2+}$and total Fe was very low in the feedwater and varied with sampling day between 1.38 ± 0.0 and 16.10 ± 0.0 µM Fe^{2+}, and 2.28 ± 0.0 and 40.73 ± 0.0 µM total Fe, and levels decreased further in all treatments. Likewise, Al, Fe and Mg concentrations with rNAu-1

treatment were close to initial concentrations, while a small increase was observed for Si over time (table 5.7). Regardless, there were no significant changes in metal concentrations resulting from treatment with rNAu-1.

Table 5.7 Results from the metal analysis using ICP-OES. 'Feedwater' refers to secondary clarifier effluent. In the experiment with rNAu-1, 'Feedwater' and all conditions in the presence of rNAu-1 contained deoxygenated secondary clarifier effluent.

Treatment conditions	Al (µM)	Fe (µM)	Si (µM)	Mg (µM)
Combined H_2O_2/NAu-1 (8 hrs contact time, 0.5 g/L NAu-1)				
Feedwater	*NA	NA	NA	NA
NAu-1	**0.43** ± 0.14	**0.46** ± 0.17	**120** ± 9.51	NA
3.4 g/m^3 H_2O_2/NAu-1	**0.40** ± 0.08	**0.38** ± 0.11	**116** ± 5.25	NA
9 g/m^3 H_2O_2/NAu-1	**0.45** ± 0.04	**0.39** ± 0.09	**114** ± 8.47	NA
340 g/m^3 H_2O_2/NAu-1	**0.42** ± 0.05	**0.09** ± 0.02	**109** ± 5.34	NA
Combined H_2O_2/NAu-1 (0.5 g/L NAu-1, 9 g/m^3 H_2O_2)				
Feedwater	**ND	**0.27** ± 0.01	**133** ± 0.50	**480** ± 20.53
NAu-1 (24 hrs)	**ND**	**0.00** ± 0.02	**150** ± 2.62	**489** ± 19.34
H_2O_2/NAu-1 (30 min)	**ND**	**0.20** ± 0.04	**135** ± 0.59	**493** ± 19.94
H_2O_2/NAu-1 (4 hrs)	**ND**	**0.12** ± 0.04	**137** ± 1.72	**489** ± 19.56
H_2O_2/NAu-1 (24 hrs)	**ND**	**ND**	**164** ± 9.63	**489** ± 19.37
rNAu-1 (0.5 g/L rNAu-1)				
Secondary clarifier effluent	**0.51** ± 0.03	**0.56** ± 0.01	**148** ± 0.08	**483** ± 1.00
Feedwater	**0.72** ± 0.21	**0.41** ± 0.01	**148** ± 0.48	**485** ± 2.07
rNAu-1 (30 min) – high O_2	**0.57** ± 0.04	**0.19** ± 0.01	**173** ± 2.88	**408** ± 9.42
rNAu-1 (4 hrs) – high O_2	**0.61** ± 0.17	**0.19** ± 0.08	**172** ± 1.02	**403** ± 4.78
rNAu-1 (8 hrs) – high O_2	**0.59** ± 0.07	**0.13** ± 0.01	**174** ± 1.58	**402** ± 3.07
rNAu-1 (24 hrs) – high O_2	**0.63** ± 0.09	**0.11** ± 0.01	**182** ± 3.40	**412** ± 9.59
rNAu-1 (8 hrs) – low O_2	**0.71** ± 0.08	**0.55** ± 0.30	**177** ± 2.60	**403** ± 4.32
rNAu-1 (24 hrs) - low O_2	**0.48** ± 0.12	**0.25** ± 0.03	**191** ± 1.14	**408** ± 8.77

*NA = not analysed
**ND = not detected

Additional experiments were conducted to quantify further physicochemical parameters associated with the H_2O_2/NAu-1 treatment using 9 g/m^3 H_2O_2 and 0.5 g/L NAu-1 and for contact

times of 4 and 8 hrs. These conditions were chosen as the most realistic for application of this AOP in a WWTP. As data in table 5.8 show, pH values gradually increased with time, which agrees with our previous observations. Values of COD declined by 5.9% and 6.9% after 4 and 8 hrs, respectively. The same trend was seen in UV absorbance at 254 nm (organic matter measurement), where 60% decrease was seen in both conditions tested. Regarding TSS, a 16.6% decrease was observed at 4 hrs, whereas a significant increase was observed after 8 hrs. Similarly, TOC also increased over time by 16.9% and 28.0% after 4 and 8 hrs, respectively, whereas TP declined by 25.4 % after 4 hrs and 26.5% after 8 hrs contact time.

Table 5.8 Physicochemical parameters tested in feedwater (secondary clarifier effluent) and at the end of combined H_2O_2/NAu-1 treatment with 4 and 8 hrs contact time.

Physicochemical parameter	Feedwater	4 hrs	8 hrs
pH	**6.9** ± 0.0	**7.6** ± 0.2	**7.9** ± 0.1
COD (mg/L)	**30.5** ± 0.2	**28.7** ± 0.6	**28.4** ± 3.5
UV absorbance (254 nm)	**0.1** ± 0.0	**0.04** ± 0.0	**0.04** ± 0.0
TSS (mg/L)	**7.5** ± 2.0	**6.2** ± 3.7	**22.1** ± 6.5
TOC (mg/L)	**10.3** ± 0.0	**12.1** ± 0.8	**13.2** ± 0.3
TP (mg/L)	**1.7** ± 0.0	**1.3** ± 0.0	**1.3** ± 0.0

5.4 Discussion

5.4.1 *H_2O_2/UV treatment*

The H_2O_2/UV treatment was tested in the laboratory to identify parameters that might affect this treatment option in reducing ARGs in treated WWTP effluents. Since UV irradiation levels in the AOP pilot plant testing (Chapter 4) were kept constant (due to time constraints at the WWTP), the effect of changes in UV dose was examined in a bench-scale experiment to test any potential effect on ARG removal. Preliminary results showed that increasing UV dose from 96 to 576 mJ/cm^2 resulted in decreases in absolute abundances of the 16S rRNA, *int*1 and *tet*M genes (figure 5.2). Log removal rates were up to 1.52 and 1.13 for 16S rRNA and *tet*M genes, respectively (table C.2), for a UV dose of 576 mJ/cm^2, whereas change in *int*1 genes were less affected than the other genes (figure 5.2). The highest log removal of *int*1 was 0.52 achieved at the 288 mJ/cm^2 UV dose (table C.2).

Similar to our results, previous studies showed decreases in ARG absolute abundances in secondary clarifier effluents by increasing UV dose (McKinney and Pruden, 2012; Guo *et al.*, 2013; Zhang *et al.*, 2015b; Zhuang *et al.*, 2015; Calero-Cáceres and Muniesa, 2016; Yoon *et al.*, 2017). Although target ARGs, UV doses and experimental set-ups were different in each study, all findings suggest that UV dose is important to ARG reduction in wastewater processes. Interestingly, higher removal rates were observed in studies where a collimated-beam system was used for the experiments. For instance, a log removal of 4.0 was achieved at a UV dose of 130 mJ/cm^2 for the genes *amp*^{R} and *kan*^{R} in a study of Yoon *et al.* (2017), and log 3 to 4 was seen at a UV range of 200-400 mJ/cm^2 for the genes *mec*A, *van*A, *tet*A and *amp*C in the work of McKinney and Pruden (2012). Likewise, a wider range of target genes (*ere*A, *ere*B, *erm*A, *erm*B, *tet*A, *tet*B, *tet*M and *tet*O) were below the LoD at a UV dose of 10 mJ/cm^2 UV (Guo *et al.*, 2013).

In contrast, work conducted without using a collimated beam system resulted in lower log removal rates, such as 1 to 2 (*bla*$_{TEM}$, *bla*$_{CTX}$, *sul*1), for a UV range between 5.94 and 178.2 mJ/cm^2 (Calero-Cáceres and Muniesa, 2016), and 1 to 3 (*tet*G, *sul*1, *int*1, 16S rRNA) when UV dose varied between 1000 and 1300 mJ/cm^2 (Zhuang *et al.*, 2015). Also, Zhang *et al.* (2015b) working on a UV spectrum (62.4, 124.8 and 294.5 mJ/cm^2) closer to the one used here, reported log removal rates up to 0.60 for *sul*1, *tet*X, *tet*Q, *int*1 and 16S rRNA, which is similar to our results; i.e., a 0.40 log removal was the highest rate achieved for the *int*1 at 294.5 mJ/cm^2. Both our preliminary data and previous studies showed that UV dose is key parameter for a treatment system based on UV alone, although the UV apparatus used also appears important. Although not proven, UV seems to be selective against bacteria carrying specific ARG types, which has been discussed before (Dodd, 2012), however there is no mechanistic explanation for this observation.

Since we observed that UV dose plays a key role in treatment, we performed the same experiment adding a constant concentration of H_2O_2 (680 g/m^3) in the system to assess if UV treatment can be enhanced by this oxidant. Similar removal rates of approximately 1 log unit were achieved for all target genes (table C.2), suggesting that, in contrast to UV alone, variation of UV dose did not affect relative gene removal when combined with H_2O_2. Comparing UV alone to H_2O_2/UV, the addition of H_2O_2 in the system enhanced 16S rRNA, *tet*M and *int*1 removal at a conventional UV dose of 96 mJ/cm^2 as reported in Michael-Kordatou *et al.* (2018), whereas H_2O_2/UV was always more effective at reducing *int*1. Hence, H_2O_2 can potentially have added value in the treatment under specific experimental conditions.

H_2O_2/UV was less selective at reducing all three target genes compared to UV alone, as similar removal rates were achieved among all genes. In fact, UV irradiation has been shown to be quite selective, with the degree of DNA damage closely depending on the organism affected (Mamane-Gravetz *et al.*, 2005), possibly because the ability of DNA repair varies among organisms (Süß *et al.*, 2009; Giannakis *et al.*, 2016). The main oxidative mechanism of UV irradiation relies on its penetration into the cytoplasm where the UV light is strongly absorbed by pyrimidine and purines nucleobases in DNA and RNA (Dodd, 2012), causing photolytic inactivation or degradation of intracellular DNA and leading to cell death. Photo-induced generation of ROS, such as $^{\bullet}OH$, singlet oxygen and superoxide radicals, has also been reported as a potential pathway resulting in DNA, RNA and cell damage (Cadet *et al.*, 2005). Consequently, the addition of H_2O_2, which mainly produces ROS, should theoretically improve the UV treatment due to the combination of oxidative pathways: UV penetration into the cytoplasm, increased production of ROS by the combination of UV with H_2O_2 and H_2O_2 penetration into the cytoplasm activating intracellular Fenton oxidative reactions (Giannakis *et al.*, 2016). However, based on the decreased selectivity and increased ROS formation in the combined H_2O_2/UV treatment compared to UV alone, it is conceivable that ROS may be predominantly involved in ARG removal in the combined treatment, yet be less selective at reducing ARGs.

Alternatively, the wastewater matrix in presence of high dissolved organic matter (DOM) and natural organic matter (NOM) (Lee *et al.*, 2016) as well as high H_2O_2 doses (Liu *et al.*, 2015) can have significant scavenging effects, limiting the effectiveness of H_2O_2/UV treatment for the removal of contaminants and pathogenic microorganisms. For example, Yoon *et al.* (2017) evaluated the effect of a constant H_2O_2 (10 mg/L) concentration in a UV system treating ARGs, where the addition of H_2O_2 had no additional effect at reducing target gene abundances, due to the potential scavenging of hydroxyl radicals by the effluent's DOM. suggesting that the wastewater matrix is important for the effectiveness of the treatment. Similarly poor ARG removal was seen by Ferro *et al.* (2016) in secondary effluents for 20 g/m^3 H_2O_2 doses and UV irradiation contact times from 30 to 240 min. In contrast, other studies reported high log removal rates up to 4 for target ARGs by a H_2O_2/UV system applying similar H_2O_2 concentrations to our study, however at higher UV doses and lower pH values (3.5) (Zhang *et al.*, 2016). Therefore, UV dose, H_2O_2 concentration and ancillary experimental conditions (e.g. DOM, pH) are important factors for the effectiveness of the combined H_2O_2/UV treatment method.

5.4.2 *H_2O_2 activation using NAu-1*

The preliminary data, which assessed a combination of 680 g/m^3 H_2O_2 with 1 g/L NAu-1 and 8 hrs contact time, showed promising results for the effectiveness of this treatment. Statistically significantly higher log removal rates were observed for *tet*M (1.31) and *int*1 (1.27) after treatment with H_2O_2/NAu-1 compared to UV alone and H_2O_2/UV. The outcome of these preliminary experiments led us to examine this new treatment option further, investigating removal rates for different H_2O_2 doses and contact times and evaluating the effect of each individual component.

Treatment with H_2O_2 alone removed target ARGs at doses of 9 and 340 g/m^3, where significant reductions in *int*1 and *tet*M gene abundances (9 g/m^3) or all target genes (340 g/m^3, Games-Howell; $p < 0.05$) were observed. The involvement of H_2O_2 in ARG removal was confirmed by measurements of the residual H_2O_2 at the end of each treatment, where decreases were seen compared to initial concentrations (tables C.20 and C.21). H_2O_2 is a known oxidant and has been examined before as a potential disinfectant at the Montreal Urban Community WWTP, although high impractical levels were required for adequate treatment (Wagner *et al.*, 2002). As has been described before, H_2O_2 can inhibit bacterial growth, cause mutations (Mishra and Imlay, 2012) and can penetrate membrane cells, initiating Fenton reactions with free ferrous iron not bound to proteins; this produces ROS that can damage intracellular components resulting in bacterial cell death (Park *et al.*, 2005). However, limiting factors include H_2O_2 scavenging by organic matter present in the wastewater matrix (Yoon *et al.*, 2017) and the activity of peroxidases and catalases inside bacterial cells (Michael-Kordatou *et al.*, 2018). Furthermore, H_2O_2 can also be converted to non-radical products, such as H_2O and O_2 (Pham *et al.*, 2012).

Similarly, NAu-1 alone was effective at significantly reducing *tet*M and *tet*Q absolute abundances after 8 hrs of treatment. Although this was the first study of the effect of NAu-1 on ARG removal, previous studies have investigated the effectiveness of clays and clay minerals against bacteria causing skin infections (Haydel *et al.*, 2008; Williams *et al.*, 2011; Morrison *et al.*, 2014; Behroozian *et al.*, 2016; Morrison *et al.*, 2016). These studies found that clays and clay minerals differ widely in their effectiveness for removing bacteria and propose several mechanisms in which clays, when hydrated, may cause damage to bacterial cells. For example, physical attraction between bacterial membranes and exfoliated clay minerals can lead to the blockage of nutrient uptake and waste removal by enveloping the cells, or even to cell death by

physically damaging the membranes (Wei *et al.*, 2011). Williams (2017) and Haydel *et al.* (2008) also suggested that clay minerals compete with bacteria for nutrients or provide toxins that affect the bacterial cell regulatory system. They also reported that clays are selective against bacterial cells, which might explain why NAu-1 was effective only in reducing significantly tetracycline genes in our experiment. It is also known that some metals can cause intra-bacterial oxidation, when solubilised at pH < 5 or pH > 9 (Williams, 2017). However, this is not likely to be the case here since pH was typically between 7 and 8 (table C.19) and dissolved Fe^{2+} and total Fe decreased (table C.22).

Although NAu-1 alone and high doses of H_2O_2 alone each decreased gene absolute abundances from feedwater, the combination H_2O_2/NAu-1 achieved significantly higher log removal rates, up to 2.34 (table C.6), compared with individual treatments (up to 0.59 for NAu-1 alone and up to 0.84 for 340 g/m^3 H_2O_2 alone; table C.6). A possible explanation for this might be a synergistic effect of combining NAu-1 with H_2O_2. NAu-1 is an iron-rich clay mineral with a Fe content of approximately 19.8 wt%, resulting in the dioctahedral sheet mainly occupied by Fe^{3+} (Keeling *et al.*, 2000). As observed for Fe oxides/hydroxides, synthetic iron-silica precipitates and Fe-bearing clays (Pham *et al.*, 2009; Pham *et al.*, 2012), H_2O_2 can be decomposed in a surface reaction with the mineral-bound Fe^{3+} to form $^{\bullet}OH$ by following a series of reactions described by the Haber-Weiss mechanism (figure C.5). However, H_2O_2 decomposition does not always lead to ROS formation, due to radical scavenging and/or via an alternative non-radical mechanism forming an intermediate, ferryl ion Fe(IV), and leading to non-radical products (Pham *et al.*, 2009). Ferryl ion has been shown to be less reactive, yet more selective, than $^{\bullet}OH$ in contaminant degradation (Voegelin and Hug, 2003) and can either react with water to produce $^{\bullet}OH$ or react directly with organic substrates oxidising them (figure C.6). In analogy, the significantly higher removal efficiencies achieved by the H_2O_2/NAu-1 can be attributed to the formation of ROS, which target proteins, lipids and nucleic acids (Cabiscol *et al.*, 2000), and/or ferryl ion, in addition to physical contact with NAu-1 and bactericidal action of H_2O_2 alone.

Based on this proposed mechanism of H_2O_2 activation by NAu-1, we explored the effect of oxidant concentration on the treatment and found an apparent threshold in H_2O_2 dose below which ARG removals were low (figure 5.3). For example, 3.4 g/m^3 had limited effect on ARG levels, whereas 9 g/m^3 H_2O_2 significantly reduced all target genes, including ARGs. Statistically significant differences in removal rates between 340 g/m^3 and 9 g/m^3 were observed only for

*int*1, but not for the ARGs and 16S rRNA gene. The absence of significant decreases in target gene concentrations when applying a high dose of 340 g/m^3 H_2O_2 is surprising, also because treatment with the same dose H_2O_2 alone achieved significantly lower target gene concentrations. At these high initial H_2O_2 doses, residual H_2O_2 remained higher after 8 hrs of treatment compared to when lower initial H_2O_2 doses were applied (table C.20), hinting that H_2O_2 may not have been activated to significant extents. However, the presence of NAu-1 consistently led to higher H_2O_2 decomposition for all H_2O_2 doses and higher initial H_2O_2 doses led to higher H_2O_2 concentration decreases (table C.20), implying that H_2O_2 was indeed activated to much higher extents at 340 vs 9 g/m^3 H_2O_2 (77.5 vs 6.5 g/m^3 H_2O_2 decrease, respectively). Considering that previous studies of heterogeneous Fenton systems found that contaminant oxidation could not be predicted from the extent of H_2O_2 decomposition (Pham *et al.*, 2012), we suspect a similar effect in our combined H_2O_2/NAu-1 treatments for gene removal and hypothesise that this effect might be due to competition of radical and non-radical pathways of H_2O_2 activation (figure C.5). Therefore, further experiments are warranted to test if a higher concentration of NAu-1 (or rather: higher NAu-1/H_2O_2 ratios) would increase conversion of H_2O_2 into ROS over formation of non-radical products, and consequently increase gene removal rates.

In our experiments, we found that also reaction time was a key parameter in the H_2O_2/NAu-1 treatment. All contact times tested, from 30 min to 24 hrs, were able to reduce gene abundances, including all ARGs and *int*1. Among these contact times, 4 and 8 hrs treatment were the most effective (i.e. resulting in the highest gene removal), although 16S rRNA genes were affected significantly only by applying 8 hrs contact time. Interestingly, different contact times were needed for each gene type, indicating that different bacteria hosts may respond differently to this treatment. Furthermore, quite long (i.e. 4-8 hrs) but not too long (24 hrs) reactions times were needed to have a significant effect on gene abundances. Even though removal rates among different contact times were not statistically different, they gradually increased from 30 min to 8 hrs and then, surprisingly, declined after 24 hrs treatment to removal rates that were significantly lower compared to 4 and 8 hrs treatments. However, residual H_2O_2 concentration at the end of both 8 and 24 hrs were very low (table C.21), indicating that H_2O_2 levels in the system were almost depleted after 8 hrs, leading to decreased production of ROS and less effective treatment. This plausible explanation is further supported by the significantly higher absolute 16S rRNA

gene concentrations after 24 hrs compared to 30 min, 4 and 8 hrs, which implies regrowth of bacterial cells, including ARB carrying *int*1 and target ARGs.

There is, however, an alternative explanation of the 24 hrs contact time data. While we observed a significant increase in absolute gene concentrations in the 'no treatment' control compared to feedwater, possibly due to the organic matter present in the wastewater matrix that enhanced bacterial cells growth (Giannakis *et al.*, 2016), absolute concentrations in the H_2O_2/NAu-1 and NAu-1 alone experiments remained at similar levels as in the feedwater and even decreased in *int*1 abundances (figure 5.7). Thus, in both reactors with added NAu-1 bacterial regrowth was partially inhibited and consequently the mechanism of gene removal is more likely to be related to the presence of NAu-1 in the system rather than H_2O_2. Consistent with this conclusion, residual H_2O_2 in reactors containing H_2O_2 alone was very low after 24 hrs and an increase in absolute gene concentrations was observed, suggesting that H_2O_2 had no effect on reducing genes. In contrast, NAu-1 alone appeared to inhibit regrowth of bacteria after 24 hrs and decreased *tet*Q absolute abundances, implying that inhibition of bacterial cells was more likely an effect of NAu-1 rather than its combination with H_2O_2. Therefore, our data are consistent with both a bacteriostatic effect of NAu-1 and a bactericidal effect of the combined H_2O_2/NAu-1 treatment, which, combined, eliminated bacteria from the final effluent.

To assess whether the combined H_2O_2/NAu-1 treatment could be practically applied in future pilot and full scale treatment systems, we also evaluated further water quality parameters in samples treated for 4 and 8 hrs contact time. Analysis of physicochemical parameters revealed that the H_2O_2/NAu-1 increased the pH after treatment. This might be a result of the reactions that take place during treatment increasing the presence of hydroxide ions (OH^-), which form part of the oxidative reaction path (Pham *et al.*, 2009). Despite the fact that H_2O_2/NAu-1 caused the treated water to become more alkaline, pH was still lower than 8 which is normally seen in European natural ecosystems such as rivers (EEA, 1994). In contrast, COD and UV absorbance at 254 nm, which are both indicators of organic material present (Mrkva, 1983), declined with treatment at 4 and 8 hrs contact time, maintaining COD concentrations inside the allowable values regulated by the European legislation for discharged water (EEC, 1991). Likewise, further reduction was observed on TP values, which was maintained <2 mg/L with both contact times. Although suspended solids were reduced by 16.6% after 4 hrs, a significant increase was seen at 8 hrs; nevertheless, this parameter was still inside the allowable limit from European regulations

(EEC, 1991). Similarly, TOC increased with both contact times, which can be possibly explained by the release of bacterial debris as a result of bacterial lysis during the oxidative treatment. Furthermore, metal analysis showed constant or even decreasing concentrations during treatment, which indicates that the use of clay minerals did not compromise the water quality. These results are also an indication that NAu-1 did not structurally change and could potentially be used in further treatment cycles, which needs to be confirmed in future studies.

5.4.3 *Treatment using reduced NAu-1*

In addition to the catalytic decomposition of H_2O_2 we explored in Section 5.4.2, ROS such as $^{\bullet}O^{2-}$, H_2O_2 and Fe(IV) can also be produced upon contact of aqueous Fe^{2+} with O_2 in a series of reactions presented in figure C.6 (Remucal and Sedlak, 2011). A similar reaction pathway has also been suggested for the oxygenation reaction of clay mineral Fe^{2+} at neutral pH (Tong *et al.*, 2016). Further work by Wang *et al.* (2017) suggests that $^{\bullet}OH$ and other ROS can subsequently react with cardiolipin, a membrane lipid, increasing membrane permeability and accumulation of soluble Fe^{2+} and/or other oxidants within the cytoplasm, initiating Fenton reactions and subsequently leading to the dissociation of the respiration complex, damaging the osmotic regulating system and finally inducing cell lysis. Thus, reduced clay minerals show great potential for *sustainable* ARG removal due to the wide range of ROS involved and their *in-situ* formation without the need for dosing of additional oxidant.

Indeed, rNAu-1 in the presence of ambient air was able to reduce target genes under all contact times tested (figure 5.10). The evaluation of contact time in the rNAu-1 treatment showed that although 4 and 24 hrs achieved higher removal rates than 30 min and 8 hrs in all target genes, the observed differences were not statistically significant. Interestingly, target gene removal was fast with log removal rates being >1.31 after only 30 minutes, suggesting that also the destruction of bacterial cells carrying these genes was fast. This finding is consistent with previous work showing fast oxidation of reduced NAu-2, a structurally similar clay mineral to NAu-1 used here, upon contact with oxygen (Wang *et al.*, 2017).

One unanticipated finding was that rNAu-1 had a significant effect on reducing target gene abundances under low oxygen conditions (i.e. in the presumed absence of ROS produced), achieving log removal rates greater than 2. This result was unexpected because formation of ROS under low oxygen conditions has not been reported before. However, as described in Section

5.2.6, O_2 was always <2 ppm inside the glovebox, resulting in ≤ 1.74 ± 0.17 mg O_2/L in the reactors at the end of treatment (table C.23). It is conceivable that the DO present might have reacted with rNAu-1 to produce ROS for reaction with bacterial cells, in which case we could suggest that rNAu-1 requires very low levels of O_2 to be effective.

There is, however, other possible explanation. For example, our results might be due to unintended clay mineral Fe^{2+} oxidation after the end of treatment, when samples were transferred out of the anoxic glovebox for filtration. Although catalase was used at the end of treatment to quench any H_2O_2 present, it is possible that clay mineral Fe^{2+} reacted with O_2 once the sample was exposed to ambient air and catalase was not able to prevent the formation of $^{\bullet}OH$ via inactivation of H_2O_2. This explanation would imply, however, that oxidation occurred through a different route not involving H_2O_2, e.g. in a reaction of a wastewater matrix component with Fe^{2+} triggering the production of lethal ROS and resulting in bacterial cell death even under low oxygen conditions. Regardless, based on our current data set, these are speculations and more investigation is warranted to first establish that clay mineral Fe^{2+} oxidation occurred under low oxygen conditions (<2 ppm) and to then elucidate further factors that initiated and/or contributed to the oxidation of a reduced clay mineral when minimal oxygen was present.

Significant differences were observed between the treatments using NAu-1, H_2O_2/NAu-1 and rNAu-1, revealing that rNAu-1 was the most effective at reducing gene abundances. Contact time had limited effect on gene abundances when using native NAu-1, where only up to 0.59 log removal was observed (table C.10). On the other hand, removal rates increased over time in the treatment with rNAu-1, where 1.98-2.94 log removal was achieved at 24 hrs (figure 5.10), showing a clear difference in the effectiveness between native NAu-1 and rNAu-1. H_2O_2/NAu-1 was also effective against the target ARGs and *int*1, where up to 1.80 log removal was achieved at 8 hrs contact time (table C.10). Nevertheless, absence of H_2O_2 in the H_2O_2/NAu-1 system proved to be a limiting factor. Therefore, these results suggested a bactericidal effect of both H_2O_2/NAu-1 and rNAu-1, which increased over time, and a bacteriostatic effect of native NAu-1 was seen, apparently by inhibiting the regrowth of bacteria.

5.4.4 *Practical implications*

Although direct comparisons cannot be made between bench- and pilot-scale studies, general conclusions can be drawn. As we saw in Chapter 4, ozonation was more effective than pilot scale

H_2O_2/UV treatment, where log removal rates up to 0.63 were achieved by applying H_2O_2 doses between 1 and 9 g/m^3 for contact times of 140 seconds. These removal efficiencies were considerably lower compared to the ones obtained in our bench-scale experiments (up to 1.10 at 288 mJ/cm^2 and 15 min contact time; table C.2). Even though a pilot plant system is more complex and several parameters can affect the treatment effectiveness, laboratory experiments have helped to elucidate key factors. We suggest, therefore, that higher log removal rates (up to 1.10) achieved here were an effect of H_2O_2 dose and contact time: higher concentrations (680 g/m^3) and longer reaction times (5-30 min) resulted in higher removal rates. In contrast, UV dose was less important as it had limited effect in the lab-scale H_2O_2/UV treatment and we additionally achieved higher removal rates with lower UV doses (96, 288 and 576 mJ/cm^2) compared to those applied in the pilot plant (650 mJ/cm^2).

Going a step further, by studying alternative oxidation processes, we saw that the oxidative mechanisms taking place during treatment are also key, significantly increasing the efficacy of the reduction of gene abundances. In fact, the combination of H_2O_2 with NAu-1 or use of reduced clay mineral, where a wider range of ROS are likely to be involved, achieved higher gene removal rates than H_2O_2/UV. Additional modes of action for gene removal were found to be plausible in the treatments using NAu-1, in either native or reduced form, as both significantly reduced relative abundances per genome of all or most of the target ARGs and *int*1. This can indicate effective reaction mechanisms against bacteria and/or selectivity to attack specific bacteria carrying the target genes. However, more sophisticated analysis, such as HT-qPCR or sequencing, could be more appropriate to elucidate these hypotheses. Although more detailed analysis is warranted to evaluate the application of this oxidation method in a real wastewater treatment system, preliminary physicochemical and molecular analysis revealed that NAu-1 has potential as an advanced treatment targeting antimicrobial resistance while improving water quality.

5.5 Conclusions

In this study, follow-up laboratory experiments were conducted to explore important factors that may affect the performance of a H_2O_2/UV system which was not as effective as ozonation in the pilot plant testing of Chapter 4. Higher H_2O_2 concentration than those tested in the AOP pilot plant and variation of the UV dose were examined and contrasted to a system including only UV irradiation. Comparison between the treatment systems showed that addition of H_2O_2 enhanced UV performance at the conventional dose of 96 mJ/cm^2 in the removal of target genes. Nevertheless, variation of UV dose had no effect on the H_2O_2/UV performance. It is noteworthy that higher removal rates were achieved in the bench-scale experiments than the AOP pilot plant. Direct comparisons between the systems cannot be made; however, higher H_2O_2 concentrations and contact times resulting in higher removal rates in the laboratory experiments might explain why H_2O_2/UV was not effective against ARGs in the pilot plant testing.

Alternative AOPs that are potentially more cost- and energy-effective were also explored in this study. NAu-1, an iron-rich clay mineral, was assessed either in combination with H_2O_2 or after reduction of the structural Fe to Fe^{2+} upon contact with oxygen at reducing ARG abundance in domestic wastewater by production of ROS. To the best of our knowledge, this is the first attempt to study the potential of clay minerals for reducing ARGs from real wastewater and the results here are promising. Preliminary data showed that the H_2O_2/NAu-1 system achieved higher removal rates compared to H_2O_2/UV or UV alone under the same experimental conditions. Therefore, further experiments varying key factors were conducted.

The main findings showed that H_2O_2 dose and contact time are important parameters for ARG abundance reduction when applying the combined H_2O_2/NAu-1 treatment. Removal rates up to 2.34 logs were achieved by increasing the H_2O_2 dose from 3.4 to 9 and 340 g/m^3 after 8 hrs treatment; while, gradual removal of target ARGs was seen by increasing contact times from 30 min to 4 and 8 hrs. Interestingly, non-significant ARG removal was observed after 24 hrs treatment. This might be a result of low H_2O_2 concentration in the system at the end of treatment implying that H_2O_2 levels are limiting factor for the process. Although H_2O_2/NAu-1 resulted in higher removal rates compared to H_2O_2 or NAu-1 alone, native NAu-1 inhibited the growth of bacterial cells after 24 hrs, indicating a different reduction mechanism without involving ROS.

Reduced NAu-1 removed more effectively genes compared to H_2O_2/NAu-1 and maintained gene removal potential even after 24 hrs treatment. Although higher contact times resulted in higher removal rates up to 2.94 logs, no statistical differences were observed suggesting a very fast reaction even at 30 min contact time. Further, oxygen levels had no effect on the activation of rNAu-1 as similar removal rates were observed in both high and low oxygen levels.

Data hint that oxidative mechanisms involved in each process are important for the effectiveness of the treatment options for decreasing ARG abundances. The most plausible mode of action of both H_2O_2/NAu-1 and rNAu-1 was bactericidal activity through production of reactive oxidizing species. However, additional bacteriostatic activity cannot be ruled out for native NAu-1, following a different reduction pathway. This study showed that clay minerals in wastewater treatment has potential as a promising alternative AOP on controlling ARB in wastewater while improvement of the water quality and no structural changes in the material during treatment suggest that this is a safe and sustainable new technology.

Chapter 6 Conclusions and Recommendations

6.1 Summary and Concluding Comments

This project studied the effectiveness of wastewater treatment at reducing the spread of ARB and ARGs from raw wastes to the environment. Specifically, four wastewater treatment technologies were evaluated (i.e. MBR, AS, GAS, and TF) for treating domestic wastewater, and contrasted in their relative ability to reduce ARG abundance and diversity in final effluents (Chapter 3). Data showed that these wastewater treatment technologies are effective at removing most ARB and ARGs of all major classes of antibiotics. However, additional treatment options were examined as sometimes biological treatment may not be adequate to satisfy local water quality needs, such as in water scarce areas and/or where reuse is needed. Therefore, two AOP technologies and their combination (i.e. O_3, H_2O_2/UV, and O_3/H_2O_2/UV) (Chapter 4), and a Fenton-like system using Fe-bearing clay minerals (Chapter 5), were also tested as additional treatment steps for secondary treatment systems.

Three suites of experiments were performed to achieve the main objectives of the project:

1. Study the performance of four different WWTPs relative to reducing ARG abundance and diversity in final effluents.
2. Evaluate the additional benefit of quaternary treatment options to a conventional WWTP.
3. Investigate an alternative AOP option using natural materials which is less energy intensive and plausibly more cost-effective.

Such a cohesive comparison, using common methods, of how a wide array of treatment processes reduce ARB and ARGs is key to understand the relative value of different WWTP options for mitigating AMR spread. Each chapter met the overall aim and objectives elucidating key factors for each treatment technology at reducing ARGs, and implicitly, ARB in final effluents. To fulfil the above objectives, over 290 different gene primers were used to quantify ARGs, MGEs and 16S rRNA (‘all’ bacteria), using both qPCR and HT-qPCR analyses.

Many wastewater treatment technologies have shown gene-to-gene and ARB specificity (Rodriguez-Mozaz *et al.*, 2015; Calero-Cáceres and Muniesa, 2016; Sousa *et al.*, 2017; Korzeniewska and Harnisz, 2018; Le *et al.*, 2018; Proia *et al.*, 2018), hence, monitoring a wide

range of ARGs is important to estimate the overall performance of a wastewater treatment. However, the novelty here is differentiating gene carriage in viable from non-viable bacterial cells using PMA as most studies have focused on ARGs in bacteria with both intact and broken membranes without distinguishing them. The importance of focusing on viable cells have been identified in recent studies (Michael-Kordatou *et al.*, 2018; Hiller *et al.*, 2019). Monitoring the patterns of ARGs in viable and total cells in parallel within different wastewater trains can contribute to understanding the fate of ARGs carried in viable cells and their potential contribution to HGT in receiving environments.

Four different full-scale WWTPs were compared (Chapter 3) and data revealed that all treatment systems reduced ARG and MGE abundances in final effluents. The relative effectiveness of each system at removing genes differed among technologies. Overall comparisons among all conventional treatment options from the most effective to the least effective at reducing ARG abundances, based on removal rates, is shown in figure 6.1 and table 6.1. Clearly, MBRs were the most effective among tested technologies at reducing ARGs, followed by AS, GAS, and then TF. This agrees with previous studies showing that MBRs were more effective compared to other treatment systems at removing specific ARGs from final effluents (Du *et al.*, 2015; Zhang *et al.*, 2015a), achieving up to 7 log removal of ARGs (Le *et al.*, 2018). MBR and AS systems also reduced significantly ARG diversities in final effluents. MGE diversity was not affected by any treatment technology; however, this was probably because of the small number of MGEs assessed.

Few studies have identified the contribution of each treatment step to the total performance of a WWTP (Mao *et al.*, 2015; Zhang *et al.*, 2018), which is important for decisions in future treatment strategies. In this study, the biological treatment step was the major contributor to ARG, MGE and 16S rRNA reduction with > 0.73 proportional contribution to the total removal, in both total and viable cells, in most WWTPs (table 6.1), whereas tertiary treatment (where applied) had lower (e.g. disc filters in TF system) or negligible contribution (e.g. GAC & chlorination in MBR system). However, secondary (GAS) and tertiary treatment (pile cloth filtration) in the GAS WWTP had the same contribution to ARGs, MGEs and 16S rRNA removal in viable cells. This indicates that each treatment step affects differently viable and total cells patterns, suggesting that the technology applied can have significant effect on the whole performance of a WWTP at removing ARGs.

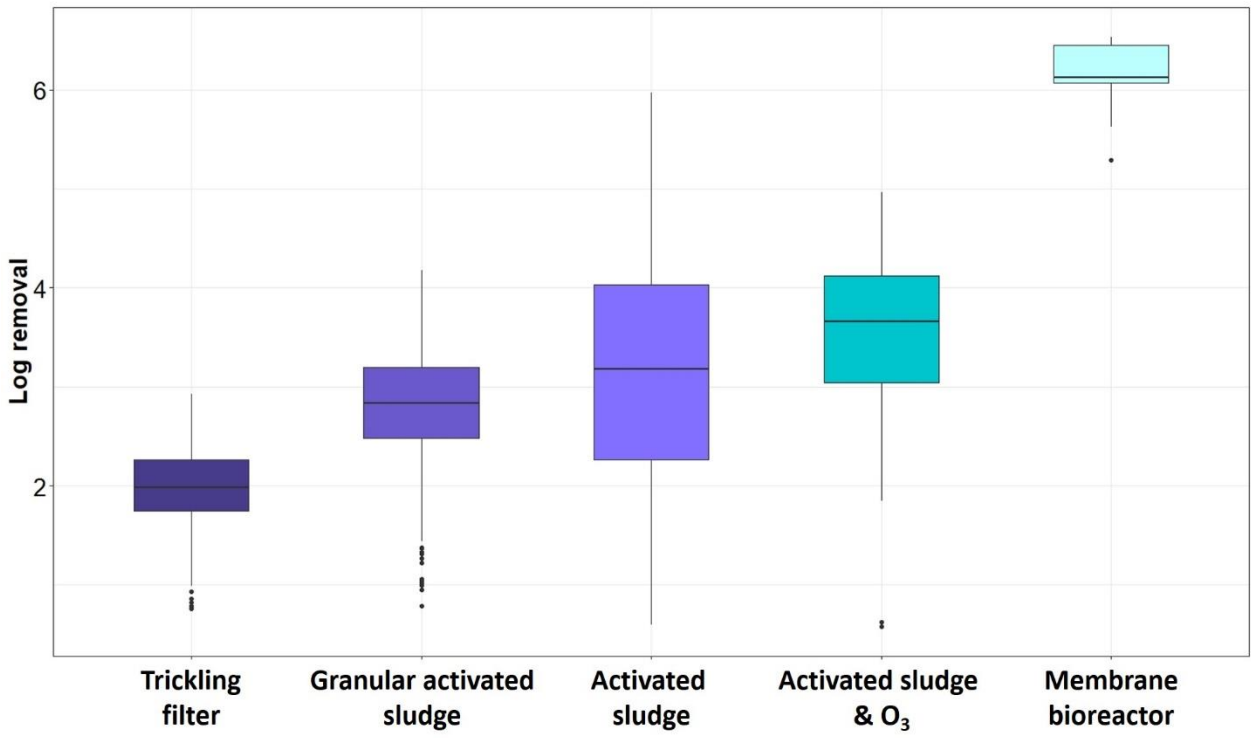

Figure 6.1 ARG removal rates of different options of wastewater treatment systems. All data refer to $\log_{10}(C_{influent}/C_{final\ effleunt})$ of ARGs in total bacteria of each treatment system. Boxes represent the first quartile and third quartile of the HT-qPCR data for total bacteria, the vertical line shows the median, whiskers extend from each quartile to minimum and maximum data points and outliers are depicted with dots. The ozone dose in the activated sludge & O_3 system is 3 g/m^3 (conventional dose), and the data of the membrane bioreactor refer to 16S rRNA removals (qPCR data) as HT-qPCR data were not available.

In general, ARG levels positively correlates with 16S rRNA ('all' bacteria), and physicochemical parameters such as pH, TOC and total nitrogen (TN), suggesting the effectiveness of a wastewater treatment system at reducing ARGs is strongly related to the ability to remove bacteria and the overall treatment performance. Although operating parameters are not available due to confidentiality issues, a positive correlation between ARGs and physicochemical parameters hint that the whole performance of a treatment system can greatly affect the reduction of ARGs. Similar conclusions have been drawn in previous studies, where positive correlations between ARGs and parameters such as COD, TN and TP were observed (Du *et al.*, 2015; Bengtsson-Palme *et al.*, 2016; Sun *et al.*, 2016). Further, the strong positive correlation between ARGs and 16S rRNA ('all' bacteria) and the fact that ARGs followed similar removal rate patterns with 16S rRNA reveal that ARG abundances decrease by removing bacteria in general. This has also been observed in previous studies suggesting that enhancing biosolids separation will improve the control of ARG releases into receiving waters (Chen and Zhang, 2013b; Yang *et*

al., 2014; Al-Jassim *et al.*, 2015; Quintela-Baluja *et al.*, 2015; Bengtsson-Palme *et al.*, 2016; Karkman *et al.*, 2016).

No obvious selection for specific ARGs through biological or physical processes was evident in any of the treatment systems. Final effluents were less diverse than influents and there was no evidence of ARG or MGE enrichment (i.e. decreases or no changes in diversity or relative abundances per genome) through treatment processes (e.g. RAS, mixed liquor) in any of the WWTPs (table 6.1), while the distribution of ARG types across treatment did not change. These data agree with other studies based on resistome analysis which observed similar patterns as here, i.e. decreases in ARG diversity and relative abundances after treatment (Yang *et al.*, 2014; Bengtsson-Palme *et al.*, 2016; Karkman *et al.*, 2016; Quintela-Baluja *et al.*, 2019). This research in accordance with other studies using resistomics challenge the dogma that WWTPs contribute to the dissemination of ARGs to the wider environment suggested by studies based on few target ARGs or ARB (Al-Jassim *et al.*, 2015; Mao *et al.*, 2015; Naquin *et al.*, 2015; Rafraf *et al.*, 2016; Korzeniewska and Harnisz, 2018; Proia *et al.*, 2018). This shows the importance of monitoring a wide range of ARGs to understand the overall performance of a WWTP.

Differentiating between viable and total bacterial populations within samples using the PMA-qPCR method has been valuable to test for selection in WWTPs. This approach demonstrated that ARG concentrations (i.e. ARGs per genome) in viable cells are lower than in total cells; this is important as it shows that ARGs carried in viable bacteria behave differently to ARGs in total cells. This also suggests that treatment did not select positively for ARGs in the viable fraction. Therefore, combining PMA pre-treatment with HT-qPCR showed that all WWTPs effectively reduced ARG releases into the receiving waters without selecting for ARGs and also highlights the importance of monitoring the viable fraction within a treatment system to understand the implications this will have on the HGT in the environment.

Table 6.1 Table summarising all conventional treatment technologies and treatment steps tested in full-scale in the thesis including overall removal rates per WWTP ($\log_{10}(C_{influent}/C_{final\ effleunt})$), contribution of each treatment step to the overall removal rates of each WWTP, the effect of each technology in the viability of bacteria and also in the relative concentration per genome. The last column shows whether relative concentrations per genome decreased significantly (↓) or it was not significantly different (-) compared to influent (WWTPs).

Technology	Treatment steps	ARG overall log removal rates of the WWTPs	Contribution of each treatment step to the total removal of ARGs in WWTPs	Effect on ARG viability (Viable cells < Total cells)	Effect on ARG relative concentration per genome compared to influent
Trickling filter (TF WWTP)	Primary settler	Total cells: 1.98 Viable cells: 1.62	*NA	Significant	NA
	TF		Total cells: 0.75 Viable cells: 0.82	Not significant	Total cells: ↓ Viable cells: ↓
	Disc filter		Total cells: 0.25 Viable cells: 0.18	Significant	Total cells: ↓ Viable cells: ↓
Granular activated sludge (GAS WWTP)	Primary settler	Total cells: 2.80 Viable cells: 3.20	NA	Significant	NA
	GAS		Total cells: 0.73 Viable cells: 0.55	Not significant	Total cells: - Viable cells: -
	Pile cloth filter		Total cells: 0.27 Viable cells: 0.45	Significant	Total cells: - Viable cells: ↓

Technology	Treatment steps	ARG overall log removal rates of the WWTPs	Contribution of each treatment step to the total removal of ARGs in WWTPs	Effect on ARG viability (Viable cells < Total cells)	Effect on ARG relative concentration per genome compared to influent
Activated sludge (AS WWTP)	Primary settler	Total cells: 3.18 Viable cells: 3.18	NA	Significant	NA
	Mixed liquor		NA	Not significant	Total cells: ↓ Viable cells: -
	Secondary clarifier		Total cells: 1.00 Viable cells: 1.00	Not significant	Total cells: ↓ Viable cells: -
	Return activated sludge		NA	Not significant	Total cells: ↓ Viable cells: -
Membrane Bioreactor (MBR WWTP)	Screens	**Total cells: 6.10 **Viable cells: 6.08	NA	Not significant	NA
	Mixed liquor		NA	Significant	Total cells: ↓ Viable cells: ↓
	MBR		**Total cells: 0.99 **Viable cells: 1.00	Target ARGs below the LoD	NA
	GAC & chlorination		**Total cells: 0.01 **Viable cells: 0.00	Target ARGs below the LoD	NA

*NA: Not analysed or not available data

**16S rRNA data

The value of different AOPs as an extension to an AS system for reducing ARG abundances and diversities was evaluated in Chapter 4. Several studies have focused on the efficacy of AOPs against ARGs and ARB in recent years (Dunlop *et al.*, 2015; Zhang *et al.*, 2016; Ferro *et al.*, 2017; Giannakis *et al.*, 2018), mainly due to the production of hydroxyl radicals and other ROS that can break bacterial cells and disintegrate genes. However, research has been based on bench scale experiments focusing on specific ARGs and ARB. In this study, testing AOPs in pilot plant scale and connecting them to the overall performance of a conventional treatment system provided a more comprehensive understanding of advanced treatment.

Results showed that although an AS WWTP followed by pile cloth filtration significantly reduced ARG abundances (table 6.2) and diversities, AOPs further enhanced removal rates. AOP effectiveness was dependent on technology type, AO dose, and ARG class, factors which have previously been reported to affect performance (Michael-Kordatou *et al.*, 2018). For instance, H_2O_2/UV was not as effective as either ozonation or O_3/H_2O_2/UV, achieving ARG removal rates up to 0.52 log with no effect on diversity. Ozonation was superior in reducing ARG abundances of total bacteria, which increased with O_3 dose. However, 3 g/m^3 O_3 (the middle dose tested and recommended dose for full-scale applications in Switzerland (Bourgin *et al.*, 2018)), was adequate for effective ARG reduction as non-significant differences in removals were observed between 3 and 9 g/m^3 O_3. In contrast, 9 g/m^3 O_3 was required to reduce MGEs. In fact, this was the only oxidant dose and treatment technology, including conventional options, which significantly reduced MGE diversity. O_3/H_2O_2/UV did not enhance reductions, either abundances or diversities, compared with O_3 alone, suggesting that O_3 was the driver oxidant in the combined system. This is promising as indicates no need for additional chemicals (H_2O_2) or use of UV irradiation in the process.

Table 6.2 Table summarising all AOP treatment technologies and treatment conditions tested in the thesis including overall removal rates per WWTP ($\log_{10}(C_{influent}/C_{feedwater})$), added value of AOPs in log removals ($\log_{10}(C_{feedwater}/C_{AOP\ effleunt})$), the effect of each technology in the viability of bacteria and also in the relative concentration per genome. The last column shows whether relative concentrations per genome decreased significantly (↓) or it was not significantly different (-) compared to feedwater. Conditions: 650 mJ/cm UV dose, 0.5g/L (r)NAu-1.

Technology	Treatment conditions	ARG overall log removal rates of the WWTPs (total cells)	Added value of AOP treatment in logs (ARG total cells)	Effect on ARG viability (Viable cells < Total cells)	Effect on ARG relative concentration per genome compared to influent (total cells)
AS WWTP & H_2O_2/UV (AOP pilot plant)	1 g/m^3 H_2O_2	2.50	0.52	*NA	↓ in major classes
	3 g/m^3 H_2O_2		0.09	Not significant	
	9 g/m^3 H_2O_2		0.16	Significant	
AS WWTP & ozonation (AOP pilot plant)	1 g/m^3 O_3	2.50	0.51	NA	↓ in major classes
	3 g/m^3 O_3		1.11	Significant	
	9 g/m^3 O_3		1.25	Significant	
AS WWTP & O_3/H_2O_2/UV (AOP pilot plant)	3 g/m^3 H_2O_2-3 g/m^3 O_3	2.50	1.02	Significant	↓ in major classes

Technology	Treatment conditions	ARG overall log removal rates of the WWTPs (total cells)	Added value of AOP treatment in logs (ARG total cells)	Effect on ARG viability (Viable cells < Total cells)	Effect on ARG relative concentration per genome compared to influent (total cells)
H_2O_2/NAu-1 (9 g/m^3 H_2O_2; lab-scale)	30 min	NA	0.59-0.76	NA	-
	4 hrs		0.94-1.49		- in most target ARGs
	8 hrs		0.81-1.18		↓
	24 hrs		0.20-0.32		-
H_2O_2/NAu-1 (8 hrs; lab-scale)	3.4 g/m^3 H_2O_2	NA	-0.21-0.37	NA	-
	340 g/m^3 H_2O_2		1.23-1.65		↓
rNAu-1 (lab-scale)	30 min	NA	1.61-1.98	NA	↓
	4 hrs		2.15-2.67		↓
	8 hrs		1.31-1.82		↓
	24 hrs		1.98-2.94		↓

*NA: Not analysed or not available

In this study the whole performance of an AS system was enhanced by ozonation to increase log removals of ARGs by up to 1.25, placing it as the second-best option at reducing ARG abundances after MBR (figure 6.1). Similar added value was seen in the study of Lüddeke *et al.* (2015), where removal rates between 0.8 and 1.1 logs were observed in a pilot plant project at a dose of 0.73 mg O_3/mg DOC and much higher contact time (20 min). Higher removal rates up to 2.55 logs have been reported in the literature; however, these have been achieved in bench scale experiments often tested with synthetic water or combined treatments, i.e. post-filtration or use of persulfate and peroxymonosulfate (Oh *et al.*, 2014; Zhuang *et al.*, 2015; Sousa *et al.*, 2017). Therefore, the observation of Graham *et al.* (2018) that a further log reduction between 1.0 and 3.0 can be achieved with an additional technology to secondary treatment depends not only on the technology type (including oxidant doses) but also the water matrix and the combination to other treatments.

Differences in observed ARG prevalence and diversity patterns confirming gene-to-gene differences (implicitly cell-to-cell differences), especially where ozone was involved, which reduced to LoD the FCA and 'Other' classes, are probably important to how ARB respond to each type of oxidative stress in advanced processes. Selective behaviour was seen before indicating that AOPs can be particularly effective against specific groups of bacteria (Wojtenko, 2001; Alexander *et al.*, 2016; Czekalski *et al.*, 2016) or in some cases select for ARGs and ARB (Luczkiewicz *et al.*, 2011; Lüddeke *et al.*, 2015; Zhuang *et al.*, 2015). Nevertheless, selection for ARGs was not evident here, as decreasing patterns of both relative concentrations per genome (table 6.2) and diversities were seen with all AOPs, including ozonation.

It is noteworthy that all AOPs reduced persistent genes in final effluents to a greater degree than conventional treatment technologies, achieving persistent gene removals between 44.6 % (H_2O_2/UV) and 72.3% (O_3). In contrast, conventional treatment reductions ranged between 16 % (TF) and 35 % (AS). Although ozonation had added value on reducing ARG abundance and diversity, MBRs clearly achieved at least two orders of magnitude higher log removals than the AS system plus ozonation (figure 6.1 & tables 6.1 and 6.2); it is reasonable to expect also lower diversities and persistent genes in MBR permeate despite the unavailability of data due to low DNA biomass. This suggests that ultrafiltration was more effective than oxidation at reducing ARGs from final effluents in this study, showing the importance of biosolids separation in the

treatment. On the other hand, MBRs produce waste that needs careful handling and further treatment, whereas technologies based on oxidation does not require post-treatment of retained solids. These characteristics of each technology should be under consideration in future decisions in water industry.

Similar to Chapter 3, PMA-qPCR and qPCR were used in tandem in Chapter 4 and it was found that detected ARGs are not always in viable cells (table 6.2), which suggests previous genetic assessments of ARGs in AOPs might have overestimated the genes available for HGT. As seen in Chapter 3, the mean proportion of ARGs in viable cells across all samples in influents and effluents of the WWTPs were 0.89 ± 0.75 and 0.84 ± 0.68, respectively, which indicates this overestimation is consequential. A noteworthy related observation is that ozonation and O_3/H_2O_2/UV appear to specifically enhance ARG reductions in viable cells, with the effluent viable to total cell ratio being close to 0.5. A < 0.5 viable/total cells ratio after treatment with chlorination was also observed in the study of Eramo *et al.* (2019) using the same approach, suggesting that disinfection is particularly effective at reducing the viable fraction, nonetheless producing non-viable cells. Although bacteria with broken cells can activate reparation mechanisms and re-produce in post-treated effluents (Alexander *et al.*, 2016; Deng *et al.*, 2019), more accurate investigation is needed about the fate of genes in viable and non-viable host cells after the treatment process (Hong *et al.*, 2018; Hiller *et al.*, 2019). While it is reasonable that any technology that can enhance the die-off of viable cells carrying ARGs will almost certainly reduce HGT of ARGs released in wastewaters.

Overall, the PMA viability test (Chapters 3 and 4) showed that each technology affects differently viable bacteria. For example, ozonation and filtration are particularly effective at reducing viable cells as ARG concentrations in viable cells were statistically lower than in total bacteria (tables 6.1 and 6.2); however, filter type plays important role on the patterns as an increasing trend on the viable to total cells ratio was observed in disc filters discharges versus a decreasing trend in the pile cloth filter discharges. In contrast, secondary settlers did not affect the viable/total cells ratios, implying that this treatment step equally removes viable and non-viable bacteria. Furthermore, data hint that operating parameters in biological systems affect ARGs in viable cells, as seen in viable/total ratios in mixed liquor of AS vs MBR systems, which decreased in MBR and did not change in AS. It is clear, therefore, that each technology and treatment step affect differently both viable and non-viable cells, while data hint the importance

of operating parameters on the total/viable ratio. This is a valuable new information that shows how the viable/total cells ratio evolve within different treatment trains that might help future decisions of water companies on implementing combination of technologies to achieve an optimum viable/total cells ratio, targeting at reductions in the viable fraction and minimising releases of non-viable cells to avoid cells reparation or HGT transformation in receiving waters.

Both AOP studies, the pilot plant (Chapter 4) and bench-scale (Chapter 5), suggest the relative efficacy of the different AOPs depends on the primary oxidative mechanism. For example, the superiority of ozonation over other options is probably due to the presence of two powerful oxidants in the process, O_3 and $^{\bullet}OH$ (Ikehata *et al.*, 2006). By combining H_2O_2/UV with ozonation, improvements in ARG and bacterial cell destruction were expected, given the suite of oxidative species, but no differences were seen, implying the reactive species in ozonation were the main oxidative drivers in ARG removal. Investigating further the H_2O_2/UV system in bench-scale experiments, I found that H_2O_2 dose and contact times are important parameters in treatment, whereas UV irradiation dose was less influential. Although bench- and pilot-scale projects cannot be compared directly, these findings may explain why H_2O_2/UV was not effective at ARG reduction in the pilot plant with a short contact time of 140 sec and H_2O_2 doses up to 9 g/m^3 which were lower than the doses and contact times tested in the bench-scale experiments; while scavenging of H_2O_2 by organic matter present in the matrix or conversion of H_2O_2 to non-radical products (H_2O and O_2) have also been reported as limiting factors in the H_2O_2/UV system (Pham *et al.*, 2012; Yoon *et al.*, 2017).

Given relative costs and performance, alternative AOP options were tested in bench-scale experiments with the aim to replace ozone, UV and/or H_2O_2 which are costly and energy-demanding processes. For this reason, Fe-bearing clay minerals which are sustainable natural materials that promote ROS formation through a Fenton-like process were tested in Chapter 5. The reason why clay minerals were chosen is because specific types of clays have shown antibacterial activity in skin and nosocomial infections (Morrison *et al.*, 2014; Behroozian *et al.*, 2016; Morrison *et al.*, 2016; Otto *et al.*, 2016; Venieri *et al.*, 2020), while they have also been applied *in situ* for remediation of organic contaminants in soil, groundwater and wastewater (Pignatello *et al.*, 2006; Krembs *et al.*, 2010). In this research, the potential of clay minerals for reducing ARGs in real wastewater has been examined for the first time to the best of our knowledge.

Specifically, NAu-1 combined with H_2O_2 or in a reduced state upon contact with O_2 showed greater ARG removal levels compared with UV alone or H_2O_2/UV in the laboratory experiments. As with any oxidation system, H_2O_2/NAu-1 effectiveness highly depended on the oxidant dose and reaction time. Higher doses of H_2O_2 and contact times resulted in greater removal rates (table 6.2). However, in H_2O_2/NAu-1 systems, depletion of H_2O_2 during treatment appeared to be limiting factor in the oxidation process as low removal rates were seen after 24 hrs treatment. Although, NAu-1 alone was not effective at reducing target ARGs, it prevented regrowth of bacteria for 24 hrs showing a bacteriostatic effect.

Reduced NAu-1 achieved higher gene removal rates compared to H_2O_2/NAu-1 and native NAu-1 alone and maintained gene removal potential even after 24 hrs treatment (table 6.2). Although higher contact times resulted in higher removal rates, no statistical differences were observed indicating a very fast reaction even at 30 min contact time. This is promising as it reduces the total reaction time for the treatment. Interestingly, oxygen levels had no effect on the activation of rNAu-1 as similar removal rates were observed in both high and low oxygen levels, which shows an oxidative pathway that have not been considered before leading to a new topic for research.

Although experiments for identifying the oxidative mechanisms of the systems including clay minerals were not conducted as they were beyond the purpose of this study, data here hint that oxidative mechanisms involved in each process are important for reducing ARG abundances. The most plausible mode of action of both rNAu-1 and H_2O_2/NAu-1 was bactericidal activity through production of reactive oxidizing species such as hydroxyl radicals, superoxide anion and ferryl ion (Voegelin and Hug, 2003; Pham *et al.*, 2009). However, additional bacteriostatic activity cannot be ruled out for native NAu-1, as physical attraction between bacterial membranes and clay minerals have shown to disrupt nutrient uptake and waste removal by enveloping the cells, or physically damage the membranes (Wei *et al.*, 2011). Also it has been suggested that clay minerals compete with bacteria for nutrients or provide toxins that affect the bacterial cell regulatory system (Haydel *et al.*, 2008; Williams, 2017).

Overall, the thesis focused on assessing conventional and advanced wastewater treatment technologies at reducing ARG releases into environment and it is aimed to assist future decisions of water industry in the UK at controlling AMR releases in the environment. The UK has established a five-year national action plan on tackling AMR (GOV.UK, 2019) which complies

with global initiatives (WHO, 2015b). According to these, sewage is identified as a ‘major concern’ and implementation of the regulation and monitoring are considered essential for safe drinking water and sewage management locally and globally. Given that AMR in drinking water or wastewater is not regulated currently, the existing regulatory regimes will be evaluated and amended accordingly in the future based on their effectiveness to prevent environmental contamination and any impact on public health. For this purpose, effort is paid on identifying potential AMR sources and risks on humans, animals and the wider environment.

In this context, the main message of this research is that WWTPs are key in reducing ARGs from wastewater if operated correctly and are an effective barrier against AMR spread to the environment. Based on findings from this study, the first step in considering approaches for increasing ARG reductions in WWTPs is how the existing systems are operated, as modifications in operating parameters (e.g. SRT, HRT, flow rates) can improve the performance. Tertiary and quaternary treatment options can enhance ARG removal; however, upgrading the whole system by adding a conventional (e.g. filtration) and/or an advanced technology (e.g. ozonation) is not always necessary, as data shows, and depends on the suite of treatment steps. For instance, GAC and chlorination following an MBR system provided little added value in terms of ARG abundances, whereas ozonation, following an AS system with pre-filtration before the oxidation, increased ARG reductions by over one log removal across the whole system. An economic evaluation was not considered here as it was beyond the objectives of this study; however, it is reasonable that MBRs or AOPs within a treatment system, will increase ARG reductions, and the overall cost. Whether this is justified depends on perceived need for additional treatment in future water legislation and the relative costs to the overall benefits this treatment will have on the environment and public health.

Upgrading the system with new technologies or modifying the treatment process is a decision depending not only in controlling antibiotic resistance bacteria and genes in wastewater. There is a holistic approach on tackling AMR in sewage pathways, including not only ARB and ARGs but also antimicrobials and metals or biocides that co-select for antibiotic resistance as reported in the UK’s five-year national action plan (GOV.UK, 2019). Furthermore, water companies need to consider other factors on re-designing treatment processes including emerging contaminants or by-products formation. For instance, the UK’s regulation on water quality complies with the EU Environmental Quality Standards amended by the Priority Substances Directive (EEC, 2013)

which has introduced a list of priority substances to be monitored with implication in future regulation. Furthermore, by-products formation during treatment is also an important parameter to consider in implementing new technologies, as carcinogenic trihalomethane and bromate production have been reported in processes such as chlorination and ozonation, respectively (Sohn *et al.*, 2004). Finally, having all previous important factors under consideration, the goal of the UK water companies for near-zero emissions by 2050 (EA, 2009) will greatly affect any treatment decisions in the future.

In this context, controlling AMR in sewage forms part of the future challenges the water industry will face and should be handled with a holistic approach. This study aimed to elucidate key characteristics of a wide range of technologies and how these affect the fate of ARGs especially in the viable fraction which promote antibiotic resistance dissemination in the wider environment. The findings of this research provide with direct information for the WWTPs assessed to the industrial sponsor and also contribute to the general knowledge revealing important information for the type of treatment technologies examined. Specifically, it is suggested that wastewater treatment reduce ARG concentrations in final effluents, while revising operating conditions should be the first consideration to improve the performance against ARB and ARGs. Upgrading the treatment systems should be considered as option depending on local needs (e.g. the use of water or sensitivity of receiving environments), ARB/ARGs and priority substances levels reported in future regulations, by-products formation, and the overall carbon footprint. Therefore, rising standards on environmental protection (EEC, 2013) is likely to affect the treatment strategy in the future raising the need for innovative solutions. For this reason, here, it is suggested that alternative advanced treatments, based on sustainable natural materials, such as clay minerals, may be of value to reduce cost and energy consumption with the aim to help water companies to achieve their future goals.

6.2 Recommendations and Future Work

Comparing different treatment technologies provided insight into important factors that influence the performance and capacity of each system against ARB and ARGs; however, this study raises several questions which remain unanswered, therefore, an array of additional work is possible.

A general recommendation is that much of future work should be conducted in a similar way to this study, with collaboration between the academic and industrial sectors. This is recommended to better leverage each sector's expertise and to inform studies according to industrial needs.

6.2.1 *AMR regulation and standardized analytical methods*

ARGs, ARB and antibiotics remain unregulated in water and wastewater legislation, and there is no agreement about how much of these can be safely released into the environment. Establishing safe limits will be complex, time consuming, work involving researchers, industry, government, and stakeholders, however it should be of high priority given the known health hazards of ARB. It is essential to prioritise research on the acute and long-term impact of WWTPs releases on the receiving environments and the public health (Barancheshme and Munir, 2018). The concentration of antibiotics and other antimicrobials should also be included in the regulatory discussion as they may affect AMR propagation (Hiller *et al.*, 2019).

Robust standardized methods analysing antibiotic resistance levels and estimating the performance of different treatments at reducing AMR levels are needed for an accurate evaluation of antibiotic resistance levels in different environments. This is a prerequisite for the risk assessment processes that will lead decisions for future regulation. Despite the large amount of accurate analytical approaches that exist, comparison among studies is challenging due to different methods used. Even when the same analytical method is used (e.g. qPCR) comparisons can be difficult due to differences in qPCR protocols, gene lengths and primer targets (Michael-Kordatou *et al.*, 2018; Hiller *et al.*, 2019). Therefore, standard analytical approaches are needed so that comparisons among studies are possible. This will help future regulation of AMR in the water and wastewater and further evaluation of the efficacy of existing and new treatment technologies.

Regarding methodological approaches, the use of PMA to distinguish between viable and non-viable bacteria is a novel aspect of this thesis that should be more widely implemented by water companies and researchers in AMR as it represents a significant improvement over current techniques. This work has shown that a simple quantification of ARGs, without differentiating viable from total bacteria, overestimates transmissible ARGs discharged from WWTPs to the environment. This has implications to HGT in receiving environments as it shows that the available ARB load for conjugation is lower than what is estimated without using PMA.

Although knowing the abundances of viable ARB is essential, further research is needed to explore how non-viable cells influence ARG dissemination in environmental systems, as data here suggest that more than half ARGs released from WWTPs are in non-viable cells. It is possible that bacteria with compromised membranes might contribute to ARG dissemination, either by reverting to a viable state through repair mechanisms or by transformation. As Hong *et al.* (2018) discussed, information regarding transformation in environmental systems is limited, therefore further research is needed to elucidate whether non-viable cells contribute to the problem by monitoring recovering rates in non-viable cells (e.g. in storage tanks for reclaimed water) or any possible transformation mechanism using model bacteria (*E. coli*) carrying ARGs in lab-scale experiments.

6.2.2 *Optimising WWTP performance*

Although there is no regulation of AMR currently, understanding further key factors that affect releases of ARB and ARG loads in discharged effluents is beneficial as it will help water companies prepare for any future adjustment of their treatment strategies to comply with water legislation.

A natural progression of this project is to establish how seasonality and other operating factors affect ARG and ARB reductions in WWTPs, as data here hint that antibiotic resistance is affected by the overall performance of the treatment system. Larger sampling campaigns across multiple seasons and with special focus on operating parameters and any correlation they may have with AMR are recommended to study this in greater detail. Additionally, there may be a need to optimise operational parameters (e.g. HRT, SRT, temperature, water flow, biomass concentration) for AMR reduction, as suggested by Barancheshme and Munir (2018). Eventually, revising operating conditions with seasons, for example, could overcome any deficiencies caused in the performance of a WWTP by climatic conditions or temporal oscillations on antibiotic usage (Golovko *et al.*, 2014).

Further research in material characteristics, pore size and operating factors of filters is recommended, as filtration appears to significantly reduce viable cells and may be more cost effective than advanced oxidation. It is also a widely used and understood technology, therefore quantifying its benefits in AMR reduction are well justified.

6.2.3 *Optimising AOPs*

Rising environmental standards and the inclusion of emerging pollutants in future legislation imply that water companies may need to modify treatment strategies in order to comply with new regulations. In this context, re-designing the whole treatment system is likely to be prohibitively expensive, and revising operating parameters may not be sufficient to meet potential new regulation, therefore, upgrading existing WWTPs with advanced treatments will be necessary, especially if the treated water will be reused.

Ozonation is already used as an additional step in wastewater treatment practice and this study revealed the potential it has at controlling a wide range of ARGs at conventional doses. Furthermore, it was revealed for the first time that ozonation is very effective at reducing ARGs in the viable fraction. Bearing in mind that the use of ozonation is justified when the treated water will be reused, I recommend studying the fate of ARGs carried in viable cells to storage tanks and also whether the presence of non-viable cells will affect bacterial regrowth.

H_2O_2/UV has been intensively examined the last decade and has been conventionally applied in a WWTP in Spain (Rodríguez-Chueca *et al.*, 2019). This AOP option has shown potential both against AMR (Michael-Kordatou *et al.*, 2018) and emerging contaminants (Rodriguez-Narvaez *et al.*, 2017) indicating a possible good option for the water industry. However, the pilot plant data in this study suggest poor performance and no synergistic effect at reducing ARGs, even when combined with ozonation. Lab experiments showed that H_2O_2 concentrations and contact time may be key to improving the effectiveness of this method, suggesting that contact time may have been the limiting factor in our pilot plant system. I recommend further pilot testing to study the role of contact time in ARGs reduction performance, with the aim of ultimately establishing more adequate contact times for use in WWTPs.

6.2.4 *Fe-bearing clay minerals: emerging potential*

One of the main concerns for the water industry when implementing advanced treatment is the high capital and operating costs. Conducting economic studies on different AOP options is challenging as related costs depends on a variety of different factors such as overall pollutants target, influent characteristics, treatment scale, type of technologies applied, water quality and

environmental standards, energy costs and even personnel salaries that varies among countries and/or region (Krzeminski *et al.*, 2019).

Considering both expenses and the goal for near-zero carbon emissions in the UK water industry, companies should invest in innovation and new, cost-effective, technologies. The potential of clay minerals at reducing ARGs from real wastewater should be studied further as the findings of the first experiments are promising. However, several questions remain un-answered and some ideas for future work are summarised below.

As data revealed, concentrations of H_2O_2 and NAu-1 are important for the treatment process. Depletion of H_2O_2 in the system can compromise treatment performance, suggesting that continuous feed with oxidant could be essential for the system. Further, 0.5 g/L NAu-1 was not able to react with the total amount of H_2O_2 at an initial concentration of 340 g/m^3. Therefore, further experiments should establish an optimum oxidant (H_2O_2) to catalyst (NAu-1) ratio, as well as the feed frequency needed to maintain this ratio constant.

An advantage of clay minerals is that it is a recyclable catalyst and can be reused. This study found no metal releases after treatment, an indication that the clay's structure did not change. I recommend further experiments to monitor the effect of clay on water quality indicators, as well to test potential changes to ARG removal rates after clay reuse. This could be combined with material characterisation such as Mössbauer spectroscopy to give insight in possible material degradation during reuse cycles.

Systems using reduced NAu-1 achieved higher removal rates than H_2O_2/NAu-1 with equal contact times. However, reduced clay minerals are more complex and further study is needed on how factors such as oxygen levels and activation mechanisms affect treatment performance. On the other hand, H_2O_2/NAu-1 or NAu-1 systems can have a more direct application in industrial projects because of their greater simplicity. Considering this I recommend further study of H_2O_2/NAu-1 or NAu-1 systems treatment options with more sophisticated microbiological/molecular and physicochemical analyses such as those executed in other parts of this project (e.g. using HT-qPCR and PMA) in order to characterise these systems in greater detail than was presented here.

The first experiments were conducted with NAu-1 in suspension, implying that in such case, an additional step would be needed to collect the catalyst from the treated water. In a real treatment

system this would potentially increase cost and/or overall treatment contact time i.e. in case a settler is required; for this reason, fixation of clay minerals in a filter column is suggested as a more appropriate solution. Further research should, therefore, include the whole design and characterisation of a clay treatment system, as well as testing the effect of physicochemical and microbiological parameters on performance.

Appendix A

A.1. PMA method development and glycerol testing

PMA was used to segregate cells that were presumptively viable vs. non-viable. In the method, glycerol was used as the buffer to preserve samples during storage at -20 ^{o}C. Therefore, preliminary experiments were performed to assess the integrity of cells preserved in storage. To do this, 400 mL of primary settled sewage was collected from a WWTP in NE England, which was segregated into four aliquots, 100 mL each. The sub-samples were centrifuged at 4200 rpm for 20 min and centrates decanted. After that, 20% glycerol was added to two samples until level at 10 mL. The pellets were resuspended and left 30 minutes at room temperature before storage at -20 ^{o}C. The other two samples were processed immediately. One sample was treated with PMA, using the method described in Section 3.2.5, whereas the other was temporarily stored at 4 ^{o}C. DNA then was extracted from both samples and stored at -20 ^{o}C until further use.

After three days, the original samples preserved in glycerol were thawed and centrifuged at 4200 rpm for 20 min. The centrate was decanted and the above process was performed with and without PMA. The resulting DNA was quantified and tested for quality, and qPCR was performed on all four samples, amplifying the 16S rRNA gene. Absolute concentrations were determined for each sample and statistical analysis was performed as described in Section 3.2.6.

As presented in Table A.1, non-significant differences were detected between samples processed the same day of collection and samples preserved in 20 % glycerol for three days, and the same trend was seen for samples either treated with PMA or not. These data confirmed that glycerol retained intact membranes, suggesting that samples could be safely stored at -20 ^{o}C. However, statistically significant differences were observed between samples with PMA versus without PMA treatment in both fresh and glycerol-added samples, showing that glycerol did not impact the influence of PMA on the samples.

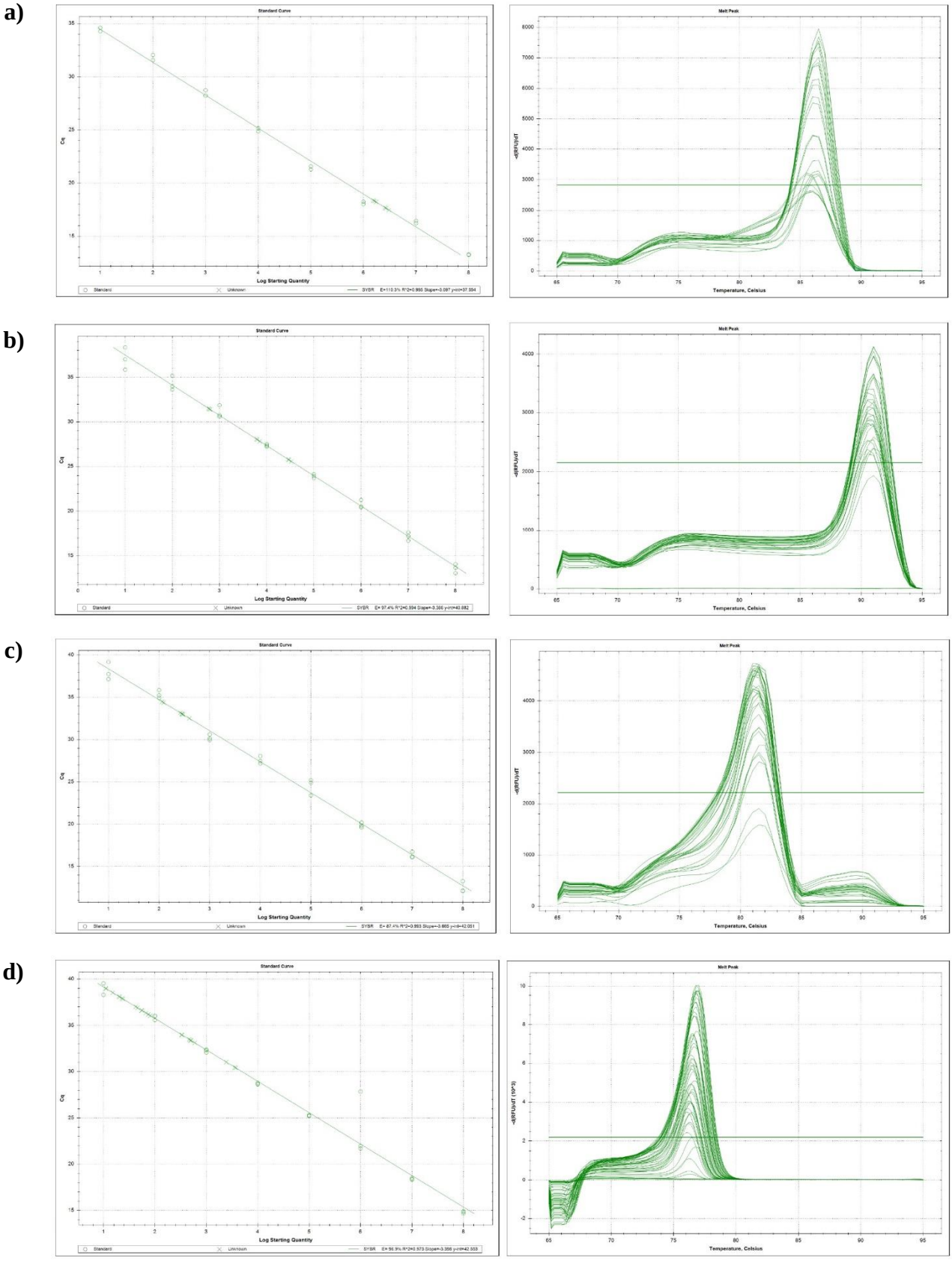

Figure A. 1 qPCR standaand rd curves and melt curves for **a)** 16s rRNA, **b)** *int*1, **c)** *bla*$_{\text{OXA-10}}$, and **d)** *tet*M.

e)

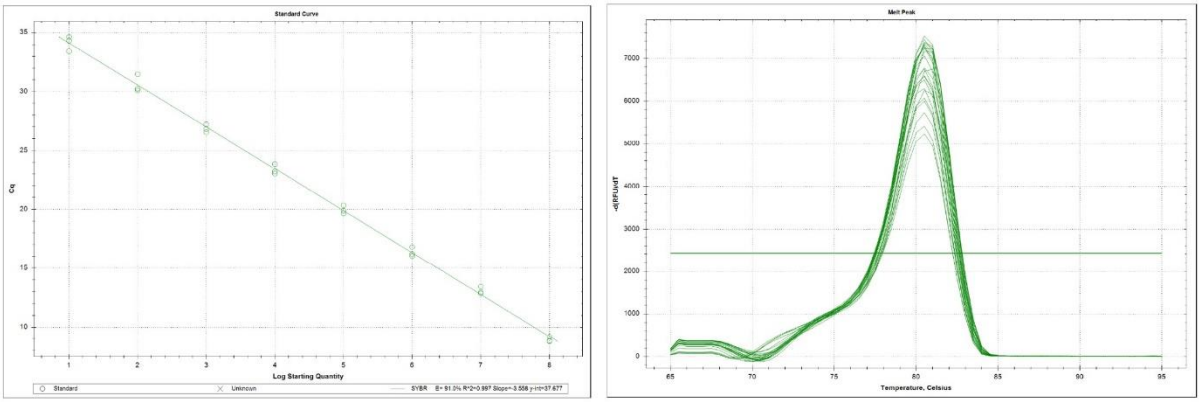

Figure A.1(cont.) As above for **e)** *tet*Q gene.

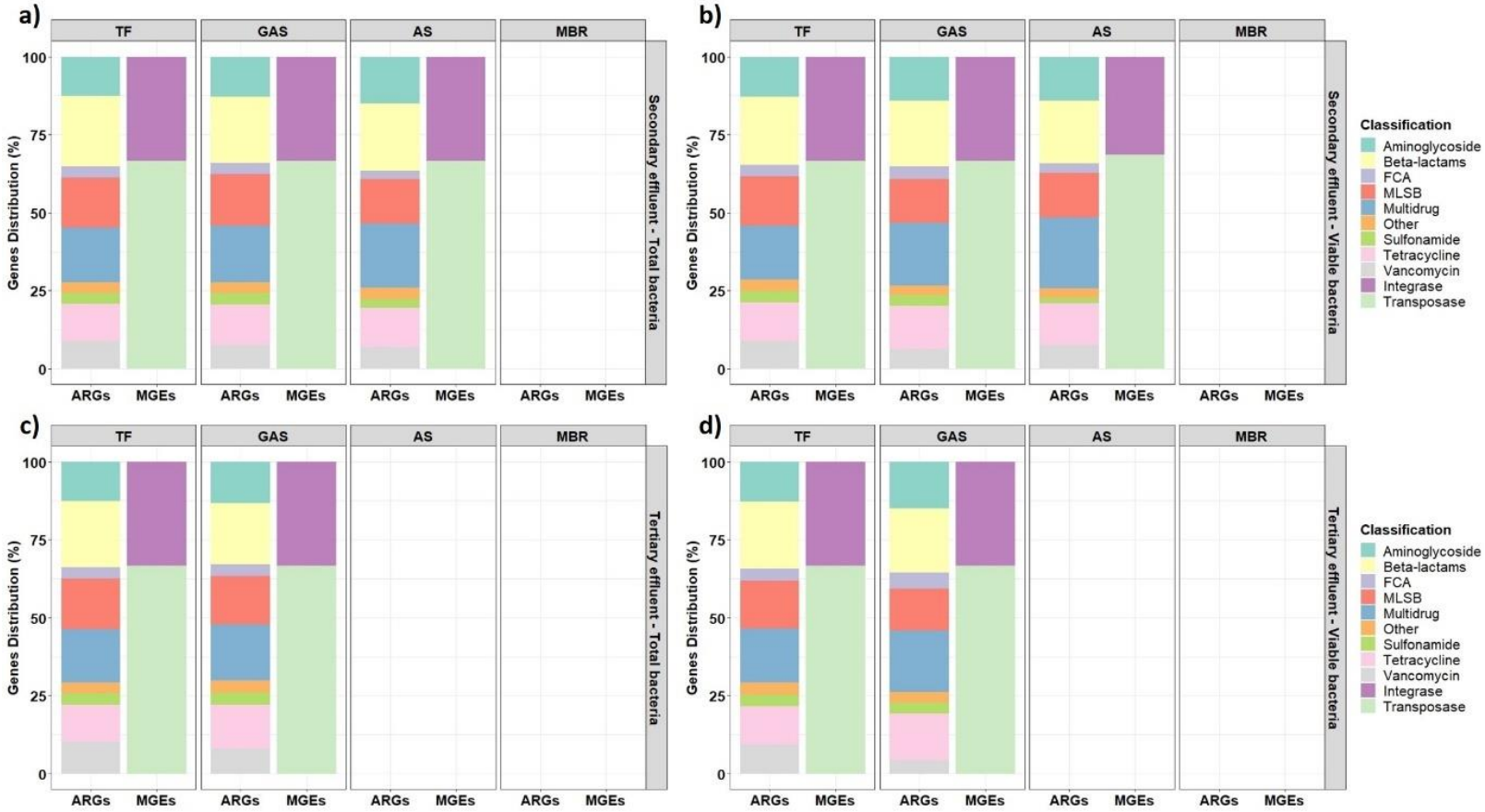

Figure A. 2 Gene distribution in secondary and tertiary effluents of a-c) total bacteria, and b-d) viable bacteria. Secondary effluent refers to TF discharge, GAS discharge, secondary clarifier effluent (AS) and MBR permeate, and tertiary effluent present the disc filter discharge (TF), pile cloth filter discharge (GAS) and GAC & chlorination discharge (MBR).

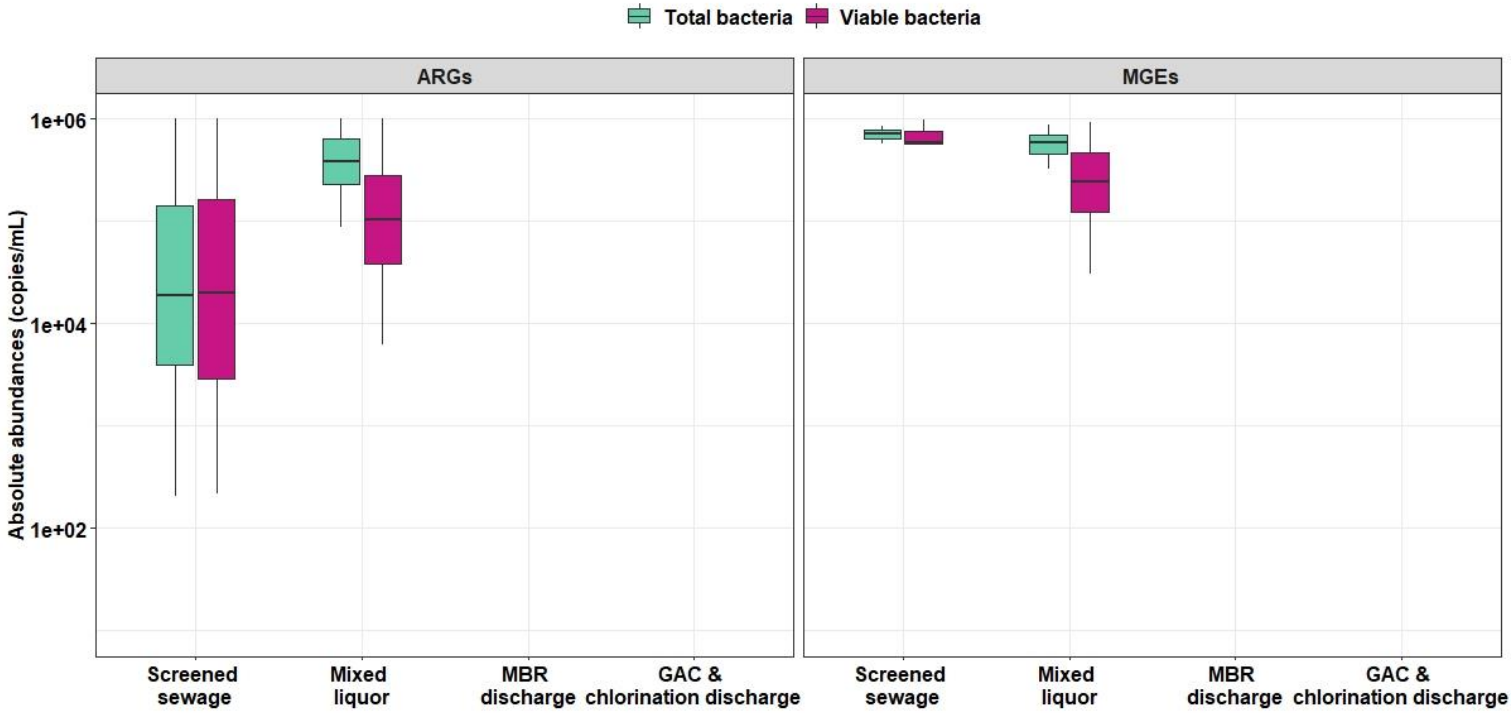

Figure A. 3 Absolute abundances grouped in ARGs and MGEs per treatment step in MBR WWTP. Boxplots include absolute concentrations of all ARGs and MGEs per treatment stage in both total and viable cells. Boxes represent the first quartile and third quartile of the data, the vertical line shows the median, whiskers extend from each quartile to minimum and maximum data points and outliers are depicted with dots.

Table A. 1 Primary settled sewage absolute concentrations (16s rRNA) of samples processed the day of collection (*fresh samples*) or after three days of storage in glycerol at -20 ºC (*glycerol samples*). Samples treated either with PMA or not are presented, as well. Statistical differences between pair samples are denoted with a significance cut-off of p = 0.05.

Fresh samples		**Glycerol samples**		**p-value**
Treated with no PMA	$1.35 \times 10^{10} \pm 8.33 \times 10^{8}$	Treated with no PMA	$1.20 \times 10^{10} \pm 1.76 \times 10^{9}$	0.29
Treated with PMA	$7.89 \times 10^{9} \pm 4.20 \times 10^{8}$	Treated with PMA	$7.17 \times 10^{9} \pm 8.72 \times 10^{7}$	0.52
Samples treated with no PMA		**Samples treated with PMA**		
Fresh samples	$1.35 \times 10^{10} \pm 8.33 \times 10^{8}$	Fresh samples	$7.89 \times 10^{9} \pm 4.20 \times 10^{8}$	< 0.01
In glycerol samples	$1.20 \times 10^{10} \pm 1.76 \times 10^{9}$	In glycerol samples	$7.17 \times 10^{9} \pm 8.72 \times 10^{7}$	< 0.01

Table A. 2 Water quality parameters of all treatment steps per WWTP. Values are averages ± standard deviation from three sampling days.

Treatment stage	*TOC (mg/L)	Conductivity (µS/cm)	pH	Suspended Solids (mg/L)	Turbidity (FTU)	**DOC (mg/L)
Primary settled sewage -TF	21.6±2.0	934.0±175.5	7.8±0.1	104.7±12.0	59.5±11.8	20.1±1.5
Trickling filter discharge -TF	13.1±1.3	829.6±130.8	7.2±0.0	50.5±17	27.0±14.9	10.6±0.5
Disc filter discharge - TF	11.3±1.9	801.0±70.9	7.3±0.1	7±3.3	4.2±0.6	11.4±2.4
Primary settled sewage – GAS	55. ±3.8	1127.6±20.5	7.6±0.1	153.3±28.7	95.3±18.9	35.1±1.4
Granular activated sludge discharge - GAS	8.6±1.3	941.3±23.0	7.0±0.1	11.0±9.2	3.9 ±2.2	7.9±1.3
Pile cloth filter discharge - GAS	8.0±0.7	930. ±32.9	7.3±0.0	3.5±2.1	0.8 ±0.4	8.7±2.7
Primary settled sewage - AS	32.8±5.4	1066.3±17.6	7.5±0.2	149.3±34.1	60 ±15.4	25.2±6.4
Biological reaction tank effluent - AS	42.8±4.6	838.3±159.9	6.8±0.0	5040.0±492.7	2685±428.7	26.0±8.2

Treatment stage	*TOC (mg/L)	Conductivity (µS/cm)	pH	Suspended Solids (mg/L)	Turbidity (FTU)	**DOC (mg/L)
Secondary clarifier effluent - AS	7.0±1.4	875.3±39.3	7.2±0.2	13±4.8	6.2 ±2.2	5.9±1.3
Return activated sludge - AS	75.5±14.5	1066.0±19.7	6.7±0.1	10593.3±1823	>4000.00	32.4±2.0
Screened sewage - MBR	51.4±7.4	1088.3±39.1	7.3±0.1	102.7±24.7	64.7±9.0	40.1±5.8
Biological reaction tank effluent - MBR	43.9±13.3	916.7±38.7	6.9±0.0	7300±367.1	> 4000.00	16.0±4.0
Membrane bioreactor permeate – MBR	6.5±0.9	900.7±17.9	7.2±0.1	<1.9	<0.09	5.8±0.5
Granular activated carbon & chlorination discharge - MBR	5.1±0.4	897.3±22.0	7.3±0.1	3.5±0.0	<0.09	5.1±0.4

*TOC: Total Organic Carbon.
**DOC: Dissolved Organic Carbon

Table A. 3 Water quality parameters of all treatment steps per WWTP. Values are averages ± standard deviation from three sampling days.

Treatment stage	Alkalinity (mg/L)	Ammoniacal Nitrogen (mg/L)	Manganese (µg/L)	NO_3^--N (mg/L)	NO_2^--N (mg/L)	Total Oxidised Nitrogen-N (mg/L)
Primary settled sewage -TF	273±16.5	23.6±4.8	56.0±22.7	3.0±1.2	1.8±0.9	4.7±1.8
Trickling filter discharge - TF	131±11.4	3.4±1.6	39.2±21.2	19.7±3.3	0.34±0.01	20.0±3.3
Disc filter discharge - TF	119±7	3.4±1.6	15.1±1.2	20.3±4.7	0.32±0.05	20.6±4.7
Primary settled sewage – GAS	415±14	30.7±3.9	39.6±1.2	<0.2	0.33±0.02	1.1±1.1
Granular activated sludge discharge - GAS	272±17.7	3.0 ±0.0	29.26±7.0	8.1±4.9	0.28±0.33	8.4±4.7
Pile cloth filter discharge - GAS	281±18.7	1.6±1.8	30.3±3.8	4.7±2.9	0.24±0.32	4.9±3.0
Primary settled sewage - AS	375±7.2	29.1±4.2	66.2±6.5	<0.2	<0.0080	<0.2
Biological reaction tank effluent - AS	242±3.8	0.07±0.07	1423±163.8	8 ±4.2	0.14±0.02	8.1±4.1
Secondary clarifier effluent – AS	244±18.5	0.09±0.07	135.4±131	10.5±0.3	0.10±0.06	10.6±0.3

Treatment stage	Alkalinity (mg/L)	Ammoniacal Nitrogen (mg/L)	Manganese (µg/L)	NO_3^--N (mg/L)	NO_2^--N (mg/L)	Total Oxidised Nitrogen-N (mg/L)
Return activated sludge - AS	267±12.0	0.46±0.4	3029±583.8	2.9±2.1	0.30±0.24	3.2±2.3
Screened sewage - MBR	346±30.3	32.7±5.5	32.3±2.4	< 0.2	1.05±0.92	1.6±1.0
Biological reaction tank effluent - MBR	210±36.0	1.5±1.7	1798±186.6	12.9±4.9	0.20±0.20	13.1±4.7
Membrane bioreactor permeate – MBR	157±3.5	<0.02	3.133±2.4	21.9±2.9	<0.0080	22.0±2.9
GAC & chlorination discharge - MBR	161±1.52	<0.02	0.8±0.0	21.1±3.6	<0.0080	21.1±3.6

Table A. 4 HT-qPCR primer list used in this study.

Gene Name	Forward Primer	Reverse Primer	Classification
16S rRNA	GGGTTGCGCTCGTTGC	ATGGYTGTCGTCAGCTCGTG	
catA1	GGGTGAGTTTCACCAGTTTTGATT	CACCTTGTCGCCTTGCGTATA	FCA
catB3	GCACTCGATGCCTTCCAAAA	AGAGCCGATCCAAACGTCAT	FCA
catB8	CACTCGACGCCTTCCAAAG	CCGAGCCTATCCAGACATCATT	FCA
cfr	GCAAAATTCAGAGCAAGTTACGAA	AAAATGACTCCCAACCTGCTTTAT	FCA
cmlA1-01	TAGGAAGCATCGGAACGTTGAT	CAGACCGAGCACGACTGTTG	FCA
cmlA1-02	AGGAAGCATCGGAACGTTGA	ACAGACCGAGCACGACTGTTG	FCA
cmx(A)	GCGATCGCCATCCTCTGT	TCGACACGGAGCCTTGGT	FCA
floR	ATTGTCTTCACGGTGTCCGTTA	CCGCGATGTCGTCGAACT	FCA
qnrA	AGGATTTCTCACGCCAGGATT	CCGCTTTCAATGAAACTGCAA	FCA
aac	CCCTGCGTTGTGGCTATGT	TTGGCCACGCCAATCC	Aminoglycoside
aac(6')I1	GACCGGATTAAGGCCGATG	CTTGCCTTGATATTCAGTTTTTATAACCA	Aminoglycoside
aac(6')-Ib(aka aacA4)-02	CGTCGCCGAGCAACTTG	CGGTACCTTGCCTCTCAAACC	Aminoglycoside
aac(6')-Ib(aka aacA4)-01	GTTTGAGAGGCAAGGTACCGTAA	GAATGCCTGGCGTGTTTGA	Aminoglycoside
aac(6')-Ib(aka aacA4)-03	AGAAGCACGCCCGACACTT	GCTCTCCATTCAGCATTGCA	Aminoglycoside
aac(6')-II	CGACCCGACTCCGAACAA	GCACGAATCCTGCCTTCTCA	Aminoglycoside
aac(6')-Iy	GCTTTGCGGATGCCTCAAT	GGAGAACAAAAATACCTTCAAGGAAA	Aminoglycoside
aacA/aphD	AGAGCCTTGGGAAGATGAAGTTT	TTGATCCATACCATAGACTATCTCATCA	Aminoglycoside
aacC	CGTCACTTATTCGATGCCCTTAC	GTCGGGCGCGGCATA	Aminoglycoside
aacC1	GGTCGTGAGTTCGGAGACGTA	GCAAGTTCCCGAGGTAATCG	Aminoglycoside
aacC2	ACGGCATTCTCGATTGCTTT	CCGAGCTTCACGTAAGCATTT	Aminoglycoside
aacC4	CGGCGTGGGACACGAT	AGGGAACCTTTGCCATCAACT	Aminoglycoside
aadA-01	GTTGTGCACGACGACATCATT	GGCTCGAAGATACCTGCAAGAA	Aminoglycoside
aadA-02	CGAGATTCTCCGCGCTGTA	GCTGCCATTCTCCAAATTGC	Aminoglycoside
aadA1	AGCTAAGCGCGAACTGCAAT	TGGCTCGAAGATACCTGCAA	Aminoglycoside
aadA-1-01	AAAAGCCCGAAGAGGAACTTG	CATCTTTCACAAAGATGTTGCTGTCT	Aminoglycoside
aadA-1-02	CGGAATTGAAAAAACTGATCGAA	ATACCGGCTGTCCGTCATTT	Aminoglycoside

Gene Name	Forward Primer	Reverse Primer	Classification
aadA2-01	ACGGCTCCGCAGTGGAT	GGCCACAGTAACCAACAAATCA	Aminoglycoside
aadA2-02	CTTGTCGTGCATGACGACATC	TCGAAGATACCCGCAAGAATG	Aminoglycoside
aadA2-03	CAATGACATTCTTGCGGGTATC	GACCTACCAAGGCAACGCTATG	Aminoglycoside
aadA5-01	ATCACGATCTTGCGATTTTGCT	CTGCGGATGGGCCTAGAAG	Aminoglycoside
aadA5-02	GTTCTTGCTCTTGCTCGCATT	GATGCTCGGCAGGCAAAC	Aminoglycoside
aadA9-01	CGCGGCAAGCCTATCTTG	CAAATCAGCGACCGCAGACT	Aminoglycoside
aadA9-02	GGATGCACGCTTGGATGAA	CCTCTAGCGGCCGGAGTATT	Aminoglycoside
aadD	CCGACAACATTTCTACCATCCTT	ACCGAAGCGCTCGTCGTATA	Aminoglycoside
aadE	TACCTTATTGCCCTTGGAAGAGTTA	GGAACTATGTCCCTTTTAATTCTACAATCT	Aminoglycoside
aph	TTTCAGCAAGTGGATCATGTTAAAAT	CCAAGCTGTTTCCACTGTTTTTC	Aminoglycoside
aph(2')-Id-02	TAAGGATATACCGACAGTTTTGGAAA	TTTAATCCCTCTTCATACCAATCCATA	Aminoglycoside
aph(2')-Id-01	TGAGCAGTATCATAAGTTGAGTGAAAAG	GACAGAACAATCAATCTCTATGGAATG	Aminoglycoside
aph6ia	CCCATCCCATGTGTAAGGAAA	GCCACCGCTTCTGCTGTAC	Aminoglycoside
aphA1(aka kanR)	TGAACAAGTCTGGAAAGAAATGCA	CCTATTAATTTCCCCTCGTCAAAAA	Aminoglycoside
spcN-01	AAAAGTTCGATGAAACACGCCTAT	TCCAGTGGTAGTCCCCGAATC	Aminoglycoside
spcN-02	CAGAATCTTCCTGAAAAGTTTGATGAA	CGCAGACACGCCGAATC	Aminoglycoside
str	AATGAGTTTTGGAGTGTCTCAACGTA	AATCAAAACCCCTATTAAAGCCAAT	Aminoglycoside
strA	CCGGTGGCATTTGAGAAAAA	GTGGCTCAACCTGCGAAAAG	Aminoglycoside
strB	GCTCGGTCGTGAGAACAATCT	CAATTTCGGTCGCCTGGTAGT	Aminoglycoside
ampC/blaDHA	TGGCCGCAGCAGAAAGA	CCGTTTTATGCACCCAGGAA	β-lactams
ampC-01	TGGCGTATCGGGTCAATGT	CTCCACGGGCCAGTTGAG	β-lactams
ampC-02	GCAGCACGCCCCGTAA	TGTACCCATGATGCGCGTACT	β-lactams
ampC-04	TCCGGTGACGCGACAGA	CAGCACGCCGGTGAAAGT	β-lactams
ampC-05	CTGTTCGAGCTGGGTTCTATAAGTAAA	CAGTATCTGGTCACCGGATCGT	β-lactams
ampC-06	CCGCTCAAGCTGGACCATAC	CCATATCCTGCACGTTGGTTT	β-lactams
ampC-07	CCGCCCAGAGCAAGGACTA	GCTCGACTTCACGCCGTAAG	β-lactams
ampC-09	CAGCCGCTGATGAAAAAATATG	CAGCGAGCCCACTTCGA	β-lactams
bla1	GCAAGTTGAAGCGAAAGAAAAGA	TACCAGTATCAATCGCATATACACCTAA	β-lactams
bla-ACC-1	CACACAGCTGATGGCTTATCTAAAA	AATAAACGCGATGGGTTCCA	β-lactams
blaCMY	CCGCGGCGAAATTAAGC	GCCACTGTTTGCCTGTCAGTT	β-lactams
blaCMY2-01	AAAGCCTCATGGGTGCATAAA	ATAGCTTTTGTTTGCCAGCATCA	β-lactams

Gene Name	Forward Primer	Reverse Primer	Classification
blaCMY2-02	GCGAGCAGCCTGAAGCA	CGGATGGGCTTGTCCTCTT	β-lactams
blaCTX-M-04	CTTGGCGTTGCGCTGAT	CGTTCATCGGCACGGTAGA	β-lactams
blaCTX-M-01	GGAGGCGTGACGGCTTTT	TTCAGTGCGATCCAGACGAA	β-lactams
blaCTX-M-02	GCCGCGGTGCTGAAGA	ATCGGATTATAGTTAACCAGGTCAGATTT	β-lactams
blaCTX-M-03	CGATACCACCACGCCGTTA	GCATTGCCCAACGTCAGATT	β-lactams
blaCTX-M-05	GCGATAACGTGGCGATGAAT	GTCGAGACGGAACGTTTCGT	β-lactams
blaCTX-M-06	CACAGTTGGTGACGTGGCTTAA	CTCCGCTGCCGGTTTTATC	β-lactams
blaGES	GCAATGTGCTCAACGTTCAAG	GTGCCTGAGTCAATTCTTTCAAAG	β-lactams
bla-L1	CACCGGGTTACCAGCTGAAG	GCGAAGCTGCGCTTGTAGTC	β-lactams
blaMOX/blaCMY	CTATGTCAATGTGCCGAAGCA	GGCTTGTCCTCTTTCGAATAGC	β-lactams
blaIMP-02	AAGGCAGCATTTCCTCTCATTTT	GGATAGATCGAGAATTAAGCCACTCT	β-lactams
blaIMP-01	AACACGGTTTGGTGGTTCTTGTA	GCGCTCCACAAACCAATTG	β-lactams
blaOCH	GGCGACTTGCGCCGTAT	TTTTCTGCTCGGCCATGAG	β-lactams
blaOKP	GCCGCCATCACCATGAG	GGTGACGTTGTCACCGATCTG	β-lactams
blaOXA1/blaOXA30	CGGATGGTTTGAAGGGTTTATTAT	TCTTGGCTTTTATGCTTGATGTTAA	β-lactams
blaOXA10-01	CGCAATTATCGGCCTAGAAACT	TTGGCTTTCCGTCCCATTT	β-lactams
blaOXA10-02	CGCAATTATCGGCCTAGAAACT	TTGGCTTTCCGTCCCATTT	β-lactams
blaOXY	CGTTCAGGCGGCAGGTT	GCCGCGATATAAGATTTGAGAATT	β-lactams
blaPAO	CGCCGTACAACCGGTGAT	GAAGTAATGCGGTTCTCCTTTCA	β-lactams
blaPER	TGCTGGTTGCTGTTTTTGTGA	CCTGCGCAATGATAGCTTCAT	β-lactams
blaPSE	TTGTGACCTATTCCCCTGTAATAGAA	TGCGAAGCACGCATCATC	β-lactams
blaROB	GCAAAGGCATGACGATTGC	CGCGCTGTTGTCGCTAAA	β-lactams
blaSFO	CCGCCGCCATCCAGTA	GGGCCGCCAAGATGCT	β-lactams
blaSHV-01	TCCCATGATGAGCACCTTTAAA	TTCGTCACCGGCATCCA	β-lactams
blaSHV-02	CTTTCCCATGATGAGCACCTTT	TCCTGCTGGCGATAGTGGAT	β-lactams
blaTEM	AGCATCTTACGGATGGCATGA	TCCTCCGATCGTTGTCAGAAGT	β-lactams
blaTLA	ACACTTTGCCATTGCTGTTTATGT	TGCAAATTTCGGCAATAATCTTT	β-lactams
blaVEB	CCCGATGCAAAGCGTTATG	GAAAGATTCCCTTTATCTATCTCAGACAA	β-lactams
blaVIM	GCACTTCTCGCGGAGATTG	CGACGGTGATGCGTACGTT	β-lactams
blaZ	GGAGATAAAGTAACAAATCCAGTTAGATATGA	TGCTTAATTTTCCATTTGCGATAAG	β-lactams
cepA	AGTTGCGCAGAACAGTCCTCTT	TCGTATCTTGCCCGTCGATAAT	β-lactams

Gene Name	Forward Primer	Reverse Primer	Classification
cfiA	GCAGCGTTGCTGGACACA	GTTCGGGATAAACGTGGTGACT	β-lactams
cfxA	TCATTCCTCGTTCAAGTTTTCAGA	TGCAGCACCAAGAGGAGATGT	β-lactams
cphA-01	GCGAGCTGCACAAGCTGAT	CGGCCCAGTCGCTCTTC	β-lactams
cphA-02	GTGCTGATGGCGAGTTTCTG	GGTGTGGTAGTTGGTGTTGATCAC	β-lactams
fox5	GGTTTGCCGCTGCAGTTC	GCGGCCAGGTGACCAA	β-lactams
mecA	GGTTACGGACAAGGTGAAATACTGAT	TGTCTTTTAATAAGTGAGGTGCGTTAATA	β-lactams
NDM1	ATTAGCCGCTGCATTGAT	CATGTCGAGATAGGAAGTG	β-lactams
pbp	CCGGTGCCATTGGTTTAGA	AAAATAGCCGCCCCAAGATT	β-lactams
pbp2x	TTTCATAAGTATCTGGACATGGAAGAA	CCAAAGGAAACTTGCTTGAGATTAG	β-lactams
Pbp5	GGCGAACTTCTAATTAATCCTATCCA	CGCCGATGACATTCTTCTTATCTT	β-lactams
penA	AGACGGTAACGTATAACTTTTTGAAAGA	GCGTGTAGCCGGCAATG	β-lactams
IS613	AGGTTCGGACTCAATGCAACA	TTCAGCACATACCGCCTTGAT	MGEs/Transposase
tnpA-01	CATCATCGGACGGACAGAATT	GTCGGAGATGTGGGTGTAGAAAGT	MGEs/Transposase
tnpA-02	GGGCGGGTCGATTGAAA	GTGGGCGGGATCTGCTT	MGEs/Transposase
tnpA-03	AATTGATGCGGACGGCTTAA	TCACCAAACTGTTTATGGAGTCGTT	MGEs/Transposase
tnpA-04	CCGATCACGGAAAGCTCAAG	GGCTCGCATGACTTCGAATC	MGEs/Transposase
tnpA-05	GCCGCACTGTCGATTTTTATC	GCGGGATCTGCCACTTCTT	MGEs/Transposase
tnpA-07	GAAACCGATGCTACAATATCCAATTT	CAGCACCGTTTGCAGTGTAAG	MGEs/Transposase
Tp614	GGAAATCAACGGCATCCAGTT	CATCCATGCGCTTTTGTCTCT	MGEs/Transposase
carB	GGAGTGAGGCTGACCGTAGAAG	ATCGGCGAAACGCACAAA	MLSB
ereA	CCTGTGGTACGGAGAATTCATGT	ACCGCATTCGCTTTGCTT	MLSB
ereB	GCTTTATTTCAGGAGGCGGAAT	TTTTAAATGCCACAGCACAGAATC	MLSB
erm(34)	GCGCGTTGACGACGATTT	TGGTCATACTCGACGGCTAGAAC	MLSB
erm(35)	TTGAAAACGATGTTGCATTAAGTCA	TCTATAATCACAACTAACCACTTGAACGT	MLSB
erm(36)	GGCGGACCGACTTGCAT	TCTGCGTTGACGACGGTTAC	MLSB
ermA	TTGAGAAGGGATTTGCGAAAAG	ATATCCATCTCCACCATTAATAGTAAACC	MLSB
ermA/ermTR	ACATTTTACCAAGGAACTTGTGGAA	GTGGCATGACATAAACCTTCATCA	MLSB
ermB	TAAAGGGCATTTAACGACGAAACT	TTTATACCTCTGTTTGTTAGGGAATTGAA	MLSB
ermC	TTTGAAATCGGCTCAGGAAAA	ATGGTCTATTTCAATGGCAGTTACG	MLSB
ermF	CAGCTTTGGTTGAACATTTACGAA	AAATTCCTAAAATCACAACCGACAA	MLSB
ermJ/ermD	GGACTCGGCAATGGTCAGAA	CCCCGAAACGCAATATAATGTT	MLSB

Gene Name	Forward Primer	Reverse Primer	Classification
ermK-01	GTTTGATATTGGCATTGTCAGAGAAA	ACCATTGCCGAGTCCACTTT	MLSB
ermK-02	GAGCCGCAAGCCCCTTT	GTGTTTCATTTGACGCGGAGTAA	MLSB
ermT-01	GTTCACTAGCACTATTTTTAATGACAGAAGT	GAAGGGTGTCTTTTTAATACAATTAACGA	MLSB
ermT-02	GTAAAATCCCTAGAGAATACTTTCATCCA	TGAGTGATATTTTTGAAGGGTGTCTT	MLSB
ermX	GCTCAGTGGTCCCCATGGT	ATCCCCCCGTCAACGTTT	MLSB
ermY	TTGTCTTTGAAAGTGAAGCAACAGT	TAACGCTAGAGAACGATTTGTATTGAG	MLSB
ImrA-01	TCGACGTGACCGTAGTGAACA	CGTGACTACCCAGGTGAGTTGA	MLSB
lnuA-01	TGACGCTCAACACACTCAAAAA	TTCATGCTTAAGTTCCATACGTGAA	MLSB
lnuB-01	TGAACATAATCCCCTCGTTTAAAGAT	TAATTGCCCTGTTTCATCGTAAATAA	MLSB
lnuB-02	AAAGGAGAAGGTGACCAATACTCTGA	GGAGCTACGTCAAACAACCAGTT	MLSB
lnuC	TGGTCAATATAACAGATGTAAACCAGATTT	CACCCCAGCCACCATCAA	MLSB
matA/mel	TAGTAGGCAAGCTCGGTGTTGA	CCTGTGCTATTTTAAGCCTTGTTTCT	MLSB
mdtA	CCTAACGGGCGTGACTTCA	TTCACCTGTTTCAAGGGTCAAA	MLSB
mefA	CCGTAGCATTGGAACAGCTTTT	AAACGGAGTATAAGAGTGCTGCAA	MLSB
mphA-01	CTGACGCGCTCCGTGTT	GGTGGTGCATGGCGATCT	MLSB
mphA-02	TGATGACCCTGCCATCGA	TTCGCGAGCCCCTCTTC	MLSB
mphB	CGCAGCGCTTGATCTTGTAG	TTACTGCATCCATACGCTGCTT	MLSB
mphC	CGTTTGAAGTACCGAATTGGAAA	GCTGCGGGTTTGCCTGTA	MLSB
msrA-01	CTGCTAACACAAGTACGATTCCAAAT	TCAAGTAAAGTTGTCTTACCTACACCATT	MLSB
msrC-01	TCAGACCGGATCGGTTGTC	CCTATTTTTTGGAGTCTTCTCTCTAATGTT	MLSB
oleC	CCCGGAGTCGATGTTCGA	GCCGAAGACGTACACGAACAG	MLSB
pikR1	TCGACATGCGTGACGAGATT	CCGCGAATTAGGCCAGAA	MLSB
pikR2	TCGTGGGCCAGGTGAAGA	TTCCCCTTGCCGGTGAA	MLSB
vatB-01	GGAAAAAGCAACTCCATCTCTTGA	TCCTGGCATAACAGTAACATTCTGA	MLSB
vatB-02	TTGGGAAAAAGCAACTCCATCT	CAATCCACACATCATTTCCAACA	MLSB
vatC-01	CGGAAATTGGGAACGATGTT	GCAATAATAGCCCCGTTTCCTA	MLSB
vatC-02	CGATGTTTGGATTGGACGAGAT	GCTGCAATAATAGCCCCGTTT	MLSB
vatE-01	GGTGCCATTATCGGAGCAAAT	TTGGATTGCCACCGACAAT	MLSB
vatE-02	GACCGTCCTACCAGGCGTAA	TTGGATTGCCACCGACAATT	MLSB
vgaA-01	CGAGTATTGTGGAAAGCAGCTAGTT	CCCGTACCGTTAGAGCCGATA	MLSB
vgaA-02	GACGGGTATTGTGGAAAGCAA	TTTCCTGTACCATTAGATCCGATAATT	MLSB

Gene Name	Forward Primer	Reverse Primer	Classification
vgb-01	AGGGAGGGTATCCATGCAGAT	ACCAAATGCGCCCGTTT	MLSB
vgbB-01	CAGCCGGATTCTGGTCCTT	TACGATCTCCATTCAATTGGGTAAA	MLSB
vgbB-02	ATACGAGCTGCCTAATAAAGGATCTT	TGTGAACCACAGGGCATTATCA	MLSB
acrA-01	CAACGATCGGACGGGTTTC	TGGCGATGCCACCGTACT	Non-specific
acrA-02	GGTCTATCACCCTACGCGCTATC	GCGCGCACGAACATACC	Non-specific
acrA-03	CAGACCCGCATCGCATATT	CGACAATTTCGCGCTCATG	Non-specific
acrA-04	TACTTTGCGCGCCATCTTC	CGTGCGCGAACGAACAT	Non-specific
acrB-01	AGTCGGTGTTCGCCGTTAAC	CAAGGAAACGAACGCAATACC	Non-specific
acrR-01	GCGCTGGAGACACGACAAC	GCCTTGCTGCGAGAACAAA	Non-specific
acrR-02	GATGATACCCCCTGCTGTGAGA	ACCAAACAAGAAGCGCAAGAA	Non-specific
adeA	CAGTTCGAGCGCCTATTTCTG	CGCCCTGACCGACCAAT	Non-specific
acrA-05	CGTGCGCGAACGAACA	ACTTTGCGCGCCATCTTC	Non-specific
acrF	GCGGCCAGGCACAAAA	TACGCTCTTCCCACGGTTTC	Non-specific
ceoA	ATCAACACGGACCAGGACAAG	GGAAAGTCCGCTCACGATGA	Non-specific
cmeA	GCAGCAAAGAAGAAGCACCAA	AGCAGGGTAAGTAAAACTAAGTGGTAAATCT	Non-specific
cmr	CGGCATCGTCAGTGGAATT	CGGTTCCGAAAAAGATGGAA	Non-specific
emrD	CTCAGCAGTATGGTGGTAAGCATT	ACCAGGCGCCGAAGAAC	Non-specific
marR-01	GCGGCGTACTGGTGAAGCTA	TGCCCTGGTCGTTGATGA	Non-specific
mdetl1	ATACAGCAGTGGATATTGGTTTAATTGT	TGCATAAGGTGAATGTTCCATGA	Non-specific
mdtE/yhiU	CGTCGGCGCACTCGTT	TCCAGACGTTGTACGGTAACCA	Non-specific
mepA	ATCGGTCGCTCTTCGTTCAC	ATAAATAGGATCGAGCTGCTGGAT	Non-specific
mexA	AGGACAACGCTATGCAACGAA	CCGGAAAGGGCCGAAAT	Non-specific
mexD	TTGCCACTGGCTTTCATGAG	CACTGCGGAGAACTGTCTGTAGA	Non-specific
mexE	GGTCAGCACCGACAAGGTCTAC	AGCTCGACGTACTTGAGGAACAC	Non-specific
mexF	CCGCGAGAAGGCCAAGA	TTGAGTTCGGCGGTGATGA	Non-specific
mtrC-01	GGACGGGAAGATGGTCCAA	CGTAGCGTTCCGGTTCGAT	Non-specific
mtrC-02	CGGAGTCCATCGACCATTTG	ATCGTCGGCAAGGAGAATCA	Non-specific
mtrD-02	GGTCGGCACGCTCTTGTC	TGAAGAATTTGCGCACCACTAC	Non-specific
mtrD-03	CCGCCAAGCCGATATAGACA	GGCCGGGTTGCCAAA	Non-specific
oprD	ATGAAGTGGAGCGCCATTG	GGCCACGGCGAACTGA	Non-specific
oprJ	ACGAGAGTGGCGTCGACAA	AAGGCGATCTCGTTGAGGAA	Non-specific

Gene Name	Forward Primer	Reverse Primer	Classification
pmrA	TTTGCAGGTTTTGTTCCTAATGC	GCAGAGCCTGATTTCTCCTTTG	Non-specific
putative multidrug	AATTTTGCCGATTATTGCTGAAA	GATTGTCATCATTCGTTTATCACCAA	Non-specific
qac	CAATAATAACCGAAATAATAGGGACAAGTT	AATAAGTGTTCCTAGTGTTGGCCATAG	Non-specific
qacA	TGGCAATAGGAGCTATGGTGTTT	AAGGTAACACTATTTTCGGTCCAAATC	Non-specific
qacA/qacB	TTTAGGCAGCCTCGCTTCA	CCGAATCCAAATAAAACCCAATAA	Non-specific
qacEdelta1-01	TCGCAACATCCGCATTAAAA	ATGGATTTCAGAACCAGAGAAAGAAA	Non-specific
qacEdelta1-02	CCCCTTCCGCCGTTGT	CGACCAGACTGCATAAGCAACA	Non-specific
qacH-01	GTGGCAGCTATCGCTTGGAT	CCAACGAACGCCCACAA	Non-specific
qacH-02	CATCGTGCTTGTGGCAGCTA	TGAACGCCCAGAAGTCTAGTTTT	Non-specific
rarD-02	TGACGCATCGCGTGATCT	AAATTTTCTGTGGCGTCTGAATC	Non-specific
sdeB	CACTACCGCTTCCGCACTTAA	TGAAAAAACGGGAAAAGTCCAT	Non-specific
tolC-01	GGCCGAGAACCTGATGCA	AGACTTACGCAATTCCGGGTTA	Non-specific
tolC-02	CAGGCAGAGAACCTGATGCA	CGCAATTCCGGGTTGCT	Non-specific
tolC-03	GCCAGGCAGAGAACCTGATG	CGCAATTCCGGGTTGCT	Non-specific
ttgA	ACGCCAATGCCAAACGATT	GTCACGGCGCAGCTTGA	Non-specific
ttgB	TCGCCCTGGATGTACACCTT	ACCATTGCCGACATCAACAAC	Non-specific
yceE/mdtG-01	TGGCACAAAATATCTGGCAGTT	TTGTGTGGCGATAAGAGCATTAG	Non-specific
yceE/mdtG-02	TTATCTGTTTTCTGCTCACCTTCTTTT	GCGTGGTGACAAACAGGCTTA	Non-specific
yceL/mdtH-01	TCGGGATGGTGGGCAAT	CGATAACCGAGCCGATGTAGA	Non-specific
yceL/mdtH-02	CGCGTGAAACCTTAAGTGCTT	AGACGGCTAAACCCCATATAGCT	Non-specific
yceL/mdtH-03	CTGCCGTTAAATGGATGTATGC	ACTCCAGCGGGCGATAGG	Non-specific
yidY/mdtL-01	GCAGTTGCATATCGCCTTCTC	CTTCCCGGCAAACAGCAT	Non-specific
yidY/mdtL-02	TGCTGATCGGGATTCTGATTG	CAGGCGCGACGAACATAAT	Non-specific
fabK	TTTCAGCTCAGCACTTTGGTCAT	AAGGCATCTTTTTCAGCCAGTTC	other
imiR	CCGGACTAGAGCTTCATGTAAGC	CCCACGCGGTACTCTTGTAAA	other
nisB	GGGAGAGTTGCCGATGTTGTA	AGCCACTCGTTAAAGGGCAAT	other
speA	GCAAGAGGTATTTGCTCAACAAGA	CAGGGTCACCCTCATAAAGAAAA	other
bacA-01	CGGCTTCGTGACCTCGTT	ACAATGCGATACCAGGCAAAT	other/bacitracin
bacA-02	TTCCACGACACGATTAAGTCATTG	CGGCTCTTTCGGCTTCAG	other/bacitracin
fosB	TCACTGTAACTAATGAAGCATTAGACCAT	CCATCTGGATCTGTAAAGTAAAGAGATC	other/fosfomycin
fosX	GATTAAGCCATATCACTTTAATTGTGAAAG	TCTCCTTCCATAATGCAAATCCA	other/fosfomycin

Gene Name	Forward Primer	Reverse Primer	Classification
nimE	TGCGCCAAGATAGGGCATA	GTCGTGAATTCGGCAGGTTTA	other/nitroimidazole
pncA	GCAATCGAGGCGGTGTTC	TTGCCGCAGCCAATTCA	other/Pyrazinamide
sat4	GAATGGGCAAAGCATAAAAACTTG	CCGATTTTGAAACCACAATTATGATA	other/streptothricin
dfrA1	GGAATGGCCCTGATATTCCA	AGTCTTGCGTCCAACCAACAG	Sulfonamide
dfrA12	CCTCTACCGAACCGTCACACA	GCGACAGCGTTGAAACAACTAC	Sulfonamide
folA	CGAGCAGTTCCTGCCAAAG	CCCAGTCATCCGGTTCATAATC	Sulfonamide
sul1	CAGCGCTATGCGCTCAAG	ATCCCGCTGCGCTGAGT	Sulfonamide
sul2	TCATCTGCCAAACTCGTCGTTA	GTCAAAGAACGCCGCAATGT	Sulfonamide
sulA/folP-01	CAGGCTCGTAAATTGATAGCAGAAG	CTTTCCTTGCGAATCGCTTT	Sulfonamide
sulA/folP-03	CACGGCTTCGGCTCATGT	TGCCATCCTGTGACTAGCTACGT	Sulfonamide
tet(32)	CCATTACTTCGGACAACGGTAGA	CAATCTCTGTGAGGGCATTTAACA	Tetracycline
tet(34)	CTTAGCGCAAACAGCAATCAGT	CGGTGATACAGCGCGTAAACT	Tetracycline
tet(35)	ACCCCATGACGTACCTGTAGAGA	CAACCCACACTGGCTACCAGTT	Tetracycline
tet(36)	AGAATACTCAGCAGAGGTCAGTTCCT	TGGTAGGTCGATAACCCGAAAAT	Tetracycline
tet(36)	TGCAGGAAAGACCTCCATTACAG	CTTTGTCCACACTTCCACGTACTATG	Tetracycline
tet(37)	GAGAACGTTGAAAAGGTGGTGAA	AACCAAGCCTGGATCAGTCTCA	Tetracycline
tetA-01	GCTGTTTGTTCTGCCGGAAA	GGTTAAGTTCCTTGAACGCAAACT	Tetracycline
tetA-02	CTCACCAGCCTGACCTCGAT	CACGTTGTTATAGAAGCCGCATAG	Tetracycline
tetB-01	AGTGCGCTTTGGATGCTGTA	AGCCCCAGTAGCTCCTGTGA	Tetracycline
tetB-02	GCCCAGTGCTGTTGTTGTCAT	TGAAAGCAAACGGCCTAAATACA	Tetracycline
tetC-01	CATATCGCAATACATGCGAAAAA	AAAGCCGCGGTAAATAGCAA	Tetracycline
tetC-02	ACTGGTAAGGTAAACGCCATTGTC	ATGCATAAACCAGCCATTGAGTAAG	Tetracycline
tetD-01	TGCCGCGTTTGATTACACA	CACCAGTGATCCCGGAGATAA	Tetracycline
tetD-02	TGTCATCGCGCTGGTGATT	CATCCGCTTCCGGGAGAT	Tetracycline
tetE	TTGGCGCTGTATGCAATGAT	CGACGACCTATGCGATCTGA	Tetracycline
tetG-01	TCAACCATTGCCGATTCGA	TGGCCCGGCAATCATG	Tetracycline
tetG-02	CATCAGCGCCGGTCTTATG	CCCCATGTAGCCGAACCA	Tetracycline
tetH	TTTGGGTCATCTTACCAGCATTAA	TTGCGCATTATCATCGACAGA	Tetracycline
tetJ	GGGTGCCGCATTAGATTACCT	TCGTCCAATGTAGAGCATCCATA	Tetracycline
tetK	CAGCAGTCATTGGAAAATTATCTGATTATA	CCTTGTACTAACCTACCAAAAATCAAAATA	Tetracycline
tetL-01	AGCCCGATTTATTCAAGGAATTG	CAAATGCTTTCCCCCTGTTCT	Tetracycline

Gene Name	Forward Primer	Reverse Primer	Classification
tetL-02	ATGGTTGTAGTTGCGCGCTATAT	ATCGCTGGACCGACTCCTT	Tetracycline
tetM-01	CATCATAGACACGCCAGGACATAT	CGCCATCTTTTGCAGAAATCA	Tetracycline
tetM-02	TAATATTGGAGTTTTAGCTCATGTTGATG	CCTCTCTGACGTTCTAAAAGCGTATTAT	Tetracycline
tetO-01	ATGTGGATACTACAACGCATGAGATT	TGCCTCCACATGATATTTTTCCT	Tetracycline
tetW-01	ATGAACATTCCCACCGTTATCTTT	ATATCGGCGGAGAGCTTATCC	Tetracycline
tetPA	AGTTGCAGATGTGTATAGTCGTAAACTATCTATT	TGCTACAAGTACGAAAACAAAACTAGAA	Tetracycline
tetPB-01	ACACCTGGACACGCTGATTTT	ACCGTCTAGAACGCGGAATG	Tetracycline
tetPB-02	TGATACACCTGGACACGCTGAT	CGTCCAAAACGCGGAATG	Tetracycline
tetPB-03	TGGGCGACAGTAGGCTTAGAA	TGACCCTACTGAAACATTAGAAATATACCT	Tetracycline
tetPB-05	CTGAAGTGGAGCGATCATTCC	CCCTCAACGGCAGAAATAACTAA	Tetracycline
tetQ	CGCCTCAGAAGTAAGTTCATACACTAAG	TCGTTCATGCGGATATTATCAGAAT	Tetracycline
tetR-02	CGCGATAGACGCCTTCGA	TCCTGACAACGAGCCTCCTT	Tetracycline
tetR-03	CGCGATGGAGCAAAAGTACAT	AGTGAAAAACCTTGTTGGCATAAAA	Tetracycline
tetS	TTAAGGACAAACTTTCTGACGACATC	TGTCTCCCATTGTTCTGGTTCA	Tetracycline
tetT	CCATATAGAGGTTCCACCAAATCC	TGACCCTATTGGTAGTGGTTCTATTG	Tetracycline
tetU-01	GTGGCAAAGCAACGGATTG	TGCGGGCTTGCAAAACTATC	Tetracycline
tetV	GCGGGAACGACGATGTATATC	CCGCTATCTCACGACCATGAT	Tetracycline
tetX	AAATTTGTTACCGACACGGAAGTT	CATAGCTGAAAAAATCCAGGACAGTT	Tetracycline
vanA	AAAAGGCTCTGAAAACGCAGTTAT	CGGCCGTTATCTTGTAAAAACAT	Vancomycin
vanB-01	TTGTCGGCGAAGTGGATCA	AGCCTTTTTCCGGCTCGTT	Vancomycin
vanC-01	ACAGGGATTGGCTATGAACCAT	TGACTGGCGATGATTTGACTATG	Vancomycin
vanC-02	CCTGCCACAATCGATCGTT	CGGCTTCATTCGGCTTGATA	Vancomycin
vanC-03	AAATCAATACTATGCCGGGCTTT	CCGACCGCTGCCATCA	Vancomycin
vanC1	AGGCGATAGCGGGTATTGAA	CAATCGTCAATTGCTCATTTCC	Vancomycin
vanC2/vanC3	TTTGACTGTCGGTGCTTGTGA	TCAATCGTTTCAGGCAATGG	Vancomycin
vanG	ATTTGAATTGGCAGGTATACAGGTTA	TGATTTGTCTTTGTCCATACATAATGC	Vancomycin
vanHB	GAGGTTTCCGAGGCGACAA	CTCTCGGCGGCAGTCGTAT	Vancomycin
vanHD	GTGGCCGATTATACCGTCATG	CGCAGGTCATTCAGGCAAT	Vancomycin
vanRA-01	CCCTTACTCCCACCGAGTTTT	TTCGTCGCCCCATATCTCAT	Vancomycin
vanRA-02	CCACTCCGGCCTTGTCATT	GCTAACCACATTCCCCTTGTTTT	Vancomycin
vanRB	GCCCTGTCGGATGACGAA	TTACATAGTCGTCTGCCTCTGCAT	Vancomycin

Gene Name	Forward Primer	Reverse Primer	Classification
vanRC	TGCGGGAAAAACTGAACGA	CCCCCCATACGGTTTTGATTA	Vancomycin
vanRC4	AGTGCTTTGGCTTATCTCGAAAA	TCCGGCAGCATCACATCTAA	Vancomycin
vanRD	TTATAATGGCAAGGATGCACTAAAGT	CGTCTACATCCGGAAGCATGA	Vancomycin
vanSA	CGCGTCATGCTTTCAAAATTC	TCCGCAGAAAGCTCAATTTGTT	Vancomycin
vanSB	GCGCGGCAAATGACAAC	TTTGCCATTTTATTCGCACTGT	Vancomycin
vanSC-01	ATCAACTGCGGGAGAAAAGTCT	TCCGCTGTTCCGCTTCTT	Vancomycin
vanSC-02	GCCATCAGCGAGTCTGATGA	CAGCTGGGATCGTTTTTCCTT	Vancomycin
vanTC-01	CACACGCATTTTTTCCCATCTAG	CAGCCAACAGATCATCAAAACAA	Vancomycin
vanTC-02	ACAGTTGCCGCTGGTGAAG	CGTGGCTGGTCGATCAAAA	Vancomycin
vanTE	GTGGTGCCAAGGAAGTTGCT	CGTAGCCACCGCAAAAAAAT	Vancomycin
vanTG	CGTGTAGCCGTTCCGTTCTT	CGGCATTACAGGTATATCTGGAAA	Vancomycin
vanWB	CGGACAAAGATACCCCCTATAAAG	AAATAGTAAATTGCTCATCTGGCACAT	Vancomycin
vanWG	ACATTTTCATTTTGGCAGCTTGTAC	CCGCCATAAGAGCCTACAATCT	Vancomycin
vanXA	CGCTAAATATGCCACTTGGGATA	TCAAAAGCGATTCAGCCAACT	Vancomycin
vanXB	AGGCACAAAATCGAAGATGCTT	GGGTATGGCTCATCAATCAACTT	Vancomycin
vanXD	TAAACCGTGTTATGGGAACGAA	GCGATAGCCGTCCCATAAGA	Vancomycin
vanYB	GGCTAAAGCGGAAGCAGAAA	GATATCCACAGCAAGACCAAGCT	Vancomycin
vanYD-01	AAGGCGATACCCTGACTGTCA	ATTGCCGGACGGAAGCA	Vancomycin
vanYD-02	CAAACGGAAGAGAGGTCACTTACA	CGGACGGTAATAGGGACTGTTC	Vancomycin
cIntI-1(class1)	GGCATCCAAGCAGCAAG	AAGCAGACTTGACCTGA	MGEs/Integrase
intI-1(clinic)	CGAACGAGTGGCGGAGGGTG	TACCCGAGAGCTTGGCACCCA	MGEs/Integrase

Table A. 5 Number of detected ARGs, transposases and integrases in both viable and total cells per treatment step. All values are averages ± standard deviation from three sampling days. 'FCA' stands for fluoroquinolone/quinolone/florfenicol/chloramphenicol/amphenicol and 'MLSB' for macrolide/lincosamide/streptogramin B.

Trickling Filter						
	Total cells			Viable cells		
Class	**Primary settled sewage**	**TF discharge**	**Disc filter discharge**	**Primary settled sewage**	**TF discharge**	**Disc filter discharge**
Total ARGs	176±5.6	162.3±7.8	166.3±8.1	167.7±11.2	156.0±6.2	154.0±0.0
Aminoglycoside	21±0.0	20.7±0.3	21.0±0.0	20.3±0.3	20.0±0.0	19.7±0.3
B-Lactams	38.3±0.8	36.3±1.2	35.3±1.4	35.0±1.5	34.0±1.1	33.0±0.0
FCA	6±0	6.0±0.0	6.0±0.0	5.7±0.3	6.0±0.0	6.0±0.0
MLSB	27.6±1.20	26.0±0.6	27.0±1.0	25.0±1.7	24.6±0.9	23.7±0.7
Non-specific	28.0±0.57	28.3±0.3	28.3±0.3	28.0±0.6	27.0±0.0	26.7±0.3
Other	7.0±0.0	5.7±0.7	6.0±0.6	7.0±0.0	5.7±0.9	6.0±0.6
Sulfonamide	6.0±0.0	5.7±0.3	6.0±0.0	6.0±0.0	5.7±0.3	5.7±0.3
Tetracycline	21.3±0.3	19.3±0.3	19.7±0.3	21.3±0.3	19.3±0.3	19.0±0.6
Vancomycin	20.7±1.2	14.3±1.8	17.0±2.3	19.3±1.8	13.7±1.4	14.3±1.4
Integrase	4.0±0.0	4.0±0.0	4.0±0.0	4.0±0.0	4.0±0.0	4.0±0.0
Transposase	8.0±0.0	8.0±0.0	8.0±0.0	8.0±0.0	8.0±0.0	8.0±0.0

Granular Activated Sludge

	Total cells			Viable cells		
Class	**Primary settled sewage**	**GAS discharge**	**Pile cloth filter discharge**	**Primary settled sewage**	**GAS discharge**	**Pile cloth filter discharge**
Total ARGs	174.67±7.77	146±6.24	137.3±16.5	166±1.00	124±8.72	77.67±67.28
Aminoglycoside	20.6±0.33	18.66±0.33	18.33±0.66	20±0	17.66±0.88	17.5±0.40
B-Lactams	34.66±1.20	31±2.08	27±2.51	34.33±0.33	26±1.73	24±0
FCA	6±0	5.33±0.33	5±0	6±0	5±0	6±0
MLSB	27.66±0.66	24±1.52	21.33±0.33	26±0.57	17.33±1.45	15.5±0.40
Non-specific	28.66±0.66	26.66±0.33	24.66±1.33	27.33±0.33	25±0.57	23±0
Other	6.66±0.33	5±0.57	5.33±0.88	5.66±0.33	3.66±0.33	4±0
Sulfonamide	6±0	5.33±0.33	5.33±0.66	6±0	4.33±0.33	4±0
Tetracycline	22±1	19±0.57	19.33±1.20	21.33±0.33	17.33±0.66	17.5±0.40
Vancomycin	22.33±1.20	11±0.57	11±2.30	19.33±1.45	7.66±0.33	5±0
Integrase	4±0	4±0	4±0	4±0	4±0	4±0
Transposase	8±0	8±0	8±0	8±0	8±0	8±0

Activated Sludge								
	Total cells				Viable cells			
Class	**Primary settled sewage**	**Mixed liquor**	**Secondary clarifier effluent**	**RAS**	**Primary settled sewage**	**Mixed liquor**	**Secondary clarifier effluent**	**RAS**
Total ARGs	179±12.1	111±17.6	117±21.00	110±17.06	179.33±3.21	111.67±4.52	110±10.82	93.67±30.37
Aminoglycoside	20.66±0.3	16.33±1.2	17.66±0.66	16.33±0.88	20.66±0.33	15.33±0.66	15.66±0.66	14.33±1.66
B-Lactams	38.33±1.5	22±1.52	25.33±3.28	22±2.30	37.33±0.33	22.33±0.88	22±1.52	20.66±1.45
FCA	6.33±0.33	4±0.57	3±0	4±0.57	6±0	4±0	3.33±0.33	3.33±0.66
MLSB	29±1.73	17.33±1.8	16.66±2.40	16±1.15	28.66±0.66	17±1	15.66±1.45	14±3.46
Non-specific	28.66±1.4	21.33±2.2	24±1.15	24.33±2.18	29.66±0.88	25±0.57	25±1.15	18±4.50
Other	6.66±0.33	4±0.57	4.33±0.66	3.33±0.66	7±0	4±0	3.33±0.66	2.66±0.66
Sulfonamide	5±0	2.33±0.33	3.33±0.66	2.33±0.33	5.66±0.33	2±0	2±0.57	2.33±0.66
Tetracycline	23±0.57	16.33±1.2	14.66±2.40	15.33±1.763	22.33±0.33	15.66±0.66	14.66±0.88	12.66±2.84
Vancomycin	21.33±1.8	7.33±1.45	8±1.73	6.33±0.66	22±0.57	6.33±0.66	8.33±1.45	5.66±2.72
Integrase	4±0	4±0	3.66±0.33	4±0	4±0	4±0	3.66±0.33	3.33±0.66
Transposase	7.33±0.66	7±1	7.33±0.66	7±1	8±0	8±0	8±0	5.66±0.88

Membrane Bioreactor

	Total cells				Viable cells			
Class	**Screened sewage**	**Mixed liquor**	**MBR permeate**	**GAC & chlorination effluent**	**Screened sewage**	**Mixed liquor**	**MBR permeate**	**GAC & chlorination effluent**
Total ARGs	184±3.00	110±21.7	Not quantified	Not quantified	179.33±5.51	95±4.36	Not quantified	Not quantified
Aminoglycoside	21±0	13.66±0.3	Not quantified	Not quantified	21±0	14±0.57	Not quantified	Not quantified
B-Lactams	40.33±0.3	24.66±2.4	Not quantified	Not quantified	38±0	21±1.15	Not quantified	Not quantified
FCA	6.66±0.33	3.33±0.33	Not quantified	Not quantified	6.66±0.33	4±0	Not quantified	Not quantified
MLSB	27.33±0.3	16.33±2.3	Not quantified	Not quantified	28.33±1.20	11.66±0.33	Not quantified	Not quantified
Non-specific	30±0.57	23±2	Not quantified	Not quantified	28.66±0.33	20±1	Not quantified	Not quantified
Other	7±0	4±1	Not quantified	Not quantified	7±0	2.66±0.66	Not quantified	Not quantified
Sulfonamide	5±0	2.33±0.88	Not quantified	Not quantified	5±0	2.33±0.33	Not quantified	Not quantified
Tetracycline	22.66±0.3	14.33±1.2	Not quantified	Not quantified	21.66±0.33	13±0	Not quantified	Not quantified
Vancomycin	24±0.57	8.33±3.17	Not quantified	Not quantified	23±1	6.33±0.33	Not quantified	Not quantified
Integrase	4±0	3.66±0.33	Not quantified	Not quantified	4±0	3.66±0.33	Not quantified	Not quantified
Transposase	8±0	7.33±0.66	Not quantified	Not quantified	8±0	6.33±0.66	Not quantified	Not quantified

Table A. 6 Multiple comparisons of means (Games-Howell post hoc test). Pairwise comparison of total number of detected ARGs and MGEs in all treatment stages of both total and viable bacteria. Statistical significance is noted by p-values; p-value less than 0.05 shows significant differences between pairs. 'FCA' stands for fluoroquinolone/quinolone/florfenicol/chloramphenicol/ amphenicol and 'MLSB' for macrolide/lincosamide/streptogramin B.

Trickling Filter – Total bacteria												
Comparison	**Total ARGs**	**Aminoglycoside**	**β-lactams**	**FCA**	**MLSB**	**Non-specific**	**Other**	**Sulfonamide**	**Tetracycline**	**Vancomycin**	**Integrase**	**Transposase**
Secondary effluent vs Primary settled sewage	0.86	0.64	0.45	NA	0.51	0.87	0.31	0.64	0.02	0.11	SN	SN
Tertiary effluent vs Primary settled sewage	0.93	*SN	0.30	NA	0.90	0.87	0.37	SN	0.05	0.44	SN	SN
Tertiary effluent vs Secondary effluent	0.99	0.64	0.86	NA	0.69	1	0.92	0.64	0.77	0.66	SN	SN
Trickling Filter – Viable bacteria												
Comparison	**Total ARGs**	**Aminoglycoside**	**β-lactams**	**FCA**	**MLSB**	**Non-specific**	**Other**	**Sulfonamide**	**Tetracycline**	**Vancomycin**	**Integrase**	**Transposase**
Secondary effluent vs Primary settled sewage	0.88	0.64	0.86	0.64	0.98	0.37	0.44	0.64	0.02	0.15	SN	SN
Tertiary effluent vs - Primary settled sewage	0.84	0.41	0.51	0.64	0.77	0.25	0.37	0.64	0.07	0.20	SN	SN

Tertiary effluent vs Secondary effluent	1	0.64	0.70	NA	0.66	0.64	0.94	1	0.87	0.94	NA	NA

Granular Activated Sludge – Total bacteria

Comparison	Total ARGs	Aminoglycoside	β-Lactams	FCA	MLSB	Non-specific	Other	Sulfonamide	Tetracycline	Vancomycin	Integrase	Transposase
Secondary effluent vs Primary settled sewage	0.48	0.02	0.39	0.31	0.22	0.14	0.16	0.31	0.14	<0.01	SN	SN
Tertiary effluent vs Primary settled sewage	0.26	0.10	0.14	SN	<0.01	0.14	0.45	0.64	0.31	0.04	SN	SN
Tertiary effluent vs Secondary effluent	0.92	0.89	0.50	0.64	0.37	0.45	0.94	1	0.96	1	SN	SN

Granular Activated Sludge – Viable bacteria

Comparison	Total ARGs	Aminoglycoside	β-Lactams	FCA	MLSB	Non-specific	Other	Sulfonamide	Tetracycline	Vancomycin	Integrase	Transposase
Secondary effluent vs Primary settled sewage	0.17	0.20	0.06	SN	0.03	0.07	0.02	0.06	0.02	0.02	SN	SN
Tertiary effluent vs Primary settled sewage	0.12	0.47	<0.01	SN	<0.01	0.01	0.06	SN	0.39	0.01	SN	SN
Tertiary effluent vs Secondary effluent	0.94	0.63	0.57	SN	0.54	0.13	0.64	0.64	0.66	0.02	SN	SN

Activated Sludge – Total bacteria												
Comparison	**Total ARGs**	**Aminoglycoside**	**β-Lactams**	**FCA**	**MLSB**	**Non-specific**	**Other**	**Sulfonamide**	**Tetracycline**	**Vancomycin**	**Integrase**	**Transposase**
Primary settled sewage vs Mixed liquor	0.03	0.15	<0.01	0.10	0.03	0.16	0.07	0.03	0.04	0.01	SN	0.99
RAS vs Mixed liquor	1	1	1	1	0.91	0.77	0.87	1	0.96	0.91	SN	1
RAS vs Primary settled sewage	0.03	0.07	0.02	0.10	0.01	0.45	0.06	0.03	0.10	0.02	SN	0.99
Secondary effluent vs Mixed liquor	0.99	0.77	0.79	0.48	0.99	0.72	0.97	0.60	0.91	0.98	0.76	0.99
Secondary effluent vs Primary settled sewage	0.07	0.08	0.11	0.02	0.05	0.20	0.14	0.29	0.16	0.01	0.76	1
Secondary effluent vs RAS	0.99	0.65	0.83	0.48	0.99	0.99	0.72	0.60	0.99	0.80	0.76	0.99
Activated Sludge – Viable bacteria												
Comparison	**Total ARGs**	**Aminoglycoside**	**β-Lactams**	**FCA**	**MLSB**	**Non-specific**	**Other**	**Sulfonamide**	**Tetracycline**	**Vancomycin**	**Integrase**	**Transposase**
Primary settled sewage vs Mixed liquor	0.03	0.01	<0.01	SN	<0.01	0.04	SN	0.02	<0.01	<0.01	SN	SN

RAS vs Mixed liquor	0.79	0.93	0.76	0.76	0.83	0.54	0.40	0.95	0.75	0.99	0.76	0.27
RAS vs Primary settled sewage	<0.01	0.14	0.01	0.13	0.11	0.27	0.05	0.06	0.18	0.05	0.76	0.27
Secondary effluent vs Mixed liquor	1	0.98	0.99	0.40	0.87	1	0.76	1	0.80	0.64	0.76	SN
Secondary effluent vs Primary settled sewage	0.03	0.02	0.01	0.03	0.01	0.11	0.07	0.03	0.01	0.01	0.76	SN
Secondary effluent vs RAS	0.83	0.87	0.91	1	0.96	0.55	0.88	0.97	0.90	0.82	0.96	0.27

Membrane Bioreactor – Total bacteria

Comparison	**Total ARGs**	**Aminoglycoside**	**β-Lactams**	**FCA**	**MLSB**	**Non-specific**	**Other**	**Sulfonamide**	**Tetracycline**	**Vancomycin**	**Integrase**	**Transposase**
Screened sewage vs Mixed liquor	< 0.01	<0.01	0.02	<0.01	0.03	0.06	0.09	0.09	0.01	0.03	0.42	0.42

Membrane Bioreactor – Viable bacteria

Comparison	**Total ARGs**	**Aminoglycoside**	**β-Lactams**	**FCA**	**MLSB**	**Non-specific**	**Other**	**Sulfonamide**	**Tetracycline**	**Vancomycin**	**Integrase**	**Transposase**
Screened sewage vs Mixed liquor	< 0.01	<0.01	<0.01	0.01	<0.01	<0.01	0.02	0.01	<0.01	<0.01	0.42	0.12

*SN = same number of genes in both pairs, therefore comparison was not possible.

Table A. 7 Pairwise comparison of the total number of detected ARGs and MGEs in all treatment stages of both total and viable bacteria. T-test was applied when data were homogeneous with normal distribution, while non-parametric test (Wilcoxon) was used when the assumptions were not met. Statistical significance is noted by p-values; p-value less than 0.05 shows statistically significant differences between pairs.

Trickling Filter		
Comparison (viable vs total)	**ARGs**	**MGEs**
Primary settled sewage	0.7341	1
Trickling filter discharge	0.8211	1
Disc filter discharge	0.5019	1
Granular Activated Sludge		
Comparison (viable vs total)	**ARGs**	**MGEs**
Primary settled sewage	0.4673	1
Granular activated sludge discharge	0.1899	1
Pile cloth filter discharge	0.2745	1

Activated Sludge		
Comparison (viable vs total)	**ARGs**	**MGEs**
Primary settled sewage	0.9585	0.8586
Mixed liquor	0.9723	0.8586
Secondary clarifier effluent	0.591	0.8059
Return activated sludge	0.3806	0.3918
Membrane Bioreactor		
Comparison (viable vs total)	**ARGs**	**MGEs**
Screened sewage	0.7873	1
Mixed liquor	0.5611	0.6643

Table A. 8 Resistance mechanisms (%) in each treatment stage of both total and viable bacteria.

Trickling Filter								
	Total cells				Viable cells			
Treatment stage	**Antibiotic deactivation**	**Cellular protection**	**Efflux pump**	**Other**	**Antibiotic deactivation**	**Cellular protection**	**Efflux pump**	**Other**
Primary settled sewage	44.00	23.11	30.67	2.22	43.12	22.94	31.65	2.29
Trickling filter discharge	45.71	21.43	31.43	1.43	45.32	21.67	31.53	1.48
Disc filter discharge	44.39	22.90	31.31	1.40	45.23	20.60	32.66	1.51

Granular Activated Sludge								
	Total cells				Viable cells			
Treatment stage	**Antibiotic deactivation**	**Cellular protection**	**Efflux pump**	**Other**	**Antibiotic deactivation**	**Cellular protection**	**Efflux pump**	**Other**
Primary settled sewage	41.52	25.45	30.36	2.68	42.99	23.83	31.31	1.87
Granular activated sludge	45.31	19.79	33.33	1.56	43.75	16.88	38.13	1.25
Pile cloth filter discharge	43.89	19.44	35.00	1.67	46.48	14.79	37.32	1.41

Activated Sludge								
	Total cells				Viable cells			
Treatment stage	**Antibiotic deactivation**	**Cellular protection**	**Efflux pump**	**Other**	**Antibiotic deactivation**	**Cellular protection**	**Efflux pump**	**Other**
Primary settled sewage	43.04	23.63	30.38	2.95	42.49	25.32	29.61	2.58
Mixed liquor	44.22	17.01	36.73	2.04	42.86	15.71	39.29	2.14
Secondary clarifier effluent	46.40	17.60	34.40	1.60	41.18	18.30	38.56	1.96
Return activated sludge	44.74	19.08	34.21	1.97	39.72	19.15	39.01	2.13

Membrane Bioreactor								
	Total cells				Viable cells			
Treatment stage	**Antibiotic deactivation**	**Cellular protection**	**Efflux pump**	**Other**	**Antibiotic deactivation**	**Cellular protection**	**Efflux pump**	**Other**
Screened sewage	42.55	24.26	30.21	2.98	42.92	24.46	30.04	2.58
Mixed liquor	43.65	16.67	38.89	0.79	43.42	19.74	34.87	1.97
MBR permeate	NA	NA	NA	NA	NA	NA	NA	NA
GAC & chlorination discahrge	NA	NA	NA	NA	NA	NA	NA	NA

Table A. 9 Presence (%) of each class in each treatment step of both total and viable bacteria.

	Total bacteria				Viable bacteria			
	Influent							
Classes	**Trickling Filter**	**Granular Activated Sludge**	**Activated Sludge**	**Membrane Bioreactor**	**Trickling Filter**	**Granular Activated Sludge**	**Activated Sludge**	**Membrane Bioreactor**
Aminoglycoside	11.93	11.83	11.55	11.41	12.13	12.05	11.52	11.71
Beta-lactams	21.78	19.85	21.42	21.92	20.87	20.68	20.82	21.19
FCA	3.41	3.44	3.54	3.62	3.38	3.61	3.35	3.72
MLSB	15.72	15.84	16.20	14.86	14.91	15.66	15.99	15.80
Multidrug	15.91	16.41	16.01	16.30	16.70	16.47	16.54	15.99
Other	3.98	3.82	3.72	3.80	4.17	3.41	3.90	3.90
Sulfonamide	3.41	3.44	2.79	2.72	3.58	3.61	3.16	2.79
Tetracycline	12.12	12.60	12.85	12.32	12.72	12.85	12.45	12.08
Vancomycin	11.74	12.79	11.92	13.04	11.53	11.65	12.27	12.83
Integrase	33.33	33.33	35.29	33.33	33.33	33.33	33.33	33.33
Transposase	66.67	66.67	64.71	66.67	66.67	66.67	66.67	66.67
	Secondary effluent							
Classes	**Tricklin g Filter**	**Granular Activated Sludge**	**Activated Sludge**	**Membrane Bioreactor**	**Trickling Filter**	**Granular Activated Sludge**	**Activated Sludge**	**Membrane Bioreactor**
Aminoglycoside	12.73	12.79	15.10	*NA	12.82	14.25	14.24	NA
Beta-lactams	22.38	21.23	21.65	NA	21.79	20.97	20.00	NA
FCA	3.70	3.65	2.56	NA	3.85	4.03	3.03	NA

	Total bacteria				Viable bacteria			
MLSB	16.02	16.44	14.25	NA	15.81	13.98	14.24	NA
Multidrug	17.45	18.26	20.51	NA	17.31	20.16	22.73	NA
Other	3.49	3.42	3.70	NA	3.63	2.96	3.03	NA
Sulfonamide	3.49	3.65	2.85	NA	3.63	3.49	1.82	NA
Tetracycline	11.91	13.01	12.54	NA	12.39	13.98	13.33	NA
Vancomycin	8.83	7.53	6.84	NA	8.76	6.18	7.58	NA
Integrase	33.33	33.33	33.33	NA	33.33	33.33	31.43	NA
Transposase	66.67	66.67	66.67	NA	66.67	66.67	68.57	NA

Tertiary effluent

Classes	Tricklin g Filter	Granular Activated Sludge	Activated Sludge	Membrane Bioreactor	Trickling Filter	Granular Activated Sludge	Activated Sludge	Membrane Bioreactor
Aminoglycoside	12.63	13.35	NA	NA	12.77	15.02	NA	NA
Beta-lactams	21.24	19.66	NA	NA	21.43	20.60	NA	NA
FCA	3.61	3.64	NA	NA	3.90	5.15	NA	NA
MLSB	16.23	15.53	NA	NA	15.37	13.30	NA	NA
Multidrug	17.03	17.96	NA	NA	17.32	19.74	NA	NA
Other	3.61	3.88	NA	NA	3.90	3.43	NA	NA
Sulfonamide	3.61	3.88	NA	NA	3.68	3.43	NA	NA
Tetracycline	11.82	14.08	NA	NA	12.34	15.02	NA	NA
Vancomycin	10.22	8.01	NA	NA	9.31	4.29	NA	NA
Integrase	33.33	33.33	NA	NA	33.33	33.33	NA	NA
Transposase	66.67	66.67	NA	NA	66.67	66.67	NA	NA

	Total bacteria				Viable bacteria			
	Final effluent							
Classes	**Tricklin g Filter**	**Granular Activated Sludge**	**Activated Sludge**	**Membrane Bioreactor**	**Trickling Filter**	**Granular Activated Sludge**	**Activated Sludge**	**Membrane Bioreactor**
Aminoglycoside	12.63	13.35	15.10	NA	12.77	15.02	14.24	NA
Beta-lactams	21.24	19.66	21.65	NA	21.43	20.60	20.00	NA
FCA	3.61	3.64	2.56	NA	3.90	5.15	3.03	NA
MLSB	16.23	15.53	14.25	NA	15.37	13.30	14.24	NA
Multidrug	17.03	17.96	20.51	NA	17.32	19.74	22.73	NA
Other	3.61	3.88	3.70	NA	3.90	3.43	3.03	NA
Sulfonamide	3.61	3.88	2.85	NA	3.68	3.43	1.82	NA
Tetracycline	11.82	14.08	12.54	NA	12.34	15.02	13.33	NA
Vancomycin	10.22	8.01	6.84	NA	9.31	4.29	7.58	NA
Integrase	33.33	33.33	33.33	NA	33.33	33.33	31.43	NA
Transposase	66.67	66.67	66.67	NA	66.67	66.67	68.57	NA

*NA = Not available data

Table A. 10 List with persistent genes in final effluents. Persistent genes refer to ARGs that entered the main WWTP and they were not removed at any treatment stage.

Persistent genes							
TF Total bacteria	**TF Viable bacteria**	**GAS Total bacteria**	**GAS Viable bacteria**	**AS Total bacteria**	**AS Viable bacteria**	**MBR Total bacteria**	**MBR Viable bacteria**
aac2ic	aac2ic	aac2ic	-	aac2ic	-	aac2ic	aac3ia
aac3ia	aac3ia	aac3ia	aac3ia	aac3ia	aac3ia	aac3ia	-
aac3iia	aac3iia	aac3iia	aac3iia	aac3iv	-	aac3iv	-
aac3iv	aac3iv	aac3iv	aac3iv	aac3vi	-	aac3vi	-
aac3vi	aac3vi	aac3vi	aac3vi	aac6ia	aac6ia	aac6ia	-
aac6ia	aac6ia	aac6ia	-	aac6ib	aac6ib	aac6ib	aac6ib
aac6ib	aac6ib	aac6ib	aac6ib	aac6ie	aac6iia	aac6iia	aac6iia
aac6ie	aac6ie	aac6ie	aac6ie	aac6iia	-	aad9	aad9
aac6iia	aac6iia	aac6iia	aac6iia	aad9	aad9	aadA5	aadA5
aad9	aad9	aad9	aad9	aadA5	aadA5	acrA	acrA
aadA5	aadA5	aadA5	aadA5	acrA	acrA	acrb	acrb
aadd	acrA	acrA	acrA	acrb	acrb	acrF	-
acrA	acrb	acrb	acrb	acrF	acrF	acrR	acrR
acrb	acrF	acrF	acrF	acrR	acrR	adea	adea
acrF	-	acrR	acrR	adea	adea	ant2ia	ant2ia
acrR	acrR	adea		ant2ia	ant2ia	ant3ia	ant3ia

Persistent genes							
TF Total bacteria	**TF Viable bacteria**	**GAS Total bacteria**	**GAS Viable bacteria**	**AS Total bacteria**	**AS Viable bacteria**	**MBR Total bacteria**	**MBR Viable bacteria**
adea	adea	ant2ia	ant2ia	ant3ia	ant3ia	ant6ia	ant6ia
ant2ia	ant2ia	ant3ia	ant3ia	ant6ia	ant6ia	-	aph2
ant3ia	ant3ia	ant6ia	ant6ia	aph3ia	-	aph3ia	aph3ia
ant6ia	ant6ia	aph3ia	aph3ia	aph3iiia	aph3iiia	-	aph3iiia
aph2	aph2	aph3iiia	aph3iiia	aph6id	aph6id	aph6id	aph6id
aph33ib	aph33ib	aph6id	aph6id	baca	bacA	baca	-
aph3ia	aph3ia	baca	baca	bacA	-	bacA	bacA
aph3iiia	aph3iiia	bacA	bacA	bl1_ampc	-	bl1_acc	bl1_acc
aph6id	aph6id	bl1_ampc	bl1_ampc	bl1_ampC	bl1_ampC	bl1_ampc	bl1_ampc
baca	baca	bl1_ampC	bl1_ampC	bl1_cmy2	bl1_cmy2	bl1_ampC	bl1_ampC
bacA	bacA	bl1_ampc/dha		bl1_ec	bl1_ec	bl1_ampc/dha	bl1_ampc/dha
bl1_acc	bl1_acc	bl1_cmy2	bl1_cmy2	bl1_ec(ampC)	-	bl1_cmy2	bl1_cmy2
bl1_ampc	bl1_ampc	bl1_ec	bl1_ec	bl1_sm	bl1_sm	bl1_ec	-
bl1_ampC	bl1_ampC	bl1_ec(ampC)	bl1_ec(ampC)	bl2_ges	bl2_ges	bl1_ec(ampC)	-
bl1_ampc/dha	-	-	bl1_mox(cmy)	bl2b_rob	bl2b_rob	bl1_mox(cmy)	bl1_mox(cmy)
bl1_cmy2	bl1_cmy2	bl1_sm	bl1_sm	bl2b_tem1	bl2b_tem1	bl1_och	-
bl1_ec	bl1_ec	bl2_ges	-	bl2be_ctxm	bl2be_ctxm	bl1_sm	bl1_sm
bl1_ec(ampC)	bl1_ec(ampC)	bl2b_rob	-	bl2be_oxy1	bl2be_oxy1	bl2a_okp	bl2_ges

Persistent genes							
TF Total bacteria	**TF Viable bacteria**	**GAS Total bacteria**	**GAS Viable bacteria**	**AS Total bacteria**	**AS Viable bacteria**	**MBR Total bacteria**	**MBR Viable bacteria**
bl1_mox(cmy)	bl1_mox(cmy)	bl2b_tem1	bl2b_tem1	bl2d_oxa1/bl2d_oxa30	bl2d_oxa1/bl2d_oxa30	bl2b_rob	bl2_veb
bl1_och	bl1_och	bl2be_ctxm	bl2be_ctxm	bl2d_oxa10	bl2d_oxa10	bl2b_tem1	bl2b_tem1
bl1_sm	bl1_sm	bl2be_oxy1	bl2be_oxy1	bl2e_cfxa	bl2e_cfxa	bl2be_ctxm	bl2be_ctxm
bl2_ges	bl2_ges	bl2be_shv2	bl2be_shv2	bl3_cpha	bl3_cpha	bl2be_oxy1	bl2be_shv2/2bl2_len
bl2_veb	bl2_veb	bl2be_shv2/2bl2_len	bl2be_shv2/2bl2_len	bl3_l	-	bl2be_shv2/2bl2_len	-
bl2a_okp	-	bl2d_oxa1/bl2d_oxa30	bl2d_oxa1/bl2d_oxa30	blaSFO	blaSFO	bl2d_oxa10	bl2d_oxa10
bl2a_pc	-	bl2d_oxa10	bl2d_oxa10	carB	-	bl2e_cfxa	bl2e_cfxa
bl2b_rob	bl2b_rob	bl2e_cepa	-	ceoA	-	bl3_cpha	bl3_cpha
bl2b_tem1	bl2b_tem1	bl2e_cfxa	bl2e_cfxa	cml_e1	cml_e1		bl3_imp
bl2be_ctxm	bl2be_ctxm	bl3_cpha	bl3_cpha	cml_e3	cml_e3	bl3_l	bl3_l
bl2be_oxy1	bl2be_oxy1	bl3_l		cmx(A)	cmx(A)	bl3_vim	-
bl2be_shv2	bl2be_shv2	blaSFO	blaSFO	dfra1	-	blaSFO	blaSFO
bl2be_shv2/2bl2_len	bl2be_shv2/2bl2_len	cata1	cata1	emrd	-	carB	catb3
bl2d_oxa1/bl2d_oxa30	bl2d_oxa1/bl2d_oxa30	catb3	catb3	erea	erea	ceoA	ceoA

Persistent genes							
TF Total bacteria	**TF Viable bacteria**	**GAS Total bacteria**	**GAS Viable bacteria**	**AS Total bacteria**	**AS Viable bacteria**	**MBR Total bacteria**	**MBR Viable bacteria**
bl2d_oxa10	bl2d_oxa10	ceoA	ceoA	erm34	erm34	cfiA	-
bl2e_cepa	bl2e_cepa	cfiA	-	erm36	-	cml_e1	cml_e1
bl2e_cfxa	bl2e_cfxa	cml_e1	cml_e1	ermb	ermb	cml_e3	cml_e3
bl3_cpha	bl3_cpha	cml_e3	cml_e3	ermf	ermf	cmx(A)	cmx(A)
bl3_imp	bl3_l	cmx(A)	cmx(A)	ermt	-	emrd	dfra1
bl3_l	-	dfra1	dfra1	ermx	ermx	ereB	erm34
blaSFO	blaSFO	dfra12	dfra12	fox5	fox5	erm36	-
carB	-	emrd	emrd	intI-1(clinic)	intI-1(clinic)	ermb	ermb
cata1	cata1	erea	erea	intI-1LC	intI-1LC	ermf	ermf
catb3	catb3	ereB	-	intI2	intI2	ermt	ermt
catb8	catb8	erm34	-	intI3	intI3	ermx	ermx
ceoA	ceoA	erm36	erm36	lnub	lnub	fabK	-
cfiA	cfiA	erma/ermTR	-	marR	marR	fox5	fox5
cml_e1	cml_e1	ermb	ermb	matA/mel	matA/mel	intI-1(clinic)	intI-1(clinic)
cml_e3	cml_e3	ermf	ermf	mdtA	mdtA	intI-1LC	intI-1LC
cmx(A)	cmx(A)	ermt	ermt	mdtE/yhiU	mdtE/yhiU	intI2	intI2
dfra1	dfra1	ermx	ermx	mefa	mefa	intI3	intI3
dfra12	dfra12	fabK		mepA	mepA	lnub	lnub

Persistent genes							
TF Total bacteria	**TF Viable bacteria**	**GAS Total bacteria**	**GAS Viable bacteria**	**AS Total bacteria**	**AS Viable bacteria**	**MBR Total bacteria**	**MBR Viable bacteria**
emrd	emrd	folA	-	mexA	mexA	lnuC	-
erea	erea	fox5	fox5	mexe	mexe	marR	marR
ereB	ereB	intI-1(clinic)	intI-1(clinic)	mphA	-	matA/mel	matA/mel
erm34	erm34	intI-1LC	intI-1LC	mtrC	mtrC	mdtA	mdtA
erm36	erm36	intI2	intI2	mtrD	mtrD	mdtE/yhiU	mdtE/yhiU
erma/ermTR	-	intI3	intI3	oleC	-	mefa	mefa
ermb	ermb	lnua	-	pbp2b/pena	-	mepA	mepA
ermc	-	lnub	lnub	pncA	-	mexA	mexA
ermf	ermf	lnuC	-	qacEdelta1	qacEdelta1	mexe	mexe
ermt	ermt	marR	-	qacH	qacH	mexf	mexf
ermx	ermx	matA/mel	matA/mel	rarD	rarD	mphA	mphA
-	fabK	mdtA	-	sat	sat	mphb	msra
folA	folA	mdtE/yhiU	mdtE/yhiU	spcN	spcN	mtrC	mtrC
fox5	fox5	mefa	mefa	sul2	sul2	mtrD	mtrD
intI-1(clinic)	intI-1(clinic)	mepA	mepA	tet32	tet32	NDM1	-
intI-1LC	intI-1LC	mexA	mexe	tet34	-	oleC	oleC
intI2	intI2	mexe	-	teta	teta	oprd	qaca
intI3	intI3	mexf	mexf	tetd	tetd	oprj	-

Persistent genes							
TF Total bacteria	**TF Viable bacteria**	**GAS Total bacteria**	**GAS Viable bacteria**	**AS Total bacteria**	**AS Viable bacteria**	**MBR Total bacteria**	**MBR Viable bacteria**
lnua	lnua	mphA	mphA	tete	tete	pbp2b/pena	-
lnub	lnub	mphb	-	tetg	-	pikR2	-
lnuC	lnuC	msrC	msrC	tetl	tetl	pncA	-
marR	marR	mtrC	-	tetm	tetm	qacEdelta1	qacEdelta1
matA/mel	matA/mel	mtrD	mtrD	teto	teto	qacH	qacH
mdtA	mdtA	oleC	oleC	tetpa	tetpa	rarD	sat
mdtE/yhiU	mdtE/yhiU	oprd	oprd	tetpb	tetpb	spcN	spcN
mefa	mefa	oprj	oprj	tetq	tetq	sul2	sul2
mepA	mepA	pbp2b/pena	-	tetR	-	tet32	tet32
mexA	mexA	Pbp5	-	tets	tets	teta	teta
mexe	mexe	pikR2	pikR2	tett	-	tetd	tetd
mexf	mexf	pncA	pncA	tetx	tetx	tete	tete
mphA	mphA	qacEdelta1	qacEdelta1	Tn21	Tn21	tetg	tetg
mphb	mphb	qacH	qacH	Tn22	-	tetl	tetl
msra	-	rarD	rarD	Tn23	Tn23	tetm	tetm
msrC	msrC	sat	sat	Tn24	Tn24	teto	teto
mtrC	mtrC	spcN	spcN	Tn25	Tn25	tetpa	tetpa
mtrD	mtrD	sul1	-	tnpA	tnpA	tetpb	tetpb

Persistent genes							
TF Total bacteria	**TF Viable bacteria**	**GAS Total bacteria**	**GAS Viable bacteria**	**AS Total bacteria**	**AS Viable bacteria**	**MBR Total bacteria**	**MBR Viable bacteria**
NDM1	-	sul2	sul2	tolc	tolc	tetq	tetq
nimE	nimE	sulA/folP		tp614	tp614	tetR	tetR
oleC	oleC	tet32	tet32	transposase	-	tets	tets
oprd	oprd	teta	teta	ttgA	ttgA	tetU	-
oprj	oprj	tetb	tetb	ttgB	ttgB	tetx	tetx
pbp	pbp	tetC	tetC	vanC	vanC	Tn21	-
pbp2b/pena	pbp2b/pena	tetd	tetd	vanC2/3	-	Tn22	Tn22
Pbp5	Pbp5	tete	tete	vanhb	vanhb	Tn23	Tn23
pikR2	pikR2	tetg	tetg	vanyb	-	Tn24	Tn24
pmrA/MdtA	-	teth	teth	vanyd	-	Tn25	Tn25
pncA	pncA	tetl	tetl	vgb	vgb	tnpA	tnpA
qaca		tetm	tetm	yceL/mdtH	yceL/mdtH	tolc	tolc
qacEdelta1	qacEdelta1	teto	teto	yidy/mdtl	yidy/mdtl	tp614	tp614
qacH	qacH	tetpa	tetpa	-	-	transposase	transposase
rarD	rarD	tetpb	tetpb	-	-	ttgA	ttgA
sat	sat	tetq	tetq	-	-	ttgB	ttgB
spcN	spcN	tetR	tetR	-	-	vana	-
sul1	sul1	tets	tets	-	-	vanC	vanC

Persistent genes							
TF Total bacteria	**TF Viable bacteria**	**GAS Total bacteria**	**GAS Viable bacteria**	**AS Total bacteria**	**AS Viable bacteria**	**MBR Total bacteria**	**MBR Viable bacteria**
sul2	sul2	tetx	tetx	-	-	vanC2/3	vanC2/3
sulA/folP	sulA/folP	Tn21	Tn21	-	-	vanhb	vanhb
tet32	tet32	Tn22	Tn22	-	-	vanhd	vanwg
tet36	tet36	Tn23	Tn23	-	-	vanrb	-
teta	teta	Tn24	Tn24	-	-	vansb	-
tetb	tetb	Tn25	Tn25	-	-	vantc	-
tetC	tetC	tnpA	tnpA	-	-	vante	-
tetd	tetd	tolc	tolc	-	-	vanyb	vanyb
tete	tete	tp614	tp614	-	-	vanyd	vanyd
tetg	tetg	transposase	transposase	-	-	vgb	vgb
teth	teth	ttgA		-	-	yceL/mdtH	yceL/mdtH
tetl	tetl	ttgB	ttgB	-	-	yidy/mdtl	yidy/mdtl
tetm	tetm	vana		-	-	-	-
teto	teto	vanC	vanC	-	-	-	-
tetpa	tetpa	vanC2/3		-	-	-	-
tetpb	tetpb	vanhb	vanhb	-	-	-	-
tetq	tetq	vanhd		-	-	-	-
tetR	tetR	vanrb		-	-	-	-

Persistent genes							
TF Total bacteria	**TF Viable bacteria**	**GAS Total bacteria**	**GAS Viable bacteria**	**AS Total bacteria**	**AS Viable bacteria**	**MBR Total bacteria**	**MBR Viable bacteria**
tets	tets	vantc	-	-	-	-	-
tett	tett	vantg	-	-	-	-	-
tetx	tetx	vanwg	vanwg	-	-	-	-
Tn21	Tn21	vanyb	-	-	-	-	-
Tn22	Tn22	vanyd	vanyd	-	-	-	-
Tn23	Tn23	vgb	-	-	-	-	-
Tn24	Tn24	yceE/mdtG	yceE/mdtG	-	-	-	-
Tn25	Tn25	yceL/mdtH	yceL/mdtH	-	-	-	-
tnpA	tnpA	yidy/mdtl	yidy/mdtl	-	-	-	-
tolc	tolc	-	-	-	-	-	-
tp614	tp614	-	-	-	-	-	-
transposase	transposase	-	-	-	-	-	-
ttgA	ttgA	-	-	-	-	-	-
ttgB	ttgB	-	-	-	-	-	-
vana	vana	-	-	-	-	-	-
vanb	-	-	-	-	-	-	-
vanC	vanC	-	-	-	-	-	-
vanC2/3	vanC2/3	-	-	-	-	-	-

Persistent genes							
TF Total bacteria	**TF Viable bacteria**	**GAS Total bacteria**	**GAS Viable bacteria**	**AS Total bacteria**	**AS Viable bacteria**	**MBR Total bacteria**	**MBR Viable bacteria**
vanhb	vanhb	-	-	-	-	-	-
vanhd	vanhd	-	-	-	-	-	-
vanrb	vanrb	-	-	-	-	-	-
vansb	vansb	-	-	-	-	-	-
vanSE	-	-	-	-	-	-	-
vantc	vantc	-	-	-	-	-	-
vantg	vantg	-	-	-	-	-	-
vanwg	vanwg	-	-	-	-	-	-
vanyb	vanyb	-	-	-	-	-	-
vanyd	vanyd	-	-	-	-	-	-
vatB	vatB	-	-	-	-	-	-
vgb	vgb	-	-	-	-	-	-
yceE/mdtG	yceE/mdtG	-	-	-	-	-	-
yceL/mdtH	yceL/mdtH	-	-	-	-	-	-
yidy/mdtl	yidy/mdtl	-	-	-	-	-	-

Table A. 11 Unique ARGs detected only in the treatment steps they are enlisted and not in other treatment locations.

Treatment stage	ARGs total cells	ARGs viable bacteria
Trickling Filter		
Disc filter discharge	erma	ermK
Disc filter discharge	vanra	NDM1
Trickling filter discharge	bl3_vim	erm35
Granular Activated Sludge		
Granular activated sludge discharge	NDM1	pbp2b/pena
Activated Sludge		
Secondary clarifier effluent	-	bl2a_iii
Membrane Bioreactor		
Mixed liquor	bl2a_iii	bl2a_iii
Mixed liquor	erm34	bl2b_rob
Mixed liquor	sul1	

Table A. 12 Absolute concentrations of ARGs and MGEs in each treatment step per WWTP. Values refer to the sum of all ARGs and MGEs per treatment stage and standard deviation (SD) represent variation from three sampling days.

Trickling Filter						
	Total cells			Viable cells		
Class	**Primary settled sewage**	**Trickling filter discharge**	**Disc filter discharge**	**Primary settled sewage**	**Trickling filter discharge**	**Disc filter discharge**
ARGs	**8.96E+07**	**3.01E+06**	**7.57E+05**	**4.40E+07**	**2.16E+06**	**6.59E+05**
SD	3.32E+07	1.93E+06	7.44E+04	2.60E+07	8.33E+05	1.16E+05
MGEs	**5.89E+07**	**1.96E+06**	**5.70E+05**	**3.11E+07**	**1.48E+06**	**5.50E+05**
SD	2.25E+07	1.19E+06	4.54E+04	2.00E+07	5.98E+05	1.32E+05

Granular Activated Sludge						
	Total cells			Viable cells		
Class	**Primary settled sewage**	**Granular activated sludge discharge**	**Pile cloth filter discharge**	**Primary settled sewage**	**Granular activated sludge discharge**	**Pile cloth filter discharge**
ARGs	**1.32E+08**	**1.22E+06**	**1.37E+05**	**4.70E+07**	**6.93E+05**	**1.25E+04**
SD	9.77E+07	5.19E+05	9.52E+04	2.34E+07	3.19E+05	1.09E+04
MGEs	**8.58E+07**	**1.40E+06**	**1.50E+05**	**3.68E+07**	**7.87E+05**	**7.33E+03**
SD	5.81E+07	5.04E+05	1.02E+05	2.09E+07	2.73E+05	6.35E+03

Activated Sludge								
	Total cells				Viable cells			
Class	**Primary settled sewage**	**Mixed liquor**	**Secondary clarifier effluent**	**RAS**	**Primary settled sewage**	**Mixed liquor**	**Secondary clarifier effluent**	**RAS**
ARGs	**7.84E+07**	**1.23E+08**	**3.45E+04**	**1.86E+08**	**3.98E+07**	**1.37E+08**	**2.24E+04**	**2.77E+08**
SD	3.50E+07	1.70E+07	2.61E+04	3.35E+07	1.10E+07	3.13E+07	7.35E+03	1.89E+08
MGEs	**5.69E+07**	**2.78E+08**	**2.61E+04**	**4.15E+08**	**3.36E+07**	**2.15E+08**	**1.69E+04**	**4.06E+08**
SD	2.48E+07	3.38E+07	1.60E+04	1.45E+08	5.74E+06	8.70E+06	1.21E+04	2.30E+08

Membrane Bioreactor								
	Total cells				Viable cells			
Class	**Screened sewage**	**Mixed liquor**	**MBR permeate**	**GAC & chlorination effluent**	**Screened sewage**	**Mixed liquor**	**MBR permeate**	**GAC & chlorination effluent**
ARGs	**1.52E+08**	**1.47E+09**	**NA**	**NA**	**1.47E+08**	**1.34E+08**	***NA**	**NA**
SD	2.37E+07	5.97E+08	-	-	9.30E+07	1.06E+08	-	-
MGEs	**1.16E+08**	**6.63E+08**	**NA**	**NA**	**1.21E+08**	**8.97E+07**	**NA**	**NA**
SD	1.37E+07	1.38E+08	-	-	7.15E+07	4.44E+07	-	-

*NA = Not available data.

Table A. 13 Multiple comparisons of means (Games-Howell post hoc test). Pairwise comparison of ARGs and MGEs absolute abundances in all treatment stages of both total and viable bacteria. Statistical significance is noted by p-values; p-value less than 0.05 shows significant differences between pairs.

Trickling Filter – Total Cells		
Comparison	**ARGs**	**MGEs**
Primary settled sewage vs Final effluent	0	2.36E-07
Secondary effluent vs Final effluent	3.43E-08	9.46E-05
Secondary effluent vs Primary settled sewage	0	3.82E-07
Tricking Filter – Viable cells		
Comparison	**ARGs**	**MGEs**
Primary settled sewage vs Final effluent	8.11E-11	1.45E-06
Secondary effluent vs Final effluent	1.12E-06	0.002904
Secondary effluent vs Primary settled sewage	2.16E-11	2.56E-06
Granular Activated Sludge – Total Cells		
Comparison	**ARGs**	**MGEs**
Primary settled sewage vs Final effluent	5.19E-11	3.24E-05
Secondary effluent vs Final effluent	4.70E-10	0.000229
Secondary effluent vs Primary settled sewage	3.19E-10	4.07E-05

Granular Activated Sludge – Viable Cells		
Comparison	**ARGs**	**MGEs**
Primary settled sewage vs Final effluent	1.75E-10	7.48E-05
Secondary effluent vs Final effluent	8.72E-12	0.000295
Secondary effluent vs Primary settled sewage	1.63E-10	0.000102

Activated Sludge – Total Cells		
Comparison	**ARGs**	**MGEs**
Primary settled sewage vs Final effluent	0	2.01E-06
Secondary effluent vs Final effluent	0	0.001989
Sludge vs Final effluent	1.14E-12	0.011829
Secondary effluent vs Primary settled sewage	1.46E-06	0.016597
Sludge vs Primary settled sewage	4.64E-09	0.027173
Sludge vs Secondary effluent	0.002765	0.472177

Activated Sludge – Viable Cells		
Comparison	**ARGs**	**MGEs**
Primary settled sewage vs Final effluent	0	2.22E-06
Secondary effluent vs Final effluent	7.83E-07	0.002706
Sludge vs Final effluent	5.65E-05	0.008709
Secondary effluent vs Primary settled sewage	7.44E-05	0.013893
Sludge vs Primary settled sewage	0.000293	0.016907
Sludge vs Secondary effluent	0.15352	0.378096

Membrane Bioreactor – Total Cells		
Comparison	**ARGs**	**MGEs**
Secondary effluent vs Primary settled sewage	0.000101	0.012237
Membrane Bioreactor – Viable Cells		
Comparison	**ARGs**	**MGEs**
Secondary effluent vs Primary settled sewage	0.145052	0.724781

Table A. 14 Pairwise comparison of ARGs and MGEs absolute abundances in all treatment stages of both total and viable bacteria. T-test was applied when data were homogeneous with normal distribution, while non-parametric test (Wilcoxon) was used when the assumptions were not met. Statistical significance is noted by p-values; p-value less than 0.05 shows statistically significant differences between pairs.

Trickling Filter		
Comparison (viable vs total)	**ARGs**	**MGEs**
Primary settled sewage	1.583e-05	0.002595
Trickling filter discharge	0.1548	0.2488
Disc filter discharge	0.03381	0.3101
Granular Activated Sludge		
Comparison (viable vs total)	**ARGs**	**MGEs**
Primary settled sewage	3.524e-05	0.001497
Granular activated sludge discharge	0.6156	0.1584
Pile cloth filter discharge	< 2.2e-16	4.035e-08

Activated Sludge		
Comparison (viable vs total)	**ARGs**	**MGEs**
Primary settled sewage	0.04528	0.01272
Mixed liquor	0.8156	0.2531
Secondary clarifier effluent	0.1021	0.1995
Return activated sludge	0.5217	0.5308
Membrane Bioreactor		
Comparison (viable vs total)	**ARGs**	**MGEs**
Screened sewage	0.3571	0.6127
Mixed liquor	< 2.2e-16	0.007289

Table A. 15 Gene absolute concentrations (± standard deviation of three sampling days) of 16S rRNA, *int*1, *tet*Q, *tet*M, bla_{OXA-10} of total and viable cells in each treatment stage of MBR plant.

Gene	Treatment stage	Average	sd	Group	Average	sd	Group
16s rRNA	**Screened sewage**	**1.66E+08**	5.09E+07	**Total bacteria**	**7.37E+07**	1.04E+07	**Viable bacteria**
	Mixed liquor	**2.07E+09**	2.47E+08		**1.47E+09**	3.99E+08	
	MBR permeate	**9.27E+01**	4.65E+01		**4.14E+01**	3.86E+01	
	Final effluent	**1.68E+02**	1.64E+02		**5.69E+01**	1.02E+01	
***int*1**	**Screened sewage**	**8.89E+06**	1.74E+06		**6.26E+06**	1.04E+06	
	Mixed liquor	**2.21E+07**	1.67E+06		**1.88E+07**	1.71E+06	
	MBR permeate	**NA**	NA		**NA**	NA	
	Final effluent	**NA**	NA		**NA**	NA	
***tet*Q**	**Screened sewage**	**3.30E+06**	2.66E+05		**1.99E+06**	5.85E+05	
	Mixed liquor	**1.69E+06**	4.12E+05		**1.21E+06**	3.70E+05	
	MBR permeate	**NA**	NA		**NA**	NA	
	Final effluent	**NA**	NA		**NA**	NA	
***tet*M**	**Screened sewage**	**1.67E+06**	1.31E+05		**8.47E+05**	2.08E+05	
	Mixed liquor	**2.48E+05**	1.79E+04		**6.15E+04**	5.66E+03	
	MBR permeate	**NA**	NA		**NA**	NA	
	Final effluent	**NA**	NA		**NA**	NA	

Gene	Treatment stage	Average	sd	Group	Average	sd	Group
bla$_{\text{OXA-10}}$	**Screened sewage**	**6.17E+05**	1.43E+05	**Total bacteria**	**2.97E+05**	9.36E+04	**Viable bacteria**
	Mixed liquor	**4.29E+04**	1.39E+04		**1.86E+04**	1.21E+04	
	MBR permeate	**NA**	NA		**NA**	NA	
	Final effluent	**NA**	NA		**NA**	NA	

Table A. 16 Multiple comparisons of means (Games-Howell post hoc test). Pairwise comparison of 16S rRNA, *int*1, *tet*Q, *tet*M and $bla_{\text{OXA-10}}$ genes absolute concentrations carried by both total and viable bacteria in each treatment step of the MBR plant. Statistical significance is noted by p-values (in bold); p-value less than 0.05 shows significant differences between pairs.

	Total bacteria					**Viable bacteria**				
Comparison	**16S**	***int*1**	***tet*M**	***tet*Q**	$\boldsymbol{bla_{\text{oxa-10}}}$	**16S**	***int*1**	***tet*M**	***tet*Q**	$\boldsymbol{bla_{\text{oxa-10}}}$
Influent vs Final Effluent	4.69E-05	*NA	NA	NA	NA	8.91E-08	NA	NA	NA	NA
MBR Effluent vs Final Effluent	0.56635	NA	NA	NA	NA	0.67116345	NA	NA	NA	NA
MBR Effluent vs Influent	4.69E-05	NA	NA	NA	NA	8.91E-08	NA	NA	NA	NA
Mixed Liquor vs Final Effluent	2.60E-08	NA	NA	NA	NA	1.85E-05	NA	NA	NA	NA
Mixed Liquor vs Influent	1.82E-08	2.04E-11	1.57E-09	1.45E-07	1.88E-06	2.70E-05	7.30E-11	3.34E-06	0.004615	1.70E-05
Mixed Liquor vs MBR Effluent	2.60E-08	NA	NA	NA	NA	1.85E-05	NA	NA	NA	NA

*NA = Not available data.

Table A. 17 Pairwise comparison between total versus viable absolute concentrations of 16S rRNA, *int*1, *tet*Q, *tet*M and $bla_{\text{OXA-10}}$. T-test was applied when data were homogeneous with normal distribution, while non-parametric test (Wilcoxon) was used when the assumptions were not met. Statistical significance is noted by p-values; p-value less than 0.05 shows statistically significant differences between pairs.

Comparisons (total vs viable)	16S rRNA	*int*1	*tet*M	*tet*Q	$bla_{\text{oxa-10}}$
Screened sewage	4.114e-05	0.00131	1.454e-08	0.0004095	3.479e-05
Mixed liquor	0.002666	0.0007421	0.0004066	4.114e-05	0.0002879
MBR permeate	0.02149	NA	NA	NA	NA
Final effluent	0.004795	NA	NA	NA	NA

Table A. 18 Removal rates, expressed as $\log_{10}(C_{influent}/C_{final\ effluent})$, per treatment technology grouped in ARGs, MGEs and 16S rRNA.

Treatment technology	**ARGs**	**MGEs**	**16S rRNA**	**ARGs**	**MGEs**	**16S rRNA**
	Total bacteria			**Viable bacteria**		
Trickling Filter	**1.98**±0.01	**2.04**±0.06	**1.57**±0.04	**1.62**±0.02	**1.71**±0.11	**1.27**±0.07
Granular Activated Sludge	**2.80**±0.02	**2.90**±0.10	**2.77**±0.13	**3.20**±0.02	**3.43**±0.08	**2.90**±0.14
Activated Sludge	**3.18**±0.04	**3.76**±0.18	**2.80**±0.07	**3.18**±0.04	**3.91**±0.18	**2.74**±0.07
Membrane Bioreactor	*NA	NA	**6.10**±0.14	NA	NA	**6.08**±0.02

*NA = Not available data

Table A. 19 Multiple comparisons of means (Games-Howell post hoc test). Pairwise comparison of ARGs, MGEs and 16S rRNA removal rates, expressed as $\log_{10}(C_{influent}/C_{final\ effluent})$, in all treatment technologies of both total and viable bacteria. Statistical significance is noted by p-values; p-value less than 0.05 shows significant differences between pairs.

Comparisons	Total bacteria			Viable bacteria		
	16S rRNA	**ARGs**	**MGEs**	**16S rRNA**	**ARGs**	**MGEs**
Granular Activated Sludge vs Activated Sludge	0.996797	4.52E-09	0.000649094	0.833206	0.927311	0.07046
Membrane Bioreactor vs Activated Sludge	3.44E-10	*NA	NA	0	NA	NA
Trickling Filter vs Activated Sludge	2.37E-08	5.29E-12	8.10E-10	2.86E-10	3.25E-10	2.75E-12
Membrane Bioreactor vs Granular Activated Sludge	4.33E-11	NA	NA	2.24E-05		
Trickling Filter vs Granular Activated Sludge	3.62E-05	0	9.59E-10	0.000303	4.12E-10	9.67E-12
Trickling Filter vs Membrane Bioreactor	9.71E-10	NA	NA	0	NA	NA

*NA = Not available data

Table A. 20 Relative abundances (gene copies/cell) of antibiotic resistance genes, transposases and integrases in both viable and total cells per treatment stage. All values are averages ± standard deviation form three sampling days.

Trickling Filter						
	Total cells			Viable cells		
Class	**Primary settled sewage**	**Trickling filter discharge**	**Disc filter discharge**	**Primary settled sewage**	**Trickling filter discharge**	**Disc filter discharge**
Total ARGs	**0.875**± 0.215	**0.427**± 0.075	**0.286**± 0.072	**0.663**± 0.096	**0.403**± 0.113	**0.239**± 0.147
Aminoglycoside	**0.097**± 0.009	**0.047**± 0.005	**0.032**± 0.004	**0.068**± 0.008	**0.045**± 0.007	**0.032**± 0.010
B-Lactams	**0.082**± 0.010	**0.041**± 0.004	**0.024**± 0.004	**0.073**± 0.008	**0.041**± 0.007	**0.028**± 0.011
FCA	**0.005**± 0.001	**0.011**± 0.001	**0.007**± 0.000	**0.004**± 0.000	**0.007**± 0.000	**0.003**± 0.000
MLSB	**0.241**± 0.055	**0.058**± 0.010	**0.047**± 0.014	**0.132**± 0.020	**0.051**± 0.0145	**0.031**± 0.015
Non-specific	**0.152**± 0.012	**0.202**± 0.011	**0.092**± 0.0008	**0.142**± 0.004	**0.197**± 0.018	**0.076**± 0.027
Other	**0.007**± 0.001	**0.003**± 0.000	**0.002**± 0.000	**0.008**± 0.001	**0.003**± 0.000	**0.002**± 0.001
Sulfonamide	**0.007**± 0.001	**0.005**± 0.000	**0.003**± 0.000	**0.006**± 0.000	**0.0056**± 0.000	**0.002**± 0.001
Tetracycline	**0.273**± 0.0427	**0.053**± 0.017	**0.072**± 0.012	**0.222**± 0.031	**0.048**± 0.019	**0.059**± 0.018

Vancomycin	**0.006**± 0.002	**0.003**± 0.000	**0.002**± 0.000	**0.004**± 0.000	**0.003**± 0.000	**0.002**± 0.000
Integrase	**0.117**± 0.0176	**0.102**± 0.004	**0.052**± 0.005	**0.070**± 0.018	**0.100**± 0.0105	**0.043**± 0.016
Transposase	**0.457**± 0.069	**0.176**± 0.019	**0.163**± 0.027	**0.383**± 0.040	**0.171**± 0.028	**0.154**± 0.056

Granular Activated Sludge

	Total cells			**Viable cells**		
Class	**Primary settled sewage**	**Granular activated sludge discharge**	**Pile cloth filter discharge**	**Primary settled sewage**	**Granular activated sludge discharge**	**Pile cloth filter discharge**
Total ARGs	**1.022**± 0.055	**0.609**± 0.077	**0.677**± 0.318	**0.955**± 0.146	**0.500**± 0.078	**0.290**± 0.270
Aminoglycoside	**0.163**± 0.022	**0.160**± 0.017	**0.107**± 0.033	**0.200**± 0.026	**0.147**± 0.013	**0.044**± 0.027
B-Lactams	**0.109**± 0.006	**0.051**± 0.003	**0.080**± 0.025	**0.164**± 0.020	**0.045**± 0.005	**0.033**± 0.019
FCA	**0.006**± 0.000	**0.005**± 0.000	**0.004**± 0.001	**0.008**± 0.000	**0.006**± 0.000	**0.002**± 0.001
MLSB	**0.359**± 0.029	**0.093**± 0.017	**0.102**± 0.022	**0.103**± 0.030	**0.051**± 0.007	**0.017**± 0.009
Non-specific	**0.163**± 0.011	**0.235**± 0.022	**0.267**± 0.093	**0.292**± 0.046	**0.198**± 0.022	**0.103**± 0.059
Other	**0.007**± 0.000	**0.001**± 0.000	**0.007**± 0.002	**0.018**± 0.005	**0.001**± 0.000	**0.005**± 0.002

Sulfonamide	**0.007**± 0.000	**0.003**± 0.000	**0.004**± 0.001	**0.009**± 0.001	**0.002**± 0.000	**0.002**± 0.001
Tetracycline	**0.200**± 0.033	**0.052**± 0.006	**0.098**± 0.025	**0.153**± 0.036	**0.044**± 0.004	**0.080**± 0.040
Vancomycin	**0.005**± 0.000	**0.004**± 0.000	**0.006**± 0.002	**0.004**± 0.001	**0.003**± 0.000	**0.001**± 0.000
Integrase	**0.144**± 0.025	**0.301**± 0.005	**0.362**± 0.161	**0.155**± 0.023	**0.225**± 0.012	**0.065**± 0.039
Transposase	**0.538**± 0.020	**0.407**± 0.021	**0.420**± 0.099	**0.552**± 0.041	**0.367**± 0.0184	**0.107**± 0.058

Activated Sludge

	Total cells				**Viable cells**			
Class	**Primary settled sewage**	**Mixed liquor**	**Secondary clarifier effluent**	**RAS**	**Primary settled sewage**	**Mixed liquor**	**Secondary clarifier effluent**	**RAS**
Total ARGs	**0.865**± 0.067	**0.238**± 0.041	**0.251**± 0.192	**0.179**± 0.043	**0.744**± 0.087	**0.362**± 0.085	**0.236**± 0.083	**0.319**± 0.155
Aminoglycoside	**0.154**± 0.017	**0.058**± 0.002	**0.021**± 0.009	**0.044**± 0.003	**0.123**± 0.011	**0.057**± 0.003	**0.022**± 0.010	**0.0524**± 0.006
B-Lactams	**0.069**± 0.009	**0.023**± 0.005	**0.025**± 0.0106	**0.022**± 0.007	**0.074**± 0.003	**0.030**± 0.001	**0.022**± 0.005	**0.023**± 0.007
FCA	**0.007**± 0.001	**0.005**± 0.000	**0.007**± 0.002	**0.003**± 0.000	**0.006**± 0.000	**0.005**± 0.000	**0.008**± 0.003	**0.004**± 0.001
MLSB	**0.327**± 0.029	**0.047**± 0.005	**0.019**± 0.007	**0.038**± 0.004	**0.222**± 0.023	**0.031**± 0.001	**0.011**± 0.003	**0.030**± 0.003
Non-specific	**0.123**±	**0.0581**±	**0.140**±	**0.044**±	**0.143**±	**0.182**±	**0.133**±	**0.162**±

	0.014	0.002	0.071	0.002	0.007	0.045	0.031	0.061
Other	**0.003**± 0.000	**0.001**± 0.000	**0.001**± 0.000	**0.000**± 0.000	**0.005**± 0.000	**0.001**± 0.000	**0.001**± 0.000	**0.001**± 0.000
Sulfonamide	**0.006**± 0.001	**0.016**± 0.000	**0.009**± 0.003	**0.008**± 0.004	**0.006**± 0.000	**0.018**± 0.000	**0.012**± 0.004	**0.015**± 0.001
Tetracycline	**0.170**± 0.031	**0.024**± 0.006	**0.018**± 0.006	**0.012**± 0.003	**0.160**± 0.030	**0.030**± 0.001	**0.019**± 0.005	**0.024**± 0.006
Vancomycin	**0.002**± 0.000	**0.003**± 0.001	**0.006**± 0.004	**0.003**± 0.001	**0.003**± 0.000	**0.005**± 0.000	**0.004**± 0.001	**0.003**± 0.001
Integrase	**0.150**± 0.032	**0.268**± 0.007	**0.094**± 0.035	**0.168**± 0.084	**0.126**± 0.026	**0.274**± 0.011	**0.074**± 0.028	**0.251**± 0.035
Transposase	**0.482**± 0.010	**0.270**± 0.038	**0.094**± 0.032	**0.233**± 0.011	**0.513**± 0.038	**0.294**± 0.004	**0.088**± 0.026	**0.238**± 0.048

Membrane Bioreactor

	Total cells				**Viable cells**			
Class	**Screened sewage**	**Mixed liquor**	**MBR permeate**	**GAC & chlorination effluent**	**Screened sewage**	**Mixed liquor**	**MBR permeate**	**GAC & chlorination effluent**
Total ARGs	**0.956**± 0.109	**3.478**± 2.017	***NA	NA	**0.879**± 0.178	**0.051**± 0.057	NA	NA
Aminoglycoside	**0.271**± 0.023	**0.123**± 0.033	NA	NA	**0.227**± 0.027	**0.001**± 0.000	NA	NA
B-Lactams	**0.104**± 0.010	**0.707**± 0.171	NA	NA	**0.115**± 0.016	**0.009**± 0.005	NA	NA
FCA	**0.015**± 0.002	**0.050**± 0.010	NA	NA	**0.015**± 0.002	**0.000**± 0.000	NA	NA
MLSB	**0.202**±	**0.182**±	NA	NA	**0.189**±	**0.004**±	NA	NA

	0.007	0.049			0.015	0.002		
Non-specific	**0.177**± 0.018	**1.850**± 0.854	NA	NA	**0.170**± 0.022	**0.026**± 0.019	NA	NA
Other	**0.003**± 0.000	**0.024**± 0.0137	NA	NA	**0.003**± 0.000	**2.5E-05**± 1.5E-05	NA	NA
Sulfonamide	**0.011**± 0.001	**0.230**± 0.092	NA	NA	**0.009**± 0.000	**0.004**± 0.001	NA	NA
Tetracycline	**0.167**± 0.009	**0.208**± 0.058	NA	NA	**0.145**± 0.022	**0.003**± 0.002	NA	NA
Vancomycin	**0.002**± 0.000	**0.099**± 0.054	NA	NA	**0.002**± 0.000	**0.001**± 0.000	NA	NA
Integrase	**0.230**± 0.022	**0.688**± 0.119	NA	NA	**0.191**± 0.021	**0.013**± 0.006	NA	NA
Transposase	**0.498**± 0.021	**0.879**± 0.219	NA	NA	**0.537**± 0.048	**0.020**± 0.009	NA	NA

*FCA stands for fluoroquinolone / quinolone / florfenicol / chloramphenicol / amphenicol.
**MLSB stands for macrolide / lincosamide / streptogramin B.
***NA = Not available data

Table A. 21 Multiple comparisons of means (Games-Howell post hoc test). Pairwise comparison of ARGs and MGEs relative concentrations (gene copies/cell) in all treatment stages of both total and viable bacteria. Statistical significance is noted by p-values; p-value less than 0.05 shows significant differences between pairs.

Trickling Filter – Total bacteria												
Comparison	**Total ARGs**	**Aminoglycoside**	**β-lactams**	***FCA**	****MLSB**	**Non specific**	**Other**	**Sulfonamide**	**Tetracycline**	**Vancomycin**	**Integrase**	**Transposase**
Secondary effluent vs Primary settled sewage	0.01	0.04	0.11	0.57	0.08	0.54	0.76	0.84	0.02	0.96	0.88	< 0.01
Tertiary effluent vs Primary settled sewage	< 0.01	< 0.01	0.01	0.76	0.05	0.14	0.18	0.49	0.04	0.42	0.05	< 0.01
Tertiary effluent vs Secondary effluent	0.03	0.35	0.17	0.84	0.85	0.04	0.31	0.81	0.63	0.64	0.11	0.95
Trickling Filter – Viable bacteria												
Comparison	**Total ARGs**	**Aminoglycoside**	**β-lactams**	**FCA**	**MLSB**	**Non specific**	**Other**	**Sulfonamide**	**Tetracycline**	**Vancomycin**	**Integrase**	**Transposase**
Secondary effluent vs Primary settled sewage	0.05	0.36	0.23	0.59	0.13	0.43	0.54	0.98	0.03	0.92	0.49	< 0.01
Tertiary effluent vs Primary settled sewage	< 0.01	0.07	0.05	0.60	0.04	0.03	0.37	0.56	0.05	0.99	0.35	< 0.01
Tertiary effluent vs Secondary effluent	0.01	0.57	0.36	0.38	0.65	0.01	0.82	0.70	0.82	0.92	0.08	0.93

Granular Activated Sludge – Total bacteria												
Comparison	**Total ARGs**	**Aminoglycoside**	**β-lactams**	**FCA**	**MLSB**	**Non specific**	**Other**	**Sulfonamide**	**Tetracycline**	**Vancomycin**	**Integrase**	**Transposase**
Secondary effluent vs Primary settled sewage	0.16	0.93	0.04	0.98	0.08	0.42	0.29	0.55	0.02	0.48	0.18	0.61
Tertiary effluent vs Primary settled sewage	0.61	0.67	0.91	0.49	0.13	0.21	0.84	0.84	0.29	0.35	0.29	0.66
Tertiary effluent vs Secondary effluent	0.57	0.46	0.091	0.83	0.91	0.86	0.06	0.90	0.08	0.85	0.91	0.99
Granular Activated Sludge – Viable bacteria												
Comparison	**Total ARGs**	**Aminoglycoside**	**β-lactams**	**FCA**	**MLSB**	**Non specific**	**Other**	**Sulfonamide**	**Tetracycline**	**Vancomycin**	**Integrase**	**Transposase**
Secondary effluent vs Primary settled sewage	0.06	0.90	0.01	0.85	0.84	0.34	0.31	0.40	0.08	0.53	0.53	0.51
Tertiary effluent vs Primary settled sewage	0.04	0.158111	0.05	< 0.01	0.24	0.17	0.81	0.47	0.99	0.71	0.37	0.01
Tertiary effluent vs Secondary effluent	0.95	0.16	0.81	0.17	0.29	0.97	0.10	0.99	0.18	0.99	0.16	0.28

Activated Sludge – Total bacteria

Comparison	Total ARGs	Aminoglycoside	β-lactams	FCA	MLSB	Non specific	Other	Sulfonamide	Tetracycline	Vancomycin	Integrase	Transposase
Primary settled sewage vs Mixed liquor	< 0.01	0.04	0.42	0.99	0.14	0.46	0.82	0.32	0.01	0.38	0.54	0.49
RAS vs Mixed liquor	0.99	0.96	0.99	0.99	0.99	0.99	0.34	0.94	0.50	0.86	0.93	0.99
RAS vs Primary settled sewage	< 0.01	0.01	0.55	0.99	0.18	0.58	0.03	0.62	<0.01	0.45	0.93	0.73
Secondary effluent vs Mixed liquor	1	0.01	0.99	0.69	0.17	0.60	0.94	0.58	0.62	0.88	0.27	0.39
Secondary effluent vs Primary settled sewage	0.02	< 0.01	0.41	0.62	0.04	0.93	0.99	0.76	<0.01	0.44	0.77	<0.01
Secondary effluent vs RAS	1	0.07	0.99	0.64	0.27	0.64	0.39	0.90	0.99	0.99	0.68	0.38

Activated Sludge – Viable bacteria

Comparison	Total ARGs	Aminoglycoside	β-lactams	FCA	MLSB	Non specific	Other	Sulfonamide	Tetracycline	Vancomycin	Integrase	Transposase
Primary settled sewage vs Mixed liquor	0.8	0.35	0.72	0.86	0.08	0.82	0.68	0.25	0.03	0.18	0.36	0.49
RAS vs Mixed liquor	0.97	0.90	0.96	0.94	1	0.99	0.98	0.87	0.91	0.86	0.99	0.99
RAS vs Primary settled sewage	0.47	0.10	0.44	0.99	0.08	0.92	0.50	0.35	0.01	0.44	0.46	0.39
Secondary effluent vs Mixed liquor	0.55	0.03	0.92	0.77	0.02	0.94	0.98	0.93	0.42	0.88	0.16	0.39

Secondary effluent vs Primary settled sewage	0.06	<0.01	0.36	0.60	0.01	0.99	0.85	0.46	<0.01	0.20	0.68	<0.01
Secondary effluent vs RAS	0.83	0.12	0.99	0.64	0.04	0.98	0.91	0.99	0.82	0.99	0.21	0.46

Membrane Bioreactor – Total bacteria

Comparison	**Total ARGs**	**Aminoglycoside**	**β-lactams**	**FCA**	**MLSB**	**Non specific**	**Other**	**Sulfonamide**	**Tetracycline**	**Vancomycin**	**Integrase**	**Transposase**
Screened sewage vs Mixed liquor	< 0.01	0.16	<0.01	0.08	0.24	0.04	0.17	0.14	0.06	0.03	0.12	0.33

Membrane Bioreactor – Viable bacteria

Comparison	**Total ARGs**	**Aminoglycoside**	**β-lactams**	**FCA**	**MLSB**	**Non specific**	**Other**	**Sulfonamide**	**Tetracycline**	**Vancomycin**	**Integrase**	**Transposase**
Screened sewage vs Mixed liquor	< 0.01	<0.01	<0.01	<0.01	<0.01	0.01	0.024	0.73	<0.01	0.43	<0.01	<0.01

*FCA stands for fluoroquinolone/quinolone/florfenicol/chloramphenicol/amphenicol.
**MLSB stand for macrolide/lincosamide/streptogramin B.

Table A. 22 Pairwise comparison of ARGs and MGEs relative concentrations (gene copies/cell) of total versus viable bacteria in all treatment stages. T-test was applied when data were homogeneous with normal distribution, while non-parametric test (Wilcoxon) was used when the assumptions were not met. Statistical significance is noted by p-values; p-value less than 0.05 shows statistically significant differences between pairs.

Trickling Filter		
Comparison (viable vs total)	**ARGs**	**MGEs**
Primary settled sewage	0.6822	0.1986
Trickling filter discharge	0.8496	0.8328
Disc filter discharge	0.3176	0.1424
Granular Activated Sludge		
Comparison (viable vs total)	**ARGs**	**MGEs**
Primary settled sewage	0.07331	0.7925
Granular activated sludge discharge	0.3045	0.6422
Pile cloth filter discharge	0.0009831	0.01321

Activated Sludge		
Comparison (viable vs total)	**ARGs**	**MGEs**
Primary settled sewage	0.1049	0.745
Mixed liquor	0.01299	0.7883
Secondary clarifier effluent	0.278	0.5828
Return activated sludge	0.179	0.5308
Membrane Bioreactor		
Comparison (viable vs total)	**ARGs**	**MGEs**
Screened sewage	0.5541	0.755
Mixed liquor	< 2.2e-16	1.523e-07

Table A. 23 Contribution of each treatment step at reducing absolute abundances of ARGs, MGEs and 16S rRNA in trickling filter, granular activated sludge, activated sludge and membrane bioreactor WWTPs of both total and viable bacteria.

Treatment stages	Total bacteria			Viable bacteria		
	ARGs	**MGEs**	**16S rRNA**	**ARGs**	**MGEs**	**16S rRNA**
Trickling filter (Secondary treatment)	**0.75**±0.22	**0.76**±0.17	**0.77**±0.14	**0.82**±0.60	**0.73**±0.19	**0.82**±0.08
Trickling filter (Tertiary treatment)	**0.25**±0.22	**0.24**±0.17	**0.23**±0.14	**0.18**±0.60	**0.27**±0.19	**0.18**±0.08
Granular activated sludge (Secondary treatment)	**0.73**±0.21	**0.69**±0.20	**0.64**±0.11	**0.55**±0.23	**0.48**±0.21	**0.46**±0.05
Granular activated sludge (Tertiary treatment)	**0.27**±0.21	**0.31**±0.20	**0.36**±0.11	**0.45**±0.23	**0.52**±0.21	**0.54**±0.05
Activated sludge (Secondary treatment)	**1.00**±0.00	**1.00**±0.00	**1.00**±0.00	**1.00**±0.00	**1.00**±0.00	**1.00**±0.00
Activated sludge (Tertiary treatment)	*NA	NA	NA	NA	NA	NA
Membrane Bioreactor (Secondary treatment)	NA	NA	**0.99**±0.02	NA	NA	**1.00**±0.00
Membrane Bioreactor (Tertiary treatment)	NA	NA	**0.01**±0.02	NA	NA	**0.00**±0.00

*NA = Not available data

Table A. 24 Ratios of gene absolute concentrations in viable cells to gene absolute concentrations in total cells in each treatment stage of the trickling filter, granular activated sludge, activated sludge, membrane bioreactor.

Treatment stages	ARGs	MGEs	16S rRNA
Trickling Filter			
Primary settled sewage	**0.70**±0.55	**0.63**±0.43	**0.69**±0.40
Trickling filter discharge	**0.97**±0.75	**0.95**±0.49	**1.04**±0.59
Disc filter discharge	**1.23**±0.71	**0.95**±0.40	**1.17**±0.33
Granular Activated Sludge			
Primary settled sewage	**0.93**±0.94	**0.74**±0.70	**0.63**±0.42
Granular activated sludge discharge	**0.78**±0.54	**0.66**±0.42	**0.70**±0.27
Pile cloth filter discharge	**0.14**±0.11	**0.09**±0.08	**0.14**±0.06
Activated Sludge			
Primary settled sewage	**0.85**±0.62	**0.61**±0.27	**0.66**±0.26
Mixed liquor	**0.83**±0.42	**0.71**±0.38	**0.73**±0.11
Secondary clarifier effluent	**0.76**±0.39	**0.63**±0.45	**0.75**±0.28
Return activated sludge	**0.92**±0.53	**0.93**±0.87	**0.75**±0.31

Membrane Bioreactor			
Screened sewage	**1.06**±0.77	**1.03**±0.60	**0.50**±0.21
Mixed liquor	**0.16**±0.23	**0.25**±0.18	**0.72**±0.21
MBR permeate	*NA	NA	**0.38**±0.26
Final effluent	NA	NA	**0.66**±0.24

*NA = Not available data

Table A. 25 Multiple comparisons of means (Games-Howell post hoc test). Pairwise comparison of ARGs, MGEs and 16S rRNA viable cells to total cells ratios in all treatment steps. Statistical significance is noted by p-values; p-value less than 0.05 shows significant differences between pairs.

Comparisons	p - value
Trickling Filter	
16S-Primary settled sewage vs 16S-Disc filter discharge	0.201240211
16S-Trickling filter discharge vs 16S-Disc filter discharge	0.999451613
16S-Trickling filter discharge vs 16S-Primary settled sewage	0.845345453
ARGs-Primary settled sewage vs ARGs-Disc filter discharge	0
ARGs-Trickling filter discharge vs ARGs-Disc filter discharge	2.14E-07
ARGs-Trickling filter discharge vs ARGs-Primary settled sewage	8.47E-11
MGEs-Primary settled sewage vs MGEs-Disc filter discharge	0.043577708
MGEs-Trickling filter discharge vs MGEs-Disc filter discharge	1
MGEs-Trickling filter discharge vs MGEs-Primary settled sewage	0.115506812
Granular Activated Sludge	
16S-Pile cloth filter discharge vs 16S-Granular activated sludge discharge	0.003250917
16S-Primary settled sewage vs 16S-Granular activated sludge discharge	0.999945552
16S-Primary settled sewage vs 16S-Pile cloth filter discharge	0.112378993
ARGs-Pile cloth filter discharge vs ARGs-Granular activated sludge discharge	2.14E-10
ARGs-Primary settled sewage vs ARGs-Granular activated sludge discharge	0.034656939
ARGs-Primary settled sewage vs ARGs-Pile cloth filter discharge	0
MGEs-Pile cloth filter discharge vs MGEs-Granular activated sludge discharge	7.91E-08

Comparisons	p - value
MGEs-Primary settled sewage vs MGEs-Granular activated sludge discharge	0.999436243
MGEs-Primary settled sewage vs MGEs-Pile cloth filter discharge	0.000114054
Activated Sludge	
16S-Primary settled sewage vs 16S-Mixed liquor	0.999534085
16S-Return activated sludge vs 16S-Mixed liquor	1
16S-Secondary effluent vs 16S-Mixed liquor	1
16S-Return activated sludge vs 16S-Primary settled sewage	0.999926464
16S-Secondary effluent vs 16S-Primary settled sewage	0.999882515
16S-Secondary effluent vs 16S-Return activated sludge	1
ARGs-Primary settled sewage vs ARGs-Mixed liquor	0.999980268
ARGs-Return activated sludge vs ARGs-Mixed liquor	0.586449157
ARGs-Secondary effluent vs ARGs-Mixed liquor	0.547274601
ARGs-Return activated sludge vs ARGs-Primary settled sewage	0.914372516
ARGs-Secondary effluent vs ARGs-Primary settled sewage	0.1979864
ARGs-Secondary effluent vs ARGs-Return activated sludge	0.00369368
MGEs-Primary settled sewage vs MGEs-Mixed liquor	0.985611392
MGEs-Return activated sludge vs MGEs-Mixed liquor	0.98507855
MGEs-Secondary effluent vs MGEs-Mixed liquor	0.999773536
MGEs-Return activated sludge vs MGEs-Primary settled sewage	0.801318288
MGEs-Secondary effluent vs MGEs-Primary settled sewage	0.999999999
MGEs-Secondary effluent vs MGEs-Return activated sludge	0.900804536

Comparisons	p - value
Membrane Bioreactor	
16S-MBR discharge vs 16S-GAC & chlorination discharge	0.476221468
16S-Mixed liquor vs 16S-GAC & chlorination discharge	0.999592599
16S-Screened sewage vs 16S-GAC & chlorination discharge	0.880374656
16S-Mixed liquor vs 16S-MBR discharge	0.1279028
16S-Screened sewage vs 16S-MBR discharge	0.963125063
16S-Screened sewage vs 16S-Mixed liquor	0.434988696
ARGs-Screened sewage vs ARGs-Mixed liquor	6.91E-13
MGEs-Screened sewage vs MGEs-Mixed liquor	1.44E-07

Appendix B

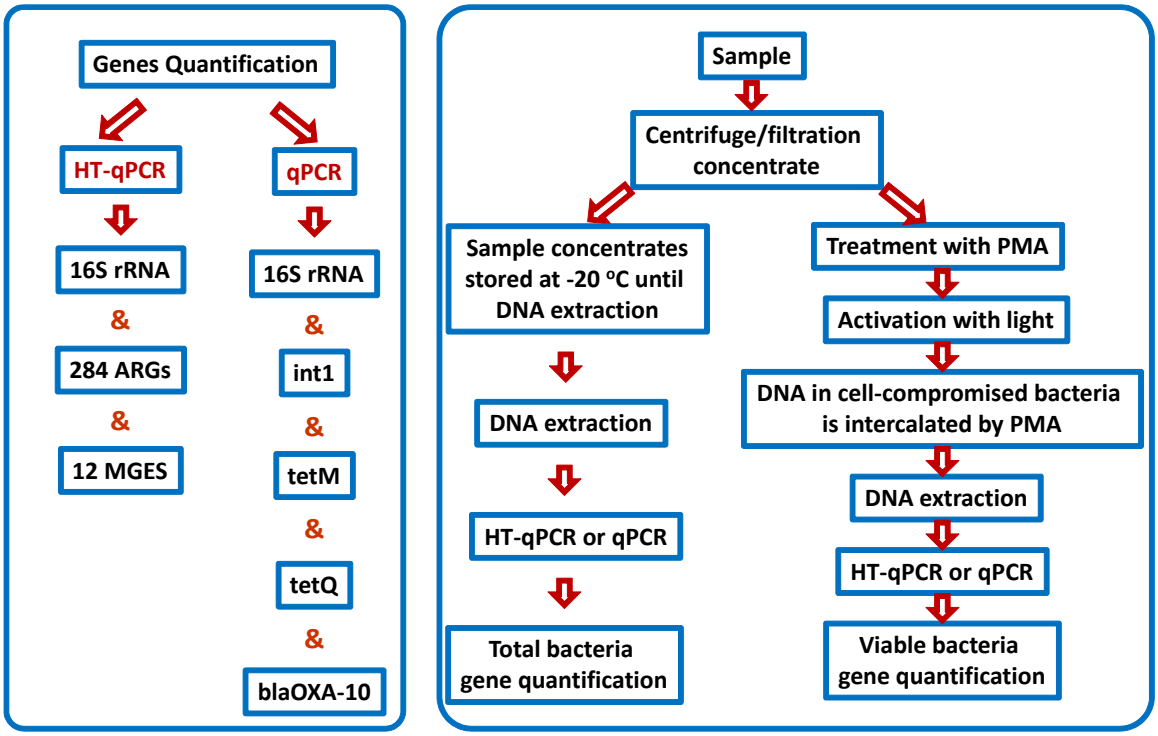

Figure B. 1 Schemes summarizing the steps followed to process the samples and subsequently quantify ARGs and MGEs. The scheme on the left depicts the two main gene quantification analyses performed in this study. The scheme on the right presents in parallel the main steps followed for the quantification of genes in viable and total (viable & non-viable) bacterial cells.

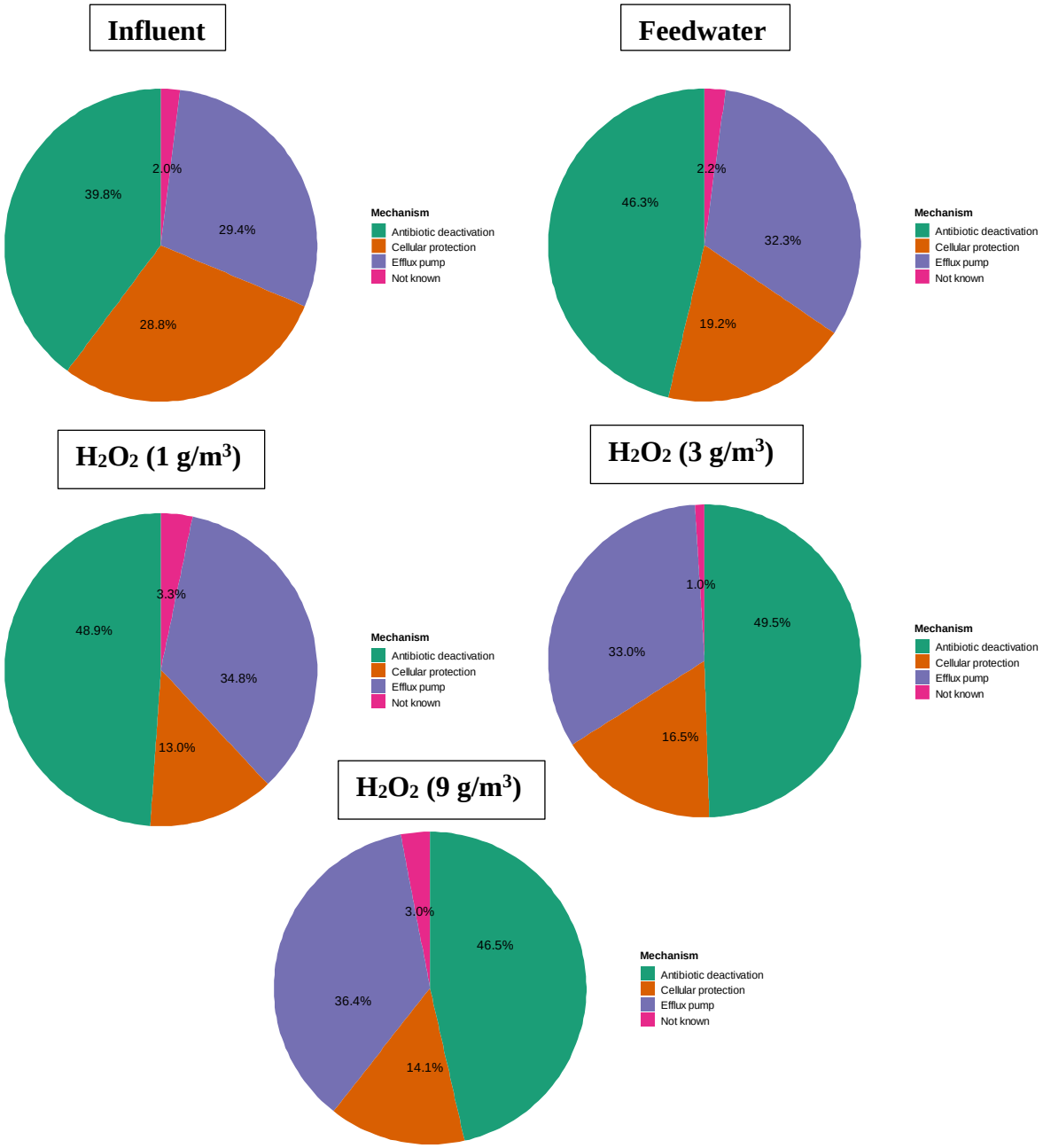

Figure B. 2 Proportion of ARGs resistance mechanisms in influent (primary settled sewage), feedwater (tertiary filtered discharge) and all AOPs conditions. H_2O_2 was combined with UV, constant at 650 mJ/cm^2, and the combination included the H_2O_2/O_3/UV at 3 g/m^3/3 g/m^3/650mJ/cm^2.

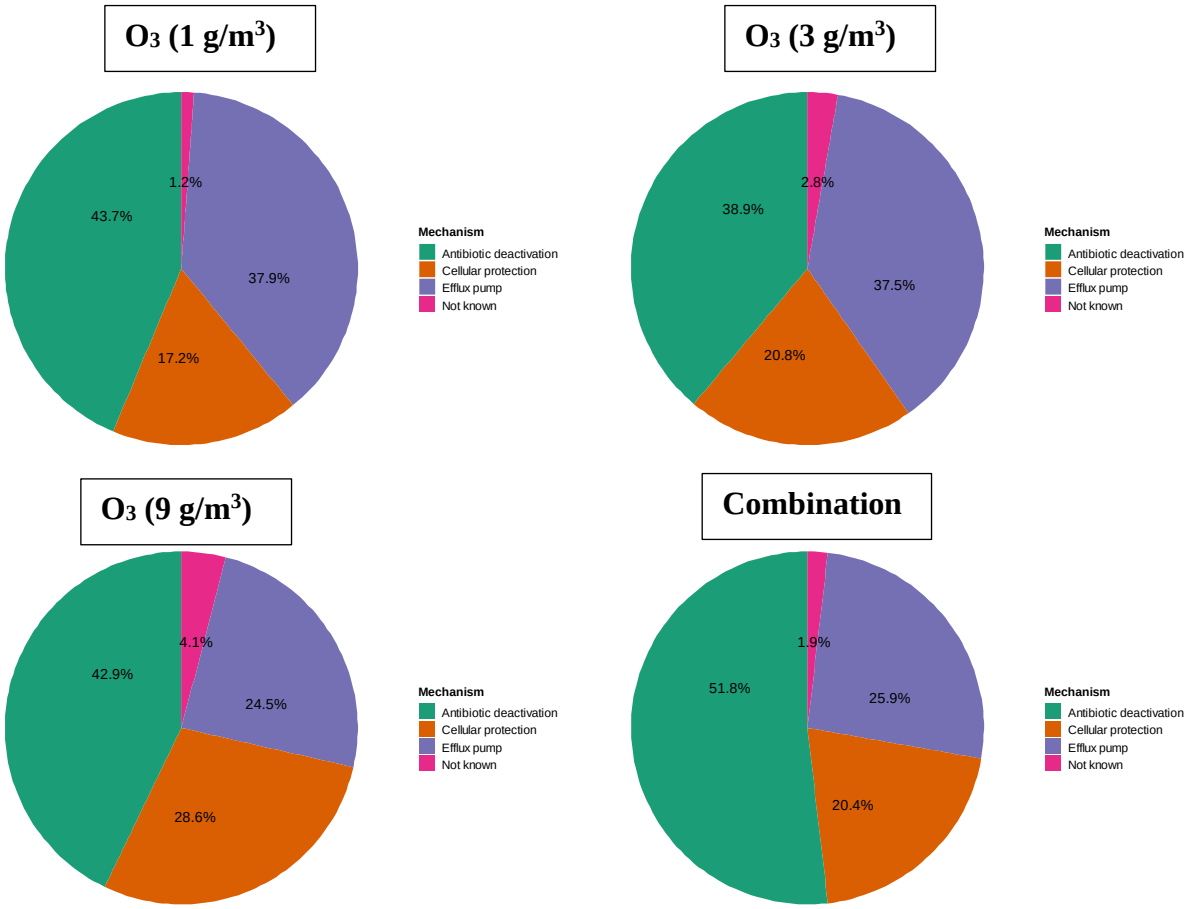

Figure B.2(cont.) Proportion of ARGs resistance mechanisms in influent (primary settled sewage), feedwater (tertiary filtered discharge) and all AOPs conditions. H_2O_2 was combined with UV, constant at 650 mJ/cm^2, and the combination included the H_2O_2/ O_3/UV at 3 g/m^3 / 3 g/m^3 / 650 mJ/cm^2.

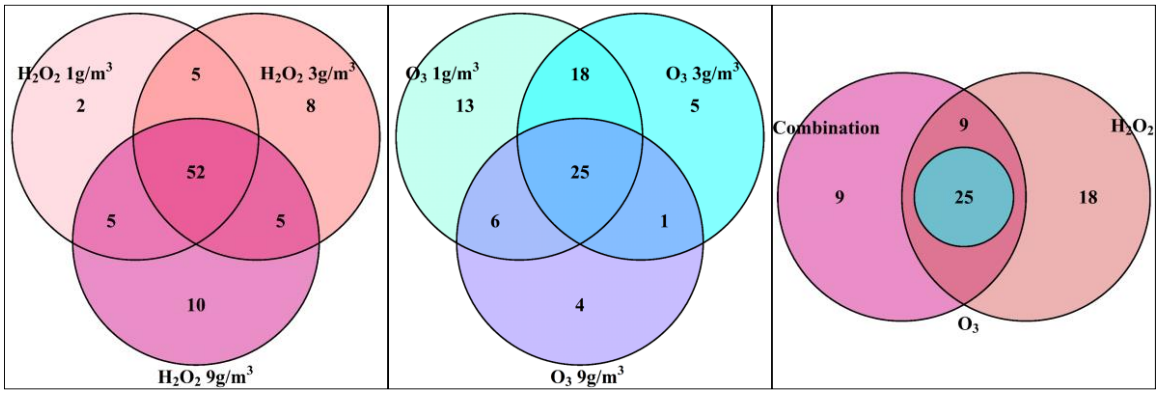

Figure B. 3 Venn diagrams showing common genes among all H_2O_2/UV and O_3 conditions as well as all AOPs from left to the right. The number located in the intersection of all treatment steps shows persistent genes that were not removed by any tested dose or advanced oxidation treatment.

Table B. 1 Water quality parameters of AOP effluents and the corresponding feedwater. Values are averages ± standard deviation from three sampling days. H_2O_2 was combined with UV, always constant at 650 mJ/cm^2 and the combination include the O_3/H_2O_2/UV at 3 g/m^3/3 g/m^3/650 mJ/cm^2.

Treatment stage	TOC (mg/L)	Conductivity (µS/cm)	pH	Suspended Solids (mg/L)	Turbidity (FTU)	UV transmission (UVT %)
Feedwater	6.00 ± 0.00	922.33 ± 17.67	7.40 ± 0.00	2.25 ± 0.35	0.65 ± 0.08	25.27 ± 0.29
Control	5.93 ± 0.12	921.67 ± 16.17	7.47 ± 0.06	<1.9	0.66 ± 0.05	25.33 ± 0.21
Feedwater	5.53 ± 0.06	913.67 ± 20.65	7.40 ± 0.00	<1.9	0.64 ± 0.03	26.30 ± 1.27
H_2O_2/UV (1 g/m^3)	5.63 ± 0.06	911.33 ± 20.31	7.47 ± 0.06	<1.9	0.58 ± 0.12	28.50 ± 1.70
Feedwater	5.73 ± 0.06	872.00 ± 18.68	7.40 ± 0.00	5.25 ± 4.60	0.56 ± 0.05	25.73 ± 1.19
H_2O_2/UV (3 g/m^3)	5.63 ± 0.15	874.00 ± 18.52	7.50 ± 0.10	2.75 ± 0.35	0.56 ± 0.03	28.00 ± 0.60
Feedwater	5.80 ± 0.00	896.00 ± 8.72	7.40 ± 0.00	3.25 ± 1.06	0.60 ± 0.10	26.43 ± 1.32
H_2O_2/UV (9 g/m^3)	5.73 ± 0.15	895.33 ± 9.24	7.40 ± 0.00	<1.9	0.66 ± 0.17	28.83 ± 1.66
Feedwater	5.67 ± 0.06	912.00 ± 21.38	7.40 ± 0.00	3.75 ± 2.47	0.60 ± 0.05	26.55 ± 1.34

Treatment stage	TOC (mg/L)	Conductivity (µS/cm)	pH	Suspended Solids (mg/L)	Turbidity (FTU)	UV transmission (UVT %)
O_3 (1 g/m^3)	5.73 ± 0.06	913.00 ± 20.42	7.50 ± 0.00	3.00 ± 1.41	0.52 ± 0.14	38.15 ± 1.06
Feedwater	5.67 ± 0.12	874.67 ± 21.01	7.37 ± 0.06	3.75 ± 0.35	0.52 ± 0.04	26.13 ± 0.47
O_3 (3 g/m^3)	5.63 ± 0.15	875.67 ± 16.01	7.50 ± 0.00	2.50 ± 0.00	0.40 ± 0.12	41.53 ± 0.68
Feedwater	5.83 ± 0.15	893.67 ± 10.79	7.33 ± 0.06	<1.9	0.61 ± 0.08	26.63 ± 1.06
O_3 (9 g/m^3)	5.80 ± 0.10	894.33 ± 9.29	7.40 ± 0.10	<1.9	0.36 ± 0.09	50.77 ± 0.60
Feedwater	5.93 ± 0.21	921.67 ± 18.93	7.37 ± 0.06	<1.9	0.65 ± 0.06	25.23 ± 0.32
Combination	5.80 ± 0.00	921.00 ± 20.07	7.50 ± 0.00	<1.9	0.50 ± 0.04	41.83 ± 0.86

Table B. 2 Water quality parameters of AOP effluents and the corresponding feedwater. Values are averages ± standard deviation from three sampling days. H_2O_2 was combined with UV, always constant at 650 mJ/cm^2 and the combination include the O_3/H_2O_2/UV at 3 g/m^3/3 g/m^3/650 mJ/cm^2.

Treatment stage	Alkalinity (mg/L)	Ammoniacal Nitrogen (mg/L)	Manganese (µg/L)	NO_3^-- N (mg/L)	NO_2^-- N (mg/L)	Total Oxidised Nitrogen - N (mg/L)
Feedwater	220.33 ± 8.62	0.03 ± 0.01	15.57 ± 1.55	22.80 ± 0.36	<0.0080	22.80 ± 0.36
Control	220.00 ± 8.54	0.03 ± 0.01	17.00 ± 1.32	22.43 ± 0.46	<0.0080	22.43 ± 0.46
Feedwater	231.00 ± 2.83	<0.02	13.87 ± 2.57	20.20 ± 0.42	<0.0080	20.35 ± 0.21
H_2O_2/UV (1 g/m^3)	230.50 ± 2.12	<0.02	12.17 ± 0.40	20.10 ± 0.42	<0.0080	20.05 ± 0.35
Feedwater	220.33 ± 14.36	<0.02	14.27 ± 2.58	19.87 ± 1.36	<0.0080	19.87 ± 1.36
H_2O_2/UV (3 g/m^3)	219.33 ± 13.43	<0.02	14.53 ± 2.67	19.70 ± 1.35	0.01 ± 0.00	19.70 ± 1.35
Feedwater	229.33 ± 12.74	1.01 ± 1.39	27.80 ± 14.52	18.27 ± 5.04	1.06 ± 1.48	18.97 ± 3.84
H_2O_2/UV (9 g/m^3)	230.00 ± 14.80	0.64 ± 1.02	28.37 ± 14.59	18.20 ± 4.54	0.08 ± 0.12	18.27 ± 4.43

Treatment stage	Alkalinity (mg/L)	Ammoniacal Nitrogen (mg/L)	Manganese (µg/L)	NO_3^-- N (mg/L)	NO_2^-- N (mg/L)	Total Oxidised Nitrogen - N (mg/L)
Feedwater	229.50 ± 0.71	<0.02	12.60 ± 0.66	20.20 ± 0.42	<0.0080	20.20 ± 0.42
O_3 (1 g/m^3)	230.00 ± 4.24	<0.02	12.20 ± 0.75	20.40 ± 0.28	0.01 ± 0.00	20.25 ± 0.49
Feedwater	219.67 ± 14.47	<0.02	12.90 ± 2.15	19.63 ± 1.40	<0.0080	19.63 ± 1.40
O_3 (3 g/m^3)	219.00 ± 11.53	0.03 ± 0.01	13.93 ± 2.81	19.80 ± 1.67	<0.0080	19.80 ± 1.67
Feedwater	229.00 ± 12.17	0.64 ± 1.01	26.23 ± 12.76	18.53 ± 4.92	<0.0080	18.60 ± 4.80
O_3 (9 g/m^3)	228.33 ± 13.65	0.97 ± 1.24	27.40 ± 11.74	18.17 ± 4.20	<0.0080	18.17 ± 4.20
Feedwater	219.00 ± 8.54	0.03 ± 0.01	15.70 ± 1.68	22.47 ± 0.25	<0.0080	22.47 ± 0.25
Combination	220.33 ± 8.50	0.08 ± 0.02	15.20 ± 0.92	22.13 ± 0.57	0.01 ± 0.00	22.20 ± 0.62

Table B. 3 Multiple comparisons of means (Games-Howell post hoc test). Pairwise comparison of total ARG and MGE absolute abundances, total ARG and MGE relative abundances normalized per bacterial genome, and ARG and MGE diversities of all primary settled sewage samples collected in 12 different sampling days. Statistical significance is noted by p-values (in bold); p-value less than 0.05 shows significant differences between pairs.

Multiple Comparisons of Means : Games-Howell post hoc test (p-values)			
Pairs	**Absolute abundances**	**Relative abundances**	**Diversities**
SS10 vs SS1	1	1	1
SS11 vs SS1	1	0.99	1
SS12 vs SS1	1	1	1
SS2 vs SS1	1	1	1
SS3 vs SS1	1	1	1
SS4 vs SS1	0.97	1	1
SS5 vs SS1	1	1	1
SS6 vs SS1	1	1	1
SS7 vs SS1	1	1	1
SS8 vs SS1	1	0.99	1
SS9 vs SS1	0.99	1	1
SS11 vs SS10	1	0.97	1
SS12 vs SS10	1	1	1
SS2 vs SS10	1	1	1
SS3 vs SS10	1	1	1
SS4 vs SS10	1	1	1
SS5 vs SS10	0.93	1	1
SS6 vs SS10	1	0.99	1
SS7 vs SS10	1	1	1
SS8 vs SS10	1	0.97	1
SS9 vs SS10	1	1	1
SS12 vs SS11	1	1	1
SS2 vs SS11	1	1	1
SS3 vs SS11	1	1	1
SS4 vs SS11	1	1	1
SS5 vs SS11	0.87	1	1
SS6 vs SS11	1	1	1
SS7 vs SS11	0.99	1	1
SS8 vs SS11	1	1	1
SS9 vs SS11	1	1	1
SS2 vs SS12	1	1	1
SS3 vs SS12	1	1	1
SS4 vs SS12	1	1	1
SS5 vs SS12	0.92	1	1

Multiple Comparisons of Means : Games-Howell post hoc test (p-values)			
Pairs	**Absolute abundances**	**Relative abundances**	**Diversities**
SS6 vs SS12	1	1	1
SS7 vs SS12	1	1	1
SS8 vs SS12	1	1	1
SS9 vs SS12	1	1	1
SS3 vs SS2	1	1	1
SS4 vs SS2	1	1	1
SS5 vs SS2	0.88	1	1
SS6 vs SS2	1	1	1
SS7 vs SS2	0.99	1	1
SS8 vs SS2	1	1	1
SS9 vs SS2	1	1	1
SS4 vs SS3	0.99	1	1
SS5 vs SS3	0.99	1	1
SS6 vs SS3	1	1	1
SS7 vs SS3	1	1	1
SS8 vs SS3	1	1	1
SS9 vs SS3	1	1	1
SS5 vs SS4	0.75	1	1
SS6 vs SS4	1	1	1
SS7 vs SS4	0.94	1	1
SS8 vs SS4	1	1	1
SS9 vs SS4	1	1	1
SS6 vs SS5	0.93	1	1
SS7 vs SS5	1	1	1
SS8 vs SS5	0.98	1	1
SS9 vs SS5	0.82	1	1
SS7 vs SS6	1	1	1
SS8 vs SS6	1	1	1
SS9 vs SS6	1	1	1
SS8 vs SS7	1	1	1
SS9 vs SS7	0.97	1	1
SS9 vs SS8	1	1	1

Table B. 4 Multiple comparisons of means (Games-Howell post hoc test). Pairwise comparison of total ARG and MGE absolute abundances, total ARG and MGE relative abundances per bacterial cell, and ARG and MGE diversities of all feedwater (tertiary filtered discharge) collected in 12 different sampling days. Statistical significance is noted by p-values (in bold); p-value less than 0.05 shows significant differences between pairs.

Multiple Comparisons of Means : Games-Howell post hoc test (p-values)			
Pairs	**Absolute abundances**	**Relative abundances**	**Diversities**
FW_C vs FW_A	0.99	1	1
FW_E vs FW_A	0.98	1	1
FW_G vs FW_A	0.64	1	1
FW_J vs FW_A	0.98	1	1
FW_K vs FW_A	0.02	1	1
FW_M vs FW_A	0.01	0.86	1
FW_O vs FW_A	<.01	1	1
FW_Q vs FW_A	<.01	0.62	1
FW_S vs FW_A	<.01	0.28	1
FW_U vs FW_A	<.01	1	1
FW_X vs FW_A	<.01	1	1
FW_E vs FW_C	1	1	1
FW_G vs FW_C	1	1	1
FW_J vs FW_C	1	1	1
FW_K vs FW_C	0.14	1	1
FW_M vs FW_C	0.04	0.98	1
FW_O vs FW_C	<.01	1	1
FW_Q vs FW_C	<.01	0.85	1
FW_S vs FW_C	<.01	0.51	1
FW_U vs FW_C	<.01	1	1
FW_X vs FW_C	<.01	1	1
FW_G vs FW_E	1	1	1
FW_J vs FW_E	1	1	1
FW_K vs FW_E	0.09	1	1
FW_M vs FW_E	0.02	0.91	1
FW_O vs FW_E	<.01	1	1
FW_Q vs FW_E	<.01	0.68	1
FW_S vs FW_E	<.01	0.3	1
FW_U vs FW_E	<.01	1	1
FW_X vs FW_E	<.01	1	1
FW_J vs FW_G	1	0.99	1
FW_K vs FW_G	0.44	1	1
FW_M vs FW_G	0.12	1	1
FW_O vs FW_G	<.01	1	1

Multiple Comparisons of Means : Games-Howell post hoc test (p-values)

Pairs	Absolute abundances	Relative abundances	Diversities
FW_Q vs FW_G	<.01	0.98	1
FW_S vs FW_G	<.01	0.75	1
FW_U vs FW_G	<.01	1	1
FW_X vs FW_G	0.02	1	1
FW_K vs FW_J	0.06	1	1
FW_M vs FW_J	0.01	0.72	1
FW_O vs FW_J	<.01	0.99	1
FW_Q vs FW_J	<.01	0.43	1
FW_S vs FW_J	<.01	0.14	1
FW_U vs FW_J	<.01	0.99	1
FW_X vs FW_J	<.01	1	1
FW_M vs FW_K	1	1	1
FW_O vs FW_K	0.05	1	1
FW_Q vs FW_K	<.01	0.97	1
FW_S vs FW_K	<.01	0.71	1
FW_U vs FW_K	<.01	1	1
FW_X vs FW_K	0.89	1	1
FW_O vs FW_M	0.18	1	1
FW_Q vs FW_M	<.01	1	1
FW_S vs FW_M	<.01	0.99	1
FW_U vs FW_M	<.01	1	1
FW_X vs FW_M	1	0.96	1
FW_Q vs FW_O	0.09	0.99	1
FW_S vs FW_O	0.11	0.89	1
FW_U vs FW_O	0.82	1	1
FW_X vs FW_O	0.62	1	1
FW_S vs FW_Q	1	1	1
FW_U vs FW_Q	0.71	0.99	1
FW_X vs FW_Q	<.01	0.77	1
FW_U vs FW_S	0.82	0.83	1
FW_X vs FW_S	<.01	0.37	1
FW_X vs FW_U	0.01	1	1

Table B. 5 Number of detected genes per treatment step grouped by ARG and MGE classes. AOPs and control values are averages ± standard deviation form three sampling days, whereas influent and feedwater are averages from twelve sampling days. H_2O_2 was combined with UV, always constant at 650 mJ/cm^2 and combination include the H_2O_2/O_3/UV (3 g/m^3/3 g/m^3/650 mJ/cm^2).

Numbers of antibiotic resistance genes, transposases and integrases										
Class	**Influent**	**Feedwater**	**Control**	**H_2O_2 (1 g/m^3)**	**H_2O_2 (3 g/m^3)**	**H_2O_2 (9 g/m^3)**	**O_3 (1 g/m^3)**	**O_3 (3 g/m^3)**	**O_3 (9 g/m^3)**	**Combination**
Total ARGs	**129.67**± 2.6	**78.22**± 1.50	**69.67**± 2.08	**64.00**± 6.08	**72.33**± 5.77	**70.00**± 2.65	**65.67**± 3.06	**50.33**± 5.69	**37.00**± 0.00	**44.00**± 0.00
***FCA**	**4.67**± 1.00	**3.11**± 0.93	**2.33**± 0.58	**4.00**± 0.00	**3.33**± 0.58	**4.00**± 1.00	**3.33**± 1.15	**1.67**± 0.58	**0.00**± 0.00	**1.00**± 0.00
Aminoglycoside	**14.44**± 1.42	**11.44**± 1.01	**11.33**± 1.53	**8.33**± 0.58	**13.33**± 2.08	**9.67**± 0.58	**8.00**± 1.73	**8.00**± 0.00	**4.00**± 0.00	**8.00**± 0.00
B-Lactams	**23.00**± 1.94	**14.44**± 1.24	**12.67**± 1.15	**13.33**± 2.89	**13.00**± 1.00	**13.67**± 1.15	**12.67**± 1.53	**7.00**± 1 .00	**5.00**± 0.00	**9.00**± 0.00
****MLSB**	**21.78**± 2.33	**10.00**± 1.00	**8.67**± 0.58	**8.67**± 0.58	**10.00**± 1.00	**9.67**± 0.58	**8.67**± 0.58	**8.00**± 0.00	**9.00**± 0.00	**7.00**± 0.00
Non specific	**18.44** ± 2.4	**16.56**± 1.33	**13.67**± 1.53	**14.00**± 1.00	**15.00**± 1.00	**13.67**± 2.52	**17.00**± 2.00	**10.00**± 2.65	**5.00**± 0.00	**5.00**± 0.00
Other	**3.00**± 0.00	**3.22**± 0.44	**3.00**± 0.00	**2.00**± 1.00	**2.00**± 0.00	**3.00**± 1.00	**1.00**± 0.00	**1.33**± 0.58	**1.00**± 0.00	**0.00**± 0.00
Sulfonamide	**4.89**± 0.33	**2.44**± 1.33	**1.00**± 0.00	**1.00**± 0.00	**2.00**± 0.00	**1.00**± 0.00	**2.00**± 0.00	**1.67**± 0.58	**1.00**± 0.00	**1.00**± 0.00
Tetracycline	**20.11**± 0.93	**13.56**± 1.33	**13.67**± 0.58	**11.67**± 0.58	**10.33**± 0.58	**12.33**± 0.58	**10.33**± 0.58	**11.33**± 1.15	**9.00**± 0.00	**11.00**± 0.00
Vancomycin	**19.33**± 1.66	**3.44**± 0.73	**3.33**± 1.15	**1.00**± 0.00	**3.33**± 1.15	**3.00**± 0.00	**2.67**± 0.58	**1.3**± 0.58	**3.00**± 0.00	**2.00**± 0.00

Integrase	**1.56**± 1.88	**3.22**± 0.97	**3.00**± 1.00	**3.33**± 1.15	**3.67**± 0.58	**2.67**± 0.58	**1.67**± 0.58	**3.00**± 0.00	**1.00**± 0.00	**3.00**± 0.00
Transposase	**7.44**± 0.53	**7.33**± 0.71	**7.00**± 0.00	**8.00**± 0.00	**7.67**± 0.58	**6.67**± 0.58	**7.67**± 0.58	**7.67**± 0.58	**5.00**± 0.00	**6.00**± 0.00

*FCA stands for fluoroquinolone / quinolone / florfenicol / chloramphenicol / amphenicol.
**MLSB stands for macrolide / lincosamide / streptogramin B.

Table B. 6 Multiple comparisons of means (Games-Howell post hoc test). Pairwise comparison of total number of detected ARGs and MGEs in influent (primary settled sewage), feedwater (tertiary filtered discharge), control (no oxidation), and all AOPs conditions. Statistical significance is denoted by p-values; p-value less than 0.05 shows significant differences between pairs. H_2O_2 was combined with UV, always constant at 650 mJ/cm^2 and the combination include the H_2O_2/O_3/UV (3 g/m^3/3 g/m^3/650 mJ/cm^2).

Multiple Comparisons of Means : Games-Howell post hoc test (p-values)												
Comparison	**Total ARGs**	**Aminoglycoside**	**β-lactams**	***FCA**	****MLSB**	**Non specific**	**Other**	**Sulfonamide**	**Tetracycline**	**Vancomycin**	**Transposase**	**Integrase**
Feedwater vs Influent	<0.01	0.02	<0.01	0.07	<0.01	0.25	1	<0.01	<0.01	<0.01	0.62	0.32
Feedwater vs Control	0.99	1	0.8	0.86	0.32	0.47	0.66	0.06	1	1	1	0.3
Feedwater vs H_2O_2 (1 g/m^3)	0.65	0.02	0.99	0.20	0.29	0.28	0.63	0.05	0.12	<0.01	0.07	0.99
Feedwater vs H_2O_2 (3 g/m^3)	0.98	0.77	0.93	0.98	0.99	0.76	0.06	0.82	0.02	0.99	0.99	0.82

Multiple Comparisons of Means : Games-Howell post hoc test (p-values)												
Comparison	**Total ARGs**	**Aminoglycoside**	**β-lactams**	***FCA**	****MLSB**	**Non specific**	**Other**	**Sulfonamide**	**Tetracycline**	**Vancomycin**	**Transposase**	**Integrase**
Secondary effluent vs H_2O_2 (9 g/m^3)	0.93	0.18	0.99	0.77	0.99	0.76	0.99	0.05	0.33	0.99	0.49	0.82
Feedwater vs O_3 (1 g/m^3)	0.77	0.32	0.92	0.99	0.29	0.98	<0.01	0.82	0.02	0.90	0.99	0.19
Feedwater vs O_3 (3 g/m^3)	0.02	0.04	<0.01	0.24	<0.01	0.24	0.12	0.77	0.26	0.14	0.99	0.81
Feedwater vs O_3 (9 g/m^3)	< 0.01	<0.01	0.01	0.02	0.02	<0.01	<0.01	0.05	0.03	0.99	0	<0.01
Feedwater vs Combination	0.01	0.04	0.04	0.06	<0.01	<0.01	<0.01	0.05	0.09	0.01	<0.01	0.81

Multiple Comparisons of Means : Games-Howell post hoc test (p-values)												
Comparison	**Total ARGs**	**Aminoglycoside**	**β-lactams**	***FCA**	****MLSB**	**Non specific**	**Other**	**Sulfonamide**	**Tetracycline**	**Vancomycin**	**Transposase**	**Integrase**
H_2O_2 (3 g/m^3) vs H_2O_2 (1 g/m^3)	0.96	0.21	0.99	0.55	0.61	0.91	1	<0.01	0.31	0.31	0.93	0.99
H_2O_2 (9 g/m^3) vs H_2O_2 (1 g/m^3)	0.99	0.31	0.99	0.99	0.55	0.99	0.91	1	0.85	<0.01	0.23	0.97
H_2O_2 (9 g/m^3) vs H_2O_2 (3 g/m^3)	1	0.37	0.99	0.96	0.99	0.98	0.74	<0.01	0.10	0.99	0.55	0.55
O_3 (3 g/m^3) vs O_3 (1 g/m^3)	0.68	0.99	0.065	0.53	0.67	0.17	0.97	0.93	0.86	0.31	1	0.23
O_3	0.11	0.25	0.06	0.17	0.93	0.04	1	<0.01	0.25	0.93	0.07	0.67

Multiple Comparisons of Means : Games-Howell post hoc test (p-values)												
Comparison	**Total ARGs**	**Aminoglycoside**	**β-lactams**	***FCA**	****MLSB**	**Non specific**	**Other**	**Sulfonamide**	**Tetracycline**	**Vancomycin**	**Transposase**	**Integrase**
(O_3 (9 g/m^3) vs O_3 (1 g/m^3))												
(O_3 (9 g/m^3) vs O_3 (3 g/m^3))	0.65	<0.01	0.31	0.18	<0.01	0.34	0.97	0.67	0.31	0.16	0.07	<0.01
(O_3 (3 g/m^3) vs H_2O_2 (3 g/m^3))	0.31	0.21	0.01	0.18	0.31	0.33	0.60	0.93	0.86	0.39	1	0.67
(H_2O_2 (3 g/m^3) vs Combination)	0.16	0.21	0.09	0.09	0.15	0.01	<0.01	<0.01	0.60	0.65	0.17	0.67
(O_3 (3 g/m^3) vs Combination)	0.98	1	0.30	0.74	<0.01	0.34	0.25	0.67	0.99	0.60	0.17	1

*FCA stands for fluoroquinolone / quinolone / florfenicol / chloramphenicol / amphenicol. **MLSB stand for macrolide / lincosamide / streptogramin B.

Table B. 7 Unique genes detected only in the treatment steps they are enlisted and not in previous treatment steps. H_2O_2 was combined with UV, always constant at 650 mJ/cm^2 and combination include the H_2O_2/O_3/UV (3 g/m^3/3 g/m^3/650 mJ/cm^2).

	Treatment stages						
Tertiary filtered discharge	**H_2O_2 (1 g/m^3)**	**H_2O_2 (3 g/m^3)**	**H_2O_2 (9 g/m^3)**	**O_3 (1 g/m^3)**	**O_3 (3 g/m^3)**	**O_3 (9 g/m^3)**	**Combination**
bl1_mox(cmy)	pncA	aac3vi	aac3vi	adea	bl2a_iii	oprd	aac3vi
bl3_imp	-	emrd	carB	bl1_sm	cmx(A)	-	-
ceoA	-	cmx(A)	-	bl3_l	mdtE/yhiU	-	-
cmx(A)	-	-	-	oprd	teta	-	-
intI-1(clinic)	-	-	-	-	pncA	-	-
pncA	-	-	-	-	-	-	-
transposase	-	-	-	-	-	-	-
vanhb	-	-	-	-	-	-	-

Table B. 8 List with persistent genes in final effluents. Persistent genes refer to ARGs that entered the main WWTP and they were not removed by any advance oxidation treatment in all H_2O_2/UV and O_3 doses, as well as in all AOPs (H_2O_2/UV, O_3, the combination). ARGs are classified according to antibiotic they encode resistance and the resistance mechanism.

H_2O_2/UV	O_3	AOPs	Class	Resistance Mechanism
aadA5	aadA5	aadA5	Aminoglycoside	Antibiotic deactivation
ant2ia	ant2ia	ant2ia	Aminoglycoside	Antibiotic deactivation
ant3ia	ant3ia	ant3ia	Aminoglycoside	Antibiotic deactivation
bl2d_oxa1/bl2d_oxa30	bl2d_oxa1/bl2d_oxa30	bl2d_oxa1/bl2d_oxa30	B-lactams	Antibiotic deactivation
bl2d_oxa10	bl2d_oxa10	bl2d_oxa10	B-lactams	Antibiotic deactivation
erea	erea	erea	MLSB	Antibiotic deactivation
ermb	ermb	ermb	MLSB	Cellular protection
ermf	ermf	ermf	MLSB	Cellular protection
lnub	lnub	lnub	MLSB	Antibiotic deactivation
qacEdelta1	qacEdelta1	qacEdelta1	Non specific	Efflux pump
qacH	qacH	qacH	Non specific	Efflux pump
sul2	sul2	sul2	Sulfonamide	Cellular protection
tet32	tet32	tet32	Tetracycline	Cellular protection
tetb	tetb	tetb	Tetracycline	Efflux pump
tetg	tetg	tetg	Tetracycline	Efflux pump
tetm	tetm	tetm	Tetracycline	Cellular protection
teto	teto	teto	Tetracycline	Cellular protection
tetpa	tetpa	tetpa	Tetracycline	Efflux pump
tetpb	tetpb	tetpb	Tetracycline	Cellular protection
tetq	tetq	tetq	Tetracycline	Cellular protection
tetx	tetx	tetx	Tetracycline	Other
Tn23	Tn23	Tn23	Transposase	Transposase
Tn24	Tn24	Tn24	Transposase	Transposase
Tn25	Tn25	Tn25	Transposase	Transposase
tnpA	tnpA	tnpA	Transposase	Transposase
aac6ib	-	-	Aminoglycoside	Antibiotic deactivation
aac6iia	-	-	Aminoglycoside	Antibiotic deactivation
acrA	-	-	Non specific	Efflux pump
acrb	-	-	Non specific	Efflux pump
acrF	-	-	Non specific	Efflux pump
acrR	-	-	Non specific	Efflux pump
aph3ia	-	-	Aminoglycoside	Antibiotic deactivation
aph3iiia	-	-	Aminoglycoside	Antibiotic deactivation
baca	-	-	Other	Antibiotic deactivation
bl1_cmy2	-	-	B-lactams	Antibiotic deactivation
bl2_ges	-	-	B-lactams	Antibiotic deactivation
bl2_veb	-	-	B-lactams	Antibiotic deactivation

H_2O_2/UV	O_3	AOPs	Class	Resistance Mechanism
bl2b_tem1	-	-	B-lactams	Antibiotic deactivation
bl2be_ctxm	-	-	B-lactams	Antibiotic deactivation
bl2e_cfxa	-	-	B-lactams	Antibiotic deactivation
catb3	-	-	FCA	Antibiotic deactivation
catb8	-	-	FCA	Antibiotic deactivation
matA/mel	-	-	MLSB	Efflux pump
tete	-	-	Tetracycline	Efflux pump
tetR	-	-	Tetracycline	Efflux pump
Tn22	-	-	Transposase	Transposase
tolc	-	-	Non specific	Efflux pump
tp614	-	-	Transposase	Transposase
ttgB	-	-	Non specific	Efflux pump
yceE/mdtG	-	-	Non specific	Efflux pump
yceL/mdtH	-	-	Non specific	Efflux pump
yidy/mdtl	-	-	Non specific	Efflux pump

Table B. 9 Comparisons of ARGs and MGEs absolute concentrations in feedwater and the corresponding influent. Standard deviation (SD) represent data from three sampling days, and significant differences between influent and feedwater were examined in pairwise comparisons using the Games-Howell post hoc test. The 'Trend' column shows whether the concentration in the feedwater was decreased significantly (↓). Statistical significance is noted by p-values (in bold); p-value less than 0.05 shows significant differences between pairs.

	Influent		**Feedwater**			
Type	**Average**	sd	**Average**	sd	**p-value**	**Trend**
ARGs	$\mathbf{1.41 \cdot 10^8}$	$3.02 \cdot 10^7$	$\mathbf{6.06 \cdot 10^5}$	$1.17 \cdot 10^5$	**< 0.01**	↓
MGEs	$\mathbf{8.59 \cdot 10^7}$	$2.15 \cdot 10^7$	$\mathbf{3.91 \cdot 10^5}$	$7.18 \cdot 10^4$	**< 0.01**	↓
ARGs	$\mathbf{1.37 \cdot 10^8}$	$7.80 \cdot 10^7$	$\mathbf{4.18 \cdot 10^5}$	$1.85 \cdot 10^5$	**< 0.01**	↓
MGEs	$\mathbf{8.14 \cdot 10^7}$	$4.73 \cdot 10^7$	$\mathbf{3.08 \cdot 10^5}$	$1.20 \cdot 10^5$	**0.01**	↓
ARGs	$\mathbf{1.43 \cdot 10^8}$	$3.46 \cdot 10^7$	$\mathbf{1.16 \cdot 10^5}$	$9.79 \cdot 10^4$	**< 0.01**	↓
MGEs	$\mathbf{8.19 \cdot 10^7}$	$2.04 \cdot 10^7$	$\mathbf{7.71 \cdot 10^4}$	$5.02 \cdot 10^4$	**0.01**	↓
ARGs	$\mathbf{1.19 \cdot 10^8}$	$3.89 \cdot 10^6$	$\mathbf{7.98 \cdot 10^4}$	$7.25 \cdot 10^4$	**< 0.01**	↓
MGEs	$\mathbf{7.57 \cdot 10^7}$	$5.33 \cdot 10^6$	$\mathbf{6.22 \cdot 10^4}$	$4.72 \cdot 10^4$	**< 0.01**	↓

Table B. 10 Data corresponding to Figure 4.5 for each treatment step and type. Minimum value (Min.), first quartile (1st Qu.) of data, median, mean, third quartile (3rd Qu.) of data and maximum value (Max.) are presented in the table in this order. H_2O_2 was combined with UV, always constant at 650 mJ/cm^2 and combination include the H_2O_2/O_3/UV (3 g/m^3/3 g/m^3/650 mJ/cm^2).

Treatment	Type	Min.	1st Qu.	Median	Mean	3rd Qu.	Max.
Feedwater	**ARGs**	-0.33	2.04	2.53	**2.50**	3.02	4.18
	MGEs	1.19	2.11	2.59	**2.64**	3.16	4.06
1 g/m^3 H_2O_2	**ARGs**	-0.21	0.31	0.48	**0.52**	0.72	1.62
	MGEs	0.17	0.35	0.65	**0.63**	0.83	1.33
3 g/m^3 H_2O_2	**ARGs**	-1.30	-0.12	0.12	**0.09**	0.30	0.90
	MGEs	-0.21	0.14	0.28	**0.29**	0.43	1.22
9 g/m^3 H_2O_2	**ARGs**	-0.73	-0.07	0.14	**0.16**	0.366	1.50
	MGEs	-0.38	0.13	0.40	**0.37**	0.59	1.17
1 g/m^3 O_3	**ARGs**	-0.28	0.32	0.47	**0.51**	0.70	1.49
	MGEs	0.00	0.41	0.59	**0.64**	0.94	1.29
3 g/m^3 O_3	**ARGs**	-0.33	0.83	1.20	**1.11**	1.45	2.24
	MGEs	0.016	0.62	1.16	**1.13**	1.68	2.27
9 g/m^3 O_3	**ARGs**	0.02	0.64	1.28	**1.25**	1.82	2.49
	MGEs	0.38	0.57	1.80	**1.47**	2.11	2.65
Combination	**ARGs**	-0.61	0.44	1.16	**1.02**	1.52	2.49
	MGEs	-0.52	0.65	1.27	**1.10**	1.57	2.17

Table B. 11 Multiple comparisons of means (Games-Howell post hoc test). Pairwise comparison of ARGs and MGEs removal rates in feedwater (tertiary filtered effluent), and all AOPs conditions. Statistical significance is denoted by p-values; p-value less than 0.05 shows significant differences between pairs. H_2O_2 was combined with UV, always constant at 650 mJ/cm^2 and combination include the H_2O_2/O_3/UV (3 g/m^3/3 g/m^3/650 mJ/cm^2).

Comparison	ARGs	MGEs
Tertiary filtered effluent vs H_2O_2 1 g/m^3	<0.01	<0.01
Tertiary filtered effluent vs H_2O_2 3 g/m^3	<0.01	<0.01
Tertiary filtered effluent vs H_2O_2 9 g/m^3	0	<0.01
Tertiary filtered effluent vs O_3 1 g/m^3	<0.01	0
Tertiary filtered effluent vs O_3 3 g/m^3	<0.01	0
Tertiary filtered effluent vs O_3 9 g/m^3	<0.01	0.03
Tertiary filtered effluent vs Combination	<0.01	<0.01
H_2O_2 3 g/m^3 vs H_2O_2 1 g/m^3	0	<0.01
H_2O_2 9 g/m^3 vs H_2O_2 1 g/m^3	0	0.19
H_2O_2 9 g/m^3 vs H_2O_2 3 g/m^3	0.51	0.99
O_3 3 g/m^3 vs O_3 1 g/m^3	<0.01	0.01
O_3 9 g/m^3 vs O_3 1 g/m^3	<0.01	0.18
O_3 9 g/m^3 vs O_3 3 g/m^3	0.77	0.94
H_2O_2 g/m^3 vs O_3 3 g/m^3	0	<0.01
Combination vs H_2O_2 3 g/m^3	<0.01	0.01
Combination vs O_3 3 g/m^3	0.98	1

Table B. 12 Relative abundances per genome in each treatment stage grouped in ARGs and MGEs classes. AOPs values are averages ± standard deviation from three sampling days, whereas influent and feedwater are averages from twelve sampling days. H_2O_2 was combined with UV, always constant at 650 mJ/cm^2 and combination include the H_2O_2/O_3/UV (3 g/m^3/3 g/m^3/650 mJ/cm^2).

Relative abundances per bacteria genome of resistance genes, transposases and integrases									
Class	**Influent**	**Feedwater**	**1 g/m^3 H_2O_2**	**3 g/m^3 H_2O_2**	**9 g/m^3 H_2O_2**	**1 g/m^3 O_3**	**3 g/m^3 O_3**	**9 g/m^3 O_3**	**Combination**
Total ARGs	**1.004** ± 0.080	**0.275** ± 0.023	**0.188** ± 0.019	**0.196** ± 0.015	**0.186** ± 0.051	**0.302** ± 0.001	**0.138** ± 0.037	**0.12** ± 0.114	**0.06** ± 0.052
***FCA**	**0.004** ± 0.003	**0.022** ± 0.001	**0.014** ± 0.003	**0.017** ± 0.013	**0.007** ± 0.004	**0.061** ± 0.051	**0.029** ± 0.020	**0.000** ± 0.000	**0.000** ± 0.000
Aminoglycoside	**0.077** ± 0.008	**0.041** ± 0.002	**0.049** ± 0.007	**0.053** ± 0.003	**0.050** ± 0.029	**0.041** ± 0.017	**0.017** ± 0.002	**0.024** ± 0.026	**0.016** ± 0.014
β-lactams	**0.05** ± 0.009	**0.03** ± 0.009	**0.022** ± 0.004	**0.024** ± 0.002	**0.024** ± 0.007	**0.036** ± 0.008	**0.016** ± 0.007	**0.010** ± 0.011	**0.011** ± 0.009
****MLSB**	**0.54** ± 0.045	**0.060** ± 0.012	**0.02** ± 0.002	**0.026** ± 0.002	**0.021** ± 0.004	**0.026** ± 0.004	**0.008** ± 0.002	**0.006** ± 0.005	**0.006** ± 0.005
Non specific	**0.092** ± 0.012	**0.067** ± 0.003	**0.039** ± 0.006	**0.035** ± 0.002	**0.037** ± 0.011	**0.087** ± 0.031	**0.016** ± 0.003	**0.034** ± 0.043	**0.011** ± 0.009
Other	**0.004** ± 0.000	**0.002** ± 0.001	**0.001** ± 0.000	**0.00** ± 0.000	**0.001** ± 0.001	**0.001** ± 0.000	**0.000** ± 0.000	**0.001** ± 0.002	**0.000** ± 0.000
Sulfonamide	**0.003** ± 0.000	**0.003** ± 0.000	**0.003** ± 0.002	**0.004** ± 0.000	**0.006** ± 0.005	**0.006** ± 0.002	**0.003** ± 0.001	**0.008** ± 0.009	**0.004** ± 0.004

Relative abundances per bacteria genome of resistance genes, transposases and integrases									
Class	**Influent**	**Feedwater**	**1 g/m^3 H_2O_2**	**3 g/m^3 H_2O_2**	**9 g/m^3 H_2O_2**	**1 g/m^3 O_3**	**3 g/m^3 O_3**	**9 g/m^3 O_3**	**Combination**
Tetracycline	**0.231** ± 0.013	**0.043** ± 0.005	**0.038** ± 0.006	**0.033** ± 0.005	**0.036** ± 0.009	**0.040** ± 0.01	**0.047** ± 0.006	**0.036** ± 0.034	**0.011** ± 0.010
Vancomycin	**0.003** ± 0.000	**0.003** ± 0.000	**0.000** ± 0.000	**0.003** ± 0.001	**0.002** ± 0.000	**0.005** ± 0.003	**0.001** ± 0.000	**0.001** ± 0.001	**0.001** ± 0.001
Integrase	**0.077** ± 0.004	**0.044** ± 0.005	**0.036** ± 0.008	**0.033** ± 0.010	**0.047** ± 0.013	**0.020** ± 0.012	**0.039** ± 0.012	**0.036** ± 0.041	**0.032** ± 0.028
Transposase	**0.552** ± 0.004	**0.126** ± 0.012	**0.080** ± 0.002	**0.084** ± 0.015	**0.085** ± 0.011	**0.105** ± 0.015	**0.069** ± 0.007	**0.109** ± 0.112	**0.071** ± 0.061

*FCA stands for fluoroquinolone / quinolone / florfenicol / chloramphenicol / amphenicol.
**MLSB stand for macrolide / lincosamide / streptogramin B

Table B. 13 Multiple comparisons of means (Games-Howell post hoc test). Pairwise comparison of relative abundances per genome comparing different treatment steps. Statistical significance is denoted by p-values; p-value less than 0.05 shows significant differences between pairs. H_2O_2 was combined with UV, always constant at 650 mJ/cm^2 and combination include the H_2O_2/O_3/UV (3 g/m^3/3 g/m^3/650 mJ/cm^2).

Comparison	Aminoglycoside	β-lactams	FCA	MLSB	Non specific	Other	Sulfonamide	Tetracycline	Vancomycin	Transposase	Integrase
Influent vs Feedwater	0.15	0.76	0.76	<0.01	0.28	<0.01	0.37	<0.01	1	<0.01	0.01
Feedwater vs Control	0.99	0.83	0.59	0.31	0.99	*NA	0.06	0.97	0.99	0.99	0.99
Feedwater vs H_2O_2 1g/m3	0.69	1	0.99	<0.01	1	0.89	0.48	0.99	0.01	<0.01	0.99
Feedwater vs H_2O_2 3g/m3	0.97	0.99	0.99	0.011	0.99	0.35	0.95	0.99	0.99	0.01	0.99
Feedwater vs H_2O_2 9g/m3	0.72	0.99	0.99	<0.01	0.99	1	0.74	1	0.99	0.01	0.70
Feedwater vs O_3 1g/m3	0.69	0.85	0.88	0.09	0.88	0.99	0.88	0.91	0.65	0.99	0.99
Feedwater vs O_3 3g/m^3	0.13	0.99	0.76	<0.01	0.70	0.41	0.89	0.99	0.99	0.24	0.99
Feedwater vs O_3 9g/m3	0.64	0.99	NA	<0.01	0.85	0.72	0.32	0.82	0.89	0.97	0.41

Comparison	Aminoglycoside	β-lactams	FCA	MLSB	Non specific	Other	Sulfonamide	Tetracycline	Vancomycin	Transposase	Integrase
Feedwater vs Combination	0.99	0.99	0.45	<0.01	0.99	NA	0.09	0.03	0.99	0.99	0.93
H_2O_2 3 g/m3 vs H_2O_2 1 g/m3	0.99	0.99	0.99	0.99	0.99	0.87	0.98	0.99	0.17	0.99	0.99
H_2O_2 9 g/m3- H_2O_2 1 g/m3	1	1	0.98	0.99	0.99	0.96	0.97	0.99	0.60	0.98	0.89
H_2O_2 9 g/m3 vs H_2O_2 3 g/m3	0.99	0.99	0.97	0.99	0.99	0.72	0.88	0.99	0.99	0.99	0.72
O_3 3g/m^3 vs O_3 1g/m3	0.05	0.99	0.99	<0.01	0.55	0.70	0.99	1	0.61	0.93	0.99
O_3 9g/m^3 vs O_3 1g/m^3	0.99	0.99	NA	<0.01	0.99	0.75	0.43	0.98	0.40	0.94	0.44
O_3 9g/m3 vs O_3 3 g/m^3	0.18	1	NA	0.99	0.73	0.62	0.37	0.99	0.99	0.83	0.45
H_2O_2 3g/m^3 vs O_3 3 g/m^3	0.12	0.99	0.86	<0.01	0.98	0.99	0.99	0.99	0.99	0.99	0.99
Combination vs H_2O_2 3 g/m^3	0.84	0.99	0.76	0.047	0.99	NA	0.19	0.13	0.98	0.98	0.93

Comparison	Aminoglycoside	β-lactams	FCA	MLSB	Non specific	Other	Sulfonamide	Tetracycline	Vancomycin	Transposase	Integrase
Combination - O_3 3 g/m^3	0.92	0.99	0.68	0.92	0.85	NA	0.12	0.63	0.99	0.95	0.99

*NA = not applied; these samples were excluded from the statistical analysis as genes were not detected in one of the samples.

Table B. 14 Pairwise comparison of genes carried in viable cells against genes carried in total cells. Samples are grouped by treatment process and gene type. T-test was applied in pair samples with normal distribution and equal variances and wilcox-test was used when the assumptions for normality and homoscedasticity were not met. Statistical significance is noted by p-values (in bold); p-value less than 0.05 shows significant differences between pairs. H_2O_2 was combined with UV, always constant at 650 mJ/cm^2 and combination include the H_2O_2/O_3/UV (3 g/m^3/3 g/m^3/650 mJ/cm^2).

Paired sample statistical comparisons (p-values)
Genes in total cells vs genes in viable cells

Treatment process	16S rRNA	*int*1	*tet*Q	*tet*M	*bla*$_{\text{OXA-10}}$
Feedwater	<0.01	0.012	<0.01	<0.01	<0.01
Control	0.042	<0.01	<0.01	<0.01	0.545
H_2O_2/UV (3 g/m^3)	0.065	0.092	<0.01	0.278	0.3401
H_2O_2/UV (9 g/m^3)	0.016	0.079	<0.01	0.016	0.015
O_3 (3 g/m^3)	<0.01	<0.01	<0.01	*NA	<0.01
O_3 (9 g/m^3)	0.261	0.081	0.034	NA	<0.01
Combination	<0.01	0.014	<0.01	NA	NA

*****NA = not applied; these samples were excluded from the statistical analysis as genes in viable cells were not detected.

Table B. 15 Comparisons of gene absolute concentrations in total and viable cells in AOPs effluents and the corresponding feedwater for 16S rRNA, *int*1, *tet*Q, *tet*M, *bla*$_{OXA-10}$. Standard deviation (SD) represent data from three sampling days and significant differences between feedwater and AO treated effluent were examined in pairwise comparisons using the Games-Howell post hoc test. Statistical significance is denoted by p-values; p-value less than 0.05 shows significant difference between pairs.

AOPs	Type	Feedwater	SD	Effluent	SD	p-value
		16S rRNA				
H_2O_2-UV [3 g/m^3 - 6500 J/m^2]	Total	**8.27E+05**	1.52E+05	**2.28E+05**	1.48E+05	**0.08**
	Viable	**3.61E+05**	2.23E+05	**9.48E+04**	8.21E+04	**0.49**
H_2O_2-UV [9 g/m^3 - 6500 J/m^2]	Total	**8.96E+05**	1.60E+05	**1.54E+05**	6.01E+04	**<0.01**
	Viable	**3.06E+05**	9.85E+04	**8.27E+04**	6.29E+04	**0.19**
O_3 [3 g/m^3]	Total	**8.27E+05**	1.52E+05	**1.84E+05**	1.76E+04	**0.23**
	Viable	**3.61E+05**	2.23E+05	**5.61E+04**	2.52E+04	**0.03**
O_3 [9 g/m^3]	Total	**8.96E+05**	1.60E+05	**5.59E+04**	2.47E+04	**<0.01**
	Viable	**3.06E+05**	9.85E+04	**4.52E+04**	1.26E+04	**0.01**
O_3 - H_2O_2-UV [3 g/m^3 – 3 g/m^3 – 6500 J/m^2]	Total	**1.19E+06**	2.25E+05	**7.06E+04**	8.06E+04	**0.01**
	Viable	**3.96E+05**	9.00E+04	**1.30E+04**	9.60E+03	**0.02**
Control	Total	**1.19E+06**	2.25E+05	**1.07E+06**	6.71E+05	**0.99**
	Viable	**3.96E+05**	9.00E+04	**4.03E+05**	2.70E+05	**1**

AOPs	Type	Feedwater	SD	Effluent	SD	p-value
		***int*1**				
H_2O_2-UV [3 g/m^3 - 6500 J/m^2]	Total	**3.04E+04**	2.13E+04	**6.42E+03**	4.29E+03	**0.53**
	Viable	**1.53E+04**	1.42E+04	**3.23E+03**	2.82E+03	**0.74**
H_2O_2-UV [9 g/m^3 - 6500 J/m^2]	Total	**1.96E+04**	5.37E+03	**2.09E+03**	1.03E+03	**<0.01**
	Viable	**9.84E+03**	6.69E+03	**1.35E+03**	5.97E+02	**0.027**
O_3 [3 g/m^3]	Total	**3.04E+04**	2.13E+04	**3.62E+03**	4.97E+02	**0.04**
	Viable	**1.53E+04**	1.42E+04	**1.42E+03**	7.98E+02	**0.06**
O_3 [9 g/m^3]	Total	**1.96E+04**	5.37E+03	**3.11E+03**	2.08E+03	**<0.01**
	Viable	**9.84E+03**	6.69E+03	**1.61E+03**	8.18E+02	**0.02**
O_3 - H_2O_2-UV [3 g/m^3 – 3 g/m^3 - 6500 J/m^2]	Total	**1.02E+04**	1.87E+03	**8.71E+02**	6.69E+02	**0.01**
	Viable	**5.51E+03**	1.63E+03	**2.71E+02**	3.14E+02	**0.04**
Control	Total	**1.02E+04**	1.87E+03	**1.74E+04**	4.13E+03	**0.03**
	Viable	**5.51E+03**	1.63E+03	**5.80E+03**	4.51E+03	**1**

AOPs	Type	Feedwater	SD	Effluent	SD	p-value
		***tet*Q**				
H_2O_2-UV [3 g/m^3 - 6500 J/m^2]	Total	**6.82E+03**	3.59E+03	**8.28E+02**	1.65E+02	**<0.01**
	Viable	**1.84E+03**	1.46E+03	**1.97E+02**	1.95E+02	**0.03**
H_2O_2-UV [9 g/m^3 - 6500 J/m^2]	Total	**1.03E+04**	2.96E+02	**5.90E+02**	1.06E+02	**<0.01**
	Viable	**1.81E+03**	4.63E+02	**2.37E+02**	5.66E+01	**<0.01**
O_3 [3 g/m^3]	Total	**6.82E+03**	3.59E+03	**3.50E+01**	1.44E+01	**<0.01**
	Viable	**1.84E+03**	1.46E+03	**5.93E+00**	4.19E+00	**0.02**
O_3 [9 g/m^3]	Total	**1.03E+04**	2.96E+02	**1.75E+01**	7.38E+00	**<0.01**
	Viable	**1.81E+03**	4.63E+02	**9.52E+00**	6.64E+00	**<0.01**
O_3 - H_2O_2-UV [3 g/m^3 – 3 g/m^3 - 6500 J/m^2]	Total	**5.28E+03**	1.44E+03	**9.00E+01**	9.45E+01	**<0.01**
	Viable	**1.60E+03**	7.67E+02	**6.12E+00**	4.17E+00	**0.037**
Control	Total	**5.28E+03**	1.44E+03	**7.68E+03**	1.35E+03	**0.11**
	Viable	**1.60E+03**	7.67E+02	**1.65E+03**	1.33E+03	**1**

AOPs	Type	Feedwater	SD	Effluent	SD	p-value
		***tet*M**				
H_2O_2-UV [3 g/m^3 - 6500 J/m^2]	Total	**9.70E+03**	4.29E+03	**9.10E+02**	1.06E+03	**0.01**
	Viable	**1.85E+03**	1.16E+03	**1.33E+02**	1.22E+02	**0.05**
H_2O_2-UV [9 g/m^3 - 6500 J/m^2]	Total	**2.47E+04**	1.38E+04	**3.95E+02**	2.42E+02	**<0.01**
	Viable	**1.89E+03**	2.08E+03	**1.52E+02**	1.26E+02	**0.04**
O_3 [3 g/m^3]	Total	**9.70E+03**	4.29E+03	**7.36E+01**	4.04E+01	**0.01**
	Viable	**1.85E+03**	1.16E+03	***NA**	NA	**NA**
O_3 [9 g/m^3]	Total	**2.47E+04**	1.38E+04	**8.91E+01**	5.94E+01	**<0.01**
	Viable	**1.89E+03**	2.08E+03	**NA**	NA	**NA**
O_3 - H_2O_2-UV [3 g/m^3 – 3 g/m^3 - 6500 J/m^2]	Total	**1.05E+04**	8.71E+03	**1.00E+02**	1.03E+02	**0.01**
	Viable	**1.30E+03**	1.54E+03	**NA**	NA	**NA**
Control	Total	**1.05E+04**	8.71E+03	**2.19E+04**	1.42E+04	**0.62**
	Viable	**1.30E+03**	1.54E+03	**1.60E+03**	1.70E+03	**0.99**

AOPs	Type	Feedwater	SD	Effluent	SD	p-value
		***bla*OXA-10**				
H_2O_2-UV [3 g/m^3 - 6500 J/m^2]	Total	**8.58E+03**	2.13E+03	**8.23E+02**	1.18E+03	**<0.01**
	Viable	**4.83E+03**	3.89E+03	**4.75E+02**	2.93E+02	**0.02**
H_2O_2-UV [9 g/m^3 - 6500 J/m^2]	Total	**1.00E+04**	5.98E+03	**4.67E+02**	5.77E+02	**0.01**
	Viable	**2.45E+03**	2.70E+03	**4.93E+01**	3.49E+01	**0.04**
O_3 [3 g/m^3]	Total	**8.58E+03**	2.13E+03	**2.74E+02**	2.56E+02	**<0.01**
	Viable	**4.83E+03**	3.89E+03	**5.00E+01**	3.22E+01	**0.02**
O_3 [9 g/m^3]	Total	**1.00E+04**	5.98E+03	**7.90E+02**	1.15E+03	**0.01**
	Viable	**2.45E+03**	2.70E+03	**3.26E+01**	1.48E+01	**0.04**
O_3 - H_2O_2-UV [3 g/m^3 – 3 g/m^3 - 6500 J/m^2]	Total	**4.72E+03**	2.86E+03	**1.71E+03**	2.38E+03	**0.55**
	Viable	**2.09E+03**	1.13E+03	**NA**	NA	**NA**
Control	Total	**4.72E+03**	2.86E+03	**5.43E+03**	5.39E+03	**0.99**
	Viable	**2.09E+03**	1.13E+03	**2.29E+03**	1.86E+03	**0.99**

*NA = not applied; these samples were excluded from the statistical analysis as genes were not detected in one of the samples.

Table B. 16 Multiple comparisons of means (Games-Howell post hoc test). Pairwise comparison of 16S rRNA, *int*1, *tet*Q, *tet*M and *bla*$_{OXA-10}$ genes absolute concentrations carried by viable bacteria in each treatment step. Statistical significance is noted by p-values (in bold); p-value less than 0.05 shows significant differences between pairs. NA (not applied): these samples were excluded from the statistical analysis as genes were not detected in one of the samples.

Pairs	**16S rRNA**	***int*1**	***tet*Q**	***tet*M**	***bla*$_{OXA-10}$**
Feedwater_3 vs Feedwater_2	0.99	0.99	1	1	0.90
Feedwater_4 vs Control	1	0.99	1	0.99	0.99
Feedwater_4 vs Feedwater_2	0.99	0.85	0.99	0.97	0.71
Feedwater_4 vs Feedwater_3	0.83	0.81	0.99	0.99	0.99
H_2O_2-UV [3 g/m^3 - 6500 J/m^2] vs Feedwater_2	0.49	0.74	0.03	0.05	0.02
H_2O_2-UV [9 g/m^3 - 6500 J/m^2] vs Feedwater_3	0.19	0.02	<0.01	0.04	0.04
H_2O_2-UV [9 g/m^3 - 6500 J/m^2] vs H_2O_2-UV [3 g/m^3 - 6500 J/m^2]	0.99	0.77	0.99	0.99	0.02
O_3- H_2O_2-UV [3 g/m^3 – 3 g/m^3 - 6500 J/m^2] vs Feedwater_4	0.02	0.04	0.03	*NA	NA
O_3- H_2O_2-UV [3 g/m^3 – 3 g/m^3 - 6500 J/m^2] vs H_2O_2-UV [3 g/m^3 - 6500 J/m^2]	0.32	0.36	0.20	NA	NA
O_3 [3 g/m^3] vs Feedwater_2	0.03	0.06	0.02	NA	0.02
O_3 [3 g/m^3] vs H2O2-UV [3 g/m^3 - 6500 J/m^2]	0.93	0.81	0.20	NA	0.03
O_3 [3 g/m^3] vs O3- H_2O_2-UV [3 g/m^3 – 3 g/m^3 - 6500 J/m^2]	0.07	0.15	1	NA	NA
O_3 [9 g/m^3] vs Feedwater_3	0.01	0.02	<0.01	NA	0.04
O_3 [9 g/m^3] vs O_3 [3 g/m^3]	0.97	0.99	0.93	NA	0.87

Table B. 17 Multiple comparisons of means (Games-Howell post hoc test). Pairwise comparison of 16S rRNA, *int*1, *tet*Q, *tet*M and bla_{OXA-10} genes absolute concentrations carried by total bacteria in each treatment step.

Pairs	**16S rRNA**	***int*1**	***tet*M**	***tet*Q**	$\boldsymbol{bla_{OXA-10}}$
Feedwater_3 vs Feedwater_2	0.99	0.96	0.35	0.44	0.99
Feedwater_4 vs Control	0.99	0.31	0.62	0.11	0.99
Feedwater_4 vs Feedwater_2	0.33	0.65	0.99	0.97	0.31
Feedwater_4 vs Feedwater_3	0.49	0.07	0.49	<0.01	0.59
H_2O_2-UV [3 g/m^3 - 6500 J/m^2] vs Feedwater_2	0.08	0.53	0.01	<0.01	<0.01
H_2O_2-UV [9 g/m^3 - 6500 J/m^2] vs Feedwater_3	<0.01	<0.01	<0.01	<0.01	0.01
H_2O_2-UV [9 g/m^3 - 6500 J/m^2] vs H_2O_2-UV [3 g/m^3 - 6500 J/m^2]	0.93	0.40	0.95	0.04	0.99
O_3- H_2O_2-UV [3 g/m^3 – 3 g/m^3 - 6500 J/m^2] vs Feedwater_4	0.01	0.01	0.01	<0.01	0.55
O_3- H_2O_2-UV [3 g/m^3 – 3 g/m^3 - 6500 J/m^2] vs H_2O_2-UV [3 g/m^3 - 6500 J/m^2]	0.41	0.02	0.73	<0.01	0.98
O_3 [3 g/m^3] vs Feedwater_2	0.23	0.04	0.01	<0.01	<0.01
O_3 [3 g/m^3] vs H_2O_2-UV [3 g/m^3 - 6500 J/m^2]	0.99	0.78	0.70	<0.01	0.88
O_3 [3 g/m^3] vs O3- H_2O_2-UV [3 g/m^3 – 3 g/m^3 - 6500 J/m^2]	0.06	<0.01	0.99	0.72	0.782
O_3 [9 g/m^3] vs Feedwater_3	<0.01	<0.01	<0.01	<0.01	0.01
O_3 [9 g/m^3] vs O_3 [3 g/m^3]	<0.01	0.99	0.99	0.10	0.92

Table B. 18 Data corresponding to Figure 4.8 for each treatment condition and gene. Minimum value (Min.), first quartile (1st Qu.) of data, median, mean, third quartile (3rd Qu.) of data and maximum value (Max.) are presented in the table in this order. . H_2O_2 was combined with UV, always constant at 650 mJ/cm^2 and combination include the H_2O_2/O_3/UV (3 g/m^3/3 g/m^3/650 mJ/cm^2).

Treatment stages	**Min.**	**1st Qu.**	**Median**	**Mean**	**3rd Qu.**	**Max.**
16s rRNA						
Feedwater	0.16	0.23	0.34	**0.39**	0.46	0.95
H_2O_2/UV (3 g/m^3)	0.18	0.32	0.48	**0.95**	0.66	3.55
H_2O_2/UV (9 g/m^3)	0.09	0.37	0.43	**0.50**	0.58	0.98
O_3 (3 g/m^3)	0.12	0.18	0.37	**0.31**	0.42	0.44
O_3 (9 g/m^3)	0.30	0.49	0.67	**1.12**	2.05	2.36
Feedwater	0.20	0.28	0.32	**0.39**	0.39	0.95
Combination	0.01	0.25	0.59	**0.48**	0.69	0.88
***bla*$_{\text{OXA-10}}$**						
Feedwater	0.03	0.12	0.21	**0.47**	0.78	1.49
H_2O_2/UV (3 g/m^3)	0.24	0.46	3.25	**5.62**	7.40	21.37

Treatment stages	**Min.**	**1st Qu.**	**Median**	**Mean**	**3rd Qu.**	**Max.**
H_2O_2/UV (9 g/m^3)	0.02	0.10	0.24	**0.49**	0.49	1.95
O_3 (3 g/m^3)	0.05	0.08	0.19	**0.69**	0.31	4.24
O_3 (9 g/m^3)	0.03	0.07	0.13	**0.17**	0.26	0.39
Feedwater	0.16	0.37	0.50	**0.52**	0.68	1.00
Combination	*NA	NA	NA	**NA**	NA	NA
***tet*M**						
Feedwater	0.00	0.017	0.16	**0.20**	0.20	0.75
H_2O_2/UV (3 g/m^3)	0.13	0.14	0.17	**1.25**	0.33	5.48
H_2O_2/UV (9 g/m^3)	0.075	0.13	0.26	**0.72**	1.11	2.71
O_3 (3 g/m^3)	NA	NA	NA	**NA**	NA	NA
O_3 (9 g/m^3)	NA	NA	NA	**NA**	NA	NA
Feedwater	0.00	0.04	0.13	**0.19**	0.17	0.75
Combination	NA	NA	NA	**NA**	NA	NA

Treatment stages	**Min.**	**1st Qu.**	**Median**	**Mean**	**3rd Qu.**	**Max.**
***tet*Q**						
Feedwater	0.11	0.13	0.18	**0.20**	0.24	0.37
H_2O_2/UV (3 g/m^3)	0.05	0.06	0.13	**0.21**	0.34	0.50
H_2O_2/UV (9 g/m^3)	0.25	0.30	0.37	**0.42**	0.55	0.67
O_3 (3 g/m^3)	0.04	0.10	0.22	**0.20**	0.27	0.40
O_3 (9 g/m^3)	0.11	0.31	0.57	**0.61**	0.88	1.25
Feedwater	0.13	0.19	0.27	**0.26**	0.33	0.37
Combination	0.00	0.10	0.16	**0.21**	0.32	0.46
***int*1**						
Feedwater	0.03	0.31	0.47	**0.44**	0.58	0.72
H_2O_2/UV (3 g/m^3)	0.17	0.30	0.56	**0.48**	0.63	0.70
H_2O_2/UV (9 g/m^3)	0.22	0.30	0.57	**0.88**	1.39	2.15
O_3 (3 g/m^3)	0.12	0.20	0.46	**0.37**	0.51	0.57

Treatment stages	Min.	1st Qu.	Median	Mean	3rd Qu.	Max.
O_3 (9 g/m^3)	0.40	0.43	0.49	**0.66**	0.57	1.91
Feedwater	0.26	0.43	0.48	**0.47**	0.57	0.60
Combination	0.00	0.27	0.55	**0.54**	0.80	1.10

*NA = not applied; these samples were excluded from the statistical analysis as genes were not detected in one of the samples.

Table B. 19 Multiple comparisons of means (Games-Howell post hoc test). Pairwise comparison of 16S rRNA, *int*1, *tet*Q, *tet*M and *bla*$_{OXA-10}$ genes viable cells/total cells ratios among treatment steps. Statistical significance is noted by p-values (in bold); p-value less than 0.05 shows significant differences between pairs.

Pairs	**16S rRNA**	***int*1**	***tet*Q**	***tet*M**	***bla*$_{OXA-10}$**
Feedwater_3 vs Feedwater_2	0.99	0.99	0.93	0.65	0.99
Feedwater_4 vs Control	0.99	0.89	0.90	0.99	0.99
Feedwater_4 vs Feedwater_2	0.99	0.88	0.94	0.57	0.99
Feedwater_4 vs Feedwater_3	0.99	0.99	0.11	0.99	0.99
H_2O_2-UV [3 g/m^3 - 6500 J/m^2] vs Feedwater_2	0.98	0.99	0.99	0.92	0.46
H_2O_2-UV [9 g/m^3 - 6500 J/m^2] vs Feedwater_3	0.85	0.75	0.01	0.39	0.99
H_2O_2-UV [9 g/m^3 - 6500 J/m^2] vs H2O2-UV [3 g/m^3 - 6500 J/m^2]	0.98	0.78	0.24	0.99	0.44
O_3- H_2O_2-UV [3 g/m^3 – 3 g/m^3 - 6500 J/m^2] vs Feedwater_4	0.94	1	0.96	*NA	NA
O_3- H_2O_2-UV [3 g/m^3 – 3 g/m^3 - 6500 J/m^2] vs H2O2-UV [3 g/m^3 - 6500 J/m^2]	0.98	0.99	1	NA	NA
O_3 [3 g/m^3] vs Feedwater_2	0.98	0.99	0.99	NA	0.99
O_3 [3 g/m^3] vs H_2O_2-UV [3 g/m^3 - 6500 J/m^2]	0.92	0.99	1	NA	0.50
O_3 [3 g/m^3] vs O_3- H_2O_2-UV [3 g/m^3 – 3 g/m^3 - 6500 J/m^2]	0.94	0.98	1	NA	NA
O_3 [9 g/m^3] vs Feedwater_3	0.27	0.96	0.16	NA	0.98
O_3 [9 g/m^3] vs O_3 [3 g/m^3]	0.23	0.783	0.24	NA	0.95

*NA = not applied; these samples were excluded from the statistical analysis as genes were under the limit of detection in one of the samples.

Appendix C

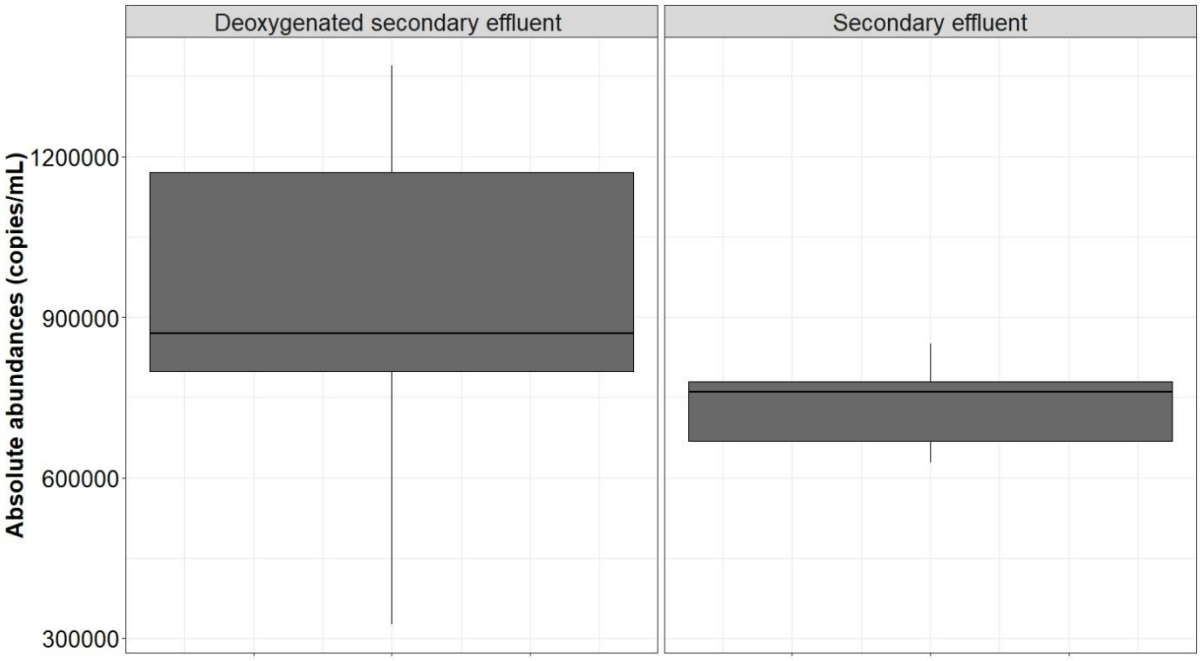

Figure C. 1 Absolute concentrations (three samples per condition) of the 16S rRNA gene in secondary clarifier effluent in both deoxygenated (left) and aerobic (right) samples.

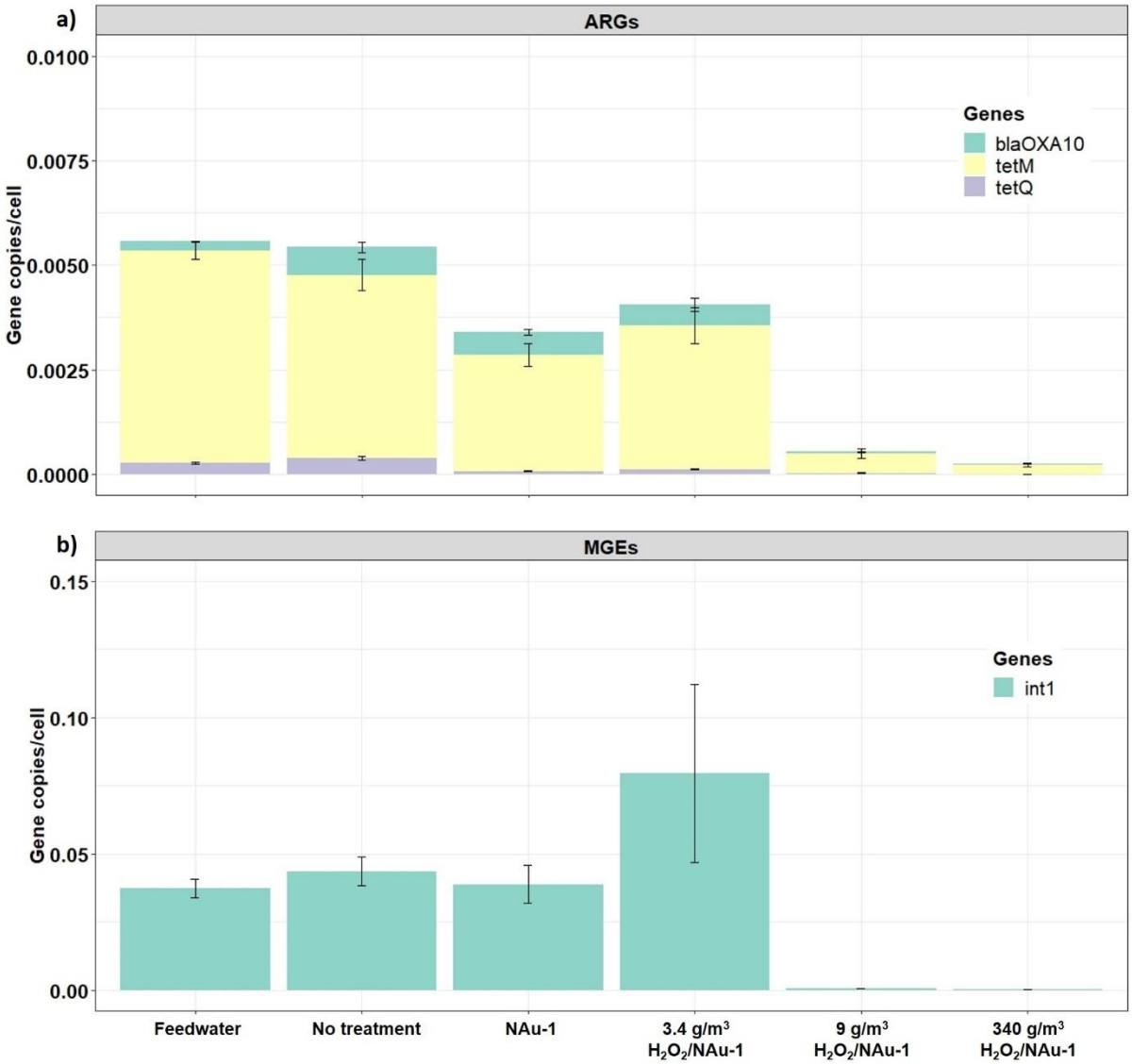

Figure C. 2 Relative abundances per genome in a) ARGs and b) MGEs in the H_2O_2/NAu-1 experiment. 'Feedwater' refers to secondary clarifier effluent and 'no treatment' to secondary clarifier effluent with no addition of H_2O_2 or NAu-1. Conditions: 0.5 g/L NAu-1, 8 hrs contact time.

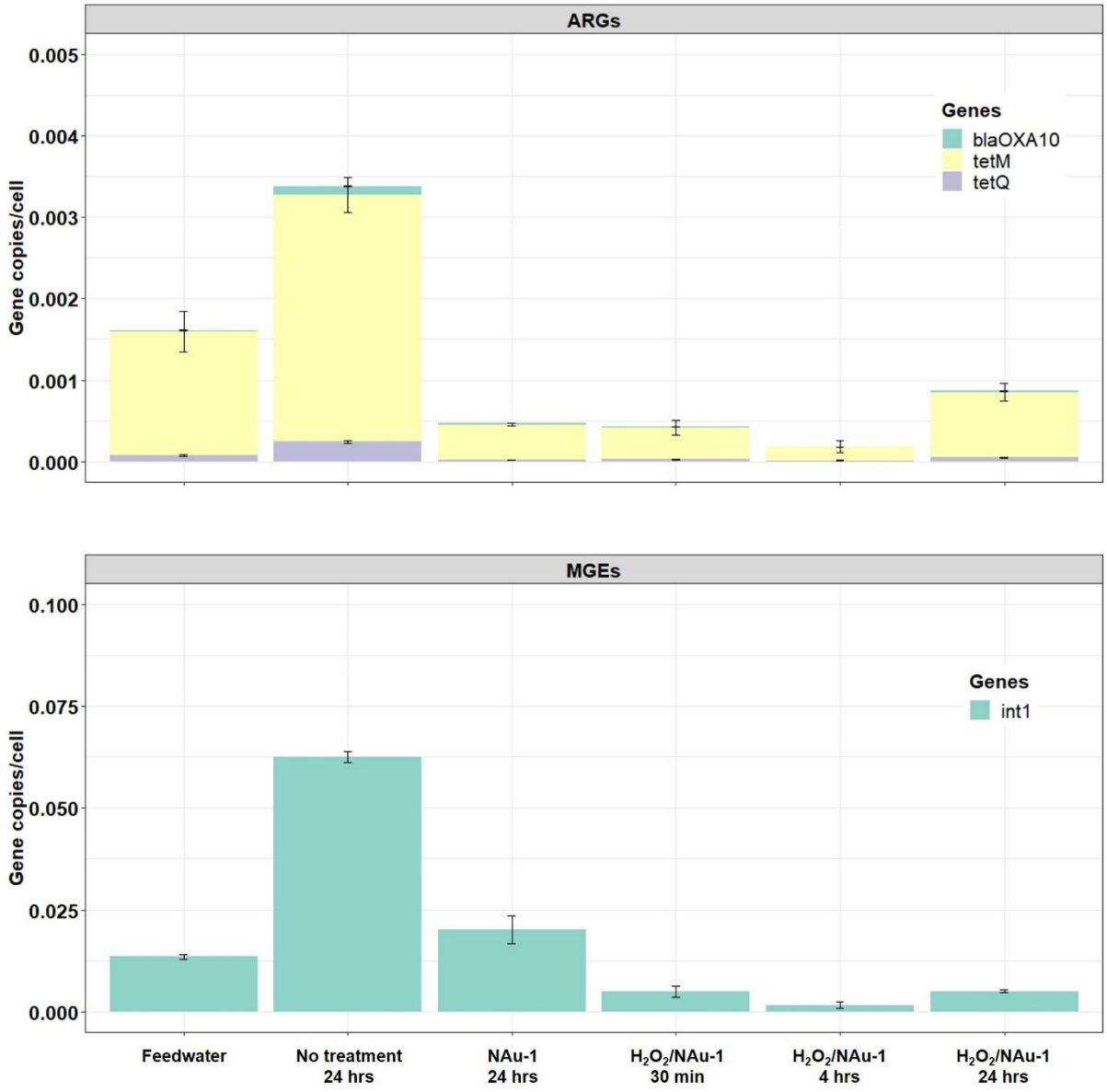

Figure C. 3 Relative abundances in a) ARGs and b) MGEs in the H_2O_2/NAu-1 experiment. 'Feedwater' refers to secondary clarifier effluent and 'no treatment' to secondary clarifier effluent with no addition of H_2O_2 or NAu-1. Conditions: 9 g/m^3 H_2O_2, 0.5 g/L NAu-1.

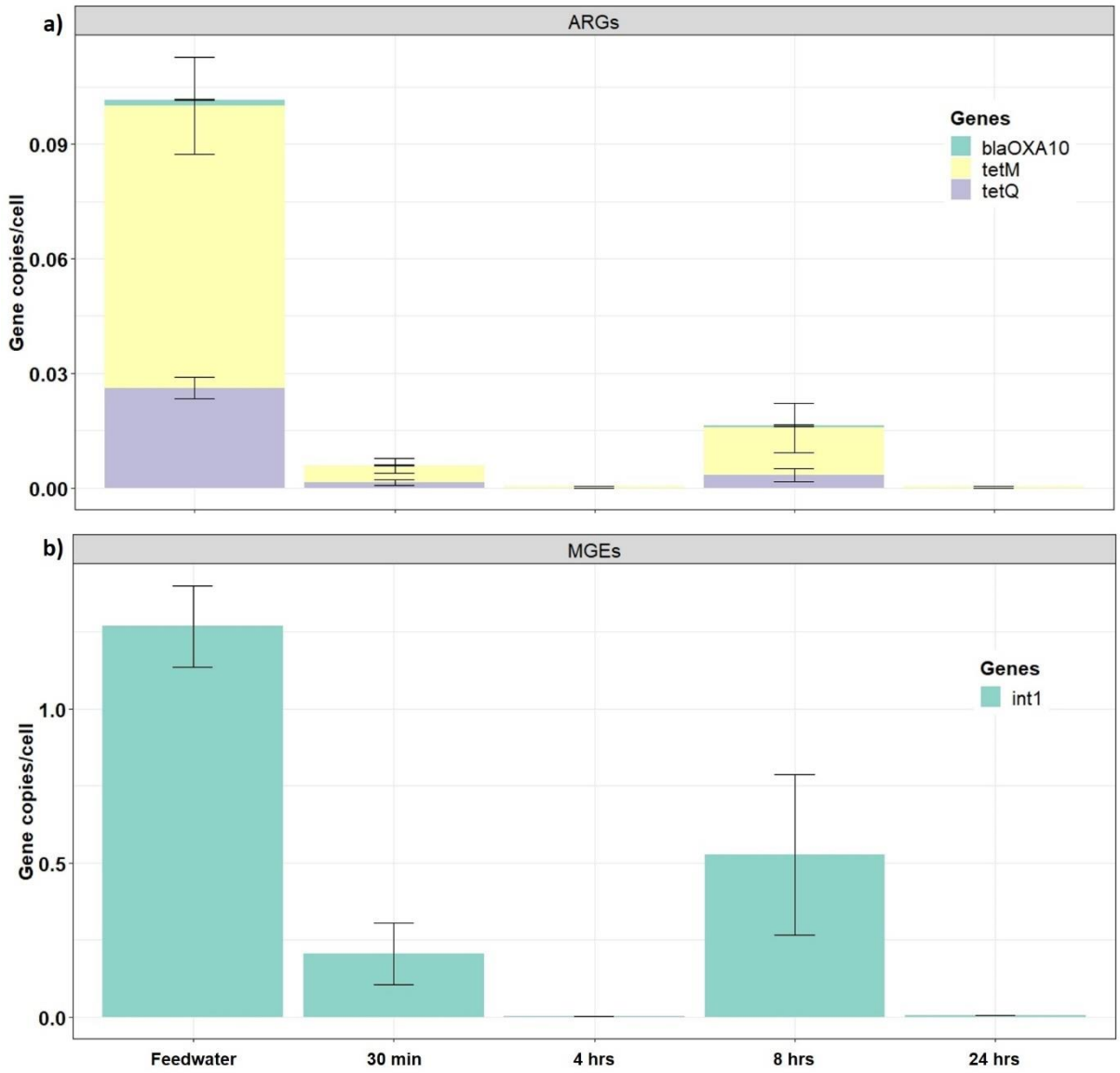

Figure C. 4 Relative abundances in a) ARGs and b) MGEs in the rNAu-1 experiment after the addition of rNAu-1 (0.5 g/L). 'Feedwater' refers to deoxygenated secondary clarifier effluent.

a)

Haber–Weiss mechanism	reaction	A possible non-radical mechanism	reaction
$\equiv Fe(III) + H_2O_2 \rightarrow \equiv Fe(II) + HO_2^{\bullet}(O2^{\bullet -}) + H^+(2H^+)$	(1)	$\equiv Fe(III) + H_2O_2 \rightarrow \equiv Fe(II) + HO_2^{\bullet} + H^+$	(1)
$\equiv Fe(II) + H_2O_2 \rightarrow \equiv Fe(III) + {}^{\bullet}OH + OH^-$	(2)	$\equiv Fe(II) + H_2O_2 \rightarrow \equiv Fe(IV) + 2OH^-$	(9)
$\equiv Fe(III) + HO_2^{\bullet}(O_2^{\bullet -}) \rightarrow \equiv Fe(II) + O_2 (+H^+)$	(3)	$\equiv Fe(IV) + H_2O_2 \rightarrow \equiv Fe(II) + O_2 + 2H^+$	(10)
$HO_2^{\bullet} \Leftrightarrow H^+ + O_2^{\bullet -}$	(4)	$\equiv Fe(IV) + \equiv Fe(II) \rightarrow 2\equiv Fe(III)$	(11)
${}^{\bullet}OH + H_2O_2 \rightarrow H_2O + HO_2^{\bullet}$	(5)	$\equiv Fe(III) + HO_2^{\bullet}(O_2^{\bullet -}) \rightarrow \equiv Fe(II) + O_2(+H^+)$	(3)
${}^{\bullet}OH + \equiv Fe(II) \rightarrow \equiv Fe(III) + OH^-$	(6)	$HO_2^{\bullet} + HO_2^{\bullet} \rightarrow H_2O_2 + O_2$	(8)
${}^{\bullet}OH + HO_2^{\bullet}(O_2^{\bullet -}) \rightarrow O_2 + H_2O (+OH^-)$	(7)		
$HO_2^{\bullet} + HO_2^{\bullet} \rightarrow H_2O_2 + O_2$	(8)		

b)

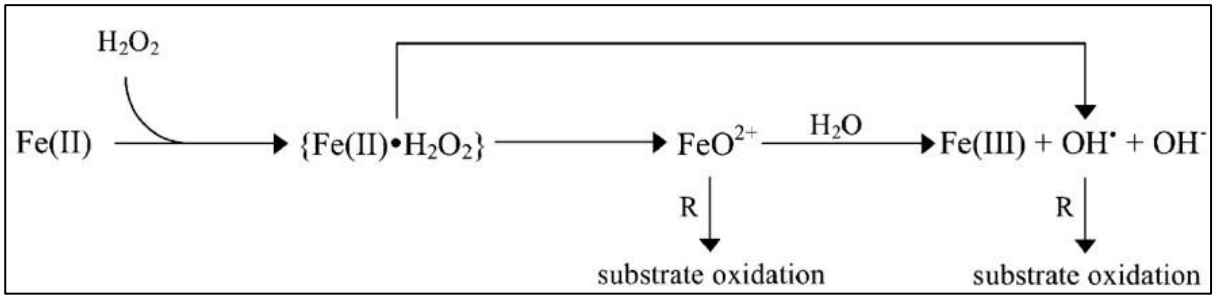

Figure C. 5 a) Proposed mechanism of H_2O_2 decomposition by reacting with mineral-bound Fe^{3+} (Pham et al., 2009) and b) possible reaction path for the formation of a less reactive and more selective oxidant, Fe(IV), from aqueous Fe^{2+} and H_2O_2 (Remucal and Sedlak, 2011).

$Fe(II) + O_2 \rightarrow Fe(III) + O_2^{-\bullet}$	(1)
$HO_2^{\bullet} \leftrightarrow H^+ + O_2^{-\bullet}$	(2)
$Fe(II) + HO_2^{\bullet} \rightarrow Fe(III) + HO_2^- \xrightarrow{H^+} Fe(III) + H_2O_2$	(3a)
$Fe(II) + O_2^{-\bullet} \rightarrow Fe(III) + O_2^{2-} \xrightarrow{2H^+} Fe(III) + H_2O_2$	(3b)
$Fe(II) + H_2O_2 \rightarrow Fe(III) + OH^- + OH^{\bullet}$	(4)

Figure C. 6 Oxidative mechanism of aqueous Fe^{2+} upon its contact with O_2 (Remucal and Sedlak, 2011), which can plausibly be relevant for the oxidation of clay mineral Fe^{2+} as suggested by Tong et al. (2016).

Table C. 1 Water quality characterization of the secondary clarifier effluent from a domestic WWTP. Values are the average ± standard deviation of all measurements taken in all experiments in this study.

Physicochemical parameter		
Dissolved oxygen	**2.5** ± 0.3	(mg/L)
Conductivity	**733.3** ± 65.4	(µS/cm)
COD	**27.4** ± 8.5	(mg/L)
TSS	**24.2** ± 7.0	(mg/L)
pH	**6.4** ± 0.3	-

Table C. 2 Removal rates ± standard deviation from three parallel reactors per condition of all target genes. Conditions: 680 g/m^3 H_2O_2, 1 g/L NAu-1.

Conditions	**16Sr RNA**	***int*1**	***tet*M**
96 mJ/cm^2 UV	**0.69** ± 0.08	**0.07** ± 0.19	**0.58** ± 0.08
288 mJ/cm^2 UV	**1.56** ± 0.50	**0.52** ± 0.47	**1.03** ± 0.52
576 mJ/cm^2 UV	**1.52** ± 0.05	**0.45** ± 0.05	**1.13** ± 0.12
H_2O_2/UV 96 mJ/cm^2	**0.89** ± 0.29	**0.99** ± 0.04	**0.79** ± 0.10
H_2O_2/UV 288 mJ/cm^2	**0.88** ± 0.05	**1.10** ± 0.12	**0.92** ± 0.12
H_2O_2/UV 576 mJ/cm^2	**1.05** ± 0.33	**1.08** ± 0.30	**0.84** ± 0.10
H_2O_2/NAu-1 (8 hrs)	**0.46** ± 0.13	**1.27** ± 0.19	**1.31** ± 0.21
NAu-1 (8 hrs)	**-0.19** ± 0.34	**0.17** ± 0.06	**0.36** ± 0.22

Table C. 3 Multiple comparisons of means (Games-Howell post hoc test). Pairwise comparison of 16S rRNA, int1 and tetM gene removal rates of all conditions tested in the preliminary UV, H_2O_2/UV and H_2O_2/NAu-1 experiments. Statistical significance is noted by p-values (in bold); p-value less than 0.05 shows significant differences between pairs.

Pairs	**16S rRNA**	***int*1**	***tet*M**
UV_576 vs UV_288	0.999999303	0.999921	0.998696
UV_96 vs UV_288	0.069953111	0.496176	0.290403
UV_96 vs UV_576	**0.003473064**	0.146976	**0.005478**
H2O2_UV_576 vs H2O2_UV_288	0.891236452	1	0.908108
H2O2_UV_96 vs H2O2_UV_288	1	0.417427	0.402772
H2O2_UV_96 vs H2O2_UV_576	0.967745348	0.977886	0.957922
NAU1_8h vs H2O2_NAU1_8h	**0.00433801**	**3.00E-07**	**1.81E-06**
H2O2_UV_96 vs UV_96	0.62933201	**0.012983**	**0.043916**
H2O2_UV_288 vs UV_288	0.168187207	0.222128	0.997976
H2O2_UV_576 vs UV_576	0.134662684	**0.002582**	0.077783
H2O2_NAU1_8h vs H2O2_UV_96	**0.029078711**	**0.027758**	**0.000624**
H2O2_NAU1_8h vs H2O2_UV_288	**3.14E-05**	0.418846	**0.011978**
H2O2_NAU1_8h vs H2O2_UV_576	0.056678145	0.760206	**0.001913**
H2O2_NAU1_8h vs UV_96	0.095389026	**0.000642**	**4.24E-05**
H2O2_NAU1_8h vs UV_288	**0.025909779**	0.0938	0.809077
H2O2_NAU1_8h vs UV_576	**1.41E-09**	**4.22E-06**	0.610032

Table C. 4 Multiple comparisons of means (Games-Howell post hoc test). Pairwise comparison of 16S rRNA, int1, bla_{OXA-10}, tetM and tetQ gene absolute abundances of all conditions tested in the H_2O_2/NAu-1 experiments with varying H_2O_2 dose. 'Feedwater' refers to secondary clarifier effluent and 'no treatment' to secondary clarifier effluent with no addition of H_2O_2 or NAu-1. Conditions: 0.5 g/L NAu-1, 8 hrs contact time. Statistical significance is noted by p-values (in bold); p-value less than 0.05 shows significant differences between pairs.

Pairs	**16S rRNA**	***int*1**	***bla*$_{OXA-10}$**	***tet*M**	***tet*Q**
No_treatment_8h vs Feedwater	1	0.98	0.10	0.81	0.73
NAu1 vs Feedwater	0.546	1	**0.02**	**<0.01**	**0.03**
Feedwater vs 3.4g/m3_H2O2_NAu1	0.98	0.91	0.65	0.09	0.09
No_treatment_8h vs 3.4g/m3_H2O2_NAu1	0.98	0.96	0.99	0.74	**0.01**
NAu1 vs 3.4g/m3_H2O2_NAu1	0.84	0.93	0.99	0.90	0.06
3.4g/m3_H2O2_NAu1 vs 3.4g/m3_H2O2	0.99	0.91	0.94	0.99	0.99
Feedwater vs 9g/m3_H2O2_NAu1	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**0.01**
No_treatment_8h vs 9g/m3_H2O2_NAu1	**<0.01**	**<0.01**	**0.01**	**<0.01**	**<0.01**
NAu1 vs 9g/m3_H2O2_NAu1	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**
9g/m3_H2O2_NAu1 vs 9g/m3_H2O2	**0.05**	**<0.01**	**<0.01**	**0.01**	**<0.01**
Feedwater vs 340g/m3_H2O2_NAu1	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**0.01**
No_treatment_8h vs 340g/m3_H2O2_NAu1	**<0.01**	**<0.01**	**0.01**	**<0.01**	**<0.01**
NAu1 vs 340g/m3_H2O2_NAu1	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**
340g/m3_H2O2_NAu1 vs 340g/m3_H2O2	**0.02**	**0.03**	**0.03**	**<0.01**	0.058
340g/m3_H2O2_NAu1 vs 9g/m3_H2O2_NAu1	0.18	**<0.01**	0.29	0.62	0.35
340g/m3_H2O2_NAu1 vs 3.4g/m3_H2O2_NAu1	**0.01**	**0.03**	**0.01**	**<0.01**	**<0.01**
9g/m3_H2O2_NAu1 vs 3.4g/m3_H2O2_NAu1	**0.01**	**0.03**	**0.01**	**<0.01**	**<0.01**
Feedwater vs 3.4g/m3_H2O2	0.99	1	0.93	0.09	0.33
Feedwater vs 9g/m3_H2O2	0.28	**0.01**	0.99	**<0.01**	0.13
Feedwater vs 340g/m3_H2O2	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**0.04**
No_treatment_8h vs NAu1	0.90	0.99	0.99	0.057	**<0.01**
No_treatment_8h vs 3.4g/m3_H2O2	0.99	0.99	0.30	0.27	**0.03**
No_treatment_8h vs 9g/m3_H2O2	0.56	**<0.01**	0.09	**0.03**	**0.01**

Pairs	**16S rRNA**	***int*1**	***bla*$_{\text{OXA-10}}$**	***tet*M**	***tet*Q**
No_treatment_8h vs 340g/m3_H2O2	**0.01**	**<0.01**	**0.02**	**<0.01**	**<0.01**

Table C. 5 Multiple comparisons of means (Games-Howell post hoc test). Pairwise comparison of 16S rRNA, int1, bla_{OXA-10}, tetM and tetQ gene removal rates of all conditions tested in the H_2O_2/UV experiments with varying H_2O_2 dose. Conditions: 0.5 g/L NAu-1, 8 hrs contact time. Statistical significance is noted by p-values (in bold); p-value less than 0.05 shows significant differences between pairs.

Pairs	**16S rRNA**	***int*1**	***bla*$_{OXA-10}$**	***tet*M**	***tet*Q**
NAu1 vs 3.4g/m3_H2O2_NAu1	0.92	1	0.91	0.90	**0.02**
NAu1 vs 9g/m3_H2O2_NAu1	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**0.01**
NAu1 vs 340g/m3_H2O2_NAu1	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**
340g/m3_H2O2_NAu1 vs 9g/m3_H2O2_NAu1	0.07	**0.01**	**0.05**	0.79	0.07
9g/m3_H2O2_NAu1 vs 3.4g/m3_H2O2_NAu1	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**
340g/m3_H2O2_NAu1 vs 3.4g/m3_H2O2_NAu1	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**

Table C. 6 Removal rates ± standard deviation from three separate reactors per condition of all target genes for the combined H_2O_2/NAu-1 treatment. 'No treatment' refers to secondary clarifier effluent with no addition of H_2O_2 or NAu-1. Conditions: 0.5 g/L NAu-1 and 8 hrs contact times.

Conditions	**16S rRNA**	***int*1**	***bla*$_{OXA-10}$**	***tet*M**	***tet*Q**
NAu-1	**0.13** ± 0.14	**0.04** ± 0.22	**-0.37** ± 0.16	**0.28** ± 0.13	**0.59** ± 0.14
3.4 g/m^3 H_2O_2/NAu-1	**-0.02** ± 0.34	**0.03** ± 0.59	**-0.21** ± 0.37	**0.20** ± 0.16	**0.37** ± 0.11
9 g/m^3 H_2O_2/NAu-1	**0.93** ± 0.30	**1.80** ± 0.17	**0.81** ± 0.29	**1.18** ± 0.40	**1.18** ± 0.36
340 g/m^3 H_2O_2/NAu-1	**1.43** ± 0.34	**2.34** ± 0.32	**1.23** ± 0.21	**1.43** ± 0.25	**1.65** ± 0.22
3.4 g/m^3 H_2O_2	**0.02** ± 0.23	**0.40** ± 0.75	**0.05** ± 0.52	**0.23** ± 0.15	**0.50** ± 0.54
9 g/m^3 H_2O_2	**0.33** ± 0.36	**0.59** ± 0.06	**0.02** ± 0.05	**0.37** ± 0.22	**0.31** ± 0.06
340 g/m^3 H_2O_2	**0.71** ± 0.25	**0.95** ± 0.45	**0.44** ± 0.33	**0.49** ± 0.11	**0.84** ± 0.93
No treatment	**0.05** ± 0.25	**-0.04** ± 0.16	**-0.41** ± 0.27	**0.08** ± 0.12	**-0.12** ± 0.17

Table C. 7 Relative abundances ± standard deviation from three separate reactors per condition of all target genes for the combined H_2O_2/NAu-1 treatment. 'Feedwater' refers to secondary clarifier effluent and 'no treatment' to secondary clarifier effluent with no addition of H_2O_2 or NAu-1. Conditions: 0.5 g/L NAu-1, 8 hrs contact time.

Conditions	***int*1**	***bla*$_{OXA-10}$**	***tet*M**	***tet*Q**
Feedwater	**3.73E-02** ± 1.00E-02	**2.21E-04** ± 2.79E-05	**5.07E-03** ± 6.19E-04	**2.72E-04** ± 9.30E-05
NAu-1	**3.88E-02** ± 2.10E-02	**5.46E-04** ± 2.06E-04	**2.78E-03** ± 7.98E-04	**7.31E-05** ± 1.97E-05
No treatment	**4.36E-02** ± 1.62E-02	**6.68E-04** ± 3.81E-04	**4.38E-03** ± 1.11E-03	**3.79E-04** ± 1.49E-04
3.4 g/m^3 H_2O_2	**3.55E-02** ± 3.38E-02	**3.03E-04** ± 2.00E-04	**3.10E-03** ± 1.02E-03	**1.42E-04** ± 1.09E-04
9 g/m^3 H_2O_2	**9.80E-03** ± 1.28E-03	**2.13E-04** ± 2.44E-05	**2.44E-03** ± 1.13E-03	**1.34E-04** ± 1.85E-05
340 g/m^3 H_2O_2	**5.98E-03** ± 4.06E-03	**9.83E-05** ± 5.74E-05	**1.70E-03** ± 5.60E-04	**9.15E-05** ± 6.43E-05
3.4 g/m^3 H_2O_2/NAu-1	**7.94E-02** ± 9.83E-02	**5.00E-04** ± 4.52E-04	**3.43E-03** ± 1.27E-03	**1.20E-04** ± 3.14E-05
9 g/m^3 H_2O_2/NAu-1	**6.30E-04** ± 2.34E-04	**4.23E-05** ± 3.09E-05	**4.70E-04** ± 3.62E-04	**2.47E-05** ± 2.16E-05
340 g/m^3 H_2O_2/NAu-1	**2.03E-04** ± 9.51E-05	**1.44E-05** ± 7.87E-06	**2.18E-04** ± 1.28E-04	**6.76E-06** ± 3.01E-06

Table C. 8 Multiple comparisons of means (Games-Howell post hoc test). Pairwise comparison of 16S rRNA, int1, bla_{OXA-10}, tetM and tetQ gene relative abundances of all conditions tested in the H_2O_2/NAu-1 experiments with varying H_2O_2 dose. 'Feedwater' refers to secondary clarifier effluent and 'no treatment' to secondary clarifier effluent with no addition of H_2O_2 or NAu-1. Conditions: 0.5 g/L NAu-1, 8 hrs contact time. Statistical significance is noted by p-values (in bold); p-value less than 0.05 shows significant differences between pairs.

Pairs	***int*1**	***bla*OXA-10**	***tet*M**	***tet*Q**
No_treatment_8h vs Feedwater	0.98	0.10	0.81	0.73
NAu1 vs Feedwater	0.99	**0.02**	**<0.01**	**0.03**
Feedwater vs 3.4g/m3_H2O2_NAu1	0.91	0.65	0.09	0.09
Feedwater vs 9g/m3_H2O2_NAu1	**<0.01**	**<0.01**	**<0.01**	**0.01**
Feedwater vs 340g/m3_H2O2_NAu1	**<0.01**	**<0.01**	**<0.01**	**0.01**

Table C. 9 Multiple comparisons of means (Games-Howell post hoc test). Pairwise comparison of 16S rRNA, int1, bla_{OXA-10}, tetM and tetQ gene absolute abundances of all conditions tested in the H_2O_2/NAu-1 experiments with varying contact time. 'Feedwater' refers to secondary clarifier effluent and 'no treatment' to secondary clarifier effluent with no addition of either H_2O_2 or NAu-1. Conditions: 0.5 g/L NAu-1, 9 g/ H_2O_2. Statistical significance is noted by p-values (in bold); p-value less than 0.05 shows significant differences between pairs.

Pairs	**16S rRNA**	***int*1**	***bla*$_{OXA-10}$**	***tet*M**	***tet*Q**
No_treatment_24h vs Feedwater	**<0.01**	**<0.01**	**<0.01**	**0.04**	**<0.01**
NAu1_24h vs Feedwater	0.77	0.60	0.99	0.15	**0.01**
H2O2_30min vs Feedwater	**<0.01**	**<0.01**	**<0.01**	0.18	**<0.01**
H2O2_4h vs Feedwater	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**
H2O2_24h vs Feedwater	**<0.01**	**<0.01**	**<0.01**	0.20	**<0.01**
H2O2_NAu1_30min vs Feedwater	0.56	**<0.01**	0.28	**0.01**	0.051
H2O2_NAu1_4h vs Feedwater	0.09	**<0.01**	**0.04**	**0.04**	**0.01**
H2O2_NAu1_24h vs Feedwater	0.99	**<0.01**	0.74	0.49	0.41
H2O2_NAu1_30min vs H2O2_30min	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**
H2O2_NAu1_4h vs H2O2_4h	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**
H2O2_NAu1_24h vs H2O2_24h	**<0.01**	**<0.01**	**<0.01**	**0.01**	**<0.01**
H2O2_NAu1_4h vs H2O2_NAu1_30min	0.66	0.50	0.46	0.63	0.70
H2O2_NAu1_30min vs H2O2_NAu1_24h	0.08	1	0.49	0.13	0.39
H2O2_NAu1_4h vs H2O2_NAu1_24h	**<0.01**	**0.02**	**<0.01**	**<0.01**	**<0.01**
No_treatment_24h vs NAu1_24h	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**
No_treatment_24h vs H2O2_NAu1_24h	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**
NAu1_24h vs H2O2_NAu1_24h	0.84	**0.02**	0.61	0.10	**<0.01**

Table C. 10 Removal rates ± standard deviation from three separate reactors per condition of all target genes for the combined H_2O_2/NAu-1 treatment. 'No treatment' refers to secondary clarifier effluent with no addition of H_2O_2 or NAu-1. Conditions: 0.5 g/L NAu-1, 9 g/m^3 H_2O_2.

Conditions	**16S rRNA**	***int*1**	***bla*$_{OXA-10}$**	***tet*M**	***tet*Q**
NAu-1 (8 hrs)	**0.13** ± 0.14	**0.04** ± 0.22	**-0.37** ± 0.16	**0.28** ± 0.13	**0.59** ± 0.14
NAu-1 (24 hrs)	**-0.13** ± 0.19	**-0.13** ± 0.22	**0.05** ± 0.28	**0.55** ± 0.05	**0.54** ± 0.07
H_2O_2/NAu-1 (30 min)	**0.40** ± 0.45	**0.96** ± 1.09	**0.59** ± 0.52	**0.76** ± 0.47	**0.65** ± 0.52
H_2O_2/NAu-1 (4 hrs)	**0.71** ± 0.40	**1.65** ± 1.12	**1.07** ± 0.56	**1.49** ± 0.78	**0.94** ± 0.41
H_2O_2/NAu-1 (8 hrs)	**0.93** ± 0.30	**1.80** ± 0.17	**0.81** ± 0.29	**1.18** ± 0.40	**1.18** ± 0.36
H_2O_2/NAu-1 (24 hrs)	**-0.02** ± 0.14	**0.44** ± 0.08	**0.21** ± 0.15	**0.32** ± 0.21	**0.20** ± 0.13
H_2O_2 (30 min)	**-0.38** ± 0.13	**-0.26** ± 0.10	**-0.38** ± 0.09	**-0.23** ± 0.12	**-0.34** ± 0.11
H_2O_2 (4 hrs)	**-0.69** ± 0.06	**-0.46** ± 0.04	**-0.61** ± 0.08	**-0.37** ± 0.06	**-0.55** ± 0.06
H_2O_2 (24 hrs)	**-0.73** ± 0.09	**-0.16** ± 0.04	**-0.49** ± 0.05	**-0.25** ±0.24	**-0.70**±0.19
No treatment (24 hrs)	**-0.86** ± 0.07	**-0.67** ± 0.03	**-0.73** ± 0.08	**-0.29** ±0.10	**-0.50**±0.09

Table C. 11 Multiple comparisons of means (Games-Howell post hoc test). Pairwise comparison of 16S rRNA, int1, bla_{OXA-10}, tetM and tetQ gene removal rates of all conditions tested in the H_2O_2/ NAu-1 experiments with varying contact time. Conditions: 0.5 g/L NAu-1, 9 g/m^3 H_2O_2. Statistical significance is noted by p-values (in bold); p-value less than 0.05 shows significant differences between pairs.

Pairs	**16S rRNA**	***bla*$_{OXA-10}$**	***int*1**	***tet*M**	***tet*Q**
NAu1_8h vs H2O2_NAu1_8h	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**0.01**
NAu1_24h vs H2O2_NAu1_24h	0.93	0.85	**<0.01**	0.15	**<0.01**
H2O2_NAu1_4h vs H2O2_NAu1_30min	0.86	0.69	0.93	0.38	0.93
H2O2_NAu1_30min vs H2O2_NAu1_8h	0.17	0.97	0.47	0.59	0.32
H2O2_NAu1_30min vs H2O2_NAu1_24h	0.27	0.55	0.88	0.32	0.36
H2O2_NAu1_4h vs H2O2_NAu1_8h	0.92	0.95	0.99	0.97	0.92
H2O2_NAu1_4h vs H2O2_NAu1_24h	**<0.01**	**0.02**	0.15	**0.03**	**<0.01**
H2O2_NAu1_24h vs H2O2_NAu1_8h	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**

Table C. 12 Relative abundances ± standard deviation from three separate reactors per condition of all target genes for the combined H_2O_2/NAu-1 treatment. 'Feedwater' refers to secondary clarifier effluent and 'no treatment' to secondary clarifier effluent with no addition of H_2O_2 or NAu-1. Conditions: 0.5 g/L NAu-1, 9 g/m^3 H_2O_2.

Conditions	*int*1	*bla*$_{OXA10}$	*tet*M	*tet*Q
Feedwater	**1.35E-02** ± 1.80E-03	**1.94E-05** ± 9.43E-06	**1.52E-03** ± 7.54E-04	**7.55E-05** ± 2.40E-05
NAu-1 (24 hrs)	**2.02E-02** ± 1.01E-02	**2.05E-05** ± 1.17E-05	**4.35E-04** ± 5.48E-05	**1.94E-05** ± 8.49E-06
H_2O_2/NAu-1 (30 min)	**4.86E-03** ± 4.04E-03	**7.84E-06** ± 5.60E-06	**3.90E-04** ± 2.72E-04	**2.81E-05** ± 2.35E-05
H_2O_2/NAu-1 (4 hrs)	**1.65E-03** ± 2.14E-03	**3.13E-06** ± 3.25E-06	**1.68E-04** ± 2.23E-04	**1.27E-05** ± 1.14E-05
H_2O_2/NAu-1 (24 hrs)	**5.01E-03** ± 8.80E-04	**1.26E-05** ± 3.77E-06	**8.03E-04** ± 3.13E-04	**4.97E-05** ± 1.52E-05
H_2O_2 (30 min)	**2.49E-02** ± 5.52E-03	**4.73E-05** ± 1.08E-05	**2.65E-03** ± 6.85E-04	**1.70E-04** ± 4.29E-05
H_2O_2 (4 hrs)	**3.90E-02** ± 3.77E-03	**8.00E-05** ± 1.58E-05	**3.62E-03** ± 4.91E-04	**2.70E-04** ± 3.94E-05
H_2O_2 (24 hrs)	**1.97E-02** ± 1.85E-03	**6.05E-05** ± 7.30E-06	**2.98E-03** ± 1.26E-03	**4.07E-04** ± 1.40E-04
No treatment (24 hrs)	**6.25E-02** ± 3.92E-03	**1.07E-04** ± 1.85E-05	**3.03E-03** ± 6.50E-04	**2.43E-04** ± 5.13E-05

Table C. 13 Multiple comparisons of means (Games-Howell post hoc test). Pairwise comparison of 16S rRNA, int1, bla_{OXA-10}, tetM and tetQ gene relative abundances of all conditions tested in the H_2O_2/NAu-1 experiments with varying contact time. 'Feedwater' refers to secondary clarifier effluent. Conditions: 0.5 g/L NAu-1, 9 g/m^3 H_2O_2. Statistical significance is noted by p-values (in bold); p-value less than 0.05 shows significant differences between pairs.

Pairs	***int*1**	***bla*$_{OXA-10}$**	***tet*M**	***tet*Q**
No_treatment_24h vs Feedwater	**6.25E-12**	**8.55E-07**	**0.041981**	**4.88E-05**
NAu1_24h vs Feedwater	0.605562	0.999999883	0.153945	**0.019846**
H2O2_NAu1_30min vs Feedwater	**0.002589**	0.283108564	0.134187	0.051626
H2O2_NAu1_4h vs Feedwater	**1.64E-06**	0.077571745	0.068	**0.010043**
H2O2_NAu1_24h vs Feedwater	**0.000309**	0.743028445	0.495123	0.418064
H2O2_NAu1_4h vs H2O2_NAu1_30min	0.507899	0.468990356	0.630054	0.704041
H2O2_NAu1_4h vs H2O2_NAu1_24h	**0.021879**	**0.000892036**	**0.004367**	**0.000856**
H2O2_NAu1_30min vs H2O2_NAu1_24h	1	0.498794621	0.139888	0.392343

Table C. 14 Multiple comparisons of means (Games-Howell post hoc test). Pairwise comparison of 16S rRNA, int1, bla_{OXA-10}, tetM and tetQ gene absolute abundances of all conditions tested in the rNAu-1 experiments varying contact time 'Effluent' refers to secondary clarifier effluent, 'feedwater' to deoxygenated secondary clarifier effluent, and 'no treatment' to deoxygenated secondary clarifier effluent with no addition of rNAu-1. rNAu-1 concentration: 0.5 g/L. Statistical significance is noted by p-values (in bold); p-value less than 0.05 shows significant differences between pairs.

Pairs	**16S rRNA**	***int1***	***blaOXA-10***	***tetM***	***tetQ***
Effluent vs Feedwater	0.173341	0.999992	0.552523788	0.997836	0.997836
No_treatment_8h vs Feedwater	0.064505	**0.01514**	0.230696324	0.208611	0.208611
No_treatment_24h vs Feedwater	0.912318	1	0.999455379	0.982195	0.982195
No_treatment_8h_GB vs Feedwater	0.995591	0.997187	0.999961271	0.999985	0.999985
No_treatment_24h_GB vs Feedwater	0.993497	0.456286	0.054407557	0.97322	0.97322
Reduced_NAu-1_8h vs Feedwater	**0.038781**	0.401928	**0.044922458**	**0.050502**	**0.050502**
Reduced_NAu1_24h vs Feedwater	**0.000262**	**0.000324**	**5.71E-05**	**0.020079**	**0.020079**
Reduced_NAu1_24h_GB vs Feedwater	**0.000222**	**0.000259**	**1.85E-05**	**0.022604**	**0.022604**
Reduced_NAu1_8h_GB vs Feedwater	**0.000276**	**0.000323**	**5.59E-05**	**0.02015**	**0.02015**
Reduced_NAu1_8h_GB vs No_treatment_8h_GB	**0.001889**	**1.98E-05**	**0.000206933**	**0.000167**	**0.000167**
Reduced_NAu1_24h_GB vs No_treatment_24h_GB	**1.71E-08**	**4.86E-07**	**2.72E-09**	**1.74E-11**	**1.74E-11**
Reduced_NAu-1_8h vs No_treatment_8h	**0.000228**	**0.007295**	**0.003308615**	**1.62E-06**	**1.62E-06**
Reduced_NAu1_24h vs No_treatment_24h	**1.71E-05**	**2.07E-06**	**1.27E-05**	**2.92E-05**	**2.92E-05**
Reduced_NAu1_8h_GB vs Reduced_NAu-1_8h	0.750524	0.688404	0.681844095	0.776003	0.776003
Reduced_NAu1_24h_GB vs Reduced_NAu1_24h	0.717557	0.719303	0.68656179	0.851298	0.851298
Reduced_NAu1_30min vs Feedwater	**0.000188**	**0.000497**	**2.19E-05**	**0.02628**	**0.02628**
Reduced_NAu1_4h vs Feedwater	**0.000258**	**0.000319**	**5.57E-05**	**0.019996**	**0.019996**
Reduced_NAu1_24h vs Reduced_NAu-1_8h	0.692095	0.689337	0.669716967	0.771835	0.771835

Pairs	16S rRNA	*int1*	*blaOXA-10*	*tetM*	*tetQ*
Reduced_NAu1_30min vs Reduced_NAu-1_8h	0.947362	0.982615	0.882813376	0.980014	0.980014
Reduced_NAu1_4h vs Reduced_NAu-1_8h	0.68517	0.684481	0.657319462	0.76777	0.76777
Reduced_NAu1_30min vs Reduced_NAu1_24h	0.698309	0.692145	0.773948113	0.62929	0.62929
Reduced_NAu1_4h vs Reduced_NAu1_24h	**0.015601**	0.307691	0.677884237	0.99992	0.99992
Reduced_NAu1_4h vs Reduced_NAu1_30min	0.674471	0.679452	0.721468325	0.614393	0.614393

Table C. 15 Removal rates ± standard deviation from three separate reactors in each condition tested in the rNAu-1 experiment with varying contact time and oxygen levels. 'No treatment' refers to deoxygenated secondary clarifier effluent with no addition of rNAu-1. NAu-1 concentration: 0.5 g/L.

Conditions	16S rRNA	int1	blaOXA-10	tetM	tetQ
No treatment 8 hrs (low O_2)	**-0.18** ± 0.10	**-0.20** ± 0.08	**-0.11** ± 0.07	**-0.21** ± 0.08	**0.03** ± 0.06
No treatment 24 hrs (low O_2)	**0.09** ± 0.09	**-0.01** ± 0.07	**0.04** ± 0.09	**0.12** ± 0.10	**0.62** ± 0.07
No treatment 8 hrs (high O_2)	**-0.04** ± 0.17	**0.06** ± 0.09	**-0.02** ± 0.12	**-0.03** ± 0.12	**0.18** ± 0.11
No treatment 24 hrs (high O_2)	**-0.05** ± 0.06	**-0.11** ± 0.08	**-0.14** ± 0.06	**0.11** ± 0.04	**0.17** ± 0.06
rNAu1 30 min (high O_2)	**1.91** ± 1.02	**1.73** ± 1.07	**1.72** ± 0.79	**1.61** ± 0.62	**1.98** ± 0.88
rNAu1 4 hrs (high O_2)	**2.67** ± 0.10	**2.57** ± 0.12	**2.15** ± 0.16	**2.35** ± 0.25	**2.67** ± 0.20
rNAu-1 8 hrs (high O_2)	**1.79** ± 1.34	**1.59** ± 1.29	**1.31** ± 1.01	**1.82** ± 1.17	**1.81** ± 1.06
rNAu1 24 hrs (high O_2)	**2.39** ± 0.11	**2.36** ± 0.20	**1.98** ± 0.17	**2.26** ± 0.19	**2.94** ± 0.24
rNAu1 8 hrs (low O_2)	**2.26** ± 0.78	**2.41** ± 0.22	**2.15** ± 0.56	**2.46** ± 0.53	**2.28** ± 0.46
rNAu1 24 hrs (low O_2)	**2.06** ± 1.04	**2.04** ± 1.10	**1.53** ± 0.73	**2.51** ± 1.16	**2.22** ± 0.81

Table C. 16 Multiple comparisons of means (Games-Howell post hoc test). Pairwise comparison of 16S rRNA, int1, bla_{OXA-10}, tetM and tetQ gene removal rates of all conditions tested in the rNAu-1 experiments with varying contact time and oxygen levels. 'GB' stands for glovebox and means that this condition was under'low oxygen' (<2 ppm O_2). rNAu-1 concentration: 0.5 g/L. Statistical significance is noted by p-values (in bold); p-value less than 0.05 shows significant differences between pairs.

Pairs	**16S rRNA**	***int*1**	***bla*$_{OXA-10}$**	***tet*M**	***tet*Q**
Reduced_NAu1_4h vs Reduced_NAu1_30min	0.51249	0.45581	0.82048027	0.11878	0.46548
Reduced_NAu1_30min vs Reduced_NAu-1_8h	1	1	0.99294247	0.99995	0.99999
Reduced_NAu1_30min vs Reduced_NAu1_24h	0.90037	0.75885	0.98611971	0.18959	0.16676
Reduced_NAu1_4h vs Reduced_NAu-1_8h	0.64026	0.48418	0.47038722	0.92402	0.42236
Reduced_NAu1_4h vs Reduced_NAu1_24h	0.06124	0.25203	0.53317114	0.99731	0.29924
Reduced_NAu1_24h vs Reduced_NAu-1_8h	0.91845	0.73756	0.69745585	0.96865	0.17834
Reduced_NAu1_8h_GB vs Reduced_NAu1_24h_GB	0.99996	0.98512	0.59392026	1	1
Reduced_NAu1_8h_GB vs Reduced_NAu1_30min	0.99704	0.68918	0.92420385	0.12542	0.99359
Reduced_NAu1_30min vs Reduced_NAu1_24h_GB	0.99999	0.99970	0.99991091	0.58778	0.99970
Reduced_NAu1_8h_GB vs Reduced_NAu1_4h	0.83996	0.67034	1	0.99981	0.40960
Reduced_NAu1_4h vs Reduced_NAu1_24h_GB	0.74702	0.88793	0.38741582	0.99998	0.81554
Reduced_NAu1_8h_GB vs Reduced_NAu-1_8h	0.99308	0.67833	0.56774611	0.87242	0.95631
Reduced_NAu1_24h_GB vs Reduced_NAu-1_8h	0.99995	0.99746	0.99992802	0.95157	0.99297
Reduced_NAu1_8h_GB vs Reduced_NAu1_24h	0.99992	0.99989	0.99329295	0.98116	0.06675
Reduced_NAu1_24h_GB-Reduced_NAu1_24h	0.98856	0.99436	0.72117840	0.99942	0.35754

Table C. 17 Relative abundances ± standard deviation from three separate reactors in each condition tested in the rNAu-1 experiment with varying contact time. 'Feedwater' refers to deoxygenated secondary clarifier effluent, and 'no treatment' to deoxygenated secondary clarifier effluent with no addition of rNAu-1. rNAu-1 concentration: 0.5 g/L.

Conditions	*int*1	*bla*$_{OXA-10}$	*tet*M	*tet*Q
Secondary clarifier effluent	**1.13E+00** ± 7.56E-01	**1.20E-03** ± 8.97E-05	**8.95E-02** ± 3.07E-02	**1.86E-02** ± 7.34E-03
Feedwater	**1.27E+00** ± 3.95E-01	**1.49E-03** ± 3.65E-04	**7.39E-02** ± 3.82E-02	**2.62E-02** ± 8.47E-03
No treatment 8 hrs (high O_2)	**2.05E+00** ± 3.53E-01	**1.95E-03** ± 3.03E-04	**1.22E-01** ± 2.21E-02	**2.44E-02** ± 3.67E-03
No treatment 24 hrs (high O_2)	**1.30E+00** ± 2.07E-01	**1.37E-03** ± 2.75E-04	**5.78E-02** ± 1.30E-02	**6.29E-03** ± 1.04E-03
No treatment 8 hrs (low O_2)	**1.12E+00** ± 2.41E-01	**1.60E-03** ± 4.68E-04	**8.20E-02** ± 2.34E-02	**1.78E-02** ± 4.02E-03
No treatment 24 hrs (low O_2)	**1.67E+00** ± 3.01E-01	**2.06E-03** ± 2.72E-04	**5.76E-02** ± 5.22E-03	**1.79E-02** ± 2.40E-03
rNAu-1 30min (high O_2)	**2.05E-01** ± 3.00E-01	**1.11E-04** ± 1.54E-04	**4.43E-03** ± 5.66E-03	**1.40E-03** ± 2.00E-03
rNAu-1 4 hrs (high O_2)	**3.58E-03** ± 1.09E-03	**1.12E-05** ± 3.65E-06	**3.81E-04** ± 2.01E-04	**6.06E-05** ± 2.81E-05
rNAu-1 8 hrs (high O_2)	**5.27E-01** ± 7.83E-01	**4.61E-04** ± 6.18E-04	**1.23E-02** ± 1.95E-02	**3.48E-03** ± 5.09E-03
rNAu-1 24 hrs (high O_2)	**6.09E-03** ± 2.49E-03	**1.67E-05** ± 7.29E-06	**4.37E-04** ± 1.71E-04	**3.43E-05** ± 1.94E-05
rNAu-1 8 hrs (low O_2)	**5.60E-03** ± 3.42E-03	**2.18E-05** ± 2.53E-05	**4.89E-04** ± 5.48E-04	**2.27E-04** ± 2.29E-04
rNAu-1 24 hrs (low O_2)	**1.02E-01** ± 1.48E-01	**1.39E-04** ± 1.83E-04	**2.18E-03** ± 3.17E-03	**6.29E-04** ± 8.66E-04

Table C. 18 Multiple comparisons of means (Games-Howell post hoc test). Pairwise comparison of 16S rRNA, int1, bla_{OXA-10}, tetM and tetQ gene relative abundances of all conditions tested in the rNAu-1 experiments with varying contact time. 'Effluent' refers to secondary clarifier effluent, 'feedwater' to deoxygenated secondary clarifier effluent, and 'no treatment' to deoxygenated secondary clarifier effluent with no addition of rNAu-1. rNAu-1 concentration: 0.5 g/L. Statistical significance is noted by p-values (in bold); p-value less than 0.05 shows significant differences between pairs.

Pairs	***int1***	***blaOXA-10***	***tetM***	***tetQ***
Effluent vs Feedwater	0.999992	0.552523788	0.997836	0.668981
Reduced_NAu1_30min vs Feedwater	**0.000497**	**2.19E-05**	**0.02628**	**0.000426**
Reduced_NAu1_4h vs Feedwater	**0.000319**	**5.57E-05**	**0.019996**	**0.000415**
Reduced_NAu-1_8h vs Feedwater	0.401928	**0.044922458**	**0.050502**	**0.000416**
Reduced_NAu1_24h vs Feedwater	**0.000324**	**5.71E-05**	**0.020079**	**0.000412**
Reduced_NAu1_4h vs Reduced_NAu1_30min	0.679452	0.721468325	0.614393	0.679898
Reduced_NAu1_30min vs Reduced_NAu-1_8h	0.982615	0.882813376	0.980014	0.984307
Reduced_NAu1_30min vs Reduced_NAu1_24h	0.692145	0.773948113	0.62929	0.659801
Reduced_NAu1_4h vs Reduced_NAu-1_8h	0.684481	0.657319462	0.76777	0.68121
Reduced_NAu1_4h vs Reduced_NAu1_24h	0.307691	0.677884237	0.99992	0.509926
Reduced_NAu1_24h vs Reduced_NAu-1_8h	0.689337	0.669716967	0.771835	0.673338
Reduced_NAu1_8h_GB vs Feedwater	**0.000323**	**5.59E-05**	**0.02015**	**0.000433**
Reduced_NAu1_24h_GB vs Feedwater	**0.000259**	**1.85E-05**	**0.022604**	**0.000452**

Table C. 19 Values of pH before (secondary clarifier effluent) and after treatment in all experiments conducted with clay minerals, including controls. 'No treatment' refers to reactors containing only secondary clarifier effluent. In the experiment with rNAu-1, all conditions included deoxygenated secondary clarifier effluent.

Conditions	Before treatment	After treatment
H_2O_2/NAu-1 testing different H_2O_2 concentrations (contact time = 8 hrs)		
No treatment	**6.25**	**6.67** ± 0.04
0.5 g/L NAu-1	**6.25**	**7.99** ± 0.08
3.4 g/m^3 H_2O_2	**6.25**	**8.10** ± 0.15
9 g/m^3 H_2O_2	**6.25**	**8.13** ± 0.04
340 g/m^3 H_2O_2	**6.25**	**8.09** ± 0.04
0.5 g/L NAu-1 / 3.4 g/m^3 H_2O_2	**6.25**	**7.92** ± 0.04
0.5 g/L NAu-1 / 9 g/m^3 H_2O_2	**6.25**	**8.05** ± 0.14
0.5 g/L NAu-1 / 340 g/m^3 H_2O_2	**6.25**	**8.14** ± 0.02
H_2O_2/NAu-1 testing different contact times (NAu-1 = 0.5 g/L & H_2O_2 = 9 g/m^3)		
No treatment (24 hrs)	**6.76**	**7.16** ± 0.09
NAu-1 (24 hrs)	**6.76**	**8.26** ± 0.04
H_2O_2 (30 min)	**6.76**	**7.62** ± 0.23
H_2O_2 (4 hrs)	**6.76**	**8.06** ± 0.08
H_2O_2 (24 hrs)	**6.76**	**8.29** ± 0.03
H_2O_2/NAu-1 (30 min)	**6.76**	**7.84** ± 0.04
H_2O_2/NAu-1 (4 hrs)	**6.76**	**7.98** ± 0.14
H_2O_2/NAu-1 (24 hrs)	**6.76**	**8.24** ± 0.04
rNAu-1 experiment testing different contact times (rNAu-1 = 0.5 g/L)		
No treatment (8 hrs) - high O_2	**7.72**	**7.78** ± 0.20
No treatment (24 hrs) - high O_2	**7.72**	**8.31** ± 0.01
rNAu-1 (30 min) - high O_2	**7.72**	**8.41** ± 0.22
rNAu-1 (4 hrs) - high O_2	**7.72**	**8.12** ± 0.27
rNAu-1 (8 hrs) - high O_2	**7.72**	**8.05** ± 0.31
rNAu-1 (24 h) - high O_2	**7.72**	**8.12** ± 0.11

Conditions	Before treatment	After treatment
No treatment (8 hrs) - low O_2	**7.72**	**8.15** ± 0.22
No treatment (24 hrs) - low O_2	**7.72**	**8.91** ± 0.15
rNAu-1 (8 hrs) - low O_2	**7.72**	**8.73** ± 0.04
rNAu-1 (24 hrs) - low O_2	**7.72**	**8.86** ± 0.18

Table C. 20 H_2O_2 (g/m^3) before and after treatment in reactors with H_2O_2 in the H_2O_2/NAu-1 experiment with varying H_2O_2 dose. Conditions: 0.5 g/L NAu-1, 8 hrs contact time.

Conditions	Before treatment	After treatment
H_2O_2	**3.4**	**1.54** ± 0.11
H_2O_2	**9**	**3.96** ± 0.00
H_2O_2	**340**	**313.62** ± 13.73
H_2O_2/NAu-1	**3.4**	**0.91** ± 0.14
H_2O_2/NAu-1	**9**	**2.53** ± 0.14
H_2O_2/NAu-1	**340**	**262.54** ± 33.81

Table C. 21 H_2O_2 (g/m^3) before and after treatment in reactors with H_2O_2 in the H_2O_2/NAu-1 experiment varying contact time. NAu-1 concentration: 0.5 g/L.

Conditions	Before treatment	After treatment
H_2O_2 (30 min)	**9**	**7.29** ± 1.36
H_2O_2 (4 hrs)	**9**	**6.19** ± 1.06
H_2O_2 (8 hrs)	**9**	**3.96** ± 0.00
H_2O_2 (24 hrs)	**9**	**2.37** ± 1.03
H_2O_2/NAu-1 (30 min)	**9**	**7.11** ± 1.84
H_2O_2/NAu-1 (4 hrs)	**9**	**4.20** ± 1.87
H_2O_2/NAu-1 (8 hrs)	**9**	**2.53** ± 0.14
H_2O_2/NAu-1 (24 hrs)	**9**	**1.68** ± 1.19

Table C. 22 Fe^{2+} and total Fe concentrations measured using the 1,10-phenanthroline method. 'Feedwater' refers to secondary clarifier effluent and 'no treatment' to secondary clarifier effluent in absence of H_2O_2 and/or NAu-1. The calibrated range for the 1,10-phenanthroline method is 1-100 µM, hence some of these measurements are below the quality assured concentration.

Treatment conditions	Fe^{2+} (µM)	total Fe (µM)
H_2O_2/NAu-1 testing different H_2O_2 concentrations (contact time = 8 hrs)		
Feedwater	**16.10** ± 0.00	**40.73** ± 0.00
0.5 g/L NAu-1	**0.73** ± 0.00	**1.06** ± 0.14
3.4 g/m^3 H_2O_2 & 0.5 g/L NAu-1	**0.65** ± 0.14	**0.98** ± 0.24
9 g/m^3 H_2O_2 & 0.5 g/L NAu-1	**0.89** ± 0.14	**1.06** ± 0.14
340 g/m^3 H_2O_2 & 0.5 g/L NAu-1	**0.49** ± 0.00	**0.85** ± 0.17
H_2O_2/NAu-1 experiment testing different contact times (NAu-1 = 0.5 g/L & H_2O_2 = 9 g/m^3)		
Feedwater	**1.38** ± 0.00	**2.28 ± 0.00**
NAu-1 (24 hrs)	**0.75** ± 0.13	**1.39 ± 0.00**
H_2O_2 /NAu-1 (30 min)	**1.00** ± 0.13	**1.86 ± 0.07**
H_2O_2 /NAu-1 (4 hrs)	**0.92** ± 0.07	**1.60 ± 0.07**
H_2O_2 /NAu-1 (24 hrs)	**0.71** ± 0.14	**1.56 ± 0.41**
rNAu-1 testing different contact times (rNAu-1 = 0.5 g/L)		
Data not available		

Table C. 23 Dissolved oxygen (mg/L) in reactors containing deoxygenated secondary clarifier effluent with or without rNAu-1 before and after treatment.

Conditions	**Before treatment**	**After treatment**
No treatment (8 hrs) - high O_2	**0**	**4.93** ± 0.91
No treatment (24 hrs) - high O_2	**0**	**5.59** ± 1.08
rNAu-1 (30 min) - high O_2	**0**	**3.38** ± 0.64
rNAu-1 (4 hrs) - high O_2	**0**	**8.37** ± 0.03
rNAu-1 (8 hrs) - high O_2	**0**	**8.46** ± 0.11
rNAu-1 (24 hrs) - high O_2	**0**	**9.17** ± 0.17
No treatment (8 hrs) - low O_2	**0**	**1.20** ± 0.04
No treatment (24 hrs) - low O_2	**0**	**1.63** ± 0.25
Reduced NAu-1 (8 hrs) - low O_2	**0**	***NM**
Reduced NAu-1 (24 hrs) - low O_2	**0**	**1.74** ± 0.17

*NM: not measured

References

Ahammad, Z.S., Sreekrishnan, T.R., Hands, C.L., Knapp, C.W. and Graham, D.W. (2014) 'Increased waterborne bla NDM-1 resistance gene abundances associated with seasonal human pilgrimages to the Upper Ganges River', *Environmental Science and Technology*, 48(5), pp. 3014-3020.

Al-Jassim, N., Ansari, M.I., Harb, M. and Hong, P.Y. (2015) 'Removal of bacterial contaminants and antibiotic resistance genes by conventional wastewater treatment processes in Saudi Arabia: Is the treated wastewater safe to reuse for agricultural irrigation?', *Water Research*, 73, pp. 277-290.

Alexander, J., Bollmann, A., Seitz, W. and Schwartz, T. (2015) 'Microbiological characterization of aquatic microbiomes targeting taxonomical marker genes and antibiotic resistance genes of opportunistic bacteria', *Science of the Total Environment*, 512-513, pp. 316-325.

Alexander, J., Knopp, G., Dötsch, A., Wieland, A. and 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, pp. 103-112.

Alrousan, D.M.A., Dunlop, P.S.M., McMurray, T.A. and Byrne, J.A. (2009) 'Photocatalytic inactivation of E. coli in surface water using immobilised nanoparticle TiO 2 films', *Water Research*, 43(1), pp. 47-54.

Anastasi, E.M., Wohlsen, T.D., Stratton, H.M. and Katouli, M. (2013) 'Survival of Escherichia coli in two sewage treatment plants using UV irradiation and chlorination for disinfection', *Water Research*, 47(17), pp. 6670-6679.

Anderson, J.C., Joudan, S., Shoichet, E., Cuscito, L.D., Alipio, A.E.C., Donaldson, C.S., Khan, S., Goltz, D.M., Rudy, M.D., Frank, R.A., Knapp, C.W., Hanson, M.L. and Wong, C.S. (2015) 'Reducing nutrients, organic micropollutants, antibiotic resistance, and toxicity in rural wastewater effluent with subsurface filtration treatment technology', *Ecological Engineering*, 84, pp. 375-385.

Andersson, D.I. and Hughes, D. (2014) 'Microbiological effects of sublethal levels of antibiotics', *Nature Reviews Microbiology*, 12(7), pp. 465-478.

APHA (2005) *Standard methods for the examination of water and waste water*. 21st edn. New York: American Public Health Association.

Arnold, K.E., Williams, N.J. and Bennett, M. (2016) "Disperse abroad in the land': The role of wildlife in the dissemination of antimicrobial resistance', *Biology Letters*, 12(8).

Arya, M., Shergill, I.S., Williamson, M., Gommersall, L., Arya, N. and Patel, H.R.H. (2005) 'Basic principles of real-time quantitative PCR', *Expert Review of Molecular Diagnostics*, 5(2), pp. 209-219.

Bae, S. and Wuertz, S. (2009) 'Discrimination of viable and dead fecal Bacteroidales bacteria by quantitative PCR with Propidium monoazide', *Applied and Environmental Microbiology*, 75(9), pp. 2940-2944.

Baquero, F., Martinez, J.L. and Canton, R. (2008) 'Antibiotics and antibiotic resistance in water environments', *Curr Opin Biotechnol*, 19(3), pp. 260-5.

Barancheshme, F. and Munir, M. (2018) 'Strategies to combat antibiotic resistance in the wastewater treatment plants', *Frontiers in Microbiology*, 8(JAN).

Barraud, O., Baclet, M.C., Denis, F. and Ploy, M.C. (2010) 'Quantitative multiplex real-time PCR for detecting class 1, 2 and 3 integrons', *Journal of Antimicrobial Chemotherapy*, 65(8), pp. 1642-1645.

Behroozian, S., Svensson, S.L. and Davies, J. (2016) 'Kisameet clay exhibits potent antibacterial activity against the ESKAPE pathogens', *mBio*, 7(1).

Bengtsson-Palme, J., Hammarén, R., Pal, C., Östman, M., Björlenius, B., Flach, C.F., Fick, J., Kristiansson, E., Tysklind, M. and Larsson, D.G.J. (2016) 'Elucidating selection processes for antibiotic resistance in sewage treatment plants using metagenomics', *Science of the Total Environment*, 572, pp. 697-712.

Berendes, D.M., Yang, P.J., Lai, A., Hu, D. and Brown, J. (2018) 'Estimation of global recoverable human and animal faecal biomass', *Nature Sustainability*, 1(11), pp. 679-685.

Berendonk, T.U., Manaia, C.M., Merlin, C., Fatta-Kassinos, D., Cytryn, E., Walsh, F., Bürgmann, H., Sørum, H., Norström, M., Pons, M.-N., Kreuzinger, N., Huovinen, P., Stefani, S., Schwartz, T., Kisand, V., Baquero, F. and Martinez, J.L. (2015) 'Tackling antibiotic resistance: the environmental framework', *Nature Reviews Microbiology*, 13, p. 310.

Biancullo, F., Moreira, N.F.F., Ribeiro, A.R., Manaia, C.M., Faria, J.L., Nunes, O.C., Castro-Silva, S.M. and Silva, A.M.T. (2019) 'Heterogeneous photocatalysis using UVA-LEDs for the removal of antibiotics and antibiotic resistant bacteria from urban wastewater treatment plant effluents', *Chemical Engineering Journal*, 367, pp. 304-313.

Bouki, C., Venieri, D. and Diamadopoulos, E. (2013) 'Detection and fate of antibiotic resistant bacteria in wastewater treatment plants: A review', *Ecotoxicology and Environmental Safety*, 91, pp. 1-9.

Bourgin, M., Beck, B., Boehler, M., Borowska, E., Fleiner, J., Salhi, E., Teichler, R., von Gunten, U., Siegrist, H. and McArdell, C.S. (2018) 'Evaluation of a full-scale wastewater treatment plant upgraded with ozonation and biological post-treatments: Abatement of micropollutants, formation of transformation products and oxidation by-products', *Water Research*, 129, pp. 486-498.

Bourgin, M., Borowska, E., Helbing, J., Hollender, J., Kaiser, H.P., Kienle, C., McArdell, C.S., Simon, E. and von Gunten, U. (2017) 'Effect of operational and water quality parameters on conventional ozonation and the advanced oxidation process O3/H2O2: Kinetics of micropollutant abatement, transformation product and bromate formation in a surface water', *Water Research*, 122, pp. 234-245.

Brigatti, M.F., Galan, E. and Theng, B.K.G. (2006) 'Chapter 2 Structures and Mineralogy of Clay Minerals', in *Developments in Clay Science*. pp. 19-86.

Brinkman, N.E., Villegas, E.N., Garland, J.L. and Keely, S.P. (2018) 'Reducing inherent biases introduced during DNA viral metagenome analyses of municipal wastewater', *PLoS ONE*, 13(4).

Bugg, T.D.H., Wright, G.D., Walsh, C.T., Dutka-Malen, S., Arthur, M. and Courvalin, P. (1991) 'Molecular Basis for Vancomycin Resistance in Enterococcus faecium BM4147: Biosynthesis of a Depsipeptide Peptidoglycan Precursor by Vancomycin Resistance Proteins VanH and VanA', *Biochemistry*, 30(43), pp. 10408-10415.

Bussiere, D.E., Muchmore, S.W., Dealwis, C.G., Schluckebier, G., Nienaber, V.L., Edalji, R.P., Walter, K.A., Ladror, U.S., Holzman, T.F. and Abad-Zapatero, C. (1998) 'Crystal structure of ErmC', an rRNA methyltransferase which mediates antibiotic resistance in bacteria', *Biochemistry*, 37(20), pp. 7103-7112.

Cabiscol, E., Tamarit, J. and Ros, J. (2000) 'Oxidative stress in bacteria and protein damage by reactive oxygen species', *International Microbiology*, 3(1), pp. 3-8.

Cadet, J., Sage, E. and Douki, T. (2005) 'Ultraviolet radiation-mediated damage to cellular DNA', *Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis*, 571(1-2 SPEC. ISS.), pp. 3-17.

Calero-Cáceres, W. and Muniesa, M. (2016) 'Persistence of naturally occurring antibiotic resistance genes in the bacteria and bacteriophage fractions of wastewater', *Water Research*, 95, pp. 11-18.

Cardinal, P., Anderson, J.C., Carlson, J.C., Low, J.E., Challis, J.K., Beattie, S.A., Bartel, C.N., Elliott, A.D., Montero, O.F., Lokesh, S., Favreau, A., Kozlova, T.A., Knapp, C.W., Hanson, M.L. and Wong, C.S. (2014) 'Macrophytes may not contribute significantly to removal of nutrients, pharmaceuticals, and antibiotic resistance in model surface constructed wetlands', *Science of the Total Environment*, 482-483(1), pp. 294-304.

Chen, H. and Zhang, M. (2013a) 'Effects of advanced treatment systems on the removal of antibiotic resistance genes in wastewater treatment plants from Hangzhou, China', *Environmental Science and Technology*, 47(15), pp. 8157-8163.

Chen, H. and Zhang, M. (2013b) 'Occurrence and removal of antibiotic resistance genes in municipal wastewater and rural domestic sewage treatment systems in eastern China', *Environment International*, 55, pp. 9-14.

Chen, J., Ying, G.G., Wei, X.D., Liu, Y.S., Liu, S.S., Hu, L.X., He, L.Y., Chen, Z.F., Chen, F.R. and Yang, Y.Q. (2016a) 'Removal of antibiotics and antibiotic resistance genes from domestic sewage by constructed wetlands: Effect of flow configuration and plant species', *Science of the Total Environment*, 571, pp. 974-982.

Chen, Q., An, X., Li, H., Su, J., Ma, Y. and Zhu, Y.G. (2016b) 'Long-term field application of sewage sludge increases the abundance of antibiotic resistance genes in soil', *Environment International*, 92-93, pp. 1-10.

Cho, M., Kim, J., Kim, J.Y., Yoon, J. and Kim, J.H. (2010) 'Mechanisms of Escherichia coli inactivation by several disinfectants', *Water Research*, 44(11), pp. 3410-3418.

Christgen, B., Yang, Y., Ahammad, S.Z., Li, B., Rodriquez, D.C., Zhang, T. and Graham, D.W. (2015) 'Metagenomics shows that low-energy anaerobic-aerobic treatment reactors reduce antibiotic resistance gene levels from domestic wastewater', *Environmental Science and Technology*, 49(4), pp. 2577-2584.

Christou, A., Agüera, A., Bayona, J.M., Cytryn, E., Fotopoulos, V., Lambropoulou, D., Manaia, C.M., Michael, C., Revitt, M., Schröder, P. and Fatta-Kassinos, D. (2017) 'The potential implications of reclaimed wastewater reuse for irrigation on the agricultural environment: The knowns and unknowns of the fate of antibiotics and antibiotic resistant bacteria and resistance genes – A review', *Water Research*, 123, pp. 448-467.

CLSI (2017) *Performance Standards for Antimicrobial Susceptibility Testing CLSI supplement M100*. 27 edn. Wayne, PA: Clinical and Laboratory Standards Institute.

Collignon, P., Beggs, J.J., Walsh, T.R., Gandra, S. and Laxminarayan, R. (2018) 'Anthropological and socioeconomic factors contributing to global antimicrobial resistance: a univariate and multivariable analysis', *The Lancet Planetary Health*, 2(9), pp. e398-e405.

Czekalski, N., Imminger, S., Salhi, E., Veljkovic, M., Kleffel, K., Drissner, D., Hammes, F., Bürgmann, H. and Von Gunten, U. (2016) 'Inactivation of Antibiotic Resistant Bacteria and Resistance Genes by Ozone: From Laboratory Experiments to Full-Scale Wastewater Treatment', *Environmental Science and Technology*, 50(21), pp. 11862-11871.

Das, T.K. (2001) 'Ultraviolet disinfection application to a wastewater treatment plant', *Clean Products and Processes*, 3(2), pp. 69-80.

Davies, J. (1994) 'Inactivation of antibiotics and the dissemination of resistance genes', *Science*, 264(5157), pp. 375-382.

Davies, J., Spiegelman, G.B. and Yim, G. (2006) 'The world of subinhibitory antibiotic concentrations', *Current Opinion in Microbiology*, 9(5), pp. 445-453.

Davis, M.F., Price, L.B., Liu, C.M.H. and Silbergeld, E.K. (2011) 'An ecological perspective on U.S. industrial poultry production: The role of anthropogenic ecosystems on the emergence of drug-resistant bacteria from agricultural environments', *Current Opinion in Microbiology*, 14(3), pp. 244-250.

Dcosta, V.M., King, C.E., Kalan, L., Morar, M., Sung, W.W.L., Schwarz, C., Froese, D., Zazula, G., Calmels, F., Debruyne, R., Golding, G.B., Poinar, H.N. and Wright, G.D. (2011) 'Antibiotic resistance is ancient', *Nature*, 477(7365), pp. 457-461.

Deng, S., Yan, X., Zhu, Q. and Liao, C. (2019) 'The utilization of reclaimed water: Possible risks arising from waterborne contaminants', *Environmental Pollution*, 254.

Destiani, R. and Templeton, M.R. (2019) 'Chlorination and ultraviolet disinfection of antibiotic-resistant bacteria and antibiotic resistance genes in drinking water', *AIMS Environmental Science*, 6(3), pp. 222-241.

Ding, C. and He, J. (2010) 'Effect of antibiotics in the environment on microbial populations', *Applied Microbiology and Biotechnology*, 87(3), pp. 925-941.

Dodd, M.C. (2012) 'Potential impacts of disinfection processes on elimination and deactivation of antibiotic resistance genes during water and wastewater treatment', *Journal of Environmental Monitoring*, 14(7), pp. 1754-1771.

Dröge, M., Pühler, A. and Selbitschka, W. (2000) 'Phenotypic and molecular characterization of conjugative antibiotic resistance plasmids isolated from bacterial communities of activated sludge', *Molecular and General Genetics*, 263(3), pp. 471-482.

Du, J., Geng, J., Ren, H., Ding, L., Xu, K. and Zhang, Y. (2015) 'Variation of antibiotic resistance genes in municipal wastewater treatment plant with A2O-MBR system', *Environmental Science and Pollution Research*, 22(5), pp. 3715-3726.

Dunlop, P.S.M., Ciavola, M., Rizzo, L., McDowell, D.A. and Byrne, J.A. (2015) 'Effect of photocatalysis on the transfer of antibiotic resistance genes in urban wastewater', *Catalysis Today*, 240(PA), pp. 55-60.

EA (2009) *EVIDENCE: A Low Carbon Water Industry in 2050*. Bristol: EA. [Online]. Available at: https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/291635/scho1209brob-e-e.pdf.

EC (2017) *A European One Health Action Plan against Antimicrobial Resistance (AMR)*. [Online]. Available at:

https://ec.europa.eu/health/amr/sites/health/files/antimicrobial_resistance/docs/amr_2017_action-plan.pdf (Accessed: 3 May 2020).

EC (2018) *Proposal for a Regulation of the European Parliament and of the Council on minimum requirements for water reuse*. [Online]. Available at: https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A52018PC0337 (Accessed: 3 May 2020).

EEA (1994) *European Rivers and Lakes*. Copenhagen: European Environment Agency.

EEC (1991) *Council Directive 91/271/EEC of 21 May 1991 concerning urban waste-water treatment*. [Online]. Available at: https://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CELEX:31991L0271:EN:HTML (Accessed: 1 March 2020).

EEC (2013) *Directive 2008/105/EC of the European Parliament and of the Council of 16 December 2008, as amended by Directive 2013/39/EU of the European Parliament and of the Council, on environmental quality standards in the field of water policy, amending and subsequently repealing Council Directives 82/176/EEC, 83/513/EEC, 84/156/EEC, 84/491/EEC, 86/280/EEC and amending Directive 2000/60/EC of the European Parliament and of the Council*. [Online]. Available at: http://ec.europa.eu/environment/water/water-dangersub/pri_substances.htm.

Eggimann, S., Truffer, B. and Maurer, M. (2015) 'To connect or not to connect? Modelling the optimal degree of centralisation for wastewater infrastructures', *Water Research*, 84, pp. 218-231.

Eischeid, A.C., Meyer, J.N. and Linden, K.G. (2009) 'UV disinfection of adenoviruses: Molecular indications of DNA damage efficiency', *Applied and Environmental Microbiology*, 75(1), pp. 23-28.

Eisenberg, G. (1943) 'Colorimetric determination of hydrogen peroxide', *Industrial & Engineering Chemistry Analytical Edition*, 15(5), pp. 327-328.

Entwistle, J., Latta, D.E., Scherer, M.M. and Neumann, A. (2019) 'Abiotic Degradation of Chlorinated Solvents by Clay Minerals and Fe(II): Evidence for Reactive Mineral Intermediates', *Environmental Science and Technology*, 53(24), pp. 14308-14318.

EPA (1983) *Treatability Manual. Volume IV: Cost Estimating*. Washington: 20460, D.C. [Online]. Available at: https://www.fs.usda.gov/Internet/FSE_DOCUMENTS/stelprdb5336483.pdf (Accessed: 3 May 2020).

EPA (2002) *Method 1600: Enterococci in Water by Membrane Filtration Using membrane-Enterococcus Indoxyl-β-D-Glucoside Agar (mEI)*. Washington: 20460, D.C. [Online]. Available at: https://www.epa.gov/sites/production/files/2015-08/documents/method_1600_2009.pdf (Accessed: 3 May 2020).

EPA (2003) *Wastewater Technology Fact Sheet Disinfection for Small Systems*. [Online]. Available at: https://www.epa.gov/sites/production/files/2015-06/documents/disinfection_small.pdf (Accessed: 3 May 2020).

Eramo, A., Morales Medina, W.R. and Fahrenfeld, N.L. (2019) 'Viability-based quantification of antibiotic resistance genes and human fecal markers in wastewater effluent and receiving waters', *Science of the Total Environment*, 656, pp. 495-502.

EUCAST (2016) *EUCAST Guidance Documents in susceptibility testing*. Available at: https://www.eucast.org/ast_of_bacteria/guidance_documents/ (Accessed: 3 May 2020).

Fang, H., Zhang, Q., Nie, X., Chen, B., Xiao, Y., Zhou, Q., Liao, W. and Liang, X. (2017) 'Occurrence and elimination of antibiotic resistance genes in a long-term operation integrated surface flow constructed wetland', *Chemosphere*, 173, pp. 99-106.

Ferro, G., Fiorentino, A., Alferez, M.C., Polo-López, M.I., Rizzo, L. and Fernández-Ibáñez, P. (2015) 'Urban wastewater disinfection for agricultural reuse: effect of solar driven AOPs in the inactivation of a multidrug resistant E. coli strain', *Applied Catalysis B: Environmental*, 178, pp. 65-73.

Ferro, G., Guarino, F., Castiglione, S. and Rizzo, L. (2016) 'Antibiotic resistance spread potential in urban wastewater effluents disinfected by UV/H2O2 process', *Science of the Total Environment*, 560-561, pp. 29-35.

Ferro, G., Guarino, F., Cicatelli, A. and Rizzo, L. (2017) 'β-lactams resistance gene quantification in an antibiotic resistant Escherichia coli water suspension treated by advanced oxidation with UV/H2O2', *Journal of Hazardous Materials*, 323, pp. 426-433.

Fiorentino, A., Ferro, G., Alferez, M.C., Polo-López, M.I., Fernández-Ibañez, P. and Rizzo, L. (2015) 'Inactivation and regrowth of multidrug resistant bacteria in urban wastewater after disinfection by solar-driven and chlorination processes', *Journal of Photochemistry and Photobiology B: Biology*, 148, pp. 43-50.

Gallup, J.M. and Ackermann, M.R. (2006) 'Addressing fluorogenic real-time qPCR inhibition using the novel custom Excel file system 'FocusField2-6GallupqPCRSet-upTool-001' to attain consistently high fidelity qPCR reactions', *Biological Procedures Online*, 8(1), pp. 87-151.

García-Fernández, I., Polo-López, M.I., Oller, I. and Fernández-Ibáñez, P. (2012) 'Bacteria and fungi inactivation using Fe 3+/sunlight, H 2O 2/sunlight and near neutral photo-Fenton: A comparative study', *Applied Catalysis B: Environmental*, 121-122, pp. 20-29.

Gatica, J. and Cytryn, E. (2013) 'Impact of treated wastewater irrigation on antibiotic resistance in the soil microbiome', *Environmental Science and Pollution Research*, 20(6), pp. 3529-3538.

Giannakis, S., Le, T.T.M., Entenza, J.M. and Pulgarin, C. (2018) 'Solar photo-Fenton disinfection of 11 antibiotic-resistant bacteria (ARB) and elimination of representative AR genes. Evidence that antibiotic resistance does not imply resistance to oxidative treatment', *Water Research*, 143, pp. 334-345.

Giannakis, S., Voumard, M., Grandjean, D., Magnet, A., De Alencastro, L.F. and Pulgarin, C. (2016) 'Micropollutant degradation, bacterial inactivation and regrowth risk in wastewater effluents: Influence of the secondary (pre)treatment on the efficiency of Advanced Oxidation Processes', *Water Research*, 102, pp. 505-515.

Golovko, O., Kumar, V., Fedorova, G., Randak, T. and Grabic, R. (2014) 'Seasonal changes in antibiotics, antidepressants/psychiatric drugs, antihistamines and lipid regulators in a wastewater treatment plant', *Chemosphere*, 111, pp. 418-426.

GOV.UK (2019) *Tackling antimicrobial resistance 2019–2024*. [Online]. Available at: https://www.gov.uk/government/publications/uk-5-year-action-plan-for-antimicrobial-resistance-2019-to-2024 (Assessed: 3 May 2020).

Graham, D.W., Bergeron, G., Bourassa, M.W., Dickson, J., Gomes, F., Howe, A., Kahn, L.H., Morley, P.S., Scott, H.M., Simjee, S., Singer, R.S., Smith, T.C., Storrs, C. and Wittum, T.E. 1441 (2019) 'Complexities in understanding antimicrobial resistance across domesticated

animal, human, and environmental systems' *Annals of the New York Academy of Sciences* [Review]. Blackwell Publishing Inc., pp. 17-30. Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6850694/pdf/NYAS-1441-17.pdf.

Graham, D.W., Collignon, P., Davies, J., Larsson, D.G.J. and Snape, J. (2014) 'Underappreciated role of regionally poor water quality on globally increasing antibiotic resistance', *Environmental Science and Technology*, 48(20), pp. 11746-11747.

Graham, D.W., Giesen, M.J. and Bunce, J.T. (2018) 'Strategic approach for prioritising local and regional sanitation interventions for reducing global antibiotic resistance', *Water (Switzerland)*, 11(1).

Graham, D.W., Knapp, C.W., Christensen, B.T., McCluskey, S. and Dolfing, J. (2016) 'Appearance of β-lactam Resistance Genes in Agricultural Soils and Clinical Isolates over the 20 th Century', *Scientific Reports*, 6.

Green, M.R. and Sambrook, J. (2012) *Molecular cloning : a laboratory manual* 4th edn. N.Y.: Cold Spring Harbor Laboratory Press.

Guggenheim, S., Adams, J.M., Bain, D.C., Bergaya, F., Brigatti, M.F., Drits, V.A., Formoso, M.L.L., Galán, E., Kogure, T. and Stanjek, H. (2006) 'Summary of recommendations of nomenclature committees relevant to clay mineralogy: Report of the Association Internationale pour l'Etude des Argiles (AIPEA) Nomenclature Committee for 2006', *Clay Minerals*, 41(4), pp. 863-877.

Guo, C., Wang, K., Hou, S., Wan, L., Lv, J., Zhang, Y., Qu, X., Chen, S. and Xu, J. (2017) 'H2O2 and/or TiO2 photocatalysis under UV irradiation for the removal of antibiotic resistant bacteria and their antibiotic resistance genes', *Journal of Hazardous Materials*, 323, pp. 710-718.

Guo, M.T. and Kong, C. (2019) 'Antibiotic resistant bacteria survived from UV disinfection: Safety concerns on genes dissemination', *Chemosphere*, 224, pp. 827-832.

Guo, M.T., Yuan, Q.B. and Yang, J. (2013) 'Ultraviolet reduction of erythromycin and tetracycline resistant heterotrophic bacteria and their resistance genes in municipal wastewater', *Chemosphere*, 93(11), pp. 2864-2868.

Guo, M.T., Yuan, Q.B. and Yang, J. (2015) 'Distinguishing effects of ultraviolet exposure and chlorination on the horizontal transfer of antibiotic resistance genes in municipal wastewater', *Environmental Science and Technology*, 49(9), pp. 5771-5778.

Han, X.M., Hu, H.W., Shi, X.Z., Wang, J.T., Han, L.L., Chen, D. and He, J.Z. (2016) 'Impacts of reclaimed water irrigation on soil antibiotic resistome in urban parks of Victoria, Australia', *Environmental Pollution*, 211, pp. 48-57.

Harms, G., Layton, A.C., Dionisi, H.M., Gregory, I.R., Garrett, V.M., Hawkins, S.A., Robinson, K.G. and Sayler, G.S. (2003) 'Real-time PCR quantification of nitrifying bacteria in a municipal wastewater treatment plant', *Environmental Science and Technology*, 37(2), pp. 343-351.

Haydel, S.E., Remenih, C.M. and Williams, L.B. (2008) 'Broad-spectrum in vitro antibacterial activities of clay minerals against antibiotic-susceptible and antibiotic-resistant bacterial pathogens', *Journal of Antimicrobial Chemotherapy*, 61(2), pp. 353-361.

Hijnen, W.A.M., Beerendonk, E.F. and Medema, G.J. (2006) 'Inactivation credit of UV radiation for viruses, bacteria and protozoan (oo)cysts in water: A review', *Water Research*, 40(1), pp. 3-22.

Hiller, C.X., Hübner, U., Fajnorova, S., Schwartz, T. and Drewes, J.E. (2019) 'Antibiotic microbial resistance (AMR) removal efficiencies by conventional and advanced wastewater treatment processes: A review', *Science of the Total Environment*, 685, pp. 596-608.

Hong, P.Y., Julian, T.R., Pype, M.L., Jiang, S.C., Nelson, K.L., Graham, D., Pruden, A. and Manaia, C.M. (2018) 'Reusing treated wastewater: Consideration of the safety aspects associated with antibiotic-resistant bacteria and antibiotic resistance genes', *Water (Switzerland)*, 10(3).

Huang, Z., Maness, P.C., Blake, D.M., Wolfrum, E.J., Smolinski, S.L. and Jacoby, W.A. (2000) 'Bactericidal mode of titanium dioxide photocatalysis', *Journal of Photochemistry and Photobiology A: Chemistry*, 130(2-3), pp. 163-170.

Hughes, D. and Andersson, D.I. (2012) 'Selection of resistance at lethal and non-lethal antibiotic concentrations', *Current Opinion in Microbiology*, 15(5), pp. 555-560.

Ikehata, K., Jodeiri Naghashkar, N. and Gamal El-Din, M. (2006) 'Degradation of aqueous pharmaceuticals by ozonation and advanced oxidation processes: A review', *Ozone: Science and Engineering*, 28(6), pp. 353-414.

Jiao, Y.N., Chen, H., Gao, R.X., Zhu, Y.G. and Rensing, C. (2017) 'Organic compounds stimulate horizontal transfer of antibiotic resistance genes in mixed wastewater treatment systems', *Chemosphere*, 184, pp. 53-61.

Jiao, Y.N., Zhou, Z.C., Chen, T., Wei, Y.Y., Zheng, J., Gao, R.X. and Chen, H. (2018) 'Biomarkers of antibiotic resistance genes during seasonal changes in wastewater treatment systems', *Environmental Pollution*, 234, pp. 79-87.

Jong, M.C., Su, J.Q., Bunce, J.T., Harwood, C.R., Snape, J.R., Zhu, Y.G. and Graham, D.W. (2018) 'Co-optimization of sponge-core bioreactors for removing total nitrogen and antibiotic resistance genes from domestic wastewater', *Science of the Total Environment*, 634, pp. 1417-1423.

Ju, F. and Zhang, T. (2015) 'Experimental Design and Bioinformatics Analysis for the Application of Metagenomics in Environmental Sciences and Biotechnology', *Environmental Science and Technology*, 49(21), pp. 12628-12640.

Kahn, L.H. (2017) 'Antimicrobial resistance: A One Health perspective', *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 111(6), pp. 255-260.

Karaolia, P., Michael-Kordatou, I., Hapeshi, E., Alexander, J., Schwartz, T. and Fatta-Kassinos, D. (2017) 'Investigation of the potential of a Membrane BioReactor followed by solar Fenton oxidation to remove antibiotic-related microcontaminants', *Chemical Engineering Journal*, 310, pp. 491-502.

Karaolia, P., Michael, I., García-Fernández, I., Agüera, A., Malato, S., Fernández-Ibáñez, P. and Fatta-Kassinos, D. (2014) 'Reduction of clarithromycin and sulfamethoxazole-resistant Enterococcus by pilot-scale solar-driven Fenton oxidation', *Science of the Total Environment*, 468-469, pp. 19-27.

Karkman, A., Johnson, T.A., Lyra, C., Stedtfeld, R.D., Tamminen, M., Tiedje, J.M. and Virta, M. (2016) 'High-throughput quantification of antibiotic resistance genes from an urban wastewater treatment plant', *FEMS Microbiology Ecology*, 92(3).

Keeling, J.L., Raven, M.D. and Gates, W.P. (2000) 'Geology and characterization of two hydrothermal nontronites from weathered metamorphic rocks at the Uley Graphite Mine, South Australia', *Clays and Clay Minerals*, 48(5), pp. 537-548.

Keen, P.L., Knapp, C.W., Hall, K.J. and Graham, D.W. (2018) 'Seasonal dynamics of tetracycline resistance gene transport in the Sumas River agricultural watershed of British Columbia, Canada', *Science of the Total Environment*, 628-629, pp. 490-498.

Kemper, N. (2008) 'Veterinary antibiotics in the aquatic and terrestrial environment', *Ecological Indicators*, 8(1), pp. 1-13.

Kennedy, K. and Collignon, P. (2010) 'Colonisation with Escherichia coli resistant to "critically important" antibiotics: A high risk for international travellers', *European Journal of Clinical Microbiology and Infectious Diseases*, 29(12), pp. 1501-1506.

Khan, G.A., Berglund, B., Khan, K.M., Lindgren, P.E. and Fick, J. (2013) 'Occurrence and Abundance of Antibiotics and Resistance Genes in Rivers, Canal and near Drug Formulation Facilities - A Study in Pakistan', *PLoS ONE*, 8(6).

Kibbee, R.J. and Örmeci, B. (2017) 'Development of a sensitive and false-positive free PMA-qPCR viability assay to quantify VBNC Escherichia coli and evaluate disinfection performance in wastewater effluent', *Journal of Microbiological Methods*, 132, pp. 139-147.

Klappenbach, J.A., Saxman, P.R., Cole, J.R. and Schmidt, T.M. (2001) 'Rrndb: The ribosomal RNA operon copy number database', *Nucleic Acids Research*, 29(1), pp. 181-184.

Knapp, C.W., Dolfing, J., Ehlert, P.A.I. and Graham, D.W. (2010) 'Evidence of increasing antibiotic resistance gene abundances in archived soils since 1940', *Environmental Science and Technology*, 44(2), pp. 580-587.

Korzeniewska, E. and Harnisz, M. (2018) 'Relationship between modification of activated sludge wastewater treatment and changes in antibiotic resistance of bacteria', *Science of the Total Environment*, 639, pp. 304-315.

Krembs, F.J., Siegrist, R.L., Crimi, M.L., Furrer, R.F. and Petri, B.G. (2010) 'ISCO for groundwater remediation: Analysis of field applications and performance', *Ground Water Monitoring and Remediation*, 30(4), pp. 42-53.

Krzeminski, P., Tomei, M.C., Karaolia, P., Langenhoff, A., Almeida, C.M.R., Felis, E., Gritten, F., Andersen, H.R., Fernandes, T., Manaia, C.M., Rizzo, L. and Fatta-Kassinos, D. (2019) 'Performance of secondary wastewater treatment methods for the removal of contaminants of emerging concern implicated in crop uptake and antibiotic resistance spread: A review', *Science of the Total Environment*, 648, pp. 1052-1081.

Laht, M., Karkman, A., Voolaid, V., Ritz, C., Tenson, T., Virta, M. and Kisand, V. (2014) 'Abundances of tetracycline, sulphonamide and beta-lactam antibiotic resistance genes in conventional wastewater treatment plants (WWTPs) with different waste load', *PLoS ONE*, 9(8).

Lamba, M., Graham, D.W. and Ahammad, S.Z. (2017) 'Hospital Wastewater Releases of Carbapenem-Resistance Pathogens and Genes in Urban India', *Environmental Science and Technology*, 51(23), pp. 13906-13912.

Lambert, P.A. (2005) 'Bacterial resistance to antibiotics: Modified target sites', *Advanced Drug Delivery Reviews*, 57(10), pp. 1471-1485.

Lavender, J.S. and Kinzelman, J.L. (2009) 'A cross comparison of QPCR to agar-based or defined substrate test methods for the determination of Escherichia coli and enterococci in municipal water quality monitoring programs', *Water Research*, 43(19), pp. 4967-4979.

Le, T.H., Ng, C., Tran, N.H., Chen, H. and Gin, K.Y.H. (2018) 'Removal of antibiotic residues, antibiotic resistant bacteria and antibiotic resistance genes in municipal wastewater by membrane bioreactor systems', *Water Research*, 145, pp. 498-508.

Lee, Y., Gerrity, D., Lee, M., Gamage, S., Pisarenko, A., Trenholm, R.A., Canonica, S., Snyder, S.A. and Von Gunten, U. (2016) 'Organic Contaminant Abatement in Reclaimed Water by UV/H2O2 and a Combined Process Consisting of O3/H2O2 Followed by UV/H2O2: Prediction of Abatement Efficiency, Energy Consumption, and Byproduct Formation', *Environmental Science and Technology*, 50(7), pp. 3809-3819.

Lee, Y. and von Gunten, U. (2010) 'Oxidative transformation of micropollutants during municipal wastewater treatment: Comparison of kinetic aspects of selective (chlorine, chlorine dioxide, ferrateVI, and ozone) and non-selective oxidants (hydroxyl radical)', *Water Research*, 44(2), pp. 555-566.

Lekkerkerker-Teunissen, K., Knol, A.H., Van Altena, L.P., Houtman, C.J., Verberk, J.Q.J.C. and Van Dijk, J.C. (2012) 'Serial ozone/peroxide/low pressure UV treatment for synergistic and effective organic micropollutant conversion', *Separation and Purification Technology*, 100, pp. 22-29.

Levy, S.B. (1992) 'Active efflux mechanisms for antimicrobial resistance', *Antimicrobial Agents and Chemotherapy*, 36(4), pp. 695-703.

Li, D., Tong, T., Zeng, S., Lin, Y., Wu, S. and He, M. (2014) 'Quantification of viable bacteria in wastewater treatment plants by using propidium monoazide combined with quantitative PCR (PMA-qPCR)', *Journal of Environmental Sciences (China)*, 26(2), pp. 299-306.

Li, J., Cheng, W., Xu, L., Jiao, Y., Baig, S.A. and Chen, H. (2016) 'Occurrence and removal of antibiotics and the corresponding resistance genes in wastewater treatment plants: effluents' influence to downstream water environment', *Environmental Science and Pollution Research*, 23(7), pp. 6826-6835.

Li, J., Cheng, W., Xu, L., Strong, P.J. and Chen, H. (2015) 'Antibiotic-resistant genes and antibiotic-resistant bacteria in the effluent of urban residential areas, hospitals, and a municipal wastewater treatment plant system', *Environmental Science and Pollution Research*, 22(6), pp. 4587-4596.

Li, N., Sheng, G.P., Lu, Y.Z., Zeng, R.J. and Yu, H.Q. (2017) 'Removal of antibiotic resistance genes from wastewater treatment plant effluent by coagulation', *Water Research*, 111, pp. 204-212.

Lin, Y.W., Li, D., Gu, A.Z., Zeng, S.Y. and He, M. (2016) 'Bacterial regrowth in water reclamation and distribution systems revealed by viable bacterial detection assays', *Chemosphere*, 144, pp. 2165-2174.

Liu, N., Sijak, S., Zheng, M., Tang, L., Xu, G. and Wu, M. (2015) 'Aquatic photolysis of florfenicol and thiamphenicol under direct UV irradiation, UV/H2O2 and UV/Fe(II) processes', *Chemical Engineering Journal*, 260, pp. 826-834.

Liu, R., Xiao, D., Guo, Y., Wang, Z. and Liu, J. (2014) 'A novel photosensitized Fenton reaction catalyzed by sandwiched iron in synthetic nontronite', *RSC Advances*, 4(25), pp. 12958-12963.

Liu, W., Zhang, Z., Yang, X., Xu, Y. and Liang, Y. (2012) 'Effects of UV irradiation and UV/chlorine co-exposure on natural organic matter in water', *Science of the Total Environment*, 414, pp. 576-584.

Liu, X., Yuan, S., Tong, M. and Liu, D. (2017) 'Oxidation of trichloroethylene by the hydroxyl radicals produced from oxygenation of reduced nontronite', *Water Research*, 113, pp. 72-79.

Lloyd, K.G., MacGregor, B.J. and Teske, A. (2010) 'Quantitative PCR methods for RNA and DNA in marine sediments: Maximizing yield while overcoming inhibition', *FEMS Microbiology Ecology*, 72(1), pp. 143-151.

Lorenz, M.G. and Wackernagel, W. (1994) 'Bacterial gene transfer by natural genetic transformation in the environment', *Microbiological Reviews*, 58(3), pp. 563-602.

Luby, E.M., Moorman, T.B. and Soupir, M.L. (2016) 'Fate and transport of tylosin-resistant bacteria and macrolide resistance genes in artificially drained agricultural fields receiving swine manure', *Science of the Total Environment*, 550, pp. 1126-1133.

Luczkiewicz, A., Jankowska, K., Bray, R., Kulbat, E., Quant, B., Sokolowska, A. and Olanczuk-Neyman, K. (2011) 'Antimicrobial resistance of fecal indicators in disinfected wastewater', *Water Science and Technology*, 64(12), pp. 2352-2361.

Lüddeke, F., Heß, S., Gallert, C., Winter, J., Güde, H. and Löffler, H. (2015) 'Removal of total and antibiotic resistant bacteria in advanced wastewater treatment by ozonation in combination with different filtering techniques', *Water Research*, 69, pp. 243-251.

Macauley, J.J., Qiang, Z., Adams, C.D., Surampalli, R. and Mormile, M.R. (2006) 'Disinfection of swine wastewater using chlorine, ultraviolet light and ozone', *Water Research*, 40(10), pp. 2017-2026.

Mamane-Gravetz, H., Linden, K.G., Cabaj, A. and Sommer, R. (2005) 'Spectral sensitivity of Bacillus subtilis spores and MS2 coliphage for validation testing of ultraviolet reactors for water disinfection', *Environmental Science and Technology*, 39(20), pp. 7845-7852.

Mao, D., Yu, S., Rysz, M., Luo, Y., Yang, F., Li, F., Hou, J., Mu, Q. and Alvarez, P.J.J. (2015) 'Prevalence and proliferation of antibiotic resistance genes in two municipal wastewater treatment plants', *Water Research*, 85, pp. 458-466.

Martinez, J.L. (2009) 'Environmental pollution by antibiotics and by antibiotic resistance determinants', *Environmental Pollution*, 157(11), pp. 2893-2902.

Mazel, D. (2006) 'Integrons: Agents of bacterial evolution', *Nature Reviews Microbiology*, 4(8), pp. 608-620.

McCann, C.M., Christgen, B., Roberts, J.A., Su, J.Q., Arnold, K.E., Gray, N.D., Zhu, Y.G. and Graham, D.W. (2019) 'Understanding drivers of antibiotic resistance genes in High Arctic soil ecosystems', *Environment International*, 125, pp. 497-504.

McKinney, C.W. and Pruden, A. (2012) 'Ultraviolet disinfection of antibiotic resistant bacteria and their antibiotic resistance genes in water and wastewater', *Environmental Science and Technology*, 46(24), pp. 13393-13400.

Michael-Kordatou, I., Karaolia, P. and Fatta-Kassinos, D. (2018) 'The role of operating parameters and oxidative damage mechanisms of advanced chemical oxidation processes in the combat against antibiotic-resistant bacteria and resistance genes present in urban wastewater', *Water Research*, 129, pp. 208-230.

Michod, R.E., Bernstein, H. and Nedelcu, A.M. (2008) 'Adaptive value of sex in microbial pathogens', *Infection, Genetics and Evolution*, 8(3), pp. 267-285.

Miller, R.V. (2001) 'Environmental bacteriophage-host interactions: Factors contribution to natural transduction', *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology*, 79(2), pp. 141-147.

Mishra, S. and Imlay, J. (2012) 'Why do bacteria use so many enzymes to scavenge hydrogen peroxide?', *Archives of Biochemistry and Biophysics*, 525(2), pp. 145-160.

Moreira, N.F.F., Sousa, J.M., Macedo, G., Ribeiro, A.R., Barreiros, L., Pedrosa, M., Faria, J.L., Pereira, M.F.R., Castro-Silva, S., Segundo, M.A., Manaia, C.M., Nunes, O.C. and Silva, A.M.T. (2016) 'Photocatalytic ozonation of urban wastewater and surface water using immobilized TiO2 with LEDs: Micropollutants, antibiotic resistance genes and estrogenic activity', *Water Research*, 94, pp. 10-22.

Morrison, K.D., Misra, R. and Williams, L.B. (2016) 'Unearthing the Antibacterial Mechanism of Medicinal Clay: A Geochemical Approach to Combating Antibiotic Resistance', *Scientific Reports*, 6.

Morrison, K.D., Underwood, J.C., Metge, D.W., Eberl, D.D. and Williams, L.B. (2014) 'Mineralogical variables that control the antibacterial effectiveness of a natural clay deposit', *Environmental Geochemistry and Health*, 36(4), pp. 613-631.

Mrkva, M. (1983) 'Evaluation of correlations between absorbance at 254 nm and COD of river waters', *Water Research*, 17(2), pp. 231-235.

Naquin, A., Shrestha, A., Sherpa, M., Nathaniel, R. and Boopathy, R. (2015) 'Presence of antibiotic resistance genes in a sewage treatment plant in Thibodaux, Louisiana, USA', *Bioresource Technology*, 188, pp. 79-83.

Negreanu, Y., Pasternak, Z., Jurkevitch, E. and Cytryn, E. (2012) 'Impact of treated wastewater irrigation on antibiotic resistance in agricultural soils', *Environmental Science and Technology*, 46(9), pp. 4800-4808.

Nesme, J., Cécillon, S., Delmont, T.O., Monier, J.M., Vogel, T.M. and Simonet, P. (2014) 'Large-scale metagenomic-based study of antibiotic resistance in the environment', *Current Biology*, 24(10), pp. 1096-1100.

Nesme, J. and Simonet, P. (2015) 'The soil resistome: A critical review on antibiotic resistance origins, ecology and dissemination potential in telluric bacteria', *Environmental Microbiology*, 17(4), pp. 913-930.

Neumann, A., Olson, T.L. and Scherer, M.M. (2013) 'Spectroscopic evidence for Fe(II)-Fe(III) electron transfer at clay mineral edge and basal sites', *Environmental Science and Technology*, 47(13), pp. 6969-6977.

Neumann, A., Petit, S. and Hofstetter, T.B. (2011) 'Evaluation of redox-active iron sites in smectites using middle and near infrared spectroscopy', *Geochimica et Cosmochimica Acta*, 75(9), pp. 2336-2355.

Nocker, A., Sossa-Fernandez, P., Burr, M.D. and Camper, A.K. (2007) 'Use of propidium monoazide for live/dead distinction in microbial ecology', *Applied and Environmental Microbiology*, 73(16), pp. 5111-5117.

Novo, A. and Manaia, C.M. (2010) 'Factors influencing antibiotic resistance burden in municipal wastewater treatment plants', *Applied Microbiology and Biotechnology*, 87(3), pp. 1157-1166.

Oh, J., Salcedo, D.E., Medriano, C.A. and Kim, S. (2014) 'Comparison of different disinfection processes in the effective removal of antibiotic-resistant bacteria and genes', *Journal of Environmental Sciences (China)*, 26(6), pp. 1238-1242.

OSTI.GOV (1988) *Smectite Dehydration and Stability: Applications to Radioactive Waste Isolationat Yucca Mountain, Nevada*.LA-21023-MS. [Online]. Available at: https://www.osti.gov/servlets/purl/60343 (Accessed: 27 July 2020).

Otto, C.C., Kilbourne, J. and Haydel, S.E. (2016) 'Natural and ion-exchanged illite clays reduce bacterial burden and inflammation in cutaneous meticillin-resistant Staphylococcus aureus infections in mice', *Journal of Medical Microbiology*, 65(1), pp. 19-27.

Oturan, M.A. and Aaron, J.J. (2014) 'Advanced oxidation processes in water/wastewater treatment: Principles and applications. A review', *Critical Reviews in Environmental Science and Technology*, 44(23), pp. 2577-2641.

Ouyang, W.Y., Huang, F.Y., Zhao, Y., Li, H. and Su, J.Q. (2015) 'Increased levels of antibiotic resistance in urban stream of Jiulongjiang River, China', *Applied Microbiology and Biotechnology*, 99(13), pp. 5697-5707.

Pang, Y.C., Xi, J.Y., Xu, Y., Huo, Z.Y. and Hu, H.Y. (2016) 'Shifts of live bacterial community in secondary effluent by chlorine disinfection revealed by Miseq high-throughput sequencing combined with propidium monoazide treatment', *Applied Microbiology and Biotechnology*, 100(14), pp. 6435-6446.

Park, S., You, X. and Imlay, J.A. (2005) 'Substantial DNA damage from submicromolar intracellular hydrogen peroxide detected in Hpx- mutants of Escherichia coli', *Proceedings of the National Academy of Sciences of the United States of America*, 102(26), pp. 9317-9322.

Peak, N., Knapp, C.W., Yang, R.K., Hanfelt, M.M., Smith, M.S., Aga, D.S. and Graham, D.W. (2007) 'Abundance of six tetracycline resistance genes in wastewater lagoons at cattle feedlots with different antibiotic use strategies', *Environmental Microbiology*, 9(1), pp. 143-151.

Pei, R., Kim, S.C., Carlson, K.H. and Pruden, A. (2006) 'Effect of River Landscape on the sediment concentrations of antibiotics and corresponding antibiotic resistance genes (ARG)', *Water Research*, 40(12), pp. 2427-2435.

Petersen, T.N., Rasmussen, S., Hasman, H., Carøe, C., Bælum, J., Charlotte Schultz, A., Bergmark, L., Svendsen, C.A., Lund, O., Sicheritz-Pontén, T. and Aarestrup, F.M. (2015) 'Meta-genomic analysis of toilet waste from long distance flights; A step towards global surveillance of infectious diseases and antimicrobial resistance', *Scientific Reports*, 5.

Pham, A.L.-T., Doyle, F.M. and Sedlak, D.L. (2012) 'Kinetics and efficiency of H2O2 activation by iron-containing minerals and aquifer materials', *Water Research*, 46(19), pp. 6454-6462.

Pham, A.L.T., Lee, C., Doyle, F.M. and Sedlak, D.L. (2009) 'A silica-supported iron oxide catalyst capable of activating hydrogen peroxide at neutral pH values', *Environmental Science and Technology*, 43(23), pp. 8930-8935.

Philippon, A., Labia, R. and Jacoby, G. (1989) 'Extended-spectrum beta-lactamases', *Antimicrobial agents and chemotherapy*, 33(8), pp. 1131-1136.

Piddock, L.J.V. (2006) 'Multidrug-resistance efflux pumps - Not just for resistance', *Nature Reviews Microbiology*, 4(8), pp. 629-636.

Pignatello, J.J., Oliveros, E. and MacKay, A. (2006) 'Advanced oxidation processes for organic contaminant destruction based on the fenton reaction and related chemistry', *Critical Reviews in Environmental Science and Technology*, 36(1), pp. 1-84.

Pomba, C., Rantala, M., Greko, C., Baptiste, K.E., Catry, B., van Duijkeren, E., Mateus, A., Moreno, M.A., Pyörälä, S., Ružauskas, M., Sanders, P., Teale, C., John Threlfall, E., Kunsagi, Z., Torren-Edo, J., Jukes, H. and Törneke, K. (2017) 'Public health risk of antimicrobial resistance transfer from companion animals', *Journal of Antimicrobial Chemotherapy*, 72(4), pp. 957-968.

Proia, L., Adriana, A., Jessica, S., Carles, B., Marinella, F., Marta, L., Luis, B.J. and Servais, P. (2018) 'Antibiotic resistance in urban and hospital wastewaters and their impact on a receiving freshwater ecosystem', *Chemosphere*, 206, pp. 70-82.

Proia, L., Von Schiller, D., Sànchez-Melsió, A., Sabater, S., Borrego, C.M., Rodríguez-Mozaz, S. and Balcázar, J.L. (2016) 'Occurrence and persistence of antibiotic resistance genes in river biofilms after wastewater inputs in small rivers', *Environmental Pollution*, 210, pp. 121-128.

Quach-Cu, J., Herrera-Lynch, B., Marciniak, C., Adams, S., Simmerman, A. and Reinke, R.A. (2018) 'The effect of primary, secondary, and tertiary wastewater treatment processes on antibiotic resistance gene (ARG) concentrations in solid and dissolved wastewater fractions', *Water (Switzerland)*, 10(1).

Quintela-Baluja, M., Abouelnaga, M., Romalde, J., Su, J.-Q., Yu, Y., Gomez, M.L., Smets, B., Zhu, Y.-G. and Graham, D.W. (2019) 'Spatial ecology of a wastewater network defines the antibiotic resistance genes in downstream receiving waters', *Water Research*.

Quintela-Baluja, M., Chan, W., Alnakip, M., Abouelnaga, M. and Graham, D.W. (2015) 'Sanitation, Water Quality, and Antibiotic Resistance Dissemination', in Méndez-Vilas, A. (ed.) *The Battle Against Microbial Pathogens: Basic Science, Technological Advances and Educational Programs*. Fomatex Research Center, pp. 965-975.

Rafraf, I.D., Lekunberri, I., Sànchez-Melsió, A., Aouni, M., Borrego, C.M. and Balcázar, J.L. (2016) 'Abundance of antibiotic resistance genes in five municipal wastewater treatment plants in the Monastir Governorate, Tunisia', *Environmental Pollution*, 219, pp. 353-358.

Remucal, C.K. and Sedlak, D.L. 1071 (2011) 'The role of iron coordination in the production of reactive oxidants from ferrous iron oxidation by oxygen and hydrogen peroxide' *ACS Symposium Series* [Conference Paper]. American Chemical Society, pp. 177-197. Available at: https://pubs.acs.org/doi/pdfplus/10.1021/bk-2011-1071.ch009.

Riquelme Breazeal, M.V., Novak, J.T., Vikesland, P.J. and Pruden, A. (2013) 'Effect of wastewater colloids on membrane removal of antibiotic resistance genes', *Water Research*, 47(1), pp. 130-140.

Rizzo, L., Fiorentino, A. and Anselmo, A. (2013a) 'Advanced treatment of urban wastewater by UV radiation: Effect on antibiotics and antibiotic-resistant E. coli strains', *Chemosphere*, 92(2), pp. 171-176.

Rizzo, L., Manaia, C., Merlin, C., Schwartz, T., Dagot, C., Ploy, M.C., Michael, I. and Fatta-Kassinos, D. (2013b) 'Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: A review', *Science of the Total Environment*, 447, pp. 345-360.

Rizzo, L., Sannino, D., Vaiano, V., Sacco, O., Scarpa, A. and Pietrogiacomi, D. (2014) 'Effect of solar simulated N-doped TiO2 photocatalysis on the inactivation and antibiotic resistance

of an E. coli strain in biologically treated urban wastewater', *Applied Catalysis B: Environmental*, 144, pp. 369-378.

Rodríguez-Chueca, J., Varella della Giustina, S., Rocha, J., Fernandes, T., Pablos, C., Encinas, Á., Barceló, D., Rodríguez-Mozaz, S., Manaia, C.M. and Marugán, J. (2019) 'Assessment of full-scale tertiary wastewater treatment by UV-C based-AOPs: Removal or persistence of antibiotics and antibiotic resistance genes?', *Science of the Total Environment*, 652, pp. 1051-1061.

Rodriguez-Mozaz, S., Chamorro, S., Marti, E., Huerta, B., Gros, M., Sànchez-Melsió, A., Borrego, C.M., Barceló, D. and Balcázar, J.L. (2015) 'Occurrence of antibiotics and antibiotic resistance genes in hospital and urban wastewaters and their impact on the receiving river', *Water Research*, 69, pp. 234-242.

Rodriguez-Narvaez, O.M., Peralta-Hernandez, J.M., Goonetilleke, A. and Bandala, E.R. (2017) 'Treatment technologies for emerging contaminants in water: A review', *Chemical Engineering Journal*, 323, pp. 361-380.

Ruppert, G., Bauer, R. and Heisler, G. (1994) 'UV-O3, UV-H2O2, UV-TiO2 and the photo-Fenton reaction - comparison of advanced oxidation processes for wastewater treatment', *Chemosphere*, 28(8), pp. 1447-1454.

Salyers, A.A., Shoemaker, N.B., Stevens, A.M. and Li, L.Y. (1995) 'Conjugative transposons: An unusual and diverse set of integrated gene transfer elements', *Microbiological Reviews*, 59(4), pp. 579-590.

Schlüter, A., Krause, L., Szczepanowski, R., Goesmann, A. and Pühler, A. (2008) 'Genetic diversity and composition of a plasmid metagenome from a wastewater treatment plant', *Journal of Biotechnology*, 136(1-2), pp. 65-76.

Sharma, V.K., Johnson, N., Cizmas, L., McDonald, T.J. and Kim, H. (2016) 'A review of the influence of treatment strategies on antibiotic resistant bacteria and antibiotic resistance genes', *Chemosphere*, 150, pp. 702-714.

Shaw, J.P., Malley Jr, J.P. and Willoughby, S.A. (2000) 'Effects of UV irradiation on organic matter', *Journal / American Water Works Association*, 92(4), pp. 157-167.

Shaw, K.J., Rather, P.N., Hare, R.S. and Miller, G.H. (1993) 'Molecular genetics of aminoglycoside resistance genes and familial relationships of the aminoglycoside-modifying enzymes', *Microbiological Reviews*, 57(1), p. 138.

Sherratt, D.J. (1974) 'Bacterial plasmids', *Cell*, 3(3), pp. 189-195.

Shoemaker, N.B., Vlamakis, H., Hayes, K. and Salyers, A.A. (2001) 'Evidence for extensive resistance gene transfer among Bacteroides spp. and among Bacteroides and other genera in the human colon', *Applied and Environmental Microbiology*, 67(2), pp. 561-568.

Sigala, J. and Unc, A. (2012) 'A PCR-DGGE approach to evaluate the impact of wastewater source on the antibiotic resistance diversity in treated wastewater effluent', *Water Science and Technology*, 65(7), pp. 1323-1331.

Smith, C.M. and Hill, V.R. (2009) 'Dead-End Hollow-Fiber Ultrafiltration for Recovery of Diverse Microbes from Water', *Applied and Environmental Microbiology*, 75(16), p. 5284.

Sohn, J., Amy, G., Cho, J., Lee, Y. and Yoon, Y. (2004) 'Disinfectant decay and disinfection by-products formation model development: Chlorination and ozonation by-products', *Water Research*, 38(10), pp. 2461-2478.

Song, M.D., Wachi, M., Doi, M., Ishino, F. and Matsuhashi, M. (1987) 'Evolution of an inducible penicillin-target protein in methicillin-resistant Staphylococcus aureus by gene fusion', *FEBS Letters*, 221(1), pp. 167-171.

Sørensen, S.J., Bailey, M., Hansen, L.H., Kroer, N. and Wuertz, S. (2005) 'Studying plasmid horizontal transfer in situ: A critical review', *Nature Reviews Microbiology*, 3(9), pp. 700-710.

Sousa, J.M., Macedo, G., Pedrosa, M., Becerra-Castro, C., Castro-Silva, S., Pereira, M.F.R., Silva, A.M.T., Nunes, O.C. and Manaia, C.M. (2017) 'Ozonation and UV254 nm radiation for the removal of microorganisms and antibiotic resistance genes from urban wastewater', *Journal of Hazardous Materials*, 323, pp. 434-441.

Spratt, B.G. (1994) 'Resistance to antibiotics mediated by target alterations', *Science*, 264(5157), pp. 388-393.

Stucki, J.W., Golden, D.C. and Roth, C.B. (1984) 'Preparation and handling of dithionite-reduced smectite suspensions', *Clays and Clay Minerals*, 32(3), pp. 191-197.

Su, J.Q., Wei, B., Ou-Yang, W.Y., Huang, F.Y., Zhao, Y., Xu, H.J. and Zhu, Y.G. (2015) 'Antibiotic Resistome and Its Association with Bacterial Communities during Sewage Sludge Composting', *Environmental Science and Technology*, 49(12), pp. 7356-7363.

Sun, Y., Shen, Y.X., Liang, P., Zhou, J., Yang, Y. and Huang, X. (2016) 'Multiple antibiotic resistance genes distribution in ten large-scale membrane bioreactors for municipal wastewater treatment', *Bioresource Technology*, 222, pp. 100-106.

Süß, J., Volz, S., Obst, U. and Schwartz, T. (2009) 'Application of a molecular biology concept for the detection of DNA damage and repair during UV disinfection', *Water Research*, 43(15), pp. 3705-3716.

Svensson, S.L., Behroozian, S., Xu, W., Surette, M.G., Li, L. and Davies, J. (2017) 'Kisameet glacial clay: An unexpected source of bacterial diversity', *mBio*, 8(3).

Tamura, H., Goto, K., Yotsuyanagi, T. and Nagayama, M. (1974) 'Spectrophotometric determination of iron(II) with 1,10-phenanthroline in the presence of large amounts of iron(III)', *Talanta*, 21(4), pp. 314-318.

Tan, S.Y. and Tatsumura, Y. (2015) 'Alexander Fleming (1881–1955): Discoverer of penicillin', *Singapore Medical Journal*, 56(7), pp. 366-367.

Tchobanoglous, G., Burton, F.L. and Stensel, H.D. (2003) *Wastewater engineering : treatment and reuse / revised by George Tchobanoglous, Franklin L. Burton, H. David Stensel*. 4th edn. Boston: McGraw-Hill.

Tombini Decol, L., López-Gálvez, F., Truchado, P., Tondo, E.C., Gil, M.I. and Allende, A. (2019) 'Suitability of chlorine dioxide as a tertiary treatment for municipal wastewater and use of reclaimed water for overhead irrigation of baby lettuce', *Food Control*, 96, pp. 186-193.

Tong, M., Yuan, S., Ma, S., Jin, M., Liu, D., Cheng, D., Liu, X., Gan, Y. and Wang, Y. (2016) 'Production of Abundant Hydroxyl Radicals from Oxygenation of Subsurface Sediments', *Environmental Science and Technology*, 50(1), pp. 214-221.

Truchado, P., Gil, M.I., Kostic and Allende, A. (2016) 'Optimization and validation of a PMA qPCR method for Escherichia coli quantification in primary production', *Food Control*, 62, pp. 150-156.

Tsai, T.M., Chang, H.H., Chang, K.C., Liu, Y.L. and Tseng, C.C. (2010) 'A comparative study of the bactericidal effect of photocatalytic oxidation by TiO2 on antibiotic-resistant and antibiotic-sensitive bacteria', *Journal of Chemical Technology and Biotechnology*, 85(12), pp. 1642-1653.

Umar, M., Roddick, F. and Fan, L. (2019) 'Moving from the traditional paradigm of pathogen inactivation to controlling antibiotic resistance in water - Role of ultraviolet irradiation', *Science of the Total Environment*, 662, pp. 923-939.

UNICEF (2017) *Progress on Drinking Water, Sanitation and Hygiene: 2017 Update and SDG Baselines*. [Online]. Available at: https://www.unicef.org/publications/index_96611.html (Accessed: 3 May 2020).

van Dijk, E.L. (2014) 'Ten years of next-generation sequencing technology', *Trends in genetics : TIG*, 30(9), pp. 418-426.

Venieri, D., Gounaki, I., Bikouvaraki, M., Binas, V., Zachopoulos, A., Kiriakidis, G. and Mantzavinos, D. (2017) 'Solar photocatalysis as disinfection technique: Inactivation of Klebsiella pneumoniae in sewage and investigation of changes in antibiotic resistance profile', *Journal of Environmental Management*, 195, pp. 140-147.

Venieri, D., Gounaki, I., Christidis, G.E., Knapp, C.W., Bouras-Vallianatos, P. and Photos-Jones, E. (2020) 'Bridging the Gaps: Bole and Terra Sigillata as Artefacts, as Simples and as Antibacterial Clays', *Minerals*, 10(4).

Voegelin, A. and Hug, S.J. (2003) 'Catalyzed oxidation of arsenic(III) by hydrogen peroxide on the surface of ferrihydrite: An in situ ATR-FTIR study', *Environmental Science and Technology*, 37(5), pp. 972-978.

Von Sonntag, C. (2001) 'Rate constants of ozone reactions with DNA, its constituents and related compounds', *Journal of the Chemical Society, Perkin Transactions 2*, (3), pp. 269-274.

Wagner, M., Brumelis, D. and Gehr, R. (2002) 'Disinfection of wastewater by hydrogen peroxide or peracetic acid: Development of procedures for measurement of residual disinfectant and application to a physicochemically treated municipal effluent', *Water Environment Research*, 74(1), pp. 33-50.

Walsh, C. (2000) 'Molecular mechanisms that confer antibacterial drug resistance', *Nature*, 406(6797), pp. 775-781.

Wan, X., Gao, M., Ye, M., Wang, Y.K., Xu, H., Wang, M. and Wang, X.H. (2018) 'Formation, characteristics and microbial community of aerobic granular sludge in the presence of sulfadiazine at environmentally relevant concentrations', *Bioresource Technology*, 250, pp. 486-494.

Wang, F.H., Qiao, M., Lv, Z.E., Guo, G.X., Jia, Y., Su, Y.H. and Zhu, Y.G. (2014a) 'Impact of reclaimed water irrigation on antibiotic resistance in public parks, Beijing, China', *Environmental Pollution*, 184, pp. 247-253.

Wang, F.H., Qiao, M., Su, J.Q., Chen, Z., Zhou, X. and Zhu, Y.G. (2014b) 'High throughput profiling of antibiotic resistance genes in urban park soils with reclaimed water irrigation', *Environmental Science and Technology*, 48(16), pp. 9079-9085.

Wang, J., Mao, D., Mu, Q. and Luo, Y. (2015) 'Fate and proliferation of typical antibiotic resistance genes in five full-scale pharmaceutical wastewater treatment plants', *Science of the Total Environment*, 526, pp. 366-373.

Wang, Q., Wang, P. and Yang, Q. (2018) 'Occurrence and diversity of antibiotic resistance in untreated hospital wastewater', *Science of the Total Environment*, 621, pp. 990-999.

Wang, S., Ma, X., Liu, Y., Yi, X., Du, G. and Li, J. (2020) 'Fate of antibiotics, antibiotic-resistant bacteria, and cell-free antibiotic-resistant genes in full-scale membrane bioreactor wastewater treatment plants', *Bioresource Technology*, 302.

Wang, X., Dong, H., Zeng, Q., Xia, Q., Zhang, L. and Zhou, Z. (2017) 'Reduced Iron-Containing Clay Minerals as Antibacterial Agents', *Environmental Science and Technology*, 51(13), pp. 7639-7647.

Webber, M.A. and Piddock, L.J.V. (2003) 'The importance of efflux pumps in bacterial antibiotic resistance', *Journal of Antimicrobial Chemotherapy*, 51(1), pp. 9-11.

Wei, J.C., Yen, Y.T., Su, H.L. and Lin, J.J. (2011) 'Inhibition of bacterial growth by the exfoliated clays and observation of physical capturing mechanism', *Journal of Physical Chemistry C*, 115(38), pp. 18770-18775.

Wen, Q., Yang, L., Duan, R. and Chen, Z. (2016) 'Monitoring and evaluation of antibiotic resistance genes in four municipal wastewater treatment plants in Harbin, Northeast China', *Environmental Pollution*, 212, pp. 34-40.

WHO (2015a) *Antimicrobial resistance: An emerging water, sanitation and hygiene issue (Briefing note)*. Switzerland: WHO Document Production Services. [Online]. Available at: https://www.who.int/water_sanitation_health/publications/antimicrobial-resistance/en/ (Accessed: 3 May 2020).

WHO (2015b) *Global action plan on antimicrobial resistance*. Switzerland: WHO Document Production Services. [Online]. Available at: https://www.who.int/antimicrobial-resistance/global-action-plan/en/ (Accessed: 3 May 2020).

WHO (2018) *Antibiotic resistance*. Available at: https://www.who.int/news-room/fact-sheets/detail/antibiotic-resistance (Accessed: 21 April 2020).

Williams, L.B. (2017) 'Geomimicry: Harnessing the antibacterial action of clays', *Clay Minerals*, 52(1), pp. 1-24.

Williams, L.B., Metge, D.W., Eberl, D.D., Harvey, R.W., Turner, A.G., Prapaipong, P. and Poret-Peterson, A.T. (2011) 'What makes a natural clay antibacterial?', *Environmental Science and Technology*, 45(8), pp. 3768-3773.

Wojtenko, I. (2001) 'Performance of ozone as a disinectant for combined sewer overflow', *Critical Reviews in Environmental Science and Technology*, 31(4), pp. 295-309.

Wright, G.D. (2007) 'The antibiotic resistome: The nexus of chemical and genetic diversity', *Nature Reviews Microbiology*, 5(3), pp. 175-186.

Xie, W.Y., McGrath, S.P., Su, J.Q., Hirsch, P.R., Clark, I.M., Shen, Q., Zhu, Y.G. and Zhao, F.J. (2016) 'Long-term impact of field applications of sewage sludge on soil antibiotic resistome', *Environmental Science and Technology*, 50(23), pp. 12602-12611.

Xiong, P. and Hu, J. (2013) 'Inactivation/reactivation of antibiotic-resistant bacteria by a novel UVA/LED/TiO2 system', *Water Research*, 47(13), pp. 4547-4555.

Xue, G., Jiang, M., Chen, H., Sun, M., Liu, Y., Li, X. and Gao, P. (2019) 'Critical review of ARGs reduction behavior in various sludge and sewage treatment processes in wastewater

treatment plants', *Critical Reviews in Environmental Science and Technology*, 49(18), pp. 1623-1674.

Yang, Y., Li, B., Zou, S., Fang, H.H.P. and Zhang, T. (2014) 'Fate of antibiotic resistance genes in sewage treatment plant revealed by metagenomic approach', *Water Research*, 62, pp. 97-106.

Yoon, Y., Chung, H.J., Wen Di, D.Y., Dodd, M.C., Hur, H.G. and Lee, Y. (2017) 'Inactivation efficiency of plasmid-encoded antibiotic resistance genes during water treatment with chlorine, UV, and UV/H2O2', *Water Research*, 123, pp. 783-793.

Young, K.D. (2006) 'The selective value of bacterial shape', *Microbiology and Molecular Biology Reviews*, 70(3), pp. 660-703.

Yu, K. and Zhang, T. (2012) 'Metagenomic and metatranscriptomic analysis of microbial community structure and gene expression of activated sludge', *PLoS ONE*, 7(5).

Zainal-Abideen, M., Aris, A., Yusof, F., Abdul-Majid, Z., Selamat, A. and Omar, S.I. (2012) 'Optimizing the coagulation process in a drinking water treatment plant - Comparison between traditional and statistical experimental design jar tests', *Water Science and Technology*, 65(3), pp. 496-503.

Zhanel, G.G. and Karlowsky, J.A. (2016) 'Kisameet clay isolated from the central coast of British Columbia, Canada, demonstrates broad-spectrum antimicrobial activity', *mBio*, 7(2).

Zhang, J., Yang, M., Zhong, H., Liu, M., Sui, Q., Zheng, L., Tong, J. and Wei, Y. (2018) 'Deciphering the factors influencing the discrepant fate of antibiotic resistance genes in sludge and water phases during municipal wastewater treatment', *Bioresource Technology*, 265, pp. 310-319.

Zhang, M.Q., Yuan, L., Li, Z.H., Zhang, H.C. and Sheng, G.P. (2019a) 'Tetracycline exposure shifted microbial communities and enriched antibiotic resistance genes in the aerobic granular sludge', *Environment International*, 130.

Zhang, S., Han, B., Gu, J., Wang, C., Wang, P., Ma, Y., Cao, J. and He, Z. (2015a) 'Fate of antibiotic resistant cultivable heterotrophic bacteria and antibiotic resistance genes in wastewater treatment processes', *Chemosphere*, 135, pp. 138-145.

Zhang, T., Hu, Y., Jiang, L., Yao, S., Lin, K., Zhou, Y. and Cui, C. (2019b) 'Removal of antibiotic resistance genes and control of horizontal transfer risk by UV, chlorination and UV/chlorination treatments of drinking water', *Chemical Engineering Journal*, 358, pp. 589-597.

Zhang, Y., Zhuang, Y., Geng, J., Ren, H., Xu, K. and Ding, L. (2016) 'Reduction of antibiotic resistance genes in municipal wastewater effluent by advanced oxidation processes', *Science of The Total Environment*, 550, pp. 184-191.

Zhang, Y., Zhuang, Y., Geng, J., Ren, H., Zhang, Y., Ding, L. and Xu, K. (2015b) 'Inactivation of antibiotic resistance genes in municipal wastewater effluent by chlorination and sequential UV/chlorination disinfection', *Science of the Total Environment*, 512-513, pp. 125-132.

Zhou, X., Qiao, M., Su, J.Q. and Zhu, Y.G. (2019) 'High-throughput characterization of antibiotic resistome in soil amended with commercial organic fertilizers', *Journal of Soils and Sediments*, 19(2), pp. 641-651.

Zhou, Z.C., Zheng, J., Wei, Y.Y., Chen, T., Dahlgren, R.A., Shang, X. and Chen, H. (2017) 'Antibiotic resistance genes in an urban river as impacted by bacterial community and physicochemical parameters', *Environmental Science and Pollution Research*, 24(30), pp. 23753-23762.

Zhu, Y.G., Johnson, T.A., Su, J.Q., Qiao, M., Guo, G.X., Stedtfeld, R.D., Hashsham, S.A. and Tiedje, J.M. (2013) 'Diverse and abundant antibiotic resistance genes in Chinese swine farms', *Proceedings of the National Academy of Sciences of the United States of America*, 110(9), pp. 3435-3440.

Zhuang, Y., Ren, H., Geng, J., Zhang, Y., Zhang, Y., Ding, L. and Xu, K. (2015) 'Inactivation of antibiotic resistance genes in municipal wastewater by chlorination, ultraviolet, and ozonation disinfection', *Environmental Science and Pollution Research*, 22(9), pp. 7037-7044.

Zou, W., Xue, B., Zhi, W., Zhao, T., Yang, D., Qiu, Z., Shen, Z., Li, J., Zhang, B. and Wang, J. (2016) 'Effects of antibiotic resistance genes on the performance and stability of different microbial aggregates in a granular sequencing batch reactor', *Journal of Hazardous Materials*, 304, pp. 343-351.