Technische Universität Dresden, Faculty of Environmental sciences
Institute of Hydrobiology, Chair of Limnology

DISSERTATION

The impact of treated wastewater irrigation on the dissemination of antibiotic resistance in soil, subsoil and groundwater environments.

Author:
MSc Ioannis Kampouris
born on 17.01.1990 in Thessaloniki, Greece

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Supervisor and Reviewer	Prof. Dr. Thomas U. Berendonk	Technische Universität Dresden
Reviewer	Dr. Edward Topp	Agriculture and Agri-Food Canada
Reviewer	Prof. Dr. Petros Samaras	International Hellenic University

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Übereinstimmungserklärung:

Die Übereinstimmung dieses Exemplars mit dem Original der Dissertation zum Thema:

„The impact of treated wastewater irrigation on the dissemination of antibiotic resistance in soil, subsoil and groundwater environments.“

zu Deutsch: „Der Einfluss der Bewässerung von gereinigtem Abwasser auf die Verbreitung von Antibiotikaresistenzen in Bodenumgebungen, Untergrundumgebungen und Grundwasserumgebungen“

wird hiermit bestätigt.

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“The day you stop learning is the day you begin decaying.”

Isaac Asimov

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AMR: Antimicrobial Resistance

ARB: Antibiotic Resistant Bacteria

ARGs: Antibiotic Resistance Genes

BWA: Braunschweig Wastewater Association

DS: Digested Sludge

FW: Freshwater

GW: Groundwater

MAR: Managed Aquifer Recharge

qPCR: quantitative polymerace chain reaction

PNEC: Predicted no effect concentration

PERMANOVA: Permutational Mulitvariate Analysis of Variance

SPW: Subsoil Pore-Water

TWW: Treated Wastewater

UWTPs: Urban Wastewater Treatment Plants

Preface

Almost two hundred years ago, Dr John Snow identified the faecal contaminated water as a source of bacterial infections during a severe cholera outbreak. Several years later, we have developed many weapons on our arsenal to reduce the bacterial infections, from simple ones such as public hygiene measures (e.g. frequent showers & hand washing, clean water), to specialised ones such as the use of antibiotics. The antibiotics inhibit the bacterial growth, thus their use has effectively helped to treat many bacterial infections, revolutionizing medicine. Successful recovery from surgical operations would be seldom and would last exponentially without their use. Yet, antimicrobial resistance (AMR) has increased globally threatening to render antibiotics useless.

However, the "golden era" of novel antibiotics development, when many novel antibiotics were discovered in a few years, belongs to the past. The bacteria developed resistance mechanisms to every single one of the antibiotics and rendered them useless. This could be reflected to an increase in the death rates, but more importantly to the increased health-care costs, which might compromise the treatment for other diseases. The Covid-19 pandemic provided such a clear paradigm on the straining of health care systems during massive parallel hospitalisation of patients. While, the misuse of antibiotics for human and veterinary was the main contributor of the increased AMR levels, other anthropogenic activities greatly contributed to AMR spread as well. Specifically, the wastewater treatment plants are considered as hotspots for AMR and agricultural practices, such as manure amendment, have been show to clearly promote AMR. Thus, the scientific community across clinical settings, environmental and agricultural sectors intensively researches on AMR, in an attempt to fully understand the AMR phenomenon.

Nevertheless, the AMR is not the only problem that currently occurs in our society. The climate change, the urbanisation and the ever increasing human population has caused an increasing freshwater scarcity. The demand for treated wastewater (TWW) irrigation has increased due to this freshwater scarcity, and is expected to increase more. Since the TWW contains a high load of antibiotics, antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs), the irrigation with TWW has raised concerns regarding AMR spread in the environment. Many studies have attempted to investigate the impact of TWW on AMR spread in crops and soil; however, the impact on deeper lying environments remains not yet elucidated. This should raise concerns, since groundwater remains the most valuable drinking water source globally. Here in this thesis, I attempted to gain further understanding on whether TWW irrigation promotes the AMR spread in the soil and the so-far neglected deeper-lying subsurface

environments. My outmost desire is that the present work will contribute to a framework of minimising the potential risks during TWW irrigation, rendering TWW irrigation as a safe and sustainable alternative for freshwater resources depletion.

Summary

The water scarcity due to climate change and the ever-increasing human population have led to an extended demand for treated wastewater (TWW) irrigation. However, conventional wastewater-treatment technologies, such as secondary biological treatment (e.g. activated sludge) do not completely remove antibiotics, antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) from TWW effluents. Thus, TWW irrigation could potentially promote the spread of antimicrobial resistance (AMR) in soil and other downstream environments.

In the present thesis, I performed several long-term sampling campaigns in a full-scale commercially operated TWW irrigated field, to elucidate whether TWW irrigation promotes the spread of ARG in soil and subsurface aquatic environments; specifically subsoil pore-water (SPW) and groundwater (GW) environments. The full-scale commercially operated field was irrigated with TWW, subjected to secondary biological treatment, and occasionally mixed with digested sludge. In addition, I used laboratory-controlled soil/SPW-microcosms irrigated with freshwater (FW) and TWW, to confirm the insights gained from the long-term sampling campaigns under controlled laboratory conditions. Samples were analysed with qPCR for the occurrence of six ARGs, the integrase gene *intI1* and the total 16S rRNA abundance. 16S rRNA amplicon sequencing was performed to identify whether TWW-related bacterial taxa increased in soil, SPW, and GW during TWW irrigation. During the GW sampling campaign, liquid chromatography tandem mass spectrometry (LC-MS/MS) was performed as well, to identify the infiltration and accumulation of antibiotics in the GW.

Specifically, TWW irrigated and non-irrigated adjacent soil displayed completely different ARG and *intI1* profiles: *sul1*, *qnrS*, $bla_{\text{OXA-58}}$, *tet*(M) and *intI1* were significantly more abundant in the TWW irrigated field soil. In contrary, $bla_{\text{CTX-M-32}}$ and bla_{TEM}, the least abundant genes in the TWW irrigation, showed higher abundance in the non-irrigated soil. In long-term sampling campaigns, ARG abundance in soil and SPW was associated with the irrigation intensity and the introduced ARG load from TWW irrigation. Controlled laboratory microcosm experiments verified observations from the field study: TWW irrigation promoted the spread of ARGs and *intI1* into soil and SPW at far elevated levels compared to FW irrigation. Furthermore, TWW irrigation mainly promoted the spread of the sulfonamide ARG *sul1* and the integrase gene *intI1* in GW. Apart from ARGs, the GW contained elevated concentrations of the sulfonamide antibiotic sulfamethoxazole and the anticonvulsant drug carbamazepine.

The GW sulfamethoxazole concentration increased over the duration of irrigation and correlated with the relative abundance of the sulfonamide ARG *sul1*. This indicates a possible contribution of the persistence of sulfonamides in GW to the successful dissemination of *sul1* in this environment.

In conclusion, irrigation with TWW subjected only to secondary wastewater treatment can promote the spread of antibiotic resistance, not only in soil but also in SPW and GW environments. Thus, further monitoring of antibiotic and ARG occurrence in soil and subsurface aquatic environments of agricultural settings remains crucial. However, performing only monitoring of full-scale systems will not be enough; it has to be combined with further research using controlled laboratory systems to mechanistically explore the effects of various biotic/abiotic factors. The combination of these two approaches can shed light onto the interplay of biotic and abiotic factors affecting the soil/SPW/GW microbiome and consequently the resistome.

Kurzfassung

Die Wasserknappheit aufgrund des Klimawandels und das weltweite Bevölkerungswachstum haben zu einer erhöhten Nachfrage nach Bewässerungsoptionen mit geklärtem Abwasser (treated wastewater: TWW) geführt. Herkömmliche Abwasserbehandlungstechnologien, wie die sekundäre biologische Behandlung (z. B. Belebtschlamm) entfernen Antibiotika, antibiotikaresistente Bakterien (ARB) und Antibiotikaresistenzgene (ARGs) jedoch nicht vollständig aus dem TWW. Somit kann die Bewässerung von Böden mit TWW möglicherweise die Ausbreitung von Antibiotikaresistenzen (AMR) im Boden und anderen nachgelagerten aquatischen Umweltkompartimenten fördern. In der vorliegenden Dissertation habe ich mehrere Langzeit-Probenahmekampagnen in einem großflächigen, kommerziell landwirtschaftlich betriebenen, mit TWW bewässerten Feld durchgeführt, um herauszufinden, ob die Bewässerung mit TWW die Verbreitung von ARG im Boden und in der unterirdischen aquatischen Umwelt fördert. Dies umfasst insbesondere den Boden selbst, das Porenwasser des Bodens (soil pore water: SPW) und das darunterliegende Grundwasser (GW). Das großflächig kommerziell betriebene Feld wurde mit TWW bewässert, welches vorher einer biologischen Sekundärbehandlung unterzogen wurde - gelegentlich wurde zusätzlich Faulschlamm beigemischt. Darüber hinaus habe ich in kontrollierten Laborexperimenten Boden- & SPW-Mikrokosmen mit Trinkwasser (fresh water: FW) und TWW bewässert, um die Erkenntnisse aus den Langzeit-Probenahmekampagnen unter kontrollierten Laborbedingungen zu bestätigen. Die Proben wurden mit qPCR auf das Vorkommen von sechs ARGs, das Integrase-Gen *intI1* und die Gesamtabundanz von 16S-rRNA analysiert. Zusätzlich wurden 16S-rRNA-Amplikon-Sequenzierungen durchgeführt, um zu testen, ob TWW-bezogene bakterielle Taxa im Boden, SPW und GW während der TWW-Bewässerung angereichert wurden. Während der GW-Probenahmekampagne wurde zudem Flüssigchromatographie-Tandem-Massenspektrometrie (LC-MS/MS) durchgeführt, um die Infiltration und Anreicherung von Antibiotika im GW zu identifizieren. Im Speziellen zeigte sich, dass TWW-bewässerter und nicht bewässerter angrenzender Boden völlig unterschiedliche ARG- und *intI1*-Profile besitzen: *sul1*, *qnrS*, bla_{OXA-58}, *tet(M)* und *intI1* waren im TWW-bewässerten Feldboden signifikant häufiger zu finden. Die ARG-Häufigkeit im Boden und in SPW korrellierte signifikant mit der Bewässerungsintensität und der absolut eingeführten ARG-Fracht aus der TWW-Bewässerung. Im Gegensatz dazu zeigten $bla_{CTX-M-32}$ und bla_{TEM}, die am seltensten vorkommenden Gene in TWW, eine höhere Häufigkeit im nicht bewässerten Boden. Kontrollierte Labor-Mikrokosmos-Experimente bestätigten die Beobachtungen aus der

Feldstudie: TWW-Bewässerung förderte die Ausbreitung von ARGs und *intI1* im Boden und SPW in weit höheren Konzentrationen als Bewässerung mit FW. Darüber hinaus förderte die TWW-Bewässerung die Verbreitung des Sulfonamid ARG *sul1* und des Integrase-Gens *intI1* im GW. Zusätzlich zu erhöhten ARG Abundanzen enthielt das GW erhöhte Konzentrationen des Sulfonamid-Antibiotikums Sulfamethoxazol und des Antikonvulsivums Carbamazepin. Die Sulfamethoxazol-Konzentration im GW stieg über die Dauer der Bewässerung an und korrelierte zusätzlich mit der relativen Häufigkeit des Sulfonamid-ARG *sul1*. Dies weist auf einen möglichen Beitrag der Persistenz von Sulfonamiden in GW zur erfolgreichen Verbreitung von *sul1* in dieser Umgebung hin. Zusammenfassend lässt sich sagen, dass die Bewässerung mit TWW, welches nur einer sekundären Abwasserbehandlung unterzogen wird, die Ausbreitung von Antibiotikaresistenzen nicht nur im Boden, sondern auch in SPW- und GW-Umgebungen fördern kann. Daher bleibt die weitere Überwachung des Vorkommens von Antibiotika und ARG in Böden und tiefergelagerten Gewässerumgebungen von landwirtschaftlich genutzten Flächen von entscheidender Bedeutung. Es reicht jedoch nicht aus, ausschliesslich Felder im großen Maßstab zu überwachen; um die Auswirkungen verschiedener biotischer/abiotischer Faktoren mechanistisch zu untersuchen bietet sich die Kombination mit Forschung unter Verwendung kontrollierter Laborsysteme an. Die Kombination dieser beiden Ansätze kann Aufschluss über das Zusammenspiel von biotischen und abiotischen Faktoren geben, welche das Mikrobiom von Böden/SPW/GW und damit ihr Resistom beeinflussen.

Chapter 1

1. Introduction and Scientific Background

1.1 Antibiotic use and the worldwide emergence of antibiotic resistance

The discovery of antibiotics has greatly revolutionized modern medicine by minimizing the deaths and treatment-costs from bacterial infections (Teillant et al., 2015). Recovery following surgical medical operations would last exponentially longer without the use of antibiotics (Teillant et al., 2015). The majority of antibiotics originated from secondary metabolites produced by fungal and bacterial strains (e.g. β-lactams, tetracyclines) (Wright, 2010a), while few of them have completely synthetic origin (e.g. sulfonamides) (Nunes et al., 2020). Slight chemical modification in these metabolites provided the commercial forms of the compounds, widely known as antibiotics (Wright, 2010a). Antibiotics target bacterial metabolic functions or basic common bacterial cell components such as nucleic-acid or protein synthesis, bacterial cell wall synthesis and bacterial membrane integrity (Wright, 2010a; Piddock, 2012; Nunes et al., 2020).

Nevertheless, antibiotic resistant bacteria (ARB) to each antibiotic emerged shortly after their introduction to the market for commercial use (Wright, 2010a; Piddock, 2012). Prescription of penicillin, the first discovered antibiotic (by Sir Alexander Fleming in 1928) started around 1940 (Piddock, 2012). By the 1950s, the first signs of resistance to penicillin treatments started to appear (Clatworthy et al., 2007). Over the next decades, novel antibiotics were discovered, such as erythromycin, tetracycline and colistin. Yet, despite the introduction of new antibiotics, resistant bacterial strains appeared shortly after the introduction of each newly discovered antibiotic (Clatworthy et al., 2007). The rapid increase of resistance reduced the treatment-performance for each antibiotic (Teillant et al., 2015). In contrast, the discovery and introduction of novel antibiotics into the market requires time and huge workload, thus remains a costly and slow process (Piddock, 2012; Theuretzbacher et al., 2017). As a consequence, pharmaceutical companies invest less on research and production for novel antibiotics, since they might become ineffective after a few years of introduction rendering the profits low (Theuretzbacher et al., 2017).

Antimicrobial resistance (AMR) has emerged so highly and rapidly that has rendered the majority of antibiotics ineffective for many opportunistic pathogens, in a relatively short time-frame (Wright, 2010a; Piddock, 2012; Teillant et al., 2015, Wright, 2010b). Estimated ARB infections reach 25,000 annually in United States of America and around 23,000 in the European Union, while 10 million deaths due to AMR are expected by 2050 annually (O'Neill,

2016). Furthermore, most bacterial infections do not immediately cause death (Teillant et al., 2015). This does not reduce the threat of AMR. Specifically, in the case that antibiotics stopped to be effective, most of the patients suffering from bacterial infections will recover eventually, but in a far slower time-period (Founou et al., 2017). This slow recovery can increase the demands for treatment costs exponentially, straining the healthcare systems and lowering their capability to effectively treat patients (Founou et al., 2017). Consequently, AMR poses a great risk to modern healthcare increasing the death rate and the treatment costs; hence, there is a necessity for measures to mitigate of AMR dissemination.

1.2 Antibiotic resistance origin and mechanisms

In nature, microorganisms frequently compete with neighbours in their habitat for limited space and resources (Hibbing et al., 2010). Several free-living bacteria and fungi produce a variety of secondary metabolites (including antibiotics), hazardous to other community members, as a strategy for survival and competition (Hibbing et al., 2010; Tyc, et al., 2017; Zhang & Straight, 2019). Antibiotic production provides a fitness advantage, by inhibiting metabolic functions of competitor bacteria. However, the natural antibiotic production resulted in adaptive responses through co-evolutionary processes (Laskaris et al., 2010). Bacteria developed various strategies to counter the fitness advantage of over-competitive antibiotic-producing microorganisms (Hibbing et al., 2010).

Antibiotic resistance genes (ARGs) provide various mechanisms of resistance to antibiotics in bacterial strains (Fig. 1.1). A common mechanism, the modification or inactivation/degradation of antibiotic compounds, can provide resistance to antibiotics by altering the antibiotic molecules (Munita & Arias, 2016). For example, β-lactamases use this mechanism by hydrolysing the amide bond of the β-lactam ring, essential for β-lactam antibiotic function (Munita & Arias, 2016). Furthermore, bacteria can protect, change, replace, or bypass of the target sites to make antibiotics inefficient. For example, the *tet*(M) gene encodes a peptide that competes with tetracycline for the same ribosomal space and alters the geometry of the binding site (Li et al., 2013). Efflux pump genes also provide resistance, by transferring the antibiotic compounds outside of the bacterial cell (Munita & Arias, 2016). The replacement or bypass of the target site happens when bacteria synthetize new compound that

accomplish similar functions to the antibiotics original target, so that the antibiotic does not affect them (Wright, 2010b; Munita & Arias, 2016; Nunes et al., 2020). The *sul1* gene confers resistance to sulfonamides via the mechanism of replacement/bypass. The sulfonamides inhibit the dihydropteroate synthase, a folate-pathway enzyme and basic part of nucleotide and protein synthesis. The *sul1* gene encodes a dihydropteroate synthase with low affinity for sulfonamides, bypassing their inhibition while keeping the folate pathway in full function (Nunes et al., 2020). Consequently, bacteria can develop a variety of mechanisms that confer resistance to various antibiotics.

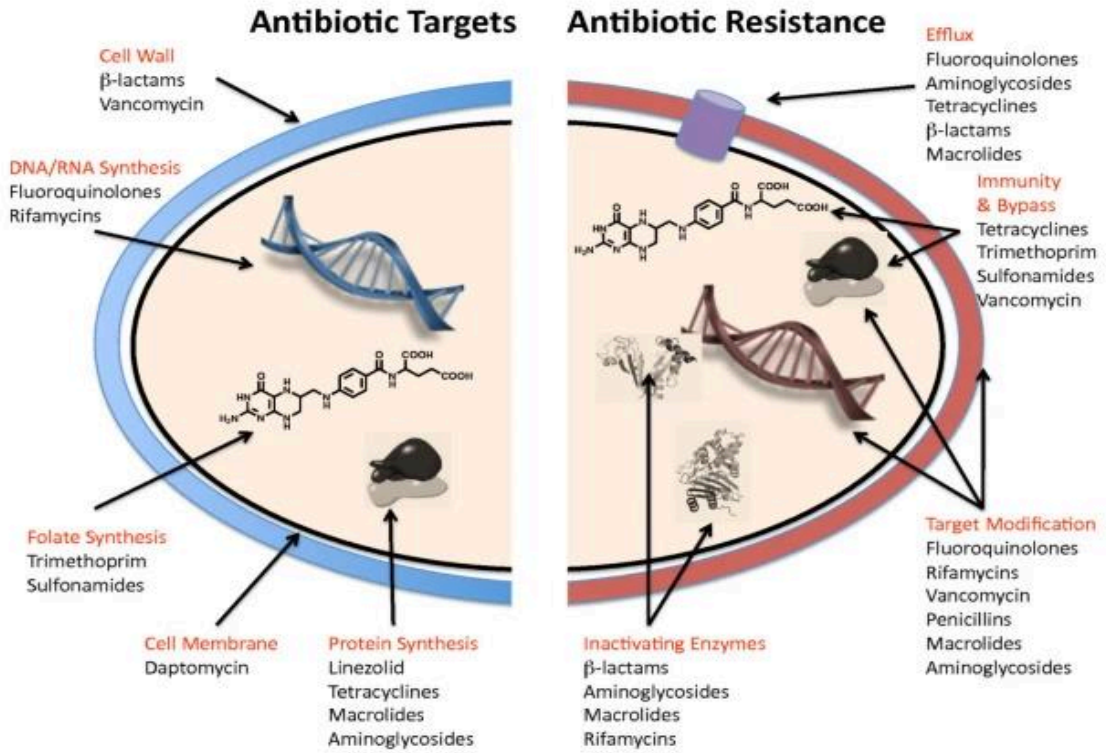

Figure 1.1: Schematic representation of antibiotic targets and common mechanisms of resistance (replicated from Wright et al., 2010b).

1.3 Resistance transmission: intrinsic and acquired resistance

Generally, antibiotic resistance is categorized into intrinsic or acquired resistance. In intrinsic resistance, the ARGs constitute a part of the physiology of specific bacterial strains (Wright, 2010a). These ARGs are located in chromosomal genes, constitutively encoding the resistance trait and are not transferred via horizontal gene transfer (HGT) (Cox & Wright, 2013). For example, the bacterial strains that produce antibiotics need to protect their metabolic functions from the self-produced antibiotic (Wright, 2010a; Laskaris et al., 2010). Non-specialised efflux pumps in the membrane of bacteria also provide intrinsic resistance, since they can lead to an effective reduction of the concentration of antibiotics in the cell (Cox & Wright, 2013).

The adaptation of resistance from bacteria, as a response to frequently used antibiotics, happens through the acquisition of genetic information (Martinez, 2009), known as acquired antibiotic resistance. Mutations of bacterial chromosomal genes or HGT of ARGs can lead to the acquisition of resistance (Palmer & Kishony, 2013). Specifically, spontaneous gene mutation and recombinatorial events may result in antibiotic resistance, even in the absence of selective pressure (D' Costa et al., 2006). Mutations confer resistance only for the individual bacterium that harbours the gene. Thus the trait disseminates vertically from the parent cell to the daughter cells (Munita & Arias, 2016). However, genetic information in bacterial cells disseminates quickly to bacterial communities through the mechanisms of HGT (Martinez, 2009; Klümper et al., 2015; Berendonk et al., 2015). Bacteria possess the capability for exchange of genetic information. The presence of a mobile ARG, even in one bacterium, might be relevant for the dissemination of resistance in a population, since it can reach either, non-pathogenic, human pathogenic or opportunistic pathogenic bacteria (Berendonk et al., 2015). Presumably, the precursors of several mobile ARGs derived from intrinsic ARGs that were captured by mobile genetic elements (MGEs) (Munita & Arias, 2016). The mobile ARGs are considered to have a greater potential risk for the spread of AMR into the environment and potentially back to human-associated microbiota (Munita & Arias, 2016).

ARG transfer mainly takes place via the mechanisms of conjugation, transduction and transformation (Fig. 1.2; Soucy et al., 2015). MGEs have a complex structure and are composed of modular units, including integrons, insertion sequences and genomic islands (Toussaint & Merlin, 2002). Conjugative plasmids are non-chromosomal circular or linear double-stranded DNA molecules capable of independent replication (Binh et al., 2008; Soucy et al., 2015). They consist of an essential backbone of genes encoding replicative functions and

a variable amount of different accessory genes including genes for antibiotic resistance, virulence or metabolic functions. Plasmids can harbour ARGs and transfer them among bacterial species via the mechanism of conjugation. During conjugation, a pair of cells is connected through a specialized mating pore (pilus), which allows the transfer of the donor-cell DNA into the recipient-cell (Schroder & Lanka, 2005; Soucy et al., 2015). ARGs can be transferred via MGEs along with other genes such as virulence genes and other metabolic genes (Toussaint & Merlin, 2002). In particular, conjugative plasmids belonging to the incompatibility (Inc) groups (IncP, IncQ, IncW and IncN), are known ARG carriers (Perry & Wright, 2013). Other MGEs, such as integrons, facilitate recombination by capturing new ARGs to plasmids or chromosomes (Boucher et al., 2007). Integrons can be part of transposons (Naas et al., 2001), which can move onto other locations of chromosomes, onto different plasmids, from plasmids onto chromosomes and vice versa (Parks & Peters, 2009). This reshuffling of genetic information through the activity of integrons and transposons increases the genetic recombination events. In addition, integrative and conjugative elements (ICE) can facilitate integration to bacterial chromosome and conjugation, favouring not only the genetic exchange and genetic recombination in parallel (Delavat et al., 2017).

Transduction happens through the transfer of bacterial viruses (bacteriophages) (Soucy et al., 2015). Bacteriophages are known as the most abundant and fast replicating life forms on earth (Clokie et al., 2011). Bacteriophages can have either single-or double-stranded RNA or DNA as genome and their size ranges up to 100 kb. They can be virulent, lysing the infected cells, or lysogenic, integrating into the genome of the host until an external signal activates the lytic cycle (Fortier & Sekulovic, 2013). Typically, in the lysogenic phase, the phage genome integrates into the host genome and replicates with it as a so-called prophage. This lysogenic conversion provides additional phenotypic properties to the new host like toxin production and therefore increases their plasticity (Soucy et al., 2015). During every phage infection event, the host cell DNA can enter into the phage virion and be later transferred into the genome of a new host via infection (Balcazar, 2014; Soucy et al., 2015). Thus, transduction relocates sections of microbial chromosomes and plays an important role in dissemination of ARGs as well (Balcazar, 2014).

Transformation is the third mechanism involved in horizontal gene transfer. In transformation, bacterial cells pick and incorporate extracellular DNA. The extracellular DNA stabilization in the new host cell happens through self-replication and DNA integration into the host chromosome via homologous recombination (Hynes et al., 2013; Soucy et al., 2015). In

comparison with conjugation, the incorporation of extracellular DNA is considered random and not consistent in genetic information incorporation, and thus a less common route of HGT for ARGs (Soucy et al., 2015). However, specific food source availability (chitin) and species-specific quorum-sensing signals can trigger transformation in *Vibrio cholerae* strains (Suckow et al., 2011). Therefore, transformation might contribute to the dissemination of MGEs and ARGs in complex bacterial communities as well.

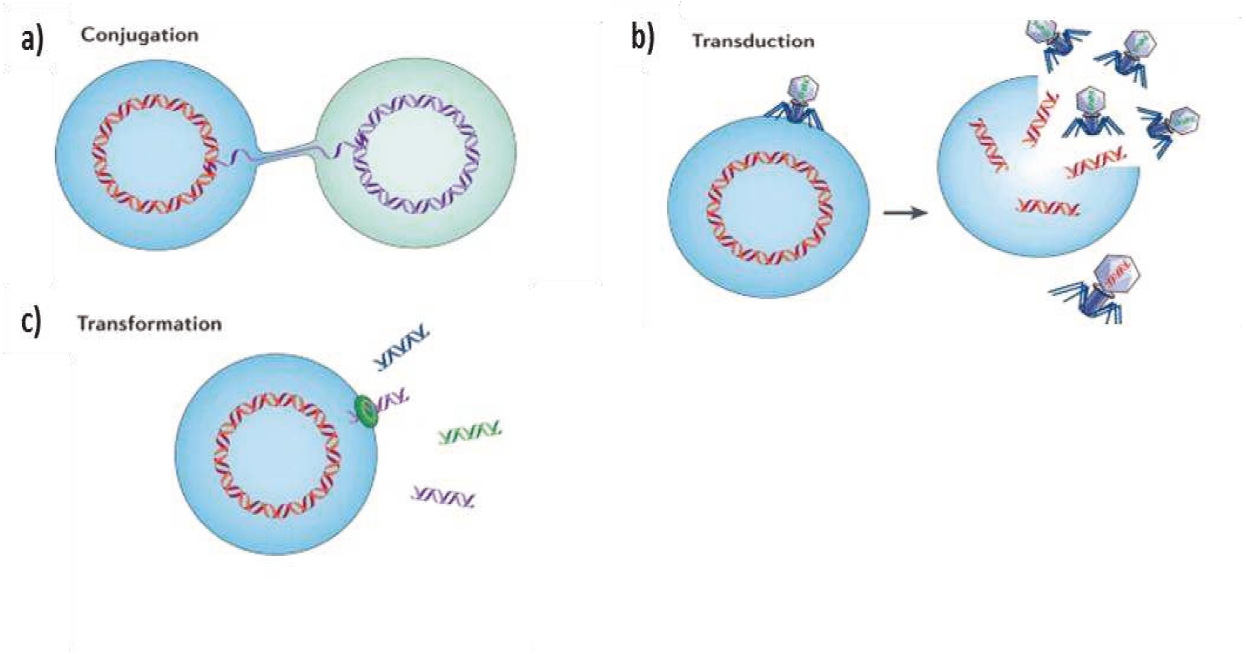

Figure 1.2: Representations the main HGT mechanisms (adapted from Sucy et al., 2015). Conjugation (a) occurs through donor–recipient cell contact, and single-stranded DNA is transferred from the donor cell to the recipient cell. Gene transfer mediated by phage is known as transduction (c). In the case of generalized transduction, any piece of genomic DNA may be loaded into the phage head; a general transducing phage is shown with host DNA (red). Specialized transduction occurs when an activated prophage loads a piece of genomic DNA neighbouring the prophage genome into the phage head together with the phage DNA (not shown). During transformation, (c) DNA is taken up from the surrounding environment.

1.4 Antibiotic resistance in soil environments

Soil favours complicated inter- and intra-kingdom mutualistic or antagonistic interactions between organisms (Hibbing et al., 2010; Tyc et al., 2016). This explains why most of the antibiotic producing organisms are derived from soil environments (Laskaris et al., 2010). Several fungal species produce the first discovered antibiotic, the β-lactam penicillin, to inhibit bacterial competition (Hibbing et al., 2010; Tyc et al., 2016). Despite the association of β-

lactams with fungi, their production is not restricted to the fungal community. *Actinobacteria* members can synthesize various β-lactams as well (Tyc et al., 2016). In addition, genes encoding β-lactam biosynthesis in fungi presumably originated from *Actinobacteria* members. *Actinobacteria* members produce a huge variety of other secondary metabolites, including several other antibiotic and antifungal metabolites (Miao et al., 2010). Specifically, most of the antibiotics derived from metabolites were originally produced by *Streptomyces* or *Actinomyces* isolates. Both fungi and *Actinobacteria* members thrive in soil, showing complex interactions with the rest of the organisms (Hibbing et al., 2010; Tyc et al., 2016). Thus, the soil environments function as natural ARG reservoirs (including intrinsic and acquired ARGs), resulted from defence mechanisms against antibiotic producing organisms. Permafrost soil, for example, contained precursors of modern β-lactamase genes, indicating AMR as an ancient phenomenon due to competition with antibiotic producing microorganism (D' Costa et al., 2011).

Nevertheless, despite the natural occurrence of ARGs in soil, ARG levels in archived soils increased from the start of commercial antibiotic usage (Knapp et al., 2011). Tetracycline, β-lactam, and macrolide ARGs showed a time-depended increasing trend in archived sampled soils from the 1940 until 2000 (Knapp et al., 2011). Furthermore, antibiotics use extends to agricultural applications (Heuer et al., 2011; Berendonk et al., 2015), since their use can counter livestock diseases and in low concentrations can promote livestock growth (Gaskins et al., 2006; Berendonk et al., 2015). The administration of antibiotics in livestock, can lead to high levels of antibiotics, ARB and ARGs in livestock faeces. Thus, manure application from livestock contains high amounts of antibiotic residues, ARB and ARGs (Heuer et al., 2011). Manure application with this high load of antibiotics, ARB and ARGs promotes AMR dissemination in soil (Heuer & Smalla, 2007). Several bacteria, including opportunistic pathogenic bacteria (e.g. *Pseudomonas* spp.), survive and thrive in soil environments, so they might disseminate from manure to soil (Leclercq et al., 2016). In addition, soil bacterial community members can receive plasmids from exogenous bacteria via conjugation (Klümper et al., 2015) and soil bacteria can easily transfer their ARGs back to human-associated pathogenic strains (Forsberg et al., 2012). Therefore, while the overuse of antibiotics remains the primary contributor for AMR increase, agricultural practices can promote the spread of ARG as well (Berendonk et al., 2015). Consequently, the routes of ARGs into soil microbiota should be tracked extensively, to provide information on factors that promote AMR spread and how to mitigate them in soil environments.

1.5 Wastewater treatment and antibiotic resistance

The urban wastewater treatment plants (UWTPs) receive high loads of microbial biomass, mainly through faecal and urine excretions; along with the rest of municipal waste or industrial waste (Michael et al., 2013; Rizzo et al., 2013; Manaia et al., 2018). Most UWTPs utilise the activated sludge process for wastewater treatment. The activated sludge process sufficiently removes the biodegradable organic matter, including organic carbon, nitrogen (N) and phosphorus (P) and suspended solids (Krzeminski et al., 2019). This process depends on the growth of bacterial biomass in the wastewater, mostly facultative or obligatory aerobic microorganisms and the suspension of microbial flocs (Manaia et al., 2018; Michael et al., 2013; Rizzo et al., 2013). Nevertheless, several plactonic bacteria remain in the liquid suspension of TWW, however, in far less abundance than in the original raw wastewater (Manaia et al., 2018). Yet, despite the efficiency of this process to reduce the organic and bacterial load, the conventional wastewater treatment does not specifically target biological pollutants such as ARB and ARG and their removal from the bacterial population (Caucci et al., 2016; Manaia et al., 2018; Cacace et al., 2019). Additionally, conventional wastewater treatment does not eliminate drug residues, including antibiotic residues (Michael et al., 2013; Rizzo et al., 2013; Alygizakis et al., 2019). Consequently, UWTPs have been considered as "hot spots" for AMR, since they release high loads of antibiotics, ARB and ARGs to the environment (Berendonk et al., 2015; Caucci et al., 2016; Manaia et al., 2018; Cacace et al., 2019).

1.6 Antibiotic resistance spread in soil microbiota as a consequence of wastewater irrigation

Water stress, due to FW resources depletion will increase substantially in the coming decades (Maaß & Grundmann, 2016). Climate change, the ever-increasing global population and the proportion of irrigated farmland have forced governments and supranational entities to rethink their water management paradigms (Paranychianakis et al., 2015). To this regard, there has

been a push to reuse wastewater, especially for agricultural irrigation purposes. TWW reuse further ensures the sustainability of modern agricultural practices (Paranychianakis et al., 2015; Maaß & Grundmann, 2016). In addition, TWW irrigation leads to the recycling of water and nutrients, promoting a green and circular economy (Maaß & Grundmann, 2016). Releasing TWW into the environment, without removing these nutrients, leads to ecological disruptions such as eutrophication (Gücker et al., 2006). Since crops need these nutrients to support their growth; the reuse of TWW could minimise the nutrient release to surface water via their absorption from the irrigated crops (Michael et al., 2013; Rizzo et al., 2013; Alygizakis et al., 2019). Germany suffers less from FW depletion since precipitation rates are higher, when compared to Mediterranean basin areas (Paranychianakis et al., 2015). However, TWW irrigation still takes place as a practice in agricultural fields near to the German city of Braunschweig, which started around 70 years ago. The sandy soil of the area nearby Braunschweig has low water holding capacity and high nutrient deficiency (Maaß & Grundmann, 2016). To counter the nutrient deficiency, the Braunschweig Wastewater Association performs wastewater irrigation and utilizes the cultivated crops for biogas (Paranychianakis et al., 2015; Maaß & Grundmann, 2016).

The frequency of epidemiological risks and concerns associated with the reuse of wastewater will rise due to the increase of TWW irrigation performance (Michael et al., 2013; Rizzo et al., 2013). Especially the occurrence of several newly emergent contaminants in TWW has raised concern. This includes metals, drug residues, but also biological contaminants related to AMR that spread during wastewater reuse practices (Michael et al., 2013; Rizzo et al., 2013; Alygizakis et al., 2019). Specifically, the impact of TWW irrigation on the ARG and *intI1* prevalence in topsoil has been investigated in full-scale agricultural systems from different countries/continents. Often these studies reported contradicting conclusions. A few studies have reported that TWW irrigation increases ARG prevalence (Wang et al., 2014; Chen et al., 2014; Han et al., 2016; Dalkmann et al., 2012; Jechalke et al., 2015). In contrast, other studies reported negligible impact of TWW irrigation on ARG prevalence in soil (Negreanu et al., 2012; Cerqueira et al., 2019a; Cerqueira et al., 2019b).

These contradicting results could be attributed to the variability of TWW quality or of tested ARGs between the different studies. Furthermore, variations occur between the soil-ecosystems of different geographical regions (Forsberg et al., 2014; Bahram et al., 2018). These variations could potentially influence the results of each study. Still, some important factors have so far been neglected (e.g. the natural ARG background of the sampled soils).

Specifically, information on two factors that could define the observed irrigation impact, the ARG load of irrigation and the irrigation intensity is missing. For example, *bla*$_{TEM}$ and *bla*$_{CTX-M}$ variants are indigenous to soil resistomes, even in the absence of anthropogenic impact (Gatica et al., 2015). Thus, as long as these ARGs occur in TWW at low abundance, the impact of TWW irrigation on their prevalence in soil might be insignificant. Furthermore, the irrigation water demand might differ for different crops or vary across different climatic seasons. Therefore, the applied ARG loads differ dramatically between high and low intensity irrigation.

1.7 Antibiotic resistance dissemination in subsurface terrestrial microbiota through treated wastewater irrigation

Apart from immediate effects on soil, anthropogenic processes can potentially affect subsurface terrestrial microbiota, located in subsoil and groundwater (GW) environments (Szekeres et al., 2018, Rossi et al., 2019). However, the majority of studies so far focused on either crops or topsoil, hence neglecting deeper lying environments like subsoil, subsoil pore-water (SPW) and GW (Negreanu et al., 2012, Cerqueira et al., 2019a, Cerqueira et al., 2019b, Cerqueira et al., 2019c, Marano et al., 2019, Wang et al., 2014; Han et al., 2016; Dalkmann et al., 2012, Jechalke et al., 2015). This raises concerns, due to the importance of GW as a drinking water resource (Szekeres et al., 2018). Presumably, anthropogenic pressure led to increasing ARG abundance in GW wells, in dependence on their proximity to urban settings (Szekeres et al., 2018). Furthermore, tetracycline and erythromycin ARGs were present in the GW of managed aquifer recharge (MAR) sites (Bockelmann et al. 2009). Contrary, the ARGs *bla*$_{TEM}$ and *qnrS* occurred in the TWW from a MAR site in Israel, but did not appear in the GW (Elkayam et al., 2018).

Nevertheless, antibiotics have been regularly detected in GW environments from Europe (Szeckeres et al., 2018), the USA (Barber et al., 2008) and Asia (Avisar et al., 2009). Among those, sulfonamides, a class of antibiotics with synthetic origin (Underwood et al., 2011), typically occur in high concentration in TWW (Johnson et al., 2015). They are able to persist in GW environments (Barber et al., 2008; Avisar et al., 2009; Underwood et al., 2011; Szeckeres et al., 2018), especially during TWW-irrigation/MAR operations (Avisar et al., 2009). The sulfonamide antibiotic sulfamethoxazole has occurred in GW samples, with

concentrations reaching up to 1,100 ng/L (Barber et al., 2008). Moreover, the spiking of antibiotics in irrigation can increase the prevalence of ARGs in the soil of water flow paths, as demonstrated in soil/subsoil microcosms (Lüneberg et al., 2018). Thus, TWW irrigation might promote the spread of ARGs in the underlying subsurface terrestrial environments, while the occurrence and accumulation of antibiotics in GW may favour the selection and the persistence of ARGs.

1.8 Outline and aims of the thesis

The present thesis was performed in the framework of the ANSWER-ITN (ANtibioticS and mobile resistance elements in WastEwater Reuse applications: risks and innovative solutions) project. This project aimed to unravel the highly complex factors driving AMR spread during urban wastewater reuse. Therefore, in the first part of this work, I attempted to elucidate the effect of TWW irrigation on the ARG spread in soil microbiota (Chapter 2). Specifically, the hypothesis that *the ARG load and irrigation intensity define the effect of TWW irrigation on ARG spread dynamics in soil* (**Hypothesis 1**) was tested using multiphase approach. This approach included sampling campaigns of a real-scale TWW-irrigated field and laboratory controlled microcosms, and analysis of samples with qPCR and sequencing.

Apart from the impact of TWW irrigation on the ARG abundance in topsoil, this thesis aimed to unravel the impact of TWW irrigation on the ARG abundance in the neglected deeper lying environments. Specifically, Chapter 3 focuses on whether *TWW irrigation increases ARG abundance in the subsoil pore-water* (**Hypothesis 2**), utilizing a similar multiphase approach. The specific environment was sampled because it is part of the water percolation process from the topsoil surface to GW, which remains the most important drinking water resource in several countries.

In Chapter 4, I extended my work by further verifying the impact of TWW irrigation on ARG spread into the even deeper GW environments. Additionally, the hypothesis that *TWW irrigation increases ARG abundance in GW through the accumulation of antibiotics in GW* (**Hypothesis 3**) was tested by combination of molecular biological and chemical analysis methods, here liquid chromatography tandem mass spectrometry to determine antibiotic concentrations in GW. The final synthesis section (Chapter 5) contains a discussion regarding the important findings, insights and conclusions gained from these three studies, along with the remaining scientific gaps that need to be addressed in future studies.

1.9 References

1) Alygizakis, N.A., Urík, J., Beretsou, V.G., Kampouris, I., Galani, A., Oswaldova, M., Berendonk, T., Oswald, P., Thomaidis, N.S., Slobodnik, J., Vrana, B., Fatta-Kassinos, D., 2020. Evaluation of chemical and biological contaminants of emerging concern in treated wastewater intended for agricultural reuse. Environ. Int. 138, 105597. https://doi.org/10.1016/j.envint.2020.105597
2) Avisar, D., Lester, Y., Ronen, D., 2009. Sulfamethoxazole contamination of a deep phreatic aquifer. Sci. Total Environ. 407, 4278–4282. https://doi.org/10.1016/j.scitotenv.2009.03.032
3) Balcazar, J.L., 2014. Bacteriophages as Vehicles for Antibiotic Resistance Genes in the Environment. PLoS Pathog. 10, 1–4. https://doi.org/10.1371/journal.ppat.1004219
4) Barber, L.B., Keefe, S.H., Leblanc, D.R., Bradley, P.M., Chapelle, F.H., Meyer, M.T., Loftin, K.A., Kolpin, D.W., Rubio, F., 2009. Fate of sulfamethoxazole, 4-nonylphenol, and 17??-estradiol in groundwater contaminated by wastewater treatment plant effluent. Environ. Sci. Technol. 43, 4843–4850. https://doi.org/10.1021/es803292v
5) Bengtsson-Palme, J., Larsson, D.G.J., 2016. Concentrations of antibiotics predicted to select for resistant bacteria: Proposed limits for environmental regulation. Environ. Int. 86, 140–149. https://doi.org/10.1016/j.envint.2015.10.015
6) 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., Martinez, J.L., 2015. Tackling antibiotic resistance: the environmental framework. Nat. Rev. Microbiol. 13, 310–317. https://doi.org/10.1038/nrmicro3439
7) Binh, C.T.T., Heuer, H., Kaupenjohann, M., Smalla, K., 2008. Piggery manure used for soil fertilization is a reservoir for transferable antibiotic resistance plasmids. FEMS Microbiol. Ecol. 66, 25–37. https://doi.org/10.1111/j.1574-6941.2008.00526.x
8) Böckelmann, U., Dörries, H.H., Ayuso-Gabella, M.N., De Marçay, M.S., Tandoi, V., Levantesi, C., Masciopinto, C., Houtte, E. Van, Szewzyk, U., Wintgens, T., Grohmann, E., 2009. Quantitative PCR monitoring of antibiotic resistance genes and bacterial pathogens in three european artificial groundwater recharge systems. Appl. Environ. Microbiol. 75, 154–163. https://doi.org/10.1128/AEM.01649-08

9) Boucher, Y., Labbate, M., Koenig, J.E., Stokes, H.W., 2007. Integrons: mobilizable platforms that promote genetic diversity in bacteria. Trends Microbiol. 15, 301–309. https://doi.org/10.1016/j.tim.2007.05.004
10) Cacace, D., Fatta-Kassinos, D., Manaia, C.M., Cytryn, E., Kreuzinger, N., Rizzo, L., Karaolia, P., Schwartz, T., Alexander, J., Merlin, C., Garelick, H., Schmitt, H., de Vries, D., Schwermer, C.U., Meric, S., Ozkal, C.B., Pons, M.N., Kneis, D., Berendonk, T.U., 2019. Antibiotic resistance genes in treated wastewater and in the receiving water bodies: A pan-European survey of urban settings. Water Res. 162, 320–330. https://doi.org/10.1016/j.watres.2019.06.039
11) Caucci, S., Karkman, A., Cacace, D., Rybicki, M., Timpel, P., Voolaid, V., Gurke, R., Virta, M., Berendonk, T.U., 2016. Seasonality of antibiotic prescriptions for outpatients and resistance genes in sewers and wastewater treatment plant outflow. FEMS Microbiol. Ecol. 92, fiw060. https://doi.org/10.1093/femsec/fiw060
12) Cerqueira, F., Matamoros, V., Bayona, J., Elsinga, G., Hornstra, L.M., Piña, B., 2019b. Distribution of antibiotic resistance genes in soils and crops. A field study in legume plants (Vicia faba L.) grown under different watering regimes. Environ. Res. 170, 16–25. https://doi.org/10.1016/j.envres.2018.12.007
13) Cerqueira, F., Matamoros, V., Bayona, J., Piña, B., 2019a. Antibiotic resistance genes distribution in microbiomes from the soil-plant-fruit continuum in commercial Lycopersicon esculentum fields under different agricultural practices. Sci. Total Environ. 652, 660–670. https://doi.org/10.1016/j.scitotenv.2018.10.268
14) Cerqueira, F., Matamoros, V., Bayona, J.M., Berendonk, T.U., Elsinga, G., Hornstra, L.M., Piña, B., 2019c. Antibiotic resistance gene distribution in agricultural fields and crops. A soil-to-food analysis. Environ. Res. 177, 108608. https://doi.org/10.1016/j.envres.2019.108608
15) Chen, C., Li, J., Chen, P., Ding, R., Zhang, P., Li, X., 2014. Occurrence of antibiotics and antibiotic resistances in soils from wastewater irrigation areas in Beijing and Tianjin, China. Environ. Pollut. 193, 94–101. https://doi.org/10.1016/j.envpol.2014.06.005
16) Clatworthy, A.E., Pierson, E., Hung, D.T., 2007. Targeting virulence: A new paradigm for antimicrobial therapy. Nat. Chem. Biol. 3, 541–548. https://doi.org/10.1038/nchembio.2007.24
17) Clokie, M.R.J., Millard, A.D., Letarov, A. V., Heaphy, S., 2011. Phages in nature. Bacteriophage 1, 31–45. https://doi.org/10.4161/bact.1.1.14942

18) Cox, G., Wright, G.D., 2013. Intrinsic antibiotic resistance: Mechanisms, origins, challenges and solutions. Int. J. Med. Microbiol. 303, 287–292. https://doi.org/10.1016/j.ijmm.2013.02.009
19) D' Costa, 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., Wright, G.D., 2011. Antibiotic resistance is ancient. Nature 477, 457–461. https://doi.org/10.1038/nature10388
20) D'Costa, V.M., McGrann, K.M., Hughes, D.W., Wright, G.., 2006. Sampling the Antibiotic Resistome. Science. 311, 374–377. https://doi.org/10.1126/science.1120800
21) Dalkmann, P., Broszat, M., Siebe, C., Willaschek, E., Sakinc, T., Huebner, J., Amelung, W., Grohmann, E., Siemens, J., 2012. Accumulation of pharmaceuticals, enterococcus, and resistance genes in soils irrigated with wastewater for zero to 100 years in central Mexico. PLoS One 7. https://doi.org/10.1371/journal.pone.0045397
22) Delavat, F., Miyazaki, R., Carraro, N., Pradervand, N., van der Meer, J.R., 2017. The hidden life of integrative and conjugative elements. FEMS Microbiol. Rev. 41, 512–537. https://doi.org/10.1093/femsre/fux008
23) Elkayam, R., Aharoni, A., Vaizel-Ohayon, D., Sued, O., Katz, Y., Negev, I., Marano, R.B.M., Cytryn, E., Shtrasler, L., Lev, O., 2018. Viral and Microbial Pathogens, Indicator Microorganisms, Microbial Source Tracking Indicators, and Antibiotic Resistance Genes in a Confined Managed Effluent Recharge System. J. Environ. Eng. 144. https://doi.org/10.1061/(ASCE)EE.1943-7870.0001334
24) Forsberg, K.J., Patel, S., Gibson, M.K., Lauber, C.L., Knight, R., Fierer, N., Dantas, G., 2014. Bacterial phylogeny structures soil resistomes across habitats. Nature 509, 612–616. https://doi.org/10.1038/nature13377
25) Forsberg, K.J., Reyes, A., Wang, B., Selleck, E.M., Sommer, M.O.A., Dantas, G., 2012. The Shared Antibiotic Resistome of Soil Bacteria and Human Pathogens. Science (80-.). 337, 1107–1111. https://doi.org/10.1126/science.1220761
26) Fortier, L.C., Sekulovic, O., 2013. Importance of prophages to evolution and virulence of bacterial pathogens. Virulence 4, 354–365. https://doi.org/10.4161/viru.24498
27) Founou, R.C., Founou, L.L., Essack, S.Y., 2017. Clinical and economic impact of antibiotic resistance in developing countries: A systematic review and meta-analysis. PLoS One 12, 1–18. https://doi.org/10.1371/journal.pone.0189621

28) Gaskins, H.R., Collier, C.T., Anderson, D.B., 2002. Antibiotics as growth promotants: Mode of action. Anim. Biotechnol. 13, 29–42. https://doi.org/10.1081/ABIO-120005768
29) Gatica, J., Yang, K., Pagaling, E., Jurkevitch, E., Yan, T., Cytryn, E., 2015. Resistance of undisturbed soil microbiomes to ceftriaxone indicates extended spectrum β-lactamase activity. Front. Microbiol. 6, 1–11. https://doi.org/10.3389/fmicb.2015.01233
30) Gücker, B., Brauns, M., Pusch, M.T., 2006. Effects of wastewater treatment plant discharge on ecosystem structure and function of lowland streams. J. North Am. Benthol. Soc. 25, 313–329. https://doi.org/10.1899/0887-3593(2006)25[313:EOWTPD]2.0.CO;2
31) Han, X.M., Hu, H.W., Shi, X.Z., Wang, J.T., Han, L.L., Chen, D., He, J.Z., 2016. Impacts of reclaimed water irrigation on soil antibiotic resistome in urban parks of Victoria, Australia. Environ. Pollut. 211, 48–57. https://doi.org/10.1016/j.envpol.2015.12.033
32) Heuer, H., Schmitt, H., Smalla, K., 2011. Antibiotic resistance gene spread due to manure application on agricultural fields. Curr. Opin. Microbiol. 14, 236–243. https://doi.org/10.1016/j.mib.2011.04.009
33) Heuer, H., Smalla, K., 2007. Manure and sulfadiazine synergistically increased bacterial antibiotic resistance in soil over at least two months. Environ. Microbiol. 9, 657–666. https://doi.org/10.1111/j.1462-2920.2006.01185.x
34) Hibbing, M.E., Fuqua, C., Parsek, M.R., Peterson, S.B., 2010. Bacterial competition: Surviving and thriving in the microbial jungle. Nat. Rev. Microbiol. 8, 15–25. https://doi.org/10.1038/nrmicro2259
35) Jechalke, S., Broszat, M., Lang, F., Siebe, C., Smalla, K., Grohmann, E., 2015. Effects of 100 years wastewater irrigation on resistance genes, class 1 integrons and IncP-1 plasmids in Mexican soil. Front. Microbiol. 6, 1–10. https://doi.org/10.3389/fmicb.2015.00163
36) Klümper, U., Riber, L., Dechesne, A., Sannazzarro, A., Hansen, L.H., Sørensen, S.J., Smets, B.F., 2015. Broad host range plasmids can invade an unexpectedly diverse fraction of a soil bacterial community. ISME J. 9, 934–945. https://doi.org/10.1038/ismej.2014.191

37) Knapp, C.W., Dolfing, J., Ehlert, P.A.I., Graham, D.W., 2010. Evidence of increasing antibiotic resistance gene abundances in archived soils since 1940. Environ. Sci. Technol. 44, 580–587. https://doi.org/10.1021/es901221x
38) 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., 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. Sci. Total Environ. 648, 1052–1081. https://doi.org/10.1016/j.scitotenv.2018.08.130
39) Laskaris, P., Tolba, S., Calvo-Bado, L., Wellington, L., 2010. Coevolution of antibiotic production and counter-resistance in soil bacteria. Environ. Microbiol. 12, 783–796. https://doi.org/10.1111/j.1462-2920.2009.02125.x
40) Leclercq, S.O., Wang, C., Sui, Z., Wu, H., Zhu, B., Deng, Y., Feng, J., 2016. A multiplayer game: species of Clostridium, Acinetobacter, and Pseudomonas are responsible for the persistence of antibiotic resistance genes in manure-treated soils. Environ. Microbiol. 18, 3494–3508. https://doi.org/10.1111/1462-2920.13337
41) Li, W., Atkinson, G.C., Thakor, N.S., Allas, U., Lu, C.C., Yan Chan, K., Tenson, T., Schulten, K., Wilson, K.S., Hauryliuk, V., Frank, J., 2013. Mechanism of tetracycline resistance by ribosomal protection protein Tet(O). Nat. Commun. 4. https://doi.org/10.1038/ncomms2470
42) Lüneberg, K., Prado, B., Broszat, M., Dalkmann, P., Díaz, D., Huebner, J., Amelung, W., López-Vidal, Y., Siemens, J., Grohmann, E., Siebe, C., 2018. Water flow paths are hotspots for the dissemination of antibiotic resistance in soil. Chemosphere 193, 1198–1206. https://doi.org/10.1016/j.chemosphere.2017.11.143
43) Maaß, O., Grundmann, P., 2016. Added-value from linking the value chains of wastewater treatment, crop production and bioenergy production: A case study on reusing wastewater and sludge in crop production in Braunschweig (Germany). Resour. Conserv. Recycl. 107, 195–211. https://doi.org/10.1016/j.resconrec.2016.01.002
44) Manaia, C.M., Macedo, G., Fatta-Kassinos, D., Nunes, O.C., 2016. Antibiotic resistance in urban aquatic environments: can it be controlled? Appl. Microbiol. Biotechnol. 100, 1543–1557. https://doi.org/10.1007/s00253-015-7202-0
45) Marano, R.B.M., Zolti, A., Jurkevitch, E., Cytryn, E., 2019. Antibiotic resistance and class 1 integron gene dynamics along effluent, reclaimed wastewater irrigated soil,

crop continua: elucidating potential risks and ecological constraints. Water Res. 164, 114906. https://doi.org/10.1016/j.watres.2019.114906

46) Martinez, J.L., 2009. Environmental pollution by antibiotics and by antibiotic resistance determinants. Environ. Pollut. 157, 2893–2902. https://doi.org/10.1016/j.envpol.2009.05.051

47) Miao, V., Davies, J., 2010. Actinobacteria: The good, the bad, and the ugly. Antonie van Leeuwenhoek, Int. J. Gen. Mol. Microbiol. 98, 143–150. https://doi.org/10.1007/s10482-010-9440-6

48) Michael, I., Rizzo, L., McArdell, C.S., Manaia, C.M., Merlin, C., Schwartz, T., Dagot, C., Fatta-Kassinos, D., 2013. Urban wastewater treatment plants as hotspots for the release of antibiotics in the environment: A review. Water Res. 47, 957–995. https://doi.org/10.1016/j.watres.2012.11.027

49) Munita, J.M., Arias C.A 2016. Mechanism of Antibiotic Resistance. In "Virulence Mechanisms of Bacterial Pathogens, 5th edition" By Kudva I.T., Nancy, C.A. Plummer P.J., Qijing Z., Tracy N.L., Bannantine J.P., Bellaire, B.H. Published June 2016. Willey

50) Naas, T., Mikami, Y., Imai, T., Poirel, L., Nordmann, P., 2001. Characterization of In53, a class 1 plasmid- and composite transposon-located integron of Escherichia coli which carries an unusual array of gene cassettes. J. Bacteriol. 183, 235–249. https://doi.org/10.1128/JB.183.1.235-249.2001

51) Negreanu, Y., Pasternak, Z., Jurkevitch, E., Cytryn, E., 2012. Impact of treated wastewater irrigation on antibiotic resistance in agricultural soils. Environ. Sci. Technol. 46, 4800–4808. https://doi.org/10.1021/es204665b

52) Nunes, O.C., Manaia, C.M., Kolvenbach, B.A., Corvini, P.F.X., 2020. Living with sulfonamides: a diverse range of mechanisms observed in bacteria. Appl. Microbiol. Biotechnol. 104, 10389–10408. https://doi.org/10.1007/s00253-020-10982-5

53) O' Neill, Pr., 2016. Tackling drug-resistant infections globally: final report and recommendations. Gov. of the Uni. King. https://apo.org.au/sites/default/files/resource-files/2016-05/apo-nid63983.pdf

54) Palmer, A.C., Kishony, R., 2013. Understanding, predicting and manipulating the genotypic evolution of antibiotic resistance. Nat. Rev. Genet. 14, 243–248. https://doi.org/10.1038/nrg3351

55) Paranychianakis, N. V., Salgot, M., Snyder, S.A., Angelakis, A.N., 2015. Water reuse in EU states: Necessity for uniform criteria to mitigate human and environmental

risks. Crit. Rev. Environ. Sci. Technol. 45, 1409–1468. https://doi.org/10.1080/10643389.2014.955629

56) Parks, A.R., Peters, J.E., 2009. Tn7 elements: Engendering diversity from chromosomes to episomes. Plasmid 61, 1–14. https://doi.org/10.1016/j.plasmid.2008.09.008

57) Perry, J.A., Wright, G.D., 2013. The antibiotic resistance "mobilome": Searching for the link between environment and clinic. Front. Microbiol. 4, 1–7. https://doi.org/10.3389/fmicb.2013.00138

58) Piddock, L.J.V., 2012. The crisis of no new antibiotics-what is the way forward? Lancet Infect. Dis. 12, 249–253. https://doi.org/10.1016/S1473-3099(11)70316-4

59) Rizzo, L., Manaia, C., Merlin, C., Schwartz, T., Dagot, C., Ploy, M.C., Michael, I., Fatta-Kassinos, D., 2013. Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: A review. Sci. Total Environ. 447, 345–360. https://doi.org/10.1016/j.scitotenv.2013.01.032

60) Rossi, D., Caracciolo, A.B., Grenni, P., Cattena, F., Di Lenola, M., Patrolecco, L., Ademollo, N., Ciannarella, R., Mascolo, G., Ghergo, S., 2019. Groundwater autochthonous microbial communities as tracers of anthropogenic pressure impacts: Example from a municipal waste treatment plant (Latium, Italy). Water 11, 1–20. https://doi.org/10.3390/w11091933

61) Soucy, S.M., Huang, J., Gogarten, J.P., 2015. Horizontal gene transfer: Building the web of life. Nat. Rev. Genet. 16, 472–482. https://doi.org/10.1038/nrg3962

62) Suckow, G., Seitz, P., Blokesch, M., 2011. Quorum sensing contributes to natural transformation of Vibrio cholerae in a species-specific manner. J. Bacteriol. 193, 4914–4924. https://doi.org/10.1128/JB.05396-11

63) Szekeres, E., Chiriac, C.M., Baricz, A., Szőke-Nagy, T., Lung, I., Soran, M.L., Rudi, K., Dragos, N., Coman, C., 2018. Investigating antibiotics, antibiotic resistance genes, and microbial contaminants in groundwater in relation to the proximity of urban areas. Environ. Pollut. 236, 734–744. https://doi.org/10.1016/j.envpol.2018.01.107

64) Teillant, A., Gandra, S., Barter, D., Morgan, D.J., Laxminarayan, R., 2015. Potential burden of antibiotic resistance on surgery and cancer chemotherapy antibiotic prophylaxis in the USA: A literature review and modelling study. Lancet Infect. Dis. 15, 1429–1437. https://doi.org/10.1016/S1473-3099(15)00270-4

65) Theuretzbacher, U., Årdal, C., Harbarth, S., 2017. Linking sustainable use policies to novel economic incentives to stimulate antibiotic research and development. Infect. Dis. Rep. 9, 28–31. https://doi.org/10.4081/idr.2017.6836
66) Toussaint, A., Merlin, C., 2002. Mobile elements as a combination of functional modules. Plasmid 47, 26–35. https://doi.org/10.1006/plas.2001.1552
67) Tyc, O., Song, C., Dickschat, J.S., Vos, M., Garbeva, P., 2017. The Ecological Role of Volatile and Soluble Secondary Metabolites Produced by Soil Bacteria. Trends Microbiol. 25, 280–292. https://doi.org/10.1016/j.tim.2016.12.002
68) Underwood, J.C., Harvey, R.W., Metge, D.W., Repert, D.A., Baumgartner, L.K., Smith, R.L., Roane, T.M., Barber, L.B., 2011. Effects of the antimicrobial sulfamethoxazole on groundwater bacterial enrichment. Environ. Sci. Technol. 45, 3096–3101. https://doi.org/10.1021/es103605e
69) Wang, F.H., Qiao, M., Su, J.Q., Chen, Z., Zhou, X., Zhu, Y.G., 2014. High throughput profiling of antibiotic resistance genes in urban park soils with reclaimed water irrigation. Env. Sci Technol 48, 9079–9085. https://doi.org/10.1021/es502615e
70) Wright, G.D., 2010a. The antibiotic resistome. Expert Opin. Drug Discov. 5, 779–788. https://doi.org/10.1517/17460441.2010.497535
71) Zhang, C., Straight, P.D., 2019. Antibiotic discovery through microbial interactions. Curr. Opin. Microbiol. 51, 64–71. https://doi.org/10.1016/j.mib.2019.06.006

Chapter 2

2. Antibiotic resistance gene load and irrigation intensity determine the impact of wastewater irrigation on antimicrobial resistance in the soil microbiome.

Ioannis D. Kampouris[a], Shelesh Agrawal[b], Laura Orschler[b], Damiano Cacace[a], Steffen Kunze[a], Thomas U.Berendonk[a], Uli Klümper[a]

[a] Institute for Hydrobiology, Technische Universität Dresden, 01217, Dresden, Germany

[b] Technische Universität Darmstadt, Institute IWAR, Chair of Wastewater Engineering, Franziska-Braun-Straße 7, 64287 Darmstadt, Germany

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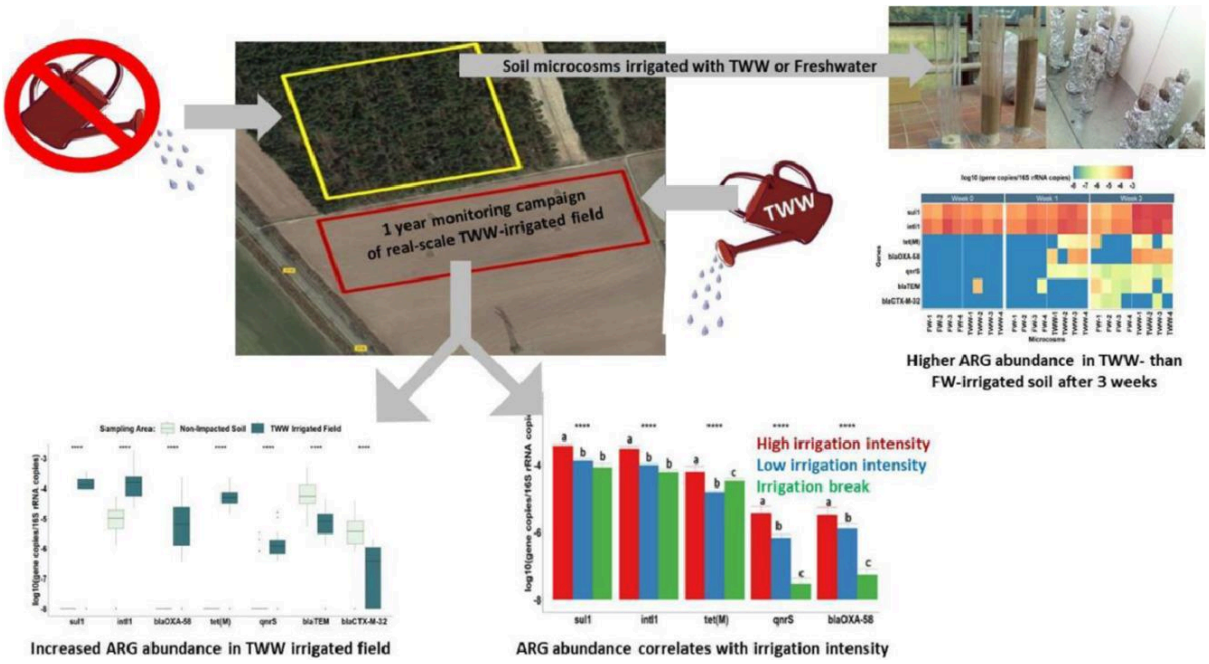
Graphical Abstract



Highlights

- Higher ARG abundance in TWW irrigated field compared to non-irrigated soil.
- ARG abundance in TWW irrigated soil positively correlates with irrigation intensity.
- Higher impact of TWW than FW irrigation on soil ARGs due to increased ARG load in TWW.
- No impact of TWW irrigation on soil microbial community density or composition.

Abstract

Treated wastewater (TWW) irrigation is a useful counter-measure against the depletion of freshwater (FW) resources. However, TWW contains several contaminants of emerging concern, such as antibiotic resistant bacteria (ARB) and antibiotic resistant genes (ARGs). Thus, TWW irrigation might promote the spread of antimicrobial resistance in soil environments. In the present work, we hypothesized that the ARG load and irrigation intensity define the effect of TWW irrigation on ARG spread dynamics in soil. This hypothesis was tested using a multiphase approach: a) comparing soil from a full-scale, commercially operated, TWW irrigated field with non-irrigated soil, b) long-term sampling of the TWW irrigated field over one year with different irrigation intensities and intercepted by irrigation breaks and c) laboratory-scale soil microcosms irrigated with TWW compared to FW. Six ARGs, the integrase gene *intI1* and the 16S rRNA were quantified using qPCR. In addition, effects of TWW irrigation on bacterial community composition of microcosm-samples were analysed with 16S rRNA amplicon sequencing. The genes *sul1, qnrS,* $bla_{\text{OXA-58}}$*, tet*(M) and *intI1* were significantly more abundant in the TWW irrigated field soil, whereas $bla_{\text{CTX–-M-32}}$ and $bla_{\text{TEM,}}$ the least abundant genes in the TWW irrigation, showed higher abundance in the non-irrigated soil. The relative abundance of *sul1, qnrS,* $bla_{\text{OXA-58}}$*, tet*(M) and *intI1* correlated with TWW irrigation intensity and decreased during irrigation breaks. Despite the decrease, the levels of these genes remained consistently higher than the non-irrigated soil indicating persistence upon their introduction into the soil. Microcosm experiments verified observations from the field study: TWW irrigation promoted the spread of ARGs and *intI1* into soil at far elevated levels compared to FW irrigation. However, the impact of TWW irrigation on 16S rRNA absolute abundance and the soil microbial community composition was negligible. In conclusion, the impact of TWW irrigation depends mainly on the introduced ARG load and the irrigation intensity.

2.1. Introduction

Wastewater reuse has recently gained popularity in arid/semi-arid areas as an action against the depletion of freshwater (FW) resources for irrigation purposes (Paranychianakis et al., 2015). The rising temperatures and lower precipitation rates are expected to increase the relevance of treated wastewater (TWW) irrigation even in northern European countries (Paranychianakis et al., 2015; Maaß et al., 2016). Despite the usefulness of TWW irrigation, concerns regarding this practice originate from the introduction of contaminants of emerging concern into the soil environment (Michael et al., 2013). These contaminants include pharmaceutical residues (Michael et al., 2013), antibiotic resistant bacteria (ARB) and antibiotic resistant genes (ARGs) (Manaia et al., 2018; Caucci et al., 2016; Pärnänen et al., 2019; Cacace et al., 2019). The high and increasing mortality rates due to infections with antibiotic resistant pathogens are a major global threat to human health (Friedman et al., 2016), recently recognised by the World Health Organisation (WHO, 2014). The continuous release of ARGs into natural and agricultural environments (Fatta-Kassinos et al., 2011; Gatica and Cytryn, 2013; Rizzo et al., 2013; Christou et al., 2017) could pose risks for human health. Specifically, resistance genes can be acquired by soil bacteria trough horizontal gene transfer (HGT) (Musovic et al., 2014; Klümper et al., 2015) and disseminate back to human-associated pathogenic strains (Forsberg et al., 2012). Therefore, identifying and monitoring of ARB/ARG sources in the environment has been prioritized recently as part of the "One-Health" strategy to combat the antimicrobial resistance (AMR) threat (Berendonk et al., 2015). Although AMR is often associated with clinical environments, the majority of the clinically-relevant ARGs originate from soil bacteria and have been mobilized into human pathogens (Cytryn, 2013; Nesme and Simonet, 2015). Despite the natural occurrence of ARGs in soil, agricultural practices (e.g. manure amendment or dairy wastewater irrigation) have been identified to significantly increase their prevalence and thus the risk for dissemination of ARGs to humans (Chen et al., 2016; Muurinen et al., 2017; Wang et al., 2018; McKinney et al., 2018; Dungan et al., 2018; Wolters et al., 2018).

The impact of TWW irrigation on the prevalence of ARGs and *intI1* in topsoil has been investigated in full-scale agricultural systems from different countries/continents, with contradicting conclusions. A few studies have reported that TWW irrigation increases ARG prevalence (Wang et al., 2014; Han et al., 2016; Dalkmann et al., 2012; Jechalke et al., 2015). Furthermore, Chen et al. (2019) reported higher relative abundance of only a subset of sulfonamide/tetracycline ARGs in TWW-irrigated soils. Nevertheless, other studies reported

minimal impact of TWW irrigation on ARG prevalence in soil (Negreanu et al., 2012; Cerqueira et al., 2019a; Cerqueira et al., 2019b). In addition, Marano et al. (2019) detected several ARGs in higher prevalence in TWW when compared to FW, but reported no enrichment of ARGs in TWW irrigated soils.

These contradicting results could be attributed to the variability of TWW quality or of tested ARGs between the different studies. In addition, variations occur between the soil ecosystems of different geographical regions (Forsberg et al., 2014; Bahram et al., 2018), which could potentially influence the results of each study. Still, we consider that some important factors have so far been neglected, when estimating the impact of TWW irrigation on soil ARG dynamics. Several ARGs (e.g. *bla*$_{\text{TEM}}$ and *bla*$_{\text{CTX–-M}}$ variants) are indigenous to soil resistomes even in the absence of anthropogenic impact (Cytryn, 2013; Nesme and Simonet, 2015; Gatica et al., 2015; Cerqueira et al., 2019c). Thus, the impact of TWW irrigation on their prevalence in soil might be negligible, as long as these ARGs occur in TWW at low abundance. For example, dairy wastewater irrigation promoted the increase of several ARGs with the exception of *bla*$_{\text{CTX-M-1}}$ (Dungan et al., 2018).

Furthermore, the irrigation water demand might differ for different crops or vary across different climatic seasons. Thus, during real-scale, commercial TWW irrigation operations, the irrigation intensity fluctuates during the year. Consequently, the applied ARG loads differ dramatically between high and low intensity irrigation. Given the high capacity for resilience of the soil microbiome towards invasion by ARB and ARGs (Bahram et al., 2018), low intensity irrigation might not alter soil ARG profiles.

In addition, the prevalence of ARGs differs between irrigation waters of different quality. For example, ARGs are not only abundant in TWW, but also well disseminated in freshwater resources used for irrigation (Cacace et al., 2019; Pantanella et al., 2020), even if usually at lower absolute abundance. The absolute ARG abundance in combination with irrigation intensity ultimately determines the ARG load that a field receives.

Accordingly, including the applied ARG load in combination with soil ARG background levels can substantially improve comparability between studies on TWW irrigation impacts. Thus, the objective of this study was to investigate the influence of TWW irrigation on the prevalence of ARGs in soil, taking into account these factors. To achieve this, we applied a multiphase approach including a long-term monitoring survey in a real-scale TWW irrigated field with

varying irrigation intensity, and controlled laboratory experiments for further validation. We hypothesized that the ARG load and irrigation intensity define the effect of TWW irrigation on ARG spread dynamics in soil. To test the hypothesis, we quantified six ARGs, *intI1* and 16S rRNA in a commercially operated TWW irrigated field and non-impacted soil to gain insights on which genes are TWW related or predominant members of the native soil resistome/microbiome.

The selection of genes was based on the framework for TWW monitoring established by the NEREUS (www.nereus-cost.eu) and ANSWER-ITN (www.answer-itn.eu) networks (Cacace et al., 2019; Rocha et al., 2018). For example, *sul1, qnrS* and *tet*(M) and $bla_{\text{OXA-58}}$ were included for their clinical importance, high occurrence rate and abundance in TWW across European countries (Caucci et al., 2016; Alygizakis et al., 2020; Cacace et al., 2019). The two final β-lactamase genes bla_{TEM} and $bla_{\text{CTX–-M-32}}$ were selected due to their clinical importance, their low abundance in TWW (Cacace et al., 2019) and their known natural prevalence in soil microbiota (Gatica et al., 2015). The integrase gene *intI1* was analysed as well, since is commonly used as a genetic marker for anthropogenic pollution (Gillings et al., 2015) and frequently part of mobile gene cassettes that carry ARGs (Gatica et al., 2016).

In addition, we aimed to gain insights into the effect of irrigation intensity and irrigation breaks on ARG and *intI1* prevalence in soil on a real-scale setting, with a temporal sampling campaign of the TWW irrigated field under various irrigation intensity periods. We further verified the results from the real-scale TWW irrigation operation with performance of FW/TWW irrigated soil microcosms under controlled laboratory conditions, while simultaneously monitoring irrigation effects on the microbial community density and composition.

2.2. Materials & methods

2.2.1 Sampling

2.2.1.1. Agricultural practice of the sampled location

Field sampling took place in a real-scale, commercially operated TWW irrigated field that belongs to Braunschweig Wastewater Association (BWA) in central Germany (N: 52.360139, E: 10.398805; Fig. S2.1 & S2.2). The soil (cambisol, sandy soil) of the nearby area of Braunschweig is deficient in nutrients and has a limited water retention capacity (Ternes et al., 2007; Maaß et al., 2016). The local area farmers use TWW, subjected only to secondary (biological) treatment to counter the nutrient-limitation and in case of high nutrient-demand, they mix TWW with digested sludge (DS). To tackle the low nutrient-retention capacity, commercial TWW irrigation has taken place in the local area for over 50 years (Ternes et al., 2007).

2.2.1.2. Sampling TWW irrigation impacted and non-impacted soil

We sampled soil not impacted by TWW irrigation adjacent to the TWW irrigated field, along with the soil of the TWW irrigated field. Comparative sampling took place only in May 2019 due to logistic/legal issues with sampling of the adjacent area for the remainder of the sampling period. The TWW irrigated field was irrigated with TWW in the three months leading up to this sampling campaign. For both sampling sites we sampled the topsoil aseptically (10–15 cm of upper soil layer, n = 12). The top 10 cm of soil, most severely exposed to changing environmental conditions and potentially containing crop debris, were removed with a sterile spade. Then a 50 mL falcon tube was driven 5 cm deep into the ground to extract a representative soil core from this slightly deeper part of the topsoil, with TWW irrigation as the defining variable on ARG abundance. Each soil core was transported to the lab on ice, homogenized with a sterile spatula and subsequent vortexing (2000 rpm x 60 s), and finally used for DNA extraction. The samples were stored at −20 °C until DNA extraction. The pH of the field soil was moderately acidic (5.97). Both soils contained more than 90% of sand. The pH of the adjacent soil was more acidic (3.77), which was expected for this soil as it hosts coniferous vegetation (Mareschal et al., 2010).

2.2.1.3. Long-term sampling of real-scale TWW irrigated field, along with the respective irrigation water

Long-term sampling of the TWW irrigated field started in October 2017 and lasted until December 2018. During this period, the field was irrigated with TWW or TWW mixed with DS (TWW & DS) at different irrigation intensities, intercepted by long irrigation breaks (Table 2.1). The total volume of irrigation water per irrigation event was 35 mm^3 $perm^2$ of the field. The field was irrigated 10–14 times per month, during periods of high intensity irrigation, resulting in 350–420 mm^3/m^2/month. In the periods of low intensity irrigation, two to three irrigation events took place per month. Thus during low irrigation intensity periods the total irrigation volume per surface was 70–105 mm^3/m^2/month. The soil samples (12 samples per time point) were taken temporally across this gradient of irrigation intensities (high irrigation intensity n = 24, low irrigation intensity n = 36, Irrigation Breaks n = 36) and subsequently handled as described in the previous section. Furthermore, samples of the respective TWW irrigation water were taken frequently (three replicates per time point, total n = 15). Bacteria from water samples were captured by filtration (polycarbonate, 0.2 µm pore size, 47 mm diameter, Sartorius, Germany) of 150 mL of irrigation water and filters were stored at −20 °C prior to DNA extraction.

Table 2.1: Conditions and sampling-dates of the TWW irrigated field soil during the temporal sampling campaign. The information given in this table is explanatory for the figures of gene abundances in the field soil (Fig. 2.3 & 2.4). TWW: Treated Wastewater, DS: Digested Sludge

Date	**Irrigation Status**	**Sampling**
October 2017	Low Intensity Irrigation (TWW)	Once
November 2017	Irrigation Break	-
December 2017	Irrigation Break	-
January 2018	Irrigation Break	-
February 2018	Irrigation Break/Start of Low Intensity irrigation (TWW)	Before and after the start of irrigation
March 2018	Low (TWW)	Once, before the start of the Irrigation Break
April 2018	Irrigation Break	-
May 2018	Irrigation Break	-
June 2018	Irrigation Break/ Start of High Intensity Irrigation (TWW & DS)	Once (before the end of Irrigation Break)
July 2018	High Intensity Irrigation (TWW & DS)	Once
August 2018	High Intensity Irrigation (TWW)	-
September 2018	High Intensity Irrigation (TWW)	Once
October 2018	Start of Irrigation Break	-
November 2018	Irrigation Break	-
December 2018	Irrigation Break	Once

2.2.2. Microcosm experiments

For microcosm experiments, we sampled the soil adjacent to the TWW irrigated field that was not previously impacted by TWW irrigation (12 samples) at 0–60 cm depth and homogenised it to create a composite sample. After air-drying at room temperature, we sieved the soil (~6 mm mesh size) and used it for microcosm experiments. The microcosms were assembled by acrylic cylinders of 66 cm total height and 4.5 cm radius (Fig. S2.3) and an inner tube (1.5 cm radius) was placed centrally to allow the collection and removal of the percolated water. Then the microcosms were filled with gravel (size ~3 mm^3) in the bottom (5 cm) and the previously homogenized soil up to 50 cm height, resulting in a total soil volume per microcosm of 2827.6 mL. The same volume of dry, sieved, homogenised and similarly packed soil was placed in the microcosms, ensuring similar bulk density.

Microcosms were divided into two groups, with four replicate microcosms per treatment: The FW-Group and TWW-Group were defined with respect to the type of irrigation water. TWW for irrigation, subjected to secondary biological treatment, was obtained from an urban wastewater treatment plant nearby the Dresden area (Kaditz, Germany; N: 51.070640, E: 13.680888). The FW was collected from a shallow well (depth ~7 m) located next to the Elbe river, in Pirna, Germany (N: 50.965905, E: 13.924034). The microcosms were irrigated with 350 mL of water, which led to saturation. Fresh irrigation water (3 L each of FW and TWW) was aseptically sampled three times per week in sterile 1 L glass bottles from July to August 2019. Sampled irrigation water was transported to the lab on ice, homogenized by shaking and used for microcosm irrigation within the day of sampling. Prior to irrigation, the residual water in the microcosms was removed.

The microcosms were placed in a controlled temperature chamber at 20 °C and with controlled light conditions with 12 h light/darkness. Both groups were initially irrigated with FW for two weeks, to stabilize and equilibrate the soil conditions. Then the TWW-Group switched to TWW irrigation for three weeks, while the FW-Group was continuously irrigated with FW. Soil samples were taken aseptically from the microcosms with a sterile spatula at 15 cm depth. This was the same depth of soil previously sampled in the TWW irrigated field to allow comparability between results obtained from field sampling and microcosm experiments. Samples were transferred in a falcon tube and were homogenised. First sampling took place at the end of the two-week FW irrigation/stabilization-period (Week 0), the second in the 1st

week after switching to TWW irrigation (Week 1) and the third in the 3rd week after switching to TWW irrigation (Week 3). In addition, bacteria were harvested from six FW and six TWW irrigation samples through filtration as described above. Specifically, filtration (500 mL) and subsequent DNA extraction was performed on both types of irrigation water samples on day 1, 5, 8, 12, 15 and 19 after switching to TWW irrigation.

2.2.3. DNA extraction, quantitative real time PCR and sequencing

DNA extractions were performed according to the manufacturer's instructions using the DNeasy PowerWater Kit for water (150 mL) and the DNeasy PowerSoil Kit (Qiagen, Germany) for soil samples (0.25 g). Quantity and quality of DNA was measured with NanoDrop (Thermo Fischer Scientific, Germany). We performed qPCR for the following genes: a) *qnrS* (protein family, which protects DNA gyrase from the inhibition of quinolones), b) bla_{TEM} (class A β-lactamase), c) *sul1* (sulfonamide resistant dihydropteroate synthase), d) $bla_{\text{CTX-M-32}}$ (class A β-lactamase, cephalosporinase), e) $bla_{\text{OXA-58}}$ (class D β-lactamase, carbapemenase), f) *tet*(M) (ribosomal protection protein that protects ribosome from the translation inhibition of tetracycline), g) *intI1* (class I integrase, this gene is associated with horizontal gene transfer and environmental pollution) and h) 16S rRNA, which is an indicator for the total microbial abundance.

The reactions were performed in a MasterCycler RealPlex (Eppendorf, Germany) at a final volume of 20 µL with 10 µL of Luna Universal qPCR Master Mix (New England Biolabs, Germany), which uses SYBR Green chemistry. Details about reagents, primers, thermal-profile and plasmid-standards are given in the Table S2.4. The template volume was 4 µL. The amount of DNA per reaction was standardized to 20 ng. The limit of detection (LOD) was set at 3 copies per reaction (according to Kralik & Ricchi, 2017). The limit of quantification (LOQ) varied among targeted genes (details are listed in Tables S2.4 & S2.5).

Standard curves with amplification efficiency 0.9–1.1 and $R^2 \geq 0.99$ were accepted. Melting curve analysis was performed to assess the amplicons' specificity. Screening for potential PCR inhibition was performed by spiking a plasmid for a gene, which was rarely detected and if so present at very low abundance in our samples ($bla_{\text{CTX–M-32}}$, spiking-concentration 4×10^6 copies/µL). No PCR inhibition was detected. The absolute abundance for soil-samples is

expressed in copies/g of dry soil and for water-samples in copies/L. The ratio of gene copies per 16S rRNA copy will be referred to as the relative abundance for the rest of the manuscript.

The FW/TWW-Group replicates were pooled (in equimolar concentrations) with final concentration of 5 ng/µL and were analyzed with the 16S Ion Metagenomics Kit™ (Thermo Fisher Scientific, Germany) for amplification and sequencing of multiple parallel variable regions. The protocols for 16S rRNA library preparation for parallel variable regions sequencing and processing of the sequences were described previously in Orschler et al. (2019). Raw fastq sequences were submitted to SRA (bioproject accession number: PRJNA668737).

2.2.4. Data processing and statistical analysis

Prior to statistical analysis, every sample that was below LOD/LOQ was placed at 1 copy/L or 1 copy/g of dry soil (absolute abundance) and 10^{-8} (relative abundance): one order of magnitude below the minimum relative abundance that we observed ~10^{-7}. Data were $\log_{10}$-transformed. We used the programing language R ((R Core Team, 2019), v. 3.5.3) for generation of graphical representations, with the packages "ggplot" (Wickham, 2016) and "ggpubr" (v. 0.2.2, Kassambara, 2019). Significant differences were assessed with the Wilcoxon rank sum test or in case of group comparisons with the Kruskal-Wallis test (package "ggpubr"). Dunn's test with Benjamini-Hochberg correction was carried out with the package "dunn's test" (v1.3.5, Dinno, 2016) for pairwise multiple comparisons. Additional statistical analysis of ARG and *intI1* profiles was performed with a PERMANOVA test ("adonis" function, method="euclidean") from the "vegan" package (v2.5–6, Oksanen et al., 2019) or "pairwiseAdonis" package (v0.3, (Martinez Arbizu et al., 2019)). For the analysis and graphical representation of bacterial community data, the package "phyloseq" was used (McMurdie & Holmes, 2013). Comparisons with p-values below 0.05 were considered as statistically significant (α=0.05).

2.3. Results

2.3.1. Non-impacted and TWW irrigated soil displayed different ARG and *intI1* profile

The ARG and *intI1* profiles were significantly different between the TWW irrigated field and the non-impacted adjacent soil (PERMANOVA, Euclidean distance, n = 12, R^2 = 0.59, p = 0.001). Specifically, *sul1, qnrS, tet*(M) and *bla*$_{\text{OXA-58}}$ were only present in the TWW irrigated field at high relative abundance (*sul1*: −3.8 ± 1.1; *qnrS*:−6.1 ± 0.9; *tet*(M): −4.8 ± 1.4; *bla*$_{\text{OXA-58}}$:−5.3 ± 0.9 $\log_{10}$ copies/16S rRNA) and not detected in the non-impacted soil, with exceptions of outliers (Fig. 2.1 & S2.4) (Wilcoxon rank sum test, p< 0.0001, n = 12). The integrase gene *intI1* was present in both types of soil with one order of magnitude higher relative abundance in the TWW irrigated field soil (Wilcoxon rank sum test, $p = 7.5 \times 10^{-11}$, n = 12). Contrary, *bla*$_{\text{TEM}}$ and *bla*$_{\text{CTX-M-32}}$ showed higher relative abundance in the non-impacted soil (−4.2 ± 0.1 and −5.7 ± 1.1 $\log_{10}$ copies/16S rRNA) in comparison with the field soil (−5.5 ± 1.1 and −6.8 ± 1 $\log_{10}$ copies/16S rRNA, Wilcoxon rank sum test, p< 0.0001, n = 12) (Fig. 2.1). However, the single time-point sampling provided little information regarding the temporal dynamics of ARGs and *intI1*, whether they increase instantly or are eliminated quickly during irrigation breaks. In addition, the two soils differed in vegetation and physicochemical conditions. Therefore, further insights into TWW irrigation effects were needed from more detailed temporal observations of the soil of the TWW irrigated field.

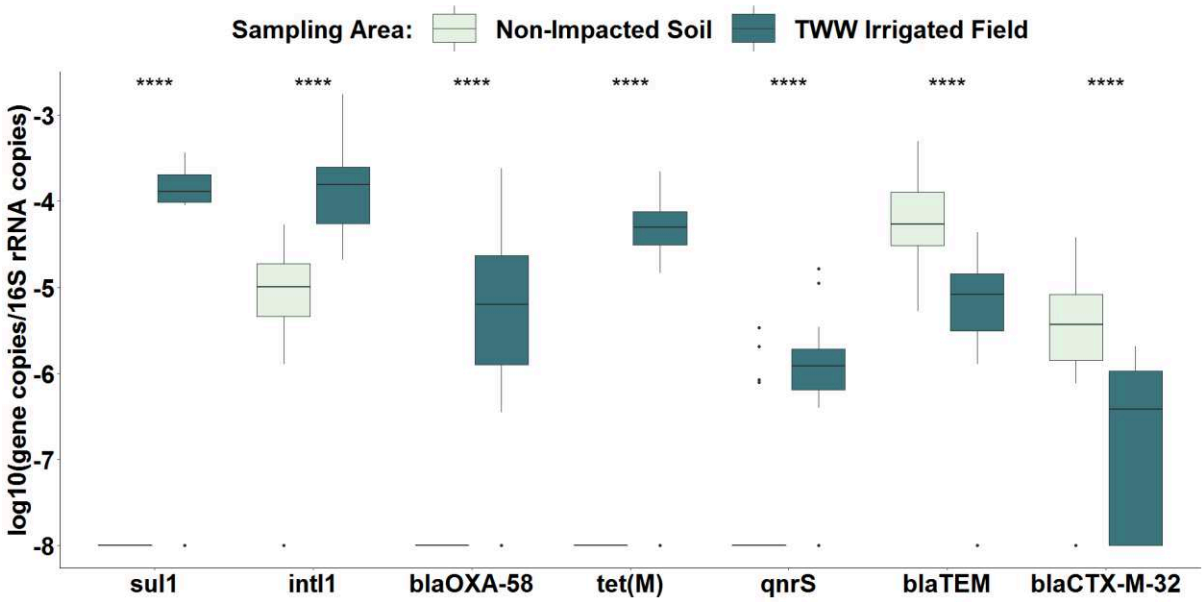


Figure 2.1: The relative abundance of ARGs and *intI1* in the irrigated field and the adjacent non-irrigated soil (Fig. S2.3). Significant differences were assessed with a Wilcoxon rank sum test (*p<0.05, **p<0.01, ***p< 0.001, ****p<0.0001, n=12). The total significance of separation between these two groups was assessed as well with a PERMANOVA test (Euclidean distance): R^2= 0.59, p<0.001, n=12.

2.3.2. The genes *sul1*, *intI1*, *qnrS*, *tet*(M) and *bla*$_{OXA-58}$ were highly abundant in TWW irrigation water

To determine if the relative abundance of ARGs and *intI1* in the TWW irrigated soil is linked to their abundance in the TWW resistome, TWW irrigation water samples were taken for a period of one year. The genes with the highest relative abundance in TWW were *intI1* (−1.8 ± 0.6 $\log_{10}$ copies/16S rRNA) and *sul1* (−2.0 ± 0.3 $\log_{10}$ copies/16S rRNA) (Fig. 2.2), those with the highest relative abundance in TWW irrigated soil (Fig 2.1). The remaining three genes detected at higher relative abundance in TWW irrigated soil were also highly abundant in TWW (*bla*$_{OXA-58}$: −3.1 ± 0.3; *qnrS*: −3.6 ± 0.6; *tet*(M): −3.8 ± 0.3 $\log_{10}$ copies/16S rRNA; Fig. 2.2). Unsurprisingly, *bla*$_{TEM}$ and *bla*$_{CTX-M-32}$ displayed the lowest abundance in the irrigation water (*bla*$_{TEM}$: −5.8 ± 0.3; *bla*$_{CTX-M-32}$: −4.7 ± 1.0 $\log_{10}$ copies/16S rRNA; Fig. 2.2). As digested sludge was added to the TWW during a few periods of the sampling campaign its influence on the TWW resistome was tested. The profile of ARGs of TWW & DS irrigation was similar to TWW irrigation, with the exception of *qnrS* and *bla*$_{OXA-58}$ where their relative abundance was more than one order of magnitude higher in TWW & DS compared to TWW (Fig. S2.5). In

conclusion, genes with higher relative abundance in the irrigation water (TWW or TWW & DS), were also found in higher relative abundance in TWW irrigated field soil when compared to the non-impacted soil, strongly indicating a direct link of the respective resistomes.

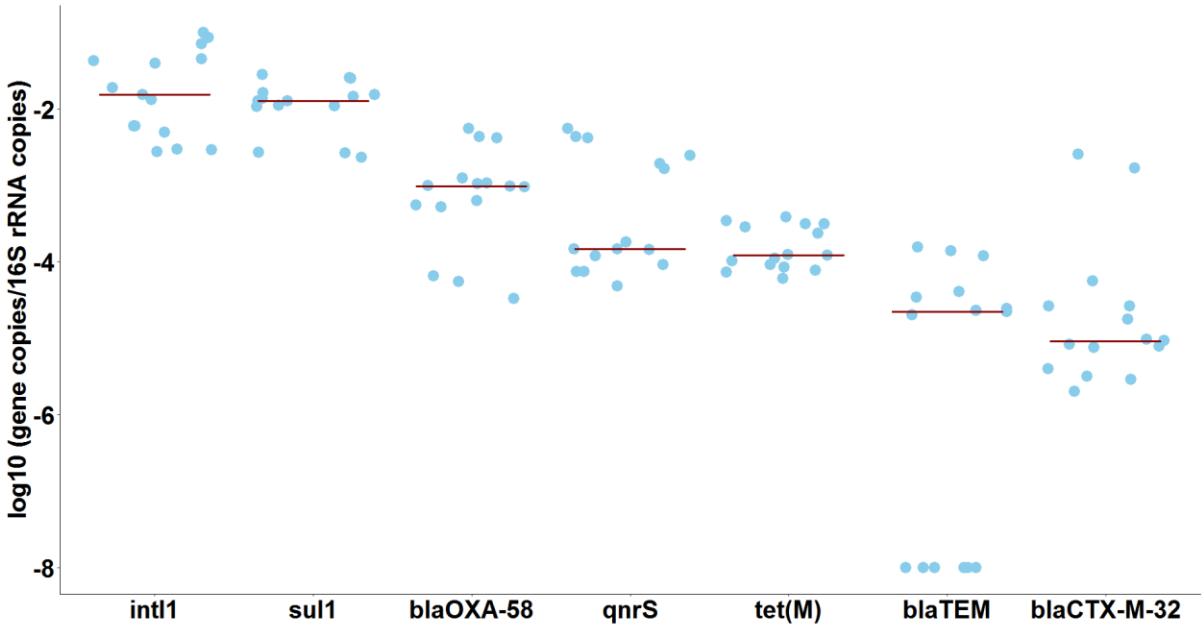


Figure 2.2: Ranking of ARGs and *intI1* based on their relative abundance in TWW irrigation water. The samples were taken over one year of irrigation operations (n=15). The crossbar represents the median relative abundance.

2.3.3. Temporal dynamics of most ARGs and *intI1* in soil correlate with TWW irrigation intensity

As the irrigation water and the receiving soil resistomes were correlated, we tested if the load of introduced ARGs played a significant role on their prevalence in the soil microbiome. To determine the temporal ARG and *intI1* dynamics in a full-scale TWW irrigated field, samples were regularly taken during different irrigation intensity periods and irrigation breaks for over a year (Table 2.1). The samples were grouped based on irrigation intensity (High, Low and Irrigation Breaks). The soil ARG and *intI1* profiles were significantly different across the three irrigation intensities (PERMANOVA, Euclidean distances with $R^2 = 0.3$, $p = 0.001$, $n =$ 24–36; Fig. 2.3A). Specifically, a separation between irrigation breaks and high irrigation intensity periods was observed (Fig. 2.3A). Pairwise PERMANOVA tests with Benjamini-

Hochberg correction revealed that all groups of irrigation intensity were indeed significantly different from each other (p<0.001, R^2 = 0.2–0.33, n = 24–36, Table 2.2).

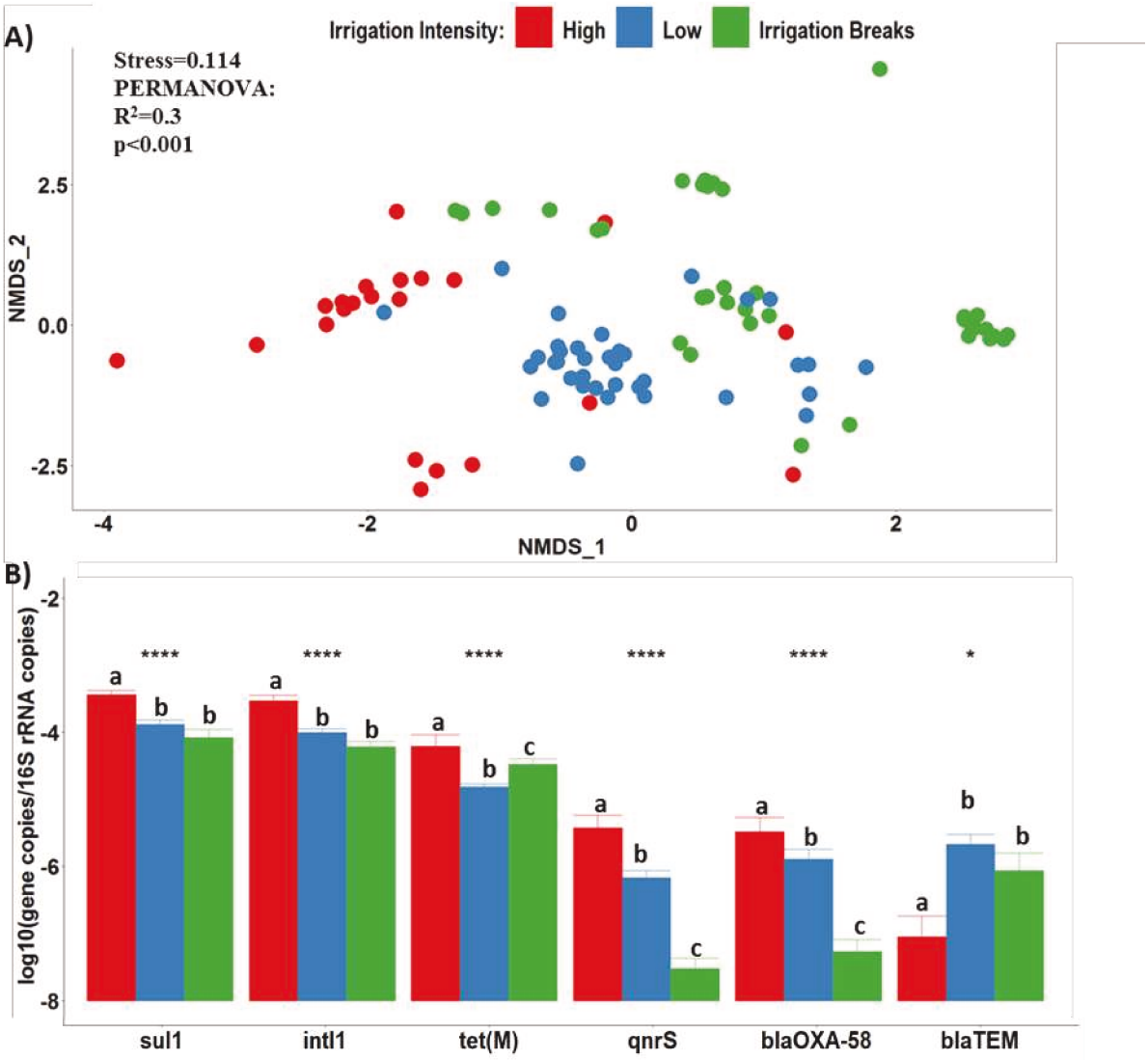


Figure 2.3: A) NMDS plot illustrating differences of the ARGs and *intI1* profiles, in the TWW irrigated field soil, during the several periods of irrigation intensity over one year (distance: Euclidean). Significance of separation was assessed with a PERMANOVA test (R^2=0.35, p<0.001), while the results for pairwise PERMANONA comparisons are shown in Table 2.2. B) The relative abundance of every individual gene during the different periods of irrigation intensity (Kruskal-Wallis test: *p<0.05, **p<0.01, ***p< 0.001, ****p<0.0001, High irrigation intensity n=24, Low irrigation intensity n=36, Irrigation breaks n=36). Letters from "a" to "c" were assigned to each none significant different groups after a Dunn's test (Benjamini-Hochberg correction). The cutoff of significance for pairwise comparison was p<0.05. The gene *bla*$_{CTX\text{-}M\text{-}32}$ was not detected during these sampling campaigns (with the exception of outliers) and was thus excluded.

Table 2.2: Pairwise comparisons with PERMANOVA test ("pairwise.adonis" function) of the relative abundance of ARGs and *intI1* (log_{10} copies/16srRNA) in the TWW irrigated field soil (Fig. 2.2A) during the different periods of irrigation intensity or irrigation breaks (Table 2.1). The p-values were adjusted with Benjamini-Hochberg correction for pairwise multiple comparisons (IB: Irrigation Breaks, LIP: Low Intensity Irrigation, MIP: Moderate Intensity Irrigation, HIP: High Intensity Irrigation).

Groups Comparisons	R^2	Adjusted p-value
IB vs LIP	0.21	0.001**
LIP vs HIP	0.2	0.001**
IB vs HIP	0.31	0.001**

Most of the genes exhibited significantly higher relative abundances during the high intensity irrigation periods, in comparison with low irrigation or irrigation breaks. The relative abundance of *sul1* was −3.4 ± 0.3 log_{10} copies/16S rRNA at high irrigation intensity, while it dropped to −3.8 ± 0.4 log_{10} copies/16S rRNA in low irrigation intensity and −4.1 ± 0.7 log_{10} copies/16S rRNA during irrigation breaks (Kruskal-Wallis Test, $p = 9.7 \times 10^{7}$, n = 24–36, Fig. 2.3B). Similarly, the relative abundance of *intI1* was significantly higher during high irrigation intensity with −3.5 ± 0.4, −4.0 ± 0.3 and −4.0 ± 0.4 log_{10} copies/16S rRNA for high, low irrigation intensity and irrigation breaks (Kruskal-Wallis Test, $p = 2.4 \times 10^{-7}$, n = 24–36, Fig. 2.3B). The genes *tet*(M), bla_{OXA-58} and *qnrS* exhibited similar trends to *sul1* and *intI1* with higher relative abundances during high intensity irrigation periods (Kruskal-Wallis Test, $p<0.001$, n = 24–36, Fig. 2.3B). However, the *tet*(M) gene showed higher relative abundance in the irrigation breaks compared to low irrigation intensity (Dunn's test, $p<0.001$, n = 36, Fig. 2.3B), mainly because its relative abundance only slightly decreased (from −3.8 ± 01 to 4.0 ± 0.2 log_{10} copies/16S rRNA) during the final tested irrigation break, which happened immediately after the high intensity irrigation periods (Fig. S2.6). Despite this observed irrigation intensity effect, the relative abundance of these five genes remained consistently significantly elevated when compared to the non-impacted soil under any irrigation scenario (PERMANOVA, R^2 = 0.8–0.6, $p<0.0001$, n = 12–36).

Contrary, the genes that occurred in low relative abundance in the TWW and were predominant in the non-impacted soil (bla_{TEM} and $bla_{CTX-M-32}$), did not display any significant effect based on TWW irrigation intensity. The relative abundance of bla_{TEM} was one order of magnitude lower during high intensity irrigation in comparison to the other irrigation periods (Fig. 2.3B).

In addition, *bla*$_{CTX-M-32}$ was not detected in most of the samples during this campaign, with the exception of outliers, independent of irrigation intensity.

When comparing the temporal dynamics of *sul1, qnrS* and *intI1* in detail, prior to the start of high intensity irrigation (June 2018), during one month (July 2018) and three months of high intensity irrigation (September 2018), an immediate increase in relative abundance was detected, visible from one month after irrigation started (June-July 2018, Fig. 2.4, Kruskal-Wallis, $p<0.001$, $n = 12$). The relative abundance of *sul1, intI1* and *qnrS* increased from −3.9 ± 0.2 to −3.4 ± 0.4, −4.6 ± 0.5 to −3.5 ± 0.5 and −7.5 ± 1.1 to −5.4 ± 0.9 $\log_{10}$ copies/16S rRNA for each gene respectively (Fig. 2.4). However, a plateau effect was observed, where the relative abundance of these three genes was not significantly impacted when re-examining it after three months of continuous high intensity irrigation (Dunn's test $p>0.05$, $n = 12$, Fig. 2.4). It is noteworthy that despite this plateau effect, the standard deviation of each gene's relative abundance was lower after three months of irrigation, which indicates improved homogenisation of the TWW irrigation effect on the field soil after a prolonged time-period.

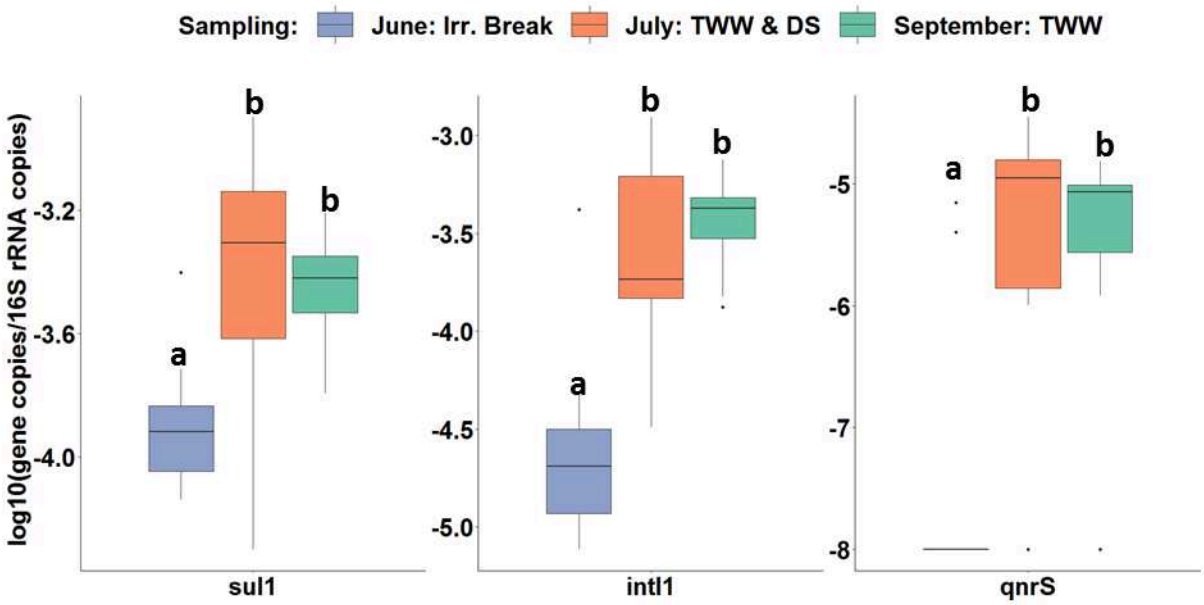


Figure 2.4: Relative abundance of *sul1, intI1* and *qnrS* during continuous high intensity irrigation. The samples were taken prior to the start of irrigation season (June) and after high intensity irrigation for one (July) and three months (September). Letters from "a" to "b" were assigned to each none significant different groups after a Dunn's test (Benjamini-Hochberg correction). The cutoff of significance for pairwise comparisons was $p<0.05$. TWW: Treated Wastewater, DS: Digested Sludge.

The long-term temporal investigation of ARG and *intI1* dynamics of the real-scale TWW irrigated field along with comparison with the non-impacted soil provided clear indication that TWW irrigation affects mainly the prevalence of TWW related genes such as *sul1, intI1, qnrS* and *tet*(M) in the soil. In addition, the genes that were absent in the non-impacted soil (*sul1, tet*(M)*, qnrS* and $bla_{\text{OXA-58}}$, Fig. 2.1) were not eliminated during irrigation breaks (Fig. 2.3B), indicating persistence of these genes, upon their introduction to the soil. However, *qnrS* and $bla_{\text{OXA-58}}$ were far less persistent than *sul1* and *tet*(M)*,* since their relative abundance decreased close to LOQ during irrigation breaks. Nevertheless, high variability of environmental conditions and agricultural management (e.g. temperature, precipitation and different crop cultivars) occurred in the investigated field across the year. Such variation is common among field studies of real-scale TWW irrigation operations. Therefore, we aimed at further confirming and extending our results using controlled laboratory conditions.

2.3.4. Higher abundance of ARGs and *intI1* in TWW irrigated soil microcosms compared to FW irrigated ones

To overcome the effect of varying environmental conditions, we performed laboratory-scale microcosm experiments with the non-irrigated soil adjacent to the TWW irrigation field. The microcosms were irrigated with either TWW or FW for three weeks. When comparing the two irrigation waters, the 16S rRNA absolute abundance was two orders of magnitude higher in TWW (9.8 ± 0.1 $\log_{10}$ copies/L), compared to FW (7.5 ± 1.1 $\log_{10}$ copies/L) (Wilcoxon rank sum test, $n = 6$, $p = 3.6 \times 10^{-5}$, Fig. S2.7A). Further, the absolute abundance of all ARGs and *intI1* was constantly more than one order of magnitude higher in TWW than in FW (Fig. S2.7A). The relative abundance of *sul1, intI1,* $bla_{\text{OXA-58}}$, *qnrS* and *tet*(M) was equally higher in TWW with significant differences ranging from one to two orders of magnitude ($p<0.05$, Wilcoxon rank sum test, $n = 6$) (Fig. S2.7B). However, the relative abundance of bla_{TEM} and $bla_{\text{CTX-M-32}}$, did not differ between the two types of irrigation water with $p>0.05$ (Wilcoxon rank sum test, $n = 6$).

Despite the log-fold difference of 16S rRNA load observed for the irrigation waters, no significant increase of 16S rRNA absolute abundance in the soil of the TWW-Group was observed over the three weeks of irrigation (Fig. 2.5A). In fact, 16S rRNA absolute abundance

in the soil of the TWW-Group did not change significantly from Week 0 to Week 3 (Week 0: 11.9 ± 0.9; Week 3: 11.9 ± 1.1 log 10 copies/g of dry soil) (Dunn's test, $n = 4$, $p>0.05$, Fig. 2.5A). Moreover, the 16S rRNA absolute abundance in the TWW-Group soil was similar to the FW-Group at Week 3 (11.8 ± 1.6 log 10 copies/g of dry soil, Fig. 2.5B) with $p>0.05$ (Dunn's test, $n = 4$). The average 16S rRNA absolute abundance per gram of soil was four orders of magnitude higher than in the TWW irrigation water (soil: 11.8 ± 0.1 $\log_{10}$ copies/g of dry soil; TWW: 6.9 ± 0.1 $\log_{10}$ copies/mL, Dunn's test, $p<0.05$, s). Thus, the bacterial load addition of TWW irrigation in the soil was relatively minor.

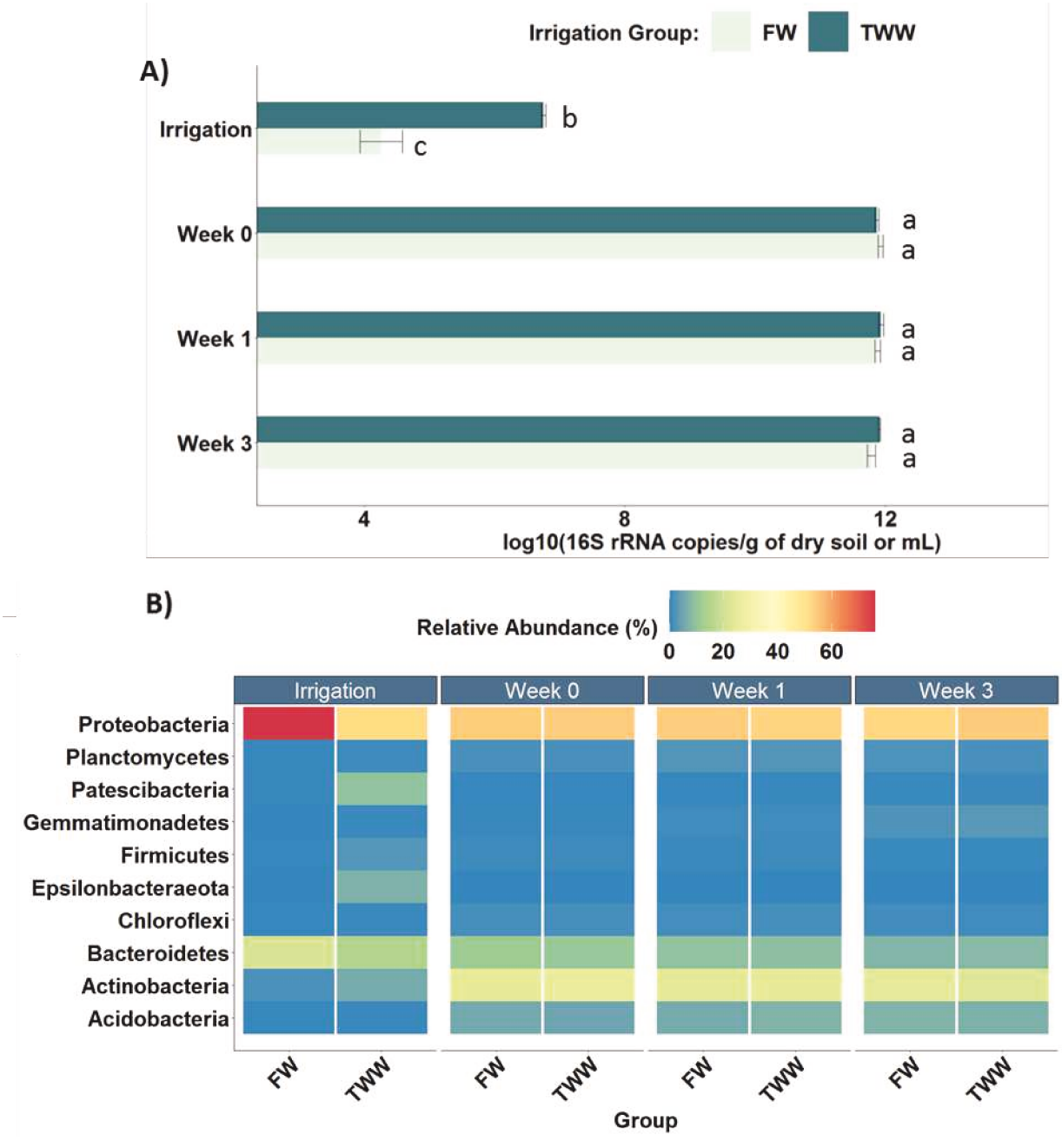


Figure 2.5: Absolute abundance of 16S rRNA gene in soil (copies/g of dry soil) of the soil microcosms and irrigation waters (copies/mL). Letters from "a" to "c" were assigned to each non-significant different groups after a Dunn's test (Benjamini-Hochberg correction) with a cutoff p<0.05. B) The relative abundance (% reads) of the 10 most abundant bacterial phyla in microcosm soil samples and irrigation waters from 16S rRNA profiling with high throughput sequencing. The two groups were irrigated for two weeks with freshwater for the equilibration of conditions before the start of experiment. We sampled at Week 0 (before the switch to TWW irrigation) at Week 1 (one week after the switch to TWW-irrigation) and at Week 3 (three weeks after the switch to TWW-Irrigation). The FW-group was irrigated in parallel only with freshwater. FW: Freshwater, TWW: Treated Wastewater.

To support the qPCR results, we sequenced microcosm samples to gain insights into if TWW irrigation altered community composition. However, no particular difference in the phyla composition of TWW/FW irrigated soil (Fig. 2.5B) was detected. The profile of FW, TWW and all soils was different on the phylum level with *Actinobacteria* and *Acidobacteria* being more prevalent in any of the soils, and *Patescibacteria* and *Firmicutes* being present mainly in the TWW (Fig. 2.5B). However, the most predominant phylum (*Proteobacteria*) was overly abundant in all types of samples (soil: 52.5–54.4%, TWW: 51.2%, FW: 75.8%) (Fig. 2.5B). Nevertheless, no increase of TWW-related phyla in the TWW-Group soil over the weeks of irrigation with TWW was detected (Fig. 2.5B), supporting the fact, that TWW irrigation addition to bacterial abundance in soil was negligible.

Despite the absence of an immediate effect of TWW irrigation on the bacterial load of the soil and its phylogenetic composition, the ARG and *intI1* profiles between the two groups of microcosms (TWW & FW) differed significantly over the three weeks of irrigation (PERMANOVA, Fig. 2.4, Euclidean distance, $R^2 = 0.63$, $p<0.001$, Table 2.3). Specifically, the genes $bla_{\text{OXA-58}}$, *intI1* and *sul1* increased in the TWW-Group after the switch to TWW irrigation at Week 1 and 3 (Fig. 2.6 & S2.8). The gene *sul1* was present in the soil of the FW-Group and the TWW-Group at Week 0 (-3.8 ± 0.2 $\log_{10}$ copies/16S rRNA) and at Week 3 it increased to -3.0 ± 0.05 $\log_{10}$ copies/16S rRNA (Kruskal-Wallis Test, $p = 0.0001$, $n = 4$, Fig. 2.6 & S2.8). In the FW-Group, *sul1* relative abundance was more than one order of magnitude lower at Week 3 (-4.5 ± 1.6 $\log_{10}$ copies/16S rRNA) compared to the TWW-Group (Fig. 2.6 & S2.8) (Wilcoxon rank sum test, $p = 0.0093$, $n = 4$), even lower than at the beginning of the experiment. The *qnrS* abundance was below LOD in both groups at Week 0, and was detected only in the TWW-Group at Week 1 (Fig. 2.6). However, it was detected at Week 3 in both groups, but remained slightly elevated in the TWW-Group (TWW-Group: -6.0 ± 0.5 $\log_{10}$ copies/16S

rRNA, FW-Group: −6.5 ± 0.6 $\log_{10}$ copies/16S rRNA; Wilcoxon rank sum test, $p = 0.049$, $n = 4$, Fig. 2.6 & S2.8).

Table 2.3: Pairwise comparisons with PERMANOVA test ("pairwise.adonis" function) of the microcosm groups over time (Fig. 2.6), along with Benjamini-Hochberg correction for pairwise multiple comparisons (FW: Freshwater irrigated, TWW: TWW irrigated, W: Week).

Groups Comparisons	R^2	Adjusted p-value
FW-W0 vs FW-W1	0.05	0.71
FW-W0 vs FW-W3	0.36	0.0015**
FW-W1 vs FW-W3	0.40	0.0015**
TWW-W0 vs TWW-W1	0.49	0.0015**
TWW-W0 vs TWW-W3	0.62	0.0015**
TWW-W1 vs TWW-W3	0.28	0.008**
FW-W0 vs TWW-W0	0.02	0.71
FW-W1 vs TWW-W1	0.65	0.0015**
FW-W3 vs TWW-W3	0.52	0.0015**

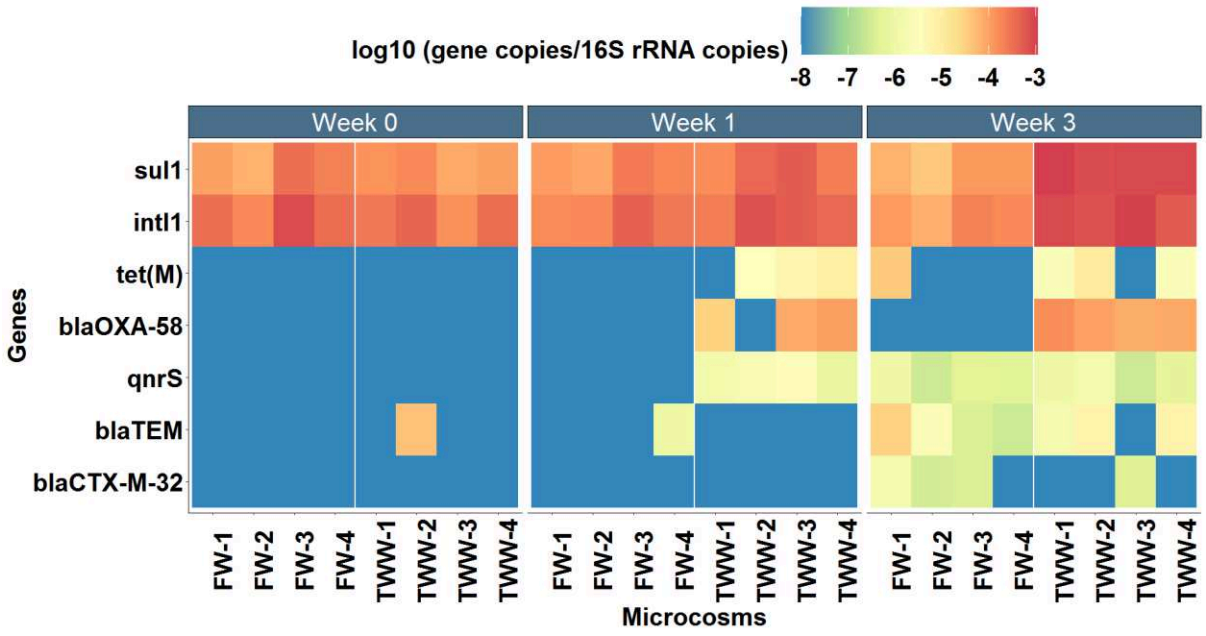


Figure 2.6: The relative abundance of ARGs and *intI1* ($\log_{10}$ copies/16srRNA) in the microcosm soil samples between the two different groups of irrigation (FW: Freshwater, TWW: Treated Wastewater). The two groups were irrigated for two weeks with freshwater for the equilibration of conditions before the start of experiment. We sampled at Week 0 (before

the switch to TWW irrigation) at Week 1 (one week after the switch to TWW-irrigation) and at Week 3 (three weeks after the switch to TWW-Irrigation). The FW-group was irrigated in parallel only with freshwater. The significance of difference was assessed with a PERMANOVA test (Euclidean distance, R^2=0.63, p <0.001). Pairwise PERMANOVA tests are given in Table 2.3. The soil that was used for the microcosms was the non-impacted by irrigation soil adjacent to the field (Fig. 2.1).

2.4. Discussion

The necessity to bridge the gap interconnecting anthropogenic and soil resistomes has been highlighted in previous studies (Nesme and Simonet, 2015; Cytryn, 2013; Smalla et al., 2018). In this study, we focused on the effects of irrigation with TWW on the soil resistome using a multiphase approach. The approach consisted of detailed time-series sampling of a full-scale, commercially operated, TWW irrigated agricultural field and controlled laboratory microcosm experiments to investigate the dynamics of ARGs and *intI1* under TWW irrigation. Our set of genes (six ARGs and *intI1*) covered resistance to a wide variety of antibiotic classes: sulfonamides, tetracyclines, quinolones, penicillins, cephalosporins and carbapenems.

The in-/decrease of ARGs and *intI1* due to TWW irrigation varied across the different genes. The gene abundances of *sul1, intI1, qnrS,* $bla_{\text{OXA-58}}$ and *tet*(M) were positively correlated with TWW irrigation intensity in the field. Contrary, bla_{TEM} and $bla_{\text{CTX-M-32}}$, which were of higher prevalence in the non-irrigated soil, did not significantly increase with TWW irrigation. In soil microcosm experiments with either FW or TWW irrigation, we confirmed that the field-observed dynamics hold true under controlled laboratory conditions. Consequently, the working hypothesis that the ARG load and irrigation intensity define the effect of TWW irrigation on ARG spread dynamics in soil was supported.

The three different experiments elucidated temporal ARG dynamics as a consequence of TWW irrigation. Significantly elevated levels of ARGs manifested in the long-term (>50 years) TWW-irrigated soil, when compared to the non-irrigated control. However, ARG abundances remain dynamic and dependent on irrigation intensity, across a one-year monitoring period. Still, throughout the year, even during irrigation breaks, ARG levels constantly remained far above background levels detected for non-irrigated soil, indicating that introduced ARGs, once established in the soil microbiome, do not disappear. First effects of TWW irrigation on ARG abundance and hence early ARG establishment in non-impacted soil can already be detected after short time spans on the week to month scale.

Previous studies have reported contradictive statements on whether they observed (Dalkmann et al., 2012; Chen et al., 2014; Wang et al., 2014; Han et al., 2016) or did not observe (Negreanu et al., 2012; Caucci et al., 2016, Cerqueira et al., 2019b, Cerqueira et al., 2019a; Marano et al., 2019) increases of ARGs in the soil due to TWW irrigation. The here reported variance of

effects across the tested genes suggests that the choice of target genes might play a key role. While the coverage of common investigated genes among the previous studies is low, studies from both contradicting groups of statements included at least some of the genes that we observed to be affected by TWW irrigation in our study (e.g. *sul1, qnrS* and *bla*$_{\text{OXA-58}}$) (Dalkmann et al., 2012; Negreanu et al., 2012; Chen et al., 2014; Caucci et al., 2016, Cerqueira et al., 2019b, Cerqueira et al., 2019a, ;, Marano et al., 2019). However, the two studies with the highest coverage of ARGs, utilizing parallel qPCR (semi-quantitative method) for a massive amount of ARGs, reported that TWW irrigation promotes the spread of at least a subset of ARGs in soil (Wang et al., 2014; Han et al., 2016), in agreement with our quantitative study.

Genes with high abundance in the TWW and commonly associated with gene mobility were particularly increased in the irrigated soil, while those associated with the native soil resistome were not following similar patterns. Among those genes introduced to the soil due to anthropogenic irrigation activity and most severely increased in abundance in irrigated soil were *sul1* and *intI1*. These two genes were the most abundant in the here used TWW, and are generally found at high levels in TWW across several European countries (Cacace et al., 2019). The ARG *sul1* confers resistance to sulfonamides, a group of synthetic antibiotics that persist through the wastewater treatment and are usually present in high concentration in TWWs (Rizzo et al., 2013; Zhang and Li, 2011). The *intI1* gene is commonly considered as an indicator of horizontal gene transfer and anthropogenic pollution (Gillings et al., 2015) due to its association with MGE. It is often present in gene cassettes that contain ARGs or other genes for various stress responses, with *sul1* frequently being part of these gene cassettes (Gillings et al., 2015). This explains the correlated increase of *sul1* and *intI1* in our experiments.

Mobile broad-host-range resistance plasmids, regularly hosting these integron cassettes, have previously been shown to transfer from human associated bacteria to a majority of indigenous soil microbial communities (Klümper et al., 2015; Klümper et al., 2017). This mobility allows the integron cassettes to persist in the soil microbiome, independent of their original TWW hosts (Gillings et al., 2015). While the relative abundance of the tested ARGs increased independent of one another, no changes in the phylogenetic structure of the communities were observed in our microcosm experiments. This indicates that horizontal gene transfer of the tested genes from the TWW to the soil community might be the main mechanism behind this increase. Furthermore, a plateau effect was observed, where these affected genes (*sul1, intI1* and *qnrS*) initially increased in abundance after one month of high intensity TWW irrigation.

However, they did not exhibit any further increase from one to three months of high intensity irrigation in the full-scale TWW irrigated field. Such plateau effects up to carrying capacity are expected in microbial ecosysytems (Dalkmann et al., 2012). Additionally, ecological barriers may limit the dissemination of resistance genes to further members of the microbial community (Gibson et al., 2015). A similar plateau effect has been reported from a field study in Mexico (Dalkmann et al., 2012) with higher abundance of *sul1* in topsoil of untreated-wastewater full-scale irrigated fields compared to rainfall-fed ones. However, the relative abundance of *sul1* in the fields irrigated for 1.5 years was similar compared to fields that were irrigated with untreated wastewater for three and even up to 100 years (Dalkmann et al., 2012). Presumably, soil previously amended with manure, with an even higher load of ARGs than TWW irrigation (Chen et al., 2016; Muurinen et al., 2017), would exhibit similar plateau effects. Thus measuring only at the peak of this plateau, could mask the impact of TWW irrigation, hence leading to no additional observable effects of TWW irrigation as reported in previous studies (Cerqueira et al., 2019c). Upon decreasing irrigation intensity, the abundance of ARGs dropped in our field study, indicating that carrying capacity of ARGs is only stable as long as fresh mobile genes are constantly provided at a high rate. Especially when there is weaker selective pressure compared to clinical environments, fitness cost of ARGs may be higher for certain bacterial species (Porse et al., 2018) and thus ARGs are lost in relatively short time. However, despite their decrease, the relative abundance of TWW-affected genes in the field remained higher during irrigation breaks than in the non-irrigated soil. Especially *sul1, intI1* and *tet*(M) showed higher persistence upon their introduction in the soil, when compared to *qnrs* and *bla*$_{\text{OXA-58}}$.

The two ARGs that were unaffected by TWW irrigation (*bla*$_{\text{CTX-M-32}}$ and *bla*$_{\text{TEM}}$) were also present in TWW but at comparably lower relative abundance. However, they were among the most abundant genes in the native soil resistome of the non-irrigated soil. Both belong to the group of β-lactamase genes, a class of genes that has frequently been reported to occur naturally in soil environments (Wolters et al., 2018; Muurinen et al., 2017; Gatica et al., 2015). The production of β-lactams by indigenous soil bacteria or fungi is speculated to be the cause of this natural prevalence of β-lactamase genes in soil bacterial communities (Nesme and Simonet, 2015; Gatica et al., 2015). The third tested β-lactamase gene, *bla*$_{\text{OXA-58}}$, was absent from the non-irrigated soil. It initially increased upon irrigation, but its relative abundance rapidly decreased during irrigation-breaks, close to LOQ levels. Therefore, contrary to its counterparts which are unaffected by irrigation, *bla*$_{\text{OXA-58}}$ was not stably maintained in the soil

community. Hence, if ARGs are already naturally and stably prevalent in the soil and might consequently be unaffected by TWW irrigation needs to be considered before prioritizing ARG targets for estimating the effects of TWW irrigation.

For all here observed effects, the quality of irrigation water remained a crucial parameter. Despite their detection, *sul1, qnrS, intI1, tet*(M) and *bla*$_{\text{OXA-58}}$ were present at lower relative abundance in FW compared to TWW used for irrigation in our microcosm experiments. It is noteworthy that *sul1* was not detected in the non-irrigated soil, whereas it was detected in both microcosm groups at Week 0, after the equilibration with FW irrigation. In addition, *qnrS* was detected in the FW-Group of microcosms at Week 3 as well. Consequently, even irrigation with higher quality water can introduce certain ARGs, such as *sul1* and *qnrS,* but at lower, hence slower rates. Presumably, if irrigation was performed with TWW subjected to advanced tertiary treatment (Michael et al., 2013) or with FW with a higher load of ARGs (due to general anthropogenic pollution), then the observable differences between FW and TWW irrigation would be eliminated. Similar studies comparing the effects of FW and TWW irrigation are currently lacking. One exception is the study by Marano et al. (2019) that highlighted differences in ARG abundance in TWW and FW but detected no irrigation effect on ARG abundance in the respective soils. In contrary, we detected all the ARGs and *intI1* in our sampled FW, while most of the ARGs were present in FW irrigated soil microcosms as well. Therefore, the estimation of ARG abundance in FW and TWW should be considered as a crucial step, prior the estimation of the irrigation impact on soil.

Consequently, the differing ARG loads, due to differing TWW quality, might thus have contributed to the contradictive statements from previous studies on the impact of TWW irrigation. However, there are other important factors to consider such as the variability of soil types, physicochemical characteristics and microbiota from different geographical regions (Forsberg et al., 2014; Bahram et al., 2018). Most of the studies, including our own, are limited to distinct geographical regions, with different climate and soil characteristics. Thus, the contrasting observations could be explained by the different geographic locations of the studies. We here show that microcosm studies with different soil types, microbial communities and different quality TWWs could provide a successful research tool to gain further insights into the exact parameters determining the effects of TWW irrigation on ARGs in soil in the future. This includes considering complex mutualistic and antagonistic interactions of the soil microbiome with different agricultural crops through for example root exudates (Chen et al.,

2019). Interactions in the rhizosphere have previously been shown to lead to either positive (Jechalke et al., 2013) or negative (Song et al., 2020) selection for ARGs in agricultural soils.

Further, soil properties might change, as TWW irrigation introduces not only bacteria and mobile resistant genes, but also high nutrient loads and chemical contaminants (e.g. heavy metals) (Ternes et al., 2007; Rusan et al., 2007). This may lead to accumulation of metals and drug residues (Elgallal et al., 2016) and induce bacterial stress, hence favouring ARG and *intI1* dissemination (Zhao et al., 2019). Consequently, we speculate that the alteration of soil properties with enrichment of metals and drug residues could have contributed partially to the observed ARG and *intI1* persistence in the soil. However, further research is necessary to elucidate the complex interplay of soil biotic and abiotic factors with TWW irrigation.

2.5 Conclusion

In summary, we demonstrated that multiphase and multifactorial experiments are a powerful tool to assess the impact of TWW irrigation on the prevalence of ARGs in agricultural soil. These include temporal field sampling, comparisons with non-impacted soil and microcosm experiments with various types of irrigation. We showed that any type of irrigation could lead to the dissemination of ARGs in soil (e.g. *sul1* and *qnrS*). Nevertheless, the impact of FW irrigation on ARG and *intI1* profile of soil was distinctively minor compared to the impact of TWW irrigation with a high load of ARGs. Our multiphase approach was a useful tool for differentiating which genes were increased by TWW irrigation (*sul1, intI1, qnrS,* and $bla_{\text{OXA-58}}$) and genes that were stable members of the indigenous soil resistome unaffected by TWW irrigation ($bla_{\text{CTX–-M-32}}$ and bla_{TEM}). Therefore, the impact of TWW irrigation depends on the ARG and *intI1* load of irrigation water, the irrigation intensity, the type of each gene and its potential link with the native soil resistome.

2.6. References

1) Alygizakis, N.A., Urík, J., Beretsou, V.G., Kampouris, I., Galani, A., Oswaldova, M., Berendonk, T., Oswald, P., Thomaidis, N.S., Slobodnik, J., Vrana, B., Fatta-Kassinos, D., 2020. Evaluation of chemical and biological contaminants of emerging concern in treated wastewater intended for agricultural reuse. Environ. Int. 138, 105597. https://doi.org/10.1016/j.envint.2020.105597
2) Bahram, M., Hildebrand, F., Forslund, S.K., Anderson, J.L., Soudzilovskaia, N.A., Bodegom, P.M., Bengtsson-Palme, J., Anslan, S., Coelho, L.P., Harend, H., Huerta-Cepas, J., Medema, M.H., Maltz, M.R., Mundra, S., Olsson, P.A., Pent, M., Põlme, S., Sunagawa, S., Ryberg, M., Tedersoo, L., Bork, P., 2018. Structure and function of the global topsoil microbiome. Nature 560, 233–237. https://doi.org/10.1038/s41586-018-0386-6
3) 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., Martinez, J.L., 2015. Tackling antibiotic resistance: the environmental framework. Nat. Rev. Microbiol. 13, 310–317. https://doi.org/10.1038/nrmicro3439
4) Cacace, D., Fatta-Kassinos, D., Manaia, C.M., Cytryn, E., Kreuzinger, N., Rizzo, L., Karaolia, P., Schwartz, T., Alexander, J., Merlin, C., Garelick, H., Schmitt, H., de Vries, D., Schwermer, C.U., Meric, S., Ozkal, C.B., Pons, M.N., Kneis, D., Berendonk, T.U., 2019. Antibiotic resistance genes in treated wastewater and in the receiving water bodies: A pan-European survey of urban settings. Water Res. 162, 320–330. https://doi.org/10.1016/j.watres.2019.06.039
5) Caucci, S., Karkman, A., Cacace, D., Rybicki, M., Timpel, P., Voolaid, V., Gurke, R., Virta, M., Berendonk, T.U., 2016. Seasonality of antibiotic prescriptions for outpatients and resistance genes in sewers and wastewater treatment plant outflow. FEMS Microbiol. Ecol. 92, fiw060. https://doi.org/10.1093/femsec/fiw060
6) Cerqueira, F., Matamoros, V., Bayona, J., Elsinga, G., Hornstra, L.M., Piña, B., 2019b. Distribution of antibiotic resistance genes in soils and crops. A field study in legume plants (Vicia faba L.) grown under different watering regimes. Environ. Res. 170, 16–25. https://doi.org/10.1016/j.envres.2018.12.007
7) Cerqueira, F., Matamoros, V., Bayona, J., Piña, B., 2019a. Antibiotic resistance genes distribution in microbiomes from the soil-plant-fruit continuum in commercial Lycopersicon esculentum fields under different agricultural practices. Sci. Total Environ. 652, 660–670. https://doi.org/10.1016/j.scitotenv.2018.10.268
8) Cerqueira, F., Matamoros, V., Bayona, J.M., Berendonk, T.U., Elsinga, G., Hornstra, L.M., Piña, B., 2019c. Antibiotic resistance gene distribution in agricultural fields and crops. A soil-to-food analysis. Environ. Res. 177, 108608. https://doi.org/10.1016/j.envres.2019.108608
9) Chen, C., Li, J., Chen, P., Ding, R., Zhang, P., Li, X., 2014. Occurrence of antibiotics and antibiotic resistances in soils from wastewater irrigation areas in Beijing and Tianjin, China. Environ. Pollut. 193, 94–101. https://doi.org/10.1016/j.envpol.2014.06.005
10) Chen, Q., An, X., Li, H., Su, J., Ma, Y., Zhu, Y.G., 2016. Long-term field application of sewage sludge increases the abundance of antibiotic resistance genes in soil. Environ. Int. 92–93, 1–10. https://doi.org/10.1016/j.envint.2016.03.026

11) Chen, Q.L., Cui, H.L., Su, J.Q., Penuelas, J., Zhu, Y.G., 2019. Antibiotic Resistomes in Plant Microbiomes. Trends Plant Sci. 24, 530–541. https://doi.org/10.1016/j.tplants.2019.02.010
12) Christou, A., Karaolia, P., Hapeshi, E., Michael, C., Fatta-Kassinos, D., 2017. Long-term wastewater irrigation of vegetables in real agricultural systems: Concentration of pharmaceuticals in soil, uptake and bioaccumulation in tomato fruits and human health risk assessment. Water Res. 109, 24–34. https://doi.org/10.1016/j.watres.2016.11.033
13) Cytryn, E., 2013. The soil resistome: The anthropogenic, the native, and the unknown. Soil Biol. Biochem. https://doi.org/10.1016/j.soilbio.2013.03.017
14) Dalkmann, P., Broszat, M., Siebe, C., Willaschek, E., Sakinc, T., Huebner, J., Amelung, W., Grohmann, E., Siemens, J., 2012. Accumulation of pharmaceuticals, enterococcus, and resistance genes in soils irrigated with wastewater for zero to 100 years in central Mexico. PLoS One 7. https://doi.org/10.1371/journal.pone.0045397
15) Dinno, A., 2016. dunn's test R package: Dunn's Test of Multiple Comparisons Using Rank Sums. R package version. 0.2.3 https://cran.r-project.org/web/packages/dunn.test/index.html
16) Dungan, R.S., McKinney, C.W., Leytem, A.B., 2018. Tracking antibiotic resistance genes in soil irrigated with dairy wastewater. Sci. Total Environ. 635, 1477–1483. https://doi.org/10.1016/j.scitotenv.2018.04.020
17) Elgallal, M., Fletcher, L., Evans, B., 2016. Assessment of potential risks associated with chemicals in wastewater used for irrigation in arid and semiarid zones: A review. Agric. Water Manag. 177, 419–431. https://doi.org/10.1016/j.agwat.2016.08.027
18) Fatta-Kassinos, D., Meric, S., Nikolaou, A., 2011. Pharmaceutical residues in environmental waters and wastewater: Current state of knowledge and future research. Anal. Bioanal. Chem. 399, 251–275. https://doi.org/10.1007/s00216-010-4300-9
19) Forsberg, K.J., Patel, S., Gibson, M.K., Lauber, C.L., Knight, R., Fierer, N., Dantas, G., 2014. Bacterial phylogeny structures soil resistomes across habitats. Nature 509, 612–616. https://doi.org/10.1038/nature13377
20) Friedman, N.D., Temkin, E., Carmeli, Y., 2016. The negative impact of antibiotic resistance. Clin. Microbiol. Infect. 22, 416–422. https://doi.org/10.1016/j.cmi.2015.12.002
21) Gatica, J., Cytryn, E., 2013. Impact of treated wastewater irrigation on antibiotic resistance in the soil microbiome. Environ. Sci. Pollut. Res. 20, 3529–3538. https://doi.org/10.1007/s11356-013-1505-4
22) Gatica, J., Yang, K., Pagaling, E., Jurkevitch, E., Yan, T., Cytryn, E., 2015. Resistance of undisturbed soil microbiomes to ceftriaxone indicates extended spectrum β-lactamase activity. Front. Microbiol. 6, 1–11. https://doi.org/10.3389/fmicb.2015.01233
23) Gatica, J., Yang, K., Pagaling, E., Jurkevitch, E., Yan, T., Cytryn, E., 2015. Resistance of undisturbed soil microbiomes to ceftriaxone indicates extended spectrum β-lactamase activity. Front. Microbiol. 6, 1–11. https://doi.org/10.3389/fmicb.2015.01233
24) Gibson, M.K., Forsberg, K.J., Dantas, G., 2015. Improved annotation of antibiotic resistance determinants reveals microbial resistomes cluster by ecology. ISME J. 9, 207–216. https://doi.org/10.1038/ismej.2014.106
25) Gillings, M.R., Gaze, W.H., Pruden, A., Smalla, K., Tiedje, J.M., Zhu, Y.G., 2015. Using the class 1 integron-integrase gene as a proxy for anthropogenic pollution. ISME J. 9, 1269–1279. https://doi.org/10.1038/ismej.2014.226

26) Han, X.M., Hu, H.W., Shi, X.Z., Wang, J.T., Han, L.L., Chen, D., He, J.Z., 2016. Impacts of reclaimed water irrigation on soil antibiotic resistome in urban parks of Victoria, Australia. Environ. Pollut. 211, 48–57. https://doi.org/10.1016/j.envpol.2015.12.033
27) Jechalke, S., Kopmann, C., Rosendahl, I., Groeneweg, J., Weichelt, V., Krögerrecklenfort, E., Brandes, N., Nordwig, M., Ding, G.C., Siemens, J., Heuer, H., Smalla, K., 2013. Increased abundance and transferability of resistance genes after field application of manure from sulfadiazine-treated pigs. Appl. Environ. Microbiol. 79, 1704–1711. https://doi.org/10.1128/AEM.03172-12
28) Jechalke, S., Radl, V., Schloter, M., Heuer, H., Smalla, K., 2016. Do drying and rewetting cycles modulate effects of sulfadiazine spiked manure in soil? FEMS Microbiol. Ecol. 92, 1–7. https://doi.org/10.1093/femsec/fiw066
29) Kassambara, A., 2019. ggpubr R Package: ggplot2-Based Publication Ready Plots R package version 0.2.3 https://CRAN.R-project.org/package=ggpubr.
30) Klümper, U., Dechesne, A., Riber, L., Brandt, K.K., Gülay, A., Sørensen, S.J., Smets, B.F., 2017. Metal stressors consistently modulate bacterial conjugal plasmid uptake potential in a phylogenetically conserved manner. ISME J. 11, 152–165. https://doi.org/10.1038/ismej.2016.98
31) Klümper, U., Riber, L., Dechesne, A., Sannazzarro, A., Hansen, L.H., Sørensen, S.J., Smets, B.F., 2015. Broad host range plasmids can invade an unexpectedly diverse fraction of a soil bacterial community. ISME J. 9, 934–945. https://doi.org/10.1038/ismej.2014.191
32) Maaß, O., Grundmann, P., 2016. Added-value from linking the value chains of wastewater treatment, crop production and bioenergy production: A case study on reusing wastewater and sludge in crop production in Braunschweig (Germany). Resour. Conserv. Recycl. 107, 195–211. https://doi.org/10.1016/j.resconrec.2016.01.002
33) Manaia, C.M., Rocha, J., Scaccia, N., Marano, R., Radu, E., Biancullo, F., Cerqueira, F., Fortunato, G., Iakovides, I.C., Zammit, I., Kampouris, I., Vaz-Moreira, I., Nunes, O.C., 2018. Antibiotic resistance in wastewater treatment plants: Tackling the black box. Environ. Int. 115, 312–324. https://doi.org/10.1016/J.ENVINT.2018.03.044
34) Marano, R.B.M., Zolti, A., Jurkevitch, E., Cytryn, E., 2019. Antibiotic resistance and class 1 integron gene dynamics along effluent, reclaimed wastewater irrigated soil, crop continua: elucidating potential risks and ecological constraints. Water Res. 164, 114906. https://doi.org/10.1016/j.watres.2019.114906
35) Mareschal, L., Bonnaud, P., Turpault, M.P., Ranger, J., 2010. Impact of common European tree species on the chemical and physicochemical properties of fine earth: An unusual pattern. Eur. J. Soil Sci. 61, 14–23. https://doi.org/10.1111/j.1365-2389.2009.01206.x
36) Martinez Arbizu, P., 2019. pairwiseAdonis: Pairwise multilevel comparison using adonis. R package version 0.3. https://github.com/pmartinezarbizu/pairwiseAdonis.
37) McKinney, C.W., Dungan, R.S., Moore, A., Leytem, A.B., 2018. Occurrence and abundance of antibiotic resistance genes in agricultural soil receiving dairy manure. FEMS Microbiol. Ecol. 94, 1–10. https://doi.org/10.1093/femsec/fiy010
38) McMurdie and Holmes, 2013. Phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. PLoS ONE 8. doi:10.1371/journal.pone.0061217
39) Michael, I., Frontistis, Z., Fatta-Kassinos, D., 2013. Removal of pharmaceuticals from environmentally relevant matrices by advanced oxidation processes (AOPs), 2nd ed,

Comprehensive Analytical Chemistry. Elsevier B.V. https://doi.org/10.1016/B978-0-444-62657-8.00011-2
40) Musovic, S., Klümper, U., Dechesne, A., Magid, J., Smets, B.F., 2014. Long-term manure exposure increases soil bacterial community potential for plasmid uptake. Environ. Microbiol. Rep. 6, 125–130. https://doi.org/10.1111/1758-2229.12138
41) Muurinen, J., Stedtfeld, R., Karkman, A., Pärnänen, K., Tiedje, J., Virta, M., 2017. Influence of Manure Application on the Environmental Resistome under Finnish Agricultural Practice with Restricted Antibiotic Use. Environ. Sci. Technol. 51, 5989–5999. https://doi.org/10.1021/acs.est.7b00551
42) Negreanu, Y., Pasternak, Z., Jurkevitch, E., Cytryn, E., 2012. Impact of treated wastewater irrigation on antibiotic resistance in agricultural soils. Environ. Sci. Technol. 46, 4800–4808. https://doi.org/10.1021/es204665b
43) Nesme, J., Simonet, P., 2015. The soil resistome: A critical review on antibiotic resistance origins, ecology and dissemination potential in telluric bacteria. Environ. Microbiol. 17, 913–930. https://doi.org/10.1111/1462-2920.12631
44) Oksanen, J., Blanchet, F.G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P.R., O'Hara, R.B., Simpson, G.L., Solymos, P.M. Stevens, H.H., Szoecs, E., Wagner H., 2019. vegan R package: an R package for community ecologists. R package version 2.5-6. https://cran.rproject.org/package=vegan
45) Orschler, L., Agrawal, S., Lackner, S., 2019. On resolving ambiguities in microbial community analysis of partial nitritation anammox reactors. Sci. Rep. 1–10. https://doi.org/10.1038/s41598-019-42882-8
46) Pantanella, F., Lekunberri, I., Gagliardi, A., Venuto, G., Sànchez-Melsió, A., Fabiani, M., Balcázar, J.L., Schippa, S., De Giusti, M., Borrego, C., Solimini, A., 2020. Effect of urban wastewater discharge on the abundance of antibiotic resistance genes and antibiotic-resistant escherichia coli in two Italian rivers. Int. J. Environ. Res. Public Health 17, 1–13. https://doi.org/10.3390/ijerph17186813
47) Paranychianakis, N. V., Salgot, M., Snyder, S.A., Angelakis, A.N., 2015. Water reuse in EU states: Necessity for uniform criteria to mitigate human and environmental risks. Crit. Rev. Environ. Sci. Technol. 45, 1409–1468. https://doi.org/10.1080/10643389.2014.955629
48) Pärnänen, K.M.M., Narciso-Da-Rocha, C., Kneis, D., Berendonk, T.U., Cacace, D., Do, T.T., Elpers, C., Fatta-Kassinos, D., Henriques, I., Jaeger, T., Karkman, A., Martinez, J.L., Michael, S.G., Michael-Kordatou, I., O'Sullivan, K., Rodriguez-Mozaz, S., Schwartz, T., Sheng, H., Sørum, H., Stedtfeld, R.D., Tiedje, J.M., Giustina, S.V. Della, Walsh, F., Vaz-Moreira, I., Virta, M., Manaia, C.M., 2019. Antibiotic resistance in European wastewater treatment plants mirrors the pattern of clinical antibiotic resistance prevalence. Sci. Adv. 5. https://doi.org/10.1126/sciadv.aau9124
49) R Core Team, R: A language and environment for statistical computing, R Foundation for Statistical Computing, Vienna, Austria (2019)
50) Reis, P.J.M., Homem, V., Alves, A., Vilar, V.J.P., Manaia, C.M., Nunes, O.C., 2018. Insights on sulfamethoxazole bio-transformation by environmental Proteobacteria isolates. J. Hazard. Mater. 358, 310–318. https://doi.org/10.1016/j.jhazmat.2018.07.012
51) Rizzo, L., Manaia, C., Merlin, C., Schwartz, T., Dagot, C., Ploy, M.C., Michael, I., Fatta-Kassinos, D., 2013. Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: A review. Sci. Total Environ. 447, 345–360. https://doi.org/10.1016/j.scitotenv.2013.01.032

52) Rocha, J., Cacace, D., Kampouris, I., Guilloteau, H., Jäger, T., Marano, R.B.M., Karaolia, P., Manaia, C.M., Merlin, C., Fatta-Kassinos, D., Cytryn, E., Berendonk, T.U., Schwartz, T., 2020. Inter-laboratory calibration of quantitative analyses of antibiotic resistance genes. J. Environ. Chem. Eng. 8, 102214. https://doi.org/10.1016/j.jece.2018.02.022
53) Rusan, M.J.M., Hinnawi, S., Rousan, L., 2007. Long term effect of wastewater irrigation of forage crops on soil and plant quality parameters. Desalination 215, 143–152. https://doi.org/10.1016/j.desal.2006.10.032
54) Song, M., Peng, K., Jiang, L., Zhang, D., Song, D., Chen, G., Xu, H., Li, Y., Luo, C., 2020. Alleviated Antibiotic-Resistant Genes in the Rhizosphere of Agricultural Soils with Low Antibiotic Concentration. J. Agric. Food Chem. 68, 2457–2466. https://doi.org/10.1021/acs.jafc.9b06634
55) Ternes, T.A., Bonerz, M., Herrmann, N., Teiser, B., Andersen, H.R., 2007. Irrigation of treated wastewater in Braunschweig, Germany: An option to remove pharmaceuticals and musk fragrances. Chemosphere 66, 894–904. https://doi.org/10.1016/j.chemosphere.2006.06.035
56) Wang, F.H., Qiao, M., Su, J.Q., Chen, Z., Zhou, X., Zhu, Y.G., 2014. High throughput profiling of antibiotic resistance genes in urban park soils with reclaimed water irrigation. Env. Sci Technol 48, 9079–9085. https://doi.org/10.1021/es502615e
57) Wang, M., Liu, P., Xiong, W., Zhou, Q., Wangxiao, J., Zeng, Z., Sun, Y., 2018. Fate of potential indicator antimicrobial resistance genes (ARGs) and bacterial community diversity in simulated manure-soil microcosms. Ecotoxicol. Environ. Saf. 147, 817–823. https://doi.org/10.1016/j.ecoenv.2017.09.055
58) Wickham, H. 2016, ggplot2: Elegant Graphics for Data Analysis, Springer.
59) Wolters, B., Fornefeld, E., Jechalke, S., Su, J.Q., Zhu, Y.G., Sørensen, S.J., Smalla, K., Jacquiod, S., 2018. Soil amendment with sewage sludge affects soil prokaryotic community composition, mobilome and resistome. FEMS Microbiol. Ecol. 95. https://doi.org/10.1093/femsec/fiy193
60) Zhang and Li, 2011. Occurrence, transformation, and fate of antibiotics in municipal wastewater treatment plants. Critical Reviews in Environmental Science and Technology 41, 951–998. doi:10.1080/10643380903392692
61) Zhao, Y., Cocerva, T., Cox, S., Tardif, S., Su, J.Q., Zhu, Y.G., Brandt, K.K., 2019. Evidence for co-selection of antibiotic resistance genes and mobile genetic elements in metal polluted urban soils. Sci. Total Environ. 656, 512–520. https://doi.org/10.1016/j.scitotenv.2018.11.372

Chapter 3

3. Treated wastewater irrigation promotes the spread of antibiotic resistance into subsoil pore-water.

Ioannis D. Kampouris[a], Uli Klümper[a], Shelesh Agrawal[b], Laura Orschler[b], Damiano Cacace[a], Steffen Kunze[a], Thomas U. Berendonk[a]

[a] Institute for Hydrobiology, Technische Universität Dresden, 01217, Dresden, Germany

[b] Technische Universität Darmstadt, Institute IWAR, Chair of Wastewater Engineering, Franziska-Braun-Straße 7, 64287 Darmstadt, Germany

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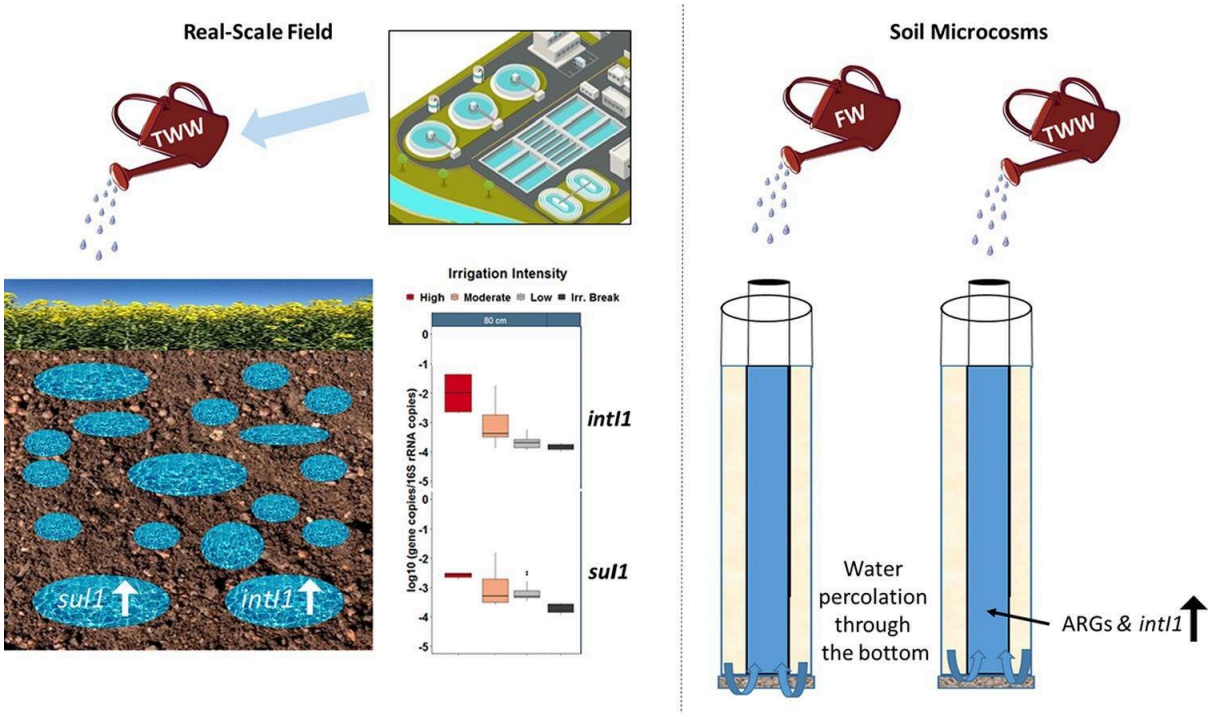
Graphical Abstract



Highlights

- TWW irrigation intensity and *sul1* & *intI1* abundance correlate in a real-scale field.
- ARGs & *intI1* increase in subsoil pore-water during TWW irrigation in mesocosms.
- No increase of ARGs & *intI1* in freshwater irrigated mesocosms.
- TWW irrigation does not affect the bacterial load of subsoil pore-water.

Abstract

In the present study, we investigated the impact of treated wastewater (TWW) irrigation on the prevalence of antibiotic resistance genes (ARGs) in subsoil pore-water, a so-far under-appreciated matrix. We hypothesized that TWW irrigation increases ARG prevalence in subsoil pore-water. This hypothesis was tested using a multiphase approach, which consisted of sampling percolated subsoil pore-water from lysimeter-wells of a real-scale TWW-irrigated field, operated for commercial farming practices, and controlled, laboratory microcosms irrigated with freshwater or TWW. We monitored the abundance of six selected ARGs (*sul1,* $bla_{\text{OXA-58}}$, *tet*(M), *qnrS*, $bla_{\text{CTX-M-32}}$ and bla_{TEM}), the *intI1* gene associated with mobile genetic elements and an indicator for anthropogenic pollution and bacterial abundance (16S rRNA gene) by qPCR. The bacterial load of subsoil pore water was independent of both, irrigation intensity in the field study and irrigation water type in the microcosms. Among the tested genes in the field study, *sul1* and *intI1* exhibited constantly higher relative abundances. Their abundance was further positively correlated with increasing irrigation intensity. Controlled microcosm experiments verified the observed field study results: the relative abundance of several genes, including *sul1* and *intI1,* increased significantly when irrigating with TWW compared to freshwater irrigation. Overall, TWW irrigation promoted the spread of ARGs and *intI1* in the subsoil pore-water, while the bacterial load was maintained. The combined results from the real-scale agricultural field and the controlled lab microcosms indicate that the dissemination of ARGs in various subsurface environments needs to be taken into account during TWW irrigation scenarios.

3.1 Introduction

Treated wastewater (TWW) irrigation and managed aquifer recharge (MAR) are cost-efficient counter-measures against freshwater resource depletion in arid and semi-arid areas (Paranychianakis et al., 2015, Ternes et al., 2007, Maaß and Grundmann, 2016). However, TWW might contain diverse antibiotic residues (As), antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) (Berendonk et al., 2015, Cacace et al., 2019, Manaia et al., 2018a, Michael et al., 2013, Pärnänen et al., 2019, Smalla et al., 2018). The abundance of As, ARB and ARGs in TWW has raised questions regarding its influence on the dissemination of antibiotic resistance through irrigation (Michael et al., 2013, Berendonk et al., 2015, Manaia et al., 2018a, Smalla et al., 2018). Previous studies from various locations (China, Australia, Israel, Spain) reported contradictive observations regarding the impact of TWW irrigation on the prevalence of ARGs in soil (Negreanu et al., 2012, Cerqueira et al., 2019a, Cerqueira et al., 2019b, Cerqueira et al., 2019c, Marano et al., 2019, Wang et al., 2014, Han et al., 2016, Dalkmann et al., 2012, Jechalke et al., 2015). Specifically, a few studies have claimed that TWW irrigation increases ARG prevalence in soil (Wang et al., 2014, Han et al., 2016, Dalkmann et al., 2012, Jechalke et al., 2015), while others from the Mediterranean Basin reported a minimal and negligible impact (Negreanu et al., 2012, Cerqueira et al., 2019b, Cerqueira et al., 2019c, Marano et al., 2019). These contradictions could be caused by several factors, such as selection of investigated ARGs, masking from soil-amendment practices, variability of soil microbiota or different A/ARB/ARG-loads present in TWW (Cacace et al., 2019, Pärnänen et al., 2019, Cerqueira et al., 2019c, Marano et al., 2019, Wang et al., 2014, Han et al., 2016, Dalkmann et al., 2012, Jechalke et al., 2015). In addition, effects of irrigation could be influenced by the initial ARG prevalence of the soil microbiome caused by either soil being a natural reservoir of ARGs (native soil resistome), due to the presence of antibiotic producing soil fungi or bacteria (Nesme & Simonet, 2015) or previous exposure to manure (Luby et al., 2016; Yang et al., 2016; Barrios et al., 2020) or biosolids (Mathews & Reinhold, 2013).

Apart from immediate effects on soil, anthropogenic processes can potentially affect the subsoil and groundwater microbiota as well (Szekeres et al., 2018, Rossi et al., 2019). Specifically, Szekeres et al. (2018) reported higher relative abundance of ARGs in groundwater wells with higher proximity to urban settings, due to general anthropogenic activity. However, the majority of studies so far have focused on either crops or topsoil, hence neglecting deeper lying

matrices like subsoil, subsoil pore-water and groundwater (Negreanu et al., 2012, Cerqueira et al., 2019a, Cerqueira et al., 2019b, Cerqueira et al., 2019c, Marano et al., 2019, Wang et al., 2014; Han et al., 2016; Dalkmann et al., 2012, Jechalke et al., 2015). Thus, how and which secondary anthropogenic activities affect the prevalence of ARGs in subsoil and groundwater environments remains a major gap of knowledge. Böckelmann et al. (2009) reported the presence of tetracycline and erythromycin ARGs in the groundwater of MAR sites, without any clear trend or impact associated with MAR. In the groundwater of a MAR site in Israel, *bla*$_{\text{TEM}}$ and *qnrS* were below the limit of quantification (LOQ) (Elkayam et al., 2018). However, Lüneberg et al. (2018) demonstrated that during TWW irrigation, the prevalence of ARGs increased in the water flow paths towards the aquifer of soil/subsoil microcosms spiked with antibiotics. Taking into account that subsoil and groundwater microbial communities from various locations exhibit high variation and complexity (Haack et al., 2004, Kumar et al., 2018, Anantharaman et al., 2016, Yan et al., 2020), the number of previous studies is insufficient to cover the gap of knowledge regarding the influence of TWW irrigation on ARG prevalence in subsoil, subsoil pore-water and groundwater microbial communities.

Thus, the objective of the present study was to systematically investigate the impact of TWW irrigation on ARG prevalence in subsoil pore-water. Our working hypothesis was that TWW irrigation increases the prevalence of ARGs in subsoil pore-water. To test this hypothesis, we sampled the percolated subsoil pore-water in lysimeter-wells of a real-scale agricultural field, regularly subjected to TWW irrigation for commercial farming of crops in the nearby area of Braunschweig, for the last 50 years. The common practice of this commercial irrigation operation utilises TWW subjected to secondary (biological) treatment to ensure a high nutrient load for the crops, since the nutrient retention of the soil is limited (Ternes et al., 2007). These crops are intended for biogas production, hence a yearly cultivation plan for maximum yield is operated (Ternes et al., 2007). Samples were taken regularly across a one-year sampling period, across different periods and intensities of irrigation and after a long irrigation break, to gain insights into how TWW irrigation affects ARG prevalence on the field scale. Samples were taken at three different depths to ensure that observed TWW irrigation effects are consistent across the full soil depths profile and not restricted mainly to upper soil layers. All samples were analysed with qPCR for the abundance of six ARGs, the gene *intI1*, which is associated with mobile genetic elements (MGE) and an indicator for anthropogenic pollution (Gillings et al., 2015), and the 16S rRNA gene.

Our diverse set of ARGs included genes occurring in high and low abundance in the environment and TWW, based on the framework for TWW monitoring of NEREUS (www.nereus-cost.eu) (Rocha et al., 2020; Cacace et al., 2019) and ANSWER-ITN (www.answer-itn.eu) networks. This set conferred resistance to several classes of antibiotics: sulphonamides, quinolones, tetracyclines and the diverse group of β-lactams (penicillins, cephalosporins and carbapenems). Apart from their inclusion in TWW monitoring networks, the clinical and environmental context for each gene accounted for their selection. The gene *sul1* was selected for its ubiquity and high abundance in TWW (Cacace et al., 2019). The respective antibiotic class that *sul1* confers resistance to (sulphonamides), is regularly detected as a pollutant at high frequency in TWW (Nikolaou et al., 2007, Barnes et al., 2008, Avisar et al., 2009, Michael et al., 2013). Furthermore, sulphonamides persistence in subsurface environments during TWW reuse (Avisar et al., 2009), including the previously examined lysimeters (Ternes et al., 2007), can result in *sul1* selection as well. The genes *qnrS* and *tet*(M), $bla_{\text{OXA-58}}$ were selected for their clinical importance and high occurrence rate in TWW in previous studies (Caucci et al., 2016; Cacace et al., 2019, Alygizakis et al., 2020). Further, bla_{OXA} genes are the most abundant β-lactamase genes located on mobile integron cassettes in European TWW (Gatica et al., 2016). The two final β-lactamase genes bla_{TEM} and $bla_{\text{CTX-M-32}}$ were included as they are clinically important but have low abundance in TWW (Cacace et al., 2019), while they have been reported to be naturally prevalent in soil microbiota (Gatica et al., 2015). The integrase gene *intI1,* a suggested marker for anthropogenic pollution impact, was analysed as well, since it is frequently part of mobile gene cassettes that carry ARGs (Gatica et al., 2016).

The interpretation of long-term, real-scale, field-based observations is however regularly affected by the variability of diverse environmental parameters, such as temperature or precipitation. To overcome this limitation, we here combined our field study with lab-based mesocosm experiments under controlled conditions. We set up subsoil pore-water mesocosms irrigated with either freshwater or TWW, to comprehensively test the validity of the insights gained from the real-scale field study, hence elucidating the impact of TWW irrigation on subsoil pore-water ARG prevalence.

3.2 Materials and methods

3.2.1. Sampling campaign of the lysimeter-wells

The sampled sandy (cambisol), agricultural field is located in Wendeburg, Germany (N: 52.359500, E: 10.399833; Fig. S3.1) and is associated with the Braunschweig Wastewater Association (BWA). The high percentage of sand in the area's soil results in low water retention, but more importantly low nutrient-retention capacity. To tackle the low nutrient-retention capacity, commercial TWW irrigation has taken place in the local area for over 50 years. Further characterization of the field site (e.g. soil composition) has been provided through previous studies (Ternes et al., 2007).

The specific field contained pre-installed lysimeter-wells that allowed the collection of percolated water from three different depths: 40, 80 and 120 cm (Fig. S3.2). Due to the high percentage of sand, the water retention time of the soil is less than 24 hrs. Every year a different schedule of TWW irrigation with various intensities of irrigation is applied to the field, according to agricultural plans of the farmers and amount of natural precipitation. The field is irrigated with TWW or TWW mixed with digested sludge (TWW & DS), collected from the Urban Wastewater Treatment Plant (UWTP) of BWA, depending on the nutrient-demand of the grown crops. We sampled soil pore-water from the lysimeter-wells during several periods of irrigation intensity for eleven months: high-intensity irrigation, intermediate-intensity irrigation, low-intensity irrigation and after a long irrigation break (Table 3.1).

Samples of percolated water were collected in sterilised 2 L glass bottles at each lysimeter-well's percolation-point (Fig. S3.2). The bottles were placed in the percolation-points prior to an irrigation event and percolated water was collected for three days after each event (4 samples per depth). At the end of the irrigation break (October 2017-February 2018), the bottles were placed on the lysimeters to equally collect percolated water from natural soil-moisture. Samples were stored on ice and transferred to the lab. Bacteria were captured by filtration of 0.1 to 0.5 L of percolated water (depending on collected volume) in triplicate per sample within 48 hrs. The filters (polycarbonate, 0.2 μm pore size, 47 mm diameter, Sartorius, Germany) were stored at −20 °C prior to DNA extraction.

Table 3.1: Sampling dates, conditions and irrigation intensity of the lysimeter-wells (TWW: Treated Wastewater, DS: Digested Sludge).

Sampling	Irrigation type	Conditions	Irrigation intensity
Apr-17	TWW & DS	Continuous and regular irrigation was initiated on the field.	High
May-17	TWW & DS	The field was irrigated continuously and regularly and was already irrigated for one month.	High
Jun-17	TWW & DS	The field was irrigated continuously and was already irrigated for two months.	High
Oct-17	TWW	The field was irrigated spontaneously.	Intermediate
Feb-18	No-Irrigation (Rainfall)	The field was not irrigated since October 2017 and was only exposed to natural precipitation.	Irrigation break
Feb-18	TWW	The field was irrigated for the first time after the irrigation break.	Low
Mar-18	TWW	The field was irrigated regularly from February.	Low

3.2.2. Subsoil pore-water microcosms

For the microcosm experiments, we sampled forest soil, located 10–20 m adjacent to the TWW irrigated field. The samples were taken from 12 individual sampling points at 0–60 cm depth. After air-drying, and sieving (~6 mm mesh size) the soil was homogenised to create a composite soil sample that was used for the microcosm experiments. Moreover, soil samples were preserved for analysis of background levels of ARGs (n = 12). Despite their close proximity, we resampled the field and analysed its composition along with the forest soil to verify that they have similar physicochemical compositions (Table S3.2). Both soils were classified as sandy soils, with sand content exceeding 90%.

Microcosms (Fig. S3.3) were assembled from cylindrical plastic tubes of 66 cm height and 4.5 cm radius, which was the largest size of microcosms we could operate, without compromising aseptic sampling conditions. The bottom of each microcosm (5 cm height) was filled with

quartz gravels (size: ~3 mm^3) and an inner tube (1.5 cm radius) was placed centrally to allow the collection of the subsoil pore-water (Fig. S3.3). Microcosms were then filled with the previously homogenized soil up to 50 cm height, resulting in a total soil volume per microcosm of 2,827.6 mL. The repacking of soil in microcosms ensured the absence of soil macropore formation frequently found in higher (40 cm), but rare in lower depths (120 cm), thus avoiding preferential flow and maximizing the contact of water with the soil. Consequently, despite their limited height of 50 cm the microcosms simulate the physical conditions of the lower depth soil rather well.

Microcosms were divided into two groups, with four replicate microcosms per treatment: The Freshwater-Group and TWW-Group were defined with respect to the type of irrigation water. TWW for irrigation was obtained from an UWTP nearby the Dresden area (Kaditz, Germany; N: 51.070640, E: 13.680888). The freshwater was collected from a shallow well (depth ~ 7 m) located next to the Elbe river, in Pirna, Germany (N: 50.965905, E: 13.924034). The microcosms were irrigated with 350 mL of water, which led to saturation. Removal of residual water and renewed irrigation were performed aseptically three times per week.

The microcosms were placed in a controlled temperature chamber at 20 °C and with controlled light conditions with 12 h light/darkness. Both groups were initially irrigated with freshwater for two weeks, to stabilise and equilibrate the soil conditions. Then the TWW-Group switched to TWW irrigation for three weeks, while the Freshwater-Group was continuously irrigated with freshwater. Subsoil pore-water samples (200 mL per microcosm) were taken aseptically from each microcosm of both groups. First sampling took place at the end of the two-week freshwater irrigation/stabilisation-period (Week 0), the second in the 1st week after switching to TWW irrigation (Week 1) and the third in the 3rd week after switching to TWW irrigation (Week 3). Bacteria were harvested from the samples through filtration as described above. Filters were frozen directly after sampling and stored at −20 °C prior to DNA extraction. TWW and freshwater samples (0.5 L, n = 6 per irrigation type) were equally filtered and stored for DNA extraction.

3.2.3. DNA extraction and quantitative real time PCR and sequencing

We used the DNeasy PowerWater Kit and the DNeasy PowerSoil Kit (Qiagen, Germany) according to the manufacturer's instructions to extract DNA from the water and soil samples,

respectively. The quantity and quality of DNA was measured with NanoDrop (Thermo Fischer Scientific, Germany). The samples were analysed with quantitative real-time PCR (qPCR) for six ARGs (*sul1, bla*$_{\text{OXA-58}}$*, tet*(M), *qnrS*, *bla*$_{\text{TEM}}$ and *bla*$_{\text{CTX-M-32}}$), *intI1* and the 16S rRNA gene. Reactions were performed in a MasterCycler RealPlex (Eppendorf, Germany) at final volume 20 µL with 10 µL of Luna Universal qPCR Master Mix (New England Biolabs, Germany). The concentration of the primers varied from 0.2 to 0.5 µM (for further details regarding genes, reagents, primers and temperature for each gene see Table S3.3). Standard curves were created during every qPCR run, with the use of the same plasmid vector and procedures as described previously (Cacace et al., 2019).

Standard curves with amplification efficiency 0.9–1.1 and $R^2 \geq 0.99$ were accepted and melting curve analysis was performed to assess the amplicons' specificity. Screening for PCR inhibition was performed by spiking a plasmid containing a gene present in low abundance in our samples (*bla*$_{\text{CTX-M-32}}$, spiking concentration $4 * 10^6$ copies/µL): no inhibition was detected in any of the samples. The absolute abundance of genes was finally expressed as gene copies/L and the relative abundance as the ratio of gene copies per copy of the 16S rRNA gene. Further, the bacterial community profile of TWW irrigation and the TWW-Group prior and after the switch to TWW irrigation was analysed with 16S rRNA sequencing of multiple variable regions (Orschler et al., 2019), to determine if TWW related bacteria, are present in subsoil pore-water after exposure to TWW irrigation. The protocols used for 16S rRNA sequencing of the samples and processing of the sequences were described previously in Orschler et al. (2019). Raw nucleotide sequences were submitted to the GenBank under the project number PRJNA665982.

3.2.4. Data processing and statistical analysis

Prior to any statistical analysis, every gene in every sample that was below LOQ was set as 1 copy/L for absolute abundance and 10^{-8} for relative abundance (one order of magnitude lower than the minimum relative abundance observed for any gene in any sample, ~10^{-7}). Data was then $\log_{10}$ -transformed before performing statistical analysis. The programing language R (R Core Team, 2019, v. 3.5.3) was used for graphical representations, with the packages "ggplot" (Whickam, 2016) and "ggpubr" (v. 0.2.2, Kassambara, 2019). Significant differences were assessed with the Wilcoxon rank sum test or in case of group comparisons with the Kruskal-Wallis test (package "ggpubr"). A post-hoc test (Dunn's test with Benjamini-Hochberg

correction) was performed with the use of the package “dunn’s test” (v1.3.5, Dinno, 2016), to assign significant differences from pairwise multiple comparisons. The difference in ARG profiles of the lysimeter-well samples were assessed with PERMANOVA tests (“adonis” function, method=“euclidean”) from the “vegan” package (v2.5–6, Oksanen et al., 2019). In addition, we performed pairwise comparisons for the field and the microcosm groups over time with the “pairwiseAdonis” package (Martinez Arbizu, 2019, v0.3, function “pairwise.adonis”, method=“euclidean”) along with Benjamini-Hochberg correction for multiple comparisons.

3.3. Results

3.3.1. Lysimeter-Well

3.3.1.1. Seasonal variation, rather than TWW irrigation affects subsoil pore-water bacterial abundance

Throughout the sampling period, the studied field site was irrigated with either TWW or TWW mixed with digested sludge (DS). The bacterial load in the irrigation water, determined through 16S rRNA gene abundance, remained stable, whether there was a mixing with DS or not: 9.8 ± 0.36 $\log_{10}$ copies/L for TWW & DS (April, May, June 2017) and 9.9 ± 0.34 $\log_{10}$ copies/L for the TWW (October 2017, February and March 2018) (Fig. 3.1) (Wilcoxon rank sum test, p = 0.16, n = 3).

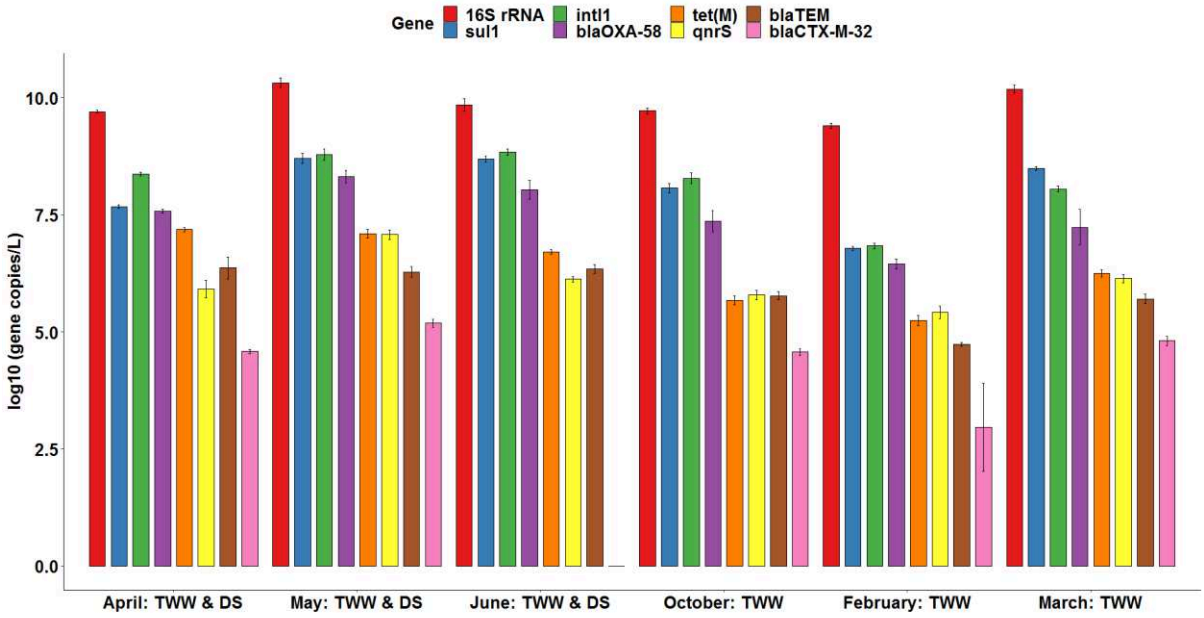


Figure 3.1: Absolute abundance (copies/L) of the selected genes in the irrigation water of Braunschweig Wastewater Association (BWA). TWW: Treated Wastewater, DS: Digested Sludge (n=3).

The absolute abundance of 16S rRNA gene copies in the pore-water samples throughout the year ranged from 8.0 to 9.8 $\log_{10}$ copies/L (Fig. 3.2), while we observed similar patterns of in-/decrease at all three different depths. During intensive irrigation periods (April, May and June 2017), the 16S absolute abundance was constantly one order of magnitude lower at each

sampled depth compared to the rest of the sampling campaign (Fig. 2). For example at 120 cm depth, the abundance of 16S rRNA gene was 7.9–8.3 $\log_{10}$ copies/L in April-June 2017 (periods of high-intensity irrigation). Yet, in October 2017, Feb. (1) 2018 (before start of TWW irrigation) and Feb. (2) 2018 (after the restart of TWW irrigation) the abundance of 16S rRNA gene was significantly higher at 9.0 ± 0.2, 8.9 ± 0.1 and 8.9 ± 0.3 $\log_{10}$ copies/L, respectively (Fig. 3.2, $p = 2.2 \times 10^{-14}$, Kruskal-Wallis, n = 4).

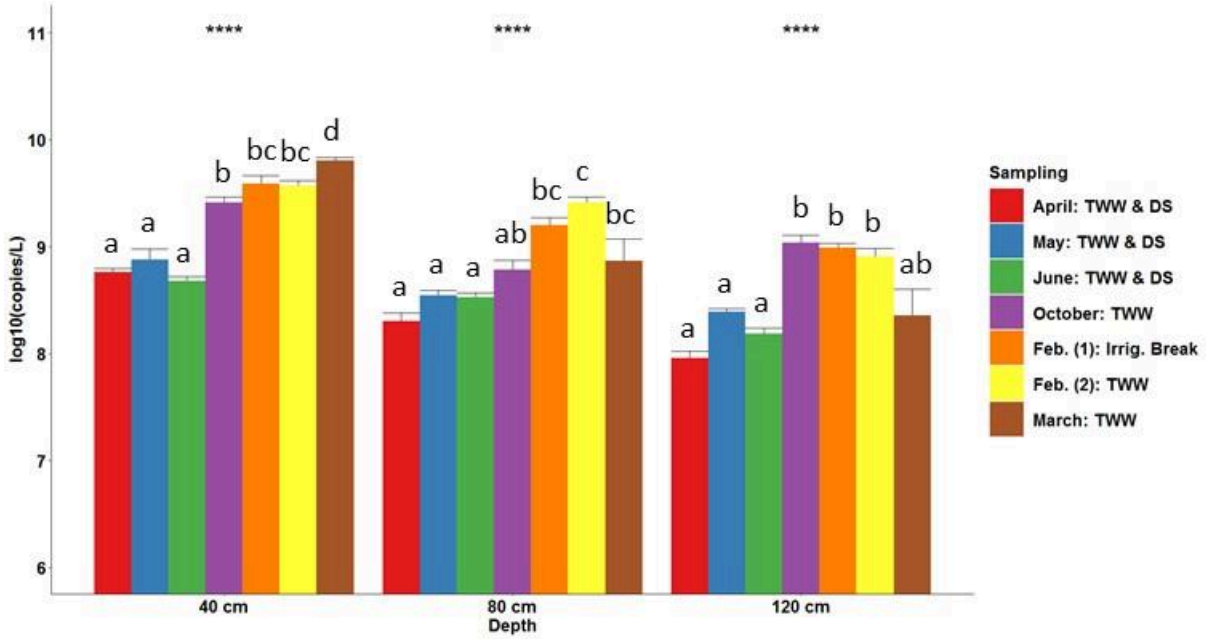


Figure 3.2: Absolute abundance ($\log_{10}$ copies/L) of 16S rRNA in percolated subsoil pore-water from the lysimeter-wells during periods of high (April, May, June), moderate (October) and low intensity irrigation (Feb. (2), March) or irrigation break (Feb. (1)). Kruskal-Wallis test: *p<0.05, p<0.01, *p< 0.001, p<0.0001, n=4. The letters "a" to "d" were assigned to non-significantly different groups after pairwise comparisons with Dunn's test along with Benjamini-Hochberg correction and cutoff p<0.05. TWW: Treated Waste Water, DS: Digested Sludge, Irrig.: Irrigation.

3.3.1.2. The relative abundance of *sul1*, *intI1*, *qnrS* and *bla*$_{\text{OXA-58}}$ correlated with TWW irrigation intensity

All investigated ARGs as well as the *intI1* gene were detected in the irrigation TWW (Fig. 3.1). The genes with highest abundance in the irrigation water were *sul1* and *intI1*. Their mean relative abundance was −1.6 and −1.8 $\log_{10}$ copies/16S rRNA, respectively. The mean relative abundance of the remaining ARGs varied from −2.9 to −5.5 $\log_{10}$ copies/16S rRNA. The rank abundance in the irrigation water was determined as *intI1* > *sul1* > *bla*$_{\text{OXA-58}}$ > *tet*(M) > *qnrS* > *bla*$_{\text{TEM}}$ > *bla*$_{\text{CTX-M-32}}$ (Fig. 3.1). In addition, all investigated genes were also present in subsoil

pore-water, with *sul1* and *intI1* again being the most abundant (*intI1:* −2.7 ± 1.0 log 10 copies/16S rRNA, *sul1*: −2.9 ± 0.5 $\log_{10}$ copies/16S rRNA, Fig. 3.3A). To determine if irrigation intensity affects the ARG profile of the subsoil pore-water, we grouped the different samples based on the intensity of irrigation during the sampling period (high, moderate, low, irrigation break). ARG profiles of each group of irrigation intensity were significantly different (pairwise PERMANOVA tests, p = 0.001 with $R^2 < 0.25$, Euclidean Distance, Benjamini-Hochberg correction) (Table 2) based on the relative abundance of ARGs and *intI1*.

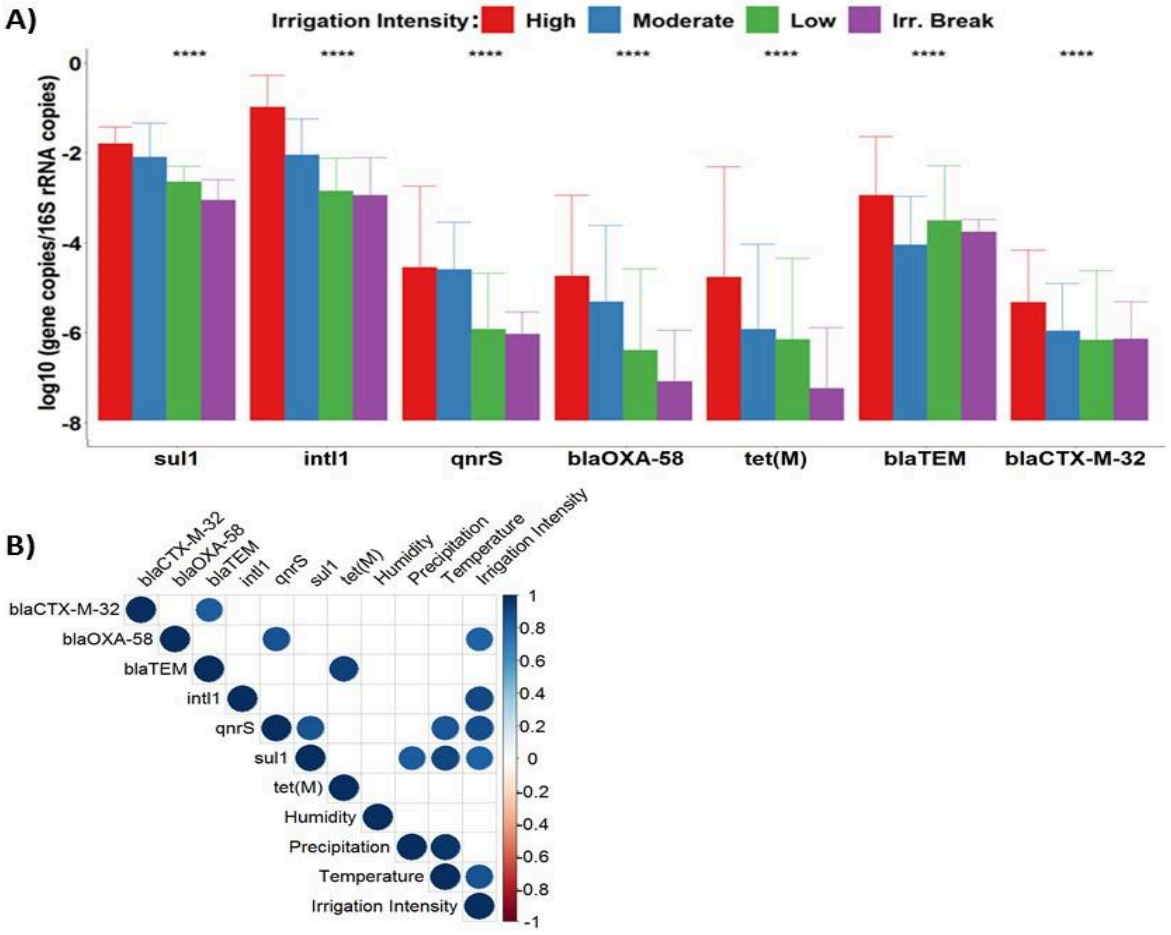


Figure 3.3: A) The relative abundances of all the tested genes from samples of all three depths assigned based on the level of irrigation intensity from Table 1. High Irrigation Intensity: April, May, June (n=36); Moderate Irrigation Intensity: October (n=12), Low Irrigation Intensity: Feb. (2), March (n=24); Sampling after a long Irrigation Break: Feb. (1) (n=12). Kruskal-Wallis test: *p<0.05, **p<0.01, ***p< 0.001, ****p<0.0001, n=4. B) Spearman correlations of the mean relative abundance of the tested genes during each sampling (Table 1, n=7), the irrigation intensity (Table 3.1) and physicochemical conditions (Precipitation, Humidity, Temperature) (Table S3.3). The level of significance cutoff was p<0.05. The dots show correlations that exceeded the significance cutoff. The blue shade represents positive while the red shade represents negative correlations.

Table 3.2: Pairwise comparisons of the lysimeters groups over periods of irrigation with PERMANOVA test (pairwise.adonis function, Euclidean distance), with Benjamini-Hochberg correction for multiple comparisons.

Pairwise Comparisons	R^2	Adjusted p-value
High Intensity Period vs Intermediate Intensity Period	0.060169	0.001
High Intensity Period vs Irrigation Break	0.214535	0.001
High Intensity Period vs Low Intensity Period	0.168915	0.001
Intermediate Intensity Period vs Irrigation Break	0.214919	0.001
Intermediate Intensity Period vs Low Intensity Period	0.075231	0.001
Irrigation Break vs Low Intensity Period	0.033387	0.001

To gain insights into which genes most significantly influenced the respective differences in ARG and *intI1* profiles, we compared the relative abundance of each gene, in relation to irrigation intensity (Fig. 3.3A). In general the mean relative abundance of all ARGs and *intI1* was 0.5 to two orders of magnitude higher during high intensity irrigation periods, when compared to irrigation breaks (Kruskal Wallis test, $p < 0.0001$, n = 12–36, Fig. 3.3A). For example, the relative abundance of *sul1* was -2.5 ± 0.3 $\log_{10}$ copies/16S rRNA during high irrigation periods (n = 36) while it decreased to -3.6 ± 0.4 $\log_{10}$ copies/16S rRNA during the irrigation break. Similar trends were observed for the *intI1*, *qrnS*, $bla_{\text{OXA-58}}$ and *tet*(M) genes (Fig. 3.3A). To ensure that the higher relative abundance of these genes was due to the change in irrigation intensity, we performed correlation analysis of the mean relative abundances of ARGs and *intI1* (Table 3.1, n = 7), with physicochemical conditions (precipitation, temperature and humidity, Table S3.3) as well as irrigation intensity. The relative abundance of *sul1, intI1, qnrS* and $bla_{\text{OXA-58}}$ correlated positively and significantly with irrigation intensity (R = 0.8–0.9, $p < 0.05$, Fig. 3.3B, n = 7). Temperature correlated strongly with *sul1* and *qnrS* relative abundance (R = 0.8–0.9, $p < 0.05$, Fig. 3.3B, n = 7). However, it correlated with irrigation intensity as well (R = 0.85. p = 0.013, Fig. 3.3B), probably because during the summer irrigation is generally higher than in winter. Precipitation correlated exclusively with temperature (R = 0.96, p = 0.00013, n = 7) and *sul1* relative abundance (R = 0.9, p = 0.005, Fig. 3.3B, n = 7). Humidity did not correlate significantly with any of the tested genes. Despite their correlation with irrigation intensity, high variation of $bla_{\text{OXA-58}}$ and *qnrS* gene abundance was detected during high intensity irrigation periods (Fig. 3.3A).This, in addition with their low abundance during irrigation breaks, indicates that their dissemination and persistence is rather limited, in comparison with *sul1* and *intI1*.

While the lysimeter-wells provided indications that TWW irrigation introduced and affected the prevalence of several ARGs in the subsoil pore-water, there was a lack of a representative field with freshwater irrigation, different crop-rotation and varying the environmental conditions for comparative studies. Such disadvantages and variation are common features among field studies targeted on real-scale commercial operations, thus insights gained from field studies should to be tested further under controlled conditions.

3.3.2. Microcosm experiments

3.3.2.1. Absolute bacterial abundance in subsoil pore-water is independent of the irrigation water and its bacterial load

By comparing the impact of TWW or freshwater irrigation, the microcosm experiments aimed at verifying the effects of TWW irrigation on ARG abundance subsoil pore-water, obtained from the field study under controlled conditions. The absolute bacterial abundance significantly differed between the two types of irrigation water (9.7 ± 0.2 $\log_{10}$ 16S rRNA gene copies/L TWW; 7.2 ± 1.0 $\log_{10}$ copies/L Freshwater; n = 6; Wilcoxon rank sum test, $p = 3.6 \times 10^{-5}$, n = 6) (Fig. S3.10A, Fig. 3.4A). The initial 16S rRNA gene abundance in the pore water of the microcosms after 3 weeks of equilibration with freshwater irrigation was 10.6 ± 0.2 $\log_{10}$ copies/L for the Freshwater-Group and 10.4 ± 0.2 $\log_{10}$ copies/L for the TWW-Group in Week 0, approximately three orders of magnitude higher than in the freshwater irrigation feed (Fig. 3.4A). No significant difference between the two groups in absolute 16S abundance was observed after irrigation switched to TWW in half of the microcosms. In both groups 16S abundance decreased to 9.8 ± 0.2 and 9.7 ± 0.3 $\log_{10}$ copies/L in Week 1 (Dunn's test, $p < 0.05$, n = 4), yet no significant difference between the two groups of microcosms was detected (Dunn's test, $p > 0.05$, n = 4). However, the Freshwater-Group displayed slightly higher 16S rRNA gene abundance than the TWW-Group in Week 3 (Freshwater: 10 ± 0.1 $\log_{10}$ copies/L; TWW: 9.7 ± 0.2 copies/L, Dunn's test, $p < 0.05$, n = 4). Therefore, continuous irrigation with higher bacterial loads through TWW application did not increase the absolute abundance of 16S rRNA gene in the pore water of the TWW-Group. On the contrary, 16S rRNA gene abundance showed peculiar and rather stochastic dynamics, since there was an almost ten-fold decrease from Week 0 to Week 1 (one-week difference) for both types of irrigation (Fig. 3.4A, Dunn's test, $p < 0.05$, n = 4).

3.3.2.2. TWW irrigation increases the relative abundance of *sul1*, *intI1*, *qnrS*, *tet*(M) and *bla*$_{OXA-58}$ in subsoil pore-water of microcosms

Upon initiation of the microcosm experiments, the ARG-profile of the soil was analysed. While, *sul1, tet*(M)*, qnrS* and *bla*$_{OXA-58}$ were below the LOQ, *bla*$_{TEM}$, *bla*$_{CTX-M-32}$ and *intI1* were present in the soil (Fig. S3.11). In addition, the ARG-profile of both selected irrigation water sources (freshwater and TWW) was analysed. All analysed genes were present in all TWW and above LOQ/LOD in approximately half of the freshwater samples. They constantly exhibited higher absolute abundances in TWW than in freshwater (Wilcoxon rank sum test, $p < 0.0001$, $n = 6$, Fig. S3.12A). The difference in the mean relative abundance of ARGs and *intI1* was at least one order of magnitude higher in TWW as well (Wilcoxon rank sum test, $p < 0.0001$, $n = 6$), with the exception of *bla*$_{CTX-M-32}$ and *bla*$_{TEM}$, where no significant difference between the two irrigation types was observed (Wilcoxon rank sum test, $p > 0.05$, $n = 6$, Fig. S3.12B). For example, the relative abundance of *sul1* differed significantly from −2.5 ± 0.2 $\log_{10}$ copies/16S rRNA in TWW to −6.8 ± 1.9 in freshwater, while no significant changes in the relative abundance of *bla*$_{CTX-M-32}$ at −5.2 ± 0.9 (TWW) & −4.5 ± 2.2 (freshwater) $\log_{10}$ copies/16S rRNA was detected.

After both groups of microcosms were initially equilibrated through freshwater irrigation, the profile of ARGs and *intI1* was identical in Week 0 (Fig. 3.4B, Table 3.3, PERMANOVA, $p > 0.05$, $n = 4$). However, after the switching half the microcosms to TWW irrigation, the ARG-profile of the two groups differed significantly in Week 1 (PERMANOVA, $p = 0.005$, $R^2 = 0.42$) and Week 3 (PERMANOVA, $p = 0.005$, $R^2 = 0.66$) (Fig. 3.4B, Table 3.3). For example, the relative abundance of *intI1* of the TWW-Group in Week 0 was −5.6 ± 0.3 $\log_{10}$ copies/16S rRNA and in Week 1 significantly increased to −5.2 ± 0.2 $\log_{10}$ copies/16S rRNA (Kruskal-Wallis test, $p = 0.003$, $n = 4$) after which it remained stable in Week 3 at −5.1 ± 0.2 $\log_{10}$ copies/16S rRNA. In the Freshwater-Group, the relative abundance of *intI1* decreased from −5.7 ± 0.3 $\log_{10}$ copies/16S rRNA in Week 0 to below LOQ after Week 3 (Kruskal-Wallis test, $p = 0.0003$, $n = 4$). Thus, continuous freshwater irrigation led to a significant reduction up to elimination of *intI1* from the subsoil pore-water. Furthermore, *sul1* was not detected in Week 0 in either group or at any time during freshwater irrigation. However, *sul1* was detected in two of the four microcosms of the TWW-Group in Week 1 and finally in all microcosms of the TWW-Group in Week 3 (−4.2 ± 0.3 $\log_{10}$ copies/16S rRNA), resulting in a significant difference of relative abundance compared to the Freshwater-Group ($p = 0.00041$, Wilcoxon

rank sum test, n = 4). Similarly, *tet*(M) was mainly detected in the TWW-Group following similar trends as *intI1* and *sul1* during the weeks of irrigation.

The gene $bla_{\text{CTX-M-32}}$ was not detected, in either group in all weeks of irrigation. In addition, bla_{TEM} was below LOQ for the majority of samples in Week 0, but its relative abundance increased to 6.5 $\log_{10}$ copies/16S rRNA in both groups of irrigation in Week 3. Therefore, TWW irrigation did not influence the dissemination of bla_{TEM} in the subsoil pore-water relative to freshwater irrigation. The genes *qnrS* and $bla_{\text{OXA-58}}$ were also below LOQ in Week 0 (both groups), but were detected in Week 1 exclusively in the TWW-Group. However, their relative abundance remained stable once detected from Week 1 to Week 3. For example, relative abundance of *qnrS* was present at -6.6 ± 0.7 $\log_{10}$ copies/16S rRNA copies/16S rRNA at Week 1 and -6.1 ± 1 $\log_{10}$ copies/16S rRNA at Week 3 ($p = 0.42$, Wilcoxon rank sum test, n = 4, Fig. 3.4B). In contrast, *qnrS* and $bla_{\text{OXA-58}}$ remained below LOQ for the majority of samples from the Freshwater-Group throughout the experiment. Therefore, TWW irrigation showed a strong influence on the dissemination of *sul1* and *intI1* and a moderate influence for *qnrS* and $bla_{\text{OXA-58}}$, which was in line with the results from the lysimeter-wells. The more controlled and consequently more sensitive microcosm experiments were able to further document effects of TWW irrigation for *tet*(M), when compared with freshwater irrigation.

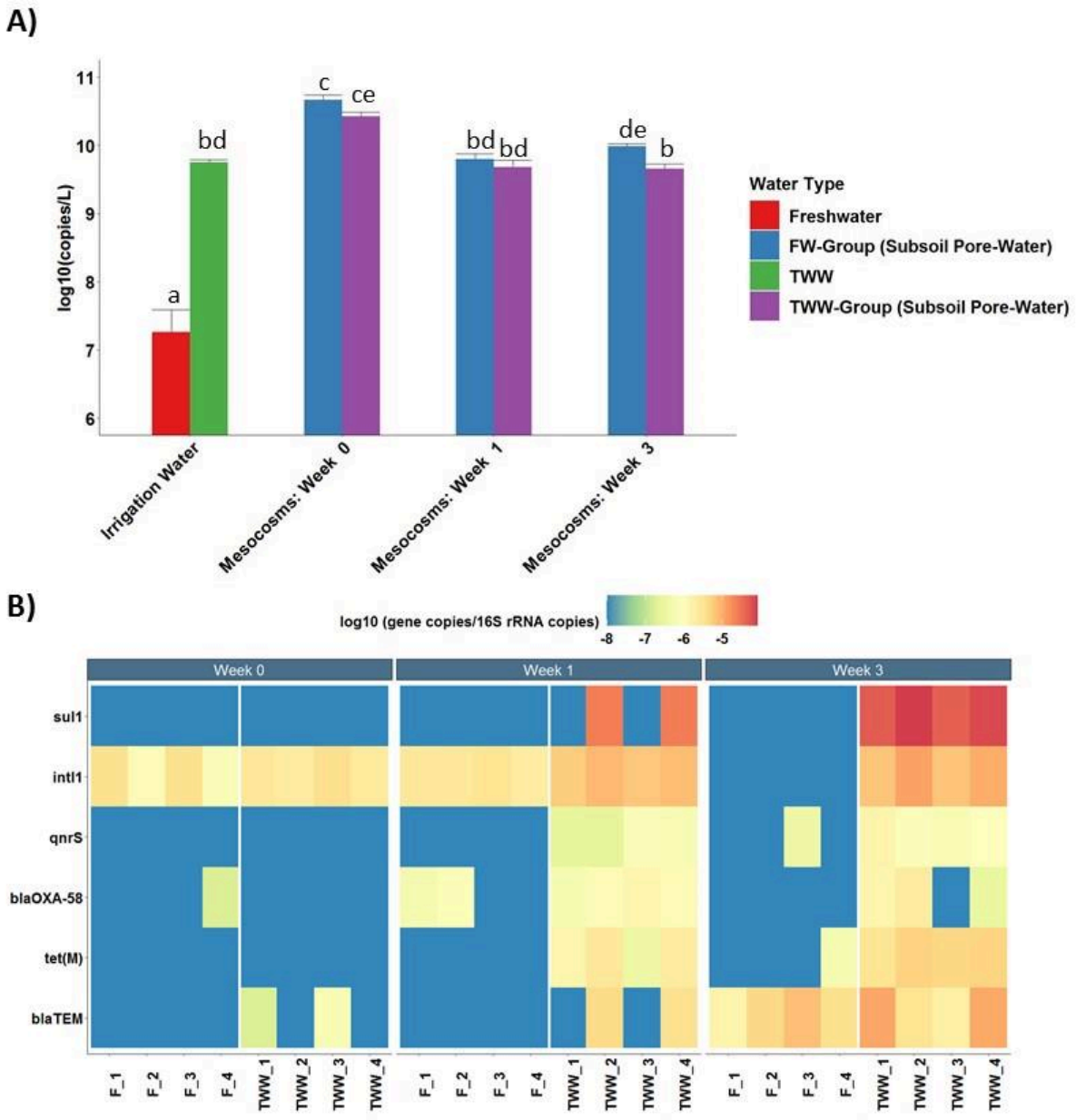


Figure 3.4: A) Absolute abundance (log_{10} gene copies/L) of 16S rRNA in the percolated subsoil pore-water of the mesocosms and the respective irrigation waters. Irrigation water: n=6; Mesocosms subsoil pore water: n=4. The letters "a" to "d" were assigned to non-significantly different groups after pairwise comparisons with Dunn's test along with Benjamini-Hochberg correction and cutoff $p<0.05$. TWW: Treated Wastewater, FW: Freshwater. B) Relative abundance of ARGs and *intI1* (log_{10} gene copies/16S rRNA) in the subsoil pore-water of mesocosms (F: Freshwater irrigated, TWW: Treated Waste Water irrigated). The gene $bla_{CTX\text{-}M\text{-}32}$ was not shown as it was below LOQ in all samples. Pairwise comparisons with PERMANOVA test, along with Benjamini-Hochberg correction, can be found in Table 3.3).

Table 3.3: Pairwise comparisons of the ARG and *intI1* profiles in the mesocosm percolated w ater with PERMANOVA test along with Benjamini-Hochberg correction for multiple compar isons (FW: Freshwater irrigated, TWW: Treated Wastewater irrigated, W: Week).

Pairwise Comparisons	R^2	Adjusted p-value
FW-W0 vs TWW-W0	0.106597	0.285
FW-W0 vs FW-W1	0.053469	0.285
FW-W0 vs TWW-W1	0.447706	0.005
FW-W0 vs FW-W3	0.715021	0.005
FW-W0 vs TWW-W3	0.689762	0.005
TWW-W0 vs FW-W1	0.133986	0.285
TWW-W0 vs TWW-W1	0.425895	0.005
TWW-W0 vs FW-W3	0.649432	0.005
TWW-W0 vs TWW-W3	0.66361	0.005
FW-W1 vs TWW-W1	0.416059	0.005
FW-W1 vs FW-W3	0.688689	0.005
FW-W1 vs TWW-W3	0.666517	0.005
TWW-W1 vs FW-W3	0.522884	0.005
TWW-W1 vs TWW-W3	0.186126	0.132
FW-W3 vs TWW-W3	0.66171	0.005

3.3.2.3. The majority of TWW-related bacterial genera do not persist in subsoil pore-water

We analysed the subsoil pore-water TWW-Group prior and after the switch to TWW irrigation, along with the respective TWW irrigation water, to confirm that TWW irrigation related bacteria do not persist during TWW irrigation, as was indicated by the qPCR results (Fig. 3.4A). The profile of relative abundance of the 40 most abundant genera was completely different, with *Pseudomonas* being the genus that was ubiquitously present in all soil pore water samples (Fig. 3.4A). Nevertheless, *Pseudomonas* showed low relative abundance in the TWW (1.6% reads) and high relative abundance in comparison with subsoil pore-water (22.8–15.4% reads) (Fig. 3.5). Despite the presence of *Pseudomonas* in the TWW irrigation, no increase was detected after one or three weeks of irrigation in *Pseudomonas* relative abundance. Furthermore, genera more highly abundant in the TWW such as *Aeromonas*, *Bacteroides* and *Flavobacterium* (Fig. 3.5) did not increase in the subsoil pore water samples due to TWW irrigation.

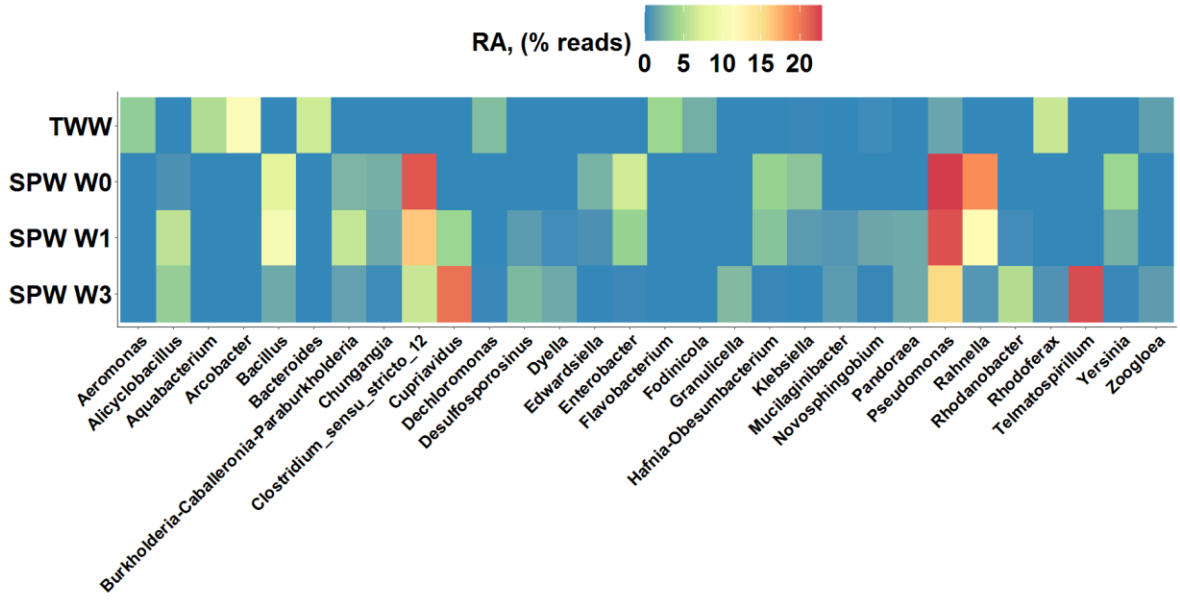


Figure 3.5: The relative abundance (% reads) of the 40 most abundant genera in the TWW irrigation and subsoil pore-water (SPW) of the TWW group, after the equilibration for two weeks with freshwater and prior to switch to TWW irrigation (W0: Week 0), one Week after the switch to TWW (W1: Week 1) and after 3 Weeks of Irrigation (W3: Week 3). Replicates were pooled for each sample.

3.4 Discussion

In this field study, the prevalence of ARGs and *intI1* in the subsoil pore-water of a real-scale agricultural field, subjected to TWW irrigation for commercial farming operation, was positively correlated with TWW irrigation intensity for the genes *sul1*, *intI1, qnrS* and *bla*$_{\text{OXA-58}}$. This trend and hence a causal link was later confirmed in controlled lab-scale microcosm experiments, overcoming the lack of representative controls (freshwater irrigated fields with lysimeter-wells) and varying environmental conditions (temperature, precipitation). Therefore, our working hypothesis that TWW irrigation promotes the spread of ARGs and *intI1* into subsoil pore-water was confirmed. To the best of our knowledge, this is the first study that investigated the impact of TWW irrigation on the profile of ARGs in this so far underappreciated matrix through the combination of real-scale TWW-irrigated field investigations and microcosm experiments.

While the TWW irrigation intensity was positively correlated with the gene abundances of *sul1*, *intI1, qnrS* and *bla*$_{\text{OXA-58}}$, we found that a few genes also correlated with temperature (*sul1* and *qnrS*) and precipitation (*sul1*). However, irrigation intensity correlated with temperature, which is to be expected since agricultural operations are more intensive during the hottest months, and precipitation correlates with temperature in this region of Germany. Using the microcosms, we demonstrated that TWW irrigation indeed induced the dissemination of *sul1*, *intI1, qnrS* and *bla*$_{\text{OXA-58}}$, without the influence of other confounding variation of physicochemical parameters.

The impact of TWW irrigation on the relative abundance of ARGs and *intI1* displayed consistent but not completely identical patterns at the three different sampled depths of our real scale field study. All depths were equally affected during high intensity irrigation, with similarly increased relative abundance of *sul1* and *intI1* at all three depths during high intensity irrigation periods. Therefore the effect of TWW irrigation is not only restricted to the subsoil pore-water of upper, but also carried through to the deeper soil layers. The main exception we observed was *intI1* only increasing in the two upper soil layers immediately upon the restart of low intensity irrigation after a prolonged irrigation break. This indicates a temporal delay in exposure to and hence effect of TWW irrigation in the deeper soil layers.

The fact that not all ARGs and *intI1* followed parallel trends of in- and decrease correlated with irrigation intensity, leads to the assumption that the abundance of ARGs in soil pore-water is not exclusively due the leaching of irrigation water, a process described previously as a main source of the microbial load in lysimeter samples (Forslund et al., 2011). Assuming such a leaching scenario would rather lead to a uniform, parallel increase of all ARGs, based on their relative abundance in irrigation water as a function of irrigation intensity. Hence, a complex combination of interactions of soil, TWW and their respective microbiomes needs to be taken into account when evaluating the effect of TWW irrigation on ARG abundance in soil pore-water. These include microbial ecological and evolutionary processes such as horizontal gene transfer of resistance genes, competition and selection dynamics, but also physico-chemical processes like transport and leaching.

A strong indicator for horizontal gene transfer between TWW and soil microbes playing a major role in the observed increase in resistance genes in the subsoil pore-water is that among the genes tested, *sul1* and *intI1* showed the highest relative abundance and strongest correlation with irrigation intensity. The integrase gene *intI1,* commonly used as an indicator for anthropogenic pollution (Gillings et al., 2015), is generally associated with MGE and usually located in close genetic proximity to ARGs or other genes connected to adaptive stress responses in plasmids or transposons (Gillings, 2017). For example, *sul1* has been frequently found as part of *intI1* gene cassettes that are located on mobile genetic elements and conjugative plasmids (Gillings et al., 2015; Gillings, 2017), which have the ability to transfer to a the majority of the diverse bacterial phyla found in soil (Klümper et al. 2015). Consequently, their parallel increase in abundance is most likely connected to them being co-located on MGEs that get co-transferred and co-selected. Unsurprisingly, these mobile genes were also highly abundant in TWW effluents from several geographical regions (Cacace et al., 2019, Pärnänen et al., 2019).

While these genes were also present in the freshwater used for irrigation of microcosms, their abundance was orders of magnitude lower, compared to TWW. However, taking into account the high horizontal mobility of *sul1* and *intI1* (Gillings et al., 2015; Gillings, 2017) we presume that long-term freshwater irrigation could in theory lead to an enrichment of *sul1* in the soil and hence the subsoil pore-water. In a previous study *sul1* was one of the ubiquitously detected ARGs in six groundwater wells (Szekeres et al. 2018), suggesting that its dissemination in the subsoil/groundwater microbiome is indeed rather successful. However, *intI1*, which was

initially detected in the soil after the two weeks of equilibration with freshwater (Week 0), was subsequently eliminated from microcosms in the Freshwater-Group after five weeks of total freshwater irrigation (Week 3), indicating that in addition to its gene transfer potential, positive selection might be needed for enrichment in the subsoil pore-water.

The presence of selective agents may cause such positive selection for ARGs and/or promote horizontal gene transfer of MGEs by inducing the bacterial stress response. These agents can for example include pharmaceutical residues (including antibiotic and non-antibiotic residues) (Lin and Gan, 2011, Dalkmann et al., 2012, Manaia et al., 2018b) or heavy metal ions (Klümper et al., 2017, Zhang et al., 2018, Zhao et al., 2019). The gene *sul1* confers resistance to sulphonamides, a class of antibiotics of synthetic origin, which have been reported to occur in high concentration in TWW (Nikolaou et al., 2007, Barnes et al., 2008, Avisar et al., 2009, Michael et al., 2013) and they persist in sub-surface environments (Barnes et al., 2008, Avisar et al., 2009). For example, sulphamethoxazole was detected in groundwater from different areas in the USA (Barnes et al., 2008) and in phreatic aquifer samples in Israel (Avisar et al., 2009). In addition, the reported maximum values of sulphamethoxazole concentrations reported for these groundwater samples was 1.1 µg/L (Barnes et al., 2008) thus exceeding the predicted no effect concentration for the selection of sulphamethoxazole resistance for environmental strains (0.5 µg/L), based on predictive models (Bengtsson-Palme & Larsson, 2016). Lüneberg et al. (2018) showed that during limited and simulated TWW irrigation events, the prevalence of *sul1* indeed increased in the water flow paths towards the aquifer of their tested soil/subsoil microcosms as a function of sulphamethoxazole concentrations.

Further, non-antibiotic pharmaceuticals of TWW origin, such as carbamazepine, can induce the mobilisation of ARGs and MGE (Wang et al., 2019). Carbamazepine is one of the most common non-antibiotic drugs, regularly found in TWW (Clara et al., 2004, Nikolaou et al., 2007, Gasser et al., 2011), while it has shown high persistence in groundwater as well (Clara et al., 2004, Gasser et al., 2011, Sui et al., 2015). The preferential increase of specific mobile ARGs and *intI1* observed in our study could probably be explained by the fact that these agents may be released by TWW irrigation and are able to persist in the subsurface environments as well. Ternes et al. (2007) reported the presence of pharmaceutical residues including sulphamethoxazole (maximum value: 0.12 µg/L) and carbamazepine (maximum value: 1.3 µg/L) in the exact lysimeter-wells of the same TWW irrigated field we studied. Based on our findings and previous results we presume that a causal link might exist between the

dissemination/invasion success of *sul1,* the introduced dose of *sul1* and the combination of selective agents, which are originally co-introduced into the subsoil/groundwater matrices through TWW irrigation. Therefore, further controlled exploration of *sul1* dissemination in subsoil/groundwater, in combination with the presence and persistence of selective agents in these matrices, is necessary to elucidate the potential mechanisms behind this link.

The subsoil pore-water in the field and microcosms showed high 16S rRNA gene absolute abundance, but was not influenced by the type or intensity of irrigation, indicating that physical processes such as direct leaching of the irrigation water into the subsoil pore-water plays only a minor role. In the field, we observed a seasonal rather than irrigation intensity based trend of 16S rRNA gene absolute abundance; the highest absolute abundance occurred during the irrigation break and low intensity irrigation. In addition, the bacterial load did not increase after the restart of irrigation in February. In the microcosms, the subsoil pore-water exhibited 3–4 orders of magnitude higher 16S rRNA gene absolute abundance than the freshwater, and still 0.5–1 orders of magnitude higher abundance than the irrigation TWW. Despite the fact that the freshwater had 2–3 orders of magnitude lower 16S rRNA gene abundance compared to TWW, no increase of 16S rRNA gene abundance in the subsoil pore-water was observed after switching to TWW irrigation.

To further examine the potential persistence and addition of TWW related bacteria to subsoil pore-water during TWW irrigation, we performed 16S rRNA sequencing on our controlled laboratory microcosms of the TWW-Group. In general, TWW related bacteria such as *Aeromonas* or *Bacteroides* did not increase after the switch to TWW irrigation. *Pseumodomonas* was ubiquitously present in both, TWW and subsoil pore-water of the TWW-Group, but showed the highest absolute abundance in the subsoil pore-water samples, with its abundance not increasing after the switch to TWW irrigation. Therefore, the higher bacterial load and diversity added through TWW irrigation did not affect the bacterial composition of the subsoil pore-water. A significant proportion of bacteria that inhabit the soil can mobilise and occupy the water matrix as well, during the percolation of water (Dibbern et al., 2014, Herrmann et al., 2019). For example *Pseudomonas* members are known to be highly abundant in soil (Silby et al., 2011), thus their higher abundance in subsoil pore-water is justified by their abundance and survival potential in the soil matrices. These indigenous soil/subsoil bacteria are well adapted to their specific niches (Marano et al., 2019, Cerqueira et al., 2019a, Cerqueira et al., 2019b, Cerqueira et al., 2019c, Bahram et al., 2018, Jansson and Hofmockel, 2018) and

thus most likely outcompete the majority of invading TWW bacteria. Hence, we demonstrated that soil bacteria are expected to make up the majority of the bacterial load in the subsoil pore-water under TWW irrigation scenarios. Nevertheless, the ubiquitous presence of *Pseudomonas* sp. lead us to hypothesize that members of this genus could be key players in horizontal gene transfer of ARGs from TWW microbiota to subsoil pore-water microbiota.

In the present study, we examined the impact of TWW irrigation on the prevalence of ARGs in subsoil pore-water, however, TWW irrigation is not the only source of antibiotics, ARB and ARGs in most agricultural fields. Specifically, biosolids (Mathews & Reinhold, 2013) and manure amendment (Luby et al., 2016; Yang et al., 2016; Barrios et al., 2020) introduce a high loads of antibiotics and ARGs (Chen et al., 2016; Jechalke et al., 2016, Zhou et al., 2017, McKinney et al., 2018; Wolters et al., 2018) and therefore might mask the impact of wastewater irrigation in this specific matrix in many cases (Cerqueira et al., 2019c). Further, only relative effects on ARG abundance can be measured as ARGs naturally occur at low levels in many environments of low anthropogenic or even pristine nature (D'Costa et al., 2011, Martínez, 2012, Gatica et al., 2016).

In fact, our diverse set of ARGs was detected in the tested freshwater, sampled from a groundwater well near an agricultural suburban area of Dresden, indicating how widespread the resistant determinants are in the environment. However, their mean relative abundance was orders of magnitude lower compared to those detected in TWW. This does not exclude the possibility that long-term irrigation with freshwater might promote their dissemination in subsoil pore-water in the long term, but it would, if at all, happen at a far slower rate than with TWW irrigation. In addition, the TWW that was used for irrigation, both in the field (BWA) as well as in the microcosms (TWW Kaditz) was only subjected to secondary treatment. Tertiary and advanced wastewater treatment can lead to a significant reduction of ARG load in the effluents (Cacace et al., 2019). Irrigation with TWW of higher quality might minimise the differences between the ARG profiles of TWW and freshwater (Michael et al., 2013, Manaia et al., 2018a, Manaia et al., 2018b) and consequently the impact of TWW irrigation observed in our study.

3.5 Conclusion

Overall, TWW irrigation increased the relative abundance of specific genes associated with antimicrobial resistance, mainly *sul1* and *intI1*. Combining microcosm approaches with long-

term studies on a regularly real-scale, commercially operated TWW irrigated field, proved a successful research tool to study irrigation effects in this so-far understudied environment. The ecology and drivers of ARGs in subsoil pore-water (and potentially other subsurface matrices) remains a major knowledge gap. Thus, we consider that further research into the mechanisms of the observed ARG dissemination in subsoil pore-water, groundwater and other subsurface matrices is necessary. Our subsoil pore-water microcosms have proven to be a successful research tool to test the influence of diverse factors on ARG dynamics such as soil type (clay, loamy, sandy), varying environmental conditions (temperature, precipitation), crops-rotation or other disturbances (e.g. soil amendment) more comprehensively in the future. This will allow generating mitigation strategies to minimize the risk associated with ARG dissemination in the subsoil pore-water and potentially in the groundwater during TWW irrigation scenarios

3.6. References

1) Alygizakis, N.A., Urík, J., Beretsou, V.G., Kampouris, I., Galani, A., Oswaldova, M., Berendonk, T., Oswald, P., Thomaidis, N.S., Slobodnik, J., Vrana, B., Fatta-Kassinos, D., 2020. Evaluation of chemical and biological contaminants of emerging concern in treated wastewater intended for agricultural reuse. Environ. Int. 138, 105597. https://doi.org/10.1016/j.envint.2020.105597
2) Anantharaman, K., Brown, C.T., Hug, L.A., Sharon, I., Castelle, C.J., Probst, A.J., Thomas, B.C., Singh, A., Wilkins, M.J., Karaoz, U., Brodie, E.L., Williams, K.H., Hubbard, S.S., Banfield, J.F., 2016. Thousands of microbial genomes shed light on interconnected biogeochemical processes in an aquifer system. Nat. Commun. 7, 1–11. https://doi.org/10.1038/ncomms13219
3) Avisar, D., Lester, Y., Ronen, D., 2009. Sulfamethoxazole contamination of a deep phreatic aquifer. Sci. Total Environ. 407, 4278–4282. https://doi.org/10.1016/j.scitotenv.2009.03.032
4) Bahram, M., Hildebrand, F., Forslund, S.K., Anderson, J.L., Soudzilovskaia, N.A., Bodegom, P.M., Bengtsson-Palme, J., Anslan, S., Coelho, L.P., Harend, H., Huerta-Cepas, J., Medema, M.H., Maltz, M.R., Mundra, S., Olsson, P.A., Pent, M., Põlme, S., Sunagawa, S., Ryberg, M., Tedersoo, L., Bork, P., 2018. Structure and function of the global topsoil microbiome. Nature 560, 233–237. https://doi.org/10.1038/s41586-018-0386-6
5) Barnes, K.K., Kolpin, D.W., Furlong, E.T., Zaugg, S.D., Meyer, M.T., Barber, L.B., 2008. A national reconnaissance of pharmaceuticals and other organic wastewater contaminants in the United States - I) Groundwater. Sci. Total Environ. 402, 192–200. https://doi.org/10.1016/j.scitotenv.2008.04.028
6) Barrios, R.E., Khuntia, H.K., Bartelt-Hunt, S.L., Gilley, J.E., Schmidt, A.M., Snow, D.D., Li, X., 2020. Fate and transport of antibiotics and antibiotic resistance genes in runoff and soil as affected by the timing of swine manure slurry application. Sci. Total Environ. 712. https://doi.org/10.1016/j.scitotenv.2020.136505
7) Bengtsson-Palme, J., Larsson, D.G.J., 2016. Concentrations of antibiotics predicted to select for resistant bacteria: Proposed limits for environmental regulation. Environ. Int. 86, 140–149. https://doi.org/10.1016/j.envint.2015.10.015
8) 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., Martinez, J.L., 2015. Tackling antibiotic resistance: the environmental framework. Nat. Rev. Microbiol. 13, 310–317. https://doi.org/10.1038/nrmicro3439
9) Böckelmann, U., Dörries, H.H., Ayuso-Gabella, M.N., De Marçay, M.S., Tandoi, V., Levantesi, C., Masciopinto, C., Houtte, E. Van, Szewzyk, U., Wintgens, T., Grohmann, E., 2009. Quantitative PCR monitoring of antibiotic resistance genes and bacterial pathogens in three european artificial groundwater recharge systems. Appl. Environ. Microbiol. 75, 154–163. https://doi.org/10.1128/AEM.01649-08
10) Cacace, D., Fatta-Kassinos, D., Manaia, C.M., Cytryn, E., Kreuzinger, N., Rizzo, L., Karaolia, P., Schwartz, T., Alexander, J., Merlin, C., Garelick, H., Schmitt, H., de Vries, D., Schwermer, C.U., Meric, S., Ozkal, C.B., Pons, M.N., Kneis, D., Berendonk, T.U., 2019. Antibiotic resistance genes in treated wastewater and in the receiving water bodies: A pan-European survey of urban settings. Water Res. 162, 320–330. https://doi.org/10.1016/j.watres.2019.06.039

11) Caucci, S., Karkman, A., Cacace, D., Rybicki, M., Timpel, P., Voolaid, V., Gurke, R., Virta, M., Berendonk, T.U., 2016. Seasonality of antibiotic prescriptions for outpatients and resistance genes in sewers and wastewater treatment plant outflow. FEMS Microbiol. Ecol. 92, fiw060. https://doi.org/10.1093/femsec/fiw060
12) Cerqueira, F., Matamoros, V., Bayona, J., Elsinga, G., Hornstra, L.M., Piña, B., 2019. Distribution of antibiotic resistance genes in soils and crops. A field study in legume plants (Vicia faba L.) grown under different watering regimes. Environ. Res. 170, 16–25. https://doi.org/10.1016/j.envres.2018.12.007
13) Cerqueira, F., Matamoros, V., Bayona, J., Piña, B., 2019. Antibiotic resistance genes distribution in microbiomes from the soil-plant-fruit continuum in commercial Lycopersicon esculentum fields under different agricultural practices. Sci. Total Environ. 652, 660–670. https://doi.org/10.1016/j.scitotenv.2018.10.268
14) Cerqueira, F., Matamoros, V., Bayona, J.M., Berendonk, T.U., Elsinga, G., Hornstra, L.M., Piña, B., 2019. Antibiotic resistance gene distribution in agricultural fields and crops. A soil-to-food analysis. Environ. Res. 177, 108608. https://doi.org/10.1016/j.envres.2019.108608
15) Chen, Q., An, X., Li, H., Su, J., Ma, Y., Zhu, Y.G., 2016. Long-term field application of sewage sludge increases the abundance of antibiotic resistance genes in soil. Environ. Int. 92–93, 1–10. https://doi.org/10.1016/j.envint.2016.03.026
16) Clara, M., Strenn, B., Kreuzinger, N., 2004. Carbamazepine as a possible anthropogenic marker in the aquatic environment: Investigations on the behaviour of Carbamazepine in wastewater treatment and during groundwater infiltration. Water Res. 38, 947–954. https://doi.org/10.1016/j.watres.2003.10.058
17) D' Costa, 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., Wright, G.D., 2011. Antibiotic resistance is ancient. Nature 477, 457–461. https://doi.org/10.1038/nature10388
18) Dalkmann, P., Broszat, M., Siebe, C., Willaschek, E., Sakinc, T., Huebner, J., Amelung, W., Grohmann, E., Siemens, J., 2012. Accumulation of pharmaceuticals, enterococcus, and resistance genes in soils irrigated with wastewater for zero to 100 years in central Mexico. PLoS One 7. https://doi.org/10.1371/journal.pone.0045397
19) Dibbern, D., Schmalwasser, A., Lueders, T., Totsche, K.U., 2014. Selective transport of plant root-associated bacterial populations in agricultural soils upon snowmelt. Soil Biol. Biochem. 69, 187–196. https://doi.org/10.1016/j.soilbio.2013.10.040
20) Dinno, 2016. dunn's test R package: Dunn's Test of Multiple Comparisons Using Rank Sums. R package version. 0.2.3 https://cran.r-project.org/web/packages/dunn.test/index.html
21) Elkayam, R., Aharoni, A., Vaizel-Ohayon, D., Sued, O., Katz, Y., Negev, I., Marano, R.B.M., Cytryn, E., Shtrasler, L., Lev, O., 2018. Viral and Microbial Pathogens, Indicator Microorganisms, Microbial Source Tracking Indicators, and Antibiotic Resistance Genes in a Confined Managed Effluent Recharge System. J. Environ. Eng. 144. https://doi.org/10.1061/(ASCE)EE.1943-7870.0001334
22) Forslund, A., Plauborg, F., Andersen, M.N., Markussen, B., Dalsgaard, A., 2011. Leaching of human pathogens in repacked soil lysimeters and contamination of potato tubers under subsurface drip irrigation in Denmark. Water Res. 45, 4367–4380. https://doi.org/10.1016/j.watres.2011.05.009
23) Gasser, G., Rona, M., Voloshenko, A., Shelkov, R., Lev, O., Elhanany, S., Lange, F.T., Scheurer, M., Pankratov, I., 2011. Evaluation of micropollutant tracers. II. Carbamazepine tracer for wastewater contamination from a nearby water recharge

system and from non-specific sources. Desalination 273, 398–404. https://doi.org/10.1016/j.desal.2011.01.058
24) Gatica, J., Tripathi, V., Green, S., Manaia, C.M., Berendonk, T., Cacace, D., Merlin, C., Kreuzinger, N., Schwartz, T., Fatta-Kassinos, D., Rizzo, L., Schwermer, C.U., Garelick, H., Jurkevitch, E., Cytryn, E., 2016. High Throughput Analysis of Integron Gene Cassettes in Wastewater Environments. Environ. Sci. Technol. 50, 11825–11836. https://doi.org/10.1021/acs.est.6b03188
25) Gatica, J., Yang, K., Pagaling, E., Jurkevitch, E., Yan, T., Cytryn, E., 2015. Resistance of undisturbed soil microbiomes to ceftriaxone indicates extended spectrum β-lactamase activity. Front. Microbiol. 6, 1–11. https://doi.org/10.3389/fmicb.2015.01233
26) Gillings, M.R., 2017. Class 1 integrons as invasive species. Curr. Opin. Microbiol. 38, 10–15. https://doi.org/10.1016/j.mib.2017.03.002
27) Gillings, M.R., Gaze, W.H., Pruden, A., Smalla, K., Tiedje, J.M., Zhu, Y.G., 2015. Using the class 1 integron-integrase gene as a proxy for anthropogenic pollution. ISME J. 9, 1269–1279. https://doi.org/10.1038/ismej.2014.226
28) Haack, S.K., Metge, D.W., Fogarty, L.R., Meyer, M.T., Barber, L.B., Harvey, R.W., Leblanc, D.R., Kolpin, D.W., 2012. Effects on groundwater microbial communities of an engineered 30-day in situ exposure to the antibiotic sulfamethoxazole. Environ. Sci. Technol. 46, 7478–7486. https://doi.org/10.1021/es3009776
29) Han, X.M., Hu, H.W., Shi, X.Z., Wang, J.T., Han, L.L., Chen, D., He, J.Z., 2016. Impacts of reclaimed water irrigation on soil antibiotic resistome in urban parks of Victoria, Australia. Environ. Pollut. 211, 48–57. https://doi.org/10.1016/j.envpol.2015.12.033
30) Herrmann, M., Wegner, C.E., Taubert, M., Geesink, P., Lehmann, K., Yan, L., Lehmann, R., Totsche, K.U., Küsel, K., 2019. Predominance of Cand. Patescibacteria in groundwater is caused by their preferential mobilization from soils and flourishing under oligotrophic conditions. Front. Microbiol. 10, 1–15. https://doi.org/10.3389/fmicb.2019.01407
31) Jansson, J.K., Hofmockel, K.S., 2018. The soil microbiome — from metagenomics to metaphenomics. Curr. Opin. Microbiol. 43, 162–168. https://doi.org/10.1016/j.mib.2018.01.013
32) Jechalke, S., Broszat, M., Lang, F., Siebe, C., Smalla, K., Grohmann, E., 2015. Effects of 100 years wastewater irrigation on resistance genes, class 1 integrons and IncP-1 plasmids in Mexican soil. Front. Microbiol. 6, 1–10. https://doi.org/10.3389/fmicb.2015.00163
33) Jechalke, S., Radl, V., Schloter, M., Heuer, H., Smalla, K., 2016. Do drying and rewetting cycles modulate effects of sulfadiazine spiked manure in soil? FEMS Microbiol. Ecol. 92, 1–7. https://doi.org/10.1093/femsec/fiw066
34) Kassambara, 2019. ggpubr R Package: ggplot2-Based Publication Ready Plots *R package version* 0.2.3 https://CRAN.R-project.org/package=ggpubr
35) Klümper, U., Dechesne, A., Riber, L., Brandt, K.K., Gülay, A., Sørensen, S.J., Smets, B.F., 2017. Metal stressors consistently modulate bacterial conjugal plasmid uptake potential in a phylogenetically conserved manner. ISME J. 11, 152–165. https://doi.org/10.1038/ismej.2016.98
36) Klümper, U., Riber, L., Dechesne, A., Sannazzarro, A., Hansen, L.H., Sørensen, S.J., Smets, B.F., 2015. Broad host range plasmids can invade an unexpectedly diverse fraction of a soil bacterial community. ISME J. 9, 934–945. https://doi.org/10.1038/ismej.2014.191

37) Kumar, S., Herrmann, M., Blohm, A., Hilke, I., Frosch, T., Trumbore, S.E., Küsel, K., 2018. Thiosulfate- and hydrogen-driven autotrophic denitrification by a microbial consortium enriched from groundwater of an oligotrophic limestone aquifer. FEMS Microbiol. Ecol. 94, 1–13. https://doi.org/10.1093/femsec/fiy141
38) Lin, K., Gan, J., 2011. Sorption and degradation of wastewater-associated non-steroidal anti-inflammatory drugs and antibiotics in soils. Chemosphere 83, 240–246. https://doi.org/10.1016/j.chemosphere.2010.12.083
39) Luby, E.M., Moorman, T.B., Soupir, M.L., 2016. Fate and transport of tylosin-resistant bacteria and macrolide resistance genes in artificially drained agricultural fields receiving swine manure. Sci. Total Environ. 550, 1126–1133. https://doi.org/10.1016/j.scitotenv.2016.01.132
40) Lüneberg, K., Prado, B., Broszat, M., Dalkmann, P., Díaz, D., Huebner, J., Amelung, W., López-Vidal, Y., Siemens, J., Grohmann, E., Siebe, C., 2018. Water flow paths are hotspots for the dissemination of antibiotic resistance in soil. Chemosphere 193, 1198–1206. https://doi.org/10.1016/j.chemosphere.2017.11.143
41) Maaß, O., Grundmann, P., 2016. Added-value from linking the value chains of wastewater treatment, crop production and bioenergy production: A case study on reusing wastewater and sludge in crop production in Braunschweig (Germany). Resour. Conserv. Recycl. 107, 195–211. https://doi.org/10.1016/j.resconrec.2016.01.002
42) Manaia, C.M., Lira, F., Martinez, J.L., Henriques, I., Narciso-da-Rocha, C., Vaz-Moreira, I., Rocha, J., Tamames, J., 2018a. Bacterial lineages putatively associated with the dissemination of antibiotic resistance genes in a full-scale urban wastewater treatment plant. Environ. Int. 118, 179–188. https://doi.org/10.1016/j.envint.2018.05.040
43) Manaia, C.M., Rocha, J., Scaccia, N., Marano, R., Radu, E., Biancullo, F., Cerqueira, F., Fortunato, G., Iakovides, I.C., Zammit, I., Kampouris, I., Vaz-Moreira, I., Nunes, O.C., 2018b. Antibiotic resistance in wastewater treatment plants: Tackling the black box. Environ. Int. 115, 312–324. https://doi.org/10.1016/j.envint.2018.03.044
44) Marano, R.B.M., Zolti, A., Jurkevitch, E., Cytryn, E., 2019. Antibiotic resistance and class 1 integron gene dynamics along effluent, reclaimed wastewater irrigated soil, crop continua: elucidating potential risks and ecological constraints. Water Res. 164, 114906. https://doi.org/10.1016/j.watres.2019.114906
45) Martinez Arbizu et al., 2019. pairwiseAdonis: Pairwise multilevel comparison using adonis. R package version 0.3. https://github.com/pmartinezarbizu/pairwiseAdonis
46) Martínez, J.L., 2012. Natural antibiotic resistance and contamination by antibiotic resistance determinants: The two ages in the evolution of resistance to antimicrobials. Front. Microbiol. 3, 2010–2012. https://doi.org/10.3389/fmicb.2012.00001
47) Mathews, S., Reinhold, D., 2013. Biosolid-borne tetracyclines and sulfonamides in plants. Environ. Sci. Pollut. Res. Int. 20, 4327–4338. https://doi.org/10.1007/s11356-013-1693-y
48) McKinney, C.W., Dungan, R.S., Moore, A., Leytem, A.B., 2018. Occurrence and abundance of antibiotic resistance genes in agricultural soil receiving dairy manure. FEMS Microbiol. Ecol. 94, 1–10. https://doi.org/10.1093/femsec/fiy010
49) Michael, I., Frontistis, Z., Fatta-Kassinos, D., 2013. Removal of pharmaceuticals from environmentally relevant matrices by advanced oxidation processes (AOPs), 2nd ed, Comprehensive Analytical Chemistry. Elsevier B.V. https://doi.org/10.1016/B978-0-444-62657-8.00011-2
50) Narciso-da-Rocha, C., Rocha, J., Vaz-Moreira, I., Lira, F., Tamames, J., Henriques, I., Martinez, J.L., Manaia, C.M., 2018. Bacterial lineages putatively associated with the

dissemination of antibiotic resistance genes in a full-scale urban wastewater treatment plant. Environ. Int. 118, 179–188. https://doi.org/10.1016/j.envint.2018.05.040
51) Negreanu, Y., Pasternak, Z., Jurkevitch, E., Cytryn, E., 2012. Impact of treated wastewater irrigation on antibiotic resistance in agricultural soils. Environ. Sci. Technol. 46, 4800–4808. https://doi.org/10.1021/es204665b
52) Nesme, J., Simonet, P., 2015. The soil resistome: A critical review on antibiotic resistance origins, ecology and dissemination potential in telluric bacteria. Environ. Microbiol. 17, 913–930. https://doi.org/10.1111/1462-2920.12631
53) Nikolaou, A., Meric, S., Fatta, D., 2007. Occurrence patterns of pharmaceuticals in water and wastewater environments. Anal. Bioanal. Chem. 387, 1225–1234. https://doi.org/10.1007/s00216-006-1035-8
54) Oksanen, J., Blanchet, F.G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P.R., O'Hara, R.B., Simpson, G.L., Solymos, P.M. Stevens, H.H., Szoecs, E., Wagner H., 2019. vegan R package: an R package for community ecologists. R package version 2.5-6. https://cran.rproject.org/package=vegan
55) Orschler, L., Agrawal, S., Lackner, S., 2019. On resolving ambiguities in microbial community analysis of partial nitritation anammox reactors. Sci. Rep. 1–10. https://doi.org/10.1038/s41598-019-42882-8
56) Paranychianakis, N. V., Salgot, M., Snyder, S.A., Angelakis, A.N., 2015. Water reuse in EU states: Necessity for uniform criteria to mitigate human and environmental risks. Crit. Rev. Environ. Sci. Technol. 45, 1409–1468. https://doi.org/10.1080/10643389.2014.955629
57) Pärnänen, K.M.M., Narciso-Da-Rocha, C., Kneis, D., Berendonk, T.U., Cacace, D., Do, T.T., Elpers, C., Fatta-Kassinos, D., Henriques, I., Jaeger, T., Karkman, A., Martinez, J.L., Michael, S.G., Michael-Kordatou, I., O'Sullivan, K., Rodriguez-Mozaz, S., Schwartz, T., Sheng, H., Sørum, H., Stedtfeld, R.D., Tiedje, J.M., Giustina, S.V. Della, Walsh, F., Vaz-Moreira, I., Virta, M., Manaia, C.M., 2019. Antibiotic resistance in European wastewater treatment plants mirrors the pattern of clinical antibiotic resistance prevalence. Sci. Adv. 5. https://doi.org/10.1126/sciadv.aau9124
58) R Core Team 2019. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/.
59) Rossi, D., Caracciolo, A.B., Grenni, P., Cattena, F., Di Lenola, M., Patrolecco, L., Ademollo, N., Ciannarella, R., Mascolo, G., Ghergo, S., 2019. Groundwater autochthonous microbial communities as tracers of anthropogenic pressure impacts: Example from a municipal waste treatment plant (Latium, Italy). Water 11, 1–20. https://doi.org/10.3390/w11091933
60) Silby, M.W., Winstanley, C., Godfrey, S.A.C., Levy, S.B., Jackson, R.W., 2011. Pseudomonas genomes: Diverse and adaptable. FEMS Microbiol. Rev. 35, 652–680. https://doi.org/10.1111/j.1574-6976.2011.00269.x
61) Smalla, K., Cook, K., Djordjevic, S.P., Klümper, U., Gillings, M., 2018. Environmental dimensions of antibiotic resistance: assessment of basic science gaps. FEMS Microbiol. Ecol. 94, 1–6. https://doi.org/10.1093/femsec/fiy195
62) Sui, Q., Cao, X., Lu, S., Zhao, W., Qiu, Z., Yu, G., 2015. Occurrence, sources and fate of pharmaceuticals and personal care products in the groundwater: A review. Emerg. Contam. 1, 14–24. https://doi.org/10.1016/j.emcon.2015.07.001
63) Szekeres, E., Chiriac, C.M., Baricz, A., Szőke-Nagy, T., Lung, I., Soran, M.L., Rudi, K., Dragos, N., Coman, C., 2018. Investigating antibiotics, antibiotic resistance genes,

and microbial contaminants in groundwater in relation to the proximity of urban areas. Environ. Pollut. 236, 734–744. https://doi.org/10.1016/j.envpol.2018.01.107
64) Ternes, T.A., Bonerz, M., Herrmann, N., Teiser, B., Andersen, H.R., 2007. Irrigation of treated wastewater in Braunschweig, Germany: An option to remove pharmaceuticals and musk fragrances. Chemosphere 66, 894–904. https://doi.org/10.1016/j.chemosphere.2006.06.035
65) Wang, F.H., Qiao, M., Su, J.Q., Chen, Z., Zhou, X., Zhu, Y.G., 2014. High throughput profiling of antibiotic resistance genes in urban park soils with reclaimed water irrigation. Env. Sci Technol 48, 9079–9085. https://doi.org/10.1021/es502615e
66) Wang, Y., Lu, J., Mao, L., Li, J., Yuan, Z., Bond, P.L., Guo, J., 2019. Antiepileptic drug carbamazepine promotes horizontal transfer of plasmid-borne multi-antibiotic resistance genes within and across bacterial genera. ISME J. 13, 509–522. https://doi.org/10.1038/s41396-018-0275-x
67) Whickham, 2016. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag, New York https://www.springer.com/gp/book/9780387981413
68) Yan, L., Herrmann, M., Kampe, B., Lehmann, R., Totsche, K.U., Küsel, K., 2020. Environmental selection shapes the formation of near-surface groundwater microbiomes. Water Res. 170, 115341. https://doi.org/10.1016/j.watres.2019.115341
69) Yang, Q., Zhang, H., Guo, Y., Tian, T., 2016. Influence of chicken manure fertilization on antibiotic-resistant bacteria in soil and the endophytic bacteria of pakchoi. Int. J. Environ. Res. Public Health 13, 1–12. https://doi.org/10.3390/ijerph13070662
70) Zhao, Y., Cocerva, T., Cox, S., Tardif, S., Su, J.Q., Zhu, Y.G., Brandt, K.K., 2019. Evidence for co-selection of antibiotic resistance genes and mobile genetic elements in metal polluted urban soils. Sci. Total Environ. 656, 512–520. https://doi.org/10.1016/j.scitotenv.2018.11.372
71) Zhou, X., Qiao, M., Wang, F.H., Zhu, Y.G., 2017. Use of commercial organic fertilizer increases the abundance of antibiotic resistance genes and antibiotics in soil. Environ. Sci. Pollut. Res. 24, 701–710. https://doi.org/10.1007/s11356-016-7854-z

Chapter 4

4. Elevated levels of antibiotic resistance in groundwater during treated wastewater irrigation associated with infiltration and accumulation of antibiotic residues.

Ioannis D. Kampouris[a], Nikiforos Alygizakis[b,c], Uli Klümper[a], Shelesh Agrawal[d], Susanne Lackner[d], Damiano Cacace[a], Steffen Kunze[a], Nikolaos Thomaidis[c], Jaroslav Slobdonik[b], Thomas U. Berendonk[a]

[a] Technische Universität Dresden, Institute of Hydrobiology, Chair of Limnology 01062, Dresden, Zellescher Weg 40, Germany

[b] Environmental Institute, Okružná 784/42, 97241 Koš, Slovak Republic

[c] Laboratory of Analytical Chemistry, Department of Chemistry, University of Athens, Panepistimiopolis Zografou, 15771 Athens, Greece

[d] Technische Universität Darmstadt, Institute IWAR, Chair of Wastewater Engineering, Franziska-Braun-Straße 7, 64287, Darmstadt, Germany

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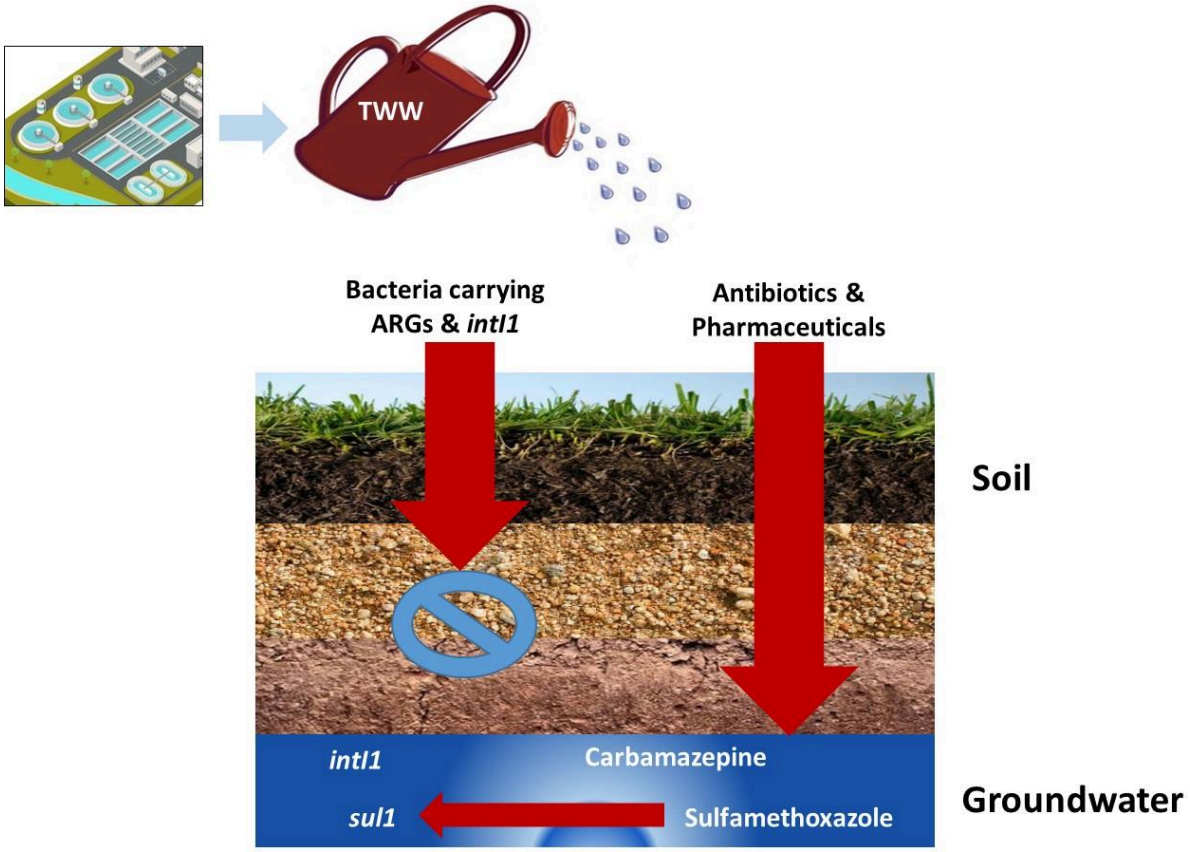
Graphical Abstract



Highlights

- Bacterial abundance in groundwater did not increase due to TWW irrigation.
- No faecal indicator bacteria were detected in GW after TWW irrigation.
- Elevated concentration of sulfamethoxazole & carbamazepine in the groundwater.
- Relative abundance of *sul1* & *intI1* in groundwater increased during TWW irrigation.
- Relative abundance of *sul1* correlated with sulfamethoxazole concentration in GW.

Abstract

Treated wastewater irrigation (TWW) releases antibiotics and antibiotic resistance genes (ARGs) into the environment and might thus promote the dissemination of antibiotic resistance in groundwater (GW). We hypothesized that TWW irrigation increases ARG abundance in GW through two potential mechanisms: the contamination of GW with ARG bacteria and the accumulation of antibiotics in GW. To test this, the GW below a real-scale TWW-irrigated field was sampled for six months. Sampling took place before, during and after high-intensity TWW irrigation. Samples were analysed with 16S rRNA amplicon sequencing, qPCR of six ARGs and integrase gene *intI1* and liquid chromatography tandem mass spectrometry to detect antibiotic and pharmaceutical residues. 16S rRNA absolute abundance in GW decreased rather than increased during long-term irrigation. Also, the relative abundance of TWW-related bacteria did not increase in GW during long-term irrigation. In contrast, long-term TWW irrigation increased the relative abundance of *sul1* and *intI1* in the GW microbiome. Furthermore, the GW contained elevated concentrations of sulfonamide antibiotics, especially sulfamethoxazole, to which sul1 confers resistance. The total sulfonamide concentrations in GW correlated with sul1 relative abundance. Consequently, TWW irrigation promoted *sul1* and *intI1* dissemination in the GW microbiome, while the accumulation of drug residues most likely contributed to their successful spread.

4.1. Introduction

Over the past years, it has become well known that pharmaceutical residues, including antibiotics, and antibiotic resistance genes (ARGs) remain in treated wastewater (TWW) in relatively high concentrations (Caucci et al., 2016; Alygizakis et al., 2020). Thus, TWW discharges have the potential to release high loads of antibiotic and ARG into the environment (Caucci et al., 2016; Berendonk et al., 2015; Cacace et al., 2019; Alygizakis et al., 2020). Therefore, concerns have emerged regarding the spread of antimicrobial resistance (AMR) from agricultural practices involving TWW, such as TWW irrigation and managed aquifer recharge (MAR) (Guo et al., 2017; Smalla et al., 2018). Specifically, for TWW irrigation, several studies have investigated the impact of TWW irrigation on the ARG prevalence in soil, crops and subsoil pore water (Wang et al., 2014; Han et al., 2016; Dalkman et al., 2012; Jechalke et al., 2015; Cerqueira et al., 2019a; Cerqueira et al., 2019b; Cerqueira et al., 2019c; Marano et al., 2019; Kampouris et al., 2021a, Kampouris et al., 2021b). However, the number of studies investigating the prevalence of ARGs in groundwater (GW) environments of TWW irrigated agricultural fields is limited. This raises concerns due to the importance of GW as a freshwater resource (Szekeres et al., 2018). For example, a previous study found that GW wells close to human settings exhibited elevated levels of ARG abundance, presumably because of anthropogenic pressures (Szekeres et al., 2018). Tetracycline and erythromycin ARGs were present in the GW of MAR sites (Bockelmann et al. 2009). On the contrary, the ARGs bla_{TEM} and *qnrS* occurred in TWW from a MAR site in Israel, but did not appear in the GW (Elkayam et al., 2018).

Furthermore, the presence of ARGs in TWW is regularly connected with the integrase gene *intI1* (Marano et al., 2019; Gatica et al., 2016), which is associated with mobile genetic elements (MGE) and horizontal gene transfer (HGT) (Gillings et al., 2015; Gillings et al., 2017). Usually, several, diverse ARGs are part of mobile class 1 integron gene cassettes

alongside *intI1* (Gatica et al., 2016). The increase of *intI1* is not only related to ARG dissemination, but also to stress experienced by bacterial communities due to anthropogenic impact (Gillings et al., 2015; Gillings et al., 2017). Moreover, stress can increase the mobility potential of MGE (Klümper et al. 2017; Wang et al. 2018; Klümper et al. 2019). For example, carbamazepine, a non-antibiotic drug that induces bacterial stress responses, which promote HGT (Wang et al. 2018), has been detected in high concentration in GW during TWW irrigation (Ternes et al., 2007; Lesser et al., 2018).

In addition to carbamazepine, antibiotics have been regularly detected in GW environments from Europe (Szeckeres et al., 2018), the USA (Barber et al., 2008) and Asia (Avisar et al., 2009; Chen et al., 2017). Among those, sulfonamides, a class of antibiotics with synthetic origin (Underwood et al., 2011), typically occur in high concentration in TWW (Johnson et al., 2015). Further they are able to persist in GW environments (Barber et al., 2008; Avisar et al., 2009; Underwood et al., 2011; Szeckeres et al., 2018), especially during TWW-irrigation or MAR operations (Avisar et al., 2009). The sulfonamide antibiotic sulfamethoxazole has occurred in GW samples, with concentrations reaching up to 1,100 ng/L (Barber et al., 2008), close to the predicted no effect concentration (PNEC) for positive selection of sulfamethoxazole-resistance (16,000 ng/L) (Bengtsson-Palme & Larsson, 2016).

Here we investigated the impact of TWW irrigation on ARG and *intI1* prevalence in the GW, at a ten-meter depth of a real-scale, commercially operated TWW irrigated field. In the same field TWW irrigation increased the ARG abundance in topsoil and subsoil pore-water microbiota, even at the depth of 1.2 m. Nevertheless, it remained unclear whether TWW irrigation can similarly affect the underlying GW environment. Such an impact would be possible through two main hypothesized mechanisms: the infiltration of ARG carrying bacteria from TWW irrigation into the GW and the accumulation of antibiotics from TWW in the GW. The impact of the hypothesized mechanisms was tested by sampling the GW below a real-

scale, commercially operated, TWW-irrigated field subjected to irrigation with secondary TWW. The GW of the TWW irrigated field was sampled over a period of six months, prior during and after irrigation ceased. Samples were analysed with qPCR, to determine the ARG and *intI1* dynamics, while 16S rRNA high throughput sequencing was performed to examine the bacterial community profiles. Further, drug residues in TWW and GW were determined with liquid chromatographic separation with tandem mass spectrometric detection (LC/MS-MS). Our results demonstrate that TWW irrigation mainly promotes the spread of *sul1* and *intI1* into the GW microbiome, while elevated concentrations of the pharmaceuticals sulfamethoxazole and carbamazepine were also a consequence.

4.2. Materials and Methods

4.2.1 Sampling

4.2.1.1 Description of the sampling area

Samples were collected from the facilities of Braunschweig Wastewater Association (BWA) (Paranychianakis et al., 2015; Ternes et al., 2007). BWA is one of the few companies that perform the commercial operation of real-scale TWW irrigation in agricultural fields in Germany. The BWA is located in Wendeburg (Lower Saxony, Germany) and performs the treatment of municipal wastewater in the local area. The Urban Wastewater Treatment Plant (UWTP) of BWA receives approximately 60,000 m^3 WW/d and has a population equivalent of 350,000. TWW is then used for irrigation of the agricultural field in question. The soil of the field (sandy soil, cambisol, N: 52.359500, E: 10.399833; Fig. S4.1) has a high percentage in sand (over 90% percent) (Ternes et al., 2007; Kampouris et al., 2021a; Kampouris et al., 2021b). The soil is deficient in nutrients and hence not suitable for systematic agricultural use. The farmers counter the lack of nutrients through TWW irrigation (Paranychianakis et al.,

2015). Further information regarding the soil and its physicochemical characteristics are given in Ternes et al. (2007) and Kampouris et al. (2021a). Irrigation is performed with TWW subjected to conventional secondary biological treatment. Occasionally, the TWW is mixed with digested sludge (TWW & DS), depending on the nutrient demand of crops (rye, maize or rapeseed) and amount of natural precipitation. The crops are used for production of biogas.

4.2.1.2 Sampling

We sampled the GW from a real-scale field irrigated with TWW/TWW & DS, which is commercially operated. The sampling started at the end of June 2018. During this time-point, the field had not been irrigated with TWW for three months. The field was then irrigated intensively with TWW & DS from the end of June 2018 to the middle of August 2018. It was switched to TWW only until October 2018, in accordance with the farmers' plan for crop-cultivation. Samples were taken prior to the start of irrigation (June 2018), after one month (July 2018) and after three months (September 2018) of high-intensity irrigation. The irrigation stopped at the start of October 2018. A final sampling was performed approximately two months after the irrigation break in December 2018. At each time-point, three samples were taken from three GW wells each (total n=9), located in the field at a depth of 10 m. One additional sample was taken from each well for drug residue profile analysis.

Water samples were stored on ice and transferred to the lab immediately. Bacteria from water samples were captured by filtration (polycarbonate, 0.2 μm pore size, 47 mm diameter, Sartorius, Germany), within 24 hours from sampling. The filtration volume was 0.5 L for irrigation water and 2.5 L for GW samples. Additionally, samples from July, September and December 2018 were stored at -20^{o}C for subsequent chemical analysis. Due to the severe heat wave during June 2018 the volume of sampled GW in June 2018 was insufficient for

performing both DNA extraction and preserving samples for chemical analysis. Thus, no additional sample from June 2018 was taken and stored for LC-MS/MS.

4.2.2 Liquid chromatography tandem mass spectrometry (LC-MS/MS)

4.2.2.1 Chemicals and reagents

Acetonitrile (ACN) and Methanol (MeOH) LC–MS grade was purchased from Merck (Darmstadt, Germany). Formic acid (FA) with purity 99% was obtained from Sigma–Aldrich, Fluka (Buchs, Switzerland). Distilled water was provided by a Milli-Q purification apparatus (Millipore Direct-Q UV, Bedford, MA, USA). Atlantic HLB-M disks were purchased from Labicom (Olomouc, Czechia) and RC syringe filters (4 mm diameter, 0.2 μm pore size) from Phenomenex (Torrance, CA, USA).

4.2.2.3 Sample preparation and instrumental analysis

The sample preparation protocol involved clean up and pre-concentration by 4000 times. Automatic solid phase extraction by HORIZON SPE-DEX 4790 was used. The conditioning and extraction program used for the preparation of the samples can be found in Table S4. The extracts were evaporated using a gentle stream of nitrogen and reconstituted to 250 μL (50:50 methanol:water). Before the analysis, the extracts were filtered through RC syringe filters of 4 mm diameter and 0.2 μm pore size.

Instrumental analysis was performed with a Thermo UHPLC Accela system connected to a triple quadrupole (TSQ Quantum Access, Thermo Electron Corporation, USA) equipped with an electrospray ionization source (Thermo IonMAX) in positive mode. Chromatographic separation was accomplished on an Atlantis T3 C18 column (100 mm x 2.1 mm, 3 μm) from

Waters (Milford, MS, USA). A constant flow rate of 100 μL min^{-1} was used. The mobile phase, the gradient elution programs and the ESI parameters are presented in Table S5. Identification and quantification were performed under selected reaction monitoring (SRM) mode. The transitions between the precursor ion and the two most abundant product ions were recorded for all target compounds. This allows to achieve four identification points per compound (2002/657/EC). SRM transitions for each substance was optimized by infusion of standard reference solutions at average concentration levels of 1 mg L^{-1}. The optimized ionization mode, fragmentation voltages and collision energies for each antibiotic (41 in total) are summarized in Table S6. Thermo LCquan 2.7 (CA, USA) was used to analyze the data from the LC-MS/MS instrument. More details about the instrumental method can be found elsewhere (Thomaidis et al., 2016).

4.2.3 DNA extraction, qPCR and sequencing

DNA extractions were performed using the DNeasy PowerWater Kit (Qiagen, Germany) according to the manufacturer's instructions. The quantity and quality of DNA was measured with NanoDrop (Thermo Fischer Scientific, Germany). The analysis with quantitative real-time PCR (qPCR) was performed for eight genes (*sul1*, *intI1*, *qnrS*, *tet*(M), $bla_{\text{OXA-58}}$, bla_{TEM}, $bla_{\text{CTX-M-32}}$ and 16S rRNA). The selection of genes was based on the framework for TWW monitoring established by the NEREUS (www.nereus-cost.eu) and ANSWER-ITN (www.answer-itn.eu) networks (Rocha et al., 2018; Cacace et al., 2019). In addition, the genes *sul1*, *qnrS* and *tet*(M) and $bla_{\text{OXA-58}}$ were selected due to their clinical importance and occurrence in high rate and abundance in TWW across European countries (Caucci et al., 2016; Cacace et al., 2019; Alygizakis et al., 2020) and TWW irrigated soil (Kampouris et al., 2021a). The two final β-lactamase genes bla_{TEM} and $bla_{\text{CTX-M-32}}$ occur in low abundance in TWW

(Cacace et al., 2019), while they have shown natural prevalence in soil microbiota (Gatica et al., 2015, Kampouris et al., 2021a). Apart from the ARGs, the integrase gene *intI1* was analysed as well. It is commonly used as a genetic marker for anthropogenic pollution (Gillings et al., 2015) and frequently part of mobile gene cassettes that carry ARGs (Gattica et al., 2016). The reactions were performed in a MasterCycler RealPlex (Eppendorf, Germany) at final volume of 20 μL with 10 μL of 2x Luna Universal qPCR Master Mix (New England Biolabs, Germany). Further details about reagents, primers and annealing temperature for each gene are given in Tables S2 & S3. Standard curves with efficiency 0.9-1.1 and $R^2 \geq 0.99$ were accepted (Table S3), and melting curve analysis was performed to assess the amplicons' specificity.

The amount of inserted DNA was 20 ng per reaction. The limit of quantification (LOQ) varied from gene to gene (4 to 4000 copies per reaction Table S2 & S3). Screening for potential PCR inhibition was performed by spiking a plasmid for a gene, which was rarely detected in the samples at very low abundance ($bla_{\text{CTX-M-32}}$, spiking concentration $4*10^6$ copies/μL). The absolute abundance was calculated from the filtrated volume, the dilution factor (copies/L) and the relative abundance of gene copies per copy of the 16S rRNA gene.

The GW/TWW-Group replicates were pooled (in equimolar concentrations) with a final concentration of 5 ng/μL and were analysed with the 16S Ion Metagenomics Kit™ (Thermo Fisher Scientific, Germany) for amplification and sequencing of multiple parallel variable regions. The protocols for 16S rRNA library preparation for parallel variable regions sequencing and processing of the sequences were described previously in Orschler et al. (2019). Raw sequencing data was submitted to sequencing read archive (SRA) (bioproject accession number: PRJNA713765).

4.2.4 Data processing and statistical analyses

Every sample below LOQ was placed at 1 copy/L for absolute abundance and 10^{-8} relative abundance (one order of magnitude below the minimum possible relative abundance ~10^{-7}). The data was $\log_{10}$ -transformed prior to any graphical representation or statistical analysis. The program R (R Core Team, 2019; v. 3.5.3) was used for graphical representations and statistical analyses, specifically the packages "ggplot" (Wickham, 2016, v.3.3) and "ggpubr" (v. 0.2.2, Kassambara, 2019) for the generation of plots. Significant differences were assessed with Wilcoxon rank sum test or Student's t-test and in case of group comparisons with the Kruskal-Wallis test (package "ggpubr"). Correlation was analysed with Kendall rank correlation (package "ggpubr"). Multiple comparisons were performed with Dunn's test and Benjamini-Hochberg correction (package "dunn's test", v1.3.5, Dinno, 2016), to assign significant differences from pairwise comparisons. For the analysis and graphical representation of bacterial community data, the package "phyloseq" (McMurdie & Holmes, 2011) was used. Comparisons with p-values below 0.05 were considered statistically significant (α=0.05).

4.3. Results

4.3.1 Bacterial abundance in groundwater did not increase due to TWW irrigation

To evaluate whether groundwater (GW) receives a high increase of bacterial abundance due to contamination through TWW irrigation, both, the irrigation waters and water from the GW wells receiving the percolated water were analyzed with qPCR. The TWW & DS irrigation water contained the highest absolute bacterial abundance in regards to 16S rRNA gene (10.0±0.1 $\log_{10}$ copies/L, Fig. 4.1A). Both, TWW irrigation water (9.7±0.2 $\log_{10}$ copies/L) and GW prior to irrigation (9.5±0.2 $\log_{10}$ copies/L) had significantly lower absolute bacterial abundance than TWW & DS irrigation water ($p<0.05$, Dunn's test, n=9, Fig. 4.1A), but were insignificantly different from one another ($p>0.05$, Dunn's test, n=9, Fig. 4.1A).

During long-term irrigation, rather than an increase, a gradual decrease of GW bacterial abundance occurred. Specifically, the absolute abundance decreased from 9.5±0.2 to 8.6±0.2 $\log_{10}$ copies/L, during the first month of irrigation (June-July 2018, $p<0.05$, Dunn's test, n=9, Fig. 4.1A). After three months of irrigation, 7.9±0.5 $\log_{10}$ copies/L, significantly less than in July 2018 ($p<0.05$, Dunn's test, n=9, Fig. 4.1A) were detected. After the two-month irrigation break (December 2018) the absolute abundance of 16S rRNA gene increased slightly but not significantly to 8.0±0.1 $\log_{10}$ copies/L compared to September 2018 ($p>0.05$, Dunn's test, n=9, Fig. 4.1A). Therefore, despite the high number of bacteria introduced into the soil through continuous, intensive TWW/TWW & DS irrigation, no increase in bacterial abundance in the underlying GW environments occurred.

To further verify the limited bacterial contamination of GW due to TWW/TWW & DS irrigation, the bacterial community profile of TWW, TWW & DS and GW samples was analyzed (Fig. 4.1B). No increase for TWW and TWW & DS related genera occurred in GW

during long-term irrigation (Fig. 4.1B). For example, *Pseudomonas* relative abundance showed a linear but non-significant reduction one month after the start of irrigation (72.6-45.4%), three months after irrigation (45.1-31.3%) and after the irrigation break (31.3-10.7 %) (Mann-Kendall test, τ=-1, p=0.089, Fig. 4.1B). Fecal indicator bacteria such as *Escherichia/Shigella* genera were present in TWW (0.1 %) and TWW & DS (0.01 %), but not detected in GW as a consequence of irrigation. Consequently, bacterial contamination of GW with TWW-related bacteria during TWW irrigation was limited.

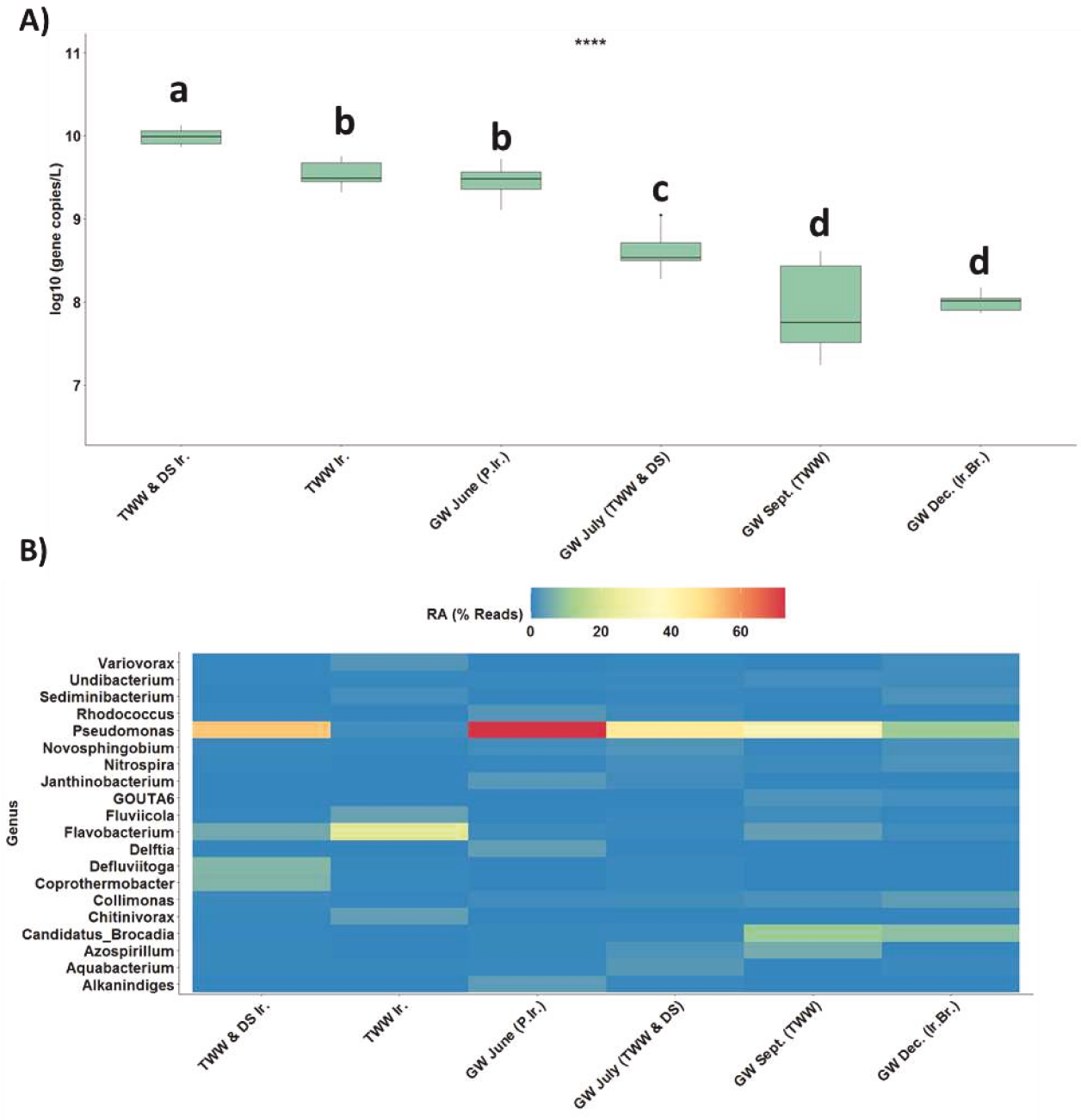


Figure 4.1: A) Absolute abundance of 16S rRNA (gene copies/L) in the groundwater and the irrigation waters, based on the qPCR analysis. Kruskal-Wallis test, $p = 1.4*10^{-12}$, n=9. Letters from "a" to "d" were assigned to non-significantly different groups after multiple pairwise comparison with Dunn's test along with Benjamini-Hochberg correction. The p-value cut-off for significance was set at 0.05. B) Relative abundance (% reads) of the 20 most abundant bacterial genera of irrigation waters and groundwater, based on the 16S amplicon sequencing. Given in parenthesis is the status of irrigation during each groundwater sampling. Ir.=Irrigation, P.Ir.=Prior Irrigation, GW=Groundwater, TWW=Treated Wastewater, DS=Digested sludge, Ir. Br.=Sampling after the two-month irrigation break.

4.3.2 Elevated concentrations of sulfamethoxazole and carbamazepine in the groundwater of the TWW irrigated field

Apart from bacteria, the TWW contained drug residues that can infiltrate into the GW. Thus, the drug-residue profile of irrigation waters and GW was analyzed. As expected, the irrigation waters contained several antibiotic and non-antibiotic residues. The most frequent class of detected antibiotics was sulfonamides, especially sulfamethoxazole detected at the highest concentration with 83.5 ng/L in TWW & DS (July 2018) and 61.85 ng/L in TWW (September 2018, Fig. 4.2). Besides sulfamethoxazole, doxycycline (tetracycline) was present in high concentrations (194.5 ng/L) in TWW & DS (July 2018) but not detected in TWW (September 2018, Fig. 4.2). Other antibiotics detected in TWW/TWW & DS included lincomycin (macrolide), metronidazole (nitroimidazole) and ofloxacin (quinolone) (Fig. 4.2). Further, a multitude of non-antibiotic pharmaceuticals (e.g. carbamazepine, ibuprofen and hydrochlorothiazide) were present in the irrigation water (Fig. 4.2).

However, only a minor fraction of the drug residues infiltrated and persisted in the GW during the irrigation periods (Fig. 4.2). The compounds detected in GW were lincomycin, metronidazole, ofloxacin, carbamazepine, ibuprofen, hydrochlorothiazide as well as several sulfonamide antibiotics (Fig. 4.2). However, the concentration of the majority of drug residues was low, close to the LOQ of the chemical method. An exception were four compounds: carbamazepine, ibuprofen, hydrochlorothiazide and sulfamethoxazole were abundant in high concentrations in the GW and were previously identified in high concentrations in irrigation water as well (Fig. 4.2). Among these four, carbamazepine and sulfamethoxazole displayed the highest concentrations in GW (Fig. 4.2). Specifically, sulfamethoxazole concentrations increased significantly during TWW irrigation from 98.2±39.8 ng/L (July 2018) to 301.9±33.8 ng/L (September 2018, Student's t-test, $p=0.0057$, $n=3$) (Fig. 4.2). Surprisingly, even after a

two months irrigation break, the concentrations remained significantly elevated but with far higher variation among replicates (406.9±204.0 ng/L, Fig. 4.2).

While carbamazepine concentrations were equally high as those detected for sulfamethoxazole, no increase with prolonged irrigation was detected, with concentrations of 272.3±185.1 ng/L (July 2018), 168.5±18.9 (September 2018) and 183.8±101.4 ng/L after the irrigation break (Fig. 4.2). Thus, sulfamethoxazole and carbamazepine co-occurred as the main drug-residue contaminants in the GW, with sulfamethoxazole increased during TWW irrigation duration. Consequently, while TWW-related bacteria did not infiltrate the GW, several drug residues did and even persisted after the irrigation break.

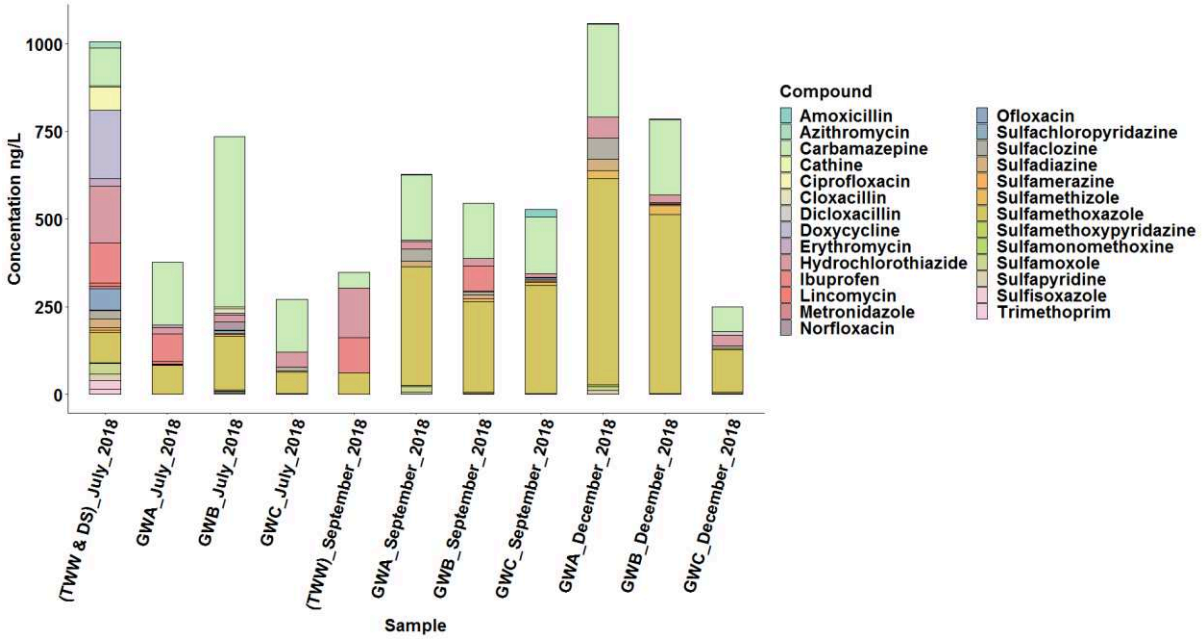


Figure 4.2: Concentration of antibiotic and non-antibiotic drug residues detected in the irrigation waters and the respective groundwater wells (GWA, GWB and GWC) from July 2018 (one month of high intensity irrigation), September 2018 (three months of high intensity irrigation) and December (two months after irrigation break). GW=Groundwater, TWW=Treated Wastewater, DS=Digested sludge.

4.3.3 TWW irrigation promotes *sul1* and *intI1* dissemination in groundwater.

To evaluate whether TWW irrigation promotes the dissemination of ARGs in the GW, irrigation waters and GW samples were analyzed by qPCR. TWW and TWW & DS irrigation contained all six tested ARGs and the integrase gene *intI1*, with the exception of the bla_{TEM} gene (Fig. S4.2). The genes *intI1* and *sul1* showed the highest relative abundance in the irrigation waters (*intI1:* -1.8±0.5 *sul1:* -2.0±0.5 $\log_{10}$ copies/16s rRNA, Fig. S4.2). The genes *qnrS,* $bla_{\text{OXA-58}}$ and *tet*(M) showed one-order of magnitude lower relative abundance than *sul1* and *intI1* (*qnrS*: -3.4±0.8, $bla_{\text{OXA-58}}$:-3.2±0.7 & *tet*(M):-3.8±0.3 $\log_{10}$ copies/16S rRNA; Fig. S4.2). The gene $bla_{\text{CTX-M-32}}$ showed the lowest abundance among detected genes in irrigation water (-4.7±0.9 $\log_{10}$ copies/16S rRNA).

Of the detected genes in irrigation water, the relative abundances of *sul1* and *intI1* increased significantly and continuously in the GW over irrigation operation (Kruskal Wallis, $p<0.0001$, n=9, Fig. 4.3A). Between June and July 2018, the relative abundance of *sul1* slightly but not significantly increased from -6.0±2.1 to -3.9±0.5 $\log_{10}$ copies/16s rRNA (Dunn's test, $p>0.05$, n=9, Fig. 4.3A). The observed increase continued after three months of irrigation to -2.8±0.8 $\log_{10}$ copies/16S rRNA, leading to a significant difference when compared to June 2018 (Dunn's test, $p<0.05$, n=9, Fig. 4.3A). Despite the irrigation break, *sul1* relative abundance remained stable at 2.9±1.0 $\log_{10}$ copies/16S rRNA and was significantly higher than prior to the irrigation-start (Dunn's test, $p<0.05$, n=9, Fig. 4.3A). Kendall rank correlation was performed with *sul1* relative abundance (average abundance per well) and total sulfonamide concentration per well. The *sul1* relative abundance correlated significantly with total sulfonamide concentration ($R=0.56$, $p=0.045$, n=9, Fig. 4.3B).

The integrase gene *intI1,* followed similar patterns in terms of relative abundance as *sul1*. The *intl1* relative abundance increased slightly but not significantly between June and July 2018, from -4.4±0.5 to -3.7±0.4 $\log_{10}$ copies/16S rRNA (Dunn's test, $p>0.05$, n=9, Fig. 4.3A). This increase continued after three months of irrigation to -2.3±0.6 $\log_{10}$ copies/16S rRNA, significantly higher than June and July 2018 samplings (Dunn's test, $p<0.05$, n=9, Fig. 4.3A). A slight decrease occurred after the two-month irrigation break to -2.9±0.8 (Fig. 4.3A). Despite this decrease, the *intI1* relative abundance in GW remained significantly higher when compared to prior irrigation GW sampling (June 2018, Dunn's test, $p<0.05$, n=9, Fig. 4.3A). However, the *intI1* relative abundance did not correlate significantly with the total sulfonamide concentrations (R=0.5, p=0.057, n=9, Fig. 4.3B).

Regarding the remaining ARGs, the relative abundance of *qnrS* displayed a limited increase during TWW irrigation. The *qnrS* relative abundance significantly increased from -7.0±1.2 to -5.8±1.6 copies/16S rRNA (Dunn's test, $p<0.05$, n=9, Fig 4.3A) and remained stable at -5.8±1.7 copies/16S rRNA, after three months of irrigation (September 2018). Other than *sul1* and *intI1*, its relative abundance decreased significantly down to -7.1±1.2 copies/16S rRNA after the two-month irrigation break (Dunn's test, $p<0.05$, n=9, Fig 4.3A). No specific patterns were observed for any of the other tested genes. In addition, the relative abundance of the remaining ARGs did not correlate with the total concentration of any of the antibiotic classes ($p>0.05$, n=9, Fig. S4.3)

Therefore, during long-term TWW irrigation *qnrS* slightly increased in the GW, while *sul1* and *intI1* relative abundance showed a stronger, consistent and significant increase. In addition, *sul1* abundance correlated with the total sulfonamide concentration, indicating a contribution of sulfonamide persistence to *sul1* dissemination.

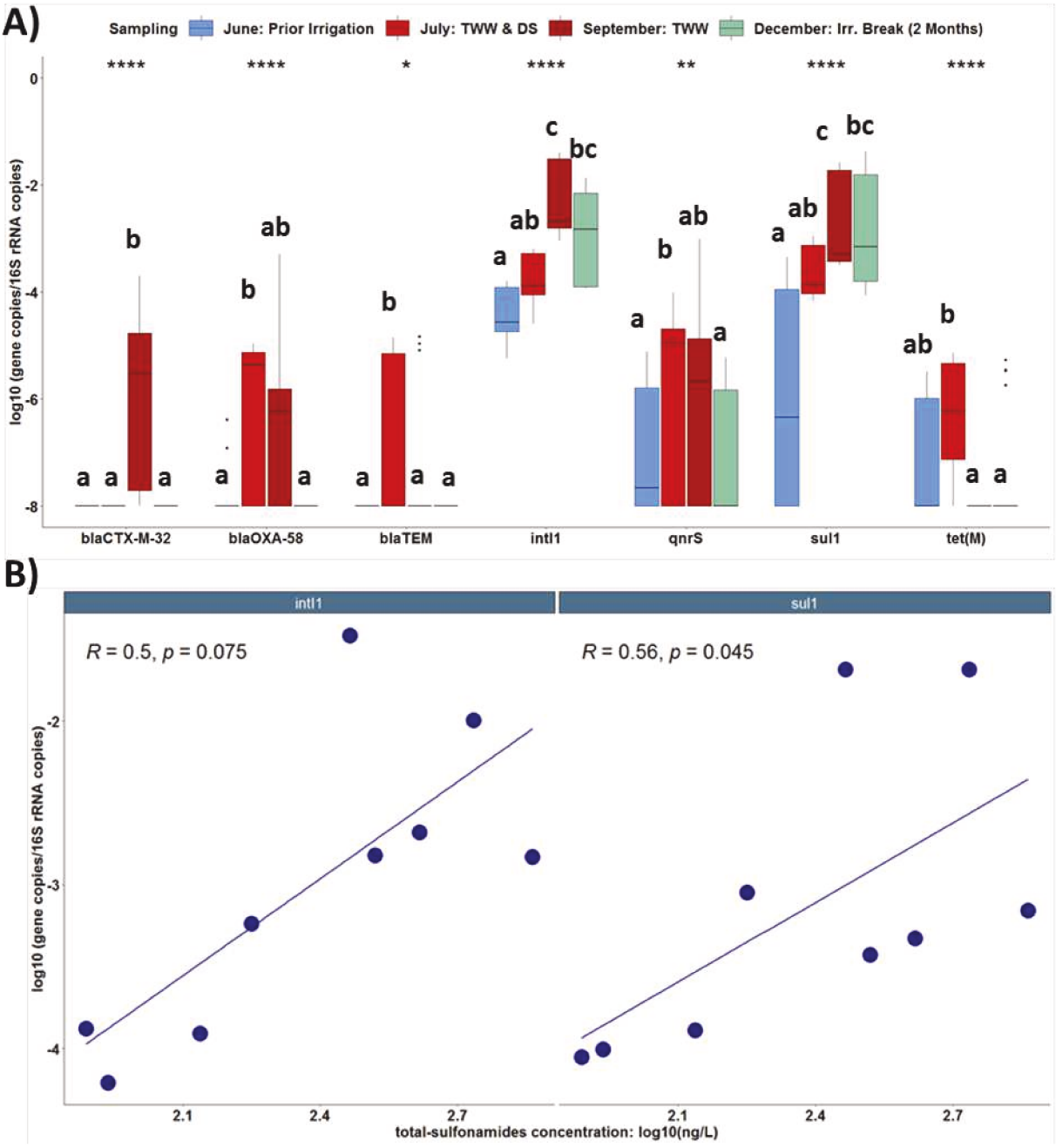


Figure 4.3: A) Log_{10} transformed relative abundance of ARGs and *intI1* to 16s rRNA gene copies. The samples were taken from the groundwater of the selected field (depth 10 m). The sampling started from June 2018 (prior irrigation) and lasted until December 2018. We sampled in one (June 2018) and three months (September 2018) of high intensity irrigation. The high intensity irrigation was continued until end of October 2018. A last sampling took place in December 2018, two months after the irrigation operation ceased. TWW=Treated Wastewater, DS= Digested Sludge; Kruskal-Wallis test *p<0.05, **p<0.01, ***p< 0.001, ****p<0.0001, n=9. Letters from "a" to "c" were assigned to non-significantly different groups after multiple pairwise comparison with Dunn's test along with Benjamini-Hochberg correction. B) Kendall rank correlation of the median *sul1* and *intI1* relative abundance of each well (Fig. 4.3A) and the total sulfonamide concentration (ng/L) of each sampled well (Fig. 4.3B) (total n=9).

4.4. Discussion

Despite the importance of groundwater (GW) environments, their ARG dynamics remain underexplored. Here, we demonstrated that TWW irrigation promoted the spread of ARGs, specifically *sul1* and *intI1*, in GW environments. After irrigation, the GW contained elevated concentrations of sulfonamides (especially sulfamethoxazole), which correlated to the relative abundance of *sul1*. Thus, TWW irrigation increased ARG abundance corresponding to the accumulation of antibiotics in GW, supporting the second hypothesized mechanism of TWW irrigation impacts on ARG in GW.

TWW, subjected to conventional biological wastewater treatment only, regularly contains high amounts of opportunistic pathogens, including fecal indicator bacteria, such as *E. coli* (Petousi et al., 2019). This has raised initial concerns regarding contamination of GW reservoirs through the bacterial load introduced by TWW irrigation (Ayni et al., 2011). In the present study, no increase in the absolute bacterial abundance of GW due to TWW irrigation was observed. Moreover, none of the fecal indicator bacteria identified in TWW was detected in the GW microbiome after irrigation. Accordingly, Elkayam et al. (2018) reported that TWW-related opportunistic pathogens do often not persist during soil passage. Absolute bacterial abundance decreased in the GW during continued TWW irrigation. To our knowledge, such log-fold fluctuations of absolute bacterial abundance prior and after long-term irrigation in GW has not been reported yet. A knowledge gap remains on whether these decreases were directly related to TWW irrigation or simply seasonal bacterial dynamics of the GW microbiome, which need to be tackled in follow up long-term studies.

Still, bacteria introduced through TWW irrigation were most likely outcompeted by indigenous microbes, and did not invade the GW microbiome. Therefore, this trend previously shown for the microbiomes of TWW irrigated soil and subsoil pore-water was confirmed for GW

microbiota as well (Kampouris et al., 2021a; Kampouris et al., 2021b). This supports further that soil filtration during TWW irrigation could serve as a suitable low-cost additional barrier option for TWW bacteria. Nevertheless, despite the incapability of TWW bacteria to persist in soil, TWW bacteria can potentially transfer their ARGs to soil bacteria (Kampouris et. al 2021a). These can then lead to ARG carrying bacteria infiltrating the subsoil (Kampouris et al., 2021b) and ultimately reach the GW.

While retention of the TWW bacteria was achieved during soil passage, the occurrence of several micro-contaminants in GW suggests that drug-residues introduced through TWW irrigation are not equally well contained. Especially sulfamethoxazole and carbamazepine showed elevated concentration, close to TWW irrigation levels. Similar elevated sulfamethoxazole concentrations have been reported in groundwater wells during a monitoring study for baseline pharmaceutical concentrations across the USA (Barnes et al., 2008) and in a phreatic aquifer of TWW irrigation operation in Israel (Avisar et al., 2009). Sulfonamides, such as sulfamethoxazole, are not fully eliminated during wastewater treatment (Göbel et al., 2005), and have previously been detected in investigations of this specific field (Ternes et al., 2007). Specifically, sulfamethoxazole occurred in elevated concentrations in the GW of the TWW irrigated field, with a sharp increase from one to three months of irrigation. Sulfamethoxazole even persisted at high levels in the GW matrix after a two-month irrigation break. This persistence can be explained by low biodegradation rates compared to other pharmaceuticals due to the synthetic nature of sulfonamides (Underwood et al., 2011). Still, observed sulfamethoxazole concentration (406.9±204.0 ng/L) remained below the PNEC values for selection of sulfamethoxazole resistance in environmental bacteria (16,000 ng/L) (Bengtsson-Palme & Larsson, 2016). Hence, positive selection for sulfonamides resistance might be possible at concentrations lower than previously suggested. However, in complex

environmental habitats it remains difficult to mechanistically disentangle the observed AMR dynamics.

The second main contaminant, carbamazepine, has been reported in GW during TWW infiltration at high concentrations as well (Clara et al., 2004), but did not significantly increase any further during irrigation. A few other drug residues (lincomycin, metronidazole, ofloxacin, carbamazepine, ibuprofen, hydrochlorothiazide and several sulfonamides) persisted TWW infiltration in the present study, however, in much lower concentrations.

Through GW contamination with drug residues, TWW irrigation promoted the dissemination of *sul1* and *intI1* in the GW microbiome. The gene *sul1* confers resistance to the detected sulfonamides through bypassing the inactivation with the encoding of a dihydropteroate synthase, which has low affinity for sulfonamides (Reis et al., 2018). The *sul1* gene is frequently present in TWW as one of the most abundant ARGs (Cacace et al., 2019; Kampouris et al., 2021). Remarkably, *sul1* relative abundance in GW increased so significantly during long-term irrigation that it reached similar levels as detected in the irrigation waters. The increase of *sul1* abundance here was directly positively correlated with both, sulfamethoxazole and total sulfonamide concentrations in the GW. Thus, the introduction and persistence of sulfonamides (and especially sulfamethoxazole) from TWW irrigation, even at concentrations lower than PNEC models (Bengtsson-Palme et al., 2016), provides a mechanistic explanation behind the successful dissemination of *sul1* in GW, compared to the other tested ARGs.

Apart from *sul1,* the integrase gene *intI1* increased as well during long-term irrigation. The *sul1* gene is frequently part of mobile *intI1* gene cassettes (Gillings et al., 2015; Gillings et al., 2017). Thus, co-selection may occur for *intl1,* due to the positive selection pressure *sul1* is subjected from sulfonamides. Furthermore, the consistent GW bacterial community profile combined with the qPCR results supports that HGT contributes to *sul1* and *intI1* dissemination,

since no significant addition of TWW-related bacteria occurred. Carbamazepine, which occurred in high concentration in GW is known to function as bacterial stressor (Wang et al., 2018). Elevated levels of carbamazepine can enhance plasmid transfer in both, laboratory experiments (Wang et al., 2018) as well as the collembolan gut microbiome (Wang et al., 2020). Thus, carbamazepine occurrence in GW along with sulfamethoxazole, might additionally accelerate the dissemination of *intI1* and *sul1*. However, further, mechanistically-oriented experiments are needed to disentangle the contributions of selection, co-selection and horizontal gene transfer connected to sulfamethoxazole and carbamazepine pollution on the *sul1* and *intI1* dynamics in GW.

TWW remains a necessary countermeasure for depleting freshwater resources in semi-arid and arid areas (Paranychianakis et al., 2015; Ternes et al., 2007; Maaß & Grundmann, 2016). GW environments are important freshwater reservoir and common freshwater resources. Since we showed here that drug residues are able to persist during soil filtration and reached the GW in high concentrations, we recommend that TWW with regards to GW quality should only be used for irrigation after the successful elimination of drug residues. Further purification of TWW with respect to these contaminants needs to be performed, to reduce their concentration and ensure minimal risks of TWW irrigation to GW environments. This purification can be achieved by low-cost measures (e.g. long hydraulic retention time) (Ejhed et al., 2018), which could further support the aspect of green and circular economy of TWW reuse.

Here, the use of TWW with high absolute bacterial abundance seem to pose little impact on GW quality. However, the overlying soil and especially crops that come into direct contact with TWW bacteria could be impacted more significantly (Libuti el a., 2018; Tripathi et al., 2019, Petousi et al., 2019). Opportunistic pathogens and pathogenic bacteria (e.g. *Salmonella* spp. or *E. coli)* may colonize crops (Sven Jechalke et al., 2015; Araújo et al., 2017), including fresh produce (Blau et al., 2018). For example, total coliform abundance has shown to be

increased on grapes of vineyards irrigated with TWW subjected to secondary treatment (Petousi et al., 2019). The occurrence and survival of enterohemorrhagic *E. coli* of faecal origin in crops has caused severe bloody diarrhea outbreaks across the world, leading to increased hospitalization and death rate (Viazis & Diez-Gonzalez, 2011). However, the accumulation of bacteria on plants is less of a problem, when irrigated crops are directly utilized for biogas production, as bacteria do not reenter the human microbiome through the food chain. Thus, in case crops and especially fresh produce are intended for food consumption, TWW irrigation with high bacterial load still poses several risks for human health (Blau et al., 2018; Petousi et al., 2019).

4.5. Conclusion

In the present study, we confirmed that TWW irrigation promotes the dissemination of the sulfonamide ARG *sul1* along with the integrase gene *intI1* to GW microbiota. Additionally, the correlation of *sul1* relative abundance to elevated total sulfonamide concentration provides a mechanistic explanation behind *sul1*'s successful dissemination. Therefore, further monitoring and reduction of sulfonamides and *sul1* in TWW could minimize the impact on GW environments during TWW irrigation. By overcoming these impacts, the proper use of TWW irrigation as a necessary countermeasure against freshwater and especially GW resources depletion can be ensured.

4.6. References

1) Alygizakis, N.A., Urík, J., Beretsou, V.G., Kampouris, I., Galani, A., Oswaldova, M., Berendonk, T., Oswald, P., Thomaidis, N.S., Slobodnik, J., Vrana, B., Fatta-Kassinos, D., 2020. Evaluation of chemical and biological contaminants of emerging concern in treated wastewater intended for agricultural reuse. Environ. Int. 138, 105597. https://doi.org/10.1016/j.envint.2020.105597
2) Araújo, S., A.T. Silva, I., Tacão, M., Patinha, C., Alves, A., Henriques, I., 2017. Characterization of antibiotic resistant and pathogenic Escherichia coli in irrigation water and vegetables in household farms. Int. J. Food Microbiol. 257, 192–200. https://doi.org/10.1016/j.ijfoodmicro.2017.06.020
3) Avisar, D., Lester, Y., Ronen, D., 2009. Sulfamethoxazole contamination of a deep phreatic aquifer. Sci. Total Environ. 407, 4278–4282. https://doi.org/10.1016/j.scitotenv.2009.03.032
4) Ayni, F. El, Cherif, S., Jrad, A., Trabelsi-Ayadi, M., 2011. Impact of Treated Wastewater Reuse on Agriculture and Aquifer Recharge in a Coastal Area: Korba Case Study. Water Resour. Manag. 25, 2251–2265. https://doi.org/10.1007/s11269-011-9805-2
5) Barber, L.B., Keefe, S.H., Leblanc, D.R., Bradley, P.M., Chapelle, F.H., Meyer, M.T., Loftin, K.A., Kolpin, D.W., Rubio, F., 2009. Fate of sulfamethoxazole, 4-nonylphenol, and 17??-estradiol in groundwater contaminated by wastewater treatment plant effluent. Environ. Sci. Technol. 43, 4843–4850. https://doi.org/10.1021/es803292v
6) Barnes, K.K., Kolpin, D.W., Furlong, E.T., Zaugg, S.D., Meyer, M.T., Barber, L.B., 2008. A national reconnaissance of pharmaceuticals and other organic wastewater contaminants in the United States - I) Groundwater. Sci. Total Environ. 402, 192–200. https://doi.org/10.1016/j.scitotenv.2008.04.028
7) Bengtsson-Palme, J., Larsson, D.G.J., 2016. Concentrations of antibiotics predicted to select for resistant bacteria: Proposed limits for environmental regulation. Environ. Int. 86, 140–149. https://doi.org/10.1016/j.envint.2015.10.015
8) 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., Martinez, J.L., 2015. Tackling antibiotic resistance: the environmental framework. Nat. Rev. Microbiol. 13, 310–317. https://doi.org/10.1038/nrmicro3439
9) Blau, K., Bettermann, A., Jechalke, S., Fornefeld, E., Vanrobaeys, Y., Stalder, T., Top, E., Smalla, K., 2018. The transferable resistome of produce. bioRxiv 9, 1–15. https://doi.org/10.1101/350629
10) Böckelmann, U., Dörries, H.H., Ayuso-Gabella, M.N., De Marçay, M.S., Tandoi, V., Levantesi, C., Masciopinto, C., Houtte, E. Van, Szewzyk, U., Wintgens, T., Grohmann, E., 2009. Quantitative PCR monitoring of antibiotic resistance genes and bacterial pathogens in three european artificial groundwater recharge systems. Appl. Environ. Microbiol. 75, 154–163. https://doi.org/10.1128/AEM.01649-08
11) Böckelmann, U., Dörries, H.H., Ayuso-Gabella, M.N., De Marçay, M.S., Tandoi, V., Levantesi, C., Masciopinto, C., Houtte, E. Van, Szewzyk, U., Wintgens, T., Grohmann, E., 2009. Quantitative PCR monitoring of antibiotic resistance genes and bacterial pathogens in three european artificial groundwater recharge systems. Appl. Environ. Microbiol. 75, 154–163. https://doi.org/10.1128/AEM.01649-08
12) Cacace, D., Fatta-Kassinos, D., Manaia, C.M., Cytryn, E., Kreuzinger, N., Rizzo, L., Karaolia, P., Schwartz, T., Alexander, J., Merlin, C., Garelick, H., Schmitt, H., de Vries,

D., Schwermer, C.U., Meric, S., Ozkal, C.B., Pons, M.N., Kneis, D., Berendonk, T.U., 2019. Antibiotic resistance genes in treated wastewater and in the receiving water bodies: A pan-European survey of urban settings. Water Res. 162, 320–330. https://doi.org/10.1016/j.watres.2019.06.039

13) Caucci, S., Karkman, A., Cacace, D., Rybicki, M., Timpel, P., Voolaid, V., Gurke, R., Virta, M., Berendonk, T.U., 2016. Seasonality of antibiotic prescriptions for outpatients and resistance genes in sewers and wastewater treatment plant outflow. FEMS Microbiol. Ecol. 92, fiw060. https://doi.org/10.1093/femsec/fiw060
14) Cerqueira, F., Matamoros, V., Bayona, J., Elsinga, G., Hornstra, L.M., Piña, B., 2019b. Distribution of antibiotic resistance genes in soils and crops. A field study in legume plants (Vicia faba L.) grown under different watering regimes. Environ. Res. 170, 16–25. https://doi.org/10.1016/j.envres.2018.12.007
15) Cerqueira, F., Matamoros, V., Bayona, J., Piña, B., 2019a. Antibiotic resistance genes distribution in microbiomes from the soil-plant-fruit continuum in commercial Lycopersicon esculentum fields under different agricultural practices. Sci. Total Environ. 652, 660–670. https://doi.org/10.1016/j.scitotenv.2018.10.268
16) Cerqueira, F., Matamoros, V., Bayona, J.M., Berendonk, T.U., Elsinga, G., Hornstra, L.M., Piña, B., 2019c. Antibiotic resistance gene distribution in agricultural fields and crops. A soil-to-food analysis. Environ. Res. 177, 108608. https://doi.org/10.1016/j.envres.2019.108608
17) Clara, M., Strenn, B., Kreuzinger, N., 2004. Carbamazepine as a possible anthropogenic marker in the aquatic environment: Investigations on the behaviour of Carbamazepine in wastewater treatment and during groundwater infiltration. Water Res. 38, 947–954. https://doi.org/10.1016/j.watres.2003.10.058
18) Dalkmann, P., Broszat, M., Siebe, C., Willaschek, E., Sakinc, T., Huebner, J., Amelung, W., Grohmann, E., Siemens, J., 2012. Accumulation of pharmaceuticals, enterococcus, and resistance genes in soils irrigated with wastewater for zero to 100 years in central Mexico. PLoS One 7. https://doi.org/10.1371/journal.pone.0045397
19) Ejhed, H., Fång, J., Hansen, K., Graae, L., Rahmberg, M., Magnér, J., Dorgeloh, E., Plaza, G., 2018. The effect of hydraulic retention time in onsite wastewater treatment and removal of pharmaceuticals, hormones and phenolic utility substances. Sci. Total Environ. 618, 250–261. https://doi.org/10.1016/j.scitotenv.2017.11.011
20) Elkayam, R., Aharoni, A., Vaizel-Ohayon, D., Sued, O., Katz, Y., Negev, I., Marano, R.B.M., Cytryn, E., Shtrasler, L., Lev, O., 2018. Viral and Microbial Pathogens, Indicator Microorganisms, Microbial Source Tracking Indicators, and Antibiotic Resistance Genes in a Confined Managed Effluent Recharge System. J. Environ. Eng. 144. https://doi.org/10.1061/(ASCE)EE.1943-7870.0001334
21) Gatica, J., Tripathi, V., Green, S., Manaia, C.M., Berendonk, T., Cacace, D., Merlin, C., Kreuzinger, N., Schwartz, T., Fatta-Kassinos, D., Rizzo, L., Schwermer, C.U., Garelick, H., Jurkevitch, E., Cytryn, E., 2016. High Throughput Analysis of Integron Gene Cassettes in Wastewater Environments. Environ. Sci. Technol. 50, 11825–11836. https://doi.org/10.1021/acs.est.6b03188
22) Gatica, J., Yang, K., Pagaling, E., Jurkevitch, E., Yan, T., Cytryn, E., 2015. Resistance of undisturbed soil microbiomes to ceftriaxone indicates extended spectrum β-lactamase activity. Front. Microbiol. 6, 1–11. https://doi.org/10.3389/fmicb.2015.01233
23) Gillings, M.R., Gaze, W.H., Pruden, A., Smalla, K., Tiedje, J.M., Zhu, Y.G., 2015. Using the class 1 integron-integrase gene as a proxy for anthropogenic pollution. ISME J. 9, 1269–1279. https://doi.org/10.1038/ismej.2014.226

24) Gillings, M.R., Gaze, W.H., Pruden, A., Smalla, K., Tiedje, J.M., Zhu, Y.G., 2015. Using the class 1 integron-integrase gene as a proxy for anthropogenic pollution. ISME J. 9, 1269–1279. https://doi.org/10.1038/ismej.2014.226
25) Guo, W., Andersen, M.N., Qi, X. bin, Li, P., Li, Z. Fan, X., Zhou, Y., 2017. Effects of reclaimed water irrigation and nitrogen fertilization on the chemical properties and microbial community of soil. J. Integr. Agric. 16, 679–690. https://doi.org/10.1016/S2095-3119(16)61391-6
26) Han, X.M., Hu, H.W., Shi, X.Z., Wang, J.T., Han, L.L., Chen, D., He, J.Z., 2016. Impacts of reclaimed water irrigation on soil antibiotic resistome in urban parks of Victoria, Australia. Environ. Pollut. 211, 48–57. https://doi.org/10.1016/j.envpol.2015.12.033
27) Jechalke, S., Broszat, M., Lang, F., Siebe, C., Smalla, K., Grohmann, E., 2015. Effects of 100 years wastewater irrigation on resistance genes, class 1 integrons and IncP-1 plasmids in Mexican soil. Front. Microbiol. 6, 1–10. https://doi.org/10.3389/fmicb.2015.00163
28) Jechalke, S., Schierstaedt, J., Becker, M., Flemer, B., Grosch, R., Smalla, K., Schikora, A., 2019. Salmonella establishment in agricultural soil and colonization of crop plants depend on soil type and plant species. Front. Microbiol. 10, 1–17. https://doi.org/10.3389/fmicb.2019.00967
29) Kampouris, I.D., Agrawal, S., Orschler, L., Cacace, D., Kunze, S., Berendonk, T.U., Klümper, U., 2021a. Antibiotic resistance gene load and irrigation intensity determine the impact of wastewater irrigation on antimicrobial resistance in the soil microbiome. Water Res. 116818. https://doi.org/10.1016/j.watres.2021.116818
30) Kampouris, I.D., Klümper, U., Agrawal, S., Orschler, L., Cacace, D., Kunze, S., Berendonk, T.U., 2021b. Treated wastewater irrigation promotes the spread of antibiotic resistance into subsoil pore-water. Environ. Int. 146. https://doi.org/10.1016/j.envint.2020.106190
31) Klümper, U., Dechesne, A., Riber, L., Brandt, K.K., Gülay, A., Sørensen, S.J., Smets, B.F., 2017. Metal stressors consistently modulate bacterial conjugal plasmid uptake potential in a phylogenetically conserved manner. ISME J. 11, 152–165. https://doi.org/10.1038/ismej.2016.98
32) Lesser, L.E., Mora, A., Moreau, C., Mahlknecht, J., Hernández-Antonio, A., Ramírez, A.I., Barrios-Piña, H., 2018. Survey of 218 organic contaminants in groundwater derived from the world's largest untreated wastewater irrigation system: Mezquital Valley, Mexico. Chemosphere 198, 510–521. https://doi.org/10.1016/j.chemosphere.2018.01.154
33) Libutti, A., Gatta, G., Gagliardi, A., Vergine, P., Pollice, A., Beneduce, L., Disciglio, G., Tarantino, E., 2018. Agro-industrial wastewater reuse for irrigation of a vegetable crop succession under Mediterranean conditions. Agric. Water Manag. 196, 1–14. https://doi.org/10.1016/j.agwat.2017.10.015
34) Maaß, O., Grundmann, P., 2016. Added-value from linking the value chains of wastewater treatment, crop production and bioenergy production: A case study on reusing wastewater and sludge in crop production in Braunschweig (Germany). Resour. Conserv. Recycl. 107, 195–211. https://doi.org/10.1016/j.resconrec.2016.01.002
35) Marano, R.B.M., Zolti, A., Jurkevitch, E., Cytryn, E., 2019. Antibiotic resistance and class 1 integron gene dynamics along effluent, reclaimed wastewater irrigated soil, crop continua: elucidating potential risks and ecological constraints. Water Res. 164, 114906. https://doi.org/10.1016/j.watres.2019.114906

36) McMurdie and Holmes, 2013. Phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. PLoS ONE 8. doi:10.1371/journal.pone.0061217
37) Orschler, L., Agrawal, S., Lackner, S., 2019. On resolving ambiguities in microbial community analysis of partial nitritation anammox reactors. Sci. Rep. 1–10. https://doi.org/10.1038/s41598-019-42882-8
38) Petousi, I., Daskalakis, G., Fountoulakis, M.S., Lydakis, D., Fletcher, L., Stentiford, E.I., Manios, T., 2019. Effects of treated wastewater irrigation on the establishment of young grapevines. Sci. Total Environ. 658, 485–492. https://doi.org/10.1016/j.scitotenv.2018.12.065
39) R Core Team, 2019. R: A language and environment for statistical computing, R Foundation for Statistical Computing, Vienna, Austria. http://www.r-project.org/index.html
40) Reis, P.J.M., Homem, V., Alves, A., Vilar, V.J.P., Manaia, C.M., Nunes, O.C., 2018. Insights on sulfamethoxazole bio-transformation by environmental Proteobacteria isolates. J. Hazard. Mater. 358, 310–318. https://doi.org/10.1016/j.jhazmat.2018.07.012
41) Rocha, J., Cacace, D., Kampouris, I., Guilloteau, H., Jäger, T., Marano, R.B.M., Karaolia, P., Manaia, C.M., Merlin, C., Fatta-Kassinos, D., Cytryn, E., Berendonk, T.U., Schwartz, T., 2020. Inter-laboratory calibration of quantitative analyses of antibiotic resistance genes. J. Environ. Chem. Eng. 8, 102214. https://doi.org/10.1016/j.jece.2018.02.022
42) Smalla, K., Cook, K., Djordjevic, S.P., Klümper, U., Gillings, M., 2018. Environmental dimensions of antibiotic resistance: assessment of basic science gaps. FEMS Microbiol. Ecol. 94, 1–6. https://doi.org/10.1093/femsec/fiy195
43) Szekeres, E., Chiriac, C.M., Baricz, A., Szőke-Nagy, T., Lung, I., Soran, M.L., Rudi, K., Dragos, N., Coman, C., 2018. Investigating antibiotics, antibiotic resistance genes, and microbial contaminants in groundwater in relation to the proximity of urban areas. Environ. Pollut. 236, 734–744. https://doi.org/10.1016/j.envpol.2018.01.107
44) Ternes, T.A., Bonerz, M., Herrmann, N., Teiser, B., Andersen, H.R., 2007. Irrigation of treated wastewater in Braunschweig, Germany: An option to remove pharmaceuticals and musk fragrances. Chemosphere 66, 894–904. https://doi.org/10.1016/j.chemosphere.2006.06.035
45) Tripathi, V.K., Rajput, T.B.S., Patel, N., Nain, L., 2019. Impact of municipal wastewater reuse through micro-irrigation system on the incidence of coliforms in selected vegetable crops. J. Environ. Manage. 251, 109532. https://doi.org/10.1016/j.jenvman.2019.109532
46) Underwood, J.C., Harvey, R.W., Metge, D.W., Repert, D.A., Baumgartner, L.K., Smith, R.L., Roane, T.M., Barber, L.B., 2011. Effects of the antimicrobial sulfamethoxazole on groundwater bacterial enrichment. Environ. Sci. Technol. 45, 3096–3101. https://doi.org/10.1021/es103605e
47) Underwood, J.C., Harvey, R.W., Metge, D.W., Repert, D.A., Baumgartner, L.K., Smith, R.L., Roane, T.M., Barber, L.B., 2011. Effects of the antimicrobial sulfamethoxazole on groundwater bacterial enrichment. Environ. Sci. Technol. 45, 3096–3101. https://doi.org/10.1021/es103605e
48) Viazis, S., Diez-Gonzalez, F., 2011. Enterohemorrhagic Escherichia coli. The Twentieth Century's Emerging Foodborne Pathogen: A Review, 1st ed, Advances in Agronomy. Elsevier Inc. https://doi.org/10.1016/B978-0-12-387689-8.00006-0

49) Wang, F.H., Qiao, M., Su, J.Q., Chen, Z., Zhou, X., Zhu, Y.G., 2014. High throughput profiling of antibiotic resistance genes in urban park soils with reclaimed water irrigation. Env. Sci Technol 48, 9079–9085. https://doi.org/10.1021/es502615e
50) Wang, Y., Lu, J., Mao, L., Li, J., Yuan, Z., Bond, P.L., Guo, J., 2019. Antiepileptic drug carbamazepine promotes horizontal transfer of plasmid-borne multi-antibiotic resistance genes within and across bacterial genera. ISME J. 13, 509–522. https://doi.org/10.1038/s41396-018-0275-x
51) Wang, Y.F., Qiao, M., Zhu, D., Zhu, Y.G., 2020. Antibiotic Resistance in the Collembolan Gut Microbiome Accelerated by the Nonantibiotic Drug Carbamazepine. Environ. Sci. Technol. 54, 10754–10762. https://doi.org/10.1021/acs.est.0c03075
52) Wickham, H. 2016, ggplot2: Elegant Graphics for Data Analysis, Springer.
53) Kassambara, A., 2019. ggpubr R Package: ggplot2-Based Publication Ready Plots R package version 0.2.3 https://CRAN.R-project.org/package=ggpubr.
54) Dinno, A., 2016. dunn's test R package: Dunn's Test of Multiple Comparisons Using Rank Sums. R package version. 0.2.3 https://cran.r-project.org/web/packages/dunn.test/index.html

Chapter 5

5. Synthesis: Integration and Future Perspectives

5.1 High intensity treated wastewater irrigation can promote the spread of antibiotic resistance in soil and other deeper-lying environments.

TWW irrigation remains a valuable alternative to counter freshwater (FW) resources depletion (Paranychianakis et al., 2015; Maaß et al., 2016). Yet, despite its necessity, the increasing use of TWW irrigation has raised concerns, especially regarding the dissemination of drug residues and biological contaminants, like antibiotic resistant (ARB) and antibiotic resistance genes (ARGs), in soil and crops (Christou et al., 2017; Krzeminski et al., 2019; Piña et al., 2020). Alternatives such as the Braunschweig Wastewater Association (BWA) model, utilize TWW irrigated crops for biogas production, while preventing the direct dissemination of ARB/ARGs through fresh produce consumption (Ternes et al., 2007; Maaß et al., 2016). However, ARGs can potentially be transferred to soil bacteria (Klümper et al., 2015) and from soil bacteria back to human-associated commensal or pathogenic bacteria (Forsberg et al., 2012). Thus, the increase in ARG levels in the soil might lead to increased transfer of ARGs to human associated microbiota, including commensal and pathogenic strains.

So far, many studies have investigated the impact of TWW irrigation on ARG abundance in soil. However, they reported contradicting statements on whether TWW irrigation promotes ARG dissemination in soil microbiota (Dalkmann et al., 2012; Chen et al., 2014; Wang et al., 2014; Han et al., 2016; Negreanu et al., 2012; Caucci et al., 2016, Cerqueira et al., 2019a, Cerqueira et al., 2019b; Marano et al., 2019). In **Chapter 2**, I attempted to fill part of the missing gaps, with investigating the most crucial factor that may have resulted in the observed differences, the total ARG load that a field receives. Since, irrigation intensity over time and the ARG load of the irrigation water affect the total ARG load that soils receive, I hypothesized that *the irrigation intensity and the ARG load define the impact of TWW irrigation in the soil microbiome* (**Hypothesis 1**). To test **Hypothesis 1**, I performed long-term sampling in a full-scale, commercially operated, TWW-irrigated field and controlled laboratory experiments.

Specifically, the long-term sampling showed that the in-/decrease of ARGs and *intI1* due to TWW irrigation varied across different genes in dependence with their abundance in the irrigation water. In the controlled soil microcosm experiments, the ARG increase remained higher during continuous TWW irrigation, for those genes that occurred in higher relative abundance in the TWW, in comparison with FW irrigation. Consequently, the **Hypothesis 1**

was strongly supported. These three different experiments in **Chapter 2** shed light on the temporal ARG dynamics in the soil as a consequence of TWW irrigation: ARG abundances remain dynamic, dependent on irrigation intensity and decreased during irrigation breaks. Despite their significant decrease during irrigation breaks, the levels of ARGs constantly remained far above background levels detected for non-irrigated soil. Therefore, once irrigation introduces ARGs in soil, they do not disappear quickly when irrigation is ceased, once they are established in soil microbiome. Furthermore, TWW irrigation affected the ARG abundance in soil already within a short time-frame. Increased ARG levels can be detected after short time spans on the week to month scale (**Chapter 2**).

Moreover, anthropogenic processes can potentially affect not only soil but also subsoil and groundwater (GW) microbiota (Szekeres et al., 2018, Rossi et al., 2019). GW reservoirs provide drinking water resources in many areas of the world; thus the spread of ARG through infiltration of TWW to GW could raise concerns. However, only a few studies have investigated the effect of TWW irrigation on the ARG abundance in subsoil/GW-related environments, up to now. One of the studies reported that TWW irrigation increased ARG abundance in the subsoil of microcosms, in dependence with the water flow paths (Lüneberg et al., 2018). Since the exact impact of irrigation on environments related to GW remained unclear, I attempted to fill the missing gaps in **Chapters 3 & 4**. Specifically, I investigated whether *TWW irrigation promotes ARG and intI1 abundance in the subsoil pore-wate*r *(SPW)* (**Hypothesis 2, Chapter 3**), using a similar multiphase approach as the one described in **Chapter 2**. The percolated SPW is a crucial part for the GW recharge. If ARGs increase in the SPW, they might reach to GW environments. To the best of my knowledge, **Chapter 3** describes the first study that investigated the impact of TWW irrigation on the profile of ARGs in this so far neglected environment.

In SPW of lysimeters, the relative abundance of *sul1*, *intI1, qnrS* and $bla_{\text{OXA-58}}$ positively correlated with the TWW irrigation intensity, during the long-term sampling (**Chapter 3**). All these genes positively correlated with irrigation intensity in the topsoil as well (**Chapter 2**), indicating a causal link. The analysis of collected SPW from controlled microcosm experiments in **Chapter 2** confirmed this causal link between soil and SPW**.** ARGs increased in SPW (**Chapter 3**), in dependence on their increase in soil (**Chapter 2**). Therefore, **Hypothesis 2** was supported, in both, full-scale TWW-irrigated field investigations and microcosm experiments. The combined results from **Chapter 2 & 3** suggest that if ARGs increase in the soil

microbiome, they will ultimately increase in SPW microbiome as well. Consequently, apart from the ARG abundance in soil, high intensity TWW irrigation can promote ARG spread in deeper lying SPW.

While TWW irrigation clearly promoted ARG spread in soil and SPW, I considered it a necessity to further investigate whether TWW irrigation can affect the even deeper lying GW as well. Thus in **Chapter 4** I sampled the GW (depth 10 m) below the TWW irrigated field over the various periods of irrigation. In GW, the impact of TWW irrigation differed slightly compared to soil and SPW. TWW irrigation mainly promoted the spread of the ARG *sul1* and the integrase gene *intI1* in GW, similarly to soil and SPW. The remainder of previously reported TWW-related ARGs (**Chapter 2 & 3**) did not increase constantly over the duration of irrigation. However, both *sul1* and *intI1* showed the highest abundance over TWW irrigation in the soil and SPW microbiota as well. Generally, over the long-term sampling campaigns and through the microcosms, I observed a consistent trend and causal link for the increase of these two genes, in all three different environments.

The correlation of *sul1* and *intI1* with irrigation intensity in these three environments strongly indicates that horizontal gene transfer (HGT) between TWW and soil/subsoil bacteria played a major role in the observed increase in ARGs in soil/SPW/GW microbiota. These two genes were the most abundant in the here used TWW irrigation, and are generally found at high levels in TWW across several European countries (Cacace et al., 2019, Pärnänen et al., 2019; Alygizakis et al., 2019). The ARG *sul1* confers resistance to sulfonamides, a group of synthetic antibiotics that persist through the wastewater treatment, usually occurring in high concentration in TWWs (Rizzo et al., 2013, Zhang and Li, 2011). The *intI1* gene is commonly considered as an indicator of horizontal gene transfer and anthropogenic pollution (Gillings et al., 2015) due to its association with MGEs. *intI1* gene cassettes often contain ARGs or other genes related to overcoming various stress conditions, with *sul1* frequently being part of these gene cassettes (Gillings et al., 2015). Furthermore, these gene cassettes can be located on MGEs and conjugative plasmids (Gillings et al., 2015; Gillings, 2017), which have the ability to transfer to a majority of the diverse bacterial phyla found in soil (Klümper et al., 2015). Consequently, their parallel increase in abundance is most likely connected to them being co-located on MGEs that get co-transferred and co-selected.

Herein, FW contained all ARGs and the integrase gene *intI1* as well, but in far lower relative abundance for most ARGs, than the TWW irrigation water. In microcosms, FW irrigation

introduced *sul1* into the soil microbiome of the non-irrigated soil. Thus, even irrigation with higher quality water can introduce certain ARGs in the soil. I assume that long-term FW irrigation in theory could lead to an enrichment of ARGs, such as *sul1,* in the soil and hence the SPW and GW microbiota. Furthermore, the TWW that was used for irrigation in the full-scale field and the microcosm experiments, was subjected only to secondary biological treatment. The use of TWW subjected to tertiary or advanced treatment would potentially reduce the ARG load of the TWW (Michael et al., 2013). Similarly, the use of FW irrigation with a higher load of ARGs (due to general anthropogenic pollution) would eliminate any observable differences of impact between FW and TWW irrigation. In conclusion, I consider that the estimation of ARG abundance in FW and TWW should be regarded as a crucial move, prior the estimation of the irrigation effect on ARG abundance in soil and deeper-lying microbiota. Furthermore, the β-lactamase genes bla_{TEM} and $bla_{\text{CTX-M-32}}$, did not significantly increase with TWW irrigation, while they showed high prevalence in the non-irrigated soil, confirming their previous association with the native soil resistome (Gatica et al., 2015). Additionally, their relative abundance did not differ significantly between TWW and FW. Presumably, these two genes are either part of the native environmental resistome or they have become naturalized after they widely spread and disseminated in the environment.

5.2 Persistence of sulfamethoxazole and carbamazepine in the groundwater during treated wastewater irrigation operation.

Environmental surveys in USA, Asia and Europe have reported that sulfonamide antibiotics and the anticonvulsant carbamazepine persist and potentially accumulate in GW (Ternes et al., 2007; Barber et al., 2008; Avisar et al., 2009; Underwood et al., 2011; Szeckeres et al., 2018; Lesser et al., 2018). Especially phreatic aquifers contained high concentrations of the antibiotic sulfamethoxazole (Avisar et al., 2009). Since *sul1* confers resistance to sulfonamides, the reported persistence of these sulfonamides could contribute to the relative success of *sul1* dissemination in GW, in comparison with the rest of the ARGs. Thus, I hypothesized that *TWW irrigation increases ARG abundance in GW through the accumulation of antibiotics in GW* (**Hypothesis 3**).

To test this, LC/MS-MS was performed on GW samples, to reveal potential connections of ARG spread and persistence of antibiotics in the GW (**Chapter 4**). Indeed, I confirmed the high persistence of the antibiotic sulfamethoxazole in GW, which increased significantly from one to three months of irrigation. In addition, the GW wells contained elevated carbamazepine concentrations, but these did not increase over the duration of irrigation. Sulfamethoxazole concentrations increased along with *sul1* relative abundance, yet their correlation was barely significant, since the increase rate differed greatly over the GW wells. Presumably, in these low concentrations of sulfamethoxazole, the selection due to antibiotics slightly contributes to *sul1*'s successful dissemination. Thus, the **Hypothesis 3** is weakly supported, by the present results. Nevertheless, these surprisingly high concentrations of sulfamethoxazole confirm the previous concerns regarding the concerning persistence and accumulation of sulfamethoxazole and carbamazepine in GW during TWW infiltration in the soil (Avisar et al., 2009; Underwood et al., 2011; Szeckeres et al., 2018; Lesser et al., 2018).

5.3 Minimal passage of treated-wastewater bacteria to groundwater due to high bacterial density in soil.

Aside from the introduction of ARGs and *intI1* into the soil, the results from this thesis shed light on whether TWW irrigation increases the total bacterial load of soil, SPW and GW environments. Specifically, the soil microcosm experiments demonstrated that even without vegetation, the watered soil contained already log-fold higher abundance than the TWW or FW irrigation water (**Chapter 2**). This high absolute abundance did not show any increase due to switching to TWW irrigation. In addition, 16S rRNA amplicon sequencing confirmed qPCR results, indicating low persistence of TWW-related bacteria in soil.

Similarly, in SPW of the TWW irrigated field, no increase in absolute bacterial abundance occurred due to TWW irrigation (**Chapter 3**). In the microcosms, the SPW contained high bacterial loads, independently of the irrigation type (FW or TWW). Presumably, the majority of bacteria in the percolated SPW originated from soil, which has log-fold higher absolute abundance (**Chapter 2**), when compared to the FW and TWW irrigation water. Furthermore, sequencing results revealed again a low introduction of TWW related bacteria in subsoil pore-water. This negligible persistence of TWW-related bacteria became apparent in the GW, where

no increase of absolute bacterial abundance occurred during TWW irrigation (**Chapter 4**). The 16s rRNA sequencing results confirmed that no TWW-related bacterial taxa increased during TWW irrigation in either, subsoil pore-water or GW. Previously, the leaching process was described as a main source of the microbial load in lysimeter samples (Forslund et al., 2011). In contrast, the results of this thesis do not confirm the assumed leaching scenarios. The negligible effect of TWW irrigation on soil/SPW/GW bacterial load, along with the increase of *intI1* gene support horizontal gene transfer as the main mechanism of ARG dissemination from TWW to soil bacteria and eventually to SPW and GW bacteria.

5.4 Absolute bacterial abundance of subsoil pore-water and groundwater fluctuates over long-term periods of irrigation.

In the present thesis, the absolute bacterial abundance of both, SPW and GW of the TWW irrigated field, showed similar fluctuations during TWW irrigation operation (**Chapter 3 & 4**). Specifically, the SPW contained a low absolute bacterial load during high intensity irrigation, which increased after long irrigation breaks in the winter. Similarly, the GW absolute bacterial abundance decreased by two orders of magnitude during TWW irrigation performance. So far, no study has explored the long-term absolute abundance dynamics of subsurface microbiota in a full-scale agricultural field. This thesis contains the first studies that investigated the SPW and GW absolute bacterial dynamics over time, in a full-scale agricultural system.

Even though these fluctuations in SPW and GW hinted at a potential toxicity-effect due to TWW irrigation, this trend was not confirmed in the SPW of the microcosms. Consequently, I consider that more research is needed to elucidate whether this was a toxicity effect to subsoil/GW microbiota, or if it was connected to their natural annual dynamics over irrigation operations. Herein I presented a valuable tool to investigate deeper-lying microbiota, the soil/SPW microcosms, which, with a few modifications, could simulate GW environments as well. Long-term experiments with these microcosms could help to understand the basic ecological dynamics of subsoil, SPW and GW microbiota and further understand the potential impact of irrigation.

5.5 Implications for agricultural operation of treated wastewater irrigation.

TWW irrigation use remains crucial for countering the FW resources depletion, while the demand for alternative FW resources will only increase in the future (Paranychianakis et al., 2015; Maaß et al., 2016). Yet identifying the potential issues that may arise due to the absence of regulations during extensive TWW irrigation can only help to improve the sustainability of TWW irrigation (Christou et al., 2017, Krzeminski et al., 2019; Piña et al., 2020). The present thesis demonstrated clearly that irrigation with TWW subjected only to secondary biological wastewater treatment could promote the ARG dissemination in downstream environments.

Nevertheless, the use of crops as biogas can prevent the potential direct exposure of humans to TWW irrigated crops (Ternes et al., 2007; Paranychianakis et al., 2015; Maaß et al., 2016). Yet, the impact was not restricted only to soil microbiota, but TWW irrigation affected the deeper lying so-far neglected microbiota. Especially the GW serves as the most important drinking water reservoir, thus special care should be given to GW environments (Christou et al., 2017, Szeckeres et al., 2018; Krzeminski et al., 2019; Piña et al., 2020). The sulfonamide ARG *sul1* showed an equally successful dissemination from topsoil to the deep GW. Generally, *sul1* is a widespread ARG, detected even in tap water (Hao et al., 2019). Even during the present thesis, the FW irrigation water, collected from a GW well near Dresden, contained the *sul1* gene (**Chapter 2 & 3**). Probably the capability of *sul1* to quickly disseminate in topsoil and GW microbiota might have assisted its wide spread in the environment. Furthermore, the GW of the TWW irrigated field contained elevated concentrations of the antibiotic sulfamethoxazole along with the anticonvulsant carbamazepine. Since GW remains the most important freshwater resources, the accumulation of these two compounds along with *sul1* might raise concerns.

Currently, the European Union lacks common guideline criteria for treatment prior to wastewater irrigation (Paranychianakis et al., 2015). The implementation of tertiary and advanced treatment could lead to a reduction in antibiotics and ARGs (Michael et al., 2013; Christou et al., 2017). For example, advanced oxidation treatment or extremely long hydraulic retention time prior to irrigation can lead to this purification (Michael et al., 2013; Christou et al., 2017; Ejhed et al., 2018). These treatments can reduce the concentration of the GW

persistent contaminants providing higher quality of TWW irrigation, however, even though TWW irrigation increased ARG abundance in the soil and deeper-lying environments, the exact risk due the spread of ARGs in soil, SPW and GW remains unknown. The same applies to the elevated sulfonamide and carbamazepine persistence in the GW. Thus, prior to the implementation of any guidelines, I consider the long-term and extensive monitoring of ARG abundance (especially sulfonamide ARGs) and sulfonamide antibiotics in soil and deeper-lying environments of agricultural settings an urgent requirement. As demonstrated here, long-term monitoring along with mechanistic experiments can provide sufficient data, which can be implemented for the generation of risk assessments in the future.

5.6 Closing Conclusions.

Overall, TWW irrigation increased the relative abundance of specific genes associated with AMR in soil, SPW and GW microbiota. The impact of TWW irrigation depended on the ARG and *intI1* load of irrigation water, the irrigation intensity, the type of each gene and its potential link with the native soil resistome. Combining microcosm approaches with long-term studies on full-scale, commercially operated, TWW irrigated fields proved to be a successful research tool to study irrigation effects not only in the soil, but also in the SPW environment. Any type of irrigation (FW or TWW) could lead to the dissemination of ARGs in soil (e.g. *sul1*) and potentially to the SPW and GW. Nevertheless, the impact of FW irrigation showed distinctively minor impact on ARG and *intI1* profile of soil and SPW microbiota, when compared to the TWW irrigation with a high ARG and *intI1* load.

Furthermore, TWW irrigation highly promoted *sul1* and *intI1* dissemination in the GW of the full-scale TWW-irrigated field. This GW contained elevated concentrations of the sulfonamide antibiotics and the anticonvulsant carbamazepine, suggesting the accumulation of these compounds due to irrigation practices. The correlation of *sul1* relative abundance to elevated total sulfonamide concentrations indicates that the accumulation of sulfonamides contributed to the successful dissemination of *sul1* in the GW. High-intensity irrigation with TWW subjected to secondary biological treatment, promotes ARG spread in soil and deeper-lying environments with great importance such as the GW reservoirs, while leading to infiltration of certain antibiotics and pharmaceuticals. The use of TWW subjected to further treatment could

reduce the release of carbamazepine, sulfamethoxazole and sulfonamide-ARGs in soil, SPW and GW environments. This would minimize the potential risks associated with AMR spread from the practice of TWW irrigation and improve the sustainability of TWW reuse.

5.7 Future Perspectives.

Herein, I performed long-term samplings, while in parallel developing soil/SPW microcosms that allowed the observation of the soil and SPW. With further modifications (e.g. higher length, enclosed system), this design has the potential to simulate GW environments as well. The use of such microcosms/mesocosms could provide a successful research tool to gain further insights into the effect of exact parameters, in the future with a more mechanistic approach. For example, the microbial communities of different soil types could be exposed in TWWs. This includes considering complex mutualistic and antagonistic interactions occurring in the soil, SPW and GW microbiota, such as influence of agricultural crops on the soil bacterial communities through root exudates (Chen et al., 2019).

Interactions in the rhizosphere have previously been shown to lead to either positive (Jechalke et al., 2013) or negative (Song et al., 2020) selection for ARGs in agricultural soils. Additionally, the effect of root exudates and crops to SPW or GW bacteria could be investigated more mechanistically, with the use of these microcosms. Nonetheless, the use of microcosm-experiments along with long-term monitoring of ARG-abundance (and/or microbiome) could find applications beyond the practice of TWW irrigation. For example, this combination could help to further mechanistically disentangle the impact of other agricultural practices as well, such as manure amendment or pesticide application, on the soil, SPW and GW microbiome or resistome. Consequently, further research, combining microcosms and long-term samplings, would help to further elucidate this complex interplay of soil/subsoil biotic and abiotic factors, with agricultural practices and ARG spread in not only soil, but also SPW and GW environments.

In addition, the GW of the real-scale TWW irrigated field contained high sulfonamide concentrations, which accumulated due to TWW irrigation. Sulfonamides, a class of synthetic antibiotics, have low biodegradation rates in the environment, with sulfamethoxazole being the prominent example (Underwood et al., 2011). This accumulation of sulfonamide antibiotics

could explain the successful spread of *sul1* gene in GW, which confers resistance to sulfonamides. This is of particular importance as *sul1*, through its association with integron cassettes, has a high co-selection potential (Nunes et al., 2020). Some environmental microorganisms are able to transform sulfonamides (Bouju et al., 2012; Reis et al., 2014). However, the transformation products may still possess antibiotic function (Achermann et al., 2018). Hence, research into the accumulation of sulfonamide antibiotics and their active transformation products could provide precise data on selection for sulfonamide resistance and co-selection of other ARGs in GW microbiota.

5.8 References

1) Achermann, S., Bianco, V., Mansfeldt, C.B., Vogler, B., Kolvenbach, B.A., Corvini, P.F.X., Fenner, K., 2018. Biotransformation of Sulfonamide Antibiotics in Activated Sludge: The Formation of Pterin-Conjugates Leads to Sustained Risk. Environ. Sci. Technol. 52, 6265–6274. https://doi.org/10.1021/acs.est.7b06716
2) Alygizakis, N.A., Urík, J., Beretsou, V.G., Kampouris, I., Galani, A., Oswaldova, M., Berendonk, T., Oswald, P., Thomaidis, N.S., Slobodnik, J., Vrana, B., Fatta-Kassinos, D., 2020. Evaluation of chemical and biological contaminants of emerging concern in treated wastewater intended for agricultural reuse. Environ. Int. 138, 105597. https://doi.org/10.1016/j.envint.2020.105597
3) Avisar, D., Lester, Y., Ronen, D., 2009. Sulfamethoxazole contamination of a deep phreatic aquifer. Sci. Total Environ. 407, 4278–4282. https://doi.org/10.1016/j.scitotenv.2009.03.032
4) Barber, L.B., Keefe, S.H., Leblanc, D.R., Bradley, P.M., Chapelle, F.H., Meyer, M.T., Loftin, K.A., Kolpin, D.W., Rubio, F., 2009. Fate of sulfamethoxazole, 4-nonylphenol, and 17??-estradiol in groundwater contaminated by wastewater treatment plant effluent. Environ. Sci. Technol. 43, 4843–4850. https://doi.org/10.1021/es803292v
5) Bouju, H., Ricken, B., Beffa, T., Corvini, P.F.X., Kolvenbach, B.A., 2012. Isolation of bacterial strains capable of sulfamethoxazole mineralization from an acclimated membrane bioreactor. Appl. Environ. Microbiol. 78, 277–279. https://doi.org/10.1128/AEM.05888-11
6) Cacace, D., Fatta-Kassinos, D., Manaia, C.M., Cytryn, E., Kreuzinger, N., Rizzo, L., Karaolia, P., Schwartz, T., Alexander, J., Merlin, C., Garelick, H., Schmitt, H., de Vries, D., Schwermer, C.U., Meric, S., Ozkal, C.B., Pons, M.N., Kneis, D., Berendonk, T.U., 2019. Antibiotic resistance genes in treated wastewater and in the receiving water bodies: A pan-European survey of urban settings. Water Res. 162, 320–330. https://doi.org/10.1016/j.watres.2019.06.039
7) Caucci, S., Karkman, A., Cacace, D., Rybicki, M., Timpel, P., Voolaid, V., Gurke, R., Virta, M., Berendonk, T.U., 2016. Seasonality of antibiotic prescriptions for outpatients and resistance genes in sewers and wastewater treatment plant outflow. FEMS Microbiol. Ecol. 92, fiw060. https://doi.org/10.1093/femsec/fiw060
8) Cerqueira, F., Matamoros, V., Bayona, J., Elsinga, G., Hornstra, L.M., Piña, B., 2019b. Distribution of antibiotic resistance genes in soils and crops. A field study in legume plants (Vicia faba L.) grown under different watering regimes. Environ. Res. 170, 16–25. https://doi.org/10.1016/j.envres.2018.12.007
9) Cerqueira, F., Matamoros, V., Bayona, J., Piña, B., 2019a. Antibiotic resistance genes distribution in microbiomes from the soil-plant-fruit continuum in commercial Lycopersicon esculentum fields under different agricultural practices. Sci. Total Environ. 652, 660–670. https://doi.org/10.1016/j.scitotenv.2018.10.268
10) Chen, C., Li, J., Chen, P., Ding, R., Zhang, P., Li, X., 2014. Occurrence of antibiotics and antibiotic resistances in soils from wastewater irrigation areas in Beijing and Tianjin, China. Environ. Pollut. 193, 94–101. https://doi.org/10.1016/j.envpol.2014.06.005
11) Chen, Q.L., Cui, H.L., Su, J.Q., Penuelas, J., Zhu, Y.G., 2019. Antibiotic Resistomes in Plant Microbiomes. Trends Plant Sci. 24, 530–541. https://doi.org/10.1016/j.tplants.2019.02.010

12) Christou, A., Karaolia, P., Hapeshi, E., Michael, C., Fatta-Kassinos, D., 2017. Long-term wastewater irrigation of vegetables in real agricultural systems: Concentration of pharmaceuticals in soil, uptake and bioaccumulation in tomato fruits and human health risk assessment. Water Res. 109, 24–34. https://doi.org/10.1016/j.watres.2016.11.033
13) Dalkmann, P., Broszat, M., Siebe, C., Willaschek, E., Sakinc, T., Huebner, J., Amelung, W., Grohmann, E., Siemens, J., 2012. Accumulation of pharmaceuticals, enterococcus, and resistance genes in soils irrigated with wastewater for zero to 100 years in central Mexico. PLoS One 7. https://doi.org/10.1371/journal.pone.0045397
14) Ejhed, H., Fång, J., Hansen, K., Graae, L., Rahmberg, M., Magnér, J., Dorgeloh, E., Plaza, G., 2018. The effect of hydraulic retention time in onsite wastewater treatment and removal of pharmaceuticals, hormones and phenolic utility substances. Sci. Total Environ. 618, 250–261. https://doi.org/10.1016/j.scitotenv.2017.11.011
15) Forsberg, K.J., Reyes, A., Wang, B., Selleck, E.M., Sommer, M.O.A., Dantas, G., 2012. The Shared Antibiotic Resistome of Soil Bacteria and Human Pathogens. Science (80-.). 337, 1107–1111. https://doi.org/10.1126/science.1220761
16) Forslund, A., Plauborg, F., Andersen, M.N., Markussen, B., Dalsgaard, A., 2011. Leaching of human pathogens in repacked soil lysimeters and contamination of potato tubers under subsurface drip irrigation in Denmark. Water Res. 45, 4367–4380. https://doi.org/10.1016/j.watres.2011.05.009
17) Gatica, J., Yang, K., Pagaling, E., Jurkevitch, E., Yan, T., Cytryn, E., 2015. Resistance of undisturbed soil microbiomes to ceftriaxone indicates extended spectrum β-lactamase activity. Front. Microbiol. 6, 1–11. https://doi.org/10.3389/fmicb.2015.01233
18) Gillings, M.R., 2017. Class 1 integrons as invasive species. Curr. Opin. Microbiol. 38, 10–15. https://doi.org/10.1016/j.mib.2017.03.002
19) Gillings, M.R., Gaze, W.H., Pruden, A., Smalla, K., Tiedje, J.M., Zhu, Y.G., 2015. Using the class 1 integron-integrase gene as a proxy for anthropogenic pollution. ISME J. 9, 1269–1279. https://doi.org/10.1038/ismej.2014.226
20) Han, X.M., Hu, H.W., Shi, X.Z., Wang, J.T., Han, L.L., Chen, D., He, J.Z., 2016. Impacts of reclaimed water irrigation on soil antibiotic resistome in urban parks of Victoria, Australia. Environ. Pollut. 211, 48–57. https://doi.org/10.1016/j.envpol.2015.12.033
21) Hao, H., Shi, D. yang, Yang, D., Yang, Z. wei, Qiu, Z. gang, Liu, W. li, Shen, Z. qiang, Yin, J., Wang, Hua ran, Li, J. wen, Wang, Hui, Jin, M., 2019. Profiling of intracellular and extracellular antibiotic resistance genes in tap water. J. Hazard. Mater. 365, 340–345. https://doi.org/10.1016/j.jhazmat.2018.11.004
22) Jechalke, S., Radl, V., Schloter, M., Heuer, H., Smalla, K., 2016. Do drying and rewetting cycles modulate effects of sulfadiazine spiked manure in soil? FEMS Microbiol. Ecol. 92, 1–7. https://doi.org/10.1093/femsec/fiw066
23) Klümper, U., Riber, L., Dechesne, A., Sannazzarro, A., Hansen, L.H., Sørensen, S.J., Smets, B.F., 2015. Broad host range plasmids can invade an unexpectedly diverse fraction of a soil bacterial community. ISME J. 9, 934–945. https://doi.org/10.1038/ismej.2014.191
24) 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., 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. Sci. Total Environ. 648, 1052–1081. https://doi.org/10.1016/j.scitotenv.2018.08.130

25) Lesser, L.E., Mora, A., Moreau, C., Mahlknecht, J., Hernández-Antonio, A., Ramírez, A.I., Barrios-Piña, H., 2018. Survey of 218 organic contaminants in groundwater derived from the world's largest untreated wastewater irrigation system: Mezquital Valley, Mexico. Chemosphere 198, 510–521. https://doi.org/10.1016/j.chemosphere.2018.01.154
26) Lüneberg, K., Prado, B., Broszat, M., Dalkmann, P., Díaz, D., Huebner, J., Amelung, W., López-Vidal, Y., Siemens, J., Grohmann, E., Siebe, C., 2018. Water flow paths are hotspots for the dissemination of antibiotic resistance in soil. Chemosphere 193, 1198–1206. https://doi.org/10.1016/j.chemosphere.2017.11.143
27) Maaß, O., Grundmann, P., 2016. Added-value from linking the value chains of wastewater treatment, crop production and bioenergy production: A case study on reusing wastewater and sludge in crop production in Braunschweig (Germany). Resour. Conserv. Recycl. 107, 195–211. https://doi.org/10.1016/j.resconrec.2016.01.002
28) Marano, R.B.M., Zolti, A., Jurkevitch, E., Cytryn, E., 2019. Antibiotic resistance and class 1 integron gene dynamics along effluent, reclaimed wastewater irrigated soil, crop continua: elucidating potential risks and ecological constraints. Water Res. 164, 114906. https://doi.org/10.1016/j.watres.2019.114906
29) Michael, I., Rizzo, L., McArdell, C.S., Manaia, C.M., Merlin, C., Schwartz, T., Dagot, C., Fatta-Kassinos, D., 2013. Urban wastewater treatment plants as hotspots for the release of antibiotics in the environment: A review. Water Res. 47, 957–995. https://doi.org/10.1016/j.watres.2012.11.027
30) Negreanu, Y., Pasternak, Z., Jurkevitch, E., Cytryn, E., 2012. Impact of treated wastewater irrigation on antibiotic resistance in agricultural soils. Environ. Sci. Technol. 46, 4800–4808. https://doi.org/10.1021/es204665b
31) Nunes, O.C., Manaia, C.M., Kolvenbach, B.A., Corvini, P.F.X., 2020. Living with sulfonamides: a diverse range of mechanisms observed in bacteria. Appl. Microbiol. Biotechnol. 104, 10389–10408. https://doi.org/10.1007/s00253-020-10982-5
32) Paranychianakis, N. V., Salgot, M., Snyder, S.A., Angelakis, A.N., 2015. Water reuse in EU states: Necessity for uniform criteria to mitigate human and environmental risks. Crit. Rev. Environ. Sci. Technol. 45, 1409–1468. https://doi.org/10.1080/10643389.2014.955629
33) Pärnänen, K.M.M., Narciso-Da-Rocha, C., Kneis, D., Berendonk, T.U., Cacace, D., Do, T.T., Elpers, C., Fatta-Kassinos, D., Henriques, I., Jaeger, T., Karkman, A., Martinez, J.L., Michael, S.G., Michael-Kordatou, I., O'Sullivan, K., Rodriguez-Mozaz, S., Schwartz, T., Sheng, H., Sørum, H., Stedtfeld, R.D., Tiedje, J.M., Giustina, S.V. Della, Walsh, F., Vaz-Moreira, I., Virta, M., Manaia, C.M., 2019. Antibiotic resistance in European wastewater treatment plants mirrors the pattern of clinical antibiotic resistance prevalence. Sci. Adv. 5. https://doi.org/10.1126/sciadv.aau9124
34) Piña, B., Bayona, J.M., Christou, A., Fatta-Kassinos, D., Guillon, E., Lambropoulou, D., Michael, C., Polesel, F., Sayen, S., 2020. On the contribution of reclaimed wastewater irrigation to the potential exposure of humans to antibiotics, antibiotic resistant bacteria and antibiotic resistance genes - NEREUS COST Action ES1403 position paper. J. Environ. Chem. Eng. 8. https://doi.org/10.1016/j.jece.2018.01.011
35) Reis, P.J.M., Reis, A.C., Ricken, B., Kolvenbach, B.A., Manaia, C.M., Corvini, P.F.X., Nunes, O.C., 2014. Biodegradation of sulfamethoxazole and other sulfonamides by Achromobacter denitrificans PR1. J. Hazard. Mater. 280, 741–749. https://doi.org/10.1016/j.jhazmat.2014.08.039

36) Rizzo, L., Manaia, C., Merlin, C., Schwartz, T., Dagot, C., Ploy, M.C., Michael, I., Fatta-Kassinos, D., 2013. Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: A review. Sci. Total Environ. 447, 345–360. https://doi.org/10.1016/j.scitotenv.2013.01.032
37) Rossi, D., Caracciolo, A.B., Grenni, P., Cattena, F., Di Lenola, M., Patrolecco, L., Ademollo, N., Ciannarella, R., Mascolo, G., Ghergo, S., 2019. Groundwater autochthonous microbial communities as tracers of anthropogenic pressure impacts: Example from a municipal waste treatment plant (Latium, Italy). Water 11, 1–20. https://doi.org/10.3390/w11091933
38) Song, M., Song, D., Jiang, L., Zhang, D., Sun, Y., Chen, G., Xu, H., Mei, W., Li, Y., Luo, C., Zhang, G., 2021. Large-scale biogeographical patterns of antibiotic resistome in the forest soils across China. J. Hazard. Mater. 403, 123990. https://doi.org/10.1016/j.jhazmat.2020.123990
39) Szekeres, E., Chiriac, C.M., Baricz, A., Szőke-Nagy, T., Lung, I., Soran, M.L., Rudi, K., Dragos, N., Coman, C., 2018. Investigating antibiotics, antibiotic resistance genes, and microbial contaminants in groundwater in relation to the proximity of urban areas. Environ. Pollut. 236, 734–744. https://doi.org/10.1016/j.envpol.2018.01.107
40) Ternes, T.A., Bonerz, M., Herrmann, N., Teiser, B., Andersen, H.R., 2007. Irrigation of treated wastewater in Braunschweig, Germany: An option to remove pharmaceuticals and musk fragrances. Chemosphere 66, 894–904. https://doi.org/10.1016/j.chemosphere.2006.06.035
41) Underwood, J.C., Harvey, R.W., Metge, D.W., Repert, D.A., Baumgartner, L.K., Smith, R.L., Roane, T.M., Barber, L.B., 2011. Effects of the antimicrobial sulfamethoxazole on groundwater bacterial enrichment. Environ. Sci. Technol. 45, 3096–3101. https://doi.org/10.1021/es103605e
42) Wang, F.H., Qiao, M., Su, J.Q., Chen, Z., Zhou, X., Zhu, Y.G., 2014. High throughput profiling of antibiotic resistance genes in urban park soils with reclaimed water irrigation. Env. Sci Technol 48, 9079–9085. https://doi.org/10.1021/es502615e
43) Zhang, T., Li, B., 2011. Occurrence, transformation, and fate of antibiotics in municipal wastewater treatment plants. Crit. Rev. Environ. Sci. Technol. 41, 951–998. https://doi.org/10.1080/10643380903392692

References to own original first author publications included in the Thesis.

First-authorship publications accepted in Peer-Review Journals:

Chapter 2 has been published in the "Water Research" Journal under the title:

Kampouris, I.D., Agrawal, S., Orschler, L., Cacace, D., Kunze, S., Berendonk, T.U., Klümper, U., 2021a. Antibiotic resistance gene load and irrigation intensity determine the impact of wastewater irrigation on antimicrobial resistance in the soil microbiome. Water Res. 116818. https://doi.org/10.1016/j.watres.2021.116818

Chapter 2 differs from the original manuscript on the following aspects: Reference list have been modify to match alphabetical order of each cited paper. Figures and table enumeration has been adjusted to a chapter-like format, using the "2." suffix (e.g. Figure 1 was converted to Figure 2.1).

Chapter 3 has been published in the "Environmental International" Journal with minor alterations under the title:

Kampouris, I.D., Klümper, U., Agrawal, S., Orschler, L., Cacace, D., Kunze, S., Berendonk, T.U., 2021b. Treated wastewater irrigation promotes the spread of antibiotic resistance into subsoil pore-water. Environ. Int. 146. https://doi.org/10.1016/j.envint.2020.106190

Chapter 3 differs from the original manuscript on the following aspects: The nomenclature of the gene *tetM* from the original publication change to *tet*(M). While both version of the nomeclture of gene occur in published studies, I considered that is more appropriated to use *tet*(M). Similarly log10 changed to $\log_{10}$ because is considere as more precise nomenclature. The final paragraph have been label as the "Conclusion" section, to match in format the paper in the previous Chapter. Reference list have been modify to match alphabetical order of each cited paper. Figures and table enumeration has been adjusted to a chapter-like format, using the "3." suffix (e.g. Figure 1 was converted to Figure 3.1).

First-authorship manuscript submitted in Peer-Review Journals for publication:

Chapter 4 has been submitted in the"Hazardous Material" Journal and currently is under review:

Kampouris, I.D., Alygizakis, N., Klümper, U., Agrawal, S., Lackner, S., Cacace, D., Kunze, S., Thomaidis, N., Slobdonik, J., Berendonk, T.U. Elevated levels of antibiotic resistance in groundwater during treated wastewater irrigation associated with infiltration and accumulation of antibiotic residues. Under review in the "Journal of Hazardous Materials".

Chapter 4 differs from the original submitted manuscript on the following aspects: The final paragraph have been label as the "Conclusion" section, to match in format the paper in the previous Chapter. Reference list have been modify to match alphabetical order of each cited paper. Figures and table enumeration has been adjusted to a chapter-like format, using the "4." suffix (e.g. Figure 1 was converted to Figure 4.1). Since the manuscript is under "review" and

"revision" process from the "Journal of Hazardous Materials", the final published version might slighlty differ than the submitted one.

All papers were published on Journals from Elsevier: Theses and dissertations which contain embedded final published articles as part of the formal submission can be posted publicly by the awarding institution with DOI links back to the formal publication on ScienceDirect (https://www.elsevier.com/about/policies/copyright)

Further Publications not included in the Thesis

First-authorship publications accepted in Peer-Review Journals:

Kampouris, I.D., Karayannakidis, P.D., Banti, D.C., Sakoula, D., Konstantinidis, D., Yiangou, M., Samaras, P.E., 2018. Evaluation of a novel quorum quenching strain for MBR biofouling mitigation. Water Res. 143, 56–65. https://doi.org/10.1016/j.watres.2018.06.030

Co-authorship in other publications accepted in Peer-Review Journals:

Alygizakis, N.A., Urík, J., Beretsou, V.G., **Kampouris, I.**, Galani, A., Oswaldova, M., Berendonk, T., Oswald, P., Thomaidis, N.S., Slobodnik, J., Vrana, B., Fatta-Kassinos, D., 2020. Evaluation of chemical and biological contaminants of emerging concern in treated wastewater intended for agricultural reuse. Environ. Int. 138, 105597. https://doi.org/10.1016/j.envint.2020.105597

Manaia, C.M., Rocha, J., Scaccia, N., Marano, R., Radu, E., Biancullo, F., Cerqueira, F., Fortunato, G., Iakovides, I.C., Zammit, I., **Kampouris, I.**, Vaz-Moreira, I., Nunes, O.C., 2018. Antibiotic resistance in wastewater treatment plants: Tackling the black box. Environ. Int. 115, 312–324. https://doi.org/10.1016/J.ENVINT.2018.03.044

Rocha, J., Cacace, D., **Kampouris, I.**, Guilloteau, H., Jäger, T., Marano, R.B.M., Karaolia, P., Manaia, C.M., Merlin, C., Fatta-Kassinos, D., Cytryn, E., Berendonk, T.U., Schwartz, T., 2020. Inter-laboratory calibration of quantitative analyses of antibiotic resistance genes. J. Environ. Chem. Eng. 8, 102214. https://doi.org/10.1016/j.jece.2018.02.022

Book Chapters:

Kampouris, I.D., Banti, D.C., Samaras, P.E., 2020. *Quorum Quenching as an Anti-biofouling Strategy for Wastewater Reuse and Biofouling Affected Industries*. In "Trends in Quorum Sensing and Quorum Quenching: New Perspectives and Applications." By V. Ravishankar Rai, Jamuna A Bai. Published May 24, 2020. CRC Press, Taylor & Francis Group.

Oral and poster presentations in conferences:

I.D. Kampouris, D. Cacace, S. Kunze, T.U. Berendonk, "The dynamics of antibiotic resistance genes in topsoil and groundwater of an agricultural area frequently irrigated with wastewater", 5th International Symposium on the Environmental Dimension of Antibiotic Resistance (EDAR-5), 9–14 June 2019, Hong Kong (Oral Presentation)

I. D. Kampouris, D. Cacace, S. Kunze, T.U. Berendonk, "Wastewater irrigation affects the prevalence of antibiotic resistance genes in topsoil and groundwater", 8th Congress of the Scientific Society MikroBioKosmos, Patra, Greece, 18-20 April, 2019. (Oral presentation)

I.D. Kampouris, S. Heß, D. Cacace, S. Kunze, T.U. Berendonk, "The effect of the wastewater irrigation on the dissemination of antibiotic resistance genes in subsoil passages", Challenges and Solutions related to Xenobiotics and Antimicrobial Resistance in the Framework of Urban Wastewater Reuse: Towards a Blue Circle Society (XENOWAC II), Limassol, Cyprus, 10-12 October 2018. (Oral presentation)

I.D. Kampouris, S. Kunze, D. Cacace, T.U. Berendonk. (2018). Evaluation of the effect of wastewater irrigation on the prevalence of ARGs in surface soil and subsoil passages. 17th International Symposium on Microbial Ecology (ISME17), Leipzig, Germany. (Poster)

Apendixes & Supplementary Material

Apendix 1

(Supplementary Material for Chapter 2)

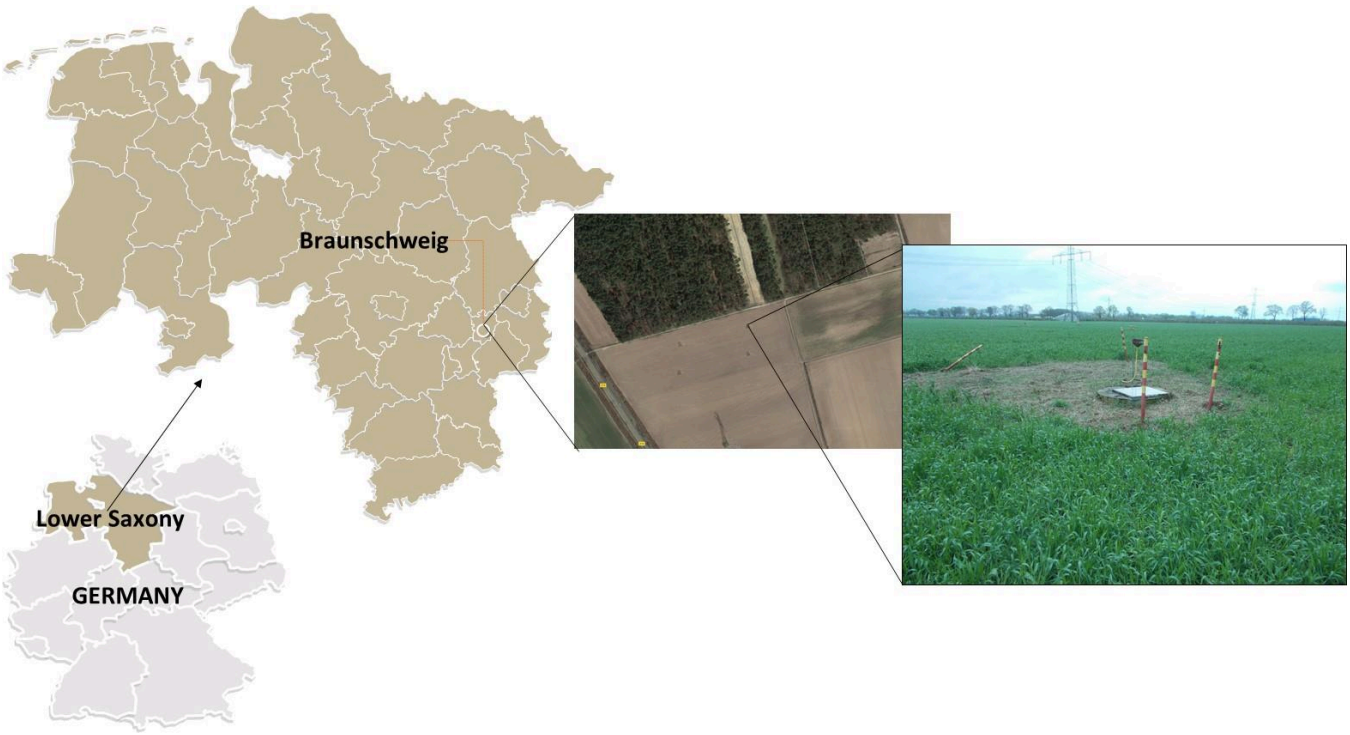


Figure S2.1: Description of the sampling location on the map.

Figure S2.2: Sampling locations: The irrigated field (red box) and the non-irrigated area adjacent to the field (yellow box). The sampling points were randomized. They are given with the specific coloured dots for each area (red: irrigated field; yellow: non-irrigated area). Samplings took place in the dotted areas with approximate deviation of 10 m.

Figure S2.3: The microcosm design and structure (Left). The aluminum foil wrapped microcosms (Right).

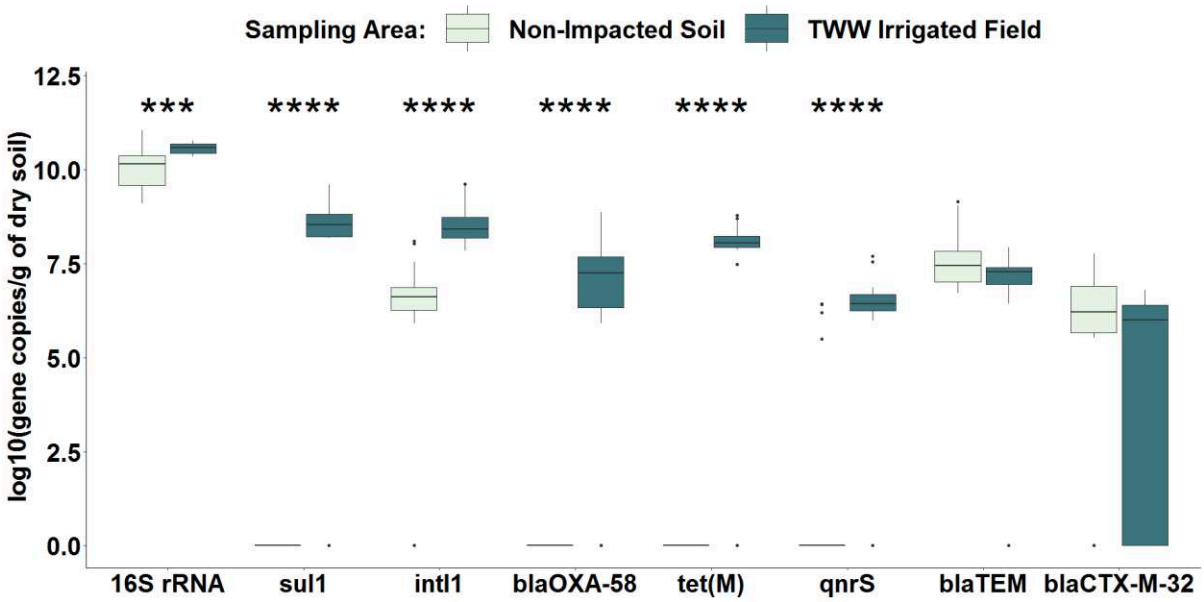


Figure S2.4: Absolute abundance of 16S rRNA, *intI1* and ARGs in the non-irrigated soil and field soil from the sampling campaign in May 2019 (Wilcoxon rank sum test *p<0.05, **p<0.01, ***p< 0.001, ****p<0.0001, n=12).

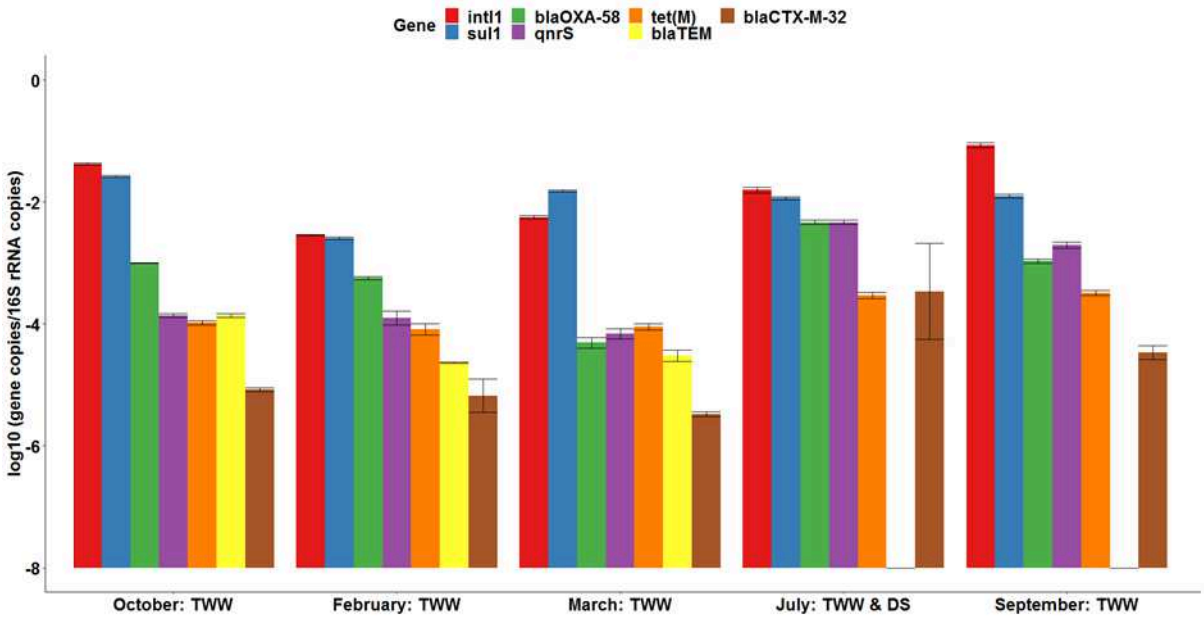


Figure S2.5: Log_{10} transformed relative abundance (copies/16S rRNA) of the ARGs and *intI1* in the irrigation water (n=3) from October 2017 to September 2018. The gene bla_{TTM} was below the LOQ in July and September 2018. Samples were not taken in June and December 2018 due to the irrigation break in these periods (Table 1). TWW: Treated Wastewater, DS: Digested Sludge.

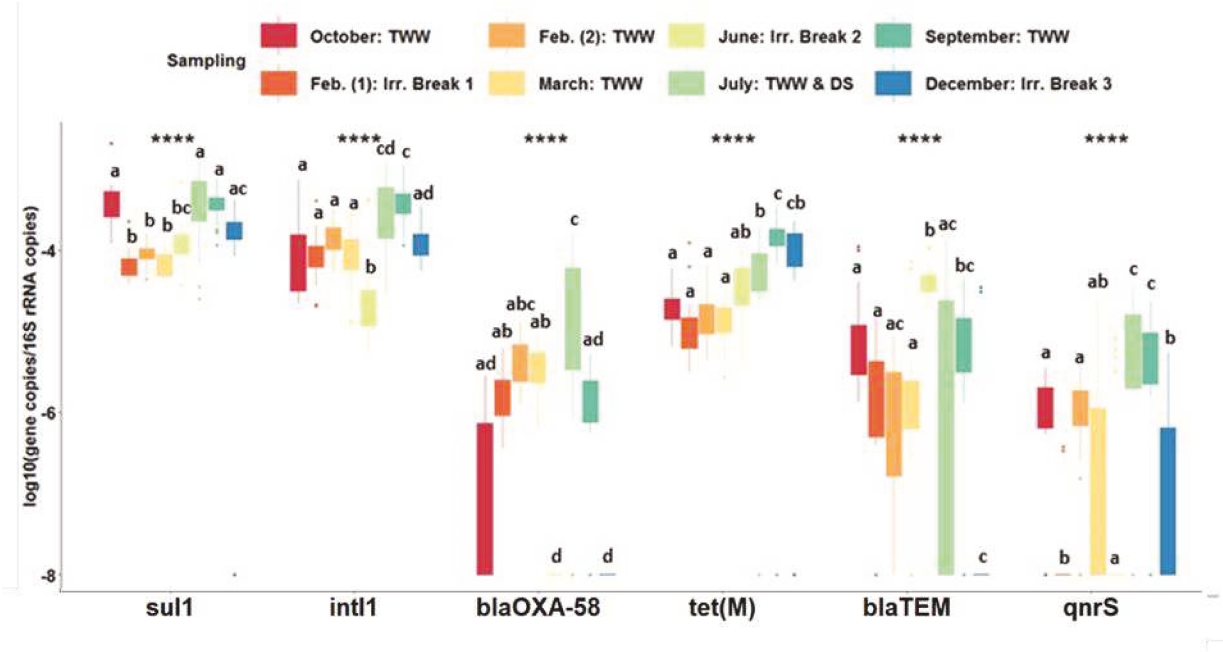


Figure S2.6: Detailed temporal dynamics of $\log_{10}$ transformed relative abundance of ARGs and *intI1* to 16S rRNA ($\log_{10}$ copies/16srRNA) in the TWW irrigated field soil during different periods of TWW/TWW & DS irrigation or irrigation breaks (Kruskal-Wallis test: *p<0.05, **p<0.01, ***p< 0.001, ****p<0.0001, n=12). Letters from "a" to "d" were assigned to each none significant different groups after a Dunn's test (Benjamini-Hochberg correction). The cutoff of significance for pairwise comparison was p<0.05. Further details for each irrigation period are given in Table 1. The samplings started from October 2017 and lasted until December 2018. The gene *bla*$_{\text{CTX-M-32}}$ was not presented because it was below LOQ/LOD in these sampling campaigns (with the exception of outliers). Irr. Break= Irrigation Break from the last sampling campaign, TWW=Treated Wastewater, DS= Digested Sludge.

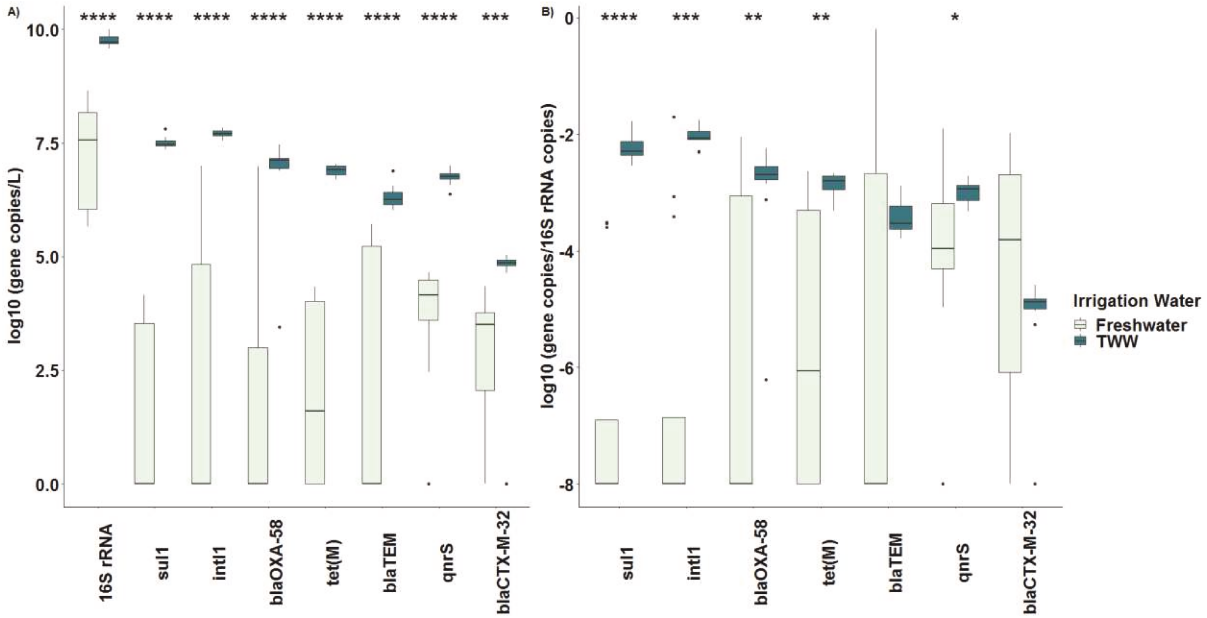


Figure S2.7: A) Absolute abundance of genes (copies/L) and B) the relative abundance (gene copies to 16S rRNA copies) of the irrigation waters, which were used for irrigation of the microcosms (Wilcoxon rank sum test *p<0.05, **p<0.01, ***p< 0.001, ****p<0.0001, n=6).

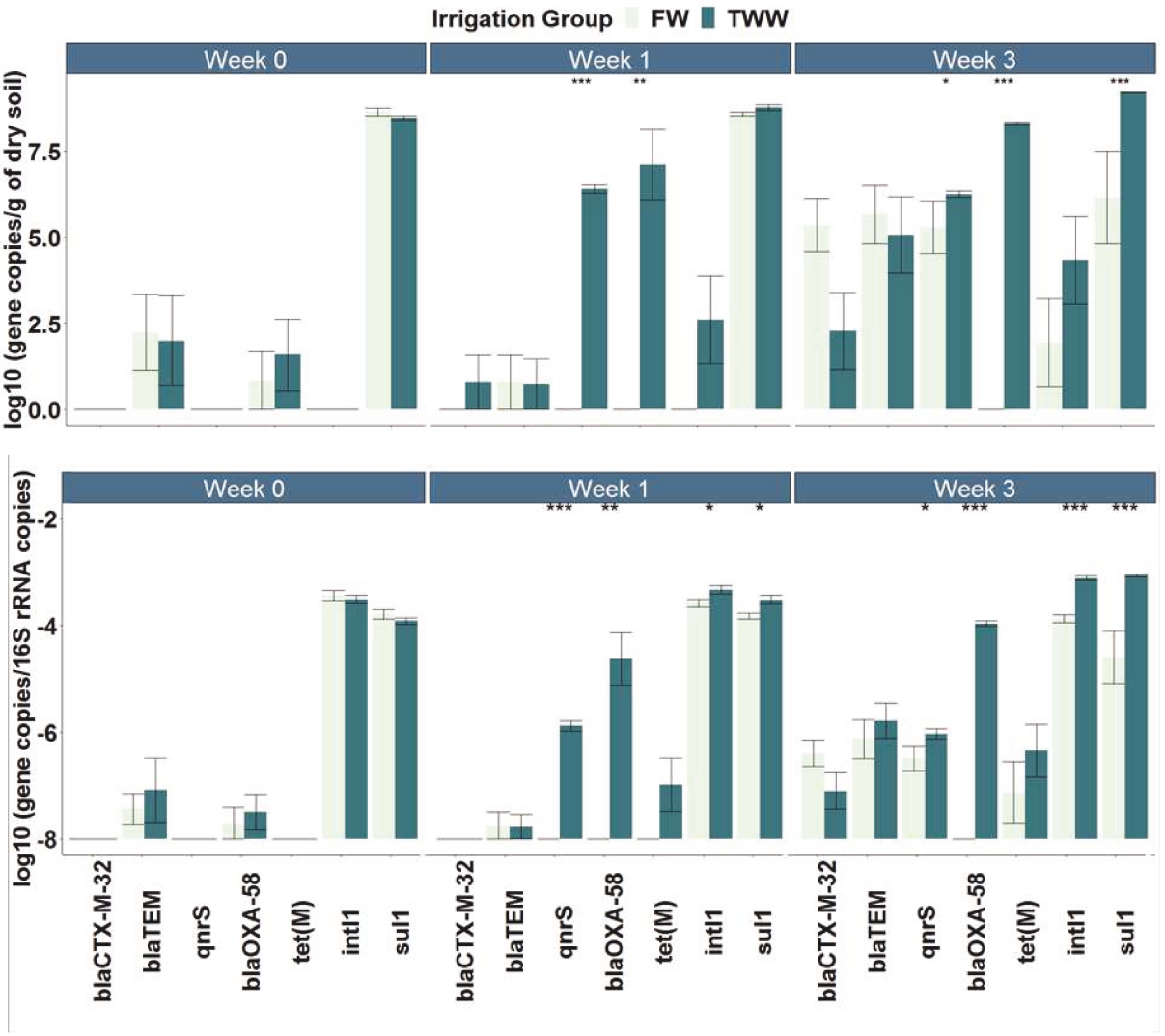


Figure S2.8: Absolute (up) and relative (down) abundance of genes in the microcosm soil samples between the two different groups of irrigation. Prior to Week 0 both of the irrigation groups were equilibrated only with freshwater irrigation for 2 weeks. At Week 1, the wastewater group was irrigated with wastewater for one week and at Week 3 for 3 weeks. In parallel we continued to irrigate the freshwater group with freshwater (Wilcoxon rank sum test: *p<0.05, **p<0.01, ***p< 0.001, ****p<0.0001, n=4).

Table S2.1: Average annual chemical oxygen demand for the TWW irrigation water in Braunschweig (TWW & DS n= 36, TWW n= 60).

	TWW		TWW & DS	
	COD-homogenised (mg/L)	COD-filtrate (mg/L)	COD-homogenised (mg/L)	COD-filtrate (mg/L)
Maximum	258	97	900	126
Average	62.87	44.53	468.25	96.53
Minimum	29	23	244	58

Table S2.2: Crops rotation of the selected field in Braunschweig.

Date	**Crop**
October_2017	Winter wheat
October 2017 - February_2018	Winter wheat
February_2018 - June_2018	Mustard
June_2018 - August_2018	Corn
August_2018 - October_2018	Rye
October_2018 - December_2018	Mustard
March_2019 - June_2019	Winter wheat

Table S2.3: Field and non-irrigated area soil characteristics

Soil Characteristics	**Non-Impacted Soil**	**Field**
Clay (<2.0µm) %	1.66	0
Fine-Silt (2.0-6.3µm) %	0.61	0.02
Medium-Silt (6.3-20µm) %	0.89	0.35
Coarse-Silt (20-63µm) %	1.69	0.44
Fine-Sand (63-200µm) %	20.2	20.3
Medium-Sand (200-630µm) %	61.5	71.1
Coarse-Sand (630-2000µm) %	11.3	5.50
pH	3.77	5.94
Corg %	4	2

Table S2.4: Extensive protocols of qPCR assays.

Target gene		**Primers sequence**	**Amplicon size**	**Conditions**	**LOQ**	**Reference**

16S rRNA	Fw	TCCTACGGGAGGCAGCAGT	195 bp	95 °C - 10 min (1 cycle); 95 °C - 15 sec, 60 °C - 1 min (40 cycles)	4000 copies per reaction	Rocha et al., 2018
	Rev	ATTACCGCGGCTGCTGG		Other: 1		
bla$_{TEM}$	Fw	TTCCTGTTTTTGCTCACCCAG	113 bp	95 ºC - 10 min (1 cycle); 95 ºC - 15 sec, 60 ºC - 1 min (40 cycles)	40-400 copies per reaction	Rocha et al., 2018
	Rev	CTCAAGGATCTTACCGCTGTTG		Other: 2		
bla$_{CTX-M-32}$	Fw	CGTCACGCTGTTGTTAGGAA	156 bp	95 ºC - 10 min (1 cycle); 95 ºC - 15 sec, 58.5 ºC – 1 min (40 cycles)	4-40 copies per reaction	Rocha et al., 2018
	Rev	CGCTCATCAGCACGATAAAG		Other: 2		
sul1	Fw	CGCACCGGAAACATCGCTGCAC	162 bp	95 ºC - 10 min (1 cycle); 95 ºC - 10 sec, 60 ºC - 1 min (40 cycles)	40-400 copies per reaction	Rocha et al., 2018
	Rev	TGAAGTTCCGCCGCAAGGCTCG		Other: 2		
qnrS	Fw	GACGTGCTAACTTGCGTG	118 bp	95 ºC - 10 min (1 cycle); 95 ºC - 15 sec, 60 ºC - 1 min (40 cycles)	4-40 copies per reaction	Rocha et al., 2018
	Rev	TGGCATTGTTGGAAACTT		Other: 2		
intI1	Fw	GATCGGTCGAATGCGTGT	196 bp	95 ºC - 10 min (1 cycle); 95 ºC - 15 sec, ºC - 1 min (40 cycles)	40-400 copies per reaction	Rocha et al., 2018
	Rev	GCCTTGATGTTACCCGAGAG		Other: 2		

bla$_{OXA-58}$	Fw	CACTTACAGGAAACTTGGGGTCG	79 bp	95 ºC - 10 min (1 cycle); 95 ºC - 15 sec, 60 ºC - 1 min (40 cycles)	4-40 copies per reaction	Cacace et al., 2019
	Rev	AGTGTGTTTAGAATGGTGATC		Other: 2		
tet(M)	Fw	GCAATTCTACTGATTTCTGC	186 bp	95 ºC - 10 min (1 cycle); 95 ºC - 15 sec, 55 ºC - 1 min (40 cycles)	40 copies per reaction	Cacace et al., 2019
	Rev	CTGTTTGATTACAATTTCCGC		Other: 3		

Other: 1* 0.5 µM primers, 2* 0.25 µM primers, 3* 0.2 µM primers and 0.1 mg/mL BSA.

Table S2.5: Efficiency, LOQ and R^2 for each amplified gene. The LOD for each reaction was set at 3 copies per reaction

Target gene	**Efficiency**	**R^2**	**Negative Control Cq**	**LOQ**
16S rRNA	93%	0.999	28.74	4000 copies per reaction
bla$_{TEM}$	96%	1	32.42	40 copies per reaction
bla$_{CTX-M-32}$	100%	0.995	-	4 copies per reaction
sul1	96%	0.999	35.75	40 copies per reaction
qnrS	98%	0.995	38.5	4 copies per reaction
intI1	95%	0.992	35.64	40 copies per reaction
bla$_{OXA-58}$	95%	0.997	-	4 copies per reaction
tet(M)	90%	0.995	39.1	4 copies per reaction

References

1) Rocha et al., 2018. Inter-laboratory calibration of quantitative analyses of antibiotic resistance genes J. Environ. Chem. Eng. (2018). doi: 10.1016/J.JECE.2018.02.022

2) Cacace et al., 2019. Antibiotic resistance genes in treated wastewater and in the receiving water bodies: A pan-European survey of urban settings. Water Research 162, 320–330. doi:10.1016/j.watres.2019.06.039

Apendix 2

(Supplementary Material for Chapter 3)

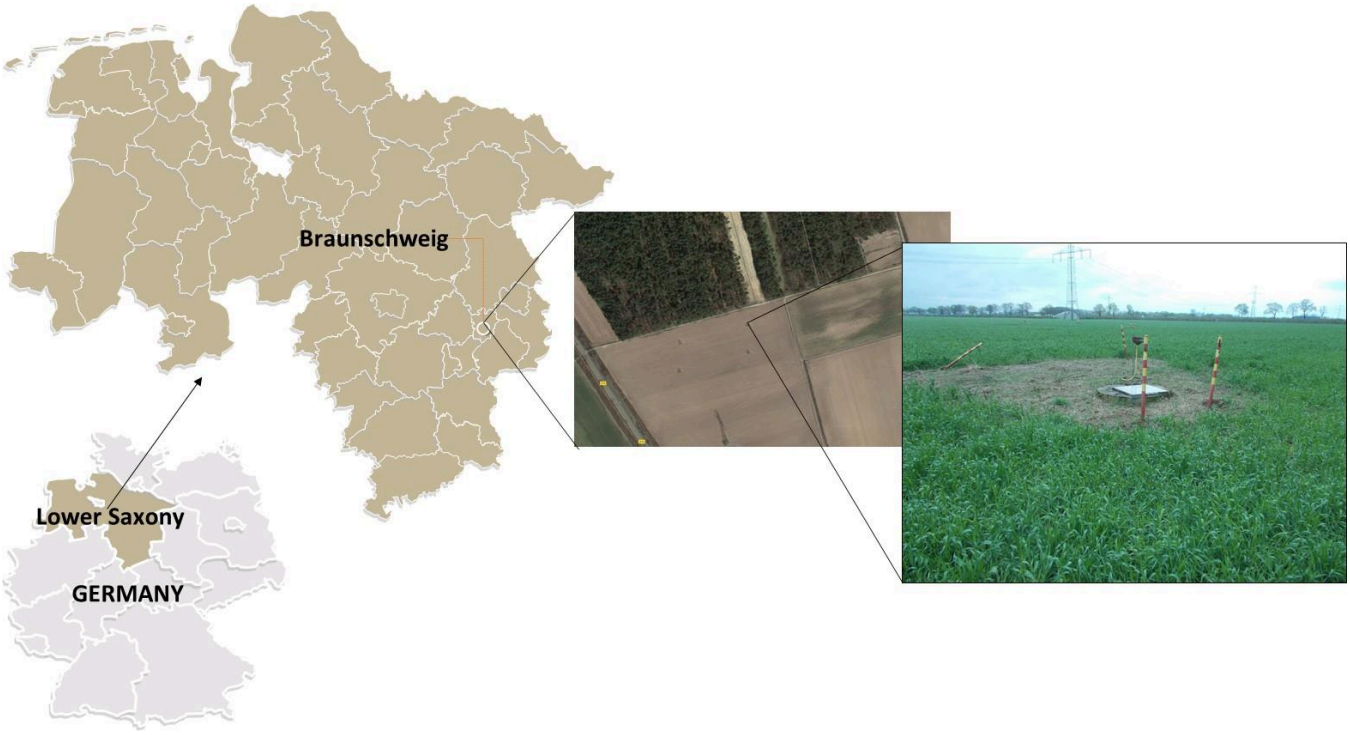


Figure S3.1: Field sampling location in the map. In the right picture, the entrance door to the Lysimeter installation.

Figure S3.2: The interior bottom of the Lysimeter wells: a tap that allows collection of percolated water at a defined depth.

Table S3.1: Average annual chemical oxygen demand for the irrigation water provided by BWA (TWW & DS n= 36, TWW n= 60).

	TWW		TWW & DS	
	COD-homogenised (mg/L)	COD-filtrate (mg/L)	COD-homogenised (mg/L)	COD-filtrate (mg/L)
Maximum	258	97	900	126
Average	62.8666667	44.5333333	468.25	96.5277778
Minimum	29	23	244	58

Table S3.2: Field and non-irrigated forest area soil characteristics.

Soil Characteristics	**Non-Irrigated Soil (Forest)**	**Field**
Clay (<2.0µm) %	1.66	0
Fine-Silt (2.0-6.3µm) %	0.61	0.02
Medium-Silt (6.3-20µm) %	0.89	0.35
Coarse-Silt (20-63µm) %	1.69	0.44

Fine-Sand (63-200µm) %	20.2	20.3
Medium-Sand (200-630µm) %	61.5	71.1
Coarse-Sand (630-2000µm) %	11.3	5.50
Large Size Grains (>2000µm)%	2.15	2.29
pH	3.77	5.94
Corg %	4	2

Table S3.3: Average humidity, temperature and total precipitation during the sampling campaign.

Sampling	Crop	Humidity (%)	Total Monthly Precipitation (L/m^2)	Average Temperature (^oC)
April 2017	Corn	70	24.4	9
May 2017	Corn	75	98.1	16
June 2017	Corn	73	115.3	19
October 2017	Rapeseed/Winter Wheat	87	88.7	13
February 2018	Winter Wheat	71	5.7	0
March 2018	Winter Wheat	70	34.9	4

Table S3.4: Tested genes, primers and protocols of qPCR assays.

Target gene		Primers sequence	Amplicon size	Conditions	Reference
16S rRNA (Indicator for microbial abundance)	Fw	TCCTACGGGAGGCAGCAGT	195 bp	95 °C - 10 min (1 cycle); 95 °C - 15 sec, 60 °C - 1 min (40 cycles)	Cacace et al., 2019
	Re	ATTACCGCGGCTGCTGG		Other: 1	
bla$_{TEM}$ (class A β-lactamase)	Fw	TTCCTGTTTTTGCTCACCCAG	113 bp	95 ºC - 10 min (1 cycle); 95 ºC - 15 sec, 60 ºC - 1 min (40 cycles)	Cacace et al., 2019

	Re	CTCAAGGATCTTACCGCTGTTG		Other: 2	
bla$_{CTX-M-32}$ (class A β-lactamase, cephalosporinase)	Fw	CGTCACGCTGTTGTTAGGAA	156 bp	95 ºC - 10 min (1 cycle); 95 ºC - 15 sec, 58.5 ºC – 1 min (40 cycles)	Cacace et al., 2019
	Re	CGCTCATCAGCACGATAAAG		Other: 2	
sul1 (sulfonamide resistant dihydropteroate synthase)	Fw	CGCACCGGAAACATCGCTGCAC	162 bp	95 ºC - 10 min (1 cycle); 95 ºC - 10 sec, 60 ºC -1 min (40 cycles)	Cacace et al., 2019
	Re	TGAAGTTCCGCCGCAAGGCTCG		Other: 2	
qnrS (protein family which protects DNA gyrase from the inhibition of quinolones)	Fw	GACGTGCTAACTTGCGTG	118 bp	95 ºC - 10 min (1 cycle); 95 ºC - 15 sec, 60 ºC - 1 min (40 cycles)	Cacace et al., 2019
	Re	TGGCATTGTTGGAAACTT		Other: 2	
intI1 (class I integrase, this gene is associated with horizontal gene transfer and environmental pollution)	Fw	GATCGGTCGAATGCGTGT	196 bp	95 ºC - 10 min (1 cycle); 95 ºC - 15 sec, ºC - 1 min (40 cycles)	Cacace et al., 2019
	Re	GCCTTGATGTTACCCGAGAG		Other: 2	

bla$_{OXA-58}$ (class D β-lactamase, carbapemenase)	Fw	CACTTACAGGAAACTTGGGGTCG	79 bp	95 ºC - 10 min (1 cycle); 95 ºC - 15 sec, 60 ºC - 1 min (40 cycles)	Cacace et al., 2019
	Re	AGTGTGTTTAGAATGGTGATC		Other: 2	
tet(M) (ribosomal protection protein that protects ribosome from the translation inhibition of tetracycline)	Fw	GCAATTCTACTGATTTCTGC	186 bp	95 ºC - 10 min (1 cycle); 95 ºC - 15 sec, 55 ºC - 1 min (40 cycles)	Cacace et al., 2019
	Re	CTGTTTGATTACAATTTCCGC		Other: 3	

Other: 1* 0.5 µM primers, 2* 0.25 µM primers, 3* 0.2 µM primers and 0.1 mg/mL BSA.

Table S3.5: Efficiency, LOQ and R^2 for each amplified gene. The LOD for each reaction was set at 3 copies per reaction

Target gene	**Efficiency**	**R^2**	**Negative Control Cq**	**LOQ**
16S rRNA	93%	0.999	28.74	4000 copies per reaction
bla$_{TEM}$	96%	1	32.42	40 copies per reaction
bla$_{CTX-M-32}$	100%	0.995	-	4 copies per reaction
sul1	96%	0.999	35.75	40 copies per reaction
qnrS	98%	0.995	38.5	4 copies per reaction
intI1	95%	0.992	35.64	40 copies per reaction

bla$_{OXA-58}$	95%	0.997	-	4 copies per reaction
tet(M)	90%	0.995	39.1	4 copies per reaction

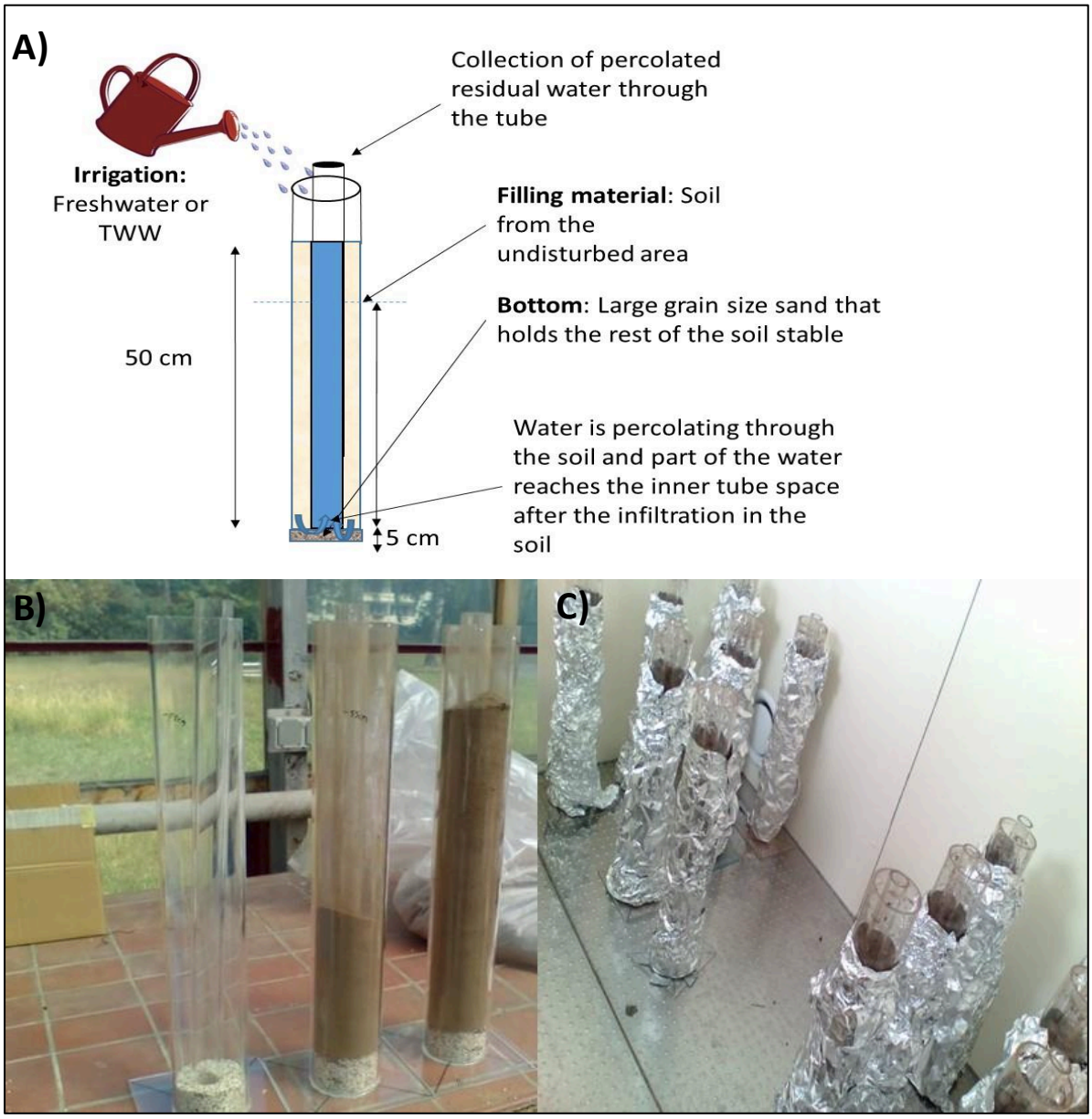


Figure S3.3: A) Schematic representation of the microcosm design and structure, irrigation and percolated residual water sampling. B) The filling of the microcosms with gravels in the bottom and sieved homogenized soil in the main tube. C) Aluminum foil wrapped microcosms in the controlled-temperature room, with 20ºC stable temperature and 12 hours light per day.

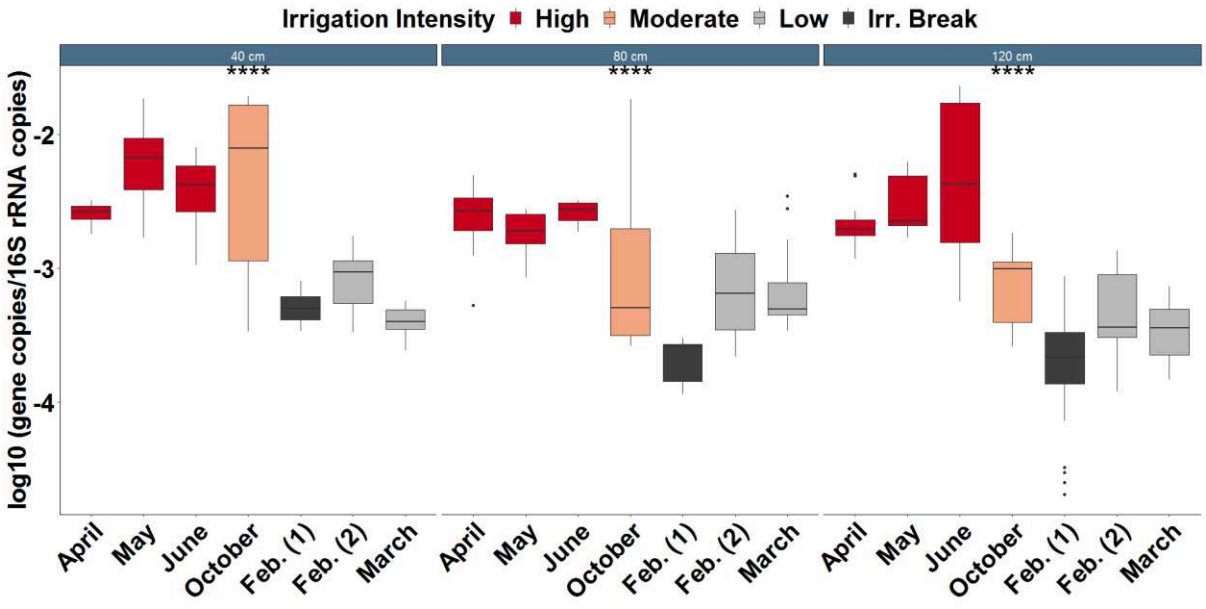


Figure S3.4: Relative abundance of *sul1* during periods of high-, moderate-, low-irrigation intensity or irrigation break (Irr. Break). Kruskal-Wallis test: *p<0.05, **p<0.01, ***p< 0.001, ****p<0.0001, n=4.

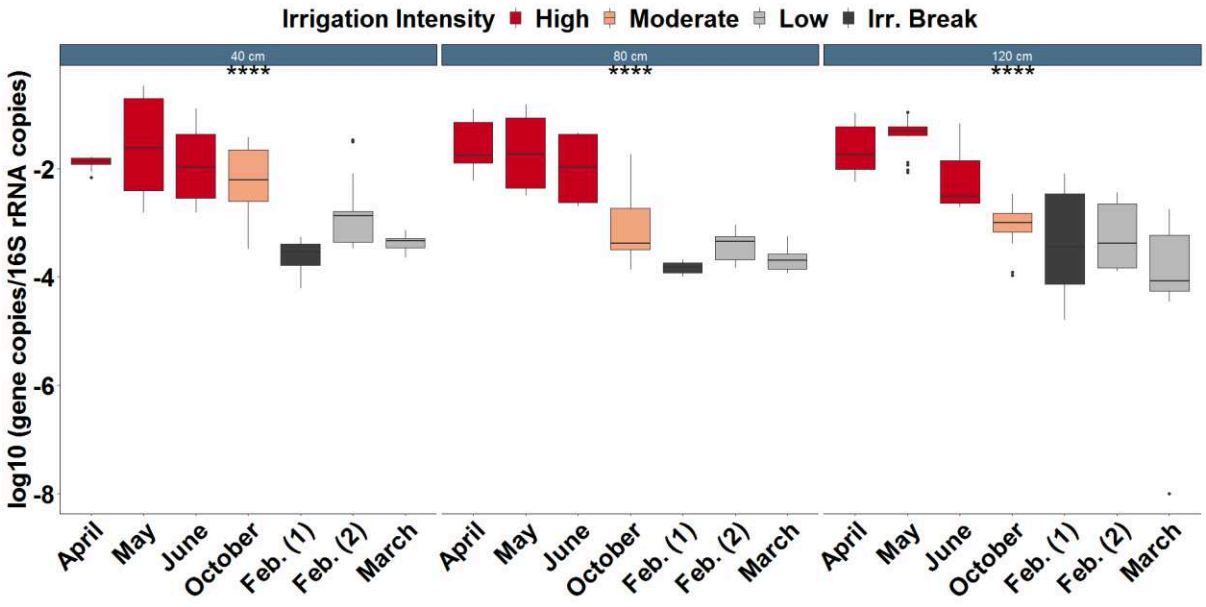


Figure S3.5: Relative abundance of *intI1* during periods of high-, moderate-, low-irrigation intensity or irrigation break (Irr. Break). Kruskal-Wallis test: *p<0.05, **p<0.01, ***p< 0.001, ****p<0.0001, n=4.

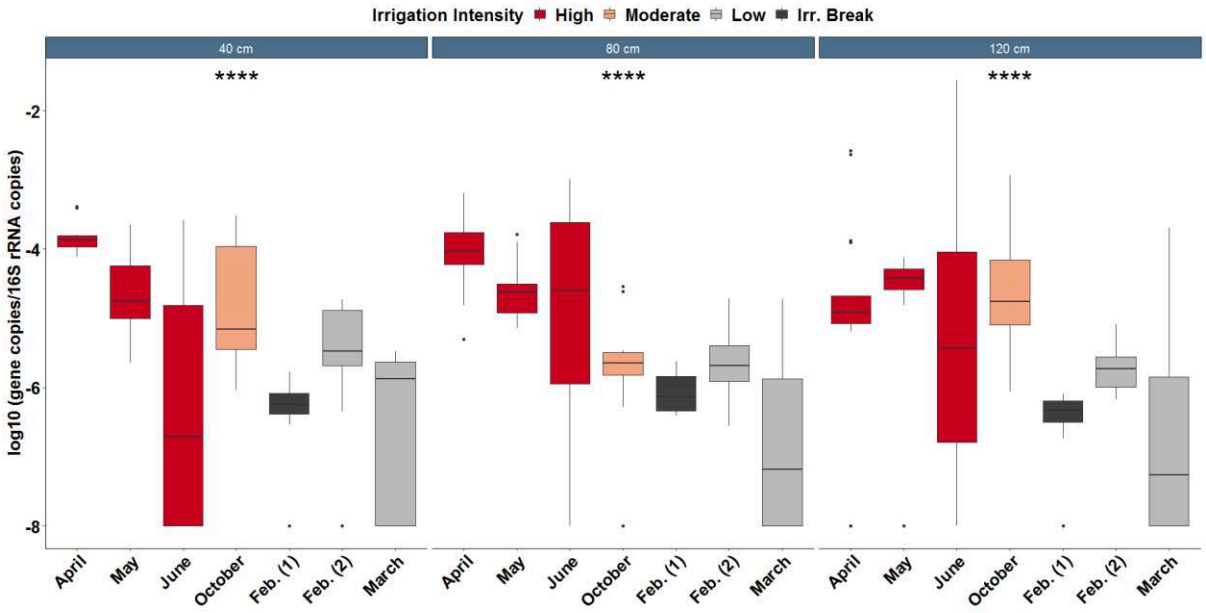


Figure S3.6: Relative abundance of *qnrS* during periods of high-, moderate-, low-irrigation intensity or irrigation break (Irr. Break). Kruskal-Wallis test: *p<0.05, **p<0.01, ***p< 0.001, ****p<0.0001, n=4.

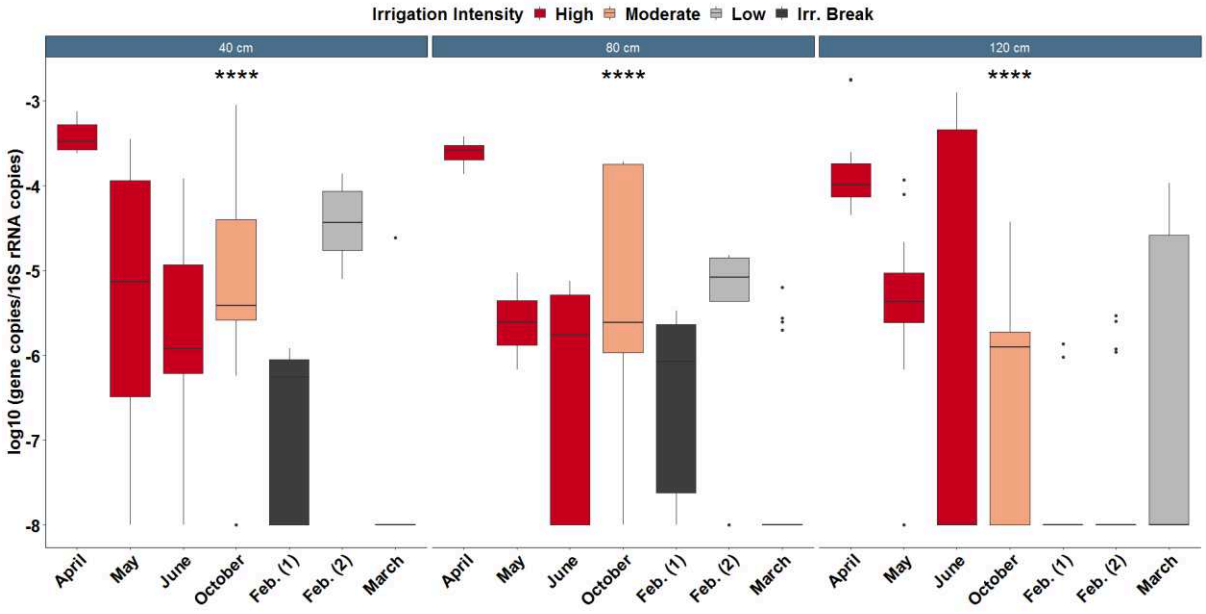


Figure S3.7: Relative abundance of *bla*$_{\text{OXA-58}}$ during periods of high-, moderate-, low-irrigation intensity or irrigation break (Irr. Break). Kruskal-Wallis test: *p<0.05, **p<0.01, ***p< 0.001, ****p<0.0001, n=4.

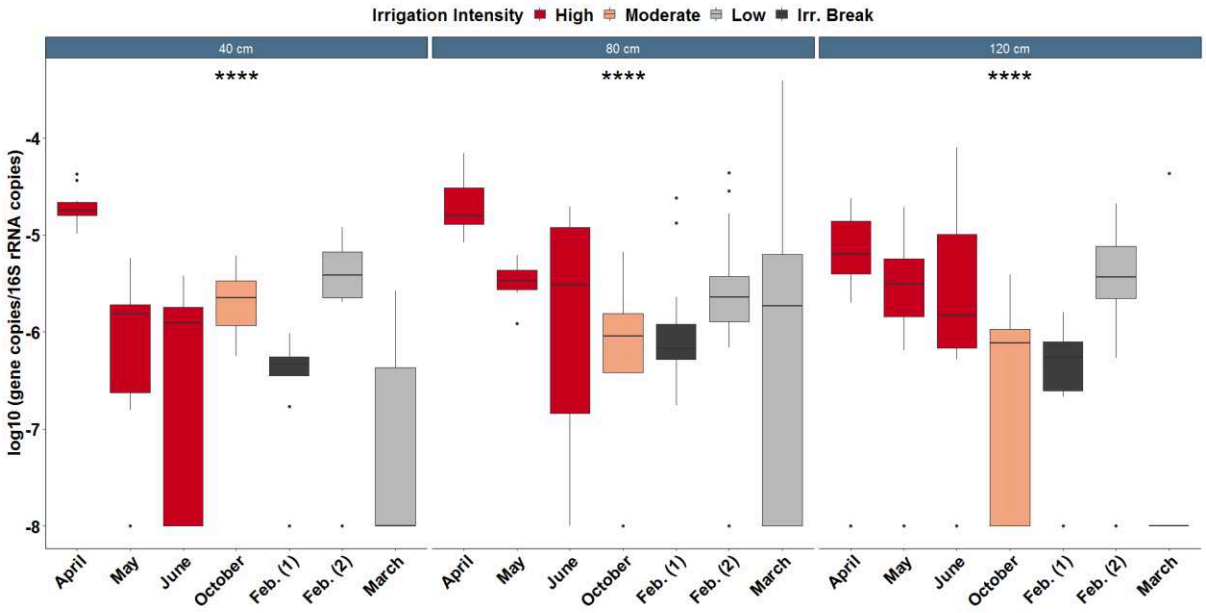


Figure S3.8: Relative abundance of *bla*$_{\text{CTX-M-32}}$ during periods of high-, moderate-, low-irrigation intensity or irrigation break (Irr. Break). Kruskal-Wallis test: *p<0.05, **p<0.01, ***p< 0.001, ****p<0.0001, n=4.

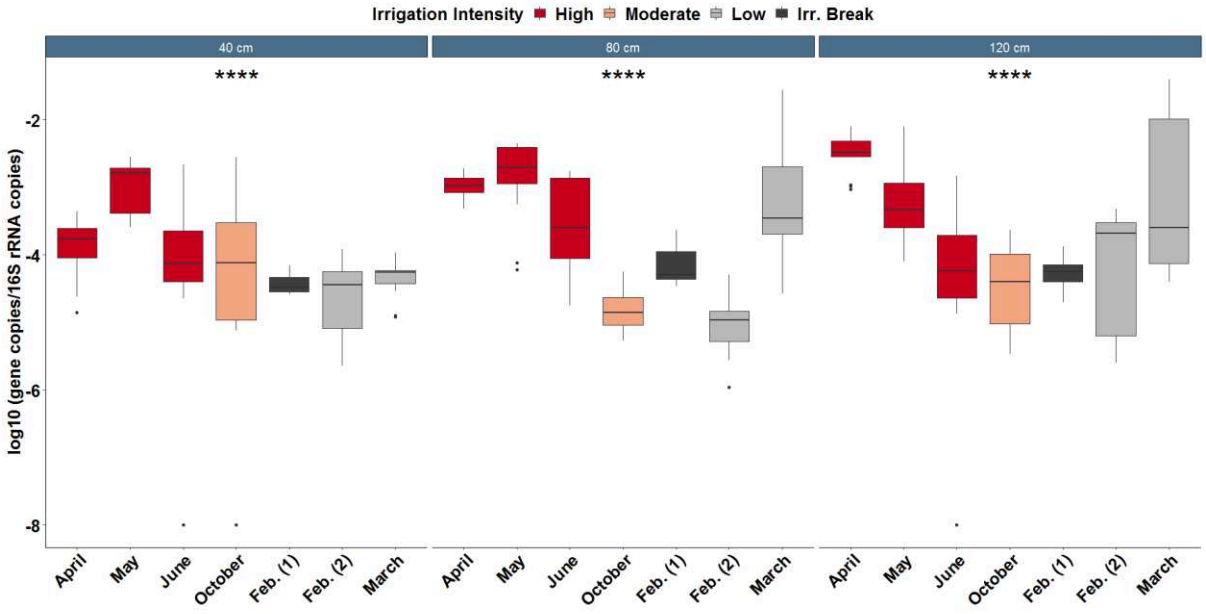


Figure S3.9: Relative abundance of *bla*$_{\text{TEM}}$ during periods of high-, moderate-, low-irrigation intensity or irrigation break (Irr. Break). Kruskal-Wallis test: *p<0.05, **p<0.01, ***p< 0.001, ****p<0.0001, n=4.

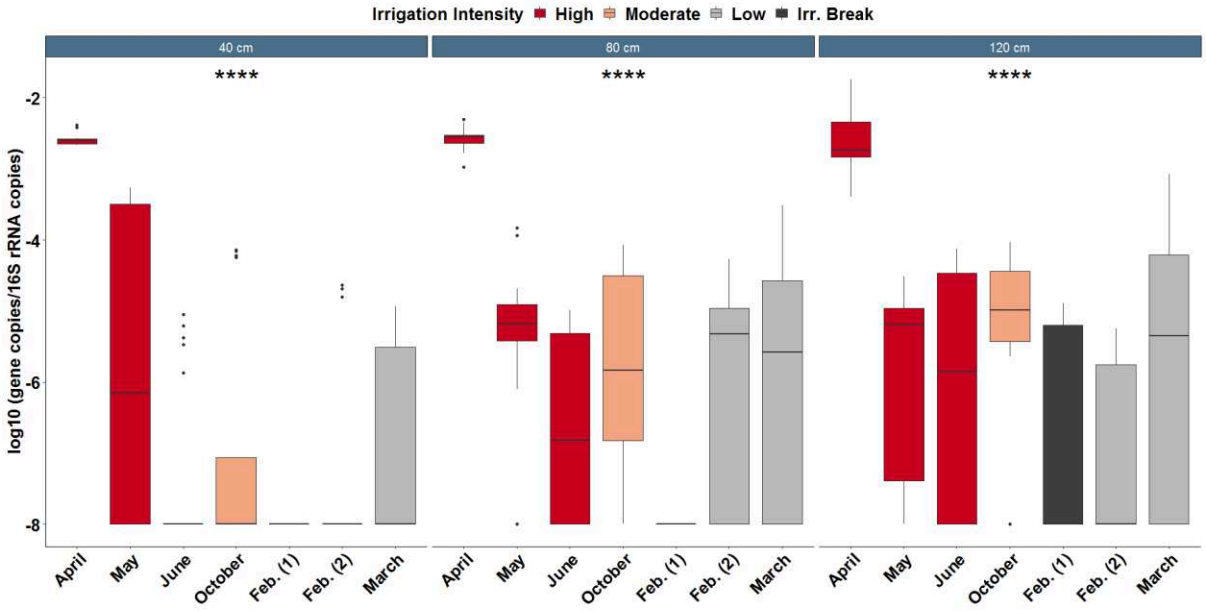


Figure S3.10: Relative abundance of *tet*(M) during periods of high-, moderate-, low-irrigation intensity or irrigation break (Irr. Break). Kruskal-Wallis test: *p<0.05, **p<0.01, ***p< 0.001, ****p<0.0001, n=4.

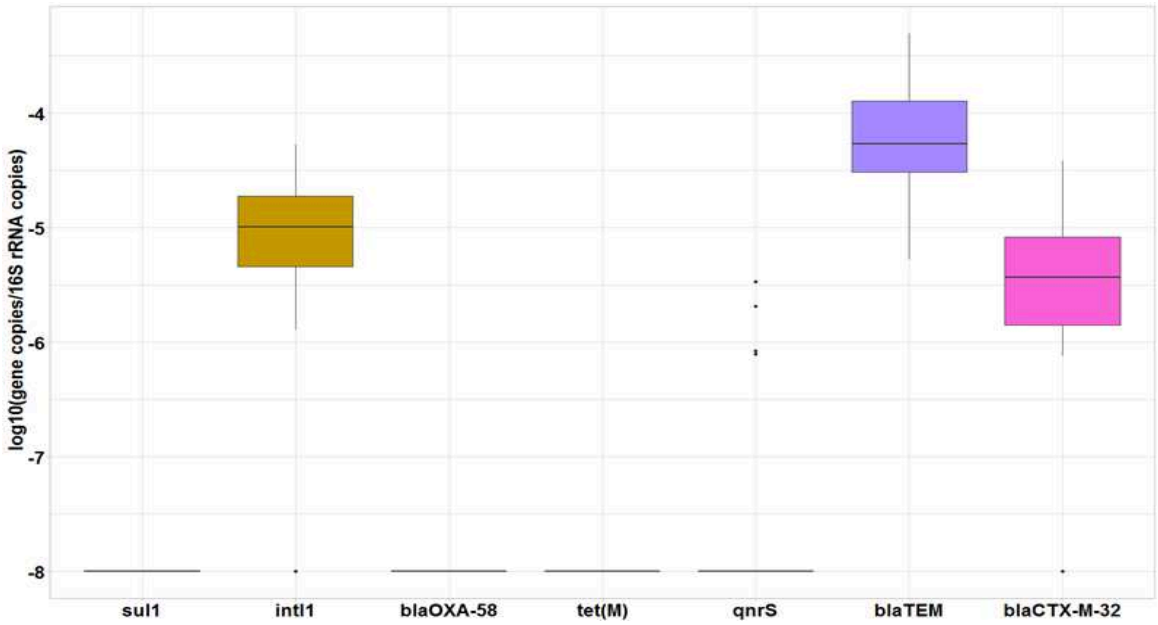


Figure S3.11: Relative abundance of ARGs and *intI1* in the soil of the forest, before it was used for microcosms experiments.

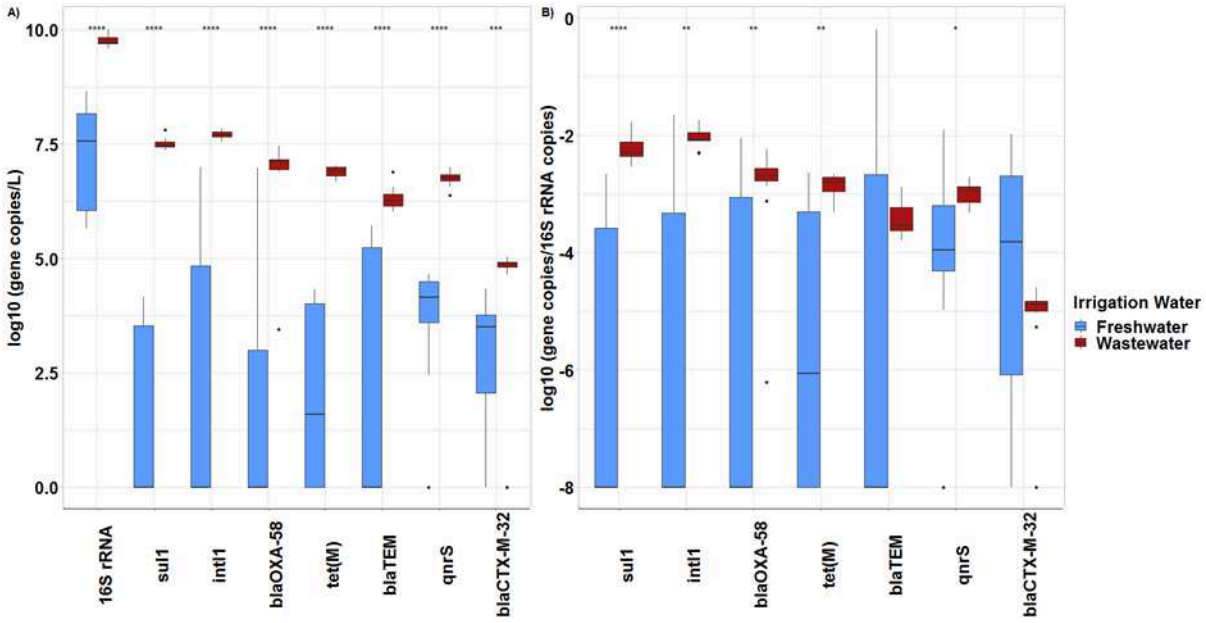


Figure S3.12: A) Absolute abundance of genes in copies/L and B) relative abundance of gene copies per 16S rRNA copy of the two types of irrigation water used for irrigation of the microcosms (Wilcoxon rank sum test *p<0.05, **p<0.01, ***p< 0.001, ****p<0.0001, n=6).

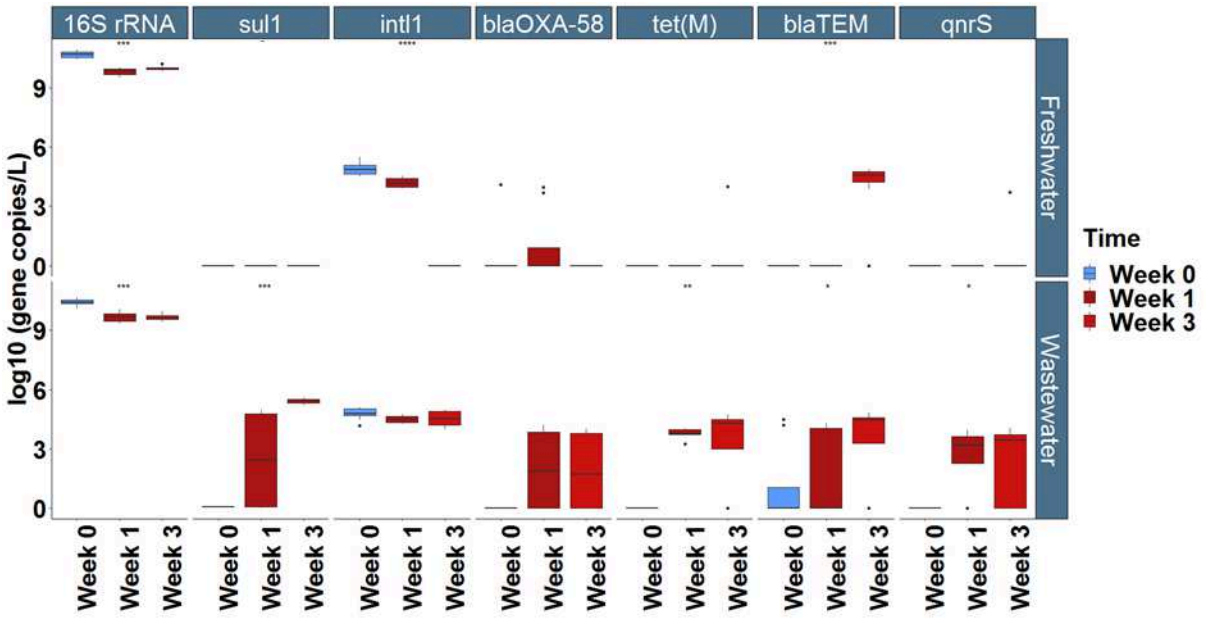


Figure S3.13: Absolute abundance of genes in the microcosm percolated pore-water samples. (Kruskal-Wallis test, *p<0.05, **p<0.01, ***p< 0.001, ****p<0.0001, n=4).

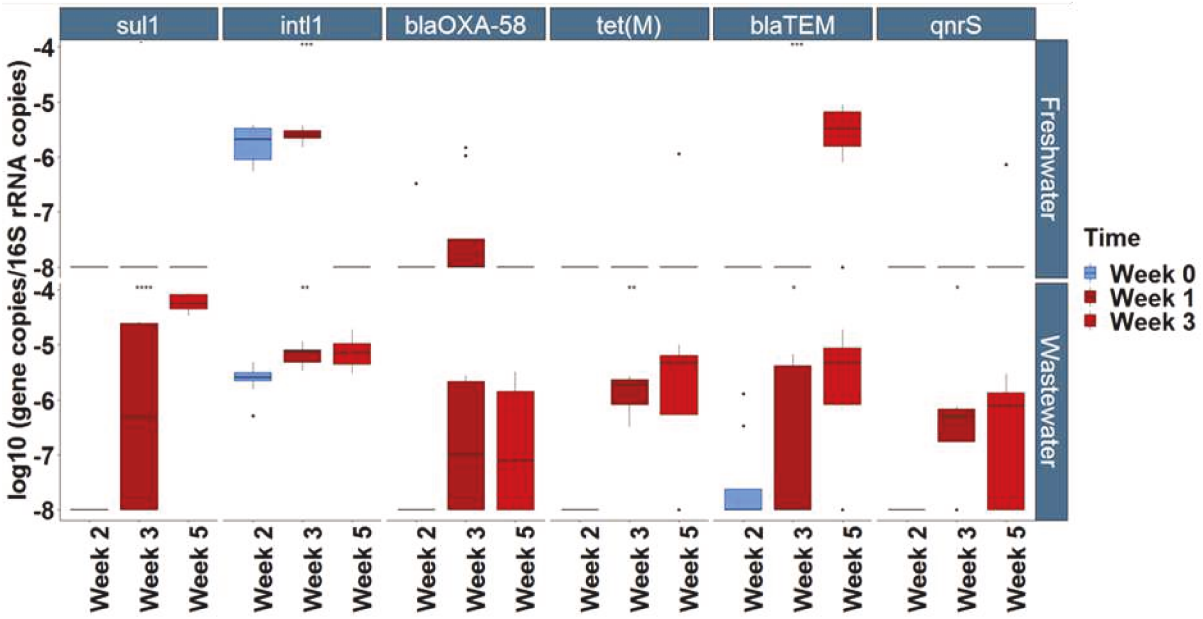


Figure S3.14: Relative abundance of genes in the microcosm percolated pore-water samples. (Kruskal-Wallis test *p<0.05, **p<0.01, ***p< 0.001, ****p<0.0001, n=4).

Apendix 3

(Supplementary Material for Chapter 4)

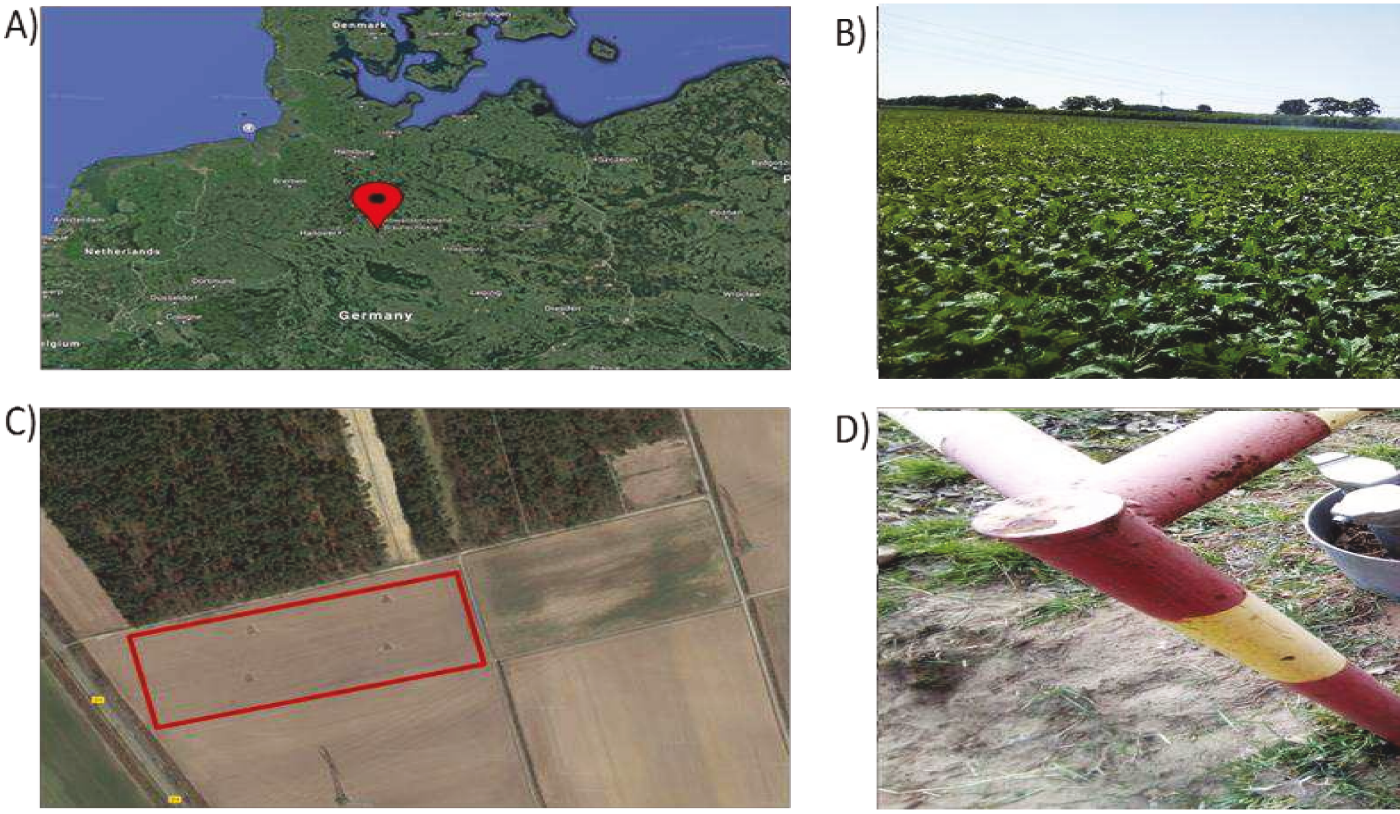


Figure S4.1: A) Location of BWA in Germany, B) Irrigation in the sampled field. C) Sampled field area (marked with red colour) and D) one of the three sampled groundwater wells (samples were taken at 10 m depth).

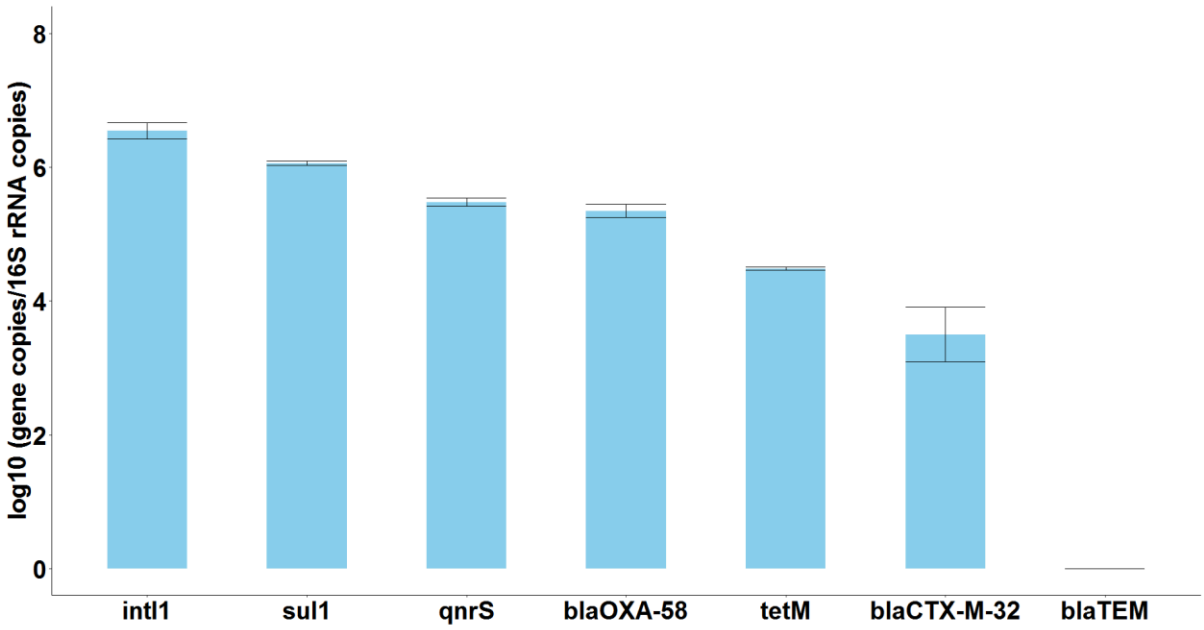


Figure S4.2: The $\log_{10}$ transformed relative abundance of ARGs and *intI1* in the irrigation water.

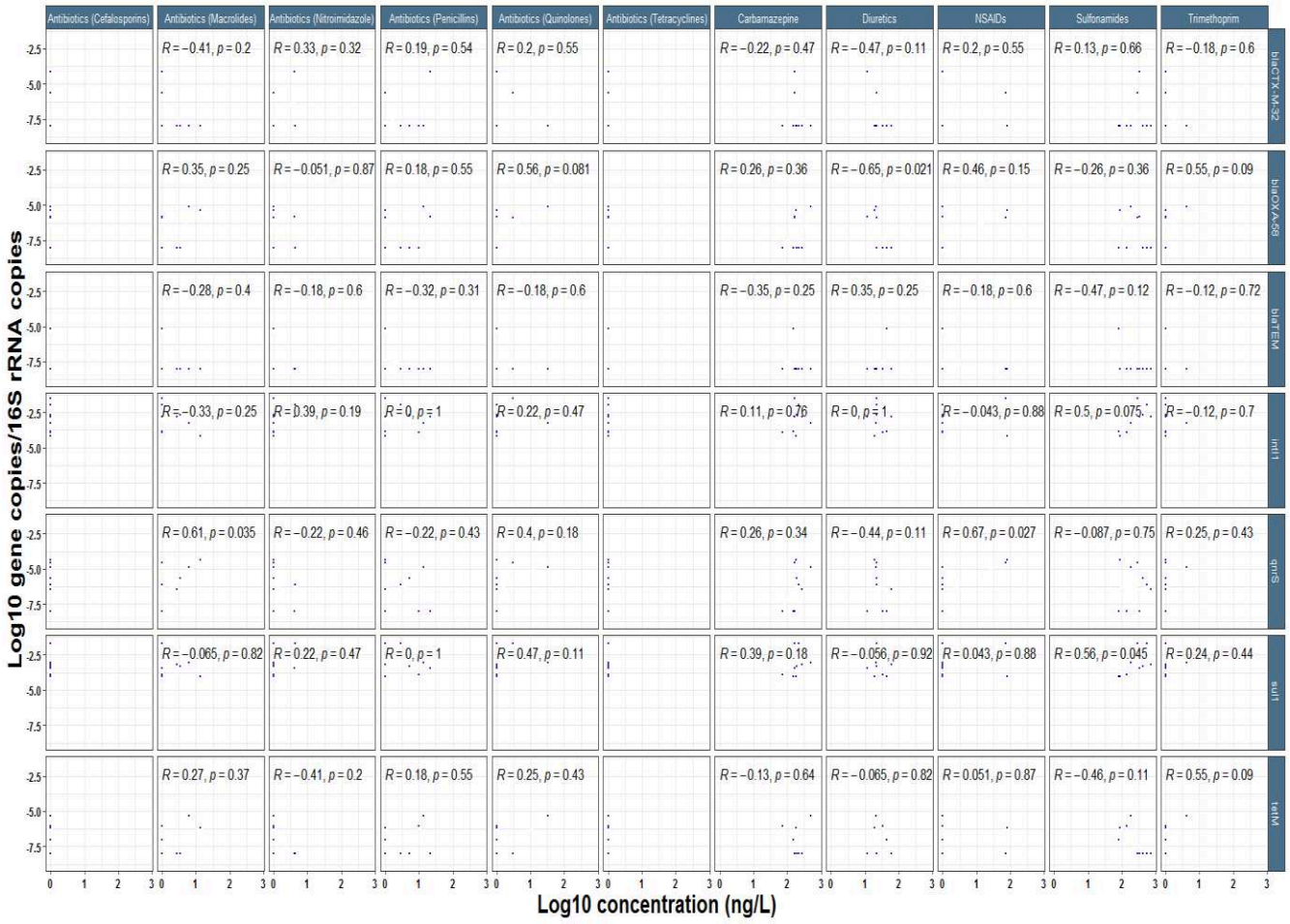


Figure S4.3: Kendall rank correlation of the median gene relative abundance for each gene per well (Fig. 4.3B) and the total drug residues concentration (ng/L) for each class of pharmaceutical residues of each sampled well (Fig 4.3B) (total n=9). Logistic issues led inefficient volume of sampled water in prior irrigation June 2018 sampling (high summer drought) and thus they were not included.

Table S4.1: Average annual chemical oxygen demand for the irrigation water in Braunschweig (TWW & DS n= 36, TWW n= 60).

	TWW		TWW & DS	
	COD-homogenised (mg/L)	COD-filtrate (mg/L)	COD-homogenised (mg/L)	COD-filtrate (mg/L)
Maximum	258	97	900	126
Average	62.8666667	44.5333333	468.25	96.5277778
Minimum	29	23	244	58

Table S4.2: Details for the tested genes and protocols of qPCR assays.

Target gene		Primers sequence	Amplicon size	Conditions	LOQ	Reference
16S rRNA (Indicator for microbial abundance)	Fw	TCCTACGGGAGGCAGCAGT	195 bp	95 °C - 10 min (1 cycle); 95 °C - 15 sec, 60 °C - 1 min (40 cycles) *	4000 copies per reaction	Rocha et al., 2018
	Re	ATTACCGCGGCTGCTGG				
bla$_{TEM}$ (class A β-lactamase)	Fw	TTCCTGTTTTTGCTCACCCAG	113 bp	95 ºC - 10 min (1 cycle); 95 ºC - 15 sec, 60 ºC - 1 min (40 cycles) **	40-400 copies per reaction	Rocha et al., 2018
	Re	CTCAAGGATCTTACCGCTGTTG				
bla$_{CTX-M-32}$ (class A β-lactamase, cephalosporinase)	Fw	CGTCACGCTGTTGTTAGGAA	156 bp	95 ºC - 10 min (1 cycle); 95 ºC - 15 sec, 58.5 ºC – 1 min (40 cycles) **	4-40 copies per reaction	Rocha et al., 2018
	Re	CGCTCATCAGCACGATAAAG				
sul1 (sulfonamide resistant dihydropteroate synthase)	Fw	CGCACCGGAAACATCGCTGCAC	162 bp	95 ºC - 10 min (1 cycle); 95 ºC - 10 sec, 60 ºC -1 min (40 cycles) **	40-400 copies per reaction	Rocha et al., 2018
	Re	TGAAGTTCCGCCGCAAGGCTCG				
qnrS (protein family which protects DNA gyrase from the inhibition of quinolones)	Fw	GACGTGCTAACTTGCGTG	118 bp	95 ºC - 10 min (1 cycle); 95 ºC - 15 sec, 60 ºC - 1 min (40 cycles) **	4-40 copies per reaction	Rocha et al., 2018
	Re	TGGCATTGTTGGAAACTT				
intI1 (class I integrase, this gene is associated with horizontal gene transfer	Fw	GATCGGTCGAATGCGTGT	196 bp	95 ºC - 10 min (1 cycle); 95 ºC - 15 sec, ºC - 1 min (40 cycles) **	40-400 copies per reaction	Rocha et al., 2018
	Re	GCCTTGATGTTACCCGAGAG				

and environmental pollution)						
bla$_{OXA-58}$ (class D β-lactamase, carbapemenase)	Fw	CACTTACAGGAAACTTGGGGTCG	79 bp	95 ºC - 10 min (1 cycle); 95 ºC - 15 sec, 60 ºC - 1 min (40 cycles) **	4-40 copies per reaction	Cacace et al., 2019
	Re	AGTGTGTTTAGAATGGTGATC				
tet(M) (ribosomal protection protein that protects ribosome from the translation inhibition of tetracycline)	Fw	GCAATTCTACTGATTTCTGC	186 bp	95 ºC - 10 min (1 cycle); 95 ºC - 15 sec, 55 ºC - 1 min (40 cycles) ***	40 copies per reaction	Cacace et al., 2019
	Re	CTGTTTGATTACAATTTCCGC				

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17 * 0.5 μM primers, ** 0.25 μM primers, *** 0.2 μM primers and 0.1 mg/mL BSA.

Table S4.3: Efficiency, LOQ and R^2 for each amplified gene. The LOD for each reaction was at 3 copies per reaction

Target gene	Efficiency	R^2	Negative Control Cq	LOQ
16S rRNA	93%	0.999	28.74	4000 copies per reaction
bla$_{TEM}$	96%	1	32.42	40 copies per reaction
bla$_{CTX-M-32}$	100%	0.995	-	4 copies per reaction
sul1	96%	0.999	35.75	40 copies per reaction
qnrS	98%	0.995	38.5	4 copies per reaction
intI1	95%	0.992	35.64	40 copies per reaction
bla$_{OXA-58}$	95%	0.997	-	4 copies per reaction
tet(M)	90%	0.995	39.1	4 copies per reaction

Table S4.4. Conditioning and extraction program used for sample preparation of wastewater samples by HORIZON SPE-DEX 4790.

		Solvent	Soak Time (sec)	AirDry Time (sec)
HLB conditioning	PreWet Cycle	Isopropanol	-	5
		Isopropanol	-	5
		Milli-Q Water	-	5
		Methanol	-	5
		Ethyl Acetate	-	5
	Rinse Cycle	Methanol	-	5
		Ethyl Acetate	-	5
Extraction	PreWet Cycle	Ethyl Acetate	120	30
		Ethyl Acetate	120	30
		Ethyl Acetate	90	30
		Methanol	120	30
		Methanol	120	30
		Methanol	60	30
		Milli-Q water	120	30
		Milli-Q water	60	30
		Milli-Q water	60	30
	Sample AirDry Cycle	Ethyl Acetate	150	60
		Ethyl Acetate	90	30
		Ethyl Acetate	90	30
		Methanol	150	60
		Methanol	90	30
		Methanol	90	30

Table S4.5: LC-MS/MS conditions in positive ionization mode and gradient elution program.

Positive Ionization Mode			
GRADIENT PROGRAM		**ESI (+) Parameters**	
Time (min)	% B	Spray Voltage	3500V
0	2	Capillary temperature	270 °C
3	2	Sheath gas	30 psi
20	100	Auxiliary (drying) gas	10 a.u.
29	100	(A) H_2O 0.01% v/v HCOOH (B) MeOH	
30	2		
45	2		

Table S4.6: Selected reaction monitoring transitions of the targeted analytes, precursor ions, product ions, collision energies and tube lens.

Analytes	Precursor Ion		Product Ion 1 (m/z)	CE (eV)	Product Ion 2	CE (eV)	Tube Lens (V)
3,4-Methylenedioxy-methamphetamine (MDMA)	180		163	9	135	17	55.6
2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP)	278		234	30	249	23	78.6
3,4-Methylenedioxyamphetamine (MDA)	194		163	12	135	20	60.1
4-OH-Omeprazole	330		182	23	149.1	25	90
6-Monoacetylmorphine (6-MAM)	328		165	35	211	25	98.9
7-amino-Flunitrazepam	284		135	27	148	26	86.8
8-OH-Mirtazapine	282		211	26	225	23	86.4
9-OH-Risperidone	427		207	28	110	39	98.6
Alprazolam	309		281	25	205	38	92.8
Amisulpride	370		241.9	26	195.9	39	84
Amitriptyline	278		233	18	191	25	68.3
Amoxicillin	366		349	8	114	22	68
Amphetamine	136		91	16	119	6	54.8
Ampicillin	350		106	27	160	14	98
Atenolol	267		145	26	190	18	94
Atorvastatin	559		440.1	22	250	42	123
Azithromycin	749		591	29	158	37	127
Benzoylecgonine (BECG)	290		168	19	105	30	82.6
Bromazepam	316		182	31	209	26	97.9
Caffeine	195		138.2	18	110.2	22	87
Carbamazepine	237		194.1	19	193.1	32	114
Cathine	152		115.1	26	91.1	17	55
Cefaclor	368		178	31	106	27	93
Cefadroxil	364		114	22	134	33	79
Cefalexin	348		158	6	106.1	30	113
Cefalexine	348		158	6	106	30	113
Cefazolin	455		156	17	323	10	79
Chloramphenicol	321		257	13	152	19	90
Chlordiazepoxide	300		227	25	241	15	113.4
Chlorpromazine	319		86	20	246	23	68.6
Chlortetracycline	479		444	20	462	15	90
Cimetidine	253		159.1	13	95.2	29	73
Ciprofloxacin	332		288	18	314	22	85
Citalopram	325		109	29	262	19	100.4
Clarithromycin	749		158	30	591	20	123
Clobazam	301		259	20	224	32	86.9

Clomipramine	315		86	18	58	39	73.8
Clonazepam	316		269.8	25	213.9	37	86
Cloxacillin	410		178	33	174	19	80
Clozapine	327		270	23	192	39	88.9
Cocaine (COC)	304		182	19	150	25	78.9
Codeine (COD)	300		215	25	165	39	112.1
Diazepam	285		193	30	154	27	84.2
Dicloxacillin	468		327	15	424	11	91
Difloxacin	400		356	20	299	27	85
Doxepin	280		107	25	235	15	101.1
Doxycycline	445		427	19	267	35	90
Duloxetine	298		44.4	12	123	45	48
Enrofloxacin	360		245	25	317	20	85
Ephedrine	166		148	12	117	24	59.1
Erythromycin	734		158	30	576	20	130
Fentanyl	337		188	24	105	34	90.8
Florfenicol	356		336	11	185	18	90
Flumequine	262		244	20	202	30	85
Flunitrazepam	314		268	25	211	33	85.6
Fluoxetine	310		44	13	117	74	61.3
Flurazepam	388		314.8	23	316.8	18	74
Gabapentin	172		154	13	137	16	64
Haloperidol	376		164.9	23	123	34	89
Heroin (HER)	370		165	45	211	30	134.1
Imipramine	281		86	17	58	35	63.6
Ketamine	238		125	28	207	13	61.6
Lacosamide	251		91.1	28	74.2	27	48
Lamotrigine	256		159	29	166	27	90.7
Levetiracetam	171		126	15	154	5	48.8
Lidocaine	235		86	17	58	32	63.6
Lincomycin	407		126	30	359	17	99
Lorazepam	321		275	23	303	14	79
Lysergic acid diethylamide (LSD)	324		223	23	208	28	77.6
Marbofloxacin	363		320	15	72	20	85
Medazepam	271		206.9	27	91.1	30	63
Methadone (METH)	310		265	15	105	28	53.3
Methamphetamine (MA)	150		91	19	119	10	50.1
Methylenedioxyethylamphetamine (MDEA)	208		163	12	135	22	63.4
Metoprolol	268		191.1	17	133.1	25	96
Metronidazole	172		128	13	82	25	69
Midazolam	326		291	27	209	33	90.3
Minocycline	458		441	19	352	29	105
Mirtazapine	266		195	24	209	23	68.3

Morphine (MOR)	286		201	25	165	33	90.6
Niflumic Acid	283		264.9	22	244.9	28	97
Nitrazepam	282		236	24	250	14	98.4
Norbuprenorphine (NBN)	414		413.4	13	186.9	36	118
Norclozapine	313		192	38	270	23	88.6
Nordiazepam	271		140	28	165	26	87.3
Norephedrine	152		134	10	117	17	59.3
Norfentanyl	233		84	17	177	15	63.3
Norfloxacin	320		276	16	233	23	91
Norketamine	224		207	12	125	29	54.6
Normirtazapine	252		195	23	193.9	38	86
Norolanzapine	299		255.9	22	197.8	38	77.2
Norsertraline	292		275	10	159	25	58.8
Nortriptyline	264		233	14	91	31	84.6
Norvenlafaxine	264		246	11	58.3	17	64
Ofloxacin	362		318	19	261	27	120
Olanzapine	313		256	23	213	29	84.1
Oxacillin	402		114	32	259	24	65
Oxazepam	287		241	22	269	14	81.8
Oxcarbazepine	313		255.9	22	197.8	38	79
Oxolinic acid	262		244	18	158	31	79
Oxycodone (OC)	316		298	19	241	29	89.8
Oxytetracycline	461		426	19	443	12	90
Paracetamol	152		93.2	22	110.2	15	84
Paroxetine	330		192	19	135	34	83.8
Prazepam	325		270.8	22	139.9	34	77
Pregabalin	160		55.3	23	124.1	15	67
Primidone	219		162	11	91	29	106.9
Progesterone	315		109.2	28	97.2	24	79
Propranolol	260		183.1	19	155.2	25	99
Quetiapine	384		252.9	22	220.9	34	82
Ranitidine	315		176	17	102.2	31	79
Remifentanil	377		317	14	113	29	76
Risperidone	411		191	30	110	42	78.1
Rivastigmine	251		206	14	58.3	33	70
Ronidazole	201		140.1	10	55.5	21	73
Sarafloxacin	386		342	18	299	27	85
Sertraline	306		275	13	159	26	53.1
Simvastatin	419		199	14	224.9	19	103
Sulfachloropyridazine	285		92	28	156	14	87
Sulfaclozine	285		92	28	156	15	87
Sulfadiazine	251		156	15	92	27	87
Sulfadimethoxine	311		156	17	108	29	87
Sulfadoxine	311		156	17	108	27	87

Sulfaguanidine	215		156	14	92	14	87
Sulfamerazine	265		156	16	172	16	87
Sulfamethizole	271		156	14	92	28	87
Sulfamethoxazole	254		156	16	108	25	87
Sulfamethoxypyridazine	281		156	13	92	29	87
Sulfamonomethoxine	281		156	13	92	29	87
Sulfamoxole	268		156	13	92	28	87
Sulfapyridine	250		156	15	184	17	87
Sulfaquinoxaline	301		156	18	92	30	87
Sulfathiazole	256		156	15	92	26	87
Sulfisoxazole	268		156	13	92	27	87
Sulpiride	342		112.1	26	213.8	31	90
Temazepam	301		255	23	283	13	88.6
Tetracycline	445		410	18	426	12	90
Tetrazepam	289		196.9	31	153.9	31	92
Theophylline	181		124.1	17	96.2	22	79
Thiamphenicol	354		290	11	185	19	90
Tiagabine	376		246.9	18	148.9	25	82
Tiamulin	494		192	21	119	33	101
Tramadol	264		58.4	15	246	8	66
Triamterene	254		237	26	104.1	36	93
Trimethoprim	291		230	25	123	30	87
Tylosin	917		174	36	772	28	148
Valsartan	436		207	28	291	16	99
Venlafaxine	278		260	12	121	28	69.8
Zolpidem	308		234.9	33	262.9	25	96
Zopiclone	389		245	17	217	31	73.8

Table S4.7: The concentration of the most abundant drug compounds and antibiotics in irrigation water samples from July 2018 (TWW & DS) and September 2018 (DS) (TWW: Treated Wastewater, DS: Digested sludge)

Compound	Type of Drug	Concentration (ng/L)	Sample
Azithromycin	Macrolide (Antibiotic)	18.3	TWW & DS
Carbamazepine	Anticonvulsant	107.2	TWW & DS
Carbamazepine	Anticonvulsant	43.4	TWW
Ciprofloxacin	Quinolone (Antibiotic)	66.4	TWW & DS
Doxycycline	Tetracycline (Antibiotic)	194.5	TWW & DS
Erythromycin	Macrolide (Antibiotic)	22.9	TWW & DS
Hydrochlorothiazide	Diuretic	160.2	TWW & DS
Hydrochlorothiazide	Diuretic	141.3	TWW
Ibuprofen	NSAID	114.5	TWW & DS
Ibuprofen	NSAID	100.3	TWW
Lincomycin	Macrolide (Antibiotic)	9.4	TWW & DS
Metronidazole	Nitroimidazole (Antibiotic)	7.5	TWW & DS
Ofloxacin	Quinolone (Antibiotic)	60.0	TWW & DS
Sulfachloropyridazine	Sulfonamide (Antibiotic)	2.5	TWW & DS
Sulfaclozine	Sulfonamide (Antibiotic)	23.8	TWW & DS
Sulfadiazine	Sulfonamide (Antibiotic)	24.1	TWW & DS
Sulfamerazine	Sulfonamide (Antibiotic)	8.26	TWW & DS
Sulfamethizole	Sulfonamide (Antibiotic)	7.27	TWW & DS
Sulfamethoxazole	Sulfonamide (Antibiotic)	85.37	TWW & DS
Sulfamethoxazole	Sulfonamide (Antibiotic)	61.85	TWW
Sulfamonomethoxine	Sulfonamide (Antibiotic)	1.32	TWW & DS
Sulfamoxole	Sulfonamide (Antibiotic)	30.23	TWW & DS

Sulfapyridine	Sulfonamide (Antibiotic)	19.49	TWW & DS
Sulfisoxazole	Sulfonamide (Antibiotic)	23.73	TWW & DS
Trimethoprim	Trimethoprim (Antibiotic)	15.01	TWW & DS

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"The most exciting phrase to hear in science, the one that heralds new discoveries, is not Eureka! (I found it!) but That's funny …"

Isaac Asimov