Impact of antibiotics and particulate matter from wastewater discharges on the abundance of antibiotic resistance genes in river sediments

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DISSERTATION

von
M.Sc. Philip Clifford Brown
aus Sigmaringen

Referent: Prof. Dr. Harald Horn
Korreferent: Prof. Dr. Johannes Gescher
Korreferent: Prof. Dr. Thomas Schwartz
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Abstract

The relationship between wastewater discharge by wastewater treatment plants (WWTPs) and the abundance of antibiotic resistance genes (ARGs) in river sediments of the receiving waterbody was investigated. The goal was to assess which component of wastewater, i.e. residuals of antibiotics (ABs), or particulate matter, has the larger impact.

First results indicated that, at environmental concentrations, the selection of ARGs by ABs might not be as significant as widely accepted. Instead, they emphasized the significance of ARG transport, and highlighted the relevance of wastewater particles and associated microorganisms. They showed that ARGs (*erm*B, *bla*$_{TEM,}$ *tet*M, *qnr*S) as well as facultative pathogenic bacteria (FPB) (*enterococci*, *Pseudomonas aeruginosa, Acinetobacter baumannii*) inside the particulate fraction of WWTP effluent are likely to remain in the riverbed of the receiving water due to sedimentation. Moreover, ARG and FPB abundances measured in the particulate fraction strongly correlated with the delta ARG and FPB abundances measured in the receiving river sediment (downstream compared to upstream) ($R^2 = 0.93$, $p < 0.05$). Yearly, several hundred tons of suspended solids are discharged by the investigated WWTP, from which approximately 50 % are particles large enough to settle within a short distance downstream of the discharge point. Apparently, the sheer amount of associated ARGs and FPB is sufficient, to increase abundances in the receiving riverbed by 0.5 to 2 log units.

To better understand the dynamics of the above described sedimentation process, a particulate wastewater fraction was obtained through sieving and filtering, and added to batch reactors, which were previously filled with natural river sediments, and tap water. Additionally, ABs (erythromycin, tetracycline, ciprofloxacin, roxithromycin, penicillin V, and sulfamethoxazole) were spiked to investigate their capability to select for resistance. The abundance of six ARGs (*erm*B, *tet*M, *bla*$_{TEM}$, *sul*1, CTX-M-32, and *qnr*S) as well as total bacteria (16S rDNA) was monitored in waters and in sediments for a duration of two months. Despite a continuous exposure to ABs

(5 µg/L), the abundance of ARGs remained unaffected. Addition of wastewater particles resulted in a sudden and strong increase of ARGs in waters (3 – 5 log units) and sediments (1 – 4 log units), however, elevated ARG levels underwent a certain and complete decay. Our results indicate that the increased ARG abundances in receiving rivers are the result of a continuous import of ARGs from wastewater discharges or sewer overflow events. They further imply that elevated ARGs do not persist in receiving rivers, if this continuous import is removed.

Zusammenfassung

In der vorliegenden Arbeit wurde der Zusammenhang zwischen der Einleitung von kommunalen Kläranlagen und der Menge an Antibiotikaresistenzgenen (ARG) in Flusssedimenten des Vorfluters untersucht. Eine wesentliche Frage war dabei, was der Treiber für die Akkumulation der ARG ist. Zur Auswahl standen dabei die Einleitungen von Antibiotikarückständen (ABs) auf der einen Seite oder die partikuläre Fracht auf der anderen Seite. Letztere ist dafür bekannt, pathogene Organismen aber auch ARG mit sich zu führen.

Die Ergebnisse einer Feldstudie zeigten zunächst, dass die Selektion von ARG durch Antibiotikarückstände möglicherweise weniger entscheidend ist, als allgemein angenommen wird. Stattdessen wiesen sie auf die zweite der oben benannten Optionen für die Verbreitung von ARG hin: ARG (*erm*B, *bla*TEM, *tet*M, *qnr*S) assoziieren zum Teil stark mit den partikulären Bestandteilen im Abwasser und gelangen damit über die Sedimentation in das Sediment der Vorfluter.

Die ARG-Menge in der sedimentierbaren Fraktion von Abwasser (Genkopien pro g Schwebstoffe, engl. total suspended solids, TSS) korrelierte dabei positiv mit der Änderung der ARG-Menge im Flusssediment des Vorfluters (Differenz der Genkopien pro g Sediment unterhalb und oberhalb der Einleitungsstelle) ($R^2 = 0,93$; $p < 0,05$).

Jährlich werden mehrere hundert Tonnen an partikulären Bestandteilen aus dem untersuchten Klärwerk in den Vorfluter eingeleitet. Ca. 50 % davon kann sich unmittelbar hinter der Einleitungsstelle absetzen. Scheinbar ist die Sedimentation dieser Masse entscheidend für die Verbreitung von ARG im Flusssediment und führt zu einer Erhöhung um 0,5 – 2 Zehnerpotenzen.

Um die Dynamik des oben beschriebenen Sedimentationsprozesses besser zu verstehen, wurde im nächsten Schritt eine Partikelfraktion aus kommunalem Abwasser extrahiert (durch Sieben und Filtration) und in Batchreaktoren gegeben, welche zuvor mit natürlichen Flusssedimenten und Leitungswasser befüllt wurden. Parallel

hierzu wurden ABs (Erythromycin, Tetrazyklin, Ciprofloxacin, Roxithromycin, Penicillin V und Sulfamethoxazol) hinzugegeben, um sie auf ihre Fähigkeit zur Selektion von ARG zu testen. Die Entwicklung der Häufigkeit von sechs ARGs (*erm*B, *tet*M, *bla*TEM, *sul*1, CTX-M-32 und *qnr*S) und die Gesamtzahl an Bakterien (16S rDNA) wurde in der Wasserphase und im Sediment über einen Zeitraum von zwei Monaten verfolgt. Trotz der relativ hohen Konzentration an ABs, welche über den gesamten Zeitraum auf konstantem Niveau gehalten wurde (5 µg/L), blieb die Häufigkeit der ARG unverändert. Die Zugabe von Abwasserpartikeln führte zu einem sofortigen, starken Anstieg an ARG in der Wasserphase (3 – 5 Zehnerpotenzen) und im Sediment (1 – 4 Zehnerpotenzen). Erhöhte ARG-Mengen gingen allerdings mit einem bestimmten und vollständigen Zerfall einher.

Die Ergebnisse sowohl aus dem Feldversuch als auch aus den gezielten Versuchen im Labor zeigen, dass die erhöhte Häufigkeit an ARG in Vorflutern durch den kontinuierlichen Eintrag von ARG aus Klärwerksabläufen verursacht wird. Sie lassen zudem vermuten, dass ARG nicht persistieren, wenn dieser Eintragspfad unterbrochen wird.

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1 Overview – theoretical background

The spread of antibiotic resistant bacteria (ARB) and ARGs has been described as one of the biggest threats facing humankind in the 21st Century ("WHO | United Nations meeting on antimicrobial resistance," 2016). It is estimated that during the time between 2014 and 2016, approximately one million people died due to antibiotic resistant infections and current projections suggest that antibiotic resistance will cause increasingly more deaths within the following decades ("WHO | United Nations meeting on antimicrobial resistance," 2016). While the circumstances leading to the transmission of resistances are complex, it is generally thought that it can be largely attributed to the excessive use of ABs in clinical or agricultural settings (Holmes et al., 2016). Unfortunately, due to the mobility of ARGs and their tendency to be transmitted, it is a major challenge to eliminate the threat once antibiotic resistance develops.

Nutrient rich environments with high bacterial numbers are ideal settings within which resistance can develop (Baquero et al., 2008). Wastewaters represent such environments and have shown to serve as point sources for ARGs and resistant pathogens (Berendonk et al., 2015; Di Cesare et al., 2016; Hembach et al., 2017). Due to the incomplete removal of ARGs and ARB in WWTPs, the release of wastewater into receiving rivers by WWTP discharges is a key mechanism by which ARGs and ARB enter the environment (Schwartz et al., 2003). This is worrisome because of gene transmission between indigenous and allochthonous bacteria by which ARGs are recruited into clinically relevant pathogens (Forsberg et al., 2012). Humans, animals, and plants are connected through the environment and may therefore exchange these pathogens, including the ARGs they carry.

In order to understand the complexity of antimicrobial resistance development, theoretical background and a comprehensive overview of the current state-of-the-art is essential. This chapter intends to describe the evolution of antimicrobial resistance and the role of ABs in accelerating this development. As so often the case with evolutionary processes, it reaches far back in time. There is, withal, one major force that is driving the evolution of early life on earth: Horizontal gene transfer.

1.1 Evolution of antimicrobial resistance and environmental relevance

All living organisms can be divided into three domains of life: *Bacteria*, *Eukarya, and Archaea* (Figure 1.1). While it is established that organisms from one domain to another (or from one kingdom to another) have evolved to be distinct from each other, archaeal, bacterial and eukaryotic genomes share certain similarities and contain genes from multiple sources. This is thought to be largely attributed to horizontal gene transfer (HGT) between species. HGT is the non-sexual transmission of genetic material between unrelated genomes, hence, HGT is gene transfer across species boundaries. Classical phylogenetic trees are based on sequence similarities of orthologous genes (Figure 1.1 A) and therefore cannot universally describe the origin of all organismal lineages. However, by defining organisms as more than just the sums of their genes, and acknowledging the evolutionary role of HGT, one might think of organismal lineages as more of an intertwined existence (Figure 1.1 B) (Doolittle, 1999). HGT it is a major and continuing force in archaeal and bacterial evolution and the primary mechanism for the spread of ARGs in bacteria.

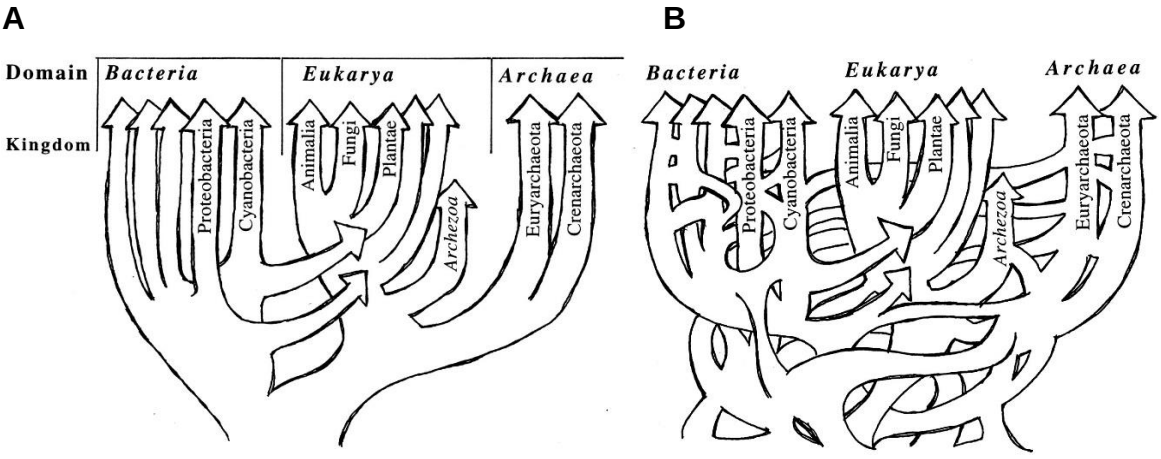

Figure 1.1: (A) The neo-Darwinian tree about the evolution of life on earth based on natural classification. The distinctive nature of the three domains is supported by differences in small subunit ribosomal ribonucleic acid (SSU rRNA) sequences. (B) An intertwined tree, which more appropriately represents the evolution of life on earth, reflects horizontal gene transfer events as major forces in archaeal, bacterial, and early eukaryotic evolution (Doolittle, 1999).

1.1.1 Origin, emergence, and human impact

Antimicrobials as well as antimicrobial resistance (AMR) emerged from microbial communities 2 billion to 40 million years ago as products of long lasting evolutionary processes (D'Costa et al., 2011; Munita and Arias, 2016). AMR emerged because of the interaction of organisms with their environment, and with naturally occurring ABs, in order to survive and compete for resources. Proof that AMR is a natural phenomenon, which predates modern medicine, can be found in the study of D'Costa et al. (2011) who found a highly diverse set of genes that confer resistance to beta-lactams, tetracyclines, and glycopeptides in DNA extracts from 30'000-year-old permafrost sediments. The authors confirmed the similarity of these ARGs to modern variants.

Contrary to the long lasting process of evolution described above, AMR may also develop fast. This is possible because of the genetic variability, high mutation rates, and short generation times of bacteria. The severity and speed with which AMR developed over the past few decades, however, was certainly provoked by human activities. The extensive use of ABs in modern medicine has accelerated the evolution of AMR to a degree that resistance mechanisms have now strongly diversified and multiplied. This is the result of the selection effect of ABs (Figure 1.2) as well as ARG transfer events between organisms (Figure 1.3). Before the ABs era, which began in the early 1940s, organisms were merely "intrinsically" resistant to one or more ABs. Today, clinically relevant pathogens that were originally susceptible to ABs are now causing problems because they have "acquired" resistances from external sources via HGT (Figure 1.3). Additionally, previously unknown variants of ARGs have emerged due to the tendency of ABs to increase mutation rates in bacteria. A good example for this are beta-lactamases. To date, more than 1'000 different variants have been described, which are the product of mutations as part of the normal process of bacterial evolution, and as part of AB-driven adaptive bacterial evolution (Munita and Arias, 2016).

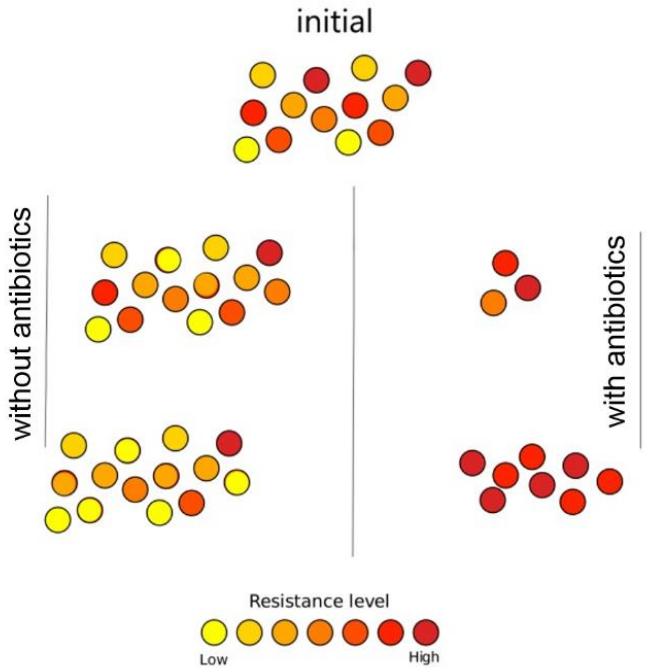

Figure 1.2: Effect of antibiotics on a microbial community. By using antibiotics, susceptible strains are killed, while intrinsically resistant bacteria survive. The intrinsically resistant bacteria can now multiply unhindered as they do not have to compete for resources (Mahapatra, 2015).

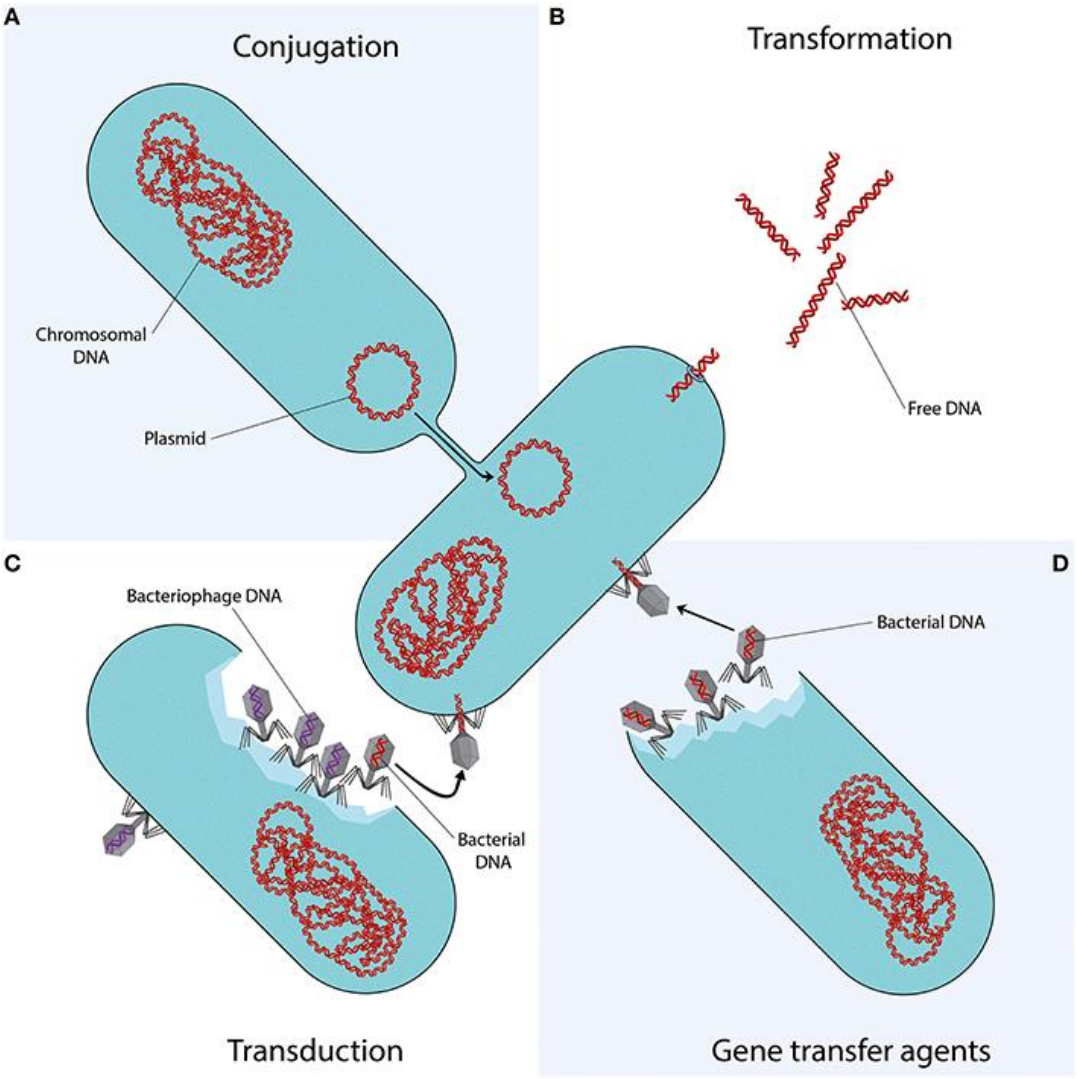

Figure 1.3: Mechanisms of horizontal gene transfer that may lead to acquired resistance in bacteria. (A) Conjugation: transfer of DNA from the donor cell to the recipient cell through surface pili or adhesins. (B) Transformation: uptake, integration, and functional expression of naked fragments of extracellular DNA. (C) Transduction: transfer of bacterial DNA through bacteriophages from a previously infected donor cell to a recipient cell. (D) Gene transfer agents: bacteriophage-like particles that carry random pieces of the producing cell's genome (von Wintersdorff et al., 2016).

1.1.2 Resistance mechanisms

Whether attributed to the normal process of bacterial evolution, or to AB-driven adaptive bacterial evolution, microorganisms have developed mechanisms to overcome the effects of ABs. These will be described in the following paragraph. Yet

beforehand, it is noteworthy that resistance to one AB class can usually be achieved through multiple biochemical pathways, while one bacterial cell may be capable of using several mechanisms of resistance to survive the effect of one AB (Munita and Arias, 2016). It is a general strategy in bacterial cell physiology to respond to environmental stressors in multiple ways.

Resistance to ABs can be achieved with the following four mechanisms: (1) modification of the AB through chemical alterations, such as acetylation (aminoglycosides, chloramphenicol, streptogramins), phosphorylation (aminoglycosides, chloramphenicol), and adenylation (aminoglycosides, lincosamides), or through destruction of the molecule (as beta-lactamases do by destroying the amide bond of the beta-lactam ring), (2) decreased AB penetration (reduced expression or alteration of membrane diffusion proteins, commonly known as porins) and AB efflux (increased expression of efflux pumps that actively extrude ABs as for example tetracyclines, beta-lactams, fluoroquinolones, and erythromycin), (3) changes in target sites through target protection (as for example mediated by the tetracycline resistance genes *tet*M and *tet*O as well as by the quinolone resistance genes *qnr*A, *qnr*B, *qnr*C, *qnr*D, *qnr*S, and *qnr*VC) or through modification of the target site (by mutation, or enzymatic alteration, as known for the enzymes encoded by the *erm* gene variants), or complete replacement of the target site (methicillin resistance *mec*A in *Staphylococcus aureus*, and vancomycin resistance *van*A in *enterococci*), and finally, (4) resistance due to global cell adaptations (for example daptomycin resistance via sequential and ordered genetic changes in cell envelope homeostasis) (Munita and Arias, 2016).

There are many resistant microorganisms that cause illness in humans. It is not within the scope of this introduction to describe them all, however, some of the most critical species and groups as well as the problematic resistance mechanisms they carry are presented in the following subsection.

1.1.3 Clinically relevant resistance genes and pathogenic bacteria

On account of transmission and accumulation of ARGs, multi-resistant pathogens emerge, against which a variety of ABs are ineffective. Clinical settings reflect this

development, within which the regular use of ABs and sub-optimal hygiene standards strongly enhanced the spread of AB resistant pathogens. Particularly problematic and hard-to-treat infections are caused by multi-resistant *Staphylococcus aureus* (MRSA), extended-spectrum beta-lactamase (ESBL)-producing *Enterobacteriaceae* (or ESBL-producing *E. coli*), vancomycin resistant *enterococci* (VRE), fluoroquinolone resistant *Pseudomonas aeruginosa* (FQRP), and the upcoming resistant *Acinetobacter baumannii* and *Klebsiella pneumoniae* strains. All of the aforementioned organisms, genera, or families are normal residents of the healthy human or animal microbiome that can become opportunistic pathogens. They are therefore also referred to as FPB (facultative pathogenic bacteria). A more extended list of problematic strains and resistance genes may be studied in the GERMAP 2015 report (Federal Office of Consumer Protection and Food Safety and Paul-Ehrlich-Gesellschaft für Chemotherapie e.V., 2016).

MRSA strains are still considered dangerous in clinical settings because they are hard to eradicate. They circulate between patients and therefore serve as dominant ARG carriers. Treatment against MRSA became more demanding as co-resistance to other important ABs was acquired by MRSA strains in recent years (Figure 1.4). It is believed that MRSA might in future contribute to the spread of new methicillin resistance (*mec*A) variants and establish the linezolid resistance *cfr* (Morales et al., 2010). Linezolid was marketed in the year 2000 (Silver, 2011).

ESBL-producing *E. coli* are troublesome as they obtained not only resistance against the third-generation cephalosporin cefotaxime (mediated by the CTX-M genes) but also against a variety of fluoroquinolones. Additionally, plasmid-mediated resistance *mcr-1* to polymyxins (colistin) was recently detected in *E. coli* isolates, showing a negative trend in the efficacy of ABs for these organisms (Liu et al., 2016, p. 1). CTX-M is a plasmid-encoded ESBL commonly found in *K. pneumoniae*, *E. coli*, and other *Enterobacteriaceae*. This enzyme was likely acquired through HGT from *Kluyvera* spp., an environmental bacterium with no major human pathogenic significance (Bonnet, 2004).

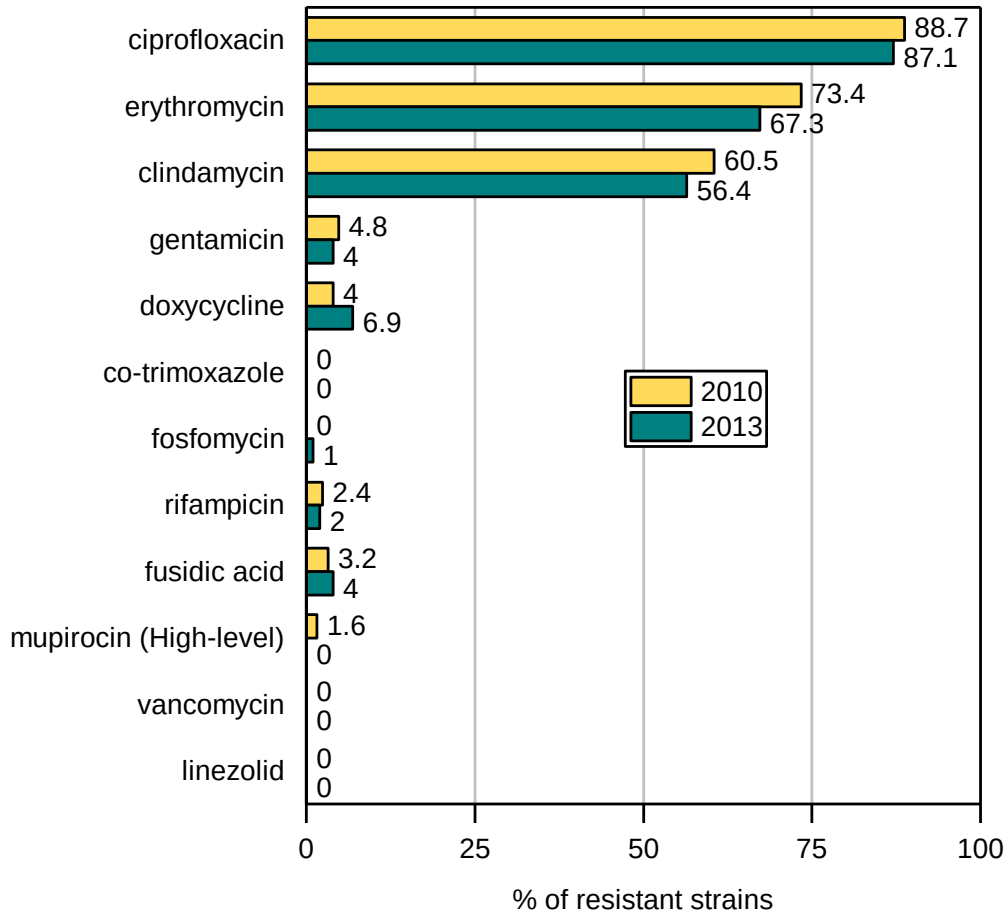

Figure 1.4: Co-resistance to important antimicrobials in MRSA (Federal Office of Consumer Protection and Food Safety and Paul-Ehrlich-Gesellschaft für Chemotherapie e.V., 2016).

Another example of acquired resistance, which causes problems, are the carbapenemases. They are clinically relevant because they are beta-lactamases with versatile hydrolytic capacities. carbapenemases have the ability to hydrolyze penicillins, cephalosporins, monobactams, and carbapenems, which renders many beta-lactams ineffective. Carbapenemases are grouped into enzyme families (SME, IMI, NMC, GES, and KPC families; OXA-type beta-lactamase families; IMP, VIM, SPM, GIM, and SIM metallo-beta-lactamase families). Carbapenemases have been detected in *Pseudomonads*, *Enterobacteriaceae*, *Klebsiella pneumonia*, and *Acinetobacter baumannii*. Some of these were isolated from animals, which proves that ARB and ARGs can be transferred between humans and animals (Fischer et al., 2012, 2013).

1.1.4 Environmental aspects of resistance development and risk to human public health

Understanding the role of the environment in the spread of AMR is necessary to identify possible countermeasures that may help interrupt ARG transfer. As indicated in the previous subsection, transfer events of ARGs into clinically relevant pathogens have occurred from either environmental bacteria with no major human pathogenic significance. This intrinsic resistance plays a crucial role in the emergence of resistance mechanisms (Bengtsson-Palme et al., 2018).

The environment contributed to the development of AMR in the past and will most probably continue to do so in the future. The environment represents an indefinite source of resistance genes, many of which are still unknown to us (Berglund et al., 2017; Hatosy and Martiny, 2015). The major environmental aspect of AMR development therefore lies in the enormous diversity of microorganisms and their intrinsic ARGs. The recruitment of novel and unknown ARGs into clinically relevant pathogens is a real threat (Bengtsson-Palme et al., 2018). New resistance mechanisms may be acquired from environmental gene reservoirs through spontaneous transfer events. This may happen at any place where humans interact with the environment and the involved transfer processes do not necessarily require the selection pressure of ABs. However, novel resistance factors are far more likely to arise when there is a selective pressure of a novel AB (Bengtsson-Palme et al., 2018).

Four steps are necessary for the recruitment of novel resistances: emergence of a novel ARG, mobilization of the ARG, transfer to a human pathogen, and dissemination of the pathogen (Martínez et al., 2015). According to Bengtsson-Palme (2018), environments in which AB concentrations are clearly above established minimal selective concentrations (Bengtsson-Palme and Larsson, 2016; Tell et al., 2019) constitute the most severe risks. These may be (1) soil microbiomes impacted by manure, (2) lake microbiomes impacted by intensive aquaculture, (3) human and animal microbiomes impacted by AB misuse or overuse (4) marine microbiomes impacted by numerous human activities in coastal areas as well as offshore (as discussed by Hatosy and Martiny, 2015), and (5) surface water and sediment microbiomes impacted by wastewaters.

Undoubtedly, the role that ABs play in the selection of ARGs is crucial. However, the question remains as to whether selection happens in proximity or distance to the application of ABs. It is therefore important to monitor AB release into the environment, review effect levels of ABs, and to measure AB concentrations in environmental matrices.

1.2 Commercially available antibiotics – consumption, effects, and residual concentrations in surface waters

ABs can be purchased in every country of the world, and their sales regulation is highly uneven from nation to nation. There is often lack in legislation or enforcement of regulating the purchase of ABs, hence, consumers may be able to buy ABs at competitive prices without having to visit a physician. This level of availability has contributed to the development of AMR as well as the emergence of multi-resistant pathogens (Ventola, 2015).

The problem resides partly in the misuse or overuse of ABs. The overuse of ABs drives the evolution of AMR due to selection effects (Read and Woods, 2014). The agricultural use of ABs as growth supplements in livestock as well as the inappropriate prescription of ABs are primary examples. Conversely, exposing bacteria to non-lethal quantities of ABs results in AB-driven adaptive responses that make the target pathogen as well as co-residing bacteria resistant due to changes in gene expression, HGT, and mutagenesis (Davies et al., 2006; Viswanathan, 2014). Low levels of ABs have shown to also contribute to strain diversification in organisms, such as *Pseudomonas aeruginosa* and *Bacteroides fragilis* (Viswanathan, 2014).

The following subsections are dedicated to describing ABs: their history, their consumption in Germany, the pathway they take after excretion from the body, and finally, their concentrations predicted to select for resistance.

1.2.1 A brief history of the antibiotic era

The modern era of ABs did not start with the discovery of penicillin by Fleming in 1928, but rather 15 years later when penicillin was first prescribed to treat infections

in the early 1940s (Mahoney, 1944). Between 1940 and 1962, a great number of ABs were discovered through screening of microbial extracts (Singh and Barrett, 2006). In fact, those discoveries led to the identification of most of the chemical structures that became ABs or served as chemical proxies for the development of subsequent generations of ABs until the early 1980s (Silver, 2011). The majority of AB classes were discovered during that time. After 1983, the development of new ABs and new AB classes started to strongly decline or even come to a halt (30-year-discovery-void) (Figure 1.5) (Silver, 2011). Today, fewer new ABs are being developed and approved. Bacterial infections are now again becoming a major threat (Brooks and Brooks, 2014). However, an enormous effort is made to develop new drugs and there have been some advancements in the recent years (Figure 1.5) (Durand et al., 2017; Ling et al., 2015; Lodhi et al., 2018; Pantel et al., 2018; Singh et al., 2017; Smith et al., 2018). For example, by using a technique that allows for cultivation of previously “uncultivable” microorganisms (via the isolation chip) (Nichols et al., 2010), Ling et al. (2015) have discovered a new and potent AB, which they named teixobactin, that is naturally produced by *Eleftheria terra*, a soil bacterium with no major human pathogenic significance. Furthermore, screening of already approved drugs, which were originally marketed for other purposes, brought about several new AB discoveries in the recent years, such as auranofin and delamanid (drug repurposing) (Zheng et al., 2018). Finally, by chemically modifying arylomycins, naturally occurring compounds with weak activity and limited spectrum, Smith et al. (2018) developed a potent AB with broad-spectrum activity.

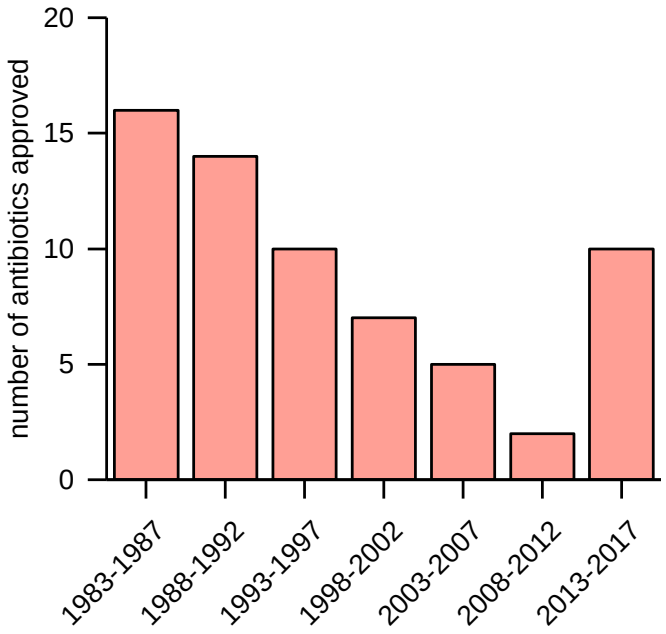

Figure 1.5: The decline of new antibiotics. The number of approved new antibiotics fell from 16 between 1983 and 1987 to 2 between 2008 and 2012, and increased back to 10 between 2013 and 2017 (adapted from Zheng et al., 2018; Andrei et al., 2018).

As for the development of AMR, the story follows an obvious pattern. Penicillin resistance became a substantial clinical problem shortly after penicillin was introduced (Finland, 1955). The first case of methicillin-resistant *Staphylococcus aureus* (MRSA) was reported soon after the introduction of methicillin (Finland, 1979). Vancomycin was introduced in 1972 for the treatment of methicillin resistance in both *S. aureus* and coagulase-negative *staphylococci* (Rubinstein and Keynan, 2014). The first cases of vancomycin resistance were reported in 1979 (Siebert et al., 1979) and 1983 (Tuazon and Miller, 1983). Resistance was eventually reported in proximity to all ABs that have been developed and deployed (Figure 1.6) (Aminov, 2010).

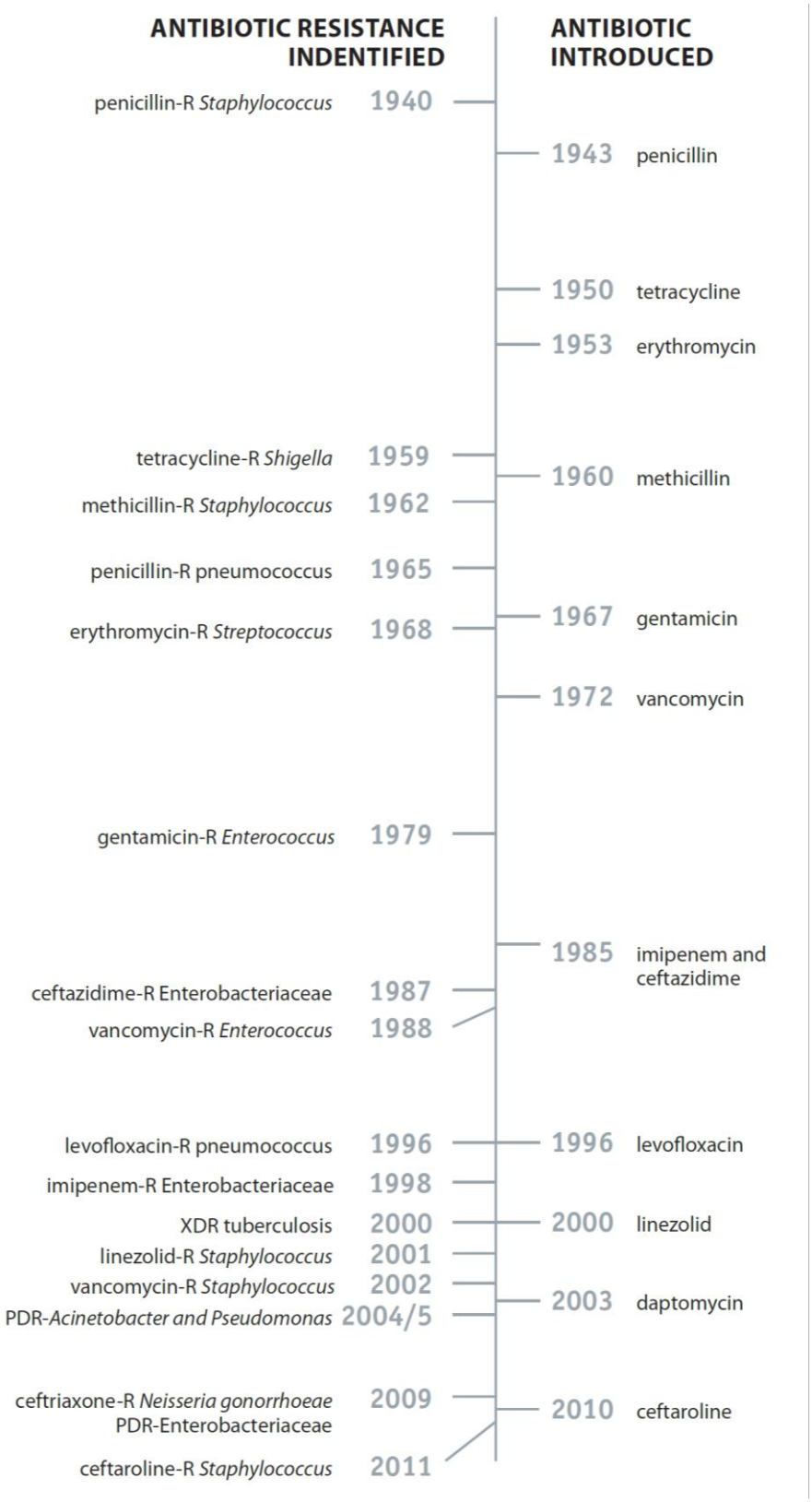

Figure 1.6: Developing antibiotic resistance: a timeline of key events. PDR = pan-drug-resistant; R = resistant; XDR = extensively drug-resistant. Dates are based on

early reports of resistance in the literature. In the case of pan-drug-resistant *Acinetobacter* and *Pseudomonas*, the date is based upon reports of health care transmission or outbreaks (CDC, 2019).

This pattern of "new AB leads to new ARG" is also referred to as the "ABs dilemma". For 80 years scientists have extensively researched and developed new ABs to be better equipped for the future. However, by doing so, new ARGs have emerged. Today, the solution is the administration of a combination of ABs. This protects the few ABs that still have an effect on highly resistant bacteria. However, the problem is often the identification of the right AB combination that needs to be applied. In hospitals, the absence of new ABs means that doctors need to do more with the arsenal of ABs currently available to them. Besides, it is also becoming increasingly challenging from an economical point of view: It is simply not feasible to invest in the development of ABs, and doing so poses a great business risk. Some major pharmaceutical companies have recently shut down their AB research (Franklin-Wallis, 2019).

1.2.2 Key antibiotic classes

Today, there are over 160 AB substances, which can be divided into 13 classes (Table 1.1) (adapted from Singh et al., 2017). AB action generally falls within one of four mechanisms, three of which involve the inhibition or regulation of enzymes involved in cell wall synthesis, nucleic acid metabolism and repair, or protein synthesis. The fourth mechanism involves the disruption of the membrane structure (Table 1.1).

Table 1.1: Major classes of antibacterial agents used in clinical therapy (adapted from Singh et al., 2017).

drug class	examples	target	pathway inhibited	spectrum
beta-lactams	meropenem, amoxicillin, penicillin V	multiple PBPs*	cell wall synthesis	broad spectrum
glycopeptides	vancomycin	lipid II	cell wall synthesis	gram-positive
sulfonamides	sulfamethoxazole	inhibit PABA†	protein synthesis	broad spectrum
macrolides	erythromycin, roxithromycin	50S RNA of ribosome	protein synthesis	gram-positive
oxazolidinones	linezolid	50S RNA of ribosome	protein synthesis	gram-positive
amphenicols	chloramphenicol	50S RNA of ribosome	protein synthesis	broad spectrum
lincosamides	clindamycin	50S RNA of ribosome	protein synthesis	gram-positive
tetracyclines	doxycycline, tigecycline, tetracycline	30S RNA of ribosome	protein synthesis	broad spectrum
aminoglycosides	gentamicin, amikacin	30S RNA of ribosome	protein synthesis	broad spectrum
fluoroquinolones	levofloxacin, ciprofloxacin	gyrase, topoisomerase IV	DNA synthesis	broad spectrum
nitroimidazoles	metronidazole	DNA	DNA synthesis	broad spectrum
lipopeptides	daptomycin	membranes	membrane integrity	gram-positive
polymyxins	colistin	membranes	membrane integrity	gram-negative

*PBPs = penicillin binding proteins

†PABA = *para*-aminobenzoic acid

1.2.3 Antibiotic consumption

ABs are consumed by people or given to animals to treat or prevent infectious diseases. In Germany, they are among the top-selling and most frequently prescribed drugs in outpatient care. From a total of 448 million defined daily doses (DDD, according to the ATC index of the World Health Organization, WHO) prescribed in Germany in 2014, basic penicillins (penicillin G, penicillin V), topical penicillins (locally administered creams), tetracyclines, macrolides, and quinolones made up the largest portion with 106, 89, 54, 42, and 35 million DDD, respectively (Federal Office of Consumer Protection and Food Safety and Paul-Ehrlich-Gesellschaft für Chemotherapie e.V., 2016). Consumption data is available for approximately 70 million people (87 % of Germany's population). Nearly 17.4 DDD per 1'000 persons per day were prescribed in 2014. For example, the population of the city of Karlsruhe is 307'755. If all the above numbers are correct, about 2 million DDD of ABs were prescribed in Karlsruhe in the year 2014.

The technical unit "DDD" was created in conjunction with the anatomical therapeutic chemical (ATC) classification of the WHO. It is defined as "the assumed average maintenance dose per day for a drug used for its main indication in adults". The values may differ from the DDD assigned for the main active ingredient, as DDDs are based on the average number of dosing intervals per day. Table 1.2 shows examples of DDDs for some ABs.

It is important to consider the DDDs as they indicate the amount of ABs used. A DDD of 48.4 million cefuroxime that was prescribed in outpatient care in Germany in 2014 therefore relates to approximately 24 t of the substance (0.5 g cefuroxime per DDD). However, this value might be slightly over- or underestimated as the prescription volume of ABs varies depending on the type of administration (oral, parenteral, intravenous, topical) and as there are also regional differences in AB consumption.

Table 1.2: Examples of defined daily doses (DDD) for antibiotics assigned by the World Health Organization ("WHOCC - ATC/DDD Index," 2018).

antibiotic	defined daily dose (oral)
amoxicillin	1 g
cefuroxime	0.5 g
ciprofloxacin	1 g
clindamycin	1.2 g
erythromycin	1 g
norfloxacin	0.8 g
penicillin V	2 g
piperacillin	14 g
roxithromycin	0.3 g
sulfamethoxazole	1.6 g
tetracycline	1 g

In Germany, the total amount of ABs used in human medicine is estimated to range between 700 and 800 t per year and the outpatient and inpatient prescription volumes account for 85 % (500 – 600 t) and 15 % (100 – 120 t) of that, respectively. The amount of ABs sold to German-based veterinarians in 2014 was 1'238 t. While "old" (classical) ABs, such as penicillins (450 t), tetracyclines (342 t), sulfonamides (121 t), macrolides (109 t), and polypeptide ABs (107 t) comprised the majority of sales (91.2 %), more recently developed fluoroquinolones (12.3 t) and third- and fourth-generation cephalosporins (3.7 t) accounted for only a small fraction of the sales (1.3 %). Fluoroquinolones and third- and fourth-generation cephalosporins are known to strongly promote the development of multi-resistance and are therefore clinically relevant. While they are rarely used in veterinary medicine, they still have a large share in total consumption in human medicine, especially in inpatient care. All of the above numbers were published in open access by the Federal Office of Consumer Protection and Food Safety and Paul-Ehrlich-Gesellschaft für Chemotherapie e.V., (2016).

1.2.4 From households to rivers

ABs are usually taken orally. Once ingested, ABs are removed from the body via drug excretion. ABs are either excreted as unchanged parent molecules, if they are sufficiently hydrophilic (aminoglycosides, beta-lactams, glycopeptides, and colistin) or as metabolites, which have to be transformed in the body from a lipophilic to a hydrophilic form first (fluoroquinolones, macrolides, tigecycline, and lincosamides) (Maddison et al., 2008). ABs are excreted via different routes, such as the kidney or in bile, sweat, and breast milk. Drug elimination rates, described by their half-lives, affect the plasma concentrations of ABs and their metabolites and thus play an important role in the prescription design (Maddison et al., 2008).

ABs are not fully degraded within the human body and a portion is excreted via urine or faeces (Gao et al., 2012). Depending on the compound, this can be up to 90 % of the administered dose (unchanged parent molecule) (Mompelat et al., 2009; Polesel et al., 2016). High excretion is observed for ciprofloxacin (50 – 80 %) and tetracycline (80 – 90 %), while lower excretion is observed for erythromycin (5 – 10 %), sulfamethoxazole (15 – 30 %), and clarithromycin (~25 %) (Mompelat et al., 2009). Through the sewage system, ABs and their metabolites are therefore transported to the local WWTPs in large amounts. However, in wastewaters, the dilution of ABs is large, hence, their concentrations lowered. Numerous studies have reported ABs concentrations in WWTP influent samples or upstream sewer pipes in the low µg/L-range, and this seems to be the general case (Bengtsson-Palme et al., 2016; Danner et al., 2019; Fatta-Kassinos et al., 2011). Within the treatment line of WWTPs, ABs are further reduced via retention in sludge or by natural degradation, however, their decay is yet incomplete and ABs or their metabolites end up entering the environment.

In parallel to the above-mentioned pathway, ABs can enter the environment also directly, without passing through WWTPs. Research has verified the occurrence of veterinary ABs in manure, agricultural fields, and surface water bodies (through AB runoff from agricultural fields) (Davis et al., 2006; Hoese et al., 2009; Kuchta and Cessna, 2009; Le et al., 2018). In Germany, approximately twice as much ABs are used in veterinary medicine than in human medicine, which is largely administered

to cows, pigs, and chicken. A fraction of this is likely to end up in surface waters through rainwater runoff from agricultural fields impacted by manure.

Concentrations of ABs in surface waters have been measured in plenteous investigations throughout the world as summarized by Danner et al. (2019) and Fatta-Kassinos (2011). Although there are several extreme cases, concentrations of ABs in European rivers are usually found in the ng/L-range, and occasionally in the low µg/L-range.

1.2.5 Predicted no effect concentrations for resistance selection

The predicted no effect concentration (PNEC) refers to the concentration of a substance below which no adverse effect on an organism or ecosystem is detected. PNECs are often used in environmental risk assessment and can be determined through toxicity testing. For ABs, this is an extensive task as bacteria show strong variation in sensitivity towards ABs. The determination of PNECs of ABs for resistance selection is therefore based on already published data of minimal inhibitory concentrations (MIC) of ABs. The MIC is the lowest concentration of an AB that inhibits visible growth of a bacteria after 24-h of incubation. Data on MIC is available at the EUCAST database (The European Committee on Antimicrobial Susceptibility Testing). Additionally, PNECs of ABs can also be determined from the minimal selective concentration (MSC). The MSC is defined as the minimal concentration at which the expression of a resistance gene provides resistant bacteria with an advantage over non-resistant bacteria of the same species (Le Page et al., 2017).

Already available PNECs in literature are based on lowest MIC and lowest MSC data, and an additional safety factor of 10 (Bengtsson-Palme and Larsson, 2016), or on toxicity endpoint data that includes the higher sensitivity of cyanobacteria towards ABs (ECHA European Chemicals Agency 2008; EU WFD European Union Water Framework Directive 2018), and an additional safety factor of 10 (Tell et al., 2019). Table 1.3 shows some of these values that were determined by Bengtsson-Palme and Larsson (2016), and Tell et al. (2019). They are the first authors to publish PNEC values of ABs for resistance selection.

Table 1.3: Predicted no effect concentrations (PNECs) for some antibiotics reported in literature [µg/L].

antibiotic	PNEC (resistance selection) according to Bengtsson-Palme and Larsson (2016)	PNEC-ENV (environmental) according to Tell et al. (2019)
amoxicillin	0.25	N/A
cefuroxime	0.5	N/A
ciprofloxacin	0.064	0.57
clindamycin	1	0.1
erythromycin	1	0.5
norfloxacin	0.5	120
penicillin V	0.064	N/A
piperacillin	0.5	N/A
roxithromycin	1	6.8
sulfamethoxazole	16	0.6
tetracycline	1	3.2

PNEC values for more than 100 AB substances were reported. They can be used as a guidance in environmental risk assessment, and for the implementation of emission limits of ABs. They represent an invaluable tool in AMR research.

1.3 Initial motivation to pursue this investigation – particulate matter from wastewater discharges and the overlooked problem in the spread of antimicrobial resistance

This investigation specifically focusses on the contribution of particulate matter from WWTP discharges in the spread of AMR. It is known for many decades that wastewater constitutes an enormous mass of suspended solids that is carried into downstream surface waters. Their removal during wastewater treatment is challenging and requires sophisticated filtration techniques that we are only now considering of implementing into the treatment lines of WWTPs (Lüddeke et al., 2015). Apart from that, untreated wastewaters may also be directly discharged into rivers, without passing through WWTPs (Qian et al., 2015). This occurs during heavy rainfall

events at combined sewer overflows when the capacity of the WWTPs (typically twice the dry weather discharge) has reached its limit. Again, there is awareness about this issue (Qian et al., 2015; Walters et al., 2014a). The problem lies not only in the fact that wastewater particles transport nutrients, such as organic carbon, nitrogen and phosphorous that may impact the microbial community (secondary productivity in river sediments), particles may also transport large sums of attached microorganisms (Walters et al., 2014a, 2014b). As these microorganisms derive from upstream wastewater treatment or sewage systems, they are likely to carry a considerable amount of ARGs. In the downstream waterway, the deposition of suspended solids may therefore impact the absolute abundance of ARGs in the sediment. Here, close cell contact may provoke gene transfer between indigenous and allochthonous bacteria, and assist the development of new resistances. All of the aforementioned issues that are linked to particle transport have not been appropriately addressed. The pathway that wastewater particles take from urban households to the river sediment needs further investigation. A hypothetical pathway is demonstrated in Figure 1.7.

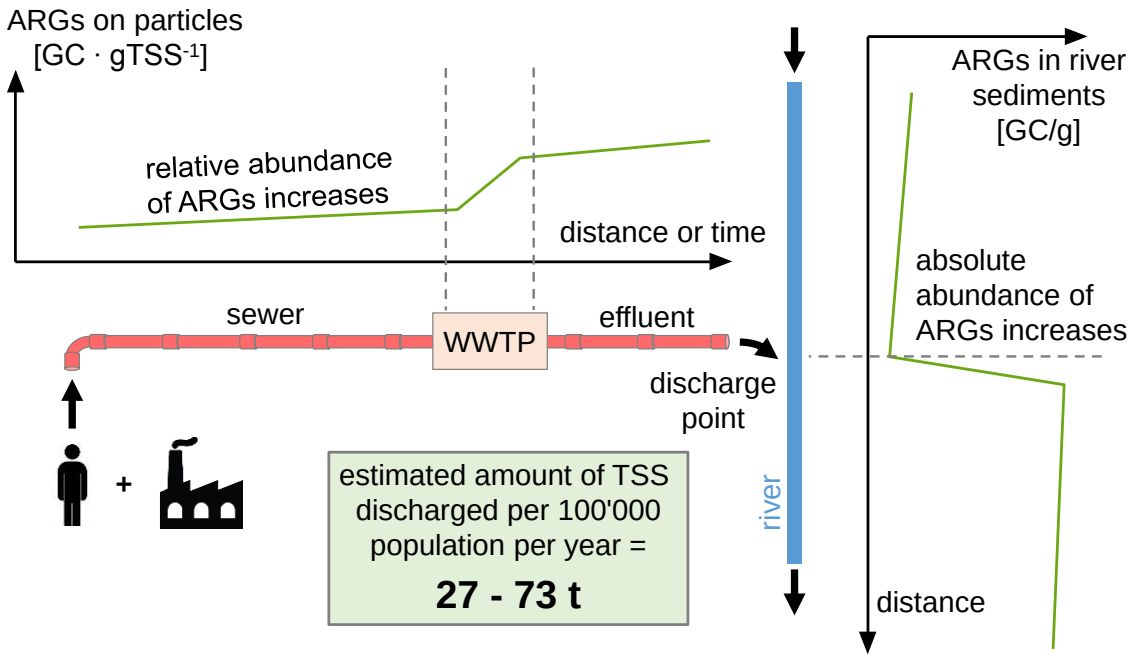

Figure 1.7: Hypothetical pathway of wastewater particles, and associated antibiotic resistance genes (ARGs), from urban households to the river sediment. The quality of particles changes along the first passage (sewer, WWTP, and effluent). Here, selection processes result in an increase of relative abundances of ARGs in the

particle-attached microbial population (as indicated in the top left graph; not part of this study) (Bengtsson-Palme et al., 2016; Alexander et al., 2016). Particles are then discharged into the receiving river at the highlighted discharge point. We estimate the load of particulate matter to be in the range of 27 – 73 t (total suspended solids, TSS) per 100’000 population per year (150 – 200 L per person per day, 5 – 10 mg of TSS per L). Our hypothesis is that, through the disposition of this mass, ARGs are directly transported to the downstream sediment, and this results in an increase of absolute ARG abundances downstream compared to upstream of the discharge point (as indicated in the graph to the right).

Taking into account all of the above, the aim of the research presented in this dissertation was to evaluate the impact of the particulate load from wastewater discharges on the abundance of ARGs in downstream rivers, and there especially in sediments. This was addressed in two studies. In the first study, presented in chapter 2, the objective was to characterize the burden of a large scale WWTP on the ARG abundances in the receiving river. Furthermore, the amount of settleable ARGs in WWTP effluents was determined through microbial partitioning. Finally, concentrations of ABs were monitored in WWTP effluents, and in the receiving river. The findings of the first study became the basis for the second study, which is presented in chapter 3. In laboratory scale, the transport dynamics of ARGs were evaluated using batch reactors, a wastewater particulate fraction, and natural river sediments. Furthermore, residual ABs were tested for their ability to select ARGs. In both studies, the major goal was to put particle-associated ARG transport into numbers, and to evaluate sedimentation potentials or removal rates. These numbers can be expressed by gene copies (GC) per g total suspended solids (TSS), which can then be extrapolated to the amount of 27 – 73 t of TSS per 100’000 population per year (Figure 1.7). For all we know, this has not been done before and might help to understand the mechanisms of AMR spread better. The results might assist in finding appropriate strategies to reduce or even eliminate the spread of AGRs by WWTPs.

2 Impact of the particulate matter from wastewater discharge on the abundance of antibiotic resistance genes and facultative pathogenic bacteria in downstream river sediments

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2.1 Introduction

The dissemination and spread of ARB and ARGs, with increasing numbers in soils and river sediments, have become an emerging environmental problem. A variety of studies report on the occurrence of ARB and ARGs as well as the presence of ABs in the effluent of WWTPs (Michael et al., 2013; Berendonk et al., 2015). Abundances of ARB and ARGs have shown to increase in rivers downstream compared to upstream of WWTP discharges (Berglund et al., 2015; Marti et al., 2013; Proia et al., 2016; Tang et al., 2016).

There are several possible explanations for this phenomenon. Previous investigations discussed selection and proliferation by sub-inhibitory concentrations of ABs as a possible mechanism (Baquero et al., 2008; Marti et al., 2013; Proia et al., 2016). Some studies tried to prove this relation by showing that low concentrations of ABs indeed have an effect on biofilm formation, host-colonization and can even induce stress responses, mutation, and HGT (Davies et al., 2006; Hathroubi et al., 2015; Molina-Quiroz et al., 2015; Wright et al., 2013). In all of the aforementioned investigations, AB concentrations ranged between 65 – 6'000 µg/L. However, in rivers impacted by WWTPs, antimicrobial compounds are usually found in the low to mid ng/L-range (Yan et al., 2013; Li et al., 2014, 2015; Burke et al., 2016). There are in fact only very few reported cases, in which AB concentrations have exceeded 1 µg/L

(Hirsch et al., 1999; Kolpin et al., 2002; Le-Minh et al., 2010). The same has been observed for river sediments (Li et al., 2018b; Zhou et al., 2012). Even in an area in China that is heavily impacted by an abnormal use of ABs in agriculture and fish farming, mean concentrations of 27 ABs were below 200 ng/g sediment (Li et al., 2018a). At such concentrations, the effect of ABs on resistance selection might not be as significant as often presumed (Ashbolt et al., 2013; Bengtsson-Palme et al., 2016; Bengtsson-Palme and Larsson, 2016; Finley et al., 2013).

For the urban water cycle this means that more attention needs to be paid to the absolute amount of ARB and ARGs transported into the receiving rivers rather than to AB concentrations. Considering the quantity of intestinal bacteria from humans and their domesticates, it seems clear that the disposal of manure has a strong impact on the distribution of ARB and ARGs (Chen et al., 2016; Jechalke et al., 2014; Zhu et al., 2017). Urban WWTPs consequently receive high loads of intestinal bacteria with a rich drug resistance gene pool (Auerbach et al., 2007; Moura et al., 2010; Rizzo et al., 2014; Wan and Chou, 2015). Despite a relatively efficient removal during the treatment process, there is still a high quantity of residual ARB and ARGs discharged into the aquatic environment (Alexander et al., 2015; Berendonk et al., 2015; Hembach et al., 2017). Until now, it is unclear to which extend WWTP borne ARGs remain in the river and there is no in-depth study that focusses on their sedimentation behavior. We know from literature that faecal indicator bacteria can associate with wastewater particles, which, depending on their size, either remain in suspension or are removed from the water column via sedimentation (Ahn, 2012; Qian et al., 2015; Walters et al., 2014b). However, we can only speculate whether or not this also concerns ARGs and to which extend. To assess the significance of settleable ARGs, WWTP effluents must be considered as a combination of settleable and suspended fractions, which should be investigated separately (Proia et al., 2018).

The objective of this study is to (1) characterize the impact of a large scale WWTP on the absolute abundances of FPB and ARGs in water and sediment of the receiving river; (2) determine the amount of settleable FPB and ARGs from WWTP efflu-

ents by separating wastewater particles and associated microbes from the suspended fraction; (3) monitor concentrations of five ABs frequently detected in the aquatic environment (erythromycin, roxithromycin, ciprofloxacin, tetracycline, and penicillin V).

2.2 Materials and methods

2.2.1 Sampling sites

Sampling was carried out at a German WWTP with a population equivalent (p.e.) of 875'000 and a high industrial impact of 50 % (*v*:*v*). The municipal sewer system has a catchment area of 46 km². On average, 35 million m³ of wastewater are treated per year, resulting in an average outflow of approximately 1.1 m³/s. The WWTP treatment line includes a primary settling stage, an activated sludge process, two biological stages, and a secondary clarifier. The plant has a total volume of approximately 70'000 m³. Hydraulic retention varies between 60'000 m³ per day in dry weather conditions and up to 340'000 m³ per day during heavy rainfall (combined sewers). The sludge age varies between 2 – 4 days. The effluent can be characterized by the measured physico-chemical parameters, i.e. COD = 46 mg/L, NH_4-N = 1.89 mg/L, $N_{anorg.}$ = 12.1 mg/L, and P_{total} = 0.67 mg/L (yearly average values, 2016). Three 24 h-composite samples were collected in sterile 5 L plastic containers at the effluent of the WWTP (Figure 2.1) in December 2017. The automated 24 h-sampler transfers individual grab samples into a cooling chamber (4 °C) to avoid microbial growth. After collection, the samples were further cooled and transported to the laboratory for immediate analysis.

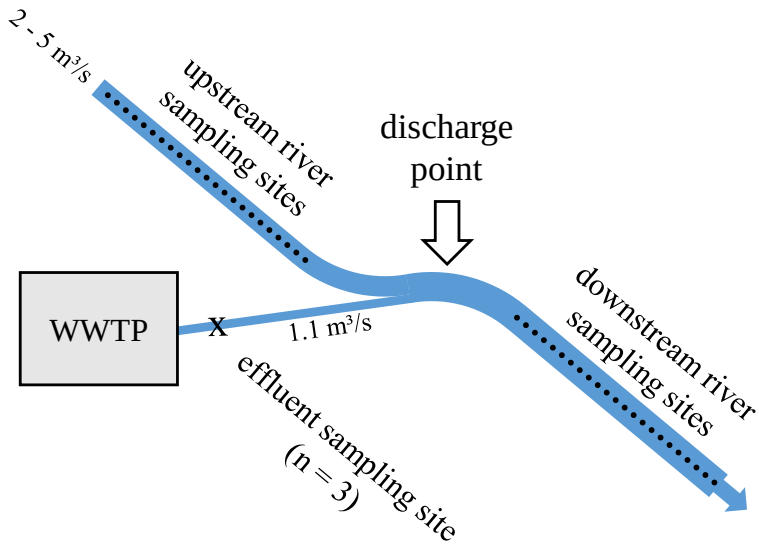

Figure 2.1: Locations of the sampling points at the effluent of the WWTP and along the receiving river.

The receiving river is a 51 km long mid-sized mountain stream with a catchment area of 450 km². 46 water and 46 sediment grab samples were collected along the lower course of the river (Figure 2.1) during a dry period in June 2017. Half of the samples were taken upstream, and the other half downstream of the WWTP discharge. The distance between each of the upstream or downstream sampling points was 40 ± 10 m. According to the State Institute for the Environment, Survey and Nature Conservation (LUBW, Karlsruhe, Germany), the volumetric flow rate of the receiving river, upstream of the discharge point, varies between 2 and 5 m³/s.

Downstream sampling points were set in a distance of at least 400 m away from the discharge point to ensure that downstream river samples were evenly mixed with the effluent. Water samples were collected in sterile 500 mL bottles (VWR, Darmstadt, Germany) and immediately stored in cooling boxes. Sediment samples were drawn with self-made cylindrical sediment core samplers (PVC tubes, Ø = 2 cm) and stored in cooling boxes. Only the upper 5 cm of the sediment cores were used for further analysis. All samples were processed within 48 h after collection.

2.2.2 Sample preparation and DNA extraction

The WWTP effluent samples and fractions were split into two aliquots, one used for the determination of TSS and the other for DNA extraction and subsequent molecular biology analysis. The TSS were analyzed by filtering a 0.1 – 1 L aliquot of sample through a tarred 0.45 µm cellulose nitrate filter (Ø = 10 cm; Sartorius AG, Goettingen, Germany). Filters plus solids were dried at 110 °C for 24 h, cooled, and weighed.

To extract bacterial DNA from wastewater or river water, 100 mL of sample was filtered through membrane filters with a 0.2 µm pore size (Whatman Nuclepore Track-Etched Membranes, Sigma-Aldrich, Munich, Germany). For the extraction of bacterial DNA from sediments, the top 5 cm of the sediment cores were homogenized using PBS at pH 7.4 (1:1; *v:v*) by shaking for 5 min at room temperature. The suspension was then centrifuged at 3'000 *g* for 10 min. Bacterial DNA was extracted from the pellet or from membrane filters using the lysing matrix E of the Fast DNA Spin Kit for Soil (MP Biomedical, Illkrich, France) following the manufacturer's instructions. The total DNA yield for each sample was measured with the Qubit Fluorimetric Quantitation, using the dsDNA BR Assay Kit according to the manufacturer's instructions (Thermo Scientific, Waldham, USA). To calculate the DNA yield per g of sediment, samples were dried at 110 °C for 24 h, cooled, and weighed. To determine the organic fraction of sediments, samples were burned at 500 °C for 2 h, cooled, and weighed.

2.2.3 Detection of facultative pathogenic bacteria and antibiotic resistance genes

Three species specific genes for FPB and ARGs were detected in WWTP effluent as well as in river water and sediment samples of the receiving river. All detection systems are listed in Table 2.1. Most of the selected targets are described to be frequently found in WWTP effluents and receiving bodies (Hembach et al., 2017; Rizzo et al., 2013), hence, their high abundances are necessary for the dynamic observation of the mentioned FPB and ARGs. To assess the impact of a WWTP on a receiving river, in terms of ARG concentrations, a dynamic observation of FPB and ARG is essential.

FPB and ARGs were quantified by a SYBR Green qPCR approach. Reactions were run in volumes of 20 µL, containing Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific, Waldham, USA), nuclease-free water (Ambion, Life technologies, Karlsbad, Germany), 0.2 µM of the respective primers (Table 2.1), and 50 ng of template DNA. The qPCR protocol comprised 10 min at 95 °C for activation of the DNA polymerase followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. All samples were assayed in duplicate, as were the standards, the positive and negative controls. To evaluate the specificity of amplification, a melting curve was recorded by raising the temperature from 60 to 95 °C (1 °C every 10 s). The Bio-Rad CFX Manager software was used for data analysis. Calibration curves for the calculation of absolute gene abundances were prepared in previous works (Hembach et al., 2017; Rocha et al., 2018), in which serial dilutions of reference strains or plasmids were generated to correlate gene copy numbers with the measured Ct values. All calibration curves are shown in the Supporting Information A (SI A) (Figure SA1 – SA7).

Table 2.1: List of primers used to detect bacteria and antibiotic resistance genes.

specificity	gene	primer	sequence (5′- 3′)	reference
enterococci	23S rDNA	ECST784F	AGAAATTCCAAACGAACTTG	Ludwig and Schleifer, 2000
		ENC854R	CAGTGCTCTACCTCCATCATT	
Pseudomonas aeruginosa	*ecf*X	ecfXRT-F	AGCGTTCGTCCTGCACAAGT	Clifford et al., 2012
		ecfXRT-R	TCCACCATGCTCAGGGAGAT	
Acinetobacter baumannii	*sec*E	secEFT	GTTGTGGCTTTAGGTTTATTATACG	Clifford et al., 2012
		secERT	AAGTTACTCGACGCAATTCG	
erythromycin resistance gene	*erm*B	ermB-F	TGAATCGAGACTTGAGTGTGCAA	(Alexander et al., 2015)
		ermB-R	GGATTCTACAAGCGTACCTT	
beta-lactamases of the TEM family	bla_{TEM}	blaTEM-F	TTCCTGTTTTTGCTCACCCAG	Rocha et al., 2018
		blaTEM-R	CTCAAGGATCTTACCGCTGTTG	
tetracycline resistance gene	*tet*M	tetM-F	GGTTTCTCTTGGATACTTAAATCAATCR	Peak et al., 2007
		tetM-R	CCAACCATAYAATCCTTGTTCRC	
Quinolone resistance gene	*qnr*S	qnrSrtF11	GACGTGCTAACTTGCGTGAT	Rocha et al., 2018
		qnrSrtR11	TGGCATTGTTGGAAACTTG	

2.2.4 Microbial partitioning of WWTP effluent samples

A previously described technique (adjusted) was used to separate wastewater particles and associated microbes (settleable fraction) from planktonic bacteria (suspended fraction) (Characklis et al., 2005). In short, 2'800 mL (4 × 700 mL) of 24 h-composite sample was spinned in a Hettich ROTANTA 460 R centrifuge with a 5624 swing-out rotor at 1'164 *g* for 10 min with the brake set to 4, while holding the temperature constant at 4 °C. 2'240 mL (4 × 140 mL) of supernatant (suspended fraction) was then carefully removed using a vacuum pump and hose. The removal of TSS, total DNA, FPB and ARGs was calculated by dividing concentrations measured in the settleable fraction by concentrations measured in the unpartitioned WWTP effluent sample.

2.2.5 Detection of antibiotics

Five ABs were quantified in water samples – erythromycin (macrolide), roxithromycin (macrolide), ciprofloxacin (fluoroquinolone), tetracycline (tetracycline), and penicillin V (penicillin). The selection of these compounds was driven by their high environmental relevance, as well as the type of ARGs related to these classes of ABs. Details concerning used chemicals can be found in SI A (Text SA1).

For the detection of ABs, 5 grab samples of each, WWTP effluent, upstream river water, and downstream river water were collected in sterile 1 L glass bottles on individual days and immediately acidified with HCl to pH 2. The samples were filtered through 0.45 µm cellulose nitrate filters (Sartorius Stedim Biotech, Germany) and a solution of Na_2EDTA (ethylenediaminetetraacetic acid disodium salt) was added to a final concentration of 1 g/L. The samples were then extracted using solid phase extraction (SPE) on the Oasis® HLB (500 mg, 6cc) cartridges (Waters, Germany) in acidic conditions (SI A, Text SA2). The performance of the SPE procedure was evaluated using deutered standards of ciprofloxacin, erythromycin, penicillin V and roxithromycin. Due to the lack of the deutered standard for tetracycline, the recovery of this compound was assessed using samples spiked with a known concentration of the AB. Detailed information about the quality control of the extraction process is depicted in SI A (Text SA3).

The concentrated samples were analyzed using an Agilent 1290 Infinity II UHPLC system coupled to an Agilent 6470 Triple Quadrupole LC/MS system via an Agilent Jet Stream electrospray ionization source. Separation was performed on an Agilent ZORBAX Eclipse Plus C-18 (50 × 2.1 mm, 1.8 µm particle size) column with a flow of two eluents - HPLC water and acetonitrile, both acidified with formic acid to the concentration of 0.05 %. The analytical method used for the quantification of ABs is shown in SI A (Table SA1). The detection of analytes was performed in dynamic Multiple Reaction Monitoring (MRM) mode. The mass spectrometry parameters used for the data acquisition are collected in SI A (Table SA2 and Table SA3).

2.2.6 Statistical analyses

The box-whisker plot was constructed for visual representation of spread and distribution of sample data. The central rectangle represents first quartile (Q1), median (Q2), and third quartile (Q3). The whiskers indicate smallest and the largest values of sample data.

The one-tailed Wilcoxon Mann-Whitney U-test was used for non-parametric data to assess whether gene abundances in downstream samples statistically significantly differed from those in upstream samples. The null hypothesis stated that gene abundances were equal or lower downstream, whereas the alternative hypothesis presumed that gene abundances were higher downstream.

The 100 % stacked-column chart was plotted to show the amount of gene abundances in different fractions of WWTP effluent samples. The mean values are shown. Error bars represent standard errors of the means.

Linear regression analysis were employed to assess correlation between imported gene abundances (WWTP effluent) and delta gene abundances in the receiving river. The values were logarithmised prior to analysis. Regression was performed without weighting and without fix of intercept. All plottings and analyses were performed using OriginPro 2017 (OriginLab Corporation, Northampton, MA, USA).

2.3 Results and discussion

2.3.1 Abundance and prevalence of facultative pathogenic bacteria and antibiotic resistance genes in the receiving river

FPB and ARGs were quantified by qPCR in river water and sediment samples upstream and downstream of a WWTP discharge. High R^2 values (average 0.999) and high efficiencies (90 – 102 %) in the qPCR standards indicated the validity of these quantifications (Figure SA1 – SA7). Absolute gene abundances were determined in gene copies (GC) per mL water or g sediment. The results are shown in Figure 2.2. All selected targets were detected. Generally, the concentrations of FPB and ARGs were significantly higher downstream compared to upstream of the WWTP (by at least 0.4 and up to 1.9 log units) in both, water samples (except the *P. aeruginosa* specific gene) and sediment samples ($p < 0.05$). A similar trend was observed for the detection frequency of FPB and ARGs (prevalence in percent of total sample number) (Table 2.2), which was, with just few exceptions, equal or higher downstream compared to upstream of the WWTP discharge. Additionally, sediment samples showed higher variation in gene abundance than water samples and ARGs were generally more abundant as well as more prevalent compared to FPB (except *qnr*S). The gene abundances did not show any growth or decay with distance to the WWTP discharge point (Figure SA8). The concentrations of FPB and ARGs remained stable within samples upstream of the WWTP discharge point (-1.4 – 0 km) as well as within samples downstream of the WWTP discharge point (0 - 1.6 km) (Figure SA8).

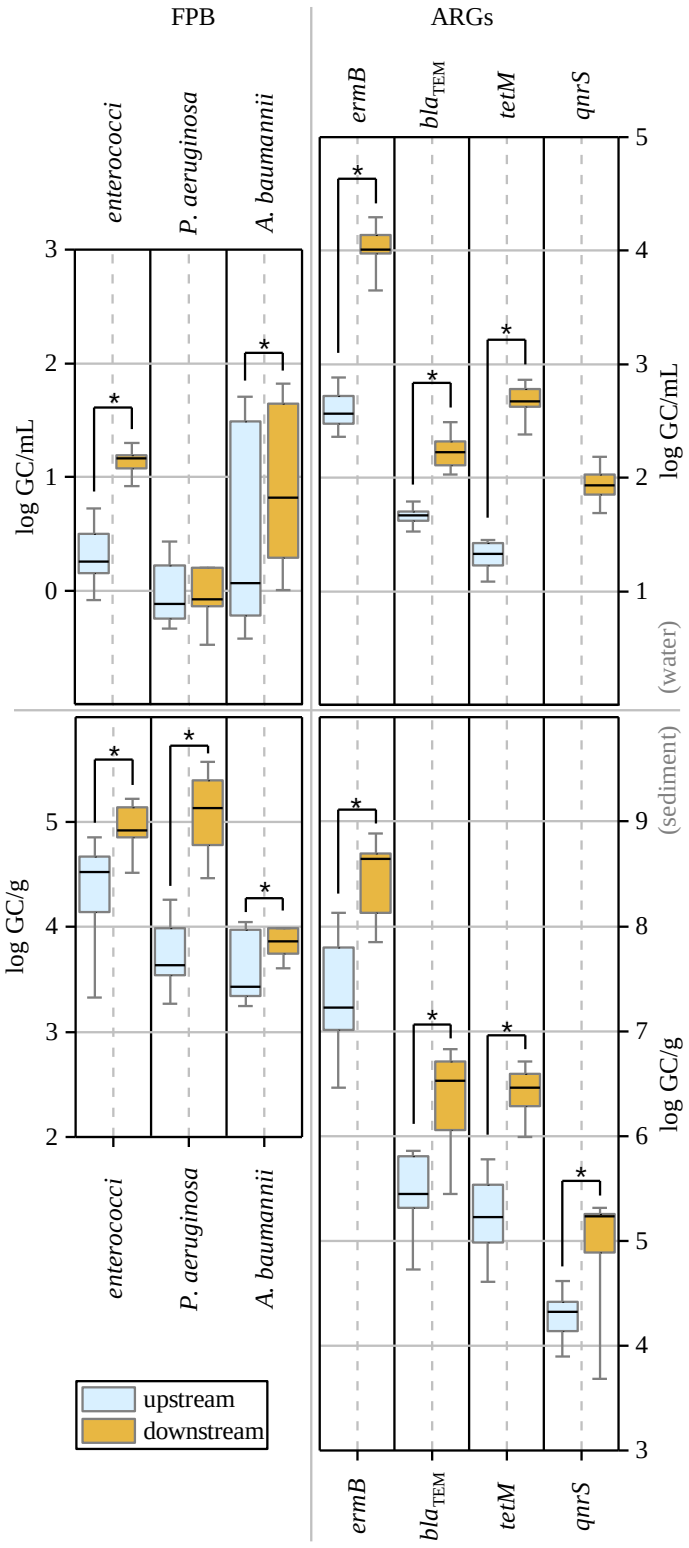

Figure 2.2: Absolute abundances of facultative pathogenic bacteria (FPB) (left) and antibiotic resistance genes (ARGs) (right) in the receiving river upstream and downstream of the WWTP discharge. GC = gene copy number. Targets were measured

in river water (top) or river sediment (bottom). One-tailed Mann-Whitney tests were performed to show statistical significance (*) i.e. $p < 0.05$.

Looking at single targets at the downstream sampling sites, *erm*B was the most abundant gene with a median of 4.01 log GC/mL water and 8.64 log GC/g sediment, followed by *bla*$_{TEM}$ (2.2 log GC/mL water; 6.5 log GC/g sediment), and *tet*M (2.7 log GC/mL water; 6.5 log GC/g sediment). All FPB as well as *qnr*S were less abundant (0 – 2 log GC/mL water; 4 – 5 log GC/g sediment). Remarkably, the abundance of the *P. aeruginosa* specific gene did not increase in the water samples but was 1.5 log units higher in the sediment downstream compared to upstream. This can be explained by *P. aeruginosa*'s rapid flagellar-mediated attachment to abiotic surfaces, its ability to grow in a range of environmental niches, and its predominant residence in biofilms (Pirnay et al., 2009). Interestingly, *qnr*S was below detection limit in upstream water, but was found at high concentrations in downstream water. The *A. baumannii* specific gene was found at low concentrations and showed high variation of abundance in river water samples.

Table 2.2: Detection frequency of genes in percent of total sample number (n = 23). n.d. = not detected.

	water		sediment	
	upstream	downstream	upstream	downstream
enterococci	57	100	96	87
P. aeruginosa	57	57	70	91
A. baumannii	48	78	39	26
*erm*B	78	100	100	100
bla$_{TEM}$	87	100	100	96
*tet*M	78	100	74	100
*qnr*S	n.d.	96	22	39

Our findings confirm results from previous investigations and clearly prove that WWTP discharges have a direct impact on the concentration of FPB and ARGs in the aquatic environment (Berglund et al., 2015; Czekalski et al., 2014; Marti et al., 2013; Proia et al., 2016; Tang et al., 2016). Compared to studies conducted in USA,

China, Italy, and Spain, ARG abundances were similar or slightly higher in water samples (LaPara et al., 2015; Jiang et al., 2013; Di Cesare et al., 2017; Rodriguez-Mozaz et al., 2015), and sediments (Calero-Cáceres et al., 2017). In this investigation however, the extent to which ARGs increased, downstream compared to upstream of the WWTP discharge, was higher. A similar before-after effect was observed in a recent study by Proia et al., (2018) who measured ARGs in the water phase of a small Belgian river (4 m^3/s) impacted by the two large-scale WWTPs of Brussels (0.36 and 1.2 million p.e.). Although they generally measured higher ARG concentrations, the contribution of WWTP effluents (p.e.) on the receiving river (per m^3/s) is similar to that described in our study. We therefore conclude that the amount by which FPB and ARG abundances increased downstream compared to upstream of the WWTP discharge is most likely correlated to the volume ratio of WWTP effluent and river water.

In addition, sediments could act as natural reservoirs, where ARGs accumulate and spread between organisms via HGT (Calero-Cáceres et al., 2017; Schwartz et al., 2003). The presence and higher abundances of the studied FPB underline the potential health risk when clinically relevant ARGs accumulate in bacteria, promoting the AB evolution in sediment systems.

2.3.2 Antibiotic concentrations in WWTP effluent and receiving river

In WWTP effluent, upstream river water, and downstream river water, only three out of five ABs were detected. In general, AB concentrations were in the low ng/L range along the course of the river. Despite their high consumption in human and veterinary medicine in Germany (Federal Office of Consumer Protection and Food Safety and Paul-Ehrlich-Gesellschaft für Chemotherapie e.V., 2016), penicillin V and tetracycline were below detection limit (LOQ = 5 ng/L). This may be explained by the instability of penicillin in aqueous media as well as the decreased mobility of tetracycline due to their strong sorption to organic substances (Gros et al., 2013; Gu et al., 2007). The remaining AB compounds (ciprofloxacin, erythromycin and roxithromycin) ranged at concentrations between 123.0 ± 42.4 and 176.7 ± 92.5 ng/L in WWTP effluent, 4.3 ± 0.8 and 15.4 ± 4.6 ng/L in upstream river water, and 27.6 ± 11.1 and 35.2 ± 17.3 ng/L in downstream river water (Table 2.3).

Table 2.3: Concentrations of ABs (ng/L) measured in WWTP effluent, upstream river water, and downstream river water. Comparison to lowest minimal inhibitory concentrations (MICs) and predicted no effect concentrations (PNECs) for bacteria (Bengtsson-Palme and Larsson, 2016). Reference values for the pseudo-partitioning coefficients $k_{d,s}$ (L/kg) (Cheng et al., 2016; Zhao et al., 2016). Expected concentration range of ABs in the sediment (ng/g).

antibiotic	WWTP effluent	river upstream	river downstream	size adjusted lowest MIC	PNEC (resistance selection)	$k_{d,s}$ value	expected conc. range in sediment
ciprofloxacin	176.7 ± 92.5	4.3 ± 0.8	35.2 ± 17.3	1´000	64	300 – 4´664	10 – 165
erythromycin	133.9 ± 34.4	10.6 ± 6.1	27.6 ± 11.1	8´000	1´000	30 – 500	0.8 – 13.8
roxithromycin	123.0 ± 42.4	15.4 ± 4.6	29.3 ± 19.0	8´000	1´000	210 – 11´400	6 – 333
penicillin V	< LOQ	< LOQ	< LOQ	500	64	-	-
tetracycline	< LOQ	< LOQ	< LOQ	16´000	1´000	113 – 5´800	-

Although the contribution of WWTP effluent to the river runoff is high (15 – 35 %; *v:v*), concentrations of ABs were low compared to those found in other WWTP-impacted rivers (Fatta-Kassinos et al., 2011). Moreover, ABs never exceeded the PNECs and were at least 28 times and up to 300 times below the lowest MIC. As stated before, at such concentrations, ABs most probably do not select for ARGs and ARB. This is in agreement with Bengtsson-Palme et al. (2016), who conducted shotgun metagenomic sequencing of samples from different steps of the treatment process in three Swedish WWTPs. Although 2 out of 16 substances were detected at concentrations predicted to be selective (900 ng/L ciprofloxacin, 4'500 ng/L tetracycline), they could not find clear evidence for selection by any particular class of AB.

In this study, the selected ABs were not quantified in river sediments due to analytical limitations. However, as discussed in previous studies, ABs can adsorb to sediments to various extend depending on pH, content of clay, silt and organic matter, as well as content of ionic components in sediments. This affinity of ABs to adsorb is usually expressed by the pseudo-partitioning coefficient ($k_{d,s}$-value) that describes the ratio between the concentration in the sediment (C_s) and in the water (C_w). To assess the range of the expected AB amount in the investigated sediments, the concentrations were calculated based on the pseudo-partitioning coefficients found in literature (Table 2.3) (Cheng et al., 2016; Zhao et al., 2016) and the concentrations of ABs detected in water. Ciprofloxacin was estimated to be in the range of 10 – 165 ng/g, whereas concentrations of macrolides ABs were calculated to be in the range of 0.8 – 13.8 ng/g and 6 – 333 ng/g for erythromycin and roxithromycin, respectively. The results are in agreement with already published values (Cheng et al., 2016; Zhao et al., 2016), however, insight into the effect on resistance selection, specifically in river sediments, is still lacking. More investigations need to be made, in order to assess the AB concentration levels that leads to an increase of resistance in the benthic zone of WWTP influenced rivers.

2.3.3 Sedimentation of wastewater particles and associated facultative pathogenic bacteria and antibiotic resistance genes

The concentrations of TSS and DNA as well as three FPB and four ARGs were measured in three 24 h-composite effluent samples (Figure 2.3, left). Centrifugation

lowered these concentrations, showing that sedimentation is likely to occur (Figure 2.3, right). This can be stated because the fraction associated with settleable particles was previously estimated through the use of a calibrated centrifugation technique (standard solutions containing glass and latex particles) (Characklis et al., 2005). TSS and DNA were removed by 56 ± 3 and 30 ± 5 %, respectively. The *enterococci* and *A. baumannii* specific genes were strongly associated with the settleable fraction and were removed from wastewater by 44 ± 10 and 57 ± 9 %, respectively. In contrast, the *P. aeruginosa* specific gene was removed only by 22 ± 3 %. Motile, flagellated *P. aeruginosa* cells are more likely to remain in suspension than non-flagellated *enterococci* or *A. baumanni* and therefore show fewer aggregation, hence, fewer settling. Finally, all ARGs were strongly associated with the settleable fraction. The resistance genes *erm*B, *bla*$_{TEM}$, *tet*M, and *qnr*S were removed by 66 ± 6, 40 ± 3, 51 ± 7, and 44 ± 2 %, respectively.

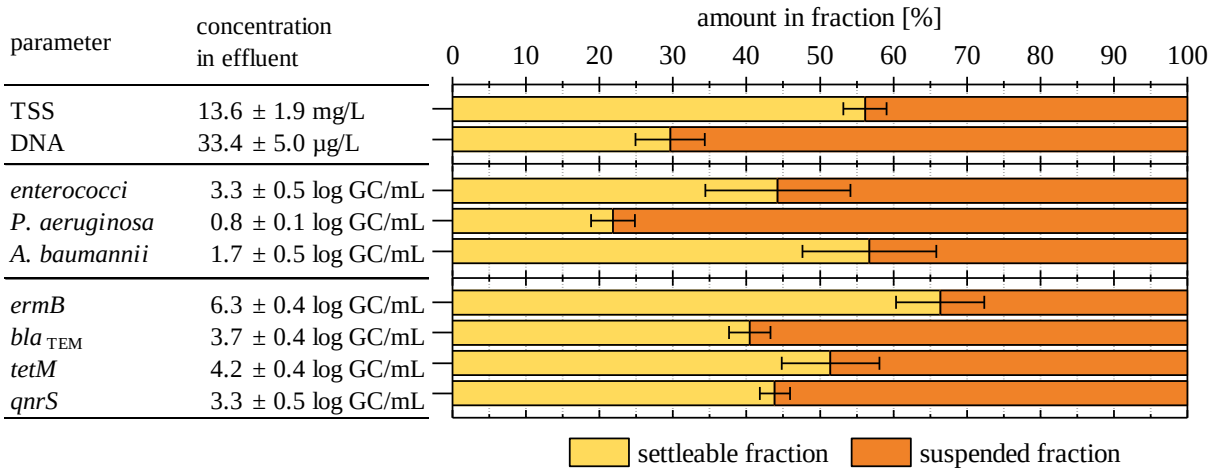

Figure 2.3: Concentrations of TSS, DNA, FPB, and ARGs measured in WWTP effluent (left), and their amount measured in settleable and suspended fractions (right). GC = gene copy number. Mean values and standard errors of the means are shown (n = 3).

The results indicate that TSS, DNA, FPB, and ARGs from WWTP effluents have the potential to settle to the receiving riverbed. The sedimentation potentials out of 1 m³ are 7.6 g of TSS, 10 mg of DNA, and from 10^6 up to 10^{12} GC of FPB and ARGs. It needs to be emphasized that the measured abundances are only an estimation, although centrifugation has shown to provide a reasonable separation of settleable

particles from the water (Characklis et al., 2005). Erosion and resuspension as well as biotic and abiotic degradation are likely to reduce the amounts. Despite a possible overestimation, the significance of settling wastewater particles becomes apparent when considering a total outflow of 35 million m³ effluent per year (480 t of suspended solids). There are several studies which investigate settling velocities for specific particle fractions (Auer and Niehaus, 1993; Battin et al., 2003; Canale et al., 1993; Cushing et al., 1993; Jamieson et al., 2005; Thomas et al., 2001). For instance, with a modelling technique, Jamieson et al. (2005) found that settling velocities for particle sizes between 45 and 75 µm varied between $2 – 3 \times 10^{-5}$ m/s in two rivers. Battin et al. (2003) performed experiments in large-scale flumes ($30 \times 0.3 \times 0.3$ m) at different flow velocities and found particle deposition velocities up to 250 times faster for organic particles 53 - 106 µm in size ($3 – 8 \times 10^{-3}$ m/s). If this is true, this particle fraction can very well settle within a short stretch downstream of the discharge point (24 – 600 m, assuming a water depth of 2 m and a flow velocity of 0.1 – 1 m/s). An organic fraction in downstream sediments of 17 ± 4 % compared to 10 ± 3 % upstream was measured in this study. This further supports the hypothesis of settling wastewater particles. Together with the values presented in Figure 2.3, a somewhat strong impact of particulate matter from wastewater discharges on the abundance of ARGs in downstream river sediments seems very probable.

Linear regression analyses of all 7 genes (*enterococci, P. aeruginosa*, *A. baumannii*, *erm*B, *bla*$_{TEM}$, *tet*M, *qnr*S) were employed to assess the association between the change of gene abundances in river sediments (downstream compared to upstream) (ΔGC/g) and the amount of gene abundances in WWTP effluents (settleable fraction only) (GC/g) (Figure 2.4). At first, there was only a moderate positive correlation ($R^2 = 0.69$, $p < 0.05$) (Figure 2.4, left). By excluding *P. aeruginosa* from the analysis, correlation became stronger ($R^2 = 0.93$, $p < 0.05$) (Figure 2.4, right). The *P. aeruginosa* specific gene was identified as an outlier. This is coherent with the results shown in Figure 2.3, in which it was demonstrated that *P. aeruginosa* does not associate with the settleable fraction. Figure 2.4 is a sedimentation based visualization, hence, there is a stronger correlation when *P. aeruginosa* is excluded. Probably, *P. aeruginosa* enters the sediment a different way. Due to flagellar cell

motility, *P. aeruginosa* might attach to the riverbed actively as free-floating organisms instead of passively settle to it via sedimentation of wastewater particles. Nevertheless, the increase of all other targets is most probably caused by the sedimentation of high amounts of FPB and ARGs from WWTP effluents. Apparently these amounts are sufficient to change abundances in the receiving river bed by 0.5 to 2 log units.

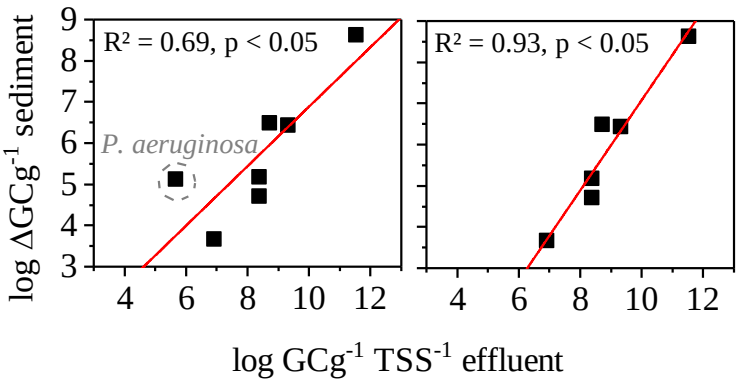

Figure 2.4: Linear regression between the change of gene abundances in river sediments (downstream compared to upstream) (ΔGC/g) and the abundance of gene copies in the settleable fraction of WWTP effluents (GC/g). Left graph includes all measured genes. The *P. aeruginosa* specific gene was excluded in the right graph. Data was logarithmised prior to regression.

2.4 Conclusion

Although it was previously shown that bacteria from WWTP effluents show the potential of settling, this study demonstrates the involvement of ARGs in this process and highlights the significance of sedimentation on the abundance of ARGs in downstream river sediments. Our findings show that the particulate load from WWTP discharges play a crucial role in the spread of FPB and ARGs in downstream sediments. Here, microbes from WWTP could potentially regrow and, despite a certain reduction during wastewater treatment, WWTP impacted rivers are colonized by resistant strains from WWTP effluents, underlining the importance of WWTP disinfection techniques. In order to contain the spread of ARGs by WWTPs, separation and removal of wastewater particles from WWTP discharges need to be considered.

2.5 Supporting Information A

The SI A contains additional information, including 8 figures, 3 tables, and 3 short text passages.

Partners at Karlsruhe Institute of Technology, Dr. Johannes Alexander, Thomas Jäger, and Norman Hembach, prepared the following calibration curves (Figures SA1 – SA7).

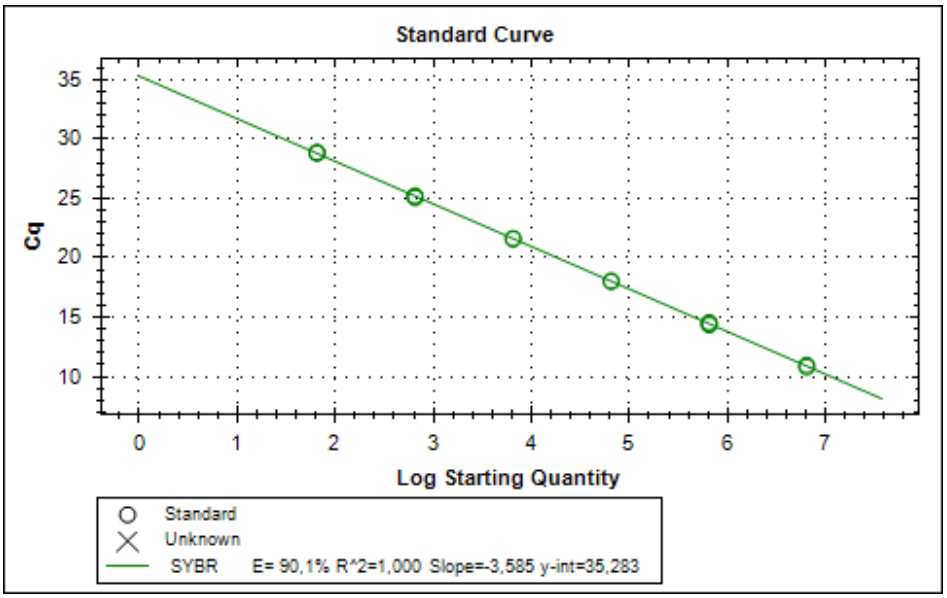

Figure SA1: Calibration curve for the *enterococci* specific 23S rDNA. The reference strain *E. faecium* DSM 20477 was used. Gene copy number (x-axis) plotted against qPCR-cycles (Cq) (y-axis). Annealing temperature: 60 °C. Fragment size: 93 bp.

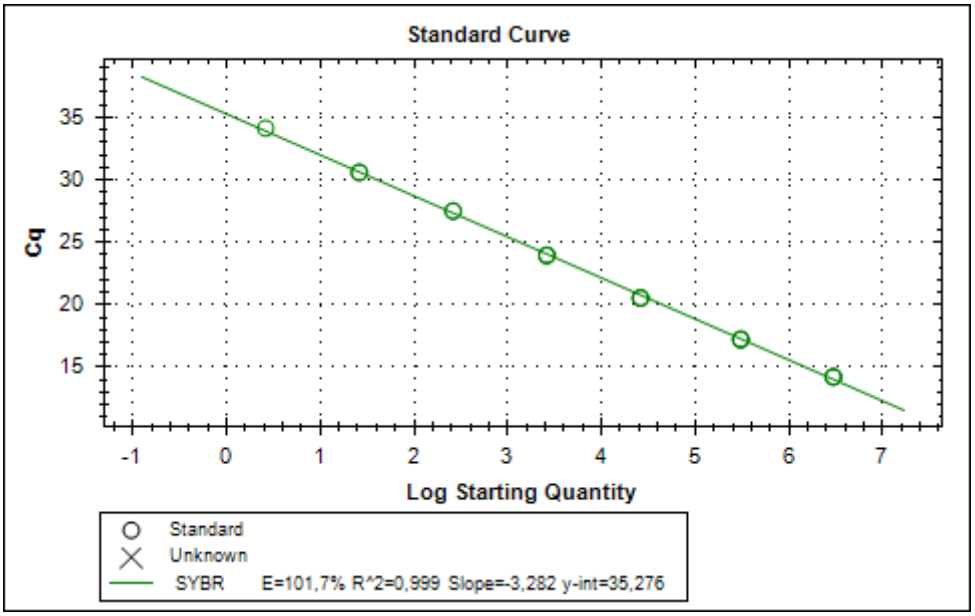

Figure SA2: Calibration curve for *ecf*X (*P. aeruginosa* DSM 1117). Gene copy number (x-axis) plotted against qPCR-cycles (Cq) (y-axis). Annealing temperature: 56 °C. Fragment size: 81 bp.

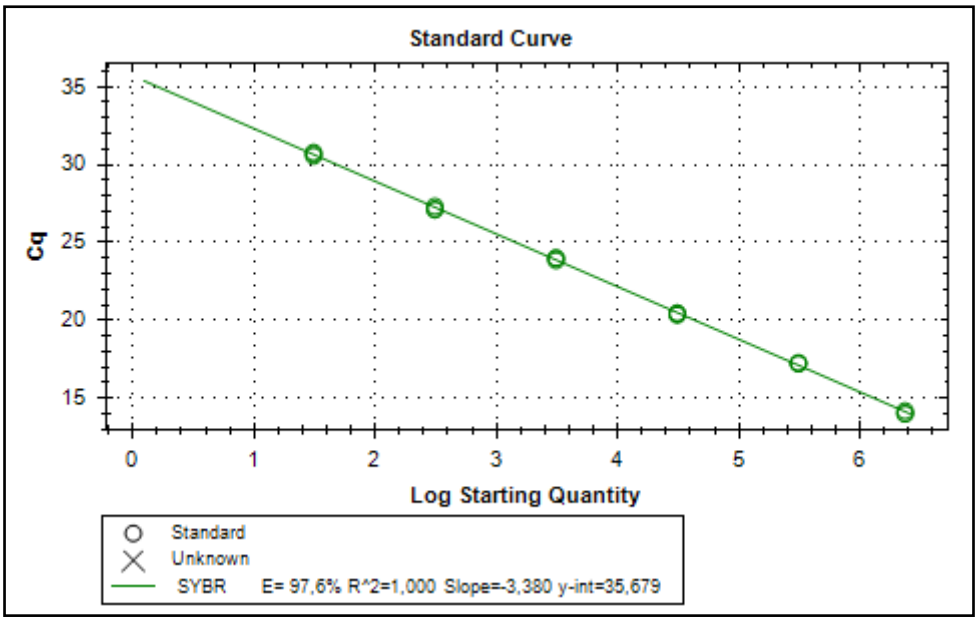

Figure SA3: Calibration curve for *secE* (*A. baumannii* DSM 30007). Gene copy number (x-axis) plotted against qPCR-cycles (Cq) (y-axis). Annealing temperature: 56 °C. Fragment size: 94 bp.

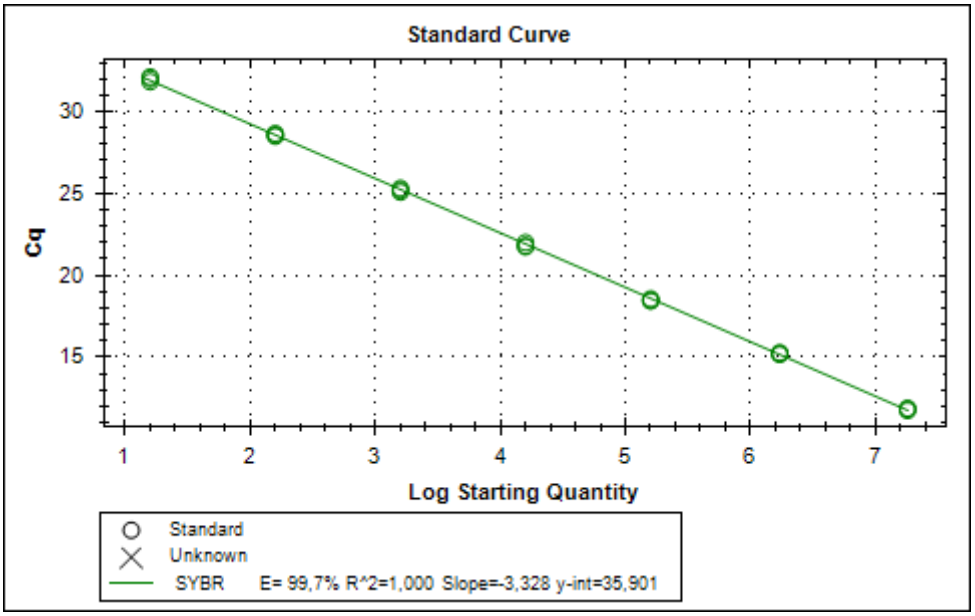

Figure SA4: Calibration curve for the erythromycin resistance gene *erm*B. The reference strain *S. hyointestinalis* DSM20770 was used. Gene copy number (x-axis) plotted against qPCR-cycles (Cq) (y-axis). Annealing temperature: 60 °C. Fragment size: 91 bp.

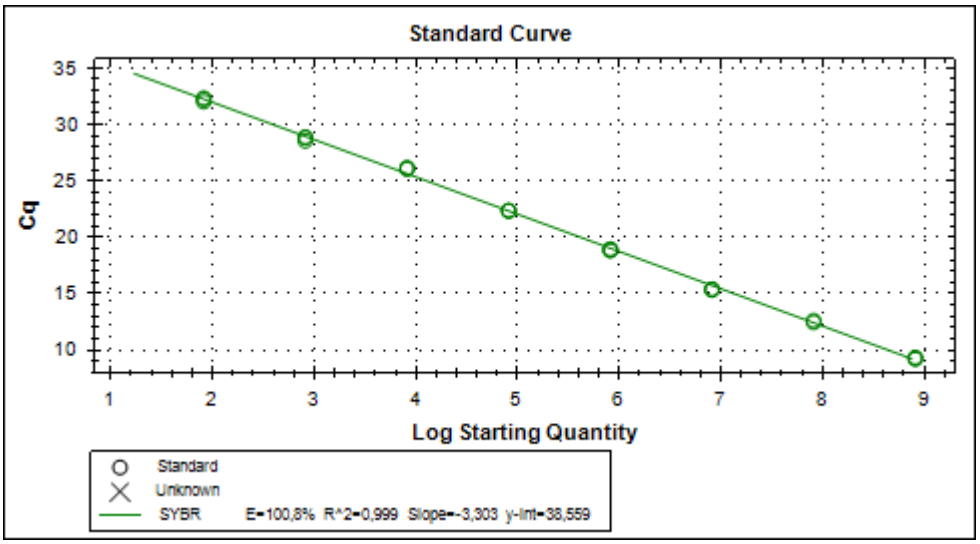

Figure SA5: Calibration curve for the beta-lactam resistance gene *bla*$_{TEM}$. The plasmid pNORM was used. Gene copy number (x-axis) plotted against qPCR-cycles (Cq) (y-axis). Annealing temperature: 60 °C. Fragment size: 112 bp.

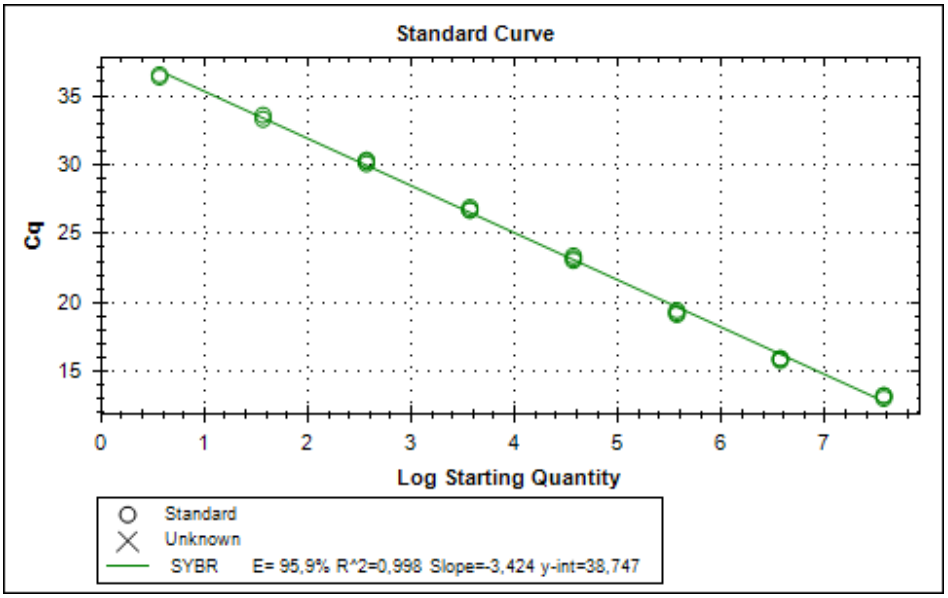

Figure SA6: Calibration curve for the tetracycline resistance gene *tet*M. The reference strain *E. coli* DH5α was used. Gene copy number (x-axis) plotted against qPCR-cycles (Cq) (y-axis). Annealing temperature: 60 °C. Fragment size: 88 bp.

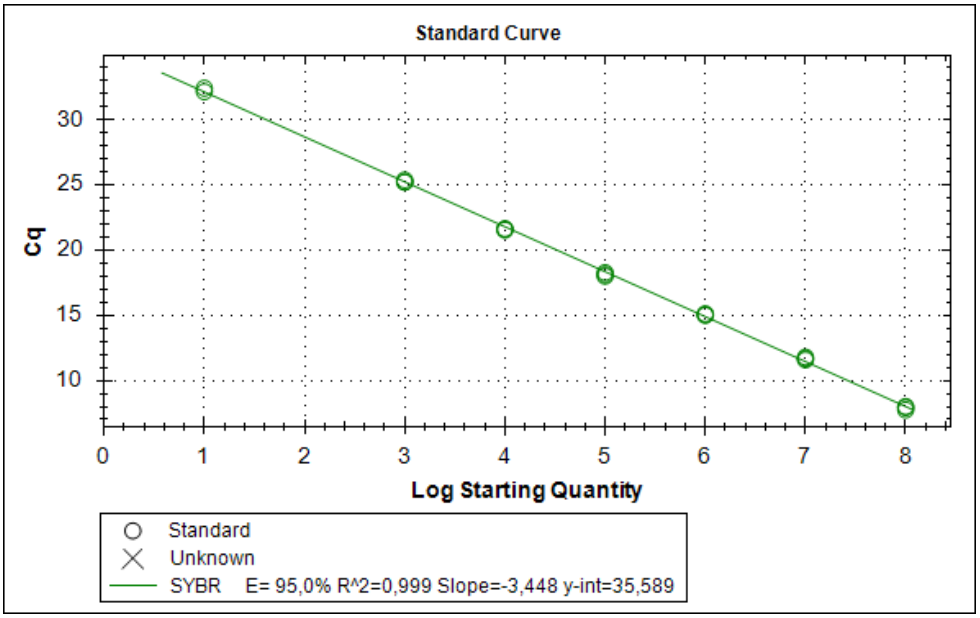

Figure SA7: Calibration curve for the quinolone resistance gene *qnr*S. Gene copy number (x-axis) plotted against qPCR-cycles (Cq) (y-axis). The plasmid pNORM was used. Annealing temperature: 60 °C. Fragment size: 118 bp.

Text SA1: Chemicals and reagents.

Erythromycin, roxithromycin, ciprofloxacin, tetracycline hydrochloride (>90 %) and penicillin V potassium salt were of analytical grade, if not mentioned differently, and

obtained from Sigma Aldrich (Germany). Pure standard of erythromycin-d_3, roxithromycin-d_7, ciprofloxacin-d_8 and penicillin V-d_5 were purchased from Toronto Research Chemicals (Canada). All solvents- methanol, acetonitrile, HPLC water- were analytical grade and were provided by VWR International (Germany). Formic acid (≥98 %) was supplied by Merck (Germany). Ethylenediaminetetraacetic acid disodium salt dihydrate ($Na_2EDTA{\cdot}2H_2O$) was obtained from Sigma Aldrich (Germany).

Text SA2: Solid phase extraction (SPE) of ABs present in water samples.

$Na_2EDTA{\cdot}2H_2O$ solution was added to the filtered acidified samples as chelating agent, to the final concentration of 1g/L (Lindsey et al., 2001). Subsequently, the samples were spiked with labeled standards to the concentration of 50 ng/L and extracted immediately. Solid Phase Extraction (SPE) was performed on Oasis® HLB (500 mg, 6cc) sorbent cartridges (Waters, Germany). The cartridges were preconditioned with 5 mL MeOH, 5 mL ACN, 10 mL HPLC water acidified with HCl to pH 2. The water samples were percolated through the cartidges at a flow rate around 5 mL/min. After percolation, the cartridges were washed with 5 mL of HPLC water and dried completely in a nitrogen flow. The analytes were eluted with 5 mL of MeOH and 5 mL of ACN and the eluate was collected into 10 mL glass vials. 100 µL of all eluates were transferred into amber 2 mL HPLC vials and diluted with 900 µL with HPLC water.

Text SA3. Recovery of ABs during SPE.

To assess possible losses during SPE, all the samples were spiked with available labeled standards of ABs (erythromycin-d_3, roxithromycin-d_7, ciprofloxacin-d_8 and penicillin V-d_5). Considering identical physico-chemical properties of labeled and non-labeled standards of the same compound, it was assumed that the interaction with SPE cartridges for both types of the AB was the same. With such approach, the recovery could be calculated for each compound in all the samples individually. The calculation was made according to the following equation: $R_{labeled\_std} = \frac{c_{measured\_labeled\_std}}{c_{spike\_labeled\_std}} \cdot 100\,\%$. Assuming identical efficiency of labeled and non-labeled standards of individual ABs, $R_{labeled\_std} = R_{non\_labeled\_std}$.

Due to the lack of labeled standard of tetracycline, the evaluation of SPE procedure was made using additional recovery samples. That means that, in parallel to water

samples of unknown AB concentration, another set of water samples represented all types of investigated matrices, i.e. WWTP effluent, river downstream and upstream, was prepared. Such samples were spiked with known concentration of tetracycline (50 ng/L), and then the measured concentration was compared with expected concentration ($c_{expected} = c_{not-spiked\ sample} + c_{spike}$), and recovery factor was calculated ($R = \frac{c_{measured}}{c_{expected}} \cdot 100\ \%$).

In general, the recovery factor was in the range 80-120 %. In the few cases when the recovery exceeded these limits, the concentration of the AB was divided by the recovery of corresponding labeled standard.

Table SA1: Analytical conditions used for quantification of selected antibiotics. Eluent A = 0.05 % Formic acid in HPLC water; Eluent B = 0.05 % Formic acid in acetonitrile; Flow = 300 µL/min; Injection volume = 50 µL.

time [min]	A [%]	B [%]
0	95	5
0.2	95	5
4.2	5	95
5.6	5	95
5.8	95	5
8.5	95	5

Table SA2: Settings of mass spectrometer used for quantification of antibiotics.

compound	RT [min]	polarity	precursor ion	quantifier	CE*	qualifier	CE*
ciprofloxacin	2.9	positive	332.1	231.0	40	245.0	24
ciprofloxacin-d_8	2.9	positive	340.2	235.0	44	296.3	20
erythromycin	3.5	positive	734.5	158.0	32	576.0	20
erythromycin-d_3	3.5	positive	737.5	161.0	32	579.4	20
penicillin V	4.0	positive	351.1	160.0	8	113.9	40
penicillin V-d_5	4.0	positive	356.1	160.0	12	229.0	16
roxithromycin	3.8	positive	837.5	158.0	36	679.4	20
roxithromycin-d_7	3.8	positive	844.6	158.0	36	686.5	20
tetracycline	3.0	positive	445.2	154.0	28	410.0	20

*collision energy

Table SA3: Source parameters used for antibiotics quantification.

gas temperature	350 ^{0}C
gas flow	8 L/min
nebulizer	25 psi
sheath gas temperature	400 ^{0}C
sheath gas flow	12 L/min
capillary	positive 5000 V; negative 4500 V
nozzle voltage	positive 500 V; negative 300 V

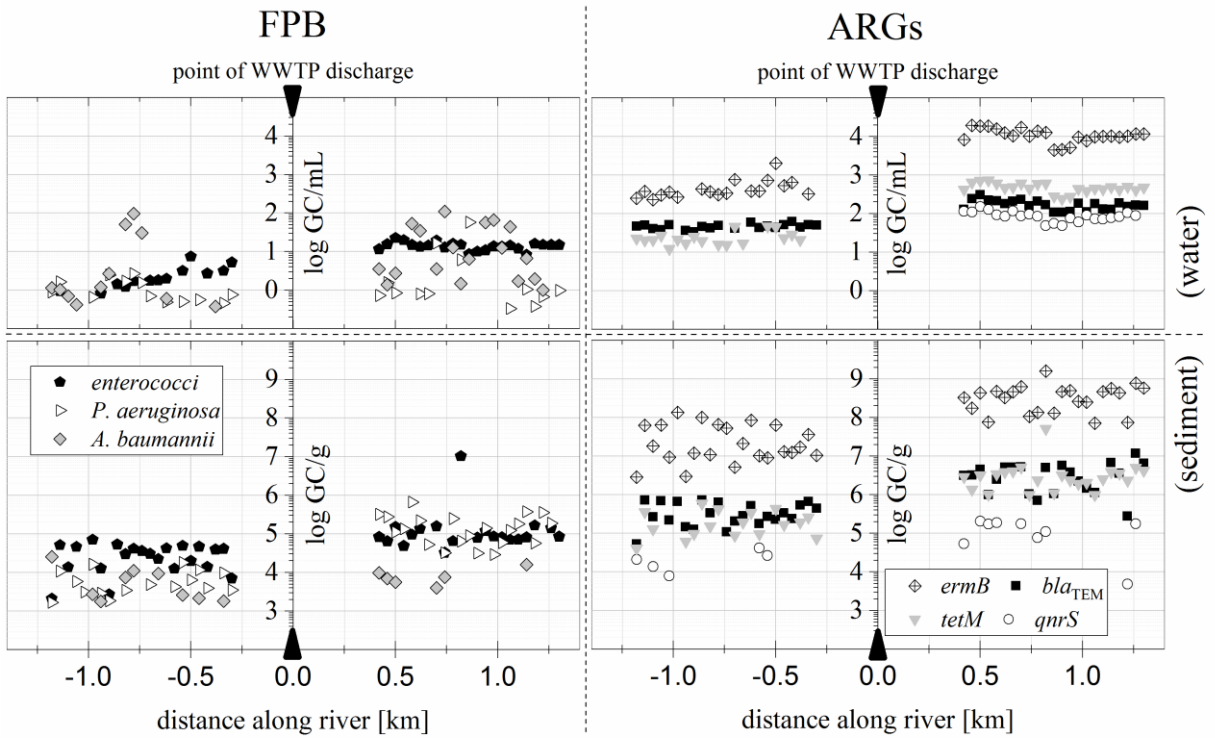

Figure SA8: Absolute abundances of facultative pathogenic bacteria (FPB) (left) and antibiotic resistance genes (ARGs) (right) in water (top) and sediment (bottom) along the receiving river. Upstream samples are shown on the left side and downstream samples are shown on right side of the y-axis (point of WWTP-discharge)

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3 Decay of elevated antibiotic resistance genes in natural river sediments after sedimentation of wastewater particles

Submitted for publication by *Brown P.C., Borowska E., Peschke R., Schwartz T., Horn H.*

3.1 Introduction

The evolution of antibiotic resistant pathogens threatens human health because of the decreasing efficiency of treatment with ABs. Although this appears to be a greater concern in the clinical area, environmental aspects of antibiotic resistance development have to be included in the health risk assessment because of HGT of ARGs between indigenous, environmental, and pathogenic bacteria (Cummings et al., 2011; Forsberg et al., 2012). It is an undeniable fact that many of the either clinically relevant bacteria or resistance mechanisms originate from the natural environment (D'Costa et al., 2011; Finley et al., 2013; Forsberg et al., 2012), and it was the anthropogenic impact on the natural environment that increased the probability of ARG recruitment into clinically relevant pathogens (Knapp et al., 2011). Humans, domesticates, wild animals, and plants are interconnected through the environment and constantly exchange microorganisms and the genes they carry. Urbanization, globalization, and the growing demand for water and food are propelling the evolution and transmission of antibiotic resistance worldwide (Bengtsson-Palme et al., 2015). It is therefore crucial to understand this process and to minimize its development by installing an efficient barrier between the allochthonous and autochthonous bacteria.

WWTPs could potentially be such a barrier. However, the removal of ARB and ARGs in conventional treatment lines with activated sludge is incomplete and the risk of ARG transmission remains (McConnell et al., 2018). Downstream rivers are often

heavily impacted by wastewater discharges containing excessive amounts of bacteria and ARGs (Brown et al., 2019; Jäger et al., 2019; Hembach et al., 2017; Alexander et al., 2015; Berendonk et al., 2015; Schwartz et al., 2003). Enrichment of ARGs in both, downstream waters, and downstream sediments, have been surveyed in plenteous research endeavours (Brown et al., 2019; Proia et al., 2018; Gatica et al., 2016; Rodriguez-Mozaz et al., 2015; Lupo et al., 2012).

Further reasons for ARG enrichment in downstream waterways are widely discussed. Residual ABs from WWTP discharges, with the potential of modulating gene expression in microorganisms, are frequently detected in surface waters (Danner et al., 2019; Berendonk et al., 2015; Michael et al., 2013), where they might provoke ARG and ARB proliferation. This can be stated because, amongst other effects, ABs at sub-inhibitory concentrations enhance HGT between bacteria and elevate other phenotypical responses. This was shown in various transcriptome analyses, as summarized in a review by Davies et al. (2006). However, the results are inconsistent. Gene expression signatures that occur as a result of AB exposure have not been clearly identified. Moreover, ABs concentrations used in the experiments (10 – 16'000 µg/L) were usually higher than those observed in the environment (in Europe and the Americas, 0.005 – 15 µg/L) (Danner et al., 2019). Experimentation was conducted withal in laboratories, under controlled conditions, using pure cultures of either *E. coli* or other representatives of *Bacteroidales* and *Enterobacterales*. Development of AMR within natural microbial communities might not be the same.

It appears that the transport of ARGs/ARBs itself is a driving factor for ARG/ARB enrichment in receiving rivers. With regard to the sheer quantity of ARGs, which are carried by WWTP effluents, this inference seems reasonable (Brown et al., 2019). An enormous mass of TSS, attached microorganisms and ARGs is released into surface waters by WWTPs every year (Brown et al., 2019; Proia et al., 2018). This comprises particles from treated wastewaters as well as from combined sewer overflows (Passerat et al., 2011). For instance, considering a TSS of 5 mg/L and a flow rate of 1 m^3/s, 150 t of particulate material would be discharged into the downstream waterway in one year. A considerable portion of this is known to settle to the riverbed

via sedimentation (Ahn, 2012; Qian et al., 2015; Walters et al., 2014b). However, the dynamics of this process is still poorly understood. It is uncertain, if and for how long wastewater particles persist in receiving waters and, according to the best of our knowledge, there is no comprehensive study that focusses on the decay of particle-attached microorganisms and their ARGs. The question remains as to what happens, if the continuous import of particulate matter from urban wastewater management is reduced.

Regression analyses have been used as evidence that either ABs (Proia et al., 2018; Pruden et al., 2012; Xu et al., 2015), or ARB/ARGs (Brown et al., 2019; Karkman et al., 2019), from WWTP discharges drive the development of ARGs in downstream rivers. A higher nutrient availability downstream compared to upstream of WWTP discharges possibly supports this progress (Saarenheimo et al., 2017; Wakelin et al., 2008). However, the effluent is known to consist of a mixture of all three components. This implies that these variables are correlated, hence, the causal inference that one of these components alone drives ARG enrichment is not valid. For all we know, ARG/ARB enrichment might be the outcome of their combination. For natural systems, such as river bacterioplankton microbiomes or benthic microbial biofilms, an experiment that specifically focuses on the change of ARG abundances, attributed to either one of the components, is missing.

With mesocosm experiments, the present study addresses this issue by evaluating the effect of either ABs, or wastewater particles, on the absolute abundance of ARGs in natural river sediments, while keeping nutrient levels constant.

3.2 Materials and methods

3.2.1 Chemicals and reagents

A list of the used chemicals and reagents with their suppliers and purity degree is depicted in the Supporting Information B (SI B) (Text SB1).

3.2.2 Sediment collection and preparation

The sediment used in this study was taken from a mid-sized mountain stream (average flow rate of 2.4 m^3/s), which we have previously examined (Brown et al., 2019). It is also the receiver of the WWTP effluent described in the sections below. 12 L of natural river sediment was collected upstream of the WWTP-discharge point, using a hand shovel and a 20 L vessel (both sterilized). The sediment was immediately transported to the laboratory and strained through a sieve with a mesh size of 5 mm to remove larger stones, debris, plant material, and animals. To ensure similar initial conditions in replicate series, the sediment was homogenized. A portion of sediment was dried at 110 °C for 24 h, cooled, and weighed.

3.2.3 Design of batch reactor experiment and sampling

The experimental setup consisted of five 10 L batch reactors (Figure 3.1 A, B) each filled with 1.5 L of sediment, and 8.5 L of tap water. More information, regarding the design of the batch reactors, can be found in the SI B (Text SB2). To assess the effect of ABs on ARG selection, erythromycin, tetracycline, ciprofloxacin, roxithromycin, penicillin V, and sulfamethoxazole were spiked to a final concentration of 5 µg/L, every 6 – 13 days. Concentrations of ABs were regularly monitored in the water phase using LC-MS/MS, as described in the section below. The ABs scenario was tested in duplicate (reactors 1 and 2, Figure 3.1 B). To assess the contribution of ABs sorption to the sediment, an additional experiment was performed, which is described in a section below.

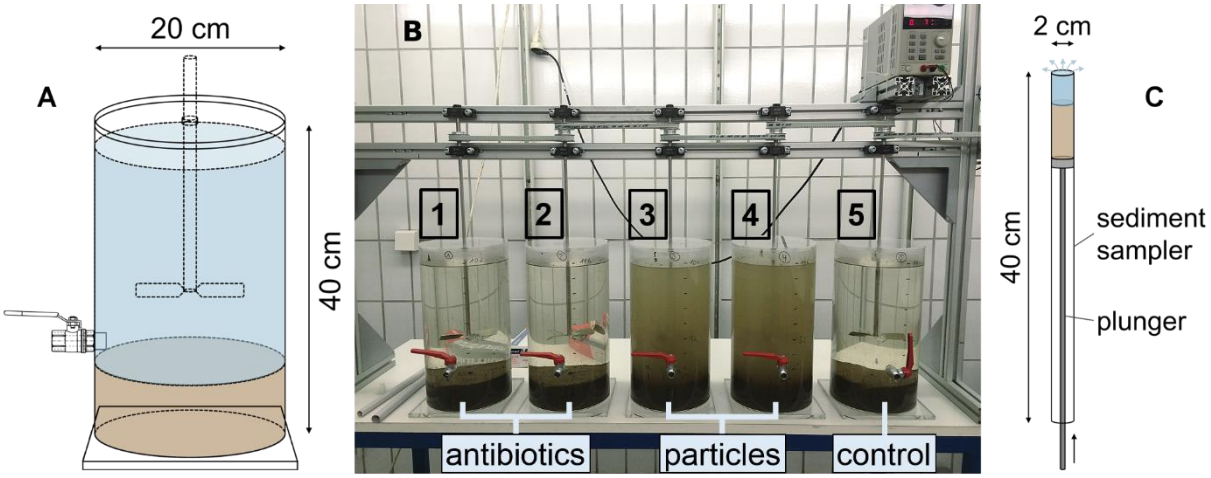


Figure 3.1: Experimental design. (A) Schematic drawing of one 10 L batch reactor. (B) Photo of experimental setup; taken after spiking wastewater particles. (C) Schematic drawing of sediment sampler.

The contribution of the transported resistances was verified by adding wastewater particles to a final concentration of 150 mg/L. Wastewater particles were obtained from a local WWTP, as described in the sections below. To observe dynamic changes in gene abundances, particles were spiked only once, at the beginning of the experiment. The propellers were operated at a slow rotation of 5 rpm to exclude turbulence (maximal Reynolds number, Re_{max} = 850) and to guarantee homogenous settling of particles onto the sediment surface. The particle scenario was tested in duplicate (reactors 3 and 4, Figure 3.1 B).

To assure equal nutrient availability in all reactors, nitrate and phosphate (1.5 mg/L NO_3-N, 0.3 mg/L PO_4-P) were regularly added to the water phase. Mesotrophic concentrations, similar to those found in the waterway downstream of the WWTP discharge (central laboratory, WWTP Karlsruhe, Germany), were met and kept at the aforementioned levels for the duration of the experiment. This was to ensure that changes in bacterial numbers could not be attributed to changes in nutrient availability. Wet-chemical analyses (Hach Lange, LCK349 and LCK339, Düsseldorf, Germany) were used to determine nitrate and phosphate concentrations.

For the analysis of ABs, water samples were taken from the middle of the water column using a glass pipette, filtered via cellulose acetate syringe filters (CHROMA-FIL® Xtra CA-45/25, Machery-Nagel, Germany) and diluted 1:10 with HPLC water. Prepared samples were placed in the amber HPLC-vials and stored at 4 °C, no longer than 4 h before analysis.

To measure ARGs and 16S rDNA, water samples were taken in volumes of 500 mL and batch reactors were re-filled with tap water to a final volume of 10 L after each sampling. Sediment samples were taken in duplicate with self-made cylindrical sediment samplers (PVC, Ø = 2 cm) (Figure 3.1 C) at the outermost positions of the batch reactors. The sediment sample was carefully separated from the supernatant water by using a plunger (Figure 3.1 C). Gene abundances were measured using qPCR after extraction of total DNA from water or sediment.

3.2.4 Wastewater sampling site

Sampling was carried out at the influent of a large-scale German WWTP with a population equivalent of 875'000 and a combined sewer system. In case of heavy rainfall, a significant amount of diluted wastewater is directly discharged into the river as combined sewer overflow. Consequently, 1 to 2 million m³ of untreated waste- and rainwater are discharged into the downstream river every year. The plant has a total tank volume (activated sludge and settling tanks) of approximately 70'000 m³. The hydraulic retention time varies between one day in dry weather conditions and less than 6 h during heavy rainfall. The influent is characterized by the following physico-chemical parameters: chemical oxygen demand (COD) = 475 mg/L, biochemical oxygen demand (BOD) = 229 mg/L, nitrogen (NH_4-N) = 26 mg/L, and phosphorous (P_{total}) = 5.9 mg/L. Over a period of three days, an amount of 20 L of 24 h-composite sample was collected in a sterile 20 L plastic container at the influent of the WWTP in August 2018. The automated 24 h-sampler transferred individual grab samples into a cooling chamber (4 °C). After collection, the samples were further cooled and transported to the laboratory for immediate analysis.

3.2.5 Wastewater particle size fraction

Wastewater particles were isolated from 20 L of raw wastewater by sieving through a mesh size of 1'000 µm and subsequent vacuum filtering through a 12 µm membrane (Sartorius, Germany). The resulting particle size fraction $12 < d_P \leq 1'000$ µm was re-suspended in a sterile 250 mL centrifuge bottle with 100 mL phosphate buffered saline (PBS, pH= 7.4). The solution was shaken and centrifuged, the supernatant was removed and the bottle re-filled with PBS. This washing-procedure was repeated 3 times. A portion of the particle solution was re-filtered through a 12 µm membrane, washed with nanopure water, dried at 110 °C for 24 h, cooled, and weighed.

3.2.6 Quantification of antibiotics by LC-MS/MS

Measurement of the selected ABs was performed using an Agilent 1290 Infinity II UHPLC system coupled to an Agilent 6470 Triple Quadrupole LC/MS system via an Agilent Jet Stream electrospray ionization source. Analytes of interest were separated on an Agilent ZORBAX Eclipse Plus C-18 column (50 × 2.1 mm, 1.8 µm particle size) using HPLC water and acetonitrile, both acidified with formic acid to the concentration of 0.05 % as mobile phases. Details of the analytical method used in the study is shown in SI B (Table SB1). The detection of analytes was performed in dynamic Multiple Reaction Monitoring (MRM) mode. To compensate for a potential matrix effect that could influence the quantitative analysis, all samples were spiked with stable-isotope-labeled internal standards of the investigated ABs. The mass spectrometry parameters used for the data acquisition are collected in the SI B (Tables SB2 and SB3). The quantification was achieved with an extrernal calibration curve.

3.2.7 Sorption of antibiotics to sediments

The sorption of ABs to river sediments was assessed in 500 mL conical flasks that were filled with 20 g of autoclaved sediment (dried) and nanopure water to a final volume of 200 mL. To distinguish the abatement driven by hydrolysis, a set of 500 mL conical flasks filled with 200 mL of nanopure water was prepared. All flasks were spiked with a mixture of ABs (erythromycin, tetracycline, roxithromycin, sulfamethoxazole, ciprofloxacin, and penicillin V) to a concentration of 5 µg/L. The bottles

were shaken (125 rpm) in the dark at 12 °C. The change of ABs concentrations in water were monitored for a duration of 1 week using LC-MS/MS. Sorption was calculated based on the loss of ABs in water attributed to the presence of sediment via an area-based balancing (integral).

3.2.8 DNA extraction

To extract total DNA from water samples, 500 mL of sample was filtered through membranes with a 0.2 µm pore size (Whatman Nuclepore Track-Etched Membranes, Sigma-Aldrich, Munich, Germany). For the extraction of total DNA from sediments, samples were homogenized using PBS at pH 7.4 by shaking for 5 min at room temperature. The suspension was then centrifuged at 3'000 *g* for 10 min. Bacterial DNA was extracted from the pellet or from membrane filters using the lysing matrix E of the Fast DNA Spin Kit for Soil (MP Biomedical, Illkrich, France) following the manufacturer's instructions. The total DNA yield for each sample was measured with the Qubit Fluorimetric Quantitation, using the dsDNA BR Assay Kit according to the manufacturer's instructions (Thermo Scientific, Waldham, USA).

3.2.9 qPCR measurement of antibiotic resistance genes and total bacteria

ARGs and total bacteria (16S rDNA) were detected in water and sediment samples of the batch reactors. The detection systems are described in previous works (Brown et al., 2019; Hembach et al., 2017; Rocha et al., 2018). Control measures were taken to ensure the quality of the qPCR data. Dilution series were carried out to eliminate threshold cycle suppression by inhibitors (1:10 or 1:100). To verify positive detections, melt curve analysis was included in every run. Measurements were excluded from further analysis, if the variance among triplicates (gene copies) were greater than 20 %. Two or more positive detections were scored as positives and the mean value of all positive technical replicates were employed in further analyses. Pooled means were calculated from biological replicates.

ARGs and 16S rDNA were quantified by a SYBR Green qPCR approach. Reactions were run in volumes of 20 µL containing Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific, Waldham, USA), nuclease-free water (Ambion, Life technologies, Karlsbad, Germany), 0.2 µM of the respective primers, and 0.5 – 50 ng of

template DNA. The qPCR protocol comprised 10 min at 95 °C for activation of the DNA polymerase followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C.

3.2.10 Statistics and determination of decay parameters

Measured gene copy numbers (GC) were log-transformed prior to plotting. To better visualize the temporal development of GC in the particle-scenario, non-linear fitting curves were generated. Additionally, reaction rate constants were calculated. For GC in water, which were influenced by two different processes, calculation was based on the rate law of a second order reaction. This is due to Qian et al. (2015) who investigated the removal of *Escherichia coli* from river water. In the first 20 hours, they could show that sedimentation had a stronger influence on the removal, while decay (inactivation) became more significant afterwards. To determine the rate constant (*k*), linear regression analyses were made after plotting 1/GC against *t*, where *k* is represented by the slope. Finally, the half-life was calculated by:

$$t_{1/2} = \frac{1}{k \cdot \mathrm{GC}_{max}}$$

(GC_{max} = maximal GC at time zero). The decay in the sediment was represented by a first order reaction. The *k*-values were determined from the slope after plotting ln(GC) against *t*. The half-life was then calculated by:

$$t_{1/2} = \frac{\ln 2}{k}$$

More detailed information regarding decay functions can be found in the SI B (Figures SB1 and SB2). Data analyses and graphs were made with OriginPro 2017 (OriginLab Corporation, Northampton, MA, USA).

3.3 Results and discussion

3.3.1 Initial and final concentrations of antibiotic resistance genes and total bacteria

ARG abundances and 16S rDNA concentrations were measured in water and sediment samples of 5 individual batch reactors with negligible relative errors between duplicates (Figure 3.2). The initial gene concentrations ($C_{initial}$) did not vary between the batch reactors (Table 3.2 and 3.3). In waters and sediments, respectively, they were measured at average concentrations of 3.60 ± 0.10 log GC/mL and 7.35 ±

0.02 log GC/g (*erm*B), 0.29 ± 0.06 log GC/mL and 5.16 ± 0.05 log GC/g (*tet*M), 1.34 ± 0.02 log GC/mL and 6.52 ± 0.01 log GC/g (bla_{TEM}), 3.32 ± 0.11 log GC/mL and 7.62 ± 0.03 log GC/g (*sul*1), 0.17 ± 0.40 log GC/mL (CTX-M-32, not detected in some water samples, not detected in sediments), 0.35 ± 0.08 log GC/mL and 3.35 ± 0.10 log GC/g (*qnr*S, not detected in some water and some sediment samples), and 6.00 ± 0.18 log GC/mL and 10.19 ± 0.01 log GC/g (16S rDNA). Compared to surface waters in Germany, Spain, Belgium, and Italy, gene abundances were low in waters, and similar in sediments (Alexander et al., 2015; Brown et al., 2019; Calero-Cáceres et al., 2017; Di Cesare et al., 2017; Proia et al., 2018). Over a period of two months, and independent from the individual treatment scenarios, total bacteria, as quantified via their 16S rDNA, equally decreased by 0.49 – 0.60 log units in the sediments of all reactors to a final average concentration of 9.64 ± 0.05 log GC/g. In parallel, the amount of bacteria increased in water columns by 0.24 – 0.90 log units to a final average concentration of 6.50 ± 0.19 log GC/mL (exclusively when compared to initial 16S rDNA concentration). A possible explanation for this increase could be proliferation or the mobilization of bacterial cells from sediments in order colonize lesser populated areas of the batch reactors, including water columns, reactor walls, and propeller surfaces. Nutrient concentrations did not vary throughout the experiment and were measured at concentrations of 1.48 ± 0.56 mg/L (NO_3-N) and 0.28 ± 0.09 mg/L (PO_4-P).

3.3.2 Hydrolysis and sorption of antibiotics

ABs were below limit of quantification (LOQ) in all batch reactors before the start of the experiment (LOQ = 0.1 – 0.25 µg/L). After addition of ABs, an average concentration of 5.1 ± 0.9 µg/L was measured (Figure 3.2). Within the following week, the 6 compounds decayed at individual rates (Table 3.1). Concentrations of tetracycline, ciprofloxacin, and penicillin V decreased by ≥ 90 % per week, probably due to the instability of penicillin in water (hydrolysis) as well as the adsorption of tetracycline and ciprofloxacin to organic substances (Belden et al., 2007; Le-Minh et al., 2010). For sulfamethoxazole, a loss of 20 ± 11 % per week was observed. The remaining ABs decreased by 18 ± 13 % (erythromycin) and by 46 ± 15 % per week (roxithromycin). ABs were not detected after addition of wastewater particles.

Table 3.1: Average decay of antibiotics in reactors after periodic spikes to 5.1 ± 0.9 µg/L.

compound	average decay per day [$\mu g \cdot L^{-1} \cdot d^{-1}$]	average removal per week [%]
tetracycline	0.71 ± 0.16	99 ± 6
sulfamethoxazole	0.14 ± 0.10	20 ± 11
erythromycin	0.13 ± 0.11	18 ± 13
roxithromycin	0.33 ± 0.16	46 ± 15
penicillin V	0.64 ± 0.17	90 ± 10
ciprofloxacin	0.65 ± 0.18	91 ± 14

Several ABs are known to adsorb to sediments, depending on pH, content of clay, silt, and organic matter, as well as content of ionic components (Cheng et al., 2016; Zhao et al., 2016). This could lead to local accumulation of ABs and potentially higher impact on the benthic biofilm. To assess the adsorption properties of compounds, a partitioning-coefficient ($K_{d,s}$) is often used, which describes the ratio between the concentration in the solid phase (sediment, soil) (C_S) and in the water phase (C_W). The values reported in literature classify ciprofloxacin (266 – 5'471 mL/g) (Mutavdžić Pavlović et al., 2017; Tang et al., 2019) and roxithromycin (667 – 2'391 mL/g) (Tang et al., 2019) as highly adsorbing compounds, tetracycline (198 – 653 mL/g) (Tang et al., 2019) as a moderately to highly adsorbing compound, and erythromycin (5 – 426 mL/g) (Radović et al., 2016; Tang et al., 2019) and sulfamethoxazole (5 – 147 mL/g) (Radović et al., 2016; Zhou and Broodbank, 2014) as poorly to moderately adsorbing compounds. This is in agreement with our findings. Sorption (µg of AB adsorbed per kg of sediment) was observed for ciprofloxacin (37 µg/kg, 73 %), roxithromycin (28 µg/kg, 56 %), and tetracycline (15 µg/kg, 30 %) (Figure SB3, page 72). Sorption was calculated based on the loss of ABs in water attributed to the presence of sediment, hence, sorption of ABs might have been overestimated. Nevertheless, the effect was clear and the results are consistent with previous works (Belden et al., 2007; Chen and Zhou, 2014; Cheng et al., 2016, 2014; Gu et al., 2007; Wunder et al., 2011). No or no clear sorption was observed for sulfamethoxazole, erythromycin, and penicillin V (Figure SB3, page 72).

3.3.3 Effect of antibiotics on absolute antibiotic resistance gene abundances and total bacteria

In water, the abundance of some ARGs was influenced by ABs (Figure 3.2, column 1). The ARGs *erm*B and *qnr*S decreased by approximately 1 log unit. ABs seemed to also have an effect on the selection of sulfamethoxazole-resistant bacteria because the *sul*1 gene was 2 log units more concentrated after 2 months. However, clear trends were not visible for the remaining targets *tet*M, *bla*$_{TEM}$, CTX-M-32, and 16S rDNA as their concentrations were similar to those observed in the control (Figure 3.2, column 3).

In sediments, no changes in absolute ARG abundances were observed. Despite the continuous exposure to ABs and sorption of ABs, all ARGs remained close to their $C_{initial}$ (Figure 3.2, column 1). The variances were below the range of 1 log unit and equally high or identical to those observed in the control. When ABs were present, the quinolone resistance gene *qnr*S was more frequently detected compared to the control, however, the results were not clear.

Researchers have previously stated that the effect of sub-inhibitory ABs on resistance selection might not be as significant as frequently speculated (Bengtsson-Palme et al., 2016; Flach et al., 2018; Brown et al., 2019; Karkman et al., 2019). Bengtsson-Palme et al. (2016) found indication for selective processes in sewage pipe biofilms, including HGT. However, they could not find clear evidence for ARG selection further downstream (WWTP), although some ABs were measured above the PNECs (Bengtsson-Palme and Larsson, 2016) (4'500 ng/L tetracycline, 900 ng/L ciprofloxacin). Flach et al. (2018) recently showed comparable results. In their study, 4'028 *E. coli* isolates were collected from a large-scale WWTP and screened for resistance to seven ABs. Enrichment of resistance was not found, although tetracycline was above PNECs (660 ng/L). It should be noted that both studies were focusing on samples taken from inside the WWTP. The dilution of effluents in receiving rivers will often be large, hence, concentrations of ABs will be lower. Nevertheless, ARGs typically increase in WWTP impacted rivers (Berendonk et al., 2015). In our previous field study, ARG abundances and ABs were measured in a river influenced by the effluent of a large-scale WWTP (Brown et al., 2019). Although

ABs in the downstream river were far below PNECs (~30 ng/L), ARGs increased by 0.5 – 2 log units downstream compared to upstream of the WWTP discharge (sediment and water). The transport of ARGs/ARB might be a more noticeable contributor in the spread of AMR than the selection by antimicrobial residues. Disinfection of effluents, rather than removal of selective agents, might be a more important measure. This is strongly supported by a recent study of Karkman et al. (2019) who found that the presence of resistance genes can mostly be explained by faecal pollution, with no clear signs of selection in the environment. They furthermore point out the importance of not making false assumptions about environmental selection of antibiotic resistance while clearly making the connection to human faecal pollution levels. Jäger et al. (2018) have modelled the distribution of ARG loads from WWTP into receiving water bodies and asserted the need to minimize the bacterial freight and ARGs at WWTPs.

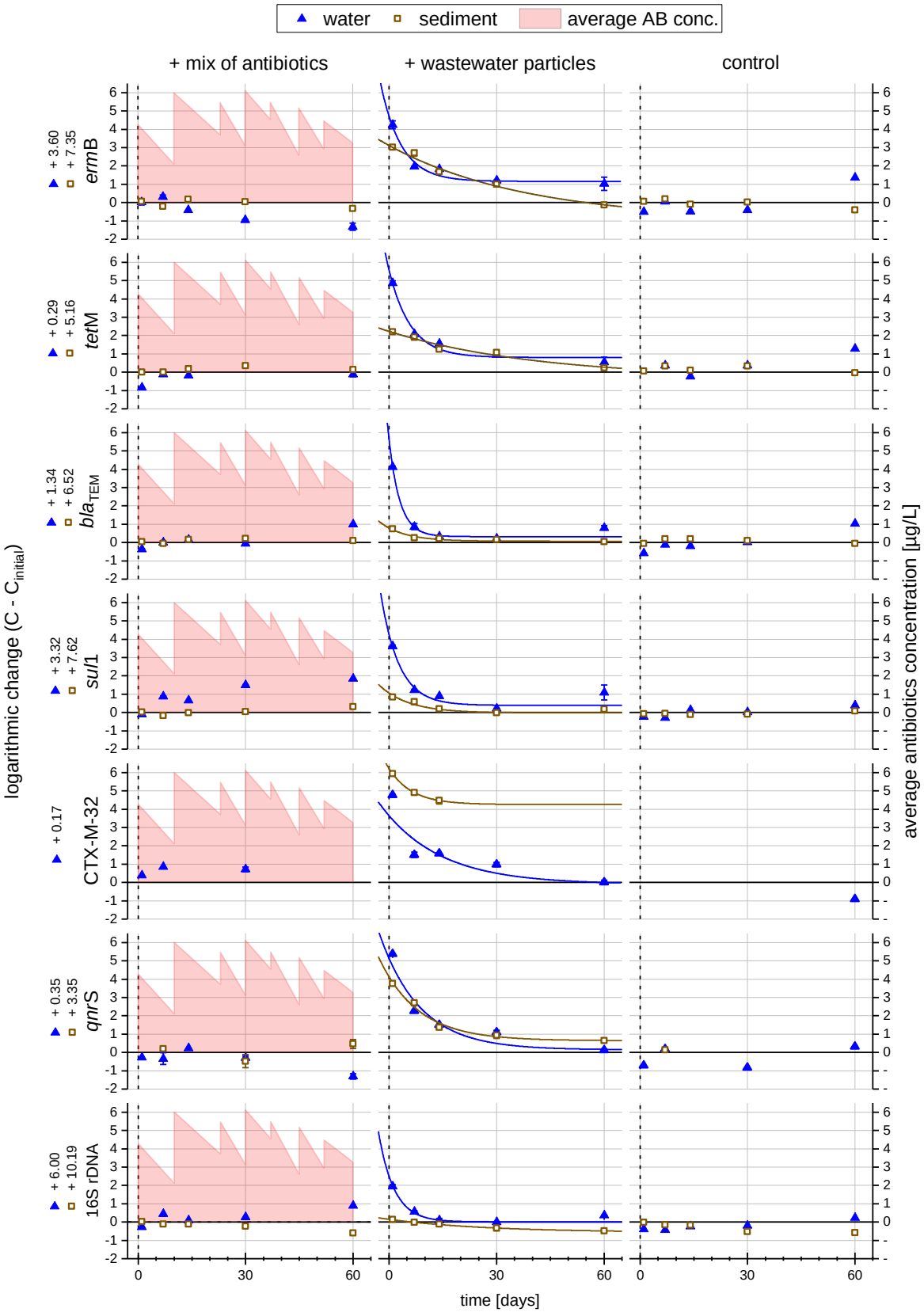


Figure 3.2: Logarithmic change of antibiotic resistance genes and 16S rDNA abundances in 2 months compared to initial concentration ($C_{initial}$) in waters (log GC/mL) and sediments (log GC/g) (left axis). $C_{initial}$ (left of axis title). Average antibiotic (AB)

concentration (right axis). Effect of antibiotics (column 1). Effect of wastewater particles (column 2). Non-linear fitting curves (column 2). Control (column 3). Error bars represent relative errors.

Our study purely focusses on absolute ARG abundances and therefore cannot identify possible modulations of gene expression profiles and functional changes that occur on a subcellular level. Several papers have used transcriptome and proteome analysis and revealed AB induced changes in gene expression patterns that can be detected in an array of genes not related to antibiotic resistance (Davies et al., 2006). These functional changes include enhanced gene transfer, increased mutation frequency, reduced toxin secretion, reduced biofilm formation, and several other responses (Davies et al., 2006). According to this, ABs concentrations 10 times below the MIC pose a risk because they might change the functionality of benthic microbial biofilms in the environment. Similar alterations might have occurred in the microbial community inside the batch reactors of this study. However, this remains unclear until further research with natural river sediments is conducted. In our study, no obvious signs for the selection of ARGs by ABs were observed, nevertheless, the possibility for AB induced genetic modulation, leading to functionality changes in the biofilm, cannot be excluded.

3.3.4 Effect of spiked wastewater particles

After addition of wastewater particles, ARG abundances increased by 3 to 5 log units in waters, and by 1 to 4 log units in sediments (Figure 3.2, column 2). The 16S rDNA concentration increased by 2 log units in water samples but remained unaffected in sediments. CTX-M-32 was not detected in sediments before the start of the experiment but was measured at high concentrations after spiking with particles (5.93 ± 0.02 log GC/g). After 2 weeks, CTX-M-32 was not detected in sediments anymore.

ARGs and 16S rDNA underwent a certain decay. After two months, at the latest, all targets were measured at concentrations similar to those observed before the start of the experiment in both, water samples, and sediments. Exceptions were *erm*B and *tet*M in water samples, which remained slightly elevated by approximately 1 log

unit after 2 months (4.48 ± 0.36 log GC/mL and 0.93 ± 0.25 log GC/mL, respectively). Nonetheless, these final concentrations were still low compared to those measured in the environment (Brown et al., 2019), and within a short time, like all other targets, spiked abundances decayed almost entirely. Within the first 14 days, elevated ARG and 16S rDNA concentrations were reduced by a total of 99.48 to 99.88 % in water samples, and by 88.16 to 99.67 % in sediments (excluding 16S rDNA abundances). In the following 6 weeks, further reduction was observed for all targets by a total of 99.72 – 100.00 % in water samples and by 91.39 – 100.02 % in sediments.

3.3.5 Decay of ARGs in water samples and sediments

To better characterize the removal of elevated gene levels, non-linear fitting curves were generated (Figure 3.2, column 2). Coefficients of determination (R^2 values) ranged between 0.82 and 0.99, indicating that the experimental data can be well explained by the fittings (SI B, Figure SB4). Theoretical maximal concentrations (C_{max}) were calculated from y-intercepts. $C_{initial}$ (before particle addition), C_{max} (after particle addition), reaction rate constants (k) and half-lives (τ) are depicted in Table 3.2 (water, based on a second order approach) and Table 3.3 (sediment, based on a first order approach). Half-lives of ARGs were generally shorter in waters than in sediments. This indicates that there are two processes occurring in waters (sedimentation and decay) and another two occurring in sediments (accumulation and decay). Via sedimentation, ARGs are transported to the sediment (Brown et al., 2019; Proia et al., 2018), leading to a fast reduction of their abundances in the water and potentially to an accumulation in the sediment. However, a subsequent increase of ARG abundances was not observed in sediments. This means that the entire dose, which was spiked at the beginning of the experiment, settled within the first few days and the decay of ARGs in sediments, after this point, was not influenced by sedimentation anymore. A narrow range of the determined decay rates in sediments (0.02 – 0.12 d^{-1}) (Table 3.3) support this hypothesis.

Table 3.2: Average initial concentrations $C_{initial}$, theoretical maximal concentration after particle addition C_{max}, and decay parameters of ARGs and 16S rDNA in water after spiking 150 mg/L of wastewater particles.

gene/target	average initial conc. $\mathbf{C_{initial}}$ [log GC·mL^{-1}]	theoretical maximal concentration $\mathbf{C_{max}}$ [log GC·mL^{-1}]	rate constant $\boldsymbol{k}$ [mL·GC^{-1}·d^{-1}]	half-life $\boldsymbol{\tau}$
*erm*B	3.60 ± 0.10	8.11	$5.83 \cdot 10^{-7}$	19 min
*tet*M	0.29 ± 0.06	5.72	$2.06 \cdot 10^{-3}$	79 s
bla$_{TEM}$	1.34 ± 0.02	6.05	$9.75 \cdot 10^{-4}$	80 s
sul1	3.32 ± 0.11	7.33	$1.14 \cdot 10^{-5}$	6 min
CTX-M-32	0.17 ± 0.40	5.52	$1.14 \cdot 10^{-2}$	23 s
*qnr*S	0.35 ± 0.08	6.23	$5.85 \cdot 10^{-3}$	9 s
16S rDNA	6.00 ± 0.18	8.14	$4.03 \cdot 10^{-8}$	4 h

An explanation for the removal of all measured targets could be high die-off due to nutrition limitation (Allocati et al., 2015), as the wastewater particles were transferred from a hypertrophic (O_2 < 3 mg/L, dissolved organic carbon, DOC > 50 mg/L, phosphate > 50 mg/L, nitrate > 2 mg/L) to a nutrient limited surrounding (O_2 > 8 mg/L, DOC < 5 mg/L, phosphate < 2 mg/L, nitrate < 2 mg/L). In wastewaters, heterotrophic bacteria are the predominant group of organisms (McLellan and Roguet, 2019; Harmsen and de Goffau, 2016; Bengtsson-Palme et al., 2016), however, in nearly every natural ecosystem, the availability of carbon/energy substrates, supporting heterotrophic growth, is critically restricted (Atashgahi et al., 2015; Egli, 2009). Though present in various forms, there, utilizable carbon substrates are scarce and hard to break down. They originate mainly from decomposition (hydrolysis) of organic matter, i.e. plant material and animal excretes (Egli, 2009). This group of carbon compounds is referred to as assimilable organic carbon (AOC). Along with the temperature, the AOC is considered to dominate and restrict growth rates of heterotrophic microbial communities (Egli, 2009; Thayanukul et al., 2013). In the present study, natural sediments were used. Comparable carbon constraints might have been the reason for the strong reduction of wastewater bacteria inside the batch reactors, where the introduced microorganisms were seemingly outcompeted by the

natural microorganisms because of their differences in specializing to two distinct groups of carbon compounds (Atashgahi et al., 2015).

Table 3.3: Average initial concentrations $C_{initial}$, theoretical maximal concentration after particle addition C_{max} and decay parameters of ARGs and 16S rDNA in sediment after spiking 150 mg/L of wastewater particles.

gene/target	average initial conc. $\mathbf{C_{initial}}$ [log GC·g^{-1}]	theoretical maximal concentration $\mathbf{C_{max}}$ [log GC·g^{-1}]	rate constant $\boldsymbol{k}$ [d^{-1}]	half-life $\boldsymbol{\tau}$
*erm*B	7.35 ± 0.02	10.46	0.12	5 d 18 h
*tet*M	5.16 ± 0.05	7.46	0.07	9 d 15 h
bla$_{TEM}$	6.52 ± 0.01	7.41	0.02	35 d 13 h
sul1	7.62 ± 0.03	8.51	0.02	30 d 8 h
CTX-M-32	-	6.12	0.11	6 d 13 h
*qnr*S	3.35 ± 0.10	7.38	0.10	6 d 15 h
16S rDNA	10.19 ± 0.01	10.35	0.02	29 d 6 h

Contradictory to the previous statements, multiple studies have shown regrowth of wastewater bacteria in downstream rivers, proving their survivability (Blasco et al., 2008; Czekalski et al., 2012; Proia et al., 2018). More importantly, along with faecal pollution, shifts in benthic microbial communities have been observed, suggesting permanent population changes in downstream waters and sediments (Wakelin et al., 2008; Saarenheimo et al., 2017; Lu and Lu, 2014). Wakelin et al. (2008) observed a strong relationship between the loading of carbon and nitrogen from a WWTP discharge into the sediment of a small river and the responses in bacterial abundance, community structure, and function. They furthermore showed significant alterations in the availability of carbon, nitrogen and phosphorous in downstream sediments, which they linked to changes in organic matter assimilation and secondary productivity in river sediments. Similar results were presented by Saarenheimo et al. (2017) who showed that WWTP effluents provided nitrogen and organic matter to boreal lakes, altering the physico-chemical properties of the sediments, which resulted in microbial community changes in lake sediments. Both studies clearly demonstrate a severe impact of the continuous discharge of particulate

organic matter from WWTP on the benthic microbial community, where the organic matter specifically acts as substrate for the introduced bacteria.

The question remains as to what happens, if the continuous import of particulate organic matter from WWTP discharges is reduced or even limited to close to zero. Perhaps the results of this study give an answer to this question. Possibly, the introduced bacteria and ARGs cannot persist and are entirely inactivated, ending up in the nutrient chain. Increased ARG abundances in downstream sediments are probably caused by the continuous import of particulate matter and associated bacteria from WWTPs. If this import is removed, ARGs/ARB are likely to decline. Possible explanations could be high die-off and degradation rates due to substrate limitation. However, wastewater-borne microorganisms and their ARGs can persist, as long as the particulate organic matter from WWTPs remains an available carbon/energy source in downstream rivers.

3.4 Supporting Information B

The SI B contains additional information, including 4 figures, 3 tables, and 2 short text passages.

Text SB1. Chemicals and reagents

Erythromycin, roxithromycin, ciprofloxacin, tetracycline hydrochloride (>90 %), sulfamethoxazole and penicillin V potassium salt were of analytical grade, if not mentioned differently, and obtained from Sigma Aldrich (Germany). Pure standard of erythromycin-d_3, roxithromycin-d_7, ciprofloxacin-d_8, sulfamethoxazole-d_4 and penicillin V-d_5 were purchased from Toronto Research Chemicals (Canada). All solvents- methanol, acetonitrile, HPLC water- were analytical grade and were provided by VWR International (Germany). Formic acid (≥98 %), phosphate buffered saline, nitrate ($NaNO_3$), and phosphate (Na_3PO_4, 96 %) was supplied by Merck (Germany).

Text SB2. Construction of batch reactors

Batch reactors with lids were constructed in PLEXIGLAS® of a 5 mm thickness by ‘Glasschmid’ (Karlsruhe, Germany). Brass ball valves were obtained from ‘Esska’

(Hamburg, Germany), stainless steel propellers (550 x 8 x 100 mm) from IKA® (Staufen, Germany), the aluminum scaffold from 'item' (Solingen, Germany), and mechanical parts, such as timing belts, pulleys, and wheel bearings from 'Mädler' (Stuttgart, Germany). The design and assembly was made by us. The propellers were electrically driven by a Korad programmable power supply from 'Reichelt' (Sande, Germany) at 4 V using a 37 mm planetary gearbox motor from 'RS Components' (Mörfelden-Walldorf, Germany).

Table SB1: Analytical conditions used for quantification of selected antibiotics. Eluent A = 0.05 % Formic acid in HPLC water; Eluent B = 0.05 % Formic acid in acetonitrile; Flow = 300 µL/min; Injection volume = 50 µL.

time [min]	A [%]	B [%]
0	95	5
0.2	95	5
4.2	5	95
5.6	5	95
5.8	95	5
8.5	95	5

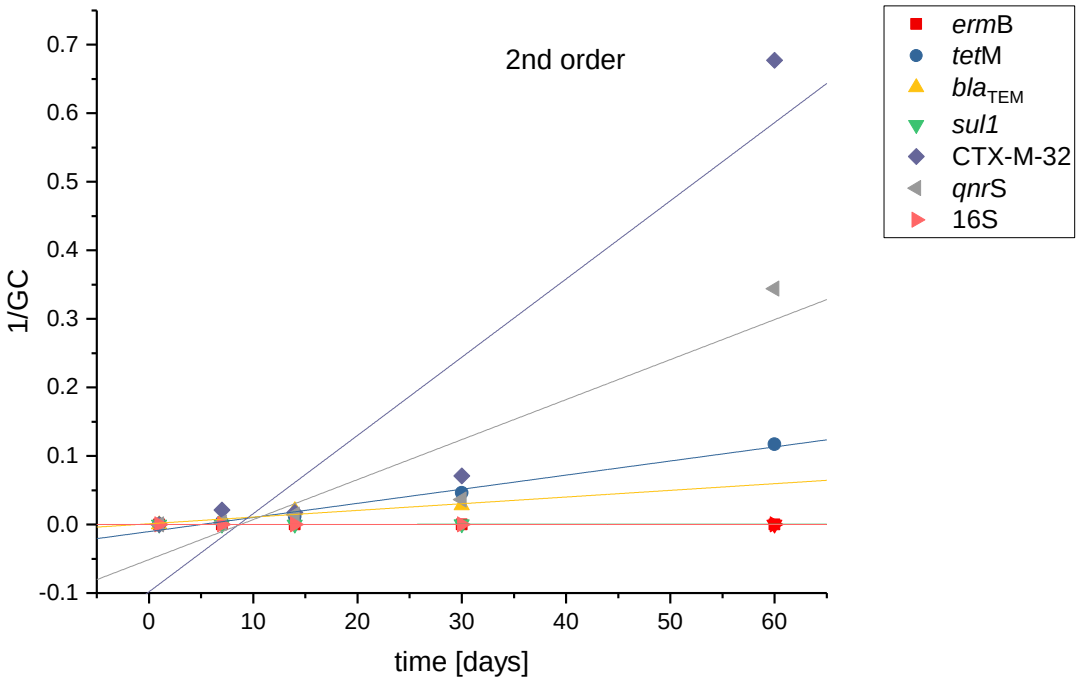
Table SB2: Settings of mass spectrometer used for quantification of ABs.

compound	RT [min]	polarity	pre-cursor ion	quanti-fier	CE* [V]	quali-fier	CE* [V]
ciprofloxacin	2.9	positive	332.1	231.0	40	245.0	24
ciprofloxacin-d_8	2.9	positive	340.2	235.0	44	296.3	20
erythromycin	3.5	positive	734.5	158.0	32	576.0	20
erythromycin-d_3	3.5	positive	737.5	161.0	32	579.4	20
penicillin V	4.0	positive	351.1	160.0	8	113.9	40
penicillin V-d_5	4.0	positive	356.1	160.0	12	229.0	16
roxithromycin	3.8	positive	837.5	158.0	36	679.4	20
roxithromycin-d_7	3.8	positive	844.6	158.0	36	686.5	20
sulfamethoxazole	4.5	positive	254.1	155.9	12	92.0	28
sulfamethoxazole-d_4	4.5	positive	258.1	156.0	36	151.1	12
tetracycline	3.0	positive	445.2	154.0	28	410.0	20

*collision energy

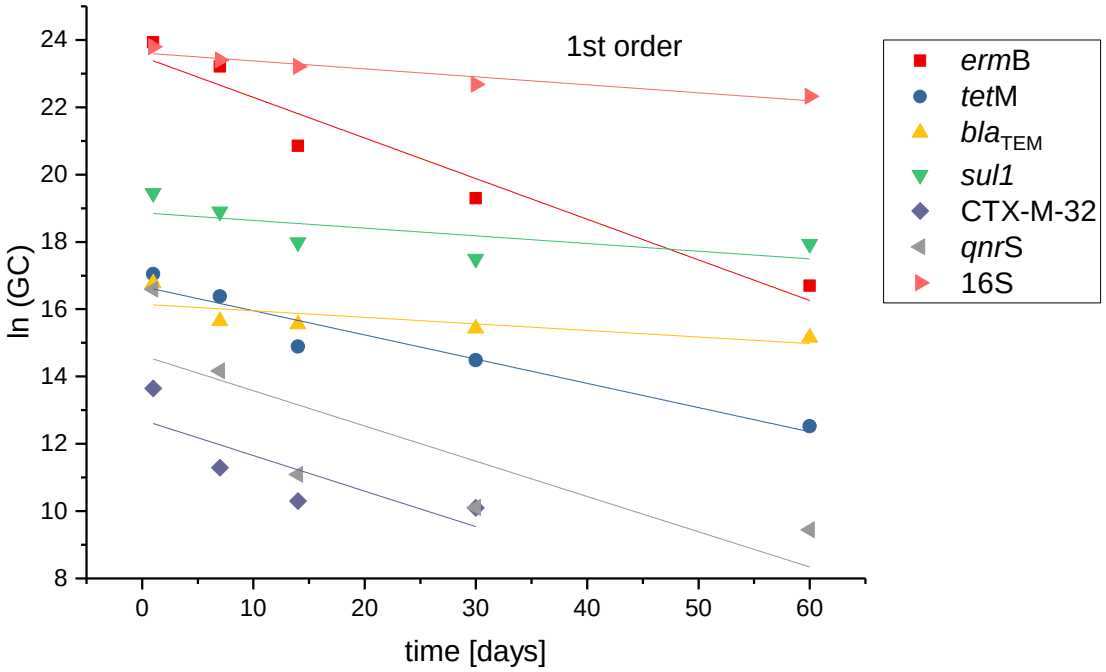
Table SB3: Source parameters used for antibiotics quantification.

gas temperature	350 ^{0}C
gas flow	8 L/min
nebulizer	25 psi
sheath gas temperature	400 ^{0}C
sheath gas flow	12 L/min
capillary	positive: 5000 V; negative 4500 V
nozzle voltage	positive 500 V; negative 300 V



--	second order decay - water						
Plot	*erm*B	*tet*M	*bla*$_{TEM}$	*sul1*	CTX-M-32	*qnr*S	16S
k [mL·GC^{-1}·d^{-1}]	6E-07 ± 7E-08	2E-03 ± 2E-04	1E-03 ± 2E-04	1E-05 ± 2E-06	1E-02 ± 3E-03	6E-03 ± 1E-03	4E-08 ± 1E-08
C_{max} [log GC/mL]	8.11	5.72	6.05	7.33	5.52	6.23	8.14
R^2	0.96	0.98	0.9	0.94	0.86	0.86	0.86
adjusted R^2	0.94	0.98	0.85	0.91	0.81	0.82	0.79

Figure SB1: Decay rate (*k*) for ARGs and 16S rDNA in water based on the rate law of a second order reaction.



	first order decay - sediments						
Plot	*erm*B	*tet*M	bla_{TEM}	*sul1*	CTX-M-32	*qnr*S	16S
k [d^{-1}]	-0.12 ± 0.02	-0.07 ± 0.01	-0.02 ± 0.01	-0.02 ± 0.01	-0.11 ± 0.05	-0.1 ± 0.04	-0.02 ± 0.00
C_{max} [log GC/g]	10.46	7.46	7.41	8.51	6.12	7.38	10.35
R^2	0.94	0.93	0.55	0.46	0.66	0.68	0.92
adjusted R^2	0.92	0.91	0.4	0.28	0.49	0.57	0.89

Figure SB2: Decay rate (*k*) for ARGs and 16S rDNA in sediment based on the rate law of a first order reaction.

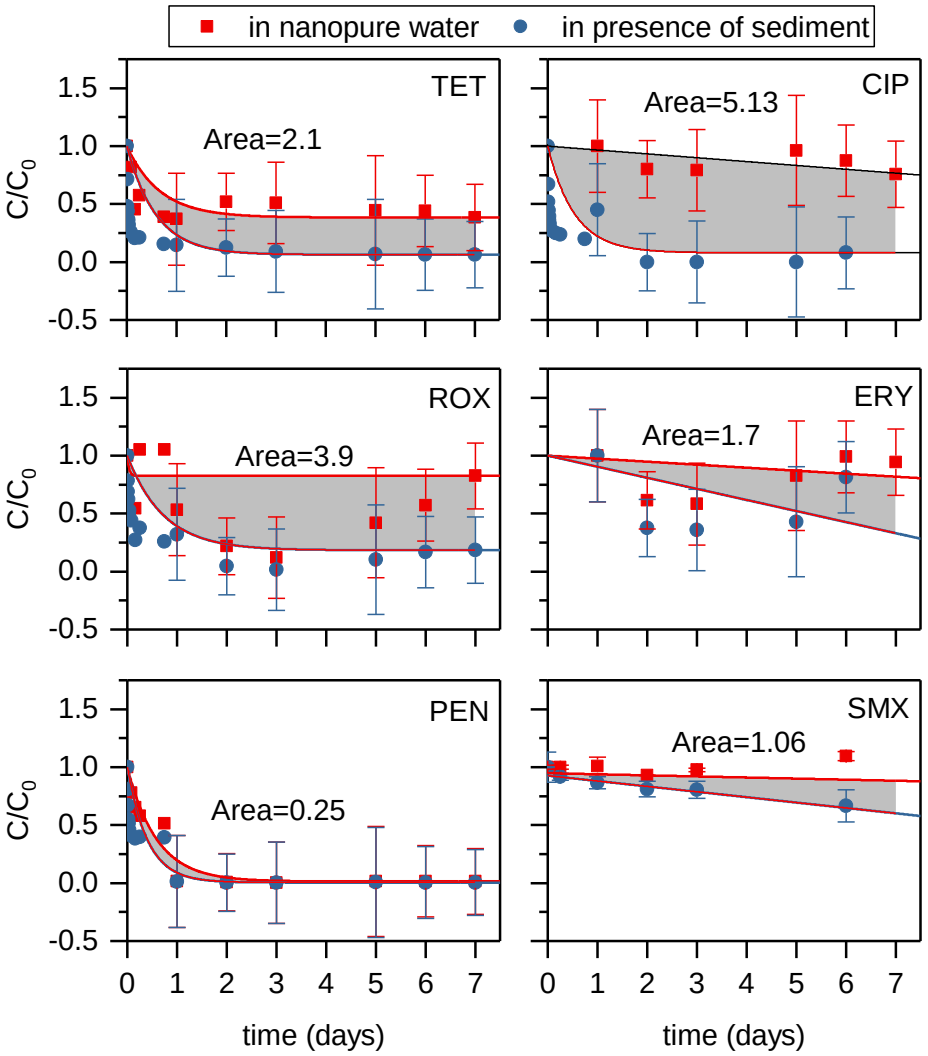
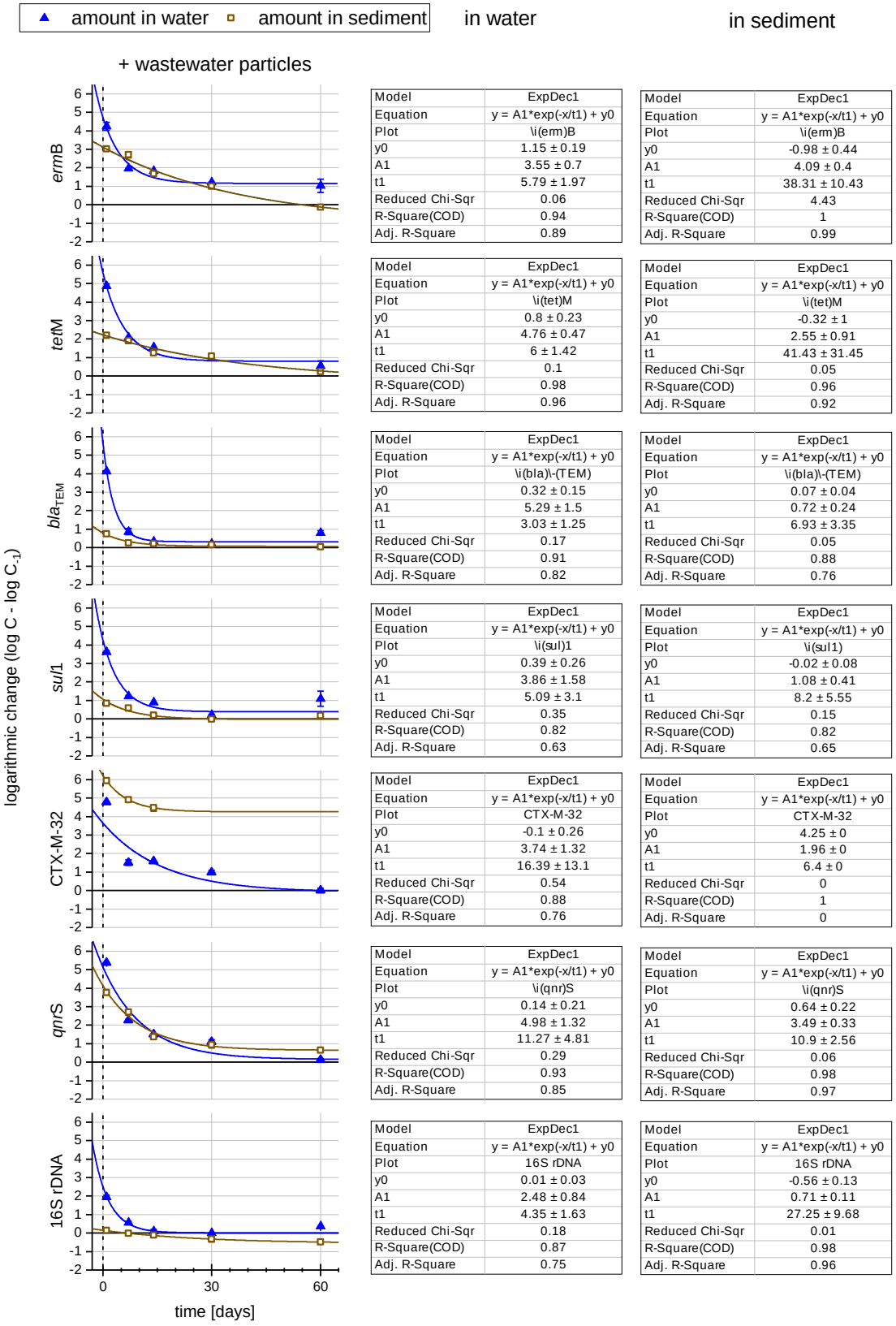


Figure SB3: Concentration ratio of antibiotics. Sorption and hydrolysis of antibiotics after spiking 1 µg of each compound to a final concentration of 5 µg/L. Integral analysis reveals difference between sorption and hydrolysis. Tests were run in triplicate. TET = tetracycline, CIP = ciprofloxacin, ROX = roxithromycin, ERY = erythromycin, PEN = penicillin V, SMX = sulfamethoxazole.



Model	ExpDec1
Equation	y = A1*exp(-x/t1) + y0
Plot	\i(erm)B
y0	1.15 ± 0.19
A1	3.55 ± 0.7
t1	5.79 ± 1.97
Reduced Chi-Sqr	0.06
R-Square(COD)	0.94
Adj. R-Square	0.89

Model	ExpDec1
Equation	y = A1*exp(-x/t1) + y0
Plot	\i(erm)B
y0	-0.98 ± 0.44
A1	4.09 ± 0.4
t1	38.31 ± 10.43
Reduced Chi-Sqr	4.43
R-Square(COD)	1
Adj. R-Square	0.99

Model	ExpDec1
Equation	y = A1*exp(-x/t1) + y0
Plot	\i(tet)M
y0	0.8 ± 0.23
A1	4.76 ± 0.47
t1	6 ± 1.42
Reduced Chi-Sqr	0.1
R-Square(COD)	0.98
Adj. R-Square	0.96

Model	ExpDec1
Equation	y = A1*exp(-x/t1) + y0
Plot	\i(tet)M
y0	-0.32 ± 1
A1	2.55 ± 0.91
t1	41.43 ± 31.45
Reduced Chi-Sqr	0.05
R-Square(COD)	0.96
Adj. R-Square	0.92

Model	ExpDec1
Equation	y = A1*exp(-x/t1) + y0
Plot	\i(bla)\-(TEM)
y0	0.32 ± 0.15
A1	5.29 ± 1.5
t1	3.03 ± 1.25
Reduced Chi-Sqr	0.17
R-Square(COD)	0.91
Adj. R-Square	0.82

Model	ExpDec1
Equation	y = A1*exp(-x/t1) + y0
Plot	\i(bla)\-(TEM)
y0	0.07 ± 0.04
A1	0.72 ± 0.24
t1	6.93 ± 3.35
Reduced Chi-Sqr	0.05
R-Square(COD)	0.88
Adj. R-Square	0.76

Model	ExpDec1
Equation	y = A1*exp(-x/t1) + y0
Plot	\i(sul)1
y0	0.39 ± 0.26
A1	3.86 ± 1.58
t1	5.09 ± 3.1
Reduced Chi-Sqr	0.35
R-Square(COD)	0.82
Adj. R-Square	0.63

Model	ExpDec1
Equation	y = A1*exp(-x/t1) + y0
Plot	\i(sul1)
y0	-0.02 ± 0.08
A1	1.08 ± 0.41
t1	8.2 ± 5.55
Reduced Chi-Sqr	0.15
R-Square(COD)	0.82
Adj. R-Square	0.65

Model	ExpDec1
Equation	y = A1*exp(-x/t1) + y0
Plot	CTX-M-32
y0	-0.1 ± 0.26
A1	3.74 ± 1.32
t1	16.39 ± 13.1
Reduced Chi-Sqr	0.54
R-Square(COD)	0.88
Adj. R-Square	0.76

Model	ExpDec1
Equation	y = A1*exp(-x/t1) + y0
Plot	CTX-M-32
y0	4.25 ± 0
A1	1.96 ± 0
t1	6.4 ± 0
Reduced Chi-Sqr	0
R-Square(COD)	1
Adj. R-Square	0

Model	ExpDec1
Equation	y = A1*exp(-x/t1) + y0
Plot	\i(qnr)S
y0	0.14 ± 0.21
A1	4.98 ± 1.32
t1	11.27 ± 4.81
Reduced Chi-Sqr	0.29
R-Square(COD)	0.93
Adj. R-Square	0.85

Model	ExpDec1
Equation	y = A1*exp(-x/t1) + y0
Plot	\i(qnr)S
y0	0.64 ± 0.22
A1	3.49 ± 0.33
t1	10.9 ± 2.56
Reduced Chi-Sqr	0.06
R-Square(COD)	0.98
Adj. R-Square	0.97

Model	ExpDec1
Equation	y = A1*exp(-x/t1) + y0
Plot	16S rDNA
y0	0.01 ± 0.03
A1	2.48 ± 0.84
t1	4.35 ± 1.63
Reduced Chi-Sqr	0.18
R-Square(COD)	0.87
Adj. R-Square	0.75

Model	ExpDec1
Equation	y = A1*exp(-x/t1) + y0
Plot	16S rDNA
y0	-0.56 ± 0.13
A1	0.71 ± 0.11
t1	27.25 ± 9.68
Reduced Chi-Sqr	0.01
R-Square(COD)	0.98
Adj. R-Square	0.96

Figure SB4: Non-linear fitting curves for ARGs and 16S rDNA in waters and sediments of batch reactors after addition of wastewater particles. Decay parameters, statistic evaluation, and used equation (tables).

4 Summary and conclusion

The aim of this dissertation was to investigate the relationship between wastewater discharge and ARG abundance in river sediments, and to assess which component of wastewater, namely residuals of ABs, or particulate matter, has the larger impact. This issue was addressed in two studies.

The field study (Chapter 2) showed the current state of an impacted river. The results indicated that particles, rather than residual ABs, were responsible for the increase of ARGs in the receiving river (0.5 – 2 log units in water and in sediment downstream compared to upstream of the discharge point). The study furthermore provided numbers for transported ARGs (10^8 – 10^{14} GC of ARGs per g of suspended solids). This highlighted the significance of the transport process as 480 t of TSS (dry mass) are discharged by the investigated WWTP per year. Through microbial partitioning of WWTP effluents, we could show that a large fraction of this is likely to settle via sedimentation. We further gave solid reasoning why sedimentation of wastewater particles can very well occur within a short stretch downstream of the discharge point (24 – 600 m, assuming a water depth of 2 m and a flow velocity of 0.1 – 1 m/s).

The second study (chapter 3), performed under controlled laboratory conditions, enabled us to differentiate between the effect of ABs, and particular matter, on the abundances of ARGs. A constant exposure to ABs at concentrations as high as 5 ug/L of individual compounds did not select for resistance. On a logarithmic scale, a clear change of ARG abundances was not detected. This was noticed for ARGs in sediments, despite strong sorption effects of ABs (15 – 37 µg/kg). Concurrently, evidence was provided that wastewater particles are indeed capable of raising ARG levels in sediments through sedimentation (by 1 – 4 log units). Wastewater particles were obtained by fractioning of wastewater (through filtering and sieving; $12 < d_P \leq 1'000$ µm fraction). This ruled out any other factors that might have affected ARG abundances, such as ABs or other substances not attached to particles or carried by the suspended fraction. Finally, increased levels of ARGs completely decayed in the water phase, and in sediments, which we attributed to the combination

of natural degradation and sedimentation in water, and natural degradation in sediments. We thereupon argued that the natural degradation can be associated with nutrient limitation as the introduced microorganisms are outcompeted by the natural microorganisms because of their differences in specializing to two distinct groups of carbon compounds.

The presented findings show that the particulate load from WWTP discharges plays a crucial role in the spread of ARGs in downstream sediments. We provided insight into the persistence of particles and attached ARB/ARGs, which helps to understand the dynamics of ARGs in wastewater-impacted rivers better. We conclude that the increased ARG abundances in downstream sediments are caused by the continuous import of particulate matter and associated bacteria from WWTPs. If this import is removed, ARGs are likely to decline. Furthermore, by removing particulate organic matter from WWTPs discharges, an essential carbon/energy source for heterotrophic bacteria is eliminated, which might lead to a microbial ecological state restoration.

However, this needs to be interpreted with great care as certain bacteria with clinical relevance may still thrive in surface waters not impacted by wastewaters, such as *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. While our results clearly show a strong decline of the imported bacteria, and ARGs, a certain persistence of FPB may be still a reality. Unlike obligate pathogenic bacteria, FPB have a highly flexible metabolism allowing them to adapt to a variety of carbon/energy sources, and nutrient levels. This means that they may very well persist in the river, long after removal of the external energy/carbon source from WWTP effluents. This problem is partly reflected in literature. With flexibility of FPB comes adjustability, and how well FPB adjust, has to be differentiated. Further research is necessary to grasp the diversity of this issue.

Yet still, if wastewater particles are not removed from WWTP discharges, numerous wastewater-borne microorganisms are directly transported into the sediment of the receiving river. HGT could lead to the exchange of ARGs between indigenous, environmental, and pathogenic bacteria. It poses a great risk to humans, if ARGs are

recruited into clinically relevant pathogens. We therefore underline the importance of WWTP disinfection to lower the discharge of resistant bacteria. More importantly, the focus should be directed towards membrane filtration techniques, such as ultra-filtration and microfiltration, to significantly reduce or even completely remove the particulate load, associated microorganisms, and ARGs.

Another concern is the environmental concentrations of ABs. It is widely accepted that residual ABs are a threat to public health and the environment because of their potential effect on resistance selection. We have tested a scenario, in which ABs exceeded the environmental concentrations in the field study by a factor of 150 (above the PNECs for resistance selection). Despite these conditions, no effect on the abundance of ARGs was observed. Further reduction of ABs, below the PNECs for resistance selection, might not help to prevent the spread of antibiotic resistance. However, we still suggest to monitor AB concentrations in WWTP effluents and to implement emission limits for ABs in conjunction with the PNECs for resistance selection suggested in literature and in this dissertation (subsection 1.2.5).

The effect of ABs must not be underestimated. There is sufficient evidence for the adverse effects of ABs and the role they play in the development of AMR in the environment. Besides, there may be other components present in wastewater that affect resistance development, such as the co-selection of ARGs by metals (not within the scope of this dissertation). However, it is highly unlikely that ARGs in river sediments can be multiplied by a noticeable factor, on a logarithmic scale, if ABs are present merely in the ng/L-range. Considering the mass transport of particles (approximately 1 t of dry mass per day), and associated ARB/ARGs (10^{14} – 10^{20} GC/t) (concentrations measured in the effluent of the investigated WWTP, Chapter 2), the inference that ARGs in sediments can be raised by several log units is not surprising. Removing the particles through microfiltration or ultrafiltration at the WWTP will eliminate this contribution entirely. As shown in Chapter 3, there is strong indication that WWTP impacted rivers can herewith be remediated.

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6 Appendix

6.1 List of publications

Brown P.C., Borowska E., Peschke, R., Schwartz T., Horn H (**2019**). Decay of elevated antibiotic resistance genes in natural river sediments after sedimentation of wastewater particles. Manuscript submitted for publication.

Brown P.C., Borowska E., Schwartz T., Horn H. (**2019**). Impact of the particulate matter from wastewater discharge on the abundance of antibiotic resistance genes and facultative pathogenic bacteria in downstream river sediments. Sci. Total Environ., 649, 1171 -1178, doi:10.1016/j.scitotenv.2018.08.394.

Könneke M, Schubert D.M., **Brown P.C.**, Hügler M., Standfest S., Schwander T., Schada von Borzykowski L., Erb T., Stahl D.A., Berg I.A. (**2014**). Ammonia-oxidizing archaea use the most energy-efficient aerobic pathway for CO_2 fixation. Proc. Natl. Acad. Sci. (USA) early edition, 111(22), 8239-8244, doi:10.1073/pnas.1402028111.

6.2 Verification of the contribution from the co-authors

Chapter 2

Title: Impact of the particulate matter from wastewater discharge on the abundance of antibiotic resistance genes and facultative pathogenic bacteria in downstream river sediments

Journal: Science of The Total Environment, (2019) 649, p. 1171-1178

Authors: Philip Clifford Brown, Ewa Borowska, Thomas Schwartz, Harald Horn

Position in the dissertation

The content of this paper has been included in Chapter 2

Contribution of Philip Clifford Brown (first author) (84 %)

- Conceived the concept
- Designed and conducted the experiments
- Analyzed and visualized the results
- Wrote the manuscript

Contribution of Ewa Borowska (second author) (8 %)

- Performed detection of antibiotics
- Wrote parts of the methodology
- Corrected the manuscript

Contribution of Thomas Schwartz (third author) (4 %)

- Designed the experiment
- Discussed the results
- Corrected the manuscript

Contribution of Harald Horn (fourth author) (4 %)

- Designed the experiment
- Discussed the results
- Corrected the manuscript

Signature of the authors:

Author	Signature
Philip Clifford Brown	
Ewa Borowska	
Thomas Schwartz	
Harald Horn	

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Chapter 3

Title: Decay of elevated antibiotic resistance genes in natural river sediments after sedimentation of wastewater particles"

Journal: Submitted for publication

Authors: Philip Clifford Brown, Ewa Borowska, Rafael Peschke, Thomas Schwartz, Harald Horn

Position in the dissertation

The content of this paper has been included in Chapter 3

Contribution of Philip Clifford Brown (first author) (80 %)

- Conceived the concept
- Designed and conducted the experiments
- Designed and constructed the batch reactors
- Analyzed and visualized the results
- Wrote the manuscript

Contribution of Ewa Borowska (second author) (7 %)

- Performed detection of antibiotics
- Wrote parts of the methodology
- Corrected the manuscript

Contribution of Rafael Peschke (third author) (2 %)

- Performed detection of antibiotics

Contribution of Thomas Schwartz (fourth author) (4 %)

- Designed the experiment
- Discussed the results
- Corrected the manuscript

Contribution of Harald Horn (fifth author) (7 %)

- Conceived the concept of the batch reactors
- Designed the experiment
- Discussed the results
- Corrected the manuscript

Signature of the authors:

Author	*Electronic Signature*
Philip Clifford Brown	
Ewa Borowska	
Rafael Peschke	
Thomas Schwartz	
Harald Horn	

7 List of abbreviations

AB	antibiotic
ABs	antibiotics
AMR	antimicrobial resistance
AOC	assimilable organic carbon
ARG	antibiotic resistance gene
ARGs	antibiotic resistance genes
ATC	anatomical therapeutic chemical
BOD	biological oxygen demand
bla$_{TEM}$	beta-lactamase gene
bp	base pairs
CE	collision energy
CTX-M-32	cefotaxime resistance
DDD	defined daily dose
DDDs	defined daily doses
DNA	deoxyribonucleic acid
DOC	dissolved organic carbon
*ecf*X	*Pseudomonas aeruginosa* specific gene
*erm*B	erythromycin resistance gene
ESBL	extended spectrum beta lactamase
FPB	facultative pathogenic bacteria
FQRP	fluoroquinolone resistant *Pseudomonas aeruginosa*
HGT	horizontal gene transfer
LC-MS/MS	liquid chromatography tandem-mass spectrometry
LOQ	limit of quantification
MIC	minimal inhibitory concentration
MRSA	methicillin resistant *Staphylococcus aureus*
MSC	minimal selective concentration
PABA	*para*-aminobenzoic acid
p.e.	population equivalents
PBPs	penicillin binding proteins
PBS	phosphate buffered saline

PNEC	predicted no effect concentration
PNECs	predicted no effect concentrations
PVC	polyvinyl chloride
*qnr*S	quinolone resistance gene
qPCR	quantitative polymerase chain reaction
rDNA	ribosomal deoxyribonucleic acid
rpm	rotations per minute
rRNA	ribosomal ribonucleic acid
*sec*E	*Acinetobacter baumannii* specific gene
*sul*1	sulfonamide resistance gene
*tet*M	tetracycline resistance gene
TSS	total suspended solids
VRE	vancomycin resistant *enterococci*
WHO	World Health Organization
WWTP	wastewater treatment plant
WWTPs	wastewater treatment plants

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