Ways of transmission of antibiotic resistant organisms in the environment and households

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Content

Abbreviations

°C	degree Celsius
µg	microgram
µL	microliter
µM	micromolar
aadA	aminoglycoside nucleotidyltransferase gene
ABR	antibiotic resistance
A. baumannii	*Acinetobacter baumannii*
AOB	activated oxygen bleach
approx.	approximately
ARG	antibiotic resistance genes
ATTC	American Type Culture Collection
BAC	Benzalkonium chloride
bla	β-lactamase gene
CAT	chloramphenicol acetyl-transferases
CCUG	Culture Collection University Of Gothenburg
CPO	carbapenemase-producing organisms
CTX-M	CefoTaXimase Munich
DNA	deoxyribonucleic acid
DW	dishwasher
e.g.	*exempli gratia* (for example)
ECDC	European Centre for Disease Prevention and Control
E. coli	*Escherichia coli*
EF	wastewater treatment plant effluent
erm	erythromycin resistance gene
ESBL	extended spectrum β-lactamase
et al.	*et alii* (and others)
EUCAST	European Committee on Antimicrobial Susceptibility Testing
GES	Guiana extended spectrum
h	hours
HGT	horizontal gene transfer
intI	integron-integrase gene
JPIAMR	Joint Programming Initiative on Antimicrobial Resistance

K. pneumoniae	*Klebsiella pneumoniae*
KPC	*Klebsiella pneumoniae* carbapenemase
LR	logarithmic reduction
M	molar
mcr	mobile colistin resistance gene
MDR	multi-drug resistance
$MgCl_2$	magnesium chloride
MGE	mobile genetic elements
min	minutes
mL	milliliters
MRSA	methicillin-resistant *Staphylococcus aureus*
NDM	New Delhi metallo-β-lactamase
QACs	quaternary ammonium compounds
qPCR	real-time quantitative polymerase-chain-reaction
PDR	pan-drug resistance
P. aeruginosa	*Pseudomonas aeruginosa*
rDNA	ribosomal DNA
RND	resistance-nodulation-cell-division
SD	shower drain
SS	sewage sludge
sul	sulfonamide resistance gene
tet	tetracycline resistance gene
TSA	tryptic soy agar
TSB	tryptic soy broth
TVC	total viable count
VIM	Verona integron-encoded metallo-β-lactamase
WHO	World Health Organization
WM	washing machine
WW	wastewater
WWTP	wastewater treatment plant
XDR	extensively drug resistance

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Abstract

The prevalence of antibiotic resistance genes (ARGs) and antibiotic resistant bacteria in the environment is steadily increasing, notably driven by the selective pressure induced by the extensive use of antibiotics in human and veterinary settings. Hence, this thesis aimed to possibly identify transfer routes by analysing the abundance of ARGs and antibiotic resistant bacteria in different environments and comparing their bacterial composition and resistomes. Moreover, elucidating the role of the domestic area and possible hotspots of antibiotic resistance in households was targeted.

The first part of this thesis focussed on the occurrence of β-lactamase, mobile colistin resistance and class 1 integron-integrase genes over a one-year-period in a wastewater treatment plant (WWTP). The negative correlation of the ARG abundance and temperature in wastewater and significant differences of the ARG level during warmer and colder seasons indicated a seasonal variation of ARGs. Although wastewater treatment reduced the ARG level and antibiotic resistant bacteria, resistance gene harbouring strains were still isolated from the final effluent. In the second part of the thesis, the abundance of the same genes as well as the prevalence of antibiotic resistant bacteria was assessed in shower drains, washing machines and dishwashers of private households. Additionally, the effect of laundering and automated dishwashing on antibiotic resistant strains was investigated. A significantly higher relative ARG abundance was observed in shower drains and phenotypic resistance of bacterial isolates correlated positively with genotypic resistance. Antibiotic resistant strains were significantly reduced during automated dishwashing as well as laundering tests and did not differ from susceptible strains. In the last part, the metagenomes of agricultural soil, wastewater treatment plant and household samples in the same geographical region were compared. The level of ARGs and mobile genetic elements was significantly lower in soils compared to WWTP and household samples with the highest level in wastewater. Both bacterial communities and resistomes were distinct for each environment revealing no correlation and only few overlaps of ARGs.

This thesis shows that ARGs might be transferred via domestic wastewater from households to WWTPs and subsequently could be released in the natural environment via the effluent. However, antibiotic resistances predominantly develop in each environment as caused by distinct environmental conditions, while a transfer between them is less likely. Thus, it might be more important to focus on the implementation of prevention measures in each individual environment than on the detection of the possible transfer of ARGs.

Zusammenfassung

Die Ausbreitung von Antibiotikaresistenzgenen (ARGs) und antibiotikaresistenten Bakterien in der Umwelt nimmt stetig zu, maßgeblich verursacht durch den Selektionsdruck ausgehend von dem intensiven Einsatz von Antibiotika in Human- und Veterinärmedizin. Daher war das Ziel dieser Arbeit potentielle Transferrouten zu identifizieren, indem das Auftreten von ARGs und resistenten Bakterienstämmen in verschiedenen Habitaten analysiert und deren bakterielle Gemeinschaft und Resistom verglichen wurden. Zudem wurden die Rolle des häuslichen Umfelds und mögliche Hotspots von Antibiotikaresistenzen im Haushalt bestimmt. Der erste Teil dieser Arbeit konzentrierte sich auf das Auftreten von β-Laktamase, mobilen Colistin-Resistenz und Klasse 1-Integron-Integrase Genen über einen einjährigen Zeitraum in einer Kläranlage. Die Korrelation der ARG-Häufigkeit und Abwasser-Temperatur sowie signifikante Unterschiede des ARG-Levels während wärmeren und kälteren Jahreszeiten deuteten auf einen saisonalen Effekt hin. Obwohl die Abwasserbehandlung die Menge von ARGs und antibiotikaresistenten Bakterien verringerte, wurden Bakterien mit diesen Genen aus dem gereinigten Abwasser isoliert. Im zweiten Teil wurde das Auftreten der gleichen Resistenzgene und antibiotikaresistenter Bakterien in Duschabflüssen, Waschmaschinen und Geschirrspülern aus Haushalten untersucht. Zusätzlich wurde der Einfluss des Waschprozesses und maschinellen Geschirrspülens auf antibiotikaresistente Bakterienstämme untersucht. Es zeigte sich eine signifikant höhere relative ARG-Häufigkeit in Duschabflüssen sowie eine Korrelation von phänotypischer und genotypischer Resistenz. Maschinelles Geschirrspülen und Waschen resultierte in signifikanten Reduktionen der antibiotikaresistenten Teststämme, die keine höhere Widerstandsfähigkeit als nicht resistente Bakterien aufwiesen. Im letzten Teil wurden die Metagenome von Boden-, Kläranlagen- und Haushaltsproben aus der gleichen Region verglichen. Im Vergleich zu Kläranlagen- und Haushaltsproben war die Anzahl von ARGs und mobilen genetischen Elementen in Böden signifikant geringer während die höchsten Werte im Abwasser nachgewiesen wurden. Sowohl die bakterielle Gemeinschaft als auch das Resistom waren für jedes Habitat spezifisch und es wurden nur wenige Gemeinsamkeiten deutlich. Diese Arbeit zeigt, dass ARGs über häusliche Abwässer in Kläranlagen gelangen und anschließend mit dem gereinigten Abwasser in die Umwelt freigesetzt werden könnten. Dennoch scheinen sich Resistenzen vorwiegend in jedem Habitat als Folge unterschiedlicher Umweltbedingungen zu entwickeln, während ein Transfer zwischen ihnen weniger wahrscheinlich ist. Daher könnte die Einführung von Präventionsmaßnahmen in jedem einzelnen Habitat von größerer Bedeutung sein als der Nachweis von möglichen Transfers von ARGs.

1 Introduction

1.1 Development of antibiotic resistance

The majority of antibiotics are produced by soil microorganisms as secondary metabolites which thus harbour antibiotic resistance mechanisms as self-defence [1,2]. Furthermore, the presence of antibiotic resistance (ABR) in environments prior to the therapeutic use of antibiotics or without anthropogenic impact has been shown, indicating that ABR occurs naturally [3,4]. However, the diversity and dissemination of antibiotic resistance determinants has dramatically increased since the introduction of penicillin, with the first penicillinase being reported in 1940 [5]. The main driver of the emergence of ABR is the selective pressure caused by overuse, agricultural use and inappropriate prescribing of antibiotics [6,7]. This selective pressure increases the development of ABR caused by spontaneous mutations and provides advantages for resistant species [8,9]. Moreover, it promotes the exchange of antibiotic resistance genes (ARGs) located on mobile genetic elements (MGEs) such as plasmids, integrons and transposons enabling vertical and horizontal gene transfer via conjugation, transformation and transduction [10,11].

The introduction of antibiotics provided an effective treatment option and thus resulted in extensive use without considering the development of resistances [7]. ABR represents an advantage to the resistant strain, if antibiotic selection pressure is present [12]. While antibiotics kill or inhibit susceptible bacteria, resistant strains are able to reproduce with less competition. Consequently, the increasing use of antibiotics leads to more selective pressure, favouring the growth of resistant bacteria. Hence, a direct connection between antibiotic consumption and the prevalence of ABR has been determined [13–15]. In addition, the unregulated sale in some countries results in cheap and easy to access antibiotics promoting overuse [7]. Klein *et al.* (2018) reported a global increase of antibiotic consumption of 65% between 2000 and 2015 [16]. Moreover, the consumption of the last-line antibiotics carbapenem and colistin increased, driven by the rising number of infections caused by antibiotic resistant bacteria [16]. Studies showed that the duration of therapy, active agent choice and treatment indications are inappropriate in 30 to 50% of the cases [6,17–19]. Antibiotics are often prescribed based on past experience and usually the pathogen causing the infection is not identified prior to antibiotic application, especially in outpatient cases [20]. This not only promotes the development of ABR, it also results in a delayed cure of the patient and in an unnecessary exposure of the human microbiota to an inappropriate antibiotic [21]. Non-prescribed antibiotic use occurs prevalent as well, most likely resulting in inappropriate drug and dosage choice.

The amount of non-prescribed use in Europe varied between 3% in northern Europe to 30% in eastern Europe, while in Asia around 58% and Africa even 100% of the antibiotics were used without prescription [22].

Besides the human consumption of antibiotics, the agricultural use for infection treatment and the use of low concentrations as growth promoter or infection prevention is also enhancing the antibiotic resistance crisis [23]. In Germany, approx. 85% of antibiotics are used in food-producing animals [15] and antibiotics such as penicillins, cephalosporins, tetracyclines, aminoglycosides, polymyxins and quinolones (Table 1) are used in both veterinary and human medicine [24]. Antibiotics have been used to control bacterial diseases in plant agriculture and in sub-therapeutic concentrations as feed additive in livestock since 1950 [25,26]. Livestock is treated with antibiotics to improve growth, overall health and quality of the products [7]. Although the use of antibiotics as growth promoter is prohibited in the EU since 2006 [27], it remains unregulated in many other countries [28]. While up to 90% of antibiotics used in food-producing animals are excreted and thus disseminated via fertilizer to soil or ground- and surface water [29], antibiotic resistant bacteria occur in meat as well [30]. Apart from livestock, antibiotic use in aquaculture is also increasing, resulting in the development of reservoirs for ARGs in fish and the aquatic environment [31].

Another factor which promotes the antibiotic resistance crisis is the lack of development of new antibiotics, which decreased to six newly developed agents between 2010 and 2014 compared to 19 between 1980 and 1984 [6]. Antibacterial research is considered not profitable enough and thus only three of the 18 major suppliers of antibiotics remain active in the field of antibacterial drug discovery [32,33]. Moreover, the inevitable risk of the emergence of resistances to newly developed agents further discourages pharmaceutical companies [6]. Although the development of antibiotics increased in the past few years again, these agents are not targeting strains with resistances to nearly all antibiotics, even though urgently needed [33]. In Europe alone, 33,000 people die each year from infections caused by antibiotic resistant bacteria and the percentages of resistant Gram-negative pathogens increased significantly between 2012 and 2015 [34]. Furthermore, the number of strains with combined resistances to various antibiotics is increasing as well, resulting in limited treatment options.

Table 1. Overview of antibiotic classes, examples for antibiotics and their mode of action.

classes of antibiotics	examples	mode of action
β-lactams	penicillins (piperacillin), cephalosporins (cefotaxime), carbapenems (imipenem)	inhibition of cell wall synthesis [35]
Aminoglycosides	streptomycin, gentamicin	inhibition of protein synthesis [36]
Polymyxins	colistin	unclear, destroys outer membrane [37]
Quinolones	ciprofloxacin, norfloxacin	inhibition of DNA replication [38]
Tetracyclines	doxycyclin, demeclocyclin	inhibition of protein synthesis [39]

1.1.1 Resistance mechanisms

In principle, bacteria can be intrinsically resistant or acquire resistances via several mechanisms. A natural or intrinsic resistance is based on a pre-existing insensitivity to an antibiotic due to certain genetic characteristics [40]. For example, vancomycin inhibits the peptidoglycan crosslinking of the cell wall in Gram-positive bacteria while Gram-negative bacteria are intrinsically resistant due to their outer membrane, which vancomycin cannot pass [41]. In contrast, acquired resistance results from spontaneous mutations with subsequent selection under the influence of antibiotic therapy or from the transfer of resistance determinants between bacteria [11,41].

Basically, there are three main strategies based on biochemical mechanisms conferring antibiotic resistance:

a. Protection of the target
b. Modification or inactivation of the antibiotic
c. Efflux pumps and reduced penetration

a. Protection of the target

A common resistance mechanism is the prevention of the action of the antibiotic by interfering with its target site. Modifications of the target structure without loss of function can influence the binding of the antibiotic. These modifications can be caused by mutations, changing the structure of the enzymes to prevent efficient binding of the antibiotic [41]. In addition, the incorporation of mobile DNA elements from the environment by transformation can lead to the

synthesis of modified enzymes with a different structure than the target to take over its tasks [11]. Another mechanism is the synthesis of an alternative target structure. The cell synthesizes an enzyme that is not inhibited by the antibiotic and either replaces or produces the original target structure. The overproduction of the target results in the bypass of the effect of the antibiotic and the cell's metabolism can be maintained [41,42]. For example, quinolones inhibit the DNA-synthesis by interfering with the DNA gyrase [43]. Quinolone resistance in *Enterobacteriaceae* is induced by the mutation of the *gyrA* gene leading to reduced binding of the antibiotic to the active site [11]. Another example for target protection is *tet*(O)- and *tet*(M)-mediated tetracycline resistance. The genes confer the production of proteins that bind to the ribosome and remove tetracycline from its binding site [42].

b. Modification or inactivation of the antibiotic

The inactivation of the antibiotic itself is caused by specific enzymes, inducing the hydrolysis or modification of the chemical structure. In case of aminoglycoside and chloramphenicol resistance, the mechanisms are based on chemical alterations [42]. The aminoglycoside modifying enzymes are nucleotidyl-transferases, acetyl-transferases or phosphoryl-transferases, which modify the molecular structure of the antibiotic by binding to its adenylyl, phosphoryl, or acetyl groups, respectively. This modification usually results in a reduced affinity of the antibiotic to the modified molecule and it thus becomes ineffective [11,41]. This has been observed inter alia in many species of the *Enterobacteriaceae*, the genus *Mycobacterium* and in *Pseudomonas aeruginosa* [44]. Chloramphenicol acetyl-transferases (CAT) acetylate the hydroxyl group of the antibiotic resulting in a modified chloramphenicol, which is unable to bind to the ribosome. The *cat* genes are usually located on MGEs and occur in Gram-positive and Gram-negative bacteria [42]. In contrast, the most common resistance mechanism to β-lactams in Gram-negative bacteria is the hydrolysis by β-lactamases, leading to the destruction of the antibacterial agent. The enzymes degrade the ester or amide bond of the β-lactam ring by the free hydroxyl group of a serine residue, or less frequently by a zinc ion, at the active site of the enzyme [45]. More than 4,000 β-lactamases have been described so far [46] hydrolysing penicillins, monobactams, cephalosporins and carbapenems.

c. Efflux pumps and reduced penetration

The reduced cellular uptake of the antibiotic is regulated by the permeability of the cell membrane. To get to the target site, antibiotics have to penetrate the cytoplasmic membrane of bacteria to induce an antibacterial effect. Compared to Gram-positive species, cells of Gram-negative bacteria are less permeable due to their additional outer membrane. However, small hydrophilic agents such as β-lactams or tetracyclines can enter the bacterial cell through porin channels in the outer membrane [41,42]. The penetration of antibiotics can be limited by reduced porin expression, replacement of the porin channels or changes in their function [42]. For example, the loss of the porin channel D2 results in imipenem resistance in *P. aeruginosa* [47]. Furthermore, antibiotic-specific efflux pumps can actively reduce the intracellular concentration of the antibiotic by transporting it to the outside of the cell. Efflux systems are usually able to transport a wide range of different antibiotics which are therefore known as multi-drug resistance (MDR) efflux pumps [11,41]. MDR efflux pumps of the resistance-nodulation-cell-division (RND) family especially occur in clinically relevant Gram-negative bacteria and can be located on MGEs. An overexpression of these efflux pumps results in high-level resistance to a wide array of antibiotics [41,42,48].

1.1.2 Mobile genetic elements and gene transfer

Genes conferring resistance to antibiotics are commonly located on MGEs such as plasmids, integrons and transposons enabling vertical and horizontal gene transfer via conjugation, transformation and transduction [10,11]. While gene transfer only occurs vertically between generations, horizontal gene transfer (HGT) describes the exchange of genes between unrelated organisms and occurs inter- and even intra-species [10,49], enabling a rapid spread of resistances.

Plasmids are self-transmissible, double-stranded circular DNA molecules [50] that exist and replicate separately from the bacterial chromosome [11,51]. Plasmids harbouring ARGs can confer resistances to all classes of antibiotics, and many are conjugative thus able to promote cell-to-cell DNA transfer [49,51]. Transposons are short DNA sequences, which are able to move inter- and intra-molecular and can change their position by moving from one plasmid to another plasmid or from plasmids to chromosomal DNA and vice versa [11,51]. Furthermore, transposons can be conjugative as well, being similar in their structure to plasmids but do not replicate [52]. In contrast, integrons are able to capture gene cassettes via site-specific

recombination. They contain an integrase gene (*intI*), a specific recombination site and a promoter [11], but do not include genes encoding proteins that induce their movement. Thus, they are usually located within plasmids or transposons, since they are not self-movable [49,53].

Conjugation is the most important form of DNA transfer between bacteria, where genetic properties are exchanged via direct cell contact. In most cases, these are conjugative plasmids on which several resistance determinants are located. Transduction is the transfer of genes by bacteriophages. The resistance determinants (bacterial DNA) are incorporated into the phage in addition to the phage's own DNA. This enables the transfer of chromosomal or extrachromosomal DNA to related bacterial strains. In contrast, transformation describes the uptake of free DNA from the environment and its incorporation into the chromosomal DNA using specific enzymes [49,54,55].

The HGT of plasmids harbouring ARGs plays a key role in the steadily increasing dissemination of ABR [56]. Especially genes encoding β-lactamases (*bla* genes) such as carbapenemases, AmpC-β-lactamases, extended spectrum β-lactamases (ESBL) or mobile colistin resistance (*mcr* genes) are located on conjugative plasmids [57–59] or within integrons or transposons [60]. For example, the widespread of the carbapenemase OXA-48 was associated with a conjugative plasmid that disseminates between *Enterobacteriaceae* in high rates [61]. The transfer of plasmids has led to the worldwide prevalence of ARGs encoding resistance to many antibiotics, especially in *Enterobacteriaceae* [55]. Resistance plasmids such as IncF, IncI, IncA/C and IncH occur most prevalent in animal, human and environmental isolates in Europe and confer resistances to inter alia carbapenems, extended spectrum β-lactams, cephalosporins, aminoglycosides, quinolones and sulfonamides [62]. In this regard, the increasing spread of plasmids harbouring genes encoding carbapenemases [63] and colistin resistance [64,65] is particularly concerning and might lead to truly pan-drug resistant pathogens, meaning a resistance to all approved and used antibiotics [66]. Besides plasmids, many ARGs are located on integrons and class 1, 2 and 3 integrons are multi-resistant integrons [60] with class 1 integrons occurring most frequently in Gram-negative pathogens such as *Escherichia*, *Klebsiella*, *Pseudomonas*, *Acinetobacter* or *Stenotrophomonas* [53,60]. Gene cassettes can be captured by integrons and include ARGs conferring resistance to inter alia β-lactams, quinolones, aminoglycosides, trimethoprim, chloramphenicol and macrolides [67]. The carbapenemases VIM (Verona integron-encoded metallo-β-lactamase) and GES (Guiana extended-spectrum) have been detected in *Pseudomonas* spp. and *Enterobacteriaceae* on class 1 integrons [58,68]. In conclusion, HGT of resistance genes is considered a major driver

of the increasing spread of ABR in Gram-negative bacteria and could lead to infections caused by pan-drug resistant pathogens without any treatment options left.

1.2 Clinically relevant resistances

The World Health Organization (WHO) has categorized antimicrobials used in human medicine according to their importance [69]. Among others, 3rd generation cephalosporins, carbapenems and colistin are critically important antimicrobials for human health, with colistin even being a reserve antibiotic that should only be used if no other treatment options are left [70]. Resistances to β-lactams and colistin are especially prevalent in Gram-negative bacteria such as *Enterobacteriaceae*, *Pseudomonas aeruginosa* or *Acinetobacter baumannii* [71,72] and can result in serious infections with limited therapeutic options [59,73,74].

1.2.1 Multi-resistant Gram-negative bacteria

Multi-drug resistance in Gram-negative bacteria is a major public health concern since microorganisms with resistances to a large number of different antibiotic classes represent a particular challenge in the treatment of diseases. Whereas Gram-positive pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA) were previously of particular importance, nosocomial infections with multi-resistant Gram-negatives have increased worldwide in recent years [66,75]. Among the clinically relevant Gram-negative bacteria are *P. aeruginosa*, *Acinetobacter* spp. as well as species of the *Enterobacteriaceae* family such as *E. coli*, *Klebsiella* and *Enterobacter* [12]. The outer membrane of Gram-negative bacteria serves as a permeability barrier, resulting in intrinsic resistance to antibiotics such as vancomycin [41]. However, acquired resistances are of greater concern, and evidence indicates that ARGs and related MGEs are often accumulated on so-called multi-resistance regions in Gram-negative bacteria, especially *Enterobacteriaceae* [76]. Thus, HGT of these multi-resistance regions enables the rapid transfer of several ARGs. The terms multi-drug resistance (MDR), extensively drug resistance (XDR) and pan-drug resistance (PDR) are defined based on international recommendations [66]. MDR describes strains with resistances to at least one agent in three or more categories of antibiotics while XDR refers to strains that remain susceptible to only one or two categories. In contrast, PDR is defined as resistance to all agents in all antimicrobial categories, thus no available antibiotic has any activity against PDR bacteria. The definitions are based on the tested strains being resistant, intermediate or non-susceptible to the antibiotics

based on clinical breakpoints of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [77]. Gram-negative bacteria, particularly *Enterobacteriaceae*, cause urinary tract infections, respiratory infections, gastrointestinal infections or sepsis, both hospital- and community-acquired [12,78]. The treatment of these infections is severely limited if strains are multi-resistant, especially regarding immunocompromised patients, and multi-resistance has led to increasing morbidity and mortality rates worldwide [79].

While the European Centre for Disease Prevention and Control (ECDC) reported a significant increase of resistances to 3rd generation cephalosporins in combination with fluoroquinolone and aminoglycoside resistance or ESBL-production in *E. coli* and *K. pneumoniae* in Europe, the prevalence of MRSA has continuously decreased. Although carbapenem resistance remained rare in *E. coli*, carbapenem- and multi-resistance significantly increased in the species *Acinetobacter* spp. and *P. aeruginosa* [34], most likely due to the increasing use of carbapenems caused by resistances to other β-lactams (such as 3rd generation cephalosporins). The main mechanism of β-lactam resistance in Gram-negative bacteria is the production of β-lactamases [68,80]. The increasing use of carbapenems to treat infections caused by MDR Gram-negatives has led to the rise in carbapenemase-producing bacteria, and thus colistin was used as an option of last resort [59]. Unfortunately, mobile colistin resistance was first reported in 2015 with increasing prevalence ever since [59,64]. Resistances to carbapenems and colistin are most concerning. Carbapenem resistance is usually caused by the production of carbapenemases which hydrolyse carbapenems [81]. Infections caused by carbapenemase-producing organisms (CPO) have high mortality rates and since many carbapenemase genes are located on MGEs, a widespread occurrence can be observed [72,73]. Even though most reports of CPO-infections originate in the clinical sector, the number of community-acquired infections with CPO is increasing as well, which is more difficult to monitor let alone control [68]. Although mobile colistin resistance mainly occurs in the environment [82–84], nosocomial infections caused by colistin resistant strains have been reported as well [85–87].

Especially worrisome is the high amount of isolates with polymyxin resistance (colistin) with high percentages of carbapenem resistance in European countries [34]. In addition, studies revealed the co-occurrence of carbapenemase or ESBL genes with *mcr* genes in clinical isolates [87–89]. This development shows that Gram-negative bacteria seem to be highly adaptable and that resistances can develop rapidly.

1.2.2 β-lactamases

β-lactamases confer resistance to some of the most important antibiotics and pose a significant threat to human health. The enzymes are produced by bacteria and are able to hydrolyse the β-lactam ring of β-lactam antibiotics, resulting in their inactivation [45]. A total of 4,944 different enzymes is known so far [46], usually classified based on amino acid similarities (Ambler classification) or functional similarities (Bush-Jacoby-Medeiros classification) [90,91]. The functional scheme classifies the different β-lactamases in four groups with multiple subgroups considering β-lactamase substrates and inhibitors (Table 2). The first group contains cephalosporinases with poor inhibition by clavulanic acid, the second group all serine-β-lactamases inhibited by active site-directed inhibitors, followed by the third group of the metallo-β-lactamases with poor inhibition by typical β-lactamase inhibitors and the last group of penicillinases without inhibition by clavulanic acid (not shown in Table 2). In contrast, the Ambler classification divides the β-lactamases into four major groups based on molecular properties, without considering enzymatic activity. The classes A, C and D comprise distinct groups of serine-β-lactamases, which catalyse the hydrolysis by a catalytic serine residue while class B metallo-β-lactamases utilize zinc ions to disrupt the β-lactam ring [45]. The Class C and D β-lactamases are also known as AmpC- and OXA-β-lactamases, respectively.

Table 2. Classification of β-lactamases.

Ambler class	**Bush-Jacoby-Medeiros group**	**hydrolytic activity**	**examples targeted in this study**
A	2a, 2b, 2be, 2br, 2c, 2e, 2f	penicillins, 1st to 3rd generation cephalosporins, carbapenems	ESBL (CTX-M-1, CTX-M-9) carbapenemases (GES, KPC)
B	3	all β-lactams except monobactams	metallo-β-lactamases (VIM, NDM)
C	1	all β-lactams except carbapenems	AmpC-β-lactamases (CMY-2, FOX, ACT, MIR, DHA)
D	2d	penicillins (oxacillins), cephalosporins, partly carbapenems	OXA-β-Lactamases (OXA-48-, OXA-23- and OXA-58-like)

AmpC-β-lactamases are able to hydrolyse all β-lactams, especially cephalosporins but no carbapenems [92] whereas OXA-β-lactamases confer resistances to penicillins, oxacillins and, in some instances, even carbapenems [93]. In Gram-negative bacteria, *ampC* genes occur frequently chromosomally encoded, and an overproduction of AmpC-β-lactamases can result in resistances to 3rd generation cephalosporins and monobactams [94]. In contrast, genes such as CMY-2, FOX, ACT, MIR and DHA are located on transferable plasmids enabling their transfer among bacteria and thus have been detected worldwide [92,95]. OXA-β-lactamases are located on plasmids and their hydrolytic activity used to be limited to penicillins, followed by cephalosporins. However, new variants conferring resistance to carbapenems such as class D carbapenemases OXA-48, OXA-23 or OXA-58 were discovered and strongly affect the treatment of infections caused by Gram-negative bacteria [93,96,97]. Although OXA genes dominate in *Acinetobacter* spp., their detection in *Enterobacteriaceae* or *Pseudomonas* spp. is of great concern, since these bacteria can cause serious infections [73,93,98]. Carbapenems are used for the treatment of serious infections caused by ESBL-producing organisms and the emergence of carbapenem-resistant Gram-negatives significantly contributes to patient mortality and morbidity [73,81]. Carbapenemases are often located on MGEs, enabling their dissemination among bacteria and include enzymes of the molecular classes A, B and D [73,99]. Among others, the GES and KPC (*Klebsiella pneumoniae* carbapenemase) genes belong to the Class A β-lactamases, with KPC being the most widespread carbapenemase with the highest clinical relevance [73,99,100]. While GES is located within a class 1 integron on a transferable plasmid [101], KPC is plasmid-mediated [102]. Both carbapenemase genes have been identified in strains of *Enterobacteriaceae*, *P. aeruginosa* and *A. baumannii* [81,99,101]. The carbapenemases of class B are the metallo-β-lactamases. Especially mobile variants such as VIM located within a class 1 integron and NDM (New Delhi metallo-β-lactamase) encoded on a plasmid with various other ARGs [103] occur predominantly worldwide in *Enterobacteriaceae*, *P. aeruginosa* and *A. baumannii* [73,104]. The extended-spectrum β-lactamases (ESBL) of the molecular class A can be chromosomal or plasmid-encoded and confer resistances to penicillins, 1st to 3rd generation cephalosporins and aztreonam [74]. In this regard, the plasmid-mediated CTX-M genes are most prevalent in both clinical and community-associated environments [74,105,106]. Furthermore, the CTX-M genes have been mobilized with a much higher frequency compared to other ESBLs from the chromosomes of *Kluyvera* spp. [107], which might explain their concerning widespread. CTX-M-β-lactamases have been identified particularly in *Enterobacteriaceae* such as *E. coli* or *K. pneumoniae* and with increasing prevalence in *P. aeruginosa* and *A. baumannii* as well [108]. Overall, β-

lactamases are evolving steadily and while carbapenems used to be the agent of choice for the treatment of infections caused by β-lactam resistant bacteria, the prevalence of carbapenemases is increasing as well [73,75].

1.2.3 Mobile colistin resistance

Although colistin was introduced in 1950, it was used rather limited in human medicine due to its nephrotoxicity and thus colistin was mainly applied in agriculture [59,64]. However, the global widespread of carbapenemase-producing bacteria has led to the increasing use of colistin as an antibiotic of last resort, resulting in the development of mobile colistin resistance (*mcr*) [59,109]. Before the first detection of *mcr* by Liu *et al.* (2016) in China [64], only chromosomally encoded colistin resistance based on decreased binding to the outer membrane caused by mutations of outer membrane proteins, overexpression of efflux pumps or the presence of a capsule was known [59,71]. Since then, many studies reported the detection of *mcr-1* and other variants across the world in clinical isolates [86,110–113]. However, *mcr*-positive isolates still occur predominantly in strains with environmental origin such as livestock [82–84] or wastewater treatment plants [114,115], leading to the assumption that the gene originates from the veterinary sector [64,116]. The *mcr* genes are located on transferable plasmids and have been detected in *Enterobacteriaceae*, with particularly high prevalence in *E. coli* [82].

The co-occurrence of *mcr* and ESBL or carbapenemase genes is of great concern. In Switzerland, the production of both *mcr-1* and a CTX-M-β-lactamase in an *E. coli* isolate from a patient was reported [117], and there is evidence that *mcr-1* and ESBL genes can be located on the same plasmid [85]. Furthermore, co-occurrence of carbapenemases with *mcr-1* was observed in both clinical and environmental isolates [89,118–121]. However, most *mcr*-producing isolates are susceptible to β-lactams such as carbapenems or cephalosporins [111,119,122] and despite its worldwide detection, the prevalence of *mcr* genes remains comparably low [59,123].

1.3 Antibiotic resistance beyond the clinic

Even though ABR in clinically relevant bacteria is a serious public health concern, ABR is ancient and occurs naturally in various environments independent of the use and dissemination of antibiotics [3,4]. However, the prevalence of ARGs and antibiotic resistant bacteria in the

environment is steadily increasing, notably driven by the selective pressure induced by the extensive use of antibiotics in human and veterinary settings. Most antibiotics are not completely metabolized and might pass the wastewater treatment [2]. Hence, ARGs, resistant bacteria and antibiotics from anthropogenic sources are discharged in the environment resulting in the proliferation of antibiotic resistant bacteria [124,125]. Furthermore, antibiotics used in agriculture can be transmitted to soils either directly or during fertilization and subsequently end up in ground- or surface water [25,126]. Especially environments with a high abundance and diversity of ARGs might act as potential sources for the transfer of ARGs to human pathogens.

1.3.1 Antibiotic resistance in the natural environment

Despite of the wide range of studies dealing with ABR in the clinical setting, current data on the occurrence, distribution and types of ARGs and antibiotic resistant bacteria in the natural environment and its importance for humans still leaves knowledge gaps that need to be filled. The Joint Programming Initiative on Antimicrobial Resistance (JPIAMR) determined critical knowledge gaps regarding ABR. These include the determination of the impact of different sources of antibiotics and resistant bacteria into the environment, the importance of the environment and anthropogenic sources on the evolution of ABR, the consequences of human and animal exposure to antibiotic resistant environmental bacteria and the efficacy of measures to reduce ABR dissemination [127]. The natural environment has been determined as a potential transmission route for antibiotic resistant bacteria [8,9,125], and studies revealed that many ARGs originate from environmental bacteria [10,128].

Soil

Soil represents a natural reservoir of ABR [1,129,130] since most antibiotics are produced by soil bacteria to eliminate competing species, which thus harbour the corresponding ARGs [131,132]. Since up to 90% of the antibiotics used in food-producing animals are excreted [29], the diverse microbial community of soil can be exposed to antibiotics via agriculture [133], surface water or groundwater [134,135], which might impact the soil resistome. Therefore, significant increases of ARGs in soils since 1940 have been observed [129,136]. Furthermore, studies showed that manure application increases the ARG abundance in soil [137,138]. McKinney *et al.* (2018) determined higher ARG levels in soils with higher manure application

rates, indicating that either higher selective pressure due to contained antibiotic residues or higher amounts of ARGs and antibiotic resistant bacteria in the manure might raise the ARG abundance [137]. However, even manure without the impact of antibiotic treatment resulted in an increase of resident soil bacteria such as *Pseudomonas* spp. harbouring β-lactamases [126] substantially changing the natural microbiome of soil. This is most likely caused by the high abundance of ARGs and antibiotic resistant bacteria in manure even in animals that never received antibiotics [139]. A study of agricultural, urban and natural soil showed that more than 80% of the isolates were resistant to up to 23 antibiotics [140]. Furthermore, ARGs and antibiotic resistant bacteria are highly prevalent in wastewater treatment plants and even after treatment, the effluent still contains ARGs and resistant bacteria [141,142] and thus are believed to be a major anthropogenic source of ARGs in soils [143].

Apart from anthropogenic impacts, the intrinsic resistome of soil can also act as a source of ABR that can be transferred to human pathogens since studies revealed evidence for the exchange of ARGs between human and soil microbiota [29,128,144]. However, Forsberg *et al.* (2014) determined a low mobility of the soil resistome, which possibly accounts for the low number of shared ARGs between soil and human pathogens [128]. A transfer of ABR from soil to humans might occur via various routes, including the food chain, water, air or direct contact [139,145]. Since antibiotic resistant bacteria can be transferred from soil to plants [146], the consumption of food might expose the human microbiome to ABR. Studies revealed the presence of antibiotic resistant bacteria on raw vegetables [145,147], which might originate from the agricultural soil at harvest. Water is the main pathway of contaminants in soil, and Lüneberg *et al.* (2018) showed that the flow paths of water are hotspots for ARGs and antibiotics, revealing a possible transfer route to groundwater [135]. Furthermore, wind and surface water can also facilitate the dissemination of ABR to other environments [139]. Although determining the relationship of clinical pathogens and soil bacteria is difficult, the possibility of a transfer remains and in some instances was already proven.

Aquatic environments

The prevalence of antibiotic resistant species is based on different influencing factors. The first to mention is the intrinsic resistome, since many waterborne bacteria possess intrinsic resistances. For example, species like *P. aeruginosa*, *P. fluorescens*, *Aeromonas* spp. or *Enterobacteriaceae* like *Morganella morganii*, *Enterobacter* spp. and *Hafnia alvei* harbour

chromosomally encoded AmpC-β-lactamases [148]. Additionally, resistance mechanisms such as efflux pumps are also frequently present in these species [47,48].

Resistant bacteria of anthropogenic origin such as wastewater treatment plants (WWTPs) are disseminated into the surrounding area, hence inducing selective pressure on environmental bacteria or even the transfer of ARGs to waterborne species. The majority of antibiotics are not completely metabolized and thus are released into the natural habitats including the receiving surface waters of WWTPs [149,150] and might even reach ground- or drinking water [151]. Both carbapenemase- and ESBL-producing *Enterobacteriaceae* have been identified in WWTP effluents and rivers [150,152]. Amos *et al.* (2014) reported a strong increase of *bla*$_{\text{CTX-M-15}}$ downstream of a WWTP in UK, and Mokracka *et al.* (2012) determined a higher frequency of isolates resistant to 1st to 3rd generation cephalosporins in the effluent compared to wastewater or sewage sludge [150,153]. Furthermore, Sabri *et al.* (2020) showed that the WWTP effluent contributed significantly to the concentration of ARGs and class 1 integrons in the receiving river, and genes released via the effluent such as *sul1, tet*(W) or *ermB* were detectable for 20 km in the river and its sediment [142]. In aquaculture, antibiotics are added directly to the water or are applied as medicated feed, exposing the aquatic microbiome to these substances [31,154]. Critically important antibiotics to human medicine such as amoxicillin or streptomycin are used in aquaculture as well [31], and studies report the widespread of ABR [154,155]. In aquatic environments, bacteria frequently form biofilms, facilitating their survival and persistence to antibiotics [156]. Moreover, biofilms favour the acquisition and spread of ARGs via HGT due to the close contact between the cells in the matrix of the biofilm [156,157]. Thus, aquatic environments represent a diverse community with a high prevalence of ABR and direct contact to other natural settings and humans [148].

1.3.2 Antibiotic resistance in wastewater treatment plants

WWTPs have been identified as hotspots for ABR and as a setting promoting HGT between bacteria [143,158–160]. Especially in the influent of WWTPs, where domestic, industrial and clinical sewage accumulates, ARGs and antibiotic resistant bacteria are highly prevalent. Studies frequently identified MDR bacteria, *bla* genes such as carbapenemase, *ampC* and ESBL genes [161–167] as well as genes conferring resistance to aminoglycoside, macrolide, rifampicin, tetracycline, trimethoprim, chloramphenicol, fluoroquinolone and sulfonamide in WWTPs [153,168,169]. Müller *et al.* (2018) isolated bacteria such as *P. aeruginosa*, *E. coli*,

K. oxytoca and *K. pneumoniae* from clinical and urban systems, determining that all isolates were resistant to 3rd generation cephalosporins, 28.21% harboured carbapenemase genes and 5.47% were XDR strains [165]. A study from Szczepanowski *et al.* (2009) determined 140 different ARGs in sewage sludge and 123 genes in WWTP effluent samples [168]. These genes included ARGs which were just reported in clinical isolates, such as *bla*$_{\text{GES-3}}$, *bla*$_{\text{CTX-M-27}}$ or the aminoglycoside resistance gene *aadA6/aadA10*. Even though the prevalence of *mcr-1* genes in WWTPs seems rather low [114,170], other *mcr* variants have been detected frequently [171], revealing a higher prevalence of *mcr* in wastewater as believed so far.

During biological treatment, activated sludge containing microorganisms is used to remove dissolved organics, and studies showed that sewage sludge is an important source of ABR as well [169,172,173]. Sewage sludge can even contain higher amounts of ARGs [169,173] and if used as fertilizer in agriculture, a transfer to the environment is possible [174]. During wastewater treatment, the amount of ARGs is usually significantly decreased, but the effluent still contains antibiotic resistant bacteria and ARGs [141,142,163]. Hence, the released effluent can affect the bacterial population of the receiving surface waters [149,175,176] or even groundwater [29,151] by introducing antibiotic resistant bacteria. Moreover, even the enrichment or selection of ARGs and antibiotic resistant bacteria during wastewater treatment is possible [169,177]. Harris *et al.* (2012) determined a higher amount of antibiotic resistant bacteria in the WWTP effluent, although the overall bacterial count was reduced, and Zhang *et al.* (2009) showed that phenotypic resistance of *Acinetobacter* spp. was significantly increased in the effluent compared to the untreated wastewater [177,178]. In addition, Mao *et al.* (2015) analysed the prevalence of 20 tetracycline, four sulfonamide, four quinolone and two macrolide resistance genes and showed that 12 of the targeted genes were detected at higher rates in the WWTP effluent [169]. However, other studies reported significantly lower amounts of ARGs and antibiotic resistant bacteria in the final effluent [173,179], indicating that the results seem to differ between WWTPs or depending on treatment technologies [159].

The microbiota of wastewater comprises human and animal commensals as well as environmental bacteria, which might have been exposed to antimicrobial substances from private households, clinical and agricultural settings [180]. Frequently detected bacterial species harbouring resistances in WWTPs are *Enterobacteriaceae* like *E. coli*, *Enterobacter* spp. or *Klebsiella* spp., *Pseudomonadaceae*, *Aeromonadaceae*, *Acinetobacter* spp. as well as enterococci and *Staphylococcus* species [159,176,181,182]. The location of ARGs on MGEs enables the transfer of genes between bacteria, and the HGT can

be promoted due to subinhibitory concentrations of antibiotics in the wastewater [183]. Especially in Gram-negative bacteria, MGEs harbouring carbapenemase or ESBL genes are prevalent. Che *et al.* (2019) performed a metagenomic analysis of WWTP samples and showed that 55% of ARGs were carried by plasmids, indicating that mobile resistance genes dominate the WWTP resistome [184]. Furthermore, the effluent revealed an even higher relative abundance of plasmids [184]. Environmental factors provided in WWTPs such as a high cell density, biofilm formation, constant temperature and pH paired with a high nutrient supply might promote HGT between bacteria, enabling the transfer of ARGs between environmental bacteria and human pathogens [143,156,185,186] and thus their dissemination in other habitats.

1.3.3 Antibiotic resistance in the domestic environment

Data on ABR in the domestic setting is limited [187–189] even though the introduction of bacteria carrying ARGs into private households by contaminated clothes, skin, food stuff or other sources and their release from households to the environment via domestic wastewater of dishwashers, washing machines and drains might occur. Since domestic wastewater must be considered as an important component of wastewater, households could also play a role in the dissemination of antibiotic resistant bacteria and ARGs.

Antibiotic resistant bacteria have been isolated from companion animals, and due to their close contact to humans, resistant bacteria can easily be transferred via direct contact or the domestic area. Many antibiotics used in veterinary medicine are the same agents used in human medicine [190], and β-lactams are one of the most important antibiotics used in veterinary care [191]. Furthermore, antibiotics critically important for human medicine such as carbapenems or vancomycin are used unauthorized in cats and dogs [192], increasing the risk of the development of carbapenem resistance in zoonotic bacteria. MDR *P. aeruginosa* and various *Enterobacteriaceae* have been isolated from pets [192,193] with increasing prevalence. MRSA occurs frequently in companion animals such as cats and dogs [194], and there is evidence for the transfer of MRSA from animals to humans and vice versa [195]. Moreover, studies report the isolation of ESBL-harbouring *Enterobacteriaceae*, especially *E. coli*, in both healthy and sick companion animals since 1986 [191,196,197]. MDR *E. coli* strains harbouring the metallo-β-lactamase gene NDM were identified in companion animals in a study in the United States [198]. Although reports of MDR bacteria in companion animals remain comparably low, their occurrence is still concerning. However, humans also represent a natural reservoir for ABR.

The intestinal microbiome comprises many bacteria harbouring various ARGs that might be transferred via HGT to potential pathogens [199,200]. Consequently, a transmission of bacteria harbouring ABR via pets or household members into the domestic environment can be assumed.

The selection of antibiotic resistant bacteria might be promoted by antibiotics and antibacterial agents used in cleaning agents or personal care products [2,201,202]. Lucassen *et al.* (2019) identified shower and bathroom sink U-bends as potential hotspots of class 1 integrons in households and isolated multi-resistant bacteria such as *P. aeruginosa* [187]. Furthermore, a study of the frequency of antibiotic resistant bacteria in households of Marshall *et al.* (2012) determined highest titers of antibiotic resistant bacteria in sink drains as well [189]. In clinical settings, sinks and even water fountains have been identified as source of nosocomial infections with antibiotic resistant bacteria [203,204] and water reticulation systems as reservoir for antibiotic residues and MDR [205,206]. These studies indicate that shower drains in households can also act as a potential source of ABR, caused by the shed of antibiotic resistant strains from humans or a selection promoted by the use of biocides, cleaning agents and personal care products.

Generally, infections can originate from domestic surfaces [207], and thus cross-contaminations from washing machines or dishwashers might occur as well [208–211]. Studies revealed the prevalent formation of biofilms in washing machines and dishwashers [212,213], and their detachment might result in contaminations of already cleaned items. Another source of cross-contamination might be the detachment of bacterial cells from dirty laundry or kitchen utensils during the cleaning process and their re-attachment to the washed clothes and dishes [210,214]. Schmithausen *et al.* (2019) already determined the transfer of an ESBL-producing *K. oxytoca* strain from a washing machine via textiles to newborns [211]. Moreover, antibiotic resistant bacteria might be more persistent during automated dishwashing or laundering, since stress response and heat intolerance have been associated with ABR [215,216] and might be induced by these cleaning processes.

1.3.4 Transfer of antibiotic resistance between different habitats

The available data on ABR in different habitats suggest that antibiotic resistant bacteria and resistant determinants may be transferred frequently between the habitats. The use of antibiotics in the clinic, humans, companion animals and livestock results in the development of ABR or the release of antibiotic residues in the environment [25,29,124,192,206]. As already mentioned above, the transfer of antibiotic resistant bacteria between pets and their owners [195] as well as livestock and farm workers [217] has been reported. Interestingly, a study of human faecal metagenomes revealed that ARGs conferring resistances to antibiotics also used in animals were most prevalent [218], possibly indicating genetic transfer. Wastewater originating from hospitals, households, agriculture and industry accumulates in WWTPs, and antibiotic resistant bacteria are released via the treated effluent into water bodies [142,149,175]. Subsequently, ABR can be transferred from surface water to soil since water has been determined as a main pathway of ABR dissemination in soils [135,139]. A transfer from agricultural soils to vegetables at harvest has been shown as well and even increased during manure application [145,219]. Antibiotic resistant bacteria are not only prevalent in manure, but also in animal-based food. Studies determined the occurrence of ARGs in retail meat [220,221] and plant-based food [147,222], which then act as transmission pathways of ABR into the domestic setting. Furthermore, humans might transfer antibiotic resistant bacteria from natural habitats in the household or become colonized after hospital stays. The occurrence of ABR in different habitats and the evidence for transfer highlights the intermingled connection between all areas. Hence, a “One Health” approach due to the complex interrelationship between humans, animals, environment and health is necessary to counteract environmental and health problems caused by ABR [223].

1.4 Objectives

The occurrence of ARGs and antibiotic resistant bacteria has been comprehensively analysed in humans, animals, food, soil, WWTPs and natural water bodies. Thus, a transfer of ARGs and resistant bacteria into private households via the water supply or as part of the human/animal microbiota and vice versa via domestic wastewater in the natural environment seems possible, but the significance of the domestic area is comparatively less understood.

To determine potential transfer routes, directions and risks, the comparison of the occurring antibiotic resistant bacteria and ARGs of different habitats is necessary. For a better understanding of ARG transfer between environments, the determination of ARGs that are prevalent in both households and other habitats is important. Since bacteria may facilitate the transfer of ARGs across ecological barriers, the identification of bacterial taxa in different settings is important as well.

Hence, the **first part** of this study aimed to identify differences in occurrence and co-occurrence of β-lactamase (*bla*), mobile colistin resistance (*mcr*) and class 1 integron-integrase (*intI1*) genes over a one-year-period in a WWTP. In the **second part**, the investigation focused on the domestic environment, aiming to enhance our understanding of ABR in households by quantifying the abundance of *bla*, *mcr* and *intI1* genes in washing machines, dishwashers and shower drains. In addition, the effect of laundering and automated dishwashing on antibiotic resistant strains was assessed to define the risk originating from laundry items and dishes. In both approaches phenotypic resistance was investigated as well since human health risks are originating from antibiotic resistant bacteria able to cause infections and do not depend on ARG abundance only. In the **third part** of this study, metagenomes of samples originating from the previously analysed WWTP and households as well as soils were compared to determine similarities and differences of the taxonomic distribution as well as the abundance and diversity of genes encoding antibiotic resistance factors such as ARGs and MGEs between the different settings.

Based on this, the thesis aimed to possibly identify environments that might act as transmission routes. Moreover, elaborating the role of the domestic area as source of antibiotic resistant bacteria and possible hotspots of ABR in households was targeted.

2 Winter is coming – Impact of temperature on the variation of β-lactamase and *mcr* genes in a wastewater treatment plant

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Own contribution

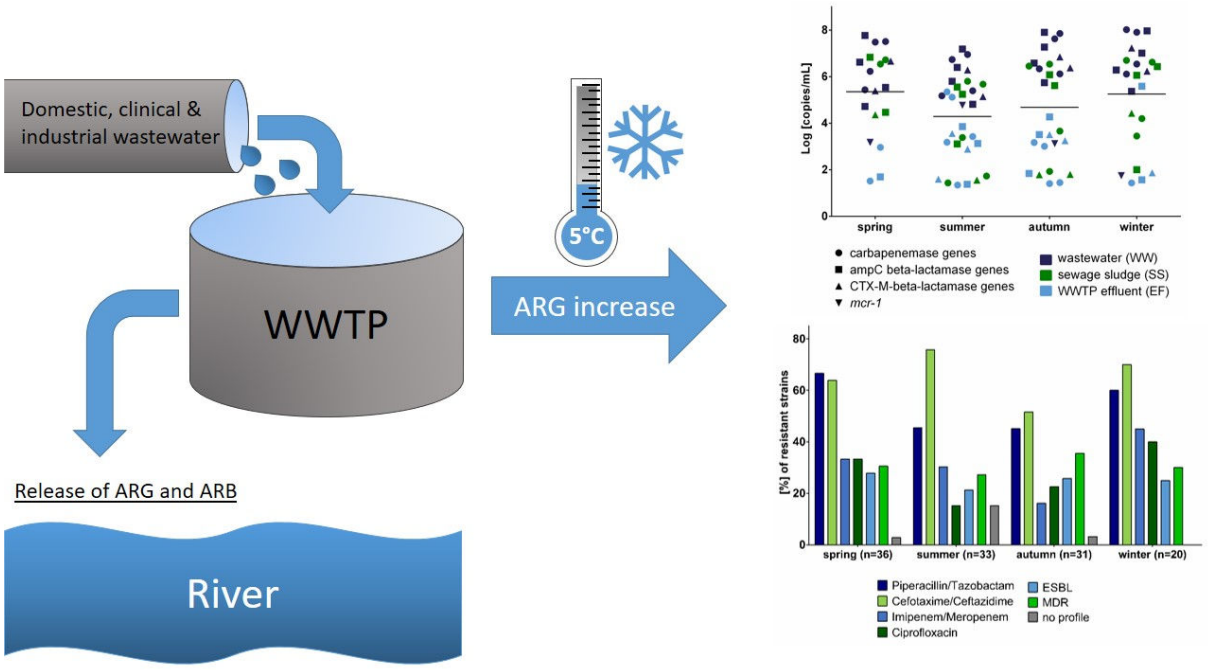
- Writing of first version of complete manuscript draft
- Sample collection and sample preparation
- Purification of total DNA
- qPCR for the detection of *bla*, *mcr*, and *intI1* genes
- Cultivation of bacteria and determination of total viable count
- Culture-based isolation of antibiotic resistant bacteria using sub-inhibitory antibiotic concentrations
- Identification and antimicrobial susceptibility testing using VITEK 2
- Statistical analysis of data

Abstract

Wastewater treatment plants (WWTP) play a key role in the dissemination of antibiotic resistance and analyzing the abundance of antibiotic resistance genes (ARGs) and resistant bacteria is necessary to evaluate the risk of proliferation caused by WWTPs. Since few studies investigated the seasonal variation of antibiotic resistance, this study aimed to determine the abundance of β-lactamase and *mcr* genes and to characterize phenotypic resistant strains in a WWTP in Germany over the seasons. Wastewater, sewage sludge and effluent samples were collected over a one year period and analysed using quantitative real-time PCR. Resistant strains were isolated, followed by identification and antibiotic susceptibility testing using VITEK 2. The results show a significantly higher occurrence of nearly all investigated ARGs in the wastewater compared to sewage sludge and effluent. ARG abundance and temperature

showed a negative correlation in wastewater and significant differences between ARG abundance during warmer and colder seasons were determined, indicating a seasonal effect. Co-occurrence of *mcr-1* and carbapenemase genes in a multi-drug resistant *Enterobacter cloacae* and *Escherichia coli* producing extended-spectrum β-lactamase (ESBL) was determined. To the best of our knowledge, this is the first detection of *mcr-1, bla*$_{VIM}$ and *bla*$_{OXA-48}$ in an ESBL-producing *E. coli*. Although wastewater treatment reduced the abundance of ARGs and resistant strains, a dissemination into the river might be possible because carbapenemase-, CTX-M- and *mcr-1*-gene harboring strains were still present in the effluent.

Graphical abstract



3 The household resistome – frequency of β-lactamases, class 1 integron and antibiotic resistant bacteria in the domestic environment and their reduction during automated dishwashing/laundering

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Own contribution

- Writing of first version of complete manuscript draft
- Sample collection and sample preparation
- Purification of total DNA
- qPCR for the detection of *bla*, *mcr*, and *intI1* genes
- Culture-based isolation of antibiotic resistant bacteria using sub-inhibitory antibiotic concentrations
- Identification and antimicrobial susceptibility testing using VITEK 2
- Performance of tests to determine the effect of laundering and automated dishwashing on antibiotic resistant strains
- Statistical analysis of data

Abstract

Households provide a habitat for bacteria originating from humans, animals, foods, contaminated clothes or other sources. Thus, bacteria carrying antibiotic resistance genes (ARGs) might be introduced via household members, animals or the water supply from these habitats into private households and vice versa. Since data on ABR in the domestic environment is limited, this study aimed to determine the abundance and correlation of β-lactamase, mobile colistin resistance and class 1 integron genes and to characterize phenotypic resistant strains in 54 private households in Germany.Additionally, the persistence of antibiotic resistant bacteria

to automated dishwashing compared to laundering was assessed. Shower drains, washing machines and dishwashers were sampled and analysed using quantitative real-time PCR. Resistant strains were isolated, followed by identification and antibiotic susceptibility testing using VITEK 2. The results show a significantly higher relative ARG abundance of 0.2367 ARG copies/16S rDNA copies in shower drains compared to dishwashers (0.1329 ARG copies/16S rDNA copies) and washing machines (0.0006 ARG copies/16S rDNA). *bla*$_{\text{CMY-2}}$, *bla*$_{\text{ACT/MIR}}$ and *bla*$_{\text{OXA-48}}$ were the most prevalent ARG and *intI1* occurred in 96.3% of the households while no *mcr* genes were detected. Several β-lactamase genes co-occurred and resistance of bacterial isolates correlated positively with genotypic resistance, with carbapenemase genes dominating across isolates. Antibiotic resistant bacteria were significantly reduced during automated dishwashing as well as laundering tests and did not differ from susceptible strains. Overall, the domestic environment might represent a potential reservoir of β-lactamase genes and β-lactam resistant bacteria with shower drains as the dominant source of ABR.

Importance

The abundance of antibiotic resistant bacteria and ARGs is steadily increasing and has been comprehensively analysed in natural environments, animals, foods or wastewater treatment plants. In this respect, β-lactams and colistin are of particular interest due to the emergence of multidrug-resistant gram-negative bacteria. Despite of the connection of private households to these environments, only few studies focused on the domestic environment so far. Therefore, the present study further investigated the occurrence of ARGs and antibiotic resistant bacteria in shower drains, washing machines and dishwashers. The analysis of the domestic environment as a potential reservoir of resistant bacteria is crucial to determine whether households contribute to the spread of ABR or might be a habitat where resistant bacteria from the natural environment, humans, food or water are selected due to the use of detergents, antimicrobial products and antibiotics. Furthermore, ABR could limit treatment options of infections arising in the domestic environment.

4 Soils, wastewater treatment plants and households have distinctive resistomes revealing little evidence of antibiotic resistance transfer between environments

Manuscript submitted

Own contribution

- Writing of first version of complete manuscript draft
- Sample collection and sample preparation (Household and WWTP samples)
- Purification of total DNA (Household and WWTP samples)
- Preparation of DNA samples for sequencing
- Statistical analysis of data

4.1 Manuscript

Soils, wastewater treatment plants and households have distinctive resistomes revealing little evidence of antibiotic resistance transfer between environments

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Keywords: antibiotic resistance, resistome, environment, household, soil, wastewater treatment plant, transfer, metagenomics

Abstract

The use of antibiotics in the clinic, humans and animals results in a release of access antibiotic residues into the environment through wastewaters and insufficient removal in wastewater treatment plants (WWTPs). This can lead to the development of antibiotic resistance through increasing bacteria enriched in antibiotic resistance genes (ARGs). However, the knowledge on the potential transfer of ARGs and their host bacteria between different environments remains largely unexplored. We sampled agricultural soils, a WWTP, dishwashers, washing machines and shower drains in the same geographical region and performed metagenomic analyses to evaluate differences and potential overlaps in bacterial communities and the resistome of different environments. Wastewater samples revealed significantly higher richness of ARGs (n=40) and mobile genetic elements (n=52) than soil and household samples. Bacterial communities differed between the environments with pronounced differences between

sub-environments of WWTP and household samples. Moreover, antibiotic resistance factors clustered distinctly as well, revealing no correlation between the different environments. Overall, only few overlaps of ARGs between the environments were observed, leading to the conclusion that antibiotic resistances predominantly develop in individual environments as caused by environmental filtering for ARGs, while a transfer between the different environments is less likely.

Introduction

Although antibiotic resistance (ABR) is prevalent even in the absence of anthropogenic impacts and prior to the therapeutic use of antibiotics [1,2], the number of antibiotic resistant bacteria has increased rapidly since the use, overuse and misuse of antibiotics in human medicine and for veterinary purposes [3,4]. This spread of ABR is a serious threat challenging not only human but also animal and environmental health. Antibiotic resistant bacteria and antibiotic resistance genes (ARGs) have been analyzed in humans, animals, food, soil, and aquatic environments and can be transferred within and between the different environments [4–11]. Studies indicate that the resistome is mainly shaped by bacterial composition [12–14] and that similarities in taxonomic community composition lead to overlaps in mobile genetic elements (MGE) and ARG prevalence [15]. Therefore, analyzing both the resistome and bacterial community of different environments is crucial to identify potential reservoirs of ABR and possible transfer routes of antibiotic resistant bacteria. Horizontal gene transfer (HGT) between bacteria promotes genetic diversity and thus the spread of ABR [16]. Gene transfer is especially mediated by MGEs such as plasmids, integrons and transposons [17,18] commonly harboring ARGs enabling vertical and horizontal gene transfer via conjugation, transformation and transduction [17,19]. Therefore, environments with a large diversity of ARGs and MGEs are potential sources for ABR. However, HGT require that the bacteria inhabit or at least shortly share the same environment [20]. Antibiotic resistant bacteria and ARGs are especially prevalent in the influent of wastewater treatment plants (WWTPs) originating from private households, industry and clinical settings. Despite of a significant decrease of ARGs during biological treatment, the effluent still contains ARGs and resistant bacteria [8,21]. Besides,

ARGs and resistant bacterial species have already been detected in ground- and drinking water [22,23], which might act as a potential transmission route to other environments such as soil. Soil represents a natural reservoir of ABR [24–26] since most antibiotics originate from soil

bacteria and fungi as a product to eliminate competing species [27,28]. Anthropogenic pollution via wastewater used for irrigation or sewage sludge as fertilizer plays a key role in the transmission of ABR and MGEs [29–32] and thus represent an important source of ARGs in soil [33]. Moreover, a transfer of bacteria harboring ARGs into private households via the water supply or as part of the human/animal microbiota and vice versa via domestic wastewater of washing machines, dishwashers and drains in natural environments seems possible, but the significance of the domestic area is comparatively less understood [34–37]. However, domestic wastewater as a component of the WWTP influent might also play a role in the dissemination of ABR. Although different environments have been identified as possible sources of ABR, their relative role in ARG transfer remains unclear. There is evidence for the transfer of ARGs from environmental bacteria to human pathogens [11,19,38] which highlights the importance of identifying the role of different habitats in the spread of ABR. Therefore, identifying bacterial taxa that are most prevalent is important, since these taxa might enable the transfer of ARGs between the environments. Furthermore, the detection of ARGs that are widespread in different environments is necessary in order to comprehend the transmission pathways of ARGs and antibiotic resistant bacteria. A metagenomic approach enables the investigation of the resistome without limitation to certain organisms. Hence, it is not surprising that in many studies metagenome analyses focusing on distinct sources such as wastewater, soil, surface water, humans and animals were performed [12,13,39,40], revealing the widespread of ABR. In this field, household are hardly studied and integrative cross-habitat studies to investigate potential transfer routes are rare.

Here we performed metagenome sequencing of soil, WWTP and household samples to identify potential ABR reservoirs and to understand possible transfer routes. We hypothesized that ABR rather develop and cluster in each distinct environment with a less frequent transfer and thus characteristic metagenome patterns can be observed in samples originating from soil, a WWTP and households in the same geographical region. The objectives of our study were (i) to identify similarities and differences of the taxonomic distribution and antibiotic resistomes of the environments under investigation, (ii) to determine the abundance and diversity of genes encoding antibiotic resistance factors such as ARGs and MGEs and (iii) to elucidate if based on similarities in micro- and resistome, a transfer between the different environments can be assumed.

Material and methods

Sample collection and preparation

Samples of the inner tubing of shower drains (SD), dishwasher sumps and sieves (DW), detergent trays and rubber door seals of washing machines (WM) were taken from households in the vicinity of Kleve, Germany between September 2018 and June 2019 in a previous study [37]. All samples were taken in households from different apartment buildings or single family houses. The surfaces of the inner tubing of SD, the sumps and sieves of DW or the detergent trays and rubber door seals of WM were sampled using a sterile cotton swab in triplicate.

Wastewater (WW), sewage sludge (SS) and effluent (EF) were collected in another study [9] at a secondary wastewater treatment plant (WWTP) in the district of Kleve each week over a one year period (May 2018 to April 2019). In this WWTP, a total of approximately 5.5 million m³ WW is treated every year resulting in 7.300 t of SS. The influent comprises approx. 53.035 households, WW from industries (5% of the WW) and the area includes three different clinics with a total of 1,280 beds. Samples were prepared as described in the previous studies [9,37], since the same samples were analyzed before using qPCR and a microbiological approach.

Soil samples were taken from a long-term experiment (since 2001) with different fertilization treatments aiming at evaluating effects on potato, yield and quality. Three treatments were selected for next generation sequencing: Treatment 1 (S1) was fertilized with mineral fertilizer, treatment 2 (S2) was fertilized with cattle manure and treatment 3 (S3) with straw and liquid cattle manure. Each different treatment was represented in four plots of 9m x 9m and five sub-samples were taken in each plot in 30 cm depth and pooled. Samples were then sieved to 2 mm prior to DNA extraction.

DNA Extraction

For purification of total DNA, the Fast DNA Spin Kit for Soil (MP Bio, Santa Ana, CA, USA) was used according to the manufacturer's instructions with the following adjustments:

In case of domestic samples (SD, DW and WM) instead of 500 mg solid material, 250 µL of suspended sample were applied to the lysing matrix tube. Samples were homogenized twice in the FastPrep-24™ instrument for 60 sec at 6.0 m s^{-1} [37]. WW, SS and EF samples were each pooled from one month and WW and SS samples were centrifuged at 4,800g/8 °C/10min.

While the pellets of WW samples were re-suspended in 200 mL of sterile 0.9% sodium chloride, SS samples were washed with 10 mL of sterile 0.9% sodium chloride twice. Samples were centrifuged and re-suspended in sodium chloride again and 500 µL of the sample were added to the lysing matrix tubes. Each 500 µL were added to the lysing matrix tubes and WW samples were homogenized as the household samples and SS samples three times for 40 s at 5.0 m s^{-1} in the FastPrep-24™ instrument. EF samples were filtered through a 0.2-µm-pore size filter which was added in small pieces to the lysing matrix tubes followed by homogenization for 60 s at 6.0 m s^{-1} [9]. DNA from soil samples (S1, S2, S3) was extracted with the following modifications [41]: of sodium phosphate buffer and "MT buffer" 950 mL and 120 mL were used, respectively. Samples were homogenized twice in FastPrep-24™ instrument for 45 s at a 6.5 m s^{-1}. After that, samples were centrifuged at 14,000g/5min at room temperature. To remove contaminants, the DNA bound to the binding matrix of the kit was washed twice with 1 mL of 5.5 M guanidine thiocyanate (Carl Roth, Karlsruhe, Germany).

Metagenomic analysis

Since the study aimed to analyze the microbial communities of different environments, the INVIEW Metagenome from Eurofins Genomics (Eurofins Genomics Germany GmbH, Ebersberg, Germany) was chosen for a NGS-based taxonomic, resistance and functional profiling of the samples. Samples were pooled and DNA quantity was properly balanced in each pool to equally represent each genome. To achieve this, the DNA concentration of the individual samples was adjusted to the same concentration and equal amounts of each sample were pooled. Since DNA concentration of domestic samples and EF samples was below 20 ng/µL, it was not possible to conduct NGS for the pools DW1 to DW4, SD1 to SD4, WM1, WW2 and EF2. Sequencing and library preparation was performed at Eurofins Genomics using Genome Sequencer Illumina Hiseq platform with the NovaSeq 6000 S2 PE150 XP system. Bioinformatic analysis was performed using established pre-built MiniKrakenDB constructed from complete bacterial, archaeal, fungal, protozoan and viral genomes in RefSeq for taxonomic profiling, screening for virulence factors and ARGs using MvirDB. In addition, paired end reads were blasted in order to find acquired ARGs using ResFinder [42]. A total of 870,593,530 high-quality sequences were acquired from soils (S1 (n=4), S2 (n=4), S3 (n=4)), WWTP (WW (n=4), SS (n=5), EF (n=4)) and households (SD (n=1), DW (n=1), WM (n=4)).

Data analysis

Statistics were performed using GraphPad Prism (GraphPad Software Inc.). Data were expressed as means (± standard error). Alpha diversity (Shannon diversity exp(H')), the number of ARGs and MGEs was not normally distributed, thus Mann-Whitney test ($p \leq 0.05$) was performed to identify significant differences between the environments. The structure of the microbial community was compared using a principal component analysis (PCA) based on Bray-Curtis dissimilarity of the different environments using ClustVis [43]. Bacterial community and ABR factor composition was compared using non-parametric Mann-Whitney test ($p \leq 0.05$). We explored the underlying relationships between antibiotic resistance factors, ARGs, MGEs and efflux pumps using PCA based on a Spearman correlation since it is robust to nonlinear relationships and outliers.

Results

Bacterial diversity and richness of species, ARGs and MGEs

The Shannon diversity of the bacterial communities was significantly lower ($p < 0.05$) in WWTP (exp(H')=256.8 ± 123.8) and household (HH) samples (exp(H')=105.2) compared to soil samples (exp(H')=614.1 ± 2.4) (Fig. 1A). In contrast, species richness was highest in WWTP samples (n=1603.9 ± 18.2) compared to soils (n=1542.7 ± 2.9). Hence, based on the Shannon diversity, species in soil samples were more equally distributed although the number of different species was highest in WWTP samples. In contrast, a significantly lower species richness and diversity was determined in HH (n=1430 ± 69.8), which varied strongly between the different sub-environments of HH samples (Fig. 1B). Diversity was both lowest (exp(H')=50.9) and highest (exp(H')=157.1) in each a WM sample. Moreover, ARG (n=12.8 ± 2.2) and MGE (n=30.3 ± 3.9) richness was highest in WM samples as well. Nearly no ARGs (n=1.8 ± 1.1) and MGEs (1.0 ± 0.9) were detected in soil samples and thus ARG and MGE richness was significantly higher in WWTP (n(ARGs)=24.3 ± 13.2 and n(MGEs)=39.9 ± 9.7) and HH samples (n(ARGs)=11.2 ± 3.1) and n(MGEs)=28.8 ± 3.9) (Fig. 1C-D). Highest counts of ARGs and MGEs occurred in wastewater (WW, n(ARGs)=40 ± 2.9 and n(MGEs)=52.3 ± 0.9) followed by sewage sludge (SS, n(ARGs)=24.2 ± 5.1 and n(MGEs)=37.8 ± 4.0) while WWTP effluent (EF, n(ARGs)=10.3 ± 4.3 and n(MGEs)=30.3 ± 4.6) and HH samples revealed a significantly lower richness.

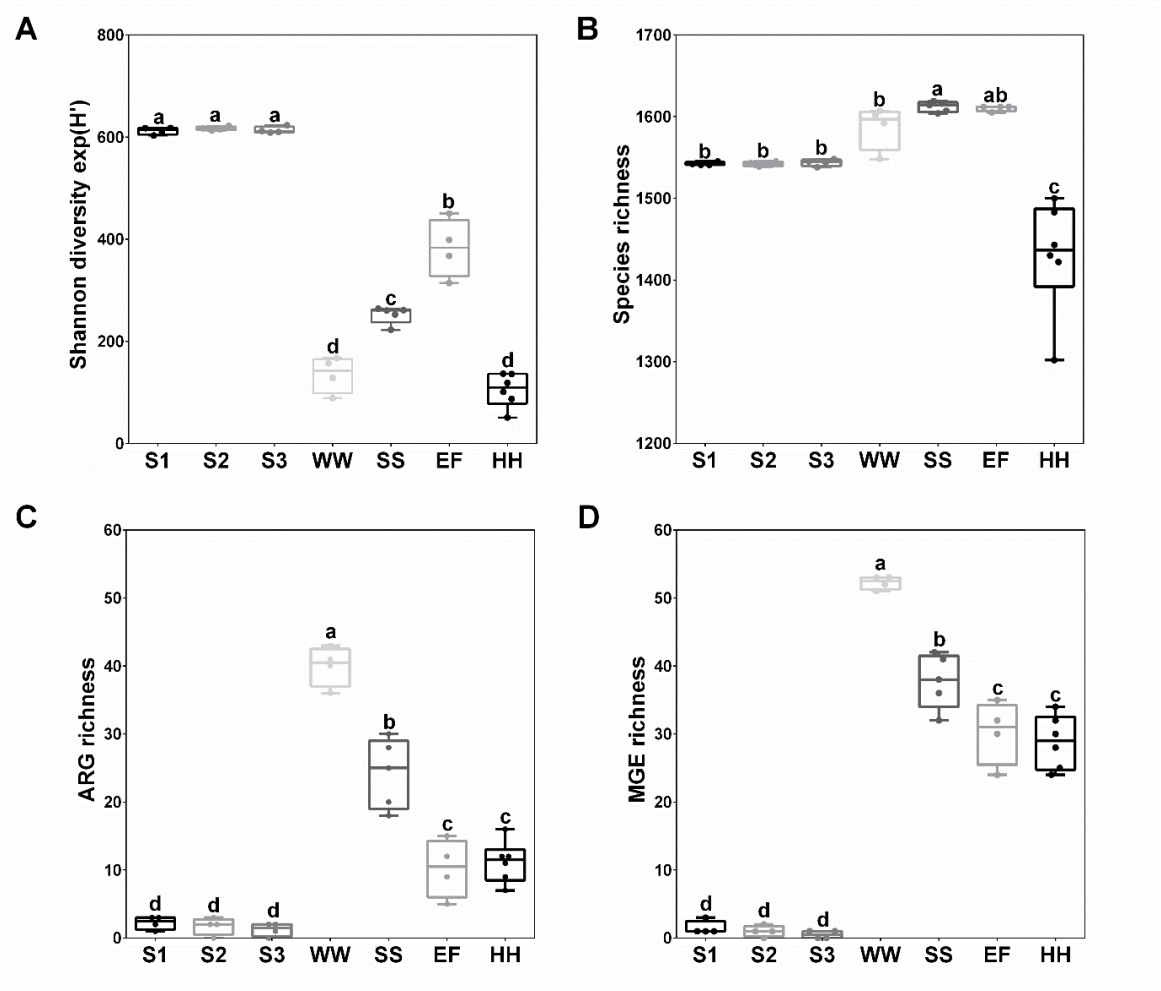

Figure 1. Exponential Shannon index (**A**), species (**B**), ARG (**C**) and MGE (**D**) richness (count of species/ARGs/MGEs) of different soils (S1, S2 S3, each n=4), wastewater (WW, n=4), sewage sludge (SS, n=5), WWTP effluent (EF, n=4) and household samples (HH, n=6). Different letters indicate significant differences calculated using non-parametric Mann-Whitney test.

Beta diversity revealed a high degree of variation between the different environments while the samples of the same environment clustered distinctly (Fig. 2), revealing that soil samples shared more species with SS (Bray-Curtis index=0.55) and EF (Bray-Curtis index=0.53) than with WW (Bray-Curtis index=0.85) or HH (Bray-Curtis index=0.75) samples. WW samples clustered closer to HH samples (Bray-Curtis index=0.73), although Bray-Curtis indices of HH and SS (0.74) were similar or even lower in case of EF samples (0.70). However, SS and EF revealed many similarities (Bray-Curtis index=0.26) while WW differed strongly from SS (Bray-Curtis index=0.70) and EF (Bray-Curtis index=0.72). Moreover, HH samples showed the highest variation within one environment, with an average Bray-Curtis index of 0.55.

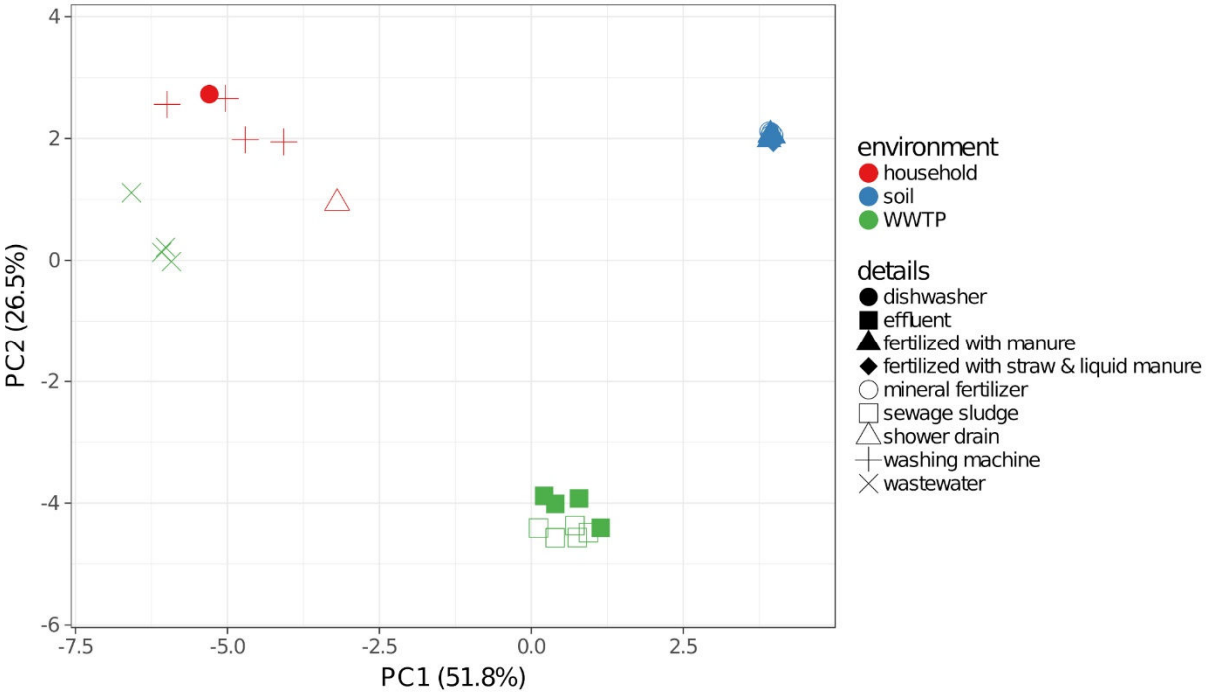

Figure 2. Principal component analysis (PCA) of the beta diversity of different soils, wastewater, sewage sludge, effluent, shower drains, dishwashers and washing machines based on Bray-Curtis dissimilarity. Unit variance scaling was applied to rows and NIPALS (Nonlinear Iterative Partial Least Squares) PCA was used to calculate principal components. X- and Y-axis show principal component 1 and principal component 2 that explain 51.8% and 26.5% of the total variance, respectively.

Community composition

In the present study, we focused on the bacterial community. The analysis revealed 32 phyla, 670 genera and 1625 species. The majority of identified bacteria belonged to the phylum *Proteobacteria* (Mean: 63.9% ± 10.1%) (Fig. 3A). *Pseudomonas* was the only genus to occur in higher fractions across all samples varying between 1.9% and 17.1% (Fig. 3B). Besides the group "others", Streptomyces (11.0% ± 0.5%) and *Bradyrhizobium* (9.2% ± 0.3%) revealed the highest percentages in soil samples. While *Aeromonas* (14.1%) was significantly more prevalent in WW followed by *Pseudomonas* (13.5%), *Nitrospira* occurred the most in SS and EF samples with significantly higher percentage in SS compared to the other environments. The percentage of *Pseudomonas* was significantly higher in washing machine (WM) samples and identified predominantly in all HH samples followed by *Mycobacterium* (8.2%), *Stenotrophomonas* (10.2%) and *Ochrobactrum* (7.4%) in shower drain (SD), dishwasher (DW) and WM, respectively. Moreover, the highest percentage of Acinetobacter was determined in WW and all HH samples (1.7 to 6.7%). Generally, more similarities between WWTP and HH

samples were determined compared to soil samples, e.g. regarding the fractions of *Pseudomonas*, *Acidovorax*, *Acinetobacter* or *Brevundimonas*.

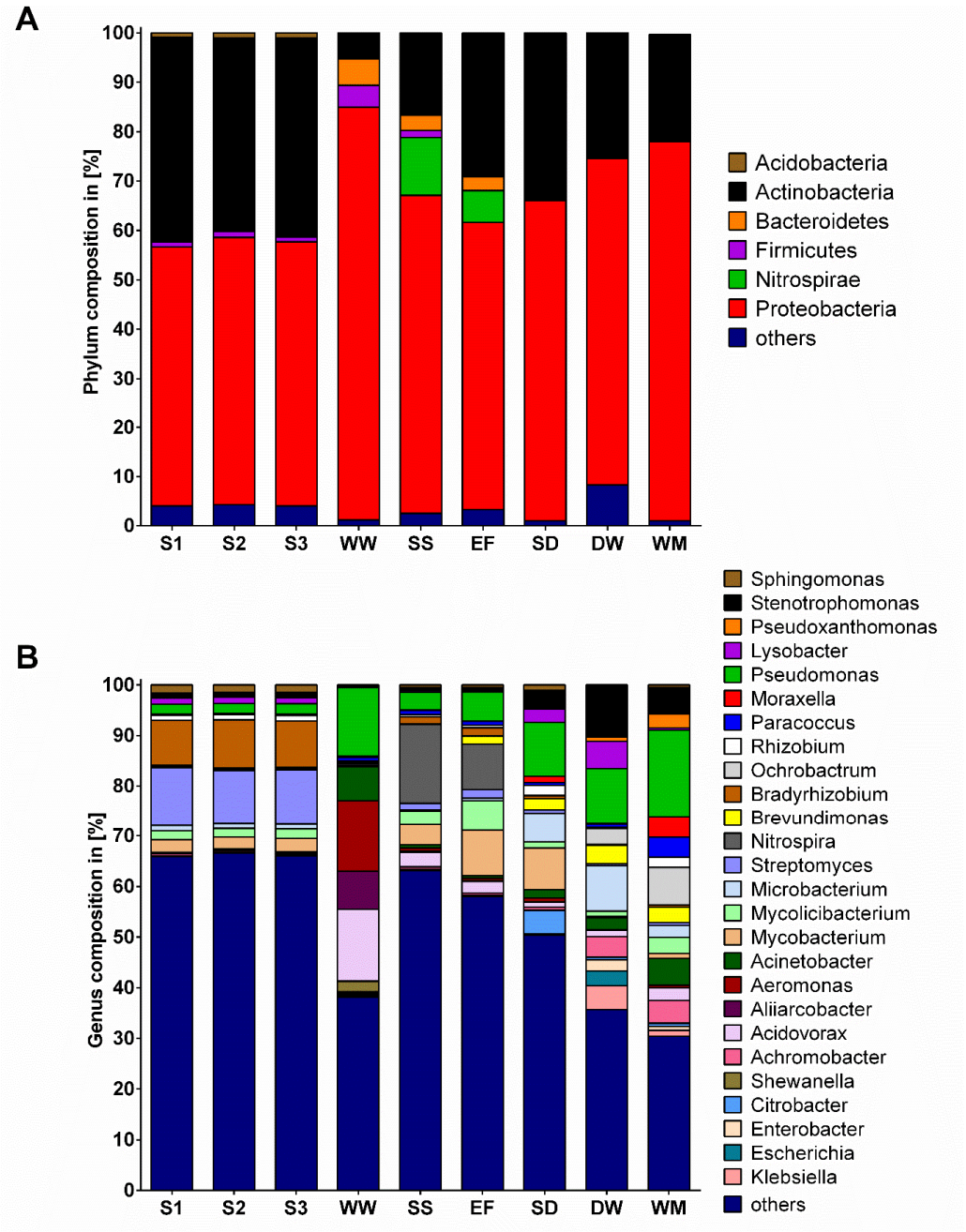

Figure 3. Bacterial composition at phylum (**A**) and genus (**B**) level of different soils (S1, S2, S3, each n=4), wastewater (WW, n=4), sewage sludge (SS, n=4), WWTP effluent (EF, n=4), shower drains (SD, n=1), dishwashers (DW, n=1) and washing machines (WM, n=4) based on next generation sequencing. To ameliorate presentation, only phyla with ≥ 1% and genera with ≥ 2% in at least one sample group are shown.

Antibiotic resistance factors

Compared to WW and HH, the soil samples revealed significantly lower reads with only approx. 3% accounting for ABR factors (Table S2), while the remaining percentages were annotated to other virulence factors (Fig. 4). The majority of the ABR factors was annotated to genes encoding efflux pumps or other resistances and tetracenomycin C resistance only occurred in soil samples whereas MGEs constituted approx. 7.8% of all ABR factors. In contrast, 41.1% ± 2.9% ABR factors were determined in WWTP samples and 23.3% ± 7.5% in HH samples, with lowest percentage in DW (Table S2). The percentage of MGEs including transposons, integrons and plasmids were significantly higher ($p \leq 0.05$) across WWTP and represented the main part of the relative composition of ABR factors in HH samples as well. Even though in all HH samples the majority of ABR factors were represented by MGEs and efflux pumps, the percentage of MGEs in WWTP samples was much higher. Moreover, resistances to aminoglycosides, β-lactams and tetracyclines were more prevalent in WWTP samples.

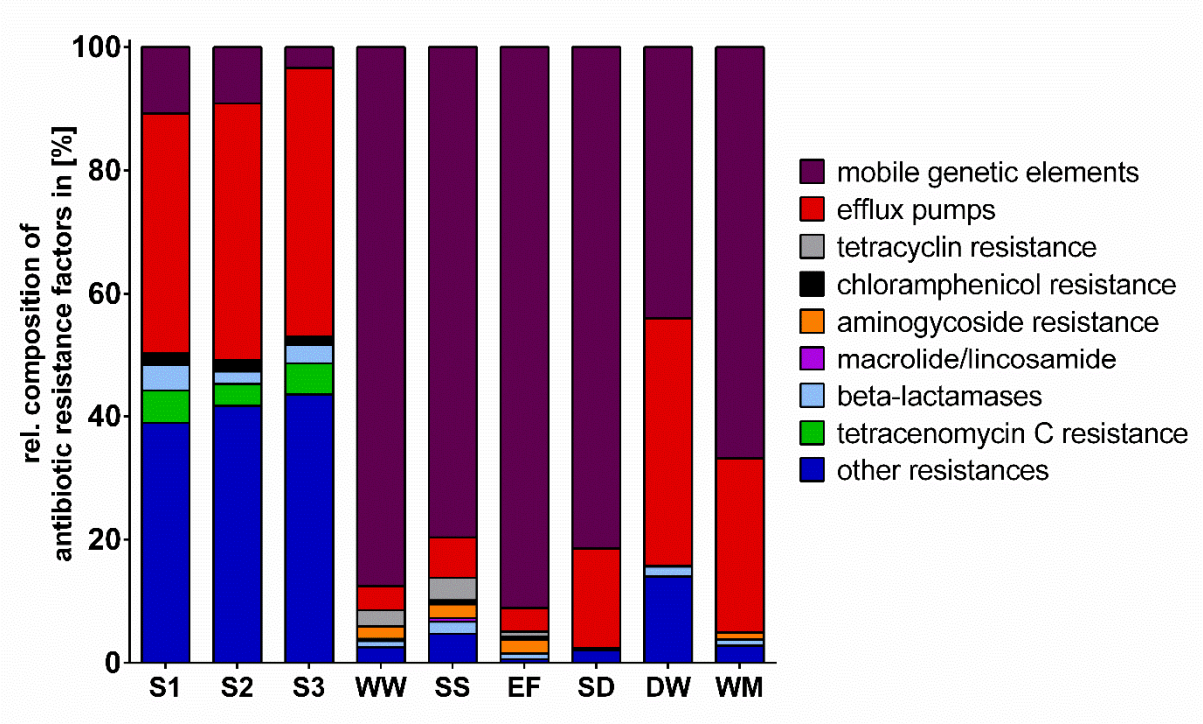

Figure 4. Relative composition of reads annotated to antibiotic resistance factors in soil samples (S1 (n=4), S2 (n=4), S3 (n=4)), WWTP (WW (n=4), SS (n=5), EF (n=4)) and HH samples (SD (n=1), DW (n=1), WM (n=4)). Sequence reads were mapped against the microbial virulence database (MvirDB) and the antibiotic resistance factors are one of seven virulence factors.

Principal component analysis (PCA) of Spearman correlation and samples of the same environment revealed distinct clustering regarding ABR factors and ARGs (Fig. 5). Genes encoding ABR factors showed only weak correlations of soil and WWTP samples (r=-0.05), soil and HH (r=1.10) as well as WWTP and HH samples (r=0.01). The ARGs also correlated weakly in soil and WWTP (r=-0.14), soil and HH (r=-0.03) and WWTP and HH samples (r=-0.16). In contrast, positive correlations of ABR factors in the different sub-environments were determined, revealing strongest correlations of WWTP samples (r=0.82) followed by HH samples (r=0.59) while soil samples showed the weakest correlation (r=0.18). Regarding MGEs, a weak positive correlation of soil and household (r=0.19), WWTP and household (r=0.25) and soil and WWTP (r=0.17) was determined. Moreover, genes encoding efflux pumps positively correlated in soil and WWTP (r=0.59), soil and HH (r=0.48) and WWTP and HH (r=0.44).

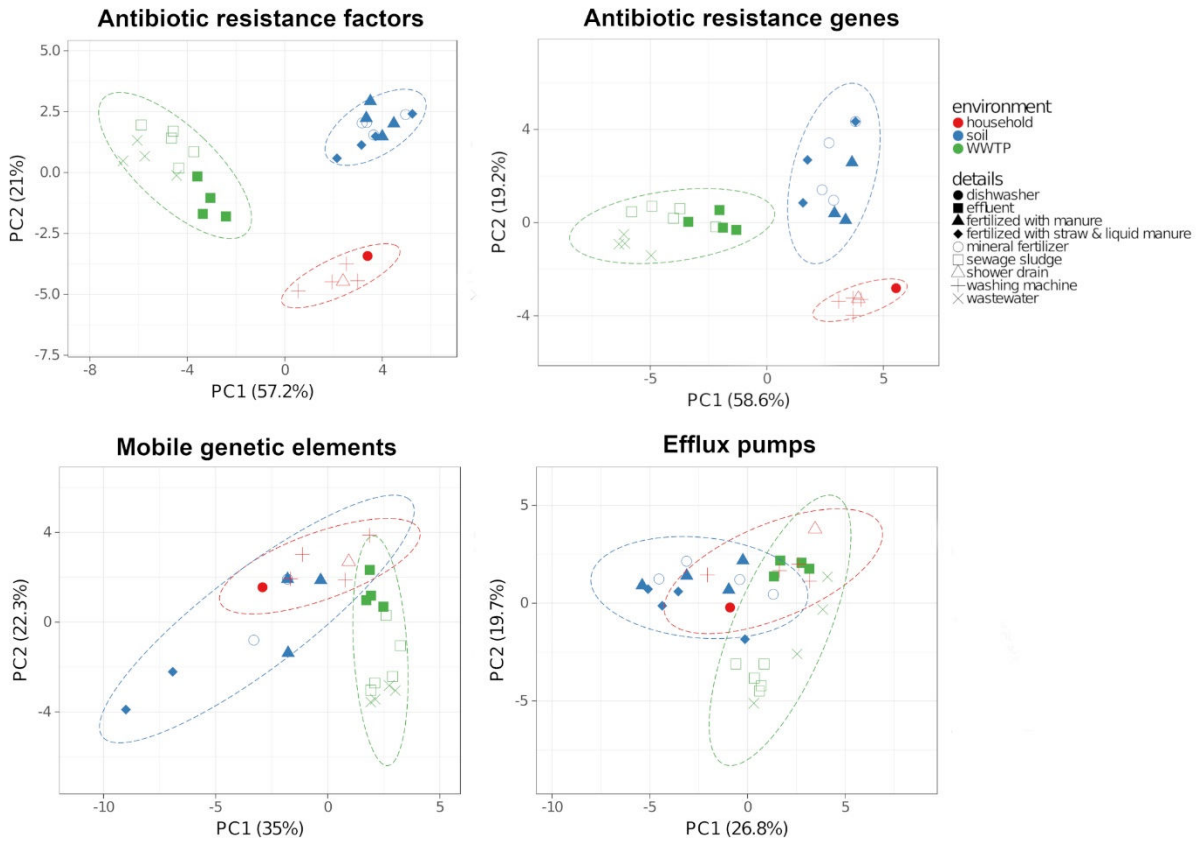

Figure 5. Principal component analysis (PCA) with 95% prediction ellipses based on Spearman correlation of the total of antibiotic resistance factors and its subgroups antibiotic resistance genes, mobile genetic elements and efflux pumps. Unit variance scaling was applied to rows and NIPALS (Nonlinear Iterative Partial Least Squares) PCA was used to calculate principal components.

The Venn diagrams created using InteractiVenn [43] of ARG and MGE annotations showed only a small set of shared genes between all three environments, resulting in an overlap of only two (ARGs) and six (MGEs) genes present in all environments (Fig. 6a-b). Most similarities were identified between WWTP and HH samples, with 34.1% of ARGs and 85.2% of MGEs in WWTP samples present in HH as well. Compared to the similarities between WWTP and HH, soil shared much lower numbers of ARGs and MGEs with the other investigated environments. However, all MGEs annotated in soil and HH samples were determined in WWTP samples. In addition to the screening for virulence factors and ARGs using MvirDB, paired end reads were blasted in order to find acquired ARGs using ResFinder (Fig. 6c). In WWTP and HH samples, an overlap of 11 ARGs was determined in these samples while only two ARGs were detected in all soil samples which did not occur in WWTP or HH.

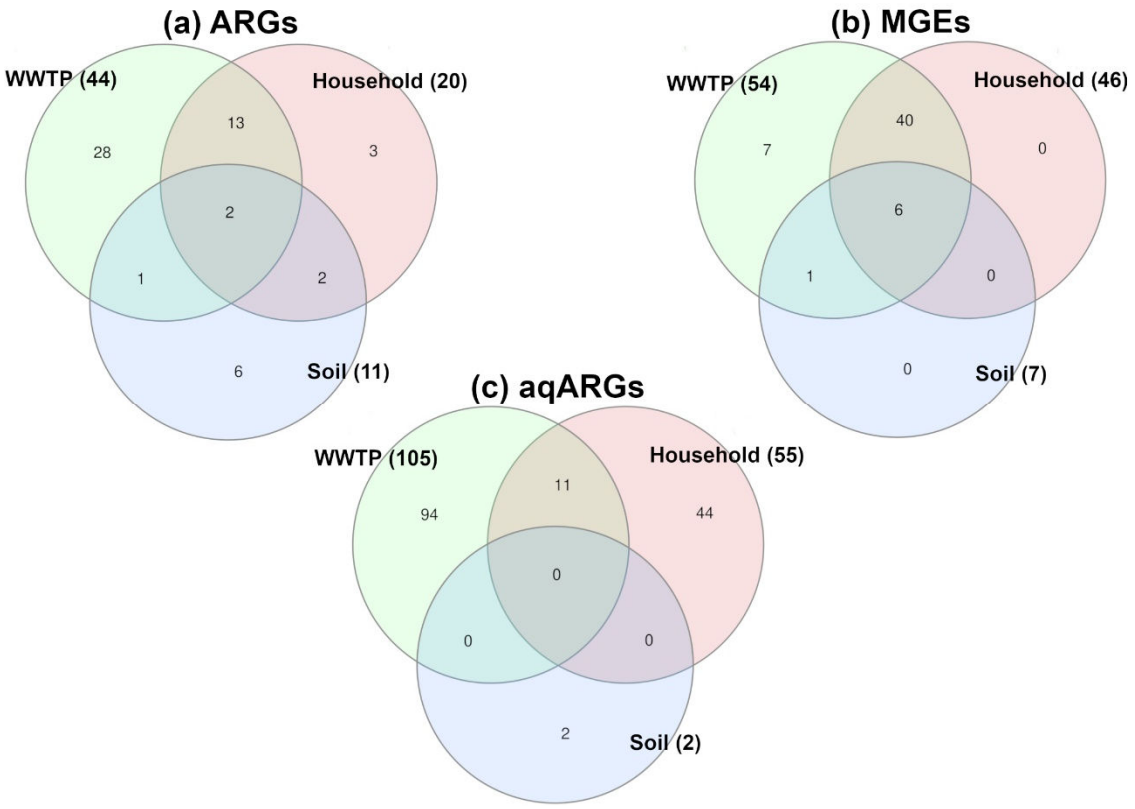

Figure 6. Venn diagram of antibiotic resistance genes (ARGs) (**a**) and mobile genetic elements (MGEs) (**b**) annotated using MvirDB as well as acquired antibiotic resistance genes (aqARGs) (**c**) annotated using Resfinder in the WWTP, soil and HH samples. The number in the bracket indicates the total number of genes annotated for each sample type.

Discussion

Distinct bacterial communities and resistomes in the different environments

The results of this cross-environmental study revealed distinct bacterial communities in the different environments, since both alpha and beta diversity of the bacterial microbiome of soil, WWTP and HH samples differed significantly. Moreover, the ABR factors were distinctive for each environment. Studies indicate that the resistome is mainly shaped by bacterial composition [12–14] since a shared taxonomy leads to overlaps in MGE and ARG prevalence [15], supporting the distinct clustering of ABR factors and ARGs. Hence, our results indicate that resistances rather seem to develop independently in each environment. This is supported by Munck *et al.* (2015), determining that less than 10% of the WWTP resistome were found in other environments [45] and Pal *et al.* (2016) showing clear differences in both ABR pattern and bacterial composition of different environments [39]. The varying environmental conditions given in each environment such as nutrient supply, temperature, pH or wet-and-dry-cycles [46–48] might lead to the distinct bacterial compositions and resistomes.

The extremely unique bacterial community and ABR pattern in WWTPs can be explained by the bacterial-beneficial environment consisting of high nutrients and a constant temperature while contaminants such as antibiotic residues, biocides or heavy metals [47,49–51] select for antibiotic resistant bacteria. This confirms previous studies showing a unique bacterial community [40,45] dominated by bacteria originating from the sewer system [40]. WWTPs are known to be hotspots for ABR [30,52], which could be supported based on species richness, diversity and proportion of ABR factors as well as ARG and MGE richness in WWTP samples.

The analyzed HH sub-environments have a considerable influx of bacteria originating from humans, animals or foodstuff and are frequently exposed to disinfecting, cleaning and personal care products [34,36,53]. Extreme conditions caused by biocides and detergents as well as the frequent change of conditions (e.g. a longer period without use followed by a cleaning cycle in WM and DW) might lead to a selection of well-adapted species and resistant bacteria [46]. This is supported by the low bacterial diversity and significantly higher ARG richness compared to soil samples. Although no comparable data on HH is available so far, studies already determined the prevalence of ABR in households [35–37], suggesting that HH might be an under-investigated reservoir of ABR while WWTPs and soil are analyzed more frequently. Despite of the distinct structure of ABR and bacterial community, the overlap of identified

ARGs and MGEs in HH and WW highlights that domestic sewage represents an important component of WW. Nonetheless, the ARG richness was significantly lower in HH and the set of shared ARGs is most likely connected to the higher diversity and richness of ARGs in WWTP samples.

The bacterial community of soil might be comparably less exposed to stressors such as antibiotic residues and industrial pollutants (WWTP) or cleaning and personal care agents (HH). It has to be kept in mind, that the soil microbiome is often dominated by fungi in terms of biomass [54,55] and that other microbial groups are frequent, such as protists [56], which were all not detected to the full extent in the present study but surely add on to the species richness and diversity of the soil microbiome. Although soil samples revealed the highest taxonomic bacterial diversity and are generally known to harbor a variety of ARGs [1,26,57], the analyzed soils revealed by far the lowest percentage of ABR factors. This is further supported by the low ARG and MGE richness determined in soil samples in our and an earlier study [39], indicating that ARG abundance can still be low in diverse microbial communities. However, our findings could also be due to database biases since the analysis is limited to genes included in MvirDB and Resfinder and generally known ARGs and many ARGs occurring in soils have been unknown before their first detection [2,24]. Forsberg *et al.* (2014) determined a low potential for HGT [12] and thus stressors such as antibiotic residues might favor the selection of existing resistant bacteria in soils rather than the acquisition of ARGs [11].

Transfer between the environments

HGT between bacteria is especially mediated by MGEs such as plasmids, integrons and transposons [17,18], which play a fundamental role in the spread and evolution of ABR [16]. Especially in WWTPs, HGT via MGEs occurs frequently [58–60] and the MGE richness determined in this study was significantly higher compared to soil and HH samples. In contrast, soil samples revealed a low abundance of MGEs and a low mobility of the soil resistome has been determined already [12,39] suggesting that HGT of ARGs is less likely and transfer is limited compared to WWTP samples [13]. HH and WWTP samples revealed more similarities regarding the genus composition, indicating that partially the same species occur and survive in the different environments. HGT between bacteria requires that they inhabit or at least shortly share the same environment [20] and is induced by substances such as antibiotics [61] which is more likely in case of HH and WWTP samples via the release of domestic sewage.

Therefore, the presence of all MGEs identified in HH samples in WWTP samples and the overlaps of ARGs as well might indicate a dissemination of antibiotic resistant bacteria from HH to WWTP. However, biofilms form frequently in the sampled sub-environments of HH and on sewer pipe surfaces [62–65] and especially detected species such as *Pseudomonas* or *Acinetobacter* are prone to biofilm formation, possibly explaining the similarities between WW and HH samples as well. Consequently, to reduce transfer of ABR, limiting the development of resistances in the HH sub-environments by e.g. reduced use of antibiotics, antibacterial agents and biocides in the HH or the occasional use of programs with higher temperatures in WM or DW to remove microbial communities are ways to reduce the spread of ABR.

Although physical forces such as water movement or wind can promote the transfer of soil bacteria [58], a dissemination from HH via sewage to WWTP would be more likely since these environments are immediately interconnected. Antibiotic resistant strains have been isolated from both the EF and the receiving surface waters downstream of WWTPs [49,66,67], highlighting that a transfer to the natural environment is possible as well. However, the application of SS as fertilizer or the irrigation with treated WW is not common in Germany and hence a transfer from the WWTP to the analyzed soil is highly unlikely. Nevertheless, to decrease the dissemination of ARGs from WWTPs, the implementation of advanced technologies such as UV-treatment or chlorination might minimize possible risks [66,68]. In contrast, all ARGs and MGEs in SS and EF were shared with WW while nearly all genes detected in SD and DW matched the WM samples (Fig. S1). This further supports the hypothesis of ABR development or gene transfer within the same environment dominating compared to the exchange between environments. While many factors need to be fulfilled for a transfer between different environments [69], a transfer between bacteria within the same environment can occur frequently. Furthermore, ABR can develop due to spontaneous mutations promoted by selective pressure of antibiotics or biocides [70], which might contribute to the distinct resistomes of the analyzed environments as well. Even though these environments can still act as reservoir for ABR since many ARGs detected in pathogens seem to have their origin in environmental bacteria [71–74], we assume that the transfer between distinct environments might occur less frequently than expected.

Conclusions

We observed distinctive resistomes and only few overlaps of ARGs between the environments, suggesting that ABR predominantly develop in individual environments as caused by the distinct environmental conditions. A transfer of ABR between the different environments is more limited to directly connected environments (e.g. HH to WWTP). The investigated environments revealed distinct bacterial communities with pronounced differences between sub-environments in WWTP and HH, supporting that resistomes are predominantly structured by bacterial phylogeny. Hence, the transfer of ARGs and antibiotic resistant bacteria between different environments might be less important than to focus on the implementation of prevention measures in each individual environment. ABR spread can be limited by reducing the development of resistances in the HH environment, to prevent a low but still possible infection risk for household members and a dissemination within the HH or via domestic sewage to the WWTP. Moreover, a possible transfer of antibiotic residues and resistant bacteria from WWTPs to terrestrial and aquatic environments should be avoided to prevent the promotion of resistance development. This could be achieved by implementing advanced technologies in WW treatment and the prevention of the use of treated WW or SS for fertilization/irrigation. However, more comprehensive studies are needed to compare the resistomes of different environments and to confirm whether or not ABR develop independently in each environment.

Acknowledgements

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4.2 Supplementary data

Sequencing reads

Table 3. Sequence reads per samples of soil (S1, S2, S3), WWTP (WW, SS, EF) and household samples (SD, DW, WM).

sample	total reads	high quality reads	sample	total reads	high quality reads
S1.1	30,155,842	29,392,172	**SS1**	27,841,748	27,184,980
S1.2	30,252,856	29,487,760	**SS2**	29,552,232	28,842,838
S1.3	33,207,516	32,049,660	**SS3**	23,844,194	23,306,058
S1.4	27,088,328	26,289,070	**SS4**	28,851,940	28,162,328
S2.1	27,874,140	27,145,314	**SS5**	45,557,500	44,544,338
S2.2	22,138,400	21,476,644	**EF1**	29,956,520	29,260,546
S2.3	36,275,070	35,326,374	**EF3**	27,685,258	27,026,562
S2.4	26,881,178	26,241,796	**EF4**	29,199,610	28,493,828
S3.1	25,784,402	25,198,700	**EF5**	30,428,190	29,768,104
S3.2	28,240,628	27,420,090	**SD5**	31,657,982	30,737,836
S3.3	25,678,742	24,967,918	**DW**	24,271,104	23,617,480
S3.4	22,515,406	21,833,202	**WM2**	32,182,966	31,423,310
WW1	31,810,164	30,987,066	**WM3**	25,906,062	25,300,232
WW3	25,286,020	24,730,394	**WM4**	27,960,538	27,110,180
WW3	23,253,200	22,800,878	**WM5**	29,590,952	28,754,122
WW5	32,467,628	31,713,750			

Reads assigned to antibiotic resistance factors

Table 4. Percentage and number of reads of virulence factor annotated to ABR factors.

sample	percentage of ABR factors	number of reads
S1	3.2%	20
S2	3.1%	18
S3	3.4%	18
WW	40.7%	8132
SS	44.1%	1150
EF	38.4%	584
SD	28.2%	6002
DW	14.6%	6532
WM	27.1%	4971

Taxonomic distribution

Table 5. Percentage of reads assigned to kingdoms in different soils (S1, S2, S3), wastewater (WW), sewage sludge (SS), effluent (EF), shower drains (SD), dishwashers (DW) and washing machines (WM) in percent.

	S1	S2	S3	WW	SS	EF	SD	DW	WM
Archaea	0.4	0.5	0.4	0.2	0.3	0.1	0.0	0.0	0.0
Bacteria	97.4	97.3	97.4	99.3	98.1	96.5	99.6	97.7	99.5
Eukaryota	0.5	0.5	0.5	0.1	0.4	1.6	0.1	0.0	0.1
Fungi	0.5	0.4	0.5	0.1	0.3	0.6	0.1	1.9	0.1
Viruses	0.0	0.0	0.0	0.1	0.0	0.1	0.0	0.1	0.1
Ambiguous	1.2	1.3	1.2	0.3	0.9	1.2	0.2	0.3	0.2

Acquired antibiotic resistance genes (Resfinder)

Table 6. Acquired antibiotic resistance genes with accession number identified via Resfinder in Wastewater (WW) samples.

WW1	Accession no.	WW3	Accession no.	WW4	Accession no.	WW5	Accession no.
aadA10	U37105	aadA11	AY144590	aadA11	AY144590	aadA11	AJ567827
aadA11	AJ567827	aadA13	AY713504	aadA13	AY713504	aadA11	AY144590
aadA11	AY144590	aadA2	NC_010870	aadA5	AF137361	aadA13	AY713504
aadA16	EU675686	aadA5	AF137361	ant(3")-Ia	X02340	aadA16	EU675686

aaDA5	AF137361	aadA6	AF140629	aph(3")-Ib	AF024602	aadA5	AF137361
aadA6	AF140629	ant(3")-Ia	X02340	aph(3')-Ia	V00359	ant(2")-Ia	X04555
ant(3´´)-Ia	X02340	aph(3")-Ib	AF024602	aph(6)-Id	M28829	ant(3")-Ia	02340
ant(6)-Ia	KF421157	aph(6)-Id	M28829	blaAER-1	U14748	ant(6)-Ia	KF421157
ant(6)-Ia	KF864551	blaAER-1	U14748	blaCARB-10	EU850412	ant(6)-Ia	KF864551
aph(3´´)-Ib	AF024602	blaGES-5	DQ236171	blaGES-5	DQ236171	aph(3")-Ib	AF024602
aph(6)-Id	M28829	blaNPS	AY027589	blaLCR-1	X56809	aph(3")-Ib	AF321551
blaAER-1	U14748	blaOXA-10	J03427	blaOXA-1	HQ170510	aph(6)-Id	M28829
blaLCR-1	X56809	blaOXA-119	DQ767903	blaOXA-10	J3427	blaAER-1	U14748
blaNPS	AY027589	blaOXA-2	DQ112222	blaOXA-119	DQ767903	blaBEL-1	DQ089809
blaOXA-10	J03427	blaOXA-20	AF024602	blaOXA-129	FJWZ01000025	blaCARB-10	EU850412
blaOXA-101	AM412777	blaOXA-205	JF800667	blaOXA-2	DQ112222	blaGES-5	DQ236171
blaOXA-119	DQ767903	blaOXA-347	ACWG01000053	blaOXA-205	JF800667	blaLCR-1	X56809
blaOXA-129	FJWZ01000025	blaOXA-392	AB901044	blaOXA-211	JN861779	blaNPS	AY027589
blaOXA-2	DQ112222	blaOXA-427	KX827604	blaOXA-296	APOH01000009	blaOXA-10	J03427
blaOXA-205	JF800667	blaVEB-1	HM370393	blaOXA-333	KF203107	blaOXA-101	AM412777
blaOXA-211	JN861779	cfxA3	AF472622	blaOXA-347	ACWG01000053	blaOXA-119	DQ767903
blaOXA-296	APOH01000009	mcr-3.6	MF598076	blaOXA-392	AB901044	blaOXA-129	FJWZ01000025
blaOXA-347	ACWG01000053	ere(D)	KP265721	blaOXA-427	KX827604	blaOXA-2	DQ112222
blaOXA-392	AB901044	erm(B)	U86375	blaVEB-1	DQ393569	blaOXA-20	AF024602
blaOXA-4	AY162283	erm(F)	M17808	cfxA3	AF472622	blaOXA-205	JF800667
blaOXA-427	KX827604	lnu(B)	JQ861959	cfxA6	GQ342996	blaOXA-211	JN861779
blaOXA-58 A	Y665723	lnu(C)	AY928180	ere(D)	KP265721	blaOXA-296	APOH01000009
blaVEB-1	HM370393	lnu(F)	EU118119	erm(B)	U86375	blaOXA-333	KF203107
cfxA3	AF472622	lsa(E)	JX560992	erm(F)	M17808	blaOXA-347	ACWG01000053
mcr-5.1	KY807921	mef(A)	AF227520	lnu(B)	JQ861959	blaOXA-35	AF315786
ere(A)	AF099140	mef(A)	AJ971089	lnu(C)	AY928180	blaOXA-392	AB901044
ere(A)	DQ157752	mef(A)	HG423652	lnu(F)	EU118119	blaOXA-4	AY162283
ere(D)	KP265721	mef©	AB571865	lnu(G)	KX470419	blaOXA-427	KX827604
erm(47)	KU612222	mph(A)	D16251	lsa(E)	JX560992	blaOXA-58	AY665723
erm(B)	U86375	mph E	DQ839391	mef(A)	HG423652	blaVEB-1	HM370393
erm(F)	M17808	mph(G)	AB571865	mef(A)	U83667	cfxA5	AY769934
lnu(B)	JQ861959	msr(D)	AF227520	mef(B)	FJ196385	ere(A)	AF099140

lnu(F)	AJ561197	msr(E)	FR751518	mef(C)	AB571865	ere(D)	KP265721
lnu(F)	EU118119	aac(6')-Ib-cr	DQ303918	mph(A)	D16251	erm(47)	KU612222
lsa(E)	JX560992	qnrS2	DQ485530	mph(E)	DQ839391	erm(B)	U86375
mef(A)	AF227520	sul1	U12338	mph(G)	AB571865	erm(F)	M17808
mef(A)	HG423652	sul2	AY034138	mph(N)	KF648874	lnu(B)	JQ861959
mef(B)	FJ196385	tet(39)	KT346360	msr(D)	AF227520	lnu(C)	AY928180
mef©	AB571865	tet(A)	AF534183	msr(D)	AF274302	lnu(F)	EU118119
mph(E)	DQ839391	tet(C)	AF055345	msr(E)	FR751518	lnu(G)	KX470419
mph(G)	AB571865	tet(E)	CP000645	aac(6')-Ib-cr	DQ303918	lsa(E)	JX560992
msr(D)	AF227520	tet(Q)	L33696	qnrS2	DQ485530	mef(A)	AF227520
msr(D)	AF274302	tet(Q)	X58717	qnrVC4	GQ891757	mef(A)	AJ971089
msr(E)	FR751518	tet(W)	AJ427422	sul1	U12338	mef(A)	HG423652
catB3	U13880	dfrA14	KF921535	sul2	AY034138	mef(B)	FJ196385
cmlA1	M64556			tet(39)	KT346360	mef(C)	AB571865
cmx	U85507			tet(A)	AJ517790	mph(A)	D16251
aac(6')-Ib-cr	DQ303918			tet(C)	AY046276	mph(E)	DQ839391
qnrS2	DQ485530			tet(E)	CP000645	mph(G)	AB571865
sul1	AY963803			tet(M)	X04388	msr(D)	AF227520
sul2	AY034138			tet(Q)	L33696	msr(D)	AF274302
tet(36)	AJ514254			tet(Q)	X58717	msr(E)	FR751518
tet(39)	KT346360			tet(W)	AJ427422	catB3	U13880
tet(A)	AJ517790			tet(X)	M37699	catQ	M55620
tet(C)	AY046276			dfrA12	AM040708	cmlA1	M64556
tet(E)	CP000645			dfrA14	DQ388123	cmx	U85507
tet(M)	X04388					aac(6')-Ib-cr	DQ303918
tet(Q)	L33696					qnrS2	DQ485530
tet(W)	AJ427422					qnrVC4	GQ891757
dfrA14	KF921535					sul1	AY963803
						sul2	AY034138
						tet(39)	KT346360
						tet(40)	FJ158002
						tet(A)	AF534183
						tet(C)	AF055345
						tet(E)	CP000645
						tet(M)	X04388
						tet(O/32/O)	FP929050
						tet(Q)	L33696
						tet(Q)	X58717
						tet(Q)	Z21523
						tet(W)	AJ427422
						tet(X)	M37699

Table 7. Acquired antibiotic resistance genes with accession number identified via Resfinder in sewage sludge (SS) samples.

SS1	Accession no.	SS2	Accession no.	SS3	Accession no.
ant(6)-Ia	KF421157	aadA11	AJ567827	aadA2	NC_010870
blaOXA-296	APOH01000009	ant(6)-I	FN594949	mph(A)	U36578
blaOXA-5	AF347074	erm(47)	KU612222	sul1	AY963803
erm(B)	U86375	erm(B)	X66468		
lnu(B)	JQ861959	lsa(E)	JX560992		
lsa(E)	JX560992	mef(A)	HG423652		
mef(A)	HG423652	mef(B)	FJ196385		
mph(E)	DQ839391	msr(D)	AF227520		
mph(N)	KF648874	msr(D)	AF274302		
msr(D)	AF227520	msr(E)	FR751518		
msr(D)	AF274302	sul1	AY963803		
msr(E)	FR751518	sul2	AY034138		
tet(M)	AM990992	catQ	M55620		
sul1	AY963803	tet(W)	AJ427422		
catQ	M55620				

SS4	Accession no.	SS5	Accession no.
blaOXA-10	J03427	aph(6)-Id	M28829
blaOXA-129	FJWZ01000025	blaOXA-5	AF347074
lnu(B)	JQ861959	erm(47)	KU612222
sul1	AY963803	erm(B)	U86375
		erm(F)	M17808
		lnu(B)	JQ861959
		lsa€	JX560992
		mef(A)	HG423652
		mph(E)	DQ839391
		msr(D)	AF227520
		msr(D)	AF274302
		msr(E)	FR751518
		sul1	AY963803
		sul2	AY034138
		EstDL136	JN242251
		catQ	M55620

Table 8. Acquired antibiotic resistance genes with accession number identified via Resfinder in WWTP effluent (EF) and soil (2-1 and 3-3) samples.

EF1	**Accession no.**	**EF3**	**Accession no.**	**S2-1**	**Accession no.**
sul1	AY963803	aph(3")-Ib	AF024602	dfrB3	FM877478
		erm(B)	AF299292		
		sul1	U12338		
		cmx	U85507		
EF4	**Accession no.**	**EF5**	**Accession no.**	**S3-3**	**Accession no.**
aadA11	AJ567827	blaOXA-20	AF024602	dfrB3	X72585
sul1	U12338	sul2	AY034138		

Table 9. Acquired antibiotic resistance genes with accession number identified via Resfinder in dishwasher (DW), shower drain (SD) and washing machine (W) samples.

DW	**Accession no.**	**SD**	**Accession no.**	**WM1-4**	**Accession no.**
aac(6')-Iih	AJ584701	aph(3")-Ib	AF024602	aac(3)-Ich	CP000490
aac(6')-Iz	AF140221	aph(3')-IIb	CP006832	aadA10	U37105
aph(3')-Iic	AM743169	aph(6)-Id	M28829	aadA11	AY144590
blaACT-14	JX440354	blaCMY-49	GQ402541	aph(3")-Ib	AF321551
blaADC-25	EF016355	blaOXA-50	AY306130	aph(6)-Id	M28829
blaEBR-1	AF416700	fosA	ACWU01000146	blaACT-12	JX440355
blaOCH-2	AJ295340	catB7	AF036933	blaCMY-25	EU515249
blaOCH-7	AJ295345	crpP	HM560971	blaOCH-7	AJ295345
blaOXA-258	HE614014	qnrB1	DQ351241	blaOXA-373	HG931732
blaOXA-417	KM220587			blaSHV-187	LN515533
blaOXY-5-1	AJ871868			blaSHV-75	AM176550
fosA	M85195			fosA	ACWO01000079
mdf(A)	Y08743			oqxA	EU370913
tet(42)	EU523697			oqxB	EU370913
				sul1	AY963803
				sul2	AY034138
				aadA10	U37105
				ant(3")-Ia	X02340
				aph(3")-Ib	AF024602
				aph(3')-Iic	AM743169
				aph(6)-Id	M28829
				blaHERA-1	AF311385
				blaOCH-4	AJ295342
				blaOXA-114c	HM056041
				blaOXA-373	HG931732
				catA1	V00622
				crpP	HM560971
				cat(pC194)	NC_002013
				catB7	AF036933

	fosA	ACWU01000146
	aadA10	U37105
	aph(3')-Iib	CP006832
	aph(6)-Id	M28829
	blaOXA-114c	HM056041
	blaOXA-134	HQ122933
	blaOXA-212	JN861780
	blaOXA-282	APQS01000019
	blaOXA-283	AYHO01000005
	blaOXA-333	KF203107
	blaOXA-488	CP017969
	blaPAO	AY083592
	aadA6	AF140629
	aph(3")-Ib	AF024602
	aph(3')-IIb	CP006832
	aph(6)-Id	M28829
	blaOCH-5	AJ295343
	blaOCH-6	AJ295344
	blaOCH-7	AJ295345
	blaOXA-114c	HM056041
	blaOXA-212	JN861780
	blaOXA-396	AY306134
	blaPAO	FJ666073
	crpP	HM560971
	sul1	U12338
	sul2	AY034138

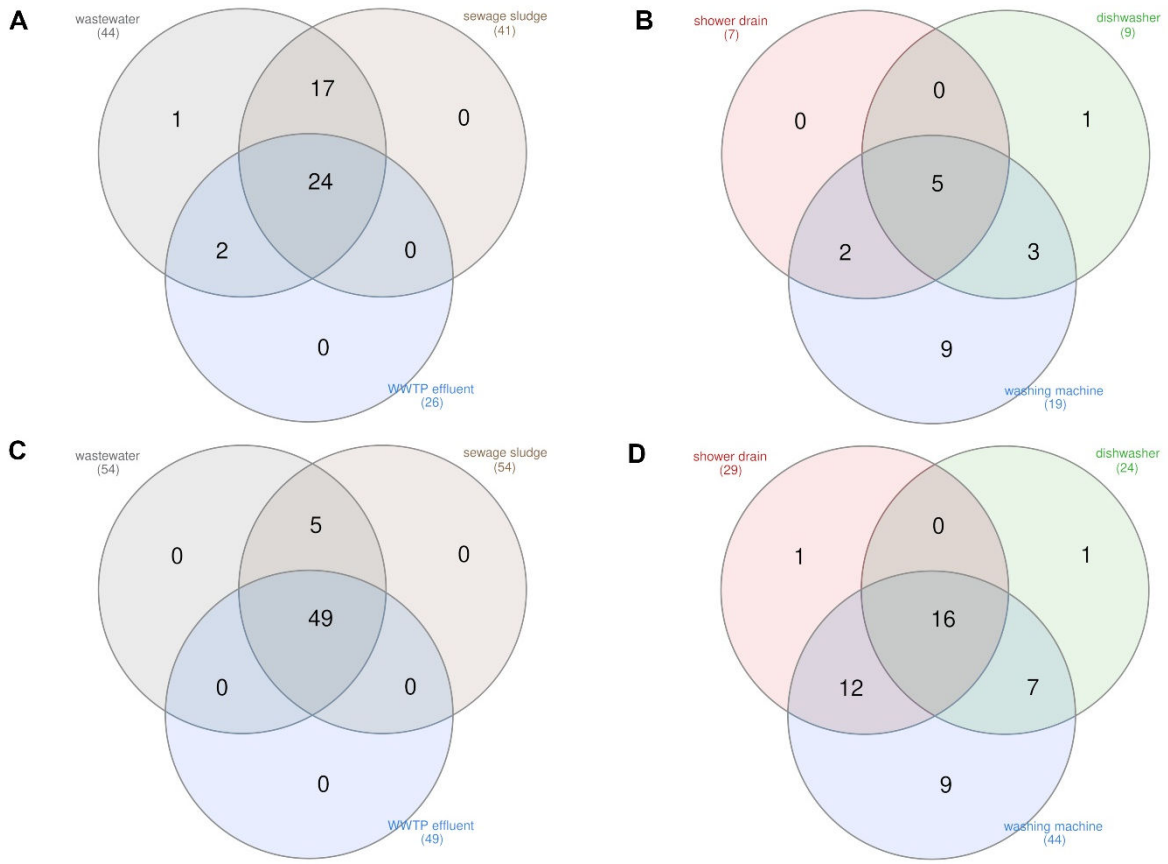

Figure 7. Venn diagram of antibiotic resistance genes and mobile genetic elements in WWTP (**A**, **C**) and household samples (**B**, **D**) annotated using MvirDB. The number in the bracket indicates the total number of genes annotated for each sample type.

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5 Discussion

5.1 Impact of wastewater treatment plants and households on the dissemination of antibiotic resistance

The study revealed a high prevalence of clinically relevant *bla* genes while *mcr-1* occurred less frequently in wastewater in the district of Kleve, Germany. We showed that wastewater treatment was apparently successful in reducing the amount of ARGs and antibiotic resistant bacteria since ARG abundance was significantly higher in the influent compared to the effluent. This corresponds well with other studies [142,173,179] although even increasing amounts of ARGs due to wastewater treatment have been reported [169,177]. However, potential pathogens such as *Pseudomonas* spp. or *Enterobacteriaceae* such as *E. coli* harbouring genes encoding resistances to carbapenems or extended spectrum cephalosporins were still isolated from the WWTP effluent. Since other studies determined the release of antibiotic resistant bacteria from WWTPs into receiving surface waters [114,165,226], it can be assumed that a dissemination of ABR regarding the investigated WWTP to the Rhine might occur as well. Furthermore, *intI1* has been identified as an indicator of pollution with heavy metals, antibiotics or personal care products and has already been used as a marker for the spread of ARGs [187,227] since it is frequently connected with carrying multiple ARGs [53,60]. Our results also revealed high concentrations of class 1 integrons, hence indicating that bacteria in WWTPs might serve as reservoirs for integrons that are released via the effluent. Koczura *et al.* (2016) already observed a significant increase of *intI1* in the receiving river of a WWTP [228], thus it can be speculated that ARGs are captured and disseminated by integrons in WWTPs and in the natural environment downstream the WWTP. Coexistence of ESBL or carbapenemase genes and *mcr-1* has been described before [87,120,122]. However, we detected an ESBL-producing *E. coli* strain carrying *mcr-1*, *bla*$_{\text{VIM}}$ and *bla*$_{\text{OXA-48}}$ and a MDR-*E. cloacae* strain harbouring *bla*$_{\text{OXA-58}}$ and *mcr-1*, which, to the best of our knowledge, did not occur so far. Moreover, the *E. coli* strain with the same phenotypic and genotypic resistances was isolated at all stages of the WWTP, indicating that the same strain possibly endured the wastewater treatment.

Interestingly, a correlation of ARG abundance and lower temperatures in wastewater was observed. It has been shown that cold stress response of bacteria caused by lower temperatures can induce an increase of ARGs and integrons due to HGT [229]. In addition, Caucci *et al.* (2016) even determined a correlation of antibiotic prescriptions and ARG levels in wastewater [230], and data of the Association of Statutory Health Insurance Physicians North

Rhine revealed a higher antibiotic consumption in colder months compared to warmer months during the sampling period [231]. The bacterial community of wastewater is usually composed of human commensal and environmental bacteria, which might have been exposed to antimicrobial agents from clinical and agricultural settings as well as private households [180]. Thus, the domestic environment and WWTPs might comprise a similar microbial composition, and the higher ARG abundance in wastewater could be connected to higher prescription rates and antibiotic use during the colder months in domestic and clinical settings connected to the analysed WWTP.

Both resistant strains of *Enterobacteriaceae* and *Pseudomonas* spp. dominated in the investigated WWTP samples and are known to harbour *bla* and *mcr* genes [58,232]. In our study of the domestic environment, these taxa also dominated apart from intrinsic resistant *Stenotrophomonas maltophilia*. Resistant strains of these genera also occur in the clinic [206,233] and the natural environment [126,148,152], indicating that bacteria of different habitats serve as reservoirs for ARGs. Moreover, although Bray-Curtis dissimilarity revealed distinct clustering of samples of the same environment, the beta diversity of wastewater samples seemed to be more similar to the microbial community of the domestic environment than sewage sludge or WWTP effluent. Domestic wastewater is a major component of the WWTP influent and might also play a role in the dissemination of ARGs and antibiotic resistant bacteria. In contrast to the WWTP, we detected no *mcr-1* genes in washing machines, dishwashers or shower drains in private households. However, *ampC* genes and $bla_{\text{OXA-48}}$ were detected most frequently and revealed high abundances in WWTP samples as well. Compared to the WWTP where domestic, industrial and clinical sewage accumulates, the abundance of MDR bacteria and ARGs in the domestic environment was much lower. This might also be due to the higher species richness in WWTP samples, since genetic diversity can be associated with taxonomic diversity and thus a diverse composition of antibiotic resistant bacteria as well [234].

Nevertheless, ARGs as well as antibiotic resistant bacteria were detected in private households, and the same genes partly occurred at all sub-environments within a household, prompting speculation that a transfer might take place. This dissemination is likely to occur by contaminated eating utensils, laundry or household members [30,147,235]. Schmithausen *et al.* (2019) determined a transmission of ESBL-producing *K. oxytoca* from a washing machine to newborns highlighting the possible infection risk posed by cross-contaminations. The isolation of clinically relevant species such as MDR-*P. aeruginosa* and ESBL-*E. coli* harbouring carbapenemase genes suggests that the domestic environment might be a potential reservoir for

the spread of β-lactam resistance as well. We showed that all antibiotic resistant test strains were reduced significantly during laundering and dishwashing, and since bacteria on textiles usually originate from the human microbiome or the environment, the infection risk should be rather low. However, bacterial counts of approx. 10^3 cfu mL^{-1} were still determined after laundering at 40 °C for 60 min using a bleach-free detergent and if a sufficient level of hygiene must be guaranteed, higher temperatures or the use of a bleach-containing detergent might be necessary [236]. Moreover, laundering at lower temperatures in combination with bleach-free liquid detergents might lead to cross-contaminations of the laundry or the washing machine itself [209,237] with antibiotic resistant bacteria, possibly enabling their transfer within the household or via the greywater to WWTPs. Hence, in critical cases such as acute infections or risk groups being nursed at home, the use of programs with higher temperatures and bleach should be preferred [208].

HGT between bacteria is especially mediated by MGEs such as plasmids, integrons and transposons [10,11] which promote genetic diversity and thus play a fundamental role in the spread and evolution of ABR [49]. In both WWTP and household samples the total ARG abundance correlated with *intI1* abundance, thus the genes could be located on class 1 integrons or other MGEs harbouring both *intI1* and ARGs. Furthermore, we showed that all identified MGEs in household samples were the same that occurred in WWTP samples, possibly enabling their transfer between these environments. However, biofilms form frequently in the sampled sub-environments of households and on sewer pipe surfaces [212,213,238,239], and particularly species such as *Pseudomonas* or *Acinetobacter* are prone to biofilm formation, possibly explaining the taxonomic similarities between wastewater and households samples as well. McLellan *et al.* (2010) determined that only small fractions of human faecal bacteria are represented in wastewater and that the sewer system is a distinct environment itself [240], indicating that on the way of wastewater to the WWTP, an independent bacterial community seems to form.

Environments with increased exposure to antibiotics, biocides or heavy metals are often highly contaminated with ARGs of clinical relevance [241]. While WWTPs have already been identified as hotspots for ABR promoting HGT between bacteria [143,158,159], the significance of the domestic environment is comparatively less understood. Our obtained results substantiate that the domestic environment represents a potential reservoir of *bla* genes and β-lactam resistant bacteria as well. The determination of the co-occurrence of *bla* genes

indicates the prevalence of bacterial species possibly harbouring multiple ARGs or bacterial communities harbouring multiple β-lactam resistant species in washing machines, dishwashers and shower drains. This is supported by the detection of various *bla* genes in resistant bacterial strains isolated from the household environment. Hence, the positive correlation of carbapenemase and *ampC* genes with carbapenem and cefotaxime or ceftazidime resistance, respectively, shows that the phenotypic resistance is most likely based on β-lactamase activity. Besides β-lactam resistance, our metagenome study of different environments also revealed inter alia the prevalence of chloramphenicol, aminoglycoside and tetracycline resistance genes in households.

Antibacterial agents are frequently present in households as part of personal care or cleaning products and might lead to bacteria resistant to these agents or even cross-resistances to antibiotics [242]. The use of triclosan has already been linked to the selection of bacteria with higher resistance to antibiotics [243], highlighting the potential of households as a reservoir of ABR. Additionally, the aim to improve the energy-efficiency of household appliances by reducing the temperature can favour microbial growth [236]. Humans and companion animals are regularly exposed to antibiotics, thus promoting the development of resistances. Therefore, on the one hand, antibiotic residues are released in the household, resulting in the selection of antibiotic resistant bacteria and on the other hand already resistant species can be introduced into the domestic environment via the human or animal microbiota. The connection of washing machines, dishwashers and shower drains to the water supply system [244] might promote the spread of ARGs and antibiotic resistant bacteria from, to and within households. Although bacterial counts are usually sufficiently reduced during drinking water treatment, the number of bacteria increases again due to the formation of biofilms in water supply systems [245]. Biofilms regularly form on inner pipe surfaces and harbour diverse microbial communities [151,212] with higher tolerance to environmental factors (such as exposure to antibiotics) and promote the acquisition and spread of ARGs via HGT [156,157]. Our results showed a higher abundance of β-lactam resistance in shower drains compared to washing machines and dishwashers. Even though biofilms occur in washing machines and dishwashers as well [212,246], higher temperatures and desiccation only enable species well-adapted to high temperatures and drying to grow [246,247] while shower drains supply a less extreme environment. Studies indicate that the resistome is mainly shaped by bacterial composition [144,248,249], and the bacterial diversity in households, despite of close clustering of the sub-environments and hence more similarities, still revealed different bacterial compositions in

shower drains, washing machines and dishwashers. Therefore, differences in the bacterial community might also be responsible for differences in ARG abundance. Furthermore, Gram-positive bacteria seem to dominate in dishwashers [213], and biofilm formation occurs more frequent in the inner parts of washing machines [212], which might explain a lower ARG abundance in these samples. Apart from biofilms, the higher level of *bla* genes in shower drains could also be linked to the shed of antibiotic resistant strains from the human body. Antibiotic resistant bacteria have been detected in the microbiota of the gastrointestinal tract [250,251], the skin [252] and the oral cavity [253], and thus a detachment of these strains during showering seems inevitable. Shower and sink drains have been associated with β-lactamases, MDR bacteria and nosocomial outbreaks [203,204] and frequently reveal a high prevalence of *Pseudomonas* spp. and coliforms [239]. We dominantly found strains of these genera paired with *bla* genes, ESBL-production or multi-drug resistance in shower drains, indicating a higher resistance potential compared to washing machines and dishwashers. This is further supported by a study of the frequency of antibiotic resistant bacteria in households performed by Marshall *et al.* (2012) determining highest amounts of antibiotic resistant bacteria in sink drains [189] and by the identification of shower and sink drains as hotspots for *intI1* by Lucassen *et al.* (2019) [187]. Hence, a transfer of antibiotic resistant bacteria located on the human body to shower drains resulting in their selective accumulation due to the exposure to biocides used in cleaning agents and personal care products [189,254] could explain the higher ABR levels in shower drains. Although the metagenome analysis determined significantly lower ARG richness in households than WWTP samples, the number of ARGs was still significantly higher compared to soil. Consequently, ARGs and antibiotic resistant bacteria might be disseminated into the environment via domestic wastewater. Our overall results suggest that households are an under-investigated reservoir of ABR, even though the human health risk should be rather low. However, further studies are needed to confirm the possible risk of ARGs and antibiotic resistant bacteria in the domestic environment.

5.2 Interrelationship of antibiotic resistance in different environments

The analysis of the resistome of different environments is a helpful tool to determine possible reservoirs of antibiotic resistant bacteria and ARGs. In this matter, identifying most prevalent bacterial taxa besides ARG abundance is important since these taxa might enable the transfer of ARGs between environments. A metagenomic approach enables the investigation of the resistome without limitation to certain organisms. Hence, it is not surprising that many studies

conducted metagenome analyses of wastewater, soil, surface water, humans and animals [144,234,240,248] revealing the widespread of ABR. However, we performed one of few studies investigating different environments at once, with households being rather neglected so far. This cross-environmental analysis revealed both distinct bacterial community compositions and resistomes in soil, WWTP and household samples.

In the three sampled environments, different factors and stressors are impacting the bacterial community, which might result in species sorting by environmental conditions and thus the observed distinct bacterial community compositions [255]. The analysed sub-environments of households have a considerable influx of bacteria originating from humans, companion animals or food and are frequently exposed to cleaning, disinfecting and personal care products [187,189,242]. Hence, the contained biocides and detergents might lead to a selection of well-adapted species and resistant bacteria. Although shower drains, washing machines and dishwashers provide a steady nutrient supply in a humid environment promoting bacterial growth and biofilm formation [239,246,256], the samples had a rather low bacterial diversity. However, Savage *et al.* (2016) determined that extreme conditions such as high temperatures or high pH in dishwashers or washing machines [247] result in a lower diversity. Bacteria in shower drains are frequently exposed to cleaning and personal care products and thus they could be considered challenged by an extreme environment as well. Although even within the household bacterial communities of the sampled sub-environments slightly differed, more similarities compared to soil or WWTP samples were observed (see 4.1 Figures 2 and 3). The differences are most likely due to varying environmental conditions such as temperature or wet-and-dry-cycles. In contrast to households, temperature, water flow or organic matter load on the one hand and antibiotic residues, biocides or heavy metals on the other hand [169,241,257] affect the bacterial community structure of WWTPs and select for antibiotic resistant bacteria. Wastewater is high in nutrients resulting in a high bacterial density and is exposed to substances such as antibiotics or heavy metals [169,257,258], forming a unique bacterial community [240,259]. The differences of effluent compared to wastewater is most likely caused by the biological treatment with sewage sludge containing nitrifying and denitrifying bacteria such as *Nitrospira* [260,261] for the removal of pollutants and xenobiotics. Soil is a complex ecosystem comprising a diverse microbiome often dominated by fungi in terms of biomass [262,263] and revealed the highest bacterial diversity in our study. Despite many environmental factors impacting the bacterial community in soils such as pH, water, nutrient availability or particle size [1], it seems to be mainly affected by pH changes [264]. Moreover, soil bacterial

communities might be comparably less exposed to stressors such as antibiotic residues and industrial pollutants or cleaning and personal care products.

The resistome is mainly shaped by bacterial composition [144,248,249] and since distinct bacterial communities of the sampled environments were observed, the resistomes revealed distinct structures as well. This is supported by the detection of nearly all ARGs and MGEs identified in shower drains and dishwashers shared with washing machine samples and all genes in sewage sludge and WWTP effluent shared with wastewater. WWTPs are hotspots for ABR [158,181] and gene transfer via MGEs [159,227,265]. Therefore, a higher prevalence of ABR in WWTP samples compared to soil was expected and is consistent with other studies [234,266]. Interestingly, ARG richness in households was significantly higher compared to soils as well. This is supported by our previous results and other studies [187–189], indicating that the household might be an under-investigated reservoir of ABR. HGT between bacteria requires that they inhabit or at least shortly share the same environment [267] and occurs more frequently between closely related bacteria [268]. Therefore, a transfer between bacteria within the same habitat or the development of resistances due to spontaneous mutations promoted by selective pressure of antibiotics or biocides [202] is more likely than a transfer from one environment to another. Soil samples revealed by far the lowest amount of ABR determinants despite a high taxonomic diversity, even though soils are known to harbour a variety of ARGs [3,125,130]. However, these findings could also arise from database biases and unknown genes in the soils. Since domestic sewage is considered an important part of wastewater, a transfer of bacteria from households to WWTPs is possible. We showed that the bacterial community of wastewater shared more similarities with the household environment compared to soil and even sewage sludge or WWTP effluent. Moreover, an overlap of ARGs and MGEs was observed. However, only small fractions of human faecal bacteria have been determined in wastewater so far [240], and biofilm formation in the sampled sub-environments of households and sewer systems [212,213,238,239] might also be responsible for these similarities. The transfer of soil bacteria can be promoted by physical forces such as water movement or wind [125], but still a dissemination from households via domestic wastewater to WWTP would be more likely since these environments are immediately connected. Hence, a transfer rather follows the flow of substances. Furthermore, the application of sewage sludge as fertilizer or the irrigation of agricultural soils with treated wastewater is not common in Germany, and thus a transfer from the WWTP to the analysed soil is highly unlikely. However, antibiotic resistant bacteria have been isolated from both the WWTP effluent and the receiving surface waters downstream of

WWTPs [149,150,169], highlighting that a transfer to the natural environment is possible. Thus, the implementation of advanced treatment technologies might decrease a possible spread of ARGs from WWTPs. For HGT between different environments many factors need to be fulfilled [269] whereas it occurs more frequently between bacteria within the same habitat. Although an actual transfer of antibiotic resistant bacteria between some settings (e.g. from households to the WWTP) might be possible, it still requires the survival of the bacteria under the new environmental conditions. Hence, ABR seem to predominantly develop in each environment as caused by the selection of bacterial species due to distinct conditions or spontaneous mutations promoted by selective pressure of antibiotics, biocides or heavy metals [202,241]. Even though the analysed environments can still act as reservoir for ABR, our results indicate that an exchange between distinct habitats might occur less frequently than expected.

5.3 Future prospects

In this thesis, different environments of the same geographical region were analysed to elucidate possible ways of transmission of antibiotic resistant bacteria with special focus on private households. While the role of WWTPs as hotspots of ABR was confirmed, ARGs and antibiotic resistant bacteria occurred frequently in private households, highlighting that the domestic environment might act as a reservoir for ABR as well. However, distinct bacterial compositions and resistomes of soils, WWTP and households substantiate that, although a frequent development of mobile resistance genes is likely [269], only few of these determinants are transferred between or established in distinct bacterial communities (Fig. 8). For a transfer between different environments many factors need to be fulfilled, and it thus might be more important to implement strategies to minimize the development of resistances within each habitat. Consequently, further studies of households are needed to confirm the possible risks of dissemination of resistant bacterial species and ARGs within households, since it is the environment closest to humans.

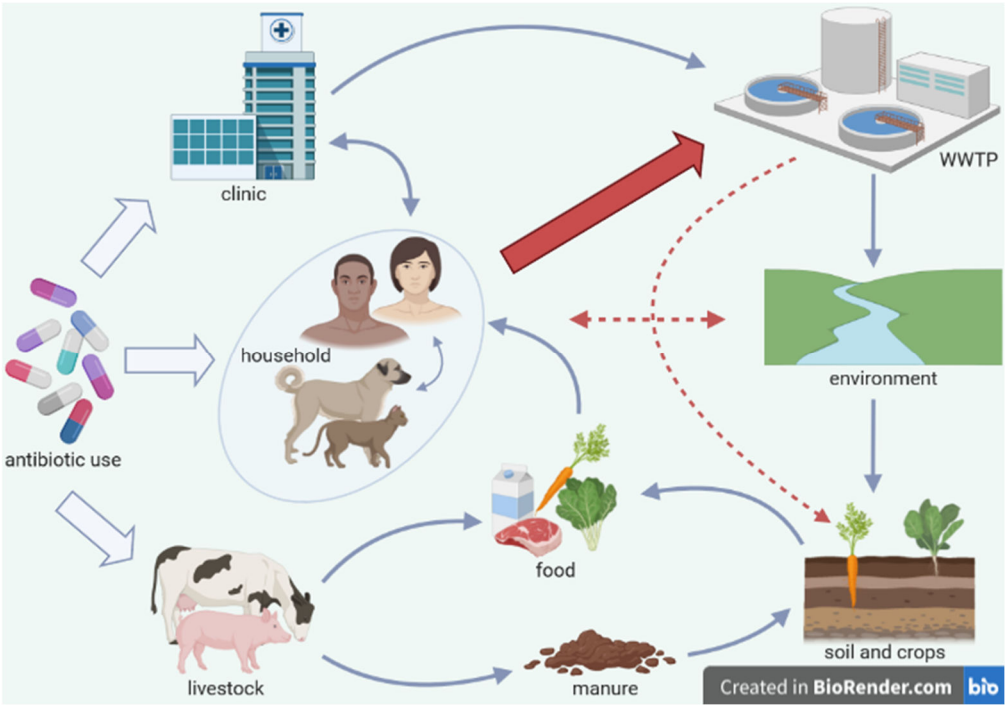

Figure 8. Potential transmission routes of antibiotic resistance between different environments. Red arrows represent the investigated routes and dashed lines highlight limited transfer (Created with BioRender.com).

To limit the development of resistances in private households, the use of antibiotics, disinfectants and biocides needs to be reduced. Antibiotics are often prescribed without identification of the pathogen to act time- and cost-efficient, promoting the development of ABR. Hence, new methods for pathogen and antibiotic resistance diagnostics are needed to enable fast and cost-effective possibilities, which would improve the treatment of infections. Since an inappropriate antibiotic leads to a delayed cure of the patient, a more efficient antibiotic therapy would result in lower consumption rates. Besides the improvement in antibiotic prescribing, the behaviour of the consumer needs to change as well. The use of antimicrobial agents is often driven by the patient's demand for an immediate action to cure the infection [270]. Moreover, consumers need to finish the antibiotic therapy as prescribed and self-prescription without advice of a physician needs to be avoided. Subsequently, a diligent use of antibiotics by both physicians and patients is necessary to limit the selective pressure on bacteria and thus to decrease the development of resistance. Additionally, the overuse of disinfectants and biocides results in the selection of ABR as well. Hence, the consumer's awareness and knowledge about the correct use of antibiotic and disinfecting agents in the home needs to be improved. This could be achieved by the publication of recommendations for

consumers by e.g. the “Verbraucherzentrale” or “Forum Waschen”. However, if a sufficient level of hygiene must be guaranteed especially in critical situation such as acute infections or pandemics (e.g. COVID-19 pandemic), the use of disinfecting agents might be necessary. Another strategy to minimize the risk of infections or ABR dissemination could be the occasional use of programs with higher temperatures in washing machines or dishwashers to achieve an efficient removal of bacteria present in these appliances. Although significant reductions of the microbial load during laundering and automated dishwashing on textiles/dishes were shown, low bacterial counts were still detected and resistant bacteria were isolated frequently from appliances in private households. In case of pre-disposed persons, such as elderly people, pregnant women, young children or immunocompromised patients, antibiotic resistances may pose an additional health risk, especially regarding the increasing number of people being nursed at home. Therefore, the occasional use of higher temperatures could help to remove bacterial communities and thus reduce a possible risk of infection and transfer.

Besides the domestic environment, prevention measures in WWTPs as melting pots of ABR are necessary to limit a possible release of antibiotic resistant bacteria via the treated wastewater. In some instances, treated wastewater and sewage sludge even contain higher ABR levels compared to the untreated sewage [169,173,177] and if used for irrigation of agricultural soils or as fertilizer, a transfer is possible [174]. Advanced treatment technologies such as UV-treatment, chlorination, ozonation or membrane filtration proved to be efficient in reducing the level of ARGs and antibiotic resistant bacteria to a level comparable to low impacted water bodies [271]. Therefore, the implementation of advanced technologies in WWTPs and further research of combinations of different treatment methods could help to efficiently reduce the release of antibiotic resistant bacteria in the surrounding environments. Furthermore, consistent regulations in agriculture regarding the use of antibiotics in livestock and sewage sludge, manure or treated wastewater on fields might further reduce the dissemination of ABR.

Annually 33,000 deaths are caused by infections with antibiotic resistant bacteria in Europe alone [34], highlighting the significance of preventing the spread and development of ABR. In this respect, this thesis gives new insights into the prevalence of β-lactam resistance in WWTPs and households and provides evidence for the distinct development of ABR in each environment induced by environmental selection resulting in distinct resistomes and only limited transfer.

6 References to chapter 1 and 5

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Research contributions

Scientific Papers

- **L. Schages**, F. Wichern, R. Kalscheuer, D. Bockmühl (2020) Winter is coming – Impact of temperature on the variation of β-lactamase and *mcr* genes in a wastewater treatment plant. Sci Total Environ 712(2020)
- **L. Schages**, R. Lucassen F. Wichern, R. Kalscheuer, D. Bockmühl (2020) The household resistome – frequency of β-lactamases, class 1 integron and antibiotic resistant bacteria in the domestic environment and their reduction during automated dishwashing/laundering. Appl Environ Microbiol 86(23)
- **L. Schages**, F. Wichern, R. Kalscheuer, D. Bockmühl (2020) Soils, wastewater treatment plants and households have distinctive resistomes revealing little evidence of antibiotic resistance transfer between environments. (submitted)

Poster Presentations

- **L. Rehberg,** D. Bockmühl: Antibiotic-resistant bacteria in domestic appliances and the impact of laundering. SEPAWA Congress, Berlin, D, October 2018
- **L. Rehberg**, F. Wichern, R. Kalscheuer, D. Bockmühl: Occurrence of β-lactamase genes in the domestic environment. 71. Jahrestagung der Deutschen Gesellschaft für Hygiene und Mikrobiologie (DGHM) e. V. Göttingen, D, March 2019

Further Publications

- R. Lucassen, **L. Rehberg**, M. Heyden, D. Bockmühl (2019) Strong correlation of total phenotypic resistance of samples from household environments and the prevalence of class 1 integrons suggests for the use of the relative prevalence of *intI1* as a screening tool for multi-resistance. PLOS ONE 14(6)
- D. Bockmühl, J. Schages. **L. Rehberg** (2019) Laundry and textile hygiene in healthcare and beyond. Microb Cell 6(7)
- M. Zinn, **L. Schages**, D. Bockmühl (2020) The toothbrush microbiome: Impact of user age, period of use and bristle material on the microbial communities of toothbrushes. Microorganisms 8(9)

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Eidesstattliche Erklärung

Ich, Frau Laura Schages, versichere an Eides Statt, dass die vorliegende Dissertation von mir selbständig und ohne unzulässige fremde Hilfe unter Beachtung der „Grundsätze zur Sicherung guter wissenschaftlicher Praxis an der Heinrich-Heine-Universität Düsseldorf“ erstellt worden ist.

Düsseldorf, den

Laura Schages