DIE UNTERSUCHUNG VON WÄSSRIGEN MATRICES AUF RÜCKSTÄNDE ANTIBIOTISCH WIRKSAMER SUBSTANZEN MITTELS LC-MS/MS

INAUGURAL – DISSERTATION

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von

Alexander Maximilian Voigt
staatlich geprüfter Lebensmittelchemiker
aus Köln

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1. **Gutachter:** Prof. Dr. rer. nat. Matthias Wüst

2. **Gutachter:** Prof. Dr. med. Dr. h.c. Martin Exner

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Summary

Due to increasing concern regarding antibiotic-resistant bacteria and the serious consequences of a predicted post-antibiotics era, an interdisciplinary research approach is being pursued by science, politics and industry to develop appropriate preventive measures against the development and spread of antibiotic-resistant bacteria. The "One-Health" action plan covers the aquatic environment as one pillar related to the spread and development of antibiotic resistance, in addition to intensive livestock farming, human and veterinary medicine.[1,2]

In this context, suitable analytical methods are required for the analysis of antibiotic residues in environmental samples at subinhibitory residual concentrations (mostly in the nanogram per litre range). These concentrations may be sufficient to generate selection pressure in favour of antibiotic-resistant bacteria or to promote the acquisition of antibiotic resistance, either via horizontal gene transfer or point mutations.[3,4] Antibiotic residues can enter the aquatic environment via different pathways (e.g. treated wastewater, sludge, digestates, the use of antibiotics in aquacultures and plant cultures).[1] Thus, screening methods that cover a broad spectrum of antibiotics (including veterinary and human pharmaceuticals) and which are sufficiently sensitive are needed.

In this dissertation the development of a novel analytical method is described, by which a large number of antibiotics can be measured simultaneously, and without enrichment of the analyte concentrations, via liquid chromatography coupled with tandem mass spectrometry. This “direct injection” method is characterized by sufficient sensitivity (limit of quantification: 3.3 ng/L – 190 ng/L) and appropriate recovery rates for surface water, drinking water, groundwater and treated wastewater (65% ± 13% – 117% ± 5%).[5]

The method developed here allows further investigation of known sources of antibiotic residues and the identification of new entry pathways of antibiotic residues into the aquatic environment. In addition, the number of analytes (45 different antibiotics from eleven different substance classes and two environmentally-relevant metabolites) allows an investigation independent of point sources (human medicine, in- and outpatient, as well as veterinary medicine).[5]

For the first time, this work showed that the wastewater in sink siphons, toilets and shower drainpipes in specific clinical areas can be a reservoir for high residual concentrations of antibiotics, especially clinically-relevant antibiotics. Such residue concentrations (up to 79 mg/L) may be sufficient to exert selection pressure in favour of antibiotic-resistant bacteria or to encourage the acquisition of antibiotic-resistant bacteria. Shower drainpipes appear to be a potential hot spot for high residual concentrations of a wide spectrum of antibiotic residues. These new findings will be taken into account in future recommendations of the German Commission for Hospital Hygiene and Infection Prevention (KRINKO) related to the hygiene requirements for wastewater-carrying systems in medical facilities. In addition, the results of this study showed that biofilms in the wastewater network can serve as storage for antibiotic residues. Accordingly, changes in regulations governing cleaning procedures or structural-functional changes in connection with the removal of biofilm or the prevention of the formation of new biofilms appear to make sense not only from a microbiological but also from a chemical point of view.[6]

Further along the course of the sewer network, the influence of clinical wastewater on municipal mixed wastewater can also be determined by the detection of specific antibiotic residues, antibiotic-resistance genes and antibiotic-resistant bacteria. For specific antibiotic-resistant bacteria (third-generation cephalosporin-resistant *Klebsiella* spp., *Enterobacter* spp., *Citrobacter* spp. or *Pseudomonas aeruginosa*), as well as antibiotic-resistance genes (bla_{OXA48}, bla_{VIM2}, bla_{NDM} and $bla_{CTX\text{-}M}$), the probability of detection was increased if residues of ciprofloxacin, ceftazidime or meropenem were detected in the wastewater simultaneously. Furthermore, noticeable differences in this probability could be observed between clinical and communal wastewater. In addition, the results of this study showed that high concentrations of residues of meropenem and ciprofloxacin may serve as markers for the presence of multidrug-resistant bacteria in wastewater.[7]

In addition to exposure to clinical or municipal wastewater via, for instance, sanitary units, the discharge of treated wastewater into certain surface waters represents a possible feedback pathway for antibiotic-resistant bacteria, antibiotic resistance genes and antibiotic residues to humans. Of particular interest, in this respect, are drinking water reservoirs or bathing waters.

In this context, a German river, which is affected by the discharge of treated wastewater from two municipal sewage treatment plants and which discharges into a drinking water reservoir, was investigated for antibiotic residues, antibiotic-resistant bacteria and antibiotic resistance genes. The results of this study showed that a significant influence of wastewater have been measured by the detection of antibiotic residues, antibiotic resistance genes and antibiotic-resistant bacteria in the river below the discharge of sewage treatment plants. However, a rapid decrease in concentrations of antibiotic residues and antibiotic-resistant bacteria along the river could be observed. Thus, no significant differences were found between the sampling points in the river above the discharge of the sewage treatment plant and those below the drinking water reservoir.[8]

The analytical method developed in this dissertation in combination with molecular biological and cultural methods seems to be suited to the future investigation of other surface waters used for drinking water production or bathing waters in order to assess the influence of wastewater discharge in relation to the burden of antibiotic residues, antibiotic-resistant bacteria and antibiotic resistance genes. On the basis of the results obtained, a database could be created which could provide a baseline for comparison and which would allow future developments and trends to be assessed and evaluated.[8]

Zusammenfassung

Vor dem Hintergrund einer zunehmenden Resistenzproblematik und der schwerwiegenden Folgen einer möglichen Post-Antibiotika-Ära steht ein interdisziplinärer Forschungsansatz bei der Entwicklung geeigneter Präventionsmaßnahmen bezüglich der Entwicklung und Verbreitung von antibiotikaresistenten Bakterien im Fokus von Forschung, Politik und Wirtschaft. Unter dem Begriff „*One-Health*“ werden neben der Human-, Veterinärmedizin und Landwirtschaft auch die aquatische Umwelt als tragende Säule der Antibiotikaresistenzprävention bzw. -forschung zusammengefasst.[1,2]

Diesbezüglich werden geeignete Analysemethoden zur Untersuchung auf Antibiotikarückstände in Umweltproben in Konzentrationsbereichen bis in den zweistelligen ng/L-Bereich benötigt. Diese subinhibitorischen Rückstandskonzentrationen können ausreichend sein, um die Verbreitung und Entwicklung von Antibiotikaresistenzen zu begünstigen.[3,4] Der Eintrag von Antibiotikarückständen in die aquatische Umwelt kann hierbei über unterschiedliche Eintrittspfade erfolgen,[1] sodass ein Bedarf an Screening-Methoden besteht, die ein möglichst breites Spektrum an Antibiotika abdecken und sich durch eine ausreichende Empfindlichkeit auszeichnen.

Die im Rahmen dieser Dissertation entwickelte Analysenmethode und deren Anwendung zeigen, dass eine Vielzahl an Antibiotikarückständen simultan und ohne Anreicherung der Analytkonzentrationen via Flüssigchromatographie gekoppelt mit Tandem-Massenspektrometrie mit ausreichender Empfindlichkeit (Bestimmungsgrenze: 3,3 ng/L – 190 ng/L) nach Direktinjektion in wässrigen Umweltproben nachgewiesen werden können. Ferner können trotz des Verzichts auf eine aufwendige Probenaufbereitung gute Wiederfindungsraten für die untersuchten Matrices Oberflächen-, Trink- und Grundwasser sowie behandeltes Abwasser erreicht werden (65% ± 13% - 117% ± 5%).[5]

Die entwickelte Multimethode erlaubt zukünftig die weitere Untersuchung bereits bekannter und die Identifizierung neuer Eintragspfade von Antibiotikarückständen in die aquatische Umwelt, wobei die Anzahl an Analyten (45 verschiedene Antibiotika aus elf unterschiedlichen Substanzklassen und zwei umweltrelevante Metaboliten) eine vom Emittenten (ambulante bzw. klinische Humanmedizin sowie Veterinärmedizin) unabhängige Untersuchung erlaubt.[5]

Bei der weiteren Charakterisierung möglicher Eintragspfade von Antibiotikarückständen konnte erstmalig gezeigt werden, dass das in Waschbeckensiphons, Toiletten und Duschabläufen bestimmter Kliniken befindliche Abwasser ein Reservoir an Rückständen von vor allem klinisch relevanten Antibiotika sein kann. Die dort zum Teil extrem hohen Rückstandskonzentrationen (bis zu 79 mg/L) könnten ausreichen, um einen Selektionsvorteil zugunsten von antibiotikaresistenten Bakterien auszuüben beziehungsweise den Neuerwerb von Antibiotikaresistenzen zu begünstigen. Ferner konnte gezeigt werden, dass vor allem in Duschabläufen ein breites Spektrum an Antibiotika in hohen Rückstandskonzentrationen nachzuweisen ist. Diese neuen Erkenntnisse werden in einer zukünftigen Empfehlung der Kommission für Krankenhaushygiene und Infektionsprävention (KRINKO Empfehlung) bezüglich der „Anforderungen der Hygiene an abwasserführende Systeme in medizinischen Einrichtungen" berücksichtigt. Darüber hinaus zeigten die Ergebnisse dieser Dissertationsschrift, dass eine Speicherung der Antibiotikarückstände im Leitungsnetz befindlichen Biofilm zu erfolgen scheint. Demnach erscheint die Entfernung des Biofilms beziehungsweise die Prävention der Ausbildung von Biofilmen in patientennahen Bereichen durch regulatorische Änderungen der Reinigungsverfahren beziehungsweise baulich-funktionellen Änderungen nicht nur aus mikrobiologischer Sicht sondern auch aus chemischer Sicht sinnvoll.[6]

Auch im weiteren Verlauf des Kanalnetzes kann ein Einfluss von klinischem Abwasser auf kommunale Mischabwässer anhand des Nachweises spezifischer Antibiotikarückstände, Antibiotikaresistenzgenen sowie antibiotikaresistenter Bakterien festgestellt werden.[7]

So ergab sich für spezifische antibiotikaresistente Bakterien (unter anderem Drittgenerations-Cephalosporin-resistente *Klebsiella* spp., *Enterobacter* spp., *Citrobacter* spp. bzw. *Pseudomonas aeruginosa*) beziehungsweise Antibiotikaresistenzgene (bla_{OXA48}, bla_{VIM2}, bla_{NDM} und $bla_{CTX\text{-}M}$) eine erhöhte Nachweiswahrscheinlichkeit, wenn parallel Rückstände an Ciprofloxacin, Ceftazidim oder Meropenem im Abwasser nachgewiesen wurden. Dies bezüglich konnten deutliche Unterschiede zwischen Krankenhausabwasser und nicht klinisch-beeinflusstem Abwasser festgestellt werden. Diese Ergebnisse legen nahe, dass hohe Rückstandskonzentrationen von Meropenem und Ciprofloxacin als Marker für das Vorhandensein von multiresistenten Bakterien in Abwasser fungieren könnten.[7]

Des Weiteren stellt der Eintrag von behandeltem Abwasser in bestimmte Oberflächengewässer einen möglichen Rückkopplungspfad von antibiotikaresistenten Bakterien, Resistenzgenen und Antibiotikarückständen zum Menschen dar. Von besonderem Interesse sind hierbei sogenannte „Schutzgüter“ wie zum Beispiel Trinkwassertalsperren oder Badegewässer.

Dies bezüglich konnte am Beispiel eines Flusses, der in eine Trinkwassertalsperre mündet und in den das behandelte Abwasser zweier kommunaler Kläranlagen eingeleitet wird, gezeigt werden, dass ein Abwassereinfluss durch den Nachweis von Antibiotikarückständen, Antibiotikaresistenzgenen und antibiotikaresistente Bakterien im Fluss nach Kläranlageneinleitung nachweisbar ist. Jedoch konnte eine rasche Abnahme der Konzentrationen an Antibiotikarückständen und antibiotikaresistenten Bakterien im Verlaufe des Fluss festgestellt werden, sodass keine signifikanten Unterschiede zwischen den Probeentnahmestellen vor Kläranlageneinleitung und nach der Trinkwassertalsperre festgestellt wurden.[8]

Die in dieser Arbeit entwickelte Analysenmethode in Verbindung mit molekularbiologischen und kulturellen Untersuchungsmethoden scheint geeignet, zukünftig weitere zur Trinkwassergewinnung dienende Oberflächengewässer oder Badegewässer auf etwaige Abwasserbelastungen zu untersuchen. Hierdurch könnte eine Datengrundlage geschaffen werden, die es erlaubt zukünftige Entwicklungen besser bewerten und einschätzen zu können.[8]

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Abkürzungsverzeichnis

3GCR	Drittgenerations-Cephalosporin-resistent
3MRGN	Multiresistente gramnegative Stäbchen mit einer Resistenz gegen 3 der 4 Antibiotikagruppen[9,10]
4MRGN	Multiresistente gramnegative Stäbchen mit einer Resistenz gegen 4 der 4 Antibiotikagruppen[9,10]
AMG	Arzneimittelgesetz
APCI	engl. *atmospheric pressure chemical ionization*
AR	Antibiotikarückstand
ARB	antibiotikaresistente Bakterien
ARG	Antibiotikaresistenzgen
BMBF	Bundesministerium für Bildung und Forschung
BVL	Bundesamt für Verbraucherschutz und Lebensmittelsicherheit
DDD	Definierte Tagesdosis (*Defined daily dose*, engl.)
DIMDI	Deutsches Institut für Medizinische Dokumentation und Information
ESBL	*Extended-spectrum beta-lactamase,* engl.
ESI	Elektrosprayionisation
GC	Gaschromatographie
HGT	Horizontaler Gentransfer
HPCIA	*Highest Priority Critically Important Antimicrobials*, engl.[11]
HPLC	Hochleistungsflüssigchromatographie
KEC	*Klebsiella* spez., *Enterobacter* spez. und *Citrobacter* spez.
KRINKO	Kommission für Krankenhaushygiene und Infektionsprävention
LC	Flüssigchromatographie (*liquid chromatography*, engl.)

LC-MS/MS	Flüssigchromatographie-Tandem-Massenspektrometrie (*liquid-chromatography-mass spectrometry/mass spectrometry*, engl.)
MDR	Multiresistent (*multi drug resistant,* engl.)[12]
MRE	Multiresistente Erreger
MRM	*Multiple reaction monitoring*, engl.
MRSA	Methicillin-resistente *Staphylococcus aureus*
MS/MS	Tandem-Massenspektrometrie
m/z	Masse-zu-Ladungs-Verhältnis
$PNEC_{Res}$	*Predicted-No-Effect-Concentration*[3], engl.
RP	Umkehrphase (*reversed-phase*, engl.)
STP	Kläranlage (*sewage treatment plant*, engl.)
VRE	Vancomycin-resistente Enterokokken
WHO	Weltgesundheitsorganisation (*World Health Organization*, engl.)
XDR	Extrem resistent (*extensively drug resistant,* engl.)[12]

Veröffentlichungen, Posterbeiträge und Vorträge

Veröffentlichungen, die essentieller Bestandteil der vorliegenden Dissertationsschrift sind:

Die nachfolgenden Arbeiten wurden bereits alle in internationalen *peer-reviewed* Zeitschriften veröffentlicht bzw. zur Veröffentlichung eingereicht. Die entsprechenden Lizenzen zum Nachdruck dieser Publikationen in der vorliegenden Dissertationsschrift wurden bei den entsprechenden Verlagen eingeholt.

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1. Theoretische Grundlagen

1.1. Antibiotisch wirksame Substanzen

Per Definition sind Antibiotika antibakteriell wirksame Substanzen, die durch Mikroorganismen biosynthetisiert, synthetisch hergestellt oder partialsynthetisch aus natürlich gewonnenen Antibiotika dargestellt werden. Untergeordnet werden Antibiotika in die Gruppe der Antiinfektiva, zu denen ebenfalls Antimykotika, Antiviralia und Antiparasitika zählen.[13]

Der übergeordnete Wirkmechanismus aller Antiinfektiva beruht auf den Erkenntnissen von Robert Koch aus dem Jahre 1881, dass sich Mikroorganismen spezifisch im Wirtsorganismus durch z.B. Methylenblau anfärben lassen.[13,14] Aufgrund der spezifischen Bindung eines Farbstoffes an gewisse Zielstrukturen im Wirtsorganismus vermutete Ehrlich, dass auch eine selektive Behandlung bzw. zielstrukturabhängige Wirkung von Substanzen erreicht werden könnte.[15] Auf Grundlage dieser Erkenntnisse postulierte Paul Ehrlich circa 10 Jahre später das sogenannte „Ehrlich‘sche Prinzip der selektiven Toxizität“, welches als das Grundprinzip des medizinischen Einsatzes von Antiinfektiva gilt.[13,15]

Ein Beispiel für die folgende systematische Suche nach antimikrobiell wirksamen Substanzen ist die Entdeckung von Salvarsan® durch Paul Ehrlich und seine Mitarbeiter Alfred Bertheim und Sachiro Hata 1909, welches sich als wirksames Medikament in der Behandlung von Spirochäten (z.B. *Treponema pallidum*, Syphilis-Erreger) und Trypanosomen (*Trypanosoma brucei*, Erreger der Schlafkrankheit) erwies.[15–17] Wie akribisch die Suche nach Antiinfektiva durchgeführt wurde, zeigte die Entdeckung bzw. der Nachweis der antibakteriellen Wirksamkeit von Sulfonamiden. Ausgehend von Ehrlich‘s Prinzip der selektiven Toxizität führte der deutsche Forscher Gerhard Domagk eine routinemäßige Überprüfung sämtlicher neuer Farbstoffe auf antibakterielle Eigenschaften ein und entdeckte so 1932 die antibiotische Wirksamkeit von Prontosil Rubrum, welches im Körper u.a. in die aktive Wirkform Sulfanilamid gespalten wird.[18]

Weitere Meilensteine stellen die Entdeckungen von Antibiotika-produzierenden Mikroorganismen wie *Penicillium chrysogenum* (produziert Benzylpenicillin, Alexander Fleming, 1928), *Streptomycetes griseus* (Streptomycin, Abraham Waksman, 1943) sowie *Streptomyces aureofaciens* (Chlortetracyclin, Benjamin Minge Duggar, 1943) dar.[13,16,19,20]

Insgesamt kann das 20. Jahrhundert als goldenes Zeitalter bezüglich der Entdeckung und Weiterentwicklung einer Vielzahl an Antibiotika angesehen werden.

Nichtsdestotrotz stellt die Suche nach neuen antibiotisch wirksamen Substanzen sowie der chemischen Modifikation bereits bekannter Antibiotika noch heute einen Forschungsschwerpunkt dar.[21] In diesem Kontext konnte 2015 mit Teixobactin ein neue antibiotisch wirksame Substanz entdeckt werden, die sich keiner bis dato bekannten Substanzklasse an Antibiotika zuordnen lässt und die eine wirksame Therapieoption gegen insbesondere multiresistente *Staphylococcus aureus* und *Mycobacterium tuberculosis* Stämme zu sein scheint.[22]

Die Einordnung der unterschiedlichen Substanzklassen kann auf Grundlage verschiedenster Eigenschaften erfolgen. So kann zwischen Antibiotika unterschieden werden, die bakteriostatisch (Hemmung der bakteriellen Zellvermehrung) oder bakterizid (Abtötung der Bakterienzelle) wirken. Ferner können antibiotisch wirksame Substanzen auf Grundlage ihres Wirkungsspektrums (grampositiv, gramnegativ, Breitband-Antibiotika etc.) sowie anhand der Applikation (intravenös, topisch, oral) unterschieden werden. Die wohl bedeutendste Unterteilung ist die auf Basis der chemischen Grundstrukturen der einzelnen Wirkstoffe vorgenommene Gliederung.[13]

Im folgenden Abschnitt werden die wichtigsten Substanzklassen inklusive ihrer Charakteristika vorgestellt. Zudem liefert Tabelle 1 einen Überblick über die wichtigsten Eigenschaften der in dieser Arbeit thematisierten Substanzklassen.

1.1.1. Beta-Lactam-Antibiotika

Eine der wohl bekanntesten Antibiotikaklassen stellen die β-Lactam-Antibiotika dar, welche als gemeinsames Strukturelement den namensgebenden β-Lactam-Ring aufweisen, der für die antibakterielle (bakterizide) Wirkung von entscheidender Bedeutung ist.[13]

Bekannte Vertreter dieser Gruppe sind die Wirkstoffe der Penicilline, Cephalosporine und Carbapeneme, die anhand von Modifikationen des β-Lactam-Ringes differenziert werden können (Abbildung 1).[13]

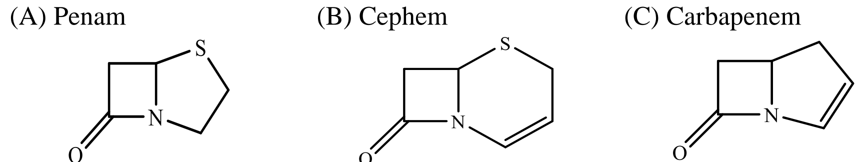

Abbildung 1 Überblick der Grundstrukturen der verschiedenen β-Lactam-Antibiotika ([A] Penicilline, [B] Cephalosporine und [C] Carbapeneme)[13]

Die bakterizide Wirkung der β-Lactam-Antibiotika beruht auf der Störung der Zellwandsynthese proliferierender Bakterienzellen. Zu Beginn des Wachstumszyklus einer Bakterienzelle muss die Stabilität der Zellwand reduziert werden, um ein Wachstum zu ermöglichen. Dies geschieht durch die hydrolytische Spaltung der Quervernetzungen der parallel angeordneten Glykanstränge, welche einen essentiellen Bestandteil der bakteriellen Zellwand (Peptidoglykanschicht) darstellt. Um die Zellwandstabilität der gewachsenen Zelle abschließend wieder herzustellen, erfolgt neben der Synthese neuer Glykanstränge eine erneute Verknüpfung der Glykanstränge bei gramnegativen Bakterien über eine Peptidbindung zwischen dem D-Alanin eines Glykanstranges und der Diaminopimelinsäure eines benachbarten Glykanstranges. Bei grampositiven Bakterien erfolgt die Quervernetzung über Glycin-Interpeptidbrücken zwischen D-Alanin und L-Lysin. Katalysiert wird diese Reaktion durch eine spezifische Transpeptidase, die als eigentlicher Angriffsort der β-Lactam-Antibiotika dient. Aufgrund der hohen strukturellen Ähnlichkeit des β-Lactam-Ringes zum D-Alanyl-D-Alanin-Dipeptids eines Glykanstranges können diese Antibiotika kompetitiv an das aktive Zentrum der Transpeptidase binden. Hierdurch wird die Quervernetzung der Glykanstränge gestört und die Stabilisierung der neusynthetisierten Peptidoglykanschicht reduziert, was letztlich zur Bakteriolyse der Bakterienzelle führt.[13,23–25]

Neben dem aus *P. chrysogenum* isolierten Penicillin G werden heutzutage hauptsächlich partialsynthetisch hergestellte Derivate der 6-Aminopenicillansäure in der Human- und Veterinärmedizin eingesetzt.[13]

Durch gezielte chemische Modifikationen ließen sich Präparate mit gewünschten Eigenschaften synthetisieren wie z.B. einer erhöhten Stabilität gegenüber niedrigen pH-Werten zur oralen Applikation (z.B. Penicillin V), einer Erhöhung der Stabilität gegenüber der Spaltung des β-Lactam-Ringes durch Penicillinasen (z.B. Flucloxacillin) oder die Erweiterung des Wirkungsspektrums (insbesondere gegenüber gramnegativen Bakterien) im Falle der Amino- (z.B. Ampicillin oder Amoxicillin) bzw. Acylpenicillinen (z.B. Mezlocillin oder Piperacillin).[13]

Zusätzlich zur chemischen Modifikation des jeweiligen Wirkstoffs kann eine Erhöhung der Stabilität gegenüber β-Lactamasen im Allgemeinen durch die Kombination eines Penicillins mit einem sogenannten β-Lactamase-Inhibitor erfolgen.[13] Als Beispiel sei die Kombination aus Piperacillin und Tazobactam genannt, die aufgrund ihrer Wirksamkeit bei der Behandlung von schweren nosokomialen Infektionen durch z.B. *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* bzw. multiresistenten Erregern (MRE) von besonderer klinischer Bedeutung ist.[13,26,27] Benzylpenicillin, Amoxicillin, Cloxacillin bzw. Phenoxymethylpenicillin sind beispielhafte Vertreter an Penicillinen, die in der Veterinärmedizin verabreicht werden.[28,29]

Bei der Gruppe der Cephalosporine handelt es sich um partialsynthetisch hergestellte β-Lactame, welche sich von der 7-Aminocephalosporansäure ableiten lassen und die anhand ihres Wirkungsspektrums in fünf Generationen untergliedert werden. Insgesamt erstreckt sich das Wirkungsspektrum je nach Cephalosporin über einen breiten Bereich im grampositiven sowie –negativen Spektrum. Vertreter der ersten (z.B. Cefaclor) bzw. zweiten Generation (z.B. Cefuroxim) zeichnen sich durch eine gute Wirksamkeit gegenüber grampositiven Bakterien aus, während Substanzen der dritten (z.B. Cefotaxim oder Ceftazidim) und vierten (u.a. Cefepim) Generation sich zudem durch eine gute Wirkung im gramnegativen Bereich charakterisieren.[13]

Es ist jedoch zu beachten, dass bei MRE, insbesondere bei *Extended-spectrum beta-lactamase* (ESBL) produzierende Bakterien und Methicillin-resistenten *S. aureus* (MRSA) Cephalosporine unwirksam sind. Eine Ausnahme stellt hierbei das 2010 in den USA zugelassene Ceftarolin (fünfte Generation) dar, welches sich durch eine gute Wirksamkeit gegenüber MRSA und Linezolid- bzw. Daptomycin-resistenten *Staphylococcus* Stämmen auszeichnet, jedoch nicht wirksam gegenüber ESBL produzierenden Stämmen ist.[13,30]

Eine Therapieoption in der Behandlung von ESBL produzierenden Bakterien stellen Carbapeneme dar. Diese β-Lactame weisen als Grundgerüst das namensgebende Carbapenem auf (Abbildung 1). Zugelassene Wirkstoffe dieser Gruppe sind Meropenem, Imipenem, Ertapenem und Doripenem, die allesamt parenteral verabreicht werden.[13]

Der wohl wichtigste Vertreter dieser Gruppe ist Meropenem, der aufgrund seiner guten Wirksamkeit gegenüber antibiotikaresistenten gramnegativen Krankheitserregern, z.B. *Escherichia coli* oder *Klebsiella* spp., als sogenanntes „Reserveantibiotikum“ eingesetzt wird, wenn wie im Falle von z.B. ESBL oder AmpC produzierenden Stämmen andere β-Lactame nicht mehr wirksam sind.[31]

1.1.2. Makrolid-Antibiotika

Eine alternative Therapieoption bei der Behandlung von grampositiven Bakterien mit Penicillin-Resistenz bzw. bei vorhandener Penicillinallergie des Patienten stellen Makrolid-Antibiotika dar. Allgemein hemmen Makrolid-Antibiotika bakteriostatisch das Zellwachstum durch die Störung der Proteinbiosynthese, indem sie die Verlängerung der Polypeptidkette (sterische Unterdrückung des Wechsels der tRNA von der A-Bindungsstelle auf die P-Bindungsstelle) verhindern. Die Grundstruktur dieser Wirkstoffe beruht auf einem heterocyclischen System (14-16-gliedriger Lactonring), an das unterschiedliche Zuckerreste glykosidisch gebunden sein können.[13]

Wichtige Vertreter dieser Substanzklasse im humanmedizinischen Bereich sind Clarithromycin, Azithromycin, Erythromycin und Roxithromycin.[13,32] Ferner werden Makrolid-Antibiotika in der Veterinärmedizin eingesetzt (u.a. Erythromycin, Tylosin und Spiramycin).[28]

1.1.3. Tetracycline

1943 isolierte Benjamin Minge Duggar das antibiotisch wirksame Chlortetracyclin aus *Streptomyces aureofaciens*, welches noch heute zu den am meisten eingesetzten Tetracyclin-Präparaten in der Veterinärmedizin in Deutschland zählt.[19]

So wurden 2018 circa 44 t an Tierärzte durch pharmazeutische Unternehmen bzw. Großhändler abgegeben, was in etwa 25 % aller abgegebenen Tetracycline bzw. 6 % der Gesamtabgabemenge (circa 722 t) an Veterinärantibiotika entspricht.[29] Weitere häufig eingesetzte Wirkstoffe sind Doxycyclin, Oxytetracyclin und Tetracyclin.[28] In der Humanmedizin werden aktuell noch Tetracyclin, Doxycyclin und Minocyclin eingesetzt, die sich lediglich durch ihre Substituenten an der in Abbildung 2 dargestellten Grundstruktur unterscheiden.[13,32]

Abbildung 2 Tetracyclin-Grundstruktur[13]

Tetracycline hemmen die Proteinbiosynthese und wirken so bakteriostatisch und werden v. a. bei der Behandlung von Infektionen durch grampositive (z.B. Pneumokokken oder Staphylokokken, inkl. MRSA) und gramnegative Bakterien (z.B. *Campylobacter jejuni*) eingesetzt. Im Vergleich zu Makrolid-Antibiotika wirken Tetracycline einen Schritt früher bei der Elongation der Polypeptidkette, da sie den Ablesevorgang zwischen Codon und Anticodon stören und so die Bindung der Aminoacyl-tRNA an der A-Stelle der 30S ribosomalen Untereinheit unterdrücken.[13]

Von besonderer Bedeutung ist Tigecyclin, welches als „Reserveantibiotikum" bei schwerwiegenden Infektionen mit MRE (z.B. Vancomycin-resistenten Enterokokken (VRE), MRSA und ESBL) als Alternative zu Carbapenemen, Vancomycin oder Linezolid dienen kann.[13,33]

1.1.4. Fluorchinolone

Eine weitere wichtige Substanzklasse stellen die synthetisch hergestellten Fluorchinolone dar, die durch die Einführung eines Fluoratoms an das Chinolon-Grundgerüstes (Abbildung 3) gezielt synthetisiert wurden.[13]

Die weitere Entwicklung der Fluorchinolone kann als Beispiel für die gezielte chemische Modifikation von synthetisch dargestellten Antibiotika dienen. Durch gezielte Veränderungen des Substitutionsmusters der Fluorchinolon-Grundstruktur, welche sich von der Chinolin-4-on-3-carbonsäure ableitet, konnte das Wirkungsspektrum erweitert bzw. die bakterizide Wirkung und die pharmakokinetischen Eigenschaften optimiert werden.[13]

Abbildung 3 Grundstruktur der Fluorchinolone[13]

Anhand des Wirkspektrums und der pharmakologischen Eigenschaften erfolgt eine Einordnung der, für die Humanmedizin zugelassenen, aktuellen Fluorchinolone in vier Gruppen. Vertreter der Gruppe 1 (z.B. Norfloxacin) werden v. a. bei der Therapie von Infektionen durch *Enterobacteriales* (ehemals *Enterobacteriaceae*) eingesetzt. Aus Gruppe 2 ist Ciprofloxacin das aktuell bedeutsamste Fluorchinolon, welches sich durch ein deutlich breiteres Wirkungsspektrum gegen gramnegative Erreger auszeichnet und v. a. bei schweren Infektionen durch *P. aeruginosa* verabreicht wird. Jedoch muss bei Ciprofloxacin die sogenannte „Pneumokokkenlücke“ beachtet werden, da dieses unwirksam bei der Behandlung von Infektionen durch Pneumokokken, Staphylokokken und Streptokokken ist. Mögliche Therapieoptionen bei Infektionen durch diese Bakterien sind u. a. Fluorchinolone der 3. bzw. 4. Gruppe (Levofloxacin bzw. Moxifloxacin), die sich v. a. durch eine verbesserte Wirksamkeit im grampositiven Bereich gegenüber Ciprofloxacin auszeichnen. Häufige Anwendung finden Fluorchinolone bei der Behandlung von Harnwegsinfekten, Atemwegsinfekten, Infektionen von Haut und Weichteilen bis hin zur Sepsis.[13,32]

Die bakterizide Wirkung der Fluorchinolone beruht auf einer Störung der räumlichen Anordnung der DNA (Verdrillung, Superspiralisierung, Entspiralisierung) während der bakteriellen Replikation bzw. Transkription.[13]

Durch die Bindung an die Untereinheit A der Topoisomerase II (Gyrase) und IV über einen ternären Fluorchinolon-Topoisomerase-DNA-Komplex verhindern Wirkstoffe dieser Substanzklasse das Wiederverschließen der DNA-Stränge. Dieser Mechanismus erklärt das charakteristische breite Wirkungsspektrum der Fluorchinolone, das sowohl grampositive (Topoisomerase IV) als auch gramnegative (Topoisomerase II) Bakterien umfasst.[13]

Neben dem humanmedizinischen Einsatz werden Fluorchinolone, deren Bedeutung für die Humanmedizin von der Weltgesundheitsorganisation (engl. *World Health Organization*, WHO) als „*critically important*“ definiert wurde, in der Veterinärmedizin eingesetzt.[11,28] Jedoch erfolgt hierbei eine strikte Trennung der Wirkstoffe zwischen veterinärmedizinischen (z.B. Enrofloxacin) und humanmedizinischen Präparaten (z.B. Ciprofloxacin).[34] Typische Wirkstoffe, die in der Veterinärmedizin eingesetzt werden, sind u.a. Enrofloxacin, Marbofloxacin und Danofloxacin.[28,35]

Ungeachtet der Resistenzsituation sind Fluorchinolone zuletzt aufgrund ihrer z.T. schwerwiegenden Nebenwirkungen (v. a. Sehnen, Muskeln, Gelenken und das Nervensystem betreffend) in die Kritik geraten.[36]

1.1.5. Sulfonamide

Seit 1935 ist sowohl in der Human- als auch in der Veterinärmedizin die Klasse der synthetisch hergestellten Sulfonamide von Bedeutung für die Behandlung von gramnegativen sowie insbesondere grampositiven Bakterien. Als Leitsubstanz dient Sulfanilamid, welches durch zahlreiche chemische Modifikationen zu einer Fülle an antibiotisch wirksamen Derivaten (s. Abbildung 4) führte und welches das antibiotisch wirksame Stoffwechselprodukt des von Gerhard Domagk entdeckten Prontosils® ist.[13]

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Abbildung 4 Grundstruktur der Sulfonamide[13]

Die antibakterielle Wirksamkeit von Sulfonamiden beruht auf der kompetitiven Hemmung der Dihydropteroinsäure-Synthetase, die im Verlauf der Folsäuresynthese für die Produktion von Dihydrofolsäure aus Dihydropterin, Glutaminsäure und p-Aminobenzoesäure verantwortlich ist. Die Störung der Folsäuresynthese und die daraus folgende gestörte Synthese von DNA begründet die bakteriostatische Wirksamkeit der Sulfonamide.[13]

In der Humanmedizin sind aufgrund von Resistenzentwicklungen und unerwünschten Nebenwirkungen aktuell allen voran Sulfamethoxazol in Kombination mit Trimethoprim (Cotrimoxazol, Verhältnis 5:1) sowie Sulfadiazin von klinischer Bedeutung.[13,32] Cotrimoxazol kann z.B. in der Therapie von Harnwegs- bzw. Atemwegsinfekten eingesetzt werden und bewährte sich zudem in der Prophylaxe von bakteriellen Infektionen bei immunsuppressiven Patienten.[37]

Im Veterinärbereich stehen weitere Wirkstoffe dieser Klasse als Therapieoption zur Verfügung. Die Abgabemengen von Sulfadiazin, Sulfamethoxazol, Sulfadimethoxin, Sulfadimidin, Sulfadoxin, Sulfamerazin, Sulfamethoxypyridazin und Formosulfathiazol müssen von pharmazeutischen Unternehmern und Großhändlern an das Deutsche Institut für Medizinische Dokumentation und Information (DIMDI) gemeldet werden.[28]

1.2. Verbrauchszahlen

Auch noch ein Jahrhundert nach den Entdeckungen von Robert Koch und Paul Ehrlich stellen Antiinfektiva, allen voran Antibiotika, eine der wichtigsten Medikamentengruppen weltweit dar.

Zur besseren Vergleichbarkeit von Verbrauchsdaten sowie zur Erfassung von zeitlichen Veränderungen der Verbrauchsmengen von Medikamenten werden definierte Tagesdosen (DDD, *defined daily dose*, engl.) in der Humanmedizin angegeben. Diese werden für jeden Arzneistoff entsprechend der jeweiligen Indikationsform (parenteral, oral etc.) sowie der angenommenen durchschnittlichen Verschreibungsdosis pro Tag ermittelt.[38]

Tabelle 1 Eigenschaften ausgewählter Antibiotikasubstanzklassen

Substanzklasse	**Wirkungstyp**[13]	**Wirkmechanismus**[13]	**Applikation**[13,39]	**Wichtige Vertreter**[11,13,28,32]	**WHO-Klassifizierung**[11]	**Einsatz-gebiete**[13,28,32]
Carbapeneme	Bakterizid*	Hemmung der Zellwandsynthese (kompetitive Hemmung der Transpeptidase)	Parenteral	Meropenem, Imipenem, Doripenem, Ertapenem	*Critically important*	Human
Cephalosporine	Bakterizid*	Hemmung der Zellwandsynthese (kompetitive Hemmung der Transpeptidase)	Parenteral, oral	Cefotaxim, Cefepim, Ceftarolin, Ceftazidim, Cefuroxim, Ceftriaxon,	*Critically important Highly important*	Human, veterinär
Fluorchinolone	Bakterizid	Hemmung der DNA Synthese (Topoisomerase II und IV)	Parenteral, oral, topisch	Ciprofloxacin, Enrofloxacin, Moxifloxacin, Levofloxacin, Ofloxacin, Nadifloxacin	*Critically important*	Human, veterinär
Glykopeptide	Bakterizid	Hemmung der Zellwandsynthese (Komplexbildung zwischen Glykopeptid und L-Lysin-D-Alanyl-D-Alanin des Mureins)	Parenteral, oral	Vancomycin	*Critically important*	Human
Lincosamide	Bakteriostatisch / bakterizid#	Hemmung der PBS (Blockade des ribosomalen tRNA-Kanals bei der Elongation)	Parenteral, oral, topisch	Clindamycin, Lincomycin	*Highly important*	Human, veterinär
Makrolide	Bakteriostatisch / bakterizid#	Hemmung der PBS (Blockade des ribosomalen tRNA-Kanals bei der Elongation)	Parenteral, oral, topisch	Erythromycin, Clarithromycin, Roxithromycin, Azithromycin, Spiramycin, Tylosin	*Critically important Highly important*	Human, veterinär
Nitroimidazole	Bakterizid	Schädigung der Bakterien-DNA (Strangbrüche durch reaktive Zwischenprodukte)	Parenteral, oral, rektal	Metronidazol	*Important*	Human, veterinär
Oxazolidinone	Bakteriostatisch / bakterizid#	Hemmung der PBS (Blockiert die Verbindung der 30S- und 50S-Untereinheit zum 70S-Initiationskomplexes)	Parenteral, oral	Linezolid	*Critically important*	Human
Penicilline	Bakterizid*	Hemmung der Zellwandsynthese (kompetitive Hemmung der Transpeptidase)	Parenteral, oral	Amoxicillin, Ampicillin, Cloxacillin, Piperacillin, Penicillin G, Penicillin V, Flucloxacillin	*Critically important Highly important*	Human, veterinär
Sulfonamide	Bakteriostatisch	Hemmung der Purinnucleotid-Synthese (Folsäuresynthese gestört)	Parenteral, oral, topisch	Sulfamethoxazol, Sulfadiazin, Sulfadimidin, Sulfamerazin, (Trimethoprim)	*Highly important*	Human, veterinär
Tetracycline	Bakteriostatisch	Hemmung der PBS (Blockiert Anticodon-Codon-Wechselwirkung)	Parenteral, oral, topisch	Chlortetracyclin, Doxycyclin, Oxytetracyclin, Tetracyclin, Tigecyclin	*Critically important Highly important*	Human, veterinär

* nur proliferierende Zellen

\# je nach Wirkstoff, Erreger und eingesetzter Konzentration

Anhand der von der WHO veröffentlichten ATC-Klassifizierung können so die Stoff-spezifischen DDD aus den verschiedensten vorhandenen Kenngrößen wie Packungsanzahl, Packungsgröße [g/Packung], Umsatz [Preis/Packung] oder Verschreibungsmenge [g] berechnet und verglichen werden.[38,39]

Der geschätzte weltweite Antibiotikaverbrauch von rund 42 Milliarden DDD für 2015 und ein prognostizierter Anstieg auf circa 120 Milliarden DDD bis 2030 unterstreichen die globale Bedeutung von Antibiotika für die öffentliche Gesundheit.[40] Aktuellen Zahlen zufolge beläuft sich die Gesamtmenge an verschriebenen Antibiotika in Deutschland auf etwa 700 t bis 800 t im humanmedizinischen Bereich, wobei circa 85% des Verordnungsvolumen auf den ambulanten Sektor fallen.[41]

Nach Angaben der Gesetzlichen Krankenkassen zählten 2015 Antibiotika mit 38,6 Mio. Verordnungen der niedergelassenen Ärzte zu den am fünfthäufigsten verordneten Medikamenten in Deutschland. Im Vergleich zum Vorjahr entspricht dies einem geringfügigen Rückgang (-1,6%). Der allgemeine Rückgang an Antibiotikaverordnungen fällt im Vergleich zur Anzahl der Verordnung im Jahre 2002 (-8,5%) deutlicher aus. Verglichen zum generellen Rückgang der Gesamtverordnungen an Antibiotika zeigt Abbildung 5 jedoch, dass für einzelne Wirkstoffgruppen zwischen den Jahren 2001 und 2015 ein deutlicher Anstieg an Verschreibungen zu verzeichnen ist. So ist eine deutliche Zunahme der Menge an verordneten Cephalosporine (+53 Mio. DDD) und Aminopenicilline (+31 Mio. DDD) zwischen 2001 und 2015 festzustellen.[32,42]

Insgesamt zählten 2015 β-Lactame {v.a. Aminopenicilline (99 Mio. DDD) und Cephalosporine (80 Mio. DDD)}, Doxycyclin (46 Mio. DDD), Makrolide/Lincosamide (62 Mio. DDD) und Fluorchinolone (34 Mio. DDD) zu den ambulant am häufigsten verschriebenen Antibiotika in Deutschland.[32]

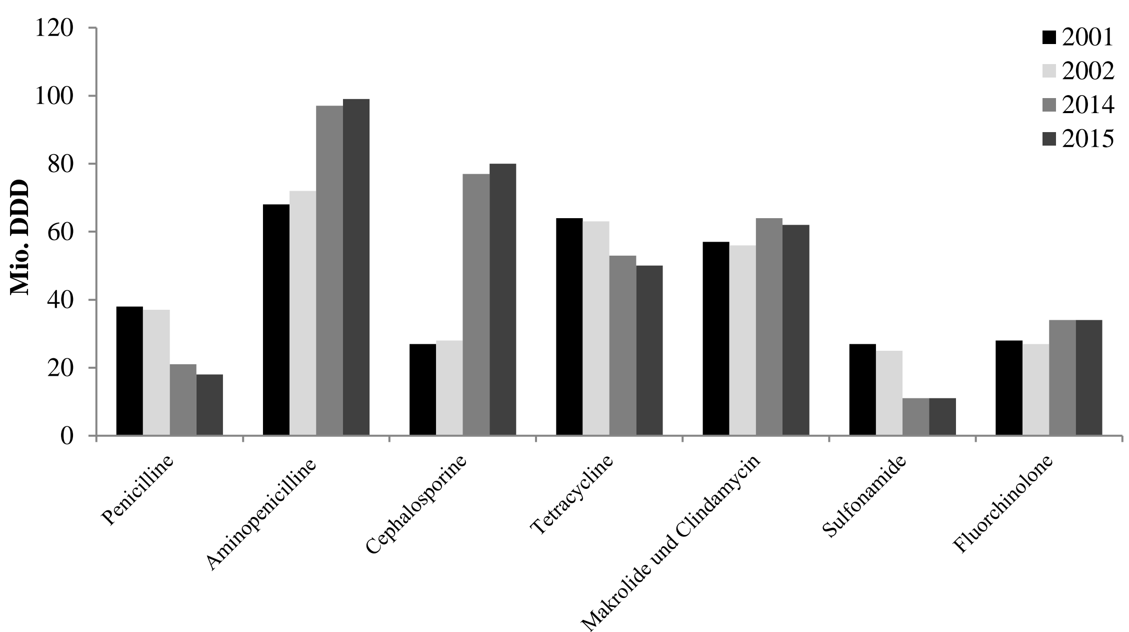

Abbildung 5 Anzahl der ambulanten Verordnungen von Antibiotika [Millionen DDD] der Jahre 2001, 2002, 2015 und 2016 in Deutschland, basierend auf den Ergebnissen des Arzneiverordnungsreports 2002 und 2016.[32,42]

Aufgrund steigender Resistenzzahlen sind die für die Klassifizierung von multiresistenten gramnegativen Erregern herangezogenen Leitsubstanzen Piperacillin/Tazobactam (Acylureidopenicilline), Ciprofloxacin (Fluorchinolone), Cefotaxim/Ceftazidim (Drittgenerations-Cephalosporine) sowie Meropenem oder Imipenem (Carbapeneme) von besonderem Interesse.[9,10] Hinzu kommen die als „Reserveantibiotika" geltenden Wirkstoffe Vancomycin und Linezolid sowie Tigecyclin und Colistin, die bei schweren multiresistenten Infektionen oftmals als letzte Therapieoption zur Verfügung stehen.[13,33] Daher ist der globale Anstieg der Antibiotika-Verbrauchsdichten (ausgedrückt in DDD/1000 Patiententage) von Carbapenemen, Colistin, Linezolid und Tigecyclin zwischen den Jahren 2000 und 2015 bedenklich.[40]

Neben dem Einsatz von Antibiotika in der Humanmedizin werden auch größere Mengen in der Veterinärmedizin eingesetzt. Trotz des Verbotes von antimikrobiellen Substanzen als Wachstums- bzw. Leistungsförderer in der Tiermast durch Artikel 5 Absatz 4 in Verbindung mit Artikel 11 Absatz 2 der VO(EG) Nr. 1831/2003 im Jahre 2006, betrug laut Angaben des Bundesamtes für Verbraucherschutz und Lebensmittelsicherheit (BVL) die Menge an therapeutisch eingesetzten Antibiotika, die von „pharmazeutischen Unternehmen und Großhändlern" 2018 an die Tierärzte abgegeben wurde, rund 722 t. Dies entspricht einem Rückgang von circa 58% im Vergleich zum Jahre 2011 (1.706 t).[43,44]

Die seit 2011 durch § 47 Abs. 1c und § 67a des Arzneimittelgesetzes (AMG) sowie der DIMDI-Arzneimittelverordnung zur Verfügung stehenden jährlichen Verkaufszahlen zeigen, dass eine Reduktion der Abgabemengen an Tetracyclinen (-68%), Penicillinen (-49%), Makroliden (-66%) und Sulfonamiden (-66%) zwischen den Jahren 2011 und 2018 stattgefunden hat (Abbildung 6).[29,44–46] Ferner konnte eine Abnahme der Therapiehäufigkeit von zur Lebensmittelgewinnung dienenden Tieren mit Antibiotika, die durch die 16. Novellierung des AMG zentral erfasst wird,[47] bei Rindern, Schweinen und Mastgeflügel in Deutschland zwischen 2014 und 2017 festgestellt werden.[28] Jedoch wurde mit der generellen Abnahme der Antibiotikamengen eine Erhöhung der Abgabemengen an Fluorchinolonen (+21%, 2011-2017) und Colistin (+7%, 2016-2017) verzeichnet.[28] Die Erhöhung der Abgabemengen dieser Antibiotika, die als besonders wertvoll für die Humanmedizin bewertet werden, sollte jedoch in Zukunft weiterhin beobachtet werden, um durch geeignete Präventionsmaßnahmen weiteren Resistenzentwicklungen vorzubeugen.[11]

In diesem Kontext sei erwähnt, dass die 2019 seitens des BVL veröffentlichten Daten zeigen, dass im Jahr 2018 für Fluorchinolone erstmals eine Reduktion der Abgabemenge im Vergleich zu 2011 erfasst werden konnte (2011: 8,2 t – 2018: 7,7 t).[44]

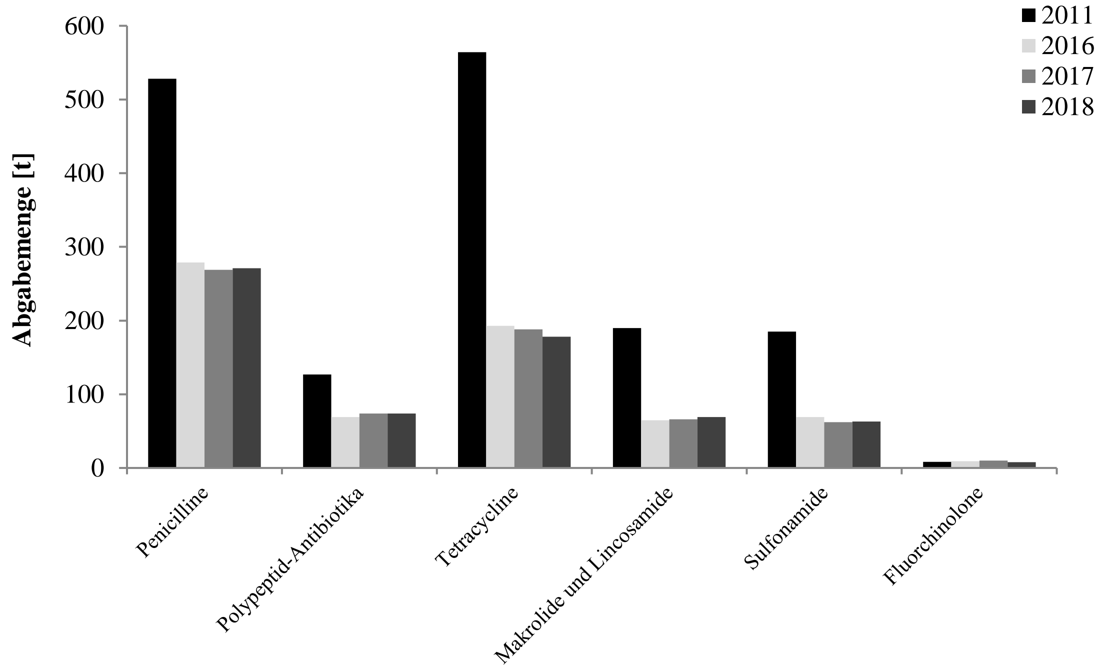

Abbildung 6 Vergleich der Menge an Antibiotika, die von der pharmazeutischen Industrie und Großhändlern an Veterinärmediziner in Deutschland in den Jahren 2011, 2016, 2017 und 2018 abgegeben wurden, modifiziert auf Basis der Auswertungen des Bundesamtes für Verbraucherschutz und Lebensmittelsicherheit (BVL, 2018 und 2019).[44,48]

1.3. Antibiotikaresistenzen

Zu Beginn dieses Kapitels sei explizit darauf hingewiesen, dass Antibiotikaresistenzen natürliche Abwehrmechanismen von Bakterien sind, um dem Selektionsdruck ausgehend von Antibiotika-produzierenden Schimmelpilzen entgegen zu wirken. So konnten verschiedenste Antibiotikaresistenzgene (ARG) bereits in Habitaten nachgewiesen werden, die bis dato keinem anthropogenen Einfluss unterlagen wie z.B. in einer Höhle in New Mexiko oder in Permafrost-Proben.[49,50] Jedoch wird dieses natürliche Gleichgewicht durch die in Abschnitt 1.2 erläuterten hohen Einsatzmengen an Antibiotika zu Gunsten von antibiotikaresistenten Bakterien (ARB) beeinflusst.[51]

Per Definition ist eine Antibiotikaresistenz gegeben, wenn die zur Therapie benötigte minimale Hemmkonzentration über der maximal medizinisch einsetzbaren Serum- bzw. Gewebekonzentration des jeweiligen Antibiotikums liegt, sodass keine bzw. eine unzureichende antibiotische Wirksamkeit in der Behandlung gegeben ist.[13]

Unterschieden wird allgemein zwischen Resistenzen, deren Entstehung bzw. Übertragung durch eine Antibiotikatherapie ausgelöst wurde (Sekundärresistenzen), sowie Resistenzen, die natürlicherweise eine Spezies charakterisieren und nicht in Folge einer Antibiotikatherapie ausgebildet wurden (Primärresistenz).[13]

Alexander Fleming erkannte bereits während seiner Studien rund um die Entdeckung des Penicillins, dass Bakterien durch subletale Konzentrationen leicht Resistenzen entwickeln können und warnte daher bereits am 11. Dezember 1945 vor den möglichen Konsequenzen, die ein unsachgemäßer Einsatz von Antibiotika nach sich ziehen würde: „*The time may come when penicillin can be bought by anyone in the shops.* […] *and by exposing his microbes to non-lethal quantities of the drug make them resistant*."[52]

Analog zur Vielzahl unterschiedlicher Wirkmechanismen, die im vorherigen Abschnitt 1.1 erläutert wurden, gibt es auch eine Vielzahl an möglichen Gegenmaßnahmen (Resistenzmechanismen), welche die Bakterienzelle treffen kann, um sich gegen die Wirkung des Antibiotikums zu schützen. Insgesamt unterscheidet man vier Hauptgruppen an Resistenzmechanismen.[13]

Zum Beispiel die Bildung spezifischer Enzyme, die den Wirkstoff in seiner Funktionsweise hemmen.[13] Als Beispiel für diese Art der Antibiotikaresistenz dienen β-Lactamasen, welche β-Lactam-Antibiotika hydrolytisch spalten können und so deren antibiotische Wirksamkeit verhindern.[13,24] Anhand ihres Substratspektrums (Cephalosporine, Penicilline, Carbapeneme bzw. Monobactame) sowie der Empfindlichkeit gegenüber β-Lactamase-Inhibitoren wie z.B. Clavulansäure oder Tazobactam erfolgt eine Klassifizierung der β-Lactamasen in insgesamt drei Haupt- bzw. 16 Untergruppen.[53] Insbesondere ESBL wie z.B. CTX-M sind von klinischer Bedeutung, da diese Enzyme zusätzlich zur Resistenz gegenüber Penicillinen und frühen Cephalosporinen auch eine Resistenz gegenüber neueren Cephalosporinen (z.B. Cefotaxim oder Ceftazidim) verursachen können.[53]

Von besonderem Interesse sind β-Lactamasen, die zudem Carbapeneme hydrolytisch spalten können. Diese können sowohl den Serin-β-Lactamasen (z.B. OXA-48, KPC, GES) als auch den Metallo-β-Lactamasen (z.B. VIM, IMP) zugeordnet werden.[53]

Aufgrund ihrer diversen Eigenschaften können Carbapenemasen bzgl. ihrer klinischen Relevanz gewichtet werden. Mögliche Unterscheidungen sind die noch vorhandene Empfindlichkeit des Organismus gegenüber Ceftazidim bzw. Tazobactam (z.B. IMI-1, GES-3/4) sowie die Unterscheidung, ob es sich um plasmidiär- (z.B. KPC, VIM, IMP) oder chromosomal-codierte (z.B. IMI-1) Carbapenemasen handelt.[53,54] Die Bedeutung von Carbapenem-Resistenzen für die Humanmedizin ist auch anhand der Meldepflicht (seit Mai 2016) für Carbapenem-resistente bzw. Carbapenemase-produzierende *Enterobacteriales* gemäß § 7 Abs. 1 Satz 1 des Infektionsschutzgesetzes ersichtlich.[55,56] Auch die WHO unterstreicht die Relevanz von Carbapenem-Resistenzen in ihrer „*Global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics*“, die u.a. Carbapenem-resistente *Acinetobacter baumannii* und *P. aeruginosa* sowie Carbapenem- und 3GCR *Enterobacteriales* umfasst.[57]

Antibiotikaresistenzen können zudem durch eine erhöhte Expression von „Effluxpumpen“ ausgebildet werden, die das Antibiotikum aus den jeweiligen Bakterienzellen heraustransportieren und so deren Wirkung verhindern.[13]

Ein weiterer Mechanismus ist die Veränderung der Zellpermeabilität durch z.B. eine reduzierte Expression von Porin-Kanälen, durch die u.a. das Antibiotikum in die Bakterienzelle gelangen kann, um das Eindringen des Wirkstoffs in die Bakterienzelle zu verhindern bzw. zu erschweren.[13,58] Das Zusammenspiel der Reduktion an Porin-Kanälen (z.B. OprD) und eine erhöhte Expression von Effluxpumpen kann z.B. eine mögliche Ursache für die Resistenz von *P.aeruginosa* gegenüber Carbapenemen (z.B. Meropenem) erklären.[59,60]

Des Weiteren kann eine Modifikation der Zielstruktur des Antibiotikums zu einer Resistenz führen, z.B. der Austausch der terminalen D-Alanyl-D-Alanin-Zielstruktur (s. Abschnitt 1.1.1) durch D-Alanyl-D-Lactat oder durch D-Alanyl-D-Serin bei VRE.[61] Die durch *mec*A vermittelte Resistenz bei MRSA kann als weiteres Beispiel für diese Art der Resistenzentwicklung angesehen werden.[62] Das Vorhandensein einer modifizierten Transpeptidase (*penicillin-binding proteins*, PBP 2a) führt zu einer erniedrigten Affinität zwischen Wirkstoff und Zielstruktur, wodurch trotz Antibiotikatherapie eine Quervernetzung der Peptidoglykanstränge und Stabilisierung der Zellwand proliferierender Zellen erfolgt.[62–64]

Insbesondere die Ausbildung von MRE stellt eine Herausforderung für die moderne Medizin dar, da der parallele Wegfall mehrerer Behandlungsoptionen insbesondere bei nosokomialen Infektionen zu Komplikationen bei der Therapie und dem Zurückgreifen auf „Reserveantibiotika" führen kann.[13] Ein besonderes Augenmerk sollte hierbei auf die sogenannte „ESKAPE"-Gruppe gelegt werden, die die Spezies ***E**nterococcus faecium*, ***S**. aureus*, ***K**. pneumoniae*, ***A**. baumannii*, ***P**. aeruginosa* und ***E**nterobacter* spp. umfasst, die v.a. mit Krankenhausaufenthalten assoziierte (nosokomiale) Infektionen hervorrufen und sich durch eine erhöhte Neigung zur Ausbildung von Mehrfachresistenzen auszeichnen.[65] Die Charakterisierung des Resistenzstatus kann anhand verschiedenster Definitionen bzw. Klassifizierungen erfolgen.[9,10,12]

Eine international anerkannte Klassifizierung gramnegativer und grampositiver Bakterien wurde von Magiorakos et al. 2011 veröffentlicht und erleichtert den globalen Vergleich epidemiologischer Daten und Statistiken.[12]

In dieser Studie wurden spezifische Antibiotikakategorien für die oben aufgeführten Bakterien entworfen, die anhand ausgebildeter Resistenzen gegen eben diese Antibiotika als multiresistent (engl. *multidrug-resistant*, MDR), extrem resistent (engl., *extensively drug resistant*, XDR) oder pan-resistent eingestuft werden. Als MDR werden demnach Bakterien klassifiziert, die gegen mindestens eine Substanz aus drei Substanzklassen resistent sind. Im Vergleich hierzu erfolgt eine Einstufung als XDR, wenn Resistenzen gegen mindestens ein Antibiotikum aus allen Substanzklassen ausgebildet wurden bzw. lediglich gegen eine bzw. zwei Substanzklassen keine Resistenz ausgebildet wurden. Pan-resistente Bakterien sind gegen alle Wirkstoffe der angegebenen Substanzklassen resistent. Berücksichtigt werden muss, dass intrinsische Resistenzen bei Bakterien nicht gewertet wurden und dass keine Gewichtung der einzelnen Substanzklassen erfolgte.[12]

Eine Klassifizierung von multiresistenten gramnegativen Bakterien mit einem stärkeren Bezug auf die klinische Relevanz der entsprechenden Antibiotikaklassen stellt das Bewertungskonzept der deutschen Kommission für Krankenhaushygiene und Infektionsprävention (KRINKO) dar. In diesem Fall werden Enterobakterien, *P. aeruginosa* und *A. baumannii* anhand ihres phänotypischen Resistenzstatuts gegenüber einer spezifischen Auswahl an Antibiotika („primäre bakterizide Therapeutika bei schweren Infektionen“) als 3MRGN (resistent gegen 3 der 4 genannten Antibiotikagruppen resistent) bzw. 4MRGN (resistent gegen 4 der 4 genannten Antibiotikagruppen resistent) klassifiziert.[9,10]

Bei den vier zur MRGN-Klassifizierung herangezogenen Substanzen bzw. Substanzklassen handelt es sich um Piperacillin, Cefotaxim/Ceftazidim, Carbapenemen (Meropenem bzw. Imipenem) und Ciprofloxacin.[9,10]

1.3.1. Entwicklung und Übertragung von Antibiotikaresistenzen

Im folgenden Abschnitt werden die verschiedenen Möglichkeiten des Erwerbs sowie der Verbreitung von Antibiotikaresistenzen bzw. ARG vorgestellt, da diese von Bedeutung für die Interaktion zwischen Antibiotikarückständen, ARG und ARB in der aquatischen Umwelt sind.

Allgemein kann zwischen zwei verschiedenen Mechanismen des Resistenzerwerbs unterschieden werden. Dem Erwerb der Antibiotikaresistenz durch eine Mutation im eigenen Genom und der Übertragung von ARG eines resistenten Organismus auf einen zuvor sensiblen Organismus via horizontalem Gentransfer (HGT).[66] Drei wesentliche Mechanismen sind beim HGT zu unterscheiden. Die Übertragung von mobilen genetischen Elementen (z.B. Plasmide) via Konjugation, die Übertragung von DNA bzw. ARG in eine Bakterienzelle über Phagen (Transduktion) und die Aufnahme freier DNA-Segmente via Transformation.[25]

Bei der Mutation handelt es sich um eine Veränderung der Nucleotidbasensequenz im Genom eines Organismus, die an nachfolgende Generationen vererbt werden kann und deren Ausmaß von der Art der Mutation abhängig ist (Punktmutation, Leserastermutation etc.).[25] Als Beispiel sei der Resistenzerwerb bzw. die Erhöhung der minimalen Hemmkonzentration gegenüber Vancomycin bei Vancomycin-*Intermediate Staphylococcus aureus* Spezies genannt, der auf Punktmutationen von Genen, die an der Zellwandsynthese beteiligt sind, zurückzuführen ist.[67]

Neben der Resistenzentwicklung durch Mutation kann ein Erwerb von Resistenzinformationen durch mobile genetische Elemente erfolgen, was eine Erweiterung des bereits im Bakterium vorhandenen Genoms bedeutet und die Übertragung von ARG zwischen verschiedenen Zellen ermöglicht. Eine entscheidende Rolle spielen hierbei Resistenz-Plasmide, die ARG tragen können. Hierbei handelt es sich um selbstreplizierende, überwiegend ringförmige DNA, die über Konjugation zwischen Bakterien der gleichen Spezies oder unterschiedlicher Spezies übertragen werden kann.[13,25]

Neben der klassischen Konjugation eines Resistenzplasmids von einer Donor- in eine Rezipientenzelle über einen Zell-Zell-Kontakt spielt die sogenannte Transposition, die Verlagerung von Genen innerhalb des Genoms, bei der Verbreitung von Mehrfachresistenzen und der Entwicklung neuer Resistenzplasmide eine entscheidende Rolle.[25,68]

So können z.B. ARG innerhalb eines Organismus zwischen Chromsom und Plasmid bzw. zwischen zwei unterschiedlichen Plasmiden über sogenannte Transposons übertragen werden.[13,25,68]

Die durch Transposition mögliche Anhäufung von ARG auf einem Resistenzplasmid kann so dazu führen, dass durch Konjugation dieses genetisch veränderten Plasmids simultan mehrere ARG auf eine andere Zelle übertragen werden können.[25,68] Ferner kann so eine Übertragung von chromosomal kodierten ARG, nach Übertragung des Transposons auf ein Plasmid, auf andere Zellen via Konjugation übertragen werden.[13] Einen Sonderfall stellen konjugative Transposons (z.B. Tn916, u.a. Übertragung von Tetracyclin-Resistenzen) dar, die ohne vorherigen Einbau in ein Plasmid in eine andere Bakterienzelle übertragen werden können, indem sie sich von der wirtseigenen DNA lösen, in die Empfängerzelle transferieren und dort in die DNA eingebaut werden.[68]

Von besonderer Bedeutung sind genetische Strukturen, die als Integrons bezeichnet werden und die auf Plasmiden bzw. Transpons lokalisiert sein können. Diese sind dazu in der Lage, sogenannte Genkassetten (freie DNA, die Resistenzgene gegen unterschiedlichste Substanzen enthalten können) spezifisch innerhalb der attI Stelle im Integron mittels Integrase zu integrieren und dadurch ggf. mehrere ARG anzuhäufen, sodass bei der Übertragung des Integron tragenden Plasmids simultan mehrere ARG auf die Rezipienten-Zelle übertragen werden.[13,68]

1.4. Umweltrelevanz

Die im vorherigen Kapitel beschriebenen Resistenzmechanismen können z.B. im Rahmen einer Antibiotikatherapie von Mensch bzw. Tier dazu führen, dass ARB im Vergleich zu sensiblen Bakterien selektiv im Wachstum begünstigt bzw. nicht abgetötet werden.[13]

Entfällt dieser Selektionsvorteil durch z.B. den Wegfall des Antibiotikums bzw. durch das Absinken der Wirkstoffkonzentration, können Antibiotikaresistenzen eine physiologische Benachteiligung („*fitness-cost*“) für ARB darstellen, wodurch diese gegenüber antibiotikasensiblen Bakterien im Wachstum benachteiligt sein können.[69,70]

Jedoch müssen Antibiotikaresistenzen nicht zwangsweise einen erhöhten Aufwand für den resistenten Organismus verursachen und können sogar trotz des fehlenden Selektionsdruckes durch Antibiotika zu einem Wachstumsvorteil für ARB gegenüber z.B. dem sensiblen Wildtyp führen, wodurch ein Erhalt der Antibiotikaresistenz begünstigt werden kann.[69,70]

Als Beispiel sei das Phänomen der Kreuzresistenzen genannt, die sich ergeben, wenn mehrere Resistenzinformationen (z.B. gegen Antibiotika, Desinfektionsmittel und Schwermetalle) auf demselben mobilen genetischen Element (z.B. Plasmid, Transposon, Integron) lokalisiert sind.[69–71] So könnte z.B. durch Schwermetallrückstände in Abwesenheit von Antibiotika die Verbreitung bzw. die Persistenz von Plasmiden, die ARG tragen, gefördert werden, sofern diese Plasmide auch Gene bzgl. einer Resistenz gegenüber Schwermetallen tragen.[72]

Baquero et al. (2008) veranschaulicht mit seiner Theorie der vier genetischen Reaktoren bzgl. der Entwicklung von Antibiotikaresistenzen, dass dem mit Antibiotika behandelten Organismus nachgeschaltete Kompartimente wie z.B. Abwasser oder Oberflächengewässer ebenfalls von Bedeutung für den Erwerb von Antibiotikaresistenzen und den genetischen Austausch zwischen verschiedenen Spezies unterschiedlicher Herkunft (Pathogene, fakultativpathogene Bakterien sowie Umweltbakterien) sind.[71] Insbesondere Abwasser wird so eine wichtige Rolle bei der Verbreitung und Entstehung von Antibiotikaresistenzen zuteil, da dieses als eine Art „Schmelztiegel“ für den genetischen Austausch von Resistenzinformationen dienen und sowohl AR, ARB als auch ARG enthalten kann.[2,73–77]

Bereits 1999 erörterte Feuerpfeil et al. die Frage, „ob Antibiotika nicht nur beim Einsatz in der Klinik oder Tierhaltung an der Selektion ARB beteiligt sind, sondern ob sie nach Eintrag in die Umwelt auch dort noch eine Zunahme antibiotikaresistenter Bakterien bewirken“.[51]

AR werden z.B. aufgrund eines unvollständigen Metabolismus im behandelten Organismus als unveränderter Wirkstoff (Ausscheidungsraten zwischen 10% und 90%), als acetylierte oder glucuronidierte Konjugate (u.a. zur Verbesserung der renalen Eliminationsrate[78]) sowie als Abbauprodukte (z.B. die Dehydration von Erythromycin zu Anhydroerythromycin bzw. die Spaltung von Meropenem in seinen ringgeöffneten Metaboliten) ins Abwasser ausgeschieden.[79–82] Im weiteren Verlauf gelangen so AR, ARB und ARG über kommunale Kläranlagenabläufe in Oberflächengewässer, da auch der Abbau von AR, ARB und ARG durch aktuelle Abwasseraufbereitungsverfahren unvollständig ist.[73,77,83]

Im Vergleich unterschiedlicher Kläranlagen zeigte sich, dass in Abhängigkeit vom jeweiligen Wirkstoff die Abbauleistungen z.T. extrem schwankten, was an unterschiedlichen Charakteristika der Kläranlage wie z.B. der Art der biologischen Klärung, der Verweilzeit des Abwassers bzw. des Klärschlamms oder der Durchführung zusätzlicher Desinfektionsverfahren (z.B. Chlorung, Ozonung, UV-Desinfektion oder modernen Membranverfahren) liegen kann.[83] So müssen unterschiedliche Abbauprozesse wie z.B. die Sorption an Klärschlamm (Fluorchinolone, Eliminationsrate: 88-92%)[84], die Hydrolyse von β-Lactam-Antibiotika (Amoxicillin, max. Abbaurate: 99%)[85] aber auch die Umwandlung von im Organismus gebildeten Konjugaten in ihre ursprüngliche Wirkform (N-Acetylsulfamethoxazol → Sulfamethoxazol)[80] bei der Bewertung der Abbauraten von AR innerhalb der Kläranlage berücksichtigt werden.[83] Im Vergleich hierzu erreichen konventionelle Kläranlagen eine Abbaurate von ARB zwischen 2 und 3 log-Stufen, die ähnlich zu der Eliminationsleistung von antibiotikasensiblen Bakterien ist.[86] Zusätzliche Aufbereitungsverfahren wie der Einsatz von Ozon bzw. Chlor oder die Verwendung von Membran-Verfahren können eine weitere Verbesserung der Eliminationsraten von ARG, ARB oder AR bewirken.[83,87,88] Jedoch ist diese Verbesserung zum einen abhängig von z.B. der eingesetzten Menge an Ozon bzw. Chlor, der Porengröße der verwendeten Membran sowie abwasserspezifischen Parametern (u.a. pH-Wert, Anteil an gelöstem organischen Kohlenstoff) und kann zwischen verschiedenen AR, ARG und ARB stark variieren.[83,87,88]

Des Weiteren können AR über z.B. die Entlastung von kommunalen Kläranlagen durch Mischwasserabschläge bei Starkregenereignissen, die Ausbringung von Gülle, Klärschlamm oder Gärresten sowie die Anwendung von Antibiotika in Aquakulturen in die Umwelt gelangen.[89–94] Diese komplexen Eintrittspfade in Verbindung mit dem unvollständigen Abbau im Organismus bzw. in den Kläranlagen und den hohen Antibiotikaverbrauchszahlen führen dazu, dass Antibiotikarückstandskonzentrationen weltweit in Oberflächengewässern (bis in den zweistelligen µg/L-Bereich) bzw. in Grundwasser (bis in den einstelligen µg/L-Bereich) nachgewiesen wurden.[1,77,85,95,96]

Es gilt jedoch zu beachten, dass der Abbau von AR nach der Kläranlage in der aquatischen Umwelt durch z.B. die Hydrolyse von β-Lactam-Antibiotika oder die Photodegradation von z.B. Tetracyclinen oder Fluorchinolonen fortschreitet.[69,75,97]

Weiterhin müssen die Sorption an organisches Material bzw. Sediment von z.B. Fluorchinolonen sowie eine Komplexierung von z.B. Tetracyclinen durch z.B. Calcium- oder Magnesium-Ionen, die die AR der wässrigen Phase entziehen, beachtet werden.[69,75,84]

Ferner konnte bereits gezeigt werden, dass solche subinhibitorischen Konzentrationen bereits ausreichen, um die Mutation neuer Antibiotikaresistenzen zu induzieren und einen Selektionsdruck zu Gunsten von ARB auszuüben.[3,98] Zudem konnte für Sulfamethoxazol und Gentamicin nachgewiesen werden, dass subinhibitorische Konzentrationen auch den Austausch von ARG via Konjugation fördern können.[4]

Dies zeigt, dass in die Umwelt eingetragene AR in Verbindung mit ARB und ARG bei der Prävention der Neuentwicklung und Verbreitung von bereits vorhandenen Antibiotikaresistenzen neben z.B. dem restriktiveren Einsatz in der Human- und Veterinärmedizin oder der Entwicklung neuartiger Wirkstoffe berücksichtigt werden müssen. Diesbezüglich wurden 2017 vier Schwerpunkte identifiziert, die zum besseren Verständnis der Resistenzentwicklung und –verbreitung weitere Forschung benötigen.[99]

Hierzu zählen die weitere Erforschung der Eintrittspfade von ARB und AR in die Umwelt, der Einfluss dieser Emittenten auf die Umwelt, die Bedeutung der Umwelt bei der Resistenzentwicklung und etwaige Rückkopplungen bzw. etwaige Auswirkungen durch resistente Umweltbakterien auf Mensch bzw. Tier sowie technische, politische und gesellschaftliche Möglichkeiten zur Reduzierung und Prävention von Antibiotikaresistenzen in der Umwelt.[99]

1.5. Zielsetzung der Arbeit

Die vorliegende Dissertation schließt sich den oben genannten Schwerpunkten an und ist ein Bestandteil des vom Bundesministerium für Bildung und Forschung (BMBF) geförderten Verbundprojektes „Biologische bzw. hygienisch-medizinische Relevanz und Kontrolle Antibiotika-resistenter Krankheitserreger in klinischen, landwirtschaftlichen und kommunalen Abwässern und deren Bedeutung in Rohwässer" („HyReKA", FKZ: 02WRS1377), welches sich das Ziel setzte, Eintrittspfade von ARB, ARG und AR von Mensch oder Tier in die Umwelt („*Source Dissemination*") sowie etwaige Rückkopplung zurück zum Menschen („*Microbial Dissemination*") zu identifizieren und geeignete Verfahren zu dessen Unterbrechung zu untersuchen.[100]

Hierzu sollte in der vorliegenden Dissertation eine schnelle und kostengünstige Multimethode mittels Flüssigchromatographie-Tandem-Massenspektrometrie (engl. *liquid-chromatography-mass spectrometry/mass spectrometry*, LC-MS/MS) entwickelt werden, die ein Screening verschiedener wässriger Matrices (u.a. Oberflächenwasser und Abwasser) auf AR ermöglicht. Ferner sollte sich diese Methode durch ein breites Spektrum an Analyten auszeichnen, um eine Vielzahl an Antibiotika, die in der Human- und Veterinärmedizin eingesetzt werden, qualitativ und quantitativ nachweisen zu können. Als Richtwert bezüglich der Empfindlichkeit, welche die Screening-Methode erreichen soll, wurden die von Bengtsson-Palme und Larsson (2016) vorgeschlagenen antibiotikaspezifischen Rückstandskonzentrationen, unterhalb derer keine Selektion zugunsten von ARB bzw. der Entwicklung von Antibiotikaresistenzen zu erwarten sind (engl. *Predicted-No-Effect-Concentration*, $PNEC_{Res}$), herangezogen.[3] Im weiteren Verlauf der Arbeit sollten verschiedene Teilaspekte des Antibiotikaeintrags in die Umwelt näher untersucht werden und etwaige „*Hotspots*“ identifiziert werden.

1.5.1. Flüssigchromatographie-Tandem-Massenspektrometrie (LC-MS/MS)

Bei chromatographischen Trennverfahren kann zwischen der Gaschromatographie (GC), die sich zur Analyse von flüchtigen Substanzen bzw. Stoffen, die sich ohne Zersetzung bei hohen Temperaturen verdampfen lassen, eignet und der Hochleistungsflüssigchromatographie (engl. *high performance liquid chromatography*, HPLC) bzw. Flüssigchromatographie (engl. *liquid chromatographie*, LC), die zur Analyse von gelösten Substanzen eingesetzt werden kann, unterschieden werden.[101,102]

Im Falle der in dieser Dissertationsschrift thematisierten AR wird überwiegend die LC als chromatographisches Trennverfahren eingesetzt.[80,81,85,95,103,104] Die Analyse von AR via GC spielt nur eine untergeordnete Rolle und ist nur selten in Publikationen wie z.B. bei der Untersuchung von Sulfonamiden nach Derivatisierung beschrieben.[105]

Bei der LC-MS/MS erfolgt die Analyse der jeweiligen Zielanalyten in zwei Schritten: Der chromatographischen Trennung der Analyten und die nachgeschaltete massenspektrometrische Detektion (Identifikation und Quantifizierung) der zeitlich voneinander getrennten Analyten.[101,106]

Das Trennprinzip der LC beruht auf unterschiedlich starken Wechselwirkungen der in der mobilen Phase gelösten Analyten und der verwendeten Trennsäule, sodass Analyten, die stärker mit der stationären Phase wechselwirken, eine längere Zeit benötigen, um den Detektor zu erreichen.[101,107]

Beeinflusst wird die Retention der jeweiligen Analyten durch das verwendete Säulenmaterial der stationären Phase (meist ein durch funktionelle Gruppen modifiziertes Kieselgel) und die Zusammensetzung der mobilen Phase sowie der daraus resultierenden Elutionskraft.[101,107] Die zwei klassischen LC-Trennverfahren stellen die Normalphasen-Chromatographie und die Umkehrphasen- (engl. *reversed-phase,* RP) Chromatographie dar.[101] Zur Trennung von Antibiotika in wässrigen Matrices wird meist im RP-Modus gearbeitet, da neuartige chemische Modifizierungen der endständigen Silanol-Gruppen des Silicagels eine optimale Trennung von Analyten mit sowohl apolaren als auch polaren Eigenschaften erlauben.[108–110]

Bei der LC-MS/MS erfolgt nach der chromatographischen Trennung der einzelnen Substanzen eine Identifikation der Analyten anhand ihrer spezifischen Masse-zu-Ladungs-Verhältnisse (m/z) mittels Massenspektrometer.[106]

Die hierzu benötigten Analytionen werden in der Ionisationsquelle erzeugt.[106,111] Bei LC-MS/MS-Kopplungen sind die Elektrospray-Ionisation (ESI) sowie die *atmospheric pressure chemical ionization* (APCI) die gängigsten Ionisationsverfahren.[111] Die Ionisation der zu untersuchenden AR via ESI hat sich in den meisten Fällen als Mittel der Wahl erwiesen.[109]

Bei dieser Technik wird das von der LC-Trennsäule kommende Eluat durch eine dünne Kapillare in die Ionisationskammer gesprüht. Da zwischen der Sprühkapillare und dem Sprayschild bzw. dem *Orifice* (Eintrittsöffnung in den Massenanalysator) eine Hochspannung anliegt (bis zu 5000 V), erfolgt unter Einwirkung eines elektrischen Feldes eine Ladungstrennung im einströmenden Eluat und die Ausbildung des sogenannten Taylor-Konus, der an der Spitze in Form eines dünnen Flüssigkeitsstrahls in Richtung des *Orifice* verläuft.[106,111]

Aufgrund des hohen Ladungsüberschusses in diesem Flüssigkeitsstrahls werden nach Überschreitung der Rayleigh-Grenze geladene Tröpfchen gebildet, die das eigentliche Spray erzeugen. Diese Tröpfchen werden nun durch Verdampfen des restlichen Lösemittels immer weiter eingedampft, sodass daraus eine immer höhere Ladungsdichte und gleichzeitig schrumpfende Oberfläche der Tröpfchen resultiert, was letztlich in der sogenannten Coulomb-Explosion endet und die sanfte Bildung der Analytionen durch die ESI erklärt.[106,111]

Nach der Überführung der in der ESI erzeugten Ionen durch die Ionenoptik in den eigentlichen Massenanalysator und den Hochvakuum-Bereich erfolgt eine Trennung der Ionen anhand ihres m/z und die Detektion des jeweiligen Analyten durch z.B. Sekundärelektronenvervielfältiger.[106,111] Bei der Analytik von AR wird häufig ein Triple-Quadrupol-Massenspektrometer als Massenfilter verwendet.[112]

Bei einem Triple-Quadrupol-Massenspektrometer handelt es sich um die räumlich versetzte Anordnung von drei Quadrupolen (Q1 bis Q3), welche jeweils aus vier hyperbolischen Metallstäben bestehen. Im Q1 wird durch Anlegen von Wechsel- bzw. Gleichspannung an die jeweils gegenüberliegenden Paare von Metallstäben ein elektrisches Feld induziert, welches die Ionen eines spezifischen m/z auf eine stabile oszillierende Flugbahn zwingen und so ein Durchqueren des Massenfilters ermöglichen. Ionen eines abweichenden m/z weichen von dieser Flugbahn ab, treffen auf die Metallstäbe und werden so neutralisiert, was die Funktion von Quadrupolen als Massenfilter erklärt. Die so gefilterten „Vorläufer-Ionen“ (engl. *precursor ions*) werden anschließend in die Kollisionszelle (Q2) beschleunigt und durch Zusammenstöße mit dem Stoßgas (z.B. Stickstoff oder Argon) nochmals in „Fragment-Ionen“ (engl. *product ions*) fragmentiert, die wiederum im Q3 (analog zu den Vorläufer-Ionen im Q1) gefiltert werden können.[106,111]

Besonders bei der quantitativen Analytik von AR erwies sich der „*multiple reaction monitoring* (MRM) *mode*“ als sensitives Detektionsverfahren.[108] Hierbei werden nur vorab definierte m/z der Vorläufer-Ionen im Q1 selektiert und in Q2 fragmentiert, wobei wiederum nur ausgewählte m/z der gebildeten Fragment-Ionen in Q3 gefiltert werden, sodass analytspezifische Massenübergänge detektiert werden, die für eine sichere Identifikation und Quantifizierung der jeweiligen Analyten verwendet werden können.[106]

2. Analytik antibiotisch wirksamer Substanzen in wässrigen Matrices

2.1. Einleitung

Die analytische Bestimmung antibiotisch wirksamer Substanzen stellt seit Mitte des 20. Jahrhunderts eine Herausforderung für die unterschiedlichsten Disziplinen der analytischen Chemie dar. Eine der ersten chemischen Untersuchungsmethoden ist die von Jelinek und Boxer im Jahre 1948 veröffentlichte fluorometrische Methode zur Bestimmung des Streptomycin-Gehaltes in Körpergewebe und Urin.[113] Neben solchen Methoden, die zur Überprüfung der Wirkstoffkonzentration im Blut dienen, werden Antibiotika zudem in Humanmaterial (z.B. Serum oder Urin) analysiert, um stoffspezifische Vorgänge der Pharmakokinetik sowie des Metabolismus aufklären zu können.[114–116]

Des Weiteren stellen Methoden zur Bestimmung von AR in Lebensmitteln tierischen Ursprungs einen Teilaspekt bei der Sicherstellung eines hohen Maßes an Verbraucherschutz und Lebensmittelsicherheit dar. Die eingesetzten Verfahren variieren hierbei zwischen qualitativen Schnelltests zur Überprüfung auf das Vorhandensein von Hemmstoffen und der quantitativen Bestimmung spezifischer AR mittels moderner LC-MS/MS Verfahren.[117–119]

Als Umweltkontaminante wurden AR (z.B. Chlortetracyclin, Sulfonamide und Chloramphenicol) erstmalig in den 70iger und 80iger Jahren des 20. Jahrhunderts in Gülle thematisiert.[120,121] Beinahe zwei Dekaden später wurden AR als Kontaminanten im aquatischen Kreislauf mit komplexen Eintragspfaden und Rückkopplungswegen zum Menschen beschrieben.[51,122–124] Aufgrund der vielschichtigen Eintrittspfade können sowohl veterinär- als auch humanmedizinische Präparate unabhängig der Tierart oder der Abwasserart (kommunales Abwasser, klinisches Abwasser, Industrieabwasser) in die aquatische Umwelt gelangen.[2]

Bereits 1999 wurde postuliert, dass subtherapeutische Konzentrationen an Antibiotika einen Einfluss auf die Selektion von Antibiotikaresistenzen sowie deren Übertragung zwischen Bakterien in der Umwelt ausüben könnten.[51]

In diesem Kontext zeigten aktuelle Studien, dass nicht nur hohe Antibiotikakonzentrationen, welche die minimale Hemmkonzentration übersteigen, einen Einfluss auf Resistenzentwicklungen sowie die Verbreitung von Antibiotikaresistenzen ausüben können.[3,98] Solche subinhibitorischen Konzentrationen konnten bereits in den unterschiedlichsten Umweltkompartimenten nachgewiesen werden und lassen eine Persistenz bereits vorhandener Antibiotikaresistenzen sowie den Neuerwerb von Antibiotikaresistenzen befürchten.[4,71,98] Der Nachweis solch niedriger Konzentrationen erfolgt zumeist nach Anreicherung der Analyten via Festphasenextraktion und anschließender Messung mittels LC-MS/MS.[108–110]

Ziel der nachfolgenden Arbeit war es, eine Multianalytmethode zur Bestimmung von Rückständen antibiotisch wirksamer Substanzen zu entwickeln, die sich sowohl durch eine einfache Probenvorbereitung als auch durch eine ausreichende Empfindlichkeit auszeichnen sollte. Ferner sollte ein breites Untersuchungsspektrum anhand einer Vielzahl von unterschiedlichen Antibiotika sowie Substanzklassen implementiert werden, um unabhängig von der Art des Emittenten mögliche „*Hotspots*“ identifizieren zu können. So unterscheiden sich die in der ambulanten Therapie, der stationären Humanmedizin sowie der Veterinärmedizin eingesetzten Präparate teils signifikant.[28,32,41] Dies kann am Beispiel der Fluorchinolone verdeutlicht werden, da Ciprofloxacin lediglich in der Humanmedizin eingesetzt werden darf.[28] Enrofloxacin hingegen ist der Veterinärmedizin vorbehalten.[28,34] Eine derartig strikte Trennung ist jedoch nicht für alle Antibiotika möglich, da zum Teil die gleichen Wirkstoffe in der Human- und Veterinärmedizin eingesetzt werden. Beispiele hierfür wären die Verwendung von Benzylpenicillin (Penicillin G) oder Erythromycin.[28,32] Zudem sollten Antibiotika, die in der Humanmedizin zur Behandlung schwerwiegender gramnegativer nosokomialer Infektionen (z.B. Meropenem, Piperacillin oder Ciprofloxacin) sowie zur Behandlung von Infektionen mit grampositiven Bakterien (Vancomycin oder Linezolid) eingesetzt werden, ins Untersuchungsspektrum aufgenommen werden.[11,13]

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Research Paper

Liquid chromatography-tandem mass spectrometry as a fast and simple method for the determination of several antibiotics in different aqueous matrices

Alexander M. Voigt,[A] *Dirk Skutlarek,*[A] *Christian Timm,*[A] *Christiane Schreiber,*[A] *Carsten Felder,*[A] *Martin Exner*[A] *and Harald A. Faerber*[A,B]

[A]Institute for Hygiene and Public Health, University Hospital Bonn, Medical Faculty, University of Bonn, Building 63, Venusberg-Campus 1, 53127 Bonn, Germany.
[B]Corresponding author. Email: harald.faerber@ukbonn.de

Environmental context. Antibiotic residues released to the environment could influence the selection of antibiotic-resistant bacteria and hence their spread within the aquatic environment. We report a multi-method approach for determining 47 antibiotics in wastewater, surface water, drinking water and groundwater. The method provides a rapid screening of water samples for common antibiotics that have the potential to alter natural bacterial populations.

Abstract. A simple and rapid liquid chromatography-electrospray ionisation-tandem mass spectrometry (LC-ESI-MS/MS) method for the determination of 47 different antibiotics in water samples was developed. A straightforward sample preparation of aqueous samples could be established using a simple dilution step with a mixture of water and acetonitrile (+ 0.8 g L^{-1} ethylenediaminetetraacetic acid (EDTA)), subsequent filtration through a hydrophilised polytetrafluoroethylene (H-PTFE) syringe filter and a subsequent direct injection. The multi-analyte method presented includes substances from eleven classes of antibiotics (penicillins, cephalosporins, carbapenems, macrolide antibiotics, lincosamides, fluoroquinolones, tetracyclines, sulfonamides, glycopeptid antibiotics, oxazolidinones and nitroimidazoles). The method is characterised by a typical dynamic range from 0.01 μg L^{-1} to a maximum of 5 μg L^{-1}, with good linearity regression coefficients ($r^2 > 0.99$) and suitable recovery rates (generally from 65 ± 13 % to 117 ± 5 %) in spiked drinking water, surface water, groundwater and simulated treated wastewater. Suitable limits of quantification between 3.3 ng L^{-1} and 190 ng L^{-1} could be obtained, which are sufficient to determine low levels of antibiotic residues in the aquatic environment. The efficiency of the developed method was tested by analysing the residual concentrations of antibiotics in a small creek in Germany ('Swistbach'). Sulfamethoxazole in combination with trimethoprim could be detected frequently, with calculated detection frequencies of 94–100 % and 29–47 %, as well as macrolide antibiotics (azithromycin (50–60 %), clarithromycin (82–94 %), clindamycin (88–100 %) and erythromycin (41–53 %). The determined concentrations were in a range between 0.01 μg L^{-1} and 0.43 μg L^{-1} downstream of the municipal wastewater treatment plants, whereas no antibiotics could be detected upstream.

Additional keywords: antibiotic residues, direct injection, environmental health, selection pressure.

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Introduction

The discovery of penicillin by Alexander Fleming in 1928 represented a breakthrough in the therapeutic treatment of bacterial infections, and is regarded as a milestone in modern medicine. However, Fleming also warned of the development of penicillin resistance and the loss of the antibacterial effect of antibiotics arising from improper use (Fleming 1945). This came true for various antibacterial agents only a few years after their initial medical applications (Saga and Yamaguchi 2009).

In general, resistance can be caused by diverse mechanisms, such as mutations, the acquisition of resistance genes through horizontal or vertical gene transfer, and efflux pumps (Baquero et al. 2008; Blanco et al. 2016; Jutkina et al. 2018). In this context, low antibiotic concentrations (< minimal inhibitory concentration, MIC) might lead to an increased probability of the development or spread of antibiotic resistance (Baquero et al. 2008; Jutkina et al. 2018).

A recent study by Bengtsson-Palme and Larsson developed a kind of risk assessment using predicted no effect concentrations (PNECs) for resistance selection. They postulated that no environmental effects are to be expected below these PNECs (Bengtsson-Palme and Larsson 2016). For many antibiotics, such concentrations are present in wastewater (WW) or animal excretions owing to the incomplete metabolism by patients or treated animals (Hirsch et al. 1999; Radke et al. 2009; Voigt et al. 2019).

Subsequently, antibiotic residues can enter the aquatic environment through incomplete degradation or the retransformation of antibiotic metabolites in sewage treatment plants (STPs) (Heberer 2002; Kümmerer 2003; Radke et al. 2009). Other entry paths include runoff or transfer through the ground passage from

manured fields (Christian et al. 2003; Kemper et al. 2008), the use of antibiotics in aquaculture (Hirsch et al. 1999), and the improper disposal of leftover medicaments through the toilet (Feuerpfeil et al. 1999; Westphal-Settele et al. 2018). Therefore, emphasis should also be placed on environmental areas (rather than merely WW and excrements) to reduce and assess the influx of antibacterial substances from anthropogenic sources into the environment and mitigate the potential promotion of resistant microorganisms.

Over the last 20 years, numerous studies have been published with the objective of determining antibiotic residues in various aqueous matrices, e.g. drinking water (DW), groundwater (GW), surface water (SW) and WW (Batt and Aga 2005; Dinh et al. 2011; Rossmann et al. 2014; Watkinson et al. 2009), as well as solid matrices such as manure, soil, sewage sludge and fermentation residues from biogas plants (Hamscher et al. 2002; Langhammer and Büning-Pfaue 1989; Ratsak et al. 2013). These investigations have differed in terms of the number of antibiotics and classes of antibiotics analysed. Various analytical methods for the detection of antibiotic residues have been described in the literature, for example, enzyme-linked immunosorbent assay (Aga et al. 2016; Kumar et al. 2004), gas chromatography (Chiavarino et al. 1998) or liquid chromatography (LC) in combination with different detectors such as UV, fluorescence or mass spectrometry (MS), which are most commonly ion trap, single quadrupole or triple quadrupole (Batt and Aga 2005; Göbel et al. 2004; Grujić et al. 2009; Kai 2003; Nakata et al. 2005). According to the current state of science, LC coupled to a tandem mass spectrometer constitutes one of the most sensitive and selective methods for the measurement of numerous antibiotics in one multi-method approach, and represents the method of choice (Aga et al. 2016; Le Bizec et al. 2009; Monteiro et al. 2017; Seifrtová et al. 2009). In general, the ionisation of antibiotics is conceivable in both electrospray ionisation (ESI), whether positive or negative, as well as by atmospheric pressure chemical ionisation (APCI), whereby ESI, despite its higher susceptibility to matrix effects, is most widely used owing to its higher sensitivity (Seifrtová et al. 2009).

Most published methods for the analysis of antibiotic residues use reversed-phase C18-columns in combination with solid phase extraction (SPE), usually with Oasis HLB cartridges for sample clean-up and the enrichment of target compounds to achieve low limits of detection (LOD) and to reduce matrix effects (Monteiro et al. 2017; Petrović et al. 2005; Seifrtová et al. 2009). However, laborious sample preparation results in higher financial costs (e.g. time to perform SPE and analysis, solvent consumption, SPE cartridges and a more sophisticated method development). In addition, a higher sample volume is required for the extraction step. Owing to the advantages of new high-end LC-MS instruments (e.g. higher flow rates and injection volumes), direct injection represents a possible alternative in which even low traces of substances can be measured without preconcentration of analytes (Busetti et al. 2012). For direct injection, a simple sample preparation, such as filtration, dilution or centrifugation, is usually used in combination with both large injection volumes (up to 5000 µL) and comparatively smaller injection volumes (10 µL to 50 µL) (Busetti et al. 2012; Campos-Mañas et al. 2017). This technique provides an alternative with significantly lower costs for the laboratory. In addition, several studies have already been published for various substance classes like pesticides (Campos-Mañas et al. 2017; Reemtsma et al. 2013), mycotoxins (Malachová et al. 2018) and for doping tests (Deventer et al. 2014) in aqueous and solid matrices.

The applicability of direct injection methods for the analysis of antibiotic residues in SW (Bayen et al. 2014; Denadai and Cass 2015), sea water (Bayen et al. 2014) and WW (Campos-Mañas et al. 2017; Oliveira et al. 2015; Vosough et al. 2015) has also been demonstrated. For example, Bayen et al. (2014) developed a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for the quantification of seven different antibiotics in seawater and freshwater with LOD below the respective PNEC for resistance selection reported by Bengtsson-Palme and Larsson (2016) using direct injection (20 µL) of the filtrated samples. Furthermore, 'dilute-and-shoot' methods can also be used to determine significantly more analytes. Therefore, Campos-Mañas et al. (2017) published a rapid LC-MS/MS method involving a low injection volume (10 µL) for the analysis of 87 organic microcontaminants, which included 18 different antibiotics with limits of quantification (LOQ) between 10 and 400 ng L^{-1}, based on a simple sample preparation (filtration with glass microfibre, PTFE syringe filters and a subsequent dilution step). Nevertheless, there seems to be a lack of direct injection methods that allow the simultaneous determination of a variety of antibiotics (number of analytes > 20), which include important substance classes like carbapenems, ureidopenicillins (e.g. piperacillin) or glycopeptide antibiotics.

Given the multitude of therapeutically used antibiotics, the aim of the research presented here was to develop a simple screening method to detect the contamination of aquatic matrices (for example DW and SW) with a broad spectrum of antibacterial agents regardless of the point source (veterinary and human medicine; clinical and outpatient). In this context, Table S1 (Supplementary Material) displays the chemical structure of all analysed antibiotics (Bryskier 1999) and their application (human or veterinary medicine) based on the online database from a collaborative work between the Division of the German Federal Ministry of Health and the German Institute for Medical Documentation and Information (DIMDI) called 'pharmnet.bund' (DIMDI 2018). The selection of target analytes was determined by the number of prescriptions and degree of therapeutic significance in human and veterinary medicine. In addition, all antimicrobial agents selected had to be detectable simultaneously with sufficient sensitivity using one chromatographic run and detection method.

Furthermore, clinically relevant antibiotics, such as linezolid (LIN), vancomycin (VANC) and meropenem (MERO), should be implemented in this method for environmental aqueous samples. These three antibiotics are classified as 'last resort antibiotics' in the treatment of e.g. serious nosocomial infections with Gram-positive bacteria (LIN, VANC) and Gram-negative bacteria (MERO) (Harris et al. 2015; Isaac et al. 2017; Stevens et al. 2002) and whose entry into the aquatic environment should be prevented, particularly with regard to the increasing resistance reports (Chalhoub et al. 2016; Miller et al. 2016; Niebel et al. 2016; WHO 2017; Zahedi Bialvaei et al. 2017). Accordingly, the proposed method would help to integrate those 'critically important antimicrobials' (WHO 2017) in the aquatic environment into an effective antibiotic resistance surveillance system. In addition, the time for sample preparation and the total analysis time should be shortened by establishing a fast and proper sample extraction method with a subsequent direct injection of the aqueous sample ('dilute-and-shoot'), whereas the PNECs proposed by Bengtsson-Palme and

Table 1. Chromatographic conditions for the separation of 47 various antibiotics and eight ISTDs
ACN, acetonitrile; MeOH, methanol; HCOOH, formic acid; MCT, multicolumn thermostat

LC-System 1290 Infinity II (Agilent Technologies – Waldbronn, Germany)		
Column	NUCLEOSHELL RP18Plus® 2 mm × 100 mm, 2.7 µm (Macherey-Nagel – Düren, Germany)	
MCT temperature	50 ± 0.8 °C	
Eluent A	Water + ACN + HCOOH (98 : 2 : 0.01, v/v/v)	
Eluent B	MeOH + ACN + HCOOH (80 : 20 : 0.1, v/v/v)	
Injection volume	20 µL	
Flow rate	0.4 mL min^{-1}	
Multisampler temperature	10 °C	
Time (min)	Eluent A (%)	Eluent B (%)
0.00	100	0
0.20	100	0
0.25	80	20
4.00	70	30
5.00	60	40
8.00	40	60
9.00	10	90
11.00	10	90
11.50	100	0
20.00	100	0

Larsson (2016) should still be undercut to evaluate the potential risk of an increased selection pressure caused by antibiotic residues within the aquatic environment.

As a first application example for the developed method, a small creek ('Swistbach') in North Rhine-Westphalia (Germany) was examined for antibiotic residues. To this end, samples were taken at six different points along the stream, starting at the source (forest area in Rhineland-Palatinate) and ending at the mouth into the next larger 'Erft' ($n = 17$) from September 2016 until June 2018.

Experimental

Chemicals and materials

All chemicals and solvents used here were purchased from VWR International GmbH (Darmstadt, Germany) and Carl Roth GmbH + Co. KG (Karlsruhe, Germany), and were of HPLC-MS grade. The analytical and internal standards (ISTD) were received from Sigma-Aldrich (Taufkirchen, Germany), Cayman Chemicals (Ann Arbor, United States), Toronto Research Chemicals Canada (Toronto, Canada) and USP Reference Standard (Basel, Switzerland). The LC columns and Xtra micropore filters (pore size of 0.45 µm) made from hydrophilised polytetrafluorethylene (H-PTFE) were obtained from Macherey-Nagel (Düren, Germany). Stock solutions of all analytes and ISTD (1000 mg L^{-1}) were prepared in an acetonitrile water solution (2+1, v/v) and stored at −20 °C. The standards were freshly prepared in a mixture of a filtered (H-PTFE filters, pore size of 0.45 µm, Macherey and Nagel, Germany) solution of DW and acetonitrile (95+5, v/v) that contained 0.4 g L^{-1} EDTA in a concentration ranging from 1 ng L^{-1} to 2500 ng L^{-1}. Fifty microlitres of a mixture of all ISTD (containing 10000 ng L^{-1}) were added to every calibration standard.

Sampling procedure and sample preparation

A total of 98 samples were collected from six different sampling sites ($n = 17$). For site A (source region), only 13 samples were collected owing to intermittent drought at the site. Each sample was taken as a qualified grab sample (five aliquots over eight minutes). The mixed samples were filled in 250-mL amber glass bottles. The samples were transported in ice (2–4 °C) to the laboratory, and prepared and measured on the same day. After dilution (1 : 1) with a solution of water and acetonitrile (95 : 5, v/v) containing 1 µg L^{-1} of each ISTD and 0.8 g L^{-1} EDTA, the diluted samples (containing 0.4 g L^{-1} EDTA and 0.5 µg L^{-1} ISTDs) were filtered through a syringe filter (H-PTFE, pore size of 0.45 µm).

High performance liquid chromatography

An Agilent LC-System 1290 Infinity II with a NUCLEOSHELL RP18Plus® column 2 mm × 100 mm, 2.7 µm, was used to separate the filtered and diluted samples. The temperature of the column oven was set to 50 °C. To minimise contamination between consecutive runs, the special Multiwash Function® from Agilent was used. Thus, the needle and the needle seat were washed with isopropanol (0.1 % formic acid), followed by acetonitrile (0.1 % formic acid), and finally with a mixture of water, acetonitrile and formic acid (98 : 2 : 0.1, v/v/v). The total washing time was 1 min. The temperature of the multisampler was set to 10 °C. The injection volume was 20 µL. The complete chromatographic conditions are shown in Table 1.

Mass spectrometry parameters

The separated antibiotics were analysed by a QTRAP® 6500+ tandem mass spectrometer from AB Sciex GmbH (Darmstadt, Germany). The target components were ionised using an ESI interface in positive mode. The scheduled multiple reaction monitoring (sMRM) mode was selected to detect the two most intensive ion transitions of each antibiotic for identification and quantification.

Therefore, the two most intensive fragment ions were identified in the product ion scans and specific MS parameters were determined. The specific MS parameters are shown in Table 2.

Table 2. Specific instrumental parameters (DP, CE, and CXP), retention time (t_R, including the respective dynamic sMRM detection window (s)), obtained precursor and fragment ions for each analyte (including abbreviation of antibiotics)

CE, collision energy; CXP, cell exit potential; DP, declustering potential; t_R, retention time

Antibiotics	CAS No.	Precursor ion	Fragment ion	t_R (min)	Window (s)	DP (V)	CE (V)	CXP (V)
Penicillins								
Amoxicillin, AMOX	26787–78–0	366.0	349.1	1.13	20	26	11	12
			207.9			26	17	12
Ampicillin, AMP	69–53–4	350.0	106.0	1.73	35	36	21	12
			173.9			36	21	20
Penicillin G, PEN-G	61–33–6	335.0	160.0	7.06	35	56	15	10
			175.9			56	17	20
Cloxacillin, CLOX	61–72–3	435.9	276.9	8.52	40	31	19	18
			160.0				17	18
Dicloxacillin, DICLOX	3116–76–5	469.9	160.0	9.06	35	51	17	18
			310.8				19	18
Flucloxacillin, FLU	5250–39–5	453.9	159.9	8.65	35	36	17	18
			294.9				19	18
Methicillin, METHI	61–32–5	381.0	165.0	6.55	45	66	23	20
			222.0				29	14
Mezlocillin, MEZLO	51481–65–3	540.0	295.8	7.14	35	76	27	18
			252.9				43	14
Nafcillin, NAF	985–16–0	415.1	199.0	8.92	45	146	17	12
			170.9				49	20
Oxacillin, OXA	66–79–5	402.0	243.1	8.12	45	36	17	16
			159.9				17	20
Penicillin V, PEN-V	87–08–1	351.0	160.0	7.75	35	46	15	20
			113.9				45	14
Piperacillin, PIP	66258–76–2	518.2	143.0	7.00	40	36	21	18
			160.0				15	18
Carbapenems								
Meropenem, MERO	96036–03–2	384.1	141.0	1.32	40	56	21	18
			68.0				79	8
Cephalosporins								
Cefaclor, CEFA	53994–73–3	368.0	173.9	1.70	40	21	19	20
			106.0				23	12
Cefotaxime, CEFO	63527–52–6	455.9	396.0	2.94	40	56	13	14
			167.0				27	20
Ceftazidime, CEFT	72558–82–8	546.9	166.8	1.44	40	46	33	20
			276.9				27	18
Sulfonamids								
Sulfachloropyridazine, SCP	80–32–0	284.9	155.9	2.70	45	26	21	18
			108.0				31	12
Sulfadiazine, SDZ	68–35–9	251.0	155.9	1.51	35	56	21	18
			108.0				31	14
Sulfadimethoxine, SDMX	122–11–2	310.9	156.0	4.73	45	76	27	18
			108.0				35	14
Sulfadimidine, SDMD	57–68–1	279.0	186.0	2.31	35	1	23	22
		124.0				29	16	
Sulfadoxine, SDX	2447–57–6	310.9	156.0	3.15	35	41	23	18
			108.0				33	14
Sulfaethoxypyridazine, SEP	963–14–4	294.9	155.9	3.76	45	61	23	33
			108.0				18	14
Sulfamethoxypyridazine, SMP	80–35–3	280.9	155.9	2.40	35	46	23	18
			108.0				33	12
Sulfamerazine, SMZ	127–79–7	265.0	107.9	1.83	35	56	33	12
			92.1				33	12
Sulfamethoxazole, SMX	723–46–6	254.0	156.0	2.85	45	51	21	18
			108.0				31	12
N4-Acetylsulfamethoxazole, N4AcSMX	21312–10–7	296.0	198.0	4.04	45	76	23	24
			134.0				31	16
Sulfathiazole, STZ	72–14–0	255.9	155.9	1.58	35	41	19	18
			92.0				33	12
Trimethoprim, TMP	738–70–5	291.0	230.0	1.47	50	111	31	14
			261.0				33	16

(*Continued*)

Table 2. (Continued)

Antibiotics	CAS No.	Precursor ion	Fragment ion	t_R (min)	Window (s)	DP (V)	CE (V)	CXP (V)
Macrolide antibiotics and lincosamides								
Azithromycin, AZI	83905–01–5	375.4	83.0	3.48	40	41	21	10
			158.1				29	8
Clarithromycin, CLA	81103–11–9	748.4	590.3	7.92	45	121	25	20
			158.0				35	18
Clindamycin, CLIN	18323–44–9	425.2	126.1	4.87	45	101	33	16
		427.2	126.1			1	35	8
Erythromycin, ERY	114–07–8	734.4	576.3	6.85	35	1	25	20
			158.1				35	18
Anhydroerythromycin, dh-ERY	23893–13–2	716.4	558.3	7.39	40	1	19	20
			158.0				35	18
Roxithromycin, ROX	80214–83–1	837.4	679.3	8.11	40	1	31	24
			158.0				39	18
Spiramycin, SPIR	8025–81–8	422.4	174.0	3.60	45	56	27	20
			100.9				21	12
Tylosin, TYL	847659–38–5	916.4	772.4	7.33	40	6	41	26
			174.0				47	20
Fluorochinolones								
Ciprofloxacin, CIP	85721–33–1	332.0	313.9	1.75	50	71	27	18
			231.0				49	12
Enrofloxacin, ENRO	93106–60–6	360.1	342.0	1.98	40	71	29	18
			315.9				25	20
Ofloxacin, OFLOX	82419–36–1	362.1	318.0	1.63	35	91	25	20
			261.0				37	14
Moxifloxacin, MOX	354812–41–2	402.0	357.7	3.40	40	111	27	22
			260.8				33	14
		Others						
Linezolid, LIN	165800–03–3	338.0	295.9	4.40	45	101	25	20
			234.9				27	14
Metronidazole, METRO	443–48–1	171.9	127.9	1.49	35	31	19	16
			82.0				31	12
Vancomycin, VANC	1404–90–6	725.0	144.1	1.16	35	56	19	16
			100.0				63	12
Tetracyclines								
Chlortetracycline, CTC	64–72–2	479.0	444.0	3.58	100	66	29	26
			461.9				23	30
Doxycycline, DOC	10592–13–9	445.2	428.2	4.26	150	1	25	28
			267.0				49	30
Oxytetracycline, OTC	2058–46–0	461.0	426.0	2.11	45	66	25	26
			443.0				17	36
Tetracycline, TC	64–75–5	445.1	410.0	2.11	45	61	27	12
			427.0				17	26
Internal standards								
CEFO-D_3	n.a.	459.0	326.7	2.94	35	66	19	40
			166.9				27	20
CIP-D_8	1130050–35–9	340.1	234.9	1.75	45	86	51	28
			295.9				25	34
CLIN-$^{13}C,D_3$	2140264–63–5	429.1	130.0	5.55	45	1	35	16
			381.1				27	20
LIN-D_3	1127120–38–0	341.1	297.0	4.40	40	111	25	16
			235.0				29	12
MERO-D_6	1217976–95–8	390.1	147.0	1.32	35	46	21	18
			113.9				31	14
PIP-D_5	n.a.	523.0	148.0	7.00	35	51	23	18
			159.8				15	20
SMXl-$^{13}C_6$	1196157–90–0	260.0	161.9	2.85	45	56	21	18
			114.0				33	14
AMP-D_5	1426173–65–0	355.0	110.9	1.73	35	51	23	14
			196.9				23	24

Table 3. Optimised MS parameters
ESI, electrospray ionisation; sMRM, scheduled multiple reaction monitoring

QTRAP® 6500+ mass spectrometer (AB Sciex - Darmstadt, Germany)	
Time	20 min
Curtain gas	45 psi
Collision gas	Medium
Ion spray voltage	5500 V
Temperature	500 °C
Ion source gas 1 (nebulizer)	80 psi
Ion source gas 2 (evaporator)	60 psi
Entrance potential	10 V
Ionisation	ESI (+)
Scan mode	sMRM

Finally, the most intense ion transitions were used as quantifiers, and the next most intense as qualifiers. To optimise the solution-specific ESI parameters, these parameters were tuned manually with a mixed standard (containing 1 μg L^{-1} of each analyte) using a flow injection analysis bypassed to the mass spectrometer. The parameters leading to the highest intensity of the total ion current (TIC) were set for the final detection method. Nitrogen was used as nebuliser, Curtain^{TM}, drying and collision (collisionally activated dissociation, CAD) gas. The parameters that provided the best compromise for all substances are shown in Table 3.

For the control of LC and MS, the software *Analyst*® (ver. 1.6.3) combined with an 'Analyst Device Driver' (ver. 1.2) was used. The received data were analysed using *Multiquant*® (ver. 3.0.2) and *Peakview*® (ver. 2.2); all software was from AB Sciex GmbH (Darmstadt, Germany).

Method assessment

Typical validation parameters, such as recovery, precision, linearity, LOD and LOQ, were defined for the developed method in several matrices. To compensate for possible treatment losses during the filtration as well as possible ion enhancements or suppressions during ionisation arising from matrix effects, isotope labelled standards were attached to the target analytes.

For the assignment of the ISTD, the specific recovery rates of each analyte and potential ISTD were investigated in different matrices using specific external calibration curves for each analyte and ISTD. Therefore, blank matrices of DW (Bonn), SW (Rhine near Bonn), GW (groundwater gauge near Bonn) and treated WW were spiked with each analyte and ISTD at four different concentration levels (0.05, 0.1, 0.5 and 2.0 μg L^{-1}) with five replicates. To estimate recovery rates for treated WW, 10 mL of the certified simulated matrix – MMW001–250ML from Sigma-Aldrich (Taufkirchen, Germany) – was filled up to 1 L with MilliQ water to simulate antibiotic-free treated WW. All spiked blank matrices were diluted and filtered as described above.

To investigate the improvement through the use of the ISTD, the final recovery rates for each analyte were determined using an ISTD-calibration. Therefore, blank matrices (DW, GW, SW and treated WW) were spiked with 500 ng L^{-1} of each analyte and ISTD and filtered through H-PTFE (pore size of 0.46 μm) filters. The final recovery was calculated through a ten-point calibration (each standard contained 500 ng L^{-1} ISTD). For a further description of the accuracy, the precision was determined and expressed as the relative standard deviation (RSD) of these spiked matrix samples.

To evaluate the sensitivity, LOD and LOQ were calculated in accordance with the German guidelines of DIN 32645:2008 11 in spiked DW matrix (containing 0.4 g L^{-1} EDTA). Therefore, individual calibration curves were measured around the assumed LOQ (ten calibrators per analyte). Data analysis was performed using the software *Valistat* (ver. 2.0, 2011) by ARVECO. The results were given to two significant decimal digits. The specific linearity was determined by the regression coefficient (r^2) of a 10-point calibration (four replicates), between 10 and 5000 ng L^{-1}, in spiked matrices to assess the dynamic range in which the target concentration was proportional to the signal intensity. Table S2 (Supplementary Material) shows typical water-chemical parameters, like pH, TOC or turbidity, to characterise the blank matrices used.

Results and discussion

Analytical conditions (LC-MS/MS)

For most analytes, single protonated molecules $[M+H]^+$ were detected in full scan mode and defined as precursor ions. For VANC (m/z = 725.0), azithromycin (AZI, m/z = 375.4) and spiramycin (SPIR, m/z = 422.4), double-charged molecular ions (= $[M+2H]^{2+}$) were observed and used as precursors. These observations are consistent with the results of previous studies (Batt and Aga 2005; Cass et al. 2001; Christian et al. 2003; Dasenaki and Thomaidis 2015; Dinh et al. 2011; Geis-Asteggiante et al. 2012; Göbel et al. 2004; la Marca et al. 2012; Lindberg et al. 2004; Monteiro et al. 2017; Sacher et al. 2001). To support the formation of the target molecules $[M+H]^+$, different ionisation modifiers (TFA and HCOOH) as well as different concentrations (0.01, 0.05, 0.1 and 0.1 % for HCOOH) were tested. A content of 0.01 % formic acid in the aqueous eluent (A) and 0.1 % formic acid in the organic eluent (B) provided the best compromise for all analytes regarding peak shape and recovery rates.

Based on the two most intense fragment ions identified, two specific mass transitions could be defined as a quantifier and qualifier for each component and sMRM could be used as the detection mode. Exceptions were sulfadoxine (SDX) and sulfadimethoxine (SDMX), which shared the same mass transitions (m/z: 310.9 > 156.0 and 310.9 > 108.0) but differed sufficiently in their retention time (Δt_R = 1.6). The substance-specific retention time (t_R) was determined by single standard injections. For the detection of such a large number of analytes, sMRM provided the best results by obtaining a sufficient number of data points for each target transition (the target sampling time was set to 0.5 s). Moreover, both MRM transitions of all targeted analytes were only measured around the defined t_R, which yielded greater sensitivity, accuracy and reproducibility.

Furthermore, it should be mentioned that under acidic conditions (pH < 7), erythromycin (ERY) rapidly degraded to its dehydrated degradation product, anhydroerythroymycin (dh-ERY) (Batt and Aga 2005; Seifrtová et al. 2009). Owing to the different types of application (oral or ointment) as well as degradation processes in the environment, the appearance of the original compound (ERY) and the degradation product (dh-ERY) was conceivable. In addition, for the detection of ERY (m/z = 734.4) and dh-ERY (m/z = 716.4), separate qualifiers and quantifiers were defined, which facilitated a distinction between the antimicrobial substance (ERY) and its

degradation product (dh-ERY). However, this required certificated ERY and dh-ERY standards that were freshly added to the stock solution, because ERY degrades to dh-ERY in certain aging processes. In addition, no distinction could be made between the stereoisomers levofloxacin and ofloxacin (OFLOX). Given that OFLOX (50 million defined daily doses (DDD) in 2015) is more widely used in Germany than levofloxacin (1 million DDD in 2015) (Schwabe and Paffrath 2016), OFLOX can be acknowledged as having positive results. However, it should be mentioned that Levofloxacin may also have been present. The overall results for the development of the MS detection mode are presented in Table 2.

Four different LC columns were tested for the chromatographic separation behaviour of the investigated antibiotics in preliminary tests pertaining to peak shape, peak intensity and chromatographic separation. It should be mentioned that the analysis of 47 substances belonging to 11 different groups of active substances with different properties did not permit the optimal separation of all analytes; rather, it always requires a compromise in favour of the best overall performance.

The best results for all target compounds were obtained for operation in the reverse-phase mode with the Nucleoshell RP18Plus® column 2 mm × 100 mm, 2.7 μm (M.-N., Düren), using a binary gradient with a solution of water, acetonitrile and formic acid (98 : 2 : 0.01, v/v/v) as eluent A, and a mixture of acetonitrile, methanol and formic acid (80 : 20 : 0.1, v/v/v) as eluent B. Owing to its hydrophobic octadecyl phase and further polar selectivity, the RP18Plus® column was suitable for the separation of various substance classes with both hydrophilic and lipophilic properties. Furthermore, all silanole groups were end-capped with additional functional groups. In addition, amphoteric tetracyclines could not bind to these groups irreversibly, which would have led to an increased fronting of the separated peaks as previously described (Seifrtová et al. 2009; Valette et al. 2004).

To find a suitable compromise for all analytes (e.g. with hydrophilic, lipophilic, cationic, basic or amphoteric properties), the binary gradient began aqueous (100 % eluent A) and was increased quickly to non-polar conditions (90 % eluent B). A mixture of methanol, acetonitrile and formic acid (here 80 : 20 : 0.1, v/v/v) was used as organic eluent, comparable to other studies, to obtain an effective concession between a sufficient peak separation and short analysis time (Batt and Aga 2005; Monteiro et al. 2017; Sacher et al. 2001; Seifrtová et al. 2009).

The final chromatographic conditions are given in Table 1. Fig. 1 shows extracted ion chromatograms (XICs) of all separated antibiotics (only quantifier) in a standard solution (0.5 μg L^{-1}).

Sample pretreatment

The aim of the present work was the development of a fast and cost-effective sample preparation for aqueous samples with regard to the analysis of several antibiotics (human and veterinary application). This could be achieved by a dilution step and a filtration of the sample through a syringe filter ('dilute-and-shoot'). For sample clean-up, the sample was diluted (1 : 1) with a solution of water and acetonitrile (95 : 5, v/v + 0.8 g L^{-1} EDTA) and then filtered through a syringe filter. To minimise filtration losses, syringe filters made of various materials (polyethersulfone, mixed cellulose ester, cellulose acetate, regenerated cellulose, polyamide, polyvinylidene difluoride, hydrophobic polytetrafluorethylene and hydrophilised polytetrafluorethylene) were tested ($n = 2$; pore size = 0.20 and 0.45 μm).

The best results were obtained for H-PTFE filters (Macherey-Nagel, Düren, Germany), which consisted of a hydrophobic membrane with additional hydrophilic properties, and are recommended for processing aqueous samples with polar and non-polar analytes. Especially for the analysis of macrolide antibiotics, a higher pore size (0.45 μm instead of 0.20 μm) was chosen, which resulted in far superior recovery rates of these antibiotics in the preliminary experiments (results not shown). For example, the recovery rates for roxithromycin, clarithromycin and ERY could be improved with 0.20-μm H-PTFE filters from 31 %, 31 % and 84 % respectively up to 64 %, 64 % and 70 %, and with 0.45-μm H-PTFE filters at the beginning of the method development.

In the presence of calcium and magnesium ions, tetracyclines tend to form chelate complexes (Kümmerer 2009; Lindsey et al. 2001), which can no longer be detected analytically by LC-MS/MS. Furthermore, previous studies have already shown that the recovery rates for macrolides can be significantly improved by the addition of EDTA to matrices with a low salt content (Batt and Aga 2005; Hirsch et al. 1999). To achieve sufficient recovery rates, the peak area of spiked SW (0.5 μg L^{-1}) with different concentrations of EDTA was determined to find the optimum EDTA concentration to bind calcium and magnesium ions and to improve the recovery of tetracyclines. The comparison of the peak areas of different tetracyclines (chlortetracycline, doxycycline, oxytetracycline and tetracycline) showed no discernible differences above 0.4 g L^{-1} EDTA in the final sample.

Method performance

After the establishment of the sample pretreatment procedure and completion of the LC method, the accuracy of the developed method was evaluated by the recovery rates, precision, dynamic range, linearity, LOD and LOQ.

Preliminary experiments showed that in both DW and SW samples, measured against a matrix-matched calibration (filtered DW with 5 % acetonitrile and 0.4 g L^{-1} EDTA), significantly better recovery rates could be achieved compared with those achieved with a solvent calibration in MilliQ water (+5 % acetonitrile and 0.4 g L^{-1} EDTA). These results are consistent with internal laboratory experience from other MS-based methods.

To determine the accuracy of the developed method, possible ion enhancement and ion suppression were investigated. Therefore, specific recovery rates for each analyte and the envisaged ISTD were determined individually in different matrices, using external calibration curves. Possible losses of analytes arising from filtration (H-PTFE, 0.45 μm) were also considered. The average ($n = 5$) recovery rates for all spiked concentrations (0.05, 0.1, 0.5 and 2.0 μg L^{-1}) as well as the overall mean recovery rate including relative standard deviations (RSDs) for DW, SW, GW and simulated treated WW are given in Table 4.

Thus, mean recovery rates ranged between 29 % and 134 % in the investigated matrices. Some macrolides, like AZI, clarithromycin (CLA) or roxithromycin (ROX), typically show a low recovery (~70 %), which is consistent with previous studies where the recovery rates of macrolide antibiotics are generally between 37 % and 124 % (Dasenaki and Thomaidis 2015; Grujić et al. 2009; Rossmann et al. 2014; Wei et al. 2014). For all analytes, RSDs ranging from 2 % to 15 % (SW: 2–11 %; GW: 2–17 %; treated WW: 2–19 %) could be achieved in DW.

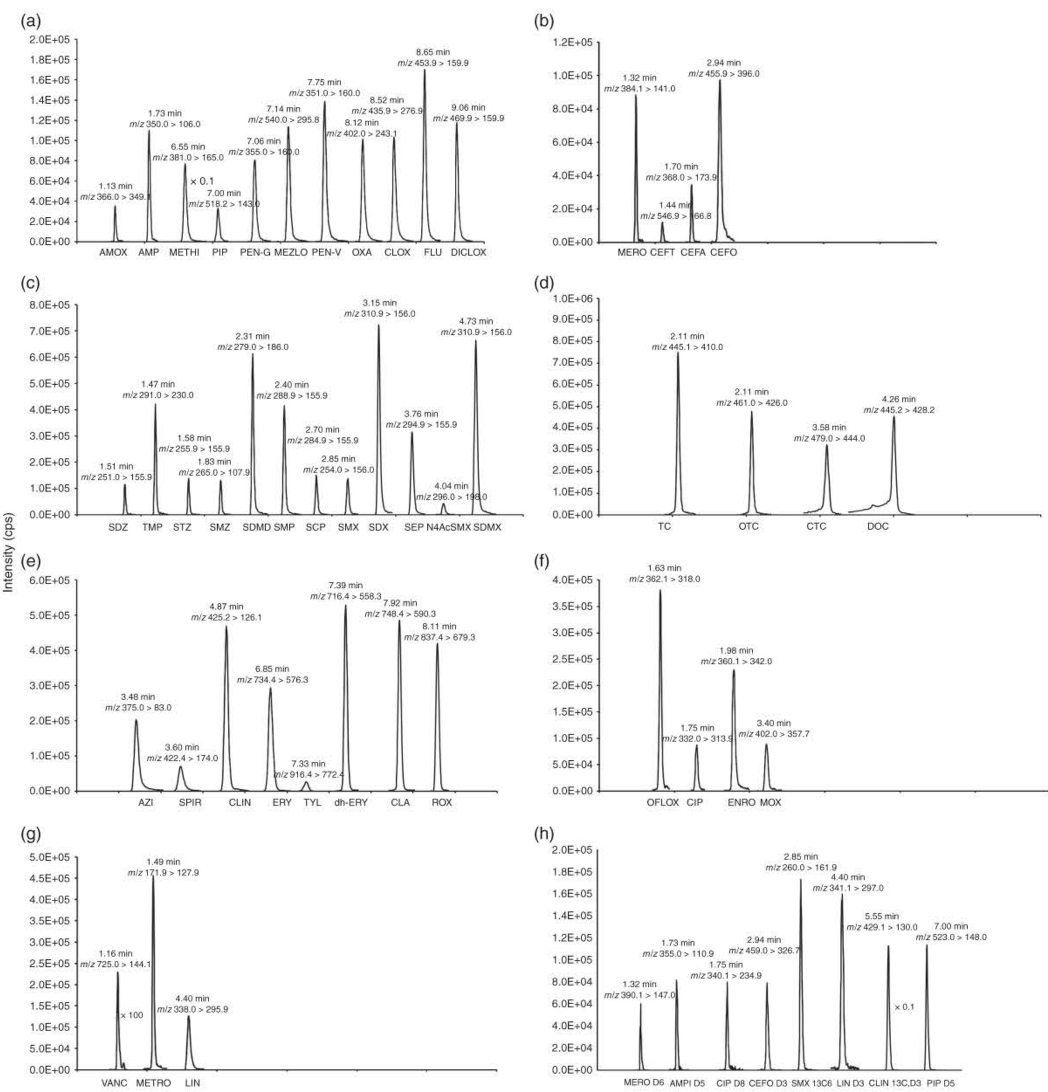

Fig. 1. Extracted ion chromatogram of all examined antibiotics (0.5 μg L^{-1} mixed standard, only precursor) sorted by their substance classes: (a) Penicillins; (b) cephalosporins and carbapenems; (c) sulfonamides and trimethoprim; (d) tetracyclines; (e) macrolide antibiotics; (f) fluoroquinolones; (g) nitroimidazoles, oxazolidinones and glycopeptides; (h) internal standards (ISTD).

The most demanding matrix was untreated WW. Owing to a strongly varying composition and associated influences on respective recovery rates, treated STP effluent as a spiking matrix was preferred. A simulated treated WW matrix from Sigma Aldrich was spiked with a standard solution of all antibiotics, as there was no WW without antibiotic residues which could be used as a blank matrix.

Generally, recovery rates between 69 % and 103 % could be achieved for treated WW for most analytes. Especially, ceftazidime (CEFT) was detected below this value (recovery = 44 %). In addition, the recovery rates obtained for all fluoroquinolones [ciprofloxacin (CIP), enrofloxacin (ENRO), ofloxacin (OFLOX) and moxifloxacin (MOX)] were extremely low. The recovery rates ranged between 25 % and 51 %, independent from the spiking concentration (RSD between 5 % and 14 %). Given the simulated matrix was characterised by a large amount of unfilterable residues (25.1 + 0.448 mg L^{-1}), one explanation could be the ability of fluoroquinolones to adsorb to organic particles (Kümmerer 2009). These results are confirmed by existing studies that demonstrate that fluorochinolones are mainly found in STP influent, owing to adsorption to (for example) sewage sludge and, therefore, their removal from the water circuit after treatment in the STP (Golet et al. 2002, 2003; Lindberg et al. 2005).

Table 4. Calculated recovery rates (0.05 µg L^{-1}, 0.1 µg L^{-1}, 0.5 µg L^{-1}, 2.0 µg L^{-1}), RSD as well as the dynamic range and linearity (r^2) in spiked drinking water, surface water, groundwater and treated wastewater

LOD, limit of detection; LOQ, limit of quantification; RSD, relative standard deviation

Analyte	0.05 µg L^{-1} (±%RSD)	0.1 µg L^{-1} (±%RSD)	0.5 µg L^{-1} (±%RSD)	2.0 µg L^{-1} (±%RSD)	Mean recovery (±%RSD)	Min. ng L^{-1}	Max. ng L^{-1}	Linearity
Drinking water								
AMOX	107 (9)	103 (4)	98 (3)	101 (1)	102 (6)	10	5000	0.99962
AMP	< LOQ	105 (7)	99 (2)	102 (2)	101 (6)	50	5000	0.99570
AZI	83 (2)	90 (5)	91 (2)	99 (3)	91 (7)	10	4000	0.99979
CEFA	95 (3)	101 (4)	97 (2)	101 (1)	99 (4)	10	5000	0.99958
CEFO	92 (4)	105 (4)	102 (1)	104 (2)	101 (6)	10	4500	0.99981
CEFT	< LOQ	102 (9)	102 (5)	104 (2)	103 (6)	50	4000	0.99980
CTC	76 (5)	105 (3)	106 (4)	110 (2)	99 (14)	10	4500	0.99973
CIP	< LOQ	155 (6)	116 (4)	120 (3)	130 (15)	50	4000	0.99965
CLA	63 (5)	71 (3)	71 (5)	76 (3)	70 (8)	10	4000	0.99978
CLIN	101 (3)	105 (2)	100 (2)	103 (1)	102 (3)	10	4000	0.99985
CLOX	85 (5)	99 (3)	95 (2)	100 (1)	95 (7)	10	5000	0.99974
dh-ERY	77 (2)	91 (3)	92 (4)	101 (2)	90 (10)	10	4000	0.99972
DICLOX	95 (4)	94 (1)	93 (1)	97 (1)	95 (3)	10	5000	0.99967
DOC	106 (7)	106 (7)	103 (2)	113 (6)	107 (6)	50	4500	0.99962
ENRO	153 (5)	138 (5)	117 (3)	128 (2)	134 (11)	50	4500	0.99936
ERY	86 (6)	72 (3)	71 (1)	75 (1)	76 (9)	10	4500	0.99966
FLU	90 (5)	95 (3)	92 (1)	98 (1)	94 (4)	10	5000	0.99974
LIN	107 (5)	110 (2)	107 (2)	115 (2)	110 (4)	10	4500	0.99980
MERO	< LOQ	122 (3)	107 (3)	106 (2)	110 (7)	50	4000	0.99959
METHI	88 (1)	90 (2)	89 (1)	94 (1)	90 (4)	10	5000	0.99979
METRO	94 (5)	94 (5)	96 (2)	97 (2)	95 (4)	10	5000	0.99980
MEZLO	98 (5)	96 (5)	94 (1)	100 (1)	97 (4)	10	5000	0.99967
MOX	< LOQ	< LOQ	112 (10)	115 (3)	113 (8)	50	4500	0.99943
N4AcSMX	< LOQ	94 (3)	89 (3)	96 (2)	93 (4)	50	5000	0.99954
NAF	94 (3)	97 (2)	92 (2)	95 (1)	95 (3)	10	5000	0.99985
OFLOX	< LOQ	135 (8)	108 (3)	107 (1)	117 (13)	50	4000	0.99912
OXA	97 (5)	97 (2)	93 (2)	99 (1)	97 (4)	10	5000	0.99956
OTC	93 (9)	109 (5)	100 (2)	104 (1)	102 (7)	10	4500	0.99984
PEN G	104 (9)	95 (2)	96 (3)	100 (1)	101 (6)	10	5000	0.99968
PEN V	95 (2)	97 (2)	93 (1)	99 (1)	96 (3)	10	5000	0.99979
PIP	94 (9)	100 (6)	98 (3)	101 (2)	98 (6)	50	5000	0.99950
ROX	65 (5)	71 (2)	70 (2)	80 (4)	72 (9)	10	4000	0.99974
SPIR	116 (5)	111 (1)	107 (3)	112 (1)	111 (4)	50	4000	0.99964
SCP	96 (4)	102 (4)	89 (3)	100 (1)	97 (6)	10	4500	0.99976
SDZ	< LOQ	< LOQ	100 (2)	99 (2)	99 (2)	50	4500	0.99943
SDMD	85 (0)	87 (1)	86 (2)	90 (2)	87 (2)	10	5000	0.99982
SDMX	84 (3)	90 (2)	87 (2)	93 (2)	89 (4)	10	5000	0.99977
SDX	68 (5)	76 (2)	72 (3)	75 (2)	73 (5)	10	5000	0.99974
SEP	88 (3)	96 (2)	95 (1)	98 (1)	94 (4)	10	5000	0.99978
SMZ	91 (3)	95 (4)	94 (2)	97 (1)	94 (4)	50	5000	0.99965
SMX	107 (4)	108 (1)	102 (2)	104 (2)	105 (3)	10	5000	0.99966
SMP	96 (2)	98 (2)	90 (1)	92 (1)	94 (4)	10	4500	0.99988
STZ	113 (2)	114 (3)	101 (4)	100 (1)	107 (7)	50	5000	0.99965
TC	94 (4)	100 (5)	100 (2)	106 (1)	100 (5)	10	4500	0.99980
TMP	89 (3)	104 (7)	90 (2)	95 (2)	95 (8)	10	4500	0.99972
TYL	< LOQ	89 (9)	80 (4)	88 (5)	85 (8)	50	4000	0.99971
VANC	< LOQ	< LOQ	101 (10)	95 (4)	98 (8)	50	5000	0.99966
SMXl $^{13}C_6$	-	-	99 (5)	-	-	-	-	-
CIP D_8	-	-	115 (6)	-	-	-	-	-
MERO D_6	-	-	104 (11)	-	-	-	-	-
AMP D_5	-	-	94 (3)	-	-	-	-	-
PIP D_5	-	-	95 (2)	-	-	-	-	-
CEFO D_3	-	-	101 (2)	-	-	-	-	-
CLIN $^{13}C,D_3$	-	-	100 (1)	-	-	-	-	-
LIN D_3	-	-	98 (2)	-	-	-	-	-
Surface water								
AMOX	97 (3)	104 (2)	97 (1)	98 (1)	99 (4)	10	5000	0.99968
AMP	< LOQ	106 (5)	94 (4)	100 (2)	100 (6)	50	5000	0.99944
AZI	74 (6)	80 (5)	85 (2)	91 (1)	82 (9)	10	4000	0.99885
CEFA	94 (3)	90 (1)	94 (1)	97 (1)	94 (3)	10	5000	0.99947

(*Continued*)

Table 4. (Continued)

Analyte	0.05 μg L^{-1} (±%RSD)	0.1 μg L^{-1} (±%RSD)	0.5 μg L^{-1} (±%RSD)	2.0 μg L^{-1} (±%RSD)	Mean recovery (±%RSD)	Min. ng L^{-1}	Max. ng L^{-1}	Linearity
CEFO	95 (1)	97 (7)	95 (1)	93 (1)	95 (4)	50	5000	0.99937
CEFT	< LOQ	103 (3)	85 (4)	88 (1)	91 (9)	50	50000	0.99906
CTC	105 (3)	106 (4)	91 (3)	94 (2)	99 (7)	100	3500	0.99621
CIP	< LOQ	109 (7)	96 (3)	92 (2)	99 (9)	100	4000	0.99293
CLA	65 (6)	76 (6)	77 (2)	76 (1)	73 (8)	10	4000	0.99945
CLIN	109 (2)	99 (1)	96 (1)	96 (1)	100 (6)	10	4000	0.99965
CLOX	86 (4)	94 (2)	95 (1)	94 (1)	92 (5)	10	5000	0.99947
dh-ERY	74 (4)	90 (2)	91 (3)	95 (1)	87 (10)	10	5000	0.99947
DICLOX	91 (5)	95 (1)	95 (1)	93 (1)	93 (3)	10	5000	0.99911
DOC	88 (2)	95 (1)	94 (4)	101 (2)	94 (5)	100	3000	0.99564
ENRO	105 (11)	97 (1)	89 (1)	91 (2)	95 (9)	100	4000	0.99251
ERY	84 (5)	80 (1)	77 (3)	74 (1)	79 (6)	10	5000	0.99961
FLU	86 (3)	93 (2)	94 (2)	92 (2)	91 (4)	10	5000	0.99957
LIN	85 (5)	89 (4)	93 (2)	94 (1)	90 (5)	50	5000	0.99917
MERO	< LOQ	111 (9)	97 (10)	93 (3)	101 (11)	50	5000	0.99887
METHI	91 (5)	100 (1)	100 (1)	97 (1)	97 (5)	10	5000	0.99967
METRO	98 (6)	93 (5)	96 (3)	92 (3)	95 (5)	50	5000	0.99865
MEZLO	89 (4)	96 (3)	95 (2)	93 (1)	93 (4)	10	5000	0.99950
MOX	< LOQ	< LOQ	94 (12)	95 (5)	95 (8)	50	4000	0.99775
N4AcSMX	< LOQ	97 (8)	104 (1)	99 (2)	100 (5)	100	5000	0.99904
NAF	101 (2)	95 (2)	94 (1)	93 (1)	95 (3)	10	5000	0.99964
OFLOX	< LOQ	112 (3)	103 (1)	98 (1)	98 (7)	100	4500	0.99188
OXA	94 (3)	99 (2)	96 (2)	96 (1)	96 (3)	10	5000	0.99960
OTC	98 (5)	96 (4)	95 (4)	93 (1)	95 (4)	100	2500	0.99572
PEN G	101 (6)	101 (1)	98 (2)	98 (1)	99 (4)	10	5000	0.99964
PEN V	90 (3)	96 (2)	96 (2)	96 (1)	95 (3)	10	5000	0.99966
PIP	94 (7)	102 (5)	99 (3)	96 (2)	98 (5)	50	5000	0.99947
ROX	69 (9)	75 (1)	78 (3)	81 (2)	76 (7)	10	4000	0.99957
SPIR	101 (2)	102 (3)	99 (2)	102 (1)	101 (2)	50	4000	0.99943
SCP	85 (2)	92 (3)	90 (3)	92 (2)	90 (4)	10	5000	0.99935
SDZ	< LOQ	< LOQ	94 (7)	96 (3)	95 (5)	50	5000	0.99928
SDMD	94 (8)	96 (3)	101 (1)	98 (2)	97 (5)	10	5000	0.99934
SDMX	89 (4)	95 (1)	98 (2)	97 (1)	95 (4)	10	5000	0.99971
SDX	85 (2)	91 (3)	92 (2)	90 (2)	90 (4)	10	5000	0.99961
SEP	88 (2)	87 (1)	91 (2)	92 (1)	90 (3)	10	5000	0.99962
SMZ	96 (11)	103 (1)	96 (5)	97 (2)	97 (6)	50	4500	0.99919
SMX	92 (2)	115 (1)	100 (2)	96 (1)	100 (9)	10	5000	0.99964
SMP	102 (4)	101 (2)	98 (1)	93 (1)	99 (4)	10	5000	0.99971
STZ	118 (10)	121 (7)	101 (1)	99 (2)	110 (11)	50	5000	0.99898
TC	87 (5)	95 (8)	95 (2)	94 (1)	93 (5)	100	3000	0.99544
TMP	99 (7)	97 (3)	90 (3)	94 (3)	95 (6)	50	5000	0.99954
TYL	< LOQ	82 (5)	88 (2)	88 (1)	86 (4)	50	4000	0.99902
VANC	< LOQ	< LOQ	93 (10)	93 (3)	93 (7)	50	5000	0.99956
SMXI $^{13}C_6$	-	-	96 (3)	-	-	-	-	-
CIP D_8	-	-	89 (2)	-	-	-	-	-
MERO D_6	-	-	95 (8)	-	-	-	-	-
AMP D_5	-	-	95 (2)	-	-	-	-	-
PIP D_5	-	-	93 (3)	-	-	-	-	-
CEFO D_3	-	-	91 (3)	-	-	-	-	-
CLIN $^{13}C,D_3$	-	-	98 (2)	-	-	-	-	-
LIN D_3	-	-	97 (2)	-	-	-	-	-
Groundwater								
AMOX	92 (4)	98 (2)	94 (1)	100 (1)	96 (4)	10	2500	0.99740
AMP	< LOQ	121 (0)	105 (5)	101 (2)	109 (9)	50	4000	0.99940
AZI	97 (4)	97 (2)	97 (1)	101 (2)	98 (3)	10	4500	0.99928
CEFA	< LOQ	95 (6)	96 (1)	97 (1)	96 (4)	10	3500	0.99912
CEFO	88 (3)	101 (4)	102 (5)	101 (2)	98 (7)	50	4000	0.99877
CEFT	59 (9)	64 (9)	69 (4)	72 (2)	68 (7)	50	3500	0.99859
CTC	101 (4)	86 (8)	71 (3)	68 (3)	81 (17)	100	3500	0.99126
CIP	< LOQ	118 (4)	89 (6)	101 (1)	103 (13)	100	4000	0.99752
CLA	82 (5)	86 (3)	84 (1)	83 (2)	84 (3)	10	4500	0.99880
CLIN	110 (3)	112 (2)	107 (1)	108 (3)	109 (3)	10	5000	0.99913

(Continued)

Table 4. (Continued)

Analyte	0.05 µg L^{-1} (±%RSD)	0.1 µg L^{-1} (±%RSD)	0.5 µg L^{-1} (±%RSD)	2.0 µg L^{-1} (±%RSD)	Mean recovery (±%RSD)	Min. ng L^{-1}	Max. ng L^{-1}	Linearity
CLOX	92 (6)	98 (5)	98 (2)	99 (1)	97 (5)	10	5000	0.99924
dh-ERY	88 (1)	93 (2)	90 (2)	90 (3)	90 (3)	10	3000	0.99939
DICLOX	94 (3)	96 (4)	93 (1)	94 (1)	94 (3)	10	5000	0.99936
DOC	96 (4)	88 (8)	83 (4)	86 (3)	85 (5)	100	4000	0.99525
ENRO	100 (4)	128 (15)	96 (6)	104 (9)	107 (15)	100	4000	0.99823
ERY	95 (2)	100 (3)	99 (1)	97 (1)	98 (3)	10	3000	0.99854
FLU	98 (7)	108 (5)	99 (1)	99 (1)	101 (6)	50	5000	0.99943
LIN	99 (4)	98 (4)	102 (2)	103 (2)	100 (3)	50	5000	0.99911
MERO	< LOQ	84 (2)	93 (10)	100 (3)	93 (9)	100	3500	0.99493
METHI	98 (2)	102 (1)	101 (1)	102 (1)	101 (2)	10	5000	0.99961
METRO	106 (2)	105 (12)	100 (8)	103 (1)	104 (7)	100	3500	0.98913
MEZLO	92 (5)	100 (1)	98 (1)	102 (1)	98 (5)	10	5000	0.99958
MOX	< LOQ	< LOQ	94 (5)	98 (5)	96 (5)	100	4000	0.99736
N4AcSMX	< LOQ	98 (2)	98 (2)	102 (2)	99 (3)	50	4500	0.99901
NAF	96 (1)	98 (4)	96 (2)	96 (1)	96 (2)	10	5000	0.99948
OFLOX	< LOQ	110 (13)	103 (5)	112 (9)	108 (9)	100	4000	0.99753
OXA	98 (5)	101 (5)	96 (2)	98 (2)	98 (4)	10	5000	0.99955
OTC	90 (6)	84 (4)	75 (7)	84 (3)	83 (8)	100	3500	0.99343
PEN G	102 (5)	101 (6)	101 (2)	101 (1)	101 (4)	10	4500	0.99901
PEN V	99 (5)	101 (1)	96 (1)	95 (1)	98 (4)	10	5000	0.99956
PIP	98 (9)	106 (2)	100 (3)	99 (2)	100 (5)	50	5000	0.99903
ROX	74 (6)	83 (2)	83 (2)	80 (2)	80 (5)	10	4000	0.99894
SPIR	109 (3)	101 (7)	90 (2)	91 (2)	98 (9)	50	5000	0.99873
SCP	101 (5)	99 (7)	103 (5)	101 (1)	101 (5)	10	5000	0.99927
SDZ	< LOQ	< LOQ	117 (3)	117 (4)	117 (3)	100	3500	0.99845
SDMD	106 (2)	94 (5)	93 (3)	101 (15)	98 (10)	10	3500	0.99905
SDMX	100 (1)	101 (2)	97 (1)	101 (1)	100 (2)	10	5000	0.99933
SDX	100 (1)	101 (2)	97 (1)	101 (1)	100 (2)	10	4500	0.99896
SEP	105 (3)	103 (4)	100 (3)	100 (1)	102 (3)	10	4500	0.99942
SMZ	122 (2)	110 (4)	102 (2)	103 (1)	109 (7)	100	4000	0.99720
SMX	97 (4)	100 (3)	98 (1)	101 (1)	99 (3)	10	4500	0.99917
SMP	106 (1)	106 (3)	102 (1)	103 (1)	104 (3)	10	5000	0.99954
STZ	95 (7)	100 (8)	104 (1)	110 (2)	102 (7)	50	2500	0.99785
TC	106 (5)	89 (6)	79 (2)	81 (2)	90 (14)	100	3500	0.99338
TMP	70 (6)	87 (5)	93 (5)	97 (2)	87 (13)	50	3500	0.99783
TYL	< LOQ	93 (4)	81 (4)	79 (4)	84 (9)	50	3000	0.99849
VANC	< LOQ	< LOQ	101 (4)	103 (2)	93 (3)	50	3000	0.99582
SMXl $^{13}C_6$	-	-	103 (2)		-	-	-	-
CIP D_8	-	-	93 (3)		-	-	-	-
MERO D_6	-	-	104 (8)		-	-	-	-
AMP D_5	-	-	102 (8)		-	-	-	-
PIP D_5	-	-	97 (1)		-	-	-	-
CEFO D_3	-	-	100 (3)		-	-	-	-
CLIN $^{13}C,D_3$	-	-	105 (1)		-	-	-	-
LIN D_3	-	-	103 (2)		-	-	-	-
Treated wastewater								
AMOX	98 (6)	103 (4)	102 (3)	104 (1)	102 (4)	10	5000	0.99976
AMP	< LOQ	95 (3)	98 (3)	95 (2)	96 (3)	50	5000	0.99953
AZI	66 (9)	74 (1)	70 (6)	78 (3)	72 (7)	10	5000	0.99968
CEFA	70 (3)	84 (3)	96 (1)	99 (1)	87 (14)	10	5000	0.99978
CEFO	100 (7)	96 (4)	91 (2)	92 (2)	95 (6)	10	5000	0.99963
CEFT	< LOQ	44 (7)	44 (4)	44 (3)	44 (4)	50	5000	0.99896
CTC	124 (2)	99 (2)	82 (2)	81 (5)	96 (19)	50	5000	0.99950
CIP	< LOQ	37 (8)	37 (5)	40 (6)	38 (7)	100	4500	0.99746
CLA	71 (5)	78 (4)	67 (5)	75 (6)	73 (7)	10	4500	0.99971
CLIN	98 (2)	96 (1)	91 (1)	93 (1)	95 (3)	10	5000	0.99980
CLOX	76 (2)	90 (3)	95 (2)	100 (6)	90 (11)	10	5000	0.99954
dh-ERY	68 (1)	71 (3)	65 (4)	70 (4)	69 (5)	10	5000	0.99932
DICLOX	93 (3)	95 (3)	94 (2)	94 (2)	94 (2)	10	5000	0.99970
DOC	104 (2)	96 (3)	92 (2)	91 (2)	96 (6)	50	5000	0.99965
ENRO	43 (8)	34 (7)	39 (5)	45 (10)	40 (13)	100	4500	0.99810
ERY	86 (3)	95 (3)	91 (2)	97 (2)	92 (5)	10	4500	0.99971
FLU	97 (5)	102 (3)	96 (2)	96 (2)	98 (4)	10	5000	0.99968

(*Continued*)

Table 4. (Continued)

Analyte	0.05 µg L^{-1} (±%RSD)	0.1 µg L^{-1} (±%RSD)	0.5 µg L^{-1} (±%RSD)	2.0 µg L^{-1} (±%RSD)	Mean recovery (±%RSD)	Min. ng L^{-1}	Max. ng L^{-1}	Linearity
LIN	91 (5)	97 (5)	92 (3)	94 (1)	94 (4)	50	5000	0.99968
MERO	< LOQ	99 (1)	101 (4)	93 (4)	98 (5)	50	5000	0.99950
METHI	98 (1)	102 (1)	100 (1)	100 (1)	100 (2)	10	5000	0.99979
METRO	86 (5)	86 (4)	82 (4)	87 (3)	85 (4)	50	5000	0.99965
MEZLO	102 (6)	104 (1)	100 (1)	100 (2)	102 (4)	10	5000	0.99968
MOX	< LOQ	< LOQ	43 (6)	51 (7)	47 (11)	100	4500	0.99763
N4AcSMX	< LOQ	91 (13)	98 (5)	102 (5)	97 (9)	100	5000	0.99913
NAF	98 (2)	102 (2)	97 (1)	99 (1)	99 (2)	10	5000	0.99790
OFLOX	< LOQ	25 (13)	29 (7)	33 (6)	29 (14)	100	4000	0.99737
OXA	98 (4)	96 (3)	95 (2)	98 (1)	97 (3)	10	5000	0.99961
OTC	98 (5)	86 (4)	82 (2)	80 (3)	86 (9)	50	5000	0.99962
PEN G	98 (5)	98 (2)	100 (1)	102 (1)	100 (3)	10	5000	0.99978
PEN V	101 (3)	99 (3)	98 (1)	99 (2)	100 (2)	10	5000	0.99975
PIP	115 (3)	103 (5)	97 (3)	98 (1)	103 (8)	50	5000	0.99967
ROX	72 (4)	84 (5)	73 (6)	82 (4)	77 (8)	10	4000	0.99919
SPIR	100 (4)	97 (2)	81 (5)	88 (3)	91 (9)	10	5000	0.99968
SCP	101 (6)	92 (2)	91 (1)	90 (1)	93 (6)	10	5000	0.99973
SDZ	< LOQ	< LOQ	86 (7)	87 (3)	86 (5)	50	5000	0.99928
SDMD	97 (4)	96 (4)	95 (2)	95 (3)	96 (3)	10	5000	0.99960
SDMX	91 (5)	96 (2)	95 (2)	96 (0)	95 (3)	10	5000	0.99984
SDX	104 (6)	102 (1)	100 (2)	101 (1)	101 (3)	10	5000	0.99978
SEP	96 (3)	101 (3)	96 (2)	96 (1)	97 (3)	10	5000	0.99980
SMZ	86 (4)	96 (5)	88 (3)	89 (2)	89 (5)	50	5000	0.99972
SMX	92 (2)	95 (2)	90 (1)	90 (1)	92 (3)	10	5000	0.99982
SMP	90 (3)	93 (1)	91 (1)	90 (1)	91 (2)	10	5000	0.99983
STZ	99 (7)	95 (7)	90 (4)	89 (1)	93 (7)	50	5000	0.99954
TC	95 (9)	86 (2)	80 (2)	79 (2)	85 (9)	50	5000	0.99963
TMP	77 (7)	67 (12)	70 (8)	74 (3)	72 (9)	10	5000	0.99966
TYL	< LOQ	79 (5)	69 (8)	77 (9)	75 (9)	50	4500	0.99824
VANC	< LOQ	< LOQ	95 (2)	99 (3)	97 (3)	100	5000	0.99961
SMXl $^{13}C_6$	-	-	89 (2)	-	-	-	-	-
CIP D_8	-	-	36 (7)	-	-	-	-	-
MERO D_6	-	-	101 (11)	-	-	-	-	-
AMP D_5	-	-	98 (3)	-	-	-	-	-
PIP D_5	-	-	99 (2)	-	-	-	-	-
CEFO D_3	-	-	95 (3)	-	-	-	-	-
CLIN $^{13}C,D_3$	-	-	92 (1)	-	-	-	-	-
LIN D_3	-	-	97 (5)	-	-	-	-	-

To compensate for higher or lower recovery rates resulting from filtration losses and possible matrix effects during ionisation (ion enhancement or suppression) and chromatography, isotopically labelled standards were used for quantification based on their respective substance classes (Batt and Aga 2005; Göbel et al. 2004; Lindberg et al. 2004; Lindsey et al. 2001). Based on the results of Table 4 and Fig. 1, the assignment of the ISTD represented a compromise between t_R, substance class and the comparison of the recovery rates of the respective analyte and the potential ISTD, which was determined with an external calibration curve. The final recovery after ISTD application was also investigated in DW, GW, SW and treated WW with a spiking concentration of 500 ng L^{-1} (4 replicates). The final recoveries using the ISTD are given in Table 5.

Improvements in recovery were observed for all analytes and matrices. Thus, the recovery ranged between 90 % and 117 % (DW), 75 % and 113 % (GW), 67 % and 113 % (SW) as well as 65 % and 115 % (treated WW). These results are in accordance with the recent studies dealing with methods for the analysis of antibiotics using direct injection (without sample preconcentration) (Bayen et al. 2014; Campos-Mañas et al. 2017). In comparison, Bayen et al. (2014) achieved good recovery rates for sulfonamides and lower recovery rates for tylosin (TYL) and lincomycin (same substance class as CLIN) in freshwater after filtration (PTFE, 0.2 µm) and quantification through the isotope dilution method. In this study, proper results could be achieved in comparison with the already published methods using SPE as sample clean-up and preconcentration (Batt and Aga 2005; Göbel et al. 2004; Grujić et al. 2009).

Generally, macrolide antibiotics (CLA, AZI and SPIR) are characterised by lower recovery rates in treated WW using CLIN-$^{13}C,D_3$ as ISTD. Thus, the specific recovery rates should be considered for the analysis of treated WW (AZI: 65 %; CLA: 73 %; SPIR: 75 %). Batt and Aga (2005) developed a LC-MS/MS method for the analysis of 13 different antibiotics (including fluoroquinolones, tetracyclines, sulfonamides and macrolides) with recovery rates ranging between 51 % and 129 % in GW, SW and WW with SPE for sample clean-up and preconcentration (factor: 500). Especially ROX and TYL were also characterised by lower recovery rates especially for GW and SW (Batt and Aga 2005). In addition, lower recovery rates were described for AZI in SW and GW depending on pH by Grujić

Table 5. Final recovery rates and the calculated RSD in spiked drinking water, surface water, groundwater and wastewater (0.5 µg L^{-1}) after filtration (H-PTFE syringe filter)

For quantification the respective internal standards were used. LOD, limit of detection; LOQ, limit of quantification; RSD, relative standard deviation; ISTD, internal standard

Analyte	Drinking water (%) ± RSD	Groundwater (%) ± RSD	Surface water (%) ± RSD	Treated wastewater (%) ± RSD
AMOX	106 ± 5	108 ± 9	113 ± 8	115 ± 3
AMP	94 ± 5	96 ± 5	95 ± 5	96 ± 4
AZI	96 ± 4	89 ± 5	91 ± 3	65 ± 13
CEFA	100 ± 3	95 ± 4	93 ± 4	96 ± 2
CEFO	99 ± 6	98 ± 4	94 ± 4	98 ± 3
CEFT	104 ± 6	75 ± 16	67 ± 6	74 ± 12
CTC	no ISTD	no ISTD	no ISTD	no ISTD
CIP	95 ± 6	100 ± 4	95 ± 8	92 ± 4
CLA	91 ± 5	84 ± 4	84 ± 1	73 ± 9
CLIN	101 ± 2	102 ± 4	101 ± 2	100 ± 2
CLOX	96 ± 1	96 ± 3	95 ± 3	93 ± 2
dh-ERY	117 ± 5	113 ± 1	105 ± 1	94 ± 3
DICLOX	92 ± 2	91 ± 5	92 ± 1	89 ± 3
DOC	no ISTD	no ISTD	no ISTD	no ISTD
ENRO	106 ± 8	102 ± 3	111 ± 6	103 ± 1
ERY	92 ± 5	82 ± 3	84 ± 1	86 ± 1
FLU	95 ± 1	96 ± 3	97 ± 2	92 ± 1
LIN	112 ± 6	106 ± 5	105 ± 6	102 ± 7
MERO	96 ± 8	95 ± 10	93 ± 7	110 ± 9
METHI	97 ± 2	96 ± 2	96 ± 2	95 ± 1
METRO	90 ± 6	95 ± 4	95 ± 10	83 ± 4
MEZLO	96 ± 2	96 ± 1	98 ± 2	95 ± 0
MOX	100 ± 6	104 ± 7	113 ± 11	99 ± 5
N4AcSMX	100 ± 10	94 ± 8	99 ± 10	108 ± 9
NAF	92 ± 2	91 ± 3	91 ± 1	90 ± 2
OFLOX	100 ± 5	106 ± 7	107 ± 6	67 ± 9
OXA	96 ± 3	96 ± 2	93 ± 3	93 ± 2
OTC	no ISTD	no ISTD	no ISTD	no ISTD
PEN G	99 ± 2	94 ± 4	94 ± 2	94 ± 1
PEN V	97 ± 3	95 ± 2	94 ± 1	92 ± 1
PIP	98 ± 2	100 ± 3	97 ± 5	97 ± 2
ROX	94 ± 6	90 ± 4	90 ± 2	84 ± 7
SPIR	100 ± 5	96 ± 2	95 ± 2	75 ± 4
SCP	100 ± 2	98 ± 2	100 ± 1	100 ± 2
SDZ	93 ± 6	100 ± 5	99 ± 4	80 ± 7
SDMD	95 ± 3	100 ± 5	96 ± 4	107 ± 2
SDMX	99 ± 2	100 ± 4	100 ± 2	106 ± 3
SDX	97 ± 3	100 ± 2	100 ± 3	103 ± 2
SEP	99 ± 2	101 ± 2	100 ± 1	104 ± 0
SMZ	94 ± 2	102 ± 1	97 ± 1	96 ± 3
SMX	98 ± 1	99 ± 1	100 ± 3	95 ± 1
SMP	98 ± 2	100 ± 3	99 ± 2	99 ± 2
STZ	91 ± 6	99 ± 1	91 ± 5	87 ± 5
TC	no ISTD	no ISTD	no ISTD	no ISTD
TMP	93 ± 3	91 ± 2	91 ± 3	80 ± 2
TYL	91 ± 5	82 ± 1	82 ± 4	80 ± 2
VANC	101 ± 7	100 ± 3	102 ± 9	111 ± 5

et al. (2009) as well as CLA and dh-ERY in WW (using TYL as surrogate ISTD) by Göbel et al. (2004). Possible improvements may be achieved by lower pH, another filter syringe material and the use of more specific ISTD than CLIN-$^{13}C,D_3$ related to the physico-chemical characteristics of macrolide antibiotics (Table S1, Supplementary Material). In contrast, a lower pH may reduce the recovery for other antibiotics and a suitable compromise has to be reached.

In addition, improvements could be achieved related to the recovery of CIP from 38 % ± 7 % to 92 % ± 4 % in treated WW using an ISTD. The usage of CIP-D_8 as ISTD for all fluoroquinolones also improved the recovery of MOX (47 % ± 11 % to 99 % ± 5 %), OFLOX (29 % ± 14 % to 67 % ± 9 %) as well as ENRO (40 % ± 13 % to 103 % ± 1 %). The precision was also improved, which was exhibited by a reduction of RSD.

Regardless of the matrix studied, the use of CEFO-D_3 appeared to be suboptimal for the quantification of CEFT, since the final recovery only yielded proper results in DW (104 % ± 6 %). In the more complex matrices, recovery rates were significantly reduced despite the usage of the ISTD

(GW: 75 % ± 16 %; SW: 67 % ± 6 %; treated WW: 74 % ± 12 %). These differences may be the result of the used syringe filter material. In preliminary tests, higher recovery rates of CEFT could be obtained using other syringe filters, e.g. X-PTFE (93 %), X-PES (96 %) or X-RC (102 %) compared with H-PTFE (84 %) in milliQ (+ACN). However, the best compromise had to be found for all analytes when selecting the syringe filter, which was determined to be H-PTFE (0.45 µm). Thus, for the other cephalosporins, this material showed a better recovery (CEFO: 105 %; CEFA: 99 %) in preliminary tests. In comparison, the usage of CEFO-D_3 provided significantly better results for the quantification of CEFA 93 % ± 4 % to 100 % ± 3 %. In addition, a better recovery could possibly be achieved by an adjustment of the pH, since Opriş et al. (2013) showed an improvement for the recovery of CEFT after sample clean-up (SPE) and pH-adjustment (pH < 3). Nevertheless, in this study CEFT (69 %) was characterised by a lower recovery than other cephalosporins (ceftriaxone: 110 %) (Opriş et al. 2013). In conclusion, the implementation of an isotopically marked standard for CEFT in this method should be considered.

Unfortunately, it was not possible to use an individual isotope-labelled analogue for each substance that would give the best results, because some could not be purchased or would have exceeded our budget. A limitation in this study was the lack of the use of isotope labelled ISTD for tetracyclines, because no labelled tetracyclines are available for purchase and the used ISTD do not cover the chemical-physical properties of tetracyclines, especially their capability to form chelate-complexes with double-charged cations (e.g. Mg, Ca).

Typically, a dynamic range between 0.01 µg L^{-1} and 5.0 µg L^{-1} could be determined in spite of the straightforward sample preparation. Good linearity (r^2 > 0.99) could be achieved for all analytes. Indeed, even treated WW was characterised by a good linear instrumental response. The specific dynamic ranges as well as linearities (±RSD) for all analytes and matrices are shown in Table 4.

As a measure of sensitivity, the LOD and LOQ were determined over a 10-point calibration around the expected LOQ in spiked DW (+ 0.4 g/L EDTA). The LOD ranged from 1.1 ng L^{-1} (sulfamethoxypyridazine, SMP) to 76 ng L^{-1} (VANC), whereas LOQ were between 3.3 ng L^{-1} (SMP) and 190 ng L^{-1} (VANC). To take into account the sample dilution (1 : 1) before filtration, all calculated LOD and LOQ were multiplied by a factor of two, giving the final LOD and LOQ for each substance. All values for LOD and LOQ are shown in Table 6. To consider the deviating recoveries in treated WW, the calculated LOD and LOQ in DW were corrected for the final recoveries (Table 5). Owing to the lack of ISTD for tetracyclines, the recovery rates given in Table 4 were used to calculate the predicted LOD and LOQ for treated WW. The results for the predicted LODs and LOQs in treated WW are shown in Table 6.

In general, the primary goal was to develop a cross-substance class screening method for quantifying as many antibiotics as possible to identify possible hotspots of antibiotic residues in the aquatic environment, as well as to evaluate the influence on the development of antibiotic resistance by selection pressure. The obtained LOD for all antibiotics were below the PNECs estimated by Bengtsson-Palme and Larsson (2016); indeed, only the LOQ for CIP (LOQ: 0.08 µg L^{-1}; PNEC: 0.064 µg L^{-1}) was above this researchers' predicted values. Accordingly, CIP can be determined at least qualitatively below its PNECs.

Related to the separation of adsorbed fluoroquinolones with sewage sludge (Golet et al. 2002, 2003; Lindberg et al. 2005), CIP should prove more significant in untreated (e.g. hospital) WW than in DW, treated STP effluent, GW and SW. Furthermore, much higher residue concentrations should be expected in raw WW even above the estimated LOQ. For a quantification of CIP in ranges between the achieved LOD and PNEC, individual analytical methods should be applied. For all other antibiotics, a potential selection pressure for resistant bacteria could be negated if the respective residue concentration is below the PNEC.

In comparison with other studies, it should be noted that SPE usually enriches the target components by a factor of up to 1000. In this case, it seems to be clear that a lower LOD and LOQ could have been achieved compared with the direct injection screening method developed in this study. Thus, published LOQs ranged between 0.5 and 64 ng L^{-1} (Monteiro et al. 2017), 0.92 and 12.66 ng L^{-1} (Wei et al. 2014), 0.8 and 245.1 ng L^{-1} (Rossmann et al. 2014) or 3.45 and 470 ng L^{-1} (Dasenaki and Thomaidis 2015) in different aqueous matrices like DW, SW or WW operating SPE for sample pre-concentration and clean-up. In addition, proper LOQs could be achieved for the analysis of antibiotics using direct injection methods as described by Bayen et al. (2014), between 0.016 and 26 ng L^{-1}, and Campos-Mañas et al. (2017), between 10 and 50 ng L^{-1}.

A multi-method approach must always consider the fact that optimum sensitivity cannot be achieved for all components. Nevertheless, further improvements in sensitivity could be achieved by e.g. increasing the injection volume to maintain the direct injection character of the developed method (Busetti et al. 2012), in addition to preconcentration of analytes using, for instance, SPE (Monteiro et al. 2017; Petrović et al. 2005; Seifrtová et al. 2009). Furthermore, sensitivity, which represents a compromise for all analytes in multi-analyte analytical methods, could be achieved by reducing the particle size from 2.7 µm to 1.8 µm operating in ultra performance liquid chromatography (UPLC) mode (Busetti et al. 2012). Moreover, it should be noted that some antibiotics, like colistin or ceftriaxone, could not be implemented in the method presented. Further research is therefore needed to extend the spectrum of antibiotics being analysed.

Sample collection

From summer 2016 to summer 2018, the entire course of a small creek named Swistbach was investigated. This creek is characterised by a length of 44 km and a catchment area of 289 km^2 (Schreiber et al. 2016). Fig. 2 shows the whole stream starting at the source (forest in Rhineland-Palatinate) up to the mouth in the Erft in North Rhine Westphalia (Western Germany). The catchment area is predominantly characterised by rural areas (without clinics and extensive livestock farming) and intensive plant cultivation (mostly fruits and sugar beets). Qualified grab samples were taken at six different sampling spots along the creek course ($n = 17$) from August 2016 to June 2018.

The results of all detected antibiotics are shown in Table 7. Generally, no antibiotic residues could be detected in the samples of sites A, B and C throughout the sampling period. Given that these sites were upstream of the discharge of the four located municipal STPs in the catchment area, these results were reasonable. Furthermore, there seemed to be no diffuse entry pathways from livestock farming with regard to the antibiotic spectrum investigated here (e.g. SPIR, TYL or sulfonamides in general).

At sites D, E and F, at least one antibiotic residue could be detected in a range from 0.01 µg L^{-1} to a maximum of

Table 6. Obtained LOD and LOQ of all investigated antibiotics in spiked drinking water (DW) and the predicted LOD and LOQ for treated WW
Calculated values [ng L^{-1}] were multiplied by a factor of two to obtain the final LOD and LOQ in the diluted (1 : 1) sample [μg L^{-1}]. All determined LOD were below the individual PNECs by Bengtsson-Palme and Larsson (2016), whereas only the LOQ of CIP was above the predicted concentration. Furthermore, the assignment of the respective ISTD to the target analytes is shown. DW, drinking water; ISTD, internal standards; LOD, limit of detection; LOQ, limit of quantification; PNEC, predicted no effect concentration; WW, wastewater; n.a., not available

Analyte (DW)	Calculated LOD (DW) [ng L^{-1}]	Calculated LOQ (DW) [ng L^{-1}]	Predicted LOQ (tWW) [ng L^{-1}]	Predcicted LOQ (tWW) [ng L^{-1}]	PNEC[A] [μg L^{-1}]	Corresponding ISTD
AMOX	6.2	16	5.4	14	0.25	AMP-D_5
AMP	30	74	31	77	0.25	AMP-D_5
AZI	3.1	9.0	4.7	14	0.25	CLIN-$^{13}C,D_3$
CEFA	5.8	17	6.0	18	0.5	CEFO-D_3
CEFO	11	28	11	29	0.125	CEFO-D_3
CEFT	33	78	44	110	0.5	CEFO-D_3
CIP	31	77	32	80	0.064	CIP-D_8
CLA	4.9	13	5.4	14	0.25	CLIN-$^{13}C,D_3$
CLIN	2.8	7.0	3.8	9.6	1	CLIN-$^{13}C,D_3$
CLOX	1.8	4.9	1.8	4.9	0.125	PIP-D_5
CTC	7.7	22	8.3	23	n.a.	n.a.
DICLOX	1.8	4.9	1.9	5.2	n.a.	PIP-D_5
DOC	3.9	12	4.3	13	2	n.a.
ENRO	11	33	12	35	0.064	CIP-D_8
ERY	2.8	7.0	2.7	6.8	1	CLIN-$^{13}C,D_3$
dh-ERY	3.6	10	4.2	12	n.a.	CLIN-$^{13}C,D_3$
FLU	1.6	4.6	1.8	5.0	n.a.	PIP-D_5
LIN	8.0	22	7.8	22	8	LIN-D_3
MERO	21	55	19	50	0.064	MERO-D_6
METHI	1.4	4.2	1.5	4.4	n.a.	PIP-D_5
METRO	7.6	21	9.2	26	0.125	AMP-D_5
MEZLO	3.8	11	4.0	11	n.a.	PIP-D_5
MOX	58	120	59	120	0.125	CIP-D_8
NAF	3.4	9.8	3.2	9.1	n.a.	PIP-D_5
OFLOX	23	59	25	66	0.5	CIP-D_8
OTC	15	42	22	63	0.5	n.a.
OXA	2.1	5.7	2.3	6.1	1	PIP-D_5
PEN-G	11	30	13	34	0.25	PIP-D_5
PEN-V	3.5	10	3.7	11	0.064	PIP-D_5
PIP	17	48	19	52	0.5	PIP-D_5
ROXI	1.9	5.1	1.9	5.3	1	CLIN-$^{13}C,D_3$
SCP	6.5	19	7.7	22	n.a.	SMXl-$^{13}C_6$
SDMD	3.8	11	5.0	14	n.a.	SMXl-$^{13}C_6$
SDMX	2.2	6.6	2.2	6.6	n.a.	SMXl-$^{13}C_6$
SDX	4.9	13	6.1	16	n.a.	SMXl-$^{13}C_6$
SDZ	47	100	44	94	n.a.	SMXl-$^{13}C_6$
SEP	5.0	13	4.7	13	n.a.	SMXl-$^{13}C_6$
SMP	1.1	3.3	1.1	3.2	n.a.	SMXl-$^{13}C_6$
SMX	3.6	10	3.5	9.9	16	SMXl-$^{13}C_6$
N4AcSMX	21	55	21	58	n.a.	SMXl-$^{13}C_6$
SMZ	18	50	19	52	n.a.	SMXl-$^{13}C_6$
SPIR	11	29	11	29	0.5	CLIN-$^{13}C,D_3$
STZ	15	42	17	49	n.a.	SMXl-$^{13}C_6$
TC	12	31	14	36	1	n.a.
TMP	19	42	23	53	0.5	SMXl-$^{13}C_6$
TYL	24	62	30	78	4	CLIN-$^{13}C,D_3$
VANC	76	190	69	170	8	AMP-D_5

[A]Predicted by Bengtsson-Palme and Larsson (2016).

0.43 μg L^{-1}. Generally, AZI, CLA, CLIN, ERY, dh-ERY, ROX, SMX and TMP were the most frequently detected antibiotics ($n >$ LOQ: D = 18–100 %; E = 12–100 %; and F = 18–100 %). The results of this study were congruent with previous studies in Germany as well as in other countries. In addition, macrolide antibiotics (especially CLA, ERY, ROX, CLIN) and SMX/TMP could also be detected in several German rivers in the 2–3 digit ng L^{-1} range (Christian et al. 2003; Faerber et al. 2003; Hirsch et al. 1999; Kümmerer 2009). In addition, CLA, CLIN, ROX, ERY, SMX and TMP were also detected in the same creek in 2002 (Christian et al. 2005).

The predominant detection of macrolide antibiotics (AZI, CLA, ERY, ROX), clindamycin (CLIN) as well as sulfamethoxazole (SMX) and its synergist trimethoprim (TMP) can be

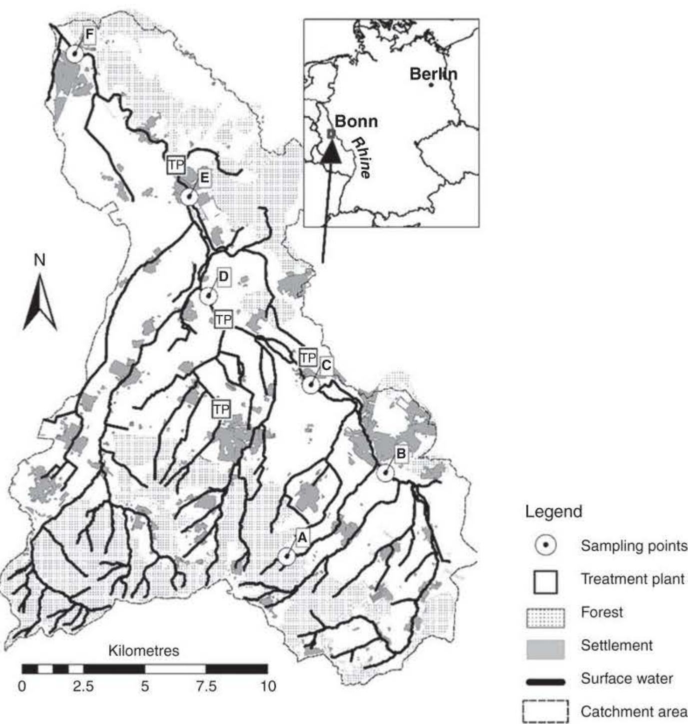

Fig. 2. Schematic overview of the Swistbach (North Rhine-Westphalia, Germany), which shows the location of all sampling sites and municipal treatment plants.

explained by the incomplete degradability of these substances in STP (Göbel et al. 2007; Heberer 2002; Hirsch et al. 1999; Kümmerer 2009; Miao et al. 2004). In addition, these antibiotics are often prescribed to outpatients and are used orally or in topical form. For example, CLIN and ERY are two of the most commonly prescribed antibiotics for the treatment of dermatological infections like inflammatory acne (Leyden 2001). SMX and TMP are usually used in combination as cotrimoxazole (5 : 1) in the treatment of urinary and lower respiratory tract infections, related to their broad active spectra (Church et al. 2015).

Only in two samples (sites E and F) were β-lactam antibiotics detected (0.09 $\mu g\ L^{-1}$ amoxicillin (AMOX) and 0.06 $\mu g\ L^{-1}$ PIP). Despite the high consumption figures of β-lactams (penicillins, carbapenems and cephalosporins) (BVL and Paul-Ehrlich-Gesellschaft für Chemotherapie e.V. 2016), such infrequent detection could be explained by the cleavage of the chemical structure (lactam-ring or penam-ring) of this antibiotic group through hydrolysis (Christian et al. 2005; Deshpande et al. 2004; Hirsch et al. 1999; Kümmerer 2009). Residues of fluoroquinolones have been rarely detected and no tetracyclines were found. These results were in line with previous studies, which showed that fluoroquinolones are readily adsorbed to organic material and are removed from the wastewater path by the separation of sewage sludge, and do not enter SW (Golet et al. 2002, 2003; Lindberg et al. 2005). With regard to tetracyclines, it could be shown that these antibiotics bind as a chelate complex to (for example) calcium and magnesium ions (Kümmerer 2009; Lindsey et al. 2001) and are removed from the aqueous phase. Therefore, they were analytically camouflaged.

Moreover, no clinically relevant antibiotics (MERO, VANC or LIN) could be detected during the sampling period, which could be explained by the absence of clinics or hospitals in the catchment area of the STP or the creek. No residues of antibiotics typically used in livestock farming (SPIR, ENRO, TYL) were detected. Finally, there was no overrun of the predicted no-effect concentrations related to the selection of antibiotic resistances according to Müller et al. (2018). The complete results are given in Table 7.

Fig. 3 shows the total amounts of antibiotic residues summarised by the sampling locations. This graph indicates the role of STP as a key source of antibiotic residues in SW in accordance with the results of previous publications (Batt et al. 2006; Göbel et al. 2007; Watkinson et al. 2009). Interestingly, resistant bacteria could be isolated from the same SW samples from Müller et al. (2018). In contrast, antibiotic-resistant bacteria were isolated upstream from the first STP discharge in a former publication by Müller et al. (2018). This was explained by heavy rainfall and the related remobilisation of sediment or animal faeces, or the possible direct discharge of WW from individual builds (related to a 'missing or faulty sewer system') (Müller et al. 2018). Overall, such entry pathways for antibiotic residues appear to be negligible, as they could not be detected upstream from the first STP discharge.

In addition, Fig. 3 shows a kind of equilibrium or balance in the stream under investigation, as the median between sampling

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Table 7. Results of the investigated creek ('Swist') sorted by sampling points (D, E and F) in µg L^{-1} (minimum, maximum and median) and the percentage of samples with residue concentrations > LOQ (frequency)

No antibiotic residues could be found at sampling sites A, B and C. LOD, limit of detection; LOQ, limit of quantification

Antibiotic	Sampling site D (*n* = 17)					Sampling site E (*n* = 17)					Sampling site F (*n* = 17)				
	Min.	Max.	Median	Frequency > LOQ (%)	Frequency > LOD (%)	Min.	Max.	Median	Frequency > LOQ (%)	Frequency > LOD (%)	Min.	Max.	Median	Frequency > LOQ (%)	Frequency > LOD (%)
AMOX	< LOD	< LOD	< LOD	-	-	< LOD	< LOD	< LOD	-	-	0.09	0.09	0.09	5.9	-
AMP	< LOD	< LOD	< LOD	-	-	< LOD	< LOD	< LOD	-	-	< LOD	< LOD	< LOD	-	-
AZI (*n* = 10)	0.04	0.14	0.07	60.0	-	0.04	0.10	0.07	50.0	-	0.04	0.12	0.09	50	-
CEFA	< LOD	< LOD	< LOD	-	-	< LOD	< LOD	< LOD	-	-	< LOD	< LOD	< LOD	-	-
CEFO	< LOD	< LOD	< LOD	-	-	< LOD	< LOD	< LOD	-	-	< LOD	< LOD	< LOD	-	-
CEFT	< LOD	< LOD	< LOD	-	-	< LOD	< LOD	< LOD	-	-	< LOD	< LOD	< LOD	-	-
CIP (*n* = 16)	0.13	0.13	0.13	12.5	-	0.09	0.09	0.09	6.3	-	< LOD	< LOQ	< LOQ	-	6.3
CLA	0.04	0.20	0.07	94.1	-	0.03	0.13	0.06	82.4	-	0.02	0.16	0.08	88.2	-
CLIN	0.02	0.15	0.06	100	-	0.02	0.12	0.07	88.2	5.9	0.02	0.10	0.06	100.0	-
CLOX	< LOD	< LOD	< LOD	-	-	< LOD	< LOD	< LOD	-	-	< LOD	< LOD	< LOD	-	-
CTC (*n* = 16)	< LOD	< LOD	< LOD	-	-	< LOD	< LOD	< LOD	-	-	< LOD	< LOD	< LOD	-	-
DOC (*n* = 16)	< LOD	< LOD	< LOD	-	-	< LOD	< LOD	< LOD	-	-	< LOD	< LOD	< LOD	-	-
dh-ERY	0.02	0.10	0.04	52.9	-	0.02	0.10	0.05	47.1	-	0.02	0.09	0.03	64.7	-
DICLOX	< LOD	< LOD	< LOD	-	-	< LOD	< LOD	< LOD	-	-	< LOD	< LOD	< LOD	-	-
ENRO (*n* = 16)	< LOD	< LOD	< LOD	-	-	< LOD	< LOD	< LOD	-	-	< LOD	< LOD	< LOD	-	-
ERY	0.01	0.10	0.02	52.9	-	0.01	0.11	0.05	41.2	-	0.01	0.07	0.03	52.9	-
FLU	< LOD	< LOD	< LOD	-	-	< LOD	< LOD	< LOD	-	-	< LOD	< LOD	< LOD	-	-
LIN (*n* = 15)	< LOD	< LOD	< LOD	-	-	< LOD	< LOD	< LOD	-	-	< LOD	< LOD	< LOD	-	-
MERO	< LOD	< LOD	< LOD	-	-	< LOD	< LOD	< LOD	-	-	< LOD	< LOD	< LOD	-	-
METHI	< LOD	< LOD	< LOD	-	-	< LOD	< LOD	< LOD	-	-	< LOD	< LOD	< LOD	-	-
METRO (*n* = 15)	< LOD	< LOD	< LOD	-	-	< LOD	< LOD	< LOD	-	-	< LOD	< LOD	< LOD	-	-
MEZLO	< LOD	< LOD	< LOD	-	-	< LOD	< LOD	< LOD	-	-	< LOD	< LOD	< LOD	-	-
MOX (*n* = 15)	< LOD	< LOD	< LOD	-	-	< LOD	< LOD	< LOD	-	-	< LOD	< LOD	< LOD	-	-
N4AcSMX (*n* = 13)	< LOD	< LOD	< LOD	-	-	< LOD	< LOD	< LOD	-	-	< LOD	< LOD	< LOD	-	-
NAF	< LOD	< LOD	< LOD	-	-	< LOD	< LOD	< LOD	-	-	< LOD	< LOD	< LOD	-	-
OFLOX (*n* = 16)	< LOD	< LOQ	< LOQ	-	6.3	< LOD	< LOQ	< LOQ	-	6.3	< LOD	< LOD	< LOD	-	-
OTC (*n* = 16)	< LOD	< LOD	< LOD	-	-	< LOD	< LOD	< LOD	-	-	< LOD	< LOD	< LOD	-	-
OXA	< LOD	< LOD	< LOD	-	-	< LOD	< LOD	< LOD	-	-	< LOD	< LOD	< LOD	-	-
PEN-G	< LOD	< LOD	< LOD	-	-	< LOD	< LOD	< LOD	-	-	< LOD	< LOD	< LOD	-	-
PEN-V	< LOD	< LOD	< LOD	-	-	< LOD	< LOD	< LOD	-	-	< LOD	< LOD	< LOD	-	-
PIP	< LOD	< LOQ	< LOQ	-	11.8	0.06	0.06	0.06	5.9	-	< LOD	< LOQ	< LOQ	-	11.8
ROX	0.05	0.06	0.05	17.7	-	0.04	0.06	0.05	11.8	-	0.03	0.05	0.05	17.7	-
SDZ	< LOD	< LOD	< LOD	-	-	< LOD	< LOD	< LOD	-	-	< LOD	< LOD	< LOD	-	-
SCP	< LOD	< LOD	< LOD	-	-	< LOD	< LOD	< LOD	-	-	< LOD	< LOD	< LOD	-	-
SDMD	< LOD	< LOD	< LOD	-	-	< LOD	< LOD	< LOD	-	-	< LOD	< LOD	< LOD	-	-
SDMX	< LOD	< LOD	< LOD	-	-	< LOD	< LOD	< LOD	-	-	< LOD	< LOD	< LOD	-	-
SDX	< LOD	< LOD	< LOD	-	-	< LOD	< LOD	< LOD	-	-	< LOD	< LOD	< LOD	-	-
SEP	< LOD	< LOD	< LOD	-	-	< LOD	< LOD	< LOD	-	-	< LOD	< LOD	< LOD	-	-
SMZ	< LOD	< LOD	< LOD	-	-	< LOD	< LOD	< LOD	-	-	< LOD	< LOD	< LOD	-	-

(*Continued*)

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Table 7. (Continued)

Antibiotic	Sampling site D ($n = 17$)					Sampling site E ($n = 17$)					Sampling site F ($n = 17$)				
	Min.	Max.	Median	Frequency > LOQ (%)	Frequency > LOD (%)	Min.	Max.	Median	Frequency > LOQ (%)	Frequency > LOD (%)	Min.	Max.	Median	Frequency > LOQ (%)	Frequency > LOD (%)
SMX	0.06	0.31	0.09	100	-	0.04	0.43	0.10	100	-	0.03	0.34	0.11	94.1	-
SMP	< LOD	< LOD	< LOD	-	-	< LOD	< LOD	< LOD	-	-	< LOD	< LOD	< LOD	-	-
SPIR	< LOD	< LOD	< LOD	-	-	< LOD	< LOD	< LOD	-	-	< LOD	< LOD	< LOD	-	-
STZ	< LOD	< LOD	< LOD	-	-	< LOD	< LOD	< LOD	-	-	< LOD	< LOD	< LOD	-	-
TC ($n = 16$)	< LOD	< LOD	< LOD	-	-	< LOD	< LOD	< LOD	-	-	< LOD	< LOD	< LOD	-	-
TMP	0.04	0.08	0.06	47.1	11.8	0.04	0.08	0.05	29.4	23.5	0.04	0.08	0.05	35.3	17.7
TYL	< LOD	< LOD	< LOD	-	-	< LOD	< LOD	< LOD	-	-	< LOD	< LOD	< LOD	-	-
VANC	< LOD	< LOD	< LOD	-	-	< LOD	< LOD	< LOD	-	-	< LOD	< LOD	< LOD	-	-

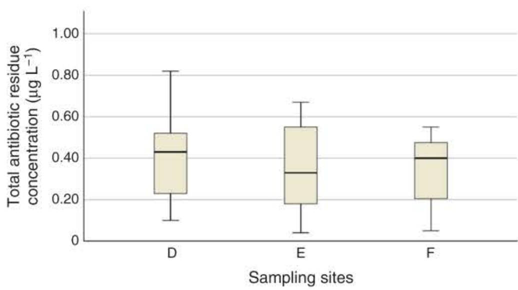

Fig. 3. Total antibiotic residue concentration sorted by sampling sites. No antibiotics could be found upstream from the first wastewater influent. At least one antibiotic residue could be detected in all samples downstream from the discharge. No antibiotic residues could be found at sampling sites A, B and C.

site D (downstream of the first STP discharge) and site F (after four STPs) did not differ significantly. Indeed, only for sampling site E (downstream of three STPs) could a lower median and a higher interquartile distance be obtained. Potential reasons for these differences may be the dilution effects of further discharging streams or field drains, as well as slow degradation processes, a counterpart to the discharges from the STPs along the river.

Conclusion

In this study, a multi-method approach without a cost-intensive and time-consuming sample preparation for the analysis of 47 different antibiotics from 11 substance groups was established. The method covered veterinary antibiotics (e.g. TYL, SPIR, ENRO or several sulfonamides) as well as outpatient (e.g. AMOX, CLA, CLIN) and inpatient human medicine (e.g. MERO, VANC, PIP), with an important role in the treatment of serious bacterial infections. Furthermore, newer antibiotics, such as LIN and metronidazole, as well as rarely published antibiotics (at least in an environmental context), like MERO, could be implied. In this case, the analysis of residues from carbapenem antibiotics (especially MERO) represents an important and current research focus, as these antibiotics are used for the treatment of serious nosocomial infections, with Gram-negative bacteria producing extended spectrum β-lactamases (Müller et al. 2018), and are classified as 'critically important antimicrobials' by the WHO (WHO 2017).

Overall, the method performance was characterised by good recovery rates, a high dynamic range with good linearity, and suitable LOD and LOQ, which could undercut the PNECs for resistance selection proposed by Bengtsson-Palme and Larsson (2016) for most antibiotics. The method developed here represents a fast and powerful analytical tool for screening DW, GW, SW and treated WW regarding antibiotic residues. An initial application demonstrates that the effluent of municipal STPs plays an important role in the release of antibiotic residues into the aquatic environment. In this case, macrolide antibiotics as well as CLIN, SMX and TMP could be detected most frequently in SW (in 2–3 digit ng L^{-1} range) influenced by WW.

It must generally be considered that the analysis of antibiotic residues is only one part of the evaluation of a sample regarding its 'resistance situation' besides the analysis of (for instance) antibiotic resistance genes and the cultivation and identification of antibiotic resistant bacteria. Based on this method, further

research projects on the subject of antibiotic residues in the environment and the influence on the potential development of antibiotic resistance will be investigated in interdisciplinary cooperation.

Supplementary material

Additional information regarding the application area of the respective antibiotics and a further characterisation of the matrices used for method assessment (DOC, pH, conductivity etc.) can be found on the Journal's website.

Conflicts of interest

The authors declare no conflicts of interest. This study complies with the guidelines of the Declaration of Helsinki (1964) by the World Medical Association (No. 160/120-HyReKA-Ethikantrag).

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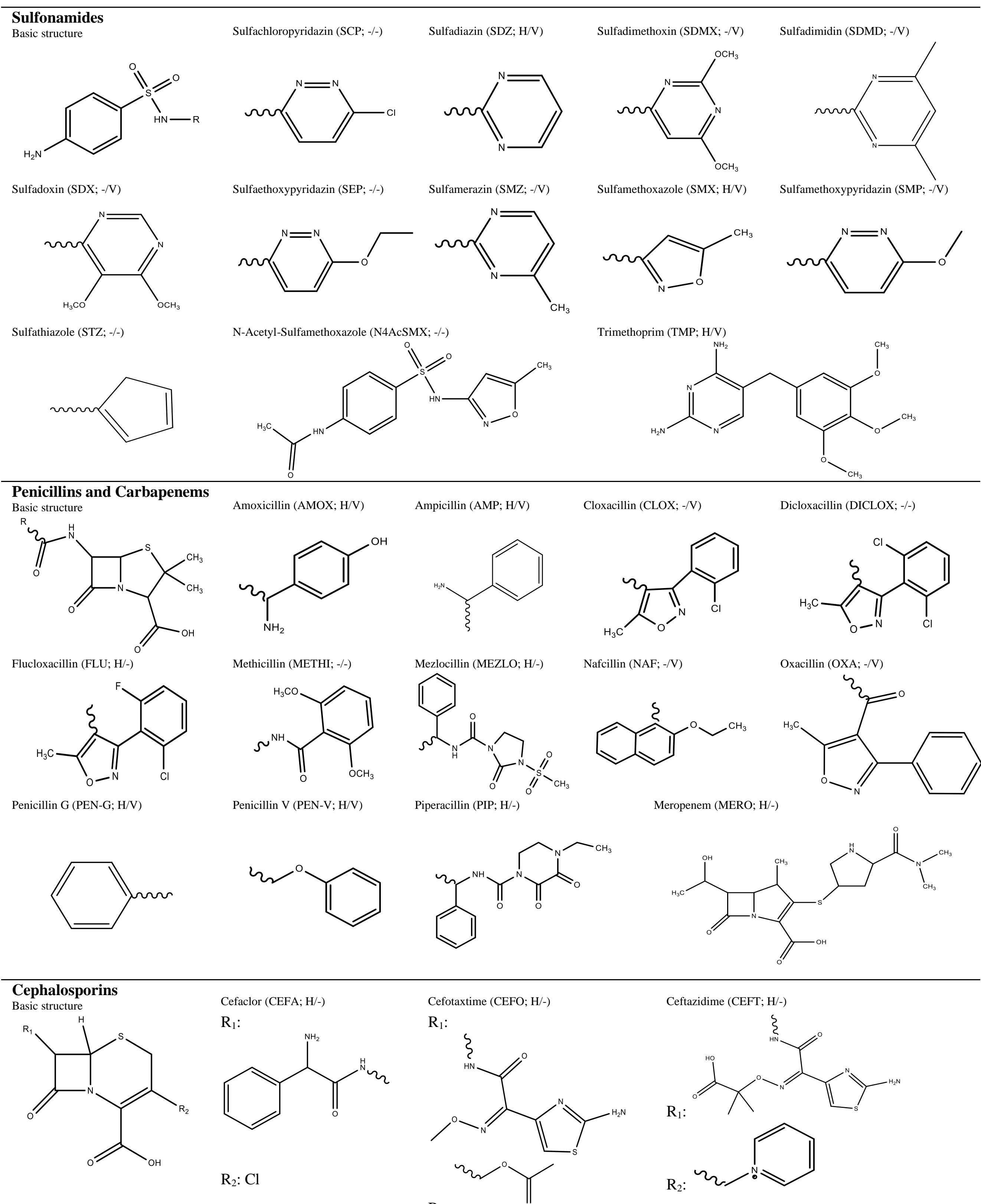
Appendix: „Liquid chromatography-tandem mass spectrometry as a fast and simple method for the determination of several antibiotics in different aqueous matrices"

Table S1: Chemical structure (Bryskier, 1999)[125] and the therapeutical field of application, human (H), veterinary medicine (V) and no marketable preparations in Germany (-), based on DIMDI (2018)[126] of the analyzed antibiotics sorted by substance classes.



Appendix: „Liquid chromatography-tandem mass spectrometry as a fast and simple method for the determination of several antibiotics in different aqueous matrices"

Table S1 (continue): Chemical structure (Bryskier, 1999)[125] and the therapeutical field of application, human (H), veterinary medicine (V) and no marketable preparations in Germany (-), based on DIMDI (2018)[126] of the analyzed antibiotics sorted by their substance classes.

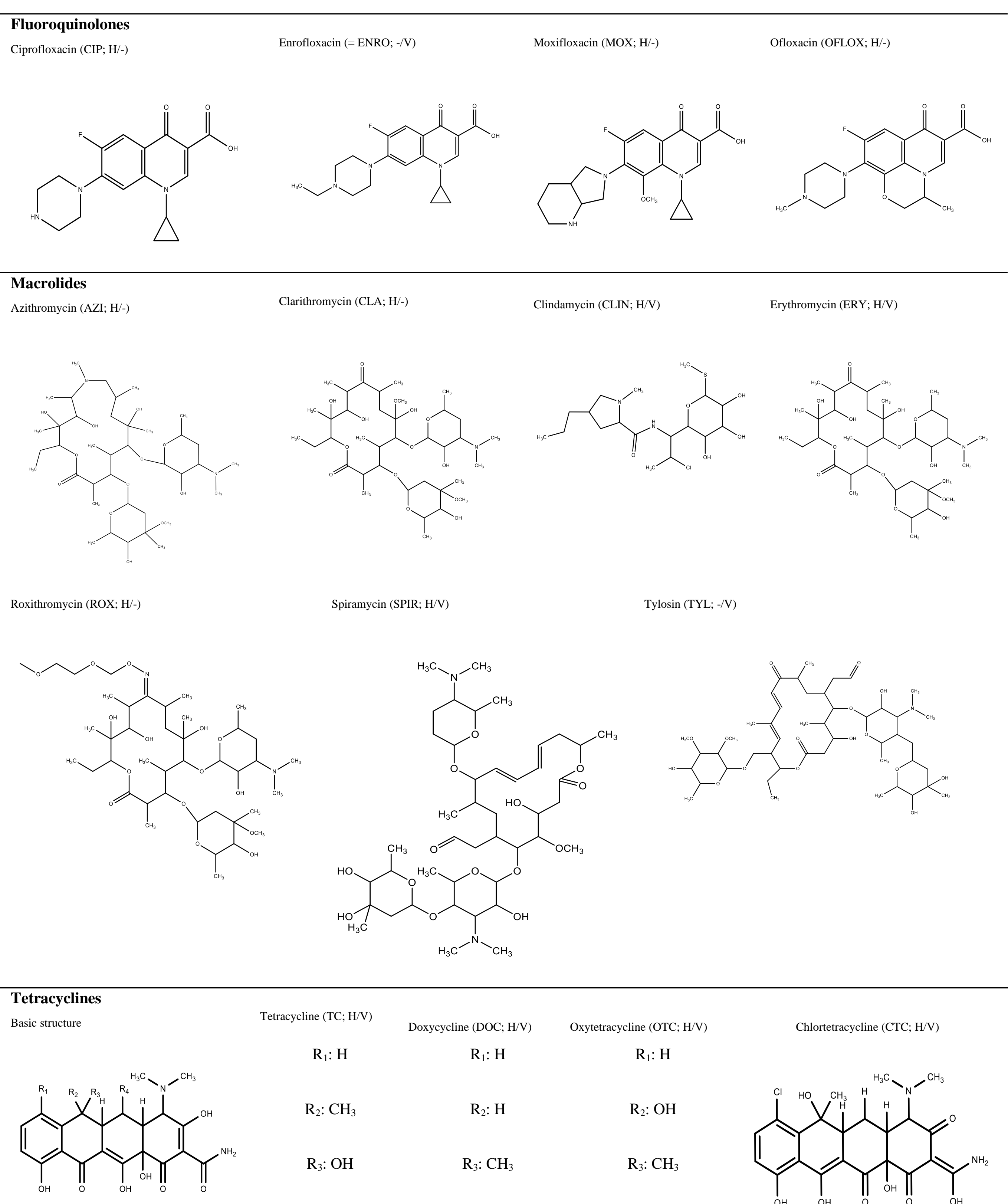


Table S2: Characterization of the blank matrices (DW, SW and GW) by specific water quality parameters

Legend: DW = drinking water; GW = groundwater; SW = surface water; TOC = total organic carbon

Parameter	DW	SW	GW
pH	8.0 ± 0.1	8.3	6.9
TOC [$mg \cdot L^{-1}$]	0.6 ± 0.2	6.8	0.64
Electric conductivity (25°C , $\mu S \cdot cm^{-1}$)	340 ± 40	579	565
Chlorid [$mg \cdot L^{-1}$]	32 ± 3	61	49
Total amount of phosphate [$mg \cdot L^{-1}$]	< 0.01	< 0.03	0.07
Sulfate [$mg \cdot L^{-1}$]	30 ± 2	64	66
Calcium [$mg \cdot L^{-1}$]	35.7 ± 4.5	59	60
Magnesium [$mg \cdot L^{-1}$]	7.5 ± 0.9	13	14
Turbidity [FNU]	< 0.10	13	0.69

2.2. Zusammenfassung

In der vorliegenden Arbeit konnte eine Direktinjektionsmethode für insgesamt 45 verschiedene Antibiotika in Trinkwasser, Grundwasser, Oberflächenwasser und Abwasser entwickelt werden.[5] Insgesamt umfasst das Untersuchungsspektrum Antibiotika aus elf verschiedenen Wirkstoffgruppen und deckt einen Großteil der eingesetzten Antibiotika ab.[5,28,32,41] Dies ermöglicht unabhängig von der Art der Eintragsquelle den Nachweis etwaiger Kontaminationen durch AR in aquatischen Matrices. Mit Anhydroerythromycin und N-Acetylsulfamethoxazole wurden zudem zwei wichtige Hauptmetaboliten ins Untersuchungsspektrum aufgenommen.[5,103,123]

Durch die Verwendung isotopenmarkierter interner Standards konnten gute Wiederfindungsraten zwischen 65% und 117% mit einer akzeptablen Präzision (relativen Standardabweichungen $\leq 16\%$) in Trinkwasser, Grundwasser, Oberflächenwasser und behandeltem Abwasser erreicht werden. Der dynamische Bereich erstreckte sich zwischen 10 ng/L und 5.000 ng/L und zeichnete sich durch eine sehr gute Linearität aus ($r > 0{,}99$). Weiterhin wird durch eine ausreichende Empfindlichkeit (Bestimmungsgrenze: 3,3 ng/L – 190 ng/L) ermöglicht, subinhibitorische Konzentrationen in der aquatischen Umwelt nachzuweisen, um mögliche Einflüsse zugunsten von ARB zu erkennen und gegebenenfalls Handlungsoptionen aussprechen zu können.[5]

Als erste Absicherung der Eignung einer Direktinjektionsmethode zur Bestimmung von Antibiotika in wässrigen Proben dient die erfolgreiche Teilnahme an einer Laborvergleichsuntersuchung (Ringversuchs 5/16 – TW S2 – Pharmazeutika in Trinkwasser), an der im Rahmen der Methodenentwicklung teilgenommen wurde.[127] Es konnte gezeigt werden, dass die Bestimmung von zehn verschiedenen Sulfonamiden sowie Trimethoprim mittels Direktinjektionsmethode valide Ergebnisse bei der Trinkwasseruntersuchung liefern kann.

Lediglich Sulfachlorpyridazin lag mit einem z-*Score* von $2{,}0 < |z_U|$ außerhalb des akzeptierten Bereiches (siehe Anhang Tabelle A).[127] Zur weiteren Bestätigung der Leistungsfähigkeit der vorgestellten Untersuchungsmethode sollte zukünftig an weiteren Laborvergleichsuntersuchungen hinsichtlich der Bestimmung von AR in verschiedensten Untersuchungsmatrices teilgenommen werden.

Eine erste Anwendung der entwickelten Methode zeigte, dass diese geeignet ist, Punktquellen zu identifizieren und erste Rückschlüsse auf die Art der Punktquelle zu ermöglichen.[5] Aufgrund der Charakteristika des untersuchten Einzugsgebietes (keine Krankenhäuser sowie intensive Viehzucht), konnten keine Rückstände klinisch relevanter Antibiotika sowie klassischer Veterinärantibiotika nachgewiesen werden.[5] Der Nachweis von Rückständen überwiegend ambulant eingesetzter Antibiotika (z.B. Makrolid-Antibiotika, Sulfamethoxazol oder Trimethoprim) in dem untersuchten Oberflächengewässer nach der Einleitung von behandeltem Abwasser bestätigt vorherige Studienergebnisse, dass kommunale Kläranlagen einen Haupteintragspfad von AR in Oberflächengewässer darstellen.[5,75,103,123,128]

Da in der vorliegenden Studie ein Oberflächengewässer ohne klinischen Einfluss zur Überprüfung der Anwendbarkeit der Methode durch Realproben untersucht wurde, sollte in weiterführenden Studien überprüft werden, ob eine besondere Belastung durch klinisches Abwasser für die aquatische Umwelt resultiert. Zudem stellt sich die Frage, ob das Vorhandensein von AR mit der Belastung durch ARB und ARG korreliert.

3. Krankenhausabwasser als potentieller „Hotspot“

3.1. Einleitung

Die Behandlung von Infektionen durch MRE stellt eine globale Herausforderung für die Öffentliche Gesundheit dar.[129] Aktuellste Studien belegen auf Grundlage der Daten des Europäischen Antibiotikaresistenz-*Surveillance*-Netzwerks (EARS-Net), dass sich die Anzahl an Infektionen mit ARB (circa 670.000 Infektionen in 2015) sowie die Anzahl der mit solchen Infektionen assoziierten Todesfällen (circa 33.000 Todesfälle in 2015) in Europa zwischen den Jahren 2007 und 2015 deutlich erhöht haben.[130]

Vor diesem Hintergrund veröffentlichte die WHO 2017 mit der sogenannten „*global priority list of antibiotic-resistant bacteria*“ eine Priorisierung der wichtigsten ARB. Als besonders kritisch wurden Carbapenem-resistente *A. baumannii* und *P. aeruginosa* Spezies sowie Carbapenem- und 3GCR *Enterobacteriales* Spezies eingestuft, bei denen die Erforschung neuer antibiotisch wirksamer Substanzen als Behandlungsoption im Fokus stehen soll.[57] Insgesamt konnte eine Erhöhung der Rate an Infektionen mit Carbapenem-resistenten *K. pneumoniae* (+ 530%), Carbapenem-resistenten *E. coli* (+ 380%), 3GCR *E. coli* (+ 300%), multiresistenten *P. aeruginosa* (+ 56%) sowie Carbapenem-resistenten *P. aeruginosa* (+ 230%) Stämmen in Europa festgestellt werden.[130]

Auch wenn sich die Gesamtzahlen an Infektionen mit MRE in Deutschland deutlich unter dem EU-Durchschnitt befindet,[130] steht die Verhinderung der Entstehung und Verbreitung von Antibiotikaresistenzen auch in Deutschland weiter im Fokus.[131] Als Grundlage für eine restriktivere Antibiotikaverschreibungspraxis (*Antibiotic Stewardship*) veröffentlichte die WHO eine Priorisierung der einzelnen Wirkstoffgruppen, um einen umsichtigeren Einsatz von Antibiotika sowohl in der Human- als auch in der Veterinärmedizin im Rahmen eines verbesserten Risikomanagements zur Reduzierung von Antibiotikaresistenzen durch den veterinärmedizinischen Einsatz von Antibiotika zu erreichen.[11]

Darüber hinaus erfolgte eine Einteilung der Antibiotika in der Humanmedizin anhand ihrer Verschreibungspraxis, ihrer Toxizität sowie ihres Wirkungsspektrums in drei unterschiedliche Gruppen gemäß der „AWaRe“ Klassifizierung (*Access, Watch and Reserve classification*).[132,133]

Neben der Suche nach neuen Behandlungsoptionen gegen die oben aufgeführten antibiotikaresistenten Erreger, der Verbesserung des *Antibiotic Stewardship* und Etablierung von *Antibiotic Resistance Surveillance*-Systemen stellt die Untersuchung des Vorkommens und der Verbreitung von MRE und AR in der Umwelt im Rahmen des sogenannten „*One-Health*“-Ansatzes eine weitere Säule nationaler und internationaler Programme dar. So streben z.B. die Deutsche Antibiotika-Resistenzstrategie (DART 2020) oder der Globale Aktionsplan der WHO die Erforschung der Entstehung und Verbreitung von Antibiotikaresistenzen sowie die Entwicklung geeigneter Präventionsmaßnahmen an.[129,131]

Seit circa zwei Dekaden stellt Krankenhausabwasser einen Forschungsschwerpunkt bei der Untersuchung des Eintrags von AR in die aquatische Umwelt dar.[75,77,95,134] Es konnte bereits gezeigt werden, dass ein breites Spektrum an AR im 1- bis 2-stelligen µg/L-Bereich in Krankenhausabwasser zu finden ist.[95] Rückstände an Ciprofloxacin z.B. konnten bis in den 3-stelligen µg/L-Bereich (max. 236,6 µg/L) in Krankenhausabwasser nachgewiesen werden.[104,135,136] Ferner zeigten Hartmann et al. (1999), dass derart hohe Rückstandskonzentrationen an Ciprofloxacin bereits genotoxische Auswirkungen auf die Bakterien-DNA in Krankenhausabwasser ausüben können.[136] Ferner konnte ebenfalls eine hohe Diversität an ARG und ARB in klinischen Abwässern nachgewiesen werden.[2,74,137–139] Aktuelle Studien konnten zeigen, dass Handwaschbecken, Duschabläufe und Toiletten klinischer Bereiche und das dort befindliche Abwasser als potentielles Erregerreservoir für ARB, insbesondere Carbapenem-resistente gramnegative Bakterien wie z.B. *P. aeruginosa*, *A. baumannii* oder *Enterobacteriales*, dienen können und in Präventionsstrategien berücksichtigt werden sollten.[140–145]

Im Rahmen der im Folgenden zusammengefassten Arbeiten wurden Sanitäreinheiten in Patientenbadezimmern unterschiedlicher klinischer Bereiche auf AR hin untersucht, die neben ARB und ARG über Patientenausscheidungen (u.a. Faeces, Urin oder Schweiß) in die jeweiligen Sanitäreinheiten eingetragen werden könnten,[123,146,147] um einen etwaigen Selektionsdruck zugunsten von ARB im unmittelbaren Umfeld höchst vulnerabler Patienten zu untersuchen. Aufgrund der erhöhten Verschreibungsmenge und Anwendungsdichte sowie deren Bedeutung für die Humanmedizin standen vor allem klinisch relevante Antibiotika, wie z.B. Meropenem, Vancomycin oder Ciprofloxacin im Fokus der Arbeiten.[11,32,41,132]

Darüber hinaus wurde überprüft, inwieweit sich Krankenhausabwasser von kommunalem Abwasser bzw. Mischabwässern hinsichtlich der Belastung durch AR, ARB und ARG unterscheidet und ob diese als spezifische Marker für klinisches Abwasser fungieren könnten. Diese Untersuchungen wurden auf Grundlage der in Kapitel II vorgestellten LC-MS/MS-Methode sowie mit molekularbiologischen und mikrobiologischen Untersuchungsverfahren, die im Rahmen des BMBF-Verbundprojektes „HyReKA“ (Förderkennzeichen 02WRS1377) entwickelt wurden, durchgeführt.[5,74,148]

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The occurrence of antimicrobial substances in toilet, sink and shower drainpipes of clinical units: A neglected source of antibiotic residues

A.M. Voigt[a,2], H.A. Faerber[a,*], G. Wilbring[a], D. Skutlarek[a], C. Felder[a], R. Mahn[b], D. Wolf[b,c], P. Brossart[b], T. Hornung[d], S. Engelhart[a], M. Exner[a], R.M. Schmithausen[a,1]

[a] Institute for Hygiene and Public Health, University Hospital Bonn, Sigmund-Freud-Str. 25, 53105 Bonn, Germany
[b] Medical Clinic, Department of Haematology and Oncology, Centre for Integrated Oncology, University Hospital Bonn, Sigmund-Freud-Str. 25, 53105 Bonn, Germany
[c] University Clinic V, Dpt. Hematology and Oncology, Medical University Innsbruck, Anichstraße 35, A-6020 Innsbruck, Austria
[d] Clinic and Polyclinic for Dermatology and Allergology, University Hospital Bonn, Sigmund-Freud-Str. 25, 53105 Bonn, Germany

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ABSTRACT

Antibiotics represent one of the most important drug groups used in the management of bacterial infections in humans and animals. Due to the increasing problem of antibiotic resistance, assurance of the antibacterial effectiveness of these substances has moved into the focus of public health. The reduction in antibiotic residues in wastewater and the environment may play a decisive role in the development of increasing rates of antibiotic resistance. The present study examines the wastewater of 31 patient rooms of various German clinics for possible residues of antibiotics, as well as the wastewater of five private households as a reference.

To the best of our knowledge, this study shows for the first time that in hospitals with high antibiotic consumption rates, residues of these drugs can be regularly detected in toilets, sink siphons and shower drains at concentrations ranging from 0.02 $\mu g \cdot L^{-1}$ to a maximum of 79 $mg \cdot L^{-1}$. After complete flushing of the wastewater siphons, antibiotics are no longer detectable, but after temporal stagnation, the concentration of the active substances in the water phases of respective siphons increases again, suggesting that antibiotics persist through the washing process in biofilms. This study demonstrates that clinical wastewater systems offer further possibilities for the optimization of antibiotic resistance surveillance.

1. Introduction

The treatment of bacterial infections remains a serious and cost-intensive factor in modern healthcare (European Commission, without year; U.S. Department of Health and Human Services - Centers for Disease Control and Prevention, April 23, 2013). According to the US Center for Disease Control and Prevention (US-CDC), at least 2 million patients in the United States of America (USA) are infected with resistant bacteria every year. Moreover, at least 23,000 patients die each year as a result of such infections (U.S. Department of Health and Human Services - Centers for Disease Control and Prevention, April 23, 2013). Cases in the European Union (EU) and the European Economic

Abbreviations: AMOX, amoxicillin; AMP, ampicillin; AZI, azithromycin; CEFA, cefaclor; CEFO, cefotaxime; CEFT, ceftazidime; CEFU, cefuroxime; CIP, ciprofloxacin; CLA, clarithromycin; CLIN, clindamycin; CTC, chlortetracycline; CLOX, cloxacillin; DDD, defined daily doses; DICLOX, dicloxacillin; DOC, doxycycline; ERY, erythromycin; dh-ERY, dehydrato-erythromycin; ENRO, enrofloxacin; ESBL, extended-spectrum beta-lactamase; FLU, flucloxacillin; LOQ, limit of quantification; LIN, linezolid; GLASS, Global Antimicrobial Resistance Surveillance System; MERO, meropenem; METHI, methicillin; METRO, metronidazole; MEZLO, mezlocillin; MIC, minimal inhibition concentration; MOX, moxifloxacin; MDRO, multi drug resistant organisms; NAF, nafcillin; OFLOX, ofloxacin; OXA, oxacillin; OTC, oxytetracycline; PEN-G, penicillin G; PEN-V, penicillin V; PIP, piperacillin; PNEC, predicted no effect concentration; RDD, recommended daily dose; ROX, roxithromycin; STP, sewage treatment plant; SH, shower drain; SI, sink siphon; SPIR, spiramycin; SCP, sulfachlorpyridazine; SDZ, sulfadiazine; SDMX, sulfadimethoxine; SDMD, sulfadimidine; SDX, sulfadoxine; SEP, sulfaethoxypyridazine; SMZ, sulfamerazine; SMX, sulfamethoxazole; N4AcSMX, N4-Acetylsulfamethoxazole; SMP, sulfathiazole; STZ, sulfamethoxypyridazine; TC, tetracycline; TMP, trimethoprim; TYL, Tylosin; US-CDC, US Center for Disease Control and Prevention; WC, water closet; WHO, World Health Organization; VANC, vancomycin

* Corresponding author. Institute for Hygiene and Public Health, Sigmund-Freud-Str. 25, 53105 Bonn, Germany. Tel.: +49 228 287 16864; fax: +49 228 287 11144.

E-mail address: harald.faerber@ukbonn.de (H.A. Faerber).

[1] Senior authorship.
[2] Main authorship.

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Area (EEA) are comparable with estimated 33,110 deaths from resistant bacteria in 2015, with mortality rates higher in the southeast than in the northwest (Cassini et al., 2018). Based on data from the World Health Organization (WHO) evaluated in the Global Antimicrobial Resistance Surveillance System (GLASS), *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Streptococcus pneumoniae* and *Salmonella* spp. were the most frequently reported antibiotic resistant bacteria (World Health Organization, January 29, 2018).

In response, national and international antibiotic resistance surveillance programs have been established (Bundesministerium für Gesundheit et al., April 2018; Qiao et al., 2018; The White House Washington, March 2015). In addition, various international programs have been set up to evaluate and share national research data, meet harmonized standards and coordinate global action against the spread and development of antibiotic resistance (European Centre for Disease Prevention and Control, 2017; World Health Organization, 2015, 2017b).

Although the antimicrobial resistance situation in Germany has not increased in recent years, it should be mentioned that it is at a significantly higher level than in the 1990s (BVL & Paul-Ehrlich-Gesellschaft für Chemotherapie e.V., 2016). In particular, Vancomycin resistant Enterobacteriaceae (VRE), methicillin-resistant Staphylococcus (MRSA) and carbapenem-resistant Enterobacteriaceae (CRE) represent current dangers for the global health system (U.S. Department of Health and Human Services - Centers for Disease Control and Prevention, April 23, 2013). Therefore, numerous studies regarding the medical importance of VRE (Cetinkaya et al., 2000; O'Driscoll and Crank, 2015; Sievert et al., 2013), MRSA (Hassoun et al., 2017; Sievert et al., 2013) and CRE (Kizny Gordon et al., 2017; Sievert et al., 2013; Walsh, 2010) have been published in recent decades. Moreover, increased global commodity flow (Iark et al., 2018; Roca et al., 2015; Teubner, 1999) and inter-continental travel (European Centre for Disease Prevention and Control, 2018; Ostholm-Balkhed et al., 2013; Tängdén et al., 2010; Wiklund et al., 2015) constitute potential risk factors. A previous study has demonstrated that approximately one quarter of the travelers (inter-continental) examined were colonized with extended-spectrum beta-lactamase- (ESBL) producing *Escherichia coli* during their journey, with hotspots including the Middle East (e.g. Egypt) and Asia (especially India) (Tängdén et al., 2010). In addition, a recent study has shown the contamination of chicken meat with ESBL-producing *Escherichia coli* (CTX-M-44) in Brazil and the possible risk of export (Iark et al., 2018).

In this context, it is interesting to note that in Germany the total amount of defined daily doses (DDD) and the number of prescriptions of antibiotics remained constant between 2005 and 2014, whereas fluorochinolones and cephalosporins are still very high (sum of prescriptions in 2014: "reserve" cephalosporins = 7.4 million DDD; "reserve" fluorochinolones = 5.6 million DDD). The total amount of antibiotics used in Germany for human medicine is approximately 700–800 t (BVL & Paul-Ehrlich-Gesellschaft für Chemotherapie e.V., 2016).

In addition to human medicine, the usage of antibiotics in veterinary medicine is another important field of application that is important for the interpretation of the total consumption of antibacterial substances (Bundesamt für Verbraucherschutz und Lebensmittelsicherheit, July 23, 2018; BVL & Paul-Ehrlich-Gesellschaft für Chemotherapie e.V., 2016; European Commission, without year; Qiao et al., 2018). In the period from 2011 to 2017, the total quantity of antibiotics (data evaluated by DIMDI (German Institute for Medical Documentation and Information) fell from 1.706 t to 733 t (Bundesamt für Verbraucherschutz und Lebensmittelsicherheit, July 23, 2018). This corresponds with a percentage of antibiotics for veterinary applications of approximately 50% of total consumption. In the USA, it is estimated that around 80% of total consumption is used for animal fattening (European Commission, without year). In China a comparable proportion of antibiotics was found in livestock farming (52%), but with a significantly higher total amount of 92,000 t in 2013 (Qiao et al., 2018).

It is important to note that the human health system, the veterinary sector and the environment are interrelated (Feuerpfeil et al., 1999; Kümmerer, 2003; Roca et al., 2015; Westphal-Settele et al., 2018) and cannot be considered separately. Rather, the whole context must be contemplated, as described in the "One-Health" concept (World Health Organization, November 12, 2018).

Thus, for example, the decreasing total quantity of prescribed antibiotics in Germany in livestock sectors, the quantities of fluoroquinolones (9.9 t, +1.7%), cephalosporins of the 3rd generation (2.3 t, +0.2%), and the large amount of polymyxin (74 t), must be viewed critically (Bundesamt für Verbraucherschutz und Lebensmittelsicherheit, July 23, 2018), because these antibiotics, which are related to the "watch" and "reserve" group, should be restricted for treating serious or life-threating infections in human patients (World Health Organization, March 2017). In general a reduction or renouncement in antibiotics in intensive livestock farming, which are defined as "critically important antimicrobials" (e.g. vancomycin, meropenem, linezolid, ciprofloxacin, ampicillin and colistin) for human medicine by the WHO, should be considered (World Health Organization, 2017a).

A good example of a more restrictive usage of antibiotics in veterinary fields is the prohibition of colistin as a potential growth stimulator in China in 2017 (Qiao et al., 2018) and the general restriction of antimicrobial substances for growth promotion in the EU since 2006 (VO (EG) Nr. 1831/2003, December 30, 2005). Furthermore, the development of "Guidelines for careful handling of antibacterial veterinary medicinal products" in 2015 may help veterinarians to find a more effective and restrictive application of antibiotics (Bundestierärztekammer (BTK), 2015).

The reduction of the release of antibiotic residues, resistant genes and antibiotic resistant bacteria into the environment should be emphasized in the development of new wastewater treatment processes and a sufficient antibiotic resistant surveillance system. This can be explained by the fact that antibiotic residues are just partially metabolized, and excreted unchanged will enter the wastewater path (Faerber et al., 2003; Kümmerer, 2003, 2009). Recent studies have demonstrated that antibiotic genes (Rizzo et al., 2013; Xu et al., 2015; Yang et al., 2014), antibiotic-resistant bacteria (Feuerpfeil et al., 1999; Müller et al., 2018; Rizzo et al., 2013) and antibiotic residues (Chang et al., 2010; Feuerpfeil et al., 1999; Kümmerer, 2001) are incompletely eliminated in sewage treatment plants and enter the (aquatic) environment.

The fate of antibiotic residues varies with the respective substance class. Tetracyclines and fluoroquinolones have a high adsorption capacity (Golet et al., 2003; Kümmerer, 2003, 2009) and are removed from the water cycle with sewage sludge. Β-lactams exhibit increased hydrolysis sensitivity regarding their β-lactam ring (Deshpande et al., 2004; Kümmerer, 2009). On the other hand, sulfonamides and macrolides tend to have a higher persistence within the wastewater treatment plant and can therefore be detected more frequently and in higher concentrations in surface waters (Faerber et al., 2003; Kümmerer, 2009).

For conventional sewage treatment plants (STP), it has been shown in former studies that a general reduction in bacteria by one to three log levels can be achieved (Koivunen and Heinonen-Tanski, 2005; Mandilara et al., 2006; Rechenburg et al., 2006; Schreiber et al., 2015). A similar reduction capacity can be achieved for antibiotic-resistant bacteria (publication in preparation).

A schematic overview of the potential clinical wastewater pathways of antibiotic residues in the aquatic environment is shown in Fig. 1.

Released into the environment, antibiotic residues may be a selection factor in aquatic matrices in favor of antibiotic-resistant bacteria (Baquero et al., 2008; Bengtsson-Palme and Larsson, 2016). Even small concentrations (< MIC, minimal inhibition concentration) of antibacterial substances can stimulate the transfer of resistance genes via horizontal gene transfer or promote the interaction of resistant

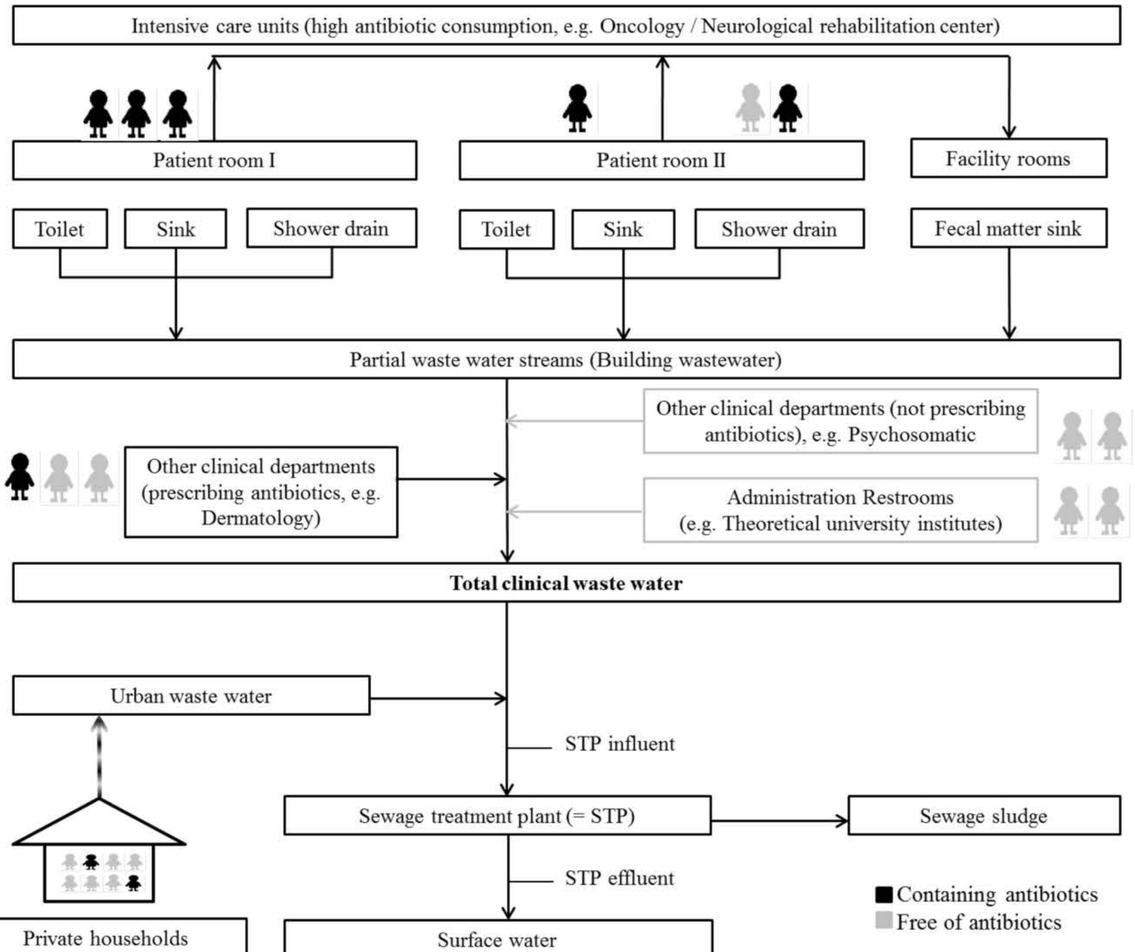

Fig. 1. Schematic overview representation of the clinical wastewater path (from the patient's room to the effluent of treated wastewater into surface water) and potential entry paths of antibiotic residues into the hospital wastewater and ultimately the aquatic environment. As expected, wastewater pathways free from antibiotics are shown in gray and wastewater pathways contaminated with antibiotics are shown in black.

wastewater bacteria with sensitive germs in the aquatic environment (Finley et al., 2013; Jutkina et al., 2018). Furthermore, it must be considered that certain environmental bacteria, like *Acinetobacter calcoaceticus*, are able to acquire genome fragments (which could include antibiotic resistance genes) (Palmen et al., 1993).

Antibiotics are detected more frequently and in higher concentrations in hospital wastewater than in municipal wastewater (Kümmerer, 2001; Watkinson et al., 2009). Some studies have already revealed high levels of contamination of total hospital wastewater with antibiotic residues through detection of ciprofloxacin (up to concentrations in the three digit $\mu g{\cdot}L^{-1}$ range) (Diwan et al., 2010; Kümmerer, 2003; Lindberg et al., 2004) or ampicillin (up to concentrations in the two digit $\mu g{\cdot}L^{-1}$ range) (Kümmerer, 2003).

In addition, clinical wastewater has been identified as a potential hotspot for antibiotic-resistant bacteria and genes (Koh et al., 2015; Müller et al., 2018; Picão et al., 2013; Schwartz et al., 2006; Simo Tchuinte et al., 2016; Zhang et al., 2014).

Therefore, an important approach to preventing the further development of resistance is the reduction of the input of these substances into the wastewater pathway. To clarify this pathway, the wastewater from various clinical areas in several German hospitals was examined in this study using a screening method by LC-MS/MS through injecting aqueous samples without complex sample preparation (filtration only). The aim was to examine the hospital wastewater more closely on the basis of its spectrum and its content of antibiotic residues, and to identify largely contaminated wastewater substreams.

2. Material and methods

2.1. Materials and chemicals

All chemicals used were purchased from VWR International GmbH (Langenfeld, Germany) and Carl Roth GmbH + Co. KG (Karlsruhe, Germany), and were HPLC-MS grade. The analytical standards were purchased from Sigma Aldrich (Taufkirchen, Germany), Biomol GmbH (Hamburg, Germany), Toronto Research Chemicals Canada (Toronto, Canada) and USP Reference Standard (Basel, Switzerland). The LC columns and micropore filters used were obtained from Macherey and Nagel (Düren, Germany).

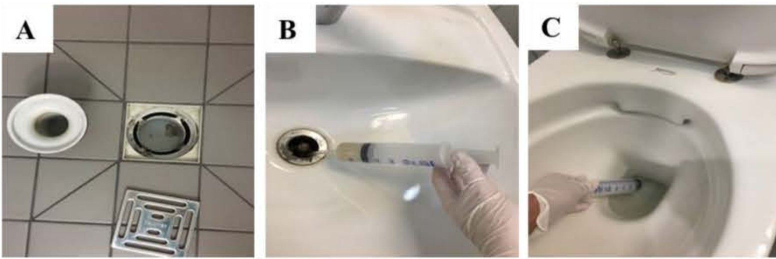

Fig. 2. Sampling spots in the oncology department: A) shower drainage B) sink and C) toilet.

2.2. Sampling sites

In the period from April 2017 to February 2018, the wastewater (defined as the aqueous phase in toilets, shower drains and washbasin siphons) of 31 patient rooms [shower drains (SH), toilet drains (WC) and washbasin siphons (SI)] in four clinical areas (oncology, neurological rehabilitation (regular ward and neuro-urology), dermatology and psychosomatics) was examined for antibiotic residues (see Fig. 2). Sanitary areas in a theoretical university institute (i.e., lecture halls, without any relations to patients) and in five private households without current use of antibiotics were investigated in the same way.

The oncology area investigated was characterized by a slightly increased amount of antibiotic consumption (recommended daily dose per 100 patient days = RDD·100 d^{-1}) in total 145.27 RDD·d^{-1}, in comparison to other hematological oncologies in Germany (based on data for 2017). For further information see Table S2.

In the case of oncology, the rooms were randomly selected to obtain a cross-section of the entire clinic (with a generally high and frequent use of antibiotics). The current administration of antibiotics was previously known (in 14 of 20 investigated rooms, see Table S1), but this did not determine whether a room was examined or not. In the case of dermatology and neurological rehabilitation, patient rooms with prior administration of antibiotics were targeted (see Table S1) in order to be compared with other clinics with a different medical specialization. Private households, the theoretical university institute and psychosomatics were selected as reference samples due to the lack of current antibiotic administration.

2.3. Sampling procedure

Sampling was undertaken in two phases (1. status quo, 2. stagnation) at several defined times (status quo, 0, 2, 4, 6, 12 and 24 h). The general sampling procedure is described below. Samples from WC were taken using a 50 mL disposable Omnifix® syringe from B. Braun Melsungen AG (Melsungen, Germany) directly from the water phase. For sampling the aqueous phase of SI and SH, additional inert tubes (length: approx. 32 cm) were used. The water was bottled, stored at 5 ± 2 °C and analyzed within 24 h.

To prevent possible falsifications of the results, contact or mobilization of the biofilm and other incrustations on the walls was strictly avoided during the sampling procedure.

2.3.1. Status quo sample

The “status quo” sample was a random grab sample (single sample) taken at a specific time in a patient's room without knowing when the last washbasin, shower or toilet was used or cleaned by the patient or staff. The systems were flushed with approximately 15 L of drinking water (antibiotic-free and filtered through inline water filters from Pall GmbH (Dreieich, Germany)) directly afterwards. (Note: preliminary tests had shown that approximately 15 L of rinsing volume were necessary so that no active substances could be detected in the water phase.)

2.3.2. Stagnation samples

When selecting the patient rooms for the stagnation tests, care was taken to ensure that the patient rooms could be locked for at least 24 h (or only the bathroom in the case of bedridden patients). This was the limiting factor for the possible number of patient rooms, which were examined (status quo sample and stagnation samples) immediately following discharge or during the hospital stay of bedridden patients. Immediately after rinsing the respective siphon, a t_0 sample (= 0 h stagnation) was drawn. At regular intervals (approx. 2 h, 4 h, 6 h, 12 h and 24 h) further stagnation samples (sample t_2-t_4, etc.) were taken analogous to the t_0 sample and analyzed as described above (one replicate per sample).

2.4. High-performance liquid chromatography

All samples were diluted 1:1 with a water-acetonitrile (95:5, v/v) mixture with 0.8 g/L Na_2-EDTA. This mixture was then cleaned using a water-wettable H-PTFE filter (0.45 µm pore size) and 20 µL were injected into the LC (direct injection). An Agilent LC-System 1290 II with a Nucleoshell RP18Plus® column 2 mm × 100 mm, 2.7 µm (Macherey & Nagel, Düren, Germany) was used to separate 48 different antibiotics. The total duration time was 15 min.

2.5. Mass spectrometry

The separated antibiotics were detected by a QTRAP® 6500 + mass spectrometer from AB Sciex GmbH (Darmstadt, Germany). The analytes were ionized by an electrospray ionization (= ESI) interface in positive mode with an ionization voltage of 5500 V. Scheduled multiple reaction monitoring (sMRM) mode was used to detect the two most intensive ion transitions of each antibiotic for identification and quantification.

3. Results

3.1. Status quo samples

3.1.1. Oncology

Between 2017 and 2018, a total of 20 patient rooms (aqueous phases of 19 SH, 20 WC and 20 SI) were examined for residues of antibiotics. The time of sampling varied between 08:00 a.m. and 04:00 p.m. (see Section II.3.1).

In the 19 SHs studied, at least one antibiotic was detected in each sample (percentage of frequency: SMX 100%, TMP 95%, N4AcSMX 84%). Residue concentrations ranged from 0.02 µg·L^{-1} to 3500 µg·L^{-1}, with PIP at 3500 µg·L^{-1}, N4AcSMX at 480 µg·L^{-1} and TMP at 260 µg·L^{-1} having the highest concentrations.

At least one antibiotic was also detected in 19 of the 20 SIs investigated (percentage of frequency: SMX 95%, TMP 90%, N4AcSMX 60%). The residue concentrations ranged from 0.04 µg·L-1 to 230 µg·L^{-1}. The highest concentrations were found for PIP (230 µg·L^{-1}), CIP (50 µg·L^{-1}) and CLA (20 µg·L^{-1}).

In total, 16 of 20 WC had antibiotic residues in the range of

0.02 μg·L^{-1} to 79,000 μg·L^{-1}, including SMX (percentage of frequency: 70%), TMP (65%) and N4AcSMX (70%). However, the highest values were found for CLA (1200 μg·L^{-1}) and PIP (79,000 μg·L^{-1}).

3.1.2. Neurological rehabilitation

Five additional patient rooms were sampled according to the same scheme in neurological rehabilitation (regular ward and neuro-urology).

In all WC (5), SI (4) and SH (5) tested, at least one antibiotic could be detected in ranges from 0.05 μg·L^{-1} to 1300 μg·L^{-1}. The number of different antibiotics was a maximum of nine (in SH) and a minimum of four (SI).

CIP and VANC (80% and 60%) could be detected most frequently in the shower drains (SI: 50% CIP and 75% PIP; WC: 60% PIP). The highest concentration detected was 1300 μg·L^{-1} MERO in a WC. All results for oncology and the neurological rehabilitation are presented in Table 2.

3.1.3. Dermatology

In the sanitary area of a single patient room, antibiotic residues in a range from 0.11 μg·L^{-1} to 39 μg·L^{-1} could be detected in all three samples. The WC showed the highest sum of antibiotic residues with 75.3 μg·L^{-1} (39 μg·L^{-1} CEFTA, 33 μg·L^{-1} CLIN and 3.30 μg·L^{-1} PIP). CLIN (1.80 μg·L^{-1}), CIP (3.80 μg·L^{-1}) and dh-ERY (0.11 μg·L^{-1}) were identified in SH. The SI showed the lowest antibiotic load; however, residues of CLIN and PIP were found there (0.35 μg·L^{-1} and 0.45 μg·L^{-1}, respectively).

3.1.4. Psychosomatics, university institute (without patient care) and private households

The final antibiotics were prescribed at least two months ago in the psychosomatics studied. None of the 18 samples (6 SH, 6 WC and 6 SI) contained antibiotic residues.

Outside the clinical areas, three two-person households, one one-person household and one four-person household, as well as a theoretical university institute, were examined. In all households studied, no antibiotics had been taken for more than a year; in the university institute this was not known due to the changing use of sanitary facilities by staff, students and visitors. None of the 17 sanitary units examined (8 WC, 6 SH, 7 SI) contained antibiotic residues. Therefore, stagnation tests were not carried out in all of these areas.

3.2. Stagnation tests

3.2.1. Oncology and dermatology

It was concluded that the frequent detection of antibiotics in the examined status quo samples may be due to insufficient flushing by the patient. Therefore, after rinsing the respective system with approximately 15 L of drinking water (see Section II.3.1), the t_0 samples were first taken. No active substances could be detected in 15 of 24 t_0 samples (sum of all sanitary units). The remaining nine samples, which exhibited small traces of antibiotics even after rinsing, were distributed over six DU (max. 1.30 μg·L^{-1} CIP, status quo: 31 μg·L^{-1} and rising up to 12 μg·L^{-1}), two WC (max. 2.30 μg·L^{-1} CIP, status quo: 98 μg·L^{-1} and rising up to 7.50 μg·L^{-1}) and one SI (= 0.28 μg·L^{-1} N4AcSMX, status quo: 3.00 μg·L^{-1} and rising during stagnation up to 2.00 μg·L^{-1}).

In order to clarify whether the siphon water may be re-contaminated by active substances adhering to the pipe system, so-called stagnation samples were taken at intervals in accordance with the above sampling scheme. In seven bathrooms in oncology and one bathroom in dermatology, the stagnation process was examined over a period of about 24 h.

In these stagnation samples (samples t_2, t_4, t_6 … t_{24}), it was shown that in the siphons of WC, SH and SI a renewed increase of the antibiotic concentrations in the previously rinsed systems could often be detected without patients influencing the system during these controlled periods.

In the stagnation waters, CIP, CLIN, CLA, PIP, LIN, METRO, SMX, TMP and VANC and the two metabolites dh-ERY and N4AcSMX could be found. The residue concentrations in the stagnation samples ranged from 0.02 μg·L^{-1} (SMX; accounted for 1.4% of the status quo value determined in this sample) to 12 μg·L^{-1} (CIP; accounted for 38.7% of the status quo value determined in this sample).

Fig. 3 and Fig. 4 show similar results (regardless of the type of sanitary unit) in the form of an increasing concentration curve, which, however, seems to partly depend on the analytes and other influencing variables (e.g. cleaning state, microbial siphon coating and time of last active substance application). Thus, Fig. 4 shows a clearly deviating stagnation behavior of CIP in the SH of dermatology.

The typical concentration curve was characterized by a steep increase in concentration within the first 2–5 h, followed by a flatter increase to a maximum residue concentration (within the first 10 h) and the achievement of an "equilibrium concentration" in which the antibiotic concentration does not change noticeably in the further course of the stagnation period. This re-occurrence was unexpected as there was no "external" supply of antibiotics during the stagnation periods, and the reason for this must be comtingent on the siphons themselves. Some stagnation curves of antibiotic residues found in the sanitary units of two patient rooms (oncology room M and dermatology) are exemplarily shown in Figs. 3 and 4.

4. Discussion

4.1. Antibiotic residues in U-bends (WC, SI and SH)

To the best of our knowledge, the results obtained in this study show for the first time the presence of antibiotic residues in SH, SI and WC in very high concentrations (up to a maximum of 79,000 μg·L^{-1}). Such residues could exert a selection pressure in the sewage system of the respective patient room in favor of resistant bacteria (Baquero et al., 2008; Bengtsson-Palme and Larsson, 2016; Finley et al., 2013; Jutkina et al., 2018).

A comparison of the different clinics demonstrated that the oncology and neurological rehabilitation clinics were frequently (and in some cases very heavily) contaminated with antibiotic residues in the sanitary units. In contrast, no residues of antibiotics could be detected in the psychosomatics clinic, where no antibiotics had been administered for at least two months. Accordingly, no residues could be found in the private households examined or in the sanitary facilities of the theoretical hospital institute. These results clearly show that the presence of antibiotic residues in WC, SI and SH correlate with high consumption levels in the respective clinics.

The increased concentrations in patient wastewater are consistent with various studies from recent years, which have repeatedly demonstrated high concentrations of antibiotic residues in the total wastewater of various hospitals (Chang et al., 2010; Diwan et al., 2010; Faerber et al., 2003; Lindberg et al., 2004; Oliveira et al., 2015; Ory et al., 2016; Watkinson et al., 2009). Furthermore, a correlation between the detected antibiotic residues and the antibiotics administered (pattern and amount) could be determined (Faerber et al., 2003; Oliveira et al., 2015; Ory et al., 2016; Watkinson et al., 2009).

In spite of the large number of detections of antibiotic residues in total hospital wastewater in recent decades, it is surprising that antibiotic residues can be detected in SI, WC and SH, because these should be antibiotic-free by regular flushing of the pipe system with drinking water, which must comply with the requirements of the German Drinking Water Ordinance (TrinkwV 2001, 21. Mai 2001).

The cause of antibiotic residues in WC is more explicable relative to the findings in SH and SI, as WC are exposed to a high concentration of antibiotic residues in the urine and feces. Potential sources of antibiotic residues in SI or SH could be the brushing of teeth, showering of the body or ejection of pulmonary secretions, as residues of PIP in saliva

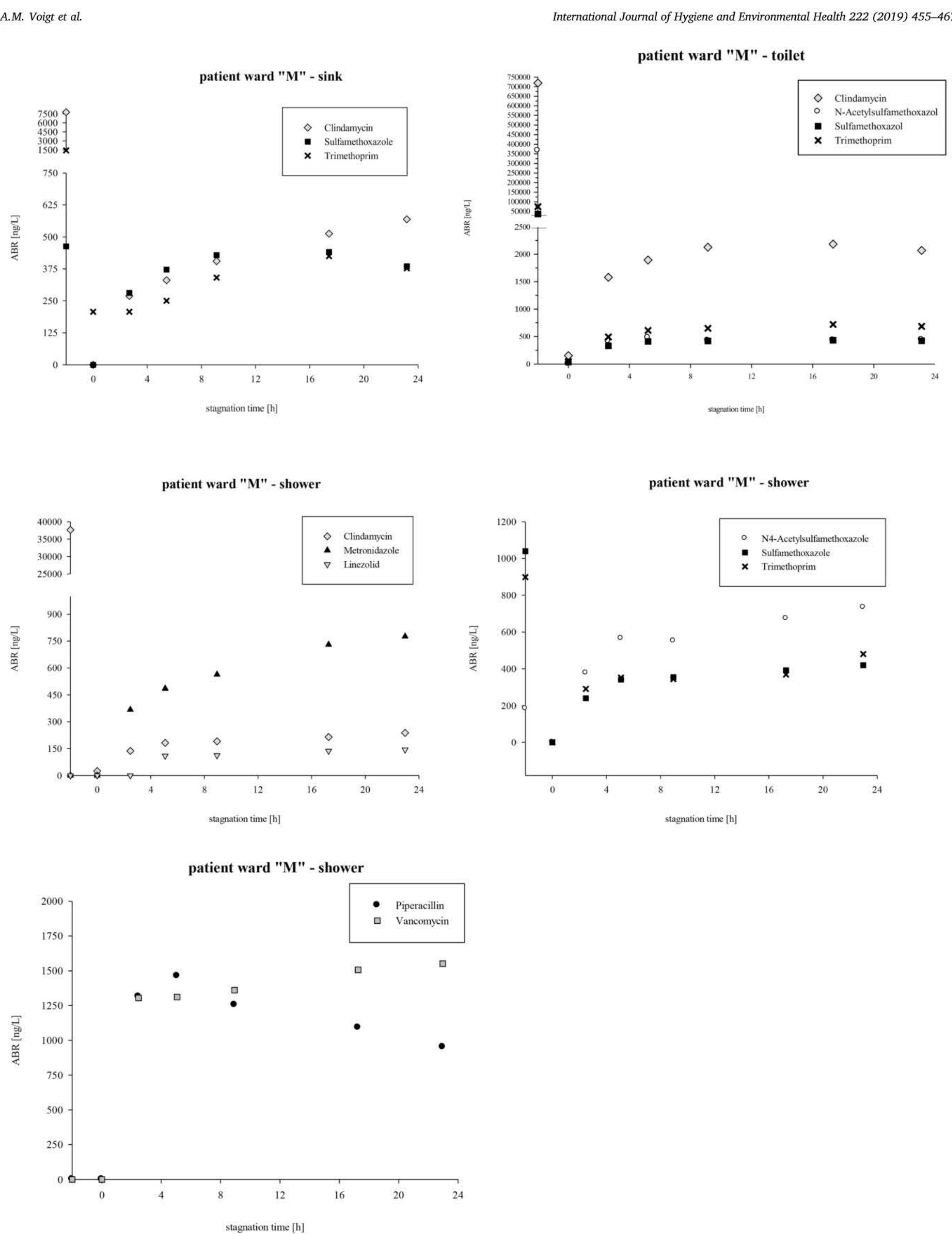

Fig. 3. Selection of typical stagnation curves for various antibiotics in different sanitation units in an oncological patient room (values on the Y-axis correspond to the status quo results for the particular antibiotic).

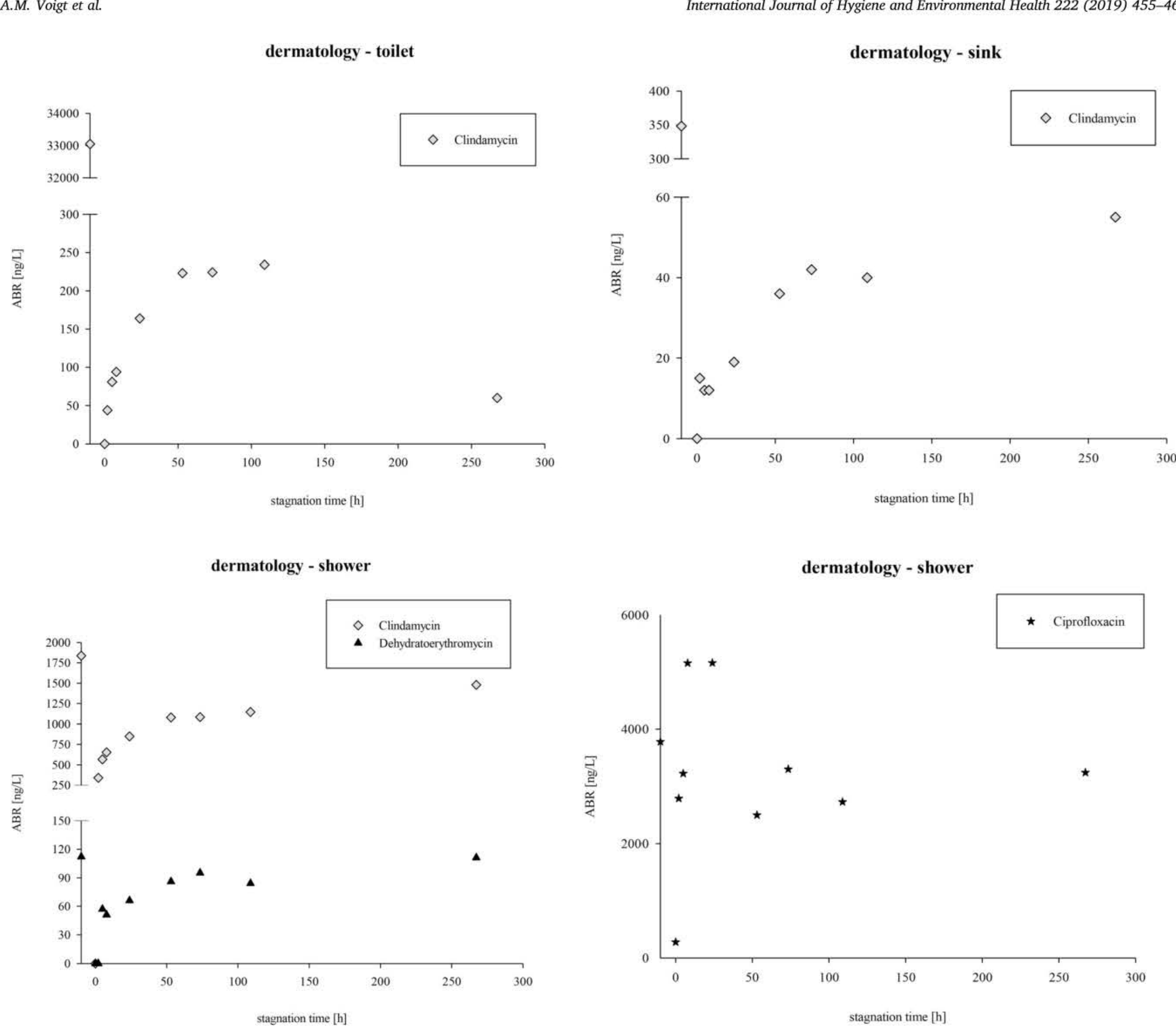

Fig. 4. Typical stagnation curves for various antibiotics in different sanitation units in a dermatological patient room (values on the Y-axis correspond to the status quo results for the particular antibiotic).

(Sagent Pharmaceuticals, 2013) or lactam antibiotics in sweat in general (Hoiby et al., 2000) have already been detected. Furthermore, improper urinating in SI and SH (based on reduced mobility) may represent another entry path. The disposal of patient wastewater into SI is also possible (Parkes and Hota, 2018).

Furthermore, this study may indicate an astonishing casuistry in dermatology. In this case, the patient was administered clindamycin (also PIP and CEFTA) immediately before sampling, which was detected in the shower. In addition, the patient defecated frequently during the inpatient stay and was therefore washed in the shower by the nursing staff, hence this path of entry of antibiotics into the SH must also be taken into account.

Even after more than 10 d of temporal stagnation (WC, SH and SI in the dermatology), significant residues of CLIN could be detected in SH (Fig. 4). This suggests that rinsing should have been conducted with a much larger amount of water. The other two antibiotics prescribed to the patient, PIP and CEFTA, were only detectable in WC and SI.

In addition to oncology, neurological rehabilitation was examined as another clinical area with high antibiotic prescription. The results of this clinic are in accordance with the findings of oncology in general, and show significant contamination of the siphon water with antibiotic residues (Table 2).

The detected antibiotic residues from oncology and dermatology are congruent with the general consumption of these clinics (Table S2) and the current room's antibiotic usage (received from the patient documentation, Table S1), respectively. A good example is the detection of PIP (20% positive samples, $0.12\,\mu g{\cdot}L^{-1}$ up to $79{,}000\,\mu g{\cdot}L^{-1}$), one of the most frequently used antibiotics in oncology (18.80 RDD·100 d^{-1}, c.f. Table S2). Furthermore, clindamycin and macrolides, LIN, carbapenems (especially MERO), PIP, narrow spectrum penicillins (especially AMPI), fluoroquinolones (especially CIP) and sulfonamides (SMX + TMP) are frequently used (RDD > 75th percentile).

In particular, the frequent evidence of SMX, TMP and CIP can be explained by the prescription practice in this clinic. As part of a general prevention of nosocomial infections, a combination of CIP and Cotrimoxazol® (TMP/SMX) is administered preventively in the first days of treatment. It is not surprising that CIP as well as SMX and TMP, which together constitute the medicine Cotrimoxazol® in a mixing ratio of 5:1, were most frequently detected in the sanitary units of oncology with 43%, 88% and 83%.

In accordance with these results, N4AcSMX (main metabolite of SMX) was found in approximately 71% of the sanitary units in

oncology. In general, 45–70% of the administered dose is excreted renally within 24 h, whereby approximately 20% is excreted as unchanged active SMX and 40% as N4AcSMX (Radke et al., 2009). This may explain the findings of SMX and N4AcSMX in WC, SH and SI via fecal entry.

However, there were also differences between the various clinics, most likely due to different prescription practices (Table 3). As a result, fewer and less common residues of SMX and TMP could generally be found. This can be explained by a lack of prevention therapy with Cotrimoxazol®. In addition, no Cotrimoxazol® was being administered in the sampled rooms at the time of sampling.

The antibiotic residues of MERO, PIP and VANC detected were consistent with the current room's antibiotic usage (antibiotic usage at the day of sampling or recently before, received from the patient documentations, Table S1). The antibiotics mentioned above are used for more serious infections (Harris et al., 2015; Isaac et al., 2017; Yang et al., 2015). This may owe to the fact that in the neurological rehabilitation of patients a treatment approach with a significantly longer inpatient stay is used, and thus a higher risk of colonization with multidrug resistant organisms (MDRO) exists (excluding other parameters like immune status, primary diseases or patient characteristics, e.g. age). Although oncological patients are more frequently treated in clinics, the length of stay is usually shorter. However, not all currently administered antibiotics could be detected in the siphons of the respective patient rooms (Table S1). This could be due to the fact that other influencing variables such as adsorption (fluoroquinolones), chelation (tetracyclines), photocatalytic degradation (e.g. fluoroquinolones, sulfonamides, tetracyclines), hydrolysis (β-lactam antibiotics) (Deshpande et al., 2004; Kümmerer, 2009) and of course the specific metabolization of the individual active substances (Kümmerer, 2009) play a role, in addition to the pure consumption figures of the examined clinics (see e.g. Table S2).

In accordance with the lack of detected β-lactams, a study from 2010 showed that in the wastewater of a clinic where fluoroquinolones (e.g. CIP) and the β-lactams ceftriaxone (126,700 mg·days^{-1}) and amoxicillin (19,150 mg·days^{-1}) accounted for the majority of prescriptions, residues of CIP could be detected in the up to three-digit μg·L^{-1} range, but no β-lactams could be detected (Diwan et al., 2010). This accords with the results of this study, as despite high β-lactam consumption rates (based on RDD·100^{-1}, c.f. Table S2), especially from MERO and AMP, lactams were only sporadically found in the sanitary units of oncology, see Table 2. This rarer detection of cephalosporins, penicillins and carbapenems (which all belong to the group of β-lactams) in oncology could be due to a reduced stability caused in the cleavage of the β-lactam ring (Deshpande et al., 2004; Kümmerer, 2009).

An exception is PIP, which was relatively common (20% in WC, 40% in SI) and partly with extremely high concentrations (up to 79,000 μg·L^{-1}). These results are plausible to the extent that PIP accounts for around 13% of the total prescriptions in oncology (based on RDD·100^{-1}, c.f. Table S2). However, this is countered by the fact that MERO, with about 14% of the antibiotic used mostly in the second line, was not found in any single oncology sample.

The comparison of the pharmacokinetics of PIP and MERO cannot explain the discrepancy between the antibiotic residues found and the prescription pattern, as the excretion pathways and rates are similar (Moon et al., 1997; Sörgel and Kinzig, 1993). On average, 50–70% of the PIP dose administered (related to the dose and other prescribed drugs) is excreted via urine and partially eliminated via feces (Sörgel and Kinzig, 1993). For comparison, MERO is excreted predominantly renally (about 72% of the dose as unchanged active substance and about 23% as inactive metabolite), as well as small amounts via feces (Moon et al., 1997).

A final explanation for this situation could not be found using the tests undertaken. However, it must always be considered that the status quo samples were random grab samples without prior knowledge of the room history. Thus, there is the possibility that the rooms examined were merely those in which MERO was not currently being used or had not been used in the recent past. Furthermore, residues of antibiotics could be detected that were not part of the antibiotics administered before the sanitary units were sampled. These results could be explained by the use of sinks or toilets by visitors or other patients who also received antibiotics. However, this does not explain the findings in SH. This provided the first indications of a kind of storage or accumulation of antibiotic residues in the control system or in the respective siphons (see Section IV.2).

For an initial interpretation of the residue concentrations found with regard to possible resistance developments, the status quo results found in this study were compared with PNEC and MIC values proposed in a recent study (Bengtsson-Palme and Larsson, 2016). The PNEC corresponds to the "predicted no effect concentrations for resistance" and the MIC to the "lowest minimal inhibitory concentration" observed for any species in the EUCAST database (Bengtsson-Palme and Larsson, 2016). It appears that in the vast majority of rooms (92%, total amount of rooms: 25), the residues of at least one antibiotic exceeded the PNEC (yellow spots) or even the MIC (red spots) (Fig. 5). Thus, by exceeding the PNEC over a long period of time, selection can be increased in favor of resistant organisms (Bengtsson-Palme and Larsson, 2016). Moreover, exceeding the MIC would mean a preference for already resistant organisms and a suppression of sensitive strains, thus providing a growth advantage for the resistant strains.

However, it should be noted that due to the high limit of quantification (LOQ) of CIP (200 ng·L^{-1}), no statements can be made regarding the influences or missing influences on resistance developments for negative samples or samples with a CIP content lower than the LOQ, since the LOQ is above the PNECs proposed by Bengtsson-Palme and Larsson (2016) of 64 ng·L^{-1}. Accordingly, for CIP only the samples with a concentration greater than the PNEC and the LOQ are highlighted in yellow, and greater than the MIC in red.

The SHs seem to have a particularly high or frequent load, as this is where most antibiotic residues were found in comparison to the three sanitary units. In addition, the sum of the SHs investigated yielded the highest number of antibiotic residues exceeding either MIC or PNEC. This may be due to insufficient flushing (too low flushing volume) of the SH after showering or alternatively to the lack of mechanical cleaning (as in toilets), as the shower drain is closed by a grid.

It is also worth mentioning that in the investigated WC (n = 20), 22 antibiotic residues exceeded the proposed MIC.

In the five rooms of neurological rehabilitation examined WC and SI seem to constitute potential hotspots, as no detected antibiotic residue in the SH exceeded the MIC. Nevertheless, the sum of SH also showed the most frequently found antibiotic residues. For a deeper comparison, however, more samples must be investigated.

Nevertheless, N4AcSMX should be considered in the evaluation of the results, even if this metabolite no longer has an antibacterial effect. Under certain conditions, a retransformation into the active starting antibiotic substance can occur (Radke et al., 2009). Therefore, occasionally extremely high concentrations of N4AcSMX, in spite of the lack of PNEC and antibacterial efficacy, should be taken into account in any risk assessment.

A further study (Sib et al., submitted) will be published soon, which will present the microbiological results of the investigated sanitary units (same status quo samples examined here) and compare the microbiological and chemical results.

4.2. *The recurrence of antibiotic residues after water stagnancy*

In addition to the general detection of antibiotic residues in patient siphons, this study has demonstrated for the first time that the residue content rises again after sufficient flushing of the system and temporal stagnation of the sanitary unit. Following adequate rinsing of the system, the antibiotic concentrations in the t_0-samples examined were

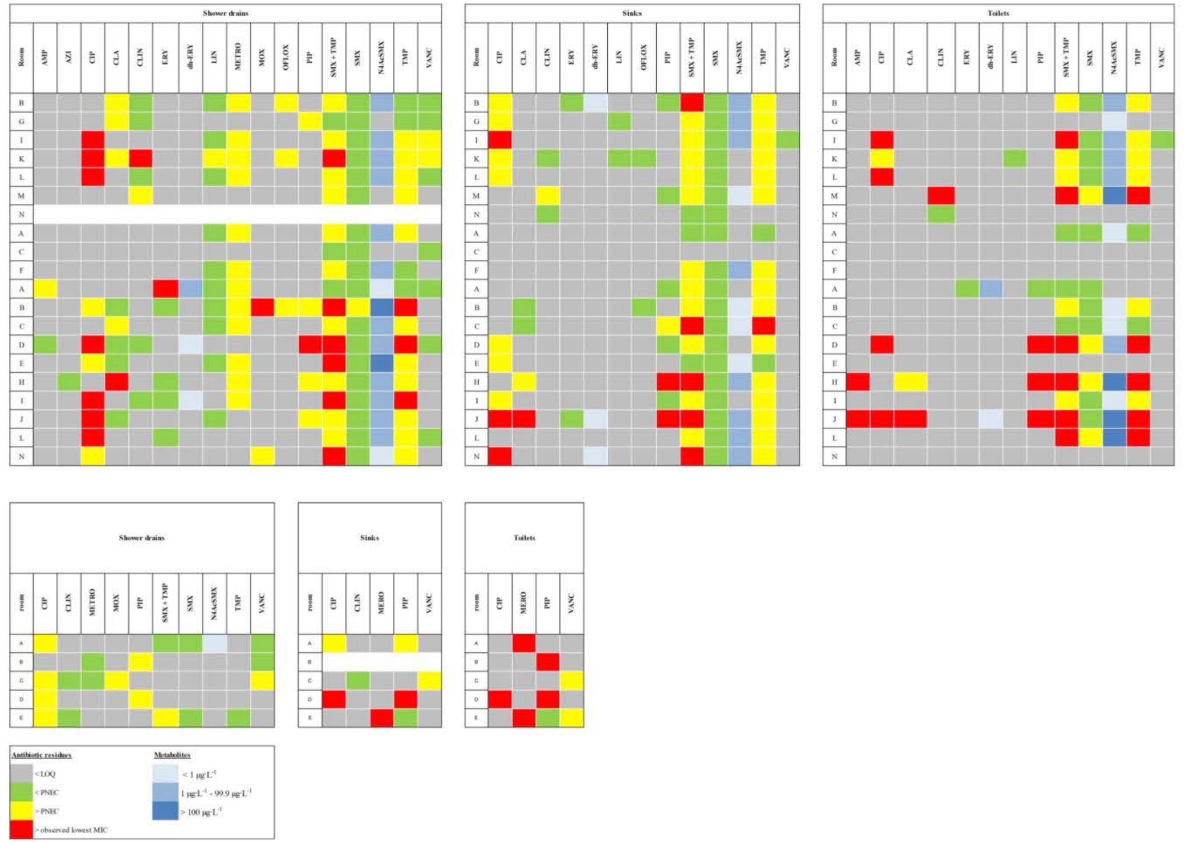

Fig. 5. Comparison of the detected antibiotic residue concentrations in several patient rooms (some rooms have multiple listings related to different sampling times) from this study with predicted PNECs and MICs (Bengtsson-Palme and Larsson, 2016) in light of a potential increase in resistance selection (first row = oncology, second row = neurological rehabilitation).

below the respective method detection limits (see Table 1).

The renewed increase in antibiotic concentrations during and after the stagnation period shows that there must be an intermediate storage of antibiotics, akin to that described for organic compounds (e.g., accumulation of nutrients or sorption of xenobiotics in biofilms) in previous publications (Flemming and Wingender, 2010), and a subsequent release of antibiotic substances into the water phase of sanitary units. However, the accumulation of antibiotic residues in the biofilm matrix of extracellular polymeric substances (Flemming and Wingender, 2010) appears to be the most plausible explanation, as the capability of antibiotics to penetrate through biofilms is already known (Anderl et al., 2000; Rodríguez-Maríñez et al., 2007; Stewart and William Costerton, 2001; Singh et al., 2010; Stewart, 2015).

Table 1
List of analyzed antibiotics (Limit of quantification (= LOQ) [$ng \cdot L^{-1}$]) sorted by their classes.

Penicillins			
AMOX (50)	AMP (200)	PEN-G (50)	CLOX (20)
DICLOX (20)	FLU (20)	METHI (10)	MEZLO (20)
NAF (20)	OXA (10)	PEN-V (20)	PIP (100)
Macrolids/lincosamides			
AZI (50)	CLA (50)	CLIN (20)	ERY (50)
dh-ERY (50)	ROX (100)	SPIR (100)	TYL (20)
Cephalosporines			
CEFA (50)	CEFO (50)	CEFT (100)	CEFU (200)
Tetracyclines			
CTC (200)	DOC (200)	OTC (200)	TC (200)
Fluorquinolones			
CIP (200)	ENR (200)	MOX (200)	OFLOX (200)
Carbapenems			
MERO (200)			
Sulfonamides/trimethoprim			
SCP (50)	SDZ (100)	SDMX (50)	SDMD (20)
SDX (50)	SEP (50)	SMZ (50)	SMX (20)
N4AcSMX (100)	SMP (10)	STZ (100)	TMP (20)
Others			
LIN (100)	VANC (100)	METRO (100)	

In addition, all systems investigated should have in common the presence of a biofilm typical for water pipes (about 95% of the biomass is located on the drain walls, and just 5% is in the water phase) (Flemming et al., 2002). Therefore, the biofilm matrix is a reservoir for antibiotic residues independent of the sanitary unit (WC, SI or SH). After flushing the system, antibiotic residues from the biofilm matrix may diffuse back into the respective waters, which could explain the noticeable increase in the concentration of antibiotics, since diffusion through biofilms has been described (Stewart, 1996, 1998).

In general there is a complex relationship between the structural properties of the individual active substances, the sorption capacity, the dose used, the period of administration, and potential biochemical reactions between antibiotics and the biofilm matrix, and therefore overall statements across substance classes are difficult to make (Stewart, 2015). Nevertheless, a comparable tendency for stagnation behavior in WC, SH and SI has been demonstrated in this study. Therefore, a recurrence of antibiotic residues during back diffusion

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Table 2

Antibiotic residues in status quo samples of WC, SH and SI of an oncology and a neurological rehabilitation unit [$\mu g{\cdot}L^{-1}$], as well as the frequency of each detected antibiotic (n [%]).

Antibiotics	Oncology								
	Toilet (n = 20)			Sink (n = 20)			Shower (n = 19)		
	min. [$\mu g{\cdot}L^{-1}$]	max. [$\mu g{\cdot}L^{-1}$]	frequency [%]	min. [$\mu g{\cdot}L^{-1}$]	max. [$\mu g{\cdot}L^{-1}$]	frequency [%]	min. [$\mu g{\cdot}L^{-1}$]	max. [$\mu g{\cdot}L^{-1}$]	frequency [%]
AMP	4.80	5.80	10	–	–	–	0.74	0.74	5
AZI	–	–	–	–	–	–	0.19	0.19	5
CIP	0.58	98	25	0.24	30	50	0.34	41	53
CLA	5.10	1200	10	0.07	20	20	0.06	73	47
CLIN	0.02	720	10	0.04	7.80	15	0.02	18	37
ERY	0.83	0.83	5	0.51	0.61	10	0.07	110	26
dh-ERY	0.05	1.03	10	0.21	0.44	15	0.08	64	16
LIN	2.30	2.30	5	0.10	3.90	10	0.10	23	58
MERO	–	–	–	–	–	–	–	–	–
METRO	–	–	–	–	–	–	0.15	7.70	63
MOXI	–	–	–	–	–	–	1.30	2.00	11
OFLOX	–	–	–	0.43	0.45	10	1.40	2.50	16
PIP	0.32	79,000	20	0.12	230	40	0.70	3500	26
SMX	0.02	36	70	0.08	6.10	95	0.05	20	100
N4AcSMX	0.27	700	70	0.38	8.30	60	0.26	480	84
TMP	0.17	110	65	0.12	19	90	0.08	260	95
VANC	0.10	0.10	5	0.13	0.13	5	0.17	26	47

Antibiotics	Neurological rehabilitation								
	Toilet (n = 5)			Sink (n = 4)			Shower (n = 5)		
	min. [$\mu g{\cdot}L^{-1}$]	max. [$\mu g{\cdot}L^{-1}$]	frequency [%]	min. [$\mu g{\cdot}L^{-1}$]	max. [$\mu g{\cdot}L^{-1}$]	frequency [%]	min. [$\mu g{\cdot}L^{-1}$]	max. [$\mu g{\cdot}L^{-1}$]	frequency [%]
AMP	–	–	–	–	–	–	–	–	–
AZI	–	–	–	–	–	–	–	–	–
CIP	50	50	20	1.40	2.40	50	0.27	0.85	80
CLA	–	–	–	–	–	0	–	–	–
CLIN	–	–	–	0.06	0.06	25	0.05	0.10	40
ERY	–	–	–	–	–	–	–	–	–
dh-ERY	–	–	–	–	–	–	–	–	–
LIN	–	–	–	–	–	–	–	–	–
MERO	94	1300	40	21	21	25	–	–	–
METRO	–	–	–	–	–	–	0.44	1.20	40
MOXI	–	–	–	–	–	–	0.51	0.51	20
OFLOX	–	–	–	–	–	–	–	–	–
PIP	0.11	370	60	0.16	120	75	1.80	3.00	40
SMX	–	–	–	–	–	–	0.06	0.07	40
N4AcSMX	–	–	–	–	–	–	0.20	0.20	20
TMP	–	–	–	–	–	–	0.45	0.45	20
VANC	10	56	40	12	12	25	0.23	100	60

Table 3
Contrast in detected antibiotics [μg·L^{-1}] (sum of all antibiotics in all sanitary units) in status quo samples of the oncology and neurological rehabilitation clinics (sorted by antibiotic classes).

Sum of all detected antibiotics [μg·L^{-1}] sorted by their class	Status quo - samples	
	Oncology (n = 59)	Neuro. Rehabilitation (n = 14)
Carbapenems	–	1515
Cephalosporins	–	–
Fluoroquinolones	328	54
Lincosamid antibiotics	826	0.20
Macrolid antibiotics	1405	–
Nitroimidazoles	19	1.64
Oxazolidinones	34	–
Penicillins	115,226	507
Glycopeptide antibiotics	57	179
Sulfonamides	3412	0.77
Tetracyclines	–	–

seems to be generally independent of the sanitary units.

In SH of room M (see Fig. 3), some antibiotics (e.g. PIP, VANC or CLIN) could be found during the stagnation, although the status quo sample was negative (related to these antibiotics). In this case, it should be noted that the status quo samples (beginning of stagnation experiments) are random grab samples and that the shower may have been flushed by patients or cleaning staff immediately before sampling. Thus, the previous residue concentration could be diluted (possibly below LOQ in the status quo sample) and diffuse back into the aqueous phase during the stagnation period. However, Fig. 3 shows that the saturation phase can take different lengths of time depending on the sanitary unit or analyte. Thus, the residue concentrations of SMX, CLIN or TMP are constant over the entire 24 h after reaching saturation. For PIP in SH of room M, however, a decrease in the residue concentration could already be observed after approximately 4–5 h, as seen in Fig. 3. In addition to the duration of the saturation phase, the different rooms and active substances also varied in terms of level of saturation concentration. These differences may be related to the time of the last application of the respective antibiotic. Depending on the length of this period, the antibiotic content could be reduced by degradation processes, rinsing processes or cleaning processes to such an extent that sufficient quantities of antibiotics can no longer diffuse back into the water (Stewart, 1998). A correlation between the status quo values and the detected residue concentrations in the saturation phase could not be determined.

Substance-specific factors should also be taken into account. The aforementioned decrease of PIP could be due to the already described high degradability of β-lactams (Deshpande et al., 2004; Kümmerer, 2009). This theory is accompanied by the fact that hydrolysis-stable antibiotics were found more frequently in the stagnation samples.

Furthermore, Fig. 4 shows that the stagnation course of CIP (SH, dermatology) did not follow the otherwise comparable rising pattern. A possible explanation for the different stagnation behavior of CIP could be the adsorption capacity of fluoroquinolones into sediments, soils and sewage sludge described in the literature (Golet et al., 2003; Kümmerer, 2003, 2009), caused in the characteristic amino and carboxylic acid group, and leading to the possibility of being positively charged as well as zwitter-ionic contingent on the pH value (at high basic pH values also being negative) (Kümmerer, 2009). Therefore a possible adsorption of CIP into the biofilm may theoretically impede both penetration into the biofilm and back diffusion from the biofilm into the aqueous phase after rinsing. Comparable results could be shown for aminoglycosides (positively charged), whose inward diffusion into a polyanionic alginate matrix at physiological pH was more retarded than the diffusion of β-Lactams (which should not be positively charged at physiological pH) (Hoyle et al., 1990). However, it must be mentioned that the penetration capacity also depends on the biofilm-forming bacteria (Stewart, 2015). In this context, the penetration of amikacin and CIP through biofilms (*Staphylococcus aureus* and *Staphylococcus epidermidis*) were not affected in comparison with the affected penetration capacity of OXA, CEFO and VANC (Singh et al., 2010). In *Pseudomonas aeruginosa*, however, it could be shown that fluorochinolones (like CIP) can penetrate readily, while aminoglycosides (such as amikacin) are retarded (Al-Fattani and Douglas, 2004).

The theory of the intermediate storage of antibiotic residues in biofilm could further explain why some antibiotics were detected that were not part of the recently applied antibiotics (c.f. Table S1). It should also be mentioned that antibiotic residues could only be detected sporadically in the samples (status quo, t_0 … t_{24}). This could be due to the following reasons:

1) An increased amount of urine, excreta, etc. in the aqueous phase of the sanitary unit due to insufficient flushing by the patient or personnel.
2) The biofilm can be mobilized during each sampling while sucking in the aqueous phase through the syringe, scratching the biofilm with the sampling utensils, unscrewing the odor traps, removing the shower outlet grids, or inserting the sampling hose through the perforated basin covers.

In spite of divergent results, a general trend could be shown in the investigated stagnation samples (eight patient rooms) by a renewed increase in antibiotic residue concentrations after previous rinsing of the system in SH, SI and WC with a comparable course.

5. Conclusions

In summary, direct patient wastewater, in addition to the total hospital effluent already identified (Chang et al., 2010; Kümmerer, 2001; Lindberg et al., 2004; Ory et al., 2016; Watkinson et al., 2009), appears to be a point source for antibiotic residues and a potential medium for resistance development. Therefore, the wastewater system of hospitals represents a neglected regulatory reservoir for antibiotic residues in the direct exposure area for vulnerable patients, particularly in areas with high antibiotic consumption rates. Biofilm in the sanitary units of patient wards may serve as a reservoir for antibiotic enrichment and subsequent back diffusion into the water medium.

However, the wastewater systems of clinics and ward areas with frequent use of antibiotics are independent risk areas for the development of antimicrobial resistance and require additional measures to reduce antibiotic resistance.

Given that the role of biofilm associated resistance mechanisms (Mah and O'Toole, 2001; Stewart, 2002; Stewart and William Costerton, 2001) and the potential risk of infections related to sanitary units (Kizny Gordon et al., 2017; Parkes and Hota, 2018) are already known, future research should focus on prevention and practice-oriented cleaning measures, i.e., mechanical removal of biofilms in running water/wastewater systems, technical solutions (e.g., thermal disinfection, chemical disinfection, sanitary equipment design and inhibition of retrograde contamination) as well as organizational solutions (rinsing, dilution, etc.) to reduce antibiotic residue concentrations and prevent further selection pressure in favor of antibiotic-resistant bacteria.

Conflicts of interest

The authors declare no conflict of interest.

This study complies with the ethical guidelines of the Declaration of Helsinki by the "World Medical Association" from 1964. The ethics committee of the Medical Faculty of the University of Bonn was involved and approved the procedures and the publication of the results (reference no. 120/16).

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Appendix A. Supplementary data

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Appendix: "The occurrence of antimicrobial substances in toilet, sink and shower drainpipes of clinical units: A neglected source of antibiotic residues"

Table S1: Comparison of the actual antibiosis (prescribed in the according patient rooms) and the detected concentrations of antibiotic residues (*status quo*-samples from T, SI and SH). Some rooms are multiple listed related for different sampling times.

hospital	room	known antibiosis (related to patient rooms)	identified residues (toilet)*,**,***	identified residues (sink)*,**,***	identified residues (shower)*,**,***
dermatology	A	AMP, sulbactam, CLIN, PIP, tazobactam, CEFT	**CEFT**, **CLIN**, **PIP**	**PIP**, **CLIN**	CIP, **CLIN**, dh-ERY
oncology	B	SMX, TMP	**N4AcSMX**, **TMP**, **SMX**	**TMP**, **N4AcSMX**, **SMX**, ERY, dh-ERY, CIP, PIP	VANC, **N4AcSMX**, LIN, OFLOX, **SMX**, METRO, **TMP**, CLA, CLIN
oncology	I	CIP, SMX, TMP, PIP, tazobactam, LIN	**CIP**, **N4AcSMX**, **TMP**, **SMX**, VANC	**CIP**, **N4AcSMX**, **TMP**, **SMX**, VANC	**CIP**, **N4AcSMX**, VANC, METRO, **TMP**, **SMX**, **LIN**
oncology	K	LIN, MERO, PIP, tazobactam, cefepime	N4AcSMX, **LIN**, TMP, CIP, SMX	**LIN**, TMP, OFLOX, CIP, CLA, SMX	VANC, **LIN**, CLIN, TMP, N4AcSMX, OFLOX, METRO, SMX, CIP, CLA, dh-ERY
oncology	L	SMX, TMP	CIP, **N4AcSMX**, **TMP**, **SMX**	**TMP**, CIP, **SMX**, **N4AcSMX**	CIP, **TMP**, **N4AcSMX**, **SMX**, VANC, LIN, METRO, CLIN, CLA
oncology	M	CLIN	**CLIN**, N4AcSMX, TMP, SMX	**CLIN**, TMP, N4AcSMX, SMX, PIP	**CLIN**, SMX, TMP, N4AcSMX
oncology	N	PIP, tazobactam, CLIN	**CLIN**	SMX, **CLIN**	n.a.
neuro. rehab.	A	MERO	**MERO**	CIP,PIP	CIP, VANC, N4AcSMX, SMX
neuro. rehab.	B	PIP, tazobactam	**PIP**	VANC, CLIN	**PIP**, VANC, METRO
neuro. rehab.	C	VANC	**VANC**	n.a.	**VANC**, METRO, CIP, MOX, CLIN
neuro. rehab.	D	PIP, tazobactam	**PIP**, CIP	**PIP**, CIP	**PIP**, CIP
neuro. rehab.	E	MERO	**MERO**, VANC, PIP	**MERO**, PIP	TMP, CIP, SMX, CLIN
oncology	A	ERY, PIP, tazobactam	**dh-ERY**, **ERY**, **PIP**, SMX	TMP, SMX, **PIP**	**ERY**, **dh-ERY**, VANC, AMP, METRO, N4AcSMX, SMX, TMP, LIN
oncology	B	none	TMP, N4AcSMX, SMX	TMP, SMX, OFLOX, N4AcSMX, CLA	N4AcSMX, TMP, SMX, OFLOX, MOX, PIP, CIP, ERY, METRO, LIN, CLA
oncology	E	none	none	CIP, NAc-SMX, SMX, TMP	NAc-SMX, SMX, TMP, METRO, LIN, CIP, CLAR
oncology	L	SMX, TMP, CIP	**N4AcSMX**, **SMX**, **TMP**	**N4AcSMX**, **TMP**, **SMX**	**CIP**, **TMP**, **N4AcSMX**, **SMX**, VANC, ERY
oncology	N	SMX, TMP, CIP	none	**CIP**, **N4AcSMX**, **TMP**, **SMX**, dh-ERY	**TMP**, **SMX**, MOX, **N4AcSMX**, **CIP**
oncology	I	SMX, TMP, CIP	**TMP**, **N4AcSMX**, **SMX**	**TMP**, **SMX**, PIP, **CIP**	**TMP**, **CIP**, **N4AcSMX**, **SMX**, METRO, ERY, dh-ERY, CLIN
oncology	C	ceftriaxone (not analyzed)	NAc-SMX, TMP, SMX	TMP, PIP, SMX, N4AcSMX, CLA	N4AcSMX, METRO, SMX, TMP, LIN, CLA
oncology	H	SMX, TMP, PIP, tazobactam, ceftriaxone (not analyzed)	**PIP**, **N4AcSMX**, **TMP**, **SMX**, AMP, CLA	**PIP**, **TMP**, **SMX**, **N4AcSMX**, CLA	CLA, **N4AcSMX**, **TMP**, **PIP**, **SMX**, METRO, AZI, ERY

* identified antibiotic residues sorted by quantity
** bold antibiotic residues are equal to prescribed antibiocs
n.a. = sampling spot was dried out (no *status quo* -sample was avaiable)

Appendix: “The occurrence of antimicrobial substances in toilet, sink and shower drainpipes of clinical units: A neglected source of antibiotic residues”

Table S2: Antibiotic consumption rate of the oncology for 2017 defined by recommended daily doses per 100 patient days (= RDD / 100 d)

Antibiotic	RDD / 100 patient days
Ciprofloxacin	20,81
Sulfamethoxazole (= Cotrimoxazole)	20,48
Trimethoprim (= Cotrimoxazole)	20,48
Piperacillin	18,80
Meropenem	15,40
Linezolid	10,93
Clarithromycin	6,40
Sultamicillin (= ampicillin + sulbactam)	5,41
Ceftriaxone	5,16
Vancomycin	4,64
Moxifloxacin	4,49
Clindamycin	2,19
Metronidazole	1,87
Levofloxacin	1,30
Ceftazidime	1,14
Colistin	0,98
Doxycyclin	0,90
Fosfomycin	0,66
Daptomycin	0,49
Tobramycin	0,42
Erythromycin	0,41
Azithromycin	0,38
Amoxicillin	0,32
Cefuroxime	0,25
Amikacin	0,23
Flucloxacillin	0,21
Penicillin V	0,14
Cefazoline	0,11
Cefpodoxime	0,09
Fidaxomicin (calc. as DDD*/100 d)	0,09
Ampicillin	0,06
Ertapenem	0,02

*DDD =defined daily doses

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Association between antibiotic residues, antibiotic resistant bacteria and antibiotic resistance genes in anthropogenic wastewater – An evaluation of clinical influences

A.M. Voigt [a, *, 1], N. Zacharias [a, 1], C. Timm [a], F. Wasser [a], E. Sib [a], D. Skutlarek [a], M. Parcina [b], R.M. Schmithausen [a], T. Schwartz [c], N. Hembach [c], A. Tiehm [d], C. Stange [d], S. Engelhart [a], G. Bierbaum [b], T. Kistemann [a], M. Exner [a], H.A. Faerber [a, 2], C. Schreiber [a, 2]

[a] *Institute for Hygiene and Public Health, University Hospital Bonn, Medical Faculty University of Bonn, Venusberg-Campus 1, Building 63, 53127, Bonn, Germany*

[b] *Institute of Immunology, Medical Microbiology and Parasitology, University Hospital Bonn, Medical Faculty University of Bonn, Venusberg-Campus 1, Building 63, 53127, Bonn, Germany*

[c] *Karlsruhe Institute of Technology (KIT), Institute of Functional Interfaces (IFG), Microbiology/Molecular Biology Department, Hermann-von-Helmholtz Platz 1, 76344, Eggenstein-Leopoldshafen, Germany*

[d] *DVGW-Technologiezentrum Wasser (TZW), Karlsruher Straße 84, 76139, Karlsruhe, Germany*

HIGHLIGHTS

- Clinically influenced wastewater differs from municipal wastewater (WW).
- Statistical associations between antibiotics and resistant bacteria are observed.
- Ciprofloxacin seems to be an indicator for the presence of ESBL-producing bacteria.
- *P. aeruginosa*, resistant against 3rd gen. cephalosporins, were mainly in clinical WW.

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ABSTRACT

The high use of antibiotics in human and veterinary medicine has led to a wide spread of antibiotics and antimicrobial resistance into the environment. In recent years, various studies have shown that antibiotic residues, resistant bacteria and resistance genes, occur in aquatic environments and that clinical wastewater seems to be a hot spot for the environmental spread of antibiotic resistance. Here a representative statistical analysis of various sampling points is presented, containing different proportions of clinically influenced wastewater. The statistical analysis contains the calculation of the odds ratios for any combination of antibiotics with resistant bacteria or resistance genes, respectively. The results were screened for an increased probability of detecting resistant bacteria, or resistance genes, with the simultaneous presence of antibiotic residues. Positive associated sets were then compared, with regards to the detected median concentration, at the investigated sampling points. All results show that the sampling points with the highest proportion of clinical wastewater always form a distinct cluster concerning resistance. The results shown in this study lead to the assumption that ciprofloxacin is a good indicator of the presence of multidrug resistant *P. aeruginosa* and extended spectrum β-lactamase (ESBL)-producing *Klebsiella* spec., *Enterobacter* spec. and *Citrobacter* spec., as it positively relates with both parameters. Furthermore, a precise relationship between carbapenemase genes and meropenem, regarding the respective sampling sites, could be obtained. These results highlight the role of clinical wastewater for the dissemination and development of multidrug resistance.

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* Corresponding author. Institute for Hygiene and Public Health, University Hospital Bonn, Venusberg-Campus 1, Building 63, D-53127, Bonn, Germany.
E-mail address: alexander.voigt@ukbonn.de (A.M. Voigt).

[1] main authorship, contributed equally.
[2] senior authorship, contributed equally.

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1. Introduction

The detection and spread of antibiotic residues (AR), antibiotic-resistant bacteria (ARB) and their resistance genes (ARG), in the aquatic environment are of interest for the general public, environmental science and political discussions.

Ninety years after the discovery of penicillin by Alexander Fleming (Fleming, 1945), a critical situation has arisen. The number of resistant bacteria has increased in recent years, while the introduction of new antibiotics into medicine has stalled, especially for infections with Gram-negative bacteria (Walsh, 2010). The WHO specified ARB as a serious threat to modern medicine (WHO, 2014) and listed specific organisms, in three priority classes, based on their virulence and resistance capabilities, e.g. carbapenem-resistant *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacterales* (priority "critical") or vancomycin-resistant *Enterococcus faecium* and methicillin-resistant *Staphylococcus aureus* (priority "high") (WHO, 2017b).

Two recent studies showed the hazard of the transmission of multidrug resistant organisms (MRO) from surface water back to humans and the potential impact on public health, the transmission of a KPC3 producing *Klebsiella pneumoniae* in Frankfurt (Germany) (Heudorf et al., 2018) and IMI2 -producing *Enterobacterales* in the south of France (Laurens et al., 2018). In contrast, another study dealing with German bathing water as a further route of exposure, did not find a critical exposure to MRO (Döhla et al., 2019). However, the aquatic environment has to be generally considered a "melting pot" for ARB, as AR and ARG are present (Baquero et al., 2008; Bengtsson-Palme and Larsson, 2016; Feuerpfeil et al., 1999; Jutkina et al., 2018; Westphal-Settele et al., 2018).

The main entry pathways of microorganisms, with ARG and trace pollutants, into the environment, are the effluents of municipal sewage treatment plants (STP) (Müller et al., 2018; Stange et al., 2019; Watkinson et al., 2009), and in the case of heavy rain fall events, the combined sewer overflow discharges (Christoffels et al., 2014; Schreiber et al., 2016), and the run-off from manured fields (Christian et al., 2003; Schmithausen et al., 2018; Schreiber et al., 2015). Antibiotics will pass treated organisms unchanged or will be excreted as metabolites or conjugates (Kümmerer, 2009).

Previous studies have detected AR in raw wastewater (WW) in the $\mu g\ L^{-1}$ range. Furthermore, in treated WW, AR concentrations ranged between the 2-digit $ng\ L^{-1}$ and the lower $\mu g\ L^{-1}$ range, as well as in surface water downstream of STP, due to an incomplete degradation of antibiotics (Kümmerer, 2009; Watkinson et al., 2009).

ARB and ARG excreted by infected, or colonized, humans or animals are only partially eliminated within the STP (Hembach et al., 2017; Müller et al., 2018) as WW treatment processes are not specifically designed for the removal of (resistant) bacteria (Schreiber, 2011). However, different types of WW (especially raw WW) should be distinguished according to their origins. Various studies have already dealt with clinical WW, consisting of ARB, AR and MRO harboring resistance genes (Koh et al., 2015; Lindberg et al., 2004; Ory et al., 2019; Picão et al., 2013; Simo Tchuinte et al., 2016; Zhang et al., 2014).

The aim of this study was to investigate the association between the occurrence of AR, ARB and ARG and whether the presence of AR might be used as a surrogate marker for the occurrence of ARB, or vice versa. To this end, the concentrations of AR and ARB, as well as ARG, were analyzed in various types of WW (clinically unaffected, and influenced), using state-of-the-art and newly developed methods. Finally, these results were related with each other in an explorative multivariate data analysis.

2. Methods and materials

2.1. Sampling sites and procedure

Between September 2016 and June 2018, various wastewater samples were compared in an urban catchment area in Germany (Case study A: "clinical-urban system") and in the rural catchment area of the river Swist in Germany (Case study B: "rural system"). The samples used for case study A were taken at a sewage disposal site of a maximum care hospital (TCWW), at a sewage disposal site, influenced by clinic/urban wastewater (CUWW), and at the two influents of the local STP (iSTP E (s) (clinically influenced) and iSTP E (n) (no clinical influence). The samples taken for case study B were from the influents and effluents of all four STPs discharging into the river Swist (iSTP A − D; eSTP A-D). Table 1 gives a review about the characteristics of the investigated sampling sites.

In a six-week cycle, 24-h automated mixed samples were taken from the sewage system and at the inlets and outlets of the sewage treatment plants (samples were stored in an automated sampler at 4 °C until collection). The samples were collected and processed in the laboratory, not more than 24 h after the finishing time (stored at 2–8 °C).

Table 1
Overview about the investigated sampling sites (abbreviation, composition, total amount of WW and the total number of inhabitants and population equivalents).

#	ID	Sampling Site	Composition	Total amount of WW	PT
Case study A					
1	CUWW	Clinical Urban WW	Mixture of clinical and urban WW	unknown	unknown
2	TCWW	Total clinical WW	Clinical WW of a maximum care hospital	27.1 m^3;/h (2016)	(>1000 beds)
3	iSTP E (s)	Influent I of STP E	Mixture of clinical and urban WW	17.5 Mio m^3/a	278,760
4	iSTP E (n)	Influent II of STP E	Urban WW, without clinical influences	17.5 Mio m^3/a	278,760
5	eSTP E	Effluent of STP E	Treated WW (mixture of clinical and urban WW)	17.5 Mio m^3/a	278,760
Case study B					
6	iSTP A	Influent of STP A	untreated municipal WW	4.5 Mio m^3/a	35,797
7	iSTP B	Influent of STP B	untreated municipal WW	2.0 Mio m^3/a	19,871
8	iSTP C	Influent of STP C	untreated municipal WW	0.5 Mio m^3/a	7705
9	iSTP D	Influent of STP D	untreated municipal WW	0.9 Mio m^3/a	10,198
10	eSTP A	Effluent of STP A	treated municipal WW	4.5 Mio m^3/a	35,797
11	eSTP B	Effluent of STP B	treated municipal WW	2.0 Mio m^3/a	19,871
12	eSTP C	Effluent of STP C	treated municipal WW	0.5 Mio m^3/a	7705
13	eSTP D	Effluent of STP D	treated municipal WW	0.9 Mio m^3/a	10,198

Legend.
STP = Sewage treatment plant.
PT = total number of inhabitants and population equivalents.
WW = Wastewater.

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2.2. Antibiotic residues

The measurement of 45 antibiotics and two metabolites, N-acetylsulfamethoxazole and anhydroerythromycin (Table S1), was performed with an Agilent 1290 Infinity™ II LC-System in combination with a QTRAP® 6500 + mass spectrometer from AB Sciex GmbH (Germany, Darmstadt). Generally, all samples were diluted (1:1) with a water-acetonitrile (95:5, v/v) mixture with 0.8 g L^{-1} Na_2-EDTA and filtered via a water-wettable H-PTFE filter (0.45 μm pore size) from Macherey and Nagel (Düren, Germany). The injection volume was 20 μl. The chromatographic separation was performed on a Nucleoshell RP18Plus® column 2 mm × 100 mm, 2.7 μm (M. & N., Düren) using a binary gradient containing a water-acetonitrile and methanol-acetonitrile solution with a total flow of 0.4 μL min^{-1}. Formic acid was used as an ion modifier to improve the ionization.

The identification and quantification were achieved by an electrospray ionization (positive mode, 5000 V) and a detection in the scheduled multiple reaction monitoring (sMRM) mode (two specific mass transitions). The complete method is published by (Voigt et al. (2019b)).

2.3. Cultivation of multi-drug-resistant bacteria

With the consideration of the Global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics (WHO, 2017b), nine different bacteria species were selected as target organisms. Microbiological parameters included Gram-negative ESBL-producing bacteria of the species *Klebsiella* spec., *Enterobacter* spec., *Citrobacter* spec. (grouped as KEC, because of morphological similarity on the used agar plates), ESBL-producing *Escherichia coli*, and *Proteus mirabilis* as well as *Pseudomonas aeruginosa* and *Acinetobacter calcoaceticus-baumannii* complex showing resistance to 3rd generation cephalosporins (3GCR), and the Gram-positive species methicillin resistant *Staphylococcus aureus* (MRSA), vancomycin resistant *Enterococcus faecium* and vancomycin resistant *Enterococcus faecalis* (VRE).

The isolation of the antibiotic resistant target bacteria was executed on CHROMagar plates (CHROMagar ESBL, CHROMagar MRSA and CHROMagar VRE; MAST Diagnostica, Germany), which are chromogenic media used for isolation and differentiation of ARB from human materials. Depending on the expected target bacteria, and background flora, 1 mL and/or a dilution of the sample was spread directly on agar plates. The plates were then incubated for 24 h at 42 °C for CHROMagar MRSA and CHROMagar ESBL and 48 h at 42 °C for CHROMagar VRE. Classification and preselection of the grown colonies was done according to Müller et al. (2018).

2.4. Identification and characterization of ARB

Final confirmation and identification of the bacterial colonies grown on the selective agar plates (chapter 2.3) was performed via matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) using the VITEK® MS mass spectrometer (bioMèrieux, Marcy l'Etoile, France) employing Myla™ software. In addition, VITEK MS-CHCA matrix (# 411,071) and disposable targets (# 410,893) were used.

Bacteria belonging to *A. calcoaceticus-baumannii* complex, *Enterobacterales* and *P. aeruginosa* were tested for antibiotic resistance towards temocillin, piperacillin, piperacillin/tazobactam, cefotaxime, ceftazidime, imipenem, meropenem, amikacin, tigecycline, chloramphenicol, fosfomycin, trimethoprim/sulfamethoxazol, ciprofloxacin, levofloxacin and colistin, utilizing the microdilution assay Micronaut-S MDR MRGN-Screening 3 system (MERLIN, Gesellschaft für mikrobiologische Diagnostika GmbH, Bornheim-Hersel, Germany). The interpretation of susceptibility status was performed according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) criteria (EUCAST, Version 9.0, 2019).

In regard to multidrug resistance, all isolates were tested for their susceptibility against different antibiotic substances. Isolates displaying resistance against three of four clinically relevant antibiotic classes, (piperacillin/tazobactam, fluoroquinolones (ciprofloxacin), third generation cephalosporins (cefotaximeand/or ceftazidime) and carbapenems (meropenem and/or imipenem), were classified as 3MRGN. All isolates that showed resistance to all of the antibiotic groups mentioned above were classified as 4MRGN. The use of piperacillin/tazobactam is a modification of the rules defined by the German Commission for Hospital Hygiene and Infection Prevention (KRINKO) which includes only piperacillin. However, it is not used as a single drug in the clinic (KRINKO, 2012; KRINKO, 2019). In case of the detection of a carbapenemase gene, the isolate was deemed 4MRGN independently of the phenotypical resistance against the tested antibiotics.

2.5. Resistance genes

2.5.1. Detection of resistance genes in the isolates

For determination of resistance genes within phenotypical carbapenem resistant isolates, via quantitative polymerase chain reaction (qPCR), three single colonies of a fresh bacterial culture were resuspended in 100 μL nuclease free water. The suspension was heated to 95 °C for 15 min and centrifuged at 14,000 g for 5 min 2 μL of the supernatant was used for the PCR reaction. The primers used for the detection via qPCR are listed in Table S2 (Annex).

2.5.2. Detection of resistance genes in the water samples

Because of a high concentration of insoluble substances, the samples were shaken and then rested for 5 min to allow the raw material to settle. The supernatant was then filtered through 47 mm polycarbonate membranes (pore size 0.2 μm, Whatman). The filtered volume was noted and later used for the calculation of the number of genes in 1 mL wastewater. The DNA extraction and purification method, for the samples of case study A, was done according to the protocol and manufacturer's instructions for the Aquadien™ kit (BioRad), including the extraction step with W2 Wash Solution. The DNA extraction and purification method, for the samples of case study B, was carried out according to the protocol and manufacturer's instructions for the FastDNA SPIN kit for soil (MP Biomedicals). The extracted DNA was stored until analysis (≤20 °C). The primer sequences used for the detection of the taxonomy and resistant genes are listed in Table S3 (Annex).

2.6. Statistical analysis

All results (positive (>limit of detection) and negative (<limit of detection)) were dichotomized and used for the first step of the statistical analysis in a fourfold table. To indicate the strength of the association between the two binary datasets, the corresponding odds ratios were calculated, which compares the odds that Y is 1 given X is 1 and Y is 1 given X is 0 (Sheskin, 2003). In this case the events of a positive finding of the analyzed bacteria were compared with the positive detection of an analyzed antibiotic in the wastewater. Then the odds ratio was given as the number of positive samples (ARB) in the group of positive antibiotic findings divided by the number of positive ARB in the group of negative antibiotic findings. If the odds ratio is larger than 1, the odds of finding a resistant bacterium in the group of positive antibiotic samples is higher than in the group of negative antibiotic samples. To prove

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that the calculated odds ratio, between two variables, was significant, a p-value was computed. If the p-value was lower than 0.05, the odds ratio was declared significant (Sheskin, 2003).

After calculation of the odds ratio for every combination of AR and ARB, significant combinations for a cluster analysis were selected. Because of the large number of colonies on the agar plates, it was not possible to determine resistance profiles for all the colonies present, so this was only done for selected isolates. In order to estimate the resistance of the population, at this sample site, against a given antibiotic (e. g. ciprofloxacin), the number of colony forming units on the agar plates was multiplied with a resistance factor (RF), as the percentage of colonies resistant to the respective AR, thus estimating the number of resistant colonies. This was done for each sampling site, each antibiotic and each bacterial species, individually. The number of colony forming units, for the respective MRGN status, was evaluated by calculating the same factor for the number of isolates with a respective MRGN status, in comparison to all isolates of the respective bacteria species, at one sampling site. To enable a direct comparison between the numeric differences of the two data sets, a z-transformation was conducted. In this case, the sampling points were compared to each other in regard to the detected median concentration of AR and number of ARB. An agglomerative hierarchical clustering analysis, with the Ward method, was used, starting with the assumption that all sampling points constitute their own cluster, then connecting the two that are most similar and proceeding until all sampling points were included. The cluster analysis is a descriptive method; therefore, it is subjective as to how many clusters are useful. To help with the interpretation a dendrogram was computed to show at what distances the clusters were generated.

3. Results and discussion

3.1. Occurrence of AR in WW

From September 2016 to June 2018, a total number of 206 WW samples (from 13 different sampling sites) were analyzed for AR, ARB and ARG, for this study.

Residues of at least one antimicrobial substance could be detected in all WW samples. Overall, the most frequently detected substance classes were macrolide antibiotics (e.g. clarithromycin, azithromycin and erythromycin), sulfonamides (sulfamethoxazole and its synergist trimethoprim) and fluoroquinolones (e.g. ciprofloxacin, ofloxacin and moxifloxacin).

The ubiquitous detection of sulfamethoxazole (and trimethoprim), with detection frequencies between 93% (88%) and 100% (100%), and residue concentrations up to 30.9 $\mu g\,L^{-1}$ (16.6 $\mu g\,L^{-1}$) for untreated WW, and 94% (50%) and 98% (77%) for treated WW with concentrations up to 1.1 $\mu g\,L^{-1}$ (0.4 $\mu g\,l^{-1}$), can be explained by the high inpatient and outpatient prescription rate in the therapy of, *inter alia*, respiratory diseases and urinary tract infections (BVL and Paul-Ehrlich-Gesellschaft für Chemotherapie e.V., 2016; Schwabe and Paffrath, 2016). Also, the frequent detection in WW effluents is in line with former studies (Kümmerer, 2009) and can be explained by the incomplete degradation during WW treatment processes (Radke et al., 2009). Comparable results could be obtained for macrolides, most notably clarithromycin and erythromycin, as well as its metabolite anhydroerythromycin and clindamycin.

The most commonly detected fluoroquinolone was ciprofloxacin, followed by ofloxacin and moxifloxacin. Residue concentrations of ciprofloxacin ranged between 0.2 $\mu g\,L^{-1}$ and 88.3 $\mu g\,L^{-1}$, in clinically influenced WW, and from 0.4 $\mu g\,L^{-1}$ up to 16.6 $\mu g\,L^{-1}$ in municipal WW, respectively. Residues of clinically relevant antibiotics, such as carbapenems or glycopeptides, could be detected in TCWW up to eSTP E. The highest concentration of, for example, meropenem (vancomycin; piperacillin) could be detected in TCWW with maximum values of 197 $\mu g\,L^{-1}$ (160 $\mu g\,L^{-1}$; 4000 $\mu g\,L^{-1}$). The obtained residue concentrations decreased within the WW pathway. Thus, meropenem (vancomycin; piperacillin) residues of about 0.2 $\mu g\,L^{-1}$ (0.7 $\mu g\,L^{-1}$; 1.1 $\mu g\,L^{-1}$) could be detected in the eSTP E. These findings can be explained by the predominantly parenteral application during the therapy of serious nosocomial infections caused by Gram-positive (e.g. vancomycin) and Gram-negative (e.g. meropenem) bacteria. The fast and strong decrease of these relevant antibiotics is caused by a dilution with uncontaminated WW and possible degradation processes. Accordingly, the reduction of β-lactams, such as meropenem, ceftazidime or piperacillin, could be explained by their instability against hydrolytic cleavage of the penam or lactam ring (Deshpande et al., 2004).

Residues of at least one tetracycline (doxycycline and tetracycline) could only be detected in 9.2% of the examined samples (19 of 206 samples). This low detection frequency could be explained by the formation of chelate complexes, with calcium or magnesium ions and the sorption to organic matter, whereby "bound" tetracyclines are analytically camouflaged and could not be detected in the aqueous phase (Christian et al., 2003; Kümmerer, 2009; Lindsey et al., 2001). Furthermore, tetracycline could only be detected in untreated WW. Another explanation is the lack of intensive livestock farming and slaughterhouses in the investigated catchment area, as tetracyclines are largely used in veterinary medicine, especially chlortetracycline and doxycycline (BVL and Paul-Ehrlich-Gesellschaft für Chemotherapie e.V., 2016; Wallmann et al., 2017). In addition, no classical veterinary antibiotics, such as enrofloxacin, spiramycin, chlortetracycline or tylosin, were detected in the sampling period. Thus, these results confirm that the catchment area can be regarded as predominantly influenced by human medical healthcare facilities. Median concentrations of the further examined AR are given in Fig. 1.

3.2. The detection of ARB in WW

Resistant bacteria were found in all WW samples (including STP effluents) in different abundances. ESBL *E. coli* could be found in 85.7% of all WW samples, taken at case study A, and in 96.4% of the WW samples of case study B. ESBL KECs (*Klebsiella* spec., *Enterobacter* spec., *Citrobacter* spec.) could be found in 88.2% and 91.4% of the WW samples in case study B, and case study A, respectively. *P. aeruginosa* 3GCR were primarily found in the clinical WW, that is, in 56–68% of the samples taken at the sampling points TCWW and CUWW, and only in up to 12% of the samples taken at the influent of the local treatment plant (iSTP E (s)). In case study B, *P. aeruginosa* 3GCR could only be detected in 11.8% of all WW samples. *Acinetobacter calcoaceticus-baumannii* complex 3GCR could be detected in 81.8% of the WW samples of case study A and in 85.3% of the WW samples of case study B. If positive, the overall concentrations varied between 3 and 5 log10 cfu/100 mL (median) in the raw WW of both case studies, to 1–2 log10 cfu/100 mL (median) in the samples of treated WW (Schreiber et al., 2019). Median concentrations of the further examined bacterial species are given in Fig. 2.

The KEC group was generated due to the similar morphology on the used agar plates. The overall results of positive detection of the KEC group in the WW samples show a relatively even distribution of the target organisms within this group, with 37.6% *Klebsiella* spec., 31.0% *Enterobacter* spec. and 31.4% *Citrobacter* spec in raw WW of case study A. a distribution of 38.1%/36.0%/25.8% in case study B.

The distribution in treated WW shifted to a slightly higher amount of *Klebsiella* spp. with 60.0%/24.0%/16.0%, in case study A, and 40.2%/35.2%/24.6% in case study B. All STP included in this

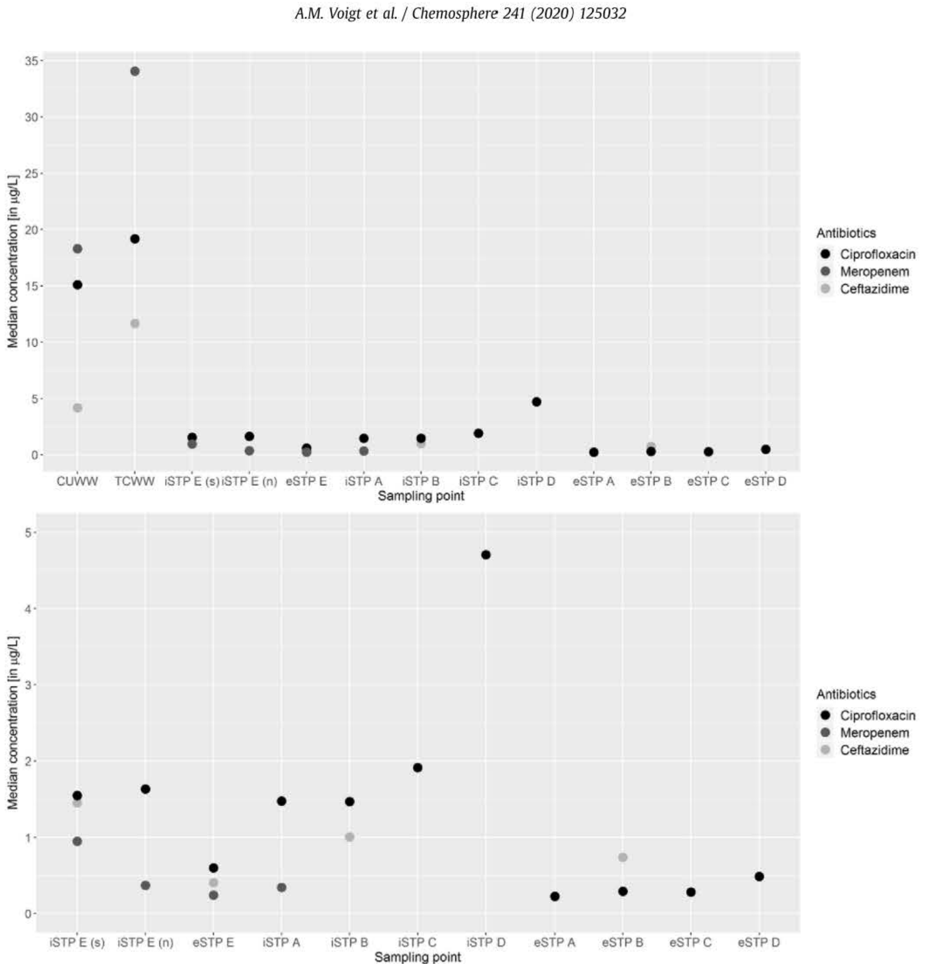

Fig. 1. Overview about the quantitative results (median, µg/L) of the AR used for statistical analysis, sorted by sampling sites (top = all sampling sites; bottom = without CUWW and TCWW).

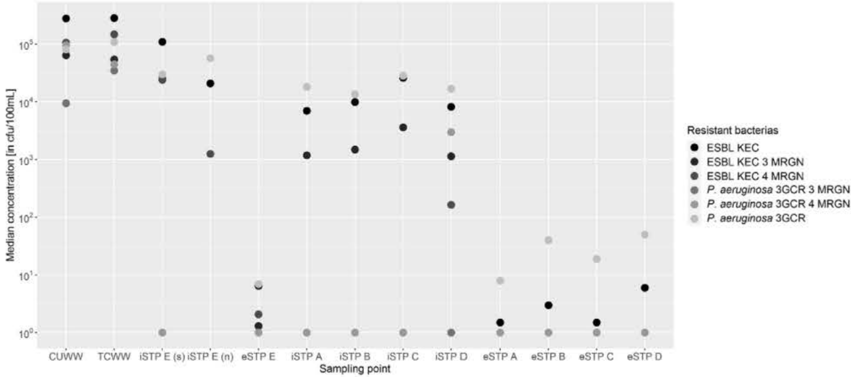

Fig. 2. Overview about the quantitative results (median, cfu/100 mL) of the ARB used for statistical analysis, sorted by sampling sites.

study use a mechanical and one or two biological sewage steps, thus the treatment process could possibly be neglected. Reasons for the differences, such as capsule formation by *Klebsiella* spp. (Amako et al., 1988) or additional influences of clinical WW need to be further evaluated and will be investigated in future studies. This study will further only refer to the KEC group without the species differentiation.

Whereas the results show that ARB could be isolated from all

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sampling sites of both case studies, the degree of multidrug resistance varied. Clinical WW was charged with a high proportion of MRO, and contained a few strains that were only susceptible to one or two antibiotic classes (extensively drug resistant (XDR) bacteria (Magiorakos et al., 2012)). In the same sampling period, case study B yielded eight carbapenemase producers and no XDR strains. Although most of these bacteria were eliminated during WW treatment processes, dissemination into surface waters is possible as single carbapenemase producers were still present in the effluent of the STP (Müller et al., 2018).

The results of this study raise the question if there is a statistical relationship, between the presence of AR and ARB, and if this relationship is influenced by the sampling site.

3.2.1. *Associations between the presence of ARB and AR*

The results of all investigated WW samples, from 13 sampling sites, were analyzed for possible associations between the presence of ARB and AR.

Altogether, 423 possible combinations were obtained for the comparison of 9 analyzed bacteria (chapter 2.3), and 47 AR, in all samples investigated. Using a 2×2 contingency table, a total of 49 significant odds ratios ($p < 0.05$) could be obtained. Subsequently, all results were selected with an increased probability of detecting ARB with simultaneous positive detection of AR ($OR > 1$). Thus, a number of 26 (= 6.1%) combinations for a significant relationship could be found.

Overall, the spectrum of resistant bacteria studied included classical fecal indicators (e.g. *E. coli* or enterococci), as well as other facultative pathogenic bacteria (like *Acinetobacter calcoaceticus-baumannii* cplx or *P. aeruginosa*). To investigate the influence of clinical WW on mixed urban WW, only Gram-negative bacteria, generally associated with forming of, or reproducing within, biofilms were considered. These bacteria may be able to reproduce themselves within the WW stream, while the number of intestinal bacteria (such as *E. coli* or intestinal enterococci) should decrease, when not further introduced. The final results of all 14 (= 3.3%) combinations are given in Table 2. The calculated minimal expected frequency, for the combination *P. aeruginosa* 3GCR/flucloxacillin, is 1.27 and therefore statistically not significant and thus, is excluded from the discussion. The minimal expected frequency of the other shown combinations is between 6 and 8.

In this context, "clinical relevance" was defined on the basis of the clinical efficacy and the clinical daily routine and prescription praxis. The combination of ESBL, KEC and N-acetylsulfamethoxazole is deemed to be not clinically relevant, since, although representatives of the KEC group are mostly sensitive to trimethoprim-sulfamethoxazole, these antibiotics are rarely used in the treatment of infections caused by bacteria from the KEC group.

3.3. *Association between ARB and AR at different sampling sites*

3.3.1. *Clinically relevant cases*

Only four (out of 423) relationships between ARB and AR could be obtained as clinically relevant (see Table 2). As predominant species, in three of four associations, *P. aeruginosa* 3GCR was observed. Furthermore, one clinically relevant relationship could be found for the KEC group.

The positive association between residual concentrations of meropenem, ceftazidime and ciprofloxacin, with *P. aeruginosa* 3GCR, seems to be clinically relevant. *P. aeruginosa* can lead to pneumonia, urinary tract infections or sepsis (Lister et al., 2009). Interestingly, the antibiotics detected here are possible options for an antibiotic therapy treating *P. aeruginosa* infections (Mutschler, 2012).

Thus, the simultaneous detection of meropenem, ceftazidime and ciprofloxacin, with *P. aeruginosa* 3GCR, is clinically relevant. These findings are substantiated by the calculated odds-ratios. So, in all the statistically evaluated samples ($N = 186$), the probability of finding *P. aeruginosa* 3GCR isolates was increased by a factor of 2.8 if ciprofloxacin residues (5.0 for meropenem and 3.2 for ceftazidime) were detected at the same time.

The obtained relationships for ceftazidime and meropenem, which are predominantly used parenterally, seem to be more associated with clinical applications (in-patient). In contrast, ciprofloxacin can be used parenterally, as well as orally, which allows for an out-patient application.

Relating to the four clinically relevant cases, Fig. 3 shows the obtained dendrograms and the scatter plots, based on the z-transformed data, in relation to the sampling sites for the concentration of AR (x-axis) and the related ARB, which show resistance against the corresponding AR (y-axis). This multivariate data analysis provides a distance between sampling points and allows clustering, based on distance, or result-based similarity of sampling points.

The highest concentrations, for either parameter, can be detected at the sampling points with the greatest proximity to clinical WW (TCWW and CUWW), which can be clearly differentiated from non-clinically influenced WW like STP A – STP D. Taking a look at the dendrograms, two additional clusters can be identified, one for raw WW and one for treated WW. Furthermore, Fig. 3 shows that for the mixed WW (clinical/urban) in iSTP E(s), a higher distance to the other STPs can be observed, with respect to ceftazidime (typical

Table 2
Positively associated parameters after the calculation of the odds ratios. Those highlighted in bold font are combinations with a clinical relevance*.

Resistant bacteria	Antibiotic	N	Odd-ratios	p-value	Substance classes	Biofilm associated	Clinical relevance
P. aeruginosa 3GCR	Vancomycin	202	2.53	0.008	Glycopeptide antibiotic	yes	no
***P. aeruginosa* 3GCR**	**Ciprofloxacin**	**186**	**2.75**	**0.034**	**Fluoroquinolone**	**yes**	**yes**
P. aeruginosa 3GCR	Metronidazole	178	3.17	0.003	Nitroimidazole	yes	no
***P. aeruginosa* 3GCR**	**Ceftazidime**	202	3.20	0.002	Cephalosporin	yes	**yes**
Pseudomonas spec. 3GCR	Ampicillin	202	3.35	0.037	Penicillin	yes	no
ESBL KEC	Erythromycin	178	3.62	0.020	Macrolide antibiotic	yes	no
P. aeruginosa 3GCR	Moxifloxacin	178	3.91	0.001	Fluoroquinolone	yes	no
ESBL KEC	**Ciprofloxacin**	**186**	**4.29**	**0.003**	**Fluoroquinolone**	**yes**	**yes**
ESBL KEC	Amoxicillin	202	4.67	0.041	Penicillin	yes	no
***P. aeruginosa* 3GCR**	**Meropenem**	**202**	**5.00**	**0.000**	**Carbapenem**	**yes**	**yes**
P. aeruginosa 3GCR	Linezolid	178	5.05	0.000	Oxazolidinone	yes	no
P. aeruginosa 3GCR	Ampicillin	202	9.41	0.000	Penicillin	yes	no
ESBL KEC	N-Acetyl-SMX**	152	12.75	0.001	Sulfonamide	yes	no
P. aeruginosa 3GCR	Flucloxacillin	202	20.79	0.006	Penicillin	yes	no

*defined on the basis of the clinical efficacy and the clinical daily routine and prescription praxis.
**SMX = sulfamethoxazole.

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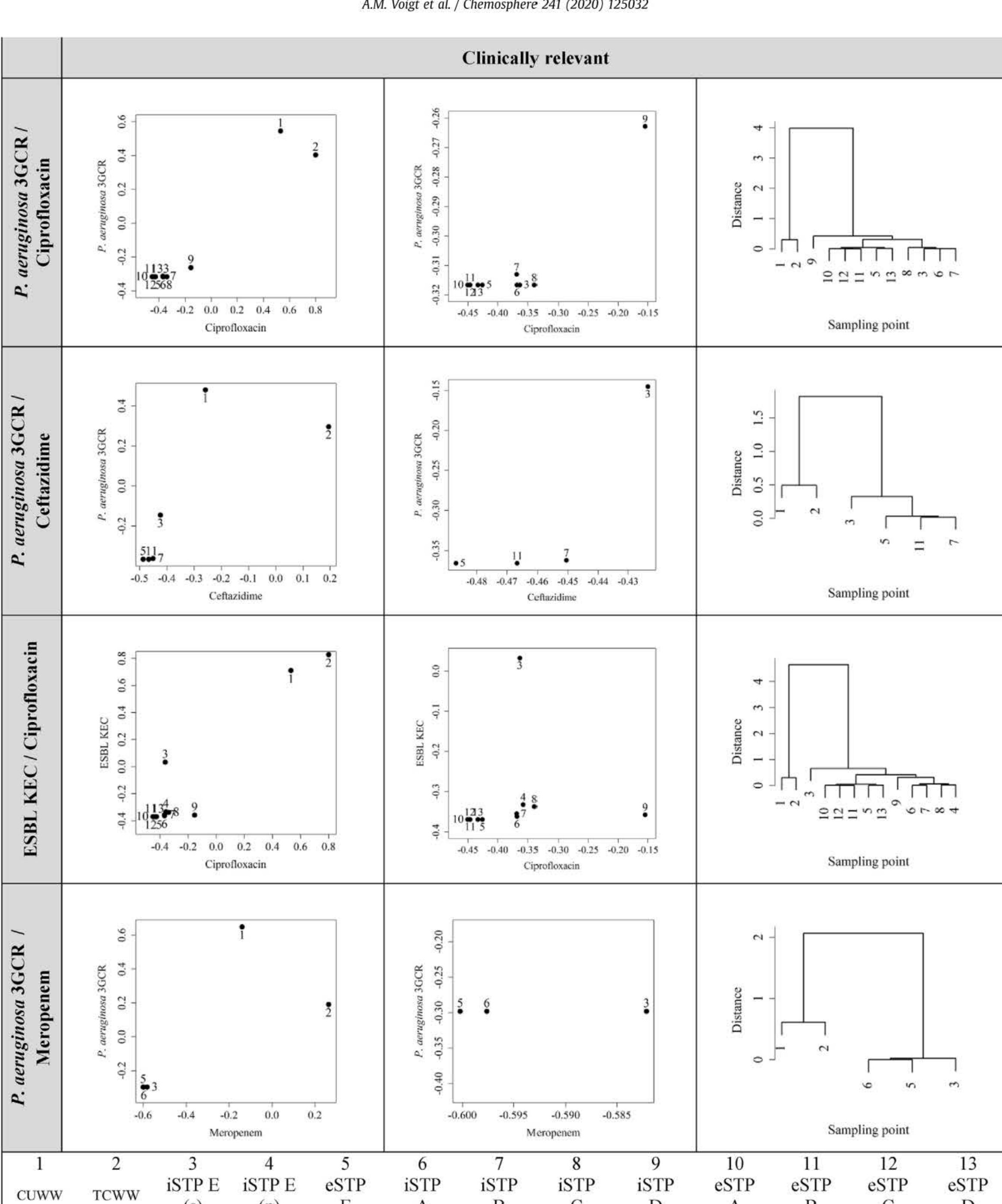

Fig. 3. Scatterplots and dendrograms of the positively related parameters with clinical relevance*. From left to right: Scatterplot containing all samples, Scatterplot without the clinic samples (TCWW and CUWW), dendrogram containing all samples.

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clinical antibiotics), in association to ceftazidime resistant *P. aeruginosa* 3GCR (RF 0.5) and ciprofloxacin in combination with ciprofloxacin resistant ESBL KEC (RF 0.75).

The scatter plots show that *P. aeruginosa* 3GCR, with resistance against the corresponding AR, is predominantly detected in the sampling points nearest to the clinic (TCWW and CUWW), with 56–68% of the samples being positive.

Interestingly, a recent study described the occurrence and spread of ST823 *P. aeruginosa* within the sanitary units in the hospital investigated in this study (Sib et al., 2019). Another study described the finding of another *P. aeruginosa* clone (ST235), which could be detected in the connected WW system (Müller et al., 2018). In this context, the high amount of ciprofloxacin, and the occurrence of *P. aeruginosa*, may lead to a further dissemination and increased selection pressure in favor of resistance development, like that described for ST235 *P. aeruginosa* (Treepong et al., 2018).

The analysis of the z-transformed data, for ciprofloxacin resistant *P. aeruginosa* 3GCR and ESBL KEC with ciprofloxacin residues, show a distinct cluster formation for the influents (8, 3, 6, 7), and effluents, of the STPs (10, 12, 11, 5, 13), which is related to the high amount of fluoroquinolone residues removed by the separation of sewage sludge, where they can be “absorbed” (Golet et al., 2002; Kümmerer, 2003; Lindberg et al., 2005). This is in line with previous publications which found less fluoroquinolone residue in STP effluent than in the respective STP influent (Golet et al., 2002, 2003; Kümmerer, 2003; Lindberg et al., 2005).

Meropenem is considered as one “last resort” antibiotic for the treatment of serious infections with Gram-negative bacteria (e.g. sepsis) (Harris et al., 2015). The prescription praxis mirrors the obtained clusters and it is possible to differentiate between TCWW and CUWW related to its meropenem content. Meropenem could not be found at all sampling sites, which leads to the display of only five of the original 13 sampling sites in Fig. 3.

This trend of a significant influence of clinical WW may be explained by the high number of prescriptions of these antibiotics, in general, and in relation to the clinics investigated here (e.g. the maximum care hospital, which is related to TCWW and CUWW). In addition, fluoroquinolones are generally the fourth most prescribed class of antibiotics, behind β-lactams, macrolides and tetracyclines, based on prescription volume in Germany (Schwabe and Paffrath, 2016). Furthermore, the total prescription volume of ciprofloxacin, in 2015, was about 9.5 million DDD (defined daily doses) in Germany (Schwabe and Paffrath, 2016), while ciprofloxacin is generally within the top 3 of the most prescribed antibiotics in German hospitals (8.2% of the total prescribed antibiotics, in RDD (recommended daily doses)) (BVL and Paul-Ehrlich-Gesellschaft für Chemotherapie e.V., 2016). The investigated maximum care hospital also used significant amounts of ciprofloxacin, as well as meropenem and ceftazidime (Voigt et al., 2019a). Interestingly, meropenem is characterized as an antibiotic with highest importance for human health (WHO, 2017a) and the prescription volume of carbapenems significantly increased, in Germany, from 76 up to 216 DDD/1000 patient days (Meyer et al., 2013).

3.3.2. Clinically not relevant cases

Of the 14 selected combinations of ARB and AR (see Table 2), one was excluded because of a calculated minimal frequency of <5, and nine were deemed clinically not relevant, because the specific therapeutic agent is not usually associated with the treatment of the corresponding ARB. All these AR could be found in different concentrations in shower, toilet and sink drains of the investigated maximum care hospital (Voigt et al., 2019a).

Vancomycin is an antimicrobial agent only effective against Gram-positive bacteria. The substance could be found in the patient sanitary rooms of the investigated hospital in concentrations ranging from 0.10 $\mu g\,L^{-1}$ up to 26 $\mu g\,L^{-1}$ (Voigt et al., 2019a). The association between the occurrence of *P. aeruginosa* 3GCR and linezolid is not clinically relevant because linezolid is used as a reserve antibiotic (last line of defense) against highly resistant Gram-positive microorganisms (e.g. vancomycin resistant enterococci) (Stevens et al., 2002).

Antibiotics of the penicillin group could be related with *Pseudomonas* spec. 3GCR, *P. aeruginosa* 3GCR and the ESBL KEC group (*Pseudomonas* spec. 3GCR/Ampicillin; *P. aeruginosa* 3GCR/Ampicillin; ESBL KEC/Amoxicillin). In general, ampicillin and amoxicillin are broad-spectrum antibiotics, which are mainly used for treatment of infections with Gram-positive bacteria, with exception of some infections with Gram-negative bacteria, but are not used against infections with *Pseudomonas* spec. Metronidazole is mainly used for treating infections with anaerobic bacteria like *Clostridium difficile* and protozoa (Zar et al., 2007). The activity spectrum of erythromycin is comparable to that of some penicillins, resulting in similar fields of application, mainly against Gram-positive bacteria. Moxifloxacin was also positively related with *P. aeruginosa* 3GCR, but it is not effective against *P. aeruginosa* infections.

The scatterplots, with the z-transformed data of the two data sets, shows, as well as the clinically relevant plots shown in Fig. 3, a very similar arrangement of the clusters, predominated by clinical WW, followed by the southern influent of STP E.

3.4. The association between ciprofloxacin residues and multidrug resistance

The treatment of MRO is more complicated and complex than the treatment of ARB, caused by the increasing limitation of therapy options due to lack of efficient antibiotic substances. Based on the results of section 3.4, ciprofloxacin is one of the most noticeable antibiotics related to the definition of MRO (3/4MRGN) by the German KRINKO (2012, 2019). The following section investigates possible relationships between the parameter's multidrug resistance and ciprofloxacin residues.

The results (Fig. S1) show that the sampling points, in direct association with clinical WW (1–3), are clearly separated from the sampling sites without clinical WW. Furthermore, it can be noted that sampling sites 1 and 2 are in contrast to each other when looking at the resistance status (3/4MRGN) of the respective bacterial species. The total clinical associated WW (TCWW) has a higher abundance of *P. aeruginosa* 3GCR with a 3MRGN status than a 4MRGN status, whereas, the abundance of 4MRGN ESBL producing KEC is higher at TCWW than CUWW. Additionally, multidrug resistant KEC can be detected, even up to the influent of the urban STP. The numbers of MRO at case study B are much lower, but even the rural sampling sites (eSTP A, iSTP D and eSTP C) show ESBL KEC with a 3MRGN status.

The dispersion of the dots on the x-axis can be described analog to the scatterplots in Fig. 3 showing ciprofloxacin, because it shows the same data. Ciprofloxacin shows a positive association with the presence of both described bacteria species. At the sampling sites with a high concentration of the AR, a high abundance of MRO could be detected.

3.5. Association between the presence of ARG and AR

Next to the abundance of ARB, the presence of ARG is an important factor for the evaluation of the emerging health risk of the dissemination of antibiotic resistance within the environment. The overall results of the association between AR and ARG are shown in Table S4.

Analogous to the analysis described in 2.6, the odds ratios for AR and ARG were performed. For the odds ratios between ARG and AR,

three clinically relevant carbapenemase genes (bla_{NDM}, bla_{VIM2} and bla_{OXA48}), and four extended spectrum β-lactamase (ESBL) genes (bla_{CTX-M}, $bla_{CTX-M32}$, bla_{CMY2} and bla_{TEM}) were chosen (n = 329). Analogous to section 3.3, odds ratios with $p > 0.05$ and an OR < 1 were excluded. This led to a new total number of 60 combinations, which corresponds to 18.2% of the initial number.

Generally, the obtained odds ratios ranged between 2.30 (ofloxacin/bla_{TEM}) and 131.54 (linezolid/bla_{VIM2}). Comparatively high values were found for odds ratios between antibiotics, like linezolid, vancomycin and ampicillin, with bla_{OXA48}, bla_{CTX-M} and bla_{VIM2}. Interestingly, a positive detection of residual concentrations of carbapenems (meropenem) correlates, *inter alia*, with the corresponding carbapenemases (bla_{NDM}, bla_{VIM2} and bla_{OXA48}). In addition, an increased detection probability can be observed for all selected ARG (odds ratios between 2.30 and 10.01, see Table S4) with positive detection of fluoroquinolone residues (ciprofloxacin, ofloxacin and moxifloxacin). Furthermore, there is a particularly high probability of finding bla_{VIM2}, bla_{CTX-M} and bla_{OXA48} simultaneously with ceftazidime, whereas no relation was found for the other β-lactamase genes (bla_{TEM} and $bla_{CTX-M-32}$). In addition, integrons, and plasmids, harbouring β-lactamase genes often contain several ARG and therefore β-lactamase genes may be selected by other antibiotics.

3.6. Relationship between ARG and AR at different sampling sites

Related to the high importance of carbapenemase genes, the KRINKO (2019) considered the sole presence of them as a criterion for the classification of the isolates as 4MRGN. As a consequence of finding that MRO are predominant at the clinical WW sampling sites, the relationships between carbapenemase genes and carbapenem residues need to be investigated for the different sampling sites. Furthermore, ceftazidime was chosen, for comparison with bla_{CTX-M}, because this third-generation cephalosporin was the only agent of this substance class which could be associated with *P. aeruginosa* 3GCR, in section 3.3, and showed a significant relationship with clinical WW 3.4.

Whereas sections 3.1–3.6 represent data for ARB and the genes investigated within the cultivated bacteria, this section deals with the ARG detected in the sampled water, regardless of their specific bacterial origin (pathogen or not).

The selection of the following investigated ARG are based on the results of section 3.6. The gene bla_{CTX-M} represents the most distributed form of ESBL and the carbapenemase genes, bla_{NDM}, bla_{VIM2} and bla_{OXA48}, were commonly detected in the area investigated in this study, as well as worldwide (Bonnet, 2004; Johnson and Woodford, 2013; Müller et al., 2018; Queenan and Bush, 2007; Walsh, 2010). Median concentrations of the further examined ARG are given in Fig. 4.

Fig. 5 shows the relationship between meropenem and bla_{NDM}, bla_{VIM2} and bla_{OXA48}, as well as between ceftazidime and bla_{CTX-M}. All scatterplots show a comparable tendency, as noticeable differences, between the sampling sites (TCWW, CUWW and iSTP E(s)) within the clinical WW of case study A, can be observed.

The positive detection of meropenem residues was the limiting factor for the association with the analyzed carbapenemases, since meropenem could only be detected in case study A (TCWW, CUWW and iSTP E(s). No residues could be detected in the rural sampling sites, as well as the eSTP E and the non-clinical influenced iSTP E(n), of case study A. The obtained clustering on the x-axis could be explained dilution and degradation processes. There are more noticeable differences between TCWW and CUWW for bla_{NDM} and bla_{OXA48}, than for bla_{VIM2}. The scatterplot for the association of ceftazidime with bla_{CTX-M} is more diffuse than that for the carbapenemases/meropenem, since an obvious cluster of clinical WW cannot be observed. The ceftazidime concentration decreased along the WW route in case study A (from 11.7 $\mu g\,L^{-1}$ down to 0.4 $\mu g\,L^{-1}$). Related to case study B, no ceftazidime residues could be detected.

The scattering of the sampling sites may be explained by the qPCR-results. Thus, bla_{CTX-M} is the most widely distributed ESBL-gene (Bonnet, 2004), whereby other entry pathways than the investigated maximum care hospital, are possible, since there are other hospitals in the investigated catchment area influencing e.g. CUWW and STP E. In addition, ceftazidime is known to exert a selection pressure in favor to the development of specific bla_{CTX-M} mutants like other cephalosporins (e.g. ceftriaxone or cefotaxime) (Bonnet, 2004). In this case, the dots on the scatterplots may further diffuse with regards to a broad spectrum of cephalosporins which also have high prescription volumes (BVL and Paul-Ehrlich-Gesellschaft für Chemotherapie e.V., 2016; Schwabe and Paffrath, 2016; Voigt et al., 2019a).

Altogether, a more precise association between the analyzed carbapenemases and meropenem, with regards to the respective sampling sites, could be obtained than for bla_{CTX-M} and ceftazidime. These results demonstrate the role of clinical WW for the

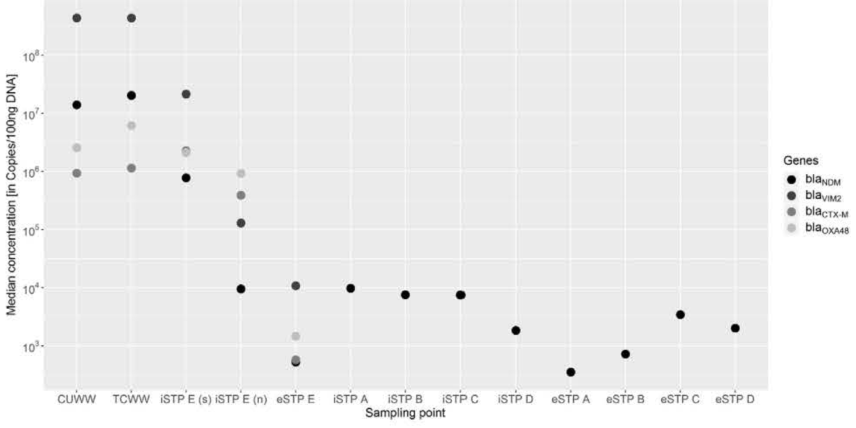

Fig. 4. Overview about the quantitative results (median, copies/100 ng DNA) of the ARG used for statistical analysis, sorted by sampling sites.

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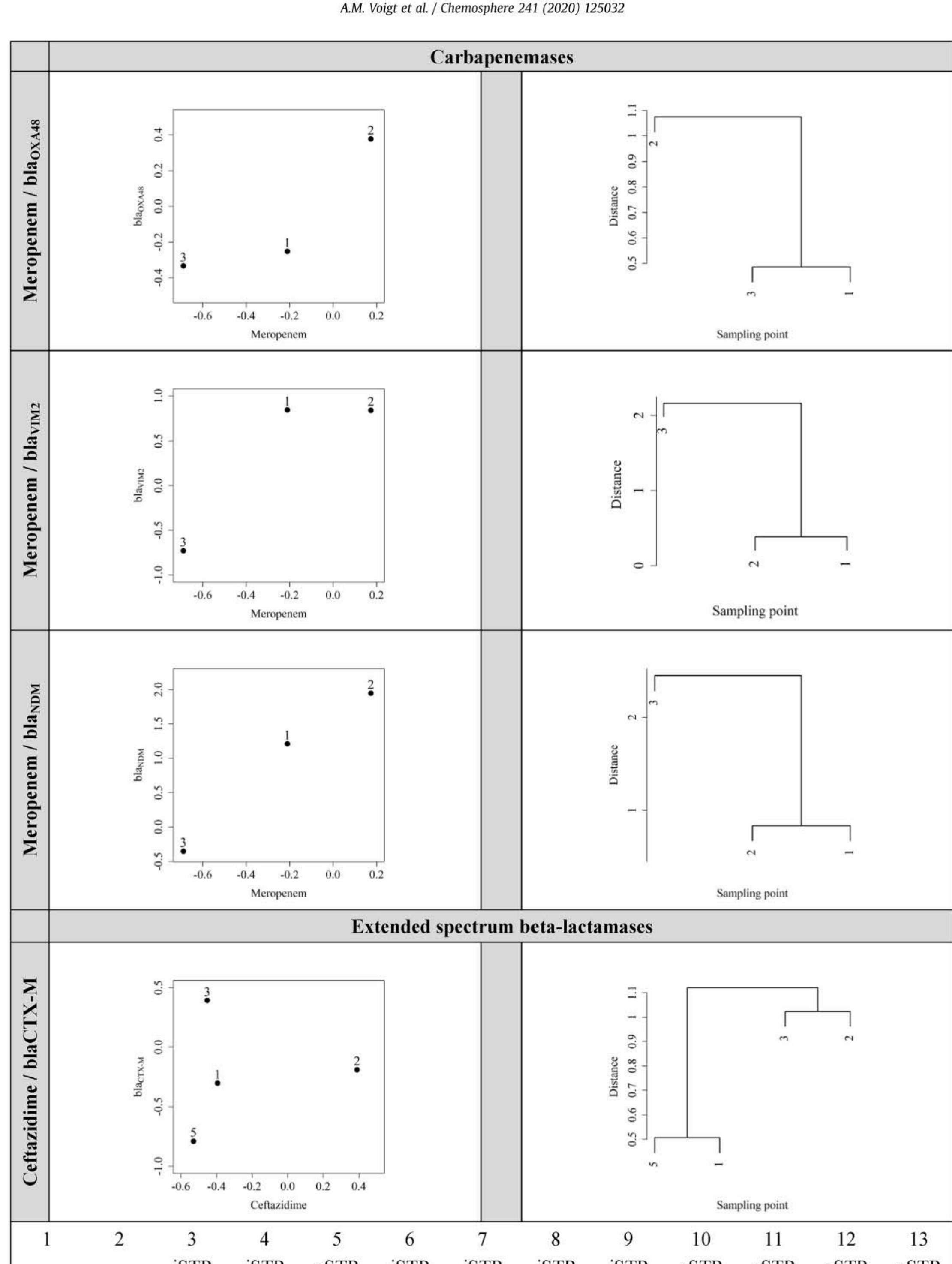

Fig. 5. Scatterplots and dendrograms of the relationship between ARG and the corresponding AR relating to all studied sampling sites (case study A and B).

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dissemination and development of serious ARG, which is in line with previous studies, which defined clinical WW as a potential "hot spot" for carbapenemases (Galler et al., 2014; Ory et al., 2019; Sib et al., 2019).

4. Conclusions

Significant relationships between ARB and the respective AR could be shown, especially between clinically used antibiotics like ciprofloxacin, meropenem and ceftazidime, in association with *P. aeruginosa* 3GCR and ESBL KEC. The scatterplots of these relationships showed a significant impact of clinical WW, which decreased within the canalization, by flow length and dilution, until the WW reached the respective STP effluent (eSTP E). In contrast, no specific clustering could be observed for the rural sampling sites (without clinical WW), only the expected distinction between influents and effluents of the municipal STPs (case study B). For the specific combination between bacteria classified as 3 or 4MRGN, and ciprofloxacin, similar clusters were obtained. Thus, clinical WW seems to be, especially, a reservoir for multidrug resistant carbapenemase producers, 3MRGN bacteria could even be detected in case study B, thus rarely. In addition, meropenem relates significantly with the respective ARG bla_{VIM2}, bla_{OXA48} and bla_{NDM} and the scatterplots show a focus on clinical WW. This may be caused by the presence of vulnerable patients, who are treated with meropenem, or are colonized with carbapenemase producing bacteria at hospitals.

Step one of the statistical analyses used 9 different bacteria species. Of which four could be seen with a significant positive association to the occurrence of at least one measured antibiotic, including MRSA and VRE. This study concentrates on the species *Pseudomonas* and the KEC group, which are capable of biofilm formation, based on the hypothesis that these bacteria are able to multiply within the sewer system and thus propagate the spread of antibiotic resistance to other pathogens. Biofilms, as a reservoir for persistence of ARB and in particular multi drug-resistant *P. aeruginosa* strains, has been shown in other studies (Ory et al., 2019; Sib et al., 2019). Despite the assumption of finding a *P. aeruginosa* 3GCR concentration through the whole WW system, because of its ability to form and reproduce in biofilms, this study showed that *P. aeruginosa* 3GCR was only present in the vicinity of the investigated hospital. Previous studies have also shown that these bacteria are present in the biofilms of the clinical wastewater network, as well as in shower drainpipes, toilets and sinks of patient rooms (Müller et al., 2018; Ory et al., 2019; Sib et al., 2019). Thus, stagnation zones of sinks, higher surface-to-volume-ratio of indoor sewage pipes, and the lower flow velocity of the WW installation within a building rather than in the connected WW canalization system, seems to promote biofilm formation and persistence of MRO.

Altogether, the results show that AR, ARB and ARG are present in high concentrations, particularly in clinical WW, and may promote the further development and spread of antibiotic resistance. Accordingly, the inclusion of a decentralized treatment of hospital WW should be discussed as an alternative of a general upgrade, of all STPs with advanced technologies. High concentrations of ciprofloxacin and/or meropenem might be considered as an indication for the presence of MRO and could be used as a surrogate signal to trigger subsequent microbiological analyses.

Declaration of competing interest

The authors declare no conflict of interest. This study complies with the guidelines of the Declaration of Helsinki (1964) by the World Medical Association (No. 160/120-HyReKA-Ethikantrag).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2019.125032.

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Appendix: „Association between antibiotic residues, antibiotic resistant bacteria and antibiotic resistance genes in anthropogenic wastewater – an evaluation of clinical influences“

Table S1 List of all investigated analytes sorted by substance classes with their respective method limit of quantification (ng/L).

Penicillins	amoxicillin (50)	ampicillin (200)	penicillin G (50)	cloxacillin (20)
	dicloxacillin (20)	flucloxacillin (20)	methicillin (10)	mezlocillin (20)
	nafcillin (20)	oxacillin (10)	penicillin V (20)	piperacillin (100)
Macrolides/ lincosamides	azithromycin (50)	clarithromycin (50)	clindamycin (20)	erythromycin (50)
	anhydroerythromycin (50)	roxithromycin (100)	spiramycin (100)	tylosin (50)
Cephalosporins	cefaclor (50)	cefotaxime (50)	ceftazidime (100)	
Tetracyclines	chlortetracycline (200)	doxycycline (200)	oxytetracycline (200)	tetracycline (200)
Fluoroquinolones	ciprofloxacin (200)	enrofloxacin (200)	moxifloxacin (200)	ofloxacin (200)
Carbapenems	meropenem (200)			
Sulfonamides / trimethoprim	sulfachlorpyridazine (50)	sulfadiazine (100)	sulfadimethoxine (50)	sulfadimidine (20)
	sulfadoxine (50)	sulfaethoxypyridazine (50)	sulfamerazine (50)	sulfamethoxazole (20)
	N4-Acetylsulfamethoxazole (100)	sulfamethoxypyridazine (10)	sulfathiazole (100)	trimethoprim (20)
Others	linezolid (100)	vancomycin (100)	metronidazole (100)	

Appendix: „Association between antibiotic residues, antibiotic resistant bacteria and antibiotic resistance genes in anthropogenic wastewater – an evaluation of clinical influences"

Table S2: Primer Sequences used for detection of carbapenemase and mcr genes. The reaction mix contained 0.125 µM of each probe, 0.25 µM of each primer, 2 x qPCR Mastermix (Biozym, Hessisch Oldendorf, Germany) and 2 µL of the DNA sample. The cycling conditions were set to 95 °C for 2 min followed by 45 cycles at 95 °C for 5 s and 60 °C for 30 s. A signal was interpreted as positive if it exceeded the threshold after the first 30 cycles.

Gene	Primer	Sequence	Reference
KPC	KPC-F	GCA GCG GCA GCA GTT TGT TGA TT	Swayne et al. (2013)[149]
	KPC-R	GTA GAC GGC CAA AAT AGG TGC	
	KPC-Probe	FAM-CAG TCG GAG ACA AAA CCG GAA CCT GC-BHQ1	
OXA_48	OXA48-F	TTC GGC CAC GGA GCA AAT CAG	
	OXA48-R	GAT GTG GGC ATA TCC ATA TTC ATC GCA	
	OXA48-Probe	HEX-CTG GCT GCG CTC CGA TAC GTG TAA CTT ATT G-BHQ1	
IMI	IMI-F	GAG GGT ATG ACT AAA TTC ATG CGG TCG A	
	IMI-R	GCA GGT GTA GAT GTG TCA CGY TCA TCG	
	IMI-Probe	Cy5-CGT TGG GAG TTA GAT C-BHQ2	
bla_{VIM}	VIM-F	GAT GAG TTG CTT TTG ATT GAT ACA GC	
	VIM-R	CCG ACK CGR TCG TCA T	
	VIM-Probe	FAM-TCG CGG AGA TTG ARA AGC AAA TTG GA-BHQ1	
bla_{NDM}	NDM-F	CCC GAC GAT TGG CCA	
	NDM-R	ATC CAG TTG AGG ATC TGG GC	
	NDM-Probe	ROX-ACC GAA TGT CTG GCA GCA CAC TTC-BHQ2	
GIM	GIM-F	TTG GTC TGA AGA AGA CAC GAA G	
	GIM-R	GTA GGA ACC GGC TTT CCT T	
	GIM-Probe	ROX-ATC GCA CTG CTG GTA TCA AGT TGC TA-BHQ2	
IMI$	IMI$-F	GCG ATG AAC GTG ACA CAT CT	Müller et al. (2018)[74]
	IMI$-R	ATC GCT TGG TAC GCT AGC AC	
	IMI$-Probe	Cy5-GAA AAC CCT TGC ACT GGG TA-BHQ2	
OXA_23	OXA-23_F	GAA GGG CGA GAA AAG GTC A	
	OXA-23_R	TCA GCA TTA CCG AAA CCA ATA C	
	OXA-23_ Probe	FAM-CGG TCT TGA TCT CAT GCA AA-BHQ1	
OXA_24	OXA-24_F	TTG GCC CCC TTA AAA TTA CAC	
	OXA-24_R	AAC ACC CAT TAC CCA TCC AC	
	OXA-24_ Probe	HEX-TGA CCT TGC ACA TAA CCG AT-BHQ1	
OXA_51	OXA-51_F	TTT TAT TTC AGC CTG CTC ACC	
	OXA-51_R	ATA CTC GGT CGA AGC ACG AG	
	OXA-51_ Probe	ROX-CAA ATC ACA GCG CTT CAA AA-BHQ2	
OXA_58	OXA-58_F	CAC GCA TTT AGA CCG AGC A	
	OXA-58_R	TGG CTT TCC ATC CCA CTT	
	OXA-58_ Probe	Cy5-AAA ACA GCT TAT ATT CCT GCA TCT-BHQ2	
mcr-1 +2	MCR1+2_F	ACA TCG ACG GCG TAT TCT GT	(unpublished)
	MCR1+2_R	TCC ATC ACG CCT TTT GAG TC	(unpublished)
	MCR1+2_Probe	Cy5-TGA TGT CGA TAC CGC CAA ATA CC-BHQ2	(unpublished)

Appendix: „Association between antibiotic residues, antibiotic resistant bacteria and antibiotic resistance genes in anthropogenic wastewater – an evaluation of clinical influences“

Table S3: Primer Sequences for the detection of resistant genes from water samples. qPCR analysis was performed according to Hembach et al. (2017).

Gene	Primer	Sequence	References
bla_{VIM2}*	vim2 FP	GAGATTCCCACGCA[C/T]TCTCTAGA	Van der Zee et al. (2014)[150]
	vim2 RP	AATGCGCAGCACCAGGATAG	
	Vim2 probe	ACGCAGTGCGCTTCGGTCCAGT	
bla_{NDM}	NDM-1 F	TTGGCCTTGCTGTCCTTG	Monteiro et al. (2012)[151]
	NDM-1FR	ACACCAGTGACAATATCACCG	
bla_{TEM}	qblaTEM-F	TTCCTGTTTTTGCTCACCCAG	Narciso-da-Rocha et al. (2014)[152]
	qblaTEM-R	CTCAAGGATCTTACCGCTGTTG	
$bla_{CTX\text{-}M}$*	S_CTX-MuF	CGCTTTGCGATGTGCAG	Paterson et al. (2003)[153]
	S_CTX-MuR	ACCGCGATATCGTTGGT	
$bla_{CTX\text{-}M\text{-}32}$	ctxm32-F	CGTCACGCTGTTGTTAGGAA	Narciso-da-Rocha et al. (2014)[152]
	ctxm32-R	CGCTCATCAGCACGATAAAG	
$bla_{CMY\text{-}2}$	CMY-2 RT-F	CGTTAATCGCACCATCACC	Kurpiel et al. (2011)[154]
	CMY-2 RT-R	CGTCTTACTAACCGATCCTAGC	
bla_{OXA48}*	OXA-48-F	TGTTTTTGGTGGCATCGAT	Monteiro et al. (2012)[151]
	OXA-48-R	GTAAMRATGCTTGGTTCGC	

*only analyzed for samples of case study A

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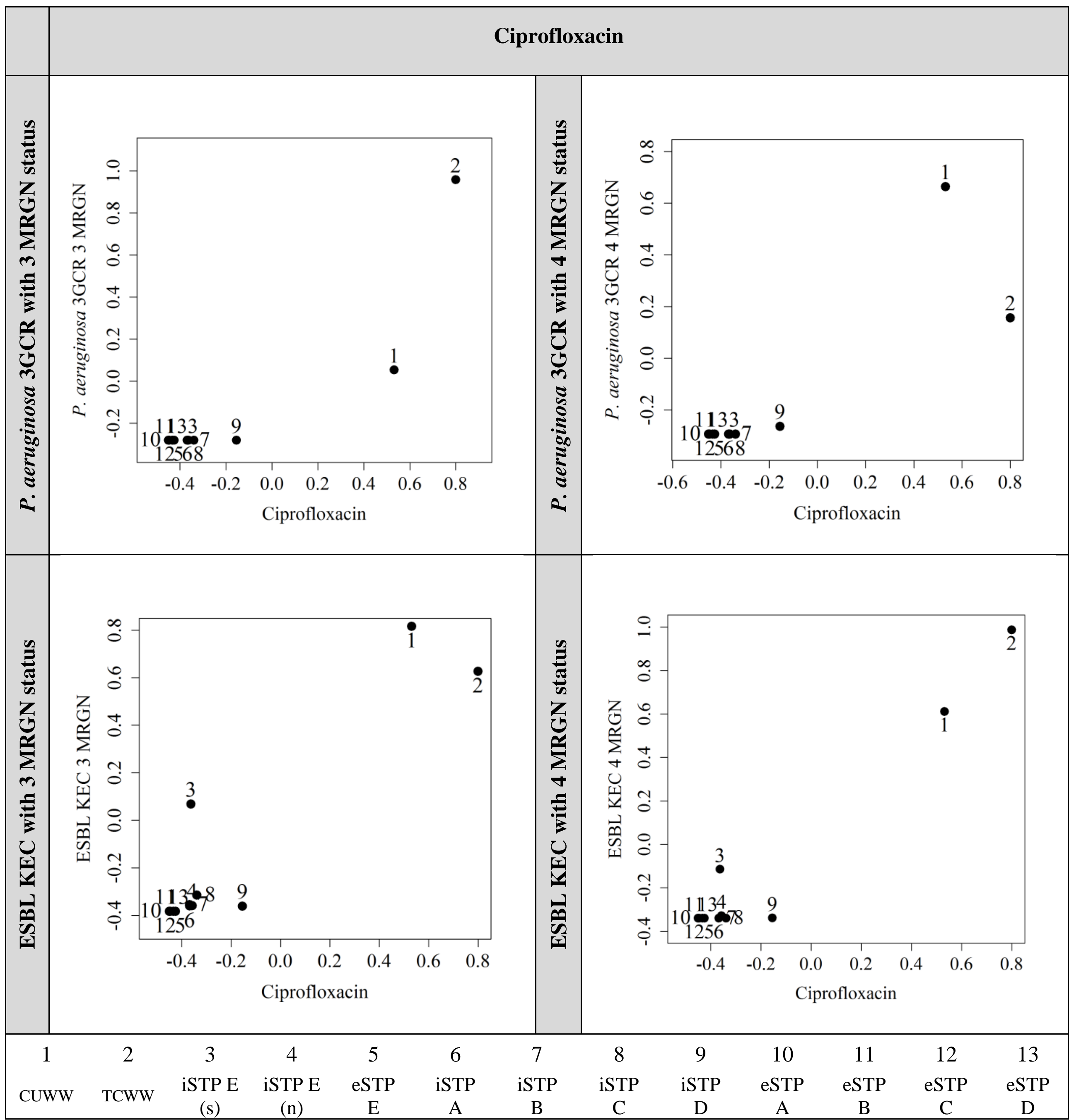

Figure S1 Scatterplots for the comparison of the sampling sites in regard to the concentration of presence and dispersion of MRO and the detected concentration of ciprofloxacin.

Legende: KEC = *Klebsiella* spez., *Enterobacter* spez. und *Citrobacter* spez. ; STP = Kläranlage (engl. *sewage treatment plant*)

Appendix: „Association between antibiotic residues, antibiotic resistant bacteria and antibiotic resistance genes in anthropogenic wastewater – an evaluation of clinical influences“

Table S4 Positive associated parameters (ARG versus AR) after the calculation of odds ratios. Those highlighted in bold fonts are combinations which will be discussed further.

Gene	Antibiotic	N	OR	p-value	Gene	Antibiotic	N	OR	p-value
bla_{NDM}	Azithromycin	127	4.25	0.013	$bla_{CTX.M.32}$	Amoxicillin	127	8.20	0.006
bla_{NDM}	Ciprofloxacin	127	3.78	0.001	$bla_{CTX.M.32}$	Ciprofloxacin	127	9.53	0.000
bla_{NDM}	Clarithromycin	127	4.96	0.005	$bla_{CTX.M.32}$	Clarithromycin	127	4.79	0.008
bla_{NDM}	N-Acetyl-SMX	127	2.98	0.009	$bla_{CTX.M.32}$	Ofloxacin	127	3.57	0.002
bla_{NDM}	Trimethoprim	127	6.30	0.000	$bla_{CTX.M.32}$	N-Acetyl-SMX	127	10.94	0.000
bla_{NDM}	**Meropenem**	**127**	**8.85**	**0.037**	$bla_{CTX.M.32}$	Meropenem	127	12.28	0.016
bla_{TEM}	Amoxicillin	127	8.22	0.044	$bla_{CTX.M.32}$	Metronidazole	127	5.65	0.007
bla_{TEM}	Ciprofloxacin	127	5.18	0.001	$bla_{CTX.M.32}$	Moxifloxacin	127	9.13	0.035
bla_{TEM}	Ofloxacin	127	2.80	0.028	bla_{CTX-M}	Ampicillin	127	41.71	0.001
bla_{TEM}	Metronidazole	127	9.12	0.034	**bla_{CTX-M}**	**Ceftazidime**	**127**	**19.11**	**0.000**
bla_{TEM}	N-Acetyl-SMX*	127	13.84	0.000	bla_{CTX-M}	Ciprofloxacin	127	4.42	0.005
bla_{TEM}	Vancomycin	127	3.46	0.033	bla_{CTX-M}	Metronidazole	127	4.56	0.001
bla_{Vim2}	Ampicillin	127	52.25	0.000	bla_{CTX-M}	Ofloxacin	127	2.84	0.013
bla_{Vim2}	Ceftazidime	127	38.22	0.000	bla_{CTX-M}	Piperacillin	127	10.00	0.000
bla_{Vim2}	Ciprofloxacin	127	3.56	0.017	bla_{CTX-M}	N-Acetyl-SMX	127	8.53	0.000
bla_{Vim2}	Metronidazole	127	4.82	0.001	bla_{CTX-M}	Meropenem	127	108.06	0.000
bla_{Vim2}	Moxifloxacin	127	10.01	0.000	bla_{CTX-M}	Moxifloxacin	127	11.48	0.000
bla_{Vim2}	Ofloxacin	127	3.14	0.010	bla_{CTX-M}	Vancomycin	127	51.67	0.000
bla_{Vim2}	Piperacillin	127	7.48	0.000	bla_{OXA48}	Ampicillin	127	44.00	0.000
bla_{Vim2}	Anhydroerythromycin	127	5.97	0.020	bla_{OXA48}	Anhydroerythromycin	127	4.23	0.026
bla_{Vim2}	Linezolid	127	131.54	0.000	**bla_{OXA48}**	**Meropenem**	**127**	**116.53**	**0.000**
bla_{Vim2}	**Meropenem**	**127**	**65.08**	**0.000**	bla_{OXA48}	Moxifloxacin	127	12.14	0.000
bla_{Vim2}	N-Acetyl-SMX*	127	9.06	0.000	bla_{OXA48}	Ceftazidime	127	15.56	0.000
bla_{Vim2}	Vancomycin	127	33.75	0.000	bla_{OXA48}	Ciprofloxacin	127	4.19	0.007
bla_{CMY2}	Amoxicillin	127	7.55	0.008	bla_{OXA48}	Metronidazole	127	4.86	0.001
bla_{CMY2}	Azithromycin	127	3.23	0.043	bla_{OXA48}	Ofloxacin	127	3.18	0.007
bla_{CMY2}	Ciprofloxacillin	127	7.83	0.000	bla_{OXA48}	Piperacillin	127	7.15	0.000
bla_{CMY2}	Ofloxacin	127	2.30	0.033	bla_{OXA48}	N-Acetyl-SMX	127	10.86	0.000
bla_{CMY2}	N-Acetyl-SMX*	127	11.85	0.000	bla_{OXA48}	Vancomycin	127	46.15	0.000
bla_{CMY2}	Trimethoprim	127	4.37	0.001					
bla_{CMY2}	Meropenem	127	11.34	0.020					

*SMX = sulfamethoxazole

3.2. Zusammenfassung

Trotz einer Vielzahl an Studien über die Untersuchung von Abwasser in Waschbeckensiphons, Toiletten und Duschabläufen klinischer Bereiche und dem Nachweis von antibiotikaresistenten Erregern,[140–145] bezogen sich Datenerhebungen auf AR stets auf das Sammelabwasser von Krankenhäusern bzw. klinisch-beeinflussten Mischabwässern.[95,104,135,136,155] Die in diesem Kapitel zusammengefassten Arbeiten behandelten neben der Untersuchung von Krankenhaus- bzw. Mischabwässern auch erstmalig die Analyse des in Waschbeckensiphons, Toiletten und Duschabläufen befindlichen Abwassers auf AR.[6,7]

Im vorliegenden Kapitel zeigt sich, dass vor allem in klinischen Bereichen mit einem hohen Antibiotika-Verbrauch Rückstände dieser Substanzen in Duschabläufen, Waschbeckensiphons und Toiletten in Konzentrationen von bis zu 79 mg/L nachzuweisen sind.[6] Das nachgewiesene Spektrum an Antibiotika konnte durch die typischerweise in den untersuchten Kliniken eingesetzten Antibiotika erklärt werden. Vor allem in der Onkologie spiegelten die häufigen Nachweise an Ciprofloxacin, Sulfamethoxazol und Trimethoprim die durchgeführte Antibiotikaprophylaxe zum Schutz vor nosokomialen Infektionen bei immunsuppressiven Patienten (Neutrophile-Wert < 500 Zellen/mL) wider.[6] Weiterhin umfasste das nachgewiesene Spektrum Rückstände klinisch relevanter Antibiotika wie Piperacillin, Vancomycin, Linezolid oder Meropenem.[6] In den Sanitäreinheiten der untersuchten Onkologie und des neurologischen Rehabilitationszentrum überschritten die gefundenen Rückstandskonzentrationen oftmals den $PNEC_{Res}$ nach Bengtsson-Palme und Larsson (2016), woraus ein erhöhter Selektionsdruck zugunsten von antibiotikaresistenten Bakterien resultieren könnte.[3,6]

Das Vorhandensein von derart hohen AR, insbesondere von klinisch relevanten Antibiotika, im unmittelbaren Patientenumfeld sollte weiter beobachtet und in Präventionsstrategien berücksichtigt werden. Ein erster Lösungsansatz wurde mit der Erhöhung des Spülvolumens auf circa 15 L getestet. Dies führte dazu, dass in den meisten Fällen keine AR mehr in den Sanitäreinheiten nachgewiesen werden konnten.[6]

Überraschenderweise konnten nach vollständiger Spülung des Systems und ausreichender zeitlicher Stagnation in Waschbecken, Toiletten und Duschabläufen erneut AR nachgewiesen werden. Eine mögliche Erklärung für dieses Phänomen könnte die Akkumulation von AR im Biofilm und eine während der Stagnation verlaufende Rückdiffusion in die wässrige Phase sein. Demnach sollte eine regelmäßige Entfernung des Biofilms und ausreichende Durchspülung klinischer Sanitäreinheiten in zukünftigen Reinigungskonzepten und baulichen Maßnahmen berücksichtigt werden.[6]

In diesem Kontext stellte sich die Frage, ob klinisches Abwasser einen signifikanten Einfluss auf Mischabwässer, die nach der Abwasseraufbereitung als geklärtes Abwasser in die aquatische Umwelt eingeleitet werden, ausübt und ob spezifische AR, ARG bzw. ARB als Marker für klinisches Abwasser fungieren können.

Diesbezüglich zeigte sich, dass ARB, AR und ARG in Gesamtkrankenhausabwasser, verschiedenen Mischabwässern, klinisch unbeeinflussten Abwässern sowie in behandelten Abwässern (Kläranlagenabläufen) nachgewiesen werden konnten. Am häufigsten wurden Rückstände an Makrolid-Antibiotika, Sulfamethoxazol, Trimethoprim sowie Ciprofloxacin bestimmt. Insgesamt wurden ESBL *E. coli* und KEC (*Klebsiella* spez., *Enterobacter* spez. und *Citrobacter* spez.) Stämme sowie 3GCR *P. aeruginosa* und *A. calcoaceticus-baumannii* Komplex Stämme in den 13 verschiedenen Abwässern nachgewiesen. Besonders auffällig waren die verglichen mit kommunalen Abwässern ohne Klinikeinfluss (circa 12%) hohen Nachweishäufigkeiten von 3GCR *P. aeruginosa* Stämmen im Krankenhausabwasser bzw. in klinisch beeinflussten Mischabwässern (56% – 68%).[7]

Für spezifische AR (Meropenem, Ciprofloxacin und Ceftazidim) konnte eine erhöhte Wahrscheinlichkeit bezüglich eines gleichzeitigen Nachweises an bestimmten ARB (3GCR *P. aeruginosa* und ESBL KEC) ermittelt werden. So zeigte sich z.B., dass die Wahrscheinlichkeit des Nachweises von 3GCR *P. aeruginosa* um einen Faktor von 2,8 erhöht ist, sofern Rückstände an Ciprofloxacin nachgewiesen werden konnten. Für ESBL KEC ergab sich sogar ein Faktor von 4,3 bei gleichzeitigem Nachweis von Ciprofloxacin.[7]

Anhand von Cluster-Analysen konnte ein signifikanter Einfluss von Krankenhausabwasser bezüglich der Belastung mit AR (Meropenem, Ciprofloxacin, Ceftazidim) und ARB (3GCR *P. aeruginosa* und ESBL KEC) auf Mischabwässer visualisiert werden. Die Gegenüberstellung der nachgewiesenen Rückstände an Ciprofloxacin und multiresistenten Bakterien (3MRGN bzw. 4MRGN) lassen vermuten, dass klinisches Abwasser vor allem ein Reservoir für MRGN darstellt.[7]

In Übereinstimmung damit zeigte der Vergleich nachgewiesener Meropenem-Rückstände mit spezifischen Carbapenemase-Genen (bla_{VIM2}, bla_{OXA48} und bla_{NDM}) deutliche Unterschiede zwischen Krankenhausabwasser und Abwasser ohne Klinikeinfluss. Abschließend konnte anhand der untersuchten Abwässer ohne Klinikeinfluss eine Unterscheidung von Kläranlagenzuläufen und Kläranlagenabflüssen festgestellt werden.[7]

Insgesamt zeigte sich, dass klinisches Abwasser nicht erst im Kanalsystem beginnt, sondern bereits in Waschbeckensiphons, Duschabläufen und Toiletten.[6] Der Nachweis von AR und deren Speichervermögen im Biofilm lassen befürchten, dass im unmittelbaren Patientenumfeld bereits ein erhöhter Selektionsdruck zugunsten ARB vorherrschen könnte.[6] Demnach sollte ein Fokus zukünftiger Studien auf möglichen Änderungen in Reinigungsverfahren, betrieblich-organisatorischen Abläufen und baulich-funktionellen Maßnahmen zur Reduktion von AR neben ARB und ARG sowie einer Reduktion der Biofilmausbildung in klinischen Sanitäreinheiten liegen. Obwohl der Einfluss von Krankenhausabwasser in Mischabwässern für spezifische AR, ARG und ARB nachgewiesen werden konnte, zeigte sich, dass dieser Einfluss im Abwasserkanalsystem deutlich auf dem Weg zur Kläranlage abnimmt.[7] Demnach könnte eine dezentrale Abwasseraufbereitung von Krankenhausabwasser eine geeignete Möglichkeit darstellen, die Persistenz ARB sowie den Neuerwerb von Antibiotikaresistenzen im Kanalnetz zu reduzieren und kommunale Kläranlagen gegebenenfalls zu entlasten.

4. Expositionsrisiken durch abwasserbeeinflusste Gewässer

4.1. Einleitung

Die Übertragungswege bzw. Rückkopplungswege von Antibiotikaresistenzen auf den Menschen sind vielschichtig.[2,51] So sind Übertragungen von ARB z.B. über kontaminierte Lebensmittel, über den Kontakt mit Nutz- bzw. Haustieren, den Kontakt mit Abwasser oder durch Kleidung (aufgrund von unzureichenden Temperaturen beim Waschen von mit ARB verunreinigter Schmutzwäsche) denkbar.[140,141,156–158]

In Kapitel III dieser Promotionsarbeit konnte gezeigt werden, dass kommunales sowie klinisches Abwasser ebenfalls ein Reservoir an ARG, ARB und AR ist und dass das in Duschabläufen, Toiletten und Waschbeckensiphons befindliche Abwasser klinischer Bereiche ein potentielles Risiko für die Besiedlung mit ARB darstellen kann.[6,7] Aufgrund der Einleitung von behandeltem Abwasser in Oberflächengewässer stellt der Kontakt mit diesen Gewässern ebenfalls ein Expositionsrisiko bzgl. der Besiedlung mit ARB dar. Insbesondere in Badegewässern oder Oberflächengewässern, die zur Trinkwassergewinnung dienen, könnte aufgrund der besonderen Nutzung des Gewässers ein erhöhtes Expositionsrisiko vorliegen.

Ziel der vorliegenden Studie war es, ein Flusssystem in Deutschland auf ARB, AR und ARG zu untersuchen, da diese Gewässer in eine Trinkwassertalsperre münden und durch die Einleitung zweier kommunaler Kläranlagen beeinflusst werden. Der Fokus der kulturellen Untersuchung auf etwaige ARB lag in dieser Studie auf fakultativ pathogenen Bakterien wie z.B. *P. aeruginosa*, *K. pneumoniae* oder *A. baumannii*. Aufgrund der besonderen Bedeutung für die öffentliche Gesundheit lag der Fokus auf Carbapenemase- und Colistin-Resistenzgenen. Ferner soll diese Studie erste Orientierungswerte bzgl. der Belastung von zur Trinkwassergewinnung dienenden Oberflächengewässern mit ARB, AR und ARG liefern. Zudem können die so gewonnen Daten dabei helfen, eine breitere Datenbasis über die aktuelle Resistenzsituation in deutschen Oberflächengewässern zu erstellen, um in zukünftigen Fragestellungen Entwicklungen frühzeitig erkennen bzw. gewonnene Ergebnisse besser bewerten zu können.

Denn insgesamt sind die Regulierungen für die Entsorgung von Abwasser vor dem Hintergrund der steigenden Resistenzproblematik noch nicht abschließend geregelt. So gilt allgemein gemäß § 41 des Infektionsschutzgesetzes, „dass Abwasser so beseitigt werden [muss], dass Gefahren für die menschliche Gesundheit durch Krankheitserreger nicht entstehen.“[55]

Die Ergebnisse könnten zudem als weitere Grundlage für die aktuell geführte Diskussion um den Ausbau kommunaler Kläranlagen dienen, da nach § 8 der Verordnung zum Schutz der Oberflächengewässer zur Trinkwassergewinnung dienende Oberflächengewässer so zu bewirtschaften sind, dass eine Verschlechterung der Qualität zu vermeiden ist und somit die benötigte Aufbereitung des Rohwasser für die Gewinnung von Trinkwasser minimiert wird.[159]

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The investigation of antibiotic residues, antibiotic resistance genes and antibiotic-resistant organisms in a drinking water reservoir system in Germany

A.M. Voigt*[,2], P. Ciorba, M. Döhla, M. Exner, C. Felder, F. Lenz-Plet, E. Sib, D. Skutlarek, R.M. Schmithausen[1], H.A. Faerber[1]

Institute for Hygiene and Public Health, Medical Faculty, University of Bonn, Building 63, Venusberg-Campus 1, 53127, Bonn, Germany

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ABSTRACT

Between August 2018 and June 2019, a river system in Germany that supplies a drinking water reservoir and is subject to the discharge from two sewage treatment plants was monitored for antibiotic residues via liquid chromatography–tandem mass spectrometry, antibiotic resistance genes (including bla_{NDM}, bla_{VIM}, bla_{OXA-48}, bla_{KPC}, bla_{GIM}, bla_{SME}, bla_{IMI}, bla_{IMP}, bla_{SPM}, bla_{SIM}, bla_{OXA-23}, bla_{OXA-24}, bla_{OXA-51}, bla_{OXA-58}, *mcr*) via qualitative real-time PCR and antibiotic-resistant bacteria [belonging to the ESKAPE-group (*Enterococcus faecium*, *Staphyhlococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter ssp.;* with resistance against Carbapenemases, Cephalosporines and Colistin) and *Escherichia coli*] based on cultivation methods followed by a characterization via MALDI-TOF MS and susceptibility testing applying microdilution.

Residues of macrolide antibiotics such as clarithromycin (up to 0.60 μg/L) and residues of sulfamethoxazole (up to 0.40 μg/L) and trimethoprim (up to 0.39 μg/L) were detected downstream of the sewage treatment plants. In addition, no antibiotic residues were detected upstream the respective sewage treatment plants, except for anhydroerythromycin (n = 1, < LOQ). In total, bla_{OXA-58} was found in 6, and *mcr* in one water sample throughout the whole sample period. No MRSA, extensively drug-resistant or pan-resistant bacteria, according to Magiorakos et al. (2011), could be isolated. In contrast, multidrug-resistant bacteria, especially *E. coli* and *Enterococcus faecium* were found.

In conclusion, sewage treatment plant effluents are point sources for antibiotic residues (p = 0.0000 and p = 0.0001) and antibiotic-resistant bacteria (p = 0.0021 and p = 0.0060) since their concentrations increased significantly after the discharge of treated wastewater into the investigated rivers. In this specific catchment area, which lacked clinical, hospitals, and intensive livestock farming, no significant bacterial or analyzed chemical (antibiotic residues) influences of the sewage treatment plants were observed in the river downstream of the drinking water reservoir during the study. Furthermore, no increased selection pressure could be expected as no measured antibiotic residues exceeded the predicted no effect concentration for antibiotic resistance selection, according to Bengtsson-Palme and Larsson (2016).

1. Introduction

Wastewater (WW) represents a reservoir of various chemical and microbiological pollutants. Typical chemical micropollutants found in WW are heavy metals, organic pollutants (e.g., pesticides, perfluorinated compounds, bisphenol A or phthalates) and residues of pharmaceuticals (e.g., x-ray contrast agents, carbamazepine or Ethinylestradiol) (Skutlarek et al., 2006; Seitz et al., 2006; Kumar et al., 2019; Heberer et al., 2002; Kolpin et al., 2002).

Recent studies focus on the spread of antibiotic residues (AR), antibiotic-resistant bacteria (ARB), and antibiotic resistance genes (ARG) from WW discharge into the aquatic environment because of increasing concern about the burden of antibiotic resistance (Larsson et al., 2018). Besides ARB and ARG, high levels of incompletely-metabolized AR

* Corresponding author.
E-mail address: alexander.voigt@ukbonn.de (A.M. Voigt).
[1] Senior authorship, contributed equally.
[2] Main authorship.

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enter the sewage system via stool and urine. The excretion rate of unmetabolized substances can differ between 10% and 90% depending on the antimicrobial substance (Heberer, 2002; Kümmerer and Henninger, 2003). In this case, antibiotic resistance in surface water constitutes a threat to public health because of possible feedback to humans (e.g., through bathing water, the food chain, and drinking water) (Döhla et al., 2019; Barker-Reid et al., 2010; Simazaki et al., 2015; Sanganyado and Gwenzi, 2019).

In Germany, the annual consumption of antibiotics ranges between 700 and 800 tonnes for both veterinary and human use (BVL and Paul-Ehrlich-Gesellschaft für Chemotherapie e.V., 2016), an indication of the high loads of AR that can enter the WW stream. Due to an incomplete degradation in sewage treatment plants (STP), AR can be released into the aquatic environment, whereas the removal rates are dependent on the respective antibiotic and substance class, respectively (Michael et al., 2013). In general residual concentrations of antibiotics can reach up to approx. 120 μg/L for untreated wastewater (Michael et al., 2013) and up to approximately 8.0 μg/L for treated wastewater, which is released into surface waters (Fatta-Kassinos et al., 2011). In addition, higher residual concentrations could be detected up to a two-digit μg/L range in clinical WW (Fatta-Kassinos et al., 2011; Voigt et al., 2020). WW systems therefore represent a potential point source for the occurrence and dissemination of ARG, ARB, and AR within the aquatic environment (Baquero et al., 2008; Jutkina et al., 2018). Currently, no regulatory standards are provided for antibiotic residues in wastewater. In this context, the requirements of the European directive 2000/60/EG focuses on the improvement of the aquatic environment through the reduction of discharges and emissions of hazardous substances and the loss of priority substances like pesticides, heavy metals, or nonylphenol. Since June 2018, specific antibiotics like azithromycin, erythromycin, clarithromycin, amoxicillin and ciprofloxacin have been added to the "watch list of substances for Union-wide monitoring in the field of water policy" in order to improve tracking the occurrence and spread of antimicrobial substances in the aquatic environment under the European Action Plan (Directive 2000/60/EC, 2014; Directive 2008/105/EC, 2013; Commission Implementing Decision (EU) 2018/840, 2018).

Additionally, previous studies showed diverse ARG and ARB in different types of WW (Mao et al., 2015; Müller et al., 2018; Schwartz et al., 2003; Sib et al., 2019). ARG can be found in the effluent microbiome and can be transferred between living microorganisms (Maestre-Carballa et al., 2019). Due to incomplete removal or elimination of ARB, AR and ARG during treatment processes, STP effluents serve as a point source for the entry of antibiotic resistance into the aquatic environment (Hembach et al., 2019; Michael et al., 2013; Rizzo et al., 2013).

Recent studies have not only shown that high concentrations of antimicrobial substances exert a selection pressure (maintenance or "de-novo" development of resistances), but also that very low, or even environmental residual concentrations can favor ARB (Bengtsson-Palme and Larsson, 2016; Gullberg et al., 2011). The aquatic environment therefore needs to be recognized as a compartment for the investigation of further exposure routes from ARB (Larsson et al., 2018). In addition to their function as on-site preflooder for STP effluents, surface waters are also used for swimming or for the production of drinking water, which results in further conceivable exposure risks of ARBs to humans. Despite this potential source of exposure, a recent study showed that only low levels of AR, ARB, and ARG could be detected in German bathing waters (Döhla et al., 2019).

About 30% of drinking water in Germany comes from surface water and bank filtration (Umweltbundesamt (UBA), 2015), and most German drinking water systems are not chlorinated, making the protection of surface water quality critical to public health. In light of Germany's approach to drinking water treatment and the German directive on protection of surface water (OGewV), surface water protection cannot be supplanted by drinking water treatment processes like advanced membrane filtration or combined processes alone (Crini and Lichtfouse, 2019; Simazaki et al., 2015; OGewV, 2016). A multi-barrier approach is required for the protection of raw and drinking water.

The present study aims to investigate the occurrence of AR, ARB, and ARG in a surface water system that is subject to the discharge of treated WW from two municipal STP and supplies approximately 1,000,000 people with purified drinking water. For this purpose, a direct injection liquid chromatography–tandem mass spectrometry (LC-MS/MS) method developed by Voigt et al. (2019b) was used to investigate residues of inpatient (e.g., meropenem, vancomycin, ciprofloxacin classified as critically important antimicrobials by the World Health Organization (WHO), 2019) as well as outpatient antibiotics in Germany (such as amoxicillin, ampicillin, macrolide antibiotics or sulfamethoxazole in combination with trimethoprim (Schwabe and Paffrath, 2016),) or antibiotics that are typically used in veterinary medicine [e.g., sulfonamides, enrofloxacin, chlortetracyclin, spiramycin or tylosin (Bundesministerium für Ernährung und Landwirtschaft - AG Antibiotikaresistenz, Hamscher et al., 2002; Trouchon and Lefebvre, 2016)]. Such a broad spectrum of analytes is critical for the investigation of the influence of "unknown" emitters (e.g., clinics, municipal STPs, intensive livestock farming, use of manure as fertilizer (Voigt et al., 2019b)). Furthermore, facultative pathogenic bacteria of the ESKAPE group (*Enterococcus faecium*, *Stahpyhlococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter ssp.*) and *E. coli* are of critical importance for the public health due to high infection rates. In addition, these bacteria often transmit or acquire plasmids that can lead to high levels of resistance towards antibiotics [multidrug-resistant (MDR), extensively drug-resistant (XDR) and pan-resistant bacteria] (Magiorakos et al., 2012; Rice, 2008). Finally, ARG (relevant carbapenemase encoding genes) were analyzed. The focus was put on carbapenemase encoding genes and *mcr* due to the fact that these are mostly plasmid-mediated and convey high levels of resistance against critically important antimicrobials like meropenem and colistin (Queenan and Bush, 2007). A combined approach of culture based and molecular methods was chosen to assign a possible resistance to a specific bacterium.

2. Method and materials

2.1. Sampling procedure and sampling sites

From August 2018 to June 2019, seven sampling sites around a drinking water reservoir were monitored (Fig. 1). A total of 48 grab samples were taken in seven sampling campaigns except for site C where samples were collected only six times (08/18; 10/18; 12/18; 02/19; 04/19; 05/19 and 06/19). The reservoir being studied is subject to the discharge of treated WW from two municipal STPs which do not treat WW from sources like hospitals or slaughterhouses. Two sampling sites (A and C) were upstream of two respective STPs, and four sampling sites (B, D, E, and F) were downstream of these STPs. The river was also analyzed downstream of the drinking water reservoir (G). A further characterization of the sampling sites is given in Table 1.

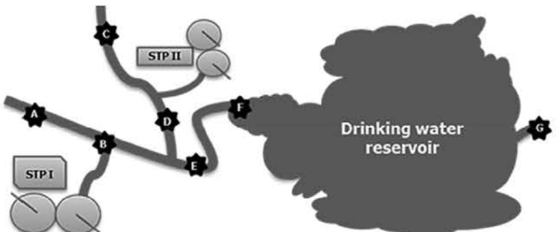

Fig. 1. Schematic overview of the investigated drinking water reservoir area and the location of the sampling sites (A to G). Legend: STP, sewage treatment plant.

Table 1
Overview of the investigated drinking water reservoir area and the location of the sampling sites (A to G). Legend: STP, sewage treatment plant.

Sampling Site	Characterization
A	Approx. 300 m upstream of the STP I (IA: ca. 9000; TAWW: 300 L/d·IA)
B	Approx. 10 m downstream of the STP I
C	Approx. 240 m upstream of the STP II (IA: ca. 3000; TAWW: 300 L/d·IA)
D	Approx. 60 m downstream of the STP II
Confluence of the two rivers	Approx. 6.5 km downstream of the STP II and approx. 6 km downstream of the STP I, respectively
E	Approx. 150 m downstream of the confluence
F	Approx. 6.5 km downstream of the confluence
G	The creek after the effluent of the drinking water reservoir

* IA = Inhabitant equivalent.
TAWW = Total amount of WW.

Amber glass bottles were filled with 250 mL of water from the sampling sites for chemical analysis. Five hundred milliliters were transferred into sterile polypropylene bottles for the cultivation of bacteria and PCR. Both types of samples were transported on ice (+4 °C) and analyzed within 24 h.

2.2. Chemical analysis

The investigation for AR was performed using a direct injection method published by Voigt et al. (2019a). The aqueous samples were then diluted with a mixture of 95% water and 5% acetonitrile (containing 0.8 g/L EDTA). The diluted samples were filtered through hydrophilized polytetrafluoroethylene (H-PTFE) syringe filters from Macherey-Nagel (Düren, Germany), and 20 µL were injected into the LC-MS/MS. For the chromatographic separation and the following detection, an Agilent 1290 Infinity II LC-system (Waldbronn, Germany) was coupled with a QTRAP® 6500 + tandem mass spectrometer from Sciex (Darmstadt, Germany). The analyzed antimicrobial substances and metabolites, including their limit of quantification (LOQ), are given in Table 2.

Meropenem was included in the analyzed spectrum of antibiotics as it is the most prescribed carbapenems in the analyzed region. In this context, much higher antibiotic consumption rates of meropenem (15.4 Recommended Daily Doses (RDD)/100 patients days) have been described than the consumption rate of ertapenem (0.02 RDD/100 patient days) (Voigt et al., 2019a). The preference of meropenem could be explained by the inactivation of imipenem due to enzymatic cleavage via renal dipeptidase which impedes the treatment with imipenem compared to meropenem (Mutschler et al., 2012). Additionally, a recent study suggests that high residual concentrations meropenem in wastewater can be used as a marker for the presence of multidrug-resistant bacteria (Voigt et al., 2020).

The obtained residual concentrations were compared to the predicted no-effect concentrations (PNEC) for resistance selection by Bengtsson-Palme and Larsson (2016). These subinhibitory concentrations serve as upper boundaries for selective concentrations. Thus, no increased selection pressure should be expected from AR in favor of ARB if the measured concentration is below the respective PNEC (Bengtsson-Palme and Larsson, 2016).

Table 2
List of the 45 analyzed antibiotics and two metabolites [dh-ERY (anhydroerythromycin) and N4SMX (N-Acetylsulfamethoxazole)] including their limit of detection (LOD) limit of quantification (LOQ) both in ng/L (Voigt et al., 2019b).

Analyte	LOD [ng/L]	LOQ [ng/L]	Analyte	LOD [ng/L]	LOQ [ng/L]
Amoxicillin	6.2	16	Ofloxacin	23	59
Ampicillin	30	74	Oxytetracycline	15	42
Azithromycin	3.1	9.0	Oxacillin	2.1	5.7
Cefaclor	5.8	17	Penicillin-G	11	30
Cefotaxime	11	28	Penicillin-V	3.5	10
Ceftazidime	33	78	Piperacillin	17	48
Ciprofloxacin	31	77	Roxithromycin	1.9	5.1
Clarithromycin	4.9	13	Sulfachlorpyridazine	6.5	19
Clindamycin	2.8	7.0	Sulfadimidine	3.8	11
Cloxacillin	1.8	4.9	Sulfadimethoxine	2.2	6.6
Chlortetracycline	7.7	22	Sulfadoxine	4.9	13
Dicloxacillin	1.8	4.9	Sulfadiazine	47	100
Doxycycline	3.9	12	Sulfaethoxypyridazine	5.0	13
Enrofloxacin	11	33	Sulfamethoxypyridazine	1.1	3.3
Erythromycin	2.8	7.0	Sulfamethoxazole	3.6	10
dh-ERY	3.6	10	N4AcSMX	21	55
Flucloxacillin	1.6	4.6	Sulfamerazine	18	50
Linezolid	8.0	22	Spiramycin	11	29
Meropenem	21	55	Sulfathiazole	15	42
Methicillin	1.4	4.2	Tetracycline	12	31
Metronidazole	7.6	21	Trimethoprim	19	42
Mezlocillin	3.8	11	Tylosin	24	62
Moxifloxacin	58	120	Vancomycin	76	190
Nafcillin	3.4	9.8			

2.3. Cultivation and identification of multidrug-resistant organisms

Depending on the water type, various methods were used for the cultivation of clinically relevant-bacteria such as vancomycin-resistant enterococci (VRE), methicillin-resistant *S. aureus* (MRSA) and MDR ESKAPE bacteria.

2.3.1. Surface water

The detection of MDR bacteria in surface water was performed via filtration of 100 mL of surface water. The filters (pore size 0.45, Ø 47 mm, MICROFIL® from Millipore, Art. No. MVHAWG 124) were plated onto selective agar plates (CHROMagar ESBL [MAST Diagnostica GmbH, Reinfeld, Germany, 201470], CHROMagar MRSA [MAST Diagnostica GmbH, Reinfeld, Germany, 201402] and CHROMagar VRE [MAST Diagnostica GmbH, Reinfeld, Germany, 201460]). One milliliter of the surface water sample was also plated out onto the selective agar plates. For the quantification of MDR gram-negative bacteria, a 1:2 dilution series was performed, of which 1 mL was plated out onto CHROMagar ESBL. All agar plates inoculated with water samples were incubated for 24 h and 48 h at 42 °C. Potential pathogenic bacteria were counted in all dilutions.

2.3.2. Sampling site B

Because of the high ratio of wastewater at sampling site B, it was necessary to modify the cultivation method. Thus, for the detection of MDR bacteria in water samples of sampling site B, 10 mL was filtered before placing the filter onto selective agar plates (CHROMagar ESBL, CHROMagar MRSA, CHROMagar VRE). One milliliter of each water sample was then plated on the selective agar plates. For the quantification of MDR gram-negative bacteria, a 1:10 dilution of the sample in 0.9% NaCl was conducted. One milliliter of each dilution step was plated onto CHROMagar ESBL. Each agar plate was incubated at 42 °C for 24 h and 48 h. After the incubation period, the bacteria were counted in all dilutions.

2.4. Characterization of MDR bacteria

One representative of each morphologically identical colony and potentially pathogenic bacteria isolated from water samples as described above was sub-cultivated on 5% sheep blood Columbia agar plates (Becton Dickinson GmbH, Germany) and incubated at 36 °C overnight. Identification was performed using VITEK MS (bioMérieux, Marcy-l'Étoile, France) with the Myla™ software.

Susceptibility testing was performed via microdilution using the Micronaut-S system (MDR MRGN-Screening 3 and MRSA/GP by MERLIN, Gesellschaft für mikrobiologische Diagnostika GmbH, Bornheim-Hersel, Germany) employing EUCAST criteria (EUCAST, 2018). A list of tested antibiotics, which are relevant for the MDR classification, is provided in Table S1.

2.5. Identification of antibiotic resistance genes

2.5.1. DNA isolation

For the isolation of bacterial DNA, 50 mL of water was centrifuged at 10,000 rpm for 10 min. The supernatant was removed up to 100 μL in which the pellet was resuspended and transferred to a precellys lysing tube (Precellys lysing kit, tough microorganism lysing VK05). One milliliter of CTAB buffer was added (MC1411, Promega GmbH, Mannheim, Germany), and samples were incubated at 95 °C with vigorous shaking. Afterward, tubes were bead beaten at 5000 rpm twice for 30 s. Forty microliters of proteinase K solution was added and incubated with shaking at 70 °C for 10 min. One milliliter of the sample was transferred into a 1.5 mL Eppendorf tubes and centrifuged at 10,000 rpm for 5 min. DNA isolation was performed using a kit (Maxwell 16 FFS nucleic acid extraction system, Promega GmbH) according to the manufacturer's instructions. The isolated DNA was stored at −20 °C until use.

2.5.2. Multiplex PCR

A qualitative analysis of resistance genes was determined using a 4-plex real-time PCR (RT-PCR) system with specific PCR primers and probes, which are listed in Table 3. Probes had been labeled with different combinations of fluorescent and Black Hole Quencher® dyes allowing multiplex PCR (Microsynth, Balgach, Switzerland) of four genes in one assay. Detection of carbapenemase genes was performed using the Biozym 2 x qPCR Mastermix (Biozym, Hessisch Oldendorf, Germany) on a CFX96 Touch Real-Time PCR Detection System (Biorad, Germany).

The PCR was set up following the manufacturer's recommendations. Each well contained 0.25 μM of each primer, 0.125 μM of each probe as well as 2 μL of the isolated bacterial DNA. The cycling conditions were set to 95 °C for 2 min, followed by 45 cycles at 95 °C for 5 s and 60 °C for 30 s.

2.6. Statistical analysis

Statistical analysis was performed using Stata 15.1 IC for Windows (StataCorp, USA). Because of the small sample sizes of each sampling site, only non-parametric analysis was used. Kruskal-Wallis tests with post hoc Dunn's tests were performed to compare the concentrations of AR and ARB between the sampling sites. A global $\alpha = 0.05$ was considered and a Bonferroni-Holm alpha-correction for the post hoc testing was performed. Associations between the concentration of AR and ARB were tested using a linear regression model that was adjusted for sampling sites and sampling campaign.

3. Results and discussion

3.1. Antibiotic residues

During the sampling period, AR could be found in the investigated surface waters downstream of two municipal STPs in a range between 0.01 μg/L and 0.60 μg/L. Residues of at least one antibiotic could be found at sampling sites B, D, E, F, and G above the respective LOQ (Table 1). At sampling site A, the metabolite anhydroerythromycin was found once below the LOQ. No AR were found at sampling site C during the entire sampling period. The complete results of the detected substances are presented in Table 4.

Macrolide antibiotics (especially azithromycin, clarithromycin, and roxithromycin) were the most frequently detected substances in addition to clindamycin, sulfamethoxazole, and trimethoprim. These results are in line with earlier studies, which showed that macrolide antibiotics, clindamycin, and sulfamethoxazole are mostly found in surface waters downstream of point sources like municipal STPs (Watkinson et al., 2009; Voigt et al., 2019b). An incomplete metabolism within treated organisms and an insufficient degradation or removal by the STP can explain the high frequency of detection of these substances (Kümmerer, 2009).

These antibiotics are also frequently prescribed in Germany in inpatient and outpatient settings (Schwabe and Paffrath, 2016). For example, clarithromycin is commonly prescribed to treat infections of the respiratory tract, gastrointestinal system, or the ear, nose and throat area (Peters and Clissold, 1992). This correlates with the high outpatient prescription volume in Germany of 15.2 million DDD (defined daily doses) in 2015 (Schwabe and Paffrath, 2016). As a result, residues of these antibiotics can be explained without hospitals and clinics discharging effluent in the catchment areas of the respective STP. Thus, the measured residues of outpatient prescribed antibiotics can be caused by the discharge of both STPs (inhabitant equivalent = 12,000; Table 1) in the investigated municipal catchment area. Moreover, no residues of clinically relevant antibiotics (e.g., meropenem, vancomycin, ciprofloxacin, linezolid and piperacillin) or veterinary antibiotics (e.g., chlortetracycline, enrofloxacin and tylosin) were detected. This might be due to a lack of hospitals in the area. In addition, the catchment area is not characterized by e.g. large-scale agriculture and intensive livestock farming, which are possible pathways of veterinary AR and ARB into the aquatic environment (Hamscher et al., 2002; Christian et al., 2003; Ratsak et al., 2013; Walczak and Xu, 2011). This does not exclude a small grassland area used as grazing land for a small group of cattle.

Despite the high prescription volume of beta-lactams in Germany (inpatient and outpatient), no residues of these antibiotics (e.g. penicillins or cephalosporins) were detected. This can be explained by high removal rates of beta-lactams within municipal STPs by hydrolysis (Deshpande et al., 2004; Michael et al., 2013). In addition, no residues of fluoroquinolones have been detected, although they belong to one of the most frequently prescribed substance classes in Germany (Schwabe and Paffrath, 2016). Former studies have also shown that these substances can be adsorbed to organic matter such as sewage or soil (Golet et al., 2003; Martinez, 2009; Kümmerer, 2009). This might explain why other studies have found that fluoroquinolones were detected more frequently and at much higher residual concentrations in STP influents than effluents (Voigt et al., 2020; Kümmerer, 2003; Lindberg et al., 2005).

In general, both the highest residual concentrations and greatest diversity of antibiotics were found at sampling site B, which was located downstream of the discharge of STP I. In comparison, the detected concentrations were ten times lower at site D, which was downstream of the discharge of STP II. The difference in antibiotic concentration might be explained by the higher amount of wastewater treated in STP I than in STP II. It should also be noted that sampling site B is located directly at the discharge point of STP I, which resulted in an "insufficient" mixing with fresh water. Fig. 2 shows the reduction of total AR concentration along the stream, and that only traces of antibiotics could be detected in the creek downstream of the drinking water reservoir. The decrease of AR between sampling sites can be explained by dilution with freshwater of other smaller branches or

Table 3
List of primers used for the detection of resistance genes, including carbapenemase encoding genes and a colistin resistance gene (*mcr*). Legend: BHQ, Black Hole Quencher®; ROX, 6-carboxyl-X-rhodamine; FAM, fluorescein; HEX, hexachloro-fluorescein; Cy5, cyanine 5.

Primer	Sequence	References
KPC-F	GCA GCG GCA GCA GTT TGT TGA TT	Swayne et al. (2013)
KPC-R	GTA GAC GGC CAA AAT AGG TGC	Swayne et al. (2013)
KPC-Probe	FAM-CAG TCG GAG ACA AAA CCG GAA CCT GC-BHQ1	Swayne et al. (2013)
OXA48-F	TTC GGC CAC GGA GCA AAT CAG	Swayne et al. (2013)
OXA48-R	GAT GTG GGC ATA TCC ATA TTC ATC GCA	Swayne et al. (2013)
OXA48-Probe	HEX-CTG GCT GCG CTC CGA TAC GTG TAA CTT ATT G-BHQ1	Swayne et al. (2013)
SME-F	TGT AGG TGA CAA RAC TGG GAG CTG TG	Swayne et al. (2013)
SME-R	GCA ATA CGT GAT GCT TCC GCA ATA G	Swayne et al. (2013)
SME-Probe	ROX-CGG CAT AAT CAT TCG CA-BHQ2	Swayne et al. (2013)
IMI-F	GAG GGT ATG ACT AAA TTC ATG CGG TCG A	Swayne et al. (2013)
IMI-R	GCA GGT GTA GAT GTG TCA CGY TCA TCG	Swayne et al. (2013)
IMI-Probe	Cy5-CGT TGG GAG TTA GAT C-BHQ2	Swayne et al. (2013)
IMP-F	CCC ACG TAT GCA TCT GAA TTA ACA AA	Swayne et al. (2013)
IMP-R	CCA AAC CAC TAC GTT ATC TTG AGT G	Swayne et al. (2013)
IMP-Probe	Cy5-CAA GCT AMA WAT TCA TTT AGC GGR GYT ARC TAT T-BHQ2	Swayne et al. (2013)
VIM-F	GAT GAG TTG CTT TTG ATT GAT ACA GC	Swayne et al. (2013)
VIM-R	CCG ACK CGR TCG TCA T	Swayne et al. (2013)
VIM-Probe	FAM-TCG CGG AGA TTG ARA AGC AAA TTG GA-BHQ1	Swayne et al. (2013)
NDM-F	CCC GAC GAT TGG CCA	Swayne et al. (2013)
NDM-R	ATC CAG TTG AGG ATC TGG GC	Swayne et al. (2013)
NDM-Probe	ROX-ACC GAA TGT CTG GCA GCA CAC TTC-BHQ2	Swayne et al. (2013)
SPM-F	AGG CAA GGT CTT CTC GTT TT	Swayne et al. (2013)
SPM-R	GCA TCT CCC AGA TAA CCA AGT T	Swayne et al. (2013)
SPM-Probe	FAM-TCG CCC GAT AAT GTC GTC GTA TAT T-BHQ1	Swayne et al. (2013)
SIM-F	CGG AAG AAG CCC AGC	Swayne et al. (2013)
SIM-R	GTT TTT TAA CGA TGC CGA ATC CCT TG	Swayne et al. (2013)
SIM-Probe	HEX-AAG GGA TCT ATC TTC ATA CAT CTT TTC AAG A-BHQ1	Swayne et al. (2013)
GIM-F	TTG GTC TGA AGA AGA CAC GAA G	Swayne et al. (2013)
GIM-R	GTA GGA ACC GGC TTT CCT T	Swayne et al. (2013)
GIM-Probe	ROX-ATC GCA CTG CTG GTA TCA AGT TGC TA-BHQ2	Swayne et al. (2013)
IMI$-F	GCG ATG AAC GTG ACA CAT CT	Müller et al. (2018)
IMI$-R	ATC GCT TGG TAC GCT AGC AC	Müller et al. (2018)
IMI$-Probe	Cy5-GAA AAC CCT TGC ACT GGG TA-BHQ2	Müller et al. (2018)
OXA-23_F	GAA GGG CGA GAA AAG GTC A	Müller et al. (2018)
OXA-23_R	TCA GCA TTA CCG AAA CCA ATA C	Müller et al. (2018)
OXA-23_ Probe	FAM-CGG TCT TGA TCT CAT GCA AA-BHQ1	Müller et al. (2018)
OXA-24_F	TTG GCC CCC TTA AAA TTA CAC	Müller et al. (2018)
OXA-24_R	AAC ACC CAT TAC CCA TCC AC	Müller et al. (2018)
OXA-24_ Probe	HEX-TGA CCT TGC ACA TAA CCG AT-BHQ1	Müller et al. (2018)
OXA-51_F	TTT TAT TTC AGC CTG CTC ACC	Müller et al. (2018)
OXA-51_R	ATA CTC GGT CGA AGC ACG AG	Müller et al. (2018)
OXA-51_ Probe	ROX-CAA ATC ACA GCG CTT CAA AA-BHQ2	Müller et al. (2018)
OXA-58_F	CAC GCA TTT AGA CCG AGC A	Müller et al. (2018)
OXA-58_R	TGG CTT TCC ATC CCA CTT	Müller et al. (2018)
OXA-58_ Probe	Cy5-AAA ACA GCT TAT ATT CCT GCA TCT-BHQ2	Müller et al. (2018)
MCRscreen 1/2_F	ACA TCG ACG GCG TAT TCT GT	Müller et al. (2018)
MCRscreen 1/2	TCC ATC ACG CCT TTT GAG TC	Müller et al. (2018)
MCRscreen 1/2	Cy5-TGA TGT CGA TAC CGC CAA ATA CC-BHQ2	Müller et al. (2018)

drainages as well as the continuous degradation within the surface water, e.g. photodegradation, hydrolysis and sorption (Kümmerer, 2009; Baquero et al., 2008).

The results of this study indicate that STP I and STP II influence the surface waters downstream of the STP effluents significantly. Thus, significant differences could be observed for the comparison of sampling site A and B ($p = 0.0000$) as well as C and D ($p = 0.0001$). Nevertheless, no significant differences could be obtained between sampling site A and F as well as C and F. Therefore, the impact of STP I and STP II seem to be reduced along the surface water, which indicates that no impact on the drinking water reservoir is expected. In addition, no differences were observed for sampling sites A compared to G and C compared to G. In conclusion, no significant differences were observed between the sampling sites upstream of the STP effluents, which are mostly characterized by forest areas, detached houses and a small number of small grasslands and sampling site G.

A comparable study showed that, despite a detection of residues of sulfamethoxazole (up to 2.00 μg/L), roxithromycin (up to 0.35 μg/L) and clindamycin (up to 0.01 μg/L) in surface water samples, no AR could be detected in drinking water obtained from reservoirs influenced by these rivers (Watkinson et al., 2009). In addition, a more recent study showed that advanced drinking water purification processes like ozonation and granular activated carbon filtration as well as conventional techniques reduced AR like sulfamethoxazole, roxithromycin and chlortetracycline (up to 45 ng/L in the source water) below their limits of detection (Simazaki et al., 2015).

It also has to be considered that oxidative purification processes (chlorination as well as ozonation) can generally lead to the formation of disinfection by-products (DBP) or transformation products (TP) of organic pollutants. In some cases, TP and DBP can be more relevant according to ecotoxicological properties than the original substances, as shown for iopamidol (used as an X-ray contrast agent) (Matsushita et al., 2015; Simazaki et al., 2015). The formation of TP of macrolide antibiotics and sulfonamides within the WW treatment process and their relevance for the aquatic environment could be demonstrated (Majewsky et al., 2014; Senta et al., 2019). Further research is needed to assess the toxicology risk of DBP and TP of antibiotics and other pharmaceuticals during advanced drinking water purification

Table 4
Detected antibiotic residues - the obtained minimal concentration (Min.), maximum concentration (Max.), median ("-" = conc. < LOD) and detection frequency ($n_{residual\ concentration > LOQ}$) during the sample campaign; in sampling sites A and C, no antibiotic residues were detected. Legend: dh-Erythromycin, anhydroerythromycin.

Sampling sites	B (n = 7)				D (n = 7)				E (n = 7)				F (n = 7)				G (n = 7)			
Antibiotic	Min. [µg/L]	Max. [µg/L]	Median [µg/L]	Frequency [%]	Min. [µg/L]	Max. [µg/L]	Median [µg/L]	Frequency [%]	Min. [µg/L]	Max. [µg/L]	Median [µg/L]	Frequency [%]	Min. [µg/L]	Max. [µg/L]	Median [µg/L]	Frequency [%]	Min. [µg/L]	Max. [µg/L]	Median [µg/L]	Frequency [%]
Azithromycin	0.08	0.55	0.14	85.7	0.02	0.22	0.02	71.4	0.01	0.03	0.02	57.1	0.02	0.02	0.02	14.3	0.01	0.02	0.02	42.9
Clarithromycin	0.05	0.60	0.22	100	0.02	0.08	0.06	57.1	0.01	0.04	0.02	85.7	0.02	0.02	0.02	14.3	–	–	–	–
Clindamycin	0.04	0.34	0.23	100	0.01	0.05	0.03	85.7	0.01	0.06	0.03	85.7	0.01	0.03	0.02	85.7	–	–	–	–
Erythromycin	0.02	0.08	0.04	85.7	0.01	0.03	0.02	57.1	0.01	0.01	0.01	28.6	–	–	–	–	0.01	0.01	0.01	14.3
dh-Erythromycin*	0.02	0.17	0.06	85.7	0.02	0.05	0.03	42.9	0.01	0.03	0.02	42.9	0.02	0.02	0.02	28.6	0.01	0.01	0.01	28.6
Roxithromycin	0.01	0.07	0.03	42.9	0.01	0.03	0.02	28.6	0.01	0.01	0.01	14.3	–	–	–	–	–	–	–	–
Sulfadoxine	0.04	0.04	0.04	14.3	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Sulfamethoxazole	0.03	0.40	0.25	100	0.01	0.17	0.04	42.9	0.01	0.09	0.02	71.4	0.02	0.08	0.05	28.6	–	–	–	–
Trimethoprim	0.06	0.39	0.16	100	0.05	0.05	0.05	14.3	–	–	–	–	–	–	–	–	–	–	–	–

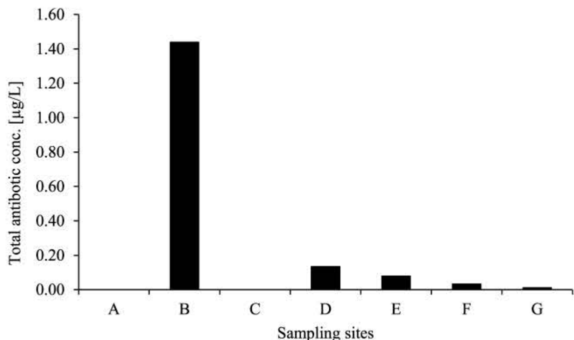

Fig. 2. Median of the total antibiotic concentration during the complete sampling period.

processes.

As a first assessment related to increased selection pressure in favor of ARB, the detected residual concentrations were compared with the PNEC by Bengtsson-Palme and Larsson (2016). The only observed exceedance of the PNECs was observed for clarithromycin (at three sampling days; PNEC 0.25 µg/L) and azithromycin (at two sampling days; PNEC 0.25 µg/L) at sampling site B. These concentrations did not reach the MIC concentration. Consequently, selection pressure for bacteria towards those antibiotic substances or classes should not be disqualified but should also not be presumed.

3.2. *Multidrug-resistant organisms*

Forty-six surface water samples from seven different sampling sites were analyzed for ARB. Samples from sampling sites A, B, E and F were collected seven times and samples from sampling sites C, D and G were collected six times. ARB could be isolated at every sampling site during the sampling period. A total of 113 different isolates of clinically relevant ARB were detected overall. In six of seven sampling points, at least one isolate (predominantly *E. coli* and VRE) could be characterized as MDR according to Magiorakos et al. (2012). No MDR bacteria could be found at sampling point C. In general, no XDR or pan-resistant bacteria could be identified over the entire sampling period (Magiorakos et al., 2012). *P. aeruginosa*, *E. coli*, *A. calcoaceticus-baumannii* (ACB) complex as well as *E. faecium* and other *Enterobacteriales* (including *Klebsiella* spp. *Citrobacter* spp. and *Enterobacter* spp.) with different resistance patterns could be isolated. MRSA was not detected in any of the samples. The distribution of ARB over the entire study and for all sampling sites is given in Fig. 3.

In analogy to the chemical analysis, the median of the total bacterial count of resistant bacteria was used for the examination of the individual sampling points. The highest concentration of ARB (335 cfu/100 mL) was found at sampling site B (directly after the discharge of the effluent from STP I). Sampling sites A and C respectively showed significantly lower concentrations of 1 cfu/100 mL and 6 cfu/100 mL since they are upstream from the discharge of STP I and II (Fig. 1). These results are in line with recent publications identifying STP effluents as a main entry pathway of ARB into surface waters (Hembach et al., 2019; Müller et al., 2018; Rizzo et al., 2013).

Overall, the contamination of ARB and residual concentration of antibiotics decreased the further each sampling point was from the point sources (effluents of STP I and STP II). In accordance with the chemical analysis, significant differences between sampling sites A and B ($p = 0.0021$) as well as C and D ($p = 0.0060$) could demonstrate the impact of treated wastewater to the receiving surface water. Thus, the total concentration of ARB at sampling site G (2 cfu/100 mL; surface water downstream of the drinking water reservoir) was comparable to upstream sampling sites (A and C) and no significant differences could

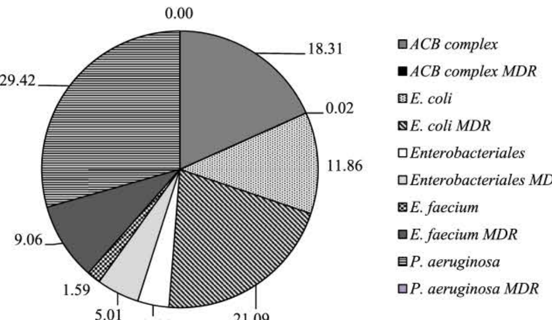

Fig. 3. Percentage of the total distribution of all antibiotic-resistant bacteria regardless of sampling date and sampling site, sorted by species and resistance status according to (Magiorakos et al., 2012). Legend: ACB complex = *Acinetobacter calcoaceticus-baumannii* complex; *E. coli* = *Escherichia coli*; *E. faecium* = *Enterococcus faecium*; *P. aeruginosa* = *Pseudomonas aeruginosa*.

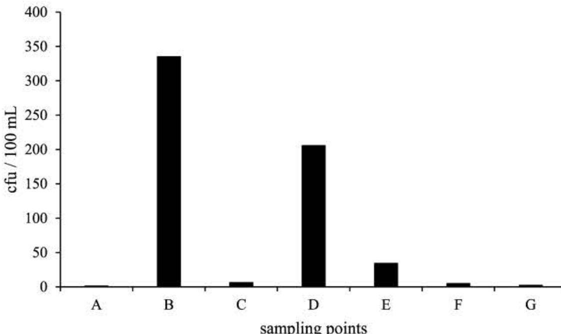

Fig. 4. Median of the total bacterial count of characterized bacteria during the complete sampling period.

be observed. In addition, no statistically significant association between AR and ARB ($F_{13,32}$ = 1.99, p = 0.0568) could be found (Table S2). However, a lack of statistical power due to small sample size and should be taken into account. The median of the total cell counts of ARB are shown in Fig. 4.

A higher concentration of MDR *E. coli* could be detected at sampling site E (947 cfu/100 mL) compared to site D (267 cfu/100 mL), which is closer to the discharge of STP I and II (Fig. 1). This diffuse entry of *E. coli* MDR, which serves as a typical fecal indicator, could be explained by a cattle pasture area located next to this sampling site. Therefore, the excrements of these animals could be an entry pathway for MDR *E. coli* as analyzed in prior studies on manure and excrement of dairy and cattle farms (Karczmarczyk et al., 2011; Walczak and Xu, 2011). Interestingly, these differences are not visible in an increasing concentration of AR. This could be explained by a missing antibiotic treatment during the sampling period. Thus, the colonized cattle would excrete MDR *E. coli*, but no AR.

Despite the presence of ARB at the different sampling sites, it has to be considered that the surface water will be further treated before it is used as fresh and drinking water. In this context, Stange et al. (2019) determined a 5.6 log reduction rate for ARB by conventional DW purification processes operating on chlorination (0.5 mg/L free chlorine). The best treatment for a reduction of ARB (5.0 log reduction) and ARG (up to 4.6 log reduction) were obtained using 1 mg/L ozone (Stange et al., 2019) however, the effectiveness of conventional and advanced disinfection methods for the reduction of ARG and ARB depended of the respective species, genes, specific disinfection methods and a possible co-selection of antibiotic resistance caused by the formation of DBPs (Sanganyado and Gwenzi, 2019).

3.2.1. Resistance patterns of MDR bacteria

The resistance data refers to all sampling points throughout the whole sampling period and characterized isolates, which is independent of the total bacterial count. In case of ACB-complex bacteria, all but one isolate displayed no MDR resistance. The MDR strain was detected in sampling site D and included resistance or intermediate resistance towards piperacillin/tazobactam (P/T), cefotaxime (CEFO), ceftazidime (CEFTA) and trimethoprim-sulfamethoxazole (T/S). For *E. coli*, 11 of 30 bacterial isolates were characterized as MDR. All 11 strains were tested resistant towards CEFO, CEFTA, and T/S. 10 of 11 isolates displayed an additional resistance or intermediate resistance towards ciprofloxacin (CIP), while one was tested resistant towards chloramphenicol (CHL). Of the other representatives of *Enterobacteriales* 12 of 24 characterized isolates were classified as MDR. Of these 12 isolates, the predominant genera were *Klebsiella* spp. (6) and *Enterobacter* spp. (5). All *Klebsiella* isolates were tested resistant for CEFO and CIP. Other resistances considered the substances P/T, CHL, and T/S. In case of *Enterobacter* spp. the resistance pattern varied widely including the substances P/T, CEFO, CEFTA, CIP, CHL, T/S and fosfomycin. No MDR isolate was identified with regard to *P. aeruginosa*. In the gram-positive spectrum 10 out of 11 *E. faecium* isolates were classified as MDR with a resistance towards ampicillin, vancomycin and daptomycin. In addition, all strains were susceptible towards high level gentamycin, tigecycline and linezolid. Most of the MDR isolates (8) were detected in sampling site B.

3.3. Antibiotic resistance genes

In total, 46 water samples were analyzed for the detection of clinically relevant resistance genes (carbapenemase genes as well as *mcr*). In seven water samples, at least one ARG could be identified. Altogether, six water samples tested positive for $bla_{OXA\text{-}58}$ at sampling site B (3), D (1) and E (2). This globally widespread gene encoding OXA-58 is mostly found in *A. baumannii* (Coelho et al., 2006; Poirel et al., 2005). The occurrence of this ARG seems to be WW associated since sampling sites B, D and E are all downstream of the respective STPs, which is in line with earlier studies (Rizzo et al., 2013).

Surprisingly, one water sample tested positive for an ARG mediating colistin resistance (*mcr*). At sampling site C, no municipal or livestock influence was expected. Possible explanations could be an entry of ARG into surface water via wild animals (e.g., birds) (Bonnedahl and Järhult, 2014) or private households that did not discharge their WW into the municipal sewage system (Müller et al., 2018). Furthermore, ARG has also been isolated from regions that have not been influenced by humans or anthropogenic activities (Bhullar et al., 2012; D'Costa et al., 2011).

The content of ARG in surface water will be further reduced during drinking water purification processes, as shown in recent studies (Sanganyado and Gwenzi, 2019; Stange et al., 2019). That said, the use of advanced purification techniques should not justify neglecting the proper protection of source water.

3.4. Consequences for risk management

A monitoring program for antibiotic-resistant pathogens is proposed as part of the water safety program using a risk assessment approach to promote drinking water quality. Such an approach should be culture-based accompanied by the analysis of AR and ARG to establish a data basis for risk assessment and risk management.

Risk assessments for antibiotic resistance in the raw water in drinking water reservoirs and surface-influenced drinking water systems differ from each other. In this study, no WW was discharged from maximum care hospitals or small clinics into the municipal STPs (I and II, Fig. 1). Therefore, the contamination with AR, ARG and ARB in water systems influenced by clinical WW may differ from the results of

this study since hospital WW is noticeably different from urban WW (Fatta-Kassinos et al., 2011; Voigt et al., 2020).

Furthermore, it must be taken into account that the detected level of ARG, ARB and AR in surface waters could change due to seasonal effects. For instance, Heß et al. (2018) showed that a resuspension of river sediment could lead to an increased bacterial abundance in the aqueous phase, since significantly higher bacterial levels were detected in the sediment compared to the aqueous phase. The specific risk of ARB and ARG input by mobilization of sediment was assessed as "moderate" (Heß et al., 2018). Therefore, mobilization of sediment by rain might cause an increased bacterial load in the stream investigated in the presented study. In addition, heavy rainfall can lead to an increased input of pollutants (e.g., sulfamethoxazole, diclofenac, carbamazepine, pesticides or heavy metals) and fecal bacteria through e.g. combined sewer overflows or the overflow of separate sanitary sewer systems, since STPs can be overloaded by the increased volume flows (Ruppelt et al., 2020; McLellan et al., 2007). For example, McLellan et al. (2007) showed that the detected level of *E. coli* in Lake Michigan was significantly higher after heavy rainfall, which was explained by the input from combined sewer overflows and sanitary sewer overflows. Future investigations of other reservoirs should take sediment mobilization as well as combined sewer overflows into account. This could not be done in the presented study, as there was no representative number of samples available due to the low rainfall events during the investigation period.

The results of this study should still provide an orientation and help inform an approach for the risk assessment in this specific issue. The basis for risk management should be the culture-based findings of antibiotic-resistant pathogens using *E. coli* as a possible indicator. Analysis of AR and ARG would give added value to the risk assessment and risk management.

4. Conclusion

In this study, surface water used for the production of drinking water was examined for ARB, AR and ARG. The highest concentrations of AR and ARB were found near the discharge points of the municipal STP. Predominantly *E. coli* and *E. faecium* could be isolated and characterized as MDR. Furthermore, no isolates were characterized as MRSA, XDR or pandrug-resistant. The concentrations of AR and ARB were also significantly reduced downstream through dilution or degradation. No significant differences could be recognized between the surface water before the STP discharge and the river beyond the drinking water reservoir. However, the detection of genes encoding for carbapenemases ($bla_{\text{OXA-58}}$) should be further observed in accordance with a proper antibiotic resistance surveillance system. Positive results may be different for other surface waters, as the catchment area in this study was not characterized by clinical WW or the influence of intensive livestock farming. Thus, in special cases, an advanced WW treatment could be necessary to reduce the entry or occurrence of ARG, ARB and AR in surface waters used for drinking water purification to advance the multi-barrier principle and to follow a hazard analysis with critical control points (HACCP) concept, respectively.

Declaration of competing interest

The authors declare no conflict of interest. This study complies with the ethical guidelines of the declaration of Helsinki by the "world medical association" from 1964. The ethics committee of the Medical Faculty of the University of Bonn was involved and approved the procedures and the publication of the results (reference no. 120/16).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2020.113449.

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Appendix: "The investigation of antibiotic residues, antibiotic resistance genes and antibiotic-resistant organisms in a drinking water reservoir system in Germany"

Bacteria	Antbiotic class	Antibiotic substance
Enterococcus spp.	Aminoglycosides	**Gentamycin (high level)**
	Glycopeptides	**Vancomycin**, **Teicoplanin**
	Glycylcylclines	**Tigecycline**
	Lipopeptides	**Daptomycin**
	Olyzolidinones	**Linezolid**
	Penicillins	**Ampicillin**
Acinetobacter baumannii	Aminogylcosides	Gentamycin, tobramycin, **amikacin**, netilmicin
	Antipseudomonal carbapenems	**Imipenem**, **meropenem**, doripenem
	Antipseudomonal fluorquinolones	**Ciprofloxacin**, **levofloxacin**
	Antipseudomonal penicillins + β-lactamase inhibitors	**Piperacillin-tazobactam**, Ticarcillin-clavulanic acid
	Extended-specrum cephalosporins	**Cefotaxime**, cetriaxone, **ceftazidime**, cefepime
	Folate pathway inhibitors	**Trimethoprim-sulfamethoxazole**
	Polymyxins	**Colistin**
Pseudomonas aeruginosa	Aminogylcosides	Gentamycin, tobramycin, **amikacin**, netilmicin
	Antipseudomonal carbapenems	**Imipenem**, **meropenem**, doripenem
	Antipseudomonal cephalosporins	**Ceftazidime**
	Antipseudomonal fluorquinolones	**Ciprofloxacin**, **levofloxacin**
	Antipseudomonal penicillins + β-lactamase inhibitors	**Piperacillin-tazobactam**, Ticarcillin-clavulanic acid
	Phosphonic acids	**Fosfomycin**
	Polymyxins	**Colistin**, Polymyxin B
Enterobacterales	Aminogylcosides	Gentamycin, tobramycin, **amikacin**, netilmicin
	Antipseudomonal penicillins + β-lactamase inhibitors	**Piperacillin-tazobactam**
	Carbapenems	Ertapenem, **imipenem**, **meropenem**, doripenem
	Extended-specrum cephalosporins	**Cefotaxime** or cetriaxone, **ceftazidime**, cefepime
	Fluorquinolones	**Ciprofloxacin**
	Folate pathway inhibitors	**Trimethoprim-sulfamethoxazole**
	Glycylcyclines	**Tigecycline**
	Phenicols	**Chloramphenicol**
	Phosphonic acids	**Fosfomycin**
	Polymyxins	**Colistin**

Table S1 Definition of multidrug-resistant (MDR) organisms according to Magiorakos et al. 2012.

Linear regression

Source	SS	df	MS
Model	1577852.28	13	121373.252
Residual	1956205.55	32	61131.4234
Total	3534057.83	45	78534.6184

Number of obs	=	46
F(13, 32)	=	1.99
Prob > F	=	0.0568
R-squared	=	0.4465
Adj R-squared	=	0.2216
Root MSE	=	247.25

ARB	Coef.	Std. Err.	t	P>t	Lower CI	Upper CI
AR	-87.28821	194.4016	-0.45	0.656	-483.2714	308.6949
Sampling site						
2	512.7121	279.4742	1.83	0.076	-56.55824	1081.982
3	20.00265	138.8801	0.14	0.886	-262.887	302.8923
4	259.8989	141.4482	1.84	0.075	-28.22161	548.0193
5	175.7085	133.4398	1.32	0.197	-96.09941	447.5164
6	57.74814	139.0239	0.42	0.681	-225.4343	340.9306
7	-5.436682	132.1836	-0.04	0.967	-274.6859	263.8125
Sampling campaign						
2	-9.088121	159.0085	-0.06	0.955	-332.9779	314.8016
3	14.29516	159.7811	0.09	0.929	-311.1682	339.7585
4	-211.2614	158.7703	-1.33	0.193	-534.6659	112.1432
5	-251.907	159.4164	-1.58	0.124	-576.6276	72.81354
6	-183.9272	158.4621	-1.16	0.254	-506.704	138.8495
7	-211.2229	164.3204	-1.29	0.208	-545.9325	123.4868
Constant term	139.0159	148.0249	0.94	0.355	-162.501	440.5328

Table S2 Results of the statistical analysis (Linear regression calculated with STATA 15.1 IC). A simple raw spearman coefficient calculation between AR and ARB leads to r_s=0.6273, p=0.000. Under stratification for sampling site, no significance in the r_s is left.

4.2. Zusammenfassung

Die in diesem Kapitel vorgestellte Arbeit beschäftigte sich mit der Untersuchung eines Oberflächengewässers, welches in eine zur Trinkwassergewinnung dienende Talsperre mündet. Flussabwärts der Einleitung zweier kommunaler Kläranlagen konnten sowohl AR, ARB als auch ARG nachgewiesen werden. Hauptsächlich konnten häufig ambulant eingesetzte bzw. verschriebene Antibiotika wie Azithromycin (max. 0,55 µg/L), Clarithromycin (max. 0,60 µg/L), Clindamycin (max. 0,34 µg/L), Sulfamethoxazol (max. 0,40 µg/L) und Trimethoprim (max. 0,39 µg/L) nachgewiesen werden. Die höchsten Rückstandskonzentrationen wurden unmittelbar nach Kläranlageneinleitung nachgewiesen. Im weiteren Flussverlauf war zu dem eine deutliche Reduzierung der Belastung mit AR festzustellen, sodass lediglich Rückstände an Makrolid-Antibiotika sowie Clindamycin im Oberflächengewässer nach der Trinkwassertalsperre sporadisch (Nachweisfrequenz < 50%) und in deutlich niedrigeren Konzentrationen (0,01 µg/L bis 0,02 µg/L) gefunden wurden.[8]

Einen vergleichbaren Trend ergaben die mikrobiologisch-kulturellen Verfahren, sodass keine signifikanten Unterschiede in der Gesamtkonzentration an ARB zwischen den Probeentnahmestellen vor den Kläranlageneinleitungen und der Probeentnahmestelle nach der Talsperre festgestellt werden konnten. In sechs von sieben Probeentnahmestellen wurden MDR Bakterien, überwiegend *E. coli* und *E. faecium* gefunden. Jedoch konnten keinerlei MRSA, XDR oder pan-resistenten Bakterien nachgewiesen werden. Ferner konnten sieben klinisch relevante ARG (sechsmal bla_{OXA58}, einmal *mcr*) über den Untersuchungszeitraum gefunden werden, wobei *mcr* oberhalb der Kläranlageneinleitungen nachgewiesen wurde.[8]

Die Ergebnisse dieser Studie können eine Orientierung bzw. eine zukünftige Hilfestellung bei der Risikobewertung von ARB, AR und ARG in Oberflächengewässer mit besonderem Expositionsrisiko (am Beispiel Trinkwassertalsperre) darstellen.

5. Gesamtfazit und Ausblick

Die in dieser Promotionsarbeit zusammengefassten Arbeiten zeigen den Bedarf an neuen instrumentellen Analyseverfahren zur simultanen Bestimmung von Rückständen antibiotisch wirksamer Substanzen. Die entwickelte LC-MS/MS-Multimethode zur Bestimmung von 45 verschiedenen Antibiotika aus elf Substanzklassen und zwei umweltrelevanten Metaboliten stellt in Zukunft in Kombination mit den in HyReKA entwickelten kulturellen Verfahren und molekulargenetischen Analyseverfahren ein geeignetes Werkzeug für die Untersuchung aquatischer Umweltproben auf eine etwaige Belastung mit AR, ARB und ARG dar.[5,74,148]

Die erreichte Empfindlichkeit der einzelnen Analyten erlaubt hierbei eine Bewertung der chemischen Untersuchungsergebnisse mit denen von Bengtsson-Palme und Larsson vorgeschlagenen $PNEC_{Res}$ bezüglich der Selektion von ARB.[3,5] Eine Ausnahme stellt hierbei Ciprofloxacin dar, da lediglich die Nachweisgrenze unterhalb des $PNEC_{Res}$ von 64 ng/L liegt ($LOD_{Ciprofloxacin}$: 31 ng/L; $LOQ_{Ciprofloxacin}$: 77 ng/L).[3,5] Des Weiteren werden die für Amoxicillin, Ciprofloxacin und Makrolid-Antibiotika gemäß Artikel 8b der RL 2008/105/EG in Verbindung mit dem Anhang des Durchführungsbeschlusses (EU) 2018/840 höchstzulässigen Nachweisgrenzen erreicht, die zur Ermittlung von hochwertigen Daten zur Risikobewertung bzw. zur Schaffung ausreichender Überwachungsdaten potentiell prioritärer Stoffe in Oberflächengewässer gefordert werden.[5,160,161]

In zukünftigen Experimenten sollte darauf aufbauend versucht werden, die Empfindlichkeit insbesondere für Ciprofloxacin zu verbessern, um auch in diesem Falle eine Bestimmungsgrenze unterhalb des spezifischen $PNEC_{Res}$ zu erreichen. Dies erscheint insbesondere für Fluorchinolone von Bedeutung, da bereits gezeigt werden konnte, dass die Einführung von Fluorchinolonen in der Human- und Veterinärmedizin vor circa 30 Jahren im Zusammenhang mit der Enwicklung und Verbreitung von z.B. Fluorchinolon-resistenten *Campylobacter* spec., *P. aeruginosa* oder *E. coli* steht.[58,162–164] Eine denkbare Möglichkeit könnte diesbezüglich die Erhöhung des Injektionsvolumens durch den Austausch der Injektionsschleife darstellen.[165,166]

Jedoch muss beachtet werden, dass eine Erhöhung des Injektionsvolumens gegebenenfalls eine Verschlechterung der Wiederfindung bewirken könnte, da neben einer größeren Menge an Analytmolekülen auch verstärkt Matrixbestandteile injiziert werden würden, wodurch die Chromatographie bzw. Ionisation der Analyten gestört werden könnte. Daher sollte zudem überprüft werden, inwiefern sich die Matrixeffekte unter Erhöhung des Injektionsvolumens verändern, z.B. durch eine Injektion von aufbereiteter Leermatrix und einer kontinuierlichen *post-column*-Flussinjektion einer Standardlösung.[167]

Nicht im Untersuchungsspektrum mit aufgenommen werden konnte Colistin, welches zu den wichtigsten und am häufigsten eingesetzten Veterinärantibiotika weltweit zählt.[5,28,44,168,169] Zudem nahm in den letzten Jahren die Bedeutung von Colistin als „Reserve-Antibiotikum" bei der Behandlung von schwerwiegenden nosokomialen Infektionen mit multiresistenten gramnegativen Bakterien, allen voran Carbapenem-resistenten Erregern, in der Humanmedizin zu.[170] Gerade vor dem Hintergrund der Entdeckung einer mobilen Plasmid-vermittelten Colistin-Resistenz (*mcr*-1) erscheint die Untersuchung von Colistin-Rückständen in der aquatischen Umwelt unabhängig des Eintragsweges (Human- oder Veterinärmedizin) als wünschenswert,[171] um etwaige Punktemittenten identifizieren und das Risiko eines resultierenden Selektionsdruckes zugunsten Colistin-resistenter Bakterien abschätzen zu können. Die Umweltrelevanz konnte bereits anhand des Nachweises des *mcr*-1 Genes in Abläufen kommunaler Kläranlagen gezeigt werden.[148] Erste Voruntersuchungen zeigten, dass Colistin nicht mit der in dieser Arbeit entwickelten Multimethode zuverlässig erfasst werden konnte. Eine mögliche Erklärung könnte der verglichen mit anderen Studien niedrige Acetonitril-Anteil (20%) im organischen Eluenten sein,[5] da in der Literatur meist 100% Acetonitril (plus ggf. Ionenmodifyer) als organischer Eluent verwendet wurde.[172,173] Ferner legen erste Ergebnisse nahe, dass es zu einer möglichen Adsorption des Analyten an Glasvials kommen könnte, was zur Folge hätte, dass bei der Analytik von Colistin auf silanisierte Vials und Probeentnahmegefäße aus Kunststoff zurückgegriffen werden müsste. In Zukunft sollte versucht werden, Colistin ins Untersuchungsspektrum zu implementieren bzw. eine zusätzliche Analysenmethode für Colistin in Umweltkompartimenten zu entwickeln, da bisherige Methoden sich auf die Untersuchung in Gülle bzw. Plasma beschränken.[172–175]

Um die Leistungsfähigkeit und Eignung der entwickelten Multimethode zur Untersuchung diverser aquatischer Matrices weiter abzusichern, sollte zudem die Teilnahme an weiteren Laborvergleichsuntersuchungen mit zertifiziertem Referenzmaterial in Zukunft durchgeführt werden.

Darüber hinaus zeigte die Untersuchung verschiedener Kläranlagenzuläufe und -abläufe für spezifische AR eine erhöhte Wahrscheinlichkeit des gleichzeitigen Nachweises spezifischer ARB bzw. ARG. Dieses Phänomen war besonders ausgeprägt für klinisch beeinflusstes Abwasser.[7]

Die Ergebnisse zeigen in Übereinstimmung mit vorherigen Studien, dass es merkbare Unterschiede zwischen Klinikabwasser und kommunalem Abwasser bezüglich der Diversität bzw. der Rückstandskonzentration antibiotisch wirksamer Substanzen gibt.[7,134,176,177] Es sollte jedoch berücksichtigt werden, dass lediglich die als klinisch relevant definierten Kombinationen aus AR und ARB bzw. ARG für die weitere statistische Datenauswertung herangezogen wurden.[7] Zudem wurde sich bei den ausgewerteten ARB nur auf Spezies fokussiert, die in der Lage sind, Biofilme auszubilden, da bei diesen davon ausgegangen wurde, dass eine längere Persistenz und Vermehrungsfähigkeit im Abwasserkanalsystem gegeben ist.[7] Daher wurden mögliche Zusammenhänge für z.B. VRE oder MRSA nicht in der vorliegenden Arbeit betrachtet. Interessanterweise konnte mit Ciprofloxacin ein Breitbandantibiotikum als Indikator für MRE in Abwasser identifiziert werden, dessen ökotoxikologisches Potential sowie eine mögliche Selektion von ARB bereits mehrfach in Studien untersucht wurde.[7,136,162,176,178]

Aufgrund der ausgeprägten Zusammenhänge bezüglich des Nachweises von AR, ARB und ARG sowie den hohen Rückstandskonzentrationen und der hohen Diversität an verschiedenen AR erscheint eine gesonderte dezentrale Aufbereitung von Krankenhausabwasser als denkbare Möglichkeit, kommunale Kläranlagen zu entlasten und den Eintrag und die Verbreitung von Antibiotikaresistenzen in die Umwelt zu reduzieren.[7] Die Vorteile einer dezentralen Aufbereitung von Krankenhausabwasser zur Reduktion des Eintrages von AR und ARG in kommunale Kläranlagen zeigt eine aktuelle Studie von Paulus et al. (2019).[179]

Für weitere Untersuchungen sollte zukünftig zu den Konzentrationen an ARG, AR und ARB die in Kläranlagen bzw. Oberflächengewässer eingetragenen Abwasserfrachten berücksichtigt werden. Für Rückstände von sowohl ambulant als auch stationär eingesetzten Antibiotika kann sich trotz einer höheren Rückstandskonzentration in Krankenhausabwasser eine deutlich höhere tägliche Abwasserfracht für kommunales Abwasser ergeben. Mögliche Größenunterschiede bezüglich der Abwassermengen zeigen die Charakteristika der in Kapitel III untersuchten Probeentnahmestellen TCWW (Krankenhausabwasser, 27,1 m^3/Stunde) und iSTP E (Zulauf der entsprechenden kommunalen Kläranlage, 17,5 Millionen m^3/Stunde).[7] Im Falle von Krankenhaus-spezifischen Antibiotika wie Meropenem, Vancomycin oder Piperacillin, die in hohen Rückstandskonzentrationen in Krankenhausabwasser und nur sporadisch und in niedrigen Konzentrationen in Mischabwässern bzw. kommunalen Abwässern nachgewiesen wurden, scheinen die eingetragenen Abwasserfrachten aus Klinikabwasser die Abwasserfrachten aus kommunalem Abwasser überschreiten zu können.[6,7] Daher sollte Krankenhausabwasser bei der Bewertung des Abwassereintrages spezifischer AR, MRE und ARG in kommunale Kläranlagen und in die aquatische Umwelt mit berücksichtigt werden.

Bei zukünftigen Untersuchungen sollten die Probeentnahmetermine jedoch repräsentativ über sämtliche Wochentage verteilt werden, um etwaige Spitzen bzw. Minima in den Rückstandskonzentrationen, die z.B. aus dem Klinikalltag (Patientenaufnahme, -entlassung etc.) resultieren können, auszugleichen. In diesem Zusammenhang zeigten die Studien von Diwan et al. (2010) sowie Lindberg et al. (2004) mögliche Schwankungsbreiten der in Krankenhausabwasser nachgewiesenen Antibiotikarückstandskonzentrationen in Abhängigkeit der Uhrzeit und Substanz.[104,135]

Ferner zeigten die Untersuchungsergebnisse der Sanitäreinheiten verschiedener Kliniken, dass bzgl. der AR nicht zwangsweise das gesamte Klinikabwasser aufzubereiten ist, sondern dass eine dezentrale Abwasseraufbereitung bei Krankenhäusern und Kliniken mit hohem Antibiotikaverbrauch gegebenenfalls ausreichend sein könnte.[6] Weiterführend sollte die Effizienz bzw. Machbarkeit einer gesonderten Behandlung einzelner Teilstränge des Krankenhausabwassers untersucht werden.

So erscheint eine dezentrale Abwasseraufbereitung bzgl. der Elimination von AR von z.B. Kliniken ohne nennenswerten Antibiotikaverbrauch (z.B. das Abwasser einer psychosomatischen Klinik) bzw. des Abwassers von Lehr- oder Verwaltungsgebäude als nicht zwingend notwendig.[6]

Die Studie von Sib et al. (2019) zeigte, dass die untersuchten Waschbeckensiphons, Duschabläufe und Toiletten darüber hinaus Reservoire an ARB bzw. MRE darstellen können.[180] Überwiegend wurden *P. aeruginosa*, *Enterobacter cloacae* Komplex, Citrobacter *freundii* sowie *K. pneumoniae* und *A. calcoaceticus-baumannii* Komplex Spezies nachgewiesen.[180] Im Einklang mit den chemischen Untersuchungsergebnissen ergaben sich deutliche Unterschiede zwischen den untersuchten klinischen Bereichen.[6,180] So konnte z.B. nur ein *C. freundii* Stamm in der untersuchten Psychosomatik, in der keinerlei AR nachgewiesen wurden, isoliert werden, der als multiresistent (3MRGN) charakterisiert werden konnte.[6,180] Deutlich mehr MRE (3MRGN, 4MRGN, ESBL) konnten in den Sanitäreinheiten der Onkologie und des neurologischen Rehabilitationszentrums nachgewiesen werden.[180]

Analog zu den chemischen Untersuchungsergebnissen legen die mikrobiologischen Ergebnisse nahe, dass Duschabläufe einen besonderen Hotspot innerhalb der untersuchten Sanitäreinheiten bezüglich des Vorkommens von AR und ARB darzustellen scheinen.[6,180] Jedoch konnte trotz des gemeinsamen Nachweises von AR, ARG und ARB keine eindeutige Korrelationen zwischen diesen in den untersuchten Sanitäreinheiten der Patientenbadezimmer festgestellt werden.[180]

Aufgrund der Vielzahl an nachzuweisenden Antibiotika sowie der hohen Rückstandskonzentrationen in Waschbeckensiphons, Duschabläufen und Toiletten klinischer Bereiche zeigte sich ein bis dato regulatorisch noch nicht berücksichtigtes Reservoir an AR in unmittelbarer Nähe zu vulnerablen Patienten.[6] Demnach werden weitere Maßnahmen zur Reduktion der AR in Sanitäreinheiten von Patientenbadezimmern parallel zur Abwasseraufbereitung in Kläranlagen als sinnvoll erachtet.

In Anlehnung an die Forderung von Ory et al. (2019) bei einer gesonderten Aufbereitung von Krankenhausabwasser die Entfernung des im Kanalnetz ausgebildeten Biofilm mit zu berücksichtigen,[177] zeigten die hier vorgestellten Arbeiten, dass eine Reduktion des Biofilm in Waschbeckensiphons, Toiletten und Duschabläufen ebenfalls sinnvoll wäre.[6]

Aufgrund der postulierten Speicherung im bzw. der zeitverzögerte Freisetzung aus dem Biofilm von AR ist eine chronische Belastung des Leitungsnetzes mit antibiotisch wirksamen Substanzen denkbar, wodurch ein Selektionsdruck zugunsten der Entstehung von Antibiotikaresistenzen bzw. deren Verbreitung ausgeübt werden könnte.[6]

Aufgrund der schnellen Ausbildung von Biofilmen in wasserführenden Leitungssystemen, erscheint eine automatisierte in regelmäßigen Intervallen statt findende Reinigungsmethode sinnvoll.[143,181] Eine mögliche Methode zur Entfernung des Biofilms und zur Unterdrückung der Neuausbildung im Leitungsnetz stellt der Einsatz von thermisch selbstdesinfizierenden Waschbeckensiphons dar.[142,143] Jedoch stehen solche Systeme aktuell nur für das Waschbecken zur Verfügung. Adäquate Alternativen müssten somit für Toiletten und Duschabläufe erst entwickelt und etabliert werden. Daher erscheint der Einsatz anderer chemischer Reinigungsmittel oder eine verstärkte mechanische Entfernung des Biofilms als mögliche Lösung, die es in Zukunft zu testen gilt.[6] In diesem Zusammenhang zeigten erste Vorversuche, dass in thermisch selbstdesinfizierenden Waschbeckensiphons ebenfalls AR im dort befindlichen Abwasser nachzuweisen sind. Unter der Annahme, dass sich dort kein Biofilm ausbilden kann,[143] erscheint dies nur möglich durch eine unzureichende Durchspülung nach Benutzung des Waschbeckens durch den Patienten. Daher erscheint eine generelle Erhöhung des Spülvolumens für besondere Bereiche wie Krankenhäuser oder Altenheime sinnvoll, um die Belastung von Waschbeckensiphons, Toiletten und Duschabläufen mit AR sowie ARG und ARB zu reduzieren.[6]

Eine wesentliche Bedeutung für die Öffentliche Gesundheit stellt die Qualität des Trinkwassers dar. Erste Orientierungswerte bezüglich der Belastung eines Flusses durch AR, ARB und ARG, der in eine Trinkwassertalsperre mündet, konnten ermittelt werden.[8]

Insgesamt gilt, dass sich, bezogen auf das in dieser Arbeit untersuchte Talsperrensystem, die Konzentrationen an AR, ARG und ARB ausgehend von den Einleitstellen der kommunalen Kläranlagen rasch im weiteren Flussverlauf reduzierten. Demnach ergaben sich keine signifikanten Unterschiede zwischen den nachgewiesenen Konzentrationen an AR und ARB an den Probeentnahmestellen vor den Einleitstellen und nach der Trinkwassertalsperre.[8]

Jedoch kann sich die Belastung anderer Oberflächengewässer stark von den hiesigen Ergebnissen unterscheiden, da im Einzugsgebiet der untersuchten Oberflächengewässer bzw. beider Kläranlagen weder Krankenhäuser noch Mastbetriebe ansässig waren und dort keine intensive Viehzucht betrieben wurde.[8] Demnach erscheint eine weitere Beobachtung der Resistenzsituation in Verbindung mit der Untersuchung auf AR und ARG in Oberflächengewässer, die zur Trinkwassergewinnung dienen, insbesondere bei starkem anthropogenem Einfluss (Krankenhausabwasser, Abwasser von Mastbetrieben, große kommunale Kläranlagen) sinnvoll. Zukünftige Untersuchungsschwerpunkte könnten daher auf stärker beeinflussten Flüssen wie der Ruhr, der Donau oder dem Rhein liegen, welche z.B. durch Uferfiltration, künstliche Grundwasseranreicherung oder unmittelbar als Oberflächengewässer zur Trinkwassergewinnung dienen.[182,183]

Abschließend sei erwähnt, dass die vorgestellten Studien überwiegend den Eintrag und die Verbreitung von AR, ARB sowie ARG aus kommunalem Abwasser und Krankenhausabwasser thematisierten. Im Sinne des „*One-Health*“-Ansatzes sollte in zukünftigen Studien weiterhin der veterinärmedizinische Einfluss auf die Verbreitung und Entstehung von Antibiotikaresistenzen sowie den Eintrag von AR in die Umwelt berücksichtigt werden. In diesem Sinne soll in einer Aufstockung des BMBF-Verbundprojektes HyReKA (FKZ: 02WRS1377C) Gülle als Reservoir für AR, ARG und ARB und die durch die Ausbringung als Wirtschaftsdünger resultierende Belastung der aquatische Umwelt untersucht werden.

Grundsätzlich sollte jedoch neben der Reduzierung des Eintrages von AR, ARB und ARG durch z.B. Kläranlagenaufrüstungen sowie restriktivere Regulierungen im Umweltsektor auch eine verantwortungsvollere Verschreibungspraxis in Human- und Veterinärmedizin angestrebt werden. So ist z.B. eine Reduzierung der Antibiotikaabgabemengen in der Veterinärmedizin nicht zu befürworten, wenn dies zur Folge hat, dass hohe Abgabemengen an Antibiotika mit einem schmalen Wirkungsspektrum durch geringere Mengen an Antibiotika mit breitem Wirkungsspektrum ausgetauscht werden. Als Beispiel hierfür dient der Vergleich der Abnahme der Gesamtabgabemengen um 972,6 t (= 57% der Gesamtabgabemenge) zwischen den Jahre 2011 bis 2017 bei einem gleichzeitigen Anstieg der spezifischen Abgabemengen an Fluorchinolonen um 1,7 t (= 21% der Gesamtabgabemenge an Fluorchinolonen) im gleichen Zeitraum.[28,29]

Gerade vor dem Hintergrund etwaiger Kreuzresistenzen ist der Einsatz von Fluorchinolonen teilweise kritisch zu sehen. So können z.B. Enrofloxacin resistente *Campylobacter* auch Resistenzen gegen andere Fluorchinolone wie Ciprofloxacin ausbilden, wodurch eine Behandlung bei Infektionen in der Humanmedizin erschwert werden kann.[184] Gerade im Fall von *Campylobacter*-Infektionen konnte gezeigt werden, dass die Übertragung durch den Umgang mit rohem Fleisch bzw. den Verzehr von nicht vollständig gegartem Geflügelfleisch eine Infektionsursache darstellt.[164]

Trotz der Trennung von Fluorchinolonen in humanmedizinische (z.B. Ciprofloxacin) und veterinärmedizinische (z.B. Enrofloxacin) Wirkstoffe kann es zu Überschneidungen bezüglich der Ursache für positive Rückstandsergebnisse kommen. Denn es konnte gezeigt werden, dass Ciprofloxacin ein Metabolit von Enrofloxacin ist.[185] So beträgt der prozentuale Anteil von Ciprofloxacin bezogen auf die Enrofloxacin-Plasmakonzentration 51% bei Schweinen, 59% bei Milchkühen und maximal 10% bei Hühnern.[34] Somit könnte der veterinärmedizinische Einsatz von Fluorchinolonen (allen voran Enrofloxacin) eine weitere Ursache bzw. Quelle für den Eintrag von Ciprofloxacin-Rückständen in die Umwelt darstellen, da in Abhängigkeit der jeweiligen Tierart neben der renalen Ausscheidung von Enrofloxacin auch Ciprofloxacin als antibiotisch wirksamer Hauptmetabolit ausgeschieden wird.[185]

In diesem Zusammenhang zeigten bis dato unveröffentlichte Ergebnisse aus dem HyReKa-Verbund, dass in Abwässern von Geflügelschlachtbetrieben sowohl Rückstände von Enrofloxacin (bis zu 83 µg/L) als auch Ciprofloxacin (bis zu 5,6 µg/L) nachzuweisen sind (Nachweisfrequenzen: 4,9% bzw. 12,2%). Eine detaillierte Veröffentlichung der Gesamtdaten soll zeitnah erfolgen. Vor diesem Hintergrund sei jedoch erwähnt, dass anhand der neuesten Abgabemengen von Antibiotika an niedergelassene Tierärzte ein Rückgang der Fluorchinolon-Abgabemengen für das Jahr 2018 (7,7 t) im Vergleich zu den vorherigen Jahren (9,9 t in 2017) zu verzeichnen war.[44] Weiterhin sollte beobachtet werden, ob sich dieser Trend in Zukunft fortsetzt.

Zukünftige Untersuchungen von Abwässern aus Schlachtbetrieben, von Gülle und Oberflächengewässer in stark landwirtschaftlich geprägten Regionen auf AR, ARB und ARG erscheinen sinnvoll, um eine weitere Datenbasis bezüglich der Resistenzsituation in der Umwelt zu schaffen. Ein Fokus könnte hierbei auf der Untersuchung von Fluorchinolon-Rückständen und etwaigen Korrelationen mit spezifischen ARB und ARG liegen, da Ciprofloxacin in dieser Dissertationsschrift bereits als möglicher Indikator für MRE in klinisch beeinflusstem kommunalem Abwasser identifiziert werden konnte.[7]

Abschließend sei erwähnt, dass ein nächster Schritt die Entwicklung einer auf Grundlage der geschaffenen Datenbasis fundierte Risikobewertung sein sollte, um die erhaltenen Daten besser interpretieren, Risiken abschätzen und mögliche Handlungsempfehlungen aussprechen zu können.

i. Anhang

Parameter	Einheit	Vorgabe	Messwert	z_U	Bewertung
Sulfadiazin	µg/L	0,8133	0,825	0,1	e
		0,1205	0,108	-1,3	e
		0,4518	0,458	0,1	e
Sulfadimidin	µg/L	0,2935	0,290	-0,1	e
		0,6604	0,640	-0,4	e
		0,1223	0,122	0,0	e
Sulfamethoxazol	µg/L	0,1081	0,116	0,6	e
		0,3243	0,342	0,5	e
		0,7298	0,757	0,4	e
Sulfaethoxypyridazin	µg/L	0,5384	0,531	-0,1	e
		0,7179	0,697	-0,2	e
		0,1196	0,122	0,1	e
Sulfamerazin	µg/L	0,3712	0,360	-0,3	e
		0,1237	0,124	0,0	e
		0,6496	0,625	-0,4	e
Sulfathiazol	µg/L	0,6550	0,652	-0,1	e
		0,1248	0,124	-0,1	e
		0,4678	0,464	-0,1	e
Sulfadoxin	µg/L	0,1290	0,134	0,2	e
		0,2903	0,294	0,1	e
		0,6674	0,684	0,1	e
Sulfamethoxypyridazin	µg/L	0,3753	0,395	0,4	e
		0,6756	0,693	0,3	e
		0,1001	0,108	0,4	e
Sulfachlorpyridazin	µg/L	0,1141	0,180	2,2	f
		0,5134	0,763	3,1	u
		0,7701	1,11	1,9	e
Sulfadimethoxin	µg/L	0,1139	0,122	0,3	e
		0,3418	0,354	0,3	e
		0,5982	0,618	0,4	e
Trimethoprim	µg/L	0,6827	0,721	0,5	e
		0,1011	0,114	1,2	e
		0,3793	0,402	0,6	e

Tabelle A: Ergebnisübersicht des Ringversuchs 5/16 – TW S2 – Pharmazeutika in Trinkwasser bereitgestellt durch das AQS Baden-Württemberg der Universität Stuttgart – Institut für Siedlungswasserbau, Wassergüte- und Abfallwirtschaft (ISWA). Die Auswertung erfolgte nach den Vorgaben der DIN 38402-A45 und den Empfehlungen des Umweltbundesamtes bezüglich der Durchführung von Ringversuchen zur Messung chemischer Parameter und Indikatorparameter zur externen Qualitätskontrolle von Trinkwasseruntersuchungsstellen. Ein Analyt gilt als erfolgreich nachgewiesen und bestimmt, sofern der z-*Score* von mindestens zwei Konzentrationsniveaus innerhalb des Toleranzbereiches von $|z_U| \leq 2,0$ liegt.[127]

Legende: e = erfolgreich ($|z_U| \leq 2,0$), f = fragwürdig ($2,0 < |z_U| < 3,0$), u = unzureichend ($|z_U| \geq 3,0$)

ii. Abbildungsverzeichnis

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iii. Tabellenverzeichnis

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