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

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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 repre sentative 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 con cerning 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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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 (Dohla 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 μg L^{-1} range. Furthermore, in treated WW, AR concentrations ranged between the 2 digit ng L^{-1} and the lower μg 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; Picao 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.

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 combi nation 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 injec tion volume was 20 μl. The chromatographic separation was per formed 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 *Staph ylococcus aureus* (MRSA), vancomycin resistant *Enterococcus fae cium* 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 spec trometer (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 resis tance towards temocillin, piperacillin, piperacillin/tazobactam, cefotaxime, ceftazidime, imipenem, meropenem, amikacin, tige cycline, chloramphenicol, fosfomycin, trimethoprim/sulfamethox azol, 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 anti biotic classes, (piperacillin/tazobactam, fluoroquinolones (cipro floxacin), 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 car bapenem resistant isolates, via quantitative polymerase chain re action (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 tax onomy 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 waste water. 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

that the calculated odds ratio, between two variables, was signifi cant, 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 col onies 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 dis tances 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. cipro floxacin, ofloxacin and moxifloxacin).

The ubiquitous detection of sulfamethoxazole (and trimetho prim), 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 ther apy 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 ob tained for macrolides, most notably clarithromycin and erythro mycin, as well as its metabolite anhydroerythromycin and clindamycin.

The most commonly detected fluoroquinolone was ciprofloxa cin, followed by ofloxacin and moxifloxacin. Residue concentra tions 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 antibi otics, 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 uncon taminated WW and possible degradation processes. Accordingly, the reduction of β lactams, such as meropenem, ceftazidime or piperacillin, could be explained by their instability against hydro lytic cleavage of the penam or lactam ring (Deshpande et al., 2004).

Residues of at least one tetracycline (doxycycline and tetracy cline) 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" tetra cyclines 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 live stock 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., *Entero bacter* 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. *Acineto bacter 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 concen trations 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

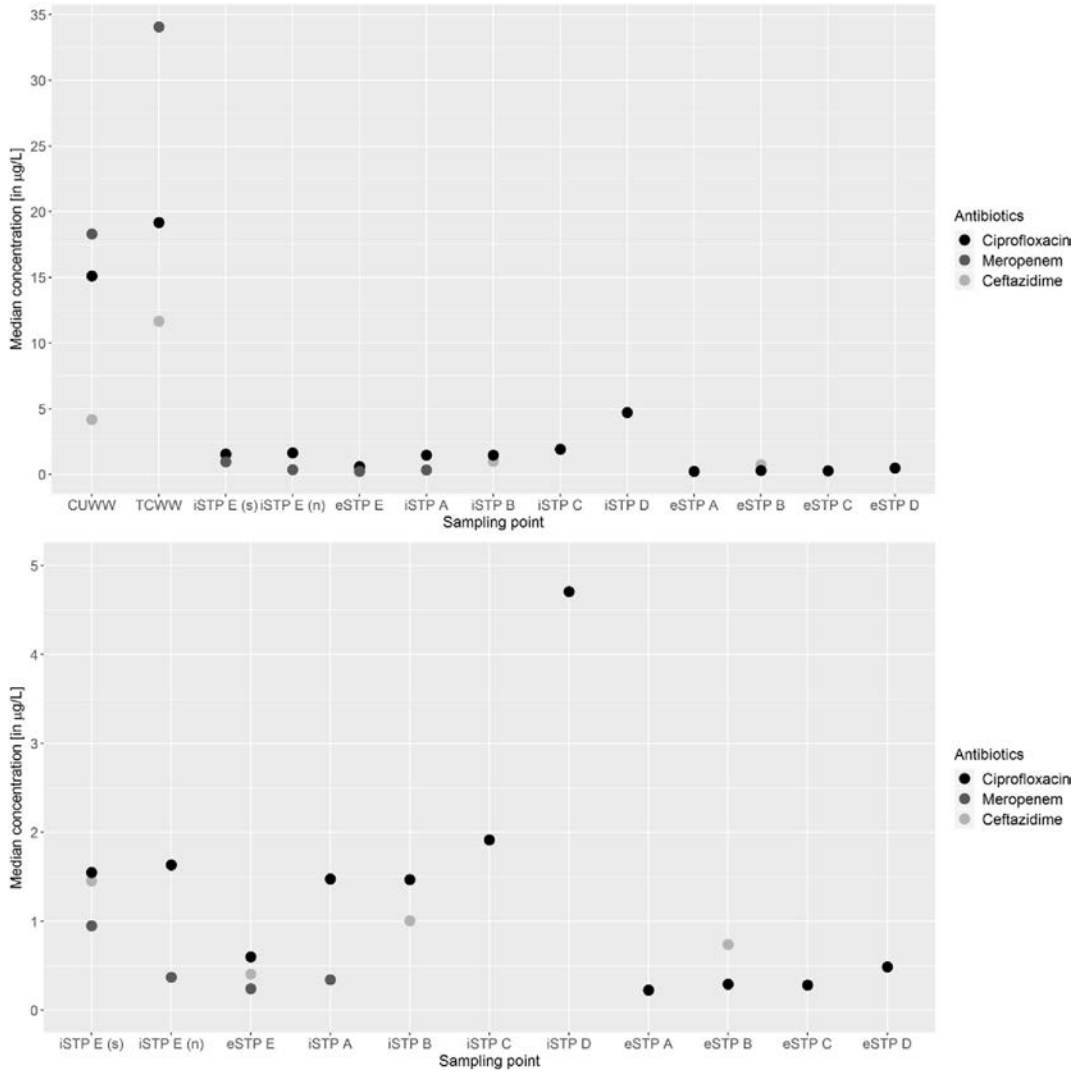

Fig. 1. Overview about the quantitative results (median, µg/L) of the AR used for statistical analysis, sorted by sampling sites (top all sampling sites; bottom without CUWW and TCWW).

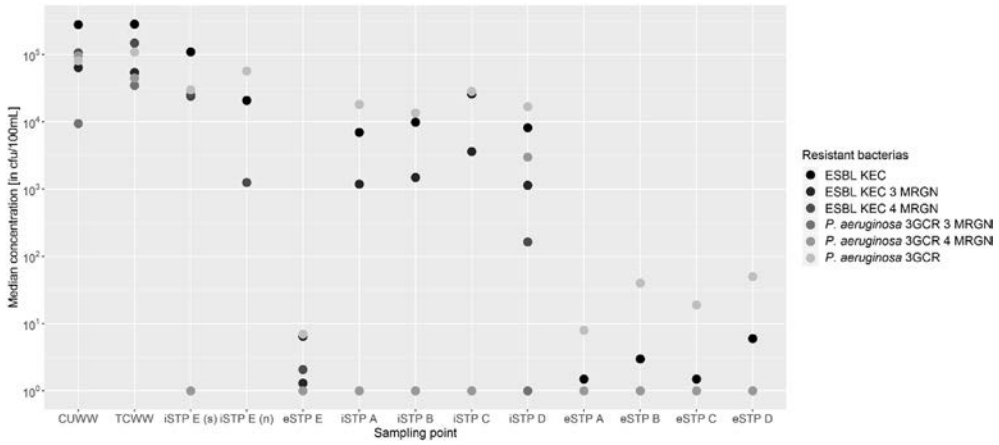

Fig. 2. Overview about the quantitative results (median, cfu/100 mL) of the ARB used for statistical analysis, sorted by sampling sites.

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

sampling sites of both case studies, the degree of multidrug resis tance 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, bio films 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 acetylsulfamethox azole is deemed to be not clinically relevant, since, although rep resentatives 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 cefta zidime) 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, cip rofloxacin 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 concen tration 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 detec ted 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.

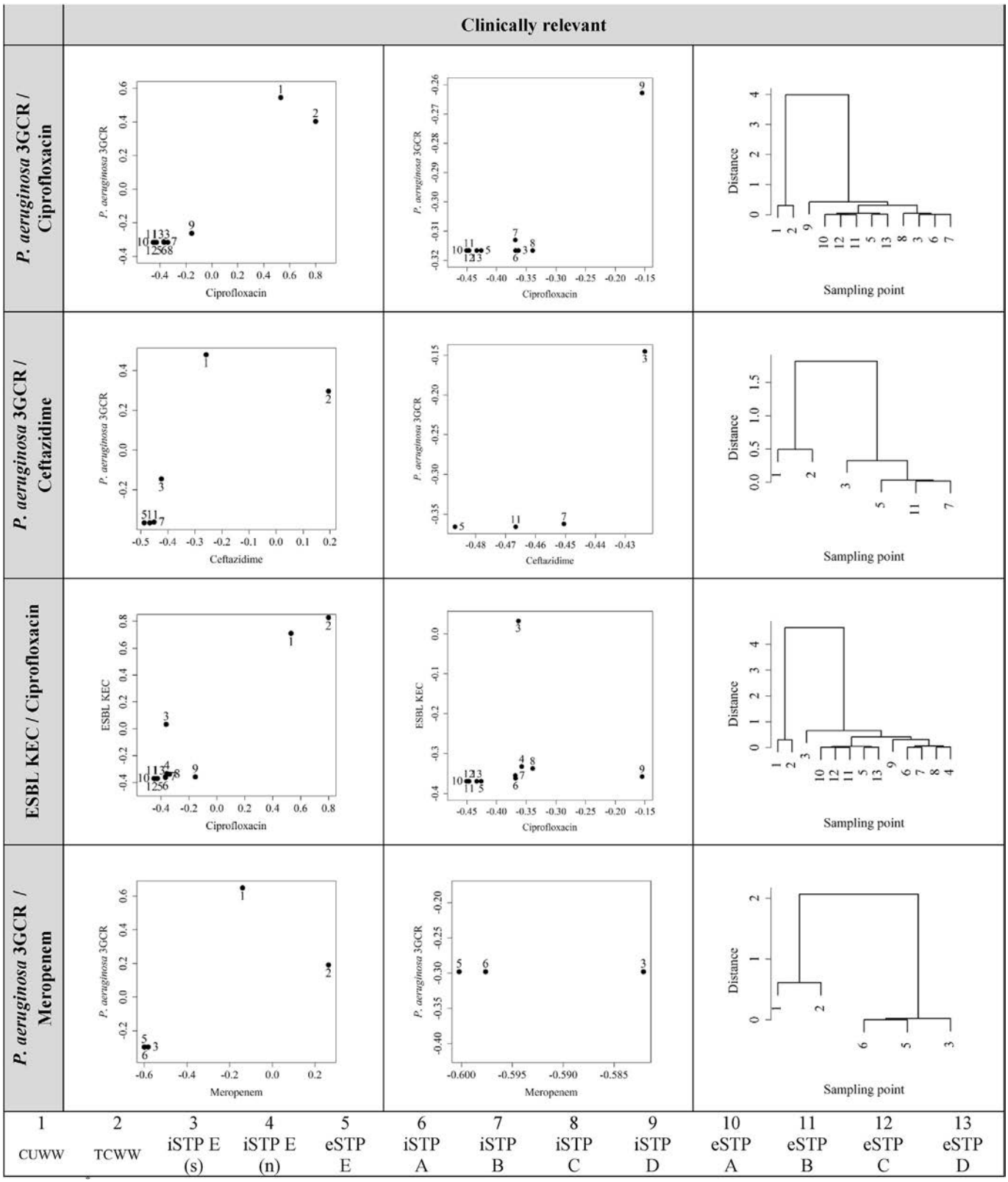

Fig. 3. Scatterplots and dendrograms of the positively related parameters with clinical relevance*. From left to right: Scatterplot containing all samples, Scatterplot without the clinic samples (TCWW and CUWW), dendrogram containing all samples.

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 concentra tions 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} simulta neously 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 carba penem 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 rela tionship 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 inves tigated 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 differ ences, 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 carba penemases/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

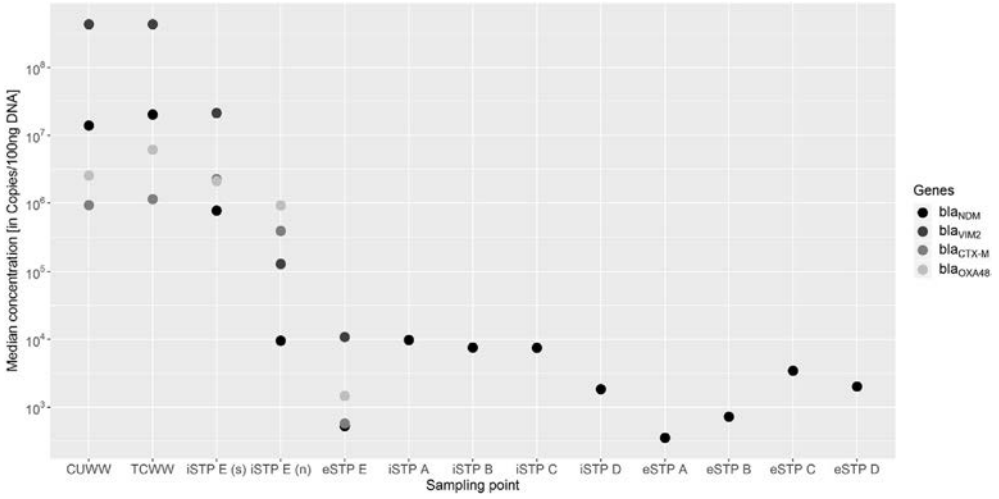

Fig. 4. Overview about the quantitative results (median, copies/100 ng DNA) of the ARG used for statistical analysis, sorted by sampling sites.

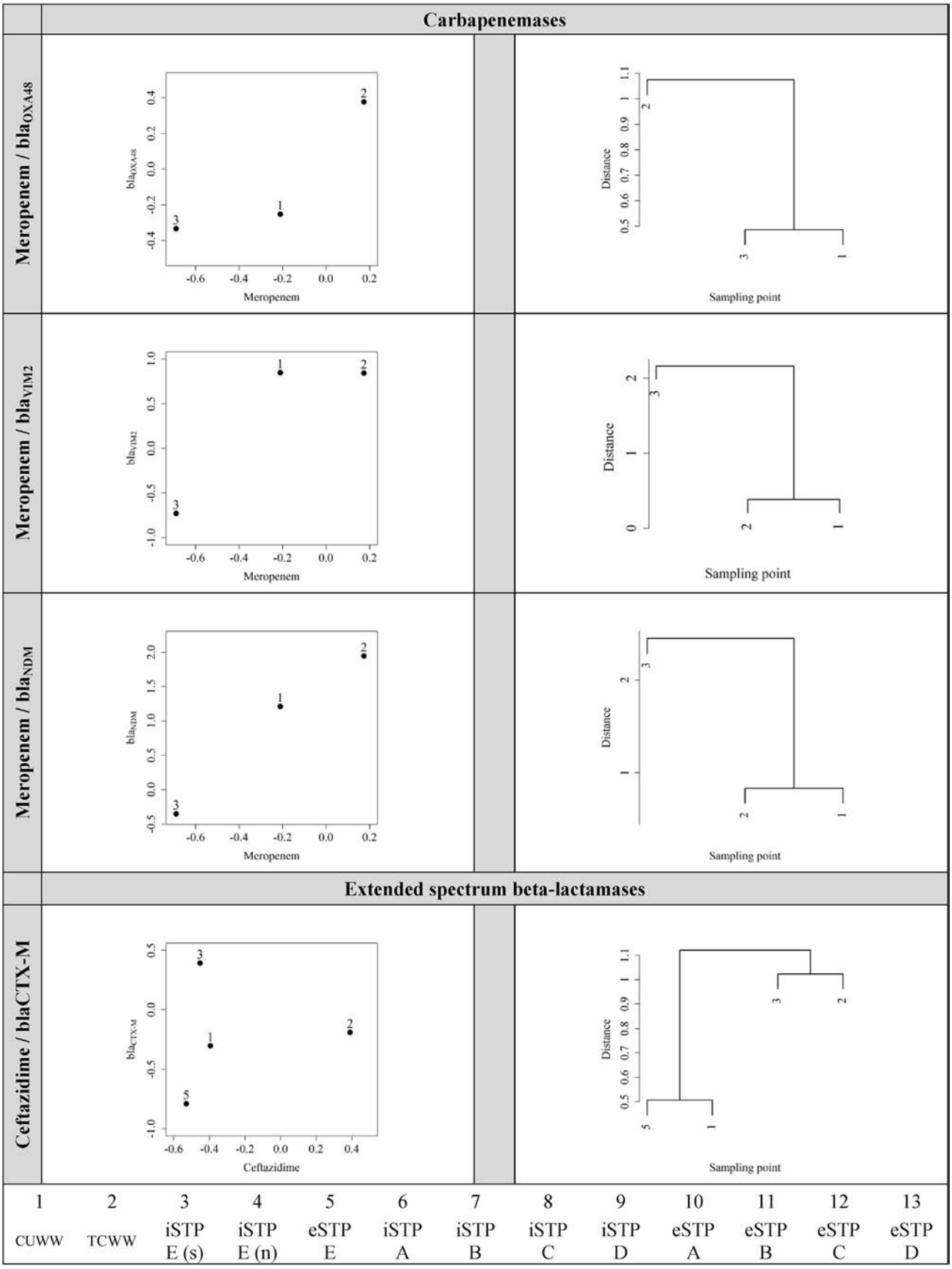

Fig. 5. Scatterplots and dendrograms of the relationship between ARG and the corresponding AR relating to all studied sampling sites (case study A and B).

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 re lationships 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 in fluents 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 carba penemase producers, 3MRGN bacteria could even be detected in case study B, thus rarely. In addition, meropenem relates signifi cantly 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 pa tient 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 cip rofloxacin 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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References

Amako, K., Meno, Y., Takade, A., 1988. Fine structures of the capsules of Klebsiella pneumoniae and Escherichia coli K1. J. Bacteriol. 170 (10), 4960 4962.

Baquero, F., Martínez, J.-L., Cantón, R., 2008. Antibiotics and antibiotic resistance in water environments. Curr. Opin. Biotechnol. 19, 260 265.

Bengtsson-Palme, J., Larsson, D.G.J., 2016. Concentrations of antibiotics predicted to select for resistant bacteria: proposed limits for environmental regulation. Environ Int 86, 140 149.

Bonnet, R., 2004. Growing group of extended-spectrum beta-lactamases: the CTX-M enzymes. Antimicrob. Agents Chemother. 48, 1 14.

BVL and Paul-Ehrlich-Gesellschaft für Chemotherapie e.V, 2016. Antiinfectives Intelligence (Rheinbach). Germap. 2015.

Christian, T., Schneider, R.J., Farber, H.A., Skutlarek, D., Meyer, M.T., et al., 2003. Determination of antibiotic residues in manure, soil, and surface waters. Acta Hydrochim. Hydrobiol. 31, 36 44.

Christoffels, E., Mertens, F.M., Kistemann, T., Schreiber, C., 2014. Retention of pharmaceutical residues and microorganisms at the Altendorf retention soil filter. Water Sci. Technol. 70, 1503 1509.

Deshpande, A.D., Baheti, K.G., Chatterjee, N.R., 2004. Degradation of beta-lactam antibiotics. Curr. Sci. 87, 1684 1695.

Dohla, M., Sib, E., Dericks, B., Grobe, S., Behringer, K., et al., 2019. Assessment of the prevalence of antibiotic-resistant bacteria and the concentration of antibiotics in EU bathing waters in Western Germany. Expo Health. https://doi.org/10.1007/s12403-019-00313-z.

EUCAST, 2019. Breakpoint Tables for Interpretation of MICs and Zone Diameters. URL:, Version 9.0. http://www.eucast.org/clinical_breakpoints/. (Accessed 11 June 2019).

Feuerpfeil, I., López-Pila, J., Schmidt, R., Schneider, E., Szewzyk, R., 1999. Antibiotikaresistente Bakterien und Antibiotika in der Umwelt. Bundesgesundheitsblatt - Gesundheitsforsch. - Gesundheitsschutz 42, 37 50.

Fleming, A., 1945. Sir Alexander Fleming Nobel Lecture. Available at. https://www.nobelprize.org/prizes/medicine/1945/fleming/lecture [verified 21 November 2018].

Galler, H., Feierl, G., Petternel, C., Reinthaler, F.F., Haas, D., et al., 2014. KPC-2 and OXA-48 carbapenemase-harbouring Enterobacteriaceae detected in an Austrian wastewater treatment plant. Clin. Microbiol. Infect. 20, O132 O134.

Golet, E.M., Alder, A.C., Giger, W., 2002. Environmental exposure and risk assessment of fluoroquinolone antibacterial agents in wastewater and river water of the Glatt Valley watershed, Switzerland. Environ. Sci. Technol. 36, 3645 3651.

Golet, E.M., Xifra, I., Siegrist, H., Alder, A.C., Giger, W., 2003. Environmental exposure assessment of fluoroquinolone antibacterial agents from sewage to soil. Environ. Sci. Technol. 37, 3243 3249.

Harris, P.N.A., Peleg, A.Y., Iredell, J., Ingram, P.R., Miyakis, S., et al., 2015. Meropenem versus piperacillin-tazobactam for definitive treatment of bloodstream infections due to ceftriaxone non-susceptible Escherichia coli and Klebsiella spp (the MERINO trial): study protocol for a randomized controlled trial. Trials 16, 24.

Hembach, N., Schmid, F., Alexander, J., Hiller, C., Rogall, E.T., et al., 2017. Occurrence of the mcr-1 colistin resistance gene and other clinically relevant antibiotic resistance genes in microbial populations at different municipal wastewater treatment plants in Germany. Front. Microbiol. 8, 1 11.

Heudorf, U., Kempf, V., Reinheimer, C., Exner, M., Schmithausen, R., et al., 2018. Klebsiella pneumoniae KPC-3 Ausbruch am Universitatsklinikum Frankfurt am Main teil II: weitere umwelthygienische Erkenntnisse (*Klebsiella pneumoniae KPC-3 outbreak at the University Hospital Frankfurt am Main - Part II: further environmental hygiene knowledge).* Gesundheitswesen 80, 383.

Johnson, A.P., Woodford, N., 2013. Global spread of antibiotic resistance: the example of New Delhi metallo-β-lactamase (NDM)-mediated carbapenem resistance. J. Med. Microbiol. 62, 499 513.

Jutkina, J., Marathe, N.P., Flach, C.-F., Larsson, D.G.J., 2018. Antibiotics and common antibacterial biocides stimulate horizontal transfer of resistance at low

concentrations. Sci. Total Environ. 616 617, 172 178.
Koh, T.H., Ko, K., Jureen, R., Deepak, R.N., Tee, N.W.S., et al., 2015. High counts of carbapenemase-producing Enterobacteriaceae in hospital sewage. Infect. Control Hosp. Epidemiol. 36, 619 621.
KRINKO, 2012. Hygienemaßnahmen bei Infektionen oder Besiedlung mit multiresistenten gramnegativen Stabchen. Empfehlung der Kommission für Kranken-haushygiene und Infektionspravention (KRINKO) beim Robert Koch-Institut (RKI) (*Hygiene measures for infections or colonization with multidrug-resistant Gram-negative rods. Recommendation of the Commission for Hospital Hygiene and Infection Prevention (KRINKO) at the Robert Koch Institute (RKI)*). Bundesgesundheitsblatt, Gesundheitsforschung, Gesundheitsschutz Volume 55, 1311 1354.
KRINKO, 2019. Erganzung zur Empfehlung der KRINKO „Hygienemaßnahmen bei Infektionen oder Besiedlung mit multiresistenten gramnegativen Stabchen" (2012) im Zusammenhang mit der von EUCAST neu definierten Kategorie „I" bei der Antibiotikaresistenzbestimmung: Konsequenzen für die Definition von MRGN.(*Supplement to the recommendation of KRINKO "Hygiene measures for infections or colonization with multidrug-resistant Gram-negative rods" (2012) in the context of the EUCAST newly defined category "I" in antibiotic resistance determination: consequences for the definition of MRGN*), Epidemiologisches Bulletin Nr. 9; February 28, 2019
Kümmerer, K., 2003. Significance of antibiotics in the environment. J. Antimicrob. Chemother. 52, 5 7.
Kümmerer, K., 2009. Antibiotics in the aquatic environment - a review - Part I. Chemosphere 75, 417 434.
Laurens, C., Jean-Pierre, H., Licznar-Fajardo, P., Hantova, S., Godreuil, S., et al., 2018. Transmission of IMI-2 carbapenemase-producing Enterobacteriaceae from river water to human. J Glob Antimicrob Re 15, 88 92.
Lindberg, R., Jarnheimer, P.A., Olsen, B., Johansson, M., Tysklind, M., 2004. Determination of antibiotic substances in hospital sewage water using solid phase extraction and liquid chromatography/mass spectrometry and group analogue internal standards. Chemosphere 57, 1479 1488.
Lindberg, R.H., Wennberg, P., Johansson, M.I., Tysklind, M., Andersson, B.A.V., 2005. Screening of human antibiotic substances and determination of weekly mass flows in five sewage treatment plants in Sweden. Environ. Sci. Technol. 39, 3421 3429.
Lindsey, M.E., Meyer, M., Thurman, E.M., 2001. Analysis of trace levels of sulfonamide and tetracycline antimicrobials in groundwater and surface water using solid-phase extraction and liquid chromatography/mass Spectrometry. Anal. Chem. 73, 4640 4646.
Lister, P.D., Wolter, D.J., Hanson, N.D., 2009. Antibacterial-resistant Pseudomonas aeruginosa: clinical impact and complex regulation of chromosomally encoded resistance mechanisms. Clin. Microbiol. Rev. 22, 582 610.
Magiorakos, A.-P., Srinivasan, A., Carey, R.B., Carmeli, Y., Falagas, M.E., et al., 2012. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clin. Microbiol. Infect. 18, 268 281.
Meyer, E., Gastmeier, P., Deja, M., Schwab, F., 2013. Antibiotic consumption and resistance: data from europe and Germany. Int J Med Microbiol : IJMM 303, 388 395.
Müller, H., Sib, E., Gajdiss, M., Klanke, U., Lenz-Plet, F., et al., 2018. Dissemination of multi-resistant Gram-negative bacteria into German wastewater and surface waters. Microb. Ecol. 94, 1 11.
Mutschler, E., 2012. Arzneimittelwirkungen (*Drug Effects*), tenth ed. Wiss. Verl.-Ges, Stuttgart).
Ory, J., Bricheux, G., Robin, F., Togola, A., Forestier, C., et al., 2019. Biofilms in hospital effluents as a potential crossroads for carbapenemase-encoding strains. Sci. Total Environ. 657, 7 15.
Picao, R.C., Cardoso, J.P., Campana, E.H., Nicoletti, A.G., Petrolini, F.V.B., et al., 2013. The route of antimicrobial resistance from the hospital effluent to the environment: focus on the occurrence of KPC-producing Aeromonas spp. and Enterobacteriaceae in sewage. Diagn. Microbiol. Infect. Dis. 76, 80 85.
Queenan, A.M., Bush, K., 2007. Carbapenemases: the versatile beta-lactamases. Clin. Microbiol. Rev. 20, 440 458 table of contents.
Radke, M., Lauwigi, C., Heinkele, G., Mürdter, T.E., Letzel, M., 2009. Fate of the antibiotic sulfamethoxazole and its two major human metabolites in a water sediment test. Environ. Sci. Technol. 43, 3135 3141.
Schmithausen, R., Schulze-Geisthoevel, S., Heinemann, C., Bierbaum, G., Exner, M., et al., 2018. Reservoirs and transmission pathways of resistant indicator bacteria in the biotope pig stable and along the food chain. Sustainability 10, 3967.
Schreiber, C., 2011. The spread of bacterial antibiotic resistance in the aquatic environment. WHOcc Newsl. on Water and Risk 18, 8 10.
Schreiber, C., Rechenburg, A., Koch, C., Christoffels, E., Claßen, T., et al., 2016. Two decades of system-based hygienic microbiological research in Swist river catchment (Germany). Environ Earth Sci 75, 217.
Schreiber, C., Rechenburg, A., Rind, E., Kistemann, T., 2015. The impact of land use on microbial surface water pollution. Int. J. Hyg Environ. Health 218, 181 187.
Schreiber, C., Zacharias, N., Müller, H., Essert, S., Timm, C., Kistemann, T., Sib, E., Gajdiss, M., Parcina, M., Voigt, A., Farber, H., Exner, M., Schmithausen, R., Bierbaum, G., 2019. Einfluss von Krankenhausabwasser auf die Verbreitung von Antibiotika-Resistenzen in der aquatischen Umwelt (*Influence of hospital effluent on the spread of antibiotic resistance in aquatic environmental drug effects*). In: Pinnekamp, J. (Ed.), 52. Essener Tagung für Wasserwirtschaft from 20.3.-22.3.2019, Aachen, vol. 250. GWA - Gewasserschutz, Wasser & Abwasser.
Schwabe, U., Paffrath, D., 2016. Arzneiverordnungs-Report 2016 (*Drug Prescription Report 2016*), first ed. Springer, Berlin.
Sheskin, D.J., 2003. Handbook of Parametric and Nonparametric Statistical Procedures, third ed. Chapman and Hall/CRC, New York.
Sib, E., Voigt, A.M., Wilbring, G., Schreiber, C., Faerber, H.A., et al., 2019. Antibiotic resistant bacteria and resistance genes in biofilms in clinical wastewater networks. Int. J. Hyg Environ. Health 222, 655 662.
Simo Tchuinte, P.L., Stalder, T., Venditti, S., Ngandjio, A., Dagot, C., et al., 2016. Characterization of class 3 integrons with oxacillinase gene cassettes in hospital sewage and sludge samples from France and Luxembourg. Int. J. Antimicrob. Agents 48, 431 434.
Stange, C., Sidhu, J.P.S., Toze, S., Tiehm, A., 2019. Comparative removal of antibiotic resistance genes during chlorination, ozonation, and UV treatment. Int. J. Hyg Environ. Health 222, 541 548.
Stevens, D.L., Herr, D., Lampiris, H., Hunt, J.L., Batts, D.H., et al., 2002. Linezolid versus vancomycin for the treatment of methicillin-resistant Staphylococcus aureus infections. Clin. Infect. Dis. 34, 1481 1490.
Treepong, P., Kos, V.N., Guyeux, C., Blanc, D.S., Bertrand, X., et al., 2018. Global emergence of the widespread Pseudomonas aeruginosa ST235 clone. Clin. Microbiol. Infect. 24, 258 266.
Voigt, A.M., Faerber, H.A., Wilbring, G., Skutlarek, D., Felder, C., et al., 2019a. The occurrence of antimicrobial substances in toilet, sink and shower drainpipes of clinical units: a neglected source of antibiotic residues. Int. J. Hyg Environ. Health 222, 455 467.
Voigt, A.M., Skutlarek, D., Timm, C., Schreiber, C., Felder, C., et al., 2019b. Liquid chromatography-tandem mass spectrometry as a fast and simple method for the determination of several antibiotics in different aqueous matrices. J Environ Chem. https://doi.org/10.1071/EN19115.
Wallmann, J., Bender, A., Bode, C., Koper, L.M., Heberer, T., 2017. Abgabemengenerfassung antimikrobiell wirksamer Stoffe in Deutschland 2016 (*Levels of emission quantification of antimicrobial substances in Germany 2016*). Dtsch. Tierarzteblatt 65 (12), 1650 1659.
Walsh, T.R., 2010. Emerging carbapenemases: a global perspective. I Int J Antimicrob Agents 36, 8 14.
Watkinson, A.J., Murby, E.J., Kolpin, D.W., Costanzo, S.D., 2009. The occurrence of antibiotics in an urban watershed: from wastewater to drinking water. Sci. Total Environ. 407, 2711 2723.
Westphal-Settele, K., Konradi, S., Balzer, F., Schonfeld, J., Schmithausen, R., 2018. Die Umwelt als Reservoir für Antibiotikaresistenzen (*The environment as a reservoir for antibiotic resistance*). Bundesgesundheitsblatt, Gesundheitsforschung, Gesundheitsschutz 61, 533 542.
WHO, 2014. Antimicrobial resistance: global report on surveillance 2014. URL: https://www.who.int/drugresistance/documents/surveillancereport/en/. (Accessed 25 May 2019).
WHO, 2017a. Critically Important Antimicrobials for Human Medicine, fifth ed. World Health Organization, Geneva).
WHO, 2017b. Global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics. URL: https://www.who.int/medicines/publications/WHO-PPL-Short_Summary_25Feb-ET_NM_WHO.pdf. (Accessed 27 May 2019).
Zar, F.A., Bakkanagari, S.R., Moorthi, K.M.L.S.T., Davis, M.B., 2007. A comparison of vancomycin and metronidazole for the treatment of Clostridium difficile-associated diarrhea, stratified by disease severity. Clin. Infect. Dis. 45, 302 307.
Zhang, C., Qiu, S., Wang, Y., Qi, L., Hao, R., et al., 2014. Higher isolation of NDM-1 producing Acinetobacter baumannii from the sewage of the hospitals in Beijing. PLoS One 8, 1 6.