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Identification of critical control points for antibiotic resistance discharge in sewers

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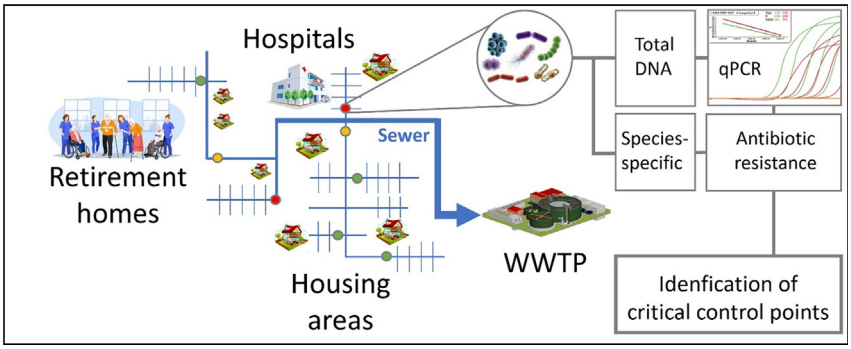
HIGHLIGHTS

- Determination of ARG load of an urban sewer system with different catchment areas
- Monitoring over 1 year identified critical control points for ARG dissemination.
- A selection of 5 out of 9 ARGs enabled comprehensive ARG burden.
- Flow volume-based analysis indicates benefit of decentralized/on-site interventions.
- Cultivation of MDR bacteria supports qPCR findings.

GRAPHICAL ABSTRACT



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ABSTRACT

Disrupting the spread of clinically relevant antibiotic resistance genes (ARGs) is one of the key components for the success of the One Health strategy. While waste water treatment plants (WWTPs) represent a final control point for daily discharges of antibiotic resistance genes (ARGs) to the aquatic environment, a decentralized upstream monitoring of wastewater feeds of selected urban drainage areas for $bla_{CTX\text{-}M32}$, $bla_{CTX\text{-}M15}$, bla_{OXA48}, $bla_{CMY\text{-}2}$, *mecA*, $bla_{NDM\text{-}1}$, bla_{KPC3}, *van*A, and *mcr*-1 representing clinically relevant ARGs has been performed. Besides hospitals, also retirement homes were found to be responsible for high levels of ARG discharges compared to housing area sewer systems. The monitoring combines qPCR-based quantifications, flow volume-based analyses, and multiple antibiotic resistance analyses of isolates. As result of the study, local actions at identified critical control points could help to prevent contaminations of larger volumes of wastewaters. This strategy will support a more cost-effective treatment compared to central actions at WWTPs, only. A polluter-pays principle should be applied by this monitoring strategy.

1. Introduction

In the future, the management of bacterial infections should not only focus on the treatment of patients, but also on the prevention of the dissemination and wide spread of antibiotic-resistant bacteria and the mobile genetic elements coding for clinically relevant antibiotic resistance genes discharged from critical local sources like hospitals (Sib et al., 2019; WHO, 2019). Hence, the surveillance of possible transmissions of antibiotic-resistant bacteria to the local population or their dissemination into the environment is an important factor and should be the focus of future surveillance strategies (Dafale and Purohit, 2016). The successful application of antibiotics in human and veterinary medicine is well recognized, but the misuse of these drugs leads to antibiotic resistance selection and evolution in pathogenic bacteria that will result in untreatable bacterial infections in the near future (O'Neil, 2014).

Antibiotic-resistant bacteria and their resistance genes are frequently detected in wastewater systems of wastewater treatment plants (WWTPs)

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and reach the aquatic environment via WWTP discharge (Alexander et al., 2020; Hembach et al., 2017). The interruption of such pathways and prevention of selecting processes using adequate strategies can effectively interfere with antibiotic resistance evolution (Bengtsson-Palme et al., 2018; Munir et al., 2011; Rizzo et al., 2013). This also includes the identification of hotspots of antibiotic resistance and multiple resistance emission in municipal sewer systems. In many cases, specific antibiotic resistance genes and also their microbial carrier organisms have already been detected in different areas of the aquatic environment (Koczura et al., 2012; Mieszkin et al., 2009; Templar et al., 2016). Furthermore, several of these resistance genes are genetically transferable to different bacterial species via horizontal gene transfer in nature (Hu et al., 2016).

For identification of critical control points in sewer systems, a specific focus of this study is directed to particularly important hygienically relevant antibiotic resistance genes, which mediate resistances that are i) directed against reserve antibiotics (antibiotics of last choice in case of infections), and ii) are detected in intermediate or low abundances in wastewater systems via genetic detection methods (Hembach et al., 2019). This group includes the carbapenem resistance genes often associated with Gram-negative facultative pathogenic bacteria (Federal Office of Consumer Protection and Food Safety, 2014) (e.g. *Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter* spp., *Pseudomonas aeruginosa*) with the resistance genes $bla_{\text{CTX-M32}}$, $bla_{\text{CTX-M15}}$, $bla_{\text{OXA-48}}$, and $bla_{\text{CMY-2}}$ (Hembach et al., 2019). Among the less frequently occurring but highly important group of antibiotic resistance genes are the methicillin resistance gene from staphylococci (*mecA*), the colistin resistance gene of Gram-negative bacteria (*mcr*-1), the vancomycin resistance gene from enterococci (*vanA*), and the especially critically evaluated mobile carbapenem resistance genes from Gram-negative bacteria ($bla_{\text{NDM-1}}$ and $bla_{\text{KPC-3}}$) (Hembach et al., 2019; Ventola, 2015). In addition, multiple resistances of extended-spectrum β-lactam (ESBL) producing Gram-negative bacteria were analyzed in all samples and resistances against three or four different classes of antibiotics were determined in some sampling points within the municipal sewer system.

2. Materials and methods

2.1. Sampling, sample preparation, and DNA extraction

Four sampling campaigns were performed from February to December in 2020 in a grab sampling manner. In the first sampling campaign hospital sewer 2 could not be sampled, otherwise each sampling campaign consists of 2 hospital sewers, 5 retirement home sewers, and 8 housing sewer samples from a city with 20,000 population equivalents leading to 59 individual samples in total with a volume of 400 mL each. It was ensured that no rain event was present before and during each sampling campaign to allow comparability between sampling campaigns and normal drainage operation. Volumes of 50 mL raw wastewater of each individual sampling point were centrifuged at 20,400 $\times g$ for 15 min (Avanti J-25, Fullerton, California), decanted, and the complete pellet was used for DNA extraction using the FastDNA Spin kit for soil utilizing the lysing matrix E and the FASTPREP Instrument (MP Biomedical, Illkirch, France), according to previous studies and stored until further analysis at −20 °C (Hembach et al., 2017). The concentration and purity of the obtained DNA were assessed using the NanoDrop 1000 Spectrophotometer (Thermo Scientific, Germany), and minimum and maximum DNA concentrations were determined between 15.5 and 153.4 ng/μL (SI Table 1).

2.2. Antibiotic resistance genes and quantitative PCR (qPCR) analysis

Antibiotic resistance genes for identification of critical control points in municipal sewer systems were chosen based on their previously described abundance (Hembach et al., 2019) and consisted of $bla_{\text{CTX-M32}}$, $bla_{\text{CTX-M15}}$, bla_{OXA48}, $bla_{\text{CMY-2}}$, *mec*A, *van*A, *mcr*-1, $bla_{\text{NDM-1}}$, bla_{KPC3} (Table S2). Additionally the facultative pathogenic bacteria enterococci (23S), *E. coli* (*ycct*), *K. pneumoniae* (*gltA*), *A. baumannii* (*secE*), *P. aeruginosa* (*ecfx*) were quantified with qPCR for additional evaluation. All qPCR systems targeting ARGs and facultative pathogenic bacteria were run in technical triplicates according to Hembach et al. (2017, 2019).

For qPCR analysis, a Bio-Rad Cycler CFX96 (CFX96 Tou.ch Deep Well Real-Time PCR Detection System, Bio-Rad, Munich, Germany) was used. The specific resistance-directed genes in the entire bacterial community were quantified utilizing an SYBR Green qPCR approach according to Hembach et al. (2019) and Jäger et al. (2018). All qPCR systems targeting ARG were analyzed for specificity and sensitivity using reference bacteria according to Hembach et al. (2019). Full primer sequences, primer efficiencies, calibration equations, amplicon size, and Limit of Detection (LOD) of each primer detection system for qPCR reaction analysis with reference bacteria are listed in the Supplementary information (SI Table 2). Gene copy calculation according to Hembach et al. (2017, 2019) was performed to determine the absolute concentration of ARG normalized to 100 mL of wastewater.

2.3. Clustering of sampling areas

To derive a general observation aiming on the identification of hotspots for antibiotic resistance dissemination, results from 59 raw wastewater samples from a sewer pipe network were divided into clusters of hospital-influenced, retirement homes, and housing areas according to their location.

Besides the logical clustering of residence based on their inhabitants and their different needs and medication, various publications also showed the distinct antibiotic resistance profile of wastewater from hospital and other health associated institutions. The selection of clusters is basing on these previously gained experiences or other publications (Alexander et al., 2020; Kumar and Pal, 2018; Paulus et al., 2019; Sib et al., 2020). The cluster of Hospital sewers consists of seven individual samples in total, the Retirement home consists of 20 independent samples each, and the Housing sewer cluster consists of 32 individual samples. Based on their abundance in the microbial community, ARGs were grouped into frequent abundant ($bla_{\text{CTX-M32}}$, $bla_{\text{CTX-M15}}$, bla_{OXA48}, $bla_{\text{CMY-2}}$), and less abundant, but critical ARGs (*mec*A, *van*A, *mcr*-1, $bla_{\text{NDM-1}}$, bla_{KPC3}). For statistical significance of the quantitative combined abundance of all frequent and critical antibiotic resistance genes, a two-sided, non-parametric Mann-Whitney *U* test was performed.

2.4. Multiple resistance determination in Gram-negative bacteria

In addition, each sample was screened for ESBL-producing Gram-negative bacteria using CHROMagar ESBL (Mast Diagnostics GmbH, Reinfeld, Germany) and the manufacturers' protocol. For this, a dilution series was prepared for each wastewater sample, which contained the following original volumes: 50 mL, 1 mL, 0.1 mL, and 0.01 mL. With the exception of the 50 mL native wastewater sample, each dilution was adjusted to a volume of 10 mL in order to ensure uniform wetting of the nitrocellulose membrane (0.45 μm, Ø 50 mm, Whatman) during the vacuum filtration process. After incubation at 36 ± 1 °C for 21 ± 3 h, a maximum of ten ESBL-producing CFUs per sample were further analyzed for multiple resistance using the "Recommendations of the Commission for Hospital Hygiene and Infection Prevention (KRINKO) and the EUCAST definition for phenotypic antibiotic resistance determination (as of February 28, 2019/ No. 9). The testing of multiple resistance bacteria was completed with the protocol for the EUCAST disc diffusion test. No further characterization of bacteria isolates for antibiotic resistance enzyme activity was carried out.

3. Results and discussion

3.1. Absolute abundance of the 16S rRNA gene in sewers with distinct influences

For a first overview about the different sampling sites and their corresponding influences, the abundance of the 16S rRNA gene, as a marker

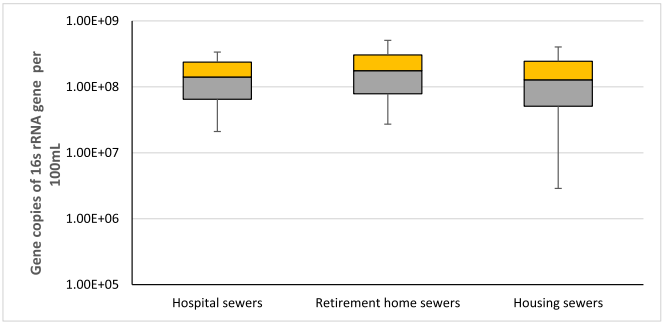

Fig. 1. Boxplot of the abundance of the 16S rRNA gene in the three different sewer wastewaters. (Hospital sewers, $n = 7$, retirement home sewers $n = 20$, and housing sewers $n = 32$). Whiskers represent minimal and maximal observed abundances.

for overall bacteria population, was calculated per 100 mL wastewater (Fig. 1).

Median values of all three clusters displayed similar concentration ranging from 7,6 × 10^7 gene copies/100 mL (Hospital sewers) to 9,7 × 10^7 gene copies/100 mL (Retirement home sewers) and 7,7 × 10^7 gene copies/100 mL (Housing sewers). While the hospital sewers were more consistent in its 16S gene copy abundance, a higher diversification in the retirement home sewers and especially in the housing sewers was observed. This can be attributed to the increasing number of sampling points compared to the hospital sewers indicating also more individual differences in discharge habits per residence of retirement homes as well as in the housing sewers.

3.2. Relative abundances of ARGs in microbial communities, structures, and diversities

The two investigated hospital sewers revealed distinct differences in ARG diversity and abundance in their microbial wastewater populations normalized to the 16S gene copy number. Here, Hospital sewer 1 was the only sampling point where all ARGs were quantified via qPCR during all sampling campaigns (Fig. 2A). In contrast, Hospital sewer 2 displayed 6 out of 9 ARGs continuously. The colistin resistance gene *mcr*-1 and the carbapenem resistance gene bla_{KPC3} were detected in only one sampling campaign with values of 1.57 × 10^{-8} and 7.68 × 10^{-5} per 16S copy number, respectively. The reason for this difference in ARG diversity may be attributed to the different hospital types. While Hospital 1 is focused more on general medical practices, the primary function of hospital 2 is characterized by its large psychiatric department.

In contrast to the specific ARG situations in hospital associated wastewaters, the $bla_{CTX-M32}$, $bla_{CTX-M15}$, bla_{CMY2}, bla_{OXA48} ARGs were found to be equally distributed in all sewer samples of the study including retirement homes and housing areas. Here, Hospital sewer 1 showed no significant difference in the abundances of these 4 ARGs, with median values ranging from 5.48 × 10^{-5} to 4.23 × 10^{-4} per 16S copy number ($p > 0.05$). In Hospital sewer 2 the ARGs $bla_{CTX-M32}$ was analyzed with 1.1 × 10^{-4} per 16S copy number, $bla_{CTX-M15}$ with 1.12 × 10^{-5} per 16S copy number, and bla_{CMY2} with 3.19 × 10^{-5} per 16S copy number. The one exception was the ARG bla_{OXA48}, which was found in significant higher abundance (2.07 × 10^{-3} per 16S copy number, $p < 0.05$). Generally, a comparable abundance of these 4 ARGs ($bla_{CTX-M32}$, $bla_{CTX-M15}$, bla_{CMY2}, bla_{OXA48}) including the dominant bla_{OXA48} abundance was also found in Retirement home sewers 3 and 5. These two retirement homes are characterized by a nursing home unit, which could explain the similar ARG pattern compared to Hospital sewer 2.

The less abundant ARGs (*mcr*-1, *mec*A, bla_{NDM-1}, *van*A, and bla_{KPC3}) were only consistently detected in Hospital sewer 1, which is associated with the main municipal hospital, indicating a continuous presence of these ARGs in the bacterial population. The highest median abundance was measured for bla_{KPC3} with 2.33 × 10^{-5} per 16S copy number followed by bla_{NDM-1} with 2.92 × 10^{-6}, *mcr*-1 with 2.23 × 10^{-6}, *mec*A with 3.26 × 10^{-7} and *van*A with 2.86 × 10^{-8} per 16S copy number. While in Hospital sewer 2 (psychiatric department) the ARGs *mcr*-1 and bla_{KPC3} were only detected sporadically, bla_{KPC3} exhibited an equivalent abundance with 7.69 × 10^{-5} per 16S copy number, which is comparable to its abundance in Hospital sewer 1. For *mcr*-1 only a low abundance with 1.57 × 10^{-8} per 16S copy number was detected in hospital sewer 2.

All 8 retirement homes sewers exhibited permanent high abundances of $bla_{CTX-M32}$, $bla_{CTX-M15}$, bla_{CMY2}, and bla_{OXA48} in their wastewater bacterial population (Fig. 2B) with median values ranging between 1.99 × 10^{-6} (bla_{CMY2}, Retirement home sewer 4) and 3.22 × 10^{-3} per 16S copy number (bla_{OXA48}, Retirement home sewer 5). The main difference of retirement home sewers regarding ARGs in bacteria populations is characterized by the diverse distribution of the less abundant detected ARGs. Here, *mcr*-1 and bla_{KPC3} displayed the lowest detection frequency, being only present during sampling campaign 2 (April 2020), or sampling campaign 3 (September 2020) with single values between 2.02 × 10^{-4} (bla_{KPC3}, Retirement home sewer 1) and 2.50 × 10^{-8} per 16S copy number

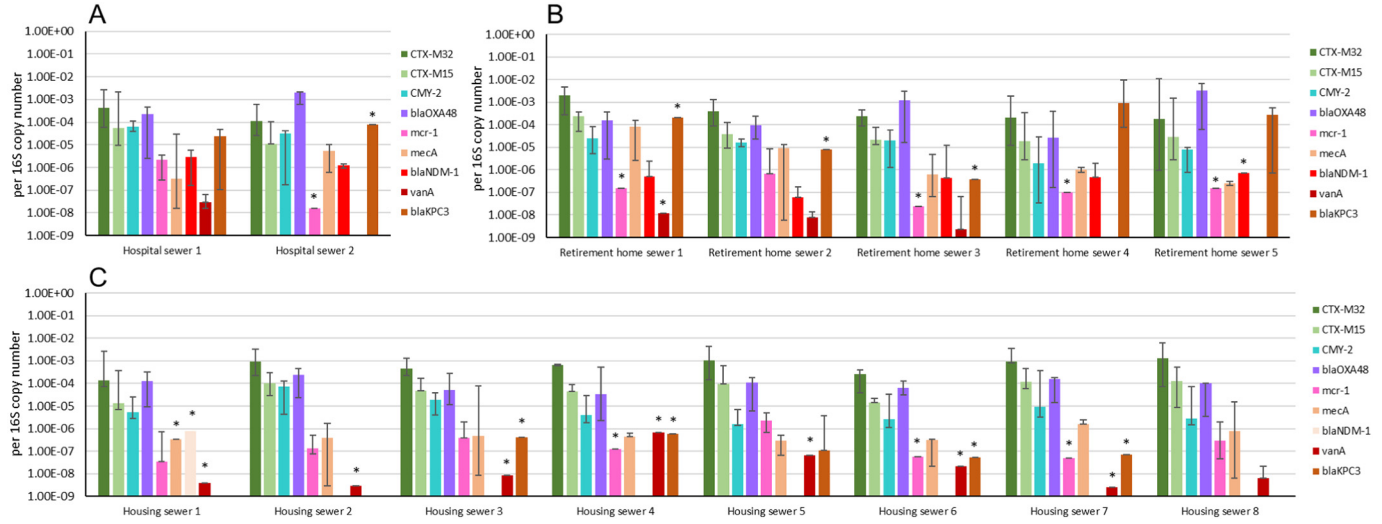

Fig. 2. Median concentration of antibiotic resistance genes (ARGs) in all investigated sewers. Whiskers show maximum and minimum values of each respective ARG. Median values for each ARG are derived from the total amount of 4 sampling campaigns. Each parameter was measured in technical triplicates. Values marked with [*] were only detected once during all sampling campaigns.

(mcr-1, Retirement home sewer 3). Noteworthy, two retirement home sewers showed low, but continuous abundances for vancomycin resistance gene *van*A with median values between 2.36 × 10^{-9} (Retirement home sewer 3) and 7.58 × 10^{-9} per 16 S copy number (Retirement home sewer 2). In Retirement home sewer 4 and 5 no *van*A genes could be detected during all four sampling campaigns. But, the staphylococcal specific methicillin ARG *mec*A was present in all retirement home sewer microbial populations with median values between 2.55 × 10^{-7} and 8.19 × 10^{-5} per 16 S copy number.

The housing sewer samples showed the lowest ARG diversities with only 4 ARGs permanently present during all sampling campaigns ($bla_{CTX\text{-}M32}$, $bla_{CTX\text{-}M15}$, bla_{CMY2}, bla_{OXA48}). Here, $bla_{CTX\text{-}M32}$ one of the most frequently detected ARGs displayed abundances between 1.33 × 10^{-4} (housing sewer 1) to 1.31 × 10^{-3} per 16S copy number (housing sewer 8). On the other side, bla_{CMY2} displayed the lowest abundance out of these 4 ARGs with median values between 1.58 × 10^{-6} (housing sewer 5) and 7.29 × 10^{-5} per 16S copy number. The less abundant ARGs (*mcr*-1, *mec*A, $bla_{NDM\text{-}1}$, *van*A, and bla_{KPC3}) were found only sporadically (Fig. 2C). Here, housing sewer 3, 4, 5, and 6 displayed conspicuous higher abundances of *van*A and bla_{KPC3} on a random basis compared to the other housing sewers reaching abundances between 8.48 × 10^{-9} (housing sewer 3) to 6.78 × 10^{-7} (housing sewer 4) per 16S copy number of *van*A and between 5.18 × 10^{-8} (housing sewer 6) and 5.84 × 10^{-7} (housing sewer 4) per 16S copy number of bla_{KPC3}. The ARG $bla_{NDM\text{-}1}$ was only detected during one sampling campaign in housing sewer 1 with 7.93 × 10^{-7} per 16S copy number.

3.3. Absolute abundances of ARGs and clustering of sampling areas

Absolute abundances of ARGs within the distinct volumes of each sewer sample were determined and divided in the categories of frequently abundant ARGs ($bla_{CTX\text{-}M32}$, $bla_{CTX\text{-}M15}$, bla_{CMY2}, bla_{OXA48}) and less abundant, but more critical ARGs (*mcr*-1, *mec*A, $bla_{NDM\text{-}1}$, *van*A, and bla_{KPC3}) (Figs. 3, 4). Furthermore, sampling areas were clustered into “Hospitals”, “Retirement homes”, and “Housing” sewers clusters to focus our findings on the identification of hotspots of antibiotic resistance dissemination.

Over the period of all sample campaigns, the hospital-influenced sewer cluster displayed a median absolute concentration for all frequently abundant ARGs of 3.95 × 10^6 gene copies per 100 mL (Fig. 3), which is derived from the individual median value of bla_{OXA48} (8.79 × 10^6 gene copies per 100 mL), $bla_{CTX\text{-}M32}$ (7.23 × 10^6 gene copies per 100 mL), $bla_{CTX\text{-}M15}$ (6.80 × 10^5 gene copies per 100 mL), and $bla_{CMY\text{-}2}$ (4.62 × 10^5 gene copies per 100 mL).

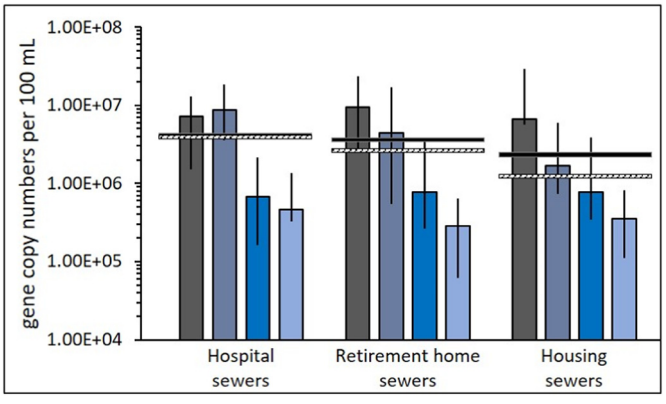

Fig. 3. Median concentration of each frequent abundant antibiotic resistance gene of all investigated sewer clusters (■ $bla_{CTX\text{-}M32}$, ■ bla_{OXA48}, ■ $bla_{CTX\text{-}M15}$, ■ $bla_{CMY\text{-}2}$). Whiskers show the upper (0.75) and lower quartiles (0.25) of each respective ARG. The mean (▬) and median (▭) value of each respective sewer cluster is derived from all frequent abundant ARG. (values and standard deviations are listed in SI Table 3).

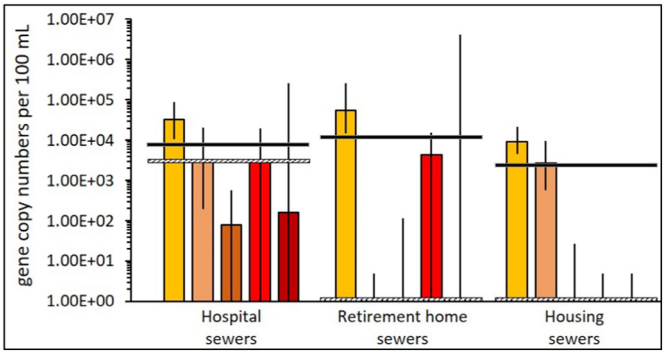

Fig. 4. Median concentration of each critical ARG in all investigated sewer clusters (■ *mecA*, ■ *mcr*-1, ■ *vanA*, ■ $bla_{NDM\text{-}1}$, ■ bla_{KPC3}). Whiskers show the upper (0.75) and lower quartiles (0.25) of each respective ARG. The mean (▬) and median (▭) value of each respective sewer cluster is derived from all critical ARG. More than half of the ARG parameter concentrations in the clusters Retirement home and Housing where equal to zero.

The median absolute concentration of all frequent abundant ARGs in the Retirement home sewer cluster was 2.59 × 10^6 gene copies per 100 mL. In contrast to the hospital-influenced sewer cluster, the highest median abundance was $bla_{CTX\text{-}32}$ (9.3 × 10^6 gene copies per 100 mL), followed by bla_{OXA48} (4.4 × 10^6 gene copies per 100 mL), $bla_{CTX\text{-}M15}$ (7.81 × 10^6 gene copies per 100 mL), and $bla_{CMY\text{-}2}$ (2.84 × 10^5 gene copies per 100 mL). A similar distribution in the ARG pattern of frequent abundant ARGs was displayed in the sewer clusters of housing areas with $bla_{CTX\text{-}M32}$ being also the most abundant ARG with a median value of 6.64 × 10^6 gene copies per 100 mL. This is followed by bla_{OXA48} with 1.68 × 10^6 gene copies per 100 mL, and $bla_{CTX\text{-}M15}$ with 7.72 × 10^5 gene copies per 100 mL. The lowest abundance in the Housing sewers cluster of this ARG category was $bla_{CMY\text{-}2}$ with 3.57 × 10^5 gene copies per 100 mL.

In the following, the mean and median values of each sewer cluster are compared for all frequently abundant ARGs under investigation. The mean value of Hospital sewers (4.29 × 10^6 gene copy numbers per 100 mL) was only 8.8% higher compared to its median value (3.95 × 10^6 gene copy numbers per 100 mL), indicating a stable and continuous presence of all four ARGs throughout all sampling campaigns (Fig. 3). The other two clusters displayed higher differences between median and mean values, with Retirement home sewers showing a median and mean ARG concentration of 2.59 × 10^6 and 3.69 × 10^6 gene copies per 100 mL, respectively, resulting in a 42.5% higher mean value difference. For the Housing sewer cluster, this difference increases to 91.84%, comparing median and mean concentrations of 1.23 × 10^6 and 2.36 × 10^6 gene copies per 100 mL. An explanation for these abundance fluctuations of the indicated categorized ARGs in sewer clusters of Retirement homes and Housing compared to hospital-influenced sewers may lie in the different prescription habits of hospital doctors compared to general practitioners. Here, Kern et al. (2006) describe the variation of outpatient antibiotics prescription in Germany to be substantially different compared to inpatient antibiotics prescriptions. Hospital doctors are found to be more stringent in antibiotics prescription which may lead to the observed more stable output of measured frequently abundant ARGs over all four sampling campaigns.

The less abundant, but critical evaluated ARGs, which are directed against reserve antibiotics (vancomycin, carbapenems, polymyxin B-type antibiotics), displayed a different pattern of abundances in the investigated sewer clusters compared to the previously described frequently abundant ARGs. These more individual differences observed are suited to identify critical control points of antibiotic resistance spread.

All five critical ARGs were only detected in the hospital-influenced sewer cluster indicating a higher ARG spectrum compared to the other sewer clusters, where only two out of five critical ARGs were consistently detected (Fig. 4). Actually, methicillin resistance gene *mec*A was found to possess the highest absolute abundance in all clustered sewer systems. A distinguishing feature for hospital-influenced sewers was the constant

abundance of the antibiotic resistance genes *vanA* and bla_{KPC3} with median values of 7.95×10^1 and 1.64×10^2 gene copies per 100 mL, respectively. In contrast, the other sewer clusters displayed only sporadic abundances of these 2 critical ARGs.

The Retirement home cluster is characterized by a high median abundance of bla_{NDM-1} (4.28×10^3 gene copies per 100 mL), which was 33.8% higher than that of the hospital-influenced sewer cluster (3.13×10^3 gene copies per 100 mL). The bla_{NDM1} was not detected in the housing sewer clusters during the sampling campaigns in February, April, and September. However, in the December sampling campaign of 2020, bla_{NDM-1} was sporadically detected also in Housing sewers (in 4 of 8 samples), which may be due to the COVID-19 pandemic-related lockdown in Germany, putting an increased number of people in home-associated self-care (Peine et al., 2020) and self-medication (Malik et al., 2020). The consequence of this home-associated self-medication during this period may be responsible for the fact that antibiotic resistances like bla_{NDM-1}, typically associated with hospital-acquired infections (Weber et al., 2019), could also be detected in housing sewers.

In the Housing sewer clusters, only *mec*A and *mcr*-1 being continuously detected. The median abundances of *mec*A and *mcr*-1 were 9.29×10^3 and 2.71×10^3 gene copies per 100 mL. In addition, there was significant difference in ARG abundance between Housing, Hospital, and Retirement home clusters ($p < 0.05$).

Comparing the different sewer clusters based on the concentration of all critical ARGs present in each cluster, the joint-mean value of critical ARGs in the Hospital sewer cluster (7.90×10^3 gene copies per 100 mL) was 157% higher compared to its joint-median value (3.07×10^3 gene copies per 100 mL). Compared to the frequently abundant ARGs of the same sewer cluster, its joint-mean value was only 8.8% higher compared to the joint-median value. This indicates a greater fluctuation of these critical ARG abundancies in the Hospital sewer cluster in contrast to the frequently abundant ARGs of this sewer cluster. For the other sewer clusters, no comparison between joint-mean and joint-median could be performed due to the absence of most of the critical ARGs in the Retirement home and Housing sewer clusters during the four sampling campaigns (Fig. 4).

Therefore, mean values for critical ARGs were used to compare these sewer clusters. The highest abundance of critical ARGs was found in the Retirement home sewer cluster with a mean value of 1.19×10^4 gene copies per 100 mL derived only from *mec*A and bla_{NDM1} abundance. It has to be mentioned that this must be discussed in a critical way since the spectrum of ARGs in the Hospital sewers cluster was higher, but with overall lower abundances of the more dominant ARGs (Fig. 4). In relation to the other sewer clusters, this means 50.6% more critical ARGs was detected in the Retirement sewer cluster compared to the Hospital sewer cluster, and 395.8% more critical ARGs compared to the Housing sewer cluster. The lowest mean value for critical ARG abundance was calculated for Housing sewers with 2.40×10^3 gene copies per 100 mL, derived only from *mec*A and *mcr*-1 abundance.

3.4. Summation of categorized ARGs for critical control point identification

Based on the results of the investigated ARGs in each sewer cluster it became apparent that the biggest difference was observed in the spectra and abundancies of critical ARGs. Therefore, to identify critical control points of ARG dissemination in urban sewer systems, the ARG diversity (quality) and the ARG concentration (quantity) are important factors to be considered. Therefore, the groups of frequently abundant ARG and hygienically critically evaluated ARGs were compared in terms of cumulated qualitative abundance and quantitative concentration in 100 mL (Fig. 5). The cumulative joint concentration of all frequent ARGs in the clusters Hospital sewers and Retirement sewers reached median values of 2.35×10^7 and 2.39×10^7 gene copies per 100 mL, respectively. The cumulative joint median values of frequently abundant ARGs in the Housing sewer clusters were 58% lower compared to the cumulative joint median values of Hospital and Retirement home sewers with 9.84×10^6 gene copies per 100 mL.

For the cumulative joint concentration of critical ARGs, the highest concentration was found in Retirement home sewers with 2.95×10^5 gene copies per 100 mL, despite of only having two out of five critical ARG parameters. The second highest cumulative joint concentration of critical ARGs was found in Hospital sewers with 6.28×10^4 gene copies per 100 mL factoring in all five ARG parameters. Housing sewers contained the least cumulative joint concentration of critical ARG with median values of 1.38×10^4 gene copies per 100 mL. This translates to 95% and 78% less critical ARG burden in Housing sewers compared to Retirement and Hospital sewers, respectively. Interestingly, in contrast to other sewer clusters, Housing sewers' cumulative joint concentration of critical ARGs displayed the lowest standard deviation (SI Table 3) out of all sewer clusters, indicating low fluctuations of critical ARG concentrations throughout all sampling campaigns.

To determine elevated levels of ARG concentrations in relation to all investigated sewer samples (hotspot identification), mean values for frequently abundant and critical ARGs were calculated and cumulative joint ARG concentration values of each individual sewer cluster were put into prospective (Fig. 5). For frequently abundant ARGs, Hospital and Retirement home sewer clusters were above the overall mean concentration (1.80×10^7 gene copies per 100 mL), while the Housing sewer cluster were below this threshold giving indications for a possible hotspot either in the Hospital or Retirement home sewer clusters. For critical ARGs, the overall mean value of 1.00×10^5 gene copies per 100 mL was only surpassed by the Retirement home sewer cluster. In addition, one of the retirement home sewer samples kept consistent elevated concentrations of

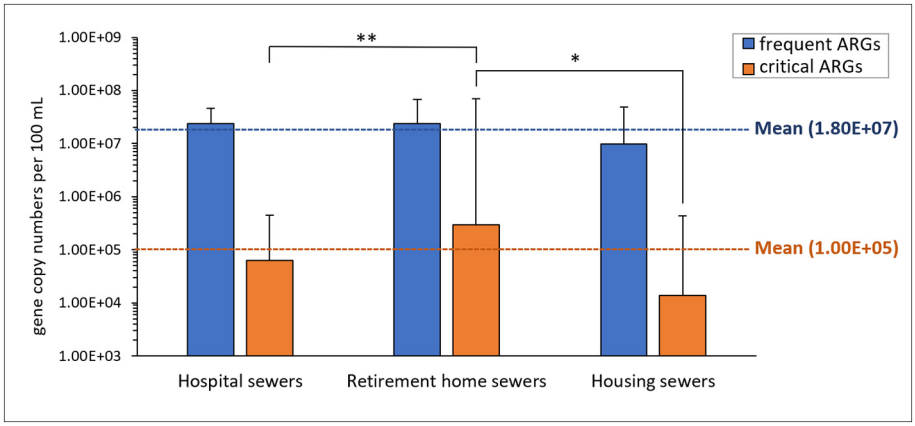

Fig. 5. Quantitative combined abundance of all frequently (blue) and critical antibiotic resistance genes (orange) in hospital sewers, retirement home sewers, and housing sewers. *$p < 0.05$, **$p > 0.05$.

Table 1
Average load of total frequent ARGs and total critical ARGs in each sewer cluster as well as in the downstream WWTP. The average flow volume of each sample cluster represents a snapshot taken during the sampling campaigns (4). No time-corresponding sampling was performed.

Sample cluster	Flow [L ∗ s^{-1}]	Time of sampling	Frequent ARGs [copies ∗ s^{-1}]	Critical ARGs [copies ∗ s^{-1}]
WWTP influent	102.75	08:33 h	1.18E+11	9.11E+07
WWTP effluent	102.75	08:27 h	4.05E+08	3.11E+08
Hospital sewers	2.56	12:20 h	7.52E+08	7.68E+06
Retirement home sewers	1.25	11:37 h	5.29E+08	2.39E+08
Housing sewers	4.75	11:32 h	1.46E+09	8.58E+06

critical ARGs leading to the highest standard deviation compared to all other sewer clusters (SI Table 3). By comparing cumulative joint concentrations of frequent and critical ARGs, the Retirement home sewer cluster was identified to contribute the most to the dissemination of ARGs. While it is important to also consider the high ARG diversity in the Hospital sewer cluster, selected retirement homes were identified to be the greatest quantitative hotspots for frequent as well as critical ARGs.

3.5. Flow volume-based analysis to estimate impact of on-site treatments

In addition, the volume flow of each wastewater system branch must also be considered as another parameter that influence subsequent contamination potentials of central wastewater volumes. Here, the average flow volume of each sewer cluster including the receiving local WWTP influent and effluent during the sampling period is displayed in Table 1. The calculations of ARGs in copies per second are based on the results presented above. For all frequently abundant ARGs ($bla_{CTX\text{-}M32}$, $bla_{CTX\text{-}M15}$, bla_{OXA48}, and $bla_{CMY\text{-}2}$) the raw wastewater of the WWTP influent displayed the highest load with 1.18×10^{11} gene copies per s. Within all investigated sewer clusters, the Housing sewers flow volume contained on average 1.46×10^{9} frequently abundant ARG copies per s. In comparison, the load of the Housing sewer cluster had on average 2-times more frequently abundant ARGs than the Hospital sewer cluster (7.52×10^{8} copies per s), and 3-times more than the total load of the Retirement home sewer cluster (5.29×10^{8} copies per s).

A different situation is presented when focusing on the total load of critical ARGs (*mecA*, $bla_{NDM\text{-}1}$, bla_{KPC3}, *van*A, and *mcr*-1) in the investigated sewer clusters. Here, the Retirement home cluster contained more than 25-times the total load of critical ARGs (2.39×10^{8} copies per s) compared to Housing (8.58×10^{6} copies per s) and Hospital sewer cluster (7.68×10^{6} copies per s). Putting the total load of critical ARGs of the different sewer clusters in prospective to the in- and effluent of the local WWTP, it becomes evident that the high ARG load found in the retirement home wastewaters (2.39×10^{8} copies per s) will be diluted by other wastewater streams (9.11×10^{7} copies per s, WWTP influent), but undergoes no additional reduction by the wastewater clarification process like observed for frequently abundant ARGs. This flow volume-based approach corresponds well with the quantitative PCR results, identifying retirement homes as well as hospitals to be important sources of clinically relevant ARGs.

These findings strongly supporting centralized actions at WWTPs to be more suited to interrupt the dissemination of frequently abundant ARGs from diffuse sources (e.g. Housing sewer cluster, Table 1). While on-site actions would effectively interrupt the dissemination of critical ARGs, which are attributed to specific hotspots like retirement homes and hospital wastewaters. Here, the contaminated flow volume is small and therefore more cost-effective to treat compared to the higher flow volumes reaching downstream WWTPs. Possible on-site treatment options for hospital wastewater were previously discussed (Paulus et al., 2019) and found to a have positive effects on the communal WWTP.

3.6. Multiple antimicrobial resistance analysis

Furthermore, cultivation experiments characterizing extended-spectrum beta-lactamase-producing (ESBL) Gram-negative bacteria in water samples of each sewer cluster. Up to 10 ESBL-producing Gram-negative bacteria from each sample (when available) were used for further phenotypical characterization, resulting in 60 isolates from the hospital cluster, 80 from the retirement housing associated sewers, and 30 isolates from the housing sewer cluster, which were tested for their resistance profile (Table 2).

The analysis revealed a total count of 32 multiple-resistant bacteria from hospital sewers, which are subdivided into 19 ESBL isolates with resistance against 3 antibiotic classes and 13 ESBL isolates being resistant towards 4 antibiotic classes (Table 2).

It is noteworthy that ESBL isolates from the Hospital sewer cluster contained the highest number of four-times multiple-resistant bacteria (i.e. 13/60). The Retirement home cluster displayed the highest total amount of analyzed multiple-resistant ESBL-producing Gram-negative bacteria (combined 3 and 4 multiple-resistant ESBL, i.e. 36). 32 out of 80 isolates were characterized to be resistant towards three antibiotic classes, but in contrast to the Hospital sewer cluster, only four out of 80 isolates conferred resistance towards four antibiotic classes. However, comparison of the total number of analyzed ESBL-producing bacteria of each sewer cluster corresponded well with the quantitative PCR results, indicating retirement homes to be an important source of clinically relevant antibiotic-resistant bacteria. Here, implementing intervention strategies could have the highest impact on the reduction of ARG/ARB loads in urban sewer systems before they reach the aquatic environment via local WWTP discharges. In addition to cultivation experiments specific taxonomic gene targets were used to quantify the abundances of facultative pathogenic bacteria being part of the ESKAPE-group defined by the WHO (World Health Organization, 2017) via qPCR according to Hembach et al. (2019). The results underlined the presence of this group of bacteria in all sewer systems in comparable gene copy quantities (Fig. S1). Hence, the taxonomic gene markers are not suitable for the identification of critical microbiological control points in sewer systems.

It could be demonstrated that the selection of appropriate biological parameters (i.e. critical ARGs) made it possible to identify hotspots of clinically relevant antibiotic resistances released into the municipal sewer system, reaching the municipal WWTP and subsequently also the receiving body (SI Table 4).

Our findings identified retirement homes to be one of the greatest quantitative hotspots for frequently as well as critically occurring ARGs. This is attributed by this clusters total load of critical ARGs being 25-times higher compared to the Housing and Hospital sewer clusters.

Table 2
Qualitative abundance of multiple-resistant ESBL-producing Gram-negative bacteria in each sewer cluster based on the total number of ESBL-producing Gram-negative bacteria analyzed.

Antibiotic class	Cephalosporines	Fluorchinolones	Carbapenems	Acylureido-penicillines	Multiple-resistant ESBL	
Test substances	Cefotaxim [5 μg] Ceftazidim [10 μg]	Ciprofloxacin [5 μg] Levofloxacin [5 μg]	Imipenem [10 μg] Meropenem [10 μg]	Piperacillin [30 μg] Tazobactam [6 μg]	Resistant to 3	Resistant to 4
Sewer cluster						
Hospital-influenced	46/60	31/60	23/60	41/60	19/60	13/60
Retirement homes	76/80	36/80	13/80	62/80	32/80	4/80
Housing area	30/30	23/30	3/30	28/30	23/30	1/30

There is a specific concern about the clinically relevant ARGs in general, but especially for the critical ARGs against the so-called reserve antibiotics as these ARGs can also pass the wastewater treatment plants reaching the receiving bodies (Hembach et al., 2017; Pärnänen et al., 2019). In addition, the Retirement home cluster displayed the highest total amount of isolated multiple-resistant ESBL-producing Gram-negative bacteria indicating also an important source of such clinically relevant antibiotic-resistant bacteria. Here, implementing on-site intervention strategies could have a high impact on the reduction of ARG/ARB loads in the urban sewer system before they reach the aquatic environment via local WWTP discharges.

This was previously demonstrated for other frequent occurring antibiotic resistances, but partially also for some of the critical antibiotic resistance genes directed against reserve antibiotics like carbapenems, colistin, and vancomycin (Alexander et al., 2020; Hembach et al., 2017, 2019). So far, such critical resistance genes have only been quantified in low abundancies in WWTP effluents, but without advanced and/or innovative interventions at hotspots, it is only a matter of time before these determinants also appear more frequently in aquatic compartments with wastewater influences. This has already happened for antibiotic resistances such as *sul*1, bla_{TEM} or *ermB* (Jäger et al., 2018). The recent study demonstrated a possible implementation of suitable parameters as a routine surveillance in a monitoring strategy, which can also help to detect shifts in the spread of clinically relevant antibiotic resistance to identify emerging critical control points.

Often, hospitals are described to be primary hotspots for the development and dissemination of antibiotic resistances and corresponding gene pools via clinic-associated pathways (Sib et al., 2019, 2020). Actually, about 7 to 12% of the patients in hospitals suffer from bacterial infections worldwide, i.e. 1.7 million people (Haque et al., 2018). Specific and targeted hygiene concepts would be helpful here in order to reduce the infection rates or outbreaks in clinics (Hota et al., 2009). The International Nosocomial Infection Control Consortiums reported that approximately 46% of all hospital-acquired infections are due to *Enterobacteriaceae* and 27% due to *Pseudomonas aeruginosa* followed by *Acinetobacter* spp. with 6%, and *Staphylococcus* spp. with 3% (Dafale and Purohit, 2016). Within the *Enterobacteriaceae*, an alarming increase in resistance to carbapenem antibiotics has been documented. Here, the most prominent carbapenemases are mediated by the resistance genes bla_{NDM-1}, bla_{OXA-23}, and bla_{KPC} indicating an alarming situation (Dafale and Purohit, 2016). Part of these genes was also detected and quantified in the present study. Ultimately, it is a matter of implementing local measures in accordance with the polluter-pays principle, in order to relieve central WWTPs and thus be able to counteract a further spread of critical antibiotic resistance genes in the aquatic environment. Although the introduction of distinct threshold values for ARB/ARGs is difficult to establish by regulations, the observed emission of these microorganismens to the environments bears the potential risk of their spread and proliferation. In addition, the horizontal gene transfer of ARGs from emitted facultative pathogens being present in WWTP effluents to indigenous aquatic bacteria and vice versa from environmentally occurring bacteria to WWTP released facultative pathogenic bacteria describe genetically dynamics, which might influence the population gene pools and drive the antibiotic resistance evolution.

This is particularly important when subjects of public protection such as water reservoirs, bathing waters, waters used for recreational purposes, and waters used in agriculture (e.g. for irrigation) are affected. Last but not least, there is also a distinct health responsibility for persons working as sewer workers in municipal wastewater systems. Colonization with antibiotic-resistant and especially multi-resistant facultative pathogenic bacteria is a critical issue when people come into direct contact with contaminated wastewater (Amarasiri et al., 2020; Rosenberg Goldstein et al., 2014).

CRediT authorship contribution statement

J.A.: data curation, manuscript preparation, conceptualization, formal analysis, project administration, validation, original draft writing, investigation.

N.H.: Formal analysis, data curation, project administration, investigation.

T.S.: Funding acquisition, conceptualization, supervision, original draft writing, writing – review and editing.

Declaration of competing interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

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References

Alexander, J., Hembach, N., Schwartz, T., 2020. Evaluation of antibiotic resistance dissemination by wastewater treatment plant effluents with different catchment areas in Germany. Sci. Rep. 101 (10), 1–9. https://doi.org/10.1038/s41598-020-65635-4.

Amarasiri, M., Sano, D., Suzuki, S., 2020. Understanding human health risks caused by antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARG) in water environments: current knowledge and questions to be answered. Crit. Rev. Environ. Sci. Technol. 50, 2016–2059. https://doi.org/10.1080/10643389.2019.1692611/FORMAT/EPUB.

Bengtsson-Palme, J., Kristiansson, E., Larsson, D.G.J., 2018. Environmental factors influencing the development and spread of antibiotic resistance. FEMS Microbiol. Rev. 42, 68–80. https://doi.org/10.1093/femsre/fux053.

Dafale, N.A., Purohit, H.J., 2016. Genomic tools for the impact assessment of 'Hotspots' for early warning of MDR threats. Biomed. Environ. Sci. 29, 656–674. https://doi.org/10.3967/bes2016.088.

Federal Office of Consumer Protection and Food Safety, 2014. Germap 2012 - Report on the Consumption of Antimicrobials And the Spread of Antimicrobial Resistance in Human an Veterinary Medicine in Germany Rheinbach.

Haque, M., Sartelli, M., McKimm, J., Abu Bakar, M., 2018. Health care-associated infections - an overview. Infect. Drug Resist. 11, 2321–2333. https://doi.org/10.2147/IDR.S177247.

Hembach, N., Schmid, F., Alexander, J., Hiller, C., Rogall, E.T., Schwartz, T., 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. https://doi.org/10.3389/fmicb.2017.01282.

Hembach, N., Alexander, J., Hiller, C., Wieland, A., Schwartz, T., 2019. Dissemination prevention of antibiotic resistant and facultative pathogenic bacteria by ultrafiltration and ozone treatment at an urban wastewater treatment plant. Sci. Rep. 9, 1–12. https://doi.org/10.1038/s41598-019-49263-1.

Hota, S., Hirji, Z., Stockton, K., Lemieux, C., Dedier, H., Wolfaardt, G., Gardam, M.A., 2009. Outbreak of multidrug-resistant Pseudomonas aeruginosa colonization and infection secondary to imperfect intensive care unit room design. Infect. Control Hosp. Epidemiol. 30, 25–33. https://doi.org/10.1086/592700.

Hu, Y., Yang, X., Li, J., Lv, N., Liu, F., Wu, J., Lin, I.Y.C., Wu, N., 2016. The bacterial mobile resistome transfer network connecting the animal and human microbiomes. 82, pp. 6672–6681. https://doi.org/10.1128/AEM.01802-16.Editor.

Jäger, T., Hembach, N., Elpers, C., Wieland, A., Alexander, J., Hiller, C., Krauter, G., Schwartz, T., 2018. Reduction of antibiotic resistant bacteria during conventional and advanced wastewater treatment, and the disseminated loads released to the environment. Front. Microbiol. 9, 1–16. https://doi.org/10.3389/fmicb.2018.02599.

Kern, W.V., De With, K., Nink, K., Steib-Bauert, M., Schröder, H., 2006. Regional variation in outpatient antibiotic prescribing in Germany. Infection 34, 269–273. https://doi.org/10.1007/s15010-006-6618-y.

Koczura, R., Mokracka, J., Jabłońska, L., Gozdecka, E., Kubek, M., Kaznowski, A., 2012. Antimicrobial resistance of integron-harboring Escherichia coli isolates from clinical samples, wastewater treatment plant and river water. Sci. Total Environ. 414, 680–685. https://doi.org/10.1016/j.scitotenv.2011.10.036.

Kumar, A., Pal, D., 2018. Antibiotic resistance and wastewater: correlation, impact and critical human health challenges. J. Environ. Chem. Eng. 6, 52–58. https://doi.org/10.1016/J.JECE.2017.11.059.

Malik, M., Tahir, M.J., Jabbar, R., Ahmed, A., Hussain, R., 2020. Self-medication during COVID-19 pandemic: challenges and opportunities. Drugs Ther. Perspect. 36, 565–567. https://doi.org/10.1007/s40267-020-00785-z.

Mieszkin, S., Furet, J.-P., Corthier, G., Gourmelon, M., 2009. Estimation of pig fecal contamination in a river catchment by real-time PCR using two pig-specific bacteroidales 16S rRNA genetic markers. Appl. Environ. Microbiol. 75, 3045–3054. https://doi.org/10.1128/AEM.02343-08.

Munir, M., Wong, K., Xagoraraki, I., 2011. Release of antibiotic resistant bacteria and genes in the effluent and biosolids of five wastewater utilities in Michigan. Water Res. 45, 681–693. https://doi.org/10.1016/j.watres.2010.08.033.

O'Neil, J., 2014. Review on antibiotic resistance. Antimicrobial resistance: tackling a crisis for the health and wealth of nations. Heal. Wealth Nations, pp. 1–16.

Pärnänen, K.M.M., Narciso-da-Rocha, C., Kneis, D., Berendonk, T.U., Cacace, D., Do, T.T., Elpers, C., Fatta-Kassinos, D., Henriques, I., Jaeger, T., Karkman, A., Martinez, J.L., Michael, S.G., Michael-Kordatou, I., O'Sullivan, K., Rodriguez-Mozaz, S., Schwartz, T., Sheng, H., Sørum, H., Stedtfeld, R.D., Tiedje, J.M., Giustina, S.V.Della, Walsh, F., Vaz-Moreira, I., Virta, M., Manaia, C.M., 2019. Antibiotic resistance in European wastewater treatment plants mirrors the pattern of clinical antibiotic resistance prevalence. Sci. Adv. 5. https://doi.org/10.1126/SCIADV.AAU9124.

Paulus, G.K., Hornstra, L.M., Alygizakis, N., Slobodnik, J., Thomaidis, N., Medema, G., 2019. The impact of on-site hospital wastewater treatment on the downstream communal wastewater system in terms of antibiotics and antibiotic resistance genes. Int. J. Hyg. Environ. Health 222, 635–644. https://doi.org/10.1016/j.ijheh.2019.01.004.

Peine, A., Paffenholz, P., Martin, L., Dohmen, S., Marx, G., Loosen, S.H., 2020. Telemedicine in Germany during the COVID-19 pandemic: multi-professional national survey. J. Med. Internet Res. 22. https://doi.org/10.2196/19745 e19745-e19745.

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

Rosenberg Goldstein, R.E., Micallef, S.A., Gibbs, S.G., George, A., Claye, E., Sapkota, A., Joseph, S.W., Sapkota, A.R., 2014. Detection of vancomycin-resistant enterococci (VRE) at four U.S. wastewater treatment plants that provide effluent for reuse. Sci. Total Environ. 466–467, 404–411. https://doi.org/10.1016/J.SCITOTENV.2013.07.039.

Sib, E., Voigt, A.M., Wilbring, G., Schreiber, C., Faerber, H.A., Skutlarek, D., Parcina, M., Mahn, R., Wolf, D., Brossart, P., Geiser, F., Engelhart, S., Exner, M., Bierbaum, G., Schmithausen, R.M., 2019. Antibiotic resistant bacteria and resistance genes in biofilms in clinical wastewater networks. Int. J. Hyg. Environ. Health 222, 655–662. https://doi.org/10.1016/j.ijheh.2019.03.006.

Sib, E., Lenz-Plet, F., Barabasch, V., Klanke, U., Savin, M., Hembach, N., Schallenberg, A., Kehl, K., Albert, C., Gajdiss, M., Zacharias, N., Müller, H., Schmithausen, R.M., Exner, M., Kreyenschmidt, J., Schreiber, C., Schwartz, T., Parčina, M., Bierbaum, G., 2020. Bacteria isolated from hospital, municipal and slaughterhouse wastewaters show characteristic, different resistance profiles. Sci. Total Environ. 746, 140894. https://doi.org/10.1016/J.SCITOTENV.2020.140894.

Templar, H.A., Dila, D.K., Bootsma, M.J., Corsi, S.R., McLellan, S.L., 2016. Quantification of human-associated fecal indicators reveal sewage from urban watersheds as a source of pollution to Lake Michigan. Water Res. 100, 556–567. https://doi.org/10.1016/j.watres.2016.05.056.

Ventola, C.L., 2015. The antibiotic resistance crisis part 1: causes and threats. 40, pp. 277–283.

Weber, R.E., Pietsch, M., Frühauf, A., Pfeifer, Y., Martin, M., Luft, D., Gatermann, S., Pfennigwerth, N., Kaase, M., Werner, G., Fuchs, S., 2019. IS26-mediated transfer of blaNDM–1 as the main route of resistance transmission during a polyclonal, multispecies outbreak in a German hospital. Front. Microbiol. 10, 1–14. https://doi.org/10.3389/fmicb.2019.02817.

WHO, 2019. No time to wait: securing the future from drug-resistant infections. Artforum Int. 54, 113–114.

World Health Organization, 2017. Global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics. Cad. Pesqui. 43, 348–365.