RESEARCH ARTICLE

Limited influence of hospital wastewater on the microbiome and resistome of wastewater in a community sewerage system

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ABSTRACT

Effluents from wastewater treatment plants (WWTPs) have been proposed to act as point sources of antibiotic-resistant bacteria (ARB) and antimicrobial resistance genes (ARGs) in the environment. Hospital sewage may contribute to the spread of ARB and ARGs as it contains the feces and urine of hospitalized patients, who are more frequently colonized with multi-drug resistant bacteria than the general population. However, whether hospital sewage noticeably contributes to the quantity and diversity of ARGs in the general sewerage system has not yet been determined.

Here, we employed culture-independent techniques, namely 16S rRNA gene sequencing and nanolitre-scale quantitative PCRs, to assess the role of hospital effluent as a point source of ARGs in the sewerage system, through comparing microbiota composition and levels of ARGs in hospital sewage with WWTP influent with and without hospital sewage. Compared to other sites, hospital sewage was richest in human-associated bacteria and contained the highest relative levels of ARGs. Yet, the relative abundance of ARGs was comparable in the influent of WWTPs with and without hospital sewage, suggesting that hospitals do not contribute importantly to the quantity and diversity of ARGs in the investigated sewerage system.

INTRODUCTION

Antibiotic-producing and antibiotic-resistant bacteria (ARB) naturally and ubiquitously occur in the environment (Anukool, Gaze and Wellington 2004; Wellington, Boxall and Cross 2013). However, human activities contribute importantly to the dissemination of resistant bacteria and resistance genes from humans and animals to the environment (Woolhouse and Ward 2013). Effluents of wastewater treatment plants (WWTPs) may represent an important source of ARB and antimicrobial resistance genes (ARGs) in the aquatic environment (Rizzo, Manaia and Merlin 2013; Wellington et al. 2013; Blaak, de Kruijf and Hamidjaja 2014; Czekalski, Gascon Diez and Burgmann 2014; Pruden 2014; Stalder, Barraud and Jove 2014; Karkman, Johnson and Lyra 2016; Karkman et al. 2017; Su, An and Li 2017). Generally, WWTPs collect municipal wastewater, but also wastewater from industry, farms and hospitals, dependent on the size

and nature of the communities connected to a single sewerage system. In hospitals, up to one third of patients receive antibiotic therapy on any given day and consequently, hospitals may be important hubs for the emergence and spread of ARB and ARGs (Vlahovic-Palcevski, Dumpis and Mitt 2007; Bush *et al.* 2011; Robert *et al.* 2012). Several studies have highlighted that multidrug-resistant nosocomial pathogens, ARGs and genetic determinants that contribute to the mobilization and dissemination of ARGs are abundant in hospital sewage, indicating that hospital sewage may play a role in the dissemination of bacteria and genetic determinants involved in antibiotic resistance (Stalder, Alrhmoun and Louvet 2013; Varela, Ferro and Vredenburg 2013; Stalder *et al.* 2014; Szekeres, Baricz and Chiriac 2017; Jin, Wang and Wang 2018).

The extent by which hospital effluent contributes to the presence of ARGs in sewerage systems is still poorly understood. To quantify the role of hospital effluent as a point source of ARGs in the sewerage system, we compared the relative levels of ARGs in hospital sewage with the WWTP influent that received the hospital sewage (urban influent) and with WWTP influent from a suburban setting that does not receive hospital effluent (suburban influent). Furthermore, relative abundance of ARGs in urban effluent and the surface water in which the urban effluent was released were determined. In addition, we investigated the microbial composition of all samples in order to investigate whether hospital effluent affected the urban sewage microbiota, and to follow the fate of intestinal microbiota as sources of ARGs along this sample gradient.

MATERIALS AND METHODS

Sampling locations

Sampling was conducted at the main hospital wastewater pipe of the University Medical Center Utrecht (UMCU), Utrecht in the Netherlands, and at two WWTP plants. One plant (termed 'urban WWTP' in this manuscript) treats wastewater of approximately 290000 inhabitants of the city of Utrecht, including the investigated hospital and two other hospitals. The other plant ('suburban WWTP' in Lopik, the Netherlands) treats wastewater of a suburban community of approximately 14000 inhabitants and does not serve a hospital (Supplementary Fig. S1). Both plants apply secondary treatment including nitrification and denitrification in activated sludge systems. Phosphorus removal is performed chemically in the urban WWTP, and biologically in the suburban WWTP. The hospital has approximately 1000 beds and 8200 employees (full-time equivalents). Additionally, some 2500 students are enrolled at the university hospital.

Sampling and DNA isolation

Samples were taken during a period of 2.5 weeks in Spring on four days (Monday 31 March 2014 = t1; Wednesday 2 April 2014 = t2; Monday 7 April 2014 = t3 and Monday 14 April 2014 = t4). Cumulative precipitation in the three days preceding each sampling date amounted to maximally 15 mm. The daily flows amount to $74800 \pm 5900 m^3$ for the urban WWTP, and $3390 \pm 380 m^3$ for the suburban WWTP during the four sampling days. The flows of the academic hospital amount to approximately $216000 m^3$ on a yearly basis, i.e. on average $590 m^3$ per day (0.8% of the influent of the urban WWTP). Exact quantification of the flows of the academic hospital is not possible, as the daily flows are not regularly registered. Flow-proportional sampling (over 24 hours) was used for sampling hospital wastewater and WWTP influent and effluent. Samples were kept at 4°C during flow-proportional sampling. For the surface water samples, grab samples (5 L) were taken at 50 cm downstream of the two effluent pipes of the urban WWTP discharging into a local river at a depth of 20 cm, in order to obtain a river sample under the direct influence of WWTP effluent. Samples were transported to the laboratory at 4°C and samples were processed the same day. The biomass of the collected water samples was concentrated for subsequent DNA extraction (the samples ranging from 4.4 Liter (urban WWTP effluent) to 0.9 Liter (hospital sewage, urban and suburban influent)). Cells and debris of sewage and surface water samples were pelleted by centrifugation (14000 *g* for 25 min). All pellets were resuspended in phosphate buffered saline (PBS; 138 mM NaCl, 2.7 mM KCl, 140 mM Na_2HPO_4, 1.8 mM KH_2PO_4, adjusted to pH 7.4 with HCl) with 20% glycerol and stored at −80 C° until DNA extraction. DNA was extracted from 200 µl of frozen samples as described previously (Godon, Zumstein and Dabert 1997).

16S rRNA gene sequencing and sequence data pre-processing

16S rRNA gene sequencing was performed on the Illumina MiSeq sequencing platform (San Diego, CA). A dual-indexing approach for multiplex 16S rRNA sequencing targeting the V3-V4 hypervariable region of the 16S rRNA gene was employed as described by (Fadrosh, Ma and Gajer 2014), using the 300 bp paired-end protocol to sequence a pool of 24 samples. Untrimmed paired-end reads were assembled using the FLASH assembler, which performs error correction during the assembly process (Magoc and Salzberg 2011). Removal of the barcodes, heterogeneity spacers, and primer sequences, resulted in a total of 1.4 million joined reads with a median length of 424 bases and a median number of 57860 joined reads per sample.

16S rRNA gene sequence data analysis

Joined reads were further analyzed using the QIIME microbial community analysis pipeline (version 1.8.0) (Caporaso, Kuczynski and Stombaugh 2010). Joined reads with a minimum of 97% similarity were assigned into operational taxonomic units (OTUs) using QIIME's open-reference OTU calling workflow. This workflow was used with the "-m usearch61" option, which uses the USEARCH algorithm (Edgar 2010) for OTU picking and UCHIME for chimeric sequence detection (Edgar, Haas and Clemente 2011). Taxonomic ranks for OTUs were assigned using the Greengenes database (version 13.8) (McDonald, Price and Goodrich 2012) with the default parameters of the script pick_open_reference_otus.py. A representative sequence of each OTU was aligned to the Greengenes core reference database (DeSantis, Hugenholtz and Larsen 2006) using the PyNAST aligner (version 1.2.2) (Caporaso, Bittinger and Bushman 2010). Highly variable parts of alignments were removed using the filter_alignment.py script, which is part of the pick_open_reference_otus.py workflow. Subsequently, filtered alignment results were used to create an approximate maximum-likelihood phylogenetic tree using FastTree (version 2.1.3) (Price, Dehal and Arkin 2010). For more accurate taxa diversity distribution (Bokulich, Subramanian and Faith 2013), OTUs to which less than 0.005% of the total number of assembled reads were mapped, were discarded using

the filter_otus_from_otu_table.py script with the parameter "–min_count_fraction 0.00005". The filtered OTU table and generated phylogenetic tree were used to assess within-sample (alpha) and between sample (beta) diversities.

Alpha- and beta-diversity of samples were assessed using QIIME's core_diversity_analyses.py workflow. For rarefaction analysis the subsampling depth threshold of 20681 was used, which was the minimum number of reads assigned to a sample. The UniFrac distance was used as input to calculate the Chao1 index as a measure of beta-diversity of the samples (Lozupone and Knight 2005). In addition to alpha- and beta-diversity analysis and visualizations, this workflow also incorporates principal coordinates analysis and visualization of sample compositions using Emperor (Vazquez-Baeza, Pirrung and Gonzalez 2013). Differences in the abundance of taxa are shown as averages over the four time points ± standard deviation resulting in six different comparisons between the different samples. The non-parametric Mann–Whitney test was used to test for significance; p values were corrected for multiple testing by the Benjamin–Hochberg procedure (Benjamini and Hochberg 1995) with a false discovery rate of 0.05. The Kruskal–Wallis test was used to test for differences in the microbiota composition between the four sampling time points at the six sites.

High-throughput qPCR

Real-Time PCR analysis was performed using the 96.96 BioMark™ Dynamic Array for Real-Time PCR (Fluidigm Corporation, San Francisco, CA, USA), according to the manufacturer's instructions, with the exception that the annealing temperature in the PCR was lowered to 56°C. DNA was first subjected to 14 cycles of Specific Target Amplification using a 0.18 μM mixture of all primer sets, excluding the 16S rRNA primer sets, in combination with the Taqman PreAmp Master Mix (Applied Biosystems), followed by a 5-fold dilution prior to loading samples onto the Biomark array for qPCR. Thermal cycling and real-time imaging was performed on the BioMark instrument, and Ct values were extracted using the BioMark Real-Time PCR analysis software. A reference sample consisting of pooled untreated wastewater DNA (hospital, urban and suburban) was loaded in a series of 4-fold dilutions and was used for the calculation of primer efficiency. All primers whose efficiency was experimentally determined to be between 80% and 120% were used to determine the normalized abundance of the target genes. The detection limit on the Biomark system was set to a Ct value of 20. In addition, to assess primer specificity, we performed melt curve analysis using the Fluidigm melting curve analysis software (http://fluidigm-melting-curve-analysis.software.informer.com/).
All PCRs were performed in triplicate and sample-primer combinations were included in the analysis only when at least two of the triplicate reactions resulted in a CT-value below the detection limit.

Other technical details of the nanolitre-scale quantitative PCRs to quantify levels of genes that confer resistance to antimicrobials (antibiotics and quaternary ammonium compounds (QACs)) were described previously (Buelow *et al.* 2017), with some modifications in the primers sequences (Supplementary Table S1).

Calculation of normalized abundance and cumulative abundance

Normalized abundance of resistance genes was calculated relative to the abundance of the 16S rRNA gene $2(-(CT_{ARG} - CT_{16S\ rRNA}))$. Data was log2 transformed for visualization by means of a heatmap that was generated using Microsoft Excel 2016 (Fig. 3). Cumulative abundance of each resistance gene family was calculated based on the sum of the normalized relative abundance $2(-(CT_{ARG} - CT_{16S\ rRNA}))$ of all genes detected within a resistance gene family. The non-parametric Mann–Whitney test was used to test for significance; P values were corrected for multiple testing by the Benjamin–Hochberg procedure (Benjamini and Hochberg 1995) with a false discovery rate of 0.05. The Kruskal–Wallis test was performed to test for differences in the resistome compositions between the four sampling time points at the six sites.

qPCR to determine absolute copy numbers of 16S rRNA genes

qPCRs for the quantification of 16S rRNA were performed with the same primers that were used in the high-throughput qPCR (Supplementary Table S1). A PCR fragment (112bp) was generated using chromosomal DNA of *E. coli* DH5α as template and serial dilutions of this fragment were used to generate a standard curve. The qPCR was performed using Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific, Leusden, The Netherlands) and a StepOnePlus instrument (Applied Biosystems, Nieuwekerk a/d IJssel, The Netherlands) with 5 ng DNA in the reaction and the following program: 95°C for 10 min, and subsequently 40 cycles of 95°C for 15 sec, 56°C for 1 min.

RESULTS

Composition of the microbiota of hospital sewage, WWTP influent, WWTP effluent and river water.

The composition of the microbiota in hospital sewage, urban and suburban WWTP influents, the effluent of the urban WWTP and the surface water in which the effluent was released was determined by multiplexed 16S rRNA gene sequencing on the Illumina MiSeq platform (Fig. 1A and Supplementary Table S2). At all sample sites, the microbiota consisted of a complex consortium of bacteria from different orders with the microbiota being most diverse in the effluent-influenced river samples and least diverse in hospital sewage (Supplementary Fig. S2). Hospital sewage contained relatively high levels (39.1 (± standard deviation of 1.9%) of anaerobic bacteria (Bifidobacteriales, Bacteroidales and Clostridiales) that are likely to originate from the human gut (Rajilic-Stojanovic and de Vos 2014). These orders were less abundant in WWTP influent (25.7%± 6.7%) and suburban WWTP influent (27.0%± 2.7%; $P < 0.05$) compared to hospital sewage. Compared to the WWTP influent, abundance of Bifidobacteriales, Bacteroidales and Clostridiales was significantly ($P < 0.05$) lower in WWTP effluent (12.1%± 2%) and effluent-influenced river water (7.0%± 1.2% for site 1 and 10.2% ± 1.4% for site 2). In contrast, bacteria that are associated with activated sludge, such as the Actinomycetales, Rhodocyclales, and Burkholderiales (Zhang, Shao and Ye 2012) became more prominent during passage through the sewerage system and WWTP (Fig. 1A and Supplementary Table S2). Principal coordinates analysis (PCoA) showed a clear distinction between the samples that were isolated prior to treatment in
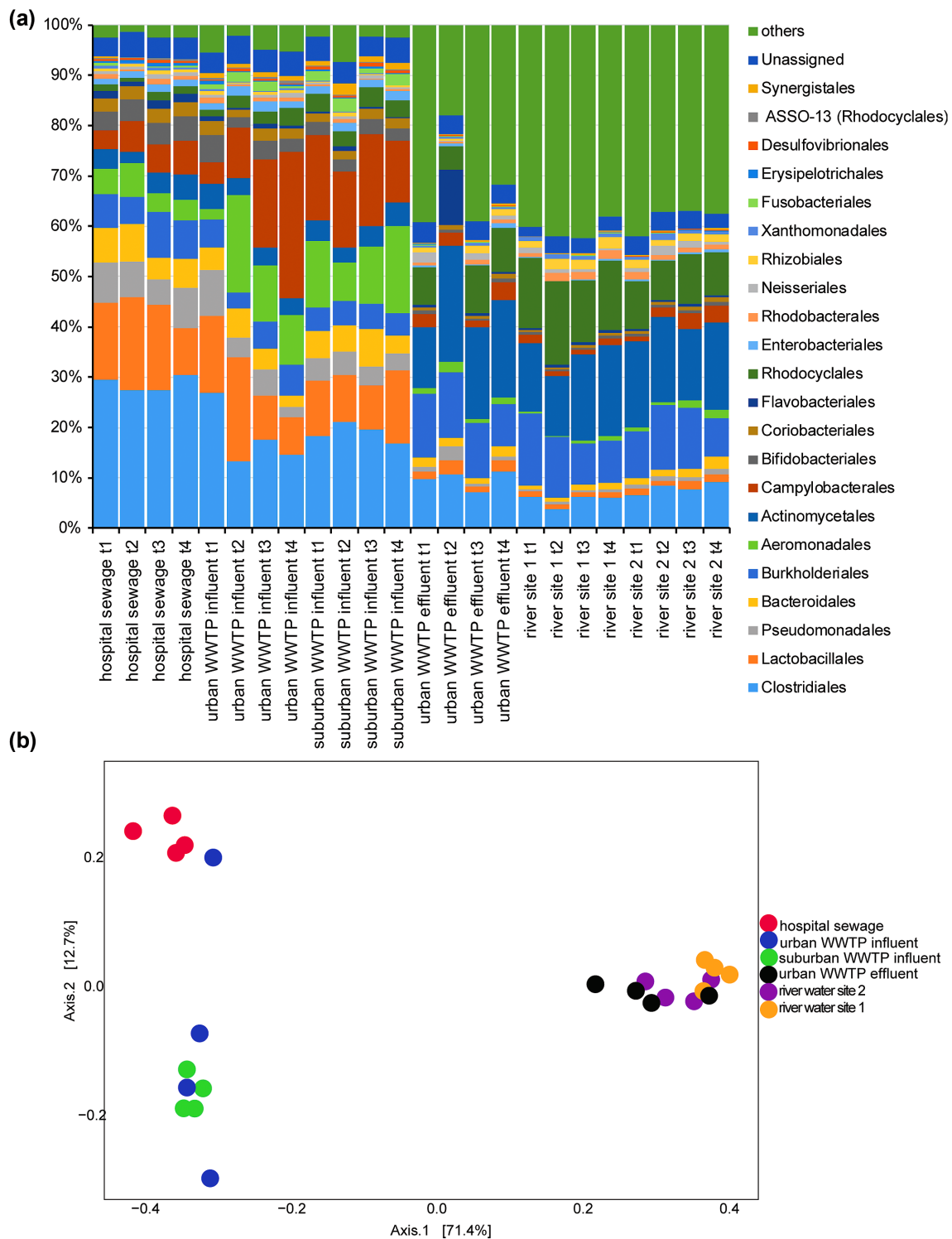
Figure 1. Microbiota composition of the sample locations at different time points. **(A)** Relative abundance of bacteria at the order level in different samples as detected by dual indexing 16S rRNA Illumina MiSeq sequencing. The 24 most abundant bacteria at the order level for all samples are depicted, where the "others" represents percentage of the remaining taxa and "Unassigned" shows percentage of OTUs that could not be assigned to any known taxonomy. The different sampling time points are indicated as t1 (Monday 31 March 2014); t2 (Wednesday 2 April 2014); t3 (Monday 7 April 2014); t4 (Monday 14 April 2014). **(B)** Principal coordinates analysis (PCoA) of microbiota composition for all different sampling locations and time points. PCoA based on the weighted UniFrac distance depicts the differences in microbiota compositions.

the WWTP and the samples of WWTP effluent and river water under direct influence of effluent (Fig. 1B). The three most abundant bacterial taxa detected in the hospital sewage were the genera *Streptococcus* (9.0%) and *Arcobacter* (6.9%) and the family Ruminococcaceae (6.3%). Both raw sewage influents (urban WWTP influent, suburban WWTP influent) clustered together and in both sites, the same three bacterial taxa were most abundant (*Arcobacter*: 17.9% in urban WWTP influent; 17.5% in suburban WWTP influent; Aeromonadaceae: 11.2% and 12.4%,respectively; Carnobacteriaceae, 9.4% and 8.3%,respectively). The comparison of urban WWTP influent with suburban WWTP influent shows that there is no significant difference in the microbiota composition between the two sewage influents (P = 0.87). The urban WWTP effluent samples were very similar to the surface water samples that were collected in close proximity of the effluent release pipes. Urban WWTP effluent shared the same three most common OTUs with one of the effluent-influenced water samples (Actinomycetales, 15.4% in urban WWTP effluent and 9.7% in effluent-influenced river site 2; Procabacteriaceae, 8.1% and 7.1%,respectively; Comamonadaceae, 7.6% and 7.7%,respectively). The surface water sample collected at the other release pipe (effluent-influenced river site 1) was slightly different and is defined by the following three most abundant OTUs: Comamonadaceae, 7.5%, Intrasporangiaceae, 6.1% and *Candidatus* Microthrix, 6.1%.

Resistome composition of hospital sewage compared to receiving urban sewage

A total of 67 ARGs were detected in the different samples, conferring resistance to 13 classes of antimicrobials. ARGs encoding efflux pumps that confer resistance to at least one of the 13 antimicrobial classes were also targeted, which resulted in the grouping and analysis of 14 ARG classes. The levels of ARGs were calculated as a normalized abundance relative to levels of the 16S rRNA gene, which provides an indication of the relative levels of ARGs within the bacterial population in each sample (Figs 2b and 3, and Supplementary Table S3). Absolute copy numbers of the 16S sRNA gene per milliliter of water were also determined as a proxy for bacterial biomass. The biomass in hospital sewage, urban WWTP influent and suburban WWTP influent were comparable (Fig. 2a). Biomass in the urban WWTP effluent and the effluent-influenced river sites was two to three logs lower compared to the untreated sewage waters (Fig.2a). Hospital sewage was found to be richer in ARGs, than the other samples. The normalized abundance of 12 out of 14 classes of ARGs was significantly (P < 0.05) higher in hospital sewage than in the urban WWTP influent, particularly so for aminoglycoside (12.0 ± 5.0-fold higher in hospital sewage), β-lactam (15.4 ± 3.6-fold higher in hospital sewage) and vancomycin resistance genes (175 ± 14-fold higher in hospital sewage, based on the three days when vancomycin resistance genes could be detected in the WWTP influent). Only the streptogramin resistance gene *vatB* was significantly less abundant (P < 0.05) in hospital sewage than in WWTP influent. The combined levels of chloramphenicol and quinolone resistance genes were not different between the sites. Seven ARGs (two aminoglycoside resistance genes, *aph(2")-Ib* and *aph(2")-I(de)*, the quinolone resistance gene *qnrA*, the erythromycin resistance gene *ermC*, the vancomycin resistance gene *vanB*, the AmpC-type β-lactamases bla_{DHA-1} and bla_{CMY-2} and the carbapenemase bla_{NDM}) were only detected in hospital sewage (Fig. 3). The carbapenemase bla_{IMP} was detected in effluent and river water samples, but not in hospital sewage

or WWTP influent. The relative abundance of ARGs in the urban WWTP influent, which receives sewage from the sampled hospital and two additional hospitals in the same city, and the suburban WWTP influent is comparable and not significantly different for any of the detected ARG families (Figs 2b and 3, and Supplementary Table S3). For nine classes of antibiotics (aminoglycosides, β-lactams, chloramphenicols, macrolides, polymyxins, puromycins, trimpethoprim, quinolones and tetracyclines), and for ARGs encoding efflux pumps, the levels of ARGs in the urban WWTP effluent were significantly (P < 0.05) lower than in the WWTP influent (ranging between a 8.0 ± 2.3-fold reduction for macrolide resistance genes to a 2.8 ± 0.9-fold reduction for β-lactam resistance genes), with the remaining classes of ARGs not changing significantly in abundance (Figs 2b and 3 and Supplementary Table S3). The levels of ARGs in WWTP effluent were comparable to the levels of ARGs in effluent-influenced river water (Figs 2b and 3 and Supplementary Table S3).

DISCUSSION

Our study demonstrates that hospital sewage harbours considerable levels of ARGs. The influents of the urban and suburban WWTPs studied here show very similar levels of ARGs, even though the urban WWTP receives sewage from a variety of sources including three hospitals, while the sub-urban WWTP does not have a hospital in its catchment area. This reflects the relatively limited effect of hospital sewage on the level of ARGs in WWTP influent and the low contribution of hospital sewage (an estimated 0.8%) to the total volume of wastewater treated in the urban WWTP that we investigated. Our study further demonstrates the capacity of WWTPs to importantly reduce the relative abundance of ARGs that are present in urban WWTP influent.

Effluents from WWTPs are thought to contribute to the dissemination of pollutants, multi-drug resistant bacteria and resistance genes in the environment (Rizzo et al. 2013; Wellington et al. 2013; Karkman et al. 2017). Particularly high levels of ARB and ARGs have previously been reported in hospital sewage (Diwan, Tamhankar and Khandal 2010; Harris, Morris and Morris 2013, 2014; Wellington et al. 2013; Berendonk et al. 2015; Rowe, Baker-Austin and Verner-Jeffreys 2017). Large amounts of antibiotics and QACs are used in hospitals and these may promote the establishment of ARB and selection of ARGs in patients and hospital wastewaters (Stalder et al. 2014; Varela, Andre and Nunes 2014; Rodriguez-Mozaz, Chamorro and Marti 2015; Barancheshme and Munir 2018). Here, we show that the relative abundance of a broad range of ARGs conferring resistance to 11 classes of antimicrobials is significantly higher in hospital sewage compared to urban and suburban WWTP sewage. In particular, genes conferring resistance to aminoglycosides, β-lactams and vancomycin are enriched in hospital sewage, presumably due to the frequent use of these classes of antibiotics in the hospital (Chandy, Naik and Charles 2014).

The most abundant bacterial taxa detected in the hospital sewage are different from those found in the urban and sub-urban WWTP influent, which are dominated by bacterial taxa (*Arcobacter*; Aeromonadaceae; Carnobacteriaceae) that are commonly found in the microbial sewerage ecosystem (Moreno, Botella and Alonso 2003; Vandewalle, Goetz and Huse 2012; Shanks, Newton and Kelty 2013; Fisher, Levican and Figueras 2014). Compared to the WWTP influent samples, several members of the human gut microbiota are significantly more abundant in hospital sewage, most probably due to the close proximity of the sampling location to the hospital sanitation systems. These human-associated taxa include the genus *Streptococcus*,
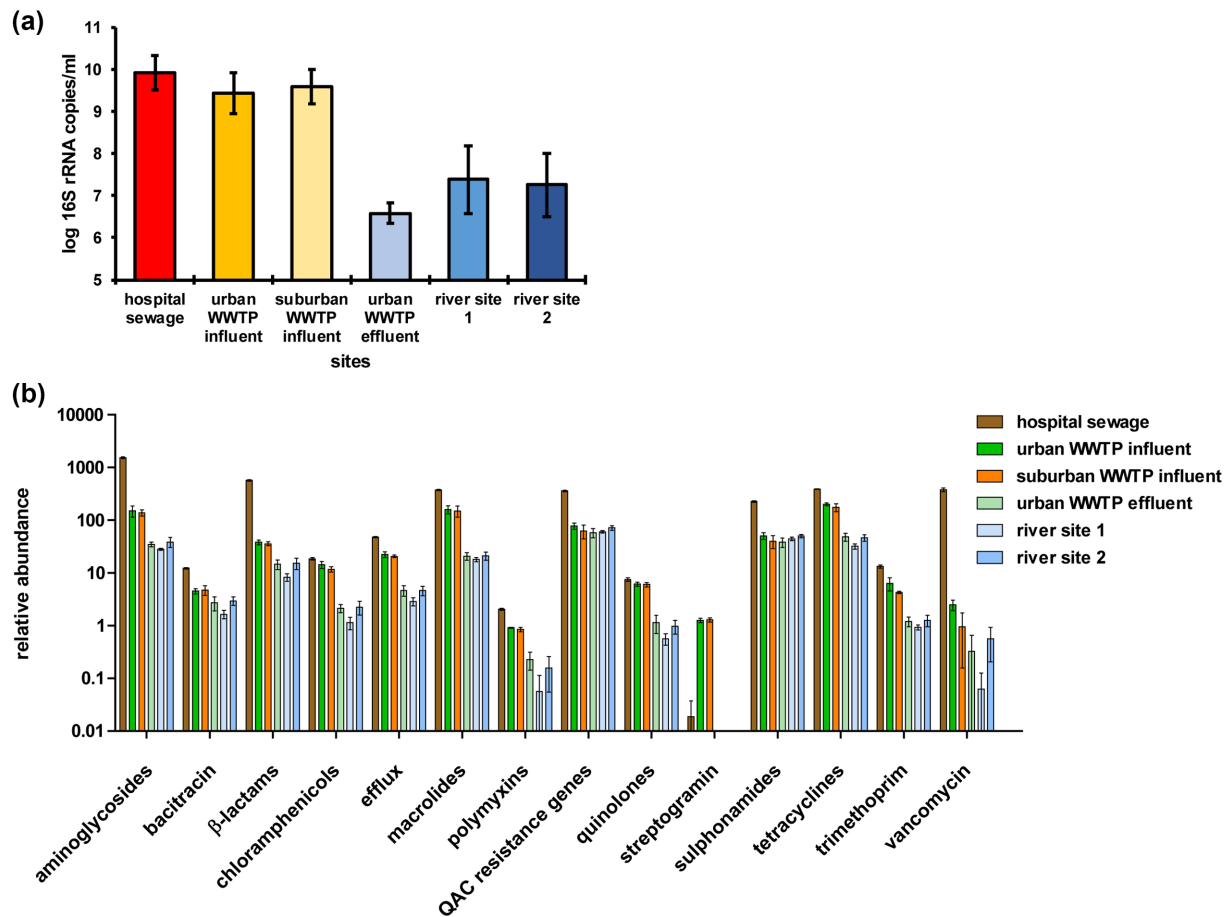
Figure 2. Biomass (copies of 16S rRNA gene/ml) and averaged relative abundance levels of ARG classes at the different sites. **(A)** Copies of 16S rRNA genes per ml as indicator for bacterial biomass averaged over the different time points (t1-t4) ± standard deviation for the individual sites. **(B)** 16S rRNA—normalized abundance of ARG classes detected in all samples. The cumulative abundance of the ARG classes detected for the different samples per site are averaged over all time points (t1-t4) and shown as an averaged fold-change ± standard deviation. ARGs are grouped according to resistance gene classes (aminoglycosides; bacitracin, ß-lactams; chloramphenicols; macrolides; efflux; polymyxins; QAC (quaternary ammonium compounds) resistance genes; quinolones; streptogramin; sulphonamides; tetracyclines; trimethoprim; vancomycin).

of which many species interact with humans either as commensals or pathogens (Kalia, Enright and Spratt 2001), and the Ruminococcaceae, which are one of the most prevalent bacterial families in the human gut (Arumugam *et al.* 2011; Lozupone, Stombaugh and Gordon 2012). These human-associated bacteria appear to be ill-suited for surviving the complex and, at least partially oxygenated, sewage environment and progressively decrease in abundance, leading to lower levels of human gut-associated bacteria in the urban WWTP influent (Pehrsson *et al.* 2016). Because most ARGs from the human microbiota appear to be carried by non-pathogenic commensal bacteria (Sommer, Dantas and Church 2009; Buelow *et al.* 2014), a general loss of human commensal bacteria in the sewerage system (Pehrsson *et al.* 2016) may contribute to a decrease in the abundance of ARGs during the passage of wastewater through the sewerage system.

The reduction of ARGs shown in urban WWTP effluent compared to WWTP influent may be explained by a further significant reduction of the relative abundance of human-associated bacterial taxa. The continuous reduction of these bacterial taxa could be mediated by their removal through sorption to activated sludge, by replacement with the bacteria that populate activated sludge, and/or by predation of protozoa during

wastewater treatment (Wen, Tutuka and Keegan 2009; Calero-Caceres, Melgarejo and Colomer-Lluch 2014). Interestingly, the presence of Procabacteriales in WWTP effluent and effluent-influenced river water may point towards a relatively high abundance of protists in these samples, as these bacteria are intracellular symbionts or pathogens of amoeba (Horn, Fritsche and Linner 2002; Greub and Raoult 2004).

Sampling for this study was limited to one single season, but was repeated on four days in dry weather conditions using mostly flow-proportional sampling as previously recommended (Ort, Lawrence and Rieckermann 2010). Microbiota and resistome profiling of our samples showed limited variation between the four sampling days for each sample, hence allowing for analysis of the treatment efficacy on the removal of ARGs relative to 16S rRNA in this particular WWTP. The reduction of the abundance of ARGs from hospital sewage to WWTP effluent highlights the importance of wastewater treatment in reducing the discharge of ARGs originating from human sources into the environment. However, the detection of bla_{IMP} in some of the WWTP effluent samples, while being non-detectable in all WWTP influent samples, suggests that this gene is present in the WWTP ecosystem and is shed into the environment through this effluent. Notably, bla_{IMP} was previously detected in the activated sludge of WWTPs in China and the USA (Yang, Zhang and Zhang
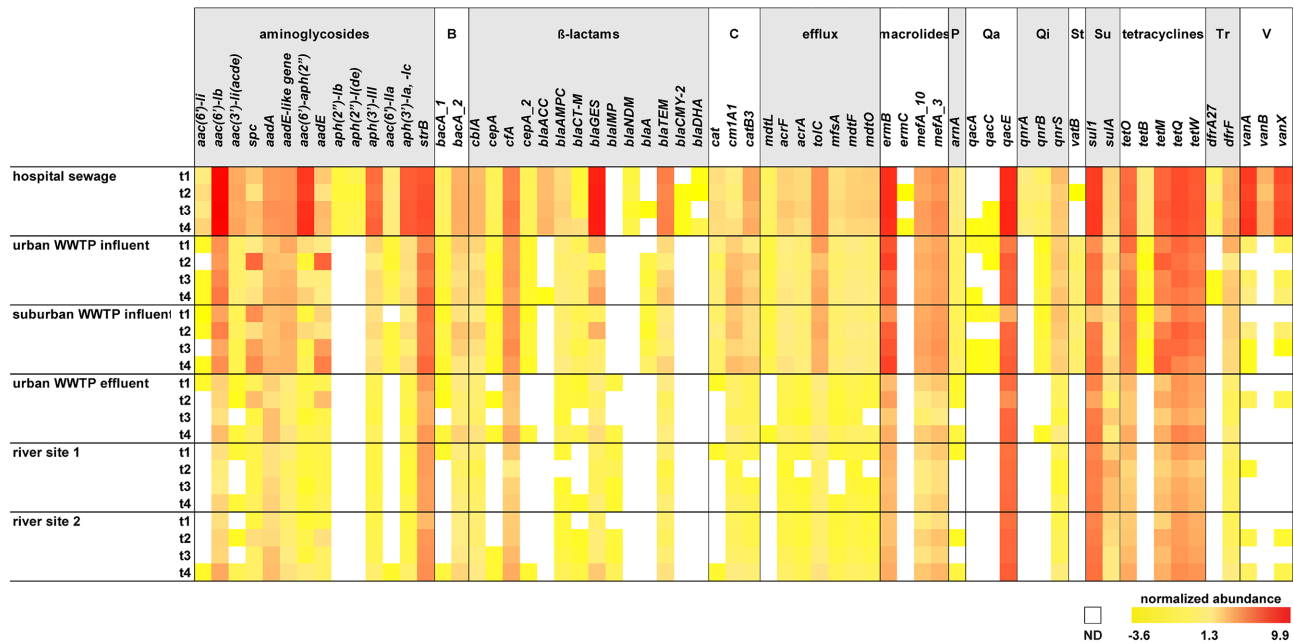
Figure 3. Relative abundance levels of individual ARGs in hospital sewage, urban and suburban WWTP influent, urban WWTP effluent and effluent-influenced river water. 16S rRNA—normalized abundance of individual ARGs detected in all samples. ARGs are grouped according to resistance gene classes (aminoglycosides; B, bacitracin, ß-lactams; C, chloramphenicols; macrolides; efflux; P, polymyxins; Qa, QAC resistance genes; Qi, quinolones; St, streptogramin; Su, sulphonamides; tetracyclines; Tr, trimethoprim; V, vancomycin). The colour scale ranges from bright red (most abundant) to bright yellow (least abundant). White blocks indicate that a resistance gene was not detected. The different sampling time points are indicated as t1 (Monday 31 March 2014); t2 (Wednesday 2 April 2014); t3 (Monday, 7 April 2014); t4 (Monday, 14 April 2014).

2012). The bla_{IMP} gene encodes a carbapenemase and is clinically mostly associated with *Pseudomonas aeruginosa* but it has also been detected in Beta- and Gammaproteobacteria of environmental origin (Riccio, Franceschini and Boschi 2000; Zhao and Hu 2011). With respect to the abundance of ARGs relative to the 16S rRNA gene, it has been debated whether sewage treatment could selectively affect the percentage of resistant bacteria within a given species, or within the total community (Rizzo *et al.* 2013; Laht, Karkman and Voolaid 2014; Alexander, Bollmann and Seitz 2015). Here, and in line with Karkman, Johnson and Lyra (2016), we observed that wastewater treatment led to a decrease in the relative abundance of the majority of ARGs. Absolute copy numbers of 16S rRNA genes per ml water are 2–3 log lower in effluent than in influent, i.e. the decrease in the abundance normalized to the 16S rRNA gene observed here translates to an even larger decrease in the absolute abundance (in copies/ml) of ARGs.

Advanced water treatment methods have been proposed as a selective measure for hospital wastewater, specifically to decrease pharmaceuticals and the release of pathogens by hospitals (Lienert *et al.* 2011). For the investigated municipal sewerage system, hospital wastewater seems to play a limited role for the level of resistance genes in the influent. Our findings suggest that—in the presence of operational WWTPs—hospital-specific sewage treatment will not lead to a substantial further reduction of the release of ARGs into influent.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online.

Availability of data and materials

The 16S rRNA sequence data that support the findings of this study have been made available at the European Nucleotide Archive (ENA) under accession number PRJEB23478.

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