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

Buelow, Elena; Bayjanov, Jumamurat R; Majoor, Eline; Willems, Rob J L; Bonten, Marc J M; Schmitt, Heike; van Schaik, Willem

DOI:
10.1093/femsec/fiy087

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

Elena Buelow[1,2*], Jumamurat R. Bayjanov[1], Eline Majoor[1], Rob J.L. Willems[1], Marc J.M. Bonten[1], Heike Schmitt[3] and Willem van Schaik[1,4]

[1]Department of Medical Microbiology, University Medical Center Utrecht, Utrecht, The Netherlands

[2]Université Limoges, INSERM, CHU Limoges, UMR 1092, Limoges, France

[3]National Institute for Public Health and the Environment (RIVM), Bilthoven, The Netherlands , and Institute for Risk Assessment Sciences, Utrecht University

[4]Institute of Microbiology and Infection, University of Birmingham, United Kingdom

*Corresponding Author: Université Limoges, INSERM, CHU Limoges, UMR 1092, Agents antimicrobiens Faculté de médecine, 2 rue du Dr Marcland, Limoges, France. Tel :+33519910564262. Email. elena.buelow@gmail.com

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 *et al.*, 2004; Wellington *et al.*, 2013). However, human activities contribute importantly to the dissemination of resistant bacteria and resistance genes from humans and animals to the environment (Woolhouse & Ward, 2013). Effluents of wastewater treatment plants (WWTPs) may represent an important source of ARB and antimicrobial resistance genes (ARGs) in the aquatic environment (Wellington *et al.*, 2013; Rizzo *et al.*, 2013; Stalder *et al.*, 2014; Pruden, 2014; Czekalski *et al.*, 2014; Blaak *et al.*, 2014; Karkman *et al.*, 2016; Karkman *et al.*, 2017; Su *et al.*, 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 *et al.*, 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 *et al.*, 2013; Varela *et al.*, 2013; Stalder *et al.*, 2014; Szekeres *et al.*, 2017; Jin *et al.*, 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 290,000 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 14,000 inhabitants and does not serve a hospital (Supplementary Figure 1). . 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 1,000 beds and 8,200 employees (full-time equivalents). Additionally, some 2,500 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 74,800 ± 5,900 m^3 for the urban WWTP, and 3,390 ± 380 m^3 for the suburban WWTP during the four sampling days. The flows of the academic hospital amount to approximately 216,000 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 minutes). 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 *et al.*, 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 *et al.*, 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 & 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 *et al.*, 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 *et al.*, 2011). Taxonomic ranks for OTUs were assigned using the Greengenes database (version 13.8) (McDonald *et al.*, 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 *et al.*, 2006) using the PyNAST aligner (version 1.2.2) (Caporaso, Bittinger *et al.*, 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 *et al.*, 2010). For more accurate taxa diversity distribution (Bokulich *et al.*, 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 & 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 *et al.*, 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 & 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, U.S.A), 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 only included in the analysis 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 1).

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 (Figure 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 & 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 1). 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 (Figure 1A and Supplementary Table 2). 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 Figure 2). 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 & 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 *et al.*, 2012) became more prominent during passage through the sewerage system and WWTP (Figure 1A and Supplementary Table 2). Principal coordinates analysis (PCoA) showed a clear distinction between the samples that were isolated prior to treatment in the WWTP and the samples of WWTP effluent and river water under direct influence of effluent (Figure 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 (Figure 2b, Figure 3, and Supplementary Table 3). 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 (Figure 2a). Biomass in the urban WWTP effluent and the effluent-influenced river sites was 2 to 3 logs lower compared to the untreated sewage waters (Figure 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 (Figure 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 (Figure 2b, Figure 3, and Supplementary Table 3). 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 (Figure 2b, Figure 3 and Supplementary Table 3). The levels of ARGs in WWTP effluent were comparable to the levels of ARGs in effluent-influenced river water (Figure 2b, Figure 3 and Supplementary Table 3).

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 *et al.*, 2010; Wellington *et al.*, 2013; Harris *et al.*, 2013; Harris *et al.*, 2014; Berendonk *et al.*, 2015; Rowe *et al.*, 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 *et al.*, 2014; Rodriguez-Mozaz *et al.*, 2015; Barancheshme & 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 *et al.*, 2014).

The most abundant bacterial taxa detected in the hospital sewage are different from those found in the urban and suburban WWTP influent, which are dominated by bacterial taxa (*Arcobacter*; Aeromonadaceae; Carnobacteriaceae) that are commonly found in the microbial sewerage ecosystem (Moreno *et al.*, 2003; Vandewalle *et al.*, 2012; Shanks *et al.*, 2013; Fisher *et al.*, 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*, of which many species interact with humans either as commensals or pathogens (Kalia *et al.*, 2001), and the Ruminococcaceae, which are one of the most prevalent bacterial families in the human gut (Arumugam *et al.*, 2011; Lozupone *et al.*, 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 *et al.*, 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 *et al.*, 2009; Calero-Caceres *et al.*, 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 *et al.*, 2002; Greub & 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 *et al.*, 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 *et al.*, 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 *et al.*, 2000; Zhao & 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 *et al.*, 2014; Alexander *et al.*, 2015). Here, and in line with (Karkman *et al.*, 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.

Funding

This work was supported by The Netherlands Organisation for Health Research and Development ZonMw (Priority Medicine Antimicrobial Resistance; grant 205100015) and by the European Union Seventh Framework Programme (FP7-HEALTH-2011-single-stage) ‘Evolution and Transfer of Antibiotic Resistance’ (EvoTAR), under grant agreement number 282004. In addition, the research of W.v.S is supported by a NWO-

VIDI grant (917.13.357) and a Royal Society Wolfson Research Merit Award (WM160092).

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.

References

Alexander J, Bollmann A, Seitz W & Schwartz T (2015) Microbiological characterization of aquatic microbiomes targeting taxonomical marker genes and antibiotic resistance genes of opportunistic bacteria Sci Total Environ **512-513:** 316-325.

Anukool U, Gaze WH & Wellington EM (2004) In situ monitoring of streptothricin production by *Streptomyces rochei F20* in soil and rhizosphere Appl Environ Microbiol **70:** 5222-5228.

Arumugam M, Raes J, Pelletier E *et al.* (2011) Enterotypes of the human gut microbiome Nature **473:** 174-180.

Barancheshme F & Munir M (2018) Strategies to Combat Antibiotic Resistance in the Wastewater Treatment Plants Front Microbiol **8:** 2603.

Benjamini Y & Hochberg Y (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. Journal of the Royal Statistical Society Series B (Methodological) **Vol. 57, No. 1:** pp. 289-300.

Berendonk TU, Manaia CM, Merlin C *et al.* (2015) Tackling antibiotic resistance: the environmental framework Nat Rev Microbiol **13:** 310-317.

Berkner S, Konradi S & Schonfeld J (2014) Antibiotic resistance and the environment--there and back again: Science & Society series on Science and Drugs EMBO Rep **15:** 740-744.

Blaak H, de Kruijf P, Hamidjaja RA, van Hoek AH, de Roda Husman AM & Schets FM (2014) Prevalence and characteristics of ESBL-producing *E. coli* in Dutch recreational waters influenced by wastewater treatment plants Vet Microbiol **171:** 448-459.

Bokulich NA, Subramanian S, Faith JJ, Gevers D, Gordon JI, Knight R, Mills DA & Caporaso JG (2013) Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing Nat Methods **10:** 57-59.

Buelow E, Bello Gonzalez TDJ, Fuentes S *et al.* (2017) Comparative gut microbiota and resistome profiling of intensive care patients receiving selective digestive tract decontamination and healthy subjects Microbiome **5:** 88-017-0309-z.

Buelow E, Gonzalez TB, Versluis D *et al.* (2014) Effects of selective digestive decontamination (SDD) on the gut resistome J Antimicrob Chemother.

Bush K, Courvalin P, Dantas G *et al.* (2011) Tackling antibiotic resistance Nat Rev Microbiol **9:** 894-896.

Calero-Caceres W, Melgarejo A, Colomer-Lluch M, Stoll C, Lucena F, Jofre J & Muniesa M (2014) Sludge as a potential important source of antibiotic resistance genes in both the bacterial and bacteriophage fractions Environ Sci Technol **48:** 7602-7611.

Caporaso JG, Bittinger K, Bushman FD, DeSantis TZ, Andersen GL & Knight R (2010) PyNAST: a flexible tool for aligning sequences to a template alignment Bioinformatics **26:** 266-267.

Caporaso JG, Kuczynski J, Stombaugh J *et al.* (2010) QIIME allows analysis of high-throughput community sequencing data Nat Methods **7:** 335-336.

Chandy SJ, Naik GS, Charles R, Jeyaseelan V, Naumova EN, Thomas K & Lundborg CS (2014) The impact of policy guidelines on hospital antibiotic use over a decade: a segmented time series analysis PLoS One **9:** e92206.

Czekalski N, Gascon Diez E & Burgmann H (2014) Wastewater as a point source of antibiotic-resistance genes in the sediment of a freshwater lake ISME J **8:** 1381-1390.

DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, Huber T, Dalevi D, Hu P & Andersen GL (2006) Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB Appl Environ Microbiol **72:** 5069-5072.

Diwan V, Tamhankar AJ, Khandal RK, Sen S, Aggarwal M, Marothi Y, Iyer RV, Sundblad-Tonderski K & Stalsby-Lundborg C (2010) Antibiotics and antibiotic-resistant

bacteria in waters associated with a hospital in Ujjain, India BMC Public Health **10:** 414-2458-10-414.

Edgar RC (2010) Search and clustering orders of magnitude faster than BLAST Bioinformatics **26:** 2460-2461.

Edgar RC, Haas BJ, Clemente JC, Quince C & Knight R (2011) UCHIME improves sensitivity and speed of chimera detection Bioinformatics **27:** 2194-2200.

Fadrosh DW, Ma B, Gajer P, Sengamalay N, Ott S, Brotman RM & Ravel J (2014) An improved dual-indexing approach for multiplexed 16S rRNA gene sequencing on the Illumina MiSeq platform Microbiome **2:** 6-2618-2-6.

Fisher JC, Levican A, Figueras MJ & McLellan SL (2014) Population dynamics and ecology of *Arcobacter* in sewage Front Microbiol **5:** 525.

Godon JJ, Zumstein E, Dabert P, Habouzit F & Moletta R (1997) Molecular microbial diversity of an anaerobic digestor as determined by small-subunit rDNA sequence analysis Appl Environ Microbiol **63:** 2802-2813.

Greub G & Raoult D (2004) Microorganisms resistant to free-living amoebae Clin Microbiol Rev **17:** 413-433.

Harris S, Morris C, Morris D, Cormican M & Cummins E (2014) Antimicrobial resistant *Escherichia coli* in the municipal wastewater system: effect of hospital effluent and environmental fate Sci Total Environ **468-469:** 1078-1085.

Harris S, Morris C, Morris D, Cormican M & Cummins E (2013) The effect of hospital effluent on antimicrobial resistant *E. coli* within a municipal wastewater system Environ Sci Process Impacts **15:** 617-622.

Horn M, Fritsche TR, Linner T, Gautom RK, Harzenetter MD & Wagner M (2002) Obligate bacterial endosymbionts of *Acanthamoeba spp.* related to the *beta-Proteobacteria*: proposal of '*Candidatus Procabacter acanthamoebae' gen. nov., sp. nov* Int J Syst Evol Microbiol **52:** 599-605.

Jin L, Wang R, Wang X, Wang Q, Zhang Y, Yin Y & Wang H (2018) Emergence of mcr-1 and carbapenemase genes in hospital sewage water in Beijing, China J Antimicrob Chemother **73:** 84-87.

Kalia A, Enright MC, Spratt BG & Bessen DE (2001) Directional gene movement from human-pathogenic to commensal-like *streptococci* Infect Immun **69:** 4858-4869.

Karkman A, Do TT, Walsh F & Virta MPJ (2017) Antibiotic-Resistance Genes in Waste Water Trends Microbiol.

Karkman A, Johnson TA, Lyra C, Stedtfeld RD, Tamminen M, Tiedje JM & Virta M (2016) High-throughput quantification of antibiotic resistance genes from an urban wastewater treatment plant FEMS Microbiol Ecol **92:** 10.1093/femsec/fiw014. Epub 2016 Jan 31.

Laht M, Karkman A, Voolaid V, Ritz C, Tenson T, Virta M & Kisand V (2014) Abundances of tetracycline, sulphonamide and beta-lactam antibiotic resistance genes

in conventional wastewater treatment plants (WWTPs) with different waste load PLoS One **9:** e103705.

Lienert J, Koller M, Konrad J, McArdell CS & Schuwirth N (2011) Multiple-criteria decision analysis reveals high stakeholder preference to remove pharmaceuticals from hospital wastewater Environ Sci Technol **45:** 3848-3857.

Lozupone C & Knight R (2005) UniFrac: a new phylogenetic method for comparing microbial communities Appl Environ Microbiol **71:** 8228-8235.

Lozupone CA, Stombaugh JI, Gordon JI, Jansson JK & Knight R (2012) Diversity, stability and resilience of the human gut microbiota Nature **489:** 220-230.

Magoc T & Salzberg SL (2011) FLASH: fast length adjustment of short reads to improve genome assemblies Bioinformatics **27:** 2957-2963.

McDonald D, Price MN, Goodrich J, Nawrocki EP, DeSantis TZ, Probst A, Andersen GL, Knight R & Hugenholtz P (2012) An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea ISME J **6:** 610-618.

Moreno Y, Botella S, Alonso JL, Ferrus MA, Hernandez M & Hernandez J (2003) Specific detection of *Arcobacter* and *Campylobacter* strains in water and sewage by PCR and fluorescent in situ hybridization Appl Environ Microbiol **69:** 1181-1186.

Ort C, Lawrence MG, Rieckermann J & Joss A (2010) Sampling for pharmaceuticals and personal care products (PPCPs) and illicit drugs in wastewater systems: are your conclusions valid? A critical review Environ Sci Technol **44:** 6024-6035.

Pehrsson EC, Tsukayama P, Patel S *et al.* (2016) Interconnected microbiomes and resistomes in low-income human habitats Nature **533:** 212-216.

Price MN, Dehal PS & Arkin AP (2010) FastTree 2--approximately maximum-likelihood trees for large alignments PLoS One **5:** e9490.

Pruden A (2014) Balancing water sustainability and public health goals in the face of growing concerns about antibiotic resistance Environ Sci Technol **48:** 5-14.

Rajilic-Stojanovic M & de Vos WM (2014) The first 1000 cultured species of the human gastrointestinal microbiota FEMS Microbiol Rev **38:** 996-1047.

Riccio ML, Franceschini N, Boschi L, Caravelli B, Cornaglia G, Fontana R, Amicosante G & Rossolini GM (2000) Characterization of the metallo-beta-lactamase determinant of *Acinetobacter baumannii AC-54/97* reveals the existence of *bla(IMP)* allelic variants carried by gene cassettes of different phylogeny Antimicrob Agents Chemother **44:** 1229-1235.

Rizzo L, Manaia C, Merlin C, Schwartz T, Dagot C, Ploy MC, 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.

Robert J, Pean Y, Varon E *et al.* (2012) Point prevalence survey of antibiotic use in French hospitals in 2009 J Antimicrob Chemother **67:** 1020-1026.

Rodriguez-Mozaz S, Chamorro S, Marti E, Huerta B, Gros M, Sanchez-Melsio A, Borrego CM, Barcelo D & Balcazar JL (2015) Occurrence of antibiotics and antibiotic

resistance genes in hospital and urban wastewaters and their impact on the receiving river Water Research **Volume 69:** 234--242.

Rowe WPM, Baker-Austin C, Verner-Jeffreys DW, Ryan JJ, Micallef C, Maskell DJ & Pearce GP (2017) Overexpression of antibiotic resistance genes in hospital effluents over time J Antimicrob Chemother **72:** 1617-1623.

Shanks OC, Newton RJ, Kelty CA, Huse SM, Sogin ML & McLellan SL (2013) Comparison of the microbial community structures of untreated wastewaters from different geographic locales Appl Environ Microbiol **79:** 2906-2913.

Sommer MO, Dantas G & Church GM (2009) Functional characterization of the antibiotic resistance reservoir in the human microflora Science **325:** 1128-1131.

Stalder T, Barraud O, Jove T, Casellas M, Gaschet M, Dagot C & Ploy MC (2014) Quantitative and qualitative impact of hospital effluent on dissemination of the integron pool ISME J **8:** 768-777.

Stalder T, Alrhmoun M, Louvet JN, Casellas M, Maftah C, Carrion C, Pons MN, Pahl O, Ploy MC & Dagot C (2013) Dynamic assessment of the floc morphology, bacterial diversity, and integron content of an activated sludge reactor processing hospital effluent Environ Sci Technol **47:** 7909-7917.

Su JQ, An XL, Li B, Chen QL, Gillings MR, Chen H, Zhang T & Zhu YG (2017) Metagenomics of urban sewage identifies an extensively shared antibiotic resistome in China Microbiome **5:** 84-017-0298-y.

Szekeres E, Baricz A, Chiriac CM *et al.* (2017) Abundance of antibiotics, antibiotic resistance genes and bacterial community composition in wastewater effluents from different Romanian hospitals Environ Pollut **225:** 304-315.

Vandewalle JL, Goetz GW, Huse SM, Morrison HG, Sogin ML, Hoffmann RG, Yan K & McLellan SL (2012) *Acinetobacter, Aeromonas* and *Trichococcus* populations dominate the microbial community within urban sewer infrastructure Environ Microbiol **14:** 2538-2552.

Varela AR, Andre S, Nunes OC & Manaia CM (2014) Insights into the relationship between antimicrobial residues and bacterial populations in a hospital-urban wastewater treatment plant system Water Res **54:** 327-336.

Varela AR, Ferro G, Vredenburg J, Yanik M, Vieira L, Rizzo L, Lameiras C & Manaia CM (2013) Vancomycin resistant *enterococci:* from the hospital effluent to the urban wastewater treatment plant Sci Total Environ **450-451:** 155-161.

Vazquez-Baeza Y, Pirrung M, Gonzalez A & Knight R (2013) EMPeror: a tool for visualizing high-throughput microbial community data Gigascience **2:** 16-217X-2-16.

Vlahovic-Palcevski V, Dumpis U, Mitt P, Gulbinovic J, Struwe J, Palcevski G, Stimac D, Lagergren A & Bergman U (2007) Benchmarking antimicrobial drug use at university hospitals in five European countries Clin Microbiol Infect **13:** 277-283.

Wellington EM, Boxall AB, Cross P *et al.* (2013) The role of the natural environment in the emergence of antibiotic resistance in gram-negative bacteria Lancet Infect Dis **13:** 155-165.

Wen Q, Tutuka C, Keegan A & Jin B (2009) Fate of pathogenic microorganisms and indicators in secondary activated sludge wastewater treatment plants J Environ Manage **90:** 1442-1447.

Woolhouse ME & Ward MJ (2013) Microbiology. Sources of antimicrobial resistance Science **341:** 1460-1461.

Yang Y, Zhang T, Zhang XX, Liang DW, Zhang M, Gao DW, Zhu HG, Huang QG & Fang HH (2012) Quantification and characterization of beta-lactam resistance genes in 15 sewage treatment plants from East Asia and North America Appl Microbiol Biotechnol **95:** 1351-1358.

Zhang T, Shao MF & Ye L (2012) 454 Pyrosequencing Reveals Bacterial Diversity of Activated Sludge from 14 Sewage Treatment Plants ISME J **6:** 1137-1147.

Zhao WH & Hu ZQ (2011) *IMP*-type metallo-beta-lactamases in Gram-negative bacilli: distribution, phylogeny, and association with integrons Crit Rev Microbiol **37:** 214-226.

Figure 1

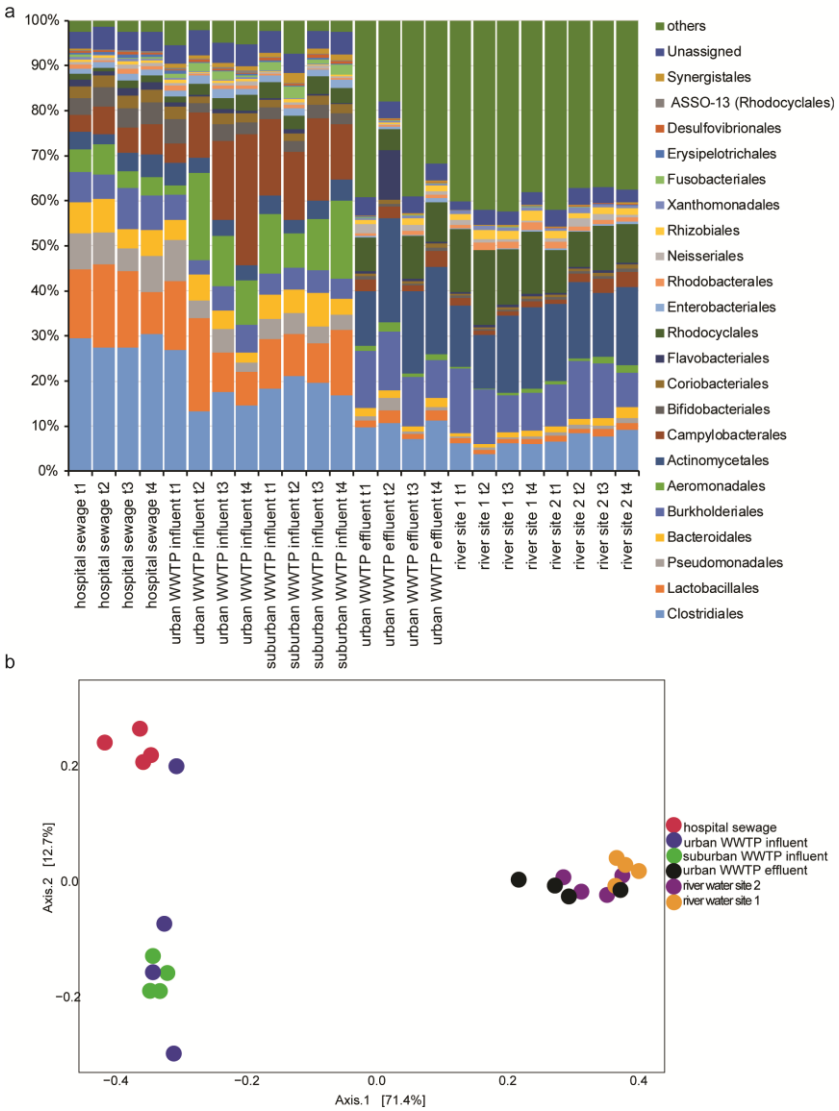

Figure 1: **Microbiota composition of the sample locations at different time points**. **a:** Relative abundance of bacteria at the order level in different samples as detected by dual indexing 16S rRNA Illumina MiSeq sequencing. The 24 most abundant bacteria at the order level for all samples are depicted, 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.

Figure 2

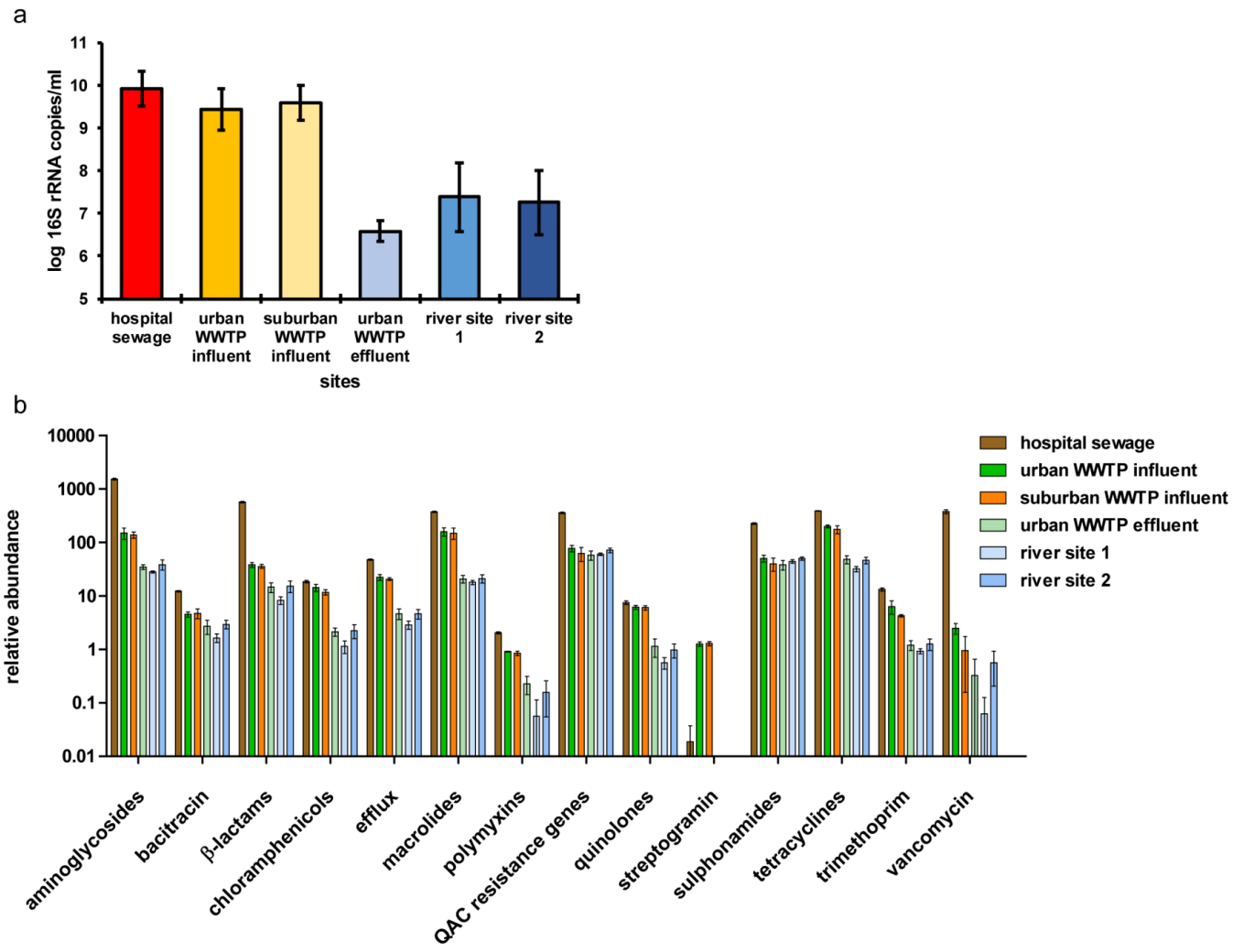

Figure 2: Biomass (copies of 16S rRNA gene/ml) and averaged relative abundance levels of ARG classes at the different sites.

a: Copies of 16S rRNA genes per ml as indicator for bacterial biomass at the different time points (t1-t4) for the individual sites. **b:**16S rRNA - normalized abundance of ARG families 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, β-lactams; chloramphenicols; macrolides; efflux; polymyxins; QAC (quaternary ammonium compounds) resistance genes; quinolones; streptogramins; sulphonamides; tetracyclines; trimethoprim; vancomycin).

Figure 3

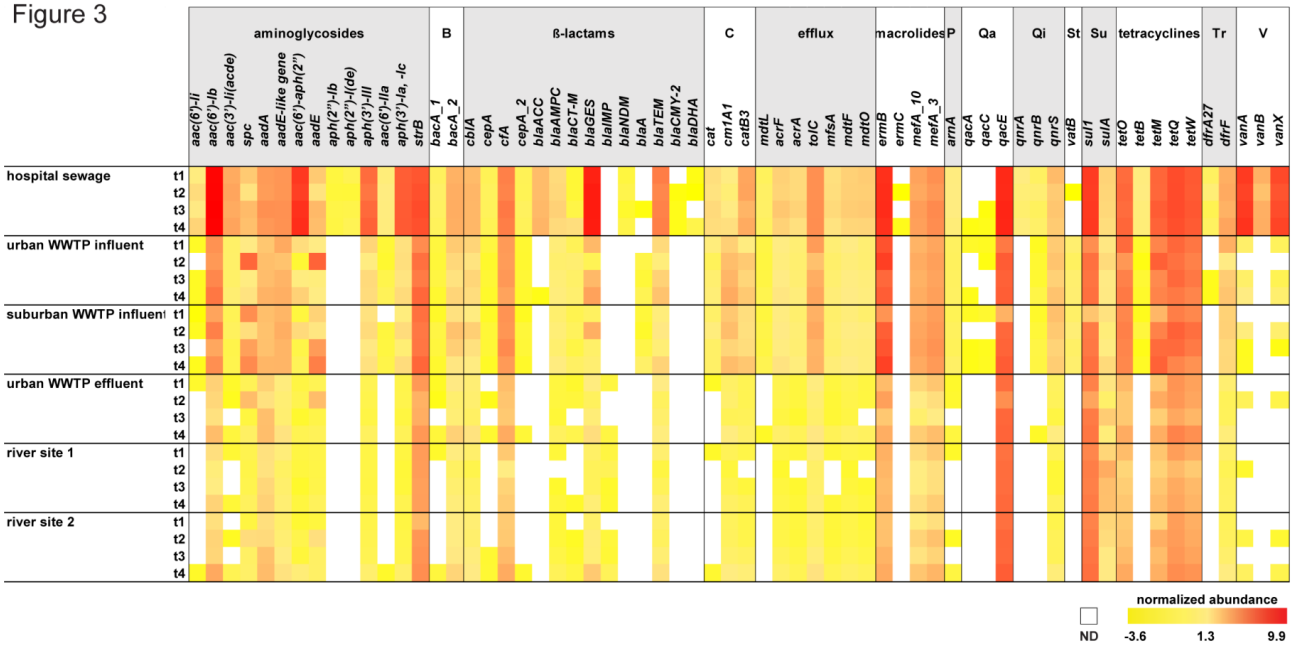

Figure 3: Relative abundance levels of individual ARGs in hospital sewage, urban and suburban WWTP influent, urban WWTP effluent and effluent-influenced river water.

16S rRNA - normalized abundance of individual ARGs detected in all samples. ARGs are grouped according to resistance gene families (aminoglycosides; B, bacitracin, β-lactams; C, chloramphenicols; macrolides; efflux; P, polymyxins; Qa, QAC resistance genes; Qi, quinolones; St, streptogramins; 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).