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1 # The microbiome and resistome of hospital sewage during passage
2 # through the community sewer system

3

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21 Abstract

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

29 Here, we employed culture-independent techniques, namely 16S rRNA and nanolitre-
30 scale quantitative PCRs, to describe the role of hospital effluent as a point source of
31 ARGs in the sewer system, through comparing microbiota composition and levels of
32 ARGs in hospital sewage with WWTP influent, WWTP effluent and the surface water in
33 which the effluent is released.

34 Compared to other sample sites, hospital sewage was richest in human-associated
35 bacteria and contained the highest relative levels of ARGs. Yet, the abundance of ARGs
36 was comparable in WWTPs with and without hospital wastewater, suggesting that
37 hospitals do not contribute to the spread of ARGs in countries with a functioning
38 sewerage system.

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39 **Introduction**

40 Antibiotic-producing and antibiotic-resistant bacteria (ARB) naturally and ubiquitously
41 occur in the environment (Anukool *et al.*, 2004; Wellington *et al.*, 2013). However,
42 human activities contribute importantly to the dissemination of resistant bacteria and
43 resistance genes from humans and animals to the environment (Woolhouse & Ward,
44 2013). Effluents of wastewater treatment plants (WWTPs) may represent an important
45 source of ARB and antimicrobial resistance genes (ARGs) in the aquatic environment
46 (LaPara *et al.*, 2011; Wellington *et al.*, 2013; Pruden *et al.*, 2013; Rizzo *et al.*, 2013;
47 Stalder *et al.*, 2014; Pruden, 2014; Czekalski *et al.*, 2014; Blaak *et al.*, 2014; Karkman *et*
48 *al.*, 2016; Karkman *et al.*, 2017). Generally, WWTPs collect municipal wastewater, but
49 also wastewater from industry, farms and hospitals, dependent on the size and nature of
50 the communities connected to a single sewer system. In hospitals, up to one third of
51 patients receive antibiotic therapy on any given day and consequently, hospitals may be
52 important hubs for the emergence and spread of ARB and ARGs (Vlahovic-Palcevski *et*
53 *al.*, 2007; Bush *et al.*, 2011; Robert *et al.*, 2012). Recent studies have highlighted that
54 multidrug-resistant nosocomial pathogens, ARGs and genetic determinants that
55 contribute to the mobilization and dissemination of ARGs are abundant in hospital
56 sewage, indicating that hospital sewage may play a role in the dissemination of bacteria
57 and genetic determinants involved in antibiotic resistance (Kummerer, 2001; Borjesson
58 *et al.*, 2009; Stalder *et al.*, 2013; Varela *et al.*, 2013; Stalder *et al.*, 2014; Brechet *et al.*,
59 2014).

60 Whether hospital effluent contributes to the presence of ARGs in the aquatic
61 environment is still poorly understood. To quantify the role of hospital effluent as a point

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62 source of ARGs in the sewer system, we compared the levels of ARGs in hospital
63 sewage with WWTP influent, WWTP effluent and the surface water in which the effluent
64 is released. In addition, we investigated the microbial composition along this sample
65 gradient, in order to follow the fate of intestinal microbiota as sources of ARGs.
66

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67 ## Materials and Methods

68

69 ### Sampling locations

70 Sampling was conducted at the main hospital wastewater pipe of the University Medical
71 Center Utrecht (UMCU), Utrecht in the Netherlands, and at two WWTP plants. One plant
72 (termed 'urban WWTP' in this manuscript) treats wastewater of approximately 290,000
73 inhabitants of the city of Utrecht, including the investigated hospital and two other
74 hospitals. The other plant ('suburban WWTP' in Lopik, the Netherlands) neither serves a
75 hospital nor a retirement or nursing home, and treats wastewater of a suburban
76 community of approximately 14,000 inhabitants (Supplementary Figure 1). Both plants
77 apply secondary treatment including nitrification and denitrification in activated sludge
78 systems. Phosphorus removal is performed chemically in the urban WWTP, and
79 biologically in the suburban WWTP. The hospital has approximately 1,000 beds and
80 8,200 employees (full-time equivalents). Additionally, some 2,500 students are enrolled
81 at the university hospital.

82 ### Sampling and DNA isolation

83 Samples were taken during a period of 2.5 weeks in spring on four days (Monday 31
84 March 2014; Wednesday 2 April 2014; Monday 7 April 2014 and Monday 14 April 2014).
85 Cumulative precipitation in the three days preceding each sampling date amounted to
86 maximally 15 mm. The daily flows amount to 74,800 ± 5,900 m^3 for the urban WWTP,
87 and 3,390 ± 380 m^3 for the suburban WWTP during the four sampling days. The flows of
88 the academic hospital amount to approximately 216,000 m^3 on a yearly basis, i.e. on
89 average 590 m^3 per day (0.8% of the influent of the urban WWTP). Exact quantification

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90 of the flows of the academic hospital is not possible as the daily flows are not regularly
91 registered. Flow-proportional sampling (over 24 hours) was used for sampling hospital
92 wastewater and WWTP influent and effluent. Samples were kept at 4°C during the flow-
93 proportional sampling. For the surface water samples, grab samples (5 L) were taken at
94 50 cm downstream of the two effluent pipes of the urban WWTP discharging into a local
95 river at a depth of 20 cm. Samples were transported to the laboratory at 4°C and
96 samples were processed the same day. Bacterial cell pellets and debris of sewage and
97 surface water samples were collected by means of centrifugation at 14000 *g* for 25
98 minutes. All pellets were resuspended in phosphate buffered saline (PBS; 138 mM
99 NaCl, 2.7 mM KCl, 140 mM Na_2HPO_4, 1.8 mM KH_2PO_4, adjusted to pH 7.4 with HCl)
100 with 20% glycerol and stored at -80 C° until DNA extraction. Metagenomic DNA was
101 extracted from 200 µl of frozen samples as described previously (Godon *et al.*, 1997).

102 **16S rRNA sequencing and sequence data pre-processing**

103 16S rRNA sequencing was performed on the Illumina MiSeq sequencing platform (San
104 Diego, CA). A dual-indexing approach for multiplex 16S rRNA sequencing targeting the
105 V3-V4 hypervariable region of the 16S rRNA gene was employed as described by
106 (Fadrosh *et al.*, 2014), using the 300 bp paired-end protocol to sequence a pool of 24
107 samples. Untrimmed paired-end reads were assembled using the FLASH assembler,
108 which performs error correction during the assembly process (Magoc & Salzberg, 2011).
109 After removal of the barcodes, heterogeneity spacers, and primer sequences, there
110 were a total of 1.4 million joined reads with a median length of 424 bases and a median
111 number of 57860 joined reads per sample.

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112 **16S rRNA sequence data analysis**

113 Joined reads were further analyzed using the QIIME microbial community analysis
114 pipeline (version 1.8.0) (Caporaso, Kuczynski *et al.*, 2010). Joined reads with a minimum
115 of 97% similarity were assigned into operational taxonomic units (OTUs) using QIIME's
116 open-reference OTU calling workflow. This workflow was used with the "-m usearch61"
117 option, which uses the USEARCH algorithm (Edgar, 2010) for OTU picking and
118 UCHIME for chimeric sequence detection (Edgar *et al.*, 2011). Taxonomic ranks for
119 OTUs were assigned using the Greengenes database (version 13.8) (McDonald *et al.*,
120 2012) with the default parameters of the script pick_open_reference_otus.py. A
121 representative sequence of each OTU was aligned to the Greengenes core reference
122 database (DeSantis *et al.*, 2006) using the PyNAST aligner (version 1.2.2) (Caporaso,
123 Bittinger *et al.*, 2010). Highly variable parts of alignments were removed using the
124 filter_alignment.py script, which is part of the pick_open_reference_otus.py workflow.
125 Subsequently, filtered alignment results were used to create an approximate maximum-
126 likelihood phylogenetic tree using FastTree (version 2.1.3) (Price *et al.*, 2010). For more
127 accurate taxa diversity distribution (Bokulich *et al.*, 2013), OTUs to which less than
128 0.005% of the total number of assembled reads were mapped, were discarded using the
129 filter_otus_from_otu_table.py script with the parameter "--min_count_fraction 0.00005".
130 The filtered OTU table and generated phylogenetic tree were used to assess within-
131 sample (alpha) and between sample (beta) diversities.

132 Alpha- and beta-diversity of samples were assessed using QIIME's
133 core_diversity_analyses.py workflow. For rarefaction analysis the subsampling depth
134 threshold of 20681 was used, which was the minimum number of reads assigned to a

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135 sample. The UniFrac distance was used as input to calculate the Chao1 index as a
136 measure of beta-diversity of the samples (C. Lozupone & Knight, 2005). In addition to
137 alpha- and beta-diversity analysis and visualizations, this workflow also incorporates
138 principal coordinates analysis and visualization of sample compositions using Emperor
139 (Vazquez-Baeza *et al.*, 2013). Differences in the abundance of taxa are shown as
140 averages over the four time points ± standard deviation resulting in six different
141 comparisons between the different samples. The non-parametric Mann-Whitney test
142 was used to test for statistical significance.

143

144 **High-throughput qPCR**

145 Real-Time PCR analysis was performed using the 96.96 BioMark™ Dynamic Array for
146 Real-Time PCR (Fluidigm Corporation, San Francisco, CA, U.S.A), according to the
147 manufacturer's instructions, with the exception that the annealing temperature in the
148 PCR was lowered to 56°C.

149 Other technical details of the nanolitre-scale quantitative PCRs to quantify levels of
150 genes that confer resistance to antimicrobials (antibiotics and disinfectants, specifically
151 quaternary ammonium compounds (QACs)) were described previously (Buelow *et al.*,
152 2017), with some modifications in the collection of primers. Primers that were negative
153 for all samples tested in the previous study, or gave unspecific amplification were
154 redesigned or replaced. The primer sequences and their targets are provided in the
155 supplementary data, updated and novel primers and targets are highlighted with a grey
156 background (Supplementary Table 1).

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158 **Calculation of normalized abundance and cumulative abundance**

159 Normalized abundance of resistance genes was calculated relative to the abundance of
160 the 16S rRNA gene ($CT_{ARG} – CT_{16S\ rRNA}$) resulting in a log2-transformed estimate of
161 ARG abundance. Cumulative abundance was calculated based on the sum of the non-
162 log2 transformed values ($2\text{^}(-(CT_{ARG} – CT_{16S\ rRNA}))$ of all genes within a resistance gene
163 family. The differences in cumulative abundance over all time points are shown as an
164 averaged fold-change ± standard deviation. The non-parametric Mann-Whitney test was
165 used to test for significance; p values were corrected for multiple testing by the
166 Benjamin-Hochberg procedure (Benjamini & Hochberg, 1995) with a false discovery rate
167 of 0.05.

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169 **Results**

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

172 The composition of the microbiota in hospital sewage, urban and suburban WWTP
173 influents, the effluent of the urban WWTP and the surface water in which the effluent
174 was released was determined by multiplexed 16S rRNA sequencing on the Illumina
175 MiSeq platform (Figure 1A and Supplementary Table 2). At all sample sites, the
176 microbiota consisted of a complex consortium of bacteria from different orders with the
177 microbiota being most diverse in the river samples and least diverse in hospital sewage
178 (Supplementary Figure 2). Hospital sewage contained relatively high levels (39.4 ± 2.5%
179 standard deviation, of the total microbiota) of anaerobic bacteria (Bifidobacteriales,
180 Bacteroidales and Clostridiales) that are likely to originate from the human gut (Rajilic-
181 Stojanovic & de Vos, 2014). These orders were less abundant in WWTP influent (25.8 ±
182 8.4%) and suburban WWTP influent (27 ± 3%; $p < 0.05$) compared to hospital sewage.
183 Compared to the WWTP influent, abundance of Bifidobacteriales, Bacteroidales and
184 Clostridiales was significantly ($p < 0.05$) lower in WWTP effluent (12.8 ± 2.5%) and river
185 water (7.0 ± 1.3% for site 1 and 10.6 ± 1.6% for site 2). In contrast, bacteria that are
186 associated with activated sludge (such as the Actinomycetales, Rhodocyclales, and
187 Burkholderiales (Zhang *et al.*, 2012)) became more prominent during passage through
188 the sewer system and WWTP (Figure 1A and Supplementary Table 2). Principal
189 coordinates analysis (PCoA) showed a clear distinction between the samples that were
190 isolated prior to treatment in the WWTP and the samples of WWTP effluent and river
191 water (Figure 1B). The three most abundant bacterial taxa detected in the hospital
192 sewage were the genera *Streptococcus* (9.0%) and *Arcobacter* (6.9%) and the family

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193 Ruminococcaceae (6.3%). Both raw sewage influents (urban WWTP influent, suburban
194 WWTP influent) clustered together and in both sites, the same three bacterial taxa were
195 most abundant (*Arcobacter*: 17.9% in urban WWTP influent; 17.5% in suburban WWTP
196 influent; Aeromonadaceae: 11.2% and 12.4% respectively; Carnobacteriaceae, 9.4%
197 and 8.3% respectively). Variation of the microbiota composition between the different
198 samples dates at the six sites was limited; with the urban WWTP influent and effluent
199 exhibiting the most pronounced fluctuations (Figure 1b). The urban WWTP effluent
200 samples were very similar to the surface water samples that were collected close to the
201 effluent release pipes. Urban WWTP effluent shared the same three most common
202 OTUs with one of the surface water samples (Actinomycetales, 15.4% in urban WWTP
203 effluent and 9.7% in river site 2; Procabacteriaceae, 8.1% and 7.1% respectively;
204 Comamonadaceae, 7.6% and 7.7% respectively). The surface water sample collected at
205 the other release pipe (river site 1) was slightly different and is defined by the following
206 three most abundant OTUs: Comamonadaceae, 7.5%, Intrasporangiaceae, 6.1% and
207 *Candidatus Microthrix*, 6.1%.

208 **Resistome composition of hospital sewage compared to receiving urban sewage**

209 A total of 67 ARGs were detected in the different samples, conferring resistance to 13
210 classes of antimicrobials. The levels of ARGs were calculated as a normalized
211 abundance relative to levels of 16S rRNA, which provides an indication of the relative
212 levels of ARGs within the bacterial population in each sample (Figure 2, Supplementary
213 Table 3). Hospital sewage was found to be enriched in ARGs, compared to the other
214 samples. The normalized abundance of 11 out of 13 classes of ARGs was significantly
215 ($p<0.05$) higher in hospital sewage than in the urban WWTP influent, particularly so for

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216 aminoglycoside (12.0 ± 5.0-fold higher in hospital sewage), β-lactam (15.4 ± 3.6-fold
217 higher in hospital sewage) and vancomycin resistance genes (175 ± 14-fold higher in
218 hospital sewage, based on the three days when vancomycin resistance genes could be
219 detected in the WWTP influent). Only one class of resistance genes (conferring
220 resistance to streptogramins) was significantly less abundant (p<0.05) in hospital
221 sewage than in WWTP influent. The combined levels of chloramphenicol and quinolone
222 resistance genes were not different between the sites. Seven ARGs (two
223 aminoglycoside resistance genes, *aph(2")-Ib* and *aph(2")-I(de),* the quinolone resistance
224 gene *qnrA,* the, erythromycin resistance gene *ermC,* the vancomycin resistance gene
225 *vanB*, the AmpC-type β-lactamases $bla_{\mathrm{DHA-1}}$ and $bla_{\mathrm{CMY-2}}$ and the carbapenemase
226 bla_{NDM}) were only detected in hospital sewage (Figure 2). The abundance of ARGs in the
227 urban WWTP influent, which receives sewage from the sampled hospital and two
228 additional hospitals in the same city, and the suburban WWTP influent is comparable
229 (Figure 2 Supplementary Table 3). For nine classes of antibiotics, (aminoglycosides, β-
230 lactams, chloramphenicols, macrolides, polymyxins, puromycins, trimpethoprim,
231 quinolones, and tetracyclines), the levels of ARGs in the urban WWTP effluent were
232 significantly (p <0.05) lower than in the WWTP influent (ranging between a 7.6 ± 2.2-fold
233 reduction for macrolide and intrinsic (efflux) resistance genes to a 2.8 ± 0.9-fold
234 reduction for β-lactam resistance genes), with the remaining classes of ARGs not
235 changing significantly in abundance (Figure 2 and Supplementary Table 3). The levels of
236 ARGs in WWTP effluent were comparable to the levels of ARGs in river water (Figure 2,
237 Supplementary Table 3).

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239 **Discussion**

240 Our study demonstrates the considerable efflux of ARGs from hospitals through sewage.
241 However, the influents of the urban and suburban WWTPs studied here show very
242 similar levels of ARGs, even though the urban WWTP receives sewage from a variety of
243 sources including three hospitals, while the sub-urban WWTP does not have a hospital
244 in its catchment area. This reflects the relatively limited effect of hospital sewage on the
245 level of ARGs in WWTP influent and the low contribution of hospital sewage (an
246 estimated 0.8%) to the total volume of wastewater treated in the urban WWTP that we
247 investigated. Our study further demonstrates the capacity of WWTPs to reduce the
248 ultimate amount of ARGs present in urban and suburban influent before entering the
249 river systems.

250 Effluents from WWTPs are thought to contribute to the dissemination of pollutants, multi-
251 drug resistant bacteria and resistance genes in the environment (Rizzo *et al.*, 2013;
252 Wellington *et al.*, 2013; Karkman *et al.*, 2017). Particularly high amounts of ARB have
253 previously been reported in hospital sewage (Diwan *et al.*, 2010; Wellington *et al.*, 2013;
254 Harris *et al.*, 2013; Harris *et al.*, 2014; Berendonk *et al.*, 2015). Large amounts of
255 antibiotics and QACs are used in hospitals and these may promote the establishment of
256 ARB and selection of ARGs in patients and hospital wastewaters (Stalder *et al.*, 2013;
257 Stalder *et al.*, 2014; Varela *et al.*, 2014). Here we show that the relative abundance of a
258 broad range of ARGs conferring resistance to 11 classes of antimicrobials is significantly
259 higher in hospital sewage compared to urban and suburban WWTP sewage. In
260 particular, genes conferring resistance to aminoglycosides, β-lactams and vancomycin

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261 are enriched in hospital sewage, presumably due to the frequent use of these classes of
262 antibiotics in the hospital (Chandy *et al.*, 2014).

263 The most abundant bacterial taxa detected in the hospital sewage are different from
264 those found in the urban and suburban WWTP influent, which are dominated by
265 bacterial families (*Arcobacter*; Aeromonadaceae; Carnobacteriaceae) that are commonly
266 found in the sewer microbiota (Moreno *et al.*, 2003; Vandewalle *et al.*, 2012; Shanks *et*
267 *al.*, 2013). Compared to the WWTP influent samples, several members of the human gut
268 microbiota are significantly more abundant in hospital sewage, most probably due to the
269 close proximity of the sampling location to the hospital sanitation systems. These
270 human-associated taxa include the genus *Streptococcus*, of which many species
271 interact with humans either as commensals or pathogens (Kalia *et al.*, 2001), and the
272 Ruminococcaceae, which are one of the most prevalent bacterial families in the human
273 gut (Arumugam *et al.*, 2011; C. A. Lozupone *et al.*, 2012). These human-associated
274 bacteria appear to be ill-suited for surviving the complex and, at least partially
275 oxygenated, sewage environment and progressively decrease in abundance, leading to
276 lower levels of human gut-associated bacteria in the urban WWTP influent. Because
277 most ARGs from the human microbiota appear to be carried by non-pathogenic
278 commensal bacteria (Sommer *et al.*, 2009; Buelow *et al.*, 2014), a general loss of human
279 commensal bacteria in the sewer system (Pehrsson *et al.*, 2016) may contribute to a
280 decrease in the abundance of ARGs during the passage of wastewater through the
281 sewer system.

282 The reduction of ARGs shown in urban WWTP effluent compared to WWTP influent may
283 be explained by the further significant reduction of the relative abundance of human-

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284 associated bacterial taxa detected in urban WWTP effluent. The continuous reduction of
285 these bacterial taxa could be mediated by their removal through sorption to activated
286 sludge, by replacement with the bacteria that populate activated sludge, and/or by
287 predation of protozoa during wastewater treatment (Wen *et al.*, 2009; Calero-Caceres *et*
288 *al.*, 2014). Interestingly, the presence of Procabacteriales in WWTP effluent and river
289 water (Figure 1A), may point towards a relatively high abundance of protists in these
290 samples, as these bacteria are intracellular symbionts or pathogens of amoeba (Horn *et*
291 *al.*, 2002; Greub & Raoult, 2004).

292 The reduction of the abundance of ARGs from hospital sewage to WWTP effluent
293 highlights the importance of wastewater treatment in reducing the discharge of ARGs
294 originating from human sources into the environment. With respect to the abundance of
295 ARG relative to 16S rRNA, it has been debated whether sewage treatment could
296 selectively affect the percentage of resistant bacteria within a given species, or within
297 the total community (Rizzo *et al.*, 2013; Laht *et al.*, 2014; Alexander *et al.*, 2015). Here,
298 and in line with (Karkman *et al.*, 2016), we observed that wastewater treatment led to a
299 decrease in the relative abundance of ARGs. A clear decrease of ARGs in absolute
300 terms (i.e. in gene copies per volume of water) along the sewage treatment has been
301 shown in a range of publications (Auerbach *et al.*, 2007; Chen & Zhang, 2013; Laht *et*
302 *al.*, 2014; Czekalski *et al.*, 2014).

303 Sampling for this study was limited to one single season, but was repeated four
304 consecutive days in dry weather conditions using mostly flow-proportional sampling as
305 recommended by Ort *et al.* (Ort *et al.*, 2010). Microbiota and resistome profiling of our
306 samples showed limited variation between the four sampling days for each sample,

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307 hence allowing for analysis of the treatment efficacy on the removal of ARGs relative to
308 16S rRNA in these particular WWTPs.

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

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318

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498

Figure 1

a

Unassigned
other
Saprospirales
BD1-5
Methylophilales
TM7-1
Acidimicrobiales
Procabacteriales
Erysipelotrichales
Rhizobiales
Fusobacteriales
Neisseriales
Rhodobacterales
Enterobacteriales
Rhodocyclales
Flavobacteriales
Coriobacteriales
Actinomycetales
Bifidobacteriales
Campylobacterales
Aeromonadales
Bacteroidales
Burkholderiales
Pseudomonadales
Lactobacillales
Clostridiales

100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%

hospital sewage t1
hospital sewage t2
hospital sewage t3
hospital sewage t4
urban WWTP influent t1
urban WWTP influent t2
urban WWTP influent t3
urban WWTP influent t4
suburban WWTP influent t1
suburban WWTP influent t2
suburban WWTP influent t3
suburban WWTP influent t4
urban WWTP effluent t1
urban WWTP effluent t2
urban WWTP effluent t3
urban WWTP effluent t4
river site 1 t1
river site 1 t2
river site 1 t3
river site 1 t4
river site 2 t1
river site 2 t2
river site 2 t3
river site 2 t4

b

Axis.2 [12.7%]
0.2
0.0
−0.2

Axis.1 [71.4%]
−0.4
−0.2
0.0
0.2
0.4

hospital sewage
urban WWTP influent
suburban WWTP influent
urban WWTP effluent
river water site 2
river water site 1

Figure 2

aminoglycosides: *aac(6')-Ii*, *aac(6')-Ib*, *aac(3')-Ii(acde)*, *spc*, *aadA*, *aadE-like gene*, *aac(6')-aph(2'')*, *aadE*, *aph(2'')-Ib*, *aph(2'')-I(de)*, *aph(3')-III*, *aac(6')-IIa*, *aph(3')-Ia, -Ic*, *strB*

B: *bacA_1*, *bacA_2*

ß-lactams: *cblA*, *cepA*, *cfA*, *cepA_2*, *blaACC*, *blaAMPC*, *blaCT-M*, *blaGES*, *blaIMP*, *blaNDM*, *blaA*, *blaTEM*, *blaCMY-2*, *blaDHA*

C: *cat*, *mdtL*, *cm1A1*, *catB3*

macrolides/ intrinsic R: *acrF*, *acrA*, *tolC*, *mfsA*, *mdtF*, *mdtO*, *ermB*, *ermC*, *mefA_10*, *mefA_3*

P: *arnA*

Qa: *qacA*, *qacC*, *qacE*

Qi: *qnrA*, *qnrB*, *qnrS*

St: *vatB*

Su: *sul1*, *sulA*

tetracyclines: *tetO*, *tetB*, *tetM*, *tetQ*, *tetW*

Tr: *dfrA27*, *dfrF*

V: *vanA*, *vanB*, *vanX*

hospital sewage: t1, t2, t3, t4

urban WWTP influent: t1, t2, t3, t4

suburban WWTP influent: t1, t2, t3, t4

urban WWTP effluent: t1, t2, t3, t4

river site 1: t1, t2, t3, t4

river site 2: t1, t2, t3, t4

ND

normalized abundance: -3.6, 1.3, 9.9

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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 “other” 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 locations and time points. PCoA based on the weighted UniFrac distance depicts the differences in microbiota compositions.

Figure 2: Abundance levels of ARGs in hospital, WWTP influent, WWTP effluent and river water.

16S rRNA - normalized abundance of ARGs detected in all samples. ARGs are grouped according to resistance gene families (aminoglycosides; B, bacitracin, β-lactams; C, chloramphenicols; macrolide / intrinsic resistance; P, polymyxins; Pu, puromycins; 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).