

Sewage from airplanes exhibits high abundance and diversity of antibiotic resistance genes

Heß, Stefanie^{b*}, Kneis, David^{a,f}, Österlund, Tobias^c, Li, Bing^d,
Kristiansson, Erik^{c,e}, Berendonk, Thomas U.^a

^a Institute of Hydrobiology, TU Dresden, Germany

^b Dept. of Microbiology, University of Helsinki, Finland

^c Mathematical Sc. Dept., Chalmers University of Technology, Gothenburg, Sweden

^d Division of Energy and Environment, Graduate School at Shenzhen, Tsinghua University, China

^e Centre for Antibiotic Resistance Research (CARE), University of Gothenburg, Sweden

^f Helmholtz-Centre for Environmental Research, Magdeburg, Germany

* Corresponding author: stefanie.hess@helsinki.fi

1 Abstract

2 Airplane sanitary facilities are shared by an international audience. We hypothesized
3 the corresponding sewage to be an extraordinary source of antibiotic resistant bacteria
4 (ARB) and resistance genes (ARG) in terms of diversity and quantity. Accordingly,
5 we analyzed ARG and ARB in airplane-borne sewage using complementary approaches:
6 metagenomics, quantitative PCR, and cultivation. For the purpose of comparison, we also
7 quantified ARG and ARB in the inlets of municipal treatment plants with and without
8 connection to airports. As expected, airplane sewage contained an extraordinary rich
9 set of mobile ARG and the genes' relative abundances were mostly increased compared
10 to typical raw sewage of municipal origin. Moreover, combined resistance against third
11 generation cephalosporins, fluoroquinolones and aminoglycosides was unusually common
12 (28.9%) among *E. coli* isolated from airplane sewage. This percentage exceeds the one
13 reported for German clinical isolates by a factor of eight. Our findings suggest that
14 airplane-borne sewage can effectively contribute to the fast and global spread of antibiotic
15 resistance.

16 **Keywords:** antibiotic resistance, airplane, sewage, wastewater, diversity

17 1 Introduction

18 Since their discovery in the 1940s, antibiotics saved millions of lives, but due to the
19 global spread of resistance genes these drugs rapidly lose their activity. This threat-
20 ens the very core of modern medicine by limiting the means to effectively cure bacterial
21 infections ([World Health Organization, 2015](#)). In the era of globalization and high mobil-
22 ity, pathogenic strains carrying antibiotic resistance genes (ARG) are spreading quickly
23 and globally ([Nordmann et al., 2011](#)). In the endeavor to decelerate or stop the spread
24 of antibiotic resistance, it is necessary to identify the hotspots and pathways of ARG
25 dissemination.

26 Airports are places where sewage of people from different parts of the world is collected
27 and, after treatment, released in the local aquatic environment. It is thus reasonable to
28 assume that airports serve as an entrance for ARG which are endemic in specific parts of
29 the world while being rare or absent in the flights' country of destination. This should be
30 reflected in a particularly high diversity of ARG in airplane-borne sewage as compared
31 to conventional municipal wastewater (**hypothesis 1**).

32 The prevalence of antibiotic resistant bacteria (ARB) is known to be linked with
33 veterinary and human antibiotic use ([World Health Organization, 2014](#)). Specifically,
34 [Forsslund et al. \(2013\)](#) found the resistance potential in human guts to be positively
35 correlated with country-specific antibiotic use. In Germany, antibiotic consumption is
36 relatively low compared to, e.g., China, India, or the USA ([Van Boeckel et al., 2014](#))
37 and it is also lower than in many member states of the EU ([European Surveillance of](#)
38 [Antimicrobial Consumption Network, 2017](#)). Consequently, airplane-borne sewage was
39 hypothesized to contain ARB and ARG in elevated abundances as compared to standard
40 municipal sewage generated by the local population (**hypothesis 2**).

41 The two hypotheses related to the diversity and abundance of ARG were tested by
42 the complementary approaches of metagenome sequencing, quantitative PCR (qPCR),
43 and cultivation, thereby providing a comprehensive picture of antimicrobial resistance
44 in studied systems. Specifically, the strength of the metagenomics approach lies in its
45 capability to detect and quantify the full spectrum of ARG with the downside of lim-
46 ited sensitivity. Quantitative PCR, on the other hand, allows even rare genes and gene
47 variants to be quantified since the target sequences are specifically amplified. However,
48 qPCR necessarily has a narrow focus dictated by the chosen primers. Finally, suscepti-
49 bility tests provide the only means to study phenotypic antibiotic resistance, including
50 multi-resistance. Unlike the other techniques, cultivation covers a small part of the mi-
51 crobial community only.

52 In this study, any of the three approaches was used to quantify antibiotic resistance
53 in untreated sewage collected from (1) airplane tanks and (2) the inlets of municipal
54 wastewater treatment plants (WWTP). The set of WWTP was chosen such that some of
55 the plants received wastewater from the nearest airport while others did not. The latter
56 distinction was made to test whether the hypothesized peculiarities of airplane-borne
57 sewage are still noticeable after mixing with "conventional" municipal wastewater.

58 2 Material and methods

59 2.1 Sampling

60 Eight grab samples of airplane-borne sewage were obtained from five different airports,
61 three of which were sampled twice (with sufficient delay to ensure independence). Three
62 of the airports had >20 million passengers p.a. in 2015 while the other two were of
63 intermediate size (>10 million passengers p.a.) or smaller. The sewage was gathered
64 from vacuum trucks collecting the waste from multiple aircrafts. Since the aircraft's

65 tanks are emptied on demand, each tank contains sewage from multiple flights. The
66 samples thus represent pooled samples integrating over space and time.

67 Incoming sewage from WWTP having a connection to airports was collected at six
68 different locations, two of which were visited twice (total number of eight samples; each
69 being a 24 h composite). At the respective plants, airport-borne sewage was known
70 to contribute at least 20% of the total inflow. These 20% represent a mixture of fecal
71 material from airplane tanks with sewage from other airport facilities such as terminal
72 buildings. Detailed data on mixing ratios and its temporal variation were unavailable to
73 us. Surface runoff from airports is generally disposed separately from sewage not least
74 because of the possible contamination with, e.g., de-icing agents.

75 Incoming sewage from WWTP without connection to airports was collected at six
76 locations. At one of the locations, samples were taken from four different sewers and
77 another plant was sampled multiple times (with sufficient delay to ensure independence).
78 The total number of 24 h composite samples was 19.

79 All samples were stored in 1 L sterile glass bottles at 4 °C and processed within 24 h.
80 Relevant meta-information is provided in Table S.1.

81 2.2 Metagenomics

82 DNA was extracted using the PowerWaterKit (MoBio, Vancouver, Canada; see Ta-
83 ble S.1 for amount and quality of DNA). All samples were shotgun-sequenced on a
84 MiSeq device (2×150 bp; GATC Biotech AG, Konstanz, Germany). Quality checking and
85 trimming (q=28, minimum length=100 bp) was performed using TrimGalore! (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/). The preprocessed
86 forward reads were then aligned to the latest (2019-03-05) `resfinder` data base (Zankari
87 et al., 2012). Specifically, we relied on the python implementation of the `resfinder`
88 script which is publicly available from the Center for Genomic Epidemiology (<http://www.genomicepidemiology.org/>). The `resfinder` script attempts to match the nu-
89 cleotide sequences contained in the samples with the sequences of known ARG as stored in
90 the `resfinder` data base. Internally, the `resfinder` script calls the Basic Local Align-
91 ment Search Tool (`blastn`; <http://blast.ncbi.nlm.nih.gov>) to compare nucleotide
92 sequences. `blastn`-reported alignments with e-values exceeding 10^{-10} were generally
93 ignored to minimize the chance of false positive hits.

94 The set of unique ARG contained in a particular sample was established from the full
95 list of resistance genes whose signatures matched with a nucleotide sequence from the
96 sample. Technically speaking, the obtained list of hits was filtered to remove any dupli-
97 cate gene identifiers. The `resfinder` data base holds information on a large number genes
98 and variants, e.g. about 2000 of the registered ARG target beta-lactams. So as to focus
99 on ARG diversity at a higher level, we generally truncated the genes' identifiers at the
100 first underscore before establishing the unique set of ARG. For example, instead of count-
101 ing the tetracycline resistance gene variants `tet(M)_7_FN433596` and `tet(M)_1_X92947`
102 as individual instances, they were commonly registered as just `tet(M)`. For the purpose
103 of additional quality assurance, a particular gene was only registered as present if its
104 signature was detected at least three times. Moreover, nucleotide sequences matching
105 the signatures of multiple ARG registered in the `resfinder` data base (ambiguous hits),
106 were ignored altogether.

109 2.3 Quantitative PCR

110 Fourteen ARG were selected for qPCR-based quantification in line with activities of
111 international research consortia investigating antimicrobial resistance in aquatic environ-
112 ments. The set of ARG includes common and rare ones with a special focus on clinically

113 relevant genes coding for carbapenemases (*bla*_{TEM}, *bla*_{CTX-M-15}, *bla*_{CTX-M-32}, *bla*_{CMY-2},
114 *bla*_{OXA-48}, *bla*_{OXA-58}, *bla*_{KPC-3}, *bla*_{NDM-1}, *bla*_{VIM-2}, *ermB*, *mecA*, *mcr1*, *tetM*, *sul1*). All
115 ARG as well as 16S rRNA genes were quantified following the same procedure as de-
116 scribed in Heß et al. (2018). The relevant references for the primers of the additionally
117 quantified ARG are the NORMAN network (www.norman-network.net) for *bla*_{TEM}, and
118 *bla*_{CTX-M-32}, the ANSWER project (www.answer-itn.eu) for *bla*_{CTX-M-15}, Kurpiel and
119 Hanson (2012) for *bla*_{CMY-2}, Monteiro et al. (2012) for *bla*_{OXA-48}, Hembach et al. (2017)
120 for *mcr1*, and Peak et al. (2007) for *tetM*. Primer sequences can be found in Table S.2. For
121 the 16S rRNA gene, *sul1* and *bla*_{CTX-M-32} the pNORM plasmid designed by Ch. Merlin
122 (University of Lorraine, France; www.norman-network.net) was used as standard. For
123 the remaining genes, standards were created by cloning the respective qPCR amplicons
124 into the pGEM-T vector (Promega, Madison, Wisconsin, USA).

125 The detection limit was 10² copies per reaction for all genes. The efficiency of these
126 assays was between 0.9 and 1 with R²>0.997 for all the runs. All genes were quantified
127 in duplicates with a standard deviation of the cycles <0.2.

128 2.4 Standardization of data and community analysis

129 In the context of this study, the diversity and abundance of ARG was to be compared
130 across samples of varying composition and dilution (toilet waste, municipal wastewater),
131 hence, standardization was required. With regard to ARG abundances, we adopted the
132 common practice of dividing absolute ARG counts by the corresponding number of 16S
133 rRNA copies (see, e.g. Pärnänen et al., 2019). The resulting numbers are referred to as
134 *relative abundances*. We applied the same standardization also to metagenomics-based
135 information on ARG diversity. Specifically, the diversity of resistance genes was expressed
136 as the number of unique ARG per 1000 copies of 16S rRNA genes.

137 The calculation of relative ARG abundances from qPCR-based data involved a qPCR-
138 based quantification of 16S rRNA genes (see Table S.2 for primer). Likewise, metagenomics-
139 based information on ARG was standardized using metagenomics-based estimates on the
140 abundance of 16S rRNA genes. The latter was extracted from the nucleotide sequences
141 using METAXA2 (Bengtsson-Palme et al., 2015b, version 2.1.3).

142 The number of 16S rRNA gene copies per bacterial cell is known to vary between
143 taxonomic groups. Consequently, a comparison of relative ARG abundances (or ARG
144 diversities) requires that the respective microbial communities are similar in terms of
145 taxonomic composition or, at least, with regard to the community-weighted average
146 number of 16S rRNA gene copies per cell. We employed METAXA2 to infer information
147 on the composition of the microbial community based on 16S rRNA. Finally, we relied
148 on the *rrnDB* data base (Stoddard et al., 2015) to compute for all metagenomics-samples
149 the expected average number of 16S rRNA copies per bacterial cell taking into account
150 taxonomic groups and their proportions. The matching of taxonomic items reported by
151 METAXA2 with items registered in *rrnDB* was successful at family level in 86% of the cases,
152 at genus level in 54% of the cases and at species level in 10% of the cases.

153 2.5 Bacteria isolation and susceptibility testing

154 *Escherichia coli* was chosen as a model organism because it is widely considered as a fecal
155 indicator and resistance levels of *E. coli* from different origins are well documented (e.g.
156 European Centre for Disease Prevention and Control, 2017; Osińska et al., 2017; Rosas
157 et al., 2015). *E. coli* is furthermore a potential pathogen and its harboring of resistance
158 genes can thus directly impact human health.

159 To obtain at least 24 *E. coli* isolates from each sample, suitable dilutions were plated
160 on mFC agar (Carl Roth, Karlsruhe, Germany). After 18 ± 2 h of incubation at 44 °C

161 blue colonies were streaked on Brilliance agar (Oxoid, Wesel, Germany) and grown
162 overnight at 37 °C to obtain pure cultures. To identify the isolates as *E. coli*, colony
163 PCR was performed as in [Heß et al. \(2018\)](#) to amplify a species specific fragment of the
164 *yccT* gene.

165 In total, 1140 *E. coli* isolates (Table S.1) were tested against 24 antibiotics which are
166 commonly used to treat the respective infections (187 isolates from airplanes, 161 and
167 362 isolates from the inflow of municipal treatment plants with and without connection
168 to an airport). The tests followed the EUCAST guidelines (agar diffusion test; [www.
169 eucast.org](http://www.eucast.org)) with *Escherichia coli* ATCC 25922 as a quality control. Applying the clinical
170 breakpoints defined by EUCAST, the isolates were classified as resistant or susceptible
171 (intermediary was counted as susceptible).

172 2.6 Statistics

173 Data analysis was conducted with R 3.4.3 ([R Core Team, 2017](#)). Relative ARG abun-
174 dances in samples of different origin were compared using the Welch test (`t.test()`)
175 with log-transformed data. A non-parametric rank sum test (`wilcox.test()`) was used
176 to test for differences in ARG richness. Proportion data were tested for significant dif-
177 ferences using Fisher’s exact test (`fisher.test()`). In case of multiple tests, p-values
178 were conservatively adjusted with the default `p.adjust()` method [Holm \(1979\)](#). p-values
179 were marked with asterisks according to the usual convention where * indicates $p \leq 0.05$,
180 ** denotes $p \leq 0.01$ and *** corresponds to $p \leq 0.001$. Bootstrap confidence inter-
181 vals (Fig. 5) were generated with `boot()` using ordinary resampling and 10^4 replicates.
182 Rarefaction curves (Fig. 3) were constructed from metagenomics data by evaluating an
183 increasing number of sequences from the whole set of sequences available for a particular
184 sample by means of sampling without replacement (R method `sample`). To minimize
185 random effects, we considered the medians of five replicate rarefaction curves per sam-
186 ple. Statistical models fitted to empirical rarefaction curves (Fig. 3) take the structure
187 of Eqn. 1 where R represents the number of different ARG, n denotes the number of
188 analyzed sequences, and R_{inf} and h are free parameters, respectively, fitted with R’s
189 default optimizer (`optim`).

$$R = R_{inf} \cdot \left(\frac{n}{n+h} \right)^{0.5} \quad (1)$$

190 Based on the overall number of sequences in a sample and the corresponding num-
191 ber of 16S rRNA copies, the variable n was adjusted individually for each sample to
192 pragmatically compensate for varying proportions of bacterial and non-bacterial DNA.

193 3 Results

194 3.1 Characterization of samples

195 In accordance with expectation, untreated wastewater sampled at WWTP inlets was
196 generally more diluted as compared to the sewage derived from airplane tanks. This is
197 reflected, for example, in the electric conductivity but also in the number of 16S rRNA
198 gene copies per volume (Table 1). In addition, airplane sewage exhibited higher values
199 of pH which is likely due to an elevated concentration of soap residues.

200 The metagenomics-derived information on 16S rRNA fragments allowed for a deeper
201 characterization of the samples in terms of the composition of the bacterial community.
202 The latter was very similar in all samples taken at the inlets of WWTP whereas airplane-
203 borne samples showed larger variations in the contribution of different bacterial orders
204 (Fig. 1). Moderate contrasts were observed between samples of different origins in terms

Table 1: Mean values of electric conductivity (EC), pH, and the concentration of 16S rRNA genes in samples of different origins. The corresponding information for individual samples can be found in Table S.1.

Origin	EC (mS cm ⁻¹)	pH	16S rRNA gene copies mL ⁻¹
Airplane	10.1	8.9	1.6×10 ⁹
WWTP, with airport	1.7	7.3	2.6×10 ⁸
WWTP, no airport	1.1	7.3	2.9×10 ⁸

205 of taxonomic diversity. For example, the signatures of 6.5 bacterial families (median) were
 206 detected in 10⁵ nucleotide sequences obtained from airplane sewage. This compares to
 207 values of 4 and 3 for wastewater from WWTP with and without connection to airports,
 208 respectively. This fact is also visible in Fig. 1 which shows a greater evenness in the
 209 contribution of taxonomic groups for airplane-borne samples as compared to WWTP
 210 samples. Characteristic differences between samples of different origins were observed,
 211 e.g., for Aeromonadales and Campylobacteriales (rare in airplane sewage but common
 212 in WWTP samples) as well as Enterobacterales and Erysipelotrichales (rare in WWTP
 213 samples but highly abundant in some airplane samples).

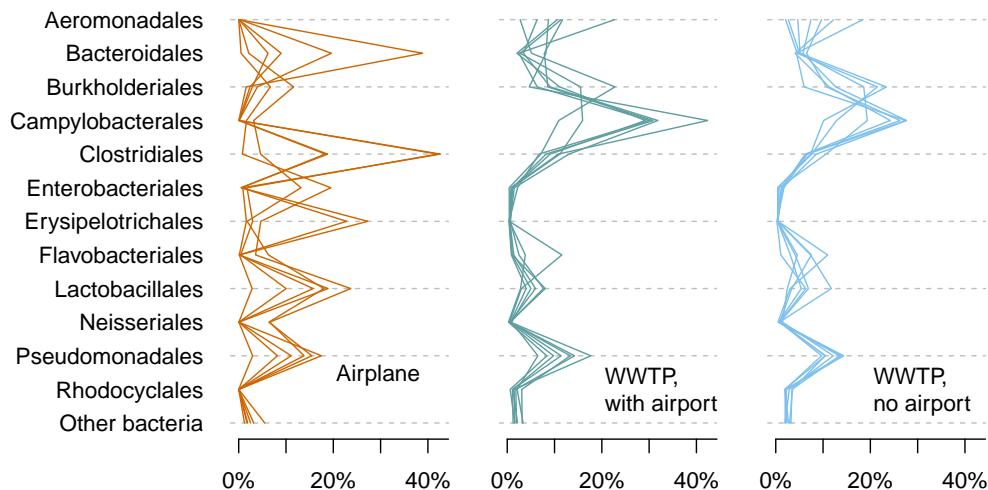


Figure 1: Percentage of 16S rRNA fragments attributable to different bacterial orders. Lines depict individual samples. Bacterial orders which did not contribute at least 5% in any of the samples were dropped for the sake of clarity.

214 In spite of the differences in taxonomic composition, the expected number of 16S
 215 rRNA genes per cell varied only little between sample of different origin. The merging
 216 of the metagenomics-based taxonomy data with information from the `rrnDB` data base
 217 resulted in a mean value of 4.9 copies of 16S rRNA genes per cell for airplane-borne
 218 samples. The respective mean values for raw wastewater with and without connection to
 219 airports were 4.5 and 4.8, respectively. The most extreme ratios observed for individual
 220 samples ranged between 4 and 5.5 and none of the differences in means fulfilled the
 221 criteria of statistical significance (ANOVA: $p > 0.23$; all $p > 0.13$ in post-hoc rank sum
 222 tests). In view of that, a standardization of ARG counts by the corresponding number
 223 of 16S rRNA genes (see Sec. 2.4) was considered appropriate. In other words, it is very
 224 unlikely that marked shifts in the relative abundance of ARG, e.g. between samples of
 225 different origin, merely reflect dissimilarities of the respective bacterial communities.

226 **3.2 Diversity of antibiotic resistance genes**

227 As outlined in Sec. 2.2, the diversity of antibiotic resistance genes was expressed as the
 228 number of unique ARG per 1000 copies of 16S rRNA genes. That measure of diversity
 229 was found to be significantly increased in airplane sewage as compared to untreated
 230 sewage sampled from the inlets of municipal WWTP (Fig. 2; $p < 0.05$, Wilcoxon rank
 231 sum test). On average, a unique ARG was detected every 580 copies of 16S rRNA genes
 232 in airplane sewage. In the WWTP samples, a unique ARG appeared every 750 copies of
 233 16S rRNA genes. WWTP samples were statistically similar in terms of ARG diversity
 234 regardless of whether the plant received sewage from an airport or not (blue vs. green
 235 boxes in Fig. 2).

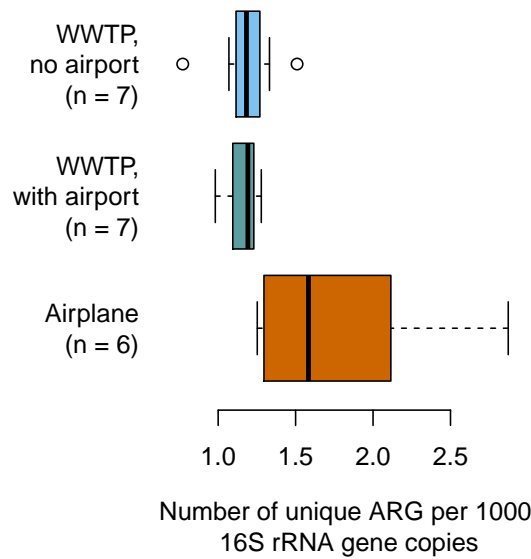


Figure 2: Diversity of antibiotic resistance genes in samples of untreated sewage collected from airplanes and WWTP with/without connection to airports. Whiskers extend to the most extreme data points not being classified as outliers.

236 The numbers on ARG diversity presented in Fig. 2 represent point estimates based
 237 on the number of sequences in each sample and the respective number of 16S rRNA gene
 238 copies. In order to verify the robustness of these estimates, we computed rarefaction
 239 curves for all samples processed with metagenomics (Fig. 3). The latter confirm that
 240 samples of airplane sewage are likely to contain a greater number different ARG in a
 241 given amount of DNA as compared to untreated wastewater. For example, the average
 242 number of unique ARG per 5×10^6 sequences was about 110 in airplane-borne samples
 243 compared to about 85 in samples taken from the inlets of WWTP (Fig. 3).

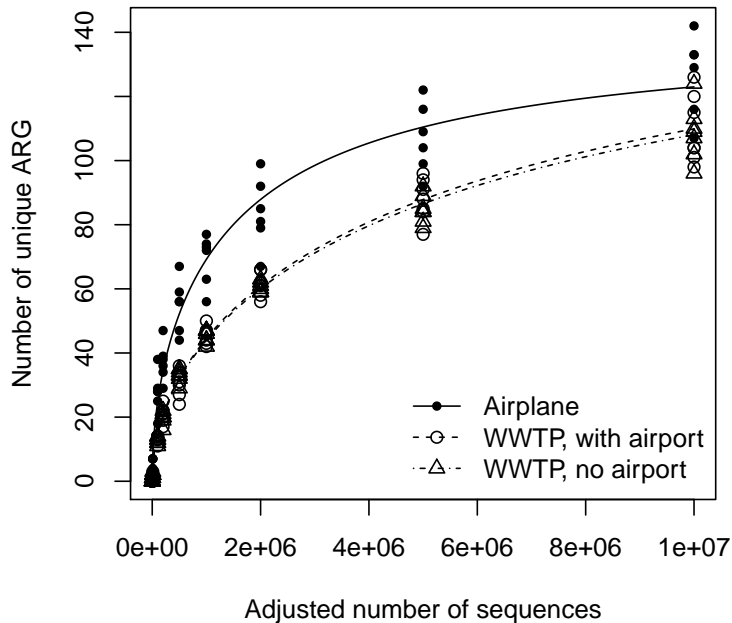


Figure 3: Rarefaction curves relating the number of unique ARG to the number of analyzed sequences (adjusted for varying abundances of 16S rRNA genes). Point symbols represent empirical estimates for individual samples. Statistical models of the form of Eqn. 1 (lines) were fitted to all points of a particular sample origin.

244 3.3 Abundance of antibiotic resistance genes

245 The metagenomics data indicate an increased relative abundance of ARG in airplane
 246 sewage as compared to sewage sampled at the inlets of WWTP (Fig. 4). The elevated
 247 prevalence in airplane-borne sewage was most pronounced for ARG directed against
 248 phenicols, sulphonamides, and tetracyclin ($p < 0.01$) as well as aminoglycosides and
 249 macrolides ($p < 0.05$, two-sided Welch test, conservatively adjusted for multiple testing).
 250 Contrary to the trend, ARG targeting colistin were more abundant in municipal sewage
 251 than in airplane sewage (value near limit of quantification). Generally, the samples taken
 252 at treatment plants with and without connection to airports (green and blue symbols in
 253 Fig. 4) did not exhibit significant differences in terms of relative abundances according
 254 to the Welch test.

255 The auxiliary qPCR-based analyses support the main outcome of the metagenomics
 256 approach depicted in Fig. 4. Specifically, the relative abundances of *sul1* (sulphonamide)
 257 and *tetM* (tetracycline) resistance genes were significantly increased in airplane samples
 258 compared to samples taken at the inlets of WWTP (Table 2). The median relative
 259 abundances differ between the two sample origins by factors of 5 (*sul1*) and 18 (*tetM*),
 260 respectively.

261 With the exception of *bla*_{VIM-2}, differences in relative abundance between airplane
 262 and WWTP samples were not statistical significant for the remaining ARG (Table 2).
 263 Especially for the β -lactamase genes, this finding is compatible with the metagenomic
 264 results (Fig. 4) according to which the total prevalence of genotypic β -lactam resistance
 265 was only slightly increased in airplane-borne samples.

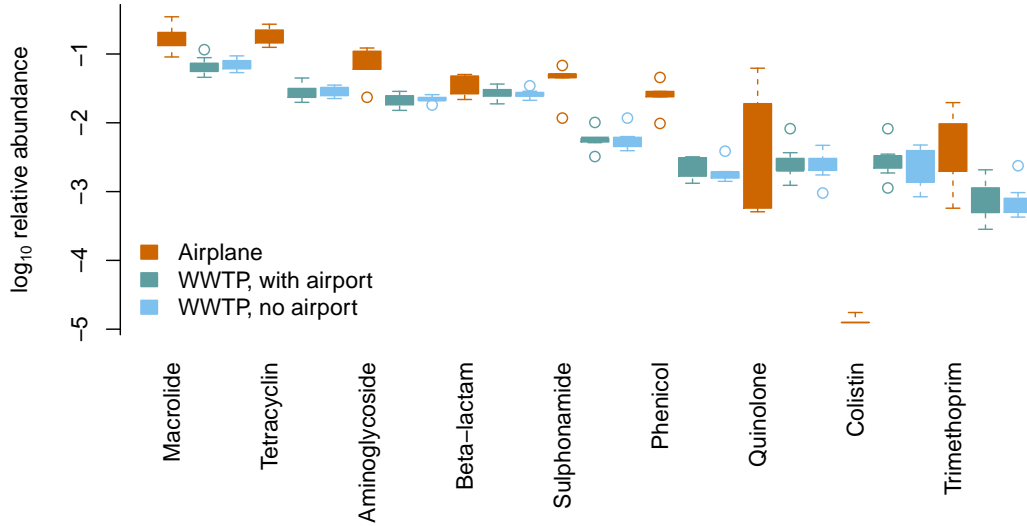


Figure 4: ARG copies per 16S rRNA gene copies in untreated sewage collected from airplanes and WWTP with/without connection to airports. The respective information on ARG and 16S rRNA genes is based on the metagenomics analysis. Genes were aggregated by target drug classes to reduce the complexity of information.

266 WWTP samples with and without contribution of airport sewage were also processed
 267 through qPCR. Like with the metagenomics approach, statistically significant differences
 268 between the two origins in terms of relative abundance could not be established (same
 269 set of ARG as in Table 2, results not shown).

Table 2: Relative abundance of resistance genes (ARG copies per 16S rRNA gene copies) in airplane sewage and the influent of wastewater treatment plants with connection to airports. Reported numbers are median values obtained by qPCR. Adjusted p-values refer to a two-sided Welch test.

Target class	ARG	Airplane (n=8)	WWTP, with airport (n=8)	adj. p-value
Colistin	<i>mcr1</i>	2.8×10^{-6}	9.7×10^{-6}	0.81
MLSB	<i>ermB</i>	2.0×10^{-2}	1.7×10^{-2}	1
β -lactam	<i>bla</i> _{KPC-3}	1.6×10^{-7}	2.0×10^{-7}	1
β -lactam	<i>bla</i> _{OXA-48}	6.5×10^{-6}	1.3×10^{-3}	0.33
β -lactam	<i>bla</i> _{OXA-58}	1.6×10^{-6}	6.1×10^{-4}	0.22
β -lactam	<i>bla</i> _{TEM}	1.1×10^{-3}	4.3×10^{-4}	1
β -lactam	<i>mecA</i>	2.7×10^{-7}	2.3×10^{-7}	1
β -lactam	<i>bla</i> _{CMY-2}	2.2×10^{-4}	1.8×10^{-5}	0.81
β -lactam	<i>bla</i> _{CTX-M-15}	3.2×10^{-4}	4.5×10^{-5}	0.81
β -lactam	<i>bla</i> _{CTX-M-32}	8.3×10^{-4}	1.3×10^{-4}	0.81
β -lactam	<i>bla</i> _{NDM-1}	1.5×10^{-5}	2.2×10^{-5}	1
β -lactam	<i>bla</i> _{VIM-2}	0	1.4×10^{-4}	0.037*
Sulphonamide	<i>sul1</i>	4.7×10^{-2}	9.2×10^{-3}	0.019*
Tetracyclin	<i>tetM</i>	2.4×10^{-2}	1.3×10^{-3}	9.4×10^{-5} ***

270 **3.4 Phenotypic resistance in *Escherichia coli***

271 The highest percentage of resistant *E. coli* isolates was generally detected in airplane
 272 samples (Table 3). The difference in proportions between airplane and WWTP sam-
 273 ples was statistically significant for 18 out of 24 antibiotics. Some Odds ratios reached
 274 values around 40, namely for two cephalosporins (cefotaxime, cefuroxime) and one of
 275 the fluoroquinolones (ciprofloxacin). At the same time, the influent of treatment plants
 276 receiving sewage from airports did not exhibit elevated proportions of resistant *E. coli*
 277 when compared to treatment plants that do not receive such input (p-values generally
 278 > 0.15 , results not shown).

Table 3: Antibiotic resistance among *E. coli* isolated from airplane sewage and the inlet of WWTP with connection to airports. Odds ratios (OR) greater than one indicate a higher level of resistance in the airplane samples compared to WWTP samples. p-values refer to Fisher’s exact test. SXT stands for the combination of trimethoprim and sulfamethoxazole also known as co-trimoxazole.

Antibiotic	Airplane	WWTP, with airport	OR	adj. p-value
Ampicillin	125:187	21:161	13.3	2.9e-24***
Amoxicillin-CA	65:187	8:161	10.1	1.3e-11***
Piperacillin	21:187	5:161	3.9	0.032*
Ticarcillin	55:187	4:161	16.3	1.3e-11***
Cefepime	7:187	0:161	Inf	0.099
Cefotaxime	97:187	4:161	41.9	1.2e-26***
Cefoxitin	9:187	1:161	8.1	0.12
Cefopodoxim	97:187	16:161	9.7	1.2e-16***
Ceftazidim	67:187	8:161	10.6	3.3e-12***
Cefuroxim	109:187	5:161	43.1	1.1e-30***
Doripenem	4:187	3:161	1.2	1
Ertapenem	4:187	2:161	1.7	1
Imipenem	83:187	7:161	17.4	3.5e-18***
Meropenem	56:186	7:161	9.4	7.8e-10***
Ciprofloxacin	94:187	4:161	39.3	2.2e-25***
Levofloxacin	82:187	8:161	14.8	4.8e-17***
Norfloxacin	31:187	10:161	3.0	0.024*
Amikacin	136:187	24:161	15.1	2.2e-27***
Gentamycin	64:187	10:161	7.8	4.1e-10***
Netilmicin	15:187	4:161	3.4	0.13
Tobramycin	126:187	17:161	17.3	1.2e-27***
Tigecyclin	4:187	1:161	3.5	1
Chloramphenicol	128:187	39:161	6.7	1.4e-15***
SXT	52:187	25:161	2.1	0.046*

279 Almost 90% of the *E. coli* isolates from airplane samples were resistant to at least
 280 one of the tested antibiotics (Fig. 5). This compares to about 45–60% in samples taken
 281 at WWTP. The peculiarity of airplane sewage is also reflected in the proportion of multi-
 282 resistant isolates. Airplane-borne *E. coli* were far more likely to be resistant against
 283 ≥ 3 classes of antibiotics than isolates obtained from raw sewage entering the WWTP
 284 (Fig. 5).

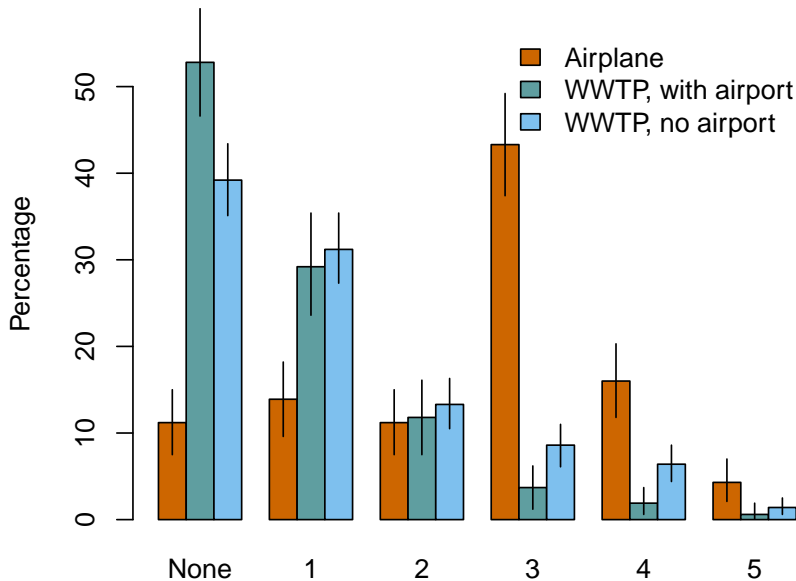


Figure 5: Prevalence of single- and multi-drug resistance among *E. coli* isolated from airplane sewage and WWTP influents with/without connection to airports. X-axis labels indicate the number of ineffective classes of drugs. The classes covered by susceptibility tests include penicillins, cephalosporines, carbapenemes, fluoroquinolones, aminoglycosides where each class is represented by 3–6 antibiotics (see 1st column of Table 3). Further drug classes represented by a single substance include tetracyclines, phenicols, and trimethoprim/sulfamethoxazole. Error bars represent 90% confidence intervals estimated by bootstrapping.

285 3.5 Methodological aspects

286 Since many of the available samples were processed in parallel through shotgun-sequencing
 287 and qPCR, there was a unique chance to compare the two approaches regarding the quan-
 288 tification of ARG. Doing so, we had to distinguish between unambiguous and potential
 289 hits in the metagenomes (Fig. 6). Unambiguous hits are those where a sample sequence
 290 (≈ 150 bp) matched just a single ARG registered in the data base (black symbols).
 291 This is in contrast to potential hits (gray symbols), where the sample sequence matched
 292 multiple related ARG among which is the particular gene of interest.

293 In general, we observed a reasonable agreement between the metagenomics and qPCR
 294 data for ARG with relative abundances greater than about 10^{-3} copies (16S rRNA gene
 295 copies)⁻¹. The correlation coefficients were > 0.98 for *sul1* and *tetM*, for example, and
 296 > 0.93 for *ermB* (based on unambiguous hits). Nevertheless, Fig. 6 also shows some
 297 characteristic mismatches between the two approaches. Most notably, there appears to
 298 be a negative bias in the metagenomics-based estimates. At relative abundances > 0.01
 299 the deviation hardly exceeds $1/2$ log unit but underestimation gets stronger as relative
 300 abundances decline. That negative bias gradually turns into failure of the metagenomics
 301 approach (symbols accumulating on the lower axis) as the signatures of rare ARG occur
 302 just by chance in the set of sample sequences (about 5×10^7 in this study).

303 However, not all of the deviations depicted in Fig. 6 should be blamed on the limi-
 304 tations of metagenomics. For example, symbols associated with *bla*_{OXA-48} and *bla*_{TEM}
 305 are far off the 1:1 line. This might well be due to unspecific amplification of primers

306 resulting in an overestimation of ARG abundances by qPCR.

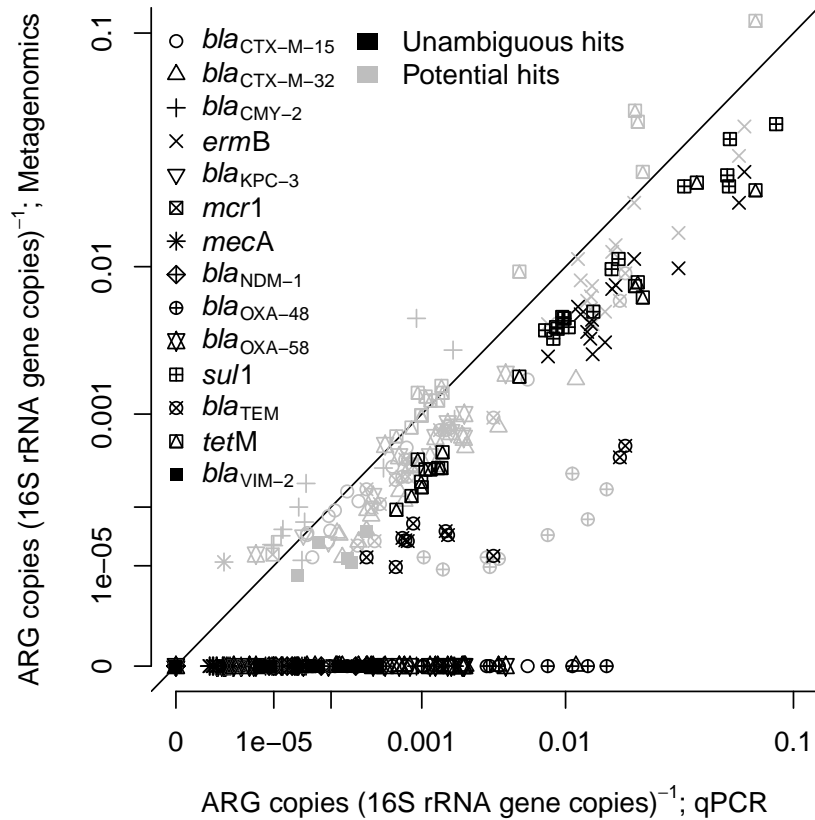


Figure 6: Comparison of relative ARG abundances obtained with qPCR and metagenomics for identical extracts of DNA. The diagonal indicates a 1:1 match. The data include raw sewage samples from all sources (airplane and WWTP). Note the custom axes resulting from square-root transformation.

307 4 Discussion

308 Cultivation and culture-independent techniques yielded a largely consistent and well
 309 differentiated picture of the prevalence of ARB/ARG in untreated wastewater of different
 310 origins. In agreement with initial expectations, sewage from airplane tanks was found to
 311 stand out from conventional sewage in terms of ARG diversity (Fig. 2). Nevertheless, the
 312 rarefaction models plotted in Fig. 3 suggest that the difference between airplane sewage
 313 and untreated municipal wastewater in terms of ARG diversity might level off for very
 314 large numbers of analyzed sequences. Considering the fact that municipal wastewater
 315 integrates bacteria from various sources besides those related to human waste (e.g. from
 316 pets, slaughterhouses, soil runoff, etc.) a convergence of the curves seems plausible. Thus,
 317 the main feature of airplane sewage appears to be the fact that a large number of different
 318 ARG can already be found in limited quantities of bacterial DNA. The mixing of human
 319 gut bacteria from different geographical backgrounds (hypothesis 1) provides a plausible
 320 explanation for this finding. Considerable variation in human resistomes is known to
 321 exist already at European level (European Centre for Disease Prevention and Control,
 322 2017; Pärnänen et al., 2019) and even larger disparities are expected on global scales. For
 323 example, Bengtsson-Palme et al. (2015a) demonstrated the import of ESBL-producing
 324 *E. coli* by travelers returning from India while no such import was observed from Africa.
 325 However, the elevated diversity of ARG in airplane sewage could also be due to alternative

326 mechanisms. For example, disparities in ARG diversity between samples of different
327 origin might – at least in parts – reflect contrasts in taxonomic diversity. In particular, the
328 elevated number of unique ARG in airplane sewage coincided with an increased diversity
329 of bacterial groups as reflected in taxonomic evenness (Fig. 1) as well as in the number of
330 bacterial families detected per 10^5 DNA sequences (Sec. 3.1). Against this background,
331 the true cause(s) of the increased ARG diversity in airplane-borne sewage are yet to be
332 explored. Instead of focusing on ARG diversity, future research might also attempt to
333 identify specific ARG which are characteristic for wastewaters of different origin. Our
334 metagenomics-based data suggest that such characteristic genes exist. For example, the
335 beta-lactamase gene *bla*_{CARB-4} was found in high relative abundances in airplane sewage
336 while it was never detected in any sample of municipal wastewater without contribution
337 from airports. Similarly, the carbapenemase resistance gene *bla*_{OXA-427} was frequently
338 detected in samples from WWTP influents while its signature was not found in any
339 sample of airplane sewage.

340 Our data clearly support the second hypothesis according to which airplane-borne
341 sewage stands out from common untreated municipal wastewater in terms of the rel-
342 ative abundance of ARG. The elevated prevalence of antibiotic resistance in airplane
343 sewage was most clearly demonstrated by drug susceptibility tests carried out on *E. coli*
344 (Table 3, Fig. 5). The high abundance of multi-resistant *E. coli* in airplane sewage is
345 particularly remarkable. For example, the proportion of isolates carrying a combined re-
346 sistance against 3rd generation cephalosporins, fluoroquinolones and aminoglycosides was
347 increased by a factor of about eight compared to German clinical isolates (28.9% com-
348 pared to 3.5%; [European Centre for Disease Prevention and Control, 2017](#)). With regard
349 to the prevalence of genotypic resistance, the clearest differentiation between wastewa-
350 ters of different origin was obtained by the metagenomics approach (Fig. 4). The median
351 relative abundance of ARG was higher in airplane-borne sewage than in conventional
352 sewage for seven out of nine classes of target drugs. Similar to the case of gene diver-
353 sity, the elevated prevalence of ARG/ARB in airplane sewage might reflect differences in
354 gut microbiomes between the local population (Germany) and the flights' or passengers'
355 countries of origin. However, storage conditions in the airplanes' wastewater tanks pro-
356 vide an alternative explanation. Those tanks typically contain disinfectants. A common
357 formulation certified for use in aircraft toilets lists alkylbenzyltrimethylammonium chlo-
358 ride, a quaternary ammonium compound, as its major ingredient. A number of potential
359 linkages between this compound and antibiotic resistance bacteria is known to exist (see
360 [SCENIHR, 2009](#), Sec. 3.8.5). The sewage tanks might thus serve as incubators that select
361 for antibiotic resistance via mechanisms of, e.g, cross- or co-resistance ([Buffet-Bataillon
362 et al., 2012](#)). The "breeding" of ARB should be particularly efficient if the tanks are
363 not completely purged, leaving a highly resistant inoculum for continued vertical and
364 horizontal ARG transfer.

365 In order to assess the potential risk associated with the special resistome of airplane
366 sewage, information on the fate of the respective ARG/ARB is required. Our data indi-
367 cate that airplane-borne sewage is strongly "diluted" upon mixing with wastewater
368 from other sources. None of the employed methods (susceptibility tests, qPCR, metage-
369 nomics) indicated significant shifts in ARG diversity or ARG/ARB prevalence between
370 samples of raw sewage acquired from WWTP with and without connection to airports.
371 Assuming that bacteria and genes do not undergo considerable retention or degradation
372 in the sewer system, that lack of significance should mainly reflect the limits of current
373 analytical methods to detect small increments in ARG/ARB abundances in the presence
374 of considerable background levels (unfavorable signal-to-noise ratio).

375 One might be tempted to conclude that airport-borne sewage is of little relevance
376 for the dissemination of ARG because of the apparently strong dilution. Furthermore,

377 airplane tanks are just one source of ARG and other hot-spots of antibiotic resistance
378 are known to exist, e. g. large health care facilities. Such reasoning, however, disregards
379 an essential property of genetic material, namely the potential for replication via hor-
380 izontal and vertical transfer. Consequently, even very small amounts of ARG released
381 into the water cycle may spread within the aquatic environment with the chance of
382 (re)emerging in human pathogens. From this point of view, the potential threat coming
383 from airport-borne sewage is not the quantity of imported ARG but the dissemination
384 of resistances which are rarely found in the local environmental systems. This relates
385 not only to rare ARG but also to combined resistances against multiple classes of drugs.
386 With the example of *E. coli*, airplane-sewage was shown to be an unusual source of highly
387 multi-resistant isolates. Nevertheless, we currently cannot provide evidence for the fact
388 that rare ARG and/or combined resistances originating from airplane-sewage actually
389 undergo considerable enrichment via selection or horizontal gene transfer in the receiv-
390 ing treatment plants. As pointed out by [Bengtsson-Palme et al. \(2016\)](#), comprehensive
391 analyses of resistant and susceptible strains in WWTP influents and effluents would be
392 necessary to specifically target that question. As a consequence of our study, we suggest
393 to first explore evolutionary processes inside airplane waste tanks and to look for options
394 that prevent (or deal with) the increased level of antibiotic resistance "begin-of-pipe".

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399 Universität Dresden.

400 Competing interests

401 The authors declare no competing interests.

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Table S.1: Characteristics of individual samples. Location names were substituted by anonymous integer codes. Volumes represent the amount of sample from which DNA was extracted.

Date	City code	Origin	Volume (mL)	DNA (ng μL^{-1})	260/280 nm ratio	260/230 nm ratio	<i>E. coli</i> isolates	Sequenced	qPCR done	pH	EC (mS cm^{-1})	16S gene copies mL^{-1}
2016-10-31	5	Airplane	10	109	1.875	1.025	0	✓	✓	9.0	9.44	6.8E+08
2016-11-14	1	Airplane	25	771	1.91	2.095	46	✓	✓	8.3	10.62	2.2E+09
2016-11-17	3	Airplane 1	17.5	322	1.885	1.84	48	✓	✓	9.0	13.00	2.2E+09
2016-11-17	3	Airplane 2	10	260	1.89	1.56	47	✓	✓	8.9	10.16	3.0E+09
2016-11-29	4	Airplane	20	136	1.86	1.27	24	✓	✓	8.9	8.23	8.5E+08
2016-12-05	5	Airplane	20	81	1.855	0.725	0	✓	✓	9.2	14.09	4.0E+08
2016-12-12	1	Airplane	25	183	1.88	1.555	22	✓	✓	8.7	4.24	8.3E+08
2017-01-31	2	Airplane	10	221	1.92	2.19	0	✓	✓	9.0	20.83	2.9E+09
2016-11-29	4	WWTP, with airport, sewer 1	65	356	1.87	1.69	23	✓	✓	7.8	1.85	5.6E+08
2016-11-29	4	WWTP, with airport, sewer 2	70	251	1.92	1.735	24	✓	✓	7.4	1.76	3.6E+08
2017-01-17	5	WWTP, with airport	100	185	1.96	1.69	23	✓	✓	6.6	2.21	9.7E+07
2017-01-31	2	WWTP, with airport	100	536	1.98	1.97	0	✓	✓	8.2	2.00	3.8E+08
2017-02-14	4	WWTP, with airport	50	88	2.17	0.2	0	✓	✓	7.5	2.35	5.5E+07
2017-02-28	3	WWTP, with airport	100	175	2.025	1.375	24	✓	✓	7.4	1.62	1.3E+08
2017-03-06	3	WWTP, with airport	100	434	1.95	1.75	24	✓	✓	6.8	0.76	2.1E+08
2017-03-14	9	WWTP, with airport	50	180	1.93	1.605	24	✓	✓	6.6	1.34	3.1E+08
2017-03-15	5	WWTP, with airport	100	222	1.9	1.55	0	✓	✓	6.9	1.26	1.8E+08
2017-05-25	8	WWTP, with airport	100	587	1.97	1.09	19	✓	✓	8.0	NA	2.6E+08
2016-09-17	1	WWTP, no airport	100	380	1.92	1.715	0	✓	✓	7.7	0.54	2.9E+08
2016-12-06	1	WWTP, no airport	100	291	1.89	1.78	21	✓	✓	7.0	1.81	3.0E+08
2017-01-03	1	WWTP, no airport	100	180	1.99	1.25	23	✓	✓	7.4	1.88	7.4E+07
2017-02-01	1	WWTP, no airport	100	310	1.99	1.51	0	✓	✓	7.7	2.01	2.1E+08
2017-02-24	1	WWTP, no airport	100	304	2.395	1.915	24	✓	✓	7.4	0.87	1.9E+08
2017-02-28	1	WWTP, no airport	100	369	1.985	1.79	0	✓	✓	7.3	1.43	1.5E+08
2017-03-07	1	WWTP, no airport	100	544	1.915	1.835	24	✓	✓	6.7	1.08	3.1E+08
2017-03-06	3	WWTP, no airport	100	567	1.955	1.74	24	✓	✓	6.7	0.73	2.8E+08
2017-03-14	9	WWTP, no airport	50	212	1.97	1.155	24	✓	✓	6.7	1.37	3.4E+08
2017-05-12	1	WWTP, no airport	100	372	1.92	1.035	0	✓	✓	6.9	1.05	2.4E+08
2017-05-25	8	WWTP, no airport, sewer 1	100	411	1.875	0.97	24	✓	✓	7.9	NA	2.0E+08

Table S.1, continued

2017-05-25	8	WWTP, no airport, sewer 2	100	453	1.895	1.86	22	✓	7.7	NA	2.9E+08
2017-05-25	8	WWTP, no airport, sewer 3	100	125	1.805	0.495	12	✓	8.0	NA	5.5E+07
2017-05-25	8	WWTP, no airport, sewer 4	100	393	1.855	1.295	23	✓	7.7	NA	1.6E+08
2017-07-11	6	WWTP, no airport	100	111	1.87	1.195	22	✓	NA	NA	8.4E+07
2017-07-27	1	WWTP, no airport	100	359	1.915	1.785	23	✓	7.4	0.82	2.6E+08
2017-08-02	1	WWTP, no airport	100	281	1.905	1.675	24	✓	NA	0.85	2.2E+08
2017-08-10	1	WWTP, no airport	100	56	1.945	0.495	24	✓	7.3	0.49	3.1E+07
2017-08-10	7	WWTP, no airport	100	91	1.96	0.68	24	✓	7.3	0.89	8.3E+07
2017-08-31	6	WWTP, no airport	100	362	1.885	1.68	24	✓	7.2	0.79	9.4E+08
2017-11-05	7	WWTP, no airport	100	365	1.89	1.68	0	✓	NA	NA	2.6E+08
2018-01-04	6	WWTP, no airport	100	347	1.94	1.98	0	✓	NA	NA	1.4E+09

Table S.2: List of qPCR primers.

Gene		Primer sequence	Amplicon size (bp)	Reference
<i>bla</i> _{TEM}	Fwd.	TTCCTGTTTTTGCTCACCCAG	112	NORMAN Network ¹
	Rev.	CTCAAGGATCTTACCGCTGTTG		
<i>ermB</i>	Fwd.	TGAATCGAGACTTGAGTGTGCAA	71	Alexander et al. (2016)
	Rev.	GGATTCTACAAGCGTACCTT		
<i>tetM</i>	Fwd.	GGTTTCTCTTGATACTTAAATCAATCR	88	Peak et al. (2007)
	Rev.	CCAACCATAYAATCCTTGTTTCRC		
<i>sul1</i>	Fwd.	CGCACCGGAAACATCGCTGCAC	161	NORMAN Network ¹
	Rev.	TGAAGTCCGCCGCAAGGCTCG		
<i>bla</i> _{CMY-2}	Fwd.	CGTTAATCGCACCATCACC	172	Kurpiel and Hanson (2012)
	Rev.	CGTCTTACTAACCGATCCTAGC		
<i>bla</i> _{CTX-M-15}	Fwd.	CTATGGCACCACCAACGATACTYM	103	ANSWER Network ²
	Rev.	ACGGCTTTCTGCCTTAGGTT		
<i>bla</i> _{CTX-M-32}	Fwd.	CGTCACGCTGTTGTTAGGAA	155	NORMAN Network ¹
	Rev.	CGCTCATCAGCAGATAAAG		
<i>bla</i> _{OXA-48}	Fwd.	TGTTTTGGTGGCATCGAT	177	Monteiro et al. (2012)
	Rev.	GTAAMRATGCTTGGTTCGC		
<i>bla</i> _{OXA-58}	Fwd.	GCAATTGCCTTTTAAACCTGA	152	Szczepanowski et al. (2009)
	Rev.	CTGCCTTTTCAACAAAACC		
<i>mecA</i>	Fwd.	CGCAACGTTCAATTTAATTTTGTTAA	91	Volkmann et al. (2004)
	Rev.	TGGTCTTTCTGCATTCCTGGA		
<i>bla</i> _{NDM-1}	Fwd.	TTGGCCTTGCTGTCCTTG	82	Monteiro et al. (2012)
	Rev.	ACACCAGTGACAATATCACCG		
<i>bla</i> _{VIM-2}	Fwd.	GAGATTCCCACGCACTCTCTAGA	93	van der Zee et al. (2014)
	Rev.	AATGGCAGCACCAGGATAG		
	probe	ACGCAGTGCCTTCGGTCCAGT		
<i>bla</i> _{KPC-3}	Fwd.	CAGCTCATTCAAGGGCTTTC	196	Szczepanowski et al. (2009)
	Rev.	GGCGCGTTATCACTGTATT		
<i>mcr1</i>	Fwd.	GGCCTGCGTATTTAAGCG	183	Hembach et al. (2017)
	Rev.	CATAGGCATTGCTGTGCGTC		
16S rRNA	Fwd.	TCCTACGGGAGGCAGCAGT	195	NORMAN Network ¹
	Rev.	ATTACCGCGCTGCTGG		

¹www.norman-network.net²www.answer-itn.eu