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Abundances of Tetracycline, Sulphonamide and Beta-Lactam Antibiotic Resistance Genes in Conventional Wastewater Treatment Plants (WWTPs) with Different Waste Load

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Abstract

Antibiotics and antibiotic resistant bacteria enter wastewater treatment plants (WWTPs), an environment where resistance genes can potentially spread and exchange between microbes. Several antibiotic resistance genes (ARGs) were quantified using qPCR in three WWTPs of decreasing capacity located in Helsinki, Tallinn, and Tartu, respectively: sulphonamide resistance genes (sul1 and sul2), tetracycline resistance genes (tetM and tetC), and resistance genes for extended spectrum beta-lactams (blaOXA-58, blaSHV-34, and blacte-M-32). To avoid inconsistencies among qPCR assays we normalised the ARG abundances with 16S rRNA gene abundances while assessing if the respective genes increased or decreased during treatment. ARGs were detected in most samples; sul1, sul2, and tetM were detected in all samples. Statistically significant differences (adjusted p < 0.01) between the inflow and effluent were detected in only four cases. Effluent values for blaOXA-58 and tetC decreased in the two larger plants while tetM decreased in the medium-sized plant. Only blaSHV-34 increased in the effluent from the medium-sized plant. In all other cases the purification process caused no significant change in the relative abundance of resistance genes, while the raw abundances fell by several orders of magnitude. Standard water quality variables (biological oxygen demand, total phosphorus and nitrogen, etc.) were weakly related or unrelated to the relative abundance of resistance genes. Based on our results there is neither considerable enrichment nor purification of antibiotic resistance genes in studied conventional WWTPs.

Introduction

Antibiotic resistance (AR) has become a worldwide problem, making infectious diseases more resilient thus making treatment more difficult and costly [1]. AR is not confined to hospital environments, and is able to spread between both human dominated and natural environments. Increased amounts of antibiotic residues [2–4] and antibiotic resistant bacteria (ARB) are found in human-related environments such as agricultural settings (e.g. farms, soil etc.) [5,6] and surface-, drinking- and wastewaters [7–11]. Wastewater treatment plants (WWTPs) receive sewage from various sources, including hospitals and households which are both important sources of antibiotics and their residues [12–14], and antibiotic resistant bacteria (ARB) [15–17]. The presence of antibiotics and antibiotic residues [18], ARB, and antibiotic resistance genes (ARG) have been confirmed in many WWTPs [19–23]. Bacteria from various environments, including human, soil, and activated sludge, are mixed in WWTPs and therefore these facilities are considered to be important “hot-spots” for AR and spread of resistance genes [18,24–26]. The presence of antibiotics, ARB, and ARG in the same setting creates an environment that selects for AR and provides an opportunity for genetic material housing ARGs to transfer between bacterial species via horizontal gene transfer [13,21,24,25,27,28]. In a metagenomic study of plasmids it was shown that numerous medically relevant ARG can be found in WWTPs [140 ARGs in a single WWTP and 123 in the effluent water] [29]. Therefore, there is a concern that resistance genes will spread in the bacterial population and further into more natural environments less impacted by human activity [30–33].

Many studies of antibiotic resistant bacteria and resistance genes have used culture-based assays, which are biased towards specific
1. Collection of total microbial community

The water samples were stored at 4°C pending filtration (within a couple of hours). Ten ml of influent water and 100 ml of effluent water were filtered through polycarbonate filters (pore size 0.22 μm, diameter 47 mm, GE Water & Process Technologies). For the last two time points at the Tallinn WWTP 200 ml of effluent water was filtered because a new treatment step (bio-filter) was added in September 2011.

1.3. DNA extraction

For the first three time points (Dec 2010; March 2011; June 2011) from the Helsinki WWTP, DNA was extracted from the samples using a MoBio PowerWater DNA isolation kit (MoBio Laboratories, Inc., CA, USA). For all other samples, the DNA was extracted using the modified bead beating and silica-membrane method (nucleic acid binding on to silica particles [41]. Method in brief: for lysis: 400 μl TE+50 μl lysozyme (30 mg/ml from egg yolk) was added to the filter and incubated at 37°C for 15 min. Fifty μl of 10% SDS and 500 μl lysin buffer BQ1 (NucleoSpin, Macherey-Nagel)+zirconium beads (0.1 mm diameter; burned at 500°C) were added and the samples were processed by a 3 min beating on a bead beater (Biospec products, Minibead beater). Theretofore, proteinase K was added followed by 15 min at 65°C with constant shaking. Five hundred μl of 96% ethanol was added and the whole volume was applied to commercial silica-membrane columns (NucleoSpin Macherey-Nagel). Finally, the total DNA was recovered in 50 μl of elution buffer BE (NucleoSpin Macherey-Nagel, 5 mM Tris/HCl pH 8.5). The concentration of extracted DNA was measured using a NanoDrop Spectrophotometer ND-1000 (absorption readings at 260 nm). The extracted DNA was stored at −20°C pending further analysis.

1.4. Detection and quantification of ARG copy number by qPCR

Seven resistance genes were surveyed: sul1, sul2, tetM, tetC, bla ESBL-34, blactx-m-32, and blaeoxa-58. For the first three time point samples (Dec 2010; March 2011; June 2011) from the Helsinki WWTP (Assay 1), qPCR was performed using a Dynamo Flash SYBR Green qPCR kit (Thermo Scientific, Lithuania) and a 7300 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). The thermal cycling conditions were as follows: 95°C for 7 min, 40 cycles at 95°C for 10 s and 72°C for 30 s. A melting curve was obtained to confirm specificity of amplification. Reactions were conducted in 10 μl volumes on 96-well plates containing 1×Dynamo Flash SYBR Green master mix, 0.3 μM of each primer and 1×ROX passive reference dye. Template DNA was used in qPCR reactions in the range 2–12 ng DNA per reaction; a fixed dilution of raw DNA extract was used. In parallel with the ARGs, the 16S rRNA gene copy numbers were quantified.

The qPCR for detecting 16S rRNA gene and ARGs for all the other samples (Assay 2) used the 7900HT Fast Real-Time PCR System (Applied Biosystems). Reactions were conducted in 10 μl volumes on 384-well plates containing 1×Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific), 0.4 μM of each primer. The two-step thermal cycling conditions for detecting 16S rRNA gene were as follows: 95°C for 10 min, 40 cycles of 95°C 15 s, 60° C 1 min. For ARGs the thermal cycling conditions were as follows: 95°C for 10 min, 40 cycles at 95°C for 15 s, 72°C for 30 s and 72°C for 30 s. Melting curves were obtained to confirm specificity of amplification. Template DNA was used in the qPCR reaction in the range 3×10⁻⁴ to 22.4 ng DNA per reaction. This was obtained by a wider range of dilutions (to avoid inhibition) of the raw DNA extraction product, three levels of 10 fold dilutions.
were used. Primers and annealing temperature, Tm (°C), are given in Table 1. Number of technical replicates in both qPCR assays was 3.

1.5. Standards used for quantification
A plasmid vector and fragments of ARGs were constructed and used as standards for quantifying the raw qPCR results. The standard plasmids were checked for the correct inserts by sequencing. In Assay 1 for the three Helsinki time points (Dec 2010; March 2011 and June 2011) the 16S rRNA gene quantification standard was genomic DNA from *E. coli* K12 (genome size 4.6 Mbp with seven copies of the rRNA operon). In Assay 2, used for all other samples, the standard for quantifying 16S rRNA gene was constructed from a 16S rRNA gene fragment from the natural aquatic *Chryseobacterium* strain isolated from Emajõgi River, which receives WWTP effluent water, and was cloned into a plasmid and validated by sequencing. Information about the plasmids used and Genbank accession numbers are given in Table 2.

1.6. Quantification and normalisation of ARGs
Standard curves (Ct per log copy number) for 16S rRNA gene and ARG quantification were obtained for each run using the plasmid constructs (or genomic *E. coli* DNA for 16S rRNA gene in Assay 1) (Table 2) in ten-fold serial dilutions. The gene copy number of a standard was determined from the plasmid/genomic DNA concentration (measured using a NanoDrop in Assay 1 or by fluorescent staining with PicoGreen (Invitrogen) and using VICTOR X3 Multilabel Plate Readers (Perkin-Elmer) in Assay 2). The ARG levels in the sample were calculated using the standard curve equation and measured Ct value, the quality control of raw Ct values for standard curve and unknown samples was done before further analysis. The limit of quantification (LOQ) was defined as the lowest point of the linear part of standard curve: Assay 1, ARGs 100 and 16S rRNA gene 1000 gene copy number per reaction; Assay 2, ARGs and 16S rRNA gene 100, except sul1 with 10 copy numbers per reaction. The negative control was the reaction mix with nuclease-free water instead of the template DNA. The negative control had always a Ct value at least 3.3 cycles lower than the smallest standard used.

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### Table 1. Primers used for detecting the target genes and the melting temperatures (Tm) used for primers.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primers</th>
<th>Tm °C</th>
<th>Ref.</th>
<th>Amplification efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S rRNA gene</td>
<td>5′-AGA GTT TGA TCC TGG CTC AG-3′</td>
<td></td>
<td>[60]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5′-CTG CTG CCC GTA GGA-3′</td>
<td>60</td>
<td>[61]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5′-CTG CTG CCT CCC GTA GG-3′</td>
<td>60</td>
<td>[61]</td>
<td></td>
</tr>
<tr>
<td>tetC</td>
<td>5′-TGC GAT GCA ATT TCT AGT C-3′</td>
<td>64</td>
<td>[62]</td>
<td>80</td>
</tr>
<tr>
<td>tetM</td>
<td>5′-GCA ATT CTA CTG ATT TCT GC-3′</td>
<td>64</td>
<td>[61]</td>
<td>90–93</td>
</tr>
<tr>
<td>sul1</td>
<td>5′-CGG GTG GTA CCT CTT GTA AGG AA-3′</td>
<td>64</td>
<td>[63]</td>
<td>80</td>
</tr>
<tr>
<td>sul2</td>
<td>5′-CGG TAT TTA AGA TTA ACC ACC CTT-3′</td>
<td>64</td>
<td>[63]</td>
<td>92–94</td>
</tr>
<tr>
<td>blactx-m-32</td>
<td>5′-CTG CTT CCA ACC TCC TCT ACC-3′</td>
<td>60</td>
<td>[63]</td>
<td>90</td>
</tr>
<tr>
<td>blashv-34</td>
<td>5′-GCA ATT GCA TGG CTA TGG AGA AG-3′</td>
<td>60</td>
<td>[63]</td>
<td>97–108</td>
</tr>
<tr>
<td>blaoxa-58</td>
<td>5′-GCA ATT GCC TTT TTA ACC TGA-3′</td>
<td>60</td>
<td>[63]</td>
<td>97–111</td>
</tr>
</tbody>
</table>

qPCR amplification efficiency is given for 16S RNA gene and for ARGs, R² of the linear range of standards was always >0.99. 
doi:10.1371/journal.pone.0103705.t001

### Table 2. Plasmids and PCR fragments used as standards.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Standard constructs</th>
<th>Accession number</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S rRNA gene</td>
<td>Assay 1 Genomic DNA from <em>E. coli</em> K12 genome size 4.6 Mbp with 7 copies of rRNA operon</td>
<td>KF737394</td>
<td>present study</td>
</tr>
<tr>
<td></td>
<td>Assay 2 PCR product cloned in plasmid PGEM-T Easy Vector System (Promega)</td>
<td>KF737394</td>
<td>present study</td>
</tr>
<tr>
<td></td>
<td>tetC pDrive (Qiagen)</td>
<td>[62]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>tetM pDrive (Qiagen)</td>
<td>[62]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>sul1 R388</td>
<td>[64]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>sul2 RSF1010</td>
<td>[64]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>blactx-m-32 pUC19</td>
<td>KF737395</td>
<td>present study</td>
</tr>
<tr>
<td></td>
<td>blashv-34 PGEM-T Easy Vector System (Promega)</td>
<td>KF737397</td>
<td>present study</td>
</tr>
<tr>
<td></td>
<td>blaoxa-58 pUC19</td>
<td>KF737396</td>
<td>present study</td>
</tr>
</tbody>
</table>

doi:10.1371/journal.pone.0103705.t002
for calculation of LOQ. Technical replicates were incorporated in the statistical analysis to reflect differences in quantification (see below for details).

1.7. Water quality variables

WWTPs in Europe analyse water quality parameters according to the EU directive for urban wastewater treatment 91/271/ECC. These parameters are monitored regularly in accredited laboratories according to standard methods. The parameters used in this study were: biochemical oxygen demand (BOD₃) - standard method EN 1899-2; total suspended solids (SS) - EN 672; total phosphorus (Ptot) – EN ISO 6878; total nitrogen (Ntot) – EN ISO 11905. In addition, the automated measurements of flow rate and water temperature in the process were recorded. We received the data for water variables from the staff of each WWTP.

1.8. Statistics

Linear mixed models were fitted to the data using the functionality of the package lme4 [42], the statistical software used was R version 3.0.1 [43]. Average levels of gene copy numbers (16S rRNA gene or ARGs) were modelled using the location of the WWTP (Helsinki, Tallinn, Tartu), sample source (IF/EF) and method protocol (Assay1/Assay2) as fixed effects and technical and biological replicates as random effects. Significance of fixed effects was assessed by an F-test using a (pre-specified) significance level of 1%. In addition, the combined effect of each of the environmental variables BOD₃, SS, Ptot, Ntot, flow rate (i.e. water discharge - WD) and temperature in inflowing versus effluent waste on gene copy numbers was described by a linear mixed model version of an analysis of covariance model. Model checking was based on residual plots and normal probability plots using the raw residuals. Models were reduced using the likelihood ratio test. A 1% or 5% significance level was used. Pairwise comparisons were evaluated based on adjusted p-values obtained using the single-step method [44]. Values below LOQ were not included in the analyses.

Principal component analysis (PCA) and its extensions to between groups (BGA) and within groups (WGA) analyses (ade4 package in R) was used to analyse the grouping of inflowing/effluent samples by monitored water quality variables (nutrients, spornins) and their resistance genes. Altogether 12 ARGs are surprisingly rarely quantified directly using community DNA in both IF and EF water samples from WWTPs. As in our study (Figure 1B), a few other studies have found from 100 to 1000 fold reductions of raw ARG copy numbers during the purification process; e.g. sul1, tetW, tetG, tetA; [52], [53], [54]. In one study, the relative abundance of sul1 increased, while sul2 decreased slightly in WWTP effluent [31]. In our study, resistance genes for “older” antibiotics (with exception of tetC) were more commonly detected. sul1, sul2, and tetM were present above LOQ in all sites and samples (Table 3 and Figure S2 in File S1). High abundances of various tetracycline resistance genes and sulphonamide resistance genes were also demonstrated in other studies [21,23]. Quantitative studies that target resistance to the newer beta-lactams in community DNA in WWTPs are almost completely absent-only blaTEM by Lachmayr et al. [38]. In addition, bla genes were quantified in the river water under the influences of wastewater but not directly in the WWTP effluent [55].

2.2. Abundance of genes (16S rRNA gene and ARGs)

To evaluate the abundance of the total bacterial community, we quantified 16S rRNA gene in the samples using qPCR. The amplification efficiency is given in Table 1. Initially, the raw gene copy numbers were used to estimate the general changes of bacterial levels during wastewater purification. The copy number of 16S rRNA gene was several orders of magnitude lower in the effluent (EF) than the inflow (IF) (Figure 1A, Table S2 in File S1). The differences between the IF and EF samples were statistically significant in all conditions (adjusted p<0.01) (Figure 1A).

The raw gene copy numbers of ARGs/ml decreased during processing in the WWTP water phase. The levels of ARGs detected in the EF were lower than IF in all three plants (Figure 1B, Table S2 in File S1). The decrease of abundance from IF to EF was statistically significant (p<0.01) for all ARGs in the large (Helsinki) WWTP. In the medium (Tallinn) WWTP, for all ARGs except blaKDE-32 (p=0.03), the decrease was statistically significant (p<0.01). In the small WWTP (Tartu), the raw abundance of ARGs decreased after purification but the decrease was statistically significant only for blaKDE-32 and tetM (p<0.01), and weakly significant for blaKDE-16 (p = 0.03). Earlier studies have demonstrated the large variation of treatment plant efficiency in removing microorganisms [50] and micropollutants [51], which also depends on the capacity of the WWTP plant. Low capacity treatment facilities are more vulnerable to changes in inflowing wastewater composition and flow rates. In addition, the WWTP in Tartu did not have biological post-filtration at the time of sampling.

ARGs are surprisingly rarely quantified directly using community DNA in both IF and EF water samples from WWTPs. As in our study (Figure 1B), a few other studies have found from 100 to 1000 fold reductions of raw ARG copy numbers during the purification process; e.g. sul1, tetW; [52]; tetC, tetA; [53], tetG, tetQ [54]. In one study, the relative abundance of sul1 increased, while sul2 decreased slightly in WWTP effluent [31]. In our study, resistance genes for “older” antibiotics (with exception of tetC) were more commonly detected. sul1, sul2, and tetM were present above LOQ in all sites and samples (Table 3 and Figure S2 in File S1). High abundances of various tetracycline resistance genes and sulphonamide resistance genes were also demonstrated in other studies [21,23]. Quantitative studies that target resistance to the newer beta-lactams in community DNA in WWTPs are almost completely absent-only blaTEM by Lachmayr et al. [38]. In addition, bla genes were quantified in the river water under the influences of wastewater but not directly in the WWTP effluent [55].

2.3. Normalised/Relative abundances of ARGs

To avoid inconsistencies among qPCR assays, including sub-optimal efficiency in some cases, we used 16S rRNA gene normalised values, and the different protocol (Assay1/Assay2) was added as an additional fixed effect into the statistical models. This type of data analysis allows one to quantify the relative changes in ARG abundances, whether more or fewer ARGs appear per microbial genome. When relative abundances were compared, statistically significant (p<0.01) differences between IF and EF were detected in only four cases (Figure 2). For blaKDE-32 and tetC in the Helsinki plant we observed a relative decrease after purification. In addition, tetM decreased in the Tallinn WWTP EF.
samples. The only increase in EF was observed for bla_{sdv-34} in the Tallinn WWTP. In all other cases the purification process had no significant effect on the relative abundances of resistance genes.

We conclude from our study that there is neither considerable enrichment (selection) nor purification of ARGs during processes in WWTPs (Figure 2) at the whole community level. Effective selection would be assumed when there are appropriate conditions, i.e. increased concentration of ABs occur. In two WWTPs studied, the measured levels of AB concentrations of several compounds were very low but measurable compared to highly labile beta-lactams [56]. This suggests that conditions could favour enrichment of at least tet and sul genes within studied WWTPs. Although, quantitative enrichment of ARGs (sul) responsible for resistance against refractory ABs with longer history of usage has

Table 3. Detection of ARGs in different WWTPs (total of all analyses per gene, n = 15).

<table>
<thead>
<tr>
<th>ARG</th>
<th>City</th>
<th>IF % (number) detected</th>
<th>EF % (number) detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>tetC</td>
<td>Helsinki</td>
<td>93 (14)</td>
<td>80 (12)</td>
</tr>
<tr>
<td></td>
<td>Tallinn</td>
<td>67 (10)</td>
<td>27 (4)</td>
</tr>
<tr>
<td></td>
<td>Tartu</td>
<td>67 (10)</td>
<td>73 (11)</td>
</tr>
<tr>
<td>bla_{sxx-58}</td>
<td>Helsinki</td>
<td>100 (15)</td>
<td>87 (13)</td>
</tr>
<tr>
<td></td>
<td>Tallinn</td>
<td>100 (15)</td>
<td>47 (7)</td>
</tr>
<tr>
<td></td>
<td>Tartu</td>
<td>87 (13)</td>
<td>80 (12)</td>
</tr>
<tr>
<td>bla_{sdv-34}</td>
<td>Helsinki</td>
<td>100 (15)</td>
<td>100 (15)</td>
</tr>
<tr>
<td></td>
<td>Tallinn</td>
<td>87 (13)</td>
<td>87 (13)</td>
</tr>
<tr>
<td></td>
<td>Tartu</td>
<td>100 (15)</td>
<td>100 (15)</td>
</tr>
<tr>
<td>bla_{sxx-m-32}</td>
<td>Helsinki</td>
<td>100 (15)</td>
<td>100 (15)</td>
</tr>
<tr>
<td></td>
<td>Tallinn</td>
<td>87 (13)</td>
<td>40 (6)</td>
</tr>
<tr>
<td></td>
<td>Tartu</td>
<td>80 (12)</td>
<td>47 (7)</td>
</tr>
</tbody>
</table>

Only genes that were sometimes not detected are given. sul1, sul2 and tetM were detected 100% in all IF and EF samples from the WWTPs.

doi:10.1371/journal.pone.0103705.t003

doi:10.1371/journal.pone.0103705.g001
been demonstrated in some studies [31], reduction or no change has been observed in most studies [21,23,40]. To date, only one study demonstrated similar case of positive selection for newer ARG possessing organisms in a WWTP, which suggests that bacteria harbouring blaTEM are released more from effluent water compared to wastewater [38].

2.4. Abundance of ARGs and treatment efficiency of wastewater

In EU countries, treatment efficiency of WWTPs is estimated by monitoring a few water quality measures, according to European directive (271/1991/EC). The compulsory parameters monitored are total nitrogen (Ntot) and phosphorus (Ptot), Biological Oxygen Demand (BOD$_7$), and suspended solids (SS). In addition, a few generic background parameters are measured in all WWTPs e.g. Water Discharge (WD) and temperature. Such water quality parameters are good for evaluating wastewater purification in the traditional sense–removal of excess nutrients, labile organic compounds etc. Obviously, the wastewater was purified of excess nutrients and organic compounds in the WWTPs studied because all parameters were up to an order of magnitude lower in EF than IF. We observed a change of between 6 to 45 fold depending on the parameter. Averages in IF: BOD$_7$ - 231; P$_{tot}$ 8; N$_{tot}$ 51 and SS 306 mg/l and in EF: BOD$_7$ 5; P$_{tot}$ 0.6; N$_{tot}$ 8 and SS 8 mg/l (Table S1B in File S1). Volumetric concentrations of nutrients, SS and labile organic compounds were higher in the IF of the small (Tartu) than in medium (Tallinn) and large (Helsinki) WWTP (Figure S4 in File S1; IF samples are strongly associated with these variables, permutation test, 1000 replicates, $p<0.01$). This could be caused by shorter solid retention time in smaller plants [57]. At the same time, the efficiency of purification in the traditional sense did not differ dramatically among plants (Figure S3 in File S1; residual differences among WWTPs disappear after decomposing the IF/EF level differences, permutation test, 1000 replicates, $p>0.05$). A relationship between the change of ARG abundances and the efficiency of nutrient removal and temperature has been reported previously (e.g. [58,59]). These studies suggest that changes in ARG abundance could depend on processes and conditions in the WWTP. However, none of these parameters are designed to estimate threats associated with the spread of either ARB or ARGs. Indeed, in our study, temperature, water discharge and concentrations of nutrients did not help in estimating the efficiency of ARG removal. This was demonstrated by the absence of the combined effect of monitored environmental variables and abundances of ARGs (Figure S4 in File S1). Moreover, a new treatment step (installation of biological post-filtration for final effluent treatment mainly for nitrogen removal) was added during the study period in the Tallinn WWTP (July 2011). However, no statistically significant effect was observed on ARG removal after this event. The Helsinki WWTP had the biological post-filtration installed throughout the study period.

In conclusion, these results force us to reject our original hypothesis. All ARGs were detected in most wastewater and effluent samples, however, the conventional WWTPs under study seem not to be important sites for changes in the relative abundance of ARGs at the whole community level: no enrichment in relative abundance was observed. Furthermore, no additional reduction of ARGs occurred; raw abundance changed in proportion to the decrease of bacterial abundance. We conclude that many unknown factors may influence the biological purification processes in conventional WWTPs and the evaluation of their relationship to ARG removal or selection requires more complex case studies.

Supporting Information

**File S1** Includes Tables S1 and S2; Figures S1, S2, S3 and S4.

(PDF)

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Author Contributions

Conceived and designed the experiments: TT MV VK. Performed the experiments: ML AK VV CR TT MV VK. Writing the manuscript: ML AK VV. Language revision: TT MV VK. Performed the statistical comparisons: AK. Performed the writing of the manuscript: ML AK VV. All authors have read and approved the final version of the manuscript.

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