This document is the accepted manuscript version of the following article:
Ju, F., Beck, K., Yin, X., Maccagnan, A., McArdell, C. S., Singer, H. P., … Bürgmann, H. (2018). Wastewater treatment plant resistomes are shaped by bacterial composition, genetic exchange, and upregulated expression in the effluent microbiomes. ISME Journal. https://doi.org/10.1038/s41396-018-0277-8

Wastewater Treatment Plant Resistomes are Shaped by Bacterial Composition, Genetic Exchange, and Upregulated Expression in the Effluent Microbiomes

Feng Ju[a, 1], Karin Beck[a], Xiaole Yin[b], Andreas Maccagnan[a], Christa S. McArdell[a], Heinz Singer[a], David R. Johnson[a], Tong Zhang[b], Helmut Bürgmann[a]*

[a] Eawag, Swiss Federal Institute of Aquatic Science and Technology, 6047 Kastanienbaum or 8600 Duebendorf, Switzerland; [b] Environmental Biotechnology Lab, Department of Civil Engineering, The University of Hong Kong, Hong Kong 999077, China

Running title: Antibiotic resistome dynamics during WWTP passage

*Corresponding author:

Dr. Helmut Bürgmann

Address: Eawag, Seestrasse 79, 6047 Kastanienbaum, Switzerland, 6047

Phone: +41 58 765 2165

Fax: +41 58 765 2168

E-mail: helmut.buergmann@eawag.ch

[1]Present address: Center for Environmental and Resources Engineering, Institute of Advanced Technology, School of Engineering, Westlake University, Hangzhou 310064, China

Abstract

Wastewater treatment plants (WWTPs) are implicated as hotspots for the dissemination of antibacterial resistance into the environment. However, the *in situ* processes governing removal, persistence, and evolution of resistance genes during wastewater treatment remain poorly understood. Here, we used quantitative metagenomic and metatranscriptomic approaches to achieve a broad-spectrum view of the flow and expression of genes related to antibacterial resistance to over 20 classes of antibiotics, 65 biocides, and 22 metals. All compartments of 12 WWTPs share persistent resistance genes with detectable transcriptional activities that were comparatively higher in the secondary effluent, where mobility genes also show higher relative abundance and expression ratios. The richness and abundance of resistance genes vary greatly across metagenomes from different treatment compartments, and their relative and absolute abundances correlate with bacterial community composition and biomass concentration. No strong drivers of resistome composition could be identified among the chemical stressors analyzed, although the sub-inhibitory concentration (hundreds of ng/L) of macrolide antibiotics in wastewater correlates with macrolide and vancomycin resistance genes. Contig-based analysis shows considerable co-localization between resistance and mobility genes and implies a history of substantial horizontal resistance transfer involving human bacterial pathogens. Based on these findings, we propose future inclusion of mobility incidence (%) and host pathogenicity of antibiotic resistance genes in their quantitative health risk ranking models with an ultimate goal to assess the biological significance of wastewater resistomes with regard to disease control in humans or domestic livestock.

Keywords: Antimicrobial Resistance; Wastewater Treatment; Quantitative Metatranscriptomics; Quantitative Metagenomics; Gene Expression, Gene Mobility

Introduction

Anthropogenic release of antibiotic resistance genes (ARGs) into environmental reservoirs has raised global public health concerns (Allen et al 2010, Berendonk et al 2015). The importance of wastewater treatment plants (WWTP) both as a barrier for resistant bacteria and as a potential hotspot for dissemination has been highlighted, although the evaluation of the risks for human health remains unresolved (Vikesland et al 2017, Bürgmann et al 2018). The increasing environmental occurrence of clinically relevant ARGs and evidence for horizontal dissemination of resistance between environmental bacteria and human pathogens demonstrate the importance of environmental resistomes (collections of resistance genes in a metagenome) (Allen et al 2010, Forsberg et al 2012, Szczepanowski et al 2009, Zurfluh et al 2013). Communal WWTPs receive diverse anthropogenic antimicrobial and microbiological contaminants including antibiotics (Michael et al 2013), biocides (Bollmann et al 2014), metals (Novo et al 2013), and human pathogens (Ju et al 2016). Metagenomic or qPCR analysis of genomic or plasmid DNA highlight the (co-)occurrence and prevalence of diverse ARGs and metal resistance genes (MRGs) in WWTPs (Czekalski et al 2014, Di Cesare et al 2016, Li et al 2015a, Li et al 2015b, Sentchilo et al 2013, Yang et al 2013), which are implicated as point sources for their release into the environment (Czekalski et al 2014, Munir et al 2011). Moreover, several PCR-based and cultivation-based studies have detected vancomycin-resistant enterococci, methicillin resistant staphylococci, and cefazolin-resistant *Enterobacteriaceae* in wastewater biofilm, as well as clinically relevant ARGs (e.g., *CTX-M*, *amp*C, *qnr* and *NDM-1*) in the final effluent (Luo et al 2013, Schwartz et al 2003, Szczepanowski et al 2009).

However, which mechanisms allow resistance genes to traverse WWTPs and how they are influenced by secondary treatment remain open questions (Fig. 1).

Environmental contaminants including metals and biocides represent widespread and recalcitrant stressors in the WWTP environment that might exert selective pressure that potentially contribute to the persistance and enrichment of antibiotic resistance determinants through selection or co-selection (Baker-Austin et al 2006, Li et al 2017, Pal et al 2014, Pal et al 2015). Although co-selection is well demonstrated at the levels of species and population (Baker-Austin et al 2006), whether the sub-inhibitory wastewater antibacterial residues may lead to trackable community resistance selection remains unclear. So far, no data is available on the extent to which resistome genes are expressed in WWTPs. Studying resistance gene expression could give important hints, which, if any, of these functions are active, and whether the activity changes across compartments or in response to environmental stressors. Importantly, determining the extent to which resistance determinants (i.e., bacteria, genes and transcripts) are selected for and horizontal gene transfer is facilitated by environmental conditions within WWTPs would inform policy decisions in risk assessment and resistance surveillance for preventing dissemination of antibacterial resistance to the environment.

In this study, we used meta-omics approaches benchmarked with mRNA internal standards and qPCR analysis to build quantitative inventories of resistome genes, specifically ARG, biocide resistance gene (BRG), and metal resistance genes (MRG) in 12 communal WWTPs, providing a highly resolved view of the flow of resistance genes and their transcription in this system (Fig. 1). In the context of this manuscript, a resistome is thus understood as the collection of these resistance genes in the metagenome of a sample. By comparing the abundance and transcription levels of resistance genes across treatment compartments, we determined the factors that best predict the composition and transcription of the resistome among a wide range of biotic and abiotic (i.e., physicochemical and operational) variables. Through gene assembly and co-localization analysis, we obtained reliable ARG identification and additional information on co-located genes to predict ARGs mobility incidence (M%) and phylogenetic distribution. The results we obtained reveal how the conventional treatment process strongly influences resistance genes and their transcriptional activities within wastewater treatment stages. Our insights provide useful guidance to the risk assessment and control strategy of WWTP discharge of resistance determinants.

Materials and Methods

A full version of the Materials and Methods are available in the Supplementary Information (SI).

Biomass and Liquid Collection

For DNA and mRNA analysis, biomass was collected from post primary clarifier influent, denitrifying and nitrifying bioreactors, and secondary clarifier effluent of 12 Swiss WWTPs between March and April 2016 (Table 1), as described in the SI. Filtered liquid samples were collected for in-lab chemical analysis (Fig. S2).

mRNA Internal Standards

mRNA internal standards were spiked immediately after cell lysis in known copy numbers to determine volume-based or biomass-based absolute copy numbers for transcript type (i.e., copies/L^{-1} or copies/g of biomass measured as volatile suspended solids). This approach circumvents the limitations of non-spiked metatranscriptomic datasets, which only provide relative abundance information (Gifford et al 2011, Satinsky et al 2012, Satinsky et al 2014). Two mRNA standards without poly(A) tails (to mimic prokaryotic and organelle mRNAs), BMS5 and BMS6, were synthesized by plasmid linearization and in vitro transcription based on a method modified from Satinsky et al., 2012 (Satinsky et al 2012), as described in the SI.

RNA Processing for Metatranscriptomes

Total RNAs were extracted from tube pellets and filters using the RNeasy Mini Kit (Qiagen, Germany) after cell lysis in a FastPrep instrument (MP Biomedicals) for 40 seconds (at the speed of 6.0 m/s) and the spiking of mRNA internal standards into the cell lysate, as described in SI. Then, the residual DNA were digested by two successive treatments with the TURBO DNA-free Kit Kit (Invitrogen, Carlsbad, CA) and mRNA was enriched from the digested total RNA samples using illumina Ribo-zero rRNA Removal Kit. cDNA libraries were generated using the rRNA-depleted RNA by NEBNext® Ultra RNA Library Prep Kit (NEB, USA) following manufacturer's instructions.

DNA Processing for Metagenomes

Genomic DNA was extracted using the FastDNA® SPIN Kit for Soil (MP Biomedicals, France), following manufacturer's instructions. The DNA extracts were then split and used for construction of metagenomic libraries, 16S rRNA gene amplification, and quantitative polymerase chain reaction (q-PCR), as described below. Metagenomic libraries were generated from 1μg DNA per sample using NEBNext® Ultra™ DNA Library Prep Kit for Illumina (NEB, USA), following manufacturer's recommendations.

16S rRNA Gene Amplification and qPCR

The V3-V4 hypervariable regions of bacterial 16S rRNA genes were amplified using genomic DNA and the forward primer 338F and reverse primer 802R (Klindworth et al 2012). Bacterial 16S rRNA gene, class 1 integron integrase gene (*intI*1), and sulfonamide resistance gene *Sul1* were quantified with qPCR using LightCycler® 480 Probes Master (Roche, Basel, Switzerland) and Roche LightCycler® 480 II. Details on the primer sets and PCR conditions used were available in the SI.

Sequencing

The constructed DNA and cDNA libraries were sequenced on the Illumina's Hiseq4000 platform using a paired-end (2 x 150) sequencing strategy at the NOVOGENE (Beijing). The 16S rRNA gene amplicons were sequenced on the Illumina's Miseq platform using a paired-end (2 x 250) sequencing strategy at the Microsynth (Switzerland).

Analytical Chemistry

Dissolved antibacterial pharmaceuticals in the samples were measured by liquid chromatography triple quad mass spectrometry with electrospray ionization. Dissolved metals were measured by high-resolution inductively coupled plasma mass spectrometry. Different forms of dissolved inorganic nitrogen and phosphate were measured using SKALAR SAN^{++} Continuous Flow Analyzer (Skalar, Breda, Netherlands). Dissolved total organic carbon was measured on a TOC-L TOC Analyzer (Shimadzu).

Bioinformatics and Statistics

The bioinformatics and statistical analysis of metagenomes, metatranscriptomes, and 16S rRNA gene amplicon data are described in detail in the SI (Fig. S2). Identification of antibiotic, biocide and metal resistance genes was based on similarity search against a concatenated protein database of The NCBI Reference Sequence Database (RefSeq release 78) (Pruitt et al 2007), The Comprehensive Antibiotic Resistance Database (CARD v1.0.1) (McArthur et al 2013), Antibiotic Resistance Genes Database (ARDB v1.1) (Liu and Pop 2009), Antibacterial Biocide and Metal Resistance Genes Database (BacMet v1.1) (Pal et al 2014) and functionally validated ARGs (Cheng et al 2012, Forsberg et al 2014, Sommer et al 2009), followed by cross validation using hmmscan search against Resfams (v1.2) (Gibson et al 2015), keyword match, and manual inspection.

Results

Gene Inventories of WWTPs

Gene inventories of microbiomes were built from influent, bioreactor and effluent metagenomes of 12 communal WWTPs (Table 1). Bioinformatics analysis of 47 metagenomes (16.6 to 22.3 million reads each) allowed us to identify 9,151,591 non-redundant open reading frames (ORFs) with contig N50 length of 1.82 kb (Dataset S1). Based on protein sequence-based homolog search coupled with string match and manual inspection (see methods), we predicted 16,554 ORFs as antibiotic resistance genes and 7,465 ORFs as biocide and/or metal resistant genes from all samples (Fig. 2a). These are carried on a total of 40,971 resistance contigs with N50 length of 18.8 kb (Dataset S2). From all resistance contigs, 7,687 ORFs co-located with resistance genes were identified as mobility indicators (iMGE) by string match of their annotations using keywords, such as transposase, plasmid, and integrase (Forsberg et al 2014).

The resistance genes were further assigned to 109 resistance 'Types' by the antibacterial agents to which they were predicted to confer resistance to (Dataset S3). The most frequent ARG types were multidrug, aminoglycoside, beta-lactam, macrolide, teicoplanin, and tetracycline (Fig. 2b), representing three classic resistance mechanisms: antibiotic efflux mainly by Resistance-Nodulation-Cell Division (RND)-type, ATP-binding cassette (ABC)-type, Major facilitator superfamily (MFS)-type multidrug efflux pumps, antibiotic inactivation (e.g., beta-lactamase), and modification of antibiotic targets.

Resistance Genes Shared by Wastewater Treatment Compartments

How many resistance genes traverse WWTPs and whether they are differentially expressed remains largely unknown, although answers to these questions are critical to address the roles of dispersal and local enrichment of antibacterial resistance within WWTPs. The use of a cross-sample mapping strategy enabled us to quantify numerous resistance genes that were present in a sample, but not successfully

assembled from its individual metagenome (Fig. S3). Based on the mapping results, a number of quantitative metagenomic and metatranscriptomic metrics were computed and used to measure the relative and absolute abundance of microbial genes and transcripts (Table S1).

Overall, we found that while each compartment harbored unique sets of ARGs (Fig. 3a) and ARG transcripts (Fig. 3b), all compartments shared 7.4(±4.1)% of ARGs and 2.6(±0.9)% ARG transcripts. This small core gene subset of the resistome (i.e., core resistome) was quite abundant (Fig. 3a). Similar results were found for the BRGs and MRGs (Table S2), as well as their gene transcripts (Table S3), revealing wastewater-driven dispersal of certain abundant and transcribed resistance genes or selective outgrowth of the bacteria carrying such genes throughout the WWTPs. Remarkably, 10.7±(2.7)% of ARGs (Fig. 3a), 9.4±(2.2)% of BRGs, and 10.5±(2.6)% of MRGs undetectable in the influent samples became subdominant in the downstream compartments (Table S2), implicating their selective enrichment within each compartment. In contrast, 70.8% of the non-redundant ARGs detected in the influent samples were no longer represented in the effluent samples.

Cross-Compartmental Differences in Resistance Gene transcription

We used quantitative meta-omic approaches to absolutely quantify gene abundance and transcription throughout communal WWTPs (Table S1). We demonstrated high reproducibility in transcript abundances in three metatranscriptomes spiked with mRNA internal standards (R^2>0.99, Fig. S4). Using the sulfonamide resistance gene *sul1* as an example, we also found strong correlations between gene abundance derived from our quantitative metagenomic approach and the qPCR method (Fig. S5). To account for the significant change in the microbial biomass concentration (Table S4), bacterial 16S rRNA gene copies (Fig. S6a) and gene concentration (Fig. S6b) across WWTP compartments ($P < 0.001$), transcript copies were scaled to biomass concentration (transcript copies per gram-of-biomas) and to gene copies of the same gene / gene type (transcript copies per gene copy) to explore differential patterns of resistance gene transcription across samples.

The absolute and relative transcript abundance metrics of the WWTP resistomes were significantly ($P < 0.05$) different across treatment compartments (Fig. 3e-f), consistent with the significant cross-compartmental variations in the relative (Fig. 3c) and absolute (Fig. S7) abundances of resistance genes. Relative to the influent and effluent, the nitrifying and denitrifying bioreactor sludge had significantly higher per-liter transcript copies of antibiotic, biocide and metal resistance genes (Fig. 3d). The strong correlations of all resistance gene categories with biomass metrics (Spearman's $r_s > 0.75$, see the network in Fig. S8) support the expectation that bacterial biomass is the main driver on the variations in the total concentration of both resistance genes and transcripts throughout the WWTPs. In contrast, the effluent and influent had significantly higher transcript copies per gram-of-biomass (Fig. 3e) and transcript copies per gene copy (i.e., expression ratio, Fig. 3f) of resistance genes, compared with the bioreactor sludge. Notably, we observed significantly higher relative abundance for both class 1 integron-integrase gene (Fig. S6a) and resistance genes (Fig. 3c) in the effluent than in the influent. These results together suggest that conventional secondary WWTPs release bacterial populations in which resistance genes and/or class 1 integrons are significantly enriched and that express these genes.

We further checked which types of antibacterial resistance genes were up-regulated and enriched in the effluent relative to the influent. Based on the relative change in the transcripts per gram-of-biomass of the most abundant resistance types for antibiotics, biocides and metals (Fig. 4a), we found that the

transcription of most resistance types increased significantly (*P<0.05) from the influent to effluent (see red bars, Fig. 4a). This pattern was most pronounced for resistance types including four antibiotic classes (tetracycline, trimethoprim, bleomycin, and polymyxin), three biocides (e.g., hydrogen peroxide), and one metal (iron). Likewise, most resistance types showed higher average transcript copies per gene copy in the effluent than the influent (Fig. 4b), suggesting that transcription of these resistance genes could be upregulated in at least a subset of all WWTPs examined. However, the lack of significant differences in the averages of transcript copies per gene copy ($P\geq$ 0.05) indicates that the increase in transcripts per gram-of-biomass largely originates with increases in the relative abundance of resistant bacteria. Indeed, the significant increase in relative abundance of most types of resistance genes, as measured by gene copies per copy of 16S rRNA gene (GP16S, Fig. 4b), agrees with the significant increase of antibiotic, biocide and metal resistance gene copies per gram-of-biomass (Fig. S7b). These results remarkably suggest substantial relative enrichment of a broad set of antibacterial resistance genes after conventional secondary wastewater treatment.

Mobility Incidence and Biotic and Abiotic Drivers of WWTP resistomes

Co-localization or co-occurrence analysis between resistance genes and mobility indicators has been used to assess resistance mobility with regard to the potential for horizontal dissemination (Forsberg et al 2012, Forsberg et al 2014, Li et al 2017, Pal et al 2015). To quantify mobility potential of resistance genes, we define "mobility incidence" (M%) as the percentage of resistance gene encoding contigs flanked with at least one co-occurring mobility indicator (iMGE) in all resistance contigs. Using resistance contigs assembled from all metagenomes, antibiotic, biocide and metal resistance genes scored a mobility incidence of 8.6%, 11% and 20%, respectively. We then classified all resistance genes by their mobility incidence. This innovative method enables the identification of resistance types, subtypes or genes that tend to be more mobilized than others in any environmental resistome. We found that in the WWTPs examined 'highly mobilized' (>95% mobility) antibiotic resistance types included sulfonamide and mercury resistance, whereas 'poorly mobilized' (<5% mobility) ones included polymyxin and nitroimidazole resistance (Table S5). At the subtype level (Table S6), we found 21 highly-mobilized subtypes encoding resistance functions to carbapenems (e.g., *OXA-58* and *OXA-181*), oxacillin (e.g., *OXA-10*), macrolides (*ermB* and *mel*), sulfonamide (*sul1*, *sul2* and *sul3*), trimethoprim (*dfrB3*), copper (*ctpG*), mercury (e.g., *merE* and *merT*), silver (*silP*), and etc.

We further compared the relative strength of biotic factors (i.e., mobility elements, biodiversity, and biomass) and abiotic factors (i.e., antimicrobials, wastewater indexes, and operational parameters) in explaining the compositional variances of WWTP resistomes (Dataset S5). Redundancy analysis showed that the variances of both resistome gene and transcript compositions in the influent and effluent were best explained exclusively by biotic variables representing genetic mobility, including *intI*, resolvase and conjugative transfer protein, suggesting that changes happen primarily in the mobilized resistome. Bacterial alpha-diversity metrics including Shannon's H and Simpson's E (Table 2) also explained part of the variances, indicating the importance of community composition. In contrast, in the nitrifying and denitrifying bioreactors (Table S7), smaller but significant parts of resistome compositional variances were explained by three nitrogen metrics, three operational parameters, two metals (i.e., cadmium and nickel), and seven pharmaceuticals (e.g., levofloxacin, trimethoprim, and sulfamethoxazole). We also identified significant positive correlations (P<0.05, Table S8) between the concentration (ng/L) of measured antibiotics (i.e., macrolides, sulfonamides, lincosamide, trimethoprim and vancomycin) and the

concentration of certain ARGs (170 instances, e.g., Fig. S9a-c) or ARG transcripts (43 instances, e.g., Fig. S10a-c). The majority of these correlations were found between an antibiotic class and ARGs (Fig. S9d-h) or ARG transcripts (Fig. S10d-f) conferring resistance to a different antibiotic class, i.e. correlations that could theoretically be derived from gene co-selection or co-expression.

Interconnected WWTP Resistomes and Microbiomes

Bacterial phylogeny structures soil resistomes (Forsberg et al. 2014). To test if this was the case in our dataset, we used ordination to follow structural variations in the resistomes (Fig. 5 and and S10) and microbiomes (Fig. S12) both between and within treatment compartments. The samples consistently clustered into three main groups by treatment compartment with bioreactor samples closely clustered together, whether the analysis was based on abundance metrics of antibiotic, biocide, and metal resistance genes (Fig. 5a-c and Fig. S11a-c) or transcripts (Fig. S11d-l). Consistent with the resistomes, the microbiomes also clustered by treatment compartment, whether a dissimilarity metric of bacterial abundance (Bray-Curtis), phylogeny (unweighted UniFrac), or both (weighted UniFrac) was used (Fig. S12). The ordinations for both resistomes and microbiomes typically showed higher within-cluster variances for the effluent samples, whereas within-cluster variances were typically smaller for the influent samples, reflecting a role of wastewater treatment in the divergence of the microbial community structure.

The structural correlations between resistome and microbiome were computed and visualized based on procrustes analyses (Fig. 5d-f). When all the treatment compartments were considered, Bray-Curtis distances calculated from abundance metrics of ARGs (d), BRGs (e) or MRGs (f) significantly ($P < 0.001$) correlated with both bacterial OTUs ($r = 0.81$-0.97, Fig. 5d-f) and taxa (i.e., at the genus, family, order, class, and phylum levels, Table S9) inferred from 16S rRNA sequence data, whether a dissimilarity metric of abundance (Bray-Curtis), phylogeny (unweighted UniFrac), or both (weighted UniFrac) was used. Likewise, Bray-Curtis distances calculated from transcript abundance metrics of all three categories of resistance genes also significantly correlated ($P < 0.001$, $r = 0.56$-0.83) with both the bacterial abundance and phylogenetic structure (Table S9). On the other hand, resistome composition within treatment compartments also significantly ($P < 0.05$) correlated with abundance and/or phylogeny-based bacterial community structure (Table S10). If horizontal gene transfer occurs at very high frequencies, we might expect increasingly weaker correlations between resistome and phylogenetic structure from inflow to effluent, but this was not observed. Combined, the resistome composition correlates with both the phylogenetic (UniFrac) and taxonomic (Bray-Curtis) distance metrics of community structure across and within treatment compartments, revealing a close relationship between resistome composition and bacterial phylogeny.

Discussion

The power of metagenomics and bioinformatics have been demonstrated in exploring diversity of environmental ARGs (Forsberg et al 2012, Li et al 2015b, Pehrsson et al 2016, Yang et al 2013, Zhu et al 2013). However, the absolute quantification of a broad-spectrum of ARGs and their transcripts remains challenging. We demonstrated the integration of metaomic approaches with mRNA internal standards and qPCR data of marker genes (e.g., 16S rRNA gene) as a powerful methodology to realize both absolute and relative quantification of a broad spectrum of microbial community genes and transcripts within a complex microbial ecosystem like WWTPs. Using these techniques, we provide extensive information on the fate and expression of the WWTP resistome genes, and influential biotic and abiotic factors.

Fate and Expression of Antibacterial Resistance Genes Our data confirm previous findings that conventional WWTPs remove the majority of bacterial cells and with it resistance genes. Previous studies have presented contradicting evidence regarding the removal versus enrichment of ARGs in WWTPs (Bengtsson-Palme et al 2016, Di Cesare et al 2016, Karkman et al 2016, Mao et al 2015, Szczepanowski et al 2009, Yang et al 2014). General conclusions remain difficult because of the discrepancies in the types of ARGs reported, abundance metrics used (i.e., relative or absolute), and/or normalization methods implemented (e.g., against 16S rRNA gene or biomass). Our data strongly supports the notion that WWTP are sites for the relative enrichment of antibacterial resistance genes and class 1 integrons, as we found a surprisingly consistent increase in the relative abundance of most resistance genes and the class 1 integron-integrase gene *IntI1*. While the relative enrichment of ARGs is also noticed in WWTPs elsewhere (Bengtsson-Palme et al 2016, Di Cesare et al 2016, Mao et al 2015), the release of class 1 integrons from wastewater systems deserves further research on their potential clinical relevance and environmental risks in the receiving environment (Gillings et al 2015).

Further, quantitative metatranscriptomics suggests that resistance genes are differentially expressed across the WWTP compartments, which provides credence to the idea that the resistance activity is influenced by environmental conditions during wastewater treatment. The constantly fluctuating physico-chemical composition of influent wastewater and rapidly changing redox conditions from one treatment compartment to the next can expose microorganisms within WWTPs to rapidly varying stress. The expression of resistance genes could thus be tied to a general stress response that is not directly linked to the presence of suspected specific stressors such as measured antibiotics or metals. The impact of such specific agents is therefore discussed in detail below. The redox contrast between denitrification and nitrification compartments at least did not result in an overall differential expression of resistance genes (Fig. 3d-f). We have further demonstrated that (i) the core resistome genes are persistent, abundant and transcribed in all the WWTP compartments and (ii) resistance genes and mobility indicators are more transcriptionally active in the secondary effluent than in activated sludge bioreactors. These findings indicate that some resistance genes and resistant bacteria are highly recalcitrant to conventional secondary treatment processes and that these facilities release abundant actively transcribed resistance genes together with mobile genetic elements into the receiving environment. It should be noted, that the expression ratios of ARGs in WWTPs we detected are far lower than one transcript per gene copy. While these values are comparable to those reported with the same methodological approach for biogeochemically relevant genes in the Amazon River Plume (Satinsky et al 2014), such values lie far below what is typically observed in organism-based studies. Further research will be needed to understand these seemingly low transcriptional activities.

Biotic and Abiotic Drivers of the WWTP Resistomes The relative roles of biotic and abiotic factors in shaping environmental resistome and facilitating resistance selection are poorly understood (Baker-Austin et al 2006, Berendonk et al 2015). We demonstrate that biotic factors including mobility elements (*intI1*, conjugal transfer protein, and resolvase) and biomass play an important role in shaping compositional variations of the influent and effluent resistomes. Class 1 integrons are central players in resistance dissemination (Gillings et al 2008, Gillings et al 2015), whose activation upon conjugative plasmid transfer allows host bacteria to rapidly develop antibiotic resistance (Baharoglu et al 2010, SLMB 2012). Plasmid mediated antibiotic and metal resistance has been reported in wastewater (Li et al 2015a, Schlüter et al 2007, Sentchilo et al 2013, Szczepanowski et al 2009). The proportions of ARGs (5.4%) and MRGs

(8.1%) in total plasmid-borne genes we identified are comparable to the levels in two other Swiss WWTPs (ARG: ~ 2.5% and 4.0%; MRG: ~ 4.5% and 12.5%) (Sentchilo et al 2013). The strong explanatory power of mobility indicators thus shows the importance of mobilized resistance in the wastewater, and supports the use of e.g. *intI1* as a general indicator of resistance (Gillings et al. 2015, Berendonk et al. 2015). However, in the activated sludge abiotic factors (i.e., inorganic nitrogen, pH, dissolved oxygen, and several antimicrobials) appear to play an additional role in shaping resistomes (Table S7). In this compartment nutrients and oxygen are substantially consumed by activated sludge biomass and may thus act as driving forces for both community and resistome composition.

Positive Correlations between Antibiotics and Resistance Positive correlations were found between certain antibiotics in wastewater and "their" resistance genes and resistance gene transcripts, as well as with resistance genes conferring resistance to a different antibiotic class. On the one hand, such positive relationships, for example those between wastewater concentrations of macrolide antibiotics, clarithromycin (150-450 ng/L) and azithromycin (50-250 ng/L) and the concentration of macrolide resistance gene *macB* (Fig. S9a-c), could be the consequence of enrichment of the resistance genes in the population based on selective pressures exerted by the antibiotic. Considering the demonstrated selection of resistant strains at very low and subinhibitory antibiotic concentration (Gullberg et al 2011) this is a reasonable expectation. However, further antibiotic susceptibility tests on wastewater isolates or experimental validation with wastewater communities are required to validate this correlation-based speculation. On the other hand, the demonstrated positive correlations between wastewater antibiotics and resistance genes or transcripts of another antibiotic class could reflect co-selection of multi-resistance on the same genetic elements, i.e. co-resistance (Baker-Austin et al 2006). The most striking examples are the strong positive correlations found between macrolide antibiotics and both vancomycin resistance genes (Fig. S9d-e) and their transcripts (Fig. S10d-e). The co-localization or adjacency of vancomycin and macrolide resistance genes on the same genomic fragments (12 instances, Dataset S2), as well as the strong positive correlations between their absolute copies in all the four WWTP compartments (R^2=0.77-0.93, Fig. S9f-i), are strong evidence for their co-selection, which also makes the induced expression of the vancomycin resistance genes by clarithromycin plausible (Fig. S10d-e). Another intriguing example is the strong positive relation found between the concentrations of sulfamethoxazole and transcripts of trimethoprim resistance gene *dfrB3* (R^2= 0.94, Fig. S10f). A general practice of combined use of trimethoprim and sulfamethoxazole in clinical settings may facilitate their co-selection. However, the observed correlations could also be inherited from selective processes in human gut bacteria of patients under treatment rather than within the WWTPs.

The above findings highlight the multi-dimensionality and complexity of environmental (co-)selection of antibiotic resistance and thus explain the inability of previous studies to assign or relate certain antibiotics to the occurrence and/or abundance of their respective resistance genes (Graham et al 2010, Looft et al 2012, Novo et al 2013, Oberlé et al 2012, Pehrsson et al 2016). In particular for the WWTP environment, one may argue that metal contaminants may also co-select for ARGs and MRGs, thus decoupling simple 'antibiotic-ARG' relationships (Baker-Austin et al 2006). However, our contig data do not support that such co-selection is common in the WWTPs, considering a very low incidence (0.6%) of an ARG and an MRG encoded on the same resistance contigs (Dataset S2). This lack of co-occurrence scenarios between MRGs and ARGs agrees with their rare co-occurrence on plasmids from natural environments (<0.7%) (Pal et al 2015). However our resistance contig-based analysis (N50 length of 18.8

kb) likely underestimates the co-selection potential if these genes are distanced on different genomic islands or multi-resistance plasmids (Baker-Austin et al 2006).

Gene Mobility Potentials of the WWTPs Resistomes The average mobility incidences (M%) of ARGs found in our WWTPs influent (10%), activated sludge (7.1%-7.8%), and effluent (9.8%) resistomes were comparable to those found in the human gut resistomes (14% of 161 contigs), where horizontal gene transfer (HGT) is implicated in facilitating resistance acquisition by human pathogens (Sommer et al 2009). In contrast, lower mobility incidence of ARGs have been reported for soils (0.8% of 4,655 contigs (Forsberg et al 2012)), where HGT is suggested to play a limited role in resistance dissemination. Remarkably, we find that of the 17,486 resistance genes shared by bacteria, the majority (93.5%) are encoded on multiple resistance contigs (2 to 46) with considerably diverging flanking regions (Dataset S6). This novel finding of a large-scale distribution of identical resistance genes on divergent contigs derived from DNA samples from different WWTPs/compartments strongly implicates a history of substantial exchange of antibacterial resistance genes.

Moreover, three further lines of evidence suggested HGT may play a more important role in the secondary clarifiers than previously appreciated: (i) an important role of *IntI1*, resolvase and conjugal transfer protein in structuring resistomes in the low-biomass clarified effluent rather than the thick activated sludge (Fig. S13), (ii) the higher per-gram-of-biomass and per gene transcriptional activities of resistance and mobility-related genes (Fig. 3e and 3f), as well as higher relative abundance of *IntI1* (Fig. S6a) and ARGs (Fig. 3c) in the secondary effluent than activated sludge, and (iii) high incidences of integrases (31%) and conjugal transfer proteins (35%) co-located with plasmid proteins on the same resistance contigs (Dataset S2). Based on these findings, we hypothesize that contrary to our original expectations, secondary clarifier suspended bacteria, which are mostly planktonic, are exposed to higher overall stress from contaminants (e.g., per-gram-of-biomass antibiotic/metal loadings). These results in both, stronger selection and more active transcription, of resistance-related genes compared with bacterial cells harbored within the protective activated sludge flocs. In flocs antibacterial resistance or detoxification can be achieved through extracellular inactivation (e.g., beta-lactam and aminoglycoside), exopolysaccharide binding (e.g., some metals and chemical toxins) and/or biodegradation or biotransformation (e.g., biodegradable pharmaceuticals).

Despite the evidence for gene mobilization within the resistomes of WWTP effluent, we have demonstrated that the resistome composition overall correlates tightly with the bacterial community phylogenetic and taxonomic composition (Fig. 5), suggesting that the changes to the species composition resulting from the wastewater treatment process strongly determine the effluent resistomes. While this finding agrees with a close connection found between antibiotic resistome and bacterial phylogeny in soils and human guts (Forsberg et al 2014, Pehrsson et al 2016), it may also reflect the existence of certain phylogenetic constraints for the horizontal dissemination of antibiotic resistance between bacterial populations.

Implications for Risk assessment and Management of Resistomes. Our data strengthens the case for using *intI1* gene abundance and concentration as a general indicator of anthropogenic impacts (Gillings et al 2015), as we could demonstrate their predictive power for WWTP resistomes and relative enrichment after wastewater treatment, in accordance with the significant increase in relative abundance of resistance genes. Using mobility incidence (M%), we are able to predict and compare the transferable potentials of resistance genes at the levels of resistance type, subtype and ecosystem, which is an important aspect for

risk ranking in resistomes (Martínez et al 2015). While the keyword-based approach used in the assessment of M% is likely to have shortcomings – for example we only test for co-localization of an appropriately annotated genetic element but do not confirm its function or if it actually confers mobility to the ARG - it proved a useful tool and provided believable rankings. Typical examples of 100% mobilized ARGs we identified from WWTP effluent include well-known acquired resistance genes such as *CTX-M*, *OXA*, and *TEM* family extended-spectrum beta-lactamases (ESBL) and *OXA* family carbapenemases. Our approach could be further improved, for example, by using a verified reference database of mobility indicators instead of keywords. Besides gene mobility, risk ranking in resistome should also consider the host pathogenicity and clinical importance of ARGs with regard to disease control in humans and/or domestic livestock (Martínez et al 2015). We demonstrate that contig-based analysis of metagenomes can again provide a basis for such assessments: For instance, we found 11 non-redundant ARGs representing a total of 138 ORFs with 100% identity to reference sequences from known clinical isolates of human pathogens (Table 3). The high occurrence frequency of these 'pathogenic' ARGs (11/12) and their transcripts (9/12) in the effluent of examined WWTPs (e.g., *sul1*, *ermB*, *ANT3*, and *cmlA*) suggests that further investigation of their fate and health risk in the receiving environment is warranted.

Additional preventative or control measures of antibiotic resistance determinants in WWTP effluents may currently not be a priority, unless direct health risks for humans are verified. Nonetheless, our data underscores that the absolute amounts of resistance bacteria and genes discharged by WWTPs into the environment is heavily dependent on the bacterial biomass remaining in the final effluent. Thus, any measure that substantially reduces bacterial biomass in the discharged effluent, such as an increase of sludge settleability in the secondary clarifiers (Novo and Manaia 2010) or membrane filtration would reduce WWTP discharge of resistance genes. In agreement with this idea, membrane bioreactors are implicated to show much higher absolute removal efficiency of some antibiotic resistance genes and bacteria than conventional WWTPs (Munir et al 2011).

Acknowledgments

This research was funded by the Eawag Discretionary Funds project (grant number: 5221.00492.009.06 DF15 / GeneFlow). We appreciate the help of M. A. Moran and her team with quantitative transcriptomics. We appreciate the help of P. Kathriner, D. Kistler, A. Minder, N. Zemp, and J.C. Walser, and thank the Genetic Diversity Centre (GDC) at the ETH Zürich for providing computational resources and support. F. Ju and H. Bürgmann received financial support from the NFP72 project 407240_167116 "Swiss River Resistome". We acknowledge valuable support by PIRE program 1545756 "Halting Environmental Antimicrobial Resistance Dissemination (HEARD)".

Author Contributions

H.B., F.J., C.M., and D.J. designed research. F.J. and K.B. performed sample collection and molecular experiments. F.J analyzed all the sequence data. T.Z. and X.L Y. assisted with the classification of antibiotic resistance genes. A.M., C.M. and S.H. conducted analytical chemistry of pharmaceuticals. F.J. and H.B. wrote the manuscript. All authors contributed to critical discussion and revisions of the manuscript.

Conflict of Interests

The authors declare no conflict of interests.

Data Availability

The sequences reported in this paper have been deposited in the Metagenomics Analysis Server (MG-RAST) with project ids of mgp83169, mgp19765, mgp19780, mgp19899, mgp19773, mgp19991, and mgp21278 (see Dataset S1 and S4 for all sample ids).

References

Allen HK, Donato J, Wang HH, Cloud-Hansen KA, Davies J, Handelsman J (2010). Call of the wild: antibiotic resistance genes in natural environments. *Nat Rev Microbiol* **8:** 251-259.

Baharoglu Z, Bikard D, Mazel D (2010). Conjugative DNA transfer induces the bacterial SOS response and promotes antibiotic resistance development through integron activation. *PLoS Genet* **6:** e1001165.

Baker-Austin C, Wright MS, Stepanauskas R, McArthur J (2006). Co-selection of antibiotic and metal resistance. *Trends Microbiol* **14:** 176-182.

Bengtsson-Palme J, Hammarén R, Pal C, Östman M, Björlenius B, Flach C-F *et al* (2016). Elucidating selection processes for antibiotic resistance in sewage treatment plants using metagenomics. *Sci Total Environ* **572:** 697-712.

Berendonk TU, Manaia CM, Merlin C, Fatta-Kassinos D, Cytryn E, Walsh F *et al* (2015). Tackling antibiotic resistance: the environmental framework. *Nat Rev Microbiol*.

Bollmann UE, Tang C, Eriksson E, Jönsson K, Vollertsen J, Bester K (2014). Biocides in urban wastewater treatment plant influent at dry and wet weather: concentrations, mass flows and possible sources. *Water Res* **60:** 64-74.

Cheng G, Hu Y, Yin Y, Yang X, Xiang C, Wang B *et al* (2012). Functional screening of antibiotic resistance genes from human gut microbiota reveals a novel gene fusion. *FEMS Microbiol Lett* **336:** 11-16.

Czekalski N, Díez EG, Bürgmann H (2014). Wastewater as a point source of antibiotic-resistance genes in the sediment of a freshwater lake. *The ISME journal* **8:** 1381-1390.

Di Cesare A, Eckert EM, D'Urso S, Bertoni R, Gillan DC, Wattiez R *et al* (2016). Co-occurrence

of integrase 1, antibiotic and heavy metal resistance genes in municipal wastewater treatment plants. *Water Res* **94:** 208-214.

Forsberg KJ, Reyes A, Wang B, Selleck EM, Sommer MO, Dantas G (2012). The shared antibiotic resistome of soil bacteria and human pathogens. *Science* **337:** 1107-1111.

Forsberg KJ, Patel S, Gibson MK, Lauber CL, Knight R, Fierer N *et al* (2014). Bacterial phylogeny structures soil resistomes across habitats. *Nature* **509:** 612.

Gibson MK, Forsberg KJ, Dantas G (2015). Improved annotation of antibiotic resistance determinants reveals microbial resistomes cluster by ecology. *The ISME journal* **9:** 207-216.

Gifford SM, Sharma S, Rinta-Kanto JM, Moran MA (2011). Quantitative analysis of a deeply sequenced marine microbial metatranscriptome. *The ISME journal* **5:** 461-472.

Gillings M, Boucher Y, Labbate M, Holmes A, Krishnan S, Holley M *et al* (2008). The evolution of class 1 integrons and the rise of antibiotic resistance. *J Bacteriol* **190:** 5095-5100.

Gillings MR, Gaze WH, Pruden A, Smalla K, Tiedje JM, Zhu Y-G (2015). Using the class 1 integron-integrase gene as a proxy for anthropogenic pollution. *The ISME journal* **9:** 1269-1279.

Graham DW, Olivares-Rieumont S, Knapp CW, Lima L, Werner D, Bowen E (2010). Antibiotic resistance gene abundances associated with waste discharges to the Almendares River near Havana, Cuba. *Environ Sci Technol* **45:** 418-424.

Gullberg E, Cao S, Berg OG, Ilbäck C, Sandegren L, Hughes D *et al* (2011). Selection of resistant bacteria at very low antibiotic concentrations. *PLoS Pathog* **7:** e1002158.

Ju F, Li B, Ma L, Wang Y, Huang D, Zhang T (2016). Antibiotic resistance genes and human bacterial pathogens: co-occurrence, removal, and enrichment in municipal sewage sludge digesters. *Water Res* **91:** 1-10.

Karkman A, Johnson TA, Lyra C, Stedtfeld RD, Tamminen M, Tiedje JM *et al* (2016). High-throughput quantification of antibiotic resistance genes from an urban wastewater treatment plant. *FEMS Microbiol Ecol* **92.**

Klindworth A, Pruesse E, Schweer T, Peplies J, Quast C, Horn M *et al* (2012). Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Res*: gks808.

Li A-D, Li L-G, Zhang T (2015a). Exploring antibiotic resistance genes and metal resistance genes in plasmid metagenomes from wastewater treatment plants. *Frontiers in microbiology* **6**.

Li B, Yang Y, Ma L, Ju F, Guo F, Tiedje JM *et al* (2015b). Metagenomic and network analysis reveal wide distribution and co-occurrence of environmental antibiotic resistance genes. *The ISME journal* **9**: 2490-2502.

Li L-G, Xia Y, Zhang T (2017). Co-occurrence of antibiotic and metal resistance genes revealed in complete genome collection. *The ISME journal* **11**: 651.

Liu B, Pop M (2009). ARDB—antibiotic resistance genes database. *Nucleic Acids Res* **37**: D443-D447.

Looft T, Johnson TA, Allen HK, Bayles DO, Alt DP, Stedtfeld RD *et al* (2012). In-feed antibiotic effects on the swine intestinal microbiome. *Proceedings of the National Academy of Sciences* **109**: 1691-1696.

Luo Y, Yang F, Mathieu J, Mao D, Wang Q, Alvarez P (2013). Proliferation of multidrug-resistant New Delhi metallo-β-lactamase genes in municipal wastewater treatment plants in northern China. *Environmental Science & Technology Letters* **1**: 26-30.

Mao D, Yu S, Rysz M, Luo Y, Yang F, Li F *et al* (2015). Prevalence and proliferation of antibiotic resistance genes in two municipal wastewater treatment plants. *Water Res* **85**: 458-466.

Martínez JL, Coque TM, Baquero F (2015). What is a resistance gene? Ranking risk in resistomes. *Nat Rev Microbiol* **13**: 116.

McArthur AG, Waglechner N, Nizam F, Yan A, Azad MA, Baylay AJ *et al* (2013). The comprehensive antibiotic resistance database. *Antimicrob Agents Chemother* **57**: 3348-3357.

Michael I, Rizzo L, McArdell C, Manaia C, Merlin C, Schwartz T *et al* (2013). Urban wastewater treatment plants as hotspots for the release of antibiotics in the environment: a review. *Water Res* **47**: 957-995.

Munir M, Wong K, Xagoraraki I (2011). Release of antibiotic resistant bacteria and genes in the effluent and biosolids of five wastewater utilities in Michigan. *Water Res* **45**: 681-693.

Novo A, Manaia CM (2010). Factors influencing antibiotic resistance burden in municipal wastewater treatment plants. *Appl Microbiol Biotechnol* **87:** 1157-1166.

Novo A, André S, Viana P, Nunes OC, Manaia CM (2013). Antibiotic resistance, antimicrobial residues and bacterial community composition in urban wastewater. *Water Res* **47:** 1875-1887.

Oberlé K, Capdeville M-J, Berthe T, Budzinski Hln, Petit F (2012). Evidence for a complex relationship between antibiotics and antibiotic-resistant Escherichia coli: from medical center patients to a receiving environment. *Environ Sci Technol* **46:** 1859-1868.

Pal C, Bengtsson-Palme J, Rensing C, Kristiansson E, Larsson DJ (2014). BacMet: antibacterial biocide and metal resistance genes database. *Nucleic Acids Res* **42:** D737-D743.

Pal C, Bengtsson-Palme J, Kristiansson E, Larsson DJ (2015). Co-occurrence of resistance genes to antibiotics, biocides and metals reveals novel insights into their co-selection potential. *BMC Genomics* **16:** 964.

Pehrsson EC, Tsukayama P, Patel S, Mejía-Bautista M, Sosa-Soto G, Navarrete KM *et al* (2016). Interconnected microbiomes and resistomes in low-income human habitats. *Nature* **533:** 212-216.

Pruitt KD, Tatusova T, Maglott DR (2007). NCBI reference sequences (RefSeq): a curated non-redundant sequence database of genomes, transcripts and proteins. *Nucleic Acids Res* **35:** D61-D65.

Satinsky BM, Gifford SM, Crump BC, Moran MA (2012). Use of internal standards for quantitative metatranscriptome and metagenome analysis. *Methods Enzymol* **531:** 237-250.

Satinsky BM, Crump BC, Smith CB, Sharma S, Zielinski BL, Doherty M *et al* (2014). Microspatial gene expression patterns in the Amazon River Plume. *Proceedings of the National Academy of Sciences* **111:** 11085-11090.

Schlüter A, Szczepanowski R, Pühler A, Top EM (2007). Genomics of IncP-1 antibiotic resistance plasmids isolated from wastewater treatment plants provides evidence for a widely accessible drug resistance gene pool. *FEMS Microbiol Rev* **31:** 449-477.

Schwartz T, Kohnen W, Jansen B, Obst U (2003). Detection of antibiotic-resistant bacteria and their resistance genes in wastewater, surface water, and drinking water biofilms. *FEMS Microbiol Ecol* **43:** 325-335.

Sentchilo V, Mayer AP, Guy L, Miyazaki R, Tringe SG, Barry K *et al* (2013). Community-wide plasmid gene mobilization and selection. *The ISME journal* **7:** 1173.

SLMB (2012). Method 333.1: determining the total cell count and ratios of high and low nucleic acid content cells in freshwater using flow cytometry. *Schweizerisches Leb*.

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

Szczepanowski R, Linke B, Krahn I, Gartemann K-H, Gützkow T, Eichler W *et al* (2009). Detection of 140 clinically relevant antibiotic-resistance genes in the plasmid metagenome of wastewater treatment plant bacteria showing reduced susceptibility to selected antibiotics. *Microbiology* **155:** 2306-2319.

Yang Y, Li B, Ju F, Zhang T (2013). Exploring variation of antibiotic resistance genes in activated sludge over a four-year period through a metagenomic approach. *Environ Sci Technol* **47:** 10197-10205.

Yang Y, Li B, Zou S, Fang HH, Zhang T (2014). Fate of antibiotic resistance genes in sewage treatment plant revealed by metagenomic approach. *Water Res* **62:** 97-106.

Zhu Y-G, Johnson TA, Su J-Q, Qiao M, Guo G-X, Stedtfeld RD *et al* (2013). Diverse and abundant antibiotic resistance genes in Chinese swine farms. *Proceedings of the National Academy of Sciences* **110:** 3435-3440.

Zurfluh K, Hächler H, Nüesch-Inderbinen M, Stephan R (2013). Characteristics of extended-spectrum β-lactamase-and carbapenemase-producing Enterobacteriaceae isolates from rivers and lakes in Switzerland. *Appl Environ Microbiol* **79:** 3021-3026.

Figure Legends

Figure 1. Key hypotheses about processes affecting the resistome (resistance gene content of the microbial metagenome) during passage of a WWTP. The WWTP consists (a) of compartments with contrasting environmental conditions including (b) changing concentrations of antibiotics, metals, and other stressors that may act as drivers on microbial community assembly and resistomes. By design (activated sludge process), and as an effect of the changing habitat conditions, we expect (c) changes in biomass per volume (piechart area) but also persistence or even enrichment of ARG-carrying bacteria (red wedge). Likewise, (d) we expect a strong shift in the composition of the microbial community as a whole, and the antibiotic resistant subset (colored, ARB). These changes are expected to correlate to changes of the resistome (e) which are here shown as metagenomic contigs (bars colored by bacterium of origin) carrying different ARGs (colored arrows). ARB and ARGs discharged with the effluent may have different origins: Some may have passed through the entire WWTP if the bacteria survive treatment (here e.g. the red bacterium), others may originate from populations of bacteria that grow in the WWTP (blue & brown bacteria). If the environmental conditions in the WWTP favor populations that carry ARGs, these ARGs may become enriched in the bacterial community of the effluent. Studying ARG transcription and changes of transcription across stages (indicated by different shades of the red bacterium) may provide clues if genes that are enriched are also active. A contig-centered analysis further allows identification of marker genes for mobile genetic elements (blue squares) occurring on the same contig as an ARG. (f) Horizontal gene transfer may act on evolutionary timescales, thus that e.g. resistance plasmids arriving with human pathogens or commensals in the inflow eventually become established also in WWTP bacteria. If horizontal transfer of ARGs would happen with such high frequency that it amounts to a mass flow on timescales relevant to the flow of biomass, shifts in the population size of the original host bacteria may no longer correlate with ARG abundance, and the resistome structure could shift independently of the phylogenetic community structure.

Figure 2. Antibiotic (ARG), biocide (BRG), and metal (MRG) resistance genes predicted from influent, bioreactors, and effluent metagenomes of 12 communal wastewater treatment plants (WWTPs). a, Percent of non-redundant open reading frames (ORFs) predicted as resistance genes (left Y-axis) and number of resistance contigs (right Y-axis) for each WWTP (Table 1). b, Number of ORFs assigned to major mechanisms for antibiotic resistance.

Figure 3. Cross-compartmental variation of the richness and abundance of genes, transcripts and mobility indicators of the WWTP resistomes. Four compartments: influent (red); denitrification (green); nitrification (cyan); effluent (purple). a-b, shared and unique percent richness (relative abundance) of ARGs (a) and ARG transcripts (b). Overall, 7.4% of ARGs and 2.6% of ARG transcripts detected in all compartments account for 26.1% of the sum for relative abundance of all ARGs (a) and 42.7% of the sum for relative abundance of all ARG transcripts (b), revealing the persistence of certain abundant resistance genes that are transcribed throughout WWTPs. c-e, gene copies per 16S rRNA gene (c), transcripts per liter (d), transcripts per gram-of-biomass (e), and transcripts per gene (f). Boxes denote the interquartile range between the 25th and 75th percentiles, respectively, the line and white diamond inside namely denote the median and average value, black dots denote outliers and asterisks indicate

685 significant different mean values (adjusted *P*: ***<0.001<**<0.01<*<0.05), compared with influent,
686 which is checked by permutational Student's t-test with 10000 simulations (n=11). For any downstream
687 compartment with significantly different means (*) with influent, there is also a significant difference
688 (*P*<0.05) between their medians (checked by Mann-Whitney U test).

689

690 **Figure 4. The relative change of transcript and gene abundance of antibiotic, biocide, and metal**
691 **resistance genes from post-primary clarifier influent to secondary effluent.** Relative change is
692 defined as the difference between effluent and influent values divided by the maximum value, thus
693 positive (negative) values indicate increase (decrease) after wastewater treatment. a, top X axis: relative
694 change (bars) in transcript copies per gram-of-biomass (TPB) from influent to effluent; bottom X axis:
695 gene copies per liter of effluent (grey circles). b, relative change in transcript copies per gene copy (TPG)
696 and gene copies per 16S rRNA gene (GP16S). The significance of mean difference in each metric
697 between influent and effluent is tested by permutational Student's t-test with 10,000 simulations (*P*:
698 ***<0.001<**<0.01<*<0.05, n = 11). The data suggests massive increases in the expression ratio, per-
699 gram-biomass transcript copies, and relative abundance of most antibacterial resistance types (see red bars
700 and cells). TPP: tetraphenylphosphonium.

701 **Figure 5. Resistome composition correlates with bacterial community composition and phylogeny**
702 **across wastewater treatment compartments**. a-c, Non-metric multidimensional scaling plots depict
703 Bray-Curtis distances between treatment compartments based on relative abundance of antibiotic (a),
704 biocide (b) and metal (c) resistance genes in the metagenomes. d-f, Procrustes analyses depict significant
705 ($P < 0.001$) and strong ($r > 0.85$) correlations between bacterial community composition (Bray-Curtis, red
706 circles) and content of antibiotic (d), biocide (e) and metal (f) resistance genes (Bray-Curtis, blue circles),
707 respectively. OTU, operational taxonomic unit. IDs were labeled for samples outside compartment-
708 defined sample clusters (Dataset S1).

a) WWTP compartments are contrasting habitats

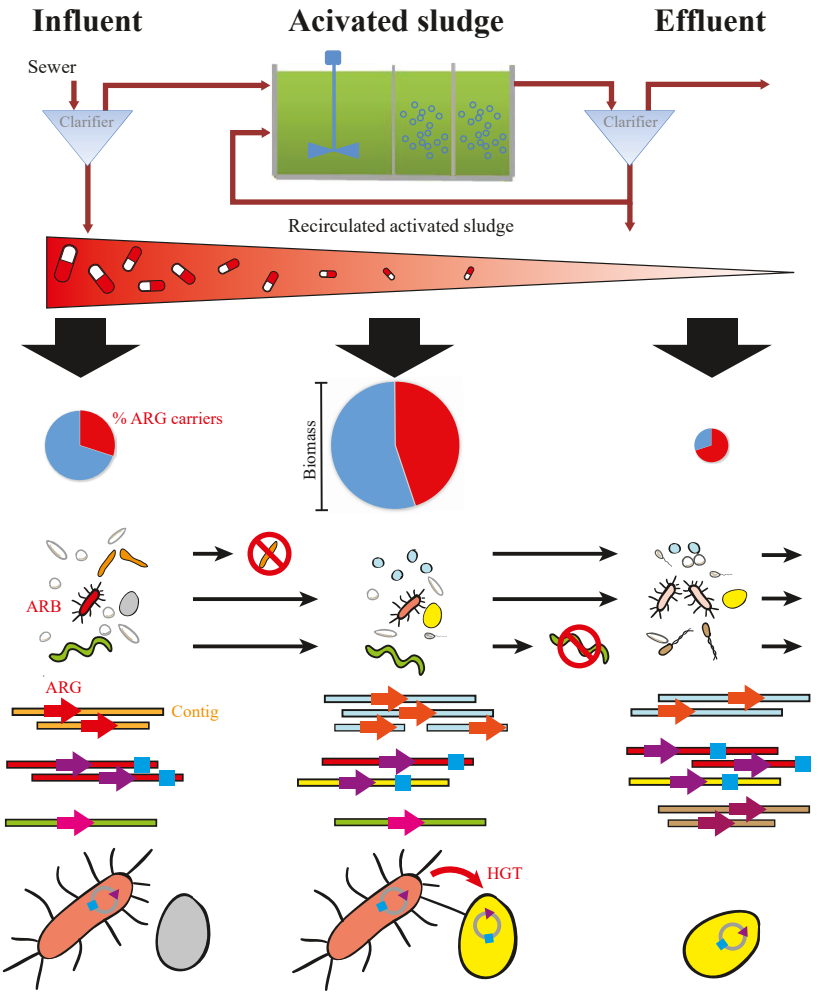
b) Changing stressor concentrations

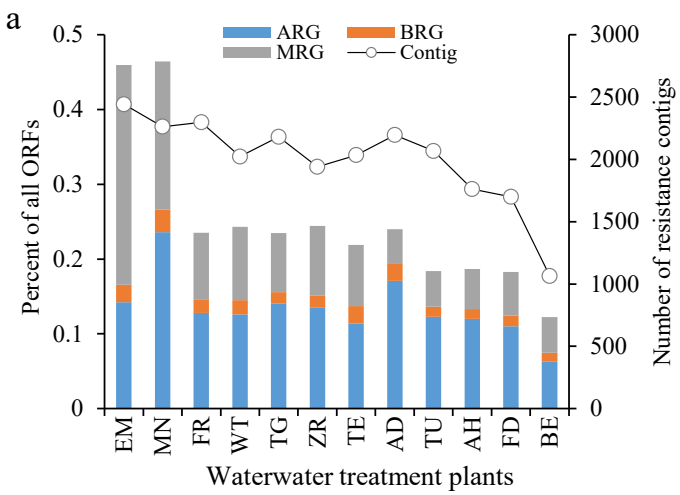
c) Biomass change and enrichment of ARG

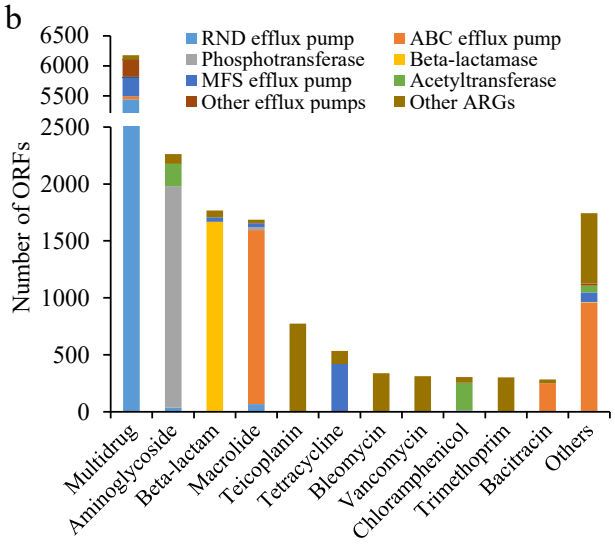
d) Community shift

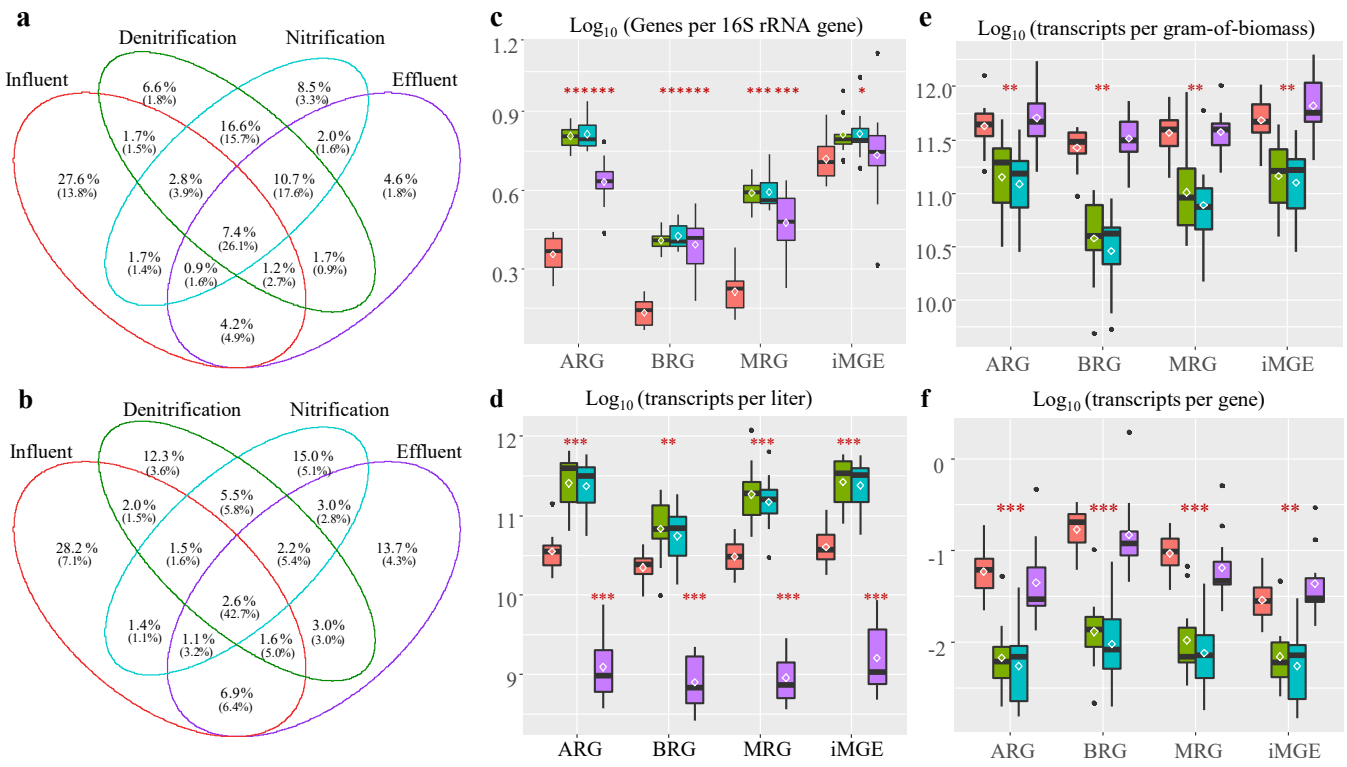
e) Resistome shift

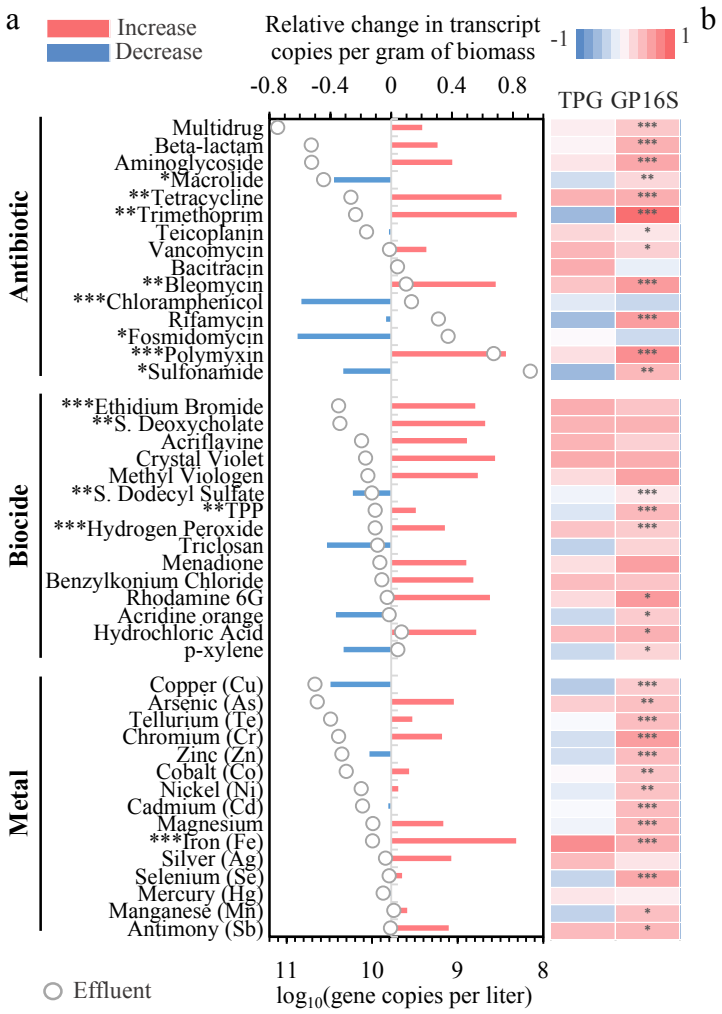
f) Horizontal gene transfer




a
ARG
BRG
MRG
Contig
Percent of all ORFs
Number of resistance contigs
0.5
0.4
0.3
0.2
0.1
0
3000
2500
2000
1500
1000
500
0
EM
MN
FR
WT
TG
ZR
TE
AD
TU
AH
FD
BE
Waterwater treatment plants


b
RND efflux pump
ABC efflux pump
Phosphotransferase
Beta-lactamase
MFS efflux pump
Acetyltransferase
Other efflux pumps
Other ARGs
Number of ORFs
6500
6000
5500
2500
2000
1500
1000
500
0
Multidrug
Aminoglycoside
Beta-lactam
Macrolide
Teicoplanin
Tetracycline
Bleomycin
Vancomycin
Chloramphenicol
Trimethoprim
Bacitracin
Others


a
Denitrification
Nitrification
Influent
Effluent
6.6% (1.8%)
8.5% (3.3%)
16.6% (15.7%)
1.7% (1.5%)
2.0% (1.6%)
27.6% (13.8%)
2.8% (3.9%)
10.7% (17.6%)
4.6% (1.8%)
7.4% (26.1%)
1.7% (1.4%)
0.9% (1.6%)
1.2% (2.7%)
1.7% (0.9%)
4.2% (4.9%)
b
Denitrification
Nitrification
Influent
Effluent
12.3% (3.6%)
15.0% (5.1%)
5.5% (5.8%)
2.0% (1.5%)
3.0% (2.8%)
28.2% (7.1%)
1.5% (1.6%)
2.2% (5.4%)
13.7% (4.3%)
2.6% (42.7%)
1.4% (1.1%)
1.1% (3.2%)
1.6% (5.0%)
3.0% (3.0%)
6.9% (6.4%)
c
Log10 (Genes per 16S rRNA gene)
1.2
0.9
0.6
0.3
ARG
BRG
MRG
iMGE
d
Log10 (transcripts per liter)
12
11
10
9
ARG
BRG
MRG
iMGE
e
Log10 (transcripts per gram-of-biomass)
12.0
11.5
11.0
10.5
10.0
ARG
BRG
MRG
iMGE
f
Log10 (transcripts per gene)
0
-1
-2
ARG
BRG
MRG
iMGE


a
Increase
Decrease
Relative change in transcript copies per gram of biomass
-1
1
b
-0.8 -0.4 0 0.4 0.8
TPG GP16S
Antibiotic
Multidrug
Beta-lactam
Aminoglycoside
*Macrolide
**Tetracycline
**Trimethoprim
Teicoplanin
Vancomycin
Bacitracin
**Bleomycin
***Chloramphenicol
Rifamycin
*Fosmidomycin
***Polymyxin
*Sulfonamide
Biocide
***Ethidium Bromide
**S. Deoxycholate
Acriflavine
Crystal Violet
Methyl Viologen
**S. Dodecyl Sulfate
**TPP
***Hydrogen Peroxide
Triclosan
Menadione
Benzylkonium Chloride
Rhodamine 6G
Acridine orange
Hydrochloric Acid
p-xylene
Metal
Copper (Cu)
Arsenic (As)
Tellurium (Te)
Chromium (Cr)
Zinc (Zn)
Cobalt (Co)
Nickel (Ni)
Cadmium (Cd)
Magnesium
***Iron (Fe)
Silver (Ag)
Selenium (Se)
Mercury (Hg)
Manganese (Mn)
Antimony (Sb)
Effluent
11 10 9 8
log10(gene copies per liter)

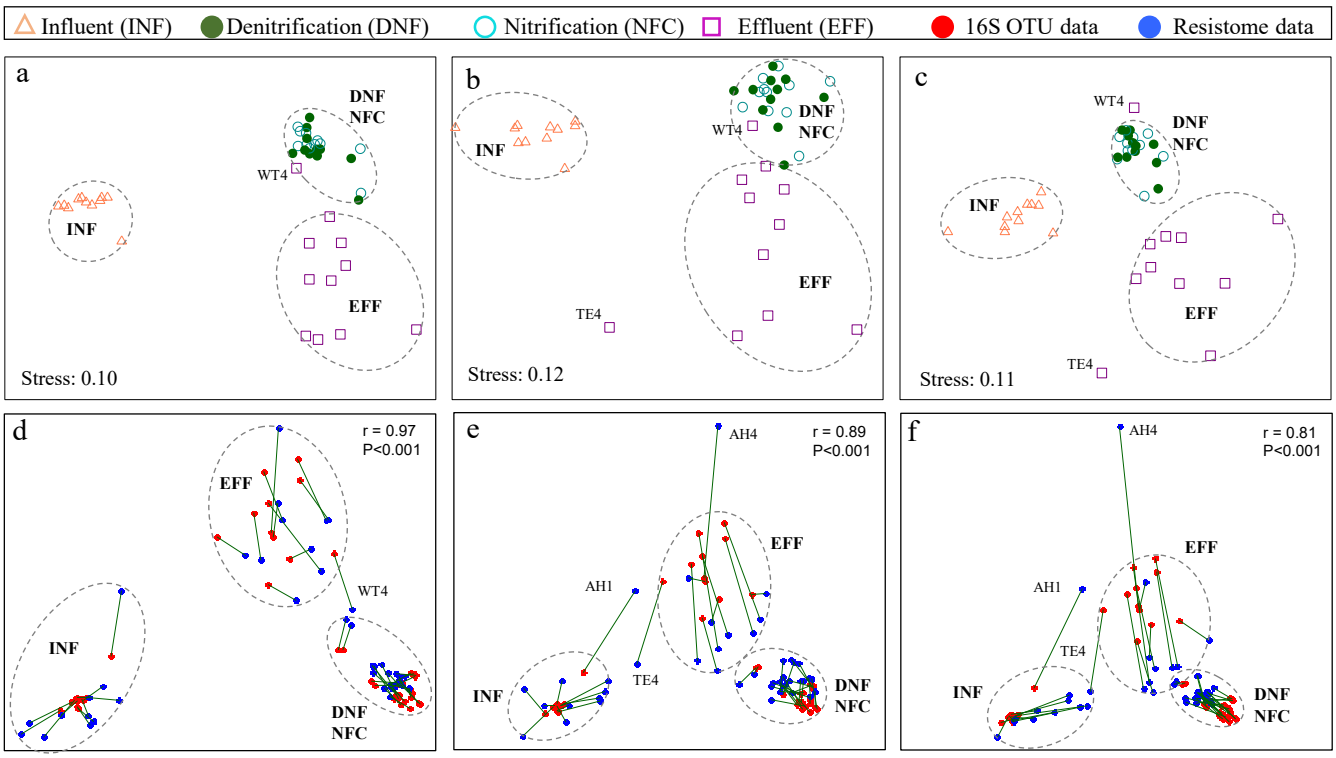

Influent (INF)
Denitrification (DNF)
Nitrification (NFC)
Effluent (EFF)
16S OTU data
Resistome data
a
DNF
NFC
WT4
INF
EFF
Stress: 0.10
b
INF
WT4
DNF
NFC
EFF
TE4
Stress: 0.12
c
WT4
DNF
NFC
INF
EFF
TE4
Stress: 0.11
d
r = 0.97
P<0.001
EFF
WT4
INF
DNF
NFC
e
AH4
r = 0.89
P<0.001
EFF
AH1
INF
TE4
DNF
NFC
f
AH4
r = 0.81
P<0.001
EFF
AH1
TE4
INF
DNF
NFC

Table 1. Treatment capacity, wastewater characteristics and operational parameters of the 12 Swiss wastewater treatment plants sampled.

Plant ID	EM	MN	TG	TE	WT	ZR	FD	AD	TU	FR	AH	BE
Plant overview												
Sampling date	2016-3-17	2016-4-4	2016-4-5	2016-4-13	2016-4-7	2016-4-12	2016-4-15	2016-4-20	2016-4-19	2016-4-28	2016-4-29	2016-4-21
Process design	INT	UPS	UPS & INT	UPS	UPS	UPS & INT	INT	UPS	UPS	UPS	UPS	Fixed-Bed
Flow rate, m^3/d	90000	14000	25000	12000	51840	200000	15250	6000	40000	25264	15000	86000
Population equivalent	210000	50000	85000	55000	130110	534000	50000	20000	132000	110000	50000	345212
Hospital beds[a]	830	0	512	253	670	3248	8	82	530	402	237	2527
Industry inflow %	10%	35%	31%	-	-	-	-	-	<10%	60%	25%	-
Industry input besides hospital wastewater	dairy, metal, etc.	dairy, food, wine, etc.	metal, food, chemical, etc.	abattoir, dairy, etc.	chemical, beer, etc.	abattoir, etc.	cosmetics food, etc.	metal, etc.	food, dairy, abattoir, etc.	dairy, etc.	food, metal, etc.	pharma-ceuticals, etc.
Hydraulic retention time, h	19.2	32.5	17.8	23.0	21.8	21.1	24.9	39.5	22.5	19.1	46.0	>2
Antimicrobials in influent/effluent [b,c]												
Macrolides, ng/L	540/660	820/720	620/410	440/390	680/430	650/520	960/480	210/170	320/190	620/300	460/440	540/390
Fluoroquinolones, ng/L	1200/160	1500/100	1400/180	2200/110	1500/130	1500/140	2200/190	990/120	1500/60	1500/200	960/91	740/390
Sulfonamides, ng/L	1400/160	1200/550	1300/150	1600/140	1600/200	1500/220	760/300	1200/300	1700/100	460/39	670/160	1300/200
Trimethoprim, ng/L	270/140	170/180	210/100	210/95	240/130	200/150	130/100	170/93	210/53	60/35	100/60	170/130
Metronidazole, ng/L	220/93	47/24	200/79	190/37	290/110	230/110	290/120	49/28	140/51	120/68	140/27	240/270
Triclosan, ng/L	660/250	340/93	740/110	290/83	460/61	690/220	630/170	480/110	410/110	680/65	350/57	470/140
Metformin, µg/L	77/1.6	78/0.38	120/0.53	81/1.6	120/0.57	90/1.2	120/2.2	110/2.7	98/0.39	60/7.2	55/4.2	47/7.1
Arsenic (As), ng/L	600/150	945/550	1615/320	900/630	505/240	800/405	885/470	865/320	680/180	1065/160	1570/585	450/415
Cadmium (Cd), ng/L	145/30	75/25	25/45	60/30	35/30	60/25	125/60	120/90	45/25	50/25	25/30	25/160
Nickel (Ni), µg/L	1.0/2.7	3.9/2.6	4.9/16.5	0.2/1.5	0.1/0.8	2.2/0.1	2.4/0.2	7.6/6.4	1.0/0.4	4.0/0.4	2.4/3.8	2.9/2.7
Copper (Cu), µg/L	6.5/4.6	48.6/2.0	13.7/3.1	9.8/1.5	10/2.9	35.2/2.5	13.5/4.1	31.6/10.3	10.3/5.4	9.4/2.5	9.9/8.0	6.2/4.4
Zinc (Zn), µg/L	58.7/49.1	64.2/131.5	44.6/106.0	84.8/139.4	75.3/149.8	48.8/49.2	80.8/18.3	179/377.6	87.2/149.8	1378/135.2	126/124.3	143.8/203.1
Nitrification Bioreactors [c]												
Sludge retention time, d	6.3	19.2	9.1	11.8	15.0	12.8	8.0	14	12.4	10.5	11.2	-
Sludge volume index, mL/g	117	99	195	102	177	224	144	152	266	132	72	-
Dissolved oxygen, mg/mL	2.20	1.78	3.31	1.90	1.25	2.47	2.08	2.65	1.05	2.04	1.70	-
pH	6.52	6.57	7.00	6.62	6.00	6.25	6.44	6.09	6.84	7.16	6.74	-
Temperature, °C	14.4	15.8	16.7	14.1	14.5	16.7	14.6	12.7	12.2	14.9	13.3	-
VSS, mg/L	2103	2374	2956	1996	1810	2114	1359	2128	2121	1052	1956	320

INT intermittent denitrification, *UPS* upstream denitrification, *VSS* volatile suspended solids

[a] Number of beds in general hospitals, in rehabilitation hospitals and psychological clinics within the plant catchment (Kuroda et al. 2016)

[b] Concentrations of antibiotic are rounded to 2 significant digits

[c] See a full list of the wastewater and operational parameters in Dataset S5

Table 2. Redundancy analysis showing percent variation in the wastewater resistome composition explained by biotic and abiotic variables. *IntI1*, class 1 integron-integrase gene; 16S, 16S ribosomal RNA gene; CTP, conjugal transfer protein-coding gene; VSS, volatile suspended solids. Only variables and values with significant constraints in the RDA tests ($P < 0.05$, 1,000 permutation) are shown, and a full list of the tested variables are available in Dataset S5.

	Gene composition						**Transcript composition**					
	Influent			Effluent			Influent			Effluent		
	ARG	BRG	MRG	ARG	BRG	MRG	ARG	BRG	MRG	ARG	BRG	MRG
Biotic variables												
IntI1/16S	34.5	28.7	27.9	27.8	22.5	32.1	24.9	18.8	18.8	33.2	16.7	18.9
Resolvase/16S	35.7	31.1	30.1	26.3	20.4	32.5	28.5	20.8	21.2	39.1	14.8	18.9
CTP/16S	34.5	29.4	28.9	28.6	24.8	35.7	25.6	20.1	19.2	42.1	17.6	21.1
Shannon's *H*				29.4	25.7	33.6				40.3	17.1	20.6
Simpson's *E*				14.2	14.1	16.1				30.8		10.4
Abiotic variables												
VSS (mg/L)	13.3	9.8	14.1	7.9	13.7	11.8		16.3		16.9		10.4
Nitrate nitrogen (mg/L)				10.8								
Total nitrogen (mg/L)				12.3								
pH	24.7	19.4										
ciprofloxacin (ng/L)			14.5						15.9			
triclosan (ng/L)								10.3				

Table 3. Non-redundant antibiotic resistance genes with 100% identity to known human bacterial pathogens. The last four columns show the number of WWTPs in which the resistance gene and its transcripts are detected in the influent or effluent compartment.

Gene ID	Length (aa)	Resistance		Number of sequence	Example of pathogen (NCBI taxon ID)	Gene		Transcript	
		Type	Subtype			Influent	Effluent	Influent	Effluent
W56_28340_1[&*#]	260	Aminoglycoside	*ANT3*	3	*A. baumannii* (509173)	12	5	10	8
W54_36555_1[&*#]	265	Aminoglycoside	*APH(3')*	9	*S. epidermidis* (176279)	12	3	7	2
W54_1320_4[&#]	144	Aminoglycoside	*sat-1*	8	*B. vulgatus* (435590)	12	5	2	0
W56_17638_3[&*#]	278	Aminoglycoside	*strB*	7	*K. pneumoniae* (272620)	12	8	8	6
W54_14042_1[&#]	281	Beta-lactam	*OXA-58*	5	*A. baumannii* (405416)	11	4	7	1
W56_739_2[&]	425	Beta-lactam	*ampG*	6	*B. vulgatus* (435590)	12	7	0	0
W70_15043_2[&*#]	420	Chloramphenicol	*cmlA*	5	*K. pneumoniae* (272620)	12	2	9	5
W60_5396_4[&#]	250	Macrolide	*ermB*	15	*E. faecalis* (226185)	12	11	12	12
W54_264_15[&]	377	Multidrug	*mexE*	12	*B. vulgatus* (435590)	12	12	1	0
W71_4945_1[&*#]	309	Sulfonamide	*sul1*	47	*S. enterica* (423368)	12	12	12	12
W56_1220_2	658	Tetracycline	*tetQ*	19	*B. fragilis* (295405)	12	12	9	4
W72_76188_2[&#]	104	Trimethoprim	*dfrA14*	2	*B. hermsii* (314723)	9	0	2	1

[&] found in human bacterial pathogens, but its resistance contigs showing < 95% global nucleotide identity (at least 5% divergence) to the pathogen sequences. [#] co-located with indicators of mobile genetic elements on the same resistance contig; * found on both genomes and plasmids of at least one pathogens