Elucidating selection processes for antibiotic resistance in sewage treatment plants using metagenomics

Johan Bengtsson-Palme, Rickard Hammarén, Chandan Pal, Marcus Östman, Berndt Björlenius, Carl-Fredrik Flach, Jerker Fick, Erik Kristiansson, Mats Tysklind, D.G. Joakim Larsson

Department of Infectious Diseases, Institute of Biomedicine, The Sahlgrenska Academy, University of Gothenburg, Guldhedsgatan 10, SE-413 46 Gothenburg, Sweden
Centre for Antibiotic Resistance Research (CARe) at University of Gothenburg, Gothenburg, Sweden
Department of Chemistry, Umeå University, Umeå, Sweden
Division of Industrial Biotechnology, School of Biotechnology, Royal Institute of Technology, Stockholm, Sweden
Department of Mathematical Sciences, Chalmers University of Technology, Gothenburg, Sweden

ABSTRACT

Sewage treatment plants (STPs) have repeatedly been suggested as “hotspots” for the emergence and dissemination of antibiotic-resistant bacteria. A critical question still unanswered is if selection pressures within STPs, caused by residual antibiotics or other co-selective agents, are sufficient to specifically promote resistance. To address this, we employed shotgun metagenomic sequencing of samples from different steps of the treatment process in three Swedish STPs. In parallel, concentrations of selected antibiotics, biocides and metals were analyzed.

We found that concentrations of tetracycline and ciprofloxacin in the influent were above predicted concentrations for resistance selection, however, there was no consistent enrichment of resistance genes to any particular class of antibiotics in the STPs, neither for biocide and metal resistance genes. The most substantial change of the bacterial communities compared to human feces occurred already in the sewage pipes, manifested by a strong shift from obligate to facultative anaerobes. Through the treatment process, resistance genes against antibiotics, biocides and metals were not reduced to the same extent as fecal bacteria. The OXA-48 gene was consistently enriched in surplus and digested sludge. We find this worrying as OXA-48, still rare in Swedish clinical isolates, provides resistance to carbapenems, one of our most critically important classes of antibiotics. Taken together, metagenomics analyses did not provide clear support for specific antibiotic resistance selection. However, stronger selective forces affecting gross taxonomic composition, and with that resistance gene abundances, limit...
1. Introduction

The development of antibiotic resistant bacteria has rapidly become a global health concern, accounting for hundreds of thousands of deaths yearly (Review on Antimicrobial Resistance, 2014). Although overuse and misuse of antibiotics for humans and animals are undoubtedly major drivers behind the development of resistance in human pathogens, there is increasing recognition of the involvement of other factors and settings in resistance emergence and dissemination (Ashbolt et al., 2013; Finley et al., 2013), necessitating a “one health” approach to resistance development. Sewage treatment plants (STPs) have been proposed as hotspots for resistance development (Berendonk et al., 2015; Berglund et al., 2015; Laht et al., 2014; Rizzo et al., 2013; Yang et al., 2014) as well as point sources for dissemination of resistance genes and resistant bacteria (LaPara et al., 2011; Pruden, 2014). To facilitate emergence of novel resistance determinants, a selection pressure from antibiotics and/or other antibacterial agents is likely critical, as it constitutes a prerequisite for fixation of mutations or gene transfer events in bacterial populations. In addition, antibiotics have the potential to increase the likelihood for such events, as they have been shown to – at sufficient concentrations – increase the rates of mutation (Chow et al., 2015; Morero et al., 2011), induce transposon activity (Barraud and Ploy, 2015; Hocquet et al., 2012), recombination (López and Blázquez, 2009), and mobilization of DNA (Johnson et al., 2015; Jutkina et al., 2016; López and Blázquez, 2009; Prudhomme et al., 2006). In addition, antibiotic selection may increase the number of donors of resistance factors, if these have a selective advantage over susceptible bacteria, in turn increasing transfer opportunities. It is known that antibiotics are present in STPs at varying concentrations (Lindberg et al., 2005; Marx et al., 2015; Michael et al., 2013), but knowledge about whether these concentrations are selective is currently lacking (Ågerstrand et al., 2015; Boxall et al., 2012). Although antibiotics concentrations seldom or never reach minimal inhibitory concentrations (MICs) for most bacteria (European Committee on Antimicrobial Susceptibility Testing, 2016; Michael et al., 2013), antibiotics can exert a selection pressure at levels far below the inhibitory concentrations (Gullberg et al., 2011; Lundström et al., 2016). We recently published predicted no-effect concentrations for resistance selection for 111 antibiotics (Bengtsson-Palme and Larsson, 2016) and thus for the first time have a comprehensive reference framework to compare measured concentrations of antibiotics to.

To understand the selection processes for antibiotic resistance in STPs it is fundamental to compare the frequencies of resistance genes and resistant bacteria coming into and leaving the treatment plants. There is conflicting evidence regarding the efficiency of resistance gene removal in STPs. A large diversity of resistance genes can be detected both in activated sludge and treated effluent (Szczepanowski et al., 2009), but most studies have reported efficient reduction of resistance gene abundances in effluent water (Al-Jassim et al., 2015; Auerbach et al., 2007; Yang et al., 2014). In contrast, Mao et al. (2015) reported little effect of sewage treatment on the relative resistance gene abundances in effluent water. The efficiency of resistance gene removal in sludge seems to be highly variable, with large differences of removal rates for different resistance genes in Chinese STPs, and some genes being enriched regardless of comparing to bacterial gene content or sample volumes (Yang et al., 2014; Zhang et al., 2015). However, antibiotic resistance genes and resistant bacteria can increase in relative abundance through the treatment process even in the absence of direct selection by a specific antibacterial substance. Both co- and cross-resistance between different types of antibiotic substances is well-known (Alekshun and Levy, 2007; Nikaido, 2009). In addition, metals and biocides have the potential to co-select for antibiotic resistance genes (Baker-Austin et al., 2006; Pal et al., 2014). The severity of the risk associated with a resistance gene finding is also highly context dependent – if a resistance gene is found on a mobile genetic element, risks for transmission increase substantially (Bengtsson-Palme and Larsson, 2015; Dantas and Sommer, 2012; Martinez et al., 2015). Thus, investigation of the regions surrounding each resistance gene is of high importance, but to gain a comprehensive picture of these regions is not straightforward, as most bacteria in the environment cannot be cultured (Amann et al., 1995) and assembly of metagenomic sequence data is difficult, particularly for resistance regions (Bengtsson-Palme et al., 2014). It is also possible that various biotic and abiotic factors in STPs shape the bacterial communities such that species carrying certain types of resistance genes increase in abundance even in the absence of any selection pressure from antibacterials. Thus, analysis of changes of resistance gene abundances should preferably be done in relation to taxonomic changes.

In this paper we aim to shed light on whether antibiotics exert a direct selection pressure for resistant bacteria in STPs. To this end, we have combined chemical measurements of antibiotics concentrations with shotgun metagenomic DNA sequencing to explore the relative abundances of antibiotic resistance genes throughout the treatment process, and interpret these abundances in relation to estimated minimal selective concentrations and genetic contexts. Furthermore, we want to assess the alternative hypothesis that increases of antibiotic resistance genes could be due to selection by other potentially selective agents than the antibiotic they confer resistance to. Therefore, we have also analyzed the concentrations of metals and biocides and the frequencies of resistance genes towards these compounds, along with changes in taxonomic composition. We find that although STPs greatly reduced the number of resistance genes per volume of water, their relative abundance per bacterial 16S rRNA was only moderately decreased. Worryingly, a few resistance genes, including the carbapenemase gene OXA-48, were enriched in the treatment process, underscoring the importance of considering dissemination in the risk assessment of STPs in relation to antibiotic resistance.

2. Materials and methods

2.1. Sample collection

Water and sludge samples were collected from three different municipal STPs: Henriksdal (Stockholm, Sweden), Kungsångsverket (Uppsala, Sweden) and Käppala (Lidingö, Sweden) (Table S1). The Henriksdal samples were collected on 2012-09-25, and the Uppsala and Käppala samples on 2012-09-26. These STPs serve between 164,000 and 782,000 persons and receive a mix of sewage from municipal, hospital and industrial sources as well as storm water (full details in Table S2). Both those days were moderately rainy, with normal or slightly elevated flow rates through the treatment plants (the latter due to the rain) and no overflow. At Henriksdal, the two water inlets (Henriksdal and Sicklå) were sampled separately, and effluent was collected both before and after the sand filter. In Uppsala, influent was sampled from both inlets, and since there is no sand-filteration of the outgoing water, the effluent was only sampled at one site. At Käppala,
the single inlet was sampled, along with effluent from before and after the sand filter. From all treatment plants, sludge was collected from primary sedimentation (primary sludge), as well as before and after mesophilic anaerobic digestion (activated surplus and digested sludge, respectively). At the Uppsala treatment plant sampling directly from the primary sludge was not possible, so a mixed sample of primary and activated sludge was instead used as the best possible compromise. From Kappala, sludge samples were also collected after dewatering treatment with Kemira KemiCond®. The Kemikond treatment is a strong oxidative Fenton type process, which increases the dry solid concentration of the sludge, oxidizes inorganic and organic substances and facilitates pathogen control. The Kemikond process consists of three steps: 1) acidification to pH 4–4.5 by addition of 94–97% sulphuric acid, 2) oxidation by addition of 50% hydrogen peroxide to the ferrous rich acidic sludge, and 3) addition of flocculants to improve dewatering. All samples were collected without compensation for retention time in the STPs, since bacterial composition of STP communities within the same treatment plant have been shown to be relatively stable over time (Saunders et al., 2015; Valentin-Vargas et al., 2012; Wang et al., 2014).

All sludge samples were taken in four replicates with about 5 g in each tube, and frozen at −20 °C. Water samples were collected over an hour in 1 l batches and mixed into 10 l cans; two cans for each sampling point. About 6–7 l were collected in each can. The water cans were kept at room temperature until brought to the lab, where they were stored at 4 °C. Separate water samples were collected for chemical analysis.

2.2. Chemical measurements

Water samples were analyzed for the presence of selected antibiotics (ciprofloxacin, clarithromycin, clindamycin, doxycycline, erythromycin, florfenicol, gentamicin, nalidixic acid, norfloxacin, ofloxacin, oxytetracycline, rifampicin, roxithromycin, sulfamethoxazole, tetracycline, and trimetoprim) using the method published by Lindberg et al. (2014). Sludge samples were analyzed for the presence of antibiotics and biocides by extraction using a beadbeater in four sequential steps with different solvents. The solvents were combined and evaporated. Samples were then analyzed on LC-MS/MS using different columns (Hypersil Gold AQ 50 × 2.1 mm, 5 μm and Hypersil Gold 50 × 2.1 mm, 3 μm) to cover the various analytical properties. Metal analysis was performed using the EPA 3051 AEC04-HP500 method for microwave assisted acid extraction. The extracts were diluted 20× and analyzed on ICP-MS (Perkin Elmer/Sciex Elan DRC-e) together with certified reference materials. A detailed description of the chemical analysis is available as Supplementary material 1.

2.3. DNA sequencing and pre-processing of sequence data

Water samples were filtered through a 1 mm sieve and centrifuged at 7500 × g (6000 rpm) in 1 l batches until a total volume of three liters had been centrifuged for effluent samples. Influent samples were diluted 200 ml to 800 ml 0.85% NaCl solution before centrifugation in a single batch (1 l of liquid in total). The resulting pellet of each batch was resuspended in 25 ml 0.85% NaCl solution, and subjected to a second round of centrifugation at 4000 × g. DNA was extracted from the pellets using the DNeasy Blood and Tissue kit (Qiagen, Hilding, Germany) including pre-centrifugation at 4000 × g filtered through a 1 mm sieve and centrifuged at 7500 × g (6000 rpm) in 1 l batches until a total volume of three liters had been centrifuged for effluent samples. Influent samples were diluted 200 ml to 800 ml 0.85% NaCl solution before centrifugation in a single batch (1 l of liquid in total). The resulting pellet of each batch was resuspended in 25 ml 0.85% NaCl solution, and subjected to a second round of centrifugation at 4000 × g. DNA was extracted from the pellets using the DNeasy Blood and Tissue kit (Qiagen, Hilding, Germany) including pre-centrifugation at 4000 × g.
count. Integron-associated integrases and ISCR transposases were identified using the Resqu database and Vmatch (see above). Conjugation systems (relaxases and type IV secretion systems) were detected using hidden Markov models (HMMs; Guglielmini et al., 2014; Smillie et al., 2010) matched to the assembled contigs using the HMMER software (hmmssearch version 3.0; Eddy, 2011), options “–E 1e-5 –cpu 16”. Coverage over the conjugation systems was calculated using the FARAO utility estimate_coverage (Hammarén et al., 2016) version 1.0.

The obtained contigs from metagenomic assemblies were annotated using the Resqu and BacMet databases for resistance genes, the NCBI plasmid genome database (Benson et al., 2014), the Pfam protein family database (Finn et al., 2014), and the above-mentioned HMMs representing conjugation systems. For Resqu and BacMet, translated ORFs were predicted using Protigal version 2.60 (default options; Hyatt et al., 2010), and those were searched using HMMER with options “hmmssearch –cpu 8 –noaln –cut_tc” against the Pfam database and the database of conjugation systems (Guglielmini et al., 2014; Smillie et al., 2010). Contigs putatively representing fully sequenced plasmids were identified using the Perna util, part of the PETkit version 1.1.1 (http://microbiology.se/software/petkit). Putative plasmids, defined as having paired-end reads overlapping the ends of the contig and either containing a conjugation system or a strong match to a known plasmid, were manually identified and annotated by BLAST searches against the full NCBI GenBank nucleotide and protein databases. All annotations were collected in a FARAO database (Hammarén et al., 2016) and the FARAO utilities were used to visualize annotated contigs.

2.6. Statistical analysis

SSU rRNA data from each sample corresponding to different species, genera, families, orders, classes and phyla were normalized to the total number of SSU sequences identified in that sample, yielding relative abundances for each taxonomic group. Principal component analysis was carried out in the R statistical program (R Development Core Team, 2011) using log-transformed SSU abundances per million reads in each library. Genera encompassing a species with a sequenced genome in the human microbiome project catalog (Human Microbiome Jumpstart Reference Strains Consortium et al., 2010; Human Microbiome Project Consortium, 2012) were considered human-associated. Significant differences of genus abundance between effluent and influent samples, as well as between primary and digested sludge, were assessed using t-tests assuming unequal variance. The p-values were corrected for multiple testing using the Benjamini-Hochberg false discovery rate (Benjamini and Hochberg, 1995) and tests with an FDR < 0.05 were considered significant.

Significant differences of log-transformed resistance gene and mobile genetic element abundances between treatment steps (incoming vs. treated water, treated vs. sand-filtered, primary vs. surplus sludge, and primary vs. digested sludge) were assessed using t-tests assuming unequal variance. Correction for multiple testing was carried out using the FDR and tests with an FDR < 0.05 were considered significant. Principal component analysis of log-transformed antibiotic resistance gene abundances was carried out using R, adding a set of fecal samples from Swedish students for comparison (only samples taken before travel were used; Bengtsson-Palme et al., 2015a). Richness of resistance genes was measured by counting the number of different gene types in the first 20,000,000 reads of each sample. Analysis of the similarity of resistance gene abundances between sample groups and samples were assessed using metaxa2.uc, part of the Metaxa2 Diversity Tools (Bengtsson-Palme et al., 2016), with the options “-g auto –table T–matrix T–filter 1 –groups”. Networks of resistance genes co-localized on the same contigs were created using Cytoscape version 3.0.2 (Shannon et al., 2003).

3. Results

3.1. Concentrations of antibiotics, metals and antibacterial biocides

We detected ten out of sixteen investigated antibiotics in the influent and seven in the effluent (Table S3). Ciprofloxacin, norfloxacin, ofloxacin, oxytetracycline and tetracycline were all efficiently removed in the treated water, while sulfamethoxazole was only partially removed. Concentrations of clarithromycin, clindamycin, fluconazole and trimethoprim were not substantially affected by the treatment process. Both ciprofloxacin (up to 910 ng/L) and tetracycline (up to 4553 ng/L) were detected at concentrations predicted to be selective (Bengtsson-Palme and Larsson, 2016) in some of the influent samples (Table S3). Notably, the concentrations of ciprofloxacin and ofloxacin were much higher in the sludge samples than in the water samples, indicating strong sorption to particles. Except for tetracycline, the concentrations of all detected antibiotics were below 1 μg/L in all water samples. Similarly, only ciprofloxacin was detected at concentrations higher than 1 mg/kg in the sludge samples.

Copper and zinc are two well-known co-selective agents that have been linked to antibiotic resistance (Seiler and Berendek, 2012). Copper was present in sludge at concentrations between 146 and 599 mg/kg, and zinc at concentrations between 294 and 1300 mg/kg. All other investigated metals had concentrations spanning a vast range between 130 μg/kg (tellurium) and 148 g/kg (iron) (Table S3). The total metal content was five to ten times higher in surplus sludge than in primary sludge, and remained higher also in digested sludge, indicating that metals accumulate in the sludge treatment process. Although this pattern was strongly influenced by the most abundant metals – iron and magnesium – no metal showed decreased concentrations throughout the treatment.

3.2. Sequencing and assembly

Metagenomic DNA sequencing yielded 70 libraries in the size range of 22.8–57.0 million paired-end reads (4.6–11.4 Gbp), resulting in a total of 517.8 Gbp of DNA analyzed (Table S4). The de novo assembly resulted in an average of 2.1 million contigs per sample (544,234–3,754,383), although the sludge samples had substantially larger numbers of contigs than water samples (Table S5). Of these contigs, only 4% were longer than 500 bp (13,272–180,350), thus, despite a large sequencing effort it seems that the assembly only scratched the surface of the total sewage treatment plant communities. This may of course influence the analysis, primarily the estimates of resistance gene richness, as the sequencing depth was not sufficient to completely saturate resistance gene accumulation in the samples (Fig. S2).

3.3. Taxonomic composition

Between 413 and 2032 bacterial SSU rRNA sequences per million reads were extracted from each library (Table S4). Importantly, influent and primary sludge systematically had higher relative SSU abundance than did other samples. Particularly, the sand-filtered water samples contained ~500 bacterial SSU sequences per million reads, suggesting a larger eukaryote or viral fraction than in the other samples. The taxonomic composition was similar within samples from the same treatment step, despite being collected from three different treatment plants (Fig. 1). However, the taxonomic composition of all STP samples was markedly different from that of human feces sampled from Swedish students (Bengtsson-Palme et al., 2015a), already in the influent (Fig. 1B). This effect was largely due to a stark shift from obligate anaerobes in feces (such as Bacteroides (22.6%) and Prevotella (7.4%)) to facultative anaerobes (such as Akinetobacter (7.0%), Streptococcus (6.7%) and...
Arcobacter (5.6%) in the sewage influent (Fig. 1C). The influent water and primary sludge were dominated by Proteobacteria, Firmicutes and Bacteroidetes (Fig. 1A), with bacteria affiliated to the Moraxellaceae, Streptococcaceae and Campylobacteraceae families standing out as typical for these incoming sample types. The combined primary and surplus sludge samples from the Uppsala treatment plant clustered in between
the primary sludge and inlet samples on one hand, and surplus sludge and treated water samples on the other hand (Fig. 1B). Notably, the composition of microbial genera in surplus sludge was similar to that of effluent water, particularly after sand-filtration. In the surplus sludge, bacterial phyla such as Acidobacteria and the nitrite oxidizers Nitrospirae started to appear, while the digested sludge showed a clear community shift, with greatly increased relative abundance of bacteria within the Chloroflexi and Spirochaetes phyla containing many anaerobes, as well as dramatically higher abundances of archaea. In addition, the Proteobacteria were greatly reduced in digested sludge, and the eukaryota were almost completely absent. Instead, large numbers of unclassifiable bacterial SSU sequences were obtained. The taxonomic composition of the Kemikond treated sludge was similar to that of the Kappala digested sludge, which it is derived from, but it should be noted that most of the DNA in the Kemikond treated sludge is expected to come from dead microorganisms due to the chemical treatment.

Specifically investigating human-associated bacteria, as defined by their presence in the HMP database, showed a vast reduction of their relative abundances from incoming water and primary sludge to outgoing samples (Figs. S3a and S4a). This reduction was, however, largely driven by only two genera: Acinetobacter (Fig. S4e) and Streptococcus (Fig. S3d), together constituting 13.6% of the identified bacteria in the inlet samples. It may therefore be more accurate to study the abundance of different bacterial genera individually to identify to what extent they are efficiently removed by the treatment process. Legionella, which contains several species associated with human infections, stood out as not being efficiently removed by the treatment process (Fig. S3f). Instead, it was significantly enriched in the effluent (adjusted p-value 0.004), also after sand-filtration. For the other human-associated bacteria, the

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**Fig. 2.** Abundance of antibiotic resistance genes in different treatment steps. (A) Total resistance gene abundance per 16S rRNA. (B) Total resistance gene abundance per mL sample volume. (C) Resistance gene abundance per 16S rRNA relative to incoming water in treated and sand-filtered water. (D) Resistance gene abundance per 16S rRNA relative to incoming water in sludge. Changes from incoming water with a false discovery rate < 0.05 are denoted with an asterisk. Error bars represent the standard error of the mean.

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enterotype drivers (Arumugam et al., 2011) Bacteroides (Fig. 54b), Prevotella (Fig. 54c), and Ruminococcus (Fig. 54d) were all reduced in the effluent, although Bacteroides and Ruminococcus were enriched in digested sludge compared to surplus sludge. It should be noted, however, that although the relative abundances of bacteria in treated and sand-filtered water increased for Legionella, its abundance per volume in treated and sand-filtered water was actually decreased slightly compared to incoming water.

3.4. Antibiotic resistance genes

The summed relative abundance per 16S rRNA of all antibiotic resistance genes decreased through the treatment process, on average by a factor of 2.7 (Fig. 2A). However, when we considered the sampling volume required to extract the same amount of DNA, the decrease of resistance genes per ml was >50-fold (Fig. 2B), similar to the decrease in bacterial CFU counts (104-fold decrease; Fig. S5). The majority of antibiotic resistance gene classes were reduced per 16S rRNA in the treatment process (Fig. 2C and D), although resistance genes against quaternary ammonium compounds and trimethoprim showed a trend to be enriched in treated and sand-filtered water compared to influent, and resistance genes against sulfonamides were enriched in digested sludge relative to primary and surplus sludge. Furthermore, trimethoprim resistance genes were enriched in surplus sludge, and lincomycin resistance genes were not reduced in digested sludge compared to incoming water. The most abundant resistance genes identified in the influent encoded resistance mechanisms against aminoglycosides, tetracyclines, macrolides, beta-lactams and lincomycin (Fig. S6a). In treated and sand-filtered water, macrolide and lincomycin resistance genes were reduced, while aminoglycoside, tetracycline, sulfonamide and beta-lactam resistance genes were not. In sludge, the most common resistance gene categories were the same, although their abundances differed somewhat from the water samples (Fig. S6b).

A few genes commonly occurred in most samples, such as the aminoglycoside resistance gene ant(3‘)-Ia and aph(3’)-Ib, the sulfonamide resistance gene sul1, and the tetracycline resistance gene tetB(P) (Tables S6 and S7), the latter which was significantly enriched throughout the treatment process (Table 1). In total, four antibiotic resistance genes were found to be significantly enriched in the effluent water compared to the influent, five genes were enriched during the sand-filteration step, and ten genes were enriched in the effluent compared to the influent and sand-filtered water.

### Table 1

<table>
<thead>
<tr>
<th>Resistance/mobility gene</th>
<th>Class</th>
<th>Average abundance per 16S rRNA</th>
<th>Fold change</th>
<th>Adjusted p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Genes enriched in effluent water compared to influent</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>aph(3’)-Ia</td>
<td>Aminoglycosides</td>
<td>3.64E-06</td>
<td>81.2×</td>
<td>0.014</td>
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<td>CTX-M</td>
<td>Beta-lactams</td>
<td>1.02E-04</td>
<td>11.5×</td>
<td>0.021</td>
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<tr>
<td>tetB(P)</td>
<td>Tetracyclines</td>
<td>2.21E-03</td>
<td>2.6×</td>
<td>0.010</td>
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<td>dfrA3</td>
<td>Trimeprithrom</td>
<td>9.30E-04</td>
<td>4.0×</td>
<td>0.015</td>
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<tr>
<td>chrA</td>
<td>Chromium (Cr)</td>
<td>8.44E-01</td>
<td>1.7×</td>
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<td>chrA1</td>
<td>Chromium (Cr)</td>
<td>8.90E-02</td>
<td>1.8×</td>
<td>0.006</td>
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<td>chrC</td>
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<td>3.49E-02</td>
<td>2.5×</td>
<td>0.002</td>
</tr>
<tr>
<td>chrF</td>
<td>Chromium (Cr)</td>
<td>2.48E-01</td>
<td>2.2×</td>
<td>0.009</td>
</tr>
<tr>
<td>cysA/artdE</td>
<td>Copper (Cu), Silver (Ag)</td>
<td>2.95E-01</td>
<td>1.5×</td>
<td>0.027</td>
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<tr>
<td>nia</td>
<td>Iron (Fe), Nickel (Ni)</td>
<td>9.34E-02</td>
<td>1.9×</td>
<td>0.007</td>
</tr>
<tr>
<td>silP</td>
<td>Silver (Ag)</td>
<td>6.14E-01</td>
<td>2.2×</td>
<td>0.002</td>
</tr>
<tr>
<td><strong>Genes enriched after sandfiltration of water</strong></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>aac(3)-I-Va</td>
<td>Aminoglycosides</td>
<td>ND</td>
<td>&gt;3.49×</td>
<td>0.006</td>
</tr>
<tr>
<td>SHV</td>
<td>Beta-lactams</td>
<td>ND</td>
<td>&gt;13.1×</td>
<td>0.006</td>
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<tr>
<td>erm(X)</td>
<td>Macrolides</td>
<td>ND</td>
<td>&gt;20.7×</td>
<td>0.008</td>
</tr>
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<td>tet(S)</td>
<td>Tetracyclines</td>
<td>ND</td>
<td>&gt;20.7×</td>
<td>0.013</td>
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<tr>
<td>vanTG</td>
<td>Vancomycin</td>
<td>ND</td>
<td>&gt;3.9×</td>
<td>0.050</td>
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<td>qoxB</td>
<td>Biocides (including QACs)</td>
<td>9.98E-02</td>
<td>3.4×</td>
<td>0.011</td>
</tr>
<tr>
<td><strong>Genes enriched in surplus compared to primary sludge</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>aac(3)-I-Llb/Iic</td>
<td>Aminoglycosides</td>
<td>4.45E-06</td>
<td>32.3×</td>
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<td>OXA-20</td>
<td>Beta-lactams</td>
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<td>4.7×</td>
<td>0.043</td>
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<td>OXA-48</td>
<td>Beta-lactams</td>
<td>ND</td>
<td>&gt;10.8×</td>
<td>0.012</td>
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<tr>
<td>erm(X)</td>
<td>Macrolides</td>
<td>ND</td>
<td>&gt;22.7×</td>
<td>0.001</td>
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<td>otr(A)</td>
<td>Tetracyclines</td>
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<td>11.3×</td>
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<td>tet(41)</td>
<td>Tetracyclines</td>
<td>8.08E-05</td>
<td>11.9×</td>
<td>0.023</td>
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<td>tet(2)</td>
<td>Tetracyclines</td>
<td>6.64E-06</td>
<td>11.3×</td>
<td>0.043</td>
</tr>
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<td>dfrA12/dfrA13/dfrA21/dfrA22/dfrA33</td>
<td>Trimerithrom</td>
<td>5.29E-05</td>
<td>5.7×</td>
<td>0.009</td>
</tr>
<tr>
<td>dfrB1/dfrB5/dfrB6/dfrB8</td>
<td>Trimerithrom</td>
<td>9.13E-06</td>
<td>50.8×</td>
<td>0.025</td>
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<td>Chromium (Cr)</td>
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<td>0.027</td>
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<tr>
<td>cmaA</td>
<td>Cobalt (Co), Nickel (Ni)</td>
<td>9.17E-02</td>
<td>1.8×</td>
<td>0.021</td>
</tr>
<tr>
<td>nccA</td>
<td>Cobalt (Co), Nickel (Ni), Cadmium (Cd)</td>
<td>8.98E-02</td>
<td>1.6×</td>
<td>0.007</td>
</tr>
<tr>
<td>intI10</td>
<td>Integrons</td>
<td>5.87E-04</td>
<td>2.5×</td>
<td>0.045</td>
</tr>
<tr>
<td>intI3</td>
<td>Integrons</td>
<td>4.06E-03</td>
<td>2.3×</td>
<td>0.045</td>
</tr>
<tr>
<td>intI6</td>
<td>Integrons</td>
<td>5.82E-03</td>
<td>1.8×</td>
<td>0.028</td>
</tr>
<tr>
<td>intI8</td>
<td>Integrons</td>
<td>1.93E-03</td>
<td>3.4×</td>
<td>0.023</td>
</tr>
<tr>
<td><strong>Genes enriched in digested compared to primary sludge</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OXA-48</td>
<td>Beta-lactams</td>
<td>ND</td>
<td>&gt;9.3×</td>
<td>0.008</td>
</tr>
<tr>
<td>lmf(F)</td>
<td>Lincomycin</td>
<td>1.54E-03</td>
<td>6.8×</td>
<td>0.023</td>
</tr>
<tr>
<td>sul3</td>
<td>Sulfonamide</td>
<td>7.75E-05</td>
<td>122.1×</td>
<td>0.012</td>
</tr>
<tr>
<td>tetB(P)</td>
<td>Tetracyclines</td>
<td>5.29E-05</td>
<td>4.4×</td>
<td>0.003</td>
</tr>
<tr>
<td>dfrA12/dfrA13/dfrA21/dfrA22/dfrA33</td>
<td>Trimerithrom</td>
<td>5.29E-05</td>
<td>4.4×</td>
<td>0.003</td>
</tr>
<tr>
<td>vanM</td>
<td>Vancomycin</td>
<td>4.94E-06</td>
<td>10.2×</td>
<td>0.018</td>
</tr>
<tr>
<td>vanRNN</td>
<td>Vancomycin</td>
<td>7.97E-04</td>
<td>3.7×</td>
<td>0.029</td>
</tr>
<tr>
<td>vanSA</td>
<td>Vancomycin</td>
<td>1.40E-04</td>
<td>5.1×</td>
<td>0.042</td>
</tr>
<tr>
<td>intI7</td>
<td>Integrons</td>
<td>3.16E-04</td>
<td>7.7×</td>
<td>0.043</td>
</tr>
</tbody>
</table>

ND = not detected. Comparisons for undetected genes were made to the highest detection limit across the samples (1E-05).
nine were enriched in surplus sludge compared to primary sludge, and eight were more abundant in digested than in primary sludge (Table 1). Notably, these resistance genes belonged to several different classes, and included genes conferring resistance to several clinically important antibiotics, such as cephalosporins (CTX-M), carbapenems (OXA-48), and tetracyclines (tetB/P), tet(S), tet(41), and tet(Z)). Generally, enrichments in relative abundance were around ten-fold or less, but in five cases enrichments were >30-fold: aph(9)-la (enriched 81-fold in effluent compared to influent), aac(3)-Iva (>34-fold after sand-filtration), aac(3)-Iib/Iic and dfrB (enriched 32-fold and 50-fold, respectively, in surplus sludge), and sul3 (122-fold in digested sludge).

The richness of antibiotic resistance genes was greatest in samples from incoming water and primary sludge, and smallest in treated and sand-filtered water (Fig. 3). Although all treatment steps except primary sludge and Kemikond (in the latter case due to too few samples) had significantly lower resistance gene richness than the inlet samples, the decreases in surplus and digested sludge were not very substantial, representing reductions in the range of 17–28%. Furthermore, the treated water samples maintained around half of the resistance genes from the inlet samples. The resistance gene profiles divided the treatment steps into three distinct groups: influent and primary sludge (including the mixed sample from Uppsala), surplus sludge and effluent (both before and after sand-filtration), and digested sludge, including Kemikond treated sludge (Fig. 4A). This mirrors the taxonomic profiles of the samples, and indeed the total resistance gene content was strongly correlated to the proportion of human-associated bacteria in each sample type (Pearson correlation coefficient 0.89; p = 0.0071). Still, when comparing the patterns of individual resistance genes, human feces was rather dissimilar from STP samples, even for influent and primary sludge (Fig. 4B). In addition, the treated water samples showed markedly larger differences in resistance gene composition within the group than incoming samples, and also showed larger variability within the biological and technical replicates (Fig. S7).

3.5. Antibacterial biocide and metal resistance genes

Similarly to antibiotic resistance genes, genes conferring resistance to biocides and metals were overall reduced in relative abundance throughout the treatment process, both in relation to bacterial SSU rRNA content and sample volume (Fig. 5). Notably, while antibiotic resistance genes were somewhat more abundant in digested than surplus sludge (Fig. 2), biocide and metal resistance genes were even further reduced in this treatment step (Fig. 5). The by far most common plasmid-borne biocide/metal resistance genes conferred resistance to arsenic, copper, mercury, silver and zinc (Tables S8 and S9). Overall, plasmid-borne biocide and metal resistance genes were 50–300 times more common than genes conferring antibiotic resistance (Fig. S8). Although most biocide and metal resistance genes decreased through the treatment process, seven metal resistance genes, conferring resistance to chromium, iron, nickel, copper and silver, were significantly enriched in effluent water compared to influent, the biocide resistance gene oqxAB was enriched after sand-filtration, and three resistance genes against...
metals were significantly more abundant in surplus than primary sludge (Table 1). All these increases were less than four-fold. In the effluent, four chromosome resistance genes belonging to the _chr_ operon ( _chrA, chrA1, chrC_ and _chrF_) were enriched, along with the _niu_ gene conferring resistance against iron and nickel, and the silver resistance genes _cysA/ybdE_ and _sllP_. In surplus and digested sludge, several chromosomal metal resistance genes were significantly enriched, particularly genes encoding arsenic, copper, tungsten and zinc resistance (Table S10).

3.6. Mobile genetic elements

We furthermore quantified the relative abundance of markers for different mobile genetic elements (MGEs) – integrases, ISCR transposases, relaxases and type IV secretion systems – in the sewage treatment samples (Fig. 6). Interestingly, these did not follow the same pattern as antibiotic, biocide and metal resistance genes (Figs. 2 and 5). Particularly, there were clear decreases of MGE markers in primary sludge (Fig. 6A), although the composition MGEs remained similar to incoming sewage (Fig. 6B and C). Furthermore there was a clear shift towards type IV secretion systems in the later treatment steps, with particular enrichment of those in class F (Fig. 6B). ISCR8 transposases dominated the integrase and ISCR transposase content, which remained very similar in all treatment steps except digested sludge and Kemikond. Surprisingly, the changes of the MGE patterns were not reflected in the number of complete plasmids obtained from the assemblies, where 14 of the 15 complete plasmids were identified in incoming water or primary sludge (Table 2), and none in the treated and sand-filtered water samples.

3.7. Co-resistance potential

In the assemblies, 776 out of 1,722,659 contigs (0.045%) were identified to carry resistance genes (583 carrying antibiotic and 216 carrying biocide and metal resistance genes). Out of these, 122 carried more than one resistance gene and could thus have co-selection potential. Most of these only contained two resistance genes on the same contig, but in eight cases six resistance genes were captured on the same DNA sequence (Fig. 59). However, seven of these eight contigs only contained the _mer_ operon conferring mercury resistance and no other resistance genes, providing no evidence for co-selection potential. There were eleven contigs carrying at least three resistance genes against both antibiotics and biocides/metals (Fig. S10). Most often, the co-occurring resistance genes were the sulfonamide resistance gene _sul1_, the _qacEdelta1_ resistance gene providing low-level resistance to quaternary ammonium compounds, and the aminoglycoside resistance gene _ant(3′)-Ia_. These genes were frequently co-located with an _intI1_ integrase gene. This corresponds well to how the abundance of the _intI1_ gene related to the abundances of _sul1_ (Pearson correlation coefficient _p_ = 0.0044) and _qacEdelta1_ (0.48; _p_ = 0.034), although the abundance of _ant(3′)-Ia_ was not significantly associated with _intI1_. In two cases there were also beta-lactamase genes localized to the same context (OXA-2 and OXA-10). Four of these contigs completely matched with >90% identity to known resistance plasmids, but most were novel variants or re-arrangements of previously characterized plasmids. These findings are also reflected in the co-localization networks (Fig. 7), where the genes constituting the _mer_ operon were frequently observed together both in incoming water samples (Fig. 7A), primary sludge (Fig. 7B; green lines), and effluent (Fig. 7C; blue lines). Apart from _qacEdelta1_, _sul1_ and _ant(3′)-Ia_ links between antibiotic resistance genes and metal/biocide resistance genes were scarce. It is also worth noting that the connections between genes were much less abundant in effluent and digested sludge samples (Fig. 7B), but also in surplus sludge (Fig. 7C, red lines), despite that MGEs were common in the latter sample type.

4. Discussion

To our best knowledge, this study represents the most comprehensive investigation to date of resistance genes against antibiotics, biocides and metals and their co-selection potential in sewage treatment plants. In some influent samples, ciprofloxacin and tetracycline were found at concentrations predicted to select for resistance. Despite this, there does not seem to be direct selection for resistance genes to any particular antibiotic classes in the STPs. Furthermore, we found limited support for co-selection between antibiotics and/or biocides and metals on the genetic level. While human-associated bacteria were reduced in the treatment process, the most important shift of taxonomy and resistance patterns compared to human feces occurred before the sewage had reached the treatment plants, likely due to the switch to aerobic conditions. At the same time, while human-associated bacteria decreased in abundance through the treatment, relative abundance and diversity of resistance genes were less affected. Importantly, some specific resistance genes, including the carbapenemase _OXA-48_, were enriched during sludge treatment and the abundances of mobile genetic elements were not significantly reduced in effluent water compared to influent.

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4.1. Selective potential of antibiotics and metals

Compared to estimated no-effect concentrations (Bengtsson-Palme and Larsson, 2016), the concentrations in incoming water of ciprofloxacin and tetracycline have potential to be selective for resistance. For tetracycline, these predicted no-effect concentrations agree well with experimentally determined minimal selective concentrations (1–10 μg/L) in complex microbial communities (Lundström et al., 2016). Notably, both ciprofloxacin and norfloxacin were detected in relatively high concentrations in sludge. These antibiotics adsorb tightly to particles (Córdova-Kreylos and Scow, 2007) and the degree to which they are bioavailable once adsorbed is not known, and hence their selective ability, regardless of measured concentration, is uncertain (Boxall et al., 2012).

The selective concentrations of metals in sludge are currently unknown, and thus we do not know if the detected metals have the potential to co-select for antibiotic resistance. This is both owing to a lack of established selective concentrations for metals, and to limited.

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knowledge of if metals bound to particles could exert a selection pressure. In addition, metals exist in a variety of chemical species (depending on pH and present ligands), with highly variable toxicity to bacteria. Consequently, it is difficult to determine whether the concentrations detected are selective. In a meta-study, concentrations of heavy metals that could co-select for antibiotic resistance have been estimated (Seiler and Berendonk, 2012). Based on these rough estimates and MICs for certain metals (Hassen et al., 1998), it seems that the concentrations of copper and zinc could have co-selective potential, while cobalt, mercury and lead are less likely to be selective. Cadmium levels definitely seem to be below concentrations selecting for resistance. For all other metals, information on inhibitory or selective concentrations is yet too scarce to aid in the interpretation of our data.

4.2. No direct selection for particular antibiotic resistance gene types

The total abundance of resistance genes per volume was reduced by around 50 times in effluent water compared to incoming water, corresponding well to the reduction in bacterial colony forming units. Thus the reduction of resistance genes observed in this and other studies (Al-Jassim et al., 2015; Yang et al., 2014) is largely driven by a reduction of total bacteria per volume, rather than by lowered numbers of resistance genes per bacterial DNA content. Indeed, the reduction of antibiotic resistance genes per 16S rRNA was less than three-fold between incoming and treated water (Fig. 2), and even smaller for resistance genes towards biocides and metals (Fig. 5). In addition, the removal of resistance genes per volume of sludge was less than ten-fold, implying that standard treatment processes are not very efficient in terms of reducing resistance gene abundances in sludge. It should be noted, however, that the efficiency of removal varied for different classes of antibiotic resistance genes. We identified several individual resistance genes that were significantly enriched during treatment (Table 1). Notably, these resistance genes belong to different classes of antibiotics, suggesting that there is no apparent selection towards a particular type of antibiotic resistance in the STPs, similar to previous findings from Finnish STPs (Laht et al., 2014). Such systematic selection would be expected to be manifested as an overall enrichment of resistance genes towards a specific class of antibiotics, as observed in controlled experiments on complex microbial communities (Lundström et al., 2016). Still, we cannot exclude selection for mutations conferring resistance, as we did not study these specifically.

A large number of resistance genes were enriched in surplus sludge compared to primary sludge. Since the surplus sludge is derived from recirculated activated sludge, which is continuously mixing with new material coming into the STP, enrichments in this sample type can be indicative of selection within the treatment plant. From this perspective, it is worrisome to note that resistance genes against important antibiotics, such as beta-lactams, macrolides and tetracyclines, were enriched in surplus sludge. We are particularly concerned about that the OXA-48 gene, conferring resistance to carbapenems – a critically important last-resort antibiotics class – was enriched in the treated sludge. Carbapenemases are only rarely found in clinical isolates from Swedish inhabitants, although the number of cases are currently on the rise, particularly for OXA-48 (Hellman et al., 2014). Interestingly, similar increases in surplus and digested sludge have been reported for the NDM-1 carbapenemase gene in Chinese STPs (Luo et al., 2014).

4.3. Co-selection for resistance between antibiotics appears limited

In effluent water, the aph(9)-la, CTX-M, tetB(P) and dfrA3 genes were accumulated (Table 1). Since the measured concentrations of trimethoprim were below predicted no-effect concentration and the long-term stability of the majority of beta-lactams is considered to be low (Längin et al., 2009), this is less likely to be due to direct selection for the CTX-M and dfrA3 genes, suggesting that co-selection could be at play in this case. The concentrations of tetracycline in the influent could very well be selective, as indicated by both theoretical calculations and experimental data (Bengtsson-Palme and Larsson, 2016; Lundström et al., 2016), and one possibility is thus that the CTX-M and dfrA3 genes have been co-selected for by tetracycline selection. On the other hand, if tetracycline co-selection was the case, we would expect a more general enrichment also of tetracycline resistance genes, or at least enrichment of some of the most sensitive markers for tetracycline selection – tet(A) and tet(G) (Huang et al., 2014; Lundström et al., 2016; Yin et al., 2015), but no such enrichments were observed.

The network of genetic co-localizations on contigs from the STPs shared features with networks from human feces, such as the strong connection between aph(3′)-Ib and aph(6)-Id, and the association of the ant(3′)-Ia and sulI resistance genes to the intI1 integrase (Bengtsson-Palme et al., 2015a). Notably, the beta-lactamases OXA-2 and OXA-10 were both detected together with sulI and qacEdelta1 on assembled contigs. None of the enriched resistance genes were found on the co-resistance contigs; instead the antibiotic resistance genes detected on contigs largely corresponded to the most abundant resistance genes. In the absence of a strong selection pressure, we would expect resistance plasmids to be in minority in a given microbial community, and in line with this expectation none of the plasmids that we could assemble completely carried any known resistance genes. However,
Fig. 7. Co-localization networks of antibiotic resistance genes (blue), biocide resistance genes (purple), metal resistance genes (orange), integrases and transposases ISCRs (green). Each link between two genes represent a co-occurrence in one sample, and thicker links correspond to co-occurrence on several contigs from the same sample. (A) Influent samples. (B) Samples from effluent (blue), sand-filtered water (cyan) and digested sludge (orange). (C) Samples from primary sludge (green) and surplus sludge (red). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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resistance regions have a tendency to break up the assembly into shorter contigs, due to that resistance genes can be highly conserved and exist in multiple contexts (Bengtsson-Palme et al., 2014). This problem was evident for many of the resistance contigs associated with integrons, where the intI1 gene was often only partially present and located at the very end of the contigs (Figs. S9 and S10). Gaining better insight into co-resistance patterns through metagenomic sequencing thus requires more specifically adapted assembly methods. That said, current evidence for co-selection of different types of antibiotic resistance genes in STPs appears to be limited.

4.4. Limited evidence for antibiotic resistance co-selection by biocides and metals

Concentrations of many metals, including arsenic, were higher in surplus sludge than in primary sludge. Arsenic resistance has been suggested to be connected to the presence of mobile antibiotic resistance genes (Farias et al., 2015; Zhu et al., 2013). Indeed, chromosomal-associated arsenic resistance genes were enriched in the surplus sludge, which could contribute to maintenance of mobile antibiotic resistance genes (Farias et al., 2015; Pal et al., 2015). Several plasmid-associated chromosome resistance genes were significantly enriched in treated effluent. These genes have, however, not been reported to frequently co-occur with antibiotic resistance genes on plasmids (Pal et al., 2015). We also found the plasmid-borne silver efflux pumps silP and cusA/ybdE to be enriched in the effluent, and silP was present in more than one copy per bacterial 16S rRNA, suggesting that silver resistance is common among bacteria released from the treatment plants. Although resistance to silver is currently not commonly associated with antibiotic resistance (Pal et al., 2015), such cases have been described (Sandegren et al., 2012; Sütterlin et al., 2014), and mobilization of silver resistance genes could in the future potentially contribute to the selection of antibiotic resistance.

The STP genetic co-localization network closely resembled the co-resistance clusters identified on plasmids (Pal et al., 2015), although considerably less densely connected. For example, the mercury resistance genes formed a highly connected cluster in both incoming water, effluent and primary sludge, and were the only metal resistance genes that were found together with antibiotic resistance genes on the same contig (aph(6)-Ib and aph(3')-Ib, both conferring aminoglycoside resistance), given the metagenomic assembly effort. Interestingly, the most abundant biocide and metal resistance genes (both plasmid-borne and chromosomal) were not associated to the co-resistance contigs to the same degree as resistance genes against antibiotics, suggesting that the connectivity of antibiotic resistance genes is higher. This corresponds to that the co-selective potential between most biocides, metals and antibiotics would be relatively limited. However, because of the limitations of metagenomic assembly, many potential co-localizations to the same plasmid may go undetected. Similarly to the plasmid network, qacEdelta1 was often connected to ant(3')-Ia, sulI and intI1 (Fig. 7). This further supports that these genes have central roles in horizontal transfer of resistance determinants, particularly on integrons (Gillings, 2014). Notably, even though qacEdelta1 was detected in most samples, there was no clear enrichment of any biocide resistance genes in digested sludge, despite biocide concentrations being markedly higher there than in surplus sludge. This suggests that other mechanisms are at play to deal with antibacterial biocides, and/or that the concentrations measured are not selective. Furthermore, it points towards the ubiquitous nature of the qacEdelta1 gene, which constitutes a part of clinical class 1 integron sequences (Gaze et al., 2005). The abundances of integrases were increased in surplus sludge compared to primary sludge. Notably, the classical intI1 integrase usually associated with clinical antibiotic resistance (Gillings, 2014), did not increase in abundance, but instead the intI3, intI6, intI8 and intI10 genes did. The intI3 integrase has occasionally been associated with beta-lactam and aminoglycoside resistance genes, although rarely so compared to intI1 and intI2. The other three integrases have not previously been associated with antibiotic resistance according to the INTEGRALL database (Moura et al., 2009). This suggests that activated sludge could indeed be a hotspot for mobilization of resistance genes, but it is still disputable whether enrichment of antibiotic resistance genes is specific, or merely a co-incidence due to co-localization with other genes.

4.5. Fecal bacteria disappear by a shift to aerobic conditions

Besides providing a potential environment for selection resistance and mobilization of resistance determinants, STPs also function as a dispersal route for human pathogens, particularly if not sufficiently treated (Anastasi et al., 2012; Frigon et al., 2013; Hendricks and Pool, 2012). In the three treatment plants we have investigated, bacteria were overall reduced by roughly a factor of 100 relative to volume as assessed by colony forming units (30-fold decrease measured by 16S rRNA abundance). The proportion of human-associated genera was reduced from >20% in the influent to <5% in the effluent and digested sludge. Furthermore, digested sludge is often treated using disinfecting techniques – either chemically or by heat – before application to e.g. agricultural soils (Kelesidis and Stasinakis, 2012). Interestingly, the proportion of human-associated bacteria was reduced by at least 40% already in the influent, compared to human feces. Particularly, the most abundant fecal bacteria were dramatically reduced by a shift from anaerobic to aerobic conditions in sewage, replacing obligate anaerobic bacteria with facultative anaerobes (Fig. 1), possibly enhanced by lower temperature. This mirrors previous results indicating that only 22% of the microbes in sewage come from the most abundant 90% of fecal bacteria (Newton et al., 2015). Thus, only bacteria that can survive oxygen exposure will be able to proliferate in STPs, and simple aerobic treatment considerably reduces the abundance of fecal bacteria. However, many important pathogens are facultative anaerobes, and those seem better suited to survive through the sewage pipes and end up in STPs. Surprisingly, despite that human-associated bacteria were efficiently reduced in the STPs, resistance gene abundances were only slightly lowered. Similarly, the resistance gene composition between the treatment steps was overall more similar than between influent and human feces (Fig. 4B), as also observed for the taxonomic composition of the samples (Fig. 1B). Finally, even though the STPs seem to efficiently reduce the numbers of pathogenic bacteria, they do not completely remove them. Particularly troublesome, the relative abundance of Legionella – a genus containing several waterborne opportunistic pathogens – increased in the treatment process. Legionella readily spreads through the water environment, but is primarily enriched in human-made aquasystems with temperatures around 35 °C (Fields et al., 2002). Increased abundance in the treated effluent suggests a potential risk for transmission, and thus sanitary treatment of the effluent, such as ozonation, may be considered.

The large dependence on abiotic factors of resistance gene frequencies in STPs suggests that attempts to establish a relationship between antibiotics concentration and resistance gene abundance through, for example, correlation analysis will be confounded. Since antibiotics concentrations and bacterial abundances are both reduced in treatment plants, strong correlations are expected due to common causes, and have little to do with selection for resistance per se. Furthermore, the influence of taxonomic composition seems to be relatively large, warranting studies of resistance frequencies within specific species, to better address if specific, clinically relevant bacteria become more or less resistant in STPs (Ferreira da Silva et al., 2007; Garcia et al., 2007; Lefkowitz and Duran, 2009). Such studies can also provide a more direct measure of selection for antibiotic resistance, less biased by other factors that can influence resistance gene abundances.
5. Conclusions

This broad and explorative analysis of resistance genes provides no clear evidence for direct selection by any particular class of antibiotics in Swedish STPs. Similarly, we find no strong evidence for selection of biocide and metal resistance, and thus co-selection of antibiotic resistance genes. Throughout the treatment process, other selective pressures, such as oxygen availability, are likely to influence the composition of resistance genes more than antibiotic selection does. Changes in resistance gene frequencies may thus to a large degree be linked to changes in taxonomic composition, and genetic analyses alone cannot be used to infer relevant selection pressures associated with risks for resistance development in STPs. Identifying appropriate mitigation strategies to avoid development and spread of resistance requires an understanding of the underlying selective processes. To gain such understanding, the resistance patterns of individual species of bacteria through the treatment process needs to be specifically investigated, which is more easily addressed using culture-dependent approaches.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2016.06.228.

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