Metagenomic Profiles of Antibiotic Resistance Genes in Activated Sludge, Dewatered Sludge and Bioaerosols

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Abstract: Wastewater treatment plants (WWTPs) have been considered hotspots for the development and dissemination of antibiotic resistance in the environment. Although researchers have reported a significant increase in bioaerosols in WWTPs, the associated bacterial taxa, antibiotic resistance genes (ARGs), and mobile genetic elements (MGEs) remain relatively unknown. In this study, we have investigated the abundance and occurrences of ARGs and MGEs, as well as the bacterial community compositions in activated sludge (AS), dewatered sludge (DS) and bioaerosols (BA) in a WWTP. In total, 153 ARG subtypes belonging to 19 ARG types were identified by the broad scanning of metagenomic profiles obtained using Illumina HiSeq. The results indicated that the total occurrences and abundances of ARGs in AS and DS samples were significantly higher than those in BA samples ($p < 0.05$). However, some specific ARG types related to sulfonamide, tetracycline, macrolide resistance were present in relatively high abundance in BA samples. Similar to many other full-scale WWTPs, the Proteobacteria (58%) and Bacteroidetes (18%) phyla were dominant in the AS and DS samples, while the Firmicutes (25%) and Actinobacteria (20%) phyla were the most dominant in the BA samples. Although the abundance of genes related to plasmids and integrons in bioaerosols were two to five times less than those in AS and DS samples, different types of MGEs were observed in BA samples. These results suggest that comprehensive analyses of resistomes in BA are required to better understand the emergence of both ARGs and MGEs in the wastewater treatment process due to the significant increase of scientific attention toward bioaerosols effects.

Keywords: antibiotic resistome; wastewater residual; wastewater treatment plant; bioaerosols; metagenomics

1. Introduction

The intensive use of antibiotics in the clinical, industrial, and agricultural sectors has encouraged the selection of antibiotic-resistant bacteria (ARB) and genes (ARGs) since the first deployment of antibiotics in the 1930s [1]. Currently, infections resistant to antimicrobial therapy are costing hundreds of thousands of lives worldwide, and the death toll is projected to further increase each year [2]. Factors influencing the environmental resistome have attracted significant interest from scientists [3,4], and it is widely recognized that wastewater treatment plants (WWTPs) are among the primary anthropogenic sources of ARB and ARGs in the natural environment [5–8]. Additionally, mobile genetic elements (MGEs) are abundant in WWTPs [9] and have been suggested to aid in the horizontal gene transfer (HGT) of ARGs within the treatment process [3,10], as well as in the receiving water [11,12]. Therefore, common byproducts of wastewater treatment processes, such as activated sludge and dewatered

sludge (hereafter referred to as WWTP byproducts), can disseminate resistomes to other environments without proper management.

The occurrence and abundance of ARB and ARGs in activated sludge [7,9,13], as well as their removal by different wastewater treatment processes [3,14–16], are undergoing continuous research. Many studies have extended their scope to the water [17], soil, and sediment [15,18] that receives the WWTP byproducts. However, the antibiotic resistance of biological aerosols (bioaerosols) generated by wastewater treatment processes has seldom been investigated. Bioaerosols have shown connections with a variety of contagious infections in people [19], and ARGs have also been discovered in urban air [20,21]. A significant increase in bioaerosols was observed on the day after the start of a new WWTP, indicating a link between the bioaerosol properties and plant operation [22]. More alarmingly, bioaerosols from WWTPs were found 10 km downwind of the plant boundaries [23]. Previous studies showed a possible connection between specific bacterial species and ARGs of WWTP samples [24,25]. Nevertheless, to the best of our knowledge, no research has comprehensively analyzed how the bacterial community and the ARG relative content change as activated sludge is processed into dewatered sludge and aerosolized into bioaerosols. In addition, few studies have simultaneously investigated the profiles of MGEs, even though they are important factors for the HGT that affects the acquisition and transfer of ARGs in the bacterial community [9,15].

The objective of this study is to preliminarily investigate the abundance and diversity of ARGs, bacterial taxa, and MGEs in WWTP byproducts and bioaerosols using a shotgun metagenomic analysis.

2. Materials and Methods

2.1. Study Area and Sampling

In total, six samples (2 activated sludges: AS, 2 dewatered sludges: DS, and 2 bioaerosols: BA) were collected from the Jungnang municipal WWTP (37°33′28.2″ N, 127°03′54.9″ E) during the winter season (January and November) in 2015 in Seoul, South Korea. The WWTP has a treatment capacity of 1.6×10^6 m³/day and serves approximately 4.1 million inhabitants. The WWTP process consists of screens, a grit chamber and a modified Ludzack-Ettinger (MLE) system, which includes an anoxic tank and an aerobic tank, followed by a secondary clarifier. Part of the activated sludge is returned to the anoxic tank from the aerobic tank. The influent biochemical oxygen demand (BOD), total nitrogen (TN) and total phosphorous (TP) were approximately 115.5–124.0 mg/L, 29–33 mg/L and 3.44–3.87 mg/L, respectively, and their average removal efficiencies were 94.3%, 58.03% and 65.4%, respectively. The influent sludge compositions were 113.8–115.7 total suspended solids (TSS) mg/L, 110.1–112.4 BOD mg/L, and 29.7–30.2 TN mg/L, while the effluent TSS, BOD and TN were 4.1–4.4 mg/L, 6.9–8.4 mg/L, and 9.7–12.8 mg/L, respectively.

Activated sludges (500 mL) were collected from an aeration tank during the MLE process. Dewatered sludges were also collected from the belt-press inside the dewatering system unit on the same day. Bioaerosols samples were collected beside the belt-press on sterilized 0.2 µm track-etched polycarbonate filters (Whatman, GE, Freiburg, Germany) using two high-volume air samplers (Model TE 5200, Tisch Environmental, Inc., Cleves, OH, USA) at a flow rate of 1.5 m³ min^{−1} for 24 h. The polycarbonate filters were autoclaved prior to sampling, and the filter holders were washed with 70% ethanol at the sampling time to avoid potential contamination [26]. The temperature and relative humidity inside the dewatering facility were 22 °C and 19%, respectively. The collected samples were immediately transferred to the laboratory in an ice box and stored at −80 °C for molecular analysis.

2.2. DNA Extraction and High-Throughput Sequencing

The FastDNA Spin Kit for Soil (MP Biomedicals, Santa Ana, CA, USA) was used to extract genomic DNA from six samples, according to the manufacturer's protocol. For the sludge and dewatered sludge samples, DNA was extracted from the cell pellet formed after centrifuging 1.5 mL of each sample at 14,000×g for 2 min. For the bioaerosol samples, gDNA was extracted from the filter pieces by a method

described in previous studies [26,27]. The quality of the extracted DNA was checked using a Qubit Fluorometer (Thermo, Wilmington, DE, USA) for the subsequent shotgun metagenome sequencing. The metagenome sequencing was performed by Macrogen (Seoul, South Korea) using the TruSeq DNA PCR-Free Kit (Invitrogen, Inc., Madison, WI, USA) according to the library protocol. Each sample was barcoded and analyzed using a 2×101 bp paired-end protocol with an Illumina HiSeq 2000 platform.

2.3. Bioinformatics Analysis

Trimmomatic (version 0.33) was used to trim adaptors and nucleotide stretches with low quality scores (default setting) from the Illumina results [28], which were then used as inputs for the downstream analysis. The diversity and abundance of ARGs and ARGs-OAP (Online Analysis Pipeline) were determined using the online tool (<http://smile.hku.hk/SARGs>), following a previously published protocol [29]. For the ARG abundances, the copies of ARGs per copy of 16S rRNA in the metagenomic data were used [27].

To characterize the MGEs in the tested samples, sequences found in the AS, DS, and BA were aligned against the integron and plasmid database using Local BLASTn. An integron database was constructed using the nucleotide sequences of all integrases available in the INTEGRALL database (<http://integrall.bio.ua.pt/>). Similarly, a plasmid database was developed on the basis of the plasmid sequences of the NCBI RefSeq database (<http://www.ncbi.nlm.nih.gov/refseq>). A read was annotated as an integron if the nucleotide sequence identity of the best BLASTn was greater than 90% with an alignment length of at least 50 bp. The plasmid-like tags in all data sets were determined by alignments with a nucleotide sequence identity above 95% over a length of at least 90 bp [30,31].

The microbial communities of the tested samples were analyzed using the default setting of MetaPhlAn2 [32]. All statistical analyses (t-test and correlation analysis) were conducted using the “vegan” package in R software. The metagenomes generated in this study are uploaded at MG-RAST (sample ID: mgm4785061.3, mgm4791578.3, and mgm4791579.3).

3. Results and Discussion

3.1. Prevalence of ARGs in AS, DS, and BA

WWTPs are regarded as hotspots for ARGs and ARB [5,9]. In this study, a metagenomic analysis of the activated sludge (AS), dewatered sludge (DS) and bioaerosols (BA) revealed 153 different ARG subtypes belonging to 19 ARG types. The AS samples contained 138 ARG subtypes representing $0.093 \pm 0.006\%$ of the total reads (Figure 1). The average values of ARGs ranged from 5.80×10^{-5} – 1.20×10^{-1} copies per 16S rRNA gene in the AS samples. This result was comparable to that obtained for DS samples, which contained 144 ARG subtypes representing $0.092 \pm 0.010\%$ of the total reads. The average values of ARGs ranged from 4.62×10^{-6} – 4.48×10^{-1} copies per 16S rRNA gene in the DS samples. In contrast, the BA samples contained only 98 ARG subtypes representing $0.025 \pm 0.004\%$ of the total reads. The average values of ARGs ranged from 7.88×10^{-7} – 3.16×10^{-3} copies per 16S rRNA gene in the BA samples. Among them, sulfonamide (*sulI*, *sulII*)-, MLS (*ermB*, *ermG*, *ermF*)- and tetracycline (*tetA*, *tetG*, *tetQ*, *tetW*)-resistance genes accounted for approximately 71% of the total identified ARGs across the three different sample types (Figure 2). Although the residual pharmaceuticals found in the influent can vary based on the purpose and service area of a WWTP [33], the influent pharmaceutical concentration at large-scale municipal WWTPs in South Korea, such as the Jungnang WWTP, correlated well with the antibiotic usage [34]. Given that sulfonamide, MLS and tetracycline are common antibiotics used in both healthcare and agriculture [35,36], continuous exposure to the residues from these antibiotics could have resulted in the high abundances of these ARGs. Similarly, genes conferring resistance to sulfonamides and MLS are frequently detected in WWTPs around the world, along with those providing resistance to tetracyclines and beta-lactams [37,38]. There are also reports of considerable increases (over two orders of magnitude) in *sul*, *erm*, and *tet* during the treatment processes [9,13,37]. Especially, resistance genes of β -Lactam were relatively predominant in the AS

samples in this study ($p < 0.05$). OXAs (Oxacillinases) genes were commonly detected in gram-negative bacteria [39]. Because OXA enzymes are usually associated with MGEs, such as integrons, insertion sequences and transposons, they potentially can be transferred between bacterial communities via HGT [40]. In addition, they were usually co-selected with other resistance determinants, such as aminoglycosides, sulfonamide, fluoroquinolones, and tetracycline. It suggests that OXA genes may be a major driver of spreading of antibiotic resistomes in the AS process [39,40].

Figure 1. The relative abundances of the antibiotic resistance genes (ARG) types in AS (activated sludge), DS (dewatered sludge), and BA (bioaerosols). MLS indicates macrolide-lincosamide-streptogramin. The error bars represent one standard deviation between two samples (January and November).

Figure 2. Broad-spectrum profiles of the ARG subtypes in AS (activated sludge), DS (dewatered sludge), and BA (bioaerosols). The abundances of the top 35 ARG subtypes (ARG per copy of 16S rRNA gene copies) are illustrated in the heat map using the normalized z-score for all samples. AS1, DS1, and BA1 indicate January samples in 2015, and AS2, DS2, and BA2 indicate November samples in 2015.

In a previous study, the total number of ARGs in DS was at least an order of magnitude higher than that in AS, with no major change in the relative compositions of ARG types [13]. In this study, the total number of ARGs found in DS was also higher than that in AS. However, the relative compositions

of ARG types were notably different before and after the dewatering process, according to a t-test ($p < 0.05$) (Figure 2). In the AS samples, *sulI*, *sulII* and *ermF* constituted 24%, 7% and 28% of the total ARG composition, respectively. However, in the DS samples, their compositions changed to 29%, 4% and 18%, respectively. Sulfonamides, with their high solubility and chemical stability, can persist in the environment for a long period of time [41], resulting in the high abundance of *sulI* and *sulII* in the WWTPs. Therefore, sulfonamide resistance is frequently detected in effluent [3,4,17], dewatered sludges [42], and surface water [6,43,44], indicating a general resilience to the wastewater treatment process. In addition, many *sulI* genes are susceptible to HGT because they are carried by the integrase gene of class 1 integrons (*intI1*), which are MGEs [45]. Ma et al. [46] reported a rebound of *sulI* after the aerobic digestion of sludge that was pretreated by thermal hydrolysis. This result not only suggests the persistence of *sulI* in a wide variety of environments, but it also indicates the possibility of HGT in the bacterial community, which is greatly aided by MGEs like *intI1* [37,38].

A lower number of ARG types and subtypes were found in the BA, comparing with AS and DS (Figure 2). Among them, *erm*, *sul*, and *tet* were dominant, constituting approximately 62% of the total ARG composition. While *sulI* was significantly decreased in the bioaerosols compared with both the AS and DS samples ($p < 0.05$), the relative abundance (16%) was still high. Although ARGs in the atmosphere have recently attracted scientific attention [21,23,47], the low number of metagenomic investigations in this field limits direct comparisons of the results obtained from this study. Nevertheless, the major profile of ARGs in the bioaerosols (*erm*, *sul*, and *tet* gene) was similar to those in sludge thickening basins [23], biological reaction basins [23], mass livestock-feeding operation facilities [48,49] and urban air [50]. These results indicate that the profiles of ARGs in BA from WWTP are commonly detected in other types of air samples, but the reason behind the dominance of *erm*, *sul*, and *tet* is still unclear. Zhou et al. [21] reported that rapid changes in physicochemical conditions may occur during and after aerosolization. These changes might either amplify or attenuate ARB [6,9,51,52], developing different ARG abundances and diversity levels in BA. ARB and ARGs in bioaerosols are particularly important because they can be easily transported by wind, spreading resistance to distant areas [49]. However, there is a major concern about the reliability of the results, because our sample size is small for interpreting the different occurrences and abundances of ARGs among the AS, DS, and BA samples. However, sometimes small samples detect more rare members and may help in the preliminary investigation of study needs by directing scientific attention toward rapidly understanding the overall trends and characteristics of the microbial communities [53]. Some previous studies [9,54] have been carried out to investigate the structures, functions, and resistomes of microbial communities from WWTPs using small sample sizes. Therefore, further investigation is quite needed to understand the cause of the enrichment of ARG abundance in the bioaerosols.

3.2. Phylogenetic Characterization of the Bacterial Community

The taxonomic profiles at the phylum and genus levels were investigated to interpret the changes in the bacterial community structure as activated sludge is pressed into dewatered sludge and then aerosolized (Figure 3). Similar to many other full-scale WWTPs, Proteobacteria (57.82%–59.58%) and Bacteroidetes (20.32%–22.24%) were dominant in the AS samples [55,56]. These phyla were dominant even after the dewatering process, but their genus-level profiles were notably different (Figure 3). The AS samples were dominated by nitrifiers (*Nitrosomonas*, 10%–11%; *Nitrospira*, 8%–9%), denitrifiers (*Dechloromonas*, 18–20%), and *Escherichia* (8%–11%), whereas *Pseudomonas* (20%–22%), *Mycobacterium* (17%–19%), and *Arthrobacter* (13%–14%) were more dominant in the DS samples. The physiochemical changes affecting activated sludge during its conversion to dewatered sludge are mechanical actions that rapidly squeeze out liquid and some biomasses [23]. The force exerted on the sludge in this process causes an unnatural shift in the environmental parameters (temperature, substrate, nutrients, etc.), which can have substantial effects on bacterial community structures and ARGs [7,37].

Figure 3. The relative abundances of the major detected phyla (a) and genera (b) in the AS, DS, and BA samples. AS1, DS1, and BA1 indicate January samples in 2015, and AS2, DS2, and BA2 indicate November samples in 2015.

According to a previous study, as much as 85.27% of the bioaerosols in the dewatering facility are emitted from sludge during dewatering [57]. However, the bacterial community structures of the bioaerosols were different from those in the WWTP byproducts, which are the most likely source. In the bioaerosols, the phyla Firmicutes (25.40%–27.12%) and Actinobacteria (20.38%–22.06%) were the most dominant, constituting a significantly higher proportion ($p < 0.05$) of the bacterial community structure than in the WWTP byproducts. Consequently, the bacterial community structure at the genus level was also different from that of the WWTP byproducts. *Mycobacterium* (25%–28%) was the most dominant genus found in the bioaerosols. This was consistent with a previous study, which revealed relatively high proportions of *Mycobacterium* in the air of sludge dewatering facilities [57]. Such a difference could also result from the rapid change in physicochemical conditions due to the aerosolization of liquids and semisolids, such as the rapid changes to which active sludge was exposed during the dewatering process. However, the different incidences of bacterial communities were not clearly demonstrated in this study due to the sample sizes. In addition, relatively fewer studies have been carried out to understand the microbial communities of bioaerosols from WWTPs. Therefore, more studies are absolutely required to better understand the emergence of both bacterial communities and ARGs in wastewater treatment processes in full-scale WWTPs.

3.3. Prevalence of Mobile Genetic Elements (MGEs)

MGEs, such as plasmids and integrons, can facilitate the HGT of ARGs linked to them. As one major player in the dissemination of antibiotic resistance, *intI1* is ubiquitous in soil, freshwater, and biofilms and is present in 1%–5% of bacterial cells [58]. In the AS, approximately $0.284 \pm 0.016\%$ and $0.023 \pm 0.020\%$ of the identified genes were related to plasmids and integrons, respectively (Figure 4).

The genes related to plasmids decreased to $0.22 \pm 0.010\%$, whereas those related to integrons increased to $0.030 \pm 0.022\%$ after the DS. In comparison, the abundances of genes related to plasmids and integrons in the BA were two to five times less than those in the AS and DS, with values of $0.112 \pm 0.007\%$ and $0.008 \pm 0.002\%$, respectively. The distribution of MGE abundance and MGE diversity were consistent with those of the ARGs of the tested samples.

Figure 4. Distribution of mobile genetic elements, including plasmids (a) and integrons (b), across the three different samples (AS (activated sludge), DS (dewatered sludge), and BA (bioaerosols)). Relative abundances of plasmid- and integron-like reads per total metagenomic sequencing read. The error bars represent one standard deviation.

Many plasmids and *intI1* were found in the WWTP byproducts and bioaerosols (Tables S1 and S2). The ARG diversity and abundance showed strong correlations with those of the plasmids, with R^2 values of 0.66 to 0.84, respectively (Table S3). However, among the major plasmids, only one was present in more than one sample (pMaq22A_1p in activated sludge and dewatered sludge). Although different ARG profiles were detected from each type of sample, the correlations between ARGs and plasmids may potentially aid in the persistence of ARGs under different environmental conditions. Plasmids conferring antibiotic resistance are known to be stable in the environment, even without antibiotic selection pressures [13,15,17,58,59]. Especially, the presence of self-transmissible plasmids, such as the atrazine catabolic plasmid pADP-1 [60], and putative conjugative plasmids, such as pAR060302 [61] and pPS1 [62], may reinforce the hypothesis of frequent plasmid transfer events between ARGs and bacterial communities during treatment processes, or these plasmids may spread through the acceleration of the ARGs mobility. Thus, the high abundance of plasmids potentially leads to the enrichment of multiple ARGs in wastewater treatment processes. Integrons are suggested to contribute to the exchange and incorporation of ARGs, resulting in the proliferation of bacterial antibiotic resistance in WWTPs [10,58,63,64]. Therefore, plasmid- and *intI1* gene-related antibiotic resistance in WWTP byproducts and bioaerosols may increase environmental and public health risks if they are released without proper treatment.

Although antibiotic resistance could spread among bacteria through HGT, the transfer rate may not be high enough to impose the change observed in the number of ARGs, unless the tested samples have been resting in the WWTP for a long period of time. The short transition and incubation time (<24 h) are unlikely to have caused a noticeable change in the resistome. Resistance found in the bacteria indigenous to the wastewater treatment environment potentially implies the occurrence of HGT between pathogens and environmental bacteria within the components of the WWTP [9,12,63]. To date, information regarding nonpathogenic environmental species, which can be important carriers of ARGs, is scarce. The potential risks of these nonpathogenic environmental species contributing to the spread of antibiotic resistance need to be assessed in future studies. A bacterial community shift via HGT was reported to be the major driver shaping the resistome of drinking water systems [64] and composting sewage sludge [65]. Therefore, it is possible that the dominant ARB listed above are counter-selected by the rapid transformation process from liquid to semisolid and then to aerosols based on their persistence under specific environmental conditions [16,63,65].

4. Conclusions

This study showed diverse ARGs and MGEs in WWTP byproducts and their connection to bioaerosols through metagenomic profiling. Although a solid conclusion cannot be made based on the limited number of samples investigated in this study, bioaerosols from the WWTP dewatering facility had different microbial community structures (Firmicutes and Acinetobacter were dominant) and ARGs (*erm*, *sul*, and *tet* genes were dominant) compared to WWTP byproducts. This indicates rapid changes in the biotic properties of activated sludge and dewatered sludge as they are aerosolized during the dewatering process. The results from this study provide new insight into the occurrence and abundance of ARGs in three different potential pollutants generated in the WWTP process, but future studies must evaluate whether the trends described in this study hold when larger sample sizes are used because of the limited robustness of our findings. In addition, further high-throughput sequencing of the metagenome and meta-transcriptome in future studies can be efficiently used as a tool to understand and build a strategy against the contamination of ARGs in WWTP processes.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2073-4441/12/6/1516/s1>, Table S1: Relative abundances of plasmids and their corresponding bacterial hosts as determined by matching metagenomic sequence reads against the NCBI plasmid genome database, Table S2: Relative abundances of the integron integrase genes and their corresponding bacterial hosts as determined by matching metagenomic sequence reads against the INTEGRALL database, Table S3: The correlation between antibiotic resistance genes (ARGs) and mobile genetic elements (MGEs).

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