

## Article

# Bacterial Communities and Antibiotic Resistance Communities in a Full-Scale Hospital Wastewater Treatment Plant by High-Throughput Pyrosequencing

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Academic Editor: Yung-Tse Hung

Received: 18 October 2016; Accepted: 1 December 2016; Published: 7 December 2016

**Abstract:** The community of whole microbes and antibiotic resistance bacteria (ARB) in hospital wastewater treatment plants (WWTP) receiving domestic wastewater (DWW) and hospital wastewater (HWW) was investigated. Samples from an influent of a secondary clarifier, at each treatment train, were characterized for the whole microbial community and ARB on the antibiotic resistance database, based on high-throughput pyrosequencing. The pyrosequencing analysis revealed that the abundance of *Bacteroidetes* in the DWW sample was higher (~1.6 times) than in the HWW sample, whereas the abundance of *Proteobacteria* in the HWW sample was greater than in the DWW sample. At the top twenty of the genus level, distinct genera were observed—*Saprospiraceae* in the DWW and *Zoogloea* in the HWW. Apart from the top twenty genera, minor genera showed various antibiotic resistance types based on the antibiotic resistance gene database.

**Keywords:** antibiotic resistance bacteria; pyrosequencing; hospital wastewater; antibiotics; microbial community

## 1. Introduction

The overuse of antibiotics in medical fields and agricultural processes resulted in a large portion of antibiotic release in water environments, which increases antibiotic resistance genes. This resistance can affect public health because pathogenic infection is not treated by certain antibiotics [1]. Thus, studies on antibiotic resistance bacteria (ARB) have been growing in number [2–4] and have been highlighted in water and wastewater environments [5,6].

Wastewater treatment plants (WWTPs) are considered as hot spots for widespread ARB or antibiotic resistance genes (ARG) because residual antibiotics and bacteria that are exposed to antibiotics are released into sewage [7]. In the biological treatment process of WWTPs, the levels of ARB/ARG can be accelerated due to the selective pressure caused by antibiotics [8]. Therefore, the rich population of bacteria in the activated sludge can disseminate selective antibiotic resistance, and biofilm formation may trigger horizontal gene transfer of ARG between different functional groups such as plasmids, integrons, transposons and bacteriophages [9,10]. The diversity and abundance of ARB were reported in drinking water, activated sludge, anaerobic sludge and agricultural waste [11–13]. These reports indicated that WWTPs are sources of ARB/ARG in receiving water, which may significantly affect human and animal health.

Previous studies on ARB focused on the sources of municipal wastewater and agricultural waste [6,7,12], while limited studies were performed concerning the abundance and diversity of ARB in hospital wastewater [14]. Hospital wastewater constitutes a major release of ARB and contains

various antibiotics, which may affect the microbial community changes in activated sludge. On the other hand, WWTPs are important receptors for antibiotic residues and ARB [7,15], which may be harmful in WWTPs when untreated effluents from health care facilities are discharged. Thus, the compositional analysis of microbial communities in hospital wastewater is important to investigate the characteristics of ARB in activated sludge and municipal wastewater [6]. Also, little is known regarding the overall microbial population in activated sludge treating hospital wastewater and the abundance of various types of ARB from activated sludge microbes.

Molecular analyses have been used to examine the abundance, diversity, and distribution of ARB in wastewater samples. For example, polymerase chain reaction (PCR), quantitative PCR, and 16S rRNA clone libraries have offered insight regarding microbial populations and ARB. However, these molecular tools have a limited ability to detect low-abundance organisms in wastewater, providing only partial information on the microbial communities in WWTPs [16]. Recently, metagenomic analysis (pyrosequencing)—a new approach [6,17] that overcomes the aforementioned drawback of conventional microbial analysis—has become more popular. A high throughput pyrosequencing analysis can generate hundreds to thousands of sequences and enhance the capacity to analyze low-abundant microorganisms [18]. This approach has been applied to various water and wastewater-related samples [19–21]. To our knowledge, there are few articles using pyrosequencing to conduct a whole microbial analysis of ARB from raw hospital wastewater collected in the activated sludge process.

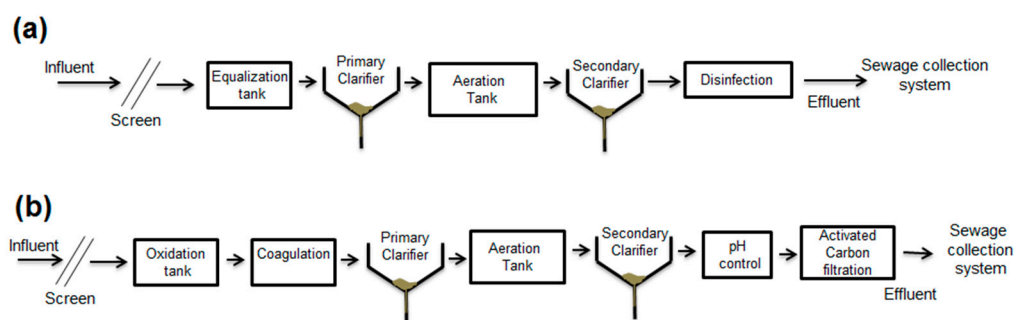
The aim of this study is to understand whole microbial compositions and ARB abundance in a full-scale hospital wastewater treatment process, while comparing the diversity of bacteria in different trains of hospital wastewater treatment. The effect on ARB communities in hospital wastewater and domestic wastewater is also analyzed. This study will increase the general understanding of the characteristics of microbial consortia in hospital wastewater receiving high levels of antibiotics, particularly the relationship between clinic wastewater treatment and domestic wastewater treatment from the hospital.

## 2. Materials and Methods

### 2.1. Description of Hospital Wastewater Treatment Plant

Samples were collected from the activated sludge process at the medical center located in Daegu, South Korea. The medical center serves a population of 2.5 million people, covering more than 35 clinics and possessing a 907 bed capacity. The wastewater treatment plant (WWTP) includes two trains: one for domestic wastewater (from human activity in the hospital) and another for hospital wastewater (from chemicals and surgery) (Figure 1). In the train for domestic wastewater treatment, wastewater passes through a screen and primary clarifier where bulk particles are removed. The wastewater is then directed to a biological treatment process providing the removal of organic matter and nitrogen compounds (nitrification), and is further decanted through a secondary clarifier in which the settled biomass is recycled to the bioreactor and discarded as wasted sludge. Chlorine disinfection is applied for the final effluent prior to discharge. In the train of hospital wastewater treatment, wastewater undergoes a primary treatment including screen, and a coagulation tank where chemicals are used to coagulate tiny particles. The primary clarifier and biological reactor contribute to the removal of solids, organics, and nitrogen. The final effluent passes through activated carbon filter systems which further treat toxic chemicals including organics and heavy metals. The domestic wastewater (DWW) has an average flow rate of 600 m<sup>3</sup>/day and average values of pH, biological oxygen demand (BOD), and suspended solids (SS) of 6.54, 191 mg/L and 52 mg/L, respectively. The DWW includes sources from mainly washing, flushing, and kitchen in the hospital, whereas the hospital wastewater (HWW) consists of chemical and surgical wastes in the hospital. The hospital wastewater (HWW) has an average flow rate of 65 m<sup>3</sup>/day and average values of pH, BOD, chemical oxygen demand (COD), SS, n-Haxane, total nitrogen (T-N), total phosphorus (T-P), Cr<sup>6+</sup>, Cu, Phenol and Pb of 7.3, 176 mg/L,

80 mg/L, 60 mg/L, 1.2 mg/L, 79.1 mg/L, 4 mg/L, 0.195 mg/L, 0.116 mg/L, 1.036 mg/L and 0.098, respectively. Treated effluent is then discharged to a sewage collection system.



**Figure 1.** Schematics of the full-scale hospital wastewater treatment plant. (a) Represents the domestic wastewater (DWW) treatment train; and (b) for the hospital wastewater (HWW) treatment train. The hospital wastewater includes clinics and chemicals.

## 2.2. Water Quality Analysis

Influent and effluent in each train were collected for the measurement of water quality. The samples were stored at 4 °C and analyzed based on the standard methods for water and wastewater [22]. The following water parameters were analyzed by local regulations: BOD and SS for the DWW; BOD, SS, chemical oxygen demand (COD as dissolved) and toxic compounds (including n-Haxane, total phosphorus, total nitrogen, Cr<sup>6+</sup>, phenol, Cu and Pb) for the HWW, respectively. The following number represents part of the number in the standard methods for water and wastewater; BOD (5210), COD (5220), SS (2540), n-Haxane (5520), total phosphorus (4500-P), total nitrogen (4500-N), Cr<sup>6+</sup> (3500-Cr<sup>6+</sup>), phenol (5530), Cu (3500-Cu) and Pb (3500-Pb).

## 2.3. Microbial Analysis

### 2.3.1. DNA Extraction

Samples (DWW and HWW) were collected at the inflow of the secondary clarifier to perform an analysis of the microbial community. Samples stored at 4 °C were immediately moved to the laboratory and prepared for pyrosequencing. Before DNA extraction, the samples were centrifuged at 7000 × g to concentrate biomass, and a 0.25 g pellet was used for DNA extraction. Genomic DNA was extracted using a Soil DNA isolation Kit (MoBioLaboratories, Inc., Carlsbad, CA, USA) according to the manufacturer's protocol. The extracted DNA samples were stored at −20 °C prior to the pyrosequencing analysis.

### 2.3.2. 454 High-Throughput Pyrosequencing

The bacterial communities in domestic wastewater and clinic wastewater of the hospital were examined using 16S rRNA gene-based pyrosequencing. All genomic DNA samples were diluted to 100 ng/μL. For PCR reaction, amplifications were performed in a final volume of 50 μL containing 10 × Taq buffer, dNTP mixture (Takara, Shiga, Japan), 10 μM of each barcoded fusion primer [23], and 2 U of Taq polymerase (ExTaq, Takara, Shiga, Japan) by a C1000 Touch thermal cycler (Bio-Rad, Hercules, CA, USA) under the following temperature conditions: initial denaturation at 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, primer annealing at 55 °C for 30 s, extension at 72 °C for 30 s and a final elongation step at 72 °C for 7 min. The PCR product was confirmed by 2% agarose gel electrophoresis and visualized under a Gel Doc system (Bio-Rad). The amplified products were purified with a QIAquick PCR purification kit (Qiagen, Valencia, CA, USA) and quantified using a PicoGreen dsDNA Assay kit (Invitrogen, Carlsbad, CA, USA). All amplicons were pooled and

purified using an AMPure bead kit (Agencourt Bioscience, Beverly, MA, USA) and then amplified on sequencing beads by emulsion PCR. The recovered beads were sequenced using a Roche/454 GS FLX system (Roche, Brandford, CT, USA).

### 2.3.3. Biodiversity Analysis and Antibiotic Resistance Gene Database

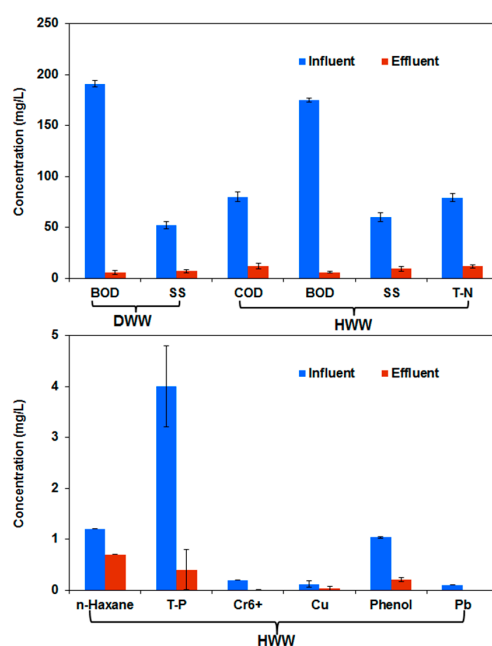
Raw sequence data was processed by previously reported literature [24]. According to the demultiplexing step, low quality reads (read length <300 bp) were removed for further quality analysis. Pairwise sequence alignment and the hmm-search program of the HMMER package [25] were used to trim primer sequences. Sequencing errors were corrected by representative sequences in clusters of trimmed sequences. Each read was assigned for taxonomic positions according to the highest similarity [26]. Sequences were then denoised and chimeras were removed. The compositions and proportions of bacteria were calculated using CL (Chun Lab.) community software (Chunlab, Inc., Seoul, Korea).

In order to match a database of antibiotic resistance bacteria with the Antibiotic Resistance Database (ARDB, version 1.1) [27], the genus data obtained from pyrosequencing analysis were used and manually searched on the ARDB.

## 3. Results and Discussion

### 3.1. Water Quality in a Full-Scale Hospital Wastewater Treatment

Water quality data for one year is shown in Figure 2. BOD and SS were measured for the DWW samples since those two parameters are included in water quality regulation. However, water parameters (including COD, BOD, SS, n-Haxane, T-N, T-P, Cr<sup>6+</sup>, Cu, Phenol and Pb) for the HWW samples were measured to meet the sewage discharge limit, for which the effluent of clinic hospital wastewater has a strict discharge limit. BOD removals were 97.0% and 96.6% for the DWW and HWW, respectively. The removal efficiency of toxic chemicals varied from 41.7% to 100%, indicating that combination of chemical–biological treatment and activated carbon adsorption effectively removes hospital wastewater. In spite of the efficient combination, 42% removal of n-Haxane and 67% removal of copper were observed, as certain compounds (i.e., metals) are resistant to the carbon adsorption process [28].



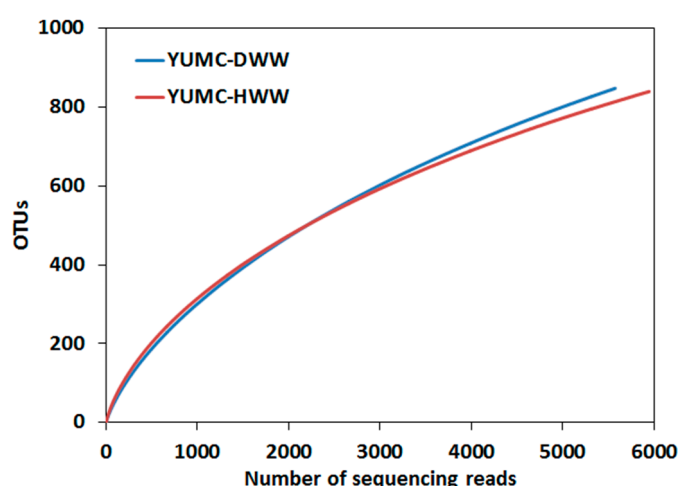
**Figure 2.** Influent and effluent characteristics of wastewater treatment. Data for one year were averaged with standard deviations. (DWW: domestic wastewater, HWW: hospital wastewater).

### 3.2. Microbial Diversity and Comparison in the DWW and the HWW

Pyrosequencing data shows 9218 and 10,990 effective sequence tags, resulting in a total of 20,118 sequences from two samples (Table 1). The rarefaction curves (plots of operational taxonomic units (OTUs) number versus sequence number) are shown in Figure 3 and are determined by a 3% nucleotide cut-off. In terms of the OTUs number, the DWW had a diversity of 848 OTUs and the HWW had 840 OTUs, indicating that the microbial community was highly complex and diverse. The estimators of Chao1 and Shannon index were used to evaluate the richness and diversity of the microbial community, respectively. Compared to the DWW sample, the HWW displayed less richness (Chao 1 estimation). The Shannon index represents not only the species richness (the number of species present) but also the abundance of each species (the evenness of the species) among all the species in the community. The HWW had greater diversity (Shannon = 5.24) than the DWW (Shannon = 4.71). These results indicate that the high-throughput sequencing method reveals a wider range of microbial diversity than conventional sequencing methods.

**Table 1.** Summary of pyrosequencing: richness and diversity of the bacterial phylotypes.

| Samples | Total Reads | Analyzed Reads | Read Length (bp) |                | Observed OTUs | Chao1 Estimation | Shannon Index | Goods Coverage |
|---------|-------------|----------------|------------------|----------------|---------------|------------------|---------------|----------------|
|         |             |                | Mean Length      | Maximum Length |               |                  |               |                |
| DWW     | 9128        | 5572           | 478              | 642            | 848           | 1493             | 4.71          | 0.921          |
| HWW     | 10,990      | 5944           | 472              | 673            | 840           | 1414             | 5.24          | 0.932          |

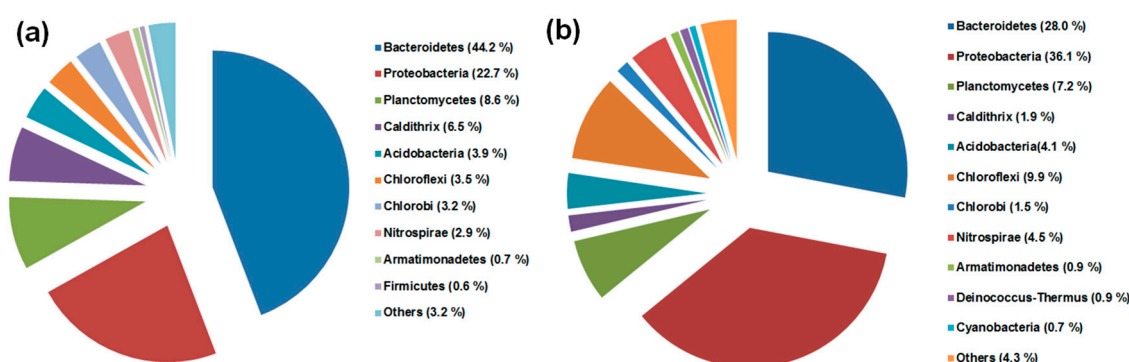


**Figure 3.** Rarefaction curves of OTUs clustered at 97% sequence identity for samples.

Figure 4a shows the microbial diversity and distribution at the phylum level in the DWW. *Bacteroidetes* (44.2%), *Proteobacteria* (22.7%), *Planctomycetes* (8.6%), *Caldithrix* (6.5%), *Acidobacteria* (3.9%), *Chloroflexi* (3.5%), *Chlorobi* (3.2%), *Nitrospirae* (2.9%), *Armatimonadetes* (0.7%), and *Firmicutes* (0.6%) were the 10 most dominant phyla; others (3.3%) accounted for less than 0.5% of the total phyla. In the HWW (Figure 4b), *Bacteroidetes* (28.0%), *Proteobacteria* (36.1%), *Planctomycetes* (7.2%), *Caldithrix* (1.9%), *Acidobacteria* (4.1%), *Chloroflexi* (9.9%), *Chlorobi* (1.5%), *Nitrospirae* (4.5%), *Armatimonadetes* (0.9%), *Deinococcus-Thermus* (0.9%) and *Cyanobacteria* (0.7%) were the eleven most abundant phyla with others (4.2%) accounting for less than 0.5% of the total. There was a difference in bacterial population and community structure in the two mixed liquors. For example, the abundance of *Bacteroidetes* in the DWW was ~1.6 times higher than in the HWW, whereas the abundance of *Proteobacteria* in the HWW was ~1.6 times higher than in the DWW, indicating that the characteristics of influent may affect the diversity and abundance of the microbial community in the activated sludge processes. Typically, the *Bacteroidetes* includes large portions of gram-negative, rod-shaped and nonsporforming bacteria

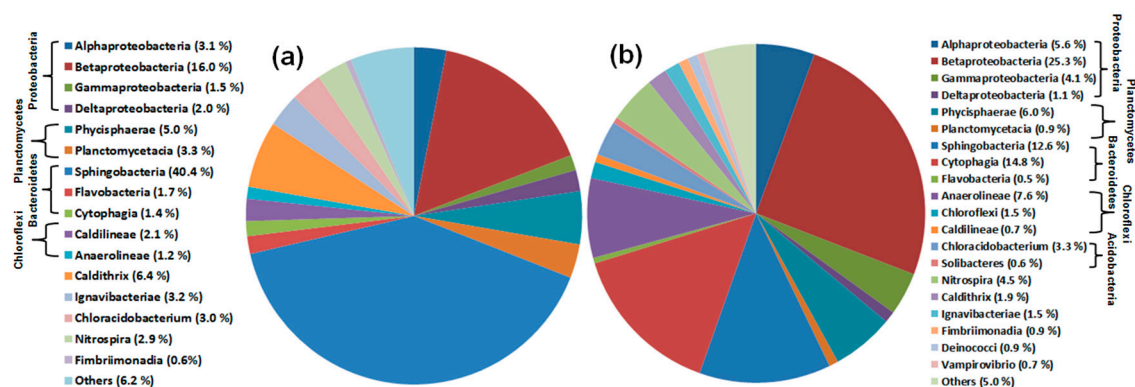


which are widely present in soil and wastewater. The *Proteobacteria* is gram-negative, pathogenic and nitrogen-fixable. In terms of antibiotic resistance properties, Yang et al. [6] reported that *Proteobacteria* was one of the dominant phyla on the fate of antibiotic resistance genes in influent, effluent, and activated sludge. In addition, some *Bacterioides* species harbored both conjugative plasmids and transposon, providing a resistance to erythromycin and tetracycline [29]. The *Planctomycetes* were present in both wastewaters, which are naturally resistant to rifampicin by peptidoglycan synthesis inhibitors including  $\beta$ -lactams, glycopeptides and D-cycloserine [30]. A higher abundance of phylum *Chloroflexi* was observed in the HWW, compared to the DWW. The phylum *Chloroflexi* was found in the downstream, receiving the treated penicillin and oxytetracycline production wastewater under long-term antibiotic pressure [31]. The phyla *Deinococcus–Thermus* and *Cyanobacteria* were only present in the HWW, indicating that those two phyla were not reported previously in hospital wastewater.



**Figure 4.** Phylum distribution of microbial communities. Minor phyla less than 0.5% of total sequences are grouped as “others”. (a) Mixed liquor from domestic wastewater (DWW); and (b) mixed liquor from hospital wastewater (HWW) in the hospital wastewater treatment plant.

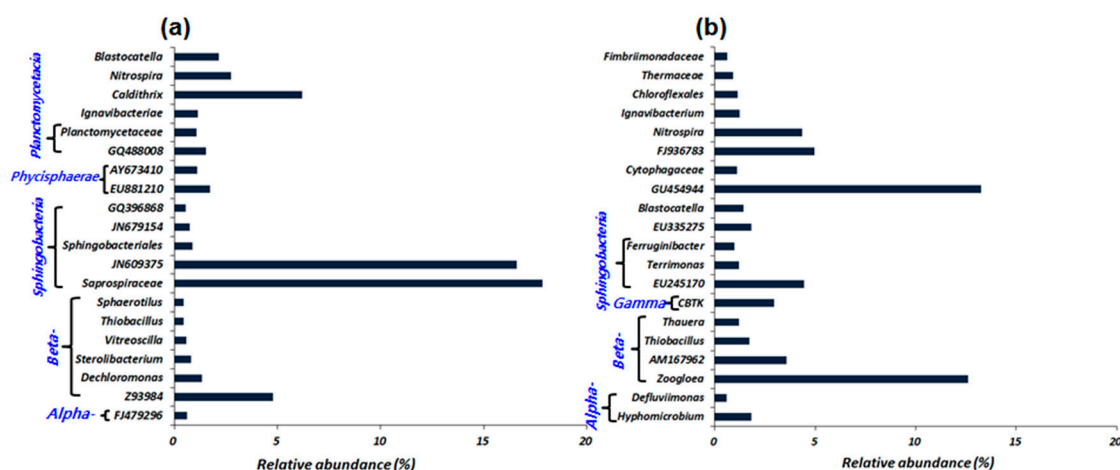
A taxonomic classification of the dominant class levels, which account for more than 0.5% of total sequences, is shown in Figure 5. High-throughput pyrosequencing detected 81 bacterial classes, belonging to 16 (DWW) and 20 (HWW) classes. The relative abundance of classes including less than 0.5% of the total is grouped as others. Among phylum *Proteobacteria*,  $\beta$ -*Proteobacteria* (16%) were dominant classes in the DWW, while  $\alpha$ - (5.6%) and  $\beta$ - (25.3%) *Proteobacteria* were profound classes in the HWW.  $\beta$ -*Proteobacteria* was the most abundant class in the activated sludge bioreactor treating pharmaceutical residues [32], which corresponds to the microbial community in the HWW. As a result, the HWW may contain high antibiotic levels affecting the richness of  $\beta$ -*Proteobacteria*. *Sphingobacteria* belonging to the phylum *Bacteroidetes* was 40.4% and 12.6% for the DWW and the HWW, respectively. *Sphingobacteria* is known to possess a tetracycline resistance gene which harbors a functional *tet* (X) gene for the degradation of tetracycline [33,34]. In addition, *Sphingobacteria* could form a biofilm and contribute to the treatment of ammonium-rich wastewater [35]. Interestingly, *Cytophagia* (14.8%) and *Anaerolineae* (7.6%) were predominant classes in the HWW, compared to the DWW (1.4% *Cytophagia* and 1.2% *Anaerolineae*). *Anaerolineae* was reported as having high resistance to antibiotics (i.e., tetracycline) [31,36], corresponding to the characteristics of the HWW receiving major antibiotics from hospital chemicals and clinics. In terms of taxonomic classification of class levels, influent characteristics seem to affect microbial richness and diversity, but further investigation is required regarding what types of antibiotics influence microbial communities.



**Figure 5.** Taxonomic classification of pyrosequences at class levels. Minor classes accounting for <0.5% of total sequences are grouped as “others”. (a) Mixed liquor from the DWW; and (b) mixed liquor from the HWW.

### 3.3. Correlation of Wastewater Type and Antibiotic Resistance Bacteria

At genus levels, a total of 374 and 334 genera were identified in the DWW and HWW, respectively. Among the identified genera, the difference among bacterial communities including top twenty abundances is shown in Figure 6. Two distinct genera were present; *Saprospiraceae* (17.8%) and JN609375 (16.6%, class *Sphingobacteria*) in the DWW, and *Zoogloea* (12.6%) and GU454944 (13.3%, class *Cytophagia*) in the HWW. *Saprospiraceae* were commonly shared by activated sludge samples [37] and were found in enhanced biological phosphorus removal [38]. To our knowledge, *Cytophagaceae* (family of GU454944) was not reported in published literature, dealing with hospital wastewater. *Zoogloea* organisms are known to possess the mechanisms to resist pharmaceuticals using their exocellular layer [39]. Therefore, it is possible that high concentrations of antibiotics in the HWW resulted in the selective microbial growth of some genera that have a resistance to certain antibiotics.



**Figure 6.** Top twenty genus levels of taxonomic classification with relative abundance: (a) Mixed liquor from the DWW; and (b) mixed liquor from the HWW.

Other than the top twenty genera, minor genera which are resistant to various antibiotics were identified from the Antibiotic Resistance Gene DataBase (ARDB), classifying ARG-like sequences using customized scripts [6]. From the ARDB, five genera in the DWW and 15 genera in the HWW were matched to the database (Table 2), suggesting that raw hospital wastewater possesses various antimicrobial properties. The genera identified in the DWW showed multi-drug resistance, for example, *Aeromonas* can be resistant to antibiotics such as tetracycline, chloramphenicol, penicillin, cephalosporin,

streptomycin and dibekacin. However, nine genera showed multi-drug resistance and six were matched to a single antibiotic resistance in the HWW. *Streptococcus*, widely known as an antibiotic resistance bacteria [40], was matched to the following antibiotics; Amikacin, butirosin, gentamicin, isepamicin, kanamycin, lividomycin, neomycin, paromomycin, ribostamycin and streptomycin. Six genera were identified to tetracycline resistance, which is highlighted because tetracycline is one of the most widely used therapeutics in human and veterinary medicine [41]. Although the database of antibiotic resistance genes is well-established, the information on the type of antibiotic resistance and its genes in hospital wastewater is very limited. Thus, the top twenty genera in Figure 6 were not able to search using the customized script of the ARDB.

Microbial communities in WWTPs are highly complex and many microorganisms are culture-dependent. Although complex microbial communities in bioreactors were examined using pyrosequencing [19,21], limited study of the ARB community was performed using conventional molecular methods. Therefore, it is necessary to understand the ARB profile and microbial distribution in WWTPs treating hospital and urban waste sources and to provide clear information concerning the control of ARB during the operation of activated sludge processes.

**Table 2.** Genus and antibiotic resistance types matched from the antibiotic resistance gene database (ARDB). Minor genera groups (<0.5% relative abundance) were matched using the ARDB.

| Samples | Genus                   | Relative Abundance (%) | Antibiotic Resistance Type                                                                                             |
|---------|-------------------------|------------------------|------------------------------------------------------------------------------------------------------------------------|
| DWW     | <i>Aeromonas</i>        | 0.084                  | Tetracycline, chloramphenicol, penicillin, cephalosporin, streptomycin, dibekacin                                      |
|         | <i>Azoarcus</i>         | 0.017                  | Bacitracin, chloramphenicol                                                                                            |
|         | <i>Enterobacter</i>     | 0.017                  | Dibekacin, fluoroquinolone, sulfonamide, tetracycline, trimethoprim                                                    |
|         | <i>Lautropia</i>        | 0.168                  | Bacitracin, chloramphenicol                                                                                            |
|         | <i>Paracaedibacter</i>  | 0.034                  | Cephalosporin, tetracycline                                                                                            |
|         | <i>Acetobacteraceae</i> | 0.018                  | Tetracycline                                                                                                           |
|         | <i>Acidovorax</i>       | 0.269                  | Spectinomycin, streptomycin, bacitracin                                                                                |
|         | <i>Bacillus</i>         | 0.054                  | Kanamycin, tobramycin                                                                                                  |
|         | <i>Blautia</i>          | 0.018                  | Tetracycline                                                                                                           |
|         | <i>Clostridiales</i>    | 0.018                  | Tetracycline                                                                                                           |
| HWW     | <i>Coproccoccus</i>     | 0.018                  | Bacitracin, tetracycline                                                                                               |
|         | <i>Enterobacter</i>     | 0.054                  | Dibekacin, fluoroquinolone, sulfonamide, tetracycline, trimethoprim                                                    |
|         | <i>Escherichia</i>      | 0.072                  | Dibekacin, amikacin, cephalosporin, cloxacillin, tetracycline                                                          |
|         | <i>Lactobacillus</i>    | 0.036                  | Bacitracin, chloramphenicol                                                                                            |
|         | <i>Parabacteroides</i>  | 0.036                  | Cephalosporin, tetracycline                                                                                            |
|         | <i>Polynucleobacter</i> | 0.018                  | Bacitracin                                                                                                             |
|         | <i>Proteus</i>          | 0.018                  | Astromicin, gentamicin, sisomicin                                                                                      |
|         | <i>Ralstonia</i>        | 0.126                  | Bacitracin                                                                                                             |
|         | <i>Streptococcus</i>    | 0.036                  | Amikacin, butirosin, gentamicin, isepamicin, kanamycin, lividomycin, neomycin, paromomycin, ribostamycin, streptomycin |
|         | <i>Thauera</i>          | 0.413                  | Bacitracin                                                                                                             |

Note: Genera of more than 0.5% relative abundance could not match with the ARDB due to the lack of database.

#### 4. Conclusions

Pyrosequencing analysis revealed broad microbial diversity and distribution in the hospital wastewater treatment plant. In total, 20,118 effective sequence tags were identified; 9218 sequence



tags for the DWW and 10,990 sequence tags for the HWW, respectively. Wastewater type affected the microbial community and ARB compositions, and hospital wastewater contains a greater diversity of ARB based on the ARDB search (i.e., <0.5% relative abundance). The combination of pyrosequencing-based methods coupled with the ARB database can provide useful information regarding antibiotic resistance communities of wastewater treatment systems.

**Acknowledgments:** This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT (Information/Communication Technology) and Future Planning (NRF-2014R1A1 A1002596).

**Author Contributions:** Youngho Ahn and Jeongdong Choi have contributed to the writing of this paper by performing the review of relevant literature. Youngho Ahn and Jeongdong Choi developed the concept of experimental idea and analyzed microbial community data.

**Conflicts of Interest:** Authors declare that there is no conflict of interest of regarding the publication of this paper.

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