Exploration of Bacterial Diversity in Hospital Sludge Extracted Using a Modified Sampling Device Through High-Throughput Sequencing Targeting the 16S rRNA Gene

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Abstract: The over-use of chlorine-based disinfectants and antibiotics decreases the effectiveness of sewage treatment, causing dominance of *Pseudomonas* in hospital sewage treatment plants. This study investigated bacterial species, abundance, and distribution in anaerobic sludge under different spatial distributions (drainpipe wall-attached sludge and floating sludge samples) in a hospital sewage treatment plant to establish a rapid method for identifying bacteria, monitoring microorganisms, and evaluating sewage treatment capacity. On April 11, 2022, 15 sludge samples were obtained from multiple points in the plant using a modified sampling device. Microbial taxon distribution and sludge sample diversity were analyzed via high-throughput sequencing of the 16S rRNA gene V3–V4 region amplicon. α-diversity, β-diversity, and relative abundance at the phylum and genus levels were calculated using QIIME2 and R software. In total, 409,705 high-quality sequences were statistically identified in 67 phyla, 165 classes, 317 orders, 449 families, 644 genera, and 1132 species. SNK-q test revealed significant differences in the dominant phyla and genera between different locations. Increasing sampling depth resulted in improved sampling performance, increased diversity and evenness of the community, but decreased *Pseudomonas* detection rate.

Key words: 16s rRNA gene sequencing; V3–V4 region amplicon; Hazardous waste; Hospital sludge; *Pseudomonas*

Introduction

Antibiotic-resistant bacteria in hospital sewage treatment stations mainly originate from inpatients, and they can enter the sewage treatment system through their body fluids and exfoliated tissue cells. *Pseudomonas aeruginosa* is a rod-shaped gram-negative aerobic bacterium and a common pathogen of the respiratory tract and urethra in hospitals. It can be isolated from the body fluids of infected patients. It has been found in all departments of hospitals, especially the intensive care unit, and there are reported cases of infection in the operating room[1]. In the hospital environment, *P. aeruginosa* can spread through all routes of transmission, including encompassing direct contact, respiratory droplets, the gastrointestinal tract, environmental surfaces, and waterborne transmission.

Several methods disinfect pathogens in hospital sewage, and chlorination is a highly recommended sewage disinfection technology widely used in tap water, hospital sewage, and other public water supply systems. The common forms of chlorine-containing disinfectants are sodium hypochlorite and chlorine. Sodium hypochlorite is a traditional disinfectant with high efficiency and economic advantages. Chlorinated disinfectants are routinely used in hospitals to sanitize medical facilities, equipment, and surfaces, including countertops, beds, floors, and laboratory desks in diagnostic and treatment departments. In addition, they can be used to sanitize the environment after cleaning patients' body fluids such as blood or interstitial fluid. For environmental disinfection in fever consulting and isolation rooms, a higher disinfectant concentration of 1000 mg/L is usually used, and a maximum concentration of 5000 mg/L is used in toilet drains[2]. The sanitization process is based on the active component hypochlorous acid, which is generated from the sanitizer once dissolved in water. This small molecule can quickly penetrate the cell membrane of bacteria, leading to death through the inhibition of critical enzymatic reactions and oxidative denaturation of proteins [3].

Bacteria injured by chlorine exposure might display enhanced antibiotic resistance when the sodium hypochlorite concentration is at the 50% lethal dose [4]. Cases of pathogens capable of repairing and regrowing even after disinfection in effluent wastewater have been documented [5]. High-concentration chlorine disinfection does not reduce antibiotic-resistance gene expression in

drug-resistant bacteria and promotes the emergence of chlorine-resistant bacterial strains carrying these genes [6].

These chlorine-tolerant pathogens should be monitored because of their survival and regeneration after chlorination, and policymakers have expressed concerns about the biosafety of chlorination-treated water. Based on the changes in the microbial community structure after hyperchlorination, researchers are exploring the effects of chlorination on the microbial community structure. They identified several chlorine-resistant strains in sewage after high-chlorination treatment[7]. They found that within 1 day after treatment, three chlorine-resistant strains (*Stenotrophomonas maltophilia*, *Citrobacter freundii*, and *Klebsiella* sp.) and two strains (*C. freundii* and *Klebsiella* sp.) with high lethal doses and regrowth inhibition were detected in the sewage. In another study, researchers detected *Pseudomonas* spp. in all samples regardless of disinfection by UV, chlorine, or hybrid treatment, and their counts could not be effectively reduced [8].

A survey conducted from April to May 2021 in ophthalmology and general hospitals in Zhejiang Province revealed considerable variations in bacterial species and abundance within influent and effluent of different hospitals [9]. The analysis focused on the phylum level of bacteria in the influent and effluent in the two hospitals, identifying four predominant phylum levels: *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, and *Actinobacteria*. Moreover, at the generic level, the eye hospital exhibited dominance of *Pseudomonas alcaligenes* (2.41%) and *Entomophila* (2.37%), whereas general hospitals reported the dominance of *Acinetobacter johnsonii* (9.30%) and *Aeromonas caviae* (4.81%).

Chlorine-resistant bacteria under long-term chlorine disinfection will significantly change the structure of microbial communities in the sewage treatment system. The peak usage of chlorine-based disinfectants and antibiotics decreases the effectiveness of sewage treatment, causing *Pseudomonas* to become the dominant genus in hospital sewage treatment plants. Antibiotic-resistant *P. aeruginosa* exhibits a resistance mechanism that prevents all known antibiotics from entering its bacterial cell body. Enzyme resistance limits the activity of antibiotics and potentially reduces the efficiency of sewage treatment. Considering that *Pseudomonas* spp., such as *P. aeruginosa*, are resistant to hybrid disinfection and they can form biofilms, particular

attention should be paid to antibiotic-resistant bacteria in sewage sludge, especially carbapenem-resistant *P. aeruginosa*, during hospital sewage treatment.

For high-throughput analysis of sewage sludge postanaerobic digestion process, biosafety protocols sterile bottles (1–2 L) are commonly used as sampling containers [6, 9, 10, 11]. However, using this size of the container to preserve environmental samples may produce false negative results owing to separation from the original sludge matrix (including humic substances, organic salts, or other chemical ingredients) for a long time after separating from the original sludge [12]. In this study, 15 sludge samples were extracted from a medical wastewater treatment facility in Shenzhen City, Guangdong Province, China, including an anaerobic–anoxic–oxidation (AAO) wastewater treatment reaction tank. During the sampling process, a new sludge sampler with a mechanical timer was utilized. The sampler performed in situ sampling of the drainpipe wall-adhered sludge and floating biomembrane sludge of an anaerobic hospital water treatment plant multiple times. This sampler helps to avoid bacterial contamination and reduces the exposure of monitoring sampling personnel to the sewage environment.

This study performed high-throughput sequencing of the 16S rRNA gene V3–V4 region to study the changes in the bacterial community composition of attached and floating sludge samples collected at different locations and depths in the anaerobic reaction tank near the inlet. In addition, we analyzed the distribution of dominant bacteria in the sludge samples divided into three groups. The sequencing depth of all sludge samples in the anaerobic reaction tank was adequate, permitting metagenomic analysis.

Materials and Methods

Sample Collection

The sludge sampling method used in this study employed a newly developed sludge sampler with waterproof materials that was equipped with a mechanical elastic drive element within its waterproof shell. The sampler works by mechanically squeezing the rubber tube inside the shell with a mechanical timing component at regular intervals, enabling the scheduled sampling of sludge. The sampling process involves inserting the sampling tube into the shell via a hose hole on the top cover. Meanwhile, the sampling device, mounted on the head of a telescopic scale rod, can

be extended up to a depth of 5 m underground. The sampling device can then be rotated and aligned with the predefined surface and subsurface position in the drainpipe wall for sampling. This method permits the effective collection of sludge samples for environmental microbiology research.

Preparation before sampling included the use of a medical particulate respirator (Winner Medical Co., Ltd, Huanggang, China), medical disposable coverall (Winner), medical inspection gloves (Winner), single-use medical rubber examination gloves (Winner), disposable caps (Winner), medical alcohol, flashlight (built-in monitoring head), goggles, waterproof shoes, brand-new sampling device, telescopic rod, test-tube rack, 4.5-mL sterile Nunc™ CryoTube™ vials (Thermo Fisher Scientific, Waltham, MA, USA), a sterile transfer pipette (Beckman-Coulter, Brea, CA, USA), and a battery-powered cooling refrigerator.

On April 11, 2022, sampling was conducted at the embedded sewage disinfection treatment facility (Shenzhen, China), which implemented an improved AAO process, and the anaerobic reaction tank could be inspected through two inspection wells. The anaerobic reaction tank was divided into closer and remote inspection wells according to the proximity to the inlet of influent sewage. With secondary protection, upon entering the hospital's sewage treatment plant, the environmental sampling and monitoring personnel, together with our group, selected the closer inspection well and collected sludge samples by lifting the inspection manhole cover through the maintenance hole located at the drain of the anaerobic tank. The liquid level in the tank drain was 3 m below the maintenance cover, and then the sludge sampler head was installed on the telescopic sampling rod. A disposable sterile sampling tube was placed inside the sampler, and the sampler was placed on the predefined site on the drainpipe wall.

Sample Categorization

A, B, and C sample groups as defined above the corresponding sits on A, B, and C plane. Six predefined sampling sites on the B plane of the pipe wall were located 1 m below the liquid sewage level, and three sampling sites on the C plane were on the floating object at the liquid sewage level. One tube of sludge sample was collected at each sampling site.

At the inlet inside the anaerobic tank drainpipe, which was regarded as the initial point of the

circumferential plane of the drainpipe wall, the circular wall plane could be divided into 12 equal points with a 30° angle per division. Point 1 corresponds to sampling sites 1 and 13, point 2 corresponds to sampling sites 2 and 14, point 3 corresponds to sampling sites 3 and 15, and points 4–12 correspond to sampling sites 4–12. The sampling angle could be calculated by multiplying the value of the sampling site name by 30°. The collected samples were labeled according to the corresponding positions of the sampling sites.

Along the pipe wall of the sewage liquid level (A plane), six samples were sampled via clockwise rotation around the wall surface of the drainpipe. The samples' names corresponded to the values of the corresponding sampling site, and they were labeled as samples 1, 2, 3, 10, 11, and 12 (Group A). After sinking 1 m below the liquid sewage level, six samples were collected at this level (B plane) and labeled as samples 4, 6, 8, 13, 14 and 15 (Group B). The three samples from the floating sludge (C plane) were labeled as samples 5, 7, and 9 (Group C).

After each sampling, the tube in the sampling device was removed, and the sludge sample inside the tube was carefully transferred to a sterile cryogenic tube, covered with the tube cap, and then placed into a double-sealed plastic bag. All specimens were stored in a −20℃ refrigerated transport box and transported to the laboratory for DNA extraction.

DNA Extraction

After thawing the cryopreserved sludge samples at room temperature, the total genomic DNA of all samples was extracted according to the instructions of the iPure® DNA extraction kit. The DNA concentration and purity were monitored using Qubit® fluorometers, and the quality of DNA extraction was monitored via 2% agarose gel.

The DNA extracts were stored in a dry-ice box and sent to Shanghai Sangon Biotech Company for experimental and molecular biology analyses. The analysis featured several steps, including DNA extraction of the entire microbiome, amplification of specific fragments, purification of amplification products, quantification of fluorescence, construction of a MiSeq library, and sequencing of the samples on the Illumina MiSeq platform, with the ultimate goal of analyzing the microorganisms found in the 15 samples.

DNA Purification and PCR Amplification

PCR amplification of bacteria in 15 sludge samples was performed by targeting the 16S rRNA gene V3–V4 region with the primer pair 341F (5′-CCTACGGGNGGCWGCAG-3′)/805R (5′-GACTACHVGGGTATCTAATCC-3′). The first PCR reaction system (30 μL) comprised 15 μL of 2× Hieff® Robust PCR Master Mix, 1 μL each of positive and reverse primers, 10–20 ng of PCR products, and 10 μL of ddH2O. The PCR program was as follows: 94°C for 3 min; five cycles of 94°C for 30 s, 45°C for 20 s, and 65°C for 30 s; 20 cycles of 94°C for 20 s, 55°C for 20 s, and 72°C for 30 s; and 72°C for 5 min. The reaction system for second-round PCR (30 μL) comprised 15 μL of 2× Hieff® Robust PCR Master Mix, 1 μL each of positive and reverse primers; 20–30 ng of PCR products, and 10 μL of ddH2O. The PCR program was as follows: 94°C for 3 min; five cycles of 94°C for 20 s, 55°C for 20 s, and 72°C for 30 s; and 72°C for 5 min.

Molecular Biology Testing

Using the Illumina MiSeq PE300 platform, PCR products were monitored via 2% agarose gel electrophoresis. The obtained raw data were uploaded to the NCBI Sequential Read Archive database under the login number PRJNA935118. High-throughput sequencing of PCR samples was performed on the Illumina MiSeq PE300 platform by Shanghai Sangon Biotech Company with a maximum read length of 300 bp per end.

Data Analysis

Cutadapt software was used to cut and filter the original sequences obtained (filter bases with a tail mass value smaller than 20, the minimum overlap length of 5 bp, and a maximum allowed mismatch rate of the overlap region of 0.2). The QIIME2 [13] software platform was used to implement subsequent analyses, remove chimeras, perform clustering in the DADA2 plug-in [14], annotate taxonomy information, and evaluate the taxonomy of community diversity and similarity [10]. The chosen depth of sequences was unified as the minor sequence depth across all samples before the diversity index calculation. The RESCRIPT-CLASSIFYING-SKLEARN algorithm was used to classify the species [15] based on the 16S rRNA gene V3–V4 region and annotate them in the SILVA rRNA database (Release 138.1, http://www.arb-silva.de). The species classification table was exported after unifying rarefaction.

The Qiime2R plug-in function in R was used to compute the Chao1, Shannon, and Simpson indices to indicate sample diversity[16]. The rarefaction curves were used to determine whether the current sequencing volume accurately represented the original community's diversity. Weighted and unweighted principal component analysis [17] among the three groups based on UniFrac distances was used to visualize the β-diversity of the overall microbial community structure.

Results

Statistical Analysis of the Sequences

Statistical analysis of the original data and clean reads obtained by quality control treatment was performed to obtain basic information about the data. In total, 989,951 effective sequences were obtained from 15 samples, and 409,705 high-quality sequences were collected after filtering and removing the chimeras. Among the samples, sample 15 had the highest number of sequences (48,677), whereas sample 9 had the least number of sequences (10,295).

By counting the ASVs, the specific composition of the microbial community in each sample at seven taxonomic levels (domain, phylum, class, order, family, genus, and species) was identified, and the numbers of taxonomic units contained in the species annotation results of these samples was counted.

Microbial Community Composition Assessment

By counting the amplicon sequence variants (ASVs) in each sample, we created the specific composition table of the microbial community. In the species annotation results of 15 sludge samples, the number of taxonomic units present at seven taxonomic levels (domain, phylum, class, order, family, genus, and species) was determined.

In total, 75 phyla, 190 classes, 408 orders, 602 families, 917 genera (genera distribution is shown in Figure 1), and 1850 species were identified. After eliminating species with fewer than two isolates, 67 phyla, 165 orders, 317 orders, 449 families, 632 genera, and 1132 species were identified.

By taking the species number of all taxa as statistical variables and performing one-way analysis of variance (ANOVA) and SNK-q test among the three groups, no significant differences in the

number of species were detected between Groups A and C at the phylum, class, order, family, genus, and species levels. However, the number of microbial units was significantly higher in Group B than in Group A/C at each classification level.

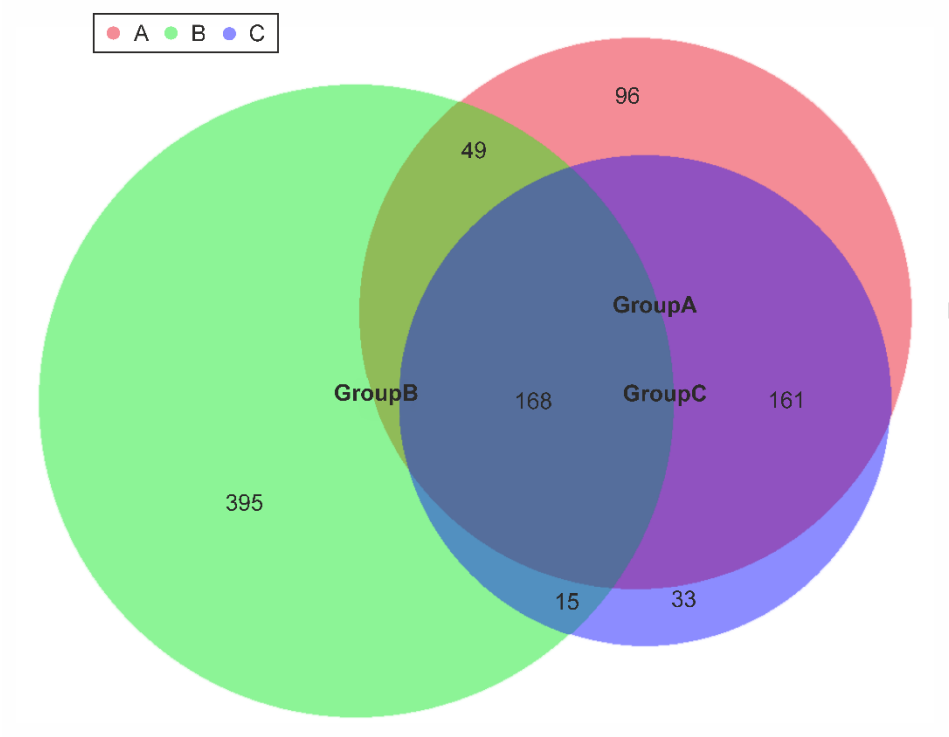

Figure 1. Venn diagram of the microbial composition at the genus level.

Phylum-Level Composition

The RDP and BLAST homologous sequence clustering methods were employed using a relative abundance greater than 0.1% as the criterion for identifying dominant phyla(relative abundance at the phylum level is shown in Figure 2).

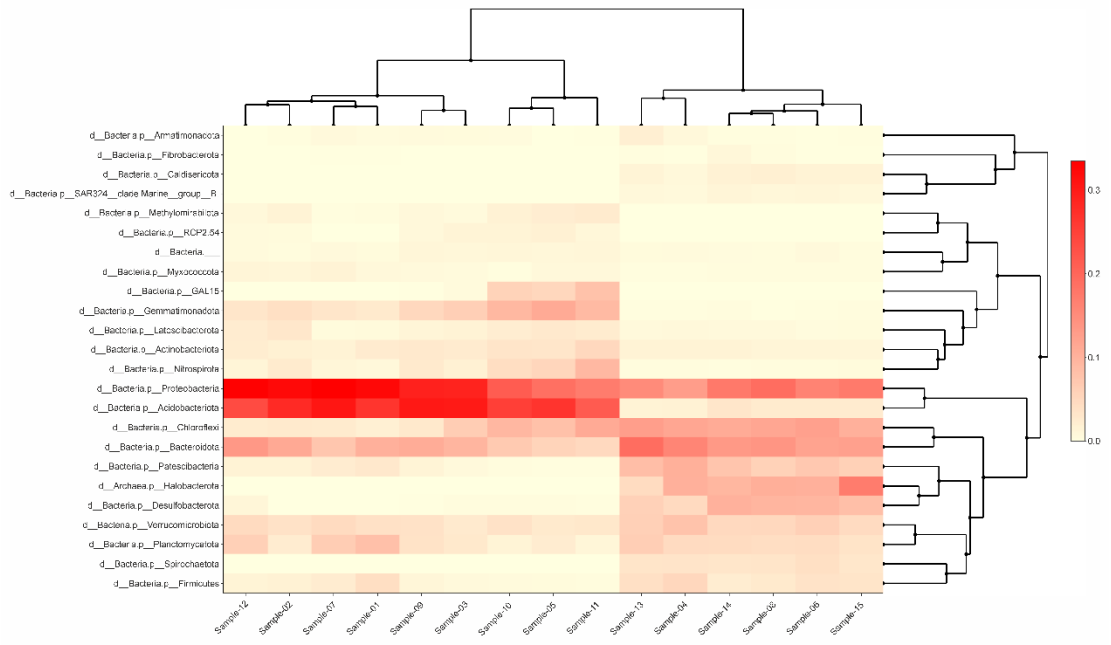

Figure 2. Bar plot of the relative abundance at the phylum level based on unified depths

NOTE: The X-axis presents the name of each sample, and the Y-axis presents the relative abundance of each taxonomic unit at the phylum level.

Fourteen phyla were identified in Group A, including *Proteobacteria*, *Acidobacteriota*, *Bacteroidota*, *Gemmatimonadota*, *Chloroflexi*, *Planctomycetota*, *Verrucomicrobiota*, *Nitrospirota*, *Actinobacteriota*, *GAL15*, *Latescibacterota*, *Firmicutes*, *Methylomirabilota*, and *Patescibacteria*, with the top three being *Proteobacteria* (27.32% average relative abundance), *Actinobacteria* (25.80%), and *Bacteroidetes* (9.27%). Notably, there was significant consistency in the prevalence and abundance of *Proteobacteria*, which emerged as the most dominant phylum in all samples in Group A.

In Group B, 13 dominant phyla were identified, namely *Proteobacteria*, *Bacteroidota*, *Chloroflexi*, *Halobacterota*, *Desulfobacterota*, *Patescibacteria*, *Verrucomicrobiota*, *Planctomycetota*, *Firmicutes*, *Spirochaetota*, *Acidobacteriota*, *Caldisericota*, and *Actinobacteriota*. The top three phyla were *Proteobacteria* (average relative abundance of 16.43%), *Bacteroidetes* (14.43%), and *Chloroflexi* (11.62%). Notably, all samples in Group B exhibited a high degree of phylum-level consistency, with *Proteobacteria* being the most prevalent and abundant phylum.

In Group C, 15 dominant phyla were identified, including *Acidobacteriota*, *Proteobacteria*,

Bacteroidota, *Gemmatimonadota*, *Chloroflexi*, *Planctomycetota*, *Verrucomicrobiota*, *Nitrospirota*, *Actinobacteriota*, *GAL15*, *Latescibacterota*, *Patescibacteria*, *Firmicutes*, *Methylomirabilota*, and *Myxococcota*. *Acidobacteriota* (29.33% average relative abundance), *Proteobacteria* (27.53%), and *Bacteroidetes* (8.16%) were three most abundant phyla. There was a consistency among samples in Group C at the phylum level, with *Acidobacteriota* being the most prevalent and abundant.

Genus-Level Composition

Among Group A samples (relative abundance at the genus level is shown in Figure 3), *RB41* was the most common and dominant genus, with a relative abundance of 7.54%. In Group B, *Methanosaeta* predominated with a relative abundance of 8.99%, whereas in Group C, *Sphingomonas* predominated with a relative abundance of 7.33%. Specifically, *Pseudomonas* was the predominant genus in samples 2, 5, 9, and 10, with relative abundances of 1.26%, 1.18%, 1.16%, and 1.05%, respectively.

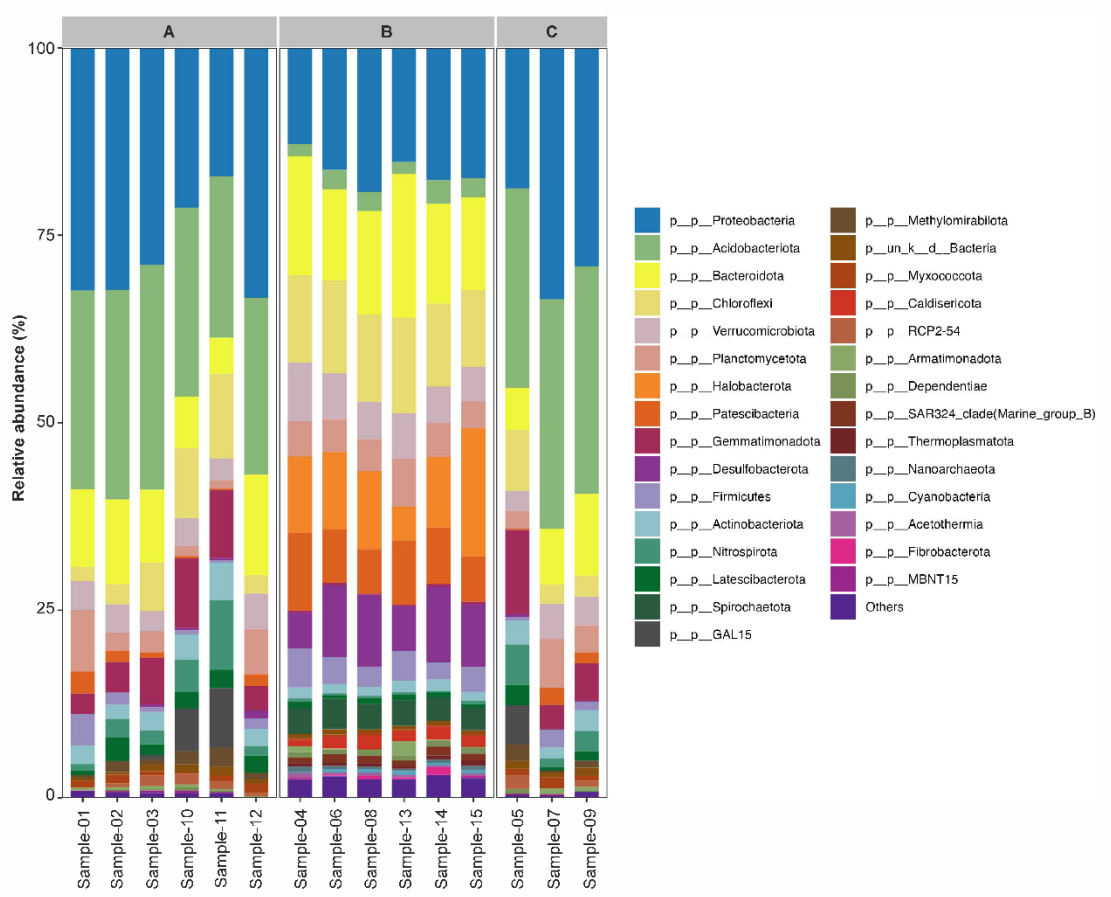

Figure 3. Bar plot of the relative abundance at the genus level based on unified depths

NOTE: The X-axis presents the name of each sample, and the Y-axis presents the relative abundance of each taxonomic unit at the genus level.

ANOVA of the abundance of *Pseudomonas* in the three groups revealed the following data: $F_{2,12} = 10.1$.. Meanwhile, the F-value table showed that $p < 0.05$. The difference in the abundance of *Pseudomonas* spp. among the three groups was statistically significant. The SNK-q test was used to analyze the relative abundance of *Pseudomonas* in the three groups, and no significant difference in abundance was identified between Groups A and C. However, the difference between Group B and Group A/C was statistically significant.

Escherichia coli, *Shigella*, *Salmonella*, *Vibrio*, *Proteus*, *Clostridium*, *Yersinia*, and *Staphylococcus* were not detected in any sludge samples. This indicates that the sewage treatment plant's function is reasonable.

The composition and distribution of microbes in each sample at the phylum level were visualized via statistical analysis of the feature table and presented in a histogram. After removing species with counts lower than two, clusters were generated according to the abundance information of the remaining species and samples. Heat maps were utilized to identify species aggregation in each sample. The outcomes at the phylum level are illustrated in Figure 4.

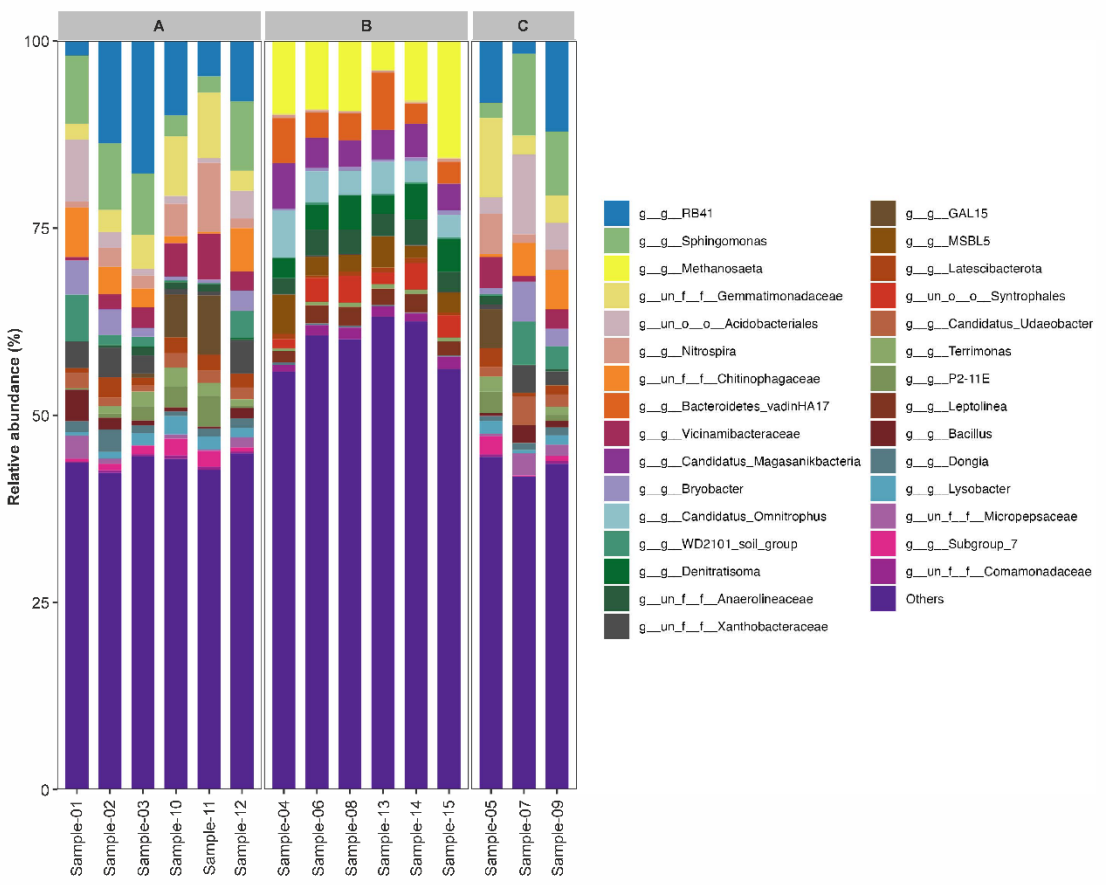

Figure 4. Hierarchical clustering analysis of frequent phyla based on the average depth of the relative abundance.

Note: The X-axis presents the name of each sample, and the Y-axis presents species annotation information.

α-Diversity Analysis

The Chao1 indices of Groups A, B, and C were 372.58 ± 55.85, 1132.92 ± 55.13, and 363.87 ± 76.91, respectively. The Shannon indices of these groups were 7.90 ± 0.26, 8.74 ± 0.12, and 7.87 ± 0.37, respectively(alpha diversity within the groups is shown in Figure 5). Group B exhibited significantly higher Chao1 and Shannon indices than Groups A and C, whereas no differences were noted between Groups A and C.

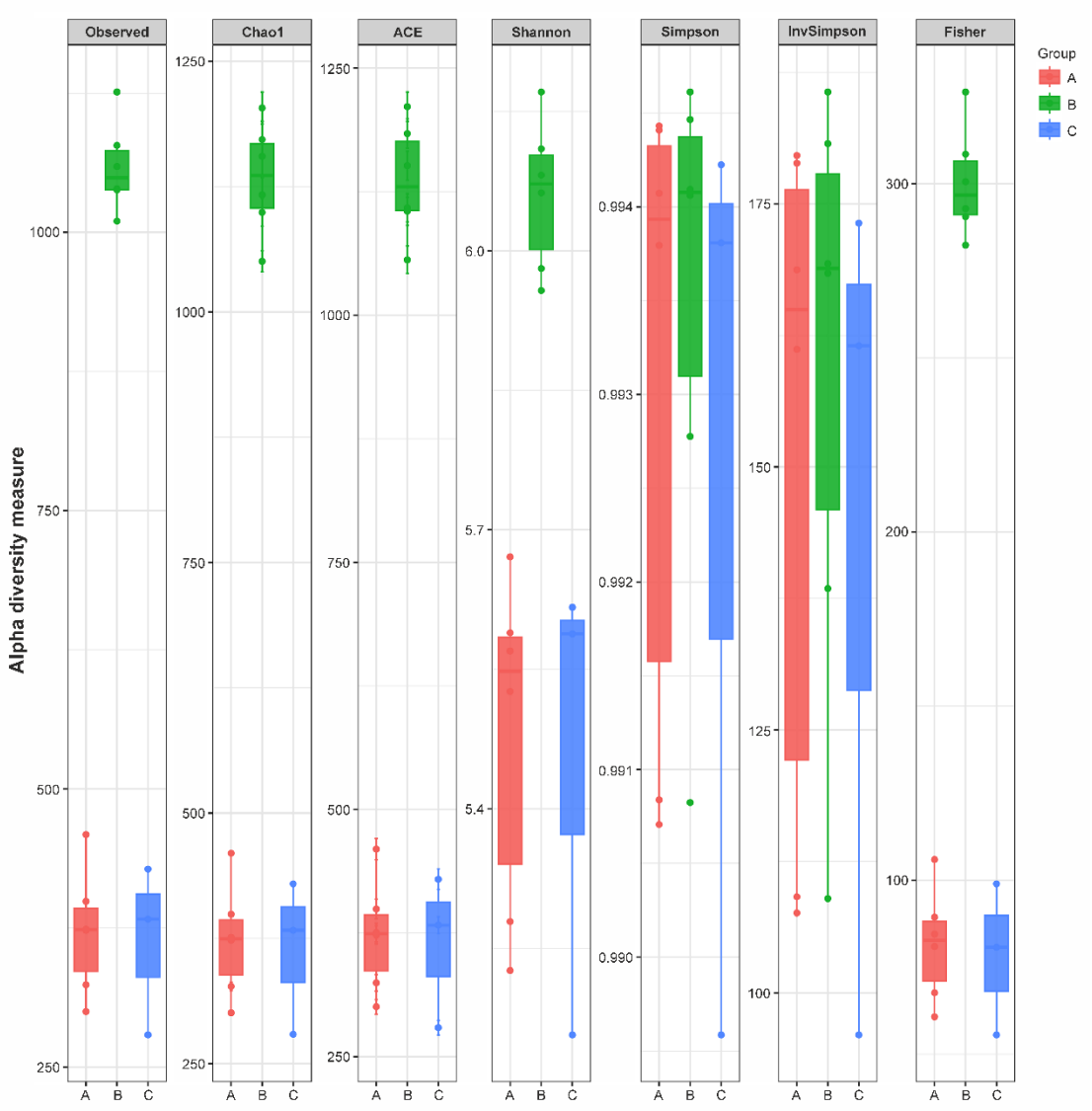

Figure 5. Box plot of alpha diversity within the groups.

The coverage index was approximately 0.98–1.00 for all 15 samples, indicating that the sequencing depth of the collected sequences sufficiently reflected the bacterial biodiversity and community characteristics in the anaerobic system.

β-Diversity Analysis

Based on the species abundance at different taxonomic levels, principal coordinate analysis (PCoA) was conducted. In the analysis, a closer position on the PCoA map between two samples indicated a more similar species composition (PCoA map is shown in Figure 6).

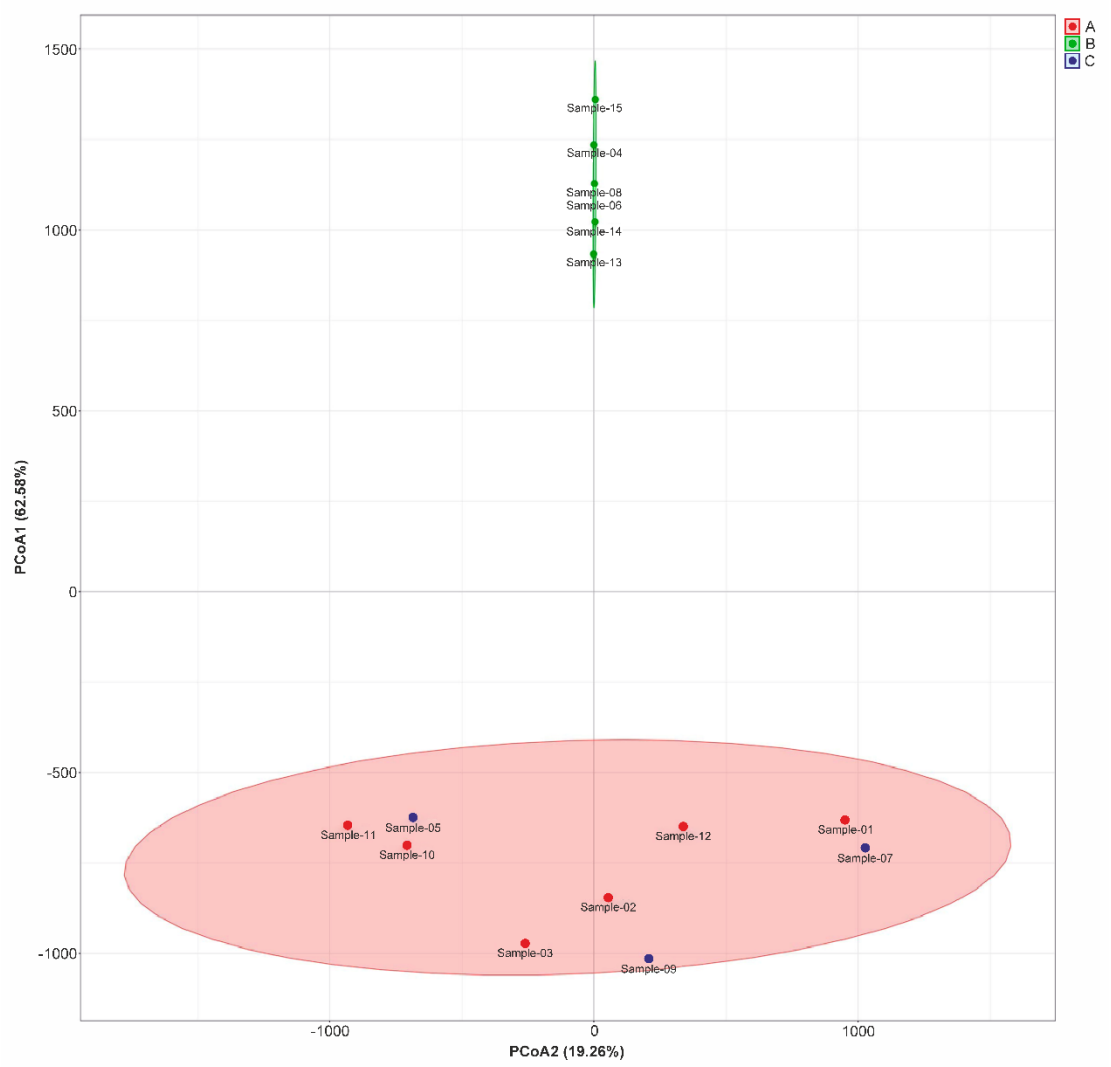

Figure 6. Principal coordinate analysis map.

The distance between Group B and Groups A and C was significantly different, but Groups A and C exhibited relatively close proximity on the map, indicating a similar bacterial community composition between these two groups, possibly attributable to the proximity of the sampling sites. Spatial location was the main factor affecting the differences among the groups, and differences in the spatial location significantly affected the sedimentation performance of bacterial communities in sediments at different depths.

Discussion

The distribution of microorganisms at seven taxonomic levels (domain, phylum, class, order, family, genus, and species) did not significantly differ between Groups A and C. However, the

number of microorganisms at each taxonomic level was higher in Group B than in Groups A and C, and the Chao1 and Shannon indices were also higher in Group B, indicating that the samples in Group B had rich biodiversity and good biological sedimentation performance. Group A had poorer sedimentation performance than Group B because its sampling site was located at the aerobic interface of the wastewater level.

Proteobacteria and *Bacteroidota* dominated Groups A and B, whereas *Acidobacteriota* dominated Group C. Comparing the bacterial species classification at the phylum level between Groups A and C indicated that the species clustering distribution overlapped to a certain extent, consistent with the results of the heat map, and *Acidobacteriota* became dominant with the expansion of floating sludge. The bacterial composition of floating sludge at the phylum level was consistent with that in the later stage of bulking sludge expansion [18].

Proteobacteria have the ability to degrade and consume complex organic matter, which is necessary for the removal and degradation of biological nitrogen, biological phosphorus, and organic pollutants in activated sludge [19]. The abundance of *Bacteroidetes* reflects the stable performance of anaerobic tanks, as anaerobic heterotrophic bacteria dominate in anaerobic reaction tanks [20]. Therefore, the microbial composition of sludge in Groups A and B was more consistent with the microbial composition of the sludge with stable degradation.

Antibiotic usage peaks in spring, and the large-scale use of chlorine-containing disinfectants might decrease the effectiveness in treating chlorine- or antibiotic-resistant bacteria in sewage treatment plants.

The sludge samples from Group A exhibit poorer sedimentation performance compared to those from Group B, whereas the pipe-attached sludge localed at the B plane is 1 meter below the A plane.

The abundance of *Pseudomonas* was 33.33% in Group A and 66.67% in Group C (the relative abundance was significantly higher than the 1% rank as positive). There were significant differences in the distribution of the relative abundance of *Pseudomonas* among the three groups, and the community distribution of *Pseudomonas* differed at different sites depths, indicating that the distribution of *Pseudomonas* is related to the depth of the sludge. In addition, the sedimentation performance of the sludge decreased as the abundance of *Pseudomonas* increased.

Considering that particular species in *Pseudomonas*, such as *P. aeruginosa*, have been reported to be resistant to disinfection or hybrid disinfection and that they can form biofilms, it is necessary to regularly remove floating biofilms from the sewage pipes and monitor their composition. The persistence of *Pseudomonas* can lead to further bulking of floating sludge. Therefore, the biggest concern regarding P. aeruginosa is the presence of virulent or antimicrobial-resistant strains. Considering the detection of carbapenem-resistant *P. aeruginosa* during municipal sewage monitoring, the effluent should be subjected to secondary chlorine disinfection and dechlorination before entering the municipal sewage network only when *Pseudomonas* is not detected.

Because *Pseudomonas* was not detected in any Group B samples, it was also suggested that *Pseudomonas* could originate from infected inpatients or the sewage pipelines upstream of the anaerobic tank. It is recommended that the sewage treatment plant periodically isolate and protect the inflow and discharge pipes of the anaerobic tank regularly and, if necessary, use an ultraviolet radiation disinfection scheme to efficiently disinfect the inlet pipes. Therefore, the attached wall sludge at the liquid level can be considered the critical sampling site for sludge monitoring and used as a conventional sampling site.

The number of high-quality sequences in the sludge samples collected by the sampling device was adequate, indicating that the specimens collected by the sampler were suitable and qualified for high-throughput sequencing of species composition and diversity analysis. The samples can also be further to analyze the antibiotic resistance of characteristic bacteria in sludge.

Conclusion

Among the sludge samples from the three groups, the microbial count was highest for sludge samples below the liquid sewage level, which exhibited rich microbial diversity and good settling performance. Meanwhile, sludge samples taken below the liquid sewage level were negative for *P. aeruginosa*, whereas the detection rate of *P aeruginosa* was relatively high in the other two groups. The efficiency of the hospital's sewage treatment plant in controlling this bacterium needs to be improved.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability statements

All data are incorporated into the article and its online supplementary material.

Author contributions

Ruiling Deng: Methodology, original draft preparation, Data curation, Writing - original draft preparation, Writing- review & editing, Formal analysis, Software.

Shu Guo: Methodology, Investigation, Conceptualization, Resources.

Haiping Tan: Funding acquisition, Project administration, Data Curation, Validation.

Wenbing Yuan: Resources of the sites.

References

1. Kerr KG, Snelling AM (2009) Pseudomonas aeruginosa: A formidable and ever-present adversary. J Hosp Infect 73:338-344. https://doi.org/10.1016/j.jhin.2009.04.020

2. Majumder A, Gupta AK, Ghosal PS, Varma M (2021) A review on hospital wastewater treatment: A special emphasis on occurrence and removal of pharmaceutically active compounds, resistant microorganisms, and SARS-CoV-2. J Environ Chem Eng 9:104812. https://doi.org/10.1016/j.jece.2020.104812

3. Russell AD (2003) Similarities and differences in the responses of microorganisms to biocides. J Antimicrob Chemother 52:750-763. https://doi.org/10.1093/jac/dkg422

4. Hou AM, Yang D, Miao J, Shi DY, Yin J, Yang ZW, Shen ZQ, Wang HR, Qiu ZG, Liu WL, Li JW, Jin M (2019) Chlorine injury enhances antibiotic resistance in Pseudomonas aeruginosa through over expression of drug efflux pumps. Water Res 156:366-371. https://doi.org/10.1016/j.watres.2019.03.035

5. Anastasi EM, Matthews B, Stratton HM, Katouli M (2012) Pathogenic Escherichia coli found in sewage treatment plants and environmental waters. Appl Environ Microbiol 78:5536-5541. https://doi.org/10.1128/AEM.00657-12

6. Makuwa S, Green E, Fosso-Kankeu E, Moroaswi V, Tlou M (2023) A snapshot of the influent and effluent bacterial populations in a wastewater treatment plant in the North-West Province, South Africa. Appl Microbiol 3:764-773. https://doi.org/10.3390/applmicrobiol3030053

7. Shekhawat SS, Kulshreshtha NM, Gupta AB (2020) Investigation of chlorine tolerance profile of dominant gram negative bacteria recovered from secondary treated wastewater in Jaipur, India. J Environ Manage 255:109827. https://doi.org/10.1016/j.jenvman.2019.109827

8. Shekhawat SS, Kulshreshtha NM, Vivekanand V, Gupta AB (2021) Impact of combined chlorine and UV technology on the bacterial diversity, antibiotic resistance genes and disinfection by-products in treated sewage. Bioresour Technol 339:125615. https://doi.org/10.1016/j.biortech.2021.125615

9. Ma XL, Dong X, Cai JB, Fu C, Yang J, Liu Y, Zhang Y, Wan T, Lin S, Lou Y, Zheng M (2022) Metagenomic analysis reveals changes in bacterial communities and antibiotic resistance genes in

an eye specialty hospital and a general hospital before and after wastewater treatment. Front Microbiol 13:848167. https://doi.org/10.3389/fmicb.2022.848167

10. Wu LW, Ning DL, Zhang B, Li Y, Zhang P, Shan X, Zhang Q, Brown MR, Li Z, Van Nostrand JD, Ling F (2019) Global diversity and biogeography of bacterial communities in wastewater treatment plants. Nat Microbiol 4:1183-1195. https://doi.org/10.1038/s41564-019-0426-5

11. Ledezma-Villanueva A, Robledo-Mahón T, Gómez-Silván C, Angeles-De Paz G, Pozo C, Manzanera M, Calvo C, Aranda E (2022) High-throughput microbial community analyses to establish a natural fungal and bacterial consortium from sewage sludge enriched with three pharmaceutical compounds. J Fungi (Basel) 8. https://doi.org/10.3390/jof8070668

12. Yang Y, Li B, Ju F, Zhang T (2013) Exploring variation of antibiotic resistance genes in activated sludge over a four-year period through a metagenomic approach. Environ Sci Technol 47:10197-10205. https://doi.org/10.1021/es4017365

13. Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, Alexander H, Alm EJ, Arumugam M, Asnicar F, Bai Y (2019) Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat Biotechnol 37:852-857. https://doi.org/10.1038/s41587-019-0209-9

14. Callahan BJ, Mcmurdie PJ, Rosen MJ, Han AW, Johnson AJ, Holmes SP (2016) DADA2: high-resolution sample inference from Illumina amplicon data. Nat Methods 13:581-583. https://doi.org/10.1038/nmeth.3869

15. Bokulich NA, Kaehler BD, Rideout JR, Dillon M, Bolyen E, Knight R, Huttley GA, Gregory Caporaso J (2018) Optimizing taxonomic classification of marker-gene amplicon sequences with

QIIME 2s q2-feature-classifier plugin. Microbiome 6:90. https://doi.org/10.1186/s40168-018-0470-z

16. Jiang Y, Guo ML, Xie JX, Chang YF, Xie JW, Chen CJ, Shen YL (2020) Characteristics of anammox granular sludge and differences in microbial community structure under different culture conditions. Huan Jing Ke Xue 41:2358-2366. https://doi.org/10.13227/j.hjkx.201911052

17. Gewers FL, Ferreira GR, Arruda HFD, Silva FN, Comin CH, Amancio DR, Costa LDF (2022) Principal component analysis: a natural approach to data exploration. ACM Comput Surv 54:1-34. https://doi.org/10.1145/3447755

18. Rivière D, Desvignes V, Pelletier E, Chaussonnerie S, Guermazi S, Weissenbach J, Li T, Camacho P, Sghir A (2009) Towards the definition of a core of microorganisms involved in anaerobic digestion of sludge. ISME J 3:700-714. https://doi.org/10.1038/ismej.2009.2

19. Nguyen HT, Le VQ, Hansen AA, Nielsen JL, Nielsen PH (2011) High diversity and abundance of putative polyphosphate-accumulating Tetrasphaera-related bacteria in activated sludge systems. FEMS Microbiol Ecol 76:256-267. https://doi.org/10.1111/j.1574-6941.2011.01049.x

20. Kampmann K, Ratering S, Kramer I, Schmidt M, Zerr W, Schnell S (2012) Unexpected stability of Bacteroidetes and Firmicutes communities in laboratory biogas reactors fed with different defined substrates. Appl Environ Microbiol 78:2106-2119. https://doi.org/10.1128/AEM.06394-11