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Posted Date: 1 March 2024

doi: 10.20944/preprints202403.0001.v1

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High-Throughput Analysis of Department-Specific Dynamic Patterns of Bacterial Communities and Antibiotic Resistance in the Hospital Indoor Environments

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Abstract: The hospital indoor environment plays a significant role in shaping the microbial exposures that humans encounter. Resistance to antibiotics is one of the mechanisms employed by bacteria to develop resilience against stress in indoor environments. The extensive usage of antibiotics has been a key factor in driving changes in the ecological function of antibiotic resistance genes (ARGs) and their acquisition by pathogenic bacteria. By integrating the *16S rRNA* Illumina sequencing and high throughput-qPCR approaches with water and air dust samples across seven departments at *Peking University Shenzhen Hospital*, China, this study yielded intriguing findings regarding the department-specific variation, transmission, and correlation of bacteria, ARGs and MGEs within the hospital indoor environment. One notable observation was that the seasonal variation played a vital role in shaping the bacterial composition in the overall hospital indoor environment across seven studied departments. Nevertheless, a similar significant seasonal impact was only observed in the blood test room and emergency departments, rather than in the other five departments. Another intriguing finding was the correlation between ARGs and MGEs abundance. This correlation was evident in the overall hospital indoor environment, which was conversely not found in the emergency, pneumology and gynecology departments. Additionally, while there was no significant relationship that occurred between bacterial communities and ARGs/MGEs within the hospital, the gynecology department demonstrated a close link between bacterial communities and the presence of ARGs/MGEs. Notably, the gynecology department also stood out as an important source of bacteria and ARGs/MGEs for other departments. This study underscores the need for careful surveillance and monitoring of antibiotic resistance, particularly in *Legionella* spp. in man-made water systems. The findings also show the importance of monitoring and understanding the genetic elements such as *Tp614* involved in gene transfer and recombination, as well as their impact on antimicrobial treatment efficacy.

Keywords: antibiotic resistance; antibiotic resistance genes; hospital indoor environment; microbial communities; high-throughput qPCR; department-specific; tap water; p-trap water; air dust; mobile gene elements

1. Introduction

Hospital indoor environments are characterized by high infective risk, firstly cause of the compromised immunologic conditions of the patients that make them vulnerable to bacterial, viral, parasitological and fungal opportunistic infections [1]. It has been estimated that around two million patients per year in the United States acquire a nosocomial infection and tragically, at least 90,000 of

 doi:10.20944/preprints202403.0001.v1

them succumb to these infections [2]. Research has demonstrated that bacteria can persist and accumulate in various locations within the hospital's indoor environment, including white coats [3], stethoscopes [4], air conditioners [5], water faucets [6], and water p-traps [7], far longer than previously believed [8]. Influenced by the bacterial cell viability and bacterial load [9], the pathogen can be transmitted through contaminated hands or gloves of healthcare workers [9], direct contact with contaminated surfaces [9], splashing of pathogen-contaminated water on sterile goods [10], and droplets for respiratory pathogens [11]. The presence of these reservoirs in the hospital environment may heighten the risk of acquiring a nosocomial infection.

Indeed, with extensive usage of antibiotic drugs on patients and routine application of antimicrobial chemicals for sanitation in hospitals, bacteria isolated from hospital environments are frequently resistant to antibiotics. Specifically, in a study conducted by Moges et al., it was found that a staggering 81.5% of the bacterial isolates from the hospital environment exhibited resistance to multiple antibiotics [12]. Similarly, Phoon et al. noted that 62.7% of the identified species such as *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Acinetobacter baumannii* in the tertiary hospital environment were multidrug-resistant (MDR) [13]. These pathogens possess the ability to withstand the effects of multiple antibiotics, greatly limiting the available treatment options and significantly increasing the risk of healthcare-associated infection. Of particular concern about the presence of antibiotic resistance is the dissemination of antibiotic resistance genes (ARGs) in the hospital environment. Many of these genes are found on transposons, integrons or plasmids, which can be mobilized and transferred to other bacteria, belonging to the same or different species [14]. These findings highlight the alarming reality of a "pre-antibiotic era".

Currently, there is a growing focus on studying the diversity, interaction and transmission of microbes and ARGs in various components of the hospital environment, including surfaces [15], air dust [5], water [16], and during different seasons [17]. These studies have shed light on the influence of several factors on the composition of microbes and ARGs in the hospital indoor environment. Factors such as patient occupancy [18,19], humidity, temperature [20], air filtration [5], chemical residues (e.g., antibiotics) [21] and room occupancy [18,19] have been identified as important contributors to microbial and ARG compositions in hospitals. However, despite the wealth of research in this area, there is still a limited number of studies that have thoroughly investigated the comprehensive patterns of the microbiome and antibiotic resistance in a department-specific manner. While numerous studies have examined microbial and antibiotic-resistance profiles in intensive care units (ICUs) [22,23], only a few studies have directed their attention to different ward-room sites [18,19] and various hospital departments [5]. Indeed, the diverse ecological interactions and conditions within different hospital sites have significant clinical implications, yet they have not been extensively explored.

Conventional methods for studying bacteria and antibiotic resistance can be time-consuming and labor-intensive. However, molecular techniques provide rapid and sensitive alternatives for these investigations, for example, *16S rRNA* amplicon sequencing uses highly conserved bacterial regions for detecting diverse bacteria, while high throughput-qPCR (HT-qPCR) is a relatively rapid and convenient method for simultaneously evaluating a large number of ARGs with low-quantity DNA samples. The objectives of this study were three-fold (workflow in Figure 1). Firstly, it aimed to identify the factors shaping the composition of bacterial communities, ARGs, and MGEs in the hospital indoor environment from a department-specific respect. Secondly, it aimed to explore the sharing phenomenon of the bacteria, ARGs and MGEs within the hospital, and to determine if these elements could be traced back to specific sources across different departments. Lastly, the study aimed to investigate department-specific correlations between bacterial communities and ARGs/MGEs, and investigate the potential bacteria associated with the spread of antibiotic resistance through the horizontal transfer within the hospital. By addressing these objectives, the study will provide a high-throughput exploration of bacterial communities, ARGs, and MGEs within the hospital indoor environment from a department-specific perspective. The results delivered may help to establish priorities to control the spread of nosocomial pathogens and the dissemination of antibiotic resistance determinants.

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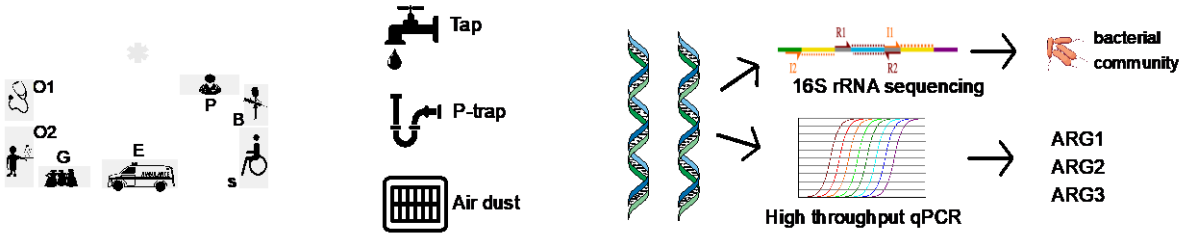

Figure 1. Overall workflow of the study. O1 is otolaryngology department. O2 is ophthalmology department. G is gynecology department. E is emergency department. S is surgery department. B is blood-test room. P is pneumology department.

2. Methods

2.1. Sampling Locations, Collections and DNA Extraction

We collected 217 samples (Table S1) in the *Peking University Shenzhen Hospital*, Shenzhen, China (22.55N, 114.10E) every month from July 2020 to January 2021. Among 217 samples, 64 samples yielded positive Ct values (Ct <31) in the HT-qPCR experiments, which were used in this study. The 64 samples constituted water and air dust samples in seven departments (ophthalmology, otolaryngology, emergency, blood test room, surgery, pneumology and gynecology) from early summer (July, August), late summer (September and October) to winter (November, December, January).

The water samples contained tap water and p-trap water (a U-shaped bend that is connected to the sink and filters water as it enters a plumbing system). Before collecting the p-trap water, the u-shaped bend of the p-trap was unplugged to allow the stored water in the bend to be discharged. Then, 500 ml of each type of water was collected in a sterile bottle. Next, bacterial cells were captured and collected by pouring the water through the filtration unit containing the sterile mixed cellulose esters (MCE) membrane with a pore size of 0.22 μm and a diameter of 47 mm. The filtration unit consisted of a funnel, a locking ring, a filter flask, and several rubber tubes connected to a water-circulation vacuum pump (-0.098Mpa). The captured membrane was promptly stored at 4°C for subsequent DNA extraction using Dneasy PowerWater Kit (QIAGEN, Germany), which was conducted within one day. As for the air dust samples, 2 filters (30cm×30cm) of the air conditioner (AC) were collected from each studied department. The filters were washed several times with 1 L of sterile reverse osmosis (RO) water, which was filtered through 0.22 μm MCE membrane to collect microbe cells for subsequent DNA extraction using Dneasy Powerwater Kit (QIAGEN). Most collected samples were subject to DNA extraction within 24h, otherwise, the temporary preservation at -20°C was applied.

2.2. HT-qPCR

ARGs and MGEs were quantified by HT-qPCR using the Takara (previously WaferGen) SmartChip Real-Time PCR System (#Cat:64022). Referring to the previous references using customed primers for detecting ARGs and MGEs in the hospital [24,25], we selected 109 gene primer sets (Table S2) for 11 major classes of antibiotics, six transposase genes, five integrase genes, five plasmid genes and one *16S rRNA* gene primer set. The 11 major classes of antibiotics are aminoglycoside, amphenicol, beta-lactam, fluoroquinolone, multidrug, macrolide/lincosamide/streptogramin B (MLSB), sulfonamide, tetracycline, trimethoprim, vancomycin, and others (peptide, phosphonic acid, and rifamycin). A non-template negative control was used for each primer, and all qPCRs were performed in triplicate. The thermal cycle process amplification was: (1) initial denaturation at 95 °C for 10 min, (2) 40 cycles of denaturation at 95 °C for 30 s; (3) annealing at 60 °C for 30 s (data collection). In the end, melting curve analyses were then automatically generated by the SmartChip qPCR

 doi:10.20944/preprints202403.0001.v1

analysis software 2.8.0.65. Only data meeting the following standard were kept for subsequent analysis: (1) amplification efficiencies within the range of 90% to 110%; (2) amplicons without multiple melting curves; (3) triplicates all within the detection limit of threshold cycle Ct of 31. ARGs and MGEs relative copies represented the gene copies to *16S rRNA* copies ratio.

We used the following formulas [24]:

(1) Gene copies = $10^{\frac{31\text{-}Ct}{10/3}}$

(2) Relative copies = $\frac{\text{Gene copies}}{16S\ rRNA\ \text{copies}}$

2.3. Bacterial 16S rRNA Sequencing of 64 Samples

Bacteria community structures were determined by *16S rRNA* gene amplicon sequencing on a HiSeq platform (Illumina, USA) with PE250 strategy. The V4 to V5 region of bacterial *16S rRNA* gene was amplified with the universal primer set 515F (5′- GTGCCAGCMGCCGCGGTAA-3′) and 907R (5′-CCGTCAATTCMTTTRAGTTT-3′) [26], and labelled with unique barcodes (6-nucleotide barcodes) for each sample (Table S1). The components of the PCR solution mix and PCR program are shown in Table S3 and Table S4. Following PCR, the *16S rRNA* amplicon samples were grouped into 3 pools and sent for Illumina pair-end sequencing, each of which contained 2000 ng DNA individually. To ensure quality control of the raw reads obtained from Illumina sequencing, Fastp software [27] was utilized The post-quality control (QC) reads, specifically those related to *16S rRNA* gene amplification, were then imported into the Quantitative Insights in Microbiology (QIIME 1) pipeline [28]. Within this pipeline, several steps were performed, including merging pair-end sequences, extracting barcodes, splitting samples, and removing amplification primers. For obtaining operational taxonomic units (OTUs), USEARCH V11 [29] was employed with a 97% similarity threshold. Subsequently, taxonomic assignments were achieved by referencing the obtained OTUs against the Silva 138.1 database [30].

2.4. Statistical Analysis

To analyze the changes and similarities in genus complexity among samples, several statistical methods and packages were employed. Principal component analysis (PCA) and analysis of similarities (ANOSIM) were conducted by the R3.5.3 VEGAN package [31] to reveal patterns and differences. The Random Forest test, implemented with the RandomForest package [32], was utilized to identify the most discriminative variables between two sample categories. In ANOSIM, the R value indicates the degree of difference between sample groups, with R = 1 representing significant dissimilarity, R = 0 indicating no difference, R > 0.75 suggesting good separation, R > 0.5 indicating differences with some overlap, and R < 0.25 representing almost no differences. Moreover, to visualize the sharing phenomenon of genus and ARGs, Venn graphs were generated by the VennDiagram package [33]. For the investigation of the correlation between ARGs and MGEs, the Mantel test was performed with “mantel” function in the Vegan package. Additionally, a pairwise correlation between the abundances of targeting genes and OTU was calculated using “corr.test” function, and it was considered statistically robust if the Spearman's correlation coefficient (ρ) was >0.6 and the P-value was <0.01. With the pairwise correlation values, the Gephi platform was used to generate networks [34]. Further, to perform the source-tracking analysis, Sourcetracker2 [35] was utilized. Notably, all graphs during the statistic analysis were generated by Rstudio3.5.3 with the ggplot2 package [36].

3. Results and Discussions

3.1. Microbial Profile

Overall sketch of microbial profile in the hospital indoor environments

From the *16S rRNA* gene amplicon sequencing, the raw data consisted of 598,1256 reads (2.99 Gbps) for all samples, and the quality-filtered (Q30) data consisted of 591,4227 reads with an average value of 92,410 reads per sample, corresponding to 1,439 different bacteria types after filtering and

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quality control. The mapped level of each taxonomic level, namely phyla, class, order, family, genus, and species, against the Silva 138.1 database [30] was 100%, 99.9%, 99.7%, 98.8%, 82.8%, and 9.98%, respectively. The limited percentage of identified bacterial species could be attributed to the short reads length that may not provide sufficient resolution for species identification. The rarefaction analysis conducted at the genus level demonstrated that the data obtained was adequate for taxonomic analysis. Despite the limitations of short reads, the analysis was able to provide reliable insights into the composition and diversity of bacterial genera within the sample (Figure S1).

At the genus level, there were 684 genera detected in total. *Dechloromonas*, *Pseudomonas* and *Flavobacterium*, *Limnohabitans*, *Ralstonia*, and *Acineotobactor* took the leading places (Figure S2a), taking 11.0%, 7.95%, 7.56%, 6.41%, 5.53% and 4.17% of the community respectively. As *Pseudomonas* is the most concerned waterborne pathogen in healthcare facilities responsible for a wide spectrum of infections in humans that can be associated with significant morbidity and mortality [37], *Dechloromonas* occurs frequently in the soil and wastewater treatment systems linked with nitrogen cycling roles [38] and *Flavobacterium* exists more in soil and freshwater that may cause disease in freshwater fish [39]. Moreover, the pathogenic *Acineotobactor* is particularly noteworthy due to the potential implications of its species (e.g. *Acinetobacter baumannii, Acinetobacter nosocomialis, Acinetobacter pittii, Acinetobacter seifertii,* and *Acinetobacter dijkshoorniae* [40]) as a source of infection in debilitated patients within the hospital. For all genera detected, there were 215 classified genera that accounted for over 0.01% of the total genera *16S rRNA* gene sequences, which would be employed for the subsequent analysis. From species-level respect, the results of predominating species (Figure S2b) are concerning, as they indicate the presence of certain pathogens in the studied environments. Among the major species, *Pseudomonas sp.* was found to be the most prevalent, accounting for 45% of the samples. This pathogen showed a particularly significant emergence in the emergency and otolaryngology departments. Another alarming pathogen was the presence of *S. maltophilia*, which accounted for 5.1%. This pathogen was most frequently detected in the pneumology department, indicating a potential association with respiratory-related conditions. Furthermore, the study identified the presence of *K. pneumoniae* in 1.8% of the samples. Although the proportion may be relatively lower compared to other pathogens, the detection of *K. pneumoniae* is still significant due to its potential to cause serious infections and its association with antibiotic resistance [41]. In the study conducted by Madebo *et al*., *P. aeruginosa* and *K. pneumoniae* were reported as the leading contaminants in the hospital as well [41]. However, another dominating pathogen of *S. aureus* did not appear in our study which could potentially be attributed to a low mapping rate at the species level during the analysis.

Seasonal changes had a department-specific impact on the microbial composition

When examining microbial alpha diversity in the indoor hospital environments, significant fluctuation was observed across different departments, as depicted in Figure 2f. The otolaryngology and surgery departments displayed the highest microbial diversity, with Shannon index values of 2.80 and 2.66, respectively. In contrast, the pneumology department exhibited the lower microbial diversity, recording the lowest Shannon index value of 1.99. To further explain such variation of alpha diversity, the Shannon index values between air dust and water within specific department were compared. Interestingly, in the otolaryngology, surgery, and pneumology departments, air dust consistently displayed higher bacterial alpha diversity. The Shannon index values for air dust were 3.23, 3.43, and 2.50, respectively. On the other hand, the water medium exhibited slightly lower Shannon index values, measuring 2.70, 2.51, and 1.61, respectively. This finding aligns with the understanding that aerosols released within the hospital environment can serve as a reservoir for a diverse range of microorganisms, contributing to the observed higher alpha diversity in air dust samples. Previous studies, such as the one by Bonadonna *et al*. [37] have also highlighted the presence of microorganisms in aerosols released through various routes within healthcare facilities.

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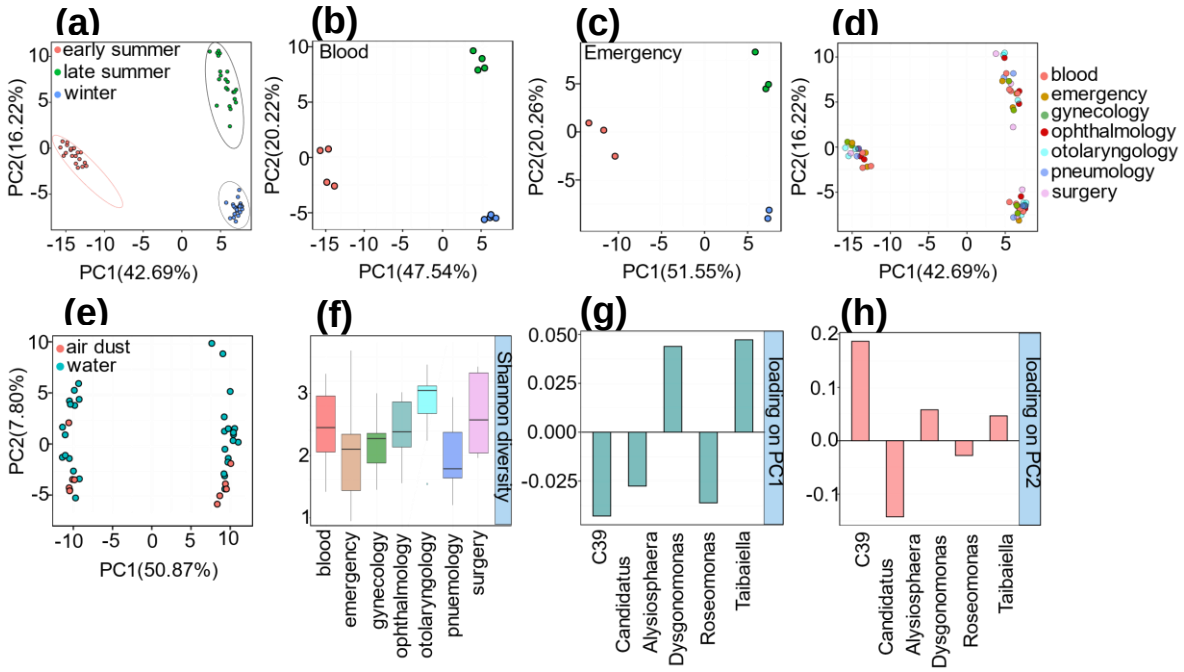

Figure 2. (**a**) PCA clusters in the studied hospital indoor environment separated by early summer, late summer and winter, of which the ellipses were drawn in 95% confidence level. (**b**) PCA clusters in the blood department separated by early summer, late summer and winter. (**c**) PCA clusters in the emergency department separated by early summer, late summer and winter. (**d**) PCA characteristics of seven departments. (**e**) PCA characteristics of two mediums. Due to the absence of air dust samples in winter, the analysis in water and air dust was conducted without including the water samples from winter. (**f**) Bacterial (genus-level) Shannon diversity in seven departments, where relative abundance was represented by the percentage of clean sequencing reads mapped to the genus level. (**g**,**h**) Five key genera contributed significantly to the observed genera compositional differentiation between early summer and late summer in the overall hospital indoor environment.

In order to assess the primary environmental factor influencing the microbial composition in the hospital indoor environments, a PCA was conducted, considering variations in seasons, mediums, and departments. The PCA results revealed the presence of three distinct clusters along the PC1 and PC2 axes, primarily separated by seasonal differences, rather than variations in mediums and departments (Figure 2a,d,e). This suggests that the microbial structure within the studied indoor hospital environment significantly differed between summer and winter. The influence of seasons was further supported by the ANOSIM result, which yielded a p-value of 1E-04 and an R-value of 0.2719. Previous research has reported that temperature and humidity can positively influence microbial diversity [42,43]. The higher temperatures and increased humidity experienced during the summer months create favorable conditions for microbial growth and activity. To gain a deeper understanding of the clusters within the summer season, the summer samples were divided into two subgroups: early summer (July and August) and late summer (September and October). Therefore, another round of PCA analysis was conducted specifically for these subgroups (Figure 2a). The results uncovered that the two summer clusters were composed of different genera components, with early summer and late summer exhibiting distinct microbial compositions. This differentiation within the summer season was strongly supported by the ANOSIM result with a p-value of 1E-04 and an R-value of 0.7346 (Table S5). To pinpoint the specific genera responsible for driving this separation between early summer and late summer, a RandomForest analysis was performed. The analysis revealed several key genera that contributed significantly to the observed differentiation (Figure 2g, 2h). These genera included *C39*, *Candidatus Alysiosphaera*, *Dysgonomonas*, *Roseomonas*, and *Taibaiella*. The variation of these genera likely played a crucial role in shaping the distinct microbial compositions observed between the early summer and late summer clusters. Among these genera,

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except for *Roseomonas*, the other four were found to be more predominant in the late summer samples. It is worth noting that *Taibaiella* exhibited the most pronounced discrimination, showing a 23-fold higher relative abundance in the late summer samples. Furthermore, it is noteworthy that certain species within the *Roseomonas* genus, e.g. *Roseomonas mucosa* are known to be opportunistic pathogens for humans [44]. Their increased relative abundance in early summer samples may suggest a potential health risk during that time. Additionally, *Dysgonomonas* bacteria are recognized as causative agents of gastroenteritis, particularly in immunocompromised individuals [45].

Nonetheless, while the beta diversity analysis demonstrated a significant influence of seasonal patterns on the overall microbial compositions within the entire indoor hospital environment, this influence was not observed uniformly across all departments. Specifically, the results of PCA and ANOSIM analyses indicated that seasonal variation significantly shaped the microbial compositions only in the blood test room and emergency departments (Figure 2b,c). In contrast, for the other five departments, the ANOSIM results indicated that the microbial compositions varied independently of seasonal changes (Table S5). Indeed, such department-specific variability can be attributed to the fact that bacteria occupy various ecological niches, such as air, water, human bodies, and medical equipment, and their responses to different stresses, including antibiotics, can vary based on the specific conditions they encounter [21]. Moreover, in addition to the diverse ecological niches that bacteria live in, the involvement of other factors in the hospital indoor environment, such as high occupancy rates and frequent movement of patients, can also contribute to different mechanisms of bacterial colonization, interaction, and evolution in the individual department. These additional factors pose further challenges to elucidating the bacterial diversity within the hospital.

Overall, the observed alpha and beta diversity patterns underscored that the bacterial community structure in the hospital indoor environments was significantly influenced by seasonal variations, despite such impact being department-specific. However, while diversity is generally considered beneficial for ecosystem health, it can also introduce challenges, particularly in healthcare settings where cross-infection is a concern for immunocompromised patients. As a result, understanding diversity dynamics can provide valuable insights into the ecological processes and environmental factors that shape microbial communities [46], contributing to a broader understanding of microbial ecology and ecosystem functioning.

Bacteria dissemination in the hospital indoor environments

Regarding the common genera present, the Venn analysis revealed several noteworthy findings in the hospital indoor environments. Across all seven departments, a total of 197 genera were shared, indicating the presence of a core microbial community within the hospital environment (Figure S3a). To explain such dissemination, the activities of healthcare occupants have a significant influence on it within healthcare facilities [47]. Through their interactions with patients and contact with various surfaces, the occupants can unintentionally facilitate the transfer of bacteria from one patient to another, thereby influencing the spread of healthcare-associated infections. In addition to the sharing communities among the department, the bacteria transfer between water and air dust samples was also observed, with 201 genera being shared between these two mediums. The implication was that bacterial dissemination occurred frequently in water and air mediums (which was also supported by the ANOSIM result in Table S5), and bacteria present in water could become aerosolized and subsequently released into the air, which enormously increased the likelihood of bacterial exposure and potential transmission to individuals within the healthcare facility [48].

To further explore the sources of the shared genera among the departments, a source-tracking analysis was conducted. During the analysis, the blood test room was considered the "sink" for the genera due to its role in receiving patients from diverse sources, while the other six departments were considered the "sources". The results of the analysis (Figure S3b) revealed that 46.8% of the genera in the blood test room originated from the other six departments, of which the gynecology and emergency departments contributed the most with 21.6% and 8.81% respectively. However, there were still 53.2% of the sources that could not be identified, indicating a substantial portion of the genera in the blood test room were outdoor origins.

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3.2. Broad-Spectrum Profile of ARGs and MGEs

MGE genes were the most abundant in the hospital indoor environments

The analysis of ARGs and MGEs revealed the detection of 107 target ARGs and MGEs across all samples. The total relative copy number per *16S rRNA* gene copy of these ARGs and MGE genes was 74.13, with an average relative copy number of 0.029. Figure S4a illustrates the relative abundance of the 107 ARGs and MGEs belonging to 14 different types in all the samples. It is witnessed that the largest proportion of detected genes was dominated by MGEs, with a total relative copy number of 26.2. This category included MDR-mobile-resistant genes, integrases, transposases, and plasmids. MDR-mobile-resistant genes had a relative copy number of 5.55, integrases had a relative copy number of 11.4, transposases had a relative copy number of 9.14, and plasmids that were represented by *tra* genes had a relative copy number of 0.060. This indicated a high likelihood of HGT risk within the hospital, as MGEs play a significant role in facilitating the transfer of ARGs. MDR-resistant genes that were not featured with the mobility accounted for the predominant share as well, with a relative copy number of 19.3. This was followed by ARGs delivering resistance to sulfonamide, MLSB and aminoglycoside, with 9.28 relative copies, 6.90 relative copies and 4.13 relative copies, respectively. These findings align with a study conducted by Y. Li et al. [49] on hospital samples collected from air-conditioning units, which also identified similar patterns of dominating drug resistance.

As for the representative ARGs and MGEs genes in studied environments, the dominating 15 representative genes were shown in Figure S4b. *merA-marko (*MDR*)*, *int1-a-marko* (integrase), *strB* (Sulfonamide resistance), *qacF/H* (MDR-mobile), *tnpA_203* (transposase), and *lnuA* (MLSB resistance) were the high-frequent representative ARGs and MGE genes spread in different environments. *merA-marko* gained the most abundance with 19.2 relative copies, followed by *int1-a-marko* and *strB* with 11.0 and 6.44 relative copies, respectively. Few representative genes were unique in certain hospital settings, such as bla_{VEB} (beta-lactam resistance) only in the blood test room, *floR* (amphenicol resistance) exclusively in the emergency department and bla_{ADC} (beta-lactam resistant) simply in the ophthalmology department.

Heterogenous ARGs/MGEs abundance and diversity in the hospital indoor environments

The different departments in the hospital indoor environments presented a heterogeneous distribution regarding the richness of ARGs and MGE genes (Figure 3a). The blood test room had the highest abundance of ARGs and MGEs, with 12.9 relative copies, while the otolaryngology department had the lowest gene copies, with 6.18 relative copies. The distribution of MGE genes exhibited a similar pattern (Figure 3b), with the blood test room showing the highest abundance of MGE genes at 4.50 relative copies, indicating a higher frequency of gene transfer events in that department. In contrast to the relatively moderate level of heterogeneity in terms of ARGs and MGEs abundance, the diversity of ARGs and MGEs displayed pronounced fluctuations across departments. The surgery samples exhibited a notable increase in diversity, with an average Shannon index of 2.26 (Figure 3c). This could be attributed to factors such as the use of antimicrobial chemicals to treat wounds and injuries [50], and the contribution of human sources from the high occupancy rates [51].

Figure 3. (**a**) Abundance of ARGs and MGEs genes in each department, where the abundance represents the relative copy number per *16S rRNA* gene copy. The five-star symbols in the bottom of the bar in the blood, ophthalmology, surgery, and otolaryngology departments represented that there was a significant correlation between ARGs and MGEs in each of those department. (**b**) Abundance of MGEs genes in each department, where the abundance represents the relative copy number per *16S rRNA* gene copy. (**c**) Boxplot of ARGs/MGEs Shannon diversity in each department.

Based on the complicated heterogeneous distribution of ARGs and MGEs abundance and diversity in the hospital indoor environments, the dominating factors that shaped their compositions were supposed to be complicated. In our study, the PCA and ANOSIM analysis indicated that none of the seasonal, medium and department variations significantly affected the components of ARGs and MGEs (Table S6). While there may be differences in the richness, diversity, and specific types of ARGs/MGEs between different environmental factors, these differences may not be strong enough to

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be statistically significant. Other factors, such as microbial community dynamics, host factors, or specific environmental conditions, could also contribute to the observed patterns.

Dissemination and department-specific correlation of ARGs and MGEs

It has been stated that MGEs can contribute to the HGT of ARGs among different microbes, and the host range of the MGEs carrying an ARG is important for determining how far it will spread [21]. Consequently, to further prove the mobility of ARGs, the Mantel test between ARGs and MGEs was conducted. The results revealed a significant correlation between the abundance of ARGs and MGE genes ($p=0.001$, $r=0.3145$) in the overall hospital indoor environment. This suggests that the abundance of ARGs was more likely to be associated with the presence of MGEs, further supporting the notion of horizontal transfer of ARGs among bacteria in the hospital. Such correlation between ARGs and MGE genes has been frequently reported in the hospital aerosol and wastewater [52–54]. Nevertheless, when the Mantel test was performed in each department individually, an intriguing finding emerged. The significant correlation between ARGs and MGEs was observed simply in the blood test room, ophthalmology, surgery, and otolaryngology departments (Figure 3a), while no significant correlation was found in the other three departments (Table S7). Therefore, it is estimated that the association between ARGs and MGEs varies across different departments within the hospital, which was presumably caused by different mechanisms of horizontal transfer and the driving forces (e.g. antibiotic stresses and the occupant's rate) behind the circulation of ARGs among departments.

Based on the ARGs' mobility indicated by the significant correlation between ARGs and MGEs, the phenomenon of sharing ARGs and MGEs in the hospital indoor environments is manifested in Figure 4a. Specifically, the indoor hospital exhibited a core set of 35 ARGs and 11 MGEs shared across the studied departments. This suggests the presence of a common pool of genes circulating within the hospital. However, it is important to note that certain departments had their unique ARGs. For example, the ophthalmology department had vancomycin-resistant genes *vanSB* and *vanWB*. The pneumology department had peptide-resistant gene *mcr-2* and MLSB-resistant gene *ermY*. Additionally, the otolaryngology department had the aminoglycoside-resistant gene *armA*. Among those unique ARGs, *vanSB*, *vanWB* and *ermY* are acknowledged as chromosome-mediated ARGs [55], consequently, their unique presence in specific departments can be attributed to their inherent mobility. In terms of *mcr-2* and *armA* gens, they have been known as mobilized resistance genes mediated by plasmids [56,57]. Hence, the presence of their unique existence in specific departments might result from their limited transferability mediated by MGEs. Besides the common ARGs and MGEs across departments, the sharing phenomenon also appeared between water and air dust. Specifically, 73 ARGs and 13 MGEs were shared between the water and air-dust samples. Among these, water samples had 20 unique genes, while air-dust samples only had one unique gene (fosfomycin-resistant gene *fosB*).

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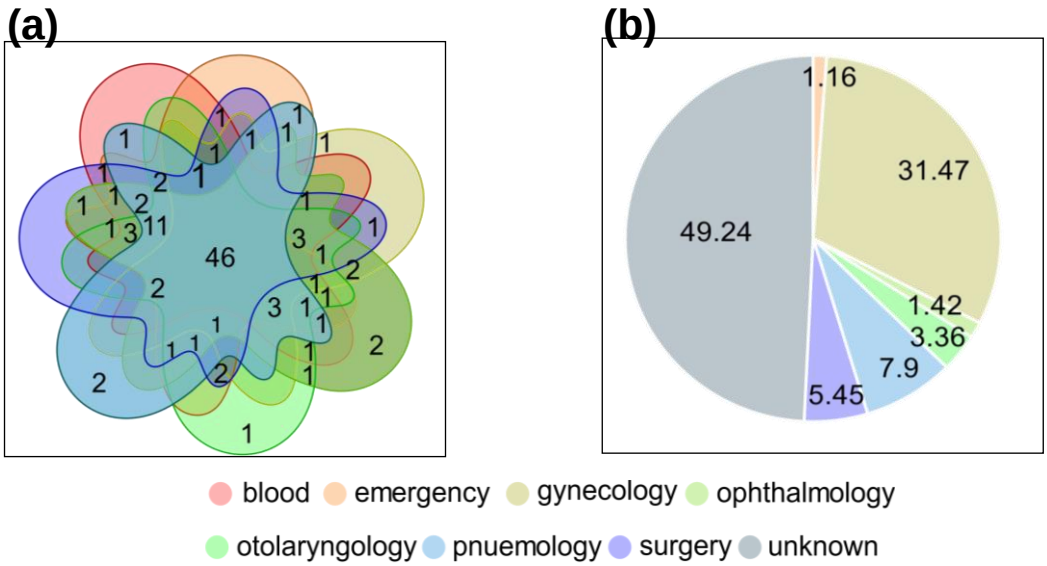

Figure 4. (**a**) Venn diagram of sharing ARGs/MGEs types in seven departments. (**b**) Source-tracking analysis of ARGs and MGEs across seven departments, where the blood department acted as "sink", and other six departments served as "sources".

To investigate the transmission pathways of ARGs and MGEs across the departments in the hospital indoor environment, a source tracking analysis was conducted. The analysis hypothesized that the blood test room acted as a "sink" for the accumulation of ARGs/MGEs due to its role in receiving patients from diverse sources, while the other six departments served as the "sources". According to the source tracking results (Figure 4b), it was found that 50.8% of ARGs and MGEs present in the blood test room originated from the other six departments. Among these, the gynecology department was identified as a significant contributor, accounting for 31.5% of the ARGs and MGEs, while the pneumology and surgery departments contributed 7.9% and 5.4% respectively. Additionally, considering the gynecology department as one of the sources that contributed 13.2% of the genera to the otolaryngology department, it suggests that the gynecology department played a non-negligible role in the dissemination of bacteria, ARGs, and MGEs. However, it is crucial to note that a substantial portion (49.2%) of the source of ARGs/MGEs could not be identified, implying the possibility of outdoor sources. A similar source-tracking study was also conducted by Li *et al.* [5] in the same hospital, which pointed out that in the air dust, the outpatient hall was one of the main ARG transmission sources to the ophthalmology and pediatrics departments.

In summary, the mobility of ARGs in the hospital indoor environments exhibited department-specific patterns. The significant correlation between ARGs and MGEs observed in the overall studied hospital indoor environments supported the sharing phenomenon of ARGs and MGEs circulating between water and air dust samples and spreading among different departments. The pathway of this mobile transmission was assumed to originate from the gynecology department and outdoor sources, eventually accumulating in the blood test room.

3.3. Co-Occurrence between Bacterial Communities and ARGs/MGEs in the Gynecology Department

In this study, ARGs were able to be found in all studied hospital indoor environments. Indeed, the use of antibiotics by humans in the hospital has been found to stimulate the acquisition of ARGs by pathogenic bacteria [58]. To investigate whether the microbial community was correlated with ARGs and MGEs composition, we used the Procrustes analysis and the Mantel test to correlate profiles. The Procrustes test shows that there was not a goodness-of-fit test (sum of squares M2 = 0.6583, r=0.5845, p =1E-06, 10000 permutations) on the basis of Bray–Curtis dissimilarity metrics, reflecting the potential inconsistency between the bacterial community composition and the composition of ARGs and MGEs. The mantel test proved this as well (r=0.03715, p=0.113). However,

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in contrast to the insignificant relationship between all detected genera and ARGs/MGEs, the pairwise Spearman's rank correlation (23,221 pairs in total) displayed the intriguing result that tetracycline-resistant gene *tet(36)* was significantly connected with nine genera ($p<0.001$, $r > 0.6$). It has been previously reported that *tet(36)* was first discovered in *Bacteroides sp.* strain and HGT of *tet(36)* was claimed to occur frequently between divergent phylogenetic groups in the farm environment [59].

Absorbingly, although there was no correlation observed between the microbial community composition and ARGs/MGEs profile in the overall studied environment, a strong connection between them was discovered specifically in the gynecology department which was supported by the Procrustes'residuals value. In general, the residuals value represents the closeness between the ARGs/MGEs and genus composition. A lower residuals value indicates a closer relationship. In the gynecology department, we found that the average residuals value of samples was significantly low, suggesting a strong association between ARGs/MGEs and microbial composition in that specific department. To further evaluate this strong correlation, Procrustes and Mantel analyses were conducted exclusively targeting the ARGs/MGEs and microbial composition in the gynecology department. Both analyses gave compatible results, showing a strong correlation between ARGs/MGEs and microbial composition (Procrustes with M^2 of 0.1007 and p of 0.0025; Mantel test with r of 0.7571 and p of 0.018).

Based on these results, we proceeded to perform pairwise Spearman's correlation analysis between nine gynecology samples containing 43 ARGs/MGEs, and 181 genera. The results showed that all 43 target genes and 181 genera had a significant relationship, with P values below 0.05 and ρ values above 0.6. Moreover, 32 ARGs and 117 genera exhibited ρ values above 0.8, with P values below 0.01. In the study conducted by Li *et al.* [60], it was hypothesized that the non-random co-occurrence patterns between ARGs and microbial taxa could indicate the possible host information of AGRs if the ARGs and the co-existed microbial taxa possessed significantly similar abundance trends among the different environments (Spearman's $\varrho>0.8$, P-value <0.01). In line with this hypothesis, those matched correlation values of 32 ARGs/MGEs and 117 genera were sent for the undirected network analysis.

The created network of the gynecology department consisted of 185 nodes (each node represented a subtype of ARGs, MGEs, or microbial taxa) and 366 edges (Figure 5). The modularity index was 0.642 (value > 0.4), suggesting that the network had a modular structure. Based on the modularity class, the entire network was separated into three major modules. Compared with a random association, clusters of nodes in the same module contained more interactions among themselves than with other nodes. Module I was the largest module comprising 82 nodes, followed by Modules II and III including 23 and 20 nodes, respectively. From the network, it was witnessed that transposase *Tp614* and aminoglycoside-resistance *sat4* in Module I had the most positive connections with 151 types of genera individually. Besides *Tp614,* MGE genes including integrase *int1-a-marko* and transposase *tnpA 201* in Module III had more positive connections with 18 and 17 types of genera as well. Consequently, the substantial number of connections involving MGEs in the network indicates the dissemination of ARGs and the potential acquisition of these ARGs by other microbes within the gynecology department. This phenomenon underscores the impact of high antibiotic selective pressure in the hospital environment. Other ARGs, such as *bacA*, *ampC* in Module III and *tetX* in Module V, which conferred resistance to bacitracin, beta-lactamase, and tetracycline, respectively, were associated with multiple candidate genera (20, 15, and 12 genera, respectively), suggesting a higher likelihood of these ARGs being carried by a diverse range of microbial hosts. However, it is important to note that while many ARGs were associated with multiple genera, only a few ARGs were found to be potentially carried by a single host. For example, the MLSB-resistant gene *msr(E)* was exclusively correlated with *Chelatococcus,* and *Paludibacter* was the only connection with MLSB-resistant gene *lnuA* as well. These observations indicate that certain ARGs may have a more restricted distribution and are less likely to be transferred between different microbial taxa.

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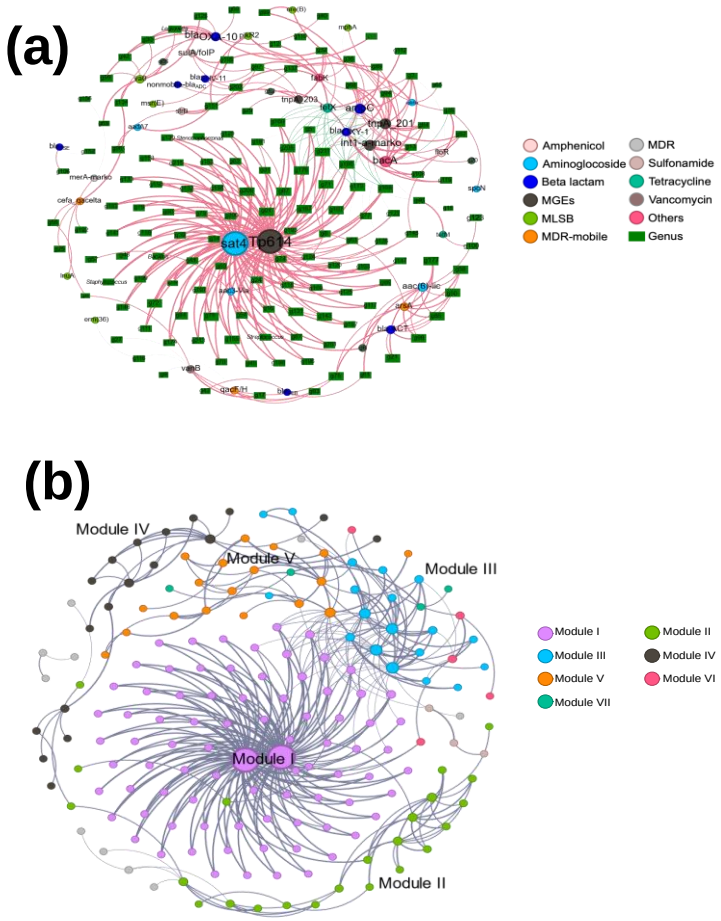

Figure 5. (**a**) Co-occurrence analysis between bacterial communities and ARGs plus MGEs in gynecology department. Circle colors represent ARGs and MGEs types, whereas all genera was represented by the dark green color. Line colors mean positive (red) or negative (blue) spearman correlation. Font/node size represents the degree of connections. Line width represent the absolute value of the correlation index, i.e. the wider the line shows, the bigger the absolute value is. (**b**) the same network as Figure(a), while the node colors here represent the Module classes.

Moreover, based on the network analysis, some concerned clinical genera appeared significantly linked to ARGs as well. *Legionella* was found to be significantly correlated with ARGs of beta-lactam-resistance *blaOXA*$_{10}$ and MLSB-resistance *va0*, which other studies have not reported. Currently, beta-lactam, fluoroquinolones, macrolides, and rifampicin are reported as the active antibiotics to which *Legionella* spp. are susceptible [61–63]. Implicitly, the resistance against beta-lactam in our study might raise concerns for the inactive therapy of antimicrobials such as amoxicillin that belongs to beta-lactam to legionellosis patients. Additionally, as the *blaOXA*$_{10}$ is a frequently encountered ARG capable of HGT in hospitals [64], the presence of beta-lactam antibiotics in the environment may promote the evolution of microbial resistance mechanisms [65]. Therefore, the potential inefficacy of beta-lactam pharmacotherapy is particularly significant and highlights the importance of considering *Legionella* spp. which colonize various man-made water systems and may be exposed to antimicrobial agents from different sources, including those produced by other microorganisms [66]. Other concerned pathogens in the network were *Bacillus*, *Staphylococcus*, *Stenotrophomonas* and *Streptococcus* correlating with *sat4* and *Tp614*. *Tp614* represents the signatures of various genetic elements involved in gene transfer and recombination. As a result, it is reasonable to estimate that *Tp614* would carry *sat4* transferred in *Bacillus*, *Staphylococcus*, *Stenotrophomonas* and *Streptococcus* which potentially impeded the antimicrobial treatment to the related infections. A similar pattern was shown by Enany and Alexander [67] that transposase *Tn5404* carried genes of *sat4* in the *Staphylococcus aureus* that was spread in the bacterial populations. Actually, *sat4* coding for aminoglycoside resistance was

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frequently found in clinical and urban wastewater carried by clinical strains [68], and the risk always arises when *sat4* formed the cluster of *aadE/ant(6)-la - sat4 - aph(3')-IIIa*, which is commonly associated with insertion elements from *Tn5405* transposons [68]. Noticeably, the network presented that *Sandaracinobacter* and *Azorhizobium* had a close loop cycle with *bla*$_{PSE}$. This implicated that *bla*$_{PSE}$ was the low-risk ARG that might not transfer in the gynecology department.

In summary, the network analysis revealed the modular structure of the gynecology department's microbial community, with specific ARGs, MGE genes, and microbial taxa forming distinct modules. MGEs played a prominent role in facilitating the dissemination of ARGs, indicating the potential for HGT among different microbial organisms. While the majority of ARGs showed correlations with a wide range of microbes, others such as *bla*$_{PSE}$ were exclusively associated with specific microbial taxa, highlighting the immobility and conservation of certain ARGs. Taken together, this finding underscores the need for careful surveillance and monitoring of antibiotic resistance, particularly in *Legionella* spp. in man-made water systems. The findings also show the importance of monitoring and understanding the genetic elements such as *Tp614* involved in gene transfer and recombination, as well as their impact on antimicrobial treatment efficacy.

4. Conclusions

Hospital indoor environments are indeed dynamic environments that harbor a diverse range of bacteria derived from both the surrounding environment and the individuals present. These factors play a critical role in determining the infectious risk faced by patients within these facilities. Considering the diverse ecological interactions and conditions within different hospital sites have significant clinical implications, this study yielded noteworthy findings by exploring the department-specific impact of variations, transmission and correlation of bacterial community, ARGs and MGEs within the hospital indoor environment. These department-specific dynamics are crucial for implementing targeted interventions and infection control measures. By recognizing the unique characteristics and contributions of each department, strategies can be developed to minimize the dissemination of antibiotic resistance and the transmission of bacteria within the hospital, leading to improved patient safety and reduced healthcare-associated infections.

Supplementary Materials: The following supporting information can be downloaded at: 10.5281/zenodo.10703376.

Author Contributions: Conceptualization, Qing Yang and Yu Xia; methodology, Qing Yang, Yuhong Sun, Bixi Zhao; software, Qing Yang; validation, Bixi Zhao; formal analysis, Qing Yang and Miao Zhang; investigation, Qing Yang; resources, Qing Yang, Zhanwen Cheng, Liming Chen, Zhenfeng Zhong and Yuhui Ye; data curation, Qing Yang; writing—original draft preparation, Qing Yang and Yu Xia; writing—review and editing, Qing Yang; visualization, Qing Yang; supervision, Yu Xia; project administration, Yu Xia; funding acquisition, Yu Xia. All authors have read and agreed to the published version of the manuscript.

Funding information: The authors are grateful to the National Key R&D Program of China (Grant No 2022YFE0103200), National Natural Science Foundation of China (42177357) for financial support.

Data availability statements: The *16S rRNA* amplicon sequencing data has been submitted to China National GeneBank (CNGB). The project ID is CNP0005378.

Acknowledgments: The authors are grateful to the National Key R&D Program of China (Grant No 2022YFE0103200),National Natural Science Foundation of China (42177357) for financial support. Also, we want to thank the State Environmental Protection Key Laboratory of Integrated Surface Water-Groundwater Pollution Control, Center for Computational Science and Engineering at Southern University of Science and Technology (SUSTech), and core research facilities at SUSTech to provide quality resources and services.

Conflicts of Interest: The authors declare no conflicts of interest.

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