Pathogenic bacteria may affectthe bacterial community structure in the sea adjacent to the sewage outlet

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Abstract

Although previous studies have analyzed bacterial diversity and community composition in marine ecosystems, some focused on the relationship between pathogenic bacteria and the composition of bacterial communities in sewage outfalls. The interactions between four pathogenic bacteria (*E. coli*, *Enterococcus*, *Staphylococcus aureus*, fecal coliforms) isolated from the sewage outfall and the bacterial diversity were investigated in the sea adjacent to the Xinghai sewage outlet, China to illustrate the influence of pathogenic bacteria on the bacterial community structure. Higher bacterial diversity was observed in the sewage outlet, and the abundance of pathogenic bacteria and bacterial diversity generally decreased with the increase in the distance from the sewage outlet. Bacterial community structure was mainly influenced by water temperature, conductivity, and dissolved oxygen in summer and winter, while pH, Oxidation-Reduction Potential and salinity were the main factors in autumn. The bacterial abundance had the most significant correlation with *E. coli*, followed by *Enterococcus* and fecal coliforms, and the least with *Staphylococcus aureus*. *E. coli* ($-0.76 < r < 0.79$) and *Enterococcus* ($-0.88 < r < 0.77$) had similar correlations to the same bacteria, in contrast to *Staphylococcus aureus* ($-0.62 < r < 0.63$). The above suggested that pathogenic bacteria may affect the bacterial community structure in the sea adjacent to the sewage outlet besides environmental factors.

Important Notes

In this study, the sewage outlet of Dalian Xinghai Bathing Beach was used as the research area, and the seasonal temporal and spatial changes of the microbial diversity and community structure in the sewage from the sewage outlet and the adjacent sea area were analyzed, and the propagation and diffusion of four significant bacteria in the sewage during the discharge process were explored. It provides a theoretical basis for the efficient management of sewage discharge.

Introduction

Marine microorganisms play a crucial role in the marine ecosystem and biogeochemistry due to their diversity, wide distribution, and strong adaptability (Lima-Mendez et al. 2015; Sunagawa et al. 2015). The abundance and community structure of microorganisms in a specific environment not only reflects the characteristics of the habitat but also explains the function and role of microorganisms in the ecosystem (Fuhrman 2009). Therefore, it is essential to understand the abundance, diversity, and community structure of microorganisms in the ocean (Gilbert et al. 2012; Bock et al. 2018). Microbial abundance and its community structure are affected by environmental conditions. Seasonal and spatial differences in environmental parameters (usually within short distances) have a significant impact on the microbial abundance and community structure (Gilbert et al. 2012; Gifford et al. 2014). Pure culture technology has been used for a long time to investigate, research, develop, and utilize microorganisms in the environment. However, imitating the specific environmental conditions for bacteria from the ocean was the main obstacle in research. The development of high-throughput sequencing technology can obtain

the microbial community structure from environmental samples, thus circumventing these challenges (Goldfeder et al. 2017).

A large number of microorganisms, especially pathogenic bacteria in land-based sewage, could cause serious environmental problems when discharged into the adjacent sea through the sewage outlet. The sea area adjacent to the sewage outlet contains relatively high levels of organic matter and nutrients (Suzuki et al. 2018), which may promote the pathogenic bacteria growth and also affect the microbial flora in the environment. Epidemiological studies suggested that sewage microorganisms from sewage outlets pose a threat to urban residents' drinking water and seafood (Human Microbiome Project 2012). Currently, the phenomenon of excessive discharge is very prominent, causing pollution that poses a severe threat to the ecological environment and microbial community of the sewage outlet and the adjacent sea areas. At the same time, land-based sewage outfall is also important for marine environmental monitoring (Teklehaimanot et al. 2014; Garcia-Aljaro et al. 2019). It is possible to understand the status and extent of the land-based sewage outlets microbial input to adjacent seas and the damage to the ecological environment of the coastal sea by assessing the amount of sewage discharged into the sea from land-based sewage outfalls in coastal waters around the world, the types and number of pathogenic bacteria, and the microorganisms community structure. Currently, the research on sewage outlets has become a global hot spot (Stewart et al. 2008; McLennan et al. 2009; Juda 2010).

E. coli, an opportunistic pathogenic bacteria (Kaper et al. 2004) causes gastrointestinal infections and other diseases in humans through contact and ingestion. It is widely distributed, and sewage discharge may introduce antibiotic resistance genes (ARGs) and drug-resistant *E. coli* into the environment (Bibbal et al. 2018; Igwaran et al. 2018). Thus exploring its abundance and influencing factors for the treatment of sewage discharge from outlets is of great significance (Jang et al. 2017). Domestic and foreign scholars reported *Enterococcus* spp. as the most effective pathogenic indicator bacteria in coastal waters (Noble et al. 2003). It is used as a fecal indicator bacteria (FIB) to monitor seawater quality and microbial pollution. Its temporal and spatial distribution and its influencing factors are closely related to water quality monitoring and public health (Tiwari et al. 2016; Tiwari et al. 2018). In 2009, the WHO designated *Enterococcus* spp. and *E. coli* as microbial indicators for water quality evaluation (WHO 2009). *Staphylococcus aureus* is distributed widely in the air, sewage, and other environments that cause a variety of diseases, such as septicemia, pneumonia, and central nervous system infections (Chambers and Deleo 2009). Fecal coliforms, as an indicator of fecal pollution, are a critical microbial evaluation index (EPA 2012). They are widely distributed, mainly used to indicate fecal pollution in seawater. The abundance of the fecal coliforms indicates the degree of fecal pollution and thereby reflects the degree of the hazard to human health (Pino-Jelcic et al. 2006; Pachepsky and Shelton 2011).

To our knowledge, few studies have analyzed the abundance of pathogenic bacteria, its community structure, and related influencing factors in the sea area adjacent to the sewage outlet (Viau and Peccia 2009; Wong et al. 2010). Thus this study aimed to explore the temporal and spatial distribution characteristics of fecal indicator bacteria (FIB) and the typical pathogenic bacteria in the sewage outlet of Xinghai Bathing Beach. It also analyzed the changes in the microbial abundance and community

structure in wastewater with seasons and locations, and the influence of pathogenic bacteria and environmental factors on its community structure. Thus it could provide a valuable reference for other sewage outfall investigations. The primary objectives of the study were as follows, (1) Evaluating cultivable microorganisms in the sewage from the sewage outfall of Dalian Xinghai Bathing Beach in different seasons, and exploring the temporal and spatial distribution of four typical pathogenic bacteria; (2) 16S rRNA gene high-throughput sequencing technology to analyze the diversity and community structure of microorganisms in sewage outfall; (3) Analyzing the correlation between community structure, environmental factors, and the pathogenic bacteria.

Material And Methods

Site description and water sampling

Xinghai Bathing Beach is one of the four largest beaches in Dalian. The sewage outlet of Xinghai Bathing Beach is located near the Shengya Ocean World. In this study, the station layout was established in the sea area near the sewage outlet, and different seasons were selected for sampling. The sampling time was July 14 in summer, September 16, in autumn, and November 5 in winter. In summer, the surface seawater (0.5 m) were collected from five sections (0 m (S1), 50 m (S2, S3, and S4), 100 m (S5, S6), 300 m (S7), and 500 m (S8)) from the sewage outlet. Autumn was similar (Fig. 1). In winter, one station (S5) was reduced at 100 m because of sampling on the shore owing to no ships.

Surface seawater samples (5 L) were collected at each station, and these samples were placed in sterile plastic barrels and transported to the laboratory at 4°C. Each surface seawater sample was divided into two parts: one stored at 4°C for physical and chemical analysis, and the other stored at − 20°C for DNA extraction and subsequent experiments.

Measurement of environmental parameters

The environmental parameters of seawater were measured using a YSI multi-parameter water quality meter. Water temperature (T), salinity (S), dissolved oxygen (DO), pH, Oxidation-Reduction Potential (ORP), and conductivity (cond) of seawater were measured *in-situ*. A total of 23 samples from different months of summer, autumn, and winter were analyzed. Sampling was carried out at different distances from the sewage outfall to study the microbial community structure and the spatial and temporal distribution of pathogenic bacteria in the sewage from the sewage outfall.

Enumeration of microorganisms

E. coli, fecal coliforms, and *Staphylococcus aureus* were enumerated using the 3M Petrifilm test kit (3M Ltd, China). The seawater sample (1 mL) was added to 9 mL of sterilized seawater for gradient dilution. The sample dilutions (10^{-1}, 10^{-2}, 10^{-3}) (1 mL) were dropped vertically on the center of the *E. coli* 3M test piece as well as the *Staphylococcus aureus* 3M test piece, and placed on the membrane with a pressure plate and pressed down gently. After removing the pressure plate, it was allowed to solidify the colloid for

more than 1 min and incubated for 24 ± 2 h at 36 ± 1°C. The colonies with pink bubbles were recorded as *E. coli* and dark purple colonies as *Staphylococcus aureus*. Similarly, the sample (5 mL) was dropped on the center of the fecal coliform 3M test piece, allowed to stand for more than 2–5 min and incubated for 24 ± 2 h at 44.5 ± 0.5°C. Red colonies with bubbles were recorded as fecal coliforms.

The concentration of Enterococci was analyzed by the membrane filtration method, according to ISO 7899–2 (ISO 2000). Diluted seawater (100 mL) was filtered through a 0.45 μm filter (Shanghai Xin Ya Purification Equipment Co., Ltd) placed on Slanetz & Bartley agar and incubated for 44 ± 4 h at 36 ± 2°C. The entire or center of a typical colony with red, maroon, or pink color was enumerated. For a typical flora, the filter was transferred onto bile-aesculin-azide agar plates and incubated at 44 ± 0.5°C for 2 h. The colonies showing tan or black in the surrounding culture medium were considered to be *Enterococcus*. Each sample was assessed in triplicate.

DNA extraction

After each seawater sample was mixed, 1 L sample was filtered through a 0.22 μm nitrocellulose filter (47 mm) (Millipore) and analyzed in triplicate. The procedure of extraction and purification of bacterial DNA was followed, as described by Kinnunen et al. (2018). The membrane filter was treated with DNase before molecular experiments to reduce the impact of exposed environmental DNA. DNeasy® PowerWater® Kit (QIAGEN, Germany) was used to extract total genomic DNA. In brief, the filter was placed into the powered bead tube, the lysate was added and vortexed. The solution was then processed to elute impurities such as RNA and protein from the system, vortexed again, and then the DNA bound to the spin filter was washed twice to purify the DNA. The extracted DNA was verified on a 1% agarose gel and stored at −20°C.

High-throughput sequencing

The water sample DNA was sent to TinyGene Bio-Tech Co., Ltd. (Shanghai) for 16S rDNA sequencing. The amplification primers for the 16S V4-V5 region gene of bacteria were 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 926R (5'-CCGTCAATTCMTTTGAGTTT-3') (Parada et al. 2015). The library was constructed using the genomic DNA as a template in a two-step PCR amplification method. The first PCR amplification 50 μL system was: 10 μL 5×Buffer, 1 μL dNTP (10 mmol/L), 1U Phusion ultra-fidelity DNA polymerase, 1 μL F/R outer primer (10 μmol/L), 5 ~ 50 ng total DNA template, and ddH2O. The second PCR amplification 40 μL system was: 8 μL 5×Buffer, 1 μL dNTP (10 mmol/L), 0.8U Phusion ultra-fidelity DNA polymerase, 1 μL F/R outer primer (10 μmol/L), 5 μL total DNA template, and ddH_2O. The first PCR conditions were: pre-denaturation at 94°C for 2 min; then denaturation at 94°C for 30 s, annealing at 56°C for 30 s, extension at 72°C for 30 s, and 23 cycles; the final extension at 72°C for 5 min. The PCR product of the first step was detected on a 2% detection agarose gel electrophoresis and recovered using AxyPrep DNA Gel Recovery Kit (AXYGEN). The second step of PCR amplification by eight cycles was performed, the Illumina high-throughput sequencing platform was utilized, and the required linker barcode was added to the target fragment. After purification, the product was subjected to quantitative quality control, and 2×300 bp sequencing was performed on the Illumina MiSeq platform.

Statistical analyses

The sequence was identified according to the barcode, and the sequence quality was controlled and filtered by UCHIME (v 4.2.40) (Edgar et al. 2011), and then spliced according to overlap relationship. The spliced sequences were subjected to quality control and filtered again, and finally, the optimized sequences were obtained. OTU cluster and species taxonomy analysis on the optimized sequence was conducted using USEARCH. Based on the OTU clustering analysis, Mothur (v1.31) software was used to analyze the OTU diversity index (alpha diversity analysis within samples), including Chao1, Shannon, and Simpson values (Schloss et al. 2009). The statistical analysis of community structure at each classification level was carried out based on the taxonomic information, and the beta diversity among samples was analyzed.

Results

Microbial diversity

The 23 samples from the sewage outlet of Xinghai Bathing Beach were sequenced in the V4-V5 region of the bacterial 16S rRNA gene by high-throughput sequencing. The number of optimized sequences obtained was 44695–53707 and clustered with 97% similarity to obtain a total of 2815 different OTUs. The OTU range between each site was between 500–1459. The highest value of OTU appeared in the C4 station, indicating higher bacterial diversity in the sewage in the winter. The A8 station 1000 m away from the sewage outlet had the least OTUs (500) in summer, and the number in winter was significantly higher than in summer.

This study statistically analyzed the alpha diversity of all samples, including OTUs, Chao, Shannon, and Simpson diversity. The coverage of all samples was above 99%, indicating that the sequencing depth can reflect the alpha diversity of sampling points. ANOVA analysis results showed that Chao and ACE at the sewage outlet were large (Fig. 2A, Fig. 2B), indicating that the species richness was higher. In addition, the Chao and ACE indexes had a decreasing trend as the distance from the sewage outlet increased, indicating that the closer the sewage outlet, the more the OTUs in the community. Similarly, the Shannon index (Fig. 2C) was highest at the sewage outlet, which decreased with the increase in distance from the outlet. The Simpson index was negatively correlated with other diversity indexes (Fig. 2D), and the value at the sewage outlet was large, indicating a high community diversity. α-diversity results showed that community diversity was inversely proportional to the distance of the sewage outlet.

β-diversity was used to analyze the content of various groups and calculate the difference between samples through Bray-Curtis. Both the presence or absence of OTU and the abundance were considered in the analysis. Figure 3A showed that there were significant differences in microbial diversity with different seasons, and Fig. 3B showed that the difference between summer and winter was the highest. However, the sewage outlet (B1) diversity in summer was more similar to winter.

Distribution of microbial community structure

The OTUs of the bacterial community in the water samples from the sewage outlets of Xinghai Bathing Beach in different seasons were clustered to obtain the main 11 phyla, 49 classes, 77 orders, 109 families, 181 genera, and 198 species. The phyla level analysis of the bacterial community structure in the sewage is shown in the Fig. 4A. The bacteria in different seasons were composed of 36 phyla, including Proteobacteria, Bacteroidetes, Actinobacteria, Cyanobacteria, among which the four dominant bacteria were: Proteobacteria, Bacteroidetes, Actinobacteria, and Firmicutes. The Proteobacteria (70.9%) dominated the sewage outlet seawater samples, followed by the Bacteroidetes (20.7%), Actinomycetes (2.7%), and Firmicutes (2.1%), and the other phyla were lower than 2%. In the three seasons, the relative abundance of the Bacteroidetes initially decreased and then increased with a decrease in temperature. In contrast, the relative abundance of Actinobacteria increased first and lowered with a decrease in temperature. The relative abundance of Firmicutes in autumn was much lower in summer and winter.

A total of 32 phyla were detected in summer, and the relative abundances of Nitrospirae and Acidobacteria closely related to sewage treatment in summer were 0.01% and 0.06%, respectively. The unique bacteria phyla in summer were Omnitrophica, the relative abundance being much higher than the other two seasons. Nitrospirae, Saccharibacteria, Yanofskybacteria, Hydrogenedentes were not detected in the summer. The rare bacteria phyla in autumn were Actinobacteria (Fig. 4A). Besides, Magasanikbacteria, Chlamydiae, Aminicenantes, Nomurabacteria, and Yanofskybacteria were not detected in sewage compared with the other two seasons. The rare bacteria phyla in winter were Deinococcus, compared with the two seasons. Most bacteria phyla were detected in sewage in winter, indicating that the highest microbial diversity in sewage from sewage outlets is in winter.

A total of 77 bacterial orders (including the unclassified parts) were detected in 23 samples. The main orders are shown in Fig. 4B. Flavobacteriales occupied the prominent position at all stations (average relative abundance 18.5%), followed by Rhodobacterales (18.1%), Oceanospirillales (16.6%), and Alteromonadales (11.7%). Through longitudinal seasonal comparison, it was found that the relative abundance of *Alteromonas* in each station in autumn was significantly lower than the other two seasons. The relative abundance of *Alteromonas* in station A8 in summer was the highest (38.3%), whereas *Rhodobacter* was the lowest (6.6%).

The detailed composition of each *in-situ* bacterial community was revealed by heat maps (Fig. 5). At the genus level, the most detectable genus among the 23 samples at the Xinghai sewage outlet was *Glaciecola* of the Alteromonadaceae family, with an average relative abundance of 8.2%. Its relative abundance was higher in summer and winter, and lower in autumn. The second detectable genus was *Marinobacterium* of Oceanospirillaceae, with an average relative abundance of 6.8%. From the perspective of longitudinal seasons, the average relative abundance of *Synechococcus* from Cyanobacteria in autumn was significantly higher than in the summer and winter. The average relative abundance of *Lentibacter* from Rhodobacterales in summer and winter was significantly higher than the autumn. The relative abundance of *Fictibacillus* in different seasons (especially autumn and winter) at each station was low (0.3%). The unclassified genera account for 43.7% of all sequences.

Temporal and spatial distribution characteristics of pathogenic bacteria abundance

To explore the changes in the abundance of fecal pollution indicator bacteria (FIB) and pathogenic bacteria in the sewage from the Xinghai sewage outlet with the season and the distance from the sewage outlet, the abundances of *E. coli*, *Enterococcus*, *Staphylococcus aureus*, and fecal coliform were determined. Figure 6 showed the changing trends of the pathogenic bacteria in different seasons. At the same distance from the sewage outlet, *S. aureus* was the most common bacteria in seawater, followed by fecal coliforms and *E. coli*, while *Enterococcus* was least in number. Water temperature and the total number of *E. coli* was found to decrease with the months (Fig. 6A). Although *Enterococcus* showed similar trends, it was more affected by temperature, and a decrease in terms of abundance was evident (Fig. 6B). The fecal coliforms showed an initial decrease in trend, which then increased, with the lowest bacterial abundance in autumn (Fig. 6C). *S. aureus* was not significantly affected by temperature, and change in the pattern was not evident (Fig. 6D). Therefore, the seasonal change in temperature is an essential factor influencing the number of microorganisms in the sewage. Besides, the optimal temperature for the survival of different microorganisms varied. Monitoring the change in the bacterial load in the water samples from different stations at the sewage outlet every month showed a decreasing trend of bacteria as the distance from the sewage outlet increased. However, some stations, such as the S6 station in winter, had exceptional circumstances, where the number of enterococci significantly increased, quite different from other stations.

Correlation between four typical pathogenic bacteria and the microbial community

Pathogenic bacteria are abundant in the sea area adjacent to the sewage outfall. In this study, *E. coli*, *Enterococcus*, *S. aureus*, and fecal coliforms were cultured, quantified, and combined with the sequencing results. Spearman's method was used to analyze the correlation between the four typical pathogenic bacterias and the different levels of community structure in sewage. At the phylum level, Tenericutes showed a significant positive correlation with *S. aureus*. Fecal coliforms were significantly positively correlated with Firmicutes and Bacteroidetes and negatively correlated with Tenericutes and Proteobacteria. Firmicutes and Bacteroidetes were also significantly positively correlated with *E. coli*, while Planctomycetes, Verrucomicrobia, Actinobacteria, and Marinimicrobia were significantly negatively correlated. Planctomycetes, Verrucomicrobia, Actinobacteria, and Marinimicrobia was found to have a strong negative correlation with *Enterococcus* (Fig. 7A).

At the order level (Fig. 7B), the bacteria that showed a significantly positive correlation with *S. aureus* were Cellvibrionales and Bdellovibrionales, while Selenomonadales and Aeromonadales were significantly negatively correlated.; Most bacteria showed a positive correlation with fecal coliforms, including Burkholderiales, Bacteroidales, Clostridiales, Aeromonadales, etc. The results showed that the negative correlation coefficient between each bacteria and the fecal coliform was small. Similarly, bacteria that showed a significant positive correlation with *E. coli* were mainly Oceanospirillales and

Flavobacteriales, etc. In addition, Acidimicrobiales, Rickettsiales, Methylophilales, Cytophagales, Rhizobiales showed a large negative correlation coefficient with *E. coli*. Similarly, *Enterococcus* also had a significant negative correlation with Acidimicrobiales, Rickettsiales, and other bacteria, and a significant positive correlation with Oceanospirillales and Bacillus.

At the genus level, the bacteria with significant positive correlation with *E. coli* were *Mesoflavibacter*, *Aquibacter*, *Salinihabitans*, and *Halioglobus*, while *Punieispirillum*, *Pelagibacter*, and *Actinomarina* of Candidatus were significantly negatively correlated (Fig. 7C). In addition, *Amylibacter*, *Balneola*, and *Fluviicola* were also significantly negatively correlated. Similarly, *Mesoflavibacter*, *Aquibacter*, *Salinihabitans*, *Halioglobu*, *Alteromonas*, *Erythrobacter*, *Psychrosphaera*, etc. were all significantly positively correlated with *Enterococcus*, while bacteria with negative correlation with enterococci were similar to that of *E. coli*. The fecal coliforms were significantly positively correlated with *Colwellia*, *Thiothrix*, *Hydrogenophaga*, etc., and had a strong negative correlation with *Synechococcus* and *Formosa*. Fewer bacteria were correlated with *S. aureus*. Among them, bacteria with negative correlations include *Lentibacter*, *Arcobacter*, etc., and bacteria with a strong positive correlation were *Synechococcus* and *Formosa*.

Influence of environmental factors on microbial community

A total of 23 samples were collected in the three seasons, summer being the tourist season. Therefore, there were a large number of tourists in the sea near the sewage outlet. The surface seawater temperature varied greatly due to the three seasons, ranging from 25.2°C to 7.8°C, salinity (S) varied slightly with seasons, ranging from 29.83 to 32.11, dissolved oxygen (DO) ranged from 12.14 to 4.24, the pH ranged from 7.7 to 8.0. The conductivity (Cond) ranged from 32011 µS/m to 47408 µS/m, and the ORP ranged from − 74.1 NTU to 45.3 NTU (Table S1). In living cells, aerobic cell potential was high, and anaerobic cell potential was low. Enzyme activity, cell assimilation ability, as well as microbial growth and development, were also affected by redox potential.

OTU abundance and the measured environmental parameters were used for redundancy analysis (RDA) to determine the specific environmental factors that can explain the composition of the bacterial community in the sewage from the sewage outlet (Fig. 8). The first two axes explain 40.16% and 9.91% of the cumulative variance, respectively. The effects of environmental factors on community structure were significantly affected by seasonal changes. In summer, water temperature, electrical conductivity, and DO were the main influencing factors, among which water temperature and electrical conductivity showed a significant positive correlation with community structure, and DO showed a significantly negative correlation. In autumn, salinity, pH, and ORP were the main variables that showed a positive correlation with community structure. The influence of environmental factors on community structure in winter was opposite to that in summer. DO was the primary determinant with a significant positive correlation with bacterial community structure, and water temperature and electrical conductivity had a significant negative correlation. It was observed that with the change in seasons, the main environmental factors affecting the bacterial community structure in sewage were different. Thus, RDA cannot explain changes

in bacterial community structure caused by unmeasured environmental variables and processes, such as virus lysis. These processes have also been proven to affect bacterial community composition.

Discussion

Seasonal distribution of bacterial community composition in the sea area adjacent to the sewage outlet

The massive discharge of sewage at the sewage outlet could bring complex biochemical changes to the adjacent seas, which can be explored preliminarily by studying the microbial community structure in the sewage. The dominant bacterial phyla in different seasons were Proteobacteria, Bacteroidetes, Actinobacteria, and Firmicutes. This result was concordant to previous studies (Raes and Bork 2008; Stewart et al. 2008; Sender et al. 2016). The bacteria phyla with the highest relative abundance in the four seasons are the Proteobacteria, which may be because the Proteobacteria is abundant in seawater. Its proportion was huge, whether it was in natural waters (such as sea bathing beach) or sewage outlet sewage (Kosek et al. 2017). Proteobacteria have a strong tolerance to environmental factors such as salinity, light, and temperature. Therefore, the external environment changes had little effect on the relative abundance of Proteobacteria in water (Hu et al. 2012; Wang et al. 2012). By observing the relative abundance changes of the Proteobacteria in different seasons, it was found that the relative abundance of *Proteus* had an increasing trend with the increase in temperature. Moreover, from autumn to winter, as the seawater temperature decreased, its relative abundance declined.

The relative abundance of Actinobacteria increased from summer to autumn and declined from autumn to winter. This may be because most Actinobacteria are mesophilic with an optimum temperature for growth ranging from 5–25 ^{o}C (Lewin et al. 2016) and limited tolerance to salinity. High salinity can affect the growth of bacteria. In summer, the temperature was higher, leading to severe water evaporation, causing the salinity of the water body to increase (Barka et al. 2016). In addition, the proportion of domestic sewage in summer was high, and the salt content was relatively large. Therefore, the relative abundance of Actinobacteria in sewage effluent in summer was low.

The relative abundance of Bacteroidetes in sewage showed a specific seasonal variation (Thomas et al. 2011). In summer and winter, its relative abundance was above 20%, which dropped to 18.26% in autumn. It may be related to the water temperature in different seasons since Bacteroides are not resistant to high temperatures in summer. A similar situation occurred in Firmicutes. Flavobacteriales (18.5%), Rhodobacterales (18.1%), Oceanospirillales (16.6%), and Alteromonadales (11.7%) showed higher relative abundances at the order level. The dominant species at the genus level was *Glaciecola* of Alteromonadaceae, which had a higher relative abundance in summer and winter, and a lower in autumn; followed by *Marinobacterium* of Oceanospirillaceae. The results of this study showed that there was no significant difference in the microbial community structure at different distances from the sewage outlet in the same season. However, the diversity index and community structure at the phylum level had a high similarity. Conversely, at the genus level, there was a significant difference in the microbial community

structure between different seasons. There were also obvious seasonal differences in the specific bacteria at the sewage outlet. There were 12 specific bacteria in summer:*Chroococcidiopsis, Cerasicoccus, Desulfococcus, Acanthopleuribacter, Amaricoccus, Aminomonas, Acetobacter, Fangia, Aquicella, Grimontia, Microbulbifer, Nannocystis*, There were the largest number of specific bacteria in autumn, with 29 species:*Geobacter, Anaerostipes, Streptococcus, Peptococcus, Zhongshania, Sphaerotilus, Odoribacter, Uliginosibacterium, Actinobacillus, Lachnospira, Chitinivorax, Pseudenhygromyxa, Aequorivita, Taibaiella, Brooklawnia, Saccharofermentans, Pelosinus, Elusimicrobium, Hoppeia, Bradyrhizobium, Petrimonas, Anaerofilum, Bacteriovorax, Propioniciclava, Roseomonas, Sedimentibacter, Desulfomonile, Rubrivirga, Acetobacter.Acetobacter* was the specific bacteria common in summer and autumn. It was worth noting that no specific bacteria were found at the sewage outlet in winter.

Pathogenic bacteria and its correlation with community structure

From the perspective of seasonal changes, pathogenic bacteria were significantly affected by seasonal changes (Kay et al. 2005). The bacterial abundance of *E. coli* decreased with the months, which may be related to temperature and light (McMinn et al. 2020). Previous studies determined the temporal and spatial variation in the abundance of enterococci (Cui et al. 2013). Understanding the survival rate of aqueous *E. coli* is essential for assessing microbial contamination and making appropriate management decisions. Previous studies have shown that the survival rate of *E. coli* was mainly dependent on temperature. (Jamieson et al. 2004; Blaustein et al. 2013) and thus tends to decrease with seasonal changes. The abundance of *E. coli* at station S8 in summer was significantly higher than the nearby stations, which may be due to the excretion of seabird feces into the sea.

Numerous epidemiological studies have shown that the outbreak period of *Enterococcus* was in the summer (Wade et al. 2008; Heaney et al. 2012), which is concordant to our study. The abundance of enterococci significantly reduced in autumn and winter. The abundance of fecal coliforms showed a decreasing trend from summer to autumn, which gradually increased in winter. *S. aureus* is widely distributed (Akanbi et al. 2017), and its abundance was higher than the other three pathogenic bacteria. However, it was the least in autumn and winter at station S2, which may be related to the reduction of DO. In autumn, the abundance of fecal coliforms at stations closer to the sewage outlet did not change significantly but decreased significantly at longer distances. The fecal coliforms at station S4 increased significantly. Studies have shown that light and solar radiation affects their abundance (Fiello et al. 2014). In winter, fecal coliforms increased due to temperature.

From the perspective of the distance from the sewage outlet, with the increase in distance from the sewage outlet, pathogenic bacteria showed an apparent decreasing trend. The abundance of *S. aureus* did not change significantly with the distance from the sewage outlet. However, fecal coliforms were significantly affected by the change in the distance from the sewage outlet. In addition, *Enterococcus*

increased initially in autumn and winter and then decreased with the increase in distance from the sewage outlet.

Pathogenic bacteria in environmental samples are diverse, which could impact the survival characteristics of the community structure (Bibby and Peccia 2013). For decades, there have been studies on the relationship between microbial community structure and ecosystem (Wittebolle et al. 2009). However, the relationship between community structure and pathogenic bacteria, in particular, is unclear. In the community structure at different levels, the bacteria with a strong correlation with *E. coli* were the most, followed by *Enterococcus* and fecal coliforms, while *S. aureus* was the least. There were significant differences in the correlation between the same species and the four pathogens. The positive and negative correlation between *E. coli* and *Enterococcus* was similar. Most bacteria that had a significantly positive or negative correlation with *E. coli* also had a significantly positive or negative correlation with *Enterococcus*, respectively. The results of the fecal coliform group and *E. coli* were similar, but there were some differences. Compared to *E. coli*, the correlation coefficient between the community structure and the fecal coliform was smaller.

In contrast, the situation of *S. aureus* was the opposite. From all levels, most bacteria that had a positive or negative correlation with these three pathogens had a negative or positive correlation with *Staphylococcus aureus*, respectively. The correlation between *E. coli* and *Enterococcus* to that of bacteria was similar, which was contrary to *S. aureus*. Therefore, our research showed that different pathogenic bacteria had a significant effect on the community structure, which varied considerably.

Environmental factors influence on bacterial communities

This study found that the conductivity at the sewage outlet was relatively high (46887μS/cm), general industrial wastewater can exceed 1000μS/cm, and water with high inorganic salts can reach 10,000μS/cm. Its high concentration was closely related to the mineral content in the water. The relationship indicated that the concentration of inorganic salts was relatively high, and there were many impurities.In addition, DO content in the seawater at the sewage outlet was lower than that of other stations. It may be that the discharge of sewage caused some nutrients to water, causing a large number of phytoplankton to grow wildly, consuming oxygen, and the oxidation of inorganic matter and the decomposition of organic matter would also be affected.In addition, the ORP in seawater varies greatly from season to season. It was reduced in summer and winter, and oxidized in autumn. The reason may be related to the sewage treatment system. This study analyzed the correlation between environmental factors and the microbial community structure, which explained the community structure of seawater to a high degree. Seasonal changes cause significant differences in the impact of environmental factors on the microbial community structure (Chen et al. 2017). In summer, water temperature and conductivity are the main influencing factors (Wang et al. 2014). In addition, previous studies have also shown that salinity is also one of the important influencing factors of microbial abundance in sewage, that affects the reproduction and growth rate of microbial flora (Thomas et al. 2011; Jeanneau et al. 2012; Mattioli et al. 2017). Studies have shown that at low-temperature conditions, the increase in salinity leads to a rise in the rate of bacterial decay in sewage, resulting in a decrease in bacterial concentration. In addition, when

the light intensity is low, the bacterial concentration is inversely proportional to the salinity (Schulz and Childers 2011). In autumn, salinity, pH, and ORP are the main environmental factors that caused changes in the microbial community structure (Elliott et al. 2006; Campbell et al. 2015). In winter, dissolved oxygen is the primary determinant of bacterial community structure. These environmental factors confirmed the change of bacterial biodiversity, indicating that the abundance of microorganisms and community structure in sewage outfall were significantly affected by environmental factors.

Overall, this experiment evaluated the diversity and community structure of microorganisms in the sewage into the sea, explored the abundance of typical pathogenic bacteria, revealed the correlation between microorganisms and community structure, and analyzed the influence of environmental factors on the community structure. Thus our study revealed the changes in the sewage microbial community structure with the season and the distance of the sewage outlet. which could guide the monitoring and treatment of seawater water quality. However, the scope of this study is limited to the sea area adjacent to the sewage outlet. It is necessary to further study whether sewage microorganisms have an impact on the water quality and organisms of the distant sea, and to further explore the functional stability of the marine ecosystem near the sewage outlet.

Declarations

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Conflict of Interest

None declared.

Author contribution

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Yunhan Fu, Jie Su,Feng Dai and Hongxia Ming. The first draft of the manuscript was written by Yunhan Fu,Tingting Shi,Yuan Jin, Daoming Guan,Jingfeng Fan and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Table

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Figures

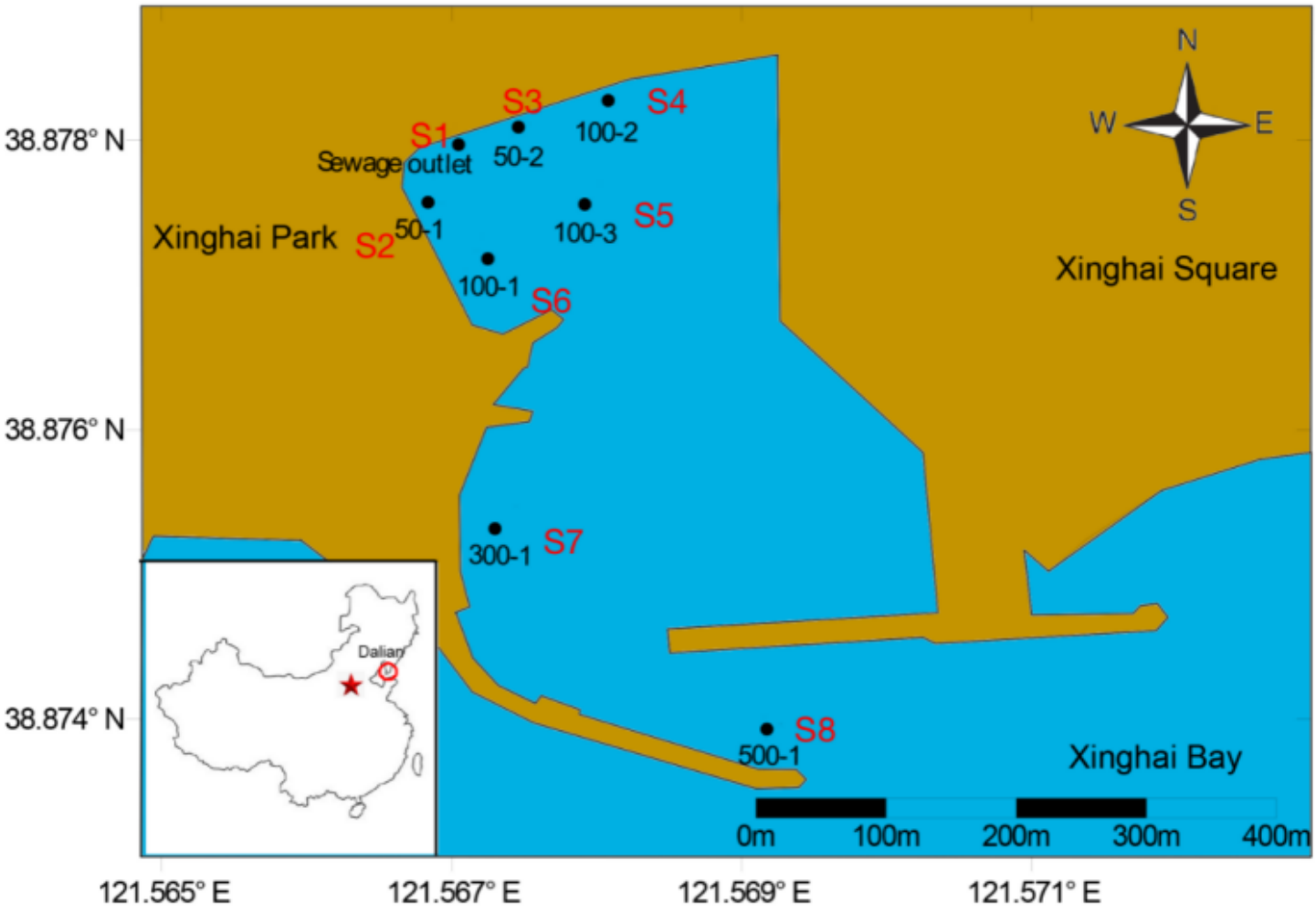

Figure 1

Sample stations at the sewage outlet of Xinghai Bathing Beach.

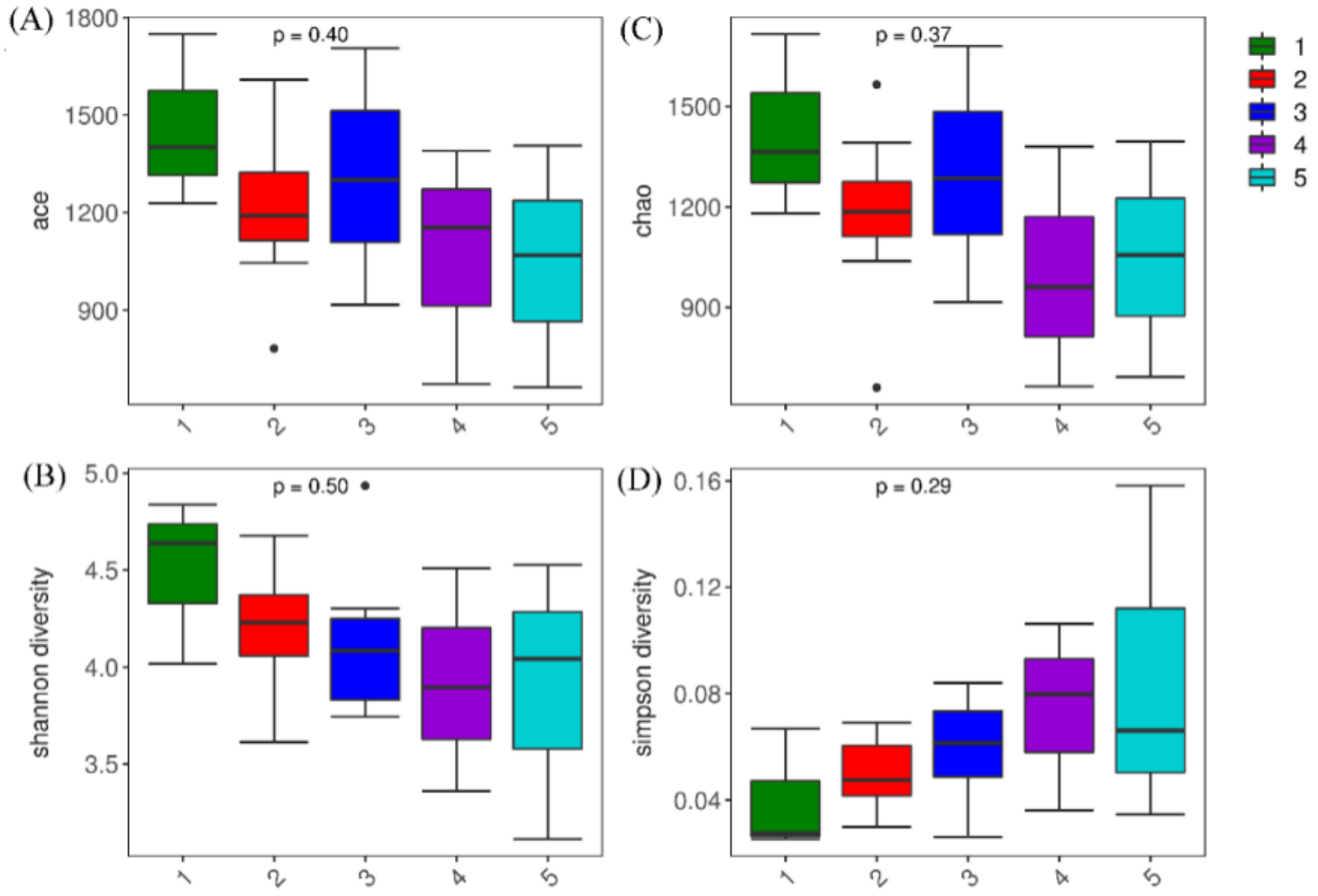

Figure 2

Four α diversity parameters of different stations at the sewage outlet of Xinghai Bathing Beach. The 1, 2, 3, 4, and 5 of the legend represented different distances from the sewage outlet (0 m, 50 m, 100 m, 300 m, 500 m).

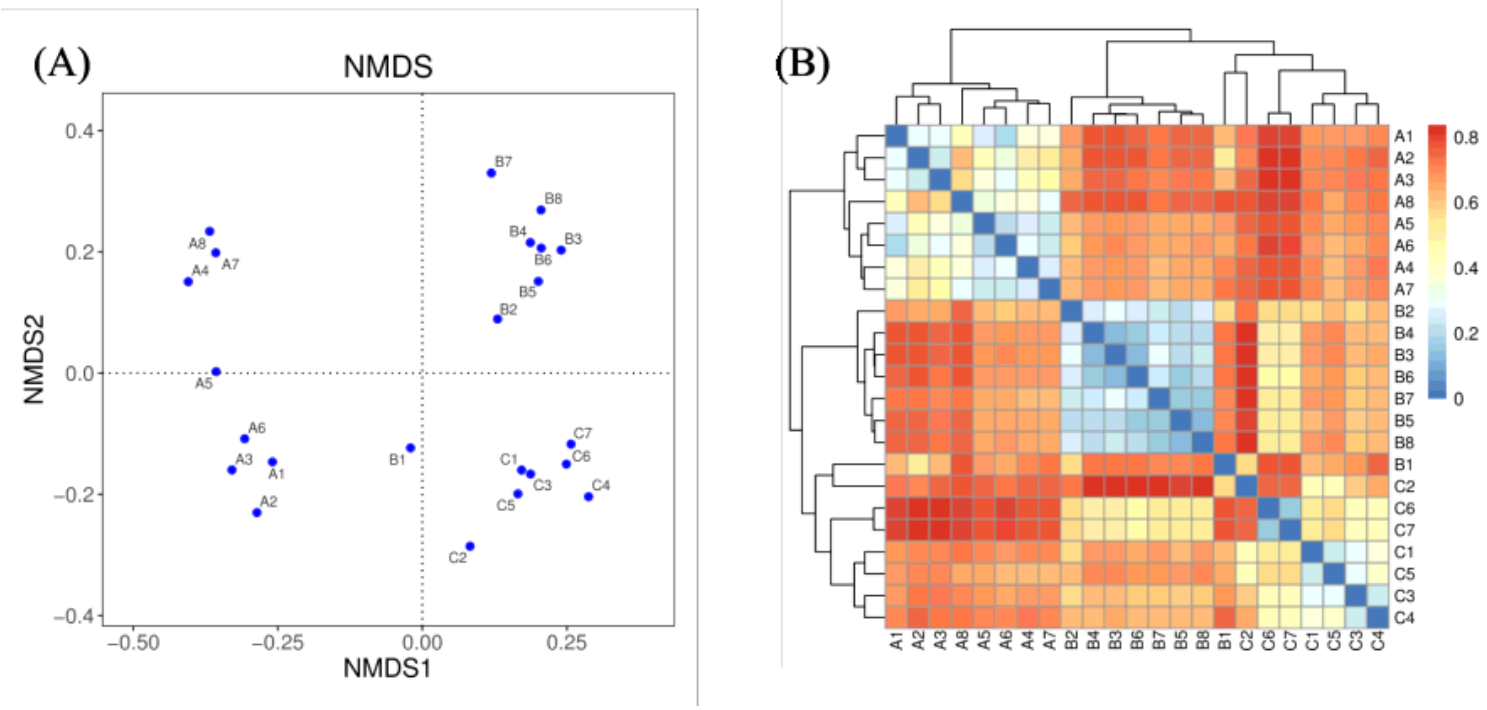

Figure 3

NMDS analysis and difference matrix heat map of sewage outlet seawater.

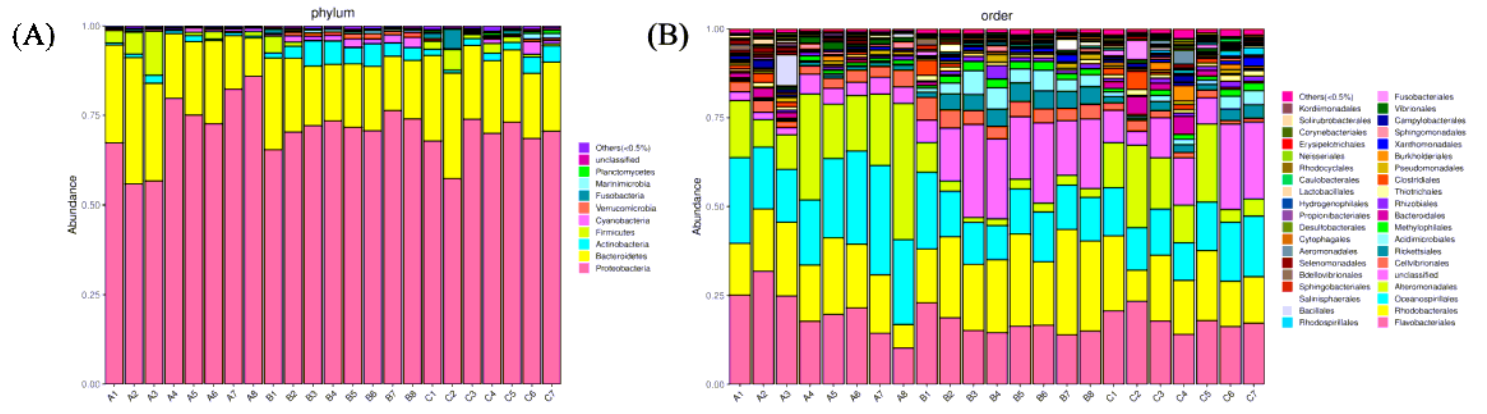

Figure 4

Relative abundances of dominant species at the phylum level and order level in sewage.

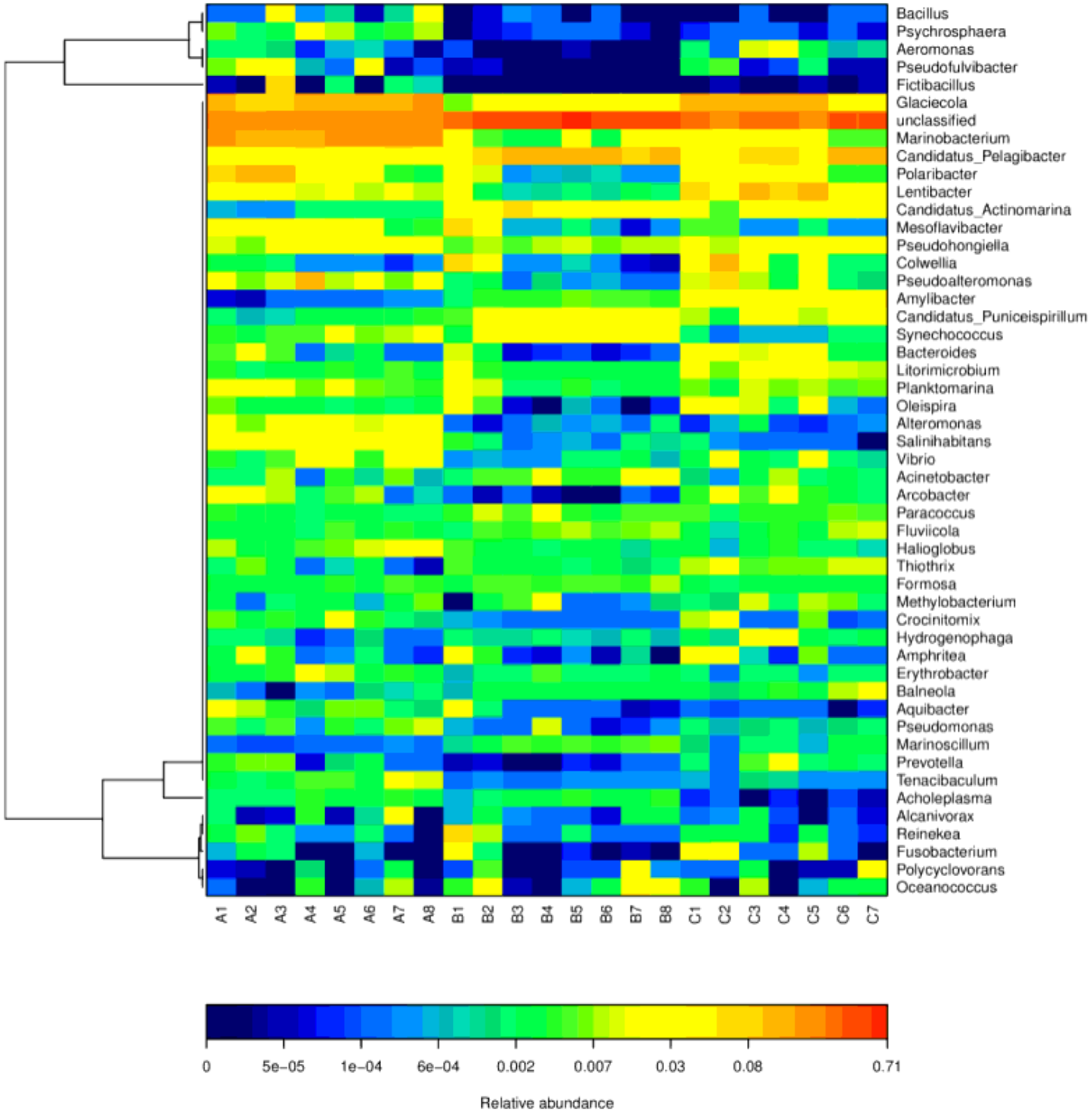

Figure 5

Relative abundances of dominant species in sewage samples.

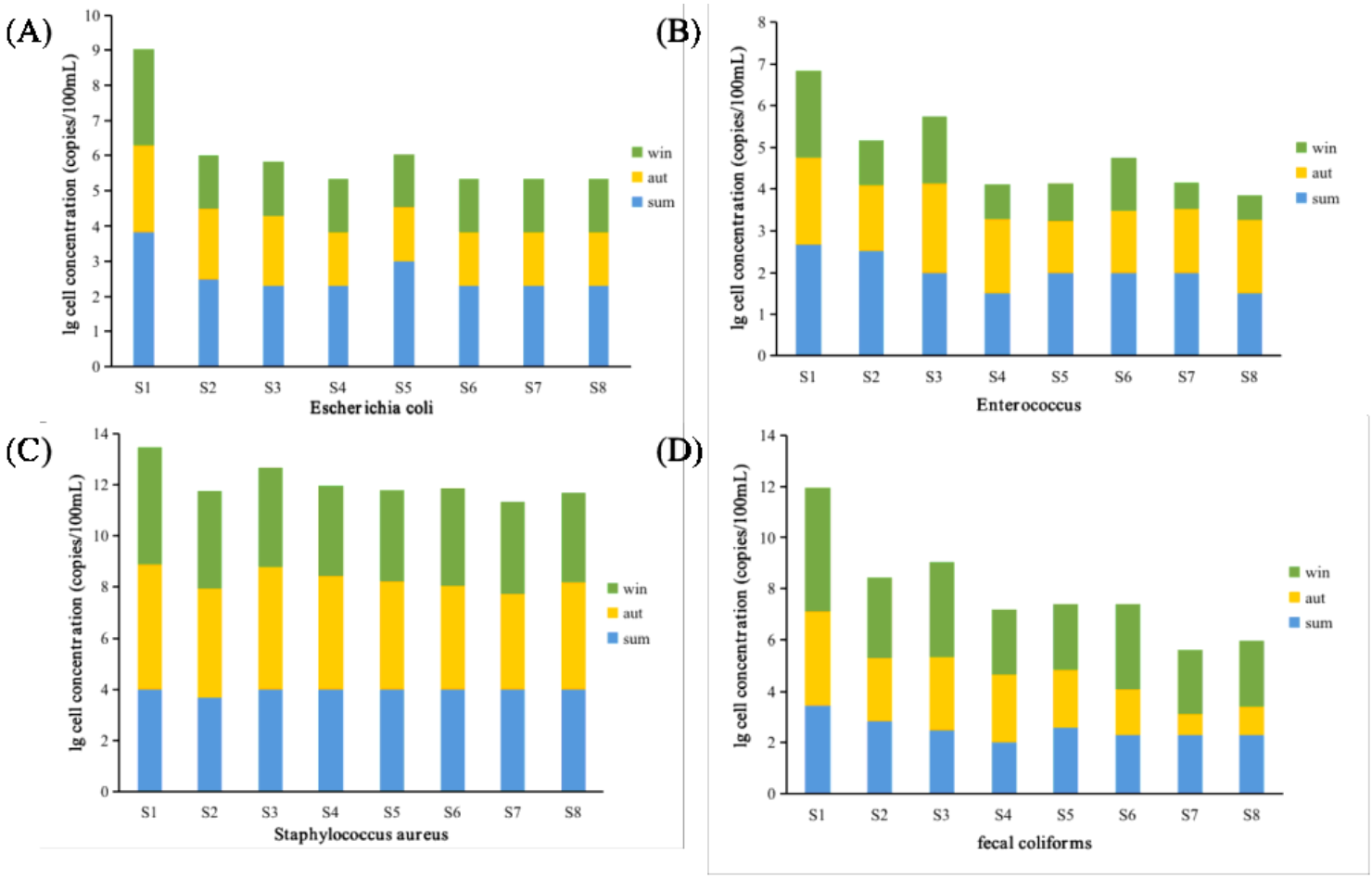

Figure 6

Temporal and spatial distribution of the abundance of the four pathogenic bacteria. SUM, AUT, and WIN represent summer, autumn, and winter, respectively.

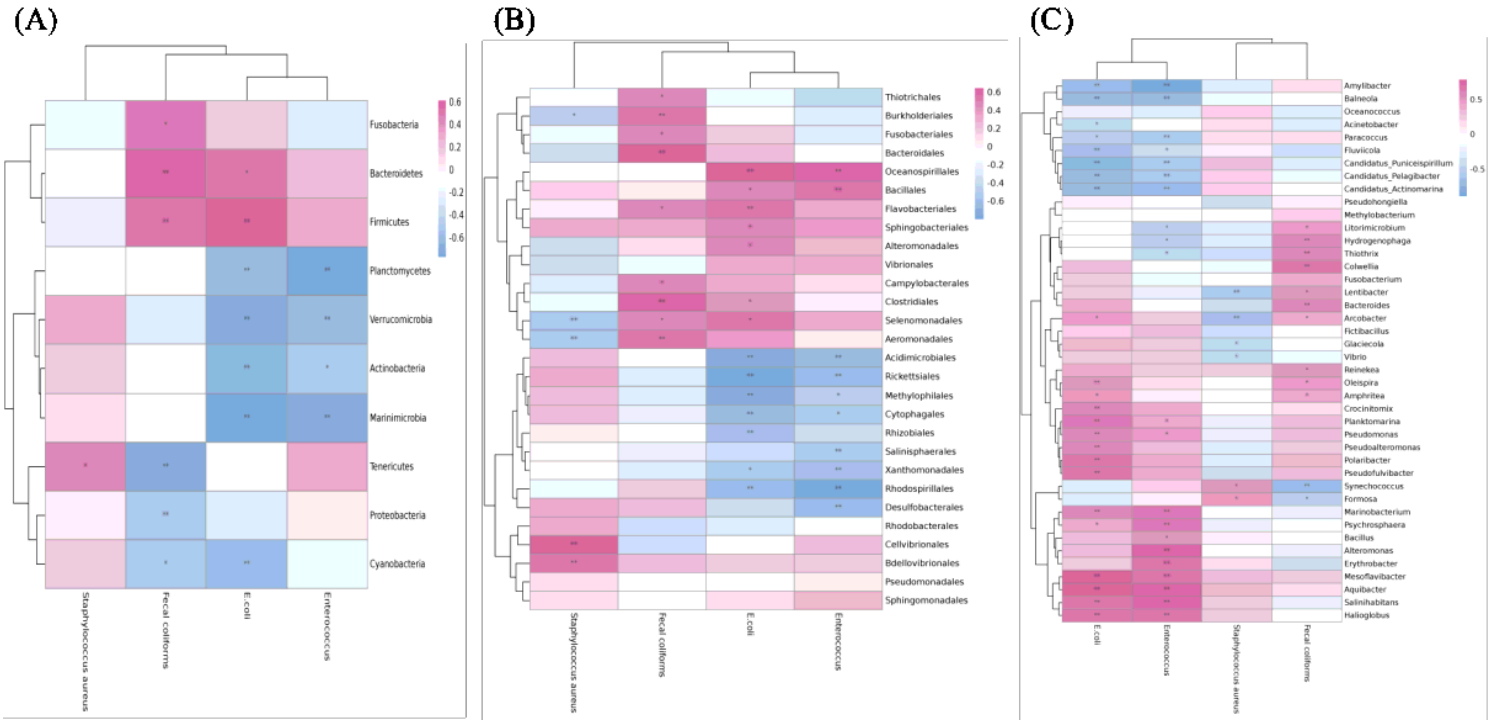

Figure 7

Heat map analysis of the correlation between the four pathogenic bacteria and the level of phylum, order, and genus community structure. The color represents the correlation coefficient: blue is a negative correlation, the pink is a positive correlation, and the darker the color, the greater the correlation coefficient. The corresponding relationship between the specific color and the correlation coefficient, as shown in the legend in the upper right corner. The * in the figure represented the *P*-value, * was 0.05 > *P* > 0.01, ** was 0.01 > *P* > 0.001, and the trees on the left and above were clustered according to the similarity of the correlation coefficient.

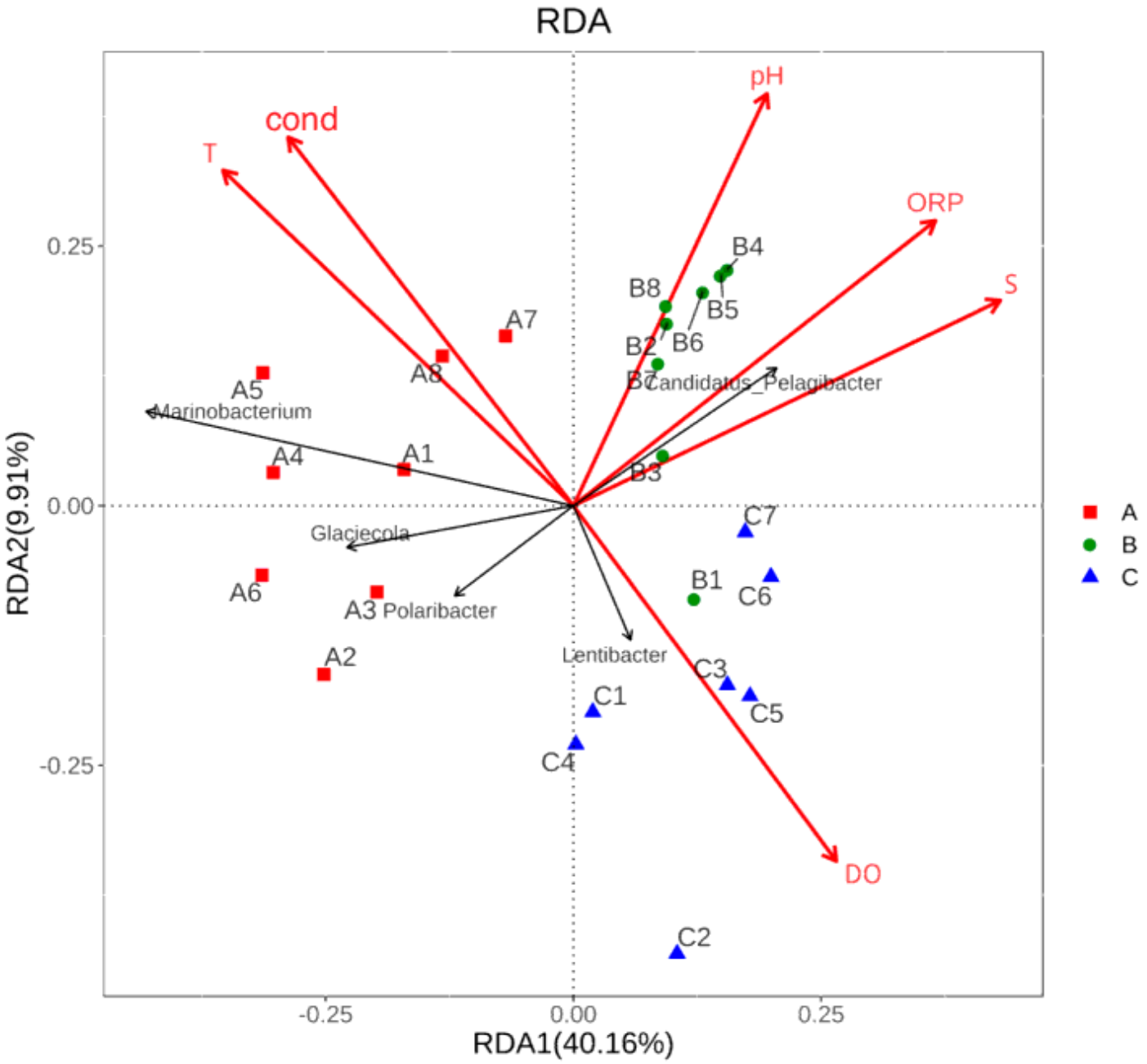

Figure 8

RDA analysis of seawater quality parameters and microbial community structure. T, cond, and S represent water temperature, conductivity, salt, respectively. Samples of categories A, B, and C represent the sampling in summer, autumn, and winter, respectively.