Composition and structure of microbial communities associated with different domestic sewage outfalls

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ABSTRACT. The diversity of microbiota in waste waters has not been thoroughly examined, despite the potential impact of microbes on effluent quality. Wastewater microbial communities harbor pathogenic bacteria, viruses, and parasites. To study microbial communities in domestic sewage outfalls, 454 pyrosequencing technology was used to investigate the composition of microbial communities associated with municipal wastewater during different seasons sampled over the course of one year. A total of 195,103 16S rRNA gene sequences were obtained from 20 samples. The R software was used to calculate the number of indices describing the alpha diversity associated with each bacterial assemblage. In this study, the α-diversity index (H', D, J), in which higher numbers represent more diversity, was found to change with seasonal cycle. The diversity of bacterial assemblages was high

in all samples, indicating that species diversity was also very high. The taxonomic composition of the assemblages varied considerably among samples, with some dominated by Proteobacteria, while others were dominated by Bacteroidetes or Firmicutes. In 2 samples, the relative prevalence of Proteobacteria exceeded 90%. α-Proteobacteria, β-proteobacteria, and γ-proteobacteria represented 90% or more of all Proteobacteria. The present characterization of wastewater from five sewage outfalls indicated the presence of some pathogenic bacteria. The γ-Proteobacteria in sewage wastefalls identified in this study included Enterobacteriaceae, Vibrionaceae, Pseudomonadaceae, *Salmonella*, *Yersinia*, *Vibrio*, and *Pseudomonas aeruginosa*.

Key words: Sewage; Bacterial diversity; 454 Pyrosequencing; Metagenomics

INTRODUCTION

Domestic sewage contains high concentrations of pollutants, including suspended solids, organics, and nutrients, which can cause a large amount of damage to marine environments (Teodoro et al., 2010). Bacteria are the most abundant and diverse group of organisms living in wastewater (Despland et al., 2012; Yu et al., 2012). The microbial diversity in sewage is very high, yet even a basic understanding of how this diversity is distributed ecologically is lacking. A better understanding of sewage microbial communities will not only provide an important framework for the design and operation of stable wastewater treatment systems, but also will enrich the study of microbial ecology.

Culture-based methods are biased in their evaluation of microbial diversity, are time-consuming, and provide information on only approximately 1% of bacteria that are amenable to cultivation (Nocker et al., 2007). As with cult uring, the primary drawbacks of molecular methods include both labor and financial costs. Recently, DNA sequencing has been revolutionized by the introduction of massively parallel sequencing systems, such as 454 pyrosequencing analysis (Margulies et al., 2005; Cristea-Fernstrom et al., 2007; Huse et al., 2007; Liu et al., 2007; Roesch et al., 2007; Sundquist et al., 2007). Pyrosequencing techniques are high-throughput analytical tools that are capable of generating large numbers of DNA reads through a massively parallel sequencing-by-synthesis approach (Margulies et al., 2005). This technology is extensively used in the analysis of microbial communities in various environmental samples (Ye et al., 2011), including the marine water column (Qian et al., 2011), terrestrial soils (Roesch et al., 2007), human distal intestinal content (Claesson et al., 2009), and wastewater treatment plant influent (McLellan et al., 2010).

The diversity of microbiota of wastewaters has not been thoroughly examined, despite the potential role that microbes have on the quality of effluent. This is particularly true in developing countries, where wastewater from residences and factories flow through a series of drains that ultimately lead to the sea. These drains, known as sewage sea outfalls, exist in various sizes and shapes (State Oceanic Administration of the People's Republic of China). In recent years, increased economic productivity has impacted sewage outfalls, notably by decreasing the water quality of nearby coastal waters (Li et al., 2011; Wang et al., 2011). Ningbo

is located in the middle coastline of Mainland China. The primary sea waters near Ningbo include the south coast of Hanzhou Bay, the mouth of the Yong River, the Xiangshan port, Sanmen Bay, and the adjacent sea areas of Beilun to Daxie. Environmental monitoring has indicated that wastewater overflow is common in the sewage draining outfalls in this area (Ningbo City Ocean and Fishery Bureau, China). Heavy pollution poses a profound threat to the ecology of the macro and microbial communities in coastal waters. Xiangshan port, located in the northern coastal area of Zhejiang, is a long, narrow, and semi-enclosed bay near the northeast and further inland to the southwest. The bay is spotted with numerous types of wastewater outlets. Because of geographical reasons, little exchange occurs between this bay and the external seawater. As a result, the bay exhibits a unique microbial diversity that is distinct from that found in open seawater (Kent et al., 2001; Li et al., 2006; Schnetzer et al., 2011). Most studies to date have focused on wastewater treatment systems themselves, while reports of the bacterial diversity associated with domestic sewage are rare. In this study, we investigated the microbial diversity associated with sewage outfalls in Xiangshan port in samples collected in different months using high-throughput pyrosequencing technology. Each sample underwent two types of treatment processes operated in parallel. This allowed comparison of community with respect to i) each type of treatment system, ii) between samples, and iii) between samples collected over an annual cycle.

MATERIAL AND METHODS

Sample collection

All samples from the 5 sewage outlets were collected in Ningbo, China in 2011. Among them, S1 was located in Xiangshan County, samples S2 and S3 were located in Ninghai County, S4 was located in Fenghua County, and S5 was located in Beilun district. Details regarding these outlets are shown in Figure 1, and all the 5 sewage outlets were near Xiangshan port. According to a study by the Ningbo City Ocean and Fishery Bureau and State Oceanic Administration of the People's Republic of China, the 5 sewage outlets have been subsumed into domestic sewage outfalls.

Untreated wastewater samples were collected in clean, pre-sterilized 5-L containers. Samples were collected during different seasons: winter (March), spring (May), summer (August), and autumn (October). For each of the 5 sites, samples collected in March were named Mar1, Mar2, Mar3, Mar4, and Mar5, respectively; samples collected in May were named May1, May2, May3, May4, and May5, respectively; samples collected in August were named Aug1, Aug2, Aug3, Aug4, and Aug5, respectively; and samples collected in October were named Oct1, Oct2, Oct3, Oct4, and Oct5, respectively. The wastewater samples collected were brought to the laboratory and filtered immediately. Seawater samples were filtered through 0.2-μm polycarbonate filters (Poretics Products; Livermore, CA, USA). Filters for DNA extraction were stored at -80°C until processing.

DNA extraction and pyrosequencing

Total genomic DNA was extracted from the filters using the Fast DNA SPIN kit for Water (Omega Bio-Tek; Norcross, GA, USA), according to manufacturer instructions. To

ensure sample quality, DNA extracts must have had an absorbance ratio at 260 to 280 nm and at 260 to 230 nm of 1.80-2.00 and >1.70, respectively. Fragments of the bacterial 16S rRNA gene were amplified by polymerase chain reaction (PCR) using the following primers: forward primer 5'-CCATCTCATCCCTGCGTGTCTCCGACTCAG and reverse primer 5'-CCTATCCCCTGTGTGCCTTGGCAGTCTCAG (Yang et al., 2012). PCR reactions were performed in a 20-µL volume containing 2 µL 10X Ex Taq Buffer (Mg^{2+}-free), 2 mM $MgCl_2$, 0.2 mM dNTP, 0.4 µM of each forward and reverse primers, 0.2 U TaKaRa Ex Taq, and 50-100 ng DNA template (TaKaRa; Shiga, Japan). Amplification was carried out with the following PCR program: 94°C for 5 min, 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min for 35 cycles. A final 10-min extension step at 72°C was also performed. PCR products were stored at 4°C. PCR products were confirmed using 2% (w/v) agarose gel. The DNA band with the correct size was excised and purified using a PCR clean-up system. Equal amounts of purified PCR products were pooled for subsequent 454 pyrosequencing.

In order to determine the composition of the bacterial communities, purified PCR products were subjected to shotgun pyrosequencing using 454 GS Junior and Titanium chemistry (454 Life Sciences-OE Biotech Company; Shanghai, China). 454 Pyrosequencing was carried out on the Titanium platform (Roche/454 Life Sciences; Basel, Switzerland). Emulsion PCR was set up according to the manufacturer protocol (McKenna et al., 2008). Each sample was tested three times.

Sequence analysis

Raw sequence data was sorted based on sample-specific barcode tags. Primers and barcodes were trimmed from sorted sequences. Raw sequences were processed through the Ribosomal Database Project II (RHD-II) pyrosequencing pipeline (Cole et al., 2009). First, ambiguous and short sequences less than 150 bp in length were removed. Second, qualified sequences were clustered into operational taxonomic units (OTUs) defined by a 3% distance level using complete-linkage clustering. Third, these sequences were assigned to phyla using the RDP-II classifier at a 50% confidence threshold (Wang et al., 2007). The sequences were clustered based on the similarity threshold of 97% sequence identities.

The R (v .2.14.2, http://www.r-project.org/) software was used to calculate a number of indices describing the alpha diversity associated with each bacterial assemblage, including the Shannon-Wiener index (H'), the Simpson's diversity index (D), and Simpson's evenness index (J). Heat maps were drawn by hierarchal clustering performed in R (g plots).

RESULTS AND DISCUSSION

As shown in marine, soil, and human gut samples, massively parallel pyrosequencing permits the exploration of microbial diversity in complex communities in unprecedented depth (Huber et al., 2007; Acosta-Martínez et al., 2008; Dethlefsen et al., 2008; Bissett et al., 2011). Pyrosequencing has afforded researchers the ability to detect the 'rare biosphere' to a greater extent than possible using traditional clone library approaches (Fabrice and Didier, 2009; Ye et al., 2011). This technology has contributed to the discovery of novel sequences that potentially represent new species (Suau et al., 1999; Im et al., 2012). In this study, we used pyrosequencing to characterize the composition and structure of bacterial populations in

5 geographically distinct sewage outfall samples, and identified shifts in the composition of these assemblages over a period of a year. A total of 195,103 sequence tags were obtained for the 20 samples analyzed in this study (Table 1). Cluster analysis was used to compare community composition and to investigate the common taxonomic classes that lead to the overall patterns in clustering. The samples were analyzed based on the composition of the microbial community and the relative number bacteria at the phylum level. The bacterial community associated with Oct5, sampled in October, was significantly different from that of other samples acquired from other outlets (Figure 1). In October, Firmicutes was the dominant class. Other samples, particularly Mar4, May2, May3, Oct4, May4, Aug5, and Oct2, showed considerable similarity and were ranked into one category. In addition, the bacterial communities associated with Mar5 and Oct3 showed the most similarity.

Table 1. Sample information, comparison of phylotype coverage and diversity estimation of the 16S rRNA gene libraries obtained from the pyrosequencing analysis.

Sample name	Good reads[a]	H'	D	J
Mar1	14083	3.72	0.81	0.76
May1	12996	4.51	0.91	0.84
Aug1	3925	4.18	0.87	0.81
Oct1	3757	5.06	0.95	0.88
Mar2	10077	3.91	0.83	0.92
May2	13878	3.92	0.83	0.81
Aug2	5432	4.31	0.89	0.83
Oct2	8672	4.26	0.88	0.81
Mar3	12858	2.52	0.72	0.57
May3	13715	4.67	0.93	0.92
Aug3	5413	4.11	0.87	0.78
Oct3	8763	4.60	0.92	0.85
Mar4	11763	2.94	0.77	0.62
May4	15446	3.05	0.79	0.61
Aug4	6973	4.04	0.87	0.71
Oct4	6650	4.17	0.88	0.75
Mar5	12391	3.86	0.81	0.86
May5	13208	4.17	0.87	0.83
Aug5	5560	5.09	0.95	0.94
Oct5	9543	4.55	0.92	0.83

[a]Reads exceeding 150 bp were considered to be good reads. H' = Shannon-Wiener index, in which higher numbers indicate more diversity. D = Simpson's Diversity index, in which higher numbers indicate more diversity. J = Simpson's Evenness index, in which high numbers indicate more evenness.
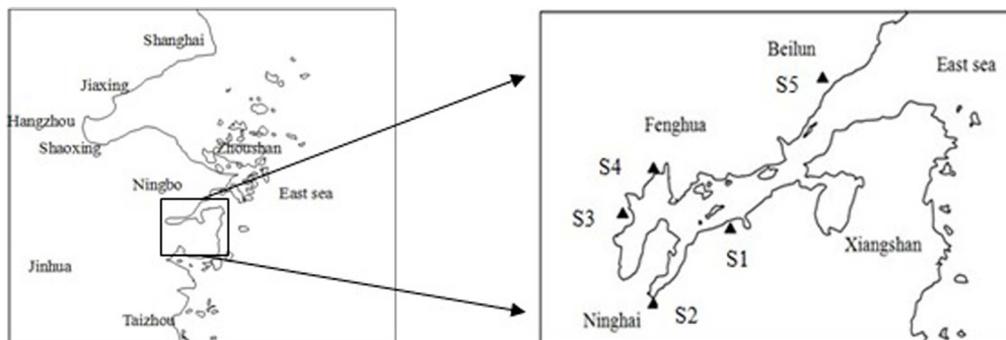

Figure 1. Map of sampling site.

Structure of bacterial communities in different samples

The composition of bacterial 16S rRNA genes associated with various sewage samples differed at the phylum level (Figure 2). In most samples, the predominant phylum was Proteobacteria, while the next most common phyla included Bacteroidetes, Acidobacteria, Cyanobacteria, and Firmicutes, in this order. In 2 samples, the relative prevalence of Proteobacteria exceeded 90% (May2, 90.2%; May3, 91.7%). Surprisingly, the percentage of Proteobacteria was only 3.8% in sample Oct5; in this sample, Firmicutes was the predominant phylum. In Mar3, sequences associated with Firmicutes represented 33% of the total sample, while the relative abundance of other taxonomic classes was lower than 10%. For the May1 sample, the relative prevalence of Verrucomicrobia was 12%, which was higher than that found in the other samples.
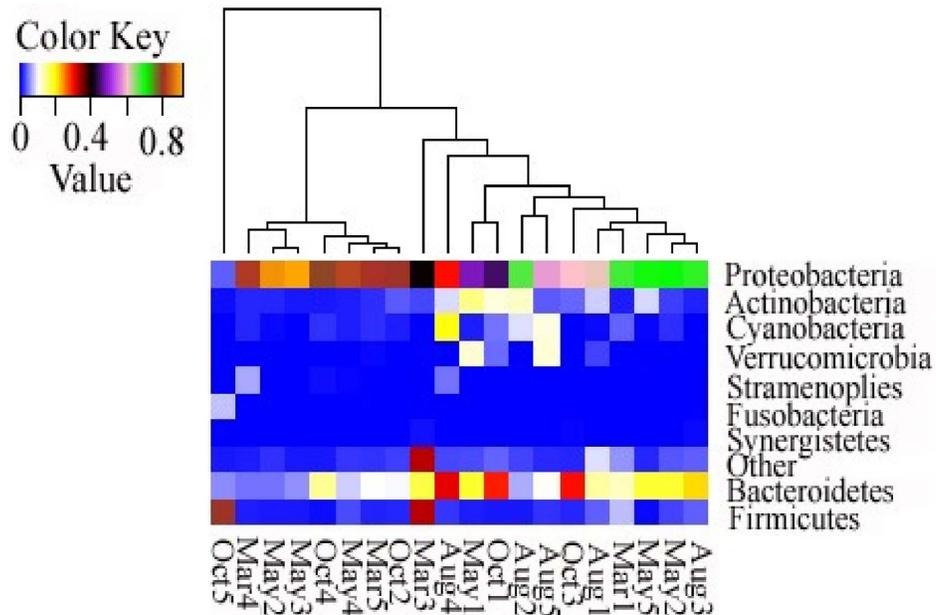

Figure 2. Clustering of sequences, defined at the phylum level, for different samples. The prevalence of microorganisms among five sewage outfalls from different month. Raw sequences were processed through the Ribosomal Database Project II (RHD-II) pyrosequencing pipeline, and the sequences were clustered based on the similarities of 97% sequence identities. The linkages of the dendrogram do not show phylogenetic relationship of the bacterial classes, but it is based on relative abundance of taxonomic profiles. The heat map color represents the relative percentage of the microbial descriptions within each sample, with the legend indicated at the upper left corner. Different colors represent relative percentage. Blue represents lower content and orange represents higher content.

The structures of microbial communities in different samples acquired during the same month were investigated in this study (Figure 2). As shown in Figure 3A, the relative abundance of Proteobacteria did not change significantly during the sampling period in sample S1, but the relative abundance of Bacteroidetes did change. In October, the percentage of this

phylum was twice that observed in March. While the abundance of sequences affiliated with Proteobacteria did not change, the composition of these sequences was distinct. For example, in March, sequences affiliated with γ-proteobacteria were dominant, where as in other months, β-proteobacteria dominated these communities. γ-Proteobacteria consists of Enterobacteri-aceae, Vibrionaceae, and Pseudomonadaceae. Some pathogenic bacteria are affiliated with γ-proteobacteria, including genera such as *Salmonella*, *Yersinia*, *Vibrio*, and *Pseudomonas*.
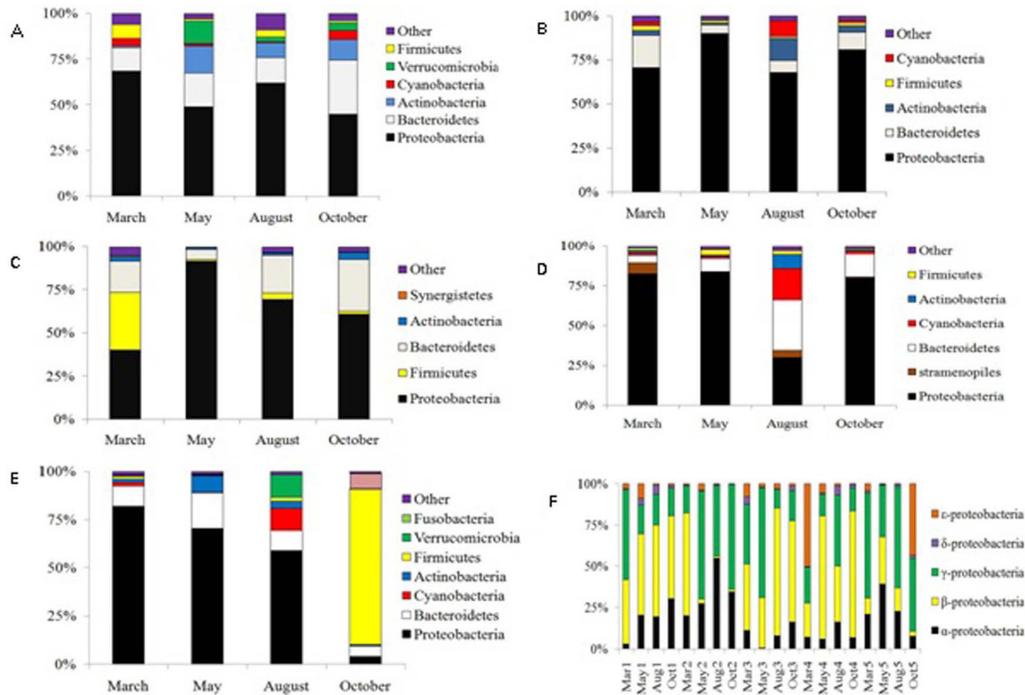

Figure 3. Relative abundance of phyla was analyzed in the five different wastewater systems analyzed in this study collected during different times of the year. **A.** sewage outfall S1; **B.** sewage outfall S2; **C.** sewage outfall S3; **D.** sewage outfall S4, and **E.** sewage outfall S5. **F.** Proteobacteria composition by class in the samples. After binning at the phylum level, bins that represented <1% of the total sequences were labeled "other".

For S2, there was little difference among the samples collected over the course of the year (e.g., S2M3, S2M5, S2M8, and S2M10). As shown in Figure 3B, sequences affiliated with the Proteobacteria were dominant in all samples, followed by Actinobacteria, Bacte-roidetes, and Firmicutes. In March, β-proteobacteria (62.3% of total sequences) was the most abundant, while in other months γ-proteobacteria were the most abundant.

The composition of the bacterial community associated with sewage outfall S3 in March (Figure 3C) was dominated by Firmicutes (33% of total sequences), which was higher than that found for the other 3 sampling times. Sequences affiliated with Proteobacteria in S3 sampled in March included α-proteobacteria (11.1% of total sequences), β-proteobacteria (40.2% of total sequences), γ-proteobacteria (36.1% of total sequences), and ε-proteobacteria (8.1% of total sequences). However, in May, γ-proteobacteria was the most abundant (67.0%

of total sequences), with a slight decrease in the abundance of β-proteobacteria (30.2% of total sequences). In August, the percentage of β-proteobacteria increased to 77.2% of total sequences, with the γ-proteobacteria representing only 11.4% of the total. In October, however, the percentage of α-proteobacteria was nearly twice as high as that in August.

The composition of bacterial assemblages associated with sewage outfall S4 also shifted during the year (Figure 3D). The percentage of sequences affiliated with Proteobacteria was always greater 80% at three of the sampling points (82.7% in March; 83.7% in May; 80.0% in October). However, in August, the percentage of sequences affiliated with the Proteobacteria was only 30.0%, while the percentage of Bacteroidetes increased to 31.5%. In March, ε-proteobacteria was the dominant group of Proteobacteria, where as for the other three months, the percentages were lower than 6%. In May and October, the percentages of β-proteobacteria were 74.4 and 76.6%, respectively.

According to a statistical analysis performed by the Ningbo City Ocean and Fishery Bureau, poor water quality was observed near the sewage outfall S5, which was the major drainage outfall. Proteobacteria was the dominant phylum in March, May, and August (Figure 3E). However, during October, Proteobacteria comprised only 3.8% of the total bacterial population, while Firmicutes was the dominant phylum. δ-Proteobacteria was the most rare group during the fourth month, making up less than 2% of the total population. During March, May, and August, ε-proteobacteria comprised 4.4, 0.5, and 0.4% of the total sample, respectively, but during October, it comprised 43.5% of the total. The change in the α-proteobacteria population was also very clear, dropping from 21.0, 39.1, and 22.5% to 7.5% during the four-month period. For β-proteobacteria, the proportion was 28.6% in May, but during October, it was only 3.1%. Although γ-proteobacteria was the dominant group during March, August, and October, its percentage differed cross these three months. During March and August, it comprised over 60% of the total population, but during October, it comprised only 44.0%.

Significant variation in the composition and structure of microbial assemblages sampled across the 5-site seasonal gradient were detected. Although findings related to Proteobacteria were consistent with the results of other examinations, such as studies of activated sludge samples and wastewater treatment plants (Wong et al., 2005; Hu et al., 2012), Proteobacteria was the predominant phylum in Mar1, Mar2, Mar4, Mar5, May1, May2, May3, May4, May5, Aug1, Aug2, Aug3, Aug5, Oct2, Oct3, and Oct4, while in Mar3 and Oct5, Firmicutes was dominant, and in Aug4, Bacteroidetes was dominant; variation in the relative abundances of individual classes of Proteobacteria were evident across the 5 sites. α-Proteobacteria, β-Proteobacteria, and γ-Proteobacteria represented 90% or more of all Proteobacteria (Figure 3F). This may have occurred because members of this phylum may originate from either wastewater or soil. The dominance of γ-Proteobacteria in the sewage wastefalls identified in this study included Enterobacteriaceae, Vibrionaceae, Pseudomonadaceae, *Salmonella*, *Yersinia*, *Vibrio*, and *Pseudomonas aeruginosa*, many of which are common in wastewaters and soils (Kent et al., 2001; Li et al., 2006; Schnetzer et al., 2011). This suggests that wastewater from sewage outfalls contains pathogenic bacteria. Through further sequence analysis, sequences belonging to pathogenic bacteria and degrading bacteria were detected. Many sequences from pathogenic bacteria were found. Further studies will be carried out on these pathogenic bacteria.

Moreover, the present characterization of wastewater from all 5 sewage out falls indicated the presence of pathogenic bacteria. α-Proteobacteria is the dominant Proteobacterial

class in the marine microbial community found at the surface of the Yellow Sea (Bai et al., 2009). In contrast, α-Proteobacteria accounted for approximately 10% of all Proteobacteria found in the present study. β-Proteobacteria, which are uncommon in seawater samples (Sogin et al., 2006; Bai et al., 2009), accounted for approximately 30% of all Proteobacteria identified in our study.

The predominance of sequences affiliated with this phylum is likely attributable to environmental factors, such as pH, phosphate levels, and Chemical Oxygen Demand (COD) (Casanovas-Massana and Blanch, 2012). The composition of the bacterial community associated with Oct5 differed from that of other samples. The predominant phylum in Oct5 was Firmicutes, which is ubiquitous in the environment (Stach et al., 2003; Urich et al., 2008). Many Firmicutes can produce endospores, which can resist dehydration and extreme environmental conditions (Li et al., 2011; Hu et al., 2012). Firmicutes are anaerobes, and this class may have become predominant because the system became anoxic. According to a statistical analysis performed by the Ningbo City Ocean and Fishery Bureau, there was considerable pollution and extreme environmental conditions at S5 in October, which likely account for the differences observed between sample Oct5 and the other samples.

The structures of the microbial communities in wastewater systems can be influenced by many factors, including salt concentration, geographical location, season, and rain, among others (Jiao et al., 2010; Jakobsson et al., 2010; Wang et al., 2011). In this study, we found that the α-diversity index (H', D, J), in which higher numbers represent more diversity, changed over the seasonal cycle. The results showed that in different seasons, the microbial diversity of sewage outfalls had a different geographic distribution: microbial diversity decreased from the inside port to the outside port. The microbial diversity in sample S5 was lower than in the other four samples, but diversity near the inside port was somewhat high. This maybe due to its location, where organic pollutants are greatly diluted. According to research performed by the Ningbo City Ocean and Fishery Bureau of China, the environment conditions were poor and the content of various organic pollutants and ammonia nitrogen materials exceeded our criteria, and this site was classified as a heavily polluted outlet (Ningbo City Ocean and Fishery Bureau). In general, diversity was found to be relatively high. The distribution of microbial diversity also differed over time. Overall, the bacterial diversity based on qualitative bacterial species and the quantities of the five sewage outfalls differed over time and space, revealing a close relationship with the classification of the sewage (Ningbo City Ocean and Fishery Bureau, China).

CONCLUSION

454-Pyrosequencing technology was used to investigate the composition of microbial communities associated with municipal wastewater in different seasons sampled over the course of one year. In the 20 samples, the taxonomic composition of the assemblages varied considerably, and the predominant phylum was Proteobacteria, followed by Bacteroidetes and Firmicutes. α-Proteobacteria, β-proteobacteria, and γ-proteobacteria represented 90% or more of all Proteobacteria. In addition, variation in the classes of Proteobacteria was evident. These results suggest that the composition of microbial communities shift in response to seasonal and spatial gradients, which likely reflects variation in the physical and chemical characteristics of the sewage.

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