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RESEARCH PAPER

Phylogenetic Diversity of Microbial Communities in Real Drinking Water Distribution Systems

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Abstract Microbial regrowth in drinking water distribution systems (DWDS) is a major concern in the water supply industry. Detailed knowledge of the microbial community in DWDS will be of great importance for assessing the microbiological risks of drinking water. The spatial heterogeneity of microbial community structures in the bulk waters of a large real DWDS was investigated using 16S rRNA clone library analysis. The results indicate that high residual chlorine in drinking water could not control microbial regrowth in DWDS. The bacterial communities in the bulk waters were spatially heterogenic, mainly composed of *Alphaproteobacteria* and *Betaproteobacteria* (or *Cyanobacteria*). Microorganisms from the genera *Acinetobacter, Sphingomonas* and *Gemella* were detected, implying there is microbiological risk from drinking water. This work provides new insight into microbial ecology in DWDS.

Keywords: microbial community diversity, *Acinetobacte, Sphingomonas*, *Gemella*, *Cyanobacteria*, drinking water, pathogen

1. Introduction

Microbial regrowth has been recognized as a major health problem within many drinking water distribution systems (DWDS). Health risks from waterborne pathogens may occur through mismanagement of freshwater resources, technological failure and/or inappropriate detection measures [1]. Maintaining a disinfectant (usually chlorine) residual is a common practice to control microbial regrowth in DWDS. However, microbial regrowth can still be promoted by the presence of organic matters and nutrients. DWDS may host a large variety of microorganisms including pathogens, which may be opportunistic, both in the bulk water and on the pipe surfaces [2]. Moreover, pathogen resistance to disinfectant can be affected by microbial community diversity and interspecies relationships [2]. Therefore, detailed knowledge of microbial communities will aid in the assessment of microbiological risks in drinking water and the subsequent development of control measures.

The presence and growth of bacteria in DWDS are typically identified based on culture-dependent methods, which may significantly underestimate the actual abundance and diversity of bacterial populations [3-5]. To circumvent the problems associated with the culture-dependent methods, molecular approaches such as denaturing gradient gel electrophoresis (DGGE) [6-8], fluorescent *in situ* hybridization (FISH) [9], terminal restriction fragment length polymorphism (TRFLP) [10], and clone library analysis [5,11], have been used for the analysis of microbial diversity in DWDS. Unfortunately, information on the microbial community diversity in DWDS remains limited. Moreover, little is known about the phylogenetic diversity of microbial communities in real DWDS because previous works using molecular tools have mainly focused on the microbial community diversity in model DWDS.

Among natural aquatic ecosystems, the microbial community is usually very sensitive to environmental perturbations [12,13]. Information about the effect of the physicochemical properties of drinking water on the microbial community is

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still scant. However, several previous works have shown that microbial communities in model DWDS can be affected by pipe materials [8,14], types of disinfectants [15], and disinfectant residual [9,16]. In large cities, DWDS are usually supplied by multiple water sources treated by several water treatment works. Therefore, spatial heterogeneity of microbial community structures in large real DWDS could be assumed due to differences of water qualities and pipe materials. In the current study, the spatial heterogeneity of bacterial community structures in real DWDS was investigated using clone library analysis. Moreover, the presence of potential pathogens in drinking water was also investigated.

2. Material and Methods

2.1. Sampling sites and water quality analysis

Four chlorinated water samples were collected from the DWDS in a large city in south China (Table 1). Residual chlorine was determined using HACH Chorine Pocket (HACH, USA). The pH values were determined using a Thermo Orion 5 STAR pH meter (THERMO, USA). Samples for dissolved organic carbon (DOC) concentration were filtered through 0.45-μm-pore-size Whatman GF/F filters and analyzed with a SHIMADZU Total Organic Carbon Analyzer (SHIMADZU, Japan). Total nitrogen (TN) was determined using a SHIMADZU Total Nitrogen Measuring Unit (SHIMADZU, Japan). Heterotrophic plate counts (HPCs) were measured according to the standard methods [17].

2.2. Bacterial clone library analysis

For analysis of bacterial community diversity in the bulk water, water samples (10 L) were filtered through 0.22-μm-pore-size membranes (diameter 50 mm; Millipore). The membrane filter was cut into quarters using a sterile scalpel and DNA from each filter was extracted with a DNA extraction kit (Mobio Laboratories, Carlsbad, USA) according to the manufacturer's protocol. 16S rRNA genes were amplified using the bacterial primers 27F (5'-GAGTTT GATCMTGGCTCAG-3') and 1492R (5'-GGTTACCTT GTTACGACTT-3') [18-20]. The PCR products were purified with a QIA quick PCR purification kit (Qiagen Inc., German) and then cloned into pGEM-T-easy Vector (Takara Corp, Japan). Clones containing an insert of the correct size were sequenced at SinoGenoMax Co., Ltd. (Beijing).

Sequences identified as chimeras by Mallard software were excluded from further analyses [21]. Chimera-free sequences with similarities of more than 98% were considered as one operational taxonomic unit (OTU). OTUs, the diversity index, and rarefaction curves were determined using distance-based OTU and a richness program (DOTUR) [22]. The taxonomic identities of the bacterial sequences were identified using "classifier" a Ribosomal Database Project (RDP) II analysis tool [23]. The 16S rRNA sequences obtained in this study were submitted to GenBank under accession numbers JQ655299 to JQ655366 (library with Sample A), JQ655367 to JQ655419 (library with Sample B), JQ613373 to JQ613442 (library with Sample C), and JQ613443 to JQ613499 (library with Sample D).

3. Results and Discussion

3.1. Heterotrophic plate count

The potential health effects of the HPC bacteria present in tap water have generated considerable concern in the drinking water industry [3]. Heterotrophic bacteria are widely used as indicators of drinking water quality and drinking water quality specifications worldwide recommend HPC limits of 100 ~ 500 CFU/mL [24]. The presence of organic matters in waters can promote bacterial regrowth in DWDS.

Table 1. Description of sampling sites

Water samples	Producers	Distance from water works (km)	HRT (h)	Pipe materials	Pipe ages (year)
A	Water Works N	10	4	Cast iron	7
B	Water Works S	8	11	Cement	20
C	Water Works X	24	5	Cast iron	20
D	Water Works X	35	9	Cast iron	12

Table 2. The parameters of four water samples

Samples	Residual chlorine (mg/L)	pH	DOC (m/L)	TN (mg/L)	HPCs (CFU/mL)
A	0.61	7.5	0.37	2.45	22
B	0.48	7.3	0.73	1.92	59
C	0.62	7.2	1.36	3.28	457
D	0.76	7.2	1.71	3.11	19

It is widely accepted that bacterial regrowth in DWDS can occur even in the presence of disinfectant in the bulk water [25]. Table 2 shows the presence of HPC bacteria in all water samples. In these samples the residual chlorine level was high (0.48 ~ 0.76 mg/L), which confirmed that there was bacterial resistance to disinfectant. The level of HPC bacteria was highest in Sample C (457 CFU/mL). Bacterial growth in distribution systems is usually found to be correlated with assimilable organic carbon (AOC) concentration [26]. However, bacterial regrowth is dependent upon a complex interaction of chemical, physical, and operational parameters [25]. In this study, the level of HPC bacteria in waters was not correlated with the levels of DOC or the total nitrogen.

3.2. Bacterial community structures

The phylum distribution of bacteria communities in the four water samples is shown in Fig. 1. Only phylum *Proteobacteria* was shared among all samples. The clones recovered from the Sample A were distributed across phyla as follows: *Alphaproteobacteria* (72%), *Betaproteobacteria* (10%), unclassified bacteria (7%), *Verrucomicrobia* (4%), *Gemmatimonadetes* (3%), *Firmicutes* (1%), and unclassified *Proteobacteria* (1%). The clone library from Sample B was also mainly composed of *Alphaproteobacteria* (62%) and *Betaproteobacteria* (28%). However, the clone library from Samples C or D was mainly represented by *Alphaproteobacteria* (49 or 79%) and *Cyanobacteria* (43 or 18%). This indicates that the major bacterial groups (with relative abundance ⩾ 10%) were different across the four samples. This shows the heterogeneity of bacterial community structures in drinking water. Moreover, these results also confirmed the presence of microbial growth in chlorinated drinking water. Numerous abiotic factors can influence biofilm formation in DWDS, including temperature, disinfectant type and residuals, organic matter, nutrient concentrations, substratum and hydraulics [27]. However, information about the effect of the physicochemical properties of drinking water on the microbial community in real DWDS is still lacking. The microbial communities in model DWDS can be affected by pipe materials [8,14], types of disinfectants [15], and disinfectant residual [9,16]. In the current study, the water samples were collected from different sites. The spatial heterogeneity of bacterial community structures in DWDS may be attributed to the differences of microorganisms in the finished water of water works, pipe materials and ages, residual chlorine, DOC and total nitrogen.

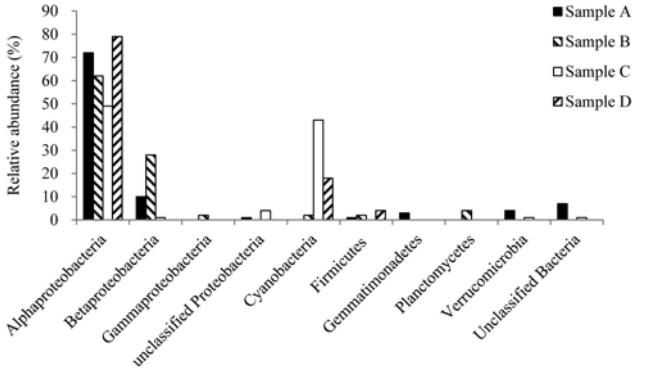

Fig. 1. Percentages of the clones affiliated with different phyla and sub-phyla to the total number of clones from Sample A-D. Clones not classified to any known phylum are included as unclassified bacteria.

Isolates from DWDS usually belonged to *Proteobacteria* [9,11].Williams *et al*. found that *Alphaproteobacteria* was the major group among the isolates from both chloraminated and chlorinated water in model DWDS [16]. However, Tokajian *et al*. (2005) found that Gram-positive bacteria and *Alpha*-, *Beta*- and *Gammaproteobacteria* constituted the major groups among the heterotrophic isolates from the bulk water of a chlorinated drinking water sample [28]. Several studies using molecular approaches have confirmed the predominance of *Proteobacteria* in DWDS [16,29-31]. *Alphaproteobacteria*, *Betaproteobacteria* and *Gammaproteobacteria* have been found to predominate in chlorinated drinking water [16,29,30], while *Betaproteobacteria* were found to be abundant in biofilms of non-chlorinated DWDS [32]. Berry *et al.* suggested that microbial community diversity in DWDS is impacted by disinfection strategy [2]. Mathieu *et al.* indicated that *Betaproteobacteria* and *Gammaproteobacteria* were dominant under high free residual chlorine concentrations (0.4 mg/L) while the *Alphaproteobacteria* population was sensitive to this oxidant level [9]. However, in this study, *Alphaproteobacteria* was the largest bacterial group in all samples, even under high free residual chlorine concentrations (0.48 ~ 0.76 mg/L). The microbial community structure in DWDS that is implied by these findings could also be impacted by other water parameters besides the disinfectant residual.

Bacterial diversity in drinking water can be affected by differences in the type of source water and treatment processes used in drinking water treatment plants [33]. Samples C and D stemmed from finished water from the same water treatment plant (Table 1), which may account for the same major bacterial groups (*Alphaproteobacteria* and *Cyanobacteria*) being found in these two samples. Moreover, to the authors' knowledge, this is the first report to show the dominance of *Cyanobacteria* in DWDS. This indicates that beside *Proteobacteria*, other phyla may also be dominant bacterial groups in DWDS. Interestingly, a recent work indicated that unclassified bacteria were the most predominant bacterial population in drinking water

Table 3. OTUs and Shannon index (calculated at 0.02 difference level) of four clone libraries

Library	Number of clones	Number of OTUs	Shannon index
Sample A	68	13	1.377
Sample B	53	18	1.902
Sample C	70	10	1.293
Sample D	57	6	0.775

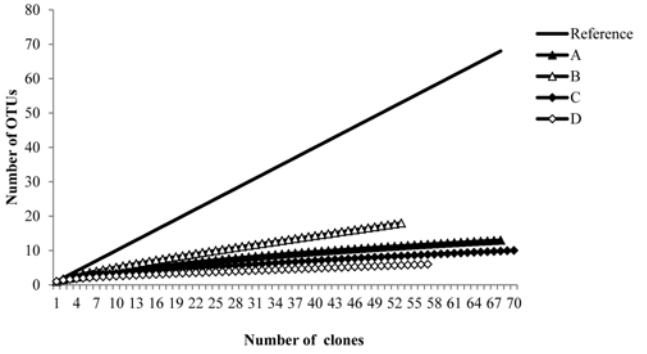

Fig. 2. Rarefaction curves indicating bacterial 16S rRNA gene richness within the four clone libraries. The observed numbers of OTUs identified by DOTUR program at 2% difference level are plotted against number of clones in libraries.

samples [5].

The OTUs and Shannon diversity index were both determined at the 2% sequence difference level by the DOTUR program (Table 3). Table 3 shows that 13, 18, 10, and 6 OTUs were obtained in three bacterial libraries constructed with Sample A, Sample B, Sample C, and Sample D, respectively. Although only 53 ~ 70 clones were selected, the rarefaction curve for each bacterial library nearly approached a plateau (Fig. 2), indicating that the microbial communities were well sampled. Table 3 shows the differences in community diversity across the four samples, indicating the spatial heterogeneity of bacterial community structures in DWDS. Moreover, the community diversity index in drinking water was not correlated with the levels of residual chlorine, DOC or total nitrogen.

3.3. Potential pathogens

Human bacterial pathogens have been regarded as an increasing threat to drinking water supplies worldwide because of the growing demand for high-quality drinking water [1]. Molecular assessment has contributed greatly to knowledge of bacterial pathogens in drinking water. In this study, one *Acinetobacter*-like sequence was detected in Sample B. The genus *Acinetobacter* comprises a complex and heterogeneous group of bacteria, many of which are capable of causing a range of opportunistic, often catheter-related, infections in humans [34]. Microorganisms from this genus have been isolated from drinking water [35,36] and from an autohydrogenotrophic pilot-plant for the denitrification of drinking water [37]. Two *Sphingomonas*-likesequences were found in Sample B. Bacteria of the genus *Sphingomonas* are environmental organisms that have recently been implicated in a variety of community-acquired and nosocomial infections [38]. *Sphingomonas* species have been isolated from the bulk water and biofilm in DWDS [7,39]. One *Gemella*-like sequence was detected in Sample D. Microorganisms of the genus *Gemella* have been linked to infectious endocarditis, bacteremia, sepsis, and abscesses [40]. However, information on the *Gemella* isolates in drinking water is still lacking. These findings also provide an illustration of the utility of molecular approaches to discover the potential pathogens in DWDS. Pathogens may exist in real DWDS even when the residual chlorine is high. Moreover, interestingly, no sequence belonging to known pathogens was detected in Sample C although its level of HPC bacteria was the highest of the samples. Therefore, HPC, as an indicator of general microbiological quality, might bias the assessment of the microbiological risk of drinking water.

4. Conclusion

Microbial regrowth could occur in DWDS despite the residual chlorine in drinking water being high. The bacterial communities in the bulk waters were mainly composed of *Alphaproteobacteria* and *Betaproteobacteria* (or *Cyanobacteria*). Heterogeneity in the bacterial community structure in DWDS was also found. However, *Alphaproteobacteria* was the largest bacterial group in water samples regardless of the sampling sites. Potential pathogens from genera *Acinetobacter, Sphingomonas*and *Gemella* were also detected, implying the microbiological risk of drinking water.

Acknowledgments

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