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RESEARCH ARTICLE

Molecular analysis of bacterial community in the tap water with different water ages of a drinking water distribution system

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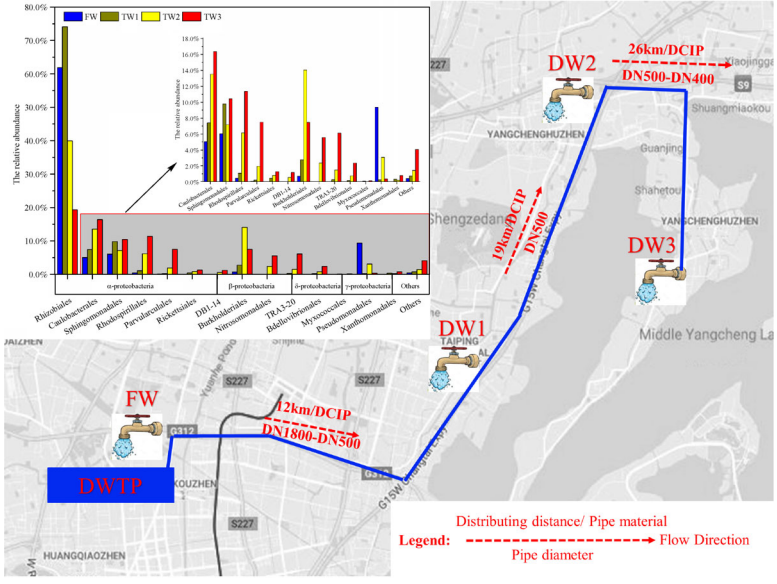
HIGHLIGHTS

- The increase of water ages drove the deterioration of drinking water quality.
- The relative abundance of Rhizobiales uniquely increase during distributing process.
- Rhizobiales order was helpful for inhibiting corrosion under high chlorine level.
- New disinfecting strategies should be developed to ensure drinking water safety.

GRAPHIC ABSTRACT



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ABSTRACT

Bacterial community in the drinking water distribution system (DWDS) was regulated by multiple environmental factors, many of which varied as a function of water age. In this study, four water samples with different water ages, including finished water (FW, 0 d) and tap water (TW) [TW1 (1 d), TW2(2 d) and TW3(3 d)], were collected along with the mains of a practical DWDS, and the bacterial community was investigated by high-throughput sequencing technique. Results indicated that the residual chlorine declined with the increase of water age, accompanied by the increase of dissolved organic matter, total bacteria counts and bacterial diversity (Shannon). For bacterial community composition, although Proteobacteria phylum (84.12%–97.6%) and Alphaproteobacteria class (67.42%–93.09%) kept dominate, an evident regular was observed at the order level. In detail, the relative abundance of most of other residual orders increased with different degrees from the start to the end of the DWDS, while a downward trend was uniquely observed in terms of Rhizobiales, who was inferred to be chlorine-resistant and be helpful for inhibiting pipes corrosion. Moreover, some OTUs were found to be closely related with species possessing pathogenicity and chlorine-resistant ability, so it was recommended that the use of agents other than chlorine or agents that can act synergically with chlorine should be developed for drinking water disinfection. This paper revealed bacterial community variations along the mains of the DWDS and the result was helpful for understanding bacterial ecology in the DWDS.

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1 Introduction

Drinking water distribution system (DWDS) is a bridge between water works and consumers. Owing to provide safe and reliable tap water (TW) for citizens, it had been evaluated as the fourth invention during the 20th century by the American Academy of Engineering [1]. However, the DWDS is complex and the TW quality is affected by natural environmental factors and operational conditions [2]. Although the finished water (FW) produced by the drinking water treatment plants (DWTP) met the national standards, some problems during the distributing process, such as discoloration [3], bacteria regrowth [4] and biofilm formation [5] were frequently reported in recent years.

The microbial safety of TW, which is directly related to human health, has always been a social concern. According to statistics, 50% of the waterborne disease outbreaks in the United States from 1995 to 2002 were ascribed to compromises in the integrity of finished tap water within DWDS [6], most of which were related to microbes. Conventionally, enteric pathogens are the primary microbial pathogens of potential concern via drinking water [7]. However, many kinds of opportunity pathogens, mainly including *Legionella pneumophila* [8], *Nontuberculosis mycobacteria* (NTM) [9,10] and *Pseudomonas aeruginosa* [11] etc. were frequently detected in recent studies, which could also trigger the outbreaks of waterborne disease in the DWDS. Therefore, getting insights into the bacterial community composition in the DWDS was beneficial for preventing the outbreak of waterborne disease.

In the DWDS, the microbial community was affected by many factors [12]. It is regrettable that many previous studies only focused on the effects of single environmental variable, such as the concentration of disinfectant [13], the pipe materials [14], while the co-effects of multiple water quality parameters on bacterial community in the full-scale DWDS were rarely concerned. It is well known that most water quality parameters varied as a function of water ages [15] in the DWDS, thus the representative bacterial community affected by multiple water quality parameters might be acquired from water samples with different water ages.

In this study, four water samples with different ages were selected along the mains of a full-scale DWDS in one city of eastern China. The water quality parameters varied among sampling points, which were very representative in the DWDS. All pipes were ductile cast iron materials, which were widely used to replace cast iron and concrete pipes in the DWDS [16]. This study was conducted with expect to acquire the response of bacterial community to the co-effects of multiple environmental factors in the DWDS, and the result was helpful for understanding bacterial ecology in the DWDS.

2 Materials and methods

2.1 The description of the DWDS

The DWTP produced about 30×10^4 m^3 drinking water every day, and the source water from Lake T was treated through pretreatment (preozonation), conventional treatment (coagulation, sedimentation and sand filtration) and advanced treatment (ozonation and biological activated carbon filtration) subsequently. To ensure the microbial safety of TW, chlorine was added in the disinfecting units, where the RC concentration of the effluent (FW) maintained at 0.8 mg/L. Then the TW was pumped to the DWDS. TW1, TW2 and TW3 were collected along the mains in this study (Fig. 1). This three sampling points were used for periodical detection by the test center of the DWTP, and the water ages of them were estimated to be 1 d, 2 d and 3 d, which was provided by the technical department of the DWTP. The pipe material of the mains was ductile cast iron material and the diameter ranged from DN400 to DN1800. The total length of the pipe was 26.1 km from the start to the end sampling points of the DWDS.

2.2 Sampling and water quality analysis

The FW was sampled according to the method used by Chao [17]. In brief, torayvino high-performance cartridge-type water purifiers (MKC-EG, Toray Industries Inc., Japan) was equipped on the FW tap in the DWTP. The FW sample was continuously filtered until it was hardly filtered out. The whole process lasted about 48 h and 430 L water sample was filtered eventually. All TW samples were collected from taps in the households close to the main pipes (within 50 m). First, the tap was opened to the maximum to drain the stagnant water in the branch pipes until stable RC concentration (PC II, HACH, USA) was acquired, then the temperature of water sample was measured on the spot and the bulk water (5 L) was collected with sterilized glass bottles. All samples were disposed within 12 h. Upon arrival at the laboratory, bulk water was used to analyze turbidity (2100Q, HACH, USA), dissolved organic carbon (DOC, TOC4100, Shimadzu, Japan), and both ammonia and COD_{Mn} were measured according to the State Standard of the People's Republic of China (GB/T5750.5-2006, GB/T 5750.7-2006), respectively. For bacterial counts, total bacteria counts (TBC) were determined by flow cytometer (FACSVerse, BD, USA). First, samples (500 μL) were pre-heated to 35°C (5 min), then stained with 5 μL SYBR® Green I (1:100 dilution in DMSO; Molecular Probes), and incubated in the dark for 15 min at 35°C in the end [18]. All parameters were detected in triplicate. The water quality of all samples was summarized in Table 1.

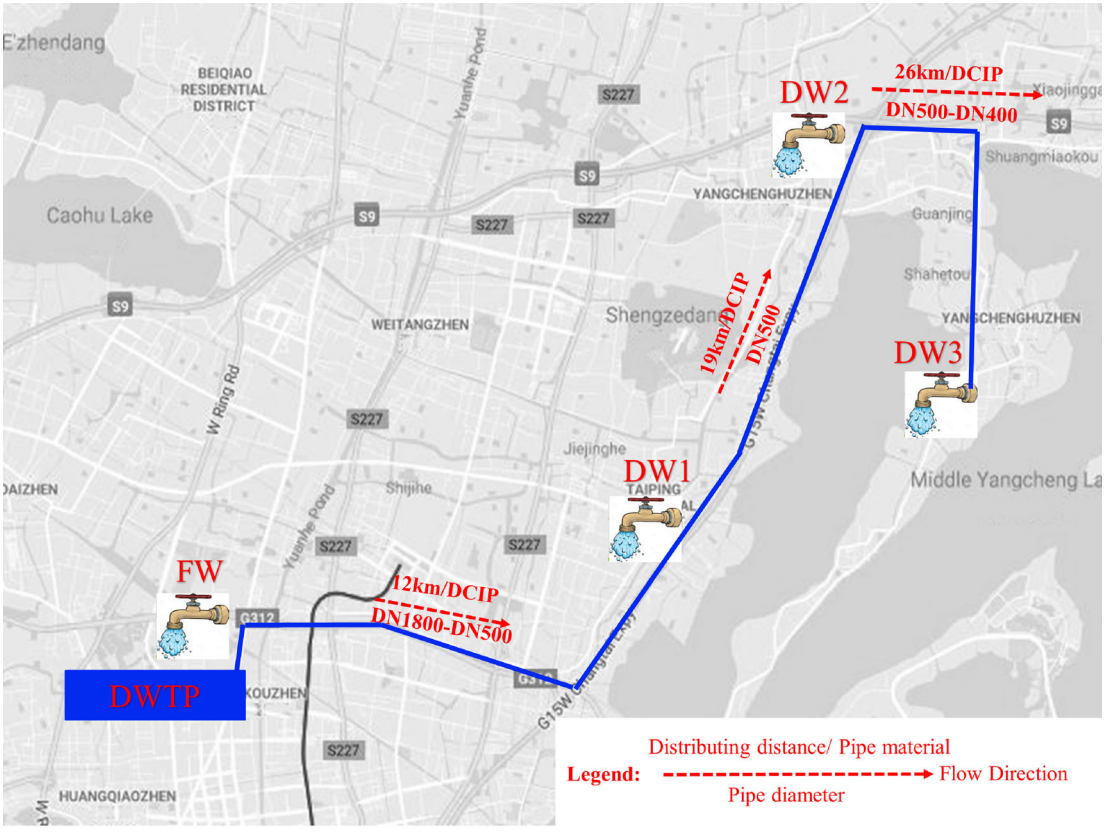

Fig. 1 The positions of the sampling points and the DWTP in the full-scale distribution system

Table 1 Water quality parameters of all samples

Parameters	FW	TW1	TW2	TW3
Temperature (°C)	22.6±0.356	20.8±0.047	16.5±0.047	18.5±0.047
Turbidity (NTU)	0.300±0.011	0.517±0.057	0.5±0.028	0.477±0.034
Ammonia (mg/L)	0.031±0.007	0.184±0.010	0.184±0.012	0.109±0.009
COD_{Mn} (mg/L)	1.29±0.082	1.78±0.062	1.67±0.04	1.76±0.036
DOC (mg/L)	1.471±0.090	1.846±0.047	1.974±0.047	2.18±0.058
TBC (cells/mL)	$1.50 \times 10^2 \pm 7$	$1.05 \times 10^4 \pm 148$	$1.46 \times 10^4 \pm 151$	$4.71 \times 10^4 \pm 211$
RC (mg/L)	0.80±0.012	0.53±0.005	0.42±0.005	0.15±0.005

2.3 DNA extraction and high-throughput sequencing

Upon reaching the laboratory, the purifier containing microbes concentrated from FW was destroyed with saw, which was sterilized by using autoclaving method. Then, the hollow fiber filter in purifier was immersed into ultrapure water (200 mL) and then treated by ultrasonication (SB-800D, Ningbo scientz biotechnology Co., LTD, China) for 30 min to detach the microbial cells [19]. The microbial mixture and TW samples (4L) were filtered with 0.22 μm polycarbonate membranes (GVWP04700, Millipore, USA) by vacuum pump (SHZ-DIII, Ling ke, China) before DNA extraction, aiming at concentrating suspended bacteria on the membrane. According to the manufacturer's protocol, total DNA was extracted immediately by using PowerWater® DNA Isolation Kit (14900-50-NF, MOBIO, USA) and 50 μL DNA sample was obtained finally. All DNA samples were preserved in a refrigerator keeping at –80°C.

The V4-V5 region of the bacteria 16S rRNA gene were amplified by PCR (95°C for 2 min, followed by 25 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s and a final extension at 72°C for 5 min) using primers 515F 5′-barcode- GTGCCAGCMGCCGCGG)-3′ and 907R 5′-CCGTCAATTCMTTTRAGTTT-3′, where barcode is an eight-base sequence unique to each sample. PCR reactions

were performed in triplicate 20 μL mixture containing 4 μL of 5 × FastPfu Buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL of each primer (5 μM), 0.4 μL of FastPfu Polymerase, and 10 ng of template DNA.

Amplicons were extracted from 2% agarose gels and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, CA, US) according to the manufacturer's instructions and quantified using QuantiFluor™ -ST (Promega, US). Purified amplicons were pooled in equimolar and paired-end sequenced (2 × 250) on an Illumina MiSeq platform according to the standard protocols.

2.4 Data analysis

Raw fastq files were demultiplexed, quality-filtered using QIIME (version 1.9.1) with the following criteria: (i) The 300 bp reads were truncated at any site receiving an average quality score < 20 over a 50 bp sliding window, discarding the truncated reads that were shorter than 50bp. (ii) exact barcode matching, 2 nucleotide mismatch in primer matching, reads containing ambiguous characters were removed. (iii) only sequences that overlap longer than 10 bp were assembled according to their overlap sequence. Reads which could not be assembled were discarded. Operational taxonomic units (OTUs) were clustered with 97% similarity cutoff using UPARSE (version 7.1 http://drive5.com/uparse/) and chimeric sequences were identified and removed using UCHIME. The taxonomy of each 16S rRNA gene sequence was analyzed by RDP Classifier (http://rdp.cme.msu.edu/) against the silva (SSU123)16S rRNA database using confidence threshold of 70% [19]. For alpha-diversity metrics, the estimated number of species based on the Chao and Shannon were calculated. For the beta-diversity metrics, principal coordinate analyses (PCoA) was conducted in QIIME.

In addition, redundancy analysis (RDA) was performed to discuss the relevance between microbial species and water quality parameters by using CANOCO (version 4.5). The correlations analysis between bacterial diversity indexes and water quality parameters was conducted with the software of Origin 9.1. MEGA 6 was applied to construct the phylogenetic tree in this study.

3 Results

3.1 Water quality

Table 1 shows the physicochemical data and bacterial counts of the water samples. Water quality varied from the start to the end of the DWDS. In detail, the RC decreased from 0.80 mg/L to 0.15 mg/L, DOC increased from 1.471 mg/L to 2.18 mg/L, and TBC increased from 1.50×10^2 cells/mL to 4.71×10^4 cells/mL, suggesting the occurrence of bacterial regrowth during the distributing process. The variations of the three water parameters were very representative in the DWDS. However, no universal tendency was observed in terms of other water quality parameters in this study. Turbidity ranged from 0.3NTU to 0.517NTU, and the concentration of ammonia increased from 0.031 mg/L to 0.184 mg/L, while COD_{Mn} changed between 1.29 mg/L and 1.78 mg/L. Generally, most of the water quality parameters met the standards for drinking water quality of China (GB 5749-2006).

3.2 Bacterial diversity

Overall, 159,787 raw reads of 16S rRNA genes with an average length of approximately 430 bp were obtained. A 97% similarity cutoff was used to delineate OTUs in the downstream analyses and 368 OTUs were acquired eventually. The sequencing results and alpha diversity indices of bacterial community are shown in Table 2.

Both species richness estimator (Chao) and species diversity index (Shannon) were used to analyze biofilm community in this study. Comparing to Chao, Shannon index not only considered the species numbers but also took species evenness into consideration. As shown in Table 2, all the values of OTU numbers, Chao and Shannon increased along the main pipes. Particularly, OTU number increased from 142 to 312, Chao increased from 165 to 325 and Shannon raised from 1.62 to 4.06 along the main pipes of the DWDS. This indicated pronounced distributing effects of the DWDS on bacterial diversity of TW.

3.3 Bacterial community

PCoA analysis indicated that community divergence between TW samples and FW was enlarged along with the main pipes (Fig. 2). The detailed analysis of community difference was concluded as follows. At the phylum level (Fig. 3), Proteobacteria dominated absolutely in all samples in terms of the relative abundance, which varied from 84.12% to 93.7% in this study. Other phylum of FW mainly embraced Bacteroidetes (14.11%), Cyanobacteria (1.52%), Acidobacteria (0.01%) and Actinobacteria (0.08%). For the sample of TW1, the relative abundance of Bacteroidetes decreased to 0.4%, while other phyla varied little. However, the relative abundance of Proteobacteria of TW2 decreased about 4% comparing to TW1, and a slight increase of *Cyanobacteria* (3%), Bacteroidetes (1.4%), Acidobacteria (1.2%) and Actinobacteria (0.08%) was observed. As for TW3, the relative abundance of Cyanobacteria, Bacteroidetes, Acidobacteria and Actinobacteria changed to be 1.3%, 0.4%, 0.6% and 1.4%, respectively.

Proteobacteria phylum was identified as the primary group of all water samples in this study, which was in consistent with previous studies [20,21]. Therefore, the community composition of Proteobacteria phylum was

Table 2 The sequencing results and alpha diversity indices of bacterial community

Sample ID	Raw reads	Average length	OTU	Chao	Shannon
FW	37478	431bp	142	165	1.62
TW1	43185	426bp	197	230	1.68
TW2	40720	431bp	273	301	3.42
TW3	38404	432bp	312	325	4.06

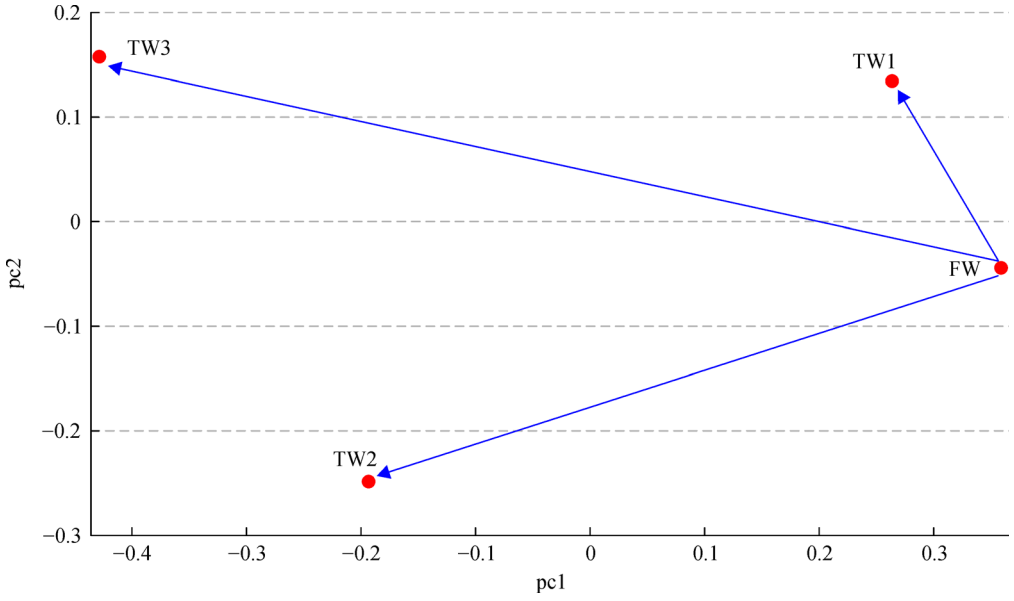

Fig. 2 PCoA analysis of FW and TW samples

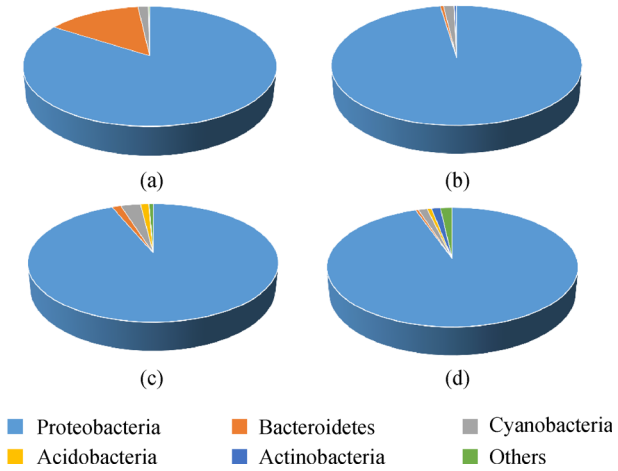

Fig. 3 Taxonomic composition of the bacterial communities of FW (a), TW1 (b), TW2(c) and TW3 (d) at the phylum level

further analyzed to reveal the bacterial community variations along the mains in the DWDS. The result was shown in Fig. 4 and distinct changes could be observed. In detail, the major class was assigned to be Alphaproteobacteria, whose relative abundance increased from 73.66% of FW to 95.43% of TW1, but continuously decreased to 72.29% of TW3. However, the relative abundance of Betaproteobacteria and Deltaproteobacteria increased with different degrees, while Gammaproteobacteria showed downward trend from the start to the end of the DWDS. At the order level, the primary bacteria was identified to be Rhizobiales, which varied from 61.89% to 74.1%. Other

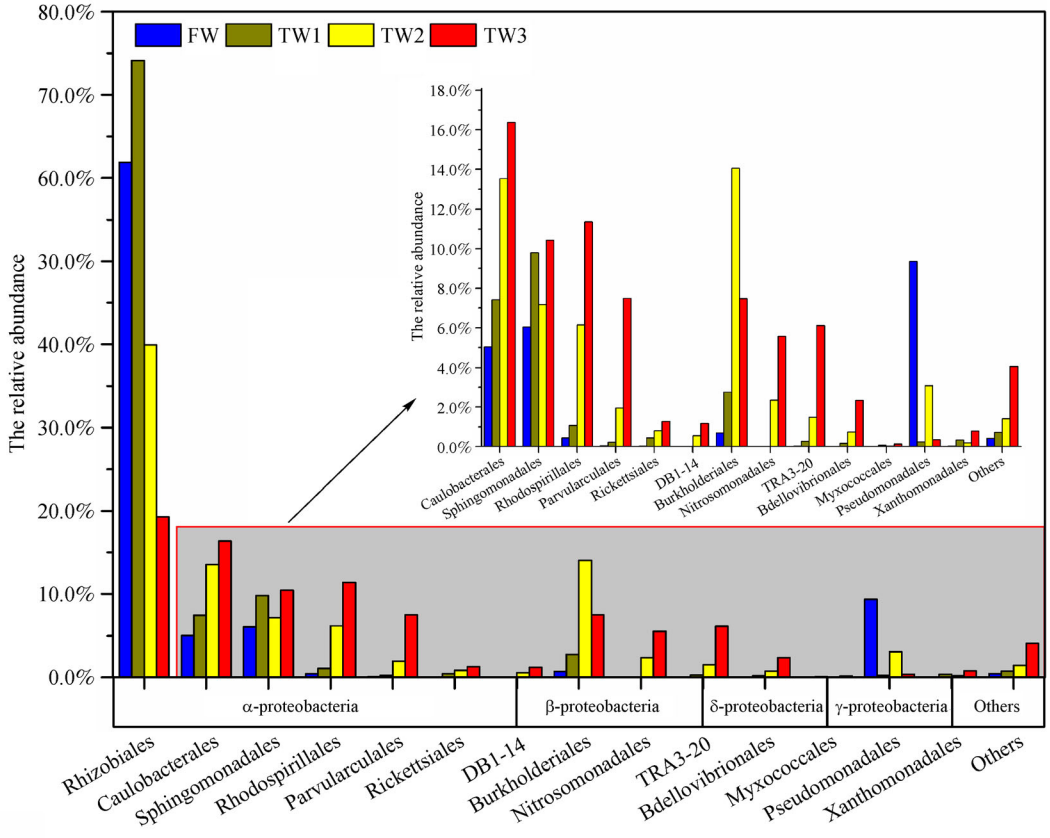

Fig. 4 The Proteobacterial composition of samples at the order level

orders, including Caulobacterales, Rhodospirillales, Parvularculales, Rickettsiales, DB1-14, Nitrosomonadales, TRA3-20, Bdellovibrionales and Xanthomonadales increased along the main pipes. In addition, a little changes of Sphingomonadales and Myxococcales were found in terms of the relative abundance, while the highest level of Burkholderiales and Pseudomonadales were observed in the sample of TW2 and FW, respectively.

At the genus level (Fig.S1), the major genus belonged to *F0723_norank* (14.92%–69.11%), *Sphingomonas* (2.65%–8.47%), *Brevundimonas* (0.44%–7.63%) *Chryseobacterium* (0.04%–14.06%) and *Woodsholea* (1.87%-6.34%). In addition, an increasing tendency was observed regarding to the number of the primary genus (>5%) from the start to the end of the DWDS. Moreover, the relative abundance of many other genera (< 5%) increased to some extent while low abundance of them were found in FW, indicating the distributing effects on bacterial community.

4 Discussion

4.1 The effects of water quality on bacterial diversity

Correlation analysis was conducted aiming at analyzing the effects of water quality on bacterial diversity, and the result was shown in Table S1 (see Supplementary material). It could be concluded that different parameters played different roles on bacterial diversity indexes. Turbidity and RC were significantly correlated with Chao ($P < 0.05$), while no significant correlation between Shannon indexes and water quality parameters was observed at 0.05 level, indicating that it might be affected by multiple environmental factors simultaneously in this study. Besides, it could be found that only temperature and RC were negatively correlated with Chao as well as Shannon, while the residual parameters showed positive correlation with it.

Chlorine was widely used for TW disinfection due to the natural oxidative capacity for inactivating bacteria [22,23], so it was doubtless that RC played a negative role on bacterial diversity indexes. TBC was positively correlated with bacterial diversity indexes, because it was the fact that the more bacteria counts (TBC), the higher bacterial diversity level (Chao and Shannon). Although they were not significantly correlated ($P < 0.05$), Chao and Shannon showed positively correlation with ammonia and DOC, which represented nitrogen source and carbon source that essential for bacteria proliferation. Besides, it was interesting that COD_{Mn} was not significantly correlated with bacterial diversity indexes. Comparing with DOC, some

inorganic reductive material was included in COD_{Mn}, which could not be utilized by bacteria. In terms of turbidity, which could reflect the concentration of suspended particles in the water, it was positively correlated with bacterial diversity indexes ($P < 0.05$). Previous studies demonstrated that particles could enhance chlorine-resistant ability of bacteria by protecting bacteria from the effects of RC [24,25]. Therefore, particles were favor for bacteria survival with the presence of chlorine in the water.

4.2 The effects of water quality on bacterial community

RDA was conducted to analyze the effects of water quality parameters on bacterial community and the result was shown in Fig. 5. The angle between microbial species and water quality indexes in the figure was on behalf of the positive (acute angle) or negative correlation (obtuse angle) between them. As shown in Fig. 5, it could be concluded that two different groups were separated. Pseudomonadales and Rhizobiales, temperature and RC belonged to one group, and the residual orders and water quality parameters were embraced in the other group. In the same group, water quality parameters were positively correlated with the relative abundance of each bacterial orders.

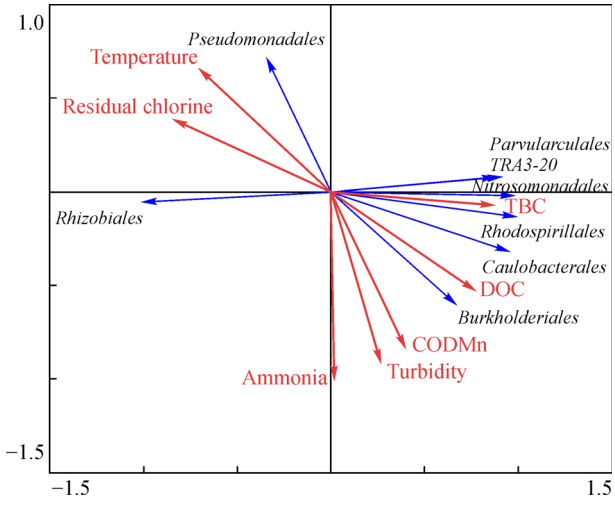

Fig. 5 RDA analysis between water quality parameters (red arrows) and the typical orders (blue arrows)

Pseudomonadales and Rhizobiales were positively correlated with temperature and RC, indicating that they were more suitable to live in the environment with high level of RC and temperature. According to the above result, Rhizobiales was the most abundant order from the start to the end of the DWDS, and a downward trend was observed in terms of the relative abundance. Also, the RC concentration decreased continuously from FW to TW3. The above results suggested that the higher level of RC, the higher relative abundance of Rhizobiales. In other words, Rhizobiales order might exhibit chlorine-resistant property. Besides, previous study had demonstrated that *Rhizobia* could produce and import siderophores (e.g. ferrichrome) that could capture iron to inhibit the iron corrosion [26]. In this study, the mains were ductile cast iron material, which could react with water and chlorine. Therefore, corrosion reaction was surely occurred. The Rhizobiales order might play an important role on inhibiting iron pipes corrosion under high chlorine level.

In fact, microbiologically influenced corrosion was one focus in the DWDS. Previous studies had reported that microbiologically influenced corrosion was enhanced by sulfate-reducing bacteria but inhibited by nitrate-reducing bacteria (NRB) during corrosion processes [27]. However, the function of specific bacteria with the presence of chlorine was still unclear. The above analysis indicated that Rhizobiales order might be responsible for inhibiting corrosion process in the DWDS with high concentration of RC. This conclusion could promote the understanding of pipe corrosion mechanism and the bacteria ecology in the DWDS, but more efforts should be spent on confirming this hypothesis.

In contrast to Pseudomonadales and Rhizobiales, Burkholderiales, Caulobacterales, Rhodospirillales, Parvularculales, TRA3-20 and Nitrosomonadales were almost positively correlated with ammonia and DOC, but negatively correlated with temperature and RC. This suggested they were chlorine sensitive orders and their growth could be promoted by nutrient substance in the DWDS. Also, Previous studies had revealed that bacteria population was regulated by carbon source [28]. Due to the low DOC level at the start of the DWDS, the analytic result indicated that carbon source was the limiting factor for their proliferation. Besides, Burkholderiales was reported as the most abundant order of Betaproteobacteria in cast iron biofilms [29], the increase of the relative abundance of Burkholderiales order along the main pipes in this study might induced by the detached biofilms in the DWDS. Moreover, Caulobacterales was found to be one of the minor groups in free chlorine treated TW [30], which was detected in this study. The above analysis was helpful for promoting the understanding of bacterial ecology in the DWDS.

4.3 Opportunistic pathogens

In this study, several genera, including *Acinetobacter, Legionella, Mycobacterium, Pseudomonas, Sphingomonas* and *Staphylococcus*, were identified in drinking water samples. It had been testified that two genera (*Acinetobacter* and *Legionella*) and some species of the residual genera had adverse effects on human health [31,32]. Therefore, typical OTUs (Table S1) representing *Mycobacterium, Pseudomonas, Sphingomonas* and *Staphylococcus* in this study were selected. Their sequences were compared with some well-known opportunistic pathogens

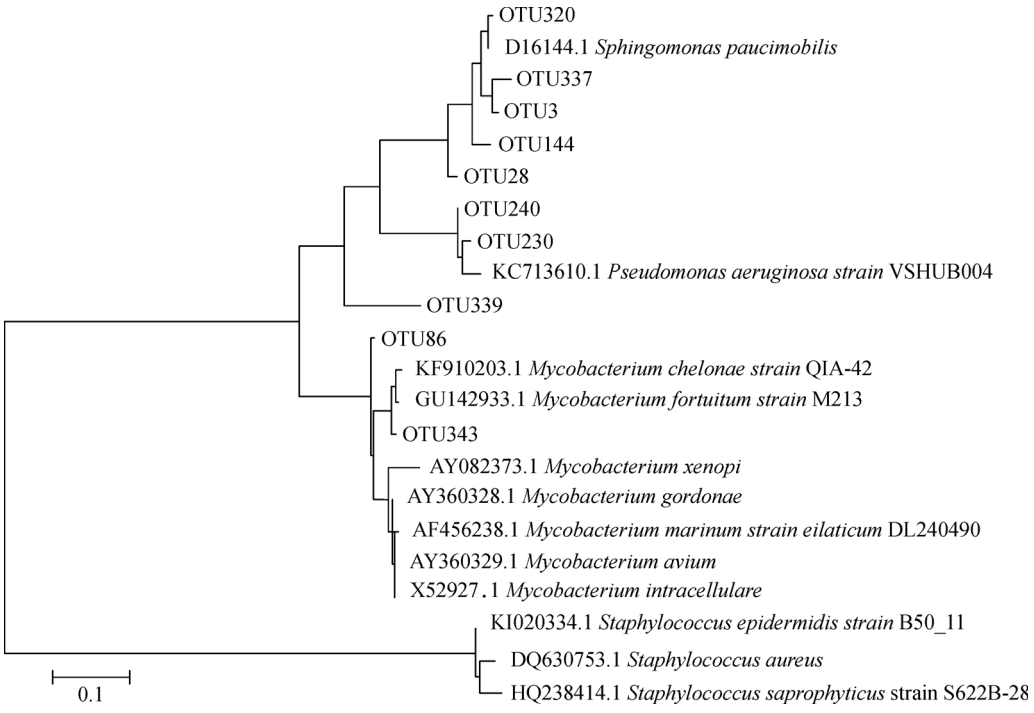

Fig. 6 The phylogenetic tree of representative OTUs and several well-known opportunistic pathogens

and the results were shown in the phylogenetic tree (Fig. 6). It could be concluded that some OTUs were closely related with *Sphingomonas paucimobilis* (OTU320), *Pseudomonas aeruginosa* (OTU230) and *Mycobacterium fortuitum* (OTU343). In addition to pathogenicity of these species, previous studies had verified the high chlorine-resistant ability of them. The CT value for 3 log inactivation of *M. fortuitum* was 600 times greater than that of *Escherichia coli* [33]. and a high level of *Sphingomonas* sp. was observed in the drinking water biofilms with 0.6–1.0mg/L of chlorine residual [34]. In the view of the fact of wide application of chlorine disinfection in practice, the use of agents other than chlorine or agents that can act synergically with chlorine should be developed in drinking water disinfection. Comparing with disinfecting technique with single disinfectant, the multi-barriers model of UV combined chloramine had been testified to be one reasonable manner for controlling the biological safety of drinking water [35]. We believe that more and more combined disinfecting methods will be proposed in the future for ensuring drinking water safety.

5 Conclusions

1) With the increase of water age in the DWDS, RC decreased while DOC and TBC increased on the contrary. Besides, under the co-effects of multiple water quality parameters, both species richness estimator (Chao) and bacterial diversity indexes (Shannon) increased along the main pipes of the DWDS.

2) For bacterial community, the dominant phylum belonged to Proteobacteria (84.12%–97.6%) and the major class was assigned to be Alphaproteobacteria (67.42%–93.09%). Rhizobiales was the most abundant order, and a downward trend was observed in terms of the relative abundance with the increase of water age, while other residual orders increased with different degrees from the start to the end of the DWDS except for Sphingomonadales, Burkholderiales and Pseudomonadales. Besides, analytic results implied that Rhizobiales order was not only chlorine-resistant but also might make contribution on inhibiting pipe corrosion in the DWDS.

3) Several typical OTUs were found to be closely related with *Sphingomonas paucimobilis* (OTU320), *Pseudomonas aeruginosa* (OTU230) and *Mycobacterium fortuitum* (OTU343). In the view of the pathogenicity and chlorine-resistant ability of these species, the use of agents other than chlorine or agents that can act synergically with chlorine should be developed in drinking water disinfection.

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