Biofilm bacterial communities and abundance in a full-scale drinking water distribution system in Shanghai

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ABSTRACT

Community diversity and abundance of biofilms from a full-scale drinking water distribution system in Shanghai were characterized by denaturing gradient gel electrophoresis (DGGE) analysis of 16S rRNA sequences and heterotrophic plate count (HPC), respectively. Bacteria affiliated to the *Beta-* and *Gamma-Proteobacteria* were dominating in both in-situ and HPC-culturable bacterial communities. Other bacteria present included members of *Alphaproteobacteria*, *Bacteroides*, *Actinobacteria*, *Nitrospirae* and *Firmicutes*. *Acidovorax*, *Ralstonia* and *Acinetobacter* were common species in biofilms. *Klebsiella pneumoniae* and *Enterobacter* sp. were detected in the local distribution system. Dissolved organic carbon (DOC), residual disinfectant and temperature were the most important factors influencing both bacterial abundance and composition. HPC for biofilm sample was not correlated with its community diversity.

Key words | biofilm, community diversity, heterotrophic plate count, PCR-DGGE, water distribution system

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INTRODUCTION

Biofilms can be found on virtually any surface contacted by water in drinking water distribution systems. Biofilms protect and support bacterial growth including pathogenic microorganisms, enhance depletion of disinfection agents and can be a potential source of various health problems (LeChevallier *et al.* 1991; Buswell *et al.* 1998; Regan *et al.* 2002). Little research has been reported on the incidence, distribution and kinds of heterotrophic bacteria present in drinking water, especially in biofilms from full-scale water distribution systems because of the difficulty of obtaining sufficient samples from real distribution systems (Kim *et al.* 2006).

Culture-based methods have been commonly used to study microbial biofilms in drinking water distribution systems in past studies (Rompre *et al.* 2002; Lee & Kim 2003). Identification of the bacteria has been focused on the detection of culturable microorganisms such as heterotrophs and coliform bacteria (LeChevallier *et al.* 1987; Reasoner *et al.* 1989). However, plate count methods are inadequate to address the whole complex problem of phylogeny of biofilm bacteria (Ludwig & Schleifer 1994; Manz 1999). Identification of bacteria by culture-based methods is limited by the ability of microorganisms to grow in different culture media and may lead to incomprehensive results. Nowadays several culture-independent methods have been developed to overcome these limitations. Advances in DNA sequencing technology have greatly enhanced the identification of bacterial isolates (Kolbert & Persing 1999). Polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) based on direct sequence analysis of 16S rRNA has been demonstrated as an effective tool to explore microbial communities for environmental samples.

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Quantification of biofilm bacteria by culture-based methods is useful since it enables quantification of indicator microorganisms related to national and international standards for drinking water quality monitoring. However, monitoring bacterial count, for example heterotrophic plate count for biofilm samples, alone is not enough to evaluate biological safety and possible health hazards in distribution systems. Inspection of bacterial community diversity is crucial to determine whether the population includes pathogens or opportunistic pathogens.

Bacterial regrowth in biofilms from a full-scale water distribution system in Shanghai was evaluated by heterotrophic plate count in our research. This paper also describes the community diversity of both in-situ and culturable bacteria in biofilms from a full-scale drinking water distribution system using PCR-DGGE and partial 16S rRNA sequencing.

METHODS

Biofilm collection and sample processing

All the samples were collected from different sites in a water distribution system of Shanghai Minhang Waterworks, which receives raw water from the Huangpu River and uses the conventional treatment process of coagulation with aluminium sulfate, sedimentation, sand filtration and chloramination disinfection. The sample collection began on October 30 2006 and was completed on 16 May 2007. Biofilms growing on the pipe wall were scraped off by sterilized spoon into sterilized wide-mouthed glass bottles. Collected samples were sent to the laboratory quickly by refrigerated transport and treated within 24 h. Samples for cultivation and DNA extraction were taken and the remaining samples were stored at −20°C.

Biofilms were treated in the laboratory by adding 5 g of sterile glass beads (diameter 2 mm) and 20 ml of sterilized phosphate buffer solution (PBS), and shaking vigorously in a rotatory mixer for 20 min and then precipitating at room temperature for 20 min after recording the wet weight (Keinanen *et al.* 2004). The supernatant liquid was used for cultivation and DNA extraction.

Heterotrophic plate count and enrichment incubation

Heterotrophic plate count was performed in triplicate by the spread plate technique using 0.15 ml of the suspensions (or an appropriate dilution) spread over the surface of either standard plate-count agar (PCA) or R2A agar. Colony-forming units (CFUs) were counted after culturing at 37°C for 48 h in nutrient-rich PCA and at 28°C for 7 days, 14 days and 28 days in nutrient-poor R2A agar (Reasoner & Geldreich 1985).

Two-millilitre extracts of each sample were inoculated into tubes with 10 ml of PCA/R2A broths to obtain the enrichment cultures. Culturable bacteria were grown in R2A/PCA broths at 28°C/37°C, respectively, in a rotary shaker set at 200 rpm. The HPC-culturable fractions were obtained from R2A/PCA broths after 7,14,28/5 days. Seven enrichment cultures from 15 samples (four samples obtained in winter and three samples in summer) were collected for subsequent DNA extraction and DGGE profiling.

DNA extraction for total and culturable bacterial communities

DNA from the total and HPC-culturable fractions was extracted for subsequent molecular analysis. In brief, treated biofilms were re-suspended in 400 μl of STET [8% sucrose; 5% Triton X-100; 50 mM Tris-HCl (pH 8.0); 50 mM EDTA (pH 8.0)]. Lysis was performed by the addition of 40 μl of 50 mg/ml lysozyme and a 30-min incubation at 37°C, followed by the addition of 10 μl of 20 mg/ml proteinase K and 25 μl of 10% sodium dodecyl sulfate and a 1-h incubation at 37°C. Then 50 μl of 5 M NaCl and 50 μl of 5% CTAB were added. The suspension was centrifuged at 14,000 × g for 10 min, and 500 μl of supernatant was recovered and transferred to a clean microcentrifuge tube. The DNA was purified by the addition of 500 μl of buffered phenol-chloroform-iso-amyl alcohol (25: 24: 1), gentle mixing and centrifuging for 10 min at 14,000 × g. From the aqueous layer, 300 μl was transferred to a clean microcentrifuge tube, and nucleic acids were precipitated by the addition of 80 μl of sodium acetate (2 M, pH 5.2) and 600 μl of ice-cold 100% ethanol, followed by 6 h of storage at −20°C. The samples were centrifuged for 10 min at 14,000 × g, supernatant was drawn off and the nucleic acid

pellet was washed once with 500 μl of ice-cold 70% ethanol. The ethanol was removed, and the tubes were placed in a vacuum desiccator until dry. The nucleic acids were dissolved in 40 μl of TE and stored at −20°C.

PCR amplification of 16S rRNA gene

Bacterial DNAs from total and HPC-culturable fractions were amplified by universal nested 16S rRNA gene-directed PCR prior to DGGE analysis. The 27F-1492R primer set was used for the first round of PCR, and this was followed by nested amplification using the 357F-GC (5′-CGCCC GCCGC GCGCG GCGGG CGGGG CGGGG GCACG GGGGG CCTAC GGGAG GCAGC AG-3′) – 518R (5′-ATTAC CGCGG CTGCT GG-3′) primer set (Hoefel *et al.* 2005). The reactions were carried out in a 50 μl volume containing 3 μl DNA template solution (DNA extracts or PCR products after the first round PCR), 5 μl 10 × buffer [100 mM Tris-HCl (pH 8.3), 500 mM KCl, 15 mM $MgCl_2$]; 2.5 U of *Taq* DNA polymerase (TaKaRa); 20 nmol of each dNTP and 20 pmol of each primer. The V3 region of 16S rRNA gene was amplified using Eppendorf PCR system. Reactions containing sterile water served as negative (no template) controls. Products were screened by gel electrophoresis.

DGGE and statistical analysis of DGGE bands

DGGE was performed using a Dcode system (Bio-Rad Laboratories Inc., USA) according to the procedure first described by Muyzer *et al.* (1993). PCR products were loaded onto an 8% (w/v) polyacrylamide gel in 1 × TAE [40 mM Tris, 20 mM acetic acid and 1 mM EDTA (pH 8.0)]. The range of denaturant was from 30 to 55% (100% denaturant contains 7 M urea and 40% formamide). The electrophoresis was run at 60°C, for 10 min at 20 V and subsequently for 4 h at 200 V. Following electrophoresis, gels were stained with a 1 × SYBR Green I solution (FMC BioProducts, USA) in 1 × TAE three times, 15 min each time. Stained gels were visualized immediately using an electrophoresis documentation and analysis system. The unweighted pair-group method with arithmetic averages (UPGMA) was used to create a dendrogram describing the pattern analysis (Sokal & Michener 1958).

Table 1 | Water quality and biofilm parameters in the distribution system

	Pipe characteristics			Water quality parameters					HPC in biofilm	
No.	Material	Distance (km)	ΦD (mm)	Temp. (°C)	pH	Total Cl_2 (mg/L)	NH_3-N (mg/L)	DOC (mg/L)	LogHPC (R)	LogTBC (P)
1	Cast iron	12.8	100	13	7.19	1.73	3.16	1.93	6.45	4.86
2	Cast iron	5.58	800	9	7.21	1.83	3.16	1.79	4.45	3.98
3	Cast iron	6.84	200	7	7.19	1.59	3.07	1.53	6.05	5.01
4	Cast iron	6.84	100	7	7.21	1.59	2.53	0.74	5.31	4.07
5	Cast iron	14.67	800	3	7.2	1.55	2.94	1.05	5.99	4.31
6	Cast iron	4.65	700	14	7.3	1.48	1.31	2.84	5.33	4.16
7	Cast iron	4.11	1,000	14	7.49	1.53	2.46	2.06	5.76	4.53
8	Steel	4.11	1,000	14	7.49	1.53	2.46	1.52	5.86	4.93
9	Cast iron	8.64	800	21	7.25	1.42	2.89	0.76	6.86	5.77
10	Cast iron	8.64	800	21	7.25	1.42	1.54	3.18	7.24	6.15
11	Cast iron	6.84	800	21	7.28	1.5	2.97	2.17	6.53	5.54
12	Cast iron	6.60	100	24	7.83	1.27	2.97	1.26	7.45	6.32
13	Cast iron	6.78	100	23	8.11	0.64	1.00	2.31	6.87	5.96
14	Cast iron	8.16	100	24	8.12	0.04	2.13	1.72	8.08	6.48
15	Cast iron	11.88	300	27	7.95	0.11	2.79	2.01	7.73	6.30

Table 2 | Correlation coefficients between HPC in biofilm samples and chemical, physical parameters in water

	NH_3-N	Total-Cl_2	NH_2-Cl	DOC	Temp.	pH
HPC	−0.412	−0.638	−0.634	0.509	0.750	0.664

Sequencing and phylogenetic analysis

Bands of 16S rRNA fragments in the DGGE gel were excised with a razor blade. The DNA was eluted overnight at 4°C in a 1.5-ml tube containing 50 μl of sterile DNA-free water. The DNA was re-amplified with GC-clamp-357F and 518R primers, and analysed by DGGE again. Amplified products that produced a DGGE band at the same position as the excised band were selected and were PCR-amplified again with primers 357F (no GC-clamp) and 518R. The PCR products were sequenced by a capillary-type automatic DNA sequencer (Invitrogen Biotech). Partial 16S rRNA sequences were compared with known sequences in the GenBank database using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). The phylogenetic trees were constructed using the program MEGA 4.0 through the neighbour joining method with the Kimura parameter, and 1000 bootstrap replications were assessed to support internal branches (Tamura *et al.* 2007). Sequences determined in this study have been deposited in the GenBank database under accession numbers EU316187-EU316214 and EU258707 to EU258733.

RESULTS AND DISCUSSION

Bacterial plate counts of biofilm samples

Fifteen biofilm samples were used for heterotrophic plate counts on both R2A and PCA plate (Table 1). The HPC in biofilm samples differed greatly. Log HPC levels on R2A agars varied from 4.5 to 8 while that on PCA media ranged from 4 to 6.5. An average 1-log disparity in HPC on PCA and R2A media was observed. The relationship between water quality parameters and HPC was shown in Table 2. Temperature, DOC and total Cl_2 were positively related to HPC. The relativity factor between DOC and HPC was the highest, which suggested an important influence of organic nutrition on bacterial regrowth in biofilms from distribution systems. Nutrition was the limiting factor for bacterial quantity in biofilms in our study and an increase in DOC would accelerate bacterial growth in biofilms.

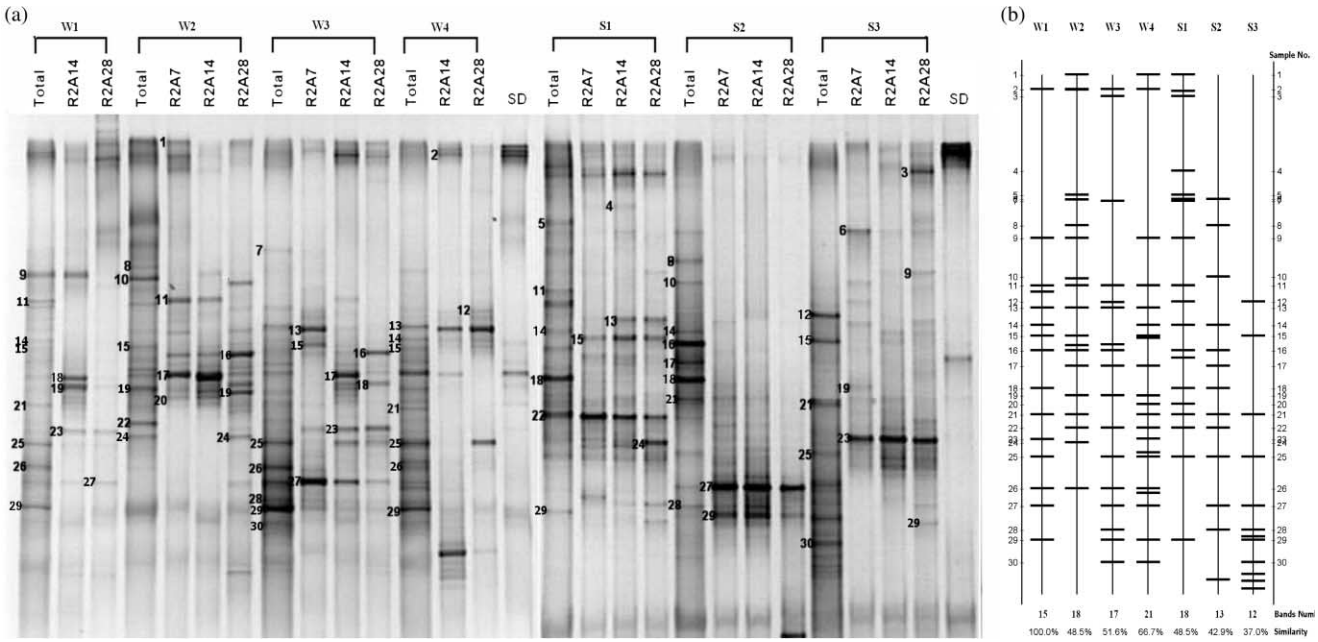

Figure 1 | DGGE profiles (30–55% denaturant) of PCR-amplified 16S rRNA fragments derived from total biofilm samples and R2A enrichment cultures (cultivated for 7, 14 and 28 days, respectively). Numbers correspond to band identities sequenced, Abbreviation: SD-reference standard for DGGE. (a) Negative image of DGGE of total and R2A-culturable bacteria, (b) schematic pattern of DGGE bands of in-situ bacterial communities using Quantity One 4.3.0 software.

Table 3 | 16S rDNA-V3 sequence analysis of bands excised from the PCR-DGGE gel of biofilm samples

DGGE band	Most closely related bacterial sequence*	Phylogenetic group	Physiological state†	DGGE band	Most closely related bacterial sequence*	Phylogenetic group	Physiological State†
B1	U. *Gamma* proteobacterium	*Gammaproteobacteria*	R2A C.	B17	*Clostridium boliviensis*	*Firmicutes*	R2A C.
B2	*Pseudomonas frederiksbergensis*	*Gammaproteobacteria*	R2A C.	B18	U. *Stenotrophomonas* sp.	*Gammaproteobacteria*	R2A C.
B3	U. freshwater bacterium	Unidentified	R2A C.	B19	*Klebsiella pneumoniae*	*Gammaproteobacteria*	R2A C.
B4	*Acinetobacter* calcoaceticus	*Gammaproteobacteria*	R2A C.	B20	*Flavobacterium johnsoniae*	CFB group	R2A C.
B5	U. bacterium	Unidentified	Not C.	B21	*Ralstonia* sp. M061122-11	*Betaproteobacteria*	Not C.
B6	*Janthinobacterium* sp. PR13	*Betaproteobacteria*	R2A C.	B22	*Acinetobacter baumannii*	*Gammaproteobacteria*	R2A C.
B8	*Acinetobacter* sp. P11-B-4	*Gammaproteobacteria*	Not C.	B23	*Devosia* sp. BD-c194	*Alphaproteobacteria*	R2A C.
B10	*Acidovorax* sp. R12-10	*Betaproteobacteria*	Not C.	B24	*Wautersia* sp. MG71	*Betaproteobacteria*	R2A C.
B11	*Desulfotomaculum* sp. Iso-W2	*Firmicutes*	R2A C.	B25	*Sanguibacter* sp. NH20	*Actinobacteria*	R2A C.
B12	*Bacillus* sp. DGV2	*Firmicutes*	R2A C.	B26	U. *Alpha* proteobacterium	*Alphaproteobacteria*	Not C.
B13	*Bacillus* sp. AUBB38	*Firmicutes*	R2A C.	B27	U. *Gamma* proteobacterium	*Gammaproteobacteria*	R2A C.
B14	U. *Cupriavidus* sp.	*Betaproteobacteria*	Not C.	B28	U. bacterium DW NsrB8	Unidentified	R2A C.
B15	U. bacterium DRY-C-G21	Unidentified	R2A C.	B29	*Paenibacillus ehimensis*	*Firmicutes*	R2A C.
B16	*Acidovorax* sp. BWLB	*Betaproteobacteria*	R2A C.	B30	U. *Nitrospira* sp.	*Nitrospirae*	Not C.

*U.: uncultured.

†Physiological state was identified by comparing DGGE profiles of in-situ community with R2A cultures; R2A C.: R2A Cultured; Not C.: Not Cultured.

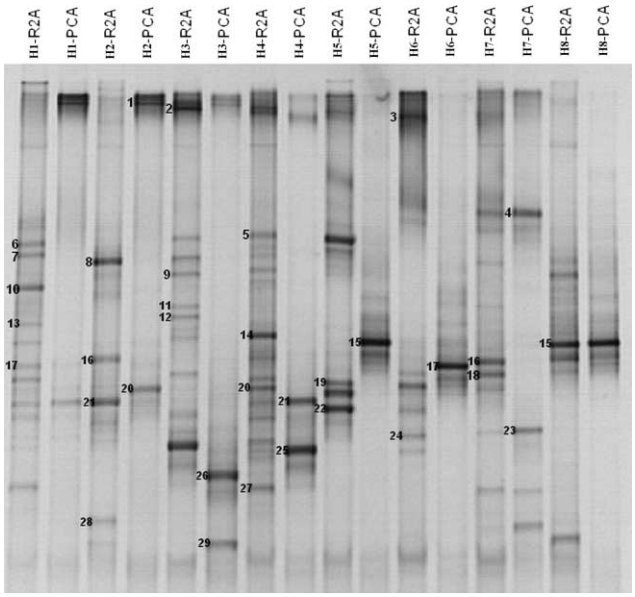

Figure 2 | DGGE profiles (30–55% denaturant) of PCR-amplified 16S rRNA fragments derived from HPC culturable bacterial fractions (using R2A/PCA media cultivating for 7/5 days) in distribution system water.

In-situ bacterial community and predominant species

In-situ bacterial community composition was analysed by PCR-DGGE. DGGE profiles of 16S rRNA fragments derived from total bacterial fractions are shown in Figure 1. DGGE analysis revealed a diverse population of bacteria. Bands on the DGGE gel were detected by the software Quantity One 4.3 followed by manual correction. A total of 30 dominant band types were detected. The number of bands per lane varied from 12 to 21. The highest bands number was observed in sample 4, which indicated higher relative abundance in microbial diversity in this sample. Among the marked bands, 14 bands occurred frequently; bands were considered frequent if they were found in more than 50% of the samples. B21 and B25 were present in six samples.

Dominant DNA bands were excised from DGGE gel and re-amplified by PCR. Twenty-eight successfully re-amplified DNA fragments were sequenced and subjected to BLAST GenBank analysis. Table 3 showed the most closely related bacterial sequence. Most of the identified bacterial species in this study have been isolated from soils, sediments, freshwater or drinking water distribution systems by other researchers (Van *et al.* 2002; Goswami *et al.* 2007; Lin *et al.* 2007). Sequence analysis revealed that common bacterial phylotypes in biofilms included members of *Alpha-*, *Beta-* and *Gamma-Proteobacteria*, *Bacteroides*, *Actinobacteria*, *Nitrospirae*, and *Firmicutes*. Overall, 64% of the populations (18 sequences of 28) were Gram-negative bacteria whereas 21% (six sequences of 28) were Gram-positive.

Although bacterial phylotype diversity varied among different samples, bacteria affiliated to *Acidovorax*, *Ralstonia*, *Acinetobacter* and *Clostridium* persisted in almost all biofilm samples. We also detected several sequences closely related to pathogenic microorganisms or opportunistic pathogens. It was found that sequence B19 was related to *Klebsiella pneumoniae*. Bands related to *Klebsiella* were found in W2, W3 and W4. All were in winter. It seemed that low temperature can't restrict the growth of potentially harmful bacteria such as *Klebsiella* in biofilms.

Phylogenetic study of HPC-culturable bacterial population

In order to study the bacterial community structure after culturing in enrichment media, samples H1–H8 were cultivated in R2A/PCA media for 7/5 days, respectively, followed by DNA extraction and PCR-DGGE analysis. Figure 2 shows the DGGE profiles of 16S rRNA fragments derived from HPC-culturable bacterial fractions after cultivating in R2A/PCA media. The biofilm samples presented quite different bacterial community structures after culturing in different media. The band numbers for culturable bacteria in R2A cultures ranged from 7 to 15, whereas the numbers in PCA cultures varied from 2 to 4. The diversity of R2A culturable bacteria was much more limited than that of in-situ bacterial population, while it was far higher than bacteria in PCA cultures.

Bands were detected in the DGGE profile of HPC-culturable bacterial communities. Closest matching sequences of 16S rRNA in the GenBank database were identified after sequencing and are given in Table 4. The predominant bacterium in R2A enrichment cultures belonged to *Proteobacteria*, in which *Beta-* and *Gamma-*subdivision occupied 38 and 29%, respectively, which was similar to in-situ bacterial community. No microbe aligned

Table 4 | 16S rDNA-V3 sequence analysis of bands excised from the PCR-DGGE gel of enrichment bacteria

DGGE band	Most closely related bacterial sequence	Phylogenetic group	Cultures	DGGE band	Most closely related bacterial sequence	Phylogenetic group	Cultures
H1	*Bacillus* sp. X5	*Firmicutes*	–, P	H15	*Bacillus thuringiensis*	*Firmicutes*	R, P
H3	*Pseudomonas tolaasii* B14	*Gammaproteobacteria*	R, P	H16	*Enterobacter* sp. Ax-4	*Gammaproteobacteria*	R, P
H4	*Pseudomonas brassicacearum*	*Gammaproteobacteria*	R, P	H17	*Bacillus subtilis*	*Firmicutes*	R, P
H5	*Janthinobacterium* sp. PR13	*Betaproteobacteria*	R, –	H19	*Enterobacter* sp. CRRI 3	*Gammaproteobacteria*	R, –
H6	*Pseudomonas mephitica*	*Betaproteobacteria*	R, –	H20	*Bacillus circulans*	*Firmicutes*	R, P
H7	*Spirillum* sp. NOX	*Betaproteobacteria*	R, –	H22	*Pantoea* sp. pfB25	*Gammaproteobacteria*	R, –
H8	*Beta* proteobacterium zj34	*Betaproteobacteria*	R, –	H23	*Bacillus thermoamylovorans*	*Firmicutes*	R, P
H9	*Oxalobacteraceae* bacterium Tf 246	*Betaproteobacteria*	R, –	H24	*Massilia aurea*	*Betaproteobacteria*	R, –
H10	*Hydrogenophaga* sp. R02-6	*Betaproteobacteria*	R, –	H25	*Acinetobacter baumannii*	*Gammaproteobacteria*	R, P
H11	*Bacillus* sp. DGV2	*Firmicutes*	R, –	H28	*Clostridium butyricum*	*Firmicutes*	R, P
H14	Uncultured *Acidovorax* sp.	*Betaproteobacteria*	R, –	H29	*Arthrobacter* sp. Pi4	*Actinobacteria*	R, P

with *Alpha-proteobacteria* was detected. In contrast, bacteria related to *Firmicutes* constituted 54% of the identified bacterial sequences in PCA media and thus represented the most prevalent group. Only *Gamma-proteobacteria* were found within PCA cultures in our study. DGGE analyses of R2A/PCA cultures of biofilm samples suggested that members of *Enterobacteriaceae* family frequently existed in biofilms of drinking water distribution systems. Sequences similar to bacteria belonging to *Enterobacter* sp. and *Pantoea* sp. were detected in both cultures. In the biofilms, the *Enterobacter* are potential producers of extra-cellular polysaccharides (EPS), which promote consolidation of the biofilm and attachment of other micro-organisms (Lehner *et al.* 2005; Kim *et al.* 2006).

Even when HPC in samples remain unchanged, great changes may occur in bacterial composition in biofilms. The bacterial diversity in W4 was much higher than in S3 as shown by higher band numbers in respective DGGE profiles. So HPC for biofilm sample was not correlated with its community diversity. Monitoring heterotrophic plate count for biofilm samples alone is not enough to evaluate biological safety and possible health hazards in distribution systems, as great changes in bacterial structure may occur.

CONCLUSIONS

It could be concluded that bacteria affiliated to the *Beta-* and *Gamma-Proteobacteria* were dominating in both in-situ and HPC-culturable bacterial communities. Other bacteria present included members of *Alphaproteobacteria*, *Bacteroides*, *Actinobacteria*, *Nitrospirae* and *Firmicutes*. *Acidovorax*, *Ralstonia* and *Acinetobacter* were common species in biofilms. *Klebsiella pneumoniae* and *Enterobacter* sp. were detected in the local distribution system. Dissolved organic carbon (DOC), residual disinfectant and temperature were the most important factors influencing both bacterial abundance and composition. Great changes in bacterial composition may occur even when bacterial counts in biofilm remained unchanged. HPC for biofilm sample was not correlated with its community diversity. So monitoring HPC in biofilm alone is not enough to evaluate biological safety and possible health hazards in distribution systems.

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