Bacterial species and variation in the biofilm of water distribution system in Harbin city

Yu Shui-li, Yu Xin-jing, Shi Wen-xin, Wang Dongguang and Qiu Xiao-xia

ABSTRACT

A model pipeline which dynamically simulates the drinking water distribution system was designed and kept running for twelve months with the tap water from Harbin. Biofilm was taken from its inner surface to isolate and identify the bacteria. Some biofilm was also taken from a real pipeline in Harbin. The results of microbiological analysis showed that there were similar dominant species both in the model and real pipeline, implying that those dominant species could grow well and form the biofilm easily under the same water quality and the same operating situation. The species vary with the length of the test period and reach a balance stage after a long test period. Some biofilm was analyzed by a scanning electron microscope for the further study of the bacterial growth. The SEM results confirmed that most of the bacterial growth on the inner surface of the pipeline were cocci and bacilli. In addition, the polymerase chain reaction (PCR) and agarose gel eletrophoresis were used to detect *Pseudomonas aeruginosa*. Biofilm samples from the model pipeline and the real distribution pipeline were analyzed by PCR. The PCR result showed that there was no *Pseudomonas aeruginosa* in both of the pipelines.

Key words | biofilm, biostability of drinking water, distribution system, dominant species, pipeline, *Pseudomonas aeruginosa*

Yu Shui-li (corresponding author)
Yu Xin-jing
Shi Wen-xin
Wang Dongguang
Qiu Xiao-xia
School of Municipal and Environmental Engineering,
Harbin Institute of Technology,
Harbin150090,
China
Tel & Fax: 0086-451-86282101
E-mail: *ysl@vip.163.com*

INTRODUCTION

Biostability of drinking water has been receiving considerable attention over the past ten years due to potential water recontamination during its distribution. The biostability of drinking water was defined as the ability of organic matter in drinking water to support heterotrophic bacterial growth (Liu *et al.* 2002). It is the maximum likelihood of the nutrient substance to support bacterial growth when the organic matter becomes the inhibitive factor of the heterotrophic bacterial growth. The higher biostability of drinking water means a lower concentration of organic matter in the distribution system.

In order to increase the biostability of drinking water, the after growth of bacteria in the distribution system must be controlled. But so far, the studies on the biota in the distribution system are very limited. The aims of this paper are to study the bacterial growth in the biofilm attached to the inner surface of the pipeline, to compare the similarity between a simulative pipeline and a real distribution system, and to assess the effect of bacterial after growth on biostability. In this paper, microbiological methods were used to isolate and identify the dominant species of bacteria. Molecular biology methods were used to detect some pathogen or significant species. Polymerase chain reaction (PCR) and agarose gel electrophoresis were used to detect *Pseudomonas aeruginosa*, a common opportunistic pathogen which has been found in the drinking water (Ma *et al.* 2001).

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METHODS

Pipeline model

Samples of biofilm were obtained from a castiron pipeline model which had been kept running with tap water for more than four months. The pipeline was 0.4 m in length and 150 mm in inside diameter (Figure 1). Some simple points, i.e. 6 castiron plates, were placed on the inner surface of the pipeline so that biofilm can grow upon them and can be taken for detection. This model began to run in March 2004 with tap water from the distribution system in Harbin city. The characteristics of tap water are shown in Table 1.

Sample taking

The biofilm samples were taken in July 2004 (running for four months), November 2004 (running for eight months) and March 2005 (running for twelve months). Sterilized absorbent cotton was used to wipe the pipe tubercles from the inner of pipeline and put into 100 ml sterile water immediately. The mixture was homogenized by shaking 15 min and then for the bacterial isolation. All glassware used was autoclaved. In addition, one sample from a real distribution system in Harbin was taken in May 2005 (when one pipeline was examined and repaired). The pipeline was 1200 mm in diameter and was running for about 5 years.

Bacterial isolation

The first and second samples were isolated without enrichment. The third sample was duplicated with one unenrichment and one enrichment. The peptone broth was used in initial enrichment which was aimed at obtaining the dominant species in rich nutrition environment. The enrichment cultures were incubated at 30 °C for 48 hours.

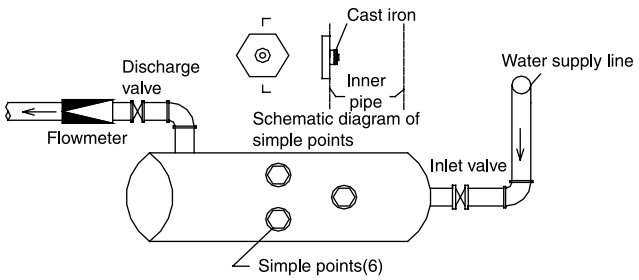


Figure 1 | The pipeline model.

Table 1 | The characteristics of tap water

Parameters	Values
pH	6.5
Residual chlorine	0.05 mg/l
Ammonia nitrogen	0.26 mg/l
Turbidity	1.0 NTU
COD_{Mn}	2.34 mg/l
TOC	2.86 mg/l
UV254	0.050
Temperature (average)	19°C

For the unenrichment sample, a dilution series from 10^{-1} to 10^{-3} was made and 0.2 ml of each dilution was taken to obtain well separated colonies using methods of pour plate and spread plate. For the enrichment sample, a dilution series from 10^{-1} to 10^{-8} was made from the homogenized enrichment culture. 0.2 ml of the 10^{-6}, 10^{-7}, 10^{-8} dilutions was taken respectively to obtain well separated colonies with the same methods. Each dilution was quadrupled with two pour plates and two spread plates. All plates were incubated at 30 °C for 1-7 days. Well separated colonies were subcultured by restreaking four times to obtain the pure cultures. The pure cultures were inoculated to medium slopes for analysis and storage (at 4 °C).

The medium used in the isolation process was nutrient agar medium which was applicable for most bacteria cultivated with the following compositions per litre: 5 g beef extract, 10 g peptone, 5 g NaCl and 15-20 g agar. The pH was adjusted to 7.0-7.2 with 1 M NaOH. The medium was sterilized at 121 °C for 30 min.

Identification of the isolates

The isolates of the model pipeline samples were identified by conventional biochemical and physiological tests (Ma *et al.* 2002). The main tests were: Gram stain, catalase,

indole, reduction of nitrate, and fermentation of glucose and aerobism tests. The isolates identification was conducted following *Bergey's Manual of Determinative Bacteriology* (Buchanan & Gibbons 1974) and the *Manual* of *Determinative Usual Bacteriology* (Li & Cai 2001).

The morphology study

The transmission electron microscopic (TEM) photos of some dominant species were taken to observe the cell shape and arrangement. The study of the morphology would be helpful for the identification.

The scanning electron microscope (SEM) of the biofilm

The scanning electron microscope (SEM) photos of the biofilm in the model pipeline were taken to observe the bacterial growth directly.

Polymerase chain reaction (PCR) and agarose gel electrophoresis

Polymerase chain reaction (PCR) is a common method of creating copies of specific fragments of DNA. PCR rapidly amplifies a single DNA molecule into many billions of molecules. The central scientific fact that makes PCR so useful is this: the genetic material of each living organism - plant or animal, bacterium or virus - possesses sequences of its nucleotide building blocks (usually DNA, sometimes RNA) that are uniquely and specifically present only in its own species. For PCR, primers must be duplicates of nucleotide sequences on either side of the piece of DNA of interest, which means that the exact order of the primers' nucleotides must already be known.

The exogenous toxin A (ETA) of *Pseudomonas aeruginosa* belongs to a family of secreted bacterial toxins which function as potent cytotoxic agents (Woods & Lglewski 1983; Allured *et al.* 1986). This paper used the primer in paper (Song *et al.* 2000) which was designed according to the ETA, and was synthesized by Shanghai GeneCore BioTechnologies Co., Ltd. The sequences list is as follows:

1, 5 -GAC AAC GCC CTC AGC ATC ACC AGC-3
2, 5 -CGC TGG CCC ATT CGC TCC AGC GCT-3

There are 5 samples in this test; T was the *Pseudomonas aeruginosa* pure culture medium, R1, R2 were the samples of the realistic distribution system, M1, M2 were the samples of model pipeline. R1, R2, M1, M2 samples were incubated at 35 °C for 48 hours in enrichment culture (*Pseudomonas aeruginosa* can survive well in this condition). To avoid failure of the extract of DNA, the culture mediums were going to the PCR and agarose gel electrophoresis directly. The PCR

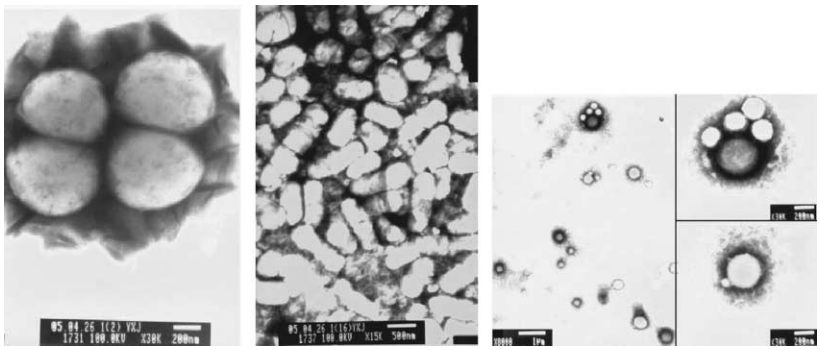
Table 2 | The results of the isolation and identification

Sample*	Stains	Totality
Sample 1	*Micrococcus roseus, Micrococcus luteus, Micrococcus varians, Curtobacterium pusillum, Terrabacter collins, Brevibacterium linens, Arthrobacter flavescens, Arthrobacter terregens, Microbacterium lactium, Microbacterium laevaniformans, Microbacterium imperiale*	11
Sample 2	*Anaerovibrio, Sphaerobacter thermophilum, Clavibacter subsp., Arthrobacter globiformis, Curtobacterium flaccumfaciens, Clavibacter subsp. michiganensis, Micrococcus roseus, Micrococcus luteus, Lactobacillaceae, Brevibacterium linens*	11
Sample 3	*Micrococcus roseus, Micrococcus luteus, Terrabacter collins, Brevibacterium linens, Arthrobacter flavescens*	5
Sample 3'	*Brevibacterium linens, Listeria denitrificans, Bacillus alcalophilus*	3
Sample 4	*Micrococcus roseus, Micrococcus varians, Curtobacterium albidum, Brevibacterium linens, Arthrobacter flavescens, Microbacterium lactium, Bacillus alcalophilus*	7

*Sample 1-3 were samples taken from the model pipeline according to time sequence. Sample 3' was the third simple which had been enriched. Sample 4 was the sample taken from the real pipeline in Harbin city.

Table 3 | Some biochemical and physiological characteristics of those stains

Strain	Gram's	Catalase	Reduction of nitrate	Indole	Fermentation of glucose	V.P	Aerobism	Color of colony
Micrococcus roseus (Micrococcus)	+	+	+	−	−	−	aerobic	pink
Micrococcus luteus (Micrococcus)	+	+	−	+	−	−	aerobic	yellow
Micrococcus varians (Micrococcus)	+	+	+	−	−	−	aerobic	light yellow
Curtobacterium pusillum (Curtobacterium)	+	+	−	−	+	−	aerobic	light yellow
Arthrobacter globiformis (Arthrobacter)	+	+	+	−	−	−	facultative anaerobe	leuco
Terrabacter collins	+	+	+	−	−	−	aerobic	white
Brevibacterium linens (Brevibacterium)	+	+	−	−	−	+/−	aerobic	orange color
Arthrobacter flavescens (Arthrobacter)	+	+	+	−	−	−	aerobic	lemon yellow
Arthrobacter terregens (Arthrobacter)	+	+	−	+	−	+	aerobic	yellow
Microbacterium lactium (Microbacterium)	+	+	−	+	−	−	aerobic	white
Microbacterium imperiale (Microbacterium)	+	+	−	+	−	−	aerobic	orange red
Anaerovibrio	−	+	−	+	−	−	anaerobe	leuco
Sphaerobacter thermophilum (Sphaerobacter)	+	+	−	+	−	−	aerobic	yellow
Clavibacter subsp. (Rathayibacter)	+	+	−	+	−	−	aerobic	white
Curtobacterium flaccumfaciens (Curtobacterium)	+	+	+	−	+	+	aerobic	orange yellow
Clavibacter subsp. michiganensis (Clavibacter)	+	+	−	+	−	−	facultative anaerobe	white
Listeria denitrificans (Listeria)	+	+	+		+		facultative anaerobe	leuco
Bacillus alcalophilus (Bacillus)	+/−	+	−		+		aerobic	white
Lactobacillaceae	+	−	−	−	+	−	facultative anaerobe	white
Microbacterium laevaniformans (Microbacterium)	+	+	−	+	−	−	aerobic	light yellow



Micrococcus roseus *Brevibacterium linens* *Arthrobacter (anaphase)*

Figure 2 | The TEM photos of some dominant species.

reaction mix consisted of 0.2μl (5U/μl) EXtag, 1.25μl 0x PCR buffer (contain magnesium ion), 1μl 2.5m M DNTP, 0.25μl Primer (each, 20m M), 1μl culture and 8.55μl DdH_2O in a total volume of 12.5μl. The reaction mixture was subjected to an initial 18 min of incubation at 95 °C to denature the DNA. This was followed by 35 cycles of amplification, which included a 15 s denaturation at 95 °C, 1 min annealing at 54 °C and 1 min extension at 72 °C. A 10 min extension at 72 °C was performed at the end of the final cycle. The gel electrophoresis use sagauoe (1%), EB staining.

RESULTS AND DISCUSSION

Dominant species isolation and identification

Table 2 shows the results of the isolation and identification of all the stains. 20 strains in total were obtained from the bioflim which was taken from the model pipeline and real pipeline. Most of them were Gram-positive bacteria with aerobic bacteria in the majority and some other facultative anaerobes. The *Micrococcus roseus, Micrococcus luteus, Terrabacter collins, Brevibacterium linens* and *Arthrobacter* were the dominant species, which were isolated and identified throughout the one-year test period. The bacteria in the real distribution system were similar to the dominant species in the model pipeline. It could be confirmed that these stains could grow well in the distribution system which convey the same pipe water for a long time.

According to the totality of species isolated from the three samples, it could be found that the number of species type was decreasing along with the formation of biofilm. It was supposed that along with the biofilm formation, bacteria that have the capacity to form biofilm took advantage over other species that cannot regulate the population density to form biofilm, which were finally eliminated by competition among bacteria.

The strains which were isolated from the enrichment culture have the features of fast reproduction and being good at absorbing nutrition. In the third sample, three dominant strains were isolated from enrichment cultures. Among these strains, *Brevibacterium linens* had also been isolated in the unenrichment samples. It could be inferred that *Brevibacterium linens* has a good adaptability to the environment either in poor or rich nutrition. For some strains, they could not have been isolated in the unenrichment samples, probably because of a small quantity in the samples due to restricted nutrition or growth competition among bacteria.

The isolates present only in unenrichment sample were supposed to be selected, often at a cost of fitness, by restricted nutrition condition over others, which could be inhibited by the condition, under the selective pressure of limited nutrition. Thus when a multiculture was inoculated on a medium of abundant nutrition, i.e. at absence of the selective pressure, other strains would take advantage over these particular species and proliferate as dominant strains. These strains undergoing limited nutrition condition should be taken seriously when studying the regrowth of bacteria in the distribution system, since such a condition usually suits the distribution system in the real world.

Some biochemical and physiological characteristics of those stains are listed in Table 3. Based on the dominant species, it was found from Table 3 that the growth of coccus was

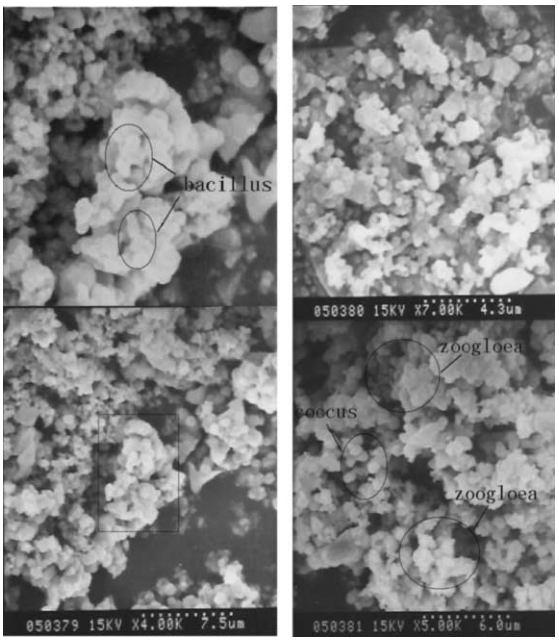
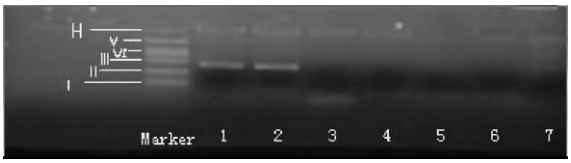


Figure 3 | The SEM photos of the biofilm.



I——100bp; VI——750bp; 1——T; 4——R2; 7——Blank
II——250bp; V——2000bp; 2——T; 5——M1;
III——500bp; H——simple pore; 3——R1;
6——M2.

Figure 4 | The agarose gel electrophoresis result of PCR products.

dependent on acetate, lactate, pyruvate, succinate, glycerin and carbohydrate (especially glucose). The bacillus nutritional requirement was complex. Many chemicals, such as carbohydrate, sugar deviant, fatty acid, dicarboxylic acid, hydroxyl acid, glycol, amino acid, heterocyclic compound, could all provide nutrition to them. Because those bacilli can adapt to a low nutrient environment, it was difficult to control its regrowth in a distribution system by means of nutrition removal.

The dominant species observation by transmission electron microscope

In order to learn more about the shape, size and other characteristics of those dominant species, the transmission electron microscope was applied in this test and some photos were taken as shown in Figure 2. From Figure 2 it can be seen that *Arthrobacter*, those globular cells in old cultures, were varied in size. Some were 2-4 times larger than others which were considered a character of *Arthrobacter*.

Biofilm observation by scanning electron microscope

To confirm the bacterial regrowth in a real distribution system, a pipe tubercles sample was taken from the model pipeline which had been running a year for groundwater transportation. The multi-porous tubercles were yellowish brown. The scanning for the tubercle sample with SEM is shown in Figure 3. The pictures showed that there were some coral-like matter and bacteria on the tubercle surface. The attached microbes were almost coccus and bacillus. This result obtained from the SEM was in agreement with the isolation results from model samples. The result also implied that the distribution system with bacterial regrowth has the potential risk of water quality deterioration, especially when the biofilm was disturbed by a sudden change of water flow velocity, pressure or direction.

Polymerase chain reaction and agarose gel electrophoresis

The agarose gel electrophoresis result of PCR products from the cultures is shown in Figure 4. Lane 1 and lane 2 were the sample of pure *P. aeruginosa* (T). The samples used for lanes 3-6 were R1, R2, M1 and M2. Lane 7 was blank for contrast.

As is shown in Figure 4, only lane 1 and lane 2 contained an amplified DNA band of about 360 bp in size. It can be confirmed that the negative result of lanes 3-6 was not due to non-specific amplification. Only the sample that contained DNA of *P. aeruginosa* was able to give positive results. It also means that the biofilm in the model pipeline and the realistic distribution system did not have the *P. aeruginosa*.

CONCLUSIONS

The results of bacterial isolation and identification from the biofilm showed that there were similar dominant species both in the model pipeline and the real pipeline in Harbin

city. It meant that those dominant species could grow well and could form the biofilm easily under the same water quality and the same operating situation. The species varied with the test period lasting and reached a balance stage after a long test period. It implied that some species would become dominant in a biofilm population because they could adapt well to the poor nutrition situation in the distribution system.

Modern analytic equipment, TEM and SEM, was used in the analysis of biofilm and tubercles samples taken from the model pipeline. The SEM results confirmed that most of the bacteria grown on the inner surface of the pipeline were cocci and bacilli. It agreed with the results of microbiological identification. The SEM result of some tubercle samples collected from the model distribution system demonstrated that the scraggly inner surface of the pipe makes the biofilm form more easily.

Pseudomonas aeruginosa was not found in the biofilm by the PCR reactions and agarose gel eletrophoresis. Pathogens have not been found in the test either. However, the large quantity of bacterial growth would probably facilitate the growth of pathogenic bacteria. Therefore this model distribution system has the potential crisis.

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