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Effect of hydraulic conditions and disinfectants on biofilm in model distribution systems

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ABSTRACT

Adopting a traditional culture method and PCR–DGGE, this paper discusses the biofilm development on a polyethylene pipeline in model water distribution systems. When chlorine is absent, different hydraulic conditions had no effect on biofilm development. Biofilm HPCs developed up to a stable level in 7 days and maintained at 10^5 CFU/cm^2. The diversity of bacteria increased as time went on, and some kinds of bacteria changed greatly: *Sphingomonas sp*. tended to excrete extracellular polymers and *Mycobacterium sp*. had a highly hydrophobic surface, gradually taking the place of some other kinds. When chlorine is present, biofilm HPCs under shear stress 0.95 N/m^2 was about a quarter of that of 0.18 N/m^2. The diversity of the bacteria decreased relatively, and *Acinetobacter baumannii*, with a good drug-fast ability, became the dominant bacterium.

Keywords: Biofilm; HPCs; Microbial community; Hydraulic condition; Chlorine; PCR–DGGE

1. Introduction

Biofilm exists universally on a water–solid common boundary in natural environments, even in human bodies. Biofilm that develops on the inner surface of drinking water pipelines has caused many serious technical and hygiene problems such as erosion of pipelines and provision of sites for pathogenic bacterial development [1]. Therefore, it is necessary to study the characteristics of development of biofilm in order to provide sufficient information to improve drinking water quality.

This paper discusses biomass and microbial community structure together to show more information on biofilm. Traditional culture methods and PCR–DGGE were adapted together to do the study. The influencing factors chosen were hydraulic condition and disinfectant because the hydraulic condition can be adjusted in real drinking water systems as there is a range of flow rate that can be chosen. And the other reason is that disinfectant is one of the most useful methods to control biofilm development. As mass transfer of disinfectant into biofilm can be influenced by hydraulic conditions, this paper also discussed the relationship of hydraulic conditions and disinfectant.

2. Materials and methods

2.1. Experimental approach

The experiments used two annular reactors operated under two different hydraulic conditions, separately

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called AR-A and AR-B. The conditions designed of the two annular reactors were the same except the rotational speed of inner drums. As a result of different rotational speeds of inner drums, shear stress of AR-A and AR-B were respectively 0.95 N/m^2 and 0.18 N/m^2. The total liquid volume in each reactor is 670 mL, and the hydraulic detention time (2 h) is controlled by the influent feed rate [2]. All exposed surfaces of the reactor system were covered by black paper and the temperature of the reactor system was controlled at 20°C, which simulated the conditions in real pipelines.

The experiments were operated for two steps. In the first instance, real water from drinking water from Tsinghua University, which was underground water sterilized by ultraviolet radiation, was supplied. The concentration of assimilable organic carbon (AOC) was 50 μg/L. The biofilm, which developed on removable polyethylene (PE) slides that were parts of an annular reactor and used for biofilm enrichment, was sampled on the 7th, 14th, 30th and 90th day from the beginning of reactor operation. Thereafter, for another new experiment, all the conditions were maintained, except that real water was added with commercially available sodium hypochlorite (5.6% w/v), and the free chlorine residual in an inflow of annular reactors was about 0. 2 mg/L. Biofilm was sampled on the 7th day.

2.2. Analytical methods

In this experiment, two methods, conventional culture and PRC–DGGE, were adopted. R_2A agar [3], especially for microorganisms in an infertile nutrient drinking water environment, was adopted as the conventional culture method. At every sampling time, two slides were taken out separately from each annular reactor in parallel. The biofilm on each slide was brushed into 2 mL 0.85% sterile brine. Subsequently, 1.5 mL of the mixed liquid was used for DNA extraction and PCR–DGGE, while 0.5 mL of that was diluted one order of magnitude step by step for the conventional culture method. The microorganism in the 1.5 mL mixed liquid was accumulated by being filtrated through sterile cellulose acetate filters (pore size 0.22 μm, Advantec). Each filter was put into a 10 mL sterile centrifugal cuvette, which was ready for DNA extraction.

2.3. Conventional cultural method

The amount of viable biofilm material was quantified in duplicate using the spread plate technique [2] on R2A agar. When there was free chlorine residual, enough $Na_2S_2O_3$ was added to consume the chlorine. This culture method shows not only the total amount of cultivable bacteria in biofilm, but also the information about the microbial community's structure. Based on the characteristics of colonies (shape, size, color, edge, wetness, transparence) and those of individuals (shape, size, gram positive or gram negative), the preponderant types of bacteria were selected, counted and separated for pure cultivation.

Every pure bacterium was enriched and DNA extraction was done. The steps of DNA extraction refer to a method by Araya Ruben et al. [4]. Then each bacterium's DNA was used for polymerase chain reaction (PCR). 16S rDNA fragments were amplified by using 8F and 1492r primers [5] as shown in Table 1. PCR amplification reactions were carried out in 50 μL of PCR mixture with 1.5 mM $MgCl_2$ and 25 pmol of each primer. Hot start PCR was performed at 95°C for 5 min. The PCR was denatured at 95°C for 1 min, and was annealed at 50°C for 1 min, followed by primer extension at 72°C for 3 min. This step was repeated for 28 cycles. Finally, an extension step was carried out at 72°C for 10 min. The PCR products were sequenced by the Sun-bio Company.

2.4. PCR–DGGE

The steps of PCR–DGGE followed the method of Araya et al. [4] with slight modification. The filter in a 10 mL sterile centrifugal cuvette was cut into pieces by sterile forfex. After DNA extraction, 16S rDNA fragments were amplified using EUBf933-GC-clamp and EUBr1387 primers as shown in Table 1. PCR amplification reactions were carried out in 50 μl of PCR mixture with 1.5 mM $MgCl_2$ and 5 pmol of each primer. Hot start PCR was performed at 95°C for 5 min. And the PCR was denatured at 95°C for 1 min and was annealed at 65°C for 0.5 min, followed by primer extension at 72°C for 1 min. This step was repeated for 30 cycles. Finally, an extension step was carried out at 72°C for 10 min.The polyacrylamide gels

Table 1
Information of primers

Name of primer	Sequence
8F	5′-AGA GTT TGA TCC TGG CTC AG-3′
1492r	5′-GGT TAC CTT GTT ACG ACT T-3′
GC-EUBf933	933-954: 5′-GCACAAGCGGTGGAGCATGTGG-3′ GC: 5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGG-3
EUBr1387	1387-1368: 5′-GCCCGGGAACGTATTCACCG-3′

(acrylamide:bisacrylamide, 37.5:1) were made with denaturing gradients ranging from 40%–60%. Electrophoresis was initially at 60°C for 20 min at 30 V, and thereafter for 12 h at 100 V. Parallel PCR amplification from the same sample was compared, obtaining identical DGGE profiles. Bands in the DGGE gel of 16S rDNA fragments were cut off and submerged in 50 μL ddH_2O at 4°C overnight. The DNA transferring into ddH_2O was re-amplified by EUBf933 and EUBr1387 primers. The products were sequenced by the Sun-bio Company.

3. Results and discussion

3.1. Biomass of biofilm

Fig. 1 shows the effects of shear stress on biofilm HPC numbers during 3 months. In Fig. 1, during different periods of development, biofilm HPC numbers on PE show few differences, which means that two shear stress has little effect on accumulation of biofilm HPCs under the experimental conditions. Peter et al. [2] suggested that biofilm accumulation might be bioreaction-limited at higher BOM conditions (500 μg/L) and mass transfer-limited at lower BOM levels. When at lower BOM levels, biofilm numbers at 2.0 N/m^2 were about one order of magnitude higher than at 0.4 N/m^2. Gagnon and Huck [6] concluded from generally similar annular reactor experiments that when easily biodegradable organic matter was 50 μg/L, there was no mass transfer-limited.

The result in Fig. 1 can be explained in two ways. On one hand, in the presence of 50 μg/L AOC supplement, no mass transfer-limited happened. On the other hand, there might be a mass transfer-limited, but the two shear stress did not have the ability to make a difference in mass transfer as the gap between 0.95 N/m^2 and 0.18 N/m^2 was limited. In addition, the effects of shear stress on biofilm HPC numbers with 0.2 mg/L of free chlorine residual on the 7th day of development (on PE substrate at 20°C) was studied. Results show that biofilm numbers at 0.95 N/m^2 shear stress (about 10^4) were about one order of magnitude lower than 0.18 N/m^2(about 10^5). The data suggest that higher shear stress accelerates mass transfer of disinfectant in biofilm.

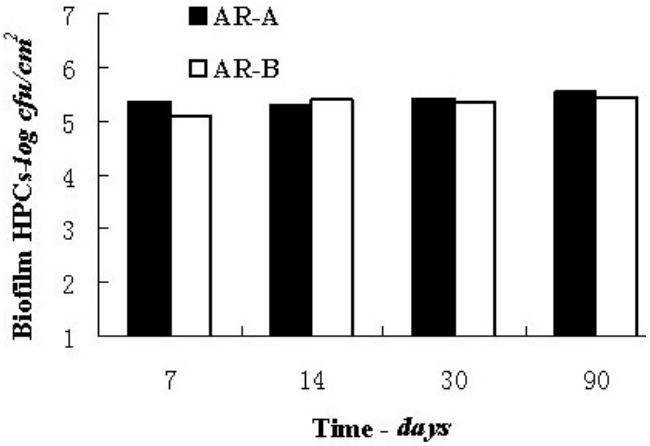

Fig. 1. Biofilm HPCs on the 7th, 14th, 30th and 90th day in the presence of 50 μg/L AOC supplement, in the absence of a disinfectant, at 0.95 N/m^2(AR-A) and 0.18 N/m^2(AR-B) shear stress and 20°C.

To sum up, the effect of hydraulic condition on the development of biofilm HPC numbers has two aspects. On one hand, the effect appears to be shear stress on biofilm. When biofilm is thin and does not overlap the viscous sublayer of current, it will not be influenced directly by shear stress of current. On the other hand, the effect appears to promote mass transfer of materials (including nutrients, disinfectants and so on) from drinking water into biofilm, which is a kind of associated effect of hydraulic condition and nutrients or disinfectants on biofilm development, especially when the concentration of those is low.

Moreover, in Fig. 1, biofilm HPCs on four different days feckly remained at 10^5 cfu/cm^2, which means biofilm HPCs had developed to a steady state around 7 days and were maintained at 10^5 cfu/cm^2 in the after days. The rule that biofilm HPCs will develop to steady state in several days was also found by other researchers [2,7]. The principle of population increase in a restricted environment in biogeocenose may be quoted to explain this phenomenon. As to biofilm, biofilm HPCs would develop to environmental maximal capacity in pipelines and be maintained around a certain value which is decided by the associated effect of nutrients, disinfectants, pipe material, temperature, and so on. The conception of environmental maximal capacity of biofilm is brought forward firstly in this paper.

3.2. Microbial community structure of biofilm

Fig. 2 shows the characteristics of microbial community structure of biofilm on four different sampling days. B1–B8 were identified by the characteristics of colonies and individuals, as shown in Table 2. In Fig. 2, the rules of change of microbial community structure in AR-A and AR-B were very similar, which meant hydraulic condition had little effect not only on biofilm HPCs but also on microbial community structure. During different periods of development, microbial community structure of biofilm on PE changed largely. At the initial period of development, the percentage of B2 in biofilm was the highest one. However, as time went on, B3 became the most powerful competitor in biofilm, and took the place of B2. On the 90th day, the main kinds of bacteria increased from 5 to 8, which showed that the diversity of bacteria in biofilm increased as time went on. B2 belongs to

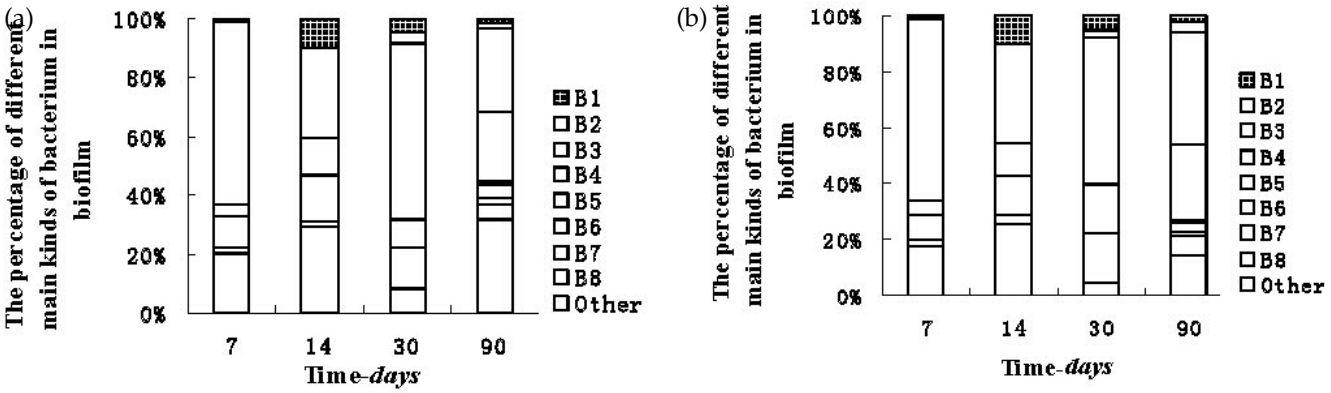

Fig. 2. Community of biofilm on the 7th, 14th, 30th and 90th day in the presence of 50 μg/L AOC supplement, in the absence of a disinfectant, at 0.95 N/m^2(AR-A-a) and 0.18 N/m^2(AR-B-b) shear stresses and 20°C.

Table 2
Characteristics of B1–B8

Name	Characteristics of colonies						Characteristics of individuals	
	Shape	Size (mm)	Color	Edge	Wetness	Transparence	Shape	Gram stain
B1	Round	2–4	White	Regular	Yes	No	Bacilliform	Negative
B2	Round	1–3	Slight yellow	Regular	Yes	No	Bacilliform	Positive
B3	Round	0.5–1.5	Bright yellow	Regular	Yes	No	Bacilliform	Negative
B4	Round	0.2–0.5	White	Regular	Yes	No	Bacilliform	Positive
B5	Concentric round	1–3	Inner round–brown Outer round-yellow	Regular	Yes	No	Bacilliform	Negative
B6	Round	1–2	Fuscous yellow	Regular	Yes	No	Bacilliform	Negative
B7	Round	2–4	Yellow associated with slight purple	Regular	Yes	No	Bacilliform	Negative
B8	Round	2–4	Slight pink	Regular	Yes	No	Bacilliform	Positive

Table 3
Results of blasting of B1–B8 and band1–band3

Bacteria/band	Organism	Similarity (%)
B1	*Mycoplana sp.*	100
B2	*Mycobacterium sp.*	100
B3	*Sphingomonas sp.*	99
B4	*Mycobacterium sp.*	100
B5	*Pseudoxanthomonas sp.*	100
B6	*Sphingomonas sp.*	98
B7	*Xanthobacter sp.*	99
B8	*Brevibacterium sp.*	99
Band 1 and Band 2	Uncultured gamma-proteobacteria	98
Band 3	*Acinetobacter sp.*	100

Mycobacterium (Table 3 and Fig. 3), which have a highly hydrophobic surface [8], while B3 belongs to *Sphingomonas* (Table 3 and Fig. 3), which tend to excrete extracellular polymers [9]. In an hydraulic environment, the development of a microbial community in biofilm is decided by the conglutinant effect between cells. In nature, most of bacteria have a drophobic surface, so the bacteria with a high drophobic surface which are easier to stick to other bacteria may be easier to live and develop in biofilm. The conglutinant effect also can be promoted by extra-cellular polymers, so the characteristic of excreting extra-cellular polymers may help the bacteria with such characteristics to develop well in biofilm. Those discussed above are only part of the reasons. As bacteria are complicated, their characteristics should be studied further to reveal the secret characteristics of biofilm development.

Fig. 4 shows the characteristics of biofilm microbial community structure on the 7th day (on PE substrate in the presence of disinfectant at 20°C). B1, B3 and B4 were the same as those in Fig. 2. In Fig. 4, with 0.2 mg/L of free chlorine residual, there were only three main kinds of bacterial in biofilm, which were obviously less than those without chlorine. Chlorine decreased not only biofilm HPCs but also diversity of bacterial in biofilm. But there

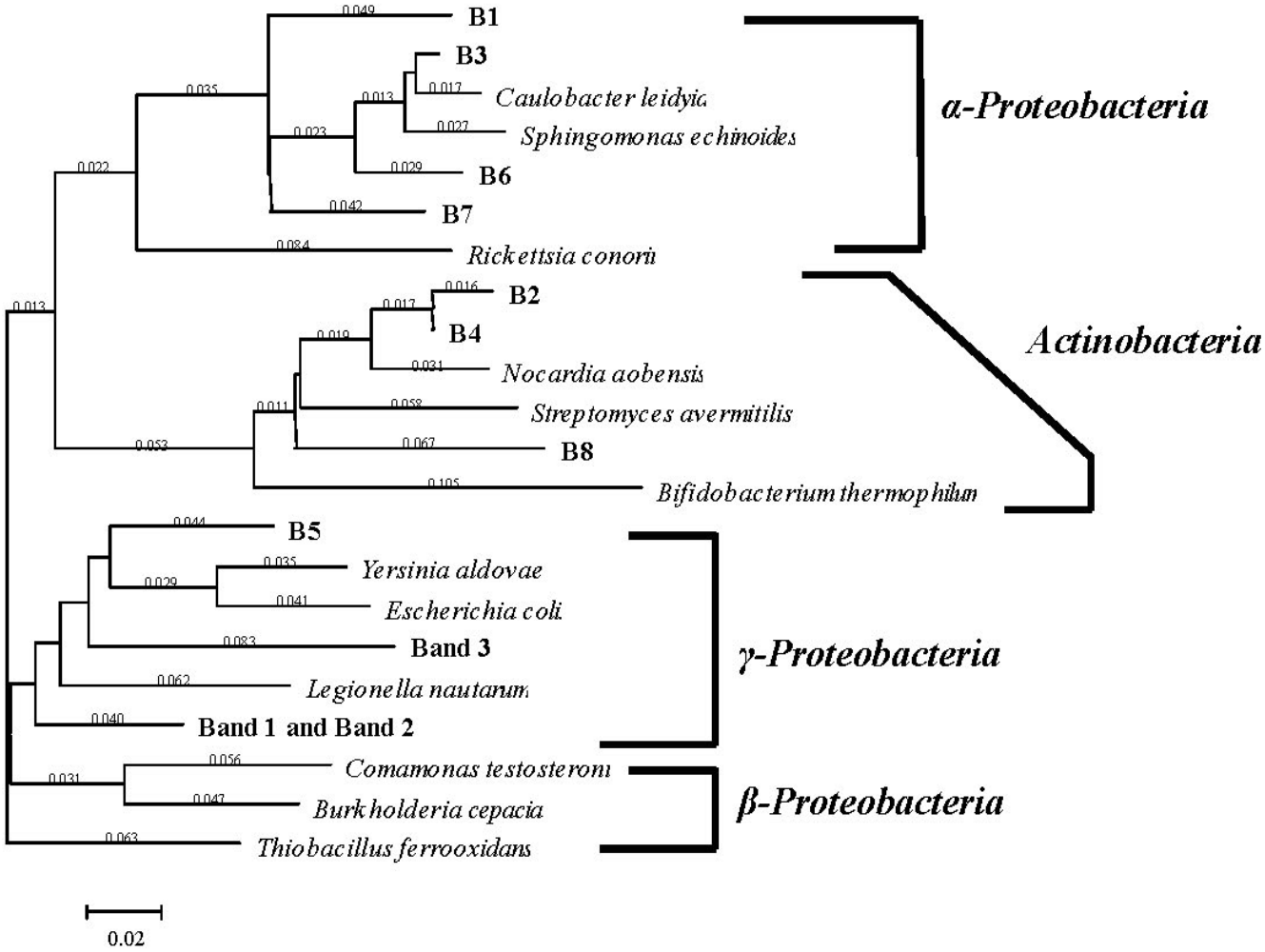

Fig. 3. Phylogenic tree of B1–B8 and band 1–band 3.

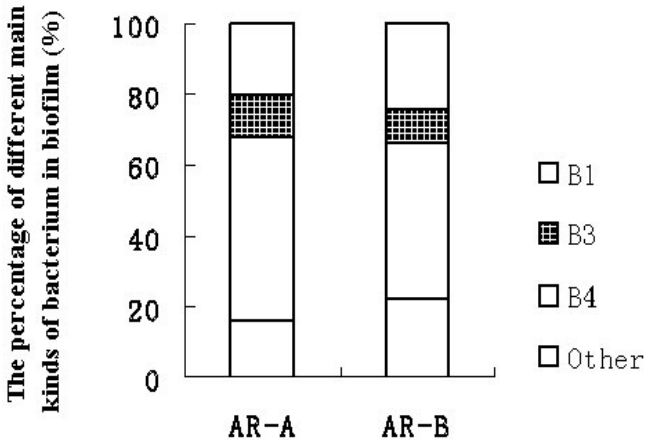

Fig. 4. Community of biofilm on the 7th day in the presence of 50 μg/L AOC and 0.2 mg/L free chlorine residual supplement, at 0.95 N/m^2 (AR-A) and 0.18 N/m^2 (AR-B) shear stresses and 20°C.

was a remarkable phenomenon that although different shear stress had effect on biofilm HPCs, they did not have an obvious effect on microbial community structure. The characteristics of microbial community structure of biofilm in AR-A and AR-B showed a similar situation. The dominant bacterium was B4, which belongs to *Mycobacterium* (Table 3 and Fig. 3). Although B4 and B2 both belong to *Mycobacterium* (Table 3 and Fig. 3), there are other differences in characteristics, which make them develop differently in biofilm.

Fig. 5 shows DGGE of PCR-amplified 16s rDNA fragments from biofilm. Firstly, it can be seen that separately in (a), (b) and (c), the distribution and brightness of bands in the strips of different shear stress are similar. This means that hydraulic conditions had little effect on the microbial community structure of biofilm. Secondly, the bands of (c) are less than those of (a) and (b), which means the diversity of bacteria decreased when chlorine

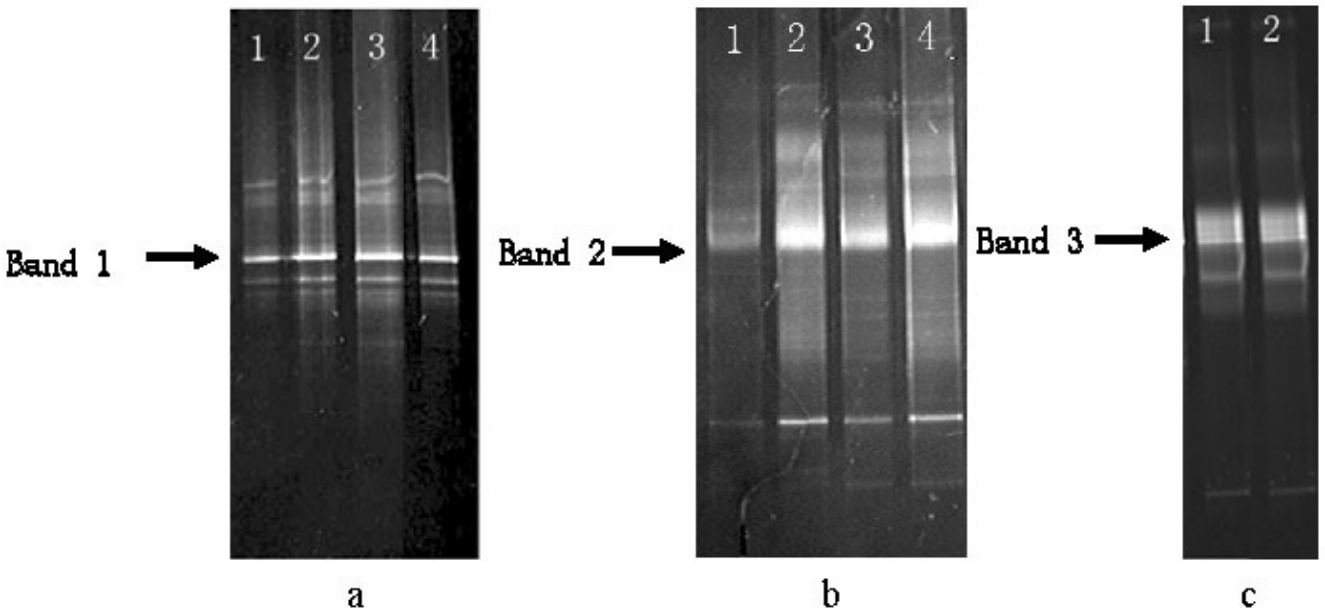

Fig. 5. DGGE of PCR-amplified 16s rDNA fragments from biofilm on the 7th day(a) in the absence of chlorine, biofilm on the 90th day(b) in the absence of chlorine, and biofilm on the 7th day(c) in the presence of 0.2 mg/L free chlorine residual. In (a) and (b), 1,2 which are parallel samples show biofilm from AR-A, while 3,4 which are parallel samples show biofilm from AR-B. In (c), 1 shows biofilm from AR-A, while 2 shows biofilm from AR-A, for the loss of parallel samples.

was added. The results talked above were same to that from traditional culture methods. Thirdly, Band 1, band 2 and band 3 separated the lightest band of (a), (b), (c) in Fig. 5. By identification (Table 3 and Fig. 3), Band 1 and band 2 were the same bacterium called uncultured gammaproteobacteria, which means the dominant bacterium did not change as time went on. Combined the results from traditional culture method and PCR–DGGE, a conclusion may be suggested as follows. The dominant bacterium that might play an important role in biofilm development did not change, and they might develop in inner layer of biofilm. But some non-dominant kinds of bacteria that may develop in outer layer of biofilm changed greatly, as *Sphingomonas sp*. tending to excrete extra-cellular polymers and *Mycobacterium sp*. having a highly hydrophobic surface took the place of some other kinds gradually. By identification (Table 3 and Fig. 3), Band 3 is the kind of bacteria called *Acinetobacter baumannii*. With good drug-fast ability, it became the dominant bacterium, which suggests that changes of conditions improve selection of bacteria of biofilm.

4. Conclusions

In conclusion, firstly, the biomass of biofilm became saturated in the accruement and the maximum was determined by the associated effect of nutrients, disinfectants, pipe material, temperature, and so on in pipelines. Therefore, a conception of environmental maximum capacity of biofilm is put forward in this paper. Secondly, with chlorine, biofilm numbers were about one order of magnitude lower at 0.95 N/m^2 shear stress than 0.18 N/m^2, which suggested that higher shear stress accelerates mass transfer of disinfectant in biofilm. Thirdly, biofilm selects different kinds of bacteria according to different environmental conditions, and some kinds of characteristics were helpful for bacteria to develop well in biofilm, such as the characteristics of tending to excrete extra-cellular polymers (*Sphingomonas sp*.) and having a highly hydrophobic surface (*Mycobacterium sp*.) and having good drug-fast ability (*Acinetobacter baumannii*).

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