

Research on Microbial Diversity of Pipeline Biofilms in Water Distribution System

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The biofilms have been described as “cities of microbes”. Some studies have suggested that pathogenic bacteria, which may not be detected by culture-dependent methods, occur in the biofilms. So a pilot drinking water distribution system is constructed and the combination of the scanning electron microscope (SEM) and the polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) is used to show the microbial growth of the biofilms in the pipe wall macroscopically and microscopically. The experiment simulates the water supply network and cultivates the biofilms for 7 months, observing the growth of the biofilms in the network entrance and end intuitively by SEM. And the microbial diversity in biofilms at different seasons and with different materials is evaluated by PCR-DGGE based on 16SrDNA. The result shows that the pipe material affects the biofilm bacteria, total bacteria and bacteria diversity; the number in the galvanized steel pipe is larger; the growth of the biofilms in the PE pipe and UPVC pipe are slowed down. The temperature is changed by the season, which is the main reason that causes the change of the diversity of bacteria. Both the hydraulic condition and residue chlorine concentration affect the growth of the biofilms in the pipe wall.

Key words: Water distribution system, Wall biofilm, Microbial diversity, SEM, PCR-DGGE.

Various undesirable water-borne microorganisms are present in water distribution systems and may cause epidemics. In most water distribution systems, the interface between the water and the pipe wall is a prime site for the accumulation of cells and organic matter, and for bacterial multiplication (Batte *et al.*, 2003). The various microorganisms living in the pipeline adsorb the inner wall of the pipe, using the organic matter as nutrient. Under the variety of the force, the microorganisms contact with the wall of the pipeline with the adhesion irreversible. As the

quantity of microorganisms grows incessantly, they finally form a complicated biocoenose, covering the inner side of pipeline. The biofilms therefore come into being (Chang *et al.*, 2008). Some studies have suggested that pathogenic bacteria, which may not be detected by culture-dependent methods, occur in biofilms (Kapley *et al.*, 2000; Gilbride *et al.*, 2006).

Presently, most of the studies over water supply networks have merely touched upon the bacteriological indexes such as AOC, the total number of bacteria, etc. However, in-depth research of the bacterial structure and quantity via molecular biology technology, which is sensitive, quick and distinctive, has seldom been seen (Liu *et al.*, 2009). Chlorine can limit bacterial regrowth, both by injuring bacteria, thereby

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preventing their growth, and by limiting the production of bacteria in the system, either in the planktonic phase or in biofilms (Lu *et al.*, 2005). However, the chlorine residual resulting from such a treatment is insufficient to kill and remove all the attached biomass. Even large doses and drastic treatments are unable to completely eradicate biofilms. Several mechanisms could explain such resistance, but the end result is that most of the biofilm remains alive.

The inability to cultivate many environmental bacteria by conventional laboratory techniques has been a significant handicap in the investigation of water microbes. One method, 16S rDNA based polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE), proposed by Muyzer *et al.* (Muyzer *et al.*, 1993), has been widely used for studying bacterial communities in numerous environments, including soils and sediments (Powell *et al.*, 2003) and lake plankton (Casamayor *et al.*, 2000; Dumestre *et al.*, 2001). It has been shown that PCR-DGGE can provide information about colony diversification and differences. This method has therefore been widely used (Li *et al.*, 2010; Zhou *et al.*, 2010).

This study aimed to monitor the microbial diversity and the degree of hygiene in biofilms, using PCR-DGGE, and to develop a useful and economic detecting technique which could be practically applied. In short, extracting total DNA from bacteria in biofilms is more rapid and accurate than traditional separation and identification

methods, and can more directly reflect the diversity of bacteria in biofilms in water supply pipes (Wu *et al.*, 2006).

This process is rendered even more complicated by the presence of debris, corrosion products and mineral deposits, and by the formation of corrosion tubercles, which provide new niches or surfaces for colonization (Batte *et al.*, 2003). Kapley *et al.* (2000) tried using a PCR technique to detect pathogenic bacteria in drinking water, since water-borne pathogens are difficult to detect using culture-dependent methods (Gilbride *et al.*, 2006). DGGE saves time and can contribute to the understanding of bacterial diversity by detecting uncultivable bacteria (Ralebitso *et al.*, 2003). PCR-DGGE was first used in microbial ecology in 1933 (Luo *et al.*, 2003). It can be used for bacterial identification purposes (Oliveira *et al.*, 2007).

MATERIAL AND METHODS

In order to obtain the diversification of the pipeline biofilms, a pilot drinking water distribution system was designed and some pre-experimental conditions were prearranged by changing system settings factitiously to simulate the actual pipe network dynamically. Three kinds of pipe materials, galvanized steel pipe, PE pipe and UPVC pipe, were chosen to compose the experimental pipe network. The sketch map of the pilot system is seen as Fig. 1.

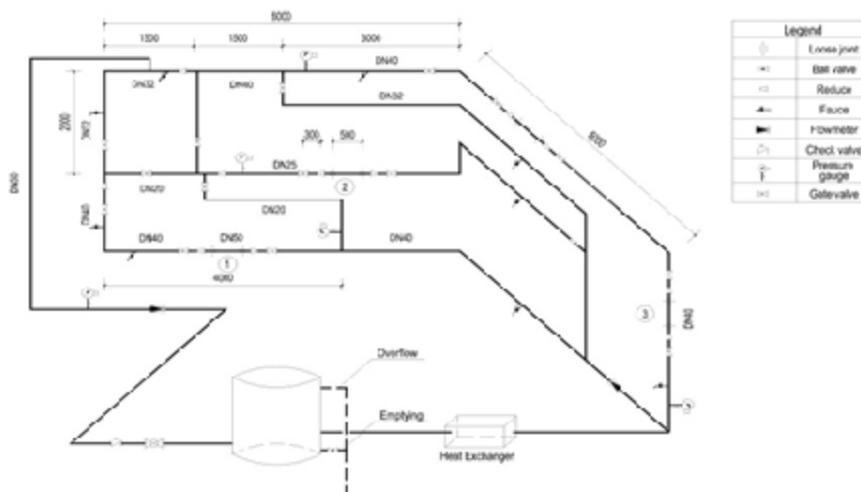


Fig. 1. Sketch map of the pilot network

The growth rate of the biofilm is slow, so samples were taken only on 20 November of the first year, 29 January and 24 April of the second year, in line with experimental requirements.

The influent factors of pipe biofilm and the influent degree of different water quality indexes will be well determined by monitoring the conventional water-quality indexes, such as temperature, turbidity, residual chlorine, total bacteria, etc.

Sample preparation

Sterile water about 150ml joined beakers that were disinfected by high temperature, and then sealed the beakers with silver paper. Three sampling points were setting in the pilot drinking water distribution system. When sampling, screwed the tracheas to take out the biofilm pipes with disinfected plier (two biofilm rings were taken out in every sampling point), and rinsed out the exterior floating bacterium with sterile water. The samples were put in the beakers and sealed them.

The pipe samples from the pilot distribution system were stored in sterile water and cut using sterile instruments, in preparation for testing. Samples were desiccated, attached to the platform and metal plated. The other parallel samples were concussed by ultrasonic, and the biofilm about 85% flaked to the water. The unfathomed bacteria liquid was the sample of the follow-up PCR-DGGE manipulation.

SEM

In order to investigate the growth of the bacteria, it is necessary to examine the biofilm without destroying it. Biofilm samples from the pilot pipeline, which had been running for 7 months, were scanned using a scanning electron microscope (SEM), which allowed non-invasive monitoring of the biofilms.

A SEM (X-650, Hitachi,JP) was used to scan the surface of pipe samples to produce the images. The energy radiated by the electron gun was 25keV. The magnification was 5000~30000.

PCR-DGGE

The study of microbial diversity using PCR-DGGE has achieved better results. The experimental conditions for water were therefore adjusted to account for the biofilm environment, and a PCR-DGGE experimental system was developed which was adapted to the bacterial diversity of biofilms.

For DGGE analysis of the PCR products, a 16S rDNA gene fragment was amplified using the following universal bacterial primer EUBf933, containing a 40 base pair –(bp) (Zeng *et al.*, 2006) GC clamp added to its 5' -end. GC-rich sequences can be incorporated into one of the primers to modify the melting behavior of the fragment of interest, such that close to 100% of all possible sequence variations can be detected (Paquin *et al.*, 1992). The discrimination rate for DNA sequences which exceed 500 bp is 50%, when using traditional DGGE electrophoresis, but this can be increased to 100% when using the “GC-clamp” (Wang *et al.*, 2004).

PCR-DGGE steps

The pipe rings were washed lightly to wipe off loose bacteria, and were then put into beakers containing 100 ml sterile water. These samples were shaken 5 times by ultrasound (the shaking time was 5 min at 5 min intervals). The shaking frequency was $< 2 \times 10^4$ Hz (as the cells would rupture and die at frequencies $> 2 \times 10^4$ Hz). The bacteria from the pipe walls became suspended in the water. Research has suggested that 85% of bacteria from the pipe walls are suspended.

The main PCR-DGGE steps were: collection of samples; extraction of the total DNA; PCR amplification; pretest study; preparation of polyacrylamide; DGGE analysis of samples.

PCR reaction system

A 16S rDNA fragment corresponding to nucleotide positions V6-V8 was amplified with primers GC-clamp-EUBf933 and EUBr1387 differential for universally conserved bacterial 16S rDNA sequences (Iwamoto *et al.*, 2000).

PCR amplification was performed in a DNA thermocycler. The temperature profile included an initial denaturation step at 94°C for 1 min followed by 35 cycles of a denaturation step at 95°C for 1 min, a primer annealing step at 55°C for 1 min, an extension step at 72°C for 2 min, and a final step at 72°C for 10min. Before DGGE analysis, the presence of PCR products was confirmed using a 1.5% agarose gel.

DGGE reaction conditions

PCR products were loaded onto a 6.5%(wt/vol) polyacrylamide gel in 1×TAE. The 6.5% polyacrylamide gel was made with denaturing gradients ranging from 40%–55% for 16S rDNA fragments. The electrophoresis was run at 60°C

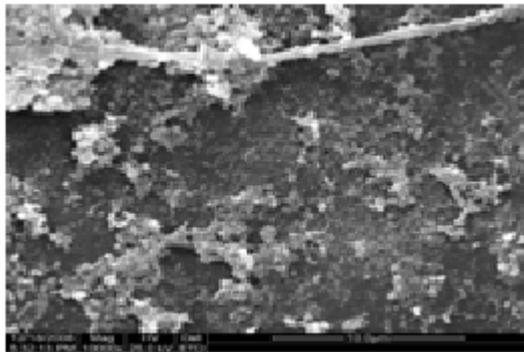
for 20min at 20V, and subsequently for 7 h at 150V. After electrophoresis, the gels were stained with silver stain.

RESULTS AND DISCUSSION

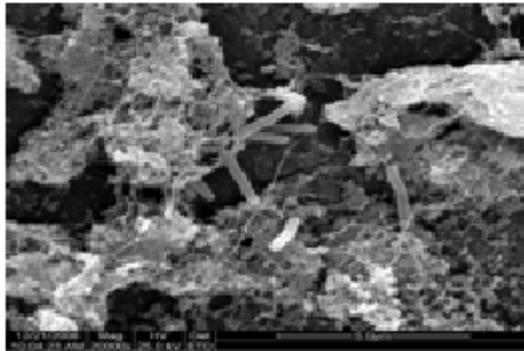
SEM

Firstly, the growth of the biofilms in the entrance and terminus of the pilot pipe were studied intuitively. Results from the three time points were similar, so the discussion and Figures 2 and 3 are based on the samples taken on 20 November. Figure 2 and Figure 3 show the entrance and terminus biofilms, respectively. Secondly, differences in the microbes at different depths of the biofilm were studied. Figure 4 shows the bacterial environment in the deep layers of the biofilm, in contrast to Fig. 2 and 3

Microbe grows in the pipe walls of most water distribution systems. The results of SEM showed that a zoogloea was formed by cells in the pipe walls. A hard, pitted surface and a multilayered structure were shown by SEM to be common

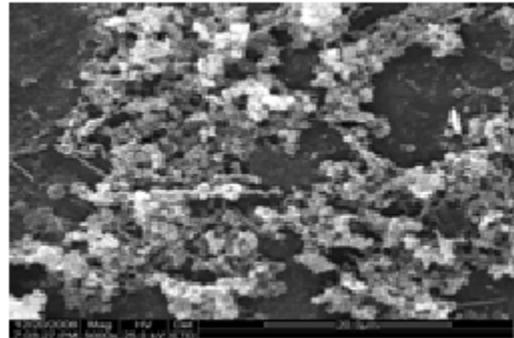


(A)

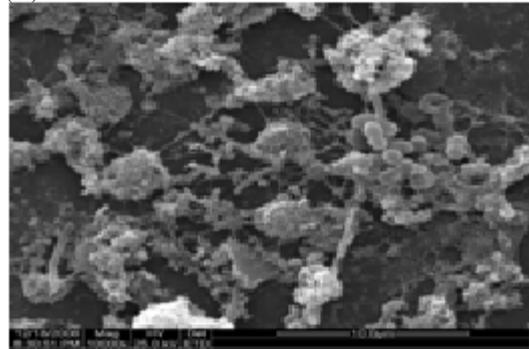


(B)

Fig. 2. Scanning results of the biofilm in entrance

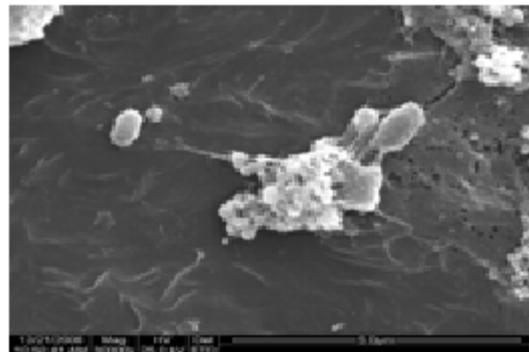


(A)

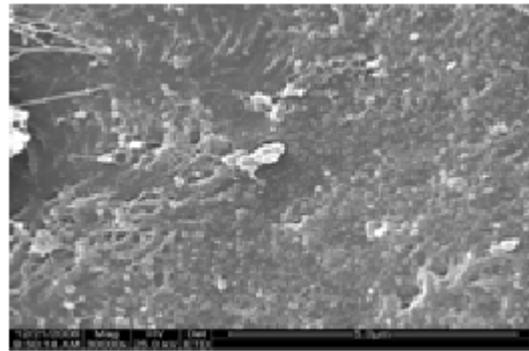


(B)

Fig. 3. Scanning results of the biofilm in terminal



(A)



(B)

Fig. 4. Scanning results of the pipe wall

characteristics of the biofilms. SEM also showed a greater microbial density in the biofilms from the entrance of the pipe, compared with the terminus. The dominant bacteria of the biofilms at the entrance were bacilli. As the flow velocity and hydraulics changed throughout the pipe, the bacilli died out gradually, whilst cocci and short bacilli increased, and short bacilli predominated at the terminus. The growth of bacteria provides favorable conditions for the development of opportunistic pathogens, which can be harmful to consumers' health. SEM samples collected in the model distribution system demonstrated that the inner wall of the pipe was not smooth, and the crevices allowed the growth of bacteria. The large quantity of bacterial growth encouraged the growth of pathogenic bacteria and water quality would deteriorate when the water flow velocity or pressure were changed suddenly.

The cellular configurations and the relationships between different components of the biofilms could be seen distinctly by SEM. Figure 4 illustrates this in the biofilm growing close to the pipe wall. Both bacilli and cocci were detected, with the bacilli growing solely in the zoogloea, but with the cocci growing collectively. There were smaller amounts and fewer species of bacteria in the deeper parts of the biofilm, and these results were also detected by SEM (as shown in Figure 4). The results indicated that most of the microbes congregated at the boundary between the pipe and the water. The nutrition for the biofilm comes from the water, and will therefore increase when the flow velocity increases. When the flow becomes stagnant at the terminus of the pilot pipe, the nutrition of the biofilm comes from this stagnant water, and the environment is therefore lacking in new nutrients. The biofilm then becomes mixed with the water, such that the microbial quantity and variety in the biofilm is similar to the water at the same point.

PCR-DGGE

After the PCR amplification, fragments of 16S rDNA V6-V8 sections of about 450 bp were obtained from all biofilm samples. Many microbial cells are generally counted on surfaces in contact with drinking water. DGGE was used to identify the dominant and unique species from the biofilms, as migration in DGGE does not depend on fragment size (Lee *et al.*, 2005).

Biofilm samples from different seasons

The water in the network comes from the municipal network, so its temperature will change throughout the seasons. The velocity of flow, turbidity, and pH will change with changes in water quantity requirements. The biofilm microbes were studied in different environments to find the main factors affecting its growth and composition, and to reduce the effects of the water on the results. Three sets each of 1-3 biofilm samples were collected from November of the first year to April of the second year at two different sites from the pilot system. Sequences present in the variable regions of the 16S rDNA gene are responsible for the different migration behavior of PCR products in the DGGE gel, and contain genetic information about the species to which each band belongs. The profiles, which contain both intense and faint bands were compared and analyzed.

The microbial diversity of the biofilms in the water distribution system was considerable, and more than 20 species of bacteria were detected. The DGGE profiles shown in Figure 5 accurately reflect the microbial growth. The potential diversity of the different biofilms can vary, so it is necessary to confirm the microbial diversity in different water distribution systems. The total bacterial numbers seen in the DGGE profiles from 29 January showed that the microbial diversity of the biofilms was greater than that of water. The temperature (d^o8°C) of the water was low and residual chlorine was present. The microbial diversity of the biofilm at the same time point was greater, suggesting that the bacteria in the biofilm were less affected by these factors, due to protection by extracellular matrix and polymers. The microbes in the biofilm could not be controlled by reducing the residual chlorine.

Figure 5 shows that samples A-G all contained band M2, indicating that the predominant bacteria were the same in the biofilm and water samples from the different areas. The brightness of band M2 in certain biofilms was greater than in water samples, proving that the nutritional environment of the biofilm provided better conditions for the multiplication of the bacteria. The profiles A and B in Figure 5 were biofilm samples from the entrance to the network. The residual chlorine and velocity of flow were similar, but the sampling temperatures were 13°C

and 8°C, respectively. The growth of microbes was slower, but the microbial diversity of profile B was greater than that of profile A. It was shown that although low temperature might slow the microbial growth and reduce the reproductive rate, it could not kill the microbes. Finally, by analogy with current concepts concerning the differentiation of biofilms as they age (Davies *et al.*, 1998; Allison *et al.*, 2000) the drinking water biofilm system is almost certainly continuously reorganized during its development.

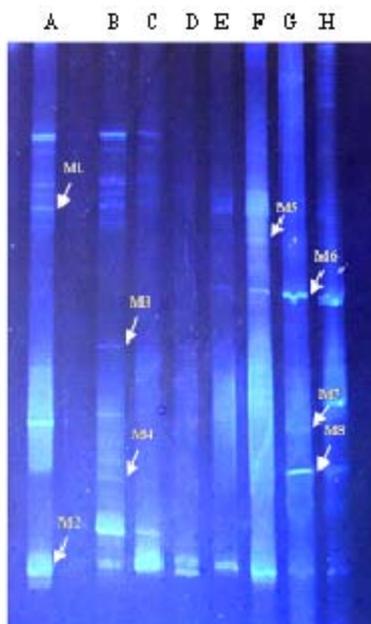


Fig. 5. Schematic representation of the DGGE gel obtained for samples submitted to different seasons and sampling sites (A, B, F-the biofilm samples of the entrance in 20 November of the first year, 29 January and 24 April of the second year; C, G-the biofilm samples of the terminal in 29 January and 24 April; D, E-the water samples of the entrance and terminal in 29 January ; H- marker.)

There were discrepancies between different biofilm samples from the same site. Each day had its special bands, for example, bands M5-M8 only existed on 24 April, whilst band M3 existed only on 29 January. This indicated that different waterpower conditions and temperatures were responsible for changes in biofilm quality at different seasons, resulting in different populations of bacteria. The temperature of the biofilm sampling site had great influence on the structure of bacteria.

The rate of microbial reproduction increased with increasing temperature. The sampling temperature on 24 April was the highest, but its microbial diversity was the lowest. This was because the water was stagnant for three months from 29 January 24 April, the residual chlorine had decreased to zero, and the temperature was higher, so the nutrients were being depleted as the microbes grew. The composition of the bacterial community changed as some bacteria became extinct, whilst others adapted metabolically and replaced the primary community.

The biofilm consisted of a mixture of microorganisms which differed in activity levels according to their positions in the aggregate. The residual chlorine at the network terminus was low, the water was stagnant, and the stagnation time was longer, so different bands occurred in the profiles. Comparison of the sampling patterns for biofilms from the two different sites indicated that the nutritional environment of the biofilm may change due to external factors. The shear stress to the biofilm will increase when the velocity of flow increases. The growth of the biofilm will be threatened by increasing velocity, but nutrients can be added to the biofilm, so the microbe species will rise because the biofilm may be firmed with the increasing of the velocity appropriately. Most of the nutrients for the biofilm come from the water, but the nutritional environment varies. Residual chlorine can slow the growth of and inactivate the bacteria suspended in the water, but the water can also bring some energy sources to the biofilm and thereby speed its growth. The terminus of the network is in a unique position. The biofilm comes into contact with stagnant water here, and the nutritional environment, microbial species and biomass of the biofilm will approach that of the water. As in profiles F and G, for example, the biofilm samples were in contact with the stagnant water, so the microbial diversity between biofilm and water was large.

It is known that many factors influence the growth of microbes in biofilms. Due to interactions between the factors, it is difficult to control the development of the biofilm by controlling just one factor. At the same time, the probability of changes in the composition of the microbial species is high, and changes in any factor could cause changes in the diversity. Changes in

environmental conditions (temperature, nutrients, toxins, ionic composition of the water, etc.) may lead to drastic shifts in bacterial population dominance.

By using DGGE analysis of microbes in biofilms at different aging periods, distinct 16S rDNA banding patterns were observed (as shown in Figure 5). Each sample had different band profiles at different aging periods, but two bands, M1 and M2, were present in all biofilm samples. For varieties A and B, there were basically no changes in band number and intensity, showing that bacterial communities remained stable. After six months aging, significant changes in the band profiles appeared. Some bands disappeared or became weaker, whilst some new bands appeared. For example, the intensities of M1 and M2 did not change obviously, but M5 and M6 appeared only in the sixth month of aging.

Comparative sequence analysis of excised DGGE bands revealed the identities of the community members. The developed PCR-DGGE strategy is a welcome tool for studying the diversity of biofilm bacteria.

Biofilm samples from different pipe materials

The water quality is directly related to the pipe material. The biofilm might develop at the pipe inner surface if the material offers suitable nutrition. In order to establish the effects of the material on the safety of drinking water, we carried out a study on the health safety aspects of galvanized steel, PE, and UPVC pipe materials. The nature of the pipe material plays a major role in the type and organization of the biomass. Through their roughness, wettability, adhesive properties etc., materials affect the adhesive efficiency of pioneers, and may act as a source of nutrients or growth factors. In this study, PE, UPVC, steel and copper materials were found to be covered with almost equal amounts of biomass, but there was much less bacterial activity on copper due to the toxicity of the released ions. Drinking water safety is becoming one of the greatest concerns in China, and more people are choosing safer water pipe materials.

The microbial diversities of the galvanized steel, PE, and UPVC pipes sampled on 29 January are shown in Figure 6.

The microbial diversity was similar in PE and UPVC pipes, with M1-M4 (as shown in

Figure 6) occurring in both, and the band M5 being near M9 in position. These profiles showed that the microbes were similar in the biofilms in PE and UPVC pipes.

Profile B is the result of monitoring the microbial diversity of the biofilm in galvanized steel pipes. These results showed a great difference from the other two materials in terms of microbial diversity. Most of the bands in B were unique to these samples, indicating low homology with the other samples. The structure of the biofilms from the galvanized steel pipes was loose and porous, with extensive extracellular matrix. This structure encouraged the growth of microbes.

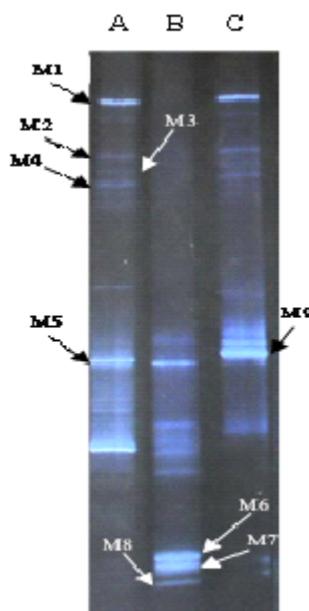


Fig. 6. Schematic representation of the DGGE gel obtained for different samples in different materials (A, B, C- galvanized steel, PE, and UPVC materials)

How iron oxy-hydroxides favour bacterial growth is not well known. It has been shown that they increase the growth of bacteria, probably through their use as nutrients.

The predominant bacterial species in the three biofilm samples were different, with those on galvanized iron pipe being most dissimilar from those on UPVC, and those on PE being intermediate between the two. The reason for this was differences between the pipe materials in terms of roughness and electrical properties of the surfaces, resulting in different nutritional environments. The

effects on the biofilms of the residual chlorine, velocity of flow and hydraulic pressure in water were different because of the diversity in the biofilm structure. All these factors contributed to differences in the microbial diversity and biomass of the biofilms.

The release of bacteria from biofilms to the water is independent of the mass and thickness of the biofilm. The pipe material does not affect water quality in real conditions, as flowing drinking water can be highly nutritive (Zacheus *et al.*, 2000) or contain disinfectants (Hallam *et al.*, 2001). It is only by dynamic testing that the effects of different materials on the bacteriological quality of drinking water can be assessed.

Prediction of the impact of the pipe material on the biofilm is difficult and needs to be tested in carefully controlled, dynamic conditions.

CONCLUSIONS

The intact biofilm on the pipe wall can be detected by SEM, allowing visualization of the growth of the biofilm and its constituent bacteria. These observations allow the detection of differences in biofilms, which can be related to the results of DGGE analyses.

The microbial biomass and species present in biofilms was found to be greater than that in water, by analyzing the DGGE profiles. The predominant bacterial species were the same, however, and the variant bacteria in the two samples were similar. Changes in microbial diversity of biofilms at different seasons were detected by changes in the bands in DGGE profiles. The change in water temperature due to the change of season was one reason for alterations in microbial diversity in the biofilms. Changes in the velocity of flow and residual chlorine can have both advantageous and disadvantageous effects on biofilms. The detection of wall biofilms on galvanized steel pipe, PE pipe and UPVC pipe showed that pipe materials could affect the predominant bacterial species, the total bacterial population and the bacterial diversity of the biofilms. The bacterial species and biomass in the galvanized steel pipe was the greatest. It has been ascertained that the regrowth of the biofilm can be reduced by the use of PE and UPVC material. Extracting total DNA from bacteria in biofilms is more rapid and accurate than traditional separation

and identification methods, and can more directly reflect the diversity and change of bacteria in biofilms. This work provides useful measures for the improvement and safety of drinking water quality.

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