Effect of sodium hypochlorite on typical biofilms formed in drinking water distribution systems

Huirong Lin, Xuan Zhu, Yuxin Wang and Xin Yu

ABSTRACT

Human health and biological safety problems resulting from urban drinking water pipe network biofilms pollution have attracted wide concern. Despite the inclusion of residual chlorine in drinking water distribution systems supplies, the bacterium is a recalcitrant human pathogen capable of forming biofilms on pipe walls and causing health risks. Typical drinking water bacterial biofilms and their response to different concentrations of chlorination was monitored. The results showed that the four bacteria all formed single biofilms susceptible to sodium hypochlorite. After 30 min disinfection, biomass and cultivability decreased with increasing concentration of disinfectant but then increased in high disinfectant doses. PMA-qPCR results indicated that it resulted in little cellular damage. Flow cytometry analysis showed that with increasing doses of disinfectant, the numbers of clusters increased and the sizes of clusters decreased. Under high disinfectant treatment, EPS was depleted by disinfectant and about 0.5–1 mg/L of residual chlorine seemed to be appropriate for drinking water treatment. This research provides an insight into the EPS protection to biofilms. Resistance of biofilms against high levels of chlorine has implications for the delivery of drinking water.

Key words | biofilms, disinfection, drinking water distribution, extracellular polymeric substances, redistribution, sodium hypochlorite

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INTRODUCTION

The presence of biofilms in drinking water systems has been a major concern to municipal water providers and consumers. Although there are usually residual concentrations of chlorine in drinking water distribution systems (DWDSs), biofilms can form even in oligotrophic conditions. Biofilms are more tolerant to antibiotics and disinfectants than their planktonic cells (Donlan & Costerton 2002; Davies 2003; Steed & Falkinham 2006; Bridier *et al.* 2011). As a result, these biofilms are a potential risk to human health as the planktonic cells in the biofilms can release to the drinking water in some conditions. Strategies aimed at preventing or interfering with the initial adhesion and subsequent biofilms formation are a considerable achievement to the control of drinking water quality as most of the bacteria in DWDSs are located at the pipe wall (Flemming *et al.* 2002).

Biofilms control in DWDSs is usually performed using a strong oxidizing agent such as chlorine, ozone or chlorine dioxide. Among these, chlorine disinfection is an effective method to prevent biofilm formation in DWDSs and assure safe drinking water. However, as the ecology of biofilms is a complex combination of physicochemical and biological parameters, the mechanisms responsible for the survival of bacteria in DWDSs are still poorly understood. Microorganisms within the biofilms can respond to these local environmental conditions in various ways. For

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example, biofilm species composition can affect the efficacy of disinfection (Behnke *et al.* 2011). Multispecies of biofilms increased their resistance to disinfection due to suitable niches of bacteria in a particular microenvironment (Burmolle *et al.* 2006; Simoes *et al.* 2010). In addition, it was reported that exposure of pure cultures of bacteria to chlorhexidine resulted in its bioaccumulation, which had an impact on the biofilms and cells, including viability, structure and biochemistry (Behnke & Camper 2012). Thus, knowledge of the efficacy of disinfectant to the biofilms is of great importance.

Biofilms which attach to the pipe surface are communities of microorganisms undergoing profound changes during their transition from planktonic (free-swimming) organisms to cells that are part of a complex, surface-attached community. Extracellular polymeric substances (EPS), known as the major constituent of biofilms, has many functions including storing nutrients for biofilms growth, promoting structural development and providing a protective barrier (Harrison *et al.* 2006, 2007; Chang *et al.* 2007). Bacteria in biofilms are considered to be a protective layer and are highly heterogeneous in their composition (Donlan & Costerton 2002; Davies 2003). It has been found that increased resistance may be the result of limited diffusion of disinfectants into the biofilms due to the EPS matrix that protected the deeper layers of cells (Debeer *et al.* 1994; Jang *et al.* 2006). The composition and viscosity of EPS changed when the biofilm was exposed to disinfectant. The disinfection efficacy of commonly used disinfectants on detached biofilm particles has not been fully investigated. Thus, in order to control the biofilm growth, it is important to study the role of EPS in the resistance of biofilms to disinfectant.

In many developing countries, high disinfectant doses were used in DWDSs due to relatively low water qualities. Are these high disinfectant doses sufficient to kill planktonic free floating cells and the cells within detached biofilm clusters? In DWDSs, EPS may affect the characteristics of biofilms and influence the efficiency of disinfection. For example, it was found that EPS could increase the resistance of biofilms and detached biofilms to chlorine (Xue *et al.* 2012). Therefore, knowledge of the efficacy of disinfectants and the role of EPS on resistance of biofilm will provide valuable information regarding minimal disinfectant residuals to control biofilms in DWDSs, thus maintaining drinking water quality. The aim of the present study was to assess the efficacy of different concentrations of sodium hypochlorite to study the resistance of biofilms to disinfection for 30 min. Four representative drinking water biofilm bacteria were chosen and exposed to sodium hypochlorite for a short time to monitor the susceptibility of biofilms. The role of extracellular polymeric substances and the redistribution of cell clusters from biofilms were also investigated and discussed.

MATERIAL AND METHODS

Biofilm formation

The four representative drinking water biofilm-isolated bacteria used throughout this work were isolated from some drinking water and related environments in southeast China and identified previously in our lab. These bacteria were: *Klebsiella* H1 from Jiulong River, *Pseudomonas* C5 from Xinglin River, *Flavobacterium* GS3 from biofilms attached to the granular activated carbon, and *Sphingomonas* Z22 from tap water in our laboratory respectively. Bacteria were grown overnight in batch cultures using 50 mL of R2A broth (25 °C, 150 rpm) and OD600 was detected. For each cellular density of about 1×10^7 cells/mL was obtained in the biofilms. The biofilm formation was carried out with the wells of sterile 96-well polyvinylchloride microtiter plates. Two hundred μL of each bacterium suspension were transferred to the wells. The plates were incubated at 25 °C for 48 h.

Disinfectant (sodium hypochlorite) treatments

The content of each well was removed and the wells were washed with 200 μL of sterile distilled water twice to remove reversibly adherent bacteria. All experiments were performed in triplicate with at least three repeats. The remaining attached bacteria on the inner walls of the wells were exposed to disinfectant solutions (free chlorine) at various concentrations (0, 0.125, 0.25, 0.5, 1, 2, 5 and 10 mg/L). The sodium hypochlorite solutions remained in contact with the biofilms for 30 min. After treatment, the disinfectant

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solutions were removed. Sodium thiosulfate solution at 0.5% (wt/vol) in sterile distilled water was used to quench the activity of the disinfectant as described previously (Simoes *et al.* 2010). The biofilms were then analyzed in terms of biomass and cultivability.

The biofilms were stained with crystal violet (CV), the dye was dissolved with ethanol. The optical density (OD) of each well was then measured at 570 nm using a microtiter plate reader (Molecular Devices, Sunnyvale, CA, USA), and biofilm biomass was presented as OD570. Cultivability was assessed in R2A in terms of cultivability using R2A plates. The numbers of colony-forming units (CFU) of attached bacteria were enumerated using gradient dilution and spread plate methods. The sodium hypochlorite effectiveness was assessed based on the cultivability and biomass. The effect of chlorination of surface charge of detached clusters was analyzed by measuring zeta potential.

Extraction and quantitative analysis of EPS concentrations

The EPS was extracted using 1 mL 0.9% NaCl containing 10 mM EDTA solution which can prevent cell lysis during harvesting. Extracellular DNA contents were determined using Qubit® fluorometer (Invitrogen) according to the instruction manual. Polysaccharides were determined by the anthrone–sulfuric acid method using glucose as the standard (Guo *et al.* 2011). Proteins were measured by the Pierce BCA Protein Assay Kits (23227; Thermo, USA).

Size and number of clusters analysis using flow cytometry

Flow cytometry (FCM) analysis was conducted after sodium hypochlorite treatment to investigate the viability of detached biofilm clusters. Two hundred μL of sterile distilled water was added into the wells to remove the adhered cells using ultrasonic waves for 3 min and filtered by 400 micron film followed by FCM (Quanta SC) analysis. Five μm-fluorescent microsphere was used as the size marker. Size distribution of the clusters and total numbers of bacteria aggregates (both active and inactive) in the samples were enumerated.

Cellular damage detection using PMA treatment and qPCR

Propidium monoazide (PMA) is highly selective in penetrating only into 'dead' bacterial cells with compromised membrane integrity but not into live cells with intact cell membranes/cell walls. So in this study, PMA-qPCR was used to determine the cellular damage of the biofilms (Nocker *et al.* 2006). After disinfectant treatment and ultrasonic, genomic DNA of each sample was extracted using a bacterial DNA extraction kit (Bioteke Corporation, Beijing, China) following the manufacturer's instructions. PMA (Biotium, Inc., Hayward, California, dissolved in 20% DMSO) was added to 50 μL of the 1 ng/μL DNA solution to reach final concentrations of 3 and 30 μM followed by light exposure for 15 min according to the instructions.

For relative quantification of DNA extracted from the biofilms, quantitative real-time PCR (qPCR) was performed in a total volume of 25 μL containing 1 μL extracted genomic DNA using SYBR® *Premix Ex Taq*™ (Takara). The primers used in this study were Eubac 534R (ATTACCGCGGCTGCTGG) and Eubac 341F (CCTACGGGAGGCAGCAG). The cycling parameters were: 30 s at 95 °C followed by 40 cycles of 5 s at 95 °C, 20 s at 52 °C and 32 s at 72 °C, then 15 s at 95 °C, 1 min at 60 °C and 15 s at 95 °C, 15 s at 60 °C. Cycle threshold (Ct) values were calculated as reported previously (Nocker *et al.* 2006).

Statistical analysis

SPSS 13.0 was used to analyze the data. The data were presented as mean ± standard deviation. Significance of differences was analyzed by one-way analysis of variance (ANOVA) test. $P < 0.05$ was considered to be statistically significant.

RESULTS

Biofilm biomass and cultivability

In order to assess the resistance of biofilms to disinfection, standard 96-well microtiter plates with CV staining were used to characterize biofilms. The biomass was defined as OD570. As shown in Figure 1, the OD570 values varied with different concentrations of residual chlorine. The

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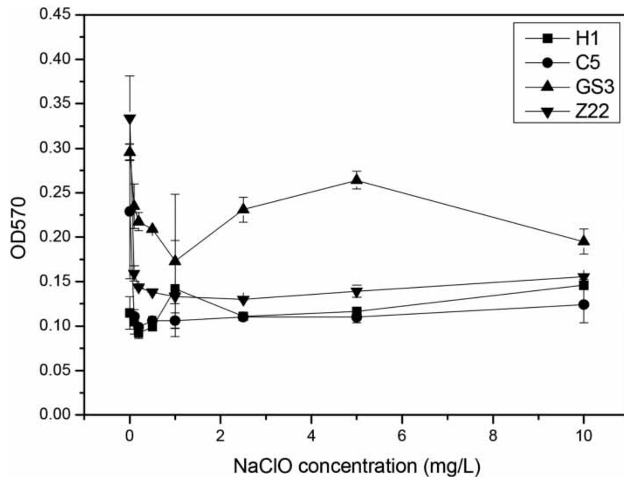

Figure 1 | Biofilm biomass after exposure to different SHC concentrations. Error bars represent standard deviations from three independent replicates.

biomass first decreased with increasing concentrations of disinfectant solutions. However, the biomass increased then for H1, GS3 and Z22 with increasing disinfectant concentrations.

The efficacy of disinfectant against biofilms of the four bacteria was determined. Total cultivability was defined as the CFU present on the R2A plates. After 30 min disinfection, all the biofilms could survive. The cell amount dropped sharply with increasing disinfectant treatment and then increased, suggesting that the cultivability decreased with increasing disinfectant concentration first, then displayed a higher number of cultivable cells (Figure 2).

Zeta potential

The zeta potential, or potential at the solid–liquid interface, is a fundamental parameter in models of electrical double layers and their associated properties (Kirby & Hasselbrink 2004a, 2004b). Zeta potential was monitored to evaluate the influence of chlorination on the surface charge of detached clusters. For the detached clusters from the biofilms after different concentrations of disinfectant treatment, there were no significant differences after the NaClO treatments (Figure 3). All the zeta potential values in different treatments were low, suggesting the high reattachment rate for the detached clusters.

Size and number of clusters after exposure to different concentrations of disinfectant solutions

EPS analysis results in this study showed that the EPS contents were below the detection threshold, as changes of EPS contents may lead to different size clusters. FCM, coupled with advancements in fluorescent dye technology, is a valuable tool for the detection of bacteria in aquatic environments. In order to indirectly understand the role of EPS, FCM was used to decide the sizes of clusters. The influence of chlorine disinfection on the redistribution of detached clusters was evaluated by monitoring the size and number of clusters by FCM. Total numbers of bacteria (both active and inactive) decreased sharply with increasing

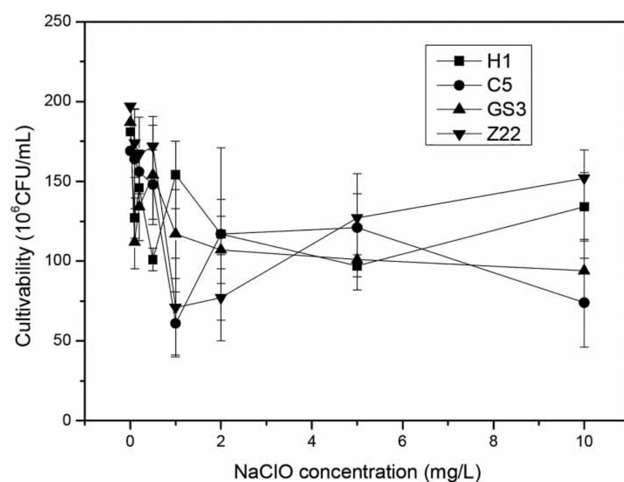

Figure 2 | Biofilms cultivability after exposure to different SHC concentrations. Error bars represent standard deviations from three independent replicates.

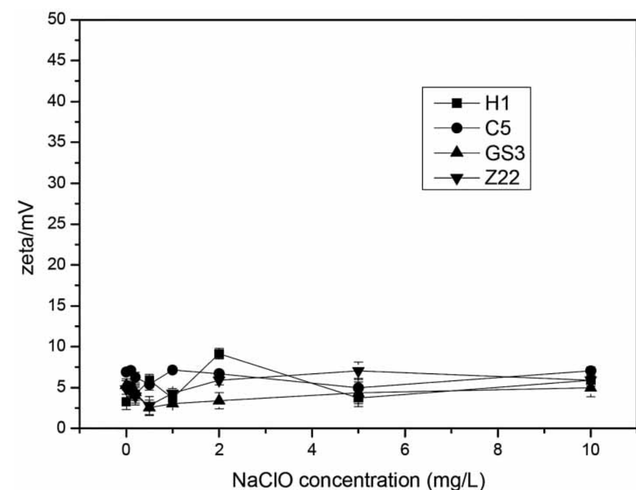

Figure 3 | Zeta potential after exposure to different SHC concentrations. Error bars represent standard deviations from three independent replicates.

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concentrations of disinfectant first, but then increased (Figure 4). Side scatter (SS) represented the degree of damaged cells. Electronic volume (EV) represented the size of clusters. As shown in Figure 5, cluster distribution varied with different concentrations of disinfectant. Even in the presence of a high concentration of chlorine, the detached clusters from all tested strains were still able to survive and form new biofilms with relatively high viability.

Cellular damage detection using PMA-qPCR

Plate count techniques are considered to be inefficient to detect the disinfectant-injured bacteria and can overestimate disinfection (Simoes *et al.* 2005). So PMA-qPCR was conducted in this study. PMA was added to a solution of genomic DNA extracted from the biofilms of different treatments. Figure 6 shows the effect of increasing disinfectant concentrations on inhibition of PCR amplification by PMA. Lower Ct values suggested higher quantities of DNA. There were no significant differences of Ct values between the samples with and without PMA treatment ($P > 0.05$ as analyzed by Paired-Samples T Test), indicating that few cells were injured in the presence of disinfectant.

DISCUSSION

The effect of different concentrations of disinfectant on biofilms

Chlorine disinfection is an important step in the biofilm control process. Disinfectants are chemical agents used to

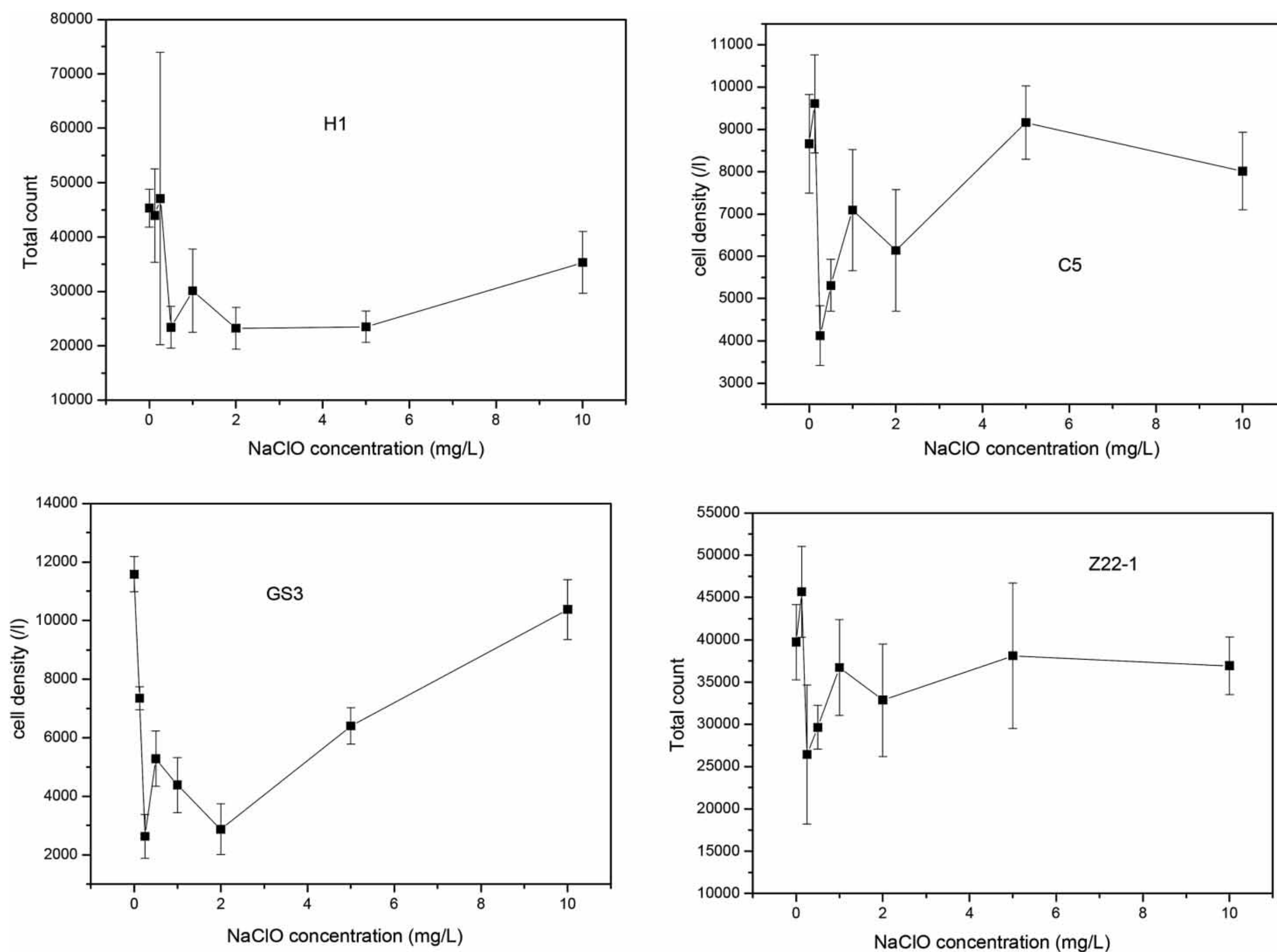

Figure 4 | Quantification of bacteria aggregates after exposure to different SHC concentrations by FCM. Error bars represent standard deviations from three independent replicates.

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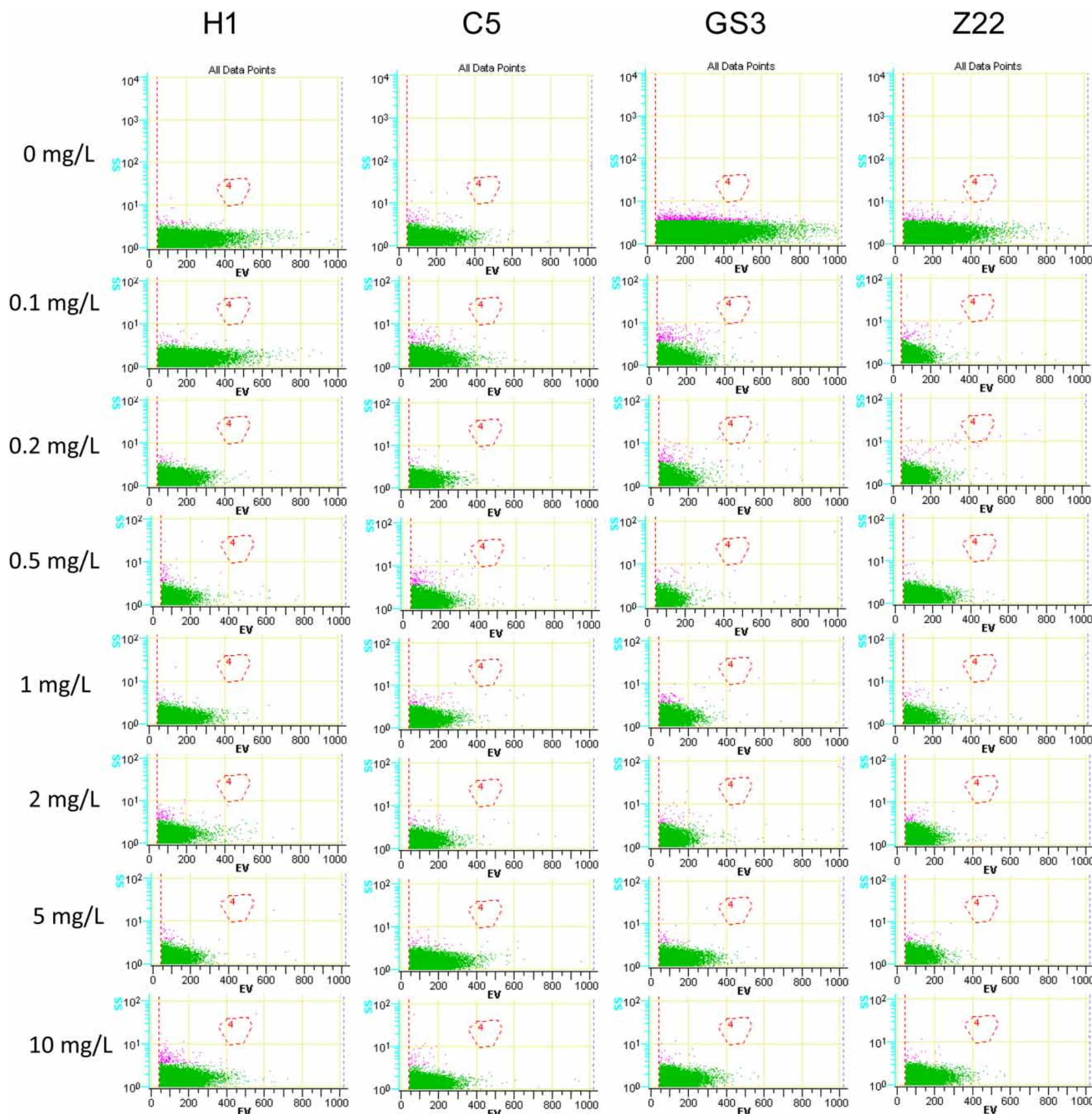

Figure 5 | Redistribution of clusters after disinfection analyzed by FCM. FCM data in dot plot: SS represented granularity the degree of damage cells. EV represented the size of clusters. The region marked 4 was the aggregation of 5 μm-fluorescent microsphere.

inactivate pathogenic microorganisms. In order to lower the potential to form harmful disinfection byproducts, residual concentrations must be kept below guidelines in DWDSs. This study considered the efficacy disinfectants to control biofilms in DWDSs. According to previous reports, *Acinetobacter, Aeromonas, Alcaligenes, Arthrobacter, Bacillus, Burkholderia, Citrobacter, Enterobacter, Flavobacterium, Klebsiella, Methylobacterium, Moraxella, Pseudomonas, Serratia, Saphylococcus, Mycobacterium, Sphingomona* and *Xanthomonas* have been found to be the predominant bacterial genera in DWDSs (Berry *et al.* 2006; Simoes *et al.* 2010). Four typical DWDS bacteria (*Klebsiella* H1, *Pseudomonas* C5, *Flavobacterium* GS3 and *Sphingomonas* Z22) were chosen for research in this study. The disinfectant concentrations used in this study were: 0, 0.125, 0.25, 0.5, 1, 2, 5 and 10 mg/L. Most of the disinfectant concentrations (less than 5 mg/L) used were those usually present in DWDSs. Our results showed that the four bacteria all

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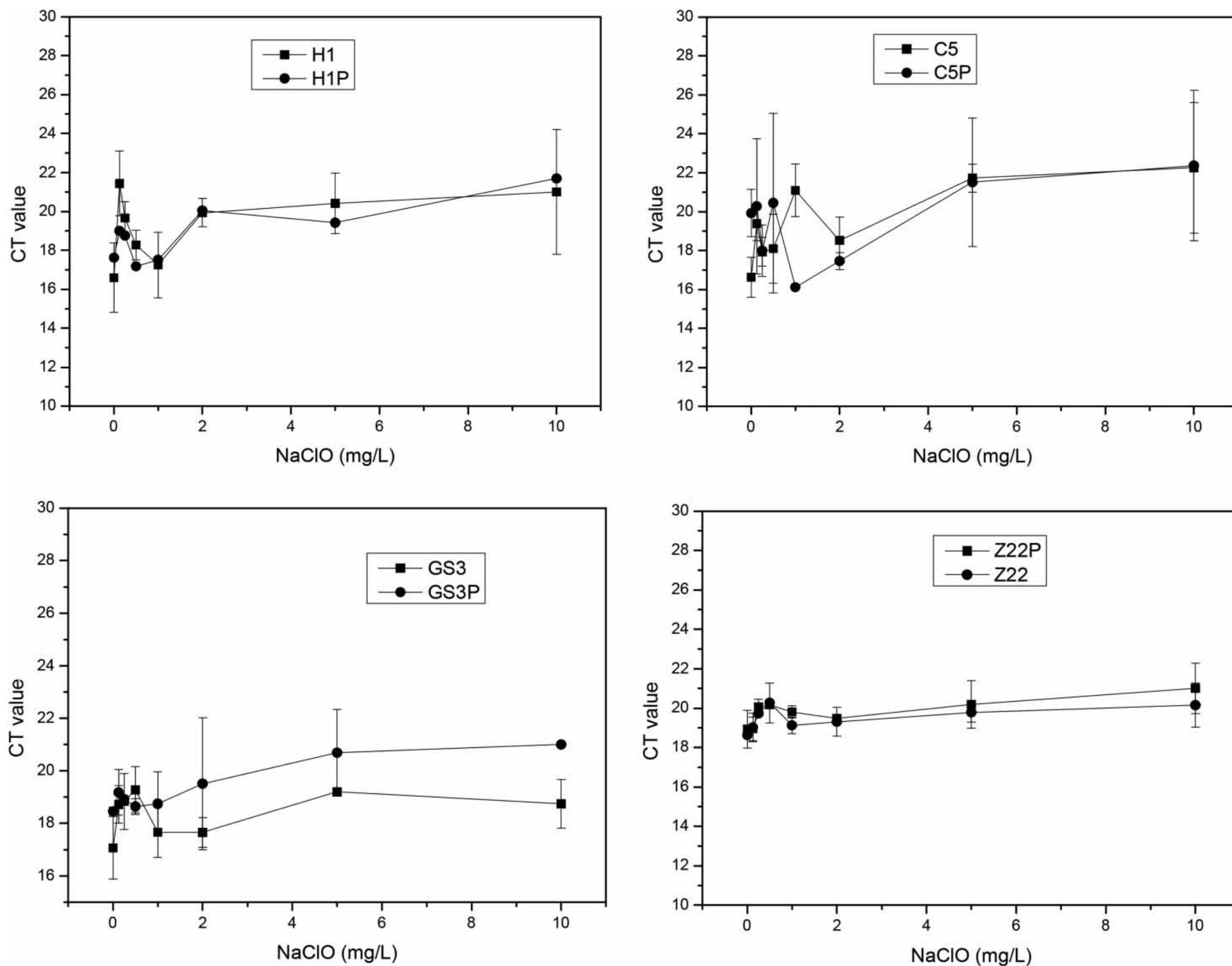

Figure 6 | Effect of increasing SHC concentrations on inhibition of PCR amplification by PMA. Error bars represent standard deviations from three independent replicates.

formed single biofilms susceptible to disinfectant. The differences between biomass (OD570 results) and cultivability (CFU results) suggested that there were viable but nonculturable cells in response to chlorine stress. Increasing concentrations of disinfectant resulted in lower biomass and cultivability first. However, biomass and cultivability increased afterwards. These result indicated that high doses of disinfectant did not certainly lead to high efficacy. It seemed that 0.5–1 mg/L sodium hypochlorite was the appropriate dose.

Our results showed that even in very high doses of residual chlorine (5 and 10 mg/L), the tested strains persisted. High doses of chlorine could not kill all the bacteria in the biofilms after 30 min disinfection. In a previous study, it was also reported that even with 4.3 mg/L of free chlorine residual coliforms may persist (Lechevallier *et al.* 1984). Our results were consistent with these results. According to the World Health Organization, 2–3 mg/L of chlorine should be added (Simoes *et al.* 2005). The maximum amount of chlorine one can use is 5 mg/L. However, bacteria could persist even in 10 mg/L of disinfectant in this study. This was consistent with a previous report which found that some Gram-positive, spore-forming bacteria were able to survive in 10 mg/L of free chlorine (Ridgway & Olson 1982). However, in another study, it was reported that almost all of the bacteria were killed under 10 mg/L of disinfectant after three 20 min sessions of disinfection (Simoes *et al.* 2010). So it seemed that the

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disinfection efficacy was dependent on not only the concentration of disinfectant used but also the time of exposure and the species of bacteria.

Even if of the same bacterial species, the biofilm cells covered by EPS are physiologically distinct from planktonic cells that are suspended in the water (Flemming & Wingender 2010; Wang et al. 2013). For planktonic cells suspended in the water, a low dose of disinfectant could lead to a sharp decrease of bacterial cells and was enough to kill the bacteria. However, for biofilms in the pipe walls, few cells were injured under various doses of disinfectant. Therefore, our results also indicated that the strategies used in drinking water treatment are not suitable for biofilms control in the pipe wall.

The role of EPS and redistribution of cell clusters from biofilms

When bacteria become attached to surfaces in suitable aqueous environments, they proliferate and form microcolonies that eventually develop into biofilms. Most of the total bacterial biomass in water distribution systems is found as biofilm. These bacterial communities produce EPS as a protective layer, which was considered to be a complex mixture of hydrated polymers that serve various purposes including protection from toxins (Harrison et al. 2006; Danhorn & Fuqua 2007). Previous studies have proven the important role of EPS to adverse conditions. For example, it was found that EPS in biofilms could enhance the resistance to heavy metals by providing more binding sites as well as serving as a protective layer against metals when metals enter the bacterial cells (Teitzel et al. 2006; Chang et al. 2007; Ueshima et al. 2008), therefore the role of EPS was considered in this study.

High doses of disinfection resulted in higher CFU in this study. Low zeta potential values detected in all of the samples indicated that a portion of the strains were still able to reattach despite the presence of disinfectant. It was reported that chlorine dioxide at 25 mg/L did not penetrate beyond a depth of 100 μm into a complex dairy biofilm that was 150–200 μm thick (Jang et al. 2006). Therefore, the EPS matrix might be the barrier for chlorine to kill the cells. In fact, the planktonic–biofilm transition is a complex and highly regulated process. Attachment is considered to be required for biofilm formation, and bacteria interact with the surface through adhesions including polysaccharides and surface proteins which were the main components of EPS, with initial contact often mediated by active motility. We detected the EPS contents after disinfection. However, they were below the detection threshold. FCM results suggest that the total numbers of clusters decreased sharply with increasing concentrations of disinfectant first, but then increased (Figure 4). Cluster distribution results indicated that in high concentrations of disinfectant, the sizes of clusters decreased and the numbers of clusters increased. These results may be due to the contribution of EPS. It was reported that chlorine disinfection of detached clusters may be dependent on cell cluster size (Behnke et al. 2011). In our study, the reaction of EPS and disinfectant may lead to the depletion of EPS matrix. As a result, attached biofilms were divided into smaller clusters under a high dose of disinfectant which then led to higher CFU results.

In addition, chlorine used in many potable waters for disinfection may cause sublethal injury of some bacteria, thereby rendering them nonculturable. For example, it was reported that more than 90% of indicator bacteria present in water systems may become injured in less than 1 week (Mcfeters et al. 1982). It is considered that their potential target sites in bacteria are the cell wall or outer membrane, the cytoplasmic membrane, functional and structural proteins, DNA, RNA and their cytosolic components (Bridier et al. 2011). However, PMA-qPCR results suggested that there was slight cell damage after a high dose of chlorine disinfection in this study. This result may also be due to the contribution of EPS. When the biofilms were subjected to disinfection, EPS can show very rapid reactions with chlorine. The EPS matrix may change with respect to diffusional properties or viscosity due to the different treatment of disinfectant. Therefore, when the biofilms were exposed to disinfectant, EPS and disinfectant reacted first in 30 min disinfection. As a result, the cells in the biofilms could survive. That was why there were still many viable cells in the high dose of disinfectant.

EPS was depleted by disinfectant so that the detached clusters from all tested strains were still able to survive and immobilize themselves downstream, forming viable new biofilms. In addition, the injured cell population during disinfection should be considered since they may recover and recolonize the surfaces (Lindsay et al. 2002).

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That was why the biomass and cultivability increased under the high dose of disinfectant in this study. Our results showed that an appropriate dose of disinfectant should be used in the drinking water treatment.

CONCLUSION

Typical drinking water bacterial biofilms (*Klebsiella*, *Pseudomonas*, *Flavobacterium* and *Sphingomonas*) and their response to different concentrations of disinfectant (0–10 mg/l) was monitored to assess the efficiency of sodium hypochlorite. The four bacteria all formed single biofilms susceptible to disinfectant. Our results showed that there was little cellular damage after 30 min disinfection. Under high disinfectant treatment, EPS was depleted by disinfectant. Attached biofilms were divided into small clusters and formed new biofilms, leading to higher cultivability. The EPS protection to biofilms provides biofilms persistent resistance against high levels of disinfectant. It is important that appropriate disinfection dosages are used to entrap the bacteria in the biofilm and control the spread of the pathogenic microbes in drinking water.

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