Monitoring the Role of Biofilm Biopolymers against Disinfectants in Water Distribution Systems

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1. PROBLEM AND RESEARCH OBJECTIVES

Biofilm formations in water distribution systems are ubiquitous. Reports from many water utilities in the US including utilities in Ohio have shown that biofilms survive in water distribution systems despite the continuing presence of disinfectants (Tuovinen and Hsu, 1982; LeChevallier et al. 1996) is great concern about the resistance of biofilms against disinfectants, the inactivation kinetics of biofilms are not well understood, especially compared to the inactivation kinetics of suspended microbial cultures (AWWA, 2007). There is not enough information for water utilities to assess and optimize disinfectant dosage to control biofilms in water distribution systems (AWWA, 2005).

One reason for this could be the complexity of biofilm EPS (Momba et al. 2000; Stewart, 2002; Stewart et al 2002). More than 80% of biofilm is comprised of EPS (Characklis and Marshall, 1990), and it is believed that these structures provide protective barriers for microorganisms (Sibille 1998; Hughes et al. 1998). However, there is still a significant knowledge gap, especially concerning the reaction kinetics of EPS with disinfectants. To date, the role of extracelluar polymeric substances (EPS) as a protective barrier against disinfectants has not been quantitatively analyzed, even though the simultaneous interaction between disinfectants and EPS is known to lead to the transport limitation of disinfectants into biofilms. Previous studies mostly focused on retarded or limited transport of disinfectants without considering reactive sites and reaction kinetics of biofilm EPS (Stewart, 2002; Stewart et al. 2002).

The principal research objective in this proposal is to monitor the role of both cell-bond EPS and biofilm EPS as protective barriers against disinfectants in water distribution systems. Physical transports of a model disinfectant and its reaction kinetics in biofilm were quantitatively studied using molecular probes and a chlorine sensitive microsensor. The reaction and disinfection kinetics of EPS was elucidated by 1) characterizing EPS components and their reaction kinetics with a model disinfectant; 2) quantifying EPS and viability of biofilm with fluorescently labeled molecular probes under a model disinfectant; 3) monitoring the transport limitation of a model disinfectant in biofilm. This study provides fundamental and effective biofilm control strategies in water distribution systems supporting research, education and local water utilities.

2. STATEMENT OF RESULTS OR BENEFITS

Water utilities in Ohio have experienced bacteria growth and biofilm formation in water distribution systems (Tuovinen and Hsu, 1982; Craun and Calderon, 2001), even under the presence of residual chlorine. However, the inactivation of biofilm in

drinking water distribution system is not well understood. One reason for our lack of understanding is strongly correlated to the complexity of biofilm EPS and their role in protecting biofilms from disinfectants. In this proposed study, this knowledge gap will be addressed. The results from the proposed study will enable local water utilities to incorporate biofilm control strategies since the results will aid in the development of effective biofilm control methods with disinfectants

3. MATERIALS/METHODOLOGY

Preparation of Buffer Solutions and Disinfectants

All disinfection experiments were conducted with chlorine demand-free (CDF) buffer (pH=7). CDF buffer was prepared by dissolving 0.54 g of Na_2HPO_4 and 0.88 g of KH_2PO_4 per liter in deionized water. The prepared buffer solution was pre-reacted with chlorine by adding sodium hypochlorite solution and allowed to stand at room temperature for one week, followed by UV light exposure for 48 hours to achieve dechlorination. When the chlorine concentration was lower than 0.01 mg/l, the buffer solution was considered to be chlorine demand free (Engelbrecht et al., 1980).

Chlorine stock solutions were prepared with Clorox bleach (The Clorox Co., Oakland, CA) and concentration were determined by the N, N-diethyl-*p*-phenylenediamine (PDP) method (Engelbrecht et al., 1980). The chlorine stock solution was diluted to 0.5 mg/l with CDF buffer solution immediately preceding the inactivation experiments. Stock chloramine solution was prepared immediately before each experiment by combining solutions of sodium hypochlorite and ammonium chloride in a 4:1 ratio (chlorine-to-ammonia-nitrogen mass ratio). To obtain the highest monochloramine yield and minimize ammonia volatilization, both solutions were pre-adjusted to a pH of 8.3. Stock chloramine solutions were diluted to a target concentration of 2 mg/l with the CDF buffer. Stock chlorine dioxide solutions were prepared from sodium chlorite ($NaClO_2$, Selective Micro Technologies, Beverly, MA, USA) (Jang et al., 2006). For inactivation tests, the ClO_2 stock solution was diluted to 0.5 mg/l before each test (Aieta et al., 1986). The concentration of all three disinfectants was selected based on residual disinfectant concentration in water distribution systems (USEPA, 1999) and measured using a DR/2700 spectrophotometer (HACH Company, Loveland, CO, USA).

Batch Experiments of planktonic cells

In this study, three *P. aeruginosa* strains were employed. The first was wild-type strain PAO1 and a well-characterized DNA sequenced strain. Two isogenic mutants of strain PAO1 were also used, (i) *algT(U)* encoding the alternative extracytoplasmic sigma factor AlgT(U) and (ii), *mucA*, encoding a cytoplasmic membrane-bound anti-sigma factor that produces copious quantities of the exopolysaccharide alginate. With these strains in hand, we examined how differences in the relative amount of EPS affected the efficacy of three common disinfectants (chlorine, chloramines, and

chlorine dioxide).

All batch experiments were performed in 250 ml amber glass bottles (Fisher Scientific, Itasca, IL) at room temperature for planktonic cells. Three amber-glass bottles were used as parallel reactors. The first bottle, containing bacterial suspension and the CDF buffer solution without disinfectant, served as a control reactor. The other two bottles contained only bacterial suspension and disinfectant solution. Experiment setup is shown in Fig 1. Microbial inactivation tests with disinfectants were performed and disinfectant decay and bacteria survival were measured simultaneously. Enumerations of viable microbial cells were performed using the heterotrophic plate count method. Serial dilutions were conducted in CDF buffer solution containing $Na_2S_2O_3$ (1 mmol/l final concentration) to quench residual disinfectants, followed by spreading 0.1 ml aliquots onto R2A media plates (Difco Laboratories, Detroit, MI). All plates were incubated at 37°C for 24 hours prior to enumeration of colony forming units (CFU).

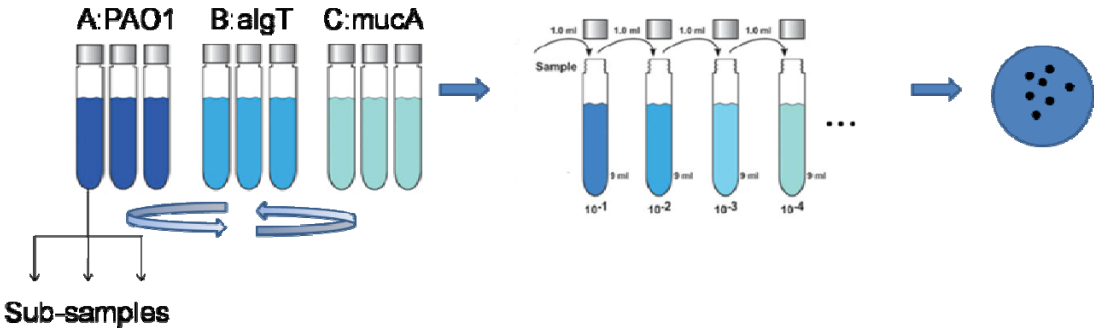

Figure 1: Batch experiment setup

Biofilm experiment

Two carboys were used as medium feeding and chlorine supply reservoirs respectively. A 0.02 strength LB broth was used as a medium to create nutrient limiting growth conditions mimicking low-carbon environment as in drinking water distribution systems. All feeds to reactors were delivered using a multichannel peristaltic pump (ISMATEC, Glattbrugg, Switzerland) and silicone tubing (Masterflex, Vernon Hills, IL). The flow cell system is shown in Fig. 2. Flow cells, tubing and solutions were sterilized at the start of each experiment. Operation and sampling of the flow cells followed aseptic technique throughout the experiments.

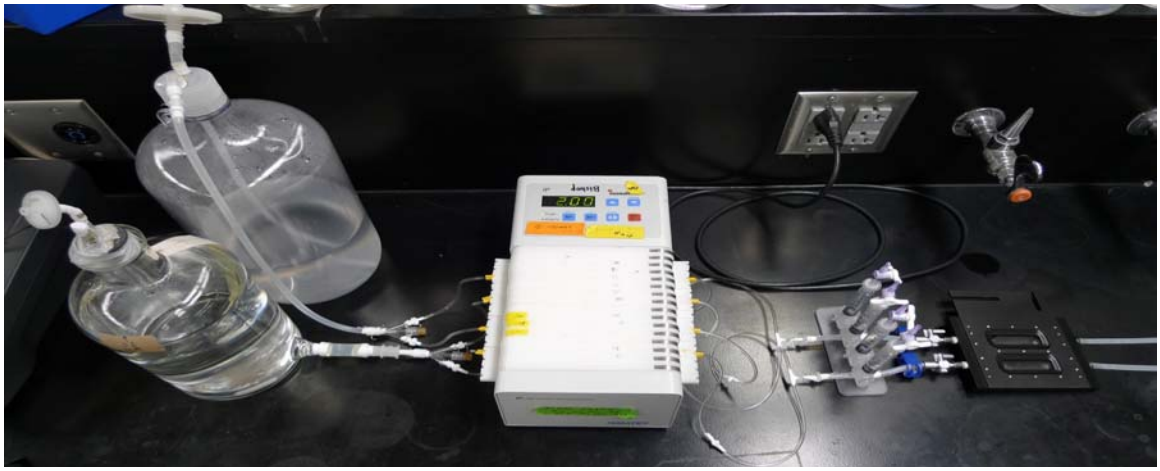

Figure 2: flow cell system setup

Biofilms were grown in continuous-culture flow cells (channel dimensions, 1.6 by 12.7 by 47.5 mm; flow rate, 0.2 ml/min) at room temperature. The flow cell contained a standard glass microscope slide on one side and a glass cover slip on the other side. Flow rate of the flow cells simulated laminar flow with an average flow velocity of 0.16 mm/s throughout each experiment. Under this flow condition, a residence time that improved biofilm formation was achieved. Channels were inoculated with bacterial suspension and incubated statically for 1 h at room temperature for initial bacterial attachment. After 1 hour, flow rate was gradually increased to 0.2 ml/min. For each experiment, the two channels in one flow cell were operated in parallel under identical conditions, the only exception being that one channel received chlorine and the other served as a non-chlorinated control.

Bacteria Cell Staining

The LIVE/DEAD Bacterial Viability Kit (*Bac*Light, InVitrogen) was applied to estimate both viable and total counts of bacteria in disinfectant treated samples. The *Bac*Light LIVE/DEAD stain is composed of two nucleic acid-binding stains: SYTO 9E and propidium iodide (PI). SYTO 9E penetrates all bacterial membranes and stains the cells green, while PI only penetrates cells with damaged membranes, while the combination of the two stains stoichiometrically produces red fluorescing cells. Total (red and green) and viable (green) cells can hence be counted simultaneously (Boulos et al., 1999). Stained solution was filtered through black polycarbonate filters for fluorescence microscopic imaging. Fluorescent images were observed at 480/500 nm for SYTO 9 and 488/617 nm for PI, respectively. For fluorescent stained cell counting, an Olympus fluorescent microscope with an 100X oil immersion objective and a TCS SP5 multi-photon laser scanning confocal microscope (Leica Microsystems) were used. Images were processed by CellCounter (Heracle Software), CellAnalyst (AssaySoft, Inc.) and COMSTAT.

EPS Extraction

All extraction procedures were performed on three separate 50 ml samples from an initial 200 ml bacterial culture. The modified EDTA extraction method described by Brown and Lester (1980) was employed (Brown et al., 1980). In this method, 100 ml of 2% EDTA (tetrasodium salt) was added to 100 ml of each culture suspension and shaken for 3 h at 4°C. High speed votex was applied both at the initial mixing of EDTA and culture suspension and after 3 h interaction. The samples were then centrifuged at 14,000 x g for 20 min at 4°C. The supernatant was analyzed to quantify EPS composition.

EPS Characterization

Total protein and polysaccharide was measured using standard colorimetric techniques. Protein concentrations were determined using the modified Lowry Protein Assay Kit (Pierce Biotechnology, Rockford, IL) with bovine serum albumin (BSA) as standard. Polysaccharide concentration was measured using the phenol-sulfuric acid method using glucose as standard (Jost Wingender, 1999).

Principle Findings and Significance

Both chlorine and chlorine dioxide are very effective disinfectants. However, these two disinfectants were heavily consumed during the initial period of inactivation. Limited by such short inactivation times and high reactivity, no significant disinfectant residual differences were observed. Chloramine was found to be a slow-acting disinfectant for the three strains. Thirty minutes of chloramine exposure were required to achieve 99% inactivation for the three strains in this study. The *mucA* mutant consumed the greatest amount of chloramine, while the *algT(U)* mutant had comparably high levels of residual chloramine. For chloramine inactivation, disinfectant residual is in inverse proportion to cell bound EPS amount. In other words, the more EPS bound to bacterial cells the more disinfectants were consumed. The result of the chloramine consumption study indicated that cell-bound EPS interacted with disinfectant during the inactivation process and part of the disinfectant consumption could be attributed to cell bound EPS. The transport limitation of disinfectant in biofilm was also monitored by microelectrode.

The results in this study indicate that the higher EPS production yields higher survival ratio and viability rate, which was confirmed by both heterogeneity plate counting and Live/Dead staining results. The mucA22 mutant with higher EPS production had a proportionally greater survival ratio and viability rate and more variation in protein and polysaccharide functional groups by interaction with disinfectants. The mucA22 mutant also had a prolonged lag time when interacting with the less reactive disinfectant chloramine.

For low disinfectants concentrations as used in water distribution system, key factor of chlorination bactericidal is not extensive membrane damage but functional

group deformation in bacteria membrane, which lead to membrane permeabilization. The acidic polysaccharide alginate, representative component in EPS of P. aeruginosa, has strong deformation after inactivation, which confirms that cell-bound EPS have high reactivity with the disinfectant used in this study. The combined results support that cell bound EPS consume disinfectant, retard bacterial membrane permeabilization, and thus decrease the susceptibility of bacteria.

REFERENCES

Tuovinen, O. H., Hsu, J. (1982). Aerobic and anaerobic microorganisms in tubercles of the Columbus, Ohio, water distribution system. *Appl. Environ. Microbiol*., 761-764.

LeChevallier, M.W., Welch, N.J., Smith, D.B. (1996b). Full-scale studies of factors related to coliform regrowth in drinking water. *Appl. Environ. Microbiol*., 62, 2201-2211.

American Water Works Association (2007). Coliforms in distribution systems: Integrated disinfection and antimicrobial resistance, AWWARF, Denver, CO.

American Water Works Association (2003). Assessment of chloramine and chlorine residual decay in the distribution system, AWWARF, Denver, CO.

Momba, M.N.B., Kfir, R., Venter, S.N., Cloete, T.E. (2000). An overview of biofilm formation in distribution systems and its impact on the deterioration of water quality, *Water SA*, 59-66.

Stewart, P.S. (2002). Mechanism of antibiotic resistance in bacterial biofilms., *Int. J. Med. Microbiol.*, 292, 107-113.

Stewart, P.S., Grab, L., Diemer, J.A. (1998). Analysis of biocide transport limitation in an artificial biofilm system., *J. Appl. Microbiol*. 495-500.

Characklis W.G., Marshall, K.C. (1990). *Biofilm*. New York, Wiley, 585-633.

Sibille, I. (1998). Biological stability in drinking water distribution systems: a review. L'Annee Biologique. 37,117-161

19. Hughes, K.A., Sutherland, I.W., Clark, J., Jones, M.V. (1998). Bacteriophage and associated polysaccharide depolymerases – novel tools for study of bacterial biofilms. *J. Appl. Microbiol.*, **85**, 583–590.

Craun, G.F., Calderon, R.L. (2001). Waterbone disease outbreaks caused by distribution system deficiencies. *JAWWA*, 64-75.

Engelbrecht, R. S., M. J. Weber, B. L. Salter and C. A. Schmidt (1980). "Comparative Inactivation of Viruses by Chlorine." Applied and Environmental Microbiology 40(2): 249-256.

Jang, A., J. Szabo, A. A. Hosni, M. Coughlin and P. L. Bishop (2006). "Measurement

of Chlorine Dioxide Penetration in Dairy Process Pipe Biofilms During Disinfection." Applied Microbiology and Biotechnology 72(2): 368-376.

Aieta, E. M. and J. D. Berg (1986). " A Review of Chlorine Dioxide in Drinking-Water Treatment." Journal American Water Works Association 78(6): 62-72.

USEPA (1999). Microbial and Disinfection Byproduct Rules Simultaneous Compliance Guidance Manual. EPA 815-R-99-015.

Haas, C. N., J. Joffe, U. Anmangandla, J. G. Jacangelo and M. Heath (1996). "Water Quality and Disinfection Kinetics." Journal American Water Works Association 88(3): 95-103.

Boulos, L., M. Prevost, B. Barbeau, J. Coallier and R. Desjardins (1999). "Live/Dead (R) Baclight (Tm): Application of a New Rapid Staining Method for Direct Enumeration of Viable and Total Bacteria in Drinking Water." Journal of Microbiological Methods 37(1): 77-86.

Brown, M. J. and J. N. Lester (1980). "Comparison of Bacterial Extracellular Polymer Extraction Methods." Applied and Environmental Microbiology 40(2): 179-185.

Jost Wingender, T. R. N., Hans-Curt Flemming (1999). Microbial Extracellular Polymeric Substances: Characterization, Structure and Function. Berlin, Springer.

Al-Qadiri, H. M., M. A. Al-Holy, M. S. Lin, N. I. Alami, A. G. Cavinato and B. A. Rasco (2006). "Rapid Detection and Identification of Pseudomonas Aeruginosa and Escherichia Coli as Pure and Mixed Cultures in Bottled Drinking Water Using Fourier Transform Infrared Spectroscopy and Multivariate Analysis." Journal of Agricultural and Food Chemistry 54(16): 5749-5754.

Al-Qadiri, H. M., M. S. Lin, A. G. Cavinato and B. A. Rasco (2006). "Fourier Transform Infrared Spectroscopy, Detection and Identification of Escherichia Coli O157 : H7 and Alicyclobacillus Strains in Apple Juice." International Journal of Food Microbiology 111(1): 73-80.

Holt, C., D. Hirst, A. Sutherland and F. Macdonald (1995). "Discrimination of Species in the Genus Listeria by Fourier-Transform Infrared-Spectroscopy and Canonical Variate Analysis." Applied and Environmental Microbiology 61(1): 377-378.

Berney, M., H. U. Weilenmann, J. Ihssen, C. Bassin and T. Egli (2006). "Specific Growth Rate Determines the Sensitivity of Escherichia Coli to Thermal, Uva, and Solar Disinfection." Applied and Environmental Microbiology 72(4): 2586-2593.

Mafart, P., O. Couvert, S. Gaillard and I. Leguerinel (2002). "On Calculating Sterility in Thermal Preservation Methods: Application of the Weibull Frequency Distribution Model." International Journal of Food Microbiology 72(1-2): 107-113.

Geeraerd, A. H., C. H. Herremans and J. F. Van Impe (2000). "Structural Model Requirements to Describe Microbial Inactivation During a Mild Heat

Treatment." International Journal of Food Microbiology 59(3): 185-209.
Rowan, N. J. (2004). "Viable but Nonculturable Forms of Food and Waterborne Bacteria: Quo Vadis?" Trends in Food Science & Technology 15(9): 462-467.
Stocks, S. M. (2004). "Mechanism and Use of the Commercially Available Viability Stain, Baclight." Cytometry Part A 61A(2): 189-195.
Barbesti, S., S. Citterio, M. Labra, M. D. Baroni, M. G. Neri and S. Sgorbati (2000). "Two and Three-Color Fluorescence Flow Cytometric Analysis of Immunoidentified Viable Bacteria." Cytometry 40(3): 214-218.
Hoefel, D., W. L. Grooby, P. T. Monis, S. Andrews and C. P. Saint (2003). "Enumeration of Water-Borne Bacteria Using Viability Assays and Flow Cytometry: A Comparison to Culture-Based Techniques." Journal of Microbiological Methods 55(3): 585-597.
Berney, M., F. Hammes, F. Bosshard, H. U. Weilenmann and T. Egli (2007). "Assessment and Interpretation of Bacterial Viability by Using the Live/Dead Baclight Kit in Combination with Flow Cytometry." Applied and Environmental Microbiology 73(10): 3283-3290.
Helm, D. and D. Naumann (1995). "Identification of Some Bacterial-Cell Components by Ft-Ir Spectroscopy." Fems Microbiology Letters 126(1): 75-79.
Schmitt, J. and H. C. Flemming (1998). "Ftir-Spectroscopy in Microbial and Material Analysis." International Biodeterioration & Biodegradation 41(1): 1-11.
Kim, H. N., Y. Hong, I. Lee, S. A. Bradford and S. L. Walker (2009). "Surface Characteristics and Adhesion Behavior of Escherichia Coli O157:H7: Role of Extracellular Macromolecules." Biomacromolecules 10(9): 2556-2564.
Ojeda, J. J., M. E. Romero-Gonzalez and S. A. Banwart (2009). "Analysis of Bacteria on Steel Surfaces Using Reflectance Micro-Fourier Transform Infrared Spectroscopy." Analytical Chemistry 81(15): 6467-6473.
Hentzer, M., G. M. Teitzel, G. J. Balzer, A. Heydorn, S. Molin, M. Givskov and M. R. Parsek (2001). "Alginate Overproduction Affects Pseudomonas Aeruginosa Biofilm Structure and Function." Journal of Bacteriology 183(18): 5395-5401.
Pereira, L., A. Sousa, H. Coelho, A. M. Amado and P. J. A. Ribeiro-Claro (2003). "Use of Ftir, Ft-Raman and (13)Gnmr Spectroscopy for Identification of Some Seaweed Phycocolloids." Biomolecular Engineering 20(4-6): 223-228.
Kansiz, M., P. Heraud, B. Wood, F. Burden, J. Beardall and D. McNaughton (1999). "Fourier Transform Infrared Microspectroscopy and Chemometrics as a Tool for the Discrimination of Cyanobacterial Strains." Phytochemistry 52(3): 407-417.
Pedone, E., S. Bartolucci, M. Rossi, F. M. Pierfederici, A. Scire, T. Cacciamani and F. Tanfani (2003). "Structural and Thermal Stability Analysis of Escherichia Coli and Alicyclobacillus Acidocaldarius Thioredoxin Revealed a Molten Globule-Like State in Thermal Denaturation Pathway of the Proteins: An Infrared Spectroscopic Study." Biochemical Journal 373: 875-883.
Sandt, C., G. D. Sockalingum, D. Aubert, H. Lepan, C. Lepouse, M. Jaussaud, A. Leon, J. M. Pinon, M. Manfait and D. Toubas (2003). "Use of

Fourier-Transform Infrared Spectroscopy for Typing of Candida Albicans Strains Isolated in Intensive Care Units." Journal of Clinical Microbiology 41(3): 954-959.