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Three-dimensional Free Chlorine and Monochloramine Biofilm Penetration: Correlating Penetration with Biofilm Activity and Viability

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Abstract

Disinfectant biofilm penetration and its effect on biofilm aerobic activity and viability are still unclear. In this study, free chlorine and monochloramine were applied until full biofilm penetration occurred, and their effects on biofilm aerobic activity and viability were investigated in three-dimensions throughout the entire biofilm depth, extending previous work where viability analysis was limited to the upper biofilm (50 μm depth), free chlorine penetration did not reach completion, and only one-dimensional (depth) profiles were obtained. The free chlorine and monochloramine biofilm concentration profiles were correlated spatially and temporally with aerobic microbial activity and cell-membrane integrity based viability using a combination of (1) microelectrode measurements for disinfectant penetration and (2) Live/Dead BacLight staining, cryo-cross-sectioning, and confocal micrographs analysis for viability measurements throughout the entire biofilm depth. Compared to monochloramine, free chlorine penetration (1) was slower, (2) led to a greater decrease in biofilm thickness from sloughing, and (3) corresponded directly with a viability decrease. In addition, biofilm heterogeneity led to minor differences in either disinfectant's biofilm penetration, and prior biofilm exposure to monochloramine provided little impact to subsequent free chlorine biofilm penetration.

Graphical abstract

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Supporting Information Available. Supporting information consists of 9 pages containing descriptions of nitrifying biofilm growth and development and biofilm viability analysis, 4 figures, and associated references. Supporting information is available free of charge at <http://pubs.acs.org/>.

INTRODUCTION

Biofilm are a self-protection growth pattern of bacteria that attach to surfaces encased by extracellular polymeric substances (EPS) and are generally considered to be more resistant to disinfection than individual planktonic cells.¹ It is estimated that approximately 95% of the total biomass in water mains is attached to surfaces, ranging from tens of microns up to 200 μm for a mature biofilm,^{2,3} while only 5% is in the water phase.⁴ Biofilm formation in drinking water distribution systems has been associated with disinfectant depletion and water quality degradation, resulting in non-compliance with existing United States (US) regulations.⁵ Particularly, biofilm in drinking water systems may harbor pathogenic microorganisms that are a public health concern.⁶ US drinking water utilities report biofilm survival and bacterial regrowth despite disinfectant presence.^{7,8} In addition, utilities using chloramines as a secondary disinfectant to comply with disinfectant by-product (DBP) regulations inherently have free ammonia present which may serve as a growth substrate for ammonia-oxidizing bacteria (AOB), resulting in nitrification that is undesirable and typically associated with biofilm.⁵ If unchecked, AOB may accelerate chloramine loss, leading to disinfectant depletion.

Once nitrification occurs, water utilities have very few options available to correct the situation (e.g., breakpoint chlorination, flushing, and increasing chloramine residual) and often convert to free chlorine for a period of time to remove the growth substrate (ammonia) and inactivate biofilm.⁵ The free chlorine application duration varies (e.g., 4–5 weeks) with 1–2 $\text{mg Cl}_2 \text{ L}^{-1}$ being a typical bulk water target. Although there are emerging biological monitoring techniques to evaluate microbial activity (e.g., ATP),⁹ they require further validation for full-scale deployment.¹⁰ Currently, the only method to assess operational responses and effectiveness of a free chlorine conversion is to measure bulk water free chlorine concentrations.

Previous research¹¹ showed that free chlorine failed to penetrate a laboratory grown nitrifying biofilm whereas monochloramine fully penetrated the biofilm. Furthermore, free chlorine penetration was similar for a biofilm that was completely penetrated with monochloramine, indicating that free chlorine gains no benefit from previous monochloramine application in terms of its ability to penetrate biofilm. Overall, the limited biofilm free chlorine penetration indicated that free chlorine bulk water concentrations provide little information on biofilm penetration and/or microbial inactivation. However, the previous study only investigated defined spots of biofilm by measuring one-dimensional

(1D) profiles, and the interpretation on biofilm viability was limited to near the biofilm surface due to the confocal laser scanning microscopy (CLSM) observation depth limitations.¹¹ Because biofilm exhibit considerable structural, chemical, and biological heterogeneity,¹² it is important to understand whether biofilm disinfectant penetration and associated impacts to aerobic activity and viability proceeds homogeneously or heterogeneously with time, requiring three-dimensional (3D) concentration surfaces.

The current study's objectives were to correlate disinfectant biofilm penetration with biofilm viability and aerobic activity throughout the entire biofilm depth; assess the impacts of biofilm heterogeneity by using 3D profiling techniques; and evaluate differences between monochloramine and free chlorine, including free chlorine penetration between a fresh biofilm and a biofilm that was previously fully penetrated with monochloramine. Relatively thick nitrifying biofilm (approximately 2,000 μm) were developed to (1) provide a clear image of how the biofilm is physically and chemically reacting with disinfectants, (2) show a well-defined stratification with high spatial resolution, (3) provide a fundamental baseline on how biofilm becomes inactivated by the different disinfectants as they penetrate, and (4) provide the necessary data for future model development and validation of a disinfectant biofilm penetration model. This study is also the first to apply cryoembedding and cross-sectioning techniques for confocal micrographs of Live/Dead BacLight to demonstrate biofilm viability progression throughout the entire biofilm depth during free chlorine and/or monochloramine application, corresponding to 3D disinfectant penetration surfaces. Overall, this research provides a better understanding of how different disinfectants (free chlorine and monochloramine) impact biofilm in terms of disinfectant penetration, aerobic activity, and viability.

MATERIALS AND METHODS

Nitrifying Biofilm Growth and Development

Using an inoculum from a distribution system experience nitrification, a mixed-culture nitrifying biofilm was developed on polycarbonate slides laid flat in an open-channel flat-plate recirculating biofilm reactor at room temperature (23 °C). Further details are provided in Supporting Information (SI).

Free Chlorine and Monochloramine Preparation

For free chlorine and monochloramine biofilm application experiments, biofilm feed media was not used. Rather, the appropriate disinfectant (free chlorine or monochloramine) was fed continuously in a 5 mM, pH 8.0 boric acid buffer in ultra-pure water (18.2 M Ω -cm, Barnstead NANOpure Diamond). Free chlorine feed solutions were prepared by adding the required amount of sodium hypochlorite solution (Fisher Scientific), whereas monochloramine feed solutions (4:1 chlorine to ammonia-nitrogen [Cl₂:N] mass ratio) were prepared by adding the required amount of sodium hypochlorite solution and ammonia stock solution (100 mg N L⁻¹). The free chlorine and monochloramine concentrations in the bulk water were verified by colorimetric test kits (free chlorine, Hach-8021; monochloramine, Hach-10200) using spectrophotometers (free chlorine, DR/2010 spectrophotometer [Hach Co.]; monochloramine, Nicolet Evolution 300 UV-Vis spectrophotometer [Thermo Electron

Co.]). During an experiment, fresh free chlorine and monochloramine solutions were used every 24 hours. Preliminary control tests (SI, Figure S1) demonstrated that free chlorine and monochloramine concentrations decreased by only 2.9% and 4.8% in 24 hours, respectively.

Free Chlorine and Monochloramine Biofilm Application

Two experiments consisting of a total of three biofilm application scenarios (Table 1) were conducted under the same conditions: pH 8.0, 8.3 mg O₂ L⁻¹, 23 °C, equivalent 10 mg Cl₂ L⁻¹ chlorine concentrations, and under continuous flow (15 mL min⁻¹; 2 min HRT, and 7.5 cm min⁻¹ surface velocity) in a separate flow/microelectrode profiling chamber. The short residence time was to ensure a constant bulk water condition during profiles (i.e., measurable concentration changes did not occur between the influent and effluent of the profiling chamber).

Experiment 1 applied free chlorine until near full biofilm penetration where biofilm penetration is defined by Equation 1. Experiment 2 consisted of two phases: Experiment 2 (Phase 1) applied monochloramine and was followed by Experiment 2 (Phase 2) which then applied free chlorine until near full biofilm penetration. The same total exposure time was applied for Experiment 1 (free chlorine) and Experiment 2, Phase 1 (monochloramine) to achieve equivalent biofilm chlorine exposure. The relatively high chlorine concentrations (10 mg Cl₂ L⁻¹) are greater than what would be used in drinking water (e.g., up to 4 mg Cl₂ L⁻¹)⁵ but were selected because of the greater biofilm thickness used.

$$\% \text{ Biofilm Penetration} = 100 * \frac{\text{Slide Surface (Substratum) Concentration}}{\text{Bulk Water Concentration}} \quad (1)$$

For each experiment, an original 15 cm long biofilm covered polycarbonate slide was taken from the flat reactor and cut into ten equal pieces (coupons), using a heavy-duty hand shears scissors. The ten coupons were inserted into the flow chamber and used for profile measurements and sacrificial samples for CLSM analyses. CLSM analysis was conducted at defined time intervals: (1) initially before disinfectant application, (2) when disinfectant reached approximately half of the bulk concentration at the biofilm base (50% penetration), (3) when disinfectant fully penetrated the biofilm (100% penetration), and (4) when complete microbial inactivation was expected. Six and three coupons were used to determine a mean biofilm density^{13, 14} before and after Experiment 2 (Phase 1), respectively. The effluent was collected for every experiment and volatile suspended solids (VSS) was measured¹⁵ to quantify biofilm sloughing from free chlorine and monochloramine application.

Microelectrode Preparation and 3D Microprofiling

Free chlorine¹⁶ and monochloramine¹⁷ microelectrodes (5–10 µm tip diameter) were used to measure free chlorine and monochloramine biofilm penetration. Control experiments (SI, Figure S2) verified that polycarbonate slides showed a minimal consumption of free chlorine (5.9%) or monochloramine (2.2%) near the slide surface. An oxygen microelectrode (OX10, Unisense A/S, Denmark) was used to measure dissolved oxygen (DO) concentrations,

allowing aerobic microbial activity to be correlated with disinfectant penetration. During an experiment, 3D concentration surfaces of free chlorine (or monochloramine) and DO were continuously collected over a small gridded area ($400\text{ }\mu\text{m} \times 400\text{ }\mu\text{m}$, $200\text{ }\mu\text{m}$ x- and y-intervals, 9 locations), using a computer controlled data acquisition and automated profiling set-up (Unisense A/S, Denmark). All profiles were measured at $50\text{ }\mu\text{m}$ intervals in the z-axis from the bulk water into the biofilm. For achievement of well-defined 3D concentration surfaces, both an anti-vibration table and a 3D micromanipulator were balanced before each experiment. The error of x- and y-axis movement was 0.0097% ($9.7\text{ }\mu\text{m}/10\text{ cm}$) and 0.007% ($7\text{ }\mu\text{m}/10\text{ cm}$), respectively. Approximately 5.3 minutes was required for one profile measurement, consisting of 65 data points from 3,200 to $0\text{ }\mu\text{m}$ ($50\text{ }\mu\text{m}$ interval) above the polycarbonate coupon base. A single data point measurement was acquired in 5 s (1 s for moving, 2 s waiting, and 2 s acquiring data).

Biofilm Viability Analysis

Biofilm viability was based on cell-membrane integrity determined using LIVE/DEAD BacLight with a CLSM where green and red fluorescing cells were deemed viable and nonviable, respectively. Further details are provided in SI.

RESULTS AND DISCUSSION

Free Chlorine Biofilm Exposure (Experiment 1)

Figure 1(a) and 1(b) show representative free chlorine and DO concentration profiles at the center ($x=200\text{ }\mu\text{m}$ and $y=200\text{ }\mu\text{m}$) of the 3D concentration surfaces (Figure 2) for Experiment 1, respectively. Before the experiment, a separate microelectrode was used to measure biofilm thickness,^{11, 18} and the initial biofilm thickness was $2,000\text{ }\mu\text{m}$ (Table 1). The free chlorine profiles demonstrated two trends with time: (1) a relatively rapid advancement of the whole free chlorine profile toward the substratum to approximately $-1,200\text{ }\mu\text{m}$, indicating biofilm sloughing, and minimal free chlorine penetration into actual biofilm (1–24 h) and (2) a slower progression of the entire profile toward the substratum from continued biofilm sloughing along with free chlorine biofilm penetration (1–16 days). The initial similar concave shapes movement towards the substratum indicated free chlorine's reaction with the biofilm surface,¹⁹ resulting in biofilm sloughing.

Approximately 7 days were required for free chlorine to reach the substratum while 84% biofilm penetration was achieved after 16 days. The free chlorine penetration was accompanied with biofilm sloughing (or detachment), resulting in a decreasing biofilm thickness from $2,000\text{ }\mu\text{m}$ to $700\text{ }\mu\text{m}$ after 16 days (Figure 1(a)). Approximately 19% of detached biomass was measured in the effluent (Table 1), indicating that free chlorine application induced biofilm sloughing and increased the amount of suspended bacteria (i.e., VSS). Figure 1(a) also shows that free chlorine was transported from the bulk through the diffusion boundary layer (DBL) and into the biofilm. The hypothetical diffusion boundary layer was determined by linear extrapolation of the substrate gradient at the biofilm surface to the bulk concentration.²⁰ During Experiment 1, DBL thickness was relatively consistent in the range of 400 to $450\text{ }\mu\text{m}$ above the biofilm surface due to a fixed flowrate. The decrease of biofilm thickness was relatively linear with time. DO profiles showed that

oxygen consumption decreased with free chlorine penetration (Figure 1(b)), indicating a decrease in the biofilm's aerobic activity. Initially, DO was fully consumed by the biofilm (0% penetration). After 2 days and with 0.1 mg Cl₂ L⁻¹ free chlorine reaching 500 µm above the substratum, DO penetration increased to 81%, and measurable microbial aerobic activity ceased after 5 days (100% DO penetration) when free chlorine nearly reached the substratum (i.e., 0.1 mg Cl₂ L⁻¹ free chlorine reaching 250 µm above the substratum, Figure 1(a)).

In our previous research, biofilm viability interpretations were limited due to the CLSM light penetration depth,¹¹ leaving questions about biofilm viability at the substratum. In this study, the new CLSM analysis method clearly demonstrated the biofilm viability throughout the entire depth with time. The confocal micrographs (Figure 3(a)) and free chlorine penetration profiles (Figure 1(a)) showed that the bacteria were viable where free chlorine did not penetrate (e.g., 5 days). Even after oxygen consumption completely ceased (i.e., 5 days), bacteria at the biofilm base were still viable and free chlorine penetration was still progressing (e.g., 7 days). The CLSM image at 13 days showed bacteria were completely nonviable throughout the depth with approximately 70% free chlorine penetration and confirmed that the biofilm thickness decreased from biofilm sloughing (Figure 3(a)). It is noted that the cryo-processing of the samples using the tissue freezing medium compressed the sample (approximately 60% at 7 days for Experiment 1), reducing biofilm thickness in CLSM analysis. Even though the bacteria were nonviable, the free chlorine continued to react with the biofilm, achieving 84% penetration at day 16.

Figure 2 displays the free chlorine and DO 3D concentration surfaces for Experiment 1 where the substratum is defined at a 0 µm distance with increasing negative distance towards and into the bulk water. Both the free chlorine and DO 3D concentration surfaces show the continued progression of the bulk water concentrations towards the substratum through a combination of biofilm loss and penetration as previously discussed. The 3D concentration surfaces also showed an evenly well-distributed homogeneous biofilm penetration and aerobic microbial activity changes throughout the biofilm depth, indicating that in this experiment, any biofilm heterogeneity did not lead to a heterogeneous free chlorine or DO penetration.

Monochloramine Biofilm Exposure (Experiment 2, Phase 1)

Figure 1(c) and 1(d) show representative monochloramine and DO concentration profiles during Experiment 2 (Phase 1) and located at the center value (x=200 µm and y=200 µm) of the 3D concentration surfaces shown in Figure 4. Figure 1(c) shows that monochloramine penetrated faster than free chlorine did in Experiment 1, achieving 90% biofilm penetration in approximately 2 days versus the 16 days it took free chlorine to penetrate 84%. DO profiles (Figure 1(d)) showed that monochloramine penetration corresponded directly with an increase of DO concentration within the biofilm, indicating the cessation of measurable aerobic microbial activity within the biofilm upon monochloramine penetration. However, CLSM images demonstrated that monochloramine full biofilm penetration did not correlate with immediate viability loss (Figure 3(b)). Even with sufficient time (e.g., 7 and 10 days) after observing monochloramine full penetration with no biofilm aerobic activity (i.e., 2

days), biofilm bacteria exhibited a mixture of viable and nonviable cells throughout the biofilm depth. After 16 days of monochloramine exposure, the biofilm was completely nonviable based on CLSM analysis. Taking these results together, this experiment showed that even though monochloramine completely penetrated the biofilm and biofilm aerobic activity ceased, it took much longer to make the biofilm nonviable based on cell membrane integrity.

The loss of microbial activity without an immediate, concurrent loss of viability may be due to the known selective reactivity of monochloramine.^{21–25} For instance, Pressman et al.²⁶ found that aerobic activity ceased in a biofilm completely penetrated by monochloramine even though complete viability loss had not occurred. Furthermore, the monochloramine penetration was not impacted by ammonia concentration. As with free chlorine, 3D monochloramine biofilm penetration and DO concentration surfaces also indicated a relatively homogeneous disinfectant penetration and DO distribution (Figure 4). Compared to free chlorine, monochloramine showed faster and farther biofilm penetration in the same time frames (e.g., 2 hrs, 6 hrs, and 2 days) and the vertical oxygen distribution showed a more compact distribution.

During monochloramine application, a fluffy slime at the biofilm surface was observed with time. The fluffy surface physically increased the actual measurable biofilm thickness in water from an initial biofilm thickness of 2,000 μm to 2,300 μm at 16 days (Table 1) and is unlike free chlorine application where free chlorine caused biofilm sloughing (or detachment) that gradually decreased the biofilm thickness. The observation of a fluffy surface during Experiment 2 (Phase 1) indicated that monochloramine oxidation impacted the biofilm matrix, creating loosely bound areas devoid of bacteria cells. Therefore, it was reasonable that confocal micrographs showed vague fluorescent images at the biofilm surface compared to those near the substratum where the boundary of the biofilm bottom was determined by the red-color stained nonviable cells (Figure 3(b)).

For 16 days, only 2% (0.7 mg) of the total biomass (31 mg) was measured in the effluent (Table 1), indicating minimal biofilm loss, but monochloramine application decreased biofilm density with time. The biofilm density decreased from an initial $65 \pm 16 \text{ mg cm}^{-3}$ ($n=6$) to $48 \pm 8.4 \text{ mg cm}^{-3}$ ($n=3$) after 5 days of monochloramine application, resulting in approximately a 27% density decrease during monochloramine application. Given that EPS forms the structure and architecture of the biofilm matrix with a proportion of 50–90% of the total organic matter,^{27, 28} the biofilm density decrease without apparent biofilm sloughing during monochloramine application may be explained by monochloramine's selective reactivity where it reacts rapidly with only four amino acids (cysteine, cystine, methionine, and tryptophan) and very slowly with DNA and RNA.^{24, 29} This selective reactivity of monochloramine can affect the inactivation efficacy in capsular EPS.³⁰ One study compared biofilm disinfection between protein-based EPS producing *Pseudomonas pituita* and polysaccharide-based EPS producing *Pseudomonas aeruginosa* strains and showed that polysaccharide EPS obstructed monochloramine reactive sites on bacterial cells, while protein EPS delayed monochloramine penetration by reaction with monochloramine. In the current research, it appears monochloramine's selective reaction with biofilm generated void spaces in the biofilm structure as shown in CLSM images (Figure 3(b)) and

decreased the biofilm density (e.g., 27% decrease) while slightly increasing its thickness (2,000 μm to 2,300 μm). In contrast, free chlorine exhibited a much broader reactivity, resulting in biofilm sloughing.

Free Chlorine Application after Monochloramine Full Penetration (Experiment 2, Phase 2)

In Experiment 2 (Phase 2), disinfectant application was switched from monochloramine to free chlorine after monochloramine achieved full biofilm penetration (Experiment 2, Phase 1). Compared to the free chlorine only application (Experiment 1), there were four different initial conditions: (1) monochloramine had already fully penetrated the biofilm; (2) there was no aerobic microbial activity as DO fully penetrated the biofilm; (3) the biofilm was already nonviable based on CLSM analysis; and (4) the biofilm was less dense and slightly thicker from monochloramine application. The results showed that these initial conditions did not affect free chlorine biofilm penetration as free chlorine still followed the same limited penetration regardless of prior monochloramine full biofilm penetration (Figure 5 and 6). The apparent lack of importance of prior monochloramine exposure likely results from the known differences in reactivity between monochloramine and free chlorine. Monochloramine more selectively reacts than free chlorine.^{21–25, 30, 31} Therefore, due to the different reactivity between free chlorine and monochloramine, free chlorine biofilm penetration appears to be independent of biofilm pretreatment with monochloramine. Considering the previous study of Lee et al.¹¹ where a thinner biofilm was used (i.e., 250 μm), it appears that the lack of dependence on prior monochloramine exposure holds for almost an order of magnitude increase in biofilm thickness in the current experiments.

With free chlorine application, biofilm thickness also decreased with time due to the biofilm sloughing as seen in Experiment 1. The 3D concentration surfaces (Figure 6) also followed previous free chlorine only application, showing homogeneous advancement toward the substratum along with the decreased biofilm thickness.

Monochloramine and Free Chlorine Comparison and Implications

The current study resolved limitations from our previous research with nitrifying biofilm^{11, 26} by successfully measuring disinfectant (free chlorine and monochloramine) and DO concentration profiles along with corresponding CLSM viability images throughout the entire biofilm depth. Overall, monochloramine and free chlorine application showed that disinfectant penetration, aerobic activity impacts, and viability progression were disinfectant specific, and in addition, similar free chlorine results were obtained whether or not the biofilm had been previously fully penetrated by monochloramine.

Biofilm are known to be heterogeneous, but the generated 3D disinfectant and DO concentration surfaces showed a homogeneous disinfectant penetration and impact to microbial activity during both free chlorine and monochloramine application. Therefore, the current results support that 1D disinfectant penetration analysis would be sufficient for investigating free chlorine or monochloramine biofilm penetration. In addition, 1D disinfectant penetration models may adequately represent penetration, simplifying future modeling efforts. Figure 7 shows a plot of free chlorine and monochloramine concentrations at 150 μm above the substratum (92.5% biofilm depth based on using an initial 2,000 μm

biofilm thickness) as a function of exposure time for each experiment, highlighting the differences seen between the two disinfectants. With the same biofilm thicknesses (2,000 μm) and equivalent bulk water chlorine concentrations (10 mg $\text{Cl}_2 \text{ L}^{-1}$), monochloramine required approximately 2 days for 90% biofilm penetration while free chlorine took over 16 days for 84% biofilm penetration regardless of whether the biofilm was previously fully penetrated by monochloramine. However, monochloramine's faster penetration did not correlate with an immediate viability and aerobic activity loss whereas free chlorine's slower biofilm penetration corresponded directly with a loss in viability and aerobic activity. SI Figure S4 shows a schematic comparison of biofilm impacts for free chlorine and monochloramine in terms of disinfectant biofilm penetration and viability. Apparent biofilm sloughing was not observed during monochloramine application and biofilm thickness was relatively consistent with time, but free chlorine seemed to react to a greater extent than monochloramine with biofilm, resulting in biofilm sloughing and a substantial decrease in biofilm thickness (2,000 to 700 μm and 2,300 μm to 750 μm) over the application period.

Even though the biofilm used in this research were thicker than typically reported for drinking water biofilm, the research results have implications to disinfectant penetration in drinking water systems. As previously mentioned, chloramine systems may temporally switch from monochloramine to free chlorine to either arrest or prevent nitrification episodes but often report a reoccurrence of nitrification after a subsequent switch back to monochloramine.⁵ This nitrification reoccurrence may result from free chlorine's limited biofilm penetration seen in the current research. Biofilm development from chlorine resistant bacteria (e.g., *Mycobacteria* spp.) in chloraminated water distribution systems^{8, 32} may further retard disinfectant penetration. The possible release of microbes due to biofilm sloughing from free chlorine presents another practical implication for water utilities which have periodic free chlorine conversions in their chloraminated drinking water distribution systems to control nitrification.

In conclusion, free chlorine penetration was directly related to microbial inactivation, indicating that if free chlorine penetration is achieved, microbial inactivation and biofilm control should be effective. Depending on the free chlorine application (i.e., exposure time, flushing procedure, and bulk water concentration) and system (i.e., pipe materials), free chlorine application may only inactivate the outer biofilm, leaving the inner biofilm unaffected by free chlorine and may require increased application times to completely inactivate biofilm during conversion from monochloramine. Specifically, in distribution systems with reactive materials, the presence of corrosion deposits may exert a disinfectant demand, delaying or preventing complete penetration, and the failure of disinfectant penetration may provide niches where bacteria and opportunistic pathogens can be sheltered from disinfectant residuals.^{8, 33, 34} Further research regarding biocorrosion (e.g., effect of corrosion deposits on disinfectant biofilm penetration) would be required to better control bacterial communities located near corrosion tubercles.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Representative Experiment 1 free chlorine (a) and DO (b) concentration profiles during free chlorine application and Experiment 2 (Phase 1) representative monochloramine (c) and DO (d) concentration profiles during monochloramine application.

Figure 2.

Experiment 1 3D free chlorine (a) and DO (b) concentration surfaces during free chlorine application. Times listed are duration of free chlorine exposure. Zero distance represents the slide surface and increasing negative distance values represent distances progressing from the slide surface into the bulk fluid.

Figure 3.

Representative CLSM microscopic images of cross-sections of cryoembedded biofilm stained with Live/Dead BacLight. Experiment 1 biofilm viability progression during free chlorine application (a) and Experiment 2 (Phase 1) biofilm viability progression during monochloramine application (b). Green color: SYTO9 stained viable cells, Red color: PI stained nonviable cells (i.e., membrane compromised cells). The images are oriented such that the image bottom represents the slide surface and the image top represents the bulk fluid. The scale bar equals 200 μm.

Figure 4.

Experiment 2 (Phase 1) 3D monochloramine (a) and DO (b) concentration surfaces during monochloramine application. Times listed are duration of monochloramine exposure. Zero distance represents the slide surface and increasing negative distance values represent distances progressing from the slide surface into the bulk fluid.

Figure 5.

Representative Experiment 2 (Phase 2) free chlorine concentration profiles during free chlorine application to a biofilm previously exposed to monochloramine for 16 days (Experiment 2, Phase 1). Zero distance represents the slide surface and increasing negative distance values represent distances progressing from the slide surface into the bulk fluid.

Figure 6.

Experiment 2 (Phase 2) 3D free chlorine concentration surfaces during free chlorine application to a biofilm previously exposed to monochloramine for 16 days (Experiment 2, Phase 1). Times listed are duration of free chlorine exposure. Zero distance represents the slide surface and increasing negative distance values represent distances progressing from the slide surface into the bulk fluid.

Figure 7.

Disinfectant concentration inside biofilm (150 µm above the slide surface) as a function of disinfection time for comparison of free chlorine and monochloramine penetration. The initial biofilm thicknesses are shown in Table 1.

Table 1

Summary of experimental conditions for disinfectant exposure

Parameter	Experiment 1		Experiment 2
	Free chlorine	Monochloramine (Phase 1)	Subsequent free chlorine (Phase 2)
Experiment time (d)	16	16	20
Disinfectant concentration (mg Cl ₂ L ⁻¹)	10	10	10
Initial biofilm thickness (μm) ¹	2,000	2,000	2,300 ⁴
Final biofilm thickness (μm) ¹	700	2,300 ⁴	750
Initial VSS (mg/slide) ²	31		31
Effluent VSS (mg/slide) ³	6.0	0.7	7.4

¹Biofilm thickness directly measured in the bulk water using a microelectrode.^{11, 18}

²Initial VSS (mg) = coupon area (1.6 cm × 1.5 cm = 2.4 cm²) × initial biofilm thickness (μm) × biofilm density (mg cm⁻³). Biofilm density (65 ± 16 mg cm⁻³) estimated from actual measurements (n=6).

³Effluent VSS determined by filtering collected effluent and normalizing by number of polycarbonate slides used.

⁴The biofilm surface became homogeneously loosened and fluffy during monochloramine application (Phase 1), increasing the initial biofilm thickness for Phase 2. The biofilm thickness was determined by measuring the distance from the end of the fluffy biofilm to the slide surface using a microelectrode.