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Article

Beyond Microbial Inactivation: Unveiling the Potential of Detachment-Promoting Agents in Water Distribution System Biofilm Control

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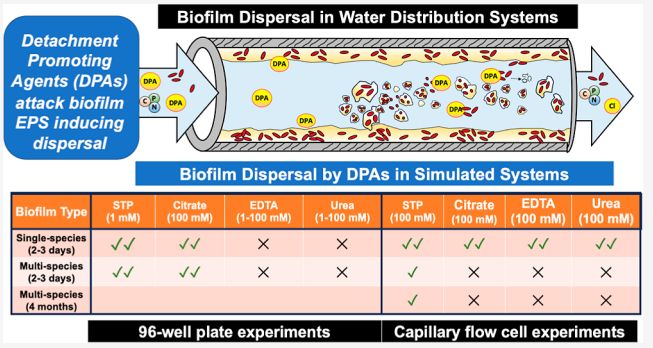
ABSTRACT: Biofilms growing on the surfaces of water distribution systems (WDS) pose a significant problem for maintaining the quality of distributed drinking water. Current approaches to biofilm control by inactivating microbial cells (e.g., residual chlorine) involve high concentrations of antimicrobials, which can cause human health hazards, produce harmful byproducts, and still allow appreciable biofilm proliferation on WDS pipes. An alternate paradigm is to instead target the biofilm extracellular polymeric substances (EPS) through "detachment promoting agents" (DPAs) to cause EPS weakening, biofilm dispersal, and eventual detachment. Four potential DPAs (sodium triphosphate [STP], ethylene diamine tetraacetate [EDTA], citrate, and urea) were tested for biofilm detachment at concentrations ranging from 1 to 100 mM for their efficacy in disrupting single-species *Staphylococcus epidermidis* biofilms and multispecies (microbial consortium derived from WDS) biofilms grown on well plates and in glass capillary flow cells. The protein and polysaccharide contents of detached biofilms in flow cell effluents were also measured. The well-plate study on 24 h-old single-species and multispecies biofilms showed significant removal (P-value < 0.002) performance with citrate and STP. Capillary flow cell experiments were conducted with a 100 mM concentration of DPAs on 72 h-old single-species biofilms, 72 h-old multispecies biofilms, and 4-month-old chlorinated multispecies biofilms. From flow cell image analyses, the maximum dispersal for *S. epidermidis* biofilms (mean ± S.E.) was observed with EDTA (45 ± 13%), while 22−28% mean biofilm removal was observed with citrate, STP, and urea treatment. Multispecies biofilms (both 72 h and 4 months) were more resistant to DPA treatments than single-species *S. epidermidis* biofilms. The maximum EPS protein in detached biofilm clusters was observed with EDTA treatment, supporting the hypothesis of biofilm dispersal due to EPS weakening. Overall, the results show the effectiveness and early promise of DPAs for biofilm dispersal, offering an additional tool in the biofilm management arsenal.



KEYWORDS: *biofilms, detachment promoting agent, S. epidermidis, multispecies, capillary flow cell*

1. INTRODUCTION

Microbial biofilms have wide-ranging applications and ramifications in water distribution systems (WDS), medicine, dentistry, the marine industry, and myriad other industrial and environmental systems.[1] With recent outbreaks of Legionnaires' disease in the United States and Europe caused by the pathogenic bacteria *Legionella pneumophila*, biofilms in WDS are attracting increasing attention and scrutiny.[2,3] It is well-known that microorganisms deposited in biofilms are more resistant to disinfectants,[4] predators,[5] and shear forces,[6] and thus biofilms may act as a reservoir for pathogenic and nonpathogenic microorganisms within the WDS.[7] Moreover, some pipe materials become corroded when exposed to biofilms.[8] A study by the U.S. EPA suggests that maintenance and replacement of existing WDS in the United States will cost around $138 B over the next 20 years, with over half of this expenditure ($77 B) required for pipe repairs and rehabilitation.[9] Typically, free chlorine or combined chlorine (i.e., monochloramine) at low doses (1−4 mg/L) is used for combating microorganisms in WDS, wherein microbes can still survive in high quantities (10^5 to 10^8 bacteria/cm^2) to form biofilms.[10] Therefore, existing strategies are inadequate, and there is a need to consider alternate strategies for biofilm management in the WDS.

Biofilms typically consist of bacteria enmeshed in a matrix of extracellular polymeric substances (EPS), which is comprised

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of various macromolecules, including polysaccharides, proteins, DNA, lipids, and humic substances.[11,12] It has been shown that these components of the biofilm matrix, along with multivalent cations, play an important role in lending structure to the biofilm matrix and in biofilm cohesiveness. Multivalent cations are often essential for biofilm cohesiveness because they can neutralize or bridge negatively charged functional groups on the EPS macromolecules. EPS can also inhibit the effectiveness of antimicrobial agents from restraining biofilm growth.[13] Given that EPS is primarily responsible for lending strength and structure to the biofilm, a paradigmatically different view of biofilm inactivation/removal is to focus on weakening the EPS matrix (anti-EPS strategies) rather than targeting the microbial cells (anti-microbial strategies).[14] Since weakening of the EPS can cause biofilm dispersal and eventual detachment, the term detachment-promoting agents (DPA) has been coined for treatments that focus on weakening the EPS matrix and facilitating the physical removal of biofilms.[15] Traditional antimicrobial chemicals primarily target the inactivation of biofilm bacteria and are often ineffective at biofilm dispersal even at high concentrations (e.g., chlorine, chlorine dioxide, chloramine, sodium hydroxide, ozone, and hydrogen peroxide).[16−19] While some of these agents may also alter the EPS (e.g., via oxidation), changes in EPS are considered secondary results and are rarely monitored or measured. In addition, the widespread health hazards associated with such antimicrobials impede their use on a mass scale, especially in drinking water distribution systems or the food industry. The use of DPAs could serve as a superior alternative over the usual practice of bacterial inactivation considering their safe usage for biofilm dispersal and detachment.

While there are several reports on the use of DPAs against biofilms, most of these studies address medical biofilms (e.g., biofilms on catheter surfaces,[20,21] in dentistry,[22−24] oral infection,[25] and medical implants[26]). For instance, the metal chelator EDTA (ethylene diamine tetra acetate) was effective in suppressing cell viability in biomedical devices[27] and increasing sensitivity to antimicrobials and cell lysis.[28,29] Over 70% decrease in biofilm growth on catheters was reported with lower concentrations (<5%) of citrate,[30,31] while similar biofilm inhibition was attained with protein denaturant urea under 500 mM dosage.[32−34] However, the use of the DPA strategy as a tool to manage WDS biofilms is still largely unexplored. In this work, we investigate the efficacy of four DPA agents against single-species *Staphylococcus epidermidis* biofilm and a drinking water-derived multispecies biofilm in WDS-relevant conditions. The main objective of this study is to elucidate the potential of DPAs in promoting biofilm dispersal (as opposed to inactivation of biofilm microbes) and the impact of DPAs on biofilm properties in the context of WDS biofilms.

We selected three environmentally benign chelating agents [i.e., EDTA, sodium citrate, and sodium triphosphate (STP)] and a protein denaturant (i.e., urea) as DPA for this study. Our selection of DPAs prioritized two key considerations: their potential to disrupt interpolymeric bonds within the biofilm's EPS matrix and their established safety in drinking water or food industry (except urea, which was selected as a known biofilm weakening agent). Notably, chelators such as EDTA, citrate, and STP are expected to compete for the multivalent cations, which are active binding agents for the negatively charged EPS molecules. This competition, according to our hypothesis, would induce a charge imbalance in the EPS, leading to electrostatic repulsion, ultimately weakening the biofilm matrix and promoting its dispersal.

A uniqueness of this work is that chemical DPA treatment of biofilms was investigated in varied biofilm growth environments, including relatively quiescent conditions (with a gentle swirl), by culturing biofilms in well plates as well as in continuous flow conditions (Reynold's number, Re = 17) by culturing biofilms in capillary flow cells (with and without residual chlorine) for both long and short durations. Direct visualization of biofilm dispersal over time allowed us to explore the temporal transformation in the biofilm matrix upon DPA exposure. Well plates, due to their operational simplicity and ability to accommodate more replicates, were used for dose−response screening of DPAs. In contrast, the use of chlorinated and nonchlorinated flow cell experiments allowed the study of simulated downscaled pipeline systems, providing a robust visual investigation of DPA efficacy against pipe biofilms grown under continuous fluid shear stress.

2. MATERIALS AND METHODS

2.1. Microorganisms and Culture. Along with an unknown multispecies culture derived from WDS, *S. epidermidis* (ATCC strain 35984) was used in this work as it is a recognized biofilm-forming species. *S. epidermidis* was maintained on tryptic soy broth (TSB) agar plates, and a single colony from an agar plate was aseptically transferred to a 250 mL flask containing 100 mL of autoclaved TSB medium. After incubating overnight at 37 °C on a shaker table (130 rpm), the inoculum (OD_{600} = 0.5−0.7) was transferred to a 96-well plate or a flow cell to initiate the growth of biofilm. For WDS multispecies culture, the inoculum was prepared by seeding 10% WDS tap water into a 250 mL flask containing 100 mL of full-strength autoclaved R2A media and incubating for 72 h at 37 °C on a shaker table (130 rpm).

2.2. Detachment Promoting Agents. DPAs selected for this study include three metal chelators (EDTA, sodium citrate, and STP) and one chaotropic agent (urea). DPA test solutions were prepared by dissolving reagent-grade chemicals in a 10% phosphate-buffered saline (PBS) solution (pH = 7). The 10% PBS solution without DPAs served as a negative control.

2.3. Well Plate Experiments. Microbial culture was grown in TSB (single species)/R2A (multispecies) media at 37 °C for 18−20 h, and then 20 mL of overnight culture was added to 180 mL of sterile TSB/R2A media for inoculation. All wells in a 96-well plate (300 μL total well capacity) were filled with 200 μL of freshly inoculated media using an 8-channel multipipettor. Then, plates were covered and incubated with shaking (∼130 rpm) at 37 °C for 24 h. At 24 h, planktonic suspensions and nutrient solutions were aspirated, and wells were rinsed with sterile water. All wells were rinsed three times in this manner, immediately before any analysis.

DPA agents (1−100 mM) or 10% PBS as a control were applied to wells immediately after rinsing. DPA solutions at concentrations 1, 10, and 100 mM were prepared by dissolving reagent-grade chemicals in a 10% PBS solution (pH = 7), and 200 μL of a DPA solution was applied and incubated in the shaker table for 1 h. After 1 h, the DPA agents were removed by rinsing the wells three times with water. Then, the wells were stained with crystal violet (CV). A 0.1% of 200 μL CV solution was added for staining the wells and shaken in a shaker table for 5 min at room temperature. After that, the

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wells were rinsed three times to remove excess stain, then filled with 200 μL of 95% ethanol and shaken for 5 min at room temperature.

Finally, light absorbance was measured in all wells, whether treated or untreated. Immediately after solubilization of the stain with 95% ethanol, the plate was vigorously shaken for 10 s and then a BioTek FL600 plate reader was used to read light absorbances at 600 nm. Higher values of absorbance indicate a higher concentration of the CV stain and, consequently, a larger biofilm biomass in the well. Replicates of 12 (multi-species)/16 (single-species) samples were taken for each DPA concentration. The treatment performance of different concentrations of DPAs was assessed by comparing the OD_{600} values with the control.

2.4. Capillary Flow Cell Experiments. Capillary flow cells are borosilicate glass capillary tubes with a square cross-section (1 mm × 1 mm × 140 mm; Biosurface Technologies, Corp., Bozeman, Mont.), which were mounted on a flow cell holder, and the entire device was then placed on the stage of an optical microscope (OMAX M434FLR) to allow monitoring of biofilm growth and detachment directly under the microscope. Following the well plate experiment, the maximum concentration of DPAs (100 mM) was applied to both short- and long-term grown biofilms.

Capillary flow cell experiments were conducted for short-term (72 h growth) or long-term (4 months) grown biofilms. For the 72 h duration short-term study, one set of biofilms was grown with *S. epidermidis* pure culture, while another set was with WDS multispecies. The long-term biofilm was seeded with a WDS multispecies inoculum and grown for 4 months under chlorinated conditions (0.25 mg/L free chlorine). For both short- and long-term biofilms, approximately 1 mL of bacterial culture (*S. epidermidis* or a multispecies consortium derived from WDS) was manually injected into the flow cell using a syringe. Both ends of the flow cell were sealed with one-way valves, and the setup was then incubated for 2 h at room temperature (25 °C) to initiate biofilm attachment.

For short-term flow cell biofilms, fresh nonchlorinated tap water with 10% media (TSB for *S. epidermidis* and R2A for multispecies culture) was sterilized (autoclaved for 20 min at 121 °C and 15 psi) and provided using a peristaltic pump (Gilson Minipuls 3) for 72 h at a flow rate of 1 mL/min (Re = 17) following initial attachment of the inoculum. The dimensionless flow parameter Reynolds number (Re) in a flow cell is calculated using the formula $Re = \rho v D/\mu$, where ρ represents the density of water (1000 kg/m^3), v denotes the flow velocity (17 mm/s), D is the flow cell dimension (1 mm), and μ signifies the dynamic viscosity of water (0.001 Pa·s). For long-term flow cell biofilms, tap water with 0.25 mg/L free chlorine and 1% R2A medium was provided at 1 mL/min for 4 months following initial attachment of the inoculum. Tap water from WDS, sterile media stock (100% R2A autoclaved for 20 min at 121 °C and 15 psi), and chlorine stock (100 mg/L free chlorine prepared from a 12.5% sodium hypochlorite solution) were mixed to provide appropriate concentrations of media and chlorine which were then supplied through a flow break (see Figure S1). The flow-break apparatus facilitated mixing while preventing back-flow contamination. To further avoid contamination, stock media was replaced every 72 h. During this biofilm accumulation phase, biomass accumulation in the flow cells was monitored and recorded on the optical microscope (OMAX M434FLR) equipped with a video camera. After reaching the maturity stage (72 h for nonchlorinated and 4 months for chlorinated biofilms), 10% PBS was pumped into the flow cell for 1 min at 1 mL/min to confirm the stability of the biofilms. Finally, biofilms in the flow cells were treated with 100 mM of each DPA solution at a flow rate of 1 mL/min for 20 min. The flow cells were viewed through a 4× objective lens (total magnification = 40×) and recorded at 30 frames/s using the video camera (OMAX A35180U3) connected to the computer. The resulting video files (resolution = 4.0 μm/pixel) were analyzed using ImageJ,[35] as discussed below.

2.5. Microscopic Image Analysis. Biofilms cultivated in capillary flow cells exhibited variable thicknesses, as indicated by a wide range of pixel intensities (0−255) in the histogram plot (see Figure S2). The underlying assumption was that darker pixels represented thicker biofilm regions, while lighter pixels corresponded to thinner biofilms. To quantify the effects of DPAs on biofilms, video files collected from the attached microscopic camera were converted to time-lapse images, and average pixel intensity was measured using the image processing software package ImageJ (ver. 1.48) following multiple steps. First, the blank flow cell image (without biofilms) was subtracted from the other time-lapse images to remove the background diffraction patterns and obtain only the images of biofilms, as well as to account for differences in optics and lighting conditions across flow cell images. After adjusting backgrounds, the average pixel weight (B) was computed for each image. As different flow cells had varied amounts of biomass and brightness before the start of the treatment, a normalized brightness value was computed to allow for comparison of biofilm disruption in different flow cells during treatment. After normalization, % of biofilm remaining at time "t" was estimated as follows

$$\%\ \text{normalized biofilm remaining at time}\ "t"\ (Z_t) = \frac{B_{\text{clean}} - B_t}{B_{\text{clean}} - B_0} \times 100$$

B_t = average brightness value at time "t", B_0 = average brightness value of the flow cell at time t = 0 (start of the treatment), and B_{clean} = average brightness value for a clean flow cell (no biomass).

Biofilm removal is proportional to the pixel intensity (B). Initial biofilm coverage on the flow cell (at t = 0) corresponds to maximum coverage and a Z_t value of 100%. From the above formula, it can be noted that at time t = 0, $B_t = B_0$, and Z_t = 100%, signifying maximum coverage of the flow cell with biofilms. If all the biofilms were removed at the end of the DPA treatment, then $B_t = B_{\text{clean}}$, and Z_t value would be 0% in this case. Thus, based on the treatment performance of DPAs, a Z_t value within 0 to 100% was achieved.

2.6. EPS Proteins and Polysaccharides Quantification in Flow Cell Effluents. While biofilms in the flow cells were exposed to the DPA solution at a flow rate of 1 mL/min for 20 min, effluent samples from DPA-treated flow cells were collected over three-time intervals (0−7 min, 7−14 min, and 14−21 min) and analyzed for total protein and polysaccharides. These data are critical to examining the EPS macromolecules in the detached biofilm clusters. Polysaccharide content in the collected biofilm effluent was measured using the phenol-sulfuric acid method.[36] Reagent-grade D-glucose, phenol, and sulfuric acid (Thermo Fisher Scientific, USA) were used for the phenol-sulfuric acid method. The calibration curve was developed using a standard glucose solution (100 mg/L)

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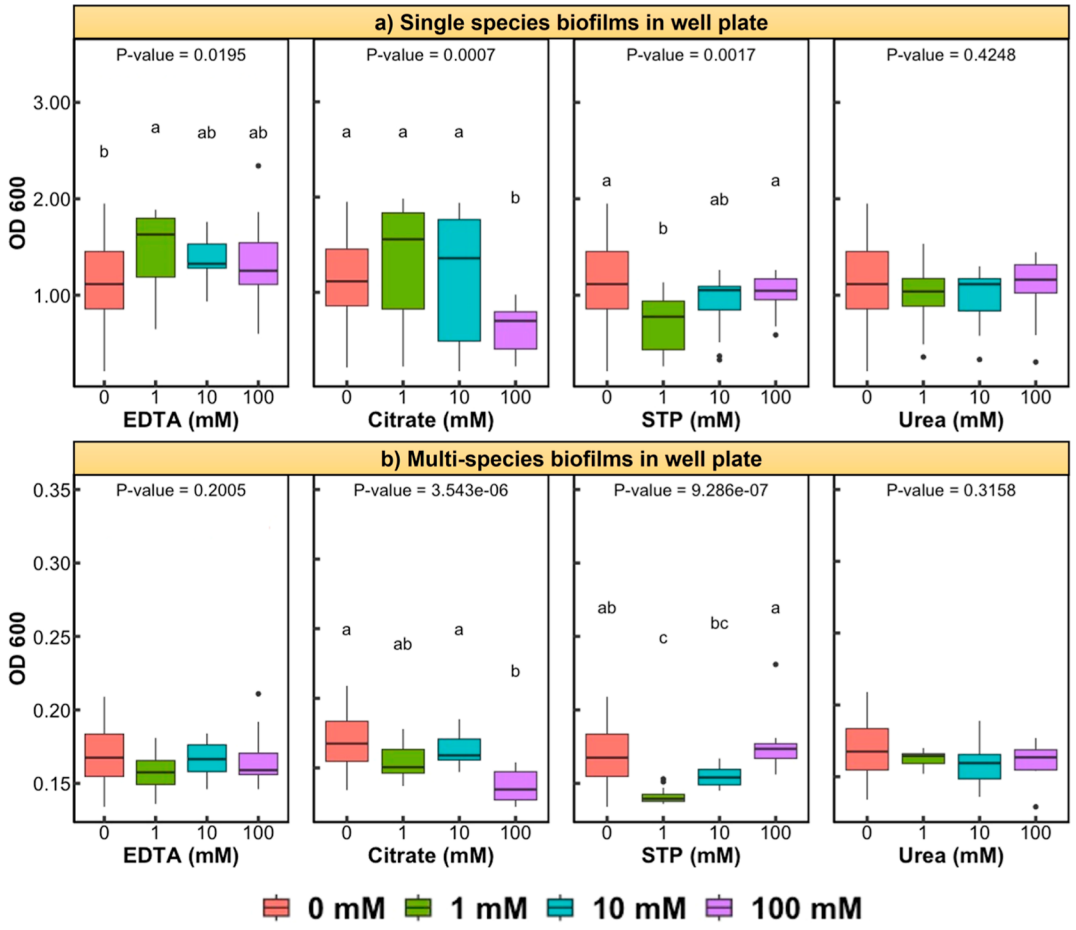

Figure 1. Optical density of well-plate biofilms at 600 nm, 1 h after treatment with DPAs at varying concentrations, (a) for single-species *S. epidermidis* biofilms and (b) for WDS multispecies biofilms. All the *P*-values shown are calculated using the Kruskal–Wallis test. Letters a,b,c denote results from post-hoc statistical comparisons (within a treatment group) at a significance level of $P < 0.05$, with values provided in Table S1.

diluted to 0–100 μg/mL. A 2 mL sample or standard solution was vortexed with 0.05 mL of 80% phenol, and then 5 mL of sulfuric acid was added to this solution rapidly. Following thorough mixing and a 10 min rest, the tubes were placed in a water bath at 25 °C for another 10 min. After final vortexing, absorbance was measured at 450 nm to form the calibration curve and determine the polysaccharide concentration. Proteins in flow cell effluent were measured using the modified Lowry assay kit (Thermo Scientific, USA) and the standard curve was prepared using 1–1500 mg/L bovine serum albumin (BSA). Briefly, 0.2 mL of the sample or standard solution was mixed with 1 mL of modified Lowry reagent at 15 s intervals. Following a 10 min rest at room temperature, 100 μL of Folin–Ciocalteu phenol reagent was added, and the tubes were incubated for another 30 min. Finally, absorbance was measured at 750 nm and used to obtain the calibration curve and determine the protein content in the flow cell effluent samples.

2.7. Data Analysis. To test for statistical significance (*P* value of <0.05) in normally distributed data, ANOVA and posthoc Tukey–Kramer tests were performed. The non-parametric Kruskal–Walli's test and posthoc Dunn's test (with Bonferroni correction or Holm's method) were used for non-normally distributed data. All data and statistical analyses were conducted using R version 4.2.2.

3. RESULTS

3.1. DPA Treatment in Well Plate Experiments. Statistically significant difference in OD_{600} was observed among different treatment concentrations of EDTA ($P = 0.0195$) for *S. epidermidis* single-species biofilms grown on well plates. Posthoc analyses revealed that EDTA treatment at 1 mM showed a significant increase in biofilm as compared to control and there was no significant difference from control at 10 and 100 mM EDTA concentrations (Figure 1a, Table S3). Higher OD_{600} values with EDTA treatment could be suggestive of the ability of *S. epidermidis* to utilize EDTA as a substrate, especially in batch reaction conditions within the well plate environment. EDTA treatment of multispecies biofilms did not show any difference from the control ($P = 0.2$). For both citrate and STP, there was an overall statistical difference in OD_{600} among various treatment concentrations ($P < 0.002$) for both *S. epidermidis* and multispecies biofilms. Additionally, posthoc analysis using Dunn's test revealed similar biofilm removal with all treatment concentrations of citrate and STP for both biofilms; only citrate at 100 mM and STP at 1 mM showed a reduction in biofilm compared to the control. Urea treatment did not show any impact on biofilm removal for either of the biofilms ($P > 0.3$).

Overall, trends in DPA treatment results for single-species well-plate biofilms were quite consistent with multispecies well-plate biofilms, suggesting that DPA agents significantly influenced biofilm dispersal regardless of the type of biofilm

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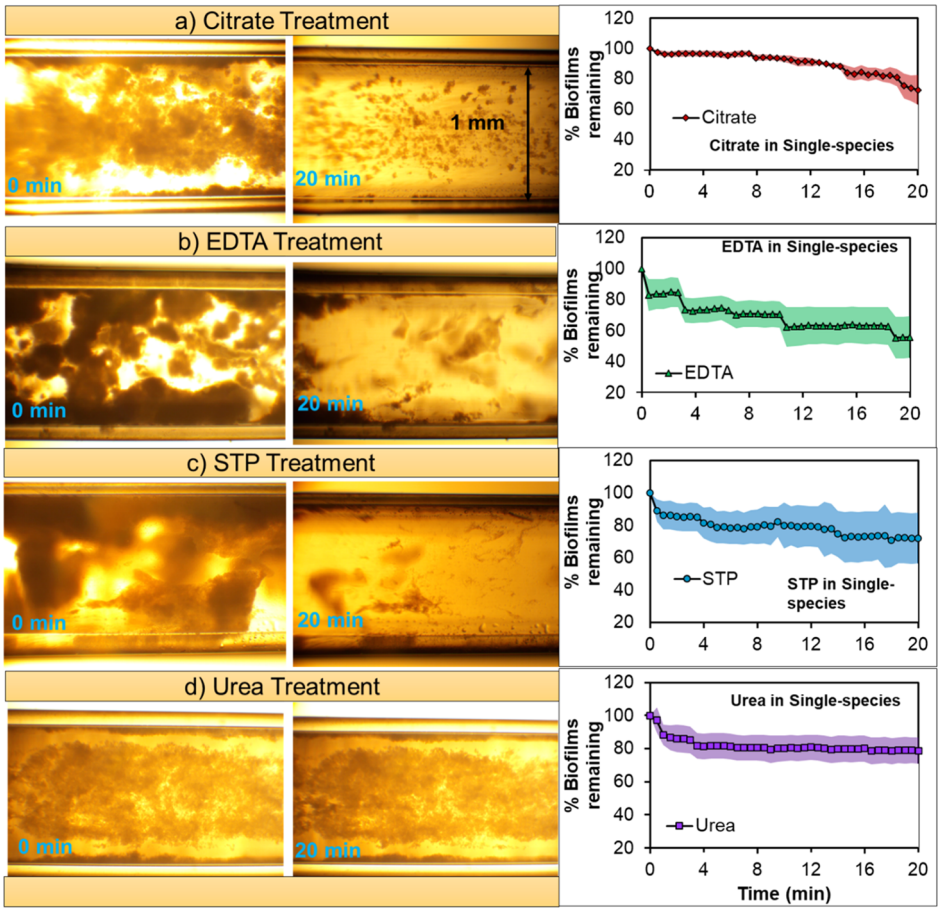

Figure 2. Time-lapse images showing removal of single species *S. epidermidis* biofilms in flow cells after treatment with DPAs (a: EDTA, b: citrate, c: STP, d: urea). 0 min image represents the state of the biofilm at the end of the 72 h growth period and beginning of DPA exposure and the 20 min image shows the biofilm state at the end of the DPA exposure. Plots on the right show the % biofilms remaining (mean ± S.E.) at different times during exposure to DPAs.

considered. Specifically, higher removal was noted with increased concentrations of citrate, while STP treatments showed the opposite trend with increasing concentration. Considering these findings and to allow experimental consistency, a 100 mM concentration of each DPA was selected and used for capillary flow cell experiments.

3.2. Effects of DPAs on Capillary Flow Cell Biofilms. *3.2.1. Flow Cell Image Analysis.* To investigate the effect of DPAs on biofilm dispersal in a flowing environment characteristic of water pipelines, we employed capillary flow cells. After 72 h/4 months of biofilm growth in the flow cells, DPA treatments were conducted (in triplicate flow cells) for 20 min at the same flow rate (1 mL/min).

At the end of the 72 h-long growth period, over ∼90% of the area of most flow cells was visibly covered with biofilms formed by the *S. epidermidis* culture (Figure 2). At the end of 20 min of DPA treatment, EDTA caused maximum mean biofilm removal, and the percentage of biofilm remaining in the flow cells (mean ± S.E.) was 72 ± 10%, 55 ± 13%, 72 ± 16%, and 78 ± 8% for citrate, EDTA, STP, and urea treatments, respectively (Figure 2). For citrate, there was a continuous and gradual reduction in biomass without any abrupt changes (Figure 2a). Similarly, STP also showed a nearly continuous suppression of biofilm (Figure 2c.). On the contrary, sudden sloughing events caused considerable biomass loss for EDTA treatment (Figure 2b). For the first 1 min of treatment, EDTA removed 20% biofilm. After 3 min, an additional 10% of the biofilm dispersed suddenly. A similar amount of sudden sloughing (∼10%) was observed at 10 min of EDTA exposure. For urea, there was continuous dispersion of biofilm for the first 4 min of treatment, and almost no removal was observed after the initial (∼20%) suppression (Figure 2d).

The comparison of images for single- and multispecies biofilms (Figures 2 and 3) indicates more compact biofilms for undefined WDS multispecies compared to single-species biofilms. Multispecies biofilms responded differently to DPA treatment than single-species biofilms. Upon exposure to DPAs, no appreciable removal (<5%) was observed for multispecies biofilm treatment with 100 mM concentrations of citrate, EDTA, and urea (Figure 3a,b,d). Treatment with STP showed around 10% reduction in biofilm coverage at the end (Figure 3c).

To closely simulate the WDS environment and longer term biofilm growth, multispecies biofilms were grown for 4 months under minimal media (1% R2A) and free residual chlorine of 0.25 mg/L. Flow cell images of biofilms from long-term growth experiments indicated a looser and thinner biofilm (low pixel intensity in the image, Figure 4) than short-term grown multispecies biofilms (Figure 3). Upon exposure to DPAs, the long-term chlorinated biofilms showed a similar removal pattern as that of short-term multispecies biofilms. There was negligible dispersal (<5% for citrate, EDTA, and urea),

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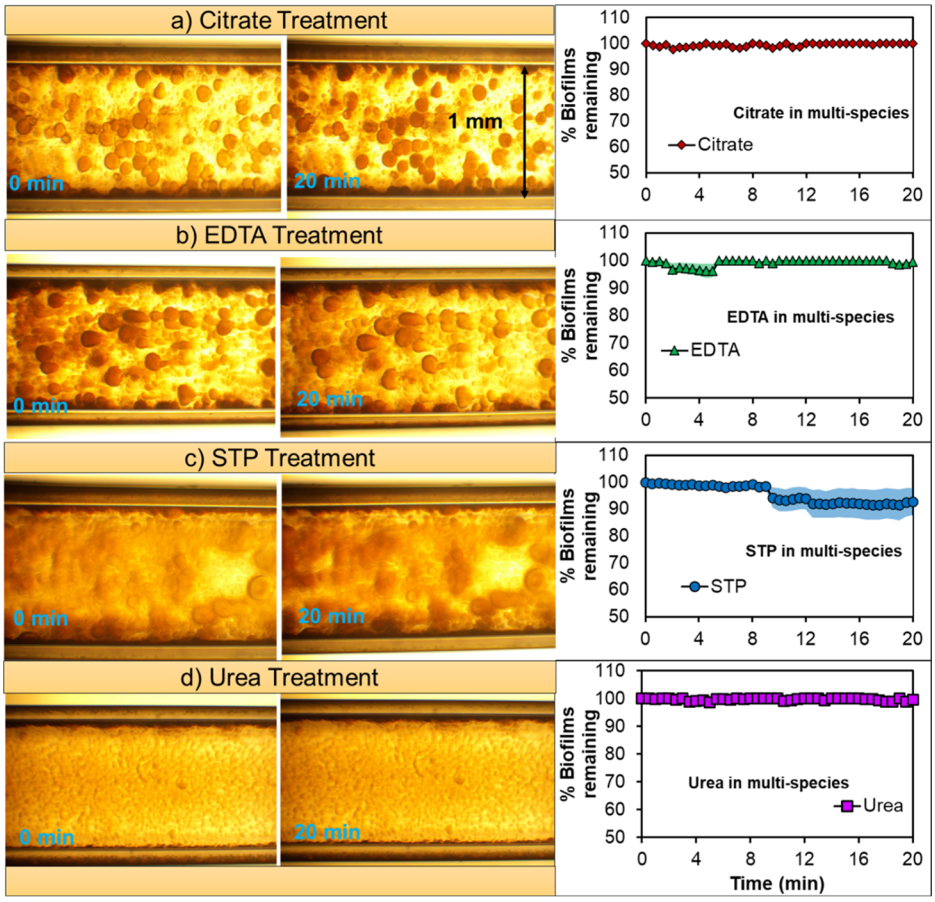

Figure 3. Time-lapse images showing the removal of WDS multispecies short-term biofilms in flow cells after treatment with DPAs (a: EDTA, b: citrate, c: STP, d: urea). 0 min image represents the state of the biofilm at the end of the 72 h growth period and beginning of DPA exposure and the 20 min image shows the biofilm state at the end of the DPA exposure. Plots on the right show the % biofilms remaining (mean ± S.E.) at different times during exposure to DPAs.

while STP showed around a 10% reduction in biofilm by the end of the experiment.

Although there is some variability in the performance of different DPAs, statistical analysis showed that for none of the DPA agents, biofilm removal differed significantly from others (Figure 5). Each of the DPAs showed a wide range of dispersal performance upon exposure to different biofilm types. In the case of single-species biofilms, the ranges of removal percentages at the end of 20 min treatment were 8−53%, 6−66%, 1−72%, and 1−38% for citrate, EDTA, STP, and urea, respectively. For short-term grown mixed culture biofilms, the dispersal range with STP was 1−22%, while it was 6−17% for long-term grown chlorinated biofilms (Figure 5). Overall, DPA interaction in single-species biofilms showed a multifold higher removal rate compared to multispecies biofilms treated with the same level of DPAs (Figure 5).

3.2.2. EPS in Flow Cell Effluents. The cumulative amounts of proteins and polysaccharides released from both single-species and multispecies biofilms showed a wide range of variability under different DPA exposure conditions (Figure 6). At 7 min, the cumulative mass of EPS (i.e., proteins and polysaccharides) was measured for a 7 mL effluent sample released from each of the flow cells (1 mL/min flow rate). The volumes were 14 mL at 14 min, and the final cumulative mass corresponded to 20 mL samples at 20 min. For single-species biofilms, the cumulative mass of polysaccharides (mean ± S.E.) was maximum for urea (2.1 ± 0.97 mg) at 20 min, followed by STP (1.67 ± 1.05 mg) and EDTA (1.67 ± 0.21 mg). A similar trend of highest urea release, followed by STP and EDTA, was observed at 7 and 14 min (Figure 6a). From Figure 6b, the maximum amount of protein released from single-species biofilms was recorded for EDTA treatment (17.86 ± 2.02 mg) after 20 min. For STP, urea, and citrate, the cumulative mass of proteins at 20 min was 10.7 ± 3.82, 8.9 ± 5.16, and 5.89 ± 5.07 mg, respectively (Figure 6b).

For WDS multispecies biofilms, cumulative amounts of polysaccharides in effluents for citrate, EDTA, and STP treatment had nearly similar values (within ∼0.2 mg at 20 min) for all three-time intervals (Figure 6c), while polysaccharide release for urea was consistently lower (Figure 6b). For cumulative protein release at 20 min, EDTA and STP treatments were dominant (∼3 mg). Urea and citrate treatment followed the same trend in protein release for all different time intervals, and it was around 1 mg at the end of 20 min treatment (Figure 6d).

As shown in Figure 7, the comparison of the average EPS release at different biofilm types showed a wide range of variability. For single species, there was no significant difference ($P > 0.05$) in polysaccharide concentrations for different DPA treatments. Although the Kruskal−Wallis test showed no significant ($P > 0.05$) effects of DPAs on protein levels, mean protein release in EDTA is apparently higher than other DPA agents in single species. For multispecies biofilms, polysaccharides released during urea treatment are significantly

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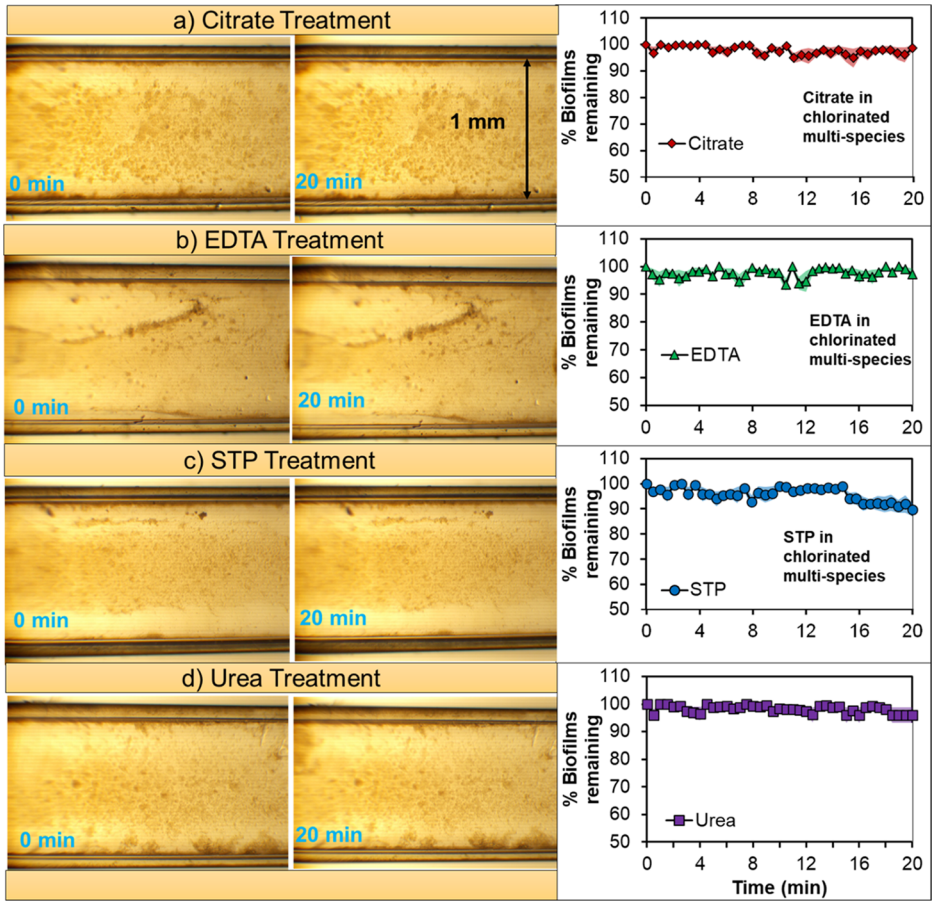

Figure 4. Time-lapse images showing the removal of multispecies long-term (4 months) chlorinated biofilms in flow cells after treatment with DPAs (a: EDTA, b: citrate, c: STP, and d: urea). 0 min image represents the state of the biofilm at the end of the 4-month growth period and the beginning of DPA exposure. The 20-min image shows the biofilm state at the end of the DPA exposure. Plots on the right show the % biofilms remaining (mean ± S.E.) at different times during exposure to DPAs.

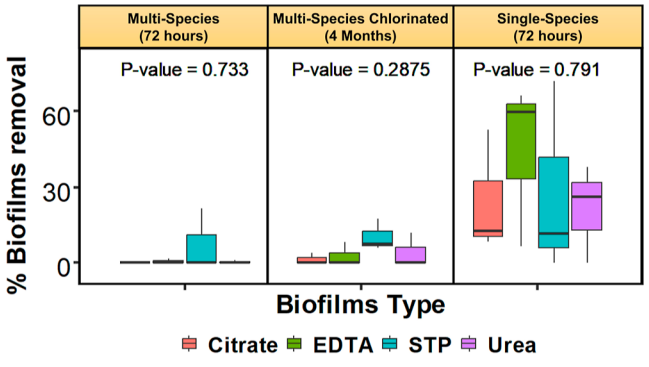

Figure 5. Percentage biomass removal from flow cells of short-term multispecies, long-term multispecies (chlorinated), and single-species *S. epidermidis* biofilms after 20 min of treatment with DPAs. The *P*-values for multispecies and chlorinated multispecies biofilms are calculated by the Kruskal–Wallis test, and the ANOVA *P*-value is used for single-species biofilms.

lower ($P < 0.05$) than others. A significantly higher ($P < 0.05$) amount of protein was released during EDTA treatment compared to citrate for multispecies biofilms. From Figure 7a,b, the median concentrations of EPS polysaccharides for single-species biofilms are 5 to 10-fold higher than the mixed species with similar DPA treatment. Similarly, the median protein levels with different DPA treatments were 2 to 5-fold higher in *S. epidermidis* biofilms compared to the corresponding mixed species (Figure 7c,d). Due to very low dispersion performance in mixed species, the EPS study was conducted only for the short-term (72 h-old) grown WDS biofilms.

4. DISCUSSION

The growth of biofilms in water distribution systems and premise plumbing is unavoidable. Many adverse impacts, including pressure loss, corrosion, and public health concerns from bacterial/pathogenic contamination necessitate the dispersal of biofilms from such wet or moist surfaces. To overcome the potential limitations of traditional antimicrobial agents, this study explores the effectiveness of nonantimicrobial chemicals on biofilm removal, where weakening of biofilm EPS bonding was considered a major removal mechanism.

Here, we investigated the effects of four DPAs on three different types of biofilms grown in capillary flow cells (72 h *S. epidermidis* biofilm, 72 h WDS derived multispecies biofilm, and 4-month WDS derived multispecies biofilm in the presence of residual chlorine). Prior to biofilm experiments using flow cells, the dose–response of DPA concentrations for biofilm dispersal were obtained using well plate experiments conducted for both single-species *S. epidermidis* biofilms and WDS multispecies biofilms. Biofilm dispersal performance (even using identical DPA exposure conditions) varied for different biofilm types. Similarly, the mass of EPS proteins and polysaccharides released during the detachment of single-

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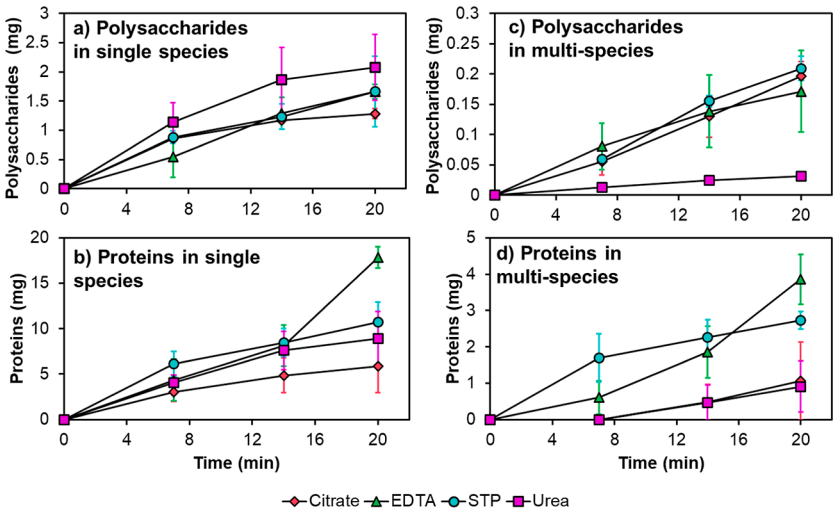

Figure 6. Cumulative amounts of (a) polysaccharides and (b) proteins released at different times from each DPA-treated flow cell for single culture *S. epidermidis* biofilms. Cumulative amounts of (c) polysaccharides and (d) proteins released from each DPA-treated flow cell for short-term WDS multispecies biofilms. Error bars show the standard error of three flow cells.

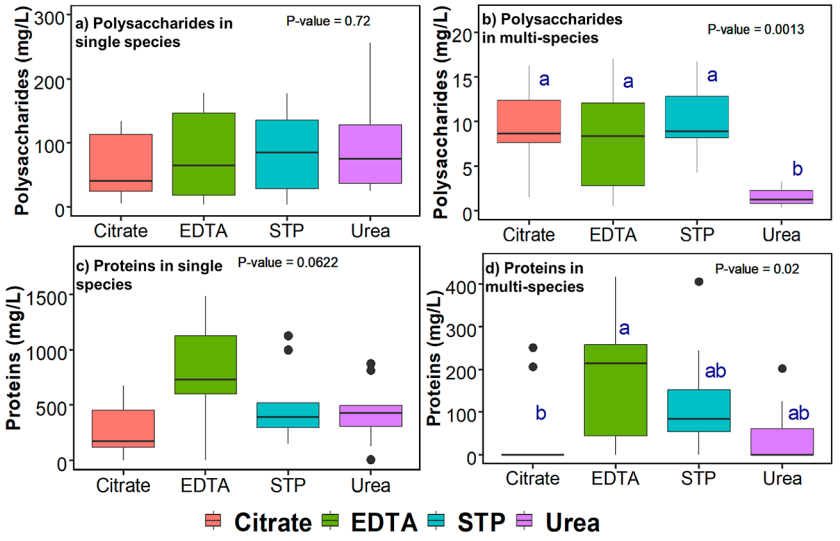

Figure 7. Amounts of polysaccharides released from DPA-treated flow cells for (a) single-species *S. epidermidis* biofilms and (b) multispecies short-term biofilms. Amounts of proteins released from DPA-treated flow cells for (c) single-species *S. epidermidis* biofilms and (d) multispecies short-term biofilms. Boxplots showing the first and third quartiles, and whiskers are extended for ±1.5 IQR. The Kruskal–Wallis tests and posthoc Dunn's test (with Holm's correction) were used to calculate statistical differences. 7 mL samples were collected from each flow cell every 7 min (1 mL/min × 7 min = 7 mL) and analyzed for protein and polysaccharide mass—for a total of three samples over 21 min. The number of samples in each box plots $n = 9$ (3 flow-cell replicates × 3 7 min samples for each flow cell). Letters a,b,c denote results from post-hoc statistical comparisons (within a treatment group) at a significance level of $P < 0.05$.

species biofilm was 4–10 times higher than that of the multispecies (Figures 6 and 7). Images of post-treatment biofilms revealed some clear structural differences between multispecies biofilms and those formed by *S. epidermidis* (Figures 2 and 3). Notably, single-species biofilms seem to have a looser, less-dense appearance (evidenced, for example, by the presence of taller and irregular biofilm structures and streamers; Figure 2) as compared to multispecies biofilms. Distinct structural and morphological characteristics in the multispecies biofilms (e.g., dark and regular biofilm flocs in Figure 3) could be due to the symbiotic mutualism between various species, even under constrained nutrition and aeration.[37,38] Similarly, biofilms grown under long-term and chlorinated conditions are different in structure than short-term multispecies biofilms (Figures 3 and 4). This indicates the implications of bacterial species and growth conditions on the structure, composition, and strength of the biofilms. In addition, the difference in growth conditions can lead to a significantly different response of the biofilms to DPA exposure.

For *S. epidermidis* biofilms in the flow cell, only the chelator EDTA was effective at inducing half of the initial biofilm removal (Figure 2b), while STP and citrate reduced the biofilm to two-thirds of the initial level with equivalent molar concentrations of 100 mM. The comparative potency of the three chelators suggests that multivalent metal ions are important in stabilizing the EPS matrix of *S. epidermidis* biofilms. The amounts of polysaccharide removal for *S. epidermidis* biofilms were similar across different treatments (Figure 7a), but the amount of protein released was highest for

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the EDTA treatment (Figure 7c). Considering that EDTA treatment was most successful against *S. epidermidis* biofilms grown in flow cells (Figure 2), it can be inferred that the protein component of *S. epidermidis* EPS is one of the primary contributors to the EPS structure and a main driver of *S. epidermidis* mechanical strength. Comparatively higher disruptions by EDTA in single-species biofilms could also be explained, in part, by its higher stability constant than other chelators. The stability constant (K^c) represents the affinity of a ligand for a specific metal ion. The stability constant values for EDTA (log K^c: Ca^{2+} = 10.6, Mg^{2+} = 8.8, and Fe^{2+} = 14.3) are higher than those for STP (log K^c: Ca^{2+} = 6.5 and Mg^{2+} = 5.8) and citrate. Previous studies concerning chelator treatments of *S. epidermidis* and other single-species biofilms yielded varied results. Some studies reported that EDTA treatment successfully reduced the biofilms. For example, Ramage et al. (2007) reported that EDTA (250 mM) treatment reduced around 31% of the biomass of a *Candida albicans* biofilm.[39] Lieleg et al. (2011) reported significant reductions in "biofilm elasticity" for the *P. aeruginosa* biofilm exposed to 25 mM EDTA and 250 mM citric acid treatments.[40] On the other hand, some studies got inconsistent results from biofilm treatment with EDTA. For example, Zenga et al. (2012) reported that EDTA delays biofilm formation;[41] however, its capacity for biofilm inhibition/reduction is inconsistent. The same study also reported that some strains of *P. aeruginosa* biofilm even increased biofilm production over control after treatment with 1−10 mM EDTA. Exposure of *S. epidermidis* and *P. aeruginosa* colony biofilms to 200 mM EDTA did not lead to any statistically significant weakening, according to rheometry testing.[33] Similarly, in the current work, we also noted varied responses of biofilm to EDTA treatment. Although the flow cell study on single-species biofilms showed superior removal performance of EDTA over other DPAs, results from the well plate study showed that there was no significant reduction in mean biofilm biomass upon EDTA exposure (compared to control) for both multispecies and single-species biofilms (Figure 1). Citrate with a 100 mM concentration has the maximum biofilm disruption in the well plate study (Figure 1).

For both short- and long-term multispecies biofilms, none of the DPAs proved their efficacy for considerable biofilm dispersal (Figures 3 and 4). However, the relatively improved efficiency of STP over other chelators in multispecies biofilm may be due to their physical or molecular reasons. STP molecules have a relatively linear structure. A smaller molecule is more likely to penetrate the compact structure of multispecies biofilm[42] and exert an influence over more of the biofilm volume. Previous studies reported that STP can also inhibit single-species biofilm formation by inactivating planktonic cells.[22,24] Lee et al. (2019) reported different biofilm inhibition percentages for different species,[22] which proved that the effect of STP varies depending on the biofilm type (and thus also the EPS type). For both single-species and multispecies biofilms, the results from our well plate study showed that biofilm removal was significantly higher at low STP concentrations (1 mM) compared to higher STP doses (10−100 mM). A similar result of a higher biomass removal rate (27.2%) at lower STP concentrations (0.025 to 0.4%) and a comparatively lower biomass removal rate (10.6%) at higher STP concentrations (0.05 to 0.4%) for *Prevotella intermedia* biofilm was reported by Jang et al. (2016).[24] At lower concentrations, STP is capable of diffusing through the EPS matrix, penetrating deeper into the biofilm matrix, and breaking it down more effectively.[24] However, future studies need to explore the exact mechanism for better performance at lower STP dosages.

Citrate showed a removal performance almost similar to that of STP for single-species biofilms in flow cells (Figure 2a,c). Also, the mean OD_{600} was significantly reduced from that of the control for single-species biofilms in the well plate study (Figure 1a). Although the flow cell study showed negligible removal by citrate for multispecies biofilms, the well plate study showed a significantly lower mean value than the control (Figure 1b). Previous studies support our result for well-plate multispecies biofilms. Yao et al. (2020) reported that sodium citrate significantly affected the microbial community and the 3D structure of early-stage multispecies biofilms.[43] Similarly, multiple studies have reported that sodium citrate inhibits the growth of *C. albicans*, *Staphylococcus aureus*, *Escherichia coli*, *S. epidermidis*, and *P. aeruginosa* biofilms.[30,31,44−47] Our results showed that biofilm removal significantly increases with increased sodium citrate concentration for both single and multispecies biofilm. Similarly, Liu et al. (2019) reported that the volume of *S. aureus* biofilm decreases with increased sodium citrate concentration.[44]

In the case of citrate and STP in flow cells of single-species, the results showed continuous suppression of biofilms until the end of treatment (Figure 2a,c), while there was intermittent sloughing of 10−20% of biofilm surface for EDTA (Figure 2b). This could be explained by the structure and composition of the chemicals. Sodium citrate (MW: 258.07 g/mol) and STP (MW: 367.82 g/mol) are the sodium salts of citric acid and phosphoric acid, respectively, and have relatively simple linear structures. On the contrary, the branched structure of EDTA (MW: 372.24 g/mol) having four carboxylic acid groups and two amine groups forms the hexadentate ligand structure, and this may offer higher steric hindrance during interaction with the EPS. These differences in structure and size of EDTA over STP and citrate might contribute to its intermittent biofilm dispersion (Figure 2). Similarly, due to the differences in texture and EPS composition in biofilm types, the branched structure of EDTA was less effective than STP for multispecies compared to single-species biofilms.

From the urea exposure of biofilms in the well plate study, the mean biofilm reduction was not significantly different from controls for both biofilm types (Figure 1). Similarly, although the effectiveness of urea was not observed for multispecies biofilms in flow cells (Figure 3d), there was some removal (22%) of *S. epidermidis* biofilm at concentrations of 100 mM urea. The maximum mass of polysaccharides released during urea treatment of *S. epidermidis* biofilms and corresponding biofilm weakening suggests that hydrogen bonding may be an important stabilizing force for the single-species biofilms. This is because polysaccharides in biofilms form a complex network within the cell surface, where hydrogen bonds are predominant for the bonding of polysaccharides containing hydroxyl groups.[48,49] Also, significant amounts (mean ± S.E.) of proteins (8.9 ± 5.16 mg) released with effluents indicate the solubilization of EPS protein during urea treatment. Since urea is a protein denaturant, its effect on biofilm may depend on EPS composition and protein content. Past studies have demonstrated biofilm weakening[33] and enhanced biofilm removal[34,50,51] upon treatment with urea at concentrations ranging from 0.2 to 2 M. For example, Chen & Stewart (2000) reported 73% biofilm protein removal from binary biofilms of

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P. aeruginosa and *Klebsiella pneumonia* using 200 mM urea.[34] Jones et al. (2011) reported that 200 mM urea was effective at disrupting *S. epidermidis* biofilms but not *P. aeruginosa* biofilms.[33] Another study reported 14.1, 18.3, 34.0, and 55.6% mean biomass removal, respectively, by 0.5, 1, 2, and 5 M urea treatments for *S. epidermidis* biofilms.[52] Therefore, biofilm reduction increases with increasing urea concentrations. Here, we used urea concentrations (1−100 mM) relatively lower than those in the reported studies. Therefore, it is not unusual to obtain an insignificant biofilm removal rate from these low-dose urea treatments. A previous study on the effects of the same strength (100 mM) urea on *S. epidermidis* biofilms grown in capillary flow cells showed almost complete removal of the biofilm.[32] The flow rate of the treatment for that study was, however, 10-fold higher (10 mL/min) than the flow rate during biofilm growth. Similar to the current study, most of the dispersal occurred in the first two min of treatment. Thus, urea could have a substantial impact on the reduction of biofilms' cohesiveness, especially when used in conjunction with the mechanical stress of flushing. Overall, *S. epidermidis* biofilms were effectively weakened by 100 mM of EDTA, citrate, and STP. Urea seems to be the least effective for single-species biofilms. Although some dispersion was achieved with STP, multispecies biofilms demonstrated higher mechanical resilience in response to all DPA treatments.

To compare the impacts of single-species and multispecies biofilm on DPA treatment, a known biofilm former and Gram-positive species *S. epidermidis* was used in this study owing to its wide application in short-term biofilm studies. As WDS often contain both Gram-positive and Gram-negative bacteria, future research could consider the effects of Gram-negative bacteria (e.g., *Pseudomonas* spp. and *Acinetobacter* spp.) on DPA interactions, which are abundant in WDS and have strong biofilm-forming potential.[53] Due to the limitation of obtaining undisturbed biofilm samples from capillary flow cells for microscopic analyses of the EPS, this study primarily used the chemical-based spectrophotometric method for EPS protein and polysaccharide content. However, there is a wide range of sensitive methods available for EPS analysis that could provide better insights into the functionalities of EPS components (e.g., peptide bonds and carbonyls) and high-resolution images of the EPS structure and interactions.[54] These include spectroscopic methods such as FTIR (Fourier transform infrared spectroscopy)[55] and NMR (nuclear magnetic resonance spectroscopy;[56] microscopic methods such as CLSM (confocal laser scanning microscopy), ESEM (environmental scanning electron microscopy), SEM (scanning electron microscopy), and AFM (atomic force microscopy); and spectral microscopy methods such as Raman microscopy. Although pre-DPA-treated biofilms were cultivated under identical growth conditions (nutrients, temperature, residual chlorine, and flow rates), some morphological variability was observed (e.g., Figure 2). This non-uniformity is likely due to the inherent heterogeneity in biofilms, which is well-recognized in the literature.[57−59] While this variability could potentially affect the treatment effectiveness of different DPAs, triplicate experiments were employed for each treatment scenario to minimize these potential influences. Widely accepted buffer PBS (10%) was used in this study as the negative control,[60,61] where the phosphate level (∼1 mM) was negligible compared to the amount of phosphate (100 mM) in STP. Thus, the competition of PBS with the chelating agent STP for binding metal ions is considered insignificant. However, the alternative buffer tris could be used in future studies to minimize this impact.

In this work, we used borosilicate glass capillaries with substantial optical clarity to allow direct visualization of biofilms. However, compared to commonly used plumbing materials [e.g., galvanized steel, ductile iron, stainless steel, copper, polyvinyl chloride (PVC), high-density polyethylene (HDPE), and cross-linked polyethylene (PEX)], initial attachment of biofilm could be delayed on the smooth surface of the glass materials, resulting in lower biofilm formation.[62] Moreover, borosilicate glass is chemically inert, thermally resistant, and corrosion-resistant, while most plumbing materials are susceptible to corrosion and may introduce trace nutrients for biofilms, impacting biofilm strength.[63,64] Many of these processes, such as corrosion, typically occur over longer time scales (months to years), raising uncertainty about their significant impact on biofilm growth and disruption, especially considering that many biofilm studies are conducted over shorter time scales (hours to days). Nevertheless, the practical application of DPAs in WDS requires research on their interactions with commonly used plumbing materials at the pilot scale (e.g., pipe-loop studies) or controlled field studies.

Overall, this work demonstrates the early promise of DPAs as an additional strategy in the biofilm management toolkit. The observations here suggest that multispecies WDS biofilms are more resistant to DPA treatment than the monospecies *S. epidermidis* biofilms. However, significant biofilm dispersal by DPAs was observed in previous studies once used in combination with other antibiofilm agents.[65,66] The concentrations of DPAs (1−100 mM) in this study are higher than what may be allowable in the WDS, and thus their direct applicability in WDS biofilm dispersal would be challenging. For instance, polyphosphate used for corrosion inhibition, red water control, and scale inhibition in WDS usually does not exceed 1 mg/L as P (32.3 μM).[67−70] The concentration of STP needed for biofilm removal (1 to 100 mM or more) is likely much greater than would be possible for routine use in drinking water pipelines. However, the higher concentrations of DPAs could be acceptable for periodic WDS flushing operations and for use in industrial settings. Going beyond the steady flow conditions (during biofilm growth and DPA exposure) used in this study, a combination of higher flow hydrodynamics (i.e., WDS flushing operation) and DPA interaction could enhance the biofilm removal performance and their efficacy for WDS biofilm control.[32] Comparing the continuous intermediate dose (1 mg/L P), this type of initial high dosing of phosphate (added during flushing) followed by continuous low dosing is also reported to be effective for better corrosion control.[69] This study is limited to 20 min of DPA exposure at 100 mM concentration. The continuous dispersal performance of EDTA, citrate, and STP suggests that a longer exposure to DPAs might lead to higher (or complete) biofilm removal. In addition, further research would be needed to explore the use of multiple DPAs concurrently, in series, or along with traditional antimicrobial treatments to determine if the DPA approach can lead to enhanced biofilm removal in pilot-scale studies.

5. CONCLUSIONS

Biofilms in WDS are unavoidable and could have many adverse impacts, including public health concerns, friction, and corrosion. To overcome the potential limitations of traditional

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antimicrobials and explore the effectiveness of nonantimicrobial chemicals on biofilm removal, we tested the impact of four DPAs (EDTA, STP, citrate, and urea) on the detachment characteristics of single-species and multispecies biofilms. The main conclusions are as follows:

i. Multispecies biofilms grown under both long-term (chlorinated) and short-term conditions demonstrated high resistance in response to DPA treatments. A very low removal rate (~10%) was achieved for STP, while for other DPAs, the removal was negligible (<5%).
ii. Monospecies *S. epidermidis* biofilms were effectively weakened by 100 mM DPA solutions, and the DPA effectiveness from the flow cell study was as follows: EDTA > STP > citrate > urea. The maximum amount of protein released during EDTA treatment could be corelated to biofilm dispersal due to EPS weakening. The well plate study showed promising biofilm dispersal with a minimum of the 1 mM STP level.
iii. Potent metal chelators like EDTA and STP may aid in biofilm removal in a variety of environments. More work is needed to investigate the benefits of multi-DPA treatments, either concurrently or in series. A combination of higher flow hydrodynamics (i.e., WDS flushing operation) and DPA interaction could enhance the biofilm removal performance and their efficacy for WDS biofilm control.
iv. The response of well-plate biofilms to DPAs was different from the response of flow-cell biofilms, likely owing to varied growth conditions in the well plates (batch conditions without shear force and fluid flow). This highlights the potential limitations of well-plate biofilms in accurately recapitulating the behavior of flow-dependent biofilms like those found in WDS environments.
v. Further work is needed to understand the long-term effects and practical implications of DPA treatments in real-world settings. This work underscores the complex nature of biofilm control and the importance of investigating interdisciplinary approaches to addressing biofouling issues in water distribution systems.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsestwater.3c00734.

Schematic of the flow cell reactor, histogram depicting the pixel intensity of biofilm images, and statistical comparisons of the performance of different DPAs in removing biofilms from well plates (PDF)

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CRediT: **Fayzul Kabir** data curation, formal analysis, visualization, writing-original draft; **Md Ibnul Hasan** formal analysis, writing-original draft; **Srijan Aggarwal** conceptualization, funding acquisition, methodology, project administration, supervision, visualization, writing-original draft, writing-review & editing.

Notes

The authors declare no competing financial interest.

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