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BIOFILM REMOVAL CAUSED BY CHEMICAL TREATMENTS

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Abstract—Biofilm protein removal by a variety of chemical treatments was investigated. Binary population biofilms of *P. aeruginosa* and *K. pneumoniae* were grown in continuous flow annular reactors for 7–9 days prior to a 1-h treatment period. Treatments that caused removal of more than 25% of the biomass (as total protein) included NaCl and $CaCl_2$, two chelating agents (EDTA and Dequest 2006), surfactants (SDS, Tween 20, and Triton X-100), a pH increase, lysozyme, hypochlorite, monochloramine, and concentrated urea. Treatments that caused little removal (less than 25%) included a control, $MgCl_2$, sucrose, nutrient upshifts and downshifts, and a pH decrease. The amount of biofilm protein removal and the reduction in viable cell numbers in the biofilm were not correlated. Some treatments caused significant killing but not much removal while other treatments caused removal with little killing. These results underscore the fact that biofilm removal and killing are distinct processes. The chemical diversity of agents that bring about biofilm removal suggests that multiple interactive forces contribute to biofilm cohesion. No pattern of differential removal of the two microbial species could be discerned. © 2000 Elsevier Science Ltd. All rights reserved

Key words—biofilm, removal, killing, biofouling

INTRODUCTION

Biocides and disinfectants have been the principal weapons used to control unwanted biofilms such as those that foul cooling water towers, oilfield produced water pipelines, or food processing plants. These agents work by killing microorganisms, a strategy that is rendered ineffective by the reduced susceptibility of biofilm microorganisms to antimicrobial challenges (Brown and Gilbert, 1993; Stewart *et al.*, 2000). Furthermore, in many systems where problematic biofilm fouling occurs, the desired end result is a clean surface rather than an inactive yet physically intact biofilm. Antimicrobial agents may achieve this indirectly by stopping growth and allowing the natural detachment process to slowly remove the biofilm. Promoting biofilm removal directly would appear to be an attractive alternative approach. This approach might also have the advantage of reducing reliance on inherently toxic control agents whose continued use is fundamentally at odds with the trend towards increasingly restrictive environmental regulations.

Much more is known about biofilm killing than is known about biofilm removal. The efficacy of biofilm control agents is most often reported in terms of a reduction in the number of colony forming units associated with a particular surface area. This measurement combines the effects of killing and removal: the number of viable microorganisms on the surface can be reduced by either the physical removal of cells from the surface or by the killing of a cell that remains attached. Plate count data do not, therefore, allow independent assessment of the extent of removal. This article reports measurements of the extent of biofilm protein removal caused by a variety of chemical treatments. Our intent was to shed light on the mechanisms that stabilize biofilm structure and the mechanisms of biofilm removal rather than to invent practical cleaning formulations. The selection of treatment chemicals reflects this intent.

MATERIALS AND METHODS

Microorganism and culture conditions

Pseudomonas aeruginosa (ERC1) and *Klebsiella pneumoniae* (KP1) were co-cultured on a minimal salts medium, pH 6.5, with 40 mg l^{-1} glucose as the sole carbon source. The medium contained 14.4 mg/l of NH_4Cl, 4.0 mg/l $MgSO_4 \cdot 7H_2O$, 382 mg/l of Na_2HPO_4, 408 mg/l of KH_2PO_4, and trace elements as detailed elsewhere (Chen *et al.*, 1993). Medium components were sterilized by autoclaving and dilution water was filter sterilized. Exper-

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iments were conducted at room temperature which was 25 ± 1°C.

Reactor system and operation

Biofilms were grown on 316L stainless steel slides in a continuous flow rotating annular reactor under aseptic conditions. Salient features of the reactor are summarized in Table 1; a detailed description of the reactor and operating conditions can be found elsewhere (Chen *et al*., 1993). Biofilm was sampled without interruption of nutrient flow or inner cylinder rotation by removing sample slides through stoppered holes in the top of the reactor. The reactor was inoculated with 1.0 ml of thawed stock culture (10^8 cells ml^{-1}) of each microorganism and grown in batch mode for 24 h before starting influent flow. The biofilm was allowed to grow for 7–9 days before a treatment was initiated. An independent reactor was operated for each experiment.

Reactor treatment and sampling

Step changes in solution chemistry were effected by switching to a continuous supply of the treatment chemical at the desired concentration and simultaneously adding several milliliters of concentrated reagent directly into the reactor. The pulse dose to the annular reactor, which is hydraulically well-mixed, was the calculated amount to instantaneously raise the bulk concentration in the reactor to the desired inlet concentration. The continuous dose duration was 60 min. The treatment solution replaced the dilution water feed to the reactor. The flow of concentrated nutrients to the reactor was maintained during treatment so that there was no change in the growth medium.

Biofilm was sampled approximately 30 min prior to the initiation of treatment and every 15 min after the step change. The total sampling period was 75–90 min or about four residence times. Effluent samples were continuously collected through a stopcock in the effluent tubing.

Analytical methods

Biofilm covered slides were removed using aseptic sampling techniques at various times during the experiments. Biofilm was scraped from the slides into 100 ml of phosphate buffer. The solution was homogenized for 0.5–1 min using a tissue homogenizer set at 20,000 min^{-1}. Viable cells were enumerated by performing serial dilutions and plating on R2A and Pseudomonas isolation agar plates using the drop plate method. The same samples were also analyzed for total protein by a modified Lowry method using Sigma kit ♯690-A. Effluent samples were also homogenized and analyzed for viable counts and for total protein. To perform the protein assay on effluent samples, they were first concentrated by centrifugation (12,000 × *g*, 4°C, 10 min) and then extracted in 2–4 ml of an extraction solution containing 10 mM Tris, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride.

The optical density at 650 nm of the reactor effluent was continuously monitored by a Spectronic 20 installed in-line. Data were logged every 60 s to a computer.

Treatment chemicals

Salts were purchased from Fisher Scientific. Surfactants and enzymes were purchased from Sigma. Lysozyme from chicken egg white had a specific activity of 50,000 units per mg protein. Dequest 2006 (aminotri(methylene-phosphonic acid), pentasodium salt) was obtained from Solutia. Monochloramine was prepared in pH 9 phosphate buffer as described elsewhere (Sanderson and Stewart, 1997).

Table 1. Annular reactor characteristics

Characteristic	Value
Fluid volume	570 ml
Flow rate	31 ml min^{-1}
Dilution rate	3.26 h^{-1}
Wetted surface area	1600 cm^{-2}
Gap width	0.8 cm
Inner cylinder diameter	10 cm
Rotation rate	100 min^{-1}
Reynolds number	4800
Material	Polycarbonate

RESULTS

The addition of various chemicals to a continuous flow biofilm reactor resulted in biofilm protein removal that ranged from 0 to 90% of the biofilm total protein content within a few hours, depending on the treatment chemistry (Table 2). Treatments that induced substantial (greater than 25%) detachment included certain salt solutions (NaCl, $CaCl_2$,), two chelating agents (EDTA and Dequest 2006), surfactants (SDS, Tween 20, and Triton X-100), a pH increase, lysozyme, hypochlorite, monochloramine, and urea (Table 2).

The amount of protein lost from the biofilm as measured by scraping sample slides was positively correlated with the integrated amount of protein

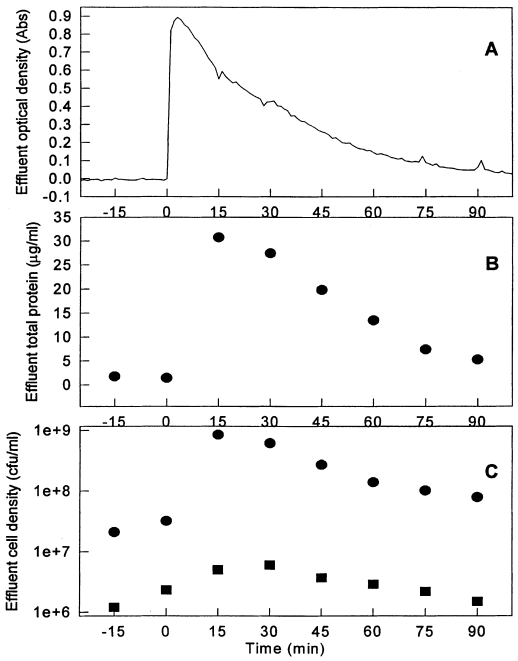

Fig. 1. Typical biomass concentration vs time measurements in the effluent of a biofilm reactor following treatment with 0.3 M NaCl. Treatment was initiated at $t = 0$ and terminated at $t = 60$ min. Biomass was determined by turbidimetry (A), total protein (B), and by colony formation on agar plates (C). In panel C, (●) denotes *P. aeruginosa* and (■) denotes *K. pneumoniae*.

released in the effluent ($r^2 = 0.61$). Although this relationship was noisy, the slope of the regressed line ($m = 0.95$) was not statistically significantly different from one ($p = 0.89$) indicating that these two measures were consistent. The percentage of biofilm protein removal value cited in Table 2 was calculated as the mean of the removal percentage measured by scraping biofilm slides and the removal percentage measured by integrating the mass of protein released in the effluent. Biofilm protein removal measured in this way reflects biomass detached from both the stainless steel sample slides and from the polycarbonate body of the reactor.

The repeatability of these experiments was assessed by performing one treatment, with 0.3 M sodium chloride addition, in triplicate. The coefficient of variation (standard deviation divided by the mean) of the six measurements of percentage of biofilm detached from these three experiments was 0.23. In duplicate treatments with SDS the coefficient of variation of all removal measurements was 0.34. This represents acceptable repeatability for a biofilm experiment. The initial level of biomass was repeatable. The mean areal protein density before treatment was 2.1 ± 0.6 mg cm^{-2} and the log mean areal cell density was 10.5 ± 0.2 cfu cm^{-2} where the uncertainty indicated is the standard deviation.

Biofilm protein removal was evidenced by the appearance of significant quantities of biomass in the reactor bulk fluid and effluent. An illustrative experimental result is plotted in Fig. 1 for a step change addition of 0.3 M NaCl. Biofilm removal was reflected in the transient increase in the concentration of biomass present in the reactor effluent as measured optically (Fig. 1(A)), as total protein (Fig. 1(B)), and by viable plate counts (Fig. 1(C)). A control experiment in which the same volume of buffer lacking NaCl was added did not cause removal as indicated by stable levels of biomass in the effluent (Fig. 2).

Treatments that caused little removal (less than 25%) included the previously mentioned control, $MgCl_2$, sucrose, nutrient upshifts and downshifts, and a pH decrease (Table 2). One of the glucose upshift experiments, in which the influent concentration of glucose was increased from 40 to 800 mg/l, actually gave rise to a negative removal value. This was probably a result of enhanced biofilm growth during the treatment due to the increased availability of the carbon source.

The amount of biofilm protein removal, quantified as the log reduction in areal protein density, was not correlated with log reduction in biofilm viable cell numbers ($r^2 = 0.05$; $p = 0.25$). For example, some treatments (e.g., monochloramine) reduced viable cell counts but gave rise to inconsistent removal. Other treatments (e.g., SDS) caused substantial removal but did not kill.

Table 2. Summary of biofilm protein removal and reduction in biofilm viable cell areal densities caused by various chemical treatments. ND denotes not determined

	Treatment	(%) mean biofilm protein removal	(%) reduction in viable cell numbers
1	Control	2	−6
2	NaCl (0.3 M)	58	50
3	NaCl (0.3 M)	40	61
4	NaCl (0.3 M)	47	59
5	NaCl (0.3 M), 4.5 h after ♯4	7	30
6	NaCl (0.3 M), 200 mg/l chloramphenicol added 3 min before	69	86
7	$CaCl_2$ (0.21 M)	48	31
8	$MgCl_2$ (0.21 M)	23	24
9	EDTA (0.01 M)	26	−49
11	Dequest 2006 (1000 mg/l)	27	ND
12	Sucrose (0.47 M)	8	−29
13	SDS (1000 mg/l)	63	32
14	SDS (1000 mg/l)	79	37
15	SDS (1000 mg/l) chloramphenicol added 3 min before	91	97
16	Triton X-100 (1000 mg/l)	48	ND
17	Tween 20 (1000 mg/l)	27	31
18	Downshift, glucose from 40 to 0 mg/l	11	ND
19	Upshift, glucose from 40 to 140 mg/l	6	ND
20	Upshift, glucose from 40 to 800 mg/l	−47	ND
21	Upshift, nitrate from 0 to 100 mg/l	11	ND
22	pH shift from 6.4 to 2.9	16	60
23	pH shift from 6.4 to 11.2	47	99.8
24	Lysozyme (500 mg/l)	40	87
25	Hypochlorite (15 mg/l, pH 6.4)	47	41
26	Hypochlorite (25 mg/l, pH 6.4)	37	10
27	Hypochlorite (15 mg/l, pH 10.9)	65	99.6
28	NH_2Cl (5 mg/l)	56	96
29	NH_2Cl (7.5 mg/l)	38	92
30	NH_2Cl (10 mg/l)	22	99.9
31	NH_2Cl (25 mg/l)	25	99.8
32	NH_2Cl (25 mg/l)	70	99.4
33	NH_2Cl (100 mg/l)	66	99.7
34	Urea (2 M)	73	ND

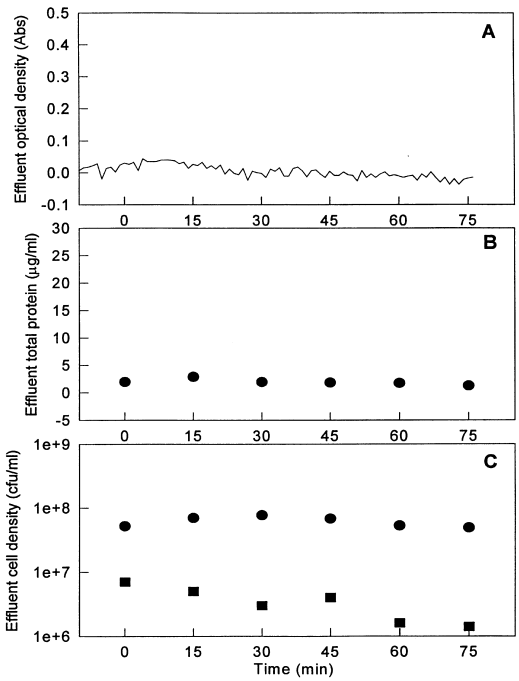

Fig. 2. Biomass concentration vs time measurements in the effluent of a biofilm reactor in an untreated control. Biomass was determined by turbidimetry (A), total protein (B), and by colony formation on agar plates (C). In panel C, (●) denotes *P. aeruginosa* and (■) denotes *K. pneumoniae*.

No preferential removal of one of the two microbial species was observed (Fig. 3).

A protein synthesis inhibitor, chloramphenicol, added 3 min before a 0.3 M NaCl or 0.1% SDS treatment, did not prevent removal. Chloramphenicol by itself did not result in detectable removal but did inhibit the growth of both microorganisms in planktonic cultures.

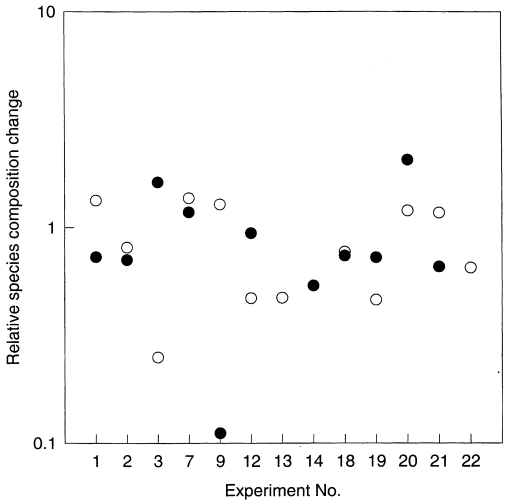

Fig. 3. Comparison of biofilm species composition before and after treatment. The *y*-axis is the ratio of the percentage of *P. aeruginosa* in the sample after treatment to the percentage of *P. aeruginosa* in the sample before treatment. Symbols indicate effluent (○) and biofilm (●) samples.

DISCUSSION

Substantial and repeatable biofilm protein removal was caused by a wide variety of chemical treatments in a two-species biofilm model system. The amount of removal and the amount of killing, as measured by protein loss and reduction in viable cell numbers, respectively, were not correlated. Some treatments removed biofilm without killing microorganisms strongly as indicated by a sharp increase in viable cell numbers in the reactor effluent during treatment (Fig. 1(C)). These observations underscore the fact that biofilm removal and killing are distinct phenomena. Agents that kill will not necessarily cause removal and agents that promote removal may or may not kill microorganisms.

The chemical diversity of agents that bring about biofilm protein removal suggests that multiple interactive forces contribute to biofilm cohesion. Salts such as sodium and calcium chloride may weaken the biofilm matrix by screening out crosslinking electrostatic interactions. These hypothesized crosslinking interactions could be between biopolymer strands carrying opposite charges or between two similar charges that are bridged by a multivalent ion. Removal by concentrated salts does not appear to be an osmotic effect since an isosmotic dose of sucrose gave rise to only about 15% of the removal caused by NaCl treatment. Rather surprisingly, treatment with $CaCl_2$ induced removal equal to that brought about by NaCl. Calcium has been hypothesized to crosslink polyanionic matrix polymers that hold the biofilm together. There is some experimental evidence showing a positive correlation between calcium concentration and the amount of biofilm accumulation (Turakhia and Characklis, 1989; Huang and Pinder, 1995). Following the hypothesis of calcium as a crosslinking agent would lead one to predict that treatment with $CaCl_2$ should actually stabilize the biofilm and should not bring about removal. A possible explanation for this is that the biofilm was already saturated with respect to calcium. In this case, increasing the calcium concentration would not drive more calcium into crosslinking binding sites. The increased ionic strength of the $CaCl_2$ would, however, function to screen electrostatic binding interactions. The measurement of biofilm protein removal following treatments with chelating agents is consistent with an important role for crosslinking multivalent cations in biofilm cohesion (Turakhia *et al.*, 1983; Caccavo *et al.*, 1996). This result agrees with that of Turakhia *et al.* (1983) who showed that immediate and substantial removal of a *P. aeruginosa* biofilm could be effected by addition of the calcium-specific

chelant ethylene glycol-bis-(β-aminoethyl ether)-*N*,*N*-tetraacetic acid (EGTA). Surfactants such as SDS, Triton X-100, and Tween 20 might disrupt hydrophobic interactions involved in crosslinking the biofilm matrix. A role for hydrogen bonding interactions in biofilm cohesion is suggested by the large amount of removal caused by treating a biofilm reactor with concentrated urea, a chaotropic agent. Enzymes such as lysozyme or proteases (Selan *et al*., 1993; Johansen *et al*., 1997) presumably degrade biofilm matrix polymers directly.

Free chlorine (pH 7–11) and monochloramine both caused biofilm protein removal at concentrations in the range of 5–100 mg Cl per liter. While the amount of biofilm removed by chlorine and monochloramine was not statistically significantly different ($p = 0.45$), monochloramine killed bacteria in the biofilm better than did free chlorine at neutral pH ($p = 0.001$). This result is consistent with other studies in which monochloramine was reported to kill biofilm bacteria more effectively than equivalent concentrations of hypochlorous acid (Stewart *et al*., 2000). It is at first counterintuitive that hypochlorite delivered at alkaline pH (treatment ♯26, Table 2) killed more bacteria than did hypochlorite at near neutral pH (treatment ♯24, Table 2). Chlorine is well known to be a more potent antibacterial agent, at least against free-floating microorganisms, at neutral pH, where hypochlorous acid predominates, compared to higher pHs, where hypochlorite ion predominates. The simplest explanation for these data is that pH 11 treatment by itself (treatment ♯22, Table 2) killed large numbers of bacteria. There is not much difference between the effect of pH 11 treatments with and without chlorine. The efficacy of chlorine treatment at neutral pH is attributed to hypochlorous acid, while the efficacy of the alkaline hypochlorite treatment is attributed to the high pH, not the halogen.

De novo protein synthesis does not appear to be required for biofilm protein removal, at least in the cases of NaCl and SDS treatments. Removal by these agents is probably chemically, rather than biologically, mediated.

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