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QUANTITATIVE LIPID BIOMARKER DETECTION OF UNCULTURABLE MICROBES AND CHLORINE EXPOSURE IN WATER DISTRIBUTION SYSTEM BIOFILMS

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Abstract—Biofilms in the drinking water distribution system can protect pathogens from disinfection and provide the inocula for periodic infestations. Assessing these biofilms can be difficult, as the plate counts of pelagic bacteria may bear little relationship to the biofilm load. Culturing the water at the outlet most often does not reflect the biofilm composition. Herein we show that analysis of polar lipid fatty acids recovered from biofilms on devices possessing a large surface area provides quantitative analysis of the viable biomass, community composition, and nutritional status that is independent of the recovery and culturability. Analysis of the polar lipid fatty acids indicated the biofilm contained a stressed and predominantly Gram-negative bacterial community. The composition was not significantly different whether collected in the summer or winter. Oxirane (epoxide) fatty acids were detected in the polar lipids of the biofilm, indicating exposure to chlorine and loss of viability within the biofilm. Tests with monocultures of *Escherichia coli* and *Sphingomonas paucimobilis* exposed to chlorine resulted in oxirane fatty acid generation and rendered them nonculturable. © 2000 Elsevier Science Ltd. All rights reserved

Key words—biofilms, biomarkers, oxiranes, epoxides, mass spectrometry, chlorine

INTRODUCTION

The presence of coliform bacteria in drinking water systems is a major public health concern, as it can indicate fecal contamination and the possibility that other virulent pathogens may be present in the water supply. If there is a loss of residual disinfectant, line breaks, back siphonage, or when there is a treatment plant failure, coliform bacteria have been cultured from drinking water. However, *Escherichia coli* and other pathogens could be present and infectious but non-culturable, particularly when the finished water is exposed to chlorine and the bacteria are injured (Colwell *et al*., 1985). Pathogens such as *E. coli, Legionella bozemanii, Mycobacterium smegmatus*, and oocysts of *Cryptosporidium parvum* readily colonize preformed biofilms developed with *Pseudomonas aeruginosa, Acidovorax* sp. and *Bacillus* sp. isolated from drinking water biofilms (Arrage and White, 1997; White *et al*., 1997). While in the biofilm, the bacteria showed a much greater resistance to chlorine than the unattached cells in finished water (comparing the viable count to the total direct count after 4 days of exposure to 5 ppm chlorine) (Arrage and White, 1997). Clearly the standard cultural methods based on recovery of viable indicators do not protect water consumers from the infectious but non-culturable pathogens (Colwell *et al*., 1985).

All living cells are surrounded by a polar lipid membrane from which the lipids can be extracted and analyzed (White *et al*., 1996a). Because the polar lipids of microbes are essentially phospholipids which have a rapid turnover, the detection of the phospholipid ester-linked fatty acids (PLFA) provides an estimate of the viable biomass (Harvey *et al*., 1987; White *et al*., 1979). Utilizing capillary gas chromatography/mass spectrometry (GC/MS) for the quantitative and qualitative identification of lipids, it is possible to sensitively define the microbial community composition in general terms

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and to quantitatively detect changes in the community (White *et al.*, 1996a, 1997). Since the lipid composition reflects defined responses to the environment during growth, the lipid analysis also provides insight into the nutritional status of the microbial community (White, 1995; White and Macnaughton, 1997). Consequently, analysis of the lipid composition of the biofilm bacteria can provide a quantitative analysis of the biofilms that serve to nurture pathogens and can provide the reservoir for intermittent release of pathogenic cells into the drinking water.

Chlorine, chloramines or chlorine dioxide are often added to finished water for the purpose of disinfection. The residual concentration of disinfectant begins to decline as soon as the water leaves the plant. The concentration of chlorine in the biofilms is typically 20% of that in the bulk water. Equilibration of the biofilm chlorine concentration with that of the bulk water does not occur even after hours of incubation as indicated by microelectrode measurements (deBeer *et al.*, 1994). Chorine reactivity with biofilm components and neutralization of chlorine show diffusion of chlorine and reduce its effects on the pathogens in the biofilm. A chlorine microelectrode sensor is impractical for use in the field due to fragility and noise sensitivity, but lipid analysis of the biofilm can be utilized to detect the effects of chlorine contact within the biofilm. Contact of Gram-negative bacteria with chlorine induces the generation of epoxide formation in the monoenoic PLFA (Phiefer, 1998). These derivatized monoenoic fatty acids in the phospholipids have been established to be oxirane fatty acids by gas chromatography/mass spectrometry through comparison of retention behavior and mass spectral fragmentation patterns with synthetically prepared standards (Smith *et al.*, 1999). Herein we describe the detection of oxirane fatty acids following the chlorine exposure of *Sphingomonas paucimobilis*-based biofilms, and in the PLFA extracted from a biofilm generated by long term exposure to drinking water. Concomitant with the appearance of the oxirane PLFA, the bacteria were shown to be no longer culturable on minimal or enriched recovery media. Oxirane fatty acid detection is suggested as a biosensor to determine the effectiveness of exposure to chlorine in drinking water distribution system biofilms. The organisms released from chlorine exposed biofilms into the drinking water which contain *E. coli* or *S. paucimobilis* are not culturable and possibly are also not infectious if oxirane fatty acids are detected. However infectivity tests of oxirane fatty acids in the polar lipids with animals were beyond the scope of this study.

MATERIALS AND METHODS

Viability of chlorine exposed bacteria

Triplicate late log phase cultures of *E. coli* and *S. paucimobilis* grown in dilute nutrient broth were suspended in 1/100 diluted nutrient broth and exposed at 25°C to 0.5 ppm of free chlorine (hypochlorite) for 10 h. To determine viability at the beginning and end of exposure utilizing the "rescue" media sequence recommended by Mirpuri *et al.* (1997), the organisms were analyzed for PLFA and spread plated with serial dilutions in triplicate on low nutrient R2A agar (Becton Dickinson, Cockeysville, MD) and high nutrient agar, Nutrient agar (Difco, Detroit, MI). Plates were incubated at 25°C, and monitored daily for 14 days.

Biofilm accumulation chambers

Three biofilm accumulation chambers (BAC) were constructed of 316 stainless steel and assembled with Swagelok fittings. They were then packed with approximately 165 g of 1 mm diameter glass beads corresponding to 1540 cm^2 of surface area. One gram contains 297 ± 7.6 single beads. Prior to packing, beads were heated to 450°C for 4 h so as to volatilize any organic residue. The packed columns were cleaned with 1:1 chloroform:methanol to dissolve and remove any lipid compounds present before experimentation.

Sphingomonas paucimobilis biofilms

Late exponential phase *S. paucimobilis* (10^9 cells) were inoculated into 10 l carboys containing 1/100 diluted nutrient broth (chlorine-free), and then pumped through the BACs. The medium was pumped at 4–6 ml/min and a biofilm accumulated for 48 h. After 48 h, filter sterilized (0.2 μM pore size) tapwater rendered free of residual chlorine by titration with 3% sodium thiosulfate was pumped at the same rate through the BAC system as a control. The control was then used for comparison purposes with BACs to which hypochlorite was added in the range of 0.5–2.0 ppm of free chlorine. After 3 days, the BAC beads and membrane filter retentates of the water exiting the BAC were analyzed for PLFA.

Biofilm recovery from tapwater

Three BACs were connected in parallel through a manifold to a laboratory shower head. Water flow was maintained for two weeks at a rate of 20 ml/min and a temperature between 36 and 38°C to select for potential pathogens. Free chlorine concentration of effluent was measured daily by the DPD colorimetric method using a Hach pocket colorimeter (cat. ♯46760-88) and DPD Free Chlorine Reagent Powder Pillows (cat. ♯21055-49). The instrument has a measurement error of ± 0.02 ppm.

Recovery of the lipids

All solvents were of GC grade and were obtained from Baxter Scientific Products (McGaw Park, IL). All glassware was washed in a 10% (v/v) Micro cleaner solution (Baxter Diagnostics, Deerfield, IL), rinsed ten times in tap water, and then ten times in deionized water. The glassware was then heated overnight in a muffle furnace at 450°C to remove carbon contamination. The BACs were opened and the glass beads transferred to a 500 ml glass separatory funnel. The lipids were then extracted from the beads using a single-phase chloroform/methanol/0.05 M aqueous phosphate buffer (pH 7.4) extraction solvent (White *et al.*, 1979). The lipids were recovered, dried under a steam of nitrogen, resuspended in chloroform, and fractionated with batch elution utilizing a silicic acid column (Gehron and White, 1982). The polar lipids were recovered, transmethylated, and analyzed by GC/MS with electron impact ionization at 70 eV (Ringelberg *et al.*, 1988). Oxirane fatty acids were defined by mass spectrometry (Smith *et al.*, 1999).

In the following text and tables phospholipid ester-linked fatty acids (PLFA) are abbreviated by the number of carbon atoms, a colon, number of unsaturations fol-

lowed by the omega symbol (ω) followed by the number of carbons from the methyl end of the molecule to the position of the unsaturation. The prefix "Cy" represents the cyclopropyl isomer of a fatty acid and "i" and "a" represent iso-branched and anteiso-branched isomers, respectively. The number preceding the abbreviation "me" indicates the position of a methyl group relative to the carbonyl carbon followed by the number of carbons in the fatty acid chain.

RESULTS

Impact of chlorine exposure on planktonic E. coli

The time zero hypochlorite exposed and control samples contained $4.5 \pm 0.5 \times 10^4$ colony forming units/ml (CFU/ml). After 10 h, the unexposed controls contained 4.8×10^4 CFU/ml and 7.6×10^4 CFU/ml on the R2A and nutrient agar spread plates, respectively. The exposed cells contained no CFU on either medium. The PLFA profile of the unexposed cultures and exposed cultures are shown in Table 1.

Impact of chlorine exposure on a Sphingomonas paucimobilis biofilm

Similarly, exposure of the *S. paucimobilis* to chlorine (0.5–1.0 ppm) resulted in the formation of 48 ± 4 mol% and for 1.5–2.0 ppm chlorine 64 ± 2 mol% oxirane PLFA in a three day exposure. In contrast, oxirane fatty acids were not detected in the PLFA extracted from the unexposed control biofilms.

Biofilm recovery from tapwater

The BAC described herein was operated at 36–38°C for 20 days in September 1996 and 23 days in November 1996 at a flow rate of 20 ± 7 ml/min. Free chlorine levels ranged between 0.09 ± 0.04 ppm in September and 0.06 ± 0.02 ppm in November. Table 2 shows the proportions of PLFA recovered from the biomass collected within the BAC.

The total viable biomass, measured as the total recoverable PLFA, ranged from 104 to 204 pmol/g beads (11.2–21.9 pmol/cm^2). This value corresponds to between 1.6×10^5 and 8.7×10^5 bacteria the size of *E. coli*/cm^2 utilizing the conversion factor of $1.4–4 \times 10^4$ cells/pmol of PLFA (White *et al.*, 1996b).

Biofilms recovered from the glass beads contained 16 and 18 carbon epoxide (oxirane) fatty acids formed in the biofilms recovered at 36–38°C representing 16.5 ± 3 (November exposure) and 26.6 ± 5 (September exposure) mol% of the total PLFA detected (Table 2). The PLFA patterns of the September biofilms differed from those taken in November primarily in that they contained a lower proportion of saturated PLFA (49.1 ± 6 mole % vs 60.5 ± 4 mole %) and higher proportion of oxirane PLFA (26.6 ± 5 vs 16.5 ± 3 mole %). The biofilms from both September and November contained approximately 7.6 mole % terminally branched saturates (indicated as * in Table 2) which likely represent Gram-positive bacteria (O'Leary and

Table 1. PLFA profiles of chlorine exposed and unexposed *Eschericia. coli.* $N = 3$, standard deviations are shown in brackets

PLFA (mol%)	Chlorine exposed	Unexposed
Saturated	47.3 (3.5)	46.7 (3.8)
Monoenoic	32.8 (0.5)	31.7 (3.6)
Cyclopropyls	19.9 (4.0)	20.9 (0.3)
Oxirane (16 and 18 C)	2.86 (1.6)	Not detected

Table 2. PLFA recovered from the biofilms generated in the BAC chambers; *indicates branched saturated PLFA indicative of Gram-positive bacteria, **indicates monoenoic and cyclopropane and oxirane PLFA derivatives indicative of Gram-negative bacteria, and Mid chain branched saturated PLFA indicative of Actinomyces (mycobacteria) and sulfate-reducing bacteria[a]

Fatty acid groups	mol%	
	November	September
Saturates		
14:0	9.02 (3.30)	4.88 (1.56)
15:0	1.41 (0.18)	1.05 (0.16)
16:0	48.17 (2.17)	40.23 (8.90)
17:0	1.55 (0.24)	1.46 (0.17)
18:0	0.23 (0.40)	0.00
20:0	0.10 (0.17)	0.80 (0.30)
21:0	0.00	0.31 (0.43)
22.0	0.00	0.38 (0.10)
Total saturates	60.48 (4.21)	49.11 (6.22)
Terminally branched saturates		
i14:0	0.50 (0.46)	0.66 (0.33)
i15:0*	3.83 (0.84)	3.34 (0.45)
i15:0*	1.76 (0.35)	1.64 (0.03)
i16:0	0.86 (0.09)	0.78 (0.06)
i17:0*	0.18 (0.31)	0.54 (0.01)
a17:0*	0.45 (0.39)	0.62 (0.02)
Total TerBr sat	7.58 (1.44)	7.58 (0.73)
Monoenoics		
cy17:0**	1.47 (0.76)	1.11 (0.21)
18:0	0.86 (0.31)	1.79 (0.42)
18:1b**	0.28 (0.50)	0.00
19:1a**	0.13 (0.22)	0.61 (0.11)
cy19:0**	3.23 (0.28)	2.44 (0.08)
Total monoenoics	5.97 (1.26)	5.95 (0.04)
Mid branched saturates		
br16:0	0.00	0.17 (0.24)
10me16$^+$	0.00	0.85 (0.20)
br17:0	1.13 (0.31)	0.00
3,10dime19:0$^+$	2.99 (0.48)	2.94 (0.09)
10me18$^+$	5.33 (0.38)	6.01 (0.39)
Total MidBr sats	9.45 (0.78)	9.97 (0.13)
Branched monoenoics		
br18:1	0.00	0.57 (0.81)
Total branched monos	0.00	0.57 (0.81)
Polyenoics		
22:6ω3	0.00	0.24(0.30)
Total polyeonics	0.00	0.24 (0.30)
Oxiranes$^+$		
Oxirane 16:0	10.04 (1.50)	12.67 (1.50)
Oxirane 18:0 a	1.12 (0.37)	3.41 (1.37)
Oxirane 18:0 b	5.36 (1.20)	10.50 (1.86)
Total oxiranes	16.52 (2.80)	26.58 (4.78)

[a]PLFA recovered from the biofilms generated in the BAC chambers exposed to tap water for 23 (November) and 20 (September) days at a rate of 20 ml/min at a water temperature between 36 and 38°C to select for potential pathogens. Free chlorine concentration of effluent was 0.24–1.4 ppm. Summer experiments were conducted in September on two columns and Fall in November 1996 using three columns in parallel. Data are given in mole %. TerBr Sat = terminally branched saturated PLFA; Mid Br Sat = mid branched saturated PLFA.

Wilkinson, 1988), and 9.7 mole % mid chain branched PLFA (indicated as + in Table 2), which were likely from actinomycetes or possibly from sulfate-reducing bacteria (White *et al.*, 1996c). The monounsaturated PLFA, the cyclopropane PLFA and the oxirane fatty acids (indicated as ** in Table 2) represent approximately 32.5 mole % in the summer and 22.5 mole % in the fall. These presumably were derived from the polar lipids of Gram-negative bacteria (Wilkinson, 1988).

DISCUSSION

The BAC provided a quantitative analysis of the biofilm formed from microbes directly as they exit the distribution system. The biofilm represents a stressed predominantly Gram-negative bacterial community (indicated by the high ratio of cyclopropane to monoenoic PLFA). When the biofilm was formed at ambient water temperatures (15–19°C) the biofilm density was 2.85 pmol of PLFA/cm^2 ($0.3–1.1 \times 10^5$ bacteria the size of *E. coli*/cm^2). At warmer temperatures (36–38°C), which would be better suited for the amplification of human pathogens, the biofilm formed on glass beads accumulated to a density of 11.2–21.9 pmol/cm^2 which corresponded to between 1.6×10^5 and 8.7×10^5 bacteria the size of *E. coli*/cm^2.

The biofilm, as assessed through signature lipid biomarker techniques (Table 2), showed the surprising presence of epoxide (oxirane) containing fatty acids which correlated with exposure to hypochlorite. Higher concentrations of hypochlorite seemed to result in greater mole % values for oxirane fatty acids. The oxirane PLFA seemed to be formed at the expense of the monoenoic PLFA. Gram-negative heterotrophic bacteria are characterized by high levels of 16 carbon monoenoic PLFA and the double bond is characteristically localized 7 carbons from the hydrophobic (ω) end of the molecule (White *et al.*, 1996c). Cyclopropane fatty acids are also formed from monoenoic PLFA in phospholipids. The monoenoic PLFA (16:1ω7c and 18:1ω7c) are increasingly converted to cyclopropane fatty acids (cy17:0 or cy19:0) in Gram-negative bacteria as microbes move from a log to a stationary phase of growth (Kivett and Cullen, 1965). The ratio of cyclopropyl to monoenoic precursor usually is less than 0.05 for log phase unstressed growth to 2.5 or greater as the stress of starvation and stationary growth phase is prolonged. In the distribution system biofilm the ratio of cy 19:0/18:1 + 18:0 oxirane is high, which indicates that cells in the distribution system biofilm are starving (Table 2). The *E. coli* incubated for 10 h in 1/100 nutrient broth demonstrated a cy17:0/16:1w7c of approximately 1.1. The added stress of chlorine exposure may increase the cyclopropane PLFA formation and definitely leads to oxirane PLFA formation thereby leading to a greater decrease of the monoenoic precursors in the phospholipid.

The PLFA recovered from the distribution system biofilm showed low levels of *iso* and *anteiso* branched fatty acids with an *iso*/*anteiso* ratio near 1. Gram-positive aerobic bacteria like *Arthrobacter* or *Micrococcus* have *iso*/*anteiso* PLFA ratios less than 1, in contrast to the anaerobic sulfate-reducing Gram-negative bacteria with ratios greater than 5 (White *et al.*, 1996c). The low levels of polyenoic PLFA indicate that there were very few microeukaryotes in the distribution system biofilm (White *et al.*, 1996c). Based on the high levels of monoenoic PLFA and the cyclopropane and oxirane PLFA in chlorine exposed cells, it is most likely that the distribution system biofilm was largely made up of Gram-negative bacteria (White *et al.*, 1996c). The presence of mid-chain branched PLFA (10 me 16:0, 10 me 18:0 (tuberculostearic acid)) indicated that Actinomycetes were present (White *et al.*, 1996c). Moreover, the presence of the multimethyl branched PLFA (a micocerosic acid) also suggested the trace amounts of *Mycobacteria* (Almeida *et al.*, 1995). *Mycobacteria* are notoriously resistant to disinfection, particularly favor older corroded portions of the distribution system where their regrowth is stimulated, and readily colonize membranes used to purify water (Geldreich, 1996). *Mycobacterium avium* is commonly found in warm water systems and is an especially serious problem to immunocompromised consumers.

Oxirane fatty acids have not yet been detected in bacteria that have not been exposed to chlorine. However, a furan ring has been found in 10,13-epoxy-11-methyloctadeca-10,12 dienoic acid in the cellular lipids of a number of marine bacteria found in the gut of fishes (Shirasaka *et al.*, 1995). Oxirane fatty acids are also found in the neutral lipids of oilseeds (Singh *et al.*, 1994), *Aeollanthus* (Dellar *et al.*, 1996), and rice (Kato *et al.*, 1993). Oxirane fatty acids are found in the rust fungi (Weete *et al.*, 1979) including the pathogen *Pneumocystis carnii* (Kaneshiro, 1998), in marine algae (Bernart *et al.*, 1993; Jiang and Gerwick, 1997), and are formed by microbes in adipocere of human cadavers exposed to microaerophilic environments (Takatori, 1996).

Signature lipid biomarker analysis can be used to detect potential pathogens, which may or may not routinely be detected by the classical plate counting methods. Evidence is presented here that pelagic *E. coli* or *S. paucimobilis* are not culturable even with dilute and enriched "rescue media" (Mirpuri *et al.*, 1997) when the oxirane PLFA are present. The presence of epoxide fatty acids establishes that the cells in the biofilm have been in contact with the biocide and provides a chemical marker for hypochlorite exposure in pelagic and biofilm microbes in water distribution systems, while they are unculturable in the classical viable count. The SLB analysis of the biofilm also showed that trace amounts of the diffi-

cult-to-culture *Mycobacteria* were present within the drinking water distribution system and that difficult to culture *Legionella* (Walker *et al*., 1993) and *Sphingomonas* were not present. There was no evidence of fecal contamination indicated by detection of coprostanol (Leeming *et al*., 1996).

CONCLUSIONS

1. Biofilms form in drinking water distribution systems.
2. Viable biomass, community composition, and nutritional/physiological status can be assessed quantitatively by signature polar lipid analysis independently of culturability and viability.
3. Analysis of the drinking water distribution systems biofilm community showed a predominantly Gram-negative bacterial community.
4. The drinking water distribution system biofilm community showed evidence of metabolic stress in the high cyclopropane PLFA content.
5. Seasonal changes and water temperatures between 15 and 38°C had no significant effect on the drinking water distribution system biofilm community composition.
6. Oxirane (epoxide) fatty acids were detected in the polar lipids of the biofilm indicating exposure to chlorine and loss of viability within the biofilm, as determined by viable plate count.
7. Tests with monocultures of *E. coli* and *S. paucimobilis* exposed to chlorine resulted in oxirane fatty acid generation in the polar lipids and rendered them noncuturable by viable plate count.

Signature lipid biomarker analysis provided no indication of *Legionella* in this biofilm. *Mycobacteria* could have been present.

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