To the Graduate Council:

I am submitting herewith a thesis written by Charles B. Phiefer entitled "Injury of Drinking Water Organisms in Biofilms and Effects Upon Ecology and Physiology." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Microbiology.

David C. White, Major Professor

We have read this thesis
and recommend its acceptance:

Accepted for the Council:

Associate Vice Chancellor and
Dean of the Graduate School

INJURY OF DRINKING WATER ORGANISMS IN BIOFILMS AND EFFECTS UPON ECOLOGY AND PHYSIOLOGY

A Thesis Presented for the Master of Science Degree
The University of Tennessee, Knoxville

Charles B. Phiefer
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DEDICATION

This thesis is dedicated to my best friend,

Joe Welteroth,

with whom I shared a love of Nature and whose fortitude, conviction, and loyalty

inspire me,

and

to Mom,

Christine Phiefer,

for unconditional love and support.

Acknowledgments

There are many people that have had a part in my progress through this endeavor, professionally and personally. I would like to thank the members of my committee, Dr. David White, Dr. Gary Sayler, and Dr. Tom Montie for their guidance throughout. I am particularly grateful to Dr. Rob Palmer, who has given generously of his time and knowledge in matters of Science. Specifically, Rob has given invaluable assistance with confocal microscopy, experimental design, and technical writing. I would also like to thank David Ringelberg and Dr. Rob Burkhalter for consultations regarding PLFA analysis and gas chromatography/mass spectroscopy. Aaron Peacock is gratefully acknowledged for help with sample processing, PLFA analysis, and thoughtful scientific discourse.

Dr. Nick McClure constructed and donated the *Sphingomonas* strain with green fluorescent protein used in confocal microscopy experiments. Dr. David Nivens permitted use of figure 3.1 and Dr. Rob Palmer, figure 6.1. National Water Research Institute of Irvine, California generously provided financial support through a fellowship.

I am eternally appreciative of the efforts of Ms. Kristin Harter, lab technician extraordinaire. Kristin has always been generous with technical

assistance, materials, and patience. Finally, thanks go to Dr. Bruce Applegate, scientist, mechanic, and friend.

Abstract

Analysis of polar lipid fatty acids has revealed that unique oxirane fatty acids are detected from chlorine exposed bacteria. These compounds are heretofore unrecognized in bacteria. Oxirane fatty acids of 16 and 18-carbon chain length were detected in water distribution system biofilms, monoculture biofilms and sloughed cells of *Sphingomonas paucimobilis*, and batch cultures of *Escherichia coli*. In control populations unexposed to chlorine oxirane fatty acids were not detected. Biofilm populations not exposed to chlorine had higher molar percentages of monounsaturated fatty acids. For *S. paucimobilis* a decrease from 92% to 7-12% monoenoic molar percentages was observed. The relative decrease in monounsaturated fatty acids correlated with formation of oxirane fatty acids. Exposure to increasing concentrations of residual free chlorine was followed by increased molar percentages of oxirane fatty acids in all experiments. Seasonal comparisons of PLFA from distribution system biofilms showed higher molar percentages of oxiranes in summer compared to winter (30% versus 20%). The maximum residual chlorine concentration in summer was 1.6 parts per million (ppm) compared to 1.2 ppm in winter. This evidence supports the hypothesis that oxirane fatty acids are formed by chlorine exposed bacterial cells. If this hypothesis is accepted, oxirane fatty acids could be used as biomarkers of

chlorination in water distribution systems even where unculturable cells are present.

Effects of chlorination upon biofilm ecology were also examined using confocal scanning laser microscopy. Biofilms of *S. paucimobilis* and *Pseudomonas aeruginosa* were formed under conditions mimicking those found in water distribution systems and chlorination effects were investigated. Order of inoculation of the two species was shown to affect distribution of cells within microcolonies. When *P. aeruginosa* was inoculated first, primarily homogeneous (single species) microcolonies were observed. Upon addition of chlorine to the bulk fluid, nearly all cells and microcolonies of that species detached. *P. aeruginosa* microcolonies and cells associated with *S. paucimobilis* microcolonies typically remained attached longer than unassociated single cells or microcolonies. When *S. paucimobilis* was inoculated first, heterogeneous (mixed species) microcolonies were observed. *S. paucimobilis* was shown to harbor *P. aeruginosa* in biofilms after chlorine exposure.

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List of Abbreviations

BAC	biofilm accumulation chamber
CFU	colony forming unit
$CHCl_3$	chloroform
CSLM	confocal scanning laser microscopy
EPS	exopolysaccharide
GC	gas chromatography
GFP	green fluorescent protein
MeOH	methanol
MidBr Sat	mid-branched saturate
MS	mass spectroscopy
NA	numerical aperture
PLFA	polar lipid fatty acid
PLFAME	polar lipid fatty acid methyl ester
SD	standard deviation
TerBr Sat	terminally-branched saturate

Chapter 1

Introduction and Purpose

In potable water distribution systems microbial activity related to biofilm formation may lead to increased resistance of microbes within that system to biocides such as chlorine (Matin and Harakeh, 1990). This is partly due to the fact that life within a biofilm affords some protection to disinfection, as compared to planktonic cells in the bulk fluid, which are more susceptible. This phenomenon leads to difficulties when trying to maintain low numbers of pathogens and indicator bacteria.

Exopolysaccharide (EPS) plays an integral role in protecting cells from biocides and harboring injured pathogens (Costerton et al., 1995). Uhlinger and White (1983) reported that the majority of phospholipid and polysaccharide accumulation occurs in the late stationary phase of growth, when cells showed maximum physiological stress. Wrangstadt et al. (1986) observed production of soluble polysaccharide when starvation induced injury in exponentially growing cells. In growing cells that were not starved, this polysaccharide was not produced. Others have also reported that enhanced rates of adhesion and correlated EPS production are observed in starved, injured cells (Marshall. 1985). This suggests that injured pathogens might produce biofilms and recover within

them.

Current public health standards in the drinking water industry rely heavily on the coliform index. This test, however, may fail to predict large numbers of pathogens within a distribution system (Ridgway and Olson, 1982). Often, coliform bacteria are cultured from drinking water if there is a loss of residual disinfectant, line breaks or back-siphonage occur, or when there is a treatment plant failure (Maul et al., 1990). However, coliforms may sometimes be isolated from distribution systems when none of the above parameters are violated and there is no indication of fecal contamination (Seligman and Reitler, 1965). Due to the fact that injured indicators are often unculturable (McFeters, 1990, pp. 478-492), the coliform index can be meaningless when mitigation is employed. Concurrently, detection of low numbers of pathogens is even more difficult (Hibler and Hancock, 1990). When these pathogens are injured and viable but unculturable, they may recover and reproduce within biofilms without detection. This introduces risk to consumers.

If coliforms and pathogens such as *Sphingomonas* and *E. coli* infect drinking water biofilm communities, then sloughing of cells into the flowing water supply can account for their presence in the absence of fecal contamination and/or treatment plant failure (Maul et al., 1990). So, if those cells are present only briefly in a water supply or are injured, then attachment, recovery, and growth

within that biofilm will cause the population to increase without being detected by culturing methods.

Observations by different workers of biofilms in drinking water distribution systems show little uniformity in distribution and community composition. Varying conditions may exist at different locations throughout a drinking water distribution network. Therefore, observations made at one time or place may not be indicative of what is happening in the system as a whole. This makes thorough analysis of such systems very difficult. The research program described below was designed to provide a better understanding of the intense, interactive, heterogeneic nature of microbial biofilms in such systems. The research described herein was performed to examine effects of cellular injury of microbial pathogens and indicator organisms within drinking water distribution system biofilms.

Chapter 2

Literature Review

Formation, Structure and Ubiquity of Biofilms

Biofilms are defined as matrix-enclosed microbial populations which are adherent to each other and attached to a surface or interface (Costerton et al., 1995). Biofilms are a layer of microorganisms embedded in or attached to exopolysaccharide (EPS) which is secreted by the resident microbes. The matrix is exposed to the bulk fluid. The EPS is composed of organic polymers and varies with the types of microbes present and environmental conditions (Allison and Sutherland, 1987).

Biofilms have a very important role in nature and technology and can be found at nearly any surface which is exposed to water regardless of the trophic state of the bulk fluid(Wilderer and Characklis, 1989, pp. 5-17). Biofilms and suspended cell aggregates in river beds, lakes, and oceans are important in the removal of contaminants from these waters (Wilderer and Characklis, 1989). Biofilms are employed during waste water treatment processes where the microbes remove organic and inorganic pollutants (Grady and Lim, 1980). Biofilms can induce corrosion processes (Little et al., 1987), infections related to artificial transplants (Khoury et al., 1992), and they may harbor pathogens in drinking water

distribution systems (Geldreich, 1996, pp.159-214).

Most microbes have the ability to attach to various surfaces and this process may be mediated by fimbriae and/or adhesins on the cell surface (Characklis and Marshall, 1990). The rate of attachment and avidity of binding depend upon microbe and substrate surface characteristics, fluid shear stress, and previous conditioning of the substratum. Attachment is followed by consolidation, which is when that initially weak adhesion is strengthened by exopolymer production. Then, growth of cells and further EPS excretion lead to formation of an established biofilm.

In water distribution systems biofilms may form a continuous layer, evenly distributed (Wilderer and Characklis, 1989, pp. 5-17). However, they are often patchy in appearance. The film contains the surface community, EPS, and organic and inorganic debris. This debris may be present due to adsorption of sediment, corrosion products, or precipitation of salts. Debris may provide metabolic substrates to the biofilm community members. In addition to providing nutrients for microbes, EPS may serve to protect biofilm bacteria from biocides, including chlorine.

Physical properties of a biofilm are significantly influenced by the nature of the EPS. These properties include diffusivity of nutrients and biocides, rheological properties such as fluid frictional resistance, and thermal conductivity (Lawrence

et al., 1994). In biofilms, EPS is typically highly hydrated and predominantly anionic. It may serve as an ion-exchange matrix increasing local concentrations of cations, such as ammonium, potassium, and heavy metals (Wolfaardt et al., 1994). In this manner, and especially under oligotrophic conditions found in distribution systems, exopolysaccharide may serve as a nutrient trap (Ophir and Gutnick, 1994). In addition, the penetration of negatively charged biocides such as chlorine and monochloramine may be hindered by the presence of EPS (Brown and Gilbert, 1993).

Effects of Chlorination in Water Distribution Systems Upon Microbial Ecology

One or more disinfectants are often applied by water utilities during treatment processes. The residual concentration of disinfectant begins to decline as soon as treated water leaves the plant (van der Wende and Characklis, 1990, pp. 249-268). This is due to reactivity of chlorine with suspended and dissolved compounds and biomass in the water, as well as biofilm and pipe material. Disinfectants oxidize substances including Fe^{2+}, sulfide, Mn^{2+}, and various organic compounds (Characklis et al., 1980). Booster stations are used in some cases to maintain or boost residual concentrations along the distribution network.

Ridgway and Olson (1982) described significant differences in the

susceptibility of microbes to chlorine and chloramine which were isolated from chlorinated versus non-chlorinated distribution systems. Selective growth of organisms which are chlorine tolerant may occur in distribution systems where mitigation is performed. Planktonic and biofilm cells isolated from systems with high concentrations of chlorine are often of only a few genera (Maki et al., 1986). Wolfe et al. (1985) showed that many isolates from these systems are chlorine-tolerant. When plated on R2A agar, "smooth" colonies of *Pseudomonas* were obtained. That phenotype was correlated with large amounts of EPS production. Although EPS production and biofilm formation in situ depends upon an array of factors, specific strains certainly have the potential to excrete significant amounts of exopolymer. A large difference in species diversity may be observed between non-chlorinated and chlorine-treated water systems (Brazos and O'Connor, 1985). Chlorination will select for growth of species which are most resistant to that biocide. Subsequently, further growth and biofilm formation may be predominated by those microbes. Chlorine tolerance may be further enhanced by mutation, transfer, and recombination of genetic material within the biofilm (Kelly et al., 1983).

DeBeer et al. (1994) observed decreasing penetration of chlorine into biofilms during disinfection using microelectrode sensors. With this sensor chlorine concentrations could be measured into the micromolar range at various

depths within a biofilm without disrupting structure. Typically, the chlorine concentration in the biofilm was 20% or less of that in the bulk fluid. Equilibration of the film chlorine concentration with the bulk fluid concentration did not occur after hours of incubation. This supports the hypothesis that diffusivity of chlorine is related not only to transient diffusion, but also reactivity within the biofilm matrix. Chlorine concentration within the film varied depending upon location of measurement. This reflects the heterogeneity of local hydrodynamics and biomass distribution.

The Viable but Nonculturable State and Coliform Index

In the United States, chlorination of potable water has been widely practiced since the turn of the century. It is the most commonly used means of maintaining microbial quality of water. Chlorination may be the major cause of bacterial injury in these systems (Camper and McFeters, 1979). The purpose of disinfection is bacterial lethality. However, conditions which lead to sub-optimal disinfectant activity may promote injury (McFeters, 1990, pp. 478-492). The most significant consequence of injury is the loss of culturability of stressed microbes upon selective media (LeChevallier and McFeters, 1984 and 1985). This can lead to an underestimation of the population of both indicator bacteria and total heterotrophs (McFeters et al., 1986).

Current public health standards rely heavily upon the coliform index. This test relies upon culturing indicator bacteria and may fail to show large numbers of opportunistic pathogens within distribution systems. Outbreaks of waterborne disease associated with detection of few or no coliforms have been observed (Seligman and Reitler, 1965; Boring et al., 1971; Craun, 1981). McFeters (1990, pp. 478-492) described municipal drinking water systems surveys where the percentage of injured coliform bacteria ranged from 43% to 99%. These results were obtained by comparing recoverability of colony forming units on m-Endo versus m-T7 agar plates. The majority of indicator bacteria in treated distribution systems may be injured, unculturable, and undetectable using accepted methods.

Chapter 3

Analysis of Warm-Water Distribution-Line Biofilms

Introduction

Distribution system biofilms can be composed of a complex community of microorganisms, making it difficult to assess what organisms are present. To compound this problem, the bacterial community members are often injured as a result of mitigation and rendered unculturable. This can eliminate the possibility of employing classical microbiological culturing methods because of the fact that the number and types of microbes recovered on agar plates likely does not represent the true biofilm community composition. Byrd et al. (1991) demonstrated that bacteria incubated in sterile drinking water and exposed to low levels of chlorine were viable but nonculturable. Environmentally hardy genera such as *Bacillus* and *Pseudomonas* showed a slower decrease in culturability over time. Experiments with monochloramine exposed biofilms of *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* by Stewart et al. (1994) revealed that microbial respiratory activity was significantly retained while culturability decreased by 99.9% after 1 hour of disinfection.

Materials and Methods

The modified Bligh/Dyer lipid extraction method (Hedrick and White, 1995) and gas chromatography/mass spectroscopy were used to analyze the polar lipid fatty acid (PLFA) composition of warm-water distribution-line biofilms. This allowed detection of groups of fatty acids which are typically associated with certain bacterial groups.

Biofilm Accumulation Chambers (BACs) (fig. 3.1) consist of stainless steel tubing 30 cm in length and 2.54 cm in diameter. These tubes are fitted with Swagelok™ adapters and couplings on both ends to which inlet and outlet valves are connected. BACs were packed with approximately 165g of lipid-free, 1mm glass beads. Beads were heated to 450°C for a minimum of six hours to oxidize organic carbon contamination on the bead surface prior to packing BACs. The packed chambers were then pre-extracted with 1:1 chloroform/methanol to dissolve and remove any such contamination from within the chamber itself. Inlet and outlet tubing were connected to the appropriate valves and the entire apparatus autoclaved to sterilize and volatilize any solvent remaining from pre-extraction.

Three BACs were connected in series to a manifold which was constructed of polypropylene tubing, union tees, and elbows. The manifold was connected to a shower head fitting and warm water (36-38°C) was allowed to flow through the system. This temperature range was selected and maintained to allow for growth

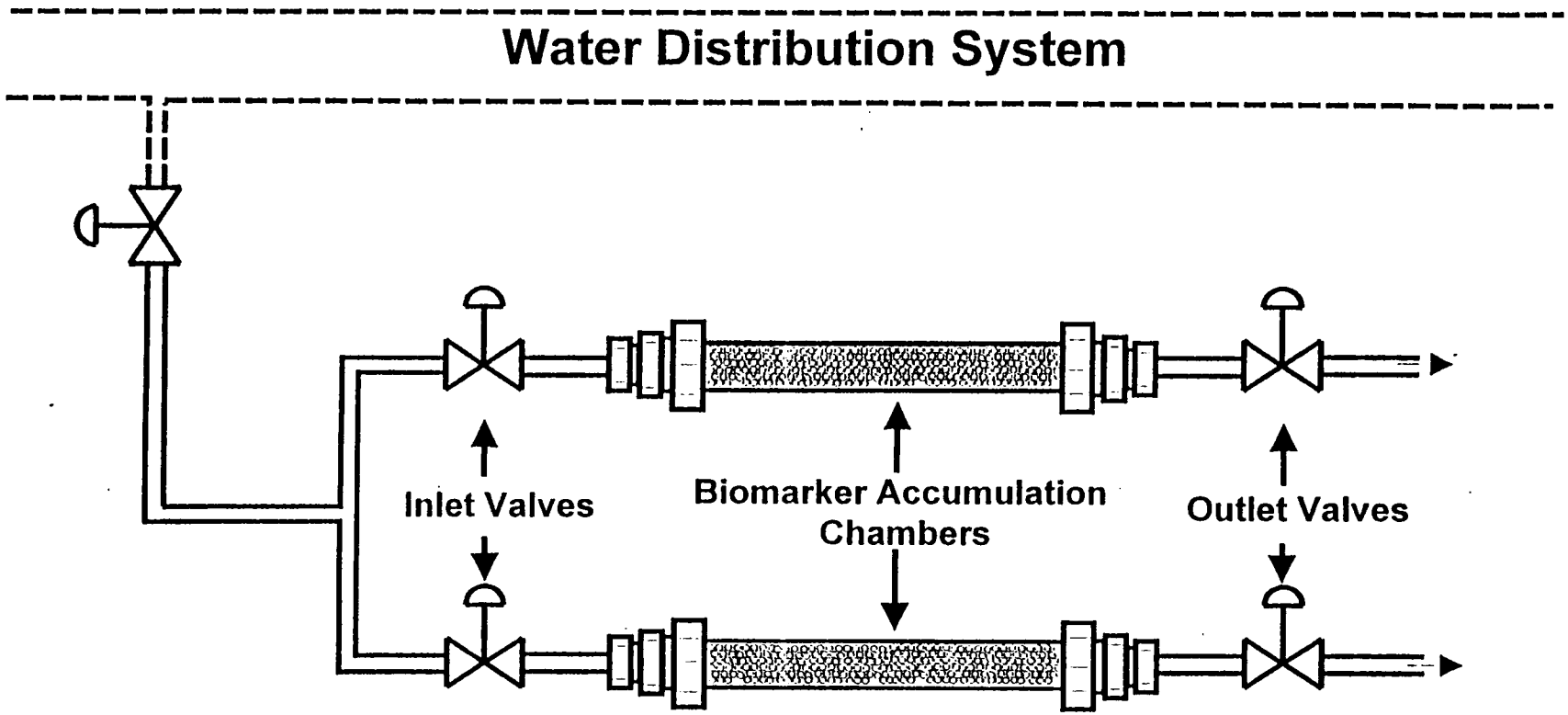


Figure 3.1. Diagram of assembled Biofilm Accumulation Chambers connected to water supply.

of potential pathogens at human body temperature. These experiments were performed twice in triplicate each trial for 20 and 23 days; once in late summer (September) and again in early winter (November). The purpose was to examine the effect of season upon biofilm community composition. Free chlorine concentration was measured from BAC effluent using the U.S. Environmental Protection Agency-approved DPD colorimetric, titrimetric method. Measurements were obtained using a Hach pocket colorimeter (cat. #46760-88) and DPD reagent powder pillows (cat. #21055-49). This method has an internal measurement error of ±0.02 ppm. Temperature and pH of the bulk fluid were also monitored.

PLFA Extraction and Analysis

A detailed description of the lipid analysis techniques used in these experiments is available (Hedrick and White, 1995). Upon takedown, BAC beads were poured into a 500 ml separatory funnel and the chamber interior was rinsed with first phase Bligh/Dyer solvents (Bligh and Dyer, 1959) modified by addition of phosphate buffer (White et al., 1979). This consists of a mixture of methanol (MeOH), chloroform ($CHCl_3$), and buffer in the ratio 2:1:0.8, respectively. Rinsing was done to remove any beads remaining in the chamber after initial pouring, as well as to ensure extraction of biofilm attached to the chamber interior. After extraction (2-18 hours), additional chloroform and water were added to

achieve a final ratio of 1:1:0.9 for $CHCl_3$:MeOH: water/buffer. Aqueous and organic phases were allowed to separate approximately 18 hours, after which the organic fraction (containing total lipid) was eluted and the solvents evaporated. This total lipid extract was fractionated into neutral lipid, glycolipid, and polar lipid classes by elution through a silicic acid (0.5 g) chromatography column with 5 ml each of $CHCl_3$, acetone, and MeOH, respectively. Polar lipid fatty acid methyl esters (PLFAME) were obtained by mild alkaline methanolysis of the polar lipid fraction and analyzed by capillary gas chromatography/mass spectrometry (Ringelberg et al., 1989). Mass spectra were determined by ChemStation (Hewlett-Packard) software. Ionization was achieved by electron impact (70eV).

Fatty Acid Nomenclature

Fatty acid names are abbreviated as the total carbon chain length followed by a colon (:), the total number of unsaturations, then an omega (ω) or "w", followed by the number of carbons to the unsaturation from the methyl end of the molecule. The prefix "Cy" or "cy" indicates a cyclopropyl isomer and "i" and "a" indicate iso-branched and anteiso-branched isomers, respectively. "Me" or "me" indicates that a mid-chain methylation is present at the position preceding that abbreviation.

Results

Poor manifold connection resulted in extremely low biomass accumulation in one chamber. Five of six chambers were included in analyses and these data are shown in Table 3.1. Saturated fatty acids are common in eubacteria (Vestal and White, 1989) and compose between 44 and 63% of the lipid profiles. Terminally branched saturates and mid-branched saturates together make up 16-18% of the profiles. Monoenoic fatty acids are Gram negative indicators (White et al., 1996a) and only compose 5-8% of the total lipid composition, which is lower than expected from a distribution system biofilm. However, oxirane fatty acids compose between 14 and 30% of the total lipid profiles. To the best of our knowledge, these compounds have only been detected in bacteria exposed to chlorine. Their detection may indicate bacterial oxidative stress. The mechanism of oxirane formation and its cause were further explored and is discussed later.

The polyenoic fatty acid 22:6w3 was detected in one replicate and likely indicates the presence of eukaryotes (Vestal and White, 1989). In addition, the signature lipid biomarker (SLB) 10me18 was detected at between 5 and 6%. This biomarker is detected in *Mycobacterium* species (Kroppenstedt, 1985), which are of importance to the drinking water industry. They are of significant health concern to water suppliers due to their infectiousness and resistance to mitigation.

A seasonal comparison of PLFA profiles illustrates that higher total oxirane

Table 3.1. Polar lipid fatty acid profiles from five water distribution system biofilms (TerBr Sat=Terminally branched saturates; MidBr Sat=Mid-branched saturates).

Fatty Acid Groups	Mole Percents				
Saturates					
14:0	5.35	10.09	11.64	3.78	5.99
15:0	1.21	1.57	1.45	0.93	1.16
16:0	46.18	50.31	47.06	45.69	33.03
17:0	1.63	1.27	1.75	1.34	1.58
18:0	0.69	0.00	0.00	0.00	0.00
20:0	0.29	0.00	0.00	0.59	1.00
21:0	0.00	0.00	0.00	0.00	0.62
22:0	0.00	0.00	0.00	0.31	0.46
Total Saturates	**55.36**	**63.24**	**61.89**	**52.65**	**43.85**
Terminally Branched Saturates					
i14:0	0.00	0.59	0.92	0.42	0.89
i15:0	2.93	3.98	4.59	3.02	3.65
a15:0	1.40	1.78	2.10	1.66	1.62
i16:0	0.94	0.77	0.86	0.82	0.74
i17:0	0.55	0.00	0.00	0.53	0.55
a17:0	0.61	0.00	0.74	0.61	0.64
Total TerBr Sat	**6.43**	**7.12**	**9.21**	**7.05**	**8.09**
Monoenoics					
cy17:0	1.42	1.44	1.56	1.25	0.96
18:1	1.22	0.76	0.62	1.49	2.09
18:1b	0.86	0.00	0.00	0.00	0.00
19:1a	0.39	0.00	0.00	0.68	0.53
cy 19:0	3.54	3.02	3.12	2.49	2.38
Total Monoenoics	**7.44**	**5.21**	**5.30**	**5.91**	**5.97**
Mid branched Saturates					
br16:0	0.00	0.00	0.00	0.00	0.34
10me16	0.00	0.00	0.00	0.71	0.98
br 17:0	1.10	0.83	1.45	0.00	0.00
3,10dime19:0	3.48	2.52	2.98	2.87	3.00
10me18	5.69	5.36	4.93	6.28	5.73
Total MidBr Sat	**10.27**	**8.71**	**9.37**	**9.86**	**10.04**
Branched Monoenoics					
br18:1	0.00	0.00	0.00	0.00	1.14
Total Branched M	**0.00**	**0.00**	**0.00**	**0.00**	**1.14**
Polyenoics					
22:6w3	0.00	0.00	0.00	0.47	0.00
Total Polyenoics	**0.00**	**0.00**	**0.00**	**0.47**	**0.00**
Oxiranes					
oxirane 16:0	11.44	10.30	8.39	11.58	13.76
oxirane 18:0 a	1.55	0.91	0.98	2.44	4.38
oxirane 18:0 b	6.71	4.50	4.86	9.21	11.84
Total Oxiranes	**19.70**	**15.71**	**14.23**	**23.24**	**29.99**

formation was detected in biofilms grown in late summer compared to early winter (fig. 3.2). The maximum residual chlorine concentration in summer was 1.6 parts per million (ppm) compared to 1.2 ppm in winter (fig. 3.3).

Oxirane fatty acids appear to be unique to chlorine-exposed bacteria and, to date, have been found with 16 and 18 Carbon chain lengths. These compounds elute at about 32.5 and 38.5 minutes (fig. 3.4) when using a typical PLFA gas chromatograph oven program. The structure and mass spectrum of 9,10 Oxirane 18:0 is illustrated in figure 3.5. The molecular ion of this compound is 312. Ions 155, 183, and 199 are detected at relatively high abundance and used to distinguish the compound from other fatty acids.

Discussion

PLFA analysis provided insight into the community structure of distribution system biofilms. Furthermore, the detection of specific lipid molecules can indicate that specific genera were present in the biofilms. Tuberculostearic acid, 10Me18, could indicate the presence of *Mycobacterium* species. This is supported by the fact that opportunistic *Mycobacteria* proliferate in warm water systems and are typically chlorine resistant.

Higher molar percentages of oxirane fatty acids grown in summer is likely explained by the fact that those biofilms were exposed to a higher residual free

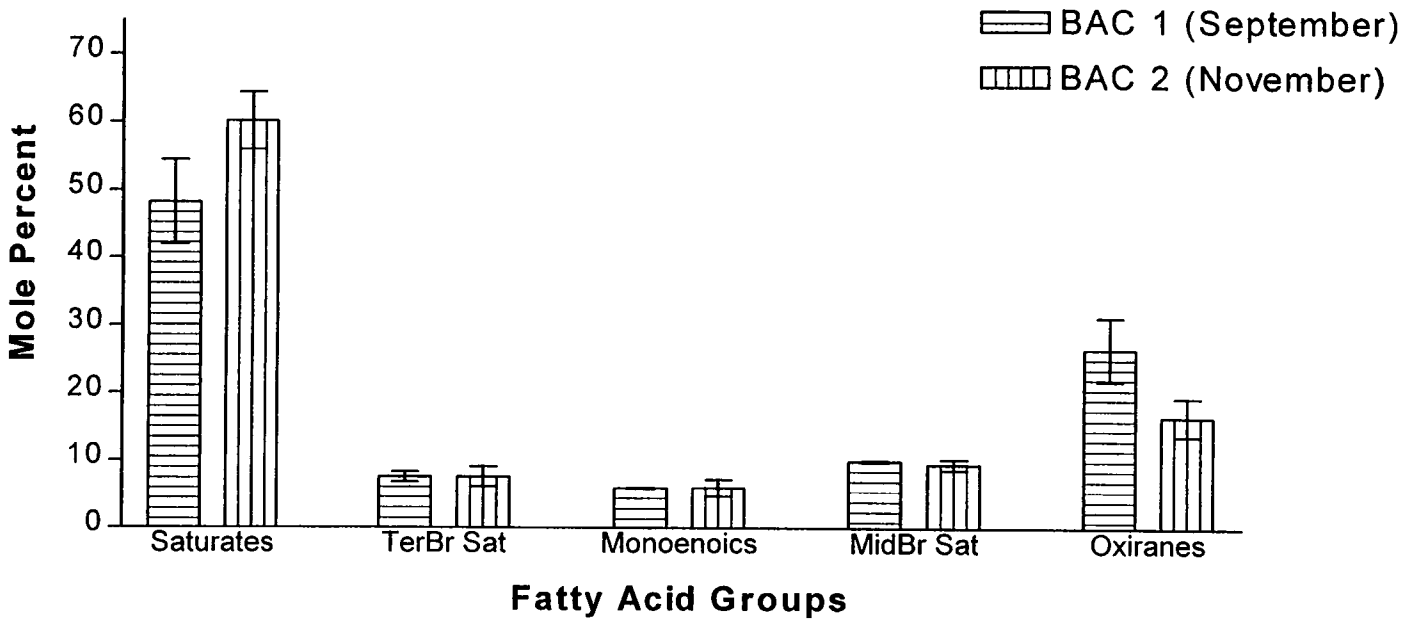


Figure 3.2. Seasonal comparison of PLFA profiles from distribution system biofilms grown in late summer compared to early winter (TerBr Sat=Terminally branched saturates; MidBr Sat=Mid-branched saturates). For September (late summer) error bars represent minimum and maximum values about the average. For November (early winter) error bars represent standard deviation about the mean.

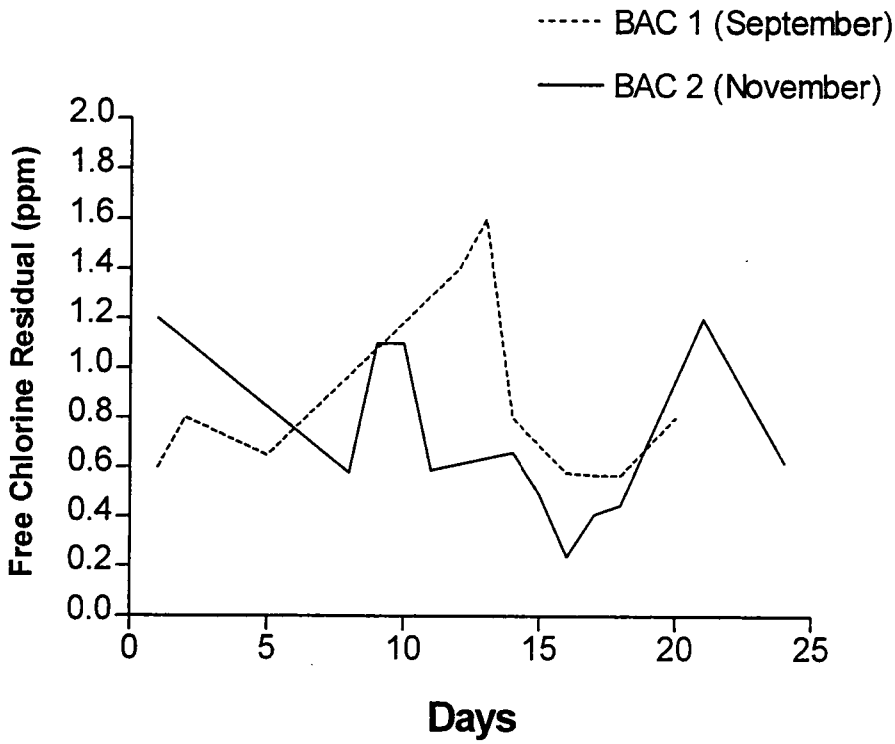


Figure 3.3. Seasonal comparison of free chlorine residuals (ppm) from distribution system biofilms.

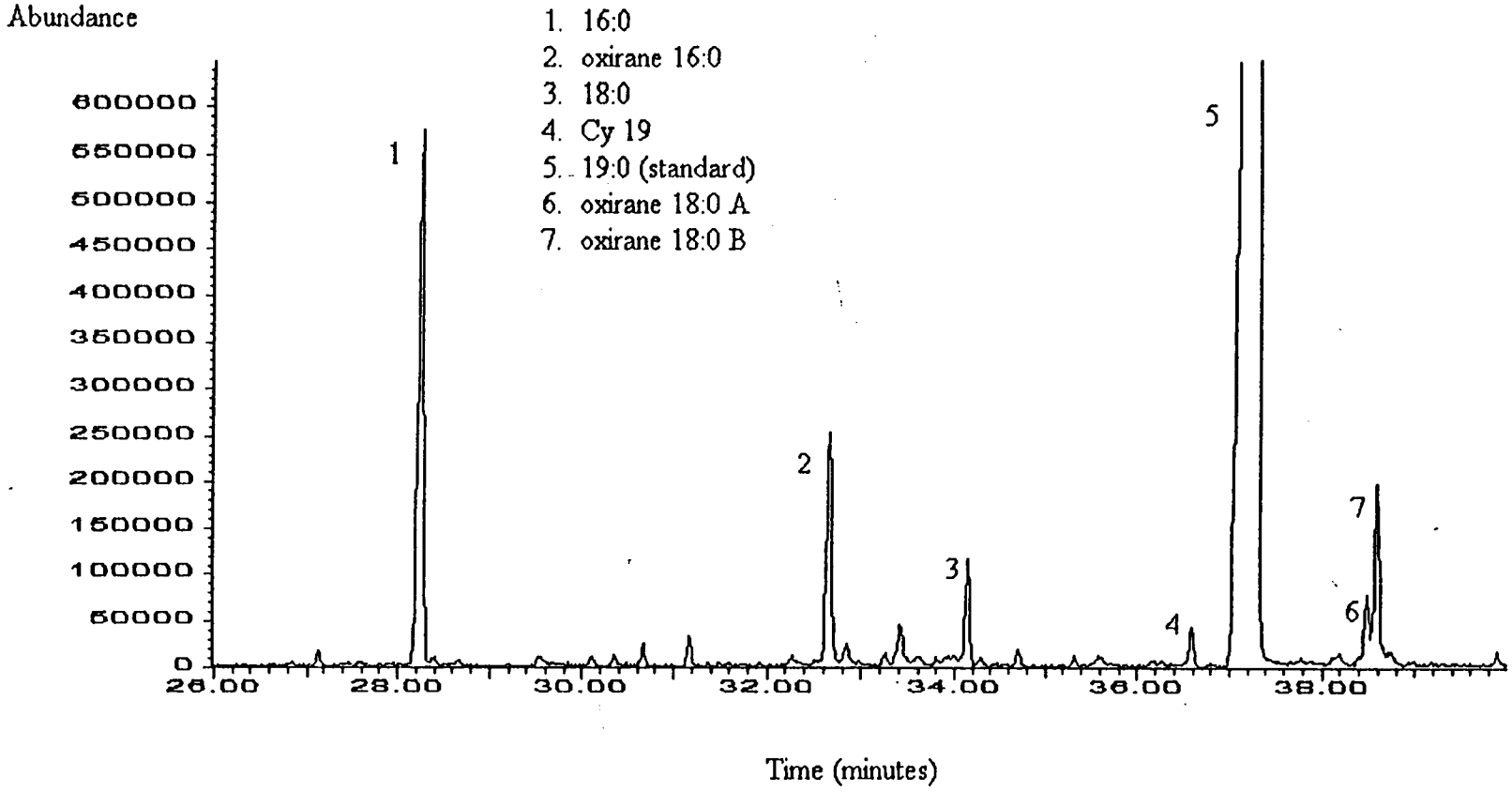


Figure 3.4. Chromatogram of PLFA obtained from a distribution system biofilm grown in late summer. Numbered peaks are identified above.

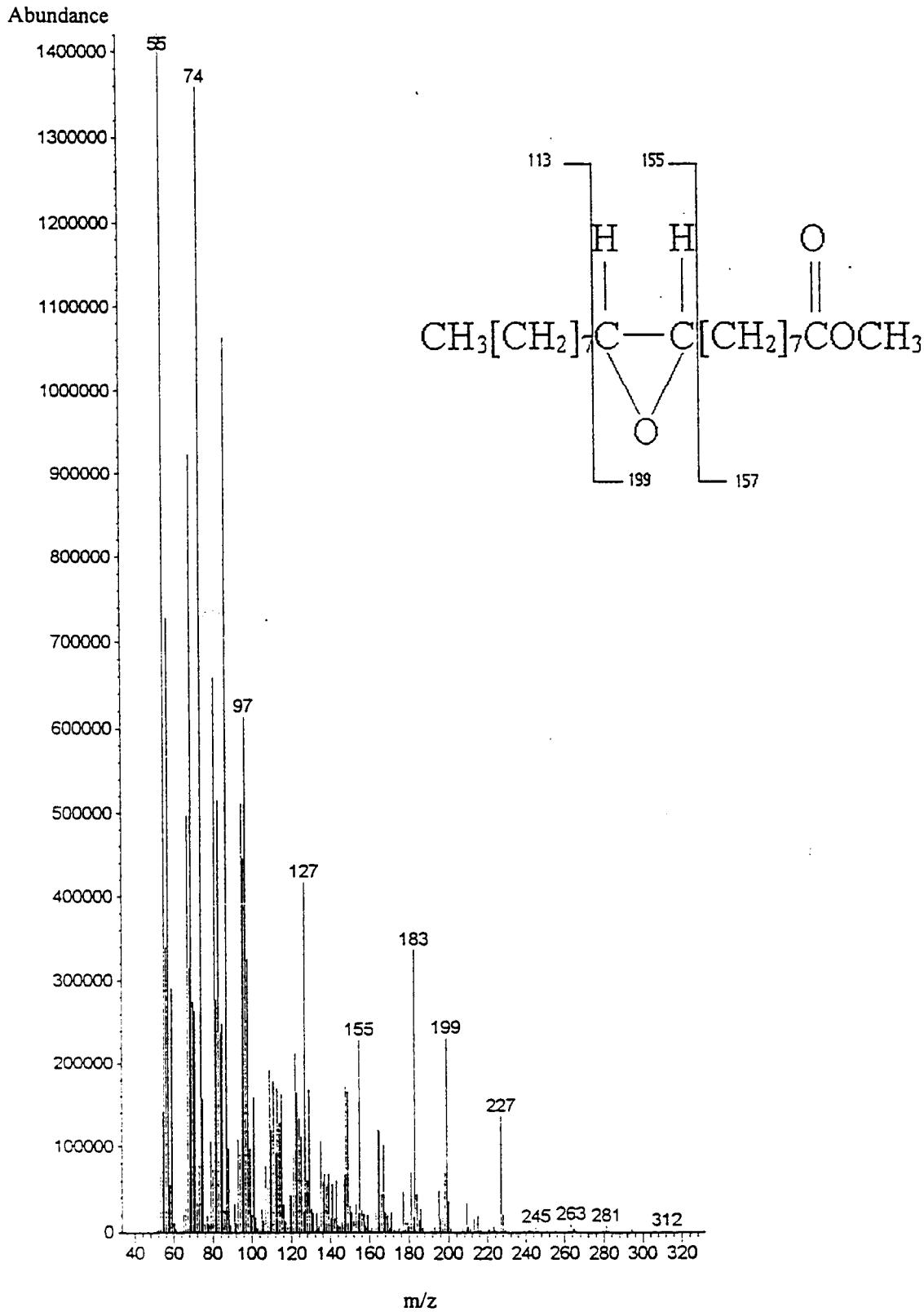


Figure 3.5. Mass spectrum and structure of 9,10 oxirane 18:0 with indicated ions. The molecular ion is 312.

chlorine concentration over the majority of the time course of these experiments. Field data from several water utilities that have experienced cyclic increases in heterotrophic bacterial populations point to a seasonal phenomenon (Geldreich, 1996, pp. 159-214). When source and/or distribution water temperatures are above 15°C, accelerated growth of microbes in the pipe environment may occur. Increased growth of microorganisms within distribution systems could correspond to elevated ambient temperature. In these cases, treatment plants might elevate the free chlorine concentration to meet federal requirements regarding detection of coliform and total heterotrophic bacteria. Higher free chlorine residuals in warmer months were noted in this study (fig. 3.3). It was initially hypothesized that oxirane fatty acids occur as a result of oxidative stress which microbes undergo in chlorinated distribution systems. The fact that higher levels of oxiranes were detected in biofilms grown in late summer was believed to be a result of higher free chlorine concentrations within the distribution system. The effect of chlorine concentration upon oxirane formation was further examined.

Chapter 4

Injury of *Escherichia coli* in Batch Culture

Introduction

Escherichia coli was chosen for injury experiments due to its importance to the drinking water industry as an indicator organism. Culturable coliform, fecal coliform, and pathogenic bacteria in municipal water systems are used as indicators of the quality of source water, effectiveness of initial treatment regimes, and maintenance of residual disinfectant (Office of Drinking Water, 1989). Often however, these indicator organisms are not detected either because they are not present in the bulk fluid or are viable but nonculturable (McFeters, 1990). Under these conditions, indicator and pathogenic bacteria may still be present within the distribution network, typically associated with biofilms coating the interior of distribution piping. Sloughing of this biofilm may occur upon line-breakage or back-siphonage (Geldreich, 1996, p.215-255). This leads to the release of microbes including pathogens into the bulk fluid whereupon deterioration of water quality has occurred and health risk has been introduced to consumers.

Experiments were performed to examine the effect of exposure to chlorine upon specific organisms. The purpose was to determine if oxirane fatty acids could be detected in a specific species under specific culture conditions. This was

done in an attempt to determine whether oxiranes are formed as a result of oxidative stress and to elucidate the mechanism of their formation.

Materials and Methods

Six *E. coli* cultures were grown to late log phase in nutrient broth. Cultures were shaken (100 rpm) during incubation (28°C). After incubation, centrifugation (2000 rpm, 20 minutes) was performed and cultures were resuspended in dilute (1/100) nutrient broth. After four hours exposure to dilute nutrient broth, spread plates were performed on nutrient rich (nutrient agar) and low nutrient (R2A) media from all six cultures. After initial spread plates were obtained, hypochlorite (as bleach) was added to three of six cultures to constitute approximately 0.5 parts per million (ppm) free chlorine. Free chlorine concentration was determined as previously described (Chapter 3) and was monitored and adjusted as needed for ten hours to maintain a residual as near as possible to 0.5 ppm. During this exposure period cells were incubated statically at room temperature. Spread plates were again performed after ten hours exposure to hypochlorite to examine the effects of chlorination upon culturability. All six cultures were then centrifuged (2000 rpm, 20 minutes), pellets lyophilized, and extracted for PLFA using the modified Bligh/Dyer method (Chapter 3).

Results

The lipid profiles obtained from these cultures are very similar with one exception. Oxiranes were detected only in the three cultures exposed to chlorine and composed 2.86±1.6% of the lipid profiles (Table 4.1). Saturated fatty acids compose 46.72±3.78% and 47.32±3.49% of the total PLFA from non-chlorine exposed and chlorine exposed cultures, respectively. The saturate 16:0 predominated in both samples, comprising approximately 40% of the profiles. Several monoenoic fatty acids were detected and constituted approximately 32% of the total PLFA. 16:1w7c and 18:1w7c were found in greatest abundance within this group. In addition, cyclopropyl 17:0 and 19:0 were detected at approximately 20% in the six samples.

The culturability of cultures unexposed to chlorine slightly increased after 10 hours incubation. This was observed on both nutrient agar and R2A (fig. 4.1). In contrast, chlorine exposed cultures which formed oxiranes were completely unculturable.

Figure 4.2 illustrates chromatograms from batch cultures exposed to zero and 0.5 ppm free chlorine, respectively. The major difference is the appearance of two peaks at 32 and 38 minutes, indicated by asterisks. These are 16- and 18-carbon oxirane fatty acids. This is the only major difference in profiles from the two groups.

Table 4.1. Lipid profiles of chlorinated and non-chlorinated *E. coli* batch cultures (SD=standard deviation).

	NON-CHLORINATED		CHLORINATED	
Saturates	AVERAGE	SD	AVERAGE	SD
12:0	0.03	0.03	0.04	0.01
14:0	4.94	0.90	4.99	0.36
15:0	0.25	0.02	0.14	0.01
16:0	39.83	4.40	40.68	3.17
17:0	0.18	0.04	0.12	0.02
18:0	1.48	0.28	1.34	0.28
TOTAL	46.72	3.78	47.32	3.49
Monoenoics				
14:1	0.17	0.07	0.14	0.05
16:1w9c	0.19	0.09	0.17	0.04
16:1w7c	16.29	2.76	17.61	0.67
16:1w7t	0.13	0.01	0.17	0.03
16:1w5c	0.36	0.05	0.33	0.01
18:1w9c	0.88	0.09	0.58	0.10
18:1w7c	13.11	0.84	13.33	0.30
18:1w7t	0.11	0.03	0.18	0.01
18:1w5c	0.06	0.02	0.06	0.01
19:1w12	0.45	0.16	0.24	0.24
TOTAL	31.74	3.58	32.81	0.49
Cyclopropyls				
cy 17:0	18.66	0.39	17.88	3.48
cy 19:0	2.23	0.14	1.96	0.59
TOTAL	20.89	0.26	19.84	4.04
Oxiranes				
oxirane 16 A			0.03	0.05
oxirane 16 B			2.02	1.16
oxirane 18 A			0.04	0.01
oxirane 18 B			0.77	0.34
TOTAL			2.86	1.57

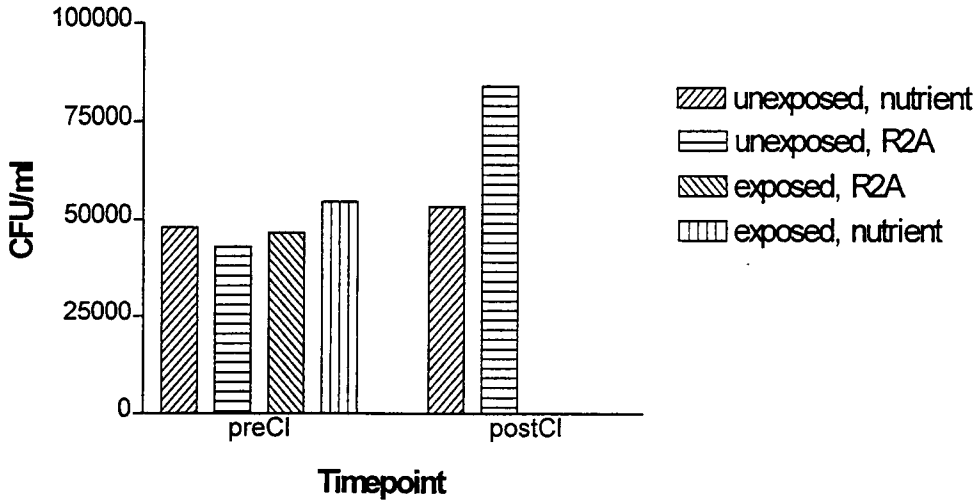


Figure 4.1. Culturability of chlorine exposed and unexposed *E. coli* batch cultures plated on nutrient versus R2A agar (preCl= pre-chlorination; postCl=post-chlorination).

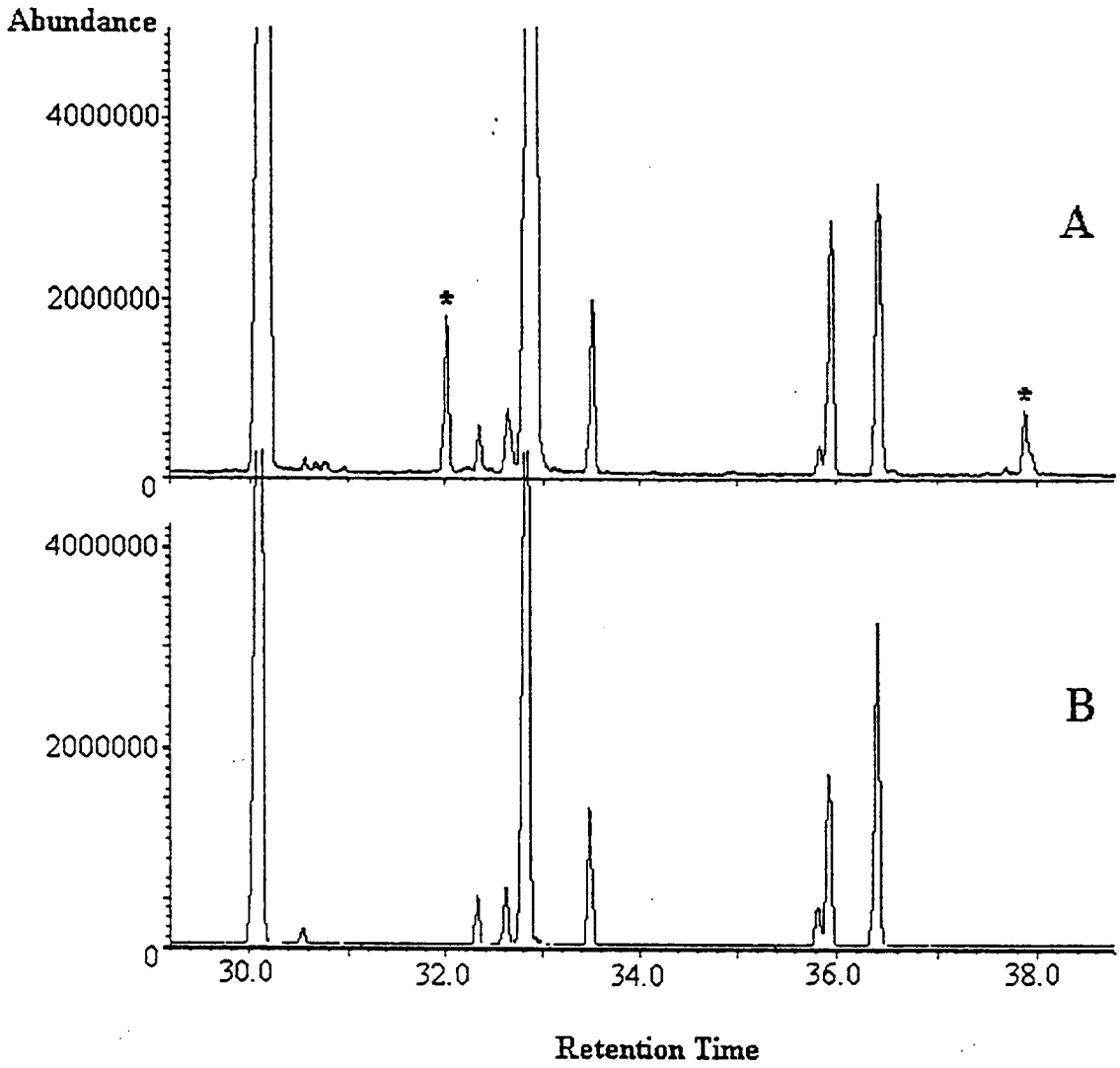


Figure 4.2. Chromatograms of PLFA obtained from chlorine exposed (A) and unexposed (B) *E. coli* batch cultures. Oxirane fatty acids of 16 and 18-carbon chain length appear at approximately 32 and 38 minutes, respectively, and are indicated by asterisks. These were only detected in chlorine exposed samples.

Discussion

These experiments illustrated that oxirane fatty acids were detected in PLFA from chlorine-exposed bacteria. Also, detection of these compounds occurred in chlorine exposed cultures which exhibited complete unculturability. This addresses the important issue of viable but nonculturable microbes within water distribution systems. That is, *E. coli* which would not be detected by traditional microbiological methods could be detected by PLFA analysis. Detection of oxirane fatty acids in distribution system biofilms and water samples may be used to determine that microbes have been exposed to chlorine when they are unculturable.

Chapter 5

Injury of *Sphingomonas paucimobilis* in Biofilms

Introduction

Sphingomonas paucimobilis is a Gram negative aerobe which possesses a single polar flagellum and is yellow-pigmented. This organism was classified as a Pseudomonad until 1990 when it was placed in the new genus *Sphingomonas* (Yabuuchi et al., 1990). The microbe is of significant importance to the drinking water industry due to its ability to cause microbially influenced corrosion (White et al., 1996b) and its potential pathogenicity (Yabuuchi et al., 1990).

In a distribution system utilizing copper piping, heating water to 64°C was shown to decrease oxygen uptake, copper liberation, and colony forming units (White et al., 1996b). In unheated controls these effects were not observed. Upon lowering the temperature of the bulk fluid all three parameters increased. Addition of 30 ng/ml of the antibiotic cefoxitin to the system again led to a decrease in corrosion, indicating that the process was microbially influenced. *Pseudomonas fluorescens* and a Sphingomonad were isolated consistently from samples of the bulk fluid. Anodic reactions involved in the process of microbially influenced corrosion likely occurred as a result of the ability of both organisms to accumulate copper within the cell wall.

S. paucimobilis has been shown to be a causative agent of wound infection (Peel et al., 1979), peritonitis (Glupezynski et al., 1984), meningitis (Hajiroussou et al., 1979), bacteremia (Morrison and Shulman, 1986), and septicemia (Slotnick et al., 1979). In addition, the microbe can cause nosocomial infections during surgical procedures and post-operatively by its ability to contaminate irrigation fluid (Faden et al., 1981), infusion fluid (Buxton et al., 1979), tube-rinsing water (Crane et al., 1981), and "sterile" surgical handwashing water (Mamiya et al., 1988). The strain of *S. paucimobilis* used in the following experiments was originally isolated from an infected hospital respirator (American Type Culture Collection, strain 29837). These experiments were performed to examine the specific effects of chlorine exposure upon PLFA composition.

Materials and Methods

Biofilm Accumulation Chambers (BACs) were prepared for experimentation as previously described (Chapter 3). Twenty-liter carboys were sterilized by autoclaving and dilute (1/100) nutrient broth was filtered (0.2μm) into these reservoirs. The sterile medium was inoculated with 10^9 late log phase cells of *S. paucimobilis* grown in nutrient broth and shake-incubated (100 rpm) at 28°C. This bulk fluid was pumped through BACs for 2-3 days allowing attachment of cells and biofilm growth to occur. Flow rate was maintained between four and six

ml/minute. Effluent was collected in a separate, sterile carboy. This initial period of growth with 1/100 nutrient broth allowed biomass to accumulate within biofilms. Therefore, PLFA analyses were not biomass limited.

After the initial growth period the bulk fluid reservoirs were aseptically changed to new sterile carboys containing filter sterilized (0.2μm) tapwater. Residual free chlorine was removed from tapwater prior to filtration by titration with 3% sodium thiosulfate. Effluent reservoirs were also changed at this time. For some experiments, the bulk fluid effluent containing planktonic cells was filtered and analyzed for PLFA. This was achieved by vacuum filtration through a lipid-free, 5 cm glass-fiber filter (~0.5μm).

Sodium hypochlorite (as chlorine bleach) was added to the sterile tapwater medium to constitute a free chlorine residual as near as possible to 1ppm. Free chlorine concentration of the bulk fluid was determined as previously described (Chapter 3). Chlorinated tapwater was pumped through BACs for an additional 2-3 days and the free chlorine concentration of BAC effluent was measured daily. Additional hypochlorite was added to the bulk fluid when the free chlorine concentration fell below 0.5 ppm. Non-chlorinated tapwater was used in control experiments. BAC beads were extracted for PLFA as previously described (Chapter 3). In addition, bulk fluid effluent containing chlorine-exposed planktonic cells was filtered and analyzed for PLFA for some experiments as

described above. PLFA compositions of chlorinated versus non-chlorinated biofilms and planktonic cells were compared.

Results and Discussion

Eleven polar lipid fatty acids were detected in *S. paucimobilis* biofilms not exposed to chlorine (Table 5.1). Saturated fatty acids composed 6.29±0.64% of lipid profiles and 16:0 predominated within this class. The largest percentage of fatty acids detected were monoenoics, comprising 91.84±0.55% of the total PLFA. 87.00% of the total profiles was 18:1w7c. The occurrence of cyclopropyl fatty acids at 1.87±0.45% suggests that the biofilm population is experiencing conditions which lead to low growth rates (Knivett and Cullen, 1965), likely caused by growth under low nutrient conditions. In contrast, four monoculture biofilms exposed to chlorine concentrations ranging 0.5-2.0 ppm contained very low levels of monoenoic fatty acids: between 7 and 12% of the total profiles (fig. 5.1). In addition, oxirane fatty acids, which were not detected in chlorine unexposed samples, comprised 58-68% of the total PLFA.

To examine the effects of free chlorine concentration upon total oxirane formation, a comparison of PLFA profiles from *S. paucimobilis* biofilms exposed to different maximum concentrations was made (fig. 5.2). Duplicate biofilms exposed to maximums of 0.5 and 1.0 ppm free chlorine (fig. 5.3) over a three day

Table 5.1. Lipid profiles obtained from *S. paucimobilis* biofilms unexposed to chlorine (SD=standard deviation).

				AVERAGE	SD
Saturates					
16:0	5.10	6.20	5.88	5.73	0.57
17:0	0.02	0.03	0.03	0.03	0.00
18:0	0.50	0.66	0.43	0.53	0.12
TOTAL	5.63	6.90	6.35	6.29	0.64
Monoenoics					
16:1w7c	2.39	1.80	2.97	2.39	0.58
16:1w5c	0.42	0.33	0.52	0.43	0.09
17:1a	0.05	0.00	0.04	0.03	0.03
17:1b	0.14	0.07	0.08	0.10	0.04
18:1w7c	87.24	87.05	86.70	87.00	0.28
18:1w5c	1.82	1.97	1.94	1.91	0.08
TOTAL	92.06	91.22	92.25	91.84	0.55
Cyclopropyls					
Cy17:0	0.82	0.67	0.70	0.73	0.08
Cy19:0	1.49	1.21	0.71	1.14	0.40
TOTAL	2.31	1.88	1.41	1.87	0.45

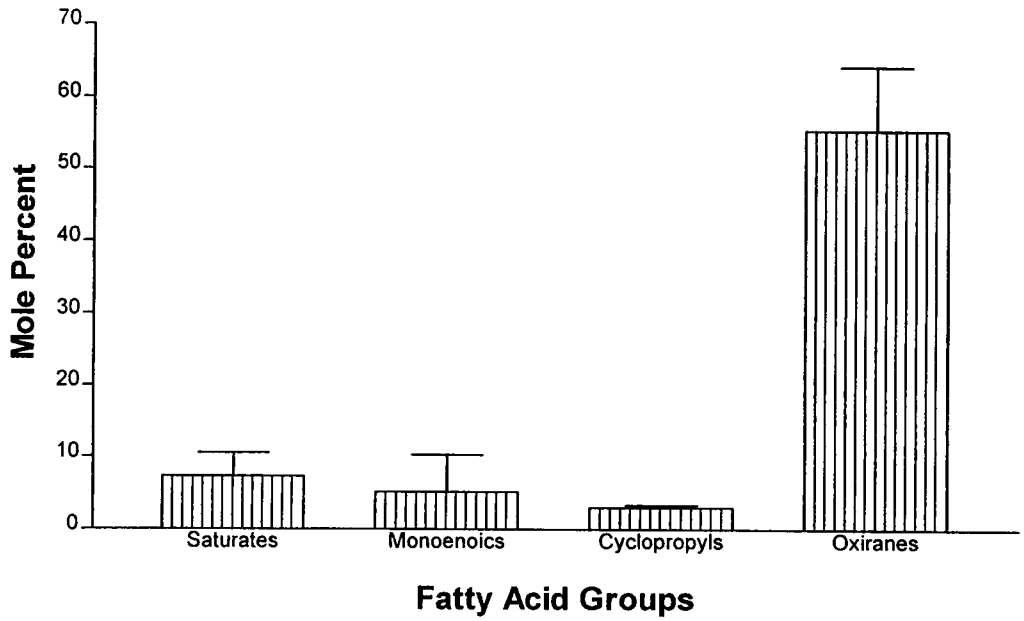


Figure 5.1. PLFA composition of four monoculture *S. paucimobilis* biofilms exposed to chlorine concentrations ranging 0.5-2.0 ppm.

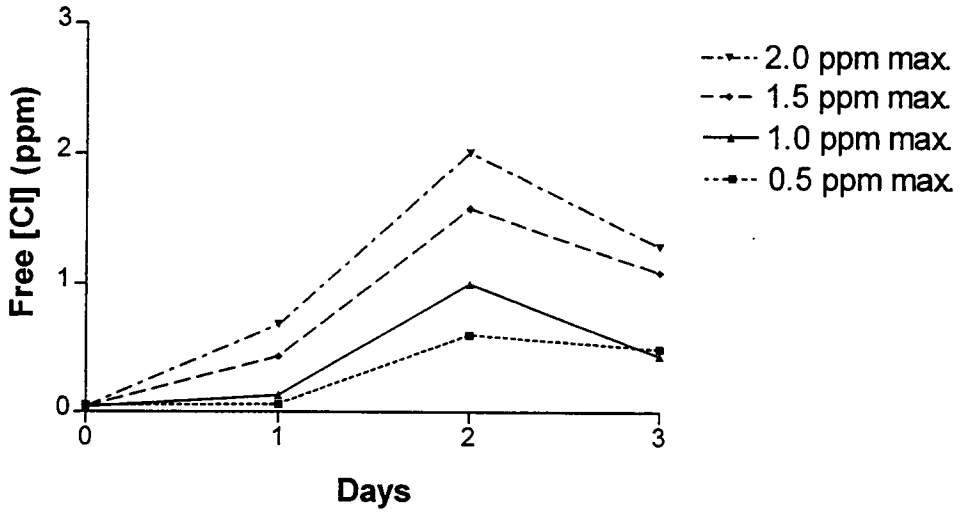


Figure 5.2. Free chlorine concentrations over time of bulk fluid from *S. paucimobilis* biofilms exposed to different maximum residuals.

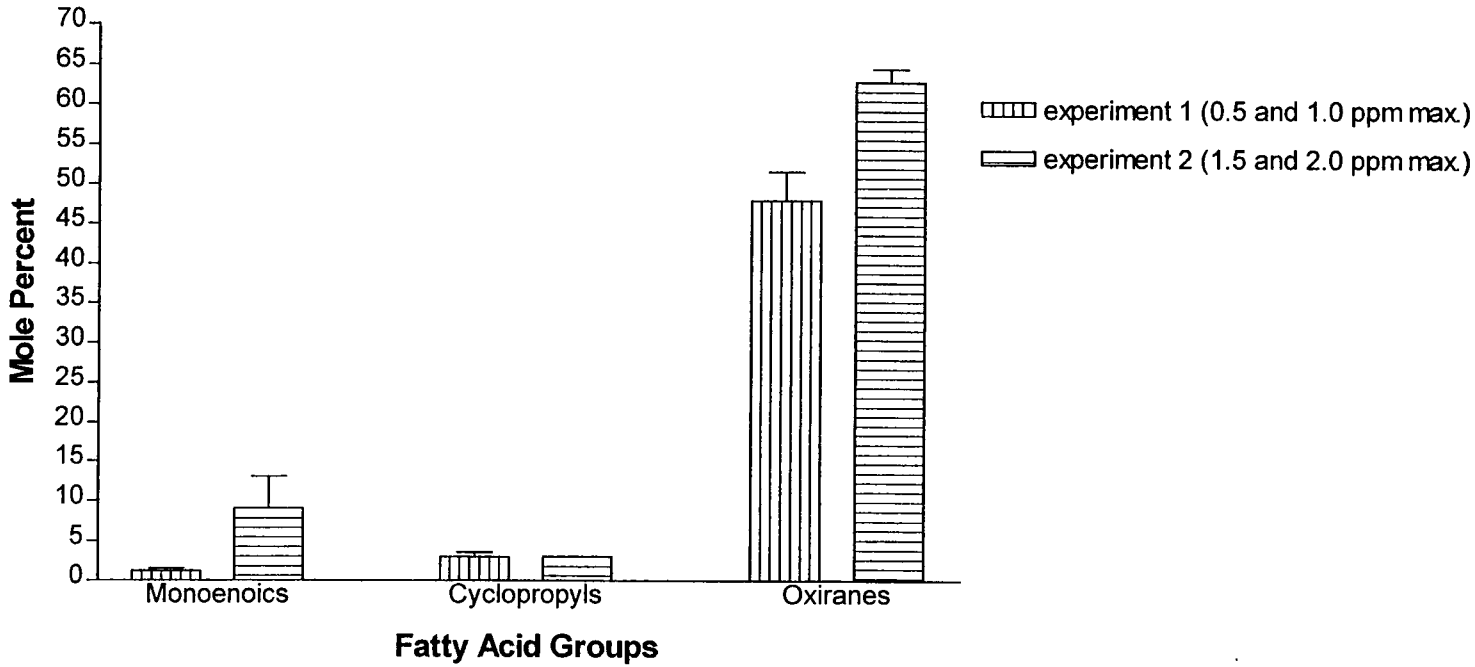


Figure 5.3. Comparison of PLFA composition of monoculture *S. paucimobilis* biofilms exposed to indicated chlorine concentrations.

period exhibited mole percentages of total oxiranes in the range 50-55%. In contrast, duplicate biofilms exposed to a higher free chlorine concentration (1.5 and 2.0 ppm maximum) (fig. 5.3) over the same length of time had total oxirane mole percentages of approximately 65%. The effect of chlorine concentration appears to be that increased free chlorine leads to increased oxirane formation. This is further supported by similar trends observed in planktonic cells from the same experiments.

As described above (materials and methods, this chapter), planktonic cells were obtained by filtration of effluent at two time points. The first was after switching bulk fluid from 1/100 nutrient broth to sterilized tapwater. The effluent collection tank was disconnected and the effluent filtered. Also, at the end of the experiment, the second (tapwater) effluent collection tank was filtered and all were extracted for PLFA. Planktonic cells sloughed from biofilms exposed to a maximum of 1.5 ppm free chlorine contained oxiranes which composed 1% of the total lipid profile (fig. 5.4). Also, planktonic cells unexposed to chlorine (1/100 nutrient broth effluent) had higher amounts of cyclopropyl fatty acids. Typically, microbes form cyclopropyl fatty acids from monoenoic precursors under low nutrient conditions (Ringelberg et al., 1989). That is, when little or no growth is occurring. It would be expected to see higher molar percentages of cyclopropyls in the chlorine-exposed planktonic cells. This is because those bacteria were

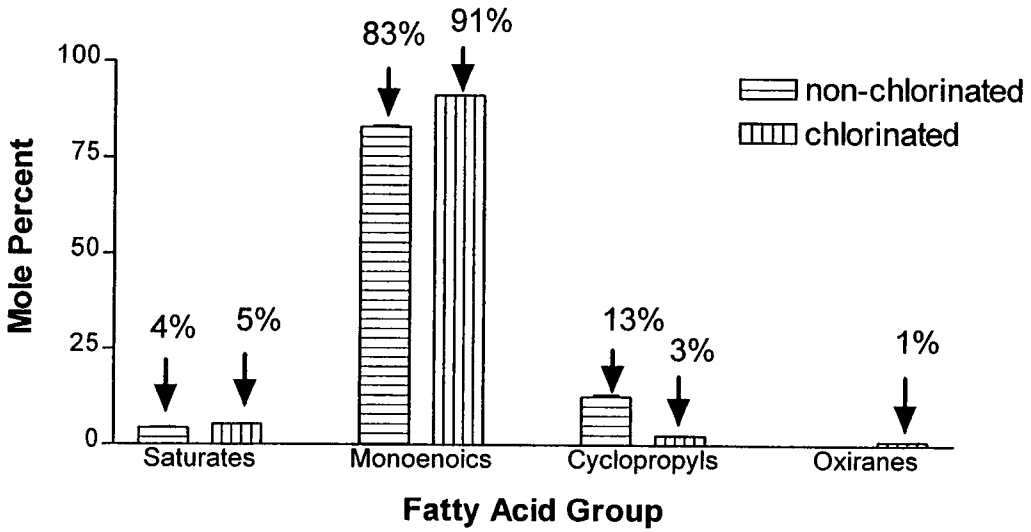


Figure 5.4. Fatty acid composition of chlorinated and non-chlorinated *S. paucimobilis* planktonic cells from a biofilm exposed to a maximum free chlorine residual of 1.5 ppm.

filtered three days after the chlorine unexposed cells and were exposed to much lower nutrient conditions. The same trend is observed in planktonic cells exposed to a maximum of 2.0 ppm free chlorine (fig. 5.5). These cells had lipid profiles consisting of 13% oxirane fatty acids. Again, it appears that increased free chlorine concentrations lead to increased oxirane formation, as was observed in biofilms.

Typically, when bacteria are exposed to low nutrient conditions or when cells are physiologically stressed by age, the ratio of cyclopropyl / monoenoic precursor increases. Also, when stressed by various toxic agents, the ratio of monoenoic fatty acids in the trans configuration increases relative to monoenoics in the cis configuration (e.g.18:1w7t/18:1w7c). A comparison of these ratios obtained from biofilms, chlorine exposed planktonic cells, and chlorine unexposed planktonic cells is shown in Table 5.2.

The ratio of cyclopropyl 19/18:1w7c decreased from biofilm experiment one to biofilm experiment two. These were grown under similar conditions and it is interesting to note that this ratio decreases in the biofilms exposed to 2.0 ppm maximum free chlorine concentration. Non-chlorinated planktonic cells from biofilm A also show a decrease in the ratio of cyclopropyl 17 compared to chlorine exposed planktonic cells (from ~1.4 to 0.3). A comparison of chlorine unexposed planktonic cells versus biofilms reveals that the relative ratio of cyclopropyl 19

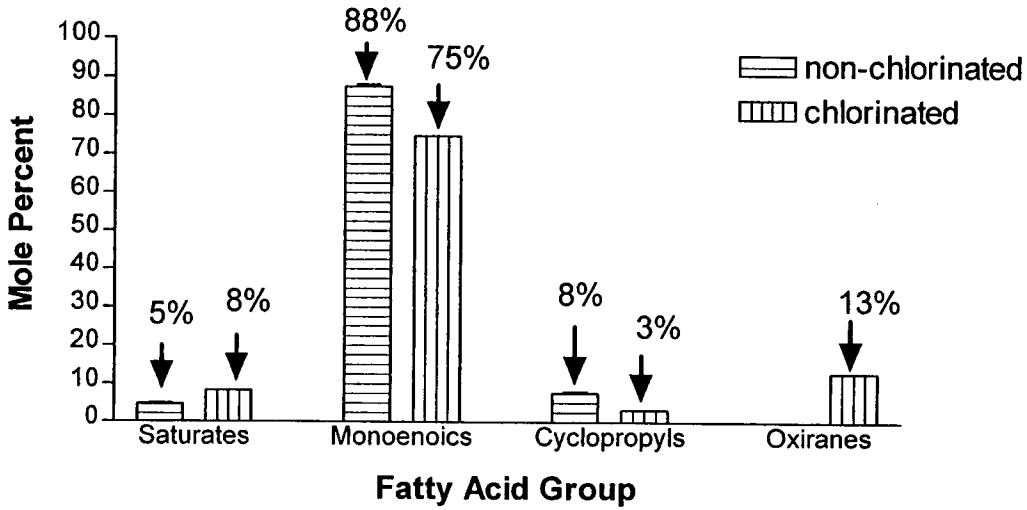


Figure 5.5. Fatty acid composition of chlorinated and non-chlorinated *S. paucimobilis* planktonic cells from a biofilm exposed to a maximum free chlorine residual of 2.0 ppm.

Table 5.2. Ratios of polar lipid fatty acids obtained from injured biofilm and planktonic cells of *S. paucimobilis* which are indicative of stress. Values are expressed as molar percentages.

	BIOFILMS			
	Experiment 1 (1ppm max.)		Experiment 2 (2ppm max)	
			A	B
18:1w7c	1.33	1.01	5.63	11.42
cy 19:0	2.61	1.82	1.87	2.31
Ratio cy19/18:1w7c	1.96	1.81	0.33	0.20
18:1w7c	1.33	1.01	5.63	11.42
18:1w7t	0.09	0.00	0.44	0.29
Ratio 7t/7c	0.06	0.00	0.08	0.03

	NON-CHLORINATED EFFLUENTS					
	A			B		
16:1w7c	1.89	2.01	1.99	2.05	2.37	2.10
cy17	2.88	2.85	2.75	1.99	2.18	1.66
Ratio cy17/16:1w7c	1.52	1.42	1.38	0.98	0.92	0.79
18:1w7c	78.20	78.74	78.62	80.90	79.65	81.66
cy19	10.23	9.61	9.66	5.83	5.90	5.65
Ratio cy19/18:1w7c	0.13	0.12	0.12	0.07	0.07	0.07
18:1w7c	78.20	78.74	78.62	80.90	79.65	81.66
18:1w7t	0.00	0.00	0.00	1.97	2.21	1.85
Ratio 7t/7c	0.00	0.00	0.00	0.02	0.03	0.02

	CHLORINATED EFFLUENTS (TAP WATER)	
	A	B
16:1w7c	5.54	0.80
cy17	1.64	1.34
Ratio cy17/16:1w7c	0.30	1.68
18:1w7c	84.51	72.05
cy19	0.89	1.97
Ratio cy19/18:1w7c	0.01	0.03
18:1w7c	84.51	72.05
18:1w7t	0.04	0.88
Ratio 7t/7c	0.00	0.01

increases in biofilms in both experiment A and B. However, the total molar percentage of cyclopropyl fatty acids (cy17 and cy19) is greater in chlorine unexposed planktonic cells than in either biofilms or chlorine exposed planktonic cells. It would be expected to detect higher total percentages of cyclopropyl fatty acids from biofilms and planktonic cells obtained at the end of the experiment and grown in tapwater compared to those obtained three days earlier and grown in 1/100 nutrient broth.

Chapter 6

Employment of Confocal Scanning Laser Microscopy to Examine Biofilm Architecture and Chlorination Effects

Introduction

Structural studies of biofilm formation and architecture can be performed by a variety of microscopic methods. Transmitted light microscopy has been used to examine wet mount preparations of biofilms (Korber et al., 1989). Electron microscopy has been shown to be a useful tool in the study of microbial biofilms as well (Eighmy et al., 1983). However, these methods are laborious and/or may introduce artifacts from sample preparation. Also, three-dimensional visualization of biofilm structure is limited due to poor penetration of light through thick films. The advent of confocal scanning laser microscopy (CSLM) in the study of attached microbes has overcome some of these limitations (Lawrence et al., 1991). High resolution and elimination of out-of-focus haze allow collection of high-quality images of thick microbiological samples. A variety of fluorescence staining techniques allow visualization of intact biofilms. In addition, horizontal (xy) and sagittal (xz) optical sections can be obtained at desired intervals through the depth of a biofilm. These scanned images can then be examined individually and processed into a single compiled image. This permits accurate reconstruction

of three-dimensional cellular relationships and overall biofilm structure from optical thin sections or slices (Lawrence et al., 1991). Image-processing techniques can be used to quantitatively analyze collected images also (Lawrence et al., 1989).

CSLM was employed to examine the biofilm ecology of two drinking water opportunists. The relationships between *Sphingomonas paucimobilis* and *Pseudomonas aeruginosa* were examined. These experiments included use of fluorescence staining to visualize cells and a genetically engineered reporter strain of *S. paucimobilis* which fluoresced without staining due to plasmid-encoded green fluorescent protein (GFP). The effects of order of inoculation of each species, trophic conditions, and addition of hypochlorite to the bulk fluid were examined.

Materials and Methods

Microscopy flowcells (fig. 6.1) were constructed from a silicone rubber (RTV 118, General Electric Co.) gasket (75 mm long x 25 mm wide x 1mm high) and glass coverslips (Palmer and Caldwell, 1995). Essentially, two glass coverslips are separated by the gasket, which is sealed between them with additional silicone. That gasket is cast in a Teflon mold that contains two raised ribs which create channels for fluid flow within the flowcell. These channels

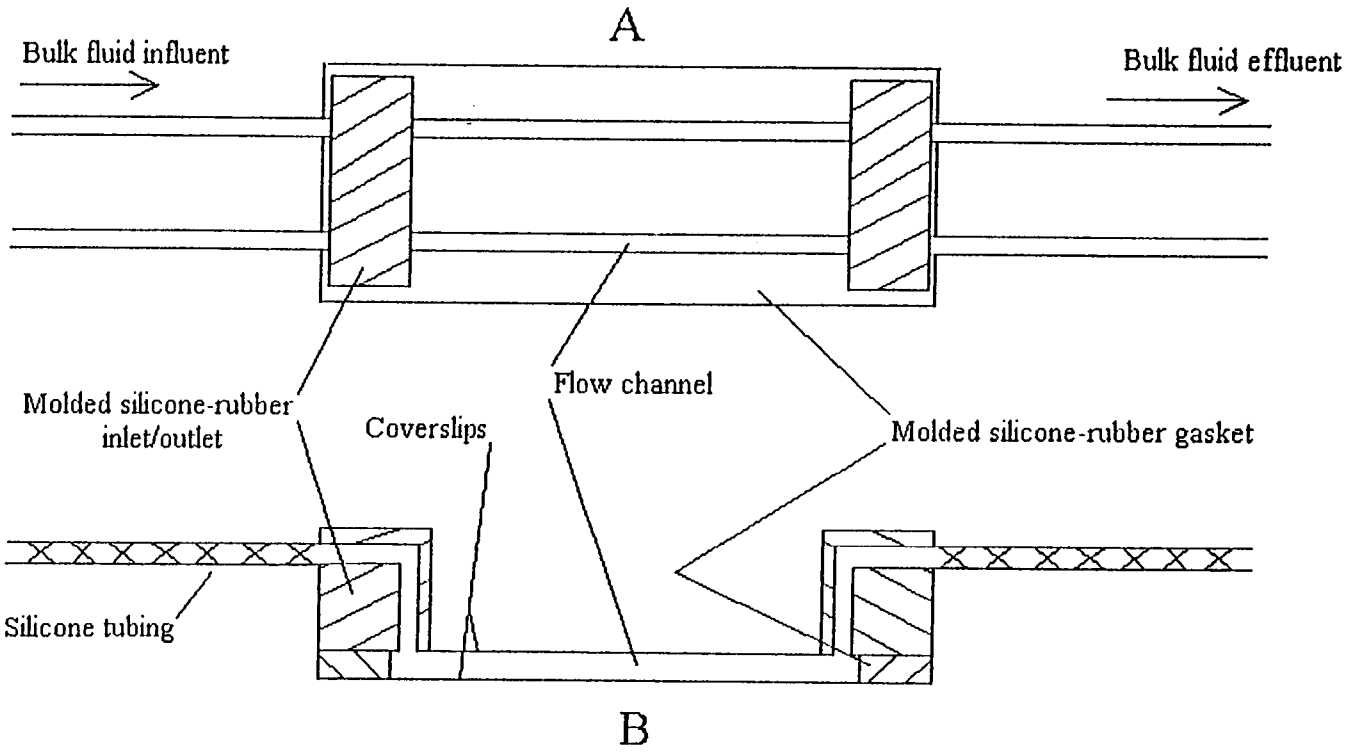


Figure 6.1. Top view of flowcell (A) and cross-section through a channel (B). The distance between the top and bottom glass surfaces is approximately 1.5 mm. A peristaltic pump (not shown) was used on the effluent end to pump the bulk fluid to a waste collector. Adapted from Palmer and Caldwell (1995).

(65 mm long x 3 mm wide x 1 mm high) are centered within the gasket. One glass coverslip has the same dimensions as the gasket and is sealed flush to the spacer edges with silicone rubber (flowcell bottom). The second coverslip is 55 mm long and is sealed flush and centered on the gasket on the top side. The resulting open-ended channels are connected to molded silicone rubber inlet/outlet adapters which contain silicone tubing connected via an L-shaped channel to the flowcell channels. Silicone tubing extending from the adapters is connected to a bulk fluid reservoir (upstream) and effluent collection reservoir (downstream). A peristaltic pump (Watson-Marlow) was employed on the downstream end to pump the bulk fluid.

The membrane stain RH795 (Molecular Probes, Eugene, Oregon, cat.#R-649) was prepared as a 1mM stock solution by dissolving with dimethyl sulfoxide. This was added to dilute (1/100) nutrient broth (0.5% v/v) prior to inoculation with *P. aeruginosa*. Genetically engineered *S. paucimobilis* containing a stable plasmid encoding green fluorescent protein (Matthysse et al., 1996) was grown in dilute (1/100) nutrient broth under selective conditions with kanamycin. Both organisms were grown to late log phase prior to centrifugation. Pellets were resuspended in filter sterilized tapwater to remove excess kanamycin or RH795 prior to inoculation into flowcells.

Flowcell channels were conditioned with bulk fluid for a minimum of 30

minutes prior to inoculation. Autoclaved, dilute (1/100) nutrient broth or filter sterilized tapwater (0.2 μm) served as bulk fluid for all experiments. Five hundred μL (~10^8 cells) of resuspended batch culture was injected into a sterile (autoclaved), pre-conditioned flowcell channel (200 μL volume). Inoculation was achieved by injection of culture into the silicone tubing extending from the molded outlet. Cells of the first species inoculated were allowed to attach for one hour under static conditions. Flow was then resumed at a rate between 50-100 μL/minute.

Twelve hours after inoculation of the first species, the second species was inoculated in a similar manner. These biculture biofilms were allowed to grow for another 12 hours before imaging. Confocal micrographs were obtained before and after addition of hypochlorite to the bulk fluid. Images were obtained with a Leica laser confocal microscope (DMR) (Heidelberg, Germany) with 100X, 1.4 NA oil-immersion lens. Argon and Krypton lasers served as excitation sources for GFP (green emission) and RH795 (red emission), respectively. These sources provided laser light of wavelengths 488 and 568 nanometers. TCS NT control software (Leica Lasertechnik, Heidelberg, Germany) permitted collection and storage of individual scanned images. Intervals between optical slices or thin sections (xy) and the location at which sagittal (xz) sections were obtained were user defined. This was achieved through TCS NT software used in conjunction with a motorized

stage on the microscope. In addition, control software can render these processed images in 2 or 3 (stereo) dimensions.

Results and Discussion

When *S. paucimobilis* is inoculated first into flowcells in which sterile tapwater served as bulk fluid, microcolonies or cell aggregates form which appear heterogeneous (fig. 6.2). That is, both species are present throughout the microcolonies but *S. paucimobilis* is predominantly found on the outer regions or periphery of those aggregates. This is likely due to the fact that *S. paucimobilis* is a slower growing microbe and is only somewhat motile. This species secretes much exopolysaccharide (EPS) (White et al., 1996) and may lend structural support to the biofilms. This is supported further by observations made when *P. aeruginosa* is inoculated first (fig. 6.3). Under these conditions microcolonies still contain both species but are much more homogeneous. That is, individual aggregates are seen within the microcolonies consisting of one or the other species. An XY scan (fig. 6.3A, arrow) shows a "pillar" or "column" of pure *S. paucimobilis* intercalated within three aggregates of pure *P. aeruginosa.* This pillar is more evident in an XZ scan (fig. 6.3B, left arrow). In addition, columns of *S. paucimobilis* appear along the edge of the microcolony (fig. 6.3B, right arrow) and are likely peripherally attached to that structure. The red horizontal line

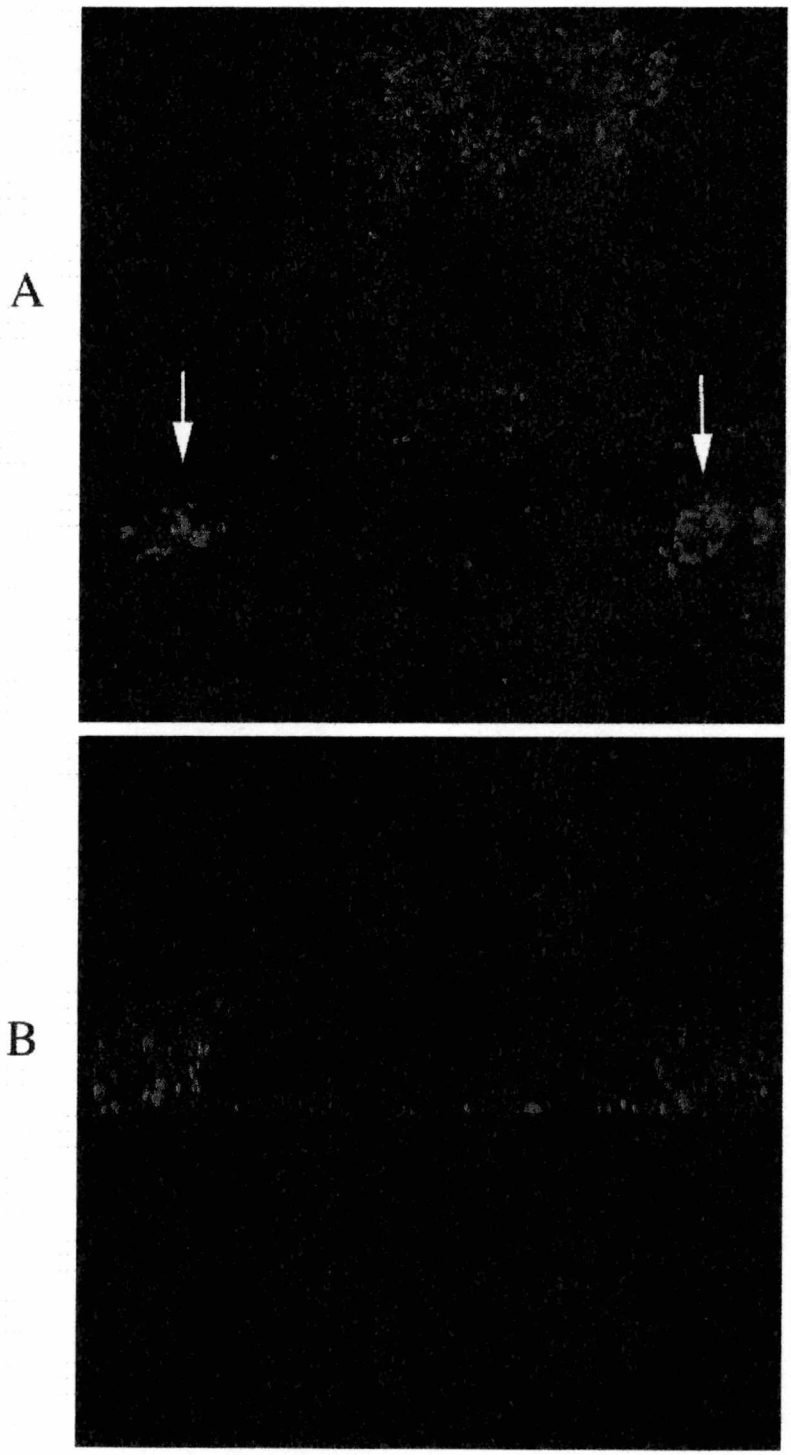


Figure 6.2. Confocal micrographs of biculture biofilms formed when *S. paucimobilis* was inoculated first. (A) Compilation of 25 horizontal slices through a mixed-species microcolony 21.4 microns high. Image size is 100 x 100 microns. *S. paucimobilis* cells are green and *P. aeruginosa* are red. (B) Sagittal (xz) section through microcolonies indicated by arrows in (A). Note the overall heterogeneity of microcolonies.

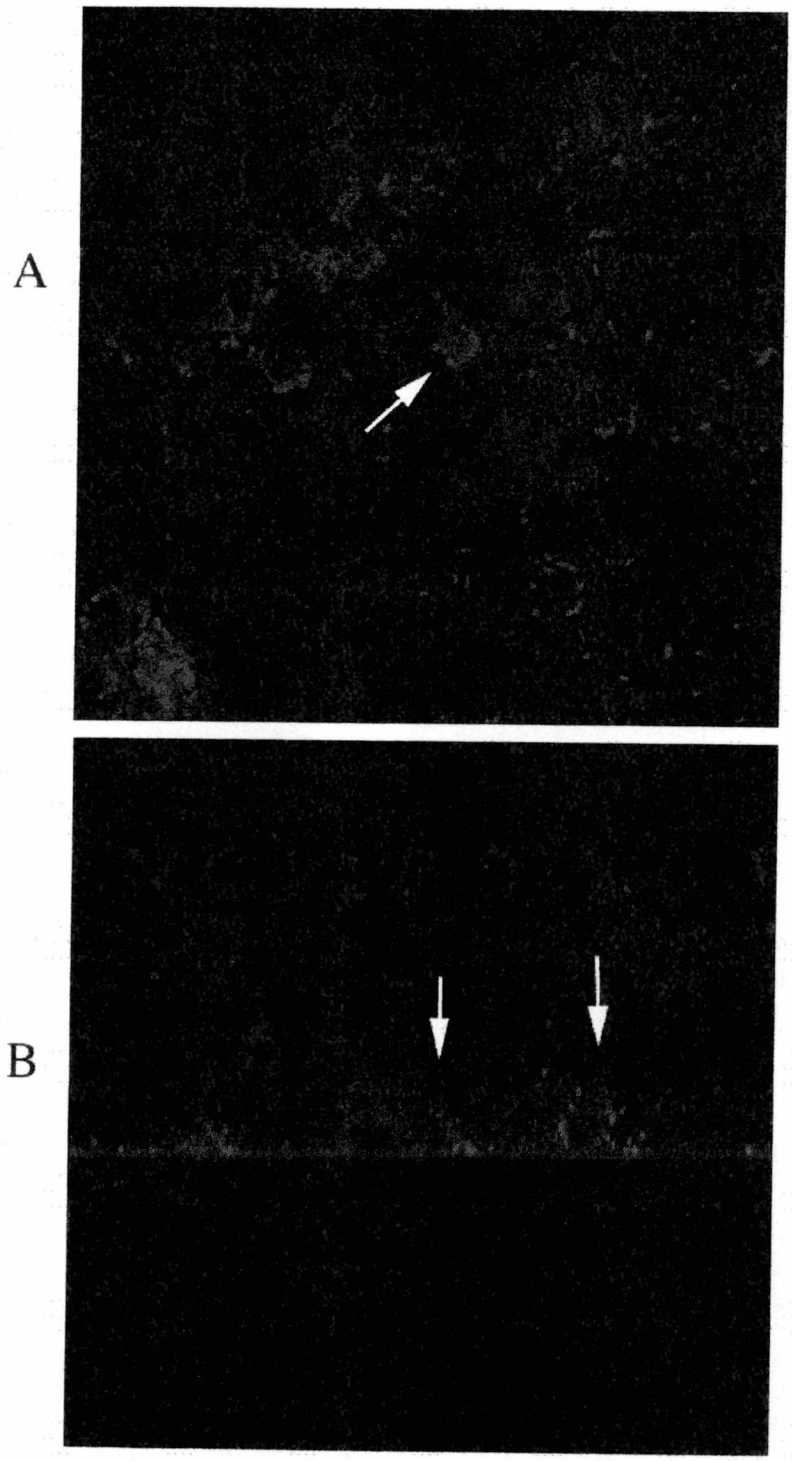


Figure 6.3. Confocal micrographs of biculture biofilms formed when *P. aeruginosa* was inoculated first. (A) Compilation of 16 horizontal slices through a mixed-species microcolony 12.6 microns high. Image size is 100 x 100 microns. GFP-containing *S. paucimobilis* cells are green and stained *P. aeruginosa* appear red. Arrow indicates pillar described in text. (B) Sagittal section through the same microcolony. Pillar (left arrow) and peripherally attached columns (right arrow) are indicated.

through the center of figure 6.3B is the glass substratum which is non-specifically stained with RH795. Upon addition of 3 ppm free chlorine to the bulk fluid and 20 minutes exposure, the large aggregates of *P. aeruginosa* detach leaving much of the *S. paucimobilis* pillars and columns present and intact (fig. 6.4, arrows). Only a few single cells of *P. aeruginosa* remain attached to the substratum. These observations led to the hypothesis that *S. paucimobilis*, though slower growing and less motile than many drinking water bacteria, may play an important role in biofilm architecture by lending structural support by EPS production.

Similar experiments were performed with dilute (1/100) nutrient broth as bulk fluid. This was done to examine how trophic conditions dictate biofilm formation and structure. Under these conditions the majority of *P. aeruginosa* cells were attached to the substratum (fig. 6.5A). Microcolony formation by that species occurred individually (fig. 6.5B, right arrow) and in association with *S. paucimobilis* (left arrow). In contrast, *S. paucimobilis* single cells attached to the substratum did not form homogeneous (single species) microcolonies. Microcolonies formed by that microbe were in association with *P. aeruginosa* attached to the outer edges. However, aggregates of *S. paucimobilis* again appeared to be intercalated within the center of these structures (as in figure 6.3) (fig. 6.5B, left arrow). The majority of *S. paucimobilis* biomass was concentrated in biofilm regions most exposed to the bulk fluid and furthest from the substratum.

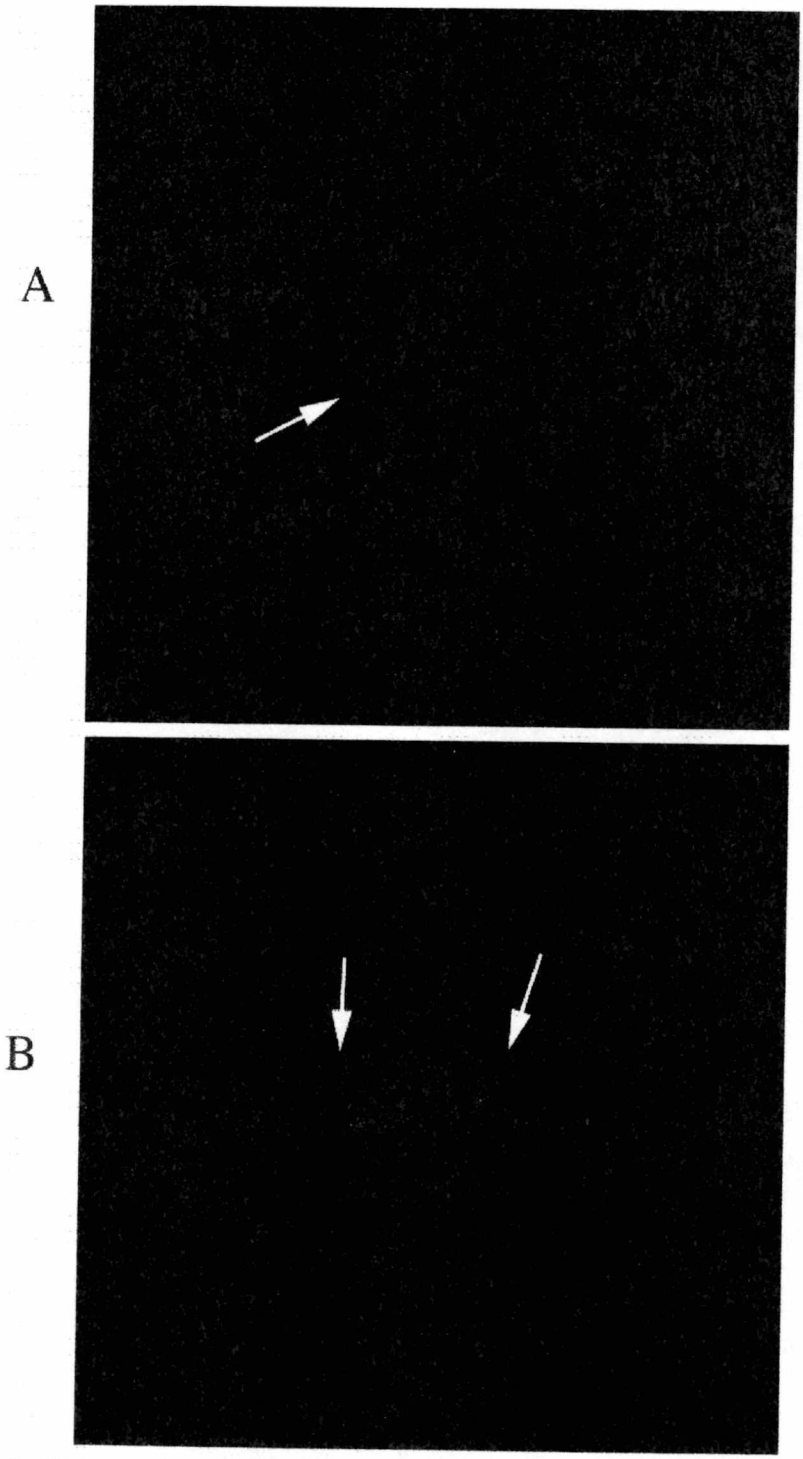


Figure 6.4. Horizontal (xy) (A) and sagittal (xz) (B) images of microcolony shown in figure 6.3 after addition of hypochlorite to the bulk fluid. The large aggregates and most single cells of *P. aeruginosa* (red cells) have detached. The majority of *S. paucimobilis* (green cells) pillars and columns are intact after 20 minutes exposure to 3.0 ppm free chlorine. Image size is 100 x 100 microns. Arrows indicate pillars and columns shown in figure 6.3.

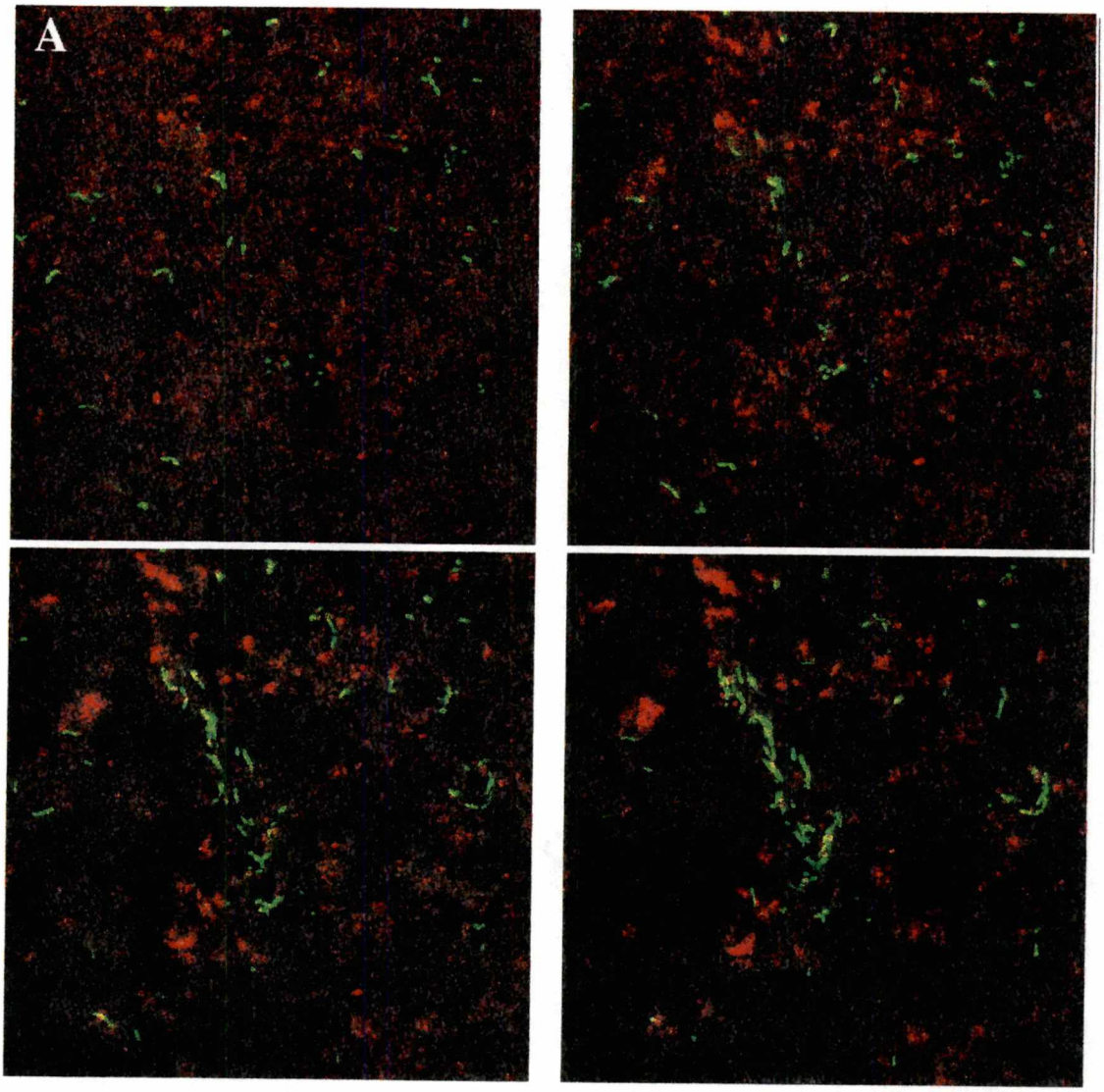


Figure 6.5. Sixteen single scans through a microcolony 13.1 microns high beginning with the substratum (A). Slices obtained at 0.873 micron intervals outward from the substratum are arranged left to right and top to bottom. Image sizes are 100 x 100 microns. Microcolonies discussed in text are indicated with arrows. The uppermost slice through the microcolony (furthest from the substratum) is indicated as well (C). Most cells of *P. aeruginosa* are at or near the substratum, in contrast to *S. paucimobilis*.

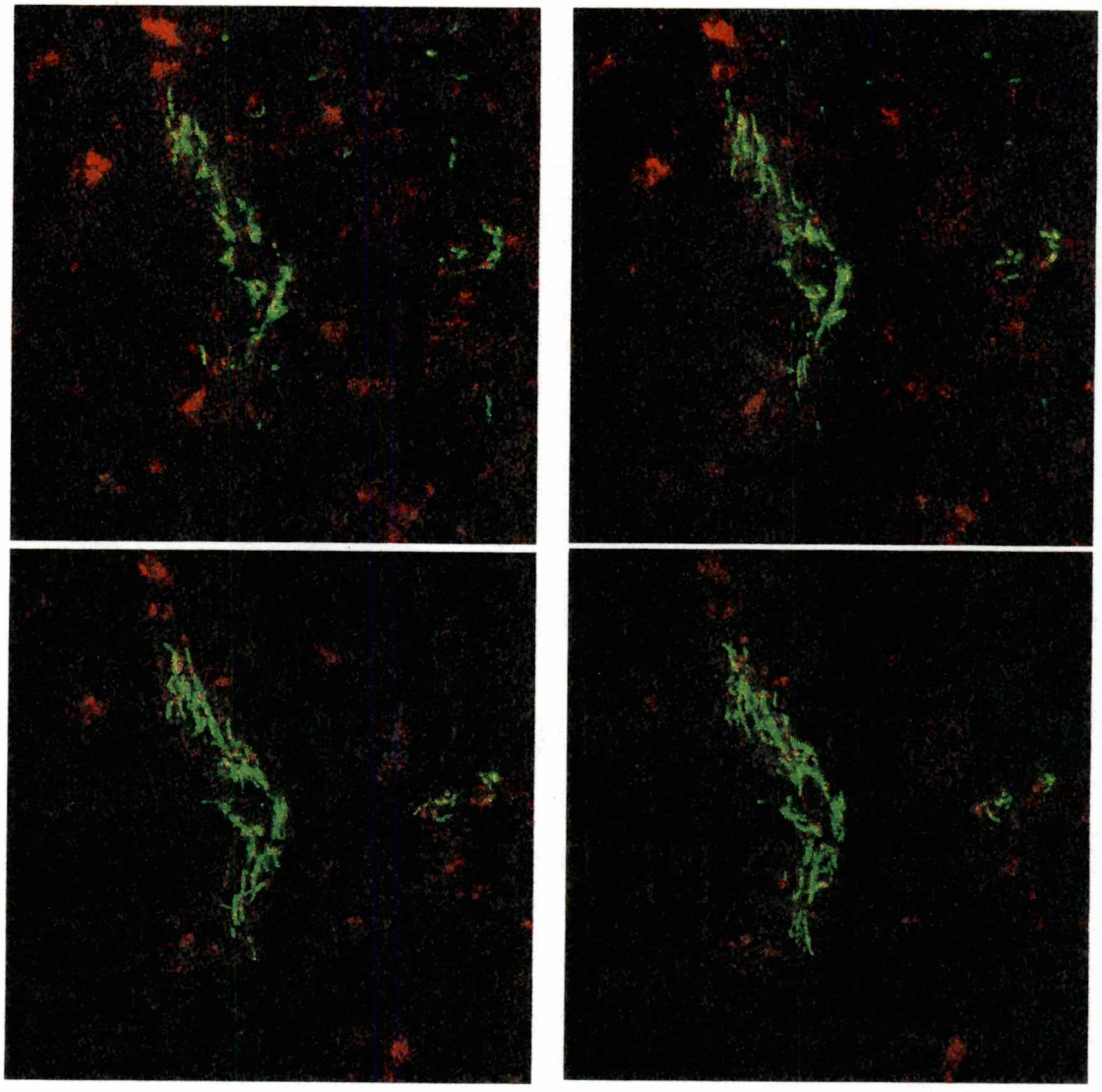


Figure 6.5. (continued)

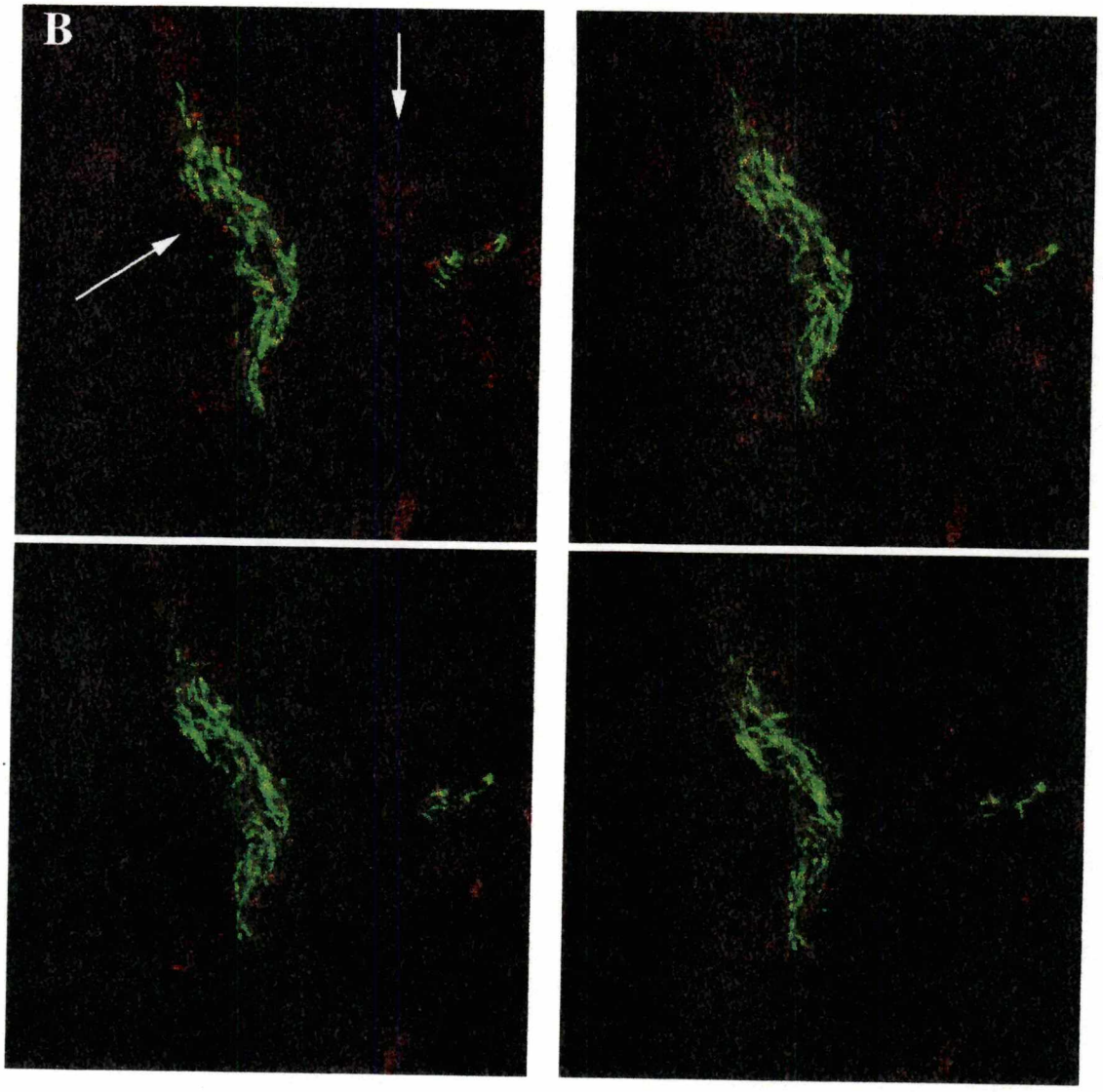


Figure 6.5. (continued)

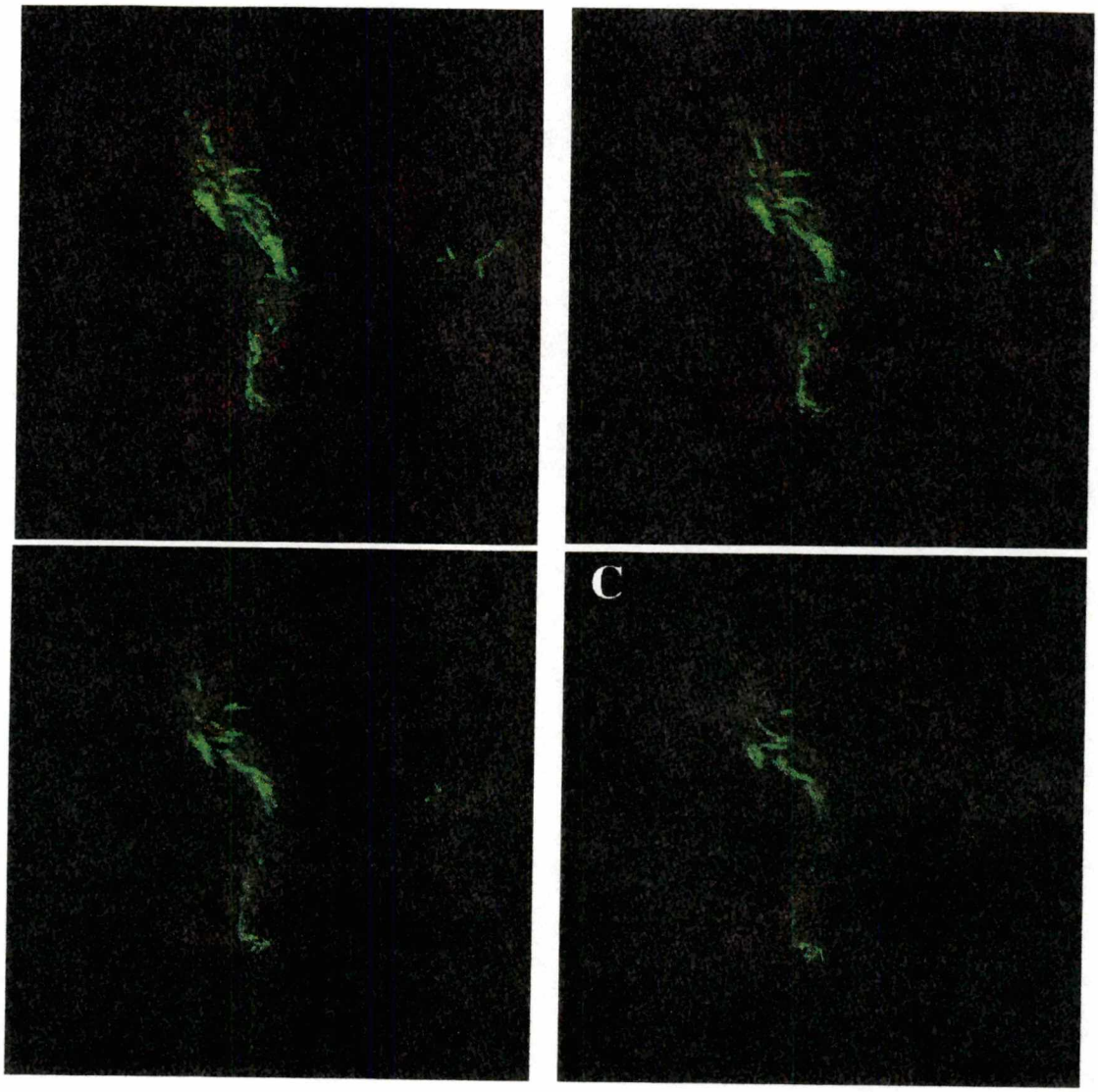


Figure 6.5. (continued)

These results show that *S. paucimobilis* cells form biofilms and microcolonies in close association with *P. aeruginosa*. In addition, it appears that *S. paucimobilis* cells find a niche in these biofilms on the upper fringes. This may be due to nutrient limitation, higher oxygen tension, and/or competition with the faster growing *P. aeruginosa*. There seems to be a clear structural role for *S. paucimobilis* in these biofilms. It is perhaps lending of structural support which causes the microbes to accumulate further out in the bulk fluid than their Pseudomonad counterparts. If this is the case than a mutualistic relationship has been observed in these biofilms whereby *S. paucimobilis* secretes EPS which helps *P. aeruginosa* cells stay attached to the microcolony surface. In turn, faster-growing *P. aeruginosa* cause *S. paucimobilis* cells to grow with more collective surface area exposed to the bulk fluid where nutrient levels and oxygen concentrations are higher.

Figure 6.6 further supports these ideas. Again, *S. paucimobilis* microcolonies tend to be larger than those formed by *P. aeruginosa* and become broader or wider as they extend out into the bulk fluid. These images also show a long (~30-40 μm), filamentous *P. aeruginosa* cell attached and completely enwrapped up and around the *S. paucimobilis* microcolony (fig. 6.6, arrows). This cell is visible in 4 of 6 scans presented and can be seen wrapping up the microcolony from the substratum.

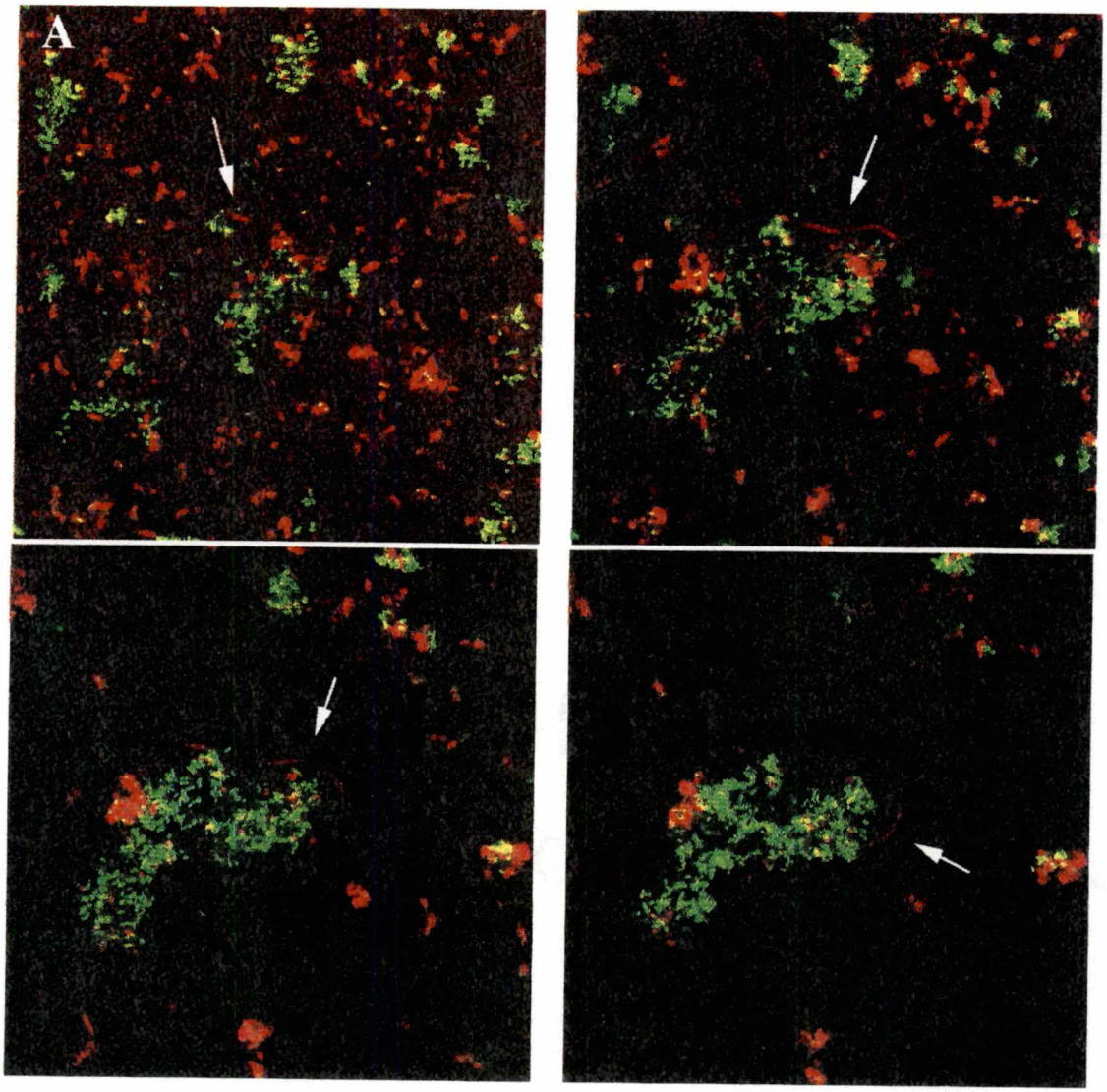


Figure 6.6. Six scans through a microcolony 10.9 microns high beginning with the substratum (A). Slices obtained at 1.36 micron intervals outward from the substratum are arranged left to right and top to bottom. Image sizes are 100 x 100 microns. *S. paucimobilis* forms microcolonies that become broader with increased height. *P. aeruginosa* form microcolonies which are typically shorter and more uniform in width. A filamentous *P. aeruginosa* cell attached to the outer edge of the *S. paucimobilis* microcolony is indicated with arrows.

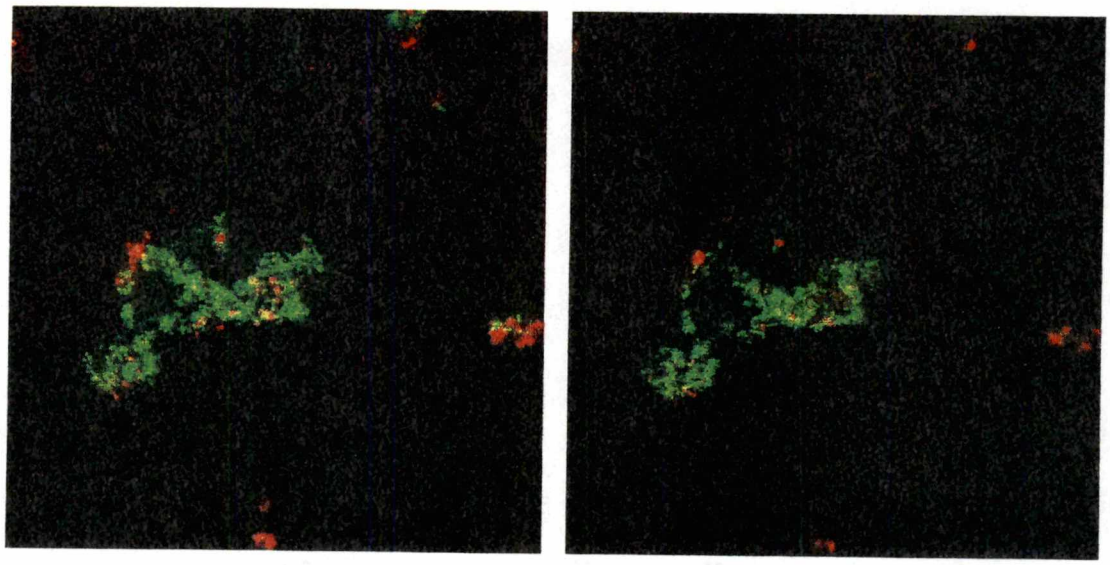


Figure 6.6. (continued)

After addition of hypochlorite (0.76 ppm free chlorine) to the bulk fluid and one hour exposure much detachment of single cells from the substratum occurred (fig. 6.7A). A microcolony composed of *P. aeruginosa* cells is shown to be attached to a larger microcolony of *S. paucimobilis* on one side. This microcolony did not detach. This may be due to the avidity with which *S. paucimobilis* microcolonies are bound even after addition of chlorine (fig. 6.4). It was shown that after exposure to 3.0 ppm free chlorine for 20 minutes large aggregates of *P. aeruginosa* detached leaving *S. paucimobilis* microcolonies intact (fig. 6.4). When the chlorine concentration is lower, it appears that *S. paucimobilis* microcolonies may harbor *P. aeruginosa* and keep biofilm structure relatively intact.

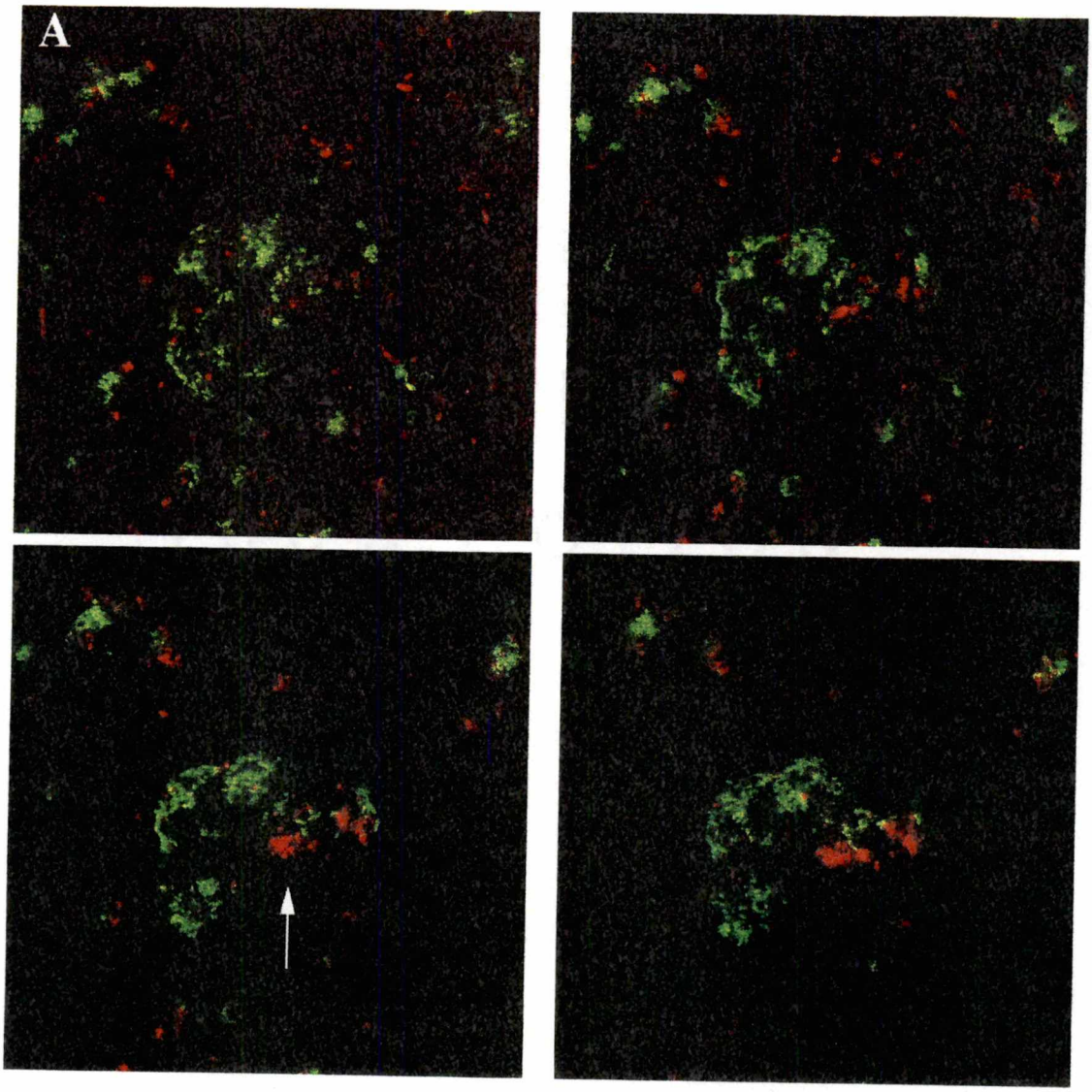


Figure 6.7. Nine single scans through two microcolonies 11.4 microns high beginning with the substratum (A) after exposure to 0.76 ppm free chlorine for one hour. Fewer single cells of *P. aeruginosa* are seen at the substratum (compared to chlorine unexposed) due to detachment. A *P. aeruginosa* microcolony (arrows) adjoined to a *S. paucimobilis* microcolony remains attached. Images obtained at 1.43 micron intervals outward from the substratum are arranged left to right and top to bottom. Image sizes are 100 x 100 microns.

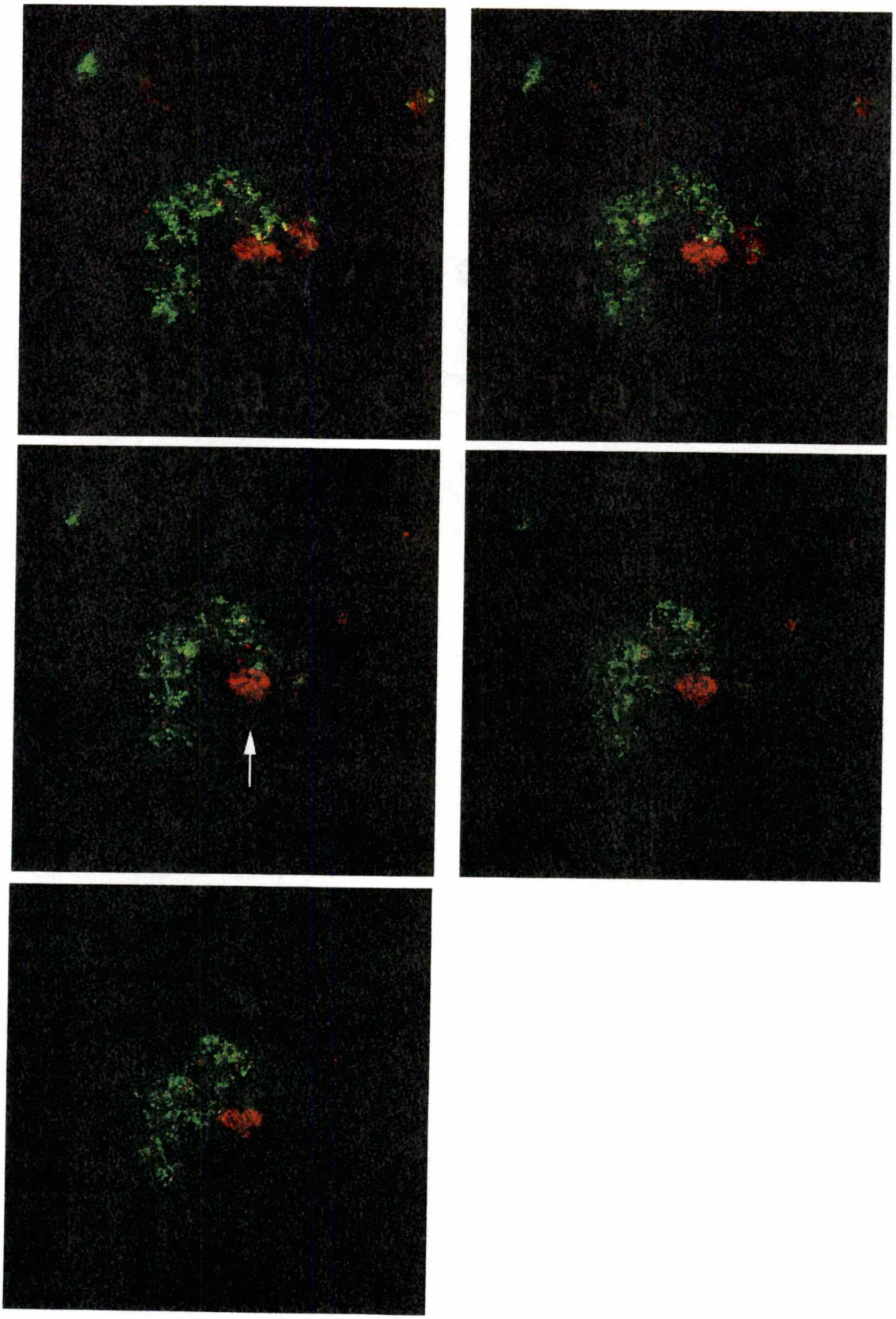


Figure 6.7. (continued).

Chapter 7

Summary and Conclusions

PLFA Analysis and Oxirane Fatty Acids

The site of cellular damage as a result of exposure to stressors in the aquatic environment is often the cytoplasmic membrane (Mossel and Corry, 1977). Frequently, loss of functions mediated within that structure are the physiological consequence of stress (Beauchat, 1978). These include leakage of cellular constituents, compromised nutrient transport and oxidative phosphorylation. Russell (1984) describes the overall cellular response to injury as a cumulative effect of many of these events which are inter-related. It is reasonable to assume that the membrane is a primary target of reactive chemicals in the environment since this structure is the outermost cellular component. Studies intended to examine the cellular effects of chlorine exposure at concentrations in the range seen in drinking water have shown that nutrient transport and intracellular ATP concentrations are greatly reduced (Camper and McFeters, 1979). Other studies have revealed that the inner membrane is the primary site of chlorine action in bacterial cells (Venkobacchar et al., 1977; Haas and Engebrecht, 1980). We believe that a heretofore unrecognized consequence of chlorine action within the membrane is the formation of oxirane fatty acids. These charged molecules could

possibly disrupt the integrity of membrane structure due to their potential reactivity, and specifically alter the structure of membrane proteins affecting their efficiency in transport processes.

We believe that PLFA analysis, especially if used in conjunction with traditional indicator and heterotroph culturing methods, may provide water servers with an idea of both the total amount of biomass or cells within the system and the degree of injury of those cells. For example, if low numbers of bacteria are detected by traditional plate count methods and high amounts of biomass are found using PLFA analysis, one could assume that many cells within the system are unculturable. In addition, if PLFA analysis reveals the presence of oxirane fatty acids, then it seems reasonable to assume that that unculturability is a result of mitigation with chlorine. Although PLFA analysis is costly and time-consuming, it could prove invaluable in cases where it is suspected that bacterial populations are being underestimated as a result of injury and this underestimation may lead to introduction of health risk to water consumers.

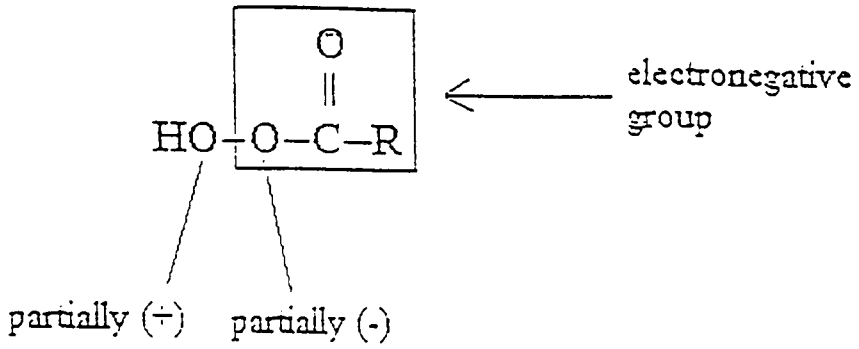
A mechanism for oxirane formation from monoenoic fatty acids was first proposed by Jonathan Lane and David Nivens after first detecting oxiranes from a cold water distribution system biofilm (unpublished data). In this mechanism, monounsaturated fatty acids are converted to halohydrin intermediates upon exposure to hypochlorite by hydroxylation and chlorination across the double

bond. Then, the halohydrin intermediate is converted to an oxirane or dioic fatty acid actively by bacteria or during the methylation process involved in preparation of lipid samples.

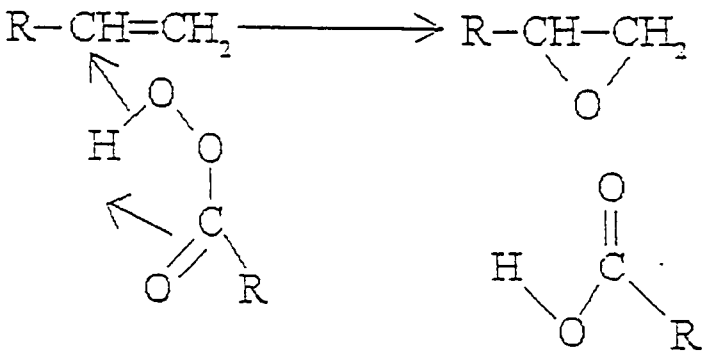
We believe that the new evidence presented here lends support to the hypothesis that the compounds are either actively formed by bacteria or spontaneously formed upon cell lysis. One possible mechanism for oxirane formation is the active conversion of halohydrin intermediates to oxiranes as proposed by Nivens and Lane (unpublished data). Halohydrin hydrogen-halide-lyase (H-lyase) is an enzyme which catalyzes the conversion of halohydrins to epoxides (Nakamura et al., 1994). The hydrogen halide is liberated. This enzyme was isolated from *Corynebacterium* sp. strain N-1074. Chlorine and bromine-containing halohydrins were shown to be actively converted to their corresponding epoxides. Enzymes involved in the dehalogenation of halohydrins have also been observed in a *Pseudomonas* sp. (Kasai et al., 1990), *Flavobacterium* sp. (Castro and Bartnicki, 1968), and *Arthrobacter* sp. strain AD2 (van der Wijngaard et al., 1989).

A well-characterized mechanism of epoxide formation is the oxidation of alkenes with peroxyacids (Loudon, 1988, p. 417-449). Peroxyacids are peroxide analogs of carboxylic acids. They contain an -O-OH group rather than an -OH group. The peroxide linkage between oxygen atoms is weak and highly reactive

due to a dipole moment resulting from partial positive and negative charges residing on adjacent Oxygen atoms as shown below:



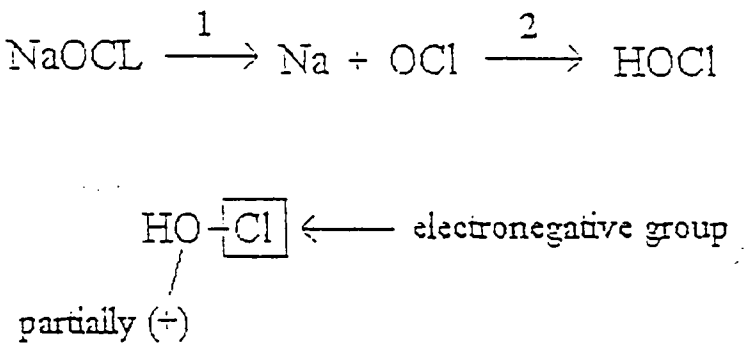
The hydroxyl group of the peroxyacid is electron-deficient. Electrophilic attack of the pi-bond of the alkene occurs, which is electron rich.



This yields epoxide and carboxylic acid products.

Oxiranes could be formed from dead cell biomass through a similar

mechanism by interaction of hypochlorous acid with monoenoic fatty acids. Upon addition of sodium hypochlorite to water, sodium and hypochlorite ions are formed (arrow 1, below). The hypochlorite quickly becomes bound by a hydrogen atom to form hypochlorous acid (arrow 2, below). Hypochlorous acid contains a dipole moment similar to that observed in peroxyacids (above). This is due to the higher electronegativity of chlorine compared to oxygen. Electrophilic attack of the alkene pi-bond by the partially positively charged oxygen could occur in a mechanism similar to that shown for peroxyacids. This would yield epoxide and hydrochloric acid products.



Epoxide formation with peroxyacids is a stereoselective reaction. That is, alkene stereochemistry is completely retained. A cis-alkene will yield a cis-substituted epoxide. A trans-alkene will yield a trans-substituted epoxide. If the proposed mechanism of oxirane formation with hypochlorous acids is true, then those substitutions should be stereoselective also. That would explain the pattern

of abundance and retention time of oxiranes of the same chain length. This pattern closely resembles that observed between monoenoic fatty acids of the same chain length with unsaturations at different positions. Bacteria unexposed to chlorine which contained distinct differences in abundance and distribution of 9-cis, 7-cis, and 5-cis monoenoic isomers of 16 and 18-carbon chain length (e.g. *S. paucimobilis* versus *E. coli*) had similarly distinct abundances and distributions of oxirane fatty acids. In each case, the 16 and 18-carbon oxirane pattern, respectively, matched the pattern of the precursor monoenoic isomer distribution in cells unexposed to chlorine.

Future experiments designed to examine the effects of drinking water disinfectants upon cellular viability should strongly consider analyzing cellular lipids using PLFA analysis. Since the physiological effect of these agents of mitigation is oxidative in nature, it seems reasonable to hypothesize that the cell membrane may be altered chemically and structurally. Detection of these alterations and correlation with culturability and potential pathogenicity may help reduce health risks to water consumers and cost of operation for water servers.

To elucidate the mechanism of oxirane formation, it would be useful to determine if H-lyase or similar enzymes are found in oxirane forming cells of *E. coli* and *S. paucimobilis*. Also, species in which oxiranes are detected could be lysed ultrasonically. Cellular viability could be determined by plating. Addition

of hypochlorous acid to the cellular debris might reveal that oxiranes are formed spontaneously upon cell lysis when HOCl is present.

Biofilm Structure and Function as Determined Using Confocal Scanning Laser Microscopy

CSLM has been used to illustrate that biofilm structure or architecture is often species specific for monoculture biofilms (Lawrence et al., 1991) and can be dictated by substrate availability for certain consortia of microbes (Wolfaardt et al., 1994b). Costerton et al. (1995) have stated that the heterogeneous nature of biofilm structure (e.g. biomass distribution, local flow mechanics) is not simply a result of microbes with various growth habits inhabiting the same niche. Monoculture biofilms often contain structural features seen in biofilms consisting of complex multi-membered communities. This suggests a direct relationship between biofilm form and function, a common theme in Nature. Function dictates structure and biofilm "structural diversity actually reflects the adaptation of unicellular organisms to a diverse range of physical, chemical, and communal circumstances on surfaces" (Costerton et al., 1995).

Those ideas have been illustrated here. Chlorine exposure may cause detachment, as was observed in *P. aeruginosa*. This could lead to relocation to a niche where conditions are more suitable for growth. In comparison, a species

which secretes large amounts of exopolymer (*S. paucimobilis*) would be expected to adhere more avidly to its substratum. Protection from that disinfectant may be afforded by the EPS. Thus, chlorination of distribution systems may dictate biofilm structure and the community composition of those biofilms. Order of inoculation or alteration of the species responsible for base-film formation was shown to drastically affect biofilm architecture by dictating the distribution of species within microcolonies. In addition, we have illustrated that a microbial species which secretes large amounts of EPS may harbor another species within biofilms.

Confocal scanning laser microscopy studies designed to examine water distribution system microbial ecology in the future should consider employing reporter strains in model systems. This eliminates the need for cellular dyes which may alter bacterial physiology and/or cause cell death during the course of experimentation. Using genetically constructed microbial reporter strains (e.g. possessing cassettes for bioluminescence or fluorescence) also eliminates problems that occur due to non-selective staining of substratum, EPS, and non-stained cells. Finally, CSLM used in conjunction with PLFA analysis may prove to be a valuable technique in understanding the architecture, community composition, physiological status and overall ecology of water distribution system biofilms.

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Vita

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