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Technical Notes

Forensic Analysis by Comprehensive Rapid Detection of Pathogens and Contamination Concentrated in Biofilms in Drinking Water Systems for Water Resource Protection and Management

David C. White,[1] Julia S. Gouffon,[1] Aaron D. Peacock,[2] Roland Geyer,[3] Anita Biernacki,[1] Greg A. Davis,[4] Marsha Pryor,[5] Mary Beth Tabacco,[6] and Kerry L. Sublette[7]

[1] *Center for Biomarker Analysis, University of Tennessee, Knoxville, TN, USA*
[2] *Center for Biomarker Analysis, University of Tennessee, Knoxville, TN, USA and Microbial Insights, Inc., Rockford, TN, USA*
[3] *Center for Biomarker Analysis, University of Tennessee, Knoxville, TN, USA and UFZ Centre for Environmental Research, Leipzig-Halle, Germany*
[4] *Microbial Insights, Inc., Rockford, TN, USA*
[5] *Pinellas County Utilities, Logan Facility, Largo, FL, USA*
[6] *Echo Technologies Inc., Boston, MA, USA*
[7] *Department of Chemical Engineering, University of Tulsa, Tulsa, OK, USA*

Clean fresh water is becoming an increasingly critical resource that will become evermore precious as more societies industrialize and population pressure increases. The critical nature of the water resource expands the vulnerability of the water supply to political and terrorist activities. With this expanded vulnerability, the importance of forensic analysis in management of water resources and protection from contamination increases. Herein, we propose monitoring the accelerated generation of drinking water microbial biofilms on "traps." The major problem with protecting water is the great dilution of the potential pathogens and toxicants. Our solution to this monitoring problem is to utilize the propensity of microbes and toxicants to concentrate at surfaces in biofilms. A logical, cost-effective method to sample pathogenic microbes and membrane-active toxicants in water is to recover biofilms from strategically placed surfaces. Modeling of the total system to maximize protection of each water system component would help identify the location of specific instrumented nodes. Nodes would be outfitted with sensors for reporting rates of general biofilm formation and some specific biofilm components. A change in biofilm at a node, or at some interval period, would signal recovery of some previously placed and colonized bio-trap beads at that node, which would then be rapidly extracted and examined for pathogen lipid, DNA biomarkers, and toxicants by tandem mass spectrometry. The lipid extraction facilitates recovery of DNA that can be used for specific microbial and virus identification. The biomarker analysis enables a much faster and comprehensive analysis than the classic microbial isolation and culture techniques currently used to protect the water supply. The total watershed collection, treatment, and distribution system would be instrumented to provide comprehensive monitoring for threat with time for purification and interdiction systems to protect the drinking water supply in the distribution system.

Keywords: biofilms, biosensors, drinking water, comprehensive protection, contamination, bioterrorism, biomarker analysis.

Introduction

Both the vulnerability of water systems to bioterrorism and the increasing pressure on a limited fresh water resource require a new recycling paradigm of turning a long considered waste into a crucial resource. The paramount consideration in water protection and reuse is safety, and the primary focus is prevention of infection and toxicity. Because typhoid is a more acutely obvious problem than cancer or birth defects, society, at least in the USA, has compromised potential long-term safety by emphasis on short-term protection from infection. Continuous pathogen suppression with biocides to control contamination in the waste discharges and drinking water distribution system leads to exposure to low levels of established toxins/carcinogens from disinfection byproducts (Weinberg and Glaze, 1996). Substitution of high-energy exposure (e.g., ultraviolet light or ozone) treatments at the water treatment facility for continuous chlorination can provide safe water that is free of disinfection byproducts but can chemically modify drinking water refractory organic

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Address correspondence to David C. White, Center for Biomarker Analysis, University of Tennessee, 10515 Research Drive, Suite 300, Knoxville, TN 37932-2575, USA. E-mail: Dwhite1@utk.edu

constituents, thereby promoting microbial regrowth in distribution systems if the water treatment or distribution system integrity allows refractory organics into the drinking water. This protection requires a much more effective water treatment.

We show that regrowth leads to biofilm formation, and the biofilms can both nurture pathogenic microbes and offer protection from biocides. The biofilms also concentrate drugs, hormones and their mimics, as well as other pharmacologically active pollutants. These microbial biofilms in the distribution system can be major threats to freshwater reuse if water used for reuse is insufficiently treated to remove most organic carbon and trace nutrients. Can a sufficiently rapid, comprehensive, and economically feasible analytical system based on biofilm recovery monitor watershed contamination inputs and outputs as well as treatment efficacy prior to and throughout the distribution system for assurance of public health safety? The system could be based on monitoring biofilms. It could incorporate four major elements: a continuously reporting biosensor network; a biofilm amplifying system; a rapid quantitative analytical system for biomarker analysis; and a system capable of manipulating interdiction and mitigation based on monitoring biofilms at strategically located nodes capable of protecting the total water system.

Microbes in nature and in drinking water distribution systems are mostly concentrated in multispecies community biofilms rather than floating freely in fluids. A logical and cost-effective method to sample water microbes is to incubate strategically placed coupons that stimulate colonization in hours to days rather than weeks. The coupons are subsequently recovered for analysis of the microbial biofilms from the watershed and treatment and distribution systems. Reproducible generation of biofilms that can be infected and colonized by pathogens and concentrate some hydrophilic drug/hormone components has been demonstrated (White et al., 1999; White, 2000; Gouffon et al., 2002).

We show lipid and DNA biomarkers from these biofilms are currently the most comprehensive and quantitative means for the rapid assessment of the presence of viable pathogenic agents and provide insight into the nutritional status of the pathogens as microbial communities that, in some circumstances, can give indications of infectivity. We report a system of sequential high-pressure solvent extraction of neutral lipids, polar lipids, and re-extraction after acid hydrolysis of the lipopolysaccharide biomarkers from biofilm coupons. The compounds are then analyzed by high-pressure liquid chromatography/electrospray/tandem mass spectrometry (HPLC/ES/MS/MS) and real-time polymerase chain reaction (PCR) analyses of DNA. This analytical system could potentially provide monitoring protection from bioterrorism via drinking water distribution systems, particularly if pathogen selection and toxin concentration in biofilm concentrating systems prove as effective as initial experiments indicate.

Methods

Biosensors

The promising prototype devices for monitoring biofilm formation on-line are described in papers from Echo Technologies, Boston, MA (Chanung et al., 2001; Chang et al., 2001a).

Amplified Sampling of Biofilms

Devices to hold the Bio-Sep® beads, which will be shown to amplify biofilm collection, may be held in stainless steel baskets that can be attached to water distribution pipes like the classical Robbins Devices for biofilm monitoring (Figure 1) or in solvent-resistant autoclavable polyfluorylalkoxy (PFA) 1.5″ long, 5/8″ diameter perforated tubes stuffed with glass wool (incinerated to remove organic carbon) (Figure 2).

The Bio-Sep® beads can be used as coupons in wells or drinking water distribution systems to induce endogenous pelagic bacteria into forming biofilms. This biofilm-forming tendency is magnified by using Bio-Sep® beads generated by K. L. Sublette, University of Tulsa, in the traps. Bio-Sep® beads are a DuPont patent transferred to the University of Tulsa in 1999. Bio-Sep® beads are 2–3 mm spherical beads consisting of 25% (w/w) aramid polymer (Nomex) and 75% (w/w) powdered activated carbon (PAC). The bulk density is about 0.16 g/cm^3 with a porosity of 74%, and adsorptive capacity is greater than 600 m^2/g. The beads are surrounded by an ultrafiltration-like membrane with a median pore diameter of 1.9 microns and with some large macropores >20 microns (Figure 3). Beads are purged

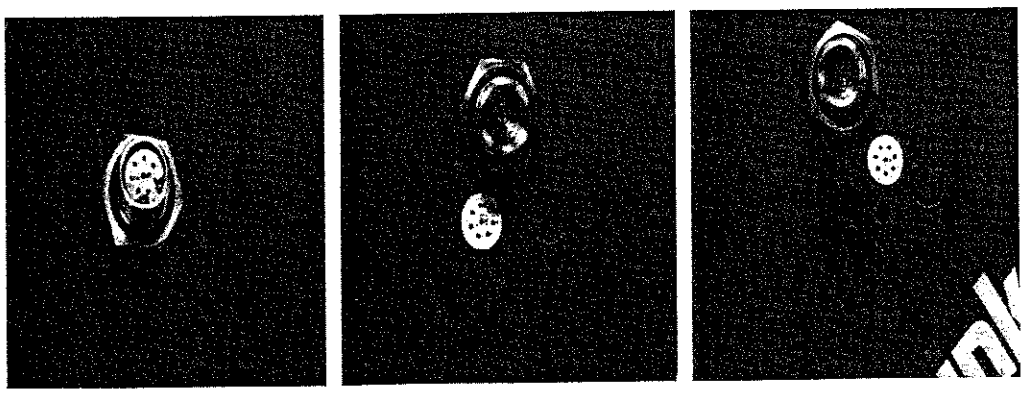

Figure 1. Half-inch diameter device with Teflon insert to hold beads in the water stream.

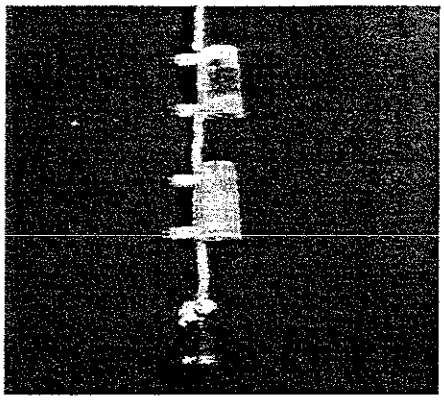

Figure 2. Bio-traps on nylon string with a coated weight. Upper trap contains Bio-Sep® beads (dark spheres) and glass wool. The lower trap contains glass wool only.

of organic carbon by incubation at 350°C for at least 5 h prior to deployment. The Nomex membrane resists formation of a surface biofilm with immobilization occurring through entrapment. It has been shown that bacteria can be immobilized inside Bio-Sep® beads by culturing the bacteria in the presence of the beads (Figure 4).

Accelerated Rapid Lipid Biomarker Analysis

Lipid Biomarker Extraction

Classical room temperature/ambient pressure extraction with methanol and chloroform takes 8–12 h to allow emulsions to settle and requires careful analytical technique by a skilled operator. Once extracted, the lipids are then separated with bulk elution on a silicic acid column into the three fractions of neutral lipid, glycolipid, and polar lipid. Each fraction is then transmethylated for analysis by capillary gas chromatography/mass spectrometry (GC/MS). These analyses require at least three working days. The GC/MS requires about 10^4 cells (picomoles/μL) to be reproducibly detected (White and Ringelberg, 1998).

Accelerated Extraction

Pressurized accelerated hot solvent extraction offers the possibility of significantly improving the speed and extraction efficiency of lipid analysis. The higher temperature increases the extraction kinetics while high pressure keeps solvents below their boiling point, thereby enabling safe rapid extraction. Initial studies have shown that using the Dionex ASE-200 Accelerated Solvent Extraction (ASE) system analysis with 2 cycles run at 80°C and 1200 PSI of chloroform:methanol:buffer enabled recovery of 3-fold more PLFA from *Bacillus* sp. spores and 2-fold more PLFA from *Aspergillus niger* spores than did the standard one-phase extraction system (MacNaughton et al., 1997). This shortened the lipid biomarker analysis time from days to about an hour. However, the lipid extract still required fractionation on silica acid columns to separate the neutral and polar lipids prior to transmethylation and GC/MS.

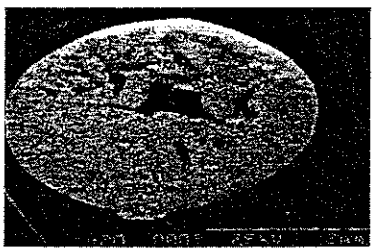

Figure 3. Cross-section of Bio-Sep® bead.

Figure 4. Bacterial biofilm colonizing inside of Bio-Sep® bead viewed by scanning electron microscopy.

Optimal ASE conditions for neutral lipids using hexane have been shown to be 40°C held at 1000 psi for 10 min for ubiquinones and 50°C for sterols and diglycerides. ASE can also recover phospholipids. Polar lipids are recovered at 50°C in methanol/chloroform/water (6/3/1, v/v) at 1000 psi. Acid hydrolysis of the lipid-extracted residue with super critical carbon dioxide ($SFECO_2$) extraction will recover the 3-0H fatty acids of the lipopolysaccharide lipid A for gram-negative bacteria, as well as archaeol and caldarchaeol from the archae. These compounds can then be analyzed by GC/MS or HPLC/ES/MS/MS. This process yields quantitative amounts of the target analytes with no carryover between the $SFECO_2$, hexane, and mixed chloroform-based steps, or after acid hydrolysis (Lytle et al., 2001a; Geyer et al., 2002a, 2002b; White et al., 2002).

In summary, the three extractions will result in the following products (Figure 5): (1) a neutral lipid fraction containing respiratory quinones, sterols, diglycerides, and DNA (the initial $SFECO_2$ extraction leaves an aqueous residue from which DNA can be recovered (Nivens and Applegate, 1997; Kehrmeyer et al., 1996)), (2) a polar lipid fraction (phospholipids), and (3) a residue for the subsequent recovery of hydroxy-fatty acids of the lipopolysaccharide (LPS-HFA), and the archaeol and caldarchaeol cores of the archaea ether lipids (R. Geyer, personal communication). The 2,6-dipicolinic acid of spores can be recovered from the lipid-extracted residue if they are prederivatized in situ (Cody et al., 2002). The extraction should take about an hour for each sample, but multiple (e.g., 4 in the prototype instrument) samples can be processed simultaneously. In addition, preliminary evidence indicates the concentration of microbial biomass in the Bio-Sep® beads facilitates enhanced recovery of both lipids and DNA when compared to subsurface sediments.

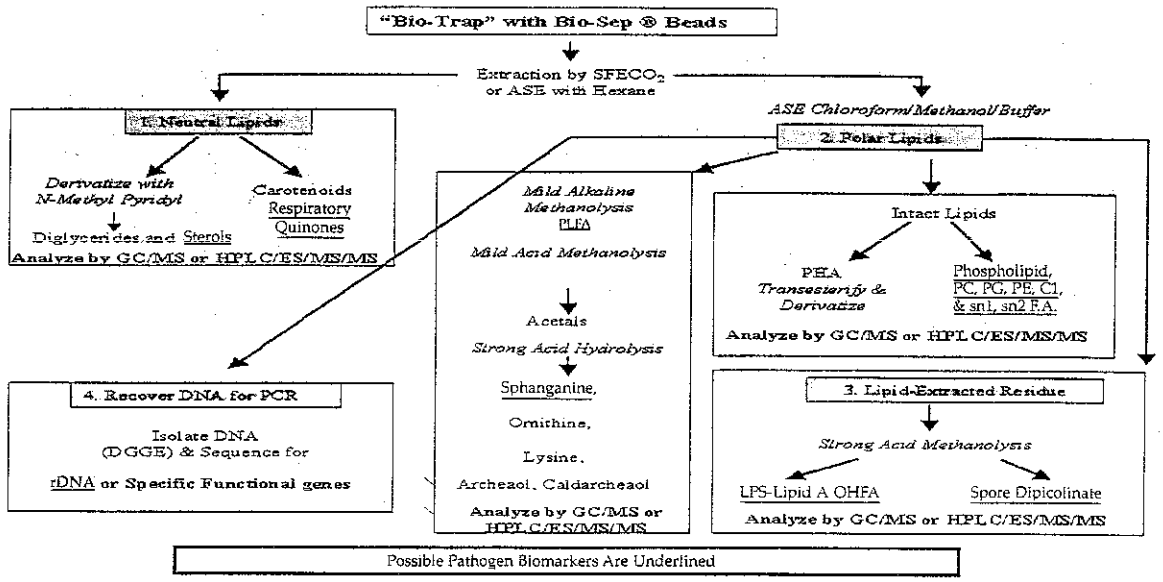

Figure 5. Diagram of the lipid biomarker analysis highlighting components with potential pathogen indicators underlined. This illustrates the analytical systems used to define the microbiota of the Bio-Sep® beads.

Respiratory Quinone Isoprenologues Analysis

Respiratory ubiquinones (UQ) are found in concentrations about 200 times less than the phospholipid ester-linked fatty acids (PLFA), or about 0.1–0.5 micromole/g dry weight. We have developed an HPLC/ES/MS/MS system for their assay directly from neutral lipids (Lytle et al., 2001a) at levels of 75 fmoles/microL. We recently established a new atmospheric pressure photoionization (APPI) (Lytle et al., 2001b) system that detects both UQ and naphthoquinones (MK) isoprenologues that recently realized sensitivities of 15 femtomoles/microL.

Sterol and Diglyceride Analysis

Electrospray (ES) analysis of other important uncharged alcohols can be based on the formation of ES active N-methylpyridyl alcohol salt. The N-methylpyridyl salt is formed from the sterol by direct reaction with 2-fluoro-1-methylpyridinium p-toluenesulfonate (Quirke et al., 1994). *Sterols* are especially valuable indicators of the microeukaryotic drinking water microbial community composition, and *diglycerides* are a measure of cell lysis (Lytle et al., 2001b; Geyer et al., 2002b).

Intact Lipid Analysis

The Center for Biomarker Analysis (CBA) has developed a method using reverse phase HPLC with a resistant C-18 column material for separation of *intact polar lipids* in a methanol solvent with 0.002% piperidine as a base with a postcolumn addition of 0.02% piperidine. This allows the detection of phospholipids as negative ions with no compromise in the efficiency of the ES ionization (Lytle et al., 2000; White et al., 2002). This has proved to be a significant advance in providing rapid (i.e., <8 min) baseline separation of intact polar lipids with ES-compatible solvents isocratically (requiring no re-equilibration on subsequent analyses). ES tandem mass spectrometry (ES/MS/MS) greatly increases the sensitivity and specificity of this environmental analysis system without any increase in the analysis time. Neutral loss scans are particularly useful for the rapid screening of targeted lipid ions, as demonstrated by Cole and Enke (1991), who showed that the phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) could be readily detected. ES/MS/MS provides great advantages in the structural analysis of phospholipids. In the negative ion mode, it has been proven possible to detect the position of each ester linked fatty acid. We have demonstrated limits of detection of 90 amoles/microL using a single molecular species of phosphatidyl glycerol with palmitic acid at *sn*-1 and oleic acid at *sn*-2 oleic acid. That detection limit is essentially equivalent to the total phospholipid in several *E. coli* cells.

Poly Beta-Hydroxyalkanoate Analysis

A particularly valuable biomarker is *poly beta-hydroxyalkanoate* (PHA), which indicates that sufficient nitrogen, phosphate, and trace nutrients are present to promote balanced growth of the in situ community. Accumulation of PHA, reflected in a higher ratio of PHA/PLFA, indicates unbalanced growth in the presence of adequate carbon and terminal electron acceptor (White et al., 1997; White and Ringelberg, 1998). We have increased the detection of PHA by high temperature/pressure extraction, hydrolysis, and detection by HPLC/ES/MS/MS (R. Geyer, personal communication).

LPS-HFA Analysis

Analysis of the acid hydrolyzed lipid-extracted residue of the neutral and polar lipid-extracted residue followed by re-extraction of the hydroxy fatty acids for GC/MS has been

described previously by White and Ringelberg (1998). The isoprenoid ether alcohols, archaeol and caldarchaeol, can also be detected with HPLC/ES/MS/MS in the hydrolysate.

DNA/Denaturing Gradient Gel Electrophoresis (DGGE) Analysis

The aqueous fraction of the lipid extract can be utilized for DNA analysis. The DNA is extracted using a standard phenol/chloroform extraction procedure with precipitation by isopropyl alcohol. The DNA is additionally purified by a glass-milk DNA purification protocol using a Gene Clean™ kit as described by the manufacturer. Next, the DNA is amplified to produce 16S rRNA gene fragments as described in Muyzer et al. (1993), with some modifications (Pinkart et al., 2002). A portion of each PCR product is analyzed by agarose gel electrophoresis. After calculating the approximate quantity of DNA, the DNA is separated on a denaturing gradient gel electrophoresis (DGGE apparatus). Denaturing gradients are formed between 30 to 65% denaturant and run at a constant temperature of 60°C in tris/ammonium acetate/EDTA (TAE) buffer. After staining the gels with ethidium bromide, gel images are captured using an Alpha Imager™ system. The central 1 mm portion of intensely fluorescing DGGE bands are excised and soaked in purified water overnight. This DNA is used as the template in a second PCR reaction. The products are purified by agarose gel electrophoresis followed by glass-milk extraction (Gene-Clean™ kit). Purified DNA is sequenced with an ABI-Prism automatic sequencer model 377 with dye terminators. Sequence identifications are performed using the BLASTN facility of the National Center for Biotechnology Information (http://ncbi.nlm.nih.gov/Blast) and the "Sequence Match" facility of the Ribosomal Database Project (http://www.cme.msu.edu/RDP/analyses.html).

The analytical scheme for the rapid comprehensive analysis of the biofilm microbiota is illustrated in Figure 5. Biomarkers indicated in red could indicate presence of pathogens.

Results and Discussion

The Utility of Biofilms as Water System Biomonitors

The generation of reproducible artificial biofilms in flowing systems allows quantitative demonstration of biofilm capacity for capturing and nurturing pathogens, and accumulating lipophylic toxicants (Gouffon et al., 2002). The reproducible generation of a biofilm trap requires strict control of three major components effecting biofilm ecology: the bulk fluid, the substratum, and the inoculum (White et al., 1999). The bulk fluid must have a chemical composition of sufficiently dilute nutrients, so that pelagic growth will not interfere with biofilm development. The substratum must be of a material that is amenable to preconditioning, thus allowing biofilms to develop. The inocula must be added as a pulse from continuous culture systems in a specific order to reproduce a consistent biofilm in terms of structure and composition (White et al., 1999). We have successfully developed a triculture biofilm composed of typical drinking water bacterial isolates: a *Pseudomonas* sp., a *Bacillus* sp., and an *Acidovorax* sp. (White et al., 1999; White 2000).

Biofilms Nurture Drinking Water Pathogens

We have shown that these reproducible biofilm tricultures effectively serve as a trap for surrogate pathogens such as *E. coli, Legionella macdadeii, Sphingomonas paucimobilis*, and *Mycobacterium pfleii* (White et al., 1999; Gouffon et al., 2002). We have also demonstrated that the biofilm offers protection from oxidative biocides, thereby allowing the trapped organisms to proliferate (Arrage and White, 1997; White et al., 1999). Exposure of our pathogen-infected biofilm to 1 and 5 ppm chlorine for 96 h showed that the pathogens in the biofilm suffer significantly less cell lysis when compared to pathogens living in pelagic fluids (as measured by the diglyceride fatty acid/phospholipid fatty acid ratio) and stress (as measured by the cyclopropane fatty acid/phospholipid fatty acid ratio) (White et al., 1999). The triculture biofilm was also shown to retain oocysts of *Cryptosporidium parvum* (White et al., 1999).

Biofilm Biosensors

A strategically placed, continuously reporting, biofilm-monitoring system could readily be incorporated into a water distribution system to serve as a sentinel monitor for system disturbances. New sensor developments can increase specificity to this on-line system. An on-line biofilm can be readily detected by measuring tryptophan fluorescence (e.g., 290 nm excitation, 340 nm emission) with a bifurcated fiberoptic device (Mittleman et al., 1992; Angel et al., 1993; Arrage et al., 1995). This technology has been incorporated into a field-capable device by Echo Technologies, Inc. (Boston, MA, USA). The device measures the fluorescence changes induced by the biofilm with time as monitored through a window in contact with the water stream. The fluorescence spectra are illustrated in Figure 6. The correlation

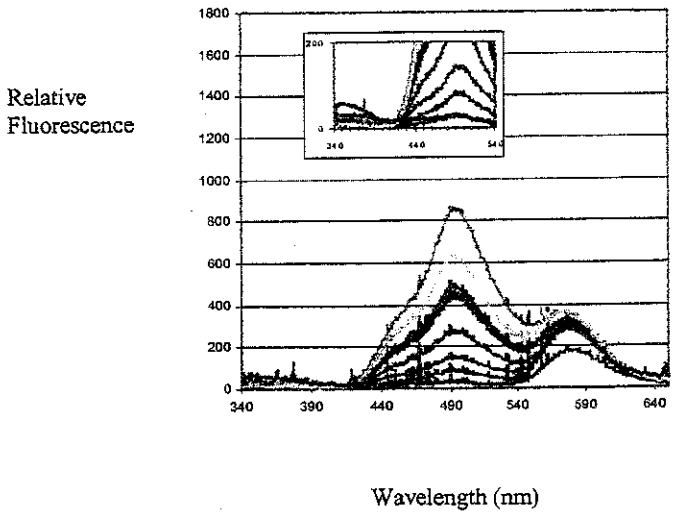

Figure 6. Fluorescence of tryptophan in biofilms formed in a flowing water system in the Echo Technologies biosensor.

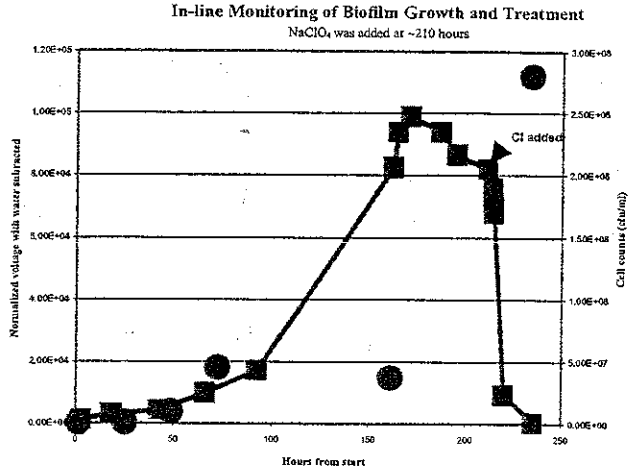

Figure 7. Comparison of fluorescence detection of biofilm(squares) on the Echo Technology biosensor and the viable cell counts (circles).

between the fluorescence yield from the biosensor and plate counts of a *Pseudomonas* incubated in dilute medium is shown in Figure 7. Figure 7 also shows a rapid decrease in both cell count and fluorescence yield induced by the application of a chlorine biocide.

Modifications of the sensor for long-term use with periodic biofilm removal and monitoring of subsequent regrowth are being tested. New developments of this technology, using the spectral response to immobilized nucleic acid stains on a distal-end fiber-optic sensor geometry, allow quantitative discrimination of changes in density of *Pseudomonas* in aerosolized or aqueous samples with a sensitivity to 10^5 cells. Utilization of nucleic acid stains on distal-end fiber-optic sensor geometry provides in situ class specificity, such as distinguishing bacteria from viruses, fungi, or spores and even between live/dead and gram-positive/gram-negative cells (Chanung et al., 2001). Adding a fourth-generation hydroxyl-terminated polyamidoamine dentrimer to the detection system both stabilizes and significantly enhances the detection sensitivity for live cells by the biosensor (Chang et al., 2001a). Continued progress can generate more specificity into the biosensor detection system proposed for the integrated water system protection system.

Analysis of the Amplified Biofilm

The most comprehensive rapid quantitative measure of biofilm-viable biomass, community composition, and nutritional/physiological status is in the lipid biomarker analysis (LBA). As a membrane marker, the LBA picks up the response of not only the in situ community gene pool but also the individual physiologic response of cells to changes in the local microniche ecosystem. It is the ability to measure the physiologic response that gives LBA its advantage over the DNA-based techniques, especially when analysis of the whole community response is the goal. LBA is not as effective as DNA analysis, however, when looking for a specific pathogen. Hence, a combination of LBA and DNA analyses provides both the specificity of the potential contamination and some indication as to the physiological status, and thus the potential infectivity, of pathogens. The concept is analogous to determining the shape of a cathedral from one brick. DNA-based techniques show you the shape of the individual bricks (mathematically, the DNA sequence is noncompressible) but do not provide much data on the community. LBA, on the other hand, provides an idea of how the whole community (the "cathedral") is shaped. As such, LBA seems to provide a "holistic" complement to the specificity of the DNA analysis. The holistic community change in the mapping of pathogens with LBA could be in estimating the potential for infectivity. Examples of the relationship of infectivity to physiological status indicated by lipid structure are the shift from culturability to nonculturability (but with retained infectivity) of the cholera bacillus, *Vibrio cholerae* (Guckert et al., 1986), and the shifts in patterns of micocerosic acids and secondary alcohols derived from the surface waxes which correlated with drug resistance in *Mycobacterium tuberculosis* (Almeida et al., 1995). Polar lipid structure modifications can also correlate with culturability. The presence of oxyrane fatty acids in the phospholipids of gram-negative bacteria on exposure to oxidative biocides indicates unculturability (Smith et al., 2000).

The determination of the total PLFA provides a quantitative measure of the viable or potentially viable biomass. Viable microbes have an intact membrane that contains phospholipids (and PLFA). Cellular enzymes hydrolyze (release) the phosphate group from phospholipids within minutes to hours of cell death, resulting in the formation of diglycerides (White et al., 1979; Pinkart et al., 2002). A careful study of subsurface sediment showed that the viable biomass determined by PLFA was equivalent (but with a much smaller standard deviation) to that estimated by intercellular adenosine triphosphate (ATP), cell wall muramic acid, and very carefully done acridine orange direct counts (AODC) (Balkwill et al., 1988).

Classical methods for microbial community analysis have severe limitations when used in environmental samples. Viable counts are dependent on microbial growth and tend to consistently underestimate the microbial community (White, 1983). Because of cell assemblages and soil aggregation, direct microscopic counts of microorganisms in environmental samples are difficult to quantify. Direct counts are highly subjective, and the analysis provides little or no indication of the in situ nutritional/metabolic status of the microbial community (Hattori and Hattori, 1976; Schallenberg et al., 1989; Tunlid and White, 1992). The modified Bligh and Dyer room-temperature solvent extraction has been used for direct quantification of lipids to estimate viable microbial biomass, community composition, and nutritional status in situ for over 15 years (White et al., 1979; Tunlid and White, 1992; Pinkart et al., 2002). Despite the fact that lipid biomarker analysis is the most satisfactory single, quantitative, and comprehensive analysis of the in situ microbial community viable biomass, community composition, and physiological status, it is not universally utilized, as this method is labor intensive and slow. The major drawback of the classical phospholipid ester-linked fatty acid (PLFA) analysis is

Table 1. Viable biofilm biomass measured as phospholipid ester-linked fatty acids (PLFA) recovered from three sites in a drinking water distribution system

	Bio-Sep® beads			Polypropylene sheet
Site	1 day exposure	3 day exposure	14 day exposure	30 days
		pmoles PLFA/g		
A	220	574	2100	1070
B	244	710	1347	672
C	308	121	917	1517

the extraction process by which lipids are recovered from environmental samples. Enhancement of lipid biomarker analysis, as reported herein, not only greatly increases the functional insights into the community dynamics by expanding the lipids that can be readily analyzed but provides this analysis more rapidly, at greater sensitivity, as well as at a lower cost, and is an ideal trigger for the more specific DNA-based analyses.

Furthermore, the in situ redox conditions can be important in the nurture of pathogens in drinking water biofilms. Redox levels reflect the terminal electron acceptor used for electron transport. For example, gram-negative bacteria and microeukaryotes form respiratory UQ when the terminal electron acceptor is oxygen (Lytle et al., 2001a, 2001b; Collins and Jones, 1981). Under anaerobic growth conditions, Gram-negative bacteria may form respiratory MK or no quinones at all. Gram-positive bacteria and archaea form respiratory MK when grown aerobically. Pathogenic *Legionella* form diagnostic long-chain UQ isoprenologues (Lytle et al., 2001a), and many aerobic pathogens contain desmethylmenaquinones (DMK) isoproenologues (Collins and Jones, 1981; White et al., 2002).

Amplified Biofilm Formation

Bio-Sep® beads were incubated at three distinct sites in a drinking water distribution system. When compared to a biofilm generated on a plastic surface in the same water, the Bio-Sep® bead-generated, biofilm-viable biomass (as measured by PLFA) was about 23% in 1 day, 43% in 3 days, and 130% in 14 days of that generated in a month on the plastic surface (Table 1). The biofilm formed in 1 day showed a preponderance of PLFA associated with gram-negative bacteria that are expected in drinking water (Smith et al., 2000) (Table 2). With time, the monoenoic PLFA in the cellular lipids increasingly formed epoxide (oxirane) containing fatty acids that correlated directly with exposure to hypochlorite. The oxirane PLFA is formed at the expense of the monoenoic PLFA indicative of gram-negative heterotrophic bacteria (Smith et al., 2000). *E. coli* or *Sphingomonas paucimobilis* containing high levels of oxirane PLFA are not culturable (Smith et al., 2000). In biofilms formed on Bio-Sep® beads, the indicators for gram-negative bacteria are diluted by bacteria with midchain branching PLFA as the exposure is prolonged. These are characteristic of *Mycobacteria* (White et al.,1996) that were detected in the DNA analysis of these biofilms (see below). Moreover, the presence of the multimethyl branched PLFA suggested trace amounts of *Mycobacteria*, which are notoriously resistant to disinfection. We have demonstrated that *Mycobacteria* actually flourish in artificial drinking water biofilms in the presence of high levels of oxidative biocides (Arrage and White, 1997; White et al., 1999).

Low levels of polyenoic PLFA, characteristic of microeukaryotes (White et al., 1996), only appeared on the 30-day plastic coupons. This indicates that there were very few microeukaryotes (algae or protozoa) in the distribution system biofilm, and it takes a while for them to accumulate (Table 2). In other tests, we have documented that *Cryptosporidium parvum* oocytes are concentrated in artificial drinking water biofilms (White et al., 1999).

rDNA Analysis of Bio-Sep® Beads and Plastic Coupon Biofilms

Biofilm samples in Tables 1 and 2 were subjected to rDNA analysis, and the amplicons were separated by DGGE. The bands were sequenced, and the phylogenetic identifications were determined (Table 3). The DGGE is illustrated in Figure 8.

The specific analyses of biofilms from the Bio-Sep® beads show clearly that a 1 day exposure is sufficient to collect potential pathogens, such as relatively slow-growing *Mycobacteria* and *Sphingomonas*, in a rich community. In the 1 day biofilm, the *Acidovorax* sp, *Ralstonia/Aquaspirillum, Methylobacterium* sp, and *Mycobacterium* sp. (mucogenicum/Chelonae

Table 2. Mole % proportions of PLFA with different times of exposure found in biofilms on Bio-Sep® beads and plastic coupons recovered from the same three sites in a drinking water distribution system as used in Table 1

	Bio-Sep® beads									Polypropylene sheet 30 days		
Exposure	1 day			3 day			14 day					
	Mole % PLFA for each sample site											
PLFA class	A	B	C	A	B	C	A	B	C	A	B	C
Monoenoic PLFA	30	25	73	36	41	82	24	18	67	49	40	67
Oxirane PLFA	70	74	26	63	58	15	70	74	31	45	43	26
Total Oxirane + Monoenoic PLFA	100	99	99	99	99	92	94	92	92	94	83	93
Mid chain Branched PLFA	0	0	1	1	1	3	6	8	8	1	0	0.5
Eukaryote Polyenoic PLFA	0	0	0	0	0	0	0	0	0	5	17	6

Table 3. Sequence results from bands excised from Figure 8. Identifications are based upon GenBank DataBase

Band	Closest match	Similarity index	Phylogenetic affiliation
A	Acidovorax sp.	100%	Beta Proteobacteria
B	Sphingomonas sp.	100%	Alpha Proteobacteria
C	Ralstonia/Aquaspirillum	100%	Beta Proteobacteria
D	Methylobacterium sp.	96%	Alpha Proteobacteria
E	Mycobacterium sp. (mucogenicum/Chelonae Like/ rhodesiae/anthrancenicum)	96%	Actinomycetes
F	Aquabacterium sp.	99%	Beta Proteobacteria
G	Sulfuricurvum kujiense	98%	Epsilon Proteobacteria
H	Methylobacterium sp.	99%	Alpha Proteobacteria
I	Methylomonas sp.	98%	Gamma Proteobacteria
J	Sulfuricum Kujiense	99%	Epsilon Proteobacteria
K	Enterobacteriaceae sp.	97%	Gamma Proteobacteria
L	Mycobacterium sp.	97%	Actinomycetes
M	Ochrobactrum anthropi	100%	Alpha Proteobacteria
N	Methylomonas sp.	98%	Gamma Proteobacteria
O	Dechloromonas	98%	Beta Proteobacteria

Like/rhodesiae/anthrancenicum) appear dominant at the three sites. The major components of the biofilm community simplifies in 3 days as seen with fewer bands. Results show the *Methylobacterium* sp. is a prominent and consistent component of the biofilm. The eubacterial community on the 30 day coupons is different and diverse. The 1 day community is remarkably similar at the three sites. This is less obvious after 3 days, and by 30 days the biofilm community has differentiated between the 3 sites.

Lipid Biomarkers Indicative of Pathogens

Neutral lipids provide a respiratory quinone profile where isoprenologues of UQ and desmethylquinones are indicators of pathogens (Collins and Jones, 1981; Lytle et al., 2001a, 2001b). Presence of cholesterol or other sterols that were detected after derivatization can provide indicators for protozoal pathogens like *Cryptosporidium* (Canuel et al., 1995). When polar lipids are extracted they can yield specific biomarkers for *Legionella* and other intercellular pathogens (White et al., 2002). Some potential threat agents have sufficient specific lipids that they can be identified from the PLFA analysis as currently performed on total polar lipids (particularly intercellular pathogens like *Coxsiella* and *Fransciella* (Nichols et al., 1985), *Legionella* (Walker et al., 1993), *Mycobacteria* (Almeida et al., 1995), and some with class-specific lipids, e.g., *Staphylococcus* spp., *Sphingomonas* spp., *Pseudomonas* spp., *Clostridia* spp. (White et al., 1995). Our newly developed methods will not only increase the speed and facility of lipid biomarker analysis but will increase the specificity by examining the position and structure of the fatty acids in each major individual polar lipid. This will greatly increase the specificity of the polar phospholipid analysis. Also, defining the head-groups and isoprenologue patterns of the respiratory quinones will also further increase this specificity.

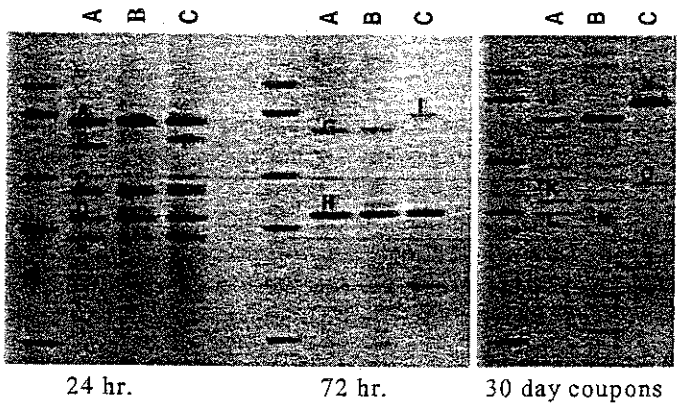

Figure 8. DGGE analysis of biofilms used in Tables 1 and 2. Bands identified in Table 3.

Bacterial spores are a special problem, as they are especially effective infectious agents that can be difficult to rapidly detect in complex environmental matrices. Recently, rapid extraction of 2,6-dipicolinic acid (DPA) from bacterial spores after in situ derivatization has been developed (Cody et al., 2002). We propose incorporation of this method into the sequential extraction system for the rapid detection of bacterial spores.

When the lipid-extracted aqueous residue is subjected to acid methanolysis, the hydroxy fatty acids can be detected and used as biomarkers for pathogens. An example is the 3-hydroxy 14 carbon fatty acids present in lipopolysacharide that are characteristic of waterborne gram-negative pathogens (White et al., 1996). Water biofilm organisms, such as *Pseudomonas*, have 3-OH 10:0 and 3-OH 12:0 as LPS hydroxy fatty acids in contrast to enteric pathogens such as *Salmonella* and *E. coli*, which contain 3-OH 14:0 (White et al., 1996; Gouffon et al., 2002). The aqueous fraction of the lipid-extracted sample can be examined for DNA by real-time PCR, should lipid biomarker indicators of pathogen be present or if system disturbance dictates. Lipid extraction facilitates recovery of DNA from biofilm

samples (Kehrmeyer et al., 1996; Nivens and Applegate, 1997). The addition of DNA analysis provides specificity in detecting microbial pathogens and waterborne viruses.

In summary, the three extractions described here will result in the following products: (1) a neutral lipid fraction containing respiratory quinones, sterols, diglycerides, and DNA, (2) a polar lipid fraction (i.e., phospholipids), and (3) a residue for the subsequent recovery of ester-linked hydroxy-fatty acids of the lipopolysaccharide. Furthermore, evidence indicates that the concentration of microbial biomass in the Bio-Sep® beads facilitates enhanced recovery of both lipids and DNA when compared to stainless steel or PVC coupons.

Biofilm Concentration of Drinking Water Organics

Many of the drugs, hormones and their mimics, and other pharmaceutically active pollutants (PAPs) that have been detected in surface waters (Halling-Sorensen et al., 1998) are hydrophobic and have activities in modifying cell membrane functions. The distribution of certain PAPs has been used in the forensic analysis of pollution point sources (Standley et al., 2000). However, the occurrence of PAPs in natural systems is often at concentrations of parts per trillion (ng/L). These low levels often require that concentration techniques be used prior to detection. PAPs, like pathogens, are difficult, expensive, and time-consuming to concentrate. We postulate that the biofilms may also serve for the concentration of hydrophobic PAP (i.e., in the lipid membranes of the constituent bacteria). Biofilms are an excellent vehicle for monitoring water systems quality since the great majority of drinking water microbes reside in biofilms. Biofilms integrate and reflect the stress to which they are exposed; are resistant to biocides, antibiotics, and toxins and readily nurture and protect pathogens (White et al., 1999). Their cellular membranes, in aggregate, are capable of concentrating lipophilic pharmaceuticals and endocrine disrupters. When the hydrophobic biocide Triclosan and the drug Viagra were exposed to a test biofilm, some unquantifiable portion of Viagra and 15% or 160 ppb of Triclosan were found in the lipids of the biofilm (White, 2000). The present limit of detection for standards of these drugs is 2 to 20 ppb. Application of immunocapture chromatography and subsequent desorption can significantly expand limits of detection with HPLC/ES/MS/MS to ppt levels reported in watershed environments. Preliminary evidence indicates that organophosphorous nerve agents can be detected with this HPLC/ES/MS/MS system (D'Agostino et al., 1999). The Bio-Sep® beads may prove an excellent concentrator of PAPs.

Risk Prediction

In the determination of risk, it is important to know the accepted levels for infectious doses of various pathogens. The dose depends not only on the quantity of organisms to which the host is exposed but on the virulence of the strain, the susceptibility of the host, and many other conditions that moderate the infectivity (Safe Drinking Water Committee, 1997). Infectious doses of selected waterborne pathogens are indicated in Table 4. These levels approximate those found by Bryan (1994), with 25 to 75% of the susceptible adult humans infected with between 10^2–10^3 *Shigella*, 10^3–10^9 *Salmonellae*, and 10^3–10^8 *Vibrio cholerae*. The biotrap, with Bio-Sep® bead-based concentration of specific biomarkers, is highly quantitative and thus will permit not only the detection of a particular pathogen but also the potential for infection (i.e., effective dose).

One limitation of the sentinel lipid biomarker analysis method is the lack of rapid specific trigger indicators for virus contamination should the virus not be associated with other clues, such as large bacterial biomass and/or large microeukaryotic

Table 4. Infectious doses of drinking water pathogens

Klebsiella pneumonia ID_{50} 3.5×10^1 and 7.9×10^5 cells/ml (1)
Salmonella typhi 38% 10^5/ml (3)
Vibrio cholerae ID_{50} 10^6/ml (4)
Shigella dysenteria 10–200 bacteria by mouth (5)
Enteropathogenic *Escherichia coli* ID_{50} 10^8 (8)
Legionella pneumophila treatment goal $< 10^3$/ml (6)
Giardia lamblia cysts 10–100 cysts in humans (7)
Cryptosporidium parvum oocysts $1-5 \times 10^2$ mice, 10 monkeys, generally 10^5-10^7 to induce consistent animal infections (2)

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biomass. Bacteriophage-induced lysis could be detected by a high diglyceride level (readily detected in the neutral lipid fraction by HPLC/ES/MS/MS), but bacterial phages are indicators of fecal contamination and the potential that pathogens could be present. Detecting viral nucleic acids in a biofilm could be a strong indicator of potential pathogen exposure.

System Modeling

An engineering model needs to be applied to define the placement of critical monitoring nodes to protect various components of the total water system to fully exploit the advances in system monitoring, accelerated biofilm generation, and rapid biomarker analysis in protecting the drinking water. The larger and more comprehensive this system becomes, the more time will be available for protection and interdiction. Rapid advances are being made in biosensors and biomarker analysis that can be readily incorporated into this model. New vortex-based efficient air samplers are now available, and the same types of rapid biomarker analyses can be integrated into the system to provide protection of both the air and water. Again, with a little more effort, the food supply could be similarly protected.

Conclusions

In this paper we have proposed a comprehensive network with biosensors and biofilm amplifying biotraps at critical nodes. The biotraps are recovered and rapidly analyzed for lipid and DNA biomarkers for control of contamination and system protection. We have established that biofilms capture and nurture pathogens, and pathogens are readily detected by lipid biomarkers recovered by sequential high pressure/temperature extraction for potentially automated mass spectral analysis.

Analysis of extracted biofilm fractions provides detection of $\sim 10^3$ cells of specific pathogens and can be coupled with immunocapture HPLC to detect specific PAPs concentrated in the biofilm. Recovery of lipids from triculture biofilms incubated 4 days, then inoculated with *E. coli* engineered to contain green fluorescent protein (GFP), showed clear differences from uninoculated controls. Biofilms containing *E. coli* showed differences in respiratory UQ isoprenologue proportions (R. Geyer, personal communication). Major differences were likewise detected in the polar lipids detected with HPLC/ES/MS/MS and with the lipopolysaccharide hydroxy-fatty acids analyzed by GC/MS. *E. coli* could be detected as discrete microcolonies by GFP fluorescence in the inoculated biofilms, and not in the uninoculated controls, by laser confocal microscopy. Similar experiments have been done with *Sphingomonas, Mycobacteria*, and *Legionella.*

The exploitation of "flash" sequential extraction, coupled to the HPLC/ES/MS/MS analytical system described herein, has just begun (Lytle et al., 2000). This system appears to provide ultrasensitive quantitative detection of viable biomass, absence or potential presence of microeukaryotes (e.g., *Cryptosporidium* oocysts), enteric bacteria, indicators of potential infectivity (e.g., cell lysis, toxin, or oxidative exposure of gram-negative bacteria), and the presence of drugs, hormones, and PAPs. The fact that the lipid extraction of sediments and biofilms facilitates recovery of DNA suitable for real-time PCR amplification makes possible the combination of lipid- and DNA-based technologies from the same sample. Rapid highly specific microprobe DNA/RNA analysis can be triggered by a positive result with the potentially automatable, ultrasensitive lipid biomarker analysis of biofilm coupons.

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