Rapid Quantitative Detection Of Pathogens & Contamination By Analysis Of Biofilms Generated On Coupons In Water Resource Management 536

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CONDENSED ABSTRACT: Clean fresh water is becoming an increasingly critical resource that will become more precious as more economies industrialize. Without new sources, recycling is the only alternative. Herein we propose accelerated generation of drinking water microbial biofilms on "traps" from which lipid biomarkers can be rapidly extracted and analyzed with tandem mass spectrometry that are independent of isolation and culture. Biofilms concentrate and nurture traces of pathogens and concentrate hydrophobic toxicants and trace pharmaceuticals that can be detected with proper application of analytical methods, thus providing quantitative forensics for tracing watershed pollution and contamination sources and monitoring treatment and distribution efficacy.

INTRODUCTION:

With the increasing pressure on a limited fresh water resource, prudent management of this resource becomes evermore critical. Freshwater reuse requires a new recycling paradigm of turning a long considered waste into a crucial resource. The paramount consideration in water reuse is safety and the primary focus is prevention of infection. Because typhoid is a more acutely obvious problem than cancer or birth defects, society has compromised potential long-term safety by emphasis on short-term protection from infection. Continuous inhibition with oxidative 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. Substitution of high-energy exposures (UV or ozone) treatments at the water treatment facility for continuous chlorination can chemically modify drinking water refractory organic constituents thereby promoting microbial regrowth in distribution systems so much more effective water treatment will be required.

Herein we show this regrowth leads to biofilm formation and these biofilms can both nurture pathogenic microbes and protect them from biocides as well as concentrate drugs, hormone and their mimics, and other pharmaceutically active pollutants. These microbial biofilms in the distribution systems can be major threats to freshwater reuse if treatment of water used for reuse is insufficient to remove most organic carbon and trace nutrients. We seek to design, develop and validate a sufficiently rapid, comprehensive, and economically feasible analytical system based on biofilm recovery to monitor watershed contamination inputs and outputs as well as treatment efficacy prior to and throughout the distribution system for assurance of public health safety.

Microbes in nature and in drinking water distribution systems are most concentrated in multi-species community biofilms rather than free in pelagic fluids. Biofilms are localized communities attached to a substratum. So a logical cost effective method to sample water microbes is to incubate strategically placed coupons that stimulate colonization and are subsequently recovered for analysis of the microbial biofilms that form in weeks in the watershed, treatment, and distribution systems. We have demonstrated reproducible generation of biofilms that can be infected and colonized by pathogens and concentrate some hydrophilic drug/hormone components.

We analyze lipid biomarkers from these biofilms as targets for the most comprehensive and quantitative means for the rapid assessment of the presence of viable pathogenic agents as well as provide insight into the nutritional status of the pathogens as microbial communities, that in some circumstances, can give indications of infectivity. We report herein 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 coupled to HPLC/electrospray/tandem mass spectrometry (HPLC/ES/MS/MS). This analytical system could potentially provide monitoring protection from bioterrorism via drinking water distribution systems particularly if pathogen selection in biofilm concentrating systems proves as effective as initial experiments indicate.

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RESULTS AND DISCUSSION:

Reproducible Generation of Drinking Water Biofilms: 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 (1). 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 pre-conditioning, 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 (1). We have successfully developed a triculture biofilm composed of typical drinking water bacterial isolates: a *Pseudomonas* sp., a *Bacillus* sp., and an *Acidovorax* sp.

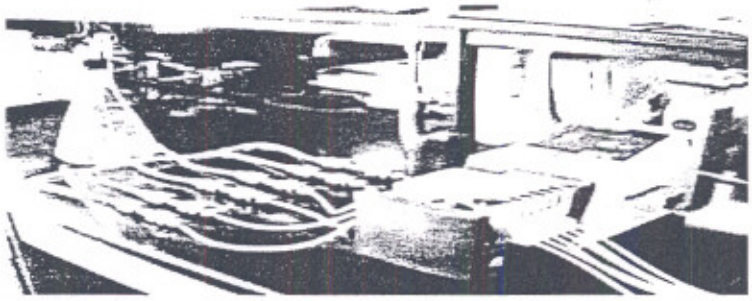

Figure 1. Once-through Biofilm development and testing device.

Biofilms Nurture Drinking Water Pathogens: We have shown that the drinking water triculture biofilms effectively serves as a trap for surrogate pathogens such as *E. coli*, *Legionella macdadeii*, *Sphingomonas paucimobilis* and *Mycobacterium pfleii*. We have also demonstrated that the biofilm offers protection from oxidative biocides, allowing the trapped organisms to proliferate (2). Exposure of our pathogen-infected biofilm to 1 and 5 ppm chlorine for 96 hours showed the pathogens suffer significantly less cell lysis (as measured by the diglyceride fatty acid/phospholipid fatty acid ratio) and stress (as measured by the cyclopropane fatty acid/phospholipid fatty acid ratio). The triculture biofilm also was shown to retain oocysts of *Cryptosporidium parvum* (1).

Selective Amplification of Pathogens in Drinking Water Pathogens: In the course of experiments in which trace amounts of pathogen surrogates were inoculated into a well-developed drinking water tri-culture biofilm, it was found that increasing the temperature from 25°C to 35°C enormously increased the pathogen to biofilm bacteria ratio.

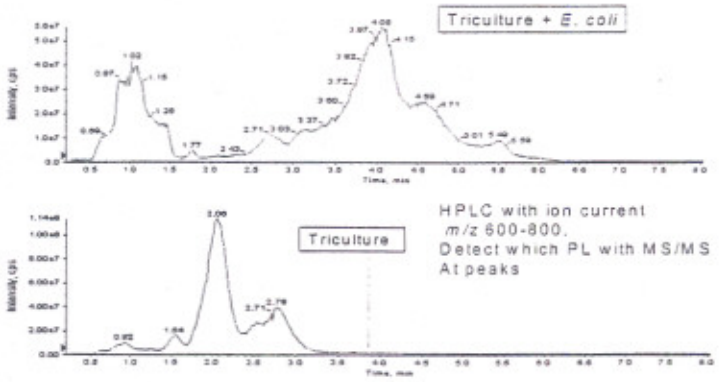

Figure 2. Ion current from polar lipids recovered from a mature triculture drinking water biofilm inoculated (top panel) with *E. coli* engineered to express GFP and un-inoculuted triculture control biofilm (lower panel) incubated at 35°C in devices as in Fig 1. Both inoculated and control triculture biofilms from coupons incubated at 25°C looked like the lower panel (data not shown). Lipids extracted from the biofilm coupons, intact polar lipids recovered and assayed by high performance liquid chromatography/electrospray/tandem mass spectrometry (HPLC/ES/MS/MS) as described (3,4).

Figure 2 shows the ion current of polar lipid extracted from a tri-culture biofilm pre- and post inoculation with and growth of a surrogate pathogen (*E. coli)* for 3 days at an incubation temperature of 35°C. The difference in the ion profile was attributed to growth of the introduced surrogate pathogen. Results showed very little growth to occur within the un-inoculated tri-culture biofilm under either the ambient or heated

conditions. In contrast, the inoculated biofilm showed *E. coli*, which contained the green fluorescent protein (GFP), to be present throughout the biofilm as discrete mini-colonies (Figure 3).

Figure 3. Confocal fluorescent microscope image of *E.coli* containing GFP microcolomies in the tri-culture biofilm.

The lipids of the surrogate pathogen (*E.coli*) and the tri-culture were readily defined and easily distinguishable from one other by analyses with HPLC/ES/MS/MS (Figure 4).

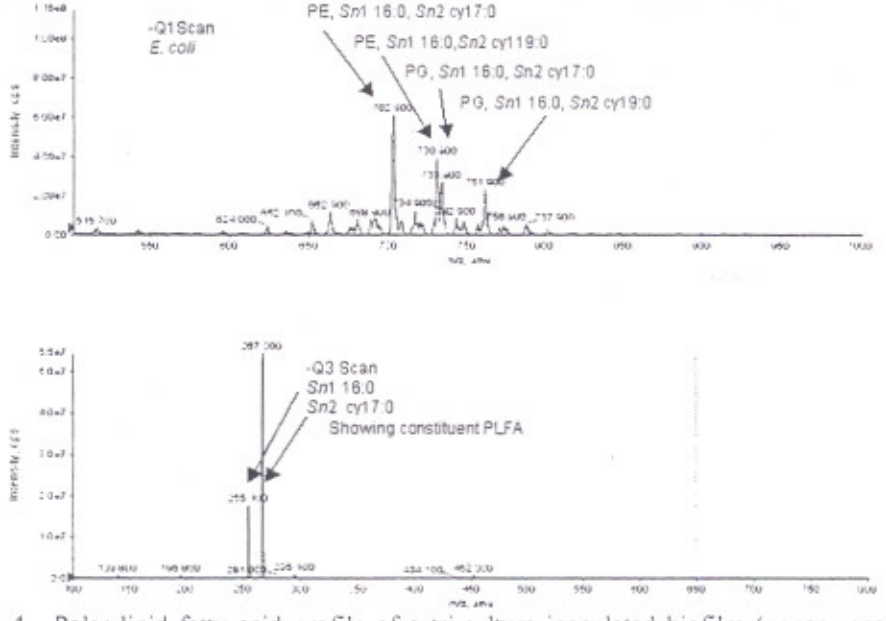

Figure 4. Polar lipid fatty acid profile of a tri-culture inoculated biofilm (upper panel) and the presence of diagnostic fatty acids for *E.coli* (lower panel). Similar uniformly dispersed microcolonies of *Sphingomonas paucimoblis* engineered to contain GFP have been demonstrated in the reproducible triculture biofilm (Gouffon et al unpublished).

Rapid Quantitative Definition of Drinking Water Biofilms by Lipid Biomarker Analysis: New methodology greatly facilitates the quantitative analysis of these biofilms with analysis potentially automatable and requiring minutes rather than days. The breakthrough involves sequential high temperature/high pressure extraction of neutral then phospholipids followed by analysis by both GC/MS, and HPLC/ES/MS/MS (4, as discussed in this symposium 5).

Biofilms accumulated on glass beads placed in a shower near the end of a drinking water distribution center for a period of two weeks, at 36-38°C, a flow rate of 20 ± 7 ml/min and with free chlorine levels between 0.09 ± 0.04 ppm. The beads contained 11.2-21.9 pmol/cm^2 total recoverable phospholipid fatty acids (PLFA) (6). This value corresponds to between 1.6 x 10^5 and 8.7 x 10^5 bacteria the size of *E. coli*/cm^2 utilizing the conversion factor of 1.4-4 × 10^4 cells/pmol of PLFA (7). The biofilm, showed the surprising presence of epoxide (oxirane) containing fatty acids that correlated directly with exposure to hypochlorite. The oxirane PLFA seemed to be formed at the expense of the monoenoic PLFA indicative of Gram-negative heterotrophic bacteria (7). Cyclopropane fatty acids are formed from monoenoic PLFA in phospholipids in gram-negative bacteria as microbes move from a log to a stationary phase of growth (7). The ratio of cyclopropyl to monoenoic precursor usually is less than 0.05 for log phase unstressed growth to 2.5 or greater as the stress of starvation and stationary growth phase is prolonged. In the distribution system biofilm, the ratio of cy 19:0/18:1 + 18:0 oxirane is high,

which indicated that cells in the distribution system biofilm were starving. The added stress of chlorine exposure increased the cyclopropane PLFA formation and leads to oxirane PLFA formation, thereby leading to a greater decrease of the monoenoic precursors in the phospholipids.

The PLFA recovered from the distribution system biofilm showed low levels of *iso* and *anteiso* branched fatty acids with an *iso/anteiso* ratio near 1. Gram-positive aerobic bacteria like *Arthrobacter* or *Micrococcus* have *iso/anteiso* PLFA ratios less than 1, in contrast to the anaerobic sulfate-reducing Gram-negative bacteria with ratios greater than 5 (7). The low levels of polyenoic PLFA indicate that there were very few microeukaryotes in the distribution system biofilm (7). The presence of mid-chain branched PLFA (10 me 16:0, 10 me 18:0) indicated that Actinomycetes were present (7). Moreover, the presence of the multi-methyl branched PLFA suggested trace amounts of *Mycobacteria*, which are notoriously resistant to disinfection, particularly favor older corroded portions of the distribution system where their regrowth is stimulated, and readily colonize membranes used to purify water.

PLFA can be used to detect potential pathogens, which may or may not routinely be detected by the classical plate counting methods. Pelagic *E. coli* or *S. paucimobilis* in biofilms or after release from the biofilm are not culturable even with dilute and enriched "rescue media" when the oxirane PLFA are present (6). The presence of epoxide fatty acids establishes that the cells in the biofilm have been in contact with the biocide and provides a chemical marker for hypochlorite exposure in pelagic and biofilm microbes in water distribution systems, while they are unculturable in the classical viable count. The lipid biomarker analysis of the biofilm also showed that trace amounts of the difficult-to-culture *Mycobacteria* were present within the drinking water distribution system and that difficult to culture *Legionella* and *Sphingomonas* were not present. There was no evidence of fecal contamination indicated by detection of coprostanol (6).

A general test for Gram-negative pathogens can be found in the 3-hydroxy fatty acids released from the lipid-extracted residue in the lipopolysaccharides (LPS) lipid A after acid hydrolysis. Nearly all serious human Gram-negative pathogens contain 3 0H 14:0 (7). (3-OH 14:0 is a 14 carbon n-saturated fatty acid with a OH 3 carbons from carboxyl end). *Pseudomonas*-type bacteria found in potable water systems are weak only occasionally opportunistic pathogens. They have 3-0H 10:0 and 3-0H 12:0 as the fatty acid components of LPS-lipid A. Analysis of the hydroxy fatty acids released from the lipid-extracted residue of the biofilm can indicate the presence of pathogens. Are dangerous pathogens present in the gleaming clean toilet bowl? Even the most fastidious toilet bowels cleaned daily contain a biofilm at the water air interface. Human feces contain 7 (0.6*)/19 (4*) 3 0H 10 +12/ 3 OH 14:0 in mol % ratios[* (mean (SD) n=15]. The toilet biofilm contained 72 (30*)/19 (4*) of 3 0H 10 +12/ 3 OH 14:0 in mol% ratios [* (mean (SD) n=15]. Toilet bowl invisible biofilms contains a ratio of pathogens to water organisms 0.37 compared to 3.8 in the feces. A biofilm with a high ratio is potentially much more dangerous to ingest.

Increasing the Effectiveness of Coupons as Biofilm Traps: Sterilized solvent-resistant autoclavable polyfluorylalkoxy (PFA) perforated tubes stuffed with glass wool (incinerated to remove organic carbon) can be used as coupons in wells or drinking water distribution systems to induce endogenous pelagic bacteria into forming biofilms. This biofilm-forming tendency can be magnified by using Bio-Sep® beads generated by K. L. Sublette, University of Tulsa in the traps. Bio-Sep® are a DuPont patent transferred to the University of Tulsa in 1999. Bio-Sep® consists of 2-3 mm spherical beads consisting of 25% aramid polymer (Nomex) and 75% 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. Beads are surrounded by an ultrafiltration-like membrane with median pore diameter is 1.9 microns with some large macropores > 20 microns. Nomex membrane resists formation of a surface biofilm with immobilization occurring through entrapment. Bacteria can be immobilized inside Bio-Sep® beads by culturing the bacteria in the presence of the beads if a carbon-absorbable species is a limiting nutrient and used to treat sulfide containing water (8). Preliminary experiments indicate the Bio-Sep® beads encouraged 10-times (~50 nmoles phospholipid fatty acids/5g trap) vs ~ 1.3 nmoles on the glass wool after incubation 1.5 months in a groundwater well field (Peacock et al unpublished).

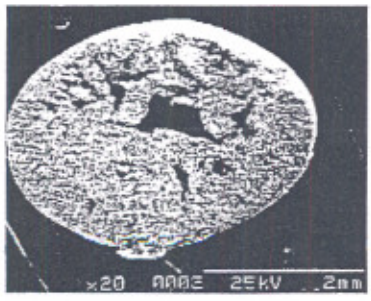

Figure 5. Cross section of Bio-Sep® bead.

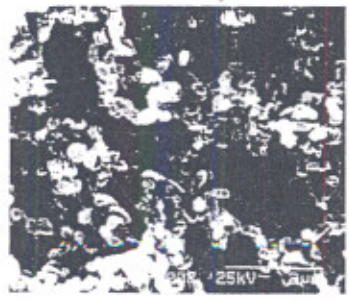

Figure 6. Bacterial biofilm colonizing inside Bio-Sep® bead

Biofilm Concentration of Drinking Water Organics: Many of the drugs, hormones and their mimics, and other pharmaceutically active pollutants (PAP) that have been detected in surface waters (9) 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 (10). 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 (1). Their cellular membranes, in aggregate, are capable of concentrating lipophilic pharmaceuticals and endocrine disrupters and the biocide Triclosan, a compound that has been detected in biofilms when it was not possible to detect it in the water column (11).

Analysis of the fractions provides detection of ~10^3 cells of specific pathogens and by coupling with immunocapture HPLC of specific pollutants concentrated in the biofilm. Recovery of lipids from triculture biofilms incubated 4 days then inoculated with *E. coli* engineered to contain green fluorescent protein showed clear differences from un-inoculated controls (Figure 2) with the presence of *E. coli* showing differences in respiratory ubiquinone isoprenologue proportions (unpublished data). 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. The *E. coli* could be detected as discrete microcolonies by GFP fluorescence in the inoculated biofilms and not in the un-inoculated controls by laser confocal microscopy (Figure 3). The hydrophobic biocide Triclosan and the drug Viagra exposed to the biofilm showed some unquantifiable portion of Viagra and 15% or 160 ppb of Triclosan were found in the lipids of the biofilm. 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.

The exploitation of "flash" sequential extraction coupled to HPLC/ES/MS/MS analytical system described herein has just begun (5). This system appears to provide ultrasensitive quantitative detection of viable biomass, absence or potential presence of microeukaryotes like *Cryptosporidium* oocysts, enteric bacteria, indicators of potential infectivity like cell lysis, toxin or oxidative exposure of Gram-negative bacteria and the presence of drugs hormones and pharmaceutically active pollutants. The fact that the lipid extraction of sediments and biofilms facilitates recovery of DNA suitable for 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 (4).

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