Session C-78

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Protecting Drinking Water: Rapid Detection of Human Fecal Contamination, Injured, and Non-Culturable Pathogenic Microbes in Water Systems.

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Abstract

The rapid, potentially- automatable extraction of filter retentates has allowed quantitative detection of the unique biomarker for human fecal contamination, coprostanol, and the signature lipid biomarkers for total cellular biomass, *viable* cellular biomass, lipopolysaccharide (endotoxin). This method may be integrated with DNA based gene probe analysis for specific strains and enzyme activities. Not only does the analysis provide for detection of injured and non-culturable microbes but it also provides biomarkers characteristic of microbes exposed to biocides and disinfectants that can be utilized to monitor effectiveness of water mitigation/treatment. The analysis schemes involve filtration of the water or direct extraction of biofilms in sidestream chambers, supercritical fluid and/or liquid extraction, derivatization, and analysis of "signature" patterns by gas chromatography/mass spectrometry. Signature lipid biomarkers of interest are diglycerides, steroids including coprostanol and its isomers, poly-ß-hydroxyalcanoates (PHA), phospholipid ester-linked fatty acids (PLFA), and the lipopolysaccharide lipid A hydroxy fatty acids. PLFA found in polar lipid fractions estimate total *viable* cellular biomass, whereas the total cellular biomass can be calculated from diglyceride/phospholipid ester-linked fatty acids ratios. Furthermore, direct evidence of mitigation/treatment effectiveness can be ascertained by detection of diglycerides, respiratory quinones, PHA, and PLFA markers indicative of metabolic stress and toxicity such as *trans* monoenoic PLFA as well as oxirane and dicarboxylic fatty acids derived from the PLFA.

Specific biomarker such as dihydroxy isobranched fatty acids derived from the LPS of *Legionella,* micocerosic acids, secondary alcohols and methyl hydroxy fatty acids from the waxes of *Mycobacteria,* steroids from *Aspergillus, Naegleria,* coordinated with DNA gene probes with or without PCR (enzymatic) amplification of non culturable organisms such as *Giardia* and *Cryptosporidiun* cysts, hydrophobic pollutants, and mycotoxins (estimated from analysis of toxins like aflatoxin) should provide a comprehensive, potentially field-utilizable, quantitative analytical system for protecting drinking water supplies and monitoring treatment effectiveness.

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Introduction

The risk of infection from water remains a serious world-wide problem. In recent years, the problem has been compounded by increased requirements for reuse of water, increased dissolved organic carbon, and an increased number of immunocompromized consumers. In developed countries, safe water requires an eternal vigilance which is currently based on viable plate counts of indicator bacteria. However, injured and non-cultured organisms are present and are potentially infectious, but not detected by the standard plate count of indicator organisms (Colwell et al, 1985). Our laboratory developed the signature lipid biomarker (SLB) analysis that allows detection of all viable microorganisms (cells possessing intact cellular membranes) associated with biofilms (recovered from strategic locations in water distribution lines) or bulk water (analyzed as filter retentates). Lipid biomarker analysis provides the microbiological community structure and the nutritional/physiological status of the community (White, 1988) and, as recently established, the SLB extraction process yields microbial DNA in a form suitable for gene probing (Kehrmeyer et al, 1996). Drawbacks to the routine use of SLB/DNA analysis to define the total microbiological community include the initial cost of equipment (gas chromatography/mass spectrometry), time-consuming sample preparation, the training of personnel, and the generation of toxic waste.

Methodology

We are developing a rapid quantitative extraction apparatus that can accelerate the extraction process and has the potential for automation that could eventually be utilized in water treatment facilities for protection against non-culturable but potentially infectious agents. Memebrane filter retentates of surfaces that can be colonized by microbes will be extracted by a sequence of treatments that will recover neutral lipids, polar lipids, and lyse microbes for extraction of nucleic acids. Supercritical fluid extraction (SFE) can replace liquid extraction/fractionation procedures (Hawthorne et al 1992). Modified supercritical fluid CO_2 will quantitatively extract ubiquinones, diglycerides, triglycerides, mycocerosic acids, and sterols. Supercritcal CO_2 has also been shown to have a lytic affect on some bacteria (Reardon et al, in preparation). Heated solvent extractants can accelerate extraction of polar lipids (Richter et al 1995) and has been demonstrated to lyse microbial cells. The RNA and DNA of the lysed cells can then be extracted with appropriate buffers for probing or enzymatic amplification and hybridization probing.

Discussion

Signature Biomarkers: Classical microbiological methods, which were successful with infectious disease, have severe limitations for the analysis of subsurface samples. Chemical analysis of signature biomarkers extracted directly from a variety of environmental samples provides a more applicable methodology for environmental microbial analysis. The applicability of signature lipid biomarker analysis (SLB), which is based on the liquid extraction and separation of microbial lipids from environmental samples, followed by quantitative analysis using gas chromatography/mass spectrometry (GC/MS has

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been established for a wide variety of environments (Tunlid and White 1991; White *1993).*

Viable biomass: Phospholipids, an important SLB class, are essential membrane components of living cells. Unlike most other biomarkers, phospholipids are typically degraded within hours following cell death (White et al 1979). This rapid degradation of the phospholipids establishes the phospholipid ester-linked fatty acids (PLFA) as ideal biomarkers for viable cells, thus, the quantification of total PLFA is an accurate measurement of living biomass. With cell death phosphatases rapidly convert the polar phospholipids into neutral lipids which survive as diglycerides which for a period of time, show the same ester-linked fatty acid pattern as did the parent phospholipid. Consequently the polar lipid content of a biofilm is a measure of the viable biomass and the diglycerides represent recently lysed cells.

Community Composition: Because different groups of microorganisms synthesize a variety of PLFA through various biochemical pathways, the PLFA can be effective taxonomic markers. Microbes form a wide variety of fatty acids with methyl branching, double bonds in *cis* or *trans* configurations, hydrolyl groups, and cyclopropane rings at various positions in the hydrocarbon chain. The patterns of phospholipid ester-linked fatty acids (PLFA) are sufficiently diverse that they can be utilized to differentiate between groups of bacteria and in some cases as signatures of specific bacteria (Tunlid and White 1991; White 1993). PLFA analysis can provide insight into the phylogenetic relationships between organisms similar to phylogenetic analysis based on the sequence homology of 16S ribosomal RNA (Guckert et al. 1991; Kohring et al. 1994).

Recently, the solvent extractions utilized for the recovery of lipids have been shown to liberate DNA as well and in a form suitable for gene probing (Kerhmeyer et al. 1996). DNA gene probing substantially increases the specificity of this *in situ* analysis and allows for detection at the strain, species, genus, or kingdom levels. DNA gene probes can also be utilized in the detection of specific metabolic activities or for the potential for these activities. The utilization of combined SLB and DNA probing technologies on the same sample allows complimentation of the limitations of the two techniques (White 1994).

Community Nutritional/Physiological Status: Knowledge of specific lipid biosynthetic pathways can provide insight into the nutritional status of the microbial community as certain fatty acids, such as *trans* monoenoic and cyclopropyl PLFA, provide indications of environmental stress (Guckert et al. 1986). Bacterial poly β-hydroxyalkanoic acid (PHA) and microeucaryotic triglyceride (Gehron et al. 1982) are endogenous storage lipids. The relative amounts of these compounds, as compared to the PLFA, provides a measure of the nutritional status of specific components of the microbial community. Many bacteria form PHA under conditions of unbalanced growth such as when a carbon source and terminal electron acceptor(s) are present but cell division is limited by the lack of some essential nutrient (Nickels et al. 1979; Findlay and White 1983). Exposure to toxic environments can lead to minicell formation and a relative increase in PLFA specific to the exposures. For example, increased

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conversion from *cis* to *trans* PLFA occurs in *Pseudomonas* species with exposure to higher concentrations of phenol in the absence of bacterial growth (Heipieper et al. 1992). Prolonged exposure to conditions inducing stationary growth phase induce the formation of cyclopropane PLFA (Guckert et al. 1986). Environments with high potential terminal electron acceptors (oxygen, nitrate) induce the formation of benzoquinones in Gram-negative bacteria in contrast to microbes respiring on organic substrates which form naphthoquinones (Hedrick and White 1986). There are other lipid biomarkers which can yield further insights into the conditions of the biofilm microniches (White 1995).

Detection of Human Fecal Contamination, Injured, and Non-Culturable Pathogenic Microbes in Water Systems: 5α-Coprostanol is metaboilic product of the bacterial reduction of cholesterol found in particularly high concentrations in human feces and its detection has been utilized to trace human fecal contamination (Hatcher and McGillivary 1979). Field tests showed that coprostanol correlated with plate counts of *E.coli* except in cases where hypochlorite was used to treat sewage effluent. With hypochlorite treatment of sewage effluent, there was a marked decrease in viable indicator bacterial counts was not reflected in the levels of coprostanol, the number of bacteria with intact cellular membranes (and potentially viable), the quantity of enteric bacterial endotoxin biomarkers, and the quantity of plasmalogen biomarkers (typical of fecal anaerobes) (Nivens et al. 1996a). Seagull feces, reportedly a possible source of the *E. coli,* did not contain coprostanol and was not the source of the contamination. Discharge/leakage of human waste into city drainage sewers that feed into the lake was a more likely source. Coprostanol gave no false negatives, its presence indicates contact with human sewage. There can be false positives since coprostanol could persist after enteric bacteria were removed. Additional biomarker measures would provide definitive evidence. There is a strong possibility that the pattern of sterol biomarkers in water particulates can be associated with specific types of fecal inputs and used in defining contamination into watersheds particularly if the lipid analysis is combined with DNA gene probe analysis.

We performed experiments that indicated the microbial biofilm formed from drinking water on a stainless steel surface was a complex community with both Gram-negative heterotrophs (with LPS indicating primarily Pseudomonas-like) and Gram-positive microbes. No *Actinomycetes, Mycobacteria,* or *Legionella* were detectable. A possible "chlorine biocide contact" marker reflecting the formation of halohydrin addition complexes to the monoenoic PLFA were detected by the recovery of dicarboxylic and oxirane fatty acids after mild alkaline methanolysis (Nivens et al 1996b).

It proved possible to quantify *Legionella pneumophila* in mixed organism biofilms in microcosms from their signature lipid fatty acids (Walker et al. 1993).

Mycobacteria contain unique lipids in their neutral lipids. Application of artificial neural network analysis to patterns of Mycobacterial neutral lipid components allowed the differentiation of 17 species from 73 strains some of

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which are difficult to achieve with current gene probe analysis (*M. tuberculosis* vs *M. bovis* or *M. avium* vs *M. intercullare*). The lipids also reflect the phenotypic properties of the strains. With this lipid analysis it was possible to pick out the pathogenic strains and to detect drug resistance in *M. tuberculosis* strains.(Almedia et al 1995).

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