Final report of Program WQ 699-524-94,
QUANTITATIVE DETECTION OF INJURED OR NON-CULTURABLE MICROORGANISMS BY SIGNATURE LIPID BIOMARKER ANALYSIS

Principal Investigators: D. C. White, D. E. Nivens, S. Reardon
Microbial Insights, Inc. 201 Center Park Dr., Knoxville, TN 37922-2105 and Center for Environmental Biotechnology, 10515 Research Drive, Suite 300, Knoxville, TN 37932-2575

Period January 1994-December 1996;

The NWRI Research Advisory Board recommended we pursue this research program in two phases. The Phase I program (two years) involves the development of extraction technology and Phase II (projected to be two years but only one year was funded) will be directed at detection/identification of the injured or non-culturable pathogenic agents and an the exploration of an automatable analytical system.

Executive Summary

The Project Goals and Objectives of the second year of Phase I were to:

Goals:
- build on the documented detection of signature lipid biomarkers from the first year by developing a rapid supercritical fluid extraction (SFE) procedure with detection by gas chromatography/mass spectrometry (GC/MS),
- Modify the extraction procedure for the recovery of DNA for gene probing or PCR amplification.

Objectives:

The objectives for this system in the Phase I program were to provide an integrated analysis of:
1) total viable cellular biomass by phospholipid ester-linked fatty acids (PLFA),
2) Total Cellular biomass by a combination of PLFA + diglycerides,
3) Total lipopolysaccharide (LPS) based on recovery of amide linked hydroxy fatty acids,
4) Total lipoid antigens based on steroids and Mycobacterial wax components,
5) Direct evidence of fecal contamination by coprostanol detection,
6) effectiveness of mitigation against microbes by detection of exposure/contact biomarkers and
7) At the request of the NWRI RAB detection of *Cryptosporidium* and *Giardia.*

The accomplishments in 1995 initially focused the research in rapid SFE extraction of lipids to be sure the development of the extractor would yield practical results. The initial extraction test matrix was from drinking water biofilms generated by running tap water trough a column with small steel beads. The control matrix was a mixed bacterial culture that did not contain coprostanol, evidence for Mycobactria, Giardia or Cryptosporidium, or Legionella and

exposure biomarkers. The exposure (to chlorinating) biomarkers consist of oxirane and dicarboxylic fatty acids with chain lengths expected if they were derived from halohydrin adducts formed from monoenoic PLFA in the cellular membranes of Gram-negative bacteria in the biofilms. The new focus was combining DNA extraction with the rapid lipid extraction to provide the complimentary insights necessary for those pathogens which do not have signature lipid biomarkers. SFE of particulate matter captured on glass fiber filters was shown to lyse cells and permit recovery of high molecular weight DNA which was not physically damaged. We also have collected sequences and made DNA probes for *E. coli*, *C. parvum* and *G. lambia*. The SFE conditions were modified to make the recovery of sterols, respiratory quinones, and diglycerides compatible with DNA recovery. Apparatus was designed to include a high-temperature lipid extraction in the sequence to recover polar lipids and more effectively lyse gram-positive cocci, bacterial spores and oocytes for DNA recovery. These are particularly difficult to lyse and are responsible for low recoveries of DNA. We have made arrangements to collaborate closely with ISCO, Inc. of Lincoln, NB in the development of a modified extraction device that will allow sequential extractions. This has been established in the development of the Dionex Accelerated solvent extraction apparatus (Richter et al. 1995).

Based on the experience and direction of the NWRI Advisory Board, the Phase II program was initiated in 1 January 1996. The program was to design, build, and test a rapid, automatable, quantitative system for detection/identification of signature biomarkers from water samples, watershed sediments, and microbial biofilms. The biomarkers were signature lipid biomarkers, and DNA suitable for probing with and without enzymatic amplification.

Specific aims were to:

1) Build, test, and validate the rapid quantitative sequential extraction apparatus (RQSEA) for use in water facilities for detection of injured and or non-culturable microbes.

2) Utilize the RQSEA to define specific watershed contamination inputs based on fecal lipid and DNA parameters.

3) Define specific lipid biomarkers for injury, and potential infectiousness based on lipid biomarkers for phenotypic status.

Accomplishments in 1996:

The system of detecting fecal contamination in watersheds was field tested at Racine WI and in New Jersey. The detection was based on the presence of coprostanol which is in particularly high proportions in human feces but is not abundant in domestic animals feces. The desire to use our research data in legal action in New Jersey and the reluctance for publicity in Racine prompted us to not pursue this part of our research.

Significant progress was made in the sequential extraction of lipids in the course of research. Neutral lipids were recovered with supercritical carbon dioxide with methanol additive. No polar lipids were released however. Polar lipids required enhanced solvent extraction with high pressure chloroform methanol. This proved markedly effective. The supercritical extraction did promote the lysis of the cells when recovered from pelagic or biofilms formed in drinking water. This lysis greatly facilitated the recovery of DNA from the cell residue with aqueous based extraction methods. In consultation with NWRI It was decided to pursue the rapid recovery of DNA as the most economically profitable application of the RQSEA technology. All discussion of the technology and research was stopped pending patent disclosures and no publications were done. NWRI patented the technology and then leased the material back to D. E. Nivens to develop independent of D. C. White and Microbial Insights. In the Fall of 1996 D. E. Nivens became increasingly difficult and refused to prepare his last quarterly report, his final report, and to prepare any papers on his research in Phase I or to communicate any data to us. We subsequently developed a rapid reproducible application of the enhanced solvent extraction and recovered 3-times the polar lipid from bacterial spores and twice the polar lipid from human tissue as the room temperature/ambient pressure methods (Macnaughton et al. 1997). We followed up on the signature lipid biomarkers in the drinking water biofilms and the oxirane fatty acids of the polar lipids as "death" biomarkers resulting from exposure to chlorine (Smith et al. 1999a, 1999b).

We independently pursued the detection of biomarkers of *Cryptosporidium* after detecting no significant biomarkers in *Giardia.* We though we had discovered the golden globe when we found an extremely unusual biomarker that appeared to be found in infectious Cryptosporidium oocysts as 10 methyl 18:0 free fatty acid. We developed methods allowing detection sensitive to a single oocyst. Unfortunately, we found out the magic free fatty aid was an artifact that occasionally contaminated the rubber bulbs used to transfer lipids (Burkhalter et al, 1998). However, we were able to differentiate between purified oocysts of *Cryptosporidium parvum*, *C. baileyi, C. muris,* and *C. serpentis* based on the patterns of polar lipid fatty acids and to show that a marked decrease in the polar to neutral lipid ratio (5.0 to 0.9) occurred with freezing to –20 C which results in loss of infectivity" (Schrum et al 1997; White et al. 1997).

Our finding that organic solvent extraction liberates microbial DNA added a powerful strategy for the detection/identification of injured or non-culturable microorganisms that could be focused on rapid detection of pathogens. Consequently NWRI pursued patenting the process. We established that supercritical fluid extraction in all the variations tested did not lyse all types of microorganisms in a biofilm, or other drinking water matrices. We subsequently applied two successive extractions one supercritical followed by one using polar solvents. This was followed by a nucleic acid extraction with EDTA/buffered saline which we have shown quantitatively recovers DNA and RNA from lysed microbial cells. The DNA and RNA was clean and a suitable template for gene probing or enzymatic amplification by PCR. These extraction will all be done in succession from the same sample by successive application of the proper extractants. Each fraction will be derivatized and analyzed by GC (or HPLC) MS for the lipids and by slot plots or polyacralymide gels for the nucleic acids.

Unanticipated Results

The occurrence of chlorite (co-) damaged fatty acids was observed during the analysis of the Knoxville tap-water biofilms. Polar lipid fatty acid analysis revealed the presence of oxirane fatty acids. It is postulated that chlorine in the tap-water attacked the unsaturated polar-lipid fatty acids to yield oxirane functional groups. This may suggest a biomarker for chlorite exposure and cell death, tat was subsequently shown to correlate with non-culturability.

It has been discovered that the rapid pressurization involved in supercritical fluid extraction lyses microbial cells. The DNA was not damaged and can be probed for specific primers, an assay could be developed to probe for the certain pathogenic microorganisms. The detection of DNA could be directly coupled to an automated instrument for the rapid detection of the signature lipid biomarkers and subsequently nucleic acid identifications.

Presentations and Papers:

Presentations in 1996:

January 16-19 Invited participant in the "Biofilm in homes in industry and in the environment" meeting at the at the S. C. Johnson Wax Corp, Racine WI. We presented papers relative to NWRI sponsored work. "Quantitative Assessment of *in situ* microniche environmental conditions and projections of metabolic activities based on signature biomarker analysis.", D. C. White, D. B. Ringelberg, and S. Alugupalli; "on-line Approaches to Industrial Research: Laser Confocal Microscopy, ATR-FTIR, and Flowcell Microbiology". R. J. Palmer Jr., D. E. Nivens, and D. C. White; "On-line monitoring of biofilm formation, stability, and pathogen ecology in drinking water protection and marine biofouling" A.A. Arrage and D.C. White.

April 26-28. Invited participant on the Scientific Advisory Board for the National Water Research Institute, in Orange City, CA. Presented reports " Quantitative detection of injured or noncultuable micro-organisms by signature lipid biomarker analysis" D. E. Nivins; and "Risk reduction in drinking water distribution systems by on-line monitoring of pathogen ecology for quantitative evaluation of mitigation procedures". A. A. Arrage and D. C. White. Chaired the session of planning programs for "Health Effects" concentrating on detecting non-culturable emerging pathogenic infections.

May 18-23. Annual meeting of the American Society for Microbiology, New Orleans, LA. Invited participant in the Symposium Application of Molecular techniques for addressing Environmental Problems. D. C. White "Lipid Biomarker Analysis for *in situ* Microbial Community Ecology" Presented Poster Arrage, A. A., J. S. Almeida, and D. C. White, "Reduction of chlorine effectiveness against *Legionella* and *E. coli* in a mixed in a mixed species biofilm

June 23-27. Invited participant in the North American Water and Environment Congress '96. Anaheim, CA June 22-27. Presented paper White, D. C., D. E. Nivens, A. A. Arrage, B. M.

Appelgate, S. R. Reardon, and G. S. Sayler. 1996. "Protecting Drinking Water: Rapid Detection of Human Fecal Contamination, Injured, and Non-Culturable Pathogenic Microbes in Water Systems.

August 4-7 Invited presentation by J. S. Almeida at the 1996 Annual Meeting of the Society of Industrial Microbiology, Research Triangle Park, NC, In the symposium "Improving our odds in the biodiversity lottery" Abstract: J. S. Almeida*, A. Sonesson, D. B. Ringelberg, D. C. White. "Application of artificial neural networks (ANN) to identify and characterize Mycobacteria based on their signature lipid biomarkers."

August 19-22. Invited participant in the International Symposium on Biodegradable Poly-beta hydroxyalkanoates '96, in Davos, Switzerland. Gave plenary lecture "Signature Lipid Biomarker Analysis For The Quantitative Assessment Of *In Situ* Environmental Microbial Ecology". D. C. White and D. B. Ringelberg.

August 24-29. Invited Participant American Chemical Society Meeting, Orlando, FL, Symposium :Application of Molecular Markers in Environmental Geochemistry. Chaired session on "Recent Biogenic Markers" and presented paper "Signature lipid biomarker analysis for the quantitative analysis of environmental microbial ecology". D. C. White and D. B. Ringelberg. Posters: "Rapid method for extraction of the fecal sterol coprostanol using supercritical fluid carbon dioxide", M. Rayner, P. Nichols, D. Nivens, D. C. White, and "Integrated lipid biomarker analysis of environmental samples" , Parlette, J., S. J. Macnaughton, J. Doll, A. K. Rose, S. Alugupalli and D. C. White.

September 23-26. Office of Naval Research Reviews of the Minimally adhesive polymers & fouling release coatings, Jacksonville, FL. I made two presentations: " Attachment, biofilm development, hydrophobicity, and surface charge of lipopolysaccharide-deficient mutants of *Pseudomonas aeruginosa*." D. C. White. C. A. Flemming, and R. J. Palmer, Jr. "Evaluation of antifouling coatings using on-line fluorescence" A. A. Arrage, J. S. Almedia, and D. C. White.

September 29-October 4. Invited participant American Society for Microbiology Conference on Microbial Biofilms, Snowbird, UT. Gave the Plenary lecture which was reported in Science **273**: 1795-1796, 1996. "Biofilms as the preferred mode of bacterial growth" D. C. White, R. J. Palmer, A. A. Arrage, C. A. Flemming, and D. B. Ringelberg. And presented posters: Kouznetsova, L. N., T. Kuritz, R. J. Palmer, Jr., and D. C. White "Effect of chemical modification of glass surfaces on bacterial adhesion"; C. B. Phiefer, R. J. Palmer, Jr., and D. C. White "Comparison of bioluminescence induction in *Vibrio harveyi* and *Photobacterium (Vibrio) fischeri* biofilms using photon-counting microscopy"; C. A Flemming, K. M. Harter, and D. C. White "Determination of cell surface charge and its role in adhesion of *Pseudomonas aeruginosa* lipopolysaccharide mutants"; T. Kuritz, L. N. Kouznetsova, C. A. Flemming, R. J.

Palmer Jr., K. M. Harter, and D. C. White "Generation and characterization of mutants of *Oceanospirillium* sp.".

October 4-8. Ninth International Symposium on Bioluminescence & Chemiluminescence, Woods Hole , MA. Gave invited papers: Palmer, R. J., C. Phiefer, R. Burlage, G. Sayler, and D. C. White. 1996. "Single Cell Bioluminescence and GFP in Biofilm Research" and Arrage, A. A., and D. C. White. 1996. "Monitoring biofilm-induced persistence of *Mycobacterium* in drinking water systems using GFP fluorescence". FEMS Microbiology Proceed Int. Bioluminescence Soc.

October 18-20. Fall meeting National Water Research Institute Scientific Review Board meeting Lincoln, NE. Presented reports on "Quantitative detection of injured or noncuturable microorganisms by signature lipid biomarker analysis" D. C. White and D. E. Nivens; "Risk reduction in drinking water distribution systems by on-line monitoring of pathogen ecology for quantitative evaluation of mitigation procedures" A. A. Arrage, C. S. Flemming and D. C. White; as well as a proposal to the health effects subcommittee "Quantitative detection, identification , determination of infectious potential and mitigation effectiveness" D. C. White

November 7-23. Invited participation in the International membrane science and technology Conference (IMSTEC'96), Sydney Australia. Invited participant in the NWRI/University of New South Wales Biofouling Workshop, Sydney Australia, where I gave the report "New and Emerging Techniques for Microbial Biofouling Biofilm Characterization and Monitoring". At the Department of Microbiology and Immunology, University of New South Wales, Sydney and the department of Microbiology, University of Tasmania, Hobart gave seminar "Signature lipid biomarkers for microbes in disease and the environment". Consulted at the department of chemical Oceanography, CSIRO, Hobart, Tasmania on recovery of potentially commercially valuable lipids from marine organisms.

Papers:

White, D. C., D. E. Nivens, A. A. Arrage, B. M. Appelgate, S. R. Reardon, and G. S. Sayler. 1996. Protecting Drinking Water: Rapid Detection of Human Fecal Contamination, Injured, and Non-Culturable Pathogenic Microbes in Water Systems. Proceedings of the North American Water and Environment Congress'96, American Soc. Civil Engineers, New York, NY.

Lane, J. R. W. R. Mayberry, and D. C. White. 1999. A method for the sequential fractionation and derivatization of fatty acids, aldehydes and long-chain bases from diester lipids, plasmalogens and sphingolipids in lipid extracts . J. Microbiol Methods. In press.

Smith, C. A, C. B. Phiefer, S. J. Macnaughton, A. Peacock, R. S. Burkhalter, R. Kirkegaard, and D. C. White. 1999. Quantitative lipid biomarker detection of unculturable microbes and chlorine exposure in water distribution system biofilms. Water Research. submitted

Smith, C. A., C. B. Phiefer, R. D. Kirkegaard, D. C. White, and R. S. Burkhalter 1999. Generation and Characterization of Epoxidated Fatty Acids in Phospholipids of Gram-negative Bacteria as a Disinfectant Biomarker. J. Microbiol. Methods. Submitted.

White, D C., R. S. Burkhalter, C. Smith, S. J. Macnaughton and K. W. Whitaker. 1998. Rapid, Potentially Automatable, Method Extract Biomarkers for HPLC/ESI/MS/MS to Detect and Identify BW Agents. Proceedings of the 1997 ERDEC Scientific Conference on Chemical and Biological Defense Research, (D. A. Berg, Ed) ERDEC-SP-063 Edgewood Area Conference Center, Aberdeen Proving Ground, MD, pp. 27-33.

White, D. C., S. Alugupalli, D. P. Schrum, S. T. Kelly, M. K Sikka, R. Fayer and E. S, Kaneshiro. 1997. Sensitive quantitative detection/identification of infectious *Cryptosporidium parvum* by signature lipid biomarker analysis. 1997 International Symposium on Waterborne Cryptosporidium Proceedings. Pp. 53-59. Amer. Water Works Assn. , Denver CO

Schrum, D.P., S. Alugupalli, S. T. Kelly, D. C. White, and R. Fayer. 1997. Structural Characterization of a "Signature" phosphatidylethanolamine as the major 10-hydroxy stearic acid containing lipid of *Cryptosporidium parvum* oocysts. Lipids **32:** 789-793.

Macnaughton, S. J., T. L. Jenkins, M. H. Wimpee, M. R. Cormier, and D. C. White. 1997. Rapid extraction of lipid biomarkers from pure culture and environmental samples using pressurized accelerated hot solvent extraction. J. Microbial Methods **31:** 19-27.

White, D. C. , D. B. Ringelberg, S. J. Macnaughton, S. Alugupalli, D. P. Schrum. 1996. Signature lipid biomarker analysis for quantitative analysis in situ of environmental microbial Ecology. ACS Symposium 671 "Application of Molecular Markers in Environmental Geochemistry", R. Eagenhouse ed. Pp. 22-34, American Chemical Society, Washington, DC.

Rayner, M., P. D. Nichols, D. E. Nivens, and D. C. White. 1996. Rapid method for extraction of the fecal sterol coprostanol using supercritical fluid carbon dioxide.. American Chemical Society Extended Abstracts, Environmental Chemistry, , August 25-23, Orlando FL, **32:** 273-276.

White, D. C. , A. A. Arrage, D. E. Nivens, P. Angell, R. J. Palmer, J. F. Rice and G. S. Sayler. 1996. Biofilm Ecology: On-line methods bring new insights into MIC and Microbial Biofouling. **10:** 3-16.

Schmitt, J. D. E. Nivens, D. C. White, and H-C. Flemming. 1996. Changes in Biofilm properties in response to sorbed substances--an ATR-FTIR study. Water Science and Technology **32:** 149-155. White, D. C., J. O . Stair, and D. B. Ringelberg. 1996. Quantitative Comparisons of *in situ* Microbial Biodiversity by Signature Biomarker Analysis. J. Indust. Microbiol. **17:** 185-196.

Nivens, D. E., R. J. Palmer, and D. C. White. 1996. Continuous non-destructive monitoring of microbial biofilms: a review of analytical techniques. J. Industrial Microbiology **15:** 263-276.

White, D. C. 1995. Chemical ecology: Possible linkage between macro-and microbial ecology. Oikos **74:** 174-181.

Nivens, D. E., R. J. Palmer, and D. C. White. 1995. Continuous non-destructive monitoring of microbial biofilms: a review of analytical techniques. J. Industrial Microbiology **15:** 263-276.

Kehrmeyer, S. R., B. M. Appelgate, H. Pinkert, D. B. Hedrick, D. C. White and G. S. Sayler. 1996. Combined lipid/DNA extraction method for environmental samples, J. Microbiological Methods. **25:** 143-153.