Final Report for Project # HRA 699-519-97

RISK REDUCTION IN DRINKING WATER DISTRIBUTION SYSTEMS BY ON-LINE MONITORING OF PATHOGEN ECOLOGY FOR QUANTITATIVE EVALUTATION OF MITIGATION PROCEDURES

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Executive Summary

This program was a continuation of the research program supported by HRA 699 510-94 which focused on the quantitative detection of drinking water biofilms by signature lipid biomarkers without the requirement for isolation and culture of the organisms.

The ecology of pathogens able to invade and colonize a drinking water biofilm was the focus of the initial efforts. We had shown a considerable "nurturing " of the pathogens by the biofilm organisms reflected in greatly increased resistance to damage by oxidative biocides like chlorine.

We utilized genetically engineered pathogen surrogates that were modified to contain green fluorescent protein (GFP) so they could be detected non-destructively by epifluorescence microscopy of the biofilm. Initial work focused on the problems with the labeled pathogen surrogates we utilized to infect the reproducible drinking water biofilms. Our experiments utilized both the analysis of the signature lipids of the biofilm and of confocal microscopy. These measurment tools showed that the incorporation of the pathogens into the biofilm significantly increased their resistance to chlorine (1, 11, 15). We also showed the biofilm concentrated oocysts of Cryptosporidium. The three main pathogen surrogates tested were: *E. coli*, *Legionella bozemanii*, and *Mycobacterium smegmatis.* Each was shown to have very different attachment/growth profiles in the biofilm. *E. coli* attaches to stainless steel and biofilm-coated substrata equally well and exhibits detectable growth over the testing period; *L. bozemanii* attachment to steel is good (although not as efficient as E. coli), but shows increased attachment in the presence of a biofilm and does not grow appreciably during testing. Preliminary results with *M. smegmatis* show poor attachment and no growth. We feel that these organisms provide a useful system to model the responses of general, bacterial, physiological types to mitigation treatments. This approach, if used to complement the testing of specific strains when needed, would provide a more

rapid evaluation of mitigation treatment efficacy under the innumerable potential environmental conditions which could be investigated (7, 8).

We studied a proprietary bismuth-containing biocide and showed it was particularly effective against biofilms. Due to the proprietary nature of the compound we could not publish the data or carry on detailed tests of the mechanism of action. Negotiations for continued testing and application are continuing.

Early in this period we felt we had defined a truly wonderful biomarker that was unique to Cryptosporidium oocysts and whose presence correlated with infectiousness (13, 14). We spent considerable effort developing this as it could have been a financial bonanza for NWRI. Unfortunately careful work with ultrasensitive equipment our unique indicator of infectiousness of *Cryposporidium parvum* oocysts was an artifact that came from the rubber pipette bulbs used in the transfer of the samples (3). Unfortunately it did not turn up in the control until late in the grant period.

We developed flow cells for the confocal microscope and the photon counting imaging microscope and utilized them in detecting the bioluminescence or fluorescence of single cells in the biofilms (2, 6, 15). We were able to modify the confocal microscope to allow examination of the same fields by attenuated total reflection Fourier transform infrared spectroscopy (12). We were able to increase the yield of lipids and decrease the time required for extraction by use of high temperature/pressure conditions (10) and to show unique lipids in Mycobacteria (9).

The two graduate students supported by fellowships, C. B. Phiefer 1996-1997, and C. A. Smith 1997-1998 showed the effectiveness of the signature lipid biomarkers in the detection of non-culturable but infectious pathogens from drinking water biofilms and the detection of "death" biomarkers in Gram-negative pathogens by oxyrane epoxide formation in the polar lipids of the organism with exposure to oxidative biocides (4, 5). Subsequently C. A. Smith made a significant breakthrough in the rapid detection of bacterial spores which was filed as a disclosure with NWRI and the University of Tennessee Office of Technology Services.

The resources available to this project were decreased by half with the withdrawal of the co-funding by the EPA and this necessitated the reduction in the scope of the effort.

New Papers resulting from research:

1. White, D. C., R. J. Palmer, Jr., M. Zinn, C. A. Smith, R. Burkhalter, S. J. Macnaughton, K. W. Whitaker and R. Kirkegaard. 1998. Manipulation of biofilm microbial ecology. Proceedings of the Eighth International Symposium on Microbial Ecology, Halifax Nova Scotia. August 9-14.

2. Palmer R. J., B. Applegate, R. Burlage, G. Sayler, and D. C. White.(1999) Heterogeneity of gene expression and activity in bacterial biofilms. In Bioluminescence and Chemiluminescence: Perspectives for the 20th century (A. Roda, M. Pazzagli, L. J. Kricka, P. E. Stanley, eds.) John Wiley & Sons. New York. In press.

3. Burkhalter, R. S., C. A. Smith, D. C. White, R. Fayer, A. B. White. 1998. The signature 10-Hydroxy Stearic Acid thought to correlate with infectivity in oocysts of *Cryptosporidium* species is an artifact. Lipids **33**: 829-833.

4. 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

5. 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

6. Palmer, R. J., C. B. Phiefer, and D. C. White. 1998. Comparison of photon flux from single cells of the bioluminescent marine bacterium *Vibrio fisheri* and *Vibrio harveyi* using photon-counting microscopy. Microbial Ecology. In press

7. Palmer, R. J., Jr., and D. C. White. 1997. Developmental biology of biofilms: implications for treatment and control. Trends in Microbiol. **5**: 435-440.

8. White, D. C, C. A. Flemming, K. T. Leung, and S. J. Macnaughton. 1998. *In situ* microbial ecology for quantitative appraisal, monitoring, and risk assessment of pollution remediation in soils, the subsurface and in biofilms. J. Microbiol. Methods **32**: 93-105.

9. Alugupali, S., M. K. Sikka, L. Larsson, and D. C White. 1998. Gas chromatography-mass spectrometry methods for the analysis of mycocerosic acids present in *Mycobacterium tuberculosis.* J. Microbiol. Methods **31**: 143-150.

10. 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.

11. White, D. C., R. D Kirkegaard, R. J. Palmer Jr., C. A. Flemming, G. Chen, K. T. Leung, C. B. Phiefer, A.A. Arrage. 1997. Microbial Biofouling and biofilm microbial ecology of biocide resistance and corrosion. Proced. Symp Biofilms in Aquatic Systems, Royal Soc. Chem. In press.

12. Suci. P. A. , K. J. Siedlecki, R. J. Palmer, Jr. D. C. White and G. G. Geesey. 1997. Combined light microscopy and attenuated total reflection Fourier transform infrared spectroscopy for integration of biofilm structure, distribution, and chemistry at solid-liquid interfaces. Appl. Eniron. Microbiol. **63:** 4600-4603.

13. 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.

14. 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

15. Arrage, A. A., and D. C. White. 1997. Monitoring biofilm-induced persistence of *Mycobacterium* in drinking water systems using GFP fluorescence. *In* Bioluminescence and Chemiluminescence: Molecular Reporting with Photons (J. W. Hastings, L. J. Kricka, and P. E. Stanley, eds.) John Wiley & Sons. New York, pp. 383-386.