Final Report for Project # HRA 699-510-94

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

Principal Investigators: D.C. White and A.A. Arrage

Microbial Insights, Inc., 201 Center Park Dr., Knoxville, TN 377922-2105 and Center for Environmental Biotechnology, Univ. of Tennessee, Knoxville, TN 37932-2575

Award Period: 1 December 1994 - 30 November 1996

Reporting Period (2 Years): 1 December 1994 - 30 November 1996

Executive Summary

The overall goal of this research program is to determine how the fate of pathogenic microorganisms in drinking water distribution systems are affected by the presence of mixed-species biofilms of drinking water bacteria. Specifically, how changes in species composition, biomass, and physiological/nutritional status of biofilms impact on the survival, colonization, propagation and release of pathogens; and how these biofilms alter the effectiveness of mitigation techniques directed against microorganisms in the bulk liquid phase.

The project goals and objectives for Year 1 were to develop a laminar-flow biofilm monitoring system which could be used to generate reproducible biofilms of drinking water isolates and begin testing the response of surrogate pathogens to mitigation (i.e. hypochlorite) in the presence of biofilms of different species composition. A stable baseline biofilm composed of three morphologically distinct drinking water isolates was generated and could be introduced into the system either sequentially or as a consortia. Surrogate pathogenic species that have been monitored in the past twelve months include an *Escherichia coli* strain transformed with the green fluorescent protein (GFP) gene and *Legionella bozemanii* . Cellular attachment and subsequent biofilm development was monitored non-destructively by fluorescence and terminal biofilm densities determined by viable and microscopic counts. We showed that exposure to chlorine decreased both the biofilm growth rate as well as cellular viability, and that the presence of a biofilm decreased the disinfecting efficacy of chlorine against both *E. coli* and *Legionella* . Release of biofilm material was monitored by collecting flowcell effluent. The effect of chlorine on *E. coli* was modeled using on-line fluorescent measurements, effluent counts and

terminal cell counts. It was shown that the decrease in *E. coli* biofilm formation in the presence of chlorine was due to decreased growth while the release *E. coli* was not affected.

The project goals and objectives of Year 2 were to continue testing biofilm effects on pathogens with the emphasis on *Legionella*, *Mycobacteria*, *Giardia* and *Cryptosporidium*. This work will include utilizing the signature lipid biomarker technique to assess the effect of chlorine on the 'viable but non-culturable' state and to distinguish between individual species components within a mixed consortia. Other objectives include examining the relationship between protozoal infection by *Legionella* and mitigation effectiveness, bacterial *Giardia* cyst-digestion, and the role of drinking water biofilms in Cryptosporidium oocyst retention and infectivity.

Project Goals and Objectives

We will monitor the attachment, development and release of pathogens (or surrogate pathogenic species) in reproducible drinking water biofilms. Selected biofilms will then be subjected to a range of environmental conditions and treatments to evaluate the effect on the retention and viability of the target organism. At the end of each experiment the biofilm and effluent will be characterized to determine biomass, community composition, and physiological status.

Objective 1: To generate reproducible biofilms composed of different drinking water bacteria by controlling the order of addition of organisms from individual continuous cultures into the laminar flow test system. Controlled parameters include media composition, flow rate, temperature, species composition and order of addition.

Objective 2: To utilize the drinking water (DW) biofilm system to study the colonization, propagation, survival, and release of the GFP-containing *E. coli* strain in a monospecies biofilm and in the presence of the DW biofilms.

Objective 3: Select DW organisms and parameters for mitigation studies.

Objective 4: Select and prioritize the mitigation techniques to be tested.

Objective 5: Study the effect of DW biofilms on the response of the GFP-containing *E. coli* to selected mitigation treatments.

Objective 6: Follow objectives 1-5 for *Legionella* species.

Objective 7: Study the effect on *Legionella* of adding amoebae species such as *Acanthamoebae* or *Hartmanella* to biofilm.

Objective 8: Follow objectives 1-5 for *Mycobacteria* spp.

Objective 9: Follow objectives 1-5 for *Giardia* cysts and *Cryptosporidium* oocysts.

Accomplishments in 1995:

Developing and optimizing the DW biofilm system has been accomplished. Up to six flowcells can be run in parallel and inoculated with a monospecies biofilm or a consortia (Objective 1). It was found that a 1:1000 dilution of Tryptic Soy Broth promoted cell attachment to stainless steel and decreased the amount of unattached biomass in flowcells running at flow rates of 10 ml/min. Three morphologically distinct bacteria isolated from corroding copper water pipes were selected for use in the baseline DW biofilm. They were identified by their PLFA profiles as an *Acidovorax* sp., *Pseudomonas* sp., and a *Bacillus* sp. (Objective 1).

The GFP-E. coli strain readily attached to stainless steel and formed a monospecies biofilm. The attachment and development of the *E. coli* biofilm was monitored by tryptophan and GFP fluorescence, and effluent samples were collected to determine release and viability of cells over time. When the *E. coli* was added to the DW biofilm, fluorescing cells were detected over a 4 day period (Objective 2).

The *E. coli* was inoculated into separate flowcells containing 0, 2, and 5 ppm chlorine (hypochlorite) to determine the mitigation effect on biofilm formation and release. Biomass levels from the untreated biofilm increased almost five-fold while biomass from treated biofilms decreased from original levels. The number of cells in effluent samples from the biocide treated flowcells was greater initially than from the untreated flowcell. After 24 h these results were reversed; a greater number of cells wee released from the untreated biofilm, most likely reflecting the denser community of attached cells in that flowcell. Modeling of *E. coli* monospecies biofilm formation showed that the effect of chlorine on biofilm formation was due to a decrease in the growth rate of attached cells. Chlorine did not affect the detachment of biofilm cells.

When *E. coli* was exposed to identical chlorine concentrations in the presence of an established DW biofilm, and increase in both total and viable cells was observed contrary to the results obtained with the monospecies biofilm. In addition to the greater overall numbers of *E. coli* cells, the number of fluorescing cells in the treated biofilms ranged from 2.6×10^7 cells/cm^2 compared to no fluorescing cells seen in the monoculture experiment. These results suggest that the presence of a DW biofilm

imparted a protective effect on the *E. coli* cells. This protective effect was observed at both concentrations of chlorine, the highest level of which is typically present in swimming pool environments (Objectives 3-5).

Studies involving the blue-fluorescent strain *Legionella bozemanii* were initiated in the fourth quarter of this year (Objective 6). Biofilm formation was monitored on-line using the bacterial autofluorescence which was more sensitive than tryptophan fluorescence. Preliminary results demonstrated that chlorine did not affect cell attachment, but no significant increase in biomass was observed over the 4 d test period. However, the presence of chlorine decreased the numbers of viable cells. No colony forming units were detected from *Legionella* biofilm exposed to 5 ppm chlorine. In the presence of a DW biofilm, viable numbers of Legionella were similar with and without chlorine exposure. These results suggest that the presence of a biofilm composed of non-pathogenic bacteria can protect *Legionella* from the effects of chlorine.

Accomplishments in 1996:

Legionella studies: Exposure to chlorine decreased the number of viable *Legionella* cells retained in the flowcell system, and this effect was mitigated in the presence of a mixed-species biofilm. Efforts are underway to improve the selective detection of *Legionella* cells using immunofluorescent stains in place of using the native fluorescent pigment. The latter fluorophore is susceptible to photobleaching which makes microscopic enumerations difficult.

Experiments were performed examining the effect of temperature (25 °C and 37°C) on *Legionella* biofilm formation and sensitivity to chlorine. On-line fluorescence results showed that temperature did not affect initial attachment of *Legionella* to stainless steel, but accelerated growth at 37°C was observed beginning approximately 72 h after initial attachment. Final *Legionella* biomass levels for both temperature regimes were equivalent by the 144 h mark. When *Legionella* was exposed to 1 ppm chlorine, there was a lag in biofilm growth at 25 °C relative to growth without chlorine. Also *Legionella* appeared to be more sensitive to chlorine at 37°C with no appreciable growth through 144 h.

We have successfully infected *Acanthamoeba castellanii* with our *Legionella* strain and recovered intracellular bacteria after a 24 h period. In flask studies, both free-living and intracellular *Legionella* can be differentiated. Infectivity rates of amoeba can be determined using a hemachrome stain which stains the intracellular bacteria. With these initial studies completed, we are now ready to examine the role of protozoal interactions with drinking water biofilms, specifically with regard to protection of *Legionella* from chlorine effects.

***Mycobacteria* studies:** We received *Mycobacteria bovis* and *M. smegmatis* strains containing the *gfp* gene from Dr. Vojo Deretic. Unlike the *E. coli* strain, GFP expression was stable in the absence of antibiotic pressure; with 80% expression efficiency in a 4 d culture. The initial experiment exposed monoculture biofilms of *M. smegmatis* to chlorine. Overall, the bacterium attached poorly to stainless steel with biofilm cell densities 2-3 orders of magnitude lower than those observed from *E. coli* and *Legionella* , and on-line GFP fluorescence near baseline levels. There was a slight decrease (less than an order of magnitude) in the number of attached cells in the presence of 5 ppm chlorine relative to 0 ppm chlorine. A current experiment introducing *M. smegmatis* to a biofilm-coated flowcell is demonstrating a 2-3x greater fluorescence signal than with *M. smegmatis* alone suggesting the biofilm is affecting cell retention.

M. smegmatis attached very poorly to a clean stainless steel surface, with cell densities of <1000 cells cm^{-2}. *M. smegmatis* attachment was significantly greater when the steel substratum was coated with a biofilm composed of drinking water isolates, with typical cell densities of 10^5 - 10^6 cells cm^{-2}. This biofilm effect was similar to the effects observed with *E. coli* and *Legionella* where initial cell retention in the flowcell was greater in the presence of a biofilm.

Lipid studies of *E. coli* and *Mycobacteria*: Lipid analyses of biofilm samples showed distinct differences between the responses of *E. coli* and *M. smegmatis* to chlorine exposure. The diglyceride:phospholipid fatty acid ratio, a measure of viability, increased with chlorine in *E. coli* monoculture biofilms (increase = less viable). There was no change in the DG:PLFA ratio vs. chlorine in the mixed biofilm community. Similarly, a change was detected in lipid metabolism of monoculture *E. coli* biofilms upon exposure to chlorine, which was typical of toxic stress and was not apparent in the mixed biofilm samples.

When *M. smegmatis* was examined using the same lipid ratios, there was an increase in nonviable cells and in stress in the monoculture biofilm relative to the mixed biofilm community, but unlike *E. coli*, chlorine did not affect these ratios. These results suggest that for *Mycobacteria*, the stress of staying attached to a surface is greater than the stress induced by exposure to chlorine.

Cryptosporidium - biofilm interactions: A pulse of ~2.5 x 10^8 oocysts/ml were injected into a sterile flowcell and a flowcell containing a drinking water biofilm. There was greater oocyst retention in the biofilm flowcell as shown by the rate of oocysts recovered from flowcell effluents:

Hrs. post-injection	Without Biofilm	With Biofilm
0.5	10,867.9	3,686.8
2	2,165.2	214.6
3	1,209.5	586.8
12	35.9	143.0
24	0.0	0.0

Biofilm material from both flowcells were examined for oocysts after 72 h. There was a 2-fold greater number of oocysts attached to the drinking water biofilm vs. the sterile stainless steel surface.

Bioluminescent reporter strain: Studies were conducted using a bioluminescent pseudomonad as a real-time biosensor for biofilm metabolic activity. The strain used was *Pseudomonas aeruginosa* UG2Lr, which contained a *luxAB/lacZY* gene cassette randomly incorporated into the chromosome by transposon (Tn7) insertion. In flask studies, bioluminescence was dependent on the concentration and time of exposure to the reaction substrate, n-decanal. The greatest amount of light was measured at n-decanal concentrations of 30 - 50 μL/100 mL. Light production peaked typically around 25 - 30 min after compound addition. Based on these results, 48 h *P. aeruginosa* UG2Lr biofilms were exposed to n-decanal at 40 μL/100 mL . Biofilm bioluminescence was measured 40 min subsequent to n-decanal addition.

Pseudomonas biofilms exposed to 5 ppm chlorine exhibited an order of magnitude decrease in bioluminescence output relative to control biofilms which were not exposed to chlorine. These results were confirmed by viable counts which were typically 3 times lower in the presence of chlorine. During the 40 min measurement period, there was no difference in on-line biofilm biomass levels (as measured by tryptophan fluorescence) although long-term biomass levels (48 h) did decrease due to chlorine-mediated inhibition of growth.

These results suggest that *lux*-containing bacterial strains which are sensitive to mitigation treatments such as chlorine may act as rapid, real-time reporters of biofilm activity. These reporter strains may be used to determine treatment efficacy even in sub-lethal concentrations of toxicants which might not affect overall biofilm densities.

Impediments to Progress

The antibiotic pressure needed to retain the plasmid bearing the GFP gene limited the ability for long term on-line monitoring of GFP fluorescence in mixed-species biofilms. Work is in progress to insert the gene into the chromosome via transposon technology to eliminate the dependence of antibiotics for gene retention and expression.

Another hindrance is a photobleaching effect seen when counting *L. bozemanii* cells under epi-fluorescence microscopy. While the level of fluorescence is adequate for on-line studies, it is somewhat difficult to enumerate single cells.

The main impediment encountered during the last twelve months has been the difficulty in working with *Legionella* in the cell injury experiment. There were two problem areas 1) cells previously exposed to chlorine did not attach well to a surface; and 2) other biofilm bacteria overgrew *Legionella* cells even on media selective for *Legionella*. To address these problems we will adjust the experimental procedure to optimize cell attachment (increase the contact time between cell inoculation and substratum) and will examine selective stains for *Legionella*.

Also, it has taken longer than expected to develop a protocol which gives consistent numbers of *Legionella*-infected amoeba. Amoeba infection rates can be replicated, but recovery of viable *Legionella* cells from amoeba have been inconsistent.

Unanticipated Results

We have begun a collaboration with Dr. Vojo Deretic and his post-doctoral researcher Laura Via using their mycobacterial strains which they have transformed to contain GFP. Unlike the E. coli strain in our possession, mycobacterial plasmids are apparently very stable and do not have as stringent a requirement for antibiotic pressure for gene retention and expression. The two strains we will be working with are *M. bovis* BCG (the tuberculosis strain) and *M. smegmatis*. The latter strain forms colonies in a matter of days while the former is a slow-growing strain requiring up to three months to achieve appreciable growth.

The three main pathogen surrogates tested to date: *E. coli*, *Legionella bozemanii*, and *Mycobacterium smegmatis* each have very different attachment/growth profiles. *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; and 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.

Recent collaborations have begun with Dr. Robert LaFrenz examining the efficacy of using pulsed UV light as a secondary treatment for drinking water. Current studies involve the optimization of the UV dose for *E. coli*, *Legionella* and *Cryptosporidium* oocysts inactivation.

Opportunities for Future Studies

Future work will continue to assess the impact of and response to mitigation procedures on multi-species drinking water biofilms. The approach will be to measure quantitatively the differences in cellular attachment, persistence, growth, and detachment of biofilms under various biocide or stress exposures in a laminar flow environment. Relationships between biofilm community members and surrogate pathogen species will also be investigated.

The focus will shift to using molecular techniques, both lipid and nucleic acid, to assess treatment efficacy in drinking water biofilms and to monitor the responses of the biofilm community and individual specie components of the biofilms.

The 'viable but non-culturable' state commonly observed in *Legionella* and other bacteria will be investigated by utilizing the SLB analysis for detection of unique lipopolysaccaride (LPS) and phospholipid fatty acid profiles which can be used to detect *Legionella* cells as well as indicate live vs. dead biomass. Increased resistance to biocide by *Legionella* cells infecting protozoa trophozooites will be studied using *Acanthamoeba*.

Interactions between biofilms and protozoa will be studied using *Giardia* cysts and *Cryptosporidium* oocysts. This will include the measurement of cyst retention in the system with and without bacterial biofilms and detachment and release of cysts under various disinfection and flow regimes. Study the interactions between protozoal cysts and bacteria (e.g. *Giardia* cysts and *Flavobacterium* Sun4 which digests cysts).

Presentations and Papers

White, D.C., R.D. Kirkegaard, R.J. Palmer, C.A. Flemming, G. Chen, K.T. Leung, C.B. Phiefer, and A.A. Arrage. 1997. The biofilm ecology of microbial biofouling, biocide resistance and corrosion. Submitted to the Royal Society of Chemistry.

Arrage, A.A. and D.C. White. 1997. Monitoring biofilm-induced persistence of *Mycobacterium* in drinking water systems using GFP fluorescence. FEMS Microbiology Proc. Inter. Bioluminescence Soc. In press.

Arrage, A.A., J.S. Almeida, and D.C. White. 1996. Reduction of chlorine effectiveness against *Legionella* and *E. coli* in a mixed-species biofilm. Poster, Amer. Soc. Microbiol. General Meeting, May 19 - 23, 1996, New Orleans, LA.

White, D.C. 1996. Biofilms as the preferred mode of bacterial growth. Keynote Lecturer, Amer. Soc. Microbiol. Conf. On Microbial Biofilms. Sept 30 - Oct 4, 1996, Snowbird, UT.

Arrage, A.A. and D.C. White. 1996. Selective detection of *Legionella* and *Mycobacteria* in mixed-species biofilms using non-destructive fluorescence monitoring. Poster, Amer. Soc. Microbiol. Conf. On Microbial Biofilms. Sept 30 - Oct 4, 1996, Snowbird, UT.

Flemming, C.A., A.A. Arrage, and D.C. White. 1996. Autofluorescence monitoring of specific bacterial populations. Workshop, Amer. Soc. Microbiol. Conf. On Microbial Biofilms. Sept 30 - Oct 4, 1996, Snowbird, UT.