Final Report

Title: Real-Time Monitoring of *E. coli* Contamination in Wyoming Surface Waters
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Problem and Research Objectives:

The state of Wyoming supports 108,767 miles of rivers and streams and 325,048 acres of lakes (USEPA website). The Clean Water Act (CWA) requires states to designate uses for surface waters such as these and monitor the quality of the water in support of its use. Under section 305(b) of the CWA, Wyoming is required to include the results of these water assessments in biennial reports submitted to the United States Environmental Protection Agency (USEPA). Fecal coliform contamination is one water quality standard tested and included in these reports. Fecal coliform monitoring is an indicator of the sanitary quality of the water and can determine the extent of fecal contamination in the water from warm-blooded animals. Fecal contamination is important from a public standpoint when the surface water's designated use includes contact recreation such as beach use, boating, or swimming (USEPA report, 1986).

Wyoming's 2002 305(b) report to USEPA included a 303(d) list of the State's surface waters with water quality impairments not allowing those reaches to support their designated use(s). Reasons for impairments include metal contamination from copper, selenium, cadmium and silver, excess siltation, high phosphate and chloride levels, high pH, habitat degradation, oil deposits, and fecal contamination. Of the 45 water reaches included in the list, 22 are for fecal coliform exceedences, with all having contact recreation designated uses. This illustrates that fecal contamination of surface waters is an important issue for the State of Wyoming.

This project is developing and proving the concept of an economical system capable of real-time detection of individual *E. coli* in surface waters in Wyoming, including streams, rivers, and lakes, in order to quantify fecal coliform contamination. Our goal is a detection limit of < 5 *E. coli* cells per 100 ml of water in 15 minutes of analysis time, with a minimum detection efficiency of 50% and a false detection rate of < 1%. We are proving the concept of a low-cost, portable testing system that screens water in the field. This system will also support a more economical testing regime than any currently in use, allowing for more frequent and comprehensive water monitoring, thus minimizing human pathogen exposure, e.g. in contact recreation. This proof of concept allows for the design and fabrication of a remote monitoring system that will automatically screen water in real time. Alternative methods necessitate the shipping of bulk water samples or concentrates to laboratories and labor-intensive screening technologies, which may include bulk water concentration, incubation, and culturing. These factors combine to impede overall routine monitoring for fecal coliforms in the field and preclude widespread, routine screening of surface waters.

Based on USGS Year I funding (in partnership with the Wyoming Water Development Commission), we developed and tested a low-cost, portable, highly sensitive, self-contained single cell detection system for *E. coli* in surface waters, which will greatly exceed the current testing procedures in both speed and reliability. Project objectives are**:** 1) low-cost detection of *E. coli* in Wyoming surface waters, 2) allows rapid (<<1hour) enumeration of *E. coli* concentration, 3) sensitive, will allow for enumeration of concentrations < 5 cell/100 ml with minimal number of false positive detections, 4) portable for field monitoring, 5) simple to use, does not require substantial training, and 6) development of proof of concept for remote monitoring.

Methodology:

The innovative technique employed in this research has the potential to detect foodborne and waterborne pathogens in real time at the level of a single bacterium in a volume of air or water through the use of specific fluorescent antibodies. The resulting fluorescence is detected with a CCD imager using a novel integration scheme called Fountain Flow (FF). Our system is called the Wyoming Biodetection System or WBS.

Our proprietary and patented (pending) WBS is based on immunofluorescence identification:

- An antibody specific to the cell species of interest is labeled with a fluorescent molecule or fluorochrome.
- The labeled antibody is mixed in solution with the cell species of interest. The antibodies attach to specific sites on the cells (antigens).
- The cells are passed in a stream of liquid toward a low-cost laser diode, which illuminates the fluorochromes causing them to fluoresce at a different wavelength.
- A low-cost CCD (charge coupled device) 2-D detector detects a burst of fluorescence emission each time a marked cell is illuminated by the laser diode while passing in front of the detector.
- The number of marked cells is then counted via computer. Antibodies can be chosen that are highly specific to the cells of interest.

In our current USGS/NSF-funded research, an operational CCD/FF apparatus was assembled and used to demonstrate the practical and economic feasibility of real time detection of *E. coli* to image and count (via computer) individual microorganisms. **The NSF-funded research focuses on detection of *E. coli* O157:H7 in ground beef, while the USGS-funded research involves detection of *E. coli* in Wyoming surface waters.** This device enabled the flow cytometry feasibility demonstrations to date and established the suitability of the FF cytometer to ***effectively detect single pathogenic microorganisms*.** Emphasis is placed on signal-to-noise enhancement. This allows for multicolor detection providing enhanced reliability using several markers. In this detection system, fluid is transported through a hole that is large enough (2-mm dia.) to prevent clogging. Our imaging technique allows automated measurement of individual cells while they transit this flow cell.

In Fountain Flow a sample of fluorescently-labeled bacteria suspended in a transparent or translucent aqueous solution flows up a tube toward imaging optics, where sample particles can be imaged onto a CCD camera and counted or measured photometrically. The imaging optics include, with a single color instrument, a filter isolating the wavelength(s) of fluorescent emission. The ideal situation occurs when a particle flowing up the flow tube is imaged in the same pixel(s).

Principal Findings and Significance:

Our US Geological Survey proposal outlined the following five goals: 1) optimize fluorescent labeling of *E. coli*, 2) perform laboratory measurements on quantified *E. coli* samples to determine the detection efficiency and sensitivity of the monitoring system, 3) test methods of removing background detritus, 4) test methods of counting quantified samples of *E. coli* in a background matrix, and 5) enumerate *E. coli* in stream and lake water samples. Progress toward these goals is listed below.

In Year I testing, our team has demonstrated success in proving the concept of the WBS. Bacteria labeling, recovery of bacteria from authentic Wyoming surface water, and enumeration efficiency of the WBS have all been successfully accomplished. Year II goals were to develop the concept to the point were it was proven to be effective in enumerating bacteria in actual surface water samples. Critical problems encountered in Year II were 1) discrimination of autofluorescing detritus in the water from

bacteria of interest, 2) testing of optimum reagents for bacteria staining, and 3) improvement of system counting reliability and accuracy.

Year I Results

Fluorescent Labeling

The fluorescent labeling of *E. coli* was the first goal accomplished. A protocol was designed using an R-phycoerythrin (R-PE) labeled antibody to *E. coli* K12, the benign test microorganisms used in our preliminary research. The antigen/antibody reaction was optimized to ensure fluorescent emission strong enough to be detected with the system with as little waste of unattached antibody as possible. This optimization can be scaled up or down according to sample size.

E. coli Detection Method and Efficiency of FF

In order to determine the efficiency of the Fountain Flow (FF) technique, comparisons were made between FF and Whipple-grid counting. For FF, samples of fixed *E. coli* K12 were labeled with propidium iodide dye and flowed through the FF system at 2.2 ml/hr. A 13-mW 475-nm laser diode was used to illuminate the stained bacteria at an illumination angle of 45 degrees. The exposure time of the CCD imaging the flow was 400 ms. Multiple frames were recorded and software used to quantify the detections made. The original overnights of the *E. coli* K12 were dilution-plated and counted with a Whipple grid to obtain the total number of cells in the sample. This number was compared with the number of cells counted by the our system, the Wyoming Biodetection System or WBS. Efficiencies of ~50% are obtained with cells and ~90% when comparisons are made with hemocytometer counts of microbeads, even when the microbeads are fainter than cells. This discrepancy is largely due to cell clumping, which doesn't significantly affect our Whipple-grid results, but has a large effect on FF counts. Typically, one ml of flowing liquid produces 900 images. This large amount of data necessitates counting in real time so that data archiving is not necessary. Detection efficiency is computed by comparing the counts from the WBS with the Whipple-grid counting method or by the mTEC filtration method (described in the next section).

Filtering Background Detritus

The USEPA introduced the mTEC enumeration method for *E. coli* in 1986. This method is currently employed by the USGS Water Resource Office in Cheyenne, WY and was demonstrated to us by USGS staff hydrologist Melanie Clark in a visit to our site. This membrane filter method provides a direct count of *E. coli* in water, based on the development of colonies that grow on the surface of the membrane filter. We first established that filtration through a 10-μm filter was necessary to remove any autofluorescing material from the water sample. We then used the mTEC method to test the efficiency of bacteria recovery after this initial filtration. An overnight culture of *E. coli* K12 was diluted by a factor of 10 using Crow Creek Reservoir (Wyoming) water as the diluent. (Because it is non-pathogenic, K12 was used in the preliminary research.) Crow Creek water simulates water samples that will be collected in the field, which contain sand, other dirt particles, and a small percentage of organic material. Fifteen ml of the sample was vacuum filtered through a 10-μm filter to remove debris, and then subsequently filtered through a 0.45-μm filter to separate bacteria from the solute. The 0.45-μm filter was then placed onto the surface of an mTEC agar plate. A second 15 ml sample, the control, was filtered only through the 0.45-μm filter. This 0.45-μm filter was also placed onto an mTec agar plate. Both plates were incubated at 35.2^{o}C for two hours and then at 44.5^{o}C for 22 hours. The colonies were enumerated the next day. Tween 80 was added to some samples to attempt to increase the recovery fraction. Similarly, sonication was also applied to some samples. **Results from spiked Crow Creek**

Reservoir water are summarized with the following mean bacteria separation efficiencies: 1) 62% (±5%) with no surfactant, 2) 33% (±2.5%) in a 2% Tween 80 solution, and 3) 33% in 2% Tween 80 following 10 minutes of sonication.

Year II Results

BACKGROUND AUTOFLUORESCENCE: THE PROBLEM AND ITS SOLUTION

During the summer and fall of 2003, it became evident that our major stumbling block in terms of enumeration of small numbers of *E. coli* in Wyoming surface waters would be autofluorescence of natural debris. Tests of water from both Crow Creek and the Laramie River, which tested negative for bacteria, produced fluorescent particles of a wide range of sizes. We first attempted to remove particles by a combination of filtration and centrifugation. All water was allowed to settle for a period of ca. 1 hour and was subsequently filtered through a 20-micron filter. What remained were autofluorescing particles that were ca. the size of a bacterium. We tried illumination of the particles with both a green and a blue laser, and the particles autofluoresced in response to illumination in both cases. The emission spectra were examined through a series of filters to determine a wavelength where emission was attenuated or absent. Unfortunately, the particles fluoresce broadly, over the entire visible spectrum from blue to deep red. Examination of the particles through an epifluorescent microscope showed that the fluorescing particles were a combination of organic particles and crystalline particles.

After discussions with several fresh water microbiologists, we chose several lines of attack. First, we attempted to bleach the particles by exposing them to intense ultraviolet light. Second, we attempted to remove the pigments in the particles by centrifugation of the suspension, treating the particulate matter with ethanol, and re-suspending the particulate matter in water. Neither of these techniques reduced fluorescence to an acceptable level.

Finally, discussions of this issue with colleagues produced the suggestion (from Huang et al., 1996 in **Biofouling**, 9:269-277) that it was possible to quench autofluorescence in particulate matter, without affecting the immunofluoresence from labeled bacteria, with the application of an inexpensive dye, crystal violet. We tried this with laser excitation in the blue and green, and found that this technique works well with Laramie River water when illuminated with a blue laser. We now believe that technique might be widely applicable for suppressing autofluorescence of particulate matter in surface waters. We plan to test this method on a variety of Wyoming surface water samples.

DEVELOPMENT OF AN ALTERNATIVE SYSTEM FOR RARE CELL DETECTION

At the same time that we examining ways of reducing autofluorescence of particulate matter in surface water samples, we developed and a technique for using our system to separate bacteria according to shape and size. This would enable differentiation of bacteria from other organic and inorganic particles by size and morphology.

CELL VIABILITY DISCRIMINATION

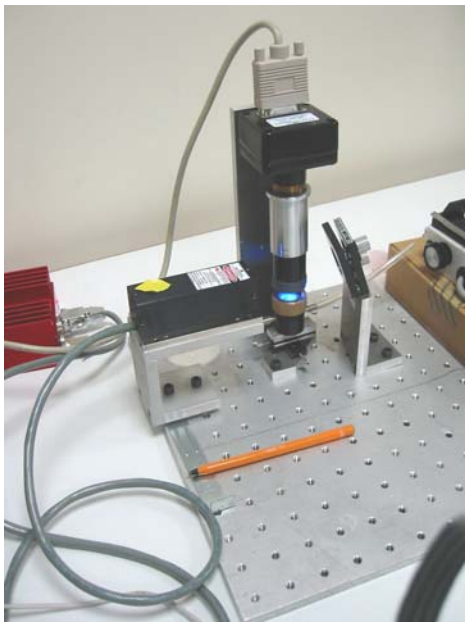
With collaborators at the University of Paris, Dr. Philippe Lebaron and Mr. Philippe Catala, we developed a staining protocol for using CV6 to label cells that are metabolizing esterase. Preliminary tests have shown that when viable cells are stained with CV6, they fluoresce very brightly in the green, and are quite easy to detect. Thus, we can not only detect cells, but measure their viability as well.

DEVELOPMENT OF AN EPIFLUORESCENT SYSTEM AND CALIBRATION TESTING

TESTING RESULTS

While the WBS prototype that we tested in Year I gave encouraging results, we were not happy with the accuracy and reliability of the results. One issue was the need more sophisticated counting software that would properly count individual bacteria once, even when the same bacterium is seen on subsequent

frames, in different positions. A second issue was the need for an epi-illuminated system that produced nearly uniform illumination of the entire orifice of the WBS. We made software and hardware modifications that successfully addressed both issues. Testing this system on 2.5 micron fluorescent Carmine beads (Molecular Probes, Eugene, OR) showed agreement between Whipple grid measurements of beads filtered onto a 0.2 micron filter and WBS measurements to within 10%. (This is approximately equal to the limitation from counting statistics for the low numbers of particles that we are counting (100-400.) Comparison of automated image counting and "hand" counting produces nearly identical results.



The Wyoming Biodetection System
with a 475 nm solid-state 13-mW laser.

SUMMARY OF GOALS FOR FOLLOW-ON RESEARCH

During the summer of 2004, we intend to finish the preparation of a manuscript to a refereed journal. This will include a description of the instrument design, but will include specific test results on the efficiency of detection of E. coli that has been inoculated at known concentrations into water from the Laramie River and/or Crow Creek. In addition, the P-I will attempt to secure funding for the version of this instrument that will use particle shape and size recognition to eliminate false counts from debris.

Students Supported:

During Year I, the P-I employed two undergraduate and two graduate students, in this research. The interaction among personnel of varying backgrounds (including microbiology, pharmacy, and physics) has provided a highly educational experience for everyone in research biodetection technology. During Year II, the P-I employed one undergraduate and one graduate student, in this research.

Publications and Patents:

Johnson, P.E., Lebaron, P., Deromedi, T., and Baudart, J., 2004. *Tests of a Fountain Flow System for Real-time Quantification of Rare Microorganism in an Aquatic Environment*. Accepted for the 2004 International Society of Analytical Cytometry, Montpellier, France.

Johnson, P.E. 2002. *Biodetection with flow cytometry: better, faster, cheaper*, Biodetection Technologies, Knowledge Press, Brookline, Massachusetts, Volume 1: 71-83.

High Throughput Analysis of Samples in a Translucent Flowing Liquid, patent pending, P.E. Johnson.

Methods for Separating Microorganisms From a Food Matrix for Biodetection, patent pending, A. Votaw and P.E. Johnson.

LED Illuminated Particle Detection Apparatus and Methods, patent pending, P.E. Johnson.