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Final Report for DOE NABIR Grant DE-FG02-01ER63264 (PI Mary Lowe)

Title: Development of a Multiplexed, Bead-Based Assessment Tool for Rapid Identification and Quantitation of Microorganisms in Field Samples

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In the past year, there were three main areas of work related to the development of a multiplexed, bead-based assay for measuring analyte DNAs in environmental PCR products. First, a field test of the bead method was conducted on sediment and groundwater samples from the acetate injection experiment at UMTRA Rifle. Second, to make the assay more effective for environmental studies, developments were made in the areas of fluorescence amplification; a new set of fluorescent beads; software for rapid postprocessing of flow cytometric data; and software for developing capture probe sequences. Third, amended microcosms were constructed from uranium-contaminated samples from the Aberdeen Proving Grounds, MD under the supervision of Rolf Halden at the Johns Hopkins School of Public Health.

Overall, a set of protocols was established for collecting groundwater and sediment samples; extracting DNA; PCR amplification of environmental DNA; executing the bead assay; flow cytometric detection; raw data processing; and quantification of individual target sequences in the PCR product. Currently, a chapter is being written by Mary Lowe, Alex Spiro, Anne Summers and Joy Wireman for a book on molecular biology protocols (Humana Press).

UMTRA Rifle field test

A 16S rDNA bead assay was developed targeting the following genera or families: *Geobacteraceae*, *Desulfococcus*, *Shewanella*, *Geothrix*, *Clostridium* (general probe), *Desulfobulbus*, *Desulfobacter*, *Desulfovibrio*, *Desulfotomaculum*, *Desulfobacterium*, and *Clostridium* isolates (ex. K39A1). This list was made in consultation with Janet Chang, DC White, Sherry Dollhopf, and Joel Kostka. The capture probe sequences were obtained from the literature, designed by Dollhopf using ARB, or designed by Lowe using in-house software (<http://probemer.cs.loyola.edu>). Preliminary measurements were conducted on microorganisms filtered on site from Rifle groundwater samples. The sample collection protocol was developed by Guibo Xie, a postdoc partially supported by this grant, under the supervision of the PI. Xie also assisted Todd Anderson (U. Mass.) with the initial setup of the injection gallery.

The field test demonstrated that with the current probes, protocols, and instrumentation, the bead method is capable of providing initial information on microbial genera within two days after receiving the samples. As many as 10 field samples can be handled at a time for a preliminary screening. We were able to monitor in "real-time" changes in the microbial community occurring two and three weeks after the start of injection, and in particular observed tremendous growth of *Geobacteraceae*, which was later confirmed by small-scale cloning. We realized that one run will probably generate new questions and the desire for further testing to confirm the findings. Therefore we believe that quantitative, environmental monitoring will most likely require multiple runs on the same sample to address all of the issues arising from prior tests. Based on our experience with the field test, improvements need to be made in the measurement of the biomass on the filters, quantification using genus-specific probes, an updated *Geobacteraceae* probe, comparative measurements of field samples from different space and time points, and sample testing throughput using robotics.

DOE Patent Clearance Granted

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Assay development

Most of our work has concentrated on quantifying selected 16S rDNA sequences in a mixture of amplicons. For this goal, a number of investigations on fluorescence characteristics of the bead system were conducted. In the past year, we showed that the DNA hybridizations in the bead reactions obey a Scatchard-type model, thereby allowing us to calculate the amount of a particular target in an environmental PCR product. This work was published in AEM (2002). In collaboration with Yu-Zhong Zhang (Molecular Probes), we developed a new set of fluorescent red and infrared microspheres. These microspheres will enable people to conduct multiplexed bead assays on standard research flow cytometers. For quantitative evaluation of biochemical parameters of hybridization reactions between capture probes and target DNAs, several types of fluorescent bead standards with labeled DNA on the bead surface were developed. The need for these standards became apparent when we encountered large variability in commercial MESF standards. To address the problem of measuring rare target sequences in environmental samples, we achieved 10-fold amplification and single DNA molecule detection on bead surfaces using novel fluorescent labels known as 3DNA dendrimers. This work was done in collaboration with Bob Getts (Genisphere).

To expedite the development and testing of bead assays, two pieces of software were written. The first, developed by Peter Kutt, can rapidly postprocess multiplexed, flow cytometric data. Unlike the software provided with our flow cytometer (Luminex 100), this software contains automated searching functions for the correct bead population, enabling us to calculate the mean and other statistical characteristics of beads with DNA on the surface. In addition, a Loyola student Scott Emrich wrote a software package called ProbeMer which enables us to optimize the capture probe sequences based on the number and position of mismatches. Based on bead tests, probes designed with this software have improved performance regarding sequence discrimination. This work was done in collaboration with Art Delcher (Loyola Computer Science Professor and staff member at Celera).

Summary of Microcosm Experiments

The following paragraph was prepared by Rolf Halden. Microcosm experiments were conducted in order to produce test samples for validating the DNA bead array tool. Preparatory work included the establishment of various analytical protocols: $U^{(VI)}$, $U^{(IV)}$ and total U by kinetic phosphorescence analysis using HCO_3^-/HNO_3 extraction and a 'wet ashing' procedures; major anions by ion chromatography; DO, pH, and Eh by electro chemistry using selective electrodes; HS^- by methylene blue method; $Fe^{(II)}$, $Fe^{(III)}$ by ferrozine method; total Fe by atomic absorption spectroscopy; protein content by Lowry method; ^{14}C -labeled carbon sources by liquid scintillation counting; community analysis by DGGE; and 16S rDNA sequence analysis using ARB software. Two sets of microcosm experiments were conducted (Aberdeen Proving Ground water/sediment_(10:1)). Test systems consisted of (A) nonsterile microcosms to which U was added (initial concentration $U^{(VI)}$: 60 and 530 ppb), (B) nonsterile microcosms that received both acetate (0.6 mM) and U, and (C) gamma-irradiated controls. Microcosms were incubated in an anaerobic glove box at ambient temperature for up to 100 days. Rapid sorption of uranium to the natural organic matter of sediments was observed (>95% in 1h). Reduction of $U^{(VI)}$ to $U^{(IV)}$ occurred in nonsterile systems only (A: 25% of U was recovered as $U^{(IV)}$ after 2 weeks; B: 34%). Split samples of microcosms were submitted for analysis by bead array. Extracted, crude DNA was amplified and analyzed by DGGE. In acetate amended sediment, at least two microbial

species had enriched from non-detectable to detectable levels as shown by DGGE analysis. However, their presence was not essential for reduction of U to occur. In summary, laboratory studies revealed that uranium strongly sorbs to NOM, yet remains bioavailable to U-transforming microorganisms. Thus, transport of U at the site likely is controlled by at least two processes: abiotic sorption and biotransformation. This is the first report of naturally occurring bioimmobilization of uranium in sediment of the Mosquito Creek Wildlife Refuge at APG.

The following paragraph on the microcosm experiments was prepared by Lowe. All samples tested with the bead method were obtained from experiments set up by Xie in the laboratory of Prof. Edward Bouwer (Johns Hopkins Department of Geography and Environmental Engineering). While processing the microcosm samples, Lowe noticed significant variation in the PCR amplification and in the bead measurements of the relative abundance of 16S rDNA target DNAs. A systematic investigation of sample handling would be required to achieve reproducible results by any molecular biology method.

Publications, abstracts, talks (copies are attached)

Spiro, A. and Lowe, M. 2002. Quantitation of DNA Sequences in Environmental PCR Products by a Multiplexed, Bead-Based Method. Appl. Environ. Microbiol. 68:1010-1013. The manuscript was edited during this grant period.

Poster and breakout session talks, DOE-NABIR PI workshop, Warrenton, Va, March 18-20, 2002 (authors M. Lowe and R. Halden).

Invited talk, Procter & Gamble (Anne Summers was also invited), June 12, 2002 (authors M. Lowe and A. Spiro)

Poster, Fifth International Weber Symposium on innovative Fluorescence Methodologies in Biochemistry and Medicine, June 25-29, 2002, Kaua'i, Hawaii (authors M. Lowe, A. Spiro, Y-Z. Zhang, and R. Getts).

Abstract for an invited talk at a Conference on Molecular Portraits of Tumors, Perigueux, France, submitted 9/02, conference will be on November 14-16, 2002 (authors M. Lowe and A. Spiro).

In progress

Chapter entitled "Multiplexed Identification and Quantification of Analyte DNAs in Environmental Samples using Microspheres and Flow Cytometry" for a book on molecular biology protocols, Humana Press, ed. Frank Spencer, authors M. Lowe, A. Spiro, A. Summers, and J. Wireman.

Paper tentatively entitled "Computational selection of Optimized DNA Oligos for Sequence Discrimination," authors S. Emrich, M. Lowe, and A. Delcher.

Paper tentatively entitled "High Sensitivity, Multiplexed, Flow Cytometric Assay for Detection of Microorganisms," authors M. Lowe, A. Spiro, Y-Z. Zhang, and R. Getts.