Final Report

Real Time Monitoring of Rates of Subsurface Microbial Activity Associated with Natural Attenuation and Electron Donor Availability

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The project was successful in developing new sensing technologies for monitoring rates of microbial activity in soils and sediments and also developed a novel proof-of-concept for monitoring the presence of bioavailable concentrations of a diversity of metabolites and toxic components in sedimentary environments. These studies led not only to publications in the peer-reviewed literature, but also two patent applications and a start-up company.

The first sensing technology that we developed is known as SMART technology. SMART is an acronym for Subsurface Microbial Activity in Real Time. With SMART technology it is possible to continuously assess the rates of microbial activity in a diversity of anaerobic soils and sediments with a real-time output of electrical current.

In the SMART approach inexpensive, but durable graphite anodes are inserted in the soil or sediment of interest or deployed within a groundwater monitoring well. The graphite anodes are connected via an electrical wire to a graphite cathode that is either suspended in the overlying aerobic water or at the ground surface. A fixed resistance is placed within the circuit between the anode and the cathode. The voltage between the anode and cathode can be continuously monitored, providing a real-time estimate of the current output. Our studies demonstrated that the current output can be directly correlated with rates of microbial activity.

For example, SMART technology was used to monitor microbial activity in sediments in which Fe(III) reduction, sulfate, reduction, or methane projection was the predominant terminal electron accepting processes. The sediments evaluated included subsurface sediments from the DOE SFA study site in Rifle, CO, as well as other sites, to provide a diverse range of geochemical conditions. Sediments with a broad range of rates of microbial metabolism were investigated. As a metric for the effectiveness of the SMART

technology, overall rates of microbial metabolism in the sediments were assessed from the metabolism of tracer quantities of [2-14C]-acetate to 14C-carbon dioxide and 14C-methane. In each sediment type there was a strong correlation between the rates of microbial metabolism measured with [2-14C]-acetate and the current output of the SMART sensors. This finding was published in *Frontiers in Microbiology*. Based on the success of these studies with the SMART sensors, and their potential for commercial application, U.S. and international patent applications were filed.

Molecular analysis of the anodes of SMART sensors indicated that a major source of the current signal from the SMART sensors could be attributed to *Geobacter* species colonizing the anodes. For example, molecular analysis demonstrated that even when methane production was the terminal electron-accepting process, *Geobacter* accounted for the majority of the bacteria comprising the anode biofilms, even though *Geobacter* would not be expected to be highly abundant in methanogenic sediments.

One caveat for converting the current output of the SMART sensors to an estimate of the rates of microbial activity was that the slope of the correlation lines between current output and microbial activity was dependent upon whether the terminal electron accepting process was Fe(III) reduction, sulfate reduction, or methane production. At comparable acetate turnover rates, currents were higher in the sediments in which sulfate-reduction or Fe(III) reduction predominated than they were in methanogenic sediments. Thus, in order to directly covert current outputs from the SMART sensors to rates of microbial metabolism it was necessary to also determine the predominant terminal electron-accepting process.

In order to better understand the differences in the current responses under different terminal electron-accepting conditions we evaluated additional sources of electrons for current production in sediments in which methane production was not the predominant terminal electron-accepting process. One hypothesis that was investigated was the possibility of long-range electron transport through the sediments contributing to additional current in addition to the current produced by the anode biofilm. In order to evaluate this hypothesis the conductivity of various sediments was investigated with a novel split electrode method. It was found that freshwater sediments were poorly conductive and that addition of the conductive mineral magnetite did not significantly enhance the measured conductivity. In contrast, more saline sediments in which sulfate reduction predominated were highly conductive and this conductivity could be attributed, at least in part, to iron-sulfur minerals. These results were published in *ISME Journal*.

We also completed several studies at the Rifle site on aspects of *Geobacter* ecology that have the potential to impact on the current output of the SMART sensors. These included the study of the function of the most abundant cytochrome at the site, which was found to be a cytochrome that specially associates with the electrically conductive pili and a study of *Geobacter* viruses, which have the potential to limit current production through their influence on *Geobacter* biofilm growth. Both of these studies led to publications in *ISME Journal*.

Further evaluation of the SMART technology in a hydrocarbon contaminated subsurface environment demonstrated that there was a higher current output from SMART sensors in monitoring wells in which there were higher concentrations of organic contaminants than in monitoring wells in an uncontaminated portion of the aquifer. These results suggest that the SMART sensors can serve as a real-time monitoring system to assess the spread of contaminants in the subsurface. This is consistent with our results that the SMART sensors were an effective monitor for the arrival of acetate to subsurface zones following the injection of acetate into the subsurface to stimulate microbial reduction of uranium.

Another application of the SMART technology is expected to be real-time monitoring of the response of the microbial community to environmental perturbations associated with climate change. There was a clear response of the SMART sensors to increased temperatures in laboratory sediment incubations. In preliminary laboratory studies with Artic soils collected at the site of DOE's NGEE Arctic experiment there was a correspondence between current production and measurements of microbial activity with [2-14C]-acetate that was comparable to that obtained from the freshwater sediments. In order to further evaluate the SMART approach, Dr. Kenneth Williams (Lawrence Berkeley National Laboratory) and Dr. Stan Wullschleger (Oak Ridge National Laboratory), installed SMART systems within two locations of "Area 0" of the Barrow Environmental Observatory in collaboration with DOE's NGEE Arctic experiment. These were a low-centered (LC) polygon and a flat-centered (FC; also referred to as transitional) polygon. The two locations are emblematic of (a) healthy, non-degraded permafrost (LC location) and (b) permafrost beginning to show signs of drying, desaturation, and degradation (FC location). Thermistors were installed at each anode location. Current and temperature were monitored autonomously and recorded at 30-minute intervals using Campbell Scientific data loggers. The results demonstrated that current production rates varied by 10-fold depending upon location, with the fully saturated, low-centered polygon center (LC1) location exhibiting the greatest current magnitude, and hence highest estimated rates of anaerobic microbial activity. These preliminary studies have demonstrated that SMART shows promise as an approach for real-time monitoring of microbial activity in remote locations, providing both depth and horizontal profiles.

With the success of the SMART sensor development we expanded the research to determine if it might be possible to produce more sophisticated real-time microbial sensors that could detect important geochemical parameters influencing microbial metabolism at the Rifle study site and in other environments. For example, our studies at the Rifle site had detected a substantial release of arsenic into the groundwater during *in situ* uranium bioremediation. In order to control such arsenic releases it would be very beneficial to have a real-time monitoring system to detect arsenic in the groundwater and provide an immediate warning when arsenic was being released. This would make it possible to more selectively control acetate inputs to limit arsenic release. With a suitable platform technology it would be possible to design sensors for a wide diversity of components of geochemical interest. Furthermore, basing the sensor technology on a microorganism has the added advantage that the sensors assess the bioavailable concentrations of these compounds.

We named this technology GeoSense because it relies on *Geobacter sulfurreducens* as the sensing organism and is designed to sense a diversity of geochemically important parameters. The concept for the GeoSense technology was that the presence of a geochemical component of interest would stimulate a *Geobacter sulfurreducens* biofilm on an electrode to produce an increased electrical signal.

One consideration for controlling current output of the sensing organism was to control the conductivity of the electrically conductive pili that *G. sulfurreducens* requires to produce high current densities on anodes. However, after further investigation of charge propagation along the pili and the possible structure of the pili, it became clear that this would not be readily feasible. A simple alternative strategy was to control the ability of the *G. sulfurreducens* to release electrons from organic substrates. This was best accomplished by placing the expression of the gene for citrate synthase, a key enzyme in the metabolism of acetate, under the control of an inducible promoter that responded to the component of interest. Proof-of-concept studies demonstrated that current production responded to well-known inducible systems, such as LacI/IPTG. We also identified regulatory systems that respond to arsenic with high sensitivity and excellent linearity. A new plasmid system that facilitates introducing new regulatory cassettes was developed. U.S. and international patent applications were filed based on these results.

Proteomics studies on the response of *Geobacter sulfurreducens* to exposure to uranium were completed and the results were published in *Microbiology*. These results serve as the foundation for identifying candidates to develop a uranium sensor with the GeoSense Technology.

With the completion of this grant the company Microbe Electric, LLC was founded to further develop and commercialize the SMART and GeoSense technologies.

Several review papers that included summaries of our research at the Rifle site were completed and published.

Patents

Real-time monitoring of subsurface microbial metabolism

Inventors	Derek Lovley and Kelly Nevin
Publication number	WO2015057800 A1
Publication type	Application
Application number	PCT/US2014/060635
Publication date	Apr 23, 2015
Filing date	Oct 15, 2014

Biosensors with a direct electrical output

Inventors	Derek Lovley, Toshiyuki Ueki, Kelly Nevin
Application number	PCT/US15/62994
Filing date	December 1, 2014

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Kenneth H Williams, John R Bargar, Jonathan R Lloyd, and Derek R Lovley. 2013. Bioremediation of uranium-contaminated groundwater: a systems approach to subsurface biogeochemistry. Current Opinion in Biotechnology 24:489-497.

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Nikhil S. Malvankar, Madeline Vargas, Kelly Nevin, Pier-Luc Tremblay, Kenneth Evans-Lutterodt, Dmytro Nykypanchuk, Eric Martz, Mark T. Tuominen, Derek R. Lovley. 2015. Structural basis for metallic-like conductivity in microbial nanowires. mBio 6:e00084-15

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