Integrated Particle Handling Methods for Multiplexed Microbial Identification and Characterization in Sediments and Groundwater

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Abstract

Molecular methods are still relatively ineffectual for monitoring community dynamics during bioremediation, due primarily to the cost, technical difficulty and retrospective nature of the analyses. For nucleic acid analyses to meaningfully contribute to bioremediation efforts they must not only contribute to the fundamental understanding of microbial ecology, but also be formatted in such a manner that in-field analysis can be achieved. The objective of this project is therefore to develop an integrated microbial and nucleic acid detection method and prototype system for the characterization and analysis of subsurface sediments, focusing on the molecular detection of metal- and sulfate-reducing bacteria and activity in sediments obtained from the Oak Ridge Field Research Center. We are meeting this objective by combining environmental molecular microbiology with renewable surface techniques, microfluidic systems and microparticle analytical chemistry. The fluidic systems are used to evaluate hypotheses on the integrated biochemistry that is necessary to directly detect 16S rRNA from metal-reducing microbial communities on a suspension microarray, without using the polymerase chain reaction (PCR). These investigations include the use of peptide nucleic acid capture and detection probes and "tunable surface" concepts to increase nucleic acid capture and detection efficiency and/or mitigate interferences due to co-extracted humic acids. The unified microparticle sample preparation method and suspension array is then used to characterize the 16S rRNA metal- and sulfate-reducing microbial community in FRC sediments before and after biostimulation.

Research Questions, Hypotheses, Milestones

- Develop automated, unified bead trapping and nucleic acid purification protocols for color-coded, 5 µm latex microspheres and subsurface sediment extracts.

  Hypothesis: Peptide nucleic acid probes will be more sensitive than identical DNA probes for the automated recovery and direct detection of metal- and sulfate-reducer 16S rRNA and mRNA from sediment extracts.

- Investigate the underlying microparticle surface chemistry required for efficient rRNA recovery *and* detection in subsurface sediment extracts.

  Hypothesis: Charge manipulation on the microbead surface (i.e. "tunable surfaces") will increase net metal- and sulfate-reducer 16S rRNA purification and direct detection efficiency from sediment extracts

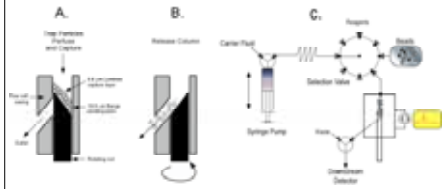
- Utilize these techniques to characterize the microbial community in metal-contaminated sediments from the Field Research Center and associated microcosm experiments, both before and after bio-stimulation.

  Hypothesis: Sulfate- and dissimilatory metal-reducing bacteria are present in FRC sediments, and they can be stimulated to immobilize uranium in situ. Microorganisms related to known strains of Geobacter and the sulfate-reducing bacteria will predominate after bio-stimulation.

Fluidic Systems

Renewable Surface Fluidics

Figure 1. We define **renewable surfaces** as derivatized microspheres that can be introduced, trapped, and released in specialized flow cells (A). While trapped, they are perfused with sample to selectively capture the desired analyte(s) and the remaining sample matrix is washed away to remove undesired components. Because new beads (hence, new interactive surfaces) are automatically introduced for each sample (B), the interactive surface does not require regeneration, and fouling of the critical bio-interactive surface does not require manual replacement of sensors or columns. Custom flow cells are integrated with simple sequential injection fluidics and hardware (C) to automate nucleic acid sample processing functions.



Suspension Arrays

Figure 2. Suspension arrays are based upon colored microparticles (A). Each color provides an identifiable address for coupling oligonucleotide or cDNA probes, with one microparticle color analogous to the traditional spot on a planar array. Nucleic acids hybridize to their complementary probes (affixed to the beads), and the beads are analyzed with a simple and inexpensive flow-cytometer (B). The flow cytometer simultaneously reads the microparticle color ("address") with one laser, and the fluorescently-tagged (and bound) target from each bead with a second laser. Increasing the number of colors/dyes that are used to prepare the microbead suspensions exponentially increases the number of nucleic acid sequences that can be analyzed simultaneously (C).

Results

Tunable Surfaces and Phosphate Extraction Buffers

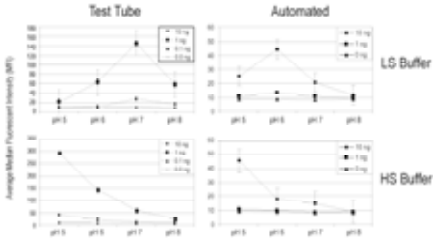


Figure 3. Detection limits of tunable surface microparticles and phosphate-based extraction and hybridization buffers for *G. chapellei* intact rRNA in both test tube and automated hybridization procedures. HS = high-salt phosphate hybridization buffer; LS = low-salt phosphate hybridization buffer. Test tube hybridizations were 15 minutes at room temperature. Total hybridization time for the automated routine is 180 sec, wherein each bolus of target rRNA is in contact with the tunable surface particles for only 30 sec. In the absence of environmental interferents on fluorescent reporters, sediment can therefore be extracted in phosphate buffer and hybridized directly to the tunable surface suspension array.

Dirt Effects on Sample Preparation and Detection

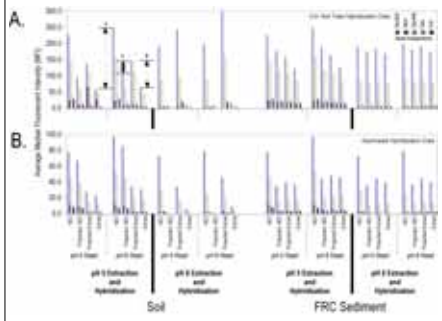
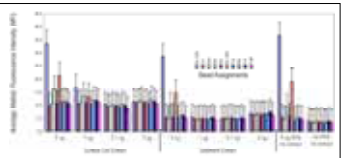


Figure 4. Integrated nucleic acid purification and flow cytometry detection in (A) manual and (B) automated formats utilizing tunable surface extraction, hybridization, and wash conditions. 100 ng fragmented *Geobacter chapellei* target RNA and chaperone probes were amended directly into each environmental extract and hybridized for 2 hours in a test tube or processed through the automated routine, both at 45°C. Signals are corrected for background bead fluorescence in the respective hybridization/wash conditions. Error bars are omitted for presentation clarity. NEC = no extract control, experimental value; projected NEC = average MFI of the no extract control (experimental value) x the % reduction in signal intensity; i.e. the signal intensity one would expect if the sediment extract alone were added to the beads. Extract = amended extract, experimental value; Projected Extract = average MFI from the amended sediment extract x (1/the % reduction in signal intensity); i.e. the expected hybridization signal assuming no quenching of or interference reading the Alexa-532 reporter. Interval "a" is the difference between average MFI in the amended extract and purified RNA hybridization control, and represents the decrease in hybridization signal due to the combined effects of fluorescence quenching and hybridization interference. Interval "b" is the difference between average MFI in the amended extract and projected extract, and is an estimate of the effect of environmental background on hybridization efficiency alone. Interval "c" is the difference between projected no extract control and projected extract , and represents an estimate of uncertainty associated with the effect of the environmental background on Alexa-532 reporting efficiency. The order of the bead assignments (from left to right) in the legend is in the same order of appearance as in the bar graph. These data show that it is possible to quantify the extent of inhibition due to residual humic acids and other soluble environmental constituents. In some environmental samples (e.g. FRC sediment), it may also be possible to directly extract sediment, hybridize and analyze the array without additional sample cleanup.

Single-tube RNA Purification, Fragmentation and Labeling

Figure 5. On-bead rRNA purification, fragmentation and labeling eliminates fluorescence quenchers at the point of bead array detection. A surface soil and FRC sediment were extracted in a guanidium-based lysis buffer. Various amounts of total RNA from *Geobacter chapellei* were amended into the unpurified extract and processed through a single-column purification, fragmentation and labeling chemistry prior to automated capture and detection on Luminex particles. Four replicate extractions and hybridizations were performed. Minimum detection limits under these conditions were approximately 5 micrograms of total RNA; importantly, on-bead rRNA purification prior to bead array hybridization eliminated any residual fluorescence quenchers from both the soil and FRC sediment extracts. Total RNA recovery from the universal chemistry was only 10-50%. Methods for automation are under continued development. The proposed chemistry and fluidic scheme should be extensible to any environmental sample.



Validating a FeRB and SRB array

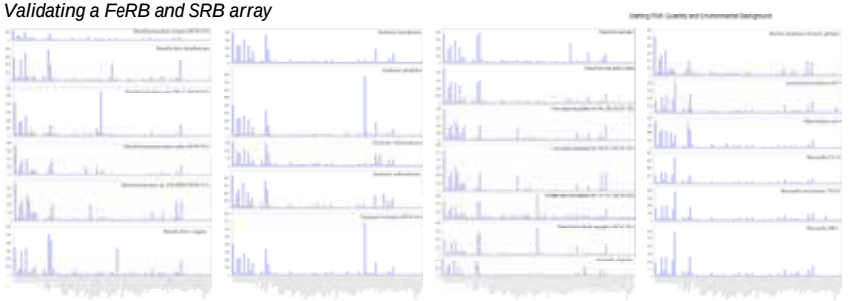


Figure 6. An 85-probe bead array was designed and constructed, containing 17 hierarchical and 68 species-specific probes in 17 genera of known iron- and sulfate-reducing bacteria. Total RNA was isolated from 24 metal- and sulfate-reducing bacteria available in the Subsurface Microbiology Culture Collection and hybridized for 2 hr at 45C in a pH 5 SSC hybridization buffer. Three replicate hybridizations were performed per isolate. Clearly, not all probes on the array are species-specific; some probes (e.g. the *Geothrix* and *Geovibrio* probes) cross-react with most targets, regardless of sequence identity to target rRNA. Hierarchical probes were also non-specific, possibly due to a failure (on our part) to design and include appropriate chaperone probes for the hierarchical target regions. On the other hand, several sets of probes were reasonably specific to the genus level, including those for *Geobacter*, *Desulfotomaculum* and *Desulfovibrio*. The implications of non-specific and unpredictable cross-hybridization are the topic of a NABIR breakout session.

Taking the Array to the Field

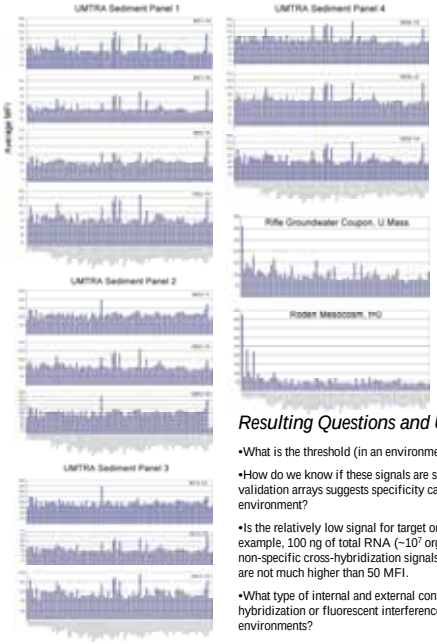


Figure 7. Five gram sediment samples from the UMTRA site (Phil Long, PI), a Rifle groundwater coupon (Todd Anderson, U. Mass) and a sediment mesocosm (E. Roden) were extracted in guanidium lysis buffer by ballistic disintegration in 0.5 g aliquots. Supernatants were pooled and ethanol precipitated at room temperature. Total nucleic acids were purified, fragmented and labeled with the single-tube chemistry (Figure 5). Nucleic acids were eluted in 10 mM sodium bicarbonate buffer (pH 8.5) at 90C. Most samples generated ~1000 ng of purified, fragmented and labeled nucleic acid (range 132 ng - 8000 ng), which was desalted in a BioRad P-30 column. Total nucleic acids were reconstituted in 14 microliters H_2O containing excess salmon sperm DNA. Proximal chaperones were added to the mix, heat denatured, and added to concentrated hybridization buffer to achieve a 2X SSC, 0.02% Tween-20 pH 5 solution in 33 microliters total volume. Nucleic acids were hybridized for 2 hrs at 45C in test tubes. Each graph represents a single 5 g aliquot of environmental sample.

These data are preliminary, and it is premature to make ecological conclusions. What we observe, however, are the following:

- Two major patterns were observed at the UMTRA site, differing primarily in the responses of the *Desulfobulbus* group probe; *Desulfotomaculum alkaliphilum*; *Desulfomicrobium norvegicum*; *Desulfovibrio desulfuricans* and *D. sulfodismutans*; *Desulfuromonas acetoxidans*; and *Desulfuromonas bakii*. The physiology of these organisms is at least consistent with UMTRA site characteristics and geochemistry.
- Interesting probe responses at the Rifle site include the substantial Universal 907 probe signal (relative to UMTRA sediments); relatively strong signals for the *Desulfovibrio* and *Desulfobacter* group probes that are consistent with increased signal at the *Desulfobacter postgatei*, and *Desulfovibrio vulgaris* probes; and relatively high signals for *Geobacter bremensis* and *Geobacter* SBD-1. The relatively high *Geobacter* signals are consistent with clone library and PCR data for the Rifle site.
- Profiles are qualitatively different between the three research areas.
- The response of the universal 907 probe was substantially different between the three research areas.

Resulting Questions and Uncertainty

- What is the threshold (in an environmental sample) where signal becomes ecologically important or meaningful?
- How do we know if these signals are specific or not given an uncharacterized environmental background? The validation arrays suggests specificity can be achieved, but how precise do we need to be when moving into the environment?
- Is the relatively low signal for target organisms related to their abundance in the environmental sample? For example, 100 ng of total RNA (~10^7 organisms) on the validation array generated average MFIs from 200-400, and non-specific cross-hybridization signals at ~25-50 MFI. Most of the signals observed in the environmental samples are not much higher than 50 MFI.
- What type of internal and external controls will be required to determine thresholds, quantify the extent of cross-hybridization or fluorescent interference, and quantify the abundance of microorganisms (or RNA) in uncharacterized environments?

Project Conclusions

1. Tunable surface chemistry enhances direct rRNA detection limits and hybridization kinetics on microparticles.
2. Chaperone probes and RNA fragmentation are necessary for hybridization specificity, but cross-hybridization cannot be predicted based upon primary nucleic acid sequence alone.
3. Integrated sample preparation and detection chemistries are now automated in a fluidic system that is compatible with in-field, autonomous monitoring applications.
4. The ecological application and interpretation of rRNA arrays still requires the development of internal and external standards and statistical models to account for unpredictable cross-hybridization in uncharacterized environmental samples.

Acknowledgements

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