**TO:** **Texas Hazardous Waste Research Center**

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**SUBJECT:** Annual Progress Report

**PROJECT NUMBER:** 513UTA0037H

**PROJECT TITLE:** Innovating on Well Biofouling Remediation: A Bacteriophage Cocktail Approach

**PROJECT PERIOD:** September 1, 2013 – July 15, 2015

**DATE:** September 15, 2014

## Project Description

The overall goal of this project is to evaluate the applicability of a bacteriophage-based therapy to control biofouling due to microbial iron oxidation in water supply wells. Biofouling can significantly reduce well yield and often requires several rehabilitation techniques to restore productivity. Groundwaters rich in ferrous iron ($Fe^{2+}$) are particularly susceptible to biofouling. Since $Fe^{2+}$ oxidation yields such a low amount of energy, iron-oxidizing bacteria (IOB) must convert large quantities of soluble $Fe^{2+}$ to insoluble ferric iron ($Fe^{3+}$) to sustain growth (Taylor et al., 1997).

## Objectives

1. Characterize biofilm samples collected from municipal groundwater supply wells that have biofouling issues due to biotic iron oxidation.
2. Isolate bacteriophage that are capable of infecting the predominant types of bacteria identified from the biofilm analyses.
3. Test the efficacy of a bacteriophage cocktail against mixed-species bacterial cultures.

## Methodology

This research is divided into three tasks, mapping to the three objectives. These are described in the following paragraphs.

### Task 1: Analysis of Biofilm Diversity and Culture of Active IOB

In this task, we first are delineating the microbial communities in several biofouled wells. Samples are collected by GSI Water Solutions, Inc. in sterile polypropylene vials and shipped overnight on ice to the University of Texas at Austin. Samples are obtained from drinking water wells with known biofouling issues and a list of the samples processed to date is shown in Table 1.

Samples are aliquoted for DNA extraction and bacteria isolation. Extracted DNA (MoBio PowerWater DNA Isolation kit) is quantified and submitted for Illumina sequencing to identify the members of these communities. Identification is based on the 16S rRNA gene using a 97% similarity cut-off. The data are analyzed by means of the QIIME open-source software (Caporaso et al., 2010) and the National Center for Biotechnology Information (NCBI) database. Beta-diversity analysis is used to compare communities across samples.

**Table 1. Description of well samples.**

| Sample ID | Description | |
|---|---|---|
| Well 6 | Samples from the City of Trousdale | 2014 Water + Biofilm sample |
| Well 7 | | 2014 Water + Biofilm sample |
| Well 8 | | 2014 Water + Biofilm sample |
| Well 9-0 | Samples from the City of Fairview | 2012 Biofilm sample - Not well preserved |
| Well 9-1 | | 2013 Water + Biofilm sample - Chlorinated |
| FH-W9-1 | | 2013 Water sample from hydrant downstream of Well 9 - Chlorinated |
| Well 9-1 EC | | Enrichment culture of Well 9-1 |
| Well 9-2 | | 2014 Water + Biofilm sample |

Next, we are enriching/isolating IOB from the well samples. To enrich for IOB, aliquots from the well samples are inoculated to enrichment media, such as Iron-Peptone (Rodina, 1965), Winodgrasky (Rodina, 1965) and gel-stabilized gradient tubes that simulate the aerobic to anoxic gradient where IOB tend to develop (Figure 1) (Emerson and Merrill, 2005). Additionally, the control strains *Sideroxydans lithotrophicus* (ATCC® 700298), *Sphaerotilus natans* (ATCC® 13338) and *Dechloromonas aromatica* (ATTC® BAA-1848) were acquired from microbiological collections and are maintained according to their specific nutritional requirements (ATCC, 2014).

### Task 2: Bacteriophage Isolation

In this task, bacteriophages capable of infecting the IOB of interest are being isolated from several diverse sources including return activated sludge (Walnut Creek Wastewater Treatment Plant, Austin, TX), untreated urban runoff (Waller Creek, Austin, TX) and water from biofouled wells. Briefly, removal of bacterial contamination and the concentration of the potential bacteriophage are achieved through a series of buffered centrifugation and membrane filtration steps. The resulting liquid sample containing bacteriophage is tested against the IOB isolates from Task 1 and the control strains.

To this end, each IOB isolate is incubated with the bacteriophage suspension in the appropriate medium (Sutherland and Wilkinson, 1965). Bacteriophage capable of infecting the bacterial populations of interest

will replicate, with a concomitant decrease in bacterial titers that can be quantified to monitor the infection process. The bacteriophage-enriched suspensions are purified from bacterial cell debris by centrifugation and/or membrane filtration, and bacteriophage stocks are maintained at 4 $^{o}$C.

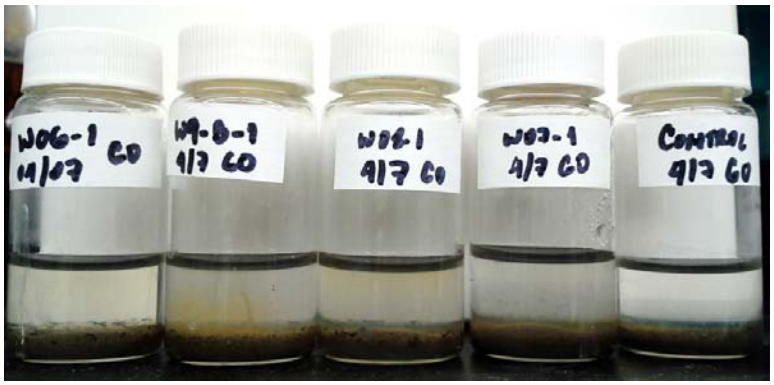


**Figure 1. Gel-stabilized gradient tube cultures.** Gel-stabilized gradient tubes inoculated with samples from biofouled wells. Tubes with IOB-containing samples (W9-B-1; W07-1; W08-1 – described in Table 1) develop a faint orange ring of biogenically produced ferric oxides within the clear (oxic) phase, right above the dark (anoxic, rich in $Fe^{2+}$) phase. Tubes inoculated with non-IOB samples (W06-01 – described in Table 1) remain unchanged as does the un-inoculated control.

### Task 3: Application of Bacteriophage Cocktail in Lab-scale Bioreactors

In this task, the efficacy of the isolated bacteriophage for lysing IOB cells will be assessed. A set of batch experiments will challenge an equal mixture of all IOBs with an equal mixture of the associated bacteriophages, according to Task 2. Additionally, an annular reactor will be used to evaluate the performance of the isolated bacteriophages on IOB biofilms. Each annular reactor will be operated for five to seven days to grow mature biofilms. Biofilm coupons will be removed from the reactor and subjected to bacteriophage treatment. In both cases, quantification of IOB will be performed after 7-10 days using propidium monoazide (PMA)-qPCR, a technique capable of distinguishing intact from compromised cell membranes (Bae and Wuertz, 2009).

## Accomplishments/Problems

As described below, Tasks 1 and 2 are well underway in the project.

In Task 1, the bacterial community analysis shows that IOB are dominant only in samples from Well 7 and Well 9 and that the most abundant genera of IOB are *Crenothrix* and *Gallionella*. As shown in Figure 2, *Crenothrix* dominates in Well 7, and *Gallionella* dominates in Well 9 (Well 9-2-A, Well 9-2-B). With respect to the non-IOB, *Corynebacterium* and *Streptococcus* tend to be the most abundant in samples from Fairview, whereas *Bradyrhizobium* and *Flavobacterium* appear more frequently in samples from Trousdale (Figure 2). All four genera are known for the production extracellular polymeric substances (EPS), a key component in biofilm development.

We will continue to work on the enrichment cultures so that we might isolate indigenous IOB to be used in Task 3. Progress has been made in that the genus *Rhodobacter* was recovered in sample Well-9-1-EC (Figure 2), which suggests that is possible to isolate IOB from these samples even when present in very low concentrations.

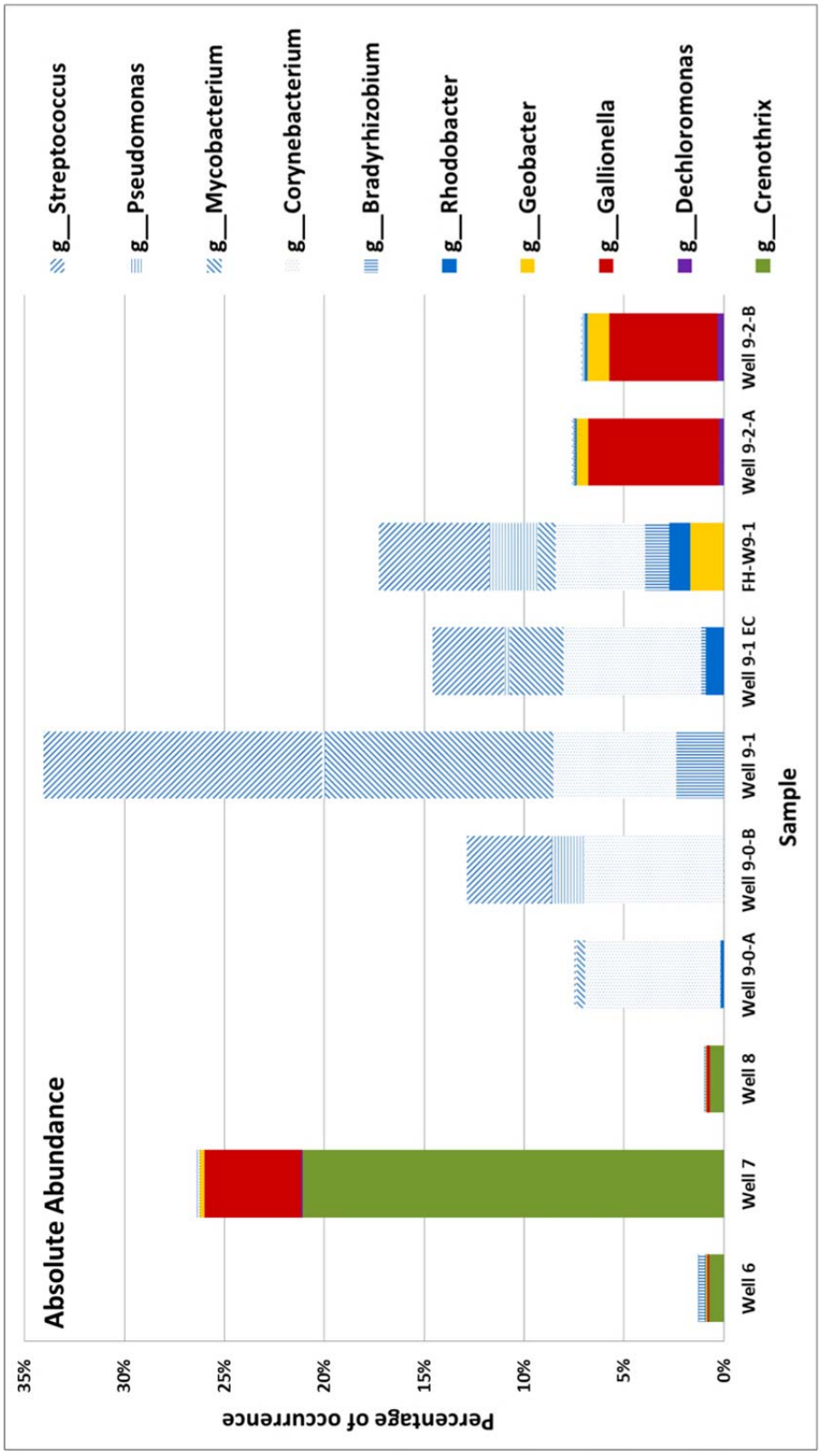


**Figure 2. Absolute abundance of relevant taxa from different well samples.** Patterned fill indicate non-IOB whereas solid fill denote occurrence of IOB taxa. Non-relevant taxa were excluded from the analysis and can be assumed to sum to 100%.

In Task 2, several bacteriophage isolation protocols are being investigated using water from well 9 as source of bacteriophage. The resulting potential bacteriophage suspensions are being tested against *Escherichia coli* (as control for the technique), *Siderooxydans lithotrophicus* (as a control for IOB) and bacterial enrichments from Well 9. So far, only *E. coli*-specific bacteriophages have been extracted, so a

more diverse source, such as return activated sludge will be tested next. We also are moving forward to include enrichments from Well 7 in the assays, as this sample is also dominated by IOB.

## Future Work

In the next year of the project, work on Tasks 1 and 2 will continue for the isolation of indigenous IOB and IOB-specific bacteriophage. Additionally, Task 3 (where a bacteriophage cocktail will be tested for its efficacy against a mixed microbial community) will commence. It is likely that these tests will be performed using a mixed community composed of the four most common IOB found across the samples: *Crenothrix*, *Gallionella*, *Geobacter* and *Rhodobacter*, and possibly some of the most frequent non-IOB, once their importance has been elucidated. If these organisms cannot readily be cultivated or purchased, closely related type strains will be used to accomplish this objective.

## List of Publications and Presentations

Building on the progress in year 1 of the project, a presentation is in preparation. We intend to present at the Molecular Genetics of Bacteria and Bacteriophages Meeting, to take place at the University of Wisconsin - Madison in 2015.

## References


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