U.S. DEPARTMENT OF ENERGY

PNNL-21611

Prepared for the U.S. Department of Energy
under Contract DE-AC05-76RL01830

Effects of Remediation Amendments on Vadose Zone Microorganisms

HM Miller
FA Tilton

August 2012

Pacific Northwest
NATIONAL LABORATORY

Proudly Operated by Battelle Since 1965

DISCLAIMER

PACIFIC NORTHWEST NATIONAL LABORATORY
operated by
BATTELLE
for the
UNITED STATES DEPARTMENT OF ENERGY
under Contract DE-AC05-76RL01830

Printed in the United States of America

Available to DOE and DOE contractors from the
Office of Scientific and Technical Information,
P.O. Box 62, Oak Ridge, TN 37831-0062;
ph: (865) 576-8401
fax: (865) 576-5728
email: reports@adonis.osti.gov

Available to the public from the National Technical Information Service,
U.S. Department of Commerce, 5285 Port Royal Rd., Springfield, VA 22161
ph: (800) 553-6847
fax: (703) 605-6900
email: orders@ntis.fedworld.gov
online ordering: http://www.ntis.gov/ordering.htm

This document was printed on recycled paper.
(9/2003)

PNNL-21611

Effects of Remediation Amendments on Vadose zone Microorganisms

HM Miller
FA Tilton

August 2012

Prepared for the U.S. Department of Energy
under Contract DE-AC05-76RL01830

Pacific Northwest National Laboratory
Richland, Washington 99352

Effects of remediation amendments on vadose zone microorganisms

Hannah Miller, Fred Tilton

Abstract

Surfactant-based foam delivery technology has been studied to remediate Hanford 200 area deep vadose zone sediment. However, the surfactants and remediation amendments have an unknown effect on indigenous subsurface microorganisms. Microbial populations are important factors to consider in remediation efforts due to their potential to alter soil geochemistry. This project focuses on measuring microbial metabolic responses to remediation amendments in batch and column studies using Deep Vadose Zone Sediments. Initial studies of the microbes from Hanford 200 area deep vadose zone sediment showed surfactants sodium dodecyl sulfate (SDS) and cocamidopropyl betaine (CAPB) and remediation amendment calcium polysulfide (CPS) had no affect on microbial growth using Biolog™ Ecoplates. To move towards a more realistic field analog, soil columns were packed with Hanford 200 Area sediment. Once microbial growth in the column was verified by observing growth of the effluent solution on tryptic soy agar plates, remedial surfactants were injected into the columns, and the resulting metabolic diversity was measured. Results suggest surfactant sodium dodecyl sulfate (SDS) stimulates microbial growth. The soil columns were also visualized using X-ray microtomography to inspect soil packing and possibly probe for evidence of biofilms. Overall, Biolog™ Ecoplates provide a rapid assay to predict effects of remediation amendments on Hanford 200 area deep vadose zone microorganisms.

1. Introduction

The Hanford 200 area soil is contaminated due to the the production of uranium and plutonium, historical releases to the land, and leaking underground waste storate tanks[1]. Pacific Northwest National Laboratory (PNNL) specializes in environmental subsurface science and has studied delivering chemical reducing agents to the subsurface via foam delivery technology (FDT). FDT utilizes surfactant-based foams to overcome preferential flow paths in the subsurface and fill pore spaces with remediation amendments[2]. However, these and other surfactants and reducing agents have an unknown effect on indigenous subsurface microorganisms and there is no standarized testing to assess these interactions. This is important because microbes have potential

[1] Oregon Department of Energy's Nuclear Safety Division. July 2009. Hanford Cleanup: The First 20 Years. Salem, OR. Grant #DE-FG06-R14780. 178 p. Available from: Oregon Department of Energy, http://oregon.gov/ENERGY/. Sponsored by U.S. Department of Energy.

[2] Kathryn L. Bailey, Fred Tilton, Danielle P. Jansik, Sarina J. Ergas, Matthew J. Marshall, Ann L. Miracle, and Dawn M. Wellman. "Growth inhibition and stimulation of *Shewanella oneidensis* MR-1 by surfactants and calcium polysulfide," Ecotoxicology and Environmental Safety. 80, 195-202 (2012).

to drive either the inhibition or enhancement of amendments effectivness toward its intended target, thereby directly and indirectly playing a significant role in subsurface remediation.

Biolog™ Ecoplates are a rapid and inexpensive tool capable of studying the effect of remediation amendments and surfactants on micoorganisms. These 96 well microplates contain 31 distinct carbon sources in triplicate. Microbes are extracted from the soil and allowed to incubate in the wells. When cells begin to utilize the carbon source and respire, a tetrazolium dye that is included with the carbon sources turns purple. This color change can be quantified using a spectrometer and indicates the average metabolism of the microorganisms. The advantage of using Biolog™ Ecoplates is they can produce results in weeks, instead of close to a year to sequence DNA, and they also give information about the entire microbial consortia in the soil instead of individual species. Although this technique does not give specific gene or protein information, it is an effective initial assay to probe the subsurface. To fully understand the soil, a combination of techniques including Biolog plates and sequencing needs to be implemented, but this study is focused on getting initial data regarding remediation amendments effects on microbial growth.[3]

This study focuses on measuring microbial metabolic responses of Hanford 200 area deep vadose zone microorganisms to remediation amendments using Biolog™ Ecoplates. After initial soil batch experiments were conducted, column methodology was developed and remediation amendments were exposed to column-packed sediment to observe their effects on microbial growth. X-ray microfocus tomography (XMT) was also used to visualize and analyze column packing and investigate technologies to visualize the formation of bio-films in standarized soil columns.

2. Materials and methods

2.1 Hanford 200 Deep Vadose Zone Sediment

The soil samples were originally collected from the BC Cribs and Trenches area of the Hanford 200 area on 3/23/2010, and then were split into smaller sections according to depth below the ground surface, and frozen at -80°C. All samples were from well C7536, spanning from 6.1 meters to 21.5 meters below the ground surface. The Columbia River Sediment and Sagebrush sediment were collected from sites near Pacific Northwest National Laboratory in sterile centrifuge tubes and then immediately inoculated into the Biolog plates. The autoclaved sediment was run at 121°C for 30 minutes.

2.2 Surfactants and remediation amendments

[3] Garland, J. L. and A. L. Mills. (1991) "Classification and characterization of heterotrophic microbial communities on the basis of patterns of community-level-sole-carbon-source utilization." *Applied and Environmental Microbiology* 57: 2351-2359.

Surfactants SDS and CAPB were donated by Stepan Co. (Northfield, IL). SDS, as CS-330 (28% w/w) solution, is an anionic surfactant commonly found in household detergents and cleansers. CAPB is a non-ionic surfactant solution (30% w/w) commonly used in cosmetics. CPS (CaS_5; 29% w/w; VGS Co., Bloomington, MN) is a remedial chemical capable of reducing Cr(VI) and Tc(VII).[4]

2.3 Microbe Extraction from Soil onto Biolog™ Ecoplates

For un-stimulated treatments, 5 grams of sediment were allowed to incubate for 24 hours at 25°C with 10mL of 50 and 250µM SDS, 20 and 50 mM CAPB, or 1.45mM CPS in a solution made with 1% PBS. After incubation, the treatments were diluted to 1:1000 and 100µL was inoculated into the 96-well Biolog™ Ecoplates. PBS was added to the 3 blank wells. For stimulated treatments, 5 grams of sediment were mixed with 50mL 20% TSB and incubated for 48 hours at 30°C. The solution was then centrifuged at 3750 rpg, the supernatant liquid was decanted, and the soil was re-suspended in 10mL of amended solutions as described for un-stimulated treatments. Control samples were made of sediment taken from similar depth intervals not treated wtih surfactants or CPS or TSB. [5]

The Biolog™ Ecoplates were checked every other day for approximately 14 days with the PowerWave HT Microplate Spectrophotometer (BioTek, Inc., Winooski, VT) at 590 nm. The number of substrates utilized (community metabolic diversity; CMD) was also counted.

2.4 Data Analysis

The average well color development (AWCD) was calculated based off optical density data. This value gives the average respiration of carbon sources by the microbial community. It is calculated as follows:

$$AWCD = \sum \frac{OD_{well} - OD_{control}}{31},$$

where OD_{well} is the absorbance of the test well and $OD_{control}$ is the absorbance of the averaged blank wells.[6] The normalized AWCD divided each value by the initial AWCD of the plates at time 0. Metabolic rate was calculated by taking the slope of the natural log of the normalized AWCD during the period of exponential growth (which was determined to be 0-6 days).

2.5 Soil Columns

All work was performed in a laminar flow hood with sterilized equipment. The column specifications were: 10 cm length by 0.765 cm diameter, 3/4" outer diameter of column, with a

[4] Kathryn L. Bailey, Fred Tilton, Danielle P. Jansik, Sarina J. Ergas, Matthew J. Marshall, Ann L. Miracle, and Dawn M. Wellman. "Growth inhibition and stimulation of *Shewanella oneidensis* MR-1 by surfactants and calcium polysulfide," Ecotoxicology and Environmental Safety. 80, 195-202 (2012).

[5] Kathryn L. Bailey (private communication).

[6] J.L. Garland. "Analytical approaches to the characterization of samples of microbial communities using patterns of potential C source utilization," Soil Biol. Biochem. 28(2), 213 (1996).

volume of 4.60 cm^3 and pore volume of 1.61cm^3. The sediments were packed via slurry packing. This method involved mixing 15 grams of sediment with 10 grams of liquid media (either water or 20% TSB), centrifuging for 5 minutes at 3750 rpg , then decanting the supernatant liquid. The saturated soil solution was then mixed and funneled into the soil column, periodically packing it.

Once the columns were packed, they were incubated in the laminar flow hood at 25°C in ring stands for 2 days, then injected with either water or a stimulant. Liquids were injected into the base of the columns via sterile syringe in the inlet tubing, and the resulting liquid pushed through the column exited through tubing coming out of the top of the column. This outlet tubing emptied into a sterile centrifuge tube so the effluent could be collected. The rate of the fluid injection was approximately 5mL per 24 hours. Once injected with liquid, the columns incubated for 2 more days in the same conditions, then were injected with 5mL of CPS, SDS, or CAPB following the same protocol as described above. The soil was sampled before being placed into the columns and after the runs were completed, the columns were deconstructed and the soil was analyzed on Biolog plates. Before deconstructing the columns, the laminar flow hood was wiped down with an ethanol solution to kill any microbes, and the equipment was also sterilized by coating with ethanol.

2.6 Data Analysis

Soil extraction experiments were conducted in triplicate, with error bars showing standard deviation. Two-way analysis of variance (ANOVA) tests (with post-hoc Bonferroni multiple comparison) were used to determine statistical significance. Soil column data is preliminary, focused mainly on method development, with only one data point.

2.7 X-ray microfocus tomography (XMT)

Some soil columns were imaged using the XMT at the Environmental Molecular Sciences Laboratory (EMSL) in collaboration with KC Carroll. This nondestructive three-dimensional imaging technique was used to probe for the existence of biofilms and also used to image sediment packing in the columns. This state-of-the-art imaging technique is rapidly developing as a useful tool for visualizing soil-microbe environments by probing their internal structure.The technique is being developed to characterize pore geometry in porous media and identify biofilm formation. It faces limitations in distinguishing between air-water interfaces and the spatial resolution is limited by the distance from the X-ray source and the type of X-ray source itself. Contrast agents are often times necessary to distiniguish between biofilms and water due to their similar densities. The XMT is a small-scale and more accessible alternative to obtaining beam-time at a synchrotron, and its full capabalities are still being explored. [7]

3. Results

3.1 Soil Extraction

[7]D. Wildenschild, J.W. Hopmans, C.M.P. Vaz, M.L. Rivers, D. Rikard, and B.S.B. Christensen. "Using X-ray computed tomography in hydrology: systems, resolutions, and limitations," J. Hydrology. 267, 285-297 (2002).

The Hanford 200 area deep vadose zone (DVZ) sediments were initially compared to different soil types, namely Columbia River Sediment (CRS) and Sagebrush (SB). These results, as shown in Figure 1, display that CRS has higher AWCD than SB, and stimulated-DVZ has higher AWCD than un-stimulated-DVZ. The autoclaved sediments, both stimulated and un-stimulated, were meant to serve as controls; however, they displayed AWCD similar to that of the SB and stimulated-DVZ.

Sediment was sampled from 6.80-8.17, 11.68-12.81 and 18.45-19.98 meters below ground surface (bgs). These depths were chosen in order to get data over the entire range of the borehole. Figure 2 shows the normalized AWCD for un-stimulated sediment exposed to remediation amendments. The highest depth, 6.80-8.17 m bgs (A), shows no statistical difference, however, the unstimulated control in both 11.68-12.81 (B) and 18.45-19.98 m bgs (C) is significant. The normalized AWCD of the controls at the aforementioned depths is lower than those of experiments exposed to SDS, CPS, and CAPB, indicated that the remediation amendments and surfactants stimulated microbial metabolism. There is not a clear trend indicating which remediation amendment or surfactant is the best stimulant, but there is a general trend of increased metabolism.

Figure 2 also displays the normalized AWCD for sediment stimulated with 20% TSB and exposed to remediation amendments is not statistically significant compared to that a stimulated control (soil not treated with any amendment). Tests were performed in triplicate and standard devation was calculated.

The average metabolic rate was also computed during the region of exponential growth. Figure 3 shows the results for both stimulated and unstimulated treatments. Table 1 shows the associated p-values based off a two-way analysis of variance (ANOVA) statistical test.

3.2 Column Studies

Most of the work on the column studies was developing methodology; thus, the full range of tests performed for the soil extractions was not possible. Figure 4 shows results from column soil before being exposed to 250uM SDS and after exposure. The SDS seems to be stimulating the metabolic activity for the sediment already stimulated with 20% TSB, however, it is not affecting metabolic activity in the unstimulated sediment. Additionally, the effluent from the columns was tested for metabolic activity. The effluent did contain microorganisms, displaying that some of the microbes in the sediment were washing off the soil as liquid was pushed through the column.

Overall, the column studies require more time incubating, which better simulates the slow processes that occur in the subsurface. There are many different parameters to change in column studies, but this project focused on small scale columns due to limited sediment samples and time constraints.

3.3 XMT Images

Figure 5 shows some images of a soil column obtained from XMT.

4. Discussion

4.1 Soil Extraction

Sediment was autoclaved to act as a control because the high heat and pressure of an autoclave is meant to kill all microorganisms present; however, the autoclaved sediment still contained microbial growth. It was initally presumed the autoclave being used was faulty. A spore test was conducted and the autoclave passed, so another autoclave was used and gave the same results. Sterile technique and equipment was used throughout; thererefore, it is hypothesized that there is an extremely resilient microorganisms present in the Hanford 200 area DVZ sediment. Previous DNA sequencing of soil in the area has revealed the presence of *Deinococcus radiodurans*, known to be radioresistant, resistant to desicattion, and overall hardy[8]. Thus, there is the possibility the surviving micoorganism is a strain of *D. radiodurans*, but further DNA sequencing has to be conducted to confirm this hypothesis.

All depths studied in this experiment showed metabolic activity using a diverse array of substrates. This infers that subsurface activities will undoubtedly come in contact with potentially active microbial communities, which has the potential to alter the efficacy of remedial treatments. Microorganisms have the potential to reduce or oxidize metals, thus affecting their subsurface mobility. Due to no statistical difference between the controls and the sediment exposed to surfactants and remediation amendments in batch studies, these amendments have no affect on microbial growth at the tested depths. However, this does not mean that some microorganisms are not being affected by the amendments because Biolog plates only give information about the big picture ecology and the entire microbial consortia. Another factor to consider is that these studies can only extract and culture an extremely tiny fraction of the entire microbial community existing in the soil, giving an incomplete view of all microorganisms present. Thus, to fully understand if specific species are thriving or dying due to these amendments, DNA sequencing, as one example would need to be conducted in concert with Biolog data to get a more comprehensive image of the subsurface microbial community.

Overall, Biolog plates were shown to be a rapid and useful screening assay and they provided initial data about remediation affects on microbial metabolism. However, the soil batch experiments do not realistically simulate the deep vadose zone environment. The actual soil is more compact with less exposed surface area, as well as more isolated from air.

4.2 Column Studies

These studies focused mainly on method development; therefore, there is less data available than the soil extraction experiments. The longer incubation time and slower flow of nutrients creates a

[8] Fred Tilton (private communication)

more realistic vadose zone environment because processes in the subsurface occur at a slow rate. The Hanford 200 area is located in a sagebrush desert environment; thus, it receives limited rain and organic nutrients[9]. From the preliminary experiment, the remediation amendment SDS stimulates metabolic activity in some sediment, but it is not consistent. More tests need to be conducted to get definitive results.

The column effluent contained metabolizing microbes, which displayed that pushing liquids through the column did strip some microbes off the soil. However, once the soil column was deconstructed and the sediment analyzed, it still contained microorganisms, showing that liquid addition to the columns was not completely displacing the microbes present. This is significant because it could lead to a build-up of microorganisms in the soil at the point where liquid collects, perhaps clogging remediation amendment flow.

4.3 XMT imaging

There was not adequate time to analyze the XMT images using sophisticated software, but the inital images were still useful. They displayed heterogeneity in the columns with non-uniform pore sizes present in vadose zone sediments. It was difficult to discern if there were biofilms present due to the low density contrast between water and biofilms.

Some of the sediment from the column was sampled on a TSA plate to determine if the x-rays killed all of the microorganisms living in the column. Surprisingly, there was growth on the plate, indicating the presence of a radiation resistant microorganism, possibly *Deinoccoccus radiodurans*, the same microbe that resisted autoclaving.[10]However, DNA sequencing has to be conducted on the sample to test this hypothesis.

5. Conclusions

Overall, metabolic signatures provide a tool to predict effects of remediation amendments on subsurface microorganisms. There is a clear potential for microbial metabolism at all depths of 200 area Deep Vadose Zone sediments. This is significant because these sediments have not been extensively studied and microbial activity has the potential to either negatively effect remediation treatments or increase their effectivenes. The remediation amendments did not hinder metabolic activity of Hanford vadose zone microorganisms, and in some instances their addition promoted metabolism. The addition of column studies creates a better field analog for microbial-remediation interactions and further work needs to be conducted to investigate if

---

[9] Oregon Department of Energy's Nuclear Safety Division. July 2009. Hanford Cleanup: The First 20 Years. Salem, OR. Grant #DE-FG06-R14780. 178 p. Available from: Oregon Department of Energy, http://oregon.gov/ENERGY/. Sponsored by U.S. Department of Energy.

[10] K. Takemoto, I. Narumi, H. Namba, and H. Kihara. "X-ray Imaging of Radioresistant *Deinococcus radiodurans*." J. of Physics: *Conf. Ser*.. 186, No. 1. (2009).

results differ from soil extraction studies. This project exhibited the resilience of deep vadose zone microorganisms and gave the unique opportunity to study Hanford microorganisms.

In the larger context of remediation and monitoring, this project provides a rapid and inexpensive protocol for monitoring microbial activity in the subsurface. These methods can be utilized for initial microbial testing of the subsurface, as well as for monitoring real-time changes in microbial metabolism. Even though this project focused on the Hanford 200 area, the techniques used can be applied to contaminated sites throughout the world.

6. Acknowledgements

I would like to thank my mentor Fred Tilton, Jim Szecsody for generously allowing me to use his soil columns, KC Carroll for allowing me to use his machine time on the XMT. Also, the scientists in RTL 520, Amoret Bunn, Dawn Wellman, Elsa Cordova, Danielle Jansik, and Hope Lee aided my project, along with my fellow interns Vincent Leray, Shauna Maple, Robert Lapierre, and Sean Ayer.

7. Figures

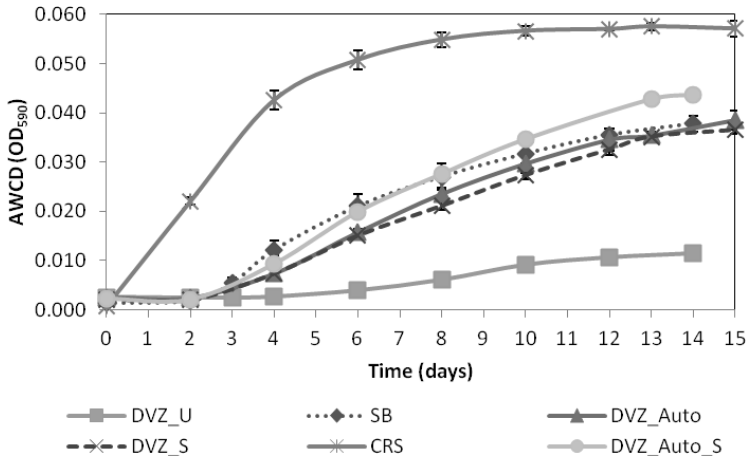

Figure 1. Average well color development (AWCD) of the microbial communities isolated from deep vadose zone (DVZ) sediment (10.04-10.58 meters bgs), the Columbia River, a sagebrush field, and autoclaved deep vadose zone sediment. Both the DVZ and Autoclaved DVZ sediments were stimulated with 20% Tryptic soy broth (TSB) and unstimulated.

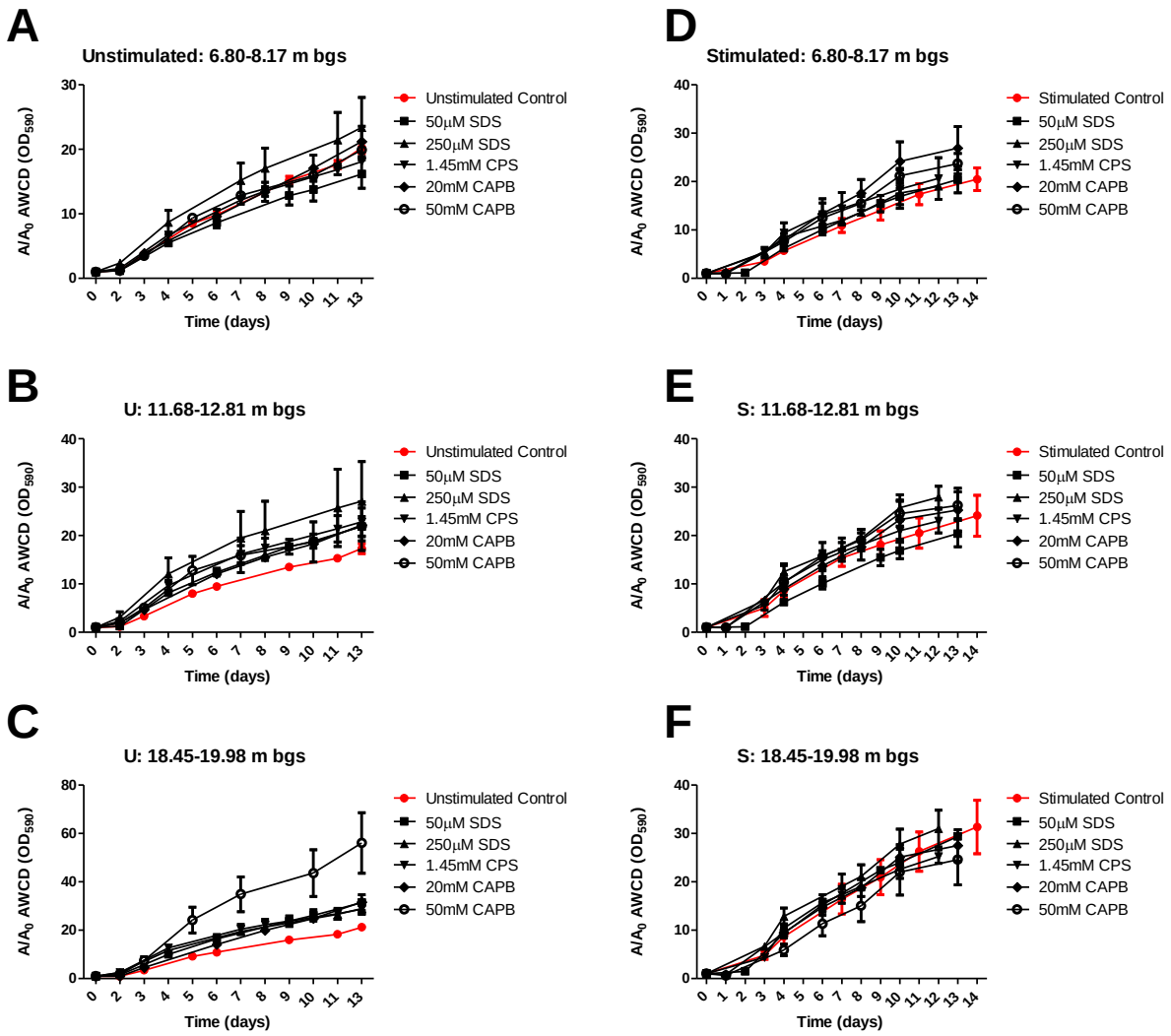

Figure 2. The normalized AWCD over time from EcoPlates inoculated with un-stimulated microbial communities isolated from well C7536 (A) indicates depth profile 6.80-8.17 meters below ground surface (bgs), (B) 11.68-12.81 m bgs, and (C) 18.45-19.98 m bgs. Experiments were run in triplicate. 20% TSB stimulated microbial communities represented at same depths in (D), (E), and (F). Experiments were run in triplicate.

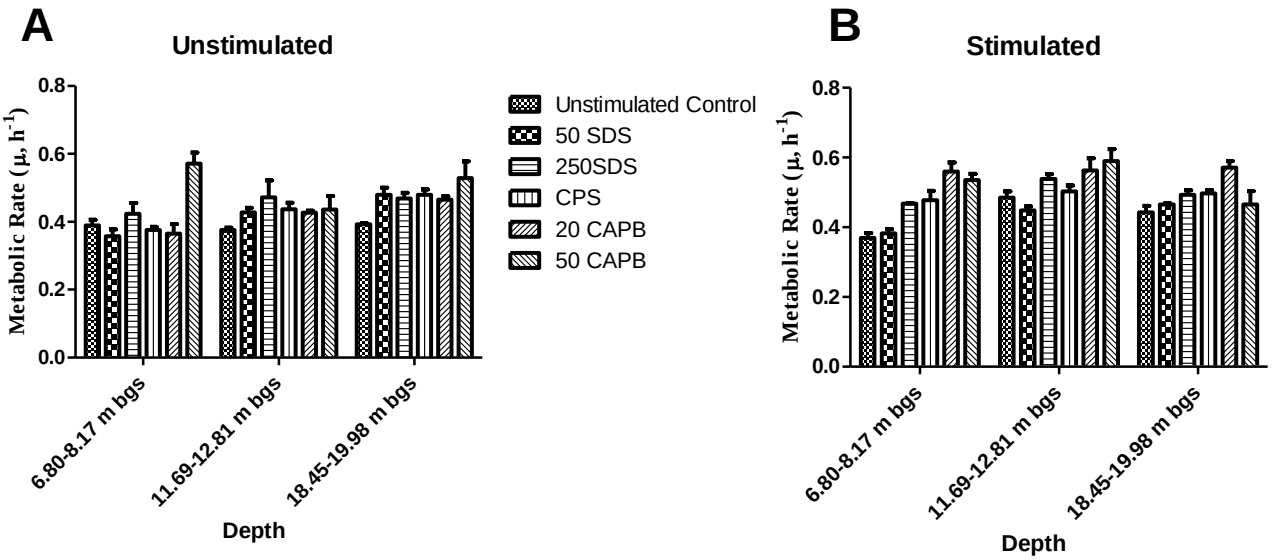

Figure 3. Average metabolic rate for unstimulated (A) and stimulated (B) sediment and treatments.

Two-way ANOVA		50 µmSDS	250µmSDS	1.45 mM CPS	20mM CAPB	50mM CAPB
Unstimulated	**6.80-8.17 m bgs**	-	P < 0.05	P < 0.05	P < 0.0001	P < 0.0001
	11.69-12.81 m bgs	-	-	-	-	P < 0.05
	18.45-19.98 m bgs	-	-	-	P < 0.01	-
Stimulated	**6.80-8.17 m bgs**	-	-	-	-	P < 0.0001
	11.69-12.81 m bgs	-	P < 0.001	-	-	-
	18.45-19.98 m bgs	P < 0.01	P < 0.05	P < 0.01	P < 0.05	P < 0.0001

Table 1. Two-way ANOVA results, comparing treatments (both unstimulated and stimulated) to various treatments with remediation amendments. "-" indicates no statistical significance, p-values indicate there is a significant difference between the control and treatment metabolic rate.

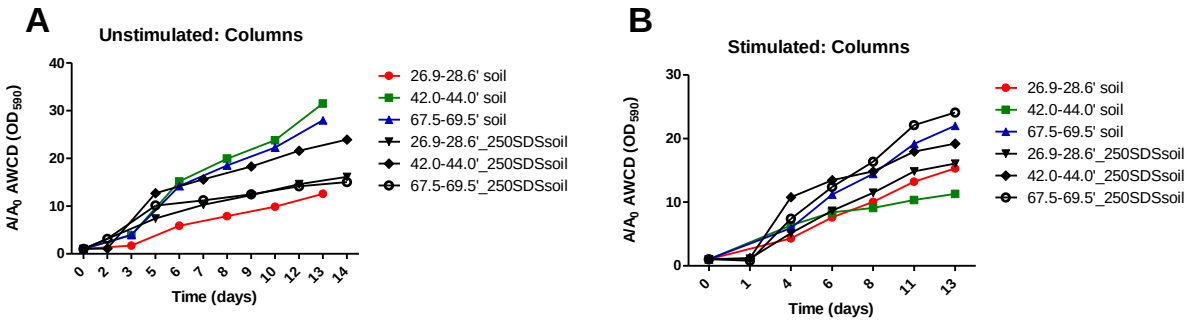

Figure 4. Unstimulated (A) and stimulated (B) sediment from various depth profiles, slurry packed in soil columns. Initial soil was sampled before put into columns, treated with 5mL of 250uM SDS, then allowed to incubate for 2 days. Soil column was then deconstructed and placed on Biolog plates, getting 250um SDS soil results.

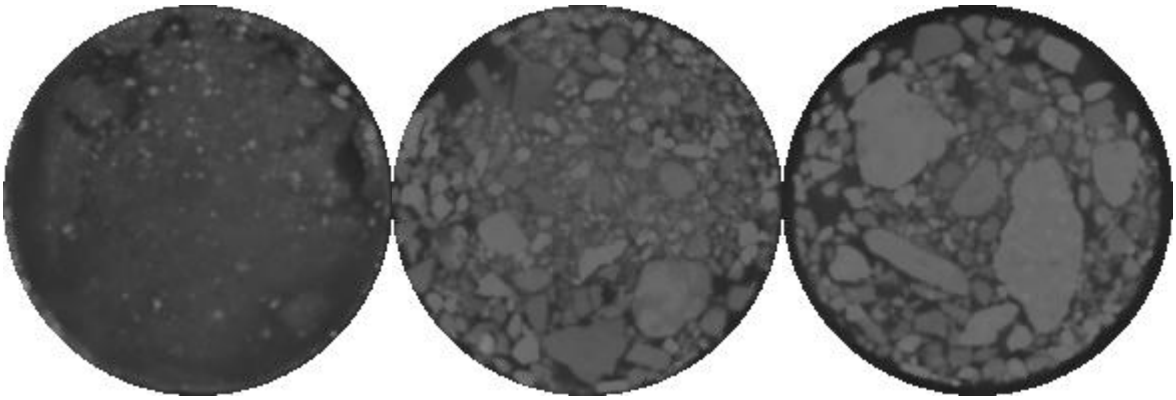

Figure 5. XMT images of soil column. The image on the left is from the top of the column, and the middle and right images are taken from various middle sections of the column.

8. References

[1] Oregon Department of Energy's Nuclear Safety Division. July 2009. Hanford Cleanup: The First 20 Years. Salem, OR. Grant #DE-FG06-R14780. 178 p. Available from: Oregon Department of Energy, http://oregon.gov/ENERGY/. Sponsored by U.S. Department of Energy.

[2] Kathryn L. Bailey, Fred Tilton, Danielle P. Jansik, Sarina J. Ergas, Matthew J. Marshall, Ann L. Miracle, and Dawn M. Wellman. "Growth inhibition and stimulation of *Shewanella oneidensis* MR-1 by surfactants and calcium polysulfide," Ecotoxicology and Environmental Safety. 80, 195-202 (2012).

[3] Garland, J. L. and A. L. Mills. "Classification and characterization of heterotrophic microbial communities on the basis of patterns of community-level-sole-carbon-source utilization," Applied and Environmental Microbiology. 57, 2351-2359 (1991).

[4] Kathryn L. Bailey, Fred Tilton, Danielle P. Jansik, Sarina J. Ergas, Matthew J. Marshall, Ann L. Miracle, and Dawn M. Wellman. "Growth inhibition and stimulation of *Shewanella oneidensis* MR-1 by surfactants and calcium polysulfide," Ecotoxicology and Environmental Safety. 80, 195-202 (2012).

[5] Kathryn L. Bailey (private communication).

[6] J.L. Garland. "Analytical approaches to the characterization of samples of microbial communities using patterns of potential C source utilization," Soil Biol. Biochem. 28(2), 213 (1996).

[7]D. Wildenschild, J.W. Hopmans, C.M.P. Vaz, M.L. Rivers, D. Rikard, and B.S.B. Christensen. "Using X-ray computed tomography in hydrology: systems, resolutions, and limitations," J. Hydrology. 267, 285-297 (2002).

[8] Fred Tilton (private communication)

[9] Oregon Department of Energy's Nuclear Safety Division. July 2009. Hanford Cleanup: The First 20 Years. Salem, OR. Grant #DE-FG06-R14780. 178 p. Available from: Oregon Department of Energy, http://oregon.gov/ENERGY/. Sponsored by U.S. Department of Energy.

[10] K. Takemoto, I. Narumi, H. Namba, and H. Kihara. "X-ray Imaging of Radioresistant *Deinococcus radiodurans*," J. of Physics: *Conf. Ser*.. 186, No. 1. (2009).