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Heavy metal influence on bioremediation

Scientists sought to isolate bacterial strains from the Nakhodka oil spill along the coast of Japan, a spill that had been undergoing bioremediation for 5 years. Scientists used the 16S rDNA sequence analysis to accomplish this. In addition, they examined the heavy metal suite, hoping to shed light on how heavy metals affect bioremediation; some heavy metals are thought to be essential for oil-degrading microorganisms while others are known to be toxic.

An oil spill can cause not only hydrocarbon contamination but also heavy metal contamination. "Many metallic compounds occur in petroleum in extremely small concentrations, such as inorganic salts, metal soaps, and organic metal-complex compounds... Major metal contaminants in petroleum oil commonly include aluminum (Al), sodium (Na), iron (Fe), nickel (Ni) and vanadium (V), with frequently smaller amounts of magnesium (Mg), tin (Sn), barium (Ba), zinc (Zn), molybdenum (Mo), calcium (Ca), copper (Cu), manganese (Mn), lead (Pb), chromium (Cr), and titanium (Ti). Metals in crude oil may enhance or inhibit oil degradation by microorganisms. Some metals may be toxic, while many are essential trace nutrients for microbial growth. Long-term exposure to heavy metals (Zn, Cu, Ni) has been found to alter microbial structure, as assessed from total soil PLFA (phospholipids fatty acids) profiles. Heavy metal contamination of soil has been shown to reduce fungal biomass and alter the composition of the fungal community" (Chaerun and others, 2004).

Sampling and analysis

Researchers obtained samples of heavy oil, sand and seawater contaminated by the Nakhodka oil spill,

and then isolated and characterized seven strains of bacteria capable of degrading hydrocarbons. Scientists performed sequence analyses of 16S ribosomal DNA (16S rDNA) on the isolates. They used an ED-XRF (Energy Dispersive-X-Ray-Fluorescence) analyzer to estimate the metallic content of heavy oil.

"To determine whether the presence of metals in heavy oil affects the isolated strains of alkane-degrading bacteria, four representative strains (selected based on sampling sites) as well as *P. aeruginosa* PAO1 and *E. coli* K-12 MG 1655 (as controls) were tested for their ability to grow on nutrient broth (NB) medium plus 1 g/l yeast extract and 0.85 percent w/v NaCl (except for *E. coli* K-12, which was grown on LB medium) amended with various concentrations (0.01, 0.1, 0.5, 1, 2 and 5 and 10 ppm) of metals (Ti, Cr, Mn, Fe, Co, Ni, Cu, Zn, Pb, Cd and V)... After one day of incubation, the bacterial growth response was determined immediately by the increase in optical density (OD₆₀₀)" (Chaerun and others, 2004).

Results

Data analyses indicated that the genus *Pseudomonas* was found in all samples and dominated the bacterial communities of the oil spill-contaminated coastal site. "This organism appears to be well adapted to hydrocarbon biodegradation in seashores and survival during the bioremediation process. Hydrocarbon-degrading communities that adapt to the Nakhodka oil spill consist mainly of alkane hydrocarbons. To date, the majority of the PAH-degrading bacteria reported belong to the genus *Pseudomonas*. Microbial communities within contaminated ecosystems tend to be dominated by those organisms capable of utilizing and/or surviving toxic contamination. As a result, genus *Pseudomonas* obtained as the most predominant bacteria in the bacterial community of contaminated sites seems to be capable of utilizing the Nakhodka oil

spill as well. The bacterial diversity may be influenced by the complexity of chemical mixtures present and the exposure. *Pseudomonas* strain may be tolerant and resilient to the metabolic products that are formed during degradation/bioremediation of the Nakhodka oil spill, so that this species was predominant among the bacterial communities at all three coastal sites and Nakhodka tanker" (Chaerun and others, 2004).

Seven isolates grew well on the spill's aliphatic hydrocarbons (C₁₄-C₂₈), heavy oil, alkane hydrocarbons such as hexane, octane, paraffin (a mixture of alkanes) and pristane (a branched alkane C₁₉), but had limited ability to grow on aromatic hydrocarbons (PAHs). "Bacteria that are capable of degrading alkanes commonly do not degrade PAHs, although metabolic pathways for both types of compounds are compatible. Also, degradation of PAHs occurs much more efficiently by means of mixed microbial consortia than by single strains" (Chaerun and others, 2004).

Chromium and other heavy metals

Chromium was the most abundant heavy metal in the oil samples, and apparently bacterial strains adapted to the Cr toxicity, even at concentrations of 5 to 10 ppm. "The ability of some strains to grow in the presence of somewhat higher concentrations of some heavy metals (V, Cr, Ni, Zn, Cd and Pb) indicated that bacterial acclimation periods ensued in metal-containing heavy oil, resulting from induction of a protein required for metal precipitation or detoxification or a genetic change or selection of a tolerant or detoxifying bacterial species or population" (Chaerun and others, 2004).

Researchers also noted that the low concentrations of heavy metals served as trace elements (micronutrients) for microbial growth in the biodegradation of some alkanes. It is possible that several anaerobic microbial species participated in bioremediation of both heavy

metals and organic pollutants, either individually or in combination. "It is also possible, however, that the indigenous microorganisms... (i.e. alkane-degrading bacteria) which are being exposed to metals may also become resistant to or capable of transforming and detoxifying heavy metals... The presence of miscellaneous pollutants in any of the oil spills may present extreme challenges to the maintenance of a phylogenetically and functionally diverse microbial community... It is suggested that both heavy metals and hydrocarbons would influence the composition of the microbial community of which genus *Pseudomonas* was a dominant member... Genus *Pseudomonas* is apparently well-equipped to cope with aliphatic hydrocarbon toxicity and may, therefore, be a primary catalyst for aliphatic hydrocarbon degradation..." (Chaerun and others, 2004).

Conclusions

Researchers concluded the following:

- after long-term (5 years) exposure to hydrocarbons and heavy metals, three alkane-degrading bacteria were isolated: *Bacillus* spp, *Pseudomonas* sp., and *Paracoccus* spp.; they may tolerate not only hydrocarbons but also heavy metals
- *Pseudomonas* species was the dominant organism during the 5-year bioremediation study and may have been associated with hydrocarbon degradation
- the bacterial community may have played a key role in hydrocarbon degradation
- elements indigenous to the spill included Si, S, Ti, Cr, Ni, Cu and Zn
- oil spills can cause not only hydrocarbon but also heavy metal (i.e., Cr) contamination

Reference

Chaerun, S.K., Tazaki, K., Asada, R. and K. Kogure, "Alkane-Degrading Bacteria and Heavy Metals from the Nakhodka Oil Spill-Polluted Seashores in the Sea of Japan After Five Years of Bioremediation," *The Science Reports of Kanazawa University*, Vol. 49, No. 1 and 2, 2004.

UTTU thanks Dr. Chaerun, kchaerun@earth.s.kanazawa-u.ac.jp, for his help on this article.



Comparison of MTBE and VOC risk in California

Williams and others (2004) evaluated MTBE and other VOC (volatile organic compound) data to determine the extent of public drinking water contamination in California and to compare the relative cancer risk from these compounds. This effort involved gathering and analyzing data on the following:

- overall detection frequency of 28 VOCs with a California maximum contaminant level (MCL) from 1985 to 2002
- annual detection frequencies of 21 VOCs found in at least 50 drinking water sources over the last 18 years
- California and federal drinking water MCLs (maximum contaminant levels) and cancer potency factor values for selected VOCs
- detected concentrations of 21 VOCs in drinking water over the last 5 years
- relative cancer risk of 12 VOCs with California potency values based on estimated drinking water exposures via ingestion, inhalation and dermal routes

Study authors obtained the California Department of

Health Services (CDHS) drinking water monitoring database, which contains 20 to 30 years of sampling data for more than 450 constituents. From this database, they selected 28 VOCs that had a primary MCL established by the State of California. "These VOCs tended to be the most frequently reported in the drinking water monitoring database. However, information about the sampling strategy for specific geographic locations or individual VOCs is not available" (Williams and others, 2004).

"For those VOCs that currently have a California-established cancer potency factor, potential drinking water exposures and associated cancer risks were estimated based on the source detection data to provide perspective on the relative health risks posed by these VOCs to water consumers. The lifetime average daily dose of each VOC was estimated by aggregating doses from three exposure pathways: ingestion of contaminated water, inhalation of volatilized vapors in the home (e.g., showering), and dermal contact during household water-related activities. (e.g., bathing). These calculations were based on standard risk assessment formulas for evaluating multimedia exposures, which are presented in detail in our previous publications" (Williams and others, 2004).

Most VOC data are based on sampling from more than 15,000 individual public drinking water sources. MTBE and a few other VOCs are based on sampling from more than 11,000 drinking water sources. The analysis includes all data from active and inactive public water supply wells as well as treated and untreated sources, but excludes data from monitoring and agricultural/irrigation wells. The study did not include any data from private wells.

Basing their findings on data analyses, study authors determined that a variety of VOCs, including MTBE, were present at some level in public drinking water

sources over the last decade. They concluded that

- “most of these chemicals are detected only infrequently and at relatively low concentrations
- the most frequently detected VOCs were chloroform, tetrachloroethylene (PCE) and trichloroethylene (TCE), which were found in about 9 to 15 percent of all sampled drinking water sources; these same chemicals were found to have the highest mean detected concentrations over the last 5 years, ranging from 13 to 15 $\mu\text{g}/\text{l}$
- “MTBE was detected in only about 1 percent of all sampled drinking water sources in California over the last 5 years, at an average concentration of 3 $\mu\text{g}/\text{l}$ (median) or 6 $\mu\text{g}/\text{l}$ (mean)”
- detection frequencies of chloroform and MTBE in drinking water appear to be lower in California than in the northeast and mid-Atlantic regions, which may be related to the sensitivity of analytical technique or inclusion of nondrinking water sources in the analyses
- “relative to the other VOCs evaluated, MTBE has the lowest estimated California cancer potency value, and was found to pose one of the least cancer risks from household exposure to contaminated drinking water”

Although study authors cite several important limitations of their analyses, they conclude that “the current evaluation suggests that the expressed concerns about MTBE in California may be unfounded, given the contrasting lack of concern over other drinking water contaminants, which pose a much greater health risk to water consumers. In fact, MTBE was generally found in only about 1 percent of all sampled drinking water sources over time, and of those sources found to contain MTBE, the overwhelming majority of detected concentrations were below California’s health-based standard. Estimated and unadjusted and adjusted

cancer risks for MTBE were also significantly less than that for other VOCs evaluated. Not only do these findings show how estimated health risks differ among common drinking water contaminants, but they also illustrate how the comparative risk assessment approach can be used to identify environmental and public health hazards or to prioritize risk management efforts” (Williams and others, 2004).

Reference

Williams, P.R.D., Benton, L., and P.J. Sheenan, “The Risk of MTBE Relative to Other VOCs in Public Drinking Water in California,” *Risk Analysis*, Vol. 24, No. 3, 2004; <http://www.blackwellpublishing.com/journal.asp?ref=0272-4332>

UTTU thanks Dr. Williams, pwilliams@chemrisk.com, for her help on this article.



Oxygen-enhanced bioremediation in a gasoline-contaminated aquifer

That the hydrologic and geochemical environment can strongly influence the success of a dissolved oxygen remedial project is not new. “There is growing concern, however, that simply adding oxygen to anoxic, fuel-contaminated groundwater does not guarantee enhanced aerobic biodegradation and successful site cleanup” (Landmeyer and Bradley, 2003). Microbiological conditions, too, must be investigated, which scientists did at a site in South Carolina.

Study site

The study site was a gasoline station in Beaufort, where a gasoline and MTBE release was detected in 1991.

Site characteristics are as follows:

- sediments of Pleistocene to Holocene age are well-sorted, eolian sands and therefore impart little to no preferential transverse groundwater flow
- depth to water ranged from 3.9 m near the former UST source to 2.7 to 0.6 m downgradient near a concrete-lined ditch
- rainwater provides about 30-45 cm/yr of aquifer recharge
- an MTBE plume extends 200 m downgradient, defined by a 100- $\mu\text{g}/\text{l}$ isoconcentration line
- a benzene plume with similar geometry to the MTBE plume exists, but it is of an order of magnitude lower concentration than the MTBE plume
- groundwater flow is rapid (minimum seepage velocity of 33 m/yr)
- MTBE and benzene biodegradation is low (-0.0001 per day) under these anoxic methanogenic conditions

Groundwater sampling

The study site had more than 40 monitoring wells, while seven additional wells were drilled. Groundwater samples were tested for

- dissolved oxygen
- dissolved hydrogen (H_2)
- ferrous iron (Fe(II))
- hydrogen sulfide (HS^-)
- methane (CH_4)
- aerobic heterotrophic and facultative anaerobic bacteria
- BTEX- and MTBE-degrading bacteria

Oxygen addition, monitoring and evaluation

Researchers added oxygen to the aquifer in the form of magnesium peroxide (MgO_2), using a high-pressure grout pump and a direct-push rig. "The MgO_2 increases levels of DO in the aquifer according to the equation, $MgO_2 + H_2O \rightarrow \frac{1}{2} O_2 + Mg(OH)_2$. Laboratory biodegradation microcosm studies of fuel compounds such as benzene and toluene, previously performed using aquifer sediments collected from the site, confirmed that the addition of oxygen increased the rate of mineralization relative to anaerobic conditions" (Landmeyer and Bradley, 2003). Oxygen was injected into two areas: the former UST source area and a downgradient area. Approximately 490 kg of ORC was added, or about 0.77 kg/m².

Field workers measured changes in dissolved oxygen concentration using a colorimetric method and in-situ sensors before and after oxygen addition. The DO (dissolved oxygen) sensors also contained additional sensors that measured specific conductance, temperature and pH.

Results

Prior to oxygen addition in the source area, the subsurface environment was anoxic. Even 10 years after source removal, benzene and toluene concentrations were above the 1 mg/l concentration, and higher than MTBE concentrations. This resulted from

- much lower rates of dissolution for the less soluble benzene and toluene
- higher sorption coefficients for these compounds compared to MTBE
- lack of complete removal of all contaminated sediments from the site

Prior to oxygen injection, there was a high concentration of ferrous iron (Fe^{2+}) "indicating the presence of microbially mediated iron-reducing conditions, and the production of Fe^{2+} was greater than the rate of Fe^{2+} oxidation." Sulfate concentrations were low here also. "These low and persistent sulfate concentrations reflected the prior depletion of sulfate due to microbially mediated sulfate-reducing conditions and the lack of infiltration of sulfate-rich rainwater (typical of many coastal areas) due to the pavement. Concentrations of hydrogen sulfide (H_2S) remained high (average 1.5 mg/l) between 1994-2001 before oxygen injection, due to the absence of oxidation from recharge... After oxygen was injected into the former UST source area in May 2001, however, concentrations of many of these reduced, inorganic species changed." Most importantly, however, the concentration of DO remained low and unchanged.

In the downgradient area where oxygen addition also occurred, following oxygen injection, MTBE and DO concentrations changed substantially:

- MTBE concentrations decreased from 19.6 to 2.66 mg/l within 50 days, and concentrations were soon near non-detect
- benzene concentration decreased from 2.8 to 0.393 mg/l
- toluene concentrations decreased from 8 to 1.5 mg/l

Assuming decreases were due to aerobic biodegradation, benzene and toluene first-order rate constants estimated from the field were, respectively, -0.0018/day and -0.019/day. Experiments from aquifer sediment microcosms using radio-labeled toluene had showed similar results.

Researchers also noted that in well two in the downgradient area during a rainfall event, "DO concentrations increased from < 0.2 mg/l to

about 8 mg/l in a shallow sensor; conversely, DO concentrations remained low (< 0.2 mg/l) and unchanged at the depth of the sensor in well 1, less than 1 m deeper than the shallow sensor (water levels in both shallow and deep wells remained unchanged during this monitoring period). Ambient, anoxic redox conditions returned rapidly in well 2 after each rainfall event ended, suggesting complete consumption of rainfall-added oxygen. Moreover, an in-situ DO sensor placed in well LB-EX-7 in the former UST source area did not exhibit any changes in DO concentrations during rainfall events" (Landmeyer and Bradley, 2003). DO decreases to ambient, low levels following rainfall at about the same rate for several wells suggested that "a biologically mediated process may be responsible for the rapid depletion in DO, rather than abiotic aquifer oxygen demand or advection of dissolved oxygen away from the wells... In each case of the three rainfall events observed, the conservative properties returned to background, pre-rainfall conditions at a different, and substantially slower rate compared to DO. Moreover, the rapid decrease in DO delivered by recharge events clearly depicts that aerobic microorganisms are capable of existence in aquifer systems rendered anoxic by gasoline contamination."

"These results extend the findings of previous studies which have shown that the atmosphere and unsaturated zone act as a source of oxygen for diffusive transport to the water table" (Landmeyer and Bradley, 2003). The depth of rainfall-induced oxygen penetration into a contaminated aquifer is a critical factor in evaluating the success of oxygen addition to enhance bioremediation.

Conclusions

Landmeyer and Bradley (2004) concluded

- groundwater collected and sampled from the former UST source area showed

- low numbers of both aerobic and facultative anaerobic microorganisms
- lower numbers of bacteria that could grow on BTEX or MTBE
- “groundwater in the downgradient plume area that received natural DO via recharge was characterized by higher numbers of both aerobic and facultative anaerobic bacteria”

“Our results suggest that the lack of recharge to groundwater in the paved UST source area led to a much larger geochemical sink for dissolved oxygen compared to groundwater in the unpaved area. This may help explain the relatively common need for multiple oxygen injections to achieve remedial goals as reported by many state UST program managers. Fortunately, the hydrologic, geochemical and microbiologic properties that can affect the outcome of a proposed oxygen addition can be readily measured. Such additional site characterization would complement the current practice at some sites of assessing the abiotic oxygen demands of the contaminated aquifer... Moreover, it may be possible to inject alternative oxidizing compounds to satisfy the abiotic oxygen demand prior to oxygen injection to enhance bioremediation. What remains to be adequately quantified at this time, however, is the length of the “lag time”, after oxygen addition in paved UST source areas, for the aquifer to become dominated by aerobic gasoline-degrading bacteria. Knowledge of the lag time would be useful in determining if additional remedial approaches, such as bioaugmentation, would be required to achieve site remediation” (Landmeyer and Bradley, 2003).

Reference

Landmeyer J.E. and P.M. Bradley, “Effect of Hydrologic and Geochemical Conditions on Oxygen-Enhanced Bioremediation in a Gasoline-Contaminated Aquifer,”

Bioremediation Journal, Vol. 7, No. 3-4, 2003; <http://journalsonline.tandf.co.uk>

UTTU thanks Dr. James Landmeyer, jlandmey@usgs.gov, for his help on this article.



Ryegrass and alfalfa effect on soil microbe population

Kirk and others (2005) recently investigated the microbial community of the rhizosphere of two plants to determine if “the microbial community structure and functional diversity in a petroleum-contaminated soil changed with the presence of plants” (Kirk and others, 2005).

Previous work has demonstrated increased pollutant degradation in planted versus non-planted systems.

“The plant rhizosphere can support a microbial community of several orders of magnitude higher than bulk soil... Plants also influence the structure of microbial communities through the release of root exudates and by providing surfaces for colonization.”

Basing their analysis on previous research showing phytoremediation potential, researchers studied the rhizosphere of the following:

- perennial ryegrass, *Lolium perenne*
- alfalfa, *Medicago sativa L.*
- mixed perennial ryegrass and alfalfa

The perennial ryegrass is a monocot with a fibrous root system, and alfalfa is a dicot with a tap root system. Kirk and others (2005) hypothesized that “the presence of plants would increase the numerical and functional diversity and alter the bacterial community structure in the rhizosphere and that this effect was dependent on plant species.”

“Plants increase the microbial numbers in the

rhizosphere, a phenomenon termed the rhizosphere effect. In phytoremediation, it is not known if this increase in microbial numbers is responsible for the increased degradation of contaminants or if plants are selectively increasing certain populations of microorganisms.”

Researchers created four microcosms with three replicates, as follows:

- perennial ryegrass
- alfalfa
- perennial ryegrass with alfalfa
- bulk soil (no plants)

Sampling procedures

For samples, scientists removed 1 to 2 grams of soil and placed the soil in an Erlenmeyer flask with 45 ml of sterile 0.1 percent sodium pyrophosphate (pH adjusted to 7) and 4 grams of sterile 3-mm diameter glass beads. Flasks were shaken for one hour at 140 rpm. For all subsequent sampling times, bulk soil was sampled using the above technique.”

Scientists removed plants from the soil and shook them to dislodge excess soil. “Roots and shoots were separated and weighed in sterile pre-weighed aluminum weighing dishes” and placed separately into Erlenmeyer flasks with the pyrophosphate solution and beads and shook at 140 rpm for 1 hour. “Roots were aseptically removed from the solution, blotted dry and weighed. The difference in root weight from the pre-and post-shaking was considered to be the amount of wet rhizosphere soil from which the microbial populations were extracted.” In addition, scientists measured soil moisture content, dry phytomass, soil pH and conductivity.

Microbial counts

Methods used to enumerate microbes were

- drop plate method of Cassidy for bacteria (2000, in Kirk and others, 2005)
- malt extract agar with chloramphenicol and Rose Bengal for total fungi enumeration
- method of Oudot (1993 in Kirk and others, 2005) for petroleum-degrading fungi
- the MPN (most probable number) to estimate numbers of aromatic and aliphatic hydrocarbon degrading bacteria present in soil samples

In addition

- Eco-Bilog plates were used to assess substrate utilization patterns for metabolic activity
- denaturing gradient gel electrophoresis (DGGE) was used for bacterial community analysis

Results

Kirk and others (2005) ascertained that by using more than one method to study the microbial population, they were able to obtain a better analysis of how plants influenced the numerical, structural and/or functional diversity of microbes in a petroleum-contaminated rhizosphere soil. The plants in this contaminated soil were stressed, which could result "...from direct toxicity of the petroleum hydrocarbons in the soil or a result of the contaminant properties. Often contaminants such as petroleum hydrocarbons alter a soil's physical and chemical properties. Hydrophobic contaminants can change the water/soil interactions that would normally occur, thereby potentially affecting oxygen transfer, available water uptake and nutrient mobility" (Kirk and others, 2005).

Researchers found, after seven weeks of incubation, that compared to bulk soil without plants:

- rhizosphere alternation occurred 233 times more with perennial ryegrass than with culturable heterotrophic bacteria and 37 times more with petroleum-degrading bacteria
- alfalfa increased the number of both culturable, aerobic heterotrophic and petroleum degrading bacteria by 5 and 8 times respectively

The effect was greater for the perennial ryegrass due largely to the ryegrass root morphology.

Other conclusions:

- both plant species increased the degrading population, but specific groups of degraders were not uniform throughout the experiment, and this might have been a sampling artifact
- as the rhizosphere matures, plants may select a more distinct group of microbes
- more research is required to determine if perennial ryegrass non-selectively increases, and alfalfa selectively increases the petroleum-degrading bacterial community in the rhizosphere
- total heterotrophic and petroleum-degrading fungal populations did not differ between plant treatments and bulk soil, although the number of fungi did increase significantly in all treatments
- fungi in the rhizosphere could be 3- to 200-fold higher in the plants versus the bulk soil, and this could be dependant on the soil, plant and sampling method
- the petroleum product used in this experiment resembled in composition a diesel fuel, and unsurprisingly, plants and soils contained "a large number of aerobic, heterotrophic bacteria"
- microbial populations did seem to be differentiating plant species
- results from DGGE (denaturing gradient gel electrophoresis) analysis suggested the plants

altered the composition of the microbial community

- alfalfa may have had "a greater influence on the composition of the dominant rhizosphere bacterial population" than the ryegrass/alfalfa mixture
- "it is possible that abundant species are present whose DNA was not easily amplified by the universal primers; it is therefore possible that a small change could reflect a significant change in the bacterial community"
- "plants can alter rhizosphere microbial populations and increase contaminant degradation or immobilization"
- "plants could selectively increase a degrading population in their rhizosphere by altering exudation, or could simply cause a non-specific increase in microbial numbers some of which may be degraders, or could have some other mechanism of protection against the toxic compound"
- some plants "when exposed to different contaminants, selectively enriched catabolic genotypes of microorganisms living within the rhizosphere"

Kirk and others (2005) conclude that "perennial ryegrass and alfalfa may utilize different mechanisms to facilitate phytoremediation. Perennial ryegrass seems to support a general increase in microbial activity and numbers in the rhizosphere... Alfalfa... seems to specifically increase the number of microorganisms capable of degrading more complex hydrocarbons." Researchers recommend a longer study with additional replicates to gain more knowledge of root exudates and how plants alter microbial populations.

Reference

Kirk, J.L., Klironomos, J.N., Lee, H. and J.T. Trevors, "The Effects of Perennial Ryegrass and Alfalfa on Microbial Abundance and Diversity in Petroleum-Contaminated Soil," *Environmental Pollution*, Vol. 133, 2005; <http://www.elsevier.com/locate/envpol>.

UTTU thanks Dr. Lee, hlee@uoguelph.ca, for his help on this article.



Potential use of stable isotope probing for bioremediation

Scientists have recently applied stable isotope probing (SIP) for identifying microorganisms involved in some biogeochemical transformations important in global cycling. "The approach has enormous potential for identifying microorganisms responsible for the degradation of natural and anthropogenic xenobiotic compounds in industrial remediation systems as well as in the natural environment" (Manefield and others, 2004). Following is a brief summary of five experimental SIP-related techniques.

The first SIP investigation, published in 1998, dealt with stable isotope-labeled methane ($^{13}\text{CH}_4$). The purpose of the investigation was to identify microorganisms responsible for the oxidation of methane in a freshwater environment. Investigators pulsed the stable isotope-labeled methane into the sediment microbial community and this effectively labeled the polar lipid-derived fatty acids (PLFAs). "Subsequently, PLFAs were extracted, separated and analyzed for ^{13}C enrichment by isotope ratio mass spectrometry. Because specific phylogenetic groups produce signature PLFA profiles, the stable isotope enrichment of certain PLFAs revealed which organisms were dominating the use of methane as a carbon source. The labeled PLFAs generated throughout

the duration of the pulse were only produced by species belonging to the genera *Methylobacter* and *Methylomicrobium*, thereby unambiguously linking these organisms with methane oxidation in this particular environment" (Manefield and others, 2004).

SIP can also involve DNA identification. "PCR-based sequence analyses of 16S rDNA genes offer high resolution, culture-independent phylogenetic information... Density centrifugation in CsCl gradients was used to separate 'heavy' (labeled) from 'natural' (unlabelled) DNA, and 16S rDNA clone libraries constructed from 'heavy' DNA were sequenced to obtain the identity of organisms assimilating the ^{13}C labeled substrate used in the study. This technique was used to attribute particular proteobacteria with methanol utilization in an oak forest soil." Thus far, this technique has not been applied to microorganisms responsible for bioremediation. A problem with this technique is that "the DNA must contain 15-20 percent ^{13}C before it can be isolated on the basis of buoyant density. Because DNA synthesis is related to replication and because bacterial replication in most environments is not optimal, the incorporation of stable isotopes from any given substrate into DNA may not be efficient enough for DNA-SIP to be applied."

A technique that eliminates this problem is RNA-SIP. This technique gives the same resolution as DNA-SIP and does not have the limitation listed above.

A group of scientists recently and successfully used fluorescence in-situ hybridization and secondary ion mass spectrometry (FISH-SIMS) to identify archaeal groups that consumed oceanic methane. This technique has not yet been applied to bioremedial situations.

Stable isotope characterization of small-subunit rRNA is another technique awaiting applications in bioremedial and ecological problems. "The primary technical challenge associated with the technique is

the isolation of sufficient quantities of rRNA for isotopic characterization" (Manefield and others, 2004).

Conclusion

Manefield and others (2004) conclude that "DNA and RNA-SIP can reveal the identity of microorganisms dominating the assimilation of a substrate. PLFA-SIP also exposed this information, albeit with less phylogenetic resolution, because all community PLFAs are analyzed for isotope content. FISH-SIMS and SSU-IRMS (small subunit-isotope ratio mass spectrometry) have the potential to provide the same information, but this would require the exhaustive and systematic application of these techniques for each phylogenetic group present in the community. If the question in mind is: Which organism assimilates this substrate? then FISH-SIMS and SSU-IRMS can be applied."

Other considerations include

- which substrate to use
- if stable isotope signatures require enrichment (amplification)
- which elements in the substrate of interest should be labeled
- "how many atoms (and at which positions) of the chosen element in the substrate of interest should be labeled"
- the cost of such a stable isotope labeled compound
- if the pulse should be administered to a community *in-situ* or administered *ex-situ*

Manefield and others (2004) state: "We believe that SIP can serve as an invaluable guide for the characterization of organisms, biochemical pathways, genes and proteins functioning in bioremediation processes *in situ*, and that such knowledge enhances our ability to manipulate these processes for industrial and environmental benefit."

Reference

Manefield, M., Whiteley, A.S. and M.J. Bailey, "What Can Stable Isotope Probing Do for Bioremediation?" *International Biodeterioration & Biodegradation*, Vol. 54, No. 2-3, <http://www.elsevier.com/locate/ibiod>

UTTU thanks Dr. Manefield, mjma@ceh.ac.uk, for his help on this article.



Relationship between iron bioavailability and dissolved hydrogen concentrations

Researchers have found a correlation between some terminal electron-accepting processes (TEAPs) and dissolved hydrogen (H_2) concentration in aquifers. They designed column laboratory experiments to measure H_2 concentrations during iron reduction, which is a TEAP. The columns contained FRC (Field Research Center) soil, *Geobacter sulfurreducens* and a chemical known as AQDS (9,10-anthraquinone-2,6-disulfonic acid). Using flow-through column experiments, scientists wanted to determine "how changes in iron bioavailability during long-term iron reduction and/or due to the addition of AQDS affect iron reduction rates and subsequent steady-state H_2 levels" (Komolos and Jaffe, 2004).

The TEAPs generally associated with bioremediation include iron reduction, denitrification, sulfate reduction and methanogenesis. Because of the importance of the TEAPs in bioremediation, to know which TEAP "is occurring at a particular time and space" can help the remediator "to understand and maximize the bioremediation/biotransformation processes."

Not only is dissolved H_2 an indicator of TEAPs in the subsurface, but in addition, specific H_2 concentrations typify different TEAPs. H_2 concentrations for the

following TEAPs are as follows:

- nitrate, < 0.1 nM
- iron reduction, 0.2-0.8 nM
- sulfate reduction, 1-4 nM
- methanogenesis, 5-15 nM

"The TEAP yielding the most energy (if that electron acceptor is present) proceeds before the TEAPs yielding the least energy because the microbial population for the higher energy-yielding TEAP is able to utilize the H_2 more efficiently, thus driving the H_2 concentration to levels that cannot be utilized by lower energy-yielding TEAPs... [this] assumes that H_2 is the sole electron donor though additional electron donors may be present during the bioremediation of a contaminated aquifer, such as the contaminant itself in the case of petroleum hydrocarbons... Theoretical analysis has shown that steady-state H_2 levels during the simultaneous utilization of H_2 and a carbon source as electron donors will remain relatively constant for a wide range of co-electron donor concentrations, thus providing an explanation as to how H_2 concentration can be used to determine TEAPs in the presence of additional electron donors."

This is what scientists have determined about TEAPs:

- the more energetically variable TEAP out-competes the less energetically favorable TEAPs
- sulfate (> 6-9 mg/l) has been shown to inhibit methanogenesis
- the presence of amorphous Fe(III) inhibits both sulfate reduction and methanogenesis; however, it has also been shown that iron reduction can occur simultaneously with sulfate reduction; Fe bioavailability may be an issue

Fe bioavailability

Scientists know that Fe bioavailability can be a

limiting factor of Fe(III) reduction in soils. "The extent of bioavailability is dependent on the chemical structure of the Fe(III) oxide, ranging from a crystalline structure (less bioavailable) to an amorphous structure (more bioavailable). Though the total amount of Fe(III) is an important parameter in characterizing soil mineralogy, bioavailable Fe(III) is key in predicting the extent of microbial iron reduction in soil. Therefore the bioavailability of the iron could limit iron reduction, and even though iron is present, it cannot be reduced fast enough to outcompete lower energy-yielding TEAPs. Electron shuttling compounds such as AQDS have been shown to increase iron bioavailability in soil...by acting as an electron shuttle between iron surfaces and iron reducing microorganisms that are not in direct contact with the surface-bound iron, which significantly increases the rate of iron reduction in soils" (Komolos and Jaffe, 2004).

Iron bioavailability may change:

- as the more bioavailable fraction of iron is being reduced and less available iron remains
- as Fe(II) sorbs onto the Fe(III) phase, which may block Fe(III) reaction sites
- as the bioavailability of iron is increased in the presence of AQDS or other electron shuttling compounds

Bacterial strain used

Researchers used *G. Sulfurreducens*, which is an "obligate anaerobic bacteria capable of iron reduction with acetate or hydrogen as electron donors." In addition, "microorganisms with 16S rDNA sequences closely related to *G. Sulfurreducens* are the predominant microorganisms in a variety of subsurface environments in which Fe(III) reduction is the predominant TEAP" (Komolos and Jaffe, 2004).

Column experiments and analytical data

The column experiments performed are described in detail in the text, as well as the analytical methods used to sample and measure H₂, Fe(II) concentration (aqueous, sorbed to soil and total extracted iron), acetate, and sulfate.

Of the columns:

- one flow-through column contained soil, only indigenous microorganisms and no AQDS
- another contained soil and AQDS
- two contained soil, *G. sulfurreducens* and a buffered solution with acetate, and one contained AQDS

Researchers performed “all column experiments at 30°C, which is significantly higher than field conditions (15°C or less, for which H₂ levels are reported), and supplied a variety of trace vitamins and minerals that may be limited in an aquifer and which would affect the biokinetic coefficients.”

The columns were sampled, sometimes destructively. One column was run for 500 days.

Results

Researchers found, in the long-term (500 days) column experiment:

- “steady-state H₂ concentrations fluctuated between 0.3 and 25 nM”; this result was achieved when the Fe(II) production rate was constant
- “Fe(II) production was first detected in the column on day 32, and Fe(II) concentrations increased until day 250 when the sorbed Fe(II) concentrations leveled out at 37.3 ± 7.8 μmol/g dry soil”
- “iron reduction appears to have been the dominant TEAP in this system throughout the duration of the

experiment, though the average H₂ concentration (3.9 nM) was higher than reported in the literature for iron-reducing zones (0.2-0.8 nM)”

- iron and sulfate reduction occurred simultaneously
- steady-state H₂ concentrations were similar to the short-term column experiment supplemented with *Geobacter sulfurreducens*

For the columns with and without AQDS:

- addition of AQDS increased iron bioavailability and the rate of iron reduction from 0.16 μmol (g soil/day) to 0.35 μmol (g soil/day)
- “addition of AQDS decreased the steady-state H₂ concentration by half, and doubled iron-reduction rate
- the column supplied with AQDS showed smaller variability in H₂ concentrations
- “Steady-state H₂ concentrations in the column supplied with AQDS were still higher than reported in the literature for iron-reducing conditions”

“More Fe(II) was produced in columns with *G. sulfurreducens* when destructively sampled after 96 days compared to the Fe(II) concentration in the long-term column without *G. sulfurreducens* at the same time point. Though the column inoculated with *G. sulfurreducens* (but not supplied AQDS) reached steady-state conditions faster than the column not supplied with *G. sulfurreducens*, the rate of iron reduction calculated in the column with *G. sulfurreducens* was similar to the rate of iron reduction calculated for the column without *G. sulfurreducens*.

“H₂ concentrations have been shown to be solely dependent upon the physiological parameters of the hydrogen-consuming microorganisms, with the steady-state H₂ concentration inversely proportional to the rate of H₂ consumption.

“The use of H₂ as an indicator of TEAPs assumes that

the availability of the electron donor(s) is limiting. This is the case when H₂ is the sole electron donor and when an additional carbon source is present at relatively low concentration. Electron donor limiting conditions would result in competition between different TEAPs with the more energetically favorable TEAP having an advantage over the less energetically favorable TEAPs. However, “when the electron donor becomes less limiting, more than one TEAP can occur simultaneously.” Results from this study have indicated that H₂ levels during iron reduction depend on iron bioavailability, yet these levels can remain constant over extended periods and be used to monitor changes in iron reduction rates in natural systems.

Reference

Komolos, J. and P.R. Jaffe, “Effect of Iron Bioavailability on Dissolved Hydrogen Concentrations During Microbial Iron Reduction,” *Biodegradation*, Vol. 15, 2004, <http://www.springerlink.com>

UTTU thanks Dr. Komolos, JKomolos@Princeton.edu, and Dr. Jaffe, Jaffe@Princeton.edu, for their help on this article.



The City of Los Angeles Former Gas Station Sites Program, part 4

Part 4 of this series on Los Angeles brownfields provides a brief introduction to the steps often taken to evaluate and remediate a former gas station site. The actual work outlined here should be supervised by an engineer, geologist or other qualified environmental professional. See appendix D (<http://www.lacity.org/ead/labf>) for guidelines on selecting a contractor.

Before undertaking a Phase I and/or Phase II

Assessment, we recommend gathering preliminary information on the site. If a professionally conducted Phase I is not affordable, much of the information normally included in a Phase I can be gathered without professional assistance, although this chapter is not meant as a guide on how to do that.

Step 1: Phase I environmental assessment

The first step in evaluating a potentially contaminated property is to conduct a Phase I environmental site assessment. As used in this context, a Phase I is a search for historical records that document the site's prior activities to identify uses that may have caused contamination and warrant further investigation. Normally it is prepared by an engineering consulting firm or other firm experienced in conducting Phase I investigations. The firm will review all prior building permits, underground tank permits, Sanborn Fire Insurance maps, state and federal lists of contaminated properties, etc. A Phase I also normally includes a visual examination on the property to look for indications of potential contamination. The firm will also make recommendations regarding future work.

Normally, a Phase I is part of an investigation preceding a property purchase or a property loan grant. Properties previously occupied by gas stations may have gasoline and motor oil contamination and may require further investigation. A Phase I of a former gas station site will often identify the following:

- when USTs were installed
- where USTs may be located
- if/when USTs were permitted and/or removed
- whether removal was conducted under a permit
- who owned the property and operated the USTs
- if previous indications of UST leaks/contamination occurred

It is important to review records not only from the current agency regulating USTs but also all past agencies potentially responsible. It may be useful to identify all potentially responsible prior owners with the economic ability to pay for tank removal and contaminant cleanup. Building department records will often include the names of prior owners, but if an owner never took out a permit, his/her name would not be included in these records. Therefore, to identify prior owners, consider asking for a Historical Chain of Title Report including certificate of occupancy permits.

Step 2: Phase II environmental site assessment

A Phase I assessment that discovers potential environmental concerns will recommend that a Phase II be conducted. A workplan for the Phase II should be based on Phase I findings and should confirm UST and piping locations. A Phase II is an onsite investigation that may involve the following activities:

- sampling and analysis of surface and subsurface soils (the latter, using soil borings)
- soil gas surveys
- groundwater sampling and testing (if at shallow depths)
- use of instrumentation to locate USTs

The exact plan for the Phase II depends on a number of site-specific variables, including depth to groundwater, and UST and piping locations.

When planning a Phase II, consider whether to do the Phase II testing first to determine if contamination exists, or combine the tank removal with the soil testing. Conducting both activities at the same time allows the sampling of soil under where the tank was located, which is less costly than soil borings from above. Deciding which approach to use depends on the circumstances. For example, if an agency identifies a

former gas station site for which the owner cannot be located, the agency may want to test for contamination first to see if the site will qualify for EAR Account funding. Identifying a mechanism to allow legal access to the site should be considered. See chapter 6 (<http://www.lacity.org/ead/labf>) on obtaining access.

On the other hand, if the priority is to minimize costs, performing the activities together may be the least expensive. If contamination is discovered, it is sometimes cheaper and less disruptive for the current occupant's business to remove contaminated soil when the hole is open rather than by other means once the hole is filled. The California Petroleum Cleanup Fund will not pay qualifying owners to move USTs but will pay for some testing and contaminant cleanup. We advise site owners to retain a consultant knowledgeable about this fund prior to UST removal so that proper procedures can be followed to enhance the likelihood that cleanup can be conducted as tanks are removed and that costs will be reimbursable by the fund. If the site owner qualifies for fund reimbursement, some consultant costs are covered. See Appendix E (<http://www.lacity.org/ead/labf>) for more information on the California Petroleum UST Fund. Appendix A lists a Web site with UST Fund information.

Site owners should obtain the appropriate UST removal permits and excavation permits, if needed. The UST regulating agency may be required to be onsite to observe tank and/or soil removal and order soil testing. Consultation with local air quality control district personnel is also recommended as the agency may also require a permit.

Site remediation

If contamination is discovered, the agency that regulates USTs will direct further action. Remediation could be as simple as removing and properly disposing of extra dirt at the direction of the UST regulating agency. If

groundwater contamination is discovered, the site will usually be referred to the Regional Water Quality Control Board for further oversight. A site with significant soil contamination may be referred to the Department of Toxic Substances Control (DTSC). If contamination is extensive, one of these agencies will request submission of a workplan for cleanup. The workplan would be prepared by an environmental consulting firm and submitted to the appropriate agency. When the workplan is approved, work begins. Again, owners seeking UST Fund reimbursements should work closely with a consultant expert in the UST Fund to assure that correct procedures are followed to ensure reimbursement for eligible owners.

Each regulating agency sets its own procedures for site remediation. Some procedures that may be required would include

- removal of gasoline floating on groundwater
- excavation of contaminated soil
- removal of soil gases with a vapor extraction system
- removal and treatment of groundwater

In rare cases, the Water Board might require testing to identify the location of groundwater contamination and verify that drinking water sources are not impacted, followed up by quarterly monitoring for a period of time. It may be possible to proceed with development of the site after surface contamination is removed and a cleanup or monitoring plan is approved.

Reference

"Guide to Resolving Environmental and Legal Issues at Abandoned and Underutilized Gas Station Sites," found under *Gas Station Program*, <http://www.lacity.org/ead/labf>.

UTTU thanks Maxine Leichter, mleichte@mailbox.lacity.org, for her help on this article.



Research notes

Bioremediation of diesel-contaminated soil by microorganisms immobilized in polyvinyl alcohol

Cunningham, C.J., Ivshina, I.B., Lozinsky, V.I., Kuyukina, M.S. and J.C. Philp, *International Biodeterioration & Biodegradation*, Vol. 54, 2004; <http://www.elsevier.com/locate/ibiod>

Biodegradable contaminants that have undergone bioaugmentation may persist in the environment because of less-than-optimal environmental conditions such as temperature, pH, electron acceptor availability and biotic factors.

Researchers suggest that delivering bioaugmentation cultures (which usually contain a consortium of microorganisms and a source of inorganic nutrient) "in an immobilized form may offer more complete and/or rapid degradation. Entrapment in a polymer matrix has been the most common method of whole cell immobilization." Benefits of immobilization include the following:

- protection from predators
- protection from extremes of pH
- protection from toxic compounds in the soil
- increased biological stability, including plasmid stability in immobilized cells
- the immobilized matrix may act as a bulking agent, facilitating oxygen transfer

Scientists can use either natural or synthetic hydrogel material for immobilization. The natural materials (agar, agarose, polyacrylamides, k-carrageenan and alginate, for example) have a low mechanical strength and durability; the synthetic materials, including

poly(carbamoyl) sulfonate, polyhydroxyethylmethacrylate, polyacrylamide and polyvinyl alcohol, have greater strength. Scientists selected PVA (polyvinyl alcohol) as their immobilization polymer because the effects of PVA-immobilized microbial cells have been studied. They also incorporated Drizit, an immobilization substratum, into one of the immobilized systems. Drizit has excellent absorbent properties and a good cell-loading capacity.

Thus, Cunningham and others (2004) sought to "produce viable microbial cells within a PVA matrix of sufficient mechanical strength for use in *ex-situ* soil biopile systems. The potential utility of the immobilized systems was compared with biostimulated and bioaugmented systems using bench scale treatment piles with relevant controls. Treatments were also compared with a commercially available bioaugmentation product. This slurry incorporated nutrients and microorganisms with applications including soil bioremediation."

Researchers set up bioaugmentation cultures using composite samples taken from the site at depths of up to 30 cm. Treatment piles "were established on large trays and incubated at 20°C in darkness using a CR/EC/600 environmental chamber." Materials added to the treatment piles, in addition to the microorganisms, included

- woodchips
- PVA
- NPK fertilizer (7 percent nitrogen, 7 percent phosphorus pentoxide (P₂O₅) and 7.5 percent potassium oxide (K₂O))

Workers manually mixed treatment piles once a week to enhance oxygenation and keep the piles moist. Samples were taken periodically and soil pH was monitored. Scientists enumerated hydrocarbon-oxidizing

microorganisms according to the 96-well microtitre plate MPN procedure and they performed phytotoxicity and bioluminescence assays.

Scientists concluded: "The immobilization product could be readily processed to any desired size, acting as a bulking agent in the treatment piles that enhanced aeration and therefore oxygen transfer. However, a size limitation may be set by oxygen transfer within the matrix. The principal enzymes involved in n-alkane and BTEX compound metabolisms are oxygenases, which have an absolute requirement for oxygen. The limited aqueous solubility of oxygen has ramifications for oxygen transfer. The theoretical oxygen demand of n-alkanes is high compared with simple carbohydrates, and with the consequence that systems containing rapidly metabolizing hydrocarbon-oxidizing bacteria can become oxygen-limited. Therefore, good diffusivity of the support matrix is essential in such an application... There remains a question over the diffusivity of n-alkanes into such systems; however, n-alkanes of the range in aged diesel are hydrophobic and have limited water solubility. It is not clear how diffusion of a hydrophobic liquid into a hydrophilic matrix is achieved. If it enters only in the dissolved state, then this mass transfer would limit biodegradation. The presence of bacterial biosurfactants may enhance mass transfer."

The contaminated soil used in this study was contaminated decades ago, thus, how immobilization "would perform in a full-scale treatment, being mechanically turned or mixed using heavy machinery, remains to be determined. It is envisaged that an immobilized system could be tailored to a particular suite of contaminants using the appropriately selected microorganisms; therefore, such a system may also be applicable to a wide variety of bioremediation scenarios" (Cunningham and others, 2004).

UTTU thanks Dr. Philp, Philp@napier.ac.uk, for his help on this article.

GC/MS-SCAN to follow the fate of crude oil components in bioreactors set to remediate contaminated soil

Ivancev-Tumbas, I., Trickovic, J., Karlovic, E., Tamas, Z., Roncevic, S., Dalmacija, B., Petrovic, O. and M. Klasnja, *International Biodeterioration & Biodegradation*, Vol. 54, 2004; <http://www.elsevier.com/locate/ibiod>

Scientists used GC/MS-SCAN (gas chromatography/mass spectrometer) analysis to identify crude oil components from contaminated soils that were set up in bioreactors. Two sets of conditions were run in the bioreactors: one with water continuously circulated and the other with water circulated for only a short period each day. In addition, one bioreactor had an adapted bacterial population, while the other was an untreated control. Bioreactor soil was sampled and analyzed over a one-year period. Added soil pollutants, which came from a refinery dumping area, included crude oil, masout, diesel, middle distillates, heavy distillates and kerosene.

"A better understanding of the chemical and biochemical processes involved in the transformation of the pollutants helps in the design of more efficient bioremediation processes... The purpose of this work was to follow the organic profiles of a soil contaminated with crude oil and oil derivatives by applying GC/MS-SCAN analysis at various stages of a biotreatment process. The treatment was carried out in bioreactors under conditions of continuous or discontinuous recirculation of water. Data provided qualitative information about the components that disappeared and those that were produced in the course of the treatments" (Ivancev-Tumbas and others, 2004).

Scientists identified 42 compounds in the original soil samples. After one year of bioremediation, the number of compounds detected decreased to about one-half of that initially observed. "The number of compounds

detected in soil samples treated in the reactor with a low flow rate varied significantly both in space and time. Thus, the number of compounds at the top of the reactor decreased during the process whereas it increased at the bottom."

Scientists attributed some of the identified compounds to "their gradual release from humic matter, or taking into account that straight-chain hydrocarbons represent components that undergo biological degradation relatively easily... However, no further investigation was carried out to determine more precisely their origin" (Ivancev-Tumbas and others, 2004).

Scientists suggest that further ecotoxicological investigation of contaminant compounds would be prudent as complex mixtures of pollutants are of environmental concern.

UTTU thanks Dr. Ivancev-Tumbas, ivana@ih.ns.ac.yu, for her help on this article.