1. Research objective

The objective of the project at large was to experiment with new methods for bioremediation of carbon tetrachloride plumes in the soils at the Hanford Site in Richland, WA. Traditionally, biostimulation occurs via pumping of liquid nutrient solution into the vadose zone, however an alternate methodology utilizes the introduction of gaseous nutrients, specifically nitrogen, phosphorus and carbon sources. The movement of liquid through the vadose zone tends to disperse contaminant plumes, and/or cause biofouling (excessive microbial growth) in the vicinity of injection wells. Alternatively, gas-phase nutrient introduction yields greater dispersion of molecules and little to no displacement of target plumes. Once vapor-phase molecules solubilize into soil water, they become bioavailable and should thus encourage colonization and degradation. The feasibility of this method of nutrient delivery was studied in an experimental laboratory system, the goal of which was to observe, *in situ,* microbial colonization in response to gaseous nutrient injection. It was hoped that these observations would aid in predictive modeling of microbial behavior in field scale bioremediation.

2. Progress & Implications

The first task was to select appropriate carbon, nitrogen and phosphorus sources to induce growth of target microorganisms. Three organisms were trialed, *P. stuzeri* KC, *P. fluorescens* HK44, and *P. butanovora*, however HK44 was finally selected due to its culturability and lux reporter system. *P. butanovora* was used as a positive control. All experiments were done in batch culture.

In initial carbon source trials, gaseous methane, ethylene, propane, propylene, and butane were pumped into the headspace of sample vials with microbes; however none of these proved successful in stimulating growth in KC or HK44. Next Konte cups of various substrates, including hexane, heptane, acetate, propionate, methylamine and diethylamine, were placed inside vials containing microorganisms. In these experiments positive growth was exhibited only by HK44 on naphthalene vapor, indicating the ability of the microbes to uptake molecules in vapor phase. Because all three organisms had previously displayed growth on liquid acetate, weaker dilutions of acetic acid were trialed, which moderated dehabilitating pH levels and resulted in growth.

The literature (e.g. Bogan, Lahner, et al. 2001) reports the success of using triethyl phosphate (TEP) and nitrous oxide (NO2) in similar gaseous nutrient induction studies. Attempts were made to grow all three organisms on liquid TEP and gaseous NO2, neither of which were successful for any organism. Eventually the goal of finding a vapor-phase phosphorus source was abandoned, and efforts were focused on experimenting with different forms of ammonium, resulting in some successes.

Once carbon and nitrogen sources had been identified in batch culture, experiments in column-type systems tested the feasibility of transporting nutrients in the vapor phase through the soil, and the microbial response as

quantified by growth. Ammonia (in the form of NH4OH) and acetate (as acetic acid) were pumped into the system in the vapor phase, while phosphorus and trace minerals were injected into the system along with microbial inoculation. At intervals, naphthalene was pulsed through the system to induce the *lux* reporter, enabling visualization of colonization patterns.

Light intensity patterns were not as expected, demonstrating "leading edge" patterns of light emission and subsequent "burn-out" moving outwards from the injection point. It appeared that the naphthalene was being trapped by microbes, slowing its diffusion and overloading colonies to the point of extinguishing the "glow". This suggested that vapor-phase naphthalene was an inappropriate indicator for the success of gaseous feeding experiments.

As a result, we next turned to experiments in microcosms which could be readily sacrificed and plated to quantify microbial growth. Although experiments were plagued by technical difficulties, it became apparent that microbes were very sensitive to acetic acid concentrations, whether this is a problem of acidification of the environment or, at the other extreme, insufficient carbon to support growth. Thus a 1-D plug-flow transport model was developed to determine appropriate initial substrate concentrations. Here another issue was illuminated, namely the high solubility of ammonia and its potential inability to travel great enough distances in the vadose zone in field conditions.

These data suggest that while gaseous nutrient injection may be a viable method of stimulating bioremediation, it is quite sensitive to substrate and target organism. The challenge is finding organisms which can utilize substrates which are volatile enough to travel significant distances, while being bioavailable and energetically favorable.

Accompanying work involved the exploration of observing gas movement through the chamber under a variety of conditions. A series of experiments was conducted using a pH indicator (0.005% w/v methyl red) to colormetrically track changes in CO_2 concentration as it moved through an abiotic sand chamber. The chamber was packed both homogenously with fine sand, and with textural inclusions of coarser sands. In some cases liquid flow was turned on, while in other the chamber was held at hydrostatic conditions. In addition to offering some insights into the movement of gases through soils, these data suggested that the chamber could be well-suited to experiments in gaseous nutrient stimulation of microbes, given alternative substrates and organisms.

3. Planned activities

Upcoming work planned should yield additional understanding of observations made up to this point.

Carbon dioxide tracking data will be strengthened by correlation of light transmittance levels to pH values, creating a calibration curve with which we can determine relative CO_2 concentration based on light intensity. Solutions of varying pH containing methyl red (0.005%) dye will be analyzed for light transmittance using a spectrophotometer (Spectronic 21) set to the wavelength of the camera filter used in capturing the images.

Additionally, the modeling software HYDRUS-2D will be used to model the behavior of CO_2 in the chamber system given the various experimental conditions (hydrostatic/flow, homogeneous/textural inclusions).

In order to investigate the cause of the failure of naphthalene to illuminate a wide swath of colonies in the gaseous feeding experiments, we will run small-scale transport tests. Microcosms will be packed with sand, and will be either inoculated or partially saturated. After vapor-phase naphthalene has been pumped into the column for an established period of time, samples at the inlet and outlet will be tested for naphthalene content, using an HP5890 gas chromatograph. Similar work will test for retardation of ammonia and acetate in the presence of simply water or microbes in liquid media.

These experiments should elucidate whether difficulties encountered in gaseous feeding trials were due to problems of transport or to substrate-organism suitability.

Approximate timeline:

July 7	calibration curve completed
July 28	HYDRUS-2D model completed
August 10	Naphthalene tracking completed
September 31	Submit Final Project Report

Bogan, B. W., L. M. Lahner, et al. (2001). "Effects of alkylphosphates and nitrous oxide on microbial degradation of polycyclic aromatic hydrocarbons." Applied and Environmental Microbiology **67**(5): 2139-2144.