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INFLUENCE OF NITRATE CONCENTRATION ON CARBON TETRACHLORIDE TRANSFORMATION BY A DENITRIFYING MICROBIAL CONSORTIUM

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Abstract—A denitrifying consortium capable of transforming carbon tetrachloride (CCl_4) was cultured from an aquifer soil isolated sample from the DOE Hanford Site in southeastern Washington State. Experiments were performed to determine the effects of nitrate concentration on CCl_4 transformation by these microbes. The microorganisms were cultured under two different experimental conditions, both of which gave similar cell growth. Under conditions where both the electron donor (acetate) and electron acceptor (nitrate) were present at all times, little CCl_4 transformation was observed. However, transformation was observed if the nitrate concentration was reduced to a level where it was periodically depleted. These results are discussed in relation to the design of an *in situ* bioremediation system.

Key words—CCl_4 biodegradation, *in situ* bioremediation, denitrification, electron donor, electron acceptor

INTRODUCTION

The Hanford Site, located in southeastern Washington State, is an area of approximately 1500 square km (600 square miles) that was selected in 1943 for producing nuclear materials in support of the United States' effort in World War II. In the 200 West area of this site, plutonium recovery processes discharged carbon tetrachloride (CCl_4)-bearing solutions to liquid waste disposal facilities. A minimum of 637 g of CCl_4 was disposed to the subsurface, primarily between 1955 and 1973, along with co-contaminants such as tributyl phosphate; lard oil; cadmium; nitrates; hydroxides; fluorides; sulfates; chloroform; and various radionuclides, including plutonium (Last and Rohay, 1991). Near the disposal site, CCl_4 vapors have been encountered in the vadose zone during well drilling operations, and groundwater contamination from CCl_4 is extensive, covering at least 7 km^2. Concentrations over 1000 times the Environmental Protection Agency's (EPA) drinking water standard of 5 ppb (μg/l) have been measured in the groundwater. In addition, nitrate concentrations up to 10 times the EPA drinking water standard of 44 ppm (mg/l) have been measured in the same area of the site. *In situ* bioremediation is one treatment method currently being developed by the U.S. Department of Energy to meet the need for cost-effective technologies to remove CCl_4 from the groundwater.

The current understanding of microbial degradation of CCl_4 is limited. However, CCl_4 biodegradation has been demonstrated with a number of different bacteria. The conditions that favor biodegradation of CCl_4 are predominantly anaerobic. For example, Bouwer and McCarty observed that cultures of sewage treatment bacteria biodegraded CCl_4 to CO_2 and other metabolites under methanogenic (Bouwer and McCarty, 1983a) and denitrifying (Bouwer and McCarty, 1983b) conditions. Sulfate-reducing microorganisms have also demonstrated the ability to destroy CCl_4 (Cobb and Bouwer, 1991; Egli *et al.*, 1988). In addition, Semprini *et al.* (1991) speculated that sulfate-reducing bacteria were responsible for the CCl_4 degradation they observed during a field test of *in situ* bioremediation. Biodegradation of CCl_4 under denitrification conditions is of particular interest at Hanford because of the occurrence of both CCl_4 and nitrate in the unconfined aquifer. The potential of stimulating microorganisms indigenous to the Hanford site to degrade both nitrate and CCl_4 has been demonstrated at the laboratory-, bench-, and pilot-scales (Brouns *et al.*, 1990; Hansen, 1990; Koegler *et al.*, 1989). However, little is known about the effects of electron donor and electron acceptor concentrations on the rates of degradation. It was observed by Lewis and Crawford (1993) that the denitrifying *Pseudomonas* strain KC degrades CCl_4 most rapidly when there is ample nitrate and electron donor and the microbes are actively growing. In contrast, Bae and Rittman (1990) demonstrated that CCl_4 degradation by a denitrifying biofilm was most rapid when the biofilm was deprived of nitrate.

The purpose of this paper is to examine the effects of nitrate concentration on the ability of the Hanford aquifer consortium to transform CCl_4. This infor-

mation is vital to designing an *in situ* bioremediation system since the concentrations of the electron donor and acceptor are the primary control variables in the remediation process, and, these concentrations will potentially affect both the spacial distribution of biomass and CCl_4 destruction.

MATERIALS AND METHODS

Reactor configuration

Batch experiments were performed to determine the effects of electron donor and acceptor levels on the transformation of CCl_4 by a native Hanford consortium. The experimental apparatus is shown schematically in Fig. 1 and described in detail by Skeen *et al.* (1993). Briefly, this reactor consists of a 1 l stainless steel vessel, fitted with gas and liquid sampling ports, a 0–30 psi Bourdon tube pressure gauge constructed entirely of stainless steel (McMaster-Carr, Santa Fe, CA), an *in situ* sterilizable pH electrode (Cole-Parmer, Chicago, IL), and an *in situ* sterilizable redox potential electrode (Cole-Parmer, Chicago, IL). An Orion 720-A dual-channel pH/mV meter (Orion, Boston, MA) was used to record the pH and the ORP. Viton or Teflon O-rings are used throughout the reactor to minimize VOC sorption to surfaces in the reactor. Before each CCl_4 degradation experiment the reactor was pressure tested to 10 psi for 60 h to ensure that it was gas tight.

Bacteria and culture conditions

The bacterial consortium used for these experiments was grown from a soil sample that was aseptically removed from a test well on the Hanford site (Brouns *et al.*, 1991). An initial bacterial stock for these experiments was prepared using 10 g of soil to inoculate 100 ml of simulated groundwater (SG) amended with acetate and buffered to pH 7.0 with 100 mM phosphate. The SG was formulated to approximate the major ion concentrations of the Hanford groundwater. In the SG, the major ion concentrations were 46 mM Na^+, 100 mM K^+, 0.06 mM Ca^{+2}, 0.6 mM Mg^{+2}, 1.6 mM SiO_3^{-2}, 1.5 mM CO_3^{-2}, 0.04 mM SO_4^{-2}, 100 mM PO_3^{-2}, 1.3 mM Cl^- and 194 mM NO_3^-. After inoculation, the culture solution was incubated on a shaker (Lab-Line, Melrose Park, IL) at 117 RPM and 27°C for 1 day. Then, 1 ml of this broth was transferred to fresh SG with acetate and allowed to grow for an additional 3 days. The resulting cell broth was mixed with an 80% glycerol solution to 50 vol.% and frozen at −70°C. This stock was used as an inoculum for the experiments reported in this paper.

Inoculation of the reactor was accomplished by adding 100 μl of the microbial stock to 20 ml of acetate-amended SG. These cells were then grown aerobically for 1 day in the shaker operated at 117 RPM and 27°C. Next, 1 ml of this material was transferred to 100 ml of fresh growth media, and after 3 days the biomass from this flask was used to inoculate the reactor. Aerobic growth processes were used to generate the biomass for these experiments because it was observed that growth was much faster under aerobic

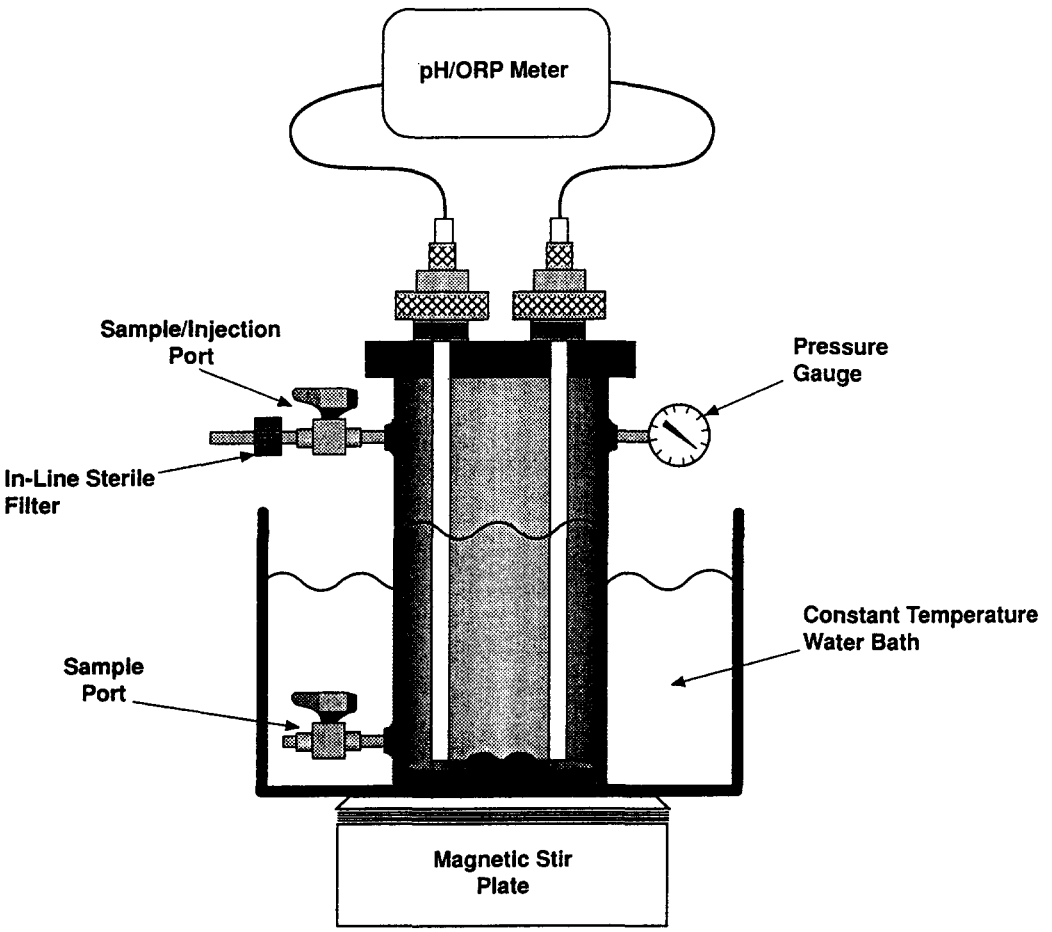

Fig. 1. Schematic of the equipment used in the batch CCl_4 degradation experiments.

conditions than under anaerobic conditions. In addition, it was shown in previous work that the microbial species in the Hanford aquifer that are responsible for CCl_4 degradation are facultative anaerobes, which were tentatively identified as *Pseudomonas stutzeri* (Hansen, 1990), and hence would not be lost during aerobic growth.

Reactor operation

Before inoculation, the reactor was sterilized in an autoclave, charged with 750 ml of sterile SG, and pressure tested at 10 psi for 60 h. A reduction in pressure would indicate a leak in the reactor, all of which were alleviated before proceeding. Nitrate was added with the SG to achieve approximately 1500 and 100 ppm for the high-nitrate and low-nitrate experiments, respectively. Once the reactor passed the pressure test, the medium was purged with nitrogen for 2 h to deoxygenate the system, and sodium acetate was added to adjust the acetate concentration to approximately 300 ppm. This acetate concentration gave a nominal acetate-to-nitrate ratio of 3.0 and 0.2 (mass/mass) for the high- and low-nitrate experiments, respectively. The observed stoichiometric acetate-to-nitrate ratio for this consortium is approximately 1.4 (mass/mass). After adding the acetate, the system was then charged with enough CCl_4 to achieve approximately 300 ppb CCl_4 in the aqueous phase. Three to 4 h were allowed after the addition of the CCl_4 to establish CCl_4 vapor–liquid equilibrium. This equilibrium time was determined from an abiotic partitioning test using the reactor. Finally, enough biomass was added to achieve a nominal cell concentration of 25 mg-DW/l.

Once inoculated, the reactor was placed in a constant temperature water bath maintained at 17°C, and mixed with a magnetic stir bar. The reactor pressure, temperature, pH and ORP were recorded daily. To measure CCl_4 levels, 2.5-ml aqueous samples were removed from the reactor using a sterile syringe containing 0.5 ml of hexane. This mixture was then dispensed directly into approximately 2 ml of hexane in a graduated cylinder that was kept in ice. The cylinder was closed with a Teflon-line cap, and the hexane volume and sample volume were recorded. The CCl_4 was extracted into the hexane by vortexing the cylinder for 30 s to mix the phases. After mixing, the phases were allowed to separate and the hexane layer was extracted and placed in a GC vial for later analysis.

Skeen *et al.* (1993) have demonstrated that the CCl_4 concentration in such reactors remains constant over an extended period if no biomass is introduced to the reactor. Thus, any losses during a test with biomass can be attributed to biological activity.

Analytical procedures

Anions and biomass were measured three times a day by withdrawing a 10-ml aqueous sample from the reactor. Biomass levels were determined by either of two methods: dry weight per unit volume (mg-DW/l), i.e. total suspended solids (TSS), or optical absorbance. The absorbance method was used if the biomass concentration was estimated to be within the range that gave a linear response. This occurred with biomass concentrations below 100 mg-DW/l. In this case, approximately 4 ml of the 10-ml sample was sonicated for 30 s and its absorbance was then measured at 450 nm. From this absorbance reading, the TSS of the biomass was determined from a standard curve. If the biomass concentration was estimated to be above 100 mg-DW/l, then the TSS was directly measured from the weight of a filtered sample that was dried at 106°C for a minimum of 1 h. A minimum drying time of 1 h was used because initial tests demonstrated that sample weight did not change with time after the sample had been dried for 1 h. In either case, the sample was filtered through a 0.45-μm membrane to remove biomass. The filtrate was collected and analyzed on an ion chromatograph to determine acetate, nitrate, nitrite, chloride and sulfate concentrations. To maintain the desired nitrate and acetate levels in the reactor, the first anion sample taken each day was analyzed and the amount of acetate and nitrate required to bring the aqueous solution in the reactor back to the desired level was added with a sterile syringe.

The gas chromatograph used in this research to determine the concentration of CCl_4 and potential degradation by-products ($CHCl_3$ and CH_2Cl_2) was an HP model 5890 Series II (Hewlett Packard, Palo Alto, CA) equipped with an electron capture detector. Nitrogen was used as the carrier gas at 6.6 ml/min. This gas chromatograph was equipped with a 30-m DB-624 column (J&W Scientific, Fulsom, CA) having a 0.53-mm i.d. The detection limit for CCl_4 was approximately 10 ppb.

Anion concentrations were determined using a Dionex 4000i (Dionex, Sunnyvale, CA) ion chromatograph. This unit was equipped with a conductivity detector and a cation suppression unit which exchanges hydrogen for the cations contained in solution. This cation exchange unit is used to remove the effect of the cations upon the conductivity of the exit stream. The eluent was 40-mM NaOH and 5% methanol which was fed at 1-ml/min. The column used was a Dionex PAX 100 anion exchange column (Dionex, Sunnyvale, CA). Tests with this column indicate that all reported anion concentrations are accurate to within 10 ppm of the indicated value.

RESULTS

Two experimental conditions were tested in this study. The first set of conditions corresponded to a high-nitrate environment where the nominal acetate and nitrate concentrations were 300 and 1500 mg/l respectively. The second set of conditions corresponded to a low-nitrate environment where the nominal acetate and nitrate concentrations were 300 and 100 mg/l, respectively. These two conditions were chosen since the high-nitrate environment would provide conditions that favor CCl_4 transformation by microorganisms which function similarly to the *Pseudomonas* sp. strain KC reported by Criddle *et al.* (1990) and Lewis and Crawford (1993). The low-nitrate conditions would allow similar growth as the high-nitrate condition, while still providing conditions that would stimulate CCl_4 transformation activity for organisms that functioned like those used by Bae and Rittmann (1990). Similar growth for the two conditions was important so the effects of cell numbers on CCl_4 transformation was the same for both tests. Although data from single experiments at each set of conditions are presented, similar behavior was observed during replicate experiments.

Figure 2 shows the aqueous CCl_4 level for the two experiments. The data presented here indicates that minimal CCl_4 is transformed under the high-nitrate conditions, but it is transformed when the nitrate concentration is lowered. No chloroform was observed in any of these experiments. The points on Fig. 2 represent the measured levels while the dashed lines are the CCl_4 concentration predicted by Henry's law if no biotic or abiotic losses occurred. This concentration was estimated using

$$C_l = \frac{m_0 - \sum_i (C_{l,i} V_{\text{sample},i})}{V_l + V_g H_c} \quad (1)$$

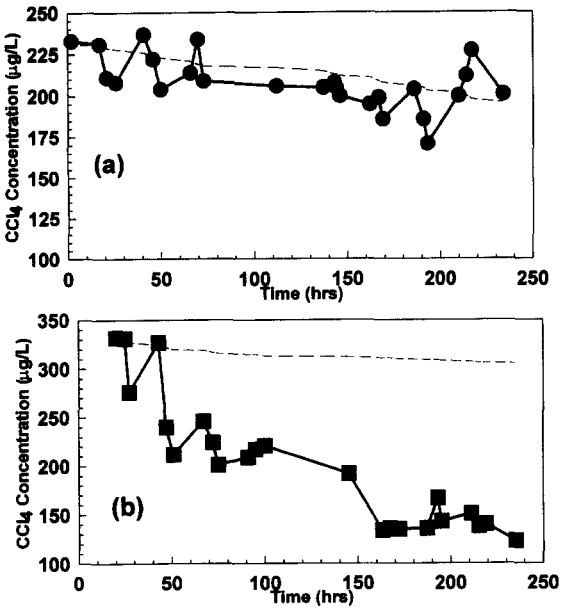

Fig. 2. Carbon tetrachloride concentration vs time for the two experiments. (a) The high-nitrate case where the little destruction occurs, and (b) the low-nitrate case.

where C_l is the aqueous CCl_4 concentration when the liquid and gas volumes of the reactor are V_l and V_g, respectively. The parameters $C_{l,i}$ and $V_{\text{sample},i}$ are the CCl_4 concentration and volume of aqueous sample removed from the reactor at the ith sampling period. The Henry's law constant (H_c) at 17°C was estimated as 0.856 (mg/l gas phase)/(mg l aqueous phase) (Gossett, 1987). The initial mass of CCl_4 added to the reactor, m_0, was estimated from Henry's law by assuming that the aqueous concentration of CCl_4 measured after 4 h was the initial equilibrium level. A 4-h equilibration time was determined with aseptic experiments where the reactor head space was sampled every 20 min.

It is intuitive that CCl_4 transformation rates depend on the amount of biomass present. However, differences in the amount of biomass can not account for the differences in the amount of CCl_4 transformed under the two conditions reported here. As shown in Fig. 3, the total amount of biomass produced over the course of the two experiments is nearly the same.

In Fig. 4, the acetate and nitrate concentration in each experiment are shown as functions of time. The arrows in this figure indicate the samples taken just after the addition of acetate and/or nitrate. It is clear from this figure that under both conditions the microbes were actively using acetate and nitrate so that periodic additions were required to return the concentrations to the desired levels. In addition, acetate is present in the reactor throughout the duration of both experiments. Conversely, only in the high-nitrate case [Fig. 4(a) and 4(c)] is nitrate present throughout the course of the experiment. In the low-nitrate case [Fig. 4(b) and [(d)], nitrate was depleted each day. Although not shown, the sulfate concentration remained constant throughout the duration of both experiments, thus indicating that it did not serve as an alternate electron acceptor.

DISCUSSION

By comparing the CCl_4 degradation achieved under the two sets of conditions used here, it is apparent that sustained CCl_4 degradation is accomplished by the Hanford denitrifying consortium only under low-nitrate conditions. These results are consistent with those of Hansen (1990), who showed that the addition of a small amount of nitrate to a nitrate-limited batch culture will stimulate CCl_4 degradation by the Hanford consortium and by two *Pseudomonas stutzeri* isolates from the consortium. This result is also consistent with that reported by Bae and Rittmann (1990) who observed CCl_4 transformation under nitrate limited conditions. Organisms which transform CCl_4 in a manner similar to the *Pseudomonas* KC reported by Lewis and Crawford (1993) would have shown active contaminant removal under the high-nitrate conditions. However, the microbes used here do not transform CCl_4 under such conditions. Hence, it appears that the CCl_4 transformation properties of the Hanford consortium are significantly different from *Pseudomonas* KC.

These findings can also be used to explain the results obtained by Semprini *et al.* (1991) during a field demonstration of *in situ* CCl_4 destruction. In that study, nitrate was continuously injected while the acetate was fed in a pulse fashion. These authors found that CCl_4 degradation occurred only at a significant distance from the injection well in a region beyond where most of the denitrification was occurring. It was speculated that CCl_4 was destroyed by a population of bacteria that were inhibited by nitrate, but could use either acetate or products from the decay of the denitrifying population as a primary substrate. Sulfate was speculated to be the electron acceptor for the CCl_4-degrading bacteria, although sulfate or sulfite levels were not measured. An

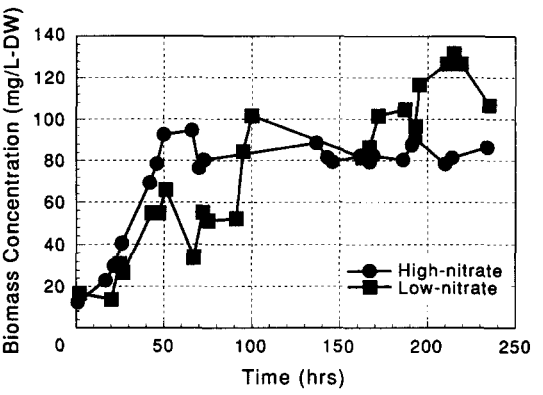

Fig. 3. Total suspended solids vs time. Similar amounts of biomass were produced under both sets of conditions.

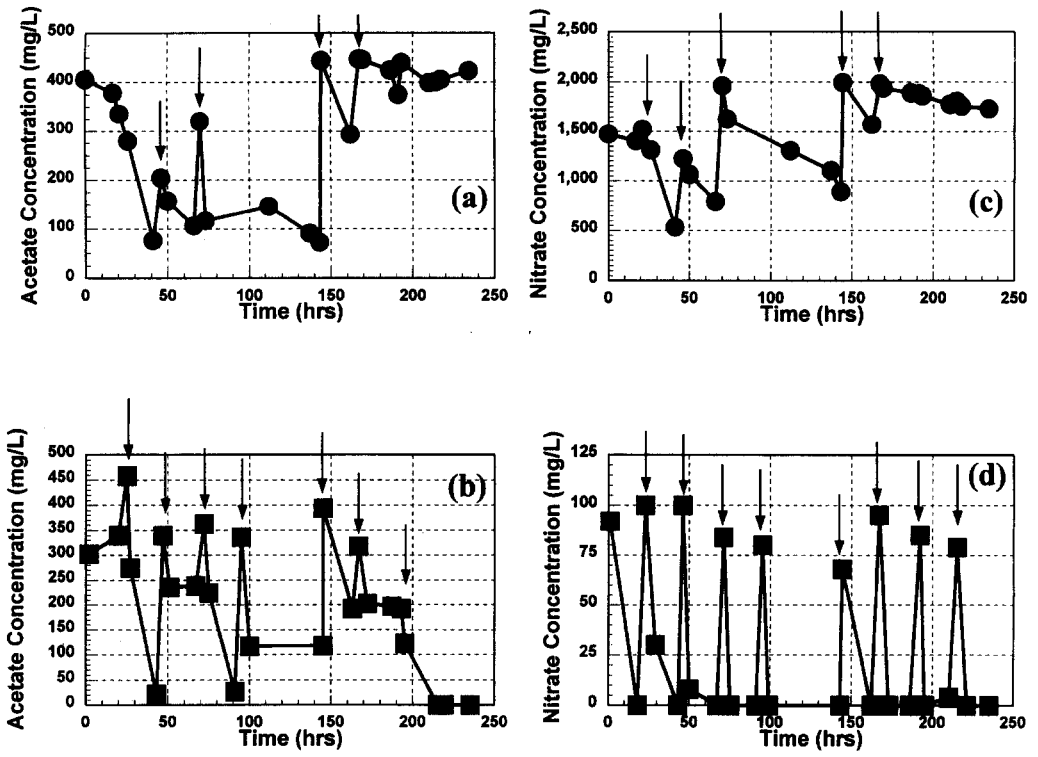

Fig. 4. Acetate and nitrate concentrations vs time for the two experimental conditions, (a) and (c) are the acetate and nitrate concentrations respectively for the high-nitrate case. (b) and (d) are the acetate and nitrate concentrations for the low-nitrate case.

alternative explanation of the behavior observed by Semprini *et al.* is that the pulse feeding strategy caused the microbes in the region where CCl_4 was degraded to be exposed to nutrients levels which cycled from low to non-existent. Exposed to such a nutrient profile, the microbes could remain in a metabolically active state similar to that observed in the low-nitrate experiments reported here.

Further evidence that the bacteria located away from the well could have periodically experienced times where little or no nitrate was present is supported by numerical simulations of the degradation

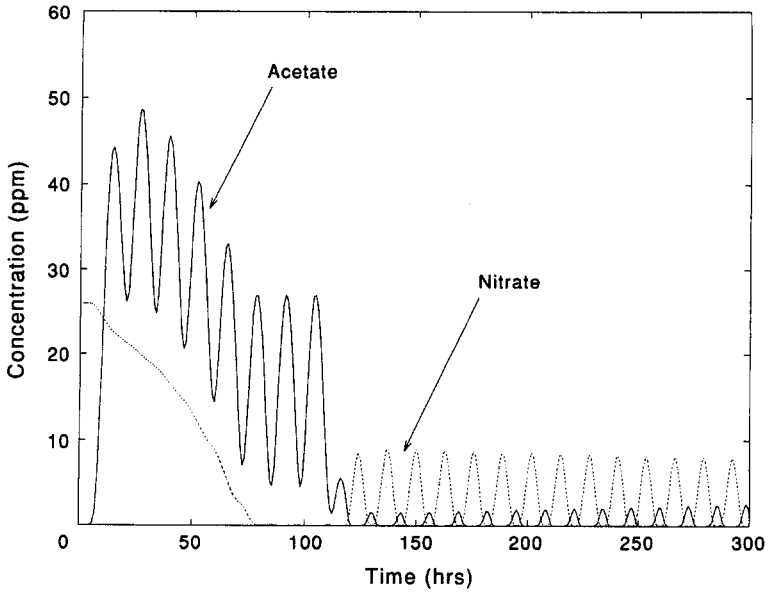

Fig. 5. Predicted acetate and nitrate levels at the end of a 2-m flow field under conditions identical to those reported for a field test of *in situ* CCl_4 bioremediation (Semprini, 1991).

process. Figure 5 shows the predicted acetate and nitrate levels at the end of a 2-m flow field under conditions identical to those reported for the field test. The predicted results were generated using microbial kinetics developed by Semprini *et al.* (1991) and accounting for fluid transport processes of advection, dispersion, and contaminant adsorption to the soil. The details of the numerical methods used to generate this data have been reported elsewhere (Schouche *et al.*, 1993). It is evident from this figure that the predicted acetate and nitrate concentrations cycle 180° out of phase. Hence, when nitrate is depleted, acetate is present, creating a condition similar to that observed in the nitrate-depleted experiments reported here.

The fact that biological transformation of CCl_4 occurs only under low-nitrate conditions will impact how *in situ* bioremediation is implemented. Nutrient feeding strategies must be devised to cycle the nitrate level to maximize the time the bacteria are in the phase where CCl_4 degradation occurs. This will most likely require a pulse addition of nutrients such as that described by Roberts *et al.* (1991). Such feeding schemes would have the added advantage of extending well life by reducing clogging caused by biomass growth near the injection well (Shouche *et al.*, 1994).

CONCLUSIONS

The results presented in this paper suggest that CCl_4 is only transformed by the Hanford consortium under low-nitrate conditions. This finding is consistent with previous results (Hansen, 1990; Bae and Rittmann, 1990). Additionally, this result has significant impact on the feeding strategy for *in situ* bioremediation. A successful feeding strategy will need to cycle the nutrients to maximize the time the microbes are in the metabolic phase in which CCl_4 is transformed. This will most likely require a pulse addition of nutrients.

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