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Influence of Nitrogen and Phosphorus on the *In Situ* Bioremediation of Trichloroethylene†

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ABSTRACT

The US Department of Energy, Office of Technology Development, has supported a field-scale *in situ* demonstration of trichloroethylene (TCE) bioremediation at the Westinghouse Savannah River Site (WSRS). Several methods were used to examine the influence of nitrogen and phosphorus species on TCE degradation during methane (CH_4) injection into contaminated sediments. Laboratory experiments using WSRS ground water revealed that the rate of acetate incorporation into microbial lipids was stimulated when triethyl-phosphate (TEP) or nitrous oxide (N_2O) was added. The trend was: $CH_4 + N_2O > CH_4 + TEP > CH_4 + N_2O + TEP > CH_4$ alone. The degree of stimulation of ^{14}C-TCE mineralization in ground water incubated for 30 d in the laboratory with added methane and nutrients increased in the order: OP = TEP > NH_3 + TEP = NH_3 > N_2O (OP, orthophosphate; NH_3, ammonia). Monitoring of WSRS ground water revealed significant differences among sampling wells over time in nutrient concentrations, nitrogen uptake, and urease activity during operations of the bioremediation demonstration. In the field, the addition of TEP + N_2O to the pulsed injection of CH_4 resulted in dramatic stimulation of TCE-degrading potentials observed in ground water enrichments. The potential to mineralize ^{14}C-TCE in ground water enriched with nutrients in the laboratory increased from <50% of the samples taken during injection of methane in the field to >90% of the samples taken during

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the injection of CH_4 + TEP + N_2O treatment. These results demonstrated the dramatic impacts of nitrogen and phosphorus supplements during the *in situ* bioremediation of chlorinated solvents.

Index Entries: Bioremediation; trichloroethylene; methane; nitrogen; phosphorus; nutrient limitation; activity; ground water; microbiology.

INTRODUCTION

Diverse microbial communities with varied metabolic capabilities are present in subsurface environments. Microbial biomass, abundance of specific populations, and activities and degradative rates of subsurface microorganisms may be constrained by properties of the subsurface environment, such as pH, Eh, permeability, water flux, and availability of electron donors, electron acceptors, and nutrients. However, the subsurface microbial community may be active and impact ground water properties by producing carbonates or volatile fatty acids, or by consuming oxygen (*1–4*).

Nutrients that can be transported by ground water may stimulate microbial activities in subsurface environments, particularly during bioremediation efforts. Physical and chemical factors have been reported to limit microbial metabolism and growth in low-permeability Eastern Coastal Plain sediments (*2,5,6*) and western US arid sediments (*7–9*). Low permeability appeared to constrain microbial mass and activities in Eastern Coastal Plain sediments, even though substantial organic matter was present (*4,5*). In contrast, microbial activities in the highly permeable sands appeared to be constrained by low concentrations of inorganic and organic nutrients (*5*). Microbial populations in semiarid western vadose sediments appeared dependent on the particulate organic carbon content of the sediments (*9*). After additions of water and minerals, the microbial densities in the organic-laden vadose sediments increased more than four orders of magnitude, with a concomitant increase in the rate of glucose mineralization (*9*). Even though these laboratory-scale experiments indicated that increased water flux and inorganic nutrient supplements result in increased microbial abundance and activities (*5,9*), direct evidence of the relationship between nitrogen and phosphorus supplements and the stimulation of methane-amended *in situ* bioremediation of chlorinated solvents is lacking.

A field-scale *in situ* demonstration of trichloroethylene (TCE) bioremediation at the Westinghouse Savannah River Site (WSRS) was designed to test the effectiveness of methane stimulation of TCE degradation in contaminated subsurface sediments (*10*). Microorganisms isolated from the site have demonstrated capabilities to degrade chlorinated organics, including TCE, dichloroethylene, vinyl chloride, and chloro-

form (*11–14*). Laboratory-scale bioreactors have verified that the addition of methane enhances TCE degradation by microorganisms native to WSRS sediments (*15,16*). Based on these results and substantiating investigations into methanotropic stimulation of TCE degradation (*17,18*), the WSRS *in situ* demonstration was initiated (*10*). If factors that constrain subsurface microbial activity could be lessened and procedures for monitoring bioremediation developed, then *in situ* metabolism may be enhanced and substantiated. Successfully implementing and demonstrating stimulation of *in situ* TCE degradation by native microoganisms could facilitate wider acceptance of bioremediation as a remediation option. The specific objectives of this research, which was part of a larger monitoring effort, were to determine nutrient limitations in the subsurface environment, and to examine the effect of nutrient amendments on microbial activity and microbial-degradative capacity in the WSRS subsurface environment.

METHODS

Materials

National Welders (Charlotte, NC) supplied all gases. Methane was >98% pure, and propane was >99.5% pure. Nitrous oxide was provided by Saddleford at University of Tennessee Veterinary Hospital. Chemicals and reagents were obtained from Sigma Chemical Company (St. Louis, MO) or from Mallinckroft, Inc. (Paris, KY). Glass-distilled solvents and reagents were purchased from J. T. Baker Chemical Co. (Phillipsburg, NJ) or from Mallinckroft. [^{3}H]-acetate (3.6 Ci/mmol), carrier-free [1,2-^{14}C]-TCE, and carrier-free [1,2-^{14}C]-tetrachloroethylene (PCE) were obtained from Du Pont NEN Research Products (Boston, MA).

Site Description

The *in situ* bioremediation demonstration at the US Department of Energy WSRS involved an area of subsurface and ground water contaminated with TCE and PCE from an abandoned process sewer line (*10*). The demonstration site consisted of a lower horizontal well located below the water table and an upper horizontal well located in the vadose zone. The lower well provided delivery of gas and nutrients at various concentrations with and without addition of nutrients (Table 1). Vacuum was applied to the upper well to encourage nutrient movement through the upper saturated zone and lower vadose zone. Several monitoring wells were installed at various locations around the demonstration site. Nutrient injection consisted of adding triethyl-phosphate (TEP) at a concentration of 0.007% and nitrous oxide (NO_2) to achieve a pulsed nutrient delivery C:N:P ratio of 20:10:1. A more detailed description of the integrated demonstration was provided by Hazen (*10*).

Table 1
Operational Campaigns

Treatment	Date	Days[a]
Air injection	3/18/92	21
1% Methane injection	4/20/92	54
4% Methane injection	8/5/92	161
Air-Methane pulse	10/23/92	240
4% Methane + nutrients	1/18/93	327
Posttreatment	4/20/93	419

[a]Days from initial start date of *in situ* demonstration.

Ground Water Sampling

Ground water was obtained twice monthly from a series of 12 monitoring wells located in and around the bioremediation site. The ground water was collected for microbiological studies according to documented WSRS well-sampling protocols (*10*). Experiments were initiated on-site, or samples were stored on ice until processed at The University of Tennessee —Knoxville or Oak Ridge National Laboratory.

Microbial Enumerations

Most probable number (MPN) techniques for methanotrophic and methanol/propane-oxidizing populations were used to evaluate changes in microbial biomass. Methanotroph enumerations were based on turbidity being exhibited over a three-to-four dilution range in a phosphate-buffered mineral salts media supplemented with 5% CH_4 (v/v, head space). Methanotrophic media were made up in 1 L of nanopure-filtered water and contained the following: NaCl, 0.9 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; $NaNO_3$, 0.5 g; $NaH_2PO_4 \cdot H_2O$, 0.044 g; K_2HPO_4, 0.26 g; trace mineral solution (*5*), 10 mL; 10X vitamin solution (*11,14*), 1 mL. The pH was adjusted to 7.1 and, after sample inoculation, methane was added at 5% of head-space volume. The same medium was used for methanol/propane oxidizers with the addition of 3 mg/L of yeast extract, 5 m*M* methanol, and 3% head-space propane plus 2% head-space CH_4.

Activity Experiments

Microbial activity was assessed by measuring acetate incorporation and mineralization of TCE and PCE in time-course experiments and enrichments as described previously (*11,14*). Isotope solutions (0.5–5 μCi) were frozen prior to use, thawed, and transferred with gas-tight syringes (Hamilton Co., Reno, NV). Ground water aliquots were inoculated in duplicate for time-course experiments. Sterile polypropylene centrifuge tubes were used for aerobic isotope-incorporation experiments. Anae-

robic crimp-top tubes (Bellco Glass, Vineland, NJ) were used for mineralization experiments. Incubations were at ambient conditions that were similar to the 21–27°C *in situ* temperatures.

Acetate incorporation experiments used 2 mL of ground water and 1 mL of sterile water containing 5 mCi [^{3}H]-acetate. Final acetate concentrations in experimental tubes were 30 ng/g. At t_0 and appropriate time-points, duplicate tubes were inhibited with 5.0 mL of a phosphate-buffered chloroform-methanol solution and frozen. Time-points of 0 and 4 h, and 1 d generally provided linear rates. The same design as that of the tritiated-acetate-incorporation experiments was used to perform nutritional studies, except that specific nutrients were added. Nutritional supplements included 0.2% CH_4 with 100 μM of either or both NO_2 and TEP.

Mineralization experiments used 10 mL of ground water and 0.5 μCi of carrier-free [1,2-^{14}C]-TCE and [1,2-^{14}C]-PCE. Enriched mineralizations used double-strength methanotrophic media (above) with the exception of phosphate, which was kept at 2 m*M*, and the additional of 60 mg/L of yeast extract and trypticase. Nutrient-amended mineralization enrichments contained 0.2% CH_4 with 100 μM of nitrogen and phosphorus compounds (N_2O), ammonia (NH_3), *ortho*-phosphate (OP), and TEP. All mineralization experiments were incubated for 30 d at room temperature, inhibited with 0.4 mL of 2*M* NaOH, and refrigerated until analyzed.

In low-nutrient subsurface samples, metabolic activity can be more accurately quantified by the rates of product accumulation than by the rates of substrate disappearance. Furthermore, transformation rate constants have been shown to be similar to turnover rate constants (*21*). Two sets of experiments were used to identify the nutrients needed to increase biological activity and to determine if inorganic nutrients could be effectively added as gases. These two experiments included acetate incorporation and TCE mineralization. The nutrients (added with CH_4) compared were TEP, OP, NH_3, and NO_2.

The urease assay was used to measure the nitrogen-scavenging activity of bacteria. Ground water (40 mL) was incubated in 1 mL of 0.1*M* phosphate buffer (pH 6.7) and 2 mL of a 10% urea solution for 2 h at 30°C. Controls consisted of distilled water. Bacteria were removed from the ground water samples by filtration through 0.2-mm pore size cellulose/acetate Easy Flow™ filter (Becton Dickinson, Lincoln Park, NJ). The amount of ammonia in each ground water sample was determined colorimetrically by using a TRAACS 800 Analyzer (Technicon, Tarrytown, NY).

Analytical Procedures

In the laboratory, samples from the acetate-incorporation experiments were extracted with chloroform-methanol, dried, and resuspended in 2.0 mL chloroform; aliquots were assayed by liquid scintillation counting to determine the amount of radioactivity incorporated into total micro-

bial lipids (*5,14*). The earliest time points yielding measurable results were used to calculate a linear rate that was extrapolated to disintegrations per minute per day. Radioactive $^{14}CO_2$ from mineralization experiments was examined by gas chromatography-gas proportional counting (*4,14*). A Shimadzu 8A gas chromatograph (GC) equipped with a thermal conductivity detector was connected to a Packard 894 gas proportional counter. One hour before analysis, tubes were acidified with 0.5 mL of 6*M* hydrochloric acid. Transformation rate constants were estimated from product accumulation rates, rather than from the rates of substrate disappearance used for turnover rate constants.

RESULTS AND DISCUSSION

Changes in Methanotrophs and Methanol/Propane Oxidizers

The numbers of methanotrophs increased >100-fold after the injection of CH_4 into the subsurface (Fig. 1A). Before CH_4 treatment, <1 methanotroph/mL was detected in ground waters from well MHT-2C. During 1% CH_4 injection, the mean number of methanotrophs observed in ground waters from well MHT-2C was 3×10^4 cells/mL. There was a less dramatic increase in methanotrophs from below detectable limits to $>10^2$ cells/mL in ground waters from well MHT-3C. With the addition of N_2O and TEP, the methanotrophic population further increased by a factor of 6. The impact from the CH_4 injection and from the CH_4 plus nutrient injection was observed in some of the wells thought to be outside the zone of influence of the horizontal wells. During posttreatment, the methanotrophic populations decreased by a factor of 2. Well MHT-2C showed a decrease in methanotrophic populations by a factor of 4 during posttreatment, whereas MHT-3C averaged 200 methanotrophs/mL, which is similar to the methanotrophic population numbers seen for this well with the CH_4 and the nutrient treatments. The difference in methanotrophic counts between the two wells, in part, may be a result of well location with respect to the horizontal wells. Well MHT-2C was influenced by both horizontal wells in that CH_4 from the injection well could be extracted via a route that took the methane near it. Well MHT-3C, however, was on the side of the injection well most distant from the extraction well, but was close enough for CH_4 to diffuse from the injection well into it.

This *in situ* bioremediation study and a study by Semprini et al. (*19*) demonstrated biostimulation of indigenous methanotrophs. At WSRS, methanotrophs isolated from ground water after the CH_4 treatment in this demonstration were predominately type II methanotrophs, and most isolates were identified as *Methylosinus* (*13*). Recent bioreactor studies suggested that type I and II methanotrophs and actinomycetes were native to WSRS soils (*20*). Methanol/propane-oxidizing populations in-

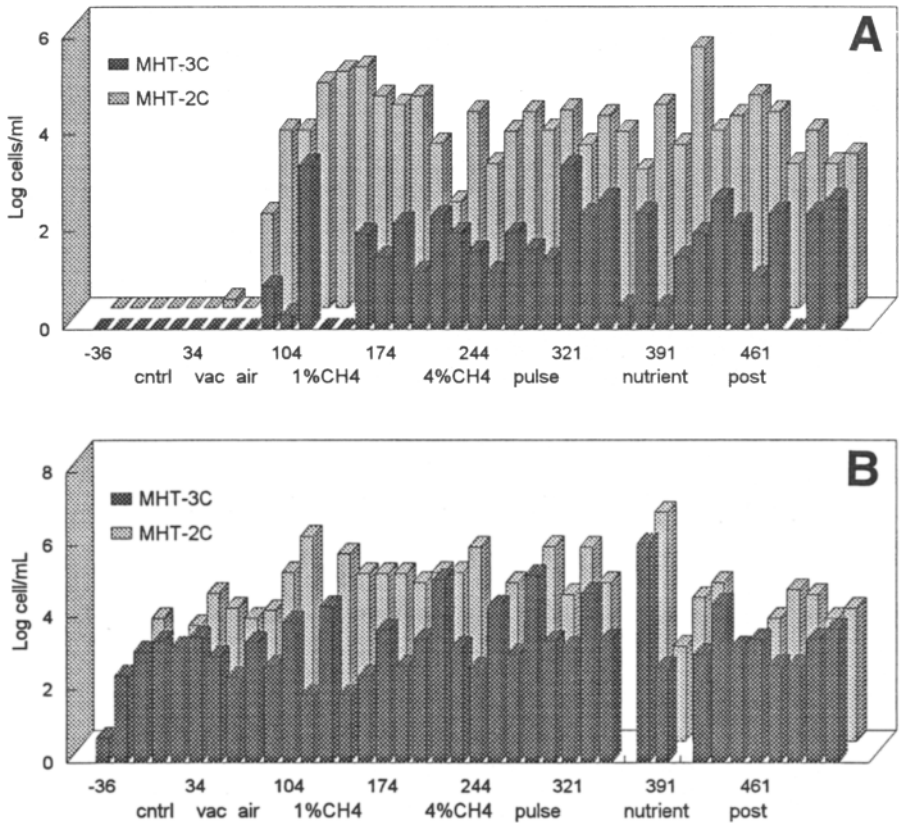

Fig. 1. Changes in methanotropic (**A**) and methanol/propane-oxidizing (**B**) populations of ground water from monitoring wells MHT-2C and MHT-3C during the various treatment campaigns. Day 0 is the start of the air-injection campaign, and all negative numbers indicate days during the control period prior to air injection.

crease with the addition of CH_4 to the subsurface (Fig. 1B). Methanol/propane oxidizers in ground water from well MHT-2C exhibited increases of >10-fold, and in ground water from well MHT-3C, there was a factor of 3 increase in methanol/propane-oxidizing populations. Based on MPN results, the size of methanol/propane-oxidizing populations was fivefold greater than the size of methanotrophic populations.

Mineralization of TCE and Acetate Incorporation with and Without Nutrients

The rate at which ground water microorganisms incorporated acetate into microbial lipids was stimulated when phosphate and NO_2 were added, suggesting that these compounds may be limiting in the subsurface (Table 2). The degree of stimulation of acetate incorporation by nutrient addition increased in the order: CH_4 alone < NO_2 + CH_4 + TEP

Table 2
Effects of Nitrogen and Phosphorus Additions
on TCE Mineralization and Acetate Incorporation Rates of Ground Water Samples

Nutrient[a]	TCE mineralization, range = 0 to ++++	Acetate incorporation
CH_4, NH_4	+++[b]	ND[c]
CH_4, OP	++++	ND
CH_4, TEP	++++	+++
CH_4, NO	++	++++
CH_4, NH_4, TEP	+++	+[d]
NH_4	+	ND
CH_4	0	0

[a]CH_4 = methane, NH_4 = ammonia, OP = inorganic *ortho*-phosphate, TEP = triethyl phosphate, and NO = nitrous oxide.
[b]A plus sign indicates mineralization or incorporation rates, which were greater than that for methane only. Multiple plus signs indicate greater rates.
[c]ND = not done.
[d]Nitrous oxide was used instead of ammonia.

< TEP + CH_4 < NO_2 + CH_4 (Table 2). Similar nutrient-amended acetate-incorporation studies demonstrated that water and phosphate stimulated acetate uptake by microorganisms in WSRS sediments to a greater degree than did other nutrients (*5*). Nitrate decreased with an increase in bacteria per milliliter in ground water analysis, whereas phosphate remained below detectable limits (Hazen et al., published results).

TEP and OP supplements enhanced TCE radioisotope mineralization equally at room temperature in 30-d experiments. The stimulation of ^{14}C-TCE mineralization increased in the following order: CH_4-only treatment < NH_3-only < NO_2 + CH_4 < NH_3 + TEP + CH_4 = NH_3 + CH_4 < TEP + CH_4 = OP + CH_4 (Table 2).

Monitoring Changes in Acetate Incorporation, TCE, and PCE Mineralization in Ground Water

TCE- and PCE-degradation capabilities were present in many of the wells during each phase of the bioremediation, and a moderate effect on the percentage of wells with PCE- and TCE-degradation capabilities was noted after nutrient addition (Fig. 2A). The increased percentage of wells with nutrient-enriched TCE and PCE mineralization during the nutrient-addition phase persisted into the posttreatment phase. These results are supported by recent column studies using microbial consortia from the contaminated site, which showed biodegradation of TCE and PCE under bulk aerobic conditions (*15,16,20,22*).

The effect of TEP and N_2O additions (Fig. 2B and C) on TCE and PCE mineralizations, expressed as the percentage radioisotope mineralized,

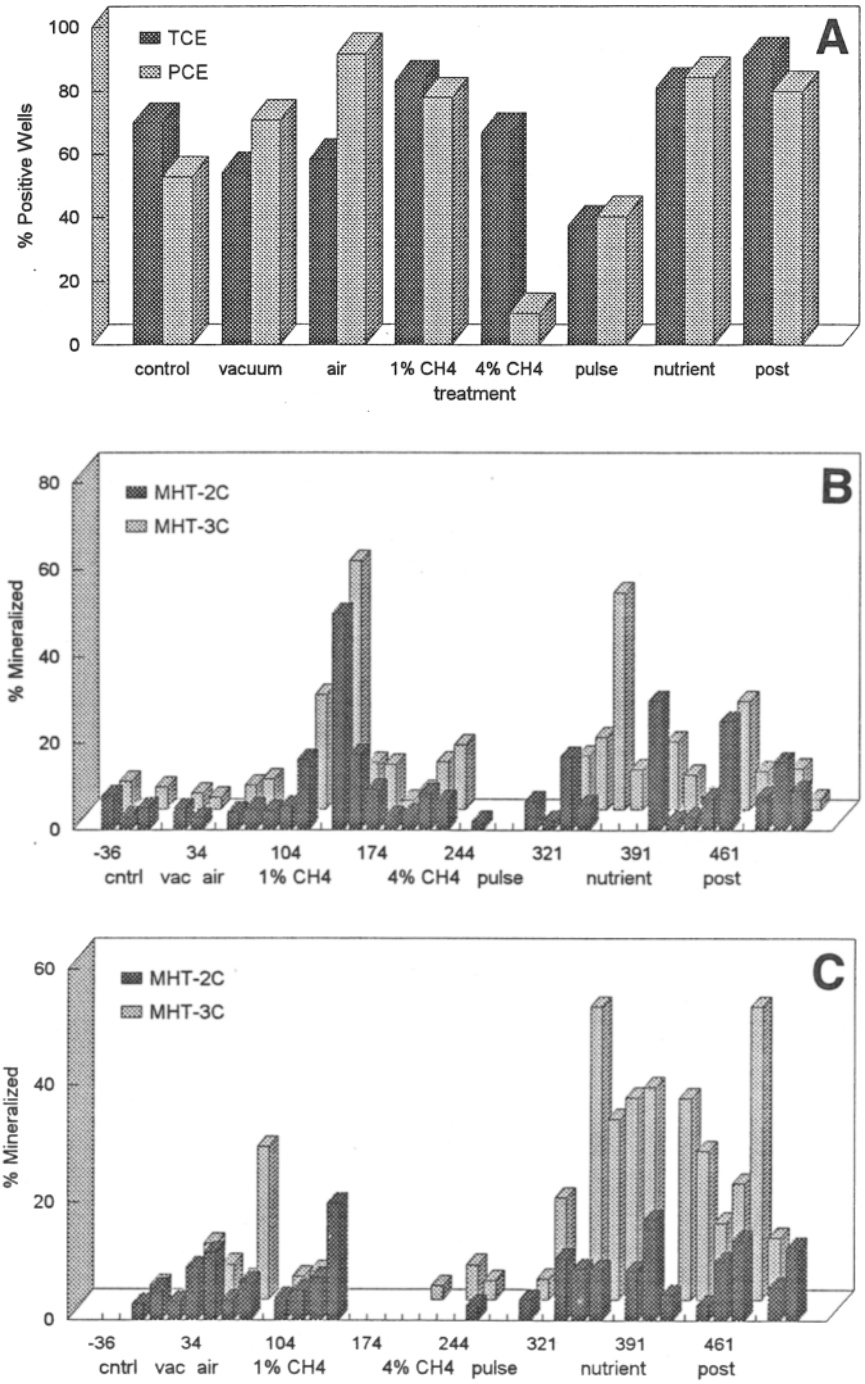

Fig. 2. Changes in TCE and PCE degradation during the various treatment campaigns. Percentage of ground water-monitoring wells exhibiting TCE and PCE degradation (**A**) and percentage of TCE (**B**) and PCE (**C**) mineralized under enriched conditions in ground water from monitoring wells MHT-2C and MHT-3C.

was dramatic; a less intense response was observed with the addition of CH_4 alone. In other phases, activity was minimal. There was also evidence that the amount of degration in some wells was dramatically increased after nutrient addition. Prior to CH_4 addition, $<10\%$ of ^{14}C-TCE was mineralized in ground water recovered from monitoring wells MHT-2C and 3C. Following CH_4 treatment, $>50\%$ ^{14}C-TCE was mineralized by ground water communities in the same wells. Enriched PCE mineralization for well MHT-3C dramatically increased from an average of $>10\%$ prior to the pulsed and nutrient injections to an average of $>30\%$ following those treatments. Increased PCE mineralizaiton for well MHT-3C compared with that exhibited by well MHT-2C may be a result of the proximity of MHT-3C to the injection well and, hence, the nutrients; MHT-2C, on the other hand, is located on the far side of the extraction well where there is little nutrient availability.

The addition of TEP and N_2O to the pulsed CH_4 delivery to the subsurface resulted in a dramatic stimulation of TCE-degrading potentials observed from enriched ground water. The potential to mineralize ^{14}C-TCE in enriched ground water increased from $<50\%$ during CH_4 treatment to $>90\%$ during the CH_4 plus nutrient treatment. PCE-degrading potential for enriched ground water continued to increase with each treatment. The potential to mineralize ^{14}C-PCE in enrichments increased from $<45\%$ of the samples during 4% CH_4 and pulse treatments to $>80\%$ during CH_4 plus nutrient treatment. The TCE and PCE degradation observed during this demonstration supports recent findings on degradation of TCE and PCE in bulk aerobic-phase sediment column studies (*15,16,20,22*).

Changes in Ground Water Nutrients, Urease Activity, and Nitrogen Uptake in Ground Water

Monitoring of WSRS ground water revealed dramatic differences among wells in NH_3 concentration, nitrogen uptake, and urease activity (e.g., Fig. 3A). Ammonia concentration was particularly variable among wells and sampling times. It is possible that these fluctuations represent changes in rates of anaerobic processes in this bulk aerobic environment.

There appeared to be few significant changes in nutrient concentrations, nitrogen uptake, and urease activity corresponding to changes in operation of the bioremediation demonstration (e.g., Fig. 3B). However, uptake of NH_3 during analysis was at a minimum during and after the nutrient-addition campaign, perhaps indicating that the bacterial populations were obtaining nitrogen from the injected NO_2. Also, NH_3 concentrations increased dramatically during the pulse-mode operation. This could indicate increased anaerobic activity during this period. Ammonia concentrations declined thereafter (Fig. 3B). There was little measurable urease activity at any time during the demonstration (Fig. 3B).

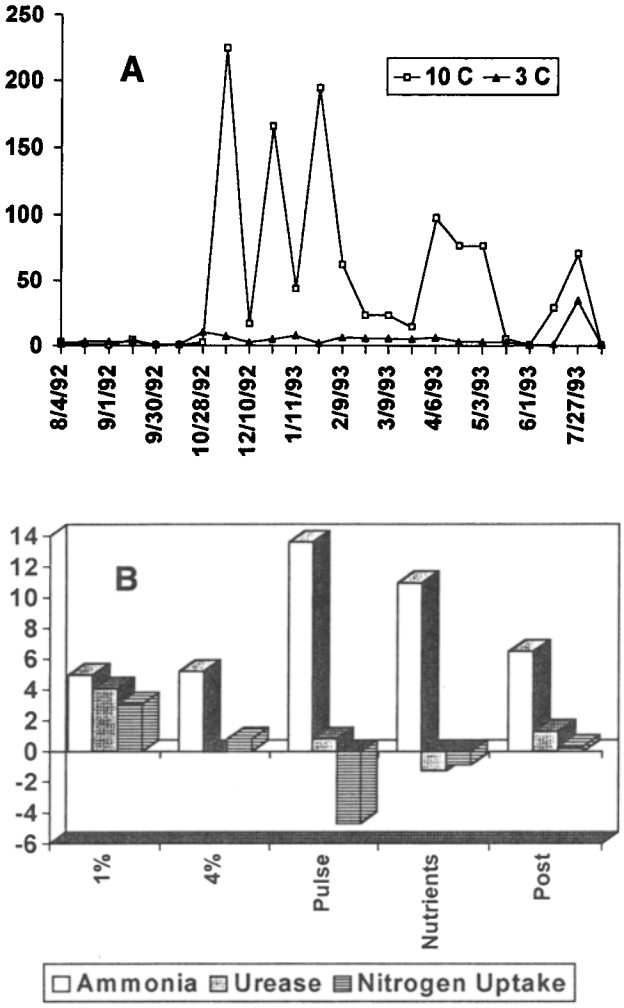

Fig. 3. Changes in measurements related to nitrogen transformations in water samples from monitoring wells. (**A**) Ammonia changes in wells MHT-10C and MHT-3C over time. (**B**) Mean changes (averaged over all wells) in ammonia concentration, urease activity, and nitrogen uptake during 1% methane addition, 4% methane addition, pulsed-methane addition, nutrient injection, and posttreatment operating campaigns.

SUMMARY

These results demonstrated dramatic impacts of nitrogen and phosphorus supplements on *in situ* bioremediation of chlorinated solvents in WSRS subsurface sediments. Injection of NO_2 and TEP as gases appeared to be an effective nutrient-delivery mechanism, and may have facilitated the distribution of the nutrients in the saturated and vadose zone sediments.

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