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# Anaerobic biodegradation of toluene under denitrifying conditions in contaminated groundwater and soil

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**Abstract**

The objectives of this study were: (1) to demonstrate nitrate loading as a control variable for the rate and extent of toluene degradation; (2) to maintain nitrate and toluene effluent concentrations below drinking water standards under continuous flow conditions; and, (3) to conduct treatability studies prior to a field demonstration delineating the effect of nitrate loading on toluene degradation. This project simulated field conditions in the laboratory by using contaminated soil and groundwater in batch and column biodegradation studies. The column experiments had continuous groundwater flow and were packed with soil from the site. Toluene concentrations approximating 50% saturation, 200 mg/l, were degraded. Carbon and nitrogen mass balances were completed. A goal during these experiments was to maintain the effluent nitrate and toluene concentrations below drinking water standards. Nitrate loading was demonstrated as a control variable for toluene degradation, with residual nitrate/nitrite concentrations in column effluents maintained below drinking water standards. The concentration of nitrate feed was varied to observe effects on toluene degradation. Reduction in influent nitrate concentrations resulted in a stoichiometric increase in column effluent toluene concentrations.

*Keywords:* Denitrification; Aromatics; In situ; Biodegradation; Toluene

## 1. Introduction

The identification and remediation of hazardous chemicals in subsurface soils and groundwater is a high priority in much of the United States. Of the major contaminants found in soils and groundwater, benzene, toluene and xylenes (BTX) are often the predominant species, especially in areas contaminated by refined petroleum products. Most of these hydrocarbons are biodegradable and are good candidates for cleanup using bioremediation technologies. To ensure effective cleanup and closure

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of a contaminated matrix, it is necessary to adopt a compatible remedial strategy and to design effective monitoring for proper characterization and assessment. In general, hydrocarbons are more readily and more completely degraded under aerobic conditions, where oxygen serves both as a reactant and as an electron sink for metabolites [1, 2]. Although less efficient thermodynamically, the anaerobic biodegradation of aromatic hydrocarbons also occurs under a variety of environmental conditions [3–5]. Anaerobic processes may be more practical for the in situ treatment of groundwater and soils, where low solubility of oxygen and poor delivery to the subsurface restricts the availability of oxygen to serve as the electron acceptor. In the absence of oxygen, denitrification has been shown to be an effective process for biodegradation with nitrate serving as the terminal electron acceptor.

The anaerobic biodegradation of toluene under denitrifying conditions in batch and column studies has been reported by a number of independent groups, utilizing pure and mixed cultures [6–19]. During biodegradation under denitrifying conditions, nitrate is reduced through a series of steps, through nitrite, nitrous oxide and finally dinitrogen gas, with the concomitant oxidation of the carbon substrate to carbon dioxide. The use of nitrate in the field may offer a cost-effective alternative to oxygen or hydrogen peroxide addition; it is highly soluble and diffuses readily in groundwater. Field experiments have had mixed results. Berry-Sparks [20], Lemon [21], and Hutchins [22] are among several, who have demonstrated successful in situ removal of toluene under denitrifying conditions. Acton [23] found no evidence of aromatic hydrocarbon (toluene) degradation in field columns remediated with nitrate. In some cases, even though toluene losses are observed, it was not possible to determine the driving reaction behind the removal [24].

The in situ remediation of a site using denitrification often involves injecting nitrate into the subsurface. Injection of nitrate can pose regulatory problems, especially if there is potential for migration into groundwater. US EPA regulations require that total nitrate and nitrite concentrations must be maintained below 10 mg/l as nitrogen, because high nitrate levels exhibit toxicity in humans [25]. In order to maintain levels below drinking water standards, designs for field implementation must ensure that only low levels of nitrate are added to the subsurface. For example, Hutchins [22] added 10 mg/l nitrate-nitrogen to remediate a site contaminated with JP-4 jet fuel. In another study, Berry-Spark [20] added 44 mg/l N as nitrate to remediate groundwater containing average BTEX concentrations of 0.8 mg/l; in another, Lemon [21] added 338 mg/l of nitrate to remediate 2.6 mg/l of toluene.

When higher levels of contamination are present, larger quantities of nitrate need to be added in order to supply sufficient nitrate to the site. If low levels of nitrate are injected in these situations, large quantities of water will also be required. This can lead to dilution and migration of the plume. In order to minimize dilution and migration and maximize degradation rates, higher levels of nitrate need to be added to the subsurface. Prior to adding higher levels of in situ nitrate, the stoichiometry for nitrate additions needs to be clearly delineated in order to prevent excess addition of nitrate to groundwater. Eq. (1) demonstrates the molar stoichiometry for the complete biodegradation of toluene the electron donor, concurrent with complete reduction of nitrate, the electron acceptor to dinitrogen gas.

$$C_7H_8 + 6NO_3 \rightarrow 7CO_2 + 4H_2O + 3N_2. \quad (1)$$

From the equation, the idealized stoichiometric ratio of toluene to nitrate for complete biodegradation is 1:6. Empirically, a sixfold increase in nitrate over toluene does not always appear to be necessary. For example, in pure cultures, Evans [15] demonstrated that smaller quantities of nitrate are sufficient; the stoichiometry of toluene oxidation to nitrate reduction was 1:4.5. In order to take advantage of the increased biodegradation rates due to nitrate additions, nitrate needs to be present in sufficient quantities so as not to be a limiting factor. However, in order not to exceed drinking water standards a tight control needs to be maintained on the nitrate additions.

The objectives of this study were: (1) to demonstrate nitrate loading as a control variable for the rate and extent of toluene degradation; (2) to maintain nitrate and toluene effluent concentrations below drinking water standards under continuous flow conditions; and, (3) to conduct treatability studies prior to a field demonstration delineating the effect of nitrate loading on toluene degradation. This project simulated field conditions in the laboratory by using contaminated soil and groundwater in batch and column biodegradation studies. The column experiments had continuous groundwater flow and were packed with soil from the site. Toluene concentrations approximating 50% saturation, 200 mg/l, were degraded. Carbon and nitrogen mass balances were completed. A goal during these experiments was to maintain the effluent nitrate and toluene concentrations below drinking water standards. Materials, soil and groundwater, used for all the described experiments were obtained from an industrial site. The primary contaminant at the site was toluene from spills or leaking underground storage tanks. Average toluene concentration in the groundwater was approximately 200 mg/l. The pH of the groundwater was 6.65; the pH of the soil was 6.1. The contaminated soil was a sandy loam with 0.45% organic matter. Toluene contamination in the soil ranged from 0 to 2400 mg/kg dry soil.

## 2. Experimental design and methods

### *2.1. Batch experiments*

Batch studies were carried out to determine the biodegradation potential for toluene degradation using field site materials. Contaminated groundwater obtained from the site was incubated in duplicate, sealed serum bottles (without soil), containing one of three toluene concentrations (115, 160, 210 mg/l). Serum bottles were sealed with Teflon-coated butyl rubber septa and aluminum crimp caps (Wheaton, NJ). All bottles were purged with helium or argon in order to maintain anoxic conditions. The groundwater was placed in each serum bottle purged with helium or argon. All serum bottles received 22 ml of inoculum, nitrate (50% excess of stoichiometric amount), nutrients and phosphate buffers [26]. Controls to monitor abiotic activity were established by adding 5 ml of 5 wt% mercuric chloride solution to

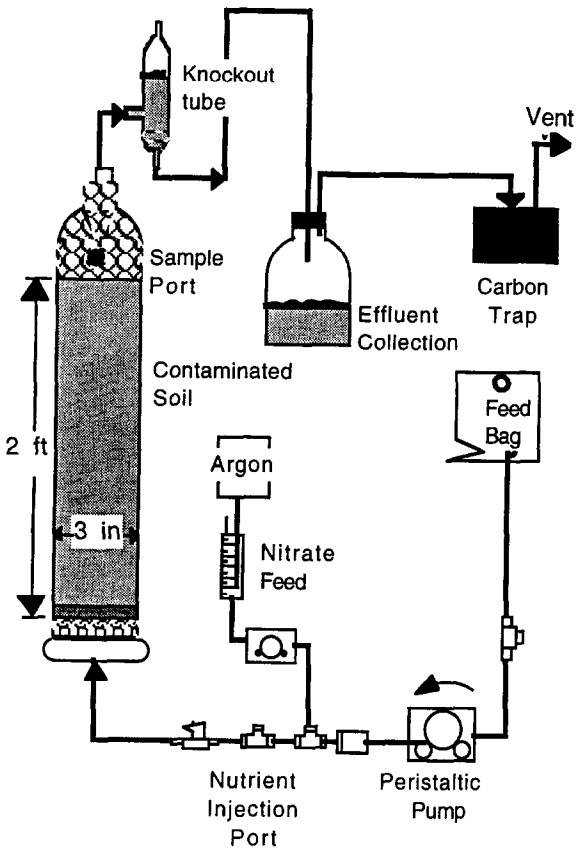


Fig. 1. Schematic of apparatus used for soil columns experiments.

the serum bottle. Samples were taken at time zero for initial toluene and nitrate concentrations. Subsequent samples were taken at 2 week intervals. Incubations were for a total of 10 weeks.

### 2.2. Column experiments

Laboratory soil column experiments were carried out to more closely simulate biodegradation of toluene under field conditions, and to observe toluene and nitrate stoichiometries. Laboratory soil columns were constructed of 3″ ID × 24″ Kimax process pipe (Lab Glass, Vineland, NJ) as depicted in Fig. 1. The sample ports were fitted with Teflon Mininert valves (Supelco, Inc., Bellefonte, PA), with 0.02″ Teflon tubes inserted into the valves. The Teflon tubes allowed a sample to be obtained from the center of the columns with little perturbation and reduced wall effects. The feed bags were five-layer aluminized gas sampling bags (Calibrated Instruments, Ardsley, NY), modified to accept Swagelok fittings. The feed bags were pre-exposed with groundwater to minimize any adsorption losses. Cassette pumps (Manostat,

Inc., NY) were used to pump both feeds into the columns. Viton tubing (1/8″ ID) was used within the cassettes. The gas produced from the system was collected in knockout tubes (Lab Glass, Vineland, NJ). Each knockout tube was filled with water; as the effluent from the column entered the tube, the gas in the effluent displaced the liquid and collected at the top. The nitrate feed was contained with a modified 60 ml plastic syringe, which was continuously purged with argon. The plunger was replaced by a stopper with two inserted 1/8″ stainless steel tubes. One tube was connected to an argon supply and extended into the nitrate solution; the second served as a vent. Nitrate feed solutions were separated from the groundwater feed to prevent bacterial growth in the feed bag. All valves, filters and fittings were stainless steel or Teflon.

Four glass columns were packed with contaminated soil from the site and received a continuous feed of contaminated groundwater for 26 weeks. Two of the columns (columns 1 and 2) were inoculated with an enriched microbial culture from the previously developed batch incubations; the other two columns (columns 3 and 4) were not inoculated. Columns 1 and 2 received a continuous feed of nitrate and nutrients in addition to the contaminated groundwater. The influent toluene concentration ranged from 100 to 250 mg/l. The nitrate feed was adjusted to deliver a toluene to nitrate molar ratio of 1:5. Two of the four columns, column 3 and 4, were not inoculated and were expected to serve as a control to monitor the breakthrough of toluene.

Each of the four columns received a continuous groundwater feed at a rate of 200 ml/d. This resulted in a superficial velocity of 6 in/d and a mean residence time of 4 d. The average toluene concentration in each feed was 200 mg/l. All feeds contained 0.6 g/l $K_2HPO_4$ and 0.1 g/l $KH_2PO_4$ for pH buffering, and 2 g/l $MgCl_2 \cdot 6H_2O$ to maintain a sodium adsorption ratio less than 2. A concentrated nitrate feed solution (24 000 mg/l) was diluted into the toluene feed to achieve an effective molar ratio of toluene to nitrate of 1:5, with an effective influent nitrate concentration of 670 mg/l. The nutrient solution contained $NH_4Cl$ and trace metals [26]. Hydraulic dispersion within the columns was quantified by tracer studies using bromide. The mean retention time within the columns was 4 d with an effective dispersion coefficient of 45 $cm^2/d$. The columns were operated in an up-flow manner. Operations were conducted so as to keep the system as anoxic as possible. The nitrate feed was boiled and purged with argon to prevent any bacterial growth in the feed and to remove any molecular oxygen. The pH of the columns was monitored weekly and was maintained between 6.5–7.0 with phosphate buffering.

A nitrate withdrawal method was utilized in order to regulate substrate utilization in columns 1 and 2. The nitrate feed to the active denitrifying columns, columns 1 and 2, was reduced from a toluene to nitrate ratio of 1:5 to 1:4, then to 1:3, for 7–10 d each. The effluent from the columns was monitored daily to determine the effects of reduced nitrate on the effluent toluene concentration.

### *2.3. Analytical methods*

The composition of the gas collected in the knockout tube was analyzed for carbon dioxide, methane, dinitrogen and nitrous oxide using a Hewlett Packard 5880A

gas chromatograph equipped with a thermal conductivity detector. Two matched packed stainless steel columns (id = 0.125 in), packed with 100/120 Carbosieve S-II packing-10 (Supelco, Bellefonte, PA), were used; helium served as the carrier gas (25 ml/min flow rate). The oven temperature was 150 °C, and the injector and detector temperatures were maintained at 210 °C. The detection limit for target gases was 0.2 μmol in an injection volume of 100 μl.

For toluene analysis, samples from the influent and effluent were extracted with equal amounts of pentane, followed by analysis using a Hewlett Packard 5890 GC, and a photoionization detector (HNU Systems – Model PI-52-02A). The pentane samples were injected onto a 5% SP-1200/1.75% Bentone 34 in 100/120 Supelcoport column (Supelco, Bellefonte, PA) 6 ft × 0.125 in. Helium served as a carrier gas with a flow of 30 ml/min. The oven temperature was isothermal at 70 °C. The detection limit for toluene was 0.055 mg/l.

For analysis of anions, influent and effluent concentrations were analyzed isocratically using a Dionex 4500i ion chromatograph. Nitrate, nitrite and bromide concentrations were determined using an Ion Pac AS9 ion exchange column, an anion micromembrane suppressor unit, and a conductivity detector. The detection limit for nitrate, bromide and nitrite was 0.5 mg/l.

## 3. Results

### *3.1. Batch incubations*

The batch experiments demonstrated that toluene degradation was feasible using contaminated field site materials. Results indicate that toluene loss was coupled with gas production and nitrate reduction. All incubations, except for the controls, showed a complete loss of toluene within 8 weeks (Fig. 2). The highest toluene concentration tested was approximately 210 mg/l (equivalent to 50% saturation). An inoculum from these batch experiments was used to inoculate the column experiments.

### *3.2. Column incubations for toluene removal, nitrate usage and gas production*

Results from the column study indicated biodegradation occurred under denitrifying conditions. Fig. 3 presents the influent and effluent toluene concentrations for columns 1 and 2, over the 35 weeks of operation. Beginning at week 20, there is an increasing trend in toluene concentration, due to a reduction of nitrate in the feed. The observed higher concentration of toluene in the effluent following week 20 demonstrates a dependency on nitrate (electron acceptor).

Fig. 4 shows total toluene mass in the influent and the effluents of columns 1 and 2, from weeks 1 to 19. Later weeks were not included in these calculations since toluene biodegradation was constrained by reduced nitrate concentrations which limited activity. From Fig. 4, it can be seen that a total of 4400 mg of toluene were fed into column 1 and only 10 mg were recovered in the effluent during the first 19 weeks of operation. This is a 99.7% reduction in the toluene entering the system. In

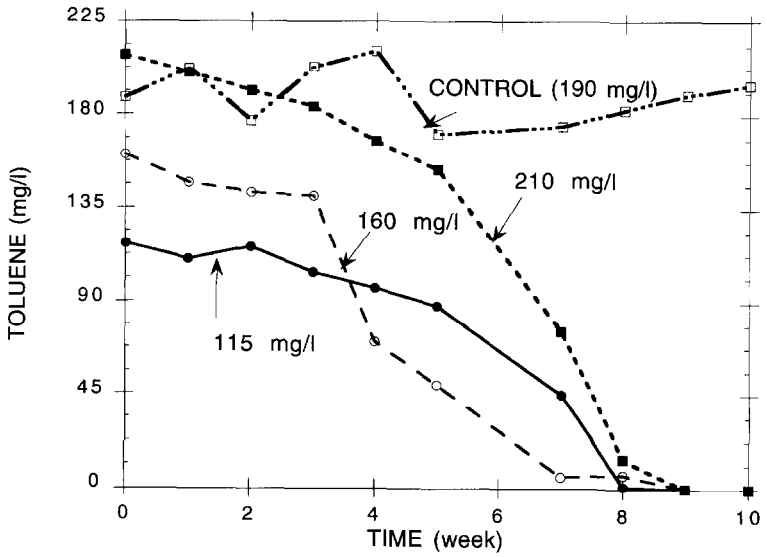


Fig. 2. Batch toluene degradation using contaminated groundwater. Toluene was degraded up to 210 mg/l.

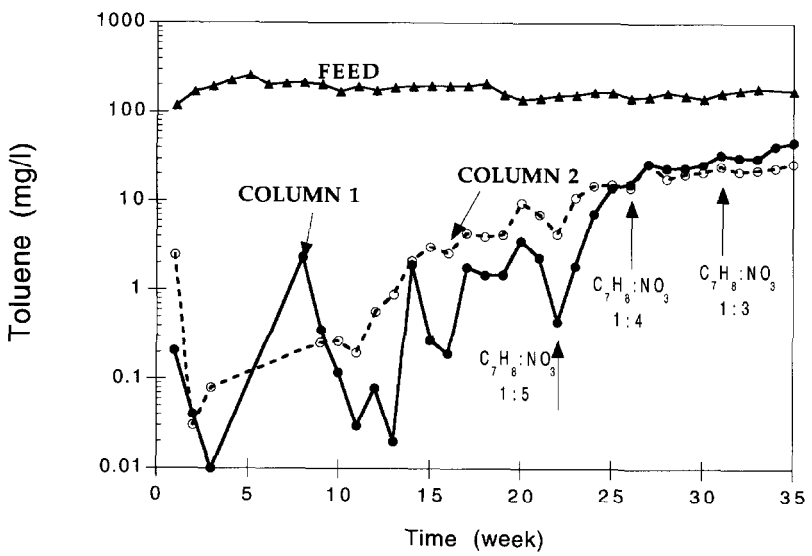


Fig. 3. Toluene influent and effluent concentrations during weeks 1–35 for columns 1 and 2, the two active denitrifying columns.

column 2, a total of 5200 mg of toluene were present in the influent, with 30 mg toluene remaining in the effluent. This represents a 99.4% reduction of the total toluene fed into the system.

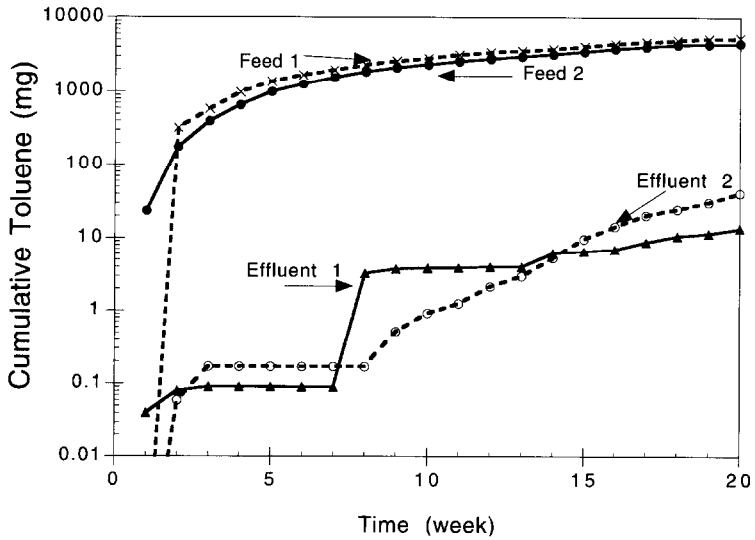


Fig. 4. Cumulative toluene influent and effluent concentrations during weeks 1–19 for columns 1 and 2, the two active denitrifying columns.

With the toluene losses, there was a concomitant reduction of nitrate in the influent by 99.9%, to an average effluent concentration of less than 0.5 mg/l. Fig. 5(a) and (b) presents influent and effluent nitrate and nitrite concentrations in columns 1 and 2, respectively, over 35 weeks of operation. Nitrate concentrations were well below the primary drinking water standards [13] during the course of the experiment, except on one occasion when the groundwater feed was interrupted during week 21 (e.g. Fig. 5(a)). A total of 27 000 mg of nitrate were fed into column 1, and a total of 800 mg nitrate and 10 mg nitrite exited the system, demonstrating an overall reduction of 97%. Column 2 received a similar loading of nitrate (27 000 mg), but exhibited higher levels of nitrate and nitrite remaining in the effluent (2000 and 120 mg, respectively). Most of the nitrate exiting column 2 was during the first week of operation, indicating that denitrifying activity was not established as rapidly as in column 1. If the first week of nitrate effluent data are removed from the calculations, then the total nitrate in the effluent is 500 mg and nitrite 90 mg. This corresponds to a reduction in nitrate of 98% in column 2. The stoichiometric ratio of toluene utilization to nitrate reduction during this interval was 1:5 for both columns.

Fig. 6(a) and (b) presents the effluent gas compositions for columns 1 and 2, respectively. Gas composition in the effluent gas for column 1 was 87.3% $N_2$, 2.9% $CH_4$, 7.7% $CO_2$, 2.1% $N_2O$; for column 2 it was 86.9% $N_2$, 3.5% $CH_4$, 8.5% $CO_2$, and 1.2% $N_2O$. The presence of nitrous oxide in the gas effluent provides additional evidence for denitrifying activity. Dissolved nitrous oxide concentrations were calculated based on the gaseous measurements, Henry's Law and published solubility coefficients [27], and closed the nitrogen mass balance. Methanogenic activity was observed in the control columns, columns 3 and 4, after 1 week. Complete

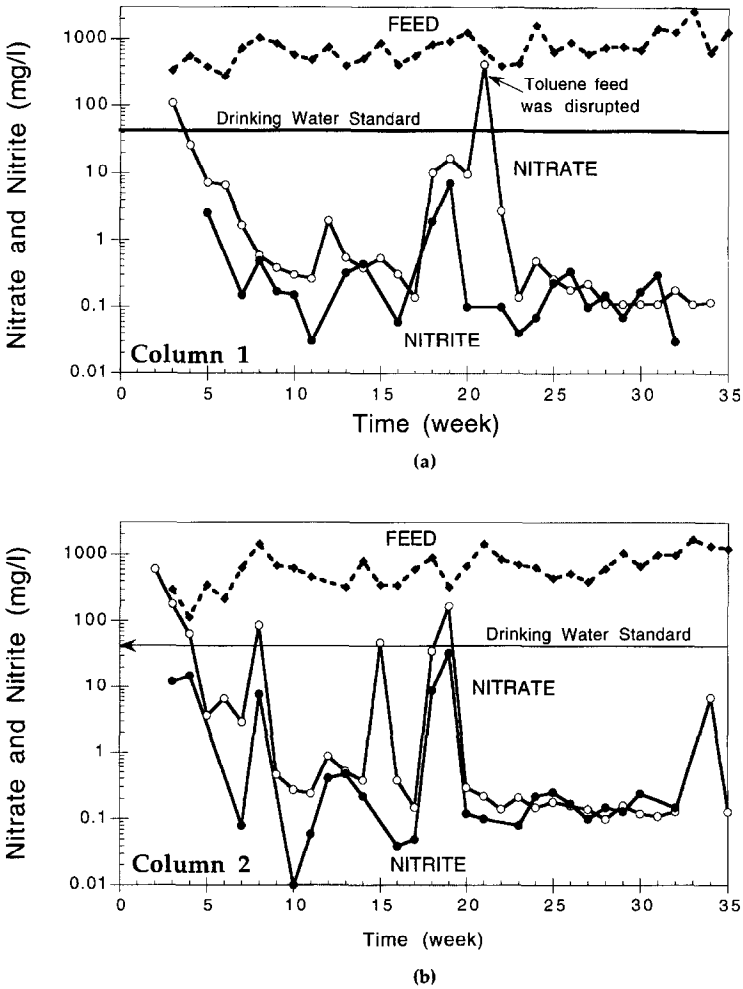


Fig. 5. (a) Nitrate influent and effluent concentrations for column 1 during weeks 1–35. (b) Nitrate influent and effluent concentrations for column 2 during weeks 1–35.

removal of toluene and gas production was observed after 1 week. The average gas composition of the control columns was 61% $CH_4$, 33% $N_2$, 5% $CO_2$ and 1% $N_2O$.

### 3.3. Carbon mass balance

A total molar carbon balance is presented in Table 1 for columns 1 and 2. The total mass balance reflects activity during a three week period, from week 16 to 18. The total carbon entering the columns includes toluene and dissolved carbon dioxide in the groundwater feed. Carbon recovery from the column effluents includes

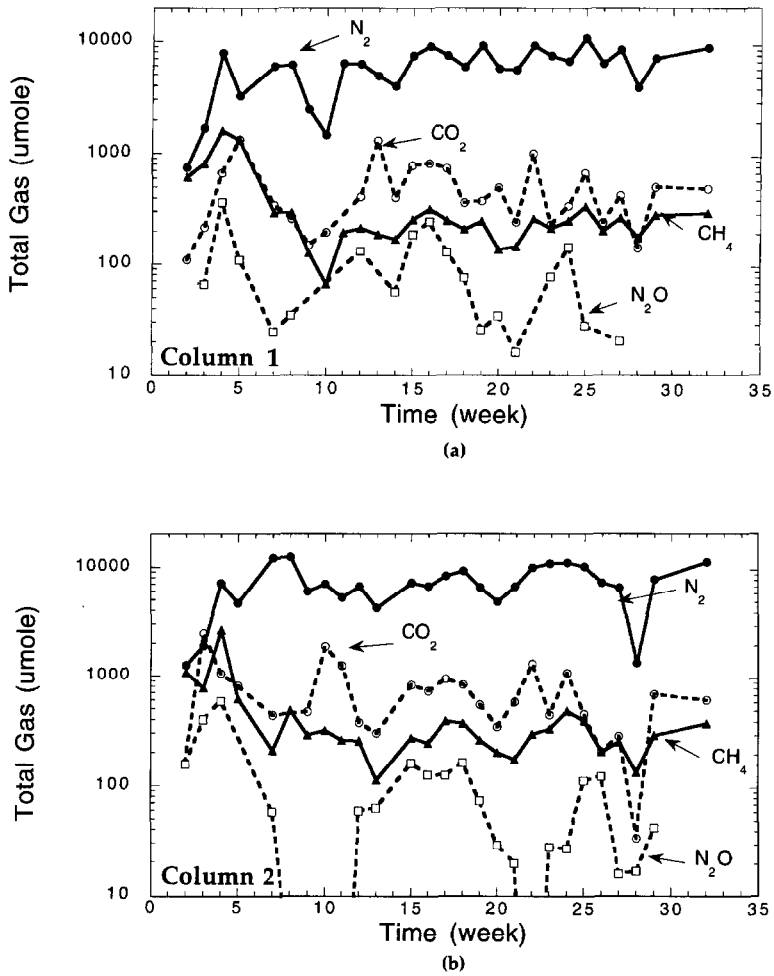


Fig. 6. (a) Weekly gas composition of column 1 during weeks 1–35. (b) Weekly gas composition of column 2 during weeks 1–35.

residual toluene and dissolved carbon dioxide in the aqueous phase, and carbon dioxide and methane in the gaseous phase. Of the total toluene carbon entering the system, 42.3% and 40.0% were accounted for as $CO_2$ in columns 1 and 2, respectively. Methane accounted for 1.4% and 1.7% of the total toluene carbon in columns 1 and 2. A cell growth term, calculated according to McCarty (1969) [28], accounted for conversion of 30% of the total toluene carbon to biomass. The carbon mass balance was closed within 25%. This error may be attributable to underestimation of the cell mass term [29] or due to the accumulation of metabolic intermediates which were not quantified [30]. Attempts to isolate the intermediates were unsuccessful.

Table 1
Carbon mass balance for soil columns 1 and 2 during weeks 16–18

| | Total $C_{in}$ (mmol) | Total $C_{out}$ (mmol) | Toluene $C^{a}_{out}$ (%) |
|---|---|---|---|
| Column 1 | | | |
| $CO_2$ | 13.5 | 36.3 | 42.3 |
| $CH_4$ | 0.0 | 0.8 | 1.4 |
| $C_7H_8$ | 53.9 | 0.2 | 0.4 |
| Estimated cell mass | 0.0 | 16.1[b] | 30.0[b] |
| Total | 67.4 | 56.8 | 74.1[c] |
| Column 2 | | | |
| $CO_2$ | 13.7 | 36.9 | 40.0 |
| $CH_4$ | 0.0 | 1.0 | 1.7 |
| $C_7H_8$ | 58.0 | 0.9 | 1.6 |
| Estimated cell mass | 0.0 | 17.6[b] | 30.0[b] |
| Total | 71.7 | 56.8 | 73.3[c] |

[a] Toluene carbon only.
[b] Estimated based on [28].
[c] Error due to cell mass term, metabolite or undefined losses.

### 3.4. Nitrogen mass balance

Table 2 summarizes the nitrogen mass balances for columns 1 and 2. The sole source of electron acceptor for the system was nitrate added as a feed solution. A total of 87.0% and 93.4% of the total nitrogen was accounted for as dinitrogen gas, in the effluent for columns 1 and 2, respectively. Nitrous oxide accounted for 14.6% and 9.6% of the total nitrogen in columns 1 and 2. Residual nitrate and nitrite were less than 0.1% and 0.001%, respectively, in both the columns. The balances accounted for 102% and 103% of the total nitrogen in columns 1 and 2, and demonstrate good recovery.

### 3.5. Verification of denitrifying activity

Verification of denitrification activity was evidenced by a decrease in the nitrate feed concentration in the feed with a concomitant increase in effluent toluene concentration. A demonstration of this dependency was tested by reducing the nitrate feed and monitoring toluene breakthrough (week 19–26). The data presented in Fig. 7 show the increase in the column effluent toluene concentrations following nitrate reduction. There was a 4 d lag period, equal to one mean residence time, before the effects of reduced nitrate loading were observed. A breakthrough of toluene was observed with each step of reduction of the nitrate feed. The stoichiometric ratio of toluene to nitrate feed was varied from 1:5 to 1:4 to 1:3. During this transition, toluene degradation decreased from 98% to 90% and finally to 83%. The overall stoichiometry of toluene utilization to nitrate reduction observed over the seven week period remained constant at 1:5. An increase in methane

Table 2
Nitrogen mass balance for columns 1 and 2 during weeks 16–18

| | Total $N_{in}$ (mmol) | Total $N_{out}$ (mmol) | $NO_3$ $N^a_{out}$ (%) |
|---|---|---|---|
| Column 1 | | | |
| $N_2$ | 13.7[b] | 45.8 | 87.0 |
| $N_2O$ | 0.0 | 5.4 | 14.6 |
| $NO_3$ | 36.8 | 0.0 | 0.1 |
| $NO_2$ | 0.0 | 0.0 | 0.0 |
| Total | 50.5 | 51.2 | 101.7 |
| Column 2 | | | |
| $N_2$ | 14.2[b] | 47.9 | 93.4 |
| $N_2O$ | 0.0 | 3.4 | 9.4 |
| $NO_3$ | 36.1 | 0.9 | 0.1 |
| $NO_2$ | 0.0 | 0.0 | 0.0 |
| Total | 50.3 | 52.2 | 102.9 |

[a] Nitrate N only.
[b] Calculated based on $N_2$ infiltration into control columns.

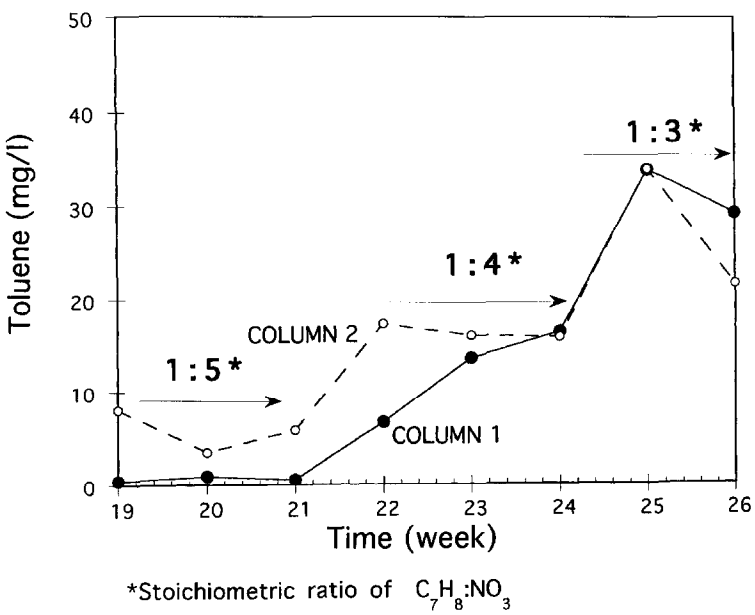


Fig. 7. Increase in effluent toluene concentrations in columns 1 and 2 during weeks 19–26, due to nitrate feed reduction. Influent toluene concentrations for both columns during this period was 200 mg/l.

production was observed as the nitrate feed was reduced, suggesting that methanogenic populations were becoming more active as the nitrate electron acceptor was depleted.

## 4. Conclusions

The utilization of nitrate was clearly linked to toluene degradation, both in the batch and soil column incubations. The coupling between the reduction of nitrate and oxidation of carbon substrates has been demonstrated by other researchers [16, 17]. Based on these earlier studies, the theoretical stoichiometric relationships used in this study demonstrated that input nitrate concentrations can be adjusted to levels which degrade the toluene, yet maintain effluent nitrate concentrations below 1 mg/l. The experimental results described here demonstrate an effluent nitrate concentration of less than 0.5 mg/l. Toluene was reduced from approximately 200 mg/l in the influent to effluent concentrations between 0.93 and 3.2 mg/l, representing greater than a 99% reduction in contaminant levels.

The carbon mass balances accounted for approximately 74% of the total carbon entering the system, with 26% of the carbon unaccounted for. The nitrogen mass balance accounted for 102% and 103% of the total nitrogen entering columns 1 and 2, respectively.

Any field implementation of a denitrifying system will require careful stoichiometric control to ensure compliance with the nitrate and nitrite regulatory limits along with consideration of additional site specific conditions. This project clearly demonstrated that nitrate and nitrite concentrations can be maintained within drinking water standards while achieving enhanced toluene degradation. With careful monitoring and control it may be feasible to add larger quantities of nitrate in situ, and enhance biodegradation rates.

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