Biodegradation **6:** 147–156, 1995.

Stoichiometry and kinetics of microbial toluene degradation under denitrifying conditions

Claus Jørgensen[1], John Flyvbjerg[1], Erik Arvin & Bjørn K. Jensen[1]
Institute of Environmental Science and Engineering, Technical University of Denmark, Building 115, DK-2800 Lyngby; [1]*Present address: VKI Water Quality Institute, Agern Allé 11, DK-2970 Hørsholm; (* Corresponding author)*

Received 12 January 1994; accepted 26 September 1994

Key words: bacteria, degradation, denitrification, kinetics, stoichiometry, toluene

Abstract

Batch experiments were carried out to investigate the stoichiometry and kinetics of microbial degradation of toluene under denitrifying conditions. The inoculum originated from a mixture of sludges from sewage treatment plants with alternating nitrification and denitrification. The culture was able to degrade toluene under anaerobic conditions in the presence of nitrate, nitrite, nitric oxide, or nitrous oxide. No degradation occurred in the absence of Noxides. The culture was also able to use oxygen, but ferric iron could not be used as an electron acceptor. In experiments with ^{14}C-labeled toluene, 34% ± 8% of the carbon was incorporated into the biomass, while 53% ± 10% was recovered as $^{14}CO_2$, and 6% ± 2% remained in the medium as nonvolatile water soluble products. The average consumption of nitrate in experiments, where all the reduced nitrate was recovered as nitrite, was 1.3 ± 0.2 mg of nitrate-N per mg of toluene. This nitrate reduction accounted for 70% of the electrons donated during the oxidation of toluene. When nitrate was reduced to nitrogen gas, the consumption was 0.7 ± 0.2 mg per mg of toluene, accounting for 97% of the donated electrons. Since the ammonia concentration decreased during degradation, dissimilatory reduction of nitrate to ammonia was not the reductive process. The degradation of toluene was modelled by classical Monod kinetics. The maximum specific rate of degradation, k, was estimated to be 0.71 mg toluene per mg of protein per hour, and the Monod saturation constant, K_s, to be 0.2 mg toluene/l. The maximum specific growth rate, μ_{max}, was estimated to be 0.1 per hour, and the yield coefficient, Y, was 0.14 mg protein per mg toluene.

Abbreviations: NVWP – Non Volatile Water-soluble Products

Introduction

Microbial degradation of aromatic hydrocarbons has often been assumed to depend on the presence of molecular oxygen, because of oxygen's dual role as a substrate in the opening of the aromatic ring and as the terminal electron acceptor (Shink 1988). Nevertheless, anaerobic degradation of aromatic hydrocarbons has been reported several times during the last decade. In particular, degradation coupled to reduction of nitrate has gained much attention because of the potential role of nitrate as an oxidizing agent in bioremediation of gasoline-polluted groundwater. The first reliable report was from Kuhn et al. (1985), who demonstrated that degradation of *m*- and *p*-xylene could be coupled to reduction of nitrate. Since then, toluene, *o*-xylene, 1,2,4-trimethylbenzene, ethylbenzene, 3-ethylmethylbenzene, and butylbenzene have been shown to be degraded by denitrification (Zeyer et al. 1986; Jensen et al. 1988; Major et al. 1988; Evans et al. 1991a,b; Hutchins et al. 1991; Seifried et al. 1991; Jørgensen et al. 1991). Benzene (Major et al. 1988) and naphthalene (Mihelcic and Luthy 1988; Al-Bashir et al. 1990; Bouwer et al. 1992) have also been reported to be degradable under nitrate reducing-conditions, but the literature is contradictory with respect to these compounds.

If denitrifying bacteria are to be used in bioremediation of gasoline-polluted groundwater, an understanding of the stoichiometry and kinetics of the degradation is required. The aim of the work presented in this paper was to determine the stoichiometry and the kinetics of toluene degradation by a mixed culture of denitrifying bacteria.

Experiments were set up to characterize the culture with respect to: (1) the interdependence of toluene degradation, nitrate reduction, microbial activity, and consumption of ammonia; (2) the growth yield and degree of mineralization during the degradation of ^{14}C-labeled toluene; (3) the capability of the culture to utilize electron acceptors other than nitrate for toluene degradation and to degrade aromatic compounds other than toluene with nitrate as electron acceptor; and (4) the kinetic parameters of toluene degradation.

Materials and methods

Chemicals

Nitric oxide was synthesized by the method of Blanchard (1946) in oxygen-free 117 ml serum bottles. Acetylene was synthesized by addition of anaerobic water to an oxygen-free 117 ml serum bottle containing calcium-carbide of technical grade and obtained from BHD Limited, Poole, England.

^{14}C-toluene was obtained from Amersham Int. plc, Buckinghamshire, England. The [Ring-1-^{14}C]toluene had a radiochemical purity of 96% and a specific activity of 3.1 MBq/mg. The [Methyl-^{14}C]toluene had a radiochemical purity of 97.7%, and a specific activity of 18.5 MBq/mg.

All other reagents were obtained from Merck, Darmstad, Germany and were of analytical grade.

Inoculum source

The inoculum used originated from sewage treatment plants with alternating nitrification and denitrification. Before the experiments described in this paper were conducted, the culture had been exposed to a mixture of alkylbenzenes and methylated phenols for more than a year under denitrifying conditions (Jensen et al. 1988).

Experimental set up

The experiments were carried out in 1.25 l bottles. Samples were taken through a stopper mounted with a valve. All materials were made in glass to prevent volatilization and minimize sorption. The culture was grown in a nutrient medium with the following composition (adapted from Platen and Schink (1989)): 161 mg/l $Na_2SO_4 \cdot 10H_2O$, 204 mg/l KH_2PO_4, 1260 mg/l $NaHCO_3$, 198 mg/l NH_4Cl, 990 mg/l NaCl, 500 mg/l KCl, 147 mg/l $CaCl_2 \cdot 2H_2O$, 407 mg/l $MgCl_2 \cdot 6H_2O$. The mineral medium and $NaNO_3$ were added to the bottles and subsequently autoclaved. A filter-sterilized vitamin solution and trace metals (Platen and Schink 1989) were added after autoclaving, and the pH was adjusted to 7.2 $\pm$ 0.1 with HCl or NaOH. Oxygen was removed to below 0.5 mg O_2/l by purging with oxygen-free N_2/CO_2 gas ($O_2 < 0.5$ ppm, $CO_2 = 300$ ppm, Hydro Gas, Rjukan, Norway), and the bottles were sealed. The remaining oxygen was reduced by addition of 10 ml of a 60 mM Na_2SO_3 solution. Finally, the inoculum and toluene from a stock solution (500 mg/l) were added to achieve the desired concentrations. The final volume was 1 l. The bottles were incubated in the dark at 20 $\pm$ 2 °C on magnetic stirrers. The experiments were performed using the inoculum described above as the first-generation inoculum. In the successive experiments, bacteria from the previous experiment were used as inoculum.

Measurements of growth yield and mineralization were carried out with ^{14}C-labeled toluene. First, to obtain an active culture 3–5 mg/l of unlabeled toluene was degraded to below the detection limit, a process that took about one week. Thereafter, 2.7 mg/l [ring-1-^{14}C]toluene (15 kBq/mg toluene) was added to 2 bottles and 2.8, 3.0, 3.4 and, 3.4 mg/l [methyl-^{14}C]toluene (220–250 kBq/mg toluene) were added to 4 bottles. Triplicate samples for measurements of $^{14}CO_2$, bacterial ^{14}C, ^{14}C-nonvolatile water-soluble products (NVWP), NO_3^-, and NO_2^-, and double samples for ^{14}C-toluene and total toluene were taken immediately after addition of labeled toluene and after 4 h of incubation. The total amount of $^{14}CO_2$ and toluene in the bottles was calculated under the assumption of equilibrium between liquid and headspace. It was assumed that the partition coefficients between air and liquid were 0.23 for toluene (Ashworth et al. 1988) and 1.12 for CO_2 (Kavanaugh and Trussel 1980). The distribution of ^{14}C between the fractions was calculated as the difference between the activities at the beginning

and at the end of the experiment relative to the activity of the degraded toluene.

Mathematical modeling of toluene degradation

The kinetics of toluene degradation was modelled by the following Monod batch equation adapted for an air-liquid system (Broholm et al. 1990):

$$\frac{dS}{dt} = -\frac{V_L}{V_L + fV_G} k \frac{S}{K_S + S} X \qquad (1)$$

where S is the toluene concentration (mg/l) , X is the biomass concentration (mg protein/l), k is the maximum specific utilization rate (mg toluene per mg of protein per hour) and K_s is the Monod saturation constant (mg toluene/l). V_L and V_G are the volumes of liquid and headspace in the bottle, and f is the partition coefficient between air and liquid for toluene. The change in V_L owing to sampling was only 5% during the experiment, and therefore V_L and V_G were assumed to be constant.

The growth rate of bacteria was modelled by the following equation:

$$\frac{dX}{dt} = Yk\frac{S}{K_S + S} X - bX \qquad (2)$$

where Y is the yield coefficient (mg protein per mg of toluene) and b is the decay constant for biomass (per hour).

The rates of nitrate consumption and nitrite production were modelled by the following equations:

$$\frac{dS_{NO_3}}{dt} = -\alpha_{NO_3} k \frac{S}{K_S + S} X \qquad (3)$$

$$\frac{dS_{NO_2}}{dt} = \alpha_{NO_2} k \frac{S}{K_S + S} X \qquad (4)$$

where S_{NO3} is the concentration of nitrate (mg NO_3-N/l), S_{NO2} is the concentration of nitrite (mg NO_2-N/l), α_{NO3} is the stoichiometric coefficient for the reduction of nitrate (mg NO_3-N per mg toluene), and α_{NO2} is the stoichiometric coefficient for the production of nitrite (mg NO_2-N per mg toluene).

Control experiments

Control experiments were carried out to estimate abiotic losses of toluene, to prove that inoculation and NO_3^- are necessary for degradation, and finally, to test whether oxygen accumulated in the biologically inactive bottles. Two bottles were used. One was not inoculated, and one was inoculated without NO_3^-. 20 ml samples were taken every half hour for 10 h. The toluene concentration in the medium decreased between 12% and 14% in the bottles, because of the volatilization of toluene into the increasing headspace. The change did not differ significantly from the decrease calculated with a theoretical partitioning model. After two weeks and after 4 months, no further decrease in the toluene concentration was observed, and no oxygen could be detected.

Analytical methods

Toluene was measured isothermically at 50 °C on a DANI 8520 gas chromatograph/FID (Merck, Denmark) equipped with a 30 m J&W DB5 capillary column. The carrier gas was N_2 at a flow of 10 ml/min. The FI-detector temperature was 275 °C. 10 ml samples were extracted in 1 ml of pentane with 4 mg heptane/l as the internal standard. Peak areas were calculated on a Shimatzu C-R3A Chromatopac integrator, and concentrations were calculated on the basis of standard samples.

NO_2^- was measured on a Technicon Autoanalyzer^{TMII} at 545 nm. NO_3^- was measured as NO_2^- after reduction with hydrazine sulphate (Standard Methods 1989). Interfering cations were removed by a cation exchanger prior to reactions. Ammonium was measured on a Technicon Autoanalyzer^{TMII} at 630 nm (DS 224).

N_2O was measured on a Carlo Erba gas chromatograph/ECD equipped with a 3 m Poropak Q glas column packed with ethylvinylbenzene, mesh size 80–100, and a mol sieve 5A reference collumn. The carrier gas was He at a flow of 30 ml/min, and the makeup gas was nitrogen at 80 ml/min. The oven temperature was 45 °C, the injection temperature was 105 °C, and the detector temperature was 300 °C. Injection took place through a 0.32 ml loop. The peak areas were calculated on a Maxima 820 Chromatographic Workstation (Millipore Corp. Massachusetts), and concentrations were calculated on the basis of standard samples.

Protein was measured at 595 nm on a Perkin Elmer Lamda 2 spectrophotometer as described by Gälli and McCarthy (1989) according to the method of Bradford (1976), with bovine serum albumin as a standard.

Oxygen was measured by the azide modification of the Winkler method (Standard Methods, 1989). pH was measured on a JK 9200 pH-meter (Buck and Holm, Denmark).

^{14}C was measured on a TRICARB 2000 Liquid Scintillation Counter (Hewlett Packard Instruments Int., Zürich) in 20 ml plastic vials. 10 ml samples were extracted into 1 ml of pentane, and the ^{14}C-toluene activity was determined from the activity in a 400 μl pentane subsample counted in 15 ml lipoluma scintillation liquid (Lumac BV, Schaesberg, Holland). 5 ml samples were filtrated through 0,2 μm cellulose nitrate filters (Satorius AG, Goettingen, Germany), and the activity associated with the biomass was determined by counting the filter in 15 ml Aqualyt scintillation liquid (JT Baker Chemicals BV, Deventer, Holland). Prior to counting, the filters were washed with 5 ml of distilled water to remove NVWP and with 100 ml of water saturated with toluene to remove labeled toluene. The filter was then left for 48 h in an uncapped scintillation vial to evaporate residual toluene. To determine the activity of NVWP, 40 μl 1M HCl was added to 3 ml of the filtered, twice diluted sample, and left for 48 h in an uncapped scintillation vial for evaporation of CO_2 and toluene, and counted in HiSafe 3 scintillation liquid (LBK, Loughborough, England). $^{14}CO_2$ from a 5 ml sample was trapped in 1 ml 1N NaOH in a double vial. In a double vial, the sample was contained in a 6 ml plastic scintillation vial, which was inserted into a capped 20 ml scintillation vial containing the NaOH. For comparison, all the $^{14}CO_2$ produced in the bottles at the end of the experiments with [methyl-^{14}C]toluene was trapped by purging air through the acidified medium and 5 successive bottles, each with 50 ml of 1 N NaOH. It was shown that the double vial trap had a 61% (SD = 3%, n = 6) efficiency.

Results

Toluene/nitrate interdependence

Duplicate experiments were set up with either nitrate or toluene in surplus. Figure 1 shows an experiment with toluene in surplus. It can be seen that the culture degraded toluene until NO_3^- and NO_2^- were depleted. Addition of NO_3^- activated the culture, and the degradation of toluene continued until NO_3^- and NO_2^- were depleted. On the basis of mass loss from 11 experiments where toluene was in surplus, it was calculated that 0.7 ± 0.2 mg NO_3^--N was reduced per mg toluene metabolized. In contrast, experiments run with NO_3^- in surplus showed that the NO_3^- reduction stopped when toluene was depleted (see Fig. 2). From 9 experiments where NO_3^- was in surplus, it was calculated that 1.3 ± 0.2 mg NO_3^--N was reduced to NO_2^- per mg of toluene metabolized.

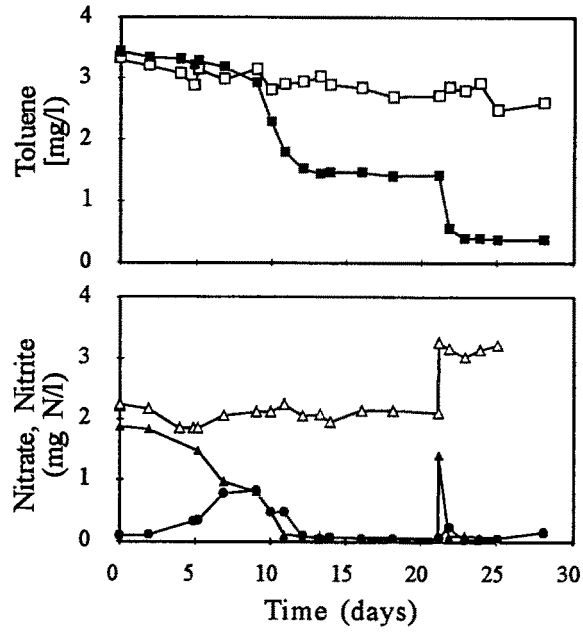

Fig. 1. Simultaneous toluene degradation, and nitrate reduction under conditions with toluene in surplus. Blank markers are from a single control experiment. Filled markers are means of two experiments. Toluene (■), Nitrate (▲), Nitrite (•). After depletion of nitrate, the culture was activated on day 21 by a subsequent addition of nitrate.

At the start of the experiments, the acetylene assay (described by Balderston et al. 1976 and Yoshinari and Knowles 1976) was used to describe the relationship between toluene and NO_3^- and to confirm denitrification as the reductive process. This attempt failed because acetylene completely inhibited toluene degradation at an acetylene concentration as low as 1.25%. In addition, control experiments without acetylene addition and with toluene in surplus (3.6 mg/l) showed that N_2O was below the detection limit (< 0.3 mg/l) during the experiment.

A duplicate experiment with low ammonia concentration in the medium (3.3 mg NH_4^+-N/l) was performed to investigate if dissimilatory nitrate reduction to ammonia took place. During degradation of toluene, the NH_4^+ concentration decreased by 0.15 mg N per mg toluene degraded.

Table 1. Distribution of ^{14}C after degradation of [ring-1-^{14}C]toluene (2 experiments) and [methyl-^{14C}]toluene (4 experiments).

CO_2	53% ± 10%
Biomass	34% ± 8%
[2]NVWP	6% ± 2%
[3] Recovery	93% ± 7%

[1] Percent of metabolized carbon. The results given are the means and standard deviations of the six experiments. [2] Non Volative Water-soluble Products. [3] Calculated as the sum of $^{14}CO_2$ + ^{14}C-biomass + ^{14}C-NVWP + ^{14}C-toluene at the end of the experiments divided by the sum at the beginning of the experiment. The sum at the beginning of the experiments constituted 98% ± 6% of the added activity.

Growth yield and mineralization

Experiments using ^{14}C-toluene and NO_3^- in surplus were carried out to compare the amount of electrons donated by the toluene and accepted by nitrate. The results of the experiments are shown in Table 1. The data from the two experiments with [ring-1-^{14}C]toluene and the four experiments with [methyl-^{14}C]toluene are averaged because they are not significantly different at the 95% level. Six percent of the metabolized ^{14}C-carbon was found as NVWP, and 53% as $^{14}CO_2$. The recovery of ^{14}C, calculated as the sum of activities of the 3 fractions and toluene at the end of the experiment divided by the sum of activities at the beginning of the experiment, was 93% ± 7%. The sum of activities at the beginning of the experiments constituted 98% ± 6% of the added activity. A control experiment where the culture was killed by addition of $HgCl_2$ showed no difference in the distribution of activity at the beginning of the experiment and after 4 h.

Utilization of different electron acceptors and donors

NO_3^-, NO_2^-, NO, N_2O, O_2, and Fe^{3+} were tested as electron acceptors for oxidation of toluene. The experiments were performed as single experiments. Toluene was added in surplus (7.7 ± 0.2 mg/l), and the nitrogen oxides were added in the same concentrations with respect to nitrogen (5 mg N/l). Ferric iron was added as ferric citrate and incubated at 30 °C. The results are shown in Table 2. All the nitrogen oxides and oxygen could be used as electron acceptors, and the degradation of toluene resulted in an increase of the ATP concentration (data not shown). The amounts of toluene degraded per mol of reduced N decreased going from NO_3^- to N_2O. Ferric iron could not be used by the culture as an electron acceptor during a two-month incubation period.

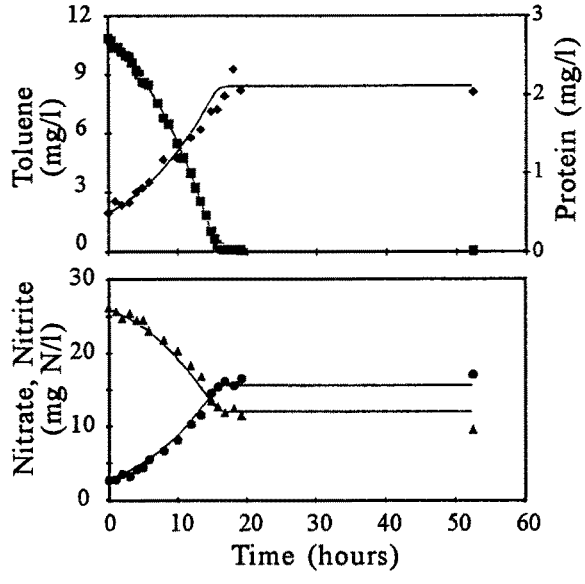

Fig. 2. Simultaneous toluene degradation, bacterial growth and nitrate reduction under conditions with nitrate in surplus. Toluene (■), Protein (means of duplicate samples) (♦), Nitrate (▲), Nitrite (•) The data of this experiment were used to estimate the parameters of Table 3 by non-linear regression analysis. The solid lines are the model predictions calculated with the parameter estimates of Table 3.

Several other aromatic compounds were tested as substrates. It was found that the culture was able to degrade phenol, p-cresol, and 2,4-dimethylphenol (initial concentrations were 1.7 mg/l, 2.1 mg/l, 1.95 mg/l, respectively) to below detection limit within 7 days, whereas 2,6-dimethylphenol (initial concentration was 1.75 mg/l) and 3,5-dimethylphenol (initial concentration was 1.77 mg/l) were not degraded in 65 days. *o*-Xylene (Jørgensen et al. 1995) and *o*-cresol (Flyvbjerg et al. 1993) were only partly degraded and only simultaneously with the degradation of toluene.

Kinetics of toluene degradation

The rates of toluene degradation, bacterial growth, and nitrate consumption under conditions with nitrate in surplus were measured in a 10.6 l bottle with an initial substrate concentration of 10.8 mg toluene/l and

Table 2. Utilization of different electron acceptors in the degradation of toluene.

E^--acceptor	NO_3^-	NO_2^-	NO	N_2O	O_2	Fe^{3+}
Toluene degraded [μmol]	89.8	57.8	19.1	24.0	85.1	0
N reduced [μmol N]	370	390	[1]360	[1]360	–	–
Toluene degraded per N reduced [mol/mol]	0.24	0.15	0.053	0.067	–	–

[1] Not measured, calculated from added volume.

Table 3. Kinetic parameters of toluene degradation by the mixed culture. The standard deviation of a parameter is a measure of how well the parameter could be estimated from the experimental data by non-linear regression analysis.

k mg toluene/ mg protein/ hour	K_s mg/l	Y mg protein/ mg toluene	*b $hour^{-1}$	**μ_{max} $hour^{1-}$	α_{NO3} mg NO_3-N/ mg toluene	α_{NO2} mg NO_2-N/ mg toluene
0.71 ± 0.04	***0.4 ± 0.2	0.14 ± 0.02	0	0.10	1.24 ± 0.05	1.14 ± 0.05

* b was fixed at 0 $hour^{-1}$ during estimation of the remaining parameters.
** μ_{max} was calculated from the relation: $\mu_{max} = Y \cdot k$
*** Additional experiments performed at toluene concentrations below 1 mg/l revealed that a more precise estimate of K_s is 0.20 ± 0.01 mg/l.

an initial concentration of active biomass at 0.5 mg protein/l.

The experimental data shown in Figure 2 were used to determine the kinetic and stoichiometric parameters of equation (1) - (4) (k, K_s, Y, b, α_{NO3}, α_{NO2}). To estimate the decay constant for biomass,b, the protein concentration was monitored for a period of 16 days after toluene was depleted (data not shown). Since there was no significant decrease in the protein concentration during that period, b was estimated to 0 per hour. The remaining parameters were determined by fitting equations (1) - (4) with the experimental data shown in Figure 2 by non-linear regression, using a least squares method as described by Bilbo (1991). The estimated values of k, K_s, Y, α_{NO3}, and α_{NO2} are shown in Table 3. The maximum specific growth rate, μ_{max}, also shown in Table 3, was calculated from Y and k after the relation $\mu_{max} = Y \cdot k$. As seen from Figure 2, the proposed model equations give a good description of the experimental data. All parameters except K_s were determined with a fairly high degree of precision as shown by the relatively low standard deviations for the parameter estimates. Furthermore, the correlation matrix of the parameter estimates (not shown in Table 3) indicates that none of the estimates are highly correlated. The poor estimate of K_s is due to relatively few measurements of the toluene concentration in the concentration range around the value of K_s.

To determine K_s more precisely, three additional experiments were performed. These experiments started with an initial biomass concentration of about 1.0 mg protein/l and initial toluene concentrations below 1.0 mg/l, which was the expected order of magnitude of K_s. The toluene degradation data from these experiments are shown in Figure 3. The protein concentrations (data not shown) were only measured in the beginning and at the end of each experiment. New values for K_s between 0.19 and 0.21 mg toluene/l were obtained by fitting the data of each toluene degradation experiment with the analytical solution to the Monod equation by non-linear regression analysis. The analytical solution to the Monod equation is obtained by solving equation (1) and (2) for b = 0, as described by Robinson (1985). Since Y and k were known with high accuracy from the data in Figure 2, these parameters were fixed at the values shown in Table 3 in the fitting process. The solid lines in Figure 3 are the model simulations using a K_s of 0.2 mg toluene/l. Thus, the experiments at low toluene concentrations revealed

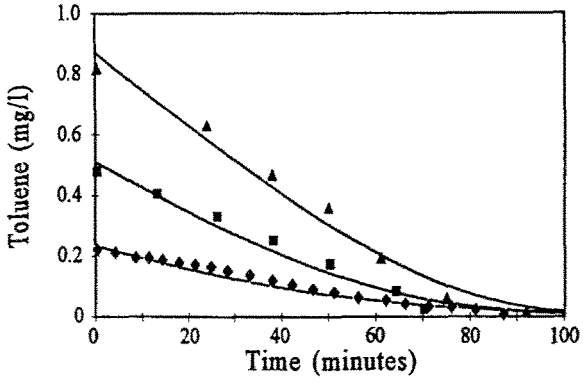

Fig. 3. Comparison of experimental data on toluene degradation at initial concentrations of 0.8 mg/l (▲), 0.5 mg/l (■), and 0.2 mg/l (◆) with model predictions (solid lines). The initial biomass concentrations were 1.1 ± 0.1 mg protein/l.

that K_s is somewhat lower than estimated in the first experiment (Table 3).

Discussion

The data depicted in Figure 1 show that the degradation of toluene in the absence of oxygen depends on the presence of nitrogen oxides as the electron acceptors. The degradation is followed by an accumulation of protein (Fig. 2), which proves that toluene removal is due to microbial action. When NO_3^- is present in surplus, the reduction in general only goes as far as NO_2^-. Accumulation of NO_2^- is typical for denitrification when the electron donor is the limiting factor (Tiedje 1988). On the basis of a NO_3^- consumption of 1.3 ± 0.2 mg NO_3^-N per mg of toluene, it can be calculated that 17.1 ± 2.6 mol of electrons were accepted per mol of toluene degraded. In contrast, when toluene was present in surplus, NO_2^- was further reduced upon NO_3^- depletion, as is to be expected when denitrification is the reductive process (Tiedje 1988). Because no N_2O accumulated when NO_2^- was reduced, it is assumed that the reduction of NO_3^- goes to N_2. In this case, where 0.7 ± 0.2 mg NO_3^--N were reduced per mg toluene degraded, it can be calculated that 23 ± 6.6 mol of electrons were accepted per mol of toluene degraded.

The measured activities in the three fractions after degradation of ^{14}C-toluene, shown in Table 2, suggest the following stoichiometry of toluene degradation, assuming a cell composition of $C_5H_7O_2N$ (McCarty 1972):

$$C_7H_8 + 12.4NO_3^- + 0.48NH_4^+ \Longrightarrow 0.48C_5H_7O_2N + 0.04C_{11}H_{10}O_4 + 4.2CO_2 + 12.4NO_2^- + 2.8H_2O + 0.48H^+ \quad (5)$$

Nonvolatile water-soluble products have been observed by Dolfing et al. (1990), and Evans et al. (1991b) found accumulation of benzylfumaric acid ($C_{11}H_{10}O_4$) from biodegradation of toluene under denitrifying conditions. Therefore the products in this equation are assumed to be benzylfumaric acid. ^{14}C not accounted for in the balance (7%) is assumed to be CO_2, because this compound is the most likely to escape from the experimental setup. According to this equation 24.7 ± 2.8 mol e^- per mol toluene are accepted by NO_3^-. The deviation is calculated by solving the redox equation using a 70% CO_2 evolution, which is the average CO_2 evolution plus the standard deviation of the CO_2 evolution. On average, the measured reduction of NO_3^- to NO_2^- accounts for about 70% of the measured donation of electrons. The difference between the two measurements differs significantly from zero at the 95% level (t-test). On the other hand, 23 ± 6.6 mol e^- is accepted per mol of toluene degraded when NO_3^- is reduced to N_2. In this case, the reduction of NO_3^- to N_2 accounts for 97% of the measured donation of electrons. An explanation to this discrepancy has not yet been identified, but some volatile organic metabolic products may have been determined as $^{14}CO_2$. In comparison, Evans et al. (1991b) found that 51% of the metabolized carbon was associated with the biomass, and 29% evolved as CO_2. On the basis of these observations, an electron yield of 22.4 mmol e^- per mmol of toluene was calculated, which is in good agreement with the results presented here.

It may be argued that oxygen was present in undetectable amounts in the bottles and was involved in initial oxidative reactions or as electron acceptor, and that the low NO_3^- consumption when NO_3^- was in excess could be explained by the presence of oxygen. This possibility, however, is negated by the fact that toluene degradation stopped when NO_3^- was depleted, and that the control experiments showed no detectable amounts of oxygen after 4 months of incubation.

Besides denitrification, dissimilatory reduction of NO_3^- to ammonia may account for the loss of nitrate in the absence of oxygen. In more reduced and carbon-

rich environments, dissimilatory reduction of nitrate to ammonia may predominate over denitrification (Cole 1990). If dissimilatory reduction of NO_3^- to ammonia was the reductive process in these experiments, NH_4^+ would have accumulated. This was not the case as the concentration of ammonia decreased during degradation. According to the stoichiometric equation, one would expect a decrease in the ammonia concentration of 0.07 mg per mg toluene degraded. This accounts for about 50% of the observed decrease.

NO_3^-, NO_2^-, and N_2O are efficient electron acceptors for most denitrifiers (Tiedje 1988), and they all worked well as electron acceptors for the culture used in this study. The experimental ratios between the amounts of degraded toluene per mol of N are 5 : 3.1 : 1.4, going from NO_3^- to N_2O. The ratios are almost proportional to the oxidation state of the N in the Noxides, and therefore also proportional to the amounts of electrons accepted if the Noxides are reduced to N_2, and the growth yields are assumed to be the same for each Noxide. This is in good agreement with the work of Koike and Hatory (1975). They found that the growth of *Pseudomonas denitrificans* per mol of electrons transferred to either NO_3^-, NO_2^-, or N_2O was the same. Dolfing et al. (1990) also found that N_2O was a very effective electron acceptor.

In addition, NO also served as electron acceptor. NO is commonly used by denitrifiers, but they usually cannot grow with NO (Zumft and Kroneck 1990), and the role of NO as electron acceptor is not fully understood (Kroneck and Zumft 1990). In this case, the degradation was accompanied by an accumulation of ATP, but conclusive measurements of growth were not conducted. The ratio of toluene degradation per mol of reduced N between NO and NO_3^- is only half of what is expected from the oxidation state, namely 1.1 : 5, indicating that NO is not as efficient as the other Noxides. The data are based, however, on single experiments, so it is not known whether the difference is significant.

As with most denitrifiers, this culture was able to use O_2 as electron acceptor and to degrade toluene aerobically, while Fe^{3+} could not be used as electron acceptor.

The degradation of toluene was completely inhibited by acetylene. The same phenomenon was observed by Hutchins (1992). He found that aqueous acetylene concentrations of 70 mg/l inhibited the degradation. In this study, inhibition occurred at 1.25%, which is equivalent to approximately 15 mg/l. Barbaro et al. 1992 also observed inhibition of toluene degradation both in laboratory experiments and in *in situ* experiments in the Borden aquifer (Ontario, Canada) conducted under denitrifying conditions. In this case, the concentration of acetylene was approximately 1%. These reports indicate that inhibition of degradation of aromatic hydrocarbons by denitrification is a widespread phenomenon.

The rate of toluene degradation and the simultaneous growth of the mixed culture were modelled according to the classical Monod kinetics. The rates of nitrate consumption and nitrite production were shown to be proportional to the rate of toluene degradation. During the bacterial growth on toluene, the yield coefficient, Y, was estimated to be 0.14 mg protein per mg toluene, and the maximum specific rate of degradation, k, was estimated to be 0.71 mg toluene per mg protein per hour. By comparison, Evans et al. (1991b) determined Y to be 0.3–0.4 mg protein/l and k to be 0.32 mg toluene per mg protein per hour in a pure culture of a toluene-degrading, denitrifying strain designated T1. However, these differences are most likely due to differences in measured protein concentrations because of the use of different protein assays as opposed to the mixed culture actually having a lower Y and a higher k than strain T1. Thus, we found that the protein assay used in this study (the method of Bradford (1976), as described by Gälli and McCarthy (1989)) only recovers about 25% of the cell dry weight, although a more realistic protein content is around 50%. If a protein content of 25% is assumed, Y of the mixed culture may be calculated to be 0.56 mg cell dry weight per mg toluene. Assuming the cells have an average chemical composition of $C_5H_7O_2N$, this yield corresponds to a carbon assimilation of 33%, which is close to the carbon assimilation actually measured.

The nitrate consumption in the kinetic experiment, α_{NO3}, was estimated to be 1.24 mg NO_3-N per mg toluene (Table 2). The nitrite production, α_{NO3}, was a little lower, 1.14 mg NO_2-N per mg toluene, since only 92% of the reduced nitrate was recovered as nitrite. The estimated nitrate consumption is in good agreement with the nitrate consumption observed in the other experiments of this study, where nitrate was in surplus. As mentioned previously, however, this nitrate consumption accounts for only about 70% of the electrons donated from toluene under the assumption of a carbon assimilation of 34% as determined in the ^{14}C-experiments.

The maximum specific growth rate, μ_{max}, of the mixed culture was calculated to be 0.10 per hour. This value is in the same order of magnitude as the μ_{max} of

the pure culture studied by Evans et al. (1991b) (0.10–0.13 per hour) and the μ_{max} of another denitrifying mixed culture that grew on phenol (0.09–0.11 per hour) (Bakker 1977).

The mixed culture was capable of degrading toluene close to the maximum rate at relatively low toluene concentrations. Therefore, it was difficult to obtain an accurate estimate of the Monod saturation constant, K_s. However, experiments performed at initial toluene concentrations below 1.0 mg/l indicate that K_s of the mixed culture is about 0.2 mg toluene/l. By comparison, Bakker (1977) found a K_s of 9 mg/l for his phenol-degrading culture. This culture, however, was adapted to substrate concentrations in the range of 25–100 mg phenol/l, whereas the mixed culture of this study was adapted to concentrations below 10 mg toluene/l.

The experiments discussed above show that the bacterial culture is able to degrade toluene by denitrification: First, toluene is degraded only in the presence of Noxides, and degradation is accompanied by accumulation of ATP and protein. In addition, NO_3^- is not reduced after depletion of toluene. Second, the amount of electrons accepted by the Noxides is in good agreement with the amount donated by toluene when NO_3^- is limiting the degradation. Third, aromatic ring cleavage is proven by evolution of $^{14}CO_2$ from [ring-1-^{14}C]toluene in the absence of oxygen. Fourth, NO_2^- and N_2O can serve as electron acceptors, and dissimilatory nitrate-reduction to NH_4^+ is not the reductive process. In addition, the degradation of toluene was well modelled by the classical Monod kinetics.

The results of this study and the literature cited in the introduction demonstrate that toluene and several other monoaromatic hydrocarbons, which are common constituents of gasoline, may be degraded if nitrate is used as the terminal electron acceptor in enhanced bioremediation of polluted aquifers. If benzene is present, however, oxygen will be needed. If nitrate is reduced to N_2, it can be expected that 0.7 mg nitrate-N will be reduced per mg of toluene oxidized. One must, nevertheless, keep in mind that naturally occurring organic matter in the aquifer may also be oxidized, leading to larger nitrate demand than would be expected from the amount of toluene present (Hutchins et al. 1991). The rates of degradation of toluene under denitrifying conditions are comparable to aerobic degradation rates (Alvarez et al. 1991), indicating that *in situ* degradation rates will also proceed at comparable rates. In addition, the low K_s-values determined in this study suggest that very low concentrations of toluene may be attained if nitrate is used as external electron acceptor in bioremediation of aquifers polluted with gasoline.

Acknowledgements

This work was supported by the Danish Center of Environmental Biotechnology, the Danish Environmental Research Programme, and the Technical University of Denmark. Invaluable assistance in the laboratory was given by Erik Mortensen and Susan K. Olsen.

References

Ashworth RA, Howe GB, Mullins ME & Rogers TN (1988) Air-water partitioning coefficients of organics in dilute aqueous solutions. J. of Hazardous Materials. 18: 25–36

Al-Bashir B, Cseh T, Leduc R & Samson R (1990) Effect of soil/contaminant interaction on the biodegradation of naphthalene in flooded soil under denitrifying conditions. Appl. Microbiol. Biotechnol. 34: 414–419

Alvarez PJJ, Anid PJ, & Vogel TM (1991) The kinetics of aerobic biodegradation of benzene and toluene in sandy aquifer material: Kinetics of benzene and toluene degradation. Biodegradation 2: 43–52

Bakker G (1977) Degradation of aromatic compounds by microorganisms in dissimilatory nitrate reduction. Ph.D dissertation. Technical University of Delft, Holland

Balderston WL, Sherr B, Payne WJ (1976) Blockage by acetylene of nitrous oxide reduction in *Pseudomonas perfectomarinus*. Appl. Environ. Microbiol. 31: 504–508

Barbaro JR, Barker JF, Lemon LA, Mayfield CI (1992) Biotransformation of BTEX under anaerobic, denitrifying conditions: Field and laboratory observations. J. Cont. Hydrol. 11: 245–272

Bilbo CM, Spliid H & Holst H (1991) DKM - A program for estimation of kinetic parameters in a biodegradation model and a systematic approach to the design of experiments. Technical Report 13/91, The Institute of Mathematical Statistics and Operations Research, Technical University of Denmark

Blanchard AA (1946) Nitric oxide. Inorganic Synthesis. 2: 126–128

Bradford M (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein, utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248–254

Broholm K, Jensen BK, Christensen TH & Olsen L (1990) Toxicity of 1,1,1-trichloroethane on a mixed culture of methane-oxidizing bacteria. Appl. Environ. Microbiol. 56: 2488–2493

Cole JA (1990) Physiology, biochemistry and genetics of nitrate dissimilation to ammonia. In: Revsbech NP & Sørensen J (eds) Denitrification in Soil and Sediment (pp. 57–76). Plenum Press, New York

Dolfing J, Zeyer J, Binder-Eicher P & Schwarzenbach RP (1990) Degradation of toluene by a *Pseudomonas* sp. in the absence of molecular oxygen. Arch. Microbiol. 154: 336–341

DS224. Water analysis. Determination of ammonia-nitrogen. Dansk Standardiseringsråd. Copenhagen, 1975

Evans PJ, Mang DT & Young LY (1991a) Degradation of toluene and m-xylene and transformation of *o*-xylene by denitrifying enrichment cultures. Appl. Environ. Microbiol. 57: 450–454

Evans PJ, Mang DT, Kim KS & Young LY (1991b) Anaerobic degradation of toluene by a denitrifying bacterium. Appl. Environ. Microbiol. 57: 1139–1145

Flyvbjerg J, Jørgensen C, Arvin E, Jensen BK, Olsen SK (1993) Biodegradation of *o*-cresol by a mixed culture of nitrate-reducing bacteria growing on toluene. Appl. Environ. Microbiol. 59: 2286–2292

Hutchins SR (1991) Biodegradation of monoaromatic hydrocarbons by aquifer microorganisms using oxygen, nitrate, or nitrous oxide as the terminal electron acceptor. Appl. Environ. Microbiol. 57: 2403–2407

Hutchins SR (1992) Inhibition of alkylbenzene biodegradation under denitrifying conditions by using the acetylene block technique. Appl. Environ. Microbiol. 58: 3395–3398

Hutchins SR, Sewell GW, Kovacs DA & Smith GA (1991) Biodegradation of aromatic hydrocarbons by aquifer microorganisms under denitrifying conditions. Environ. Sci. Technol. 25: 68–76

Jensen BK, Arvin E & Gundersen AT (1988) Biodegradation of phenolic compounds and monoaromatic hydrocarbons by a mixed wastewater culture under denitrifying conditions. In: Organic contaminants in wastewater, sludge, and sediments: occurrence, fate, and disposal. (pp 150–157) Quaghebeur D, Temmerman I, Angeletti G (eds). Elsevier Science Publ, Essex, England

Jørgensen C, Jensen BK, Arvin E & Mortensen E (1991) Biodegradation of toluene by a denitrifying enrichment culture. In: Hinchee RE & Olfenbuttel RF (eds) *In situ* Bioreclamation (pp 480–487). Butterworth-Heinamann, Stoneham MA, USA

Jørgensen C, Nielsen B, Jensen BK, Mortensen E (1995) Transformation of *o*-xylene to *o*-methyl benzoic acid by a denitrifying enrichment culture using toluene as the primary substrate. Biodegradation. 6: 141–146 (this issue)

Kavanaugh MC & Trussel RR (1980) Design of aeration towers to strip volatile contamints from drinking water. J. AWWA. 72: 684–692

Koike I & Hatory A (1975) Energy yield of denitrification: An estimate from growth yield in continuous cultures of *Pseudomonas denitrificans* under nitrate, nitrite, and nitrous oxide limited conditions. J. Gen. Microbiol. 88: 11–19

Kroneck PMH & Zumft WG (1990) Bioinorganic aspects of denitrification: Structures and reactions of NxOy compounds and their interaction with iron and copper proteins. In: Revsbech NP & Sørensen J (eds) Denitrification in Soil and Sediment. (pp 1–20). Plenum Press, New York

Kuhn EP, Colberg PJ, Schnoor JL, Wanner O, Zehnder AJB & Schwarzenbach RP (1985) Microbial transformations of substituted benzenes during infiltration of river water to groundwater: Laboratory column studies. Environ. Sci. Technol. 19: 961–968

Major DW, Mayfield CI & Barker JF (1988) Biotransformation of benzene by denitrification in aquifer sand. Ground Water. 26: 8–14

McCarty PL (1972) Energetics of organic matter degradation. In: Mitchell R (ed) Water Pollution Microbiology (pp 91–118). Wiley Interscience, New York

Mihelcic JR & Luthy RG (1988) Microbial degradation of acenaphthene and naphthalene under denitrifying conditions in soil-water systems. Appl. Environ. Microbiol. 54: 1188–1198

Platen H & Schink B (1989) Anaerobic degradation of acetone and higher ketones via carboxylation by newly isolated denitrifying bacteria. J General Microbiol. 135: 883–891

Shink B (1988) Principles and limits of anaerobic degradation: Environmental and technological aspects. In: Zehnder AJB (ed) Biology of anaerobic microorganisms. Ecological and applied microbiology (pp 771–846). John Wiley and Sons, New York

Standard Methods (1989) Standard methods for the examination of water and wastewater. 17^{th} edition. Clesceri LS, Greenberg AE & Trussell RR (eds) APHA, AWWA, WPCF, Washington D.C.

Tiedje JM (1988) Ecology of denitrification and dissimilatory nitrate reduction to ammonium. In: Zehnder AJB (ed) Biology of anaerobic microorganisms (pp 179–244). John Wiley and Sons, New York

Yoshinary T, Knowles R (1976) Acetylene inhibition of nitrous oxide reduction by denitrifying bacteria. Biochem. Biophys. Res. Comm. 69: 705–710

Zeyer J, Kuhn EP & Schwarzenbach RP (1986) Rapid microbial mineralization of 1,3-dimethylbenzene in the absence of oxygen. Appl. Environ. Microbiol. 52: 944–947

Zumft WG & Kroneck PMH (1990) Metabolism of nitrous oxide. In: Revsbech NP & Sørensen J (eds) Denitrification in Soil and Sediment (pp 37–55). Plenum Press, New York