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Effect of carbon:nitrogen ratio on kinetics of phenol biodegradation by *Acinetobacter johnsonii* in saturated sand

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Abstract

In polluted soil or ground water, inorganic nutrients such as nitrogen may be limiting, so that Monod kinetics for carbon limitation may not describe microbial growth and contaminant biodegradation rates. To test this hypothesis we measured $^{14}CO_2$ evolved by a pure culture of *Acinetobacter johnsonii* degrading 120 μg ^{14}C-phenol per ml in saturated sand with molar carbon:nitrogen (CN) ratios ranging from 1.5 to 560. We fit kinetics models to the data using non-linear least squares regression. Phenol disappearance and population growth were also measured at CN1.5 and CN560.

After a 5- to 10-hour lag period, most of the $^{14}CO_2$ evolution curves at all CN ratios displayed a sigmoidal shape, suggesting that the microbial populations grew. As CN ratio increased, the initial rate of $^{14}CO_2$ evolution decreased. Cell growth and phenol consumption occurred at both CN1.5 and CN560, and showed the same trends as the $^{14}CO_2$ data. A kinetics model assuming population growth limited by a single substrate best fit the $^{14}CO_2$ evolution data for CN1.5. At intermediate to high CN ratios, the data were best fit by a model originally formulated to describe no-growth metabolism of one substrate coupled with microbial growth on a second substrate. We suggest that this dual-substrate model describes linear growth on phenol while nitrogen is available and first-order metabolism of phenol without growth after nitrogen is depleted.

Introduction

Monod kinetics are often used to model biodegradation in carbon-limited laboratory experiments in which all other nutrients are present in excess (Robinson & Tiedje 1983; Simkins & Alexander 1984). Simkins & Alexander (1984) used the integrated form of the Monod growth equation to derive a family of six models in which substrate disappearance is a function of initial substrate concentration, population density, maximum specific growth rate and half-saturation constant. Two assumptions of the Monod family of models are that cell yield is constant and independent of substrate concentration, and that only one nutrient, e.g., carbon, limits growth.

In soil or ground water polluted by organic contaminants, essential nutrients such as oxygen or nitrogen may limit metabolic rates due to low concentrations and/or mass transfer limitations. Monod kinetics may not adequately predict microbial growth and contaminant biodegradation rates in these environments. Several alternative approaches, reviewed below, have been adopted for predicting growth and biodegradation kinetics.

To model growth in systems limited by more than one nutrient, researchers have developed kinetic expressions consisting of two or more single-nutrient Monod equations multiplied together (Mankad & Bungay 1988; Mankad & Nauman 1992; Megee et al. 1972). Such dual-Monod expressions have been included in field-scale models describing transport and

fate of carbon substrates and oxygen (Borden & Bedient 1986; Molz et al. 1986). Rifai & Bedient (1990) compared two conceptual models to determine when it is necessary to use dual-nutrient kinetics to model biodegradation in ground water.

Alternative approaches to dual-Monod models include the three-half-order models (Brunner & Focht 1984; Focht & Brunner 1985). The linear three-half-order model contains terms for linear growth, first-order metabolism without growth, and zero-order mineralization of soil organic matter or residual ^{14}C (e.g. metabolism of dead labeled cells). The exponential three-half-order model is similar except that it assumes exponential growth and eliminates the first-order term.

Schmidt et al. (1985) developed a family of dual-substrate models based on logistic, linear or exponential growth and the relationship between carbon substrate concentration and the Michaelis-Menten half-saturation constant. These models were originally formulated to describe no-growth metabolism of one substrate coupled with microbial growth on a second substrate. The differential form of the linear growth, low concentration model (VII) is equivalent to that of the linear-growth three-half-order model (equation 6 of Brunner & Focht 1984), but the dual-substrate models do not contain a zero-order term. Schmidt & Gier (1989) stated that the dual-substrate models are applicable to situations in which factors in addition to carbon substrate limit growth. These models have not been tested for conditions when the growth-limiting nutrient is not a carbon substrate.

To simulate a polluted sand aquifer containing a high concentration of carbon relative to nitrogen, we measured $^{14}CO_2$ evolved by a pure culture of *Acinetobacter johnsonii* degrading 120 μg ^{14}C-phenol per ml at several nitrate-N levels. The batch experiments were designed to test several hypotheses: *i*) as molar carbon:nitrogen (CN) ratios increase and cultures become more N-limited, growth and biodegradation rates will decrease; *ii*) at low CN ratios, an actively growing population mineralizes phenol, and the biodegradation kinetics will reflect this growth; *iii*) at high CN ratios, growth is restricted and there will be a significant component of non-growth metabolism, resulting in non-growth or mixed-order biodegradation kinetics.

Materials and methods

Strains and media

Acinetobacter johnsonii genospecies 7 was isolated from a sample of Yolo silt loam soil (north central California USA) that had been enriched with 50 μg g^{-1} toluene. We confirmed that *A. johnsonii* metabolizes phenol by transferring a colony from a tryptic soy agar (TSA) plate into liquid mineral medium containing 100 μg ml^{-1} phenol, growing the culture to turbidity, and making three successive transfers into fresh medium containing 100 to 120 μg phenol per ml. The microorganism was identified using the BIOLOG (Biolog, Inc., Hayward, CA) metabolic assay identification system (M. Fuller, pers. comm.). *Acinetobacter* is a Gram-negative obligate aerobe that is capable of using either ammonium or nitrate for its nitrogen source (Juni 1984). We chose nitrate for this study because it is more common than ammonium in ground water.

The sterile mineral medium used in all experiments contained (in mg per liter of deionized NANOpure (Barnstead, Dubuque, IA) filtered water): NaH_2PO_4 (501), K_2HPO_4 (996), $MgSO_4 \cdot 7H_2O$ (139), CaCl2 · $2H_2O$ (16), KNO_3 (506, unless noted), $FeSO_4 \cdot 7H_2O$ (1), H_3BO_3 (0.3), $CoCl_2 \cdot 6H_2O$ (0.2), $ZnSO_4 \cdot 7H_2O$ (0.1), $MnCl_2 \cdot 4H_2O$ (0.03), $Na_2MoO_4 \cdot 2H_2O$ (0.03), $NiCl_2 \cdot 6H_2O$ (0.02), and $CuCl_2 \cdot 2H_2O$ (0.01). Final pH was 6.9. The sterile phosphate buffer solution used to wash cells consisted of 501 mg NaH_2PO_4 and 996 mg K_2HPO_4 per liter of deionized NANOpure-filtered water.

Phenol stock solution was prepared by dissolving solid reagent-grade phenol (Aldrich Chemical Company, Inc., Milwaukee, WI) in NANOpure-filtered water and filter-sterilizing. Uniformly ring-labeled ^{14}C-phenol (specific activity, 130 mCi $mmol^{-1}$, purity 98.6%) was obtained from Amersham Corporation (Arlington Heights, IL).

Plates used to store and count cells were made with 3 g Bacto® trypic soy broth and 15 g Bacto® agar (Difco Laboratories, Detroit, MI) per liter of NANOpure-filtered water.

Inoculum preparation

We harvested *A. johnsonii* cells from stationary-phase liquid cultures by centrifugation (4° C, 15 minutes, 10000 rpm, SA-600 rotor, Sorvall RC-5B centrifuge, DuPont Company, Newtown, CT) and resuspended

them in sterile phosphate buffer. Cells were centrifuged and resuspended twice more to remove excess nutrients before inoculation. Individual inoculum densities ranged from 2.9E6 to 6.5E6 cells ml^{-1}, as determined by plate counts using a modified drop plate method (Hoben & Somasegaran 1982).

Measurement of phenol mineralization and phenol remaining

To measure $^{14}CO_2$ evolved during phenol mineralization, we performed four biodegradation experiments, designated A–D, using 120 μg non-labeled phenol and 40,000–50,000 dpm of ^{14}C-phenol per ml of mineral medium. The CN ratio ranged from 1.5 to 150 in A, from 1.5 to 530 in experiments B and C and from 1.5 to 560 in D. These CN ratios represent nitrate-N concentrations of 70 μg ml^{-1} for CN1.5, and 0.2 to 1.4 μg ml^{-1} for CN ratios 560 to 75, respectively. Experiments A–C included sterile controls, in which sterility was verified by plating. Each treatment was run in duplicate or triplicate.

We constructed respiration flasks from wide-mouth pint glass Mason jars (70610-00518, Kerr Glass Mfg. Corp., Los Angeles, CA). Each jar held two small glass containers, one for the sample and one for the base trap. Two stainless steel fittings were inserted through holes drilled into the jar lid and glued in place. A 6-inch, 14-gauge Luer-hub stainless steel pipetting needle (Perfektum 7942, Popper & Sons, Inc., New Hyde Park, NY) fitted with tubing at the bottom was used to sample the base trap, which contained 1 ml of 0.5N NaOH. A 1.5-inch, 20-gauge needle (Perfektum 7058, Popper & Sons, Inc., New Hyde Park, NY) allowed air exchange during sampling to maintain atmospheric pressure inside the respiration flask. Rubber sleeve stoppers capped the two needles to minimize gas exchange when not sampling. We calculated that the closed jar contained more than enough oxygen to allow mineralization of all added phenol.

Samples consisted of 5 g of Monterey sand (# 0/30, RMC Lonestar, Pleasanton, CA) saturated with 1.3 or 1.4 ml of mineral medium containing phenol and *A. johnsonii* cells. Monterey sand, dredged from offshore California, consists primarily of quartz with a small amount of ferromagnesian minerals and rock fragments. The sand was sieved to include a range of sizes from 425 to 850 μm. To remove organic matter, sand was bleached with Clorox (The Clorox Company, Oakland, CA) adjusted to pH 9.6, in an 80° C steam bath. This treatment effectively oxidized extraneous organic matter that could otherwise sustain a microbial population (B.L. Hoyle, unpubl.). The sand was autoclaved three times for one hour before each experiment, and incubated for a day or two between autoclavings to kill any spore-forming bacteria. Samples were incubated without shaking at 25° C ± 2° C.

To sample the flasks, the base was periodically withdrawn from the traps through the steel pipetting needles, put into scintillation vials, and replaced with fresh base. After adding scintillation fluid and waiting for chemiluminescence to subside, the vials were counted with a liquid scintillation counter (model LS 60000IC, Beckman Instruments, Inc., Fullerton, CA). Cumulative dpm were calculated for each time point, and converted to percent $^{14}CO_2$ evolved based on the total dpm initially added to each flask.

To measure cell growth and remaining phenol in sand at CN ratios 1.5 and 560, an experiment was performed concurrently with experiment D. Samples were incubated under identical conditions to the mineralization experiment. We measured the concentration of phenol remaining using a spectrophotometric technique (method 5530 D, American Public Health Association 1989) based on the method of Martin (1949).

Measurement of population growth

For experiments A–C, a liquid culture of each CN ratio was incubated at 25° C ± 2° C without shaking. Cells were counted by plating at the beginning of each experiment and at 24 hours. In the growth experiment performed with experiment D, we extracted cells from the sand by adding phosphate buffer and vortexing for 20 seconds. For CN ratios greater than 1.5 we also calculated the 24-hour population density using the growth-rate parameter estimates generated by the lin-low model (Schmidt et al. 1985, described above) in the linear growth equation (compare Schmidt et al. 1985):

$$N_{24} = N_0(1 + mt) \qquad (1)$$

where N_0 represents the measured initial cell density and m is the linear growth rate.

Kinetics modeling

We used two approaches to model the phenol biodegradation data and test hypotheses *ii* and *iii* above. First, we assumed that a single process controlled phenol

mineralization, and that either a growth or a no-growth model would fit the data. Alternatively, we conjectured that the biodegradation data reflected a combination of microbial processes, and that a mixed-order kinetic model would best fit the curves.

The single-substrate models that we fit to the data include the first-order and logistic models from the Monod family of models (Simkins & Alexander 1984) (Appendix A). The first-order model of Simkins & Alexander (1984), which contains a population density term in the expression for the rate constant, is really a pseudo-first-order equation (Paris & Rogers 1986). For convenience, we refer to it as the first-order model. The logistic model contains the parameter X_0, which represents the percent of the initial substrate necessary to produce the initial population density. We used the mean cell yield on phenol, 5.5E5 cells μg^{-1} (B.L. Hoyle, unpubl.), to convert X_0 into population density.

The dual-substrate models (Schmidt et al. 1985) incorporate terms that can describe both growth and no-growth conditions. We fit the exponential-growth, low substrate concentration (here called exp-low) and linear-growth, low substrate concentration (here called lin-low) models to the data (Appendix A). Both models are valid for initial substrate concentrations less than the half-saturation constant, K_m. The lin-low model collapses to the first-order model when the growth rate approaches zero.

Before modeling, we averaged the replicates for each CN treatment and eliminated the later straight-line portion of the data. We believe that the later data represent zero-order metabolism of dead radiolabeled cells. Comparison of the parameters estimated for mean curves with those modeled for the individual replicates showed that there was little difference between them, except for the three CN560 replicate curves in experiment D. A BASIC computer program (Hess et al. 1990), which uses the Levenburg-Marquardt method of least squares non-linear regression, was used to model the data. To determine goodness of a model fit, we calculated the Akaike Information Criterion (AIC) (Parkin et al. 1991; Yamaoka et al. 1978), which can be used to compare models with different numbers of parameters.

Results

Measurement of phenol mineralization

The trends in $^{14}CO_2$ evolution were similar for all four experiments. As the CN ratio increased, the initial rates of $^{14}CO_2$ evolution decreased (Fig. 1). The initial rate of $^{14}CO_2$ evolution for both CN1.5 and CN560 in experiment D was somewhat slower than for experiments A–C, but the initial population density was about half as great as in A–C (Table 1). All curves exhibited a 5- to 10-hour lag period, and most displayed a sigmoidal shape, suggesting that population increase had occurred. None of the curves was symmetrical about its inflection point. As the experiments progressed, $^{14}CO_2$ production leveled off in the C-limited (CN1.5) and less N-limited treatments (CN75/79 to CN150/160). However, $^{14}CO_2$ continued to evolve from the more N-limited treatments (CN150/160 to CN530/560), and eventually reached or exceeded the extent of $^{14}CO_2$ evolution in the lower CN ratio treatments (all data not shown). For the N-limited treatments, there was more variation between experiments as CN ratio increased. The two CN75/79 curves are nearly identical, while the three CN530/560 curves, representing extreme N-limitation, are the most different from each other.

Volatilization of phenol into the base trap occurred in all sterile controls. To determine whether volatilization influenced the shape of the biodegradation curves, we estimated how much $^{14}CO_2$ would evolve if available phenol volatilized at the same rate as in the sterile controls (B.L. Hoyle, unpubl.). We assumed that ^{14}C from biodegraded phenol was partitioned evenly between cells and $^{14}CO_2$. The resulting calculated $^{14}CO_2$ evolution curves were similar to the measured curves, especially for the first 50 to 100 hours of incubation. We concluded that phenol was degraded before significant volatilization could occur in the biologically active samples, and thus modeled the data without correction.

Measurement of population growth

Cell growth occurred in all treatments as shown by plate counts of the liquid cultures made during experiments A–C and sand cultures in experiment D (Table 1). Population density increased an order of magnitude or more in the CN1.5 samples, but increased progressively less as nitrogen became more limiting. The cell densities calculated using equation (1) compare reasonably well with the population densities measured at

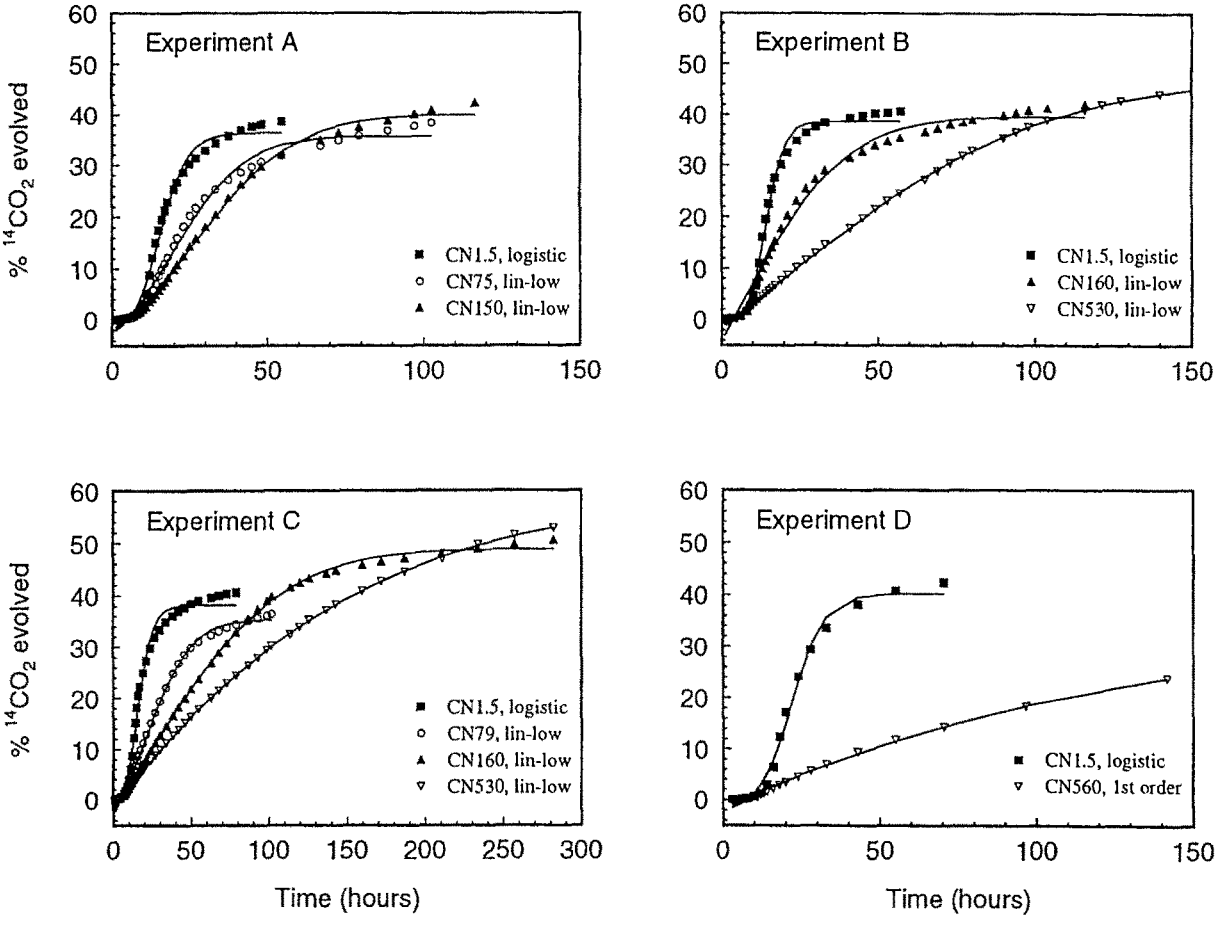

Fig. 1. Evolution of $^{14}CO_2$ over time in the four replicate experiments A–D. Symbols represent the measured data and lines the kinetics models fit using non-linear regression. As CN ratio increased, the initial rate of phenol mineralization decreased. The single-substrate logistic model best fit the CN1.5 curves, and the dual-substrate lin-low model best fit the N-limited curves.

24 hours for the N-limited treatments (Table 1). Cell growth in sand at CN1.5 and CN560 followed the same trends as the $^{14}CO_2$ evolution curves in experiment D (Fig. 2). The initial growth rate in the CN560 samples was slightly slower than in the CN1.5 samples, and the population reached stationary phase several hours sooner.

Phenol disappearance

The trends in phenol disappearance in experiment D were similar to those observed for cell growth and $^{14}CO_2$ evolution (Fig. 2). As CN ratio increased from CN1.5 to CN560, the rate of phenol disappearance decreased. In the CN1.5 treatment, the onset of stationary phase at 25 hours coincided with the nearly complete consumption of phenol. There was a slight lag between phenol disappearance and evolution of $^{14}CO_2$; only a little more than half of the total measured $^{14}CO_2$ had evolved after 25 hours. In the CN560 treatment, cells continued to consume phenol after the onset of stationary phase at 16 hours, so that 50% of the phenol had been used after 70 hours.

Kinetics modeling

The modeled kinetics curves fit the measured $^{14}CO_2$ evolution data well (Fig. 1). Both the growth rates and rate constants for the N-limited treatments decreased as CN ratio increased (Table 2). The logistic growth model fit the carbon-limited CN1.5 curves better than the exp-low model, although the measured $^{14}CO_2$ production rate declined faster than predicted by the logistic model and did not reach an asymptote as quickly as

Table 1. Summary of growth data for *Acinetobacter johnsonii*.

Exp–CN	cells ml^{-1} Initial	cells ml^{-1} Measured after 24 h	cells ml^{-1} Calculated after 24 h[1]	Modeled Growth Rate (hr^{-1})
A–1.5	4.5E6	1.1E8	NA[2]	NA
B–1.5	6.4E6	6.8E7	NA	NA
C–1.5	6.3E6	5.7E7	NA	NA
D–1.5	3.3E6	8.3E7	NA	NA
A–75	3.4E6	4.1E7	1.8E7	0.1793
C–79	5.5E6	4.2E7	1.8E7	0.0977
A–150	4.5E6	1.5E7	1.9E7	0.1310
B–160	6.3E6	1.2E7	1.1E7	0.0330
C–160	5.2E6	1.4E7	7.0E6	0.0146
B–530	6.5E6	3.1E6	8.8E6	0.0148
C–530	5.3E6	4.8E6	5.5E6	0.0017
D–560	2.9E6	1.1E7	NA	NA

[1] Population density calculated using linear growth rate and initial cell density in equation (1).
[2] Not applicable, N_{24} not calculated.

Table 2. Parameter estimates ± standard deviations from the statistically preferred models.

Exp–CN	Model	k[1]	X_0 (cells ml^{-1})	Growth Rate (hr^{-1})
A–1.5	logistic	0.0021 ± 0.0002	2.3E6 ± 6.6E5	NA[2]
B–1.5	logistic	0.0032 ± 0.0002	6.0E5 ± 1.9E5	NA
C–1.5	logistic	0.0018 ± 0.0002	3.3E6 ± 9.9E5	NA
D–1.5	logistic	0.0018 ± 0.0002	1.0E6 ± 4.2E5	NA
A–75	lin-low	0.0098 ± 0.0029	NA	0.1793 ± 0.0716
C–79	lin-low	0.0114 ± 0.0013	NA	0.0977 ± 0.0173
A–150	lin-low	0.0070 ± 0.0011	NA	0.1310 ± 0.0286
B–160	lin-low	0.0257 ± 0.0028	NA	0.0330 ± 0.0106
C–160	lin-low	0.0093 ± 0.0003	NA	0.0146 ± 0.0013
B–530	lin-low	0.0094 ± 0.0002	NA	0.0148 ± 0.0011
C–530	lin-low	0.0068 ± 0.0001	NA	0.0017 ± 0.0003
D–560avg	1st order	0.0079 ± 0.0006	NA	NA
D–560-1	1st order	0.0094 ± 0.0008	NA	NA
D–560-2	lin-low	0.0079 ± 0.0004	NA	0.0113 ± 0.0037
D–560-3	lin-low	0.0095 ± 0.0006	NA	0.0111 ± 0.0044

[1] k_4 (ml $\mu g^{-1} hr^{-1}$) for logistic model, k_1 (hr^{-1}) for lin-low model.
[2] Not applicable.

the model predicted. Diffusion limitations, either of oxygen, phenol, or waste products, may have retarded phenol mineralization after longer incubation times, resulting in asymmetrical curves. When converted to population density, X_0 was two to three times less than the measured initial cell densities for experiments A, C, and D, and approximately an order of magnitude lower for experiment B (Tables 1, 2).

As CN ratio increased and the cultures became more N-limited, the lin-low model provided a statistically better fit to the data than the first-order model for all curves except for CN560 in experiment D (Tables

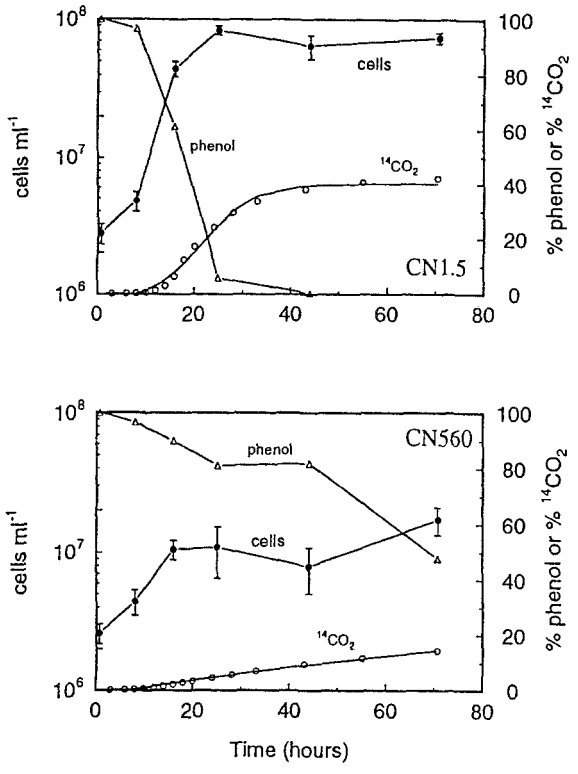

Fig. 2. Effect of CN ratio on growth of *A. johnsonii*, phenol disappearance and $^{14}CO_2$ evolution for CN ratios 1.5 and 560, experiment D. Error bars on growth curves represent 95% confidence intervals.

Table 3. Parameter estimates ± standard deviations from the alternative choice models for CN500.

Exp–CN	Model	k (hr^{-1})[1]	Growth Rate (hr^{-1})
B–530	1st order	0.0101 ± 0.0004	NA[2]
C–530	1st order	0.0066 ± 0.0001	NA
D–560avg	lin-low	0.0090 ± 0.0005	0.0071 ± 0.0038
D–560-1	lin-low	0.0082 ± 0.1094	- 0.0020 ± 0.1189
D–560-2	1st order	0.0067 ± 0.0006	NA
D–560-3	1st order	0.0089 ± 0.0007	NA

[1] k_l for lin-low model, k_3 for first-order model.
[2] Not applicable.

2, 3). The linear growth rate, m, decreased two orders of magnitude from 0.1793 to 0.0017 hr^{-1} as CN ratio increased from 75/79 to 530. However, the rate constant, k_l, varied less than half an order of magnitude, from 0.0257 to 0.0068 hr^{-1}.

Both the lin-low and first-order models visually fit the CN530/560 data for experiments C and D. Based on the AIC, the lin-low model provided a better fit to the CN530 data in C, but not to the CN560 data in D. The lin-low model was the preferred fit for two of the CN560 replicates in experiment D, but the first-order model provided a slightly better fit to the mean curve. Rate constants for the lin-low and first-order models are about the same (Tables 2, 3), suggesting that the lin-low model is collapsing to a first-order equation as the growth rate becomes small. The continued consumption and mineralization of phenol during the stationary phase in the CN560 treatment support our hypothesis of first-order metabolism after use of available nitrogen for cell growth.

Discussion

Relevance of study to polluted soil and ground water

The U.S. Environmental Protection Agency (USEPA) recommends that laboratory treatability studies be performed to estimate rates and extents of pollutant mineralization and nutrient requirements prior to designing ground water bioremediation projects (Sims et al. 1992). Phenol, a USEPA priority pollutant, is a common contaminant of soil and ground water, either through direct release (reviewed in Babich & Davis 1981) or as a by-product of degradation of other compounds. In a survey of 358 hazardous waste sites, phenol occurred in 27% of the sites at an average concentration of 511 $\mu g\ ml^{-1}$ (Eckel et al. 1985). Recommended limits for phenol in drinking water are 0.3 $\mu g\ ml^{-1}$ to prevent undesirable taste and odor and 3.5 $\mu g\ ml^{-1}$ to protect public health (Lederer 1985).

In this study we measured biodegradation of phenol under a range of N-limited conditions similar to those that might occur in shallow aerobic aquifers. In the N-limited treatments, nitrate-N concentrations ranged from 0.20 to 1.4 $\mu g\ ml^{-1}$ for CN ratios 560 to 75, respectively. The C-limited treatment, CN1.5, contained 70 $\mu g\ ml^{-1}$ nitrate-N, a concentration that could occur with sewage or agricultural contamination (reviewed in Keeney 1986; Weil et al. 1990). The initial population densities of *A. johnsonii*, 2.9E6 to 6.5E6 cells ml^{-1} (5.8E5 to 1.3E6 cells per g sand dry wt.), are similar to bacterial populations measured in shallow aquifers (reviewed in Ghiorse & Wilson 1988; Sinclair et al. 1990). *Acinetobacter* is a common member of ground-water microbial communities. The genus comprised more than half of the 514 isolates obtained

from ground water sampled from two wells in Tucson, Arizona USA (Stetzenbach et al. 1986).

Interpretation of modeling results

Phenol was biodegraded at all nitrogen levels in our experiments. The differences among treatments are in accordance with our hypotheses that N-limitation results in reduced population levels, slower biodegradation rates, and mixed-order kinetics. The single-substrate logistic model fit only the carbon-limited treatment, CN1.5. The dual-substrate lin-low model, in which N-limited growth occurred at a linear rate, best described most of the other curves. Growth rates and rate constants both declined with decreasing nitrogen. However, the decrease in growth rates was more pronounced than the drop in rate constants, suggesting that growth rate is more sensitive to nitrate-N status than is the rate constant.

We suggest that the lin-low model represents a 'dual-process' model in which N-limited bacteria utilize phenol and nitrate-N to produce new cells as long as nitrate-N is available. After the nitrate-N is depleted, the population ceases to grow and metabolizes phenol according to first-order kinetics. This interpretation is in accordance with Schmidt & Gier (1989) and the assumptions of the linear-growth three-half-order model (Brunner & Focht 1984), which includes terms for growth and no-growth metabolism. To our knowledge, this is the first time the dual-substrate models (Schmidt et al. 1985) have been used to model biodegradation kinetics of a system in which nitrogen is a limiting nutrient.

Effects of nitrogen addition on biodegradation

Nutrient addition is a common strategy to accelerate *in situ* bioremediation of ground water (reviewed by Lee et al. 1988). We have investigated pollutant biodegradation over a wider range of CN ratios than usually considered in nitrogen amendment experiments. Laboratory studies evaluating the effects of nitrogen addition on substrate biodegradation in soil (Allison & Cover 1960; Dibble & Bartha 1979; Lerch et al. 1992), aquifer sediments (Swindoll et al. 1988), and pond and river water (Paris & Rogers 1986) have been conducted over CN ratios ranging from less than 1 to 350. We compare our results with these studies below.

Allison & Cover (1960) put shortleaf-pine sawdust (CN400) or wheat straw (CN125) into sandy loam soil that had been leached to remove nitrate, and added enough NH_4NO_3 to bring the CN ratios of the added sawdust to 26, 53, 105 or 350, and of the wheat straw to 25, 50, or 99. For both carbon substrates, the initial rates of CO_2 evolution increased as CN ratio decreased, just as we measured faster $^{14}CO_2$ evolution in the lower CN treatments. By the end of their experiments, more CO_2 had evolved from the treatments with less added N, which agrees with our observation of higher $^{14}CO_2$ production from the higher CN treatments.

Dibble & Bartha (1979) added NH_4NO_3 in three CN ratios, 15, 60 and 300, and phosphorus and potassium in varying amounts, to soil amended with air-dried oil sludge containing 240 mg extractable hydrocarbons per g sludge. More CO_2 was evolved in the CN60 and CN300 treatments (23.3 and 24.0%, respectively) than either the untreated control or the CN15 treatment (13.5 and 13.9%, respectively) for the first half of the experiment. We also observed more CO_2 production at higher CN ratios. After additional sludge was added to the soil, more CO_2 evolved from the CN60 treatment (38.5%), than from the other treatments (22.1 to 30.0%).

In soil amended with sewage sludges having CN ratios ranging from 4.1 to 26.9, both the rate and extent of the percent CO_2 evolved decreased as CN of the sludges increased (Lerch et al. 1992). The presence of heavy metals in the highest CN ratio sludge may have contributed in part to its low rate of CO_2 production. While we also observed decreased rates of CO_2 evolution with increased CN ratio, we found an increased extent of percent $^{14}CO_2$ evolution for CN ratios above 150.

Swindoll et al. (1988) found that adding nitrogen to slurries of Lula aquifer sediments resulted in variable responses with respect to biodegradation of added organic contaminants. For example, adding either 0.1 $\mu g\ g^{-1}$ or 1.0 $\mu g\ g^{-1}$ NH_4Cl to a slurry containing 425 ng *p*-nitrophenol (PNP) per g similarly increased the rate and extent of biodegradation and decreased the acclimation time. The CN ratios of the two treatments were 9.6 and 1.0, respectively, not considering nitrogen contributed by the PNP. Nitrogen addition was not always necessary to achieve biodegradation; a lower concentration of PNP (113 ng g^{-1}) was mineralized equally well with or without added nitrogen (Swindoll et al. 1988). Although this result is consistent with our observation of phenol biodegradation at extremely low nitrate-N levels, we used substrate concentrations three orders of magnitude higher than Swindoll et al. (1988).

Paris & Rogers (1986) measured the effect of nutrient amendment on biodegradation of several xenobiotic compounds, including phenol, at added concentrations of 1 μg ml^{-1} or less in pond and river waters. The waters, which contained low concentrations of nitrogen, were amended with 235 μg ml^{-1} NH_4-N and other inorganic nutrients. Pseudo-first-order rate constants for phenol biodegradation ranged from 0.003 to 0.009 hr^{-1} in the unamended waters and from 0.028 to 0.690 hr^{-1} in the nutrient-amended waters. These rate constants are similar to the pseudo-first-order rate constants, 0.0068 to 0.0257 hr^{-1}, that we modeled for the N-limited treatments CN75 to CN560.

Other potential sources of nitrogen

We have observed mineralization of phenol under very low added-N conditions. Two possible sources of extraneous nitrogen include the Monterey sand used as the porous medium and the cells themselves. In Monterey sand of smaller grain size than that used here (250 to 425 μm in diameter), Rößner et al. (in press) observed that small amounts of nitrogen from the sand enhanced microbial growth. They used mineral medium without nitrogen to extract nitrogen from sands and measured from 0.2 to 0.9 μg N per g of sand. The sand used in experiment D was rinsed and soaked longer than the sand used in experiments A–C, which might explain the somewhat slower rate of $^{14}CO_2$ evolution.

Turnover of dead cells may also furnish nitrogen to the survivors. Yields of active cells upon harvesting were typically 50 to 75% of the number of cells living in the cultures just prior to centrifugation. Therefore, a considerable pool of organic nitrogen may be associated with dead cells that were added to the sand along with live cells. Assuming that the average cell contains 0.14 g N per g dry weight, and that the cell dry weight is 29% of the cell wet weight of 1E-12 g, each cell would contain 4.06E-14 g nitrogen. The average initial population density for the three CN530/560 experiments was 5E6 cells ml^{-1}. If harvest yield were 50%, as many as 5E6 dead cells ml^{-1} could also have been in the inoculum. These dead cells would contribute approximately 0.2 μg N ml^{-1}, which is the same concentration of N as in the CN530/560 mineral medium.

Conclusions

We have examined the effect of systematic N-limitation on biodegradation of the USEPA priority pollutant phenol. For our system, kinetic model parameter estimates for rate constants and growth rates declined as CN ratio increased. Phenol biodegradation proceeded under extremely low added-N conditions, suggesting that in some aquifers it may be possible to minimize N-enhancement during *in situ* bioremediation. The single-substrate logistic model described the kinetics of phenol biodegradation only for the C-limited condition, CN1.5. We found that the dual-substrate models (Schmidt et al. 1985) described phenol biodegradation for most of our N-limited systems. Only when N became extremely limiting, at CN560, did the dual-substrate model collapse to the single-substrate no-growth first-order model. These results suggest that trace amounts of nutrients may be extremely important to oligotrophic microbial communities.

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Appendix A. Kinetics model equations

Abbreviations

S = substrate concentration at time, t

S_0 = initial substrate concentration

K_m = Michaelis-Menten half-saturation constant

μ_{max} = maximum specific growth rate

X_0 = initial population density expressed in terms of substrate concentration

m = linear growth rate

Monod family models (Simkins & Alexander 1984)

first order

$- dS/dt = k_3 S$

$S = S_0 \exp(- k_3 t)$

$k_3 = \mu_{max} X_o / K_m$

logistic

$- dS/dt = k_4 S(S_0 + X_0 - S)$

$S = (S_0 + X_0) / 1 + (X_0/S_0)\exp[k_4(S_0 + X_0)t]$

$k_4 = \mu_{max}/K_m$

Dual-substrate models (Schmidt et al. 1985)

Exponential growth and low concentrations of test substrate

$- dS/dt = k_1 S \exp(rt)$

$S = S_0 \exp\{- (k_1/r)[\exp(rt) - 1]\}$

$k_1 = \mu_{max} X_0 / K_m = k_3$ of Simkins & Alexander (1984)

Linear growth and low concentrations of test substrate

$- dS/dt = k_1 S(1 + mt)$

$S = S_0 \exp[- k_1 (t + mt^2/2)]$

$k_1 = \mu_{max} X_0 / K_m = k_3$ of Simkins & Alexander (1984)

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