Supplemental Substrate Enhancement of 2,4-Dinitrophenol Mineralization by a Bacterial Consortium

T. F. HESS,¹ S. K. SCHMIDT,^{2*} J. SILVERSTEIN,¹ AND B. HOWE²

Department of Civil, Environmental and Architectural Engineering¹ and Department of Environmental, Population and Organismic Biology,² University of Colorado, Boulder, Colorado 80309

Received 28 November 1989/Accepted 21 February 1990

A *Janthinobacterium* sp. and an actinomycete, both capable of mineralizing 2,4-dinitrophenol (DNP), were used to construct a consortium to mineralize DNP in nonaxenic bench-scale sequencing batch reactors (SBRs). Average K_m values for DNP mineralization by pure cultures of the *Janthinobacterium* sp. and the actinomycete were 0.01 and 0.13 $\mu\text{g/ml}$, respectively, and the average maximum specific growth rate (μ_{max}) values for them were 0.06 and 0.23/h, respectively. In the presence of NH_4Cl , nitrite accumulation in pure culture experiments and in the SBRs was stoichiometric to initial DNP concentration and the addition of nitrogen enhanced DNP mineralization in the SBRs. Mineralization of 10 μg of DNP per ml was further enhanced in SBRs by the addition of glucose at concentrations of 100 and 500 $\mu\text{g/ml}$ but not at 10 $\mu\text{g/ml}$. Possible mechanisms for this enhanced DNP mineralization in SBRs were suggested by kinetic analyses and biomass measurements. Average μ_{max} values for DNP mineralization in the presence of 0, 10, 100, and 500 μg of glucose per ml were 0.33, 0.13, 0.42, and 0.59/h, respectively. In addition, there was greater standing biomass in reactors amended with glucose. At steady-state operation, all SBRs contained heterogeneous microbial communities but only one organism, an actinomycete, that was capable of mineralizing DNP. This research demonstrates the usefulness of supplemental substrates for enhancing the degradation of toxic chemicals in bioreactors that contain heterogeneous microbial communities.

Nitrophenols are used in industry as intermediates in the production of dyes, explosives, and pesticides. Because of their widespread use, nitrophenols occur as contaminants in industrial effluents and hence in natural waters (6). The U.S. Environmental Protection Agency lists several mono-nitrophenols and dinitrophenols on its "Priority Pollutants List" and recommends restricting their concentrations in natural waters to below 10 ng/ml (26). Because the addition of one or more nitro groups to the phenol molecule reduces the electron density of the aromatic ring, electrophilic attack of the compounds by microorganisms is impeded (2, 25) and the compounds may accumulate in the environment.

While past research has revealed a variety of organisms that can utilize mono-nitrophenols as sources of carbon and energy (14, 24, 27, 28), few researchers have reported on microorganisms that can mineralize dinitrophenols (2, 9). In recent work with dinitrophenols, 2,6-dinitrophenol was found to be degraded only under nitrogen-limiting conditions in the presence of more readily usable carbon sources (2).

Several studies have demonstrated the effectiveness of supplemental substrates for enhancing the rate of biodegradation of toxic chemicals by pure cultures of bacteria (10, 20). Schmidt et al. (20) showed that glucose could increase the growth rate of *p*-nitrophenol-mineralizing bacteria and thereby increase the rate of *p*-nitrophenol mineralization. However, the usefulness of this type of enhancement may be limited by heterogeneous systems where multiple populations of microorganisms compete with the degrading organisms for the supplemental substrate. Such a lack of enhancement has been seen in attempts to increase the rate of biodegradation of nitrophenols in soil (22).

Biodegradation of other phenolic compounds has been shown to be inhibited by glucose in aquatic treatment systems (12, 17). In batch cultures of mixed populations,

glucose inhibited the degradation of 2,4-dichlorophenoxyacetic acid even though the two substrates were used simultaneously. Likewise, glucose inhibited the degradation of phenol by a heterogeneous population that had previously been acclimated to phenol, but inhibition was reduced if an inoculum that was acclimated to phenol and glucose was used (17).

The present study was undertaken to optimize conditions for the biodegradation of 2,4-dinitrophenol (DNP) by a bacterial consortium in sequencing batch reactors (SBRs). SBR technology was previously described by Irvine and Busch (8) and is used for wastewater treatment in industry and to a lesser extent in domestic wastewater treatment. The approach of the present study was to select for organisms that could degrade DNP over a broad range of concentrations, to determine which supplemental substrates enhanced degradation in pure cultures, and then to inoculate SBRs with these organisms. The SBRs were run with different concentrations of supplemental substrate until steady-state conditions were reached and before assessing the effects of the supplemental substrate on DNP mineralization. This approach allowed for acclimation of the SBR cultures to the experimental conditions and demonstrated that the effects were reproducible and stable.

MATERIALS AND METHODS

Cultures. An actinomycete and a *Janthinobacterium* sp. isolated from a freshwater stream and forest soil (19), respectively, were used for all experimental work. These organisms were isolated by using techniques specifically designed to maintain their ability to degrade low concentrations of xenobiotic chemicals. Neither organism was ever grown on high concentrations of chemicals or cultured on rich laboratory media such as nutrient agar. The identification of organisms was done by using the standard techniques described by Holt and Krieg (7). Several other techniques

* Corresponding author.

FIG. 1. Schematic diagram of SBR and associated pumping equipment.

were also used to identify the bacteria. The number and position of bacterial flagella were determined by using the technique of M. E. Heimbrosok, W. L. L. Lang, and G. Campbell (Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, R22, p. 240), and the capacity of the organisms to metabolize other organic compounds as sole sources of carbon and energy was tested by using the auxanographic technique of Parke and Ornston (13). Results of the auxanographic study were used in part to identify possible supplemental substrates for enhanced degradation studies. Analysis of cell wall amino acids was performed by using the technique of Schaal (18). Final identification of the *Janthinobacterium* sp. was done by analysis of cell wall fatty acids by the techniques of Fieldhouse and Miller (5). Analysis of all of the above test results for the actinomycete bacterium was inconclusive for the genus or species level of identification.

Pure culture experiments. The actinomycete did not require any exogenously supplied growth factors and was grown in a liquid medium containing different concentrations of reagent grade DNP (Fluka Chemical Co., Ronkonkoma, N.Y.) as a source of carbon and energy in addition to 0.48 g of Na_2HPO_4 , 0.5 g of KH_2PO_4 , 0.04 g of NH_4Cl , 0.01 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 0.01 g of CaCl_2 per liter of solution. The *Janthinobacterium* sp. was grown in the same liquid medium except that yeast extract, at a concentration of 5 $\mu\text{g}/\text{ml}$, was added to supply the growth factors that this auxotrophic organism needed for growth. The pH of all solutions was 6.8, and no change in pH occurred as a result of DNP mineralization.

Pure culture experiments were conducted aerobically in 250-ml glass-stoppered Erlenmeyer flasks containing 100 ml of liquid medium incubated at $24 \pm 2^\circ\text{C}$. ^{14}C -labeled DNP was added to the experimental flasks to give 10,000 dpm/ml. Unlabeled DNP was added to flasks containing the labeled chemical to attain higher substrate concentrations. The L-[U- ^{14}C]DNP had a specific activity of 0.1 mCi/mmol and was purchased from Sigma Chemical Co., St. Louis, Mo.

SBR experiments. Bench-scale SBRs used in this study (Fig. 1) were operated with 4 liters of liquid volume. Each reactor had an initial DNP concentration of 10 $\mu\text{g}/\text{ml}$. Three parallel reactors were used in each set of experiments,

providing a control (no supplemental glucose) and two concentrations of glucose (10 and 100 $\mu\text{g}/\text{ml}$). SBRs were operated on a 48-h cycle. The length of each component of the cycle was as follows: fill, 48 min; react, 44 h 47 min; settle, 1 h 46 min; decant, 39 min.

Organisms were grown in the SBRs with liquid media similar to that described for pure culture studies minus the yeast extract. The inorganic salts solution, excluding NH_4Cl , was kept in a 100-liter tank for use during the fill cycle. DNP, glucose, and NH_4Cl were combined in a concentrated sterile solution that was used as the carbon feed during the fill cycle. This carbon feed was made and stored separately for each reactor.

SBRs were initially inoculated with cultures of both the actinomycete and the *Janthinobacterium* sp. A 100-ml volume of a pure culture of each organism, that had been grown to stationary phase on 10 μg of DNP and 100 μg of glucose per ml, was added to each reactor. No attempt was made to keep the reactor contents free of contaminating organisms after inoculation.

The inorganic salts solution was pumped during the fill cycle by variable-speed peristaltic pumps and during the decant cycle by constant-speed peristaltic pumps (Cole-Parmer, Chicago, Ill.). Carbon source feed was provided by microdispensing meter pumps with synchronous drives (Fluid Metering, Inc., Oyster Bay, N.Y.). Carbon source feed and inorganic salts solution feed were mixed in a T-connection during the fill cycle prior to entrance into the reactor. The carbon source was not dispensed until the latter portion of the fill cycle to avoid excessive degradation prior to sampling during the react cycle. All electrical equipment, pumps, and air solenoid valves were operated by programmable Chronrol timers (Linburg Enterprises, San Diego, Calif.).

Each reactor was constructed from an 8-in. (ca. 20-cm) (inside diameter) clear acrylic cylinder glued to a 0.5-in.-thick (ca. 1.3-cm) acrylic bottom plate. A reactor lid was constructed from a 0.5-in.-thick (ca. 1.3-cm) acrylic plate with a machined groove and a gasket to provide for an air-tight system. Bulkhead penetrations in the lid were made for decant and aeration tubes in addition to a Tenax-charged

(Alltech Associates, Inc., Deerfield, Ill.) gas-trap tube to capture any volatilized DNP. Penetrations in the reactor wall provided for a feed tube and sample port for syringe withdrawals of reactor contents. Each reactor was 16 in. (ca. 41 cm) high, and the operating volume of 4 liters filled about half of the available volume. Reactors were aerated by a dual sparger connected to a source of compressed air. Filtered air was supplied at 6 lb/in² from a spring-activated pressure valve and metered at 100 ml/min by a combination flow meter-needle valve (Cole-Parmer). Positive control of air was accomplished by the use of air solenoid valves. Mixing of the reactor contents was provided by a 1-in. (ca. 2.5-cm) magnetic stir bar. A 0.5-in. (ca. 1.3-cm) air gap between the magnetic stir plate and reactor prevented any heating of reactor fluid during operation. The three reactors were placed in a fume hood to vent exhaust gas from the gas traps, and reactor contents remained at a constant $22 \pm 2^\circ\text{C}$.

A gas trap was installed on each reactor to quantify any amount of DNP that volatilized during the reaction cycles. A trap consisted of a Pyrex tube (10 mm [inside diameter] by 20 cm) with a fritted glass insert (25- to 50- μm pore size). One gram of 60/80-mesh Tenax was placed on top of the frit surface and held in place by glass wool plugs. The trap was then secured in the reactor with a bead of silicone rubber sealant around the perimeter of the tube. The tubes and Tenax, both washed in petroleum ether, were kept sealed with Teflon plugs until the silicone sealant had cured and a fill cycle of the reactor had occurred.

Measurement of mineralization. Mineralization of DNP in pure culture was measured by the technique of Schmidt et al. (20). In this procedure, 2.5-ml samples were vacuum filtered through 0.2- μm -pore-size polycarbonate filters (Nuclepore Corp., Pleasanton, Calif.), and 1-ml subsamples were added to 4-ml Omni scintillation vials (Wheaton Industries, Millville, N.J.) along with 2.5 ml of ScintiVerse II scintillation cocktail (Fisher Scientific Co., Pittsburgh, Pa.). Radioactivity was determined by using a model LS-3133T liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.). Disappearance of DNP was also measured by using a DMS 100 spectrophotometer (Varian, Palo Alto, Calif.) at 260 nm. Samples (6 ml) were withdrawn from the center of each reactor at regular intervals with a syringe and a 6-in. (ca. 15-cm), 20-gauge needle. The samples were vacuum filtered through 0.2- μm -pore-size polycarbonate filters and acidified with 1 drop of concentrated H₂SO₄. The DNP content of samples was determined at 260 nm on a Varian DMS 100 spectrophotometer. Disappearance of DNP was confirmed by gas chromatography-mass spectrometry on a model 5988A mass spectrometer (Hewlett Packard Co., Palo Alto, Calif.).

Mineralization was also assessed by measuring ¹⁴CO₂ evolution in a manner similar to that used by Zeyer and Kearney (27). Evolved ¹⁴CO₂ was captured in 1 ml of 0.5 N NaOH at timed intervals in a sidearm biometer flask. Any remaining ¹⁴CO₂ in solution at the end of the experiment was evolved by acidification with concentrated H₂SO₄. The NaOH samples were added to scintillation vials with 2.5 ml of scintillation cocktail, and radioactivity was determined by using the liquid scintillation counter.

Nitrite was determined as described previously (1). Sample sizes and all reagents were reduced by a factor of 10. Analyses were carried out on a Varian DMS 100 spectrophotometer at 543 nm. The original nitrite in solution was subtracted from the nitrite measured during the experiment to obtain the measurement of nitrite accumulation during DNP mineralization.

Data analysis. Data from the mineralization experiments were analyzed by nonlinear regression techniques as described by Simkins and Alexander (23). An MS-DOS-based microcomputer was used to run LMFIT, an author-written computer program that minimizes the least square of difference between data and various model curves by the method of Levenberg and Marquardt as described by Dennis and Schnabel (4). The computer program requires the user to enter a model and analytical partial derivatives of the dependent variable with respect to each parameter. There is also a provision for specifying only the model type, with partial derivatives being estimated numerically within the program via central-difference gradients (4). The program output includes parameter estimates and their standard deviations. Results from both analytical and numerical partial-derivative forms of the program show no significant differences in output.

Model discrimination was based on a comparison of residual sums of squares between different model fits to data. A particular model was considered an accurate fit of the data set only if the difference between it and a model with fewer parameters was significant at the 95% confidence level in a standard F-test (15). Models were also judged for accuracy of fit based on their standard error of the estimated parameters (15). If the standard error of the estimated parameter was greater than 50%, the model was deemed an inferior fit to that data set (15). The significance of differences between data sets for measurements of suspended solids and times of degradation were also analyzed with the F-test at the 95% confidence level.

Theoretical considerations. The logistic model was previously used to describe the growth of organisms that degraded low concentrations of single xenobiotic compounds (23). A substrate-based form of the logistic model, derived from conditional restraints of the more general Monod model, was presented by Simkins and Alexander (23). For the purposes of this paper, however, a more mechanistic development of the population-based logistic model into a substrate-based model is presented below. The advantage of this reparameterized model is that estimates of the maximum specific growth rate, μ_{max} , can be obtained directly from nonlinear regression analysis, whereas μ_{max} is contained within a derived parameter in the formulation of Simkins and Alexander (23). Thus, the model presented below is a reparameterized version of a previously published model. An additional parameter, K_s , can be calculated from the use of the logistic equation presented below in consort with the logistic equation of Simkins and Alexander (23). To calculate K_s , estimates of μ_{max} from the logistic equation presented herein may be divided by estimates of K_4 (which equals μ_{max}/K_s).

Development of the substrate-based logistic model begins with the population-based expression (3, 11):

$$dB/dt = (\mu_{\text{max}} B) (1 - B/B_{\text{max}}) \quad (1)$$

where B is the population density, B_{max} is the maximum population density achievable in that environment, and μ_{max} is the maximum specific growth rate. Equation 1 can be integrated to obtain

$$B = B_{\text{max}} / \{1 + [(B_{\text{max}} - B_0)/B_0]e^{-\mu_{\text{max}}t}\} \quad (2)$$

To show changes in population density due to changes in substrate concentration, a mass balance is used (16):

$$S_0 + qB_0 = S + qB \quad (3)$$

where S_0 is the initial substrate concentration, B_0 is the

initial population density, and q is the cell quota or inverse yield. Following the work of Simkins and Alexander (23), q can be considered invariant with time and substrate concentration, and therefore qB is replaced with X , the amount of substrate required to produce a population density B , and likewise qB_0 is replaced by X_0 . These substitutions result in the following equations:

$$X = X_{\max} / \{1 + [(X_{\max} - X_0)/X_0]e^{-\mu_{\max}t}\} \quad (4)$$

and

$$S_0 + X_0 = S + X \quad (5)$$

It follows that maximum achievable population, $X_{\max}$, will always be a function of the original population present, X_0 , plus the original substrate present, S_0 , that can be used during growth to create new cells, thus:

$$X_{\max} = X_0 + S_0 \quad (6)$$

When equations 5 and 6 are substituted into equation 4, the substrate-based logistic model results:

$$S = (S_0 + X_0) [(S_0 e^{-\mu_{\max}t}) / (X_0 + S_0 e^{-\mu_{\max}t})] \quad (7)$$

Additional models used to analyze data for pure culture, single-substrate experiments have been described by Simkins and Alexander (23). Models used for analyses of data from supplemental substrate-enhanced degradation have been described previously by Schmidt et al. (21). The curves of DNP mineralization for cells growing on the supplemental substrate, glucose, were best fit by their model IV (21):

$$S = S_0 e^{-(k_1/\mu_{\max}) [\exp(\mu_{\max}t) - 1]} \quad (8)$$

where $k_1 = V_{\max}B_0/K_m$, $V_{\max}$ is the maximum specific reaction rate, and all other terms are as described above.

RESULTS

Characterization of isolates. The actinomycete, strain S, was a long, nonmotile, gram-positive rod which formed catalase but was oxidase negative (Table 1). The presence of tuberculostearic acid and other factors indicated that this organism may be a coryneform bacterium, but genus-level identification was not possible. The *Janthinobacterium* sp., strain T, was a short, motile, gram-negative rod with more than one polar flagellum and was oxidase and catalase positive. Neither organism grew at 41°C. The results of tests for carbon source utilization are shown in Table 1.

Pure culture mineralization of DNP. Both bacterial isolates were able to mineralize DNP as their sole source of carbon and energy. In addition, the actinomycete was able to utilize DNP as its nitrogen source. To determine the various kinetic parameters of DNP mineralization, solutions containing different concentrations of DNP were inoculated with the individual bacterial isolates. Mineralization of DNP at 0.5 and 0.1 µg/ml by nongrowing cells of the actinomycete and *Janthinobacterium* sp. are shown in Fig. 2 and 3, respectively. These curves of mineralization were best fit by the Michaelis-Menten model to give the parameters shown in Table 2. To verify the values for kinetic constants, two replicate experiments were conducted at two different DNP concentrations for the actinomycete, 0.5 and 1.0 µg/ml, and two replicate experiments were conducted for the *Janthinobacterium* sp. at one DNP concentration, 0.1 µg/ml (Table 2).

Experiments were also done to ascertain growth parameters, such as $\mu_{\max}$, for both organisms. Two replicate experiments at 0.1 µg of DNP per ml were conducted for the

TABLE 1. Characteristics of the actinomycete and the *Janthinobacterium* sp.

Characteristic	Actinomycete	<i>Janthinobacterium</i> sp.
Morphology	Rod	Rod
Gram stain	+	—
Flagella	None	Polar >1
Oxidase	—	+
Catalase	+	+
Fluorescent pigment	—	—
Arginine dihydrolase	—	ND ^a
Growth at 41°C	—	—
PHB production ^b	+	ND
Denitrification	—	ND
Growth on:		
Acetate	+	+
Anthranilate	+	+
Arabinose	+	+
Arginine	+	+
Aspartate	+	+
Benzoate	+	+
Betaine	+	+
Caffeate	+	+
Catechol	+	+
trans-Cinnamate	+	—
Citrate	+	+
p-Coumarate	+	+
Ferulate	+	+
Formate	—	—
Fumarate	+	+
Gallate	+	—
Gluconate	+	+
Glucose	+	+
Glutamate	—	—
Glycerol	—	ND
Glycine	—	+
m-Hydroxybenzoate	+	+
p-Hydroxybenzoate	+	+
DL-Kynurenine	—	+
Lactose	—	—
D-Malate	+	+
D-Mannose	—	+
1-Naphthol	+	—
m-Nitrophenol	—	—
o-Nitrophenol	—	—
p-Nitrophenol	—	+
Phenol	+	+
Protocatechuate	+	+
Quinate	+	+
Rhamnose	+	+
Salicin	—	—
Salicylate	—	+
Saligenin	—	—
L-Serine	+	+
Succinate	+	+
Sucrose	+	—
Syringate	—	—
p-Toluate	+	—
Trehalose	+	+
Uric acid	+	—
Vanillate	+	+
Vanillin	+	+

^a ND, Not determined.

^b PHB, Poly-β-hydroxybutyrate.

actinomycete, and two different experiments at 1.0 and 0.5 µg of DNP per ml were done for the *Janthinobacterium* sp. The model of best fit for the actinomycete data was the logistic equation, whereas the logarithmic equation (22) described the data from the experiments with the *Janthinobacterium* sp. (data not shown). Parameters that were esti-

FIG. 2. Mineralization of 0.5 µg of DNP per ml by nongrowing cells of an actinomycete. The mineralization curve was fit by the Michaelis-Menten equation.

FIG. 3. Mineralization of 0.1 µg of DNP per ml by nongrowing cells of a *Janthinobacterium* sp. The mineralization curve was fit by the Michaelis-Menten equation.

ated by nonlinear regression analyses of data from the growth experiments are shown in Table 3.

Nitrite accumulation during DNP mineralization, in the presence of added nitrogen as NH_4Cl , is shown for the actinomycete (Fig. 4). Cells for the experiment were previously grown on 2.5 µg of DNP per ml and then amended with either 1 or 5 µg of DNP per ml. Stoichiometric amounts of nitrite were accumulated in each case: 14.3 µM NO_2^- per 7.7 µM DNP in the 1-µg/ml amended flask and 52.4 µM NO_2^- per 27.5 µM DNP in the 5-µg/ml flask (Fig. 4). The resulting NO_2^- -to-DNP molar ratios were 1.85 and 1.91, respectively. Production of $^{14}\text{CO}_2$ from ^{14}C -DNP mineralization was measured for the actinomycete. A replicated experiment showed $53 \pm 1\%$ of total labeled DNP recovered as $^{14}\text{CO}_2$.

DNP mineralization in SBRs. A preliminary experiment was conducted in two SBRs inoculated with both pure culture strains (actinomycete and *Janthinobacterium*, sp.) to ascertain the effects of added nitrogen on DNP mineralization. Concentrations of DNP and glucose were 4 and 10 µg/ml, respectively. One reactor was run with the complete mineral salts medium, and the other was run with the same medium without the addition of NH_4Cl . The presence of added nitrogen caused a slight enhancement of DNP mineralization (Fig. 5). The Michaelis-Menten model best represented both DNP mineralization data sets. Parameters from this model fit were as follows: (i) for the SBR with externally added nitrogen source, $K_m = 1.85 \pm 0.29$ µg/ml and rate constant = 1.51 ± 0.12 µg/ml per h; (ii) for the SBR without externally added nitrogen source, $K_m = 1.59 \pm 0.26$ µg/ml and rate constant = 1.01 ± 0.07 µg/ml per h. In a subsequent experiment (data not shown), using the same DNP and glucose concentrations, the maximum nitrite evolution in the SBR with external nitrogen added was approximately in stoichiometric amounts to initial DNP (37.8 µM NO_2^- and 20.1 µM DNP), the NO_2^- -to-DNP molar ratio being approx-

imately 1.88. Maximum nitrite evolution in the SBR without external nitrogen added was considerably less than that with added nitrogen (7.1 µM versus 37.8 µM), presumably because of the use of nitrite from DNP as a nitrogen source. Two replicate experiments were conducted for the measurement of $^{14}\text{CO}_2$ produced from mineralization of ^{14}C -DNP in samples from the SBRs. Results showed that $37 \pm 4\%$ of total labeled DNP was recovered as $^{14}\text{CO}_2$.

In subsequent experiments, three SBRs were run in parallel for 3 months, each containing equal concentrations of DNP (10 µg/ml), different concentrations of glucose as a supplemental substrate, and complete mineral salts solution including NH_4Cl . Experiments measuring DNP removal rate profiles were conducted during a single reaction cycle every 2 to 3 weeks. Results of the first experiments differed from

TABLE 2. Comparison of separate nonlinear regression estimates of parameters of the Michaelis-Menten model in replicate experiments with different initial substrate concentrations

Organism	S_0 (µg/ml)	K_m (µg/ml)	Rate constant (µg/ml per h)
Actinomycete	0.52 ± 0.006	0.12 ± 0.030	0.34 ± 0.026
	1.06 ± 0.011	0.14 ± 0.043	0.49 ± 0.032
<i>Janthinobacterium</i> sp.	0.10 ± 0.001	0.01 ± 0.002	0.14 ± 0.006

FIG. 4. Production of nitrite (NO_2^-) and the disappearance of DNP in cultures of the actinomycete at DNP concentrations of 1 (A) and 5 (B) µg/ml.

TABLE 3. Nonlinear regression estimates of growth-related kinetic parameters in experiments with different initial substrate concentrations and different initial population sizes^a

Organism/model	S_0 ($\mu\text{g/ml}$)	X_0 ($\mu\text{g/ml}$)	$\mu_{\max}/\text{h}$	K_s ($\mu\text{g/ml}$)
Actinomycete/logistic	0.10 ± 0.001	0.02 ± 0.003	0.23 ± 0.017	0.12 ± 0.004
	0.10 ± 0.001	0.01 ± 0.002	0.23 ± 0.010	0.11 ± 0.001
<i>Janthinobacterium</i> sp./logarithmic	2.00 ± 0.017	4.14 ± 1.267	0.07 ± 0.019	NA ^a
	0.10 ± 0.0004	0.04 ± 0.008	0.06 ± 0.010	NA

^a NA, Not applicable.

later results presumably because of non-steady-state conditions prevailing when the reactors were first set up. Figure 6 shows the results of two representative SBR experiments. The first experiment (Fig. 6A) was conducted after 2 weeks of operation when the reactors had not reached steady-state conditions. The second experiment (Fig. 6B) is more representative of later experiments and shows a marked enhancement of DNP mineralization in the presence of 100 μg of glucose per ml but no enhancement in the presence of 10 μg of glucose per ml.

To determine if DNP mineralization could be further enhanced, the reactor that had been run with 10 μg of glucose per ml was converted to run at a glucose concentration of 500 $\mu\text{g/ml}$ while the other two reactors continued to run at glucose concentrations of 0 and 100 $\mu\text{g/ml}$ (Fig. 7). The reactors were allowed to run for eight cycles at these concentrations before the experiment shown in Fig. 7 was performed. The higher concentration of glucose (500 $\mu\text{g/ml}$) caused an even greater stimulation of DNP mineralization than did 100 μg of glucose per ml (Fig. 7).

The curves of DNP mineralization for all controls (no glucose added) were best fit by the logistic growth model. Curves of DNP mineralization with supplemental substrate additions were all best fit by Model IV of Schmidt et al. (21). Table 4 presents the estimates of the model parameters from nonlinear regression analyses for all SBR results shown in Fig. 6 and 7. In addition to population estimates from the logistic model, biomass was measured on a dry-weight basis for each reactor at the completion of an operating cycle for each experiment. Measurements of suspended solids at the end of each cycle and average times for complete DNP degradation in each reactor are shown in Table 5. The differences among the measurements of suspended solids were shown to be significant ($P = 0.05$) by using the standard F-test; however, there was no significant difference between the DNP-degradation times in the presence of 0 and 10 μg of glucose per ml ($P = 0.05$).

FIG. 5. Mineralization of 4 μg of DNP per ml in the presence of 10 μg of glucose per ml and in the presence or absence of NH_4Cl in SBRs. Both curves were fit by the Michaelis-Menten equation.

DISCUSSION

Preliminary experiments with pure cultures of bacteria were used to select two strains of bacteria that could be used to construct a consortium with the ability to degrade DNP over the wide range of concentrations that would be encountered during operation of SBRs. The K_m and $\mu_{\max}$ values for each organism (Tables 2 and 3) indicated that the actinomycete should be more efficient at mineralizing relatively high concentrations of DNP whereas the *Janthinobacterium* sp. should be able to mineralize much lower concentrations of DNP. However, only the actinomycete could be reisolated from the reactor population after the SBRs had been run for many cycles. This may have been because the concentration of DNP used (10 $\mu\text{g/ml}$) was closer to the K_m of the actinomycete than that of the *Janthinobacterium* sp. It may also have been that the filamentous growth of the actinomycete favored its retention in the sludge particles in the reactors. Further evidence that the *Janthinobacterium* sp. was washed out is that the $\mu_{\max}$ of the population that

FIG. 6. Mineralization of 10 μg of DNP per ml in the presence of 0, 10, or 100 μg of glucose per ml in SBRs during experiments 4 (A) and 7 (B) (see Table 4). The mineralization curves were fit by nonlinear regression.

TABLE 4. Comparison of separate experiments run during different reaction cycles of continuously operating SBRs^a

Expt	Glucose concn (μg/ml)	Model ^b	Rate constant	X_0 (μg/ml)	$\mu_{\max}$ /h
4	0	Logistic	0.03 ± 0.001^c	1.1 ± 0.095	0.33 ± 0.009
7	0	Logistic	0.03 ± 0.001^c	1.1 ± 0.076	0.36 ± 0.007
8	0	Logistic	0.03 ± 0.001^c	1.4 ± 0.054	0.31 ± 0.004
4	10	IV	0.05 ± 0.001^d	NA ^e	0.12 ± 0.005
7	10	IV	0.07 ± 0.002^d	NA	0.14 ± 0.004
6	10	IV	0.08 ± 0.002^d	NA	0.14 ± 0.004
4	100	IV	0.11 ± 0.007^d	NA	0.33 ± 0.020
7	100	IV	0.08 ± 0.002^d	NA	0.49 ± 0.007
8	100	IV	0.07 ± 0.004^d	NA	0.44 ± 0.016
8	500	IV	0.11 ± 0.007^d	NA	0.59 ± 0.026

^a All experiments were conducted at an initial DNP concentration of 10 μg/ml.

^b Model IV is shown as equation 8 in Materials and Methods.

^c Rate constant, $K_4 = \mu_{\max}/K_s$, expressed in micrograms per ml per h (23).

^d Rate constant, $K_1 = V_{\max} B_0/K_s$, expressed in units per h (21).

^e NA, Not applicable.

mineralized DNP in the SBRs containing only DNP ranged from 0.31 to 0.36/h (Table 4), whereas the $\mu_{\max}$ for the *Janthinobacterium* sp. was 0.07/h (Table 3). It is of interest that, although a diverse microbial community developed in the SBRs, no other DNP-mineralizing microorganisms seem to have invaded the reactors. Microscopic observations indicated the presence of bacterial and fungal populations as well as the presence of active predators, including a variety of protozoa and rotifers.

The logistic model was effective for fitting data both in pure cultures and SBRs when a single substrate was being mineralized (Tables 3 and 4). It is important to note that the logistic model used in this study fit the data as well as the logistic model used by Simkins and Alexander (23). Residual sums of squares and the parameters X_0 and S_0 were identical for both model fits to the same data sets, thus verifying the model as used in this paper. Values of K_s derived from both models in consort (Table 3) were in close agreement with K_m values from nongrowth experiments (Table 2). Values for K_m and K_s for a given organism should be equivalent if the growth rate is limited by the mineralization rate (20).

Nitrite evolution in both pure culture and the activated sludge cultures was approximately in stoichiometric propor-

tion to the initial DNP concentration. The respective molar ratios of NO_2^- to DNP, 1.88 and 1.90, are similar to those presented for mineralization of 2,6-dinitrophenol (2). Other possible sources of nitrite were not accounted for in the SBRs, but nitrite accumulation is not the only evidence for DNP mineralization presented in this study. Other evidence for DNP mineralization includes DNP disappearance, $^{14}\text{CO}_2$ production from ^{14}C -DNP, and the fact that the SBR with no added glucose maintained an active DNP-degrading population for over 60 consecutive cycles of operation. Sixty cycles of operation would have resulted in a 2,400-fold dilution of the original inoculum, and thus growth on DNP was necessary to maintain the active population.

Unlike the effects of nitrogen in previous studies of dinitrophenol mineralization (2), readily available sources of nitrogen did not inhibit DNP mineralization in the present study. This is probably because the organisms used in this study were enriched for under conditions of carbon rather than nitrogen limitation. As a result, the actinomycete could use DNP as its sole source of carbon and energy as well as nitrogen. Rather than inhibiting DNP mineralization in the SBRs, ammonia actually stimulated DNP mineralization (Fig. 5), increasing the rate constant for DNP mineralization by approximately 50%.

Further stimulation of DNP degradation was seen in the presence of glucose at concentrations of 100 μg/ml or higher (Fig. 6 and 7). These results were very reproducible after the reactors had run for at least 30 cycles, presumably because the reactors had reached steady-state operation. This is

FIG. 7. Mineralization of 10 μg of DNP per ml in the presence of 0, 100, and 500 μg of glucose per ml in SBRs during experiment 8 (see Table 4). The mineralization curves were fit by nonlinear regression.

TABLE 5. Average concentration of suspended solids and time to 90% DNP mineralization for SBR experiments at four supplemental substrate (glucose) concentrations^a

Glucose concn (μg/ml)	Suspended solids concn (μg/ml)	Time to 90% degradation (h)
0	32 ± 13	20 ± 3^b
10	78 ± 39	19 ± 4^b
100	397 ± 132	8 ± 1
500	958 ± 192	6

^a All experiments were conducted at an initial DNP concentration of 10 μg/ml.

^b Data not significantly different at $P = 0.05$. All others were significantly different at $P = 0.05$.

indicated by the consistency of kinetic parameter estimates between experiments 7 and 8 for 0 and 100 μg of added glucose per ml and experiments 6 and 7 for 10 μg of added glucose per ml (Table 4). This type of enhanced degradation during steady-state reactor operation is similar to the results which LaPat-Polasko et al. (10) obtained for methylene chloride degradation in biofilm reactors. It is interesting to note that μ_{max} values from the SBRs with no glucose were almost 2.5 times higher than μ_{max} values from the SBRs with 10 μg of added glucose per ml. This may indicate that the DNP-mineralizing organisms are not able to compete with the total population for glucose at concentrations of 10 $\mu\text{g}/\text{ml}$ or lower. At concentrations of 100 $\mu\text{g}/\text{ml}$ and higher, however, enough glucose was evidently available to stimulate the DNP-mineralizing population.

A possible mechanism underlying the enhancement of DNP mineralization was indicated by kinetic analyses of the DNP-depletion curves and the resultant maximum growth rates. Once steady-state operation of the SBRs was achieved (Table 4, experiments 7 and 8), the estimated value for μ_{max} averaged 0.47/h in the presence of 100 μg of glucose per ml and 0.34/h in the absence of added glucose. In addition, the μ_{max} for the single experiment run at a glucose concentration of 500 $\mu\text{g}/\text{ml}$ indicated that the DNP-mineralizing population was growing with a μ_{max} of 0.59/h. This correlation between enhanced DNP mineralization and enhanced growth rate is consistent with the work of Schmidt et al. (20) who showed that enhancement of *p*-nitrophenol mineralization was caused by an increased growth rate of *p*-nitrophenol-mineralizing bacteria in the presence of glucose. It is also evident (Table 5) that the overall population of microorganisms was much greater in the reactors with 100 and 500 μg of glucose per ml than in the reactor with no added glucose. Thus, the carryover of a high population of DNP degraders may also be responsible for the enhanced degradation of DNP in the presence of glucose. Further work is needed to clarify the mechanism of this enhancement of DNP mineralization.

ACKNOWLEDGMENTS

This work was supported by a grant from the Colorado Water Resources Research Institute and grant CES8802749 from the National Science Foundation.

We thank P. Radehaus for reading a draft of the manuscript and M. Gier and M. Hermann for technical assistance.

LITERATURE CITED

- American Public Health Association. 1985. Standard methods for the examination of water and wastewater, 16th ed. American Public Health Association, Washington, D.C.
- Bruhn, C., H. Lenke, and H. J. Knackmuss. 1987. Nitrosubstituted aromatic compounds as nitrogen source for bacteria. *Appl. Environ. Microbiol.* **53**:208–210.
- Bull, A. T., M. E. Bushell, T. G. Mason, and J. H. Slater. 1975. Growth of filamentous fungi in batch culture: a comparison of the Monod and logistic models. *Proc. Soc. Gen. Microbiol.* **3**:62–63.
- Dennis, J. E., Jr., and R. B. Schnabel. 1983. Numerical methods for unconstrained optimization and nonlinear equations. Prentice-Hall, Inc., Englewood Cliffs, N.J.
- Fieldhouse, D. J., and L. Miller. 1986. Coryneform cellular fatty acid analysis as a taxonomic aid. In *Gas chromatography*, Technical Paper No. 119. Hewlett-Packard Co., Palo Alto, Calif.
- Hallas, L. E., and M. Alexander. 1983. Microbial transformation of nitroaromatic compounds in sewage effluent. *Appl. Environ. Microbiol.* **45**:1234–1241.
- Holt, J. G., and N. R. Krieg (ed.). 1984. *Bergey's manual of determinative bacteriology*, 9th ed., vol. 1. The Williams & Wilkins Co., Baltimore.
- Irvine, R. L., and A. W. Busch. 1979. Sequencing batch biological reactors—an overview. *J. Water Pollut. Control Fed.* **51**:235–243.
- Jensen, H. L., and G. Lautrup-Larsen. 1967. Microorganisms that decompose nitro-aromatic compounds, with special reference to dinitro-orthocresol. *Acta Agric. Scand.* **17**:115–125.
- LaPat-Polasko, L. T., P. L. McCarty, and A. J. B. Zehnder. 1984. Secondary substrate utilization of methylene chloride by an isolated strain of *Pseudomonas* sp. *Appl. Environ. Microbiol.* **47**:825–830.
- Odum, E. P. 1971. *Fundamentals of ecology*, 3rd ed. Saunders College Publishing, Philadelphia.
- Papanastasiou, A. C., and W. J. Maier. 1982. Kinetics of biodegradation of 2,4-dichlorophenoxyacetate in the presence of glucose. *Biotechnol. Bioeng.* **24**:2001–2011.
- Parke, D., and L. N. Ornston. 1984. Nutritional diversity of Rhizobiaceae revealed by auxanography. *J. Gen. Microbiol.* **130**:1743–1750.
- Raymond, D. G. M., and M. Alexander. 1971. Microbial metabolism and cometabolism of nitrophenols. *Pestic. Biochem. Physiol.* **1**:123–130.
- Robinson, J. A. 1986. Approaches and limits to modeling microbiological processes, p. 20–29. In F. Megusar and M. Gantar (ed.), *Proceedings of the 4th International Symposium on Microbial Ecology*. Slovene Society for Microbiology, Ljubljana, Yugoslavia.
- Robinson, J. A., and J. M. Tiedje. 1983. Nonlinear estimation of Monod growth parameters from a single substrate depletion curve. *Appl. Environ. Microbiol.* **45**:1453–1456.
- Rozich, A. F., and R. J. Colvin. 1986. Effects of glucose on phenol biodegradation by heterogeneous populations. *Biotechnol. Bioeng.* **28**:965–971.
- Schaal, K. P. 1984. Identification of clinically significant actinomycetes and related bacteria using chemical techniques, p. 359–381. In M. Goodfellow and D. E. Minnikin (ed.), *Chemical methods in bacterial systematics*. Society for Applied Bacteriology Technical Series No. 20. Academic Press, Inc. (London) Ltd., London.
- Schmidt, S. K., and M. Gier. 1989. Dynamics of microbial populations in soil: indigenous microorganisms degrading 2,4-dinitrophenol. *Microb. Ecol.* **18**:285–296.
- Schmidt, S. K., K. M. Scow, and M. Alexander. 1987. Kinetics of *p*-nitrophenol mineralization by a *Pseudomonas* sp.: effects of second substrates. *Appl. Environ. Microbiol.* **53**:2617–2623.
- Schmidt, S. K., S. Simkins, and M. Alexander. 1985. Models for the kinetics of biodegradation of organic compounds not supporting growth. *Appl. Environ. Microbiol.* **50**:323–331.
- Scow, K. M., S. K. Schmidt, and M. Alexander. 1989. Kinetics of biodegradation of mixtures of substrates in soil. *Soil Biol. Biochem.* **21**:703–708.
- Simkins, S., and M. Alexander. 1984. Models for mineralization kinetics with the variables of substrate concentration and population density. *Appl. Environ. Microbiol.* **47**:1299–1306.
- Spain, J. C., O. Wyss, and D. T. Gibson. 1979. Enzymatic oxidation of *p*-nitrophenol. *Biochem. Biophys. Res. Commun.* **88**:631–641.
- Thiele, J., R. Muller, and F. Lingens. 1988. Enzymatic dehalogenation of chlorinated nitroaromatic compounds. *Appl. Environ. Microbiol.* **54**:1199–1202.
- U.S. Environmental Protection Agency. 1976. Water quality criteria. U.S. Environmental Protection Agency, Washington, D.C.
- Zeyer, J., and P. C. Kearney. 1984. Degradation of *o*-nitrophenol and *m*-nitrophenol by a *Pseudomonas putida*. *J. Agric. Food Chem.* **32**:238–242.
- Zeyer, J., H. P. Kocher, and K. N. Timmis. 1986. Influence of *para*-substituents on the oxidative metabolism of *o*-nitrophenols by *Pseudomonas putida* B2. *Appl. Environ. Microbiol.* **52**:334–339.