ENHANCED MICROBIAL RECLAMATION OF WATER POLLUTED WITH TOXIC ORGANIC CHEMICALS

by

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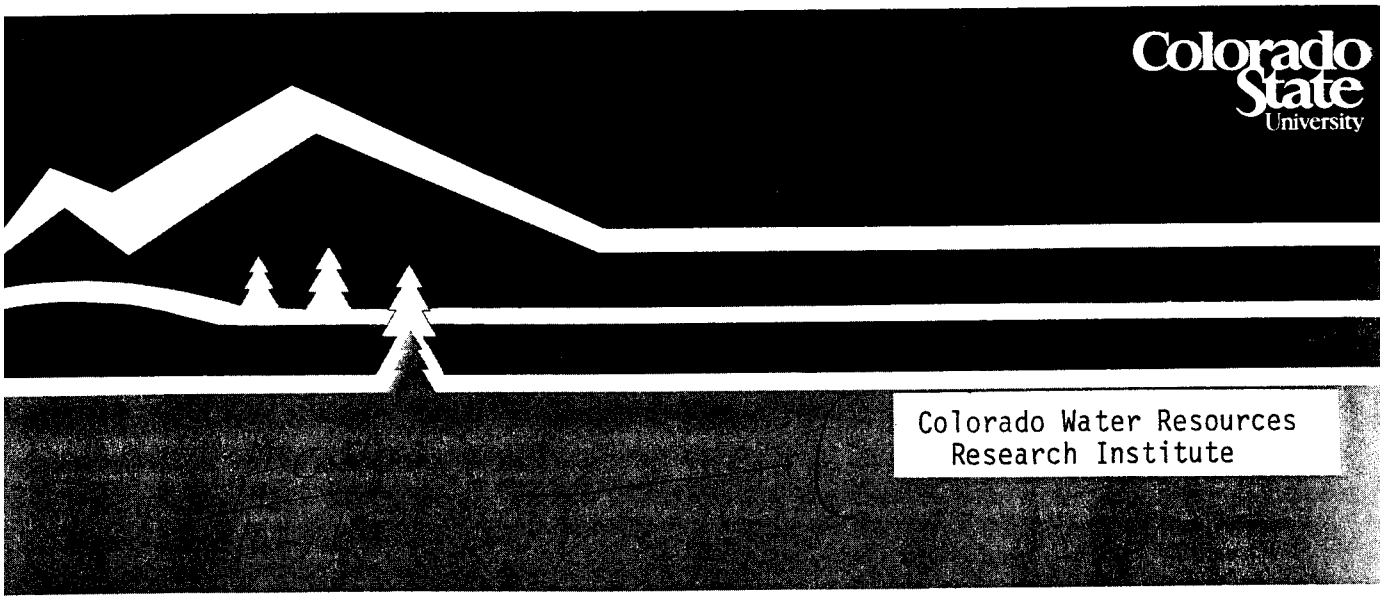

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Enhanced Microbial Reclamation of Water Polluted with Toxic Organic Chemicals

by

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NONTECHNICAL SUMMARY

The breakdown of hazardous chemical wastes, such as 2,4-dinitrophenol, was greatly enhanced in treatment systems employing specially selected strains of bacteria. Optimal conditions for the destruction of toxic chemicals by these bacteria were determined in Sequencing Batch Reactors, a type of biological treatment system. Addition of ammonia and glucose to the reactors greatly enhanced the destruction of the chemicals tested. This enhancement was attributed to the increased rate of growth of the bacteria, which normally grow very slowly on the toxic chemicals tested. The technology developed as a result of this study has great promise in the future cleanup of contaminated sites in Colorado and elsewhere.

ABSTRACT

The present study was undertaken to optimize conditions for the biodegradation of 2,4-dinitrophenol (DNP) and other toxic organic chemicals in sequencing batch reactors (SBRs). Two bacterial strains were isolated that can efficiently degrade DNP. These bacterial strains were inoculated into SBRs with different concentrations of supplemental substrates and the reactors were operated until steady-state conditions were reached. This approach allowed for acclimation of the SBR cultures to the experimental conditions and demonstrated that the effects were reproducible and stable. It was determined that glucose at concentrations of 100 μg/ml and higher greatly stimulated the destruction of DNP in the SBRs. Other supplemental substrates had little or no effect except ammonia which slightly stimulated DNP breakdown. The technology developed as a result of this study has great promise in the future cleanup of contaminated sites in Colorado and elsewhere.

INTRODUCTION AND BACKGROUND

This research was undertaken to develop an inexpensive and easy to operate microbiological system to reclaim waters contaminated with toxic organic chemicals. Groundwater pollution is a major environmental and public health problem in Colorado and solving this problem is of great importance to the quality of life and the future economic growth of the region. Inexpensive methods for removing toxic chemicals from water are needed to address the large present problem and to prevent

future buildup of these compounds. Thus, potential direct users of this technology include industries that create toxic organic wastes and agencies and companies involved in the cleanup of already contaminated sites such as the Broderick Wood Products site in Adams County, Colorado, the Snytex Chemical Inc. site near Lyons, and the Rocky Mountain Arsenal.. The technology developed as a result of this funding can also be applied to the removal of contaminating organic compounds from municipal drinking water sources. During the performance period of this research significant new information on the enhancement of microbial removal of toxic organic compounds was obtained. Research was aimed at optimizing operating parameters such as microbial species composition, type and concentration of stimulating carbon compounds, effect of cell recycle, and retention time in the reactor.

Nitrophenols are widely 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 (Hallas and Alexander, 1983). The U.S. Environmental Protection Agency lists several mono-nitrophenols and dinitrophenols on it's Priority Pollutants list and recommends restricting their concentrations in natural waters at or below 10 ng/ml (USEPA, Water Quality Criteria, 1976). Because the addition of one or more nitro groups to the phenol molecule reduces electron density of the aromatic ring, electrophilic attack of the compounds by microorganisms is impeded (Bruhn et al, 1987; Thiele et al., 1988) and the compounds may accumulate and persist in the environment.

While past research has revealed a variety of organisms that can utilize mono-nitrophenols as carbon and energy sources (Raymond and Alexander, 1979; Spain et al., 1979; Zeyer and Kearney, 1984; Zeyer et al., 1986), few researchers have reported on microorganisms that can mineralize dinitrophenols (Bruhn et al., 1987; Jensen and Lautrup-Larsen, 1967). In recent work done with dinitrophenols, 2,6-dinitrophenol (2,6-DNP) was found to be degraded only under nitrogen-limiting conditions in the presence of more readily usable carbon sources (Bruhn et al., 1987).

Several recent studies have demonstrated the effectiveness of supplemental substrates for enhancing the rate of biodegradation of toxic chemicals by pure cultures of bacteria (Schmidt et al., 1987; LaPat-Polasko et al., 1984). Schmidt et al (1987) showed that glucose could increase the growth rate of *p*-nitrophenol (PNP) mineralizing bacteria and thereby increase the rate of PNP mineralization. However the usefulness of this type of enhancement may be limited in 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 (Scow et al., 1989).

Biodegradation of other phenolic compounds has been shown to be inhibited by glucose in aquatic treatment systems (Papanastasiou and Maier, 1982; Rozich and Calvin, 1986). In batch cultures of mixed population, 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 (Rozich and Calvin, 1986).

The present study was undertaken to optimize conditions for the biodegradation of 2,4-dinitrophenol (DNP) in sequencing batch reactors (SBRs). Sequencing batch reactor technology has been previously described by Irvine and Busch (1979) and is primarily 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, determine what supplemental substrates enhanced degradation in pure cultures and then to inoculate SBRs with these organisms. The SBRs were run with different concentrations of supplementary substrate until steady-state conditions were reached 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 fresh water stream and forest soil respectively were used for all experimental work. These organisms were isolated using techniques specifically designed to maintain their abilities to degrade low concentrations of xenobiotic chemicals. Both organisms were never grown on high concentrations of chemicals and were never cultured on rich laboratory media such as nutrient agar. Identification of organisms was done using standard techniques as described in Holt and Krieg (1984). In addition, several other techniques were also used to identify the bacteria. The number and position of

bacterial flagella were determined using the technique of Heimbrook et al. (1986. Abst. Am. Soc. Microbiol. p. 240) and the capacity of the organisms to metabolize other organic compounds as sole sources of carbon and energy was tested using the auxanographic technique of Parke and Ornston (1984). 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 using the technique of Schaal [19]. Final identification of the *Janthinobacterium* sp. was done by analysis of cell wall fatty acids by the techniques of Fieldhouse and Miller (1986). Analysis of all the above materials for the actinomycete bacterium has so far been inconclusive for genus/species level identification.

Medium. The actinomycete did not require any exogenously supplied growth factors and was grown in a liquid medium containing different concentrations of reagent grade 2,4-dintrophenol (DNP) (Fluka Chemical Company, Ronkonkoma, New York) as a carbon and energy source in addition to 0.48 gm Na_2HPO_4; 0.5 gm KH_2PO_4; 0.04 gm NH_4Cl; 0.01 gm $MgSO_4 \cdot 7H_2O$ and 0.01 gm $CaCl_2$ per liter of solution. The *Janthinobacterium* sp. was grown in the same liquid medium except that yeast extract, at a concentration of 5 μg per ml, was added to supply the growth factors that this auxotrophic organism needed for growth.

Pure culture experiments were conducted in 250 ml glass-stoppered Erlenmeyer flasks containing one hundred ml of liquid medium and incubated at 24 ± 2° 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 [UL-^{14}C] 2,4-dinitrophenol had a specific activity of 0.1 mCi/mmol and was purchased from Sigma Chemical Company, St. Louis, Mo.

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

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, Illinois). Carbon source feed, DNP and glucose, and the nitrogen source feed was provided by micro-dispensing meter pumps with synchronous drives (Fluid Metering, Inc., Oyster Bay, New York). Carbon source feed

and inorganic salt solution feed were mixed in a tee 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 Chrontrol timers (Linburg Enterprises, San Diego, California).

Analytical Methods. Nitrite was determined as described in Standard Methods for the Examination of Water and Wastewater, 16th ed. (1985). Sample sizes and all added reagents were reduced by a factor of ten. Analyses were carried out on a Varian DMS 100 spectrophotometer (Varian Associates, Palo Alto, Calif.) at 543 nm. The original nitrite in solution was subtracted from the nitrite measured during the experiment to obtain measurements of nitrite accumulation during DNP mineralization.

Total organic carbon was determined by the combustion-infrared method as described in Standard Methods (1985) using a Beckman 915-B TOC autoanalyzer (Rosemount Inc., Minneapolis, Minn.). Acidified glucose solutions were used as reference standards.

Measurement of Mineralization. Mineralization of DNP in pure culture was measured by the technique of Schmidt et. al. (1987). In this procedure 2.5 ml samples were removed from the experimental flasks at regular intervals, vacuum filtered through 0.2 μm pore-size polycarbonate filters (Nucleopore, Pleasanton, Calif.) One ml subsamples were added to 4 ml Omni scintillation vials (Wheaton Co., Millville, N.J.) along with 2.5 ml of ScintVerse II scintillation cocktail (Fisher Scientific, Pittsburgh, Pa.) and radioactivity was determined by a model LS-3133T liquid scintillation counter (Beckman Instruments, Inc., Irvine, Calif.). Disappearance of DNP was also measured using a Varian DMS 100 spectrophotometer (Varian Associates, Palo Alto, Calif.) at 260 nm.

Mineralization in SBR experiments was measured in a manner similar to that used with pure cultures. Six ml samples were withdrawn from the center of each reactor at regular intervals by syringe and 6-inch, 20 ga. needle. The samples were vacuum filtered through 0.2 μm pore-size polycarbonate filters and acidified with 1 drop of concentrated H_2SO_4. DNP content of samples was determined at 260 nm on an ultraviolet spectrophotometer. Disappearance of DNP was confirmed by gas chromatograph-mass spectrometry on a model 5988A mass spectrometer (Hewlett Packard, Palo Alto, California).

Measurement of $^{14}CO_2$ was determined in a manner similar to Zeyer and Kearney (1984). Evolved $^{14}CO_2$ was captured in one ml of 0.5 N NaOH at timed intervals in a side-arm biometer flask closed to the atmosphere. Any remaining $^{14}CO_2$ in solution at the end of the experiment was evolved by acidification using concentrated H_2SO_4. The NaOH samples were added to scintillation vials along with 2.5 ml of scintillation cocktail and radioactivity was determined by a liquid scintillation counter.

SBR Experiments. Bench scale SBR's used in this study were operated with four liters of liquid volume. Experiments were conducted at a DNP concentration 10 µg/ml. Three reactors were used per experimental setup, providing a control (no supplemental glucose) and two concentrations of glucose, 10 µg/ml and 100 µg/ml.

Reactors were constructed from 8-inch I.D. clear acrylic plastic cylinder glued to a 1/2-inch acrylic bottom plate. Reactor lids were constructed from 1/2-inch acrylic plate with a machined groove and gasket to provide for an air-tight system. Bulkhead penetrations in the lid were made for decant and aeration tubes in addition to a Tenex®-charged (Alltech Assoc., Inc., Deerfield, Illinois) gas trap tube to capture any volatilized DNP. Penetrations in the reactor cylinder wall provided for a feed tube and sample port for syringe withdrawals of reactor contents. Each reactor was 16 inches in height with an operating volume of four liters. Aeration of the reactor contents was via a dual sparger air tube connected to a compressed air source. Filtered air was supplied at 6 psi from a spring activated pressure valve and metered at 100 ml/minute by a combination flow meter/needle valve (Cole Parmer Instrument Co., Oakbrook, Illinois). Positive on/off control of air was accomplished by the use of air solenoid valves. Mixing of the reactor contents was provided by a one inch stir bar and magnetic stirrer placed under the reactor. A 1/2-inch air gap between the stirrer 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 ° C.

Gas traps were installed on the reactors to quantify any amounts of DNP volatilizing during the reaction cycles. The traps consisted of 10 mm I.D. x 20 cm pyrex tubes with a 25-50 µm pore size fritted glass insert. One gram of 60/80 sieve Tenax® was placed on top of the frit surface and held in place by glass wool plugs. The traps were then secured in the reactors 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 reactors occurred.

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

SBRs were operated on a 48 hour cycle. The length of each component of the cycle was as follows: fill, 48 minutes; react, 44 hours, 47 minutes; settle, 1 hour, 46 minutes; decant, 39 minutes.

Data Analysis. Results from the mineralization experiments were analyzed by nonlinear regression techniques as described by Simkins and Alexander [26]. An MS-DOS based microcomputer was used to run LMFIT, an author written computer program that minimizes the least square of differences between data and various model curves by the method of Levenberg and Marquardt as described by Dennis and Schnabel (1983). The computer program requires the user to input a model and analytical partial derivatives of the dependent variable with respect to each parameter. There is also provision for inputting only the model with partial derivatives being calculated numerically within the program via central difference gradients. Parameter estimates and their standard deviations are part of the program output. Results from both analytical and numerical partial derivative forms of the program show no significant differences in output. Numerical partial derivatives are useful for complex models or models that contain many parameters.

Model discrimination was based on 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 (Robinson, 1986). Models were also judged for accuracy of fit based on their standard error of the estimated parameters (Robinson, 1986). If the standard error of the estimated parameter was greater than 50%, the model was deemed an inferior fit to that data set (Robinson, 1986). Significance of differences between data sets for suspended solids measurements and times of degradation were also analyzed with the F test at the 95% confidence level.

Theoretical Considerations. The logistic model has previously been used to describe growth of organisms degrading low concentrations of single xenobiotic

compounds (Simkins and Alexander, 1984). A substrate-based form of the logistic model, derived from conditional restraints of the more general Monod model was presented by Simkins and Alexander (1984). For the purposes of this paper, however, a more mechanistic development of the population-based logistic model into a substrate-based model was used. The advantage of this 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 (1984). An additional parameter, K_S, can be calculated from the use of the logistic equation presented below in consort with Simkins and Alexanders' (1984) logistic equation. Estimates of K4 (which equals μ_{max}/K_S), from Simkins and Alexander (1984), may be divided by estimates of μ_{max} from the logistic equation presented herein to obtain K_S.

Development of the substrate-based logistic model begins with the population-based expression (Bull et al., 1975; Odum, 1971):

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

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 :

$$B = B_{max}/(1+[(B_{max}-B_O)/B_O]e^{-\mu_{max}\, t}) \qquad 2)$$

To show changes in population density due to changes in substrate concentration a mass balance is used (Robinson and Tiedje, 1983):

$$S_O + qB_O = S + qB \qquad 3)$$

where S_O is the initial substrate concentration, B_O is the initial population density and q is the cell quota or inverse yield. Following the work of Simkins and Alexander (1984), 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_O is replaced by X_O. These substitutions result in the following equations:

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

and

$$S_o + X_o = S + X \quad 5)$$

It follows that maximum acheivable population, X_{max}, will always be based on the original population present, X_o, plus the original substrate present, S_o, that can be used during growth to create new cells, thus:

$$X_{max} = X_o + S_o \quad 6)$$

When equation 5) is solved for X, substituted along with equation 6) into equation 4), and simplified, the substrate-based logistic model results:

$$S = (S_o + X_o)((S_o\, e^{-\mu_{max}\, t})/(X_o + S_o\, e^{-\mu_{max}\, t})) \quad 7)$$

Additional models used for pure culture, single substrate experiments were those of Simkins and Alexander [26].

Models used for analyses of data from enhanced degradation experiments with the addition of a supplemental substrate were those described previously by Schmidt et al. [23]. The curves of DNP mineralization for cells growing on the supplemental substrate, glucose, were best fit by Model IV (Schmidt):

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

where all terms are as previously described with the addition of $k_1 = V_{max}X_o/K_m$, V_{max} being the maximum specific reaction rate.

RESULTS

Characterization of Isolates. The actinomycete, strain S, is a long, nonmotile, gram positive rod which forms catalase but is oxidase negative (Table 1). The presence of tuberculostearic acid and other factors indicate this organism may be a coryneform bacterium but genus-level identification was not possible. The *Janthinobacterium* sp., strain T, is a short, motile, gram negative rod with more than one polar flagella, is oxidase positive and does not produce catalase. Neither

Table 1. Characteristics of actinomycete and *Janthinobacterium* sp.

Characteristic	Actinomycete	*Janthinobacterium* sp
Morphology	rod	rod
Gram stain	+	-
Flagella	none	polar >1
Oxidase	-	+
Catalase	+	-
Fluorescent pigment	-	-
Arginine dihydrolase	-	ND[1]
Growth at 41° C	-	-
PHB production	+	ND
Denitrification	-	ND
Growth on:		
Acetate	+	+
Anthranalate	+	+
Arabanose	+	+
Arginine	+	+
Aspartate	+	+
Benzoate	+	+
Betaine	+	+
Caffeate	+	+
Catechol	+	+
Trans-Cinnamate	+	-
Citrate	+	+
P-Coumerate	+	+
Ferulate	+	+
Formate	-	-
Fumarate	+	+
Gallate	+	-
Gluconate	+	+
Glucose	+	+
Glutamate	-	-
Glycerol	-	ND
Glycine	-	+
M-Hydroxybenzoate	+	+
P-Hydroxybenzoate	+	+
DL-Kynurenine	-	+
Lactose	-	-
D-Malate	+	+
D-Mannose	-	+
1-Napthol	+	-
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	+	+
Xylose	-	ND

[1]ND - Not Determined

organism grows at 41° C. The results of tests for carbon source utilization are shown in Table 1. The *Janthinobacterium* sp. was originally thought to be a *Pseudomonas* sp. but subsequent analysis of cell wall fatty acid composition confirmed the organism as a *Janthinobacterium* sp.

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 non-growing cells of the actinomycete and *Janthinobacterium* sp. are shown in Figures 1 and 2 respectively. These curves of mineralization were best fit by the Michaelis-Menten model to give the parameters shown in Table 2. In order 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 at one DNP concentration, 0.1 μg/ml, were conducted for the *Janthinobacterium* sp. (Table 2).

Experiments were also conducted to ascertain growth parameters, such as μ_{max}, for both organisms. Two replicate experiments at 0.1 μg DNP per ml were conducted for the actinomycete and two different experiments at 1.0 and 0.5 μg DNP per ml were done for the *Janthinobacterium* sp.. The models of best fit for the actinomycete data were the logistic equation while the logarithmic equation (Simkins and Alexander, 1984) described the data from the experiments with the *Janthinobacterium* sp. (data not shown). Parameters estimated 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 in Figure 3. Cells for the experiment were previously grown on 2.5 μg of DNP per ml and 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 (Figure 5). The resulting NO_2^- : DNP molar ratios were 1.85 and 1.91 respectively. Production of $^{14}CO_2$ from ^{14}C-DNP mineralization was measured for the actinomycete. A replicated experiment showed 53 ± 1 % of total labeled DNP recovered as CO_2.

DNP Mineralization in SBRs. A preliminary experiment was conducted in two SBRs inoculated with both pure culture strains to ascertain the effects of added

FIGURE 2. Mineralization of 0.1 μg of DNP per ml by non-growing cells of a *Janthinobacterium* sp.. The mineralization curve was fit by the Michaelis-Menten equation.

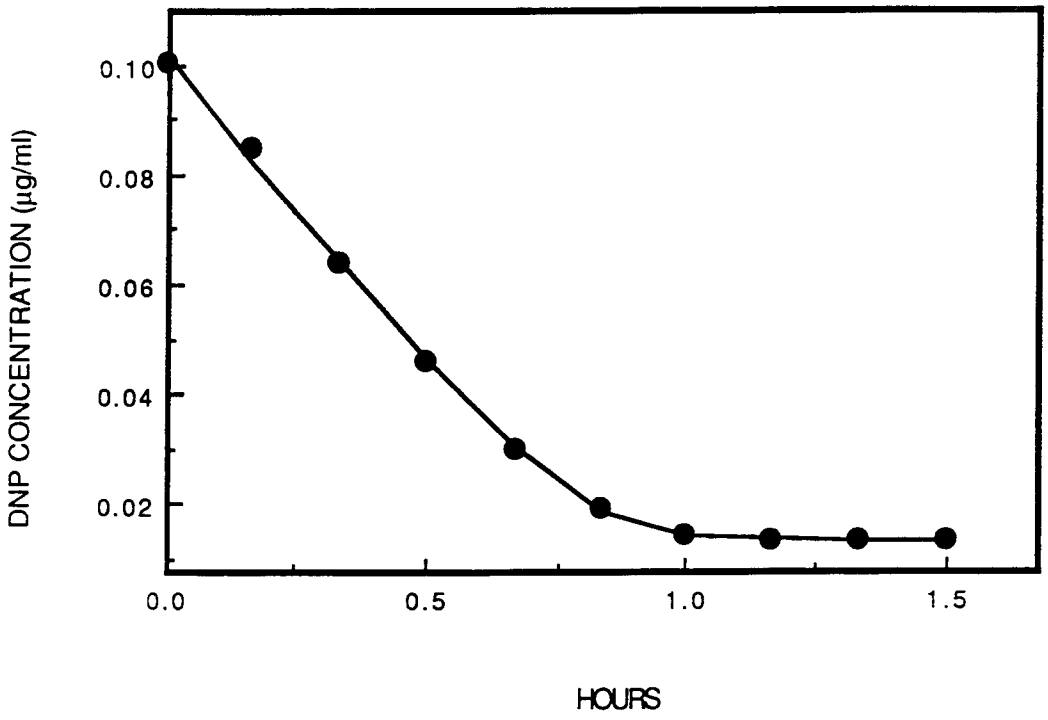


FIGURE 1. Mineralization of 0.5 μg of DNP per ml by non-growing cells of an actinomycete. The mineralization curve was fit by the Michaelis-Menten equation.

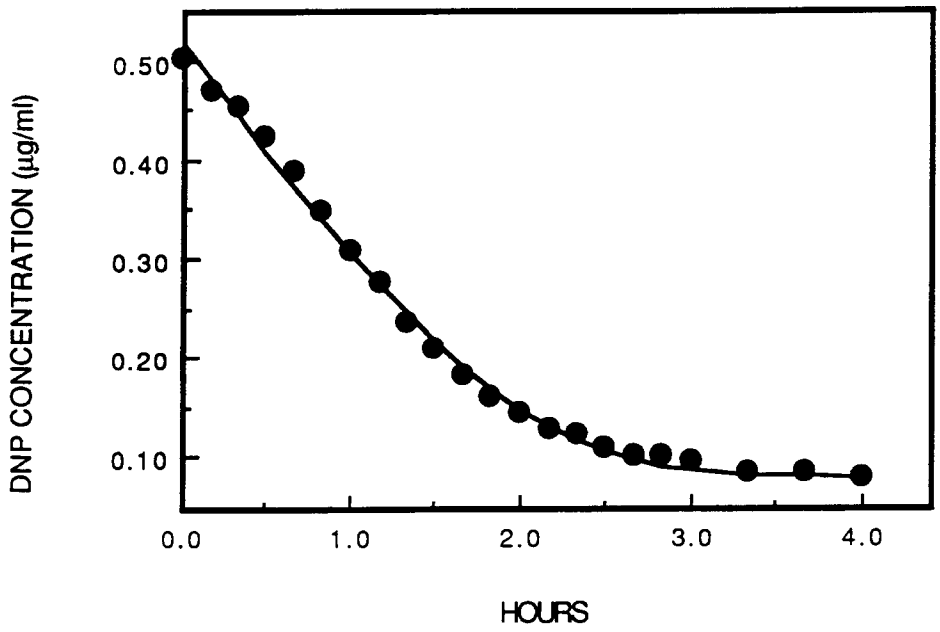


FIGURE 3. Production of nitrite and the disappearance of DNP in cultures of the actinomycete at DNP concentrations of 1 μg/ml (A) and 5 μg/ml (B).

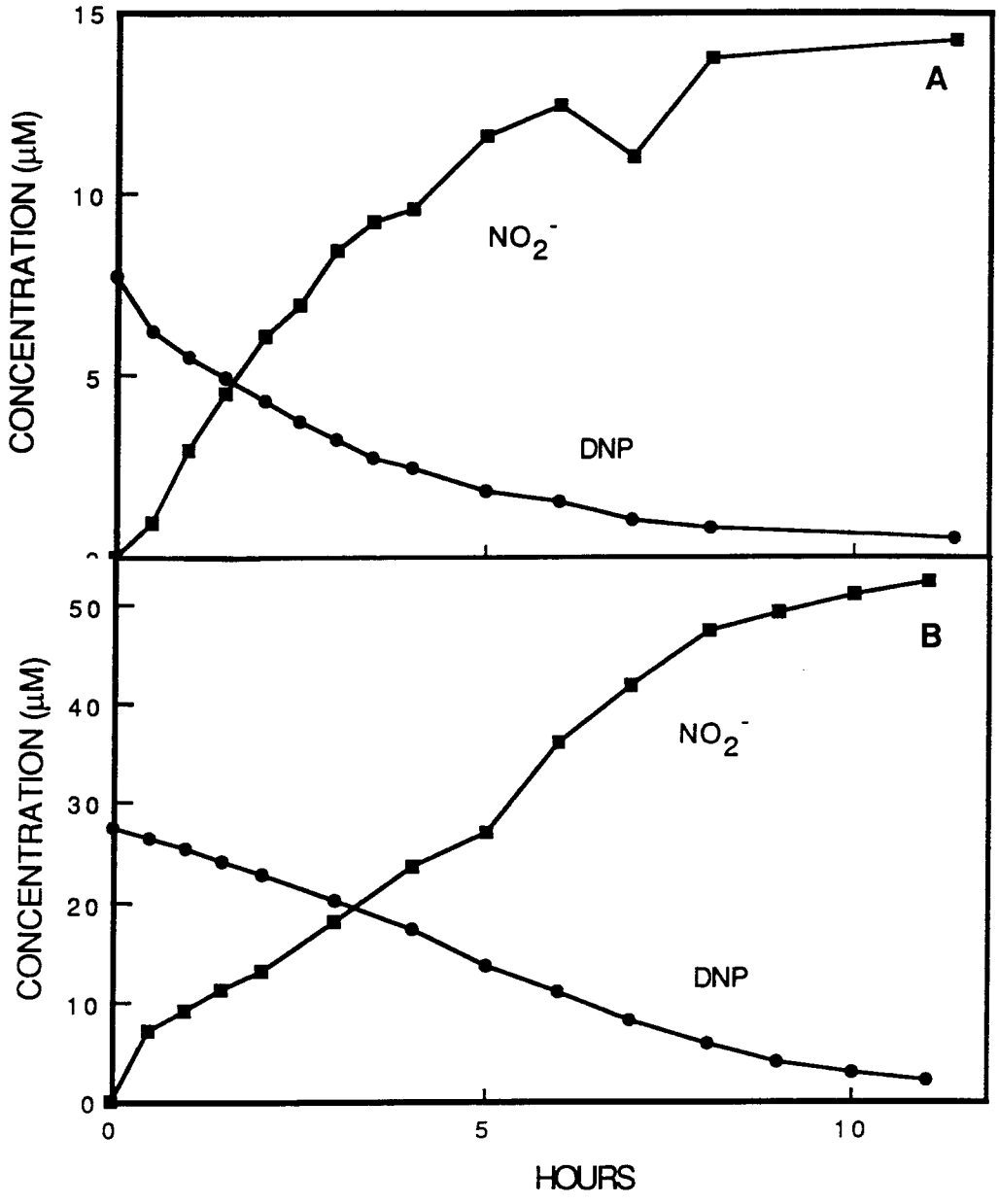


Table 2. Comparison of separate nonlinear regression estimates of parameters of the Michaelis-Menten model in replicate experiments and between experiments in which the substrate concentration was varied.

Organism	S_o (μg/ml)	K_m (μg/ml)	Rate constant (μg/ml·hr^{-1})
Actinomycete	0.52±0.006	0.12±0.030	0.33±0.027
	0.52±0.005	0.13±0.027	0.35±0.025
	1.06±0.012	0.16±0.049	0.50±0.037
	1.06±0.010	0.13±0.036	0.47±0.026
Janthinobacterium	0.10±0.001	0.01±0.002	0.14±0.005
	0.10±0.001	0.01±0.002	0.13±0.006

Table 3. Comparison of separate nonlinear regression estimates of kinetic parameters in different experiments in which the substrate concentration and population size were varied.

Organism	Model	S_o (μg/ml)	X_o (μg/ml)	μ_{max} (hr^{-1})	K_s[a] (μg/ml)
Actinomycete	Logistic	0.10±0.001	0.02±0.003	0.23±0.017	0.12±0.004
Actinomycete	Logistic	0.10±0.001	0.01±0.002	0.23±0.010	0.11±0.001
Janthin	Log	2.00±0.017	4.14±1.267	0.07±0.019	NA[b]
Janthino	Log	0.50±0.002	0.20±0.042	0.06±0.010	NA

[a] Derived from Simkins and Alexander (1984) and present logistic equation, $K_s = \mu_{max}/K_4$ (See Materials and Methods)

[b] Not Applicable

nitrogen on DNP mineralization. Concentrations of DNP and glucose were 4 μg/ml 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 (Figure 4). The Michaelis-Menten model represented both DNP mineralization data sets best. Parameters from this model fit were as follows: for the SBR with externally added nitrogen source, K_m = 1.85 ± 0.290 μg/ml, rate constant = 1.51 ± 0.117 $\mu g/ml \cdot hr^{-1}$; and for the SBR without externally added nitrogen source, K_m = 1.59 ± 0.258 μg/ml, rate constant = 1.01 ± 0.072.$\mu g/ml \cdot hr^{-1}$. In a following experiment (data not shown), using the same DNP and glucose concentrations, the maximal nitrite evolution in the SBR with external nitrogen added was approximately in stoichiometric amounts to initial DNP (37.8 μM NO_2^- and 20.1μM DNP), the NO_2^- : DNP molar ratio being approximately 1.88. Maximal nitrite evolution in the SBR without external nitrogen added was considerably less (7.1 μM versus 37.8 μM) due to use of the nitrite from the DNP as a nitrogen source.

In subsequent experiments, three SBRs were run in parallel, each containing equal concentrations of DNP (10 μg/ml) but differing concentrations of glucose as a supplemental substrate and complete mineral salts solution including NH_4Cl. DNP removal experiments were conducted during single reaction cycles every two to three weeks during the three months of operation of the reactors. Results of the first experiments were more variable than later results presumably due to non-steady state conditions prevailing when the reactors were first set up. Figure 5 shows the results from two representative SBR experiments. The first experiment depicted (Figure 5a) was conducted after two weeks of operation presumably when the reactors had not reached steady-state conditions. The second experiment shown (Figure 5b) 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 effect 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 changed to a glucose concentration of 500 μg/ml while the other two reactors continued to run at glucose concentrations of 0 and 100 μg/ml (Figure 6). The reactors were allowed to run for eight cycles at these concentrations before the experiment shown in Figure 6 was done. This higher concentration of glucose caused an even greater stimulation of DNP mineralization than did 100 μg of glucose per ml (Figure 6).

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

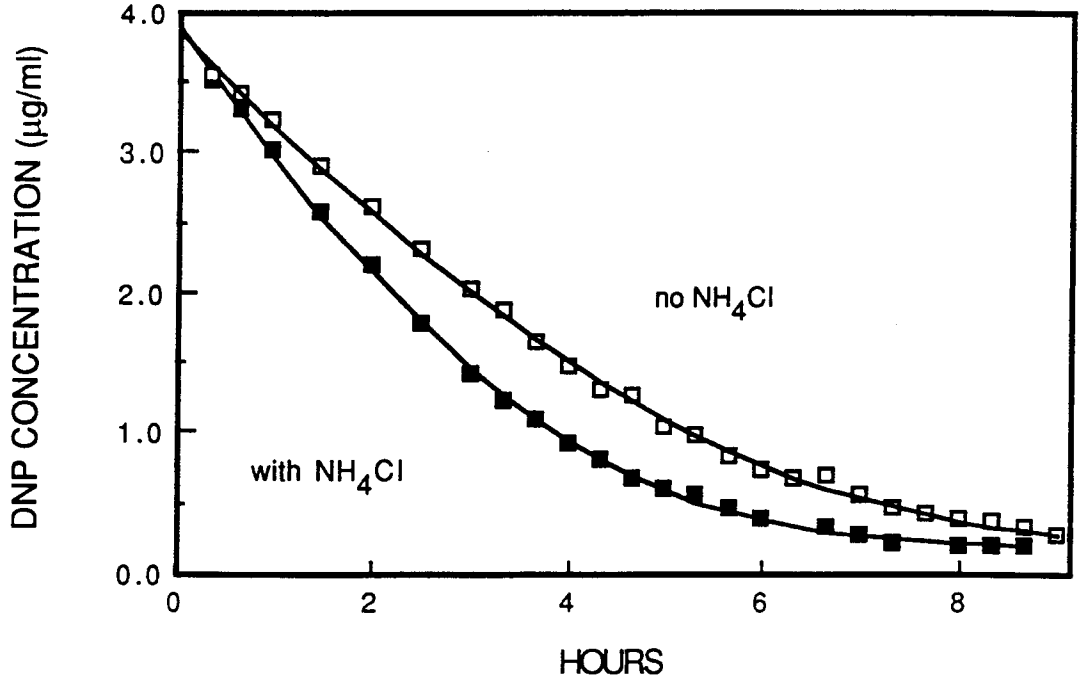


FIGURE 5. Mineralization of 10 µg of DNP per ml in the presence of different concentrations of glucose in SBRs in experiment no. 4 (A) and experiment no. 7 (B). The mineralization curves were fit by nonlinear regression.

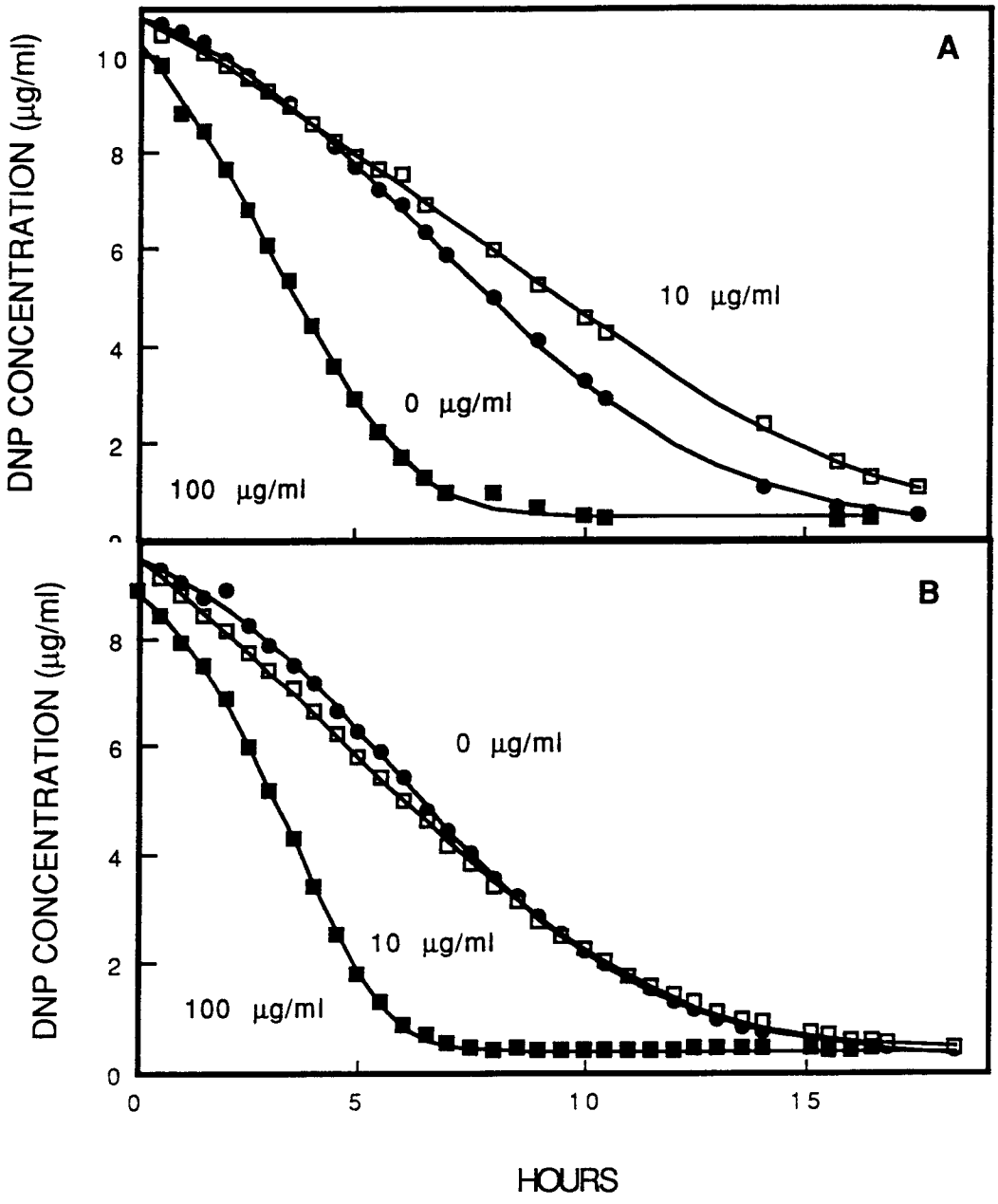
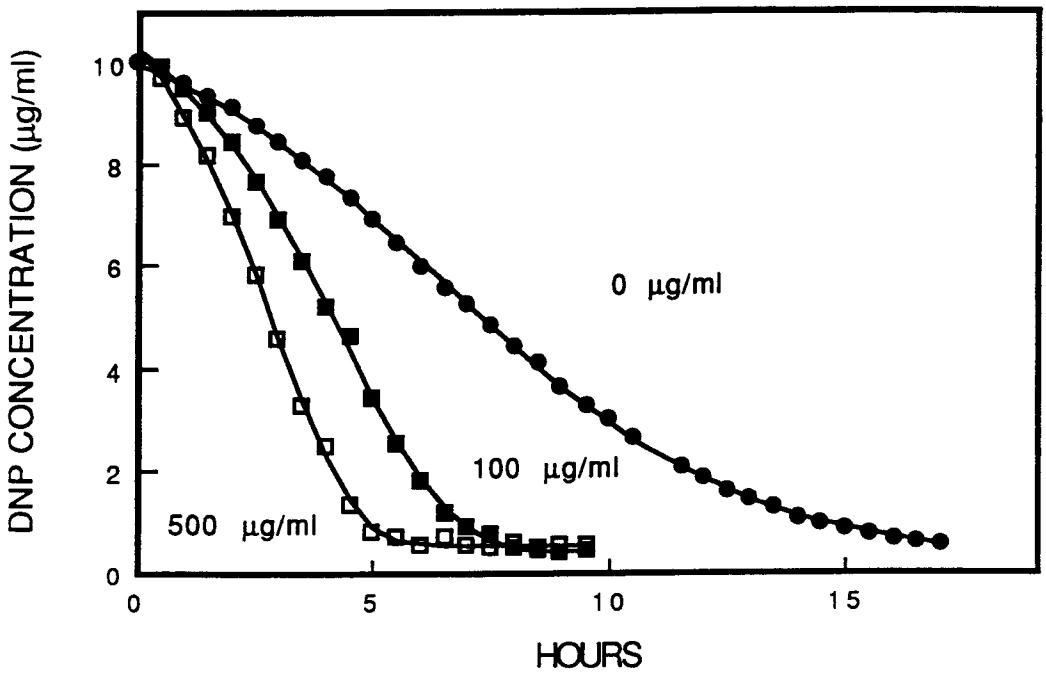


FIGURE 6. Mineralization of 10 µg of DNP per ml in the presence of different concentrations of glucose in SBRs in experiment no. 8. The mineralization curves were fit by nonlinear regression.



The curves of DNP mineralization for all controls (no glucose added) were 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. (1985). Table 4 presents the estimates of the model parameters from nonlinear regression analyses for all SBR results shown in Figures 5 and 6. 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. Suspended solids measurements at the end of each cycle and average times for complete DNP degradation in each reactor are shown in Table 5. The difference between all suspended solids measurements was shown to be significant (P=0.05) in a standard F test, however there was no significant difference between the degradation times for 0 and 10 μg glucose per ml (P=0.05).

Two replicated experiments were conducted for measurement of $^{14}CO_2$ produced from mineralization of ^{14}C-DNP in sludge samples from the SBRs. Results showed 37 ± 4 % of total labeled DNP was recovered as CO_2.

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 each cycle of the SBRs. The K_m and μ_{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. would be able to mineralize much lower concentrations of DNP. After running the SBRs for many cycles, however, only the actinomycete could be reisolated from the reactors. This may have been because the concentration of DNP used (10 μg/ml) was closer to the K_m of the actinomycete than that of the *Janthinobacterium* sp.. It may also be that the filamentous growth habitat 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 μ_{max} of the population mineralizing DNP in the SBRs containing only DNP ranged from 0.31 to 0.36 (Table 4), whereas the μ_{max} for the *Janthinobacterium* sp. was 0.07 (Table 3). It is of interest that although a diverse microbial community developed in the SBRs, no other DNP degraders seem to have invaded the reactors. Microscopic observations indicated the

Table 4. Comparison of separate experiments run during different reaction cycles of continuously operating SBRs. All experiments were conducted at a DNP concentration of10 μg/ml.

Experiment No.	Glucose (μg/ml)	Model	Rate constant	X_o (μg/ml)	μ_{max} (hr^{-1})
4	0	Logistic	0.03±0.001[a]	1.1±0.095	0.33±0.009
7	0	Logistic	0.03±0.001[a]	1.1±0.076	0.36±0.007
8	0	Logistic	0.03±0.001[a]	1.4±0.054	0.31±0.004
4	10	IV	0.05±0.001[b]	NA[c]	0.12±0.005
7	10	IV	0.07±0.002[b]	NA	0.14±0.004
6	10	IV	0.08±0.002[b]	NA	0.14±0.004
4	100	IV	0.11±0.007[b]	NA	0.33±0.020
7	100	IV	0.08±0.002[b]	NA	0.49±0.007
8	100	IV	0.07±0.004[b]	NA	0.44±0.016
8	500	IV	0.11±0.007[b]	NA	0.59±0.026

[a]Rate constant, K_4, with units of ml/μg · hr^{-1}

[b]Rate constant, K_1, with units of hr^{-1}

[c]Not Applicable

Table 5. Average suspended solids concentrations and times to 90 % DNP degradation for all SBR experiments at four supplemental substrate (glucose) concentrations. All experiments were conducted at a DNP concentration of 10 µg/ml.

Glucose Concentration (µg/ml)	Suspended Solids (µg/ml)	Time to 90 % Degradation (hours)
0	32±13	20±3*
10	78±39	19±4*
100	397±132	8±1
500	958±192	6

* Data not shown to be significantly different at P=0.05. All others were significantly different at P=0.05.

presence of diverse 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 of Simkins and Alexander (1984). Residual sums of squares and the parameters X_o and S_o 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 non-growth 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 (Schmidt et al., 1987).

Nitrite evolution in both pure culture and the activated sludge cultures was almost stoichiometric to 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-DNP (Bruhn et al, 1987). Other evidence for DNP mineralization include $^{14}CO_2$ 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 2400 fold dilution of the original inoculum and thus growth on DNP was necessary to maintain the active population.

Unlike previous studies of dinitrophenol mineralization (Bruhn et al., 1987), 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 it's sole source of carbon and energy as well as nitrogen. Rather than inhibiting DNP mineralization in the SBRs, ammonia actually stimulated DNP mineralization (Figure 4), increasing the rate constant for DNP mineralization by approximately 50 percent.

Further stimulation of DNP degradation was seen in the presence of glucose as a supplementary carbon and energy source (Figures 5 and 6). 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 indicated by the consistancy of kinetic parameter estimates between experiment Nos. 7 and 8 for 0 and 100 μg of added glucose per ml and experiment Nos. 6 and 7 for 10 μg of added glucose per ml. This type of enhanced degradation during steady-state reactor operation is similar to the results LaPat-Polasko et al. (1984) obtained in biofilm reactors.

A possible mechanism underlying the enhancement of DNP mineralization was indicated by kinetic analyses of the DNP-depletion curves and the resultant maximal growth rates. Once steady-state operation of the SBRs was achieved (experiments 7 and 8), the estimated value for μ_{max} averaged 0.47 hr^{-1} in the presence of 100 µg of glucose per ml and 0.34 hr^{-1} in the absence of added glucose. In addition, the μ_{max} for the single experiment run at a glucose concentration of 500 µg/ml indicated the DNP mineralizing population was growing with a μ_{max} of 0.59 hr^{-1}. This correlation between enhanced DNP mineralization and enhanced growth rate is consistent with the work of Schmidt et al. (1987) who showed that enhancement of PNP mineralization was caused by an increased growth rate of PNP-mineralizing bacteria in the presence of glucose. It is also evident from the data presented in 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. It is interesting to note that μ_{max} values from the SBR's with no glucose were almost 2.5 times higher than μ_{max} values from the SBR's with 10 µg/ml of added glucose. This may indicate that the DNP mineralizing organisms are not able to compete with the total population for glucose at concentrations of 10 µg/ml or lower. Concentrations of glucose less than initial DNP concentrations may actually inhibit growth on DNP and hence no particular enhancement effect would be observed. Thus the carry over 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.

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