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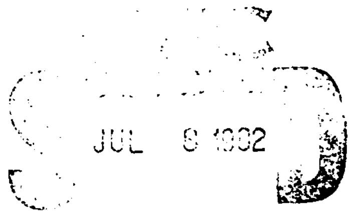
MICROBIAL INTERACTIONS WITH SEVERAL MUNITIONS COMPOUNDS: 1,3-DINITROBENZENE, 1,3,5-TRINITROBENZENE, AND 3,5-DINITROANILINE

WAYNE R. MITCHELL, Ph.D.
WILLIAM H. DENNIS, Sc.D.
ELIZABETH P. BURROWS, Ph.D.

U S ARMY MEDICAL BIOENGINEERING RESEARCH & DEVELOPMENT LABORATORY
Fort Detrick
Frederick, Maryland 21701

MAY 1982



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Wayne R. Mitchell, Ph.D.
William H. Dennis, Sc.D.
Elizabeth P. Burrows, Ph.D.

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The microbial degradation of 1,3-dinitrobenzene was complete or near complete in Tennessee River water but not in all other environmental water sources tested. Microorganisms from the Tennessee River could utilize 1,3-dinitrobenzene as a sole carbon source for growth and could mineralize the compound. The half-life of 1,3-dinitrobenzene at $25^\circ C$ and 10^6 colony-forming units per milliliter was approximately 1 day in Tennessee River water samples and slightly less than 10 days in the presence of enrichment cultures

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20. Abstract (continued)

developed from the samples. The differences in the measured half-lives likely reflects basic microbiological differences in two approaches used to obtain half-lives.

Decreases in the concentrations of 1,3,5-trinitrobenzene and 3,5-dinitroaniline mediated by microorganisms in Tennessee River water samples were incomplete and unsustained. Maximum reductions in the concentrations of the compounds required the presence of sediments and even then were slow. Neither compound would serve as a carbon source for microbial growth, but both compounds were metabolized in the presence of added nutrients. The metabolism of the compounds did not lead to their ultimate biodegradation. Nitro group reduction was seen to occur with both 1,3,5-trinitrobenzene and 3,5-dinitroaniline in the presence of added nutrients and laboratory cultures of Tennessee River microorganisms and also occurred with 1,3,5-trinitrobenzene in Tennessee River water samples.

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INTRODUCTION

More than 30 nitroaromatic compounds are formed as by-products during the manufacture of the explosive 2,4,6-trinitrotoluene (TNT).[1,2] Three of the by-products have been identified as 1,3-dinitrobenzene (1,3-DNB), 1,3,5-trinitrobenzene (1,3,5-TNB), and 3,5-dinitroaniline (3,5-DiNA).[1] All three compounds have been shown to be mutagens in Ames Tests, with 1,3,5-TNB and 3,5-DiNA reported as being highly mutagenic.[3] Toxic effects to humans following occupational exposure to 1,3-DNB are well known,[4-8] and both 1,3-DNB and 1,3,5-TNB are toxic to a diversity of animal and microbial species.[9]

The compounds have been detected in condensed wastewater discharges from a TNT production facility.[1] Since both 1,3-DNB and 1,3,5-TNB can be detected in the TNT product, they can also enter the environment in association with effluents from munitions loading and blending operations.[9,10] Moreover, photoconversion of discharged TNT or 2,4-dinitrotoluene to 1,3,5-TNB and 1,3-DNB, respectively, can contribute these pollutants to the environment.[9]

Microbiological studies with 1,3-DNB or 1,3,5-TNB have led to the conclusion that they are resistant to biodegradation.[9] Mixed cultures of phenol-adapted bacteria, with pseudomonads as the predominent flora, have exhibited only low levels of respiration when incubated in the presence of 1,3-DNB or 1,3,5-TNB.[11] Resistance of 1,3-DNB to biodegradation has also been reported following 64 days incubation of the compound with a mixed population of soil microorganisms.[12] Disappearances of both 1,3-DNB and 1,3,5-TNB have been noted following their treatment in a two-stage aerated reactor composed of *Azotobacter agilis* in the first stage and activated sludge in the second;[13] however, the degree to which the disappearance is due to microbial transformation or ultimate biodegradation of the compounds is unknown. Experiments conducted in cell-free extracts of *Veillonella alkalescens* have indicated that nitro group reduction occurs readily for 1,3,5-TNB, but less readily when 1,3-DNB is incubated with the extracts.[14] Arylamine formation has been reported for both 1,3-DNB and 1,3,5-TNB following incubation of the compounds with whole cells and extracts of *Nocardia* species.[15] Similar studies have not been reported for 3,5-DiNA, although nitro group reduction has been reported for the isomer 2,4-DiNA in *Veillonella* extracts.[14]

The objective of the study is to provide an integral part of the scientific data base necessary to establish water quality criteria in a context of hazard assessment. Toward that end, the study addresses the rate and degree to which microorganisms in environmental water samples, particularly those in the vicinity of the Volunteer Army Ammunition Plant, will degrade 1,3-DNB, 1,3,5-TNB, and 3,5-DiNA.

MATERIALS AND METHODS

WATER SAMPLES

Water samples were collected aseptically from the Harrison Bay region of the Tennessee River approximately 1 mile downstream from the Volunteer Army Ammunition Plant, Chattanooga, Tennessee. Similar results were obtained with

samples collected in December 1979 and November 1980. Biodegradation studies were initiated within 48 hours of sample collection. In several experiments, environmental water samples from the vicinity of Fort Detrick were used in 1,3-DNB biodegradation screening. The sites of the local water sources are listed in Table 15.

SCREENING FOR BIODEGRADATION OF 1,3-DNB, 1,3,5-TNB, AND 3,5-DINA

Bulk sediments (0.6-1.5 percent v/v) were removed from a portion of the water samples by settling for 1 hour followed by filtration of the supernatant fluid through two layers of a fine mesh polyester cloth. Biodegradation screening was carried out in shake flasks which contained 200 mL of the water samples (with or without sediments), test chemicals at the desired concentrations, and buffer (0.2 percent Na_2HPO_4, 0.01 percent $(NH_4)_2SO_4$, pH 7.0). Flasks were incubated with agitation for 6 weeks at 25°C. Aliquots (4 mL) were withdrawn throughout the course of the experiment and were assayed for the test chemicals by gas chromatography. Numbers of total bacteria were determined (in triplicate) following the plating of serial dilutions for 4 days at 25°C on Standard Methods Agar (Difco). Matched controls for the experimental flasks consisted of water samples which had been autoclaved for 15 minutes prior to incubation with the appropriate test chemical.

Parallel experiments were also conducted for 1,3,5-TNB and 3,5-DINA with water samples which had first been centrifuged at 12,000 XG for 15 minutes in order to concentrate microbial activity threefold. Conditions of incubation, sampling, and analysis were identical with those for the unconcentrated samples.

At the end of the incubation period, 100 mL aliquots were removed from the shake flasks and extracted twice with methylene chloride (25 mL). The organic extracts were evaporated to dryness for product studies.

MICROBIAL ENRICHMENT CULTURES

Aliquots (5 mL) from primary screening flasks showing test compound degradation were used to inoculate shake flasks containing 10 and 20 μg/mL of the appropriate chemical in 95 mL buffered salts medium (BSM) containing trace elements.[16] For 1,3,5-TNB and 3,5-DINA, additional flasks which contained 1-, 5-, 10-, and 50-fold increments of 50 μg/mL glucose and 10 μg/mL yeast extract were inoculated. Incubation was at 25°C and flasks were monitored for the disappearance of their test compounds by gas chromatography and ultraviolet spectrophotometry. Following degradation of the chemicals, the process was repeated stepwise using higher concentrations of the test compound. At the end of the series of transfers, the cultures were centrifuged at 16,000 XG and the microbial pellet was frozen at -70°C in BSM with 5 percent dimethyl sulfoxide (to be used for later study).

KINETIC STUDIES WITH ENRICHMENT MICROORGANISMS

Enrichment microorganisms were grown in 10 to 12 shake flasks containing 130 μg/mL 1,3-DNB in BSM buffer. Bacterial cells were washed three times by centrifugation and resuspension in buffer and were rested for 4 hours in a 25°C shaker bath. Before use, the bacteria were recovered by centrifugation, concentrated in one-tenth volume fresh buffer, and the test compound was

added. Incubation was for 2 hours at 25°C, and samples were removed for plating on Standard Methods agar at 0, 1, and 2 hours. One milliliter samples for chemical analysis were removed at various times during the 2-hour experiment and pipetted directly into frozen vials to stop the reaction. Gas chromatographic analysis employing a nitrogen-phosphorus detector was performed on the thawed samples following their extraction with an organic solvent.

CARBON DIOXIDE EVOLUTION

The production of CO_2 from 50 µg/mL unlabeled 1,3-DNB was measured by the method of Gledhill[17] using 10 mL 0.3 N $Ba(OH)_2$ traps. The same method was used to assess the mineralization of 10 µg/mL ^{14}C-1,3,5-TNB (0.54 µCi/mg) in the presence of 500 µg/ml glucose and 100 µg/mL yeast extract, except that liberated CO_2 was collected in 10 mL of 0.7 N KOH. Total CO_2 was determined by titrating a 5 mL sample of KOH with 0.5 N HCl to the phenolphthalein end point after precipitation of dissolved CO_2 with 30 mL 0.3 N $Ba(OH)_2$. Labeled CO_2 production was determined by liquid scintillation counting of 1 mL samples of the KOH.

The mineralization of 25 µg/mL ^{14}C-3,5-DiNA (0.35 µCi/mg) in the presence of 2,500 µg/mL glucose and 500 µg/mL yeast extract was measured by the method of Sturm,[18] adapted to facilitate the continuous gassing of 100 mL cultures and the capture of evolved CO_2 in two 20 mL 0.5 N KOH traps. Total CO_2 and radioactive CO_2 were determined as for ^{14}C-1,3,5-TNB.

All test compounds were chemically stable to the conditions employed in measuring CO_2 evolution. Gassing for mineralization studies was accomplished with 30 percent oxygen in nitrogen.

KINETICS OF MICROBIAL DEGRADATION

Second-order rate constants (k) for microbial degradation at 25°C were calculated as previously described.[16,19] Pseudo first-order rate constants (k[B]) were obtained from regression curves of multiple data points and are shown in the text. Bacterial levels (B) during intervals of pseudo first-order degradation were determined from plate counts (triplicate) on Standard Methods Agar. Second-order rate constants were obtained by dividing the pseudo first-order rate constant by the bacterial level. For purposes of discussion in the text, empirically determined second-order rate constants at assumed bacterial levels of 10^6 colony forming units (CFU) per mL are used to estimate chemical half-lives ($T_{1/2}$). The half-life is directly derivable from the second-order rate constant at given bacterial levels and can be expressed by the equation

$$T_{1/2} = \frac{\ln 2}{kB}, \text{ where B is assumed to be } 10^6 \text{ CFU/mL.}$$

BIOADSORPTION OF 1,3-DNB, 1,3,5-TNB, AND 3,5-DiNA

Biosorption studies were conducted with 3-day Standard Methods broth cultures of _Azotobacter beijerinckii_ (ATCC19366), _Bacillus cereus_ (ATCC11778), _Escherichia coli_ (ATCC 9637), and _Serratia marcescens_ (ATCC 13880) as previously described.[16] Briefly, the cultures were washed three times, rested, and concentrated by centrifugation at 16,000 XG for 10 min. Before use, they were

resuspended in 0.05 percent potassium phosphate buffer (pH 7) to an optical density (420 nm) of four units per mL. Equal volumes of the cultures were mixed and 4 mL volumes were added to weighed centrifuge tubes containing either 2 mL phosphate buffer alone or the appropriate test compound in the buffer. Following 1 hour incubation at 25°C, bacterial cells were removed by centrifugation and supernatant phases were carefully decanted. Tubes containing bacteria alone were air dried overnight at 90°C and weighed. Pellets resulting from tubes containing the test compound were resuspended in buffer and extracted along with their original supernatants. Biosorption studies with heat killed cells were conducted in the same manner except that the original bacterial mixture was held at 100°C for 15 minutes. In all cases, studies were conducted with triplicate sets of live or heat killed cells. The biosorption partition coefficient (Kp) was determined from the ratio of test chemical adsorbed per unit weight bacteria to test chemical unabsorbed per unit volume supernatant:

$$Kp = \frac{\mu g \text{ absorbed/gram bacteria}}{\mu g \text{ chemical/mL supernatant}}.$$

ANALYTICAL METHODS

For gas chromatographic analysis, samples (between 1 and 5 mL) containing from 1 to 130 µg/mL 1,3-DNB, 1,3,5-TNB or 3,5-DINA were extracted with 1 mL of methylene chloride containing an internal standard. The extracts were analyzed using a HP5830 gas chromatograph equipped with a flame ionization detector. A 6 ft column containing 3 percent OV-1 on GAS CHROM Q was used for all analyses. Isothermal conditions were usually employed with a hydrocarbon internal standard as follows: for 1,3-DNB (140°C, dodecane), for 1,3,5-TNB (175°C, pentadecane) for 3,5-DINA (215°C, octadecane). In studies where the concentrations of 1,3-DNB fell below 1 mg/L, a nitrogen-phosphorus detector was employed and ethyl acetate was substituted for CH_2Cl_2 as the extracting solvent.

When levels of 1,3-DNB in cultures were monitored by ultraviolet absorbance, bacterial cells were first removed by centrifugation at 16,000 XG for 10 min and aliquots were scanned from 210 to 360 nm with a Beckman Model Acta CV ultraviolet spectrophotometer. In all cases, uninoculated controls containing 1,3-DNB and cultures containing no added test compound were scanned with the experimental samples.

Gas chromatography/mass spectrometry (GC/MS) was conducted using a Hewlett Packard model 5840A GC/5985B MS system.

Thin-layer chromatographic (TLC) separations were performed on Eastman Chromagram[R] sheets (silica gel with fluorescent indicator) or on Merck silica gel F-254 plates (0.25 mm thickness, for preparative separations) and developed with ethyl acetate-hexane (1:1).

High-performance liquid chromatography (HPLC) of aqueous samples containing 3,5-DINA was conducted against authentic standards using a µBondapack C18 reversed-phase column (Waters Associates, Milford, MA). The mobile phase was 40 percent methanol in water at a flow rate of 1.7 mL/min. The effluent was monitored at 220 nm.

Total organic carbon (TOC) was measured using a Beckman Model 915 Total Organic Carbon Analyzer.

CHEMICALS

Unlabeled 1,3-DNB was prepared by the nitration of benzene according to the method of Wertheim.[20] Unlabeled and ^{14}C-ring labeled 1,3,5-TNB (0.54 µCi/mg) were prepared from 2,4,6-TNT as described by Vogel.[21] The 2,4,6-TNT starting material was synthesized by nitrating either unlabeled or labeled (3.4 mCi/mmol) toluene essentially as previously described.[22] Following two recrystallizations from ethanol, gas chromatographic analysis indicated that the 1,3-DNB or 1,3,5-TNB preparations were greater than 99.9 percent pure.

Unlabeled 3,5-DiNA was obtained commercially (Aldrich Chemical Co.). Labeled ^{14}C-3,5-DiNA (0.35 µCi/mg) was synthesized by the reduction of ^{14}C-1,3,5-TNB as described by Nicolet[23] and purified by elution from a small column of silica gel with methylene chloride. Unlabeled 3,5-diaminonitrobenzene was synthesized from 1,3,5-TNB as a by-product of the same reaction[23] and was eluted from the silica gel column with methylene chloride/ether (1:1/v:v) after 3,5-DiNA.

ISOTOPE MEASUREMENTS

Radioactivity measurements were made by dissolving aqueous 1 mL samples in 15 mL Instagel (Packard Instrument Co., Inc.) and by counting at maximum carbon 14 efficiency in a Packard Tricarb liquid scintillation spectrometer. All samples were in triplicate, and all were corrected for background and quenching.

RESULTS

SCREENING FOR BIODEGRADATION

1,3-DNB

The results of 21 days incubation of 5 µg/mL 1,3-DNB with Tennessee River water samples from which bulk sediments were removed are shown in Figure 1. The rate of removal of 1,3-DNB reached a maximum following 10 days of incubation in nonsterilized samples and proceeded until day 15, at which time the test compound was either completely removed or below the limit of detection (0.05 µg/mL). Such changes were not seen in water which was autoclaved prior to incubation. Significant measurable increases in microbial numbers were not detected from the 10th to 15th days of incubation, and plate counts of microorganisms during the interval averaged 8.1×10^5 CFU/mL.

A regression curve ($R^2 = 0.98$) for all chemical data obtained during the interval of maximum 1,3-DNB removal is shown in Figure 1 (insert). The curve is consistent with the pseudo first-order disappearance of 1,3-DNB with a rate constant (slope) of 2×10^{-2} hr^{-1}. Using the value of 8.1×10^5 CFU/mL for microbial levels during the interval, a second-order rate constant of 3.7×10^{-8} mL $cell^{-1}$ hr^{-1} can be calculated for the disappearance of 1,3-DNB.

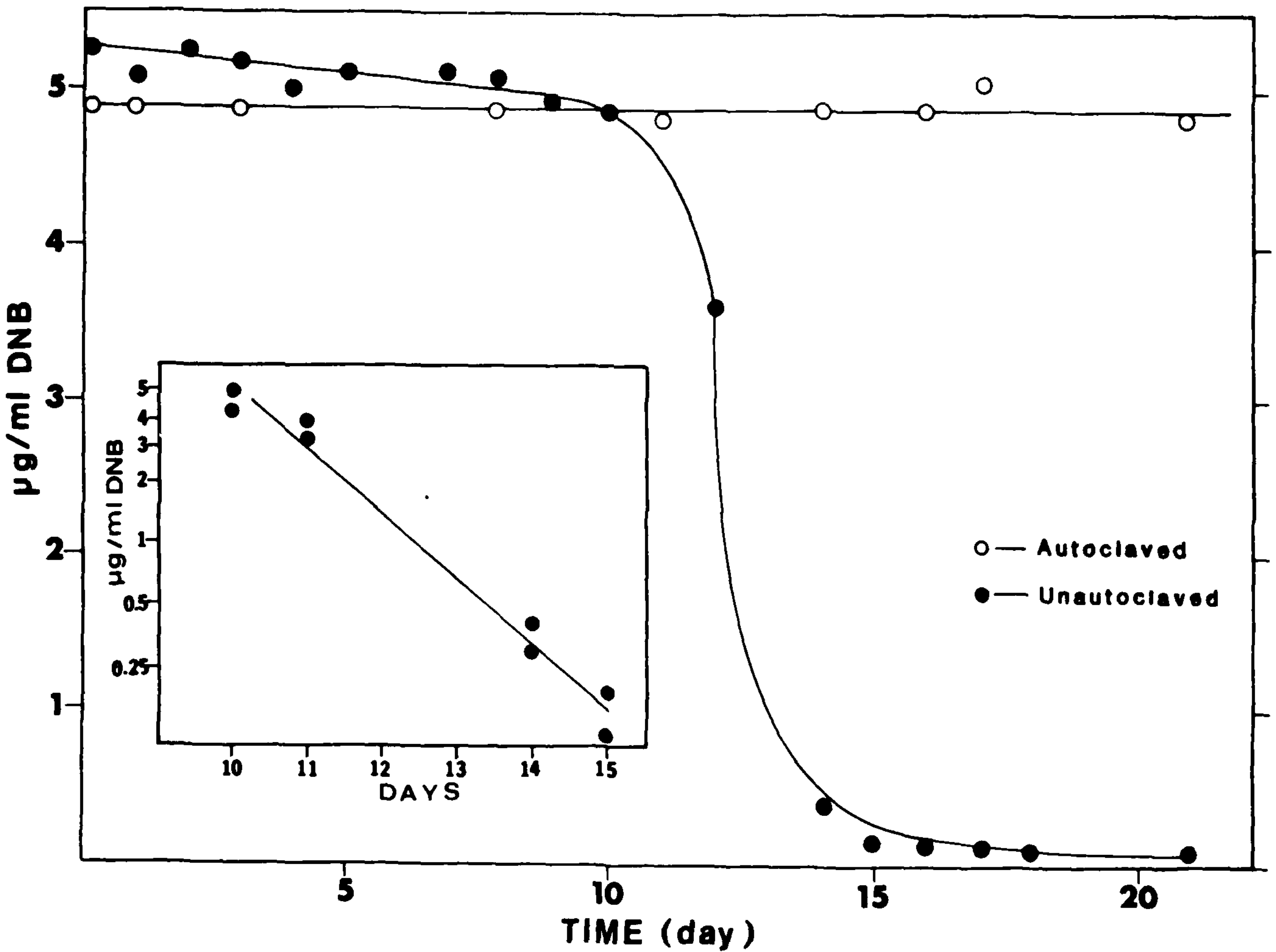

Figure 1. Incubation of 1,3-DNB with Tennessee River water.

1,3,5-TNB and 3,5 DiNA

To screen for the biodegradation of 1,3,5-TNB and 3,5-DiNA, buffered Tennessee River water samples with and without sediments were incubated for 21 days with 10.5 and 14 μg/mL of the test chemicals. Microorganisms in a second set of samples were first concentrated threefold by centrifugation and incubated as before. For each experimental flask, a control flask containing the test compound and sterilized sample water was co-incubated.

Results following 19 days of incubation are shown in Table 1. A slight decrease in the concentration of 1,3,5-TNB was seen in samples from which sediments were removed, but maximal levels of decrease (9 and 24 percent) required the presence of sediments. Decreases in the concentration of 3,5-DiNA were not seen unless sediments were present, in which case they were 33 and 52 percent. For both 1,3,5-TNB and 3,5-DiNA, concentration of microorganisms and sediments by centrifugation prior to incubation resulted in increased levels of degradation. Degradation of the test chemicals was not seen following their incubation with sterilized Tennessee River water samples.

TABLE 1. DECREASES IN 1,3,5-TNB AND 3,5-DiNA FOLLOWING 19 DAYS INCUBATION IN TENNESSEE RIVER WATER

Test Compound	Water Sample	% Decrease	
		Experimental	Sterile Control
1,3,5-TNB (≈10 μg/mL)	Filtered	6% (±1)	0
	With sediments	9% (±2)	0
	Filtered (threefold concentrated)	4% (±2)	0
	With sediments (threefold concentrated)	24% (±2)	0
3,5-DiNA (≈14.5 μg/mL)	Filtered	0	0
	With sediments	33% (±9)	0
	Filtered (threefold concentrated)	0	0
	With sediments (threefold concentrated)	52% (±2)	0

Data for the 21-day incubation of 1,3,5-TNB in sediment-containing waters concentrated threefold by centrifugation and a matched control are shown in Figure 2, panel A. Reduction in the concentration of 1,3,5-TNB by 24 percent occurred without a lag and proceeded for approximately 2 weeks, after which time the concentration of the test compound was unchanged. Levels of microorganisms in the sample remained essentially constant from the second to the ninth day of the experiment at 4.7×10^6 CFU/mL ($\pm 0.4 \times 10^6$). A regression curve ($R^2 = 0.914$) for triplicate data points within the interval is shown in Figure 3. The pseudo first-order rate constant estimated from the slope of the curve is 8×10^{-4} hr^{-1}. From this value and the number of microorganisms

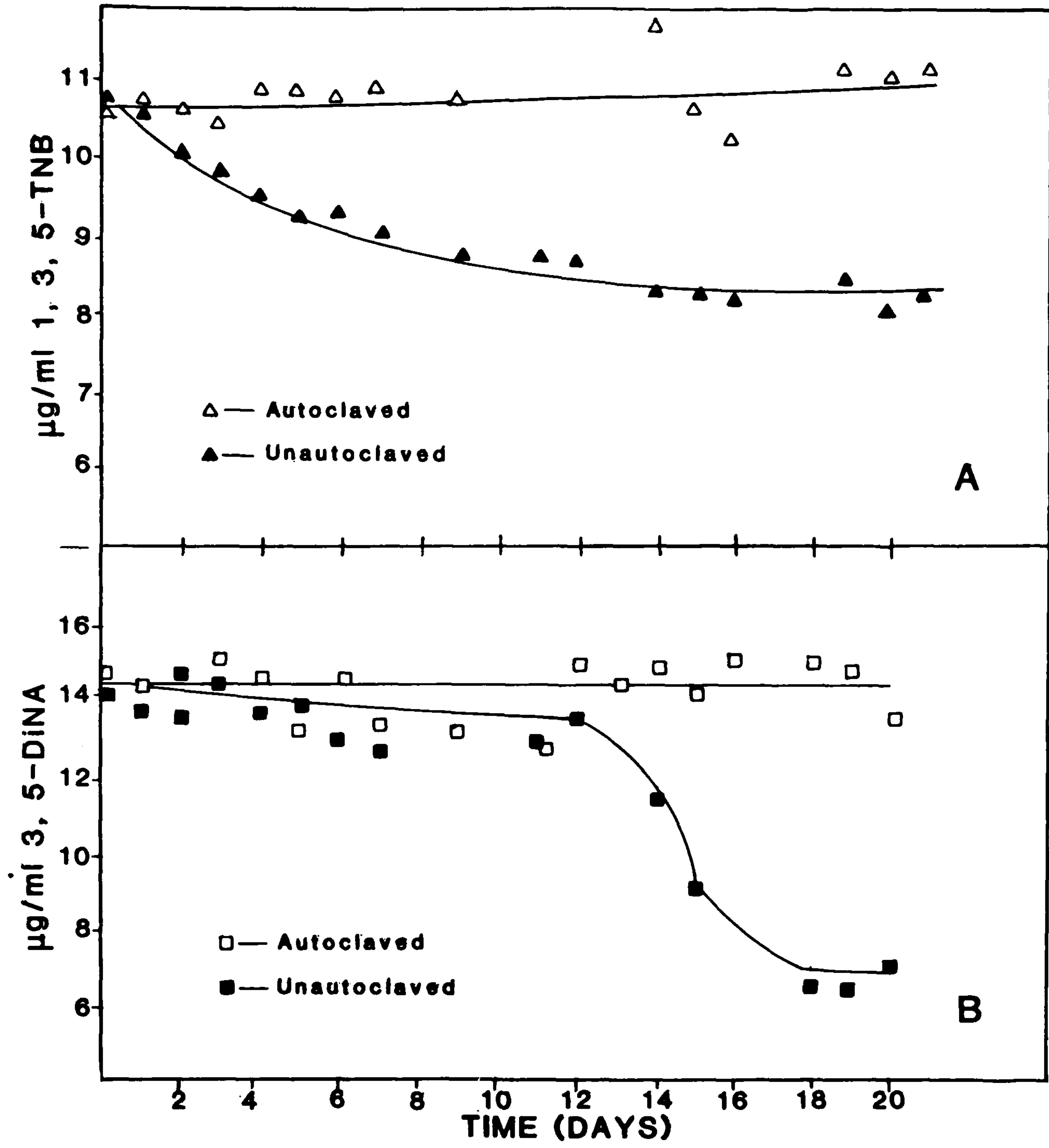

Figure 2. Incubation of 1,3,5-TNB and 3,5-DiNA with concentrated Tennessee River water containing sediments.

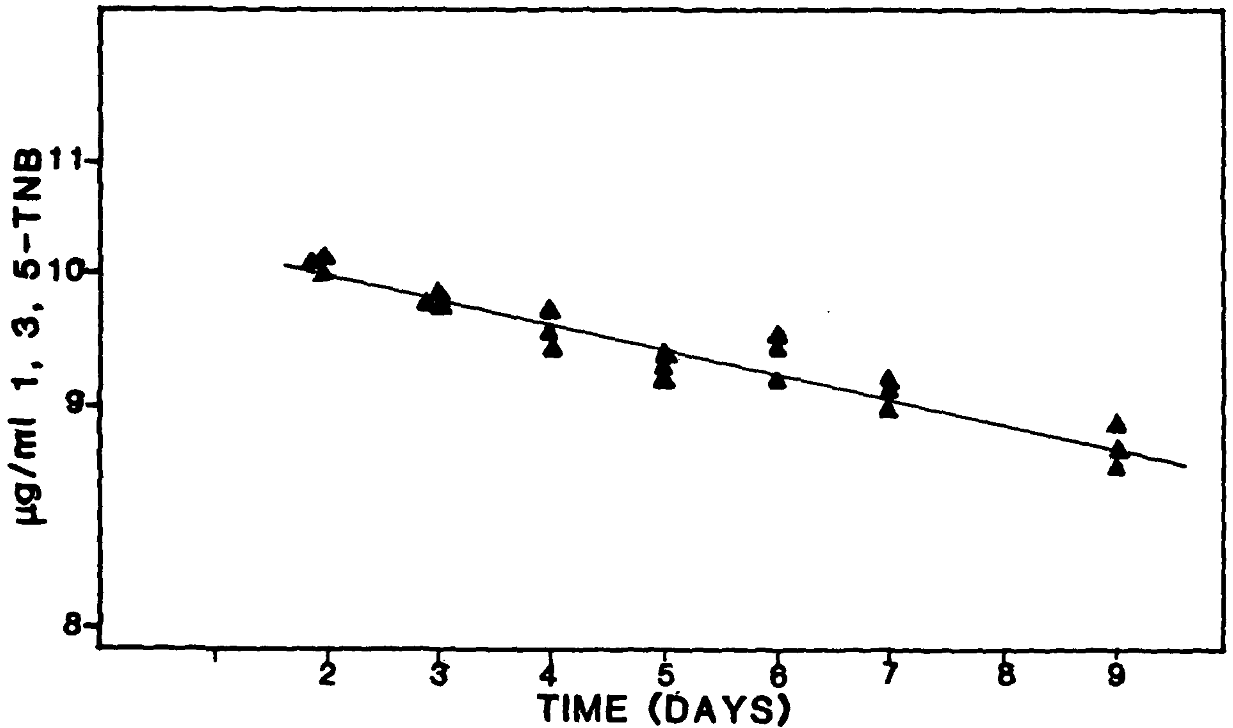

Figure 3. Degradation of 1,3,5-TNB in Tennessee River water.

during the period of change, the second-order rate constant for 1,3,5-TNB disappearance is calculated to be 1.7×10^{-10} mL $cell^{-1}$ hr^{-1}.

The pattern of 3,5-DiNA removal from threefold concentrated Tennessee River water with sediments is shown in Figure 2, panel B. Reduction in the concentration of the test chemical occurred following a lag of 12 days and proceeded until day 18, at which time 3,5-DiNA was approximately half its starting concentration. Microbial numbers fell from 1.6×10^6 CFU/mL ($\pm 0.4 \times 10^6$) to 6.4×10^5 CFU/mL ($\pm 10^5$) during the interval of change. Since microbial levels dropped during the period of test chemical decrease, the reduction in the concentration of 3,5-DiNA cannot be considered to be pseudo first-order with respect to the microbial population, and a rate constant cannot be derived from the data.

MICROBIOLOGICAL ENRICHMENTS

1,3-DNB

To develop a biodegrading culture for 1,3-DNB, a 5 percent (v/v) inoculum of material from a river water screening flask showing degradation was added to shake flasks containing the test chemical at 10 and 20 µg/mL in BSM buffer. After incubation, an aliquot from the highest concentration showing degradation was transferred to flasks containing the same and higher concentrations of 1,3-DNB. In this manner, microorganisms from the primary enrichment culture were sequentially passed through 10, 20, 40, 60, 100, and 130 µg/mL 1,3-DNB solutions. In all cases, degradation of the test chemical occurred, and visible turbidity was noted at 40 to 60 µg/mL following disappearance of the test chemical. Attempts to pass microorganisms to 1,3-DNB concentrations higher than 130 µg/mL were unsuccessful. At 180 µg/mL disappearance of the compound was 4 times slower than at 130 µg/mL and at 450 µg/mL the compound was not degraded at all.

To demonstrate microbial growth on 1,3-DNB as a sole carbon source, the 130 µg/mL enrichment culture was washed twice by centrifugation and resuspended in BSM buffer, and shake flasks containing 130 µg/mL 1,3-DNB and no test compound were inoculated at a bacterial level of 10^5 CFU/mL. Results are shown in Figure 4. Incubation in the presence of 1,3-DNB resulted in a 30- to 40-fold increase in bacterial numbers relative to the control. The compound was degraded to the lower limit of gas chromatographic detection (1 µg/mL) under the conditions employed for the analysis of high 1,3-DNB starting concentrations. The initial 24-hour growth period seen in both experimental and control flasks is atypical of bacterial growth curves but has previously been reported in experiments conducted to demonstrate growth of microorganisms on xenobiotic compounds.[24] The phenomenon could result from nutrient carryover in the inoculum, a round of growth on pooled nutrients in the lag phase cells comprising the inoculum, and/or growth on trace organic contaminants on glassware or in reagents.

Thus, 1,3-DNB at 130 µg/mL serves as a carbon source for growth of enrichment microorganisms. Subsequent experiments have shown that growth of the enrichment culture was slightly faster on 100 µg/mL 1,3-DNB, although cell yields relative to controls were essentially the same.

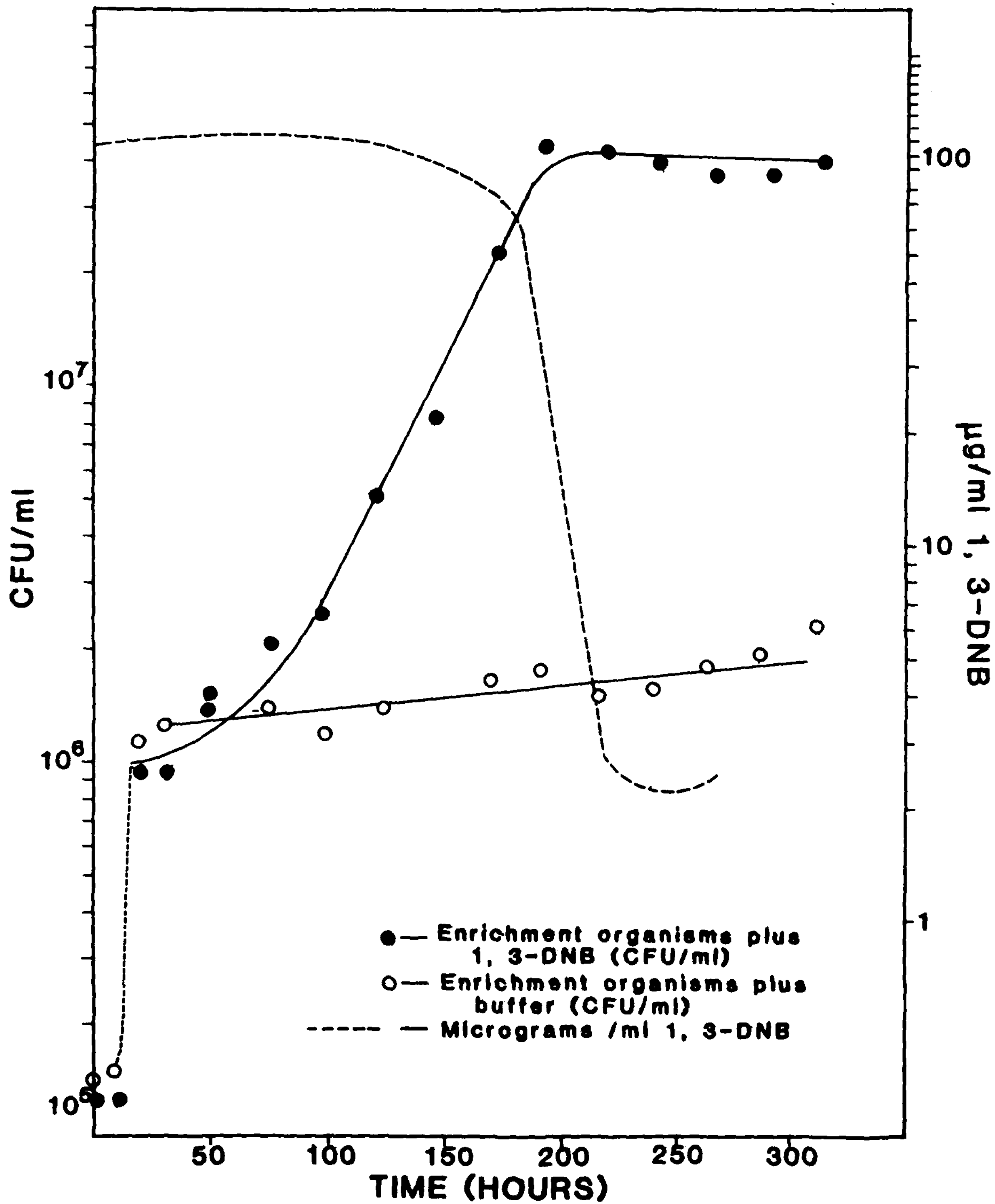

Figure 4. Growth of enrichment microorganisms on 1,3-DNB as a sole carbon source.

To determine the rate at which enrichment microorganisms were capable of removing 1,3-DNB from laboratory cultures, pseudo first-order kinetic studies at high bacterial levels were conducted as described in Materials and Methods. Enrichment microorganisms were washed, rested for 4 hours, and concentrated in fresh BSM buffer before their addition to shake flasks containing 1,3-DNB.

Results of a typical experiment conducted with 1,3-DNB at a starting concentration of 10 µg/mL are shown in Figure 5. Closed circles are results obtained with a starting bacterial concentration of 6.2×10^8 CFU/mL, and open circles are results obtained in a parallel experiment with bacteria at 2.42×10^8 CFU/mL. Following an acceleration phase of approximately 40 minutes during which the rate of 1,3-DNB uptake is constantly increasing, the rate becomes exponential for the remainder of the 2-hour experiment. Solid lines are regression curves drawn for data from the exponential phase of 1,3-DNB decline; whereas, broken lines represent an approximation of the acceleration phase. The number of bacteria did not increase significantly, if at all, in the course of the experiment as is shown in Table 2.

TABLE 2. BACTERIAL COUNTS DURING PSEUDO FIRST-ORDER BIODEGRADATION STUDIES WITH 1,3-DNB

Time (minutes)	Experiment 1 (CFU/mL)	Experiment 2 (CFU/mL)
0	6.5×10^8	2.42×10^8
60	7.6×10^8	2.27×10^8
120	6.8×10^8	2.88×10^8

The results of five such experiments conducted with different 1,3-DNB concentrations and bacterial levels are shown in Table 3. Since in all cases significant increases in bacteria did not occur, bacterial numbers measured at 0, 1, and 2 hours were averaged for the calculation of the second-order rate constant. Likewise, since an acceleration phase for 1,3-DNB uptake was seen in all experiments, the slopes of regression curves obtained after 40 minutes of incubation were used to determine pseudo first-order rate constants. The closeness of fit (R^2, Table 3) for all regression curves used in the determination of the pseudo first-order rate constants was 0.97 or greater. Based on the data of Table 3, the average value for the second-order disappearance of 1,3-DNB in the presence of enrichment microoganisms is 4.99×10^{-11} mL cell^{-1} min^{-1} ($1\sigma = 1.39 \times 10^{-11}$) or 2.99×10^{-9} mL cell^{-1} hr^{-1} ($1\sigma = 8.34 \times 10^{-10}$).

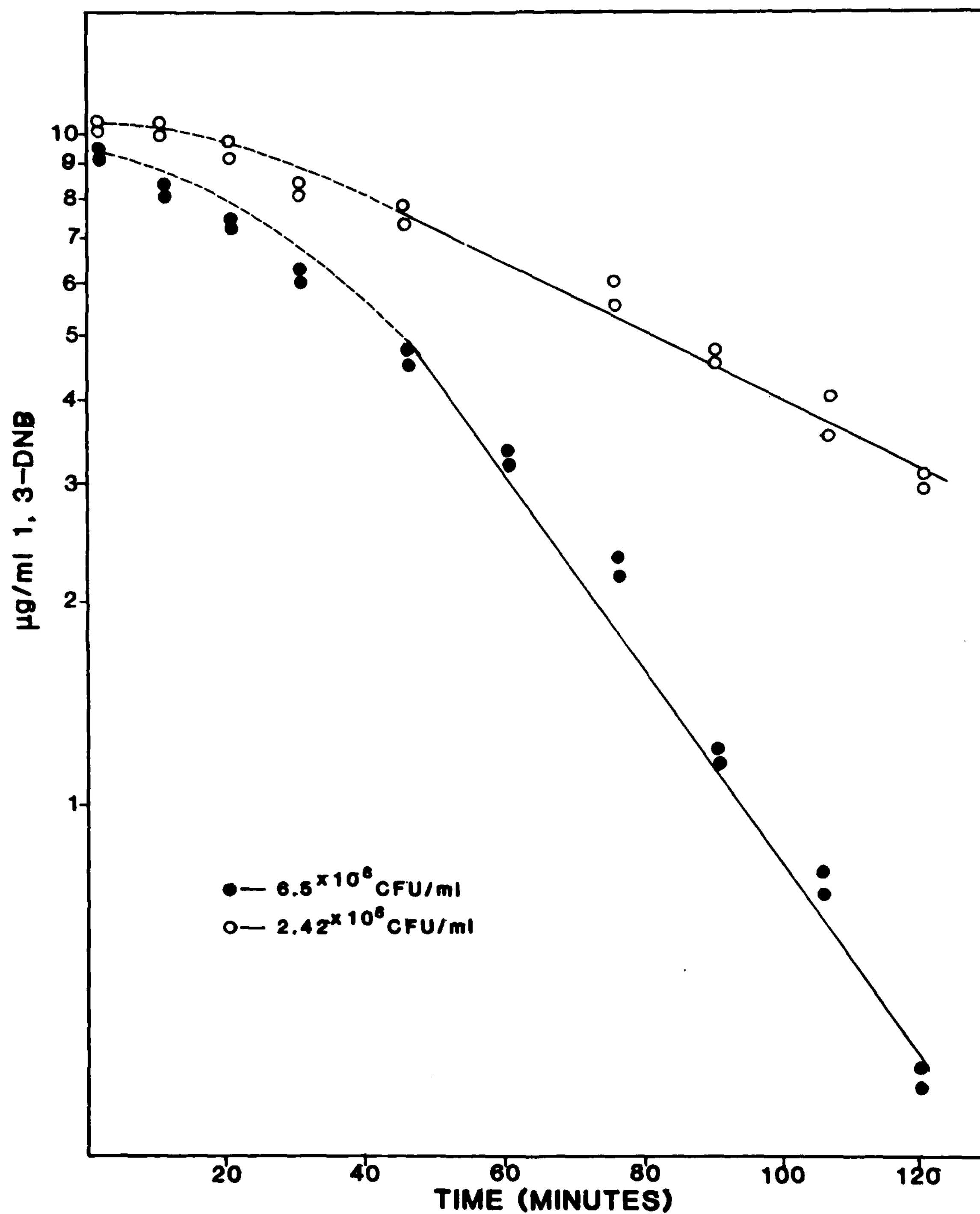

Figure 5. Uptake of 1,3-DNB by enrichment microorganisms.

TABLE 3. COMPOSITE RATE CONSTANT DETERMINATIONS FROM 1,3-DNB UPTAKE CURVES

Experiment	1,3-DNB	CFU/mL (10^{-8})	R^2	Rate Constant: Pseudo 1st-Order (min^{-1})	Rate Constant: 2nd-Order (mL $cell^{-1}$ min^{-1})
1	10	6.97	0.982	0.033	4.73×10^{-11}
2	10	2.52	0.976	0.012	4.76×10^{-11}
3	18	4.36	0.986	0.017	3.9×10^{-11}
4	20	2.16	0.993	0.009	4.16×10^{-11}
5	12	2.71	0.996	0.02	7.38×10^{-11}

Average second order rate constant = 4.99×10^{-11} mL $cell^{-1}$ min^{-1} ($1\sigma = 1.39 \times 10^{-11}$).

1,3,5-TNB

To determine if 1,3,5-TNB would serve as a sole carbon source for microbial growth, shake flasks containing BSM buffer and 8 µg/mL or no added test compound were inoculated with aliquots (5 percent v/v) of a 1,3-TNB primary screening flask (with sediments) showing 20 percent degradation. In addition, flasks containing the test chemical and various increments of 50 µg/mL glucose - 10 µg/mL yeast extract (1X GYE) were inoculated with the same material. Samples were removed at various times after the start of the experiment and were measured for microbial growth (optical density at 420 nm) and levels of 1,3,5-TNB.

The results of the study are shown in Table 4. No reduction in the concentration of 1,3,5-TNB is seen to occur when microorganisms from primary screening are reinoculated into shake flasks containing the test chemical alone (Table 4, No GYE). In all cases where incubation was carried out in the presence of the test chemical and added glucose-yeast extract, some degradation of 1,3,5-TNB took place. The rate and degree to which the compound was removed increased with increasing concentrations of glucose-yeast extract up to 10X GYE, in which case the concentration was reduced to levels below the limit of gas chromatographic detection after 22 hours incubation.

The decreases seen in the table did not appear to result from adsorption of 1,3,5-TNB by increased biomass at higher glucose-yeast extract levels; the mixing of 10 µg/mL of the compound with enrichment microorganisms grown to an optical density at 420 nm of 2.7 on 50X GYE followed by centrifugation, resulted in its quantitative recovery in the supernatant. Thus, the decreases seen in the concentration of the compound appear to result from microbial metabolic activity.

TABLE 4. EFFECT OF GLUCOSE YEAST EXTRACT ON 1,3,5-TNB DEGRADATION IN MICROBIAL ENRICHMENT CULTURES

Time (hours)	0		5.5		22		29		47		120		168	
	OD[a]	TNB[b]	OD	TNB	OD	TNB	OD	TNB	OD	TNB	OD	TNB	OD	TNB
No GYE	0.05	8.13	0.05	7.9	0.04	8.1	0.04	8.2	0.03	9.1	0.03	8.4	0.03	7.8
1 X GYE[c]	0.05	7.8	0.05	8.1	0.15	7.5	0.13	7.4	0.13	5.6	0.12	5.7	0.16	5.1
5 X GYE	0.06	8.1	0.06	7.5	0.5	4.2	0.68	4.26	0.51	3.2	0.5	1.0	0.48	0.74
10 X GYE	0.06	7.3	0.07	7.68	0.78	ND	1.0	ND	1.0	ND	0.9	ND	0.72	ND
50 X GYE	0.14	7.8	0.15	7.8	2.6	ND	3.1	ND	3.26	ND	3.4	ND	2.6	ND
50 X GYE	0.14	ND[d]	0.15	ND	3.0	ND	3.5	ND	3.1	ND	3.1	ND	Clumps	ND

a. Optical density 420 nM.
b. Micrograms per milliliter.
c. 50 μg/mL glucose - 10 μg/mL yeast extract.
d. Less than 0.5 μg/mL.

To determine the concentration range over which 1,3,5-TNB could be degraded, enrichment microorganisms grown in the presence of 8 μg/mL 1,3,5-TNB and 10X GYE (Table 4) were used to inoculate a series of flasks containing various concentrations of the test chemical and 10X GYE. Results are shown in Table 5. All starting concentrations of the compound, ranging from 16 to 200 μg/mL, show some decrease in the course of the 144-hour experiment, and the highest test compound level showing near complete disappearance was 53 μg/mL (Table 5, flasks 7).

TABLE 5. EFFECT OF ENRICHMENT MICROORGANISMS ON VARYING CONCENTRATIONS OF 1,3,5-TNB IN 10X GYE[a] MEDIUM

Time	5		20		40		120		144	
(hours)	OD[b]	TNB[c]	OD	TNB	OD	TNB	OD	TNB	OD	TNB
Flask 1	0.08	ND[d]	0.86	ND	0.72	ND	0.6	ND	0.6	ND
Flask 3	0.07	16.25	0.82	ND	0.69	ND	0.63	ND	0.65	ND
Flask 5	0.04	30.7	1.03	15.0	0.73	10.6	0.66	ND	0.69	ND
Flask 7	0.05	53.0	1.1	28.9	0.78	16.2	0.76	ND	0.78	ND
Flask 9	0.04	105.0	0.99	81.0	0.9	57.8	0.76	29	0.76	24.3
Flask 11	0.03	202.0	0.89	171.4	0.84	170.0	0.81	162	0.79	149.9

a. 10X GYE is 500 ppm glucose 100 ppm yeast extract.
b. OD is optical density at 420 nm.
c. Micrograms per milliliter 1,3,5-TNB.
d. Less than 1 μg/mL.

From the results of Tables 4 and 5, the incorporation of glucose-yeast extract in microbial enrichments results in a population of microorganisms with the metabolic capacity to interact with 1,3,5-TNB and reduce its concentration.

To rule out the possibility that long-term exposure of this population to the chemical would yield organisms capable of using it as a sole carbon source for growth, enrichment microorganisms were passed weekly for 12 weeks in medium containing 50 μg/mL 1,3,5-TNB and 10X GYE. The resulting culture was then used to inoculate flasks containing 50 μg/mL 1,3-TNB with and without added 10X GYE. Results of the experiment were consistent with those of Table 4; no increase in biomass or decrease in the test compound occurred unless glucose-yeast extract was present.

While 1,3,5-TNB did not serve as a sole carbon source for growth of enrichment microorganisms, the data of Tables 4 and 5 indicate that considerable reductions in the concentration of the compound can occur in the presence

of exogenous utilizable carbon. To determine if the decreases observed result in the mineralization of the compound, 10 µg/mL ^{14}C-1,3,5-TNB was incubated with microorganisms in the presence of 10X GYE, and the culture was measured for the liberation of $^{14}CO_2$. Inoculum consisted of enrichment microorganisms grown on 10X GYE in the presence of 50 µg/mL 1,3,5-TNB, and the experiment was conducted in carbon dioxide trapping flasks as described in Methods. Values for endogenous CO_2 production by the inoculum in matched controls were subtracted from the experimental samples. Increases in biomass were monitored by measuring the optical density of aliquots at 420 nm.

Results of the experiment are shown in Table 6. During the course of 3 days incubation, biomass increased more than tenfold and 83 percent of the 1,3,5-TNB disappeared from the culture. Approximately one-third of the total organic carbon (in the form of glucose-yeast extract) was converted to CO_2. Despite increases in biomass, conversion of glucose-yeast extract to CO_2, and disappearance of 1,3,5-TNB, no significant amount of the isotope was converted to $^{14}CO_2$. Yet almost all of the label could be recovered from the culture, and almost all of the label remained in the supernatant when the bacteria were separated by centrifugation at the end of the experiment. Thus the metabolism of 1,3,5-TNB by enrichment microorganisms in the presence of glucose-yeast extract results in a transformation of the test chemical to noncell-associated products but not in mineralization.

TABLE 6. EFFECT OF ENRICHMENT MICROORGANISMS ON ^{14}C-1,3,5-TNB

Time (hr)	OD 420 nm	TNB (µg/mL)	Percent Theoretical Maximum CO_2 Production		Isotope Recovery From Culture (DPM/mL)
			TOC	TNB	
0	0.052	10.44 (1σ = 0.2)	-	-	12664 (1σ = 141)
72	0.75	1.8 (1σ = 0.2)	32[a]	0.12[b]	12211 (1σ = 117) Cell pellet 752 (1σ = 31) Supernatant 11,346 (1σ = 108)

a. $\frac{1.2 \text{ milli equivalents } CO_2 \text{ trapped}}{3.74 \text{ milli equivalents } CO_2 \text{ total}} \times 100\%$

b. $\frac{2.35 \times 10^3 \text{ DPM trapped}}{1.96 \times 10^6 \text{ DPM total}} \times 100\%$

3,5-DiNA

To determine if 3,5-DiNA would serve as a carbon source for growth, enrichment microorganisms from a primary screening flask (containing sediments) which showed 25 percent 3,5-DiNA degradation were added to a series of flasks containing approximately 15 µg/mL 3,5-DiNA in BSM buffer. Several of the flasks contained 1-, 5-, 10-, and 50-fold increments of 50 µg/mL glucose and 10 µg/mL yeast extract (1X GYE). During the incubation of the cultures at 25°C, samples were removed and measured for levels of the test compound and bacterial growth (optical density, 420 nm).

Results are shown in Table 7. Reinoculation of primary screening microorganisms into medium containing 3,5-DiNA as a sole carbon source for growth results in little, if any, decrease in the level of the test chemical (Table 7, No GYE). Even in the presence of 1-, 5-, and 10-fold increases of glucose-yeast extract, substantial metabolism of 3,5-DiNA did not occur. Yet when the level of glucose-yeast extract was increased 50-fold, more than 90 percent of the initial 3,5-DiNA disappeared from the culture (Table 7, 50X GYE). Since the mixing of enrichment microorganisms grown on 50X GYE with the compound did not result in its adsorption to the bacteria, the decreases observed in Table 7 result from microbial metabolism. Like 1,3,5-TNB, 3,5-DiNA did not serve as a sole source for microbial growth, and its disappearance required the presence of exogenous metabolizable carbon; however, the near complete disappearance of 3,5-DiNA required higher concentrations of added glucose-yeast extract than did that of 1,3,5-TNB.

To confirm and extend the results, enrichment microorganisms from Table 7 were added to shake flasks containing 50X GYE and varying levels of the test compound. Results are shown in Table 8. All concentrations tested showed decreases in 3,5-DiNA, and the highest starting level showing near complete disappearance was 129 µg/mL (flasks 9, Table 8).

Enrichment microorganisms from the experiment of Table 8 (flask 7) were passed weekly for 9 weeks in medium containing 3,5-DiNA at 50 µg/mL and 50X GYE. The resulting culture was then used as inoculum for flasks containing either 50 µg/mL DiNA or 50 µg/mL DiNA plus 50X GYE. Results were consistent with the data of Table 7; increases in biomass and decreases in the concentration of 3,5-DiNA did not occur unless glucose-yeast extract was present. Thus, while the capability of microorganisms to metabolize 3,5-DiNA is maintained following serial passage in the laboratory, the same capability does not result in the development of microorganisms which can utilize 3,5-DiNA as a carbon source for growth.

As is indicated by the data of Tables 7 and 8, the metabolism of 3,5-DiNA by enrichment microorganisms in the presence of glucose-yeast extract, like that of 1,3,5-TNB, results in considerable decreases in the concentration of the compound. To determine if ^{14}C-3,5-DiNA was mineralized by enrichment microorganisms in the presence of glucose-yeast extract, the method of Sturm[18] was modified to facilitate the continuous gasing of duplicate 100-mL cultures, and the collection of effluent carbon dioxide. The experimental flask contained in BSM buffer: microorganisms from the experiment of Table 8, 25 µg/ml ^{14}C 3,5-DiNA (0.35 µCi/mg), and 50X GYE. A control flask consisted of 3,5-DiNA at the same concentration plus inoculum, but no glucose-yeast extract. Values for endogenous CO_2 production by the control were subtracted from the experimental sample.

Results of the study are shown in Table 9. Three days incubation of enrichment microorganisms in the presence of 50X GYE and labeled 3,5-DiNA resulted in a greater than 95 percent decrease in the concentration of the chemical. Turbidity increased more than sixfold in the experimental flask, and more than 50 percent of the total organic carbon present in the form of glucose-yeast extract was converted to CO_2. Despite these changes, less than 0.2 percent of the label was converted to $^{14}CO_2$. Thus, while metabolizable carbon was converted to carbon dioxide and 3,5-DiNA was degraded in the experimental flask, appreciable mineralization of the test compound did not

TABLE 7. EFFECT OF GLUCOSE YEAST EXTRACT ON 3,5-DINA DEGRADATION IN MICROBIAL ENRICHMENT CULTURES

Time (hour)	0		5.5		23		28		50		77		102	
	OD[a]	DINA[b]	OD	DINA	OD	DINA	OD	DINA	OD	DINA	OD	DINA	OD	DINA
No GYE	0.18	14.8	0.18	14.3	0.19	12.0	0.2	14.8	0.18	14.2	0.19	14.8	0.19	14.0
1 X GYE[c]	0.19	15.1	0.19	13.6	0.28	14.7	0.3	9.9	0.27	13.4	0.28	11.9	0.29	13.7
5 X GYE	0.18	15.8	0.2	12.4	0.6	14.3	0.65	12.3	0.6	13.5	0.61	11.23	0.58	12.0
10 X GYE	0.19	14.1	0.21	12.2	0.86	12.9	0.89	11.5	0.88	13.2	0.9	11.2	0.9	12.5
50 X GYE	0.27	14.7	0.27	14.0	1.4	10.2	1.7	5.7	1.8	1.48	1.9	0.84	1.8	0.87
50 X GYE	0.12	ND[d]	0.13	ND	1.5	ND	1.9	ND	1.8	ND	1.9	ND	1.8	ND

a. Optical density 420 nm.
b. Microgram per milliliter.
c. 50 µg/mL glucose - 10 µg/mL yeast extract.
d. Less than 0.5 µg/mL.

TABLE 8. EFFECT OF ENRICHMENT MICROORGANISMS ON VARYING CONCENTRATIONS OF 3,5-DiNA IN 50X GYE MEDIUM[a]

Time	0		5		27		96		144		196	
(hour)	OD[b]	DiNA[c]	OD	DiNA	OD	DiNA	OD	DiNA	OD	DiNA	OD	DiNA
Flask 1	0.14	ND[d]	0.95	ND	3.3	ND	3.6	ND	3.8	ND	3.5	ND
Flask 3	0.1	18	0.8	15.3	3.3	6.0	3.2	ND[e]	3.0	ND[e]	3.3	ND[e]
Flask 5	0.18	36	1.1	35.0	3.3	5.6	3.3	ND[e]	3.4	ND[e]	3.3	ND[e]
Flask 7	0.21	62	0.82	57.0	2.3	6.6	3.0	ND[e]	2.6	ND[e]	2.6	ND[e]
Flask 9	0.25	129	0.76	126.5	1.6	20.1	2.1	ND[e]	1.7	ND[e]	1.3	ND[e]
Flask 11	0.25	230	0.5	210.9	1.4	120.5	2.1	52.4	1.6	51.3	1.5	44.5

a. 50X GYE is 2,300 μg/mL glucose and 500 μg/mL yeast extract.
b. OD is optical density at 420 nm.
c. Micrograms per milliliter 3,5-DiNA.
d. Less than 1 μg/mL 3,5-DiNA.
e. New substance (3,5-diaminonitrobenzene) detected.

TABLE 9. EFFECT OF ENRICHMENT MICROORGANISMS ON ^{14}C-3,5-DINA

Time (hr)	OD 420 nm	DINA (μg/mL)	Percent Theoretical Maximum CO_2 Production TOC	DINA	Isotope Recovery From Culture (DPM/mL)
0	0.23	25.3 (1σ = 1.5)	-	-	14,403 (1σ = 500)
72	1.4	<1	58.2[a]	0.15[b]	14,655 (1σ = 188) Pellet 436 (1σ = 5) Supernatant 14,107 (1σ = 106)

a. $\frac{4.92 \text{ milli equivalents } CO_2 \text{ trapped}}{8.45 \text{ milli equivalents } CO_2 \text{ total}} \times 100\%$

b. $\frac{1.6 \times 10^3 \text{ DPM trapped}}{1.07 \times 10^6 \text{ DPM total}} \times 100\%$

occur. Following the separation of bacteria from the culture medium by centrifugation after 72-hours incubation, almost all of the carbon-14 label was recovered in the supernatant; thus, the transformation product or products of 3,5-DiNA are noncell-associated.

PRODUCTS ASSOCIATED WITH MICROBIAL INTERACTIONS

1,3-DNB

Because 1,3-DNB served as a carbon source for the development of an enrichment culture (Fig. 4), it was appropriate to determine the degree to which enrichment microorganisms mineralized the compound. Triplicate carbon dioxide trapping flasks which contained 50 μg/mL 1,3-DNB or no added test compound in BSM buffer were inoculated with enrichment microorganisms grown on 130 μg/mL 1,3-DNB as described in Methods. To minimize carbon dioxide losses which could occur during repeated sampling and gassing of the cultures, flasks were sparged only at 0 hr and at 67 hr, and samples were taken only at the beginning and end of the experiment.

Results are shown in Table 10. In the course of the 144-hr experiment, the test chemical was degraded to below the limit of gas chromatographic detection in all experimental flasks (Table 10, flasks Ia-c). Concentrations of bacteria in the experimental flasks increased more than 10 times those in the controls. Levels of carbon dioxide production within the sets of experimental or control flasks are in close agreement: 0.83 mMoles CO_2 (1σ = 0.028) were produced in the experimental series Ia-c, while 0.12 mMoles CO_2 (1σ = 0.0029) were produced in the control series IIa-c. As is shown in the calculation of Table 10, 77.5 percent of the 1,3-DNB carbon in the experiment was converted to carbon dioxide. Over the course of six mineralization experiments conducted at various levels of inoculum and substrate, the conversion of 1,3-DNB to carbon dioxide ranged from 50 to 80 percent. Taken together, the results indicate that the principal products associated with the interaction of enrichment microorganisms and 1,3-DNB are biomass and carbon dioxide, and that most of the 1,3-DNB molecule is ultimately biodegradable.

TABLE 10. REPLICATE SAMPLES FOR 1,3-DNB MINERALIZATION

	Flasks					
	Ia	Ib	Ic	IIa	IIb	IIc
0 Hours						
DNB (ppm)	52	51.8	50.5	ND[a]	ND	ND
Cells (CFU/mL)	1.94×10^5	2.04×10^5	1.76×10^5	2.08×10^5	1.89×10^5	2.02×10^5
CO_2 (mMoles)	--	--	--	--	--	--
144 Hours						
DNB (ppm)	ND	ND	ND	ND	ND	ND
Cells (CFU/mL)	3.7×10^7	2.6×10^7	2.9×10^7	2.3×10^6	2.1×10^6	2.1×10^6
CO_2 (mMoles)	0.8205	0.86	0.806	0.119	0.114	0.119

Mean CO_2 Experimental Flask I.................0.8288 mMoles ($1\sigma = 0.028$)
Endogenous Flask II..................0.1173 mMoles ($1\sigma = 0.0029$)

$$\% \text{ Theoretical Maximum} = \frac{(0.8288-0.1173) \text{ mMoles}}{0.917 \text{ mMoles}^b} \times 100\%$$

$$= 77.5\% \ (1\sigma_{max} \ 3.1\%)$$

a. Less than 0.5 µg/mL.
b. Theoretical maximum (avg.) =

$$(51.4 \text{ mg/L 1,3-DNB}) \ (0.5 \text{ L}) \left(\frac{1 \text{ mMoles 1,3-DNB}}{168 \text{ mg}}\right) \left(\frac{6 \text{ mMoles } CO_2}{\text{mMoles 1,3-DNB}}\right) =$$

$$0.917 \text{ mMoles } CO_2$$

1,3,5-TNB

In the course of the enrichment studies for 1,3,5-TNB shown in Tables 4 and 5, a slight yellowing was seen to occur in the medium of enrichment microorganisms grown in the presence of the test chemical and glucose yeast extract (10X GYE) which was not observed when the bacteria were grown on the nutrients alone. Following high-performance liquid chromatography of aqueous samples or TLC of extracted samples, material with the same elution characteristics as authentic 3,5-DiNA standards was found to be present. Its identity as 3,5-DiNA was later confirmed by GC/MS (m/e 183,M^+).

To further demonstrate the presence of 3,5-DiNA in microbial enrichments, samples from the experiment of Table 5 were analyzed and quantitated by gas chromatography using authentic 3,5-DiNA standards. Results of the analyses are shown in Table 11 for samples taken at 40-hours incubation. All samples tested following the incubation of microorganisms in the presence of 1,3,5-TNB and glucose-yeast extract showed the presence of 3,5-DiNA (Table 11; flasks 3 through 11). Prior to incubation of the same cultures, only traces (< 0.2 µg/mL) of the compound could be detected. Since traces of 3,5-DiNA were also present in the control flask containing no 1,3,5-TNB (Table 11, flask 1), it is likely that these traces were present in the inoculum for the study. (Inoculum consisted of primary screening microorganisms grown on glucose-yeast extract in the presence of 1,3,5-TNB as described in the experiment of

TABLE 11. PRODUCTION OF 3,5-DiNA FROM 1,3,5-TNB FOLLOWING 40 HOURS INCUBATION OF ENRICHMENT MICROORGANISMS WITH 10X GYE[a]

Sample (from Table 5)	3,5-DiNA (μg/mL)	μMoles 3,5-DiNA/mL (equivalent)	μMoles 1,3,5-TNB Consumed (Table 5)	% Converted $\frac{\mu M\ 3,5\text{-DiNA}}{\mu M\ 1,3,5\text{-TNB}} \times 100$
Flask 1	Trace[b]	--	None	--
Flask 3	5.8	0.032	0.076	42.1%
Flask 5	7.3	0.04	0.094	42.5%
Flask 7	3.5	0.019	0.17	11.2%
Flask 9	6.4	0.035	0.22	15.9%
Flask 11	3.5	0.019	0.15	12.7%

a. 500 μg/mL glucose - 100 μg/mL yeast extract.
b. Less than or equal to 0.22 μg/mL 3,5-DiNA equivalent.

Table 5.) The conversion of 1,3,5-TNB to 3,5-DiNA is not quantitative. At lower test compound concentrations roughly 40 percent of the chemical was converted to the product (flasks 3 and 5), and at higher concentrations 15 percent or less of the 1,3,5-TNB was converted (flasks 7, 9, and 11). Nor did the product accumulate during continued incubation of the cultures. Incubation of flask 7 for an additional 100 hours or flasks 9 and 11 for 120 hours resulted in no increases in 3,5-DiNA levels over those shown in Table 11, despite continued decreases in the 1,3,5-TNB concentration (Table 5).

To determine if a similar transformation takes place in environmental water samples, the Tennessee River water samples shown in Table 1 which had been incubated with 10 μg/mL 1,3,5-TNB for 21 days were analyzed for the presence of 3,5-DiNA. Aliquots (100 mL) of the samples or their sterilized matched controls were extracted twice with methylene chloride, evaporated to dryness, and concentrated 100-fold by dissolving in 1 mL of the solvent prior to gas chromatography. Results of a typical analysis are shown in Figure 6. Gas chromatographic profiles in the figures are those for a threefold concentrated water sample (with sediments) which had been autoclaved (Panel A) or not autoclaved (Panel B) before incubation with 1,3,5-TNB. As is indicated in the figure, 3,5-DiNA was found in the experimental sample but not in the sterile control. Moreover, the mass spectrum of the product of Figure 6 was the same as authentic 3,5-DiNA (m/e = 183).

As can be seen in Table 12, 3,5-DiNA was detected in all experimental samples from the primary screening phase, whether or not organisms were first concentrated, and whether or not sediments were present. Matched sterile controls did not contain the compound. Levels of 3,5-DiNA extracted from the environmental samples following incubation for 21 days were variable as is shown in the table, but in all cases, measurable amounts of the chemical were synthesized from 1,3,5-TNB.

3,5-DiNA

During the course of microbial enrichments with glucose-yeast extract (50X GYE) and the various concentrations of 3,5-DiNA in Table 8, a pronounced darkening was seen to develop in the cultures but not in controls containing only glucose-yeast extract (Table 8; flask 1 versus flasks 3 through 9). Gas chromatographic analysis of the same preparations indicated the presence of a product synthesized by microbial action on 3,5-DiNA in the presence of glucose-yeast extract. Thin-layer chromatography of the extracted contents of flask 9 (Table 8) confirmed the presence of newly synthesized compounds, at least one of which tended to accumulate with continued incubation. Based on the appearance of the thin-layer plates, two minor product spots also occurred early in the experiment (27 to 96 hours) but decreased thereafter. Similar materials were not observed on TLC of cultural extracts following growth on glucose-yeast extract alone.

To identify the principal products of microbial action on 3,5-DiNA, enrichment microorganisms were grown on glucose-yeast extract (50X GYE) in the presence of 180 μg/mL 3,5-DiNA for 196 hours, and the culture was extracted with ethyl acetate. Products in the extract were separated by preparative TLC and analyzed by GC/MS. Results of the analysis indicated that, other than unmodified 3,5-DiNA, the principal compound on the thin-layer plate was 3,5-diaminonitrobenzene (m/e = 153, M^+) (m/e = 107, M^+-NO_2). In addition to the

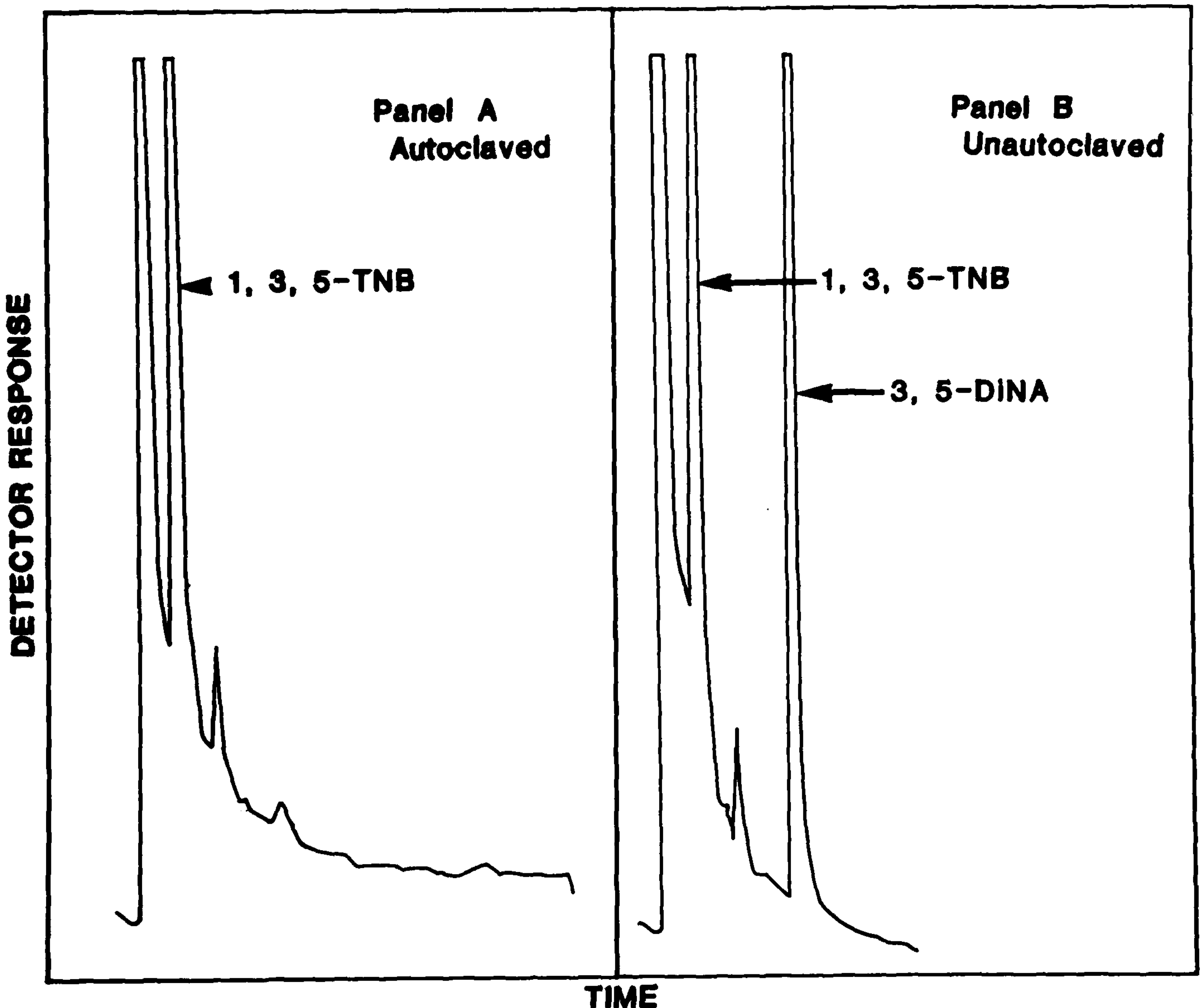

Figure 6. Gas chromatograms of 1,3,5-TNB and product 3,5-DINA after incubation in Tennessee River water with sediments.

TABLE 12. 3,5-DINA PRODUCTION FROM 1,3,5-TNB IN ENVIRONMENTAL SAMPLES

Sample (from Table 1)	µg DINA Extracted/ 100 mL Sample[a]	µMoles DINA/ 100 mL	µMoles TNB Consumed/ 100 mL (from Table 1)	% Converted $\frac{\mu\text{Moles DINA}}{\mu\text{Moles TNB}} \times 100$
Filtered	10	0.055	0.318	17.3%
With sediments	28	0.153	0.426	35.9%
Filtered (threefold concentrated)	18	0.098	0.205	47.8%
With sediments (threefold concentrated)	78	0.426	2.62	16.3%

a. Standard deviation for 3,5-DINA analysis was ±21% in this series. Extraction efficiencies were from 75 to nearly 100% based on values obtained from matched sterile controls.

major product, two minor products isolated from the thin-layer plate were tentatively identified as 3-nitro-5-aminoacetanilide (m/e = 195, M^+) and a nitro, amino substituted N-methylindoline (m/e = 193, M^+).

Under the conditions of gas chromatographic analysis described in Materials and Methods, the flame ionization detector response to authentic 3,5-diaminonitrobenzene is 90 percent that for 3,5-DiNA. Thus, the levels of the product in the chromatograms of Table 8 could be calculated from the ratio of the corrected 3,5-diaminonitrobenzene and 3,5-DiNA peak areas and the known concentration of 3,5-DiNA in the appropriate sample. Conversion of 3,5-DiNA to 3,5-diaminonitrobenzene for glucose-yeast extract enrichment cultures containing either a low (18 µg/mL) or high (129 µg/mL) 3,5-DiNA starting concentration are shown in Table 13. Levels of 3,5-DiNA consumed were determined from the data of Table 8, and amounts of 3,5-diaminonitrobenzene formed were determined as described above. As is shown in Table 13, the result of microbial action on both high and low starting concentrations of 3,5-DiNA is the conversion of more than half of the test compound to 3,5-diaminonitrobenzene. At the termination of the experiment, roughly 70 and 90 percent of the 3,5-DiNA was transformed to 3,5-diaminonitrobenzene for the low and high starting concentrations, respectively.

BIOADSORPTION OF 1,3,5-TNB, 1,3-DNB, AND 3,5-DiNA

Bioadsorption studies for the three compounds were conducted using 3-day cultures of *Escherichia coli*, *Bacillus cereus*, *Serratia marcescens*, and *Azotobacter beijerinckii*. Bacterial cells were washed by repeated centrifugation and resuspension in buffer, rested for 4 hours, and equal optical densities were combined such that the final mixed suspension was four OD_{420} units per mL. Test chemicals were added to live or heat-killed cells for 1 hr at 25°C, after which time the bacteria were removed by centrifugation. Supernatants, cell pellets, and controls consisting of the test compound without added cells were analyzed by gas chromatography for levels of the various chemicals. All tests were in triplicate.

Results for the bioadsorption studies are shown in Table 14. Biosorption coefficients for all three compounds were less than 10 in the presence of live cells and only slightly higher than this with heat-killed cells. Biosorption coefficients of even greater magnitude than those shown in Table 14 have been previously interpreted as an indication of insignificant bioadsorption.[24]

TABLE 13. CONVERSION OF 3,5-DINA TO 3,5-DIAMINONITROBENZENE BY ENRICHMENT MICROORGANISMS

Time (hr)	0	27	96	144	196
Flask 3 (18 μg/mL 3,5-DINA + 50X GYE)					
3,5-DINA Consumed[a] (μg/mL)	0	12	18	18	18
3,5-diaminonitrobenzene formed (μg/mL)	ND[b]	ND	7.7	9.1	10.1
Mole percent converted	-	-	51.0	61.0	67.0
Flask 9 (129 μg/mL 3,5-DINA + 50X GYE)					
3,5-DINA consumed[a] (μg/mL)	0	109.0	129.0	129.0	129.0
3,5-diaminonitrobenzene formed (μg/mL)	ND	11.2	59.0	96.4	97.1
Mole percent converted	-	10.0	55.0	89.0	90.0

a. From Table 8.
b. Less than 2 μg/mL.

TABLE 14. BIOADSORPTION OF 1,3,5-TNB, 1,3-DNB, AND 3,5-DINA[a]

Test Compound	Initial Concentration (μg/mL)	Cell Level (mg/mL)[b]	Biosorption Coefficient[c]
TNB			
Viable cells	134	1.2	6.53 (±0.48)
Heat-killed cells	134	0.9	7.89 (±0.54)
DNB			
Viable cells	115	1.0	4.3 (±0.02)
Heat-killed cells	115	0.71	13.1 (±0.21)
DINA			
Viable cells	106	1.1	1.82 (±0.1)
Heat-killed cells	106	0.8	7.7 (±2.5)

a. Triplicate determinations.
b. Dry weight cell pellets.
c. $\frac{\mu g \text{ adsorbed/gram cell}}{\mu g/mL \text{ supernatant}}$.

DISCUSSION

Previous microbiological studies with 1,3-DNB have led to the conclusion that the compound should resist biodegradation.[9] However, the incubation of the compound with Tennessee River water in the present study has resulted in its disappearance after 15 days, a result not seen in sterile controls. The microbially mediated disappearance of the compound took place following a lag of approximately 10 days, and the nature of the disappearance was consistent with a first-order decline in its concentration after microbial acclimation. Since under the conditions employed biomass (B) remained constant, a second-order rate constant (k) of 3.7×10^{-8} mL $cell^{-1}$ hr^{-1} can be calculated from the pseudo first-order rate constant (kB) as previously described.[19] Based on the relationship $T_{1/2} = \ln 2/kB$ and the calculated second-order rate constant, the half-life ($T_{1/2}$) of 1,3-DNB at an assumed level of 10^6 cells/mL would be approximately 1 day at 25°C following acclimation.

The microbially mediated disappearance of 1,3-DNB from Tennessee River samples after 15-days incubation was reproducible for two sampling periods almost a year apart. However, the application of the identical screening method to other water sources indicated that the result could not be generalized to all sites. A summary of the results obtained from screening various water sources for 1,3-DNB degradation throughout the course of the project is presented in Table 15. As the summary indicates, 1,3-DNB was reproducibly degraded in Tennessee River samples (with or without sediments) and in a Carroll Creek sample (with sediments). Degradation was not detected in water from other sources tested, including samples taken 5 miles downstream of the Carroll Creek site showing degradation. Thus, not only can the capability to degrade 1,3-DNB by microorganisms vary with the water source, it can also vary from site to site within the same source. The reproducible degradation of 1,3-DNB in Tennessee River samples could be explained by the adaptation of microorganisms in the vicinity of the Volunteer Army Ammunition Plant to discharges containing the compound.

Primary screening for the biodegradation of 1,3,5-TNB and 3,5-DINA was conducted with Tennessee River water taken at the same time and place as that used for 1,3-DNB screening. Unlike the disappearance of 1,3-DNB, only partial decreases in the concentrations of 1,3,5-TNB and 3,5-DINA were seen to occur (Table 1). Although slight decreases were noted in the concentration of 1,3,5-TNB in samples from which sediments were removed, maximum degradation of 1,3,5-TNB required the presence of sediments, and no decrease in the concentration of 3,5-DINA was detected unless sediments were present. The patterns of partial decrease for 1,3,5-TNB and 3,5-DINA in the unconcentrated samples of Table 1 were identical for sampling periods nearly a year apart.

Even for water samples in which microorganisms and sediments were first concentrated threefold by centrifugation, only partial decreases of 24 and 52 percent were seen for 1,3,5-TNB and 3,5-DINA following incubation. Not only did the decreases observed for 1,3,5-TNB and 3,5-DINA differ from 1,3-DNB, but from the data of Figure 2, it can be seen that the nature of their decreases differed one from the other. The reduction in the concentration of 1,3,5-TNB occurred without an appreciable lag and proceeded for approximately 1 week, whereas that for 3,5-DINA occurred following a 12-day lag and proceeded until the 18th day. Continued incubation of similar samples for as long as 6 weeks

TABLE 15. SUMMARY OF 1,3-DNB DEGRADATION IN VARIOUS SOURCE WATERS

Date	Site	DNB Concentration (μg/mL)	% Reduction (method)	Time (days)
24 Oct 79	Carroll Creek, Montevue Lane			
	-Sediments	10	None (UV, GC)	29
	+Sediments	10	100 (UV, GC)	9-13
5 Dec 79	Tennessee River Volunteer AAP			
	-Sediments	10	100 (UV, GC)	12-15
	+Sediments	10	100 (UV, GC)	12-15
18 Nov 80	Tennessee River Volunteer AAP			
	-Sediments	5	100 (GC)	12-14
	+Sediments	16	>94 (GC) (26% CO_2 Evolution)	<16
1 Jun 80	Monocacy River Biggs Ford Road			
	-Sediments	50	None (UV)	30
16 Jul 81	Carroll Creek Montevue Lane			
	-Sediments	10	None (UV)	28
	+Sediments	10	>90 (UV)	10-12
16 Jul 81	Carroll Creek Wisner Street			
	-Sediments	10	None (UV)	28
	+Sediments	10	None (UV)	28
16 Jul 81	Monocacy River Biggs Ford Road			
	-Sediments	10	None (UV)	28
	+Sediments	10	None (UV)	28
16 Jul 81	Monocacy River Jug Bridge			
	-Sediments	10	None (UV)	28
	+Sediments	10	None (UV)	28

resulted in no subsequent reductions in the concentrations of the test compounds.

It would be expected from the data of Figure 2 (panel A) that the microbially mediated removal of 1,3,5-TNB from environmental waters would be extremely slow. Based on those data, the second-order rate constant for the disappearance of the compound is 1.7×10^{-10} mL $cell^{-1}$ hr^{-1}. Thus, at bacterial levels of 10^6 cells/mL, the half-life of the compound would be estimated to be greater than 170 days. Since in sediment-free waters the rate would be expected to be even slower, it appears unlikely that microorganisms would play a key role in the clearance of 1,3,5-TNB from environmental waters. However, the interactions between microorganisms and 1,3,5-TNB were significant; in all environmental samples tested, at least some of the test compound was transformed to 3,5-DINA (Table 12).

The microbially mediated removal of 3,5-DINA in the concentrated water samples of Figure 2 (panel B) appears to be more complex than that for 1,3,5-TNB. Since microbial levels decreased during the period of change, it cannot be considered pseudo first-order with respect to the microbial population. Because the decrease in 3,5-DINA either does not occur or is undetectable in sediment-free waters, it is unlikely that it would be removed from Tennessee River water microbiologically, although some interactions to reduce the concentration of the compound could take place at sites where sediment levels were high (i.e. river bottom-river interface).

Laboratory studies conducted with the mixed culture developed from 1,3-DNB primary screening indicated that the compound would serve as a sole carbon source for growth (Fig. 4). Mineralization studies conducted with the culture and 1,3-DNB showed that most of the compound was converted to carbon dioxide; therefore, most of the 1,3-DNB molecule is suceptible to microbial metabolism. To achieve 100 percent mineralization a compound must first be converted to carbon dioxide and biomass, and the biomass must be completely degraded to carbon dioxide; the test system employed could not be used to assess the ultimate mineralization of every carbon atom in the 1,3-DNB molecule.[17] Nevertheless, in view of the fact that most of the compound was mineralized with concomitant increases in biomass, it is likely that all portions of the molecule are susceptible to microbial metabolism.

Results of laboratory studies with the mixed culture were consistent with primary enrichments showing the complete or near complete disappearance of 1,3-DNB. However, kinetic studies conducted with high levels of the mixed culture indicated that the second-order rate constant for 1,3-DNB uptake was 2.99×10^{-9} mL $cell^{-1}$ hr^{-1} ($1\sigma = 8.34 \times 10^{-10}$) based on five separate determinations. From the rate constant, a half-life of 9.7 (±2) days can be estimated for 1,3-DNB in the culture at $25^{o}C$ and 10^6 cells/mL. The more rapid rate of 1,3-DNB removal in the primary screening culture (half-life approximately 1 day) could result from enhanced biodegradation by microorganisms in a more natural metabolic state. Natural carbon or nitrogen sources or cometabolites[25] could be present in the environmental samples which were not duplicated in enrichment culture medium. Alternatively, the enrichment process could have resulted in an imbalance of the original biodegrading population such that more rapid biodegraders, or microbial species contributing growth factors to biodegraders, may not have been selected. Regardless of the nature

of the differences between primary and enrichment cultures, both methods agree that the half-life of 1,3-DNB at 10^6 cells/mL is less than 10 days at 25°C.

Unlike 1,3-DNB, utilization of 1,3,5-TNB and 3,5-DINA as sole carbon sources did not occur when media were inoculated with the corresponding primary screening microorganisms. However, with the incorporation of usable nutrients in the form of glucose-yeast extract into their medium, the microorganisms demonstrated the metabolic potential to interact with the test compounds and to decrease their concentrations (Tables 4, 5, 7, and 8). Either more biomass or greater metabolic activity was necessary for 3,5-DINA than for 1,3,5-TNB since higher levels of the added nutrients were necessary in 3,5-DINA cultures to effect roughly equivalent decreases. Despite the continued passage of the enrichment microorganisms for many weeks in the presence of the test compounds and glucose-yeast extract, the removal of the nutrients from the cultures resulted in the loss of the capability to metabolize the compounds. Therefore, even with microorganisms nonspecifically enriched on glucose-yeast extract but capable of metabolizing 1,3,5-TNB and 3,5-DINA, exposure to the compounds over a prolonged period of time did not result in cultures capable of using them as sole carbon sources for growth.

Moreover, the decreases of 1,3,5-TNB and 3,5-DINA in the presence of glucose-yeast extract-grown microorganisms cannot be equated with ultimate biodegradation. Mineralization studies conducted with isotopically labeled test compounds and glucose-yeast extract cultures resulted in the conversion of the nutrients, but not the test compounds, to carbon dioxide (Tables 6 and 10). Since in the same experiments, more than 80 and 90 percent, respectively, of labeled 1,3,5-TNB and 3,5-DINA were metabolized to noncell associated products, the interaction of nonspecifically enriched microorganisms results in the transformation rather than biodegradation of the compounds.

The recovery of nitro-reduction products from the cultures is consistent with the interpretation that the compounds are transformed. The production of 3,5-DINA from 1,3,5-TNB is not surprising (Table 11). Previous studies have indicated that 1,3,5-TNB undergoes nitro-reduction readily in the presence of cell-free extracts of Veillonella alkalescens and a strain of Nocardia.[14,15] However, since less than half of the 1,3,5-TNB metabolized in the current study is converted to 3,5-DINA (Table 11), the transformation must also result in other products. The fact that the 3,5-DINA did not accumulate during continued incubation (Table 11) could be interpreted as evidence for its further transformation at low concentrations; alternatively, sufficient metabolic potential for the transformation of slight amounts of 1,3,5-TNB to 3,5-DINA may only exist in the culture during earlier phases of the incubation period. Although it is not presumed that glucose-yeast extract grown enrichment populations duplicate those in environmental samples, results have indicated that the transformation of 1,3,5-TNB to 3,5-DINA has taken place in all primary screening cultures tested (Fig. 6, Table 12).

The formation of 3,5-diaminonitrobenzene from 3,5-DINA by nonspecifically enriched microorganisms (Table 13) is consistent with the view that 3,5-DINA is also transformed. Unlike the formation of 3,5-DINA from 1,3,5-TNB, 3,5-diaminonitrobenzene accounted for most of the 3,5-DINA transformed by enrichment microorganisms. However, the presence of the minor products noted during the transformation may be indicative of metabolic intermediates, suggesting that more than just nitro-reduction is taking place in nonspecifically

enriched cells. Attempts to demonstrate the presence of 3,5-diaminonitrobenzene following the incubation of Tennessee River water samples with 3,5-DiNA were unsuccessful despite 33 and 52 percent decreases in the concentration of the compound (Table 1). These results could be explained by the formation of other products in the primary screening cultures, or by the instability of 3,5-diaminonitrobenzene in the extracted environmental water samples.

Taken together, the results of primary screening and enrichment culture experiments with 1,3,5-TNB and 3,5-DiNA suggest that the compounds are resistant to biodegradation by Tennessee river microorganisms with the metabolic capability to interact with them. Such an interpretation is consistent with the fact that the incubation of the two more highly substituted nitro compounds with the same Tennessee River sample that completely degraded 1,3-DNB (Fig. 1), led only to their partial and unsustained decreases in concentration (Fig. 2). Results for 1,3,5-TNB are, therefore, consistent with the previous conclusion that it should resist biodegradation,[9] and the same conclusion appears to apply to 3,5-DiNA.

SUMMARY AND CONCLUSIONS

1. The microbially mediated degradation of 1,3-dinitrobenzene was complete or nearly complete in Tennessee River water at 25°C.

2. Results of microbial degradation seen with Tennessee River water and 1,3-dinitrobenzene could not be generalized to all environmental water sources.

3. Enrichment microorganisms from the Tennessee River utilized 1,3-dinitrobenzene as a carbon source for growth and could mineralize the compound. The compound is therefore biodegradable.

4. The half-life of 1,3-dinitrobenzene in the presence of Tennessee River water samples, or in enrichment cultures developed from those samples, was less than 10 days at 25°C and 10^6 CFU/mL.

5. Differences between microbial rate constants for 1,3-dinitrobenzene degradation obtained directly from environmental samples or from enrichment cultures may reflect fundamental microbiological differences in two approaches used to obtain those rate constants.

6. Screening of Tennessee River water samples for the microbial degradation of 1,3,5-trinitrobenzene or 3,5-dinitroaniline resulted in only partial and unsustained decreases in their concentrations.

7. The transformation of 1,3,5-trinitrobenzene to 3,5-dinitroaniline took place during screening of Tennessee River water samples for the biodegradation of the former compound.

8. Neither 1,3,5-trinitrobenzene nor 3,5-dinitroaniline served as carbon sources for growth of microorganisms with the metabolic potential to decrease the concentrations of the compounds in primary screening or enrichment cultures.

9. Microbially mediated decreases in the concentrations of 1,3,5-trinitrobenzene and 3,5-dinitroaniline in the presence of exogenous nutrients resulted in the transformation rather than ultimate biodegradation of the compounds.

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