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Biodegradation of *N*-Nitrosodimethylamine in Aqueous and Soil Systems

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N-Nitrosodimethylamine (NDMA) was mineralized by microorganisms in aqueous and soil systems. Initial rates of mineralization (micrograms per milliliter per day) were calculated for a wide range of initial concentrations of NDMA (micrograms per milliliter to picograms per milliliter). Log-log plots of the data were fitted with both linear and nonlinear least-squares analyses; however, linear models provided better fits for the kinetic data in all cases. The slopes of the linear fits were not significantly different than 1.0 ($P < 0.05$); thus, first-order reaction kinetics were in effect over the range of concentrations tested, and saturation kinetics were not achieved. Rate constants (day^{-1}) and total percent mineralized increased with decreasing initial concentrations of NDMA. Rates of mineralization were reduced in aqueous systems when supplemental carbon was available, whereas in soils, percentages of organic matter and supplemental carbon had little effect on rates of mineralization. Implications of these results for predictions of rates and threshold limits of mineralization activity in natural systems are discussed. A laboratory scale simulated trickling filter containing an activated charcoal bed provided a suitable environment for mineralization of NDMA at concentrations of 50 and 100 μg/ml on a continuous basis. NDMA was not toxic to natural populations of microorganisms at concentrations up to 10 mg/ml. Using high-pressure liquid chromatography coupled with radioactivity detection, we identified formaldehyde and methylamine as intermediates produced during the biodegradation of NDMA.

N-Nitrosodimethylamine (NDMA) has been identified as an undesirable contaminant in foods, alcoholic beverages, cosmetics, pesticides, and in a variety of other industrial and environmental settings. NDMA is toxic, a potent carcinogen, very soluble in water, and relatively stable.

The metabolism of NDMA by mammalian cells has been documented, with nitrogen gas, formaldehyde, formic acid, methylamine, *N*-methylhydrazine, *N*-methylhydroxylamine, and *N,N*-dimethylhydrazine identified as intermediates in various in vitro studies (4, 7, 9, 10, 12).

Microbial metabolism of NDMA in aqueous and soil systems has not been clearly established. Reports have demonstrated a wide range of findings from rapid rates of degradation to relatively slow or no degradation, depending on study conditions (3, 5, 6, 11, 14, 15, 17).

Wastewater contamination with NDMA has been reported (8). NDMA was detected in the microgram per milliliter range in wastewater from an ammonia recovery distillation column used during the manufacture of hexahydro-1,3,5-trinitro-1,3,5-triazine.

It is the purpose of this work to elucidate the capabilities of microorganisms to mineralize NDMA in aqueous and soil systems under a variety of culture conditions. Microbial intermediates will be identified. This information will provide insight into the potential for development of biological treatment schemes to handle NDMA-laden wastewaters.

MATERIALS AND METHODS

Chemicals. *N*-[*methyl*-^{14}C]NDMA (lot 1337-123; 47 mCi/mmol; >99% pure), formaldehyde (^{14}C-labeled; lot 1757-071; 10 mCi/mmol; 1% aqueous solution; approximately 95% pure), *N*-[*methyl*-^{14}C]dimethylamine hydrochloride (lot 1625-093; 35.9 mCi/mmol; in methanol; 99% pure), and *N*-[*methyl*-^{14}C]methylamine hydrochloride (lot 1397-208; 46 mCi/mmol; in ethanol; 99% pure) were purchased from New England Nuclear Corp., Boston, Mass. Unlabeled NDMA (>99% pure) was purchased from Aldrich Chemical Co., Inc., Milwaukee, Wis.

High-pressure liquid chromatography. Quantitative analysis of NDMA was performed on a Waters Associates, Inc., (Milford, Mass.) high-pressure liquid chromatograph (HPLC) equipped with two model 6000A solvent pumps, a model 441 wavelength absorbance detector set at 254 nm, a model 730 data module, and a model 721 system controller. Separations were accomplished on a C-18 reverse-phase stainless steel column (3.9 by 30 cm) and a C-8 or C-18 10-μm radial-pack cartridge used with an RCM-100 radial compression module. The mobile phase was distilled, deionized water flowing at 2.5 ml/min. Retention time was 3.2 min in the stainless steel column, injection volumes were varied up to 200 μl, and full-scale absorbance units were varied down to 0.005. To identify potential intermediates produced from NDMA, we coupled a radioactivity detector (FLO-ONE HS; Radiomatic Instruments Chemical Co., Tampa, Fla.) to the HPLC system. The use of ^{14}C-labeled NDMA results in the formation of ^{14}C-labeled intermediates detectable with this detector. Unlike NDMA, most of these intermediates are not detectable by UV absorbance. The correlation of retention times with those of the ^{14}C-labeled standard provided our initial indication of the identities of intermediates. The radioactivity detector was fitted with a 2.5-ml cell and received a total flow of 5.0 ml/min (1.0 ml from the HPLC and 4.0 ml of Flo Scint III scintillation cocktail; Radiomatic). Peaks on the radioactivity chromatograms eluted approximately 1.3 min after the corresponding peaks on the HPLC UV detector in cases, such as that of [^{14}C]NDMA, in which the compound was detectable on both detectors. Background was in the range of 45 to

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55 dpm, and counting efficiency for the radioactivity detector was between 75 and 85%.

Thin-layer chromatography. Hydrazine and substituted hydrazines (1,1-dimethylhydrazine and methylhydrazine) were detected on plastic-backed cellulose plates without fluorescent indicator (Eastman Kodak Co., Rochester, N.Y.). The developing solvent system was ethanol-water-hydrochloric acid (130:40:30). The hydrazines were visualized with a Folin-Ciocalteu reagent and then exposed to ammonia fumes to develop the blue color. Samples from batch studies with and without hydrochloric acid were cochromatographed along with the standards as hydrochloride salts. Acidified fractions were also concentrated 100-fold with a rotary evaporator at 60°C and then chromatographed.

To detect low concentrations of the hydrazines, we chromatographed samples from batch studies initiated with [^{14}C]NDMA along with the unlabeled hydrazine standard. The chromatograms of the unknowns, potentially containing ^{14}C-labeled intermediates, were scraped off the plastic backing in 22 equal linear increments and counted for radioactivity in a Tri-Carb model 3255 liquid scintillation counter (Packard Instrument Co., Inc., Rockville, Md.). The R_fs of the hydrazine standards were then correlated with the R_fs of peaks of radioactivity.

Mass spectrometry. Mass spectrometry was performed on a TAGA 6000 triple-stage quadrupole mass spectrometer (SCIEX, Thornhill, Ontario, Canada). Analysis of NDMA batch cultures was accomplished directly from filtered (0.45-μm [pore size] membrane filters) aqueous samples. Standards (NDMA, methylamine, dimethylamine, and formaldehyde) were also analyzed. The equipment is well suited to the analysis of very low concentrations of volatile compounds in aqueous solutions. The results from these analyses provided confirmation for preliminary identifications of intermediates determined by HPLC-radioactivity.

Batch studies. A series of batch studies in aqueous and soil systems with ^{14}C-labeled NDMA were initiated to evaluate the kinetics of mineralization under a variety of incubation conditions. The vessels were either 250- or 500-ml modified Erlenmeyer flasks with a 24/40 ground glass adapter containing two glass tubes modified to hold sodium hydroxide and hydrochloric acid traps, each containing 1.0 ml of a 1.0 N solution. The stationary flasks were kept at room temperature and covered with foil.

Trapping solutions were dissolved in Aquasol 2 scintillation cocktail (New England Nuclear), and radioactivity was assayed in a Packard model 3255 Tri-Carb liquid scintillation counter. Each vial was corrected for quench with an external standard, and most samples were counted for 20 min.

Basal salts consisted of the following amounts per liter: $NH_4H_2PO_4$, 2.0 g; K_2HPO_4, 1.0 g; KH_2PO_4, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaCl_2$, 0.1 g; and NaCl, 0.1 g. The basal salts were used at a concentration of 4.4 g/liter. $NH_4H_2PO_4$ was omitted for certain incubations. Mineral salts for the growth of fungi consisted of the following ingredients per liter: $NH_4H_2PO_4$, 1.0 g; KH_2PO_4, 0.6 g; K_2HPO_4, 0.4 g; Na_2HPO_4, 0.3 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaCl_2$, 0.7 g; $FeSO_4 \cdot 7H_2O$, 0.012 g; $ZnSO_4 \cdot 7H_2O$, 6.6 mg; $MnSO_4 \cdot H_2O$, 5.0 mg; $CoCl_2 \cdot H_2O$, 1.0 g; $CuSO_4 \cdot 5H_2O$, 1.0 mg; and thiamine hydrochloride, 0.1 mg. These salts were used at a concentration of 3.226 g/liter.

We added ^{14}C-labeled NDMA to batch study flasks along with unlabeled NDMA to provide the range of concentrations and activity required. For aqueous studies, between 5.00×10^{-6} and 1.56×10^{-3} μCi/ml was added to the incubation media, and for soil incubations the range was 3.80×10^{-4} to 3.24×10^{-3} μCi/g (based on oven dry weight of soil).

Lake water was collected periodically from Lake Cochituate, Natick, Mass., and passed through filter paper before use. In general, the lake water had a pH between 6 and 7 and a total organic carbon content below 25 ppm (25 μg/ml). The soil used in these experiments was topsoil purchased from a local garden shop and passed through a 3.35-mm (pore size) no. 6 sieve. The soil had a pH of 6.4 and was supplemented with equal parts of sand (sea sand, washed and ignited [pH 7.9]; Fisher Scientific Co., Pittsburgh, Pa.) and clay (bentonite; Sigma Chemical Co., St. Louis, Mo.) as required for organic matter adjustments. Ashing of soil, sediment, or sand samples was performed by incineration at 550°C overnight. Granular activated cocoanut charcoal, 6/14 mesh, was purchased from Fisher Scientific. For sterile soil, sand, or sediment systems, flasks were autoclaved for 30- or 45-min runs on 3 consecutive days.

Batch and continuous cultures were inoculated with organisms from activated sludge (Marlborough Easterly sewage treatment plant, Marlborough, Mass.), anaerobic sludge digest (Nut Island sewage treatment plant, Boston, Mass.), and garden soil. Samples (1 ml) of the two sludges were combined with 1 g of soil, diluted with 50 ml of lake water, mixed, and gravity filtered through filter paper, and 100 μl was used for inoculum in batch cultures and 1 ml was used in continuous cultures. This inoculum contained between 2.3×10^3 and 3.5×10^3 CFU/ml.

Trickling filters. Laboratory scale simulated trickling filters were constructed of glass cylinders (45.4 by 7.5 cm in diameter) containing 114.5 g of granular activated coconut charcoal (6/14 mesh) as the stationary bed. The beds were supported by polyethylene screening (925-μm mesh). Nutrient solution, delivered dropwise to the top of the columns via a Rainin Rabbit peristaltic pump, consisted of the following per liter: $NH_4H_2PO_4$, 2 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaCl_2$, 0.1 g; NaCl, 0.1 g; and NDMA, 100 or 50 mg. The sterile control column also received 1.0% mercuric chloride in the nutrient feed. After 300 days, both the active and sterile columns received a one-time dose of [^{14}C]NDMA (2.36 μCi each). During the next 203 days, the total radioactivity recovered in column effluents, as well as in sodium hydroxide traps (10 ml of a 1 N solution), was determined. Complete recovery of $^{14}CO_2$ in the alkaline traps from the effluent gases was not possible with the system design already in place. However, despite this limitation, partial recovery of $^{14}CO_2$ provided a qualitative evaluation of the sterile and active systems.

Samples of microbial slime growing on the active charcoal column were removed, and attempts were made to isolate active NDMA-degrading colonies. Standard microbiological techniques were used to isolate pure cultures and were followed by incubation with [^{14}C]NDMA. Base traps were used to trap $^{14}CO_2$ as a measure of activity. Incubations were either directly on agar petri plates or in liquid batch culture. Growth media included the same influent medium used for the trickling filters, but with 1.5% granulated agar (Difco). The NDMA, usually 100 μg/ml, was added in agar overlay medium. Growth was usually very sparse on the trickling filter media. All isolates were transferred to assure viability on the defined media on which they were originally grown.

Samples for HPLC analysis were centrifuged at 12,000 rpm for 10 min and then passed through a Swinny stainless steel syringe-type filter with a 13-mm diameter, 0.45 μm (pore size) membrane filter.

Cell viability. Concentrations of NDMA of 1 ng and 1 and

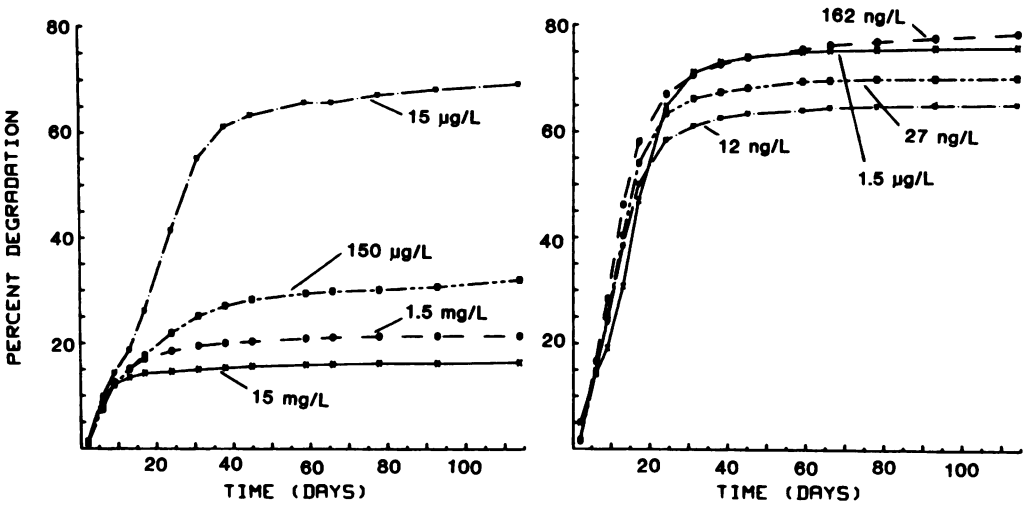

FIG. 1. Mineralization of NDMA at various concentrations in lake water supplemented with basal salts.

1,000 µg/ml in filtered lake water with natural (field) populations of microorganisms were incubated for 24 h. Controls with NDMA were run concurrently. Samples (100 ml) were withdrawn initially and at 1 and 24 h, run in a dilution series, and then spread on both nutrient agar (Difco) and filtered lake water supplemented with glucose (1 ppb [1 ng/ml]) and 1.5% granulated agar (Difco). Each plate was replicated twice.

Sorption on charcoal. We evaluated granular activated coconut charcoal (6/14 mesh; Fisher) with NDMA to determine sorption capacity. Solutions of NDMA (5, 50, 500, 5,000, and 10,000 µg/ml, each in 1 liter of distilled, deionized water) were tested with and without (control) charcoal (10 g). Samples from these solutions were withdrawn periodically, filtered through a 0.45-µm (pore size) membrane filter, and analyzed by HPLC.

RESULTS

Batch studies—aqueous. To determine the relationship between initial rate of mineralization (micrograms per milliliter per day) and concentration of NDMA, we analyzed kinetic data based on the Michaelis-Menten enzyme kinetics model. Initial rates of mineralization were determined for the apparent linear portions of the ^{14}C recovery curves over a range of concentrations of NDMA in batch studies. Log-log plots of the initial rates versus initial concentration of NDMA were used to fit both linear and nonlinear least-squares models to the data, and a comparison of the estimated standard errors for these models was used to determine best fit; in all cases the linear model provided a better fit for the data. Since the slope of the linear model represents the order of the reaction, and in a first-order kinetic model the rate of substrate utilization is proportional to the first power of the substrate concentration, the log-log plot should give a slope equal to one. This would reflect substrate concentrations well below K_m, and first-order rate constants can be calculated. However, if saturation kinetics were found, then the slope of the log-log linear model would be significantly less than 1.0. In these studies, in all cases, reaction rates (apparent initial rates of mineralization) were first order with respect to substrate concentration over the range studied.

In general, the percent mineralized (total percent of initial ^{14}C recovered as $^{14}CO_2$) increased with decreasing initial concentrations of NDMA (Table 1), with the exception of nutrient broth, in which the percent mineralized was reduced at all comparable concentrations. At concentrations in the low microgram-per-liter range and below, there was a significant increase (t test, $P < 0.05$) in percent mineralized both in lake water with salts (71.9 ± 5.3 versus 23.3 ± 8.0%) and lake water with salts and glucose (57.4 ± 8.0 versus 24.7 ± 6.5%).

No lag phase was evident in lake water with salts and salts

TABLE 1. Mineralization of NDMA during 114 days

Initial concn (µg/ml)	NDMA mineralized in:					
	Lake water with salts		Lake water with salts and glucose		Lake water with nutrient broth	
	%	$\bar{x}$ ± SD	%	$\bar{x}$ ± SD	%	$\bar{x}$ ± SD
1.50×10^{1}	16.4	23.3 ± 8.0	18.4	24.7 ± 6.5		
1.50×10^{0}	21.4		24.3			
1.50×10^{-1}	32.0		31.4			
1.50×10^{-2}	69.0	71.9 ± 5.3	48.0	57.4 ± 8.0	27.1	24.4 ± 7.1
1.51×10^{-3}	76.1		67.2		19.1	
1.62×10^{-4}	78.6		55.4		35.2	
2.72×10^{-5}	70.5		58.8		17.4	
1.22×10^{-5}	65.5		90.7[a]		23.0	

[a] Excluded from calculation of $\bar{x}$.

TABLE 2. Initial rates of mineralization (micrograms per milliliter per day) during the apparent linear portions of the $^{14}CO_2$ recovery curves (10 days)

Initial concn (µg/ml)	Lake water with salts	Lake water with salts and glucose	Lake water with nutrient broth
1.50×10^{1}	1.70×10^{-1}	1.10×10^{-1}	
1.50×10^{0}	2.00×10^{-2}	1.00×10^{-2}	
1.50×10^{-1}	1.93×10^{-3}	1.71×10^{-3}	
1.50×10^{-2}	1.38×10^{-4}	1.81×10^{-4}	2.93×10^{-5}
1.51×10^{-3}	3.46×10^{-5}	2.16×10^{-5}	8.38×10^{-7}
1.62×10^{-4}	6.55×10^{-6}	3.92×10^{-6}	3.65×10^{-7}
2.72×10^{-5}	9.59×10^{-7}	6.25×10^{-7}	1.77×10^{-8}
1.22×10^{-5}	4.02×10^{-7}	3.88×10^{-7}	1.66×10^{-8}

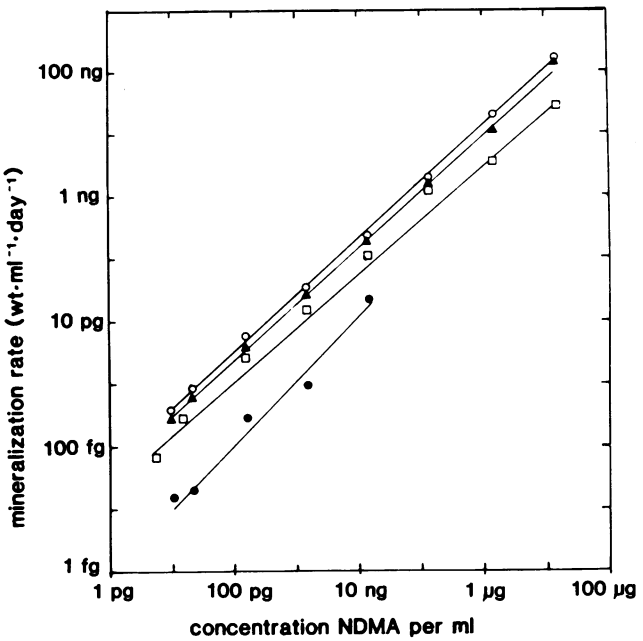

FIG. 2. Initial rates of mineralization over a range of concentrations of NDMA in aqueous batch culture. Symbols: ○, lake water with salts; ●, nutrient broth; □, lake water; ▲, lake water with salts and glucose.

with glucose media (Fig. 1). Initial rates of mineralization were calculated from the 0- to 10-day period of the $^{14}CO_2$ recovery curves (Table 2), and all correlation coefficients were between 0.95 and 0.99. These initial rates of mineralization were plotted (log-log) against the initial concentration of NDMA, and the linear model fitted to the data produced slopes of 0.907, 0.894, and 1.038 for lake water with salts, lake water with salts and glucose, and nutrient broth, respectively (Fig. 2). The corresponding estimated standard errors were 0.081, 0.068, and 0.314. At the 95% confidence level, these values were not significantly different than 1.0, indicating validity for a first-order fit and a lack of saturation kinetics at the concentrations evaluated. In addition, nonlinear regression least-squares analysis for the same data produced higher estimated standard errors than the first-order linear model, indicating a poorer fit. Rate constants (day^{-1}) increased significantly (t test, $P < 0.05$) as the initial concentration of NDMA decreased (Table 3). There was a 3.7-fold increase between the highest and lowest concentration groupings in lake water with salts and glucose and a corresponding 2.7-fold change in lake water with salts. The rate constants were higher for lake water with salts than for lake water with glucose at all comparable concentrations; however, in nutrient broth they were reduced 1 to 2 orders of magnitude. In general, supplemental carbon was utilized preferentially to NDMA, and inhibition of NDMA-degrading activity in the batch systems occurred subsequent to this initial degradation, thus prohibiting utilization of NDMA after the initial supplemental carbon was exhausted.

A separate experiment, run over 89 days in lake water with concentrations of NDMA from 6.78 pg to 30 µg/ml, was performed to further evaluate the effect of NDMA concentration on mineralization kinetics. As before, the percent mineralized increased with decreasing initial concentration (Table 4). All correlation coefficients for the linear regressions fitted for the apparent initial rates of mineralization (first 20 days) were above 0.95. The log-log plot of initial rates versus concentration was fitted with a linear model, and the slope was not significantly different than 1.0 ($P < 0.05$) (Fig. 2). The linear model provided a better fit than the nonlinear model fitted to the same data based on comparisons of estimated standard errors. Rate constants (day^{-1}) increased as the initial concentration of NDMA decreased, with an 8.4-fold increase from the lowest grouping of concentrations to the highest (Table 4; t test, $P < 0.05$).

In other aqueous batch studies conducted with 10 ng of NDMA per ml, supplemental carbon was further evaluated for its role in mineralization kinetics. Glucose additions of 0, 0.1, 0.5, 1.0, 5.0, and 50.0 µg/ml in lake water were evaluated. As the initial concentration of glucose increased, the percent mineralized decreased from an upper range of 67.1 to 70.2% with 0.0 and 0.1 µg of glucose per ml, respectively, to a low of 14.7% at 50.0 µg of glucose per ml. The apparent initial rates of mineralization (micrograms per milliliter per day) were highest for unsupplemented lake water (6.71×10^{-4}), 4.7-fold less for the 0.1- to 5.0-µg of glucose per ml grouping, and 4.42×10^{-5} for 50 µg of glucose per ml. Rate constants (day^{-1}) followed a similar pattern, ranging from a high of 5.59×10^{-2} in incubations without glucose to a low of 3.64×10^{-3} with 50 µg of glucose per ml. All indicators of mineralization activity decreased as the concentration of glucose was increased, again reflecting preferential utilization of supplemental carbon.

In a separate study with 10 ng of NDMA per ml in lake water supplemented with salts and glucose (1.0 g/liter) or nutrient broth (4.0 g/liter), a chemical reducing agent, 0.025% sodium sulfide, was added to establish anaerobic conditions. The supplemental carbon was preferentially uti-

TABLE 3. Rate constants (day^{-1}) for mineralization of NDMA

Initial concn (µg/ml)	NDMA mineralized (day^{-1}) in:					
	Lake water with salts		Lake water with salts and glucose		Lake water with nutrient broth	
	Rate constant	$\bar{x}$ ± SD[a]	Rate constant	$\bar{x}$ ± SD[a]	Rate constant	$\bar{x}$ ± SD
1.50×10^{1}	0.0113	0.0133 ± 0.0019	0.0073	0.0070 ± 0.0004		
1.50×10^{0}	0.0133		0.0067			
1.50×10^{-1}	0.0129		0.0114	0.0126 ± 0.0051		
1.50×10^{-2}	0.0159		0.0121		2.00×10^{-3}	$1.40 \times 10^{-3} \pm 7.60 \times 10^{-4}$
1.50×10^{-3}	0.0229	0.0362 ± 0.0037	0.0143		6.00×10^{-4}	
1.62×10^{-4}	0.0404		0.0242	0.0263 ± 0.0047	2.30×10^{-3}	
2.72×10^{-5}	0.0353		0.0230		7.00×10^{-4}	
1.22×10^{-5}	0.0330		0.0318		1.40×10^{-3}	

[a] $\bar{x}$ ± Standard deviation for bracket grouping.

TABLE 4. Mineralization of NDMA in lake water

Initial concn (μg/ml)	Extent of mineralization after 89 days		Initial rate of mineralization (μg/ml per day) during 20 days	Rate constant	$\bar{x}$ ± SD
	%	$\bar{x}$ ± SD			
3.00×10^{1}	2.8	3.2 ± 0.5	3.00×10^{-2}	1.00×10^{-3}	$1.09 \times 10^{-3} \pm 1.34 \times 10^{-4}$
3.00×10^{0}	3.5		3.59×10^{-3}	1.19×10^{-3}	
3.00×10^{-1}	24.8	23.5 ± 5.9	2.13×10^{-3}	7.10×10^{-3}	$5.35 \times 10^{-3} \pm 1.69 \times 10^{-3}$
3.00×10^{-2}	17.1		1.12×10^{-4}	3.73×10^{-3}	
3.00×10^{-3}	28.7		1.57×10^{-5}	5.22×10^{-3}	
3.07×10^{-4}	39.4	39.8 ± 1.3	3.20×10^{-6}	1.04×10^{-2}	$9.17 \times 10^{-3} \pm 1.18 \times 10^{-3}$
3.68×10^{-5}	41.2		2.96×10^{-7}	8.05×10^{-3}	
6.78×10^{-6}	38.7		6.15×10^{-8}	9.07×10^{-3}	

lized in all cases, while the presence of the reducing agent had no effect on this activity. NDMA was mineralized by microorganisms in lake water supplemented with salts at the highest rate, with or without sodium sulfide. When lake water was supplemented with glucose or nutrient broth, the percent mineralized and initial rates of mineralization (12 days) were greatly reduced. The percent mineralized during 90 days was 60 to 64% for basal salts with or without the reducing agent and only 10 to 14% with supplemental carbon with or without the reducing agent. Initial rates of mineralization were diminished 1 to 2 orders of magnitude with supplemental carbon present, with or without the reducing agent, in comparison with lake water with salts alone.

Residual radioactivity in the media after the 90 days of incubation correlated well with the mineralization results. In all cases there was about a 17 to 35% discrepancy in the total counts recovered (counts trapped plus residual radioactivity in the media). This difference was most likely due to the production of volatile gases, such as methane, not recovered by the trapping system, as well as volatilization of NDMA and intermediates, such as formaldehyde, from the systems. In sterile flasks in this same experiment, approximately 17 to 28% of the radioactivity was not recovered.

Batch studies—soil. The effect of concentration of NDMA on mineralization kinetics in soil was studied in batch incubations run for 98 days at concentrations ranging from 10 ng to 10 mg/g of soil (Table 5, Fig. 3). No lag phase was noted before the onset of initial mineralization activity (Fig. 4). The apparent initial rates of mineralization (micrograms per gram per day) were calculated for the first 7 days of incubation; correlation coefficients were all above 0.95. The percent mineralized and rate constants (day^{-1}) were highest for the two lowest concentrations evaluated (1 μg and 10 ng/g of soil). The slope of the linear fit for the log-log plot of initial rate of mineralization versus initial concentration was 0.857, which was not significantly different than 1.0 at the 95% confidence interval (the standard error of the estimate was 0.182). This first-order fit was better than the nonlinear fit from a statistical standpoint, as was found with the aqueous systems.

The effect of soil organic matter on NDMA mineralization kinetics was studied with 100 μg of NDMA per g of soil. At 35.0, 10.5, 5.3, and 2.6% organic matter, the percents mineralized over 119 days were 40.0, 36.0, 54.3, and 60.9%, respectively; the apparent initial rates of mineralization (30 days) were 9.60×10^{-4}, 1.42×10^{-3}, 1.41×10^{-3}, and 1.01×10^{-3}, respectively; and the rate constants (day^{-1}) were an order of magnitude higher than the apparent initial rates of mineralization. The percentage of organic matter did not have a significant effect on the mineralization kinetics of NDMA in soil.

The influence of soil moisture on the mineralization kinetics of NDMA was studied over 74 days with 100 μg of NDMA per g of soil. Almost all activity was inhibited in dry soil, whereas the percentage mineralized was similar at all other moisture levels (between 47.6 and 57.0% mineralized at 25, 50, 75, and 100% moisture levels). Apparent initial

TABLE 5. Mineralization of NDMA in soil

Initial concn (μg/g of soil)	Extent of mineralization (%) after 98 days	Initial rate of mineralization (μg/ml per day) during 7 days	Rate constant
10,000.00	28.6	1.64×10^{2}	1.64×10^{-2}
1,000.00	24.4	1.37×10^{1}	1.37×10^{-2}
100.00	29.0	1.36×10^{0}	1.36×10^{-2}
1.00	55.3	2.00×10^{-2}	2.00×10^{-2}
0.01	78.4	7.02×10^{-4}	7.02×10^{-2}

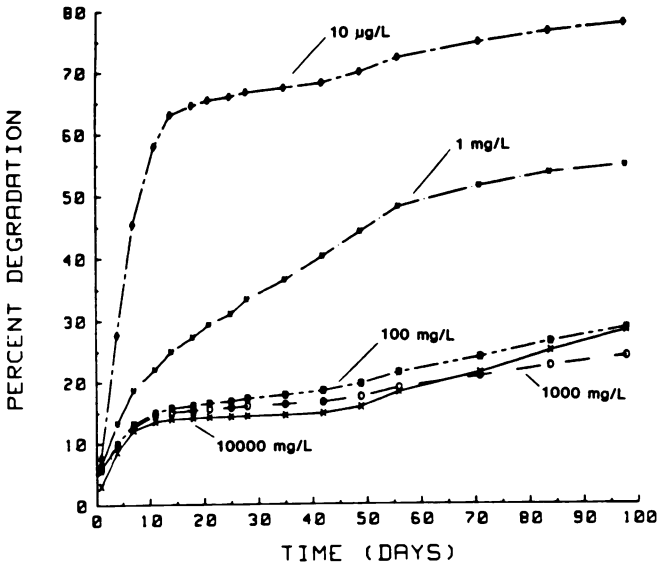

FIG. 3. Mineralization of NDMA at various concentrations in soil.

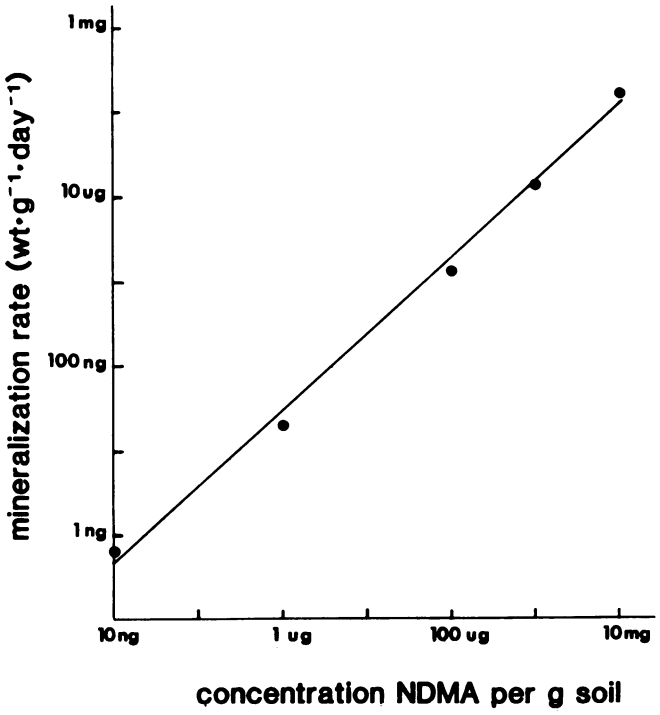

FIG. 4. Initial rates of mineralization over a range of concentrations of NDMA in soil batch culture.

rates of mineralization were higher at the 25% moisture level (1.0×10^{-2}) compared with the three highest moisture levels (between 3.0×10^{-3} and 4.0×10^{-3} for 50, 75, and 100% moisture levels).

In a separate experiment, supplemental salts, glucose, or cellulose was added to soil containing 1 μg of NDMA per g of soil. The mineralization kinetics in these different incubations were not significantly different as reflected by apparent initial rates of mineralization and rate constants.

Trickling filter. The trickling filter configuration provided a suitable environment for mineralization of NDMA. Figure 5 illustrates the results from the charcoal column run over 500 days. After reaching sorption equilibrium, NDMA passed through the sterile column unchanged, whereas on the active column almost all of the NDMA fed onto the column was removed. The addition of [^{14}C]NDMA to the columns provided conclusive evidence that NDMA was mineralized on the active column and not just volatilized or sorbed (Fig. 6). After 203 days, a total of 95.9% of the original [^{14}C]NDMA added to the sterile column had been recovered in the effluent, whereas only 2.7% of the total was recovered in the effluent from the active column. During the same time frame, 6.5% of the total added [^{14}C]NDMA was recovered as $^{14}CO_2$ in the alkaline traps from the active column (this figure represents a low estimate because complete collection of $^{14}CO_2$ from these systems was not possible) compared with 0.2% from the sterile column.

Figure 7 illustrates results obtained in an attempt to isolate NDMA-degrading microorganisms from the active charcoal column. During 20 days of incubation on plates containing either isolated colonies or the mixed population, $^{14}CO_2$ was recovered from [^{14}C]NDMA. The results showed that the various isolates had different capabilities toward mineralizing NDMA. In separate experiments, incubation of the mixed population isolated from the charcoal column in batch culture with the same influent medium as fed onto the columns (both with and without charcoal added to the medium) did not result in NDMA-mineralizing activity. Despite the use of isolates from the column, the incubation conditions could not be duplicated in aqueous batch studies to encourage the same activity that was found on the column. Furthermore, it was noted that the addition of charcoal to aqueous batch cultures almost completely inhibited all NDMA-degrading activity.

Cell viability. Total viable colony counts increased in both media after exposure to different concentrations of NDMA during 24 h. There was no indication of a toxic or inhibitory effect of NDMA at concentrations up to 1,000 μg/ml.

Thin-layer chromatography. The following R_f values for standards of hydrazine and hydrazine derivatives were obtained as a result of thin-layer chromatographic analysis: hydrazine, 0.33; 1,1-dimethylhydrazine, 0.76; and methylhydrazine, 0.58. NDMA had an R_f of 0.80 but did not produce blue color on visualization. None of the samples from batch culture systems (chromatographed direct, acidified, or concentrated) showed evidence of hydrazine intermediates. Investigations for radioactively labeled spots on chromatograms corresponding to the standards also did not produce evidence for these hydrazines.

HPLC. Initial evidence for the identity of intermediates was obtained from analyses performed on the coupled HPLC-radioactivity detector system. The retention times (minutes) of ^{14}C-labeled standards on the radioactivity detector were 3.9 to 4.7, 3.1 to 3.5, 5.5 to 5.7, and 2.3 to 2.9 for NDMA, methylamine, dimethylamine, and formaldehyde, respectively, whereas only NDMA was detectable under UV at 254 nm. Results from analysis of samples from batch cultures with [^{14}C]NDMA provided evidence for intermediates with retention times which correlated with the methylamine and formaldehyde standards. Both UV and radioactivity chromatograms illustrating these findings are shown in Fig. 8.

Mass spectrometry. To confirm results from the HPLC-

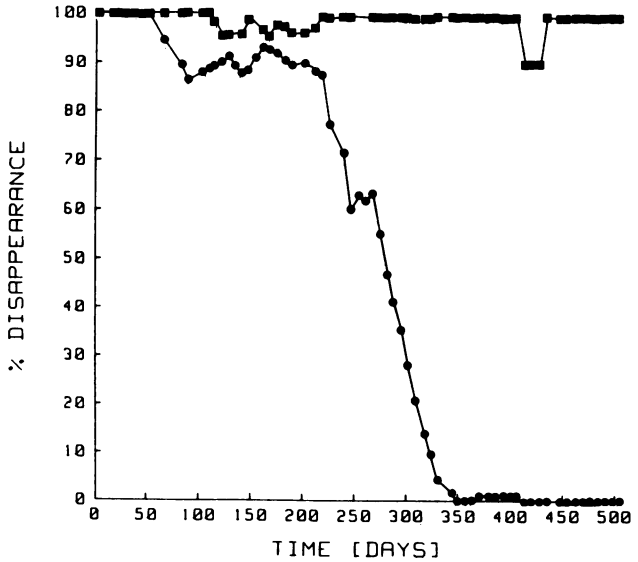

FIG. 5. Disappearance of NDMA in laboratory scale trickling filters containing granular activated coconut charcoal. Symbols: ■, active column; ●, sterile column.

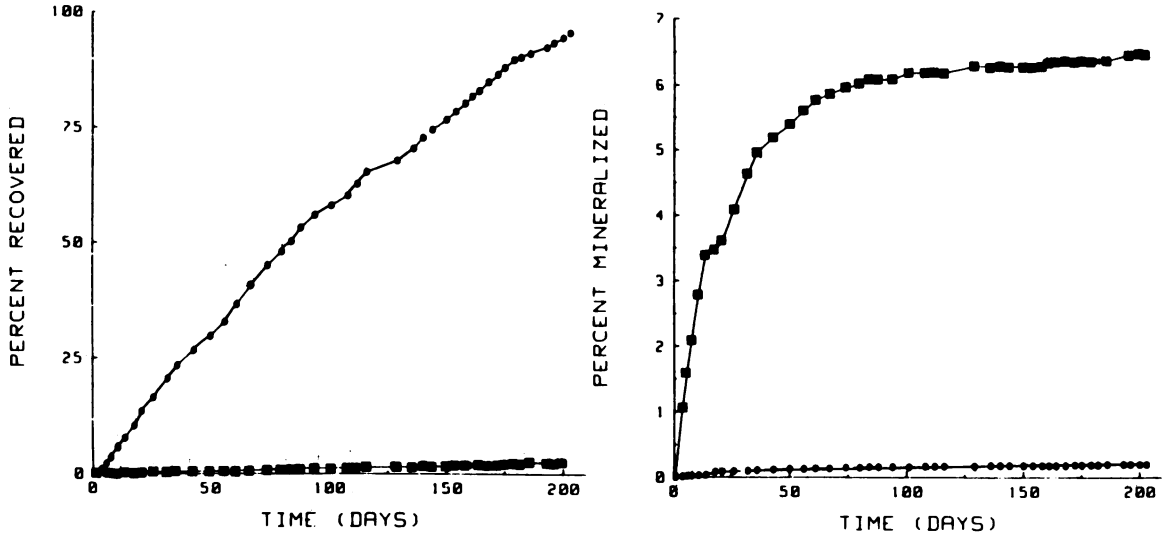

FIG. 6. Recovery of radioactivity from charcoal trickling filters. Symbols: ●, sterile column; ■, active column. The left graph shows recovery in column effluents, and the right graph shows recovery in base traps.

radioactivity detector system, we analyzed batch culture samples initiated with [^{14}C]NDMA on a SCIEX mass spectrometer. The results of these analyses confirmed the presence of formaldehyde. In separate studies, both NDMA and [^{14}C]NDMA (approximately 50 to 100 pg/ml) in sterile distilled water were stable over 4 weeks at room temperature.

Sorption of charcoal. The sorption capacity of charcoal under the experimental conditions of this study is illustrated in Fig. 9. The data for the equilibrium concentration of NDMA and the sorption of NDMA by charcoal were transformed by log manipulations. Approximately 110 mg of NDMA was retained by 1.0 g of charcoal, corresponding to 11 weight percent absorption.

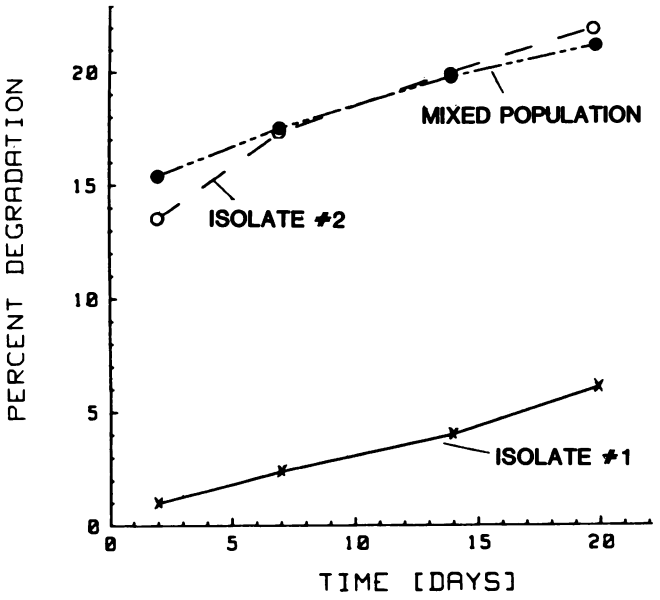

FIG. 7. Recovery of radioactivity from incubations of isolates from the active charcoal trickling filter column (0.42 μCi of [^{14}C]NDMA per plate). Medium consisted of the trickling filter influent without glucose, 1.5% agar, and 100 μg of NDMA per ml in an overlay. Sterile controls with 1.0% mercuric chloride were run concurrently.

DISCUSSION

Mineralization of [^{14}C]NDMA is determined by the evolution of $^{14}CO_2$, which is collected in alkaline traps. The recovery of ^{14}C in base traps as the measure of mineralization assumes that most of this ^{14}C is represented by $^{14}CO_2$. In general, only background quantities of ^{14}C were recovered in the base traps in corresponding sterile controls, representing some volatility. If NDMA were biotransformed and not completely mineralized, some of the possible metabolites (methylamine, dimethylamine, and hydrazines) would be retained in the acid traps, while formaldehyde, methanol, and methane would not be collected in either trap. In general, only small percentages of the total radioactivity were recovered in the acid traps. The above findings indicate that recovery of ^{14}C in the base traps provided an accurate measure of mineralization.

With NDMA, the time frame for apparent initial rates of mineralization was in days, indicating slow metabolism compared with much shorter time frames for more readily metabolized compounds. In general, no significant lag phase was apparent in most of the batch studies at the sampling frequency used, which was in consonance with initial rates of mineralization.

In the application of kinetic models, measurements of biomass are often considered as a significant variable. In our attempts to isolate NDMA-degrading organisms, it was very difficult to achieve and maintain significant colony growth on NDMA-containing media. If supplemental carbon was added to the medium, growth on NDMA could not be distinguished from growth on supplemental carbon. We therefore excluded consideration of NDMA-degrading population measurements by assuming equivalent initial populations in all cases. If measurements of microbial populations are included in rate calculations, only NDMA-degrading organisms should be considered. All experiments were initiated in a similar manner with regard to the inoculum, and therefore the initial populations should be similar. In addition, growth-related metabolism should result in a sigmoidal curve representing the reduction in substrate concentration.

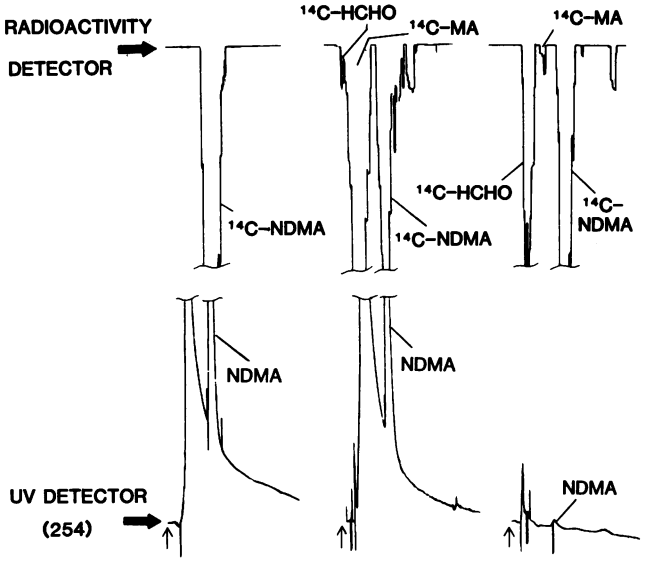

FIG. 8. HPLC chromatograms illustrating the UV and radioactivity tracings of NDMA, formaldehyde (HCHO), and methylamine (MA). The first run (left) is an NDMA standard, and the next two tracings are representative of batch culture samples.

Since none of the substrate concentration reduction curves generated with NDMA were sigmoidal, growth of the microorganisms in the batch systems was unlikely.

A number of reports have discussed the role of low concentrations of aromatic or aliphatic compounds on rates of degradation (1, 2, 17, 19). The importance of carrying out laboratory studies at realistic concentrations of a chemical, in light of the levels expected or found in natural systems, has been recognized. It can be argued that studies carried out over a broad range of concentrations are necessary to fully assess all possible levels of environmental contamination and make extrapolations to natural systems. For the range of concentrations tested, the rate of mineralization of NDMA from aqueous and soil systems was directly proportional to the initial concentration of NDMA, as would be predicted for a linear fit with Michaelis-Menten kinetics. The application of nonlinear regression analysis manipulations of the data resulted in poorer fits than the linear model, and slopes for the log-log plots of initial mineralization rate versus concentration of NDMA were not statistically different than 1.0, which is indicative of first-order kinetics. For applications of Michaelis-Menten kinetics to achieve saturation and thus calculate V_{max}, high concentrations of the compound under study may have to be used. With NDMA, we studied a 7 order-of-magnitude range (picograms to micrograms per milliliter) of concentrations; however, saturation kinetics were not attained, and therefore V_{max} could not be determined. Degradation kinetics at higher initial concentrations were not pursued, because levels of contamination in natural systems were not expected to exceed those evaluated. Although the slopes of the linear fits for the kinetic data from aqueous systems were less than 1.0, the scatter of the data was such that these slopes could not be distinguished from first-order fits on a statistical basis.

Measurements of mineralization rates were calculated from the apparent initial rates of $^{14}CO_2$ release and increased with increasing substrate concentrations. The percentage mineralized however, which decreased with increasing substrate concentrations, may be influenced by a number of factors other than just the initial substrate concentration. These other factors, theoretically, should not come into play during the initial reactions but may do so, especially over the term of the batch incubations. These factors may include a buildup of inhibitory products, secondary changes in the media, such as a change in pH, effects on cell wall or transport mechanisms, activities of two or more ecologically and physiologically distinct populations of microorganisms with optimal activities at different concentrations of NDMA, or other unidentified effects. In the case of NDMA, formaldehyde was a prominent intermediate and could be speculated to be responsible for inhibiting activity as the concentration of NDMA increased (as reflected by the percentage mineralized). This would represent extreme sensitivity to low concentrations if this were the case, since the initial concentration of NDMA, where significant formaldehyde was detected, was in the nanogram-per-milliliter range.

In soils, it is often postulated that different kinetic models may apply, owing to the fact that nutrients and the compounds under study are not distributed in a homogeneous manner. This was not the case with NDMA. A linear relationship was found between initial mineralization rate and initial concentration of NDMA. Apparently, the high solubility of NDMA overcomes the problems of diffusibility throughout water films covering soil particles.

A number of authors have discussed the role of ecologically distinct natural populations of microorganisms (eutrophic, mesotrophic, oligotrophic) in degradation processes. Subba-Rao et al. (17) have suggested that these populations respond differently when exposed to different concentrations of a compound. Rates of mineralization may increase at low substrate concentrations because of a shift in the compound-utilizing population. Rubin and Alexander (16) found that trace levels of certain compounds in eutrophic environments may not be metabolized. Wang et al. (19) postulated a role for eutrophic and oligotrophic popula-

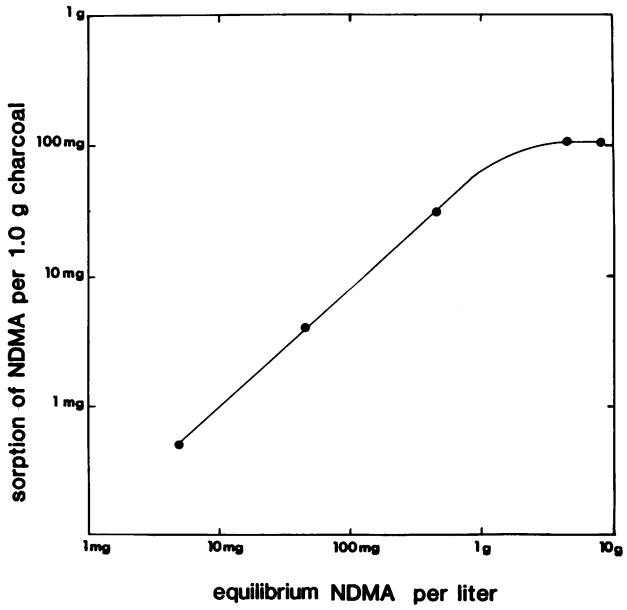

FIG. 9. Sorption of NDMA by granular activated coconut charcoal.

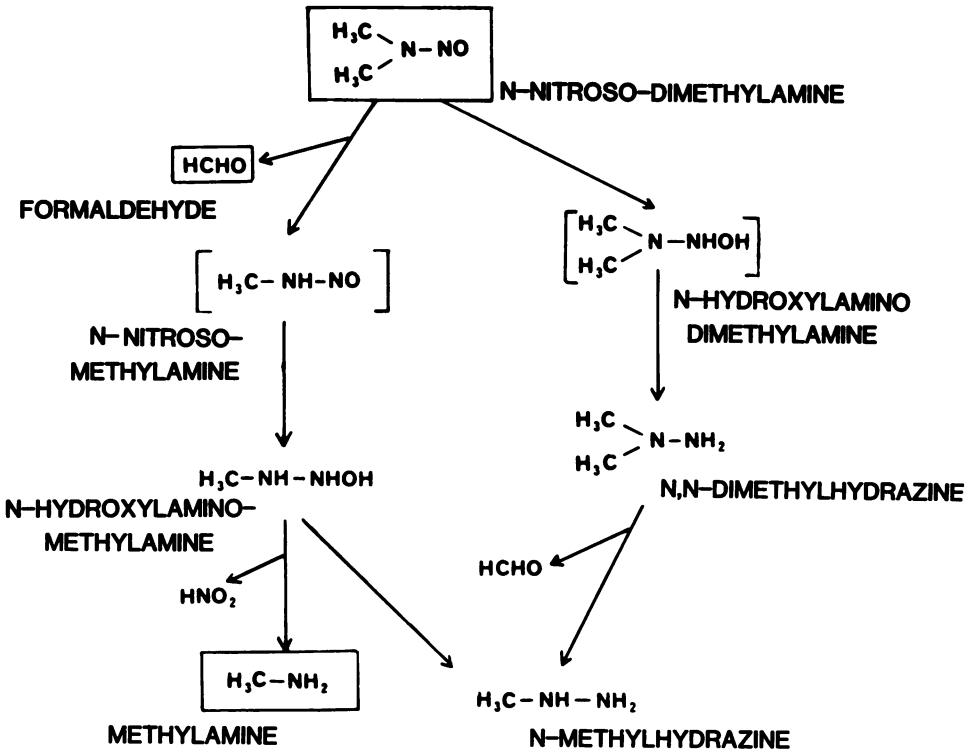

FIG. 10. Summary biodegradation scheme for NDMA. These pathways were identified by Grilli and Prodi (4) in mammalian systems. Bracketed compounds are postulated intermediates. Evidence for the oxidative pathway in microbial systems is highlighted by boxed compounds, which were identified during microbial degradation of NDMA in this study.

tions in lake water in cometabolism versus mineralization of isopropyl *N*-phenylcarbamate. Those authors reported that even a single population may cometabolize or mineralize an organic substrate, depending on its concentration. Our studies on cell viability at various concentrations of NDMA, when grown on minimal and nutrient-rich media, indicated no such shift between oligotrophic and eutrophic populations.

We showed that the rate of mineralization of NDMA is dependent on concentration over the range of concentrations tested. At concentrations above those tested, the curve will eventually become zero order with respect to concentration as saturation is reached. Although a lower percentage was mineralized at the higher concentrations on a weight basis, a greater amount of NDMA was mineralized. We found no indication for a threshold level below which mineralization activity ceased. Linear extrapolations of mineralization curves, such as Fig. 2, to zero rates of mineralization would provide a theoretical minimum concentration at which degradation ceases. However, no experimental evaluation at such low concentrations was undertaken, because material of very high specific activity and large incubation vessels are required to ensure that sufficient material is present to accurately detect mineralization activity (as determined by recovery of $^{14}CO_2$) below the picogram-per-milliliter concentration.

Some authors have predicted the existence of minimum threshold concentrations below which there is no mineralization activity (1, 2, 16). This absence of activity is usually attributed to insufficient carbon for minimal maintenance energy requirements as well as the physical location disparity between substrate and cell and their respective movements. It is our contention that for biodegradable substrates in natural systems this threshold would only occur at such low concentrations that the level of the compound would be insignificant in terms of environmental contamination. In natural systems other energy sources are present, and activity, however minimal, will be present over a finite period. Mineralization activity, even at extremely low concentrations, would still occur until the scenario of one cell and one molecule of substrate was reached. However, at that point the concentration would have no significance in terms of natural systems and the prediction of rates. This situation would not apply, however, if other environmental factors were inhibitory to mineralization activity, regardless of the concentration of NDMA. This is also one of the primary reasons why it is difficult to extrapolate laboratory findings to rates in natural systems.

A threshold level of nutrients would be required for microorganisms undergoing growth and reproduction. The point in question is whether substrates will be metabolized when the microorganisms are in maintenance or starvation phases. As summarized by Morita (13) for starvation studies with marine microorganisms, many survival strategies have developed to assure long-term survival in ultraoligotrophic environments. Some of these survival strategies are focused on priming the cells for utilization of available substrates when the compounds finally do appear in the surrounding environment. It is reasonable to assume that similar strategies, as well as unidentified mechanisms, are at work in all environments that would provide similar survival mechanisms and rapid response to substrate availability, regardless of the low concentration present. Based on these mechanisms, it is difficult to conceive of a threshold concentration

of substrate below which metabolism would cease when based solely on the concentration of substrate.

Laboratory scale trickling filter. Experiments with a laboratory scale simulated trickling filter with a charcoal bed showed potential for degradation of NDMA. NDMA was mineralized by this column and almost completely removed under continuous-flow conditions from the feed which contained either 50 or 100 μg of the nitrosamine per ml. Apparently the charcoal bed and column configuration provided a suitable environment to promote the degradation of NDMA, possibly because of enhancement of NDMA-degrading microorganisms, a change in NDMA configuration on sorption to charcoal which results in enhanced susceptibility to catabolic enzymes, or simply sorption to charcoal to perhaps provide for longer retention time in combination with the column conditions. When the same type of charcoal was added to batch cultures, there was virtually complete cessation of mineralization of NDMA. This may indicate a significant role for the column environment in terms of the activity found with the trickling filter. Also, similar columns run with an inert polymer support did not stimulate NDMA-degrading activity. In separate studies, it was found that NDMA did not bind to soil humic acid.

Biotransformation pathway. Grilli and Prodi (4) identified the products of metabolism of NDMA in rats. Oxidative dealkylation with rat liver microsomes led to the formation of formaldehyde, formic acid, methylamine, and *N*-methylhydrazine. With the soluble cell enzyme extract, reductive reactions led to the formation of *N*-methylhydroxylamine and *N*,*N*-dimethylhydrazine (Fig. 10). We found no evidence for a reductive pathway in bacterial cells. No hydrazine or hydrazine derivatives were detected. Evidence was found for formaldehyde and methylamine as intermediates as indicated.

Treatment options. We demonstrated that NDMA is biodegradable; however, at concentrations in the upper nanogram or microgram per milliliter range, only limited mineralization of NDMA was achieved in batch cultures. The influence of other environmental factors on rates of mineralization, the possibility of selective strain improvement, and the fact that it is difficult to extrapolate laboratory kinetic data to the natural environment should all be considered for a complete determination of the potential for biological approaches to the treatment of NDMA. The laboratory scale simulated trickling filter appears to offer a unique environment for activity and therefore may have some potential applications in this regard.

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LITERATURE CITED

1. **Boethling, R. S., and M. Alexander.** 1979. Effect of concentration of organic chemicals on their biodegradation by natural microbial communities. Appl. Environ. Microbiol. **6**:1211–1216.
2. **Boethling, R. S., and M. Alexander.** 1979. Microbial degradation of organic compounds at trace levels. Environ. Sci. Technol. **8**:989–991.
3. **Fochtman, E. G., and W. Eisenberg.** 1979. Treatability of carcinogenic and other hazardous organic compounds. National Technical Information Service, Springfield, Va.
4. **Grilli, A., and G. Prodi.** 1979. Identification of dimethylnitrosamine metabolites *in vitro*. Gann **66**:473–480.
5. **Harada, K.** 1980. Microbial degradation of nitrosamines. II. Effect of the conditions of growth and enzymatic reaction on the nitrosamine breakdown. Bull. Jpn. Soc. Sci. Fish. **6**:723–726.
6. **Harada, K., and K. Yamada.** 1979. Microbial degradation of nitrosamines. I. Inducible breakdown of nitrosamines. Bull. Jpn. Soc. Sci. Fish. **7**:925–928.
7. **Jensen, D. E., P. D. Lottikar, and P. N. Magee.** 1981. The *in vitro* methylation of DNA by microsomally activated dimethylnitrosamine and its correlation with formaldehyde production. Carcinogenesis **4**:349–354.
8. **Kobylinski, E. A., and B. W. Peterman.** 1979. Evaluation of ozone oxidation and UV degradation of dimethylnitrosamine. U.S. Army Medical Research and Development Center, Fort Detrick, Md.
9. **Lake, B. G., J. C. Phillips, C. E. Heading, and S. D. Gangolli.** 1976. Studies on the *in vitro* metabolism of dimethylnitrosamine by rat liver. Toxicology **5**:297–309.
10. **Lottikar, P. D., W. J. Baldy, and E. N. Dwyer.** 1975. Dimethylnitrosamine demethylation by reconstituted liver microsomal cytochrome P-450 enzyme system. Biochem. J. **152**:705–708.
11. **Mallik, M. A., and K. Testai.** 1981. Transformation of nitrosamines in soil and *in vitro* by soil microorganisms. Bull. Environ. Contam. Toxicol. **27**:115–121.
12. **Milstein, S., and J. B. Guttenplan.** 1979. Near quantitative production of molecular nitrogen from metabolism of dimethylnitrosamine. Biochem. Biophys. Res. Commun. **1**:337–342.
13. **Morita, R. I.** 1983. Feast or famine in the deep sea. Dev. Ind. Microbiol. **25**:5–16.
14. **Mosier, A. R., and S. Torbit.** 1976. Synthesis and stability of dimethylnitrosamine in cattle manure. J. Environ. Qual. **4**:465–468.
15. **Oliver, J. E., P. C. Kearney, and A. Konston.** 1979. Degradation of herbicide-related nitrosamines in aerobic soils. J. Agric. Food Chem. **4**:887–891.
16. **Rubin, H. E., and M. Alexander.** 1983. Effect of nutrients on rates of mineralization of trace concentrations of phenol and *p*-nitrophenol. Environ. Sci. Technol. **2**:104–107.
17. **Subba-Rao, R. V., H. E. Rubin, and M. Alexander.** 1982. Kinetics and extent of mineralization of organic chemicals at trace levels in freshwater and sewage. Appl. Environ. Microbiol. **5**:1139–1150.
18. **Tate, R. L., and M. Alexander.** 1975. Stability of nitrosamines in samples of lake water, soil and sewage. J. Natl. Cancer Inst. **2**:327–330.
19. **Wang, Y.-S., R. V. Subba-Rao, and M. Alexander.** 1984. Effects of substrate concentration and organic and inorganic compounds on the occurrence and rate of mineralization and cometabolism. Appl. Environ. Microbiol. **6**:1195–1200.