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FORMATION OF NITROSAMINES UNDER DENITRIFICATION CONDITIONS

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The formation of nitrosamines under denitrification conditions was evaluated as part of the Army's program on pollution abatement. Various amines (dimethyl-, diethanol-, dipropyl- and dibutyl-) were evaluated in batch and/or continuous culture microbial systems under anaerobic conditions with high nitrate loads. With the exception of dimethylamine, the amines do not form detectable concentrations of the corresponding nitrosamines in the alkaline denitrification system. N-nitrosodimethylamine (NDMA) is detected in part per billion concentrations in many of these systems. The formation of NDMA is apparently not biologically

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20. (Cont'd) mediated in the batch systems, however, the reason for its formation in the continuous systems is not clear. The contribution of microbially produced catalysts and the role of the intracellular environment in these reactions is discussed.

PREFACE

Ammonia stripping columns at Radford Army Ammunition Plant have indicated a potential for formation of N-nitrosodimethylamine in waste effluents. This nitrosamine is highly toxic and carcinogenic. Microbiological denitrification systems are being recommended to degrade anaerobically munition waste compounds as well as to reduce high nitrate levels. The use of these systems is a cost-effective method to lower pollution levels to desired criteria limits.

Nitrosamines are generally formed chemically by the reaction of nitrite with secondary or tertiary amines under acidic conditions. There is a potential for nitrosamine formation in a denitrification system due to the presence of nitrite, which is an intermediate during microbial denitrification. The gravity of this problem must be evaluated to determine the risks involved in this biological treatment approach.

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FORMATION OF NITROSAMINES UNDER DENITRIFICATION CONDITIONS

INTRODUCTION

The hazards associated with nitrosamine contamination have been extensively documented for many products, including those of the pharmaceutical, agricultural, beverage and food, and tobacco industries.[1] Health concerns are due to the carcinogenic and mutagenic activity of nitrosamines.[2]

Numerous studies have documented nitrosamine formation under a variety of environmental conditions, with the majority of work being done on the formation of N-nitrosodimethylamine (NDMA). Two routes of nitrosamine formation, microbial and chemical, have been documented.

Microbial Formation

Formation of nitrosamines in aqueous and soil systems as a result of biological mediation generally occurs due to the reaction of a secondary or tertiary amine with nitrite. These reactions may be catalyzed directly through enzymatic reactions or indirectly through the effects of secondary metabolites, such as a microbially mediated change in pH[3] or microbially mediated formation of complex organic matter, which in turn catalyzes nitrosation reactions.[4] In general, microbially mediated reactions in aqueous and

[1] Schank, R. C. Occurrence of N-nitroso compounds in the environment. 155-183. In R. C. Schank (ed.) Mycotoxins and N-nitroso compounds: Environmental Risks. Vol. 1. CRC Press, Inc., Boca Raton, FL, 1981.

[2] Schank, R. C. and P. N. Magee. Toxicity and carcinogenicity of N-nitroso compounds. 185-217. In R. C. Schank (ed.) Mycotoxins and N-nitroso Compounds: Environmental Risks. Vol. 1. CRC Press, Inc., Boca Raton, FL, 1981.

[3] Pancholy, S. K. and M. A. Mallik. Microbial formation of carcinogenic dimethylnitrosamine. Proc. Okla. Acad. Sci. 58: 98-101, 1978.

[4] Mills, A. L. and M. Alexander. Factors affecting dimethylnitrosamine formation in samples of soil and water. J. Environ. Qual., 5(4): 437-440, 1976.

soil systems occur over a broad range of pH from approximately 4.0 to 8.0.

Ayanaba et al.,[5] evaluated the potential for NDMA formation in soils and found that NDMA was formed in soils amended with amines (secondary or tertiary) and nitrite or nitrate. No alkaline soil conditions were evaluated.

In biological systems, Hawksworth and Hill[6] studied the formation of nitrosamines by human intestinal microorganisms at physiological pH levels. Kunisaki and Hayashi[7] determined the enzymatic formation of NDMA by E. coli with nitrite and secondary amines. The optimum pH for this reaction was 8.0. Yang et al.,[8] proposed a nonenzymatic mechanism for microbial acceleration of nitrosamine formation which was active in the particulate cell fraction, while Ayanaba and Alexander[9] claimed a similar reaction that was enzymatically mediated by the soluble portion of cell extracts at pH 7.5.

5 Ayanaba, A., W. Verstraete, and M. Alexander. Formation of dimethylnitrosamine, a carcinogen and mutagen, in soils treated with nitrogen compounds. Soil Sci. Soc. Am. Proc. 37: 565-568, 1973.

6 Hawksworth, G. M. and M. J. Hill. Bacteria and the N-nitrosation of secondary amines. Brit. J. Cancer. 25: 520-526, 1971.

7 Kunisaki, N. and M. Hayashi. Formation of N-nitrosamines from secondary amines and nitrite by resting cells of Escherichia coli B. Appl. Environ. Microbiol. 37(2): 279-282, 1979.

8 Yang, H. S., J. D. Okun, and M. C. Archer. Nonenzymatic microbial acceleration of nitrosamine formation. J. Agric. Food Chem. 25(5): 1181-1183, 1977.

9 Ayanaba, A. and M. Alexander. Microbial formation of nitrosamines in vitro. Appl. Microbiol. 25(6): 862-868, 1973.

Ayanaba and Alexander[10] also reported the formation of NDMA in sewage and lake water after the addition of dimethyl- and trimethylamines and nitrate. The reaction was considered to be microbially mediated and a greater concentration of NDMA is formed with increasing acidity. The authors discussed the question of nitrite accumulation (thought to be rare) during nitrification, denitrification, and microbial reduction of nitrate to ammonium.

Mills and Alexander[11] described the formation of NDMA from dimethylamine and nitrite by Pseudamonas stutzeri during growth and stationary growth phases and implicated a heat-stable fraction as the reaction catalyst. A number of other organisms were found capable of nitrosation reactions during resting cell studies. Greene et al.,[12] demonstrated NDMA formation in soils amended with sewage, dimethylamine and nitrite at pH 4.7 and 5.6. The importance of precursor concentration and soil moisture was evaluated.

Chemical Formation

The chemical formation of nitrosamines occurs optimally under acidic conditions primarily through reaction of secondary or tertiary amines with nitrite. A reaction with primary amines has also been described. The reaction kinetics of chemically mediated reactions were investigated by

[10] Ayanaba, A. and M. Alexander. Transformations of methylamines and formation of a hazardous product, dimethylnitrosamine, in samples of treated sewage and lake water. J. Environ. Qual. 3(1): 83-89, 1974.

[11] Mills, A. L. and M. Alexander. N-nitrosamine formation by cultures of several microorganisms. Appl. Environ. Microbiol. 31(6): 892-895, 1976.

[12] Greene, S, M. Alexander, and D. Leggett. Formation of N-nitrosodimethylamine during treatment of municipal waste water by simulated land application. J. Environ. Quality 10(3): 416-421, 1981.

Mirvish.[13,14] Specific chemical nitrosation reactions in the pH range 6.4 to 11.0 have also been reported.[15] In these reactions, formaldehyde, trichloroacetaldehyde, and thiocyanate can catalyze the reaction.[15,16] Casado et al.,[17] have looked at the kinetics of the reaction catalyzed by formaldehyde.

Previous investigations have demonstrated that nitrosamine formation can be chemically or microbially mediated. These studies have also shown that catalysts such as formaldehyde can accelerate reaction rates, thereby allowing chemically mediated nitrosamine formation under alkaline conditions.

The two major reactants in these studies are secondary amines and nitrite. Secondary amines are found throughout the animal and plant kingdoms and are also formed during the decomposition of natural and synthetic organic residues. Nitrite may arise in the environment during the microbial transformations of ammonia or nitrate or through manmade production.

[13] Mirvish, S. S. Kinetics of dimethylamine nitrosation in relation to nitrosamine carcinogenesis. J. Natl. Cancer Inst. 44: 633-639, 1970.

[14] Mirvish, S. S. Formation of N-nitroso compounds: chemistry, kinetics, and in vivo occurrence. Toxicol. Appl. Pharm. 31: 325-351, 1975.

[15] Keefer, L. K. and P. P. Roller. N-nitrosation by nitrite ion in neutral and basic medium. Science. 181: 1245-1247, 1973.

[16] Obiedzinski, M. W., J. S. Wishnok, and S. R. Tennebaum. N-nitroso compounds from reactions of nitrite with methylamine. Food Cosmet. Toxicol. 18: 585-589, 1980.

[17] Casado, J., A. Castro, M. A. L. Quintela, and J. V. Tato. Kinetic studies on the formation of N-nitroso compounds. V. Formation of dimethylnitrosamine in aqueous solution: effect of formaldehyde. Seit. Phys. Chemie. 127: 179-192, 1981.

Significant concentrations of NDMA have been detected in wastewaters from ammonia stripping columns at Radford Army Ammunition Plant. Amines and high nitrate concentrations are present in effluents from munitions manufacturing, loading, assembly, and packing plants. Anaerobic denitrification systems are efficient at reducing high nitrate loads and degrading some of these amines, however, the potential formation of nitrosamines during the denitrification process is an important concern.

MATERIALS AND METHODS

Chemicals

Dimethylamine (DMA), dipropylamine (DPA), dibutylamine (DBA), diethanolamine (DElA), and N-nitrosodipropylamine (NDPA) were purchased from Eastman Kodak, Rochester, NY. N-nitrosodimethylamine (NDMA) was purchased from Aldrich Chemical Co., Milwaukee, WI. N-nitrosodiethanolamine (NDElA) was purchased from Columbia Organic Chemical Co., Columbia, SC. Trimethylammonium nitrate (TMAN) and triethanolammonium nitrate (TEAN) were provided by Ballistics Research Laboratory, Aberdeen Proving Ground, MD. TMAN and TEAN are new liquid propellants under study in our pollution abatement program.

Batch Cultures

Two batch culture experiments were designed to explore the breakdown of DMA and possible biotransformation to NDMA in denitrification systems. The first DMA batch experiment consisted on 10 250-mL screw-top Erlenmeyer flasks containing the components as listed in Table 1. Flasks 1 through 6 were autoclaved prior to inoculation, while flasks 7 through 10 were filter sterilized through a 0.45 μm pore size membrane filter. The effects of

sterilization procedure, pH, and nitrogen salt were investigated in this first set of experiments. The initial pH of the solutions was asjusted with 1 N sodium hydroxide or 1 N hydrochloric acid. Active flasks were inoculated with cells from a continuous denitrification system. The cell pellet was harvested by centrifugation at 12,000 rpm for five minutes, resuspended in 0.85% potassium chloride, centrifuged, and resuspended.

Table 1. Experimental Protocol for the First DMA Batch Denitrification Experiment[a,b]

Flask	Initial Ph	KNO_3(g/L)	KNO_2(g/L)	DMA(mg/L)	Active (+) Sterile (-)
1	7.5	2.0	---	100	+
2	7.5	---	1.0	100	+
3	5.5	2.0	---	100	+
4	5.5	---	1.0	100	+
5	7.5	2.0	---	0	+
6	7.5	---	1.0	0	+
7	7.5	2.0	---	100	-
8	7.5	---	1.0	100	-
9	5.5	2.0	---	100	-
10	5.5	---	1.0	100	-

[a] All flasks included 1.32 mL/L methanol, 3.5 g/L K_2HPO_4 and 300 mg/L trace salts.

[b] Trace salts included $MgSO_4 \cdot 7H_2O$, 500 mg/L; NaCl, 50 mg/L; $CaCl_2$, 15 mg/L; $FeCl_3 \cdot 6H_2O$, 10 mg/L; $CuSO_4 \cdot 5H_2O$; 10 mg/L; $MnSO_4 \cdot H_2O$; 10 mg/L; and $NaMoO_4 \cdot 2H_2O$, 1 mg/L.

The second DMA batch experiment was run under the conditions as indicated in Table 2 and was run in order to evaluate further potential nitrosamine formation at alkaline pH. Cells used to inoculate acitve systems were obtained as described for the first experiment. Filter sterilized DMA (0.45 μm pore size membrane filter) was added after autoclaving.

Table 2. Experimental Protocol for the Second DMA Batch Denitrification Experiment[a]

Flask	Initial pH	KNO_3(g/L)	KNO_2(g/L)	DMA(mg/L)	Active (+) Sterile (-)
1	7.5	2.0	---	100	+
2	7.5	2.0	---	100	-
3	7.5	2.0	---	0	+
4	7.5	2.0	---	0	-
5	7.5	---	1.0	100	+
6	7.5	---	1.0	100	-
7	7.5	---	1.0	0	+
8	7.5	---	1.0	0	-
9	7.5	---	---	100	+
10	7.5	---	---	100	-
11	7.5	---	---	0	+
12	7.5	---	---	0	-

[a] All flasks contained methanol, 1.32 mL/L; K_2HPO_4, 3.5 g/L; and trace salts, 595 mg/L (see Table 1).

The DPA batch experiment was run as described for the second DMA batch experiment (Table 3). Flasks containing DPA were filter-sterilized as before. The inoculum consisted of cells harvested from an active denitrification system containing DPA, DBA, AND DE1A (100 mg per L each) in the influent medium.

Table 3. Experimental Protocol for the DPA Batch Denitrification Experiment[a]

Flask	Initial pH	KNO_3(g/L)	KNO_2(g/L)	DPA(mg/L)	Active (+) Sterile (-)
1	7.5	2.0	---	100	+
2	7.5	---	1.0	100	+
3	5.5	2.0	---	100	+
4	5.5	---	1.0	0	+
5	7.5	2.0	---	0	+
6	7.5	---	1.0	0	+
7	7.5	2.0	---	100	-
8	7.5	---	1.0	100	-
9	5.5	2.0	---	100	-
10	5.5	---	1.0	100	-
11	5.5	2.0	---	0	+
12	5.5	---	1.0	0	+

[a] All flasks contain methanol, 1.32 mL/L; K_2HPO_4, 3.5 g/L; and trace salts, 300 mg/L (see Table 1) in lake water.

Continuous Cultures

A continuous denitrification system with DPA, DBA, DE1A, 100 mg per L each was run in a 500-mL reaction vessel. The vessel was a modified Erlenmeyer flask fitted with a 24/40 ground glass joint and overflow tube.

A 35-cm glass tube, 5 mm I.D., was suspended in the reaction vessel by a teflon adapter to deliver nutrient solution to the bottom of the reaction vessel. The total volume of the reaction vessel was 553 mL. The vessel contents were stirred slowly and effluent was collected from the overflow line. Nutrient solution was delivered to the reaction vessel by a Rabbit peristaltic pump (Rainin Co., Woburn, MA) with a four-channel head. A series of in-line break tubes was used to prevent medium contamination. The nutrient solution, per L consisted of methanol; 1.4 mL, KNO_3; 2.05 g, K_2HPO_4; 0.87 g, trace salts (see Table 1); 595 mg, DPA; 100 mg, DBA; 100 mg, and DEIA; 100 mg. The system was operational for 123 days, during which time the pH ranged between 7.0 and 8.9. The retention time was 3 to 5 days throughout the study and denitrification was near 99% efficient when the system was operating without mechanical problems.

Continuous denitrification systems were also run individually with DMA, TMAN and TEAN as the secondary or tertiary amines of interest. The system with TMAN contained per L, KNO_3, 4.93 g; K_2HPO_4, 0.87 g; trace salts, 595 mg; methanol, 4.1 mL; and TMAN, 100 ppm. After 87 days methanol, the electron donor, was replaced with sodium acetate, 6.97 g per L for the remaining 43 days. The retention time in the system was 4 days for 57 days, 2 days for the next 26 days, 1 day for the next 10 days, and 4 days for the remaining period. With methanol, the TMAN system denitrified at better than 99% when operating at a 4-day retention time but the efficiency decreased outside of this range. With sodium acetate, the system denitrified at a 71% to 98% efficiency. The pH of the system ranged from 7.6 to 8.9 with methanol and 8.7 to 9.2 with acetate.

The denitrification system with TEAN ran for 164 days and contained

per L, KNO_3, 2.05 g; K_2HPO_4, 0.87 g; trace salts, 595 mg; and methanol, 1.4 mL. The system influent contained 1,000 ppm TEAN for 98 days and 500 ppm for the remaining 66 days. The retention time was approximately 10 days for the first 27 days, 4 days for the next 47 days, 10 days for the next 31 days and 7 to 8 days for the remaining period. During 164 days the system generally denitrified above 95% and the pH of the system ranged between 7.6 and 8.1.

The denitrification system with DMA contained per L, methanol, 10.55 mL; KNO_3, 4.04 g; K_2HPO_4, 6.96 g; trace salts, 595 mg; and DMA, 100 ppm. The DMA was filter sterilized and added subsequent to autoclaving. The system was run for 67 days. The retention time was 4 to 5 days for the first 43 days and 2 to 3 days for the remaining period. The system denitrified efficiently when mechanical operation was smooth and the pH of the system ranged from 7.8 to 8.5.

Nitrate Determination

Nitrate concentrations were determined either on an Orion (Cambridge, MA) ion-analyzer Model 901, using an Orion nitrate specific ion electrode (Model 93-07) and Orion double junction reference electrode (Model 90-02) or on a Corning (Medfield, MA) pH meter, Model 130.

Sample Preparation

Samples, 5 mL to 10 mL of influent or effluent from the continuous systems and 2 mL samples from the batch systems, were taken one to four times weekly. The samples were centrifuged at 12,000 rpm for five minutes and the supernatant was passed through a Swinny stainless steel syringe-type filter holder containing a 0.45 μm pore size membrane filter prior to injection.

High Performance Liquid Chromatography

Quantitative analysis of nitrosamines was performed on a Water's Associates (Milford, MA) high performance liquid chromatograph (HPLC) equipped with two Model 6000A solvent delivery pumps, a Model 441 detector set at 254 nm, a Model 720 System Controller and a Model 730 Data Module. All nitrosamine analyses were on a μBondapak C-18 reverse phase stainless steel column (Water's Associates).

NDMA was analyzed with distilled, deionized and degassed water flowing at 2.5 mL per minute as the mobile phase. Injection volumes varied up to 200 μL, sensitivity varied from 0.02 to 0.005 absorbance units full scale (aufs), and the retention time was 5.3 minutes. The detection limit was 1 ppb (μg/L).

NDPA was analyzed with 25% acetonitrile 75% water (v/v) as the mobile phase flowing at 2.5 mL per minute. Injection volumes varied up to 200 μL and sensitivity from 0.02 to 0.005 aufs. The retention time was five minutes.

NDELA was analyzed with water containing 0.1 M K_2HPO_4 as the mobile phase flowing at 1.5 mL per minute through two C-18 μBondapak stainless steel reverse phase columns in sequence. Injection volumes varied up to 200 μL, sensitivity from 0.02 to 0.005 aufs, and the retention time was around seven minutes. The detection limit was 1.0 ppb.

Mixtures containing NDPA and NDBA were analyzed on a programmed gradient run. For a 14-minute run time, the initial 6 minutes was with 35% acetonitrile 65% water (v/v). The acetonitrile concentration was increased to 45% from 6 to 7 minutes and remained at this concentration until 12

minutes, at which time the concentration was decreased to 35% by the 14th minute. Injection volumes ranged up to 200 uL and sensitivity was set between 0.02 to 0.005 aufs. The retention times were 4 to 5 minutes and 10 minutes for NDPA and NDBA, respectively. The detection limits were 20 ppb for both NDPA and NDBA.

Gas Chromatography

Gas chromatographic (GC) analyses were performed on a Hewlett-Packard Model 5840A GC equipped with a flame ionization detector (FID). Nitrogen carrier gas flowed at 45 mL per minute and injection volumes were 2 µL. DPA, DBA, and methanol were determined on a 3.05 m by 2 mm (ID) silanized glass column containing 10% Carbowax 20M and 2% KOH on 80/100 Chromasorb WAW. Injector, oven, and FID temperatures were 150°C, 30°C, and 230°C, respectively. Elution times were three minutes for DPA and DBA, and seven minutes for methanol, respectively.

NDMA could also be analyzed under these conditions, however, the detection limit was 10 ppm, significantly higher than the 1 ppb level obtained by HPLC.

A sensitive GC method for NDELA was unsuccessfully sought. The initial conditions were with the Carbowax 20M, KOH, Chromasorb WAW column described previously. The injector, oven, and FID temperatures were 150°C, 110°C, and 250°C, respectively. Under these conditions NDELA eluted too rapidly from the column. At 90°C and then 60°C oven temperatures, improved retention times were found, but peak shoulders indicated interfering compounds. Another column, a 1.8 m by 2 mm ID silanized glass column containing 4% Carbowax 20M and 0.8% KOH on Carbopak B, was examined. Injection, FID, and oven temperatures

were 150°C, 250°C, and 80°C or 60°C, respectively. Under these conditions NDEIA could not be separated from the water peak.

Gas Chromatography/Mass Spectroscopy (GC/MS)

Gas chromatographic Mass Spectrometer analyses on NDPA (standard and product isolated from culture) was performed on a Finnegan Model 4000 system in the EI mode with a 25 m by 0.32 mm ID FS DB-1 capillary column. The GC temperature was programmed from 60°C to 150°C at 13°C per minute. Mass numbers from m/z 20 to m/z 280 were scanned. Injection volumes were 1 µL.

Nitrosamine Specific Detector

Thermo Electron Corp. (Waltham, MA) analyzed a total of five samples, stock TMAN and TEAN, influent and effluent samples from the continuous denitrification system containing three amines (DEIA, DPA and DBA), and an effluent sample from the denitrification system with TEAN.

Volatile nitrosamines were analyzed on a GC-TEA Model 543 Analyzer (Thermo Electron Corp.). The GC was a Hewlett-Packard 5710A and 7671A automatic sampler with a 0.64 cm ID by 182 cm long glass column containing 10% Carbowax with 0.5% KOH on Chromosorb WHP 80/100 mesh. Helium carrier gas flowed at 20 mL per minute and the column temperature was programmed from 140°C to 170°C at 4°C per minute. Volatile nitrosamines were also analyzed by HPLC-TEA with a Model 502 Analyzer (Thermo Electron Corp.). The solvent, acetone/isooctane (7/93), flowed at 2 µL per min through a 10 µm, 4.6 mm by 250 mm Lichrosorb Si60 column. Nonvolatile nitrosamines were analyzed on the same instrument but with the solvent isooctane/dichloromethane/methanol (60/33/7) flowing at 2 mL per min through a 10 µm, 4.6 mm by 250 mm Ultrasil/NH_2 column.

Sample preparation for GC involved extraction with dichloromethane and solvent concentration. Samples for HPLC were directly injected or diluted with acetone prior to injection. The detection limits were NDEIA, 60 ppb; NDMA, 2 ppb; and NDPA, 2-50 ppb depending on sample preparation.

RESULTS

NDMA was detected in samples from the first DMA batch experiment. At pH 5.5 and 7.5 with nitrate salt, minimal levels of NDMA were detected by HPLC, whether or not the flasks were active or sterile. Actual concentrations detected ranged from below detection limits (1 ppb) up to about 30 ppb, but for the most part concentrations were below about 5 ppb. However, when nitrite salts were substituted for nitrate salts, higher concentrations of NDMA were detected (Figure 1). At pH 7.5, these levels ranged up to around 100 ppb, while at pH 5.5 concentrations near 2.5 ppm were measured. Since the formation of NDMA in this experiment appears to be chemically mediated, the role of pH as a factor in NDMA formation is evident, with greater formation of the nitrosamine at the acidic pH. The reaction is catalyzed through nonbiological mechanisms because similar patterns of nitrosamine formation are found in both active and sterile flasks. These experiments were terminated after 33 days.

Similar results were found in the second DMA batch experiment with nitrite salts at pH 7.5 (Figure 2). Only minimum levels of NDMA were detected and there was little difference between active and sterile systems.

Final measurements taken from the DPA batch culture experiment are presented in Table 4. GC determinations of final DPA concentrations are presented in Table 5. In general, little of the initial DPA was metabolized over the 36

days, while methanol levels were significantly reudced in active systems and only slightly reduced in sterile systems. Apparently, the methanol was utilized as the electron donor to drive denitrification while DPA was not as readily utilized. The active flasks, 1, 3, 5, and 11, demonstrated over 50% denitrification, whereas the sterile flasks showed no denitrification activity.

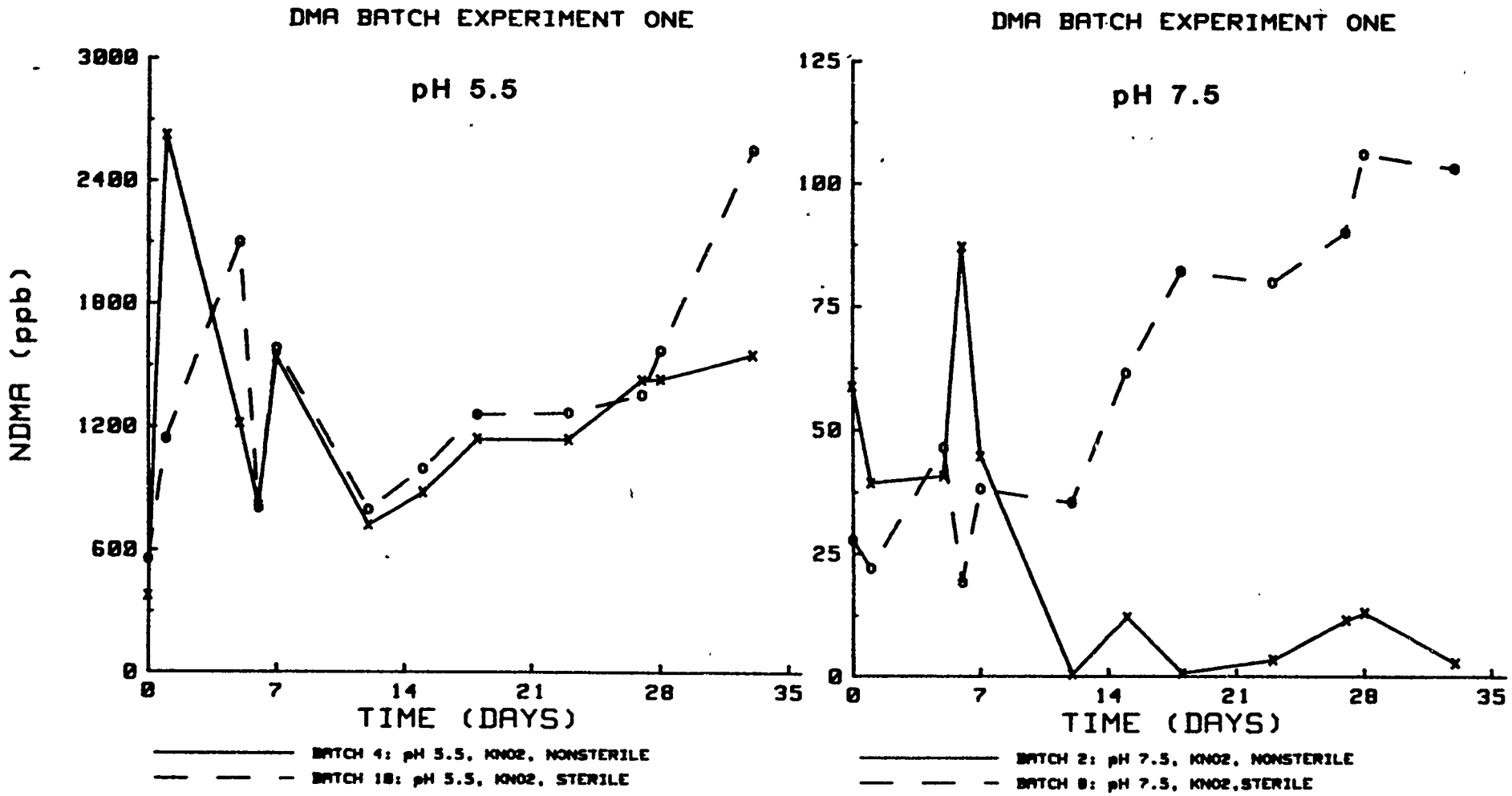


Figure 1. Formation of NDMA in the first DMA batch experiment with nitrite

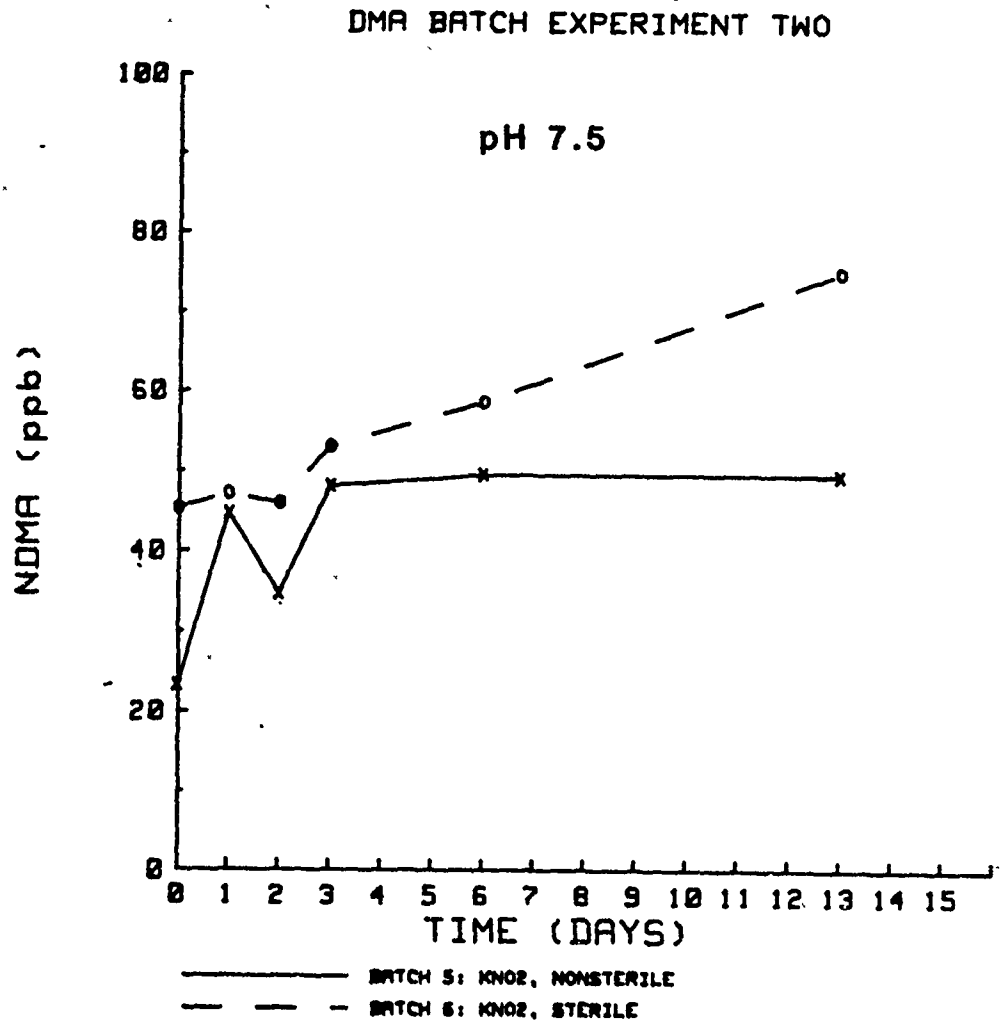


Figure 2. Formation of NDMA in the second DMA batch denitrification experiment at pH 7.5

NDPA was detected in two flasks, 4 and 10, which contained nitrite salt at pH 5.5 under sterile and active conditions, respectively. The levels of NDPA detected are listed in Table 6 and there appears to be no difference between the active and sterile flasks.

Table 4. Denitrification Efficiency and pH Measurements taken from DPA Batch Systems[a]

Flask	Initial pH	Final pH	Final nitrate Concentration (ppm)	Percent Denitrification
1	7.5	8.6	613	50
2	7.5	7.9	-[b]	-
3	5.5	6.8	188	85
4	5.5	5.9	-	-
5	7.5	7.6	260	79
6	7.5	7.6	-	-
7	7.5	8.2	1499	0
8	7.5	8.9	-	-
9	5.5	6.2	1280	0
10	5.5	6.1	-	-
11	5.5	7.1	386	68
12	5.5	6.0	-	-

[a] The initial nitrate concentration was 1,200 ppm

[b] Flasks initiated with nitrite salts

Table 5. GC Analysis for DPA and Methanol Levels in Batch Denitrification Systems

Flask	DPA[a] Final Concentration (ppm)	DPA[a] % Breakdown	Methanol[b] Final Concentration (ppm)	Methanol[b] % Breakdown
1	113.8	0	378.3	62.2%
2	98.1	1.9	65.7	93.4
3	57.0	43.0	2.1	99.8
4	123.2	0	757.5	24.2
5	0	-	43.6	95.6
6	0	-	34.3	96.6
7	98.5	1.5	731.5	26.8
8	87.6	12.4	483.7	51.6
9	99.4	0.6	883.3	11.7
10	102.5	0	668.8	33.1
11	0	-	6.7	99.3
12	0	-	756.8	24.3

[a] Initial concentration was 100 ppm

[b] Initial concentration was 1,000 ppm

Table 6. NDPA Formation in the DPA Batch Denitrification Experiment

Days	Flask (ppm) 4	10
0	B.D.[a]	B.D.
6	B.D.	trace[b]
13	trace	trace
21	0.19	0.19
30	1.10	1.06
37	0.69	0.69

[a] Below detection (20 ppb)

[b] Could not be quantified

The presence of NDPA was confirmed by GC/MS analysis. The NDPA peak was collected from HPLC, the acetonitrile evaporated, and the remaining aqueous solution was passed through a C-18 SEP-PAK (Water's Assoc., Milford, MA). The GC/MS analysis revealed m/z 70 and m/z 43 as the most prominent ions. At high concentration the parent ion m/z 130 is detected. Concentrations of 430 ppb and 300 ppb were detected in the two samples (flasks 4 and 10). At the conclusion of the experiment, the contents of the remaining flasks from this batch DPA experiment were concentrated with the SEP-PAK cartridge as described. No NDPA was detected in any of these flasks after concentration and analysis by HPLC.

Once established, the continuous-denitrification system with the three amines denitrified at 70% to 99% efficiency. During the three months of activity, the average influent concentrations of DPA and DBA were 135 and 149 ppm, respectively, as determined by GC. The effluent concentrations ranged between

87 ppm and 119 ppm for DPA and 34 ppm and 83 ppm for DBA. This is equivalent to a 35.6% to 12.5% degradation of DPA and 77.2% to 44.3% degradation of DBA. Analysis by HPLC revealed no detectable NDPA or NDBA (>20 ppb) in influent or effluent samples over the duration of the experiment. In an attempt to detect very low levels of these nitrosamines, one liter of effluent was reduced to a 10-mL volume in a rotary evaporator, and in another case, one liter of effluent was passed through C-18 SEP-PAK cartridges and eluted with one mL methanol. In neither case were any nitrosamines detected despite the 100- and 1,000-fold concentrations of samples, respectively. NDEIA was monitored by HPLC and peaks corresponding to the retention time of a standard sample of NDEIA were identified and when the unknown was spiked with a standard, only one peak was still evident on the chromatograms. However, upon analysis of this apparent NDEIA peak by GC/MS, no evidence for NDEIA was found. Similarly, results of analyses by Thermo Electron Corp. also confirmed that no detectable NDPA, or NDBA or NDEIA were present in this continuous flow denitrification system. However, NDMA was detected in influent and effluent samples from this system as indicated in Table 7. The NDMA was identified by HPLC and confirmed by results from Thermo Electron Corp. From combined effluent samples (days 4, 12, 15, 16) 19 ppb and 53 ppb NDMA were detected in influent and effluent samples, respectively. The results in Table 8 indicate a very variable concentration of NDMA in both influent and effluent samples.

Table 7. NDMA from the Continuous Denitrification System

Days	Influent NDMA (ppb)	Effluent NDMA (ppb)
12	139.0	1120.0
24	123.0	58.2
33	B.D.[a]	133.5
54	B.D.	B.D.
60	B.D.	37.7
66	B.D.	7.5
68	B.D.	18.3
73	trace	137.5
87	trace	165.1
89	B.D.	B.D.

[a] Below detection (1 ppb)

HPLC analysis of influent samples from the TEAN continuous flow denitrification system indicated contamination with low ppb levels of NDElA, while effluent samples appeared free of detectable NDElA. The presence of NDElA in the influent was due to the contaminated stock solution of TEAN. The stock TEAN (50% by weight) contained 8.4 ppm NDElA as determined by analysis by Thermo Electron. The fact that NDElA was not detectable in effluent samples from the TEAN system indicates that the concentration of NDElA was reduced by passage through the denitrification system, presumably due to biodegradation.

Table 8. Concentration of NDELA in Samples from the Continuous Denitrification System with TEAN

Influent	Effluent
3 to 17	N.D.[b]

[a] ppb

[b] Not detected (< 1 ppb)

Influent and effluent samples from the denitrification system with TMAN contained significant concentrations of NDMA (Figure 3). The presence of NDMA in the influent was due to contamination of stock TMAN with 2.8 ppm NDMA. Upon passage through the denitrification system there was little reduction in NDMA concentration indicating that this nitrosamine was not degraded. At the same time, there was a variable increase in NDMA concentrations in effluent samples compared with influent concentrations, probably indicating NDMA formation under these conditions.

In the denitrification system with DMA there was little degradation of NDMA. No conclusion regarding NDMA formation can be made because of significant contamination of influent samples with NDMA due to contaminated DMA stock solutions. The NDMA contamination of stock DMA may have arisen during product manufacture or storage.

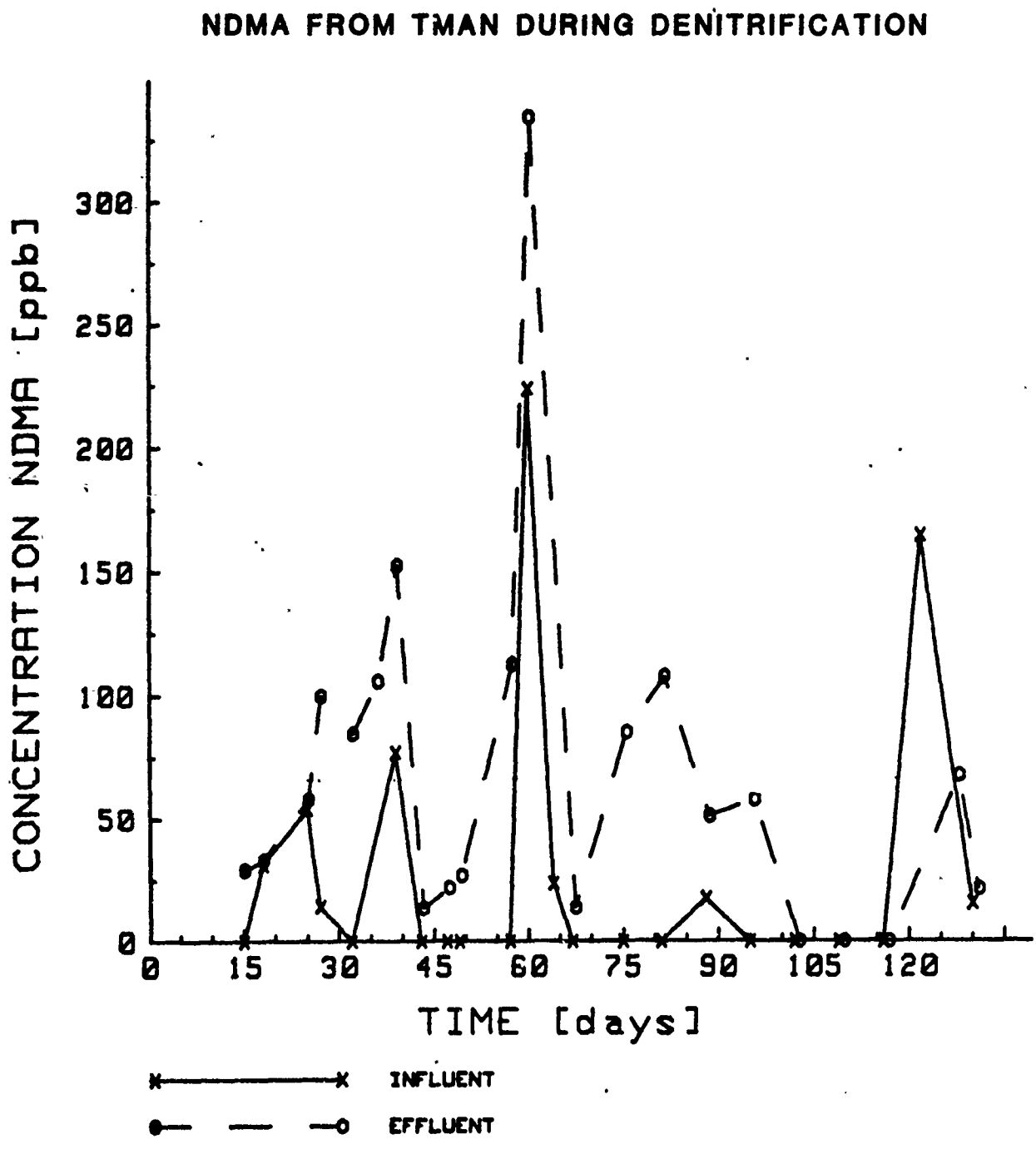


Figure 3. NDMA detected in influent and effluent samples from the denitrification system containing TMAN

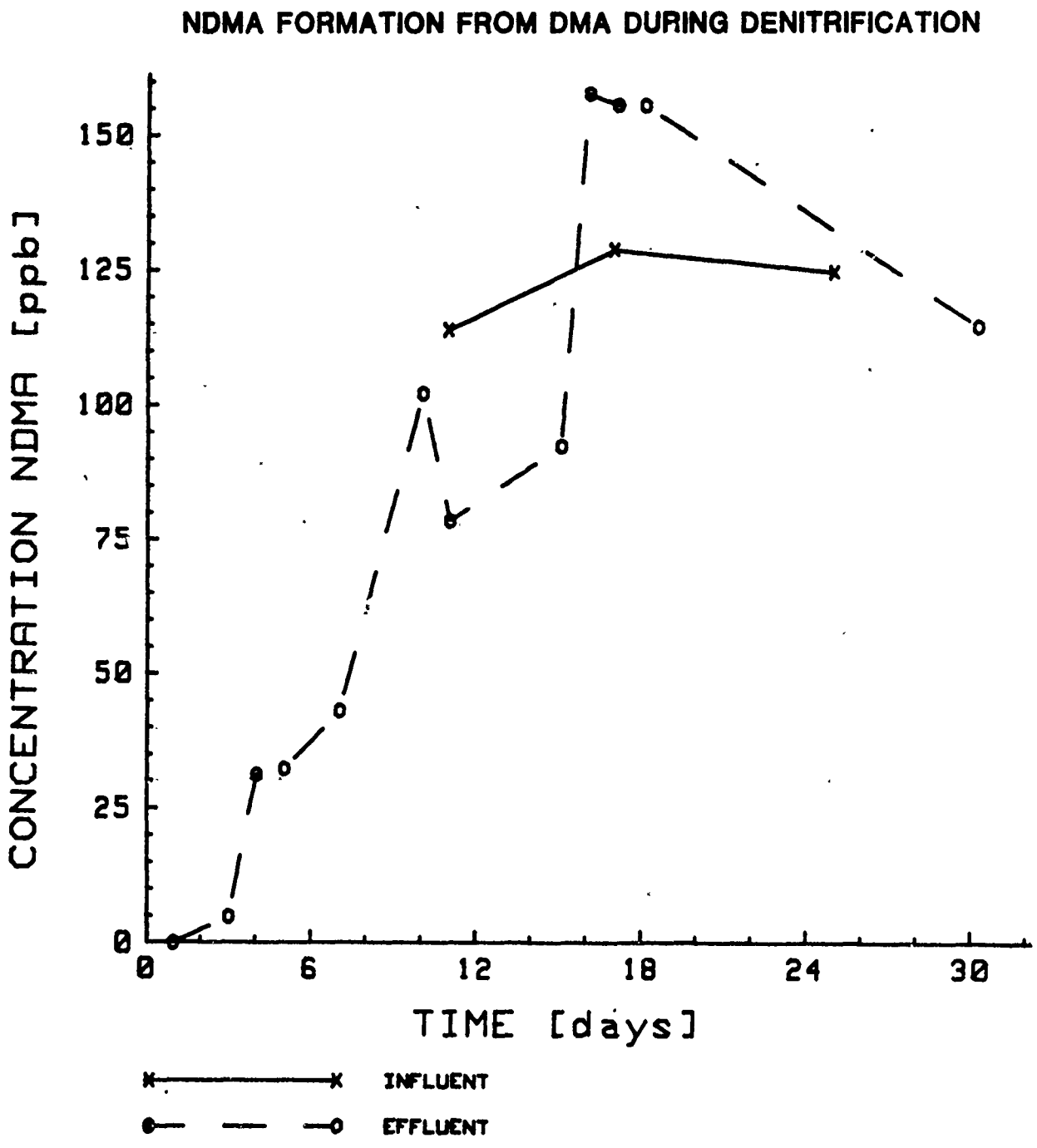


Figure 4. Formation of NDMA from DMA in influent and effluent samples from the denitrification system

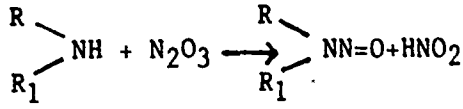
DISCUSSION

Results from batch culture studies indicate that low concentrations of NDMA but not NDPA can potentially form under denitrification conditions (alkaline pH, anaerobic, high nitrate). The formation of NDMA is not biologically mediated in these systems because for the most part concentrations formed in the biologically active systems are no higher than those in the sterile batches maintained under the same conditions. In some batch systems under acid conditions, much higher concentrations of nitrosamines formed than in comparable alkaline denitrification batch systems. This result would be expected based on previous work in the literature regarding the effect of pH on chemically mediated nitrosation reactions. For example, Mirvish[13,14] has demonstrated that the reaction rate for formation of NDMA increases 10-fold for each unit drop in pH down to pH 3.36. The overall reaction rate is proportional to the concentration of free amine and the square of the concentration of nitrous acid (equations 1 and 2 show the effect of pH on the reaction rate). The active nitrosating species is not the nitrous acid, which formed from nitrite, but nitrous anhydride, or a number of other compounds. The nitrosation reactions are illustrated in equation 3 and 4.

$$NO_2^- + H_2O \underset{OH^-}{\overset{H+}{\longleftrightarrow}} HNO_2 + OH^- \qquad (1)$$

$$\begin{matrix} R \\ \diagdown \\ \quad NH \\ \diagup \\ R_1 \end{matrix} + H_2O \underset{OH^-}{\overset{H+}{\longleftrightarrow}} \begin{matrix} R \\ \diagdown \\ \quad NH_2^+ \\ \diagup \\ R_1 \end{matrix} + OH^- \qquad (2)$$

$$2HNO_2 \longleftrightarrow N_2O_3 + H_2O \qquad (3)$$



(4)

In general, attempts to simulate denitrification in batch systems met with only limited success, as evidenced by the low denitrification efficiency. This outcome contrasts with the continuous flow denitrification systems, where efficiency of denitrification was high throughout most of the study.

The results from the continuous studies indicate that there is little potential for the formation of NDElA, NDPA, or NDBA under denitrification conditions. This fact supports the results from batch culture experiments; where no evidence was found for the formation of NDPA at alkaline pH. However, low concentrations of NDMA were detected in effluents from the continuous denitrification system with three amines (DElA, DPA, DBA) in the influent.

It is clear from these studies that of the amines tested NDElA, DPA, and DBA do not tend to form their corresponding nitrosamines in an alkaline environment under denitrification conditions, while NDMA is detected under these conditions in both batch and continuous cultures. The concentrations of NDMA found under denitrification conditions are considerably lower than those formed under acidic conditions. The fact that NDMA was detected in ppb concentrations is a cause for concern, but the following mitigating circumstances should be noted: NDMA was present as a contaminant in influent samples and its concentration in the output was variable. This result leaves some question regarding the extent and mode of NDMA formation within the

continuous systems. From the batch culture studies it is clear that the formation of NDMA is not biologically mediated, but once formed it is recalcitrant to biodegradation. This distinction can not readily be made for the continuous system.

Chemically mediated nitrosation reactions at alkaline pH have been reported that utilize catalysts such as formaldehyde.[15] Without these catalysts there is no detectable nitrosamine formation above pH 7.5. In this connection formaldehyde has been identified as a degradation product during the biotransformation of trimethylamine by Hyphomicrobium (Figure 5).[18]

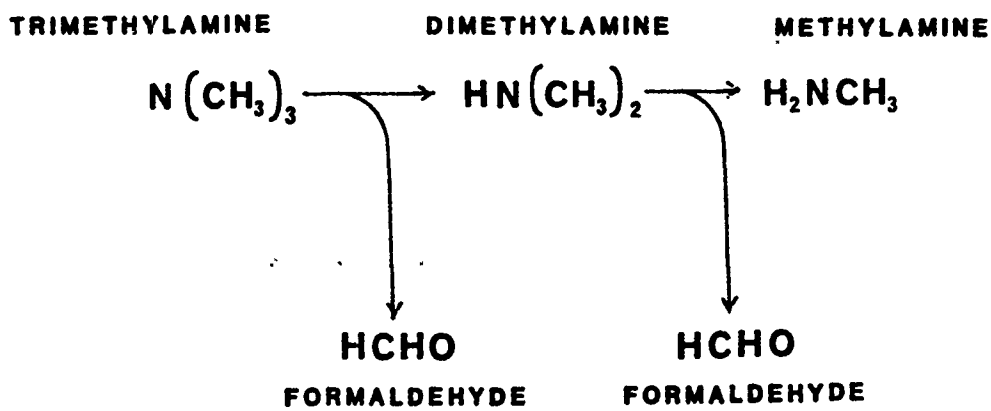


Figure 5. Formation of formaldehyde during biotransformation of trimethylamine and dimethylamine by Hyphomicrobium (from Harder et al., 1978).

[18] Harder, W. and M. M. Attwood. Biology, physiology and biochemistry of Hyphomicrobia. p. 303-359. In A. H. Rose and J. G. Morris (eds.) Advances in Mycrobial Physiology. Vol. 17. Academic Press, NY, 1978.

In our denitrification system with TMAN, Hyphomicrobium sp. was identified as the predominant microorganism. However, formaldehyde was not detected in effluents from the system using the assay system described by McCormick, et al.[19] Despite the failure to detect formaldehyde in the continuous system, it is most probably present as a transitory intermediate and therefore able to catalyze production of the low concentrations of NDMA detected. Another possible explanation is the presence of alternative catalysts to mediate the reaction.

The formation of the nitrosamines in the continuous denitrification system does require microbial mediation in that nitrate must be enzymatically reduced to the active nitrite species. This biotransformation occurs intracellularly.[20]

The nitrosation reaction that presumably must occur intracellularly as well, due to the location of the nitrate reduction activity and subsequent nitrite reductase, may not be subject to the alkaline pH of the surrounding medium environment. The intracellular environment presumably harbors a more favorable chemical and physiological environment for the formation of nitrosamines whether or not catalysts like formaldehyde are actively produced.

[19] McCormick, N. G., J. H. Cornell, and A. M. Kaplan. Biodegradation of hexahydro-1,3,5-trinitro-1,3,5-triazine. Appl. Environ. Microbiol. 42(5): 817-823, 1981.

[20] Knowles, R. Denitrification. Microbiol. Reviews. 46(1): 43-70, 1982.

CONCLUSIONS

The potential for nitrosamine formation in denitrification systems was evaluated. The nitrosamines corresponding to the amines of interest (diethanol-dipropyl- and dibutyl-) were not detected in these biological treatment systems. However, N-nitrosodimethylamine (NDMA) was detected in ppb concentrations in many of these studies. The significance of NDMA is not clear due to the formation or presence of NDMA in some influent samples and the variable concentrations detected in the denitrification systems. However, formation of this nitrosamine, even at ppb concentrations, is of significant concern. Batch studies indicated that the formation of NDMA was not biologically mediated at alkaline pH. A mechanism for its generation by reaction involving catalysis by microbially produced formaldehyde is suggested for the continuous flow denitrification system. The influence of the intracellular environment in these transformations is discussed.

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