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THE FATE OF HEXAHYDRO-1,3,5-TRINITRO-1,3,5-TRIAZINE (RDX) AND RELATED COMPOUNDS IN ANAEROBIC DENITRIFYING CONTINUOUS CULTURE SYSTEMS USING SIMULATED WASTE WATER

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CONTAMINATION	WASTE WATER	RDX
MICROBIOLOGY		HMX

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

This study presents findings that indicate the potential for biological treatment of waste waters from the manufacture of RDX/HMX. It is demonstrated that a continuous culture system operating under anaerobic conditions is active in decreasing the concentrations of RDX, HMX, SEX, and TAX as well as high concentrations of nitrate from simulated waste waters.

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PREFACE

Waste water resulting from the manufacture of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) contains low levels of these compounds, their acetylated derivatives and higher concentrations of nitrate. The objective of the present investigation is to determine the feasibility of a proposed process involving the concurrent removal of these contaminants using microbiological denitrifying systems. This report describes studies conducted on the biological fate of these compounds and on denitrification in anaerobically operated continuous cultures using simulated waste streams.

This work was supported bv the U.S. Army Toxic and Hazardous Materials Agency under project number P112.02.01.

We wish to thank Mr. Savas Danos, Mrs. Julia M. Geller, Ms. Debbie Rollins, Mr. Todd Peltonen, and Mr. Barry Greene for their technical assistance.

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INTRODUCTION

During the production of the two cyclic nitramine explosives, hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) (Fig. 1), various compounds are released into the environment via the waste streams that result either from the manufacture and blending processes or from load, assembly and pack operations. In addition to the principal products, other compounds present in waste waters include the N-acetylated derivatives of RDX and HMX (AcRDX and AcHMX, respectively*), as well as acetic acid, cyclohexanone, and nitrate.

The literature on the biodegradation of RDX and related compounds is not extensive. Soli[1] reported that the disappearance of RDX brought about by mixed cultures of purple photosynthetic bacteria was favored by anaerobic conditions. Jackson et al.[2] reported that RDX disappeared in a chemostat under anoxic conditions and further proposed that any planned treatment facility for RDX/HMX waste water should contain an anaerobic denitrification step. The addition of river sediments appeared to stimulate the disappearance of RDX in samples of river water containing either 5.5 or 11.5 ppm of RDX[3]. Spanggord et al.[4] reported that no RDX transformation occurred anaerobically in Holston River water (Holston, TN) in the absence of added yeast extract, and that a cometabolic process was probably involved in RDX biodegradation. Our laboratory has reported that the biotransformation of RDX in a nutrient rich medium occurs only under anaerobic conditions[5] .

Since munitions waste streams almost always contain nitrate it would be desirable to simultaneously remove nitrate and other pollutants by a microbial denitrification step. Accordingly, this study of the effect of denitrifying microorganisms on mixtures of RDX, HMX, AcRDX, AcHMX and nitrate, in continuous culture, using various nutrient media, was undertaken.

MATERIALS AND METHODS

Cultures and Media: Biodegradation studies were carried out in nutrient broth (Difco), in a medium consisting of 0.005 M K_2HPO_4 supplemented with 0.3% (v/v) of beet-sugar molasses, or in a basal salts medium consisting of 0.87 g of K_2HPO_4, 0.50 g of $MgSO_4 \cdot 7H_2O$, 0.05 g of NaCl, 0.015 g of $CaCl_2$, 0.01 g of $FeCl_3 \cdot 6H_2O$, 0.01 g of $CuSO_4 \cdot 7H_2O$, 0.01 g of $MnSO_4 \cdot H_2O$, and 0.002 g of Na_2MoO_4 per liter, supplemented with methanol. To these media were added 0.04 M KNO_3 for a nitrate concentration of 2480 mg/L (ppm). Inocula for the anaerobically incubated continuous culture systems were prepared by diluting anaerobic

*Abbreviations used: hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX); octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX); hexahydro-1-N-acetyl-3,5,-dinitro-1,3,5-trazine (AcRDX, TAX); octahydro-N-acetyl-3,5,7-trinitro-1,3,5,7-tetrazocine (AcHMX, SEX).

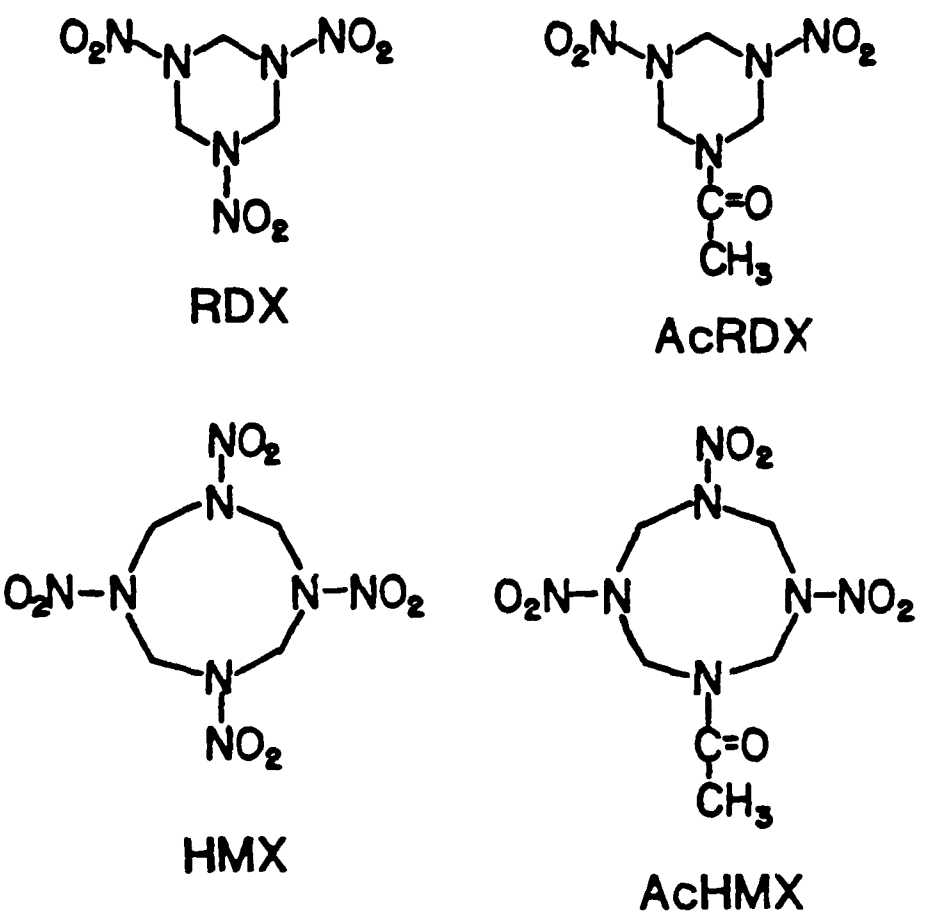

Figure 1. Chemical formulae for RDX, HMX, AcRDX and AcHMX.

sewage sludge, obtained from the Nut Island Sewage Treatment Plant, Boston, MA, with two volumes of distilled water and filtering through glass wool. A 2% (v/v) inoculum was used.

Continuous Culture Studies: Fig. 2 describes a continuous culture system (Type FL). A two-liter flask containing the nutrient medium sufficient for two liters was dissolved in one liter of water and sterilized by autoclaving. A mixture of explosives consisting of 30 mg of RDX/L, 10 mg of HMX/L, 20 mg of AcRDX/L and 20 mg of AcHMX/L was added to a second 2-liter flask and dissolved in about 20 mL of acetone with warming. The acetone was evaporated by a stream of nitrogen, leaving a thin film of material with greatly increased surface area on the inside surface of the flask. One liter of hot water in a 2-liter flask, previously sterilized by autoclaving, was poured into the flask containing the compounds and the contents were brought to a boil while stirred vigorously until all the compounds were in solution. The solution was filtered through 0.45 u pore size Nylon-66 membrane filters (Rainin Instrument Co., Inc.. Woburn, MA) into a sterile flask, allowed to cool to approximately 50° C and aseptically added to the liter of sterile double strength nutrient medium. This provided two liters of nutrient medium in reservoir A. Rainin Rabbit peristaltic pump (B) (Rainin Instrument Co., Inc., Woburn, MA) was used to pump the fluid through several medium break tubes (C) into the stage 1 reaction vessel (D) and from there into the stage 2 reaction vessel (E). The effluent was collected and monitored daily.

A second type of continuous culture system (Type NB) used a New Brunswick Model C30 Bioflo Fermenter (New Brunswick Scientific Co., Inc., Edison, NJ). As with the Type FL system the influent medium was prepared in two batches and aseptically combined in the influent reservoir. Sufficient nutrients for 7 liters were dissolved in 5 liters of water, and the appropriate amounts of explosives mixture for 7 liters were dissolved as described above in a total of 2 liters. The solution of explosives so prepared was aseptically added to 5 liters of nutrient medium to provide 7 liters of the complete medium.

With each system (FL and NB) the pump speed was adjusted to provide the desired retention time (the number of days required to collect a volume of effluent equivalent to the total volume of the reaction vessel(s).

Liquid Chromatography: Samples of effluent were centrifuged at 10,000 rpm for 5 min to clarify the solution and filtered through membrane filters as described above. The filtered samples were injected without further treatment into a Waters Model 2000A Liquid Chromatograph equipped with a uBondapak C_{18} column (Waters Associates, Inc., Milford, MA). The solvent system used to monitor the disappearance of RDX, HMX, AcRDX and AcHMX was 10% methanol in water; solvent flow was 2.5 mL/min; UV detector at 230 nm.

Nitrate Determination: The concentration of nitrate was measured with an Orion Model 901 Ionalyzer using an Orion Model 93-07 nitrate electrode (Orion Research, Inc., Cambridge, MA).

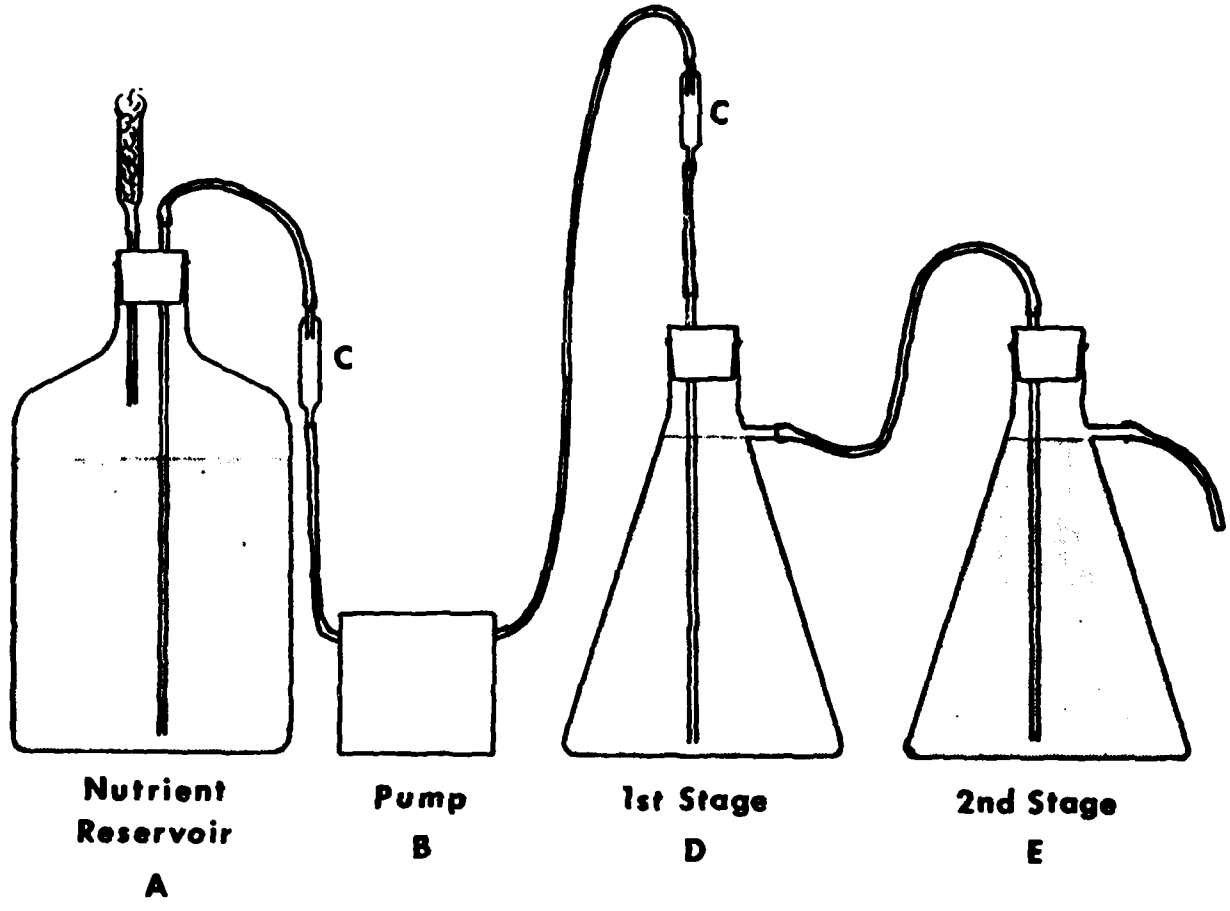

Figure 2. Type-FL continuous culture; (A) nutrient reservoir; (B) peristaltic pump; (C) medium break-tubes; (D) 1st stage reaction flask; (E) 2nd stage reaction flask.

Determination of Hydrazines: The procedure is a modification of a method reported by Abdou et al[6], which takes advantage of the reaction between salicylaldehyde and hydrazine. To a small test tube were added 0.9 mL of abs. EtOH, 0.1 mL of glacial acetic acid, 0.1 mL of sample, and 0.1 mL of 0.2 M ethanolic solution of salicylaldehyde. The contents were mixed and heated at 60°C for 20 min, 2.8 mL of abs. EtOH were added, the mixture was filtered through a Nylon-66 filter, 0.45 um pore size, and injected into the HPLC. The solvent used was 70% methanol in water for hydrazine, 50% methanol in water for 1,1-dimethylhydrazine, and the hydrazones were detected at 293 nm.

Extraction of Hydrazines: Approximately two liters of effluent were collected over a period of three weeks. The combined effluent was centrifuged at 10,000 rpm for 15 min to remove cells, and the supernatant fluid was adjusted to pH 10 with NaOH. The solution was distilled in a rotary evaporator (Brinkmann Instruments Co., Westbury, NY) at 50°C under 15 mm Hg (2.0 kPa). The distillate was collected in dilute HCl, evaporated to dryness in the same manner and taken up in a small amount of water for analysis.

Sediment Experiment: Table 1 shows the composition of the various media used in the sediment experiment. The basal salts recipe used was the one described above minus phosphate. The other components were: KNO_3, 0.04 M (2480 mg/L) for the first sediment experiment or 0.008 M (500 mg/L) for the second sediment experiment; K_2HPO_4, 0.04 M; Na_2S, 0.1 g/L; methanol, 1.69 mL/L; glucose, 1.87 g/L: sodium acetate, 1.7 g/L; $NaHCO_3$, 3.5 g/L; RDX, 15 mg/L; and either no sediment or 50 g of sediment per bottle. The components were dissolved in lake water and the bottles were filled to the neck for a total volume of 160 mL. Sediment #1 was a sandy sediment and sediment #2 was a rich organic sediment.

Oxidation-Reduction Potential (E_h) Measurements: E_h measurements were conducted with an Orion Model 901 Ionalyzer using an Orion Model 96-78 platinum redox electrode.

Gas Chromatography: Analyses of head space gases were performed on a Perkin-Elmer Model 3920 Gas Chromatograph equipped with a thermal conductivity detector. Helium carrier gas flowed at 20 mL per min through a nickel column (180 cm X 0.32 cm) packed with 100/120 Carbosieve S (Supelco, Inc., Bellefonte, PA). The detector temperature was 250°C and the column temperature was programmed at 8°C per min from 55°C to 175°C. For enhancement of the response to methane, a flame ionization detector was used.

Total Organic Carbon (TOC): TOC measurements were conducted on a Beckman Model 915B Tocamaster (Beckman Instruments, Inc., Carlsbad, CA). Samples were clarified by centrifugation, filtered through membrane filters, and acidified to pH 2 to liberate inorganic CO_2. Organic carbon was determined by injection of 20 uL of the acidified sample, conversion to CO_2 at 950°C, and detection with an IR detector.

RDX, HMX, AcRDX, and AcHMX: RDX and HMX were obtained from Holston Army Ammunition Plant (Holston, TN) as desensitized slurries in 50% ethanol/50% water. For use, small amounts were recrystallized from acetone. AcRDX and AcHMX were obtained through the auspices of Dr. David Rosenblatt, USAMBRDL, Fort Detrick, MD.

Table 1. Composition of Sediment Culture Bottles

Additions to bottle	Bottle Number											
	1	2	3	4	5	6	7	8	9	10	11	12
KNO_3	+	+	+	+	+	+	+	+	-	+	+	+
Basal salts	-	-	+	-	+	+	-	+	+	+	+	+
K_2HPO_4	-	+	-	+	+	-	-	+	+	+	+	+
Methanol	-	-	-	+	-	+	+	+	+	-	-	-
Glucose	-	-	-	-	-	-	-	-	-	+	-	-
Sodium acetate	-	-	-	-	-	-	-	-	-	-	+	-
$NaHCO_3$	-	-	-	-	-	-	-	-	-	-	-	+
Na_2S	+	+	+	+	+	+	+	+	+	+	+	+
RDX	+	+	+	+	+	+	+	+	+	+	+	+
Sediment	No sediment, sediment #1, or sediment #2											

RESULTS

Type-NB Continuous Culture Systems: The results of a continuous culture system (Type-NB) are presented in Figs. 3 to 7. RDX disappeared completely from a molasses or nutrient broth based system as seen in Figs. 3 to 5. The efficiency toward AcRDX, HMX and AcHMX declined in that order in a molasses system (Fig. 3), but increased for all compounds when the nutrient was changed from molasses to nutrient broth (Fig. 4). Activity toward both AcRDX and HMX reached > 90% disappearance from day 40 on (Fig. 4). After the nutrient broth was diluted to 50% of full strength at day 80 (Fig. 5) the efficiency declined to values similar to those observed with the molasses-based system. The retention times for the nutrient broth system varied between 10 and 14 days. The oxidation reduction potential (E_h) remained at -300 to -350 mV during the entire course of the experiment. Complete denitrification was observed at all times in this system (Figs. 6 and 7). The head space gas from the system described in Fig. 4 which had been actively carrying out 99% denitrification of 2400 ppm of nitrate with an E_h consistently around -350 mV, was examined for the presence of N_2, CO_2, CH_4, O_2 and N_2O. Only nitrogen and CO_2 were detected (Fig. 8).

Type-FL Continuous Culture System: The results of a second type of continuous culture system (Type-FL) using molasses as nutrient are presented in Figs. 9 to 11. From day 100 to day 140 (Fig. 9) the system was operated with only RDX and HMX in the influent. At that time shipment of AcRDX and AcHMX was received and these two compounds were added to the system (Fig. 9).

Disappearance of RDX increased to about 90% to 100%. Efficiency toward HMX increased to a high of ca. 85% disappearance at day 157 but then declined. A decline was also noted in the case of AcRDX and AcHMX. The highs noted for HMX, AcRDX and AcHMX may be partly due to the fact that the pump was not operating over a weekend on days 145 to 157, thus resulting in an artificially high value due to the increase in retention time to infinity. As the pumping operation was restarted, the system gradually reverted back to more realistic values.

At day 190 the Type-FL system was switched from molasses to the nutrient broth system illustrated in Fig. 10. RDX achieved a uniform 100% disappearance, but the other components did not attain the high values observed in the Type-NB nutrient broth system described in Fig. 4. Although the E_h was ca. -350 mV throughout this experiment and denitrification was 100%, the oscillations observed in Fig. 10 appeared to vary according to the fluctuations in the retention times (Fig. 11). The lowest efficiency was observed with AcHMX, which achieved a high of ca. 20% in the Type-FL nutrient broth system (Fig. 10).

A second Type-FL system using molasses as nutrient was studied. Fig. 12 shows that after an acclimation period of about three weeks the efficiency toward HMX, AcRDX and AcHMX increased gradually to day 75 (indicated by arrow B), at which point the supply of AcHMX and AcRDX was depleted. The percent disappearance of HMX continued to increase and at day 120 (arrow C) the nutrient was changed to 10% of full strength nutrient broth (0.8 g/L). A

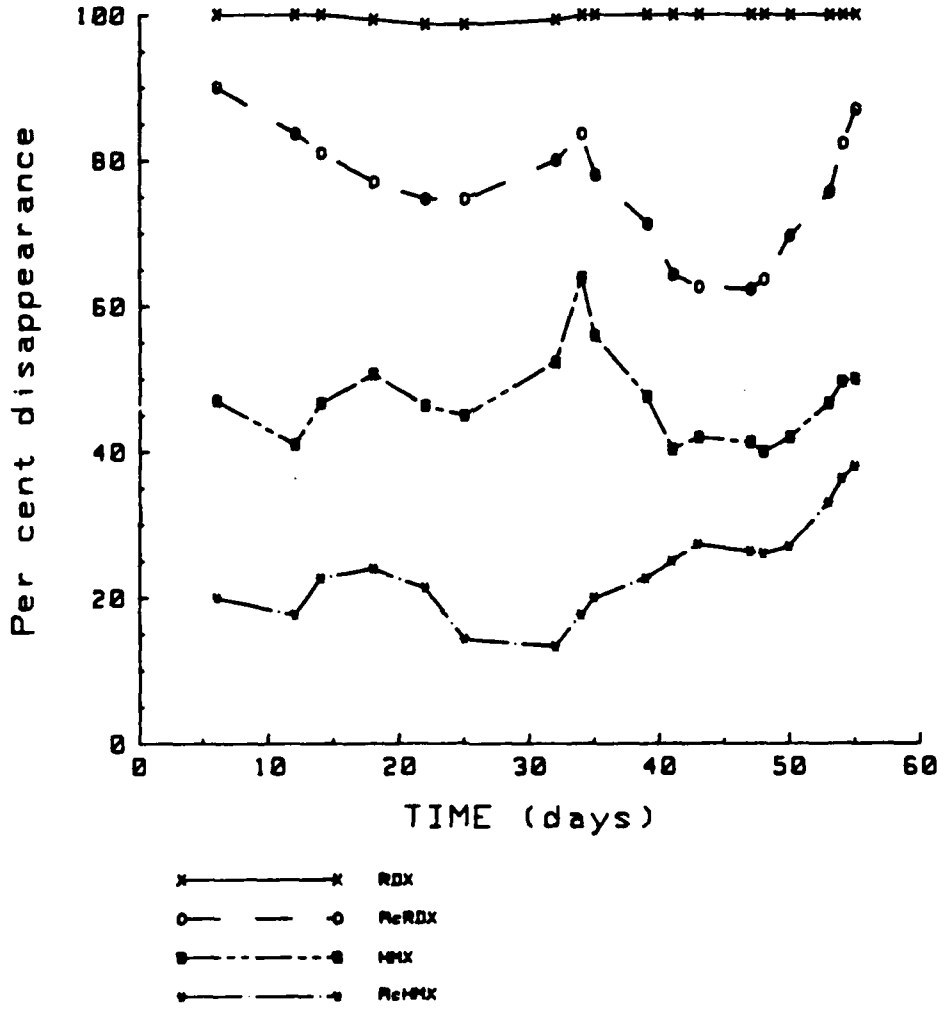

Figure 3. Disappearance of RDX, HMX, AcRDX and AcHMX in type-NB continuous culture system using 0.3% (v/v) molasses as nutrient.

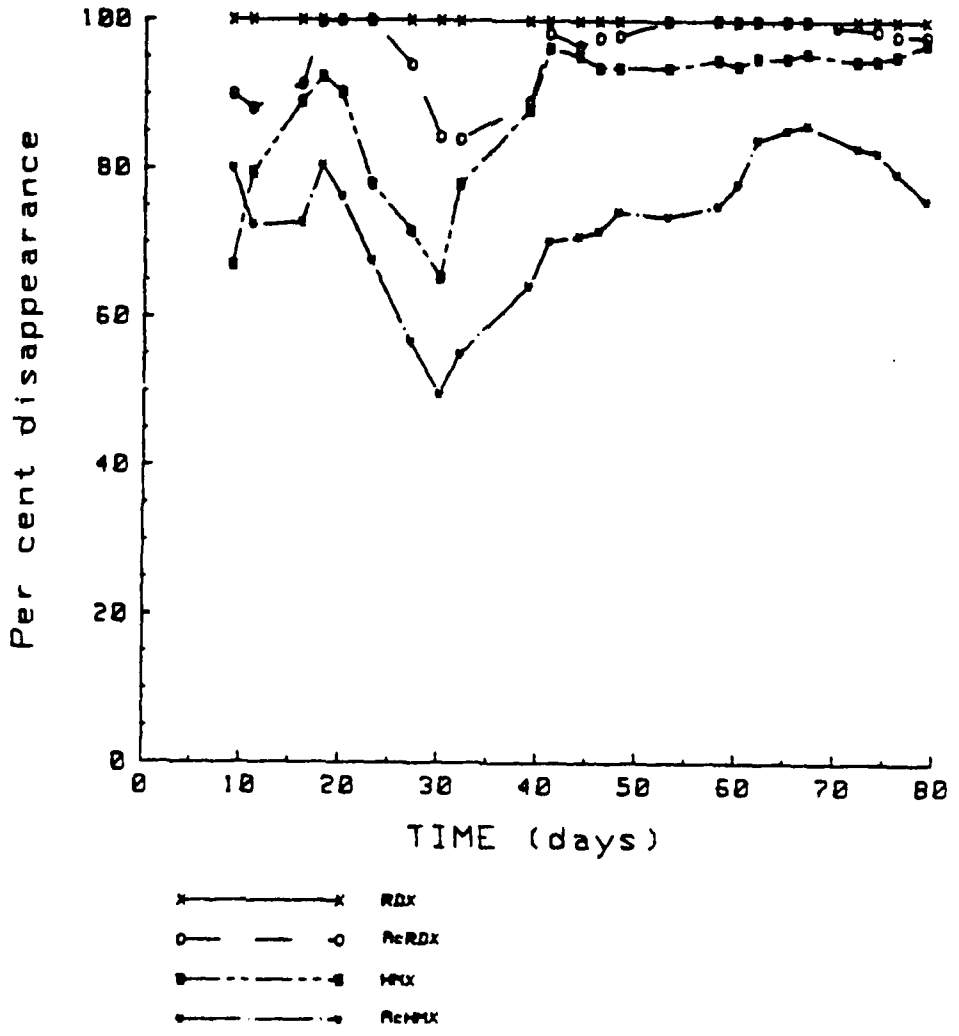

Figure 4. Disappearance of RDX, HMX, AcRDX and AcHMX in type-NB continuous culture system using 0.8% nutrient broth as nutrient.

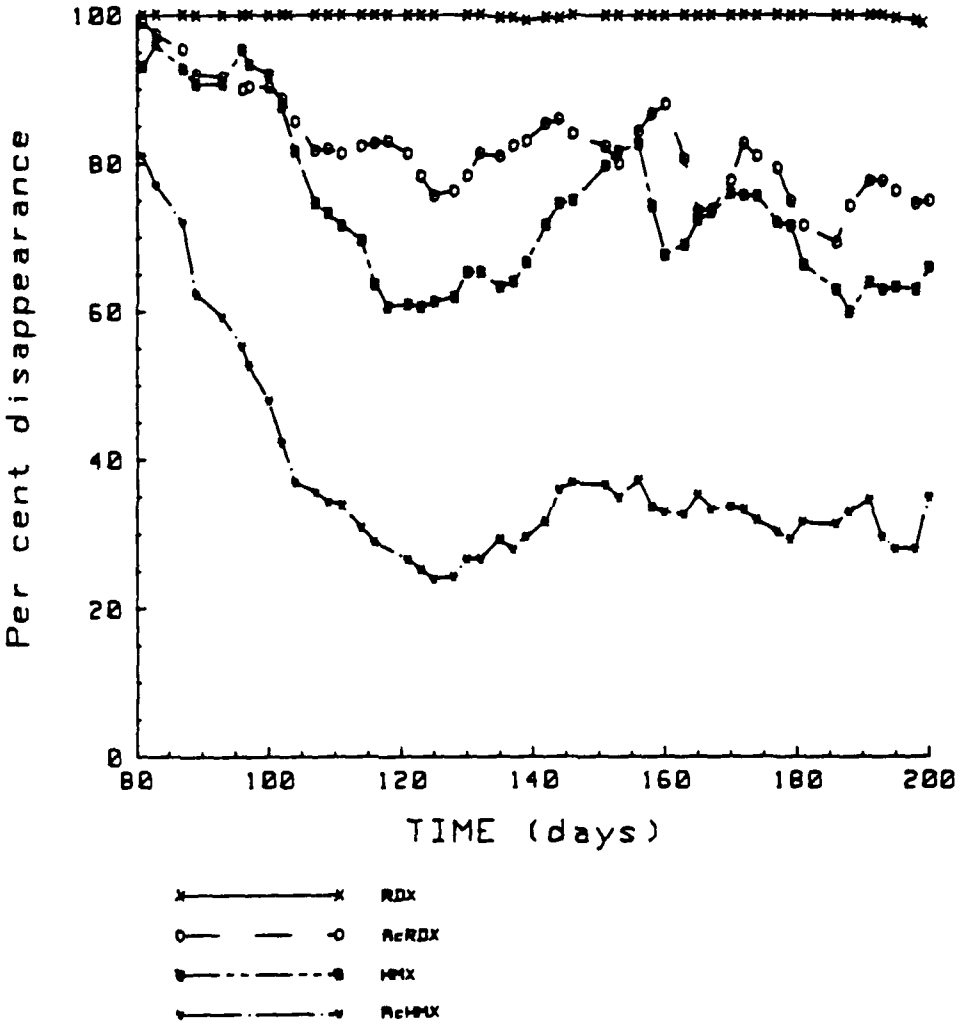

Figure 5. Disappearance of RDX, HMX, AcRDX and AcHMX in type-NB continuous culture system using 0.4% nutrient broth as nutrient.

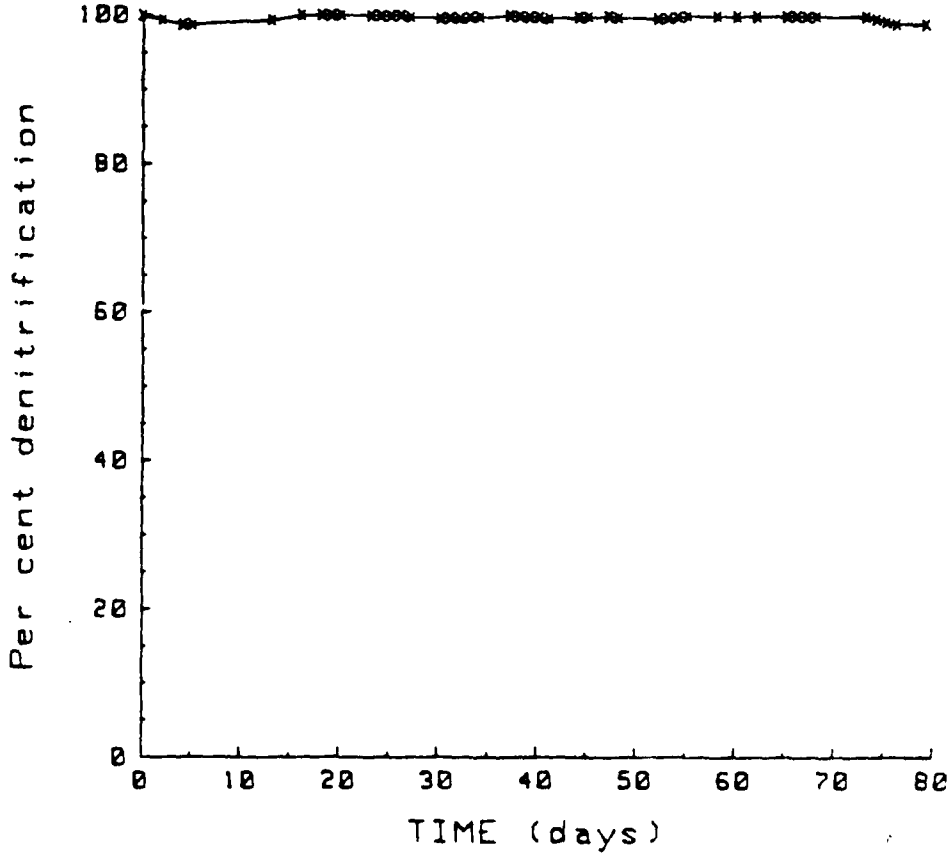

Figure 6. Percent denitrification in 0.8% nutrient broth type-NB continuous culture system.

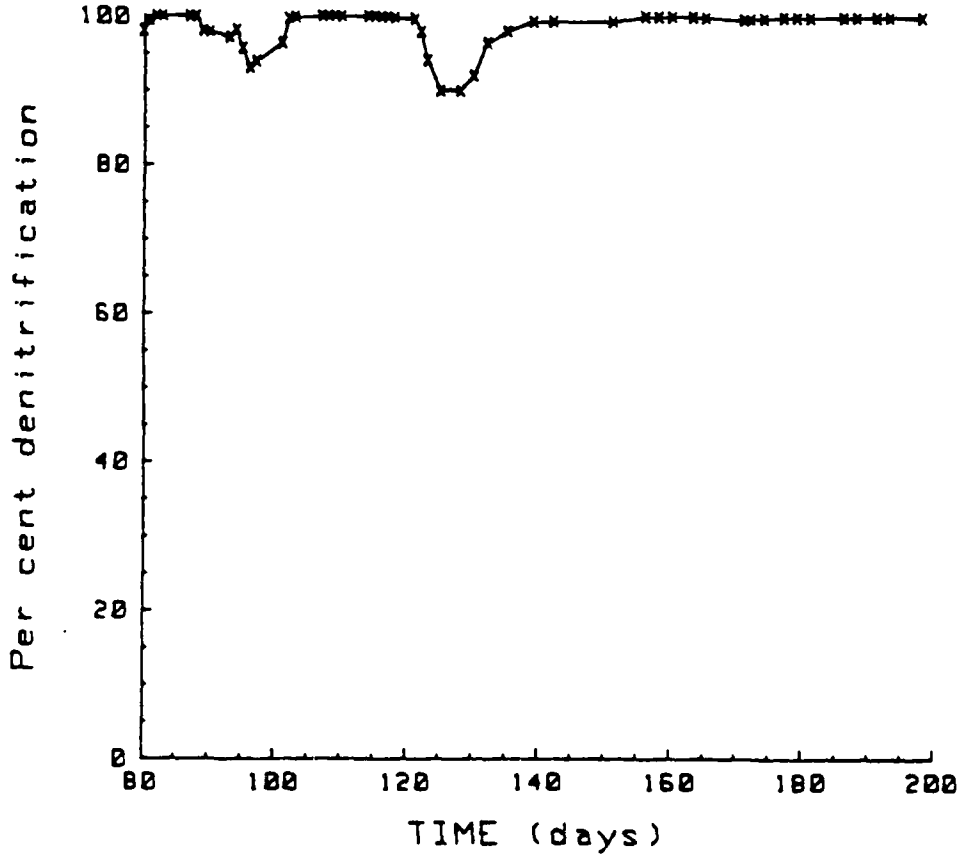

Figure 7. Percent denitrification in 0.4% nutrient broth type-NB continuous culture system.

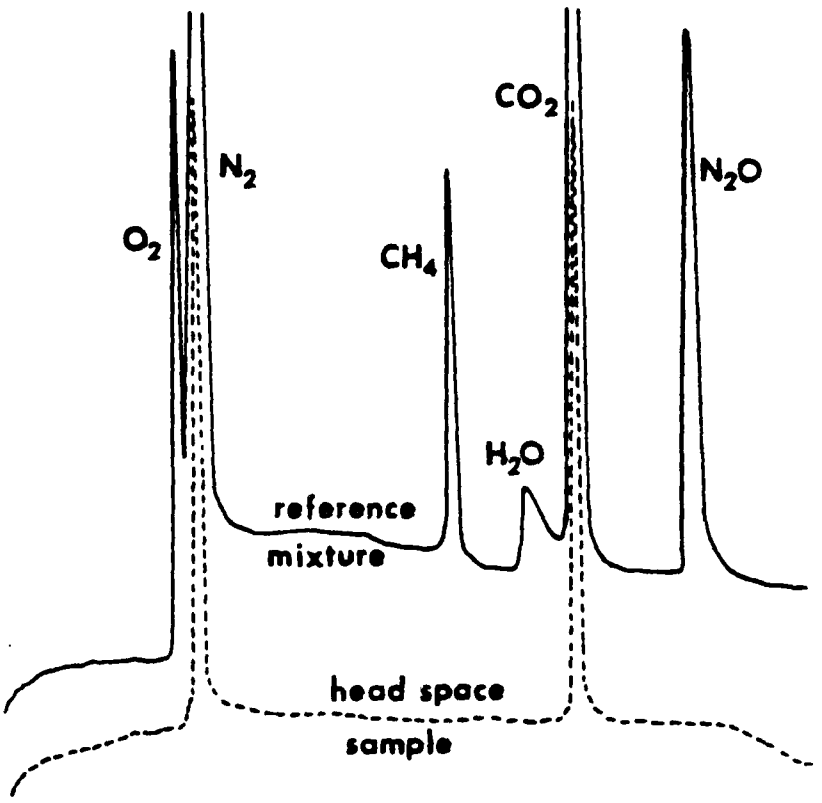

Figure 8. Gas chromatographic (GC) analysis of headspace gas from 0.8% nutrient broth type-NB continuous culture system.

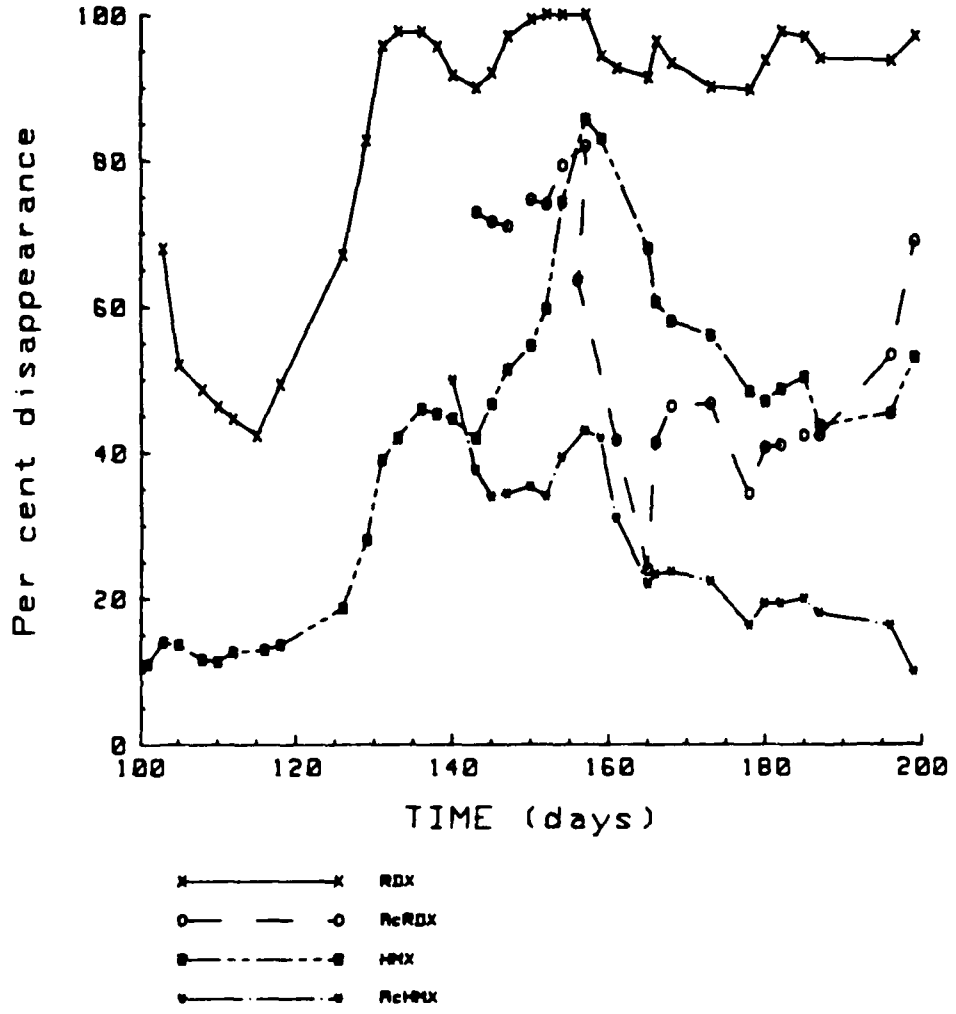

Figure 9. Disappearance of RDX, HMX, AcRDX and AcHMX in 0.3% molasses type-FL continuous culture system.

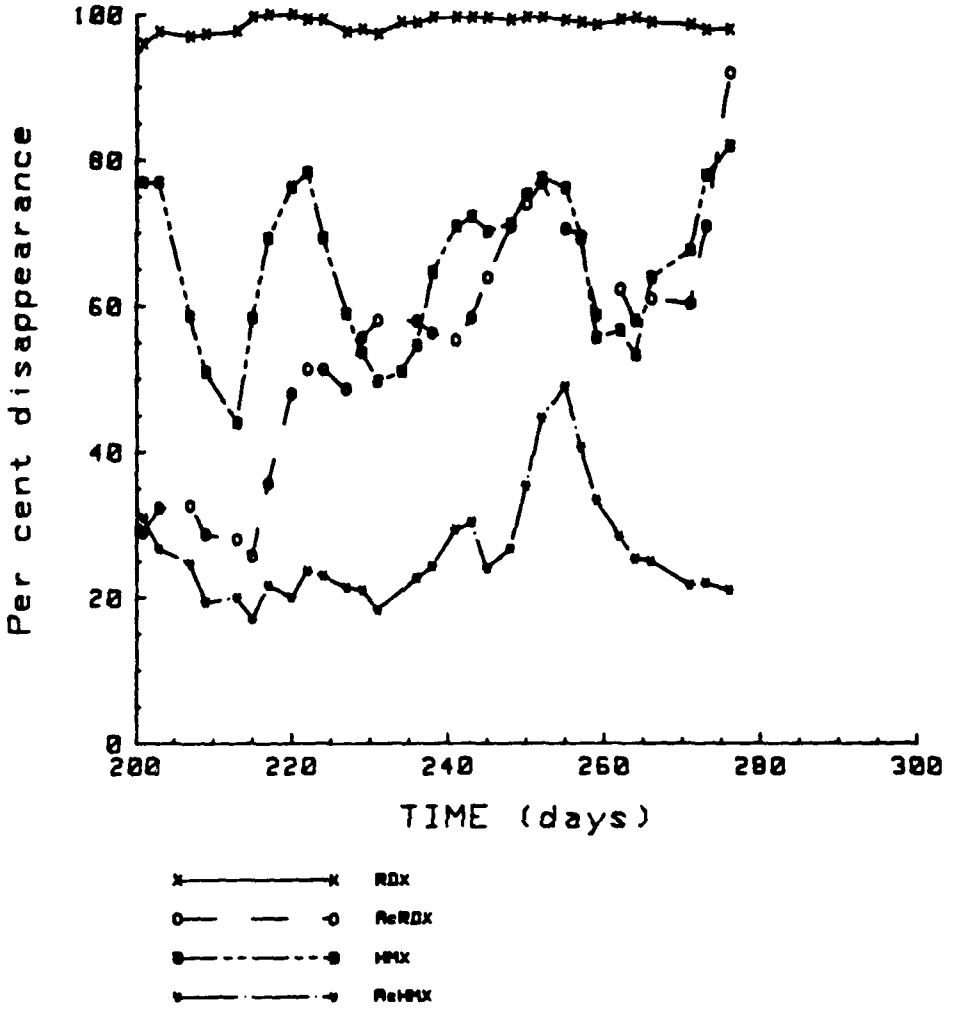

Figure 10. Disappearance of RDX, HMX, AcRDX and AcHMX in 0.8% nutrient broth type-FL continuous culture system.

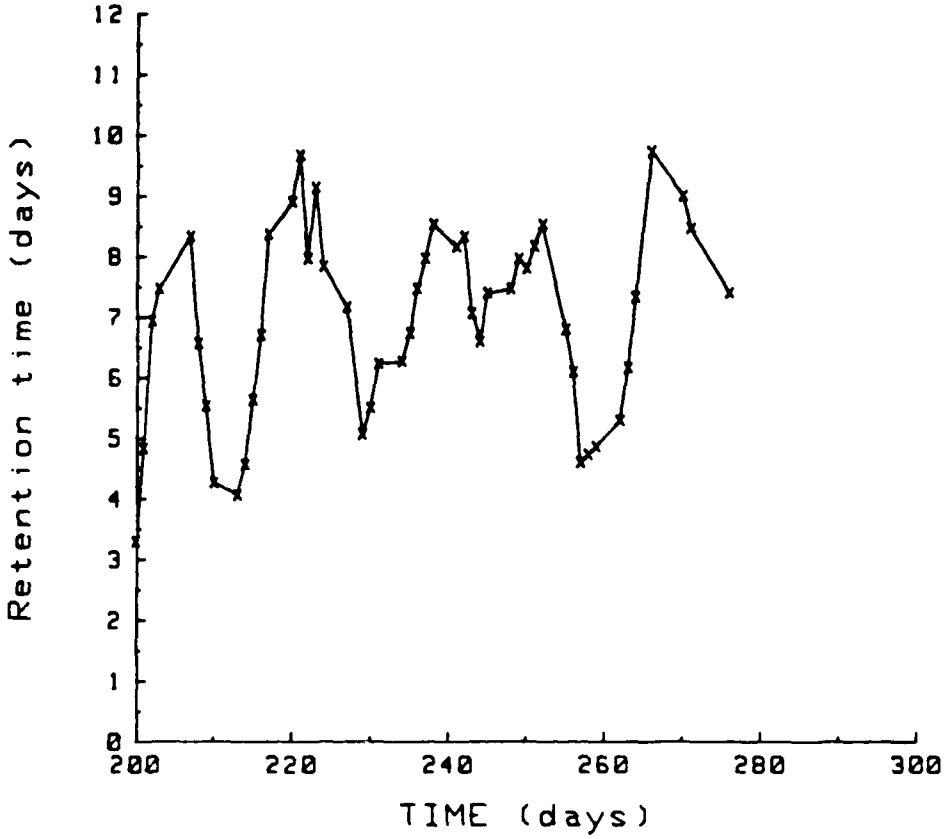

Figure 11. Average retention times for 0.8% nutrient broth type-FL continuous culture system.

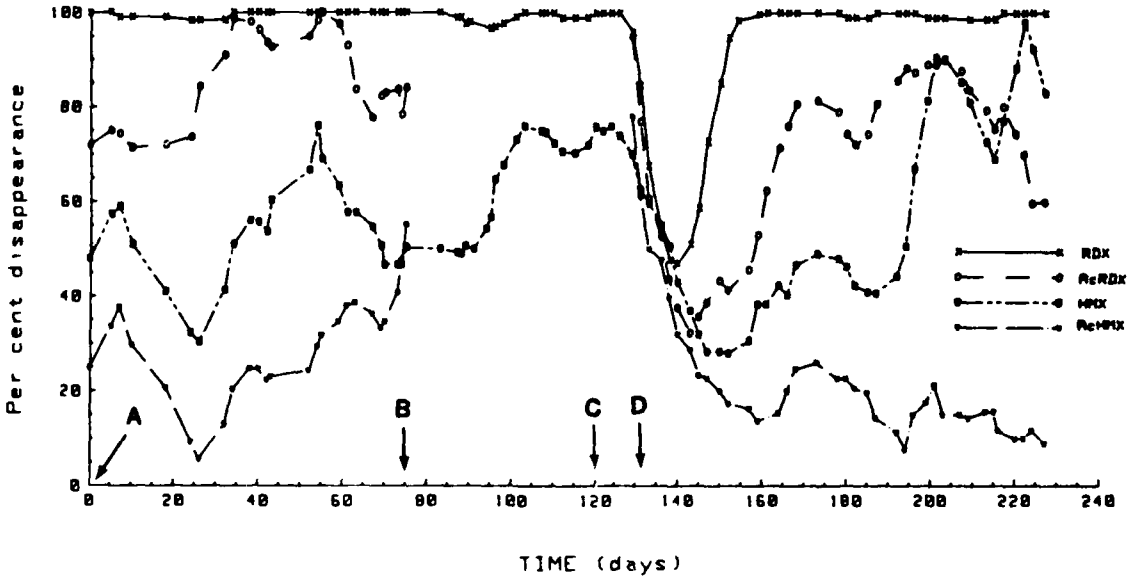

Figure 12. Disappearance of RDX, HMX, AcRDX, and AcHMX in a 0.3% molasses type-FL continuous culture system. (A) 0.3% molasses; (B) AcRDX and AcHMX discontinued; (C) 0.8% nutrient broth; and (D) AcRDX and AcHMX influent resumed, 0.3% molasses.

rather abrupt decrease in efficiency was noted which was also evident in the drop in percent denitrification (Fig. 13). At day 132 (arrow D) the influent feed containing AcRDX and AcHMX was resumed with a change from diluted nutrient broth back to molasses. A dramatic increase was noted in percent disappearance in RDX up to 100%, as well as in HMX and AcRDX. The efficiency of the system toward AcHMX continued to decrease to around 10%. There appeared to be no correlation between the minor fluctuations in retention times (Fig. 14) and the swings from a low percent disappearance to higher values observed in Fig. 12. During the course of the experiment the E_h remained around -350 mV.

Hydrolyzed Sludge Experiment: The results obtained with a Type-FL system using 20% acid-hydrolyzed sludge as nutrient source are reported in Fig. 15, point A. With the exception of AcHMX, the other components of the mixture increased rather rapidly to high values in percent disappearance. At around day 30 the percent disappearance of HMX, AcRDX and AcHMX dropped. At the same time the percent denitrification decreased from ca. 90% to ca. 40% as seen in Fig. 16, even though the E_h values remained around -350 mV during the entire experiment.

At day 86, point (B), the 20% acid-hydrolyzed sludge was adjusted to 10%. An almost immediate decrease in efficiency was noted which continued until day 120. While the decrease in denitrification during the period from day 20 to day 55 may reflect the fall-off in efficiency toward RDX, AcRDX, HMX and AcHMX during that period of time, there was an abrupt increase in denitrification after switching to 10% hydrolyzed sludge (Fig. 16). The decrease in percent disappearance of compounds continued until day 120 after which there was a general increase in overall efficiency up to day 163 (Fig. 15, point C), when the amount of nutrient was adjusted to 30% acid-hydrolyzed sludge. Here as before at the higher nutrient values the percent denitrification began to decrease while the efficiency toward the explosives increased to about the same as that observed with 0.8% nutrient broth. At no time did the E_h rise above -300 mV and the retention times oscillated slightly between 14 and 18 days.

One Type-FL system was set up containing 20% alkaline-hydrolyzed sludge. The kinetics of disappearance of the four compounds are seen in Fig. 17 and the percent denitrification in Fig. 18. The gradual decrease in values to about day 20 represents an acclimation of the new system to steady state values. It is apparent that alkaline hydrolysis does not promote the efficiency of the system as well as the use of acid-hydrolyzed sludge. The system equilibrated to about 40% disappearance of AcHMX, about 80% HMX, and 100% for both AcRDX and RDX. The E_h remained between -250 mV to -300 mV during the course of this experiment.

At the end of the experiment described in Fig. 5 (day 200) the nutrient source was changed to 10% unhydrolyzed sludge. The retention time was allowed to remain at the usual 10 to 14 days. Figs. 19 to 21 show the effects of using unhydrolyzed sludge. Over a period of 30 days the percent disappearance of compounds dropped to zero (Fig. 19), the percent denitrification dropped to zero (Fig. 20) and the E_h increased from around -300 mV to positive values (Fig. 21).

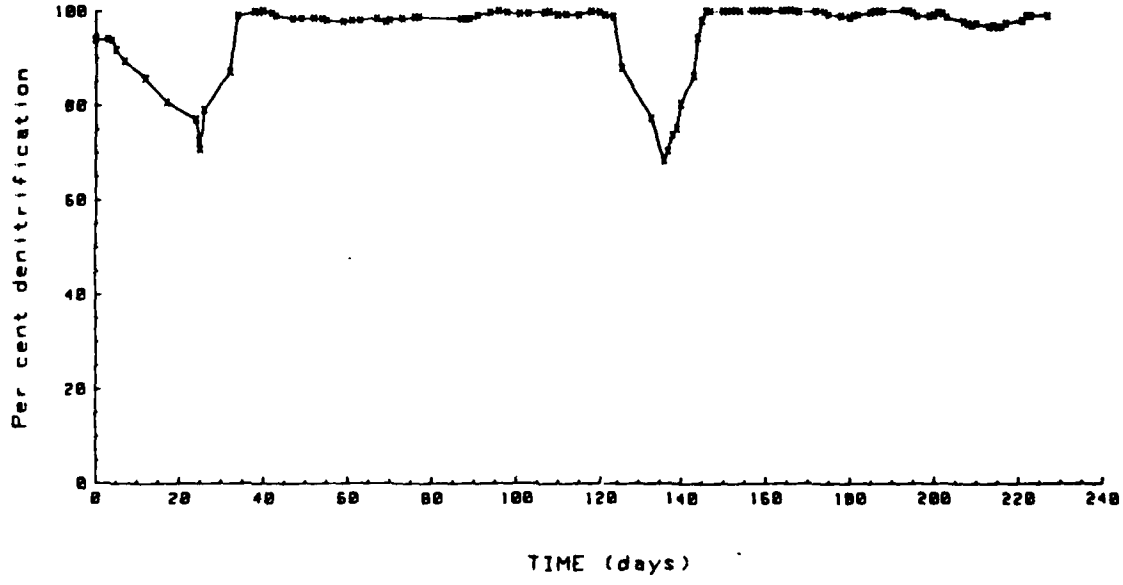

Figure 13. Percent denitrification in the system described in Figure 12.

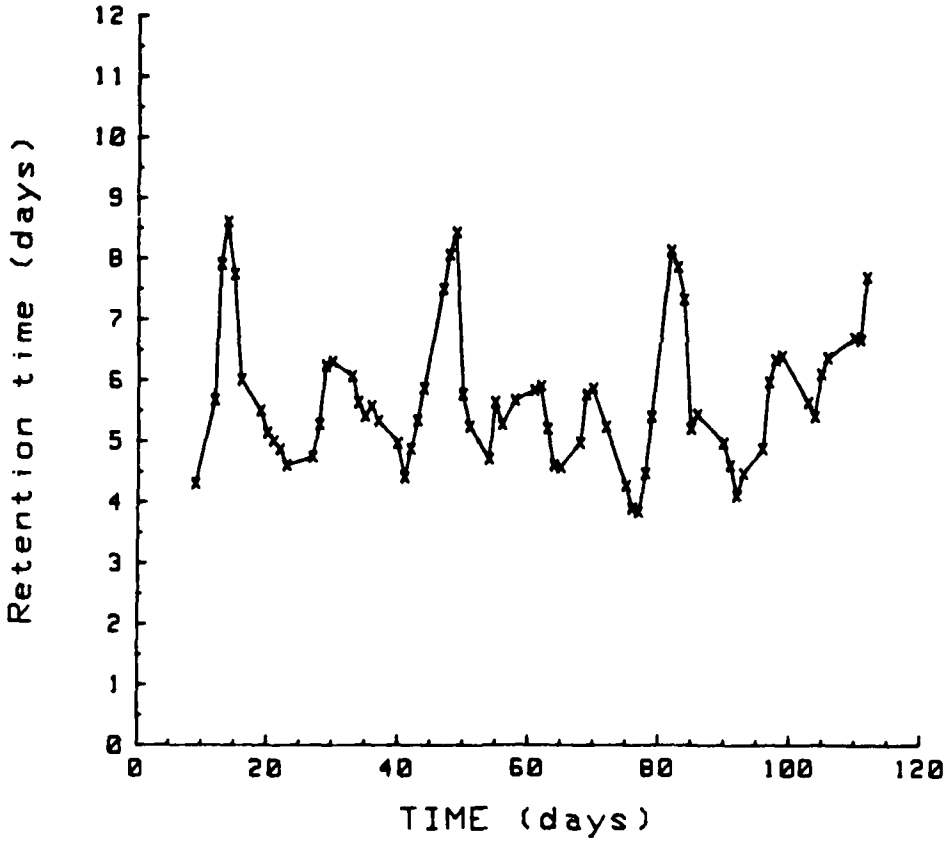

Figure 14. Average retention times for the first 120 days of the system describe in Figure 12.

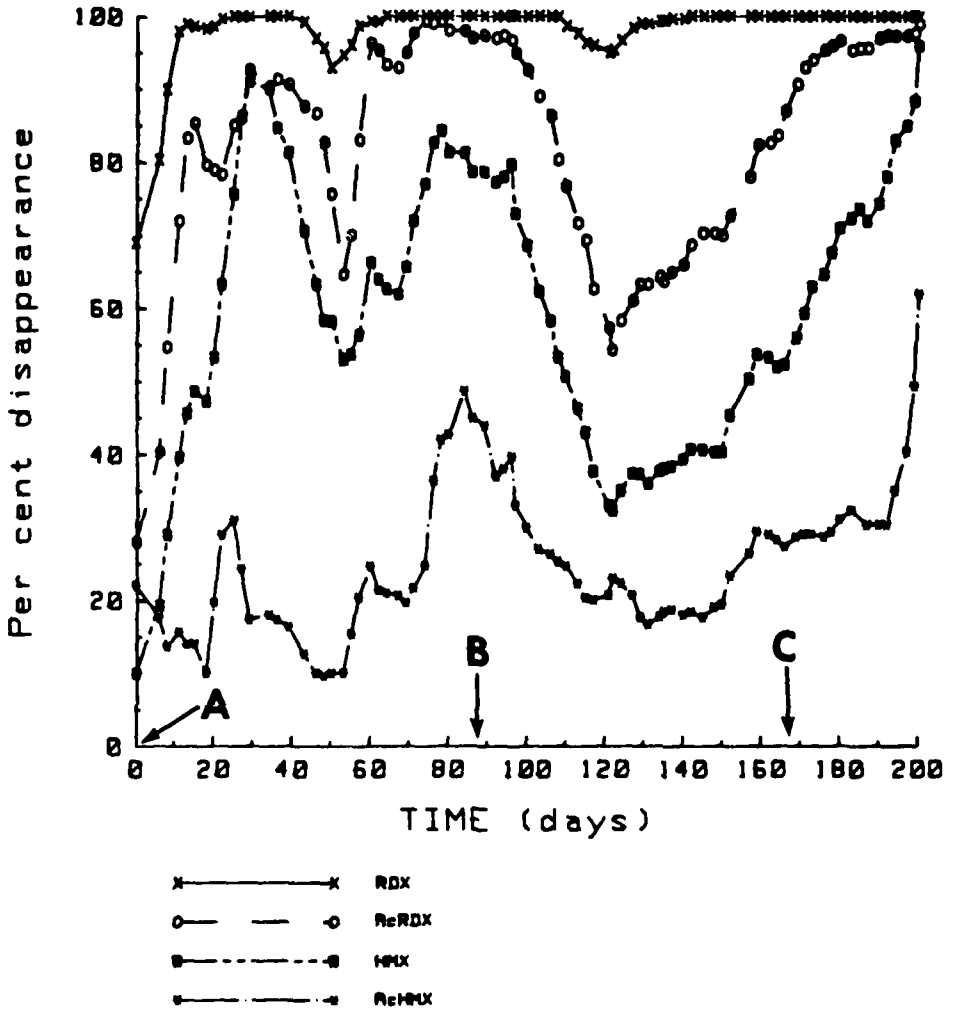

Figure 15. Disappearance of RCX, HMX, AcRDX, AcHMX in a type-FL acid-hydrolyzed sludge continuous culture system. (A) 20% hydrolyzed sludge; (B) 10% hydrolyzed sludge; (C) 30% hydrolyzed sludge.

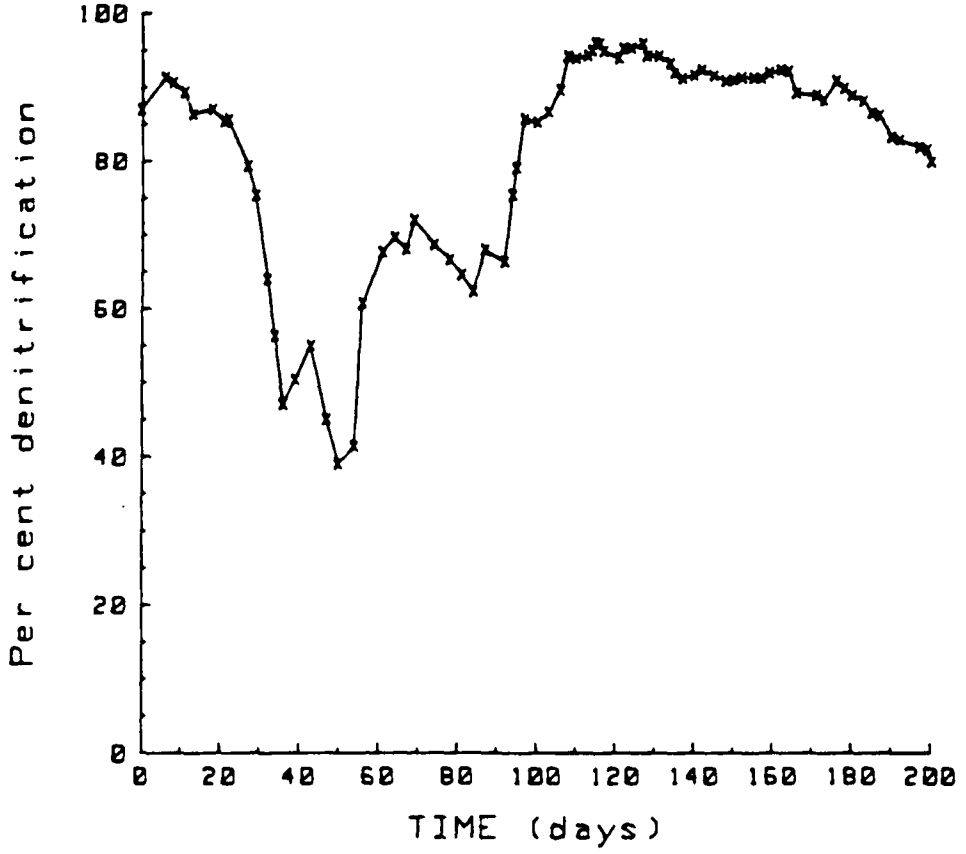

Figure 16. Percent denitrification of system described in Figure 15.

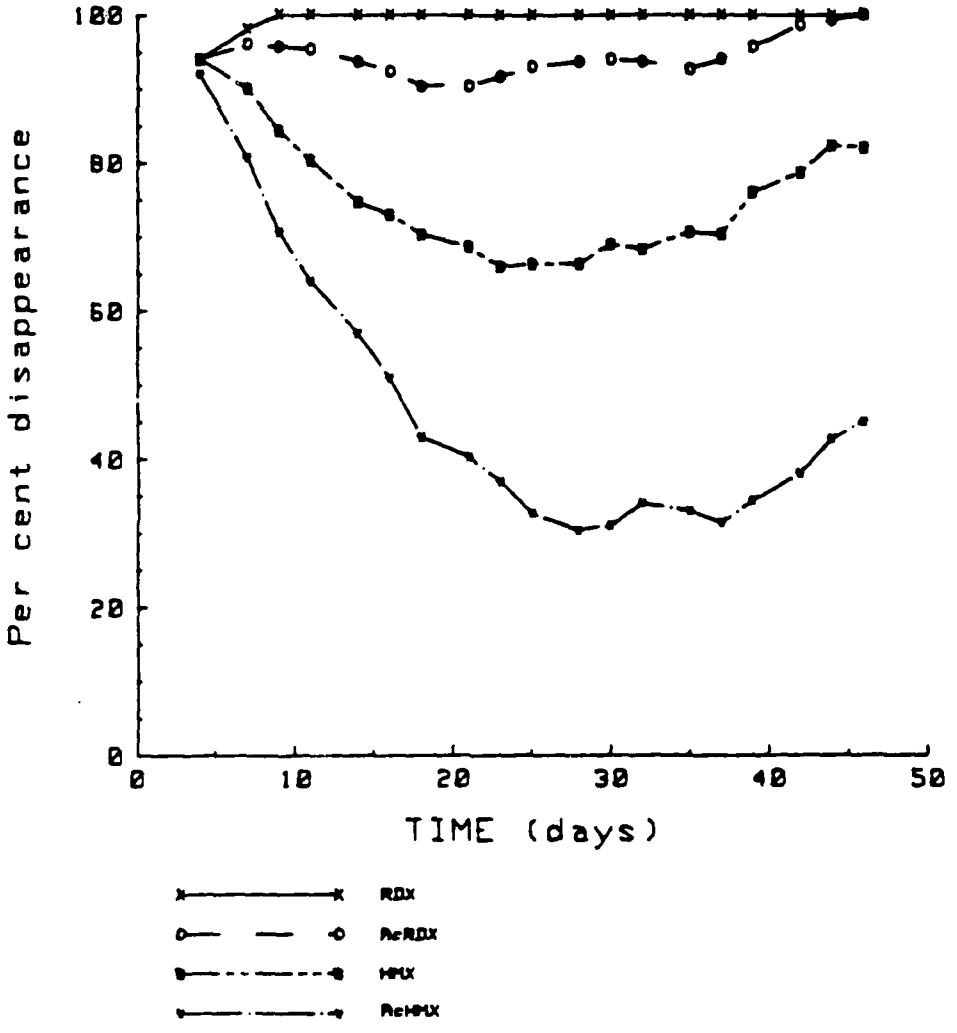

Figure 17. Disappearance of RDX, HMX, AcRDX and AcHMX in a type-FL 20% alkali-hydrolyzed sludge continuous culture system.

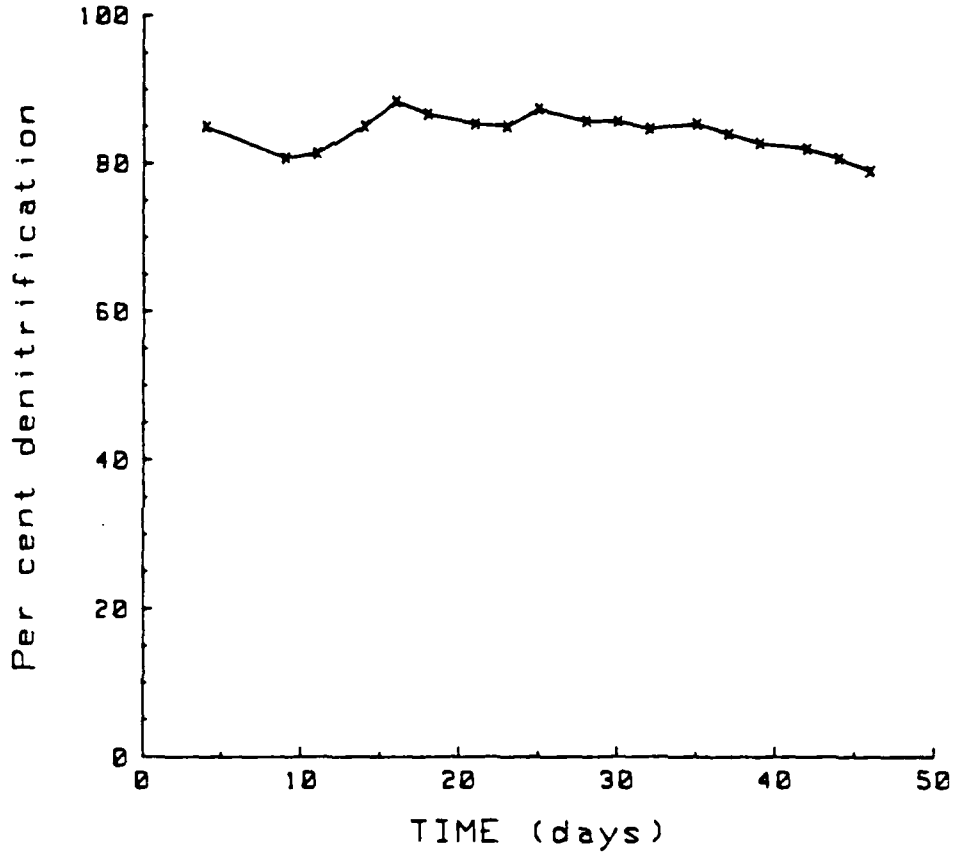

Figure 18. Percent denitrification in the 20% alkali-hydrolyzed sludge system.

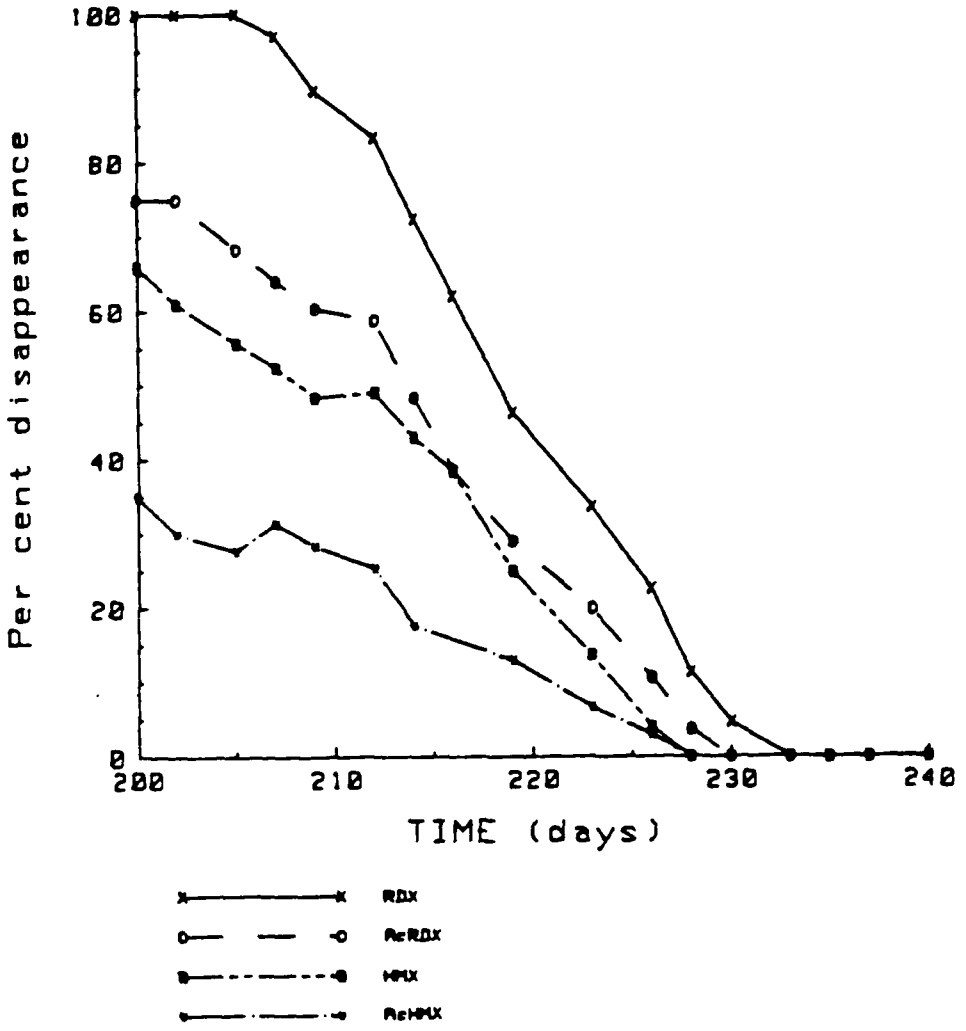

Figure 19. Disappearance of RDX, HMX, AcRDX and AcHMX in a type-FL continuous culture system using 20% unhydrolyzed sludge as nutrient.

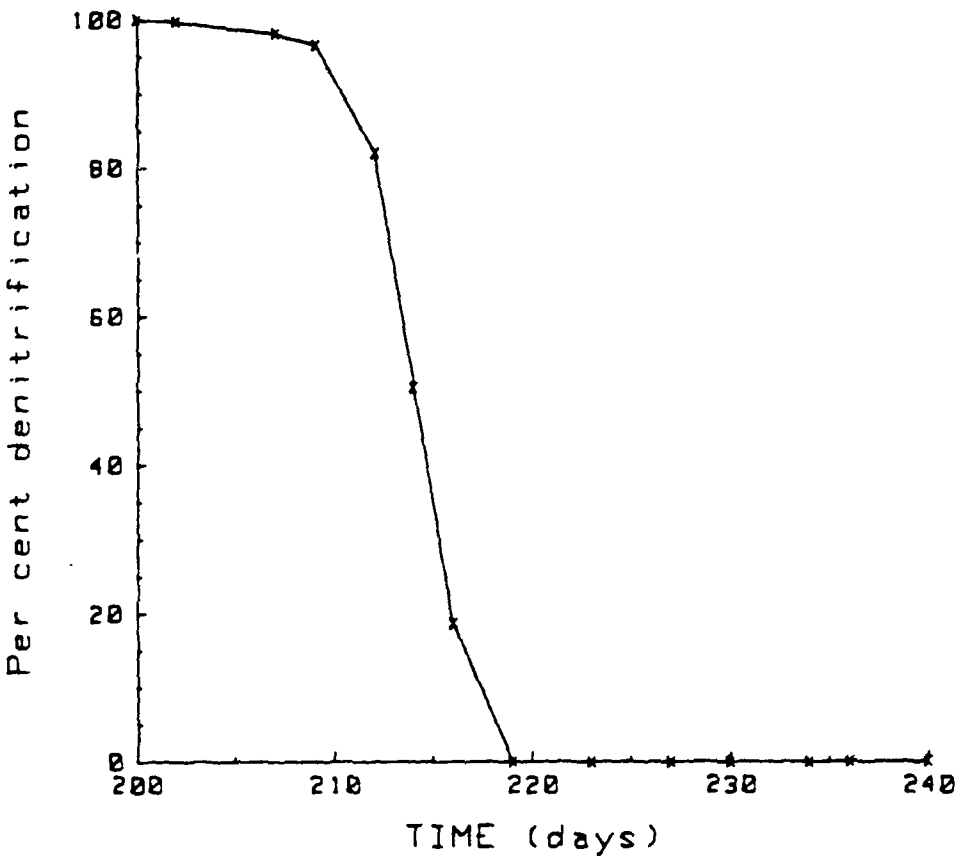

Figure 20. Percent denitrification of unhydrolyzed sludge system described in Figure 19.

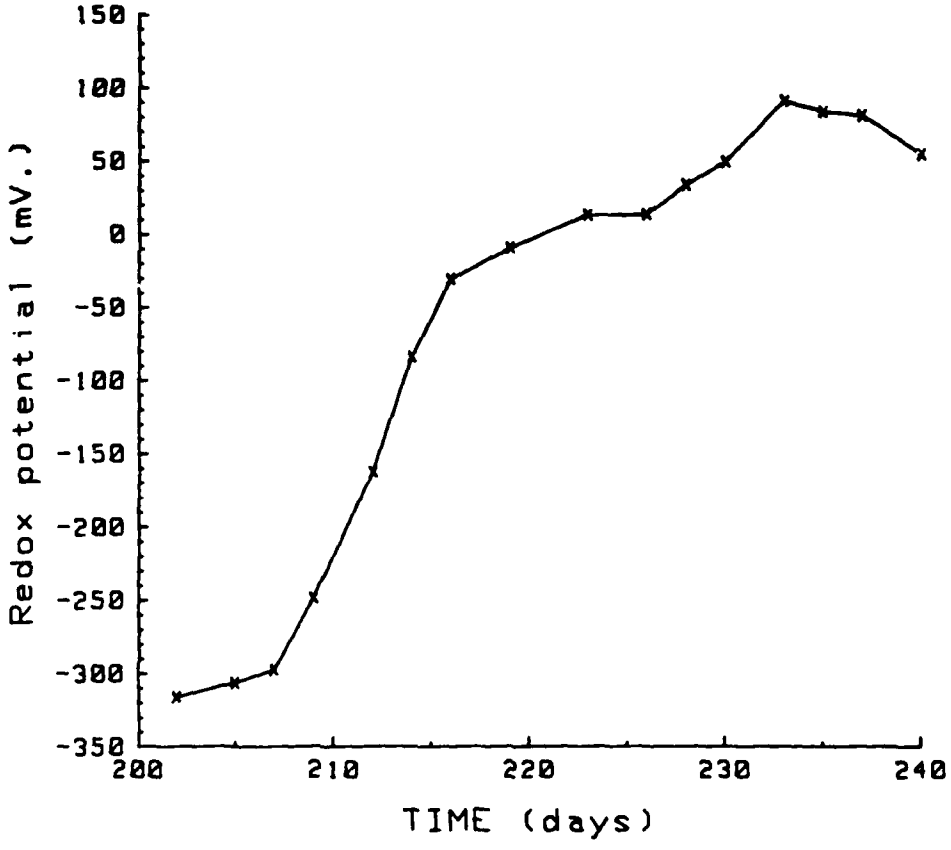

Figure 21. Redox potential of unhydrolyzed sludge system described in Figure 19.

Basal Salts-Methanol Studies: Two continuous culture Type-FL systems were established to more closely correspond to actual waste waters. While the concentrations of explosives used in the simulated mixture of RDX, HMX, AcRDX and AcHMX were realistic, the use of molasses, nutrient broth and hydrolyzed sludge as nutrient supplements was not realistic. Accordingly a basal salts (see Methods) medium containing methanol to achieve a 1.8 C/N ratio was used. Fig. 22 shows results obtained from such a system. After allowing the system to equilibrate and acclimate, methanol was added at day 9 (point A). The system continued to perform with low efficiency (Fig. 22), the percent denitrification (Fig. 23), which was high at the start, declined to ca. 40%, The high denitrification at the beginning corresponded to an E_h value of ca. -100 mV. As the efficiency of the system fell, the E_h increased to between -50 mV to 0 mV (Fig. 24). At day 48 (Figure 22, point B) the carbon source was changed from methanol to glucose (1250 ppm). This resulted in a general increase in percent disappearance and in percent denitrification, corresponding to a slight decrease in E_h to ca. -50 mV. However, as the E_h rose to ca. +50 mV over the period from day 45 to day 61, the denitrification began a steady decline concomitantly with percent disappearance of compounds. No appreciable changes were noted during the remainder of the experiment.

The next series of figures describes the results from a methanol basal salts Type-FL continuous culture system. Low efficiency was observed toward RDX and HMX (Fig. 25) and continued as AcRDX and AcHMX were added to the system at day 19. In an effort to rejuvenate the system, 10 mg/L of yeast extract were added at day 50 (point A) but this appeared to have no effect. Fig. 26 shows the inconsistent denitrification values obtained. There does not appear to be any correlation between the E_h (Fig. 27) and the efficiency of the system. Fig. 28 presents data from a methanol basal salts system in which several different carbon sources were tried. At day 9 (point A) 1200 mg/L of acetate as sodium acetate were added in place of methanol. This had no appreciable effect on the system. To this system was added yeast extract (20 mg/L) at day 48 (point B). A burst of activity seemed to accompany this addition, but then declined shortly thereafter. At day 62 (point C) the yeast extract was increased to 20 mg/L but this had little effect. The efficiency toward denitrification never exceeded 40% (Fig. 29). The E_h was brought to negative mv values (Fig. 30) by several additions of 0.025% sodium sulfide at day 76 and 83 (Fig. 27, points D and E), but the system did not increase in efficiency.

Sediment Studies: The results reported in Figs. 31, 32 and 33 show that under anaerobic conditions the rate of disappearance of RDX is greatest with the rich organic sediment and the best carbon source is methanol. The amount of each carbon source was calculated to provide approximately 500 mg of carbon per liter except for glucose which provided 750 mg/L. Complete disappearance of RDX was achieved in the methanol system in three weeks whereas in the other systems the RDX was only 75% to 85% biotransformed in five weeks. Acetate seemed to stimulate the sandy sediment (Fig. 31d). In the absence of methanol, the addition of phosphate stimulated RDX disappearance in sandy sediment but not much change was noted in organic sediment (Fig. 32b, 32d and 32e). Complete denitrification of 2480 mg/L of nitrate was attained in the more active systems (i.e., those showing greater activity toward disappearance of RDX).

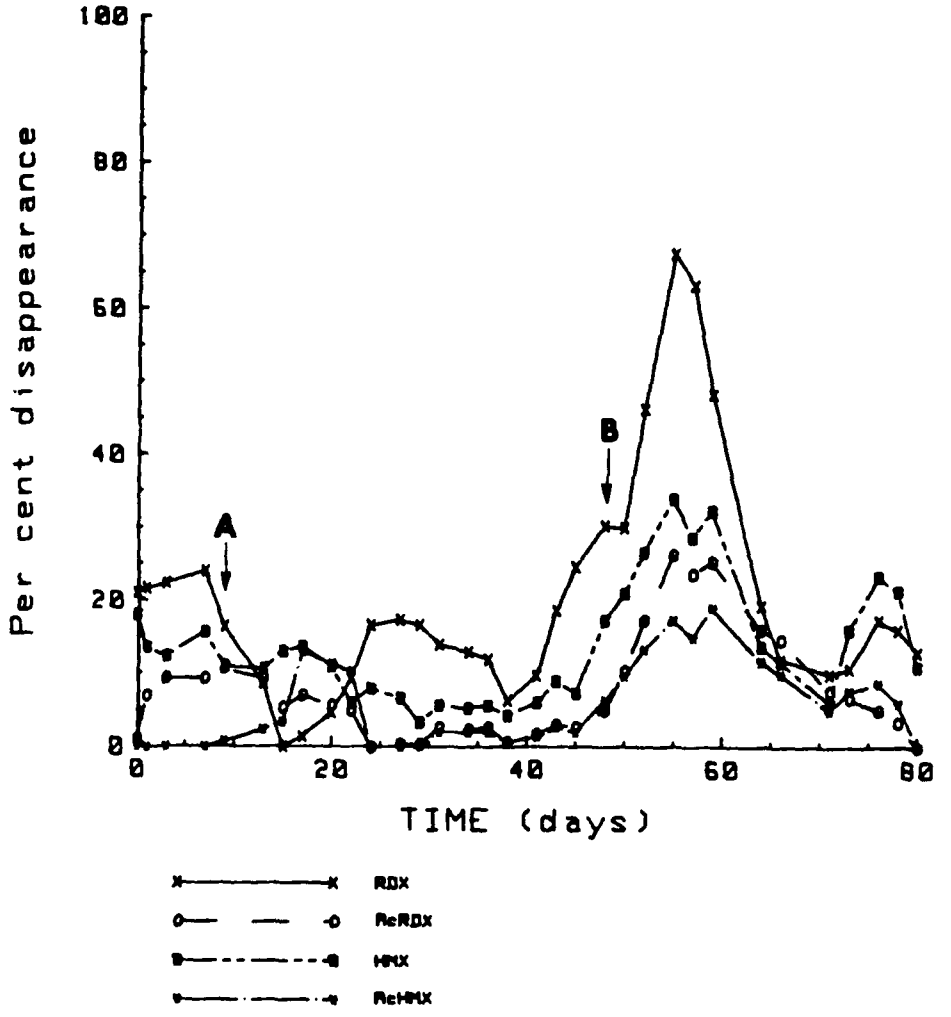

Figure 22. Disappearance of RDX, HMX, AcRDX and AcHMX in a type-FL continuous culture system containing basal salts-methanol as nutrient. (A) methanol as nutrient; (B) glucose as nutrient

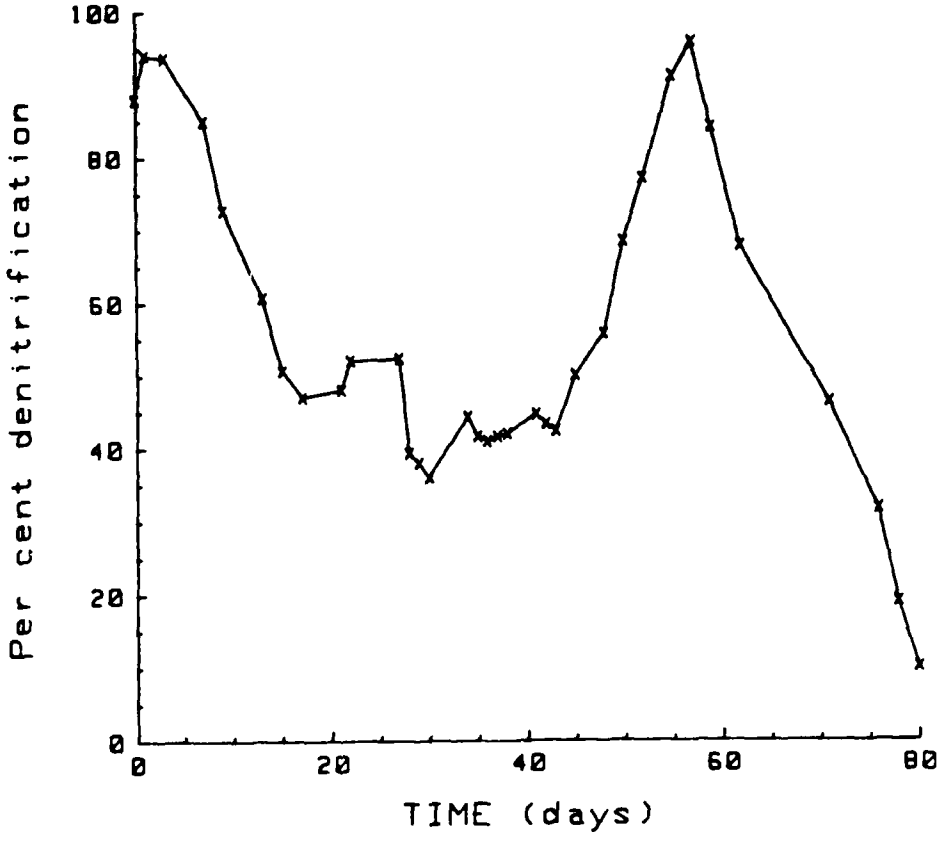

Figure 23. Percent denitrification in basal salts-methanol system described in Figure 22.

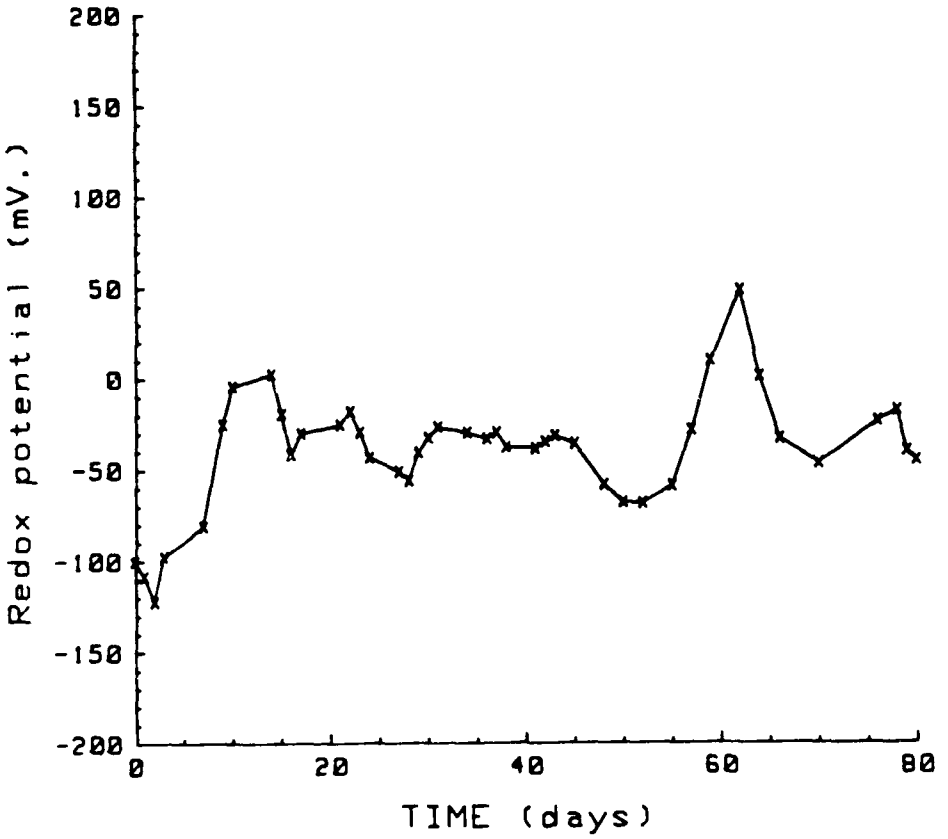

Figure 24. Redox potential of basal salts-methanol system described in Figure 22.

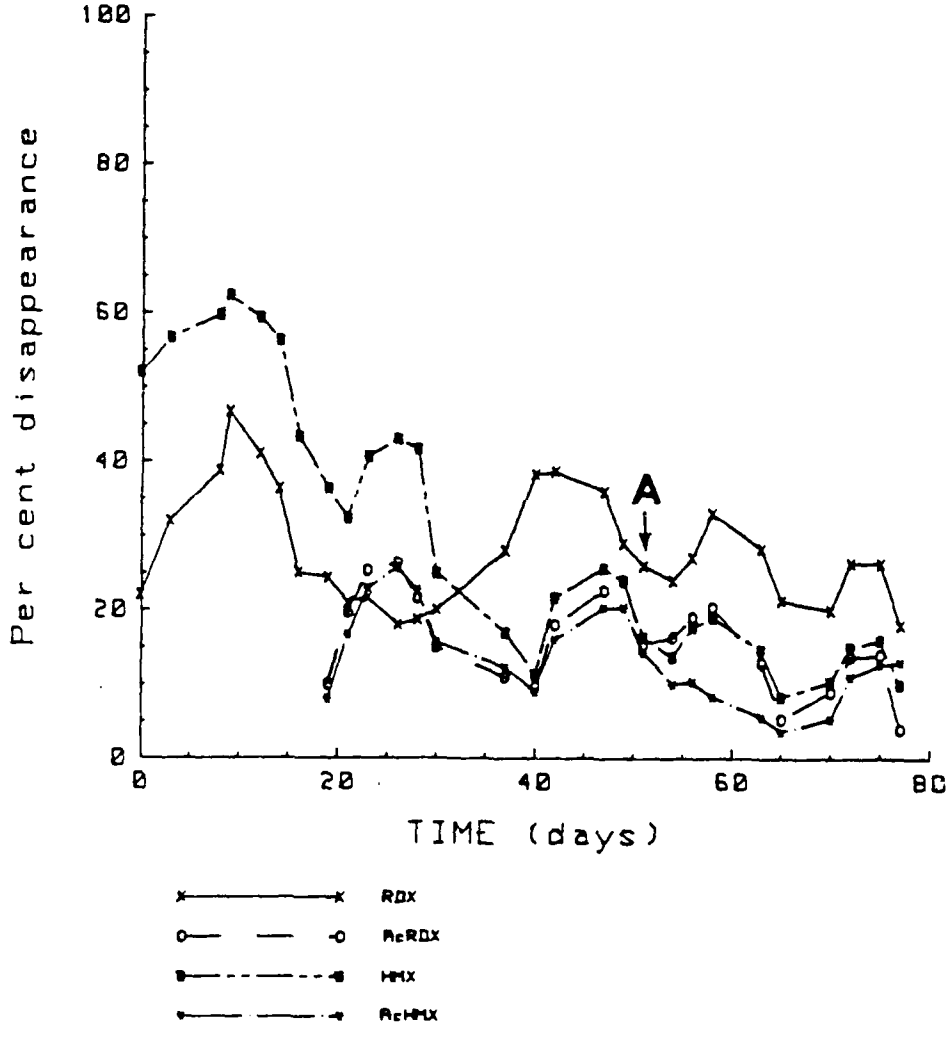

Figure 25. Disappearance of RDX, HMX, AcRDX and AcHMX in a type-FL continuous culture system containing basal salts-methanol; yeast extract added at arrow.

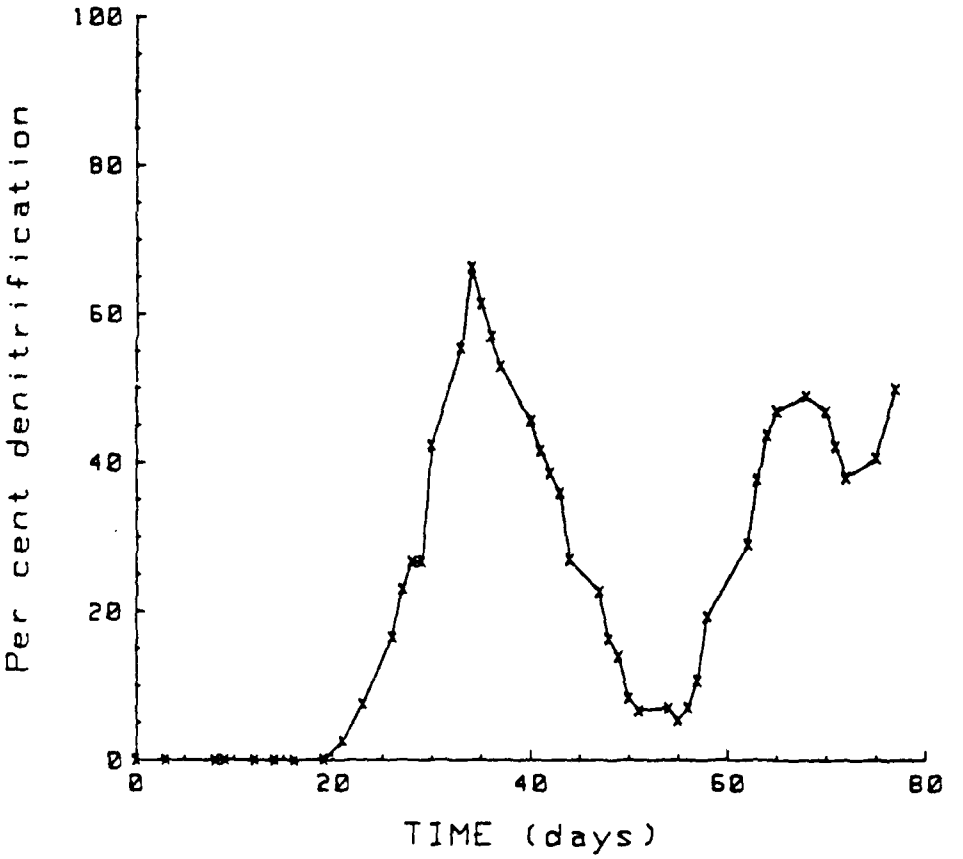

Figure 26. Percent denitrification in basal salts-methanol system described in Figure 25.

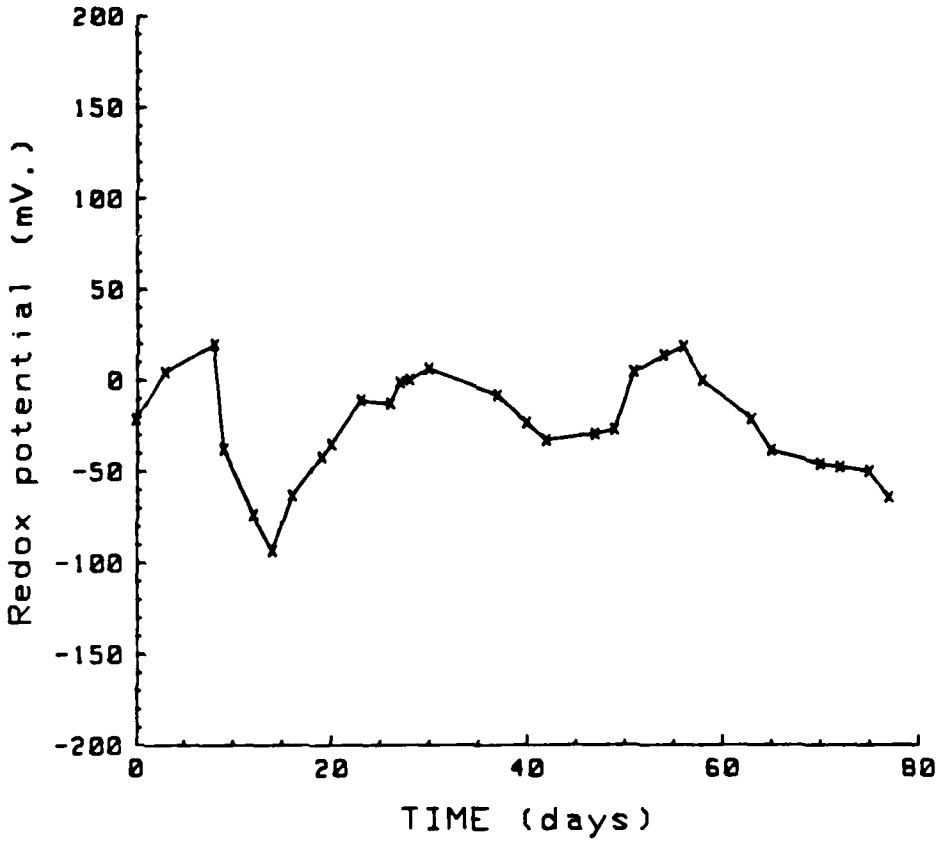

Figure 27. Redox potential of basal salts-methanol system described in Figure 25.

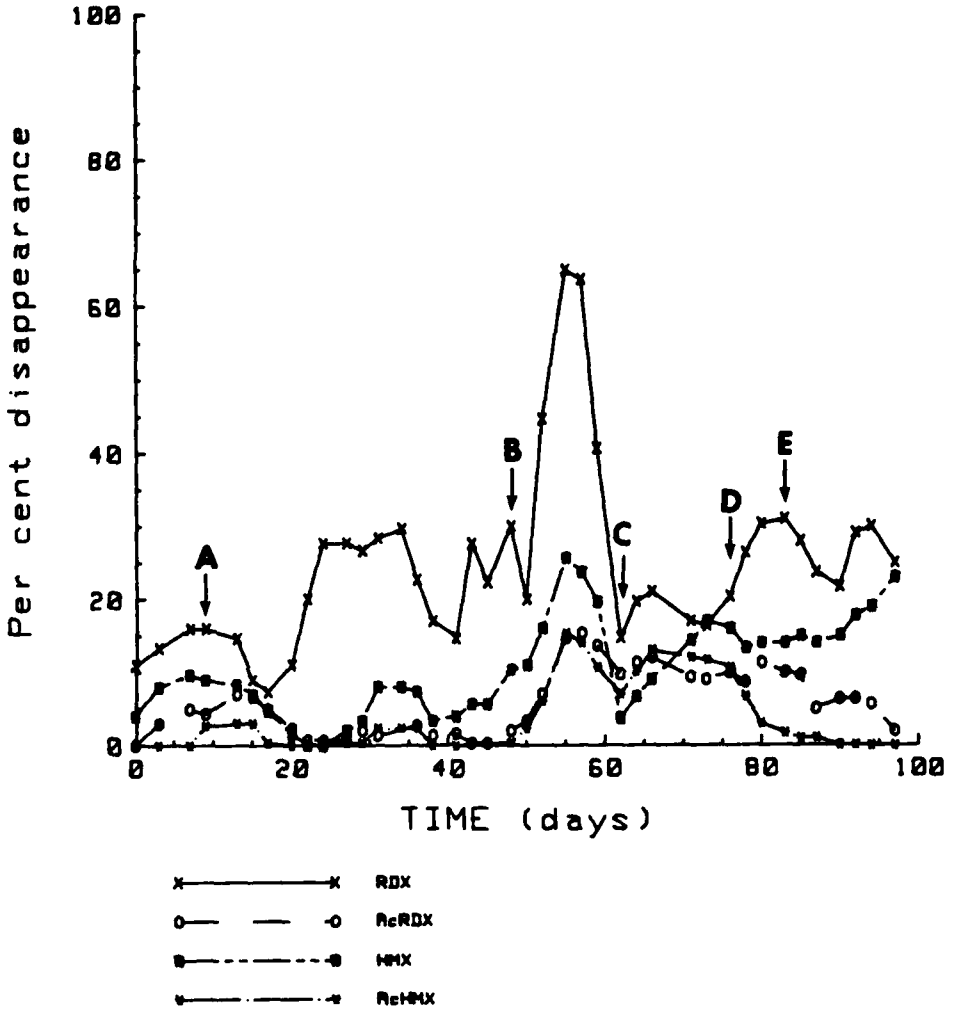

Figure 28. Effect of different supplements on disappearance of RDX, HMX, AcRDX, AcHMX in a type-FL basal salts-methanol continuous culture system: (A) acetate added; (B) yeast extract added (20 ug/mL); (C) yeast extract added (200 ug/mL); (D) & (E) sulfide added (0.025%).

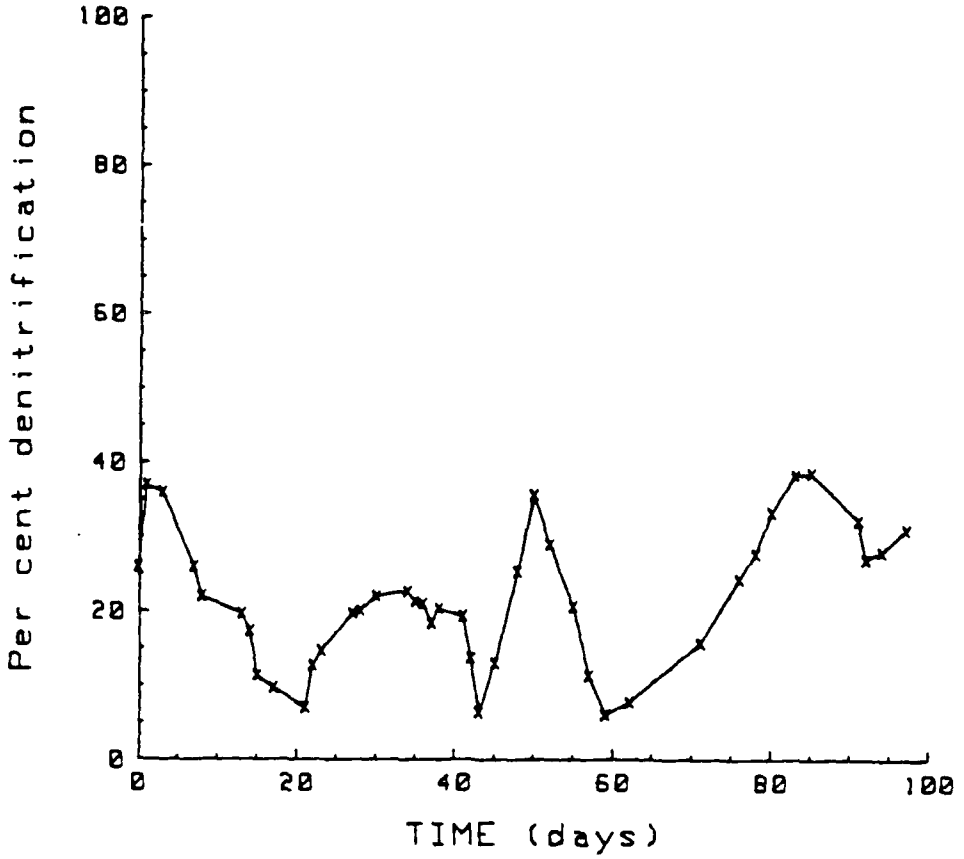

Figure 29. Percent denitrification in basal salts-methanol system described in Figure 28.

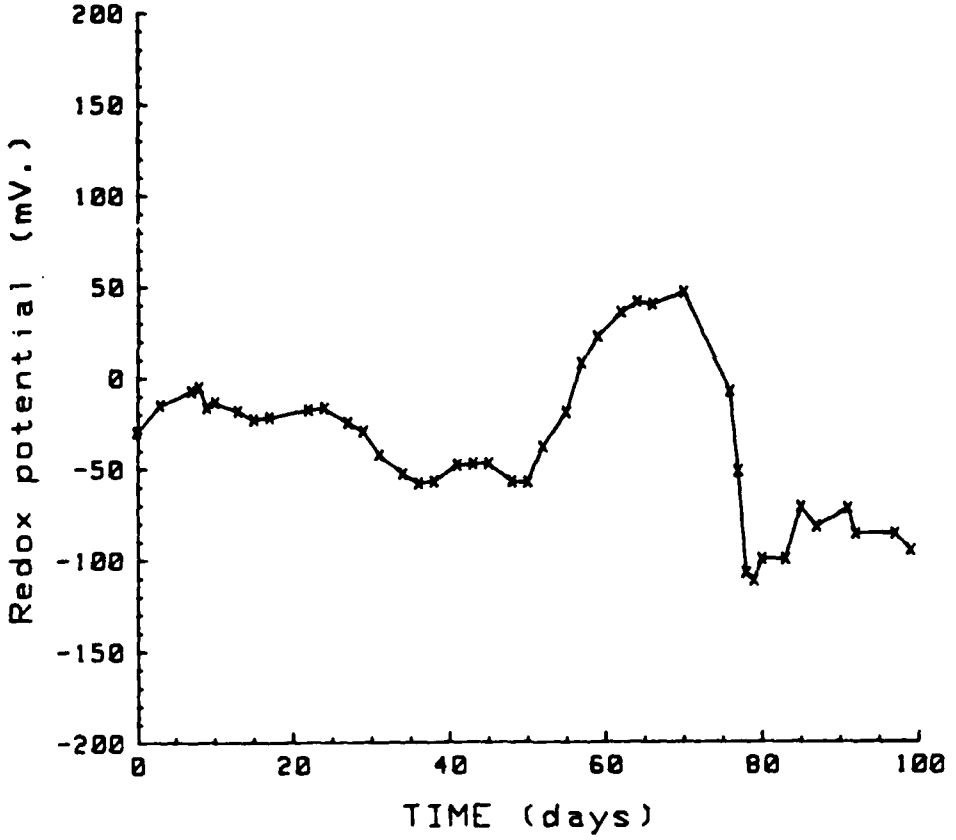

Figure 30. Redox potential of basal salts-methanol system described in Figure 28.

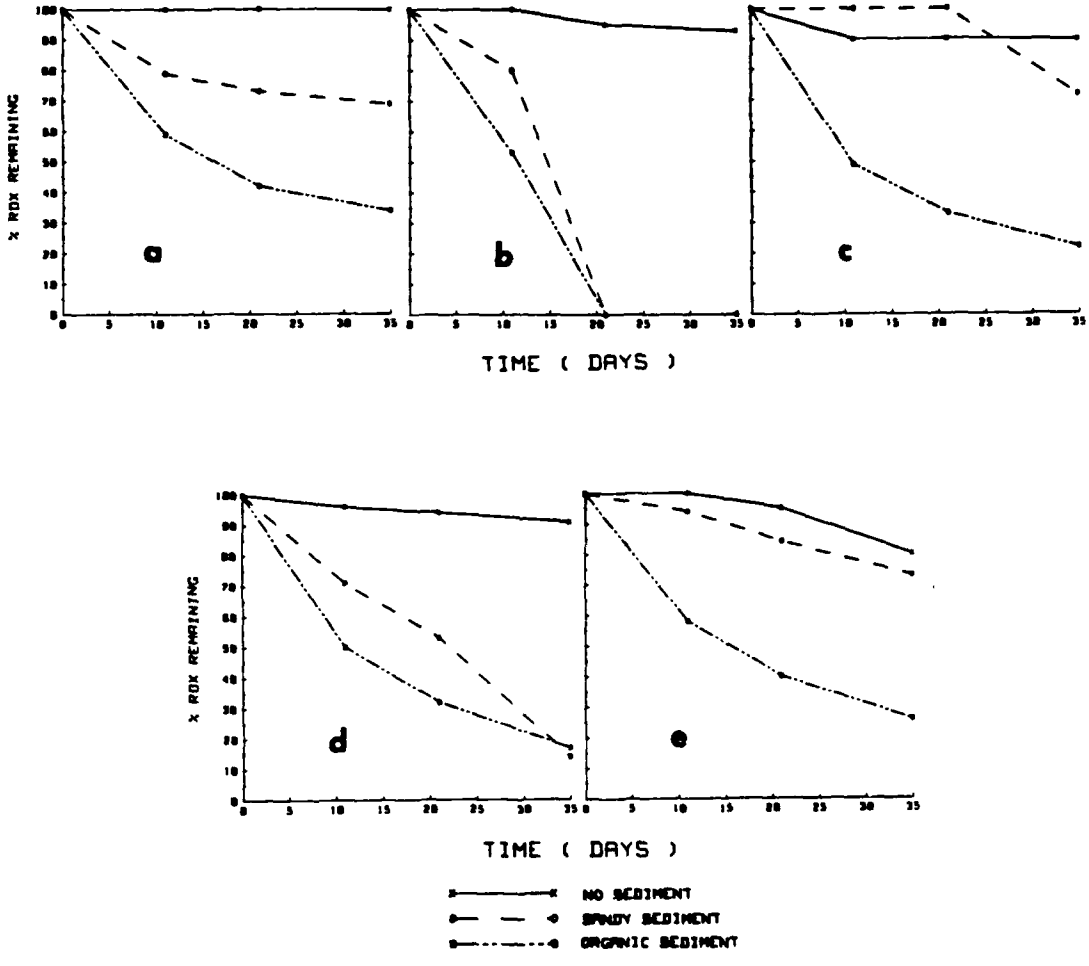

Figure 31. Effect of carbon source and sediment on the disappearance of RDX: (A) no carbon source, (B) methanol, (C) glucose, (D) acetate, (E) bicarbonate.

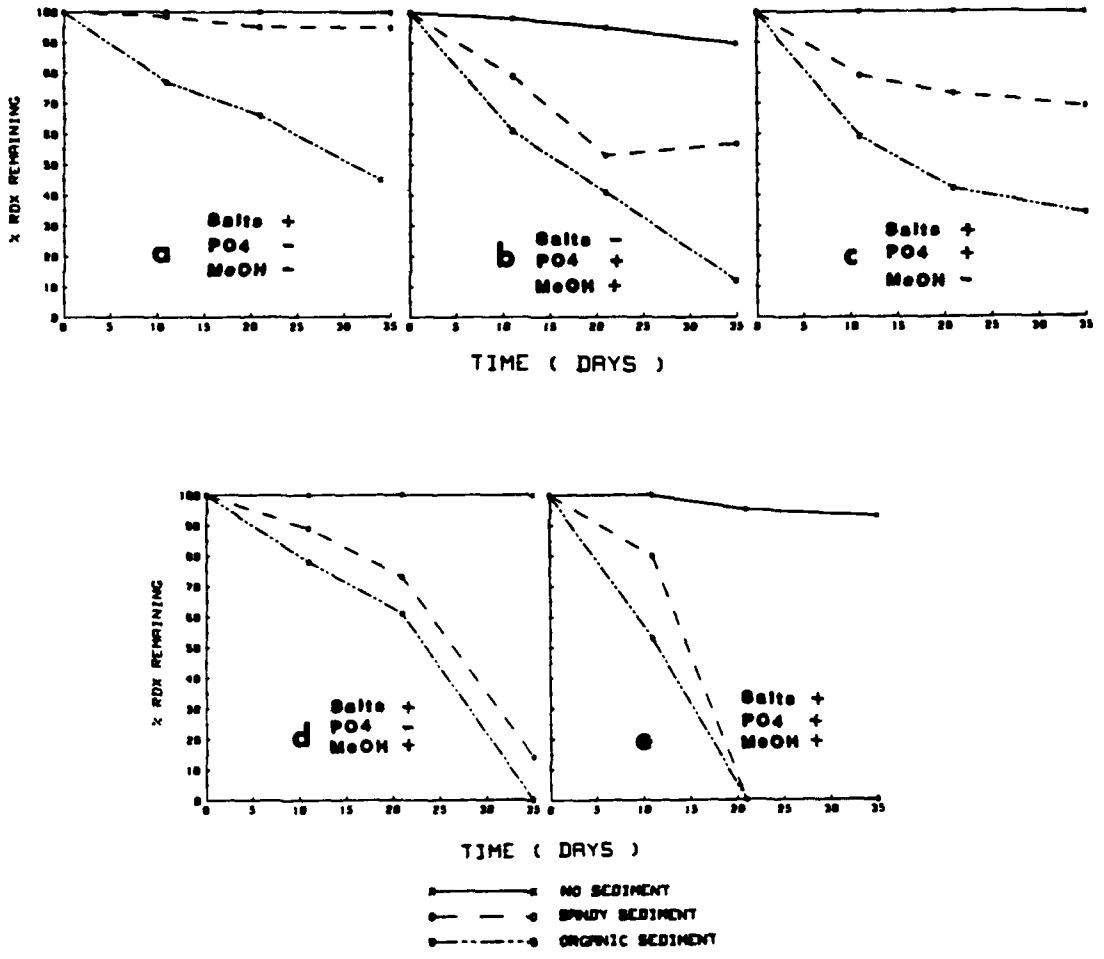

Figure 32. Effect of basal salts, phosphate, and methanol on the disappearance of RDX in presence and absence of sediment.

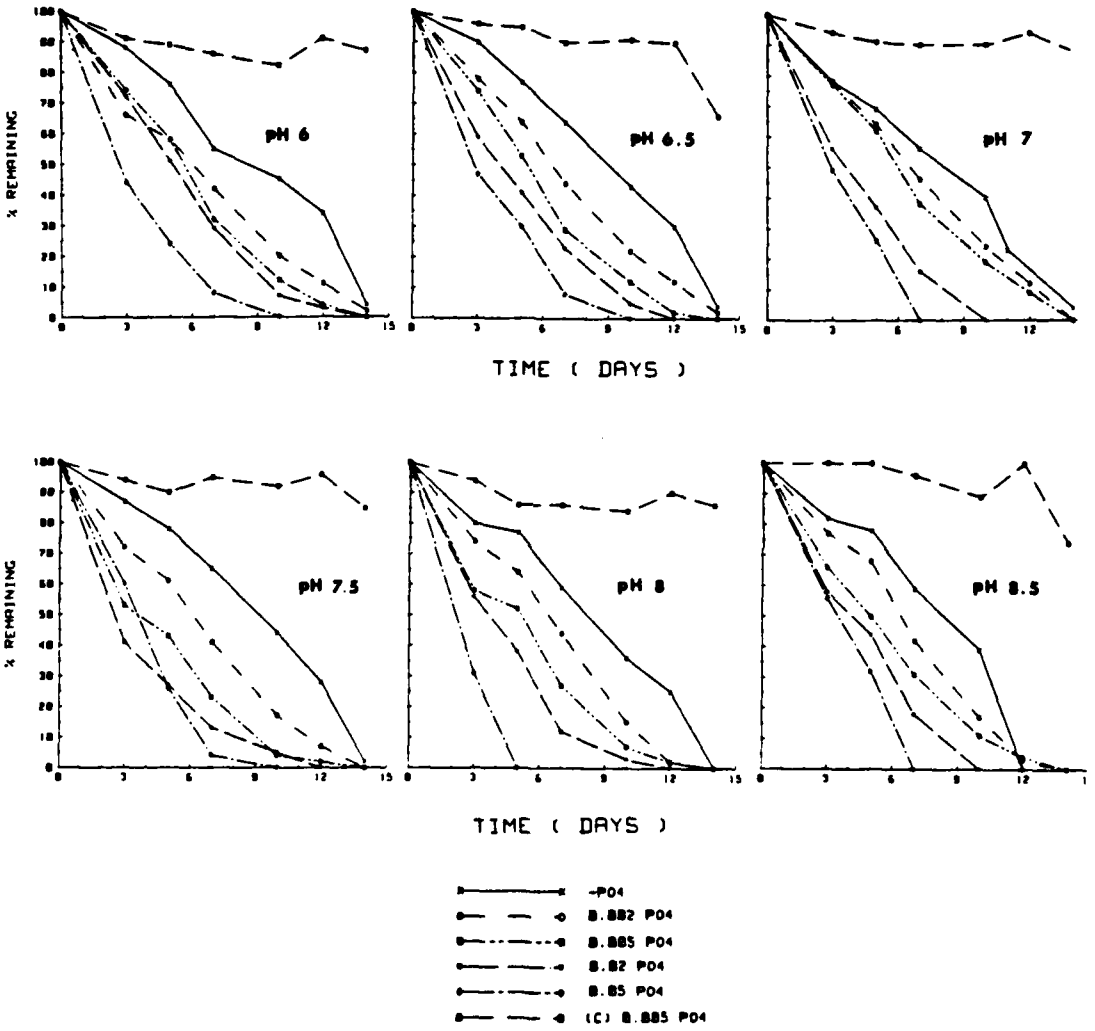

Figure 33. Effect of phosphate concentration and pH on RDX disappearance in the presence of a rich organic sediment.

The effect of pH on the disappearance of RDX using a phosphate buffered, rich organic sediment system is shown in Fig. 33. The lower concentrations of phosphate were inadequate to buffer the system at the initial desired pH. The pH data are more accurate at the higher 0.02 M and 0.05 M phosphate levels. The curve labeled (c) represents an uninoculated control in the absence of sediment. In this sediment experiment the E_h at day 1 was -350 mV but had risen to slightly positive values by day 4. Flushing the head space with N_2 after all subsequent measurements was found to reestablish the initial low E_h and the anaerobic conditions were sustained for the duration of the experiment by following this practice. Complete denitrification of 500 mg/L of nitrate was observed in all bottles except in the uninoculated controls.

DISCUSSION

This study was initiated to determine (1) whether a mixture of compounds found in RDX/HMX manufacturing waste waters could be successfully biotransformed into innocuous products under continuous culture conditions, (2) whether high concentrations of nitrate could be reduced to low levels by an active denitrification process, and (3) whether interactions occurred between intermediates that might alter the course of biotransformation.

The results indicate that the nitramines were transformed with relative reaction rates, RDX AcRDX HMX AcHMX. With all media except basal salts there was complete disappearance of RDX and the same or almost complete disappearance of AcRDX. The eight-membered ring compounds, HMX and AcHMX, were more resistant to biotransformation. The activities of the various systems appeared to be proportional to the concentration of available carbon in the medium. The total organic carbon (TOC) values of the various media are presented in Table 2. The highest activity was observed in 0.8% nutrient broth media with the next highest activity in 30% acid hydrolyzed sludge.

When the TOC was reduced to low levels as present in a basal salts-methanol medium (Table 2) the efficiency with respect to all measured parameters (i.e., disappearance of nitramines, E_h, denitrification) was significantly reduced. RDX disappearance varied between 20% and 60%, and activity toward the other compounds was even less. In the case of the methanol system, the E_h increased to positive values, whereas the E_h of the nutrient broth systems, the hydrolyzed sludge systems, and the molasses system was always between -300 mV to -400 mV. When the system was readjusted to negative E_h values by the addition of sulfide, the lower E_h was transient, and again rose to positive values.

Sikka et al.[3] and Spanggord et al.[4] have reported that the presence of sediments favors the disappearance of RDX. River waters normally are aerobic, but lakes, ponds and other shallow, standing waters have sediments rich in organic materials. Supplementation of the basal salts methanol system with sediments contributed to a higher rate of disappearance of the test compound, RDX. When first measured, the E_h had risen from the initial low value, but the addition of 0.01% sulfide and the protocol of flushing the head space with N_2

after each sampling reestablished and maintained a negative E_h. Higher efficiency was observed when methanol served as a carbon source than when glucose, acetate, or carbonate were used.

Intermediates which were observed routinely in batch culture studies during the biodegradation of RDX, HMX, AcRDX and AcHMX, were never detected in effluent samples from continuous cultures. The results from batch culture studies on the metabolic pathways of biodegradation of HMX, AcRDX and AcHMX are the object of a separate report.[7] In one instance effluent was collected and concentrated 2000 fold in order to confirm the presence of hydrazine (found in trace amounts in batch culture studies of RDX), but no hydrazine was detected.

A positive correlation between denitrification and redox potential was found. When the E_h was between -200 mV to -400 mV the efficiency of denitrification was > 99%. The finding of only nitrogen and carbon dioxide and the absence of oxygen in the head space gas sample taken from a Type-NB nutrient broth continuous culture system is consistent with the low E_h observed (-350 mV) in the actively denitrifying systems (99%). It also supports the concept that the presumably facultative denitrifying organisms require strictly anaerobic conditions (i.e., low E_h, complete absence of molecular oxygen) in order to carry out the process of denitrification. In this regard, Bazylinski and Blakemore[8] recently reported that nitrate reduction in Aquaspirillum magnetotacticum appears to have an absolute requirement for O_2. In our experiments, as the E_h rose to -100 mV and to positive values of E_h, there was always a variable and marked decrease in percent denitrification.

Table 2. Total Organic Carbon (TOC) Levels

Present in Various Influent Nutrient Solutions

Nutrient solution	TOC (mg carbon per liter)
Nutrient broth (0.8%)	3135
" " (0.4%)	1566
Alkaline hydrolyzed sludge (20%)	1511
Acid hydrolyzed sludge (30%)	2049
" " " (20%)	1743
" " " (10%)	780
Molasses (0.3%)	1513
Basal salts-methanol (0.14%)	227

An actively denitrifying system was capable of denitrifying up to 2500 mg of nitrate/L (564 mg of nitrate nitrogen/L). Amounts of nitrate found in waste waters rarely range above 500 mg of nitrate/L so the level of denitrification found in the active systems was more than sufficient to reduce very high nitrate concentrations to acceptable levels. However, attempts to increase the levels of nitrate removal from a basal salts-methanol systems by the addition of various supplemental carbon sources (Figs. 25 and 28) were unsuccessful.

The stimulation in the rate of disappearance of RDX by added phosphate, basal salts, and rich organic sediments suggests that the process may proceed naturally at a low rate wherever local anaerobic habitats exist in the environment such as may be present in sediments. It is expected that a waste water treatment facility operating in an anaerobic mode would be able to handle the biotransformation of RDX and AcRDX easily, perhaps aided by the addition of supplements. Successful denitrification should be attainable providing a sufficiently low E_h can be maintained. Due to their much lower rates of disappearance, the biotransformation of HMX and AcHMX would require longer contact times in the system.

CONCLUSIONS

This study has demonstrated conclusively that mixtures of compounds present in waste waters resulting from the manufacture of RDX/HMX can be successfully removed in a continuous treatment process provided the system is operated in an anaerobic mode. The present study extends ~~the~~ previously reported findings with RDX to HMX and the two acetylated derivatives, AcRDX and AcHMX. Nitrate, in concentrations up to 2400 ppm, is removed by the same continuous system; the lower the E_h the more efficient the removal. Removal efficiencies were also correlated with TOC values, with the eight-membered ring compounds, HMX and AcHMX, being brought up to their highest levels of disappearance by systems having the highest TOC values. This suggests that an anaerobic treatment facility designed to remove these compounds may require the addition of supplemental nutrients to maintain a high efficiency biotransformation. No buildup of transformation products was observed in any of the systems studied. It appeared that if a compound was acted on at all no detectable products remained. From extrapolation of these data it may be expected that treatment of an actual waste stream would be expected to remove all of the RDX and most of the AcRDX, while leaving some percentage of HMX and AcHMX untouched. However, these estimates could be fine-tuned by altering the average retention time of the system and/or providing supplemental nutrients.

RECOMMENDATIONS

(1) Any biological treatment system for RDX/HMX waste water should operate in the anerobic mode.
(2) A full-scale treatment system should consider the need for supplemental nutrients for proper operation.
(3) Based on these studies, conditions should be adjusted in a large scale treatment facility so that no transformation intermediates are discharged.
(4) Any treatment facility should have capability of on-line monitoring of the treated waste stream for intermediates and end-products.

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