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Biodegradation of Munitions Compounds by a Sulfate Reducing Bacterial Enrichment Culture

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Running Title: **Degradation of Munitions Compounds by SRB**

ABSTRACT

The degradation of several munitions compounds was studied. The compounds included 2,4,6-trinitrotoluene (TNT), hexahydro-1,3,5-trinitro-1,3,5-triazine, octahydro-1,3,5,7-tetranitro-1,3,5,7-tetraazocine, 2,4,6-trinitrobenzene (TNB), and 2,4-dinitrotoluene. All of the compounds studied were degraded by the sulfate reducing bacterial (SRB) enrichment culture. The SRB culture did not use the munitions compounds as their sole source of carbon. However, all the munitions compounds tested served as the sole source of nitrogen for the SRB culture. Degradation of munitions compounds was achieved by a co-metabolic process. The SRB culture used a variety of carbon sources including pyruvate, ethanol, formate, lactate, and H_2-CO_2. The SRB culture was an incomplete oxidizer, unable to carry out the terminal oxidation of organic substrates to CO_2 as the sole product, and it did not use acetate or methanol as a carbon source. In addition to serving as nitrogen sources, the munitions compounds also served as electron acceptors in the absence of sulfate. A soil slurry experiment with 5% and 10% munitions compounds-contaminated soil showed that the contaminant TNT was metabolized by the SRB culture in the presence of pyruvate as electron donor. This culture may be useful in decontaminating munitions compounds-contaminated soil and water under anaerobic conditions.

INTRODUCTION

Soil and water in most U.S. military facilities are contaminated with explosive chemicals, mainly because of the manufacture, loading, and disposal of explosives and propellants. This contamination problem may increase in future because of demilitarization and disposal of unwanted weapon systems. Disposal of obsolete explosives is a problem for the military and the associated industries because of the polluting effect of explosives in the environment (1).

2,4,6-Trinitrotoluene (TNT) is the major explosive contaminant in many military sites. Soil and water contamination by TNT has been reported previously (2,3), and its toxicity is well documented (4-6). Disposal of munitions wastes in the past occurred by dumping in the deep sea, dumping at specified landfill areas, and sometimes incineration, when quantities were small (7). All of these methods can potentially have very serious effects on the ecosystem. Incineration causes air pollution, and disposal on land may lead to soil and ground water contamination that will affect aquatic life forms, humans, and animals.

Biological removal of explosives from waste water has been proven feasible (8). Biotransformation of TNT by aerobic bacteria in the laboratory has frequently been reported (9-15). Biodegradation of 2,4-dinitrotoluene by a Pseudomonas sp. has been reported to occur via 4-methyl-5-nitrocatechol in a dioxygenase-mediated reaction (16). White rot fungus has been shown to mineralize radiolabeled TNT (17). Valli et al. (18) found that 2,4-dinitrotoluene was completely degraded by the white rot fungus, Phanerochaete chrysosporium. Under anaerobic conditions, the sulfate reducing bacterium Desulfovibrio sp. transformed TNT to toluene (19-21) by reduction followed by reductive deamination. Gorontzy et al. (22) reported that under anaerobic conditions, methanogenic bacteria reduced

nitrophenols and nitrobenzoic acids. McCormick et al. (23) observed the degradation of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) under anaerobic conditions in sewage sludge. However, there has been very few reports on the degradation of RDX, Octahydro-1,3,5,7-tetranitro-1,3,5,7-tetraazocine (HMX), 2,4,6-trinitrobenzene (TNB) or other munitions compounds. Most of the work on TNT biotransformation has involved aerobic bacteria. Here we report the degradation of various munitions compounds in contaminated soil by a sulfate reducing bacterial (SRB) enrichment culture under anaerobic conditions. The results could be of interest in the anaerobic treatment of munitions contaminated soil and water.

EXPERIMENTAL SECTION

Enrichment Culture and Growth Conditions. The anaerobic techniques described by Balch and Wolfe and Daniels et al. (24,25) were used throughout this study. The medium used for the enrichment consisted of the following components (mM): KH_2PO_4 (2.94), K_2HPO_4 (1.15), NH_4Cl (9.35), NaCl (10.27), $MgCl_2$ (0.5), $CaCl_2$ (0.34), Na_2SO_4 (20.0), pyruvate (30.0), and yeast extract (0.1 g/L). After preparation, medium was dispensed into bottles and made anaerobic as described by Daniels et al. (25) and autoclaved. For the carbon source experiment, the medium described above was used with the pyruvate replaced by different carbon substrates.

A mud sample was collected from an uncontaminated creek near Argonne National Laboratory, Argonne, Illinois. A portion of the sample (5 mL) was added to 100 mL stoppered bottles (Bellco Glass, Vineland, NJ) containing 50 mL of the medium and pressurized to 20 psi with N_2-CO_2 (80:20 V/V). The bottles were incubated at room temperature (20-22°C) in a gyratory shaker set at 150 rpm. The cultures showing substantial growth were transferred into the same medium. After five more

transfers into the same medium, the enrichment culture was used for the experiment.

Munitions Compounds Study. The SRB enrichment culture was tested for its ability to metabolize various munitions compounds. The medium described above was used. After preparation, the medium was distributed to sterile anaerobic bottles containing 50 or 100 ppm of a munitions compound (TNT, TNB, DNT, RDX, or HMX). Because of the relative insolubility of munitions compounds in water, weighed amounts of chemicals in sterile empty anaerobic bottles were dissolved in small amounts of acetonitrile. The acetonitrile was evaporated under a stream of nitrogen, leaving a thin film of material on the inside surface of each bottle. The anaerobic medium was then poured into the bottle containing the deposit and stirred vigorously until the solution was attained. The bottles were made anaerobic as described by Daniels et al. (25). The gas phase in each bottle was N_2-CO_2 (80:20 V/V). A 5% inoculum (of a previously grown midlog-phase culture) was used in all experiments. Experiments were conducted in duplicates, and duplicate heat-inactivated (autoclaved) controls were used. The bottles were covered with aluminum foil to prevent photodegradation of munitions compounds. In the experiments with munitions compounds as the sole carbon or nitrogen source, the medium described above minus yeast extract was used without pyruvate or ammonium chloride, respectively. In these experiments munitions compounds (0.5 mM) served as carbon or nitrogen source. The cultures were incubated at room temperature (22-24°C) in a gyratory shaker. All data are the averages of duplicate culture bottles. Sodium sulfide was not included in the medium to eliminate any chemical reduction reactions (22).

In an experiment with all the munitions compounds supplied together as a mixture, 100 ppm each of TNT, TNB, and 2,4-dinitrotoluene (DNT) and 50 ppm each of RDX and HMX were

added to the medium. In this study pyruvate served as the electron donor and sulfate served as the electron acceptor. In the experiment where munitions compounds were used as electron acceptor, sulfate was not included in the medium.

Soil Slurry Studies. To determine whether the SRB enrichment culture could metabolize munitions compounds present in the contaminated soil, an experiment was conducted with contaminated soil collected from the Joliet Army Ammunition Plant, Joliet, Illinois. The major contaminant in the soil, TNT, ranged from 10,000 to 20,000 mg/kg of soil. The concentrations of RDX, HMX, TNB, and DNT ranged from 50 to 300 mg/kg of soil (Table I). The soil had a total organic content of 4-5% including the contaminants. The medium described above was prepared in 100 mL anaerobic bottles. Contaminated soil (5-10%) was added to the bottles with the medium. The bottles were autoclaved and a 5% inoculum was added. In the control bottles the cells were heat inactivated (autoclaved). In this experiment pyruvate (30 mM) served as electron donor and sulfate (20 mM) as electron acceptor. Duplicate culture bottles were maintained in each experiment. A sample of the soil slurry was analyzed periodically for TNT concentration.

Analytical Methods. Levels of munitions compounds were analyzed by high performance liquid chromatography (HPLC) on a Waters Associates (Milford, MA) liquid chromatograph equipped with two model 6000A solvent pumps, a model 490E programmable multiwavelength detector set at 254 nm, a data module, and a model 600E system controller. The mobile phase was methanol:water (50:50 V/V). Aliquots of 50 µL were injected into a Supelco C-18 column at 4°C. The flow rate of the solvent was 1.5 mL/min. Munitions compounds in soil slurry were extracted by mixing a 3 mL slurry sample and 3 mL of acetonitrile on a vortex mixer for 1 min and then centrifuging at 5000 rpm for 10 min. The supernatant was filtered through

0.45 μm filter paper and the filtrate was used for the HPLC analysis.

Bacterial growth was monitored by measuring the culture turbidity at 600 nm in a Spectronic 20 spectrophotometer (Milton Roy, Rochester, NY).

Scanning Electron Microscopy (SEM). A Hitachi S-4800 Scanning electron microscope was used. Samples were fixed for 30 min in phosphate buffer containing 2.5% glutaraldehyde. After being washed and rinsed in the same buffer, samples were dehydrated in a graded ethanol series and dried at the critical point with liquid CO_2 and a critical point drier. Samples were sputter coated with gold.

Chemicals. The structures of the munitions compounds used in this study are given in Fig. 1. The TNT was obtained from Chem service Inc., Westchester, PA., while TNB, DNT, RDX and HMX were from the Naval Surface Warfare Center, Indian Head, MD. The other chemicals were of reagent grade.

RESULTS

Characteristics of SRB Enrichment Culture: Cells of the enrichment culture were vibroid rods, which were highly motile. The cells were about 0.8 μm by 2.5 μm in size (Fig. 2). Since growth did not occur in ethanol, pyruvate or lactate media exposed to oxygen we conclude this enrichment culture was strictly anaerobic. The enrichment culture did not grow in the absence of sulfate in the medium. The cells contained desulfoviridin as indicated by red fluorescence in the alkaline cell culture. Cytochrome C3 and desulfoviridin are electron carriers typical for sulfate reducing bacteria (26). The ability of the SRB enrichment culture to use a wide variety of substrates was tested. The results, presented in Table II, show that it used pyruvate, ethanol, formate, and

H_2-CO_2. The culture did not grow on methanol, acetate, or long chain fatty acids. These results indicate that the SRB enrichment culture is an incomplete oxidizer, unable to carry out the terminal oxidation of organic substrates to CO_2 as the sole product.

Removal of Munitions Compounds. The SRB enrichment culture was incubated with various munitions compounds, with pyruvate as the electron donor and sulfate as the electron acceptor. A heat-inactivated controls were used. Munitions compounds were added at 100 ppm (TNT, TNB, and DNT) or 50 ppm (RDX and HMX). The lower concentrations of RDX and HMX were used because of their limited availability.

The disappearance of munitions compounds in culture samples was monitored by using HPLC. As Fig. 3 shows, the added TNT (100 ppm) disappeared within 10 days in cultures that received pyruvate (30 mM) as the main substrate, whereas in the control bottles the TNT concentration remained at the original level of 100 ppm throughout the experiment. Similarly, TNB and DNT disappeared from the culture medium within 10 days of incubation. However, the disappearance of RDX and HMX took longer. The level of RDX in the medium dropped to 0 ppm from 50 ppm on the 21st day of incubation and the HMX concentration dropped from 50 ppm to 8 ppm on day 21 (Fig.3).

The growth of the SRB enrichment culture under different growth conditions is shown in Fig.4. Maximum growth was observed with pyruvate as the main substrate and ammonium chloride as nitrogen source. No growth occurred in the cultures with TNT as a sole source of carbon and energy. However, when TNT was the sole source of nitrogen with pyruvate as carbon source, growth was significant. Similar results were observed when TNB, DNT, RDX or HMX served either as the carbon source or as the nitrogen source (data not shown). Complete removal of munitions compounds occurred when they served as the

nitrogen source. On the other hand, no removal of munitions compounds occurred in cultures with the munitions compounds as the sole carbon source.

The effect of a carbon source other than pyruvate on the metabolism of munitions compounds is summarized in Table III. Apart from pyruvate, lactate served as the best substrate for TNT, RDX, and HMX metabolism followed by H_2+CO_2, ethanol, and formate for this SRB enrichment culture. In the absence of sulfate, TNT, RDX or HMX might have served as electron acceptor, as evidenced by the removal of these compounds from the culture medium (Table III).

Treatment of the Mixture of Explosive Chemicals. The soil and water samples were contaminated with a mixture of munitions compounds, not a single compound. Thus, we tested all the munitions compounds together, with pyruvate as the electron donor and sulfate as the electron acceptor. The concentrations of the explosive compounds were 100 ppm each of TNT, TNB, DNT and 50 ppm each of RDX and HMX. Figure 5 shows the HPLC elution profiles of these compounds at various stages in the incubation. Figure 5A represents day 0. The retention times under the chromatographic conditions used were 2.5, 3.6, 4.9, 7.9, 9.3 min for HMX, RDX, TNB, TNT, and DNT, respectively. Pyruvate eluted at 1.7 min. On day 0, the peaks representing these compounds were very prominent. By the 10th day of incubation TNT, TNB, and DNT were significantly metabolized, and the removal of RDX and HMX was moderate. Some new peaks had appeared as a result of metabolite production from these compounds (Fig. 5B). On day 20, removal of TNT, TNB, and DNT was complete, and the concentrations of RDX and HMX were less than 10 ppm. The HMX peak was still prominent on day 20, mainly because a metabolite of RDX co-elutes at the same retention time as HMX. We observed this metabolite peak at 2.5 min during the metabolism of RDX alone. In the control chromatogram the various peaks representing the munitions

compounds were present at the same intensity on day 20 as on day 0. Control chromatogram is not shown.

The concentrations of munitions compounds in this experiment are given in Fig. 6. In the mixture of munitions compounds at various concentrations, TNB disappeared first, followed by TNT and DNT. Removal of these compounds was complete within 20 days of incubation. Removal of RDX and HMX during the first week of incubation was insignificant. After 10 days, the concentrations of RDX and HMX began to drop, eventually reaching 0 ppm on day 30. No accurate measurement of HMX in the cocktail mixture could be made because of the interference by the RDX metabolite, which co-eluted at the same retention time as HMX. However, we observed a significant drop in the concentration of HMX by day 30, and the intensity of peak corresponding to HMX and the RDX metabolite dropped significantly after 25 days of incubation. The concentrations of munitions compounds in the control remained at the initial levels throughout the experiment (data not given). This experiment showed that the SRB enrichment culture could remove all of the munitions compounds in a mixture like that found at a contaminated site.

Treatment of Contaminated Soil Slurry. To evaluate the applicability of the SRB enrichment culture for the bioremediation of munitions- contaminated soil, an experiment was conducted with soil (5% and 10%) contaminated with munitions compounds. The concentrations of contaminants in the soil are presented in Table I. The major contaminant was TNT, and levels of the other compounds were significantly low. Therefore, in this experiment we monitored only the concentration of TNT. The SRB medium described above was placed in a 100 mL anaerobic bottles with pyruvate as electron donor and sulfate as electron acceptor. In one set of culture bottles contaminated soil was added to a proportion of 5% and in another set contaminated soil was used at 10%. The

experiment was conducted in duplicate. A 5% inoculum (pregrown midlog-phase culture) was added to both sets. Duplicate set of heat-inactivated (autoclaved) control bottles were used to monitor the abiotic removal of TNT. TNT was extracted from the soil slurry by using acetonitrile as described in the Experimental Section. The results represent averages for two culture bottles.

Figure 7A gives the results for the 5% soil slurry experiment. The initial TNT concentration in the slurry was about 1000 ppm. In the experimental bottles the TNT concentration dropped steadily and eventually reached 0 ppm. In the control bottles the concentration of TNT remained at 1000 ppm throughout the study. Figure 7B presents the result of 10% soil slurry experiment. The TNT initial level of 2,050 to 2,150 ppm did not drop in the experimental bottles during the first week of incubation. On day 8 we reinoculated the experimental bottles with 5% inoculum. The concentration of TNT after reinoculation dropped steadily, reaching 0 ppm on day 45. This experiment showed that the SRB enrichment culture can survive high concentrations of TNT. The study also showed that treatment of slurry at levels above 5% requires a longer incubation time and may require reinoculation of bacteria. This preliminary experiment demonstrated that successful removal of TNT is possible if an appropriate electron donor and electron acceptor are added to the enrichment culture.

DISCUSSION

This study showed that a sulfate reducing enrichment culture isolated from a creek mud sample can remove various munitions compounds under different growth conditions. This is the first report on a sulfate reducing bacteria that can remove explosive chemicals including TNT, RDX, HMX, TNB, and DNT. The SRB culture removed these compounds not only under laboratory

conditions but also in a soil slurry. This study is preliminary in the sense that we only demonstrated the removal of compounds and we did not measure production of various metabolites, identify the metabolic pathway, or demonstrate mineralization of the compounds. However, the success of the preliminary experiment will encourage us and others in the field to study more extensively on the role of sulfate reducing bacteria in the removal of munitions compounds.

Most of the work done during the 1970s dealt with aerobic degradation of TNT (9,10,15) and had very limited success, mainly because the nitro substituents on the aromatic ring are resistant to electrophilic attack by oxygenases (27). This property is attributed to the electron withdrawing character of nitro groups on the aromatic nucleus (28). Polynitroaromatic compounds are subject to reduction of the nitro groups because of their electrophilic character. Many investigators have reported reduction reactions that generate biologically inert materials such as azo, azoxy, and polymeric compounds (29,30).

The recent interest in the bioremediation of munitions compounds encouraged many investigators to evaluate various biological agents and treatment methods. The work of Spiker et al. (31) showed that P. chrysosporium is not a good candidate for bioremediation of TNT contaminated sites containing high concentration of explosives because of its high sensitivity to the contaminants. Duque et al. (32) successfully constructed a Pseudomonas hybrid strain that mineralized TNT. However, the degree of mineralization was not significant, and azoxy and amino intermediates observed in the culture are considered as dead end products.

The bioremediation of TNT contaminated soil by composting (33) has many limitations because the large amounts of additives needed restrict the volume of soil treated. Funk et al. (34) showed potential removal of TNT under anaerobic

conditions. The present study showed that the SRB enrichment culture can remove various munitions compounds by using them as a nitrogen source and also as electron acceptor in the absence of sulfate. In a previous study, we demonstrated that a Desulfovibrio sp. (B strain) used TNT as its sole source of nitrogen (19,20). This isolate first reduced TNT to triaminotoluene, which was reductively deaminated to toluene. In the process the bacteria used the ammonia released from the original TNT molecules as a nitrogen source for growth. A similar reductive deamination process was reported for the degradation of aniline by Desulfobacterium anilini (35). In the current study, a similar mechanism might have been used by the SRB enrichment culture. A detailed experiment is currently underway to study the metabolic pathway of munitions compounds by the SRB enrichment culture.

This report on the removal of various munitions compounds by the SRB enrichment culture under laboratory conditions and in a soil slurry may have significant implications for the decontamination of TNT contaminated soil. Most munitions contamination is in the surface layer of soil, which can be excavated and treated in an anaerobic soil slurry reactor that might successfully remove various contaminants. The added advantage of the SRB enrichment culture is its ability to use munitions compounds as an electron acceptor, eliminating the need for added sulfate in a large scale treatment system. Further research is needed to assess the possibility of using continuous or semi-continuous modes of slurry reactor operation.

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Table I: Concentrations of Munitions Compounds in the Contaminated Soil

Compounds	Concentration range (mg/kg)
TNT	10,000-20,000
TNB	175-300
DNT	50-200
RDX	50-125
HMX	50-100

Table II: Substrates Tested for Growth of the SRB Enrichment Culture

Substrates utilized[a]	Growth[b] (A_{600})	Substrates not utilized
H_2-CO_2	0.80	Acetate
Ethanol	0.76	Methanol
Lactate	0.82	Propionic acid
Formate	0.64	Iso-butyric acid
Fumarate	0.60	Phenol
Malate	0.60	Catecol
Pyruvate	0.96	Benzoate
n-Butanol	0.65	Benzyl alcohol
Glycerol	0.36	Vaniline

[a] Substrate concentrations except for H_2-CO_2 were at 30 mM. The gas H_2-CO_2 was fed every week in the H_2-CO_2 bottles. The gas phase in the other bottles was N_2-CO_2.

[b] Growth after 10 days of incubation at room temperature (22-24°C). Data represent averages of two readings.

Table III: Effect of Different Carbon Sources on Metabolism of Munitions Compounds by the SRB Enrichment Culture[a]

Carbon source	% TNT[c] metabolized		% RDX[c] metabolized		% HMX[c] metabolized	
	With SO_4	Without SO_4	With SO_4	Without SO_4	With SO_4	Without SO_4
Pyruvate	100	100	96	92	90	90
Ethanol	96	92	92	90	86	84
Formate	78	70	68	64	56	50
Lactate	100	100	98	95	88	90
H_2-CO_2[b]	100	98	90	90	80	71

[a] Data were collected after 21 days of incubation. Data represent means of two values.

[b] The gas phase was H_2-CO_2 (20 psi) fed every other day.

[c] Initial concentration of TNT was 100 ppm and the initial concentration of HMX and RDX was 50 ppm.

FIGURE LEGENDS

Figure 1. Structures of munitions compounds.

Figure 2. Scanning electron micrograph of the SRB enrichment culture.

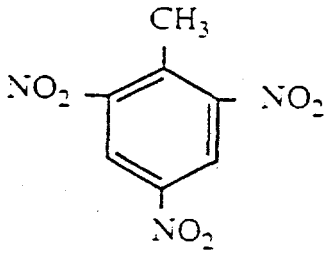
Figure 3. Removal of various munitions compounds by the SRB enrichment culture. (A) TNT; (B) TNB; (C) DNT; (D) RDX; (E) HMX. Pyruvate (30 mM) served as the electron donor and sulfate (20 mM) served as the electron acceptor. Controls were heat inactivated by autoclaving after inoculation. O, experimental; Δ, heat inactivated control.

Figure 4. Growth of SRB enrichment culture under different growth conditions. O, pyruvate as the sole carbon source with ammonium chloride as nitrogen source; ▲, TNT as the sole nitrogen source with pyruvate as carbon source; □, TNT as the sole carbon source with ammonium chloride as nitrogen source; Δ, heat inactivated control.

Figure 5. HPLC elution profiles on various days of incubation of the SRB enrichment culture containing munitions compounds. A, day 0; B, day 10; C, day 20.

Figure 6. Concentrations of munitions compounds in the SRB enrichment culture. Pyruvate (30 mM) served as electron donor. and sulfate (20 mM) served as electron acceptor. Δ, TNT; O, TNB; □, DNT; ●, RDX; ▲, HMX.

Figure 7. TNT concentration in (A) 5% soil slurry and (B) 10% soil slurry. Pyruvate (30 mM) was the electron donor and sulfate (20 mM) the electron acceptor. Control bottles were autoclaved after inoculation. Δ, control; O, experimental bottle. In the 10% soil slurry experiment, bacteria were reinoculated on day 8.



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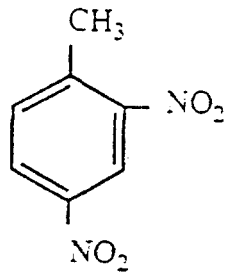

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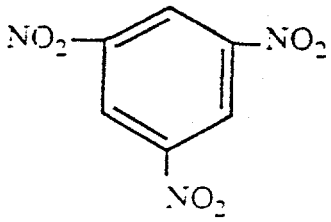
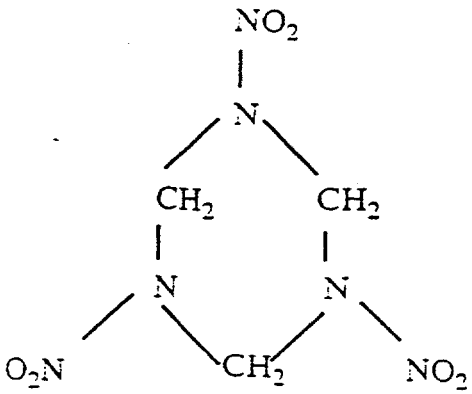
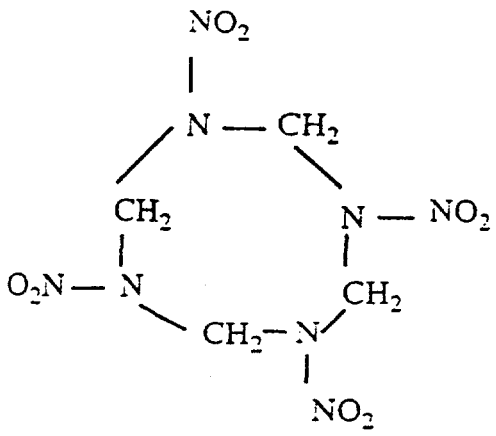


2,4,6-Trinitrobenzene



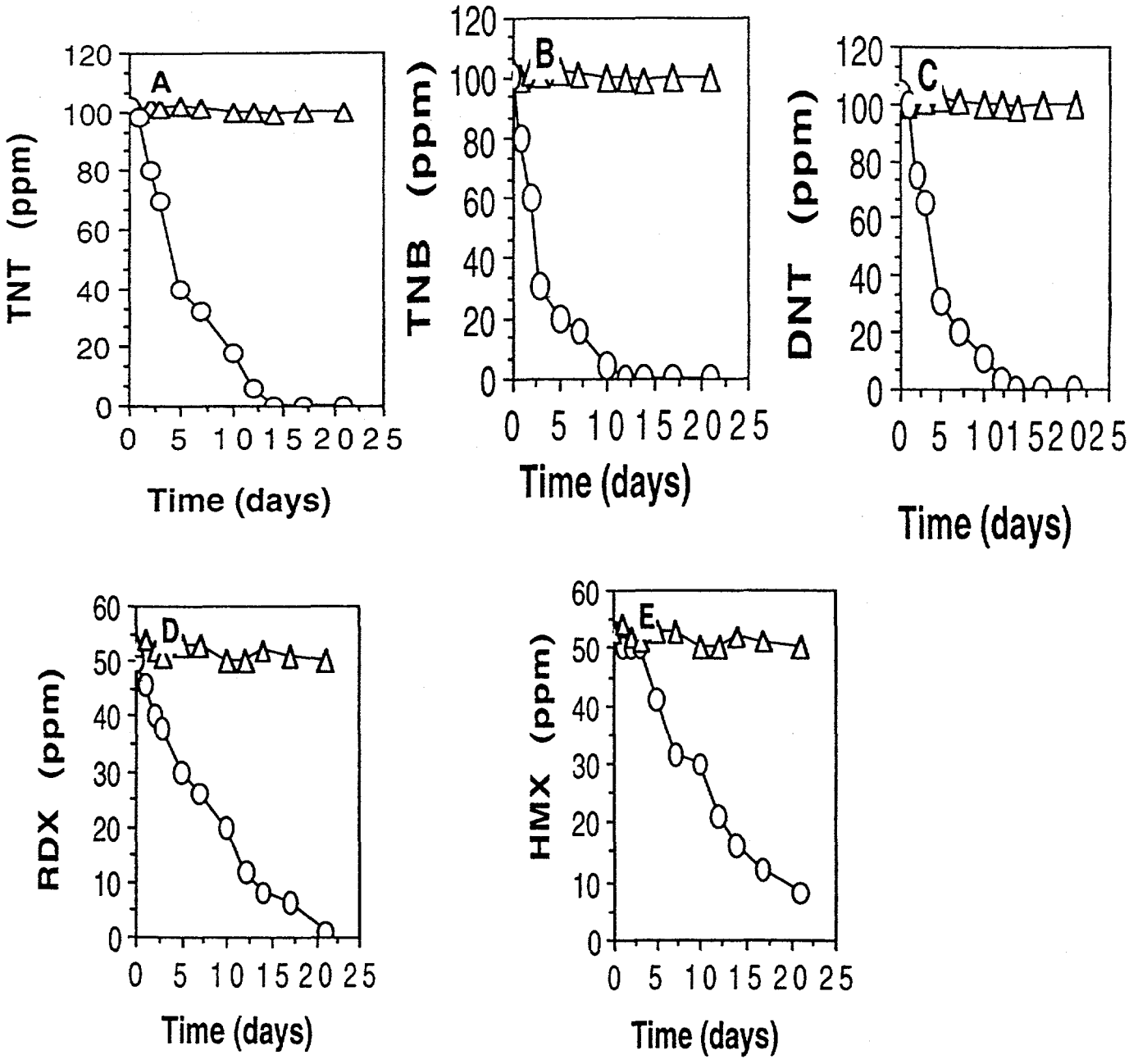
RDX

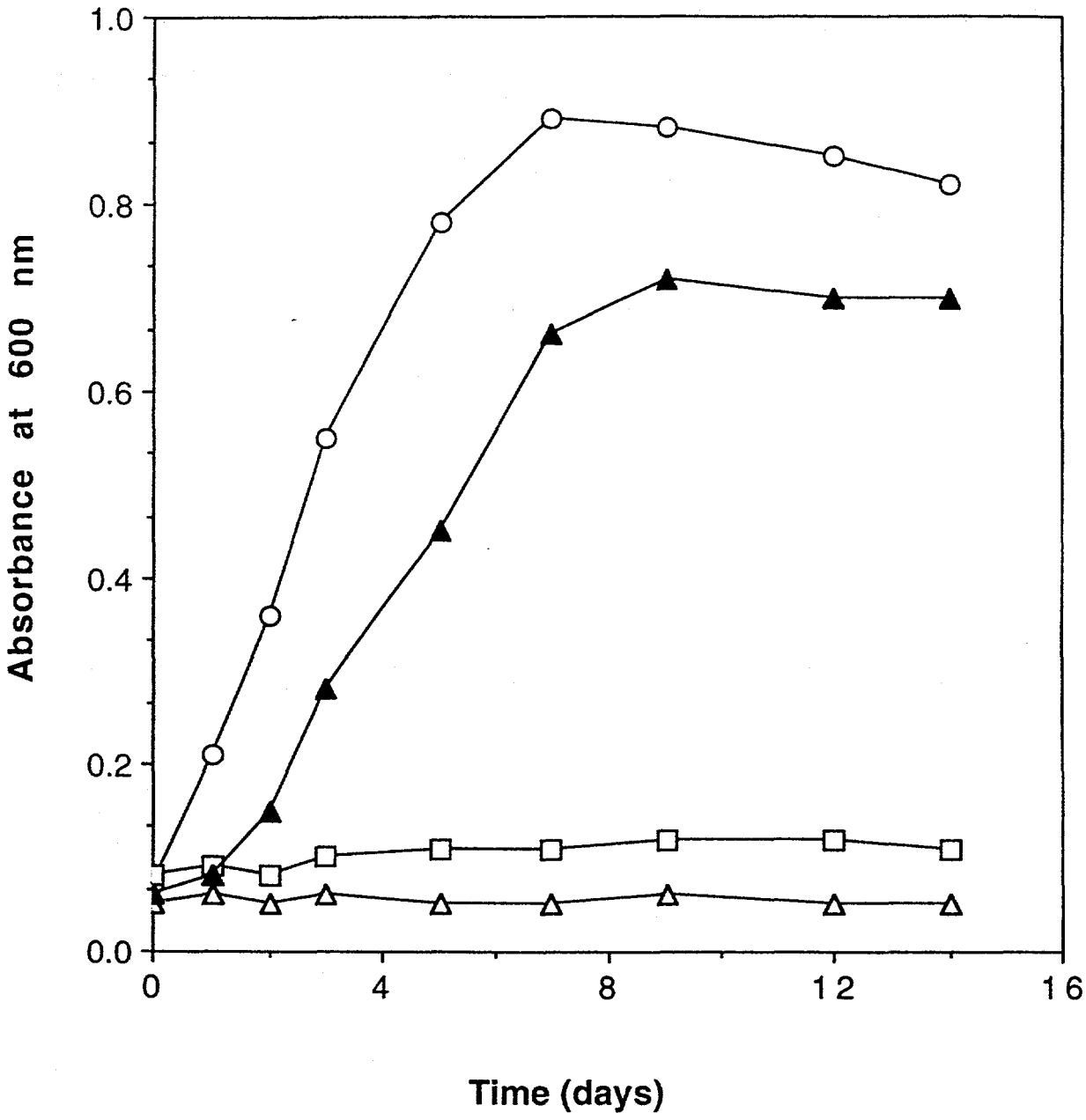


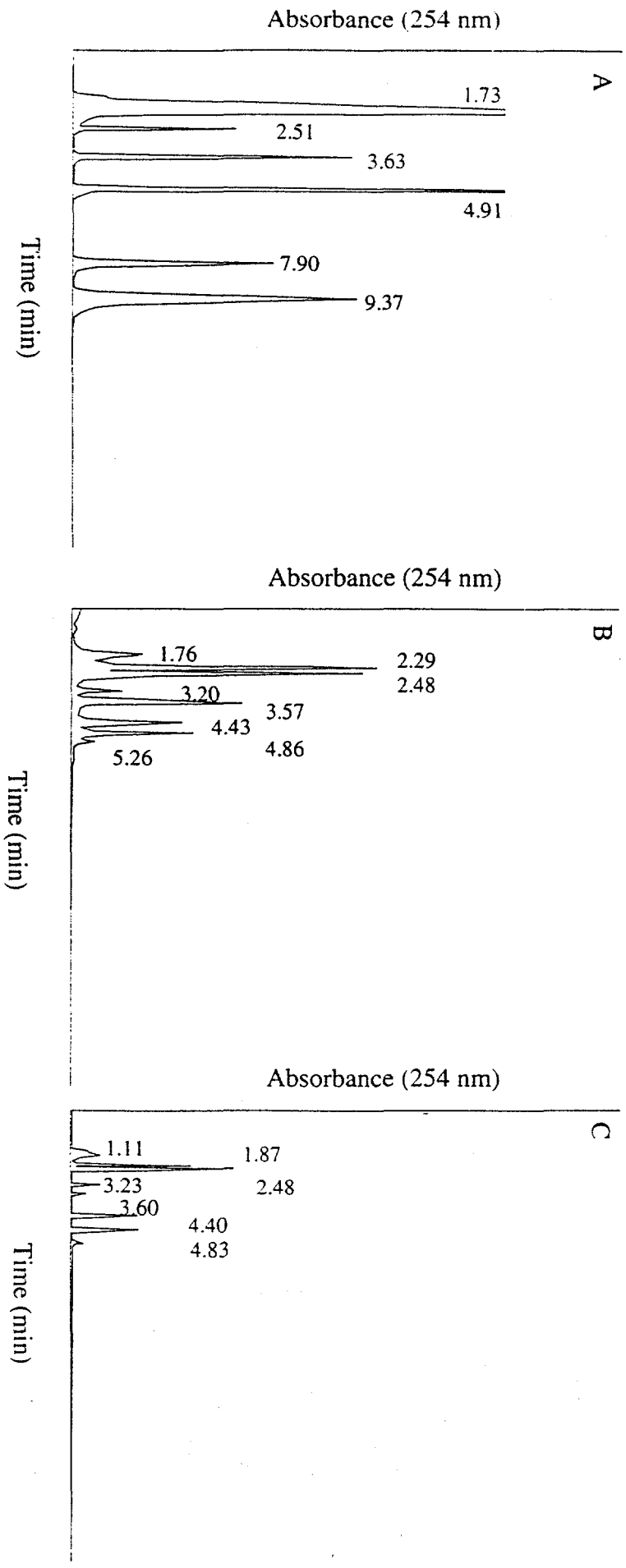
HMX

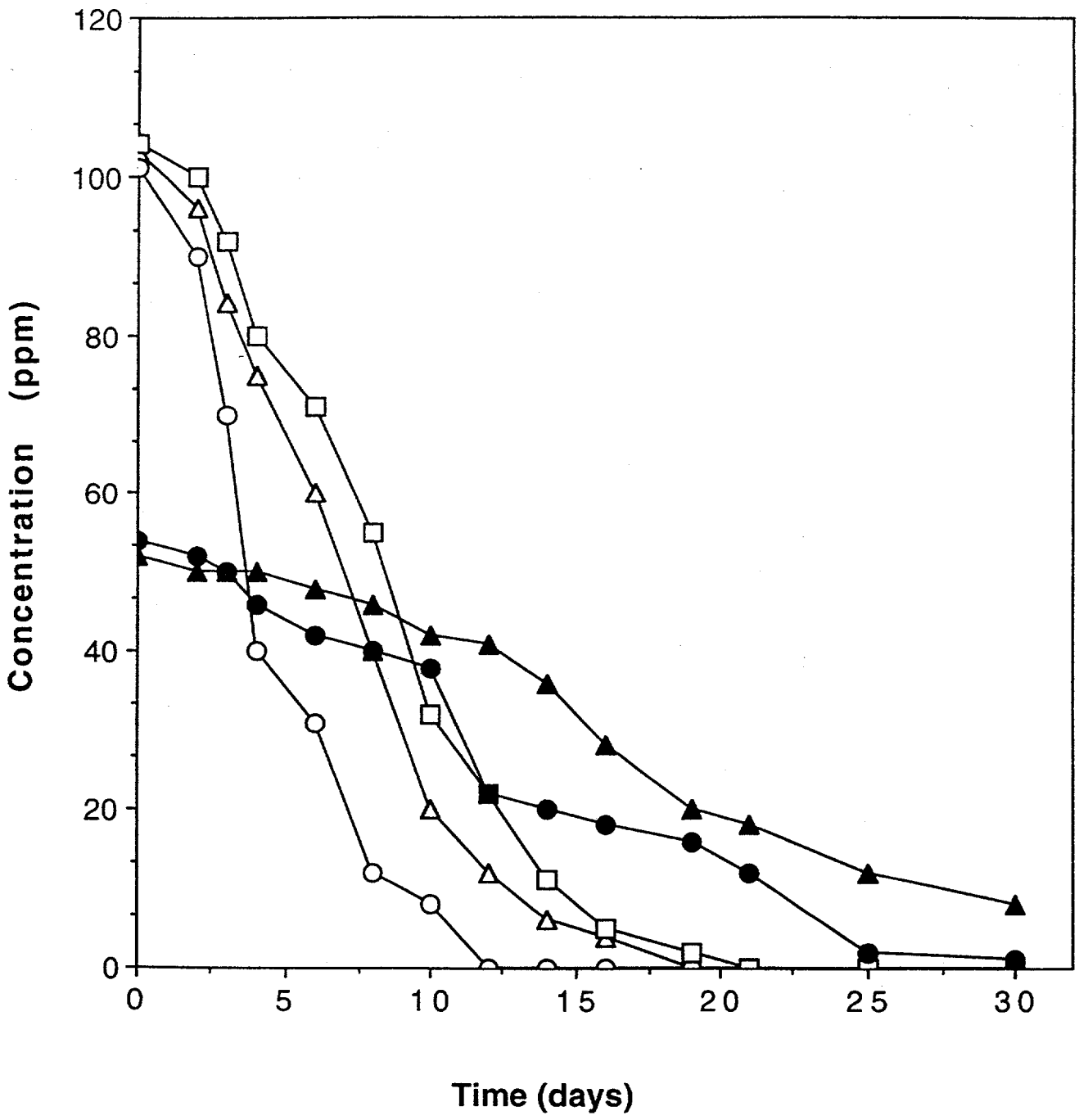
Figure 1. Structures of munitions compounds.

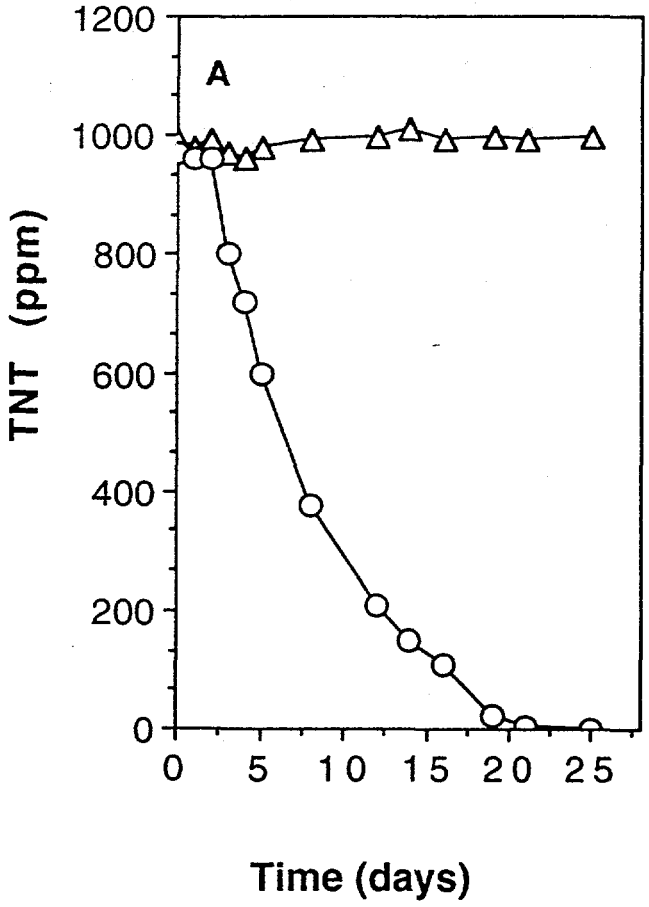
Figure 2. Scanning electron micrograph of the SFB enrichment culture.

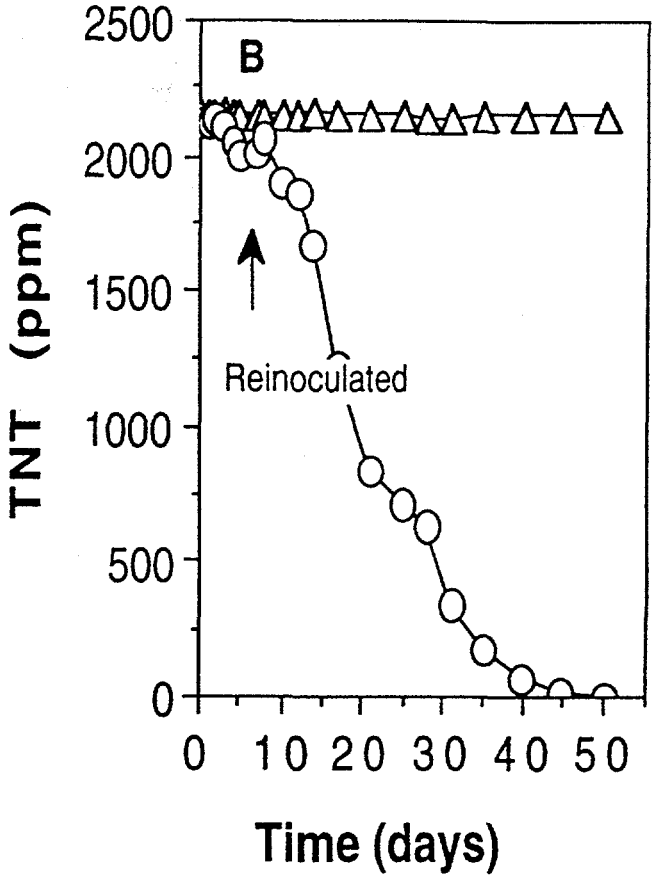

A
TNT (ppm)
120
100
80
60
40
20
0
0 5 10 15 20 25
Time (days)
B
TNB (ppm)
120
100
80
60
40
20
0
0 5 10 15 20 25
Time (days)
C
DNT (ppm)
120
100
80
60
40
20
0
0 5 10 15 20 25
Time (days)
D
RDX (ppm)
60
50
40
30
20
10
0
0 5 10 15 20 25
Time (days)
E
HMX (ppm)
60
50
40
30
20
10
0
0 5 10 15 20 25
Time (days)


1.0
0.8
0.6
0.4
0.2
0.0
Absorbance at 600 nm
0
4
8
12
16
Time (days)


Absorbance (254 nm)
A
1.73
2.51
3.63
4.91
7.90
9.37
Time (min)
Absorbance (254 nm)
B
1.76
2.29
2.48
3.20
3.57
4.43
4.86
5.26
Time (min)
Absorbance (254 nm)
C
1.11
1.87
3.23
2.48
3.60
4.40
4.83
Time (min)


120
100
80
60
40
20
0
Concentration (ppm)
0
5
10
15
20
25
30
Time (days)


A
1200
1000
800
600
400
200
0
TNT (ppm)
0 5 10 15 20 25
Time (days)


B
2500
2000
1500
1000
500
0
TNT (ppm)
Reinoculated
0 10 20 30 40 50
Time (days)