

Screening Test and Isolation Procedure for TNT-Degrading Microorganisms

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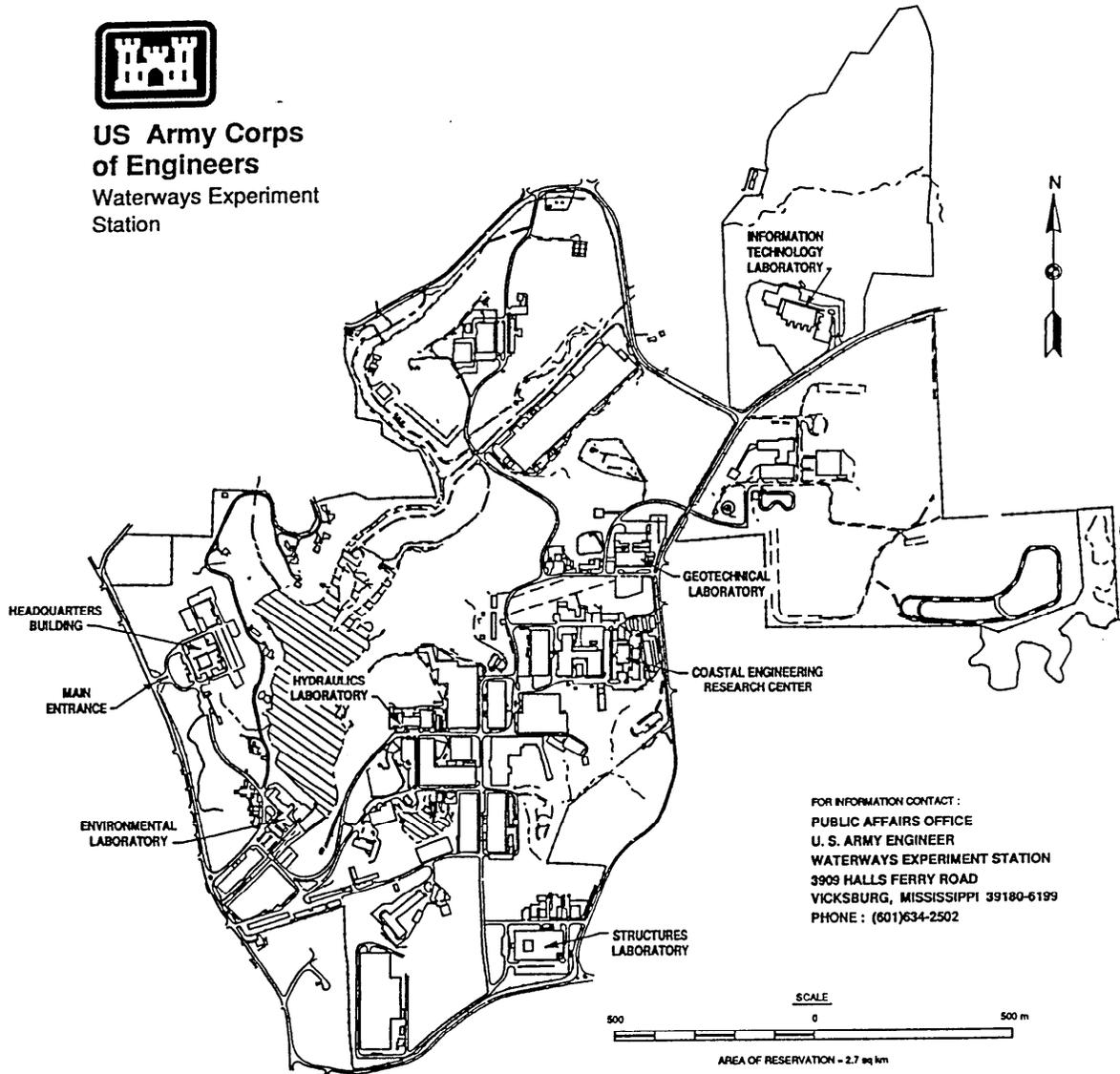
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Preface

The studies reported herein were conducted by the Environmental Laboratory (EL) of the U.S. Army Engineer Waterways Experiment Station (WES), Vicksburg, MS. The research was sponsored by the Department of Army Installation Restoration Research Program (IRRP), Environmental Quality and Technology, Basic 6.1 Research Work Unit, titled "Microbiology and Degradation of Explosives and Energetics," Project No. AH68-BR-001. Dr. Clem Myer was the IRRP Coordinator at the Directorate of Research and Development, Headquarters, U.S. Army Corps of Engineers (HQUSACE). Dr. Bob York of the U.S. Army Environmental Center and Mr. Jim Baliff of the Environmental Restoration Division, Directorate of Military Programs, HQUSACE, served as the IRRP Overview Committee. Dr. John Cullinane, WES, was the IRRP Program Manager.

The study was conducted by Drs. Douglas Gunnison and Judith C. Pennington and Ms. Cynthia B. Price of the Ecosystem Processes and Effects Branch (EPEB), Environmental Processes and Effects Division (EPED), EL, and Mr. Glenn B. Myrick, student contractor. Technical advice was given by Mr. Mark Zappi of the Environmental Engineering Division (EED), EL, and Dr. Patricia Unkefer of the Department of Energy Los Alamos National Laboratory, Los Alamos, NM. Analytical chemistry was provided by Ms. Karen Myers of the Analytical Laboratory Group, EED. Additional analytical chemistries were conducted by the Analytical Chemistry Laboratory of the Missouri River Division Laboratories and by Arthur D. Little, Inc., Cambridge, MA.

The study was conducted under the direct supervision of Dr. Richard E. Price, Acting Chief, EPEB, and under the general supervision of Mr. Donald L. Robey, Chief, EPED, and Dr. John Harrison, Director, EL.

At the time of publication of this report, Director of WES was Dr. Robert W. Whalin. Commander was COL Leonard G. Hassell, EN.

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LIST OF ABBREVIATIONS

CO ₂	carbon dioxide
HEIP	Hastings East Industrial Park
RDX	hexahydro-1,3,5-trinitro-1,3,5-triazine
TNT	trinitrotoluene
2-A-DNT	2-amino-4,6-dinitrotoluene
4-A-DNT	4-amino-2,6-dinitrotoluene
2,4-DNT	2,4-dinitrotoluene
2,6-DNT	2,6-dinitrotoluene
2,4-A-6NT	2,4-diamino-6-nitrotoluene
2,6-A-4NT	2,6-diamino-4-nitrotoluene

SCREENING TEST AND ISOLATION PROCEDURE
FOR TNT-DEGRADING MICROORGANISMS

PART I: INTRODUCTION

Background and Rationale

Bioremediation may utilize either of two mechanisms of microbial attack upon contaminants--transformation or mineralization. Transformation is the modification of the molecular structure of the contaminant to yield other organic compounds. If transformation products are determined to be environmentally and toxicologically safe and stable, bioremediation by transformation is sufficient. Mineralization is the degradation of the contaminant to yield innocuous inorganic constituents. These include carbon dioxide or methane, various forms of inorganic nitrogen, and water. Complete mineralization to products already known to be safe eliminates the need for additional testing.

While mineralization is desirable for bioremediation of 2,4,6-trinitrotoluene (TNT)-contaminated soils, for many years only TNT transformation pathways were reported. However, evidence for TNT mineralization under suitable conditions has been documented in the recent literature. Earlier work by Soviet scientists suggested a potential for complete mineralization through the readily degradable intermediates phloroglucine and pyrogallol (Selivanovskaya, Akhmetova, and Naumova 1986; Naumova, Selivanovskaya, and Cherepneva 1988; Naumova, Selivanovskaya, and Mingatina 1988). Research by Chinese scientists revealed microbial production of reductase and dehydrogenase active on TNT (Li, Yang, and Yang 1987). Recent work has suggested that mineralization proceeds through the 1,3,5- and 1,2,3-trihydroxybenzenes, which are subsequently degraded to yield carbon dioxide and water (see reviews by Higson 1992 and Golovleva et al. 1992).

Other investigations have also demonstrated a possibility for complete mineralization, although pathways have not been delineated. For example, Fernando, Bumpus, and Aust (1990) found extensive biodegradation of TNT over a 90-day period mediated by the white rot fungus *Phanerochaete chrysosporium*. These investigators found mineralization of 18.4 to 19.6 percent of initially added radiolabeled TNT in soil and liquid cultures, respectively. Spanggord et al. (1992) reported the initial steps in a pathway for complete

biodegradation of 2,4-dinitrotoluene (DNT) by a *Pseudomonas*, which used DNT as the sole source of carbon and energy.

If complete microbial mineralization of TNT is possible, utilization of this process for the treatment of TNT-contaminated soils will be beneficial. Biological treatment will be most easily conducted, if microbial populations naturally present in TNT-contaminated soil or sediment can be stimulated to mineralize TNT. Screening of TNT-contaminated soils for microbial populations amenable to development for TNT degradation requires that several tests be developed. These include cost-effective procedures to (a) readily demonstrate the presence of microbial activity effective in TNT mineralization within a soil proposed for biological treatment, (b) rapidly determine the nutritional and environmental conditions required by effective microorganisms to increase the rate and extent of their mineralization activities within the soil treatment system, and (c) quickly isolate and characterize microorganisms or microbial consortia to determine specific roles of nutrients, cometabolites, and environmental conditions in regulating pathways for mineralization and by-product release under various biotreatment scenarios.

Objectives

The objectives of the study were to develop a rapid, easily applied method to screen soils and sediments for the presence of microorganisms able to mineralize TNT, and to verify mineralization activity in soil and isolate microbial cultures active in mineralizing TNT.

PART II: METHODS AND MATERIALS

Screening Procedure

Candidate soils for screening test development were selected based on a known history of exposure to TNT contamination. Soils requiring biological treatment for TNT removal generally have been contaminated for many years. Soils with a long history of TNT contamination are likely to have microbial populations adapted to the utilization of this compound. Sources of the soils used in this work are listed in Table 1.

Soil collection and handling

Soils were obtained from areas around munitions plants having a known history of exposure to TNT contamination. Upon collection, soils were sieved through 0.5-cm mesh netting to remove rocks and possible large chunks of TNT before placement into 5- to 55-gal (19- to 208-l) containers for shipment to the U.S. Army Engineer Waterways Experiment Station (WES). In some cases, sieving was not performed until soils were received at WES. Upon arrival at WES, soils were stored at 4 °C until used. Samples of each soil were analyzed to determine particle size, organic matter content, and TNT and selected degradation products. Dry weight was assessed by determining weight loss from 10 g of soil after drying for 12 hr at 105 °C.

Soil priming

Certain aspects of the molecular structure of TNT may preclude microbial attack. Alternatively, the TNT molecule may be readily mineralized once initial transformations have been made to the molecular structure. To provide a variety of different opportunities to stimulate microbial attack on TNT, soils were treated with a variety of compounds to "prime" their microbial inhabitants for TNT utilization. Priming substrates were selected based on their similarity to the TNT molecule and/or because other investigations indicated that some of these materials might stimulate degradational activity (see, for example, Hallas and Alexander 1983).

Soils were primed to stimulate TNT degradational activity for the screening test using the procedure described below. The procedure was designed to detect microbial activity against TNT, rather than a quantitative technique designed to provide reproducible degradation rates. Therefore, only one replicate was prepared for each treatment. Thirty grams (dry weight) of

soil was placed into each of 16 sterile 125-ml Erlenmeyer flasks. To each flask was added 0.25 g of TNT.

Pairs of flasks received 0.5 g of one of the following treatments, termed "priming substrates": (a) sodium acetate, (b) sodium succinate, (c) dinitroaniline, (d) dinitrophenol, (e) dinitro-*o*-cresol, (f) toluene, (g) sawdust, or (h) no substrate. Soils in each flask were mixed to incorporate amendments. One flask of each pair was plugged with sterile foam or cotton and incubated at 30 °C under static conditions. To provide an aerobic aqueous environment, the other flask of each pair received 30 ml of sterile distilled water and was incubated on a gyrorotary shaker at 30 °C and 150 rpm.

Soil screening

Primed soils from each flask were screened for TNT degradation activity by inoculation onto crystalline lawns of TNT prepared as follows. Underlays of 15 ml of sterile basal salts medium (Table 2) containing 20 g/ℓ agar, 1.0 g/ℓ yeast extract, and 10 g/ℓ of sodium acetate, sodium succinate, or glucose (termed "cometabolites") were poured into petri dishes and allowed to solidify. Cometabolites were selected based on a known or suspected ability to support TNT degradation. A solution of 2 g of hydrated TNT (Eastman Organic Chemicals, Rochester, NY) dissolved in 10 ml of reagent grade acetone was prepared; 3.0 ml of this was spread evenly over the surface of each underlay (at this concentration, the acetone would plasmolyze any microbial cell contacted, providing sterilization of the TNT). The acetone was permitted to evaporate to dryness, leaving a sterile layer of TNT crystals deposited evenly over the surface of the underlay. Plates were preincubated for a minimum of 3 days to ensure complete removal of acetone fumes and to detect growth of any contaminating microorganisms. Plates were then inoculated by placing a clump of a primed soil onto the center of each plate.

As indicated in Table 3, soils primed with sodium acetate or sodium succinate were placed onto crystalline lawns containing sodium acetate or sodium succinate, respectively, in their underlays. Soils containing no priming substrate, toluene, dinitroaniline, dinitrophenol, or dinitro-*o*-cresol were placed onto separate lawns containing one of each of the three cometabolites in their underlays. Soils primed with sawdust were placed only onto lawns containing glucose in their underlays. After 2 to 4 weeks incubation at 30 °C, plates were scored for the presence or absence of growth, color product, and TNT removal as indicated by the formation of a clear zone surrounding

the soil clump. Plates were also examined for the release of colored metabolites into the agar.

Isolation and Development of Native TNT-Degrading Species

Because of time constraints, soils from the Hastings East Industrial Park (HEIP) site were not initially processed in the full screening test, but were screened using succinate and acetate as both priming substrates and cometabolites. The HEIP soils were selected for isolation and development of native TNT-degrading species for the following reasons. HEIP soils had the highest levels of TNT contamination of any of the soils received at the time this portion of the investigation was initiated (reaching levels of approximately 9,330 mg/kg) (Table 4). Also, the HEIP sites were selected because they remained moist most of the time. The combination of high TNT levels, moisture, and prolonged history of exposure to TNT contamination made these soils likely hosts for microorganisms adapted to TNT degradation, and some of the HEIP soils were found to contain microorganisms that were especially vigorous in transforming TNT.

Determination of activity in HEIP soils was conducted in a series of steps, starting with an examination of TNT removal and by-product accumulation in slurries of the soil. Mineralization was then verified by determining release of $^{14}\text{CO}_2$ from slurries spiked with ^{14}C -labeled TNT. From here, the study moved to liquids containing mixed cultures of isolates obtained from these soils for confirmation of product removal.

TNT removal in slurries

To each of ten 500-ml Erlenmeyer flasks was added the equivalent of 75 g dry weight of HEIP TS-Flem-2 soil (Table 4). The following treatments were added to paired sets of flasks:

- Treatment 1: 10 g of $(\text{NH}_4)_3\text{PO}_4$ + 1.0 g of sodium succinate
- Treatment 2: 10 g of $(\text{NH}_4)_3\text{PO}_4$ + 1.0 g of sodium acetate
- Treatment 3: 1.0 g of sodium succinate
- Treatment 4: 1.0 g of sodium acetate
- Treatment 5: 10 g of $(\text{NH}_4)_3\text{PO}_4$ + 4 g of HgCl_2

[Note that HgCl_2 was used at a strength of 1.6 percent (wt/vol). This concentration removes most, but not necessarily all, biological activity from the soil. Higher levels of HgCl_2 might remove all activity, but these concentrations tend to interfere with chemical determinations. Other means of

sterilization (autoclaving, gamma irradiation) have unknown effects on the soil organic matter and/or destroy TNT and were unacceptable.]

Each flask then received sufficient deionized distilled water to bring the volume to 250 ml (i.e., a 30 percent (wt/vol) slurry). This gave final ammonium phosphate concentrations of 4 percent and cometabolite (sodium succinate or sodium acetate) concentrations of 0.4 percent.

Following initial shaking to thoroughly mix flask contents, 50 ml of slurry was removed from each flask and frozen as the time 0 sample. Flasks were incubated in a G-25 shaker at 25 °C and 150 rpm. At 1-week intervals, flasks were recharged with the cometabolite, if present, using aseptic techniques. At 30 days, flasks were removed from the shaker, and 50-ml samples were removed and frozen until shipped for analysis.

Confirmation of TNT mineralization

Mineralization was confirmed by adding radiolabeled TNT to soil slurries and following the emission of radiolabeled CO₂. Mineralization of TNT in slurries of soil from the Hastings site was determined using a modification of the procedure of Dobbins and Pfaender (1988). Slurries containing 1 g TS-Flem-2 soil in 22 ml distilled deionized water with 0.8 percent sodium acetate were spiked with 10 µg [ring-U-¹⁴C] TNT.* Slurries were prepared in triplicate and incubated at room temperature on a gyrorotary shaker at 150 rpm.

To determine formation of possible radiolabeled volatile products, vials containing the slurries were vented through traps containing 20- to 50-mesh XAD-4 Amberlite resin (Mallinkrodt). To ensure that carbon dioxide was not lost through the vent, XAD traps were followed by two 10-ml, 1N KOH backup traps in series. Vials containing the slurries were shaken for 24 hr, at which time the cometabolite was added to a final concentration of 20 g/l. Cultures were sacrificed at 1, 2, and 4 weeks following addition of cometabolite. Upon sacrifice, slurries were acidified with H₃PO₄, and the evolved CO₂ was collected in 1N KOH over 48 hr. One milliliter of the KOH from each trap was placed into 15 ml of Ultima Gold scintillation cocktail (Packard Instruments, Inc., Downers Grove, IL) and counted for 10 min on a Packard 2500 TR Liquid Scintillation Analyzer.

* Specific activity 21.58 mCi/mM per gram dry weight of soil (Sigma Chemical Company, St. Louis, MO). Radiological purity was >98 percent, as determined by radio-high pressure liquid chromatography (HPLC); chemical purity was >98 percent, as determined by ultraviolet-HPLC.

To determine mass balances, radiolabeled material remaining in the liquid phase was determined by centrifuging 5.0 ml of the overlying water and counting 1.0 ml of the supernatant in 15 ml of scintillation cocktail. Residual radiation in the soil phase was established by burning 0.2-g samples of the soil in both wet and dry states on a Packard model 307 Sample Oxidizer and trapping the CO₂ released in a mixture of 10 ml Permafluor plus 10 ml Carbo-Sorb (Packard Instruments, Inc.). Radiation in the XAD resin traps was determined following extraction of the resin with three 5-ml portions of hexane; these were subsequently concentrated to 1 ml and counted in scintillation cocktail.

Isolation procedure

TNT-degrading isolates were obtained by enrichment culture conducted with soils from several different locations within the HEIP site. Thirty-gram samples of soil from selected positive screening tests were placed into sterile flasks and amended with 1 percent (wt/wt) cometabolite (either sodium acetate or sodium succinate) and 5.0 ml of distilled water. Flasks were plugged and incubated at 25 °C under static conditions.

After 1 and 4 weeks of incubation, samples of the acetate- or succinate-primed soil from each flask were diluted in sterile tap water and plated onto basal salts medium (Table 2) solidified with 15 g/ℓ agar and containing 100 mg/ℓ TNT, 0.5 g/ℓ yeast extract, and the cometabolite at a concentration of 20 g/ℓ. Plates were incubated at 25 °C for several days. Representative colonies were picked from each plate and transferred to liquid culture media containing 100 mg/ℓ TNT and 1.5 percent of the cometabolite. Cultures were incubated for 10 days at 25 °C on a gyrorotary shaker at 150 rpm.

Initial effectiveness of cultures in TNT destruction was evaluated by examining culture supernatants for loss of TNT and appearance of known TNT transformation by-products (2,4- and 2,6-dinitrotoluenes; 2-amino-4,6-dinitrotoluene; 4-amino-2,6-dinitrotoluene; 1,3,5-trinitrobenzene). Cultures found to be effective in removing or lowering the concentrations of these compounds were plated onto the same solid medium as used for the initial isolations. Isolates obtained in this manner were identified using the BIOLOG identification procedure (BIOLOG, Inc., Hayward, CA). BIOLOG is a microbial identification system utilizing a series of biochemical tests in liquid for metabolic characterization of bacteria. The results of these tests are subsequently entered into a computer program. The program provides an identifica-

tion of the organism based on a statistical comparison to an existing database for environmental isolates.

Evaluation of TNT removal and by-product formation in liquid culture

Several cultures from the HEIP site exhibiting loss of TNT were studied to determine maximum removal of TNT and TNT transformation by-product formation over a 10-day incubation period. Ten milliliters of each liquid culture was transferred to 500-ml Erlenmeyer flasks containing 250 ml of fresh liquid culture medium, as described above. Following 5 days of incubation at 25 °C and 150 rpm on a gyrorotary shaker, each culture was aseptically recharged with 1 percent fresh cometabolite and incubated for an additional 5 days. At 10 days, cultures were frozen until analyzed for TNT and TNT degradation products by HPLC.

Growth on TNT Versus Cometabolic Destruction

Cultural evaluation

To determine whether microorganisms living in soils from the Hastings site had the ability to utilize TNT as the sole source of carbon and energy, individual isolates growing on BSM containing TNT and yeast extract with sodium acetate as the cometabolite were cultured on the following media:

- a. BSM salts with agar and 100 mg/ℓ TNT, but no acetate or yeast extract.
- b. BSM salts with agar and 100 mg/ℓ TNT plus acetate, but no yeast extract.
- c. BSM salts with agar and 100 mg/ℓ TNT plus yeast extract, but no acetate (0 percent).
- d. BSM salts with agar and 100 mg/ℓ TNT and yeast extract, plus 1 g/ℓ acetate (0.1 percent).
- e. BSM salts with agar and 100 mg/ℓ TNT and yeast extract, plus 5 g/ℓ acetate (0.5 percent).
- f. BSM salts with agar and 100 mg/ℓ TNT and yeast extract, plus 10 g/ℓ acetate (1.0 percent).

Evaluation of mineralization by individual microbial isolates

Mineralization by individual microbial isolates was determined by inoculation and growth of microorganisms in 25 ml of liquid BSM containing 10 µg U-ring ¹⁴C-radiolabeled TNT. The microorganisms and consortia used were

selected from those isolates from the HEIP site giving the best removal of TNT and least accumulation of undesirable by-products. Microorganisms or consortia and radiolabeled TNT were placed together with 0.5 g/l yeast extract and 20 g/l of the appropriate cometabolite in a 50-ml Erlenmeyer flask containing a screw cap with Teflon septum.

Flasks were incubated at room temperature on a gyrorotary shaker at 150 rpm. Individual flasks were attached to a metered air supply containing a KOH scrubber to remove carbon dioxide. Air was introduced to the flasks through a syringe needle that reached into the top one third of the flask. Air exited the flask through a second syringe needle attached to a 1-ml plastic syringe packed with Fluorosil 60/80 mesh and three 15-ml scintillation bottles, each containing 10 ml of 1N KOH. KOH traps were changed and monitored every 3 days for the appearance of $^{14}\text{CO}_2$. Fluorosil traps were extracted with three 10-ml portions of acetonitrile, and 1 ml of the extract was counted in 15 ml of Ultima Gold.

Chemical Analysis

Soils were analyzed for particle size composition using the method of Patrick (1958). Total organic carbon content in soil samples was determined by dry combustion (Allison 1965). Soil moisture content was determined gravimetrically following oven-drying for 12 hr at 105 °C.

The presence of TNT and its degradation products was determined by the U.S. Environmental Protection Agency (EPA) SW 846, Method 8330. The method requires extraction with acetonitrile and analysis by HPLC. In ALG, analyses were performed on a Waters High Pressure Liquid Chromatograph having a 600 MS System Controller/Solvent Delivery System, a 700 Satellite WIST Injector, and a 991 MS Photodiode Array Detector. Separation was accomplished using a Supelco LC-18 25-cm by 4.6-mm column having a pore size of 5 μm eluted with 1:1 methanol water at a flow rate of 1.2 ml/min. Analytes were confirmed using a Supelco LC-CN 25-cm by 4.6-mm column.

TNT and TNT by-product analyses were also conducted at the U.S. Army Engineer Division, Missouri River, laboratories in Omaha, NE, and by Arthur D. Little, Inc., Cambridge, MA, also using EPA SW 846, Method 8330. USATHAMA SARM analytical reference materials were used for quantification of the explosives and TNT degradation by-products. Detection limits for all analytical

laboratories are included in Appendix A. All radiolabeled samples were counted on a Packard 2500 TR Liquid Scintillation Analyzer (Packard Instruments, Inc.).

PART III: RESULTS AND DISCUSSION

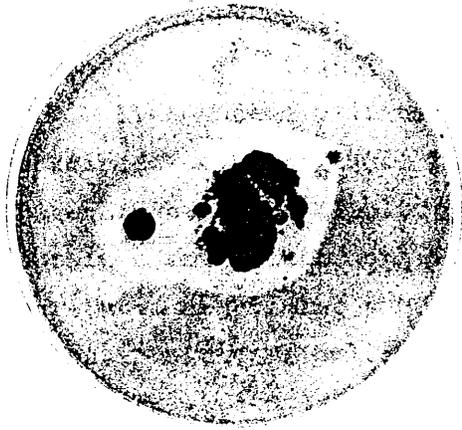
Soil Characteristics

Particle size contents of soils subjected to the full screening test as well as an average for all of the HEIP soils are presented in Table 5. Most of the soils contained predominantly sand (Iowa Army Ammunition Plant (AAP), Lone Star AAP, Sub Base Bangor) or equal concentrations of sand and silt (McAlester AAP and Radford AAP). The remaining soils were predominantly silt. Caddo Lake sediment contained one-third clay, the highest clay content of the soils used in this study.

TNT in soils ranged in concentration from <0.93 mg/kg (Caddo Lake) to several thousand milligrams per kilogram (HEIP) (Table 6). McAlester, Sub Base Bangor, and Lone Star soils contained detectable levels of 4-amino-2,6-dinitrotoluene, while McAlester, Sub Base Bangor, HEIP, and Louisiana soils contained detectable levels of 2,4-dinitrotoluene. Octahydro-1,3,5,7-trinitro-1,3,5-tetrazocine (HMX) and hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) were each found in Iowa, Lone Star (J), Louisiana, and McAlester soils. RDX only was found in the HEIP soil. Louisiana, McAlester, Sub Base Bangor, and HEIP soils all contained detectable levels of trinitrobenzene (TNB). Detectable levels of dinitrobenzene (DNB) and Tetryl were found in the HEIP and Sub Base Bangor (J) soils.

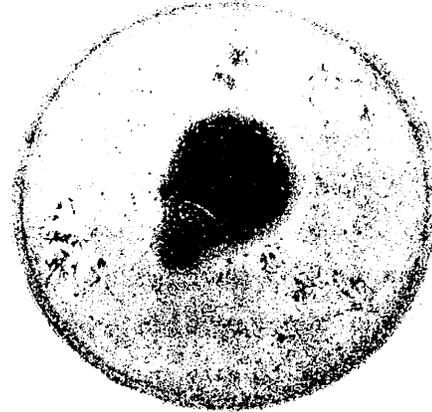
Screening Test

The screening test showed only the presence of TNT-removing activity. Because the screening test relies on a visual determination of clearing around the clump of soil being tested, the test does not provide quantitation of the amount of TNT removed or the by-products produced. Results for positive screening tests and various color combinations typical for the plates are shown in Figure 1. Compared to the initial uninoculated lawn of TNT (Figure 1a), positive plates had zones of clearing immediately surrounding the soil clump (see especially Figures 1b and 1c). While most plates exhibited little or no color change with respect to uninoculated plates (Figure 1a), others turned bright yellow to orange (Figures 1b and 1c), indicating possible accumulations of either 2-amino-4,6-dinitrotoluene, dinitrophenols, or



a. Crystalline lawn of TNT before inoculation with soil

b. Strongly positive test. Note clearing of TNT around soil clump



c. Weakly positive test. Zone of clearing barely past perimeter of soil clump



d. Positive test with yellowish-orange color change. Note profuse growth of microorganisms around and away from soil clump



e. Weak positive with red pigment production. Clearing barely visible around edges of growth

Figure 1. Typical plates from screening test

catechols.* Some plates turned red to maroon, suggesting photolytic, chemical, or biochemical reduction of TNT to 2,4-diamino-6-nitrotoluene* (Figure 1d).

Screening results for seven soils generally exhibited no correlation between static or shaking incubations and the number of TNT-active plates (Table 7, Sheets 1-6); nearly equal numbers of positive plates were obtained with each method. Thus, 55 of 108 positive results were obtained with shaking, while 53 of 108 positives were obtained under static conditions. Positive recoveries were often slightly higher at 1 week of incubation than at week 4. However, the positive results obtained at 4 weeks often occurred in different soils from those at week 1.

Even when a priming substrate was lacking, positive results were obtained for Caddo Lake, Lone Star, Louisiana, McAlester, Radford, and Sub Base Bangor soils (Table 7, Sheet 1). Positive results were also often obtained when dinitroaniline, dinitro-*o*-cresol, dinitrophenol, and toluene were used as priming substrates (Sheets 2-5). Some, but generally less frequent, positive results were obtained when sawdust, sodium acetate, and sodium succinate were used as priming substrates (Sheets 6-8). In the cases of sodium acetate and sodium succinate, the same compounds served as both priming substrate and cometabolite.

Red and dark red color production in the screening test occurred primarily in the runs made with Caddo Lake, Louisiana, McAlester, Radford, and Sub Base Bangor soils (Table 7, Sheets 1-5, 7-8). Iowa and Lone Star soils produced color changes much less frequently than the preceding soils. Although the reason for this is not apparent from the data, the possibility exists that these results were the result of differences in characteristics of microbial populations at different locations.

Caddo Lake and McAlester soils produced the highest numbers of positive results in the screening test, followed by Radford and Louisiana soils (Table 8). Somewhat fewer positives were obtained with Lone Star and Sub Base Bangor soils, while the Iowa soil produced only one positive result. The number of positives was anticipated to be related to the amount of TNT contamination present in the soil. However, this was not the case. Caddo Lake sediment contained no TNT or TNT degradation products, but yielded the highest

* Personal Communication, 1992, R. J. Spanggord, SRI International, Menlo Park, CA.

number of plates that were positive for TNT activity. However, the distribution and levels of TNT contamination to which this sediment was exposed in the past are unknown. For example, Caddo Lake sediment was taken in an area immediately downstream of Longhorn Army Ammunition (AAP) plant. While the sediments do not show detectable levels of TNT, the area has a long history of potential exposure to the compound brought in with waters leaving the AAP property. Therefore, the microbial population in the sediment may retain TNT activity developed in past exposure. Radford soil contained the next lowest level of TNT, but also gave a substantial number of positives. Louisiana, McAlester, and HEIP soils, all of which contained high levels of TNT and moderate levels of other explosives, produced positive results in the screening test.

When results of the screening test were compared with the priming substrate used, the numbers of positives obtained (greatest to smallest) were toluene > dinitroaniline = dinitro-*o*-cresol = none > dinitrophenol >> succinate > acetate = sawdust (Table 8). Results for priming substrates used in the screening test were interpreted according to molecular structures (see Figure 2). Structures similar to TNT and some of its proposed degradation

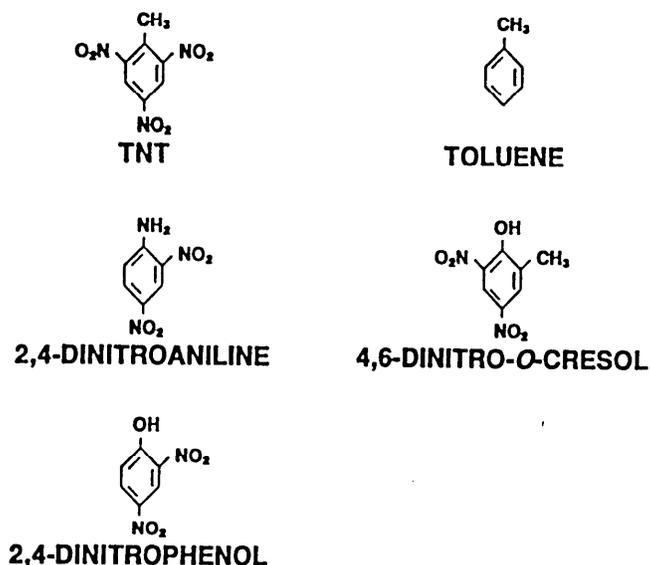


Figure 2. Structure of TNT and compounds used as priming substrates for TNT degradation in the screening protocol

products often elicited positive results in the screening test. The priming substrate producing the highest numbers of positives in the screening test,

toluene, is the parent molecule for TNT, while the highly successful priming substrate dinitroaniline differs from TNT in the presence of an amino group and the lack of a methyl and one nitro group. Dinitro-*o*-cresol, also highly successful in producing positive results, is similar to the catechols; the latter are suggested as a potential component of a degradation pathway for dinitrotoluene (Spangord et al. 1991).

The fact that unprimed soils were as effective in eliciting activity as those primed with dinitro-*o*-cresol and dinitroaniline was unexpected. Priming may not be required for some of these soils to exhibit positive results. In addition, TNT was added to all the soils treatments. If a sizable population of microorganisms active on TNT was already present, addition of TNT may have been sufficient to stimulate or support continued activity. Alternatively, a residual level of TNT contamination may be required to obtain positive results with some unprimed soils. However, this was not supported by the data. Unprimed soils exhibiting positive screening test results contained TNT concentrations varying from 0 to 18,400 mg/kg.

Much of the current literature suggests that microorganisms effective in TNT removal may be carrying out their activity via cometabolism. The term cometabolism was examined by Horvath (1972) and refers to a microbially mediated alteration in the chemical structure of a contaminant molecule so that the molecule is changed, but not to an extent sufficient to provide the responsible microorganism with energy or nutrients. However, once certain changes have been made in the original structure of the contaminant, other microorganisms may then be able to degrade the modified compound, obtaining nutrients and energy in the process. Because the contaminant does not provide the responsible microbe with energy or nutrients, another compound, the cometabolite, must provide the energy for growth. The use of cometabolites as substrates to support growth of microorganisms active in TNT removal may be necessary, based on the results of ongoing investigations in the area. For this reason, cometabolites were supplied, once the soils were primed with the same or other compounds, as described above. The success of the cometabolites used in the screening test followed the order sodium succinate > glucose > sodium acetate (Table 8).

Isolation and Development of Native
TNT-Degrading Microorganisms

TNT removal in slurries

Table 9 presents the data obtained from the study of TNT removal by indigenous microorganisms in HEIP soil under bioslurry treatment conditions. Note that for this study, total DNT was analyzed in water only, while the 2,4-DNT and 2,6-DNT isomers were separated in sediment. Table 9 presents this information as total DNT, regardless of the manner of determination. The 2-amino-4,6-dinitrotoluene and 4-amino-2,6-dinitrotoluene data are presented as the sum of the two compounds (2A+4A).

Acetate treatment dropped the aqueous phase TNT level from 169 to 45.5 mg/l and the soil level from 12,000 to 4,650 mg/kg in 4 weeks. Accumulations of 2A+4A occurred in the aqueous but not in the soil phase. Succinate treatment decreased the aqueous phase TNT level from 154 to 50.2 mg/l and the soil level from 12,400 to 6,425 mg/kg in 4 weeks. Accumulations of 2A+4A and the diaminonitrotoluenes in the aqueous phase were nearly identical to those obtained in the acetate treatment. In like manner, these were not detected in the soil phase. An apparent 25-percent decrease in the soil TNT level from 12,400 to 9,330 mg/kg also occurred in the mercuric chloride treated slurry. However, the aqueous phase concentration increased from 53.7 to 106 mg/l, and no DNT or 2A+4A accumulated in the aqueous or soil phases. This suggests that the decrease observed in the mercuric chloride soil was a result of the heterogeneous distribution of TNT in the soil, rather than a decrease due to biotransformation.

Neither of the treatments containing cometabolite and ammonium phosphate showed evidence of TNT biotransformation. The concentration of ammonium used in this study may have been high enough to prevent or inhibit microbial activity.

Confirmation of TNT mineralization

Figure 3 depicts cumulative mineralization occurring over a 4-week incubation of the Hastings soil proposed for treatment (TS-Flem-2) in a slurry containing 10 μg [U-ring ^{14}C] TNT/l, plus a single charge of acetate added at the start of the study. As indicated by Figure 3, approximately 15 percent of the added ^{14}C label appeared as $^{14}\text{CO}_2$. That this was CO_2 and not an organic ^{14}C -labeled by-product is substantiated by the following indirect evidence.

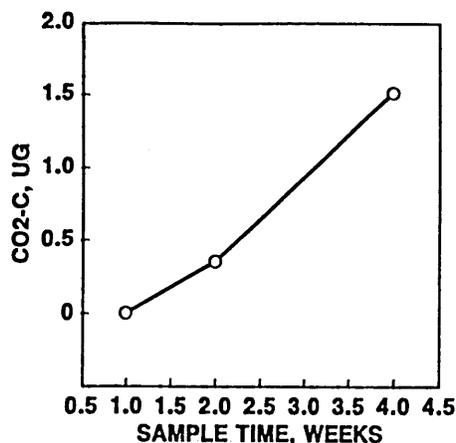


Figure 3. Cumulative mineralization (over a 4-week period) of radiolabeled TNT in HEIP soil maintained under slurry treatment conditions

No radiolabeled compound appeared in the XAD resin traps attached to the incubation vessels. Addition of phosphoric acid to the soil slurry strongly pushes the equation $\text{CO}_2 + \text{H}_2\text{O} \rightarrow \text{H}_2\text{CO}_3 \rightarrow \text{H}^+ + \text{HCO}_3^- \rightarrow 2\text{H}^+ + \text{CO}_3^{2-}$ to the left, promoting escape of CO_2 into the headspace. The presence in the headspace of a trap containing KOH pulls the equation in the opposite direction and, by removing CO_2 from the atmosphere in the vessel headspace, creates a concentration gradient favoring additional movement of CO_2 into the headspace.

A mass balance computation was performed to determine the ability to account for all of the radiolabeled carbon added. Radiolabeled material present in the aqueous phase of the slurry at the end of the study was determined by direct count of an aliquot of the water. Radiolabeled material remaining in the soil phase was recovered by burning the soil and trapping the evolved $^{14}\text{CO}_2$. The distribution of the carbon-14 label was as follows:

<u>Component</u>	<u>Percent</u>
CO ₂ trap	15.0
XAD trap	<00.1
Aqueous phase	66.6
Soil phase	<u>16.6</u>
Total	98.2

The amount of TNT mineralized may have been limited in the test system used in this study. Additional amounts of TNT may be needed to support more extensive mineralization than was observed here. Also, except for the small

opening provided to vent excessive gas pressures through the XAD trap and the KOH backup traps, the system was closed to the atmosphere and to entry of oxygen. Therefore, mineralization may have been oxygen-limited. In addition, because this was a sealed system, additional acetate was not added during the course of incubation. Thus, there was also a potential for mineralization to become cometabolite-limited.

The nature of the radiolabeled compound(s) remaining in the soil and solution phases at the end of the assay was not determined. TNT has a high affinity for soil, and for this reason, TNT may not have been the principal radiolabeled compound remaining in this phase at the end of incubation. If TNT was the principal remaining compound, the ability to retain TNT in the solution phase may reflect the high dilution of the soil in this particular slurry (1 part soil to 22 parts water). This might be expected, based on the fact that rapid desorption has been reported for TNT bound to soils after a short exposure period, when the contaminated soil was subject to several sequential desorption cycles (Pennington and Patrick 1990).

Isolation of native TNT-degrading microorganisms

To demonstrate that soils exhibiting positive results in the screening test harbor microorganisms effective in removing TNT, the active microorganisms were grown in enrichment cultures designed to stimulate profuse growth of TNT-degrading microorganisms. The TNT-degraders were subsequently isolated in mixed and pure cultures.

Results of the screening test indicated that TNT-degrading microorganisms were present and that acetate, glucose, or succinate would support this activity. Samples were taken from these soils and placed into liquid cultures using either acetate or succinate as a cometabolite. Microorganisms growing in these liquid cultures were isolated and used for TNT degradation studies.

Data on TNT degradation occurring in liquid cultures developed from each of the HEIP soils exhibited virtually the entire range of possible degradational activities (Table 10). After 2 weeks incubation, the culture from TS-Flem-3R soil with acetate as cometabolite retained 96.8 percent (96.8 mg of TNT/l) of the 100 mg TNT/l initially present. Other cultures removed all of the TNT, but accumulated substantial amounts of an isomeric mixture of 2-amino-4,6-dinitrotoluene (2A) and 4-amino-2,6-dinitrotoluene (4A). Most notable were the mixed cultures obtained from the following soil sources:

- (a) TS-SS-27 supported on acetate, (b) TS-SB-20 supported on succinate, (c) TS-Flem-3 supported on acetate, (d) TS-Flem-3R supported on succinate, and (e) TS-Flem-2 supported on acetate.

Two cultures did particularly well. The culture from TS-SS-27 on acetate removed all TNT without by-product accumulation (the by-products tested to below detection limits), while TS-SB-20 removed all TNT and the dinitro by-products, leaving only a trace (1.86 mg/l) of the amino-dinitro by-products (2A+4A). A compound exhibiting retention time similar to RDX appeared in two of the treatments (3.146 mg/l in TS-Flem-3R treated with acetate and 2.329 mg/l in TS-SS-27 treated with acetate), although RDX was not reported initially in the soil. Whether this compound was RDX or some other by-product cochromatographing with RDX is not known at present. Isolation of pure cultures from several of the mixtures indicated that *Pseudomonas aeruginosa* was the predominant microorganism in each consortium (Table 11).

Evaluation of direct TNT utilization versus destruction via cometabolism

TNT degradation occurred in soil slurries and in mixed cultures. To determine whether individual microorganisms were responsible for the observed degradation, several of the isolates listed in Table 11 were cultured on media with TNT alone and in combination with yeast extract (trace vitamin source) and acetate (as the cometabolite). None of the isolates was able to utilize TNT as a sole carbon and energy source (Table 12). Several isolates were able to subsist on low levels of yeast extract with no acetate, and two produced good growth on acetate with no yeast extract. Since the latter two isolates (*Pseudomonas aeruginosa* from the TS-Flem-2 site) are the same species isolated from the same site on two different cometabolites, the isolates are possibly the same organism. Based on the results of this brief survey, cometabolism, rather than direct attack, appears to be required for some portion of the microbial attack on TNT in HEIP soil from the TS-SS-27 and TS-Flem-2 sites.

Evaluation of mineralization by individual isolates

Growth of individual isolates in culture with radiolabeled TNT yielded no $^{14}\text{CO}_2$ production over a 2-week period, and most of the original radiolabel was recovered in the aqueous phase. This further substantiates the results of the preceding section, i.e., TNT mineralization occurs via a cometabolic pathway, not by direct attack on TNT by any one organism.

PART IV: CONCLUSIONS AND RECOMMENDATIONS

A screening test was developed that effectively demonstrates the presence of microorganisms capable of removing TNT from soils. Positive activity for TNT removal was found with and without priming substrates (when toluene, dinitroaniline, dinitro-*o*-cresol, dinitrophenol, acetate, sawdust, succinate, or no compound was used as a priming substrate). Removal of TNT was supported by glucose, succinate, and acetate as cometabolites. Toluene produced the highest number of positive results, followed by substantial but somewhat less frequent positive results with dinitroaniline, dinitro-*o*-cresol, and no priming substrate. Use of succinate and acetate as both priming substrates and cometabolites produced a much lower number of positives, as did the use of sawdust as a priming substrate with glucose as a cometabolite. Until more experience is gained with the screening test, the following combinations of screening substrates and cometabolites are recommended: (a) acetate and succinate as combined priming substrates and cometabolites and (b) toluene, dinitroaniline, and dinitro-*o*-cresol as priming substrates with acetate, succinate, and glucose as cometabolites.

The purpose of the screening test is to determine the presence of microorganisms active against TNT in a TNT-contaminated soil. However, the detection of activity does not ensure that the activity will be sufficient for biotreatment. A more detailed understanding of the environmental conditions supporting degradation will permit optimization of the degradation process to produce maximum effectiveness of the isolates or consortia.

Studies with individual isolates indicate that cometabolism, rather than direct attack on the TNT molecule by an individual microorganism, is probably responsible for TNT mineralization. This is supported by the following facts: (a) mineralization of radiolabeled TNT was demonstrated in slurries; (b) microbial consortia were able to remove TNT and degradation by-products from a solution containing acetate and/or succinate, while pure cultures of the dominant organisms in the consortia were unable to grow using TNT as a sole carbon and energy source; and (c) these same pure cultures were unable to produce $^{14}\text{CO}_2$ from radiolabeled TNT.

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Table 1

Sources of Soils Tested

<u>Source</u>	<u>Abbreviated Form Used in Text</u>	<u>Location</u>
Caddo Lake (downstream of Longhorn Army Ammunition Plant)	Caddo Lake	Red River, western Louisiana - eastern Texas Border
Hastings East Industrial Park	HEIP	Hastings, NE
Iowa Army Ammunition Plant	Iowa AAP	Burlington, IA
Lone Star Army Ammunition Plant	Lone Star AAP	Texarkana, TX
Louisiana Army Ammunition Plant	Louisiana AAP	Bossier City, LA
McAlester Army Ammunition Plant	McAlester AAP	McAlester, OK
Radford Army Ammunition Plant	Radford AAP	Radford, VA
U.S. Naval Complex, Bangor Submarine Base	Sub Base Bangor	Seattle, WA

Table 2

Basal Salts Medium for Isolation and Growth of
Bacteria Degrading Explosives*,**

<u>Component</u>	<u>Chemical Formulation</u>	<u>Amount</u> <u>g</u>	<u>10× Strength</u> <u>g</u>
Ammonium sulfate	(NH ₄) ₂ SO ₄	0.4	4.0
Potassium phosphate, dibasic	K ₂ HPO ₄	0.1	1.0
Potassium phosphate, monobasic	KH ₂ PO ₄	0.05	0.5
Magnesium sulfate, heptahydrate	MgSO ₄ *7H ₂ O	0.05	0.5
Manganese chloride, tetrahydrate	MnCl ₂ *4H ₂ O	0.02	0.2
Calcium chloride, dihydrate	CaCl ₂ *2H ₂ O	0.005	0.05
Ferrous chloride, tetrahydrate	FeCl ₂ *4H ₂ O	0.005	0.05
Calcium carbonate	CaCO ₃	0.2	Omit
(Tap water)		(To 1 ℓ)	(To 1 ℓ)

* Modified from Aaronson's medium for enrichment and isolation of *Pseudo-*
monas capable of oxidizing naphthalene (Aaronson 1970).

** For normal-strength medium, add each of the ingredients in the order listed to at least 800 ml of tap water with stirring. Wait until the last ingredient added has dissolved before adding the next. Filter the final medium through a fine filter paper or a 0.45- μ m micropore filter before adding any organic ingredients or agar, and sterilizing. For the 10× medium, again add the ingredients, except for CaCO₃, to at least 800 ml of tap water. Store in refrigerator. Since no organics will be added to the 10× medium, it should be good for several weeks. When ready to use, dilute 100 ml of the 10× to 1 ℓ with tap water, add 0.2 g of CaCO₃ with stirring, and filter as for normal-strength medium. Then add any remaining ingredients and sterilize.

Table 3
Treatment Combinations Used in the Screening Test

<u>Priming Substrate</u>	<u>Cometabolite</u>		
	<u>Sodium Acetate</u>	<u>Sodium Succinate</u>	<u>Glucose</u>
None	X	X	X
Toluene	X	X	X
Dinitroaniline	X	X	X
Dinitrophenol	X	X	X
Dinitro- <i>o</i> -cresol	X	X	X
Sawdust	--	--	X
Sodium acetate	X	--	--
Sodium succinate	--	X	--

* Symbols are defined as follows: X = combination of priming substrate and cometabolite tested for each soil; -- = combination of priming substrate and cometabolite not tested.

Table 4
Summary Information on HEIP Soils

<u>Sample ID</u>	<u>Sample Site Description</u>	<u>TNT Concentration mg/kg</u>
TS-Flem-1	Flemings Pond	5,000-6,000*
TS-Flem-2	Flemings Pond	7,070
TS-Flem-3	Flemings Pond	255
TS-Flem-3R	Flemings Pond (near TS-Flem-3)	5,000-6,000*
TS-SB-27	Soil boring SB-27 (surface)	9,330
TS-SB-20	Soil boring SB-20 (surface)	167
TS-BG	Site background soil	None

* Expected, rather than determined. In reality, TS-Flem-1 had concentrations exceeding 20,000 mg/kg.

Table 5
Particle Size Analysis of Soils

<u>Source of Soil</u>	<u>Percent Sand</u>	<u>Percent Silt</u>	<u>Percent Clay</u>
Caddo Lake	13.5	55.0	32.5
HEIP	33.0	54.0	13.0
Iowa AAP	62.5	25.0	12.5
Lone Star AAP (Texas)	67.5	20.0	12.5
Louisiana AAP	35.0	47.5	17.5
McAlester AAP	42.5	42.5	15.0
Radford AAP	45.0	45.0	10.0
Sub Base Bangor	77.5	22.5	0

Table 6
Explosives Content (mg/kg) of Soils

Source	TNT	2-A-DNT	4-A-DNT	2,4-DNT	HMX	RDX	TNB	DNB	Tetryl
Caddo Lake	<0.93	N/D*	N/D	<0.74	<0.76	<0.44	<0.35	<0.30	<1.04
HEIP**	18,572	13.8	>	14.3>>	U	19.3	30.9	1.73	5.41
Iowa AAP	1.56	U†	U	U	2.94	24.7	U	U	U
Lone Star AAP	3.04	U	2.59	U	2.40	0.599J‡	U	U	U
Louisiana AAP	377	N/D	N/D	0.513	471	2,470	1.07	U	U
McAlester AAP	3,480	U	142.0	2.48	256.0	787.0	2.50	U	U
Radford Arsenal	1.24	U	U	U	U	U	0.225J	U	U
Sub Base Bangor	9.45	U	11.9	0.348	U	U	0.667	0.174J	0.975

Note: Abbreviations used for TNT compounds are identified in the text (see pages 3 and 12).

* Not determined.

** Hastings East Industrial Part--combination of TS-Flem-1 and TS-BG soils.

> 2-A-DNT and 4-A-DNT detected and reported as 2-A-DNT only.

>> 2,4-DNT and 2,6-DNT detected and reported as 2,4-DNT only.

† Below detection. Detection limits are given in Appendix A.

‡‡ Values determined at 10 percent below the detection limit.

Table 7

Results of Screening Test

Soil	Cometabolite	Activity											
		Week 1				Week 4				Static			
		Shaking	Growth	TNT removal	TNT removal	Shaking	Growth	TNT removal	TNT removal	Shaking	Growth	TNT removal	TNT removal
		No Priming Substrate											
Caddo Lake	Glucose	+	-	-	-	+	+	-	-	+	+	-	-
	Na acetate	+	-	-	-	+	+	+	+	+	+	+	+
	Na succinate	+	-	-	-	+	+	+	+	+	+	+	+
Iowa	Glucose	+	-	-	-	+	+	-	-	+	+	-	-
	Na acetate	+	-	-	-	+	+	-	-	+	+	-	-
	Na succinate	+	-	-	-	+	+	-	-	+	+	-	-
Lone Star	Glucose	+	+	+	-	+	+	+	+	+	+	+	+
	Na acetate	-	-	-	-	-	-	-	-	-	-	-	-
	Na succinate	+	+	+	-	+	+	+	+	+	+	+	+
Louisiana	Glucose	+	-	-	-	+	+	-	-	+	+	-	-
	Na acetate	+	-	-	-	+	+	+	+	+	+	+	+
	Na succinate	+	+	+	-	+	+	+	+	+	+	+	+
McAlester	Glucose	+	-	-	-	+	+	-	-	+	+	-	-
	Na acetate	-	-	-	-	-	-	-	-	-	-	-	-
	Na succinate	+	+	+	-	+	+	+	+	+	+	+	+
Radford	Glucose	+	+	+	+	+	+	+	+	+	+	+	+
	Na acetate	+	+	+	-	+	+	+	+	+	+	+	+
	Na succinate	+	+	+	-	+	+	+	+	+	+	+	+
Sub Base Bangor	Glucose	+	+	+	+	+	+	+	+	+	+	+	+
	Na acetate	+	+	+	-	+	+	+	+	+	+	+	+
	Na succinate	+	+	+	-	+	+	+	+	+	+	+	+

(Continued)

Note: + = positive result obtained; - = negative result; +Wk = weakly positive; Rd = red color; DkRd = dark red color; Rst = rust. For details, see text (pages 12-17).

Table 7 (Continued)

Soil	Cometabolite	Activity											
		Week 1						Week 4					
		Shaking		Static		Shaking		Static		Shaking		Static	
		Growth	TNT removal	Growth	TNT removal	Growth	TNT removal	Growth	TNT removal	Growth	TNT removal	Growth	TNT removal
<u>Priming Substrate: Dinitroaniline</u>													
Caddo Lake	Glucose	+	-	+	-	+	-	+	-	+	-	+	-
	Na acetate	+Rd	+	+Rd	-	+Rd	-	-Rd	-	-Rd	-	+	+Wk
	Na succinate	+Rd	-	+Rd	-	+Rd	-	+Rd	-	+Rd	-	+Rd	+Wk
Iowa	Glucose	-	-	-	-	-	-	-	-	-	-	+	-
	Na acetate	-	-	-	-	-	-	+	-	-	-	+	-
	Na succinate	+DKRd	-	+DKRd	-	+DKRd	-	+	-	+	-	+	-
Lone Star	Glucose	-	-	+	-	+	-	+	-	+	-	+	+
	Na acetate	-	-	-	-	-	-	+	-	-	-	+	+Wk
	Na succinate	+	+	-DKRd	-	-DKRd	-	+	-	-	-	-Rd	-
Louisiana	Glucose	+	-	+	-	+	-	+	-	+	-	+	-
	Na acetate	+	+Wk	+	-	+	-	-	-	-	-	-	-
	Na succinate	+DKRd	+	+Rd	-	+Rd	-	+Rd	-	+Rd	-	+Rd	-
McAlester	Glucose	+	+	+	+	+	+	+	-	+	-	+	-
	Na acetate	-DKRd	-	-DKRd	-	-DKRd	-	-Rd	-	-Rd	-	+DKRd	-
	Na succinate	+Rd	+	+DKRd	+	+DKRd	+	+	-	+	-	+	-
Radford	Glucose	+	+	+	+	+	+	+	-	+	-	+	-
	Na acetate	+	+	+DKRd	-	+DKRd	-	+DKRd	-	+DKRd	-	+	-
	Na succinate	+	+	+	+	+	+	+DKRd	-	+DKRd	-	+DKRd	-
Sub Base Bangor	Glucose	-	-	-	-	-	-	+	-	+	-	+	-
	Na acetate	-	-	-	-	-	-	+DKRd	-	+DKRd	-	+DKRd	-
	Na succinate	+	+	-DKRd	-	-DKRd	-	-DKRd	-	-DKRd	-	-DKRd	-

(Continued)

Table 7 (Continued)

Soil	Cometabolite	Activity										
		Week 1					Week 4					
		Shaking	Static	Shaking			Static	Shaking			Static	
		Growth	TNT removal	Growth	TNT removal	Growth	TNT removal	Growth	TNT removal	Growth	TNT removal	
Priming Substrate: Dinitro-o-cresol												
Caddo Lake	Glucose	+	+	+	-	+	+	+	+	+	+	+Wk
	Na acetate	+Rd	-	+Rd	-	+Rd	-	+Rd	-	+Rd	-	+Wk
	Na succinate	+Rd	+	+Rd	-	+Rd	-	+Rd	-	+Rd	-	+
Iowa	Glucose	-	-	-	-	+	-	-	-	+	-	-
	Na acetate	-	-	-	-	+	-	-	-	-	-	-
	Na succinate	-	-	-	-	+	-	-	-	+	-	-
Lone Star	Glucose	-	-	-	-	-	-	-	-	-	-	-
	Na acetate	-	-	-	-	-	-	-	-	-	-	-
	Na succinate	-	-	-	-	-	-	-	-	-	-	-
Louisiana	Glucose	+	-	+	-	+	-	+	-	+	-	-
	Na acetate	+	-	+	+	+	-	+	-	+	-	-
	Na succinate	+DKRd	+	+Rd	-	+Rd	-	+Rd	-	+Rd	-	+
McAlester	Glucose	+	+	+	-	+	-	+	-	+	-	-
	Na acetate	-DKRd	-	-	-	-DKRd	-	-DKRd	-	-DKRd	-	-
	Na succinate	+DKRd	+	+DKRd	+	+DKRd	+	+DKRd	+	+DKRd	+	-
Radford	Glucose	+	+	+	+	+	+	+	+	+	+	+Wk
	Na acetate	+	+	+DKRd	-	+DKRd	-	+DKRd	-	+DKRd	-	-
	Na succinate	+DKRd	-	+DKRd	-	+DKRd	-	+DKRd	-	+DKRd	-	-
Sub Base Bangor	Glucose	-	-	-	-	+	-	+	-	+	-	-
	Na acetate	-	-	+	-	-	-	-	-	-	-	-
	Na succinate	+	-	+DKRd	-	+DKRd	-	+DKRd	-	+DKRd	-	-

(Continued)

Table 7 (Continued)

Soil	Cometabolite	Activity												
		Week 1						Week 4						
		Shaking	Static	Growth	TNT removal	TNT removal	Static	Shaking	Static	Growth	TNT removal	TNT removal	Static	
Priming Substrate: Dinitrophenol														
Caddo Lake	Glucose	+	-	+	-	+	-	+	-	+	-	-	+	-
	Na acetate	+Rd	-	+Rd	-	+Rd	-	+Rd	-	+Rd	-	+	+Rd	+
	Na succinate	+Rd	+	+Rd	-	+Rd	-	+Rd	-	+Rd	-	-	+Rd	+
Iowa	Glucose	-	-	-	-	+	-	+	-	+	-	-	+	-
	Na acetate	-	-	-	-	+	-	+	-	+	-	-	+	-
	Na succinate	+	-	+	-	+	-	+	-	+	-	-	+	-
Lone Star	Glucose	-	-	-	-	-	-	-	-	-	-	-	-	-
	Na acetate	-	-	-	-	-	-	-	-	-	-	-	-	-
	Na succinate	-	-	-	-	-	-	-	-	-	-	-	-	-
Louisiana	Glucose	+	+	+	-	+	-	+	-	+	-	-	+	-
	Na acetate	-	-	-	-	-	-	-	-	-	-	-	-	-
	Na succinate	-Rd	-	+Rd	-	+Rd	-	+Rd	-	+Rd	-	+	+Rd	-
McAlester	Glucose	+	+	+	+	+	+	+	+	+	+	+	+	-
	Na acetate	-DKRd	-	-	-	-	-	-Rd	-	-DKRd	-	-	-DKRd	-
	Na succinate	+DKRd	+	+DKRd	+	+DKRd	+	+	+	+	+	+	+	-
Radford	Glucose	+	+	+	+	+	+	+	+	+	+	+	+	-
	Na acetate	+	-	+	-	+	-	+DKRd	-	+DKRd	-	-	+DKRd	-
	Na succinate	+DKRd	-	+DKRd	-	+DKRd	-	+DKRd	-	+DKRd	-	-	+DKRd	-
Sub Base Bangor	Glucose	-	-	-	-	-	-	-	-	-	-	-	+	-
	Na acetate	-	-	-	-	-	-	-	-	-	-	-	-	-
	Na succinate	+	+	+	-	+	-	+	-	+	-	-	-DKRd	-

(Continued)

Table 7 (Continued)

Soil	Cometabolite	Activity											
		Week 1						Week 4					
		Shaking	Growth	TNT removal	Static	TNT removal	Static	Shaking	Growth	TNT removal	Static	TNT removal	Static
		Priming Substrate: Toluene											
Caddo Lake	Glucose	-	-	-	-	-	+	+Wk	+	+	+	+	-
	Na acetate	+	-	+Rd	-	-	+Rd	+	+Rd	+	+Rd	+	+
	Na succinate	+Rd	-	+Rd	-	-	+Rd	+Wk	+Rd	+	+Rd	+	+
Iowa	Glucose	+	-	+	-	-	+	-	+	+	+	-	-
	Na acetate	+	-	+	-	-	+	-	+	+	+	-	-
	Na succinate	+	-	+	-	-	+	-	+	+	+	-	-
Lone Star	Glucose	+	-	+	-	-	-	-	-	+	+	+	+
	Na acetate	-	-	-	-	-	+	-	+	+	+	+	+Wk
	Na succinate	-DkRd	-	+	+Wk	-	+	-	+	+	+	+	+Wk
Louisiana	Glucose	+	-	+	-	-	+Rst	-	+Rst	-	+Rst	-	-
	Na acetate	+	-	+	-	-	+	-	-	-	-	-	-
	Na succinate	+DkRd	+	+Rd	+Wk	-	+Rd	+Wk	+Rd	+	+Rd	+	+Wk
McAlester	Glucose	+	+	-	-	-	+	-	+	+	+	-	-
	Na acetate	+DkRd	+	+DkRd	+	-	-Rd	-	-Rd	+	+Rd	-	-
	Na succinate	+DkRd	+	+DkRd	+	-	+	-	+	+	+DkRd	-	-
Radford	Glucose	+	+	+	+	-	+	-	+	+	+	-	-
	Na acetate	+DkRd	-	+	-	-	+DkRd	-	+DkRd	+	+	+	+
	Na succinate	+	+	+DkRd	-	-	-DkRd	-	-DkRd	-	-DkRd	-	-
Sub Base	Glucose	+	-	+	-	-	+	-	+	+	+	-	-
Bangor	Na acetate	-	-	-	-	-	+	-	+	+	+DkRd	-	-
	Na succinate	+	+Wk	+DkRd	-	-	+	+DkRd	+	+DkRd	-DkRd	-	-

(Continued)

Table 7 (Continued)

Soil	Cometabolite	Activity							
		Week 1				Week 4			
		Shaking Growth	TNT removal	Static Growth	Static TNT removal	Shaking Growth	TNT removal	Static Growth	Static TNT removal
		Priming Substrate: Sawdust							
Caddo Lake	Glucose	+	-	+	-	+	-	+	-
Iowa	Glucose	+	-	+	-	+	-	+	+
Lone Star	Glucose	+	-	+	-	+	-	+	-
Louisiana	Glucose	+	-	+	-	+	-	+	-
McAlester	Glucose	+	-	+	+	+	-	+	-
Radford	Glucose	-	-	+	+	+	-	+	-
Sub Base Bangor	Glucose	+	+	+	+	+	-	+	-

(Continued)

Table 7 (Continued)

Soil	Cometabolite	Activity													
		Week 1						Week 4							
		Shaking Growth	TNT removal	Static Growth	TNT removal	Shaking Growth	TNT removal	Static Growth	TNT removal	Shaking Growth	TNT removal	Static Growth	TNT removal		
		Priming Substrate: Sodium Acetate													
Caddo Lake	Na acetate	+Rd	-	-	-Rd	-	-	-	-	-	-	+Rd	-	-	+Wk
Iowa	Na acetate	+	-	+	+	-	-	-	-	-	-	+	-	-	-
Lone Star	Na acetate	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Louisiana	Na acetate	+	-	+	-	-	-	-	-	-	-	-	-	-	-
McAlester	Na acetate	-DkRd	-	-DkRd	-	-	-	-Rd	-	-	-	+	-	-	-
Radford	Na acetate	+DkRd	-	+DkRd	-	-	-	+DkRd	-	-	-	+	-	-	-
Sub Base Bangor	Na acetate	+	+Wk	+	-	+	+Wk	-	-	-	-	+DkRd	-	-	+Wk

(Continued)

Table 8

Comparison of Numbers of Positives Obtained in the Screening Test

<u>Parameter</u>	<u>Number of Positive Results</u>	<u>Percent of Total</u>
<u>Soil</u>		
Caddo Lake	23	21.3
Iowa AAP	1	0.92
Lone Star AAP	14	13.0
Louisiana AAP	17	15.7
McAlester AAP	21	19.4
Radford AAP	19	17.6
Sub Base Bangor	<u>13</u>	<u>12.0</u>
	108	100.0
<u>Priming substrate</u>		
Acetate	4	3.7
Dinitroaniline	19	17.6
Dinitro-o-cresol	19	17.6
Dinitrophenol	13	12.0
None	19	17.6
Sawdust	4	3.7
Succinate	6	5.6
Toluene	<u>24</u>	<u>22.2</u>
Total	108	100.0
<u>Cometabolite</u>		
Glucose	34	31.5
Sodium acetate	23	21.3
Sodium succinate	<u>51</u>	<u>47.2</u>
Total	108	100.0

Table 9

TNT Removal from Slurries of TS-Flem-2Soil by Indigenous Microorganisms

<u>Treatment</u>	<u>TNT</u>	<u>Total DNT</u>	<u>Compound*</u>			
			<u>2A+4A</u>	<u>RDX</u>	<u>TNB</u>	<u>DNB</u>
<u>Treatment 1 - Acetate as a Cometabolite, No Ammonium Phosphate Added</u>						
Water - Day 0	169	U**	2.94	U	4.83	U
Water - Week 4	45.5	2.37	31.3	4.49	52.4	U
Soil - Day 0	12,000	U	U	U	26.0	U
Soil - Week 4	4,650	U	U	17.0	71.0	U
<u>Treatment 2 - Succinate as a Cometabolite, No Ammonium Phosphate Added</u>						
Water - Day 0	154	U	3.88	U	3.77	U
Water - Week 4	50.2	2.52	36.1	5.30	73.3	U
Soil - Day 0	12,400	U	U	U	29.0	U
Soil - Week 4	6,425	U	U	U	98.0	U
<u>Treatment 3 - Poisoned with Mercuric Chloride, No Ammonium Phosphate or Cometabolite Added</u>						
HgCl ₂ -Water - Day 0	53.7	U	1.45	U	U	U
HgCl ₂ -Water - Week 4	106	U	U	U	1.16	U
HgCl ₂ -Soil - Day 0	12,400	U	U	U	41.0	U
HgCl ₂ -Soil - Week 4	9,330	U	U	U	88.0	U

(Continued)

* Results are averages of two replicates. Water concentrations are expressed in milligrams per liter. Soil concentrations given are in milligrams per kilogram. The sum of 2,4-DNT and 2,6-DNT is reported as Total DNT.

** Below detection limit. Detection limits are given in Appendix A.

Table 9 (Concluded)

Treatment	Compound*					
	TNT	Total DNT	2A+4A	RDX	TNB	DNB
<u>Treatment 4 - Acetate as a Cometabolite with Ammonium Phosphate as a Nutrient Source</u>						
Water - Day 0	89.3	U	2.00	U	0.375	U
Water - Week 4	77.5	U	5.86	U	U	U
Soil - Day 0	14,600	U	U	U	42.0	U
Soil - Week 4	12,500	U	U	U	79.0	U
<u>Treatment 5 - Succinate as a Cometabolite with Ammonium Phosphate as a Nutrient Source</u>						
Water - Day 0	79.6	U	1.89	U	U	U
Water - Week 4	48.8	U	7.52	U	0.385	
0.260						
Soil - Day 0	16,300	U	U	U	36.0	U
Soil - Week 4	11,400	U	U	U	73.0	U

Table 10

Amount of TNT and TNT Degradation Products Remaining After 2 Weeks of
Incubation in Mixed Liquid Cultures of Microorganisms

Isolated from HEIP Soil

<u>Soil Source</u>	<u>Cometabolite</u>	<u>TNT</u>	<u>Total DNT**</u>	<u>Compound, mg/ℓ*</u>		
				<u>Total Monoamino-DNT></u>	<u>TNB</u>	<u>Tetryl</u>
TS-Flem-3R	Acetate	96.8	0.158	1.07	0.917	P>>
TS-Flem-3R	Succinate	U‡	U	9.17	0.070	U
TS-Flem-3	Acetate	U	0.157	12.4	0.081	1.571
TS-Flem-3	Succinate	U	0.064	4.18	U	0.532
TS-SB-20	Acetate	U	U	1.86	U	U
TS-SB-20	Succinate	U	0.077	12.6	U	0.095
TS-SS-27	Acetate	U	U	U	U	U
TS-SS-27	Succinate	0.028	0.052	15.6	U	U
TS-Flem-2	Acetate	U	U	6.27	U	0.441
TS-Flem-2	Succinate	U	U	2.02	U	0.287

* Initial TNT concentration was 100 mg/ℓ. Not observed in any cultures: HMX, DNB.

** The sum of 2,4-DNT and 2,6-DNT is reported as Total DNT.

> The sum of 2-A-4,6-DNT and 4-A-2,6-DNT is reported as Total Monoamino-DNT.

>> Unknown compound equivalent to 0.348 mg/ℓ as Tetryl.

‡ Below detection limit. Detection limits are given in Appendix A.

Table 11

Identity of Representative Microorganisms Isolated from Consortia
from the Hastings East Industrial Park Soils

<u>Soil Source</u>	<u>Identity of Microorganism</u>	<u>Cometabolite</u>
TS-Flem-2	<i>Pseudomonas aeruginosa</i>	Sodium succinate
	<i>Pseudomonas putida</i> b.	Sodium acetate
	<i>Pseudomonas aeruginosa</i>	Sodium acetate
SS-20	<i>Alcaligenes denitrificans</i>	Sodium succinate
	<i>Enterobacter cloacae</i> b.	Sodium acetate
TS-Flem-3	<i>Pseudomonas aeruginosa</i>	Sodium succinate
	<i>Pseudomonas aeruginosa</i>	Sodium acetate
TS-Flem-3R	<i>Pseudomonas aeruginosa</i>	Sodium succinate
	<i>Pseudomonas aeruginosa</i>	Sodium acetate
TS-SS-27	<i>Pseudomonas aeruginosa</i>	Sodium succinate
	<i>Pseudomonas aeruginosa</i>	Sodium acetate

Table 12

Ability of Representative Microorganisms Isolated from Consortia
from HEIP Soils to Grow on Media Containing TNT With and
Without Yeast Extract as a Vitamin Source and
Sodium Acetate as Cometabolite

<u>Organism and Source</u>	<u>Medium</u>	<u>Growth*</u>
<i>Pseudomonas aeruginosa</i> from TS-SS-27 on acetate	BSM + TNT, no yeast extract or acetate	NG
	BSM + TNT + 2% acetate, no yeast extract	PG
	BSM + TNT + yeast extract, no acetate	FG, Lt col
	BSM + TNT + yeast extract, 0.1% acetate	FG, Lt col
	BSM + TNT + yeast extract, 0.5% acetate	GG, Mod col
	BSM + TNT + yeast extract, 1.0% acetate	GG, Dk col
<i>Pseudomonas aeruginosa</i> from TS-Flem-2 on succinate	BSM + TNT, no yeast extract or acetate	NG
	BSM + TNT + 2% acetate, no yeast extract	GG, Dk col
	BSM + TNT + yeast extract, no acetate	GG, Clr col
	BSM + TNT + yeast extract, 0.1% acetate	GG, Clr col
	BSM + TNT + yeast extract, 0.5% acetate	GG, Lt col
	BSM + TNT + yeast extract, 1.0% acetate	EG, Dk col
<i>Pseudomonas aeruginosa</i> from TS-Flem-2 on acetate	BSM + TNT, no yeast extract or acetate	NG
	BSM + TNT + 2% acetate, no yeast extract	GG, Dk col
	BSM + TNT + yeast extract, no acetate	GG, Clr col
	BSM + TNT + yeast extract, 0.1% acetate	GG, Clr col
	BSM + TNT + yeast extract, 0.5% acetate	GG, Lt col
	BSM + TNT + yeast extract, 1.0% acetate	EG, Dk col
<i>Pseudomonas putida</i> from TS-Flem-2 on acetate	BSM + TNT, no yeast extract or acetate	NG
	BSM + TNT + 2% acetate, no yeast extract	NG
	BSM + TNT + yeast extract, no acetate	NG
	BSM + TNT + yeast extract, 0.1% acetate	PG, Dk col
	BSM + TNT + yeast extract, 0.5% acetate	NG
	BSM + TNT + yeast extract, 1.0% acetate	NG

* Abbreviations: NG = no growth, PG = poor growth; FG = fair growth; GG = good growth; and EG = excellent growth. Lt col = light coloration; Mod col = moderate coloration; Clr col = clear, no coloration; Dk col = dark coloration.

APPENDIX A: DETECTION LIMITS FOR EXPLOSIVE COMPOUNDS ANALYZED

Table A1
Detection Limits for Explosive Compounds Analyzed
in This Study by MRD Laboratories

<u>Compound</u>	<u>Detection in Soil</u> <u>mg/kg</u>	<u>Detection in Water</u> <u>µg/l</u>
HMX	2.20	1.00
RDX	1.00	0.84
TNB	0.25	0.26
DNB	0.25	0.11
Tetryl	0.65	0.55
TNT	0.25	0.11
2,4-DNT	0.25	0.02
2,6-DNT	0.26	0.31
2-A-4,6-DNT	0.25	0.03
4-A-2,6-DNT	0.26	0.06

Table A2
Detection Limits For Explosive Compounds Analyzed in
This Study by Arthur D. Little Laboratories

<u>Compound</u>	<u>Detection in Soil</u> <u>mg/kg</u>
HMX	0.755
RDX	0.445
1,3,5-TNB	0.352
1,3-DNB	0.304
Tetryl	1.04
TNT	0.931
2,4-DNT	0.744
2,6-DNT	0.830
2-A-4,6-DNT	1.59
4-A-2,6-DNT	1.04

Table A3
Detection Limits for Explosive Compounds Analyzed
in This Study by WES Analytical Laboratory*

<u>Compound</u>	<u>Detection in Soil</u> <u>mg/kg</u>	<u>Detection in Water</u> <u>µg/l</u>
HMX	2.2	20
RDX	1.0	20
TNB	0.25	20
DNB	0.25	20
Tetryl	0.65	20
TNT	0.25	20
2,4-DNT	0.25	20
2,6-DNT	0.26	20
2-A-4,6-DNT	0.25	20
4-A-2,6-DNT	0.25	20

* These limits are for undiluted samples only.

REPORT DOCUMENTATION PAGE

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13. ABSTRACT (Maximum 200 words) A test was developed to screen soils and sediments for the presence of microbial activity against 2,4,6-trinitrotoluene (TNT). TNT removal was observed with and without the use of chemicals to stimulate microbial activity. In addition to a small amount of TNT added to each soil sample, chemical treatments included toluene, dinitroaniline, dinitro- <i>o</i> -cresol, dinitrophenol, sodium acetate, sawdust, or sodium succinate. Following 1 to 4 weeks of incubation in static and slurry modes, individual soil treatments were plated onto a crystalline lawn of TNT overlying a basal salts agar containing one of three cometabolites (acetate, glucose, or succinate). Activity against TNT was detected by visual observation of TNT clearing around the soil sample. The screening procedure was applied to soils from seven military installations. Positive results were obtained for each soil, although the amount of activity varied from soil to soil. Based on these results, the following combinations of screening substrates and cometabolites are recommended: (a) acetate and succinate as combined priming substrates and <div style="text-align: right;">(Continued)</div>				
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13. (Concluded).

cometabolites and (b) toluene, dinitroaniline, and dinitro-*o*-cresol as priming substrates with acetate, succinate, and glucose as cometabolites.

Detailed studies were conducted with Hastings East Industrial Park soils to isolate and develop cultures of native TNT-degrading populations. Mixed cultures of organisms obtained from primed soil from the TS-Flem-2 site were able to remove significant levels of TNT, with accumulations of 2-amino-4,6-dinitrotoluene + 4-amino-2,6-dinitrotoluene in the aqueous but not the soil phase. Studies of the mineralization of radiolabeled TNT in a dilute aqueous slurry of the same soil in a closed system indicated conversion of 15 percent of the radiolabeled TNT to $^{14}\text{CO}_2$ over a 4-week period. A mass balance conducted on the test system accounted for over 98 percent of the original radio-label. Studies with individual isolates indicated that cometabolism, rather than direct attack on the TNT by an individual microorganism, is probably responsible for the observed TNT mineralization.

14. (Concluded).

Biodegradation
Biotransformation
Biotreatment
Explosive
Microorganism

Mineralization
Phenaerochaete
Pseudomonas
Screening test