



**US Army Corps  
of Engineers**  
Waterways Experiment  
Station

*Installation Restoration Research Program*

# **Alternative Methods for Biological Destruction of TNT: A Preliminary Feasibility Assessment of Enzymatic Degradation**

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# **Alternative Methods for Biological Destruction of TNT: A Preliminary Feasibility Assessment of Enzymatic Degradation**

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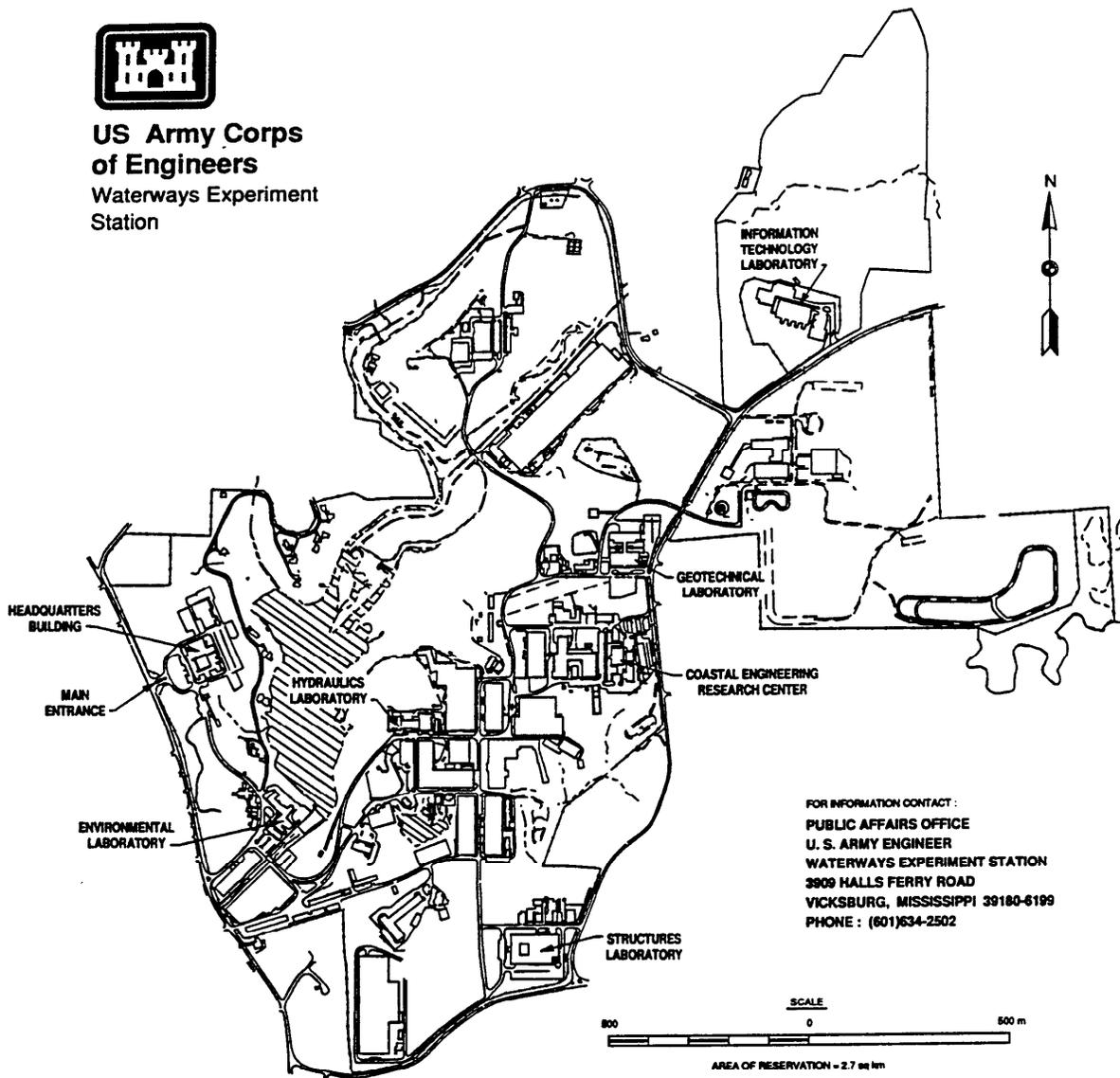
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# Preface

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The studies reported herein were conducted by the Environmental Research Laboratory, U.S. Environmental Protection Agency, Athens, GA (ERL-Athens), Technical Applications, Inc. (TAI), Athens, GA, and the Environmental Laboratory (EL), 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, Project No. AH68. Research at ERL-Athens was conducted under Military Interagency Purchase Request Numbers W81EWF-2-MO83 and W81EWF-2-MO85 from WES. 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, U.S. Army Environmental Center, and Mr. Jim Baliff, 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. N. Lee Wolfe, ERL-Athens, Tse-Yaun Ou and Laura Carreira, TAI, and Douglas Gunnison of the Ecosystem Processes and Effects Branch (EPEB), Environmental Processes and Effects Division (EPED), EL. Technical reviews of the report were provided by Drs. Judith C. Pennington, James M. Brannon, and William M. Davis, EPEB.

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 W. Keeley, Director, EL.

At the time of publication of this report, Director of WES was Dr. Robert W. Whalin. Commander was COL Bruce K. Howard, EN. Director of ERL-Athens was Dr. Rosemarie Russo.

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# 1 Introduction

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## Background and Rationale

Bioremediation may take advantage of 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 a contaminant to yield innocuous inorganic constituents. These include carbon dioxide or methane, various inorganic salts, and water. Complete mineralization to such products 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. For example, 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 enzymes active on TNT (Li, Yang, and Yang 1987).

Recent work has suggested that aerobic mineralization of TNT 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)). By contrast, work by Funk et al. (1993) revealed that anaerobic conditions may promote biodegradation of nitroaromatic contaminants in soils. Parent TNT molecules disappeared within 4 days in soil cultures, and by 30 days, nearly all of the initial radiolabel appeared as 4-amino-2,6-dinitrotoluene (4A2,6DNT) and 2,4-diamino-6-nitrotoluene (2,4A6NT). Residual label was found in other fractions and as a trace amount of carbon dioxide (CO<sub>2</sub>). Funk et al. (1993) suggested that addition of an aerobic stage following an initial

anaerobic stage might accelerate degradation of 2,4,6-trihydroxytoluene (phloroglucinol or MPG) and *p*-cresol, identified as intermediates in the second stage of degradation, to carbon dioxide.

Anaerobic or aerobic biological treatment may be acceptable if minimal manipulation of the soil is required and if complete mineralization of TNT is possible. The report herein presents an approach being evaluated to obtain biological destruction of TNT. This approach uses a combination of microbial and enzymatic processes found to be effective in reduction of TNT (Carreira and Wolfe 1992). Previous work at the Environmental Research Laboratory - Athens (ERL-Athens), U.S. Environmental Protection Agency, Athens, GA, on environmental transformation of nitroaromatic compounds has resulted in the isolation and characterization of a redox enzyme (a nitrate reductase) from sediments and from aquatic plants.

The study herein was undertaken to examine the ability of the reductase enzyme to remediate TNT-contaminated soils using the following rationale: During the treatment process, native soil microorganisms remove all traces of oxygen, thereby creating anaerobic conditions. This is necessary because the enzyme reduces nitroaromatic compounds to their corresponding amino compounds under anaerobic conditions. At this point, the incubation regime is changed to aerobic conditions to permit the resident microorganisms to complete the destruction process. Here, the nitroaromatic amines are microbially oxidized to catechols, following which fission is expected to occur. During the time this process was being studied, the plant source of the sediment nitrate reductase enzyme was identified with the use of monoclonal antibodies. The authors then incubated untreated samples of the same TNT-contaminated soils with the plant under aerobic conditions since the plant and associated nitrate reductase enzyme appear to function well under aerobic conditions.

## Objectives

The objectives of these studies were to evaluate the feasibility of using a nitrate reductase originally isolated from pond sediment as part of a new biotreatment process to reduce TNT to various monoamino, diamino, and triamino toluenes, which could subsequently be subjected to bench-scale bioslurry treatment at the U.S. Army Engineer Waterways Experiment Station (WES). During the course of the study, the source of the nitrate reductase was identified as the aquatic plant stonewort (*Nitella*). An investigation was subsequently undertaken to determine the ability to use the intact plant to destroy TNT. The results of this work are also reported.

## 2 Methods and Materials

---

### Enzyme Preparation and Purification

The nitrate reductase enzyme was extracted from sediment and purified using a procedure developed at ERL-Athens (patent pending). Sediment was obtained from a small pond near Athens, GA (Carreira and Wolfe 1992). Sediment (420 g oven dry weight (ODW)) was added to 6,000 ml of a solution containing 20-percent glycerol in 0.5 M KCl. This mixture was mixed gently for 5 min at room temperature and then centrifuged at  $5,000 \times g$  for 20 min at 6 °C. The resulting supernatant was decanted, and the crude enzyme was salted out of solution using 85-percent ammonium sulfate. The resulting enzyme precipitate was collected by centrifugation as above, providing a 20-fold increase in activity over the initial extract. This preparation was termed the “enzyme extract.”

Monoclonal antibodies were used to identify the native source of the nitrate reductase activity in sediment. Prior to production of monoclonal antibodies, the enzyme was purified by column chromatography. The extract preparation was applied to a phenyl sepharose column in 1.7-M ammonium sulfate in 20-mM phosphate buffer at pH 7. An elution gradient was provided by gradually decreasing the concentration of ammonium sulfate until only 20-mM phosphate buffer remained. Each fraction (1 column volume) was assayed for activity with  $10^{-6}$  M TNT. The active enzyme fraction was then subjected to ion-exchange chromatography by elution through a diethylaminoethyl (DEAE) cellulose column using 20-mM phosphate buffer at pH 8.6. The preparation was further purified by molecular sieve chromatography on a Sephadex CL-6B column (Pharmacia LKB, Uppsala, Sweden) eluted with 20-mM phosphate buffer at pH 7.0. This column has an exclusion range of 10,000 to 4,000,000 daltons, meaning that compounds above and below this range are retained on the column. The enzyme extract obtained from this column had a molecular weight of 300,000 to 600,000 daltons as determined by molecular sizing on additional Sephadex columns. Final polishing was performed with a metal affinity column containing zinc on the amino diacetic acid side chain. The preparation was loaded on the column in 0.5 M KCl and eluted with a gradient initially containing only 0.5 M KCl, then finally containing 3 M ammonium sulfate in 0.5 M KCl. The purified preparation

showed a single band upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

## **Immunoassay to Determine Native Sources of Nitrate Reductase**

The purified nitrate reductase was used to obtain the corresponding monoclonal antibody following standard immunological procedures (Harlow and Lane 1988). This procedure was carried out in conjunction with the University of Georgia monoclonal laboratory facility, Athens, GA. Field sampling and analysis with the immunoassay technique indicated that the plant stonewort was the source of the high nitrate reductase activity. Results of studies with TNT indicated that this plant had a great capacity to reduce TNT to triaminotoluene (TAT) using the pathway proposed in Figure 1. Further reduction of TAT to an unidentified product is very rapid. Products other than the single unknown product and the amino biotransformation compounds were not evident in the analyses.

## **Soil Collection and Handling**

Soils were obtained from areas around munitions plants having a known history of TNT contamination (Table 1). 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 containers for shipment to 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. Soils were homogenized by hand mixing and passage through a 4-mm sieve. Subsamples were analyzed for particle size distribution. Samples for shipment to ERL-Athens were transported in coolers packed with ice and shipped by overnight express. Upon arrival at ERL-Athens, subsamples of each soil were analyzed to determine organic matter content, metals, TNT, and selected biotransformation products.

## **Treatment 1 - Enzyme Extract as a Remediator for TNT-Contaminated Soils**

Only the enzyme extract was used for this study. For control treatments, 5.0 g of soil from each site were weighed into individual screw cap test tubes to which was added 9.0 ml of distilled water. For test samples, approximately 195 g of each soil were placed into 0.5-L plastic bottles and mixed with 350 ml of protein extract (approximately 1 mg/L of total protein). The six samples for each treatment were placed on a low speed

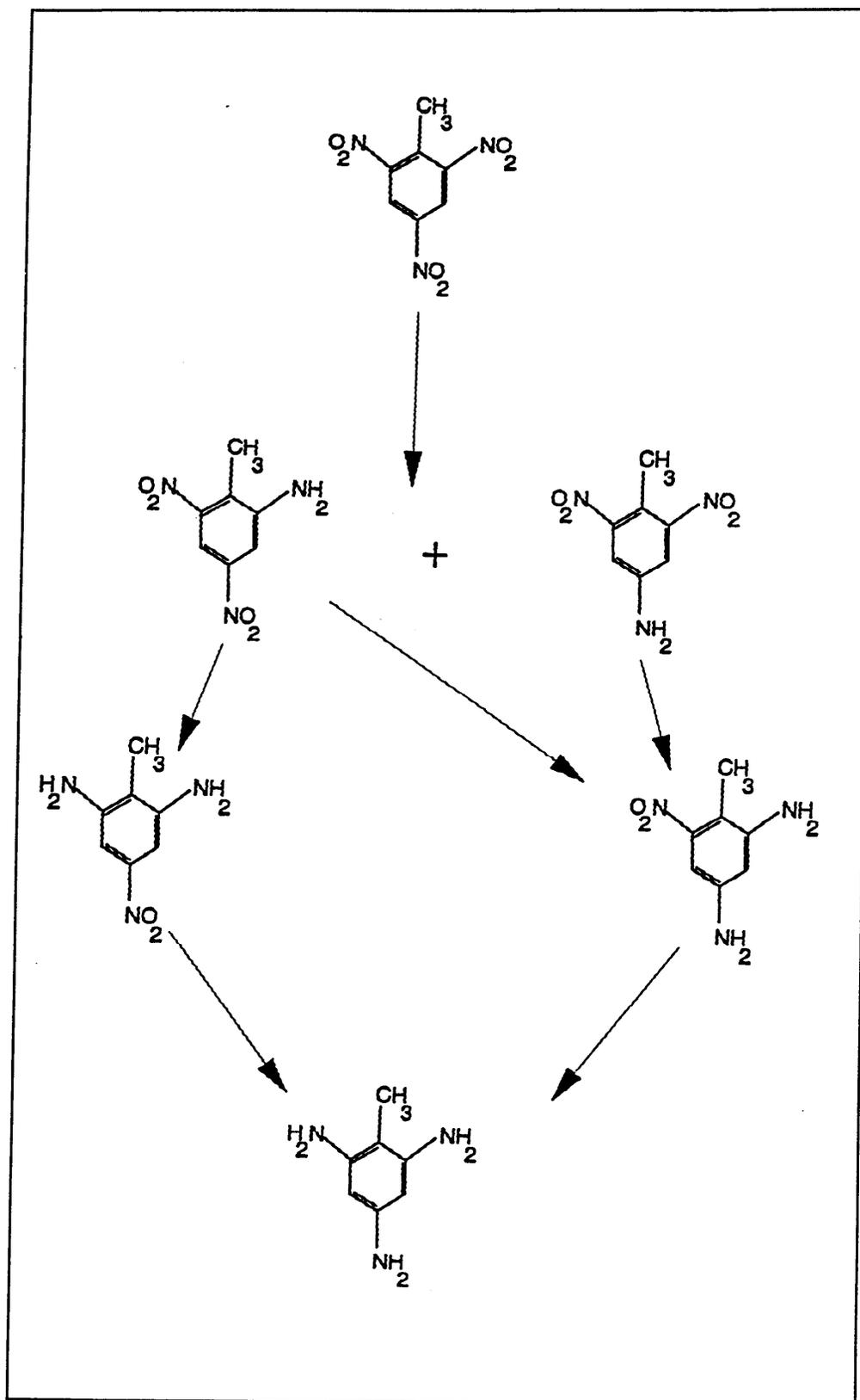


Figure 1. The proposed mechanism of TNT reduction with sediment-extracted enzyme and aquatic weed stonewort

Source	Abbreviated Form Used in Text	Location
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
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

reciprocating shaker in a glove box under a nitrogen atmosphere. One-half-ml samples were collected from each treatment at 1 and 5 days of incubation for control treatments and at 1, 5, and 7 days of incubation for test treatments. Aqueous and soil phases were separated by centrifugation in an Eppendorf Model 5415C Microcentrifuge (Eppendorf-Netheler-Hinz GMBH, Germany) for 14 min at  $16,000 \times g$  and  $4^\circ\text{C}$ .

Following 1 week of incubation, the soil-protein extract mixtures were returned to WES. Initially, these samples were to be placed into a one-tenth scale version of the WES bioslurry reactor (Zappi et al. 1993). However, these units were not available at the time of the test. To simulate the effect of bioslurry treatment, each slurry was placed into a 500-ml Erlenmeyer flask and plugged with a foam stopper. Slurries were incubated for 30 days on a gyrorotary shaker at 150 rpm and  $30^\circ\text{C}$ . The samples were then placed back into their bottles, sealed with a screw cap, and returned to ERL-Athens for analysis. Analytical samples were taken immediately upon return to ERL-Athens. The remaining material was incubated for an additional month to verify continuous reactions.

## **Treatment 2 - Stonewort as a Remediator for TNT-Contaminated Soils**

Stonewort was identified as the source of the nitrate reductase enzyme in sediment. For this reason, whole plant material was used as a concentrated source of the nitrate reductase enzyme. Stonewort was collected from Beaver Dam, a small watershed pond near Athens, GA.

Stoneworts are members of the Chlorophyta (green algae) of which there are three orders: the Charales, Charophyceae, and Charophyta. *Nitella*, the stonewort used in this work is one of the Charophyceae. Algae in this order are macroscopic and branched with nodal and internodal

cells (Aulbach-Smith and de Kozlowski 1990). Stoneworts were collected from the pond, where they grow in shallow warm water attached to the bottom sediment with a shallow root system. Plant material collected during the summer months had vigorous nitrate reductase activity. Plant material collected during the spring months lacked strong nitrate reductase activity; activity in these plants was induced by exposing intact, healthy plants to  $10^{-5}$  M nitrate for 1 day under a plant light.

### Low-TNT concentration samples

Control treatments were established as follows. Ten grams of each soil containing a low-TNT concentration (<10.0 mg/kg) (Table 2). These were added to individual 125-ml Erlenmeyer flasks. Fifty milliliters of distilled water was also added to each flask. The concentrations of TNT in these samples are given in Table 2. Active treatments contained 10.0 g of each of the same soils in separate 125-ml Erlenmeyer flasks to which were added 50 ml of distilled water and 10 g of intact stonewort plant material.

<b>Table 2</b>			
<b>Initial TNT and Aminonitrotoluene Compounds in Soil Samples</b>			
<b>Soil</b>	<b>Concentration of Constituent, mg/ki<sup>1,2</sup></b>		
	<b>TNT</b>	<b>Aminodinitro Compounds</b>	<b>Diaminonitro Compounds</b>
Radford AAP	1.0	0.3	0.7
Lone Star AAP	2.2	5.3	3.0
Iowa AAP	0.9	2.0	10
Sub Base Bangor	6.6	15	0.9
McAlester AAP	5,427	213	2,363
HEIP	1,210	30	ND <sup>3</sup>

<sup>1</sup> Based on high performance liquid chromatography analysis.  
<sup>2</sup> Ratio of soil to acetonitrile used is 1:1 (grams/milliliter).  
<sup>3</sup> ND = Not detected (detection limit is 0.01 ppm).

### High-TNT concentration samples

Control treatments for the high-TNT samples (>1,000 mg/kg) (Table 2) were prepared by adding 10.0 g each soil into individual 250-ml Erlenmeyer flasks and mixing with 100 ml of distilled water. Active treatments contained 10.0 g of the same soils in individual 250-ml Erlenmeyer flasks mixed with 100 ml of distilled water and 30.0 g of intact stonewort plant material.

## Incubation conditions

All samples were mixed under aerobic conditions. The mixture was incubated aerobically under static conditions at room temperature. One-half-ml samples were removed at 0, 3, 7, 11, and 43 days of incubation.

## Chemical Analysis

Soils were analyzed for particle size distribution using the method of Patrick (1958). Total organic carbon (TOC) content was determined with a DC-85A high temperature TOC analyzer (Dohrmann Inc., Santa Clara, CA). Metals were measured with a Plasma II Inductively-Coupled Plasma-Emission Spectrometer (Perkin-Elmer, Norwalk, CT). Soil moisture content was determined gravimetrically following oven drying for 12 hr at 105 °C.

For chemical extraction of soils contaminated with low levels of TNT (Radford AAP, Lone Star AAP, Iowa AAP, and Sub Base Bangor), a ratio of 1 g of soil:3 ml of water was used. For soils contaminated with high levels of TNT (McAlester AAP and HEIP), a ratio of 1 g soil:10 ml of water was used. The pH and Eh of the treated samples was determined using an Accumet Ion Specific Electrode/pH Meter (Fisher Scientific, Inc., Pittsburg, PA). Control and treated samples were centrifuged in the Eppendorf Model 5415C Microcentrifuge in 1.5-ml capped tubes at  $16,000 \times g$  for 15 min. The resulting supernatants were decanted and set aside for analysis. Soils were extracted with acetonitrile. Supernatants and soils were then analyzed for TNT and several TNT-biotransformation products, including 2-amino-4,6-dinitrotoluene (2A46DNT), 4-amino-2,6-dinitrotoluene (4A26DNT), 2,4,6-triaminotoluene (TAT), 2,4-diamino-6-nitrotoluene (2,4A6NT), and 2,6-diamino-4-nitrotoluene (2,6A4NT).

For Treatment 1 and all of Treatment 2 samples, except for Day 43, the presence of TNT and its degradation products was determined by EPA Method SWA 846 Number 8330 (U.S. Environmental Protection Agency (USEPA) 1990). The method requires extraction with acetonitrile followed by high performance liquid chromatography (HPLC) analysis. Analyses were performed on a Waters HPLC (Waters Instruments, Millford, MA) equipped with a variable wavelength ultraviolet (UV) detector. Separation was accomplished using a PRP-1 250  $\times$  4.1 mm column with an injection volume of 50  $\mu$ l. Samples were eluted with a mobile phase composed of 35-percent water (pH adjusted to 10 with NaOH) and 65-percent acetonitrile at a flow rate of 1.0 ml/min and detected at a wavelength of 238 nm.

Samples for Day 43 of Treatment 2 were analyzed with a micellar electrokinetic capillary electrophoresis system (MECE) using a Spectra Phoresis 1000 System (Spectra Physics, Inc., Piscataway, NJ) having a UV detector. This system is more sensitive than HPLC and separates the

mono- and diamino- structural isomers (Figures 2 and 3). Separation for MECE analysis was accomplished using a 70-cm  $\times$  75- $\mu$ m (ID) fused silica column. The mobile phase employed was 25-mM sodium dodecyl sulfate (SDS) in a 2.5-mM borax buffer (pH 8.6). The hydrodynamic injection mode was used, usually lasting for 2 sec (5.37 nl/sec). Running voltage was 20 kV, and the UV detection wavelength was 220 nm.

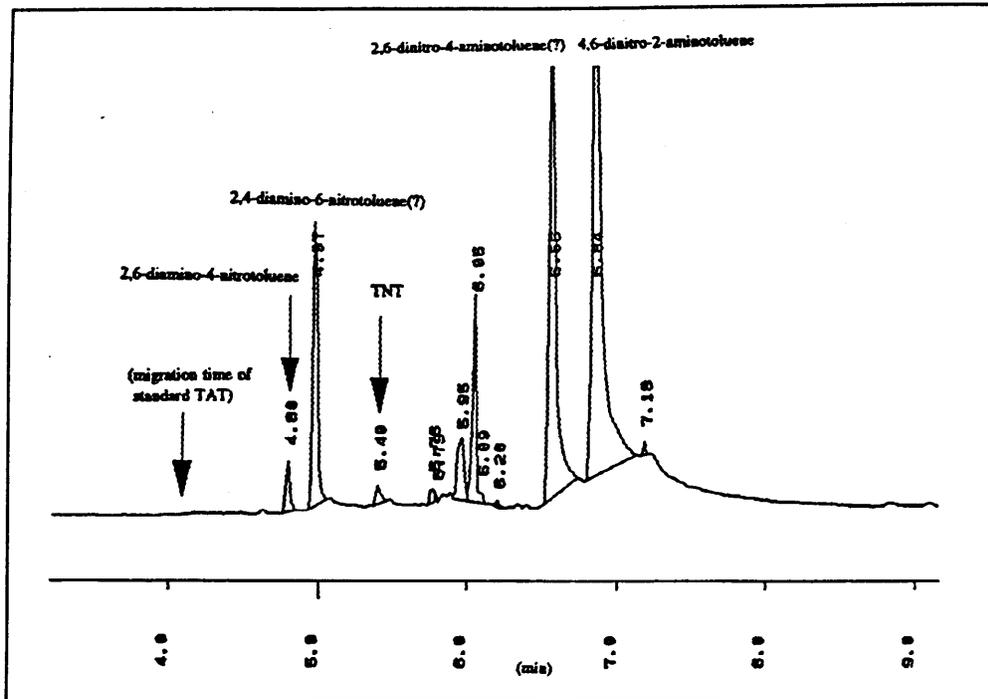


Figure 2. Electropherogram of Hastings soil supernatant treated with aquatic weed stonewort

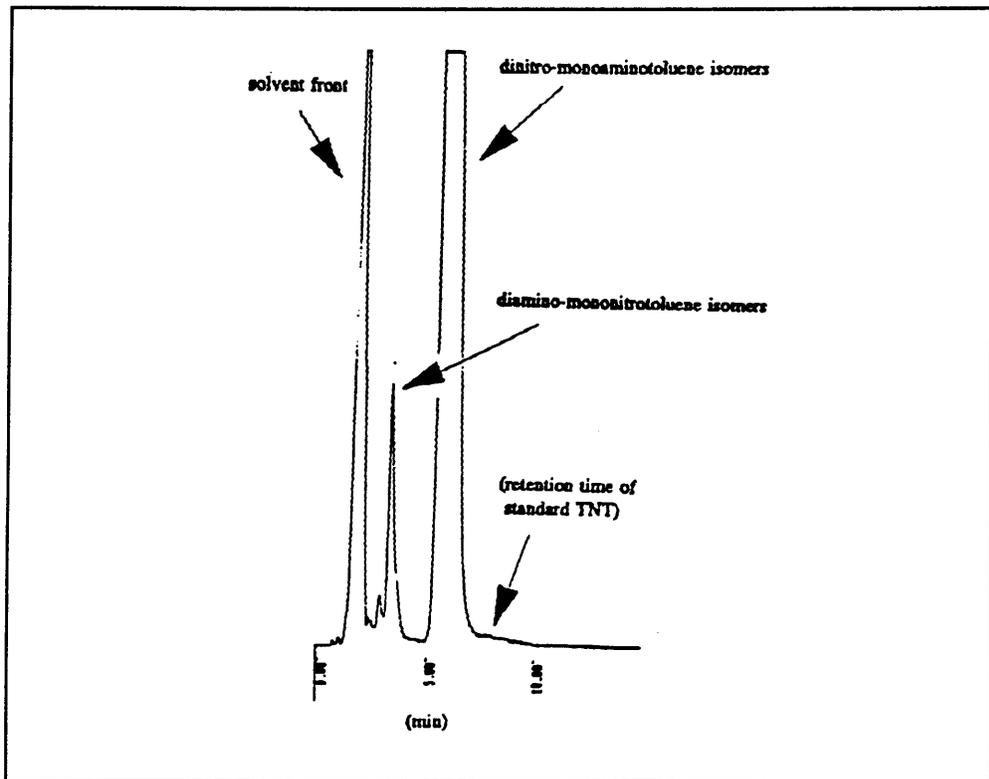


Figure 3. LC chromatogram of Hastings soil supernatant treated with aquatic weed stonewort

## 3 Results and Discussion

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### Characteristics of Contaminated Soils

Characteristics of soils used in the experimentation are listed in Table 3. All of the soils examined contained primarily sand and silt. The highest clay contents were found in McAlester AAP and HEIP soils. Slurries made with Lone Star AAP soil had a pH of 4.8. Slurries made with each of the other soils had pH values varying between 6 and 7. In other studies conducted at ERL-Athens, enzyme extracts from sediment or from stonewort were found to have significant reducing activity between pH 5.5 and 8.5 (Carreira and Wolfe 1992). The only material falling outside of this range was the Lone Star AAP slurry, which had a pH of 4.8. No attempt was made to adjust this pH.

Organic carbon (OC) content of the soils varied from 0.13 to 2.05 percent (Table 3). The rate of TNT disappearance from sediment is correlated with soil OC content (Wolfe and Delgado 1993), although some of this removal may result from irreversible binding to the organic phase. The OC levels observed for these soils were not anticipated to cause any treatment problems.

Soil metal concentration data for Fe, Pb, and Zn are also presented in Table 3. Other mechanistic studies with the enzyme have indicated that Fe can enhance TNT degradation, while Pb and Zn inhibit TNT reduction (Ou, Carreira, and Wolfe 1992).

### Enzyme Extract Treatment Results

#### Effect of enzyme extract

Table 2 presents the initial TNT concentrations for each of the six soil samples. The variation in TNT levels within a given sample is approximately 50 percent. This is likely due to sample heterogeneity, especially in samples passing a 4-mm sieve.

**Table 3**  
**Characteristics of Contaminated Soil Samples**

Soil	Initial pH <sup>1</sup>	Total Organic Carbon percent	Metal Content, mg/kg			Particle Size, percent <sup>2</sup>		
			Fe	Pb	Zn	>50 µm	50 - 2.0 µm	<2.0 µm
Radford AAP	7.6	1.9	206	<0.5	1.3	45.0	45.0	10.0
Lone Star AAP	4.8	0.35	53.6	<0.08	0.67	67.5	20.0	12.5
Iowa AAP	7.8	0.13	95.8	<0.25	0.20	62.5	25.0	12.5
Sub Base Bangor	6.0	1.2	106	<0.05	0.26	77.5	22.5	0
McAlester AAP	6.7	1.2	574	<0.07	0.39	42.5	42.5	15.0
HEIP	7.2	2.05	112	<0.08	0.66	33.0	54.0	13.0

<sup>1</sup> pH of a 1:1 (grams/milliliter) soil-to-water slurry.

<sup>2</sup> Soils were sieved prior to particle size analysis.

The initial and final pH and Eh measurements of all six soil slurries remediated with the enzyme extract are given in Table 4. The pH increases with time. Generally, Eh levels of the slurries, except for Lone Star AAP, became negative with time. This is a consequence of microbial activity in the soil slurry. All oxygen is first consumed, followed by reduction of inorganic nitrate, available manganic manganese, then ferric iron, and finally carbon dioxide (Yoshida 1975). The initial pH of the Lone Star AAP soil was low enough to inhibit the activity of all but the most acid-tolerant of microorganisms. Hence, the Eh of this soil remained in the oxidized range throughout the entire study.

<b>Table 4</b> <b>The pH and Oxidation-Reduction Potential (Eh) of Soils Treated with Extracted Enzyme as a Function of Treatment Time</b>			
<b>Soil</b>	<b>Time, days</b>	<b>pH<sup>1</sup></b>	<b>Eh, mV (Vs Ag/AgCl)<sup>2</sup></b>
Radford AAP	0	4 to 5	32
	3	7	-510
	5	5 to 6	-245
	7	5 to 6	-177
Lone Star AAP	0	4	156
	3	4 to 5	147
	5	4 to 5	107
	7	4 to 5	174
Iowa AAP	0	4 to 5	67
	3	7	-101
	5	6	-543
	7	5 to 6	-312
Sub Base Bangor	0	4 to 5	84
	3	5	-411
	5	6	-455
	7	5 to 6	-365
McAlester AAP	0	5	75
	3	6	-82
	5	7	-97
	7	6 to 7	-107
HEIP	0	4 to 5	52
	3	7	-112
	5	7	5
	7	6 to 7	-25

<sup>1</sup> pH values were determined with Sigma pH test strips.  
<sup>2</sup> Eh values were determined with Corning combination platinum electrodes.

The concentration of TNT remaining in the soils after 1 to 7 days of treatment with the sediment enzyme extract and the controls (no enzyme) are presented as a function of time for both the supernatant and the solid phases in Table 5. Initial concentrations of TNT and TNT by-products in the aqueous phase were 0 since pure distilled water was added at time 0. TNT levels remained at or near the detection limit (0.25 mg/kg except where otherwise noted in the tables) in the aqueous phases of the low-TNT soils. Some reduction activity occurred, however, as the monoaminodinitrotoluenes and diaminomononitrotoluenes appeared at detectable levels in the Lone Star, Iowa, and Sub Base Bangor supernatants. By contrast, no removal of TNT from the supernatants of the soils containing high-TNT concentrations was apparent (McAlester AAP and HEIP); in fact, TNT levels increased with time. This may be a reflection of the microbial processes occurring in the soil phase resulting in increased solubilization of TNT.

Soil phases of Radford, Iowa, and Sub Base Bangor soils exhibited strong removal of TNT (Table 5, Page 2). However, the enzyme did not appear to assist with the removal of TNT from any of the low-TNT treatments over and above that achieved in their respective controls (native soil microorganisms only). In addition, accumulations of the ADNT and DANT compounds were generally very similar in the soil phase of each of the low-TNT level samples.

Removal of TNT from soil phases of the high-TNT soils was also very similar in the enzyme-treated and control samples. In one case, the enzyme-treated HEIP soil, accumulation of ADNT compounds in the enzyme-treated sample exceeded that of the control.

In summary, the enzyme treatment alone made no appreciable difference in TNT removal and very limited difference in ADNT formation over the corresponding levels present in the controls. This means that removal of TNT was likely being carried out by the native soil microflora. Some of the failure to remove higher levels of TNT in the enzyme treatments may have been the result of the short incubation time.

### **Effect of bioreactor treatment**

A bioslurry reactor promotes biodegradation by enhancing the contact between soil particles, water, nutrients, dissolved gases, contaminants, and the degrading microorganisms through the combined actions of stirring and aeration. A small shake flask microcosm simulating the most important aspects of bioslurry treatment, enhanced soil-water contact and aeration, was employed. In this phase of the treatment, the native microorganisms presumably used the enzymatically produced amino compounds as sources of carbon and energy. Enzymatic reduction of nitro groups to amino groups alters the electron-withdrawing properties of the nitro substituents by reducing them to yield amino groups that are weak electron donors. Reduction of the nitro groups is expected to contribute to the

**Table 5  
Concentration of TNT and Aminonitrotoluene Compounds in Supernatants (mg/L) and Soil Phases (mg/kg) During  
1 Week of Treatment with Enzyme Extract<sup>1</sup>**

Soil	Time, days	TNT		ADNT Compounds		DANT Compounds	
		Control	Treated	Control	Treated	Control	Treated
<b>Supernatants</b>							
Radford AAP	T <sub>0</sub>	0	0			0	0
	1	ND <sup>2</sup>	ND	ND	ND	ND	ND
	5	ND	ND	ND	ND	ND	ND
	7	NA	ND	NA	ND	NA	ND
	T <sub>0</sub>	0	0	0	0	0	0
	1	ND	ND	0.5	0.5	0.5	0.89
	5	ND	0.3	0.44	0.72	0.5	1.25
Iowa AAP	7	NA	ND	NA	0.45	NA	0.51
	T <sub>0</sub>	0	0	0	0	0	0
	1	ND	ND	ND	ND	4.6	7.3
	5	ND	ND	0.6	ND	6.3	7.2
	7	NA	ND	NA	ND	NA	2.9
	T <sub>0</sub>	0	0	0	0	0	0
	1	0.38	ND	1.1	1.1	ND	ND
Sub Base Bangor	5	ND	ND	1.1	0.63	0.4	ND
	7	NA	ND	NA	0.63	NA	ND
	T <sub>0</sub>	0	0	0	0	0	0
	1	68.4	37.4	ND	ND	23.4	ND
	5	97.3	64	23	16.3	34.8	30
	7	NA	70	NA	18.7	NA	33.5
	T <sub>0</sub>	0	0	0	0	0	0
McAlester AAP	1	65.6	61.3	21.8	6.7	0.3	0.1
	5	91.1	63.8	6.6	11.1	0.8	0.84
	7	NA	68	NA	223	NA	1.9

(Continued)

<sup>1</sup> Analysis with HPLC.  
<sup>2</sup> Abbreviations: ND = Not detected—sample variation around ± 50 percent at a detection limit of 0.01 ppm; NA = Not analyzed.

Table 5 (Concluded)												
Soil	Time, days	TNT		ADNT Compounds		DANT Compounds						
		Control	Treated	Control	Treated	Control	Treated					
Soil Phases												
Radford AAP	T <sub>0</sub>	1.0	1.0	0.3	0.3	0.3	0.7					
	1	0.7	0.5	1.3	0.3	0.3	ND					
	5	0.4	0.41	ND	0.1	0.1	ND					
	7	NA	0.52	NA	0.8	0.8	ND					
Lone Star AAP	T <sub>0</sub>	2.2	2.2	5.3	5.3	5.3	3.0					
	1	1.0	0.8	3.24	1.46	1.46	0.1					
	5	0.9	1.1	2.9	1.45	1.45	0.8					
	7	NA	1.8	NA	3.7	3.7	2.4					
Iowa AAP	T <sub>0</sub>	0.9	0.9	2.0	2.0	2.0	10					
	1	0.22	0.14	1.06	1.0	1.0	4.7					
	5	0.23	0.04	1.84	0.87	0.87	4.8					
	7	NA	ND	NA	0.68	0.68	4.0					
Sub Base Bangor	T <sub>0</sub>	6.6	6.6	15	15	15	0.9					
	1	2.38	1.08	19.7	24.0	24.0	ND					
	5	1.0	0.14	18.1	11.0	11.0	ND					
	7	NA	1.6	NA	14.7	14.7	ND					
McAlester AAP	T <sub>0</sub>	5,427	5,427	213	213	213	2,463					
	1	4,351	3,283	194	224	224	724					
	5	3,808	2,647	192	155	155	554					
	7	NA	3,962	NA	193	193	715					
HEIP	T <sub>0</sub>	8,723	8,723	18.8	18.8	18.8	2.4					
	1	1,550	3,930	6.4	63.4	63.4	3.5					
	5	1,448	3,110	13.8	122.5	122.5	1.2					
	7	NA	2,152	NA	738	738	5.3					

instability of the new monoaminotoluenes, diaminotoluenes, and triaminotoluenes. However, most past evidence has indicated that the monoaminonitro and diaminonitro compounds seem to be refractory to microbial degradation.

Following biotreatment at WES, all six TNT-contaminated soil slurries were returned to ERL-Athens for analysis and additional incubation. The results are presented in Table 6. Compared with Table 5, the TNT levels in the McAlester and Hastings supernatants dropped markedly, while the ADNT and DANT levels all increased substantially. Also compared with Table 5, the TNT levels in the McAlester and HEIP soils underwent extensive decreases. For the McAlester AAP samples, aminodinitrotoluenes (both isomers) and diaminomononitrotoluenes were generally the same or higher at the end of the posttreatment phase than at the end of the first week of enzyme incubation. For the HEIP soil, ADNT and DANT levels also increased with time in the soil phase between the completion of the 1-week enzyme treatment (Table 5) and the initial sample of the posttreatment stage (Table 6). However, the DANT levels apparently decreased over the course of the posttreatment incubation period in the HEIP soil. The observed TNT reductions could be due to continued enzymatic activity or microbial activity during biotreatment or both. Since the controls were not incubated aerobically, no means existed to distinguish the effect of the enzyme in this portion of the treatment.

## Remediation by Stonewort

In a time course study, the stonewort plant was used as a remediator. The results are presented in Table 7. The concentrations of TNT and its reduction products in the supernatant and solid phases decreased significantly by Day 43. For the four soils contaminated with low levels of TNT (Radford AAP, Iowa AAP, Lone Star AAP, and Submarine Base Bangor), no detectable TNT remained in the supernatants, even at time 0, and no detectable TNT remained in the solid phase at Day 43. Analysis of the stonewort indicated that none of the TNT or aminotoluenes were accumulated by the plant tissues. The nitrate reductase activity was so high in the intact plant that TNT was transformed as rapidly as it moved into the aqueous phase. In the McAlester AAP soil, essentially no TNT remained after 43 days. However, it is not apparent why the initial TNT level in the McAlester soil used for the stonewort study was so low. WES did provide a second batch of McAlester soil for the stonewort study, so it is possible that the samples had different initial TNT levels. In the HEIP sample, the supernatant concentration was reduced from approximately 70 mg/L to 1 mg/L, and the solid phase level decreased from approximately 8,723 mg/kg to 11 mg/kg. Concentrations of the aminodinitro- and diaminonitro- isomers also declined from their peak levels, although some of these levels rose before falling.

**Table 6  
TNT and Aminonitrotoluene Compounds in Treatment 1 Samples Following Aerobic Bioslurry Treatment at WES<sup>1</sup>**

Soil	Posttreatment Time <sup>2</sup> , days	Supernatant Concentration, mg/L			Sediment Concentration, mg/kg		
		TNT	ADNT Compounds	DANT Compounds	TNT	ADNT Compounds	DANT Compounds
Radford AAP	1	ND <sup>3</sup>	ND	ND	0.43	0.6	ND
	30	ND	ND	ND	ND	ND	ND
Lone Star AAP	1	ND	0.9	ND	ND	0.7	0.75
	30	ND	0.33	0.88	ND1	2.65	ND
Iowa AAP	1	ND	ND	6.6	ND	0.79	4.5
	30	ND	ND	4.9	ND	ND	4.9
Sub Base Bangor	1	ND	0.43	ND	0.4	3.5	0.4
	30	ND	ND	ND	ND	0.27	ND
McAlester AAP	1	0.5	167	87	ND	1,268	628
	8	1.2	178	43	5	1,616	748
	30	1.5	177	40.6	52.9	2,567	1,239
HEIP	1	ND	358	1.8	5	2,352	32
	8	ND	382	2.5	5	2,250	28
	30	0.26	349	3.9	24.8	2,891	8.4

<sup>1</sup> Analysis with HPLC.

<sup>2</sup> Thirty-day samples were incubated an extra month following treatment at WES to verify continuous reactions.

<sup>3</sup> ND = Not detected (detection limit is 0.01 mg/kg).

Table 7 TNT and Aminonitrotoluene Compounds in Supernatants and Sediments Following Treatment with Stonewort <sup>a</sup>									
Soil	Treatment Time, days	Supernatant Concentration, mg/L			Sediment Concentration, mg/kg				
		TNT	ADNT Compounds	DANT Compounds	TNT	ADNT Compounds	DANT Compounds		
Radford AAP	0	ND	0.18	0.02	0.66	2.5	ND		
	3	ND	ND	0.02	0.12	0.12	ND		
	7	ND	ND	0.05	0.27	ND	ND		
	11	ND	ND	0.02	0.27	ND	ND		
	29	ND	ND	ND	0.44	0.25	ND		
	43 <sup>b</sup>	ND	ND	ND	ND	1.25 <sup>c</sup>	ND		
Lone Star AAP	0	ND	0.09	0.03	0.6	3.06	1.01		
	3	ND	0.03	0.15	0.14	0.37	0.24		
	7	ND	ND	0.22	0.08	0.37	0.06		
	11	ND	ND	0.12	0.14	0.36	ND		
	29	ND	ND	0.02	ND	0.21	ND		
	43 <sup>b</sup>	ND	ND	ND	ND	1.86 <sup>c</sup>	ND		

(Sheet 1 of 3)

<sup>a</sup> Analysis with HPLC. ND = not detected (detection limit is 0.01 mg/kg).  
<sup>b</sup> Analysis with MECE.  
<sup>c</sup> 4ADNT.  
<sup>d</sup> 2ADNT.  
<sup>e</sup> 2,6A4NT.  
<sup>f</sup> 2,4A6NT.

Table 7 (Continued)											
Soil	Posttreatment Time <sup>2</sup> , days	Supernatant Concentration, mg/L			Sediment Concentration, mg/kg						
		TNT	ADNT Compounds	DANT Compounds	TNT	ADNT Compounds	DANT Compounds				
Iowa AAP	0	ND	0.75	3.0	0.4	2.24	3.4				
	3	ND	ND	2.2	0.44	1.25	1.26				
	7	ND	ND	1.04	0.21	0.92	0.49				
	11	ND	ND	0.69	0.11	1.08	0.45				
	29	ND	ND	0.11	0.02	0.63	0.12				
	43 <sup>b</sup>	ND	ND	ND	ND	1.69 <sup>c</sup>	ND				
Sub Base Bangor	0	ND	0.7	0.02	1.3	9.4	ND				
	3	ND	ND	0.04	ND	0.36	ND				
	7	ND	ND	0.02	0.07	0.12	ND				
	11	ND	ND	0.01	0.05	0.06	ND				
	29	ND	ND	ND	ND	0.21	ND				
	43 <sup>b</sup>	ND	ND	ND	ND	1.84 <sup>c</sup>	ND				
McAlester AAP	0	49.4	4.1	9.2	606	60.6	44				
	3	0.02	10	32.2	12	111	46				
	7	ND	0.02	37.9	6.2	2.4	25				
	11	ND	ND	26.4	3.1	2.0	14.5				
	29	ND	ND	2.48	0.33	1.4	7.14				
	43 <sup>b</sup>	0.73	ND	ND	ND	1.81 <sup>c</sup>	ND				

(Sheet 2 of 3)

Table 7 (Concluded)									
Soil	Posttreatment Time <sup>2</sup> , days	Supernatant Concentration, mg/L			Sediment Concentration, mg/kg				
		TNT	ADNT Compounds	DANT Compounds	TNT	ADNT Compounds	DANT Compounds		
HEIP	0	70.2	3.4	0.2	8,723	18.8	2.4		
	3	27.3	86.6	0.08	4,635	356	2.0		
	7	24.5	183	4.7	3,210	964	4.1		
	11	23.1	227	4.4	2,003	1,056	5.0		
	29	19.8	367	4.41	215	1,869	8.4		
	43 <sup>b</sup>	1.18	52.8 <sup>d</sup>	1.47 <sup>e</sup>	10.8	435 <sup>d</sup>	ND		
			90.6 <sup>c</sup>	9.2 <sup>f</sup>		746 <sup>c</sup>			

(Sheet 3 of 3)

Triaminotoluene could not be detected at Day 43 when analyzed by MECE in samples treated with stonewort. The limit of detection for TAT in the aqueous samples and extracts was approximately 0.01 ppm. Based on other studies with substituted anilines, the TAT was likely oxidized rapidly by autoxidation or microbial activity to yield catechol. Catechols are known to be readily transformed to innocuous products by further microbial degradation (see Bayly and Barbour 1984).

One of the samples from Treatment 1 (HEIP) was mixed with stonewort under aerobic conditions. The initial levels of monoaminodinitrotoluenes in this treatment system were 62 mg/L and 3,910 mg/kg in the supernatant and soil phases, respectively. After 3 weeks of Treatment 2 with stonewort, only 5.5 mg/L and 358 mg/kg of these compounds remained in the supernatant and soil phases, respectively.

MECE was used to separate and quantify TNT and its degradation products (Table 7). Compared with the retention time of standard samples, both monoaminodinitrotoluene isomers and both diaminomononitrotoluene isomers were separated (Figure 3). Also, in preliminary testing, TAT was easily detected without interference (e.g., eluted with solvent front). This degree of separation is not achievable with HPLC. The rate-limiting step in TNT remediation in these systems may be mass transport. Thus, the remediation process can possibly be accelerated by mixing.

## 4 Conclusions and Recommendations

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The nitrate reductase enzyme in purified form demonstrated very little potential to remediate soils contaminated with low levels of TNT over that achieved with the native microflora alone.

However, the nitrate reductase in intact stonewort has a high potential to remediate soils contaminated with high levels of TNT. Stonewort was effective in reducing TNT from as much as 8,723 mg/kg to 10.8 mg/kg in 43 days in the HEIP soil, and was also effective in all five remaining soils. Stonewort treatment will be especially useful for soil surface bioremediation. The treatment area can be flooded and the plant added to the soil surface with only periodic turning of the soil required for continued treatment. In addition, stonewort is effective in indirectly regulating the pH of the soil-water system and eliminating poisoning of the nitrate reductase by Pb and Zn. This means that treatment with stonewort is applicable to soils having a wide range of pH values and soils contaminated with Pb and Zn. (Note added during revision: Since the original draft of this report, Dr. Wolfe has demonstrated that the triaminotoluene produced by the action of the stonewort plant enzyme undergoes ring fission to form ketones and lactones. These compounds are commonly degraded to CO<sub>2</sub> by a variety of microorganisms.) Based on this, it is recommended that use of this plant be transitioned to the 6.2 level for use at the pilot scale prior to potential application in the field.

Future investigations should focus on parameters pertinent to scale-up of the remediation technology. Recommendations include the following:

- a. Develop a procedure for mass culturing of stonewort for use in bioremediation of contaminated soils.
- b. Quantify the optimum amount of stonewort required to destroy TNT in contaminated soils.
- c. Determine the mass balance to identify any environmentally important products of TNT.

- d. Evaluate any potential soil toxicity resulting from the remediation process.**

# References

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- Aulbach-Smith, C. A., and de Kozlowski, S. J. (1990). *Aquatic and wetland plants of South Carolina*. South Carolina Water Resources Commission, Columbia, SC.
- Bayly, R. C., and Barbour, M. C. (1984). "The degradation of aromatic compounds by the meta and gentisate pathways." *Microbial degradation of organic compounds*. D. T. Gibson, ed., Marcel Dekker, Inc., New York, 253-295.
- Carreira, L. H., and Wolfe, N. L. (1992). "Isolation and characterization of a sediment component that reduces 2,4,6-trinitrotoluene," presented at the *Symposium on Oxidation-Reduction Transformations of Inorganic and Organic Species in the Environment*, 203rd ACS National Meeting Held in San Francisco, CA, 5-10 April 1992.
- Funk, S. B., Roberts, D. J., Crawford, D. L., and Crawford, R. L. (1993). "Initial-phase optimization for bioremediation of munition compound-contaminated soils," *Applied and Environmental Microbiology* 59, 2171-2177.
- Golovleva, L. A., Aliyeva, R. M., Naumova, R. P., and Gvozdyak, P. I. (1992). "Microbial bioconversion of pollutants," *Reviews of Environmental Contamination and Toxicology* 124, 41-78.
- Harlow, E., and Lane, D., ed. (1988). *Antibodies, a laboratory manual*, Cold Spring Harbor Laboratory.
- Higson, F. K. (1992). "Microbial degradation of nitroaromatic compounds," *Advances in Applied Microbiology* 37, 1-19.
- Li, W., Yang, Y., and Yang, H. (1987). "TNT-degrading enzyme of *Citrobacter freundii* and its regulation," *Acta Microbiologica Sinica* 29, 117-122.
- Naumova, R. P., Selivanovskaya, S. Y., and Cherepneva, I. Y. (1988). "Transformation of 2,4,6-trinitrotoluene in *Pseudomonas fluorescens* in the case of oxygen and nitrate respiration," *Mikrobiologiya* 57, 493-497.

- Naumova, R. P., Selivanovskaya, S. Y., and Mingatina, F. A. (1988). "Possibility of deep bacterial destruction of 2,4,6-trinitrotoluene," *Mikrobiologiya* 57, 218-222.
- Ou, T. -Y., Carreira, L. H., and Wolfe, N. L. (1992). "Nitroreduction of TNT in contaminated soils," presented at the *Symposium on Oxidation-Reduction Transformations of Inorganic and Organic Species in the Environment*, 203rd ACS National Meeting Held in San Francisco, CA, 5-10 April 1992.
- Patrick, W. H., Jr. (1958). "Modification of method of particle size analysis," *Proceedings of the Soil Science Society of America* 4, 366-367.
- Selivanovskaya, S. Y., Akhmetova, D. Z., and Naumova, R. P. (1986). "Terminal steps in preparatory metabolism of 2,4,6-trinitrotoluene in *Pseudomonas fluorescens*," *Mikrobiologiya* 55, 1040-1041.
- U.S. Environmental Protection Agency. (1990). *Test methods for evaluating solid wastes*. SW-846, 3d ed., November 1990 revision, Office of Solid Waste and Emergency Response, Washington, DC.
- Wolfe, N. L., and Delgado, M. C. (1993). "Structure-activity relationships for the reduction of *p*-substituted nitrobenzenes in anaerobic sediments," Manuscript in preparation.
- Yoshida, T. (1975). "Microbial metabolism of flooded soils." *Soil Biochemistry*. Vol 3, E. A. Paul and A. D. McLaren, ed., Marcel Dekker, Inc., New York, 83-122.
- Zappi, M., Gunnison, D., Pennington, J., Cullinane, M. J., Teeter, C., Brannon, J. M., and Myers, T. (1993). "Technical approach for in situ biological treatment research: Bench-scale experiments," TR IRRP-93-3, U.S. Army Engineer Waterways Experiment Station, Vicksburg, MS.

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