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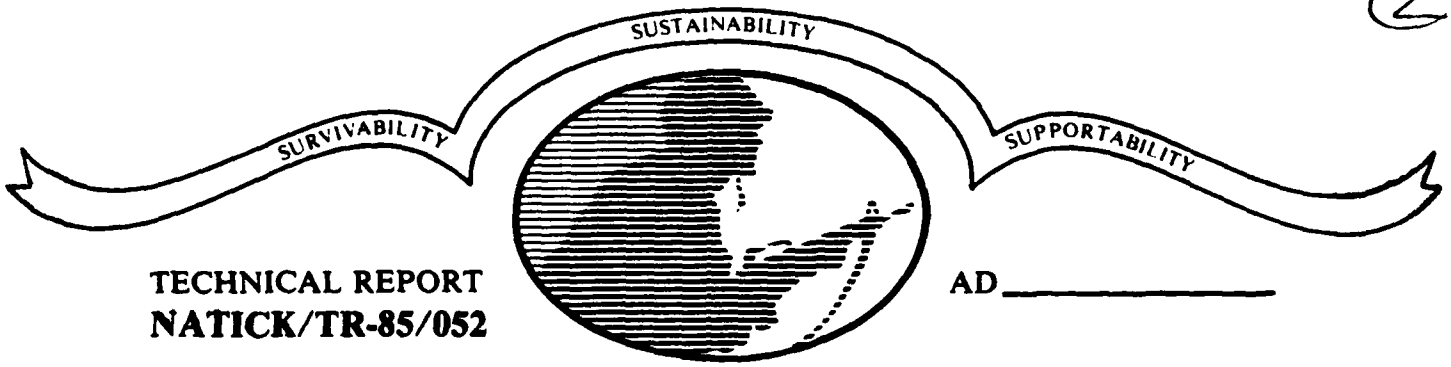

TECHNICAL REPORT
NATICK/TR-85/052

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EFFECTS OF ENVIRONMENTAL FACTORS ON THE TRANSFORMATION OF 2, 4, 6 - TRINITROTOLUENE IN SOILS

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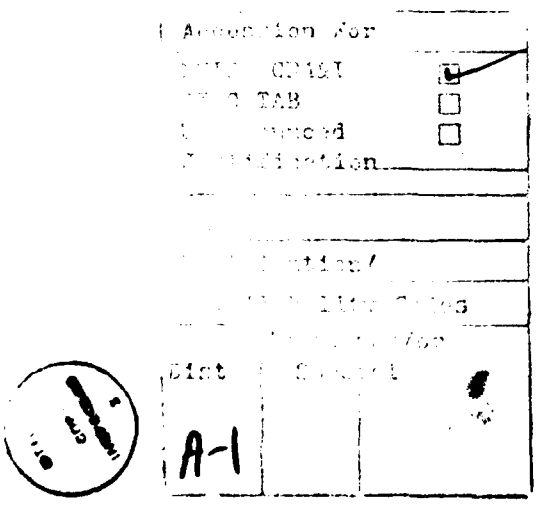

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The effects of a variety of environmental factors on the fate of TNT in soils was determined. Soxhlet extraction with acetone was found to efficiently recover TNT and its transformation products from soil. HPLC-coupled radioactivity detection was effective for quantifying the various transformation products from complex soil extracts. The initial concentration of TNT had the greatest effect on extraction efficiency, with highest recoveries of ^{14}C-labelled material in the tubes with the highest initial concentration of TNT.

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Conversely, the highest percent of unextractable or bound material resided in cubes with the lowest initial concentration of TNT, where microbial activity was also highest.

Of the environmental factors evaluated, the initial concentration of TNT had the greatest overall effect on the rates of transformation. Activity was greatest at the 0.1% level and progressively lower at 1.0% and then 10.0% TNT levels. The production of monoamines (2-amino-4,6-dinitrotoluene and 4-amino-2,6-dinitrotoluene) and diamines (2,4-diamino-6-nitrotoluene and 2,6-diamino-4-nitrotoluene) was greatest in incubations with the lowest initial concentrations of TNT. In terms of main effects, after initial concentrations of TNT, the presence or absence of organisms and the incubation temperature have the greatest effects on the response variables evaluated. Moisture level was less important and the percent organic matter and the oxygen level were the least significant of the environmental factors studied. First order interactions between some of these environmental factors were also identified.

These results, when extrapolated to potential scenarios such as lagoon entombments, would indicate that the more concentrated the TNT, the fewer the organisms, the colder the temperature, and the dryer the soil the longer the TNT will remain in an unchanged state. Originator supplied keywords included)

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PREFACE

There are concerns with the long term-environmental fate of TNT in soils and lagoons. Currently, Milan Army Ammunition Plant, Milan, TN, UMATILLA Army Depot, and other sites are experiencing problems of contamination of aquifers due to the leaching of TNT wastes originally stored above ground in lagoons and settling ponds. A capping program for contaminated lagoons is one alternative proposed by the Army for dealing with this problem. This approach would "isolate" the contaminants from the environment. However, there is not enough information available on the biological, physical, or chemical changes that TNT, RDX or DNT might undergo under these environmental constraints (or for that matter, in soils in general) in order to evaluate properly this alternative. Information on the influence of environmental factors on rates of degradation will be critical in order to provide a sound scientific basis upon which to assess the feasibility of entombment for these contaminated soils.

This work was supported with funds from the U.S. Army Toxic and Hazardous Materials Agency under project AF25, R896.04.0142, 33214155000. The research was performed in 1984. We wish to thank Patricia Riley, Steven LaRosa and Jennifer Pierce of Natick R&D Center for their technical assistance.

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EFFECTS OF ENVIRONMENTAL FACTORS ON THE TRANSFORMATION OF 2,4,6-TRINITROTOLUENE IN SOILS

INTRODUCTION

The environmental fate of 2,4,6-trinitrotoluene (TNT) in aqueous and composting systems has been well documented. [1-8] There is only limited information available on the fate of TNT in soils[7,8] although some research has been undertaken to assess the leaching of TNT through soils, including aspects of biotransformation. [9,10,11]

TNT contaminates soils[12] and ground water[13] and the problems associated with this contamination will persist. Therefore, it is essential to understand the long-term fate of TNT in soil systems. The objective of this study was to determine the effects of environmental factors on the kinetics of TNT degradation in soil. Factors such as soil moisture, soil organic matter, temperature, oxygen level, time of incubation, concentration of TNT, and biological vs. nonbiological factors will be considered.

TNT represents an environmental hazard because of its toxicity to humans and other organisms, its mutagenicity in the Ames Test and its explosive nature.[12,14-19] It is important to note that the microbial reduction products formed from TNT also pose significant environmental hazards due to mutagenic and toxic effects.[14,20] The study results will provide a scientific basis upon which to address questions concerning the long-term fate of TNT in soils. This information in turn will provide some guidance as to the long-term potential hazards associated with the contaminated soil.

The first phase of this work consisted of the development of appropriate analytical methods to analyze and handle the compounds (TNT and potential microbial intermediates) and the large number of samples required to address the objectives. This analytical work consisted of two phases: first, to extract the compounds from the soil, and second, to separate and quantify the compounds present in the extract. To this end, the information available in the literature was first reviewed and is summarized below.

In general, methods currently in use for accurate quantitation of TNT and its metabolites in soils involve the use of an organic solvent to solubilize these compounds, sometimes by means of Soxhlet extraction. The extraction step is then often followed by further extraction to remove interfering compounds from the first solvent and/or a concentration step to improve detection. Detection methods used are by ^{63}Ni electron capture or nitrogen-phosphorous flame ionization detector (FID) gas chromatography (GC). Summaries of some specific methods are included below to give an overview of the range of the procedural details.

(1) Determination of 2,4-Dinitrotoluene (2,4-DNT), 2,6-Dinitrotoluene (2,6-DNT), TNT and Tetryl in Soil: (USATHAMA METHOD 3G, [21] revised February 3, 1981). Soil samples are air-dried and sieved through a 30-mesh screen. After remixing, a 10-g portion of sample is weighed into a 250-mL bottle and extracted with 100 mL of acetone for 1 hour on a mechanical shaker. After settling, a 2-mL aliquot is transferred to a sample vial and refrigerated until ready for analysis. The compounds are detected by ^{63}Ni electron capture

GC. Detection limits are reported as: 2,4-DNT 0.51 ppm, TNT 1.9 ppm, and tetryl 1.1 ppm. Interferences present in some soils may increase these values by as much as a factor of 10.

(2) Nitroaromatics in Soil: (USATHAMA METHOD 6P).[22] Dry sediment on an aluminum pan for 1 to 4 days in a 32°C incubator. Pulverize in a mortar and pestle and transfer 75 g into a Soxhlet apparatus between layers of pre-extracted glass wool. Extract 8 hours with methylene chloride. Concentrate the extract to about 10 mL in a Kuderna-Danish apparatus. Inject 2 uL of the sample into a GC equipped with a nitrogen-phosphorous detector and a 30 SE-52 fused silica capillary column. Detection limits are given as follows in ug/g (ppm): nitrobenzene 3.1, 2,4-DNT 3.2, 2,6-DNT 3.1, 1,3-dinitrobenzene (1,3-DNB) 3.6, 1,3,5-trinitrobenzene (1,3,5-TNB) 4.8, TNT 2.5, tetryl 3.8. Interferences are reported to vary considerably.

(3) Determination of 2-Nitrotoluene (2-NT), 2,6-DNT, 2,4-DNT, TNT, 1,3,5-TNB, Cyclonite (RDX), Tetryl, and Dimethylaniline (DMA) in Soil: (USATHAMA Method 5P).[23] Weigh 5 g of air-dried soil into a 50 mL centrifuge tube. Add 20 mL of 0.01 N sodium hydroxide, mix, and extract with 15 mL of toluene:methanol (2:1) by shaking vigorously for 3 minutes. Centrifuge at medium speed (no specifics were given) for 10 minutes. Decant through a glass funnel containing anhydrous sodium sulfate and repeat, combining four extracts in all. Inject 5 uL into a ^{63}Ni electron capture detector equipped GC. Detection limits were given in ug/g as: 2-NT 1.9, 2,6-DNT 1.4, 2,4-DNT 4.7, TNT 0.84, 1,3,5-TNB, 1.5, RDX 1.6, DMA 7.3, and tetryl 2.0. Interferences should be determined routinely to check their effect on the analysis.

(4) USATHAMA Method used to Determine the Concentrations of Nitrobenzene (NB), 2,4-DNT, 2,6-DNT, 1,3-DNB, 1,3,5-TNB, TNT, and Tetryl in Soil Samples:[24] Samples of soil (approx 5 g) are dried to constant weight and extracted by mixing on a wrist-action shaker for 1 hour with 100 mL of toluene:acetone (50:50). The liquid is decanted through filter paper. The sediment is rinsed twice more with 25 mL of the toluene-acetone, filtered and pooled with the first extraction. The pooled toluene:acetone is then extracted twice with 25 mL of organic-free water. The pooled organic-free water is extracted with 5 mL of toluene and discarded. The toluene is pooled with the remaining material and then passed through a funnel containing a layer of anhydrous sodium sulfate. The volume is adjusted to 100 mL and a 1 mL portion is transferred to a sample vial for analysis by GC (electron capture). Detection limits are as follows in ug/g: NB 0.475, 2,4-DNT 0.090, 2,6-DNT 0.108, 1,3-DNB 0.160, 1,3,5-TNB 0.417, TNT 0.073, Tetryl 0.362. No interferences were encountered.

Methods similar to those shown above do not meet the needs of a study involving large numbers of samples because they consume large amounts of both time and/or solvent. The frequent transfer of soil extract among various pieces of equipment, as occurs with procedures like these above, increases the potential for loss or contamination of the sample. In addition, the use of GC for detection of these compounds presents a problem because separation of mixtures of some of the TNT metabolites is poor. Separation of these mixtures

is possible using high performance liquid chromatography (HPLC).[25] However, the complex materials that are often present in soil extraction mixtures may interfere too much with the HPLC's UV detector.

We propose to solve these problems by the use of ring-labelled ^{14}C-TNT and a radioactivity flow detector coupled to the HPLC. This approach virtually eliminates interference from materials that aren't derived from the TNT ring structure, both because that structure is the only source for ^{14}C, and because the flow detector can only "see" radioactively labelled compounds. The combined use of HPLC with radioactive flow detection will result in the coupling of the best available separation technology to a highly specific and sensitive means of detection.

MATERIALS AND METHODS

Literature Review.

To review available information on the extraction and quantitation of TNT and its potential microbial intermediates from soil, a comprehensive literature search was conducted using our own files, the library facilities at Natick Research & Development Center, and the Hazardous Materials Technical Center (Rockville, MD). Data bases searched included the following: Chemical Abstracts (1960 to present); Toxline (1965 to present), Agricol (1970 to present), Aqualine (1974 to present), ASFA, Conference Papers (1973 to present). ENVIROLINE (1971 to present). ENV. BIBL. (1973 to present), Pollution Abstracts (1970 to present), Life Sciences Collection, National Technical Information Service (1964 to present), Water Resource Abstracts, Defense Technical Information Center, and the 1982 3rd Annual Meeting of the Society of Environmental Toxicology and Chemistry.

Chemicals.

TNT, ^{14}C-uniformly ring-labelled (URL) 10 mCi/mmole, was purchased from California Bionuclear Corp., Sun Valley, CA. TNT, unlabelled, was purchased from Eastman Chemical, Rochester, NY.

Environmental Factors.

A list of environmental variables evaluated in this study is presented in Table 1. Screw capped polypropylene Oak Ridge centrifuge tubes, 50 mL capacity, were used to house the 15 g of soil for each combination of environmental factors. With this approach the entire tube contents were extracted for subsequent analysis, including centrifugation, thereby minimizing handling and transfer steps.

Soil organic loads, represented by 2.2% and 10.5% organic matter, were made by mixing garden loam with a 50/50 mixture of bentonite and sand. The pH of the soil was 6.5.

TABLE 1. Environmental Factors under Evaluation

A. Internal variables

I. Soil organic matter

1. 2.2%
2. 10.5%

II. Concentrations of TNT

1. 0.1%
2. 1.0%
3. 10.0%

III. Oxygen level

1. ambient
2. flushed with nitrogen
3. flushed with nitrogen and chemically reduced[a]

IV. Moisture

1. air dried
2. 50% of field capacity
3. field capacity

B. External variables

I. Temperature

1. room temperature
2. 10°C

II. Time of incubation

1. 6 months
2. 12 months
3. 24 months

C. Independent variable

I. Organisms

1. sterile
2. active[b]

---

[a]0.025% Sodium sulfide
[b]Mixed inoculum

TNT final concentrations of 0.1%, 1.0% and 10.0% (weight/weight TNT/soil) were solubilized in acetone. The cold TNT was spiked with ^{14}C-URL TNT (6.26 uCi, 7.57 uCi, and 4.59 uCi for the 0.1%, 1.0% and 10% concentrations, respectively). The concentrations of TNT in acetone were such that 2.0 mL of acetone solution were added to each tube to produce final concentrations of 0.1% and 1.0%, and 4.0 mL of acetone solution were added to achieve the 10% final concentration of TNT in the soil.

Three treatments were used to produce different levels of oxygenation in the tubes. One-third of the tubes were flushed with high purity air, one third with high purity nitrogen, and the final third, again with nitrogen but with sodium sulfide, 0.025% based on dry weight of soil, also added as a reducing agent. All tubes were flushed until all traces of the acetone (added with the TNT) were gone from the soil.

Three levels of moisture were evaluated, air-dried soil, soil with 4.5 mL distilled water for each 15 g of soil (50% of field capacity), and soil with 9 mL of distilled water for each 15 g of soil (field capacity).

The inoculum was made by diluting 50 g garden soil and 100 g of soil from an area near the perimeter of a munitions waste lagoon in 5 L of filtered lake water. The vessel was stirred overnight and the liquid was decanted and centrifuged at 24,000 x G in a GSA rotor for 1 hour. The resulting pellets were pooled and resuspended into 50 mL of freshly filtered lake water. A 50 uL inoculum was used for half the tubes while the other half was autoclaved for use as sterile controls. Autoclaving was accomplished with 20 minute sterilization runs on three consecutive days.

One-half of the tubes were incubated at room temperature (averaged 21°C, ranged from 17°C to 25°C) while the other half were placed in a controlled temperature incubator set at 10°C. Sufficient samples were set up, 648, in order to have sample sets for all combinations of conditions at six month, one year, and two-year intervals. All tubes were incubated in the dark.

All factors were varied against all other factors for a total of: (2 soil organic loads) x (3 TNT concentrations) x (3 oxygen treatments) x (3 moisture treatments) x (2 biological conditions) x (2 temperatures) x (3 time periods) = 648 total tubes. The factors were assigned to one of three categories. The internal factors were considered to be organic load, TNT concentration, oxygen treatment, and moisture treatment. The external factors were considered to be time and temperature. The biological condition was treated as an independent factor during the setup of the experiment. The set of tubes comprised of all combinations of the internal factors (54 tubes) was considered to be one "block". The whole experiment consisted of 12 "blocks". [A set of 6 "blocks" for all inoculated combinations of the external factors and another set of 6 "blocks" for the equivalently incubated sterile "blocks".] Each tube within a "block" has one corresponding tube in every other "block".

The levels of the internal factors within each set of corresponding tubes are identical. The differences between these tubes is that each is being incubated with a different time, temperature and biological condition

combination. A computer was used to re-sort the tubes into sets of 54 by randomly choosing from among the corresponding tubes to create "artificial blocks" that were used during setup of the experiment. This was done because the experiment required 8 days to setup and we were concerned that, because different technicians were available at different times, variations in technique might be interpreted as effects of the internal factors. The experiment was set up one group at a time using secondary computer sorts to pool the tube numbers from each artificial block with similar levels of a factor. A technician would then add the factor at the specified level to all the appropriate tubes in that group.

Extraction.

After six months of incubation one set (216 tubes) of the centrifuge tubes was extracted with 10 mL of high purity acetone. The tubes were tightly capped, inverted, and shaken to insure adequate migration of the acetone into the soil. The tubes were then uncapped, placed into an ice bath, and sonicated for 60 seconds at a power load of 35 and duty cycle of 55% with a Branson Model 350 Sonifier (Danbury, CT) fitted with a microtip. After centrifugation the tubes were recapped and centrifuged for 30 minutes at 6500 g with refrigeration at 10°C to 20°C. After centrifugation the supernatant was filtered through a 0.5 um Millex-SR filter unit (Millipore, Bedford, MA) containing a polytetrafluoro-ethylene filter. The filtrate volume was measured and then stored in the dark at -20°C. An aliquot of the final extract was counted for radioactivity in a Packard Model 3255 Tri Carb Liquid Scintillation Counter and another aliquot was analyzed by HPLC. After these series of solvent extractions, the soils were air-dried and then oven-dried at 105°C. Aliquots of the soil samples were then counted for radioactivity.

After 11 months of incubation the second set of 216 tubes was extracted, this time using continuous extraction with acetone in Soxhlet extractors for 12 hours. The extract was filtered through a 0.5 um Miller-SR filter unit containing a polytetrafluoroethylene filter. The volume of extract was measured and then stored in the dark at -20°C. An aliquot of the final extract was counted for radioactivity and another sample analyzed by HPLC. The soil samples were treated as before.

In one experiment, some of the soil samples were first extracted with the acetone as described for Soxhlet extractors, and then successively extracted with toluene and methylene chloride.

High Performance Liquid Chromatography.

Soil extracts were analyzed on a Waters' Associates (Milford, MA) HPLC system equipped with two model 6000A solvent delivery pumps, a model 441 variable wavelength detector set at 229 nm, a model 730 data module, and a model 721 system controller. A radioactivity detector, Flo-One Model HS/F1.3 (Radiomatic, Tampa, FL) was coupled to the HPLC system.

All analyses were performed on a uBondapak C-18 reverse phase stainless steel column, 30 cm by 3.9 mm (Waters' Associates) using a methanol/water pro-

grammed gradient run modified from the previously described method.[23] The gradient was run at a solvent flow-rate of 2.0 mL per minute and the total run time was 30 minutes. The gradient steps were as follows: initial conditions at 40% methanol/water, at 3.5 minutes the methanol concentration was changed to 45% using curve 11, at 14 minutes the methanol concentration was changed to 80.2% using curve 11, at 25 minutes the methanol concentration was changed back to 40% using curve 11, and a five minutes equilibration delay followed this last change.

The radioactivity Flo-One detector utilized a cell volume of 2.5 mL with a total flow-rate of 3.5 mL per minute. The flow from the model 441 HPLC detector was 2.0 mL, and this was split 50% in order to send 1.0 mL per minute into the radioactivity Flo-One detector. Flo Scint II (Radiomatic) scintillation cocktail was pumped into the detector at a flow-rate of 2.5 mL per minute and mixed with the effluent solvent coming from the HPLC on a continuous basis. The background radioactivity for the system was around 55 disintegrations per minute (dpm) and the efficiency of detection was around 60% throughout the gradient run.

Detection of Intermediates.

The detection of potential biotransformation products from TNT was accomplished using the HPLC system and radioactivity detector as described above. The retention times of the various intermediates on the model 441 ultraviolet detector at 229 nm are listed in Table 2. The corresponding retention times in the radioactivity detector are about 1.4 minutes later. The appearance of intermediates in the soil extracts was based on correspondence to retention times of the standards.

TABLE 2. Retention Times of Potential TNT Transformation Products in the Gradient Analysis.

COMPOUND	RETENTION TIME (Minutes)
1. 2,6-diamino-4-nitrotoluene	2.70 - 2.80
2. 2,4-diamino-6-nitrotoluene	3.00 - 3.10
3. 4-hydroxylamino-2,6-dinitrotoluene	10.90 - 11.10
4. 2,4,6-trinitrotoluene	11.30 - 11.40
5. 4-amino-2,6-dinitrotoluene	13.50 - 13.60
6. 2-amino-4,6-dinitrotoluene	13.90 - 14.00
7. 4,4',6,6'-tetranitro-2,2'-azoxytoluene	19.90 - 20.00
8. 2,2',6,6'-tetranitro-4,4'-azoxytoluene	21.00 - 21.10

Computer Analysis.

Analysis of variance was performed on a UNIVAC 1106 mainframe computer.

RESULTS

Block Design.

The initial block design was analyzed for variability to determine if there were any problems with the experimental set-up itself. Analysis of variance (ANOVA) found no significant differences between blocks. This result indicates that the blocks were designed and initiated on an equivalent basis and not biased from time zero.

Analysis of Six-Month Incubations.

The factors or predicator variables were assigned codes as in Table 3.

TABLE 3. Codes Assigned to Predictor Variables

CODE		DESCRIPTION	LEVELS
OM	=	Organic matter	(2 levels; 2.2% and 10.5%)
NT	=	*Concentration of* TNT	(3 levels; 0.1%, 1.0%, 10%)
OX	=	Oxygen	(3 levels; ambient, nitrogen flushed, nitrogen flushed and chemically reduced)
MO	=	Moisture	(3 levels; air dry, 50% field capacity, 100% field capacity)
TE	=	Incubation temperature	(2 levels; 10°C, 20°C)
TI[1]	=	Time of incubation	(3 levels; 6 months, 12 months, 24 months)
OR	-	Organisms	(2 levels; active, sterile)

The six response variables were chosen based on retention time regions on the radioactivity chromatograms, except for extraction efficiency, Table 4.

TABLE 4. Response Variables for Six-Month Incubations.

CODE	DESCRIPTION (retention time)	CORRESPONDING STANDARDS
EF	Extraction efficiency (^{14}C)	---
D (1-3)	percent ^{14}C recovered (1-3 min)	2,6DA; 2,4DA
D (6-8)	percent ^{14}C recovered (6-8 min)	unknown
D (10-12)	percent ^{14}C recovered (10-12 min)	TNT, 2A, 4A, 4OHA
D (14-16)	percent ^{14}C recovered (14-16 min)	4A, 2A
D (20-24)	percent ^{14}C recovered (20-24 min)	2,2'AZ; 4,4'AZ

The data for each of the response variables had to be transformed to a Gaussian distribution in order to run ANOVA. The transformations were as follows:

$$(EF)^1 = \log_{10}(EF)$$
$$[D(10\text{-}12)]^1 = \log_{10}[D(10\text{-}12)]$$
$$[D(1\text{-}3)]^1 = \log_{10}[1 + D(1\text{-}3)]$$
$$[D(6\text{-}8)]^1 = \log_{10}[1 + D(6\text{-}8)]$$
$$[D(14\text{-}16)]^1 = \log_{10}[1 + D(14\text{-}16)]$$
$$[D(20\text{-}24)]^1 = \log_{10}[D(20\text{-}24)]$$

Two types of analysis were performed on the data: (1) ANOVA, omitting all interactions above the first order and (2) ANOVA, omitting the variable with the least effect in (1) and looking at all interactions. Main effects represent the influence of each factor taken separately on the results.

Tables 5 to 10 contain the ANOVA data for the main effect and first order interactions. Table 11 contains a summary of the direction of the main effects for each response variable and each statistically significant predictor variable.

After omitting the variable with the least effect, ANOVA was repeated to look for all interactions, including higher orders. Only a few statistically significant second order interactions and one third order interaction were found. These findings will be used in looking towards the one-year sampling to see if these trends continue.

Extraction and Analysis.

The data on the percent recovery of TNT and potential transformation products from the six-month analysis were confounded by the high loading of the radioactivity and UV detectors during the analysis. This was necessary in order to detect low concentrations of some of the products. In the 11-month

analyses, lower loadings were run to provide better resolution of compounds which elute near each other, as well as still detecting intermediates present at low concentrations. Despite this problem, statistically significant information was generated from the six-month analysis which provided valuable guidance for the follow-on analyses at 11 months.

Six-Month Incubations.

For extraction efficiency of the radioactive materials, the initial concentration of TNT was the factor which had the strongest effect (0.1% and 1.0% were less efficiently recovered than the 10%), followed by moisture content (the dry and 50% moisture tubes were both more efficiently extracted than the 100% tubes). Overall, extraction efficiencies were below the high efficiency expected based on our initial (time-zero) analyses. Continued solvent extraction of the soils has shown that the removal of the radioactivity after six months of incubation is more variable and more difficult. It was determined that Soxhlet extraction was required for the 11-month data.

In determining microbial activity in biotransforming TNT, we have concentrated on the formation of the diamino (2,4-diamino-6-nitrotoluene, and 2,6-diamino-4-nitrotoluene) and azoxy (2,2',6,6'-tetranitro-4,4'-azoxytoluene and 4,4',6,6'-tetranitro-2,2'-azoxytoluene) compounds.

For formation of the diamino compounds, the factors that had the strongest main effects were moisture level and initial concentration of TNT. Moisture content of the soil was the strongest factor (air-dried soil contained the lowest, while the 50% and 100% soils showed progressively higher significant increase in concentrations of the diamino compound). The initial concentration of TNT showed lowest levels in the 10% tubes, higher levels of the diamino compounds in the 1.0% tubes, and statistically higher concentrations of the diaminos in the 0.1% tubes. Lesser effects were found due to the factors of soil organic matter and oxygen levels.

For formation of azoxy derivatives, the initial concentration of TNT had the strongest effect (statistically significant higher percentage of the azoxy compounds formed in the 0.1% and 1.0% tubes than in the 10% tubes). Moisture content was the next strongest factor (greater percentage of azoxy compounds formed in the air-dried and 50% soils than in the 100%). None of the other factors showed statistically significant main effects.

The data on recovery of TNT and the monoaminos (2-amino-4,6-dinitrotoluene and 4-amino-2,6-dinitrotoluene) are difficult to evaluate because of the problem discussed above. However, by choosing the larger retention time peaks in this region, we can assume only the monoamino derivatives are present and use these results as an indicator of activity. This analysis showed soil organic matter as having the strongest main effect (statistically significant higher percent recovery in the 10.5% than the 2.2% soil) followed by the initial concentration of TNT (higher recoveries in the 0.1% and 1.0% TNT tubes, lowest in the 10%), and soil moisture level (higher percent recovered in the 50% and 100% moisture containing tubes compared to the air dried tubes).

TABLE 5. ANOVA Matrix with Response Variable EF (Extraction Efficiency)[a]

	OM	NT	OX	MO	TE	OR
OM	_[b]	-	-	-	-	4 (4.03,0.0463)
NT		2[c] (33.19,0.0000)[d]	-	1 (6.80,0.0000)	-	3 (3.17,0.0443)
OX			-	-	-	-
MO				2 (28.17,0.0000)	-	2 (5.16,0.0067)
TE					2 (19.03,0.0000)	-
OR						4 (10.71,0.0013)

[a]Main effects are along the central diagonal, first-order interactions are outside this diagonal.

[b]Not significant (below 95% confidence limits).

[c]Numbers are used to rank the relative importance of the factors along main effects, and separately for first-order interactions.

[d]F, value, tail probability. 0.05 is the cut-off level for significance at 95% confidence. The lower the tail probability the stronger the effect.

NOTE: See Table 3 for code descriptions.

TABLE 6. ANOVA Matrix with Response Variable D(10-12), Percent Radioactivity Recovered in the 10 to 12 Minute Retention Time.[a]

	OM	NT	OX	MO	TE	OR
OM	_[b]	-	-	5 (4.34,0.0145)	7 (4.67,0.0322)	-
NT		2[c] (59.61,0.0000)[d]	-	2 (12.82,0.0000)	4 (5.63,0.0043)	6 (3.93,0.0215)
OX			5 (4.12,0.0180)	-	-	-
MO				2 (12.15,0.0000)	-	2 (63.45,0.0000)
TE					4 (12.08,0.0007)	2 (73.67,0.0000)
OR						2 (20.47,0.0000)

[a]Main effects are along the central diagonal, first-order interactions are outside this diagonal.

[b]Not significant (below 95% confidence limits).

[c]Numbers are used to rank the relative importance of the factors along main effects, and separately for first-order interactions.

[d]F value, tail probability. 0.05 is the cut-off level for significance at 95% confidence. The lower the tail probability the stronger the effect.

NOTE: See Table 3 for code descriptions.

TABLE 7. ANOVA Matrix with Response Variable D(1-3), Percent Radioactivity Recovered in the 1 to 3 Minute Retention Time.[a]

	OM	NT	OX	MO	TE	OR
OM	3[b] (4.99,0.0276)[c]	_[d]	-	2 (5.01,0.0083)	-	-
NT		1.5 (70.48,0.0000)	-	-	-	-
OX			4 (3.31,0.0404)	-	-	-
MO				1.5 (121.06,0.0000)	-	1 (8.97,0.0002)
TE					-	-
OR						-

[a]Main effects are along the central diagonal, first-order interactions are outside this diagonal.

[b]Numbers are used to rank the relative importance of the factors along main effects, and separately for first-order interactions.

[c]F value, tail probability. 0.05 is the cut-off level for significance at 95% confidence. The lower the tail probability the stronger the effect.

[d]Not significant (below 95% confidence limits).

NOTE: See Table 3 for code descriptions.

TABLE 8. ANOVA Matrix with Response Variable D(6-8), Percent Radioactivity Recovered in the 6 to 8 Minute Retention Time.[a]

	OM	NT	OX	MO	TE	OR
OM	2[b] (36.26,0.0000)[c]	3 (4.08,0.0188)	-[d]	-	-	-
NT		2 (23.48,0.0000)	-	-	-	-
OX			-	-	-	-
MO				2 (10.64,0.0000)	-	2 (5.31,0.0059)
TE					5 (4.61,0.0335)	1 (7.94,0.0055)
OR						4 (6.77,0.0102)

[a]Main effects are along the central diagonal, first order interactions are outside this diagonal.

[b]Numbers are used to rank the relative importance of the factors along main effects, and separately for first-order interactions.

[c]F value, tail probability. 0.05 is the cut-off level for significance at 95% confidence. The lower the tail probability the stronger the effect.

[d]Not significant (below 95% confidence limits).

NOTE: See Table 3 for code descriptions.

TABLE 9. ANOVA Matrix with Response Variable D(14-16), Percent Radioactivity Recovered in the 14 to 16 Minute Retention Time.[a]

	OM	NT	OX	MO	TE	OR
OM	1[b] (18.33,0.0000)[c]	_[d]	-	-	-	-
NT		2.5 (9.08,0.0002)	-	2 (4.26,0.0031)	-	-
OX			4 (4.65,0.0116)	-	-	-
MO				2.5 (9.02,0.0002)	-	1 (11.17,0.0000)
TE					-	-
OR						5 (5.81,0.0176)

[a]Main effects are along the central diagonal, first-order interactions are outside this diagonal.

[b]Number are used to rank the relative importance of the factors along main effects, and separately for first-order interactions.

[c]F value, tail probability. 0.05 is the cut-off level for significance at 95% confidence. The lower the tail probability the stronger the effect.

[d]Not significant (below 95% confidence limits).

NOTE: See Table 3 for code descriptions.

TABLE 10. ANOVA Matrix with Response Variable D(20-24), Percent Radioactivity Recovered in the 20 to 24 Minute Retention Time.[a]

	OM	NT	OX	MO	TE	OR
OM	_[b]	-	-	-	4 (5.63,0.0187)	-
NT		1[c] (6.36,0.0022)[d]	6 (2.47,0.0464)	3 (3.79,0.0056)	-	5 (4.06,0.0189)
OX			-	-	-	-
MO				2 (4.05,0.0191)	-	1.5 (10.93,0.000)
TE					-	1.5 (17.85,0.000)
OR						-

[a]Main effects are along the central diagonal, first-order interactions are outside this diagonal.

[b]Not significant (below 95% confidence limits).

[c]Numbers are used to rank the relative importance of the factors along main effects, and separately for first-order interactions.

[d]F value, tail probability. 0.05 is the cut-off level for significance at 95% confidence. The lower the tail probability the stronger the effect.

NOTE: See Table 3 for code descriptions.

TABLE 11. Summary of Main Effects for Statistically Significant Results.

RESPONSE VARIABLE See table 4	VARIABLE (Weight) See table 3	DESCRIPTION
EF	NT (2)	(0.1%-1.0%)<10%
	MO (2)	100%<(50% - 0%)
	TE (2)	20°C<10°C
	OR (4)	Sterile<active
D(10-12)	NT (2)	10%<1.0%<0.1%
	MO (2)	(100%-50%)<0%
	OR (2)	Sterile<active
	TE (4)	20°C<10°C
	OX (5)	Chem. red.<N_2<air
D(1-3)	MO (1.5)	0%<50%<100%
	NT (1.5)	10%<1.0%<0.1%
	OM (3)	10.5%<2.2%
	OX (4)	Air<N_2<chem. red.
D(6-8)	OM (2)	10.5%<2.2%
	NT (2)	10%<(1.0%-0.1%)
	MO (2)	(100%-50%)<0%
	OR (4)	Active<sterile
	TE (5)	10°C<20°C
D(14-16)	OM (1)	2.2%<10.5%
	NT (2.5)	10%<(1.0%-0.1%)
	MO (2.5)	0%<(50%-100%)
	OX (4)	Air<(N_2-chem. red.)
	OR (5)	Active<sterile
D(20-24)	NT (1)	10%<(1.0%-0.1%)
	MO (2)	100%<(50%-0%)

Analysis of 11-Month Incubations.

The predictor variables and the codes assigned are the same as described in Table 3. The response variables for the 11-month extractions were revised from those presented in Table 4 to include those presented in Table 12. As before, the response variables D through J were based on retention time regions on the radioactivity chromatograms.

TABLE 12. Response Variables for 11-Month Incubations

CODE	DESCRIPTION (Retention Time)	CORRESPONDING STANDARDS
A	Percent ^{14}C recovery in soil	-----
B	Extraction efficiency (^{14}C)	-----
C	Percent ^{14}C recovered (0-2 min)	2,4DA, 2,6DA
D	Percent ^{14}C recovered (2-5 min)	2,4DA, 2,6DA
E	Percent ^{14}C recovered (5-7 min)	unknown
F	Percent ^{14}C recovered (7-9 min)	unknown
G	Percent ^{14}C recovered (9-11 min)	TNT, 4OHA
H	Percent ^{14}C recovered (11-14 min)	4A, 2A
I	Percent ^{14}C recovered (14-18 min)	unknown
J	Percent ^{14}C recovered (18-23 min)	2,2'AZ, 4,4'AZ

As before, the data had to be transformed to a Gaussian distribution required for ANOVA. The transformations for the response variables are listed in Table 13. The transformed data were analyzed by ANOVA for main effects and first-order interactions, and the statistically significant results are presented in Table 14. Main effects are along the central diagonal boxes.

Table 13. Transformations of Response Variables for the 11-Month Incubations

RESPONSE VARIABLE See table 12	CONDITION OF DATA	TRANSFORMATION
A	long-tailed on right	$X_A=\log_{10}(A)$
B	good	none required
C[a,b]	49 non-zero points	$X_C=\log_{10}(\log_{10}C)$
D[a,b]	29 non-zero points	$X_D=\log_{10}(D)$
E[a,c]	49 non-zero points	$X_E=\log_{10}(E)$
F[a,b]	27 non-zero points	$X_F=3-\log_{10}(1000-F)$
G[a]	192 non-zero points	$X_G=3-\log_{10}(1002-G)$
H[a]	99 non-zero points	$X_H=1-H^{-1/4}$
I[a,b]	13 non-zero points	none used
J[a]	113 non-zero points	$X_j=\log_{10}(J)$

[a]Only non-zero values were analyzed.

[b]Only main effects could be found.

[c]Bimodal distribution, main effects only.

TABLE 14. Summary of ANOVA Results for the 11-Month Incubations.[a,b,c]

	OM	NT	OX	MO	TE	OR
OM (2)	A3					
NT (3)	A8 H1	A1 F2 B1 G2 C1 H1				
OX (3)	A4		G4			
MO (3)	A3	A7 G3 B2		A4 H3 D1		
TE (2)		G4	A6		A2 F1 B3 G3	
OR (2)	A5	A1 G2 B1		A2 G1 J1		A5 G1 B2 H2

[a]Entries in boxes depict main effects; others are first-order interactions.

[b]The entry A3 in row MO and column OM means that the data for response variable A had an MO X OM interaction, which was the third strongest interaction for A statistically.

[c]The entries A4, D1, H3 in the box for row MO and column MO are main effects, which represent that MO had the 4th strongest main effect on A, the strongest main effect on D, and the 3rd strongest main effect on H.

NOTE: See Table 3 and Table 12 for code descriptions.

In terms of procedure, a main problem with data sets (C through J) was the large number of zeros (below detection), making it difficult to normalize the data for ANOVA. Therefore the zeros had to be discarded, thus treating them as missing data.

The ANOVAs (Table 14) showed that the initial TNT concentration had the greatest effect, and organic matter and oxygen the least on rates of transformation, but all variables had some effect. The response variables A, B and G were the most often affected, while E, I and J were the least influenced by the test variables. Response variable A exhibited main effects from all the test variables except oxygen.

In some cases, C through F and I, insufficient data remained after discarding zeros to perform ANOVA with interactions (see Table 13). Therefore, ANOVA was carried out only for main effects in such instances. Variable E was bimodal due to five high data points. Variable I, because of the limited number of data points, could be evaluated only for main effects.

Table 15 contains a summary of the directions of the main effects for the individual response variables. For example, for concentration of TNT in all cases where all three initial concentrations (0.1%, 1.0% and 10.0%) were statistically different from each other, a higher percent was unextracted from the soil at 0.1% than at 1.0% and then 10.0%. Therefore, as would be expected, the reverse effect was found for extraction efficiency. For response variable G (TNT recovery), a higher amount of TNT was detected from the 10% tubes than the 1.0% and then the 0.1% tubes, while the inverse occurred with response variable H (2A and 4A). This result indicates that in the incubations with the lower initial concentration of TNT, a greater percent was transformed to the monoamino derivatives, while as the initial concentration of TNT increased, this transformation was adversely affected.

The data presented in Table 16 represent the means for the results for the various response variables for statistically significant values. The directional trends noted in Table 15 are assigned actual values in Table 16. For example, TNT recovery from the incubations after 11 months (response variable G) is shown to be higher in tubes originated with 10% TNT and lowest in tubes originated with 0.1%. As would be expected, a high amount of the monoamino derivatives (H) are found in the 0.1% tubes as well as diamino derivatives (C). The most biotransformation activity occurred in the 0.1% TNT tubes and the higher percentage of ^{14}C "bound" to the soil (response variable A) is located in these incubations as well. Lowest recoveries in the solvent extract were also with the 0.1% TNT incubations.

Figure 1 illustrates some of the first-order interactions. The interaction of TNT concentration with organic matter on recovery of ^{14}C in response variable H is presented. This illustrates that at the low initial concentration of TNT (0.1%), a high percent organic matter (10.5%) promotes high percent recovery in H, while at the highest concentration of TNT (10.0%) the reverse is true. The same sorts of

TABLE 15. Details of Main Effects from 11-Month Incubations.[a,b,c]

RESPONSE VARIABLES	OM (2)	NT (3)	OX (3)	MO (3)	TE (2)	OR (2)
A	2 1	1 2 3		1 2,3	2 1	2 1
B		3 2 1			1 2	1 2
C		1 2,3				
D				3 1,2		
E		no main effects				
F		3 1,2			2 1	
G		3 2 1	1 2 3		2 1	1 2
H		1 2 3		2 3,1		2 1
I		no main effects				
J		no main effects				

[a]Statistically significant differences; some control variables were only tested at two levels.

[b]For example, for response variable A, NT=1 gave the highest value, NT=3 the lowest, and NT=2 intermediate.

[c]For example, for response variable D, MO=3 gave higher values than MO=1 or MO=2, which were not significantly different.

NOTE: See Table 3 and Table 12 for code descriptions.

TABLE 16. Means of Re-Transformed Variables with Statistical Significance from 11-Month Incubations[a]

RESPONSE VARIABLES
See Table 12

CONTROL VARIABLE See Table 3	LEVEL	A	B	C	D	E	F	G	H	I	J
OM	1	38.7	-	-	-	-	-	-	-	-	-
	2	55.6									
NT	1	90.8	489.4	26.3	-	-	838.7	900.7	73.6	-	-
	2	46.2	699.0	13.8			844.0	962.9	21.0		
	3	24.3	1391.9	8.1[b]			972.4	987.6	10.4		
OX	1	-	-	-	-	-	-	972.0	-	-	-
	2							966.5			
	3							950.5			
MO	1	59.8	-	-	13.2	-	-	-	18.8	-	-
	2	41.9			19.1				46.9		
	3	39.9			55.1				31.7		
TE	1	34.4	921.8	-	-	-	921.5	941.9	-	-	-
	2	63.2	798.3				-[b]	976.3			
OR	1	43.6	949.9	-	-	-	-	977.4	15.9	-	-
	2	49.7	770.2					939.5	43.9		

[a]Note that values are re-transformations back to original data of the means for the transformed data. They are not the means of the original values.

[b]Based on very few data values.

extrapolations can be made with the Figure's second graph (right) shown for the interaction of organisms with moisture on response variable J.

DISCUSSION

Six-Month Incubations.

Some conclusions could be drawn from the statistical analysis of the six-month data. TNT was definitely transformed in the soil and it appeared that the initial concentration of TNT and the moisture level were the most critical factors of those evaluated in determining the rate of transformation. In addition, the difficulties in extraction efficiency and loading rates on the HPLC were corrected for the 11-month extracts.

Soil Recovery - Extraction Efficiency.

A separate study was run to evaluate the efficiency of Soxhlet extraction with acetone for removal of ^{14}C from the soil after 11 months of incubation. A series of solvents: acetone, toluene and methylene chloride, were used to continuously extract ^{14}C-labelled material from some of the incubation tubes (two each with 0.1%, 1.0%, and 10.0% TNT). Percent recoveries indicated that after the acetone extraction, on a percentage basis of the total ^{14}C, 0.29 $\pm$ 0.20% and 0.66 $\pm$ 0.35% was recovered in the toluene and methylene chloride extracts, respectively. These results indicate that the acetone extraction procedure did an efficient job of removing almost all of the extractable ^{14}C-labelled material that was *not "bound" to the soil*.

Eleven-Month Incubations.

Soxhlet extractions overcame the poor recovery found with the six-month analysis. Extraction efficiencies were most affected by the initial concentration of TNT, as had been found in the six-month analysis (0.1% was less efficiently extracted than 1.0% and then 10.0%). Sterility and incubation temperatures had lesser effects on extraction efficiency and the remaining predictor variables had no significant effects on recovery.

The initial concentration of TNT also had an effect on ^{14}C remaining in the soil (0.1% TNT containing soils retained the most, while 1.0% and 10.0% TNT containing soils retained sucessively less).

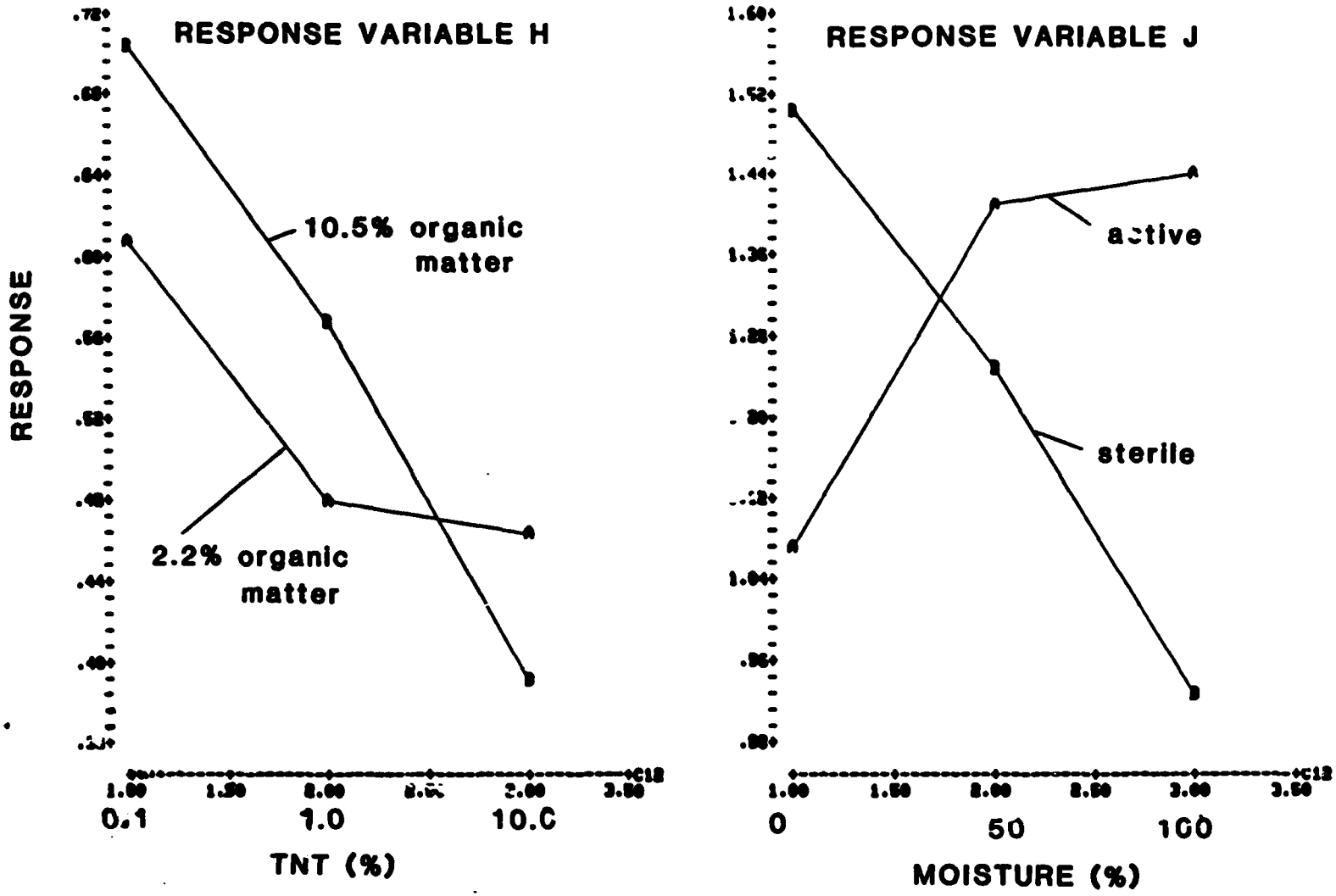

Figure 1. Illustrations for first-order interactions. A = TNT and organic matter on recovery data (H). (left graph), and activity and moisture on recovery data (J) (right graph).

The transformation of TNT in soil was such that higher amounts of biotransformation products were recovered in those incubations initiated with lower concentrations of TNT (0.1%). However, even with 10% TNT there was evidence of some biotransformation. This may be explained by the fact that although 10% TNT was present, TNT has limited solubility in water and therefore only approximately 150 mg/L would actually be in solution at any one time. However, some inhibition is expressed at these higher concentrations as reflected by the decrease in activity.

It is also clear that as biotransformation activity increased the amount of material unextractable or "bound" to the soil residue increased. This supports the notion that biotransformation products of TNT are more reactive with soil fractions than TNT itself.

The statistically significant main effects and first order interactions resulting from the 11-month incubations can be summarized as follows (referencing environmental factor codes from Table 1, response variable codes from Table 12, main effects from Tables 14, 15 and 16, and first order interactions from Table 14):

For main effects: (1) extraction efficiency (response variable B) - the concentration of TNT (NT) had the strongest effect on this response variable with greatest extraction efficiency from the 10% TNT tubes and progressively less with the 1.0% and 0.1% tubes. The presence of organisms (OR) had the next strongest effect on extraction efficiency with greater recovery from sterile than active tubes. Temperature (TE) had the third strongest effect on this response variable with greater extraction efficiency from tubes maintained at room temperature than at 10°C.

(2) Material bound or unextractable from the soil (response variable A) - The concentration of TNT (NT) had the strongest effect on this response variable with greatest material bound in the 0.1% TNT tubes and progressively less in the 1% and 10% TNT tubes. Temperature (TE) had the next strongest effect with greatest material bound at 10°C than at room temperature. Percent organic matter (OM) had the third strongest effect with a greater amount bound in the 10.5% tubes than the 2.2% tubes. Moisture (MO) had the fourth strongest effect on this response variable and organisms (OR) the fifth strongest effect.

(3) TNT recovery (response variable G) - The presence of organisms (OR) was the most significant factor, with greater recovery of TNT in the sterile vs. the active systems. The initial concentration of TNT (NT) was the second most significant factor, with greatest recovery in the 10% TNT tubes and successively less in the 1% and 0.1% TNT tubes. Temperature (TE) had the third strongest effect on this response variable with greater recovery of TNT from the tubes maintained at 10°C than at room temperature. Oxygen had the fourth strongest effect.

(4) Monoamines (response variable H) - The concentration of TNT (NT) had the greatest effect on the formation of monoamines, with greatest production of monoamines in the 0.1%, than the 1% and 10% TNT tubes. Organisms had the second strongest effect with greater formation of monoamines in the active

than sterile systems. Moisture had the third strongest effect with greatest formation at 50% than at 0 or 100%.

(5) Diaminos (response variables C and D) - The concentration of TNT (NT) had the greatest effect on the production of the diaminos, with the highest levels produced in the 0.1% tubes and lesser amounts in the 1% and 10% TNT tubes. Moisture (MO) had the second strongest effect, with greatest production of diaminos in the 100% tubes and less in the 50% and dry tubes.

For the first order interactions: (1) extraction efficiency (response variable B) - The strongest interaction was between TNT concentration (NT) and the presence or absence of organisms (OR). The second strongest interaction was between the concentration of TNT (NT) and moisture (MO).

(2) Bound material (response variable A) - The strongest interaction was between the concentration of TNT (NT) and the presence or absence of organisms. The second strongest interaction was between moisture (MO) and organic matter (OM).

(3) TNT recovery (response variable G) - The strongest interaction was between moisture (MO) and organisms (OR), the second strongest was between TNT concentration (NT) and organisms (OR), and the third strongest was between TNT concentration (NT) and moisture (MO).

CONCLUSIONS

Soxhlet extraction with acetone was found to remove efficiently TNT and its transformation products from soil. HPLC-coupled radioactivity detection was found effective for quantifying the various transformation products from complex soil extracts. Of the environmental factors evaluated, the initial concentration of TNT had the greatest overall effect on the rates of transformation. Activity was greatest at the 0.1% level and progressively lower at 1.0% and then 10.0% TNT level. The production of monoamines (2-amino-4,6-dinitrotoluene and 4-amino-2,6-dinitrotoluene) and diamines was greatest in incubations with the lowest initial concentration of TNT. In terms of main effects, after initial TNT concentration, the presence or absence of organisms and the incubation temperature had the greatest effects on the response variables evaluated. Moisture level was less important and the percent organic matter and the oxygen level were insignificant in terms of the environmental factors studied. First-order interactions between some of the environmental factors were also identified.

The initial concentration of TNT also had the greatest effect on extraction efficiency, with highest recoveries of ^{14}C-labelled material in the tubes with the highest initial concentration of TNT. Conversely, the highest percent unextractable or bound material resided in tubes with the lowest initial concentration of TNT, where microbial activity was also highest.

These results, when extrapolated to potential scenarios such as lagoon entombments, would indicate that the more concentrated the TNT, the fewer the active organisms, the colder the temperature, and the dryer the soil the longer the TNT will remain in an unaltered state.

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