IMPACT OF A MODEL SOIL ON THE BIOTRANSFORMATION OF

2,4,6-TRINITROTOLUENE AND ITS AMINE METABOLITES

by

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Diane Kathryn Walker

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ABSTRACT

The end of the Cold War resulted in the closure of many sites where explosives were manufactured, processed, and stored, and packaging practices left behind highly contaminated surface waters, groundwater and soils. Chief among the explosives contaminating these sites is the xenobiotic, 2,4,6-trinitrotoluene (TNT) whose electron-withdrawing nitro-groups make this aromatic compound highly resistant to biodegradation. An alternative option to mineralization as a bioremediation strategy, however, is immobilization. TNT can be biotransformed under reducing conditions to 2,4,6-triaminotoluene (TAT), a compound that researchers are currently investigating due to its potential to become irreversibly bound to soil components. The objectives of this research were to conduct TNT biotransformation studies under planktonic conditions and compare the results to those under slurry conditions. These objectives would also contribute to the overall goals of a DEPSCoR-sponsored project entitled "Biofilm-Induced Changes in Soil Organic Matter Structure and the Resulting Impact on the Bioavailability of Sorbed 2,4,6-Trinitrotoluene and its Amine Metabolites".

Three experiments were performed for this research. The first was to create a model soil that was both well-characterized yet chemically representative of real soil constituents. The second was to add TNT to actively growing *Desulfovibrio* sp. strain SHV and monitor TAT formation. The third was a combination of the first two experiments, adding strain SHV to the model soil to both observe TAT formation and its disappearance from solution over time.

The results of the TNT biotransformation studies indicated that TNT was transformed to TAT, which became the dominant metabolite in four weeks under planktonic conditions. Under slurry conditions, TAT became the dominant metabolite in two days and disappeared from solution by day three.

INTRODUCTION

Background

The research described in this thesis was designed to meet a portion of a project sponsored by the Defense of Defense Experimental Program to Stimulate Competitive Research (DEPSCoR) undertaken by the Center for Biofilm Engineering to study the influence of biofilm-induced changes in soil organic matter structure on the bioavailability of 2,4,6-trinitrotoluene and its amine metabolites. Fundamental among the many interfacial processes in the soil environment is the activity of microbial biofilms localized at the soil-water interface. Since biofilms can change the chemical microenvironment at this interface, they may, in turn, influence the bioavailability of organic chemicals in the soil organic matter (SOM) matrix (Lewandowski 1997). The goal of the DEPSCoR-sponsored project was to investigate whether changes in solution chemistries (i.e., ionic strength, divalent cations, and biological surface-active compounds) change the structure of soil organic matter and, in turn, influence the bioavailability of SOM-bound nitro/amino-aromatic (NAT) compounds (i.e., 2,4,6-trinitrotoluene, 2,6-diaminotitrotoluene, and 2,4,6-triaminotoluene). The results could then be compared to results obtained using a biofilm system to infer that chemical changes at the soil-water interface resulting from biofilm activity influence the bioavailability of SOM-associated NATs via induced changes of SOM structure. One phase of this project was to conduct TNT biotransformation

experiments under planktonic conditions and compare the results to the biotransformation of TNT in a slurry system after creating a model soil. The impact of the model soil on TNT biotransformation was the goal for this thesis.

TNT Contamination

2,4,6-Trinitrotoluene (TNT) is a xenobiotic compound that contaminates many military sites in both the U.S. and Europe, arising from munitions manufacturing, handling, and disposal operations dating back to World War I (Spain 2000 and Reiger 1995). Figures 1 and 2 show primary military sites where explosives were manufactured. Process wastewater was often discharged on the ground or in lagoons, which then leached explosives into soils and groundwater (Boopathy 1998). Of all the nitroaromatic compounds (NACs) that have been found in both soils and sediments, TNT is the most abundant, with concentrations as high as 60% by weight (Elovitz 1999). Human exposure to TNT occurs through eating, drinking, touching, or inhaling contaminated soil, water, food, or air. Acute health effects reported in people exposed to TNT include anemia, abnormal liver function; and, after long-term exposure (>365 days), skin irritation and cataracts may develop. Studies conducted with laboratory rats ingesting TNT for long periods found that the rats developed tumors of the urinary bladder, a finding which led the Environmental Protection Agency (EPA) to list TNT as a possible human carcinogen. This substance has

been found in at least 20 of the 1,430 EPA National Priorities List sites and

represents a significant remediation challenge.

Figure 1. Explosive-contaminated manufacturing, processing, and storage
sites in the U.S. Army Ammunition Plant (AAP); Army Depot (AD); Air Force
Base (AFB); Naval Submarine Base (NSB); Ordnance Works (OW); and Proving
Ground (PG). (Adapted from U.S. Army Environmental Center, 1996).
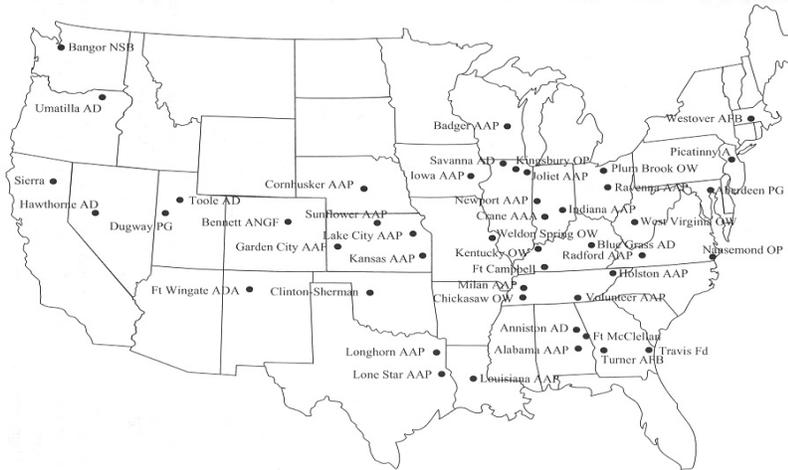
Figure 2. Explosives-contaminated manufacturing, processing, and
storage sites in the Federal Republic of Germany. Sites shown processed more
than 1700 tons of explosives per month. Numbers in parentheses are tons of
TNT per month. (Data from Pruess, 1987).

In the 1970's, several reports were published regarding the toxicity and mutagenicity of TNT. Won (1976) investigated the toxicity of TNT after finding what they referred to as pseudomonad-like organisms capable of transforming but not completely mineralizing the compound.   They found that TNT in concentrations as low as 10 mg/L were toxic to fresh water algae, tide pool copepods and oyster larvae, and mutagenic to *Salmonella typhimurium*. Smock (1976) found that TNT in concentrations higher than 2 mg/L was lethal to fathead minnows within a four-day period. Liu (1992) determined that nitroaromatics with strong electron withdrawing groups, such as 1,3-dinitrobenzene and TNT, are metabolized by rat erythrocytes, with up to 40% of these compounds becoming covalently bound to erythrocytic macromolecules.

Although numerous studies, as demonstrated in the above examples, were performed to test the toxicity and mutagenicity of TNT and its metabolites to mammals, fish and plants, it is also important to understand the effects this contaminant might have on populations perhaps experiencing its longest exposure – soil microorganisms. Klausmeier (1974) studied the effect of TNT on microorganisms and found that most fungi, yeasts, actinomycetes, and Gram-positive bacteria were able to grow at TNT concentrations less that 20 mg/L, but that growth was inhibited by concentrations of TNT greater that 50 mg/L. Gong (1999) investigated microbial activity in the laboratory using TNT-spiked garden soils of two contrasting textures (sandy loam vs. clayey loam) and compared the results with field soils collected at a TNT manufacturing facility.   It was

determined that toxicity was comparable in both systems. Table 1 below summarizes these TNT toxicity findings.

Table 1. TNT concentrations reported to negatively affect organisms.

Research Group	LOEC[1] TNT Concentration	Organism	Result
Klausmeier et al. 1974	50 mg/L	fungi	growth inhibited
		yeast	growth inhibited
		actinomyctes	growth inhibited
		most Gram-positive bacteria	growth inhibited
Won et al. 1976	10 mg/L	*Salmonella typhimurium*	mutagenic
		pseudomonad-like organisms	toxic
		fresh water algae	toxic
		tide pool copepods	toxic
		oyster larvae	toxic
Smock et al. 1976	2 mg/L	fathead minnow	lethal
Liu et al. 1992	%[2]	laboratory rats	carcinogenic
Gong et al. 1999	40 mg/L	soil microorganisms from samples taken at abandoned TNT manufacturing facility	IC50[3]

[1]LOEC = lowest observable effect concentration
[2]Up to 40% becomes covalently bound to rat erythrocytes macromolecules
[3]IC50 = 50% inhibition concentration

Although TNT has been shown to negatively affect numerous organisms, the resulting amine transformation products (i.e., aminodinitrotoluenes (ADNT) and diaminonitrotoluenes (DANT)) can be highly reactive and may constitute an even greater environmental concern than TNT itself (Gong 1999). Therefore, the ultimate environmental fate of TNT in both natural and engineered systems depends not only on the behavior of TNT, but also on the fate of the reduced aromatic amines.

Role of Soil Organic Matter

Past research devoted to the biotransformation of TNT has shown that it is readily degraded under reducing conditions (Preuss 1995), while the reduced amine products become more readily sorbed to organic matter in the soil with increasingly reduced forms.   Thus, soil organic matter (SOM) has a significant impact on the sorption and subsequent bioavailability of TNT and its amine metabolites. Murphy (1994) states that the specific structure of humic substances is affected by SOM density and soil water properties such as pH, ionic strength, and divalent cation concentration. The structure of the humic substances in solution may or may not be maintained when sorbed to the solid mineral phase. That is, the type and/or degree of binding (i.e., specific point attachments) may vary and affect the physical configuration of humic acids, thus modifying surface sorptive properties.

SOM configuration and the properties of TNT and its metabolites interact to influence the bioavailability of these sorbed compounds. Hydrophobic organic contaminants (HOCs), including TNT and its amine metabolites, often exhibit limited bioavailability and, thus, can persist in the subsurface for extended periods of time. SOM is one of the primary adsorbents for synthetic organic chemicals in the environment; the most studied SOM constituents are the humic and fulvic acids, which can be isolated by simple differential extraction techniques. Humic acids (HA) exist as variably coiled organic macromolecules that primarily exhibit carboxylic, phenolic, hydroxyl, and carbonyl functional

groups. HOCs (e.g., nitroaromatics) bind to this organic fraction of the soil primarily through covalent and ion binding mechanisms (Schwarzenbach 1993). However, nitroaromatics also exhibit an adsorption affinity to clays and iron oxides through dipole-dipole interactions, London-van der Waals forces, hydrogen bonding, and other intermolecular interaction mechanisms (Schwarzenbach 1993). Haderlein (1993) describes the interactions of nitroaromatic compounds with minerals as a result of hydrophobic interactions or by the formation of coplanar electron-acceptor complexes. Consequently, several authors have investigated the mechanisms of interaction between TNT and its degradation products with the soil and biomass (Lenke 1998, Achtnich 1999, Drzyzga 1999 and Knicker 1999). Mechanisms leading to irreversible binding and enhanced long-term stability of the resulting bound complexes in soil are presently receiving considerable attention, as knowledge about such mechanisms could be used to enhance natural attenuation strategies.

The chemical characteristics of the aqueous environment thus impact the structure of SOM, which subsequently may affect the sorption characteristics of many organic contaminants (Gauthier 1987 and Murphy 1994). It is important to realize that both the extent and the type of distribution of a contaminant between aqueous and solid phase(s) may determine its *availability* as well as its *reactivity* in abiotic and microbial transformations.

TNT Transformation Pathways

Microbial metabolism of aromatic compounds often involves an initial oxidation step, which eventually leads to cleavage of the aromatic ring and complete oxidation. However, TNT is usually considered resistant to oxidation by oxygenase enzymes of aerobic bacteria due to the presence of the electron-withdrawing nitro groups on the aromatic ring (Schwarzenbach 2000). Thus, reductive transformation of TNT to aromatic amines has been the strategy of most remediation schemes.

TNT Transformation under Aerobic Conditions

Most reported experiments performed under aerobic conditions have found little or insignificant mineralization of TNT. Those reports of complete aerobic degradation of TNT include Duque (1993), who worked with a recombinant pseudomonad constructed from a TNT-contaminated soil isolate. In addition, Hawari (1999) found that TNT was mineralized by lignin peridoxidase produced by the white-rot bacidiomycete, *Phanerochaete chrysosporium*, a common soil fungus. However, the majority of reported aerobic transformations seem to be cometabolic reactions leading to incomplete biotransformation and accumulation of metabolic products such as ADNT and DANT.

TNT Transformation under Anaerobic Conditions

Research has shown that TNT is readily reduced abiotically, to ADNT and DANT (Barrows 1996 and Brannon 1998). However, in the presence of certain anaerobes, complete biotic reduction of TNT to 2,4,6-triaminotoluene (TAT) has been observed (Pennington 1995, Rieger 1995, Daun 1998, and Lenke 1998). A commonly depicted pathway of the reduction of TNT is represented by Figure 3.

Figure 3. Complete reduction of TNT and intermediates (main degradative route shown with solid arrows).
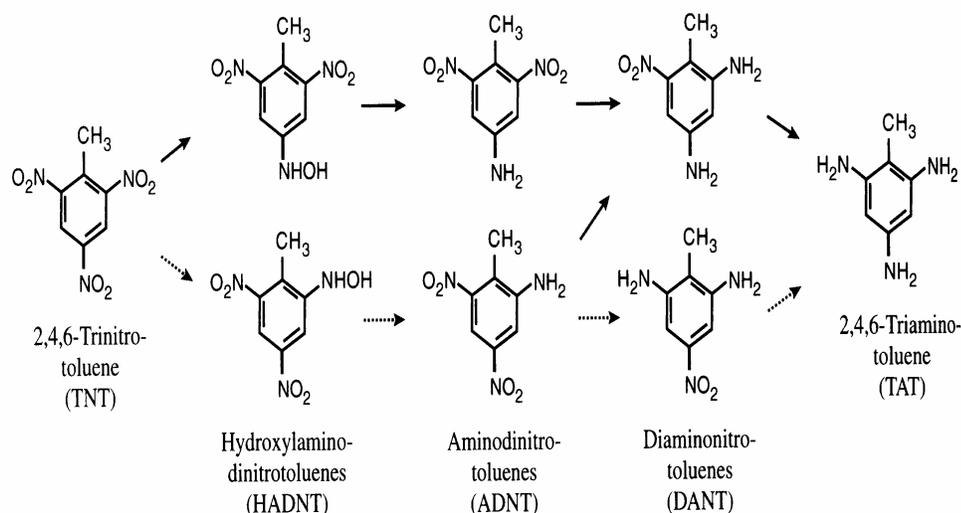
Adapted from Lenke (2000).

The initial putative reductive reaction is postulated for electron-deficient xenobiotics, particularly in anoxic systems which harbor an impressive array of microbial species capable of reductive biotransformations. Only under anaerobic conditions has the complete reduction of TNT to TAT been observed. Importantly, however, TNT does not serve as a growth substrate for

microorganisms (Spain 1995); therefore, the biotransformation mechanism is thought to be cometabolic in nature. Due to the occurrence of reducing microbial species in anoxic systems, the anaerobic biotransformation of TNT to TAT by methanogenic, nitrate-reducing, and sulfate-reducing bacteria has been investigated by several research groups. Findings suggest that intermediate compounds as well as end products in the TNT reduction pathway vary depending upon bacterial utilization of the energy sources and electron acceptors available in a given system. Sulfate-reducing bacteria (SRB) are reported to be the most successful at complete reduction of TNT to TAT. Pruess (1993) observed TAT formation through the intermediate 2,4-diamino-6-hydroxylaminotoluene (DAHAT) when pyruvate and sulfate were provided to a consortium of sulfate-reducing bacteria. They also determined that dissimilatory sulfite reductase (also referred to as desulfoviridin) was responsible for the reduction of DAHAT to TAT. Boopathy (1993) also used pyruvate as the electron donor and sulfate as the electron acceptor and observed the formation of ADNT, DANT, TAT, and ultimately, toluene by *Desulfovibrio* sp. (B strain). Similarly, Drzyzga (1998), using lactate as the electron donor instead of pyruvate, also identified desulfoviridin as the catalyst in TAT formation.

SRB are considered to be among the oldest living organisms on Earth (Postgate 1984). These microbes are ubiquitous in anoxic marine and terrestrial environments and contribute significantly to the global sulfur cycle. SRB are often the primary organisms responsible for carbon mobilization in non-polluted

ecosystems; and in polluted environments, SRB often become dominant as oxygen is depleted by aerobes and facultatively aerobic, fermenting organisms (Singleton, 1993).

Although all SRB are unified by a common metabolic ability, they are in fact a very diverse group of organisms. As SRB reduce sulfate, hydrogen sulfide is produced at concentrations favorable for the rapid transformation of TNT. SRB also produce desulfoviridin, the enzyme that has been determined to mediate the final step (DANT to TAT) in the nitro reduction process of TNT. Of this very diverse group of microbes, *Desulfovibrio* sp. have most often been utilized in TNT biotransformation studies. The research conducted for this thesis followed the protocols developed by Drzyzga (1998) during their studies with *Desulfovibrio* sp. strain SHV for planktonic degradation of TNT to TAT.

TNT Bioremediation

Incineration is the most effective and widely used remediation technology implemented for TNT-contaminated soil in the U.S. and abroad (Spain 2000); but it is expensive due to the high costs of soil excavation, transportation, and energy inputs. Increased public awareness of the toxicity and risk associated with TNT, in addition to the high cost for incineration, encouraged researchers to develop cost-effective TNT bioremediation technologies. Currently, no *in situ* microbially-based technologies achieve complete mineralization of TNT. Similarly, *ex situ* studies involving bioremediation technologies for explosives, such as composting

(Williams 1992, Griest 1993 and 1995, Jarvis 1998, Bruns-Nagel 2000), bioslurry reactors (Harvey 1997, Boopathy 1998c, Lenke 1998, Guiot 2000), and soil-flooded biopiles (Greer 1997), report the disappearance of the compound to undetectable levels, yet little or no mineralization is observed. With further research into TNT transformation, a bioremediation strategy can perhaps be developed to enhance the natural attenuation of TNT, either by complete mineralization to CO_2, by irreversible sorption to soil components, or by conversion to non-toxic, aqueous phase products.

Research Plans

This project described in this thesis was designed to meet a portion of a DEPSCoR-sponsored project undertaken by the Center for Biofilm Engineering to study the influence of biofilm-induced changes in soil organic matter structure on the bioavailability of 2,4,6-trinitrotoluene and its amine metabolites. For this thesis, planktonic studies were performed to measure TNT biotransformation and these results were then compared to TNT biotransformation in a slurry system containing a model soil. This research has provided important information to the overall DEPSCoR project by developing a model soil and reactor systems for potential use in future biofilm studies.

Initially, a microorganism was needed that was capable of degrading TNT to the metabolites of interest (i.e., ADNT, DANT and TAT). These bacteria should have the enzymes required to reduce TNT to TAT; they should be able to

do so within a reasonably short time, should be available for purchase and should have been previously characterized. *Desulfovibrio* sp. strain SHV was chosen for its ability to reduce TNT to TAT through the ADNT and DANT intermediates set forth by the DEPSCoR project. It had been shown to do so within a week under batch conditions, and had been previously characterized and made available by Drzyzga (1998) through the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany.

A series of planktonic experiments were performed to verify the biotransformation of TNT observed by Drzyzga. A model soil was then developed to compare the biotransformation of TNT in a slurry system.

Proposed are four hypotheses:

1. *Confirm that under anaerobic, planktonic conditions, 2,4,6-triaminotoluene (TAT) becomes the dominant metabolite in three days during the biotransformation of 2,4,6-trinitrotoluene (TNT).*

Experiments performed followed the protocol set forth by Drzyzga (1998) in which TAT was found to become the dominant metabolite in three days under anaerobic, planktonic conditions.

2. *The Langmuir sorption isotherm is appropriate to describe the sorption of humic acid to goethite when creating a model soil.*

Humic acid dissolved in an electrolyte solution (calcium perchlorate) was added to goethite to create a model soil. Known volumes and concentrations of humic acid were added and allowed to equilibrate until maximum sorption was reached. Through carbon analysis, total humic acid as carbon was measured to determine sorption capacity of goethite.

3. 2,4,6-Trinitrotoluene (TNT) can be biotransformed anaerobically to 2,4,6-triaminotoluene (TAT) in the presence of a model soil.

By combining the planktonic system with the model soil, a batch slurry system was created to observe the biotransformation of TNT in the presence of model soil. "Readily available" (aqueous phase) TNT and metabolite concentrations were measured from supernatant.

4. 2,4,6-Triaminotoluene (TAT) irreversibly binds to a model soil.

An extraction of the batch slurry was performed using acetonitrile and "potentially available" TNT and metabolites from the sorbed phase were measured in the supernatant. If no TNT was measured in the supernatant, it was considered to be irreversibly bound to the model soil.

MATERIALS AND METHODS

Introduction

The purpose of this research was to determine factors that might influence the biotransformation of TNT and its amine metabolites. To do this, three sets of experiments were conducted for this project. First, growth experiments were conducted in anaerobic batch reactors to measure planktonic transformation of the compounds over time. TNT and sodium lactate served as cosubstrates, while sodium sulfate served as the added electron acceptor. Sodium sulfide was included in the medium to create the reduced environment required for maximum activity of *Desulfovibrio* sp. strain SHV, which was the sulfate-reducing bacterium utilized throughout these studies. Second, a model soil of known sorption capacity was created with α-FeO(OH) (goethite) as the mineral surface and a generic humic acid representing native soil organic matter. Finally, the first two experiments were combined; and TNT biotransformation was examined with the model soil in biotic batch slurry reactors. Chapter 2 describes the experimental materials and methods employed to meet the goals of this project.

Model Soil

Sorption experiments using goethite as a model mineral surface and dissolved humic acid as a model for soil organic matter were performed according to Murphy (1992 and 1994) to characterize the model soil used in TNT biotransformation studies.

Model Soil Preparation

The model soil was prepared in 250 ml Nalgene Teflon FEP bottles (Fisher Scientific) that had been rinsed in a 1% HCl solution followed by several rinses of reverse osmosis purified water at 17.4 MΩ-cm from a Barnsted Nanopure Apparatus. Alpha-FeO(OH) (goethite) was purchased from Alfa Aesar (Ward Hill, MA) and would serve as the mineral surface for soil experiments. Calcium perchlorate electrolyte ($Ca(ClO_4)_2$) would provide both ionic strength and divalent cations in the model soil and was purchased from Aldrich Chemical Company (Saint Louis, MO).   Following the procedures outlined in Murphy (1994), a concentration of 0.0017 M $Ca(ClO_4)_2$ was prepared and adjusted to pH 7.8 with 2 N NaOH/1 N HCl and added to 2 g goethite for a combined pH of 5.6. The pH was then decreased with 1 N HCl to 4.5 in preparation for humic acid addition. Representing the organic matter in association with soil, Elliot humic acid was obtained from the International Humic Substance Society (Saint Paul, MN) (Table 2).   A 10,000 mg/L HA stock solution was prepared by thoroughly mixing 1 g of humic acid with 100 ml electrolyte and pH adjusting to 6.8 with

NaOH. This solution was allowed to equilibrate overnight and readjusted as necessary. A standard series of humic acid solutions were then made from the stock solution for humic acid sorption.

<u>Humic Acid Sorption</u>

<u>Supply preparation.</u> To ensure a carbon free environment, plastic syringes and needles used to remove samples for total organic carbon measurements were rinsed with 30 mL of 1% HCl, one time with 30 mL of intermediate Nanopure water and two times with 30 mL of fresh Nanopure water. To rinse the 0.2-um nylon syringe filters used, a pre-rinsed 30 mL syringe was filled with fresh Nanopure water and purged through the nylon filter. Ten drops of sample were passed through the filter to standardize it before aliquoting a 3 ml sample into 5 ml glass Shimadzu vials for carbon measurements. Cleaning preparations for glassware included dishwashing, acid bath soaking overnight in a Nochromix® 36 N sulfuric acid solution from Godax Laboratories and baking at 550° C for 3 hours using a Cress Electric Furnace. The baked glassware was allowed to cool down slowly (5 hrs) and then stored in a low-dust area. Glass syringes used for TNT were rinsed with methanol, followed by a thorough rinsing with Nanopure water.

<u>Humic acid coating.</u> To provide the soil organic matter for the system, a stepwise addition of HA stock solution was followed, allowing the system to come to equilibrium until maximum loading of the mineral soil was reached. Between additions, bottles were centrifuged for 20 minutes at 2980 rpm in a Sorvall RC5C

Centrifuge. Portions of the supernatant (8 ml) were removed to measure total organic carbon. Once maximum sorption was reached, the soil was rinsed with electrolyte to obtain desorption data of the humic acid. The bottles were again centrifuged at 2980 RPM for 20 minutes. The supernatant was decanted into a cleaned graduated cylinder and 200 mL of electrolyte were added to the Teflon bottles. The solution was agitated and then placed on a Glas-Col rotator Model 099A (Terre Haute, IN) for 8-12 hours prior to TOC measurement. The same steps were repeated for the necessary second desorption. KHP calibration standards were used with the Shimadzu Total Organic Carbon Analyzer, Model TOC-5000A (Columbia, MD), to determine organic carbon content (humic acid as carbon or HA-C) of the model soil. The model soil was then considered ready for experimental use.

TNT Sorption

Once the model soil was created, TNT prepared in electrolyte solution was added to "contaminate" the soil. The mixture was placed on a rotator and covered with aluminum foil to prevent photodegradation of TNT. After 24 hours, the supernatant was extracted and filtered through 0.2-um nylon syringe filters into HPLC vials for TNT analysis.

Table 2. Properties of Mineral Sorbent, Humic Acid and TNT.

Properties of Mineral Sorbent

pH_{zpc} 7.8

Properties of Humic Acid
%

C	H	O	N	S	P	H_2O	Ash
58.33	3.61	34.35	4.19	0.42	0.25	8.3	0.86

Properties of 2,4,6-Trinitrotoluene

	MW	solubility (mg/L)	log K_{ow}
2,4,6-trinitrotoluene	227	120	1.60

Adapted from Sparks 1995

Chemicals

2,4,6-Trinitrotoluene (min. 30wt% water) was obtained from Chem Service in Chester, PA with 99% purity. 2-amino-4,6-dinitrotoluene, 4-amino-2,6-dinitrotoluene, 2,4-diamino-6-nitrotoluene, and 2,4,6-triaminonitrotoluene·4HCl were purchased from SRI International (Menlo Park, CA). HPLC standards of 2,4,6-trinitrotoluene, 2-amino-4,6-dinitrotoluene, 4-amino-2,6-dinitrotoluene and 2,4-diamino-6-nitrotoluene were obtained from Supelco (Bellefonte, CA). All chemicals had purities of more than 98%. Uniformly ^{14}C-ring-labeled TNT with a specific activity of 21.6 mCi/mmol was purchased from ChemSyn Laboratories (Lenexa, KS).

Radioactivity measurements

The ^{14}C activity was determined by using a Packard Tri-Carb Liquid Scintillation Counter (LSC), Model 1900A from Meriden, Connecticut. The counting time was 5 minutes.   All determinations in the LSC were performed as triplicate measurements of duplicate samples. In all cases, 1 ml of sample was mixed with 9 ml Packard Ultima Gold liquid scintillation cocktail.   The determination of the total ^{14}C in the biomass was conducted by combustion of the cell pellets in a Biological Material Oxidizer model OX-500 from Harvey under the following conditions: temperature of catalyst, 685^oC; combustion temperature, 900^oC; nitrogen flow, 350 cc/min; oxygen flow, 350 cc/min; combustion time cycle, 4 min. The released $^{14}CO_2$ was absorbed in 15 ml of Carbo-Sorb of which 1 ml was analyzed in LSC. Mass balance studies employing ^{14}C-TNT were conducted to quantify the extent of TNT metabolite sorption to characterize the final product distribution during the batch slurry experiments. For the latter, an HPLC fractionation was carried out by sampling the HPLC eluant (1 ml/min) directly into scintillation vials, which were then filled with 9 ml of scintillation cocktail (total volume, 10 ml) prior to counting in LSC. For these fractionation experiments, 25 μl-aliquots were injected onto the HPLC column.

Analytical Procedures

Identifications of levels of TNT, 2ADNT, 4ADNT, 26DANT and TAT in the culture fluids were determined by reversed-phase HPLC with a liquid chromatograph, which included a Hewlett Packard (Palo Alto, CA) Model 1090

pump and UV-VIS diode array detector. 10-μl aliquots were injected into a Supelco C18 column (25 cm by 4 mm). The solvent flow rate was set at 0.5 ml/min. Samples were prepared in an octanesulfonic acid buffer, and the mobile phase was a 70:30% water-methanol gradient. Peak detection was performed at 254 nm and the three-dimensional field bandwidth was recorded from 190 nm to 600 nm. Nitro- and aminoaromatics were determined by comparison of the retention times and the UV spectra with those of an external standard containing the above mentioned components. Peak integration was calculated by using ChemStation software for LC and LC/MS Systems from Agilent Technologies (Palo Alto, CA) (Rev.A.08.01).

TNT Transformation Experiments

A microorganism was needed that was capable of transforming TNT to the metabolites of interest in order to meet the goals of this project. These metabolites of interest include ADNT, DANT and TAT. The bacteria should have the enzyme required for the formation of TAT; they should be able to do so within a reasonably short time frame, should be available for purchase and should have been characterized previously. *Desulfovibrio* sp. strain SHV was chosen for its ability to reduce TNT to TAT via the ADNT and DANT intermediates. It had been shown to do so within a week under batch conditions, and had been previously characterized and made available by Drzyzga (1998) through the Deutsche

Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany.

<u>Enrichment and Inoculum Preservation</u>

For enrichment of the sulfate-reducing bacteria (SRB), Desulfovibrio SHV medium 735 was prepared both to maintain cultures and to perform TNT degradation experiments. This defined medium consisted of 7 parts, labeled A-G, prepared separately (Table 2). Solution A was prepared in a 2 L Ehrlenmeyer flask with a butyl-rubber stopper plumbed for anaerobic use. The solution was boiled for 30 minutes and allowed to cool under a gas mixture of 80% N_2 + 20% CO_2 according to the Hungate Method (Ljungdahl and Wiegel, 1986; Miller and Wolin, 1974). The remaining solutions were added in alphabetical order. To transfer the medium to 50 mL serum vials, a 30 mL syringe was used to pull the medium through sample port tubing. A 20 ga. needle was then attached to the syringe and the medium was injected into vials that had previously been purged with the gas mixture, sterilized and evacuated. The vials were then stored at 4° C until needed.

To maintain a supply of SRB, frozen cultures of *Desulfovibrio* sp. strain SHV were preserved with 20% (v/v) glycerol. The cryo-preservation vials were then stored at -70^oC. Curved rod-shaped morphology was noted under microscopic examination.

To verify the presence of the enzyme previous research had found to be responsible for the biological transformation step of TNT to TAT, a 2% NaOH

solution was prepared and added to a small volume of actively growing SRB. This solution was placed on a slide under a source of 378nm light. A red coloration indicated the presence of dissimilatory sulfite reductase (desulfoviridin), the enzyme produced by SRB that transforms DANT to TAT. Drzyzga documents further characterization of strain SHV (1998).

Planktonic Experiments

Strain SHV was cultivated anaerobically in vials containing Desulfovibrio SHV Medium (Table 2). To a medium volume of 50 ml in each vial, 5 ml of a pregrown culture (55 ml total volume) was added prior to incubation at 36° C in the dark. Following the Drzyzga (1998) protocol, at an optical density of 0.15, the serum vials were spiked with 45 mg/L TNT, and sampled periodically to measure TNT concentration by HPLC analysis.

Hydrogen Sulfide Analysis

The Spectronic® Genesys color spectrophotometer (Rochester, NY) was used to determine sulfide concentrations at an absorbance of 670 nm according to the methylene blue method. A five-point sulfide standard curve using $Na_2S \cdot 9H_2O$ was employed for calculating sulfide concentrations.

Optical Density Analysis

The Spectronic® Genesys color spectrophotometer (Rochester, NY) was also used to determine optical density at an absorbance of 578nm.

Table 3. Desulfovibrio SHV Medium Composition.

Constituent			Concentration	
Solution A:	Na_2SO_4		3.00	g
	KH_2PO_4		0.20	g
	NH_4Cl		0.30	g
	NaCl		1.00	g
	$MgCl_2x6H_2O$		0.40	g
	KCl		0.50	g
	$CaCl_2x2H_2O$		0.15	g
	Resazurin		1.00	mg
	Distilled water		870	mL
Solution B:	Trace element solution		1.00	mL
	HCl (25%; 7.7 M)	10	mL	
	$FeCl_2x4H_2O$	1.5	g	
	$ZnCl_2$	70	mg	
	$MnCl_2x4H_2O$	100	mg	
	H_3BO_3	6.0	mg	
	$CoCl_2x6H_2O$	190	mg	
	$CuCl_2x2H_2O$	2.0	mg	
	$NiCl_2x6H_2O$	24	mg	
	$Na_2MoO_4x2H_2O$	36	mg	
	Distilled water	990	mL	
Solution C:	$NaHCO_3$		5.00	g
	Distilled water		100	mL
Solution D:	Na-lactate		4	g
	Distilled water		10	mL
Solution E:	Vitamin solution		10	mL
	Vitamin B_{12}	100	mg	
	p-Aminobenzoic acid	80	mg	
	D(+)-Biotin	20	mg	
	Nicotinic acid	200	mg	
	Calcium panthothenate	100	mg	
	Pyridoxine hydrochloride	300	mg	
	Thiamine-HClx2H₂O	200	mg	
	Distilled water	1	L	
Solution F:	Selenite-tungstate solution		1	mL
	$Na_2SeO_3x5H_2O$	0.5	g	
	$Na_2WO_4x2H_2O$	4	mg	
	Distilled water	1	L	
Solution G:	Na_2Sx9H_2O		0.4	g
	Distilled water		10	mL

Batch Slurry Experiments

Batch slurry experiments were conducted to determine the affects of soil organic matter on the biotransformation rate of TNT and to quantify the dependence of biotransformation kinetics of TNT on the presence of soil organic matter.

A destructive sampling procedure in duplicate was followed for this experiment. The TNT model soil prepared in Experiment 2 served as slurry, with humic acid-free slurries serving as negative controls.

Three days prior to inoculating the slurries, eight 50 ml vials (400 ml total) of fresh media were inoculated with 10% SHV to have an actively growing culture ready ($OD_{578} > 0.15$).

Ten milliliter Kimax$^®$ brand centrifuge tubes were used for slurry reactors. The mass of each cleaned and baked centrifuge tube reactor was recorded prior to slurry addition. Ten milliliter aliquots were transferred into each batch reactor for a 1:10 solid/liquid ratio (w/v) (i.e., 1g solid/10ml liquid) using a MeOH/Nanopure rinsed glass pipette and pipette bulb. All reactors were spun down for 20 min at 1040xg. The supernatant was decanted into 10 ml glass graduated cylinders and its volume recorded. This supernatant was then transferred to baked scintillation vials from which samples were removed for initial TNT concentration analysis by HPLC.

At this point, all but four of the reactors received either 10 ml of medium or 10 ml of medium with actively growing strain SHV and were then thoroughly

shaken to resuspend the soil and placed on a Glas-Col Laboratory Rotator (Model 099A) at 18 rotations per minute prior to sampling. The protocol followed for these remaining reactors was to centrifuge each for 20 minutes at 1040xg at their respective sampling times, decant supernatant, record volume, and transfer supernatant into scintillation vials. These samples would give the "readily available" or aqueous phase concentration of TNT metabolites. Ten milliliters of Fisher Scientific acetonitrile (CH_3CN) was then added to each reactor, shaken thoroughly, and returned to the rotator for 24 hours. The above process was then repeated; however, these samples were analyzed as "potentially available", i.e., sorbed TNT metabolite concentration. After this extraction step, the reactors were dried in a Fisher Scientific Model 655F Isotemp Oven for 24h at 105° C. The reactors were weighed, and the difference between this and the initial weight provided the mass of soil. The experiment was conducted until TAT accumulated or no TNT/metabolites were detected in the supernatant.

EXPERIMENTAL RESULTS

Hypothesis 1

1. Confirm that under anaerobic, planktonic conditions, TAT becomes the dominant metabolite in three days time during the biotransformation of TNT.

The sulfate-reducing bacterium *Desulfovibrio* sp. strain SHV (DSMZ No. 10291) was reported to biotransform TNT to TAT (Drzyzga 1998), with TAT becoming the predominant metabolite within three days. Anaerobic, planktonic experiments were conducted to confirm these findings as a basis for further studies. Following the protocol reported by Drzyzga (1998), *Desulfovibrio* sp. strain SHV was allowed to reach an optical density of 0.15 prior to TNT injection into reactors. Immediately after injecting approximately 250 μM of TNT dissolved in methanol into each reactor, only 10 μM of TNT was detected due to rapid transformation in this highly reduced environment. ADNT dominates the solution at the initial sample time, yet it too is rapidly transformed to DANT. Figure 4 shows that TNT is indeed transformed to TAT; however, four weeks were required to obtain these results. Table 4 provides a summary of TNT and metabolite concentrations as determined by HPLC. The expected results of TAT predominance were not confirmed by these findings and steps were taken to identify the reasons for such a substantial difference between expected results and those actually obtained.

A typical sulfate-reducing bacteria (SRB) medium contains enough iron to

turn it black--a direct indicator of iron sulfide formation and indirect indicator of

SRB growth.

Figure 4. *Desulfovibrio* sp. strain SHV biotransformation of 2,4,6-trinitrotoluene. The data are the means of duplicate measurements of duplicate incubations.
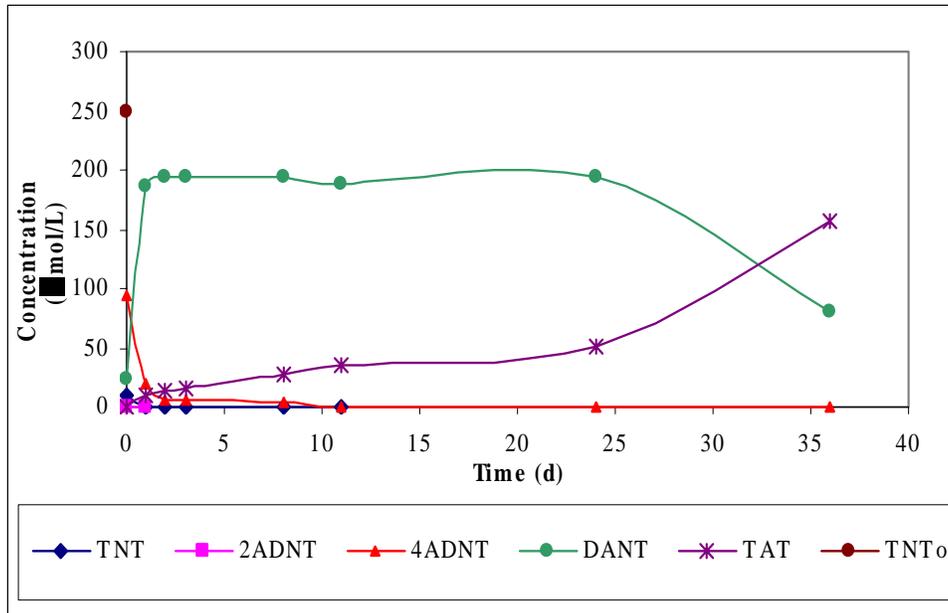

Table 4. Summary of concentrations of TNT and its amine metabolites as determined by HPLC in the culture fluids of *Desulfovibrio* sp. strain SHV. The data are the means of duplicate measurements of duplicate incubations. TNT_o = 250 μM.

	Immediately after TNT addition (μM)	after 1 d (μM)	after 3 d (μM)	after 4 wk (μM)
TNT	9	0	0	0
4ADNT	95	19	4	0
2ADNT	0	0	0	0
DANT	24	182	196	83
TAT	0	11	27	167

Strain SHV medium was prepared with a low iron concentration (see Medium Composition) such that the medium became turbid with high bacterial numbers but remained nearly clear so that any discoloration was readily apparent. Once TNT was injected into a reactor, the medium turned either yellow (bacteria present) or pink (medium only) within seconds of its addition. This was initially contributed to a bad batch of TNT, but continued to be the observed result after a fresh supply was acquired. Since rezasurin was one of the ingredients in the medium, (a chemical often added to anaerobic systems that serves as an oxygen/redox indicator), redox measurements were taken and confirmed that for the pink reactors, redox potential was not as low as the other reactors (Table 5).

Table 5. Redox potential (mV), color, pH and sulfide concentrations (mg/L) in anaerobic batch reactors. a and b are duplicates. SHV = *Desulfovibrio* sp. strain SHV. Negative sulfide numbers were measured in yellow discolored reactors.

	Redox	Color	pH	H_2S-S
SHV/TNT$_a$	-375	yellow	7.6	-
SHV/TNT$_b$	-375	yellow	7.6	-
SHV$_a$	-360	clear	7.6	209
SHV$_b$	-360	clear	7.5	212
Medium only$_a$	-315	clear	8.1	52
Medium only$_b$	-310	clear	7.9	58
Medium/TNT$_a$	-330	yellow	7.9	-
Medium/TNT$_b$	-105	pink	8.1	12

Other researchers have described seeing a yellow color during their TNT studies (Fiorella 1997), although not under anaerobic conditions, which in their

cases indicated the formation of hydroxylaminodinitrotoluene (HADNT).   Strict measures were taken to ensure anaerobic conditions in these experiments, including sparging the methanol in which the TNT was dissolved prior to its injection into each reactor, evacuating anaerobic reactors and purging each with gas mixture, and working inside an anaerobic tent. This experiment was repeated numerous times with similar results.   The following picture shows the reactors with and without bacteria after the addition of TNT (Figure 5).

Figure 5. Planktonic reactors with *Desulfovibrio* sp. strain SHV and TNT (right) and without TNT (left).

In an effort to identify whether the source of the yellow discoloration was related to this strain of bacteria, the experiment was repeated with *Desulfovibrio africanus* and an SRB oil-field consortium. The yellow discoloration appeared in both cases, and as the results depicted in Figures 6 and 7 demonstrate, TNT-

degradation capabilities of these sulfate-reducers were not able to confirm that TAT became the predominant metabolite. *Desulfovibrio* sp. strain SHV was used for the remainder of this project.

In addition to TNT and metabolite analysis, *Desulfovibrio* sp. strain SHV was also analyzed for growth indicators including optical density, sulfide production, most probable number (MPN), and total cell counts. Optical density (OD) is one method used by researchers (Drzyzga 1994) to measure bacterial growth (Figure 8). For these studies, the yellow discoloration in the reactors after the addition of TNT caused OD measurements to drop to negative readings and was therefore not useful for growth determination.

Figure 6. Biotransformation of 2,4,6-Trinitrotoluene by *Desulfovibrio africanus.*
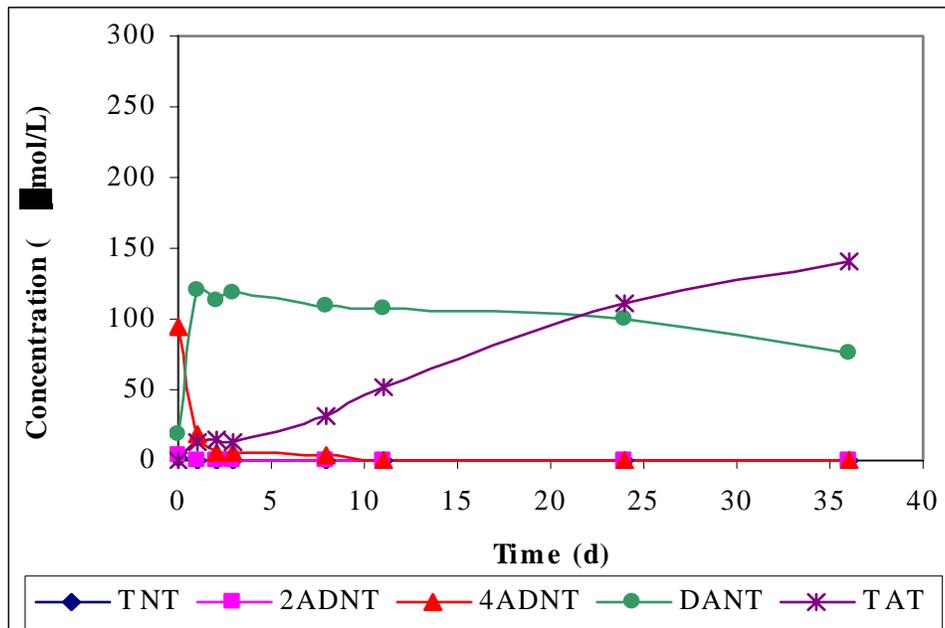The data are the means of duplicate measurements of duplicate incubations.

Figure 7. Biotransformation of 2,4,6-Trinitrotoluene by an oilfield consortium of sulfate-reducing bacteria. The data are the means of duplicate measurements of duplicate incubations.
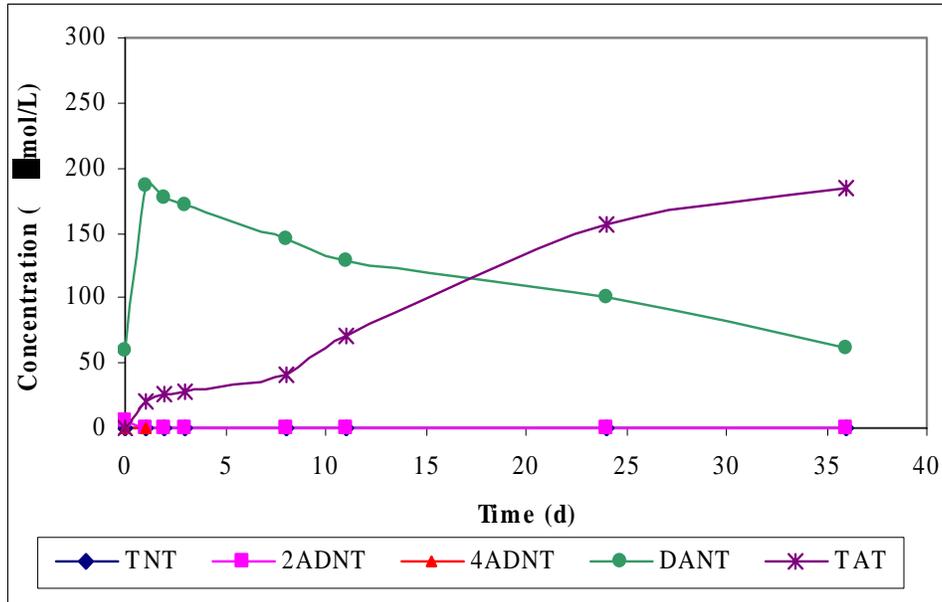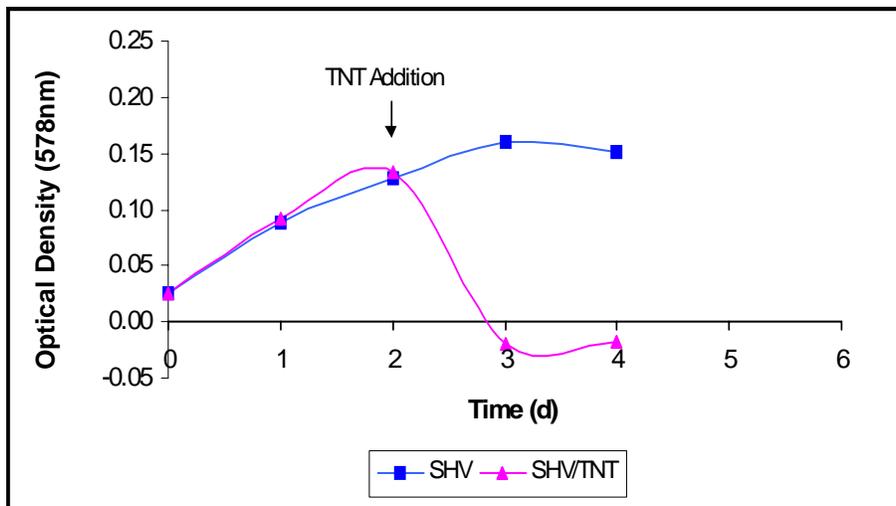

Figure 8. Optical density measurements (OD_{578} nm) from batch reactors of *Desulfovibrio* sp. strain SHV with and without the addition of TNT. The data are the means of duplicate measurements of duplicate incubations.

Likewise, colorimetric analysis of increasing sulfide concentrations as a measurement for growth met with interference by the discoloration as well (Figure 9).

Figure 9.   Sulfide measurements (Abs670 nm) from batch reactors of *Desulfovibrio* sp. strain SHV with and without the addition of TNT. The data are the means of duplicate measurements of duplicate incubations.
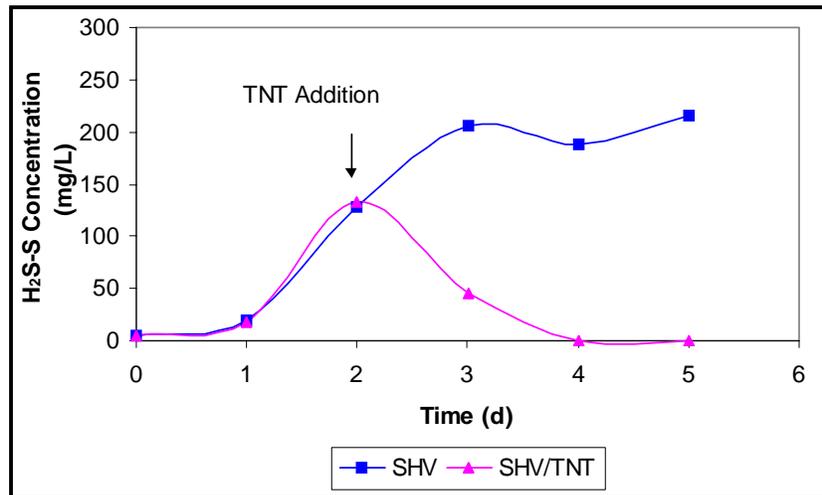
MPN and total cell counts (Figures 10 and 11) were made at the beginning of the experiment and suggest that TNT addition briefly inhibited cell growth. Addition of TNT caused a brief delay in growth after which cell numbers increased to levels identical to those observed in reactors without the addition of TNT.   It was initially believed that TAT formation was slow due to low bacterial numbers which would imply that the concentration of the enzyme responsible for the biotransformation of TNT (i.e., DANT) was therefore low. The MPN and total cell count results suggest otherwise. However, since MPN and total cell counts

were not taken later on in the experiment when TAT evolution became evident, this cannot be confirmed.

Figure 10. Most Probable Number (MPN) from batch reactors of *Desulfovibrio* sp. strain SHV with and without the addition of TNT. The data are the means of duplicate measurements of duplicate incubations.
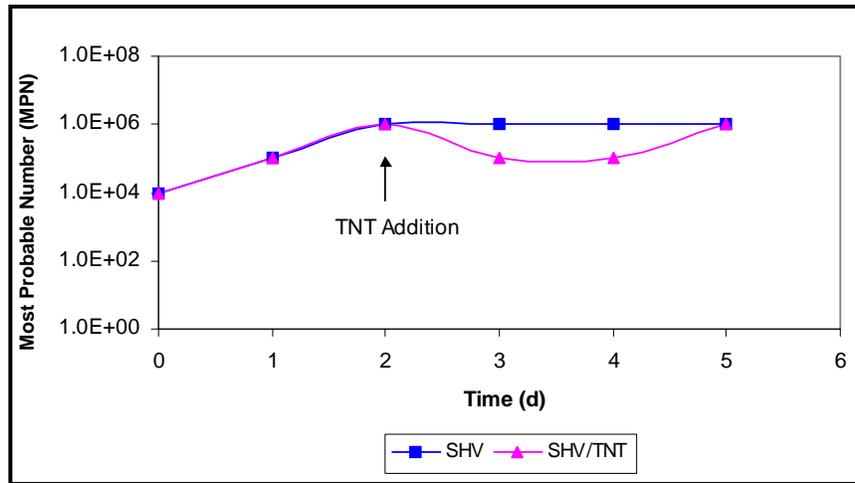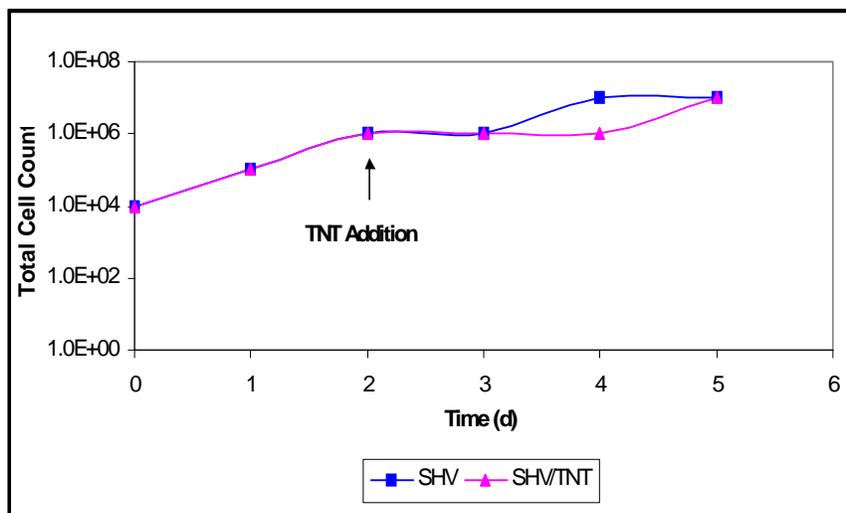

Figure 11. Total Cell Counts from batch reactors of *Desulfovibrio* sp. strain SHV with and without the addition of TNT. The data are the means of duplicate measurements of duplicate incubations.

Hypothesis 2

2. The Langmuir sorption isotherm is appropriate to describe the sorption of humic acid to goethite when creating a model soil.

Sorption experiments with goethite as mineral surface and humic acid representing soil organic matter were performed according to Murphy (1992 and 1994) to create the model soil to be used in TNT biotransformation studies. The first step was to dissolve humic acid in electrolyte solution, then add the mixture to goethite and measure increasing sorption until the sorption capacity of the goethite was reached. These data points are represented by squares in Figure 12. At maximum sorption capacity, the goethite was rinsed in electrolyte to determine whether any humic acid would desorb. Desorption data points are represented by circles in Figure 12.

Figure 12. Sorption isotherm of α-FeOO(H) coated with Elliot humic acid. ■ Represents adsorption samples from slurry. ● Represents desorption samples from slurry.
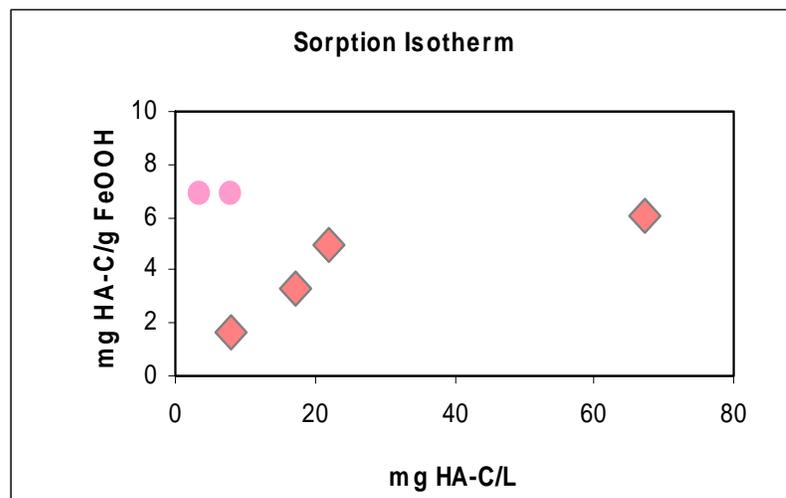
These results suggest that by approximately 6 mg of humic acid as carbon maximum adsorption per gram of goethite is reached. This follows the Langmuir sorption isotherm that was developed with the concept that solid surfaces have a limited number of sorption sites, which, once filled, will no longer sorb solute from solution. In equation form, the Langmuir sorption isotherm can be expressed as

$$\frac{C}{C*} = \frac{1}{\alpha\beta} + \frac{C}{\beta}$$

Where

C = concentration of humic acid as carbon in solution (mg HA-C/L)

$C*$ = concentration of humic acid as carbon sorbed per unit solid (mg HA-C/g FeOOH)

α = adsorption constant related to the binding energy (L/mg)

β = maximum amount of solute that can be absorbed by the solid (mg/g)

The Langmuir sorption isotherm can be made linear by plotting $C/C*$ versus C from which the adsorption constant (the slope of the line divided by the intercept), α, and the maximum solute amount (the reciprocal of the slope of the line), β, can be calculated (Figure 13). The line is statistically valid as demonstrated by an R^2 value of 0.931 and supporting the hypothesis that humic acid sorption to goethite can be represented by a Langmuir sorption isotherm.

Figure 1**3**. Linearized sorption isotherm fitting a Langmuir model.

Model Soil - Linearized Sorption Isotherm

$y = 0.1168x + 3.0793$

$R^2 = 0.931$

$\alpha = 0.038$ L/mg

$\beta = 8.56$ mg/g

C/C*

C
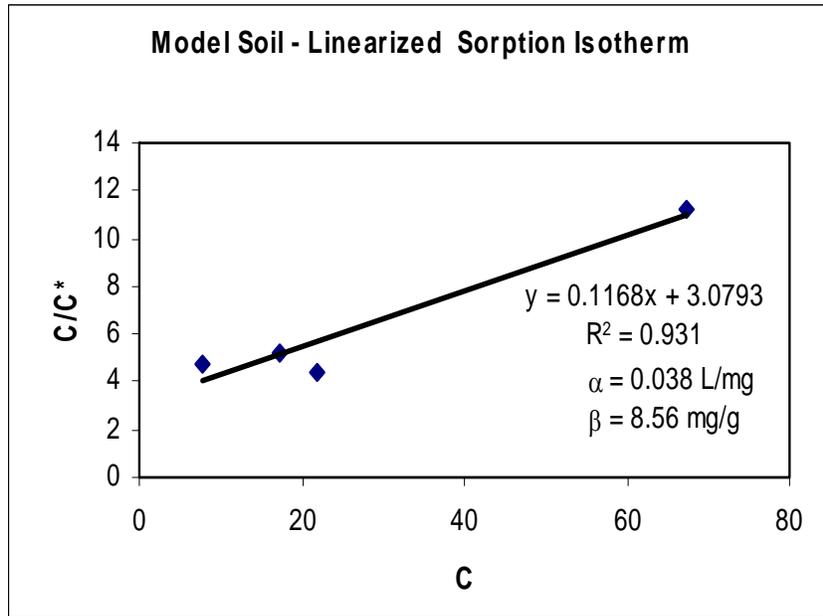
<u>Hypotheses 3</u>

3. 2,4,6-Trinitrotoluene (TNT) can be biotransformed anaerobically to 2,4,6-triaminotoluene (TAT) in the presence of a model soil.

The first two experiments were combined to create slurry conditions under which hypothesis three could be tested. *Desulfovibrio* sp. strain SHV was added to the model soil to observe the biotransformation of 2,4,6-trinitrotoluene in the presence of a model soil.

The procedure followed for these experiments was to prepare the model soil (humic acid, electrolyte and goethite) and mineral only controls (electrolyte and goethite--no humic acid), add 250 μM TNT (as in the planktonic experiments) and allow the system to equilibrate over a 24-hour period (Figure 14).

Figure 14. Batch slurry reactors used in TNT degradation studies.
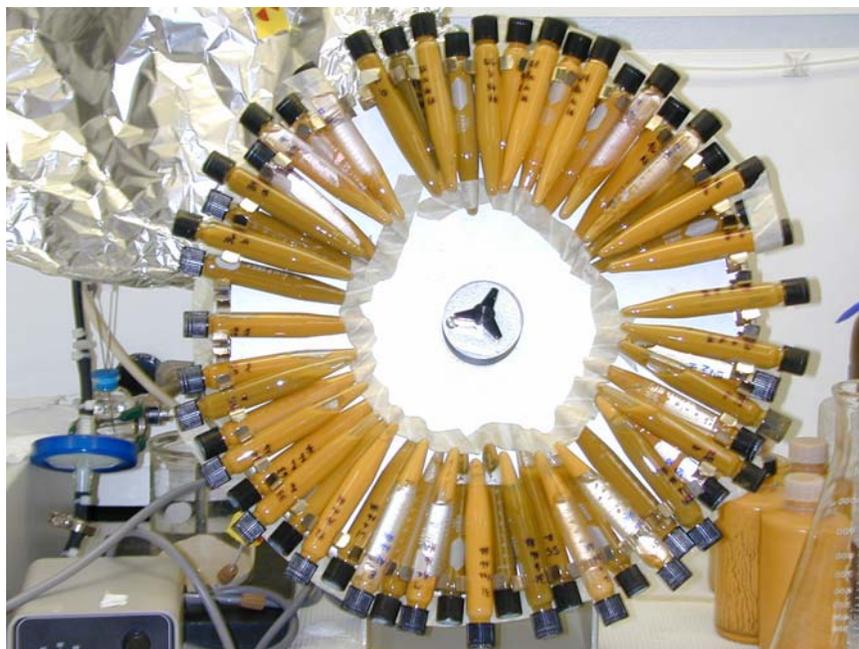
The reactors were centrifuged and the supernatant was decanted and tested for initial TNT concentrations, and by difference, the sorbed TNT concentrations were determined. HPLC analysis of the supernatant indicated that reactors with model soil (i.e., reactors with humic acid) sorbed approximately 30% TNT or 75 μM (70% of the TNT remained in aqueous phase); while controls without humic acid sorbed approximately 20% of the added TNT (50 μM).

To begin the biotransformation studies, 10-ml of either medium with actively growing *Desulfovibrio* sp. strain SHV or abiotic controls containing medium only were added to the reactors. Reactors were centrifuged and the supernatant was analyzed for TNT and metabolites.   Figures 15 and 16 depict the rapid biotransformation of TNT to DANT and TAT in the first hour as well as the eventual disappearance of TAT in solution by day three.

Figure 15. Batch slurry results of the biotransformation of 2,4,6-trinitrotoluene contaminated model soil by *Desulfovibrio* sp. strain SHV.
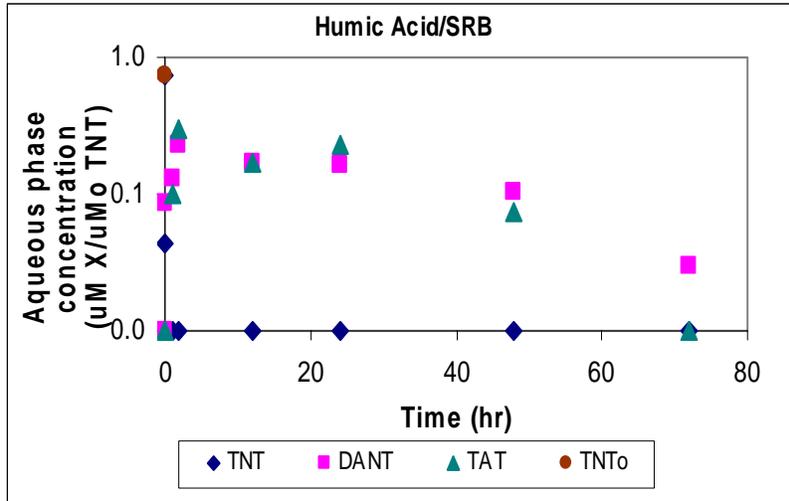
Unlike the planktonic experiments where TAT became the predominant metabolite in solution only after four weeks time, under slurry conditions (both with humic acid and without), TAT became dominant in the first 24 hours.   This more rapid biotransformation is believed to be due to the lower initial TNT concentration in the slurry experiments.   Recall that in the planktonic experiments, 250 μM TNT was injected into a reactor containing actively growing bacteria (OD = 0.15), whereas in the slurry, although it too initially received 250 μM TNT, after equilibrium the slurry retained only 30% TNT (model soil) and therefore only 75 μM TNT was in the slurry system at the time when bacteria (OD = 0.15) were added.   This means that the ratio of bacteria (and therefore the TAT-forming catalyst) to the concentration of TNT was much greater in the slurry experiments.

Figure 16. Control - No Humic Acid. Batch slurry results of the biotransformation of 2,4,6-trinitrotoluene contaminated soil by *Desulfovibrio* sp. strain SHV.
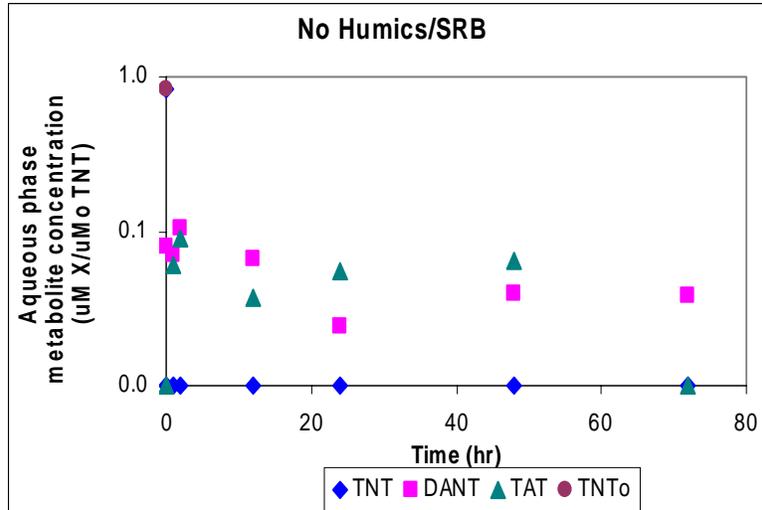
Abiotic controls with and without humic acid (Figures 17 and 18) demonstrated that TNT was reduced to DANT but that when no bacteria were present, no TAT formed. These results support the hypothesis that TNT can be biotransformed to TAT in the presence of a model soil.

Figure 17. Control - Abiotic. Batch slurry results of the transformation of 2,4,6-trinitrotoluene contaminated model soil.
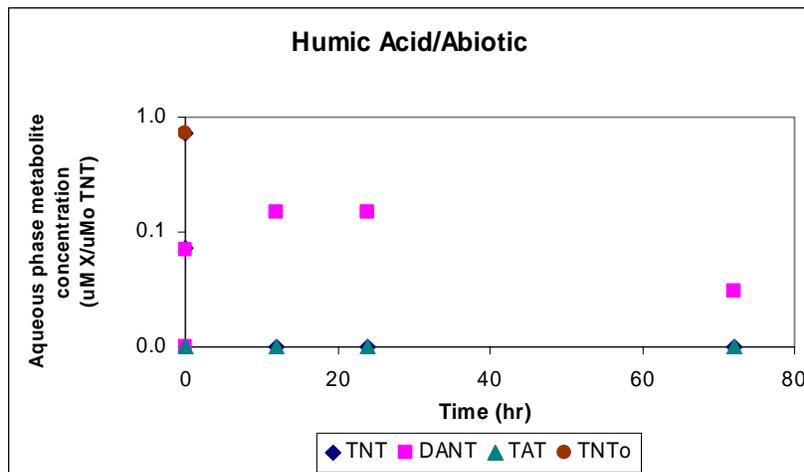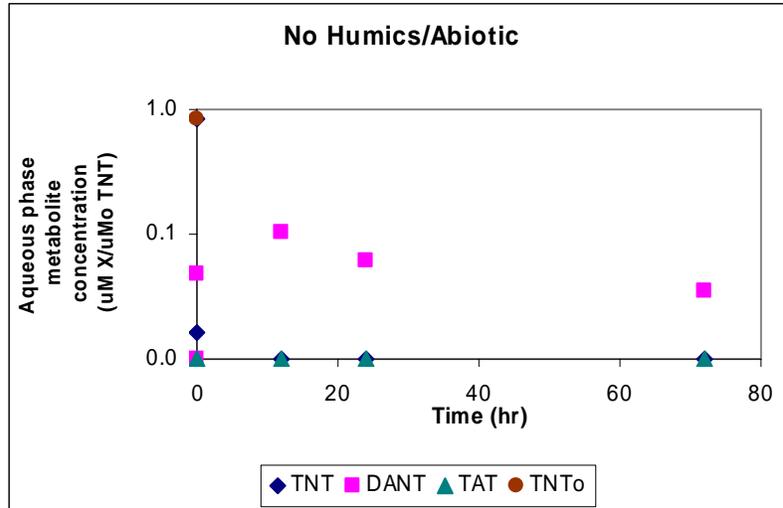
Figure 18. Control - Abiotic. Batch slurry results of the transformation of 2,4,6-trinitrotoluene contaminated model soil.

No Humics/Abiotic

4. 2,4,6-Triaminotoluene (TAT) irreversibly binds to a model soil.

The next step with the slurry reactors was to test the hypothesis that TAT irreversibly binds to a model soil. This meant that acetonitrile was added to each reactor to perform extractions for "potentially-available" metabolites (i.e., sorbed metabolites). Twenty-four hours after acetonitrile addition, reactors were centrifuged and the supernatant measured for TAT. Results in Figure 19 indicate that some of the sorbed TNT had not been available for transformation in the first two hours, but was readily extracted by the acetonitrile. This TNT would therefore be potentially available for further biotransformation. DANT was readily desorbed by the acetonitrile, while TAT was not. This suggests that TAT became irreversibly bound to the model soil.

Figure 19. Extraction results of sorbed TNT and metabolites in biotic model soil slurry.
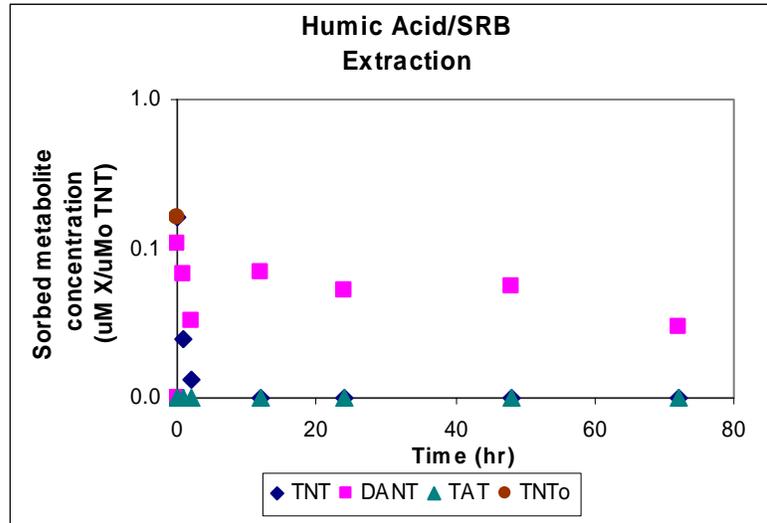
Other researchers have also observed this irreversible binding (Lenke 2000, Li 1997), and believe this sorption occurs through covalent binding. To determine the influence of soil organic matter on TNT and metabolite sorption, humic acid was omitted in control reactors as mentioned previously. In Figure 20 it was again observed that no TAT was detectable in solution after the extraction procedure.

Figure 20.   Control - No Humic Acid. Extraction results of sorbed TNT and metabolites in a biotic soil slurry.
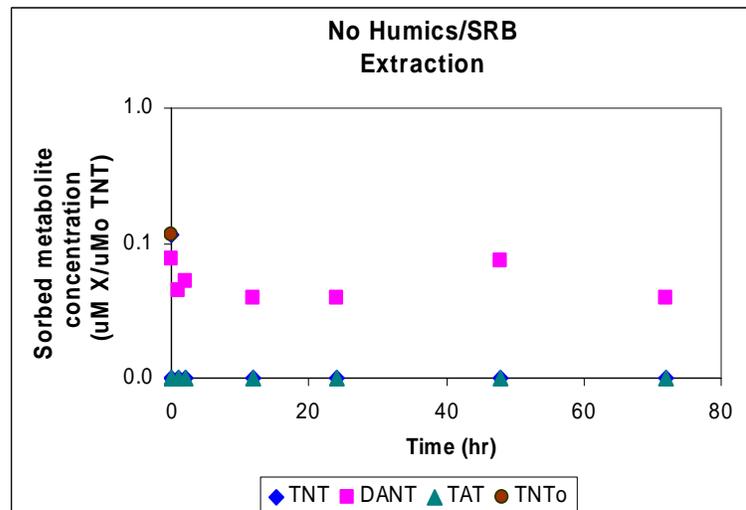
In addition, uniformly labeled ^{14}C-TNT was added to the model soil such that radiolabel counts could be measured in the irreversibly bound phase. A number of observations can be made about the results depicted in Figure 21.

Figure 21. Average ^{14}C-TNT counts (dpm) for model soil with *Desulfovibrio* sp. strain SHV. Initial ^{14}C-TNT added (•), bacteria/ humic acid (▲), humic acid/abiotic (■), bacteria/no humic acid ( ) and no humic acid/abiotic ( ).
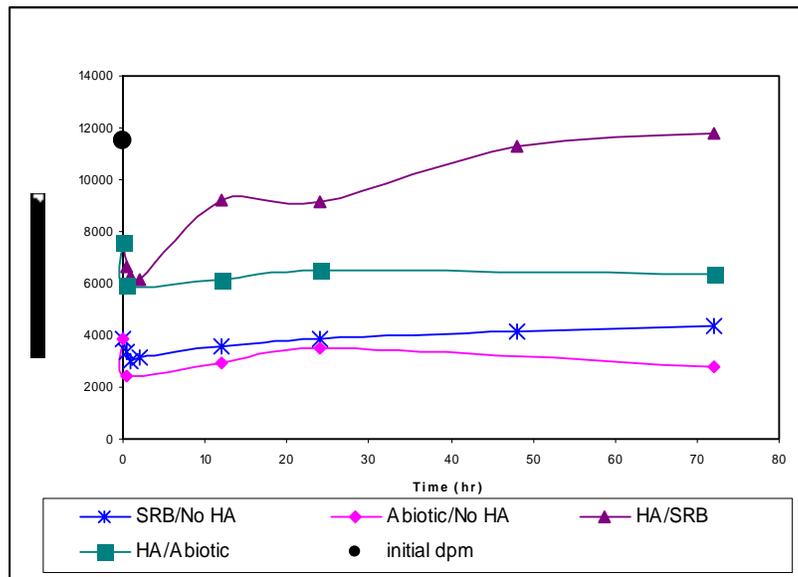
First, more TNT was sorbed under slurry conditions when humic acid was present versus when humic acid was omitted (upper two lines on graph versus lower two lines). Second, more sorption occurs when bacteria are present (uppermost line of top two lines and uppermost line of bottom two lines). This suggests that the biotransformation products (i.e., TAT) are more sorptive than abiotic transformation products. Third, that metabolites become more sorptive over time and finally, that full recovery of the final data point where TAT was not detected in solution when humic acid was present (Figure 15) provides further

evidence of irreversibly binding. That the same full recovery does not occur when humic acid was absent (Figure 16) suggests that the final measurement depicted in Figure 16 was suspect.

CONCLUSIONS AND RECOMMENDATIONS

The goal of this project was to provide useful information toward the DEPSCoR-sponsored study into biofilm-induced changes of soil organic matter structure and the resulting impact on bioavailability of TNT and its amine metabolites. The first objective of this study was to confirm the TNT-transforming ability of a known TNT-transforming bacterium. A model soil was created to which the bacteria were added in order to observe any differences in TNT biotransformation due to the presence of the model soil.   These experiments provided a foundation from which to build toward the overall goal of the DEPSCoR project.

The first hypothesis to be tested for this project was to determine whether this lab could reproduce the biotransformation of TNT to TAT within three days by *Desulfovibrio* sp. strain SHV. Although TAT did develop, it did not do so within three days as previously reported in the literature, but rather after four weeks, and based on the results presented, does not meet the first hypothesis.

The reasons for these differences remain unclear; however, it is believed to be due to whatever was causing the yellow discoloration in the medium upon TNT addition. Although a yellow discoloration has been reported in the literature (Fiorella 1997), it was only observed under aerobic conditions as an accumulating, but unidentified metabolite. Drzyzga (personal communication) also described a yellow color in their experiments, but only if oxygen had been introduced to the system. Strict measures were taken during these experiments,

to ensure oxygen was not introduced into the system, including sparging all medium solutions, sparging the methanol in which TNT was dissolved, displacing any oxygen present in sampling needles and working in an anaerobic tent. In addition, no unidentified peaks were observed during HPLC analysis during the planktonic experiments to indicate the presence of an unsought-after metabolite.

Trinitrotoluene is known to rapidly transform to diaminonitrotoluene (DANT) abiotically under reducing conditions, and by fortuitous reduction by non-specific nitroreductases produced by both aerobic and anaerobic bacteria. Further reduction of nitro groups, however, occurs only under anaerobic conditions and takes place when the enzyme desulfoviridin is present to complete the biotransformation of DANT to triaminotoluene by sulfate-reducing bacteria. When TAT did not become the predominant metabolite in solution after five days into the planktonic experiment, a test was performed on *Desulfovibrio* sp. strain SHV to ensure the presence of the enzyme. Under ultraviolet light, a red fluorescence indicated the chromophore of the pigment desulfoviridin (Postgate 1984) was indeed being produced. For this reason the planktonic experiments were carried out until TAT dominance was observed. In the end, the yellow discoloration could merely have been caused by old or contaminated laboratory chemicals from which the medium was made and it is recommended that any future experiments with this system be done with a new supply of chemicals.

The model soil was created using goethite as the mineral surface and humic acid in an electrolyte solution serving as soil organic matter. The amount of humic acid sorbed to the mineral surface reached a maximum value suggesting that all the sorption sites on the goethite had been filled--a response seen when developing a Langmuir sorption isotherm from experimental data. This met the anticipated result of the second proposed hypothesis.

Once the model soil was created, bacteria were added to form a slurry to test the third proposed hypothesis that 2,4,6-trinitrotoluene could be biotransformed to 2,4,6-triaminotoluene in the presence of a model soil. The results indicated that this hypothesis was not only true for a model soil, but also for a non-humic acid-containing goethite and electrolyte slurry. TAT was formed within the first hour of the experiment and became the predominant metabolite within 24 hours as compared to the four weeks observed under planktonic conditions. This is most likely due to lower initial TNT concentrations. A more useful experiment that can be performed in the future would be either to add comparable TNT concentrations to planktonic reactors or attempt to sorb higher concentrations to slurry reactors.

The slurry experiment also showed that the fourth hypothesis was met when TAT was not detected after extractions with acetonitrile indicating that 2,4,6-triaminotoluene became irreversibly bound to both the model soil and goethite/electrolyte slurry. Radiolabeled results provided further evidence for

irreversible binding of TAT with the model soil; however, this conclusion cannot be made for the control reactors without humic acid.

The information collected throughout the course of this study could prove useful in the continuation of the DEPSCoR project on biofilm-induced changes in soil organic matter structure and its impact on the bioavailability of TNT and its amine metabolites. A model soil system was developed within which changes might be made to the solution chemistry, i.e., pH, ionic strength, divalent cation concentrations, and biosurfactant addition. These changes may alter the structure of the humic acid to either elongated or coiled forms, which in turn may lead to different observed biotransformation rates of TNT. A system could then be designed to allow biofilm growth on the model soil to compare the biotransformation of TNT and infer from the results the impact of the biofilm on the bioavailability of TNT.

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