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2,4,6-trinitrotoluene (TNT) has been widely used in the production of explosives. Since its development, manufacturing products and wastes containing TNT have contributed to environmental contamination. Concerns about toxic health effects arise from evidence linking extensive occupational contact to TNT with increased incidence of serious diseases.

In vitro TNT biotransformation based upon the incubation of whole rumen fluid of fisturated sheep and goat was investigated. TNT destruction was completed within one hour. The metabolites were designated as 2,4-diamono, 6-nitrotoluene, 2,4,6-triaminotoluene and other unknowns.

Feasibility of TNT degradation by ruminal microorganisms in TNT inoculated soil was also examined. The initial TNT(120-130 mg/l) was completely transformed within 5 days during which time rumen fluid was sequentially added. Sixty five percent of the initial TNT disappeared in the control and was not recoverable from the soil, indicating irreversible TNT transformation by soil organic matter.

Isolation of microbial species that were responsible for TNT destruction was attempted through batch culture enrichment in order to avoid the complexities of the rumen content and defines the mechanisms. A nitrate-reducing bacteria (*G.8*), isolated from fisturated goat, was grown with lactate and nitrate to provide carbon and energy sources, utilizing 2,4,6-trinitrotoluene (TNT). The reduction of the nitro group followed by deaminization was identified as the main mechanism of TNT destruction by the isolate, producing toluene as the end product (requiring 3.76 mM of nitrate/mM of lactate). This transformation was dependent on the presence of nitrate or nitrite as the electron acceptor. Nitrates within the medium stimulated the TNT para-position nitro group, whereas nitrites catalyzed the deaminization and oxidation processes of the amino group. In the absence of other electron acceptor contributions, the transformation of the ortho-position nitro group was rapid.

When placed in a fixed-film bioreactor the isolate succeeded in building a homogenous cell matrix on the packed materials. Cell metabolism was maintained for over 8 months on the synthetic medium. Metabolites of TNT in the bioreactor effluents were identified as 4-amino-2,6-dinitrotoluene, and 2-amino-4,6-dinitrotoluene and negligible amounts of 2,6-dinitrotoluene (requiring 1.05 mM of nitrate/mM of lactate).

In Vitro Anaerobic Trinitrotoluene(TNT) Degradation with Rumen Fluid and An Isolate, *G.8*

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ABBREVIATIONS

Compounds

TNT	2,4,6-trinitrotoluene
MADNT	monoamino-dinitrotoluene
DAMAT	diamino-mononitrotoluene
HADNT	hydroxylamino-dinitrotoluene
DAHAT	diamino-hydroxylaminotoluene
2A46DNT	2-amino-4,6-dinitrotoluene
4A26DNT	4-amino-4,6-dinitrotoluene
24DA6NT	2,4-diamino-6-nitrotoluene
26DA4NT	2,6-diamino-4-nitrotoluene
TAT	2,4,6-triaminotoluene
26DNT	2,6-dinitrotoluene
24DNT	2,4-dinitrotoluene
2A6NT	2-amino-6-nitrotoluene
2NT	2-nitrotoluene
2AT	2-aminotoluene

Instruments

GC/MS	gas chromatography/ mass spectrometry
HPLC	high performance liquid chromatography
IC	ion chromatography

PREFACE

This article is a compilation of three articles prepared for publication. Chapter 3 and Chapter 4 were presented to *Applied and Environmental Microbiology*. Chapter 5 was prepared for publication in *Biodegradation*. Citations in the text refer to references listed at the end of each chapter. These references are collected into a comprehensive bibliography at the end of the thesis. Some repetition may be noted because each chapter stands alone. Chapter 1 is a complete review of previous research for TNT biodegradation. Each chapter has its own introduction but it is recommended that Chapter 1 be read for complete understanding of TNT transformation history.

In Vitro Anaerobic Trinitrotoluene(TNT) Degradation with Rumen Fluid and An Isolate, *G.8*

Chapter 1. Introduction

1.1 Background

Since World War II, 2,4,6-trinitrotoluene (TNT) has been widely used in the production of explosives because of its low boiling point, stability, low sensitivity to impact, and its relatively safe methods of manufacture. Since this development, manufacturing products and wastes containing TNT have contributed to contaminated environments (23). The contamination of over 1,100 military facilities potentially contaminated with munitions waste will require that greater than 1,000,000 cubic yards of contaminated soil be treated (2)

Explosive wastes are also toxic to some aquatic organisms (9,27). Concerns about toxic health effects arise from evidence linking extensive occupational contact to TNT with increased incidence of aplastic anemia, liver damage, dermatitis, ocular disorders, and gastrointestinal distress. With respect to these concerns, initiatives undertaken to assess the content of existing waste disposal sites and to search for environmentally acceptable ways to remediate contaminated soils have been supported by military and other government agencies (23).

The most practical approach to the remediation of explosives is currently soil incineration, but it can be a costly, energy-intensive process that destroys much of the soil, leaving ash as the primary residue with estimates approaching $800/ton (13). Chemical treatment requires the exercise of rigid controls to avoid the discharge of unreacted materials and is not practical in many situations. It is believed that the

biological remediation of explosives is the most economical and reliable method of response to this problem at a cost that ranges of \$30-\$150/yd^3 (21, 22). Composting, one of the biological remediation methods applied to explosives, has been proven to be effective and is competitive with incineration methods (30), but the procedure requires long incubation times, additional carbon supply, use of a bulking agent, a source of inoculum, and a nutrient supply, thus raising the operational costs.

The biological removal of explosives has been intensively studied. Nonetheless, little is known about the characteristics of the consortium responsible for degrading TNT or the limiting factors affecting TNT transformation by cometabolism. From a review of the literature, the present study has sought to identify the biotransformation products of nitroaromatic compounds, principally TNT, under different conditions, as well as to arrive at a means to describe the dominant mechanisms of TNT remediation. This chapter is thus presented in two parts, TNT degradation under, respectively, aerobic and anaerobic conditions, followed by a discussion section that critically reviews the short comming of both the known aerobic and anaerobic pathways. This study should also help to provide a basis for the development of bioremediation technologies as well as comprehensive understanding of the effects of biological treatment.

1.2 TNT degradation under aerobic conditions

Since the initiation of the present study, various organisms, including *P. chrysosporium, Pseudomonas,* or other isolates from contaminated sites have been used for the aerobic degradation of TNT. TNT oxidation processes under aerobic

system require the complete mineralization of TNT as a carbon or energy source. Previous studies of the aerobic biodegradation of explosives are summarized below.

Osmon and Klausmeier (22) attempted to isolate organisms capable of using TNT, RDX, or ammonium picrate for growth. TNT was degraded by microoganisms as a cometabolism, but this was not true of either RDX or ammonium picrate. TNT degradation intermediates accumulated transiently in the growth medium but the identification of these intermediates was not provided. The addition of yeast extract was found to stimulate TNT degradation.

Klausmeier et al. (15) investigated the effect of TNT on the growth of various microorganisms. Most organisms, including fungi, yeasts, actinomycetes, and gram positive bacteria, were tolerant of less than 20 ppm of TNT. With the exception of gram negative bacteria, quantities greater than 50 ppm of TNT inhibited or severely prevented the growth of bacteria.

Won et al. (32) provided evidence that microbes could oxidize TNT through three *Pseudomonad* organisms isolated from mud and water ammunition samples. TNT oxidation was effective at pH 6.5 to 7.2 in a basal salts medium, but the system required the addition of glucose or nitrogenous substances (i.e., a yeast extract) to achieve accelerated transformation. The presence of nitrites in the mineral salt medium was detected, indicating the removal of the nitro group from the ring. In a medium supplemented with a 0.5% yeast extract, 100 mg/l of TNT was completely transformed to intermediates such as MADNT, DAMNT, or azoxy compounds. It

was suspected that the azoxy compounds were formed from the coupling reactions of the corresponding hydroxylamines.

McCormick et al.(18) investigated biotransformation under both aerobic and anaerobic conditions with cell-free extract, resting cells, or growing cultures. Under aerobic conditions, two of three nitro groups with 100 ppm of TNT were reduced to the intermediates. A small amount of azoxy compounds was detected with a nonenzymatic reaction of 4HA26DNT. To reduce each nitro group to an amino group, 3 moles of hydrogen were required.

Carpenter et al. (7) investigated the fate of TNT in an activated sludge system, whereas no significant CO_2 amounts were produced after 3 to 5 days of incubation. The radioactive carbon initially added to the medium was divided equally between the floc and supernatant. It was concluded that aromatic amines arising from the biotransformation of TNT underwent condensation reactions with the carboxylate groups of cellular components, thus leading to the formation of polyamides. The formation of the polyamides then strongly resisted further biotransformation of the TNT.

Kaplan and Kaplan (14) examined the biotransformation of ^{14}C-TNT by thermophilic microorganisms in a compost system, 2A46DNT, 4A26DNT, 26DA4NT, 2 4DA6NT, and azoxy compounds were detected in solvent extracts for 91-day periods of incubation. The reduction of the para-positioned nitro groups was preferred to ortho-positioned groups, and there was no evidence of ring cleavage.

Fernando et al. (12) used a white-rot fungus, *Phanerochaete chrysosporium*, to observe the biodegradation of TNT. A liquid culture of 35.4 % 1.3 mg/l ^{14}C-TNT was degraded to $^{14}CO_2$ in 18 days; and 18.4 % ^{14}C-TNT was converted to $^{14}CO_2$ in 90 days in soil cultures. In both cases, 15% of the initial ^{14}C-TNT remained in the medium after even 90 daysof incubation. The intermediates proved to be more polar than TNT, but were not identified.

Kulpa et al. (16) investigated TNT degradation by isolates from TNT contaminated soils. In the presence of succinate, 3.1% of the initial ^{14}C-TNT was converted to $^{14}CO_2$, and 8-12% was converted to biomass under a cometabolic process. A major intermediate was produced during eight days incubation, and was accumulated over a 10-days period with small minor intermediates. However, the intermediate under investigation was not identified.

Schackmann and Muller (26) investigated the reduction of nitroaromatic compounds with *Pseudomonas* sp. The reduction mechanism was identified as the aminization of the nitro group on the aromatic ring via nitroso and hydroxyamino compounds. In the case of TNT, two MADNT and DAMNT were obtained, but TAT was not detected. In crude cell-free extracts, the enzyme was inactivated by dialysis and was reactivated by the addition of NADH or NADPH.

Williams et al. (30) investigated the clean-up of sediments contaminated with explosives in thermophilic- and mesophilic-aerated static piles. For the breakdown of TNT, half lives were 12 days under thermophilic conditions and 22 days under mesophilic conditions. It was concluded that composting was a feasible

bioremediation approach, subject to evaluation of the effects of hazardous materials such as heavy metals and volatile organic compounds on the microorganism prior to the execution of composting procedures.

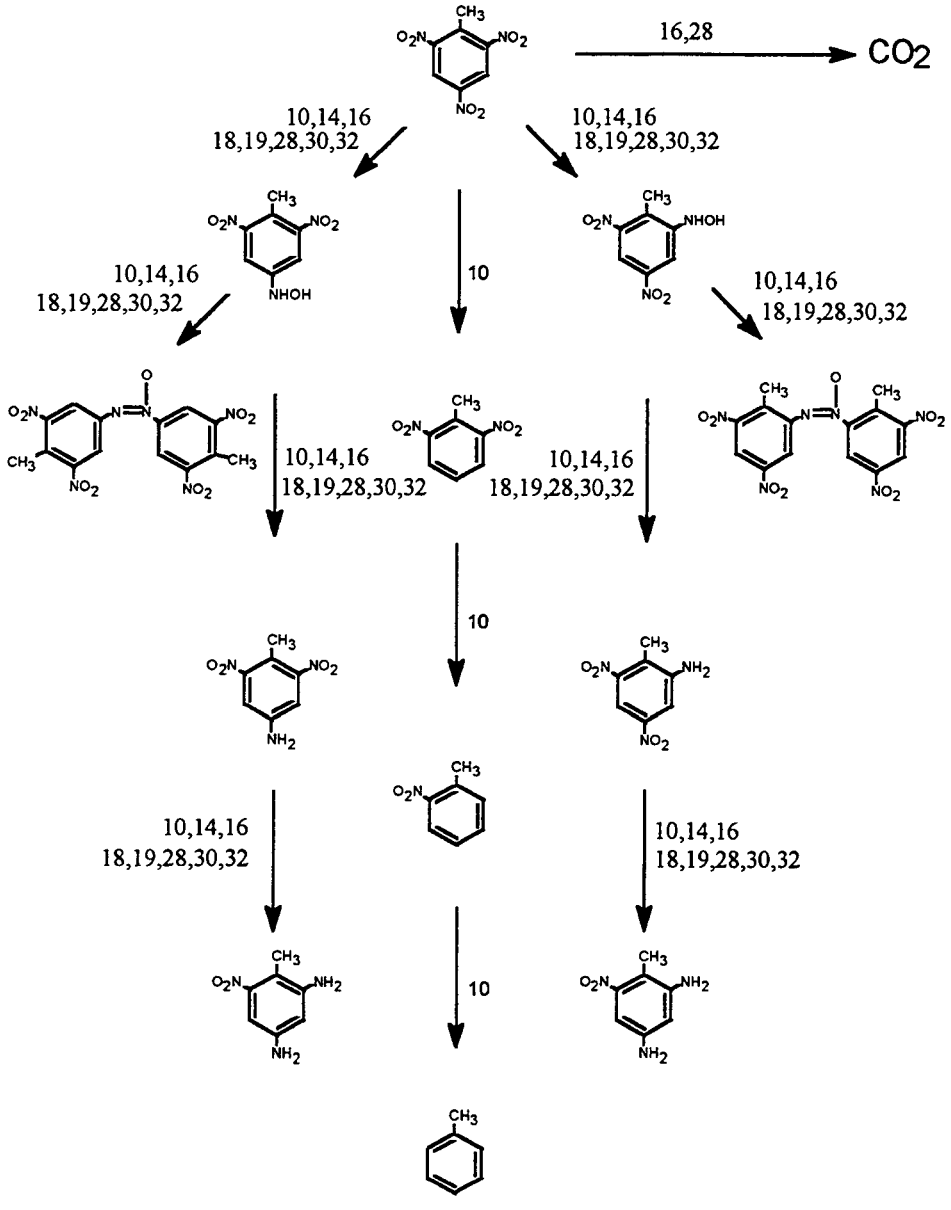
Spiker et al.(28) investigated the bioremediation of explosives using the white-rot fungus, *phanerochaete chrysosporium.* The fungus was unable to tolerate substances containing greater than 0.02 % (wt/vol), 24 ppm TNT in a malt extract broth. Spore-inoculated cultures mineralized 10% 5 ppm ^{14}C-TNT in 27 days at 37°C.

Duque et al. (10) found that a bacteria, *Pseudomonas sp.*, strain C1S1, grew on TNT. Growth occurred with fructose as a carbon source, accumulating nitrites from TNT nitro groups by the hydrogenation of NAD(P)H. Another isolate, *Pseudomonas sp.*, clone A, grew faster on TNT, but did not accumulate nitrites. These two strains used TNT as a nitrogen source, transforming TNT into 2,4- and 2,6-DNT, 2NT, and toluene. MADNT, DAMNT, and small amounts of azoxy compounds resulting from the condensation of TNT intermediates were also found.

Michels et al. (19) examed the negative effect of increased TNT concentration on the rate of degradation using liginolytic cultures of *P. chrysosporium*. A 30% degradation rate in the presence of 0.36 mg/l ^{14}C-TNT was decreased to 5% degradation during a four-day incubation in the presence of 20.36 mg/l of ^{14}C-TNT. The veratryl alcohol oxydase activity of *P. chrysosporium* was inhibited by the condensation of HADNT, but was not affected in the medium containing MADNT. This finding demonstrated that the conversion of HADNT to MADNT was a rate limiting step.

In summary of the aerobic system, the bacteria isolated from explosives-contaminated sites or mixed cultures produced small amounts of CO_2 in ranges from zero to 3.1%. Composting did not present any evidence of ring cleavage and the MADNT, DAMNT, and azoxy compounds were unaffected. It was a promising result that the lignin pereoxide of the white-rot fungus, *P. chrysosporium*, oxidized from 10-35% of initial the TNT to CO_2, while at the same time proving too sensitive to explosives concentrations to be applied at contaminated sites. The fungus was unable to tolerate amounts greater than 20ppm of TNT (15,28). The inhibition of the veratryl oxidase activity of lignin peroxidase by HADNT should affect the production of CO_2 with respect to TNT concentrations (19).

Integrated pathways for aerobic conditions are presented in Figure 1.1, which indicates that TNT was slowly reduced to amino, diamino, and azoxy compounds for various microorganic species, including bacteria and fungi. The reduction of the nitro group on the aromatic ring should take place in oxygen-insensitive enzymes that can catalyze obiligate two-electron reduction of the substrate (6). The most serious problems experienced for the aerobic treatment of explosives are as follows: 1) TNT intermediates are easily polymerized by the nonenzymatic coupling processes of HADNT (8, 32), and are tightly bound to such organic materials as azoxy compounds; and 2) early stage TNT reduction products are left in the contaminated sites. More critical reviews of the papers are presented in discussion section.



Numbers next to arrows refer to references.

Figure 1.1 Integrated pathways of TNT degradation under aerobic conditions.

1.3 TNT degradation under anaerobic conditions

The degradation of explosives under anaerobic conditions has been intensively studied within past the few years. Various microorganisms, including denitrifing, sulfate reducing, methanogenic, and specific enzymes, were applied to examine the feasibility of explosives degradation under anaerobic conditions. Principal findings are as summarized below.

McCormick et al. (18) investigated TNT reduction by cell-free extracts, resting cells, and growing cultures. The cell-free extracts and resting cells reduced three nitro groups to corresponding amino groups under strctly anaerobic conditions. Growing cultures of *V. alkalescens* and *E. coli.* produced 2,4DA6NT. In the case of nitrotoluene, the reduction of a para-positioned nitro group was followed by the ortho-positioned group. The nitro reductase separated from *V. alkalescens* consisted of hydrogenase and ferredoxin-like materials, but whether the nitro reduction took place solely via hydrogenase or ferredoxin was not conclusively demonstrated.

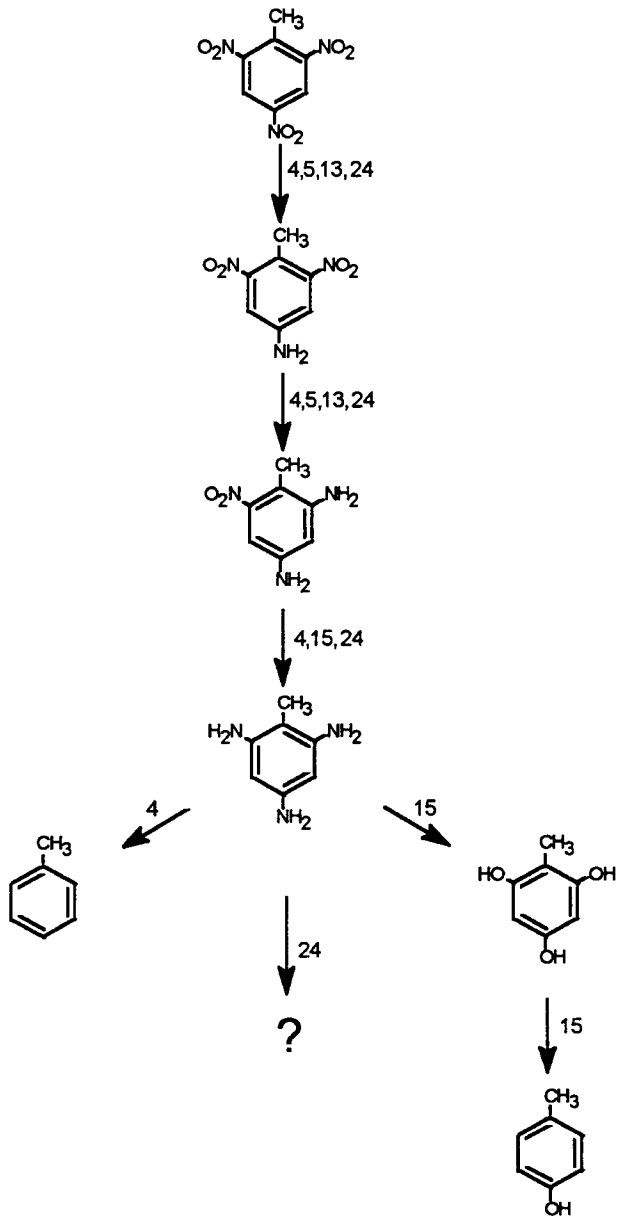
Boopathy et al. (5) examined the anaerobic removal of TNT under different electron accepting conditions in a soil-bacterial consortium. Under nitrate reducing conditions, 82% of the TNT was removed from contaminants with 100 ppm of TNT. Under sulfate reducing conditions, 30% of 100 ppm was removed. Using CO_2 as an electron acceptor, 35 % of 100 ppm TNT was removed. In these experiments, TNT did not serve as a carbon or primarily energy source, but its removal was achieved by co-metabolism with the energy source. The TNT intermediate reductions were 4A26DNT and 2A46DNT, wherein the former was predominant.

Boopathy and Kulpa (3,4) used a sulfate-reducing bacteria, *Desulfovibrio sp.* (B strain), isolated in an anaerobic reactor to study the degradation of TNT. The isolate used TNT as a nitrogen source in the presence of pyruvate and sulfate and/or as an energy source in the absence of sulfate. It was reported that TNT was first converted to 4A26DNT and to 24DA6NT. These intermediates were further converted to toluene by a reductive deamination process via triaminotoluene during 45 days of incubation.

Funk et al. (13) examined the bioremediation of soils contaminated with explosives under anaerobic conditions. Removal of TNT from the soil cultures occurred within four days, and both 4A26DNT and 24DA6NT were produced from TNT within 10 days under optimum conditions (30°C at pH 6.5). After 90 days of incubation, the removal of TNT, 4A26DNT, and 24DA6NT were accomplished, leaving several unidentified metabolites. Evidence of hydroxylation products such as p-cresol and 2,4,6-trihydroxytoluene was detected.

Preuss et al. (24) isolated a sulfate-reducing bacterium using TNT as the sole nitrogen source. An organism grown with pyruvate and sulfate as the energy source served to reduce TNT to triaminotoluene (TAT). The reduction of DAMNT to TAT was a rate-limiting step and was conducted only under anaerobic conditions. The reduction of DANT to DAHAT was initiated by unspecified hydrogenase enzymes, and further reduction to TAT was catalyzed by sulfite reductase inhibited by carbon monoxide.

In summary of the anaerobic conditions, there was no evidence of ring cleavage, but TNT reduction rates were faster than when exposed to aerobic conditions. The polymerization of HADNT was negligible or was not detected. Furthermore, complete TNT reduction to TAT was accomplished only for anaerobic conditions (24). Sulfite reductase, nitrite reductase, or hydrogenase catalyzed the reduction of nitro groups. The reduction of para-positioned nitrogroups was followed by ortho-positioned groups. As observed by Boopathy and Kulpa (5), the TNT removal rate under conditions of denitrification was higher than other conditions when TNT was involved in cometabolism. Figure 1.2 represents integrated pathways of TNT degradation for different energy sources exposed to anaerobic processes. Desirable results for the anaerobic degradation of explosives include deaminization processes when TNT acted as the nitrogen source (3, 4, 10), and hydroxylation of nitro groups of TNT by unspecified enzymes (13, 25).



Numbers next to arrows refer to references.

Figure 1.2 Integrated pathways of TNT degradation under anaerobic conditions.

1.4 Conclusions for both aerobic and anaerobic processes

a. The reduction of nitro groups to amino groups proceed through the nitroso- and hydroxylamino-compounds under both aerobic and anaerobic conditions.

b. TNT is readily reduced to amino compounds under both conditons, but the complete reduction of TNT nitro groups (i.e., the third nitro group) occurs only under strictly anaerobic conditions.

c. Reduction of nitro groups to amino groups takes place faster under anaerobic than aerobic conditions.

d. Production of highly reactive hydroxylamino intermediates leads to the formation of azoxy linkages as dimerization or polymerization under aerobic conditions. This indicates that rapid reduction under anaerobic conditions prevents the formation of azoxy compounds.

e. Nitro group removal from the aromatic ring occurs when microorganisms are able to utilize TNT as a nitrogen and/or carbon source.

f. Reduction of nitro groups to amino groups usually occurs first for para-positioned nitro groups, followed by ortho-positioned groups.

g. Several studies using white-rot fungus presented mineralization of TNT (i.e., up to 35%) to CO_2 in aqueous cultures containing low concentrations of TNT.

h. No evidence of ring cleavage was found under anaerobic conditions.

I. No mass balance study has been accomplished for both aerobic and anaerobic conditions.

1.5 Discussion

Based upon aerobic conditions, the production of unstable hydroxyamino intermediates leads to the formation of azoxy compounds and in turn to polymerization. The polymerized compounds are more resistant to biological attack and and are strongly bound to organic materials. Furthermore, the uncompleted reduction of nitro groups leaves intermediates such as MADNT and DAMNT, which can be more toxic to animals and fish than the parent compounds. *P. chrysosporium* is the most promising agent of TNT degradation under aerobic conditions, but since this fungus is highly sensitive to high TNT concentartions (i.e., >20 ppm), there are limiting factors in its ability to act as an agent of TNT bioremediation for contaminated sites. Since many contaminated soils contain grams per kilogram quantities of explosives (30), it may prove to difficult to protect the fungus.

Under anaerobic conditions, even when there is no evidence of the mineralization of explosives, the most promising explosives degradation agents, as established in several studies (5, 10, 13), proved to be nitrogen-free end products such as toluene or p-cresol. These agents are known to be readily degraded under either aerobic or anaerobic conditions by the hydroxylation of an adjacent ring position (11). In addition, anaerobic conditions provide a more rapid reduction rate with less evidence for the polymerization of intermediates than provided under aerobic conditions (24). The problems of this transformation, including p-cresol formation as a TNT intermediate under anaerobic conditions, are specialized and are not likely to provide a major pathway under natural conditions. To overcome these problems and

draw advantage from anaerobic conditions, isolation of the specific microorganism that is responsible for TNT transformation should be conducted.

For both anaerobic and aerobic conditions, the analytical methods used for most TNT transformation intermediates have been well developed (29), but their utility for the further reduction of TAT products under anaerobic conditions and of the oxidation of TNT to CO_2 under aerobic conditions remains in issue, leaving evidence that these processes are more polar than their parent compounds.

1.6 Statement of purpose

Based upon the previous results of TNT biodegradation, TNT transformations with ruminal microorganisms and an isolate, G.8, are investigated. This study is directed to: 1) identify pathways of TNT transformation with incubation of rumen fluid.; 2) explore the feasibility of using ruminal microorganisms for the decontamination of TNT contaminated soil; 3) isolate a pure culture to avoid complexities of rumen contents matrix; 4) determine TNT transformation pathways by the isolate.; 4) investigate the effects of the different energy sources (i.e. lactate, acetate and formate as electron donor; nitrate, nitrite and absence of primary electron acceptor) on TNT transformations in a batch and continuous flow bioreactor.

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Chapter 2. The Fate of ^{14}C-Trinitrotoluene(TNT) Biodegradation by Sheep Rumen Fluids

2.1 Abstract

In vitro TNT biotransformation with incubation of whole rumen fluid(WRF) of fisturated sheep was investigated. TNT transformation was completed within one hour. The metabolites are designated as 24DA6NT, TAT, and Unknowns. Efforts to identify the unknowns of the rumen fluid metabolites continue.

2.2 Introduction

A number of studies has been conducted for TNT bioremediation because of its toxicity and public concerns. Under aerobic conditions, the bacteria isolated from explosives-contaminated sites or mixed cultures produced small amounts of $^{14}CO_2$ from ^{14}C-TNT. Composting did not present any evidence of ring cleavage and monoaminodinitrotoluene (MADNT), diaminomononitrotoluene (DAMNT), and azoxy compounds were unaffected. The fungus was unable to tolerate amounts greater than 20ppm of TNT (10,17). Under anaerobic conditions, there was no evidence of ring cleavage, but TNT reduction rates were faster than when exposed to aerobic conditions. The polymerization of the TNT metaboliotes was negligible or was not detected. Furthermore, complete TNT reduction to triaminotoluene (TAT) was accomplished only for anaerobic conditions (16). Desirable results for the anaerobic degradation of TNT include deaminization processes, when TNT acted as

the nitrogen source (2,5), and hydroxylation of nitro groups of TNT by unspecified enzymes (7).

The predominant rumen bacteria are strict anaerobes with redox potential as -420mV in rumen contents (8). Rumen bacteria has shown high potential degradablity of antibiotic and xenobiotic compounds. For example, anaerobic lignocellulose degradation was presented in relation to the digestion of ruminant in an early study (15). The destruction of some pesticides such as parathion, p-nitrophonol and thiono isomers also was presented by Cook (4). The degradation of biphenyl type compounds such as dehydrodivanillin was obtained by Wei (19). Wachenheim (18) found the complete biotransformation of nitrogen containing heterocyclic plant toxics such as pyrrolizidine alkaloids.

The destruction capacities of rumen bacteria should be most promising for the feasibility of biodegrading TNT contaminated sites because of the strict anaerobic conditions and high degradation capacities of environmentally persistant compounds.

In this experiment, the fate of TNT was investigated with incubation of whole rumen. The purposes of this study is the identification of specific metabolites to define the pathway of TNT destruction by ruminal microorganism.

2.3 Materials and methods

2.3.1 Chemicals

Radiolabeled TNT (ring labeled) was purchased from Chensyn Science Laboratories (Lenexa, KS), and non-radioactive TNT was obtained from Chem

Service. Two diaminomononitrotoluene congeners (24DA6NT and 26DA4NT) were obtained from International Chem. Two monoaminodinitrotoluene congeners (2A46DNT and 4A26DNT) and TAT were purchased from Aldrich Chemical Co. All chemicals for experimentation were HPLC (high performance liquid chromatography) grade.

2.3.2 Medium

The rumen contents for the study of in vitro TNT biotransformation were collected from healthy rumen-cannulated ewes at the Animal Isolation Lab, Oregon State University, Corvallis, OR. Approximately 40 grams of combined ruminal solids and liquor were removed and transported to the laboratory. The ruminal contents were anaerobically blended with a 90% buffer solution for three minutes in a mechanical blender. The blended ruminal contents were transferred into 150 ml serum bottles under an argon gas flow, and 10 mg of TNT in ethanol was added to each serum bottle containing 100 ml of growth medium and stoppered with a rubber stopper. The in vitro assay buffer contained 4.0 grams of Na_2CO_3, 0.57 grams of KCl, 9.3 grams of Na_2HPO_4, 0.47 grams of NaCl, 0.1 grams of $MgSO_4 \cdot 7H_20$, 0.04 grams of $CaCl_2 \cdot 2H_2O$, 2.64 grams of $(NH_4)_2SO_4$, and 0.002 grams of resazurin placed in a one-liter container of deoxygenazed water. Bottles containing a 10 % rumen fluid mixture and 100 ppm of TNT were incubated at 38°C and sampled at time intervals. Aliquots of a sample were indexed and frozen at -4°C until analysis by HPLC or other methods.

2.3.3 Analysis of the experiment

The gradient HPLC system used an Alltech RP-100 (150 mm × 4.6 mm) column. Mobile phase A was 10% methanol in 5 mM solution of H_3PO_4. Mobile phase B was 90 % methanol in a 5 mM solution of K_3PO_4. The flow rate was one ml/min. The linear gradient was started one minute following injection and reached 100 % B at 21 minutes and was maintained there for 29 minutes. The outflow from the column was first flowed through a UV detector set at 221 nm (Beckman model 160) and then into a Radiomatic A140 radiochemography detector. The A140 was configured for a 500 ml flow cell, and liquid scintillant (Packard Flo-Scint V) was mixed with it in a ratio of 4:1. Data from both detectors were collected using a Perkin-Elmer PE7500 data system running *Chrom III* software. A manual injection valve was used to inject 200 ml of the sample. Sample preparation was confined to centrifugation of sedimentary solids and injection of the supernate.

Mass spectrometry analysis was performed using a Hewlett Packard model 5988A connected to a 5890 Gas chromatography (GC). The GC column was an XTI-5 fused silica capillary column. Heliumn, at flow rate of 20 ml/min, was used as the carrier gas. A 3 ml aliquot of chloroform extract was injected into the gas chromatographer (with injection port temperature of 250°C). The column was held at 45°C for two minutes and was ramped to 120°C at a rate of 10°C/min. It was held at this temperature for one minute, and then ramped to 260°C at a rate of 20°C/min. The mass spectrometer ion source was turned four minutes after the initiation of the temperature program. The interface line to the ion source was held at 280°C

throughout the run. The mass spectrometer was operated using an ionizing voltage of 70 eV and an ionizing current of 300 mA.

2.4 Results

Incubation of the tubes at 38°C containing 13.8% of ^{14}C-TNT and non-radio labelled TNT in 127 mg/l of total concentration was terminated by freezing the tubes. One ml tube samples were centrifuged (18000 xg) for six minutes and analyzed by HPLC. The chromatogram of the analysis is illustrated in Figure 2.1 (minutes intervals) and Figure 2.2 (day intervals). The ^{14}C-TNT completely disappeared within one hour, leaving MADNT and DAMNT as intermediates. After five days incubation, ^{14}C-TNT added to the supernatant was transformed into unidentified products which were retained at 2, 15, and 16.5 minutes, respectively, in the HPLC system.

To identify the transformation products of TNT degradation by the ruminal organism, only non-radio labeled TNT was added to a 10% rumen fluid with glucose as a carbon source, and incubated at 30°C for two days. The supernant was extracted with $CHCl_3$(1:1). The condensed extract was injected into GC/MS.

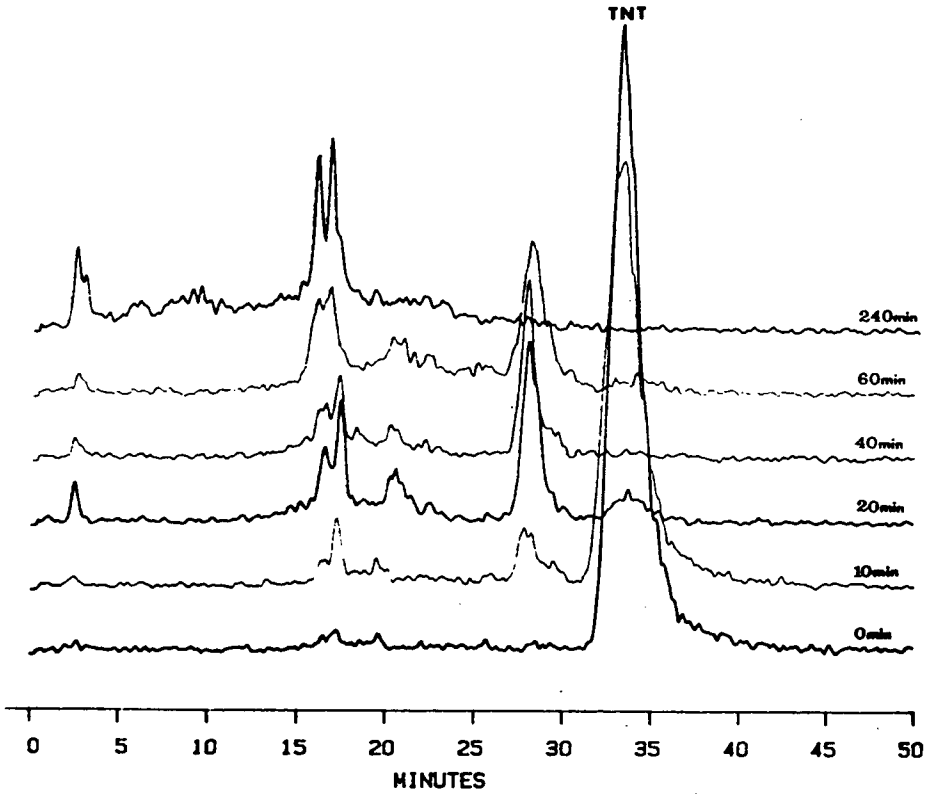


Figure 2.1 Chromatogram of Whole Rumen Fluid (WRF) Incubated with ^{14}C-TNT in minutes scale.

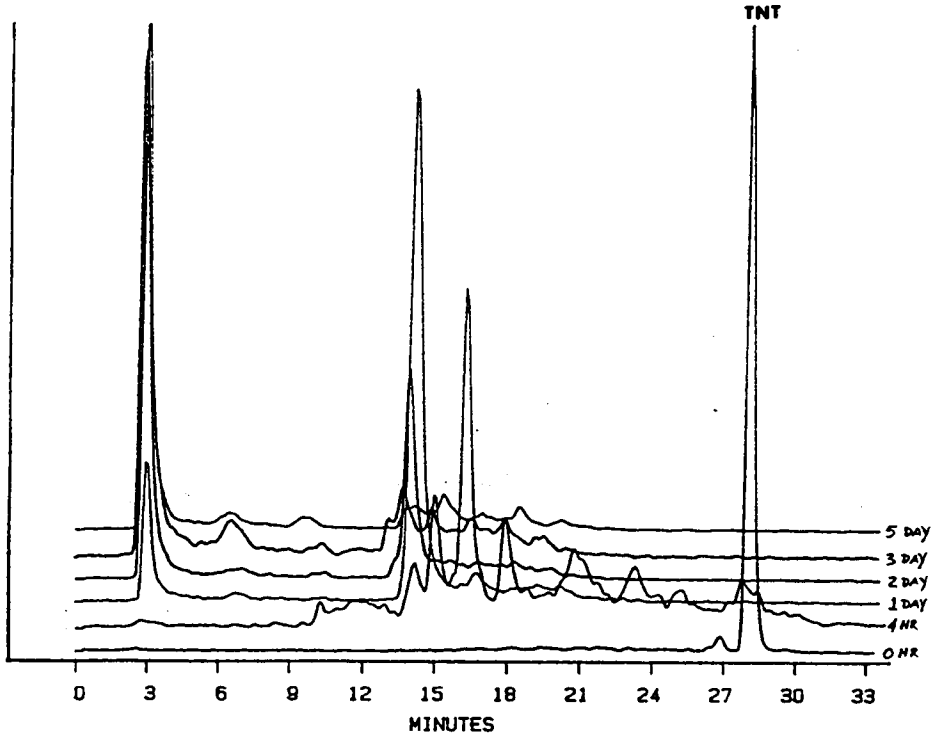
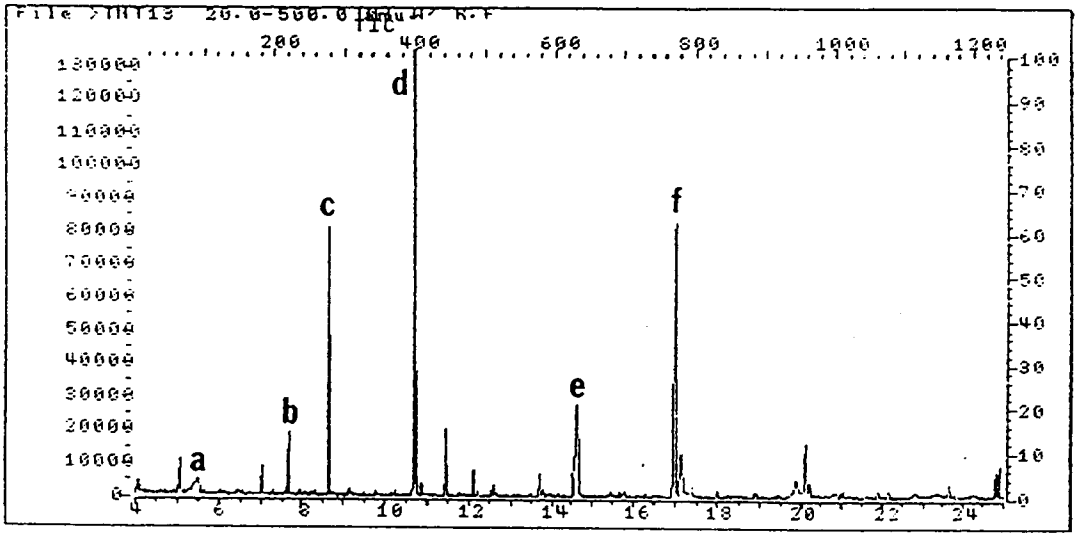


Figure 2.2 Chromatogram of Whole Rumen Fluid (WRF) Incubated with ^{14}C-TNT in days scale.



a	butanoic acid	**c**	p-cresol	**e**	TAT
b	phenol	**d**	indole	**f**	24DA6NT

Figure 2.3 GC/MS chromatogram of TNT metablites in Whole Rumen Fluid (WRF).

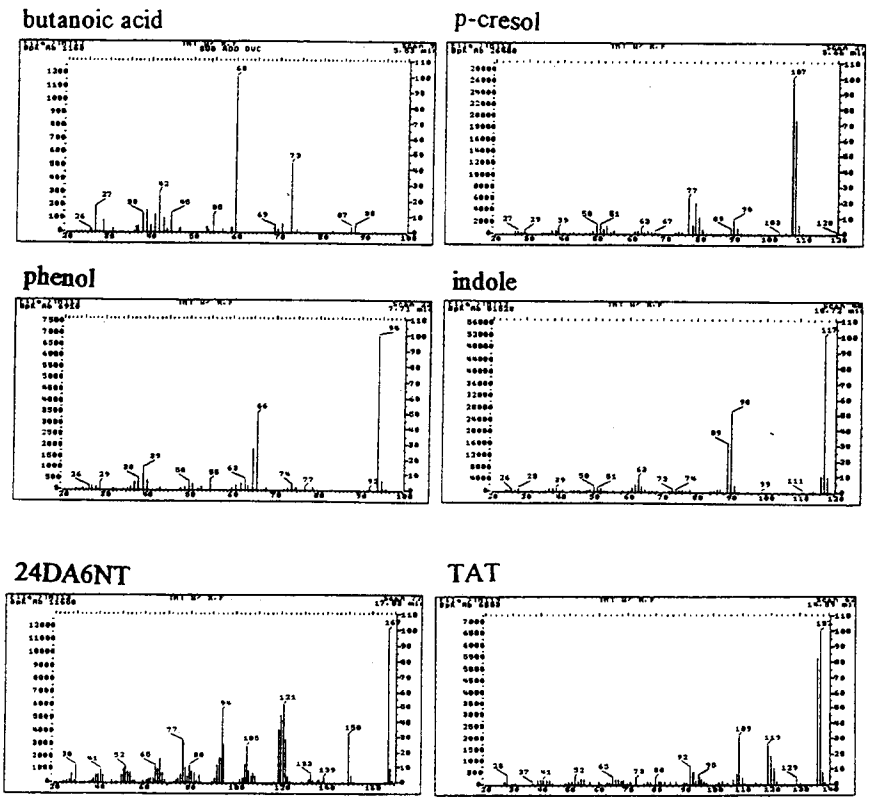


Figure 2.4 Mass spectrum of TNT metablites in Whole Rumen Fluid (WRF) Extract.

The major peaks of the GC/MS chromatogram were identified as p-cresol, phenol, indole, 24DA6NT, TAT, and butanoic acid, of which TAT and 24DA6NT have been identified as TNT intermediates (3,16) and p-cresol has been detected as a TNT intermediate in anaerobic cultures (7). The chromatogram and mass spectrum of each peak of the extract are shown in Figure 2.3 and Figure 2.4.

In the same manner discribed above, a blank sample was incubated and extracted with $CHCl_3$. The major peaks of the extract injection to GC/MS were identified as p-cresol, phenol, indole, and butanoic acid. When the retention time (3 minute) of TAT standard in the HPLC system is considered, the metabolite peak at 2 minute of Figure 2.1a is suspected as a more polar compound than TAT. From examination of TNT degradation for WRF, results indicated that the complete conversion of TNT occurred in less than one hour and that the TNT transformation extended futher than than for TAT. The identification of the end product is currently under investigation with evidence provided from GC/MS analysis.

2.5 Conclusions and discussion

The process of identifying in vitro metabolites for TNT degradation for rumen fluids has not been completed. To this point in the investigation, results are promising, leaving 4A26DNT, 24DA6NT, TAT, or other more polar end products than TAT as intermediates. The fast conversion rate of TNT demonstrated in these experiments represents the merits of TNT degradation by ruminal bacteria, as well as the tolerance of rumen bacteria to high TNT concentrations (> 100 mg/l).

Table 2.1 Incubation periods of the microbes for complete TNT conversions.

Year	Investigator(s)	Condition	Microorganism	Incubation	Concentration
1972	Osmon & Klausmeier.	Ae	Sewage Sludge	6days	100 ppm
1973	Klausmeier et al.	Ae	Gram negative	?	50 ppm
1974	Won et al.	Ae	Pseudomonas	1day	100 ppm
1976	McCormick et al.	Ae	E. coli	?	50 ppm
1982	Kaplan & Kaplan	Ae	Composting	91days	?
1990	Fernando et al.	Ae	White rot fungus	18 days	1.3ppm
1991	Kulpa et al.	Ae	Soil bacteria	70 days	100 ppm
1991	Williams et al.	Ae	Composting	60 days	12000 mg/kg
1992	Spiker et al.	Ae	White rot fungus	27 days	20 ppm
1993	Duque et al.	Ae	Pseudomonas	28 days	100 ppm
1994	Michels & Gottschalk	Ae	White rot fungus	4 days	20.36 ppm
1976	McCormick et al.	An	E.coli,cell extract	?	50 ppm
1992	Boopathy & Kulpa	An	Denitrifying	30 days	100 ppm
1993	Boopathy et al.	An	Sulfate reducing	8 days	100 ppm
1993	Preuss et al.	An	Sulfate reducing	?	45 ppm
1993	Funk et al.	An	Soil bacteria	24 days	120 ppm

These results represent the most rapid rates of conversion among all TNT biodegradation studies conducted heretofore (Table 2.1). However, the utilization of rumen microbials for TNT degradation is a complicated process since bacteria capable of TNT degradation should be interdependent, and the rumen fluid matrices are complex in this sense. The utilization of the isolate is desirable and feasible for TNT degradation.

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Chapter 3. Feasibility of Trinitrotoluene (TNT) Contaminated Soils Remediation with Ruminal Microorganisms

3.1 Abstract

The feasibility of TNT degradation by ruminal microorganisms in TNT inoculated soil was examined. Thirty-five percent of the TNT was biotransformed within 5 days during which time rumen fluid was sequentially added. The remaining TNT was not recoverable from the soil, indicating irreversible TNT transformation by soil organic matter.

3.2 Introduction

2,4,6-trinitrotoluene (TNT) exposure potentially can cause adverse human health effects which has generated strong public attention (5, 10). More than 1,100 military facilities, each potentially contaminated with munitions wastes, are expected to require treatment of more than one million cubic yards of contaminated soils (1). The cost associated with remediation of these sites has been estimated to be more than $1.5 billion (8). Biological remediation may provide the most economic and reliable solution for remediation of explosive-contaminated soils (8, 9). The polymerization of TNT intermediates experienced during aerobic incubation results in azoxy compounds that are tightly bound to soil organic materials, highly stable, and difficult to treat (8). TNT degradation under anaerobic conditions appears to both avoid polymerization and achieve complete reduction of nitro groups, although ring cleavage has not been

observed (2, 3, 6, 11). Recently, ruminal organisms demonstrated the ability to destroy some pesticides, such as parathion and p-nitrophenol; biphenyl-type compounds; thiono isomers; and nitrogen-containing heterocyclic plant toxins such as the pyrrolizidine alkaloids (4, 12, 14). Based upon the results of these previous studies, the present research was directed at the feasibility of using ruminal microorganisms for the decontamination of TNT-contaminated soils.

3.3 Experiment

For this experiment, an amity-series soils (somewhat poorly drained mollisol) were collected and dried at 105 °C for one day. TNT was injected into the dried soil. Combined ruminal solids and liquid were removed from the rumen of healthy cannulated ewes and filtered with cheese cloth under an argon stream. The *in vitro* assay buffer was identical that used by Wachenhiem et al. (12). Sample preparation was conducted in an anaerobic glove box (5% H_2:95%CO_2). The bottle sets designated as A, B, C, and D received 1 ml inoculations of buffer, buffer, autoclaved rumen fluid, and rumen fluid, respectively, for 5 days (Table 3.1). Aliquots of sample supernants were collected at time zero and after 5 days of incubation. The soil mixture for samples B, C, and D were centrifuged for 10 min (3000 xg) and then extracted with methanol in a Soxhlet extractor for 3 days. The supernant and methanol extracts were stored at -4 °C for analysis. TNT was measured by high-performance-liquid-chromatography (HPLC), using two analytical columns (LC-18 and LC-CN) connected in series (13).

3.4 Results

TNT concentrations for each condition at time 0 and 5 days are shown in Figure 3.1. Four bottles were prepared for each condition. One hundred percent of the initial TNT was recovered from A, but TNT disappearances from the other conditions varied from 65% to 100 %. TNT was not detected in the methanol extract from the soil. The final normalized concentrations, C/C_o, of A, B, C, and D replicates were averaged and measured as 1.01, 0.36, 0.31, and 0, respectively. The results indicate that about 65% of the initial TNT was sorbed or reacted to the soil. The losses of TNT in B and C were similar (p-value: 0.3215), representing no abiotic TNT transformation by rumen fluid. One hundred percent recovery of TNT in A showed TNT stability in the buffer solution during the experiment. The disappearance of TNT in D showed that the remaining 35% of initial TNT was transformed by the attack of ruminal microorganisms.

3.5 Conclusions

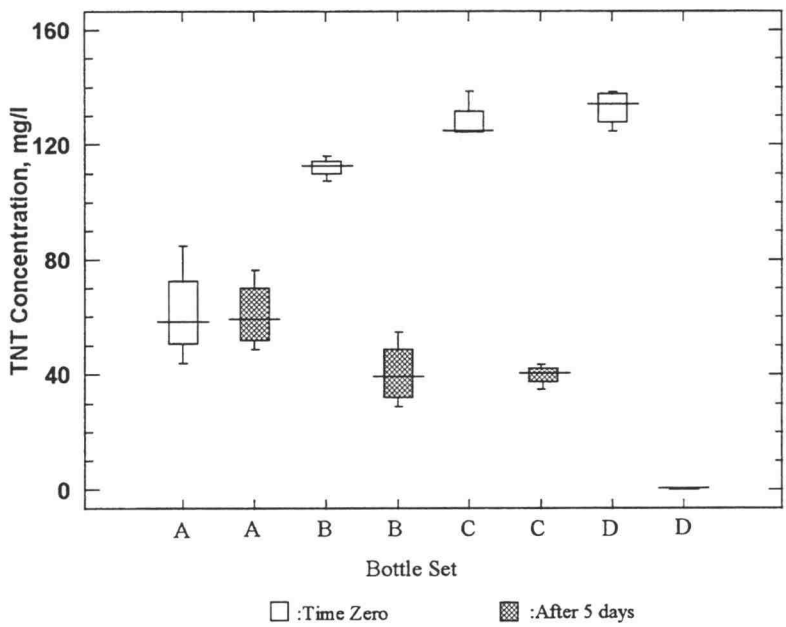
These results indicated that ruminal microorganisms can maintain their metabolisms as well as remediate TNT contaminated soils. This note verifies ruminal animals as another source of microorganism for the fast and reliable decontamination of TNT-contaminated soils or groundwater.

Table 3.1 Sample conditions for examining the feasibility of rumen fluids

Bottle Set	Buffer (ml)	Soil (grams)	Autoclaved Rumen Fluids (ml)	Rumen Fluids (ml)
A	10+5*			
B	10+5*	5		
C	10	5	+5*	
D	10	5		+5*

*: 1 ml of the additives are injected into each bottle each day for 5 days.

The sorption of TNT by soil is known to depend upon donor or acceptor group on the soil organic matter, not necessarily the organic matter fraction (7). In this study, TNT was not detected in soil extracts, indicating irreversible reaction of TNT with the donor or acceptor portion of soil organic matter or irreversible sorption. Suggested future studies include metabolic pathways of the ruminal consortium responsible for TNT degradation and the effect of soil characteristics on TNT sorption or transformation.



: A box encloses the middle 50% of the data values. A cross line in the box is the median of four data points. (Box-and-Whisker Plot of STATGRAPHICS Statistical Graphic Software)

Figure 3.1 The effects of ruminal microorganisms on TNT degradation.

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Chapter 4. Trinitrotoluene (TNT) Transformation Pathways under Denitrification Conditions by Ruminal Microorganism, *G.8*

4.1 Abstract

A nitrate-reducing bacteria, *G.8*, was isolated from ruminal microorganisms and grown with lactate and nitrate as primary energy sources, utilizing 2,4,6-trinitrotoluene (TNT). In the presence of nitrate within the medium, nitrite depletion was occurred and the reduction of the nitro group, followed by deaminization, was identified as the principal mechanism of TNT destruction by the isolate. This transformation was dependent on whether nitrate or nitrite was present as the electron acceptor. Nitrates within the medium stimulated the TNT para-position nitro group, whereas nitrites catalyzed the deaminization and hydroxylation processes of the amino group. In the absence of other terminal electron acceptor contributions, the transformation of the ortho-position nitro group was rapid. Thus, the control of energy sources in the medium can be used to produce nitrogen-free compounds such as o-cresol or toluene from TNT. These findings are helpful for understanding the process of TNT biodegradation and may potentially assist the development of a decontamination process for TNT-contaminated soil or ground water.

4.2 Introduction

2,4,6-trinitrotoluene (TNT) has been widely used for the production of explosives because of its low boiling point, high stability, low impact sensitivity, and safe manufacture (26). TNT is known to produce adverse health effects from occupational exposure including increased incidences of aplastic anemia, liver damage, dermatitis, ocular disorders, and gastrointestinal distress (10, 26). In addition, TNT is of ecological concern based on its toxicity to certain aquatic organisms (28). More than 1,100 military facilities, each potentially contaminated with munitions waste, are expected to require treatment of more than one million cubic yards of contaminated soils (1). The cost associated with remediation of these sites has been estimated to be in excess of $1.5 billion (22).

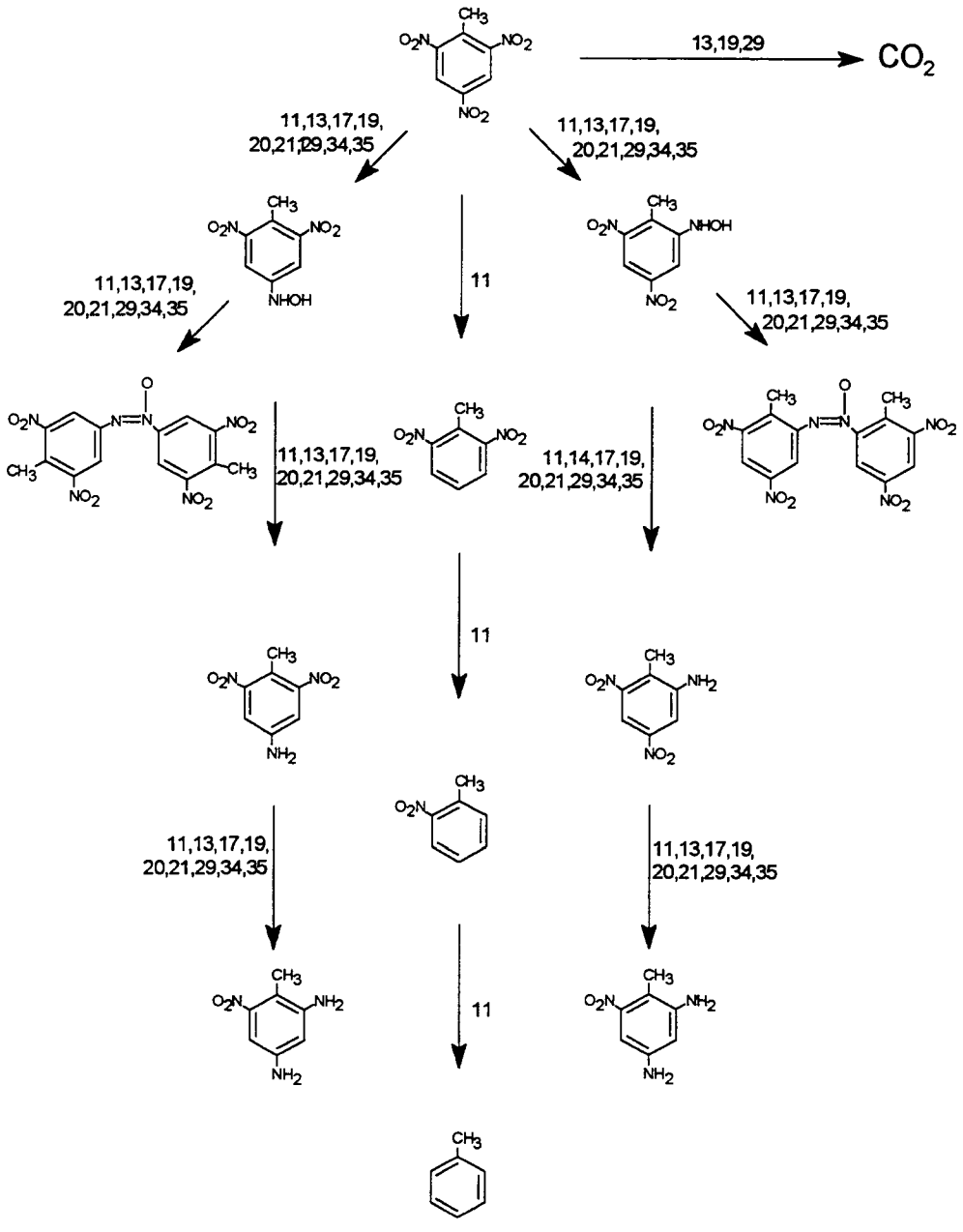
Incineration is an attractive technology to remediate explosives because of its high treatment efficiency and wide applicability. However, incineration is costly (estimated at $800/ton) and energy-intensive, and results in large volumes of end product (14). Chemical oxidation of explosive wastes is often difficult because of the need to contain unreacted materials. Biological remediation may provide an economical and reliable solution for remediation of explosive-contaminated soils (22, 23, 25). However, biological methods may require long times for treatment, and the addition of organic substrates, bulking agents, inoculum, and nutrients (34).

4.2.1 Aerobic processes

Various organisms, including *Phanerochate chrysosporium, Pseudomonas sp.*, or other isolates from contaminated sites, have been used for aerobic or anaerobic

degradation of TNT. Aerobic bacteria isolated from explosive-contaminated sites or mixed cultures has produced small amounts of mineralization of TNT in the concentration range from zero to 3.1%. Composting was shown to result in no ring cleavage or degradation of the monoaminodinitrotoluene(MADNT) congeners, diaminomononitrotoluene (DAMNT) congeners, and azoxy compounds. The white-rot fungus *Phanerochate chrysosporium* was able to oxidize TNT to CO_2 with concentration reductions of from 10 to 35%. It was unable to tolerate greater than 20 ppm of TNT (18, 29). In addition, the veratryl oxidase activity of lignin peroxidase is inhibited by hydroxylaminodinitrotoluene (HADNT), a metabolic product of TNT degradation (21).

The integrated pathways of TNT degradation for aerobic conditions indicates that TNT is slowly reduced to amino, diamino, and azoxy compounds by various microorganic species including bacteria and fungi (Figure 4.1). It is expected that the reduction of the nitro group on the aromatic ring takes place in oxygen-insensitive enzymes with the ability to catalyze obiligate two-electron substrate reductions (6). The most serious problems experienced from aerobic degradation of TNT is that the intermediates are easily polymerized by the nonenzymatic HADNT coupling processes (7, 35). These intermediate azoxy compounds are tightly bound to soil organic materials resulting in high stability and difficult treatment.



Numbers next to arrows refer to references.

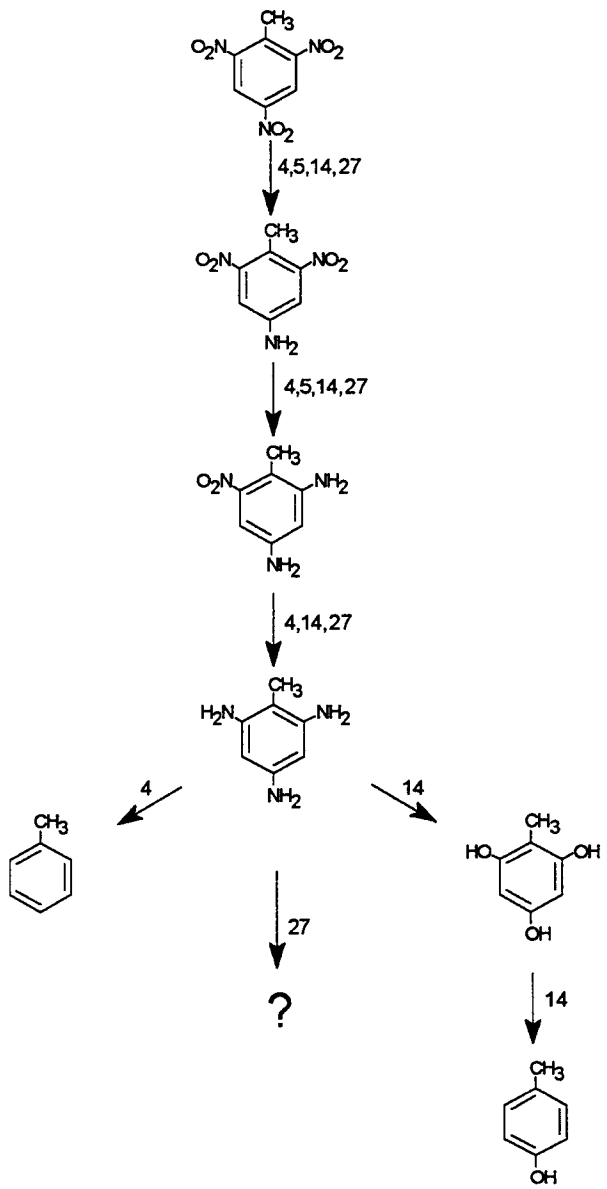
Figure 4.1. Summary of previously observed trinitrotoluene (TNT) transformation pathways under aerobic condition.

4.2.2 Anaerobic processes

Recently TNT degradation under anaerobic conditions has also been the subject of intense study. Various microorganisms, including denitrifying, sulfate reducing, methanogenic, and specific enzymes have been used to examine the feasibility of TNT degradation under anaerobic conditions. Complete TNT reduction to TAT has been accomplished only under anaerobic conditions (27). In these processes, the reduction of para-positioned nitro groups was followed by the reduction of ortho-positioned groups. TNT degradation pathways for different anaerobic electron acceptors and donors are shown in Figure 4.2. Results from the anaerobic degradation of explosives have included deaminization of TNT intermediates with subsequent use of the ammonia as a nitrogen source (4, 11), and the replacement of TNT nitro groups by hydroxyl groups using unspecified enzymes resulting in the production of p-cresol (14). As observed by Boopathy and Kulpa (3), TNT removal rates under denitrification conditions were higher than for conditions using either sulfate or carbon dioxide as the electron acceptor.

In summary, anaerobic degradation is promising for treatment of explosive wastes, resulting in nitrogen-free end products such as toluene or p-cresol (4, 14). These compounds are known to readily degrade under aerobic conditions and possibly anaerobic conditions by the hydroxylation of adjacent ring position (12). In addition, anaerobic conditions provide more rapid reduction rates with less evidence for the

polymerization of intermediates than aerobic conditions (27). Ring cleavage has not been observed.



Numbers next to arrows refer to references.

Figure 4.2. Summary of previously observed trinitrotoluene (TNT) transformation pathways under anaerobic conditions.

Recently, researchers have studied ruminal microorganisms in relation to their ability to degrade xenobiotic compounds. Many of these organisms are strict anaerobes with optimal redox potentials as low as -420 mV (16). Ruminal organisms have been shown capable of destroying some pesticides, such as parathion, p-nitrophenol, and biphenyl-type compounds; thiono isomers, (8, 33); and nitrogen-containing heterocyclic plant toxins such as the pyrrolizidine alkaloids (31). Many of these compounds have structures similar to TNT.

A TNT-degrading ruminal microorganism has been isolated from goat rumen fluid with successive enrichments on triaminotoluene (TAT) and TNT. The isolate, designated *G.8*, utilizes nitrate and lactate as the primary energy source. *G.8* was able to tolerate and metabolite levels of TNT up to the saturation point of 125 mg/l.

4.2.3 Objectives

Based upon the results of previous studies, the present research was directed at understanding the process of TNT degradation by the denitrifying ruminal microorganism *G.8*. The objectives of this study were: 1) to identify specific metabolites and define TNT destruction pathways and 2) to understand TNT and the metabolites transformation mechanisms on the different primary electron acceptors.

4.3 Materials and methods

4.3.1 Chemicals

TNT was obtained from Chem Service (West chester, PA), two monoaminodinitrotoluene congeners (2-amino 4,6-dinitrotoluene and 4-amino 2,6-dinitrotoluene) was from the Radian Corporation (Austin, TX). Toluene, and o-cresol were purchased from the Aldrich Chemical Co (Milwaukee, WI). Two dinitrotoluene congeners (2,4-dinitrotoluene and 2,6-dinitrotoluene), 2-amino,6-nitrotoluene, 2-nitro toluene, and 2-aminotoluene were purchased from Jassen Chemical (Gardena, CA).

4.3.2 Growth medium

The media used to incubate the isolate in serum bottles consisted of (mg/l) $CH_3CHOHCOONa$ (500), $MnSO_4.H_2O$ (8.5) $FeSO_4.7H_2O$ (10), KNO_3 (2000), $ZnSO_4.7H_2O$ (5), $CaCl_2.2H_20$ (24), $Na_2HPO_4.H_2O$ (2550), H_3BO_3 (1.5), $NaMo_4.2H_2O$ (1.5), $NaH_2PO_4.H_2O$ (975), $CoCl_2.6H_2O$ (0.6), Na_2EDTA (21.5), yeast extract (100), $CuCl_2.2H_2O$ (0.05), $MgSO_4.7H_2O$ (30), and $NiCl_2.6H_2O$ (1). The nutrients were mixed and well-stirred. pH was adjusted to 7.0 with 0.1 N NaOH, then boiled for 3 minutes under argon gas flow and dispensed into serum bottles, which were then stoppered and autoclaved for 25 minutes. To prevent the precipitation of salts from the medium, 0.3 ml of 800 ppm $CaCl_2.2H_2O$ was injected following autoclaving. An 0.2 % inoculum was used for all experiments. Serum bottles were cultivated anaerobically at 37^oC for given periods of time using a mechanical shaker.

Ethanol was used to prevent contamination at the contact points for the inoculant and samples, and sample aliquots were indexed and frozen at -14°C.

4.3.3 Analytical methods

TNT, 4-amino,2,6-dinitrotoluene (4A26DNT), 2,6-dinitrotoluene (26DNT), 2-amino,6-nitrotoluene (2A6NT), and 2-nitrotoluene (2NT) were measured by high performance liquid chromatography (HPLC), using two analytical columns (LC-18 and LC-CN) connected in series and eluted isocratically at 1.0 ml/min with water-methanol-tetrahydrofuran (60.5:25:14.5) (32). 2NT, 2-aminotoluene (2AT), o-cresol, and toluene were measured using an LC-18 column isocratically at 1.0 ml/min with water-methanol (1:2). Column effluent outflows for all compounds utilized a UV detector (DIONEX 2000i) set at 250 ηm except toluene which was set at 210 ηm. A manual injection valve was used to inject 50 µl samples. The samples were centrifuged (2000 ×g, for 5 min) to remove sediments or flocs prior injection into the HPLC system.

Gas chromatography/mass spectrometry (GC/MS) analysis was performed using a Hewlett-Packard Model 5988A connected to a Model 5890 gas chromatography (GC) with a XTI-5 fused-silica capillary column. Helium at a flow rate of 20 ml/min was used as the carrier gas. Aliquots of 3 ml of methylene-chloride extract was injected into the GC (injection port temperature, 250°C). The column was maintained at 40°C for 2 minutes and then ramped to 120°C at a rate of 10°C/min, maintained at 120°C for 1 min, and then ramped to 260°C at a rate of

20°C/min. Four minutes following temperature program initiation, the mass spectrometer ion source was turned on and the interface line to the ion source was held at 280°C throughout the run. Prior to running samples, the mass spectrometer was calibrated and tuned using perfluro-tributylamine as the calibration compound.

A Dionex 4000i ion chromatography (IC) with a HPIC-CS3 anion column was used to measure anions such as nitrate and nitrite. The mobile phase contained 0.191 grams of Na_2CO_3 and 0.143 grams of $NaHCO_3$ in one liter of distilled water. For regeneration of the column, 0.07% H_2SO_4 was used. The flow rate was 2 ml/min. The outflow from the column was analyzed on a conductivity detector. An autosampler was used to inject 50 μl from autosampler vials containing 500 μl of sample, which was filtered through a 0.25 μm TPFE syringe filter prior to loading the autosampler vials.

Culture turbidity in the serum tubes was measured using a spectrophotometer at 660 ηm absorbance (Bausch and Lomb, Spectronic 20D) for cell-growth analysis. Blank samples (incubated in the same manner described for the samples, but without bacteria inoculation) were prepared to compensate for other sources of absorbance increments.

4.3.4 Microorganism

A TNT degrader was isolated from rumen fluid with successive enrichment of TNT and TAT. First, 10 grams of ruminal content were collected from a fistulated goat and anaerobically blended with 90 ml of the Modified McDougall's buffer (31). This mixture was then anaerobically centrifuged for 5 minutes at 4°C (16000 ×g). The

supernatant was anaerobically transferred to GPT100 (1.5 grams of glucose, 1.5 grams of maltose, 1.5 grams of cellobrose, 5.0 grams of trytone, 1.0 grams of yeast extract, 10 % clarified rumen fluid, and minerals in one liter) and dispensed to the tubes containing 100 mg/l of TNT. The tubes were incubated stagnant at 38°C. After four days, a 10 % inoculum was transferred to fresh GPT100. After 12 days, a 10 % inoculum was transferred to DTAT (semidefined TAT medium containing sodium formate, 10 % clarified rumen fluid, and minerals with 100 mg/l of TAT). After three days incubation, 10 % was transferred to fresh DTAT. The DTAT medium was modified (D3YN) by adding 20 mM KNO_3, 0.1 % yeast extract and 100 mg/l of TAT. This medium was inoculated from the third transfer of the goat enrichment in DTAT. After the 20th transfer of the goat enrichment in D3YN, the transfer was diluted in D3YN to 10^{-6} and spread to D3YN plates solidified with 1.6 % agar, prepared anaerobically in the grove box. The plates were placed in equilibrated anaerobic jars in the grove box and incubated at 38°C. This medium After one day, large, well-isolated, dark and red colonies were shown on the 10^{-7} dilution of D3YN agar. A colony was chosen, incubated to D3YN broth, and incubated for three days. The D3YN isolates were restreaked to D3TN agar (100 mg/l TNT in place of TAT), GPT100 agar, and DTP agar (7.5 mM TAT, minerals, vitamins and 2% agar). After five days incubation, there were tiny colonies on DTP agar that, when streaked to D3YN agar, formed large, red colonies that eventually developed into microcolonies. The isolated microorganisms from a fistulated goat was named *G.8*. The gram negative *G.8* was

identified based fatty acid analysis as a *Escherichia Coli* with similarity index (SI) of 91.4 percents. A single morphology of *G.8* are illustrated in Figure 4.3.

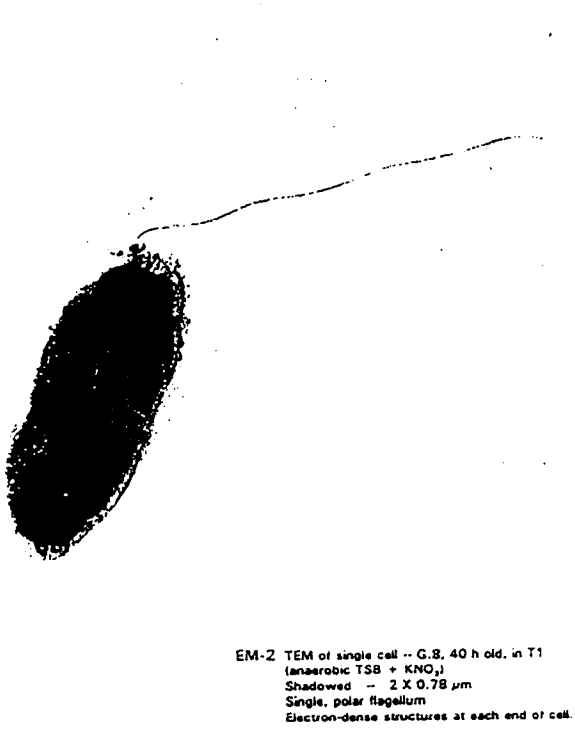


Figure 4.3. Transmission electron micrograph of G.8 isolated from goat rumen fluid.

4.3.5 *G.8* growth experiments with variable electron acceptors

The purpose of these experiments were to investigate the role of TNT and other terminal electron acceptors on the growth of *G.8*. Effects of the electron acceptor on the *G.8* growth were monitored by optical density. The compositions and concentrations of the growth medium are shown in Table 4.1. All growth medium in Table 4.1 contained 100 mg/l of yeast extract that could potentially act as energy source in addition to the lactate (15).

Table 4.1. Energy source compositions and concentrations in the growth medium.

Medium	Electron donor (mM)	Electron Acceptor (mM)
A	Lactate (4.50)	Nitrate (19.78)
B	Lactate (4.50)	Nitrite (19.78)
C	Lactate (4.50)	TNT (0.44)
D	Lactate (4.50)	Nitrate (19.78) + TNT (0.44)
E		Nitrate (19.78)
F	TNT (0.44)	Nitrate (19.78)
G	Lactate (4.50)	Ammonia (19.78)

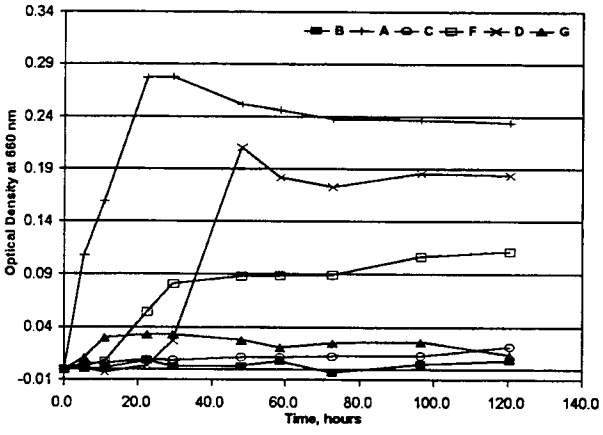
Note: The concentrations of yeast extract and minerals in the medium are identical as discribed in Materials and Methods except listed above.

4.4 Results

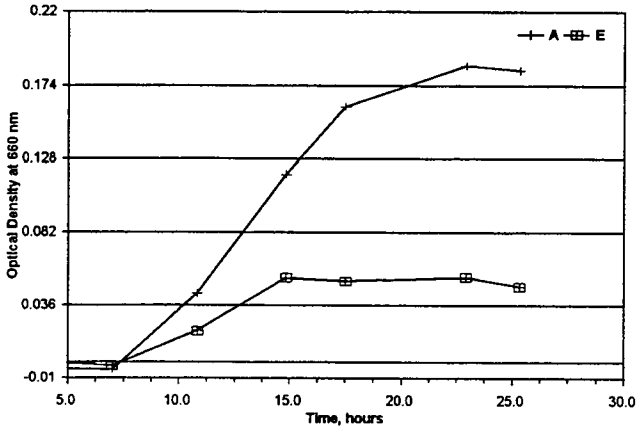
G.8 growth was monitored by optical density using different energy sources including nitrates, nitrites, or TNT to investigate the relation of TNT to *G.8* metabolism (Figure 4.4). *G.8* growth occurred in the presence of only nitrate, but not on nitrite, or TNT, indicating that nitrate serves as a viable terminal electron acceptor. Fermentation was not observed with the presence of lactate only in the medium. *G.8* growth was detected in the absence of lactate (TNT and nitrate present), suggesting that TNT could have been potentially used as the carbon source and electron donor (GRAPH A of Figure 4.4). From the other experiment (GRAPH B of Figure 4.4), *G.8* growth was observed in the medium without lactate (only nitrate present) as much as in the medium containing TNT and nitrate, probably from the use of the yeast extract as the electron donor and carbon source.

Additional experiments were conducted to investigate the influence of TNT on the utilization of primary energy source with *G.8* growth. GRAPH A and B of Figure 4.5 illustrate primary energy source concentrations without TNT and with 20 mg/l of TNT, respectively. In GRAPH A of Figure 4.5, nitrate was converted to nitrite completely within 10 hours, followed by further reduction of the nitrite. In GRAPH B of Figure 4.5, nitrate was converted to nitrite and then less transformation of the nitrite in the medium was observed when TNT is present.

GRAPH A



GRAPH B



The growth of *G.8* was monitored by the optical density with 660 ηm wavelength. Data was the average of the duplicate samples. Capitals represent the composition of the energy source; A: lactate and nitrate, B: lactate and nitrite, C: lactate and TNT, D: lactate, nitrate, and TNT, E: nitrate, F: TNT and Nitrate, G: lactate and ammonia. 100 mg/l of yeast extract was added into the medium in addition to the concentration of the energy sources (Table 1).

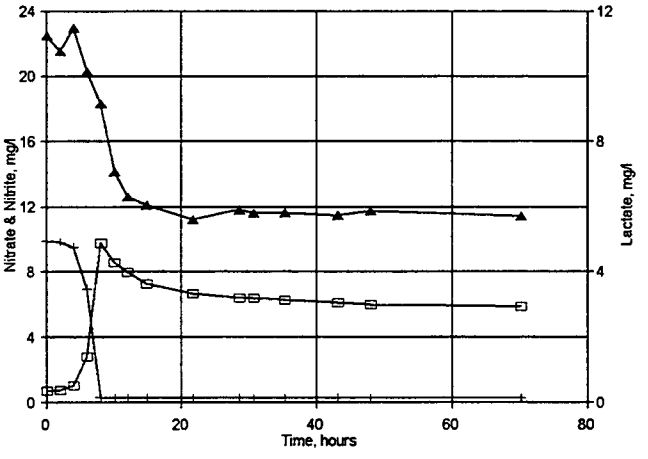
Figure 4.4. The effects of *G.8* growth with different combinations of the energy sources.

In the preliminary experiments (GRAPH A of Figure 4.4), *G.8* growth was not observed with nitrite or TNT as the primary electron acceptor, indicating *G.8* does not initially utilize the nitrite or TNT. When TNT was absent from the medium, further transformation of the nitrite from nitrate was observed (GRAPH A of Figure 4.5) and less transformation of the nitrite with presence of TNT in the medium (GRAPH B of Figure 4.5). This results demonstrate that nitrite or TNT conversions takes place in the presence of nitrate. It suppose that the nitrite and TNT appear to compete as cometabolic electron acceptors, with the TNT being preferrentially used.

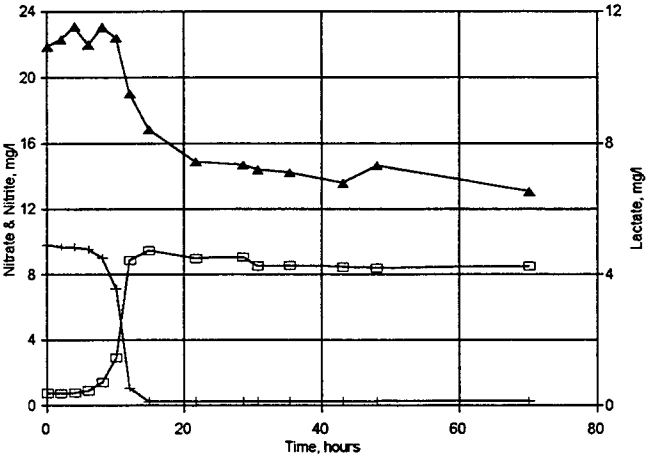
4.5 *G.8* experiments to determine metabolic pathways

Based on evidence of co-metabolic TNT transformation, we decided to attempt to identify the TNT biotransformation pathways and limiting factors. TNT and each of its transformation products were incubated individually with a *G.8* medium containing lactate as the electron donor and nitrate or nitrite as the primary electron acceptor. A medium containing lactate and TNT metabolites was also prepared. Energy source combinations in the medium were as listed in Table 4.2

GRAPH A



GRAPH B



Symbols: ⟶▲⟵, Lactate as Carbon ⟶□⟵, Nitrite as Nitrogen ⟶+⟵, Nitrate as Nitrogen GRAPH A (top figure) represents the utilization of lactate and nitrate with G.8 incubation in the absence of TNT and GRAPH B (bottom figure) is in the presence of 20 mg/l TNT.

Figure 4.5. Effects of trinitrotoluene (TNT) in the medium on lactate and nitrate transformation.

Table 4.2. Energy source composition in each bottle set.

Bottle set	Lactate	Nitrate	Nitrite	TNT or metabolites
Bottle A	O	O	X	O
Bottle B	O	X	O	O
Bottle C	O	X	X	O

Note: O = present and X = not present.

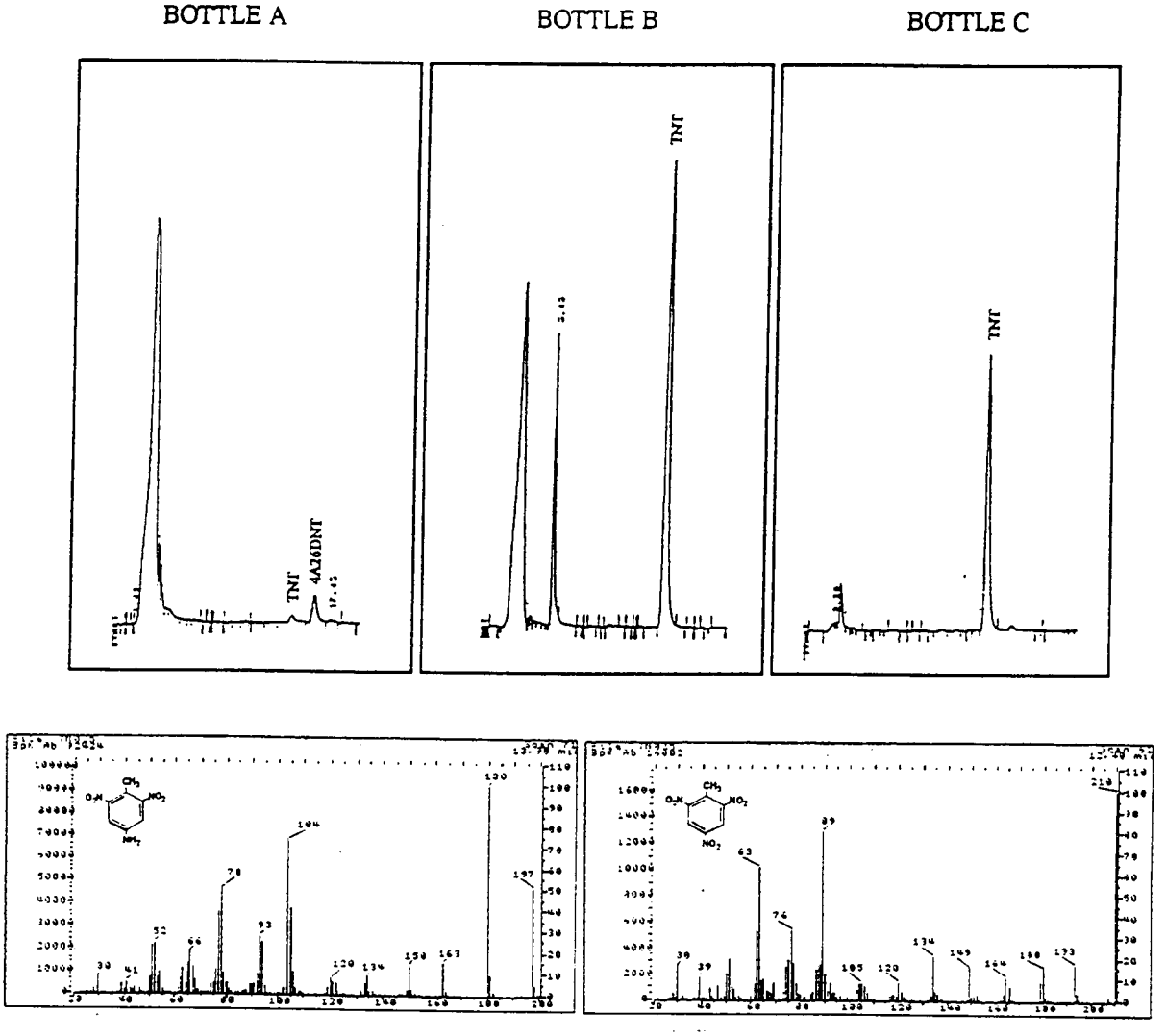
Minerals and yeast extract concentrations in each bottle set are identical as discribed in Material and Methods. The concentrations of lactate , nitrate, and nitrite in the medium are 4.50 mM, 19.78 mM, and 19.78 mM respectively. TNT and the metabolites concentration in the medium was set as 20 mg/l.

4.5.1 TNT transformation

G.8 was incubated for three days in serum bottles containing 20 mg/l of TNT. From Bottle A (containing lactate and nitrate as primary energy sources), the metabolites were identified as 4A26DNT. The complete TNT transformation in Bottle A and the non-transformations of TNT in Bottles B (containing lactate and nitrite as primary energy sources) and C (containing only lactate) showed that TNT is co-metabolically degraded in the presence of nitrate, and not degraded in the presence of nitrite. The HPLC chromatograms and mass spectrums of TNT and its transformation product are shown in Figure 4.6.

4.5.2 4A26DNT transformation

G.8 was incubated for eight days in serum bottles containing 20 mg/l of 4A26DNT. In Bottle B, 4A26DNT was reoxidized to TNT and deaminated to 26DNT. In Bottle A, only negligible amounts of TNT and 26DNT were detected. No 4A26DNT transformation occurred in Bottle C. Nitrites in the medium appeared to stimulate the *G.8* reduction and oxidation systems. The HPLC chromatograms and mass spectrums of 4A26DNT and its transformation products are shown in Figure 4.7.



The compositions of each bottle set are listed in Table 2.

Figure 4.6. HPLC chromatograms and mass spectrums (BOTTLE A) of TNT and biotransformation product with *G.8* incubation for three days.

4.5.3 26DNT transformation

In the same manner described for the 4A26DNT incubations, 26DNT was incubated for 20 days with *G.8* in Bottles A, B, and C. The transformation of 26DNT to 2A6NT was not detected or was negligible in Bottles A and B. The reduction of the 26DNT ortho-position nitro group was detected in bottle C, suggesting that *G.8* utilized 26DNT as a primary electron acceptor. The HPLC chromatograms and mass spectrums of 26DNT and the transformation products are shown in Figure 4.8.

4.5.4 2A6NT transformation

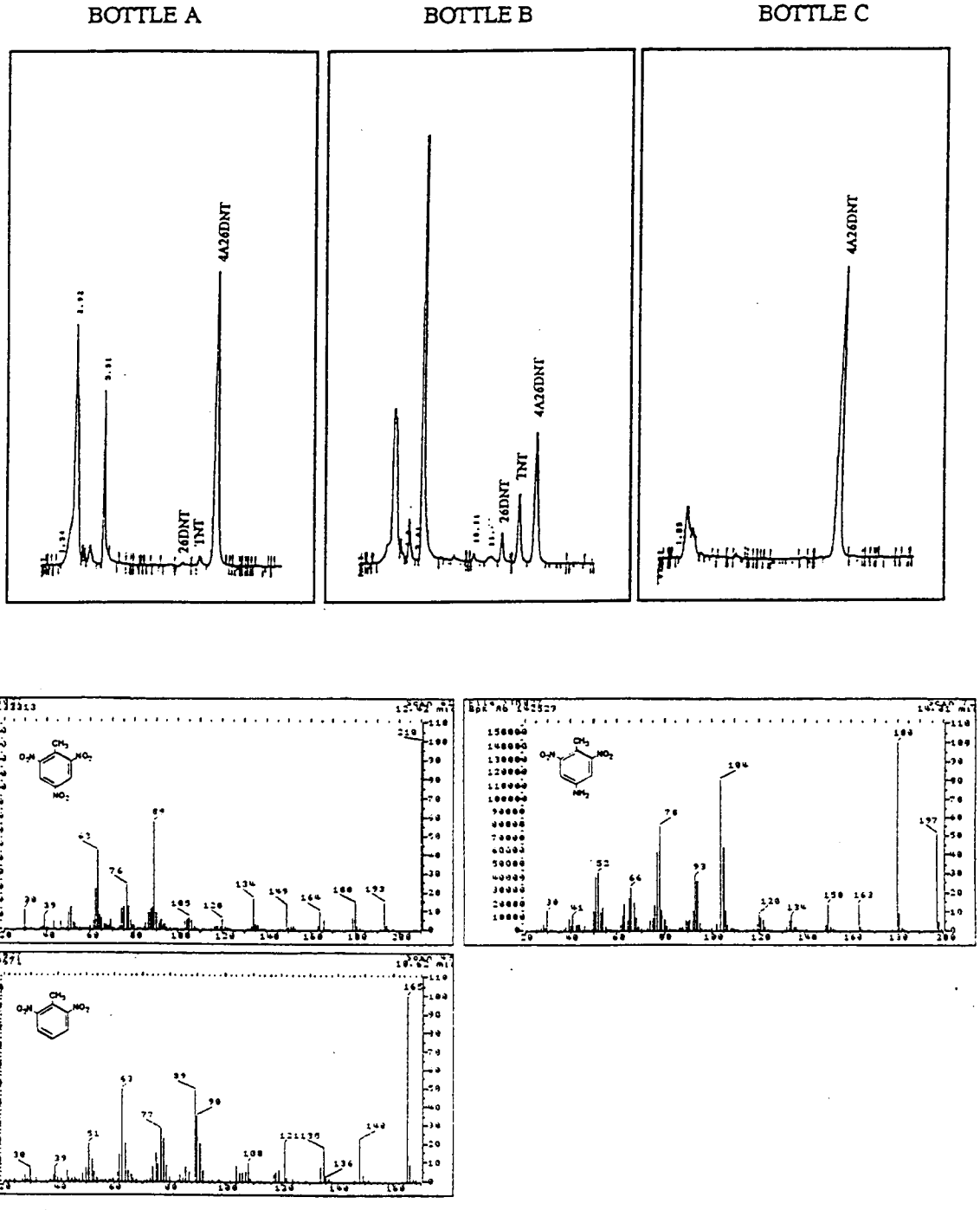
During 18 days of incubation, 2A6NT in Bottle B was transformed to 2NT and 26DNT. Oxidation and reduction of 2A6NT trends were identical to those for the 4A26DNT incubation. The absence of a primary electron acceptor in Bottle C did not stimulate 2A6NT transformation, and the oxidation of 2A6NT was faster than the reduction processes. The HPLC chromatograms and mass spectrums of 2A6NT and the transformation products are shown in Figure 4.9.

4.5.5 2NT and 2AT transformations

During 18 days of incubation, 2NT metabolites were not detected from either HPLC or GC/MS analysis (Figure 4.10), but it was noteworthy that 2-aminotoluene (2AT) completely disappeared from Bottles A and B (Figure 4.11) , suggesting that 2AT transformed more rapidly than 2NT. It is reasonable to suppose that the 2AT transformation products converted rapidly to other metabolites, but that the transformation of 2NT to 2AT was too slow. The 2AT transformation products were

identified as o-cresol, toluene, and small amounts of 2NT. These trends for amino group transformation were identical to those for 4A26DNT and 2A6NT.

TNT transformation pathways were established with series connections of each transformation products presented above. Proposed pathways for the TNT degradation are shown in Figure 4.12. The TNT biotransformation steps shown are the reduction process, wherein the nitro group is reduced to an amino group, followed by deaminization and oxidation when the amino group was present. As observed previously, para-nitro group reduction was the most susceptible transformation (11, 14, 21). The detection of toluene had been established previously under anaerobic or aerobic conditions for different pathways (4, 11).



The compositions of each bottle set are listed in Table 2.

Figure 4.7. HPLC chromatograms and mass spectrums (BOTTLE B) of 4A26DNT and biotransformation products with *G.8* incubation for eight days.

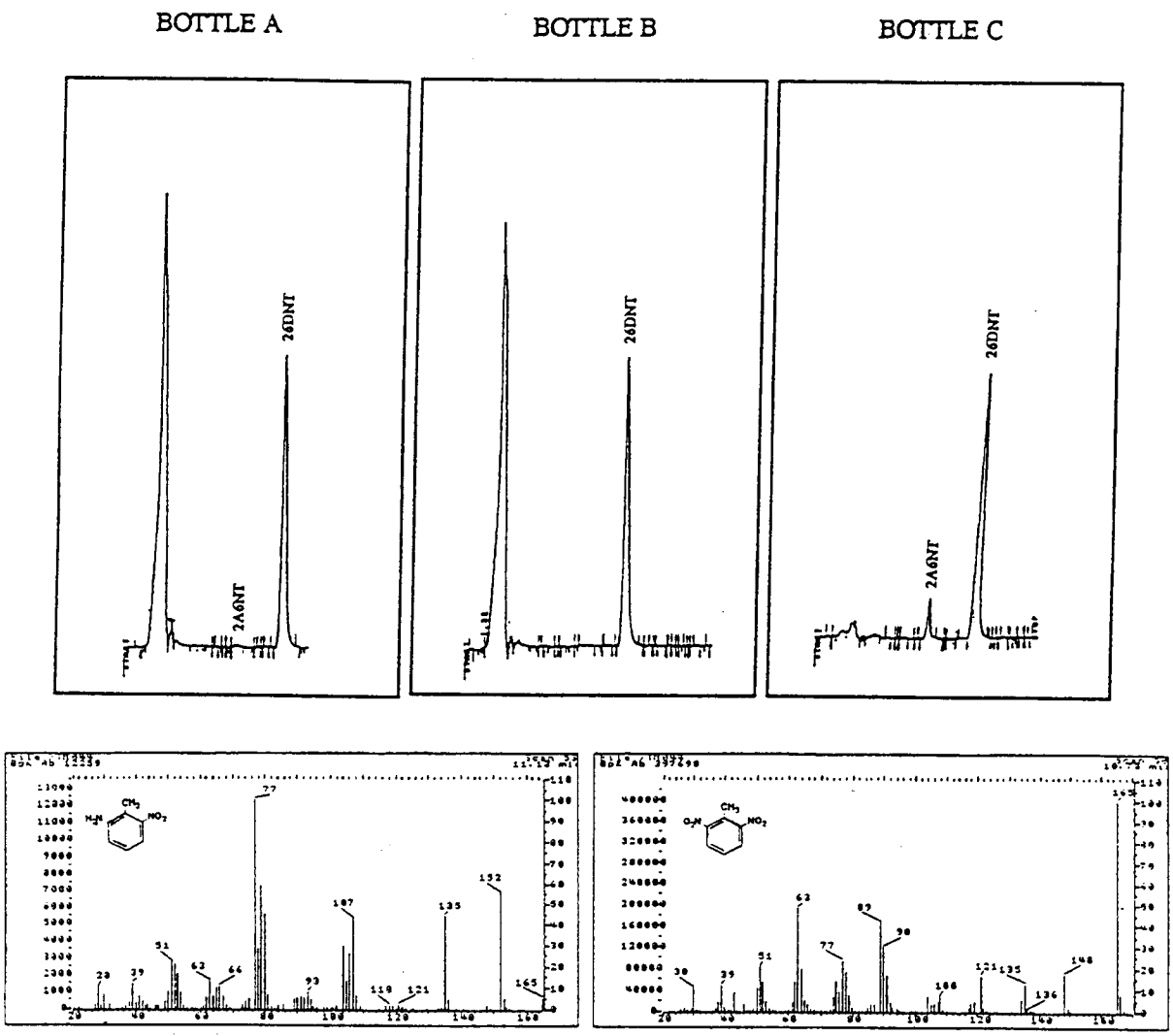
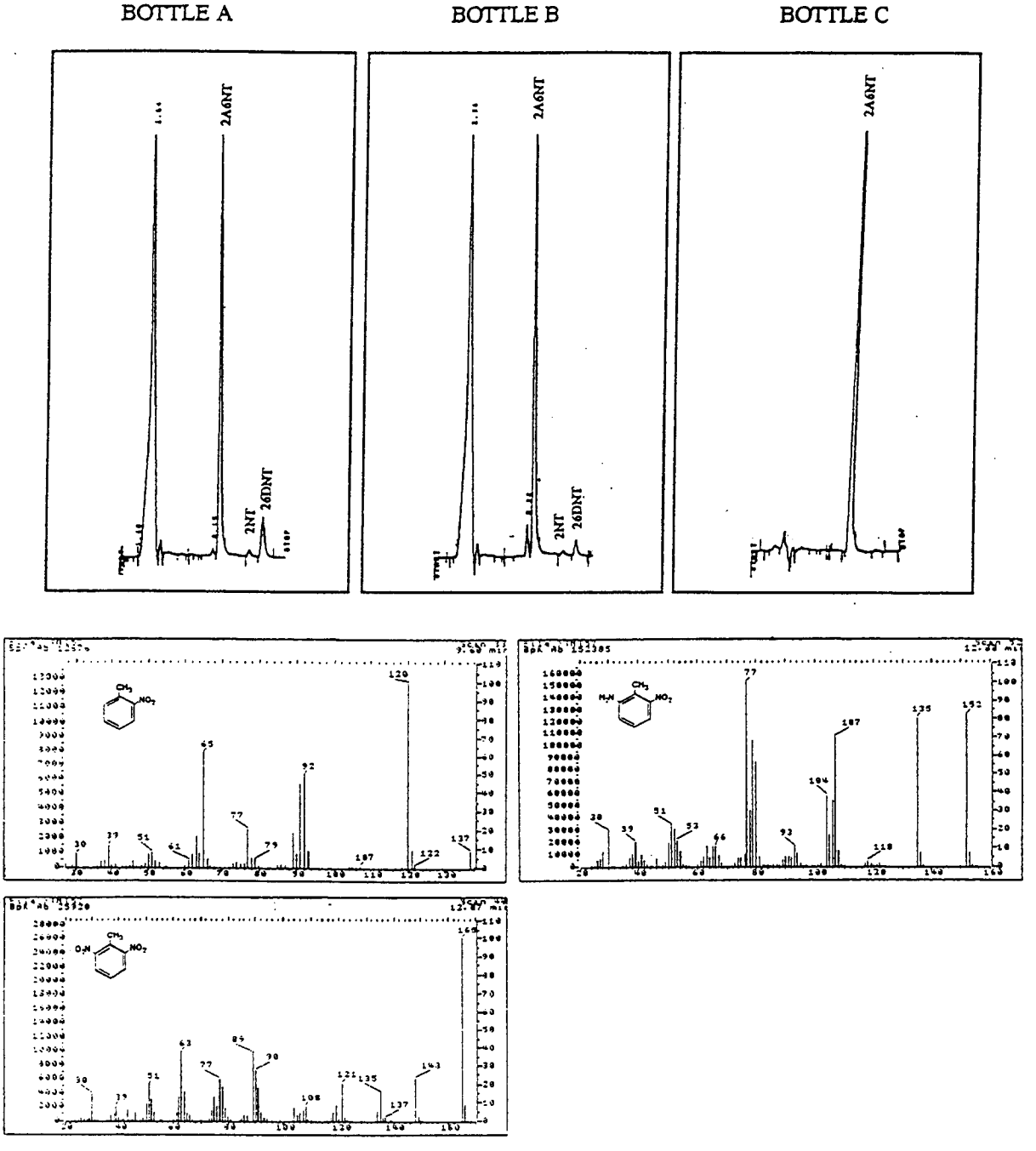
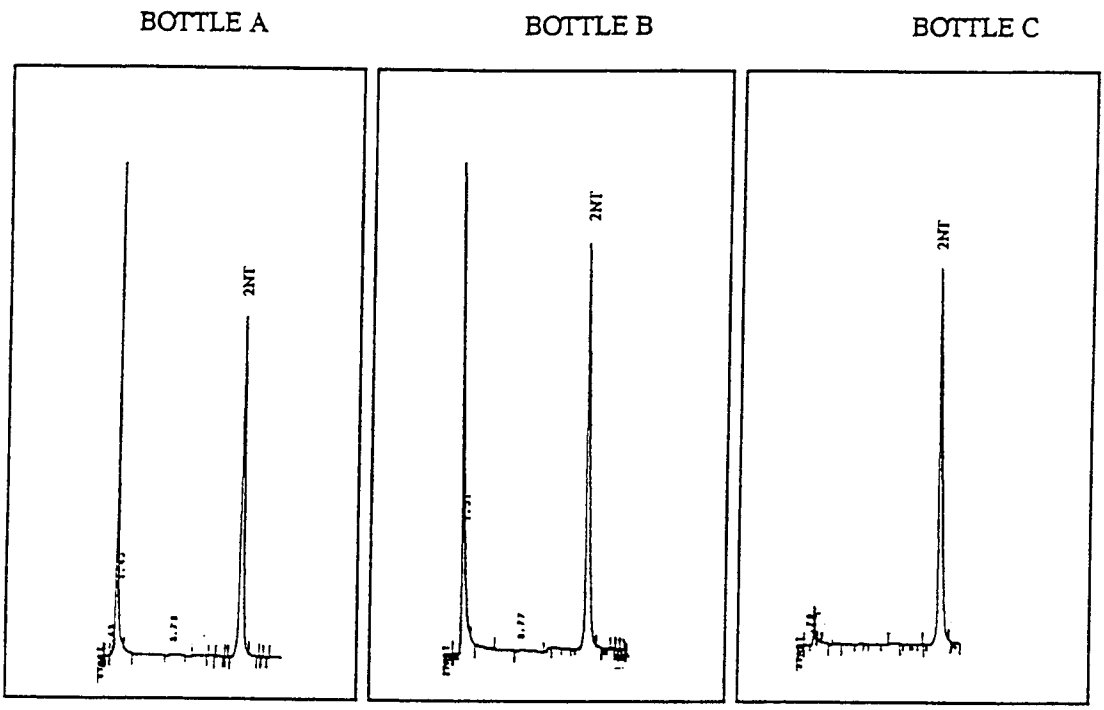


Figure 4.8. HPLC chromatograms and mass spectrums (BOTTLE C) of 26DNT and biotransformation products with *G.8* incubation for twenty days.



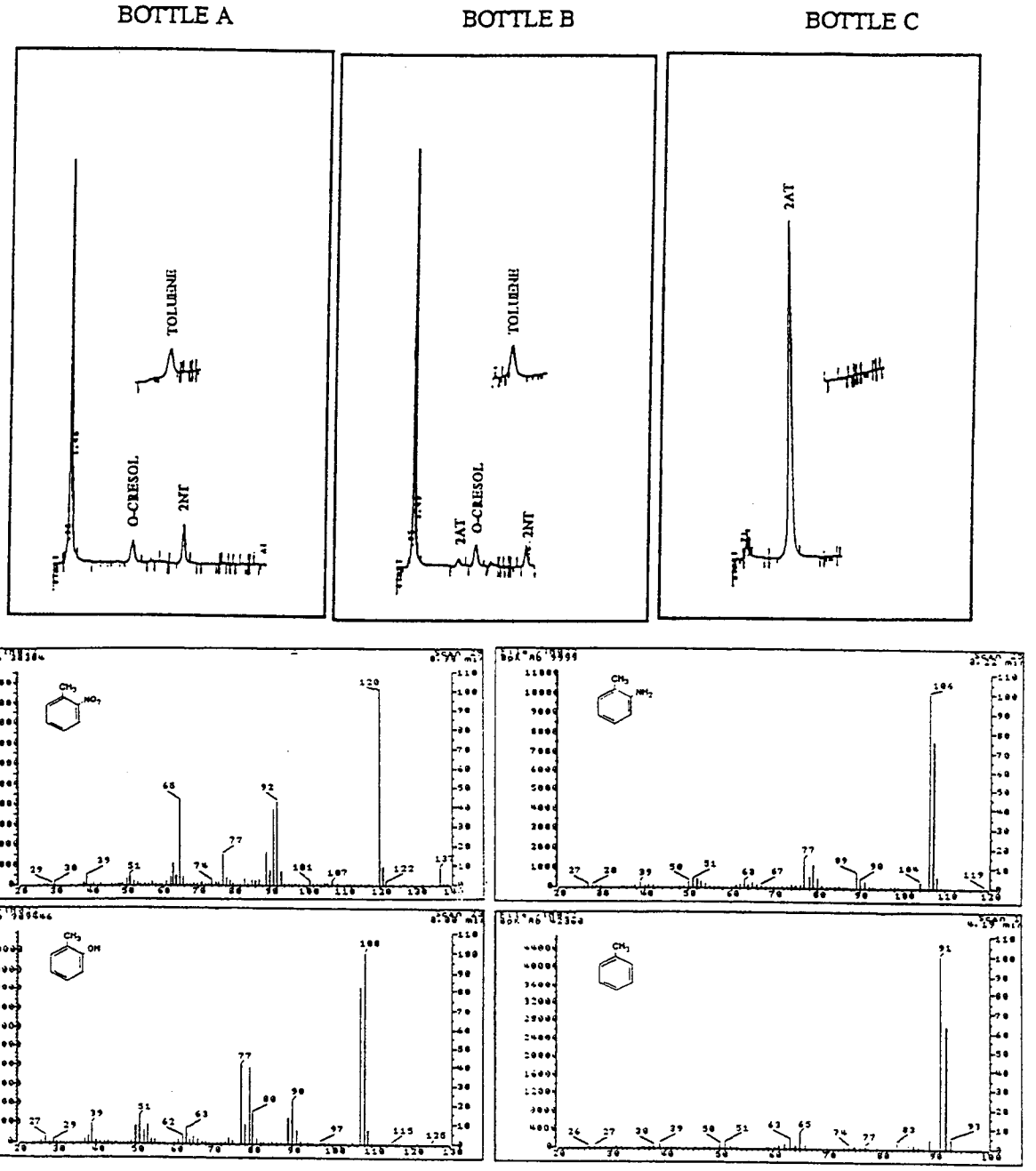
The compositions of each bottle set are listed in Table 2.

Figure 4.9. HPLC chromatograms and mass spectrums (BOTTLE A) of 2A6NT and biotransformation products with *G.8* incubation for eighteen days.



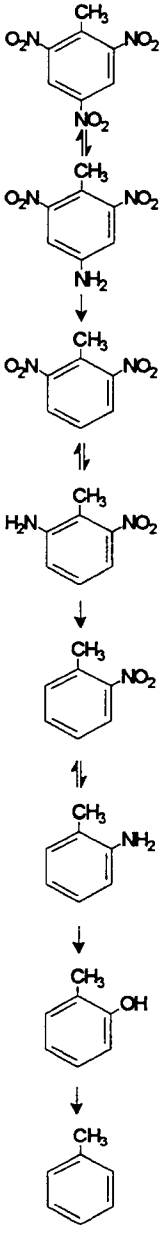
The compositions of each bottle set are listed in Table 2.

Figure 4.10. HPLC chromatograms of 2NT biotransformation products with *G.8* incubation for eighteen days.



The compositions of each bottle set are listed in Table 2. Toluene was measured at 210 ηm UV wavelength, otherwise 250ηm

Figure 4.11. HPLC chromatograms and mass spectrums (BOTTLE B) of 2AT and biotransformation products with *G.8* incubation for eighteen days.



The pathway was established with series connection of the identified transformation products by GC/MS and HPLC presented in Figure 4.6 through 4.10.

Figure 4.12. Proposed pathways for TNT biotransformation with *G.8* incubation.

4.5.6 Time course TNT transformation

TNT and metabolite concentrations in serum bottles containing nitrate and lactate as energy sources were monitored to trace the fate of TNT in the medium. TNT was added at 0.018 mM and removed at 90% efficiency after 12 hours, producing 4A26DNT, 2A46DNT, and 26DNT. Progress curves for TNT transformations are shown in Figure 4.13. The amounts of 2A46DNT in the medium inoculated with *G.8* was identical to amounts produced in the control medium. Further reduction of the 26DNT was not detected.

4.6 Conclusions

The *G.8* isolate was capable of reducing the TNT nitro group to an amino group and was also involved in deaminization as a co-metabolite, resulting in nitrogen free compounds such as toluene or o-cresol. A previous report (3) had indicated that TNT was both a nitrogen and primary electron acceptor, but a second study (5) reported that TNT transformation occurred only by a co-metabolism. Our results support co-metabolic transformation.

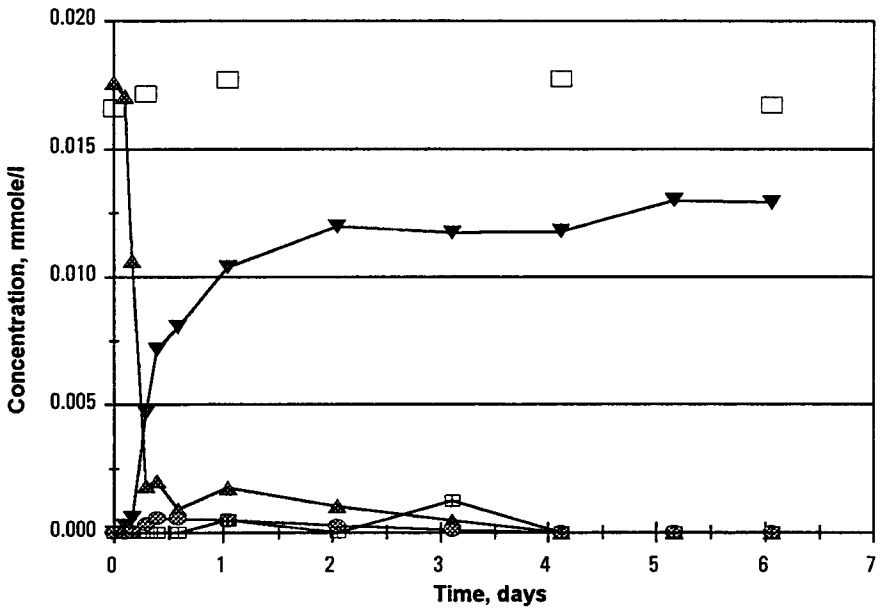
The reduction (deaminization) and oxidation (hydroxylation) reactions took place simultaneously when the amino group was present. From previous studies, it had been reported that oxidation and reduction processes occurred jointly subject to anaerobic conditions. Vogel et al. (30) demonstrated that *Cytochrome* P450 could mediate both the oxidation and reduction reactions for degradation of halogenated aliphatic compounds. For *Pseudomonas* sp. strain PN-1, it was also found that oxidation steps proceeded under conditions of denitrification (2). CO_2 and chloroform

formations from carbon tetrachloride in the culture of *Escherichia Coli* K-12 was found by Criddle et al. (9). These mechanisms are coincident with the present mechanisms of *G.8*.

The presence of nitrates in the medium stimulated the reduction of para-positioned nitro groups, and nitrites stimulated the deaminization and hydroxylation processes. The absence of such primary energy sources as nitrates or nitrites stimulated the reduction of the ortho-positioned nitro groups.

It is assumed that the incomplete TNT transformations shown in Figure 4.13 are related to inadequate combinations of the terminal electron acceptors. As shown in Figure 4.5 and Figure 4.13, nitrate consumption in the medium was complete while TNT was transformed to 4A26DNT and 26DNT. For certain periods of time, nitrite accumulated in the medium does not support *G.8* metabolism (GRAPH A of Figure 4.4) and subject to termination of 26DNT reduction process, as was indicated at 26DNT transformations with presence of nitrite in the medium (BOTTLE B of Figure 4.8).

Degradation of compounds is dependent upon the characteristics of the parent compounds, the microbial consortium, and environment factors (24). In the experiments described, the patterns of TNT metabolite transformation were dependent



Symbols: TNT Control, TNT, 4A26DNT, 2A46DNT, 26DNT

Figure 4.13. Progress curves for TNT transformations with lactate and nitrate as primary energy sources in *G.8* cultures.

From these findings, it is concluded that an appropriately designed sequencing reactor system, or other alternatives to control such energy source as nitrates, or nitrites in the TNT degradation system, could result in obtaining the full transformation of TNT to o-cresol or toluene. As indicated by Preuss et al. (27), the formation of TAT in the TNT degradation system increases the complexity of the TNT decontamination process. It is desirable that the proposed pathways, unlike the other pathways, do not include the production of TAT.

Though the full transformation of TNT was not achieved from the experimental processes described, it is promising that clear pathways and limiting factors were identified for the degradation of TNT. As appropriate systems are acquired, it is believed that these pathways will have a significant impact on the decontamination of TNT-contaminated soils or groundwater since the degradation of o-cresol or toluene under aerobic or anaerobic conditions has been well-documented (12).

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Chapter 5. Feasibility of Trinitrotoluene (TNT) Bioremediation with An Ruminal Bacteria *G.8* Isolated from Goat Rumen

5.1 Abstract

Application of ruminal microorganisms has high potential for bioremediation of antibiotic and xenobiotic compounds; however, application of these microorganism can be complicated due to their relatively short lives outside of the host. In this study, the fate of TNT was investigated in the presence of a ruminal microorganism, ***G.8***, isolated from a mixed culture obtained from a fisturated goat. Both batch and column studies were considered. In the batch system, reduction of the nitro group, under nitrate reducing conditions (requiring 3.76 mM of nitrate/mM of lactate) resulted in accumulating of nitrite. Unlike the batch system, full reduction of nitrate to ammonia (requiring 1.05 mM of nitrate/mM of lactate) was observed in the column experiment. Among three redox situations considered, the presence of nitrite resulted in the least transformation of TNT among nitrate, nitrite, or absence of primary electron acceptor. This is the first report of a ruminal microorganism maintaining complete transformation of TNT to 4A26DNT and 2A46DNTon a synthetic media for over 8 months. This is especially encourging for the application of ruminal microorganisms in the

decontamination of TNT or other xenobiotic contaminated soil and groundwater.

5.2 Introduction

2,4,6-trinitrotoluene (TNT) exposure can potentially cause adverse human health effects and has generated strong public attention. (11, 20). More than 1,100 military facilities, each potentially contaminated with munitions wastes, are expected to require treatment of more than one million cubic yards of contaminated soil (2). The cost associated with remediation of these sites has been estimated to be more than 1.5 billion dollars(16).

Biological remediation potentially provides the most economic and reliable solution for remediation of explosive-contaminated soil or groundwater (16,18). Some biological processes were shown to be capable of complete mineralization of TNT; however were unable to tolerate greater than 20 ppm (14, 23). Incubation periods for complete TNT transformation ranged from 8 to 91 days and varied depending on the microbial population (3, 4, 11, 12, 23, 27).

Ruminal organisms have demonstrated the ability to destroy some pesticides, including parathion, p-nitrophenol; biphenyl-type compounds; thiono isomers; and nitrogen-containing heterocyclic plant toxins such as the pyrrolizidine alkaloids under anaerobic conditions (7, 24, 26). In spite of the great potential of ruminal microorganisms for the destruction of xenobiotic compounds, application of ruminal

microorganism in full-scale bioremediation systems is limited because the cells are short-lived outside of the host (21).

A TNT-degrading ruminal microorganism has been isolated from goat rumen fluid with successive enrichments on triaminotoluene (TAT) and TNT (8). The isolate, designated ***G.8***, utilizes nitrate as the electron acceptor and several electron donors including lactate, succinate, propionate, butanediol, acetate, and formate. ***G.8*** was able to tolerate and metabolize levels of TNT up to the saturation point of 125 mg/l in the presence of nitrate.

5.2.1 Objectives

This study is has been directed at the feasibility of this isolate to degrade TNT in batch and laboratory-scale, fixed-film column experiments. Application of ***G.8*** for TNT bioremediation was analyzed with respect to the following concerns: 1) Is the isolate ***G.8*** sufficiently able to metabolize TNT with synthetic medium in a contineous flow column reactor; 2) Does TNT transformations be stimulated in the presence of different primary substrates (including lactate, acetate, or formate as electron donors and nitrate, nitrite, or absence of primary electron acceptor).

5.3 Materials and methods

5.3.1 Chemicals

TNT was obtained from Chem Service, two monoaminodinitrotoluene congeners (2-amino 4,6-dinitrotoluene and 4-amino 2,6-dinitrotoluene) were purchased from the Radian Corporation. All solvents were HPLC grade.

5.3.2 Medium

The media used to incubate the isolate for the batch experiment consisted of the components listed in Table 5.1. All medium were prepared under argon gas flow and were autoclaved for 25 minutes. To prevent precipitation of salts, 0.3 ml of 800 ppm $CaCl_2.2H_2O$ was injected following autoclaving. An 0.2% inoculum was used for batch experiments. Serum bottles containing the inoculated medium were cultivated anaerobically on a mechanical shaker. The temperature for batch and column experiments were maintained at 37°C. Ethanol was used to sterilize the contamination at the contact points of the instruments used to innoculate and sample the serum bottles. Sample aliquits were indexed and stored at -4°C prior to analysis.

5.3.3 Analysis of the experiment

TNT, 4-amino,2,6-dinitrotoluene (4A26DNT), 2-amino,4,6-dinitrotoluene (2A46DNT) were measured by high performance liquid chromatography (HPLC), using two analytical columns (LC-18 and LC-CN) connected in series and eluted isocratically at 1.0 ml/min with water-methanol-tetrahydrofuran (60.5:25:14.5) (25).

Gas chromatography/mass spectrometry (GC/MS) analysis was performed using a Hewlett-Packard 5988A MS coupled to a 5890 GC with a XTI-5 fused-silica capillary column. Helium at a flow rate of 20 ml/min was used as the carrier gas. Aliquots were extracted into $CHCl_3$ extract before injection into the GC (injection port temperature, 250°C). The oven was maintained at 40°C for 2 minutes and then ramped to 120°C at a rate of 10°C/min, maintained at 120°C for 1 min, and then ramped to 260°C at a rate of 20°C/min. Four minutes after the temperature program was initiated, the mass spectrometer ion source was turned on; the interface line to the ion source was held at 280°C throughout the run.

A Dionex 4000i ion chromatography (IC) with a HPIC-CS3 anion column was used to measure anions such as nitrate and nitrite. The mobile phase contained 0.191grams of Na_2CO_3 and 0.143 grams of $NaHCO_3$ in one liter of distilled water. For regeneration of the column, 0.07% H_2SO_4 was used. The flow rate was 2 ml/min. The outflow from the column was analyzed on a conductivity detector. An autosampler was used to inject 50µl from auto sampler vials containing 500µl of sample, which was filtered through a 0.25 µm TPFE syringe filter prior to loading the autosampler vials.

For analysis of cell growth, culture turbidity in serum tubes was measured by absorbance at 660 nm with a spectrophotometer (Bausch and Lomb). Blank sample, incubated just as the sample but with no inoculation of bacteria, was prepared to compensate for any other source of absorbance increments.

Table 5.1 Concentration of nutrients for batch and column experiment.

Compounds	mg/l	Compounds	mg/l	Compounds	mg/l
$CH_3CHOHCOONa$	36.	$MnSO_4.H_2O$	8.5	$FeSO_4.7H_2O$	10.
KNO_3	76.	$ZnSO_4.7H_2O$	5.	$CaCl_2.2H_20.$	24.
$Na_2HPO_4.H_2O$	2550.	H_3BO_3	1.5	$NaMo_4.2H_2O$	1.5
$NaH_2PO_4.H_2O$	975.	$CoCl_2.6H_2O$	0.6	Na_2EDTA	21.5
Yeast extract	100.	$CuCl_2.2H_2O$	0.05		
$MgSO_4.7H_2O$	30.	$NiCl_2.6H_2O$	1.		

Head space gas analysis was performed on a Fisher Gas Partitioner Model 25V. The gas partitioner used two column in series. A molecular sieve 13X (45/60 mesh) column connected to a 30% of Bis12 Ethoxyethyl Adipate and 70% of chromosorb pwu(60/80 mesh) column. Helium at flow rate of 80 ml/min was used for carrier gas. A sample volumn of 200 µl was injected.

5.3.4 Fixed-film bioreactor

A glass column, 2.5 cm diameter by 15 cm, packed with 3 mm glass beads was used to provide one-dimensional flow in a porous matrix. Bulk flow in the reactor was established by pumping the feed solution at the end of the reactor to prevent reoxygenation of the prepared feed solution (1). The peristaltic, bulk-flow pump provided a wide range of dispensing rates (i.e., from 0.02 ml/min to 22 ml/min). All equipment was autoclaved prior to assembly to prevent the growth of foreign microorganisms in the reactors. Two columns reactors, Reactor A and Reactor B,

with identical physical properties were connected in a series and set in a vertical direction. The feed solution was pumped in from the bottom to the top. The locations of sampling ports are shown in Figure 5.1.

A slight positive argon gas pressure was applied to the feed reservoir to prevent penetration of oxygen from the surrounding environment. The physical properties of the reactor are as shown in Table 5.2. The morphology of ***G.8*** was insured to microscopic examination each day from samples of the effluent solution. At the moment of final analysis, the effluents were streaked on the agar plate against contamination from foreign microorganisms.

5.3.5 Microorganisms

Isolation of the pure ruminal culture ***G.8*** was conducted by the Department of Veterinary Medicine at Oregon State University. Detailed procedures are described in Craig (8).

5.4 Results

5.4.1 Characteristics of *G.8* in batch experiment

Nitrate in different media containing several TNT concentrations, 0, 50, and 100 mg/l was monitored (Figure 2). Nitrate was converted to nitrite, and further conversion of nitrite was observed when TNT was absent from the medium; but nitrite conversion was not observed when TNT was present. This shows that nitrite was in competition with the TNT nitrigroup as the electron acceptor. Higher TNT concentrations showed longer lag periods for nitrate reduction. No nitrogen or

nitrogen oxide was observed in the head space of the batch reactor, meaning that nitrite was reduced to ammonia.

Based on the experiment results, the overall energetic equation for the medium containing TNT was established as Equation 1 (15). The ratio of lactate to nitrate was calculated to be 3.76 mM of nitrate per mM of lactate.

Table 5.2. Total physical properties of fixed-film bioreactor.

Total volume	140.0 ml
Porosity	0.4
Flow rate	27.0 ml/day
Detention time	2.07 day

The ratio (3.76 mM nitrate/mM lactate) obtained from equation 1 was used to determine the optimum ratio of lactate to nitrate for continuous flow column experiment.

$$0.209\ CH_3CHOHCOO^- + 0.787\ NO_3^- + 0.036\ H^+ =$$

$$0.209\ HCO_3^- + 0.036\ C_5H_7O_2N + 0.310\ H_2O + 0.239\ CO_2 + 0.752\ NO_2^- \quad 1)$$

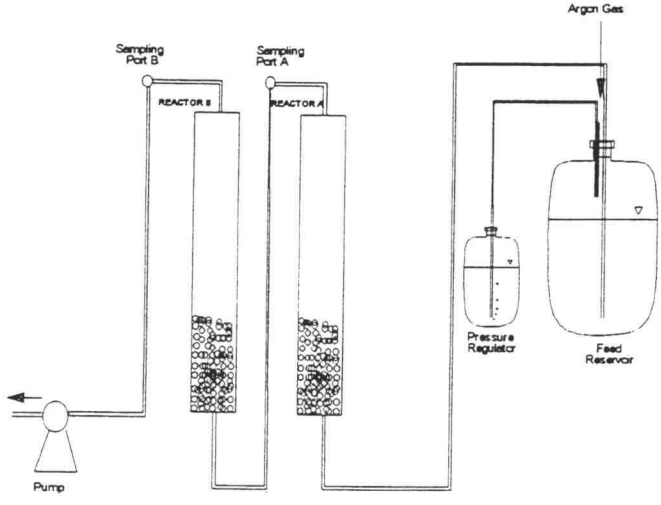


Figure 5.1 Schematic diagram of fixed film bioreactor.

5.4.2 Continuous flow fixed-film bioreactor experiments

The feed solution for the column experiment was composed of 2.31 mM of nitrate/ mM of lactate in order to set nitrate as the limiting terminal electron acceptor. Feed was initially pumped through the reactor without inoculation for 8 days. During this days, the nitrate concentration in the effluent was measured to investigate abiotic transformation of nitrate in the reactor. The nitrate concentration in the effluent reached that of the influent after 4 days and reamined there, indicating that abiotic transformation of nitrate was not occuring (Figure 5.3). 17 days after inoculation of the reactor with *G.8*, nitrate concentration at the sampling ports A and B were measured (Figure 5.4). The transformation trends of nitrate at the sampling ports A and B were similar with the batch experiment (Figure 5.1). TNT and metabolites in the chloroform extracts of the effluents were identified as 4A26DNT and 2A46DNT (Figure 5.5).

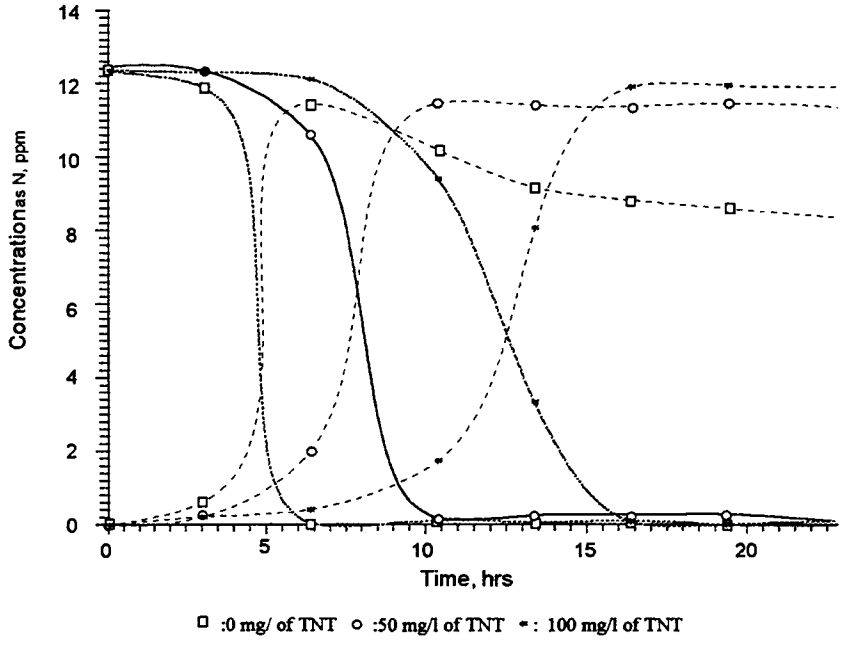
Unlike the batch experiment, complete disappearance of the primary electron acceptor was established when 20 mg/l of TNT was present in feeding solution. 85 days after feeding the medium containing 20 mg/l of TNT to the bioreactor, the effect of TNT transformation was investigated with various combinations of primary electron donors and acceptors (Table 5.3).

The different energy source combinations at Table 5.2 were applied at the times shown in Figure 6. Feed solution containing lactate and nitrate was reapplied at

various times. Figure 6 also shows the TNT and metabolites concentration with time. First, feed solution containing lactate and nitrate through the reactor was changed to acetate and nitrate and to formate and nitrate during 120-133 days (changing terminal electron donor). TNT concentration in the effluent increased while 4A26DNT was decreased, suggesting less cell activity with acetate or formate as electron donor. Second, media containing lactate, acetate, and formate as electron donors and nitrite as the primary electron acceptor were fed setpwise through the reactor during 150-170 days (changing terminal electron acceptor). Less transformation of TNT in the effluent was observed and 4A26DNT concentration decreased. 2A46DNT production was not effected. Finally, the feed solution containing only lactate, acetate, or formate was introduced to the reactor during 204-230 days (in the absence of primary electron acceptors). 2A46DNT in the effluents increased, indicating acclimation of ***G.8*** stimulated transformation of the ortho position nitro group. 4A26DNT and 2A46DNT concentrations increased for a while and then decreased, representing decreasing transformation activity of the cells with time.

Table 5.3 Characteristics of each feeding solution.(mM).

	Lactate	Acetate	Formate
NO_3^--N	0.751 \ 0.324	0.751 \ 0.324	0.751 \ 0.324
NO_2^--N	0.751 \ 0.324	0.751 \ 0.324	0.751 \ 0.324
pH initial	7.0	7.0	7.0



Line fitting was established using cubic spline technique. : ——— :Nitrate
····· :Nitrite

Figure 5.2 Nitrate conversions in different media containing several concentrations of TNT (0, 50, and 100 mg/l) and 11.7 mg/l of lactate as carbon.

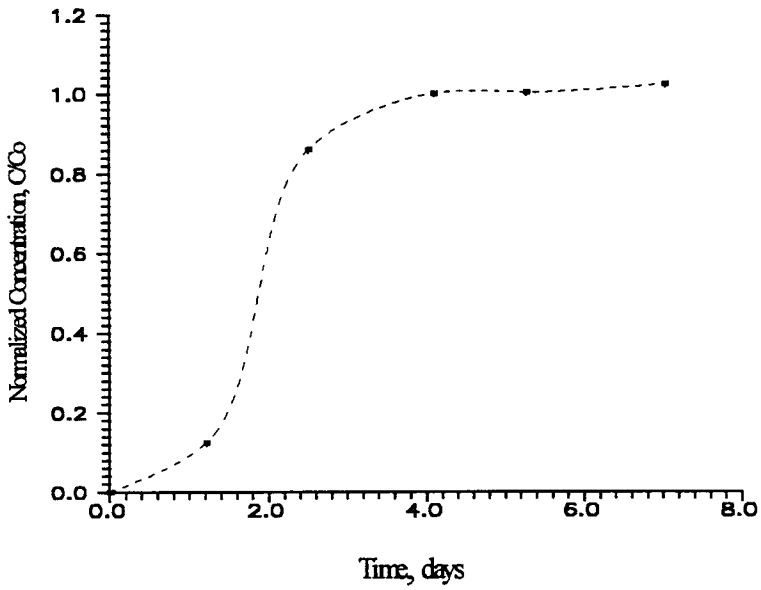


Figure 5.3 Breakthrough curve of nitrate through reactor

5.5 Discussions

In this experiment, TNT transformation by a pure cultures, isolated from rumen fluid of the goat, was investigated under denitrifying conditions because a previous report (5) indicated that the removal of TNT was faster under theseconditions than under other anaerobic conditions such a sulfate reducing conditions or methanogenesis.

The batch experiments showed that the presence of TNT depresses the growth of the isolate. Less transformation of nitrite was observed with higher concentration of TNT, indicating competition of nitrite and nitro group reduction for electron acceptor. It was reported that reduction of the nitro group was performed by nitrite reductase of the sulfate reducer, *Desulfovibrio Sp.* (4). Accumulation of nitrite in the medium of batch experiment could be explained by preferential attacks of nitrite reductase on the nitro group instead of nitrite. Possibly, nitro group reduction can generate more potential energy than reduction of nitrite. The energy potential of NO_2^- /NH_3^+ is -7.852 Kcal/ mole while the reduction of nitro group to amino group is calculated as about -9.386 Kcal/mole (13, 15, 19).

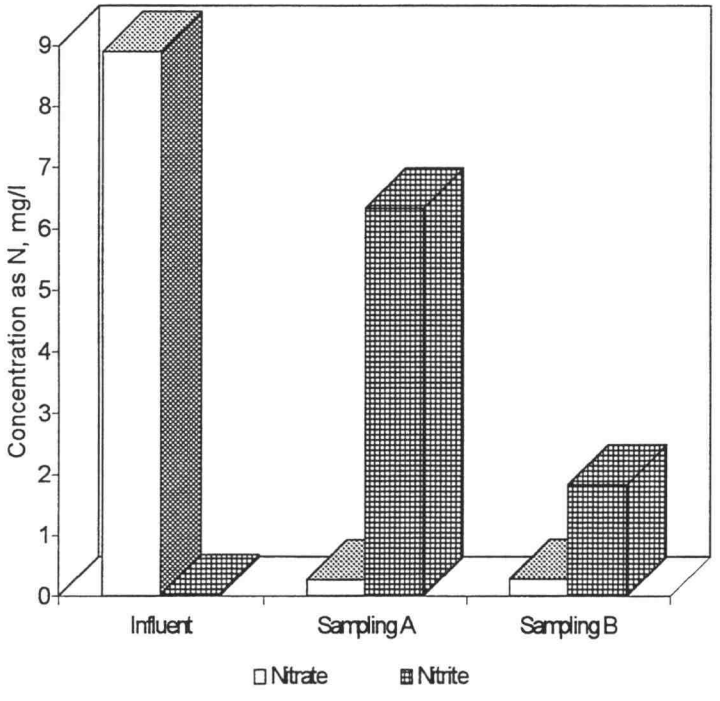


Figure 5.4 Nitrate concentrations at Sampling Port A and B before feeding TNT through reactor.

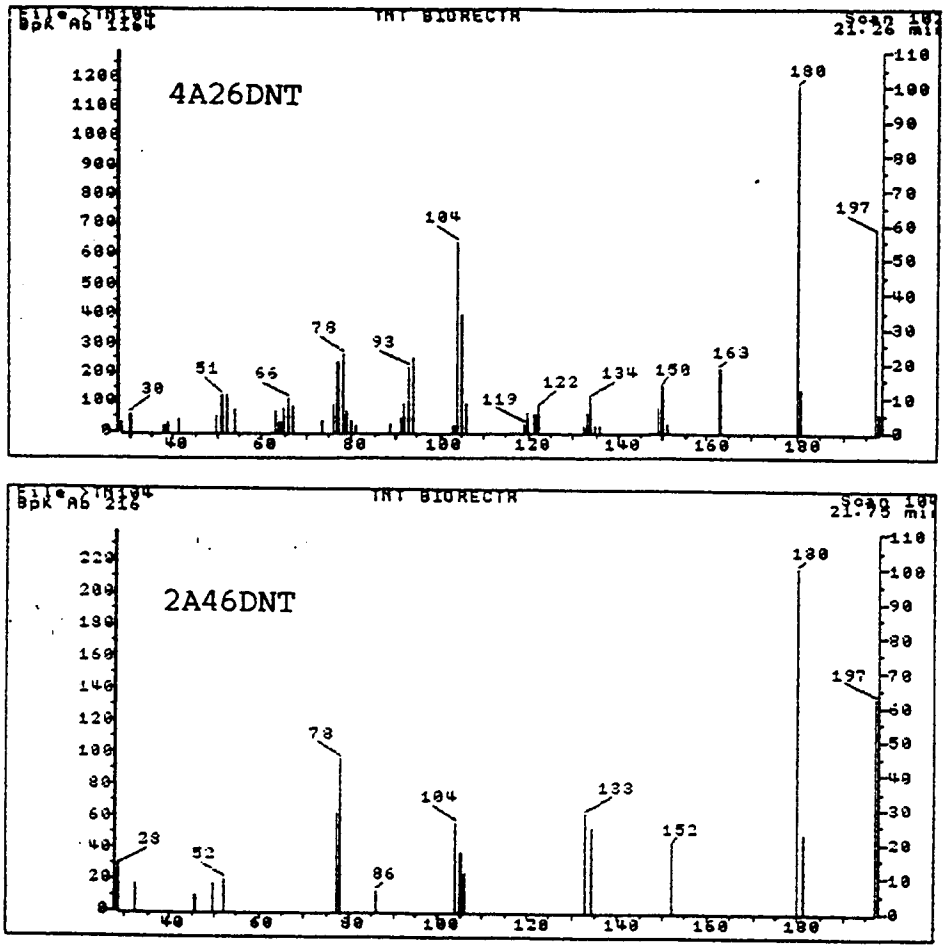


Figure 5.5 Mass spectrums of the metabolites in the effluents of the reactor.

The column experiment showed that complete transformation of TNT occured with a 2 day of residence time, leaving 2A46DNT and 4A26DNT as metabolites. Primary electron acceptors such as nitrate or nitrite were not detected in the column effluent while nitrite accumulation was observed, with identical medium (Figure 5.2), in the batch experiment. Complete disappearance of primary electron acceptors indicates that accumulation of the biomass in the reactor stimulates reduction of the electron acceptor. Based on the complete transformation of nitrate to ammonia, the energetic equation for the column study was constructed at equation 2 (15). Estrella (10) indicated that the biodegradation rate parameters calculated for batch experiments were significantly different from those estimated from column experiments. Equations 1 and 2 verified different biodegradation rate with different environments.

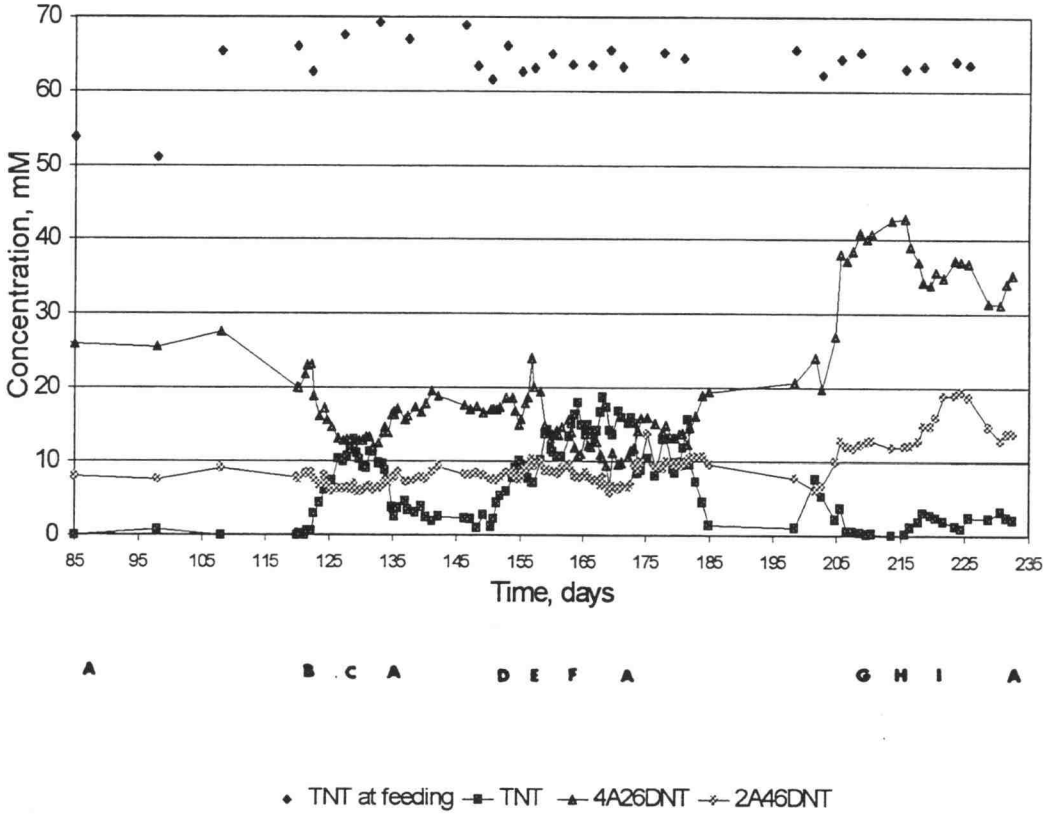
$$0.164\ CH_3CHOHCOO^- + 0.156\ NO_3^- + 0.277\ H^+ =$$

$$0.100\ H_2O + 0.149\ CO_2 + 0.121\ NH_4^+ + 0.163\ HCO_3^- + 0.036\ C_5H_7O_2N \quad 2)$$

Changing the electron donor from lactate to acetate to formate does not stimulate the reduction of TNT (Figure 5.6). It is suspected that lower levels of TNT transformation in the medium containing acetate and formate are caused by a lower C/N ratio in that medium to the medium containing lactate. Changing the electron acceptor in the feed solution from nitrate to nitrite showed least TNT transformation among the feed solution combination listed in Table 5.2. It can be concluded that nitrite in the feed solution does not support TNT transformation. In the absence of electron acceptor, TNT transformation was almost the same as when nitrate was present. As indicated by Boopathy (4, 6), TNT transformation was with other terminal

electron donors or acceptors. Long term acclimation the microorganisms to the compounds result in enzyme induction processes (17). Increasing 2A46DNT and 4A26DNT concentration in the column effluent at 200 to 220 days (Figure 5.6) confirmed the induction processes with long term acclimation of the microorganism. Possibly, the decrease in 4A26DNT and 2A46DNT concentration after 220 days verified that the reducing activity of the cells generated by induction processes were gradually decreased with continuous feeding without primary electron acceptor.

In spite of the useful merits of ruminal microbiota to degrade xenobiotic compounds such as pesticides or plant toxins, maintenance of microbial activity in an apparatus for continuous culture was not satisfactory by this time. Quinn (21) indicates the maintenance of ruminal microorganism activity at the outside of the host requires rigorous control of the physical environment. Rufener (22) found the an applicable system to keep ruminal microbial activity in continuous culture for extended periods of time (3-10 days), producing fatty acids, CO_2, and CH_4. In this experiment, continuous flow plug flow system showed that an isolate from ruminal microorganisms of the goat could maintain activity by continuous feeding of substrate for over 8 months. This finding could be an encouragement for the application of ruminants into full scale bioremediation projects.



Capitals represents the energy sources combination of feeding solution containing 20 mg/l of TNT; (A:lactate and nitrate, B:acetate and nitrate, C:formate and nitrate, D:lactate and nitrite, E:Acetate and nitrite, F:formate and nitrite, G:lactate, H:acetate, and I:formate).

Figure 5.6 TNT and metabolites concentrations at the effluents of bioreactor.

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Chapter 6. Conclusions

TNT transformation was investigated by incubation of ruminal microorganisms and an isolate, G.8 on various conditions. Both ruminal microorganisms and an isolate have shown fast and complete TNT transformation. Based on the results presented herein, the following conclusions are appropriate:

a. Ruminal microorganism has shown most rapid rates of conversion(> 1 day) among all TNT biodegradation studies conducted heretofore and maintained their metabolism with saturation points of 120 mg/l.

b. The pathways of In vitro TNT transformation are designated as reduction of the para position nitrogroup followed by the ortho position nitrogroup of TNT, leaving 4A26DNT, 24DA6NT, TAT and more reduced end products than TAT as intermediates.

c. Ruminal microorganisms can maintain their metabolism as well as decontaminate TNT contaminated soil, providing another source of microorganism for the fast and reliable bioremediation of TNT contaminated soil or groundwater.

c. The isolate that utilized nitrate and lactate as carbon and energy sources was able to tolerate and metabolize levels of TNT upto the saturation points of 125 mg/l.

d. TNT transformation pathways with incubation of G.8 were identified as reduction of para position nitrogroup followed by deaminization, leaving toluene as end product.

e. Nitrite conversion occured with the presence of nitrate and TNT transformation was competed with the nitrite conversion. Nitrates within the medium

stimulated the para position nitro group, whereas nitrites catalyzed the deaminization and the oxidation of the amino group. In the absence of other electron acceptors, the transformation of ortho position was rapid.

f. In the batch system, accumulation of nitrite was observed (requiring 3.76 mM of nitrate/mM of lactate), whereas in the column studies further transformation of nitrite was acheived (requiring 1.05 mM of nitrate/ mM of lactate).

g. Long term incubation (> 8 months) of the isolate in the continuous flow bioreactor provides great possibilities for the application of ruminal microorganisms for the decontamination of TNT contaminated sites.

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APPENDIX

I. Isolation of A Rumen Bacteria from Rumen Contents

I.1. General

Isolation of a pure culture from goat rumen contents are conducted by Department of Veterinary Medicine at Oregon State University with successive enrichment of TNT and TAT.

I.2. Methods

First, 10 grams of ruminal content were collected from fisturated goats and anaerobically blended with 90 ml of McDougall's buffer. This mixture was then anaerobically centrifuged (16000 xG, 4°C, 5 min). The supernatant was anaerobically transferred (10% inoculum) to GPT100 (general purpose growth medium with reduced carbohydrate concentrations), and added to tubes containing TNT (i.e., TNT dissolved in isopropanol and then solvent evaporated) for a final TNT concentration of 100 ppm. The tubes were incubated stagnant at 38°C. After four days, a 10% inoculum was transferred to fresh GPT100.

After 12 days, a 10% inoculum was transferred to DTAT (semidefined TAT medium containing sodium formate, 10% clarified rumen fluid, and minerals with one mg/ml of TAT). After three days incubation, 10 % was transferred to fresh DTAT. The DTAT medium was modified (D3YN) by adding 20 mM KNO3, 0.1 % yeast extract and 1 mg/ml of TAT. This medium was inoculated from the third transfer of the goat enrichment in DTAT. After the 20th transfer of the goat enrichment in

D3YN, the transfer was diluted in D3YN to 10^{-6} and spread to D3YN plates solidified with 1.6% agar, prepared anaerobically in the Grove box. The plates were placed in equilibrated anaerobic jars in the Grove box and incubated at 38°C. The goat enrichment was also plated to a defined medium with TAT as the sole carbon source. This medium contained 7.5 mM TAT, minerals, helium, vitamins and 2% noble agar (DTP agar). After one day, large, well-isolated, dark and red colonies were shown on the 10^{-7} dilution of D3YN agar.

A colony was chosen, incubated to D3YN broth, and incubated for three days. The D3YN isolates were restreaked to D3TN agar (100 ppm TNT in place of TAT), GPT100 agar, and DTP agar (defined medium, with TNT as the sole carbon source). After five days incubation, there were tiny colonies on DTP agar that, when streaked to D3YN agar, formed large, red colonies that eventually developed into microcolonies. The isolated microcroorganisms from a fistulated goat was named G.8. A single morphology and the colony shapes of G.8 are illustrated in Figure I.1.

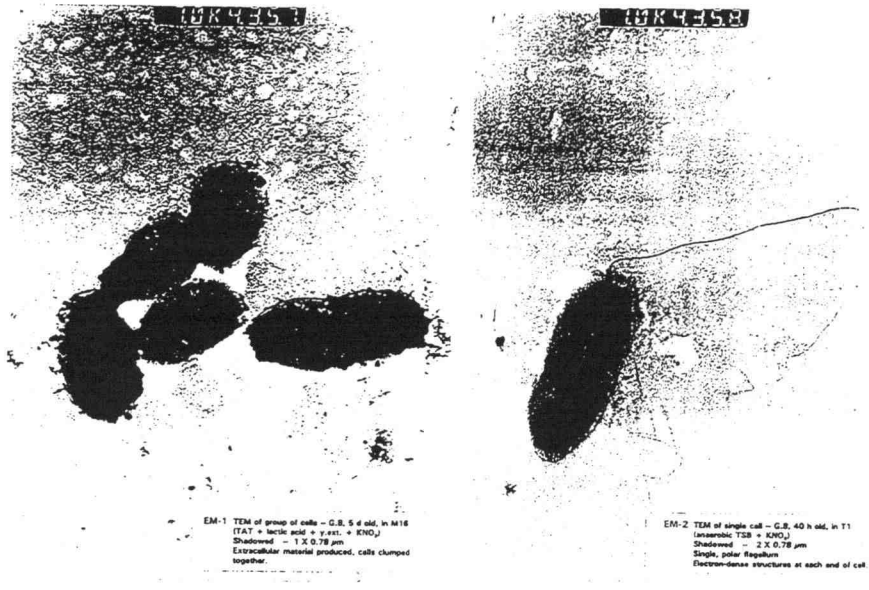


Figure I.1 Electron microscope of G.8 in a single and group cell.

II. Analytical Methods

II.1 Analytical Methods for TNT and Metabolites

Two analytical columns, LC-18 and LC-CN, in which two columns were connected in series and eluted isocratically at 1.5 ml/min with water-methanol-tetrahydrofuran (ratio = 60.5:25:14.5) for the analysis of TNT, 4A26DNT, 2A46DNT, 24DA6NT, 26DA4NT, 26DNT, 2A6NT, and 2NT. The outflow from the column flows was through a UV detector set at 250 nm (DIONEX 2000I). LC-18 column was eluted at 1.0 ml/min with water-metanol (ratio=3:1) for the analysis of 2NT, 2AT, o-cresol, and toluene. For toluene anlysis, UV detector set at 210 ηm. The sample was centrifuged (2000 xg, for 5 min) to remove sediment or floc and the supenates were injected, using a manual injection valve, with 50 ml of sample. The retention times (RT) and relative retention times (RRT) for each compound are listed in Table II.1.

Table II.1. TNT Derivatives Retention Times for HPLC System.

Compounds	R.T.(min)	Column	R.R.T.(min)	UV (ηM)
Toluene	16.89	C18	N.A.	210
o-cresol	4.32	C18	N.A.	250
2-amino toluene	3.69	C18	N.A.	250
2-nitrotoluene	14.50	CN-C18	0.77	250
2-amino,6-nitro toluene	13.23	CN-C18	0.70	250
2,6-dinitrotoluene	17.18	CN-C18	0.91	250
2-amino 4,6-dinitro toluene	27.93	CN-C18	1.49	250
4-amino 2,6-dinitro toluene	25.11	CN-C18	1.34	250
2,4,6-trinitro toluene	20.43	CN-C18	1.09	250
2,4-diamino 6-nitro toluene	12.06	CN-C18	0.64	250
2,6-diamino 4-nitro toluene	10.82	CN-C18	0.58	250
3-nitro o-xylene*	18.80	CN-C18	1.00	250

* Internal Standard

II.2 ^{14}C-TNT Analytical Method

The second gradient HPLC system used an Alltech RP-100 (150 mm × 4.6 mm) column. Mobile phase A was 10% methanol in 5 mM solution of H_3PO_4. Mobile phase B was 90 % methanol in a 5 mM solution of K_3PO_4. The flow rate was one ml/min. The linear gradient was started one minute following injection and reached 100 % B at 21 minutes and was maintained there for 29 minutes. The outflow from the column was first flowed through a UV detector set at 221 nm (Beckman model 160) and then into a Radiomatic A140 radiochemography detector. The A140 was configured for a 500 ml flow cell, and liquid scintillant (Packard Flo-Scint V) was mixed with it in a ratio of 4:1. Data from both detectors were collected using a Perkin-Elmer PE7500 data system running *Chrom III* software. A manual injection valve was used to inject 200 ml of sample. Sample preparation was confined to contrifugation of sedimentary solids and injection of the supernate.

II.3 GC/MS Analysis for TNT and Intermediates

Mass spectrometry analysis was performed using a Hewlet Packard model 5988A connected to a 5890 Gas chromatographer (GC). The GC column was an XTI-5 fused silica capillary column. Heliumn, at flow rate of 20 ml/min, was used as the carrier gas. A 3 ml aliquot of chloroform extract was injected into the gas chromatographer (with injection port temperature of 250°C). The column was held 45 °C for two minutes and was ramped to 120°C at a rate of 10°C/min, held at this temperature for one min, and then ramped to 260°C at a rate of 20°C/min. The mass spectrometer ion source was turned four minutes after initiation of the temperature program. The interface line to the ion source was held at 280°C throughout the run. The mass spectrometer was operated using an ionizing voltage of 70 eV and an ionizing current of 300 mA. The mass spectrometer was calibrated and tuned using perfluro-tributylamine (FC-43) as the calibration compound prior to running samples. The retention times (RT) and relative retention times (RRT) for each compound are listed in Table II.2.

Table II.2 TNT Derivatives Retention Times for GC/MS System.

Compounds	Retention Time (min)	Relative Retention Time (min)
Toluene	4.90	0.344
Aniline	8.90	0.625
2-aminotoluene	10.60	0.744
2-nitrotoluene	12.15	0.853
2,6-diaminotoluene	15.85	1.112
2,6-dinitrotoluene	16.85	1.182
2-amino6-nitrotlouene	17.55	1.231
Trinitrotoluene	19.15	1.344
Triaminotoluene	19.17	1.345
2,4-diamono,6-nitrotoluene	20.50	1.438
4-amino,2,6-dinitrotoluene	20.70	1.452
2,6-diamino,6-nitrotoluene	21.35	1.498
2-amino,4,6-dinitrotoluene	21.40	1.502
o-nitroxylene	14.25	1.000

II.4 Ion Chromatography for Anions Analysis

A Dionex 4000i ion chromatography (IC) with a HPIC-CS3 anion column was used to measure anions such as nitrate and nitrite. The mobile phase contained 0.191grams of Na_2CO_3 and 0.143 grams of $NaHCO_3$ in one liter of distilled water. For regeneration of the column, 0.07% H_2SO_4 was used. The flow rate was 2 ml/min. The outflow from the column was analyzed on a conductivity detector. An autosampler was used to inject 50µl from auto sampler vials containing 500µl of sample, which was filtered through a 0.25 µm TPFE syringe filter prior to loading the autosampler vials. The retention times (RT) for each compound are listed in Table II.3.

Table II.3 Retention Time of anions for Ion Chromatography

Compounds	Retention Times(min)
Lactate	1.41
Nitrate	2.38
Nitrite	1.58

II.5. Gas Partitioner for Head Space Analysis

Head space gas analysis was performed on a Fisher Gas Partitioner Model 25V. The gas partitioner used two column in series. A molecular sieve 13X (45/60 mesh) column connected to a 30% of Bis12 Ethoxyethyl Adipate and 70% of chromosorb pwu(60/80 mesh) column. Helium at flow rate of 80 ml/min was used for carrier gas. A sample volumn of 200 μl was injected. The retention times (RT) for each compound are listed in Table II.4.

Table II.4 Retention Times of each compound for Gas Partitioner

Compounds	Retention Time (min)
CO_2	0.83
O_2	2.21
N_2	3.54
CH_4	5.55

III. TNT Extraction Methods and Media Preparation.

III.1. TNT Extraction in Water for GC/MS Analysis

III.1.1. Scope

This test method covers the qualitative analysis of Gas Chromatography/ Mass Spectrometry (GC/MS) for approximately 100 mg/l of TNT and its metabolites in water (Trinitrotoluene, Monoaminodinitrotoluene, DiaminomononitroToluene, Diaminotoluene, Dinitrotoluene and Aniline).

III.1.2. Apparatus

Nitrogen evaporator

Test tube, 10 ml Teflon lined cap

Syringes, 100 µl and 10 µl

Pipette, 1 ml

Centrifuge

Mechanical shaker

III.1.3. Reagents

HPLC grade Chloroform

III.1.4. Extraction procedure

1. Place a 10 ml test tube and rinse the tube at least twice times with $CHCl_3$.

2. Place 100 µl of sample in the tube (centrifuge the sample, if necessary, before taking sample).

3. Add 500 ul of $CHCl_3$ to the tube and shake well the tube for 20 minute.

4. Centrifuge the tube to separate the water and extract solvent layers for 5 minutes (approximately 2000 xg).

5. Take exactly 100 ul from the bottom layer.

6. Inject 3 μl of the extract to GC/MS (if sensitivity is too low, concentrate the residue of the extract to about 3 μl under nitrogen gas flow and inject to GC/MS).

III.2. Minimal Medium Preparation

III.2.1. Scope

This method describes a preparation procedure of the growth medium in serum tubes.

III.2.2. Procedure

1. Place 0.5 grams of KNO_3, 0.02 grams of yeast extract, 0.1 gram of sodium lactate, 10 ml of 10X salts and 220 ml of distilled water in 500 ml of a boiling flask...
2. Boil the flask under argon gas flow for 3 minutes.
3. Cool the flask to room temperature and add TNT into the flask under argon flow.
4. Adjust pH to 7.0 with NaOH or HCl.
5. Stopper and sonicate the flask for 5 minutes.
6. Dispense 9 ml of the medium into serum tubes.
7. Autoclave the tubes for at least 15 minutes and cool to room temperature.
8. Add 0.5 ml of $CaCl_2$ $2H_2O$ per tubes.

Table III.1 Trace metal compositions in 500 ml of water

Compounds	grams
Na_2EDTA	0.2150
$FeSO_4\ _7H_2O$	0.1000
$MnSO_4\ H_2O$	0.0850
$ZnSO_4\ 7H_2O$	0.0050
H_3BO_3	0.0150
$CoCl_2\ 6H_2O$	0.0060
$CuCl_2\ 6H_2O$	0.0005
$NiCl_2\ _6H_2O$	0.0010
$NaMO_4\ 2H_2O$	0.0150

Table III.2 10X salts composition in 500 ml of water

Compounds	Grams
$NaH_2PO_4\ H_2O$	9.750
Na_2HPO_4	25.50
$MgSO_4\ 7H_2O$	0.30
Trace Metals	50 ml

III.3. Plug Reactor Medium Preparation

III.3.1. Scope

This method describes the preparation procedure of continuous flow bioreactor medium.

III.3.2. Procedure

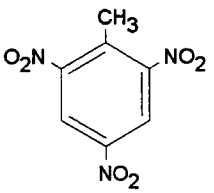
1. Place a 500 ml boiling flask (Mark the flask as Flask A).
2. Add 18 mg of sodium Lactate, 38 mg of potassium nitrate, 50 mg of yeast extract and 25 ml of 10X salts to the flask.
3. Add 250 ml of distilled water to the flask.
4. Place another flask with 350 ml of distilled water (Mark the flask as Flask B).
5. Boil the flasks for 3 minutes to remove dissolved oxygen in water under argon gas flow.
6. Cool them to room temperature (use ice cubes if needed).
7. Transfer the contents of Flask A to 500 ml graduated cylinder under argon gas flow.
8. Pour the deoxygenated water (Flask B) to fill up the graduated cylinder with exactly 500 ml.
9. Dispense 99 ml of the medium in the graduated cylinder to each serum bottles under argon gas flow (TNT or TAT should be in the bottle if necessary).
10. Autoclave the bottles for 20 minutes and cool to room temperature.
11. Inject 1 ml of $CaCl_2$-$2H_2O$ to each serum bottle.

IV. TNT and Metabolites Chemical Structure and Physical Properties

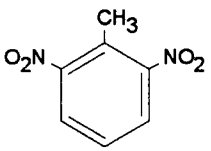
Table IV.1 Physical Properties of TNT and Its Metabolites

	CAS	M.W. (g/mole)	Density (g/cm^3)	M.P. (°C)	B.P. (°C)	Solubility (mg/l)	Absorbance (l)	G_f (Kcal/ mole)
TNT	118967	227.1	1.654	80.0	280 exp	150.0	225.0 (4.36)	50.92
2A46DNT	35572782	197.0	-	176.0	-	-	-	-
4A26DNT	19406510	197.0	-	174.0	-	7.7	-	-
24DA6NT	6629294	167.0	-	124.0	-	-	-	-
26DA4NT	59229753	167.0	-	-	-	-	-	-
TAT	28554043	137.0	-	-	-	-	-	-
26DNT	606202	182.1	1.283	70.0	300.0	300.0	241.0 (3.95)	-
24DNT	121142	182.1	1.321	71.0	300.0	300.0	252.0 (4.15)	40.94
2A6NT	603838	152.2	1.366	100.0	-	-	235.0 (4.2)	-
2NT	88722	137.1	1.163	-9.3	221.7	650.0	259.0 (3.72)	-
2AT	84673	121.2	0.973	-	207.7	15000	232.5 (3.88)	-
Toluene	108883	92.1	0.866	-95.0	110.6	492.0	207.0 (3.97)	29.23

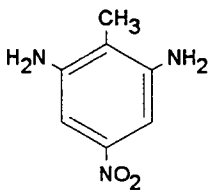
Sources: *Handbook of Chemistry and Physics*, 60th ed. (1979); Seidell, *Solubility of Organic Compounds* (1940); Dean, *Lange's Handbook of Chemistry*, 11th ed., McGraw-Hill; Lenchitz et al. (1971).



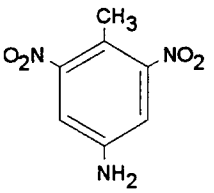
2,4,6-trinitrotoluene(TNT)



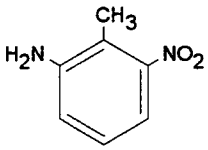
2,6-dinitrotoluene(26DNT)



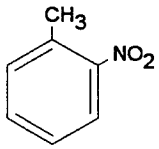
2,6-diamino,6-nitrotoluene(26DA4N



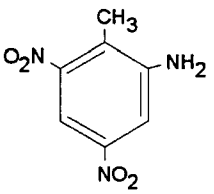
4-amino,2,6-dinitrotoluene(4A26DNT)



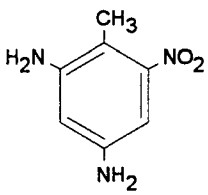
2-amino,6-nitrotoluene(2A6NT)



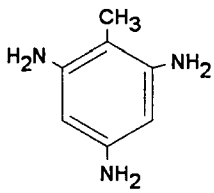
2-nitrotoluene(2NT)



2-amino,4,6-dinitrotoluene(2A46DNT)



2,4-diamino,6-nitrotoluene(24DA6NT)



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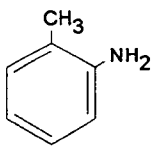
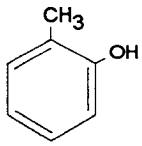
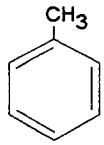


2-aminotoluene(2AT)



o-cresol



toluene

Figure IV.1 Chemical structure of TNT and its metabolites