ANAEROBIC DEGRADATION INTERMEDIATES OF 2,4,6 - TRINITROTOLUENE (TNT) UNDER DIFFERENT ELECTRON ACCEPTING CONDITIONS

By

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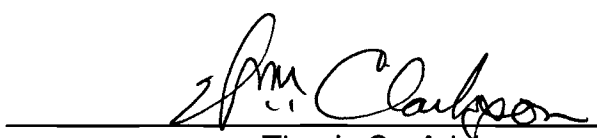

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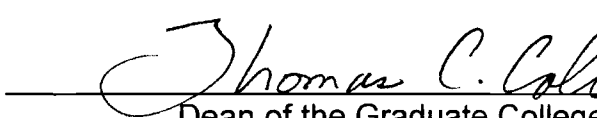

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CHAPTER I

INTRODUCTION

2,4,6- Trinitrotoluene (TNT) does not occur naturally in the environment. TNT enters the environment in the waste waters and solid wastes resulting from the manufacture of the compound, the processing and destruction of bombs and grenades, and the recycling of explosives. The compound moves in surface water and through soils to groundwater. In surface water, TNT is rapidly broken down into other chemical compounds by photolysis. The estimated half life of TNT in surface waters is 0.16 - 1.28 hours, based on the rate of photolysis and photooxidation in sunlit natural waters (*Howard et al. 1991).* The fate of TNT in groundwater remains the focus of a number of researchers. Even 50 years after World War II, TNT and its intermediates can be found in large amounts in soils at former ammunition factories. This indicates a high degree of persistence of these compounds. Recent studies by McKone and Layton (1986) identified consumption of contaminated water and ingestion of contaminated fruits and vegetables as potentially the most important exposure pathway for residents or workers near sites contaminated with TNT.

The biodegradation of TNT is rendered difficult because of the presence of the three nitro groups. However, TNT is not totally refractory to biodegradation, and a number of microorganisms have been isolated which are

capable of biotransforming TNT due to a cometabolic process. Complete mineralization in small amounts has been observed by very few authors using radiolabelled TNT. In various studies under aerobic and anaerobic conditions, amino compounds and often azoxy compounds have been identified as intermediates and byproducts in the transformation. These compounds are even more toxic than TNT itself and often result in dead-end metabolites (*Kaplan and Kaplan 1982*). However, biodegradation has shown promise in the treatment of TNT-contaminated soils (*Fernando et al. 1990; Funk et al. 1993*). Bioremediation of these soils therefore is an important alternative to the costly physical / chemical methods of incineration or wet air oxidation.

Redox conditions in the natural environment are always in a dynamic state. Shifts between oxygen-rich to oxygen-depleted conditions are very possible in the soils near the surface. Anaerobic conditions commonly exist in soils as one goes deeper. Over years contaminants may move down to the anoxic regions. Under these conditions the role of the alternate electron acceptors such as sulfate, nitrate and organic compounds must be considered.

Thus, the main focus of this research was :

1. To try to identify the initial degradation products of TNT under different electron acceptor conditions - methanogenic, denitrifying and sulfate reducing.

2. Determine the fate of the intermediates under these conditions.

CHAPTER II

LITERATURE REVIEW

2.1 SOURCES OF TNT

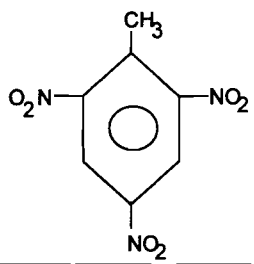
TNT is prepared by the nitration of toluene with a mixture of nitric acid and sulfuric acid (*Fisher and Taylor 1983; Sax and Lewis 1987*). Toluene is nitrated in a three step operation by using increasing temperatures and mixed acid concentrations to successively introduce nitro groups to form mononitrotoluene (MNT), dinitrotoluene (DNT), and trinitrotoluene (*Mark et al. 1980*). Byproducts formed include asymmetrical isomers of TNT, and oxidation products such as tetranitromethane, nitrobenzoic acid, nitrocresol, and partially nitrated toluenes (*Hamilton and Hardy 1974; Mark et al. 1980*). The asymmetrical TNT isomers are removed by washing with an aqueous sodium sulfite solution known as Sellite (*Fisher and Taylor 1983; Mark et al. 1980; Sax and Lewis 1987*). In the full-scale production of TNT, the collected wash solutions containing Sellite waters and spent acids are usually combined and routed to settling ponds, remaining there for varying periods. The waste waters have been classified as hazardous by EPA (*Tsai 1991*). In the US, TNT production is limited to military arsenals, and under SARA section 313 (Superfund Amendment and Reauthorization Act), TNT releases are not required to be reported (*HSDB 1990*).

The physical and chemical characteristics of TNT are shown in Table I. TNT is classified as a high explosive and is used in bombs, grenades, for filling shells and airborne demolition bombs. In addition to military use, small amounts are used for industrial explosive applications such as deep well and underwater blasting (*HSDB 1990*). Other industrial uses include use as a chemical intermediate in the manufacture of dyes, photographic chemicals, fungicides, insecticides, herbicides, and pharmaceuticals (*Sax and Lewis 1987; Walker and Kaplan 1992*).

TNT is released to the atmosphere and to soils as a result of open detonation and open burning techniques used in the demilitarization of munitions. Also, soil contamination can occur due to spills, disposal of solid waste (landfilling of waste generated during kiln incineration), and leaching of inadequately sealed impoundments (*Army 1986*). TNT has been identified in at least 19 of the 1,300 hazardous waste sites listed on the EPA National Priorities List (NPL). TNT has been detected in surface soil samples at an average concentration of 1.3% (13,000 mg/kg) at the US Department of Energy's Weldon Spring Site, MO, at 4% (40,000 mg/kg) at the West Virginia Ordnance Works, Mason County, West Virginia, and at various other TNT manufacturing sites (*Haroun et al. 1990; Krauss et al. 1985*).

Aqueous effluents of explosives production facilities (red water) and ammunition load, assemble, and pack (LAP) plants (pink water) contain large quantities of TNT. LAP facilities use large volumes of hot water to wash off residual explosives from equipment, rejected shells and interior surfaces of facilities (*Harvey et al. 1990; Won et al. 1974*). TNT has been detected in surface and groundwater samples collected in the vicinity of munitions facilities.

Table I
Chemical and Physical Properties of TNT

Chemical name	2,4,6-Trinitrotoluene
Synonym(s)	sym-Trinitrotoluene ; tolit ; 1-methyl-2,4,6-Trinitrobenzene ; trilit ; 2-methyl-1,3,5-Trinitrobenzene ; alpha - TNT ; TNT ; alpha - trinitrotoluol ; tritol ; trotyl oil (*HSDB 1990*).
Chemical Formula	$C_7H_5N_3O_6$
Chemical Structure	
Molecular Weight	227.13 gms / mole *(Budavari et al. 1989)*
Color	Yellow *(Budavari et al. 1989)*
Physical State	Monoclinic needles *(Budavari et al. 1989)*
Melting Point	80.1°C *(Budavari et al. 1989)*
Boiling Point	240°C (explodes) (*HSDB 1990*)
Density (at 20°C / 4)	1.654 *(Budavari et al. 1989)*
Odor	Odorless
Solubility : Water at 20°C Organic Solvent(s)	130 mg / L Soluble in acetone and benzene; soluble in alcohol and ether (*HSDB 1990*)
Vapor Pressure at 20°C	1.99×10^{-4} mm Hg (*HSDB 1990*)
Henry's Law Constant at 20°C	4.57×10^{-7} atm / m^3·mole (*HSDB 1990*)

2.2 TOXIC CHARACTERISTICS OF TNT

The toxic characteristics of TNT were first observed during its wide-spread use in World War I. Numerous adverse health effects such as anemia, liver function abnormalities; morphocytosis, toxic jaundice and respiratory complications have been observed at exposure levels below 1.5 mg/m^3 in air (*Hathaway 1977*). Toxic hepatitis caused by inhalation exposure to TNT is the main toxic effect of TNT and was the cause of a large number of deaths during the war. The reported oral LD_{Lo} (Lethal Dose, Low) value for humans is 28 g/kg·day (*Sax and Lewis 1987*) and LD_{50} (Lethal Dose, 50% Kill) values are 1010 and 1320 mg/kg·day for male rats and 820 and 795 mg/kg·day for female rats. Also, TNT has been reported to be toxic to bluegill fish with an LC_{50} (Lethal Concentration, 50% Kill) of 2.3 to 2.8 mg/L (*Anon. 1971*). A mean tolerance limit of 2.0 to 3.0 mg TNT/L has been observed for a number of freshwater fish (*Osmon and Klausmeier 1972*). Adverse gastrointestinal effects were observed in dogs after intermediate oral exposure. Anemia is one of the major signs of TNT toxicity and is probably induced through an oxidizing process mediated by TNT and/or its metabolites (*Dilley et al. 1982*). Discoloration of the urine is among the first indications of TNT intoxication in humans. The color of urine changes from normal amber to a deep red. A cross-sectional study performed in two plants located in Henan Province, China, in 1990 indicated that exposed male workers complained of more sexual disorders such as impotence, loss of libido and sexual hypothesia than the control group (*Yi et al. 1993*).

No Minimal Risk Level (MRL) has been derived for TNT. EPA (*IRIS 1992*) assigned TNT a reference dose (operationally derived from the No Observed Adverse Effect Level) of 5.00×10^{-4} with an uncertainty factor of 1000 based on liver effects observed in dogs. It also assigned TNT a weight of evidence

carcinogenic classification of C, which indicates that it is a possible human carcinogen.

The Drinking Water Equivalent Level (DWEL), a lifetime exposure at which adverse health effects would not be expected to occur, is 20 μg/L (*EPA 1989*). The acceptable daily intake concentration is calculated as 44.25 μg/L. The criterion to protect human health has been estimated at 135 μg/L and to protect aquatic life is estimated at 557 μg/L.

2.3 CURRENT TECHNOLOGY FOR TREATMENT OF TNT WASTES

Various remediation technologies are currently being tested for their applicability to TNT contaminated soils and water. Hao and coworkers (1993) examined the possibility of wet air oxidation (WAO) of red water. They demonstrated effective treatment of diluted red water at 340°C and 0.8 Mpa $Po_{2(25°C)}$. The treated red water had adverse effects on the efficiency of enriched Nitrosomonas in converting ammonium to nitrate, indicating toxicity. Supercritical water oxidation of DNT is reported by Li et al. (1993) at high temperatures (above 374°C) and pressures (27.6Mpa). However, such operating conditions make the treatment uneconomical.

Removal of explosives from contaminated groundwater using granular activated carbon (GAC) systems has been successfully reported by Wujcik et al. (1992). The process involved air stripping to remove volatiles from groundwater before feeding into GAC columns. However, effluent criteria of 40μg/L of TNT were not met. Though 99.5% TNT removal at concentrations of 121.8 mg/L has

been reported (*Ruchhoff et al. 1945*), regeneration of spent carbon is difficult to achieve chemically and hazardous thermally (*Walsh et al. 1973*).

Preuss and coworkers (1993) reported 25 to 40% TNT loss due to chemical reactions, mainly reduction due to addition of sulfide. Han (1993) reported the transformation of TNT by sodium sulfide only in the presence of microbial exudates. Similar findings have been reported by Cho et al. (1995) who studied Co^{+3}-centered porphyrin-catalyzed reduction of TNT, nitrotoluenes and dinitrotoluenes. Though the process could be used for pretreatment of waste waters, the high costs of porphyrins and reductants (Dithiothreitol (DTT), sodium dithionite and sodium sulfide) make the application expensive.

Incineration is the only available proven technology for the remediation of explosive-contamiated soils (*Funk et al. 1993*). However, high mobilization and capital costs, high energy costs, air emissions and toxic solid waste production make this an expensive alternative. Composting, though effective, has the disadvantage of long incubation times and large quantities of additives required for very small volumes of contaminated soil (*Funk et al. 1993*). Also, complete mineralization of TNT has not been reported.

In the light of the above discussion, bioremediation of TNT in contaminated soils and groundwater is an important treatment alternative. Physical and chemical treatments for disposal of explosive wastes are often costly and impractical. In addition, they require rigid control to prevent further contamination. Biological treatment, if possible, would offer many advantages. However, microbial reduction of TNT poses severe problems because of the toxicity exhibited by the intermediates (*Kaplan and Kaplan 1982*). TNT and its intermediates are highly sorptive, binding to humic fractions in soil. This prevents

or limits their biodegradation by soil microorganisms. Any bioremediation system must therefore satisfy biodegradation requirements of all contaminants (mono-, di-, and trinitrotoluene and also aminonitrotoluene) and their metabolic products.

In this thesis, the commonly occuring intermediates have been abbreviated and their full names have been shown in the nomenclature. The terms biodegradation, biotransformation and mineralization are used as explained below. Biodegradation includes both biotransformation and mineralization. Biotransformation indicates modification of the target chemical, such as change in a functional group (e.g. 2,4,6-TNT → 2-ADNT), but not resulting in mineralization. Mineralization refers to degradation of target chemical to form methane, carbon dioxide, water and biomass as final products and hence the return of compounds into normal geochemical carbon and nitrogen cycles (*Walker and Kaplan 1992*).

2.4 AEROBIC DEGRADATION OF TNT

Traxler and coworkers (1974) have reported aerobic utilization of TNT as a sole nitrogen and carbon source. Using ^{14}C-TNT, they reported ring cleavage and incorporation of TNT into cellular material and release of carbon from TNT as CO_2. The authors reported better microbial transformation of TNT in the presence of yeast extract. Similar observations with yeast extract have been reported by other workers (*Won et al. 1974; Osmon and Klausmeier 1972*).

Boopathy and coworkers (1994b) isolated four Pseudomonas spp. capable of transforming TNT by a co-metabolic process. Cultures with TNT as sole carbon source did not show any growth. Also, radiolabelling experiments

indicated mineralization of ^{14}C-TNT by production of ^{14}C-CO_2 and the presence of ^{14}C in cell biomass as TCA-precipitable material. The main intermediates identified were 2-ADNT and its isomer 4-ADNT. The reduction of the third nitro group was not observed. Similar results have been reported by other authors (*Won et al. 1974*). Boopathy and coworkers (1994b) reported that nitrite production was observed after all TNT had transformed, indicating nitrite release from intermediates, and therefore assumed the third unidentified intermediate to be 2-amino-6-nitrotoulene. Based on their findings, they suggest the following pathway: reduction of TNT to HADNT isomers which are reduced to form ADNT. This is similar to the pathway proposed by Won and coworkers (1974). The ADNTs are further reduced to form ANTs by cleavage of a nitro group. Won and coworkers (1974) proposed reduction of ADNT to DANT. The major intermediates identified were the isomers ADNT, tetranitroazoxytoluenes and DANT. The azoxy compounds may not be the products of direct TNT metabolism but substances formed from the abiotic coupling reactions of the corresponding hydroxylamines.

Fernando and coworkers (1990) investigated the biodegradation of TNT by the wood-rotting (white-rot) fungus Phanerochaete chrysosporium in soil and liquid cultures. In liquid cultures the authors reported that about 36% of ^{14}C-TNT was detected as ^{14}C-CO_2 at an initial concentration of 1.3 mg/L, and only 3.3% of initial TNT was recovered after 18 days. When the concentration was increased to 100 mg/L, approximately 20% of initial TNT was converted to $^{14}CO_2$ in 90 days and 85% degraded. Unidentified intermediates which were more polar than TNT were detected. In soil degradation studies, approximately 18.4% of initial TNT (10,000 mg/kg) was converted to $^{14}CO_2$ during a 90 day incubation period with 14.9% TNT remaining undegraded. Parish (1977) reported transformation of

TNT by 98 of the 190 fungi screened. No ring cleavage was evidenced with radiolabelled TNT. The main intermediates identified were 4-ADNT and 4-4'-Az. Tsai (1991) also reported bioconversion of TNT by a white rot fungal system or an extracellular enzyme preparation exhibiting ligninase activity.

Bumpus and Tatarko (1994) further investigated TNT degradation by Phanerochaete chrysosporium in a fixed-film silicone membrane bioreactor. The main intermediates identified were 4-ADNT and 2-ADNT. Further degradation of these intermediates into unknown metabolites was observed at rates slower than that for TNT. 4-HADNT, an expected intermediate, tested positive for inhibition of lignin perioxidase activity. Thus, even though TNT biotransforms rapidly, the pathway usually results in the formation of dead-end metabolites.

In another study by Spiker and coworkers (1992) using the same fungi, mineralization of 10% of TNT in solution (initial concentration 5 ppm) in spore-inoculated cultures at 37°C was observed in 27 days. No significant mineralization was observed at TNT concentrations greater than 15 ppm. No mineralization was observed in mycelium-inoculated cultures and though mycelium-inoculated cultures could degrade 100 ppm TNT, further growth was inhibited at TNT concentrations above 20 ppm. The fungus was found to be completely inhibited by even small amounts of contaminated soil from a representative contaminated munitions processing site and sensitive to pure TNT and to a TNT-, HMX-, and RDX- containing extract.

Duque and coworkers (1993) isolated Pseudomonas sp. strain C1S1 which was able to grow on TNT, DNT and 2-nitrotoluene as nitrogen sources. Optimum growth with TNT at 100 mg/L and with fructose as the carbon source was observed at 30-32°C and in the pH range between 5.5 and 9. The strain did

not fix nitrogen. Nitrite reductase activity was inhibited, indicated by an increase in NO_2^- concentrations, and TNT utilization was impaired in low concentrations (below 20 mg/L). 2,4-DNT, 2,6-DNT, 2-NT and toluene were identified in the 28-day old reactors. This indicates a cleavage of the nitro group. Identification of toluene as an intermediate is interesting since a degradation pathway for toluene is already established. The strain was also capable of reducing nitro groups to amino groups via hydroxylamines. Azoxy dimers were also observed.

Duque and coworkers (1993) further isolated a derivative strain, Pseudomonas sp. Clone A which grew on TNT and did not accumulate NO_2^-. The main intermediates identified were 4-ADNT, 2-ADNT as well as the azoxy dimer in addition to the intermediates identified in the supernatants from cultures of the parental strain mentioned above. The Pseudomonas sp. Clone A was used as a recipient for the self transmissible P. putida TOL plasmid, and their ability to grow on TNT as sole carbon and nitrogen source was observed.

Removal of nitro groups from aromatic rings is also reported by Lenke and Knackmuss (1992), who investigated bacterial utilization of picric acid as a nitrogen source. They report the formation of a Meisenheimer-complex which was further converted by cells with concomitant release of nitrite. Duque and coworkers (1993) suggest a similar reaction sequence for removal of the nitro groups. Though no Meisenheimer complex was detected (indicated amongst others by a color change from yellow to orange-red), the authors report the possibility of its formation due to nucleophilic attack by H^-, which could be supplied in vivo by NAD(P)H, on the aromatic ring of TNT.

2,4-DANT, one of the main intermediates in the biotransformation of TNT, was studied for its degradation pathway by Naumova and coworkers (1988).

Pseudomonas fluorescens B - 3468, capable of using 2,4-DANT as its sole nitrogen source, was used for the study. Production of nitrogen-free TNT metabolites phloroglucine and pyrogallol preceded by NADH-dependent deamination is reported. NADH in combination with FAD were the important cofactors influencing the deamination process. The presence of the above mentioned intermediates and absence of nitrate and nitrite ions indicate release of nitro groups as ammonium.

2.5 ANAEROBIC DEGRADATION OF TNT

Funk and coworkers (1993) suggested a two stage mechanism for the anaerobic metabolism of TNT. The first stage is a reductive stage in which TNT is reduced to its amino derivatives and the second stage involves the degradation to non-aromatic compounds. The first stage reduction has been observed and reported by various researchers (*McCormick et al. 1976*; *Boopathy et al. 1993; Preuss et al. 1993*).

McCormick and coworkers (1976) suggested two possible transformation pathways for nitroaromatic compounds. One is the reduction of a nitro group to an amino group followed by an oxidative deamination to a phenol with release of ammonia. The other involves the release of a nitro group as nitrite with simultaneous formation of a phenol. Absence of nitrite in reactors suggested the first pathway as dominant. However, no phenolic intermediate was observed. The number of nitro groups reduced depends upon the reducing potential of the system. The authors proposed the following pathway :

(1) $R\text{-}NO_2 \rightarrow R\text{-}NO + H_2$ (R-NO : Nitroso Compound)

(2) R-NO $\rightarrow$ R-NHOH (R-NHOH : Hydroxylamino Compound)

(3) RNHOH $\rightarrow$ $R\text{-}NH_2 + H_20$

The authors showed that TNT was reduced by hydrogen, in the presence of an enzyme preparation from Veillonella alkalescens, to TAT; 3 moles of hydrogen are required to reduce each nitro group to the amino group. No nitro compounds were detected; however the hydroxylamino compound was identified, and all major metabolic products of TNT originate from the hydroxylamino compound. The main intermediates observed were 4-HADNT, 4,4'-Az, 2-ADNT, and 2,2'Az. Various other researchers have reported similar findings (*Walker and Kaplan 1992; Carpenter et al. 1978; Greene et al. 1985*). As stated earlier, the azoxy compounds could be present due to dimerization reactions.

Boopathy and coworkers (1993) studied the anaerobic transformation of TNT under different electron accepting conditions. Results showed maximum TNT removal under nitrate-reducing conditions (82% of original TNT concentration). Under sulfate reducing conditions and methanogenic conditions (with CO_2 as electron acceptor and H_2 as electron donor), moderate transformation of TNT was observed (30% and 35% respectively). No TNT transformation was observed in absence of a primary substrate, indicating a co-metabolic process. Various authors have suggested a co-metabolic process for TNT biotransformation (*Boopathy et al. 1993; Boopathy et al. 1994a; Boopathy et al. 1994b; Preuss et al. 1993; Han 1993; Osmon & Klausmeier 1972*).

In a different study, Boopathy and Kulpa (1992) isolated a sulfate-reducing bacterium which was capable of using TNT as its sole nitrogen source.

Under nitrogen limiting conditions, 100% removal of TNT was observed within 8 days of incubation. The main intermediates identified were the DANTs. DANT was presumed to be further converted to toluene via TAT by reductive deamination. However, TAT was not detected in the reactors. Toluene was observed in the reactors after 45 days of incubation. Tests conducted by the authors indicated that the isolate could use TNT as a sole nitrogen source and also as an electron acceptor in the absence of sulfate.

Preuss and coworkers (1993) also studied the use of TNT as a sole nitrogen source for sulfate-reducing bacteria. The main intermediate identified was 2,4-DANT formed via 2-ADNT and 4-ADNT. The bacteria used TNT as the sole nitrogen source, as evidenced by significant growth when no other nitrogen source was added. These results are similar to those reported by Boopathy and Kulpa (1992), who identified the sulfidogenic bacteria as Desulfovibrio sp. (B Strain). Results indicated that the conversion of 2-4-DANT to TAT was the rate-limiting step in microbial TNT reduction. 2,4-DAHAT was formed as an intermediate in this reaction. DAHAT is further co-metabolically reduced to form TAT. The sulfidogenic isolate converted TAT only under aerobic conditions. A Pseudomonas strain using glucose as its energy and carbon source was isolated and was found capable of transforming TAT. TAT transformation products were not identified. Toluene production was not observed by Preuss et al. (1993).

Roberts and coworkers (1994) report accumulation of 4-ADNT to a concentration of 50 mg/L in pilot scale studies of TNT degradation under anaerobic conditions. The accumulation resulted in a reduction in the rate and extent of TNT degradation. Experiments with various carbon sources resulted in the choice of glucose as the most favorable. In laboratory studies, Roberts and coworkers (1994) report rapid conversion of TAT to MPG which was further

dehydroxylated to p-cresol in the presence of yeast extract. Han (1993) reported TNT biotransformation under anaerobic conditions with peptone and glucose as primary substrates. Various transitional intermediates were observed. One of the intermediates (tentatively identified as 2,4-DANT) accumulated in the reactors. Denitrifying conditions were introduced in the reactor at this stage. The intermediate was transformed completely without detection of any other intermediates. On the basis of this finding, Han (1993) suggested that a denitrifying stage following an anaerobic stage may lead to complete degradation of TNT.

Osmon and Klausmeier (1972) isolated various microorganisms from TNT-contaminated soil which were capable of degrading TNT. The majority of these organisms were similar to pseudomonads. A pure culture of Pseudomonas aeruginosa was used to study TNT degradation under different conditions. The authors report transient accumulation of TNT intermediates. The degradation of TNT was found to be a co-metabolic process using glucose as the primary substrate.

Funk and coworkers (1993) suggest that the para-nitro group is usually the first to be reduced, followed by reduction of one of the ortho-groups, producing DANT isomers. The reductive pathway identified is TNT $\rightarrow$ 4-ADNT $\rightarrow$ 2,4-DANT $\rightarrow$ TAT. The next compound detected was trihydroxytoluene (methylphloroglucinol, MPG) which was dehydroxylated to p-cresol. The authors examined the effect of pH and temperature on the reductive stage. They suggest maintenance of low to neutral pH to minimize polymerization of intermediates and an optimum temperature range of 20°C to 37°C. Improved biotransformation was observed when 25mM ammonium with 50mM potassium phosphate buffer was added. Since the end products identified are MPG and p-cresol, the authors

suggest the addition of an aerobic stage following the anaerobic stage, since both MPG and p-cresol are known to degrade faster under aerobic conditions than under anaerobic conditions.

Mondecar and coworkers (1994) have reported biotransformation of TNT using microbial mats. The authors report that the mats contained a number of microbial species and that unique chemistry involved with the laminated structure of the mats might have resulted in oxic and anoxic zones in close proximity and thus supporting aerobic and anaerobic bacteria. Mats exposed to 100 mg/L TNT showed greater than 99 % degradation in 6 days. Metabolites detected included 2-ADNT, 4-ADNT, 2,4-DNT, and 2,6-DNT. The authors suggest that low concentrations of the metabolites indicate continuing transformation of these metabolites.

2.6 SUMMARY

The main intermediates observed and reported by various authors have been summarized and presented in Table II. In conclusion, even though the biotransformation of TNT is widely reported and studied, complete mineralization of the compound and the degradation pathway still remain the concern of many researchers. The wide range of results regarding the environmental fate of TNT, as seen in the above literature, encourages further research in the field. Various different treatment processes have been proposed. Han (1993) proposed an anaerobic process followed by a denitrifying stage, whereas Funk and coworkers (1993) propose an anaerobic stage followed by an aerobic stage. The aim of this reseach shall be to study the degradation of TNT under three different anaerobic electron acceptor conditions.

Table II
Summary of Intermediates Reported

Authors	Reactor Conditions	Microbial Species	Intermediates Identified
Boopathy et al. (1994a)	Aerobic	Pseudomonas spp	2-ADNT, 4-ADNT
Boopathy et al. (1994b)	Aerobic	Four Pseudomonas spp : 1. acidovorans 2. fluorescens 3. medocina 4. aeruginosa	2-ADNT, 4-ADNT, 2-ANT(tentative), ^{14}C-CO_2
Won et al. (1974)	Aerobic	Pseudomonas	2,2'-Az ; 2,2',4,4'-Az ; 2-ADNT ; 4-HADNT ; DANT
Parish (1977)	Aerobic	Fungal Systems	4-ADNT; 4,4'-Az
Bumpus and Tatarko (1994)	Aerobic	Phanerochaete chrysosporium	4-ADNT; 2-ADNT
Duque et al. (1993)	Aerobic	Pseudomonas spp Clone A	2,4-DNT; 2,6-DNT; 2-NT; Toluene; ADNTs; Azoxy Dimers
McCormick et al. (1976)	Anaerobic	Enzyme prep. of Veillonella alkascens	4-HADNT; 4,4'-Az; 2-ADNT; 2,2'-Az; TAT
Boopathy et al. (1993)	H_2:CO_2 Mehanogenic Denitrifying Sulfate Reducing	Soil Bacterial Consortium	4-ADNT, 2-ADNT
Preuss et al. (1993)	Sulfate Reducing	Sufidogenic Isolate	2,4-DANT; 2-ADNT; 4-ADNT; 2,4-DAHAT; TAT
Funk et al. (1993)	Anaerobic	Methanogenic Culture	4-ADNT; 2,4-DANT; TAT; MPG; p-cresol
Naumova et al. (1988)	Anaerobic[1]	Pseudomonas fluorescens B-3468	2,4-DANT; Pyrogallol; Phloroglucine
Boopathy et al. (1992)	Sulfate Reducing	Desulfovibrio sp. (B Strain)	DANT; TAT; Toluene

1 Sulfate present in the medium

CHAPTER III

MATERIALS AND METHODS

3.1 SEED REACTORS

Seed reactors were set up in 160 mL vials with a reactor volume of 120 mL. They were set-up using microbial cultures acclimated to TNT during previous experiments. Seed cultures were started using digested sludge supernatant from the Stillwater Waste Water Treatment plant, and leachate and contaminated soil from the aquifer adjacent to the landfill at Norman, OK. The following three types of seed cultures were incubated and maintained for use in the test reactors: methanogenic, sulfate reducing, and denitrifying. The TNT concentration in the reactors was gradually increased to 100 mg/L, which is near the saturation concentration found in ground water. Two sets of seed reactors under each condition were maintained, each fed 100 mg/L TNT.

Other carbon sources initially added to the seed reactors included yeast extract, peptone, sodium acetate or sodium lactate. Concentrations of carbon sources were gradually reduced over time to observe the effect on TNT metabolism. Initial concentrations for peptone and yeast extract were 0.83 mg/L and 0.42 mg/L respectively. The final conditions in the seed reactors are as shown in the following tables. Once these conditions were achieved, no further changes were made.

3.2 TEST REACTORS

Test reactors were set up using inoculum from each respective seed reactor. Three types of test reactors were set up under each condition - one batch reactor and two controls. Each type of reactor was duplicated, as shown in Table III. The bacterial control reactor was set up to monitor any abiotic TNT reactions in the reactor. Here the reactor contents are the same as those of the main reactors. After set up, the bacterial controls were autoclaved at 248 °C and 15 psi for 30 minutes in order to insure sterilization. The TNT control reactor, with no TNT added to it, was set up to monitor any other biological or chemical products that could occur be produced due to the other carbon sources. The above two controls shall therefore help in quantifying any unknown peaks or abiotic TNT loss.

Table III
Reactor Details

Reactor Condition	TNT Concentration mg/L	Remark
METHANOGENIC		
M1, M2	100	Batch Reactor
BM1, BM2	100	Bacterial Control Reactor
TM1, TM2	0	TNT Control Reactor
DENITRIFYING		
D1, D2	100	Batch Reactor
BD1, BD2	100	Bacterial Control Reactor
TD1, TD2	0	TNT Control Reactor
SULFATE REDUCING		
S1, S2	100	Batch Reactor
BS1, BS2	100	Bacterial Control Reactor
TS1, TS2	0	TNT Control Reactor

The test reactors were set up in 500 mL bottles or flasks and the reactor volume was 400 mL. The pH of the methanogenic, denitrifying, and sulfate reducing reactors was adjusted to 7.0, 7.9, and 6.9 respectively with 10% HCl or NaOH after all the ingredients shown in Table IV were added. The reactors were then purged with argon for 15 to 30 minutes and the gas phase was also filled with argon. The reactors were incubated at 37°C. The proportions of the different solutions fed to the reactors is as shown in Table IV. The concentrated enrichment medium is shown in Table V. The same enrichment medium was used for all the reactors. The substrate solution contained the electron donor, electron acceptor and TNT. The substrate solution recipe was different for each condition and is shown in Table VI. All concentrations in Table V and Table VI are the final concentrations in the reactors.

Table IV
Reactor Recipes

	Batch & TNT Controls (mL)	Bacterial Controls (mL)	Seed (mL)	Remark
Enrichment Medium	40	40	12	Table V
Substrate Solution	335	335	80	Table VI
Inoculum	3	0	15	From seed reactor
Trace Metal Solution	3	3	1	Table VII
2-Aminobenzoate	3	3	1	6 gm/L
Landfill Leachate	0	0	2	from Norman landfill
Total Volume (mL)	400	400	120	

Table V
Enrichment Medium Recipe

Compound	g/L	mM
Peptone	0.1	
Yeast Extract	0.3	
NH_4Cl	0.4	7.48
NaCl	0.05	0.85
$CaCl_2$	0.04	0.36
$MgCl_2$	0.01	0.1
$NaHCO_3$	0.5	5.95
K_2HPO_4	0.348	2.0
KH_2PO_4	0.272	2.0

Table VI
Substrate Solution Recipe

Reactor Condition	Batch & Bacterial Controls		TNT Controls		Seed Reactors	
	g/l	mM	g/l	mM	g/l	mM
METHANOGENIC						
Sodium Acetate	2.87	35	2.87	35	2.87	35
Sodium Sulfide	0.01	0.128	0.01	0.128	0.01	0.128
TNT	0.1	0.44	0	0	0.1	0.44
DENITRIFYING						
Sodium Acetate	2.87	35	2.87	35	2.87	35
Potassium Nitrate	2.02	20	2.02	20	2.02	20
Sodium Sulfate	0.04	0.28	0.04	0.28	0.04	0.28
TNT	0.1	0.44	0	0	0.1	0.44
SULFATE REDUCING						
Sodium Lactate	3.92	35	3.92	35	3.92	35
Sodium Sulfate	2.84	20	2.84	20	2.84	20
TNT	0.1	0.44	0	0	0.1	0..44

The trace metal solution was adapted from Vishniac and Santer (1957) and is as shown in Table VII.

Table VII
Trace Metal Solution

Compound	mg/L
$FeSO_4$-$7H_2O$	200
$ZnSO_4$-$7H_2O$	10
$MnCl_2$-$4H_2O$	3
$CoCl_2$-$6H_2O$	20
$CuCl_2$-$2H_2O$	1
$NiCl_2$-$6H_2O$	2
Na_2MoO_4-$2H_2O$	3

The seed reactors were maintained in the growth phase by transferring the cultures into their respective fresh media on observance of turbidity. The seed reactors were not routinely monitored for TNT transformation. All other reactors were monitored on a regular basis for TNT transformation.

The biotransformation of TNT was monitored by taking samples at short intervals in the early stages of the experiment (1-3 days) and at longer intervals thereafter.

3.3 ANALYTICAL METHODS

3.3.1 Sampling and Sample Preparation

Samples of 3 - 4 mL were taken by introducing an equal volume of argon into the reactors. The samples were filtered using a Gelman Syringe Type Filter

holder assembly and a 0.2 μm Supor-200, 25 mm membrane filter. The first few mL of the filtrate were wasted and the remaining filtrate was collected and diluted for HPLC analysis.

3.3.2 Measurement of Sulfate, Nitrite and Nitrate

A Dionex ion chromatograph, series 20001/sp, was used to identify the anions as per the procedure outlined in Standard Methods, Section 429 (*APHA et al., 1985*). Nitrogen was used to pressurize the ion chromatograph systems. An IonPac AS4A-SC 4 mm analytical column was used. The eluent concentration was 1.8 mM Na_2CO_3 / 1.7 mM $NaHCO_3$, and the flow rate was maintained at 2.0 mL/min. A 25 mN H_2SO_4 solution was used as column regenerant. Standard solutions of known concentrations of anions were used for calibration. The results were integrated on a Hewlett Packard 3380A integrator.

3.3.3 Measurement of Sulfide

Sulfide was detected in the reactors using the Iodometric method described in the Standard Methods (*APHA et al., 1985*), Section 427D.

3.3.4 Measurement of pH

A model 900 Acumet pH meter (Fisher Scientific Co.) was used to measure pH. The pH meter was regularly checked for calibration using standard solutions of pH 4.0, 7.0, and 10.0 obtained from the HACH Company.

3.3.5 Analysis and Identification of TNT and its Intermediates

TNT and its intermediates were analyzed by high performance liquid chromatography (HPLC) with a Beckman liquid chromatograph equipped with two model 127 solvent pumps, a model 166 absorbance detector set at 254 nm

and a System Gold controller. The mobile phase was methanol-water (45 : 55 v/v). The flow rate was 1.5 mL/min. 20 μL samples were injected onto a Beckman C-18 Reverse phase, Ultrasphere ODS, 5 μm particle diameter, 4.6 mm x 25 cm column. The output was collected and integrated on a HP 3396 Series II integrator. The retention times for the various compounds and unidentified peaks are as listed in Table VIII below.

Table VIII
Retention Times for Compounds on HPLC

Compound Name or Unidentified Peak	Retention Time (min.)	Compound Name or Unidentified Peak	Retention Time (min.)
TNT	10.2	**Denitrifying**	
2-ADNT	11.7	D1 (ADNT)	11.2
4-ADNT	11.5	D2	4.4
2,6-DNT	12.5	D3	5.3
2,4-DNT	12.9	D4	2.5
o-Toluidine	6.5	D5	10.4
p-Toluidine	11.6		
p-NT	17.2		
Toluene	35		
Methanogenic		**Sulfate Reducing**	
M1	9.0	S1	14.4
M2	16	S2	16.2
M3	9.3	S3	9.3
M4 (ADNT)	11.2	S4	9.0
M5	14.4	S5 (ADNT)	11.2
		S6	13.5
		S7	2.2
		S8	2.3
		S9	2.5
		S10	7.4

The unidentified peaks were labeled according to their order of appearance in each set of reactors. Therefore, M1 and S1 are not necessarily the same intermediates. For comparison, retention times shown in Table VIII should be checked. Also, 4-ADNT and 2-ADNT could not be separated on the HPLC by the method used. A common peak eluting at about 11.2 minutes was obtained. The molar absorptivities for these two compounds was not the same. Therefore, all concentrations of ADNTs have been expressed as equivalent concentrations of both 4-ADNT and 2-ADNT and therefore represents the range of the concentration in which the ADNTs were present.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 PRELIMINARY STUDIES

Preliminary experiments were conducted to study the preferred conditions for TNT transformation. A series of 120 mL reactors was set up with contents as described previously and monitored for 6 days. The results obtained were used to optimize the conditions for TNT transformation in the subsequent test reactors. These preliminary reactors were set up with yeast extract and peptone concentrations of 0.05 g/L each and additional primary substrate (sodium acetate or sodium lactate) concentrations of 25mM. Under these conditions, TNT transformation was very slow. TNT reduction after 6 days of incubation under each of the different conditions was as follows: methanogenic 16.4% , denitrifying 34% and sulfate reducing 18%. The results of these experiments are shown in Figure 1.

Prior to these preliminary experiments, the microorganisms were acclimated and enriched over a 4 month period. During this process, all other carbon sources (peptone, yeast extract, acetate or lactate) in the reactors were gradually reduced. Various authors have reported TNT transformation to trace quantities under comparable conditions within 7 to 8 days of incubation. However, a reduction in TNT transformation rates has been reported in the presence low yeast extract concentrations by various authors (*Osmon and*

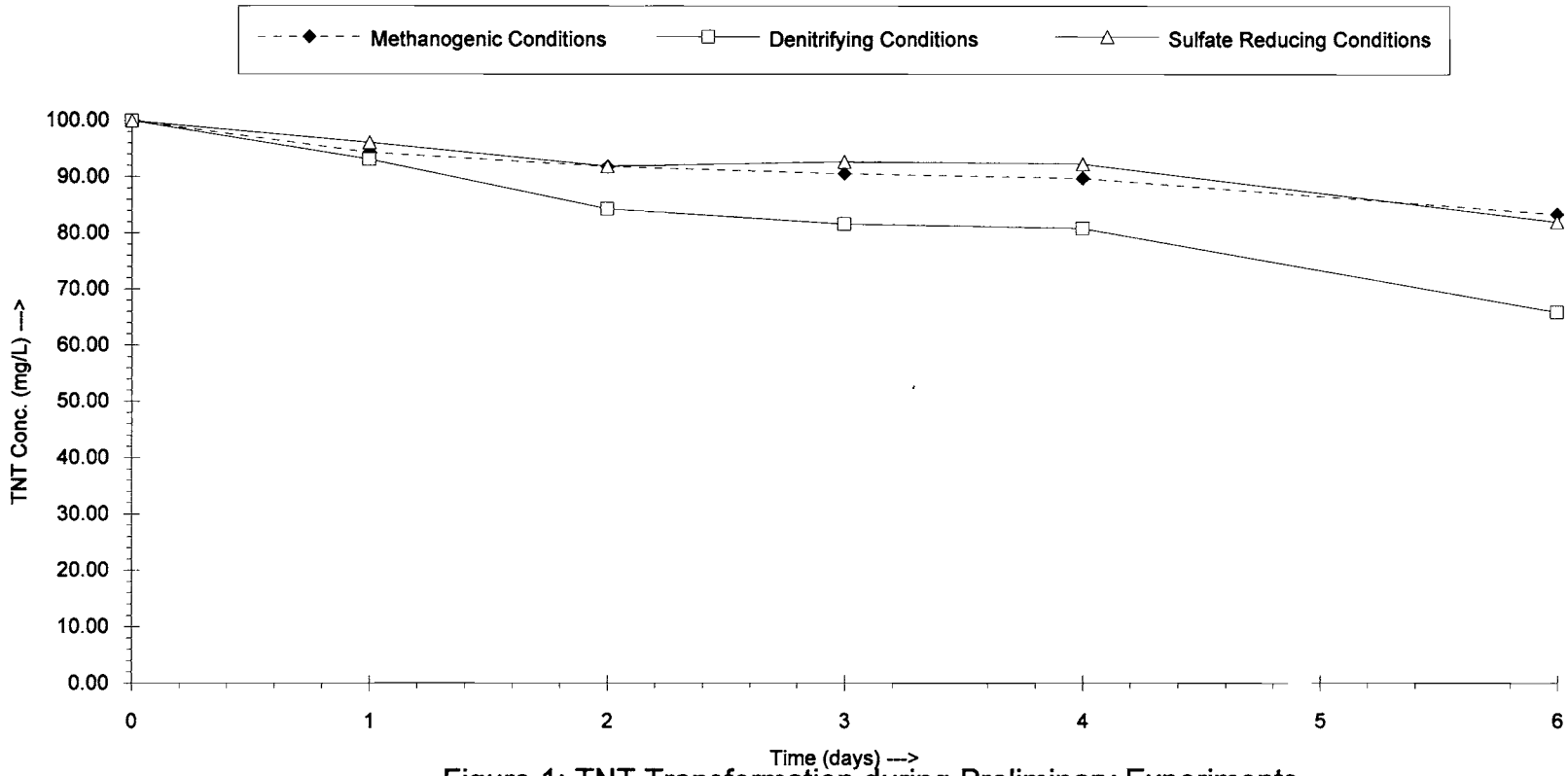


Figure 1: TNT Transformation during Preliminary Experiments

Klausmeier, 1972; Won and coworkers, 1974). Considering the results obtained and the findings reported by various researchers, the yeast extract concentration was increased to 0.3 g/L in the reactors. The seed reactors showed improved TNT transformation due to increased yeast extract concentration (data not shown). Yeast extract, which is a water soluble extract of autolyzed yeast cells and a source of amino acids, peptides, water soluble vitamins and carbohydrates, could be the supplier of important mediators in the electron transfer process and appears to have a definite positive effect on TNT transformation at concentrations above a minimum threshold - in this experiment above 0.05 g/L.

Based on the results of the preliminary studies, the test reactors were set up using yeast extract, peptone and primary substrate (sodium acetate or sodium lactate) concentrations of 0.3 g/L, 0.1 g/L and 35 mM respectively. 99.2% TNT transformation was observed under methanogenic conditions and 100% under denitrifying and sulfate reducing conditions. In a similar study by Boopathy and coworkers (1993), much less TNT transformation was reported (82% under denitrifying, 30% under sulfate reeducing and 35% under H_2:CO_2 methanogenic conditions). One of the major differences in the culture medium was the presence of yeast extract in the current test reactors. The results obtained from these reactors are presented in the following sections. All the test reactors were duplicated. The HPLC peaks of the intermediate compounds were monitored by comparing the chromatograms of the controls and batch reactors. For methanogenic conditions, the results shown are the data obtained form reactor M1. Reactor M2 was contaminated early in the study due to opening of the cover and hence introduction of air into the headspace. The reactor showed a different degradation pattern as compared to M1. The results obtained are

included in the appendix for review. For denitrifying and sulfate reducing conditions, the results shown are the data obtained from one of the duplicate reactors since both reactors showed similar degradation patterns. All results are shown in the appendix. Due to non-availability of standards, concentrations of all intermediates are expressed as peak areas unless otherwise specified.

4.2 METHANOGENIC REACTORS

TNT degradation under methanogenic conditions was relatively slow. 97.5% of TNT was converted in 29 days and 99.2% by the 44th day. Figure 2 shows the transformation of TNT and the appearance of the intermediates. As Figure 2 shows, 9% of TNT could not be accounted for at time 0 as compared to the bacterial control. This could be due to reduction of TNT by sodium sulfide which was added as a reductant. Sulfide is a powerful oxygen scavenger and has been reported to reduce TNT in the presence of microbial exudates (*Han 1993; Cho et al.1995*). Han (1993) observed that certain concentrations of microbial exudates and sulfide significantly influenced TNT transformation when acting together, but not separately. 7% abiotic TNT transformation was observed at sulfide concentration of 6 mM in the presence of microbial exudate. Preuss and coworkers (1993) reported 25 to 40% of TNT was transformed by sulfide prior to inoculation with a sulfate reducing culture. TNT was reduced via 4-ADNT and 2-ADNT to 2,4-DANT by sulfide. However, in the test reactors no reduction of TNT by sulfide was observed without addition of inoculum indicating the possibility of TNT reduction due to combined action of sulfide and microorganisms.

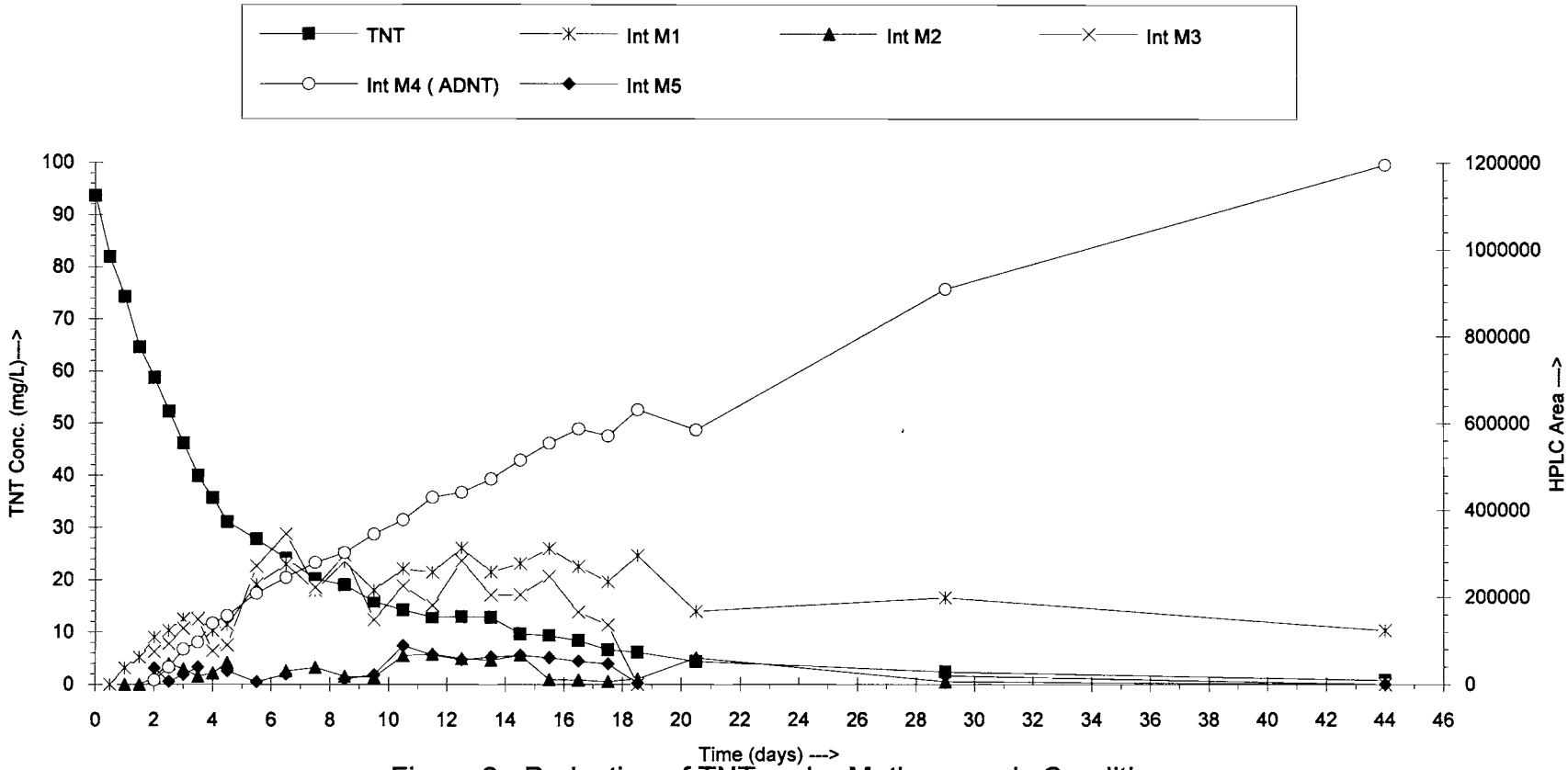


Figure 2 : Reduction of TNT under Methanogenic Conditions

Under methanogenic conditions, TNT reduced to form a series of intermediates. The HPLC peaks of a total of 5 initial intermediates were observed. The ADNTs were the fourth intermediates to be observed. Intermediate pairs M1 & M3 and M2 & M5 closely followed each other. Intermediates M1 and M4 (ADNT) continued to persist.even after 29 days of incubation. 15% abiotic reduction of initial TNT concentration was observed in the killed controls. No peaks were observed in these reactors.

These results are similar to those reported by Han (1993). Under anaerobic conditions with peptone as the primary substrate, the author reported that 5 intermediate products appeared during stepwise transformation. Of the five intermediates labelled, Int # 5 (tentatively identified as 2,4-DANT) persisted whereas all others were transient. In another test by the author using glucose as the primary substrate, Han (1993) observed the same number of products but different appearance and decay patterns. Int # 5 accumulated as in the previous case. With glucose as the primary substrate, Han (1993) reported reduction in pH resulting in suppression of activity in the reactors. No such reduction in pH was observed in the current test reactors. Alkalinity tests showed that sufficient buffering capacity was present in the reactors. The pH in the reactors remained in the neutral range (6.8-7.2) throughout the study. The acetate in the reactors was reduced from the initial concentration of 2870 mg/L to approximately 50 mg/L on day 33. Also, limited sampling for methane in the reactors indicated that no methane was present in the head space of the test and control reactors after 25 days of incubation. This could be due to leakage out of the reactors or the presence of incomplete fermentation reactions.

Intermediates M2, M3, and M5 were transient and transformed by the 19th day, whereas M1 persisted for a long time and M4 (ADNT) continued to

build up in the reactors to a maximum concentration of 34.6 mg/L as 2-ADNT or 46.9 mg/L as 4-ADNT. The reduction of a nitro group to an amino group proceeds in a series of 2 electron transfers with nitroso and hydroxylamines as intermediates. Under acidic conditions, the nitroso compounds are rapidly reduced to an amine with the corresponding hydroxylamine as an intermediate. Reduction under alkaline conditions gives rise to an azoxy compound resulting from a dimerization process between the nitroso compound and the corresponding hydroxylamine. The biological fate of azoxy compounds under aerobic and anaerobic conditions is not very well known though the soluble azo dyes are known to be susceptible to biological attack. Duque and coworkers (1993) report accumulation of azoxy dimers under aerobic conditions, whereas Won and coworkers (1974) report rapid transformation of the azoxy dimers under aerobic conditions.

Tentative identification of the intermediates has been made on the basis of the polarity of these compounds which would affect the retention times on the reverse phase column. The more polar compounds would elute faster than the less polar compounds. Accordingly, M1 and M3 have been tentatively identified as the HADNT isomers, M2 and M5 as the azoxy dimers and M4 has been positively identified as ADNT (*Kaplan and Kaplan 1982*). If these identifications are correct, the azoxy isomers disappeared without any further trace. The nitroso intermediates were not detected. The transformation pathway could have branched out from the HADNT to the ADNT and the azoxy isomers as shown in Figure 3. This could explain the low concentration of ADNT observed in the reactors even when 97.5% of the TNT had transformed.

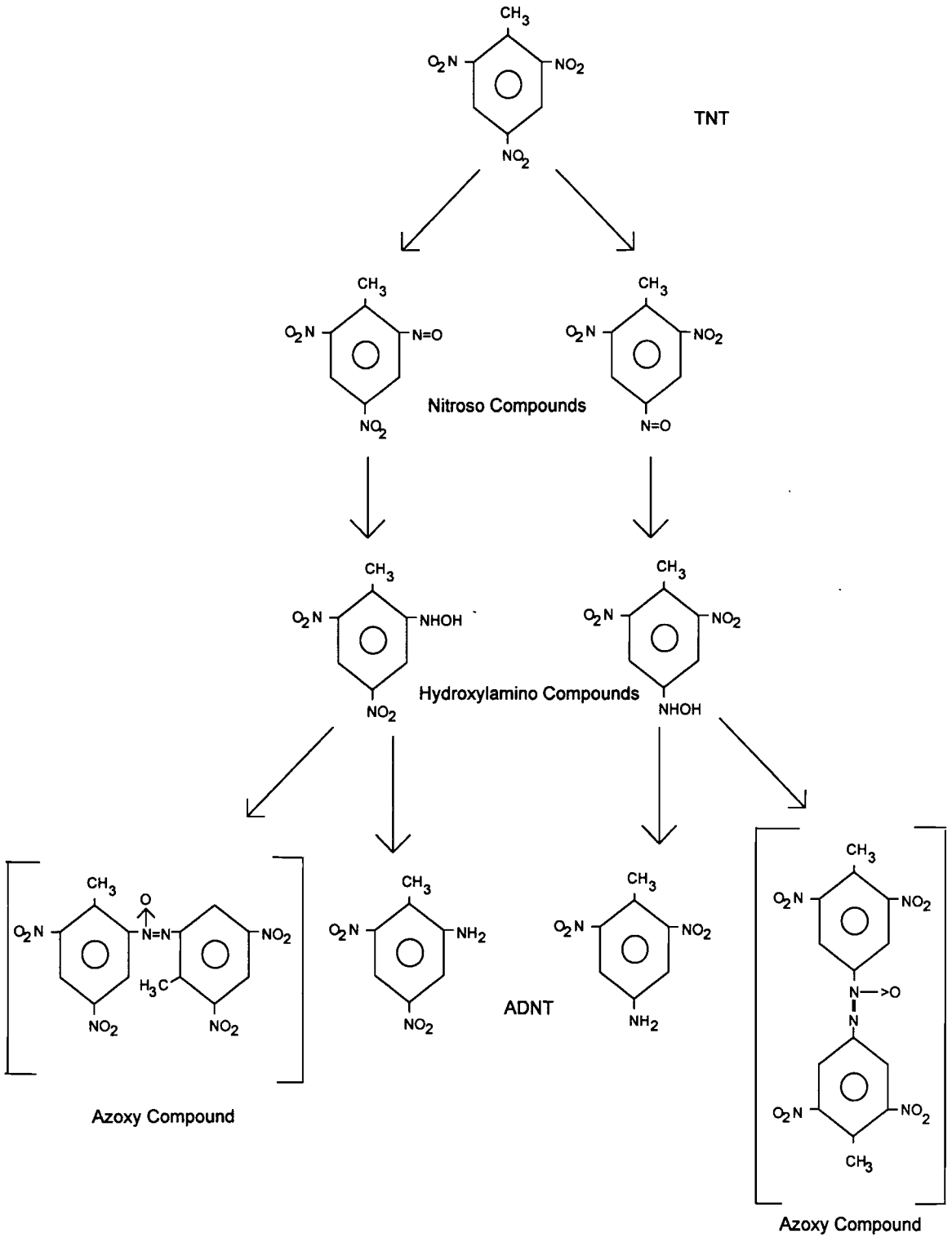


Figure 3 : Proposed TNT Reduction Pathway Under Methanogenic Conditions (*McCormick et al. 1976*)

4.3 DENITRIFYING REACTORS

Figures 4A and 4B show the disappearance of TNT, and the transient nature of the intermediates, under denitrifying conditions. Figure 4A shows the transformation of TNT and the appearance of ADNT (shown as mg/L of 2-ADNT concentration on the left axis). Figure 4B shows all the intermediates. Intermediates D2 and D3 were observed in very small quantities and transformed by the 4th day. TNT was reduced by 90% (from a starting concentration of 100 mg/L) in 2.5 days, and 100% was removed by the 6th day. ADNT started to appear as soon as TNT started to transform. Two transient intermediates (D2 and D3) were observed in small quantities and, on the basis of their retention times on the HPLC appear to be more polar than ADNT. They both disappeared before ADNT had transformed completely. The maximum ADNT concentration observed in the reactors was 23 mg/L as 2-ADNT and 31 mg/L as 4-ADNT on the 5th day. No nitrate was detected in the reactors after 29 days (initial concentration 20 mM).

On day 2, the reactors had changed color from a light orange-yellow to orange-red and on day 3 they had a deep red-brown color. Vorbeck and coworkers (1994) have reported the accumulation of a TNT metabolite imparting a characterisitic red-brown color during aerobic TNT transformation. They identified the intermediate as a hybrid Meisenheimer complex (Figure 5). The other intermediate observed by Vorbeck and coworkers (1994) was 4-ADNT. Stoichiometric analysis showed that only 5% of TNT was reduced to 4-ADNT whereas 40% accumulated as the Meisenheimer complex. Lenke and Knackmuss (1992) report the formation of a Meisenheimer complex during the aerobic degradation of picric acid. The nitro group is typically an electron

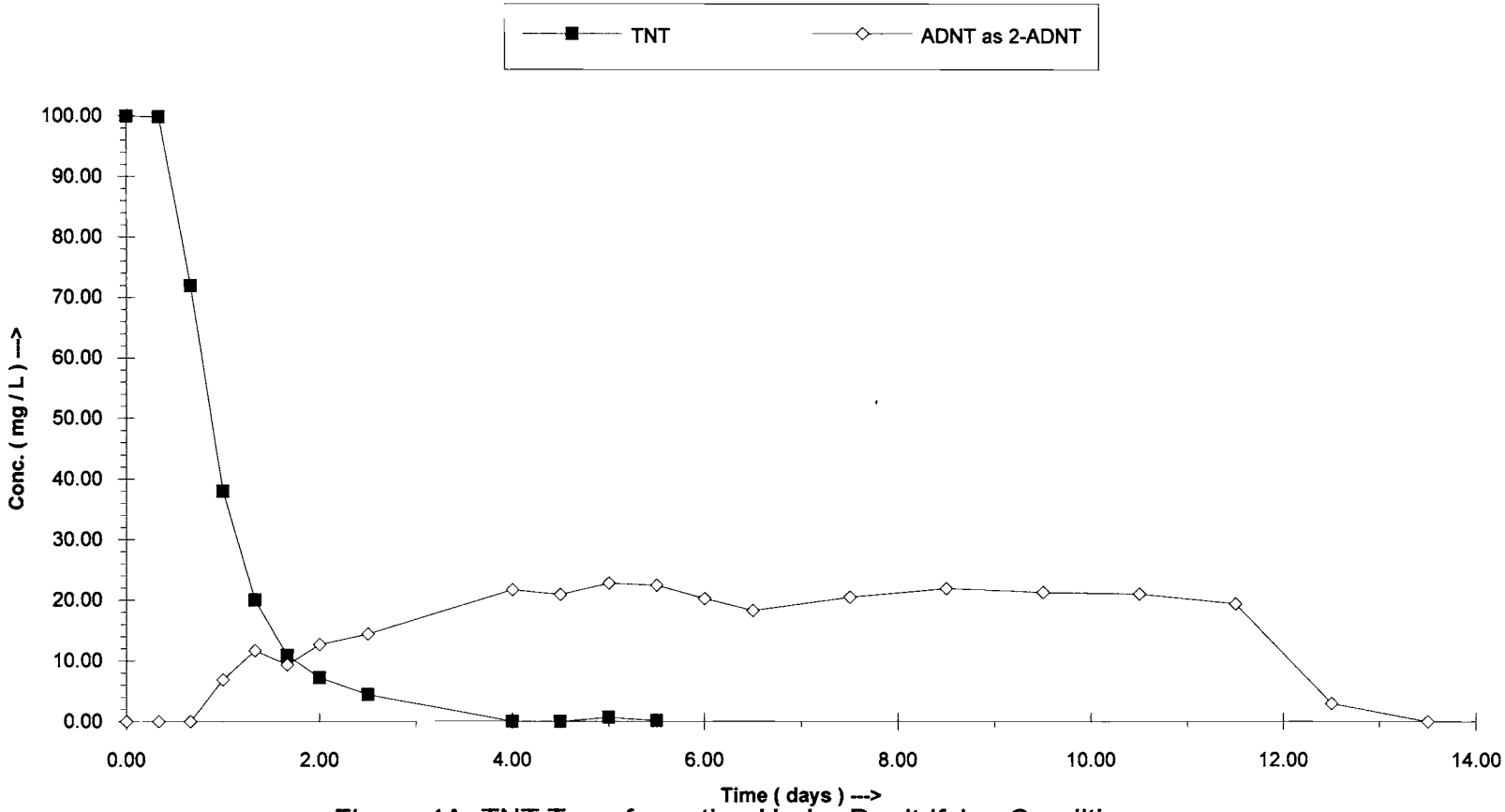


Figure 4A: TNT Transformation Under Denitrifying Conditions

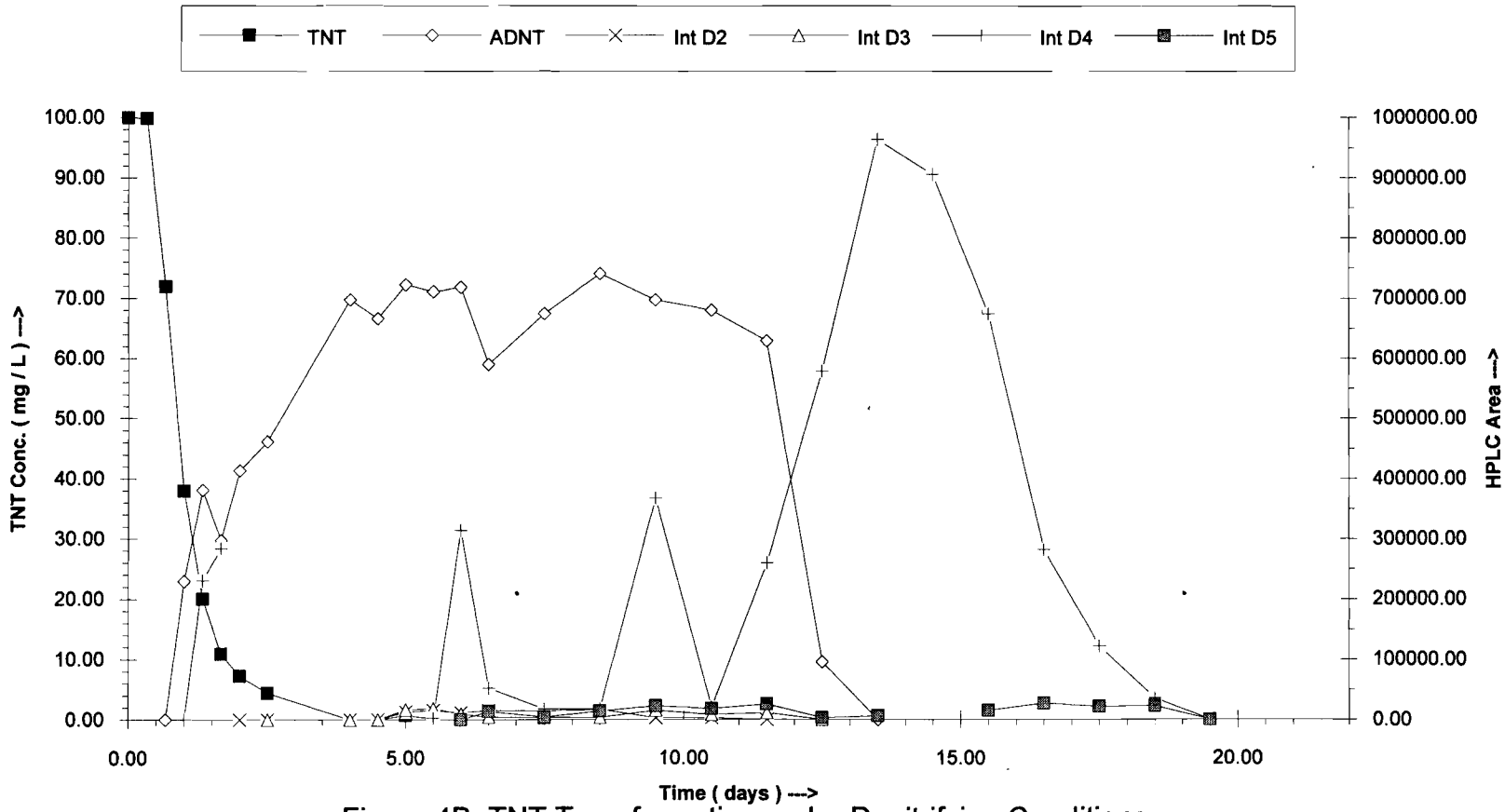


Figure 4B: TNT Transformation under Denitrifying Conditions

attracting group. In nucleophilic substitution, an electron donating species (Y^-) supplies the electrons for a new C-Y bond. This nucleophilic substitution occurs by the addition-elimination mechanism. The intermediate involved is stable and is characterized by strong coloration which is characteristic for charge transfer and Meisenheimer complexes. Duque and coworkers (1993) also report the possibility of formation of the complex subsequently resulting in cleavage of nitro groups during aerobic degradation of TNT. The main intermediates identified were the dinitrotoluenes, nitrotoluenes and toluene. None of these intermediates were identified in the current test reactors.

The degradation of TNT under denitrifying conditions could be following two different initial reductive pathways, one resulting in the reduction of one nitro group on TNT to an amino group and the other pathway responsible for the nucleophilic attack leading to the formation of the Meisenheimer complex. These possible pathways are as shown in Figure 5.

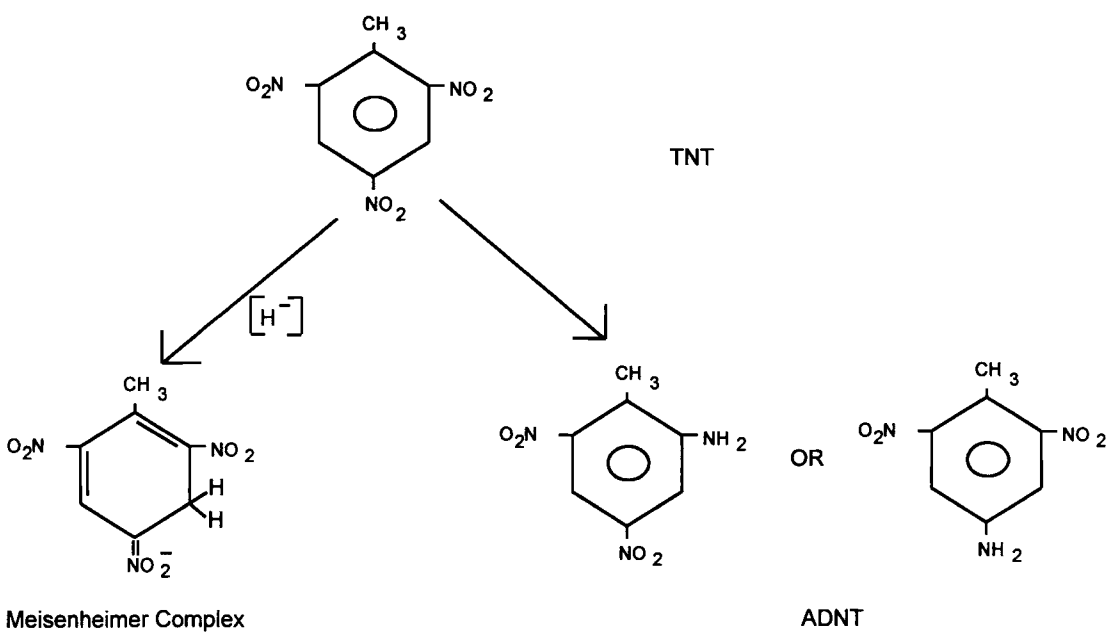


Figure 5 : Possible Initial Degradation Pathways of TNT under Denitrifying Conditions (*Vorbeck et al. 1994*)

Intermediates D4 and D5 were observed since the 6th day of incubation. Intermediate D4 was highly unstable when present along with ADNT. It showed a sharp increase and decrease in concentration when ADNT was present in substantial quantities. Concentration of D4 increased sharply once ADNT disappeared on the 12th day and then gradually decreased to 0 on the 19th day. It is possible that intermediate D4 was a product of ADNT transformation and must be transforming quickly itself. Intermediate D5 was seen in very small quantities. This intermediate had a retention time of 10.4 ± 0.1 min which is nearly identical to that of TNT (10.2 ± 0.1). It was first seen 2 days after TNT disappeared and continued to appear in small concentrations until the 19th day. Both D4 and D5 transformed by the 19th day and no other intermediate was observed. 10% abiotic reduction of TNT was observed.

Boopathy and coworkers (1993) reported maximum TNT removal under nitrate reducing conditions (82%) as compared to TNT transformation under H_2:CO_2 methanogenic (35%) and sulfate reducing conditions (30%). Han (1993) reported accumulation of unidentified TNT degradation products under anaerobic conditions. On introduction of denitrifying conditions in the reactor at this stage, all the intermediates transformed without any further intermediates being observed. Therefore, denitrifiers appear to play an important role in the reduction of TNT and conversion of its metabolites.

4.4 SULFATE REDUCING REACTORS

The enriched sulfate reducing cultures were also used to study the biotransformation of TNT. Figure 6A, 6B, and 6C show the transformation of TNT and the production of its metabolic intermediates. Figure 6A, 6B, and 6C are

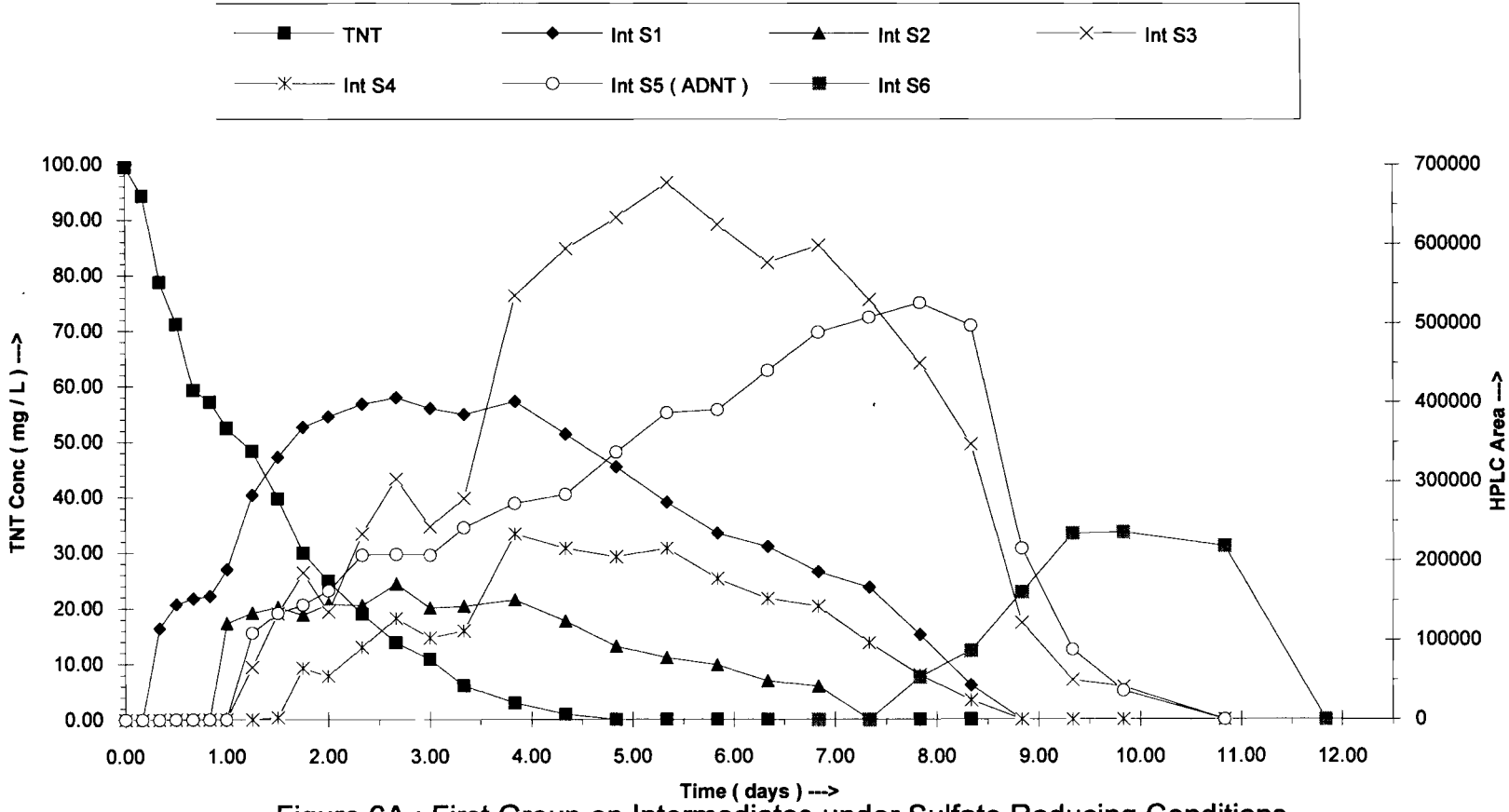


Figure 6A : First Group on Intermediates under Sulfate Reducing Conditions

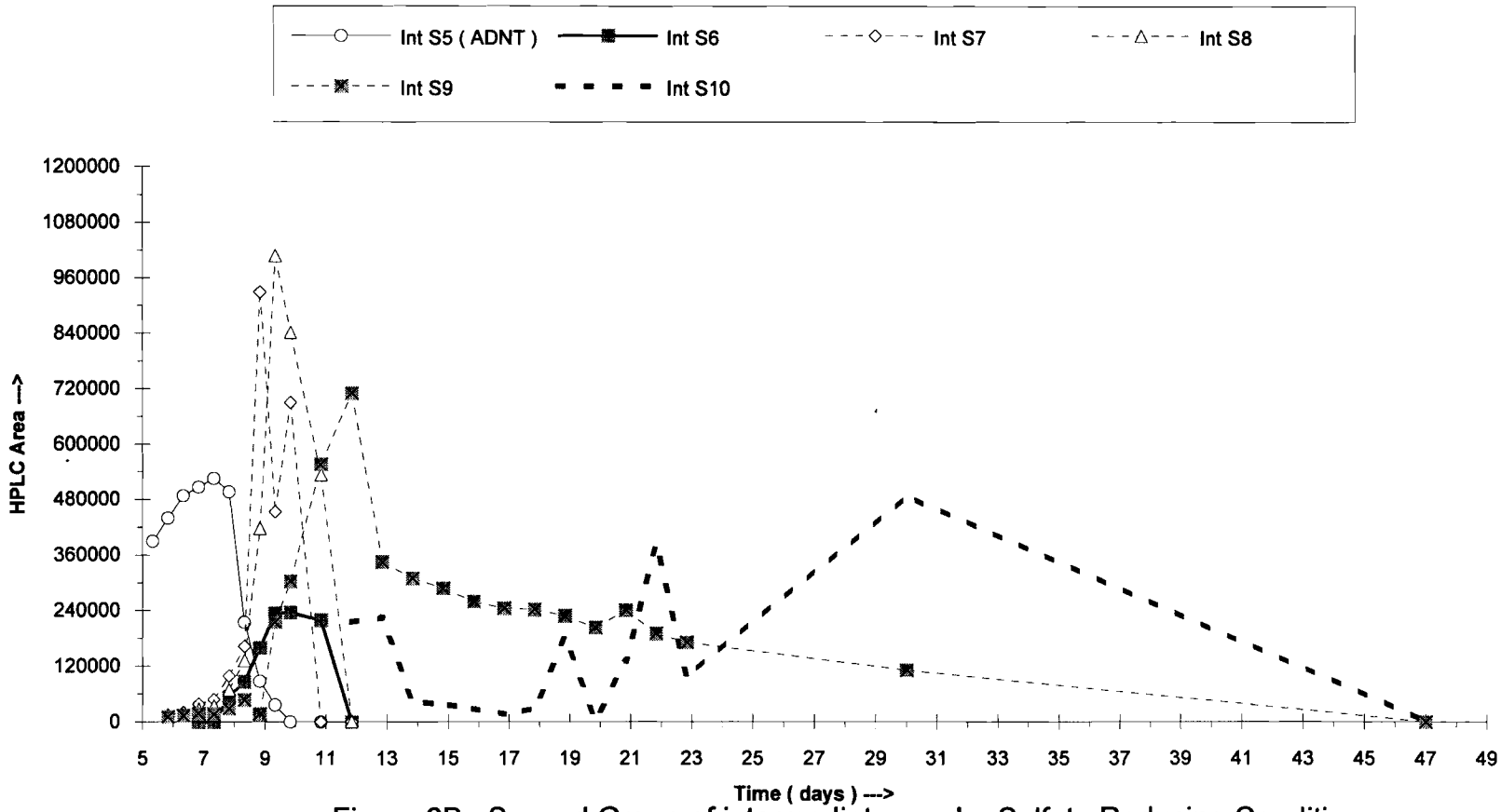


Figure 6B : Second Group of intermediates under Sulfate Reducing Conditions

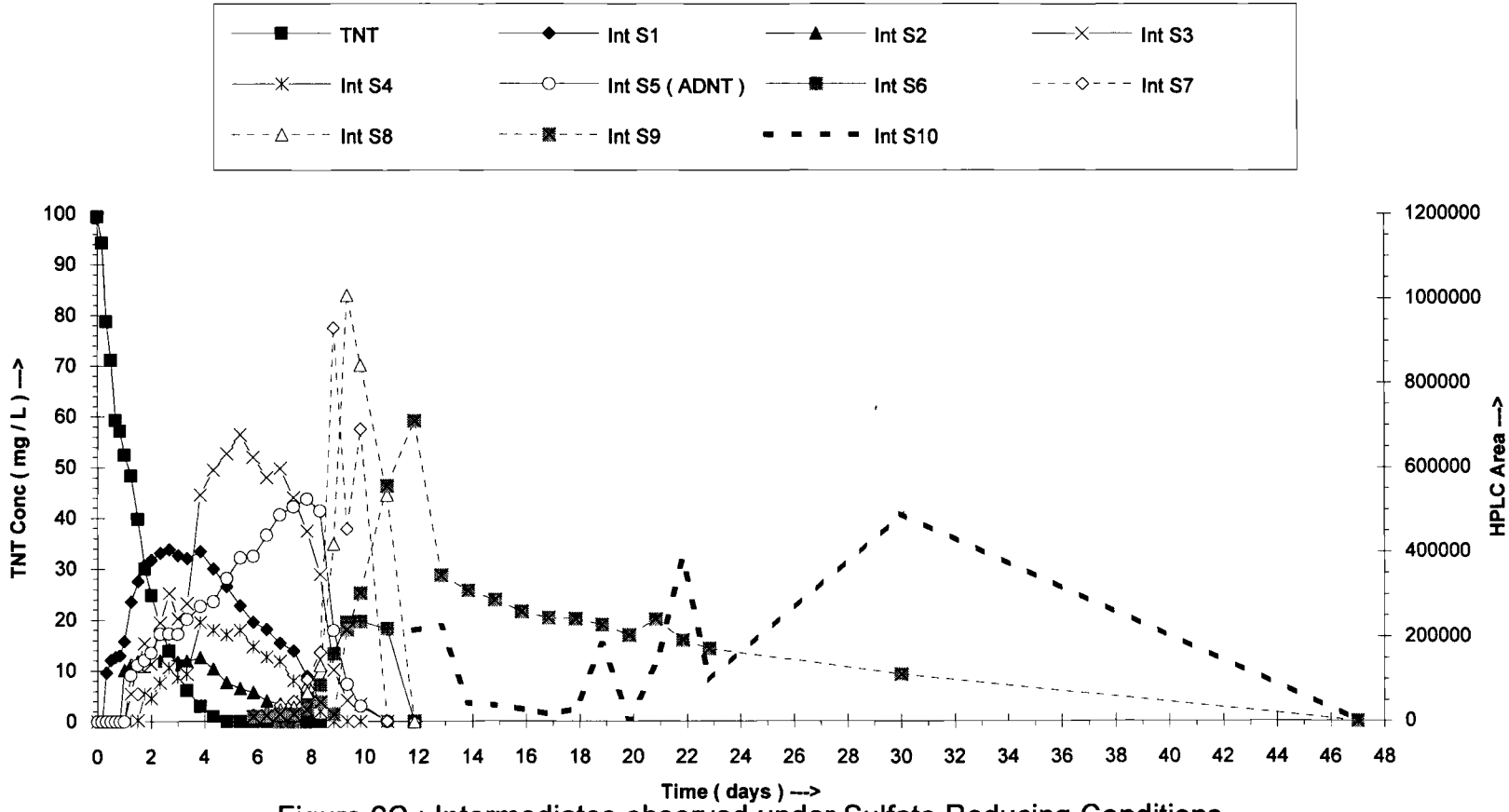


Figure 6C : Intermediates observed under Sulfate Reducing Conditions

separated to show the individual intermediate products more clearly. The X and Y axis scale for all three figures have been adjusted to show all intermediates. Figure 6A shows the first group of intermediates which disappeared by the 11th day. Figure 6B shows the second group of intermediates and Figure 6C shows all the intermediates. 15 to 20 mg/L of sulfide was detected in the reactors on the 45th day.

Figure 6A shows that TNT was reduced to trace quantities within 5 days of incubation and all of the TNT was transformed within 9 days. Intermediate S1 started appearing as soon as TNT reduction was observed. Intermediates S2, S3, S4, and S5 appeared successively, and intermediate S6 started building up at around 7 days as S1, S2, and S4 had disappeared and S3 and S5 were beginning to transform. Intermediate S5 has been identified as ADNT. This is very similar to the pathway observed under methanogenic conditions. The retention times of all intermediates observed match those for the intermediates under methanogenic conditions. However, the order of appearance is not the same and hence intermediate M1 is not the same as intermediate S1. Intermediate S5 is the ADNT and based on the tentative identification, intermediates S3 and S4 are the HADNT isomers and intermediates S1 and S2 are the azoxy dimers. It is possible that the initial reactions of HADNT occured at much faster rates under sulfate reducing conditions than under methanogenic conditions with the formation of the azoxy dimers from the nitroso and hydroxylamino compounds being favored over reduction of HADNT to ADNT. The HADNT isomers accumulated to detectable levels later in these reactors. Under sulfate reducing conditions, intermediate S3 built up to a large concentration around the fifth day and then persisted for some time until S5 (ADNT) increased. The concentrations for both S3 and S5 fell rapidly thereafter,

resulting in the appearance of a second group of intermediates. The maximum concentration of the ADNTs observed in the sulfate reducing reactors was 15.2 mg/L as 2-ADNT or 20.63 mg/L as 4-ADNT.

As Figure 6B shows, the second group of intermediates started to appear as ADNT was disappearing. Three transient intermediates (S6, S7, and S8) were observed. Intermediates S9 and S10 persisted in the reactors for a long time. No intermediates were detected in the reactors on the 47th day. Intermediates S7 and S8 followed each other very closely. Reduction in S7 and S8 concentrations coincided with an increase in the concentrations of S9 with maximum S9 concentrations on day 12 when all of S7 and S8 had transformed. Intermediates S7 and S8 were probably very polar, as indicated by their low retention times. Intermediate S6 disappeared by the 12th day and a new intermediate S10 was observed then. All the intermediates transformed by the 47th day. No positive identification has been made on any of the second group of intermediates.

Transient intermediates have also been reported by Boopathy and Kulpa (1992) and Preuss and coworkers (1993) under sulfate reducing conditions. Boopathy and Kulpa (1992) observed DANT as the main intermediate. Under nitrogen rich conditions (in the presence of other nitrogen sources), no other intermediate was observed even though the DANT was transformed. However, under nitrogen-limiting conditions (2 mM ammonium and 100 ppm TNT as nitrogen source), they observed toluene at a concentration of 98 ppm in the reactors. They assume TAT production from DANT, though it was not observed. This is similar to the results obtained in this study. Nitrogen rich conditions (7.48 mM ammonium and 100 ppm TNT) were used. All intermediates transformed with no other intermediates observed. Toluene was not detected in the reactors. Preuss and coworkers (1993) studied TNT transformation by a sulfate-reducing

bacterium using TNT as its sole nitrogen source. They proposed the degradation pathway as : TNT → DANT → DAHAT → TAT. They identified the reduction of DAHAT to TAT as the rate limiting step. Thus, both the above papers refer to utilization of TNT and its intermediates as a sole nitrogen source.

Table IX below shows the intermediates observed under the different electron acceptor conditions.

Table IX

Comparison of Intermediates under Different Electron Acceptor Conditions

Retention Time (min)	Methanogenic Conditions	Denitrifying Conditions	Sulfate Reducing Conditions
2.5		D4	S9
9.0	M1		S4
9.3	M3		S3
11.2	M4	D1	S5
14.4	M5		S1
16.0	M2		S2

The above table shows that the ADNTs (M4, D1 and S5) were the only common intermediates under all three conditions. Intermediate S9 (maximum HPLC area of 709704 on day 11) seen late in the sulfate reducing reactors has the same retention time as D4 (maximum HPLC area of 963440 on day 13.5). It is therefore possible that the degradation pathway for TNT under denitrifying and sulfate reducing conditions is the same, though with much faster rates seen under denitrifying conditions. In the sulfate reducing reactors all individual intermediates built up to detectable amounts and were then transformed.

All the intermediates under denitrifying and sulfate reducing conditions transformed without any new intermediates seen. It is possible that the further transformation products were not detectable under the current HPLC method

adopted. Since the current method is suited towards detection of double bonds in the ring compounds, this indicates the possibility of ring cleavage.

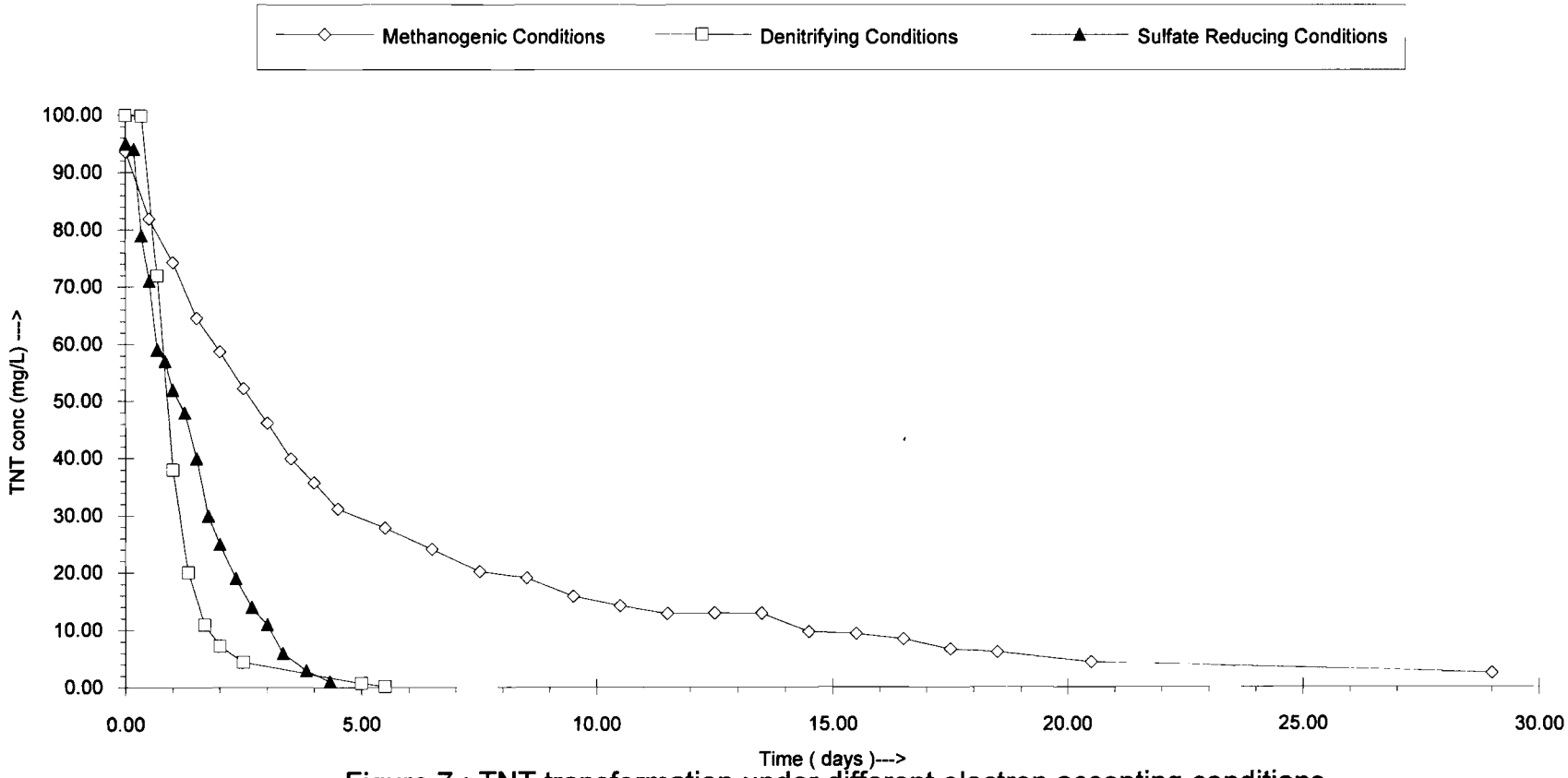
4.5 COMPARISON OF TNT TRANSFORMATION UNDER DIFFERENT ELECTRON ACCEPTING CONDITIONS

Figure 7 shows the transformation of TNT in the test reactors under different electron accepting conditions. Assuming, for the sake of comparison, that TNT transformation is a pseudo first-order reaction and evaluating the rate constant (k) accordingly for the different electron accepting conditions, maximum transformation rates are observed under denitrifying conditions, followed by sulfate reducing and then methanogenic conditions. The rate constants are as shown in Table X, and Figure 8 shows the first order plot for TNT biotransformation rates.

Table X
TNT Transformation Rates

Reactor Conditions	Rate Constant (per day)	R Square (from regression)
Methanogenic	0.13	0.97
Denitrifying	1.13	0.97
Sulfate Reducing	0.94	0.95

Under anaerobic conditions with glucose as the primary substrate and argon as the gas phase, Han (1993) observed a transformation rate of 2.72 /day. Comparison of the reactor performance on the basis of the above table should consider the following test conditions. All the reactors were set up under similar conditions. The enrichment medium (which contains the yeast extract, peptone and other nutrients) used for all reactors was the same, and the concentration of


Methanogenic Conditions
Denitrifying Conditions
Sulfate Reducing Conditions
TNT conc (mg/L) --->
100.00
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
0.00
5.00
10.00
15.00
20.00
25.00
30.00
Time (days)--->
Figure 7 : TNT transformation under different electron accepting conditions

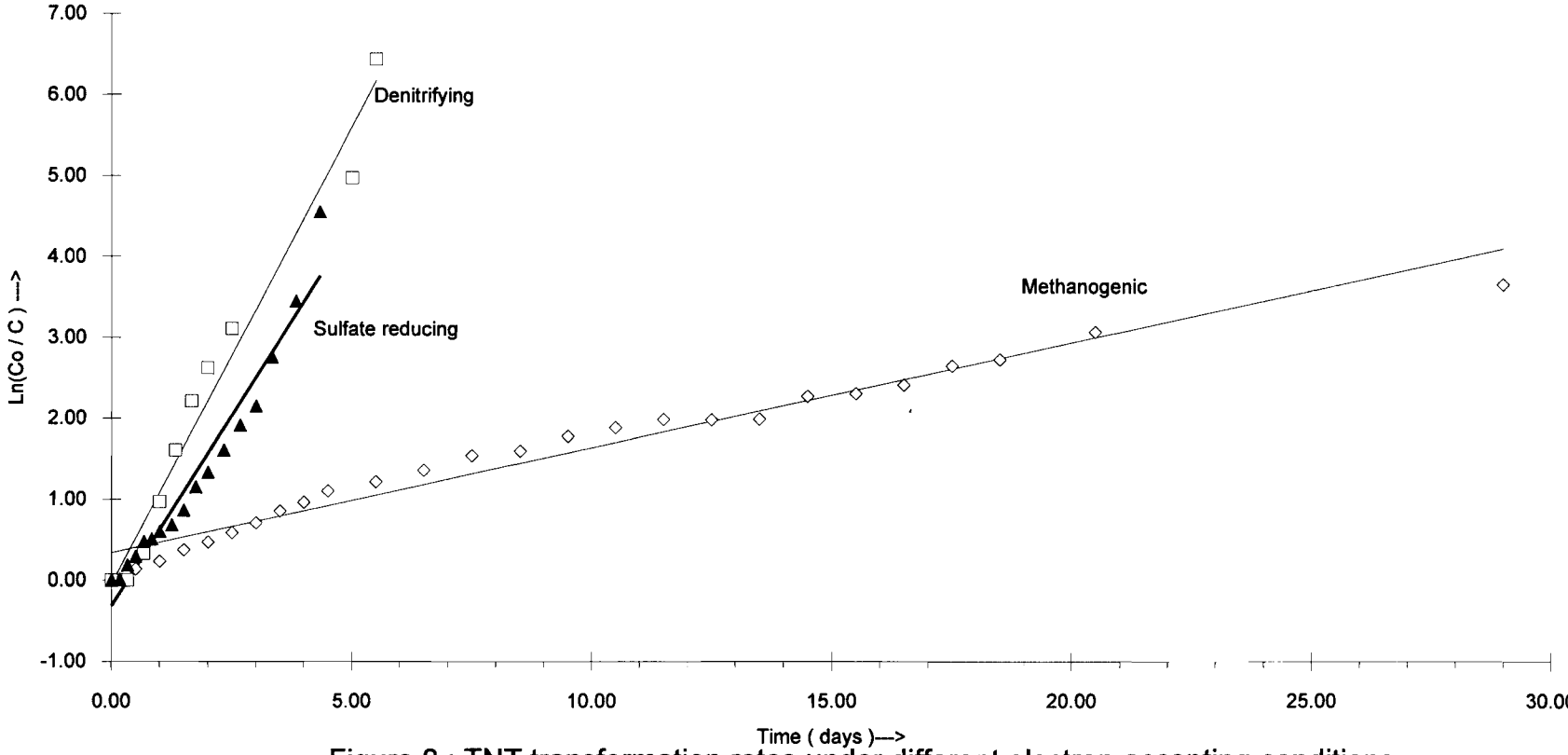


Figure 8 : TNT transformation rates under different electron accepting conditions

the additional primary substrate (sodium acetate or sodium lactate) was 35 mM under all conditions. Though the inoculum added to all reactors was the same in quantity (3 mL), the number of active microorganisms added to the reactors was not necessarily comparable. Also, no attempt was made to measure microbial density or growth under any condition. Therefore, the rate constant values serve primarily for comparison of the test reactors. Further studies would be required to thoroughly compare rate constants under different electron accepting conditions.

4.6 RECOMMENDATIONS FOR FUTURE STUDY

Based upon the findings of this study, the following is a list of recommendations for future studies investigating the biotransformation of TNT for the treatment of contaminated soils and aquifers:

1. Investigate the mechanism by which yeast extract affects TNT degradation.

2. Since degradation has been observed in the current reactors, investigate TNT reduction kinetics under similar conditions varying concentrations of organic compounds besides TNT.

3. Conduct studies closely simulating aquifer conditions, with the goal of subsequent development of a field technique.

4. Isolation and identification of bacteria involved under each condition.

5. Use of ^{14}C ring radiolabelled TNT to stoichiometrically account for all TNT degraded. Study incorporation of TNT carbon into cells, evolution as CO_2 gas, and conversion into various other intermediates known or unknown.

6. GC-MS analysis of intermediates and comparison with spectra of known possible degradation products.

7. To study the toxicity of the final products of TNT transformation.

CHAPTER V

CONCLUSIONS

The major aim of this research was to study the anaerobic removal of TNT under different electron acceptor conditions. Methanogenic, denitrifying, and sulfate reducing reactors were set up and bacteria were slowly acclimated to 100 mg/L TNT over a 4 month period.

In the preliminary study, yeast extract concentration were shown to have a significant effect on TNT reduction. Extremely slow TNT reduction was observed at yeast extract concentrations below 0.3 g/L. Yeast extract appears to be a potential supplier of important mediators in the electron transfer process.

99.2% TNT removal under methanogenic and 100% under denitrifying and sulfate reducing conditions was observed. No lag period was observed during the initial stage of biotransformation under any of the three conditions. This demonstrates that the culture used for inoculation was already well adapted to TNT.

Under methanogenic conditions, the reaction appears to stop with the accumulation of ADNT. A total of 5 intermediates were observed. Four of the five intermediates were hypothesized to be HADNT isomers and azoxy dimers, and

the fifth was identified as ADNT. Reduction of TNT by sulfide was observed only when the inoculum was added.

Under denitrifying conditions, TNT reduction was extremely fast and the major intermediate identified was ADNT. ADNT transformed to unknown intermediates, all of which disappeared by the 19th day. No persistent intermediate products were observed.

Under sulfate reducing conditions, the initial TNT concentration of 100 mg/L was biotransformed completely to various intermediates during 9 days of incubation. Two distinct degradation stages were observed. The first stage is similar to that observed under methanogenic conditions. During the second stage another group of different transient intermediates was observed.

Intermediate S9 was also observed under denitrifying conditions (D4). Since D4 is the same as S9, the degradation pathways under denitrifying and sulfate reducing conditions appear to be the same, with much faster transformation rates of the intermediates under denitrifying conditions.

Since no other intermediates were detected on the HPLC, ring cleavage might have occurred resulting in non-aromatic compounds.

A comparison of transformation rates shows faster TNT transformation under denitrifying conditions, followed by sulfate reducing conditions. Biotransformation under methanogenic conditions was comparatively slower in the current test reactors.

The study shows that TNT biodegradation under different anaerobic electron accepting conditions can be accomplished. Extrapolation of the results

of this study can be made to field applications in TNT degradation in slurry reactors, lagoons or in-situ bioremediation. As mentioned earlier, TNT has been identified at various sites- previous munition factories, superfund sites etc. Anaerobic conditions commonly exist in the soils. This would result in a wide range of microbial species in any natural soil environment with the facultative denitrifiers and a consortium of sulfate reducers and methanogens. This study has shown that these different microorganisms have the ability to adapt to TNT. A number of variables influence the degradation process including nonhomogenous distribution of material, pH, nutrient availability, and moisture content. Lack of proper conditions could result in the pollutants being stable for long periods of time. Therefore in any field application, it would be a major advantage to study the local microorganisms by enriching them and eventually using them in addition to any added inoculum by enhancing the conditions for biodegradation.

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APPENDICES

APPENDIX A

INTERMEDIATES OBSERVED IN REACTOR M1

Time (hrs)	Time (days)	TNT RT 10.2 min (mg/L)	Int M1 RT 9.0 (HPLC area)	Int M2 RT 16 min (HPLC area)	Int M3 RT 9.3 min (HPLC area)	Int M4		Int M5 RT 14.4 min (HPLC area)
						RT 11.2 min (HPLC area)	(mg/L as 2-ADNT)	
0	0.00	93.62						
12	0.50	81.92	0					
24	1.00	74.27	37660	0				
36	1.50	64.57	62220	0				
48	2.00	58.72	108360	16720	74730	9800	0.28	37720
60	2.50	52.25	123200	47400	93450	39650	1.15	6980
72	3.00	46.18	149810	35430	128540	80880	2.34	22890
84	3.50	39.93	151630	18550	149150	97040	2.81	39600
96	4.00	35.76	123580	25980	77130	140640	4.07	
108	4.50	31.14	136590	49880	90130	157760	4.56	31240
132	5.50	27.79	229740		272370	209450	6.06	6150
156	6.50	24.09	276230	30860	345970	245950	7.11	23410
180	7.50	20.21	215680	38620	222550	280590	8.12	
204	8.50	19.04	282460	18550	297300	302390	8.75	12750
228	9.50	15.85	216170	17120	148690	345270	9.99	22030
252	10.50	14.22	265772	66432	226676	378436	10.95	88948
276	11.50	12.86	257548	69004	181772	429728	12.43	68064
300	12.50	12.93	312428	59544	283648	441336	12.77	56576
324	13.50	12.85	258228	55164	205516	471784	13.65	62384
348	14.50	9.65	277536	67068	205900	514572	14.88	66516
372	15.50	9.36	312316	11128	247856	553940	16.02	61644
396	16.50	8.38	270836	9676	165936	587424	16.99	53160
420	17.50	6.63	235556	6644	136460	571256	16.52	46968
444	18.50	6.13	296212	12424	0	631040	18.25	2652
492	20.50	4.37	167484	60796		584308	16.90	
	29.00	2.41	198880	6344		909244	26.30	20436
	44.00	0.79	123464	0		1195228	34.57	0

APPENDIX B

INTERMEDIATES OBSERVED IN REACTOR M2

Time (hrs)	Time (days)	TNT RT 10.2 min (mg/L)	Int # 1 RT 9.0 min (HPLC Area)	Int # 2 RT 16 min (HPLC Area)	Int # 3 RT 9.3 min (HPLC Area)	Int # 4		Int # 5 RT 14.4 min (HPLC Area)	Int # 6 RT 8.3 min (HPLC Area)	Int # 7 RT 7.4 min (HPLC Area)	Int # 8 RT 4.4 min (HPLC Area)	Int # 9 RT 5.3 min (HPLC Area)
						RT 11.2 min (HPLC Area)	(mg/L as 2-ADNT)					
0	0.00	88.78										
12	0.50	86.28	0									
24	1.00	75.97	32490	13970								
36	1.50	65.91	32950									
48	2.00	58.88	90840		38550	23780	0.69	44160				
60	2.50	53.19	140290	6230	96100	6280	0.18	21920				
72	3.00	42.84	141680	76600	130350	11460	0.33	51740				
84	3.50	34.73	155940	17840	169330	122650	3.55	70890				
96	4.00	26.75	49250	25980	77130	23090	0.67					
108	4.50	28.29	173200	8440	136040	193860	5.61	13440				
132	5.50	22.07	230470		265080	251510	7.28	10750				
156	6.50	18.71	309160	19130	410390	316580	9.16	75790				
180	7.50	12.09	242950	19620	272140	382150	11.05					
204	8.50	7.82	318810	24040	341780	496810	14.37	60820				
228	9.50	5.66	283700		239070	532540	15.40	40300				
252	10.50	4.08	324368	84216	331628	592396	17.14	91176	22620	4900	3304	8780
276	11.50	1.57	312340	73456	272132	613976	17.76	79256	12560	3960		6500
300	12.50	2.58	348576	74420	351628	699344	20.23	79640	23784	4588	13312	
324	13.50	2.38	334088		331008	746436	21.59	76844	14336	4696		12824
348	14.50	0.85	317920	80916	301708	831188	24.04	70632	17280	7476	5172	12696
372	15.50	0.21	343180	63728	301348	845972	24.47	67880	11320	8220	4516	10816
396	16.50	0.51	306452		255816	888412	25.70	60672	18576	10548	8560	3220
420	17.50	0.71	274288	66848	224220	858628	24.84	68264	13732	7408	9160	10228
444	18.50	0.00	232692	60904	176976	925868	26.78	46008	12068	4712	3944	12088

APPENDIX C

INTERMEDIATES OBSERVED IN REACTOR D1

Time (hrs)	Time (days)	TNT RT 10.2 min (mg/L)	Int D1		Int D2 RT 4.4 min (HPLC Area)	Int D3 RT 5.3 min (HPLC Area)	Int D4 RT 2.5 min (HPLC Area)	Int D5 RT 10.4 min (HPLC Area)
			RT 11.2 min (HPLC Area)	(mg/L as 2-ADNT)				
0	0.00	99.97						
8	0.33	99.83						
16	0.67	71.95		0.00				
24	1.00	37.96	229770	6.93			0	
32	1.33	20.07	381272	11.70			230520	
40	1.67	10.94	297352	9.38			284144	
48	2.00	7.25	413360	12.72	0			
60	2.50	4.45	461308	14.44	0	0		
96	4.00	0.00	697664	21.80	0	0		
108	4.50	0.00	666216	20.98	0	0		
120	5.00	0.69	722108	22.85	12840	16456		
132	5.50	0.16	710928	22.53	15388	18700	2708	
144	6.00	0.00	718376	20.31	11944	10116	313844	0
156	6.50		589780	18.29	15660	4875	52480	14030
180	7.50		674380	20.56	14580	3640	18188	4468
204	8.50		740796	21.99	17640	4016	18696	14324
228	9.50		697240	21.34	3028	15664	367416	22836
252	10.50		680304	21.09	3208	8704	20988	18492
276	11.50		628744	19.49	0	11688	259844	25904
300	12.50		95936	2.97		0	578608	3432
324	13.50		0	0.00			963440	6680
348	14.50						905536	
372	15.50						673448	15712
396	16.50						281804	27636
420	17.50						122412	22056
444	18.50						36048	22836
468	19.50						0	0

APPENDIX D

INTERMEDIATES OBSERVED IN REACTOR D2

Time (hrs)	Time (days)	TNT RT 10.2 min (mg/L)	Int D1		Int D2 RT 4.4 min (HPLC Area)	Int D3 RT 5.3 min (HPLC Area)	Int D4 RT 2.5 min (HPLC Area)	Int D5 RT 10.4 min (HPLC Area)
			RT 11.2 min (HPLC Area)	(mg/L as 2-ADNT)				
0	0.00	95.55						
8	0.33	91.63						
16	0.67	65.07	0	0.00				
24	1.00	33.93	231560	6.98				
32	1.33	23.69	326900	10.03				
40	1.67	15.73	308972	9.74	0	0		
48	2.00	11.77	324628	9.99	0	0		
60	2.50	8.93	441624	13.82	0	0		
96	4.00	2.22	496968	15.53	0	0		
108	4.50	1.62	496692	15.64	0	0		
120	5.00	0.73	603528	19.10	19860	10848		
132	5.50	0.41	572288	18.14	14704	19704	10084	
144	6.00	0.00	482508	13.64	17472	14976	20724	26644
156	6.50		487535	15.12	13095	15940	37870	9380
180	7.50		555840	16.95	17020	12424	50248	21756
204	8.50		577104	17.13	15912	9876	26516	25412
228	9.50		469668	14.38	3292	12556	51872	33840
252	10.50		433372	13.44	0	5184	122392	31312
276	11.50		17508	0.54		0	597700	4568
300	12.50		0	0.00			614880	12940
324	13.50						563044	6756
348	14.50						507744	0
372	15.50						143932	
396	16.50						81848	
420	17.50						552200	
444	18.50						353848	
468	19.50						0	

APPENDIX E

INTERMEDIATES OBSERVED IN REACTOR S1

Time (hrs)	Time (days)	TNT RT 10.2 min	Int S1 RT 14.4 min (HPLC Area)	Int S2 RT 16.2 min (HPLC Area)	Int S3 RT 9.3 min (HPLC Area)	Int S4 RT 9.08 min (HPLC Area)	Int S5	
							RT 11.2 min (HPLC Area)	(mg/L as 2-ADNT)
0	0.00	99.42	0					
4	0.17	94.27	0					
8	0.33	78.76	115050					
12	0.50	71.19	145150	0	0	0		
16	0.67	59.30	152330	0	0	0		
20	0.83	57.15	155600	0	0	0	0	0
24	1.00	52.47	189380	121600	0	0	0	0
30	1.25	48.32	283000	134570	66080	0	109520	3.17
36	1.50	39.77	330840	141860	133980	2950	134090	3.88
42	1.75	30.01	368430	132050	184920	65100	144850	4.19
48	2.00	24.88	381220	145680	135670	54870	162700	4.71
56	2.33	18.95	397110	143640	233500	91110	207040	5.99
64	2.67	13.85	405530	170750	302700	127340	208200	6.02
72	3.00	10.83	391810	140490	242810	103080	206750	5.98
80	3.33	6.08	384220	142640	278470	111950	241490	6.99
92	3.83	2.96	400965	150915	534660	233590	271970	7.87
104	4.33	0.99	359810	123990	593725	215515	283695	8.21
116	4.83	0.00	318770	92355	633255	204800	337690	9.77
128	5.33	0.00	273664	77840	676952	215612	386616	11.18
140	5.83	0.00	234524	68652	624172	177032	390304	11.29
152	6.33	0.00	217496	48828	576108	152136	439956	12.73
164	6.83	0.00	185628	42180	597840	142404	488056	14.12
176	7.33	0.00	166748	0	529428	96512	507200	14.67
188	7.83	0.00	106856	0	449080	55816	525504	15.20
200	8.33	0.00	42956	0	347232	24460	496968	14.38
212	8.83	0.00	0	0	121680	0	215032	6.22
224	9.33				49972	0	88244	2.55
236	9.83				41292	0	36272	1.05
260	10.83				0	0	0	0

Time (hrs)	Time (days)	Int S6 RT 13.5 min (HPLC Area)	Int S7 RT 2.2 min (HPLC Area)	Int S8 RT 2.3 min (HPLC Area)	Int S9 RT 2.5 min (HPLC Area)	Int S10 RT 7.4 min (HPLC Area)
140	6		15008		11280	
152	6		20264	15044	13812	
164	7	0	38292	27680	18080	
176	7	0	47692	34268	17324	
188	8	53764	99112	69660	29184	
200	8	86700	163052	132808	47240	
212	9	160176	929600	418180	16836	
224	9	233896	453608	1007176	216156	
236	10	235964	689524	841308	303368	
260	11	218984	0	533668	555928	
284	12	0	0	0	709704	215852
308	13				344924	224700
332	14				309332	44052
356	15				287608	37660
380	16				259288	28760
404	17				244652	16984
428	18				242264	29060
452	19				228492	181876
476	20				203772	5000
500	21				240864	131132
524	22				190880	382476
548	23				171344	95752
	30				111108	489020
	47				0	0

APPENDIX F

INTERMEDIATES OBSERVED IN REACTOR S2

Time (hrs)	Time (days)	TNT RT 10.2 min (mg/L)	Int S1 RT 14.4 min (HPLC Area)	Int S2 RT 16.2 min (HPLC Area)	Int S3 RT 9.3 min (HPLC Area)	Int S4 RT 9.08 min (HPLC Area)	Int S5	
							RT 11.2 min (HPLC Area)	(mg/L as 2-ADNT)
0	0	98.62						
4	0.17	92.66						
8	0.33	81.68	0					
12	0.50	75.09	114150	0				
16	0.67	70.07	141200	0	0	0		
20	0.83	65.82	157220	0	0	0	0	
24	1.00	58.81	210750	115870	0	0	0	
30	1.25	55.09	260490	144850	0	55190	107240	3.10
36	1.50	47.06	277840	166200	97400	65250	108480	3.14
42	1.75	39.88	304040	168900	136840	95760	135150	3.91
48	2.00	36.57	305950	164410	133800	94010	150390	4.35
56	2.33	31.81	345980	195650	193720	134360	167080	4.83
64	2.67	25.77	340910	193700	236680	149450	208410	6.03
72	3.00	22.68	329200	189710	226390	144850	199840	5.78
80	3.33	18.69	316280	152180	251860	165680	218700	6.33
92	3.83	12.03	318600	173925	358075	243265	257935	7.46
104	4.33	6.19	322150	166325	491645	286000	279000	8.07
116	4.83	2.47	309335	143085	555450	293760	333825	9.66
128	5.33	0	268116	124496	584656	269472	373848	10.81
140	5.83	0	230884	106540	545808	206608	429504	12.42
152	6.33	0	169960	48592	494636	149540	522320	15.11
164	6.83	0	93528	0	355468	62076	503992	14.58
176	7.33	0	0	0	209352	0	472860	13.68
188	7.83	0	0	0	112276	0	263340	7.62
200	8.33	0	0	0	47532	0	136392	3.95
212	8.83	0	0	0	0	0	42392	1.23
224	9.33						0	0

Time (hrs)	Time (days)	Int S6 RT 13.5 min (HPLC Area)	Int S7 RT 2.2 min (HPLC Area)	Int S8 RT 2.3 min (HPLC Area)	Int S9 RT 2.5 min (HPLC Area)	Int S10 RT 7.4 min (HPLC Area)
140	5.83		20484		14048	
152	6.33		71984	45804	29240	
164	6.83	74072	142980	97292	39512	
176	7.33	125456	219292	203272	71456	
188	7.83	211956	208576	352056	136612	
200	8.33	267416	154396	462976	246396	
212	8.83	289380	480660	917632	458312	
224	9.33	177048	589256	600372	876988	
236	9.83	0	874960	0	1020620	
284	11.83		878372		357824	74088
308	12.83		0		333632	265592
332	13.83				328300	73744
356	14.83				294168	41100
380	15.83				279908	8456
404	16.83				272828	288243
428	17.83				263124	59628
452	18.83				242096	54836
476	19.83				226504	216572
500	20.83				268612	19016
524	21.83				178336	398572
548	22.83				207184	79056
	30.00				119560	150572
	47.00				0	0

VITA

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