BIODEGRADATION OF NITROAROMATIC COMPOUNDS IN TNT MUNITIONS WASTES UNDER DIFFERENT METABOLIC REGIMES

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CHAPTER I

INTRODUCTION

1.1 Background

Selected nitroaromatic compounds such as 2,4,6-trinitrotoluene (TNT) and derivatives of TNT are introduced into the environment from munitions manufacturing plants and disposal sites. Surface and subsurface waters can be contaminated by discharging plant wastewater containing these compounds into holding lagoons, rivers, or streams (Pereira et al., 1979).

Toxic effects have been noted for bluegill fish at 2.3 to 2.8 mg TNT/L. An LC_{50} and a mean tolerance limit of 2.0 to 3.0 mg TNT/L has been reported for a number of freshwater fish (Osman and Klausmeier, 1972). In humans, liver damage and anemia have been reported on workers exposed to explosives through large-scale manufacturing and handling operations (McCormick et al., 1976; Pereira et al., 1979).

Biological processing is widely studied and has been successfully adapted to removal of hazardous pollutants from industrial effluents and groundwaters. The ultimate removal of hazardous contaminants by biodegradation is effected by converting organic wastes into biomass, harmless intermediates, or byproducts of microbial metabolism such as

CO_2, H_2O, CH_4, and inorganic salts.

The substituted aromatic compounds can be metabolized to produce derivatives which are highly mutagenic, xenobiotic, or carcinogenic. Some nitroaromatic compounds are believed to be persistent and possibly toxic because of the inhibitory effects of attached nitro or amino groups on hydroxylation enzymes. Under certain environmental conditions, these compounds are thus rendered metabolically recalcitrant to microbially mediated reactions (McCormick et al., 1976; Fewson, 1981).

The complete destruction of TNT has been a continuous concern among researchers. In recent years, considerable attention has been focused on microbiological degradation of aromatic compounds. Accordingly, nitroaromatic compounds such as TNT and its derivatives have been studied under a variety of conditions and methods by many researchers. TNT has been found to be a quite persistent xenobiotic compound and resistant to biodegradation by most bacteria and fungi. However, anaerobic degradation of 2-aminobenzoate under denitrifying conditions was reported by Braun and Gibson (1984). Also, anaerobic microbial metabolism tends to form of a single group of compounds from a variety of nitrogenous xenobiotics. Therefore, it is energy-efficient and facilitates certain critical reactions such as reduction of nitro groups. Because oxygen is limited in groundwater, microbial activity may be dominated by anaerobic and facultative organisms (Kobayashi and Rittmann, 1982; Braun

and Gibson, 1984).

The possibility exists for treatment of TNT-containing munitions manufacturing plant wastewaters or bioremediation of munitions waste residuals under varying redox conditions in the absence of free oxygen. The reduced metabolic products tend to be more toxic than the parent compound and the disposal of TNT-containing wastewater requires more effective and efficient methods. Thus, treatment by complete biodegradation, if feasible, would offer great advantages.

Accordingly, this research focuses on determining the feasibility of microbial processes for the transformation and mineralization of TNT and its metabolic intermediate products in the presence of various primary substrates and electron acceptors. The scope of the study is to evaluate the feasibility of complete biodegradation of selected nitroaromatic compounds such as TNT and its derivatives at concentrations that occur in the environment (0 - 100 mg/L). Specific objectives of this research are to:

(1) Determine the fate and most probable transformation products of selected nitroaromatic compounds under aerobic, anaerobic, and denitrifying conditions.

(2) Screen for promising primary substrates which can effect cometabolic degradation of nitroaromatics under each metabolic regime.

(3) Quantify the cometabolic conversion rate for microbial transformation of nitroaromatic contaminants under aerobic, anaerobic, and denitrifying conditions.

(4) Test the influence of abiotic factors on the conversion of nitroaromatics, and the influence of abiotic processes on microbially mediated nitroaromatic bioconversions.

(5) Develop conceptual systems of biological processes for the treatment of munition plant wastewater and bioremediation under combined two stage metabolic regimes through batch feasibility studies.

CHAPTER II

LITERATURE REVIEW

2.1 Selected Nitroaromatic Compounds

2.1.1 Source

Contamination of surface and subsurface water by selected nitroaromatic compounds arouses concern about their environmental impact and solutions for remediation of sites contaminated by these toxic compounds. Nitroaromatic compounds are produced on a massive scale as intermediates in the manufacture of dyes, pesticides, plastics, and explosives (Hallas et al., 1983; Hess et al., 1990). Wastewater discharged from dye manufacturing plants contributed 19 million pounds of nitrotoluene into natural waters (Higson, 1992). The chemical industries use nitrobenzene, nitrotoluenes, nitrophenols, and nitrobenzoates to manufacture polyurethane foams, elastomers, and industrial solvents. In the beginning of the 20th century, 2,4,6-trinitrotoluene(TNT) became a major compound to be used extensively in explosives. Because of the widespread use of TNT and its discharge in wastewater, soil and groundwater contamination by TNT is a by-product of explosives manufacturing plants, ammunition loading plants,

and disposal facilities. TNT and its derivatives are manufactured chemically through a three-stage process of toluene nitration in ammunition manufacturing plants. Wastewater commonly referred to as red water from these plants includes sodium sulfite (sellite) solution used to remove the symmetrical TNT isomers and various oxidation products (Nay et al., 1974). At shell- and bomb-loading plants, wastewaters generated from washing and steam cleaning the kettles used for melting TNT were allowed to overflow from collecting tanks to open drainage ditches which led into local streams or surface water (Ruchhoft et al., 1945).

Wastewaters discharged into disposal sites such as holding lagoons and disposal beds percolate into shallow groundwaters. The maximum determined concentration 620 ug TNT/L was detected in shallow groundwaters beneath and downgradient from the disposal beds at the Hawthorne Naval Ammunition Depot, Nevada. Also, TNT, 2,4-dinitrotoluene, and two metabolic degradation products such as 4-amino-2,6-dinitrotoluene and 2-amino-4,6-dinitrotoluene were isolated and characterized at the same site (Pereira et al., 1979).

2.1.2 Toxic Effects

Some researchers observed the health and ecological toxic effects of TNT and its metabolic intermediates to aquatic life forms. They reported that TNT and its metabolites affect growth or death rates of fresh water

unicellular green algae (Selenastrum capricornutum), tidepool copepods (Tigriopus californicus), and oyster larvae (Crassostrea gigas) at concentrations as low as 2.5 ppm (Won et al., 1976). Some nitroaromatics, which are detected in urban air, the exhaust of gasoline engines, and fly ash from coal-fired power plants, were shown to be mutagenic for Salmonella typhimurium strains (Wang et al., 1980).

For a number of freshwater fish a mean tolerance limit of 2.0 to 3.0 mg α-TNT/L was noted (Osmon and Klausmeier, 1972). The growth of actinomycetes, gram-positive bacteria, yeasts, and fungi were severely limited at concentrations of greater than 50 mg TNT/L. Concentrations of 2 to 3 mg TNT/L were shown to be toxic to fathead minnows (Pimephales promelas) and bluegills (Lepomis macrochirus) (Smock et al., 1976; Fernando et al., 1990).

In humans and mammals, exposure to TNT caused toxic hepatitis and aplastic anemia characterized by a significant decrease in the number of leukocytes, erythrocytes, and reticulocytes (McConnell and Flinn, 1946). Gastrointestinal disorders, morphocytosis, anemia, and toxic jaundice caused by liver damage appeared in workers engaged in large-scale manufacturing and handling operations (Bueding and Jolliffe, 1946, Voegtlin et al., 1919). From a group of 154 workers who were exposed to dinitrotoluene, some major symptoms were observed as follows: unpleasant metallic taste, weakness, headache, and dizziness. One half of the group also showed

the development of intoxication, as shown by pallor, cyanosis, low-grade anemia, and jaundice (McGee et al., 1942).

Some nitroaromatics were converted to aminoaromatics which were found to induce bladder cancer in workers at a dyestuff factory (Clayson, 1962). TNT is rated as a toxic hazard: moderate irritant, moderate allergen, high ingestion, high inhalation, and moderate skin absorption, where moderate and high designate the potential of toxicity effects on the skin or mucous membranes (Sax, 1957).

2.2 Microbial degradation of nitroaromatic compounds

Degradation of nitroaromatic compounds is of great concern because of their widespread use and potential toxicity. The majority of nitroaromatic compounds and products of their metabolism have been shown to be highly toxic and inhibitory. Also, some of metabolic products of nitroaromatic compounds, hydroxylamino compounds, often form azoxy compounds by nonenzymatic polymerization under the presence of oxygen and have shown to be resistant to microbial degradation under most conditions. Hence, it is important to identify the microbial transformations of nitroaromatics and their metabolic products.

2.2.1 Aerobic systems

In aerobic degradation of nitroaromatic compounds, the detachment of aromatic ring substituents is often required before conversion of aromatic rings into the aromatic nucleus of hydroxylated intermediates containing adjacent hydroxyl groups that mediate ring cleavage. Aromatic compounds such as benzoates, phenols, and anilines are also oxygenated and converted to catechols by mono- or dioxygenases.

Cartwright and Cain (1959) suggested that p-hydroxybenzoate was the intermediate of p-nitrobenzoate incubated with a washed-cell suspension of Nocardia erythropolis. The p-hydroxybenzoate was oxidized to protocatechuate or via dihydroxy compound by Pseudomonas fluorescens (Sleeper and Stanier, 1950). Much ammonia and traces of nitrite were produced from the incubation with ortho or para-nitrobenzoate. However, more nitrite was released from the incubation with meta-nitrobenzoate (Cain, 1958). The following overall reactions were suggested:

$$C_7H_5O_4N + 5.5\ O_2 \longrightarrow 7\ CO_2 + NH_3 + H_2O$$

for para- and ortho-nitrobenzoic acid, and

$$C_7H_5O_4N + 7\ O_2 \longrightarrow 7\ CO_2 + HNO_2 + 2\ H_2O$$

for meta-nitrobenzoic acid.

When dinitrophenol (2,6 DNP) was incubated with succinate as carbon source, approximately 2 mol of NO_2^- per mol of 2,6 DNP was produced by Pseudomonas sp. strain N26-8

(Lenke and Knackmuss, 1987). Under an atmosphere of argon, 2,6 DNP transformation and nitrite release were not detected in 50 mM phosphate buffer, suggesting that the nitrite release was O_2 dependent. However, the reaction with 2,5 DNP produced nitrite and two other intermediate metabolites such as 2-amino-5-nitrophenol and 5-amino-2-nitrophenol.

2,4-dinitrotoluene was shown to be utilized by Phanerochaete chrysosporium (Valli et al., 1992). This dinitroaromatic compound was metabolized by lignin peroxidase (LiP), manganese peroxidase (MnP), and crude intracellular cell extracts through the following multistep pathway:

(1) 2,4-dinitrotoluene --(reduced)--> 2-amino-4-nitrotoluene --(oxidized by MnP)--> 4-nitro-1,2-benzoquinone + methanol

(2a) 4-nitro-1,2-benzoquinone --(reduced)--> 4-nitro-1,2-hydroquinone --(methylated)--> 1,2-dimethoxy-4-nitrobenzene

(2b) 4-nitro-1,2-hydroquinone --(oxidized by MnP)--> nitrite + 2-hydroxybenzoquinone --(reduced)--> 1,2,4-trihydroxybenzene

(3) 1,2-dimethoxy-4-nitrobenzene --(oxidized by LiP)--> nitrite + methanol + 2-methoxy-1,4-benzoquinone

(4) 2-methoxy-1,4-benzoquinone ---(reduced)---> 2-methoxy-1,4-hydroquinone ---(oxidized by LiP and MnP)--->

4-hydroxy-1,2-benzoquinone ---(reduced)---> 1,2,4-trihydroxybenzene

(5) 1,2,4-trihydroxybenzene ---(ring cleaved)---> ß-ketoadipic acid (reduced by cell extracts)

In this pathway, the first nitro group was reduced to 2-amino-4-nitrotoluene, which became a substrate for peroxidase-catalyzed oxidation. The second nitro group was removed from the ring by MnP oxidation. Thus, before the ring was cleaved by 1,2-dioxygenase, the two nitro groups were removed from the aromatic ring.

The extensive degradation of TNT to several intermediates by Pseudomonas-like bacteria was demonstrated by Won et al.(1974). TNT disappeared rapidly in aerobically incubated cultures within a day. Reduced TNT metabolites such as 2,2'6,6'-tetranitro-4-azoxytoluene, 2,2'4,4'-tetranitro-6-azoxytoluene, 4,6-dinitro-2-aminotoluene, 2,6-dinitro-4-hydroxyl-aminotoluene, and nitrodiaminotoluene were produced by Pseudomonas-like cultures. However, the azoxy compounds may not be direct TNT metabolic products because the corresponding hydroxylamines can be coupled abiotically to form these compounds (Channon et al., 1944, Oren et al., 1991).

Hence, the following pathway can be suggested:

TNT ---(reduced)---> 2,6-dinitro-4-hydroxylaminotoluene or its isomer 2,4-dinitro-6-hydroxylaminotoluene ---(reduced)---> 2,6-dinitro-4-aminotoluene or 2,4-dinitro-6-aminotoluene.

Under aerobic conditions, Schackmann and Muller (1991) studied reduction of nitroaromatic compounds by Pseudomonas species CBS3, P. putida, P. acidovorans, P. aeruginosa. They detected anilines from the reduction of mononitro compounds and 4-chloro-1,3-diaminobenzene from the reduction of chloro-2,4-dinitrobenzene by Pseudomonas sp. CBS3. Also, two monoaminodinitrotoluenes and one diaminomononitrotoluene were observed from the reduction of 2,4,6-trinitrotoluene. These investigators obtained similar reaction results for the reduction of nitro compounds under an argon gas atmosphere and observed the same nitro reducing behavior from other Pseudomonas species tested. Therefore, they suggested that avoiding reduction reactions by the prevailing Pseudomonads would be advantageous to the aerobic treatment of soils and wastewaters contaminated by nitroaromatic compounds, because under aerobic conditions aromatic amines produced from reduction reactions easily form polymerization products such as azoxy compounds instead of releasing nitro groups as nitrite to cleave an aromatic ring through hydroxylation.

The formation of polyamides in an activated-sludge system acting on ^{14}C-labeled TNT was reported by Carpenter and co-workers (1978). The determined radioactivity in the lipid fraction was not incorporated into individual triglycerides or phosphatides. Also, the individual amino acids from the protein fraction did not contain ^{14}C. After 3 to 5 days incubation, only low levels of $^{14}CO_2$ could be detected in this system. Thus, the biotransformation porducts of TNT reacted with the lipid, protein, and carbohydrate constituents of some cellular fractions to form cross-linked polymers. Also, the mineralization of TNT by Phanerochaete chrysosporium was studied but intermediate metabolites were revealed as incomplete reaction products (Fernando et al., 1990; Sublette et al., 1992).

2.2.2 Anaerobic systems

Anaerobic degradations of nitroaromatic compounds begin with reduction of the nitro group to an amino group. This reduction is well established in anaerobic environments.

Boyd et al.(1983) observed temporary inhibition of CH_4 gas production from anaerobic biodegradation of nitrophenol isomers in 10 % diluted sewage sludge due to reduction of the nitro group to an amino group, whereas in aerobic systems the nitro group was released as nitrite from nitrophenols (Zeyer et al., 1986). Although the inhibition of CH_4 production was only temporary, the nitrophenols tend to inhibit the methanogenic step in anaerobic systems. Oren

et al.(1991) found that the nitro group of p-nitrophenol was only reduced to aminophenol by the halophilic anaerobic eubacteria, Haloanaerobium praevalens and Sporohalobacter marismortui.

Nitroreduction of nitropyrenes by human intestinal microbial flora such as Clostridium sp. was also observed by Rafii et al.(1991). Aromatic amines such as 1-aminopyrene, 6-aminochrysene, and 4-aminobenzoic acid were detected as major metabolic products from the corresponding nitro aromatic compounds by HPLC, TLC, and biochemical tests.

In the literature, the di- and tri-nitro aromatic compounds such as 2,4-dinitrophenol, 2,4-dinitrotoluene, and 2,4,6-trinitrotoluene were often described to be transformed in anaerobic biological systems into a variety of products without cleavage of the aromatic ring. Among these, nitroso compounds were suggested as one of the major intermediate products from the reduction of 2,4-dinitrotoluene and 2,4,6-trinitrotoluene. Thus, investigations have focused on the fate of these nitroaromatic compounds in microbial transformations (Carpenter et al.,1978; McCormick et al., 1976; and Won et al., 1974). Nitroso intermediates were isolated and identified in anaerobic culture conditions by Liu et al.(1984).

By intestinal microflora, 2,4-dinitrotoluene (DNT) was also metabolized to aminonitrotoluene. In this process aminonitrotoluene was subsequently reduced to diaminotoluene but a nitroso intermediate was also detected (Guest et al.,

1982). Only five organisms of the 190 fungi representing 98 genera studied by Parrish (1977) showed the ability to transform DNT while 183 were active in transforming TNT.

McCormick et al.(1976) investigated the bacterial transformation of 40 mono-, di-, and trinitroaromatic compounds with enzyme preparations of the strict anaerobe Veillonella alkalescens. They described inhibitory effects of pendant nitro groups on hydroxylation enzymes. The nitro groups were reduced by both aerobic and anaerobic systems and the release of nitrite was found not to be a major pathway. Thus, nitro groups were reduced via the following proposed pathway by hydrogen:

$$R\text{-}NO_2 \xrightarrow{H_2} R\text{-}NO \xrightarrow{H_2} R\text{-}NHOH \xrightarrow{H_2} R\text{-}NH_2.$$

In biological degradation of nitroaromatic compounds, nitro groups are converted into hydroxyl groups via oxidative deamination of reduced nitro groups, producing phenol with release of ammonia, or nitro groups are released as nitrite accompanied by the formation of phenol. Vogel et al.(1986), studied anaerobic oxidation of toluene and benzene to cresol and phenol, respectively. In this detailed study, the oxygen incorporated into the aromatic ring to form phenol is assumed to be derived from water. In this process, benzene was described to be degraded via phenol, and eventually to methane and carbon dioxide by a mixed methanogenic culture. Benzene was also mineralized by aquifer microorganisms under strictly anaerobic conditions,

presumably by sulfate reducing microorganisms, in studies by Edwards and Grbic-Galic (1992).

Phenol has been found to be a common metabolite in the anaerobic metabolism of benzene and many other compounds. Phenol was metabolized further via a reductive pathway described for a methanogenic culture by Balba and Evans (1980):

Phenol --------> Cyclohexanol -----> Cyclohexanone ----> 2-hydroxycyclohexanone -------> adipic acid.

Under methanogenic conditions benzoic acid produced from carboxylation of phenol was also detected as an intermediate by Bisaillon et al.(1991). For the carboxylation of phenol, cometabolic substrates such as yeast extract or tryptophan and proteose peptone were found to be required (Bechard et al., 1990; Sharak-Genthner et al., 1989, 1990).

In other attempts to test the carboxylation of phenol, a gas phase of 80 % H_2 or N_2 and 20 % CO_2 in phenol-degrading culture was used by Knoll and Winter (1989). Benzoic acid as a phenol metabolite was also tested under denitrifying and ferric-reducing conditions. Shlomi et al. (1978) proposed pathways for the anaerobic degradation of benzoic acid leading to ring cleavage by a methanogenic organisms as follows:

Benzoic acid ------> Cyclohex-1-enecarboxylic acid ------> 2-hydroxycyclohexane carboxylic acid -----> 2-oxocyclohexane carboxylic acid -------> pimelic acid.

In the presence of bicarbonate, Tschech and Fuchs (1987) detected 4-hydroxybenzoate as an intermediate from the anaerobic degradation of phenol by pure cultures of two newly obtained isolates of nitrate-reducing Pseudomonas. The dissimilatory iron-reducing organism, GS-15, showed the ability to degrade phenol to CO_2. Lovley and Lonergan (1990) detected 4-hydroxybenzoate as a transient intermediate in a phenol-degrading culture medium of GS-15.

Cresol was found as a metabolite formed during the anaerobic biodegradation of tyrosine and toluene by methanogenic organisms (Balba and Evans, 1980; Vogel and Grbic-Galic, 1986). Of the three cresol isomers, ortho-cresol showed the strongest resistance to anaerobic biodegradation and para-cresol was oxidized to 4-hydroxybenzoate under denitrifying conditions (Bossert et al., 1986). The 4-hydroxybenzoate alcohol and 4-hydroxybenzaldehyde were detected as transient intermediates in the formation of 4-hydroxybenzoate. Under sulfate-reducing conditions, Smolenski and Suflita (1987) also reported the same intermediates of para-cresol as Bossert et al.(1986), and detected oxidized intermediates in denitrifying conditions.

Thus, a variety of results have been demonstrated in

degradation studies of nitroaromatic compounds. This suggests the important role of environmental conditions and existing alternative substrates on metabolic activities.

2.2.3 Denitrifying systems

Aminoaromatic compounds, formed from the reduction of nitroaromatic compounds, are highly reactive and easily polymerize to form azoxy compounds in the presence of oxygen. In aerobic soil environments, these compounds may react with humic acids to form persistent immobilized products which make further degradation extremely difficult (Carpenter et al.,1978; Schackmann and Muller, 1991). Thus, for complete oxidation of aminoaromatic compounds, anoxic conditions were favored and may lead to improved processes for eliminating these contaminants (Braun and Gibson, 1984).

Schennen et al.(1985) reported that 2-fluorobenzoate was utilized by denitrifying bacteria of the genus Pseudomonas as the sole carbon and energy source. The benzoyl-CoA synthetase, which was induced after anaerobic growth on benzoate, was involved in degradation of 2-fluorobenzoate. Thus, fluoride ion was released stoichiometrically with the reduction of dissolved organic carbon, which indicated total degradation of 2-fluorobenzoate.

Taylor et al.(1970) tested biodegradation of benzoate and p-hydroxybenzoate with facultatively anaerobic microorganisms by nitrate respiration. They reported the

following overall equation for complete benzoate oxidation:

$C_7H_6O_2 + 6KNO_3 \text{ ------> } 7CO_2 + 3N_2 + 6KOH$

Benzoate was also metabolized by Moraxella sp. under denitrifying conditions. Williams and Evans (1975) detected cyclohexanecarboxylate and 2-hydroxycyclohexanecarboxylate as intermediates in their culture medium. When ring labelled ^{14}C benzoate was used, 2-hydroxycyclohexanecarboxylate and adipate were detected as labelled intermediates. However, when the carboxyl group of benzoate was labelled with ^{14}C, adipate showed no radioactivity. Accordingly, they inferred that a decarboxylation presumably occurred at some point in the pathway between these two intermediates. Thus, the following pathway was identified:

Benzoate ----> cyclohexanecarboxylate ------> cyclohex-1-enecarboxylate -----> 2-hydroxycyclohexanecarboxylate ----------> 2-oxocyclohexanecarboxylate ------> cyclohexanone ----------> adipate.

Thus in the literature, mineralization of benzoates and phenols, which posses functional substituents containing oxygen, has been shown to occur via ring reduced cyclohexane before hydrolytic ring cleavage (Taylor et al., 1970; Taylor and Heeb, 1972; Williams and Evans, 1975). To reduce the ring structure, a modification or removal of ring substituents by dehydroxylation, demethoxylation, or dechlorination was required. However, in anoxic conditions

oxygenases are inactive, so anaerobic mineralization of aromatic hydrocarbons such as toluenes, which contain no functional substituents active in mediating hydration of aromatic rings, has not been clearly described by this type of mechanism (Zeyer et al.,1986).

In a later study (Zeyer et al., 1988), these researchers again attempted to explain the toluene degradation pathway by isotope-dilution experiments. Benzoate was detected as an intermediate. Three theoretically possible pathways were considered: aromatic ring reduction, hydroxylation of the methyl group or aromatic ring hydroxylation. The reaction transforming toluene to p-cresol proved to be very slow, and the mechanism of anaerobic degradation of toluene remained obscure.

However, p-hydroxybenzoate has been reported as an intermediate of toluene degradation (Schocher et al.,1991; Lovley and Lonergan,1990; Evans et al.,1991a). After the degradation test, using fluoroacetate, of toluene by pure cultures of denitrifying bacteria (strain T and K172), Schocher et al.(1991) concluded that the initial catabolic reaction in the degradation of toluene occurred through the oxidation of the methyl group to form benzoate.

Unlike the previous reports in the literature, Evans et al.(1992) suggested different intermediates and a pathway for toluene degradation. Benzylsuccinic acid and benzylfumaric acid were detected as two dead-end

metabolites, while 2-methylbenzyl-succinic acid and 2-methylbenzyl-fumaric acid were also produced in o-xylene transformation by growing on succinic acid. Succinyl-CoA or acetyl-CoA was identified as key to the enzymatic attack on the methyl group to form benzylsuccinyl-CoA or phenylpropionyl-CoA, respectively. Thus, they proposed a pathway for toluene mineralization as follows:

Toluene ---(acetyl-CoA)---> phenylpropionyl-CoA ---
---(ß-oxidation)---> benzoyl-CoA ------> ring cleavage.

In denitrifying conditions, anaerobic oxidation of toluene and xylenes was observed to be stoichiometrically dependent on nitrate reduction, and the degradation of o-xylene was dependent on the metabolism of toluene (Evans et al.,1991a, 1991b). Braun and Gibson (1984) observed that three Pseudomonas sp. (strains KB 740, KB 820, and KB 650) degrade 2-aminobenzoate anoxically to CO_2, N_2, and NH_4^+ under denitrifying conditions. They proposed an anoxic reaction for complete degradation as follows:

$C_7H_7O_2N + 1.8H_2O + 5.6NO_3^- \rightarrow 7CO_2 + NH_4^+ + 2.8N_2 + 6.6\ OH^-$

Thus, they suggested an anaerobic system for the complete oxidation of an aminoaromatic compound.

The effect of limited oxygen on the degradation of benzene and alkylbenzene under denitrifying conditions was reported by Hutchins (1991). In the presence of oxygen these compounds showed continuous biodegradation under denitrifying conditions, but once oxygen was depleted, biodegradation ceased. These results suggest that partial

aerobic degradation might be required initially, under certain reaction conditions.

Dolfing et al.(1990) observed that toluene degradation was severely inhibited by FeS. They inferred that sulfide inhibited the nitrous oxide reductase but not the nitrate reductase. Similar results were reported by Sorensen et al. (1980). Sulfide inhibition with 0.3 mM [H_2S] led to increased accumulation of NO and NO_2^-. Strong and partial inhibition for NO_2^- and NO reduction, respectively, were suggested during nitrate respiration by Pseudomonas fluorescens resting cells. They also noted that this finding was applicable to three other denitrifiers studied.

In previous studies, sulfide was also observed to inhibit the further reduction of N_2O more than the reduction of NO_3^- and NO_2^- to N_2O (Meyers, 1972). Due to precipitation of S_2^- with metal ions in soil, the inhibitory effect of sulfide was observed to be greater in aqueous systems than in soil systems. Na_2S showed complete inhibition of the reduction of N_2O to nitrogen gas at a concentration of 8 mM (Tam and Knowles, 1979).

2.2.4. The influence of abiotic factors

In anaerobic laboratory systems including homogeneous solutions of hydroquinones, Tratnyek and Macalady (1989) tested direct abiotic reduction of nitro groups of nitroaromatic pesticides to amines. According to their

results, they suggested that nitro groups of nitroaromatic pollutants can be reduced by hydroquinones in natural environments.

TNT was reduced into chemical reaction products under the influence of hydrogen sulfide in solvents. The following reduction products were obtained: 6-nitro-2,4-diaminotoluene, 2,6-dinitro-4-aminotoluene, 2,4-dinitro-6-aminotoluene, and 2,6-dinitro-4-hydroxylaminotoluene (Brady et al., 1929).

Schwarzenbach et al.(1990) described the reduction of nitroaromatic compounds mediated by quinone and iron porphyrin in homogeneous aqueous solution containing reduced sulfur species. They observed that quinone and iron porphyrin mediated effectively the reduction of nitroaromatic compounds with sulfur as bulk electron donor. They also noted that general predictions for nitroaromatic compounds about relative reactivity may be difficult in natural systems because their reduction rate is affected by substituents and may greatly differ between two systems with different electron carriers.

The mediation of electron transfer between hydrogen sulfide and various nitrobenzenes was investigated with Streptomyces griseoflavus Tu 2484 exudates by Glaus et al.(1992). Pseudo-first-order kinetics were observed and a linear free energy relationship was found. No competition between various compounds was observed during mediated reduction of nitroaromaic compounds by Streptomyces sp.

exudates. Cinnaquinone and dicinnaquinone were identified as two major exudate components of a culture of strain Tu 2484 and were assumed to be responsible for most mediated reduction effects.

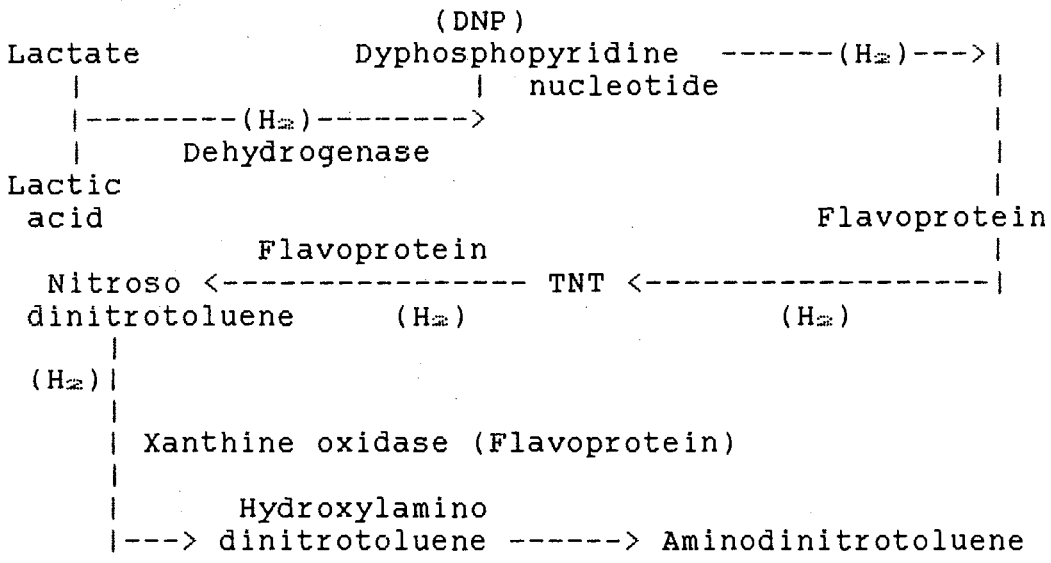
2.3 TNT biodegradation

TNT is much less amenable to biodegradation than aromatics containing a single nitro group or an activating hydroxyl and carboxyl group because three nitro groups attached on an aromatic ring establish steric hindrance and electronic deactivation (Higson, 1992). So far, the mechanism of TNT biodegradation is not well known but is the subject of current research to identify microbial metabolites and probable pathways to complete degradation.

Early studies focused on TNT metabolism in humans and higher animals. Westfall (1943) showed that a succinic dehydrogenase enzyme preparation reduced TNT to 2,6-dinitro-4-aminotoluene.

With the enzyme extracted from pig liver tissue, TNT metabolism was studied, in vitro, by Bueding and Jolliffe (1946). They reported that one nitro group of TNT was reduced to an amino group by several steps of reduction. For each of these steps, a different hydrogen-transferring system was involved. Lactate was used as an electron-donating substrate, and dehydrogenase, diphosphopyridine nucleotide (DPN), and flavoprotein were involved in transferring the donated electron to TNT. Hence,

flavoprotein initiated TNT reduction to the nitroso stage. Then xanthine oxidase, another flavoprotein, catalyzed the next reduction to hydroxylamine stage. Thus, they proposed a TNT reduction mechanism by animal tissue as follows:



2,4-diamino-6-nitrotoluene (2,4-DA), a metabolic intermediate of TNT, was tested as the sole nitrogen source for Pseudomonas fluorescens B-3468 by Naumova et al.(1988). After the NADH-dependent deamination, nitrogen free metabolites of 2,4-DA such as phloroglucine and pyrogallol were produced, then nitrogen was released as ammonium. No nitrite and nitrate ion was detected. During the process of the transformation of all three nitro groups, NADH and NADPH in combination with FAD were involved in deamination and hydroxylation in the ring where each amino group was separated. Once all three nitro groups were separated, phloroglucine was detected as a metabolic product. Phloroglucine also proceeded to ring cleavage through pyrogallol, as reported by Walker and Taylor (1983).

Pyrocatechase was believed to participate in the breakdown of the aromatic ring.

Channon et al. (1944) tested the existence of a reductive biodegradation mechanism by feeding TNT to rabbits, rat, or human volunteers. TNT was excreted in the urine as transformed products such as 2,6-dinitro-4-amino-toluene, 2,4-dinitro-6-aminotoluene, or 2,2'6,6'-tetranitro-4,4'-azoxytoluene, or as glucuronide conjugates. They proposed the following reduction pathway:

$CH_3C_6H_2(NO_2)_3$ --> $CH_3C_6H_2(NO_2)_2NHOH$ --> $CH_3C_6H_2(NO_2)_2NH_2$

A pseudomonad (N.C.I.B., 9771) was isolated from garden soil perfused with 3,5-dinitro-o-cresol mineral salts medium by Tewfik et al. (1966). This microorganism metabolized the herbicide in pure culture. They proposed the following reduction pathway: 3,5-dinitro-o-cresol ---(1)---> 3-amino-5-nitro-o-cresol ---(2)---> 3-methyl-5-nitro-catechol ---(3)---> 3-methyl-5-amino-catechol ---(4)---> 2,3,5-trihydroxy-toluene ---(5)---> ring cleavage. The reaction (1) was stimulated by $FADH_2$, Mn and Fe^{2+} ions. Reaction (2) proceeded to (3) in the presence of excess $NADH_2$.

Osmon and Klausmeier (1972) investigated microbial degradation of TNT, cyclonite (RDX), and ammonium picrate. TNT only showed degradation with addition of yeast extract to basal medium containing 100 mg TNT/L after 2 to 4 days incubation. The majority of TNT degrading microorganisms in this study were pseudomonads. These investigators also tested TNT biodegradability in a pure culture (Pseudomonas

aeruginosa) but found that the dissimilation rate was lower, and metabolites accumulated transiently in the medium.

Nay et al. (1974) reported biological treatability of TNT manufacturing wastewater. They found that the α-TNT form predominates in wastewater and exerts BOD, but the ultimate BOD rapidly decreased with increasing TNT loading due to toxicity effects.

Metabolism of 2,4-dinitrotoluene by intestinal microorganisms from rat, mouse, and man was observed under anaerobic conditions by Guest et al. (1982). The 2- and 4-nitro groups were reduced to diamino groups via nitroso and hydroxylamino intermediate groups.

Degradation products from nitroaromatics and aminoaromatics form persistent macromolecules under aerobic conditions by polymerization. In contrast, anaerobic conditions promote the reduction of nitroaromatics to amino compounds and further reactions.

However, Fernando et al. (1990) investigated biodegradation of TNT by the white rot fungus Phanerochaete chrysosporium. They reported that about 35% of the TNT was metabolized to CO2 in 18 days at an initial concentration of 1.3 mg/L. Also, when the concentration was increased to typical wastewater levels (100 mg/L), they observed that 85 % of the TNT was degraded by white rot fungus, but only about 20% of initial TNT was completely mineralized to CO_2 in 90 days.

P. chrysosporium was also found by Tsai (1991) to

partially degrade red water from TNT manufacture. Pretreatment of the waste by UV exposure increased the degree of biodegradation by the white rot fungus.

TNT in the pink water obtained from a US Army munitions plant was tested for degradation by white rot fungus immobilized in a rotating biological contactor (RBC). Sublette et al. (1992) reported about 90% TNT removal from a simulated pink water. However, in their effluent from the treatment system, TNT intermediate products were still detected, and they failed to identify components (TNT intermediate products) by HPLC separation.

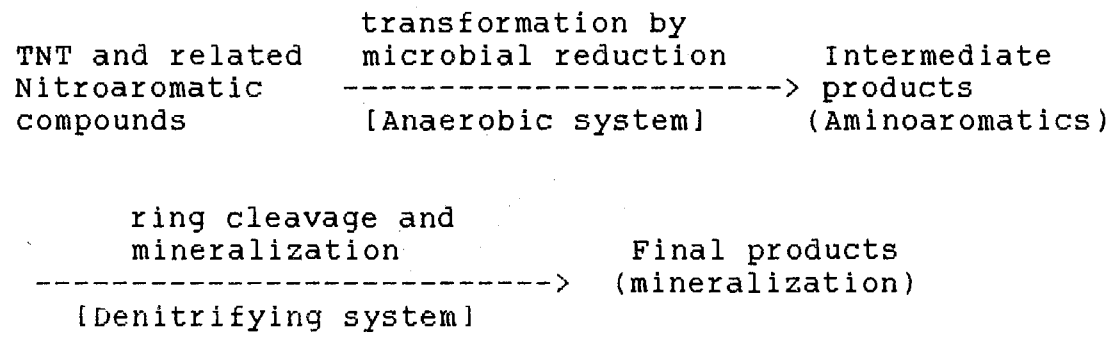
In summary, the metabolic fate of selected nitroaromatic compounds has been investigated by many researchers. Nonetheless, aromatic ring cleavage with further degradation leading to mineralization was not involved except in a few cases reported (Naumova et al.,1988; Fernando et al.,1990). However, with white rot fungus, TNT mineralization was achieved completely under some conditions, though TNT intermediate products were still found in the effluent from treatment systems (Tsai, 1991; Sublette et al., 1992).

Selected nitroaromatic compounds have exhibited a toxic effect on various life forms. Their nitro groups have an electrophilic character, so the electron density of the aromatic ring is reduced and electrophilic attack of oxygenases and oxidative degradation are hindered. Nitro reductase is a different enzyme from nitrite reductase, and

its existence determines the availability of the nitrogen of nitro-compounds through metabolic routes (Tewfik and Evans, 1966).

On the other hand, mineralization of ring substituted aromatic compounds under denitrifying conditions was reported (Taylor et al.,1970; Williams and Evans, 1975; Evans et al.,1992). Also, 2-aminobenzoate was reported to have completely degraded under denitrifying conditions by Braun and Gibson (1984).

Accordingly, an anaerobic/anoxic model system can be proposed for the complete biodegradation of TNT and its intermediate compounds with aromatic ring cleavage as follows:



CHAPTER III

MATERIALS AND METHODS

3.1 Batch Cultures

Test tube microcosm batch cultures or microorganisms from currently running reactors were used to inoculate test vials and reactors for static biodegradation studies. The following three types of batch cultures were incubated and maintained to be used: anaerobic cultures, aerobic cultures, denitrifying cultures. All glassware and materials involved in testing were sterilized by autoclaving at 121 °C and 15 lb/in^2 of steam pressure for over 20 minutes.

3.1.1 Anaerobic cultures

Isolation of organisms from soil was carried out by an enrichment culture technique with Hungate tubes. The enriched medium was adjusted to pH 7.2 with phosphate buffer solution. The medium was purged of oxygen by spiking with argon gas. Enriched microcosm batch cultures were acclimated to 100 mg TNT/L and maintained for inoculation in 40 mL test vials or 250 mL Erlenmeyer flasks, stoppered and fitted with glass tubes for gas exchange and sampling. TNT was obtained from Chemical Service, Inc. (West Chester, Pa.).
The enrichment medium is shown in Table I.

TABLE I

RECIPE FOR ENRICHED MEDIUM

Chemical compounds	Amounts	
Peptone	20	g/L
Yeast extract	10	g/L
Sodium lactate	10	mL/L
NaCl	5	g/L
K_2HPO_4	2.5	g/L
Sodium thioglycolate	1	g/L

3.1.2 Aerobic cultures

A 2.5L glass bottle was stoppered, fitted with glass tubes, and filled with 1.5L enriched medium containing 100 mg TNT/L. The medium was supplemented with 1 % yeast extract and 2 % glucose. Activated sludge from municipal wastewater treatment plant in Stillwater was maintained in a 25 L reactor by aeration. It was transferred into the 2.5 L glass bottle to be acclimated. This bottle was aerated to sustain the residual oxygen concentration level above 0.5 mg/L. Cultures were maintained at 35-37 °C in a temperature controlled water bath. Some inocula were taken out through a glass tube when inoculations were necessary.

3.1.3 Denitrifying cultures

Denitrifying cultures were isolated from a bench scale reactor, currently running in the laboratory for another concurrent denitrification study, then acclimated to 2-aminobenzoate. The cultures were maintained in 40 mL test vials or 250 mL Erlenmeyer flasks filled with 200 mL mineral

medium (Table II, adapted from Taylor et al., 1970) for inoculation.

TABLE II

RECIPE FOR MINERAL MEDIUM

Chemical compounds	Amounts
2-aminobenzoate	0.7 g/L
KNO_3	2.0 g/L
$MgSO_4$-$7H_2O$	0.1 g/L
$CaCl_2$	0.05 g/L
Na_2HPO_4-$7H_2O$	7.9 g/L
KH_2PO_4	1.5 g/L
trace metal sol'n	10.0 mL/L

The pH was adjusted to 7.9 with 0.04 M KH_2PO_4-K_2HPO_4 buffer solution. Trace metal solution (Table III) was adapted from Vishniac and Santer(1957).

TABLE III

RECIPE FOR TRACE METAL SOLUTION

Chemical compounds	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 gas phase was filled with argon gas and cultures were incubated at 37 °C in an incubator. When the media in test vials became turbid, it was transferred into 250 mL Erlenmeyer flasks containing fresh medium. The flasks were

maintained at 37 °C in the incubator for later inoculation to test reactors.

3.2 Laboratory Tests for TNT Biodegradation

In each test 5 sets of reactors (1 control with no inoculum seeded, 1 blank with no TNT included, and 3 test reactors) were used. The batch static microcosm studies shown in Table IV were conducted to investigate feasibility of microbial processes suited to the application of complete TNT biodegradation.

TABLE IV

BATCH STATIC MICROCOSM TESTS

System condition	TNT(mg/L)	Reactor	Atmosphere
Preliminary test	20, 70, 100	40 mL test vial	Ar, H_2, N_2
Aerobic	100	250 mL flask (Erlenmeyer) 2.5 L glass bottle	Air
Anaerobic	100	250 mL flask (Erlenmeyer)	Ar
Denitrifying	Metabolic products from previous reaction	250 mL flask (Erlenmeyer)	Ar
Abiotic	20, 30	120 mL serum bottle	Ar

3.2.1 Preliminary tests

Different levels of TNT concentrations were tested under various gas phase conditions to find TNT concentration and gas atmosphere effects. Concentrations of 20, 70, and

100 mg TNT/L were added into each 30 mL enriched medium (Table V) and incubated at 37 °C. The gas atmospheres were replaced with hydrogen, nitrogen, and argon gases to limit oxygen content for anaerobic conditions. Experiments were conducted for 12 days duration.

TABLE V

RECIPE FOR MEDIUM OF PRELIMINARY TESTS

Chemical compound	Amounts	
Peptone	2.0	g/L
Yeast Extract	1.0	g/L
Sodium Lactate	0.5	mL/L
Sodium Chloride	0.25	g/L
K_2HPO_4	0.125	g/L
Sodium thioglycolate	0.05	g/L

3.2.2 Anaerobic biotransformation tests

For these experiments 250 mL Erlenmeyer flasks were charged with 200 mL enriched medium containing 100 mg TNT/L and 1 mL of anaerobic inoculum. Gas atmosphere was replaced with argon gas. All of the flasks were placed in an incubator set at 37 °C. Glucose was also included in the synthetic medium in some flasks instead of peptone to test the effects of different primary substrates.

Samples were taken at shorter intervals for the early stage of experiments (1-3 days) and at longer intervals when reaching steady state. Experiments were conducted for 10-25 day periods. When experiments were completed, the media

containing TNT intermediate products were transferred into other flasks prepared for the continuous denitrifying tests.

3.2.3 Aerobic biotransformation tests

Enriched medium (1.5 L) was transferred into each 2.5 L bottle and aerated. The tubes for gas exchange were plugged with cotton to limit evaporation, and 1-2 mL inoculum was added from a reactor maintained for the acclimated aerobic cultures. The incubating bottles of these experiments were maintained at 35-37 °C in a water bath.

The effects of different primary substrates (glucose and peptone) were also investigated. Experiments were performed for 5-15 day incubation periods.

3.2.4 Complete biodegradation tests

These experiments were carried out by transferring the intermediate products from anaerobic and aerobic reactors to static denitrifying reactors. For the continuous denitrifying test, 150 - 200 mL medium containing reaction products was transferred into each prepared 250 mL Erlenmeyer flask, and argon gas was added for the gas atmosphere. The following nutrients required for denitrifying microorganisms, shown in Table VI, were added into the reactors.

TABLE VI

NUTRIENTS FOR DENITRIFYING CONDITIONS

Chemical compound	Amounts
KNO_3	2.0 g/L
$MgSO_4$-$7H_2O$	0.1 g/L
$CaCl_2$	0.05 g/L
Na_2HPO_4-$7H_2O$	7.9 g/L
KH_2PO_4	1.5 g/L
Trace metal solution	10.0 mL/L

Inoculum (2-3 mL) from denitrifying cultures acclimated to 2-aminobenzoate was seeded into each reactor containing the transferred intermediate products from initial anaerobic or aerobic reaction conditions. The reactors were incubated at 37 °C in an incubator. To investigate pH effects and electron acceptor requirements, KNO_3 was excluded from the medium and pH was also raised by Na_2HPO_4-$7H_2O$ when necessary.

3.2.5 Test for the influence of abiotic factors

Portions of the medium from reactors at the completion of the complete TNT biodegradation studies were sampled and filtered for the test of abiotic electron transfer mediation effects. The cellular materials in the medium were removed by filtration through 0.45 µm followed by 0.2 µm membrane filters. Sterilization was confirmed by agar plating of the filtrate.

Forty-mL test vials were used and filled to 30 mL with

medium filtrate and TNT solution, and pH was adjusted to 7.0 - 7.2 with a 40 mM [K_2HPO_4-KH_2PO_4] buffer solution. A stock sulfide solution (400 mM Na_2S-$9H_2O$) was used to provide 6.0 mM [S^{-2}]. From 50 % - 80 % filtrates (v/v) were tested to determine reaction rate coefficients. Argon gas was transferred into the gas phase of test vials to sustain anaerobic conditions. To check microbial contamination, agar plates were also used.

Sulfide effects on denitrifying conditions were tested with 120 mL serum bottles. The media from test vials were transferred into serum bottles in which denitrifying conditions were promoted. Gas phase was also filled with argon gas.

3.3 Analytical Methods

3.3.1 Sampling and sample preparation

Samples of 0.5-1 mL volume were taken into 2 mL sampling vials over adequate intervals to monitor the transformation of TNT into reduced intermediates and disappearance of intermediates over time. Each sample was prepared by either filtration with 0.45 µm membrane filter or centrifugation at 2800 rpm for 30-40 minutes.

3.3.2 Measurement of pH

A model 900 Accumet pH meter (Fisher Scientific Co.) was used to determine pH. Calibration was checked with standard solutions of pH 4, 7, and 10 (HACH Company), at

regular intervals.

3.3.3 Measurement of dissolved oxygen content

The dissolved oxygen content for aerobic incubations was measured with a model 97-08-00 O_2 probe (Orion Research Inc.) and YSI meter. The concentration was double checked periodically during the experiments by the procedure suggested in Standard Methods (APHA et al., 1985), Section 421 C. The deviation between the probe and chemical measurements was always less than 0.15 mg O_2/L.

3.3.4 Measurement of nitrate and nitrite

To measure these anions, a Dionex ion chromatograph, series 2000i/sp, was used according to the techniques of the Standard Methods, Section 429 (APHA et al., 1985). Nitrogen was used to pressurize ion chromatograph systems. An IonPac AS4A-SC 4mm Analytical Column was used. Eluent concentration was 1.8 mM Na_2CO_3/1.7 mM $NaHCO_3$, and flow rate was maintained at 2.0 mL/min. H_2SO_4 (25 mN) was used to regenerate the column at a flow rate of 5 mL/min. Stock standard solutions for anions were used, but fresh new solutions were also prepared whenever necessary.

3.3.5 Measurement of sulfide

Sulfide concentrations were analyzed by the method described in Standard Methods (APHA et al., 1985), Section 421 B and 427 D.

3.3.6 Separation and determination of TNT and derivatives by HPLC

TNT and its intermediate products in all samples were separated and determined by HPLC using a system equipped with a Waters Associates absorbance detector 440, Beckman model 110A gradient pump, and Hewlett Packard 3380A integrator. A reverse phase column (Rsil C-18; 4.6 x 250 mm; particle diameter, 5um; Beckman Instruments, Inc.) was used to separate intermediates.

The mobile phase, water-methanol (50:50 v/v), was eluted isocratically at a flow rate of 2 mL/min. The elution was monitored at 254 nm wavelength. A 20 µL volume of each prepared sample was injected into the HPLC. Quantification was performed with a Hewlett Packard 3380A integrator based upon the peak response factor. The separated peaks were determined based upon retention time matching with TNT as an internal standard. Analytical test conditions used by Kaplan and Kaplan (1982) were duplicated as closely as possible in this study.

The determined peaks were identified by labelling numbers according to the order of appearance from the transformation reactions in each test condition. Thus, the structures of intermediate products with matching numbers from the different systems are not necessarily the same.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Preliminary studies for static anaerobic tests

Preliminary experiments were conducted to investigate adequate conditions for TNT biotransformation tests. Different levels of TNT concentrations were tested under various gas phase conditions. Gas atmospheres of testing reactors or bottles containing TNT and culture media were filled with hydrogen, nitrogen, and argon gas to impose anaerobic conditions by limiting oxygen contents.

4.1.1 Conditions under hydrogen gas atmosphere

Figure 1 illustrates the biotransformation of 20 mg/L TNT and its metabolic production of intermediate products under the condition of hydrogen gas atmosphere. The control reactor remained at 20.37 mgTNT/L (± 0.55 mg/L standard deviation), while all of the initial concentration of TNT was transformed in the test reactors during one day incubation. The first transformation intermediate product, designated as INT#1, started to appear as soon as TNT conversion had begun. However, this product was transient, and a succession of other transformation products emerged from INT#1.

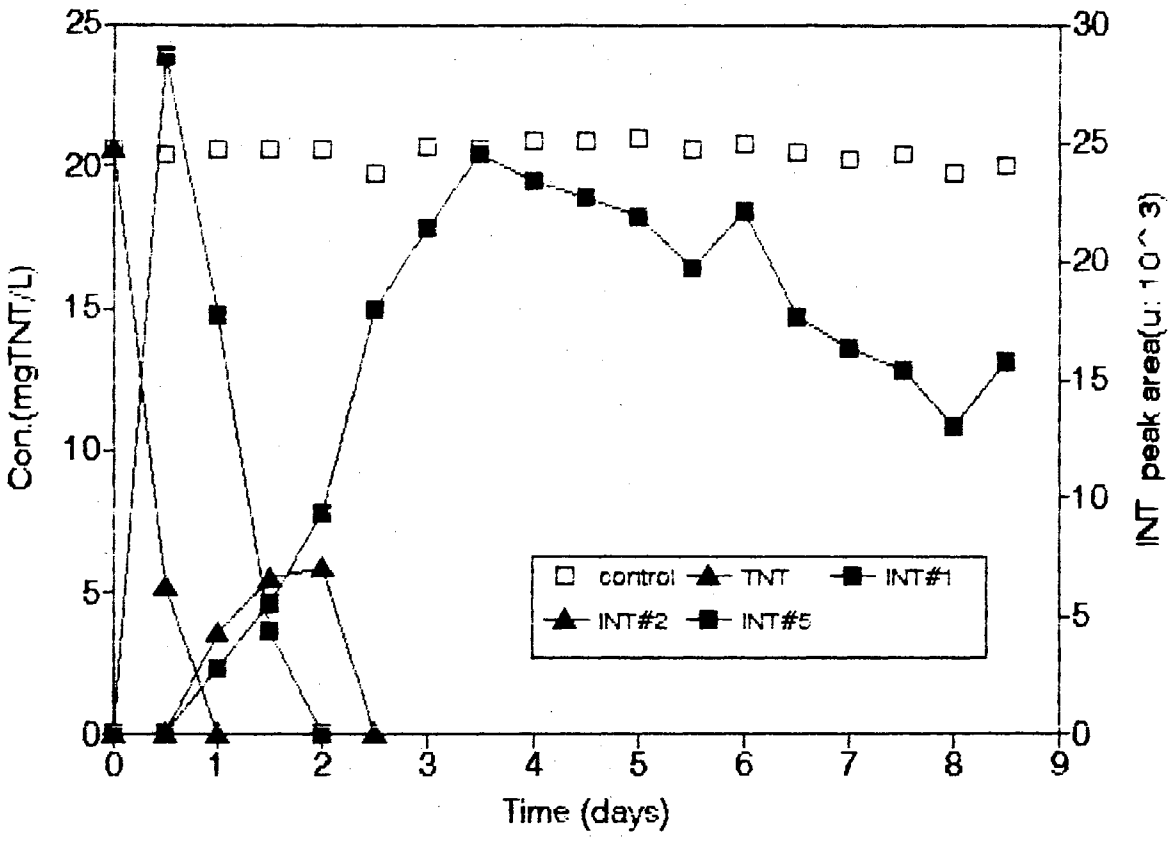


Figure 1. Plot of TNT biotransformation and its transformation products. Gas phase: H_2
Initial TNT concentration: 20 mgTNT/L

* note: control and TNT go with left y-axis
Intermediates(INT) go with right y-axis

On the other hand the profile of TNT biotransformation, depicted in Figure 1, shows a persistent product (INT#5) emerging after about 9 days incubation. Similar results were obtained from 70 mgTNT/L and 100 mgTNT/L tests explained in Figures 2 and 3, respectively. Table VII shows that the initial concentrations of 20, 70, and 100 mgTNT/L were transformed into first intermediate in about 1, 2, and 3 days of incubation, respectively. Three transformation products of TNT were detected from each of three incubations under hydrogen gas atmosphere. The rates of TNT conversion are shown as the ratio of TNT remaining to initial concentrations in Figure 4. All three cases showed similar results for 20, 70, and 100 mgTNT/L initial concentrations, respectively.

TABLE VII

TNT BIOTRANSFORMATION UNDER H_2 GAS ATMOSPHERE

Incubation Time(days)	TNT initial concentrations (mg/L)		
	20	70	100
0	20.0	70.0	100.0
0.5	5.2	62.4	94.4
1.0	0.0	31.7	77.8
1.5		11.1	56.2
2.0		---	30.3
2.5		trace	6.0
3.0			1.9
3.5			trace

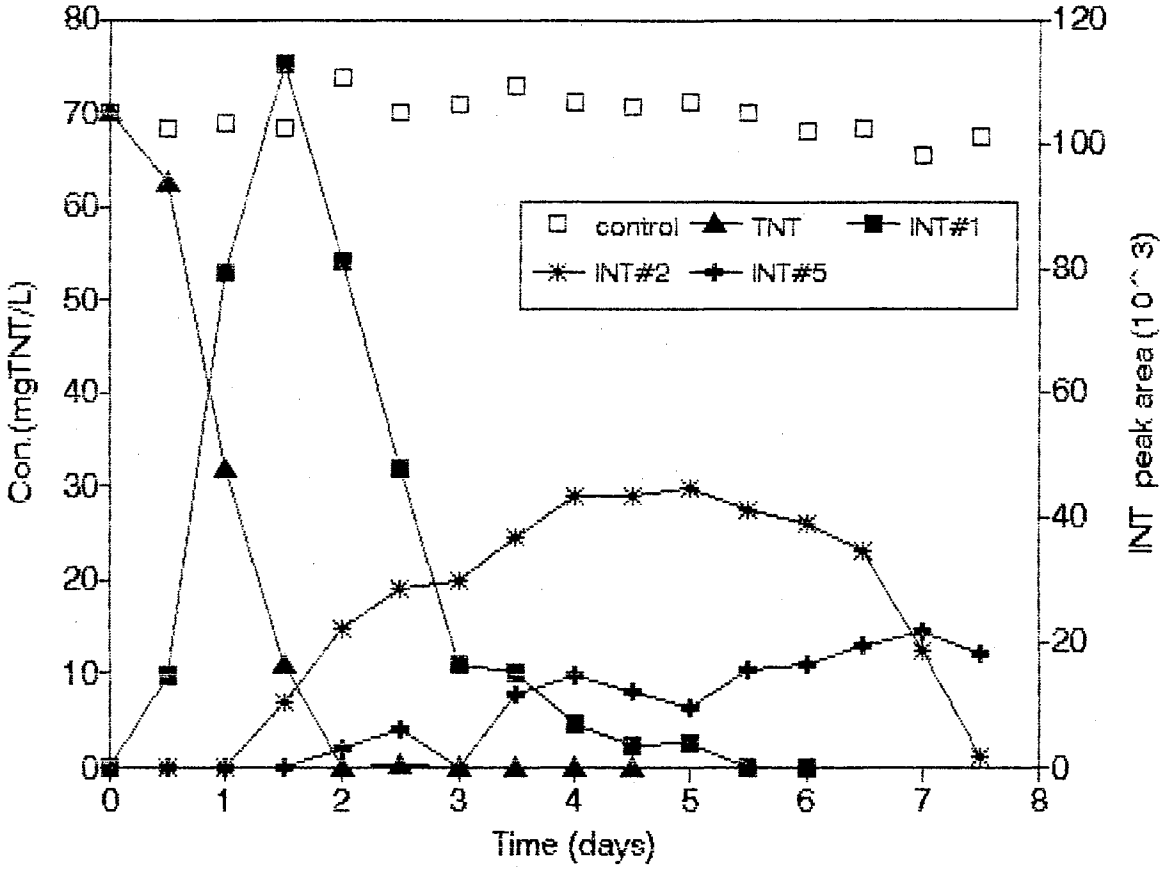


Figure 2. Plot of TNT biotransformation and its transformation products. Gas phase: H_2 Initial TNT concentration: 70 mgTNT/L

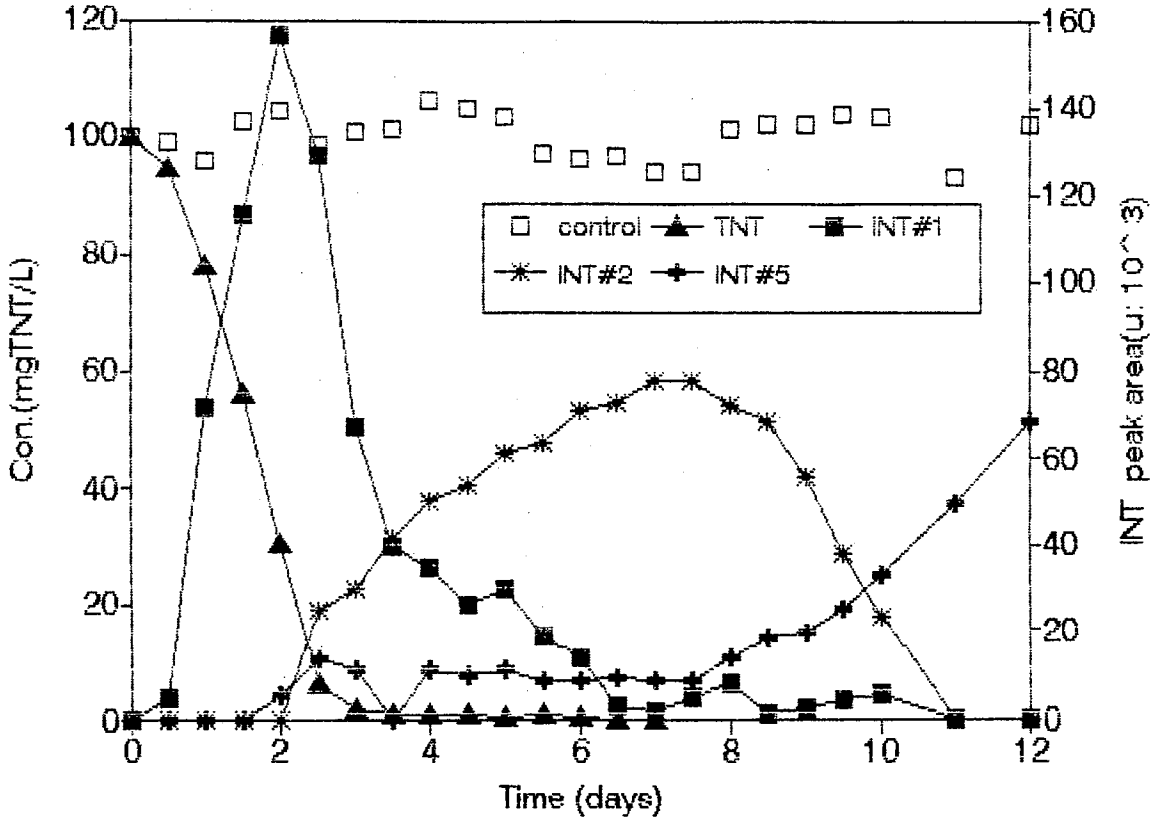


Figure 3. Plot of TNT biotransformation and its transformation products. Gas phase: H_2 Initial TNT concentration: 100 mgTNT/L

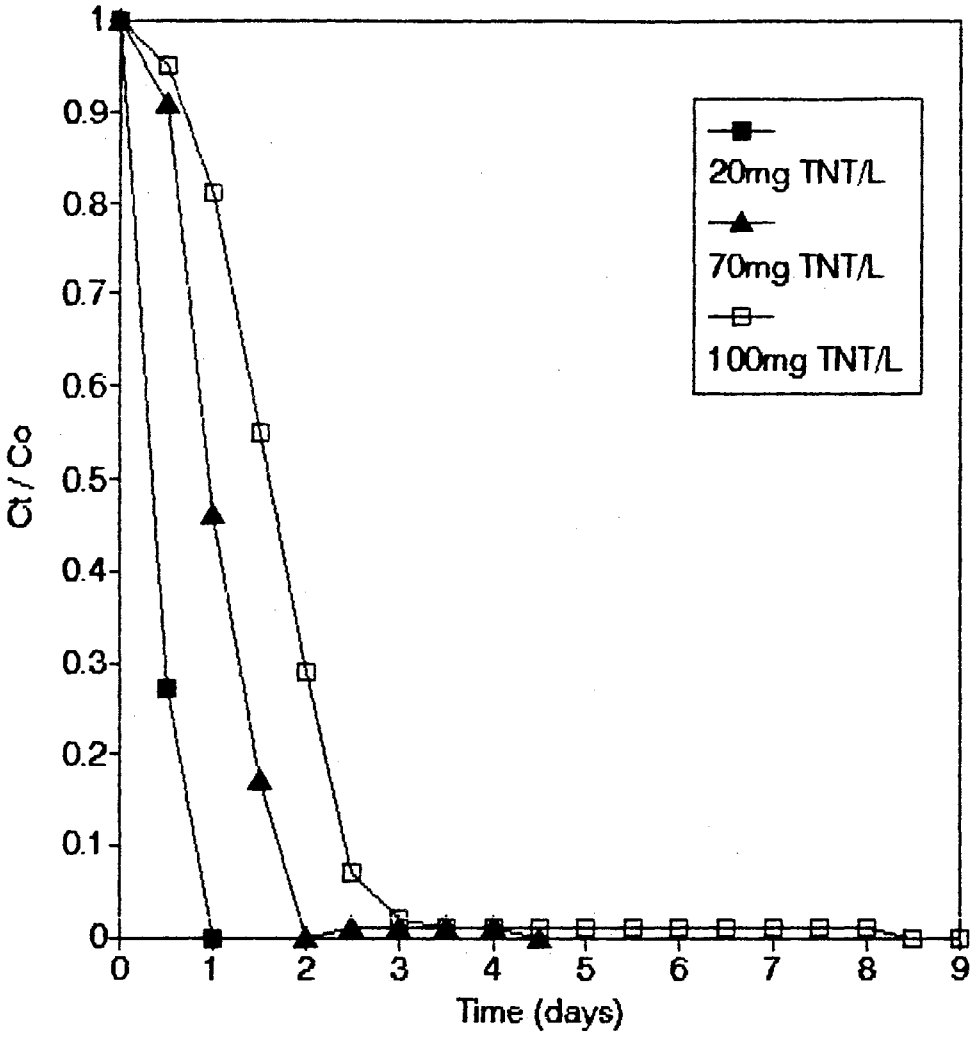


Figure 4. Plot of initial TNT biotransformation rates with different initial concentrations. Gas phase: H_2

4.1.2 Conditions under nitrogen gas atmosphere

Compared to conditions under hydrogen gas atmosphere, the disappearance of TNT and occurrence of its biotransformation products were similar in conditions tested under nitrogen gas atmosphere. The first product also appeared after the beginning of TNT conversion but immediately was transformed into second product. Figures 5 and 6 show that all three intermediates isolated by HPLC analysis were shown from incubation with 20 and 100 mgTNT/L initial concentrations. As shown by Table VIII for TNT biotransformation, all of the 20 and 100 mgTNT/L initial concentrations were depleted in 1 and 2 days incubations, respectively. A product (INT#5) arose slowly and was then persistent in both cases.

TABLE VIII

TNT BIOTRANSFORMATION UNDER N_2 GAS ATMOSPHERE

Incubation Time(days)	TNT initial concentrations (mg/L)	
	20	100
0	20.0	100.0
0.5	1.2	96.4
1.0	0.0	81.7
1.5		6.3
2.0		0.6
2.5		0.0

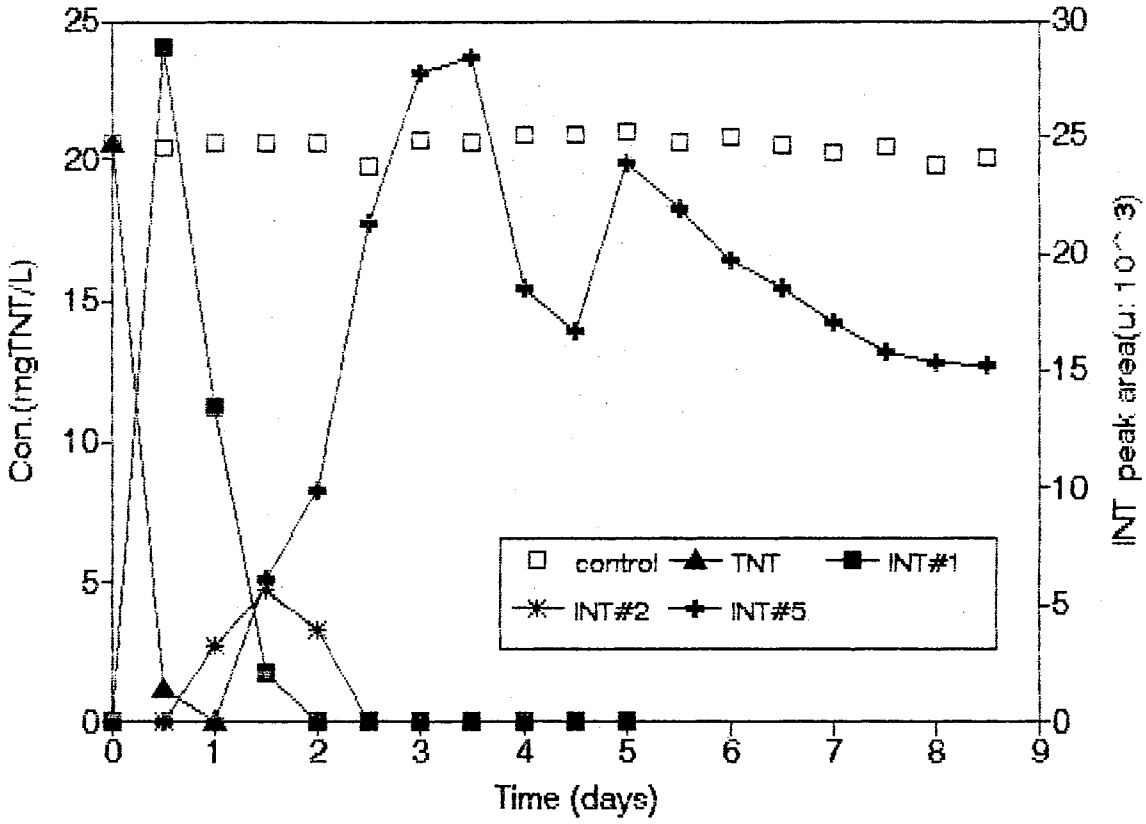


Figure 5. Plot of TNT biotransformation and its transformation products. Gas phase: N_2 Initial TNT concentration: 20 mgTNT/L

[control, TNT: left y-axis; INT: right y-axis]

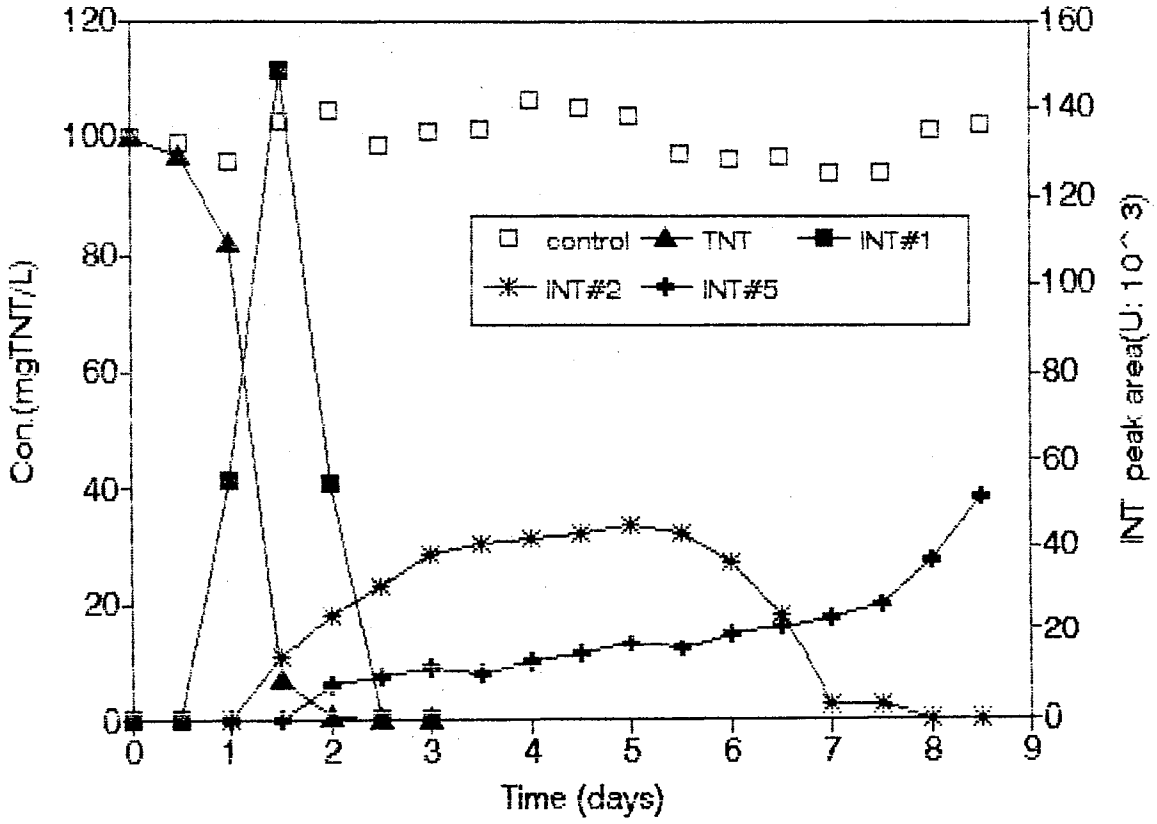


Figure 6. Plot of TNT biotransformation and its transformation products. Gas phase: N_2 Initial TNT concentration: 100 mgTNT/L

4.1.3 Conditions under argon gas atmosphere

TNT biotransformation and occurrence of its metabolic products are shown in Figures 7 and 8. As illustrated in the previous sections, the same transformation product species were also detected under argon gas atmosphere incubation. Within a day or two, all initial TNT concentrations incubated under this condition disappeared as given in Table IX. Also, an intermediate product (INT#5) accumulated in both tests during the incubation.

TABLE IX

TNT BIOTRANSFORMATION UNDER Argon GAS ATMOSPHERE

Incubation Time(days)	TNT initial concentrations (mg/L)	
	20	100
0	20.0	100.0
0.5	4.5	96.2
1.0	0.0	91.0
1.5		11.1
2.0		0.5
2.5		trace

TNT conversion rates based on the ratio remaining to initial concentrations are calculated and plotted in Figure 9. Two conditions, under nitrogen and argon gas atmospheres, are depicted. Also, two ranges of concentrations were tested for each condition. Higher initial concentrations resulted in slightly longer times for formation of reaction intermediates, but the differences do not appear to be significant.

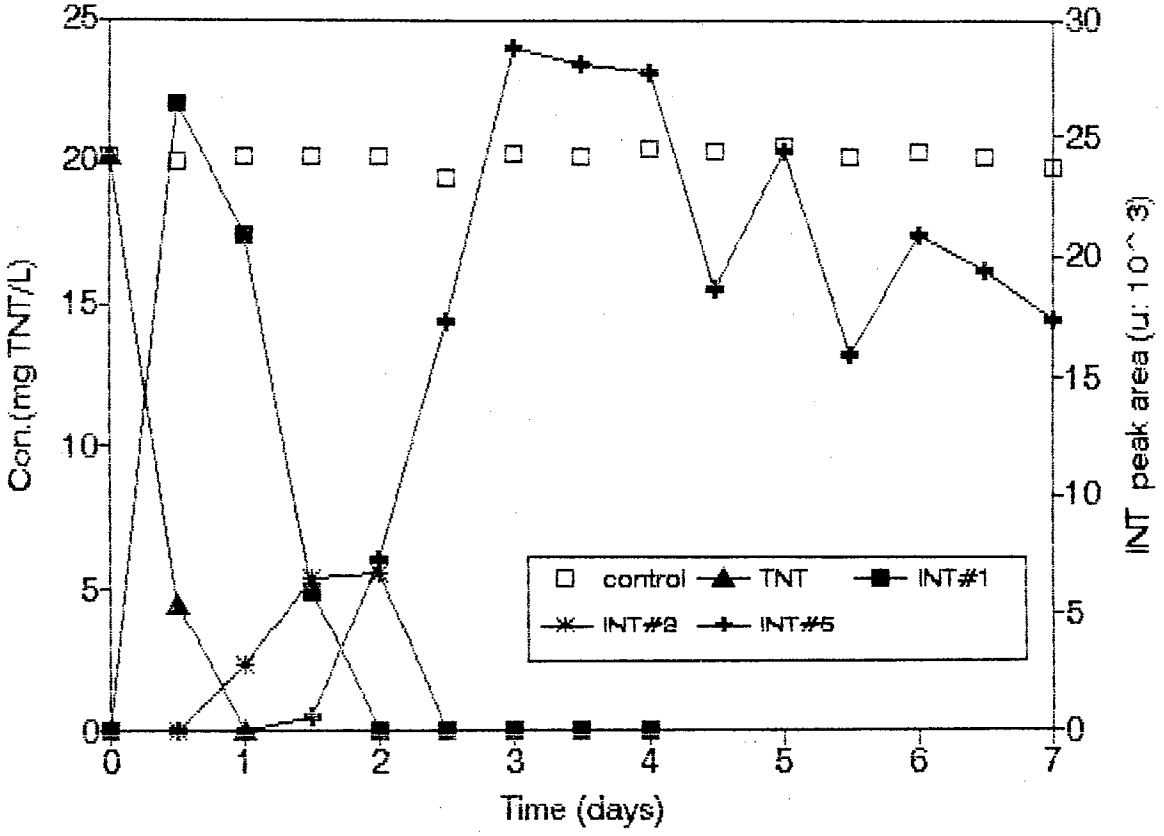


Figure 7. Plot of TNT biotransformation and its transformation products. Gas phase: Ar Initial TNT concentration: 20 mgTNT/L

[control, TNT: left y-axis; INT: right y-axis]

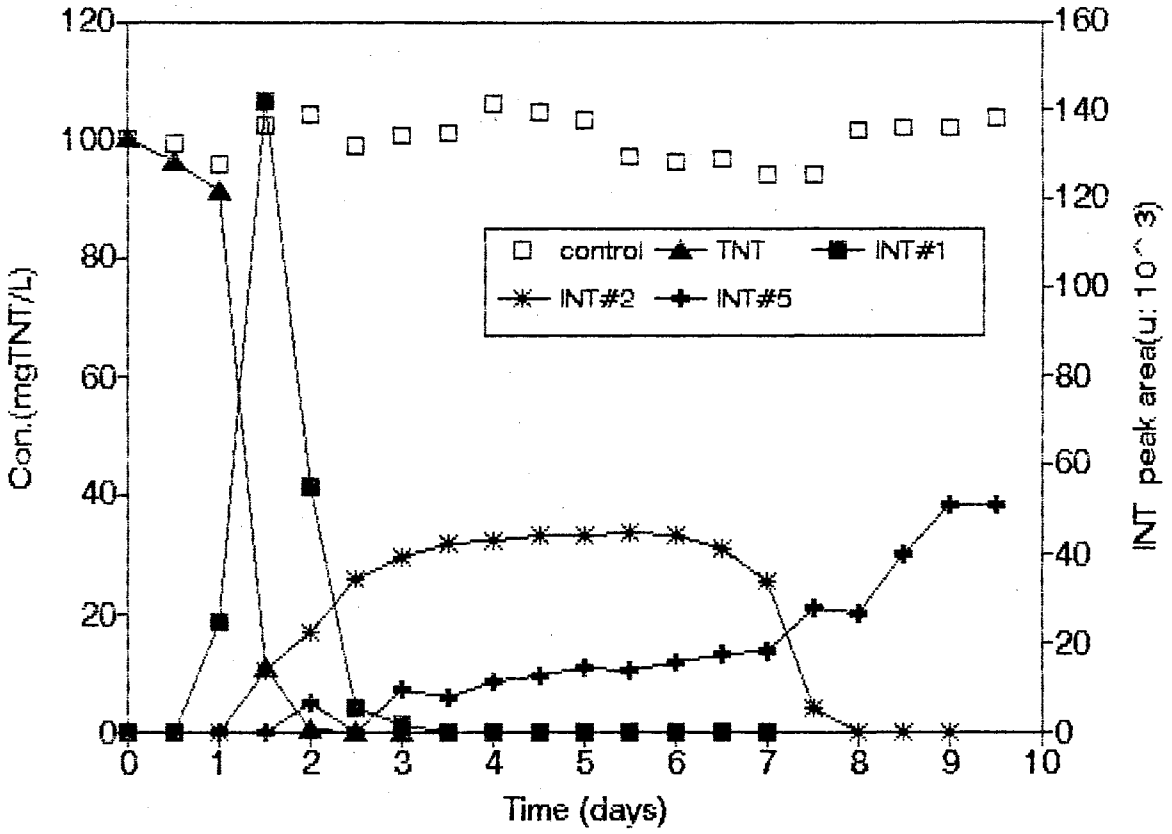


Figure 8. Plot of TNT biotransformation and its transformation products. Gas phase: Ar Initial TNT concentration: 100 mgTNT/L

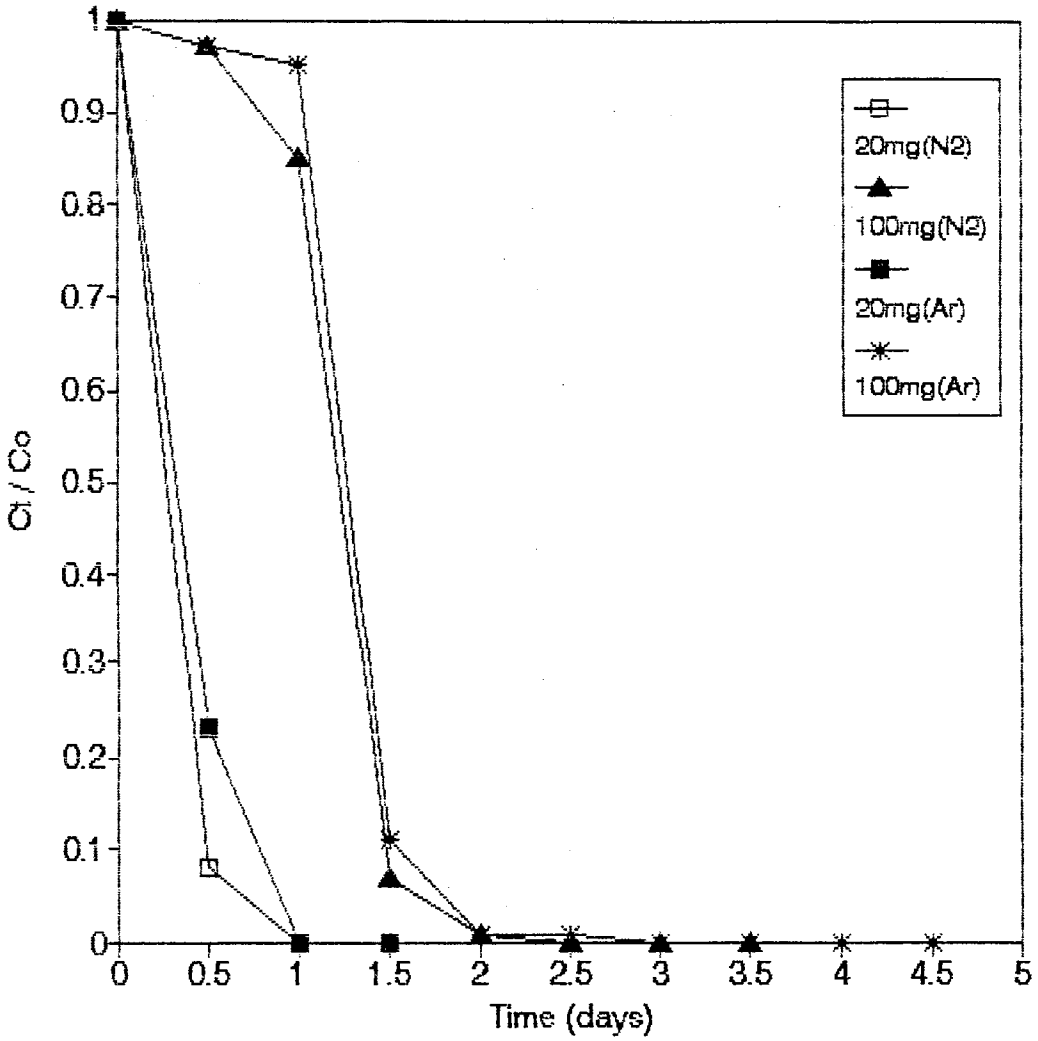


Figure 9. Plot of TNT biotransformation rates. Gas phase: N_2 and Ar, Initial TNT concentration: 20 and 100 mg/L

In Figure 10, TNT conversion rates for all three conditions are plotted as a function of time. All relative reaction rates did not appear to differ much in both initial concentration ranges. However, to quantify differences in transformation rates of TNT into intermediate metabolites for all three conditions, the relative rates were linearized and plotted for linear regression analysis in Figure 11. Thus, the coefficients were calculated and given in Table X.

TABLE X

TNT BIOTRANSFORMATION RATE COEFFICIENTS FOR 100mg/L INITIAL CONCENTRATION

Atmosphere Incubation Conditions	Transformation Coefficients (day^{-1})
Hydrogen gas	1.441
Nitrogen gas	2.319
Argon gas	2.722

From these data, the rate under argon gas was the highest, 2.722 day^{-1}, among the three test conditions. On the other hand, the rate under hydrogen gas atmosphere was lowest, contrary to the expectation of this study based on results reported in the literature.

McCormick et al.(1976) reported that with enzyme preparations from Veillonella alkalescens, TNT was reduced by hydrogen. A variety of other nitroaromatic compounds also showed nitro group reductions under similar conditions. The

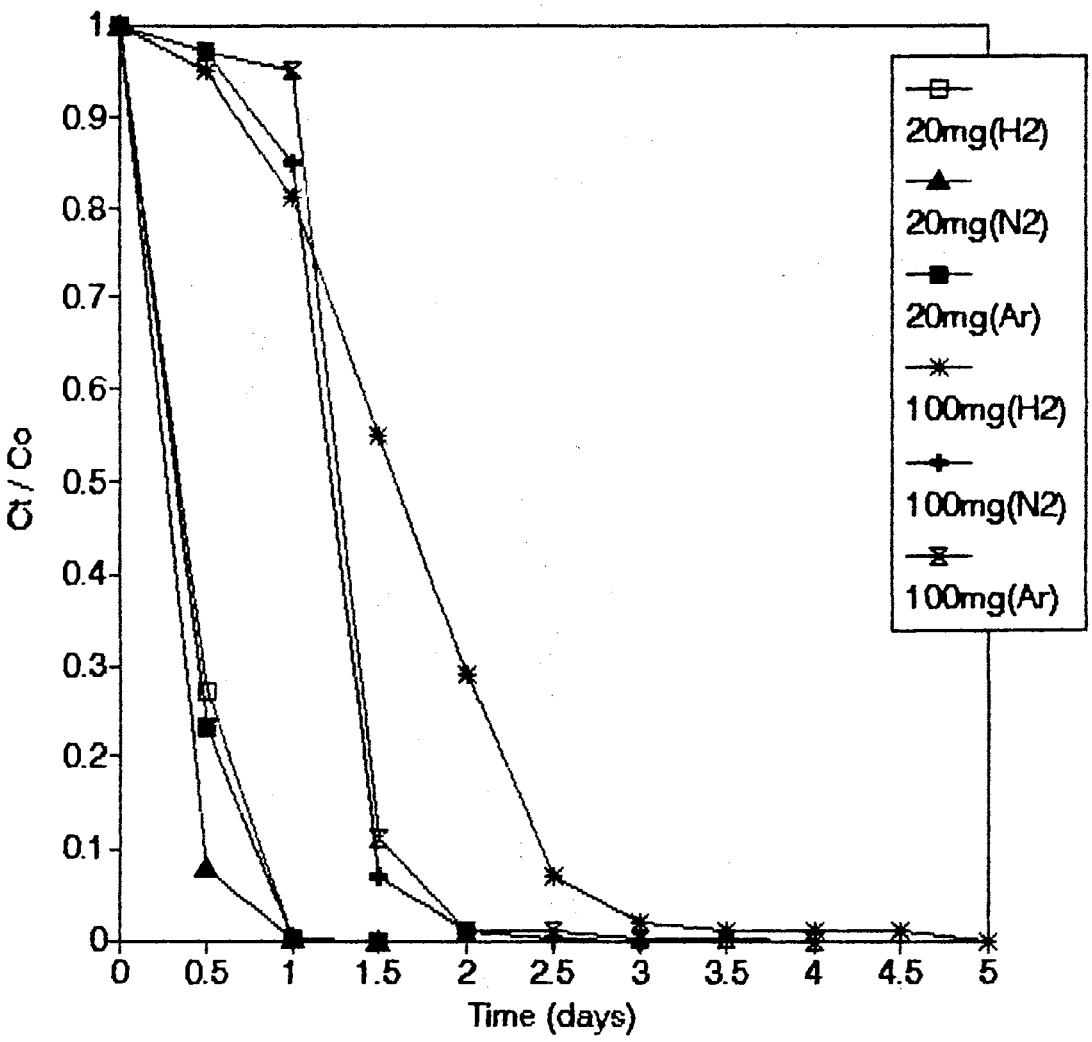


Figure 10. Comparison of TNT biotransformation rates under three different gas atmosphere. Gas phase: H_2, N_2, and Ar

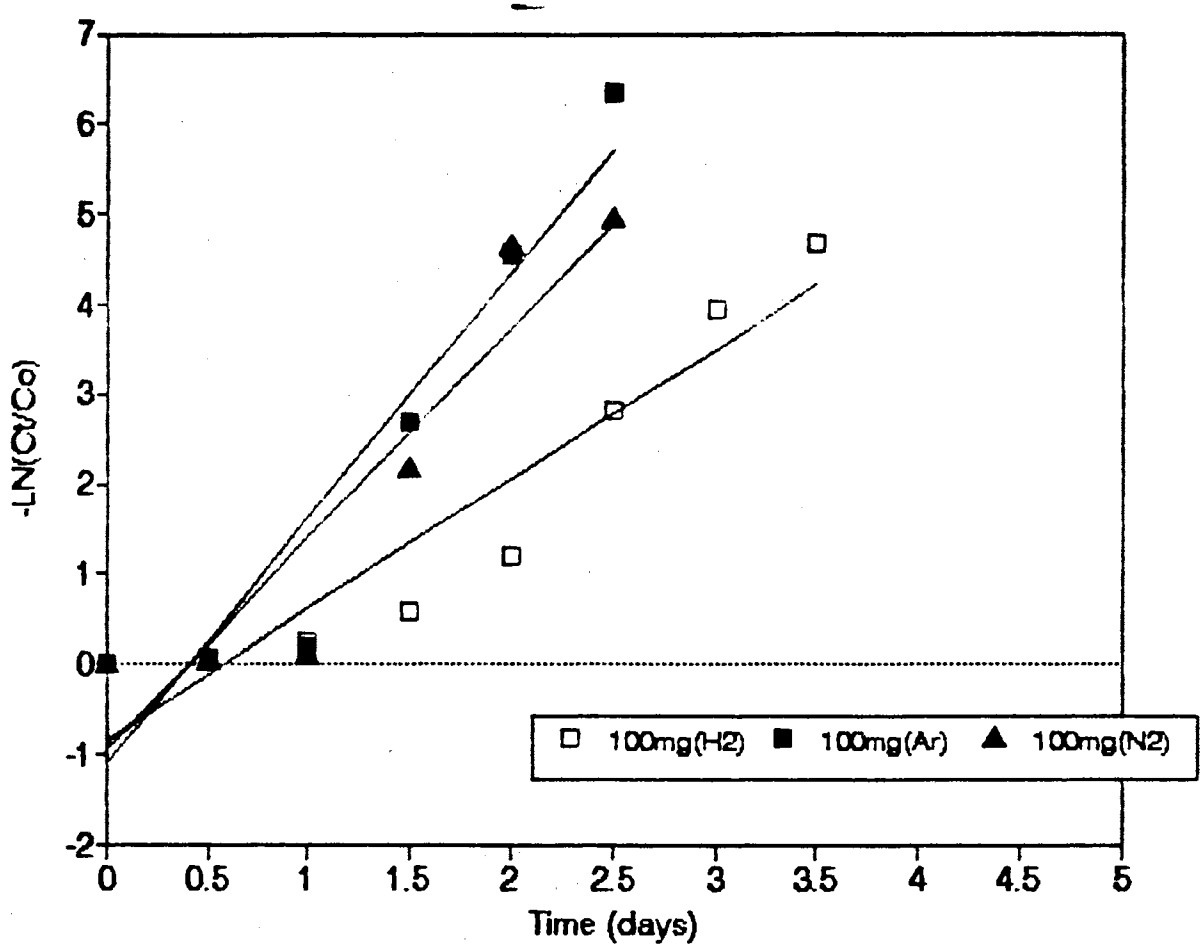


Figure 11. Linear regression plot for TNT biotransformation rate. Gas phase: H_2, N_2, and Ar. Initial TNT concentration: 100 mgTNT/L

test results indicated that nitro-reductase of *V.alkalescens* consisted of hydrogenase and a ferredoxin-like material.

Thus, with the presence of hydrogen gas as an electron donor, TNT biotransformation rate was expected to be enhanced under a hydrogen gas atmosphere. However, Nies and Vogel (1991) showed that the hydrogen transferred to the aromatic ring by incorporation in reductive dechlorination of polychlorinated biphenyls (PCBs) was the proton (H^+) from water. Also, electrons were presumed to be donated by the reduced organic substrate (acetone). Similar observations were also reported by Mohn and Tiedje (1990). Their results indicated that reduced ferrous iron and protons from water could be the source of electrons and hydrogen, respectively, for reductive dechlorination of 3-chlorobenzoate.

Thus, in this study hydrogen engaged for the amination of nitro groups on TNT aromatic ring could come from water. The fact that similar results were observed under nitrogen and argon gas atmosphere also supports this suggestion.

Also, nitrogen gas is evolved in denitrifying conditions and could constitute an interference in later stages of experimentation. Accordingly, argon gas atmosphere was selected to impose anaerobic and anoxic gas phase conditions for the rest of the study.

Concentrations of TNT over 50 mg/L were reported to be inhibitory to some bacteria, yeasts, and fungal (Klausmeier et al., 1974). Therefore, the effects of high

concentrations of TNT on its biotransformation was also examined. However, no inhibition or rate decrease from the higher TNT concentration was recognized because, as Figure 10 shows, reaction rate was relatively independent of concentration. Also, since 100 mgTNT/L represents the approximate solubility limit under ambient conditions, this is the soluble TNT concentration often encountered in natural environments or TNT wastewater streams. Therefore, this initial concentrtion range was selected for subsequent incubation conditions for this study.

4.2 Anaerobic TNT Biotransformation Tests

For these tests initial 100 mgTNT/L concentration was prepared in 250 mL Erlenmeyer flasks containing enriched medium. Peptone or glucose was used as primary substrate for mixed anaerobic cultures. Argon gas was used in the gas phase to maintain anaerobic conditions.

4.2.1 Peptone as a primary substrate

Peptone was used as a relatively complex primary carbon source for cometabolic reaction by mixed anaerobic culture. The test results from a single reactor have been plotted in two graphs because of too many dependent variables to be shown clearly in one graph.

The initial concentration of 100 mgTNT/L decreased by 99.3 ± 1.0 % within 5 days incubation periods as shown in Figures 12A and 12B. No lag period was observed during the initial stage of TNT biotransformation. Therefore, this demonstrates that the subculture, inoculated for this test, has already been adapted to TNT enriched medium. From the profile plotted in Figures 12A and 12B (separated to show individual products more clearly), five intermediate products appear during stepwise transformation. Several transformation intermediates, labled INT#1 through INT#4, appeared and decayed. Thereafter, a persistent product (INT#5), tentatively identified as 2,4-diamino-6-nitrotoluene by HPLC analysis, was detected.

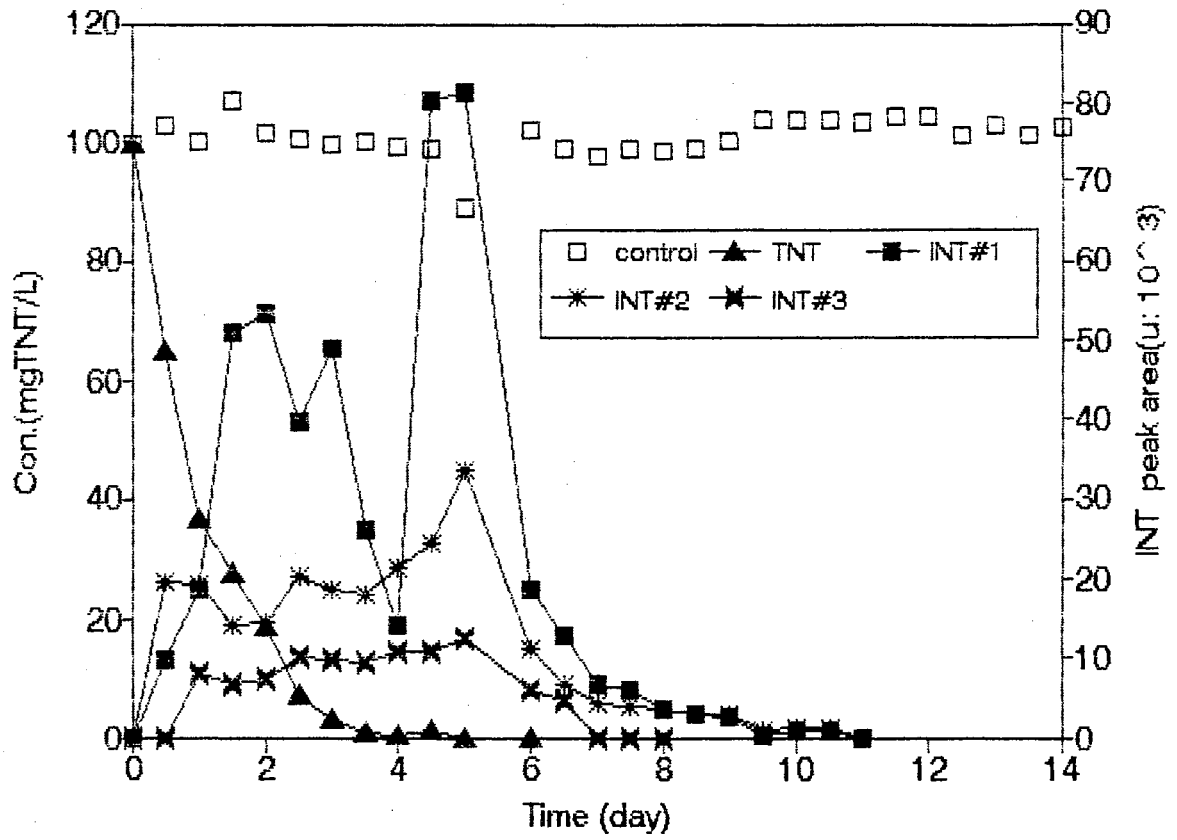


Figure 12A. TNT biotransformation under anaerobic condition. Primary substrate: peptone.

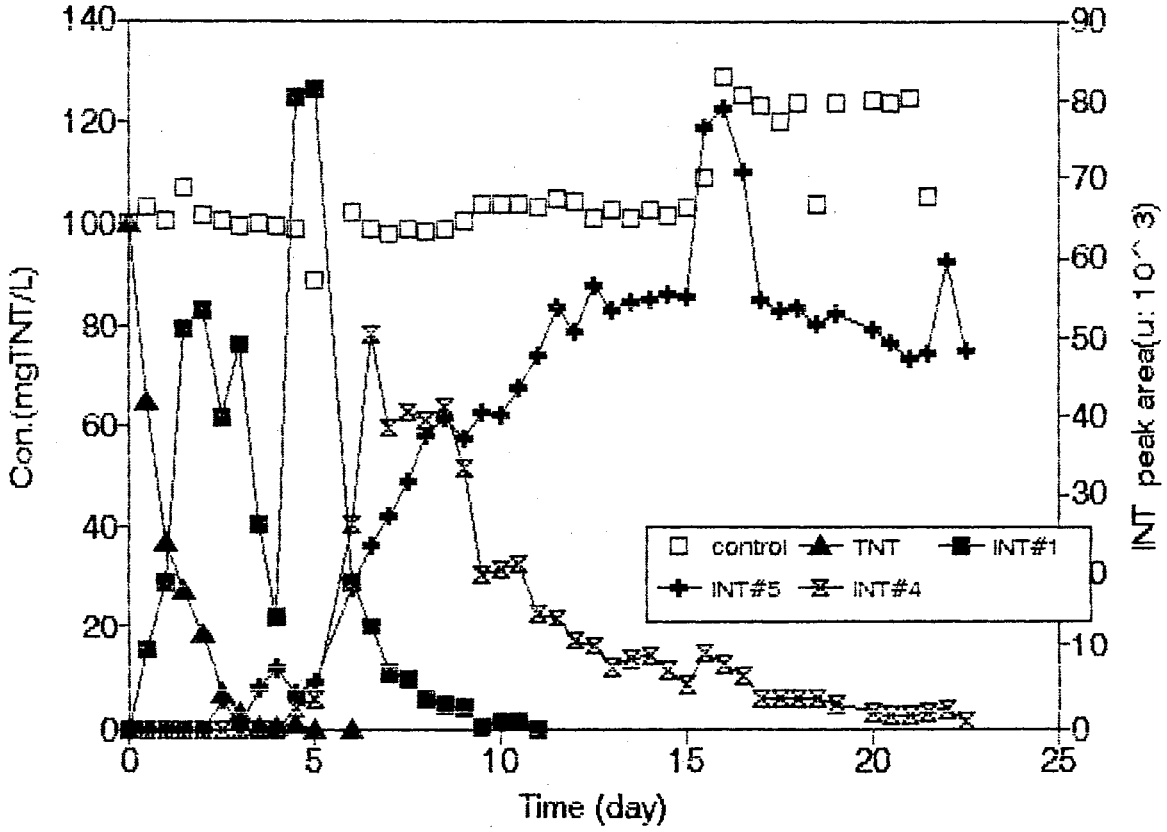


Figure 12B. Continued TNT biotransformation under anaerobic condition.
Primary substrate: peptone

4.2.2 Glucose as a primary substrate

To investigate the effects of a different, simpler primary substrate on cometabolic TNT biotransformation, glucose was also added to TNT enriched medium. The results are depicted in Figures 13 and 14.

During the first incubation as shown in Figure 13, the buffer capacity of the medium was not sufficient to maintain the pH of the system. The neutral initial pH of the medium was lowered to around pH 4 during 3 to 4 days incubation period. Therefore, the activity of the system was apparently suppressed, and all transient intermediates were maintained at steady concentrations. However, TNT was converted faster in this test than with peptone as a primary substrate. Also, several intermediates (INT#2 & 3 and INT#4 & 5) were not separated completely by HPLC analyses, so they were plotted together in Figures 13 and 14.

To control pH, the system was adjusted with 40 mM K_2HPO_4-KH_2PO_4 buffer solution. The profile of intermediate transformation products from the adjusted system is illustrated in Figure 14. From both tests (Figures 13 and 14) the same number of products were detected, but their appearance and decay patterns were quite different. In Figure 13, all intermediates steadily maintained concentration upon formation. However, in Figure 14, INT#2 and 3 disappeared, and at least one product accumulated (INT#5, or a combination of INT#4 and 5).

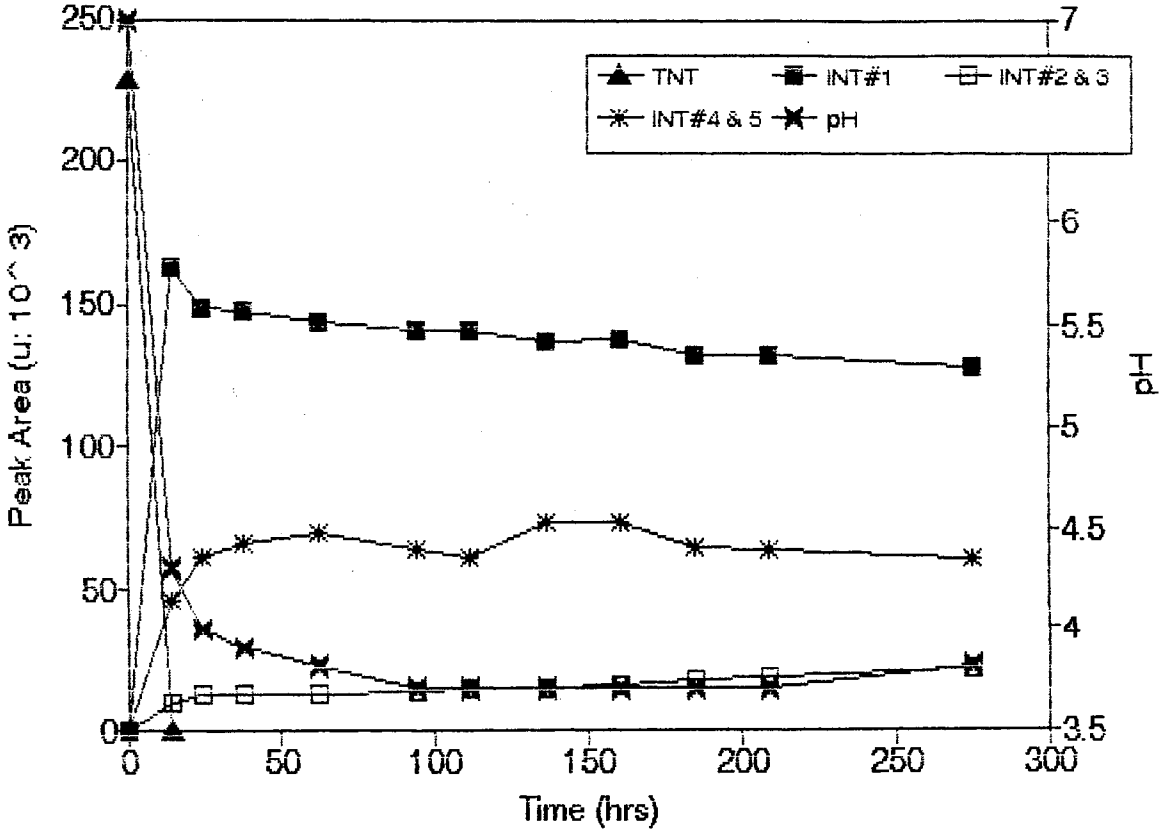


Figure 13. TNT biotransformation under anaerobic condition(A); pH depression contributes to stability of intermediates.
Primary substrate: glucose

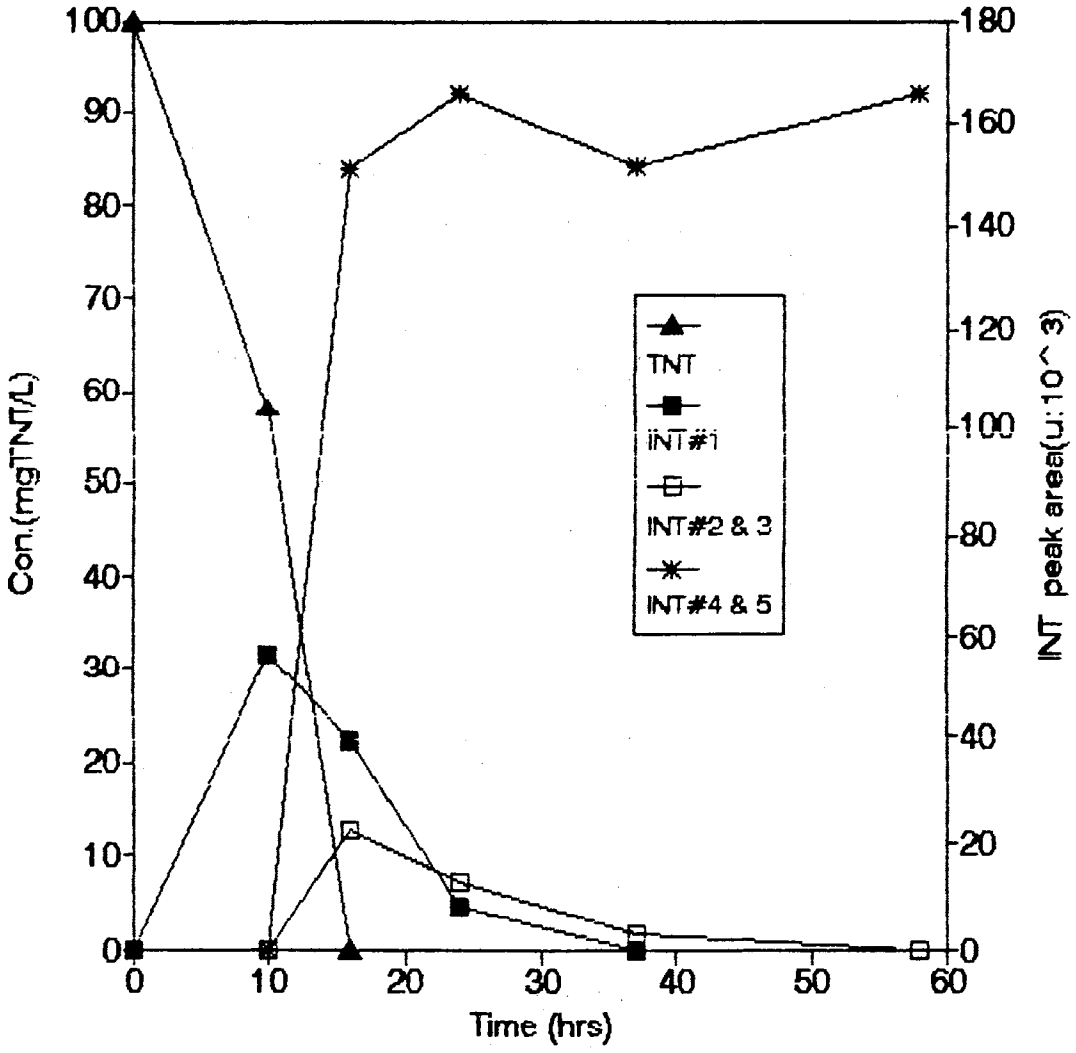


Figure 14. TNT biotransformation under anaerobic condition(B) with pH control.
Primary substrate: glucose

[TNT: left y-axis; INT: right y-axis]

In summary, profiles of TNT biotransformation with peptone and glucose as primary substrates by anaerobic mixed cultures were illustrated in Figures 12A, 12B, 13, and 14. From these tests, 5 intermediate transformation products were detected. The reduction of nitro groups to amino groups on the TNT aromatic ring is dependent on the reducing potential of the test systems (McCormick et al., 1976).

In the literature reported thus far, biotransformation of TNT produces a number of reductive intermediate products (Channon et al.,1944; Won et al.,1974; McCormick et al.,1976 and 1978; Carpenter et al.,1978; Fernando et al.,1990; Sublette et al.,1992). Biological reduction of nitroaromatic compounds under anaerobic and aerobic conditions proceeds through the nitroso and hydroxylamino compounds (Liu et al.,1984). However, the nitroso compounds are not detected in culture media because the nitroso compounds are transformed quickly in anaerobic conditions (Channon et al.,1944; McCormick et al.,1976 and 1978).

Thus, in this test the intermediate reduction products (INT#1, INT#2 and 3, and INT#4 and 5) are tentatively identified as hydroxylamino-, monoamino-, and diamino-toluene compounds through the HPLC analysis described by Kaplan and Kaplan (1982).

Primary substrates support growth of microbial cultures in batch reactors. Peptone and glucose as primary substrates had a very different effect on system pH and transformation kinetics. TNT was converted to the final persistent

metabolites through transient intermediate products, but cometabolic TNT biotransformation products remained the same under the both test conditions. In the case of peptone as a primary substrate (Figures 12A and 12B), intermediate #5 (diamino-toluene compound) appears last and persists at a constant concentrtion during 20 days incubation period. TNT biotransformations with different primary substrates are listed in Table XI.

TABLE XI

TNT BIOTRANSFORMATIONS WITH DIFFERENT PRIMARY SUBSTRATES

Incubation Time(days)	TNT conversion with different primary substrates (mg/L)		
	Peptone	Glucose(I)	Glucose(II)
0	100.0	100.0	100.0
0.4	---	---	57.7
0.5	64.8	trace	---
0.7	---		trace
1.0	37.0		
1.5	27.4		
2.0	18.7		
2.5	7.3		
3.0	3.3		
3.5	0.8		
4.0	trace		

Table XI illustrates that TNT biotransformation was much faster with glucose as a primary substrate than that with peptone. However, as shown in Figure 13, the pH of the system dropped below 4 in the initial phase of the reaction. Therefore, the pH of the system with glucose as a primary

substrate requires much greater buffer capacity because of the mixture of acids which was produced from glucose fermentation under anaerobic conditions. Thus, the entire reaction became stationary, and all intermediate products maintained their concentration level during 11 days incubation period.

Glucose at concentrations of 100 and 500 mg/L was observed to enhance the mineralization of 10 mg 2,4-dinitrophenol(DNP)/L (Hess et al., 1990). However, 1000 mg glucose/L showed inhibition to DNP degradation (Hess et al., 1993). Anaerobic fermentation of glucose results in the production of a mixture of acid metabolites (Moat and Foster, 1988). Acid production from glucose has been shown to reduce the number of viable cells of Pseudomonas-like organisms (Won et al., 1974).

Thus, inhibition by acid byproducts of glucose could result in stationary reaction conditions and no further biotransformation of intermediate products in this test. To investigate these findings, the system was adjusted with 40 mM phosphate buffer solution, then tested again. As presented in Figure 14, the system proceeded through a series of intermediates, but reduced diaminotoluene products (INT#4 and 5) accumulated at steady concentration levels.

Accordingly, further treatment for complete degradation of persistent intermediates is necessary due to incomplete degradation of TNT by anaerobic mixed cultures alone.

4.3 Aerobic TNT Biotransformation Tests

The initial 100 mgTNT/L was incubated under aerobic conditions with peptone and glucose as primary substrates. Activated sludge was transferred into reactors for acclimation and maintained in the water bath at constant temperature of 35 - 37 °C. The tests were conducted at the same time for both primary substrates. After the first set of tests was performed for both primary substrates, the second set of tests was conducted with aerobic mixed subcultures inoculated from the previous cultures.

4.3.1 Peptone as a primary substrate

The results of this test were plotted in Figures 15 and 16. In the initial set of tests as shown in Figure 15, a short lag phase was noted during the first 2 days incubation. Four transient intermediate products appeared and persisted at consistent levels of concentration after day 7.

In Figure 16, no lag phase in the initial reaction of TNT biotransformation was recognized. This illustrates that the subculture of activated sludge was already acclimated to TNT. Also, the TNT conversion rate was increased (note time scale), so most of the TNT disappeared during a one day incubation period. One additional intermediate product was detected, and only one product (INT#6) appeared to be persistent to further transformation within about 6 days duration of this test.

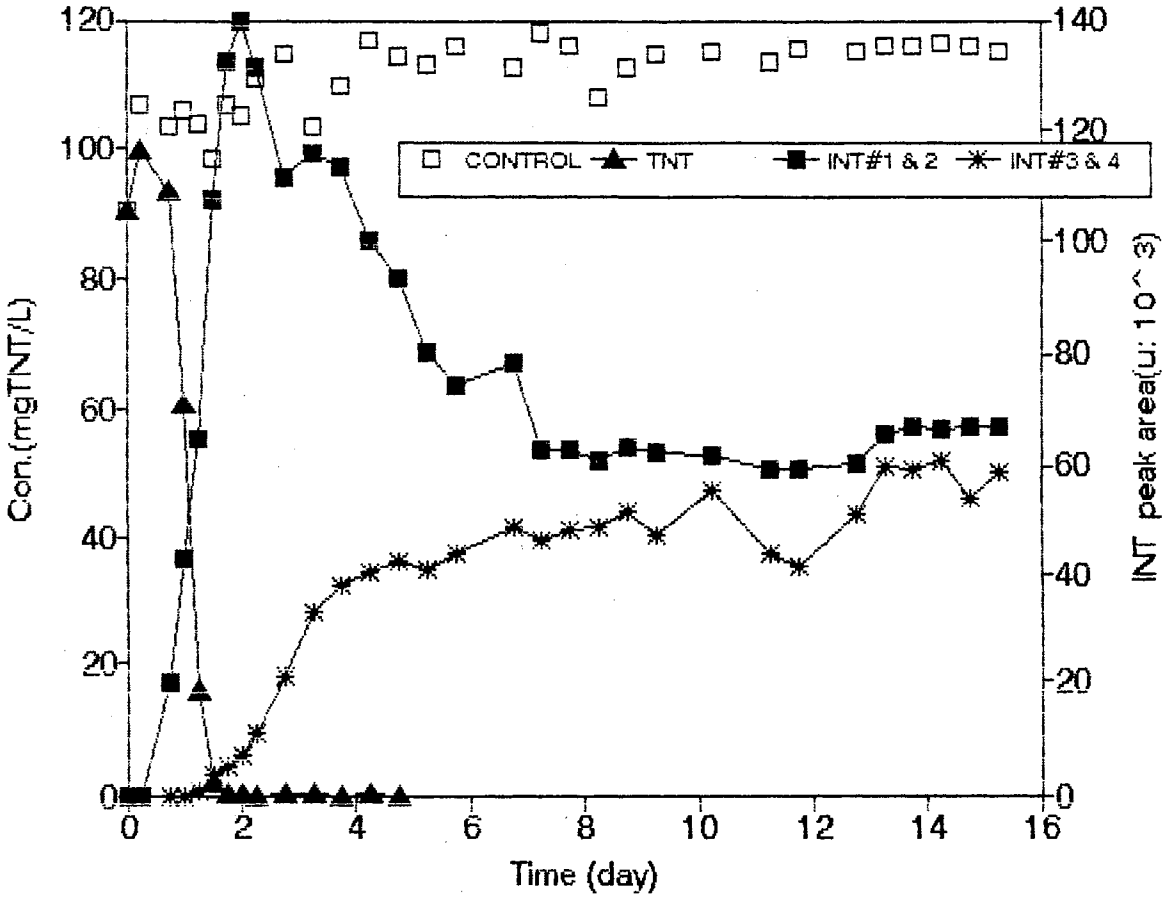


Figure 15. TNT biotransformation under aerobic condition(A). Initial batch test results. Primary substrate: peptone

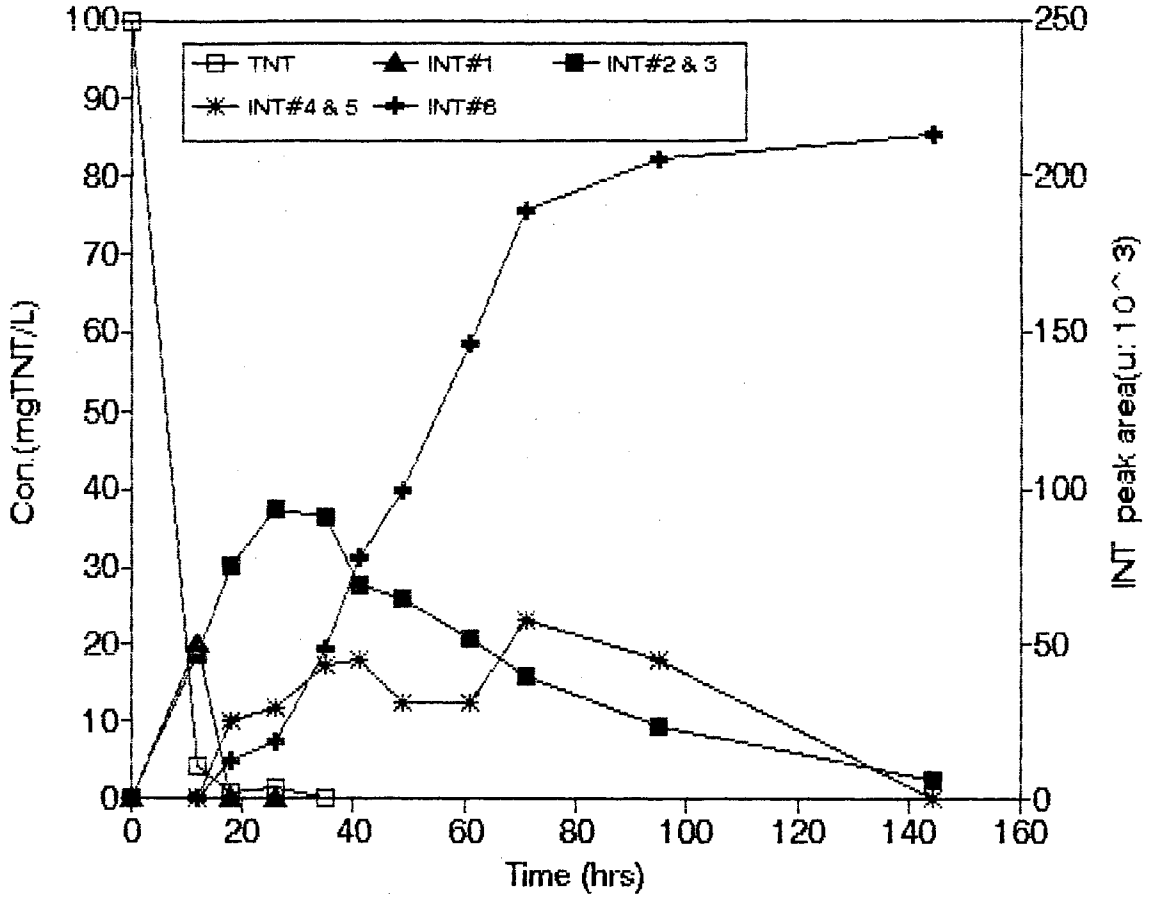


Figure 16. TNT biotransformation under aerobic condition(B). Second batch study with acclimated inoculum. Primary substrate: peptone

4.3.2 Glucose as a primary substrate

The results of this test were similar to the results with peptone as primary substrate in aerobic conditions. However, the profile for intermediates plotted in Figure 17 shows a different pattern and number of transformation products. A short lag phase was noted in the first set of tests with glucose, as explained in the previous section describing the peptone tests. Also, the TNT conversion rate by aerobic subculture was much faster than that by aerobic mixed culture as shown by comparison of Figures 17 and 18. The compared TNT transformation rates are listed in Table XII. Figure 17 shows that one group of intermediates (INT#2 & 3) accumulated and was persistent to further transformation or decay. The first intermediate (INT#1) appeared and was transformed into subsequent products as in the anaerobic reactions.

In Figure 18, the plot for TNT biotransformation with acclimated inoculum shows that all initial concentration of TNT was converted into the first product during one day incubation period. Also, no lag phase was observed for TNT conversion. More intermediate products appeared throughout the incubation period. This illustrates that the subculture was already acclimated to TNT and produced more intermediates. In this test, INT#6 persisted at high concentration and INT#4 and/or #5 at much lower concentrations after about 70 hours incubation.

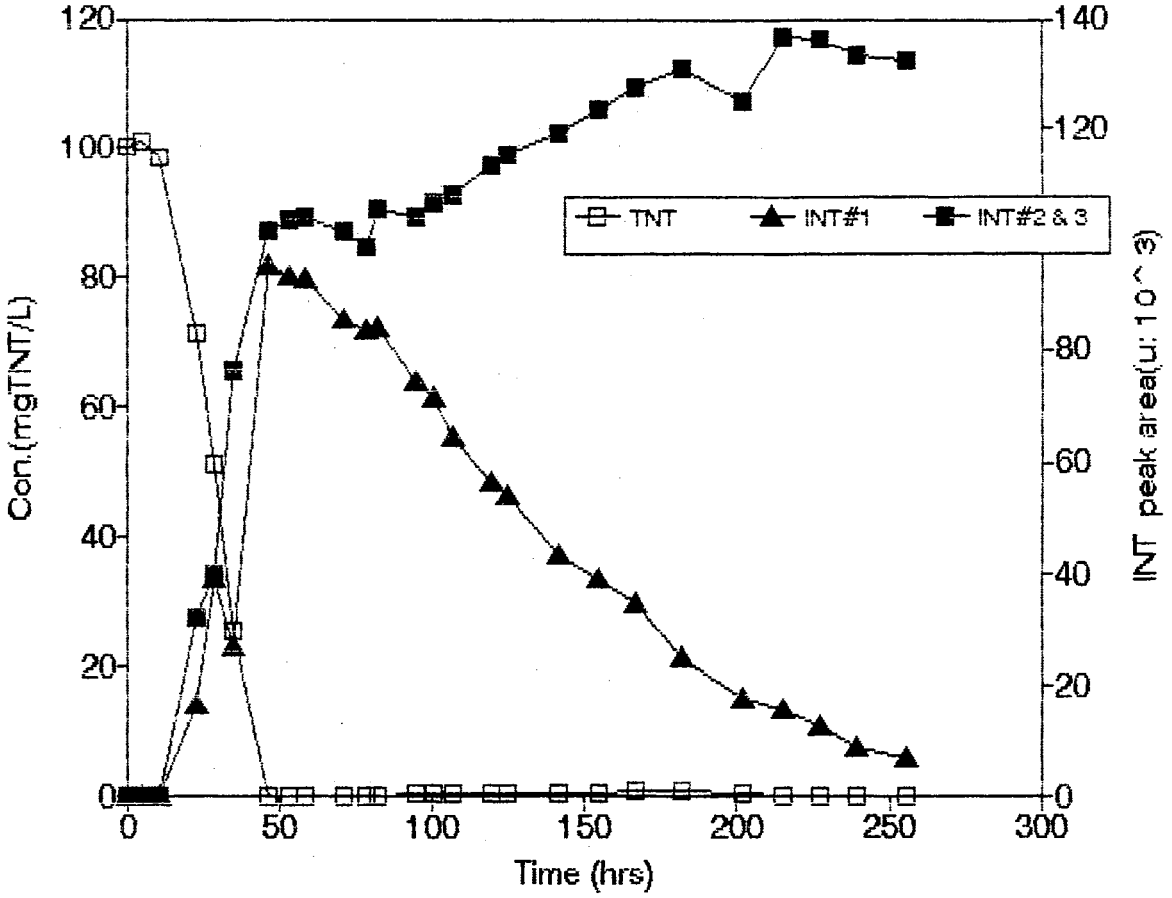


Figure 17. TNT biotransformation under aerobic condition(A). Initial batch test results. Primary substrate: glucose

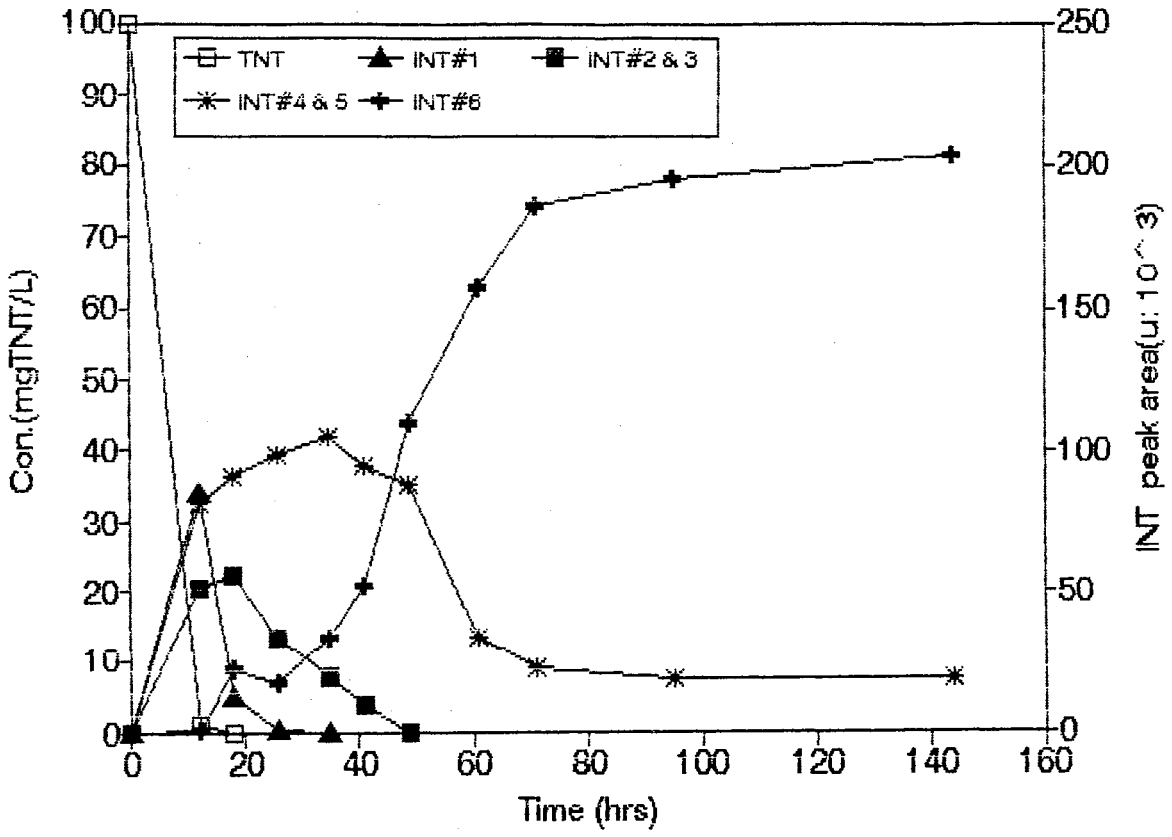


Figure 18. TNT biotransformation under aerobic condition(B).
Second batch study with acclimated inoculum.
Primary substrate: glucose

The rate of TNT biotransformation was dependent on the culture conditions of acclimation. The comparison of TNT conversion in each condition is listed in Table XII.

TABLE XII

TNT BIOTRANSFORMATION WITH DIFFERENT PRIMARY SUBSTRATES AND INOCULUM CONDITIONS

Incubation	TNT conversion with different primary substrates (mg/L)			
	Activated sludge		Subculture	
Time(hrs)	Peptone	Glucose	Peptone	Glucose
0	99.2	99.8	99.8	99.8
5	---	101.2	---	---
6	99.8	---	---	---
10.5	---	98.4	---	---
12	---	---	4.3	1.0
18	93.2	---	1.0	trace
22.5	---	71.1	trace	
24	61.2	---		
28.5	---	51.5		
30	15.7	---		
34.5	---	25.4		
36	2.4	---		
42	0.5	---		
46.5	trace	trace		

In summary, profiles of TNT biotransformation with peptone and glucose as primary substrates under aerobic conditions show that intermediate transformation products are formed sequentially. From 3 to 6 intermediate products appeared, but some are always persistent to further aerobic degradation or transformation in each case.

The nitro groups on the TNT molecule can be released as nitrite or reduced by aerobic systems, but the former case

is not the major pathway by pseudomonads which are predominant in soil and water environments (McCormick et al., 1976).

However, the appearance of various intermediates is not consistent among tests, unlike the anaerobic systems. This could suggest that, under aerobic conditions, the hydroxylamino compound may be nonenzymatically oxidized to the azoxy compound (Channon et al., 1944). Also, in the presence of oxygen, transformation products of nitroaromatic or aminoaromatic compounds tend to polymerize to recalcitrant macromolecules (Braun and Gibson, 1984; Carpenter et al., 1978).

Thus, a distinctive intermediate product not noted under anaerobic reaction conditions (INT#6) occurs and maintains its concentration as shown in Figures 16 and 18. Therefore, the formation of polyamides could be the reason for unexpected appearance of resistant transformation products (Carpenter et al., 1978).

The reaction with the subculture from the first test as inoculum resulted in the production of a greater number of intermediates and faster TNT conversion rates. However, in these aerobic systems, various persistent transformation intermediates also accumulate as in the anaerobic systems. Accordingly, further treatment is necessary to achieve complete degradation of TNT and its derivatives.

4.4 Tests for The Degradation of TNT Biotransformation Products under Combined Denitrifying Conditions

In this scheme of tests, all media previously incubated under anaerobic and aerobic conditions were transferred into denitrifying conditions to investigate further degradation of the accumulated persistent products. The purpose of this phase of experiments was to test the effects on TNT biotransformation under combined anaerobic(or aerobic)/ anoxic conditions.

4.4.1 Tests with the products from anaerobic conditions

The medium containing TNT biotransformation products was incubated again under denitrifying conditions to induce further degradation. Figure 19 illustrates the disappearance of persistent intermediate (INT#5) which was produced from anaerobic TNT biotransformation with peptone as a primary substrate. The accumulated intermediate product (INT#5) immediately started to disappear after the introduction of denitrifying conditions to the system. On the other hand, under continuous anaerobic incubation, the product was maintained at a constant level of concentration.

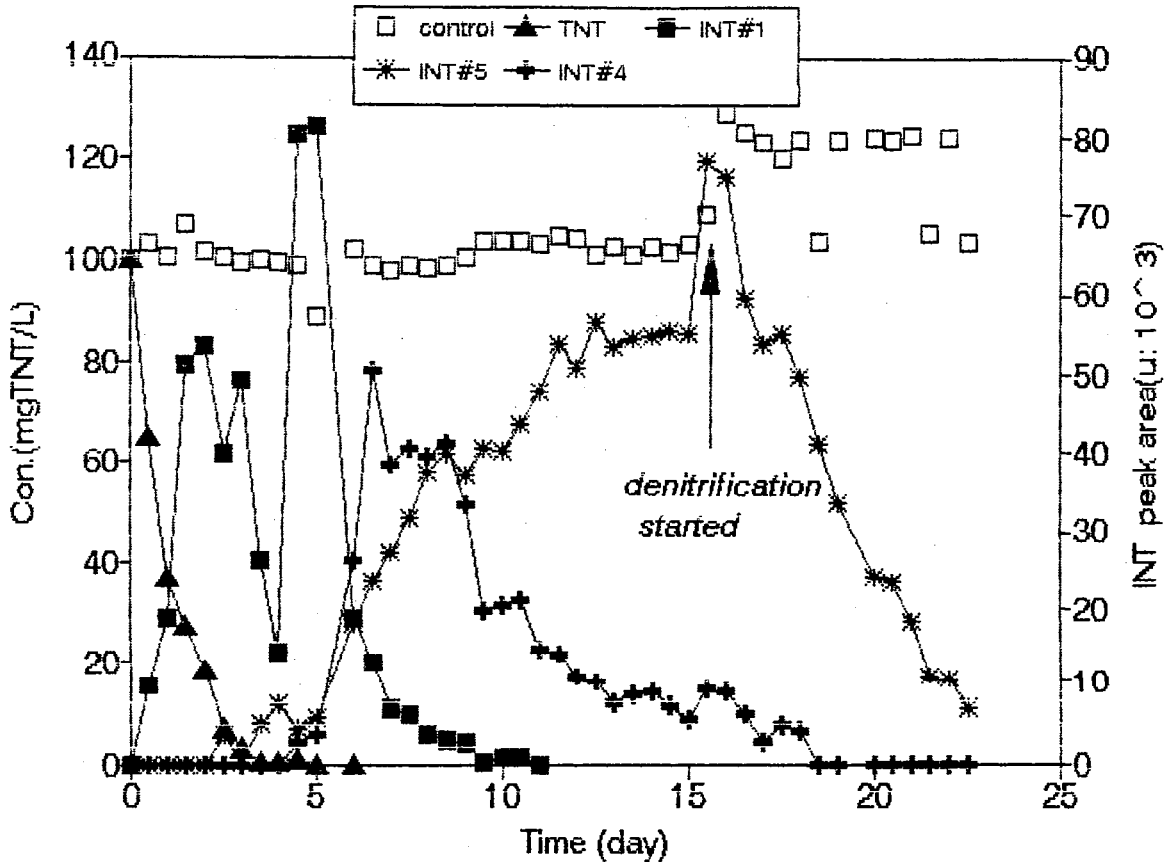


Figure 19. Further degradation of accumulated transient transformation products under consecutive anaerobic/anoxic conditions(A). Primary substrate: peptone

Both reactions are plotted together in Figure 20 to show the difference between these two test conditions. One other intermediate (INT#4) showing some accumulation was also plotted.

The glucose medium was also incubated under denitrifying conditions after being tested under anaerobic conditions. The reaction profiles are illustrated in Figure 21. Five intermediates appeared during the anaerobic incubation period (about 8 days incubation). However, two intermediates (INT#4 & 5) were persistent to further degradation for about the last 6 days of the incubation period. The pH of the system was decreased but buffered during anaerobic incubation. During the extended period of time after denitrification started, all of the accumulated intermediates showed complete degradation.

In summary, all persistent intermediate products resulting from anaerobic TNT biotransformation with peptone and glucose as primary substrates were degraded under denitrifying conditions during a subsequent, consecutive incubation period. In the literature, under denitrifying conditions, mineralization of aromatic compounds with ring

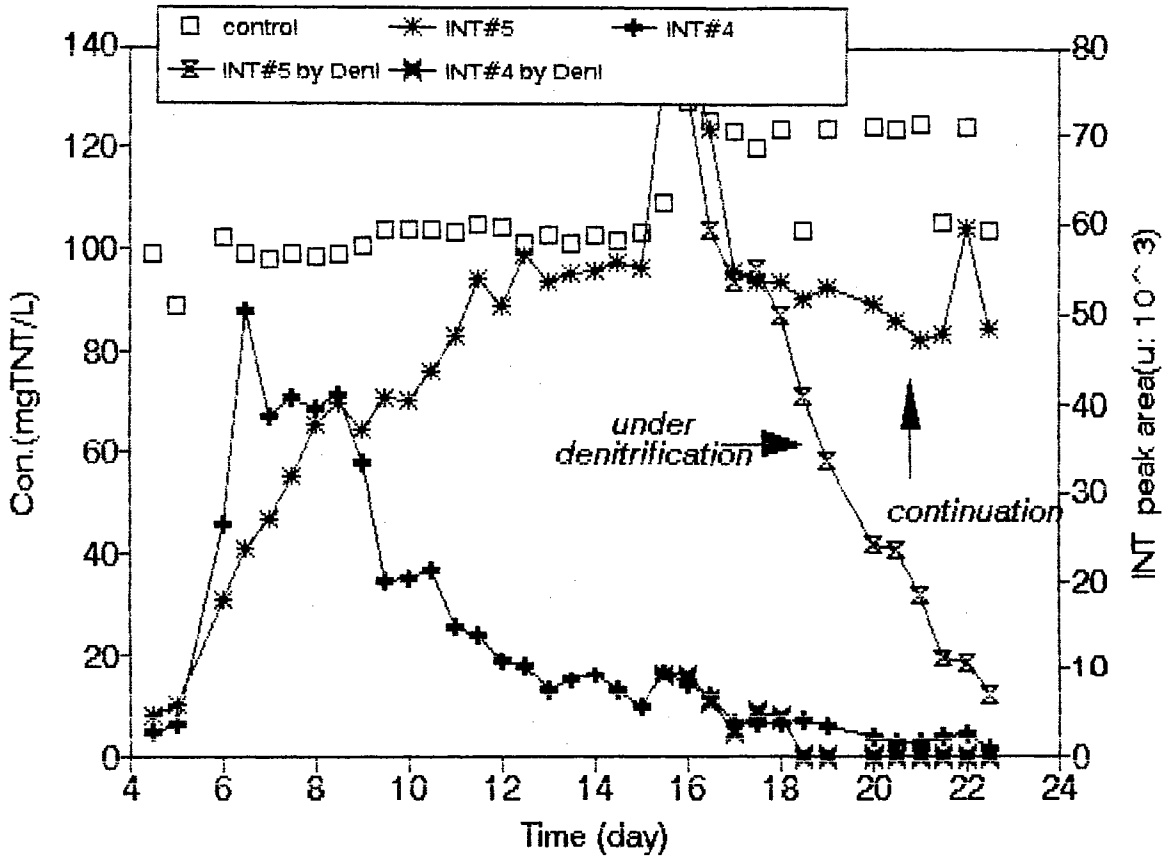


Figure 20. Further degradation of accumulated transient transformation products under consecutive anaerobic/anoxic conditions(B). Comparison with continuous anaerobic culture. Primary substrate: peptone

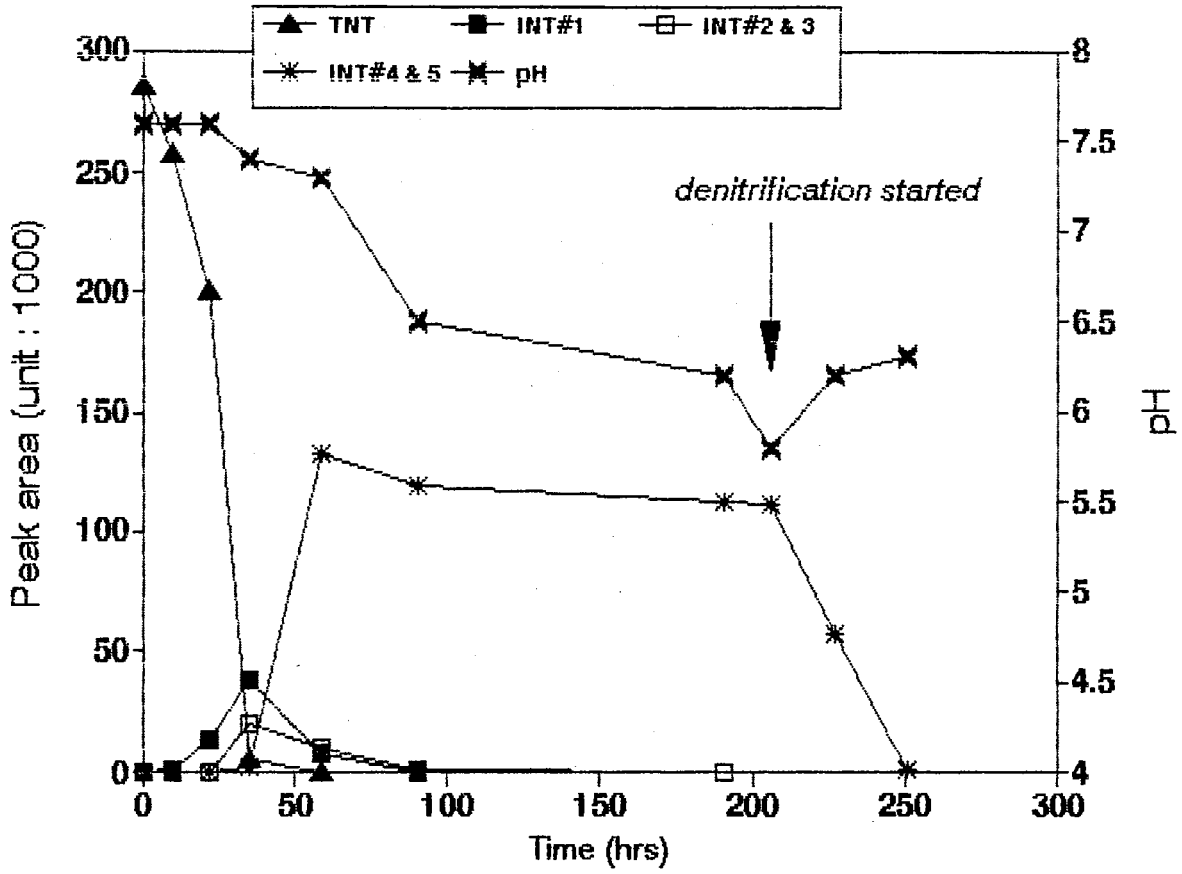


Figure 21. Further degradation of accumulated transient transformation products under consecutive anaerobic/anoxic conditions. Primary substrate: glucose

substituents such as methyl, carboxyl, hydroxyl, and amino groups is reported (Schocher et al., 1991; Schennen et al., 1985; Braun and Gibson, 1984). Therefore, in this test the complete degradation was not dependent on the primary substrates but was dependent on the system conditions.

During the second stage tests of incubation of persistent intermediates from anaerobic systems under denitrifying conditions, no new intermediate products were produced. The complete degradation of 2,4-diamino-6-nitrotoluene, one of the persistent TNT intermediate products, was also reported by Naumova et al.(1988). They reported that complete degradation was observed through NADH-dependent deamination by an extract of induced cells of Pseudomonas sp..

Thus, denitrifying systems were successfully applied to further degradation of accumulated intermediates from anaerobic systems. The complete degradation of TNT was shown to be feasible by this sequence of reaction conditions.

4.4.2 Tests with the products from aerobic conditions

Some portion of the medium containing aerobic TNT biotransformation products was transferred from the test reactors to examine the feasibility of further degradation via denitrifying activity. Also, the effects of the two different primary substrates on denitrification were monitored during an extended, continuous incubation period.

The results are plotted in Figure 22 for the case of peptone as a primary substrate. In this test, denitrification started on the sixth day of aerobic incubation. In Figure 22 the concentration of accumulated intermediate product appears steady after initially decreasing to a certain level. This shows that the accumulated product in this case is more persistent to further degradation than that accumulated in the anaerobic test.

In Figure 23, the accumulated product from the glucose experiment also persists after being decreased somewhat when denitrification was started. This initial drop in concentration may not be due to any biological activity, but could be explained as the result of dilution from the experimental procedure. From the results of both tests, the consecutive incubation under denitrifying conditions showed no promise of further degradation of accumulated persistent intermediate products of aerobic incubation.

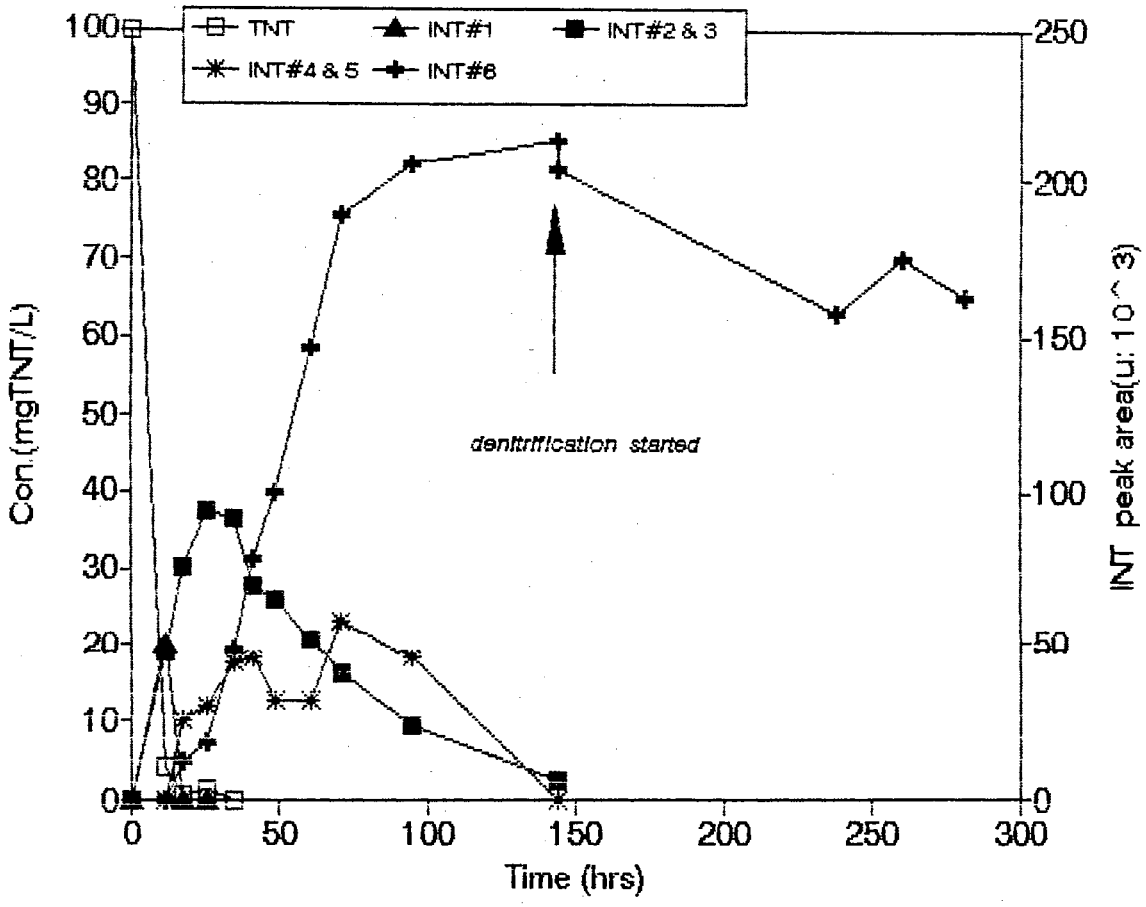


Figure 22. Further degradation of accumulated transient transformation products under consecutive aerobic/anoxic conditions. Primary substrate: peptone

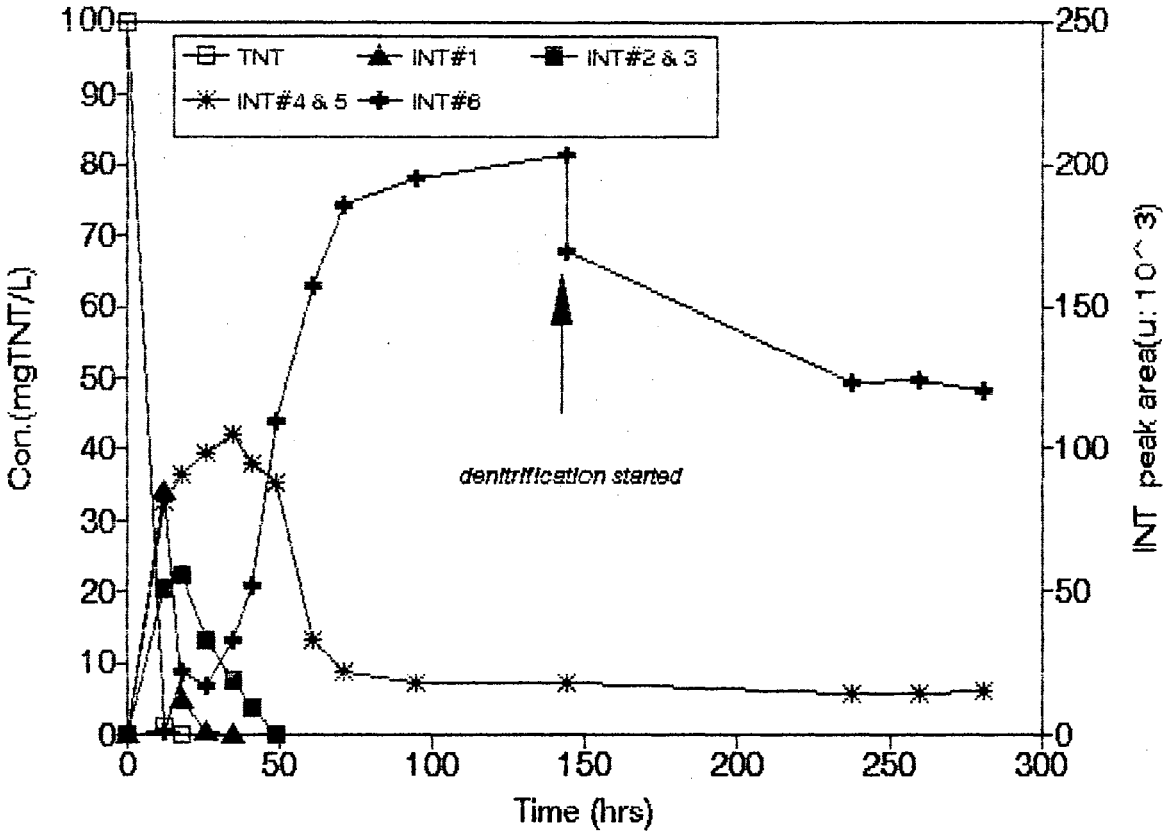


Figure 23. Further degradation of accumulated transient transformation products under consecutive aerobic/anoxic conditions. Primary substrate: glucose

In the literature, in the presence of oxygen, aminoaromatic compounds and transformation products of nitroaromatic compounds are reported to be easily polymerized to form recalcitrant macromolecules (Braun and Gibson, 1984; Carpenter et al., 1978). Also, hydroxylamino compounds tend to be oxidized to azoxy compounds (Channon et al., 1944). In this study, recalcitrant intermediate products are formed during TNT biotransformation under aerobic conditions, and denitrification is shown to be ineffective to degrade persistent products further.

So far, the complete degradation of TNT and its derivatives were tested through combined two stage anaerobic(or aerobic)/denitrifying systems. The further degradation of persistent intermediate products from the aerobic system was not found to be feasible by consecutive denitrification, but the further degradation of persistent intermediates from anaerobic systems showed promise of effective treatment for TNT and its derivatives by complete degradation.

4.5 Tests of Abiotic and Cell-free Factors Influencing Anaerobic/Anoxic TNT Biodegradation

Further tests were performed to study the influence of abiotic factors such as pH variations and availability of alternate electron acceptors on the anaerobic / denitrification system. Glucose was used as primary substrate because of its rapid anaerobic fermentation rates and acid metabolites production in previous experiments.

4.5.1 Anaerobic/denitrification with and without pH adjustment

Enriched medium containing 100 mgTNT/L and nutrients for anaerobic and denitrifying conditions was prepared and inoculated with both cultures to test the activity of a combined, single reactor system. Figure 24 illustrates the profiles of TNT transformation into several intermediate products. The results show that the same intermediate products were formed, as observed in previous experiments, under anaerobic conditions. The accumulated product concentrations (INT#4 and/or 5) decreased somewhat between about 24 and 36 hours incubation but soon sustained a steady concentration level.

It was felt that organic acid production from glucose fermentation may have suppressed denitrification activity. Because decreasing pH tends to decrease the overall denitrification rate, and a low pH of 3.5 was reported to

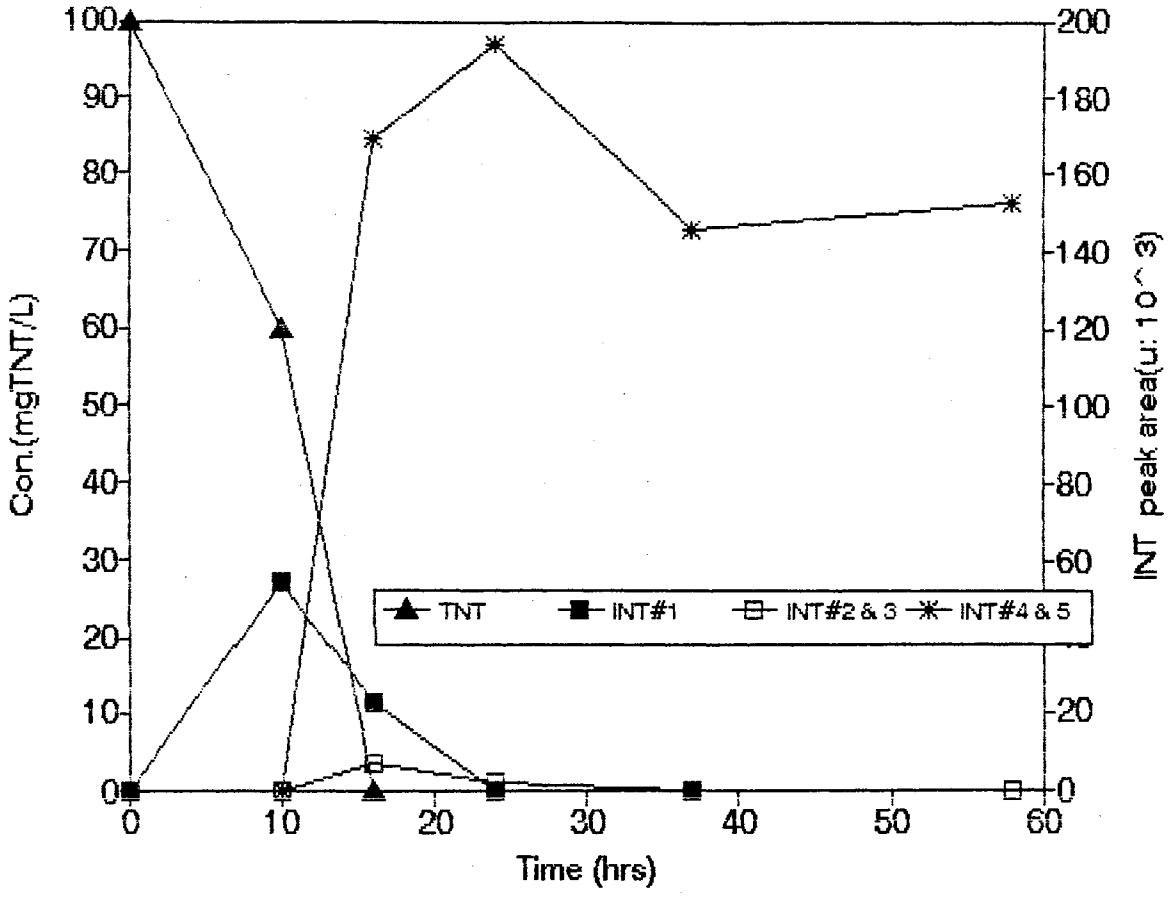


Figure 24. Anaerobic activity in the presence of nitrate with glucose as primary substrate for TNT biotransformation, and no pH adjustment.

prevent the occurrence of denitrification (Knowles, 1982). Therefore, the pH of the system was monitored and raised to investigate its influence to the biotransformation reactions. The results are plotted in Figure 25.

Figure 25 shows the same reaction profiles as in Figure 24 up to 60 hr, when pH was adjusted by the addition of 4M phosphate buffer to the system. Raising pH to above 6.2 allowed the further degradation of persistent intermediate products (INT#4 & 5) by denitrification. Extending the incubation time in these studies demonstrated that denitrification activity resulted in complete degradation of TNT intermediates within 90 hours (3.5 days) from the time pH was raised to >6. The optimum pH range for denitrification is known to be 7.0 to 8.0 (Muller et al., 1980). To confirm this observation, a system in which only nitrate was excluded was incubated under the same conditions. After 2.5 days (60 hr) incubation, similar results were noted: INT#4 and 5 accumulated to the same level, and pH was driven from about 7 to 4.5.

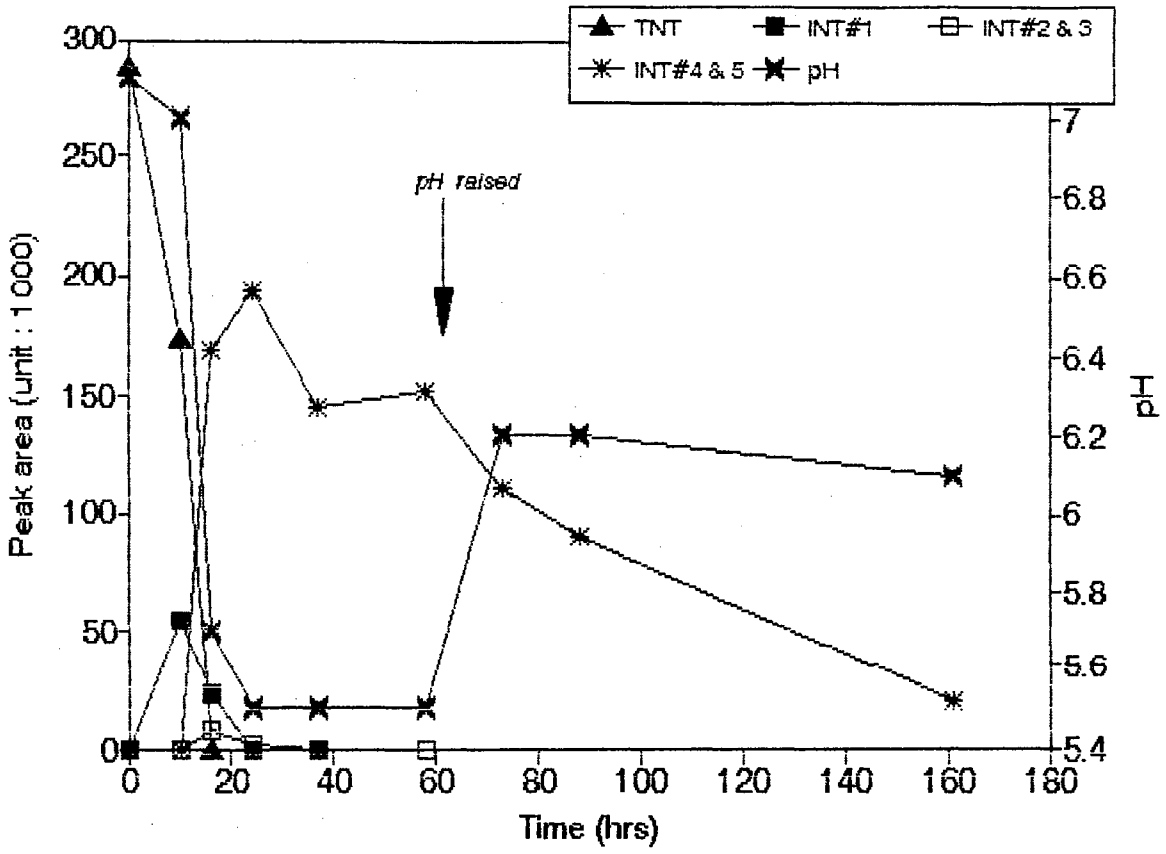


Figure 25. Effect of pH adjustment on anaerobic/anoxic (nitrate-reducing) activity with glucose as primary substrate for TNT biotransformation.

However, to initiate denitrification in this test, nitrate was added without raising pH to test any influence by introducing the electron acceptor. As shown in Figure 27, the concentration of accumulated intermediate products decreased somewhat more than before over about a 30 hr period after nitrate was added to the system. At this point, the persistent intermediates leveled off in concentration. Incubation was continued for an additional 10 days, as illustrated in Figure 27, but no further reactions were observed. This could be explained by the fact that at low pH, the nitrogen oxide reductases which reduce N_2O are inhibited (Focht, 1974). Therefore, the pH was raised again at this point from 4.4 to 5.8. After the pH adjustment, the persistent intermediate product concentration decreased completely during 1 additional day incubation as shown in Figure 27. Thus, although denitrifying conditions were induced by the addition of nitrate at 60 hours, complete degradation of accumulated products was still dependent on raising the pH of the system.

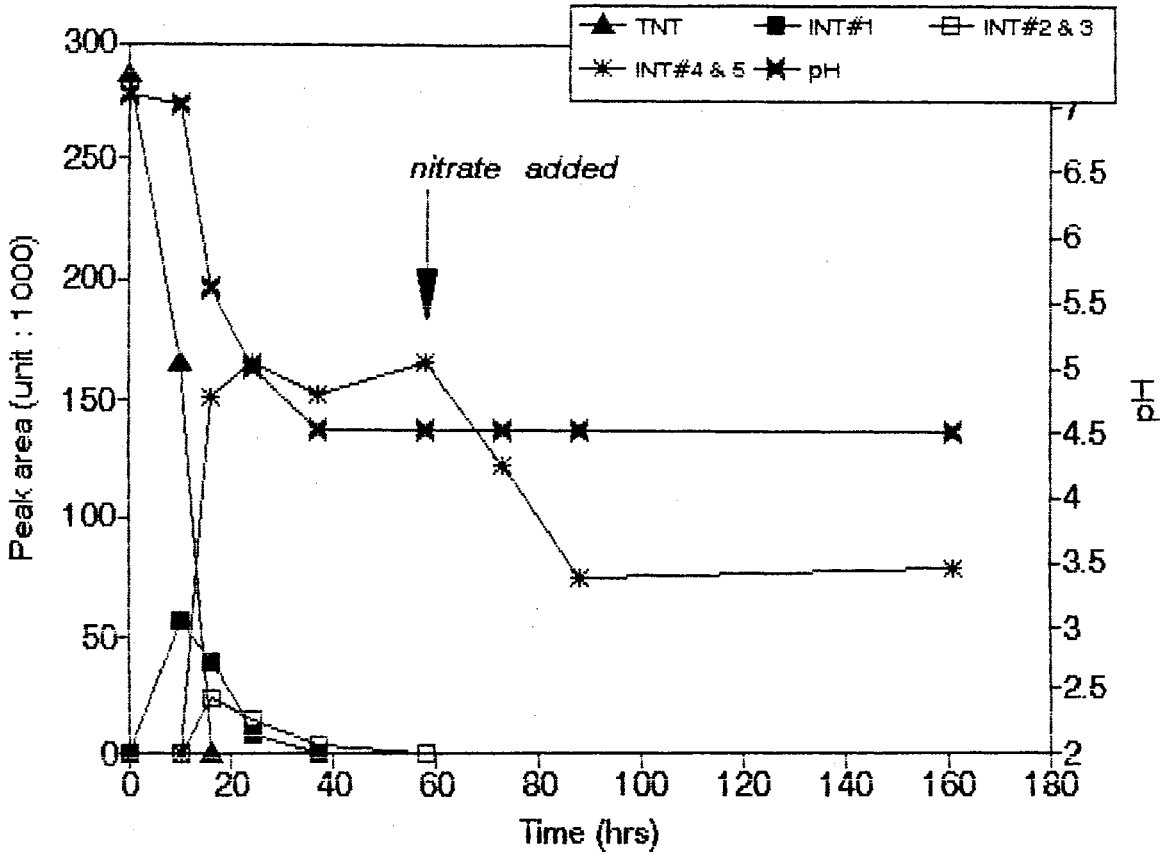


Figure 26. Nitrate addition to the anaerobic reactor with glucose as primary substrate for TNT biotransformation.

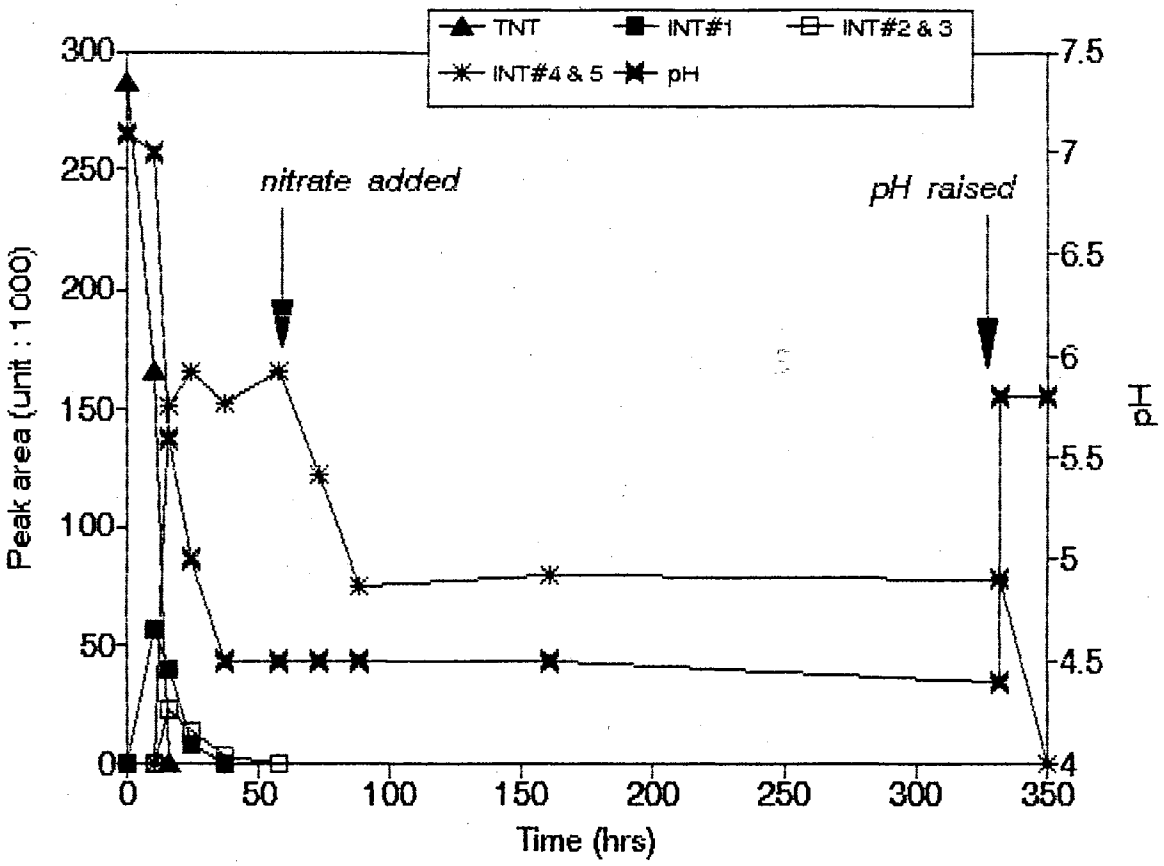


Figure 27. Nitrate addition and pH influence on extended anaerobic/anoxic incubation with glucose as primary substrate for TNT biotransformation.

4.5.2 The influence of sulfide and filtrate to system

In recent studies, Glaus et al.(1992) reported that the exudate of a bacterial culture mediated the abiotic reduction of nitroaromatic compounds in the presence of hydrogen sulfide as a bulk electron donor. The exudate mediated the electron transfer from bulk electron donor to nitro groups of the aromatic ring. Therefore, laboratory tests were performed to investigate the influence of similar effects on TNT biotransformation under the conditions of this study.

In Figure 28, TNT transformation test results with 70 % filtrate (v/v) or 5.4 mM sulfide are plotted. To remove cellular materials, portions of medium from reactors at the completion of complete TNT biodegradation studies were sampled and filtered through 0.45 µm followed by 0.2 µm membrane filters. 70 % filtrate was prepared by filling test vials 70 % with filtered medium and 30 % with phosphate buffer solution to 100 % total volume. 30 mgTNT/L was mixed only with 70 % filtrate medium and incubated under anaerobic conditions, but the data plot shows no distinguishable reaction. Sulfide was reported to inhibit denitrification at concentrations of 0.3 mM through 8 mM [H_2S] (Dolfing et al.,1990; Sorensen et al.,1980; Meyers, 1972; Tam and Knowles, 1979). Therefore, 100 mgTNT/L was also mixed only

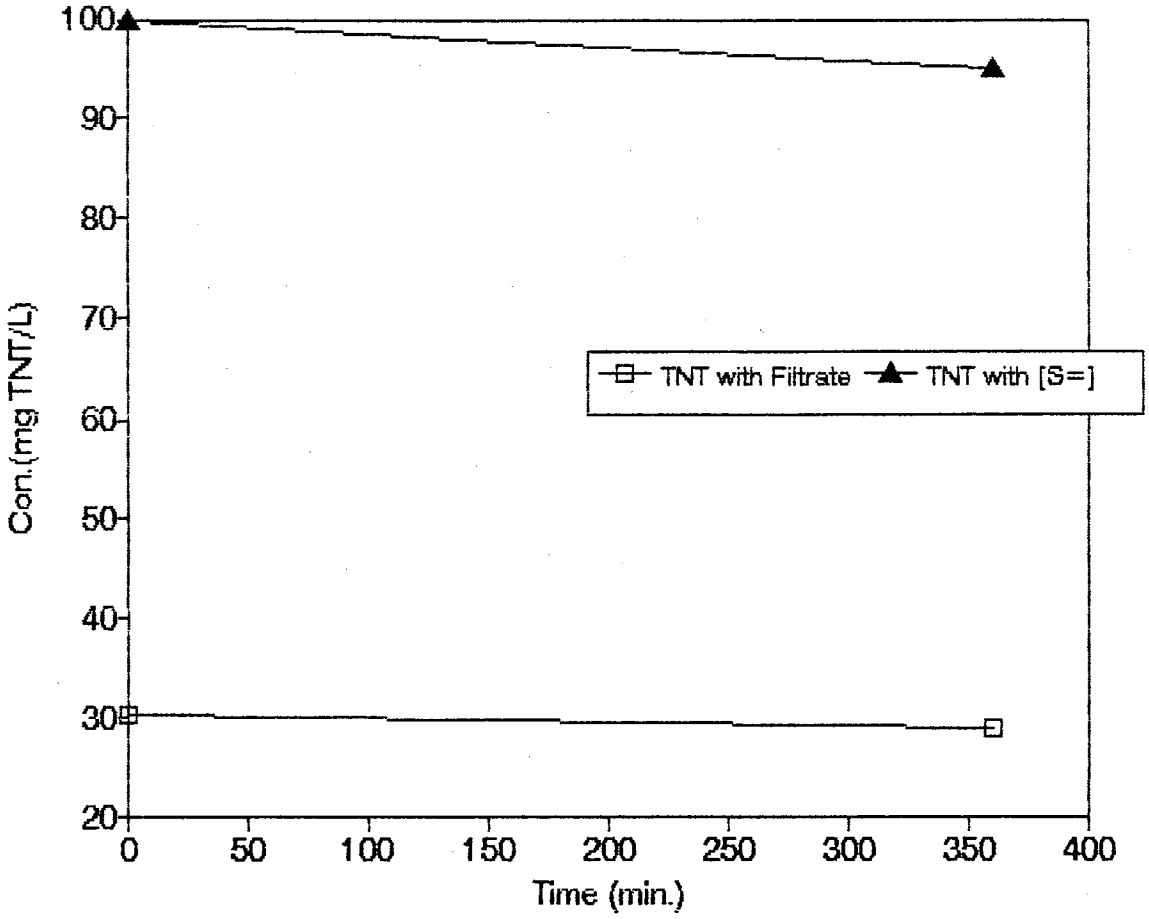


Figure 28. Plot of TNT transformation in the presence of either filtrate(exduate of microbially active system) or sulfide ions. Filtrate: 70 % , $[S^{2-}]$: 5.4 mM , pH: 7.2

with 5.4 mM sulfide. Without filtrate present, sulfide transformed only about 5 mgTNT/L during 6 hours test period.

Figure 29 shows a plot of data resulting from an extended reaction time with filtrate alone. Although TNT was incubated for 5 days , no active transformation was observed.

20 mgTNT/L was then reacted with a combination of 6.0 mM sulfide and 70 % filtrate. All TNT concentration added to the system was transformed into other intermediate products as shown in the plot of TNT biotransformation data shown in Figure 30. Three persistent intermediates formed under these test conditions. Out of three intermediates detected, one intermediate (INT#3) appeared from the start of the reaction, initially at a steady concentration. As the reaction proceeded further the concentration began to rise. This suggests that INT#3 could be an accumulated persistent product left in filtrate from the anaerobic reactor medium which was tested previously. Thus, one persistent intermediate (INT#3) from abiotic reaction was identical to a reduced intermediate product left in filtrate from the microbially active anaerobic reactor, but the other two products formed by abiotic reduction in this test could be different.

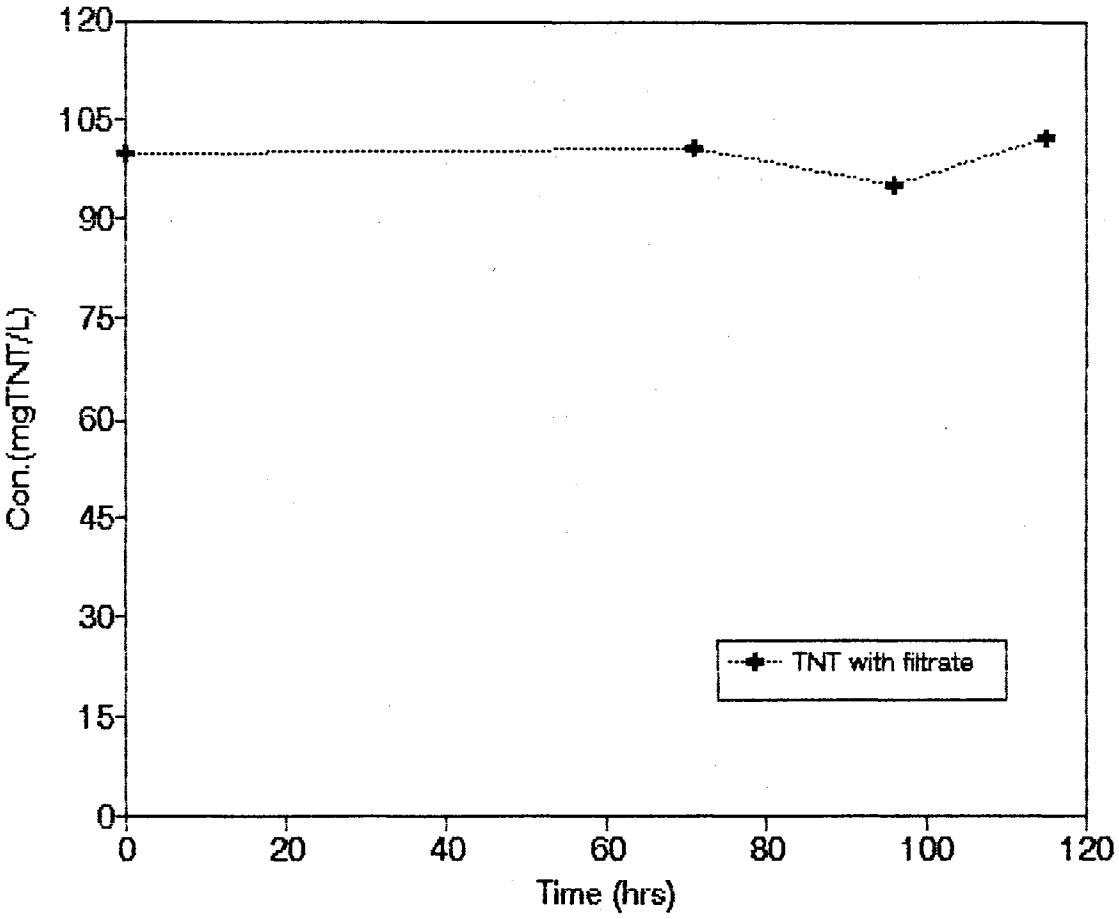


Figure 29. Plot of TNT transformation in the presence of filtrate (exudate of microbially active system). TNT: 100 mgTNT/L.

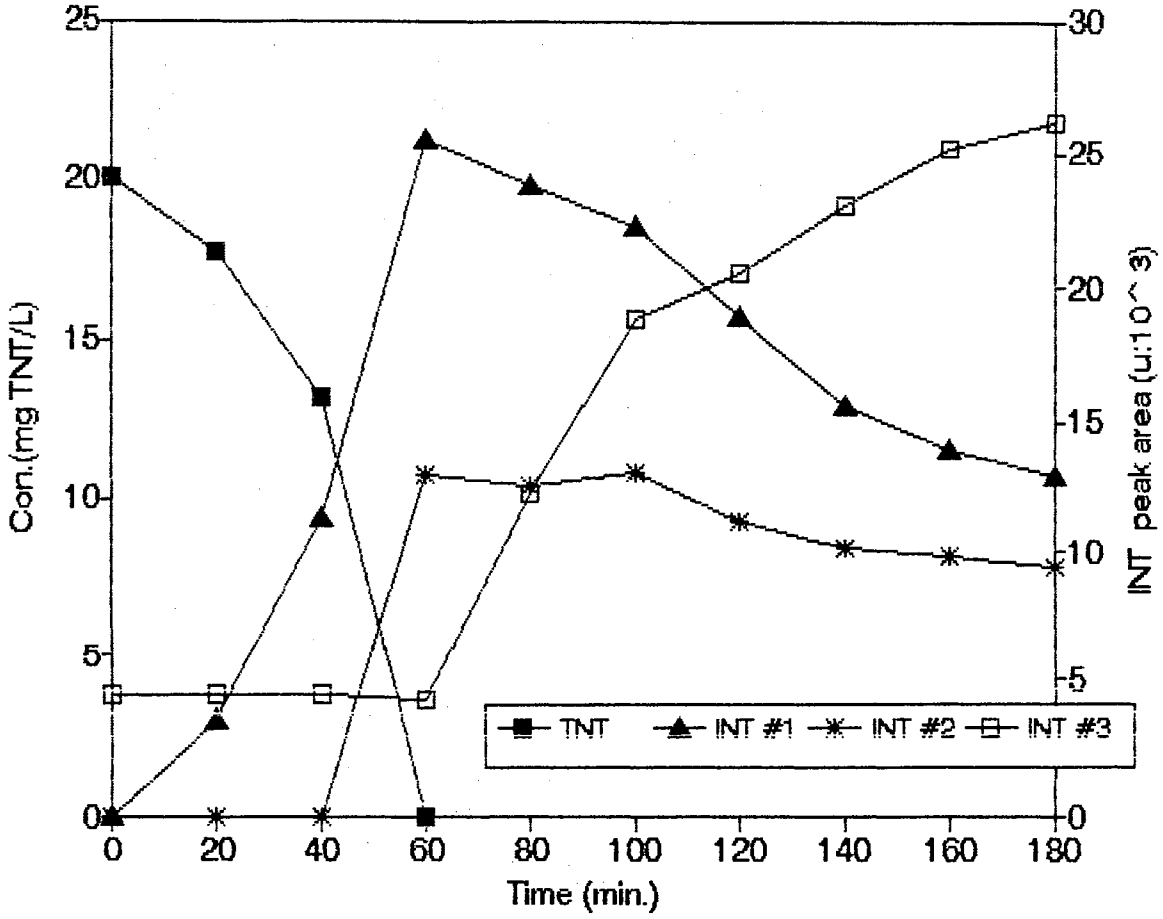


Figure 30. Plot for abiotic TNT transformation.
Filtrate: 70 %, [S^{2-}]: 6.0 mM, pH: 7.0

Abiotic transformation of 20 mgTNT/L was tested in the presence of 50, 60, 70, and 80 % filtrate with 6.0 mM sulfide at pH 7.0. As illustrated in Figure 31, the higher concentrations (70 and 80 % filtrate) show significant TNT transformation rates, while 50 and 60 % filtrate concentrations produced much slower rates.

In Figure 32, linear regression was plotted to estimate transformation rate coefficients for the reaction with 50 and 60 % filtrate. Pseudo-first-order analysis for the reaction was applied. The values calculated from this analysis are 0.001 and 0.002 min^{-1} for 50 and 60 % filtrate medium, respectively. These values are an order of magnitude less reactive than the reported values of Glaus et al. (1992).

The data illustrating the influence of different concentrations of sulfide to TNT transformations are plotted in Figure 33; 3.0 mM sulfide showed no enhancement of TNT transformation rate. However, when the concentration was raised from 3.0 mM to 5.4 mM, the reaction rate increased to a rate similar to that shown in the plot for 6.0 mM initial sulfide concentration. TNT transformation by 12.9 mM sulfide shows the fastest reaction rate among concentrations tested. Thus, tests determined that sulfide concentration has a significant influence on TNT transformation by cell-free medium exudates.

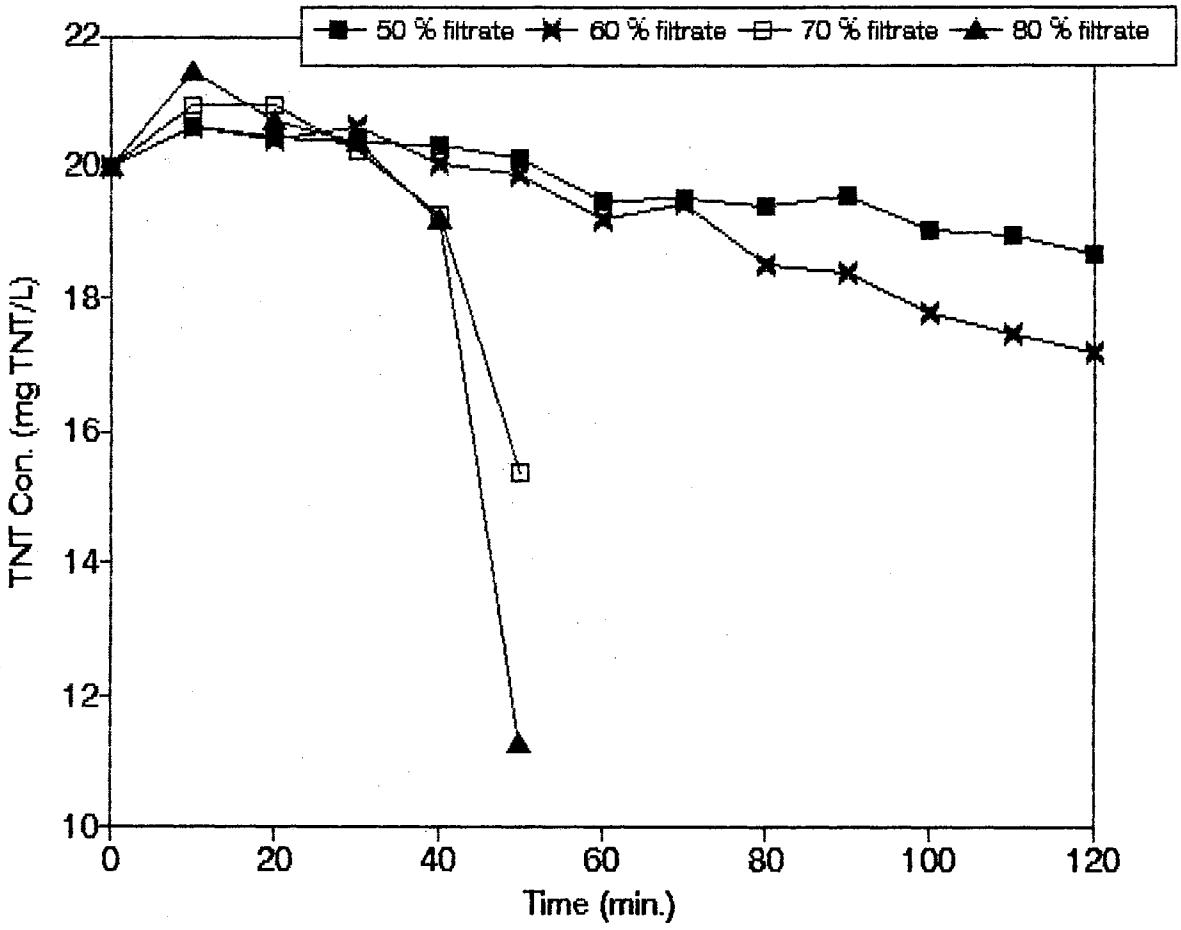


Figure 31. TNT transformation in the presence of different filtrate concentrations. $[S^{2-}]$: 6.0 mM.

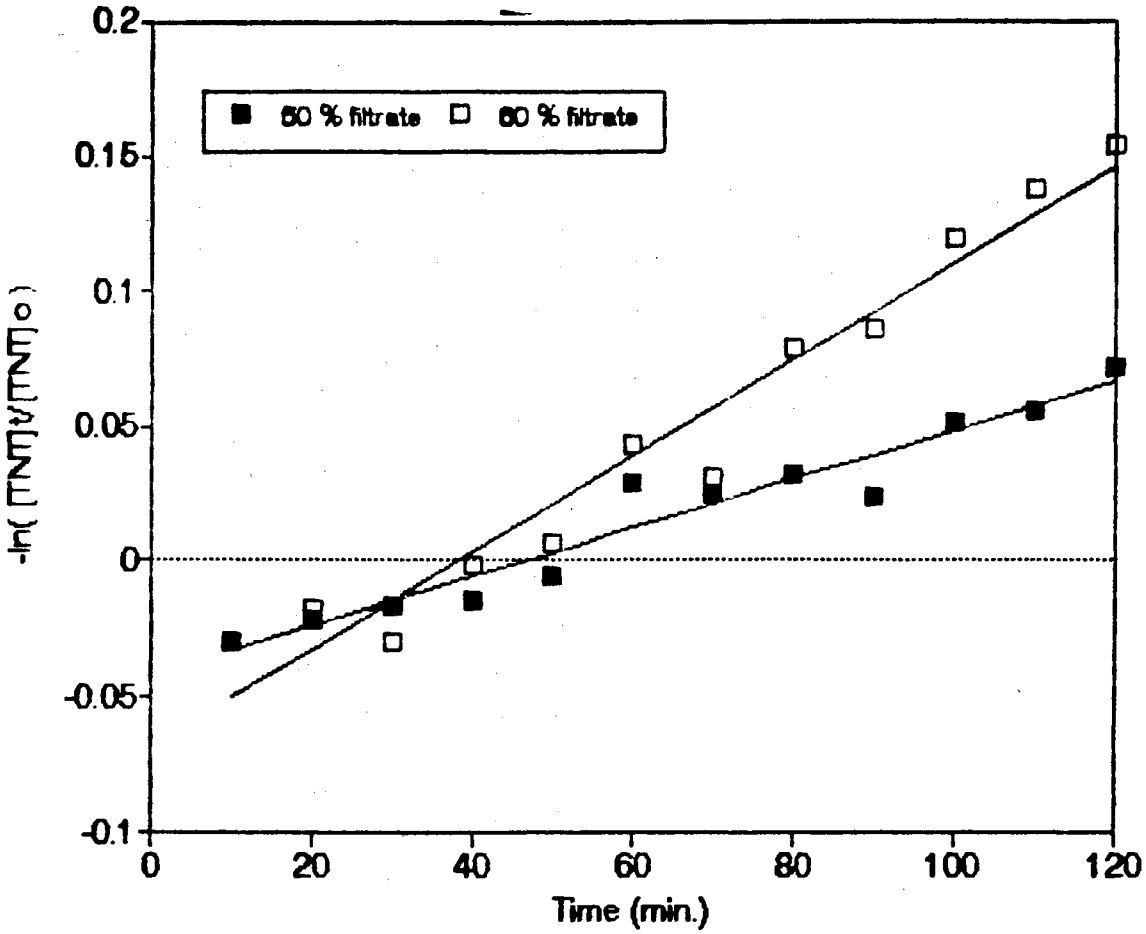


Figure 32. Linear regression fit for TNT transformation. [$S^{=}$] : 6.0 mM

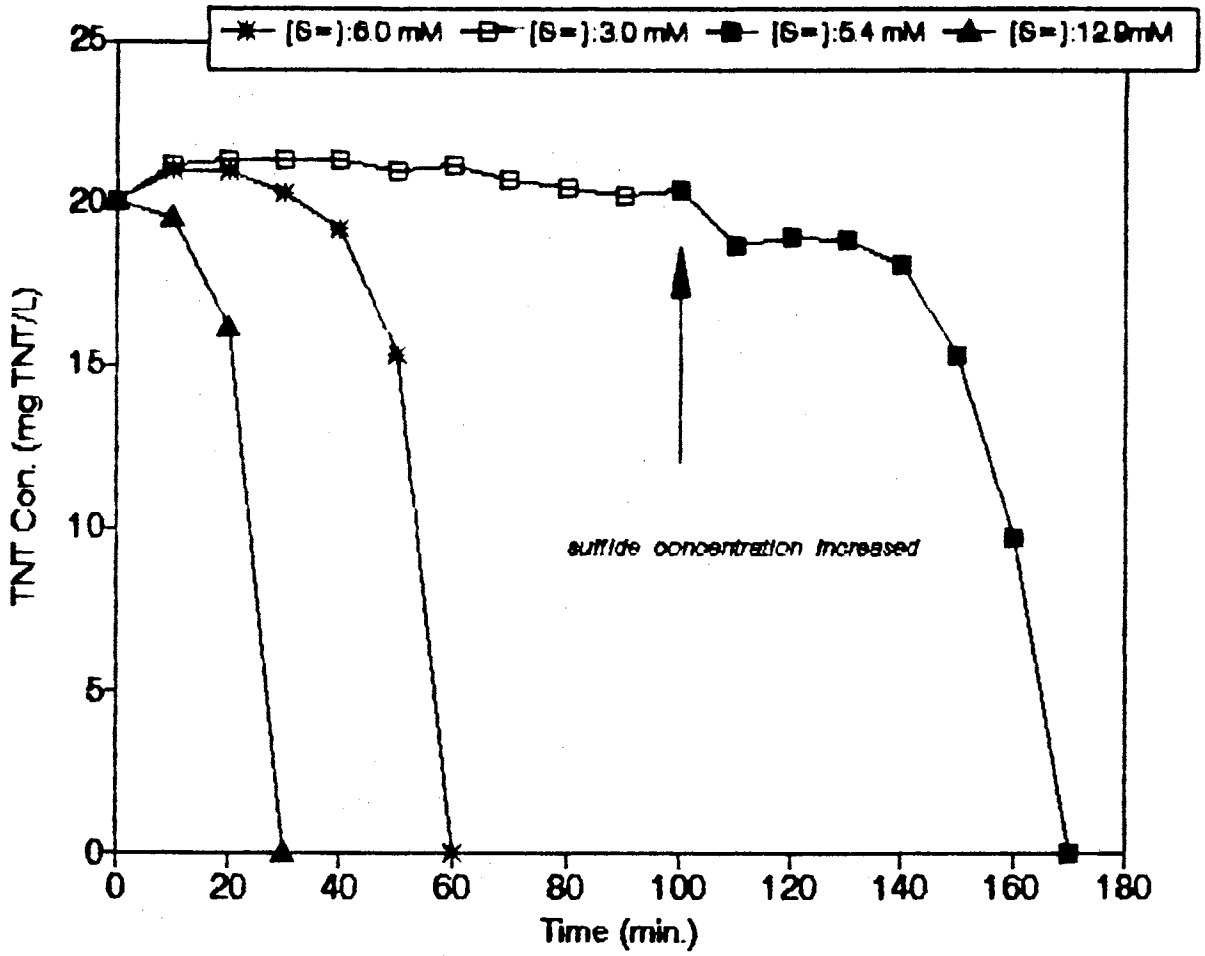


Figure 33. Sulfide influence on TNT transformation rate in presence of 70 % filtrate (exudate of microbially active system).

These test results showed that certain concentrations of filtrate (microbial exudates) and sulfide significantly influenced TNT transformation when acting together, but not separately. Only partial TNT transformation was observed. The comparison of TNT transformation rates by variable sulfide and filtrate concentrations is shown in Table XIII.

TABLE XIII

THE COMPARISON OF TNT TRANSFORMATION RATE UNDER VARIABLE SULFIDE AND FILTRATE CONCENTRATIONS

	Abiotic TNT transformation (mg/L)							
Incubation Time(min.)	Filtrate (%),[$S^{=}$]:6 mM				Sulfide (mM), Fil.:70%			
	50	60	70	80	3.0	5.4	6.0	12.9
0	20.0	20.0	20.0	20.0	20.0		20.0	20.0
10	20.6	20.6	20.9	21.5	21.2		20.9	19.5
20	20.4	20.4	20.9	20.7	21.3		20.9	16.2
30	20.3	20.6	20.2	20.3	21.3		20.2	0.0
40	20.3	20.0	19.3	19.2	21.3		19.3	
50	20.1	19.9	15.3	11.2	21.0		15.3	
60	19.4	19.2	0.0	0.0	21.1		0.0	
70	19.5	19.4			20.7			
80	19.4	18.5			20.5			
90	19.5	18.4			20.2			
100	19.0	17.8			20.4	20.4		
110	18.9	17.4				18.7		
120	18.6	17.2				18.9		
130						18.9		
140						18.1		
150						15.4		
160						9.7		
170						0.0		

4.5.3 Tests for sulfide inhibition to denitrification

In the literature, sulfide was reported to cause some inhibition effects on the reduction of nitrogen oxides in denitrification (Knowles, 1982). Therefore, the influence of 6.0 mM sulfide on denitrifying conditions was tested, and results are illustrated in Figures 34 and 35.

From the plot of Figure 34, suppression of nitrate reduction is shown. Nitrate was completely consumed during 50 hours incubation in the system with no sulfide. On the other hand, nitrate reduction was slow, and nitrite concentration accumulated in the system which contained 6.0 mM sulfide.

The inhibiting influence to TNT transformation products is also illustrated in Figure 35. The accumulated persisting products from anaerobic conditions were incubated continuously under denitrifying conditions except control which is continuation of abiotic reaction. The control maintains its concentration level of persisting product because it was not transferred to denitrifying condition. However, the accumulated product by TNT biotransformation reaction shows fast degradation under consecutive denitrifying condition. The other accumulated product, from the filtrate and sulfide-mediated reaction, shows somewhat inhibited rate of degradation compared to the degradation of the anaerobic biotransformation product because of sulfide inhibition.

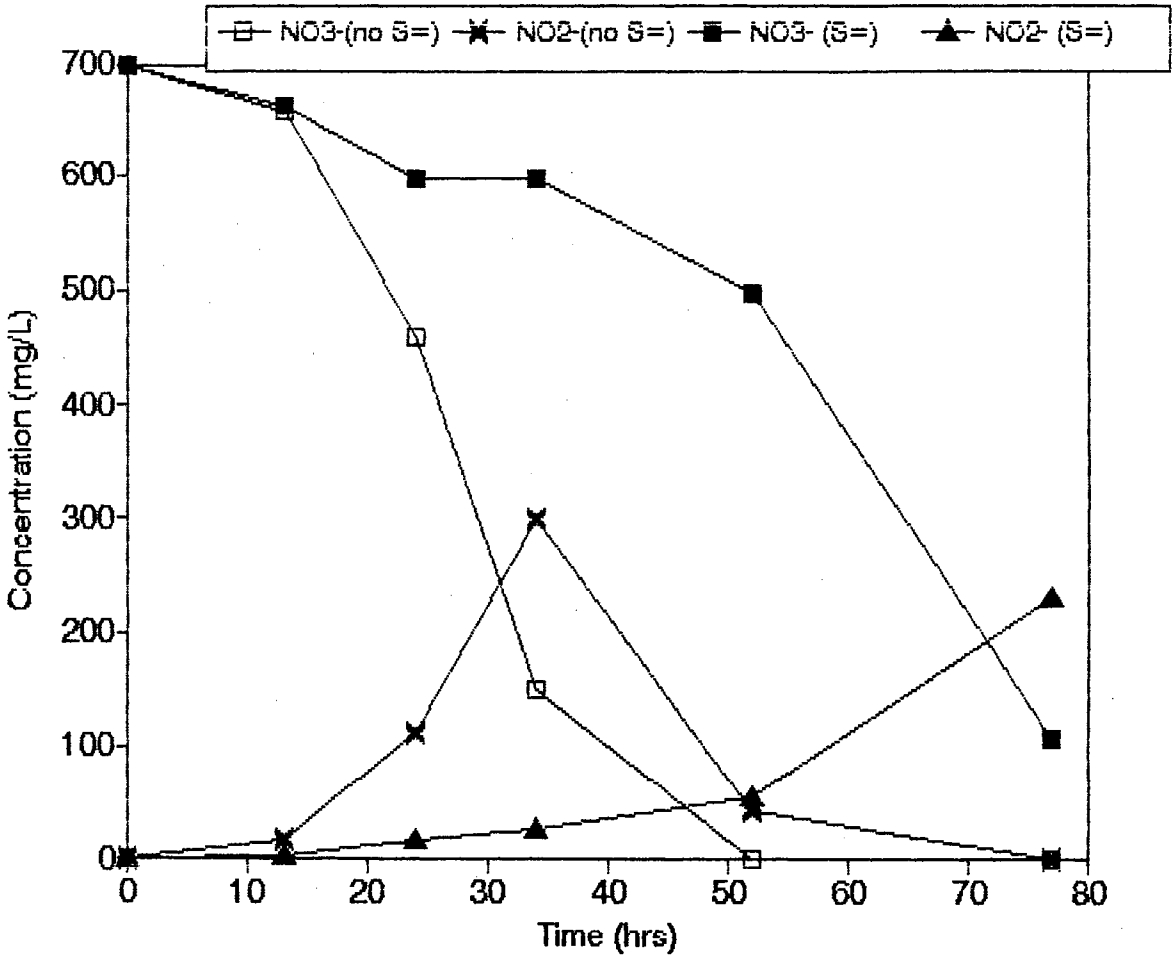


Figure 34. Sulfide effect on denitrification. 6.0 mM sulfide added to one system, and results compared to a sulfide-free denitrification system.

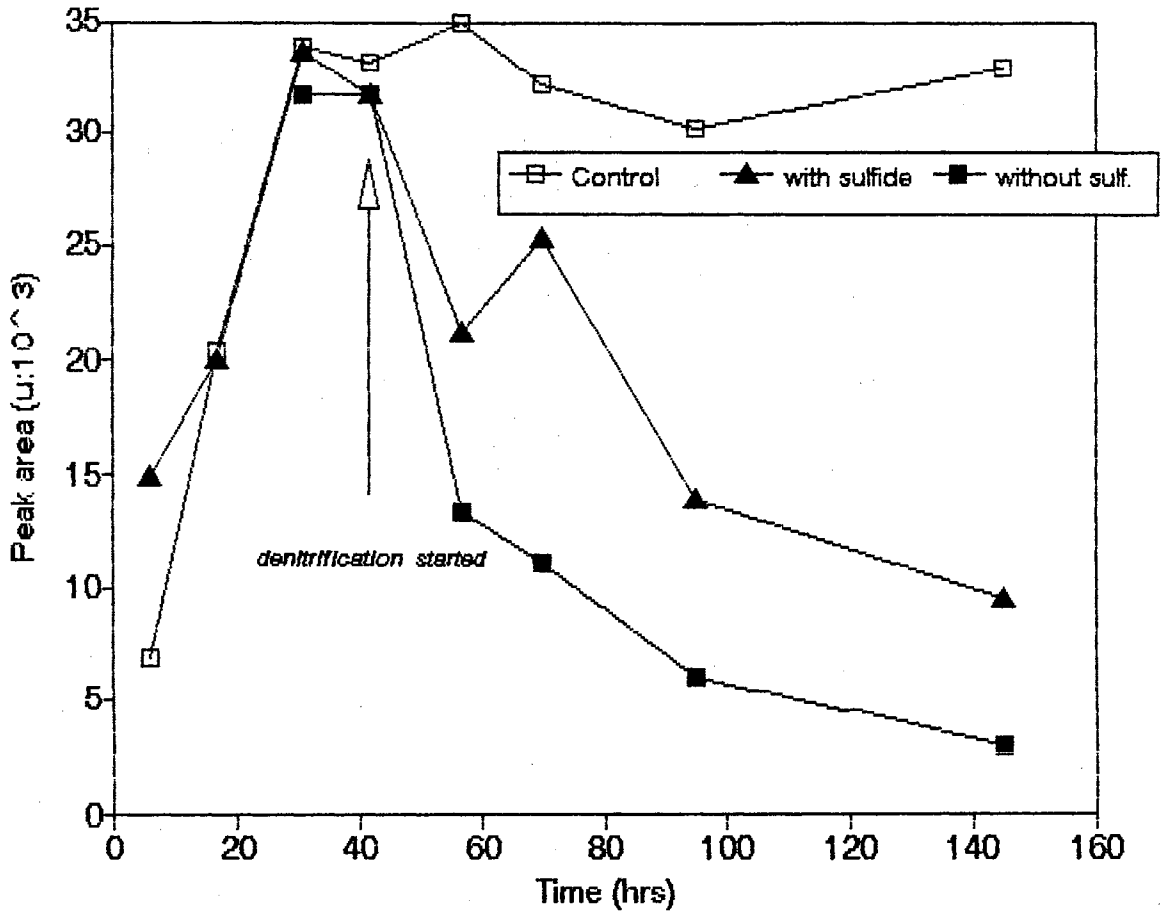


Figure 35. Sulfide inhibition to consecutive denitrification. Comparison for the degradation of persistent products from anaerobic or abiotic reaction.

CHAPTER V

CONCLUSIONS

A major effort in this study has been to determine the metabolic fate of TNT under various biotransformation regimes and to test the feasibility of further degradation of persistent intermediate transformation products.

In the preliminary study, argon gas showed somewhat higher rates initial TNT degradation than other gases (N_2 and H_2) used to impose anaerobic conditions in test reactors. Initial concentration of 100 mgTNT/L was depleted microbially within three days incubation and showed no inhibition to microbial activity of test cultures.

Under aerobic and anaerobic conditions, various persistent intermediate transformation products were detected and accumulated in the systems. Thus, a sequential two stage biotransformation approach was used, but the persistent products from aerobic conditions were not completely degraded under denitrifying conditions. However, the accumulated products under anaerobic conditions were degraded completely under consecutive denitrifying conditions. Therefore, the developed process for complete degradation of TNT by the sequential stage of denitrifying conditions following anaerobic incubation indicates a promising treatment scheme

for wastewater, groundwater, or soils contaminated by munition compounds (TNT or DNT).

Also, to evaluate abiotic influence on the developed biotransformation regime, pH, electron acceptor (NO_3^-), and abiotic TNT transformation by sulfide and filtrate of active microbial medium were tested. The test system depended more on pH than electron acceptor. TNT was only transformed in the absence of active microbial cells when sulfide and filtrate acted together. However, 6.0 mM sulfide showed inhibition to denitrifying reactions. Abiotic transformation parameters alone could not be applied to TNT degradation, but require activity of microbial exudates. Microbial sequence of anaerobic/denitrifying conditions is a more effective biotransformation regime for the complete degradation of TNT.

5.1 Summary of major findings

A number of detailed conclusions that can be drawn from the results obtained in this study are summarized as follows:

1. Initial concentration of 100 mg/L of TNT was biotransformed to various intermediate products under anaerobic conditions during 2 days of incubation. Intermediate products were tentatively identified as hydroxylamino-, monoamino-, and diaminotoluene compounds. Peptone and glucose served as effective primary substrates. TNT biotransformation rate with peptone was somewhat slower than with glucose, but reaction with glucose showed inhibition from acid fermentation

products which lowered pH. Accumulated intermediate diaminotoluene compounds were recalcitrant to further degradation in the anaerobic system.

2. Initial concentration of 100 mg/L of TNT was also biotransformed to various intermediate products under aerobic conditions. No specific difference was observed between peptone and glucose as primary substrate. A variety of strongly persistent intermediate products accumulated, presumably due to the formation of azoxy compounds via polymerization reactions which are known to be favored in an aerobic reaction medium.

3. Under consecutive denitrifying conditions, the persistent intermediate products of anaerobic reactions were completely degraded within a further 5 days incubation period. However, products from aerobic system were recalcitrant to further degradation under denitrifying conditions. Therefore, this sequential two stage scheme is more adequate to the treatment of contamination by munition compounds in the anaerobic environment.

4. In the anaerobic/anoxic system, pH showed more effective impact to denitrification than nitrate addition, because nitrate reduction was suppressed by the low pH in denitrifying reaction. The optimum pH for denitrification is known to be 7.0 to 8.0 (Muller et al., 1980), but significant activity was noted at pH between 5 and 6 in this study.

5. Sulfide showed inhibition to denitrification; 6.0 mM sulfide slowed denitrification and further degradation of

persistent products by denitrification.

6. The transformation of initial concentrations of 20, 30, and 100 mgTNT/L was abiotically mediated by microbial medium filtrate and sulfide. Three intermediates were detected but one persistent product accumulated. Further degradation of this persistent product was also tested under consecutive denitrifying conditions, but the reaction was partially inhibited. Also, TNT was not transformed by filtered microbially active exudates alone. Abiotic TNT transformation required coexistence of filtrate and sulfide. Accordingly, TNT bioconversion in this study was only influenced by pH of the system, and abiotic mediation showed no influence on biotransformation because no sulfide existed in the biological test reactor medium.

CHAPTER VI

COMMENTS FOR FUTURE WORK

Based upon the findings of this study, the following comments are suggested for future studies involving the application of complete biotransformation of TNT to the treatment of munition wastes:

(1) Isolate and identify the TNT transformation intermediate products to find bioconversion pathway.

(2) Investigate TNT reduction kinetics.

(3) Isolate microorganisms which have the ability to reduce TNT and its derivatives.

(4) Investigate the mechanism of sulfide inhibition to denitrification.

(5) Test the pH control for glucose as a primary substrate.

(6) Test the kinetic parameters for peptone to be used efficiently as a primary substrate, or identify other primary substrate with favorable growth kinetics for nitroaromatics-degrading microorganisms.

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