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Treatment and Biodegradation of the High Explosive 2,4,6-Trinitrotoluene (TNT): A Literature Review

A thesis submitted in partial satisfaction of the
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in Civil Engineering

by

Sandeep Ojha

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The thesis of Sandeep Ojha is approved.

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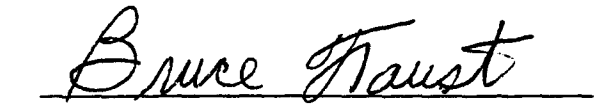

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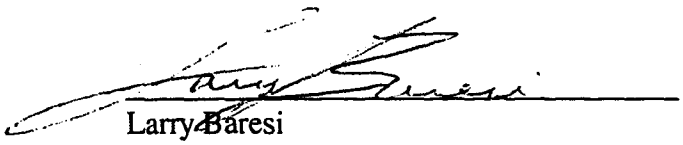

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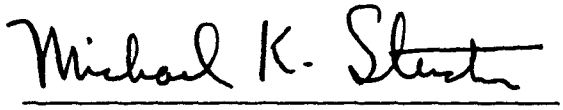

Michael K. Stenstrom, Committee Chair

University of California, Los Angeles

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ABSTRACT OF THESIS

Treatment and Biodegradation of the High Explosive 2,4,6-Trinitrotoluene (TNT): A Literature Review

by

Sandeep Ojha

Master of Science in Civil Engineering

University of California, Los Angeles, 1997

Professor Michael K. Stenstrom, Chair

TNT is the most widely used military explosive. Manufacturing of TNT, munitions load, assembly, and pack operations, and demilitarization of surplus ammunition have significantly contaminated soil and groundwater at current and former U.S. Department of Defense (DOD) and U.S. Department of Energy sites (DOE). As of 1995, the DOD has identified more than 1,200 sites which require remediation.

Traditional techniques, such as open burning (OB), incineration, and activated carbon have been used to treat contaminated soil and groundwater. However these "first generation" and "second generation" techniques do not always mineralize TNT, but often transfer it to another media, such as adsorption to activated carbon. A large amount of

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research has been performed to find "third generation" techniques. Researchers are exploring methods such as phytoremediation, composting, advanced oxidation, and photocatalytic degradation as a possible means of mineralizing the TNT in contaminated soils and groundwater. A combination of chemical and biological treatment, which employs a variety of microbial consortia, may provide a cost-effective and environmentally acceptable method for cleanup of contaminated sites.

This thesis reviews the literature of the high explosive, TNT. A brief background, including chemical properties and toxicological effects are presented. Emphasis is placed upon physical, chemical and biological treatment techniques, and research results which may be successful candidates to replace "first generation" technology.

1 INTRODUCTION

TNT (2,4,6-trinitrotoluene) is the most widely used military explosive. This is partly because of the relatively safe methods of manufacturing TNT, its stability, a low melting point, a low sensitivity to impact, friction, and high temperature (Yinon 1990). TNT was discovered by Wilbrand in 1863 when he added toluene to a mixture of nitric acid and sulfuric acid producing a yellow odorless, solid (U.S Department of Health Services 1995). By 1902, TNT was used primarily as an explosive for military shells, bombs, and grenades by the German Military.

During the two World Wars, many other countries began producing millions of tons for use as a pure explosive or as an ingredient in binary explosives (Yinon 1990). As cited by the U.S. Department of Health Services (USDHS 1995), the most common binary mixtures of TNT are cyclotols (mixtures with RDX [hexahydro-1,3,5-trinitro-1,3,5-triazine]), octols (mixtures with HMX [octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine]), amatols (mixtures with nitrate), and tritonals (mixture with aluminum). TNT is also used for industrial purposes such as underwater blasting and as a chemical intermediate in the manufacture of dyestuffs and photographic chemicals.

Disposal of TNT and its degradation products from munitions plants and disposal sites presents a dangerous contamination problem. Manufacturing of TNT, munitions load, assembly, and pack operations (LAP), and destruction of ammunition plants at the end of World War II have contaminated many sites around

the world. A single manufacturing plant can generate as much as 500,000 gallons of wastewater per day containing TNT as well as other nitrocompuonds (Yinon 1990). TNT moves in surface water and through soils contaminating the groundwater. In surface water, 2,4,6-trinitrotoluene can be rapidly broken down into other chemical compounds by sunlight (USDHS 1995). Microorganisms, however, break down TNT more slowly in groundwater and sediments.

The past practices have significantly contaminated soil and groundwater at many current and former U.S. Department of Defense (DOD) and U.S. Department of Energy (DOE) sites. The DOD has identified over 1200 sites with explosives contamination requiring remediation with at least 87% containing contaminated groundwater (Schmelling *et al.* 1995). Furthermore, 2,4,6-trinitrotoluene exists on at least 20 of the 1397 hazardous waste sites on the EPA National Priorities List (USDHS 1995).

The concern over TNT release into the environment exists because of its toxicity to humans as well as many different organisms. It is a mutagen and is listed as priority pollutant by the U.S. Environmental Protection Agency (USEPA) (Schmelling 1995). Exposure to humans can occur primarily from drinking contaminated water, or eating contaminated fruits and vegetables that may have been cultivated in contaminated soils. TNT has been measured at waste disposal sites in groundwater at 0.32 parts of 2,4,6-trinitrotoluene per million parts of water (ppm) and in soil up to 13,000 ppm (USDHS 1995). Research shows that animals force fed a low concentration of 2,4,6-trinitrotoluene over a period of 15-364 days had severe

problems in their immune system and to their spleen. Therefore, it is designated as a hazardous waste and agencies such as the U.S. EPA require strict regulations for disposal of 2,4,6-trinitrotoluene.

For many years, traditional techniques for munitions disposal at DOD sites included destructive methods such as dumping in the sea and incineration. Although incineration, open burning (OB) and open detonation (OD) have been effectively used, concerns of noise, air emission, costs, regulatory requirements, poor public perception, and the deposition of residues in soils have pushed researchers to explore other treatment possibilities. Activated carbon is often used to treat process waters at munitions plants as well as to remediate explosives contaminated groundwater (Schmelling *et al.* 1995). However, this technique requires that the adsorption media be regenerated by incineration. Researchers are exploring other methods of remediation such as utilizing phytoremediation or *sulfate-reducing* microorganisms in the degradation of 2,4,6-trinitrotoluene. These techniques may be cost-effective and more environmentally acceptable to cleanup contaminated sites.

This thesis reviews current literature on the high explosive 2,4,6-trinitrotoluene. TNT manufacturing, it's chemical properties and innovative physical, chemical and biological treatment techniques are reviewed.

2 BACKGROUND

2.1 Regulations and Toxicological Effects

TNT has been widely used since World War I, when its toxicity was first seen to effect exposed workers in manufacturing plants. As cited by the USDHS (1995), many adverse health effects such as anemia, liver function and abnormalities, respiratory complications, and possibly aplastic anemia have been observed at exposure levels below the former standard of 1.5 milligrams of TNT per cubic meter of air (mg/m^3). As researchers became more aware of the toxicological effects during the early 40's, the incidence of exposure reduced dramatically. Protective gear for ammunition workers and improved ventilation system at the facilities were used.

The U.S. government has developed regulations and guidelines for TNT to protect the public and workers exposed to the substance. The Department of Transportation (DOT) regulates the packaging, labeling and transfer of TNT because it is designated as an explosive. It is flammable and toxic, and poses a threat while being transported. The Occupational Safety and Health Administration (OSHA) regulates levels of hazardous material in the workplace. The maximum allowable amount of TNT in the air in a room during an 8-hour workday, 40-hour workweek is 0.5 mg/m^3 (USDHS 1995). The EPA designates TNT as a classification C weight-

of-evidence carcinogenic, which characterizes TNT as a possible human carcinogenic (USDHS 1995). The Drinking Water Equivalent Level (DWEL), a lifetime exposure at which adverse health effects are not expected to occur, is 29 μg/l for TNT, as cited by the USDHS (1995). The DWEL is a conservative estimate because there is a lack of appropriate data for the determination of the One-day Health Advisory and the Ten-day Health Advisory (USDHS 1995).

Although the general public may not be exposed to TNT, people who live near demilitarization plants, munitions fabrication plants, and areas where open burning and open detonation occur may be affected. Reducing peak absorption following exposure by introducing fresh air, removing contaminated clothing, and flushing skin or eyes with running water are simple methods for initial protection. Increasing the cellular concentrations of antioxidants such as glutathione, gluatathione peroxidase, and vitamin E could reduce liver damage caused by TNT (USDHS 1995).

2.2 Classification and General Characteristics of TNT

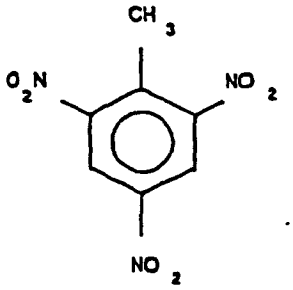
Characteristic	Information	Reference
Chemical name	2,4,6-Trinitrotoluene	HSDB 1990
Synonym(s)	sym-trinitotoluene; 1-methyl-2,4,6-trinitrobenzene; 2-methyl-1,3,5-trinitrobenzene; alpha-TNT; TNT; alpha-trinitrotoluol; tolit; tritol; trotyl oil; trilit	HSDB 1990
Registered trade name(s)	No data	
Chemical formula	$C_7H_5N_3O_6$	Budavari et al. 1989
Chemical structure		Sax and Lewis 1987
Identification numbers:		
CAS registry	118-96-7	Budavari et al. 1989
NIOSH RTECS	XU0175000	HSDB 1990
EPA hazardous waste	No data	
OHM/TADS	7217371	HSDB 1990
DOT/UN/NA/IMCO shipping	TNT, dry or wetted with <30% water (UN 0209/IMO 1.1) TNT, wetted with >30% water (UN 1356/IMO 4.1)	HSDB 1990
HSDB	1146	HSDB 1990
NCI	C56155	HSDB 1990

Table 2.1
Chemical Identity of 2,4,6-Trinitrotoluene. From USDHS (1995)

Property	Information	Reference
Molecular weight	227.13	Budavari et al. 1989
Color	Yellow	Budavari et al. 1989
Physical state	Monoclinic needles	Budavari et al. 1989
Melting point	80.1°C	Budavari et al. 1989
Boiling point	240°C (explodes)	HSDB 1990
Specific gravity	1.654	Budavari et al. 1989
Odor	Odorless	NIOSH 1990
Odor Threshold:		
Water	No data	
Air	No data	
Solubility:		
Water at 20°C	130 mg/L	HSDB 1990
Organic solvent(s)	Soluble in acetone and benzene; soluble in alcohol and ether	Budavari et al. 1989
Partition coefficients:		
Log K_{ow}	1.60; 2.2 (measured)–2.7 (estimated)	HSDB 1990; Spanggord et al. 1985
K_{oc}	300 (estimated)–1,100 (measured)	Spanggord et al. 1985
Vapor pressure at 20°C	1.99×10^{-4} mmHg	HSDB 1990
Henry's law constant:		
at 20°C	4.57×10^{-7} atm m^3/mole	HSDB 1990
at 30°C	No data	HSDB 1994
Autoignition temperature	No data	HSDB 1994
Flashpoint	Explodes	NIOSH 1994
Flammability and Reactivity	4.4	HSDB 1994
Conversion factors	1 ppm = 9.28 mg/m^3 1 mg/m^3 = 0.108 ppm	NIOSH 1973
Explosive temperature	464°F	HSDB 1994
Explosive limits	No data	NIOSH 1990

Table 2.2
Chemical Properties of 2,4,6-Trinitrotoluene. From USDHS (1995)

Characteristic		Information		Reference
Specific Heat:	20^0 C	0.328 cal/g^0 C		Yinon 1990
	40^0 C	0.345 cal/g^0 C		
	80^0 C	0.374 cal/g^0 C		
Isomers:	2,4,6-(α-),(TNT)	melting point	80.65^0C	Yinon 1990
	2,3,4-(β-),	melting point	112^0C	
	2,4,5-(γ-),	melting point	104^0C	
	3,4,5-(δ-),	melting point	137.5^0C	
	2,3,5-(ε-),	melting point	97.2^0C	
	2,3,6-(η-),	melting point	111^0 C	

Table 2.2 (cont.)
Chemical and Physical Properties of 2,4,6-Trinitrotoluene.
From Yinon (1990)

TNT is relatively insensitive to impact, friction, shock, and electrostatic energy (Yinon 1990). Confined, molten TNT is much more sensitive to impact, approaching the impact of mercury fulminate (Yinon 1990). It can also be detonated by a less severe force when confined between metal surfaces such as threads of a bolt, as cited by Yinon (1990). In thin unconfined layers it usually burns without detonation. Additional information regarding the chemical identity and the physical and chemical properties of TNT is summarized in Table 2.1 and 2.2.

2.3 Production and Characterization of TNT Wastewaters

TNT has not been produced commercially in the United States since the mid-1980's. In 1985, 9.2 million pounds of TNT were imported by the U.S. Army Armament Material Command. TNT is one of the few pure explosives that can be fabricated directly by melting and casting into a desired shape (Dobratz 1981). It is melted while solid ingredients are slowly stirred in. The molten mixture is then vacuum-cast into a mold which is carefully cooled to reduce cracking problems.

Manufacturing plants use a universal process to produce TNT (Fig. 2.1). Liquid toluene is nitrated in a three-step operation with increasing temperatures and addition of mixed nitric and sulfuric acid concentrations to sequentially form nitro groups mononitrotoluene (MNT), dinitrotoluene (DNT), and trinitrotoluene (TNT) (USDHS 1995). The unwanted isomers and residual dinitrated species are removed from the reaction by a treatment with sodium sulfite solution, "sellite", which forms water-soluble sulfonate derivatives. The remaining α-TNT is washed out and cast molten onto a flaker belt for pack-out (Yinon 1990).

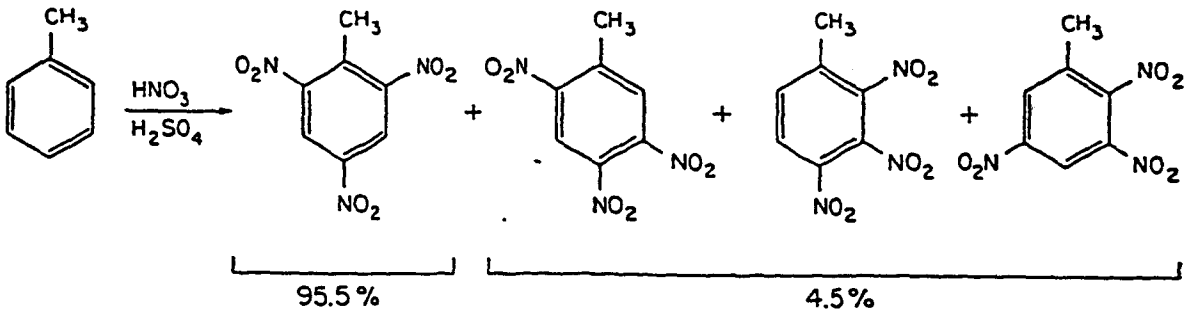


Fig. 2.1 Manufacturing of TNT. From Yinon (1990)

Oxygen-rich products can be added to TNT to form binary explosives which can increase explosive power. As discussed earlier, binary mixtures include the octols, cyclotols, pentolites, tetrytols, amatols, and picratols (addition of ammonium picrate with TNT). Mixtures of TNT-based aluminized explosives include trinotals (TNT + Al), ammonals and minols (TNT + ammonium nitrate + AL), and torpexes and HBX (TNT + RDX + Al) (Yinon 1990). Although the rate of detonation is decreased by adding aluminum in ternary mixes, the blast effect of the explosive increases (Yinon 1990).

The wastes generated during TNT manufacture contain three components: Neutralized spent acids, "Red water", a primary waste formed during the sellite purification of TNT, and "Pink water", a waste formed when purified TNT is water washed after sellite purification (Yinon 1990). The mixed waste is identified by its acidity, yellow to red color, and strong odor. "Red water" waste is a strong red, odorless, mixture with a pH of 8.0 (Yinon 1990).

The primary reaction products from the sellite process are salts of dinitrotoluene and sulfonic acids. Other than α-TNT, 14 other isomers are possible from the five-parent trinitrotoluene isomers (Yinon 1990). Organic constituents of "red water" include smaller fragments, dissolved TNT and complex unidentified dye bodies, formed due to the photolysis of TNT by sunlight, as cited by Yinon (1990). Unreacted sulfite plus nitrite and nitrate formed in the extraction reactions are part of the inorganic constituents. Although "Red water" moderately varies in composition,

it typically contains NaS_2SO_3, $NaSO_4$, $NaNO_2$, $NaNO_3$, sulfonited nitro compounds and solids.

In the finishing process, TNT is dried, flaked, and packaged. A large amount of wash-down water is used to clean equipment and to clean the interior of finishing plant buildings. "Pink water" emerges as a colorless, high-acidic, saturated solution of up to 150 ppm TNT. It is referred to as "pink water" because it turns pink in sunlight by photolysis of dissolved TNT forming complex dye-like molecules and also because the water is neutralizing with sodium carbonate. However, both "red" and "pink water" are not solely identified by their color.

As cited by Yinon (1990) examination of "red water", "pink water", and neutralized spent acids during a one-year study indicated over 30 nitroaromatic compounds in the mixtures. Components were identified by mass spectrometry using a GC/MS system. The Gas Chromatography profiles showed 2,4-dinitrotoluene, 2,6-dinitrotoluene, and 1,3-dintirobenzene as major effluent components, with approximately half of the 32 identified compounds occurring in more than 50% of the analyzed samples. The three major compounds represented approximately 75% by weight of the total compounds (Yinon 1990). N-nitrosomorpholine, a carcinogen was also identified which may have formed by nitrosation of morpholine. Morpholine is used as an algicide in water-cooling towers at certain munitions plants (Yinon 1990).

2.4 Transport and Partitioning

TNT may not partition from surface waters to the atmosphere based on the low vapor pressure of 1.99×10^{-4} mmHg at 20^0 C and high water solubility of 130 mg/L at 20^0 C given in the previous table (USDHS 1995). Insufficient volatilization from aqueous solution was detected in air stripping tests on raw and neutralized wastewater samples, where 8-10 % of TNT was lost during an 18-day test period, as cited by the USDHS (1995). Furthermore, USDHS (1995) cites volatilization half-lives of 10,000 days for ponds, streams, and lakes. A volatilization half-life of 119 days was assessed for a 20^0 C, 1 meter deep model river, flowing at 1m/s, with a wind speed of 3 m/s (USDHS 1995).

TNT will not significantly partition from surface waters to sediment or strongly sorb to soil particulates based on estimated values of 300-1,100 for the soil organic carbon adsorption coefficient (K_{oc}), which is the ratio of the amount a chemical adsorbed per unit weight of organic carbon in the soil or sediment to the concentration of the chemical in solution at equilibrium. This was established in short-term laboratory adsorption/desorption tests and long-term lysimeter studies. 24 hour laboratory batch adsorption/desorption tests were conducted using uncontaminated surface soils collected from 13 Army Ammunition Plants. As cited by the USDHS (1995), the average adsorption coefficient (k_d) for all soils tested had a value of 4 units, which indicates limited sorption ability. Adsorption was consistently lower under oxidizing conditions than under reducing conditions. Most

of the TNT adsorbed was desorbed after multiple extractions of the test soils. The pH did not affect 2,4,6-trinitrotoluene adsorption/desorption or transformation and 4-ADNT and 2-ADNT were both detected under oxidized and reduced conditions

In long-term lysimeter studies, addition of ^{14}C[TNT] to the top three inches of soils in columns with four different soil types ranging in texture from fine to course was examined. The lysimeter was regularly irrigated during the 6-month test period and column leachate samples were taken every two weeks. At the end of the test period, the soil columns were sectioned for analysis. Neither TNT nor its typical biodegradation products were detected in the leachate samples. Analysis of the samples with high ^{14}C activity indicated only highly polar, nonvolatile products which could not be separated or identified. However, 2-ADNT and 4-ADNT were identified in the soil columns with concentrations ranging in the soil columns from 0.01% to 6 % of the radiolabel 2,4,6-trinitrotoluene added to the columns (USDHS 1995).

In other mobility tests with sediments, ring-labeled ^{14}C[TNT] was added to unsterilized sediments collected from two farm ponds upstream from an Army Ammunition Plant site in Syracuse, NY and the Holston River, TN. TNT was not extensively sorbed in 24 tests. Partition coefficients varied with pH and temperature. Desorption of TNT or its breakdown products occurred slowly while steady state conditions were reached after 92 hours in just one sample.

The log octanol/water partition coefficient (k_{ow}) values of 2.2-2.7 suggests that TNT will not bioconcentrate to high levels (i.e. concentrations $\geq$ 1,000 times

media concentrations) in the tissues of exposed plants and animals or biomagnify in terrrestrial aquatic food chains. The USDHS (1995) cites a report where limited bioconcentration was examined in aquatic bioassays with water fleas Daphnia Magna, worms, algae and blue gill sunfish. Bioconcentration factors (BCFs) in 96-hour static tests were 209 for the water flea, 202 for the worms, 453 for algae, 9.5 for fish muscle, and 338 for fish viscera, as cited by the USDHS (1995).

2.5 Solubility

Crystalline TNT persists in soils and can exist as chunks of weathered crystals, as tiny crystals embedded in the soil matrix, or as TNT molecules absorbed on the soil surface (Ro *et al.* 1996). Visible amounts of neat (pure) TNT materials can be hazardous for detonation at open burn/open detonation (OB/OD) cites and are a source of contamination. Environmental fate and mass transport characteristics must be understood for risk assessment and implementation of particular treatment techniques.

Solubility determines the maximum concentration of TNT in the aqueous phase and provides information about the driving force for mass transfer. A precise estimation of temperature is important for successfully modeling the transport process because temperature strongly effects the solubility of TNT (Ro *et al.* 1996). Studies cited by Ro *et al.* (1996) review TNT literature citations, which report TNT

solubility from 85.8 mg/L to 100 mg/L at 21^0 C. Accurate measurements are necessary for TNT solubility for research purposes and for planing site remediation processes.

Ro *et al.* (1996) investigated the effects of temperature and pH on the solubility of both reference TNT and neat TNT, developed a semi-empirical correlation for TNT solubility as a function of an acceptable range of temperature, and compared the solubility of field neat TNT to reference TNT. A large weathered crystalline piece of TNT, obtained from the Alabama Army Ammunition Plant (AAAP), was crushed into different size groups of semidried particles and referred to as field neat TNT. 500 mg of TNT and 100 mL of deionized water was added to nine 100-250 mL amber bottles while temperature was maintained using a constant-temperature water recirculator (Ro *et al.* 1996).

Analysis of solubility as a function of pH and temperature indicated that TNT solubility increased from approximately 52 mg/L at 6^0 C to 205 mg/L at 42^0 C for reference TNT. The solubility of the field neat TNT was similar to reference TNT. At elevated temperatures, TNT solubility decreased when pH increased to 9.3. At different pH's, the solubility of reference TNT and field neat TNT was approximately 101.5 mg/L at 25^0 C. At 42^0 C, TNT solubility doubled to 198 mg/L at pH levels of 4.0 and 6.8. At a pH of 9.3, measurements varied from 32.3 mg/L to 60.5 mg/L.

As cited by Ro *et al.* (1996), an equation relating the solubility and temperature of an ideal solution can be derived from Raoult's law of an ideal

solution. If the solid is transformed into a hypothetical, supercooled liquid, and enthalpy of fusion does not change significantly with a small change in temperature, the following equation can be derived using Clausius-Clapeyron equation:

$$\ln (X) = -H_{fusion}/RT(1/T - 1/T_m) \qquad \text{equation (1)}$$

where,

X	is	mole fraction
H_{fusion}	is	enthalpy of fusion
T_m	is	melting point
R	is	universal gas constant
T	is	absolute temperature

The previous equation implies the temperature dependency of solubility (S) as

$$\ln S = A - B/T$$

Where A and B are empirical constants (Ro *et al.* 1996).

The data show the applicability for both reference and field neat TNT solubilities. Both fitted to the above equation well with R^2 values of 0.97 for reference and 0.96 field neat TNT solubility (Fig. 2.2). The correlation for the temperature range from 6^0 C to 42^0 C were statistically equal at the significance level of 0.05. The semi-empirical TNT solubility correlation based on the reference TNT was

$$\ln\{S/(mg/L)\} = 16.12 - 3413/(T/K) \qquad \text{for } 279\ K < T\ 315\ K$$

equation (2)

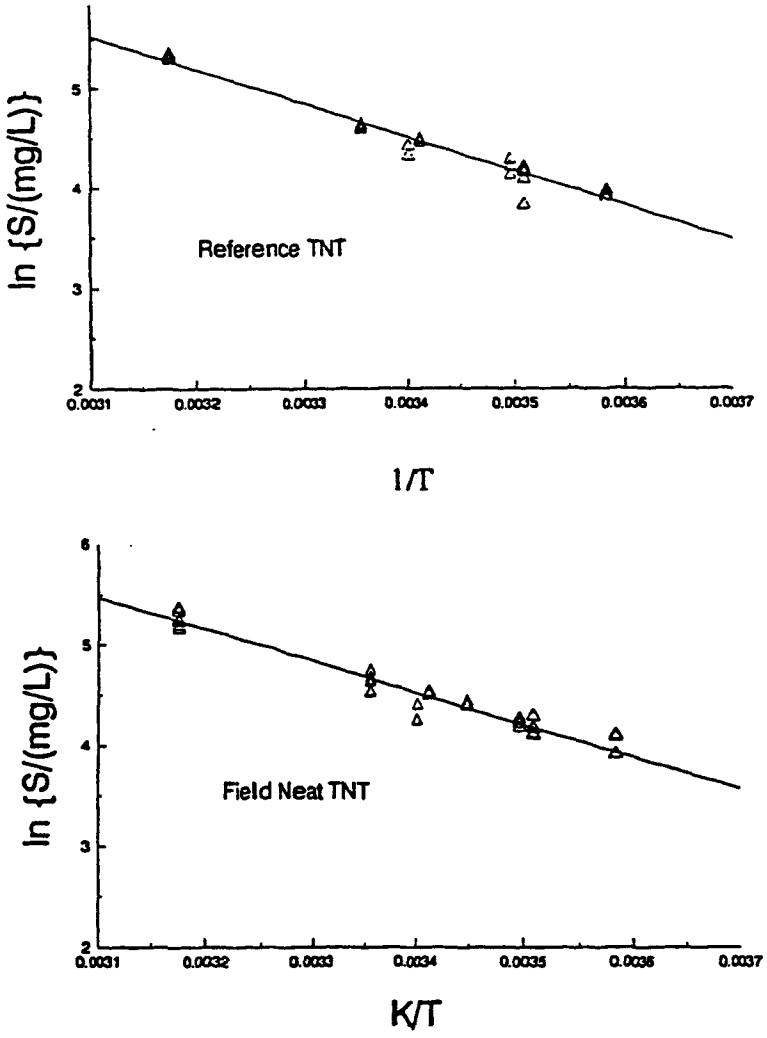


Fig. 2.2 ln S vs. 1/T. From Ro *et al.* (1996)

The solubility found in the study was lower than some of the cited literature although the value of 100 mg/L at 25^0 C reported by the *Merck Index* and *Lange's*

Handbook of Chemistry were within the range 106 and 91 mg/L solubility predicted from equation (2)

2.6 Release in Air, Water, and Soil

TNT is released to the atmosphere due to open detonation and open burning techniques used in the demilitarization of munitions. Furthermore, gases and particulates can be released to the atmosphere from the disposal of munitions TNT in rotary kiln incinerators. Dusts and vapor are also discharged into indoor air and processing facilities during manufacturing of TNT.

TNT released into the atmosphere is degraded by direct photolysis. Estimates of the photolytic half-life of TNT range from 3.7 to 11.3 hours in distilled water (USDHS 1995). As cited by the USDHS (1995), the approximate rate of photooxidation half-life of TNT in the atmosphere is 18.4 days to 184 days. These were based on the estimated rate constant for reaction with hydroxyl radicals in the atmosphere (USDHS 1995).

TNT can be transformed in surface waters by microbial metabolism even though this process is slower than photolysis. Under anaerobic and aerobic environments, the predicted biodegradation half-life of TNT in surface water can range from 1 to 6 months. The estimate is based on aerobic liver die-away test data with unacclimated microorganisms, as cited by the (USDHS 1995). Microbial degradation is slower due to increased toxicity of TNT to aquatic organisms in the

presence of the near ultra-violet component of sunlight (USDHS 1995). Microorganisms such as *Phanerochaete chrysosporium* and Pseudomonads, discussed in later sections, are able to degrade TNT under varying environmental conditions.

TNT buried in soil or on the surface can persist for many years. As cited by the USDHS, smaller amounts may undergo photolysis in surface soils to trinitrobenzene and trinitrobenzaldehyde. Yet, environmental factors such as the effects of soil organic matter content, TNT and oxygen concentration, incubation period, and microbial activity can hinder transformation of 2,4,6-trintirotoluene in soils. These factors were analyzed using ring-labeled ^{14}C[TNT] by the Army in 1985 (USDHS 1985). A pH of 6.5 was maintained in samples which were examined after 6 months and 11 months of incubation. The maximum rate of transformation occurred in soils with low concentration of TNT (0.1%) while the least rate of transformation occurred in soils with high concentrations of TNT (10%). Results indicated that TNT intermediates existed at low concentrations in the soil. The degradation products included 2-amino-4,6-dinitrotoluene, 4-amino-2,6-dinitrotoluene, and diamines. As biotransformation increased, the amount of nonextractable radioactive residue increased, which suggests that the metabolites of TNT sorbed to soils better than the parent compound (USDHS 1995). The initial TNT concentration and the soil moisture level controlled the rate of transformation. The presence or microbial activity and incubation temperature had less effect, and soil organic matter and oxygen concentration had no effect.

2.7 Chemistry of TNT

The chemistry of the nitro group is a resonance hybrid (Fig. 2.3). The polarization of the nitrogen-oxygen bond causes the nitrogen atom to carry a partial positive charge and serve as an electrophile because the oxygen atoms are more electronegative than the nitrogen atom. Therefore, the predominant reaction of the nitro group in biological systems is reduction occurring by either one electron or two electron mechanisms. (Fig. 2.3) Research on TNT bioremediation has primarily focused on reductive or anaerobic environments rather than on oxidative or aerobic environments. The initial step of biotransformation involves reducing the nitro groups to amino groups in both anaerobic and aerobic conditions. The nitro group in the para position is initially reduced by most microorganisms. Further nitro group reduction occurs at either ortho position to create dinitrotoluene isomers. Complete reduction of all three nitro groups is restricted to anaerobic environments (Shelley *et al.* 1996). The sequence of reactions of the nitro group reduced to amino groups produces highly reactive intermediates such as nitroso and hydroxylamino groups which are electrophiles.

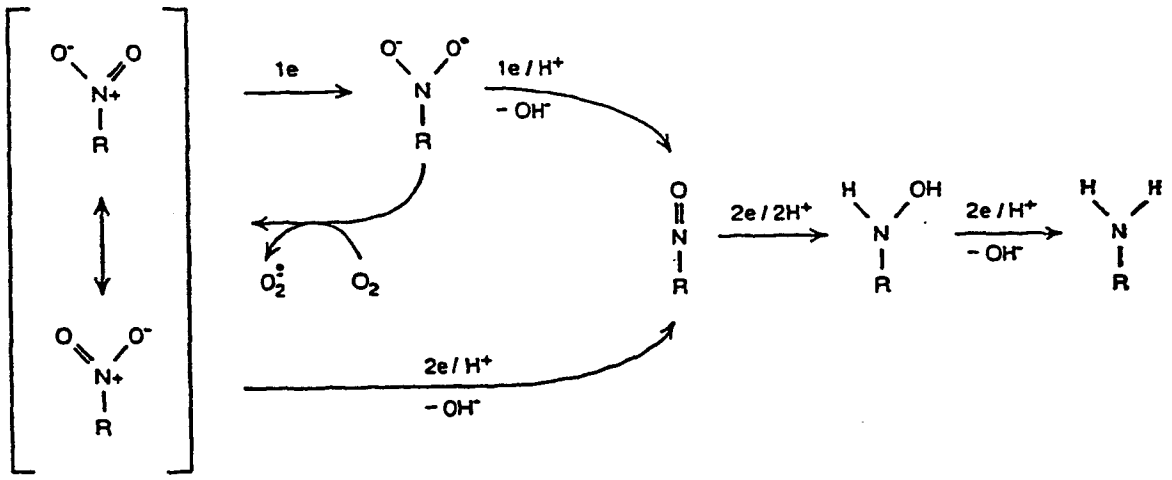


Fig. 2.3 Reduction of nitro groups by one-electron or two-electron mechanisms. From Spain 1995

The one-electron reduction creates a nitro radical anion, which is oxidized to the initial material by molecular oxygen with the coupled production of superoxide (Spain 1995). The enzymes that catalyze one-electron reduction of the nitro group are designated "oxygen sensitive." Anaerobic bacteria, facultative bacteria, as well as plants and animals contain these enzymes.

Reduction of the nitro group by sequential addition of pairs of electrons is "oxygen insensitive" because radicals are not formed. Nitroreductase enzymes transform nitro groups to either hydroxylamines or amines by the addition of electron pairs donated by reduced pyridine nucleotides (Spain 1995). Although nitroso derivatives are difficult to detect due to their instability and reactivity, they also

occur in the reaction pathway. Furthermore, it is the high reactivity associated with the nitroso and hydroxylamino intermediates which causes much of the toxicity and carcinogenity attributed to nitroaromatic compounds. Both intermediates can easily react with a variety of biological materials and can undergo condensation reactions. Spain (1995) cites partial reduction of the nitro group in the presence of oxygen leading to the nonenzymatic production of azoxy compounds as condensation products.

The ease of reduction of the aromatic nitro group depends on the nature of the other substituents on the ring and on the reducing potential of the environment (Spain 1995). Electron-withdrawing groups activate the molecule for reduction of the nitro group, whereas electron donating groups make the ring more susceptible to electrophilic attack. In nitrotoluenes, the probability of reduction increases and the probability of electrophilic attack decreases as the number of nitro groups increases (Spain 1995). Therefore, the reduction of one nitro group of TNT is very rapid under a variety of conditions, including those in aerobic bacteria. In contrast, reduction of 2-amino-4,6-dinitrotoluene (ADNT) requires a lower redox potential, and reduction of 2,4-diamino-6-nitrotoluene (DANT) requires a redox potential below -200mV, because the electron-donating properties of the amino groups lower the electron deficiency of the molecule. (Spain 1995).

Aerobic degradation of nitroaromatic compounds involve an oxidative attack on the ring structure by electrophilic oxygenases (Shelley *et al.* 1996). There are less reaction steps and faster reaction rates that occur under aerobic degradation.

3 MICROBIAL TRANSFORMATIONS

3.1 Biotransformation and Mineralization

Bioremediation can utilize either biotransformation or mineralization by microbial attack upon TNT. Transformation is the modification of the molecular structure of the contaminant to yield other organic compounds (Wolfe 1994). If the transformation products are environmentally and toxicologically safe and stable, bioremediation by transformation is sufficient. Mineralization is the conversion of toxic compounds to yield innocuous inorganic constituents such as CO_2, H_2O, energy, and various metabolites

through enzymatic reactions (Wolfe 1994). If complete mineralization is reached to these simple products, further testing is eliminated. Several bacteria, discussed in following sections, can biodegrade TNT and use the compound as a nitrogen, carbon, and energy source under both aerobic and anaerobic conditions. TNT can be transformed, mineralized, or conjugated into more complex products by organisms such as *Desulfovibrio* sp. (strain B) or *Pseudomonas*.

TNT transformation pathways have been thoroughly examined even though mineralization is more desirable for bioremediation of TNT contaminated soils. Wolfe (1994) cites recent work where aerobic mineralization of TNT occurs through the 1,3,5- and 1,2,3-trihydroxybenzenes, which are subsequently degraded to yield

H_2O and CO_2. Funk *et al.* (1993) reports that anaerobic conditions may promote biodegradation of nitroaromatic contaminants in soils. Parent molecules disappeared within 4 days in soil cultures at temperatures ranging 20^0 C to 37^0 C , and by 30^{th} day, most of the initial radiolabel appeared as 4-amino-2,6-dinitrotoluene (4A2,6DNT) and 2,4-diamino-6-nitrotoluene (2,4A6NT). Residual label was found in other fractions and as a trace amount of CO_2. Adding a second aerobic stage following the initial anaerobic stage would allow the second stage intermediates, 2,4,6-trihydroxytoluene {phloroglucinol or MPG} and *p*-cresol to degrade at a faster rate to CO_2. Further analysis regarding specific organisms is reviewed in the following sections.

3.2 Biodegradation By Fungi

The white rot fungus, *Phanerochaete chrysosporium,* has been evaluated more extensively than any other fungal species for remediating explosive wastes. *P. chrysosporium* is a lignin degrading fungus which produces a complex system of extracellular peroxidases, small organic molecules, and hydrogen peroxide (Spain 1995). The lignolytic system is nonspecific and can biodegrade recalcitrant compounds including nitroaromatics such as TNT.

Generally, the fungal degradation of TNT involves the reduction of a least one nitro group of TNT (Spain 1995). Mycelia of *P. chrysosporium* reduce TNT to a

mixture of 2-amino-4,6-dinitrotoluene, 4-amino-2,5-dinitrotoluene, and 2,4-diamino-6-nitotoluene (Spain *et al.* 1995). Under ligninolytic conditions mineralization is extensive and the amino compounds disappear. Live, intact mycelia are required for the reduction of TNT. Furthermore, Spain (1995) cites that the extracellular enzymes and enzymes in cell extracts cannot catalyze the reaction when added with reduced pyridine nucleotides. The reduction reaction is closely coupled to the proton export system used by the fungus to maintain an external pH of 4.5 (Spain 1995).

Fernando *et al.* (1990) found *P. chrysosporium* to degrade ring labeled [^{14}C]TNT sorbed to soils. Degradation of TNT was evaluated by adjusting the TNT concentration similar to contamination levels encountered in the environment during a 24-day period. *P. chrysosporium* mineralized 35% of the [^{14}C]TNT during 12 days of incubation. Glucose added to the cultures on 18th day did not further reduce [^{14}C]TNT to $^{14}CO_2$. Mass balance analysis indicates that 35.4 $\pm$ 3.5% of the total radioactivity emerged as $^{14}CO_2$, 25.1% was present as water-soluble metabolites, 15.7% was detected in methyl chloride fraction, and 17.3% was associated with mycelial fraction (Fernando *et al.* 1990).

Analysis was also performed on [$^{14}CO_2$]TNT soil mixed with corncobs previously inoculated with *P. chrysosporium.* After 30 days of incubation, 6.3% of the TNT was recovered as $^{14}CO_2$. An extra 63.6% of the radioactivity was recovered in acetonitrile extracts, while 25.2 % was unextractable. In the acetonitrile extract, only 2.2% of the radiolabel was in the form of undegraded TNT (Fernando *et al.* 1990).

After 30-, 60-, and 90-day incubation periods, liquid and soil cultures containing 100 mg/L of TNT and 10,000-mg/kg loadings of ring-labeled ^{14}C[TNT] were extracted and mass balances were calculated. Results of mass balance analysis of 100 mg of TNT per liter of contaminated liquid cultures indicated that 19.6 $\pm$ 3.5% of the recovered radioactivity transformed to $^{14}CO_2$, 22.7% bound to methylene chloride extract, 50.1% existed as water-soluble compounds, and 2.2% bound to the fungal material after a period of 90 days of incubation. A total mass recovery of 94.6% was achieved.

After 90 days of incubation, 18.4 $\pm$ 2.9% of the radioactivity was $^{14}CO_2$, 62.6% in the form of metabolites was present in the acetonitrile extract fraction, and 11.5% bound to soil/fungal matrix. The total mass recovery was 92.5% after a period of 90 days. The concentration of residual undegraded 2,4,6-trinitrotoluene in the 90-day acetronitrile extract was only 14.9% versus 99% activity found in control samples.

Fernando *et al.* (1990) determined that the wood-rotting fungus could treat water, soils, sediments and other materials contaminated by TNT in a short period of time. Compared with liquid cultures, soil cultures converted less [$^{14}CO_2$]TNT to $^{14}CO_2$. However, mineralization of [$^{14}CO_2$]TNT in liquid cultures virtually stopped after 15 days while mineralization in soil cultures continued slowly throughout the 90 days of incubation. Fernando *et al.* (1990) believe that mineralization in soil-corncob matrices can be extended if the incubation period is increased.

The study suggests that *P. chrysosporium* will be useful in the biodegradation in waste treatment systems. Average concentrations of TNT such as 20 mg/liter in effluent and 10,000 mg/kg in soil found at contaminated were not lethal to the fungus in the experiments. Furthermore, pilot-scale studies, at sites such as the Naval Submarine Base, Bangor, Washington, using *P. chrysosporium* reduced a concentration of 1,844 ppm to 1,087 ppm in 120 days (EPA Handbook 1993). The overall degradation was 41% at the former ordnance open burn/open detonation area.

Although *P. chrysosporium* lowered levels of TNT at the Naval Submarine Station, factors such as TNT's toxicity to the fungus, competition from the native bacterial populations, chemical sorption, and the inability to meet risk-based cleanup levels were further evaluated by Spiker *et al.* (1992). Analysis of the survival of *P. chrysosporium* under increasing concentrations of TNT were explored. Uncontaminated soil and contaminated soil containing 12,000 ppm of TNT was collected from the U.S. Army Munitions Depot. The toxicity to *P. chrysosporium* was examined by adding 50 mL of malt extract broth with 0.02 to 2.0% wt./vol of the initial munitions-containing soil.

The highest release of $^{14}CO_2$ occurred at 5 ppm of TNT cultures containing a spore inoculum at time zero in the study (Fig 3.1) and the majority of the $^{14}CO_2$ released was after the 14^{th} day of incubation. Little TNT mineralization occurred at concentrations greater than 15 ppm. The samples containing more inhibiting levels showed mass balance recoveries of 85.8 $\pm$ 7.6% of total radioactivity. In samples

from media containing levels greater than 15 ppm, 100% of the radioactivity was recovered.

TNT mineralization by cultures that were inoculated with mycelia and incubated for 24 days showed no $^{14}CO_2$ release at any concentration tested (Fig 3.1). Reduction of TNT concentrations could be due to the adsorption of TNT by mycelia because lower concentrations of TNT existed wherever mycelia were present. Spiker *et al.* (1992) believes some TNT could have been mineralized and/or transformed to a product not detected by the spectrometric assay. Additionally, TNT losses could also be attributed to adsorption of TNT onto soil amendments, such as corn cobs and straw (EPA Handbook 1993).

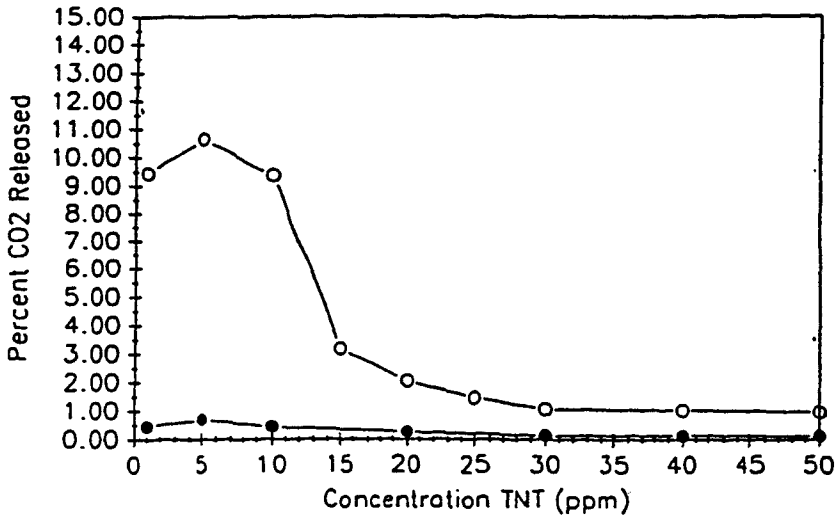


Fig. 3.1 TNT added to spore inoculum: TNT added to pregrown mycelia. From Spiker *et al.* (1992)

Uncontaminated soil did not inhibit the growth of *P. chrysosporium* at any concentration of TNT. The fungus, however, was completely inhibited by small amounts of contaminated soil. Spiker *et al.* (1992) believes that *P. chrysosporium* cannot bioremediate TNT-contaminated sites containing high concentrations of explosives because of its extreme sensitivity to the contaminants. However, the use of a matrix that immobilizes and protects *P. chrysosporium* from exposure to TNT would allow the extracellular enzymes to attack TNT and mineralize a higher percentage of the TNT present (Spiker *et al.* 1992)

Michels and Gottschalk (1994) also examined the effect of increasing concentration of TNT to the rate of degradation by ligninolytic cultures of *P. chrysosporium.* They studied the ability of *P. chrysosporium* to mineralize TNT in the concentration range of 0.36 to 20.36 mg/L. Thirty percent of the 0.36 mg/L of [^{14}C] TNT reduced to CO_2 while 5% of the 20.36 mg/L reduced to CO_2 in 4 days. An NADH-dependent nitroreductase in extracts prepared from *P. chrysosporium* reduced TNT in the preliminary steps. Ligninolytic cultures of *P. chrysosporium* mineralized TNT at an appreciable rate, while nonligninolytic cultures primarily produced the amino derivatives and azoxy condensation products (Michels and Gottschalk 1994).

Under nonligninolytic conditions, TNT is initially reduced to 4-amino-2,6-dinitrotoluene through the nitroso and hydroxyylamino intermediates (Fig 3.2). The 4-amino-2,6-dinitrotoluene is slowly converted to 4-formamido-2,6-dinitrotoluene, which is reduced to 2-amino-4-formamido-6-nitrotoluene and finally converted to

2,4-Diamino-6-nitrototoluene (Spain 1995). Direct evidence did not exist for the reduction of 4-Amino-2,6-DNT to 2,4-Diamino-6-NT.

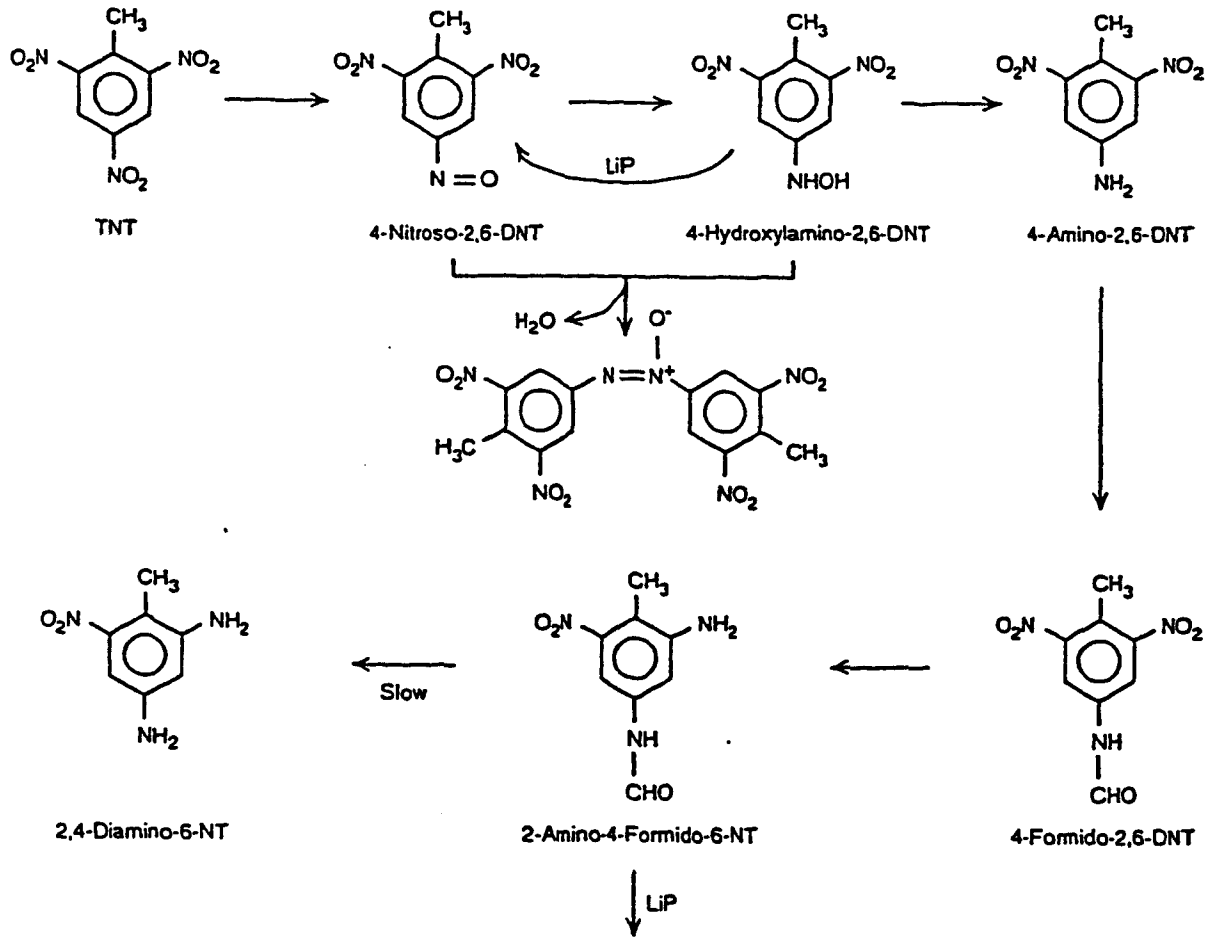


Fig. 3.2 Transformation of TNT by *P. Chrysosporium.* 4-Hydroxylamino-2,6-DNT and 2-amiono-4-formamido-6-nitrotoluene are substrates for lignin peroxidase. From Spain (1995)

Analysis reveals that the mechanism of toxicity of TNT to *P. chrysosporium* is not by the parent compound (TNT) and its amino derivatives. 4-Hydroxylamino-2,6-dinitrotoluene decreases the rate of mineralization by affecting lignin peroxidase. 4-hydroxylamino-2,6-dinitrotoluene and 2-hydroxylamino-4,6-dinitrotoluene inhibit veratryl alcohol oxidation activity of lignin peroxidase.

The conversion of veratryl alcohol to veratryl aldehyde is necessary for producing organic radicals involved in the oxidation of chemicals which are not primary substrates for the lignin peroxidase (Spain 1995). The hydroxylamino compounds are favorable substrates for the enzyme which competitively inhibit the oxidation of veratryl alcohol. The oxidation of hydroxylaminodinitrotoluenes led to the accumulation of azoxy condensation products.

The data presented by Michels and Gottschalk (1994) are important for the design of bioremediation processes for TNT contaminated soil. A method of using *P. chrysosporium* where 4-hydroxylamino-2,6-nitrotoluenes is not produced should be a primary target. Otherwise, corresponding nitrosos compounds are formed, as well as azoxy compounds generated by condensation of the nitroso and hydroxylamino-dinitrotoluenes (Michels & Gottschalk 1994). Conversion to azoxy compounds in ineffective because these compounds are even less soluble and resistant to degradation than TNT.

3.2a "Red Water" treatment

Untreated "red water" cannot be directly discharged into water ways and sewer systems. Tsai (1991) investigated the effectiveness of treating "red water" with *P. chrysosporium.* "Red water" samples obtained were pretreated with ultraviolet (UV) light and incubated under various conditions with fungal culture or crude extracellular enzyme prepared from the culture. The treated samples were analyzed for decolorization and ligninase activity by UV spectral analysis, high performance liquid chromatography (HPLC) metabolite analysis, and bacterial toxicity screen (Tsai 1991). Red color was reduced in all treated samples. UV spectral analysis showed a progressive decrease in the aromatic region (200-300 nm).

The data form the HPLC analyses showed that fungal enzymes altered red water components. HPLC analysis diminished TNT, 2,4-dinitrotoltuene, and 2,6-dinitrotoluene peaks in "red water" samples treated with concentrated fungal enzyme preparation. The same treatment also reduced the toxicity of the "red water" as tested by a bioassay. The preliminary results suggest that the *P. chrysosporium* is effective in biodegrading and reducing biotoxicity of "red water" in one week under controlled laboratory conditions with the whole fungal culture or with the extracellular enzyme preparation containing ligninase activity (Tsai 1991).

3.3 Anaerobic Biodegradation

TNT in some contaminated sites has leached deep into the soil to anaerobic areas and into groundwater. Boopathy *et al.* (1993) explored whether TNT could be removed anaerobically under different electron accepting conditions. The anaerobic soil bacteria was analyzed for removal of TNT in the presence of various electron acceptors. Of the three different electron accepting conditions, nitrate, sulfate, and methanogenic, a large amount of TNT was removed in the enrichment culture that used nitrate as an electron acceptor (Fig 3.3).

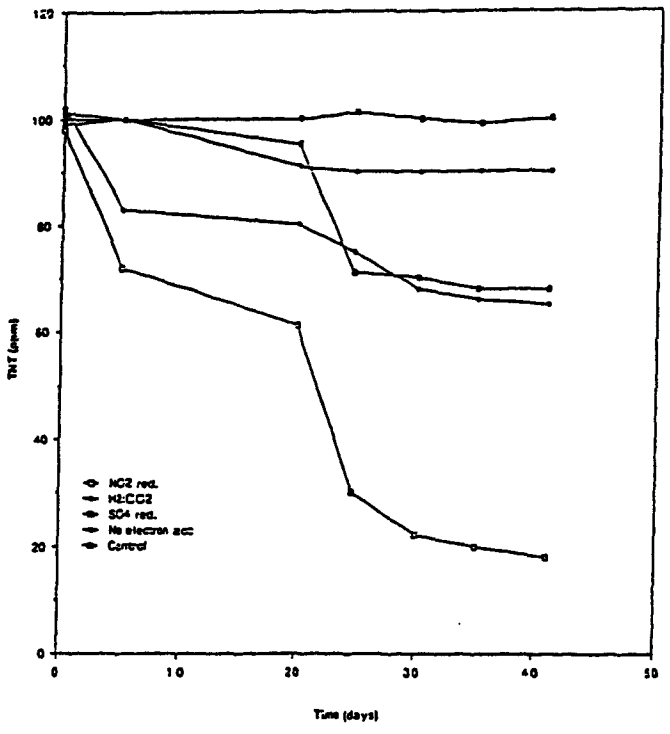


Fig. 3.3 Concentration of TNT in anaerobic bacterial consortium enriched under different electron accepting conditions.
From Boopathy *et al.* (1993)

Under sulfate reducing and H_2:CO_2 methanogenic conditions, average removal of TNT occurred while under acetotrophic conditions, where no external electron acceptor other than TNT was present, TNT concentration remained at 100 ppm. The nitrate reducing enrichment culture transformed 80% of the original 100 ppm of TNT within 40 days of incubation while sulfate reducing conditions and H_2:CO_2 methanogenic conditions only removed 30% of the TNT (Boopathy *et al.* 1993). Bacterial growth took place in flasks that held primary substrates such as lactate or acetate, which indicates that the anaerobic population cannot grow on TNT as the sole source of carbon and energy, but could transform TNT by co-metabolism with another carbon source (Boopathy *et al.* 1993). Furthermore, under nitrate reducing conditions, the enzyme nitrite reductase acted on the nitrite group of TNT and reduced it to an amino group. Similarly, under sulfate reducing conditions the activity of sulfite reductase enzyme reacted with TNT and reduced it to an amino compound.

The pH was maintained between 6.8 and 7.2 in all culture bottles throughout the study and bacterial growth was examined by the protein content of the cultures. The highest growth was detected in the nitrate reducing conditions at 90 mg/L on day 40 of incubation. Growth was lower under sulfate reducing and H_2:CO_2 methanogenic conditions. Boopathy *et al.* (1993) believe that the soil was comprised of large numbers of nitrate reducing bacteria and the activities depended on the availability of other carbon sources and electron acceptors. Examination of the soil

indicated that it was composed of various microbiological forms of gram-negative rods as well as some vibrio shaped organisms (Boopathy *et al.* 1993).

The HPLC analysis of the culture supernatant showed the presence of at least two intermediate products. A fragmentation profile similar to that of 4-amino-2,6-dinitrotoluene (4ADNT) and 2-amino-4,6-dinitrototoluene (2ADNT) was detected. These intermediates persisted in the culture media even after 60 days of incubation.

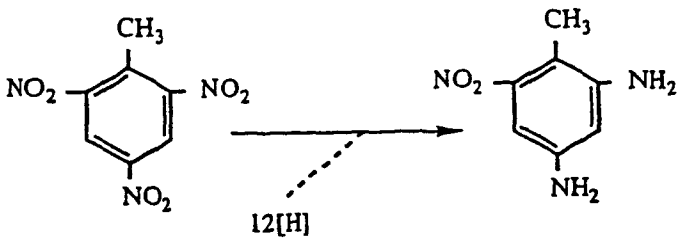
The study suggests that TNT contaminated soil collected from the anaerobic-aerobic zone could be transformed to amino compound intermediates by anaerobic bacteria under nitrate reducing, sulfate reducing and methanogenic conditions. Although the amino intermediates detected may be toxic to many organisms, explosive TNT is reduced to non-explosive amino compounds. However, soil samples collected in the study represent only one area of the contaminated site which probably contained site-specific organisms. Other contaminated areas at site probably have unique bacterial populations reacting differently with TNT. Therefore, Boopathy *et al.* (1993) proposed isolating an anaerobic bacterium that would completely degrade TNT to CO_2 and be applied to any contaminated area.

Boopathy and Kulpa (1994) examined a methanogenic bacteria for detoxification of TNT. A strain of *Desulfovibrio, Methanococcus* sp. (B strain), was studied extensively with 100 ppm TNT because of its ability to transform nitroaromatic compounds. An HPLC at an optimal pH range of 6.8 -7.2 and at a temperature of 30^0 C was used to examine the TNT in the culture sample. The H_2-CO_2 carbon substrate culture transferred 100 ppm of 2,4,6-trinitrotoluene within 40

days. TNT concentration in the cultures containing formate declined to 2 ppm after 60 days of incubation. In the heat-inactivated killed control and in cultures using TNT as a sole carbon and energy source for nine months, TNT concentration remained the same.

The culture conditions in the heat-inactivated controls were similar to the experimental cultures with a carry-over sulfide concentration of 0.1mM from the inoculum. Reduction in TNT concentration to the presence of reductant sulfide did not occur (Boopathy and Kulpa 1994). Boopathy and Kulpa (1994) believe that TNT degradation was mediated by *Methanococcus* sp. (B Strain) because of low concentrations of sulfide. Furthermore, experiments show that TNT metabolism was a co-metabolic process requiring either formate or H_2-CO_2 as the primary substrate and that the reduction of TNT was due in part to the growing cells (Boopathy and Kulpa 1994).

Mass spectral analyses of the culture samples showed one intermediate, which was identified as 2,4-diamino-6-nitrotoluene. 97 ppm of 2,4-diamino-6-nitrotoluene was produced from 100 ppm of TNT. This intermediate was not further degraded by *Methanococcus* sp. Strain B (Figure 3.4). Boopathy and Kulpa (1994) suggest that TNT could be used as an electron sink under anaerobic conditions by methanogens as they discovered with sulfate reducing bacteria.



Trinitrotoluene 2,4-diamino-6-nitrotoluene

Fig. 3.4 Metabolism of TNT by *Methanococcus* sp. Strain B. TNT was an electron acceptor for this isolate.
From Boopathy *et al.* (1993)

Preuss *et al.* (1992) isolated a strain of *Desulfovibrio* from sewage sludge of a digestor (Germany) by selective enrichment using TNT as the nitrogen source, pyruvate as the carbon source, and sulfate as the terminal electron acceptor. The strain fixes atmospheric nitrogen and can also use ammonia as its nitrogen source (Preuss *et al.* 1995). Under growth conditions the sulfide in the medium chemically reduces TNT to DANT. The DANT is then reduced by the *Desulfovibrio* sp. to TAT, which subsequently disappears from the culture fluid (Spain 1995).

Suspensions of cells containing little or no sulfide catalyze the reduction of TNT to TAT when pyruvate is supplied as the electron donor (Spain 1995). The rate of reduction of each successive nitro group decreases dramatically because amino groups restrain the molecule for further reduction. The reduction of TNT to DANT occurs by nonspecific enzymes that reduce low-potential electron carriers such as ferredoxin (Preuss *et al.* 1992). The mechanism of conversion of DANT to TAT was further analyzed because it may be the limiting step in the overall process.

Hydrogen, pyruvate, or carbon monoxide can serve as electron donors for the reduction of DANT by cell suspensions of *Desulfovibrio* sp. The ferredoxin-reducing enzymes hydrogenase, pyruvate-ferredoxin-oxidoreductase, and carbon monoxide-dehydrogenase can reduce DANT. When hydrogen or pyruvate are the electron donors, DANT is reduced to TAT. However, DANT is only partially reduced, to 2,4-diamino-6-hydroxylaminotoluene (DAHAT) when CO is the electron donor. Moreover, when pyruvate is the electron donor and CO is present, TAT reduction stops and DAHAT begins to form. Similarly, when hydroxylamine is included in cell suspensions using hydrogen as the electron donor and CO is present, inhibition results.

DANT reduction with cell suspension analysis lead Preuss *et al.* (1992) to believe that sulfite reductase may be responsible for the reduction of DAHAT to TAT, because sulfite reductase is also inhibited by carbon monoxide. *Desulfovibrio* sp. was inhibited by carbon monoxide, DAHAT, and hydroxylamine. Results indicate that sulfite reductase is responsible for DAHAT conversion to TAT which is not in agreement Boopathy *et al.* (1993) because in his study TAT was not metabolized by the *Desulfovibrio* sp. The overall scheme of TNT reduction by the *Desulfovibrio* sp. is outlined in figure 3.5. (SPAIN 1995)

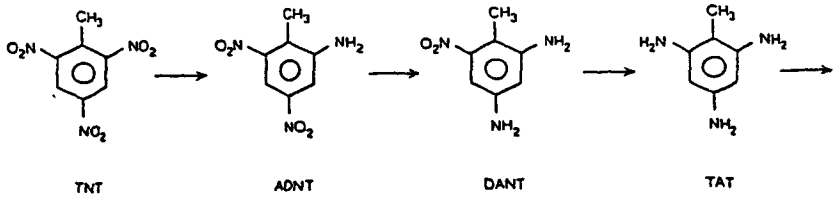


Fig. 3.5 Reduction of TNT. From Spain (1995)

The microbial conversion of TAT by cell suspensions of isolate occurred only under aerobic conditions. TAT breakdown under aerobic conditions by trace elements present in the cells probably occurred due to a catalysis, since autoclaved cells also mediated the conversion of TAT (Preuss *et al.* 1995). Trace elements are also catalyzed the conversion of TAT in the presence of oxygen and in the absence of cells. An effective TAT conversion rate of 420 nmol/l X min was observed by using magnesium as a catalyst. The rate of TAT conversion depended on the cell concentration and was higher than the maximum rate of magnesium catalysis by a factor of up to 5 (Preuss *et al.* 1995). Preuss *et al.* concluded that cellular components might be involved in TAT conversion and the products of conversion are not known. In aerobic cell suspensions, one third of the amino groups were released as ammonia.

An isolate which could use TAT as the only source of nitrogen under anaerobic conditions was obtained. Research suggested that the isolate was a species

of *Pantoea* which converted TNT at pH values lower than 6 to unknown products in the presence of glucose, which was used as the energy and carbon source. Furthermore, 0.4mM TAT released 0.5 mM NH_4^+ which indicates that the conversion of TAT by the isolate was a nonspecific reaction due to the chemical instability of TAT rather than to microbial metabolism (Preuss *et al.* 1992). The analysis of TAT degradation was performed to show that TAT can be further transformed.

Even though *Methanococcus* sp. (B strain) reduces TNT to intermediates that may be more toxic, the soil is already highly contaminated with mono-, di, and trinitrotoluene, as well as with aminonitro compounds. Boopathy and Kulpa (1994) suggest that sulfate reducers, nitrate reducers, and other anaerobic bacteria used in a mixed culture system will strengthen the degradation process under anaerobic conditions. Furthermore, Preuss *et al.* (1992) believes that the most efficient decontamination procedure should lead to a convergent degradation pathway for the biodegradation of all nitro compounds present in the soil by reduction. Although sulfidogen organisms can quickly reduce the nitro groups, further analysis regarding TAT degradation to products such as CO_2 must be performed.

3.4 Clostridium

Clostridia have also been studied because of their ability to reduce nitroaromatic compounds. As cited by Spain (1995), hydrogenase from *Clostridiumn pasteruianum* and carbon monoxide dehydrogenase from *Clostridium thermoaceticum* reduce DANT to DAHAT when ferredoxin is included in reaction mixture or, with reduced ferredoxin or methyl viologen in the absence of enzymes. The enzymes are only present in this process to reduce ferredoxin and are not specific for the nitroaromatic compound (Spain 1995). Consequently, the extensive distribution of non-specific, oxygen-sensitive nitroreductases in biological systems reflected the wide distribution of redox enzymes. Considerable reduction for these circumstances occur because many nitroaromatic compounds can be reduced nonenzymatically by a variety of reductants including iron (II), sulfhydryl compounds, and small electron carriers such as ferredoxin (Spain 1995). However, as cited by Spain (1995), the Clostridia slowly convert DAHAT to TAT and probably require action specific enzymes such as the ones used by *Desulfovibrio* sp.

Crawford *et al.* 1994 isolated pure cultures of obligate anaerobes that degrade TNT. A gram-positive, rod-shaped bacterium capable of growth containing 30 ppm mineralized TNT. An APE-AN-IDENT culture identification test and motilitiy and an rRNA gene sequence indicated *Clostridium bifermentans* as the closest match. Biodegradation of TNT of strictly anaerobic bacterial co-culture was observed and preliminary evidence suggested that the co-culture degrades TNT fermentatively,

producing volatile organic acids and metabolized TNT efficiently when a co-substrate was provided. Apparently, the agar in the original isolation medium provided contaminating nutrients sufficient for formation of colonies in which growth was primarily at the expense of TNT (Crawford *et al.* 1994).

Results indicated that a 10% *clostridial* inoculum with a 3% (w/v) addition of starch removed 100 ppm of TNT from the soil in 24 hours at room temperature. Intermediates including 4-amino2,6-dinitrotoluene, and 2,4-diamino-6-nitrtoluene were detected in the early phases of degradation, but were then removed. Higher concentration were not examined in the study. Since the solubility of TNT in aqueous solution is about 100 mg/liter, it must be solubilized as it is degraded. Therefore, concentrations of TNT above solubility are not a problem in terms of toxicity to the clostridia in the system.

The studies described indicate that reduction of the nitrogroup is the major reaction controlling the behavior and fate of nitroaromatic compounds in ecosystems that contain bacteria. The reactions that convert TNT to TAT can be catalyzed by a variety of bacteria., but analysis of metabolism of TAT is presently a controversial topic among researchers.

3.5 Ruminal Bacteria

The research concerning biological transformation of TNT, using soil, sediment, or sludge bacteria, results in slow, ineffective treatments. Organisms becoming sensitive to low levels of TNT and metabolism rates varying from three days to three months to biotransform 100 ppm of TNT are just some of the problems with bioremediation. As discussed, organisms such as *Desulfovibrio* sp. and aerobic *pseudomonads* incompletely biotransform TNT. As cited by Craig *et al.* (1994), other organisms such as *P. chrysosporium* and *V. alkalescenes* produce azoxy compounds as end products, which may be more toxic and persistent than TNT (Craig *et al.*1994).

Recently, researchers have studied ruminal microorganisms in relation to their ability to degrade xenobiotics. The rumen, a foregut organ of some herbivorous animals, contains a bacterial consortium which ferments materials that enter the animal's digestive system (Craig *et al.* 1994). Ruminal bacteria are capable of biotransforming toxic compounds found in plants at very fast rates, as well as other pollutants with chemical similarities to TNT and other ammunitions. Craig *et al.* (1994) conducted a study where the rumen system was used to obtain a new isolate (G.8), which transformed TNT at rates faster than previously reported without any detectable accumulation of toxic intermediates or end products.

A TNT-degrading ruminal microorganism was isolated from goat rumen fluid with successive enrichments on triaminotoluene (TAT) and TNT. The G.8 isolate

utilizes nitrate and lactate as the primary energy source as cited by Lee *et al.* (1995). G.8 tolerated metabolite levels of TNT up to the saturation point of 125 mg/l. Rumen samples were collected form goats and ewes through rumen canals and buffered with sodium carbonated under aerobic conditions

Research indicated that a consortium of ruminal bacteria was capable of biotransforming TNT. Disappearance of TNT incubated with G.8 is slower than the disappearance of TNT with ruminal fluid, but both systems are faster than those previously reported. MADNT and DAMNT were observed as transient intermediates in the analysis while toxic azoxy compounds were not detected in the analysis of this bacteria (Craig *et al.* 1994). Although the pure isolate G.8 cannot biotransform TNT as completely as the full consortium, experiments indicate that it is a key participant in the process. The ability of G.8 to reductively deaminate aromatic compounds, and in some circumstances to de-methylate, is an important step in the overall biotransformation pathway of TNT (Craig *et al.* 1994) Thus, the rapid TNT transformation is promising for early development of a TNT bioremediation system.

Based on results from their previous studies, Lee, Williamson and Craig (1994) also tried to understand the process of TNT degradation by the denitrifying ruminal microorganisms G.8. The study identifies specific metabolites, defines TNT destruction pathways, and examines transformation mechanisms on the different primary electron acceptor.

G.8 growth was monitored by optical density using different electron acceptor energy sources such as nitrates, nitrites, or TNT to investigate the relation of TNT to G.8 metabolism. Nitrate was the only energy source that G.8 grew upon. Additionally, experiments observed the influence of TNT by utilizing the primary energy source with G.8 growth. Nitrate was converted to nitrite completely followed by further reduction of the nitrite (Lee *et al.* 1995).

TNT and each of its transformation products were incubated individually with a G.8 medium containing lactate as the electron donor and nitrate or nitrite as the primary electron acceptor for identifying the biotransformation pathways and limiting factors (Lee *et al.* 1995). TNT transformation pathways were established with a series of connections as shown in the figure 3.6.

The G.8 isolate reduced the nitro group to an amino group and was also involved in deaminization as a co-metabolite, resulting in nitrogen free compounds such as toluene or o-cresol (Lee *et al.* 1995). The reduction (deaminization) and oxidation (hydroxylation) reactions occurred simultaneously when the amino group was present.

As seen in earlier discussions, degradation of compounds is dependent on the characteristics of the parent compounds, the microbial consortium and environmental

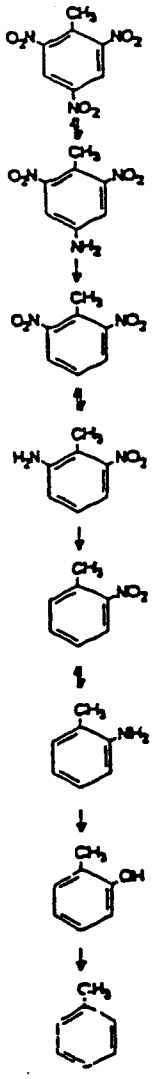


Fig. 3.6 Proposed Pathways for TNT biotransformation with G.8 incubation. Pathways established with series of connection of the identified transformation products by GC/MS and HPLC.
From Lee *et al.* (1995)

factors. The patterns of TNT metabolite transformation in the experiments performed by Lee *et al.* 1995 were dependent on the type electron acceptors. The presence of nitrates in the medium stimulated the reduction of para-positioned nitrogroups, and nitrites stimulated the deaminization and hydroxylation processes

(Lee *et al.* 1995). The absence of primary energy sources as nitrates or nitrites stimulated the reduction of the ortho-positioned nitro groups.

3.6 Actinomycetes

As cited by Grisby *et al.* (1996), *Actinomycetes* are important decomposers in composting systems. The tolerance of *Actinomycete* cultures from both TNT-contaminated and uncontaminated environments was examined and analyzed to determine if TNT transformations were fundamental or induced by exposure to TNT.

Thirty one Streptomycetes were isolated for the uncontaminated areas. Sixteen actinomycetes including ESA 1 and ESA 16, were isolated from TNT-contaminated soil. TNT tolerance of the isolates was tested with two set of plates containing 25, 50, 75, and 100 mg of TNT per liter with brain heart infusion agar (BHI) and yeast malt extract agar (YMA).

TNT tolerant *Actinomycetes* isolated from TNT-contaminated or TNT-free soils reacted very similarly in test conditions. As cited by Grisby *et al.* (1996), TNT tolerance of soil organisms was not a function of the TNT content of their native soil, but that concentrations of TNT>50 mg/liter prevented or severely limited the growth of most fungi, yeasts, *Actinomycetes*, and gram-positive bacteria. Grisby *et al.* (1996) indicates that a few *Actinomycetes* grew on YMA at 75 mg/liter while only *Streptomycetes chromofuscus* A11 and *Actinomycetes* ESA 1 grew at 100 ppm.

However, on the richer BHI Agar all the isolates grew up to the maximum concentration of 100 PPM. None of the 31 *Actinomycetes* grew when TNT was the sole carbon and nitrogen source for the four different TNT concentration plates. This indicated that a nutrient-rich growth environment can help overcome the toxicity or inhibitory effects of TNT toward *Actinomycetes.*

Grisby *et al.* (1996) showed that no significant mineralization occurred when an *Actinomycete* indigenous to a TNT-contaminated site and one never exposed to explosives were grown in six different laboratory media. The actinomycetes, *S. chromofuscus* A11 and ESA 1, did transform TNT to 2,4DA6NT and 4A2,6DNT. Although, a higher rate of transformation was achieved in the BHI medium, where a rich supply of nutrients and oxygen was present, data indicate that even if conditions were improved, the costs incurred would limit the use of the organism. Furthermore, transformation of TNT actinomycete-rich aerobic environments like composts lead into intermediates which are known to form recalcitrant polymers.

3.7 Aerobic Biodegradation

In contrast to nonspecific metabolism by fungi and anaerobes, aerobic bacteria can specifically use nitroaromatic compounds by deriving nitrogen, carbon, and energy form degradation and as using as growth substrates. Bacteria that can mineralize 2,4-DNT have been isolated from a variety of contaminated soils and studied in pure cultures. 2,4-dinitrotoluene (2,4-DNT) is a by-product of the

manufacture of TNT and is also used extensively as an intermediate synthesis of toluene diisocyanate (Spain 1995). As cited by Spain (1995), mineralization of a nitroaromatic compound through a dioxygenase initial attack was reported as a result of examination of *Pseudomonas* sp. strain grown on 2,4-DNT. The dioxygenase enzyme that catalyzes the initial reaction is constitutive and has a broad substrate range that is similar to napthalene dioxygenase. It adds hydroxyl groups to the 4 and 5 positions on the ring of 2,4-DNT, and the nitro group is eliminated nonenzymatically as nitrite (Fig 3.7).

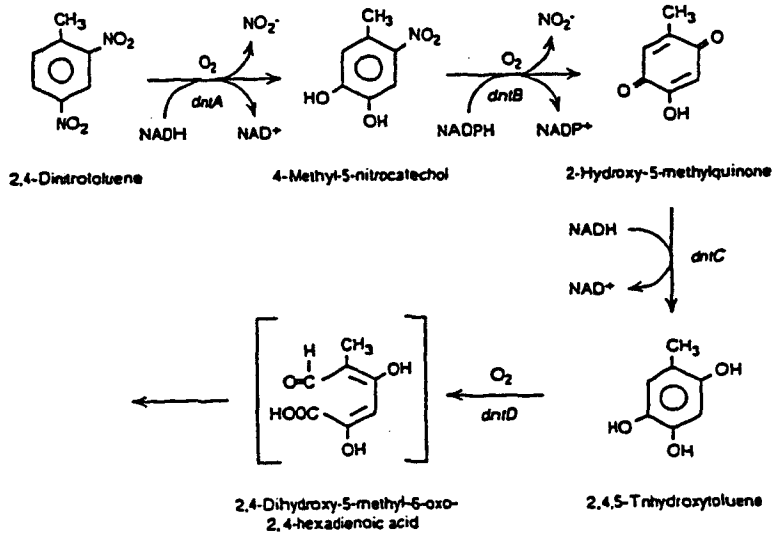


Fig. 3.7 Biodegradation of 2,4-dinitrotoluene by *Pseudomonas* sp. strain. From Spain (1995)

3.8 Pseudomonads

Pseudomonad bacteria (*Pseudomonas* sp.) reduce TNT under aerobic conditions in laboratory studies to monoaminodinitrotoluenes and diaminomononitrotoluene. *Pseudomonads* isolated from mud and water samples collected at U.S. naval ammunition Dept. at McAlester, Oklahoma, biotransformed 2,4,6-trinitrotoluene in laboratory studies. As cited by the USDHS (1995), TNT was degraded within 24 hours in cultures supplemented with yeast extracts with complete dissimilation occurring in the most active isolate within 24 hours. Degradation products identified include 2,2'6,6'-tetranitro-4,4'-azoxytoluene; 9,4'6,6'-tetranitro-2,2'-azoxytoluene; 2-amino-4,6-dinitrotolune; 4-hydroxylamino-2,6-dinitrotolune, and nitrodiamintolune. *Pseudomonads* isolated from Narragansett Bay, sediments, raw sewage, and boiler plant effluents were able to utilize ring labeled [^{14}C]TNT as a sole carbon source in laboratory degradation studies, as cited by the USDHS, (1995). TNT transformation varied with the concentration of the test compound in the medium. 2,2,6,6-Tetranitro-4,4-azoxytoluene was the only transformation product identified. The transformation removed the nitro groups from the aromatic ring because nitrite was identified in the medium. The recovery of 0.8%-1.2% of the label was in the form of $^{14}CO_2$.

Pseudomonas aeruginosa strain Ma01 was analyzed for metabolism of TNT. *P. aeruginosa* cultures grown in an anaerobic environment with TNT and succinate as co-metabolites did not produce large amounts of the TNT reduction products. *P.*

aeruginosa was also able to degrade purified aminodinitrotoluene isomers in aerobic conditions. Cultures metabolizing TNT produced large amounts of the ADNT's and did not further metabolize them. The data implied that *P. aeruginosa* used O_2 to oxidize the aminodinitrotoluene products of TNT nitroreduction. However, products of ADNT were not identified. Kitts *et al.* (1995) believe that using a combination of aerobic and anaerobic conditions will efficiently degrade mixtures of RDX and TNT.

Microbial inocula isolated from sewage treatment plant effluents, wastewater from a TNT ordnance loading facility, soil suspension, and aquarium water were capable of degrading TNT in yeast extract solution in shake flask cultures over 38^0 C over six days. TNT concentrations were reduced from the initial loading of 100 mg/l to 0-6 mg/l over the six day incubation period. Transformation did not occur in cultures containing only TNT and mineral salts. Inocula isolated from raw sewage was ineffective in transforming TNT and inocula isolated from sewage sludge digestor liquor reduced TNT concentrations by 64% over the test period. TNT also degraded in tests with pure culture of *P. aeruginosa* when glucose and nitrogen in the form of mineral salts were added to the culture medium.

In the research done by Duque *et al.* (1993), another *Pseudomonas* isolate bacterium which uses TNT, 2,4-dinitrotoluene and 2,6-dinitrotoluene and 2-nitrotoluene as its sole nitrogen sources was studied. The isolate accumulated NO_2^- in the culture medium, and TNT transformation was inhibited at concentrations below 20 mg/L. *Pseudomonas* sp. strain C1S1. *Pseudomonas* sp. clone A, a

derivative of this strain able to grow faster on TNT, were isolated after a series of enrichments in batch culture (Duque *et al.* 1993).

Growth of *Pseudomonas* sp. clone A was faster than the parental strain at both high and low concentrations of TNT. Furthermore, no accumulation of nitrite occurred in culture supernatants because it contained higher nitrite reductase activity (Duque *et al.* 1993). Nitrite reductase activity was influenced by TNT in both *Pseudomonas* sp. strain C1S1 and its derivative. Both the parental *Pseudomonas* strain and its derivative utilized TNT as a nitrogen source by eliminating nitro groups and the producing 2,4- and 2,6-dinitrotoluene, 2-nitrotoluene, and toluene.

As cited in the paper, the removal of the first nitro group occurred after nucleophilic attach by a hydrogen ion on the aromatic ring of TNT, as in the chemical reaction described Meissenheimer. The hydrogen ion can be supplied *in vivo* by NAD(P)H. A nitro group at either the *ortho* position or the *para* position was eliminated from the TNT ring because both 2,4-dinitrtoluene and 2,6-dinitrotoluene were present. Removal of the second nitro group from 2,6-nitrotoluene and 2,4-dinitrotoluene may yield 2-nitrototoluene because *p*-nitrotoluene was not detected in cultures of bacteria grown on TNT or 2,4-dinitrototoluene. Duque *et al.* (1993) believes that the removal of the nitro group at the para position of 2,4-dinitrotoluene is more likely than on 2,6-nitrotoluene.

Under anaerobic conditions *Pseudomonas* sp. clone A did not use TNT as a carbon source. This is because the key enzymes such as toluene dioxygenase, benzoate dioxygenase, and catechol 2,3-dioxygenase in the oxidation of toluene

require O_2 as a substrate. However, removal of the nitro groups from TNT took place as predicted by the reaction involved in the formation of Meissenheimer complex.

Both strains were able to reduce nitro groups on the TNT ring through hydroxylamine to amino groups, leading to monoamino-dinitroluenes and diamino-dinitrotoluenes which were resistant to biological attack by the *Pseudomonas* strains isolated. Further reduction of the nitro groups to amino groups probably occurs through successive addition of two electrons even though the nitroso intermediates have not been identified. Duque *et al.* (1993) found nitro groups on the aromatic ring are reduced by nonspecific reductases on TNT as observed with other microbes.

The aerobic transformation of TNT is a very time consuming process and reduction of nitrotoluenes leads to aminotoluenes, a disadvantage for the use of the system in for mineralization. However, *Pseudomonas* strains combined with other microorganisms which can destroy resulting metabolites may provide a successful treatment system TNT degradation.

3.9 Degradation in an Aerobic Reactor

Collie *et al.* (1994) monitored the metabolism and detoxification TNT in liquid phase bioreactors by placing pure TNT in a liquid medium with four different bacterial strains. A concentration of 70 mg//L of TNT placed in wastewater was periodically sampled and extracted out of the bioreactor liquid for mutagenicity testing and byproduct identification using an HPLC.

All test cultures including one *Enterobacter*, one *Pseudomonad*, and two *Alcaligenes* were isolated from aged soil contaminated with munitions and grown in carbon- and nitrogen-limited media. The four aerobic bacterial species transformed TNT to different intermediates at different rates of time. 2-Amino-4,6-dinitrotoluene and 4-amino-2,6dinitrotoluene were identified in all reactors at varying concentrations after 12 hours of incubation. *Alcaligenes* 1-15 transformed 98.13 $\pm$ 0.86% of the initial TNT after being placed in the reactor for 48 hours. *Enterobacter* 1-5, *Pseudomonas* 1-7, and *Alcaligenes* 1-18 transformed 27.68 $\pm$ 4.79%, 54.54 $\pm$ 3.49%, and 21.95 $\pm$ 4.15%, respectively.

The concentrations of 2-amino-4,6-dinitrotoluene and 4-amino-2,6-dinitrotoluene in the *Alcaligenes* 1-15 reactor after 48 hours was at a concentration of 4.17 $\pm$ 0.17 mg/L and 10.20 $\pm$ 0.11 mg/L, respectively. The three other reactors showed lower concentrations of 4,6-dinitrotoluene and 4-amino-2,6-dinitrotoluene

because the transformation rate was slower. Furthermore, 2,4-diamino-6-nitrotoluene was detected at low levels in just the *Alcaligenes* 1-15 reactor.

Collie *et al.* (1995) observed the bacterial strains utilizing distinct pathways for transforming TNT. The I-15 extracts decreased in TNT concentration steadily while monoaminodinitrotoluene metabolites increased and then fell, followed by a gradual increase in diaminonitrotoluene (Collie *et al.* 1995). Furthermore, no relationship existed between TNT metabolite formation and mutagenicity. Results indicated that as the mutagenicity of all bioreactor extracts decreased due to reduction of TNT concentration, all TNT intermediates were less mutagenic than TNT (Collie *et al.* 1995) However, no correlation was shown between mutagenic potential and the identified metabolites.

Each aerobic bacteria did affect the mutagenicity of TNT. A combination microbial mutagenicity assay and chemical analysis provided essential information which unattainable with either method alone (Collie *et al.* 1995). Collie *et al.* (1995) suggests further studies characterize the genotoxic interactions of TNT metabolites and examine using a varied group aerobic bacteria, because preliminary results verify reduction of the genotoxicity of TNT.

3.10 Summary of Mineralization Pathways

Glycolysis and other biological pathways are controlled by enzymes that are rate-limiting. ATP synthesis can be regulated by these enzymes at different steps. As cited by Shelley *et al.* (1996) cells obtains much of their energy and elemental carbon from these three steps which, in turn produces large negative Gibbs free energy changes. TNT is also used in biological systems for nitrogen and carbon sources and in the degradation pathways can possibly contain enzyme-controlled steps. Although these steps are not fully understood, Shelley *et al.* (1996) believes that the pathway steps with large negative Gibbs free energy changes in TNT biodegradation are potentially rate controlling. TNT biodegradation pathways are briefly reviewed from previous sections. The pathways are initially anaerobic; however, aerobic microorganisms can be utilized for ring cleavage and final mineralization of TNT intermediates (Shelley *et al.* 1996).

3.10a TNT pathway A

Desulfovibrio sp. (B strain) metabolized TNT with toluene as the main metabolic product (Boopathy *et al.* 1993). The first three steps in the pathway reduced the nitro groups to the corresponding 2,4,6-triaminotoluene. Toluene is then obtained by reductive deamination of 2,4,6-triaminonitrotoluene.

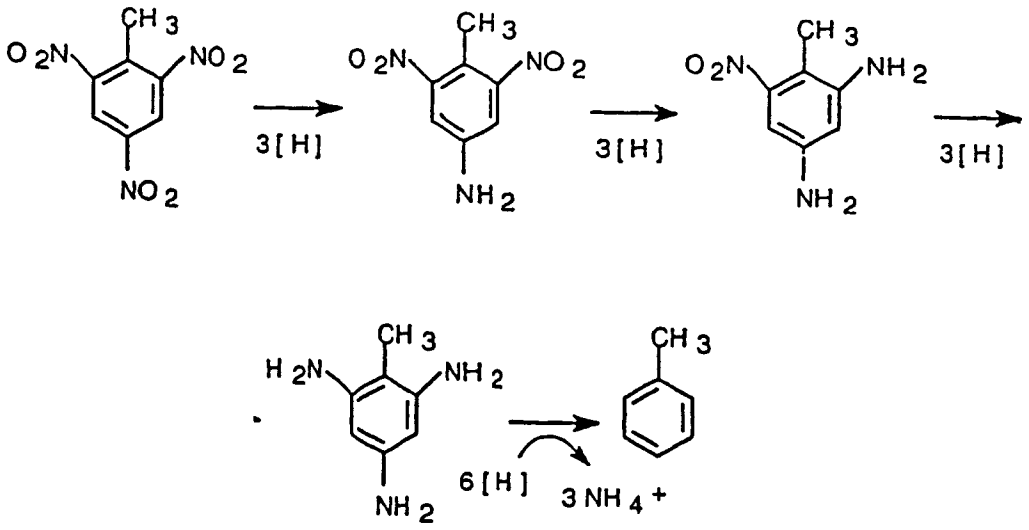
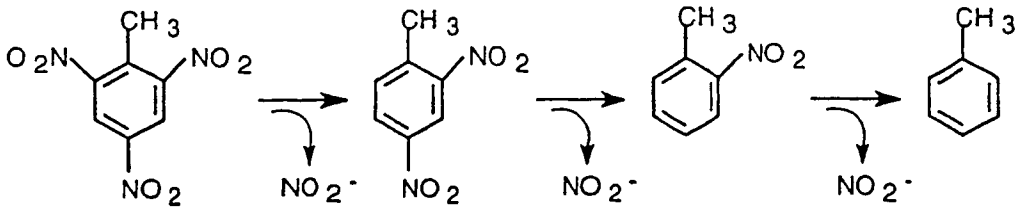


Fig. 3.8 TNT pathway A. From Shelley *et al.* (1996)

3.10b TNT pathway B

The isolation of two *Pseudomonas* hybrid sp. strain C2S2 and sp. clone A metabolized TNT to toluene (Duque *et al.* 1993). Three nitro groups on TNT are removed as nitrite ions. As cited by Shelley *et al.* (1996), these *Pseudomonas* strains remove nitro groups similar to a reaction on picric acid by the bacterium, *Rhodococcus erthroplis* HL 24-2. The nitro group removal involves the formation of a hydrided Meisenheimer complex. A hydride-Meisenheimer complex is the primary metabolite in aerobic TNT bioconversion (Spain 1995). Toluene is recovered after the three nitro groups on TNT are removed.



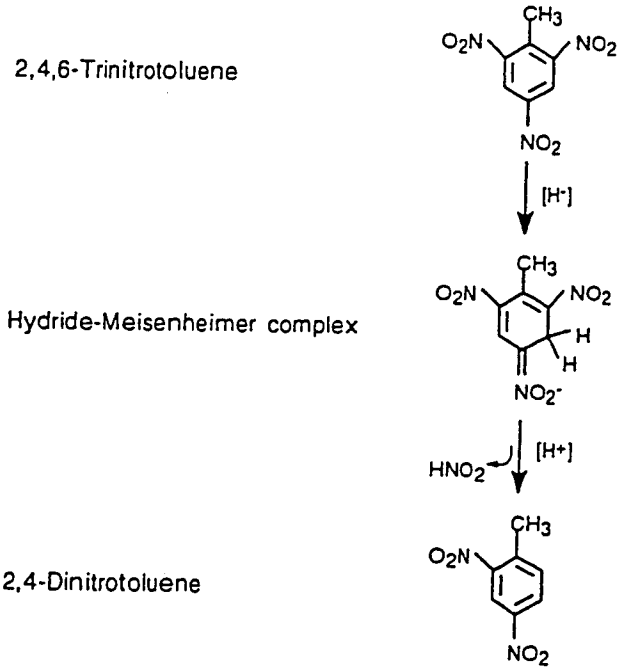


Fig. 3.9 TNT pathway B including proposed mechanism for nitro group removal by hydride-Meisenheimer Complex, from Shelley *et al.* (1996)

3.10c Toluene pathways A, B, C

Toluene, which is the metabolite from pathways A and B, is transformed into TCA cycle intermediates under both aerobic and anaerobic conditions. Aerobic catabolism occurs through direct ring attack by oxygen-dependent enzymes in

pathway A to form 3-methyl catechol. Another oxygen dependent enzyme causes ring cleavage of the methyl catechol intermediate as cited by Shelley *et al.* (1996). The second degradation approach involves methyl group hydroxylation in pathway B to form benzoate while catechol is created after another oxygenase reaction. Ring cleavage occurs through the meta cleavage pathway using plasmid-encoded enzymes, as cited by Shelley *et al.* (1996).

Anaerobic degradation of toluene occurs by ring substitution of CO_2 to form toluate, by ring substitution of H_2O to form *p*-cresol, or by hydroxylation of the methyl group to form benzoate, as cited by Shelley *et al.* (1996). Denitrifying bacteria such as *Pseudomonas*, favor anaerobic biodegradation through methyl group hydroxylation by pathway C, as cited by Shelley *et al.* 1996 Aerobic biodegradation is beneficial and bioremediation of toluene will probably occur under aerobic conditions. However, thermodynamic comparison must occur through an anaerobic pathway such as methyl group hydroxylation. Under anaerobic conditions, ring cleavage is simplified by a hydration reaction while further catabolism of the cleaved ring involves the incorporation of three coenzyme A molecules.

TNT pathway C

Funk *et al.* (1995) reports native bacteria from TNT-contaminated soil inoculated with dinoseb-degrading soil or anaerobic TNT degrading methanogenic bacteria can biodegrade triaminotoluene. The stepwise reduction of the TNT nitro groups yield triaminotoluene as previously outlined in TNT pathway A. The

anaerobic pathway for triaminotoluene biodegradation continues through methyl phloroglucinol and *p*-cresol (Funk *et al.* 1995).

(c) TNT Pathway C

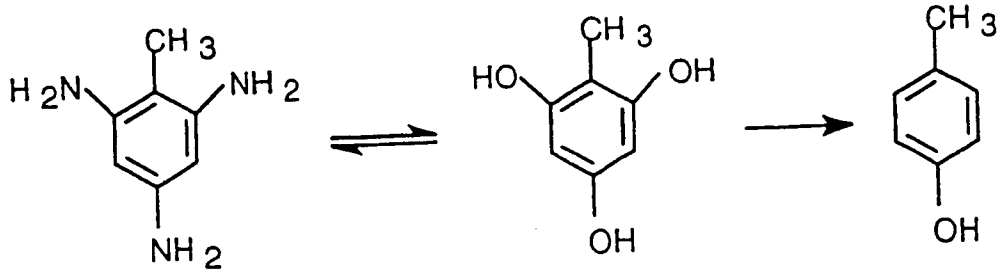


Fig. 3.10 TNT pathway C. From Shelley *et al.* (1996)

3.10 e *p*-cresol Pathways A, B, C

Organisms which can transform *p*-cresol from pathway C into TCA cycle intermediates exist under both aerobic and anaerobic conditions. Catabolism of *p*-cresol occurs by monoxygenase directly attacking the aromatic ring forming 4-methyl catechol, as in pathway A and B. Ring cleavage and mineralization occur through the meta-cleavage pathway, as cited by Shelley *et al.* (1996). The second degradation approach involves methyl group hydroxlyation forming form 4-hydroxybenzoate, as in pathway B. Protocatechueate is produced after another oxygenase reaction occurs and is mineralized through the 3-oxoadipate (ortho-cleavage) pathway as cited by Shelley *et al.* (1996). Anaerobic degradation of *p*-cresol is believed to occur through methyl group hydroxylation to form 4-hydroxybenzoated (*p*-cresol pathway C). Reaction with coenzyme-A creates 4-

hydroxylbenzoyl-CoA, which is dehydroxylated to yield a key intermediate, benzoyl-CoA. Further catabolism involves ring cleavage through a hydration reaction and the incorporation of three coenzyme A molecules Shelley *et al.* 1996).

3.11 Thermodynamic Analysis

Shelley *et al.* (1996) proposed that rate-controlling steps in the outlined TNT biodegradation and mineralization pathways will have large negative Gibbs free energy changes. The biological standard Gibbs free energy change is given by:

$$\Delta G^{o\prime} = -RT \ln K$$

where the equilibrium constant, K, represents the activities of the products divided by the reactants with each activity raised to the power of its stoichiometric coefficient. (Shelley *et al.* 1996).

The Gibbs free energy of formation for a compound is the free energy required to create the compound from its elements (Shelley *et al.* 1996). The difference in Gibbs free energies of formation between the products and the reactants of a reaction represents the standard Gibbs free energy ΔG^{o}, for that reaction. The Gibbs free energy of formation at the biological standard state is unknown for many biochemical compounds. A procedure is necessary for assessing the Gibbs free energy of formation for TNT biodegradation intermediates.

Using the reported TNT biodegradation pathways, the group contribution method was applied to each step. Group contribution methods requires that biochemical compounds be decomposed into functional groups, such as hydroxyls. Each functional group is assigned a value based on the group's partial contribution to the total thermodynamic property of the compound. Functional group values were obtained using linear regression based on biochemical compounds with known Gibbs free energies of formation. The biochemical group contribution method cited by Shelley *et al.* (1996) provided a precise procedure for assessing the standard Gibbs free energy change values. The overall Gibbs free energy changes for the TNT biodegradation pathways are given in table 3.1.

Table 3.1 Overall $\Delta G^{o\prime}$ from TNT to citrate.

Pathway	Total Gibbs Free energy (kcal/mol)
TNT pathway A + toluene pathway A	-543.0
TNT pathway A + toluene pathway B	-744.6
TNT pathway A + toluene pathway C	-336.0
TNT pathway B + toluene pathway A	-227.7
TNT pathway B + toluene pathway B	-429.3
TNT pathway B + toluene pathway C	-20.7
TNT pathway C + *p*-cresol pathway A	-484.7
TNT pathway C + *p*-cresol pathway B	-690.3
TNT pathway C + *p*-cresol pathway C	-286.8

From Shelley *et al.* (1996)

All possible pathways were used to generate the table because a variety of microorganisms can degrade TNT and its intermediates. Although TNT pathways A, B, and C are initially anaerobic, these pathways combined with their corresponding aerobic toluene or *p*-cresol pathways hold the largest overall negative free energy changes. TNT degradation released more free energy in the aerobic reactions than anaerobic reactions (Shelley *et al.* 1996).

Within each initial TNT degradation pathway, the pathway combinations containing toluene pathway B and *p*-cresol pathway B are the routes used most often by microorganisms. As cited in the paper, microorganisms appear to utilize only those enzyme-catalyzed processes that produce the greatest possible energy return for the cell. Biodegradation of TNT may provide a greater energy source for microorganisms than more typical sources such as glucose or organic acids.

From a thermodynamic perspective, further research should concentrate on smaller $-\Delta G^{o\prime}$ rate controlling steps within TNT pathway C and *p*-cresol pathway B as well as methods of increasing the enzyme levels for rate controlling steps (Shelley *et al.* 1996). Enzymes would therefore, increase forward reaction rate and overall TNT mineralization.

4 MICROBIAL TREATMENT PROCESSES

4.1 COMPOSTING

Composting technology is based on an empirical approach to manage biodegradation of organic wastes (Woodward 1990). Historically, composting was used to increase the rate of biodegradation of a variety of organic wastes and recently has been recognized as a potential treatment of hazardous wastes, such as TNT. Traditionally, composting includes the use of thermophilic conditions and thermophilic microbial consortia to biodegrade in an enriched matrix of nutrients and moisture. The process primarily depends on the exothermic reactions of the microbial consortium and the insulating properties of the compost matrix.

The composting process is divided into four major microbiologically important phases controlled by temperature: (1) the mesophilic phase, (2) the thermophilic phase, (3) the cooling phase, and (4) the maturation phase (Adrian *et al.* 1995). The process begins in the mesophilic temperature range of 20 to 45^0 C. As microbial respiration increases during matter decomposition, the temperature increases and enters the thermophilic temperature range of 45^0 C or greater. The largest amount of organic matter decomposition and biomass formation occurs in the thermophilic temperature range. As organic matter limits microbial respiration, the composting process enters a cooling phase followed by a maturation stage of the

compost. As cited by Adrian *et al.* 1995, a mature compost stabilizes when organic matter for microbial respiration is not available.

The operation is enhanced if parameters such as oxygen, moisture, pH, carbon-to-nitrogen ratio, and temperature are monitored. Moisture and temperature must be maintained between 40% and 60% of water-holding capacities and 25 and 60^0 C, respectively (Adrian *et al.* 1995). As discussed by Maleki (1994), initial pH should be close to neutral, ranging between 6 and 8 and initial carbon-to-nitrogen ratio should be maintained between 26 and 65. The most important parameter is the availability of oxygen and aeration levels must remain between 5% and 15% for optimizing the composting process (Adrian *et al.* 1995).

4.1a Relevant Studies

Early studies provided mixed results on the completeness of degradation. Osmon and Andrews (1978) conducted simple land farming experiments on small plots amended with TNT. Although appropriate regulating controls such as pH, moisture, nutrient availability and mixing technique were not well defined, the study did have promising results. Of the 10 treatment regimes, five achieved 65% or better reduction of TNT in 10 weeks of incubation. 10% dry weight TNT disappeared in spite of the fact that the metabolic pathways in the experiments were not analyzed.

In follow-up-work, Kaplan and Kaplan (1982) found that thermophilic biotransformation of TNT followed the same transformation scheme as mesophiles.

Nitro groups are reduced to amino groups without aromatic ring cleavage. Intermediates from the degradation of ^{14}C[TNT] accumulate in an insoluble humic-like fraction. Kaplan and Kaplan (1982) concluded that composting offered no advantage over mesophilic biodegradation because the thermophilic metabolic pathways produced the same toxic and mutagenic intermediates as the mesophilic ones (Kaplan and Kaplan 1982). Composting incorporates these into the insoluble humic fraction. The USDHS (1995) cites a bench-scale composting study, consisting of 10% TNT, where removal of TNT occurred in 55 days, and many of the toxic transformation products formed in activated sludge and soil were undetected. Furthermore, the USDHS (1995) cites field studies which show that composting is effective in removing TNT from contaminated lagoon sediments under both thermophilic and mesophilic conditions.

Greene *et al.* (1985) conducted soil column biodegradation studies with simulated "pinkwater". Explosive concentrations, total organic carbon, nitrates, nitrites, ammonia, pH, redox potential, and biotransformation products were monitored. Results indicated that land treatment was an unacceptable treatment option for "pinkwater" because there is a lack of biodegradation of some constituents of "pinkwater", generation and accumulation of potentially toxic intermediates and reaction products, and the potential for additional groundwater and soil contamination (Greene *et al.* 1985). Greene *et al.* (1985) concluded that further studies should analyze the C:N ratio to account for the C:N ratio contributed by TNT

because microorganisms would have to use nitrogen from TNT rather than producing undesirable intermediates.

Caton *et al.* 1994 investigated the distribution of ^{14}C-activity of ^{14}C-TNT into free and insoluble fractions from field-scale composting, and the potential environmental availability of the transformed product. Soil contaminated with explosives was supplemented with ^{14}C labeled TNT and composted in a field static pile composting experiment. After 90 days of composting, the distribution of ^{14}C-activity fractions from acetontitirile extraction and filtration, alkaline hydrolysis, and combustion of the residue showed that the bulk of the ^{14}C-activity transformed the products of the ^{14}C-TNT, accumulated in a noextractable, but hydrolyzable fraction (Caton *et al.* 1994)

Griest *et al.* 1994 conducted experiments such as the simulated "1000-year" acid rain leaching test and UV irradiation on field composting systems to analyze the ultimate fate of the explosives and their environmental availability in the compost. The matrix studied contained 10% by volume of contaminated soil form the UMDA at Umatilla, Oregon. The compost consisted of 33% cow manure, 22% alfalfa, 22% saw dust, 17% potato waste, and 6% apple pomace. 400 g of compost was added with .18 mCi of ^{14}C-TNT. A scaled down version of the USEPA Synthetic Precipitation Leaching test was adapted for small samples of the compost. Three 0.5 aliquots of 90-day ^{14}C-TNT compost were weighted into three 40 ml volume vials and added with 10 mL of pH 5 water. The vials were capped and tumbled for 18 hours and compost residues were obtained after centrifugation.

Results indicated that even after "1000" years of simulated acid rain leaching, less than 10% of the TNT transformation products would be released form the composted soil. Griest *et al.* (1994) suggests that the potential toxicological impact of the small percentage is probably insignificant, although it was not established in the study. Results from the study also indicate that exposure to UV light had little effect on the leachability of the TNT transformed products.

In the study done by Breuiting *et al.* (1995), two composting systems were compared on a laboratory scale for possible solubilization of the contaminants from the humin matrix under severe conditions. Breuting *et al.* (1995) believes that nitrotoluenes are loosely held to the humin matrix leaving compost systems ecotoxicologically sensitive. The study explored a securer composting procedure for detoxifying TNT-containing soils.

Two different composts containing highly contaminated soil were examined for degradation or fixation of TNT and its metabolite to the humin matrix. Compost 1 was aerated for 28 days while compost 2 was initially flooded with tap-water for 65 days and after drainage, aerated for 97 days. Both composts contained 3.5 kg of up to 20g of TNT/kg dry weight and 3 kg of chopped sugar beet. The overall compost moisture was stabilized to 60% during the aerobic phase of both composts (Breitung *et al.* 1995).

In aerated compost 1, TNT declined by 92% although, approximately 25 % of its original concentration was recovered by an acidic treatment. Compost 2 was slower and contained 4-amino-2,6-dinitrotoluene (4A2DNT) and 2-amino-

4,6dinitrotoluene (2A4DNT), which only declined in the subsequent aerobic phase. In compost 1, TNT was rapidly reduced from 5.6g to 2g TNT/kg during 28 days while 4A2DNT and 2A4DNT remained stable

The paper cites the humic substances model proposed by Ziechmann, in which humic substances are characterized as a complex containing inner core structure and outer edges, which are connected to non-humic organic compounds. In this complex, space is left to accommodate and integrate small molecules such as TNT. Therefore, a treatment with acid liberated 25% of the non-covalently bound and un-metabolized TNT. The study provided no conclusions of the fate of the remaining 75% of the TNT in compost 1.

No firm bindings of aromatic nitro compounds to humic substances have been reported. Breitung *et al.* (1995) suggests that TNT must be initially converted into its amino derivatives of a covalent integration into the humus matrix because the binding to this matrix is more feasible than that of a nitrogroup.

The results presented by Breuting *et al.* (1995) are a promising starting point for improvement on optimizing the anaerobic pre-phase in compost 2 by decreasing the time of TNT reduction to its amino derivatives and increasing the metabolism rate of these compounds in the subsequent aerobic phase (Breuting *et al.* 1995). The toxicity experiments should be expanded by using a variety of cellular systems to analyze the entirely unknown toxicity and mutangenicity of TNT metabolites. Furthermore, the microbial organisms implemented for TNT metabolism in the soil should be more closely studied. This may help represent a convenient and

manageable procedure to remove poisonous matter from contaminated soils (Breuting *et al.* 1995).

4.1b Implementation

Four methods of composting have been explored by the Department of Defense (DOD):

1. static-pile composting
2. in-vessel composting
3. mechanically agitated, in-vessel composting
4. windrow composting

In static pile composting, material is excavated, placed in a pile under protective shelter, and mixed with degradable carbon sources. The pile undergoes forced aeration to maintain aerobic and thermophilic conditions, which increases the growth of microorganisms. Bulking agents, such as cow manure or vegetable waste are added to increase biodegradation. In-vessel is similar to static-pile composting except that the compost pile is placed in a vessel. In a mechanically agitated, in-vessel composting system, contaminated material is aerated and blended with carbon-source materials in a mechanical composter. The difference of windrow composting and static pile composting is that the compost is aerated by a mechanical mixer, rather than a forced air system (EPA Handbook 1993). Figure 4.1 shows general diagrams of each composting method.

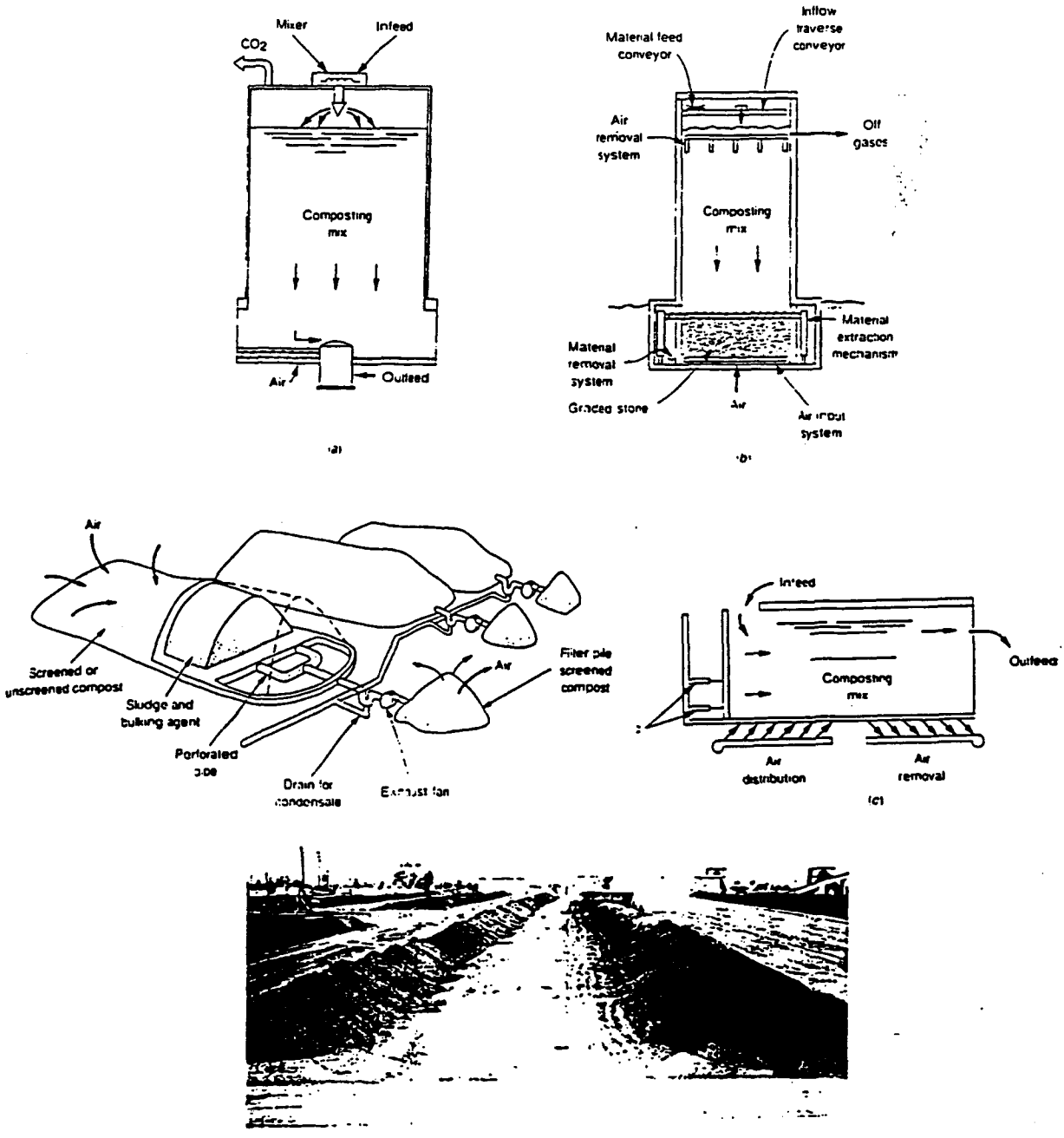


Fig 4.1 Examples of (1) plug-flow-in-vessel composting reactors a, b, c (2) aerated static pile (3) and Windrow Composting.
From Tchobanoglous and Burton (1991)

The U.S. Army began a series of demonstration studies in 1988 at the Louisiana Army Ammunition Plant (LAAP) to determine the effectiveness of composting of lagoon sediments contaminated with TNT, RDX, and HMX. Sediment from LAAP containing 56,800 mg/kg of TNT was added to a compost mix containing straw/horse manure, alfalfa, and horse feed with a temperature inside the pile at 55^0 C (USDHS 1995). Laboratory studies demonstrated the transformation of TNT because $^{14}CO_2$ was formed from radiolabeled parent substrates (Adrian *et al.* 1995).

Field scale studies were conducted to evaluate the effect of temperature on the process. Lagoon sediments contaminated with the explosives were mixed with horse manure, straw, alfalfa, horse feed, and fertilizer and composted for 153 days. The total explosive concentrations in both the mesophilic and thermophilic composts dramatically decreased to less than 400 mg/kg. After 22 weeks, the total explosive content of the compost reduced by 99%. However, results indicate that static-pile composting is not cost effective for remediating large volumes of explosives wastes. In another study, the Army analyzed a commercially available mechanically agitated composter with use of an inexpensive carbon-source material. Because of the high initial costs, the method was also determined to be economically infeasible.

The U.S. Army also examined the effectiveness of windrow composting by using cow manure, sawdust, and potato wastes at the Umatilla Depot Activity. Temperatures were maintained at 55^0 C and compost was turned daily. 98% of the explosives was reduced within 20 days and some of the TNT had been mineralized.

Radiolabeled TNT tests indicated strong binding between TNT and the humic compost. Moreover, initial costs were relatively low and windrow composting determined to be an economically feasible alternative. It was put into operation at the Utamilla Army Depot for remedial action of 300 tons per day of TNT washout lagoons (USEPA Handbook 1993).

The Department of Defense has evaluated composting systems to treat explosive wastes and found that, unlike incineration, composting generates an enriched product that can sustain vegetation. Furthermore, the technology is cost-effective, compared to physical destruction methods and is perceived by the public as an environmentally friendly. Once cleanup levels are achieved the compost material can be returned to the site and covered with a soil cap. Composting also provides both aerobic and anaerobic treatment for treating a wide range of waste.

Although composting is encouraging, the contaminated soil must be excavated and transported, supplemental carbon must be added, and in some cases, suitable microbial population must be inoculated and maintained in the contaminated soil. Composting also requires long treatment periods and composting of unfamiliar contaminants can generate toxic by-products. Furthermore, the ultimate fate of TNT during composting of contaminated soils is not yet clear. As cited by Caton *et al.* 1994, ^{14}C labeled TNT radio tracer studies showed very little mineralization of the TNT into CO_2. Monoaminodinitro- and diaminomononitrotoluenes and other metabolites are found, but they do not account for the decreased TNT concentration.

The bulk of the TNT is in an insoluble "bound" fraction, which resists extraction with organic solvents.

4.2 BIOSLURRY TREATMENT

Bioslurry is an engineering arrangement of other more widely used biotreatment approaches such as landfarming and composting that are used for decontamination of soils and sludges (Zappi *et al.* 1994). Bioslurry is similar to other biotreatment processes in terms of microbiological interactions and contaminant pathways. However, bioslurry systems are capable of increasing the degradation rate of contaminants by increasing the availability of contaminant, electron acceptors, nutrients, and other microbial consortia (Zappi *et al.* 1994). Mixing soil in a water slurry at a soil-water loading of 20%-50% [w/w] will increase biodegradation. Mass transfer limitations associated with biotreatment of contaminated soils are also reduced (Zappi *et al.* 1994). For aerobic systems, oxygen levels are maintained by diffusing air or oxygen to the soil/water slurry. These operational features allow for efficient rates and completeness of reactions in a biological system (Zappi *et al.* 1994).

The study performed by Zappi *et al.* (1994) analyzed TNT degradation using native soil aerobic bacteria. Aerobic bioslurry treatment evaluated contamination

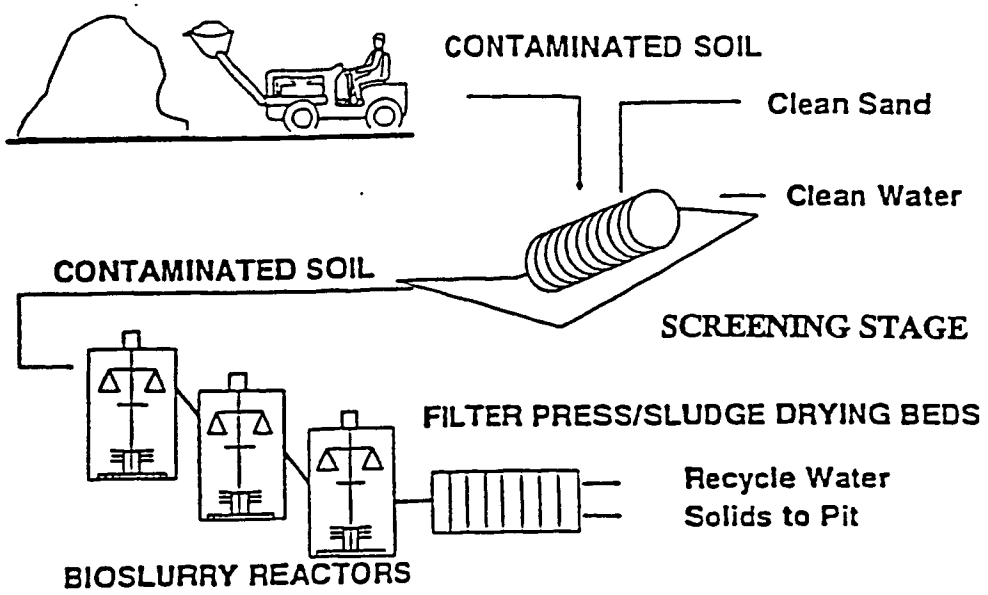
levels of 18,000 mg/kg of TNT using five liter bench-scale bioslurry reactors in the study (Fig 4.2).

Exotic and native consortia were used for aerobic mineralization of TNT with as high as 25% conversion using acetate as a co-metabolite. Commercially available surfactants were also evaluated and considered food-grade (green) surfactants (Zappi *et al.* 1994). Tween 80 at a 3% dose (w/w soil) improved the apparent desorption rate of TNT. Based on the analysis, four treatment conditions were evaluated in the bench scale reactors: 1) Acetate amendments, 2) Acetate with ammonia and phosphate amendments, 3) Acetate and Surfactant amendments, and 4) Acetate, Surfactant, with ammonia and phosphate amendments (Zappi *et al.* 1994). Acetate was dosed at a 1% (w/w soil) weekly while ammonia and phosphate were dosed on an as need basis at 50 mg/l and 10 mg/l concentration, respectively. Each treatment condition was monitored for a period of 11 weeks and soil samples were collected every other week for microbial enumeration for total heterotrophs and acetate/TNT degraders (Zappi *et al.* 1994). Oxygen uptake rates (OUR's) were also measured twice a week for an assessment of aerobic microbial activity occurring within each week.

OUR's in all four treatment conditions remained at levels higher than 20 mg/l/hour, indicating high aerobic activity, and then gradually decreased over time to 5 mg/l/hour by the end of the study. The reactors remained under aerobic conditions throughout the study and, after the fourth week the anaerobes began to use the explosives as a source of activity maintenance (Zappi *et al.* 1994).

The surfactant amended reactor had higher TNT removal rates compared to those without surfactant amending. Also, the surfactant amended bioreactors showed a faster rate and yield return of aminodinitrotoluene production which gradually decreased in concentration after the ninth week of incubation. A steady reduction of TNB concentration over time was reached in the seventh week to below detection levels in the surfactant amended bioreactor.

The results indicate that the native microbial consortia had considerable activity toward TNT when amended with acetate and surfactant. The addition of surfactant, Tween 80, improved the desorption rate of TNT by more than 400%. This enhancement to TNT desorption was reflected by a dramatic improvement in TNT biodegradation. The addition of surfactant improved the rate of by-product degradation and 100% decrease in TNT half-life. OUR and microbial enumeration data showed that the mechanisms associated with TNT loss were likely to be abiotic. Therefore, Zappi *et al.* (1994) suggests that the developed aerobic biotreatment process for remediation of explosives-contaminated soils and sediment should be considered for treatment purposes.



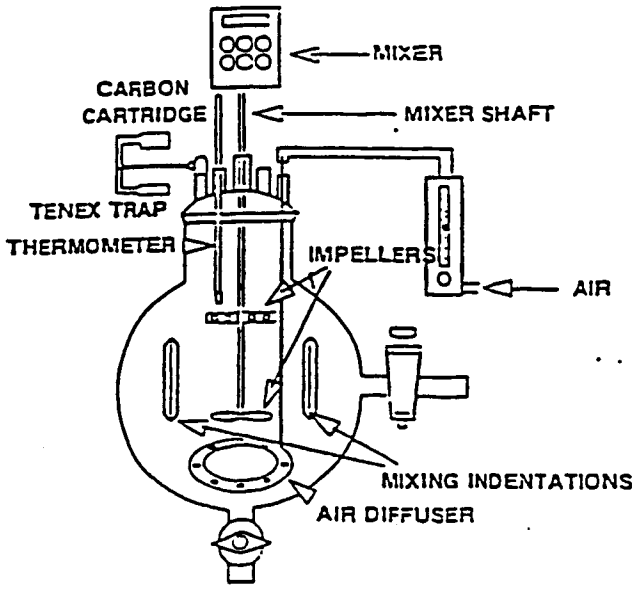


Fig. 4.2 Implementation of Bioslurry Treatment, and a Bench Scale Bioslurry Reactor.
From Zappi *et al.* (1994)

4.3 J.R. Simplot Ex-Situ Anaerobic Technology

The J.R. Simplot Ex-Situ Bioremediation Technology, also known as the J.R. Simplot Anaerobic Bioremediation Process (SA-BRE^{TM}), claims to anaerobically degrade nitroaromatic and energetic compounds with total destruction of toxic intermediate compounds at completion of the treatment (Jackson and Hunter, 1995). The technology was evaluated and conducted under the Superfund Innovative Technology Evaluation (SITE) program on soils contaminated with TNT at the Weldon Spring Ordnance Works (WSOW).

The J.R. Simplot Company developed a procedure that treats soils contaminated with nitroaromatic compounds by enhancing naturally selected anaerobic microorganisms. Although, these microorganisms did not primarily exist at the WSOW site, TNT degraders were added to the slurry after the process became anaerobic (Jackson and Hunter 1995). The Simplot process is initiated under aerobic conditions, but anaerobic conditions quickly occur enabling the microbes to degrade TNT and destroy the toxic intermediate compounds. Furthermore, the report suggests that any soil type can be treated if the treatment slurry is thoroughly mixed with the carbon-based nutrients (Jackson and Hunter 1995).

In the analysis, the Simplot Technology utilized a small-volume portable tank as the bioreactor. The dimensions of the Bioreactor were 40 ft x 8 ft x 8.5 ft which allowed a holding capacity 20,000 gallons. Contaminated soil was inoculated with microorganisms which existed in 0.02 m^3 WSOW soil previously remediated in

another treatability study. Addition of water to the bioreactor with contaminated soil provided a 1 L of water to 1 kg of soil ratio while addition J.R. Simplot company potato starch by-product and pH-regulating buffers energized the aerobic microorganisms so that oxygen was consumed from the soil. Figure 4.3 shows the Simplot process flow diagram for the SITE Demonstration.

Laboratory conditions revealed that diffusion of TNT from the solid phase to the liquid phase was the rate limiting step within the process (Jackson and Hunter 1995). Furthermore, temperatures had to be maintained to avoid problems under freezing conditions. Results indicated that a rock crushing device may be necessary to crush the debris if the size is larger than 38.1 mm. Also, the presence of high concentrations of metals may be toxic to the microorganisms. However, the process is sulfate-reducing

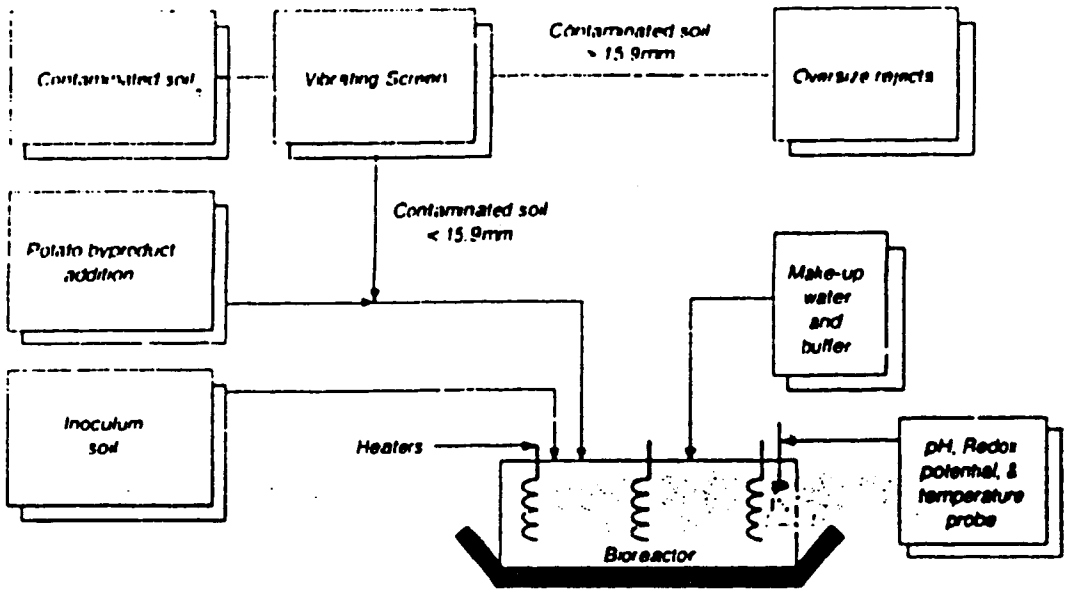


Fig. 4.3 J.R. Simplot Process Flow Diagram
From Jackson and Hunter (1995)

which can reduce toxic metals to their less toxic sulfide form (Jackson and Hunter 1995).

In the demonstration, 23 m^3 of WSOW soil was excavated and screened to remove all oversized material. 41 samples were taken to determine the average TNT concentration, analysis of metals, pesticides, relative toxicity, and grain size distributions. Three initial samples which were collected from the mixture and placed in the bioreactor which indicated a pre-treatment slurry concentration of 1500 mg/kg. The final stage of sampling occurred 9 months after initial loading. From the samples collected the final slurry concentration was at 8.7 mg/kg. Based on the data, the calculated Reduction Efficiency was 99.4% and the intermediate compounds formed by the biodegradation of TNT were below the analytical detection limit (Jackson and Hunter, 1995). Moreover, other derivatives of TNT biodegradation were undetected in the slurry.

A full-scale analysis on anaerobic bioremediation of TNT in soil was demonstrated near Weldon Springs, MO by Funk *et al.* (1995). This SITE demonstration was the a second effort to test the Simplot bioremediation technique with a few changes made to the process to maintain optimal biological activity. Sulfuric acid was used to lower the pH as the slurry mixture became alkaline. The pH was maintained at 7.6 so that TNT could not polymerize and become completely insoluble (Funk *et al.* 1995).

A 75,000-L, portable steel bioreactor with three top-mounted, low speed, high shear mixers was loaded with approximately 23 m^3 of TNT-contaminated soil in

the form of a 50:50 soil: water slurry which involved the homogenization of 50/50 (w/v) soil, 1-2% starch, and a phosphate buffer mixture. The slurry included a starchy carbon source and was buffered with phosphate to near-neutral pH. Native bacteria depleted the oxygen which made the slurry anaerobic within 1-2 days. Anaerobes then degraded the TNT (3000mg/kg) in approximately eleven weeks.

The temperature and redox were continually monitored by computer-coupled instruments on the bioreactor. Although degradation demonstrations suggest a wide temperature range, results suggest that optimal treatment occurs at 20^0 C. Furthermore, E_h was lowered by the oxidative utilization of starch by the heterotrophics present in the bioslurry (Funk *et al.* 1995).

The final samples were analyzed for 2,4-diamino-6-nitrotoluene, 2,4-diamino-4-nitrotoluene, 2,4-6-triaminotoluene, methylphloroglucinol (MPG), *p*-cresol, 2,4,6-trinitrotoluene and 4-amino-2,6-dinitrotoluene. TAT, MPG, and 26DA4DNT were not detected. Analyses of the final samples showed that TNT and 4A26DNT were removed. However, less than 50 ppm of 2,4-diamino-6-nitrotoluene existed while some samples contained detectable levels of *p*-cresol.

Superfund Innovative Technology Evaluation (SITE) program's demonstration completed the EPA's second effort to test Simplot's bioremediation technique on 2,4,6-trinitrotoluene. Bioremediation of TNT is achieved using large-scale batches. Treatability studies have been performed on soil from several other sites contaminated with TNT and other explosives. Estimates on capital costs and operating costs were calculated at $\$147/m^3$. Furthermore, the University of Idaho, in

cooperation with the J.R. Simplot company, continues to design improvements on the Simplot process and expand its applicability specific sites. Funk *et al.* (1995) believes that the process can be beneficial for the treatment of a variety of nitroaromatic compounds that are resistant to the traditional aerobic process and will make it an economically competitive alternative to incineration for the waste management of TNT-contaminated soils.

4.4 In-Situ Biodegradation Treatment

An effective in-situ bioremediation system can avoid high costs which result with techniques such as anaerobic bioslurry or composting. Research has concentrated mainly on microbial transformation of nitroaromatic compounds while the capability of the native microbial communities to degrade nitroaromatic compounds in-situ has not been thoroughly explored. Bradley *et al.* (1994) studied the potential for in-situ biodegradation of TNT by microbial communities indigenous to the surface soils and aquifer materials at an inactive munitions site near Weldon Springs, Missouri.

The U.S. Army detected concentrations of TNT in soil at greater than 30 mg/kg and at levels of up to 19 μg/liter in the underlying aquifer. This suggests that microbial degradation has not occurred extensively in the area. The ability of microorganisms native to Weldon Springs to transform TNT, 2,4-DNT, and 2,6-

DNT were investigated by using different types of soils in the area. Surface soil (red tank soil), uncontaminated surface soil (top soil), fractured carbonate bedrock material (carbonate) and material from a semi-consolidated, water bearing zone (residuum) were implemented in the study.

Bradley *et al.* (1994) found that microbial communities are capable of transforming TNT, 2,4-DNT, and 2,6-DNT. The detection limit for TNT, 2,4-DNT, and 2,6-DNT was documented at 0.05μM. In most cases the source compound completely disappeared from the dissolved phase producing amino-dinitrotoluene and amino-mononitrotoluene intermediates. For surface soil, TNT was completely removed within 22 days from an initial dissolved concentration of 100 μmol/L (Bradley and Chapelle 1995). In the carbonate and residuum treatments the initial concentrations of 2,6-DNT was reduced to 90% and 60%, respectively. Moreover, notable degradation of added ring-labeled [^{14}C]TNT to $^{14}CO_2$ occurred within 35 days of incubation (Bradley and Chapelle 1995). Approximately 11% of the [^{14}C]TNT was mineralized in microcosms containing top soil and 1% and 6.5% were mineralized in residuum and red tank soil microcosms, respectively.

Environmental factors must be governing microbial degradation at Weldon Springs because munitions activity has not occurred at the site for the last fifty years. In-situ bioremediation can be effective if the conditions which influence microbial TNT degradation are identified. Bradley and Chapelle (1995) conducted another study to evaluate environmental factors such as the effects of carbon substrate availability, soil moisture content, TNT concentration, and oxygen condition on TNT

mineralization by the microorganisms indigenous in the explosives-contaminated soil.

Surface soil was collected within an inactive wash house area which indicated TNT concentration at less than 1μmol/kg. The effect of carbon substrate addition on aerobic TNT mineralization was investigated by using cellobiose and syringate, which modeled naturally occurring cellulose and lignin-type carbon supplements.

Research indicated that mineralization by red tank soil microorganisms by adding carbon substrate substantially inhibited TNT. The addition of the two natural carbon substrates carbon substrates raised the total amount of CO_2 produced in the red tank microcosms. The addition of 2000 μmol/kg of cellobiose and syringate reduced TNT mineralization 66% and 42%, respectively. The data indicate that TNT mineralization is not accelerated by the addition of complex carbon substrates (Bradley and Chapelle 1995).

TNT mineralization was examined at different soil moisture levels because the red tank soil community must endure extreme weather conditions. Soil microcosms were prepared with soil moisture contents of 7%, 15%, 28%, and 52% by weight. Initial soil moisture, 28% water, did not extensively affect TNT mineralization. However, soil drying inhibited TNT mineralization under both aerobic and anaerobic conditions. Furthermore, TNT mineralization was stunted at each moisture level in anaerobic microcosms, while TNT mineralization increased in aerobic microcosms where soil moisture content was dried and remoisturized

(Bradley and Chapelle 1995). Typical summer weather at Weldon Springs can, therefore, negatively effect in-situ biodegradation of TNT and may be the reason why TNT persists in spite of the presence of a native microbial community capable of degrading TNT (Bradley and Chapelle 1995).

Toxic events were analyzed to see if growth of organisms were affected. The soil was collected from an area where no detectable TNT contamination existed and the study examined only short-term effects on contaminant exposure on an unacclimated community (Bradley and Chapelle 1995). A decline in activity with increasing TNT concentrations in microcosms from the uncontaminated topsoil was observed, with even 1 μmol/kg of TNT significantly inhibiting microbial activity (Bradley and Chapelle 1995). However, CO_2 production continued at concentrations up to 1000 μmol/kg.

TNT concentrations in the range of 1-100 μmol/kg accelerated activity in the red tank microcosms. TNT concentration of 100 μmol/kg did reduce microbial activity in the sediment below the rate observed at 10 μmol/kg (Bradley and Chapelle 1995). At a concentration of 1000 μmol/kg, microbial activity was completely inhibited. The response of the contaminated red tank microcosms and the uncontaminated topsoil microcosms indicates that the lack of toxicity of nitroaromatic concentrations at levels of 100 μmol/kg is connected to the adjustment of the red tank microbial communities to the nitroaromatics contamination (Bradley and Chapelle 1995).

Mineralization by red tank microorganisms was also investigated by using concentrations of TNT ranging from 0.05 to 100 μmol/kg. Under aerobic conditions, the recovery of [^{14}C] TNT activity as $^{14}CO_2$ after a 60 day incubation period was approximately 11% in microcosms. The recovery of the radiolabel was reduced to 5.5 ± 1.6% at a TNT concentration of 250 μmol/kg, 1.7 ± 0.1% at 500 μmol/kg, and 0 ± 0% at 1000 μmol/kg. These tests determined if elevated TNT concentrations contributed to the persistence of TNT at the site and identified the level to which soil concentrations should be adjusted to optimize microbial degradation (Bradley and Chapelle 1995)

Mineralization of TNT by red tank microorganisms incubated with an aerobic headspace was compared with mineralization occurring under strict anaerobic conditions to understand the effect of oxygen. Additional microcosms with a headspace of O_2-amended air were constructed so that the diffuse supply of O_2 to the microbial population could increase. For all TNT treatments, the highest recovery of [^{14}C] TNT activity occurred in microcosms that contained an air headspace (Figure 4.4). Under strict anaerobic conditions, $^{14}CO_2$ was recovered to less than 1%. Strict anaerobic conditions, therefore, cannot mineralize TNT into CO_2 in the soil.

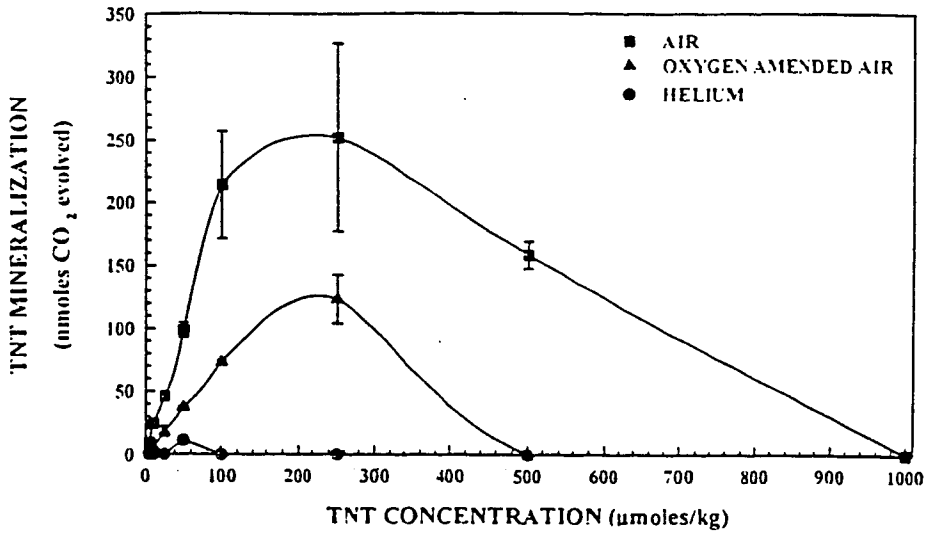


Fig. 4.4 Effect of TNT concentration on mineralization of TNT in microcosms containing red tank soil incubate under anaerobic, aerobic or oxygen-amended conditions
From Bradley and Chapelle (1995)

Increasing the headspace concentration increases the diffusive flux of O_2 to the microbial community, which reduces TNT mineralization in microcosms with an unamended air headspace (Bradley and Chapelle 1995). This indicates that red tank microorganism work well under microaerobic conditions. TNT mineralization observed in the O_2-amended microcosms reflects a shift toward formation of recalcitrant compounds such as azoxytetranitrotoluene because only CO_2 was analyzed during mineralization. The results comply with previous discussions that indicated mineralization is preferred under a combination heterogeneous conditions of anaerobic and aerobic treatment.

Minimal manipulation of soil for in-situ treatment is the most important factor of concern. In-situ treatments are less expensive than other technologies

producing low contaminant concentration. Yet, these contaminates may be more mobile intermediates (Bradley and Chapelle 1995). Furthermore, even though studies suggest anaerobic and aerobic treatment be used in conjunction to provide successful results, introducing oxygen beneath the subsurface can be a limiting factor for aerobic biodegradation. Also, biodegradation usually involves co-metabolism with another nutrient source which may difficult to deliver in an in-situ setting. These steps increases the economic costs of the process which may render it as infeasible. Finally, the effectiveness of in-situ treatment is difficult to verify both during and after treatment because it takes place beneath the surface (EPA Handbook 1993).

5 PHYSICAL AND CHEMICAL TREATMENT PROCESSES

Thousands of pounds of explosives are treated by the U. S. military and by the DOE each year. Demilitarization is the use of various technologies to process munitions so they are no longer suitable for military applications. Explosives are demilitarized because weapons containing the explosives have become obsolete, the chemical compounds have deteriorated, and because the U.S. is part of an international commitment to reduce arsenal buildup. Demilitarization of munitions involves a number of destructive and nondestructive techniques. Destructive methods include incineration, open detonation, and open burning. Nondestructive methods primarily recover various components for reuse or sale (USDHS 1995). As of 1986, destructive methods were predominantly used at the various Army Ammunitions Plants in the United States. Much of the DOE's work is done at the Pantex Plant near Amarillo, Texas. Mason & Hanger - Silas Mason Co., Inc. operate the area where weapons are dismantled and the parts are treated, demilitarized, and or sanitized so the are not operable (Phelan *et al.* 1994).

5.1 Open Burn/Open Detonation

Historically, the most frequently used method of disposal for munitions production has been open burning or open detonation. Open burn (OB) and open

detonation (OD) operations are most commonly implemented by the Department of Defense (DOD) and some private companies to destroy unusable or unstable munitions and explosives materials. OB operations are characterized by a self-sustained combustion ignited by an external source, such as flame, heat, or detonation wave which does not yield in detonation (US EPA Handbook 1993). In OD operations, detonable explosives and munitions are destroyed by a detonation initiated by a disposable charge. The US EPA Handbook (1993) reviews procedures that are employed and methods recently developed for quantifying the level of hazardous emissions from OB/OD operations.

OB and OD can be initiated by either electric or burning ignition systems. The electric system provides better control of the initiation. In an electric system, the current heats a bridge wire igniting a primary explosive which, in turn, ignites or detonates the material (US EPA Handbook 1993). Usually, OB operations are conducted in open pits which are at a minimum of 4 ft. deep with sloped sides to prevent cave-in. OD operations are also performed in pits that are 4 ft. deep which include a covering of 2 ft. of soil to minimize the risk associated with fragmentation of shrapnel. OB areas must withstand accidental detonation of any or all explosives being destroyed. After each detonation, the surrounding area is inspected for unexploded materials. Lumps of explosive materials are returned to the pit for further detonation.

Weather conditions affect the location and timing of OB/OD operations. OB/OD operations are performed only when prevailing winds carry sparks, flame,

smoke, and toxic fumes away from neighboring facilities. The optimum wind speed is 4 to 15 mph because the wind remains consistent in direction and dissipates smoke fairly quick. OB/OD operations are never performed during sand, snow, or electrical storms which might cause early detonation.

Open burning or open detonation has been practiced for many years because it was considered the best available "first generation" demonstrated technology (Adrian *et al.* 1995). It is economical and renders the explosives unsuitable for military use. As of 1995, the use of OB/OD requires a Resource Conservation and Recovery Act (RCRA) subpart X permit, which is only granted case by case. Stringent environmental regulations have effectively decreased this process increasing efforts for exploration of alternative methods.

5.2 Incineration

Before 1981, wastewater containing explosives were commonly disposed in evaporation lagoons. Army munitions plants typically contained 6 to 10 unlined lagoons, ½ acre or more in size receiving wastes for over forty years. Because nitroaromatic compounds and nitramine explosives have marginal solubility, accumulation of 40% explosive by weight in the upper sediment is possible in some areas. As cited by Major and Amos (1992), explosives at high concentration in

submerged sediments can also support the formation of semi-pure crystals of explosive which may grow to several inches in diameter.

Leaching of explosives into aquifers causing contamination is probable. Even though the concentration of explosive residues decreases sharply with depth, 3 to 5 feet of soil must still be excavated and remediated using technology such as incineration to meet cleanup criteria. The Army Environmental Center (AEC) of the Department of Defense at Aberdeen Proving Ground, Maryland oversees large-scale incineration of munitions explosive waste, and explosive contaminated soils as part of remedial actions at Army Sites in the U.S. In the US EPA Handbook (1993) various devices used for incineration and sites where incineration has been applied to explosives contaminated soils were examined.

Incineration processes treat explosives-contaminated soil and debris as well as sites with mixture of media, and bulk explosives. The army considers less than 10% explosives by weight to be a nonexplosive operation. Soil with less than 10% explosive by weight is defined by the AEC to be non-reactive (US EPA Handbook 1993). The Army's first pilot-scale use of incineration used soil up to almost 40% explosive by weight. This was allowed because of a previous study of pure TNT where no detonation occurred in a deactivation furnace and because the explosives being fed to the unsealed kiln were unconfined (US EPA Handbook 1993). Although, the pilot-scale test experienced no detonation problems, the Army's full scale incineration projects have incorporated a blending step to reduce the explosives concentrations below the 10% limit prior to feeding (US EPA Handbook 1993). The

blending step requires the approval and preparation by the Army and DOD safety offices of a site plan/safety submission including an explosives hazard analysis.

5.2a Rotary Kiln Incinerator

The incinerators commonly used are two stage systems consisting of a rotary kiln primary combustion chamber and a jet type secondary burner (US EPA Handbook 1993). The efficiency of removal of explosive residue from soil by incineration is dependent on the temperature of the primary chamber, the abundance of oxygen, and the residence time. Requirements vary with soil type and moisture, but temperatures in excess of 1200^0 F and quantities of oxygen 100% to 200% greater than the amount required to combust the organics in the soil are usual required for adequate performance (Major & Amos 1992). Shortages of oxygen during incineration of explosives contaminated soils produce reducing conditions which allows for the accumulation of unwanted organic compounds in the ash.

The rotary kiln incinerator is used primarily by the Army to treat explosives-contaminated soil. Soil enters into a primary combustion chamber where organic constituents are destroyed. Temperatures of the gases range from 800 to $1,200^0$ F while temperature of the soil ranges from 600 to 800^0 F during a retention time of approximately 30 minutes. Off gases from the primary chamber pass into a secondary combustion chamber destroying any residual organic. $2,000^0$ C gases from the secondary combustion chamber pass into a quench tank where they are cooled down to 200^0 C (US EPA Handbook 1993). The gases then pass through a

Venturi scrubber and a series of baghouse filters removing particulates and acid gases before release into the atmosphere. The treated product of the rotary kiln incineration is usually treated soil which drops from the primary combustion chamber after organic contaminants are destroyed. The soil is passed through a wet quench or water spray for remoisturization and transported to an interim storage area for further chemical analysis (US EPA Handbook 1993).

The rotary kiln incinerator process was used at the Cornhusker Army Ammunition Plant (CAAP) in Grand Island, Nebraska, where 58 explosives wastewater washout cesspools and leaching pits existed. The 10-feet-deep pits created a contaminated groundwater plume that extended into adjacent residential areas. The Army selected to incinerate contaminated soils and sludge from the cesspools and leaching pits by establishing two criteria. Soil was excavated to a particular depth based on health risk concerns while incineration had to meet specific nondetection levels for each contaminant (US EPA Handbook 1993). In 1988, the project successfully treated 40,000 tons at an average of $260 per ton. However, problems such as the overloading of the quench tank by slag that fell from the walls of the secondary combustion chamber, air infiltration through the air lock in the feed system, and severe environmental temperatures hindered the process.

The Army also opted to incinerate at the Louisiana Army ammunition Plant (LAAP) in Shreevport, Louisiana where wastewater lagoons at the site created plumes of TNT and RDX. The incineration system used at CAAP was transported to LAAP. Modifications were made to the quench to allow workers to clean it without

entering the tank, and to correct the build up of soil on the secondary combustion chamber as well as jamming of clayey wet feed to the feed system. Furthermore, the Army required that concentrations of all contaminants must total less than 100 ppm after 1 foot of lagoon had been excavated (EPA Handbook 1993). The excavation and incineration criteria for TNT was set at <5 ppm and <1.3 ppm, respectively. This project successfully incinerated 102,00 tons of soil at an average cost of $330 per ton.

The incineration of explosive-contaminated soils and sediments removes hazardous material leaving a nearly equivalent amount of incinerated soil. Major & Amos (1992) cite that incinerated soil and other residues from incineration of listed hazardous waste are still considered a hazardous waste, as defined by the "derived from Rule," (40 CFR 261.3), even though all substances which caused the soil to be hazardous have been eliminated. The incinerated soil is delisted only if it is proven to be free of harmful substances. Delisting is important because the facilities can discard the incinerator wastes by land deposition at the AOC or at an off site landfill (Major & Amos 1992).

Incineration, at long residence times and a high temperatures, is effective at reducing levels of organics to below nondetection levels (US EPA Handbook 1993). It is a successful technology offering equipment to fit the sizes of needs at any site. EPA's land Disposal Restrictions (LDRs) specify incineration as a best demonstrated available technology (BDAT) for wastes which must be incinerated prior to land disposal (US EPA Handbook 1993).

Incineration, however, has many disadvantages. Cost for mobilization and demobilization ranges around $1 to $2 million dollars (US EPA Handbook 1993). Safety concerns such as the exposure of explosive material to open flame, erecting and operating the equipment at high temperature may pose major safety problems. Air and noise emissions can generate public concern and anger. Incineration of combustible material produces a volume reduction, which can lead to higher concentration of inorganic contaminants in the ash product and create leachability problems (US EPA Handbook 1993). Some soils may require pretreatment by aeration and tilling to reduce moisture levels and viscosity. As discussed earlier, high temperatures and excess oxygen conditions are necessary for optimal combustion. Yet, limitations of fuel, high rates of slag formation, and generation of unwanted oxides of nitrogen require moderate condition than those required for optimal conditions, and products of incomplete combustion are sometimes generated (US EPA Handbook 1993). Rigid control is required in order to avoid discharge of unreacted materials.

Incineration is a "second generation technology" which is not extensively used because of the above difficulties. "Third generation" alternatives, such as UV oxidation, wet air oxidation, and supercritical water oxidation are under consideration and will only be applied if they can comply with future regulations at minimal costs. (Adrian *et al.* 1995).

5.3 Activated Carbon

Activated carbon has been used as a substrate for efficiently removing high explosives from aqueous and gaseous waste streams. Every Army Ammunition Plant has used a type of granular activated carbon (GAC) system to treat process waters from manufacturing plants, "pink water," or from contaminated ground water. GAC is very effective at removing a wide range of explosives-contaminants from water even though it cannot treat "red water." However, GAC is a transfer technology and carbon adsorption media can only be partially regenerated. The Army conducted isotherm tests and pilot-scale studies for continuous flow column GAC.

Isotherm testing is a technique for initial screening of particular wastewater prior to GAC treatment (US EPA Handbook 1993). 6 to 10 aliquots of wastewater are measured into containers and stirred or shaken for a period of time. Different amounts of pulverized carbon is added into each container and the mixture is filtered and analyzed. The results indicate the relative adsorbability of the explosives, the adsorbtion capacity and exhaustion rate of the carbon, and the maximum degree of removal of explosives (US EPA Handbook 1993).

The Army conducted pilot-scale studies of continuous flow column GAC equipment at Milan Army Ammunition Plant. The groundwater was contaminated with seven types of explosives. Treatment effectively removed every type of explosive while 2,4-DNT and 2,6-DNT were removed to below detection levels.

The GAC system consisted of eight 4.25-in diameter columns. The first column was a test column which operated in series with the second column. The second column was a back-up column used to remove contaminants when contaminant breakthrough occurred in the 1st column. The fill depth varied in each column from 2 to 4 feet. Larger fill depths required as much as 70,000 to 80,000 gallons of water to be pumped through the system to get breakthrough (US EPA Handbook 1993).

Atakim 830 carbon was selected based on data obtained from Isotherm analysis. Four flow rates ranging from 0.2 to 1.0 gpm were implemented in the study. The concentration of total explosives in the effluent ranged from 600 to 900 µg/l. Influent and effluent concentrations indicated that GAC in continuous-flow columns produced low levels of concentrations for all explosives, generally in the low parts per billion range during the early portions of the test.

Although TNT was reduced in the effluent by the Army, the contaminant was only transferred onto the activated carbon. Carbon saturated with high explosives is listed as a solid hazardous waste and has to be stored because appropriate technologies for its treatment are not yet available. Furthermore, the purchase of activated carbon and its disposal after one use is very expensive. GAC could be only be defined as "successful" if a technology existed where 100% of the activated carbon can be regenerated and reused.

Researchers have found a favorable treatment scheme for high explosives in process waters implementing existing carbon adsorbtion technology at the Pantex Plant near Amarillo, Texas. Removal of high explosives occurs similar to

the process discussed above. In this scheme however, the carbon is regenerated for reuse, using a solvent. The solvent/high explosive mixture is then processed through a biological system which degrades high explosives.

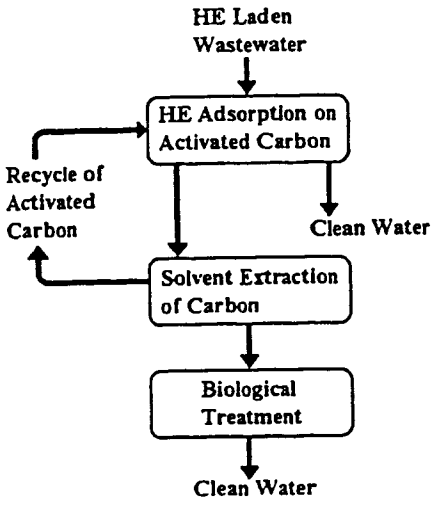


Fig. 5.1 Treatment Scheme Which Regenerates Activated Carbon from Goodfellow and Ramirez, (1994)

A prototype demonstration system designed at UCLA allowed testing at each stage of the treatment process. Six columns, four with activated carbon, two with plastic saddles for microorganisms exist in the design. The system can be operated in the up-flow or down-flow modes (Goodfellow and Ramirez 1994). The wastewater is pumped through the columns at a rate of 2 to 4 gpm.

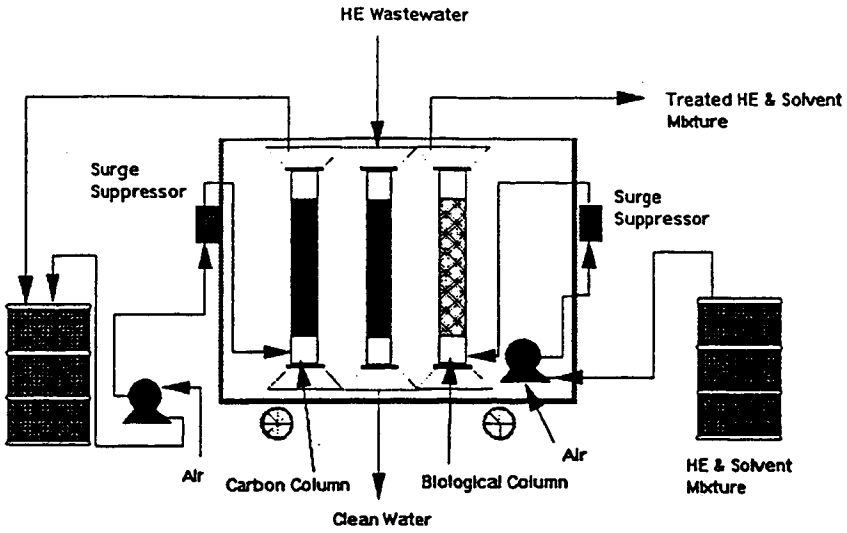


Fig. 5.2 Biological Demonstration System
from Goodfellow and Ramirez, (1994)

HMX was added to a 55 gallon drum, with inoculated bacteria, a solution of nitrates, ethanol, and other minerals and nutrients. The mixture was pumped through the system and the analysis of samples indicates potential for degradation HMX. Although the study does not directly investigate aqueous TNT systems, information regarding the nature of the process with HMX provides valuable insight for implementation on explosives such as TNT. Further tests are proposed which will include operation analysis of a single pass-through mode and analysis of each portion of the system.

A combination of chemical and biological treatment systems can effectively meet technical requirements of removing high explosives (Knezovich *et al.* 1994). With simple operation methods, waste minimization and associated costs will be reduced. Further analysis of other treatment alternatives, such as phytoremdiation

and advanced oxidation treatment discussed in following sections, coupled with conventional techniques may provide successful results.

5.4 Photolysis

Exposure of TNT as a solid or in solution to strong sunlight or ultraviolet radiation results in the formation of decomposition products. The aqueous solution first turns pink and after four to six hours changes into a rusty-orange colored solution referred to as "pink water" (USDHS 1995). The process described refers to photolysis, which is an important fate of TNT in aqueous systems. The estimated half life of TNT in surface waters is 0.16 to 1.28 hours based on the rate of photolysis and photooxidation in sunlit natural waters (USDHS 1995).

Phototransformation of TNT in surface waters can occur through direct and indirect photolysis. Direct photolysis is quick with a half-life of 14-48 hours and the rate increases in natural waters because of humic acids that indirectly effect photolysis. In sunlit natural waters, photolysis of TNT occurs 10-100 times faster than in distilled water, with the half-lives in some natural waters occurring at less than 0.5 hour (USDHS 1995). Phototransformation in natural surface waters increases because of the complexation of TNT and natural organics, or by an indirect mechanism where light adsorbed by natural organic constituents is transferred to TNT (USDHS 1995). It can also occur because of the chemical trapping by humic acids of the reactive intermediate phototransformation products.

In laboratory studies, the pH of surface waters minimally influences the rate of transformation containing few natural organics (USDHS 1995). TNT is more persistent in deep calm water bodies or systems where sunlight is weak. As cited by the USDHS (1995), 2,4,6-trinitrotolulene photodecomposition compounds such as dinitroanthrils, trinitrobenzaldehyde, trinitrobenzyl alcohol, trinitrobenzene, nitoanilines, condensed azo and azoxy derivatives, and 1,3,5-nitrobenzene were identified. (USDHS 1995).

5.5 Photocatalytic Degradation

Photocatalysis causes the formation of electron/hole pairs in semiconductor particles that are suspended in contaminated water.

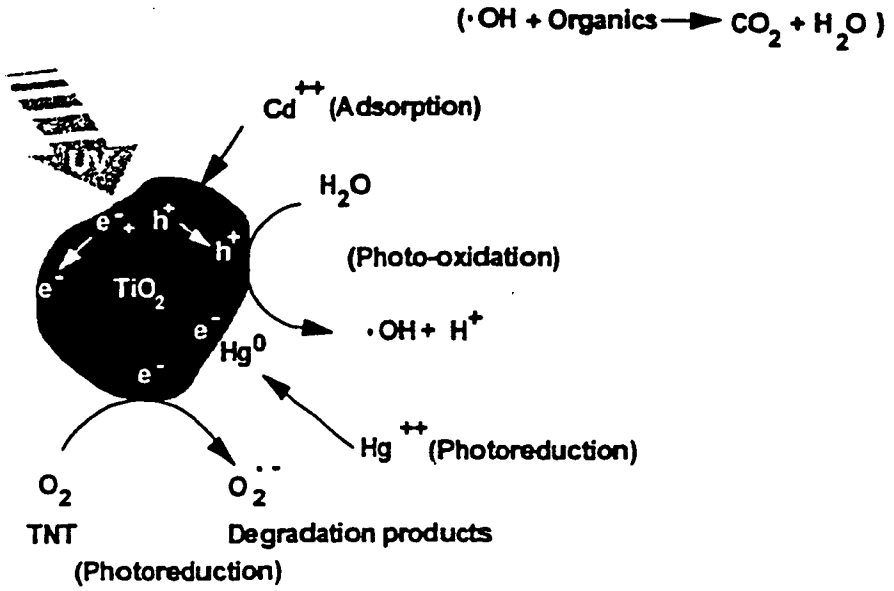


Fig. 5.3 Schematic diagram of the basic chemistry of semiconductor photocatalysis
from Prairie *et al.* 1994

These electron/hole pairs recombine to produce thermal energy, or migrate to the particle surface where interaction occurs with the external environment (Prairie *et al.* 1994). The holes reacting with the water produce hydroxyl radicals which oxidize dissolved organics into water, carbon dioxide, and dilute mineral acids (Prairie *et al.* 1994). The electrons react with dissolved oxygen forming more hydroxyl radicals, or reactions occur with other dissolved oxidants including contaminants such as TNT. Titanium dioxide (TiO_2) a commonly used catalyst because it is effective, inexpensive, stable, and non-toxic. In addition, TiO_2 is activated at near UV light (390 nm or less) allowing direct use of ultraviolet energy in sunlight (Prairie *et al.* 1994).

Although photocatalytic degradation successfully treats a variety of organic chemicals, minimal research has occurred with photocatalysis for munitions waste treatment. Prairie *et al.* (1994) cites a preliminary study where 50 ppm TNT in 800 mL of pure water was mineralized within 2 hr exposure to light from a 450 W mercury lamp using TiO_2 as the photocatalyst. Although the study seemed to be effective for the treatment of TNT-contaminated waters, Prairie *et al.* (1994) wanted to verify the effectiveness and investigated titanium (TiO_2) photocatalysis as a feasible approach for treating aqueous munitions waste.

In the study conducted by Prairie *et al.* (1994), actual "pinkwater" was obtained form the Louisiana Army Ammunition Plant, which consisted of 95 mg/l of TNT, 48 m/l of RDX, and 65 mg/l of TOC in city water. The photocatalyst, Tioxide

Tilcom HAC (270 m^2/g) was suspended in solution at 1 g/L and allowed to equilibrate for 15 minutes before illumination. One 100-W-UV spot lamp and one Oriel 1000 W ozone-free Hg (Xe) arc lamp with a photofeedback controller was used in the experiment. HPLC data (Fig. 5.5) for the anaerobic treatment of the 50% diluted pink water successfully demonstrated the potential for photocatalysis for treating aqueous munitions wasted containing TNT, and/or RDX, and "pinkwater" (Prairie *et al.* 1994).

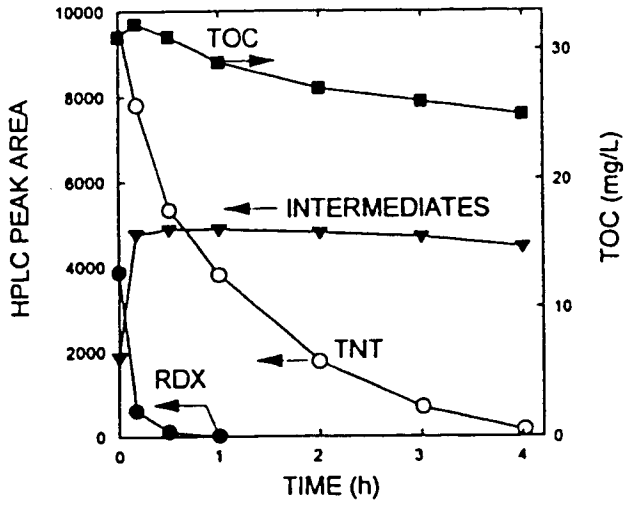


Fig. 5.4 The anaerobic photocatalytic degradation of "pinkwater" (diluted to 50%) from the Louisiana Army Ammunition Plant. HPLC peak areas for intermediates represent the sum of all intermediates times 10^{-1}. HPLC peak area is directly proportional to concentration. 100W lam, 1g/L Tio_2, N_2 purge.
From Prairie *et al.* 1994

Photocatalysis is unique because of its ability to carry out reduction in addition to oxidation (Prairie *et al.* 1994). Earlier work on the photocatalytic oxidation of TNT in a closed vessel resulted in the formation of ammonia as one of the reaction products (Prairie *et al.* 1994). To explore the reduction of TNT in detail, Prairie *et al.* (1994) carried out experiments on 90 mg/l TNT in deionized water using a batch photocatalytic reactor purged with nitrogen which was analyzed for complete mineralization of TNT through reduction processes. 0.7 mM EDTA, the sacrificial reductant (hole scavengers), was used in each 300 mL of 90 mg/l TNT solution loaded with 1 g/L titanium dioxide powder and examined under a 1000 W hg/Xe arc lamp at selected time intervals.

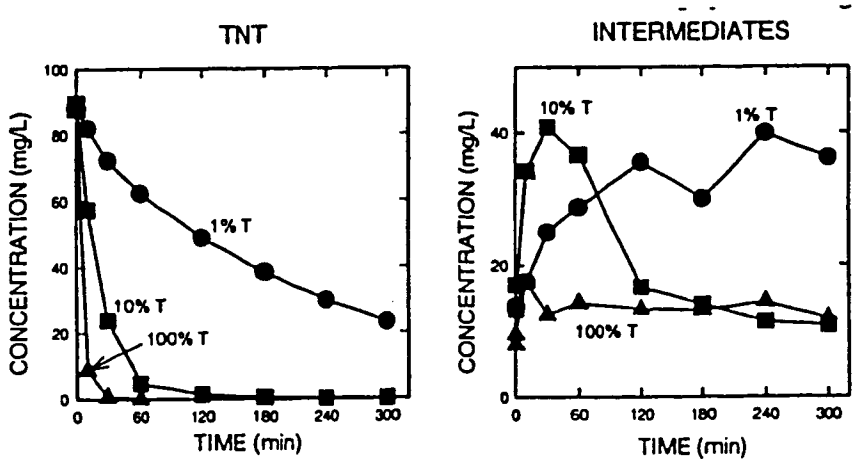


Fig. 5.5 The anaerobic degradation of TNT in a batch photocatalytic reactor using 0.7 mM EDTA as the hole scavenger. %T designates the fraction of the 1000 W *Hg/Xe* arc lamp beam that passes through the neutral density filter. 300 mL solution 1 g/L TiO_2, N_2 purged. From Prairie *et al.* (1994)

Prairie *et al.* (1994) cites previous work which suggests that photoelectrochemical metal reduction depends on the type of organic hole scavenger used. The results indicate that degradation of TNT was a reductive process. However, even without additional organic, TNT degradation utilized both oxidation and reduction processes. Prairie *et al.* (1994) believe that TNT is reduced to form intermediates that are further degraded by oxidation. Photocatalytic degradation of TNT occurs through a complex network of oxidation and reduction reactions which may all be kinetically related and should be further examined (Prairie *et al.* 1994).

Dillert *et al.* (1995) also studied light induced degradation of various nitro aromatics including TNT under different experimental conditions by varying the pH of the suspensions and the light intensity in irradiated TiO_2 suspensions. The photocatalyst, TiO_2 Degussa P25, was synthesized with TNT by nitrating 2,4-dinitrotoluene with a HNO_3-H_2SO_4 solution at 80^0 C and recrystallzed twice from ethanol.

The change in concentration of six typical nitroaromatics pollutants *vs.* irradiation time was graphed and the rate of disappearance of the organic solute was determined to fit a first order rate law. The reactivity of the compounds decreased with increasing numbers of nitro groups on the aromatic ring (Dillert *et al.* 1995). The methyl group of the toluenes enhanced the reactivity. The order of reactivity which shows influence of nitro groups towards the attack of an electrophilic reagent was as follows: Nitrotoluenes > nitrobenzenes > dinitrotoluenes > dinitrobenznenes > 2products ,4,6-trintirotoluene > 1,3,5-trinitrobenzene (Dillert *et al.* 1995).

The data showed inhomogenous degradation of TNT in an acidic solution. However, at a pH of 7 and 11, reaction rates of 0.84 and 0.92 $\mu molL^{-1}min^{-1}$ at a light intensity to 520 μmol $photonsL^{-1}min^{-1}$ were observed. The degradation rates of 2,4,6-trinitrotoluene are in the same order of magnitude as in TiO_2 suspensions (Dillert *et al.* 1995). These results also show that photocatalysis effectively treats water contaminated with TNT.

Schmelling and Gray (1995) also presented an analysis of the photodegradation of TNT in a TiO_2 slurry reactor. The rates and extent of TNT transformation and mineralization were compared for photocatalytic and direct photolytic reactions under conditions of varying light energies and in the presence and absence of oxygen. These studies evaluated the efficiency of TiO_2 photocatalysis as a process for the remediation of TNT contaminated water and examined the photocatalytic behavior of nitroaromatic compounds.

Certain initial organic transformation products were identified for both photocatalytic and photolytic reactions. Nitrate, nitrite, and ammonium ions were analyzed and the possibility of semiconductor sensitization by colored compounds were considered. TNT transformed rapidly under each set of photochemical conditions, but destruction was faster and complete with TiO_2 photocatalysis. Transformation-by-products were destroyed under oxygenated photocatalytic conditions and seemed more refractory under direct photolytic conditions.

Mass balances performed on carbon and nitrogen showed that the TiO_2 photocatalyst, in the presence of oxygen and near UV radiation ($\lambda > 340$ nm),

mineralized 90% of the TNT and 35% of the total nitrogen was recovered as ammonium ion after 120 minutes.

Among the large number of organic transformation products produced photocatalytically, trinitrobenzoic acid, trinitrobenzene, and trintirotphenol were identified as oxidative intermediate species and dinitroaniline as a reduction product (Schmelling & Gray 1995). The photocatalytic transformation of TNT in the study involved both oxidative and reductive steps and sensitization by colored compounds played no detectable role on degradation.

TiO_2 photocatalysis using near UV radiation may be highly effective in the remediation of TNT contaminated waters because the process achieves almost complete mineralization of TNT and the refractory intermediates known to inhibit biodegradation do not accumulate. Furthermore, this process has the potential to be adapted to solar radiation which may offer an economic advantage over other advanced oxidation process (Schmelling & Gray 1995). Therefore, photocatalysis shows tremendous promise for the safe, effective treatment of water contaminated with low concentrations of TNT.

5.6 Ultraviolet Oxidation

Ultraviolet (UV) oxidation is not used extensively for remediating water with contaminated explosives. Yet, UV oxidation is an effective treatment for explosives-

contaminated water and, unlike carbon treatment, destroys target compounds rather than transferring them to another easily disposable medium (US EPA Handbook 1993). In the US EPA Handbook (1993) a pilot-scale study of UV oxidation is examined as well as the types of explosives-contaminated water that can be treated.

UV oxidation can be used to treat water from the demilitarization of munitions and for groundwater contaminated from the disposal of these waters. In the 1970's, Milan Army Ammunition Plant (MAAP) was the site of munitions washout operations where process waters were placed in O-Line Ponds until the early 1980's. Although, the water was drained and the lagoons were closed with a multi-media cap to eliminate hydraulic loading on the contaminated sediments, analysis indicated that a contaminated groundwater plume was migrating from the site. Anyanwu *et al.* (1993) conducted a study for the Army which focused on optimizing the performance of a full-scale UV oxidation system which could be possibly selected as a treatment technique. The study consisted of both bench- and pilot-scale studies.

The objectives of the bench-scale treatability tests were to determine the general effectiveness of the technology, and to select design and operating conditions for the pilot-scale treatability testing program. Bench-scale UV oxidation tests were conducted on 15 gallons of contaminated water from a site to evaluate the effects of UV exposure time, ozone diffusion rate, ozone dosage, hydrogen peroxide dosage and pH on the oxidation process (Anyanwu *et al.* 1993). The bench-scale system consisted of a 2,4-L-reactor with a single 40-watt UV bulb. Ozone was diffused

through the reactor at rates ranging from 2.8 to 15.0 mg/l/s, while a 35% H_2O_2 by volume was also applied. The pH ranged from 4.5 to 8.5, and dropped due to the production of organic acids during treatment. The concentration of all explosives in the influent was 57,500 µg/L, with TNT, RDX, HMX, and tetryl in the highest concentrations (US EPA Handbook 1993). Residence times varied from 40 to 200 minutes per treatment batch. The tests indicated that UV radiation degraded explosives while longer residence times improved reduction of contaminant levels. H_2O_2 levels were not effective for contaminant degradation while UV oxidation was efficient at a pH of seven or greater.

Analytical results indicated that the level of 1,3,5-TNB concentration, which is an intermediate product of UV oxidation of TNT, was the rate controlling compound. Although 1,3,5-TNB was oxidized throughout the process, the concentration of 1,3,5-TNB in the UV-oxidation effluent increased as the other explosives under went oxidation, and then decompose (Anyanwu *et al.* 1993). The treatment goal of 1,3,5-TNB was not reached even after 180 minutes of UV exposure while other compounds were destroyed within 80-160 minutes. Optimal destruction of 1,3,5-TNB was achieved at an exposure time of 200 minutes, an ozone dosage of 750 mg/, and a pH of 7. All treatment goals were achieved and the conditions were used as an initial point for the pilot scale treatability tests.

The pilot scale plant was implemented to obtain design data and operational costs for a full-scale 500-gpm, UV oxidation system. UV oxidation tests were conducted in a 650-gallon stainless steel, Ultrox P-650 system, consisting of six

reaction chambers. Each chamber contained 12, 63-watt, low pressure, UV lamps, and a cooling system to prevent temperature increases during the long exposure times (EPA Handbook 1993). Operation of the treatment system occurred in a recycle batch mode where each 650-gallon batch was recycled six or seven times. The total concentration of explosives was about 20,656 µg/l and the pH of the water was kept at 7 to 11 during the treatment. Tests were conducted at ozone doses ranging from 1.11 to 3.33 (mg/l)/minute and water retention times ranging from 40 to 210 minutes. The pilot scale study indicated that UV oxidation was effective at a pH of 9 and an ozone dosage of 3.3 (mg/L)/minute. Residence times greater than 180 minutes combined with high ozone doses destroyed all of the explosives, including 1,3,5-TNB (US EPA Handbook 1993). Phototoxicity tests showed that the effluent was toxic because of leaching of metals from bronze impellers within the equipment.

Although UV-oxidation is capable of achieving the desired treatment goals, the UV exposure time required to destroy 1,3,5-TNB, the rate controlling compound, is almost twice as long as the exposure time required for all other explosives. Anyanwu *et al.* (1993) suggests that all the explosives, except for 1,3,5-TNB, should be degraded to below their treatment goals in the UV-oxidation reactor. This reduces the reactor size by half, which decreases the ozone dosage and the UV exposure time as well as the capital and operational costs. Anyanwu *et al.* (1993) suggests that the UV-oxidation followed by secondary treatment with GAC to

remove residual 1,3,5-TNB may offer the best economical results for the contaminated groundwater at the Milan Army Ammunitions Plant.

Ultraviolet treatment is an innovative developing technology and among methods used for degradation of TNT in contaminated water, the addition of ultraviolet (UV) irradiation and an oxidizing agent such as ozone is more effective. Although Anyanwu *et al.* 1993 suggests additional treatment to save time and costs, in theory the synergistic effects of UV radiation and ozonation is the basis of development of the technology because the primary advantage of UV/ozone oxidation process is the failure to generate process residuals which require additional treatment. Therefore, the UV/ozone oxidation process should be further evaluated and implemented for site specific areas.

5.7 Wet Air Oxidation

Wet air oxidation is a high temperature, high pressure, liquid-phase oxidation process (US EPA Handbook 1993). The technology is used in municipal wastewater treatment for treating dilute solutions of 5-10 % solids or organic matter. Tests were conducted and minimally used on a large scale basis for treating explosive wastes. Typically, in a wet air oxidation system, contaminated slurries are pumped into a heat exchanger where temperatures are between 150 to 350^0 C, and then into a

reactor where they are treated at pressures of 1,000 to 1,800 psi (US EPA Handbook 1993).

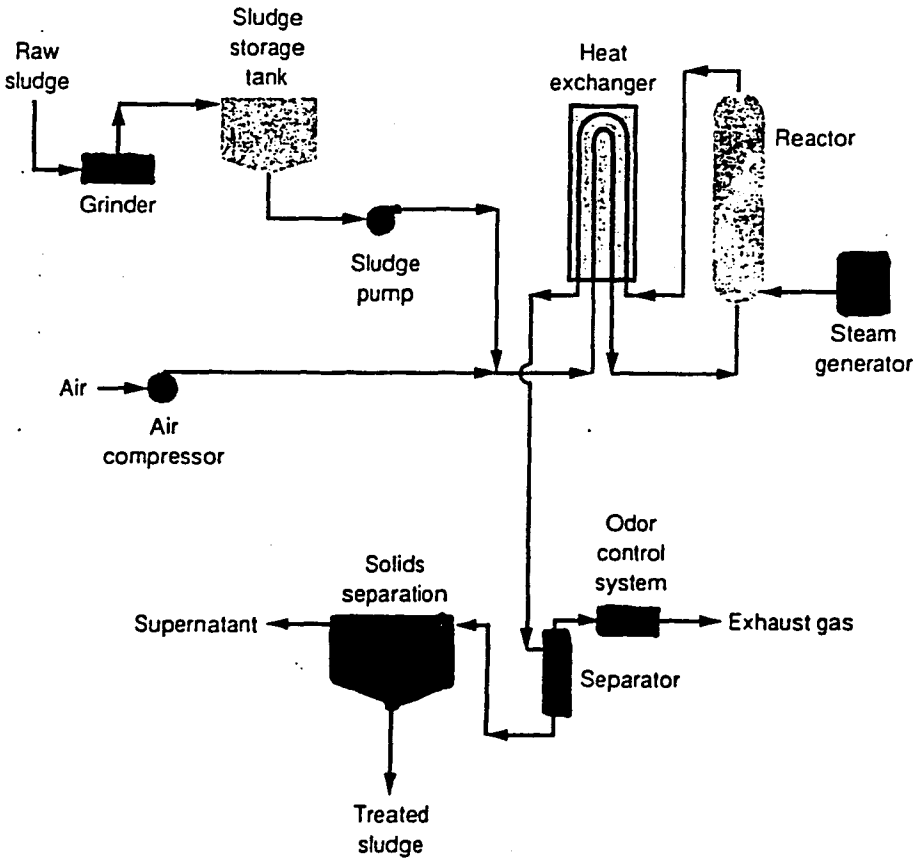


Fig. 5.6 Typical Wet Air Oxidation Process
From Tchobanoglous and Burton, (1991)

The Army identified wet air oxidation as technically or economically infeasible and a series of laboratory studies were conducted in 1982. Wet air oxidation (WAO) was applied to lagoon slurries containing 10% explosives contamination with added chemical catalysts. The technology was effective for

treating RDX, but produced hazardous byproducts from TNT degradation. Furthermore, lagoon slurries had to be diluted prior to treatment and NO_x, CO, and CO_2 from the oxidation process had to undergo a separate treatment process (US EPA handbook 1993).

While the Army conducted their studies, Hao *et al.* (1993) was also in the process of examining the WAO technology on "red water." As discussed earlier, "Red water" has a complicated nature which cannot be treated by conventional treatments such as ozonation, GAC, or biological treatment. Preliminary results by Hao *et al.* (1991) show that "red water" (1:1000) is effectively treated. At 340^0 C, and a pressure of 14.8 Mpa, almost 100% reductions were achieved in total organic carbon (TOC) and chemical oxygen demand (COD) in a 1-hour reaction time (Hao *et al.* 1993). However, upper limits on temperature and pressure for treatment of "red water" were established.

Different operating conditions for WAO were further explored by Hao *et al.* (1993) for effective application. The factors affecting WAO efficiency were examined by utilizing the process under various operating conditions and subjecting the treated "red water" to toxicity tests. The tests analyzed heterotrophic COD degradation and autotrophic oxidation of ammonium to nitrite by enriched *Nitrosomonas* (Hao *et al.* 1993).

The batch WAO experiments used diluted "red water" (1:100) at five different temperatures and three different pressures. The WAO efficiency to remove "red water" contaminants was primarily a function of temperature and somewhat

related to the oxygen pressure (Hao *et al.* 1993). At the upper temperature limit of 320^0 C and P_{o2} = 1.31 Mpa, only 8 mg/l of COD, 30 mg/l of TOC, 38 mg /l of acetic acid , and 127 mg/l of TVS remained after a 1-hour reaction while below detection levels of byproducts such as 1,3-DNB and nitrobenzene were detected. As a result of desulfonation of organic compounds significant amounts of inorganic sulfate accumulated in the WAO effluent (Hao *et al.* 1993). Nitroaromatic compounds other than nitrobenzene peaked between 230 and 260^0 C.

Based on the results, the compounds formed during WAO of red water, specifically to DNT sulfonates are as follows:

2,4-DNT-3-SO_3Na

-------> 2,4-DNT + Na_2SO_4 -----> 1,3-DNB---> acetic acid---> CO_2

2,4-DNT-5-SO_3Na

A preliminary toxicity study on treated "red water" (320^0 C and P_{o2} = 1.31 Mpa) showed minimal effect on the heterotrophic AS culture for up to a fraction of the treated red water (Hao *et al.* 1993). However, the efficiency of the enriched *nitrosomonas* culture in converting ammonium into nitrite was adversely effected in the presence of treated water (Hao *et al.* 1993). A 12% fraction of the treated red water sample reduced the nitrite production rate by half. Up to 1% and 4% fractions of diluted red water (1:100), with and without addition of $NaNO_3$ and Na_2SO_4, respectively, did not exert any visible effects on the *nitrosomonas* culture. Therefore, the organic byproducts formed during the WAO of "red water" may be responsible for the observed toxic effects and direct discharge of WAO treated red

water into the receiving water could be toxic on the aquatic life, as reported by Hao *et al* (1993).

5.8 Advanced Oxidation Techniques

Many researchers have devoted time to developing advanced oxidation technologies for destroying hazardous wastes. Some technologies rely on nonthermal processes to generate highly reactive hydroxyl radicals directly in aqueous solution. However, major engineering challenges exist in designing nonthermal industrial oxidation technologies that are cost-effective and verastile.

Willberg *et al.* (1996) suggests a possible approach of using existing technologies that have proven to be successful in industrial applications. As cited in the paper, the Electrohydraulic discharge (EHD) method is a non-thermal process based on pulsed-power technology. EHD injects energy into an aqueous solution through a plasma channel formed by a high-current/high-voltage electrical discharge between two submersed electrodes (Willberg *et al.* 1996). Besides the mechanical effect, three primary physical processes can also generate significant oxidative chemistry. The EHD-induced plasma channel is a high-temperature (14,000-15,000 K) blackbody radiation source with a maximum emitance in the vacuum UV region of the spectrum. The radiation photolyzes and ionizes water in the layer immediately surrounding the plasma channel, generating highly reactive hydroxyl radicals. UV radiation escapes into the bulk of the solution, photolyzing any chrosphores present. Also, the shockwave can indirectly initiate pyrolytic and free radical reaction through

electrohydraulic cavitation. And finally, cooling plasma channel forms an oscillating stream bubble, which may lead to the formation of supercritical water phase in which organic substrates may undergo oxidation (Fig. 5.8).

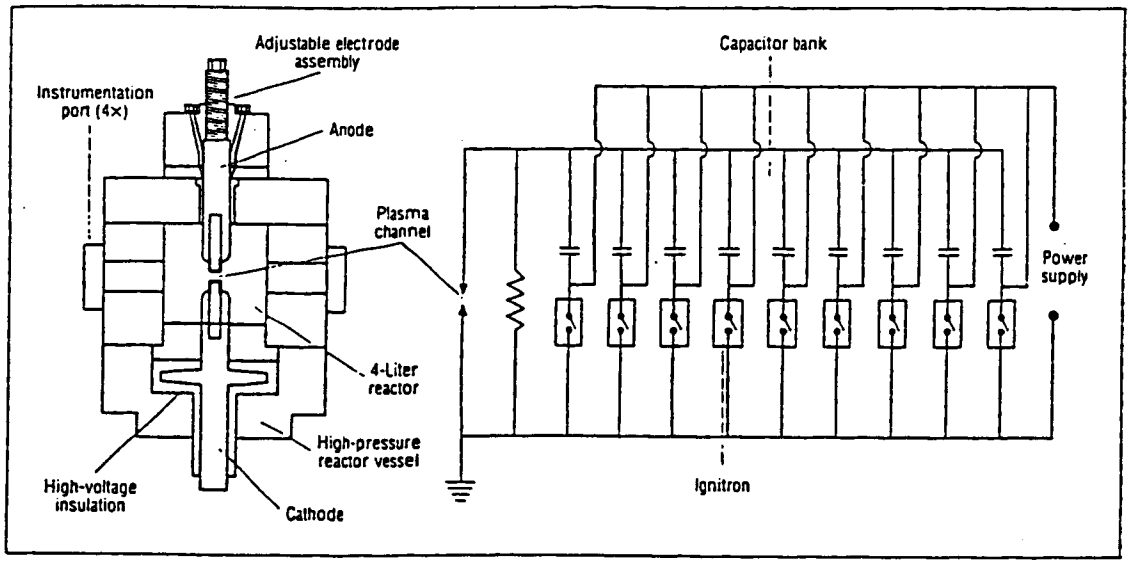


Fig. 5.7 The EHD system contains two major components: a reaction chamber (left) and a pulsed-powe electrical discharge circuit (right). From Willberg *et al.* 1996

These processes can be localized or extended. Localized effects occur in the immediate vicinity of the plasma channel with thermal oxidation occurring within the plasma channel, vacuum UV photolysis occurring at the surface of the plasma channel, and supercritical oxidation occurring within subsequent steam bubble (Willberg *et al.* 1996) These processes are limited to the combined volumes of the plasma channel and steam bubble. Extended effects primarily result from UV radiation and the intense shockwave, which radiate out into the bulk of the solution.

The two major components of an EHD system are a pulsed-power electrical circuit and a reaction chamber. The electronics package used in Willberg's laboratory is a specialized capacitor discharge circuit designed specifically with low inductance (250-300 nH) and large capacitance (135 μF) to generate short-duration, high-energy pulses. It is capable of delivering a 20-μs pulse with a total energy of 25 kJ and peak power of up to 1 GW. Fast ignitron switches trigger the discharges.

The EHD reactor used during research was designed to investigate chemical effects of underwater discharges as well as to withstand the extreme electrical and mechanical stresses experienced during an EHD event providing reproducible conditions from more than 100,000 discharges. TNT was chosen because it is relatively resistant to degradation with UV photolysis and dark reactions. In the experiments, 3.5 L of 159 μM TNT solution was treated with 260 7-kJ EHDs. The solution was sparged with an ozone-oxygen gas mixture to maintain 90 μM steady-

state ozone concentration during the EHD treatment because it is more effective to photloyze ozone in solution to generate reactive radicals (Willberg *et al.* 1996).

The EHD-ozone process degraded the TNT in solution to less than detection limits (<1μM) over the duration of a 260-discharge experiment. The overall TNT degradation kinetics can be fit to $k_A = 1.0 \pm 0.1$ μM and the total organic carbon content of the solution was reduced by 34% from 18 to 12 ppm (Willberg *et al.* 1996).

The EHD-ozone treatment resulted in 34% of the original organic material being mineralized. The rate of mineralization was slow at first. However, it accelerated after the first 150 EHDs when the TNT concentration was reduced to 22 μM. This acceleration indicates that oxidative intermediates are more prone to hydroxyl radical attack than the parent compound (Willberg *et al.* 1996). Because the rate of disappearance of total organic carbon increases over time, complete mineralization of TNT should occur if the number of discharges is increased.

Although the EHD process has not been evaluated as extensively as many other oxidation technologies, it is a promising technology for treating hazardous liquid wastes. The dependability of pulsed-power technology has worked successfully for a variety of industrial process. Presently, work is conducted in the laboratory at bench scale and further analysis and demonstration of the EHD process will make it an emerging waste treatment technology.

5.9 Low Temperature Thermal Desorption

Low Temperature Thermal Desorption (LTTD) technology was originally developed for treating aqueous slurries contaminated with volatile organic compounds (VOCs). The technology was tested for treating explosives-contaminated slurries where the slurry is fed into the system at 200^0 to 300^0 C into a hot oil heating chamber and treated under elevated pressures. Emissions from the system are treated in an afterburner.

The Army conducted a laboratory-scale study on low-temperature thermal desorption of explosive wastes in 1982. LTTD achieved a 95% destruction and removal efficiency (DRE) in 20 minutes. However, there was no discussion as to the specific explosives that were treated by the technique. Further information about the process since 1982 was not supplied in the article, although pilot-scale engineering and cost analysis technology of the technique were planned for research as of September 1993 (EPA Handbook 1993).

5.10 Alkaline Hydrolysis

Alkaline hydrolysis breaks down high explosives to organic and inorganic salts, soluble organic compounds, and nitrogen gases such as ammonia or nitrous oxide (Bishop *et al.* 1996). Typical base solutions (1-8 M) include sodium hydroxide, potassium hydroxide, ammonium hydroxide, and sodium carbonate. A relatively low pressure and temperature allows for easy control in a closed reactor. Although the end products may still be considered a hazardous waste, further treatment can be applied.

The Pantex Plant near Amarillo, Texas has collaborated with Los Alamos National Laboratory to use base hydrolysis as a demilitarization and sanitization process for large pieces of explosives and for managing explosive wastes. Explosives are immersed in concentrated aqueous sodium hydroxide and the mixture heated with stirrers. The base attacks the heterocyclic compounds to produce nitrogen oxides, formate, and other minor products (Phelan *et al.* 1994).

TNT's ability to nucleophilic attack has been reported since 1899. This reactivity with bases is due to the electron withdrawing nature of the nitro groups (Jones *et al.* 1982). Research regarding alkaline hydrolysis of TNT and other nitroaromatics is presently analyzed using alcoholic media and focuses on the identification of intermediate species. Although this does not directly benefit

aqueous TNT systems, information regarding the nature of the reaction and formation of possible by-products provides valuable insight.

As cited by Priestley (1996), the hydrolysis of TNT and other nitroaromatics results in the formation of highly colored solutions. When TNT reacts with alkalis, a significant change yields red or brown colored addition products. An organic substance of TNT, not yet positively identified, can be separated from these colored products by using inorganic acids, as cited by Priestley (1996).

Many experiments reacting TNT with various bases are implemented at or below room temperature. While examining the conversion of hydrogen in TNT, Buncel *et al.* (1968) observed the exchange of methyl protons of TNT in basic medium (90% dimethylfomamide-10% D_2O). The solution rapidly discolored and unreacted substrate was unrecoverable because it is extensively decomposed when temperature is increased to 100^0 C (Buncel *et al.*1968).

As cited by Priestley (1996), the formation of highly colored solutions when TNT is reacted with strong bases occurs because the intermediate, 2,4,6-trinitrobenzyl anion (TNT), is produced. TNT also undergoes photolytic reactions in alkaline conditions. The replacement of a nitrogroup by hydroxyl group is accelerated by exposure to light in basic media, as cited by Priestley (1996). Treatment techniques that use UV radiation to remove TNT from solution are being further examined. Y. Okamoto and J. Wang (1977) studied surfactants such as 4-dodoecyldiethlenetriamine, which enhanced the rate of color formation involving reactions of TNT and bases in aqueous solutions.

During the 1980's, the U.S. Military examined alkaline hydrolysis process to treat soils and sediments contaminated by TNT. Experiments testing several chemical methods using Fenton's reagent and sulfide as desensitizing reagents removed TNT and RDX under alkaline conditions (pH>10). As cited by Priestley (1996), this occurred independently of the concentration of any desensitizing chemicals as well as in their absence (Kubarewicz *et al.* 1985). Temperatures ranging from 3^0 C to 30^0 C did not effect the results while the Microtox Analyzer showed an increase in toxicity of the liquid phase after treatment.

Recently, T. Spontarelli at Los Alamos National Laboratory analyzed the hydrolysis of many explosive and propellant compounds. Although the study focuses on HMX-based explosives, analysis shows that TNT is completely degraded to a non-energetic substance through alkaline hydrolysis (Spontarelli *et al.* 1993). Base hydrolysis of TNT produces a very dark, water-soluble product which was not identified in the study. As demonstrated with HMX hydrolysis products, Spontarelli *et al.* (1993) suggests that the hydrolysate can be thermally treated using super critical water oxidation to mineralize and produce more readily degradable products.

Dell'Orco *et al.* (1995) conducted experiments implementing a combination of base hydrolysis/hydrothermal treatment of Composition B-3 explosive, 60% RDX and 40% TNT. The study demonstrates the conversion of the explosive to non-energetic, aqueous organic components within reaction times of four hours between temperatures of 80^0 C to 90^0 C. Bench- and large-scale methods provided similar

results producing gas-phase products of N_2O and NH_3 and major aqueous components of formate and hexamine.

The hydrothermal processing of Composition B-3 hydrolysate was conducted in the system outlined in Figure 5.10. Composition B-3 hydrolysate reacted at temperatures ranging from 300^0 C to 485^0C and pressures ranging from 660 bar to 1.2 kbar (Dell'Orco *et al.* 1995). The hydrolysate was initially diluted 2:1 based on prior knowledge of $NaOH/Na_2CO_3$ phase behavior. Hydrogen peroxide was implemented as the oxidant which provided excess oxygen. The organic carbon converted to CO_2/HCO_3^- within two minutes at a temperature of 400^0 C. However, temperature had to be increased to 450^0 C to convert aqueous nitrate/nitrite/ammonia to gaseous N_2/N_2O (Dell'Orco *et al.* 1995). The results suggest that hydrothermal processing is economical, efficient and an acceptable treatment method for Composition B-3 base hydrolysate.

Dell'Orco *et al.* (1996) further analyzed base hydrolysis/hydrothermal oxidation of TNT in a study which primarily focused on HMX. A high-temperature hydrolysis of 22g of TNT in a 220 ml solution of 1.5 M NaOH was performed. The black end product was evaporated to dryness and investigated for its thermal stability. Gas and heat was emitted by the substance as it approached a temperature of 120^0 C. A different thermal analysis (DTA) showed a possible melt at approximately 125^0 C and a decomposition exotherm immediately after the endotherm (Dell'Orco *et al.* 1996). The energetic hydrolysis product was further decomposed by treating it with an acid or a solution of calcium. The black material

forms a precipitate which had an increased thermal stability. The newly formed salt can be safely pyrolyzed or incinerated (Dell'Orco *et al.* 1996).

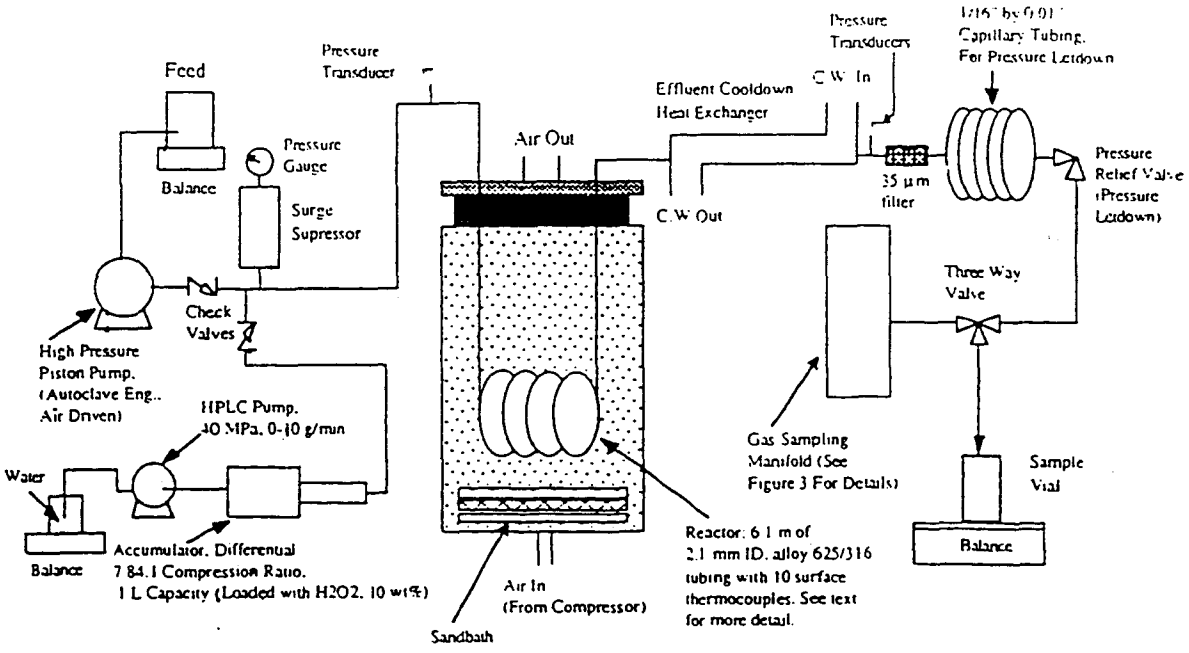


Fig. 5.8 Hydrothermal system used to investigate kinetics of Composition B-3 Hydrolysate.
From Dell'Orco *et al.* (1995), "Base Hydrolysis and Hydrothermal Processing of Compositon B-3 Explosive"

As cited by Priestley (1996), a recent joint study conducted by the Fraunhofer IITB - Aussenstelle fuer Prozessotimierung Berlin, BC Berlin - Consult GmB\bH, and the the Analytisches Zentrum Berlin-Adlershof examined the alkaline hydrolysis

base (1.4 g TNT/g NaOH), TNT was fully and irreversibly degraded within four hours at temperatures ranging from 60-100^0 C (Fraunhofer *et al.* 1995). An extensive analysis of the deep black hydorolysate was not conducted. The hydrolysate was further treated by increasing temperature into the range 150-350 ^{0} C, with optimum resulting above 200 ^{0}C. A solid was separated and the remaining liquid was treated both anaerobically and aerobically. Attempts to biologically degrade the hydrolysate were unsuccessful (without thermal treatment); however, denitrification was observed when co-substrate was added (Fraunhofer *et al.* 1995).

The hydrolysis of TNT is partially mineralized at 80 ^{0}C with at least one mole of nitrite per mole of TNT existing in the hydrolysate (Saupe *et al.* 1996). Additionally, 10% of the TNT-C as inorganic C and small amounts of ammonium were identified. Microfiltration of the hydrolysate showed that 60% of the products had molecular weights in excess of 30 kDa.

Little progress has been made in identifying the products that exist after TNT hydrolysis. Some products such as anilines (i.e. N-methylaninlin), dinitrobenzenes, nitoanalines, toluol, Ethylbenzol, and various long chain saturated hydrocarbons, such as hexadecane and tetradecane have been recognized. The nature of the hydrolysis reaction complicates the classification of possible products and reaction mechanisms. The chemistry produces all reduced forms of CH_3 and NO_2 which can result in anthranils, alcohols, and adehydes, as well as nitroso, nitrtil, azo and azoxy compounds among others (Reimer 1995).

of precipitation of metals (Raskin *et al.* 1994). Phytostabilization is the use of plants to reduce the availability of toxics in soils and prevent their entry into ground water and food chains.

The study done by Schneider *et al.* (1996) examines the uptake of explosives by existing vegetation in soils contaminated with TNT and RDX in three areas at the Iowa Army Ammunition Plant (IAAP). Plant material and soil from the root zone were sampled and separated by species at different locations in each area to determine explosives uptake under natural environment conditions.

Standard methods enforced by the EPA were used to determine the concentrations of explosives, their derivatives and metabolites in the soil sample, and for analyzing plant materials. TNT was not detected in the above ground portion of most of the plants. Low concentrations of TNT existed in two of the 35 plants in the sample area with levels of 10.7 mg/kg of 4A-DNT and 4.5 mg/kg of 2A-DNT present in only plant roots. Analysis showed that vegetation growing on TNT-contaminated soils should not be considered to be a health hazard. However, soil and plant roots may contain other TNT degradation products that may be toxic and unhealthy for consumption.

Although, the previous study only discusses health concerns of TNT and its byproducts, researchers have investigated possible applications of phytoremediation. A new approach involves rigorous pathway analyses, mass balance determinations, and identification of specific enzymes that breakdown TNT. The proteins which reduce TNT were isolated and the immuno-specific assays were developed by

purifying the enzymes from reactive sediments and injecting the extract into the spleen of a laboratory mouse to develop antibodies (McCutcheon *et al.* 1995). These antibodies were used in tests to identify the specific enzymes. Four sediment-derived proteins were traced back to plants.

Pathway analyses of metabolites, mass balances, and radiolabeled studies showed that TNT is completely and rapidly degraded by specifically the nitroreductase and laccase enzymes. The aromatic ring is broken and the carbon in the ring fragments is incorporated into new plant fiber, as part of the natural lignification process. (McCutcheon *et al.* 1995). Half-lives for TNT degradation were reached in one hour or less under ideal laboratory conditions.

A number of aquatic plants containing the nitroreductase enzyme were identified for biodegradation. Research indicates that they can adapt to high metals concentration, low pH, and high toxicity while remaining vigorous in the presence of toxic soil concentrations and keeping dissolved concentrations of TNT low enough for tadpoles and snails to thrive in overlying waters. McCutcheon *et al.* (1995) believes that nitroreductase is an ancient, nonspecific, versatile enzyme which has evolved in plants with the adaptive mechanisms to protect their enzyme systems so that they can survive under harsh conditions. The submerged and emergent aquatic plants Algae nitella and spirogyra, blue-green algae, Anthrocerotae, duckweed, and hydrilla and a few terrestrial plants (poplar and sycamore) containing the nitroreductase enzyme were identified for remediation of TNT. Both emergent and

submergent varieties of the parrot feather was the most efficient for remediation of TNT.

An important step is that the reduction of TNT by nitroreductase is not sensitive to the presence of oxygen. Thus, anaerobic-aerobic cycling is not required. Furthermore, the recent discovery that poplar trees and other terrestrial plants contain nitroreductase opens the possibilities to ecological engineering of created wetlands. A variety of crops and trees to clean up hazardous waste sites could be a solution for TNT remediation (McCutcheon *et al.* 1995).

As cited by McCutcheon *et al.* (1995), on going studies by researchers such as Young (1995) show rapid removal of TNT and all metabolites from water, and reasonable removal of TNT from soil. In batch pilot studies conducted at the demobilized Alabama Army Ammunition Plant in Childesburg, AL and at Auburn University, TX, TNT concentrations in flooded basins with soils starting at approximately at 5000 mg/L quickly reached saturation. Within two weeks of the introduction of plants, TNT levels dropped below detection. Within several weeks, parrot feather can root in previously sterile soil.

The objectives of the study done by Wolfe *et al.* (1994) were to evaluate the feasibility of using a redox enzyme, nitrate reductase, originally isolated from a pond sediment as part of a new biotreatment process to reduce TNT to various monamino, diamino, and triamino-toluenes, which could be subjected to bench-scale bioslurry treatment at the U.S. Army Engineer Waterways Experiment Station. The study examined the ability of the reductase enzyme to remediate TNT-contaminated soil

using the following rationale: Native soil microorganisms deplete oxygen, which is crucial for the nitroreductase enzyme to reduce nitroaromatic compounds to their corresponding amino compounds under anaerobic conditions (Fig. 5.9). The incubation regime changes to allow the microorganisms to complete the destruction process. The nitroaromatic amines are microbially oxidized to catechols and subsequently, fission occurs. (Wolfe *et al.* 1994).

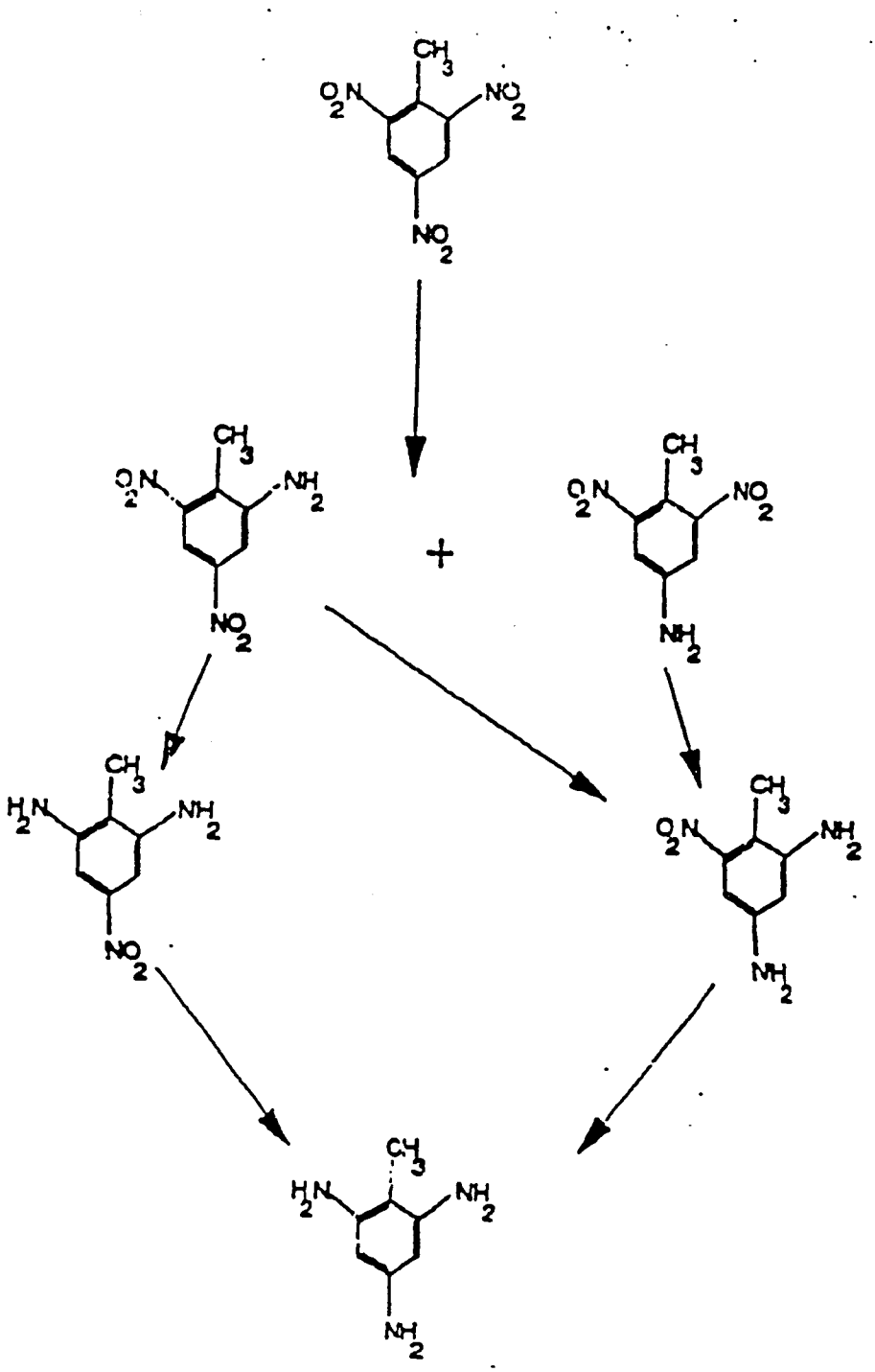


Fig. 5.9 Proposed mechanism of TNT reduction with sediment extracted enzyme and aquatic weed stonewort
Wolfe (1994)

The aquatic plant stonewort (*Nitella*) was identified as the source of nitrate reductase. Stonewort are members of the *Chlorophyta* (green algae) family (Wolfe *et al.* 1994). The entire plant material of *Nitella* was used as a concentrated plant source of the nitrate reductase enzyme. In the six soil samples studied, the pure enzyme treatment had minimal effects on TNT removal and very limited difference in ADNT formation over the corresponding levels present in the controls. Wolfe *et al.* (1994) believes removal of TNT was most likely being carried out by the native soil microflora and the failure to remove higher levels of TNT in the enzyme treatments could be due to the short incubation time.

Further investigation in the study using intact stonewort plant was effective in reducing TNT. The nitrate reductase reduced TNT from 8,723 mg/kg to 10.8 mg/kg in 43 days in the soil. Four soil samples collected from Radford AAP, Iowa AAP, Lone Star AAP, and Submarine Base Bangor contaminated with low levels of TNT, no detectable TNT in the solid phase remained after 43 days.

Analysis of stonewort indicated that none of the TNT or aminotoluenes accumulated in the plant tissues. The nitrate reductase activity was so high in the intact plant that TNT was transformed as rapidly as it moved into the aqueous phase (Wolfe *et al.* 1994). Furthermore, TAT was not detected at day 43 due to the fact that TAT was oxidized rapidly by autooxidation or microbial activity to yield catechol (Wolfe *et al.* 1994).

Stonewort treatment is useful for soil surface bioremediation. The treatment area can be flooded and the plant added to the soil surface with only periodic turning

of the soil required for continued treatment. Furthermore, Stonewort indirectly regulates the pH of the soil-water system and eliminates the poisoning of the nitrate reductase by the elements Pb and Zn which allows stonewort to be applied to a wide range of conditions (Wolfe *et al.* 1994

Van Beelen and Burris (1995) studied the reduction of TNT by NADPH, which is catalyzed by enzymes extracted from aquatic sediments. Their focus analyzed the catalysis by extracellular enzymes separately from other processes occurring in soils and sediments.

Enzymes were extracted from aquatic sediments. Some of these enzymes reduced TNT with NADPH as an electron acceptor under aerobic conditions. The origin of these enzymes was unclear because the enzymes were extracted from a sediment containing plant roots and microorganisms. Results indicated that aquatic plants were a source of TNT-reducing enzymes. Chemical reduction was not a rate-limiting process in the assays as the enzymatic activity was inhibited by heating or the addition of proteases.

Enzymatic activity was further characterized partial purifications of enzymes from the sediment extracts showed that several proteins may be capable of reducing TNT with NADPH present. The enzymes did not require flavins and preferred NADPH over NADH as the electron donor (Van Beelen and Burris 1995). The pH was around neutral and the optimum temperature was in the range of 37-45^0 C and addition of dithiothreitol improved the stability of the enyzmes. The enzymes reduced TNT to 2-amino-4,6-dinitorotoluene (2A4DNT), 4-amino-2,6-

dinitorotoluene (4A2DNT), 2,4-diamino-6-nitrotoluene, and 2,6-diamino-4-nitrotoluene under aerobic conditions with NADPH.

When used as a starting substrate, the amino dinitrotoluenes were not reduced by the enzymes. Van Beelen and Burris (1995) suggest that the hydroxylamino-dinitrotoluenes are reduced to dihydroxylaminenitrotoluenes prior to the formation of the diaminonitrototluenes (Van Beelen and Burris 1995). The enzymes appear to be present in the both freshwater and saltwater sediments. TNT reduction by sediment enzymes can be a significant environmental fate process for TNT in aquatic systems.

Although phytoremediation is not a remedy for cleanup, many great strengths exist. Lightly managed ecosystems, which are more highly evolved than bacterial cultures to control pH and metals concentrations, can be used to completely break down and incorporate contaminant molecules into new plant biomass (McCutcheon *et al.* 1995). Fast and efficient degradation of TNT occurs leaving aesthetic ecosystems in place of sterile soils produced by incineration. As cited by McCutcheon *et al.* (1995), current risk based cleanup and drinking water standards are also achieved in most cases. Furthermore, overall costs can be minimized because of reduced costs of disposal of treated residual soils, compared to bioslurries and other situ treatments. However, longer times may be required to achieve strict cleanup standards compared to incineration. Also, a lightly managed ecosystem may involve limited control and release of intermediates if the process does not work as intended.

Yet, despite these obstacles, McCutcheon *et al.* (1995) suggests that innovative remediation research should begin to focus on plant systems. The most likely use of plants will be in a diverse ecosystem that should also include the right types of bacteria and fungi to assure appropriate nutrient cycling for plants and as well as aid in the degradation of TNT (McCutcheon *et al.* 1995).

5.12 Reuse/Recycle Options

Recovery and reuse technology for energetic material, including explosives, is available in production-scale facilities, which handle amounts greater than 100,000 lb. Recovery/reuse options are important for explosives wastes because new recovery methods and potential uses for reclaimed materials are continually being developed. Overall remediation costs are reduced because destruction costs are eliminated and the recoverable value of reclaimed materials increases. Furthermore, the EPA's treatment hierarchy favors recovery/reuse options over destruction technologies because reuse/recovery is more environmentally sound. The Army has reviewed the types of explosive waste and media that can be recovered/reused, the available recovery/reuse technologies, the institutions using the process, and the advantages and limitations of recovery/reuse (US EPA Handbook 1993).

A technically difficult aspect of energetic material recovery/reuse is separating energetic ingredients from inert components. However, recovery methods

for TNT-based explosives are well established and involve melt and steam-out processes (US EPA Handbook 1993). The process liquefies TNT so that it can be poured out of the munitions. TNT melt and steam-out facilities exist at the Western Demilitarization Facility in Hawthorne, Nevada.

Once energetic materials have been separated from inert materials reuse is direct. Reuse applications includes the addition of recovered TNT as sensitizing agents and blast enhancers for slurry and emulsion explosives used in the mining quarry industries. According to the Institute of Manufactures of Explosives, hundreds of millions of pounds of slurries and emulsion explosives are used. Applying 5-30% recovered explosives in slurries could be a significant market depending on the availability and cost.

Ingredient recovery from explosive compositions is the least advanced reuse technology. Ingredient recovery is not difficult but depends on an economic or environmental driving force to recover individual ingredients (EPA Handbook 1993). Furthermore, many military programs have a "no change" policy that restricts changes in materials used in ordnance manufacture. The "no change" policy is being revamped under pressures, but ingredient recovery will continue to have resistance from risk-averse program managers (US EPA Handbook 1993).

One recovery/reuse approach proposed for energetic contaminants in soils and sludges is solvent extraction by burning of the extract with other fuels to proved energy. The Army originally considered this technology to be infeasible for treating explosives-contaminated soils. It can potentially treat explosives-contaminated soils

if a few lingering technical issues can be resolved The Army Environmental Center (AEC) has demonstrated that low levels of smokeless powder, TNT can be used as to supplement boiler fuel. This energy recovery approach also could be applied to extracted energetic materials, using the AEC studies as a guide to the sensitivity and fuel value of the materials.

In 1982, the Army conducted laboratory-scale solvent extraction tests on explosives-contaminated lagoon samples from a number of sites. Each sample was washed with a solution of 90% acetone and 10% water. This process achieved greater than 99% contaminant removals. In 1985, the Army conducted a pilot-scale engineering analysis to determine the feasibility of full-scale extraction. Analysis indicated that reducing the number of required washes and as well as recovering and reusing acetone will only allow this method to be economically viable (US EPA Handbook 1993). Currently, the only available technology for recovering acetone is distillation, which exposes acetone to heat and pressure. Exposing a solvent that has been used to extract explosives contaminant raises major safety considerations (US EPA Handbook 1993). The distillation column used to recover acetone is sometimes referred to as an "acetone rocket." However the Army believes that full scale solvent extraction would be cost-effective if a safe, efficient, alternative recovery method were developed.

Recovery and reuse of energetic materials should be a goal in every remediation effort. The Environmental Protection Agency places this option higher than destruction technologies on the preferred treatment scale. Although the

Louisiana Army Ammunition Plant's steam-out facility for TNT-based explosives is well established and production-scale methods have been operational for decades, Each situation requires a cost/risk/benefit assessment for implementation

6 SUMMARY AND RECOMMENDATIONS

This thesis has thoroughly reviewed the literature on TNT. Initially stated objectives of TNT manufacturing, its chemical properties and innovative physical, chemical and biological treatment techniques provide ample information and references for future research work.

TNT, the toxic explosive chemical, is efficiently remediated by various available techniques and methods. Even though the overall environmental impact of TNT contamination has not been fully determined, the bioamplification of the pollutant in the food chain and the consequential threat to human health is a concern to researchers.

Table 6.1 provides a summary of the information concerning transformation and degradation of TNT. Boopathy and Kulpa *et al.* (1993), and Preuss (1994) show that sulfate-reducing bacteria such as *Desulfovibrio* strains can use TNT as an electron acceptor and/or nitrogen source while using compounds such as lactate and pyruvate as carbon sources. TNT is reduced by these strains sequentially through monamino and DANT to TAT, which may accumulate or be reductively deaminated toluene. Furthermore, despite limited available work regarding aerobic biodegradation, it is more promising. Aerobes, such as *Pseudomonads*, can convert TNT into other intermediates as well as completely biodegrade 2,4-DNT. However, future work is required to firmly establish the centrality among aerobes and anaerobes.

Table 6.1 Summary of Available Microorganisms for TNT Remediation

Organism	Comments	Reference
P. chrysosporium	Evaluated more extensively than any other fungal species. Research indicates mineralization of TNT does occur. However, small amounts of contaminated soil inhibit the fungus.	Spain *et al.* 1995 Fernando *et al.* 1990 Spiker *et al.* 1992
Methanococcus sp. B strain	TNT is reduced to intermediates that may be more toxic. A mixed culture system will strengthen the degradation process under anaerobic conditions	Boopathy *et al.* 1993 Preuss et al. 1992 Spain 1995
Clostridia	Have been studied because of their ability to reduce nitroaromatic compounds. Clostridia bacteria slowly convert DAHAT to TAT and will probably require action specific enzymes.	Crawford *et al.* 1994 Spain 1995
Ruminal Bacteria	A full consortium was capable of biotransforming TNT. Although the pure isolate G.8 cannot biotransform TNT as completely as the full consortium, it is a key participant in the process.	Craig *et al.* 1994
Actinomycetes	Research indicated that no significant mineralization occurred when an actinomycete indigenous to a TNT-contaminated site. *S. chromofuscus* A11 and ESA 1 transformed TNT to 2,4,DA6NT and 4A2,6DNT.	Grisby *et al.* 1996
Pseudomonads	Can reduce TNT under aerobic conditions. Several strains, such as *P. aeruginosa* showed promising results. Research suggests that Pseudomonad strains combined with other microorganisms may provide successful treatment systems for TNT degradation.	Kitts *et al.* 1995 Duque *et al.* 1993
Alcaligenes 1-15	Research indicates the transformation of TNT into 2A4,6DNT and 4A2,6DNT being placed in an aerobic reactor for 48 hours. The mutagenicity of TNT was affected by the bacteria.	Collie *et al.* 1995

Rapid progress continues so that researchers can understand the biodegradation of nitroaromatic compounds. As a result of recent studies, the following areas must be fully determined.

- Many intermediates must still be identified, such as those between triaminotoluene and toluene in *Desulfovibrio* strains.

- Reduction of TNT by anaerobes is well-established, yet areas of uncertainty such as the metabolism of triaminotoluene remain.

- The initial steps in the pathways catalyzed by white rot fungi are clear and the mechanism of toxicity is better understood. However, very little is known about how reduced metabolites of TNT are mineralized by the fungus.

- Aerobic bacteria, such as *Pseudomonads,* can convert nitroaromatic compounds into intermediates that can serve as growth substrates. The mechanisms of the reactions, their regulation, and the structure of the enzymes are areas that must be further researched.

- A limited amount is known about the molecular biology of the systems. Further analysis of the molecular basis will allow their capabilities to be enhanced and exploited for practical purposes.

Table 6.2 Summary of Available Treatment Processes

Process	Comments	Reference
Composting	A cost-effective, environmentally safe technology which generates an enriched product that can sustain vegetation. However, composting requires long treatment periods and the ultimate fate of TNT is not yet clear. Further investigation is required.	US EPA Handbook 1993 Adrian *et al.* 1995 Caton *et al.* 1994
Bioslurry Treatment	Capable of increasing the degradation rate of TNT by increasing the availability of contaminants, electron acceptors, nutrients, and other microbial consortia when the engineering arrangement of other widely used bio-treatment methods are strategically configured in the design.	Zappi *et al.* 1994
J.R. Simplot Technology	A procedure which enhances naturally selected an-aerobic organisms. Bioremediation of TNT is achieved even though further design improvements are necessary to make it economically competitive.	Jackson & Hunter 1995 Funk *et al.* 1995
In-Situ Biodegradation	An effective system which can avoid high costs incurred with other techniques. Research indicates mineralization of TNT with microbial consortia at Weldon Springs, MI. However, some environmental factors did inhibit TNT mineralization.	Bradley & Chapelle 1995 US EPA Handbook 1993
OB/OD	Practiced for many years, considered the best available "first generation" demonstrated technology. Requires a RCRA subpart X permit for application. Stringent environmental regulations have effectively decreased the process of OB/OD.	US EPA Handbook 1993
Incineration	At long residence times and high temperatures, success-fully reduces levels of TNT to nondetection levels. However, safety concerns, air and noise problems, and public perception have limited this "second generation technology" from extensive use.	US EPA Handbook 1993
Activated Carbon	GAC has been efficiently applied at every Army Ammunition Plant. However, GAC is a transfer technology and carbon adsorption media is only partially regenerated. Researchers are exploring a combination of chemical and biological treatments to fully regenerate and reuse the activated carbon.	Goodfellow & Ramirez 1995 US EPA Handbook 1993

Table 6.2 (cont.) Summary of Available Treatment Processes

Process	Comments	Reference
Photocatalytic Degradation	Studies examining degradation of "pinkwater" with the application of the photocatalyst, TiO_2 suggest a potential for effectively remediating TNT contaminated waters because the process achieves almost complete mineralization of TNT and the refractory intermediates	Prairie *et al.* 1994 Dillert *et. al* 1995 Schmelling & Gray 1995
Ultraviolet Oxidation	Although it can effectively treat and destroy target compounds, it is not extensively used for treating explosives contaminated water. Research indicates 1,3,5-TNB, an intermediate product of TNT, is rate controlling and must require additional treatment to achieve successful economical results. Further evaluation is necessary before the technique can be implemented.	US EPA Handbook 1993 Anyanwu *et al.* 1993
Wet Air Oxidation	Although the Army identified the process as technically or economically infeasible, Recent work suggests that "red water" can be effectively treated. Based on analysis, treatment of "red water" formed DNT sulfonates which would need further treatment	Hao *et al.* 1993 US EPA Handbook 1993
Advanced Oxidation Techniques	Although not evaluated as extensively as other technologies, it is a promising for treating hazardous. Liquid wastes. Further, analysis and demonstration will make it an emerging treatment technology.	Willberg *et al.* 1996
Low Temperature Thermal Desorption	The Army conducted a laboratory-scale study and achieved 95% destruction and removal. However, no discussion was given regarding the specific explosives that were treated.	US EPA Handbook 1993
Phytoremediation	A new technological approach which uses specially selected and engineered explosives-accumulating plants. Studies show that the nitroreductase enzyme found in the plants attributes to the reduction of TNT. Phytoremediation, along with other treatment techniques, can be applied in a system to give successful results.	McCutheon *et al.* 1995 Raskin *et al.* 1994 Schneider *et al.* 1996
Reuse/Recyle Options	EPA's treatment hierarchy favors this technique over other methods. Recovery and reuse should be be a goal in every remediation effort and has been examined by the Army.	US EPA Handbook 1993
Alkaline Hydrolysis	A promising treatment technology which currently is used as a pretreatment process followed by a form of thermal treatment. They hydrolysate product must be further analyzed for effective application of the treatment.	Priestley *et al.* 1996 Dell'Orco *et al.* 1995 Spontarelli *et al.* 1993

Despite the obstacles for removal of TNT, innovative remediation research should begin to focus towards a diverse ecosystem which should include the right types of bacteria, fungi and plants to assure appropriate nutrient cycling (McCutcheon *et al.* 1995). Additionally, successful physical and chemical treatment processes, such as alkaline hydrolysis should be coupled with the biological treatment methods to provide successful application.

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8 APPENDIX

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