Anaerobic and Aerobic Transformation of TNT

C.F. Kulpa¹, R. Boopathy², and J. Manning²
¹Department of Biological Sciences, University of Notre Dame, Notre Dame, IN 46556. ²Environmental Research Division, Argonne National Laboratory, Argonne, IL 60439.

ABSTRACT

Most studies on the microbial metabolism of nitroaromatic compounds have used pure cultures of aerobic microorganisms. In many cases, attempts to degrade nitroaromatics under aerobic conditions by pure cultures result in no mineralization and only superficial modifications of the structure. However, mixed culture systems properly operated result in the transformation of 2,4,6-trinitrotoluene (TNT) and in some cases mineralization of TNT occurs. In this paper, the mixed culture system is described with emphasis on intermediates and the characteristics of the aerobic microbial process including the necessity for a co-substrate. The possibility of removing TNT under aerobic/anoxic conditions is described in detail.

Another option for the biodegradation of TNT and nitroaromatics is under anaerobic, sulfate reducing conditions. In this instance, the nitroaromatic compounds undergo a series of reductions with the formation of amino compounds. TNT under sulfate reducing conditions is reduced to triaminotoluene presumably by the enzyme nitrite reductase, which is commonly found in many Desulfovibrio spp. The removal of nitro groups from TNT is achieved by a series of reductive reactions with the formation of ammonia and toluene by Desulfovibrio sp. (B strain). These metabolic processes could be applied to other nitroaromatic compounds like nitrobenzene, nitrobenzoic acids, nitrophenols, and aniline. The data supporting the anaerobic transformation of TNT under different growth conditions are reviewed in this report.

INTRODUCTION

As a result of manufacture and widespread use of pesticides, explosives, dyes, plastics, and pharmaceuticals by modern society, large quantities of nitroaromatic compounds have been released into the environment. TNT (2,4,6-trinitrotoluene) is a primary military explosive that was widely used in the past because of its low melting point; its stability; its low sensitivity to impact, friction, and high temperature; and its relative safe methods of manufacture (Urbanski, 1984). Disposal of explosives during manufacturing and testing has resulted in extensive contamination of soil (Klausmeier et al. 1973). Concerns about the environmental fate of TNT residues have intensified because the recent vegetation of contaminated plots could allow TNT, TNT metabolites, and plant-produced TNT intermediates to be introduced into the food chain (Harvey et al. 1990).
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Biological removal of TNT is feasible. Biotransformation of nitroaromatic compounds by aerobic bacteria has been reported by many workers (Carpenter et al. 1978; McCormick et al. 1976; Schackmann and Muller, 1991; Won et al. 1974; Boopathy et al. 1994). Under anaerobic conditions, nitroaromatic compounds are metabolized by sulfate reducing and methanogenic bacteria (Boopathy and Kulpa, 1992; 1993; Boopathy, 1994). Fungal metabolism of nitroaromatics has been demonstrated (Fernando et al. 1990). Most of these works show superficial modification or transformation of TNT. In the present study, we demonstrate how, by changing the electron accepting conditions various bacteria can biotransform and mineralize TNT in a mixed culture system. The result showed that under aerobic/anoxic conditions, soil bacteria mineralized TNT significantly.

EXPERIMENTAL

**Microorganisms.** The aerobic soil bacteria, *Pseudomonas* spp. were isolated from the TNT contaminated soil collected from the Joliet Army Ammunition Plant, Joliet, Illinois, USA. The anaerobic sulfate reducing bacterium (SRB), *Desulfovibrio* sp. (B strain) was isolated from an anaerobic digester treating furfural containing wastewater (Boopathy and Daniels, 1991)

**Growth Conditions.** The aerobic soil bacteria were cultivated in a heterotrophic medium containing the following components: K$_2$HPO$_4$ (7.0 g/L), KH$_2$PO$_4$ (3.0 g/L), MgSO$_4$ (0.1 g/L), NaCl (0.1 g/L), (NH$_4$)$_2$SO$_4$ (0.25 g/L), yeast extract (0.05 g/L), succinate (5.0 g/L), and TNT (100 mg/L). All culture flasks were incubated in a gyratory shaker at 150 rpm kept at ambient temperature (20-22°C). The SRB was grown in a SRB medium described by Boopathy and Kulpa, (1992).

**Co-metabolic Screening Study.** To develop a (preferably inexpensive) co-substrate for field applications, various co-substrates were screened with the aerobic bacteria in a consortium. For co-metabolic study, the heterotrophic medium described above was used except the carbon source which was substituted with various growth substrate. For each carbon source, duplicate culture flasks were incubated. Growth and TNT concentration were monitored every day.

**Aerobic/Anoxic Soil Slurry Reactor.** Two 0.5 L aerobic/anoxic soil slurry reactors were designed. The reactor set up is shown in Fig. 1. TNT-contaminated soil was collected at the Joliet Army Ammunition Plant, Joliet, Illinois. The TNT concentration in the soil ranged from 10,000 to 20,000 mg/kg (Table 1). The reactors were operated semi-continuously. The reactors were started with 15% (w/v) of TNT-contaminated soil. Molasses (0.3%) (Grandma's molasses, Mott's USA, Cadbury Beverages Inc., Stamford, Connecticut) served as the carbon source. Air was pumped through a diffuser for 15 to 30 min each day. After a two week stabilization period, 10% (w/v) of the contaminated soil was replaced every week, with the addition of molasses. Concentrations of TNT and its metabolites were monitored.
Carbon-14 Mineralization Studies in the Slurry Reactor. After seven months of reactor operation, 100 ml of soil slurry was taken from each reactor. The soil slurry was incubated with \([^{14}C]\) TNT (uniformly ring labeled) to establish mass balance and to determine the production of metabolites including \(^{14}CO_2\). The \([^{14}C]\)TNT was added to the soil slurry in respirometer flasks at the level of 20,000 cpm/ml (Bartha and Pramer, 1965). The control flasks contained autoclaved soil slurry. Samples were withdrawn periodically, and the quantity of TNT converted to biomass was determined as trichloroacetic acid (TCA)-precipitable material (Mans and Novelli, 1961) by using a liquid scintillation spectrometer (Beckmen Model LS 5000 TD). The CO\(_2\) evolved from degradation of \([^{14}C]\) TNT by the soil bacteria was monitored according to the method described by Bartha and Pramer (1965). The experiment was conducted in duplicate.

The TNT metabolites were analyzed by collecting fractions every 30 seconds after passage through the HPLC column. The radioactivity in each fraction was measured by using a liquid scintillation counter.

**Analyses.** The water soluble TNT and the soil bound TNT were analyzed using HPLC according to the method described by Manning et al. (1995). The bacterial growth was monitored by measuring the culture turbidity at 600 nm with a Spectronic 20 spectrophotometer. The TNT metabolites were identified using GC/MS as per the method described earlier (Manning et al. 1995).

**Chemicals.** Radiolabeled TNT (uniformly ring labeled; specific activity 21.58 mCi/mm, 98.5% pure) was purchased from Chemsyn Science Laboratory, Lenexa, KS. The non-radioactive TNT (98% pure) was obtained from Chem Service Inc., Westchester, Pennsylvania. All other chemicals were of reagent grade.

**RESULTS**

**Aerobic Study:**

**Co-metabolic Screening.** The performance of the aerobic soil bacterial consortium in transforming TNT in the presence of various substrates is presented in Table 2. The specific growth rate of the culture growing on molasses was three to six times higher than those of growing on succinate, citrate, malic acid, acetate, sucrose, or glucose. Similarly, the specific transformation rate of TNT in the molasses fed culture was higher by an order of magnitude than those of cultures fed with other carbon sources. No growth or TNT transformation was observed in the killed control or in the cultures that received TNT as the sole source of carbon and energy. The molasses fed culture took only 12 h to transform 100 mg/L of TNT, whereas the succinate fed culture took 105 h, and the other cultures needed 130 h to transform 80-100 mg/L of TNT. The concentration of substrate used was significantly less for molasses (0.3%) than for the other substrates (0.5%).

These results indicate that molasses is a very good substrate, stimulating bacterial growth and increasing the rate of TNT transformation. This experiment proved that TNT transformation is achieved by co-metabolic process. The molasses used in this experiment was commercial black strap molasses. The complex composition of molasses (with sugars, amino acids, and proteins) makes its conducive to the growth of many types of bacteria in the consortium.
Aerobic Pure Culture Study. Four aerobic bacteria were isolated from the TNT-contaminated soil consortium. All four isolates were gram-negative rods identified as *Pseudomonas* spp. (Table 3). Figure 2 shows the growth curves for the isolates. The isolates were cultured in heterotrophic medium with 0.5% succinate (5.0 g/L) and TNT (100 mg/L). Isolates 1 and 2 grew rapidly and reached a maximum optical density of 1.10 at 600 nm within 31 h of incubation. Isolate 4 grew slowly, with a lag period of 24 h, and reached maximal growth after 140 h of incubation. The TNT concentration dropped rapidly with all four isolates. TNT was completely transformed within 4 days of incubation by isolate 4. Isolates 1, 2, and 3 needed eight days to transform 100% of the TNT (Fig. 3).

Radiolabeling experiments showed that isolate 3 used 13% of the [$^{14}$C] TNT to make cellular material. Isolates 1 and 2 converted about 8% of the [$^{14}$C] TNT to biomass, while isolate 4 converted about 6% of the [$^{14}$C] TNT to TCA-precipitable material. Approximately 1% of [$^{14}$C] TNT was converted to $^{14}$CO$_2$ by isolate 4. The average TNT mass balance for all the isolates is summed up in Table 4. There was very minimum mineralization. Most of the TNT was converted to amino intermediates. This pure culture experiment showed that the aerobic bacteria could transform TNT significantly but not mineralize it.

Aerobic/Anoxic Soil Slurry Reactor. As described in the experimental section, two soil slurry reactors were operated under aerobic/anoxic condition. The dissolved oxygen (D.O) in the reactor varied significantly according to the depth. The D.O was around 3 mg/L at the surface and it decreased with depth and at the bottom 2 cm of the reactor the D.O was 0 mg/L.

Performance of the Reactor. The distribution of TNT in the soil at Joliet Army Ammunition Plant is not homogeneous. Some parts of the contaminated site have high TNT concentration, whereas other parts have lower concentration. This could explain why the TNT concentration at the beginning of the experiment was so high (nearly 8000 mg/kg) (Fig. 4). The soil used as replacement material generally had a TNT concentration of 6000-8000 mg/kg. The concentrations of TNT and its metabolites 4-amino-2,6-dinitrotoluene and 2-amino-4,6-dinitrotoluene are given in Fig. 4.

The first two weeks of reactor operation were in the typical batch mode, and the concentration of TNT in the reactor dropped during this period. On day 15 the level of TNT was less than 1000 mg/kg. After two weeks of operation, a 10% volume of contaminated soil was added to the reactor. Thereafter 10% of the soil slurry was replaced every week with contaminated soil. Whenever soil was added, the concentration of TNT increased, then decreased on the next day. This rise and fall of TNT levels was seen throughout the first two months of reactor operation. After two months of operation, the TNT concentration in the soil dropped steadily, eventually falling below the detection limit (0.5 mg/kg) on the 95th day. The concentrations of the TNT metabolites, 4-amino-2,6-dinitrotoluene and 2-amino-4,6-dinitrotoluene were also monitored throughout the reactor operation. As Fig. 4 shows, the concentration of intermediates ranged from 0 to 2100 mg/kg, generally increasing upon soil addition and
decreasing on the next day. After four months of continuous operation, the concentration of TNT metabolites was less than 20 mg/kg of soil.

The radiolabeling studies with the reactor biomass demonstrated the mineralization of TNT. The $^{14}$CO$_2$ increased gradually from 0.1% on day 0 to 23% on day 14. In the killed control, less than 0.3% $^{14}$CO$_2$ was observed. This observation clearly denotes that the TNT was mineralized by the soil bacteria in the soil slurry system, and its explains the disappearance of the amino compounds from the reactor.

Fig. 5 shows the distribution of radiolabeled TNT after 30 days of incubation. Typically 29% of TNT was converted to biomass as TCA precipitable material, 24% to CO$_2$, 28% was observed as the major metabolite which was identified as 2,3-butanediol, and the rest was distributed as various reduction products.

**Anaerobic Experiment.** Under anaerobic conditions, a SRB Desulfovibrio sp. (B strain) was used to biotransform TNT. The bacteria was grown as described by Boopathy and Kulpa (1992). The SRB was grown under various growth conditions. Depending on the growth conditions, TNT was transformed to various metabolites (Table 5). The significant finding of this experiment was this SRB used TNT as the sole nitrogen source and removed the nitro groups from TNT molecule and in the end formed toluene as the major metabolite. This SRB however did not mineralize TNT and it left the ring intact. This SRB also used other nitroaromatic compounds such as nitrophenol, nitrobenzene and aniline (Boopathy and Kulpa, 1993) as the nitrogen source.

**DISCUSSION**

This study showed that under aerobic conditions, pure cultures of *Pseudomonas* were unable to mineralize TNT; however, the pure cultures significantly transformed TNT to amino compounds. Under anaerobic conditions, the SRB Desulfovibrio sp. (B strain) used TNT as the sole source of nitrogen and converted it to toluene. This bacterium also did not mineralize TNT.

The aerobic/anoxic soil slurry reactor is an effective method for remediating TNT and other munition compounds present in the contaminated soil. The operation of laboratory scale soil slurry reactors over 200 days showed that 100% removal of TNT can be achieved. During start up, a two week stabilization period was needed before further loading of soil in the reactors. Operation of the reactors was highly successful in the semi-continuous mode. TNT was also removed in the batch mode of operation. However, TNT metabolites (amino compounds) persisted during the batch operation. For successful removal of TNT and its metabolites, semi-continuous operation is required. Throughout the study no reactor failure occurred because of overloading or the accumulation of toxic metabolites. The radiolabeled study showed a very reasonable mass balance.

This study showed that the pure cultures of either aerobic or anaerobic bacteria did not mineralize TNT. However, a mixed culture of bacteria under an aerobic/anoxic system effectively removed TNT from soil. This study also indicated that the natural soil bacteria present in contaminated soil can cause extensive transformation and
degradation of TNT in a reasonable time under optimum conditions. Degradation was demonstrated by mineralization of [14C]TNT, production of metabolites and the presence of [14C]TNT in the cell biomass as TCA-precipitable material. Each of the biological system reported in the literature as acting on TNT catalyzed the reduction of at least one nitro group (Won et al. 1974; McCormick et al. 1976; Schackmann and Muller, 1991). In the present study the soil bacteria produced amino intermediates and also were able to mineralize TNT significantly. The radiolabeling study showed that the biomass from prolonged reactor operation over 200 days converted [14C]TNT to 14CO2. Because the ring carbon of TNT were uniformly labeled, conversion to CO2 clearly denotes ring cleavage. The mechanism of ring cleavage is not clear. However, the first step was the reduction process as reported before by many workers (McCormick et al. 1976; Won et al. 1974; Boopathy et al. 1994). The soil slurry reactor system seems to be very promising. The advantage of slurry reactor is its simplicity of operation. The method needs only mixing, an intermittent supply of air, and a carbon source. Molasses is an inexpensive carbon source that could be used in a large scale operation at low cost. The frequency of soil replacement in the system can be determined on a site specific basis, depending on the concentration of TNT in the contaminated soil.

REFERENCES


FIGURE 1 Schematic Diagram of the Soil Slurry Reactor

TABLE 1 Explosives Concentrations in Soil

<table>
<thead>
<tr>
<th>Explosive</th>
<th>Concentration Range (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNT</td>
<td>10,000-20,000</td>
</tr>
<tr>
<td>Trinitrobenzene</td>
<td>175-300</td>
</tr>
<tr>
<td>2,4-Dinitrotoluene</td>
<td>50-200</td>
</tr>
<tr>
<td>RDX</td>
<td>50-125</td>
</tr>
<tr>
<td>HMX</td>
<td>50-100</td>
</tr>
<tr>
<td>Trinitrobenzaldehyde</td>
<td>50-150</td>
</tr>
</tbody>
</table>

*Abbreviations: HMX, octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine; RDX, hexahydro-1,3,5-trinitro-1,3,5-triazine; TNT, 2,4,6-trinitrotoluene.*
Table 2 Transformation of TNT by Bacterial Cultures with Different Co-substrates

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Specific Growth Rate (h⁻¹)</th>
<th>TNT Concentration (mg/L)</th>
<th>Percent TNT Transformed</th>
<th>Specific Transformation Rate (mg/L/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Initial</td>
<td>Final</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>-</td>
<td>100</td>
<td>99</td>
<td>1</td>
</tr>
<tr>
<td>TNT alone</td>
<td>-</td>
<td>100</td>
<td>98</td>
<td>2</td>
</tr>
<tr>
<td>Malic acid</td>
<td>0.044</td>
<td>100</td>
<td>11.8</td>
<td>88.2</td>
</tr>
<tr>
<td>Acetate</td>
<td>0.034</td>
<td>100</td>
<td>14.08</td>
<td>85.92</td>
</tr>
<tr>
<td>Citrate</td>
<td>0.032</td>
<td>100</td>
<td>5.63</td>
<td>94.37</td>
</tr>
<tr>
<td>Sucrose</td>
<td>0.030</td>
<td>100</td>
<td>10.79</td>
<td>89.21</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.034</td>
<td>100</td>
<td>7.41</td>
<td>92.57</td>
</tr>
<tr>
<td>Succinate</td>
<td>0.050</td>
<td>100</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Molasses</td>
<td>0.18</td>
<td>100</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

* All substrates except molasses were at 0.5% concentration. Molasses concentration was 0.3%.

b Specific growth rates at the exponential phase of growth were calculated from plots of \( \lambda_{growth} \) versus time for the respective cultures.

c Specific transformation rates were calculated from the amount of TNT transformed and the time required for the transformation.
Table 3 Pure Cultures Isolated from the Soil Consortium

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Organisms</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Pseudomonas acidovorans</em></td>
</tr>
<tr>
<td>2</td>
<td><em>Pseudomonas fluorescens</em></td>
</tr>
<tr>
<td>3</td>
<td><em>Pseudomonas mendocina</em></td>
</tr>
<tr>
<td>4</td>
<td><em>Pseudomonas aeruginosa</em></td>
</tr>
</tbody>
</table>

Table 4 Mass Balance for TNT Metabolism by *Pseudomonas* spp

<table>
<thead>
<tr>
<th>14C TNT recovered</th>
<th>% TNT</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO₂</td>
<td>1</td>
</tr>
<tr>
<td>Biomass</td>
<td>8</td>
</tr>
<tr>
<td>Metabolites</td>
<td>87</td>
</tr>
<tr>
<td>% TNT remaining</td>
<td>4</td>
</tr>
<tr>
<td>Growth Conditions</td>
<td>Metabolites Produced</td>
</tr>
<tr>
<td>----------------------------------------------------------------------------------</td>
<td>----------------------------------------------------------</td>
</tr>
<tr>
<td>Pyruvate as electron donor, Sulfate as electron acceptor, ammonium as nitrogen source in the presence of TNT</td>
<td>2-Amino-4,6-dinitrotoluene, 4-Amino-2,6-dinitrotoluene, 2,4-Diamino-6-nitrotoluene</td>
</tr>
<tr>
<td>Pyruvate as electron donor, TNT as electron acceptor and ammonium as nitrogen source</td>
<td>2,4-Diamino-6-nitrotoluene</td>
</tr>
<tr>
<td>TNT as sole source of carbon and energy, sulfate as electron acceptor, ammonium as nitrogen source</td>
<td>4-Amino-2,6-dinitrotoluene, 2,4-Diamino-6-nitrotoluene</td>
</tr>
<tr>
<td>Pyruvate as electron donor, Sulfate as electron acceptor, TNT as nitrogen source</td>
<td>2-Amino-4,6-dinitrotoluene, 4-Amino-2,6-dinitrotoluene, 2,4-Diamino-6-nitrotoluene, Toluene</td>
</tr>
</tbody>
</table>
Figure 2 Growth of different Isolates
Figure 3 Metabolism of TNT by Different Isolates
Figure 4 Concentrations of TNT and its Metabolites in the Reactor
2,3-Butane diol (28%)  
TCA (23%)  
CO₂ (28%)  

2-Amino-4,6-Dinitrotoluene (1%)  
4-Amino-2,6-Dinitrotoluene (1%)  
2,4-Diamino-6-Nitrotoluene (3%)  
Unknown (8%)  
Soil Fraction (8%)  

Figure 5 Distribution of Radiolabel after 30 days (Average Recovery 90-95%)