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Anaerobic transformation of 2,4,6-TNT by bovine ruminal microbes

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Abstract

Degradation of TNT by bovine rumen fluid, a novel source of anaerobic microbes, was investigated. Whole rumen fluid contents were spiked with TNT and incubated for a 24 h time period. Supernatant samples taken at 0, 1, 2, 4, and 24 h were analyzed by reverse-phase HPLC with diode array detection. Within 1 h, TNT was not detectable and reduction products of TNT including 2-hydroxyl-amino-4,6-dinitrotoluene, 4-hydroxylamino-2,6-dinitrotoluene, and 4-amino-2,6-dinitrotoluene were present with smaller amounts of diamino-nitrotoluenes. Within 2 h, only the diamino and dihydroxyamino-nitrotoluene products remained. After 4 h, 2,4-diamino-6-nitrotoluene and 2,4-dihydroxyamino-6-nitrotoluene were the only known molecular species left. At 24 h known UV absorbing metabolites were no longer detected, suggesting further transformation such as complete reduction to triaminotoluene or destruction of the aromatic ring of TNT may have occurred. TNT was not transformed at 24 h in autoclaved and buffered controls. This study presents the first direct evidence of biodegradation of TNT by ruminal microbes.
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Keywords: TNT; Bioremediation; Biodegradation; Biotransformation; Ruminal microbes

Much work has been done to find cost effective ways of remediating the highly toxic and persistent explosive compound trinitrotoluene (TNT) from contaminated soils and groundwater [1–3]. Over 700,000 cubic yards of soil and 10 billion gallons of ground water require treatment at tremendous costs to the Department of Defense. TNT is the primary contaminant at these sites, along with dinitrotoluene (DNT) and other nitro substituted explosives (i.e., RDX and HMX). The ex situ methods of incineration and composting have been widely used in the United States for soil remediation, while contaminated groundwater has been treated primarily by pumping and carbon sorption [1]. The relatively high cost of ex situ remediation techniques has led to the search for in situ methods of degrading or transforming TNT. The development of successful in situ TNT bioremediation schemes could provide less disruptive and more cost effective techniques of solving the problems of TNT contamination in soil and groundwater.

Efforts have focused on developing methods to treat TNT biologically. A variety of ecosystems have been examined for microbes able to transform TNT as previously reported [1–3]. The highly electrophilic nature of TNT lends itself to transformations by reductive pathways. The mechanism of reduction of the nitro group to the amine does not appear to be widely used by aerobic bacteria for productive metabolism. Biotransformation of TNT by microbes occurs most commonly under anaerobic conditions through the sequential reduction of the nitro groups to amine groups to form aminodinitrotoluenes, diaminonitrotoluenes, triaminotoluene, and azoxy dimers along with other unknown products. The extent of TNT metabolism appears to be dependent on the type of culture preparation (cell extract, resting cells, and growing culture), the bacterial strain, and the environmental conditions (carbon dioxide, hydrogen).

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Degradation of TNT has been investigated in several anaerobic systems including activated sludges, soil slurries, sulfate reducing species, methanogens, denitrifying bacteria, and clostridia pure cultures [4–11].

Ruminant animals possess a highly reductive anaerobic environment in their rumen [12,13]. Anaerobic microbes in the rumen metabolize complex plant material to make fatty acids and amino acids for utilization by the host. The hypothesis is anaerobic microbes in the rumen are able to reduce synthetic complex molecules to benign non-toxic moieties. Examples of xenobiotic transformations by rumen microbes have been reported for toxicants in forages such as pyrrolizine alkaloids, fumonisin, and alflatoxin [14–17]. The plant toxicant nitropropionic acid was shown to undergo reduction to the corresponding amine [18]. This study looked at the ability of bovine ruminal microbes to degrade TNT under anaerobic conditions.

Materials and methods

Microbial sources and collection. Rumen contents from fistulated cows were collected approximately 3 h after feeding into 1 L plastic jars with no remaining air space. The jars were sealed and transported to the laboratory within 30 min. All procedures were performed using strict anaerobic techniques. Thirty grams of rumen contents was strained through cheesecloth and blended with 70 ml of modified McDougall's buffer, which is similar to the bicarbonate buffer from the salivary glands of the ruminant. Details of buffer preparation and the handling of rumen contents have been previously described [19]. In summary, a modified McDougall's buffer was prepared anaerobically and equilibrated with oxygen-free N_2:CO_2 (70:30) prior to use.

Chemicals and materials. 2,4,6-Trinitrotoluene (TNT), 4-amino-2,6-dinitrotoluene (4-A-2,6-DNT), and 2-amino-4,6-dinitrotoluene (2-A-4,6-DNT) were purchased from Chem Service (West Chester, PA). 2,4-Diamino-6-nitrotoluene (2,4-DA-6-NT) and 2,6-diamino-4-nitrotoluene (2,6-DA-4-NT) were purchased from AccuStandard (New Haven, CT). Solvents were HPLC grade and were purchased from Fisher Scientific (Tustin, CA). Reagents were of analytical grade and were purchased from Sigma–Aldrich (St. Louis, MO). A Millipore (Bedford, MA) reverse osmosis water purification system was used to generate Milli-Q (resistance >18 μmohls) quality water for all aqueous solutions.

Culture conditions and sampling protocols. Transformation experiments were conducted in 10 ml serum vials containing 8 ml of the rumen fluid and McDougall's buffer mixture under anaerobic conditions. TNT dissolved in methanol (10 mg/ml) was added to a final concentration of 100 mg/L. The vials were purged with N_2/CO_2 prior to being sealed with black rubber serum stoppers and aluminum crimp closures. The vials were incubated at 38 °C, protected from light while agitating at 200 rpm on a Model G24 Environmental Incubator Shaker (New Brunswick Scientific). Samples were withdrawn from duplicate vials at the appropriate time intervals and analyzed immediately for TNT and metabolic products. Controls were prepared with: (a) autoclaved rumen fluid/McDougall's buffer mixture and TNT; (b) McDougall's buffer mixture and TNT; and (c) rumen fluid.

Analytical methods. HPLC analyses were carried out by a modification of the method described by Khan et al. Separations were performed using a guard column hand packed with Pellicular C8 material and a Nova-Pak C8 analytical column (150 mm × 3.9 mm id, 4 μm particle size, Waters, Milford, MA). The column was eluted under isocratic conditions with water and 2-propanol (82:18) at a flow rate of 1 ml/min with a total run time of 22 min.

The HPLC system consisted of a solvent delivery system (Perkin–Elmer Series 200 Pump) equipped with a sample injector (Perkin–Elmer ISS 200 autosampler) and photodiode array detector monitoring at 230 nm and scanning between 200 and 360 nm (Perkin–Elmer 235C). Detector output was captured via an analog to digital converter (PE Nelson 600 Series LINK interface) connected to a computer equipped with Turbochrom Workstation and TurboScan 200 software.

Metabolite identification was based on comparison of retention times and UV/Vis spectra with standards (Fig. 1) and data for the hydroxylaminodinitrotoluenes collected in the laboratories at Rice University. Typical detection limit was 0.2 ppm per component.

Samples from reaction mixtures were centrifuged for 5 min at 16,000*g* and 0.100 ml of the supernatant was immediately injected onto the HPLC system. Samples were analyzed immediately following collection to minimize abiotic degradation of intermediates. A total of six sets of experiments were run with two serum bottles inoculated in each experiment. Duplicate sets of samples were also collected for autoclaved controls, buffer controls, and blank rumen fluid controls. Statistical analyses were mean, standard deviation, and coefficient of variation.

Results

Incubations of TNT with ruminal microbes were monitored by reverse-phase HPLC with photodiode array detection. Figs. 2 and 3 show representative time course analyses of the TNT metabolism with bovine rumen microbes and the autoclaved control, respectively. Comparison of TNT at 0 h and after 24 h incubation in live ruminal microbial cultures shows the complete disappearance of the TNT whereas the autoclaved control showed little change of TNT concentration after 24 h.

A typical separation of TNT and standards of aminodinitrotoluenes and diaminonitrotoluenes is shown in Fig. 1. The identity of the hydroxylamine derivatives was confirmed with identical analytical procedures in the Laboratory at Rice University, as these standards were not available at Oregon State University. The identity of peak E has been tentatively assigned as 2,6-DA-4-NT based on identical retention time with an authentic standard in the chromatographic system. However, the UV spectra of the peak E component in some analyses differ slightly from the standard. This may be due to lack of resolution of components near the void volume of the chromatographic analysis, suggesting additional analytical techniques such as MS and NMR may be prudent for unequivocal identification.

Fig. 4 illustrates the time course appearance and disappearance of the TNT degradation products summarized from replicate incubations. After 1 h incubation, the main molecular compound was 4-hydroxylamino-2,6-dinitrotoluene (4-HA-2,6-DNT) with slightly lower amounts of 2-hydroxylamino-4,6-dinitrotoluene (2-HA-4,6-DNT) and 4-amino-2,6-dinitrotoluene (4-A-2,6-DNT). After 2 h incubation, these aforementioned compounds were gone and the predominant molecules

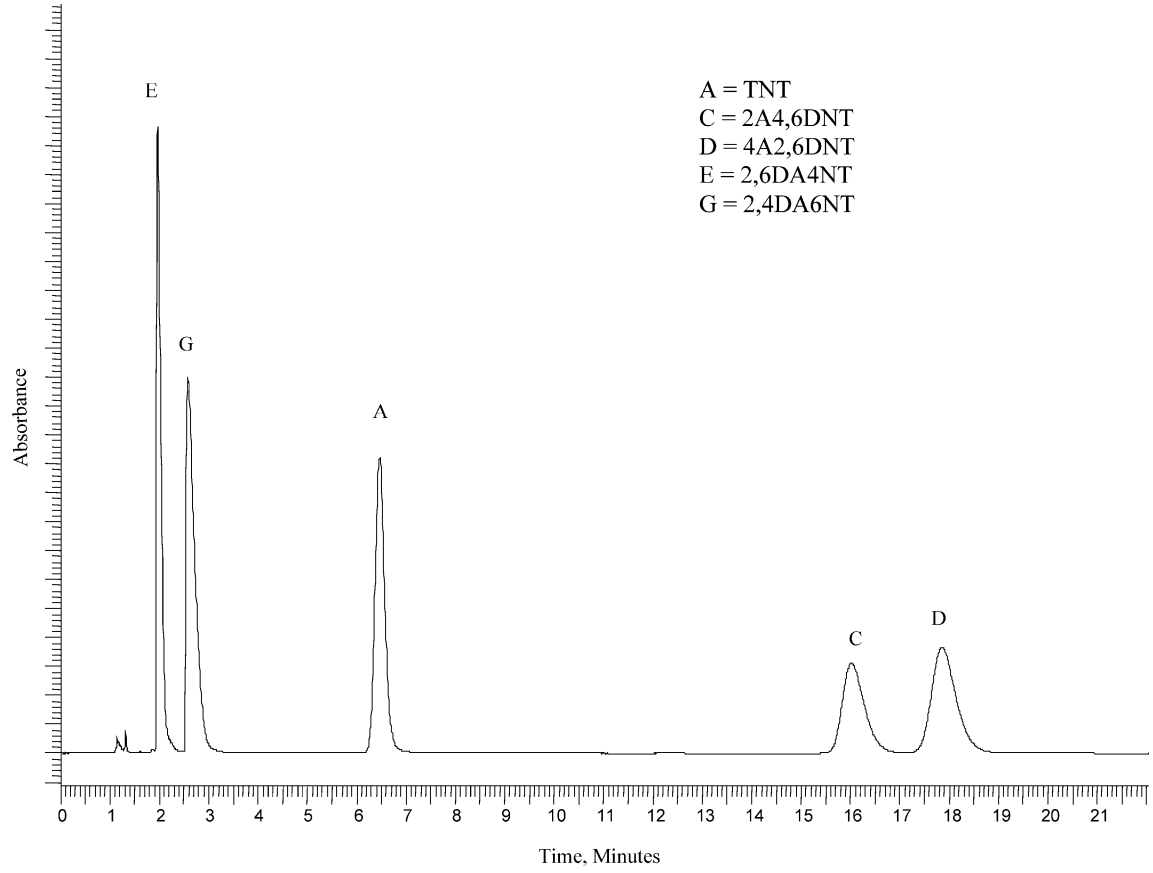

Fig. 1. HPLC chromatogram of TNT and selected metabolite standards. A, 2,4,6-trinitrotoluene (TNT); C, 2-amino-4,6-dinitrotoluene (2-A-4,6-DNT); D, 4-amino-2,6-dinitrotoluene (4-A-2,6-DNT); E, 2,6-diamino-4-nitrotoluene (2,6-DA-4-NT); and G, 2,4-diamino-6-nitrotoluene (2,6-DA-4-NT).

were 2,4-diamino-6-nitrotoluene (2,4-DA-6-NT) and 2,4-dihydroxyamino-6-nitrotoluene (2,4-DHA-6-NT). By 4 h, the 2,4-DA-6-NT and 2,4-DHA-6-NT were the only degradation products found. At 24 h, all the known TNT biodegradation products were no longer detectable.

Discussion

Ruminants possess a complex stomach system comprised of four compartments, the first and the largest of which is the rumen. Continuous anaerobic fermentation takes place in the rumen before digestion in other parts of the stomach and the intestines. This fermentation is accomplished by communities of symbiotic microorganisms: protozoa, bacteria, and fungi. Once established the rumen microbial community is relatively stable and will only change due to changes in the nutrients available

The rumen is a highly reductive environment with little available oxygen present [20]. Typically, the oxidation–reduction potential (E_h) of the soil environment under anaerobic conditions is between −200 and −300 mV [21] whereas the redox potential of the rumen environment ranges between −250 and −450 mV [12,22]. The redox potential decreases as oxygen is depleted from the environment. Anaerobic conditions for sulfate reduction occur at a range of −100 to −350 mV and methanogenesis at −200 to −350 mV [23]. Although researchers have predominantly utilized anaerobic microbes for remediation of TNT isolated from soil, compost or waste facility sludge, an advantage of searching the rumen for novel anaerobic microbes capable of degrading such electrophilic compounds such as TNT is its highly reductive environment.

In view of the considerable body of knowledge about reduction of nitroaromatic compounds by anaerobic microbes [24], this study finds that rumen microorganisms under anaerobic conditions readily transform the nitro groups of TNT to the corresponding amine group which is not surprising. However, the rate of disappearance of TNT by rumen microorganisms within 1 h was considerably faster than the 15 h reported for the disappearance of TNT using microorganisms in a sludge supplemented with molasses as a carbon source under anaerobic conditions [25]. In addition, these experiments indicate the disappearance of all monoamino- and

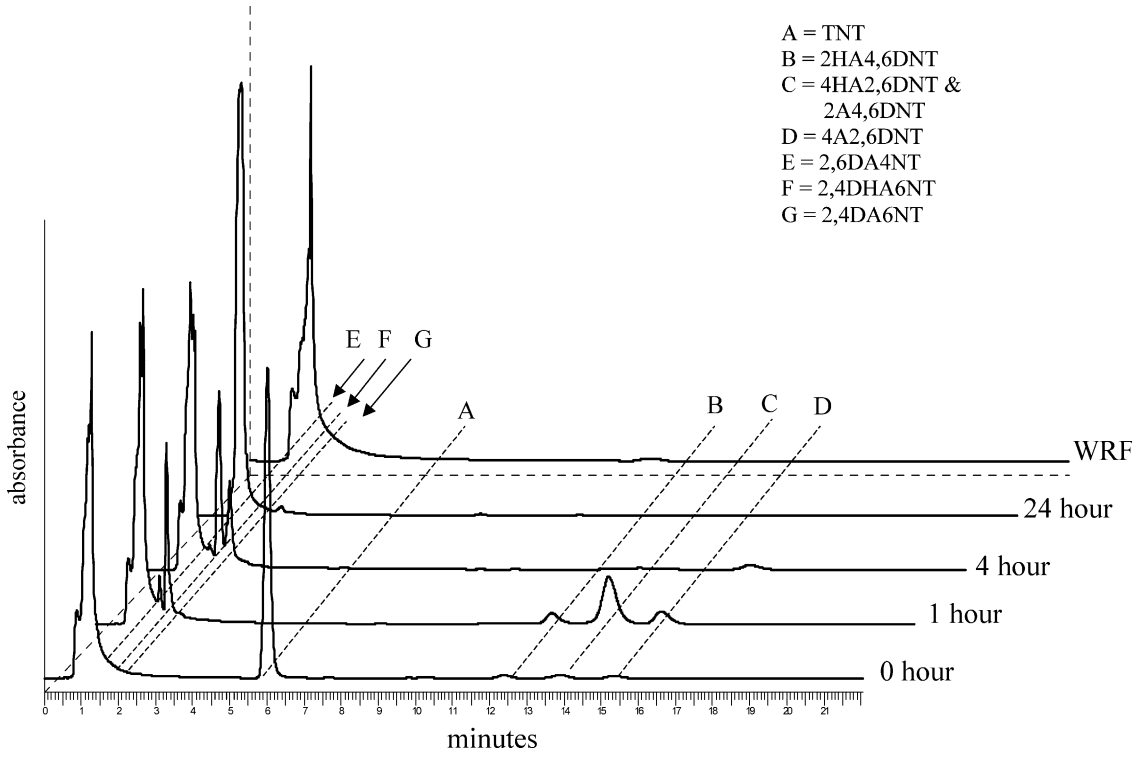

Fig. 2. Time course HPLC chromatograms of bovine rumen fluid blended with McDougall's buffer (30:70) spiked with trinitrotoluene (TNT) analyzed 0, 1, 4, and 24 h after mixing. A, 2,4,6-trinitrotoluene (TNT); B, 2-hydroxylamino-4,6-dinitrotoluene (2-HA-4,6-DNT); C, 4-hydroxylamino-2,6-dinitrotoluene (4-HA-2,6-DNT); and 2-amino-4,6-dinitrotoluene (2-A-4,6-DNT); D, 4-amino-2,6-dinitrotoluene (4-A-2,6-DNT); E, 2,6-diamino-4-nitrotoluene (2,6-DA-4-NT); F, 2,4-dihydroxyamino-6-nitrotoluene (2,4-DHA-6-NT); and G, 2,4-diamino-6-nitrotoluene (2,4-DA-6-NT). WRF is whole rumen fluid used as a negative control.

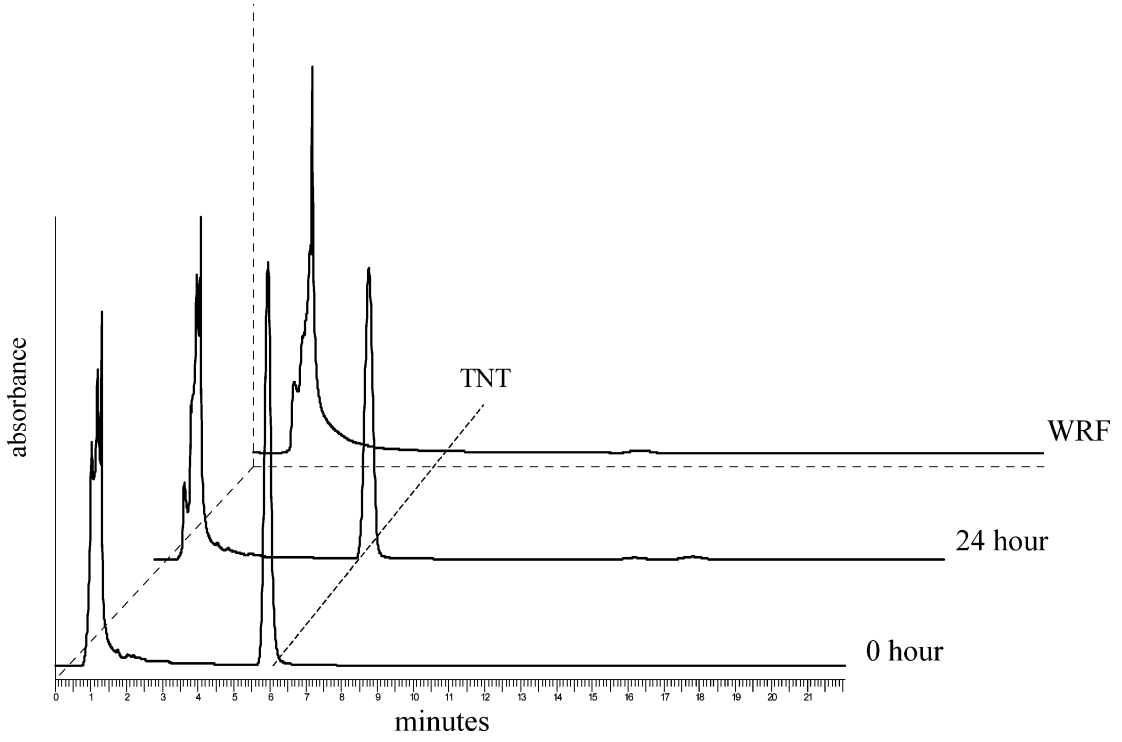

Fig. 3. HPLC chromatograms of bovine rumen fluid blended with McDougall's buffer (30:70) autoclaved prior to addition of trinitrotoluene (TNT) analyzed 0 and 24 h after mixing. WRF is whole rumen fluid used as a negative control.

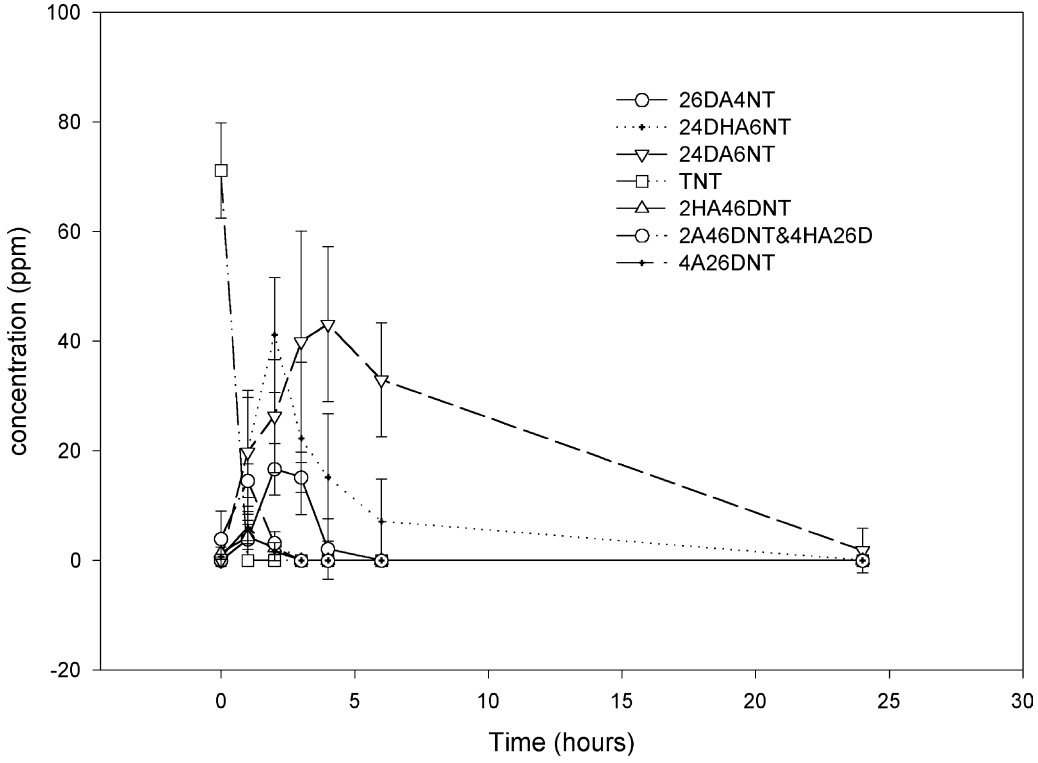

Fig. 4. Time course of TNT metabolism in bovine rumen fluid culture under anaerobic conditions. The data are means of measurements from at least six of incubations: 2,6-diamino-4-nitrotoluene (2,6-DA-4-NT); 2,4-dihydroxyamino-6-nitrotoluene (2,4-DHA-6-NT); 2,4-diamino-6-nitrotoluene (2,4-DA-6-NT); 2,4,6-trinitrotoluene (TNT); 4-hydroxyamino-2,6-dinitrotoluene; 2-hydroxylamino-4,6-dinitrotoluene (2-HA-4,6-DNT); 2-amino-4,6-dinitrotoluene (2-A-4,6-DNT) and (4-HA-2,6-D); and 4-amino-2,6-dinitrotoluene (4-A-2,6-DNT). The controls without rumen fluid and with autoclaved rumen fluid showed no TNT transformation products.

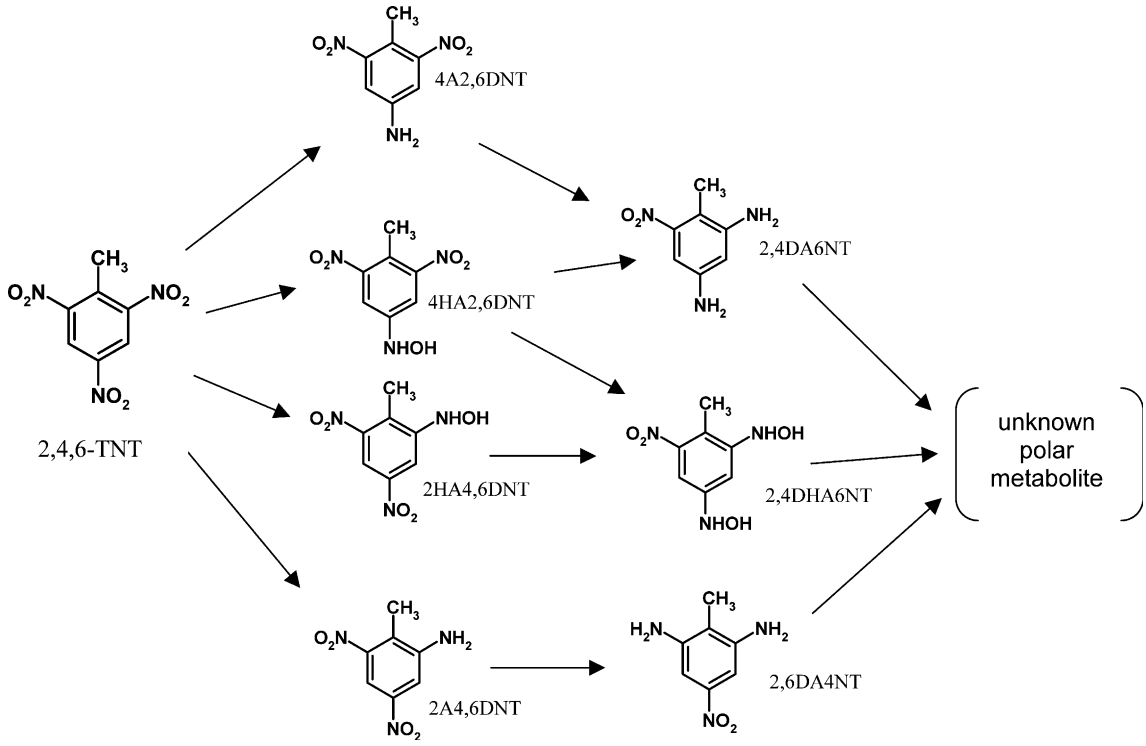

Fig. 5. Proposed transformation pathway of 2,4,6-trinitrotoluene by rumen microbes under anaerobic conditions.

diamino-substituted toluenes formed due to reduction of the nitro groups. A proposed TNT transformation pathway is shown in Fig. 5. Furthermore, ruminants have anaerobic microbes that assimilate nitrogen and amines into amino acids to be utilized by the microbial biomass [18,26–28].

The proposed pathway indicates formation of polar products as indicated from a non-retained peak in the reverse-phase HPLC separation (Fig. 2). Although the identity of the unknown polar component has not been established, the possibility of complete reduction of all TNT nitro groups to triaminotoluene and/or subsequent ring cleavage is consistent with experimental observations. The reduction and mineralization of aromatic compounds by microorganisms under anaerobic conditions have been described [12,25,29,30]. Several possible degradation pathways have been reported including reductive removal of the nitro group from the aromatic ring or removal by means of substitution reactions [31]. In this study, a similar pathway of TNT degradation was used as published by Hughes using *Clostridium* sp. [8]. TNT metabolism by ruminant microbes may result in products further transformed than those previously reported [32].

Plant toxicants such as pyrrolizidine alkaloids are nitrogenous ring compounds and are metabolized by ruminal microbes which renders these molecules nontoxic to the ruminant [14,19]. In a similar fashion, TNT biodegradation in vivo may also be degraded to a nontoxic moiety and, as a consequence, no aromatic molecule is absorbed systemically. With ruminal transport time of 16–20 h, the rapid degradation of TNT by ruminal microbes in vivo would protect the ruminant.

In summary, this study used a unique set of microbes from ruminants and found a TNT degradation pathway similar to previously reported studies [5,33,34]. However, the microbes from the rumen under anaerobic conditions appear to rapidly reduce TNT to triaminotoluene (TAT) or other ring fission products. Other studies utilizing radiolabeled TNT and supporting spectroscopic data from NMR and MS may be necessary to unequivocally identify these polar transformation products.

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