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Title: **Bioremediation of High Explosives**

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BIOREMEDIATION OF HIGH EXPLOSIVES

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Manufacture and use of high explosives has resulted in contamination of ground water and soils throughout the world. The use of biological methods for remediation of high explosives contamination has received considerable attention in recent years.

Biodegradation is most easily studied using organisms in liquid cultures. However, explosives are not very water soluble. TNT, RDX and HMX saturate 20°C water at 130, 50 and 10 mg/L respectively. Thus, the amount of explosive that can be degraded in liquid culture is quite small. However, these experiments are useful for gathering basic information about the biochemical pathways of biodegradation, identifying appropriate organisms and obtaining rates of degradation. Our laboratory has investigated all three major areas of explosives bioremediation: explosives in solution, explosives in soil, and the disposal of bulk explosives from demilitarization operations. We investigated the three explosives most commonly used in modern high explosive formulations: 2,4,6-trinitrotoluene (TNT), hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX).

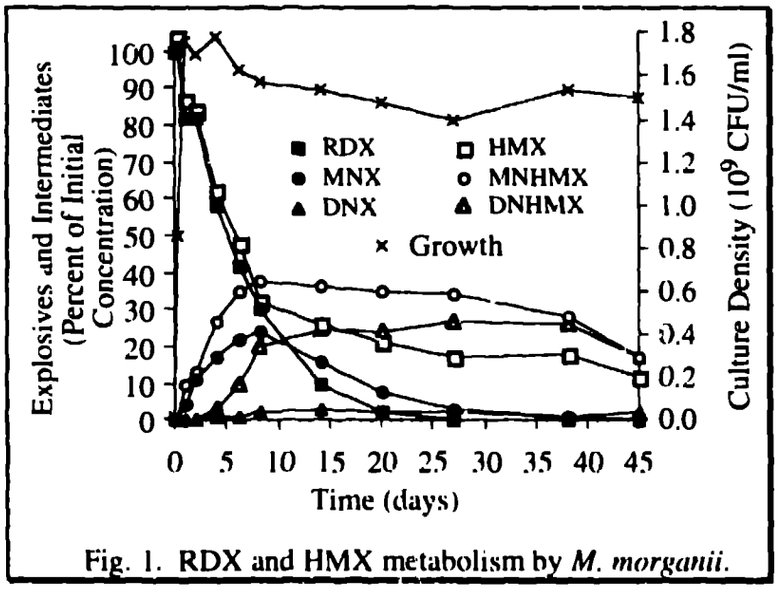

Fig. 1. RDX and HMX metabolism by *M. morganii*.

The common chemical feature of all these explosives is the nitro group. Thus, most organisms which degrade explosives start by biochemically altering, or removing the nitro substituents. The most common biotransformation of explosives is reduction of the nitro groups. This is a step wise process of reduction, i.e.: $R\text{-}NO_2$ -> R-NO -> R-NHOH -> $R\text{-}NH_2$. Further degradation then depends on the rest of the explosives' chemical structure. The ability to reduce nitro groups on organic compounds is quite broadly distributed among microorganisms; examples include *Pseudomonas*, *Enterobacter*, *Streptomyces*, and *Phanerochaete* (2, 4, 6, 12). Therefore, any bioremediation of explosives which might occur with a natural mixture of microorganisms will include some nitro group reduction. Consequently, our laboratory has specifically investigated the nitroreduction process, how it affects the efficiency of explosives bioremediation and possible organisms which might be employed to deal with intermediates of nitroreduction.

RDX and HMX in Solution

Previous reports indicated that the nitramine explosives RDX and HMX were readily transformed by mixtures of bacteria under anaerobic conditions (9). Using explosives-contaminated soil as an inoculum, we isolated three enteric bacterial strains (*Citrobacter freundii*, *Providencia rettgeri* and *Morganella morganii*) capable of transforming both nitramine explosives (8). *M. morganii* was the only strain capable of transforming a mixture of HMX and RDX (Fig. 1). In all cases, the explosives were only transformed after culture aeration was halted. Between 5% and 10% of the radiolabel from [^{14}C]-RDX was captured as CO_2 after 45 days of incubation (8). The major transformation products observed were identical to those reported in mixed culture experiments (9). They included the mono-, di-, and trinitroso-RDX derivatives (MNX, DNX and TNX) and the mono- and dinitroso-HMX

derivatives (MNHMX and DNHMX).

The 'classical' nitroreductase of enteric bacteria is well characterized for reducing nitro groups on nitroaromatic compounds. Because the nitramine reducing bacteria isolated were all enterics it was hypothesized that the 'classical' nitroreductase may also be responsible for nitramine reduction. All three bacterial strains mentioned above were also able to reduce the nitro groups on TNT. In fact, a single mutation in *M. morganii* resulted in the loss of both RDX and TNT nitroreductase activities (Table 1). We also used an *Enterobacter cloacae* nitroreductase gene cloned into the *Escherichia coli* strain TB1 (Bryant et al 1991) to show that another 'classical' enteric nitroreductase was able to reduce both substrates (Table 1).

Table 1. Nitroreductase activity with TNT and RDX in different bacterial strains.

Bacterial Strain	RDX*	TNT†
M. morganii strain B2 (wild type)	0.010	37.5
M. morganii strain B2M10 (nitroreductase mutant)	< 0.0001	< 0.1
E. coli strain TB1 (nitroreductase not induced)	0.006	182
E. coli strain TB1 (nitroreductase induced)	0.030	1301

* Rate of RDX disappearance in culture during the first 4 days of incubation. Units are nmol·min^{-1}·(mg protein)$^{-1}$.

† Rate of TNT nitroreduction as measured by NADPH oxidation in cell free extract. Units are nmol·min^{-1}·(mg protein)$^{-1}$.

Bioremediation of TNT and RDX mixtures may be complicated because the same enzyme reduces nitro groups on both explosives. For example, conditions which favor biodegradation of RDX will also favor nitroreduction of TNT. While nitroreduction of RDX appears to lead to complete biodegradation the same may not be true for TNT.

TNT in Solution

Most microbial transformations of TNT involve the reduction of the nitro groups to produce 2-amino-4,6-dinitrotoluene (2ADNT), 4-amino-2,6-dinitrotoluene (4ADNT), diaminonitrotoluene isomers (DANTs) and triaminotoluene (TAT). Polymeric azoxy compounds, tetranitroazoxytoluene isomers (TNATs), can also be formed by the abiotic condensation of partially reduced nitro groups on two TNT molecules. Therefore, attempts to degrade TNT using natural heterogeneous cultures often results in the accumulation of these aminonitrotoluenes (5, 7, 10). These compounds are not readily biodegradable and few studies have focused on their metabolism.

Mycobacterium vaccae strain JOB-5 was investigated for metabolism of TNT (13). High concentrations of cells and the presence of propane as a co-metabolite were necessary to obtain complete transformation of TNT (Fig. 2). Two novel intermediates of TNT metabolism were isolated from cultures of *M. vaccae* growing with propane as energy source: 4-amino-2,6-dinitrobenzoic acid and 2,4-diamino-6-nitrobenzyl-methylether. These data imply that *M. vaccae* oxidized the methyl group of TNT whether or not the nitro groups were reduced to amines. In addition, *M. vaccae* incorporated TNT carbons into its lipids. Fractionation of phospholipids from cells incubated with [^{14}C]-TNT showed co-migration of radiolabel with several phospholipids (13). These data indicate that complete mineralization is not necessary to accomplish complete remediation of TNT contaminated solutions.

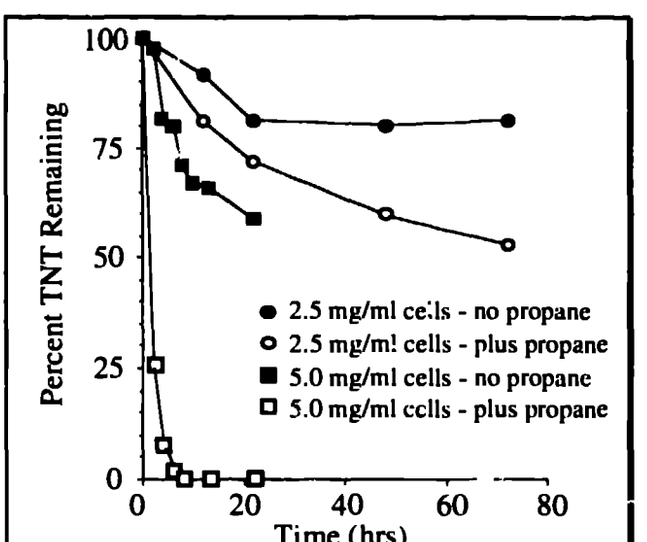

Fig. 2. Effect of cell density and propane on TNT metabolism by *M. vaccae*.

Pseudomonas aeruginosa strain MA01 was also investigated for metabolism of TNT (1). *P. aeruginosa* cultures grown aerobically with TNT and succinate as co-metabolites did not produce large amounts of the usual TNT reduction products (Fig. 3). *P. aeruginosa* was also able to degrade purified aminodinitrotoluene isomers (ADNTs) in aerobic conditions. Anaerobically grown cultures metabolizing TNT produced large amounts of the ADNTs and did not further metabolize them. These data imply that *P. aeruginosa* used O_2 to oxidize the aminodinitrotoluene products of TNT

nitroreduction. Products of ADNT oxidation by *P.. aeruginosa* are currently being identified.

These two soil bacteria appeared capable of oxidatively metabolizing the TNT nitroreduction products which inevitably accumulate during mixed culture growth in the presence of TNT. This suggests that a regimen of alternating aerobic and anaerobic growth conditions may be successful for degrading mixtures of RDX and TNT, if bacteria similar to those described above are present in the mixed culture.

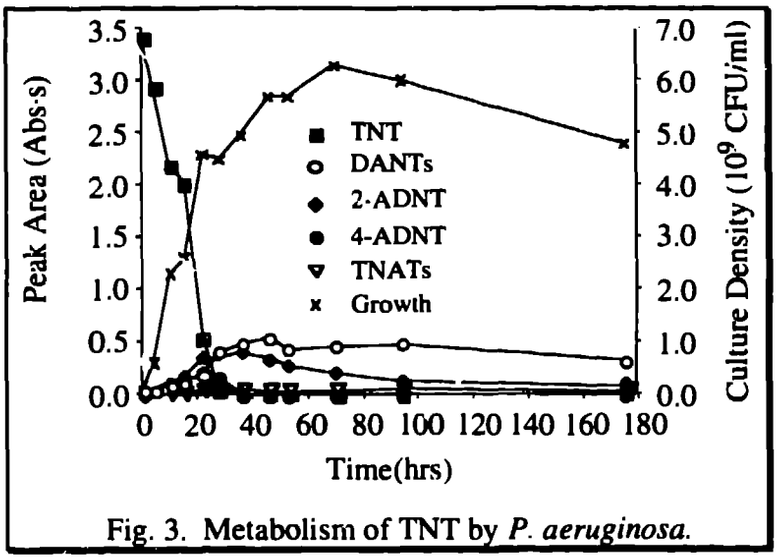

Fig. 3. Metabolism of TNT by *P. aeruginosa.*

RDX in Soils

TNT and RDX are nearly insoluble in water. However, many soils are contaminated with more than 1% (w/w) of these explosives. We used a soil containing 1.6% RDX to test a soil slurry bioremediation process in parallel microcosms (11). Each microcosm consisted of 0.4 g soil plus 3.2 ml of a nutrient solution in a 15 ml serum vial. These vials were stoppered and each head space flushed with N_2. All of the soil slurry microcosms were rotated (20 rpm) at 20°C. We tested two sources of nutrients: corn steep and nutrient broth (Difco). Two microcosm cohorts (300 vials per cohort) had only nutrient added. Another cohort had nutrient broth and 0.1% horse manure added. A final cohort had nutrient broth and 10^9 cells/ml of *M. morganii* added. At each experimental time point three microcosms were sacrificed from each of the four conditions to determine soluble and soil-bound explosive concentrations (11).

All four conditions resulted in about the same rate of RDX removal from soil. Fig. 4 is an example from the nutrient broth and horse manure cohort of microcosms. Although some MNX and DNX accumulated over time, the most abundant intermediate of RDX degradation was TNX. Under all conditions, The maximum concentration of TNX was about half of the total RDX available. TNX took much longer to disappear than RDX. In fact, the viable cell count began to fall as TNX concentrations increased (Fig. 4). After 70 days, the remaining microcosms were fed additional nutrients to increase the viable cell count. Removal of TNX from the soil slurry took over 160 days. However, the rate of RDX transformation in the soil slurries (about 0.22 mg RDX/day/10^9 cells) was over ten times faster than for a single strain (about 0.017 mg RDX/day/10^9 cells).

This study showed that biotransformation of insoluble compounds in soils can produce large amounts of soluble and still toxic chemicals which can leach into the surounding environment. Therefore, contained soil slurries may be the best way to use bioremediation with soils that are highly contaminated with explosives. It also was apparent that large amounts of nutrients may be necessary for bioremediation of heavily contaminated soils.

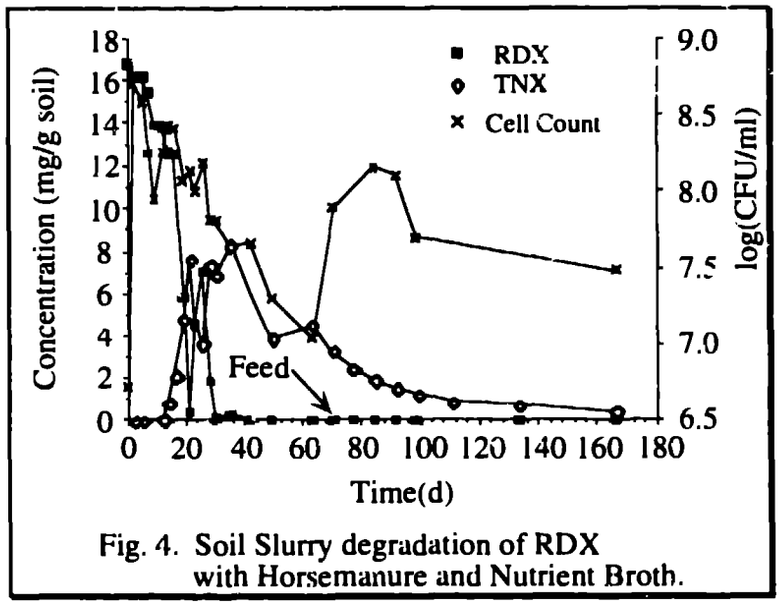

Fig. 4. Soil Slurry degradation of RDX with Horsemanure and Nutrient Broth.

Bulk Explosives

The EPA recently outlawed open air burning of explosives which was the primary method of disposal for bulk explosives. Howeve:, there are tons of bulk explosives currently being demilitarized that require safe disposal. In collaboration with others at Los Alamos National Laboratory (3), a

• combination of chemical and biological methods for disposal of bulk explosive formulations was investigated. For example, the explosive formulation PBX9404 was hydrolyzed in boiling base, resulting in a solution of nitrite and formate (3). A biological reactor was then employed to remove the toxic levels of nitrite via denitrification and incorporate formate and additional carbon into biomass (Fig. 5).

Several pure cultures and several mixed cultures were investigated for their ability to grow and denitrify PBX9404-hydrolysate. The high concentration of both nitrite and sodium ions in the hydrolysate make an inhospitable environment for microbial growth. Consequently, some dilution of the original hydrolysate solution was necessary to promote growth. Of the pure cultures tested (*Thiosphaera pantotropha* , *Bacillus halodenitrificans* , *Nitrobacter agilis* , *Pseudomonas nautica* , and *Paracoccus halodenitrificans*) none were able to both grow and denitrify solutions of even one tenth strength hydrolysate. Only the mixed cultures met with success (Table 2). These findings indicate that the combination of chemical and biological methods we described may be a safe and cost effective method for disposing of large amounts of bulk explosives.

Table 2. Cultures which completely denitrified the PBX9404-hydrolysate.

Culture Source	Growth*	Denitrify†
Waste Treatment Plant I	1:1	3
Waste Treatment Plant II	1:3	5
DeNitro® Bacterial Mix	1:10	13

* Lowest dilution of hydrolysate at which cultures grew.

† Time in days required to remove all detectable nitrite from the culture medium at the stated dilution.

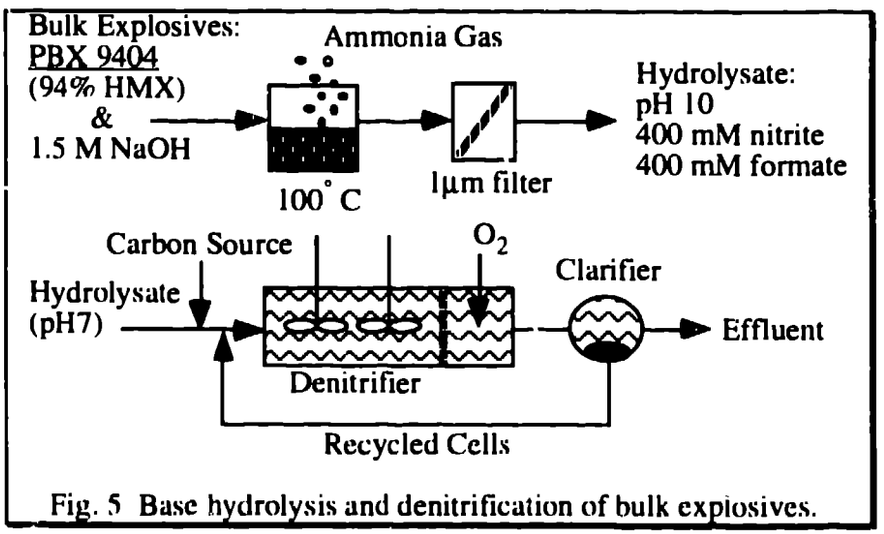

Fig. 5 Base hydrolysis and denitrification of bulk explosives.

Conclusions

Anaerobic conditions and plenty of nutrients will generally result in RDX degradation through nitro group reduction. On the other hand nitro group reduction does not necessarily result in complete degradation of TNT. However, TNT and some TNT nitroreduction products can be oxidized by some aerobic bacteria. Given the correct mix of aerobic and anaerobic conditions it may be possible to fully degrade mixtures of RDX and TNT with native bacteria in soils contaminated with these explosives. In soil slurries, bacterial mixtures appear to degrade RDX at rates faster than the single strains we isolated. In addition, no single strain we tested could denitrify the PBX9404 hydrolysate while a mixed bacterial culture succeeded. Perhaps a diverse microbial population is important for obtaining rapid and efficient biodegradation of nitro compounds, whether it is done *in situ* or in more controlled conditions.

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