APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 1992, p. 3199–3202
0099-2240/92/093199-04$02.00/0

Vol. 58, No. 9

Influence of 2,4,6-Trinitrotoluene (TNT) Concentration on the Degradation of TNT in Explosive-Contaminated Soils by the White Rot Fungus *Phanerochaete chrysosporium*†

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The ability of *Phanerochaete chrysosporium* to bioremediate TNT (2,4,6-trinitrotoluene) in a soil containing 12,000 ppm of TNT and the explosives RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine; 3,000 ppm) and HMX (octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine; 300 ppm) was investigated. The fungus did not grow in malt extract broth containing more than 0.02% (wt/vol; 24 ppm of TNT) soil. Pure TNT or explosives extracted from the soil were degraded by *P. chrysosporium* spore-inoculated cultures at TNT concentrations of up to 20 ppm. Mycelium-inoculated cultures degraded 100 ppm of TNT, but further growth was inhibited above 20 ppm. In malt extract broth, spore-inoculated cultures mineralized 10% of added [^{14}C]TNT (5 ppm) in 27 days at 37°C. No mineralization occurred during [^{14}C]TNT biotransformation by mycelium-inoculated cultures, although the TNT was transformed.

At many military sites, TNT (2,4,6-trinitrotoluene) wastes from munitions production and management have been found to contaminate water, soil, and sediments. TNT has been shown to cause liver damage and anemia in humans (7). It is harmful to rats, mice, fish, and marine life (8, 10). TNT also inhibits the growth of many fungi, yeasts, actinomycetes, and gram-positive bacteria (5). TNT has been shown to be a mutagen by the Ames test (11). Currently, alternative methods for remediation of TNT-contaminated soils are being studied.

Phanerochaete chrysosporium, a well-known lignin-degrading fungus, has been the organism of choice for many investigators studying the remediation of hazardous wastes. *P. chrysosporium* possesses nonspecific extracellular peroxidases that enable it to degrade lignin. These nonspecific enzymes also enable the organism to degrade other recalcitrant compounds (1).

TNT-contaminated soils may be amenable to cleanup through bioremediation. Sublette et al. (9) have proposed a system for cleanup of TNT-contaminated water that uses *P. chrysosporium* immobilized on rotating disks, while Fernando et al. (3) have suggested a similar process that uses *P. chrysosporium* immobilized on corn cobs. Both approaches appear to be promising bioremediation techniques.

Here we report the results of studies of the feasibility of cleanup of TNT-contaminated soil by *P. chrysosporium*. We examined the survival of the fungus under increasing concentrations of TNT, with initial exposure both at the spore stage and after mycelial growth had been established. Mineralization by *P. chrysosporium* of TNT at different concentrations was also investigated.

P. chrysosporium BKM-F-1767 (ATCC 24725) was used. The fungus was grown at 37°C and maintained on 0.2% (wt/vol) yeast extract–2.0% (wt/vol) malt extract agar. Spores for use as an inoculum were collected by washing slants with sterile distilled water and were stored at 4°C.

For tolerance and toxicity studies, spores were inoculated directly into 250-ml flasks containing 50 ml of 1.5% (wt/vol) malt extract broth. The cultures were incubated while stationary at 37°C and gassed at 3-day intervals with medical-grade oxygen until harvest or transfer to new medium. Spores were inoculated directly into medium containing TNT or into medium only. The spores inoculated into TNT-free medium were allowed to germinate and grow for 7 days, and the mycelia were then transferred to new medium containing TNT or TNT plus other waste site contaminants. All experiments were done in replicates of three.

For mineralization and TNT degradation studies, spores were inoculated directly into 100-ml serum bottles containing 30 ml of a 1.5% (wt/vol) concentration of either TNT-supplemented or TNT-free malt extract broth. Bottles were sealed with red-rubber-sleeved stoppers. The cultures were incubated while stationary at 37°C and gassed at 4-day intervals with medical-grade oxygen until harvest or transfer to new medium containing malt extract broth and TNT.

Uncontaminated and contaminated soil containing TNT and other contaminants was collected from the U.S. Army munitions depot at Umatilla, Oreg. After receipt, the soil was sieved and stored at 4°C. The soil contained 12,000 ppm of TNT, 3,000 ppm of RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine), and 300 ppm of HMX (octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine). These parts per million values were supplied to us by the U.S. Army and were representative of the average concentrations of TNT, RDX, and HMX in the soil. Analysis of the homogeneous, sieved soil for TNT by the spectrophotometric TNT assay (see below) confirmed the 12,000-ppm TNT value. Extracts of the soil contained the same contaminants, as shown by high-performance liquid chromatography analysis (data not shown). Extracts were prepared by methanol extraction of rocks present in the soil.

Radiolabeled TNT (^{14}C ring labeled) was obtained from the U.S. Army Toxic and Hazardous Material Agency, Aberdeen Proving Ground, Md. The radiolabeled TNT was further purified to ≥95% purity by preparative thin-layer chromatography. The thin-layer chromatography solvent system contained benzene-formic acid (1.0 ml:1 drop). Pu-

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† A publication of the Idaho Agricultural Experiment Station.

rity was determined by counting the percentage of initial radioactivity spotted on the thin-layer chromatography plate which was still present in the isolated TNT spot after chromatography. Nonradioactive TNT was purchased from Chem Service, Inc. (West Chester, Pa.).

To examine the toxicity of the munitions-containing soil to growth of *P. chrysosporium*, contaminated soil (12,000-ppm initial TNT concentration) in various concentrations (0.02 to 2.0%, wt/vol) was added to separate flasks containing 50 ml of malt extract broth, and the flasks were autoclaved (121°C, 30 min). These flasks were inoculated with spore suspension, as were inoculated controls containing either uncontaminated soil or no soil. Three replicates of all flasks were incubated and gassed as described above, and mycelial growth was measured gravimetrically.

To examine the toxicity of the munitions extract and pure TNT, spores or mycelia were also inoculated into 50 ml of malt extract broth containing various concentrations of either the TNT extract or pure TNT. Concentrations of TNT ranged from 0 to 250 ppm. The cultures were gassed with oxygen every 3 days, with a total incubation time of 7 days at 37°C under stationary conditions. Uninoculated controls containing 50 ppm of extracted TNT or pure TNT were treated similarly. Growth was estimated gravimetrically by measuring mycelial dry weight at harvest.

Concentrations of TNT were determined by a sensitive colorimetric assay similar to the field method developed by Jenkins (4). One milliliter of sample was added to 1 ml of acetone, and 5 to 10 μl of dilute potassium hydroxide was added to make the solution alkaline. An excess amount (approximately 5 mg) of sodium sulfite was then added to stabilize the reaction product. After a 5-min incubation at room temperature, the mixture's A_{462} was read.

For determinations of [^{14}C]TNT mineralization by *P. chrysosporium*, the experimental methods were similar to those used in the nonradioactive pure TNT work described above. *P. chrysosporium* was examined for its ability to mineralize [^{14}C]TNT (7.12 Ci/mol) at various concentrations, whether it was exposed to TNT as spores or as vegetative mycelium. Concentrations of TNT for spore-inoculated cultures varied from 0 to 50 ppm and contained 9.6×10^4 dpm of [^{14}C]TNT at the time of inoculation. Concentrations of TNT for vegetative cultures varied from 0 to 100 ppm and contained 8.1×10^4 dpm of [^{14}C]TNT at time zero. During the incubation, $^{14}CO_2$ was trapped at 4-day intervals in conjunction with oxygen flushes for a total of 24 days. The method for trapping was similar to that described by Fedorak et al. (2). Oxygen was flushed at 500 cm^3/min into each culture for 5 min; any gas released was bubbled through three sequential traps. The first trap was an organic solvent trap containing toluene-based cocktail (Econofluor-2; Du Pont, Boston, Mass.). The remaining two traps contained 10 ml of scintillation cocktail (Eco-lite; ICN Biomedicals, Irvine, Calif.) with 1 ml NaOH or Econofluor-2 with 1 ml of Carbo-Sorb (Packard, Meriden, Conn.). $^{14}CO_2$ present in the cocktails was quantified by liquid scintillation counting.

For determination of residual distribution of TNT or [^{14}C]TNT between the mycelial and aqueous phases of cultures, mycelium was removed from the culture medium by centrifugation. In the ^{14}C studies, the amount of ^{14}C present in the mycelium versus that still residing in the aqueous phase was determined by liquid scintillation counting. In the experiments with unlabeled TNT, the mycelium was removed before the acetone extraction step of the spectrophotometric TNT assay used to determine the amount of TNT present in each fraction.

TABLE 1. Tolerance and degradation of TNT by a *P. chrysosporium* spore inoculum after exposure to pure TNT[a]

TNT concn (ppm)		Dry wt of mycelia (mg) (mean ± SD)
Initial	Final (mean ± SD)	
0		16.5 ± 2.7
1	0.74 ± 0.44	14.6 ± 5.0
5	0.88 ± 0.74	12.5 ± 3.3
10	2.10 ± 0.95	8.8 ± 0.2
15	3.15 ± 0.23	6.3 ± 0.9
20	3.48 ± 1.39	6.0 ± 0.8
30	12.94 ± 4.88	3.6 ± 2.4
40	29.36 ± 1.03	2.1 ± 0.8
50	40.21 ± 0.45	1.5 ± 0.5
75	53.29 ± 1.64	2.6 ± 0.2
100	76.03 ± 2.80	3.1 ± 0.8
50[b]	44.46 ± 2.10	

[a] After 7 days of incubation.
[b] Control experiment.

Complete inhibition of the growth of *P. chrysosporium* was observed in inoculated flasks containing contaminated soil at all soil concentrations examined down to 0.02% (wt/vol), at which the medium contained 24 ppm of TNT plus HMX and RDX. Uncontaminated soil showed no apparent inhibition of growth up to the highest level tested (2.0%, wt/vol).

To determine whether the contaminated soil from the Umatilla depot might itself have contained some growth-inhibiting contaminant other than the TNT, growth inhibition by pure TNT and growth inhibition by rock extract containing TNT, RDX, and HMX were compared in a further study. TNT was quite toxic to the fungus (Table 1). Rock extract had a similar effect on the growth of *P. chrysosporium* (data not shown). The fungus was unable to tolerate concentrations of TNT or rock extract containing >10 to 20 ppm of TNT, as determined by mycelial dry weight yields (Table 1). At TNT concentrations of >20 ppm, there was little growth from the spore inoculum.

There was little additional growth of active mycelium that was transferred into cultures containing >80 ppm of pure TNT (Table 2). The rock extract had a similar growth-inhibitory effect which correlated with TNT concentration (data not shown).

In degradation experiments in which spores were added directly to contaminated media, a depletion in TNT was shown at levels of 20 ppm or less, as determined by colorimetric assay of the culture media after mycelial harvest (Table 1). This depletion was directly correlated with mycelial dry weight. In experiments in which already growing mycelia were added to contaminated media, TNT levels in the media dropped immediately and dramatically with either the extract or pure TNT, as determined by colorimetric assay of the culture media at harvest (Table 2). Uninoculated controls showed no change in TNT concentration over the 7-day incubation period. Assays for TNT present in mycelia recovered from these flasks showed that a considerable amount of TNT had been adsorbed by the mycelia (data not shown).

Mineralization studies (Fig. 1) showed the highest $^{14}CO_2$ release (10%) at 5 ppm of TNT for cultures containing a spore inoculum at time zero. The majority of the $^{14}CO_2$

TABLE 2. Tolerance and degradation of TNT by *P. chrysosporium* pregrown mycelia after exposure to pure TNT[a]

TNT concn (ppm)		Final dry wt of mycelia (mg) (mean ± SD)[b]
Initial[c]	Final (mean ± SD)	
0		20.0 ± 1.4
0.87	1.75 ± 0.26	18.0 ± 1.7
4.29	0.49 ± 0.59	16.5 ± 0.7
8.58	1.32 ± 0.43	19.0 ± 2.8
12.86	1.17 ± 0.38	19.7 ± 2.1
21.44	3.42 ± 1.78	16.3 ± 1.5
25.73	3.34 ± 0.40	16.5 ± 2.1
34.30	4.20 ± 0.99	19.0 ± 1.7
42.88	6.96 ± 0.91	16.0 ± 1.0
64.32	16.04 ± 1.94	13.0 ± 1.0
85.76	35.83 ± 7.47	10.0 ± 0.0
50.00[d]	53.76 ± 0.50	

[a] After 7 days of incubation.
[b] The dry weight of mycelia at time zero was 11.3 ± 0.8 mg.
[c] The initial TNT concentration was adjusted for the dilution created by the addition of mycelia at time zero.
[d] Control experiment.

released was released after day 14 of incubation. There was no significant TNT mineralization at concentrations of greater than 15 ppm. The samples containing tolerable levels of TNT (15 ppm) showed mass balance recoveries of 85.8% ± 7.6% of total radioactivity. With these samples, it was necessary to examine the medium containing radiolabeled products in a water-based cocktail in order to locate the soluble radioactivity. Only 2.8% ± 1.1% of the total radioactivity was found in the mycelia. This radioactivity could be counted in an organic-based cocktail. In samples from media containing intolerable levels of TNT (greater than 15 ppm), 100% of the radioactivity was recovered, and all was countable in the organic-based cocktail. The results indicate that a portion of the TNT had been transformed into more water-soluble metabolites.

TNT mineralization by cultures that were inoculated with mycelia and incubated for a total of 24 days showed no $^{14}CO_2$ release at any concentration tested (1 to 100 ppm), although the parental TNT was removed from the culture medium. In the samples that contained pregrown mycelia, the radioactivity present in the medium was countable in a water-based scintillation cocktail. The total recovery was 80.9% ± 7.5% of the initial ^{14}C. The mycelia from medium with 1- to 10-ppm initial concentrations of TNT contained 3.0% ± 1.2% of the total radioactivity. The mycelia from medium with 20- to 100-ppm concentrations of TNT contained 14.2% ± 4.0% of the total radioactivity.

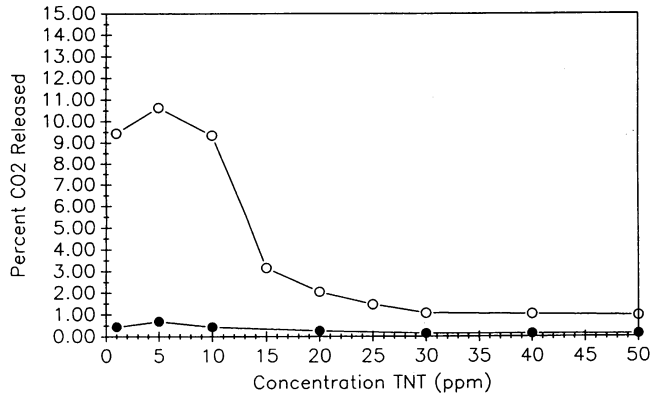

FIG. 1. Percent mineralization of [^{14}C]TNT after 24 days by *P. chrysosporium* at various initial TNT concentrations. ○, TNT added to spore inoculum; ●, TNT added to pregrown mycelia.

Tables 1 and 2 show that wherever mycelia were present, there was a substantial reduction in the TNT concentration in the medium. This was likely due to the adsorption of TNT by mycelia. Actual mineralization of some TNT and/or conversion of TNT to a product not detected by the spectrometric assay are alternative explanations for the reduction of TNT. Figure 1 shows that the reduction of TNT was not due to its complete mineralization. There was, however, clearly some transformation of the TNT to more water-soluble metabolites, since it was necessary to trap the radiolabeled products in an aqueous cocktail when mycelia were present in cultures.

P. chrysosporium was unable to tolerate the levels of TNT found in the contaminated soil. The fungus was completely inhibited by even small amounts of the contaminated soil from a representative contaminated munitions-processing site. Similar observations were reported by Lamar et al. (6), who found that *P. chrysosporium* was sensitive to pentachlorophenol levels as low as 5 ppm. Bumpus et al. (1) presented evidence that *P. chrysosporium* is capable of the complete degradation of highly chlorinated PCBs (polychlorinated biphenyls), but mineralization was examined only at very low concentrations (0.05 ppm). The fungus was also very sensitive to pure TNT and to the TNT-, HMX-, and RDX-containing extract. The degree of sensitivity was similar to that observed for contaminated soil. The specific toxicity and biodegradability of HMX and RDX were not examined in this study. However, the primary factor affecting growth of *P. chrysosporium* was TNT concentration.

The present results indicate that *P. chrysosporium* is probably not a good candidate for bioremediation of TNT-contaminated sites containing high concentrations of explosives because of its high sensitivity to the contaminants. However, the use of a matrix that immobilizes and protects *P. chrysosporium* from exposure to TNT, allowing the extracellular enzymes to attack the contaminant, may be of use for bioremediation of TNT. If a method that enabled *P. chrysosporium* to tolerate higher concentrations of TNT were found, the fungus might also be able to mineralize a higher percentage of the TNT present. Since many contaminated soils contain gram per kilogram quantities of explosives, protection of the fungus may prove to be very difficult.

This work was supported by Battelle Pacific Northwest Laboratories, Richland, Wash. (contract 097710-A-L2), and by the Idaho Agricultural Experiment Station.

We are grateful to Zemin Wang and Denise Newton for technical assistance with the ^{14}C experiments. We thank Deborah Roberts for her technical and editorial advice on the manuscript.

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