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Fungal interactions with the explosive RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine)

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The bacterially mediated, anaerobic biodegradation of the explosive RDX (hexahydro 1,3,5 trinitro-1,3,5-triazine) is well established. Reports of successful mineralization of RDX by white rot fungi, and the enhanced transformation of RDX in stirred as compared to static composts, led us to study the possible aerobic role of several filamentous fungi in RDX biodegradation. *Cladosporium resinae*, *Cunninghamella echinulata* var *elegans*, *Cyathus pallidus* and *Phanerochaete chrysosporium* were grown in the presence of 50 and 100 μg ml^{-1} of RDX on a vegetable juice agar. Little inhibition of radial growth was observed, while control cultures with TNT exhibited substantial inhibition. When 100 μg ml^{-1} of RDX was added to pre-grown mycelia in a nonlignolytic liquid medium, between 12 and 31% was lost after 3 days. In similar experiments using ^{14}C-RDX, most of the label remained in the organic fraction, and little or none was found in the aqueous fraction, the volatile fraction or incorporated into cell walls. Although disappearance of RDX was observed for all four species tested, there was no evidence of mineralization. Mixed cultures of microorganisms, including both bacteria and fungi, merit further study as agents for the decontamination of munitions-contaminated soils.

Keywords: RDX; explosives; fungal bioremediation

Introduction

The heterocyclic nitramine hexahydro-1,3,5-trinitro-1,3,5-triazine is a high explosive also called cyclotrimethylenetrinitramine, cyclonite, hexogen, or RDX (the British code name for Research Department or Royal Demolition Explosive) [19]. The most important military high explosive in the USA, it may be formulated in a large number of ways, eg alone (Composition A), in a slurry with TNT (Composition B), or in a mixture with various plasticizers (Composition C) [27,28]. Most of the explosives used in World War II, Korea, and Vietnam contained TNT and RDX in varying proportions. Since these compounds are toxic as well as explosive, they represent a toxicological legacy of formidable proportions, especially at military installations where they are manufactured and processed.

In animal tests, RDX causes convulsions and death in dogs, swine, and other experimental animals [20,29] and enhances the toxicity of TNT in mice, rats, and dogs [1]. In human beings, convulsions resembling epileptic seizures have been reported among munitions workers [9,16,20]. A rather surprising amount of adverse human exposure to RDX occurred during the Vietnam War when soldiers used composition C-4 to heat food [12,25] and ingested it for recreational purposes to mimic the effects of ethanol and cannabis [14,22]. On the other hand, no negative health effects were observed in a large study of American munitions workers exposed to inhalation levels of up to 1.5 mg m^{-3} [6]. Summaries of toxicological studies and recommended health advisory data, much of it culled from inaccessible government reports, were summarized by Rosenblatt *et al* [19] and McLellan *et al* [16].

There has been considerable success in detoxifying waste water containing RDX by treatments with ultraviolet light-hydrogen peroxide, ion exchange resins, chemical coagulation, and adsorption onto activated charcoal [16], as well as biologically with anaerobic sewage sludge [15,17]. In addition, laboratory studies have shown mineralization of RDX in aqueous systems by pure cultures of the white rot fungus, *Phanerochaete chrysosporium* [2], and by three different species of enterobacteria isolated from munitions-impacted soil at Los Alamos, NM [13]. However, the treatment of RDX-contaminated soils remains problematic, and the cost and negative environmental consequences of incineration have made biological degradation look increasingly attractive.

In early studies, Osmon and Klausmeier [18] reported some disappearance of RDX during soil enrichment studies, but composting is the method which currently has the most promise for treatment of RDX-contaminated soils and sludges. Isbister *et al* [8] found mineralization of ^{14}C-RDX in laboratory- and green house-scale compost systems. In field studies, Williams *et al* [30] reported significant loss of RDX recovery in both mesophilic and thermophilic aerobic static piles and Griest *et al* [4] showed similar reductions in both static-pile and mechanically-stirred composts.

Filamentous fungi are major components of composts, and are among the major decomposers in nature. In this preliminary study, we have compared the RDX tolerance of *P. chrysosporium* to three other species of fungi on a vegetable juice medium that supports good growth and sporulation of fungi [5]. In addition, we have assayed the level of extractable RDX after the exposure of pre-grown mycelia to relatively high levels of RDX (100 μg ml^{-1}) in

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liquid culture for 3 days. High performance liquid chromatography (HPLC) and scintillation counting of ^{14}C-labeled RDX were compared as methods for determining the fate of extractable RDX and its putative transformation products.

Materials and methods

Strains and culture conditions

Four fungal species were tested: *Phanerochaete chrysosporium* Burdsall (ATCC 24725), and *Cyathus pallidus* Berk. & Curt. (collected in Caribbean National Forest, El Verde, Puerto Rico), both lignin-degrading basidiomycetes; *Cunninghamella echinulata* var *elegans* (Lendner) Lunn et Shipton (ATCC 22762), a zygomycete (usually called *C. elegans*); and *Cladosporium resinae* (Lindau) de Vries (ATCC 22712), the anamorphic state of an ascomycete.

All cultures were grown in a vegetable juice medium formulated with 50 ml V8 vegetable juice (Campbell Soup Co, Camden, NJ, USA), 10 g sucrose, and 1000 ml deionized water, pH 5.5 [5]. Stock cultures were maintained on vegetable juice agar solidified with 2% agar. For long term storage, agar plugs were stored in vials under sterile distilled water. To estimate tolerance of RDX by fungi, 5-mm plugs of agar cultures were inoculated on plates of V8-sucrose agar containing 0, 50 or 100 μg RDX ml^{-1}. Positive controls contained 50 μg TNT ml^{-1}. Radial growth was measured every three days until the colony outgrew the plate. Each fungus-RDX combination was replicated five times.

To test for biodegradation and biotransformation of RDX, 50 ml of V8-sucrose media in 250-ml Erlenmeyer flasks were inoculated with approximately 0.05 g of macerated agar plugs in 0.5 ml sterile water. Cultures were incubated for 48 h on a shaker at 24° C and 200 rpm. Then each culture received 5 mg unlabeled RDX plus ^{14}C-RDX with a total activity of 5×10^5 dpm per culture. Cultures and uninoculated controls were extracted after 3 or 14 days of additional incubation at 24° C and shaking at 200 rpm.

Extraction and determination of RDX

Both unlabeled and ^{14}C-ring-labeled RDX were synthesized by K Horvath, Department of Chemistry, Tulane University, USA [7]. The purity of ^{14}C-RDX was determined by thin layer chromatography to be 97.5%, and its specific activity was 1.35 mCi mg^{-1}.

After 3 days incubation, 100-μl aliquots of culture filtrates were sampled. Then the entire culture, of liquid medium and mycelia, was pooled and ground together in an explosion-proof blender with 50 ml acetone, left to stand for 15 min, and filtered through filter paper. Extracts were partitioned in a separatory funnel with 100 ml methylene chloride. Organic fractions were concentrated overnight by evaporation in a chemical hood, resuspended in 5 ml acetone, and stored at −20° C.

Concentrations of RDX in culture filtrates and in organic and aqueous fractions of total cultures were determined by HPLC. Samples of 100 μl were injected into a Waters 600E liquid chromatograph (Milford, MA, USA) equipped with a DeltaPak C-18 column, 3.9 × 150 mm. The mobile phase was 20% methanol, flow rate was 0.7 ml min^{-1}, and detection was at 230 nm using a Waters 991 detector [15]. Retention time for RDX was 9.4 min. To correlate RDX concentration with absorbance at 230 nm, a standard curve was generated using from 5 to 250 μg RDX ml^{-1} in acetone ($r^2 = 0.96$).

Fate of ^{14}C-RDX

Liquid scintillation counting was used to determine how much of the label was found in culture filtrates, in headspace gases, and in organic and aqueous fractions. Counts were made in 10 ml Ecoscint scintillation cocktail (National Diagnostics, Manville, NJ, USA) in a Beckman LS7000 (Fullerton, CA, USA) scintillation counter. To estimate the amount of ^{14}C extractable from cell wall material, blended cultures were filtered and pieces of filter paper were soaked in 1 ml 95% EtOH : conc HCl (95 : 5) for 15 min, after which scintillation cocktail was added. Six replicates were extracted and counted for each fungus. Presence of ^{14}C in CO_2 and volatile organics was tested by pulling headspace gases through two traps containing 10 ml Corbosolve CO_2 absorbent and one trap containing 10 ml scintillation cocktail, and counting as described above.

Data analysis

Differences among fungi and controls in amount of RDX remaining, were tested for significance using analysis of variance (ANOVA). When significant differences were found between treatments, post hoc pairwise comparisons were made using Fisher's LSD tests. All statistics were done with the SYSTAT software package (SYSTAT, Inc, Evanston, IL, USA).

Results

Fungal tolerance of RDX

RDX had little visible effect on radial growth of fungi (Figure 1). At 3 days, *P. chrysosporium* had a mean colony diameter of 4.4 cm on control plates; on 50 μg ml^{-1} and 100 μg ml^{-1} RDX mean diameters were 3.7 and 3.5 cm, respectively, but differences were not significant. In contrast, when grown on 50 μg ml^{-1} TNT, mean colony diameter was 0.6 cm. Similarly, radial growth and appearance of the other three fungi was not significantly affected by 50 or 100 μg ml^{-1} RDX, but was greatly restricted by 50 μg ml^{-1} TNT. *C. resinae* grew the most slowly of the four fungi, and showed the least inhibition of growth by TNT.

Extractable RDX as determined by HPLC

All fungi had significantly less RDX in organic extracts than did controls, and all four fungi grew well in V8-sucrose medium with dry weights ranging from 25–97 mg culture^{-1}. For *P. chrysosporium*, the positive control, 22% of the RDX was not recovered in concentrated organic extracts. For the other three species tested, 12–31% of added RDX was not recovered. In contrast, all of the RDX added to control cultures without mycelium was recovered (Table 1). HPLC detection of organic fractions at 230 and 245 nm did not reveal any obvious RDX metabolites, nor did HPLC detection of 100-μl aliquots of culture filtrates prior to extraction, nor of aqueous fractions after extraction, show any new peaks. The only peaks other than RDX con-

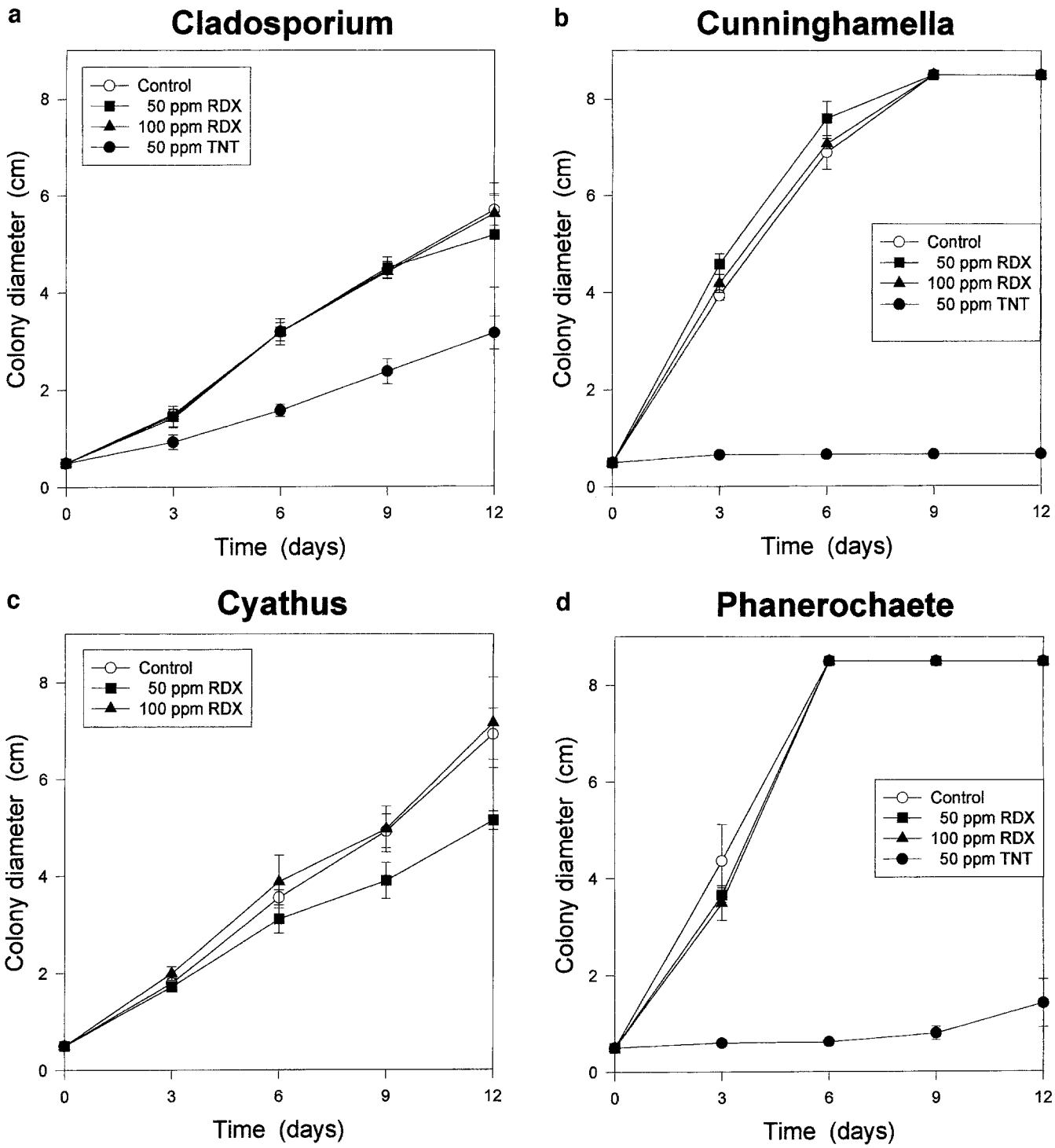

Figure 1 Radial growth of fungi on media containing RDX. (a) *Cladosporium resinae*. (b) *Cunninghamella echinulata* var *elegans*. (c) *Cyathus pallidus*. (d) *Phanerochaete chrysosporium*. A TNT control was not conducted with *C. pallidus*

stituting above 1% of the total absorbance at 230 nm were also present in control cultures to which no RDX had been added. In cases where experiments were continued for 14 days, the amounts of extractable RDX were the same and no detectable biotransformation products were observed (data not shown).

Fate of ^{14}C-RDX

In order to determine if the loss of RDX was due to mineralization, these experiments were repeated with the ^{14}C-labeled RDX delivered with 100 μg unlabeled RDX ml^{-1} as carrier. These results are presented in Table 2. Recovery of label ranged from 96% for *C. resinae* and *C. pallidus*

Table 1 RDX extracted from fungal cultures as determined by HPLC[1]

Fungus	Dry wt mycelium (mg)[2]	pH	RDX[3] (μg ml^{-1})	% Disappearance of RDX
C. resinae	57	6.3 ± 0.1	69.4 ± 9.8 c	31
C. echinulata	97	5.4 ± 0.6	88.2 ± 13.3 b	12
C. pallidus	63	5.3 ± 1	79.1 ± 11.4 bc	21
P. chrysosporium	25	4.6 ± 0.1	77.5 ± 7.6 bc	22
Control	0	5.5 ± 0	108.5 ± 5.8 a	0

[1]Mycelia were grown for two days in shake culture, then RDX was added to a final concentration of 100 μg RDX ml^{-1} and cultures were incubated for an additional 72 h
[2]Dry weight of pre-grown mycelium before addition of 100 μg RDX ml^{-1}. Dry weights performed on parallel cultures
[3]Determined from concentrated organic fractions. RDX in aliquots of unconcentrated aqueous fractions was below the limit of detection. Means are followed by letters if an ANOVA found significant variation among treatments. Any two means in a single column followed by a shared letter are not significantly different as determined by LSD tests. μg mycelium and pH measurements were done on parallel cultures. Experiments were replicated four times

Table 2 Distribution of ^{14}C-label from fungal cultures after three days of incubation with ^{14}C-RDX[1]

Fungus	^{14}C-label (dpm × 10^3)					
	Organic fraction	Aqueous fraction	Filter paper	Volatile and CO_2 traps	Total[3]	% Recovery
C. resinae	442 ± 45 a	28 ± 9 ab	8 ± 4	ND[2]	478 ± 44 a	96
C. echinulata	432 ± 71 a	22 ± 4 bc	9 ± 3	ND	463 ± 74 a	93
C. pallidus	442 ± 43 a	25 ± 4 abc	13 ± 9	ND	481 ± 40 a	96
P. chrysosporium	346 ± 114 b	22 ± 3 c	12 ± 5	ND	380 ± 117 b	76
Control	470 ± 46 a	31 ± 6 a	8 ± 3	ND	508 ± 44 a	100

[1]RDX was added to pregrown mycelia in 50 ml of medium. Each culture initially received 5 × 10^5 dpm of ^{14}C-RDX. See Methods for details
[2]ND = none detected
[3]Means are followed by letters if an ANOVA found significant variation among treatments. Any two means in a single column followed by a shared letter are not significantly different as determined by LSD tests. μg mycelium and pH measurements were done on parallel cultures. Experiments were replicated four times

to 76% for *P. chrysosporium*. Recovery of label from controls was 100%. No $^{14}CO_2$ or ^{14}C-volatiles were detected in traps. The amount of ^{14}C in the aqueous fraction of fungal cultures was less than that of control cultures, indicating an absence of polar RDX metabolites (Table 2). Furthermore, no fungus had significant amounts of label in the mycelial fragments trapped on filter paper.

Differences in amount of label in organic fractions were significant ($F = 2.76$, $P < 0.05$, model d.f.=4, error d.f.=26), however, only *P. chrysosporium* was significantly different from controls. Similarly, the amount of RDX in organic fractions measured by HPLC varied significantly; *P. chrysosporium* had 73% as much RDX as the controls ($F = 4.77$, $P < 0.01$, d.f.=4.21) (Table 1). The remaining label and RDX were not accounted for.

In summary, we were unable to obtain evidence of mineralization or volatilization of RDX, or of incorporation of RDX into mycelia, by any of the four species of fungi. No polar RDX metabolites were revealed by HPLC or scintillation counting. For *P. chrysosporium* we recovered less label in organic fractions, less label overall, and less RDX in organic fractions than we did for the inoculated controls. All other fungi also had significantly less recoverable RDX in organic fractions than the controls, although they did not have significantly less label.

Discussion

Several studies have demonstrated reduction in RDX and other munitions after composting [4,8,30] with stirred composts giving greater reduction than static piles. Although no attempt was made to identify microbial populations in these studies, visual examination of composts by Williams *et al* [30], showed heavy fungal growth in mesophilic piles with 'fungal growth penetrated into the central regions of all particles examined' [30, p 141]. These findings, coupled with reports that the white rot fungus, *P. chrysosporium*, can mineralize ^{14}C-RDX [2], made us optimistic that fungal-mediated, aerobic, biotransformation and biodegradation of RDX could provide a supplemental method for treating munitions-contaminated soils. A nutrient-poor, nonlignolytic medium was used in order to approximate field conditions.

The anaerobic destruction of RDX is well established and proposed degradative pathways have been published [10,11,15]. Anaerobic treatment of RDX-contaminated

water is used by the military [17], and destruction of RDX by anaerobic composting has also been reported [3]. In recent laboratory trials under conditions of oxygen depletion, pure cultures of *Morganella morganii* and *Providencia rettgeri*, isolated from nitramine-contaminated soil from the Los Alamos National Laboratory, completely transformed RDX, while *Citrobacter freundii* partly transformed RDX [13]. The successful mineralization of RDX by the white rot, *P. chrysosporium* [2], and the enhanced transformation of RDX in stirred as compared to static composts [4] showed that biological RDX degradation could also be mediated by fungi in an aerobic environment. In soil amended with corn cobs, 76% of the label from ^{14}C-RDX was recovered as CO_2 after 30 days of incubation with *P. chrysosporium*; in liquid culture 67% of the label from ^{14}C RDX was recovered during this time period [2]. A method for treating the waste water generated by munitions loading, assembly, and packing operations has been developed using *P. chrysosporium* immobilized on the disks of a rotating biological contractor [23]. However, the field applicability of white rot-mediated munitions biodegradation has been questioned. In tests in which TNT- and RDX-contaminated soil was added to liquid medium, growth of *P. chrysosporium* was completely inhibited [21].

We have tested the RDX tolerance of three taxonomically distinct species of filamentous fungi, and compared them to the RDX tolerance of *P. chrysosporium*. Ability to grow on comparative levels of TNT was also studied. RDX at 50 and 100 μg L^{-1} did not inhibit the radial growth of *P. chrysosporium*, *C. resinae*, *C. echinulata*, or *C. pallidus* while TNT gave significant inhibition for the three species tested. Solubility of RDX in water is approximately 60 μg ml^{-1} [19] so at 100 μg ml^{-1} some of the RDX would be expected to be precipitated out of the solution.

Growth of enterobacteria in liquid media was not inhibited by similar concentrations of RDX, although RDX affected the ability of bacteria to metabolize HMX, a related compound [13]. RDX is not active in the Ames Test [24], in a dominant lethal assay with rats, nor in an unscheduled DNA synthesis assay with human fibroblasts [16]. In humans and other mammals, RDX is far less toxic than TNT [6,16,19,20], although mixtures of TNT and RDX produce symptoms more severe than TNT alone [1]. The purposeful ingestion of RDX by soldiers in Vietnam [14,22] attests to the relative lack of toxicity by RDX. It should be noted that in animal studies, the acute oral LD_{50} of RDX is related to its physical form and the way in which it is suspended or dissolved; finely ground powders are more toxic than coarse preparations [20]. Our data, conducted with a powdery form of RDX, indicate that, like bacteria and mammals, fungi are relatively resistant to RDX.

After three days in a nonlignolytic medium, all four fungi displayed significant reductions in levels of extractable RDX (Table 1). *C. echinulata*, which grew the best, gave the least reduction; *C. resinae*, which also grew well, gave the most reduction in extractable RDX. The two wood rotting fungi, *C. pallidus* and *P. chrysosporium*, gave intermediate levels. However, when calculated as disappearance of RDX per weight of mycelium, *P. chrysosporium* was the most effective. When the fate of ^{14}C-RDX was followed with scintillation counting, most of the label remained in the organic fractions. Similar loss of extractable RDX has been noted in experiments with composting [4,8,30].

These organisms clearly modify RDX or its distribution in some way, but we were unable to detect the missing RDX. Similarly, Kitts *et al* [13] demonstrated mineralization of RDX by enterobacteria, and conversion of up to 62% to polar metabolites, but were unable to account for 25–35% of the label originally added. In contrast, Fernando and Aust [2] recovered 93.7% of label fed to a *Phanerochaete* as ^{14}C-RDX. In the present study, the fact that neither HPLC nor scintillation counting could account for up to 25% of the parent compound is puzzling. Liquid scintillation data would show the presence of organic metabolites with retention times and absorbance spectra very close to RDX. If incorporation into cell walls was involved, at least some of it should be liberated by soaking in EtOH : HCl. In their studies, Fernando and Aust [2] used 0.02 μg ml^{-1} RDX, whereas we used 100 μg ml^{-1}. Mineralization of 0.02 μg ml^{-1} RDX would not have been detectable in our system, and it is not clear that the level of degradation obtained by Fernando and Aust would have been sustained at higher levels of RDX. We recognize that mere disappearance of a compound is not sufficient evidence of biodegradation, and that too many scientists invoke 'disappearase' [26] to explain loss of target compounds. Nevertheless, we believe that our preliminary studies demonstrate that several fungi transform RDX and that environmental scientists should not limit their mycological horizons to *P. chrysosporium*. Many fungal species have the potential for supplementing existing bacteria-based approaches to munitions bioremediation. Ultimately, for the biological treatment of hazardous wastes to become a reliable technology, microbiologists will have to abandon their preference for working with pure cultures and utilize complex consortia of soil microorganisms of the sort that are already so successful in nature.

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