0273-2289/93/3940—0715$03.40

# Fungal Degradation of Organophosphorous Insecticides†

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## ABSTRACT

Organophosphorous insecticides are used extensively in agriculture. As a group, they are easily degraded by bacteria in the environment. However, a number of them have half-lives of several months. Little is known about their biodegradation by fungi. We showed that *Phanerochaete chrysosporium* mineralized chlorpyrifos, fonofos, and terbufos (27.5, 12.2, and 26.6%, respectively) during an 18-d incubation in nutrient nitrogen-limited cultures. Results demonstrated that the chlorinated pyridinyl ring of chlorpyrifos and the phenyl ring of fonofos undergo cleavage during biodegradation by the fungus. The usefulness of *P. chrysosporium* for bioremediation is discussed.

**Index Entries:** *Phanerochaete chrysosporium*; organophosphorous insecticides; fungi; biodegradation.

## INTRODUCTION

Organophosphorus insecticides are used extensively in agriculture to treat a variety of soil pests, including corn rootworms, cut worms, and wire worms, and to control orchard pests, such as the apple flea weevil,

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the plum curculio, and the coddling moth (*1,2*). Many of these compounds are also effective as topical sprays, and are used to control household pests and aerial insects on field crops and ornamentals (*1,2*). Some organophosphorus insecticides are also used in veterinary medicine to control ticks and fleas (*1,2*).

Many organophosphorous insecticides function as cholinesterase inhibitors (*1,2*). They are typically quite lethal to insects and are often relatively innocuous to fish, mammals, and birds. Also, compared to certain other effective insecticides (DDT, chlordane, and dieldrin, for example), they are not very persistent in the environment. Some organophosphorous insecticides are, however, moderately persistent and have half-lives in soil of several months (*2–4*). Furthermore, substantial amounts of relatively stable and biologically active metabolites may persist in soils either as free metabolite or tightly bound to soil (*3,4*). Mineralization by soil microorganisms appears to be a significant mode of organophosphorous insecticide removal (*3,4*).

The wood-rotting fungus *Phanerochaete chrysosporium* is able to degrade a wide variety of structurally diverse organopollutants to carbon dioxide (*5–10*). Because of its unique biodegradative abilities, considerable interest exists in its role and that of other lignin-degrading fungi in the degradation of agricultural chemicals and their metabolites. The present investigation shows that *P. chrysosporium* can mediate extensive degradation of three representative organophosphorous insecticides.

## METHODS

### Fungus

*P. chrysosporium* BKM-F-1767 was obtained from the US Department of Agriculture Forest Products Laboratory, Madison, WI. The fungus was stored at room temperature on malt agar slants prior to use.

### Radiochemicals

The following radiolabeled insecticides were a gift from Joel R. Coats and L. Somasundaram, Iowa State University, to S. N. Kakar: [2,6-phenyl-$^{14}$C]chlorpyrifos, [U-ring-$^{14}$C]fonofos, and [methylene-$^{14}$C]terbufos. The trivial names of the three organophosphorous insecticides are used as a matter of convenience. The following names of these insecticides are recognized by the Chemical Abstracts Service (CAS): chlorpyrifos, phosphorothioic acid *O,O*-diethyl *O*-(3,5,6-trichloro-2-pyridinyl) ester; fonofos, ethylphosphonodithioic acid *O*-ethyl *S*-phenyl ester; terbufos, phosphorodithioic acid *S*-{[(1,1-dimethylethyl)thio]methyl} *O,O*-diethyl ester. The radiochemical purity of [$^{14}$C]-chlorpyrifos and [$^{14}$C]-fonofos was >98%, whereas the radiochemical purity of [$^{14}$C]-terbufos was 96%. Structures of these compounds are presented in Fig. 1.

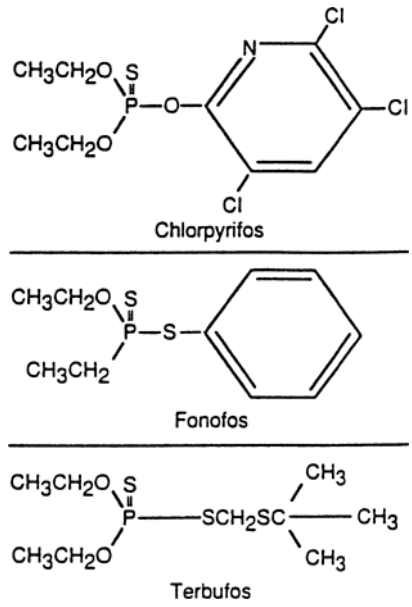


Fig. 1. Structures of organophosphorous insecticides used in this investigation.

## Experimental Conditions

Biodegradation studies were performed using stationary cultures of *P. chrysosporium* grown in the nutrient nitrogen-limited medium described by Fenn and Kirk (*11*). Briefly, this medium consists of 56 m*M* glucose, 1.2 m*M* ammonium tartrate, trace elements (*12*), and thiamine (1 mg/L) in 20 m*M* dimethylsuccinate (sodium) buffer, pH 4.2 (*11*). Cultures were initiated by addition of a spore suspension as described by Kirk et al. (*12*). Controls received 1 mL of formaldehyde (36% [w/w]) to prevent microbial growth. All cultures were incubated at 37°C.

Cultures (10 mL) were grown in sealed 250-mL Wheaton bottles in which the Teflon™-lined cap was modified by drilling two holes in the top of the cap into which a short and a long piece of glass tubing were inserted. The longer piece of glass tubing extended into the bottle until it was approx 3 cm above the fungal mat. Epoxy glue was used to hold the tubing in place. These modified caps served as gas-exchange manifolds. Wheaton bottles so modified have been used to demonstrate [$^{14}$C]-carbon dioxide evolution from [$^{14}$C]-labeled organopollutants in several studies (*5–9*). However, it was recently found that 500-mL Erlenmeyer flasks with ground-glass joints fitted with impingers (Ace Glass), originally designed for air sampling, could also be used for gas exchange. In experiments in which 500-mL Erlenmeyer flasks were used, it was necessary to increase the volume of the cultures to 20 mL, so that the fungal mat completely covered the bottom of the flask.

Radiolabeled compounds were added to cultures immediately following inoculation with fungal spores. Cultures were grown in 250-mL Wheaton bottles or 500 mL Erlenmeyer flasks equipped for gas exchange as described above. During the first 3 d of incubation, cultures were grown under ambient atmosphere. On day 3 and at 3-d intervals thereafter, cultures were flushed with oxygen for 20 (Wheaton bottles) or 30 (Erlenmeyer flasks) min, and the $CO_2$ evolved was trapped in 10 mL of a solution containing ethanolamine-methanol-Safety Solve scintillation cocktail (Research Products International Corp., Mt. Prospect, IL) (1:4:5). The amount of [$^{14}$C]-$CO_2$ trapped was determined by liquid scintillation spectrometry. Mass balance analyses were performed on cultures of *P. chrysosporium* that had been incubated with [$^{14}$C]-chlorpyrifos, [$^{14}$C]-fonofos, or [$^{14}$C]-terbufos for 18 or 21 d. Cultures (three or five replicates for each compound) were pooled, and the individual culture flasks were rinsed with water (40 mL total), which was added to the pooled cultures. The pooled cultures were then extracted three times with methylene chloride (100 mL total). Particulate material (mycelium) was separated from the aqueous fraction by filtration through glass wool. Samples of the particulate material and 1-mL aliquots of the methylene chloride extracts and the aqueous fractions were placed in scintillation vials containing 10 mL of Safety Solve. Radioactivity was determined by liquid scintillation spectrometry. For TLC, the solvent was removed from the methylene chloride extracts by evaporation under nitrogen. The residue was redissolved in a minimal amount of methylene chloride. TLC of [$^{14}$C]-chlorpyrifos (in hexane:acetone:glacial acetic acid [20:40:1]), [$^{14}$C]-fonofos (in chloroform: ethylacetate:hexane [2:2:1]), and [$^{14}$C]-terbufos (in nitromethane:acetonitrile:toluene [3:8:9]) was performed as recommended (J. R. Coats and L. Somasundaram, personal communication) using precoated silica gel 60 G F-254 plates (5×20 cm (aluminum-backed); thickness, 250 μm; E. Merck AG, Darmstadt, Germany). Following TLC, the elution solvents were evaporated under air. TLC plates were cut into 1-cm strips, which were then moistened with water and placed in scintillation vials containing 20 mL of Safety Solve. With this procedure, the silica gel was desorbed from the aluminum backing and settled on the bottom of the scintillation vial. Radioactivity was then determined by liquid scintillation spectrometry.

## RESULTS

The ability of *P. chrysosporium* to degrade [$^{14}$C]-chlorpyrifos, [$^{14}$C]-fonofos, and [$^{14}$C]-terbufos is illustrated in Table 1 and Figs. 2–7. In all cases, substantial amounts (27.5, 12.2, and 26.6%, respectively) of [$^{14}$C]-chlorpyrifos, [$^{14}$C]-fonofos, and [$^{14}$C]-terbufos were degraded to [$^{14}$C]-$CO_2$ during the 18-d incubation with nutrient nitrogen-limited cultures of this fungus. Only the aromatic ring carbons of [$^{14}$C]-chlorpyrifos and [$^{14}$C]-

Table 1
Mass Balance of $^{14}C$-Radioactivity from *P. chrysosporium* Incubated with $^{14}C$-Chlorpyrifos, $^{14}C$-Fonofos, and $^{14}C$-Terbufos for 18 D[a]

| Compound | %Recovery | Methylene chloride fraction | Aqueous fraction | Particulate | $^{14}CO_2$ |
|---|---|---|---|---|---|
| Chlorpyrifos | 45.1 | 9.0 | 4.4 | 4.2 | 27.5 |
| Fonofos | 78.2 | 13.1 | 50.8 | 2.1 | 12.2 |
| Terbufos | 60.9 | 6.9 | 22.9 | 4.5 | 26.6 |

[a]The initial concentrations of chlorpyrifos, fonofos, and terbufos were 3.5, 8.5, and 1.4 μM, respectively. All data are expressed as percent.

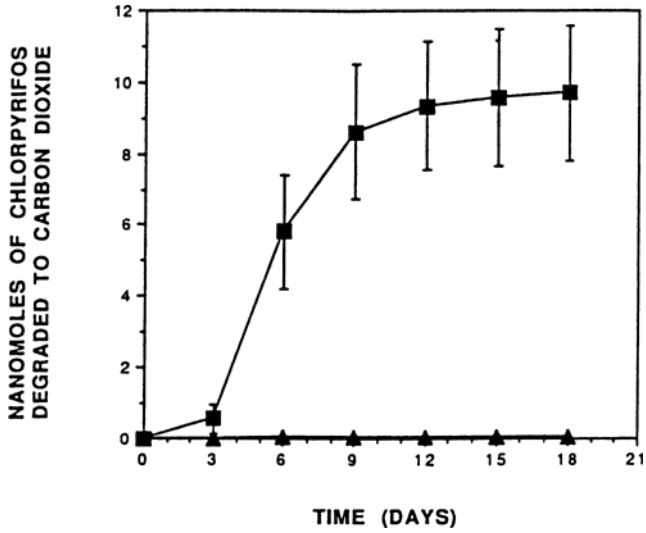


Fig. 2. Mineralization of [$^{14}C$]-chlorpyrifos by *P. chrysosporium*. The initial concentration of [$^{14}C$]-chlorpyrifos was 3.5 μ*M*. Each point (closed squares) represents the mean of five determinations ± the SD. An uninoculated culture served as the control (closed triangles).

fonofos were labeled. Thus, these results demonstrated that the chlorinated pyridinyl ring of [$^{14}C$]-chlorpyrifos and the phenyl ring of [$^{14}C$]-fonofos undergo ring cleavage during biodegradation by *P. chrysosporium*. Degradation was also assessed by mass balance analysis. After incubation, cultures were extracted with methylene chloride. Results (Table 1) show that considerable amounts of all of these compounds were converted to metabolites that partitioned into the aqueous phase. Similarly, TLC revealed the presence of several metabolites of these compounds in the methylene chloride fractions. Considerable nonbiological degradation

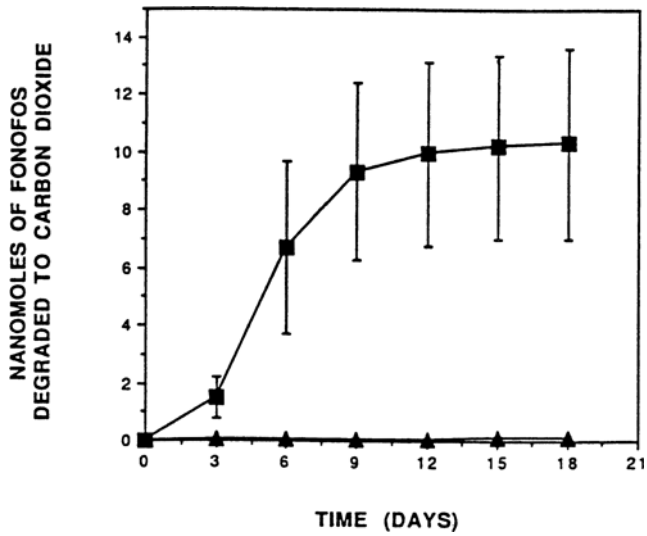


Fig. 3. Mineralization of [$^{14}$C]-fonofos by *P. chrysosporium*. The initial concentration of [$^{14}$C]-fonofos was 8.5 $\mu M$. Each point (closed squares) represents the mean of five determinations ± the SD. An uninoculated culture served as the control (closed triangles).

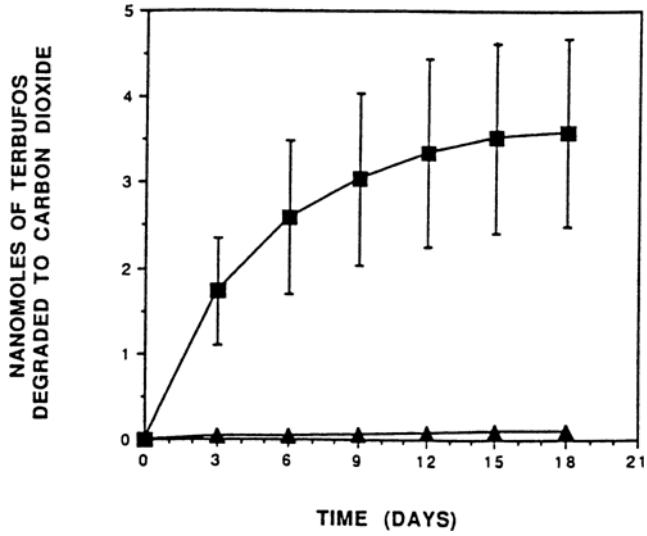


Fig. 4. Mineralization of [$^{14}$C]-terbufos by *P. chrysosporium*. The initial concentration of [$^{14}$C]-terbufos was 1.4 $\mu M$. Each point (closed squares) represents the mean of five determinations ± the SD. An uninoculated culture served as the control (closed triangles).

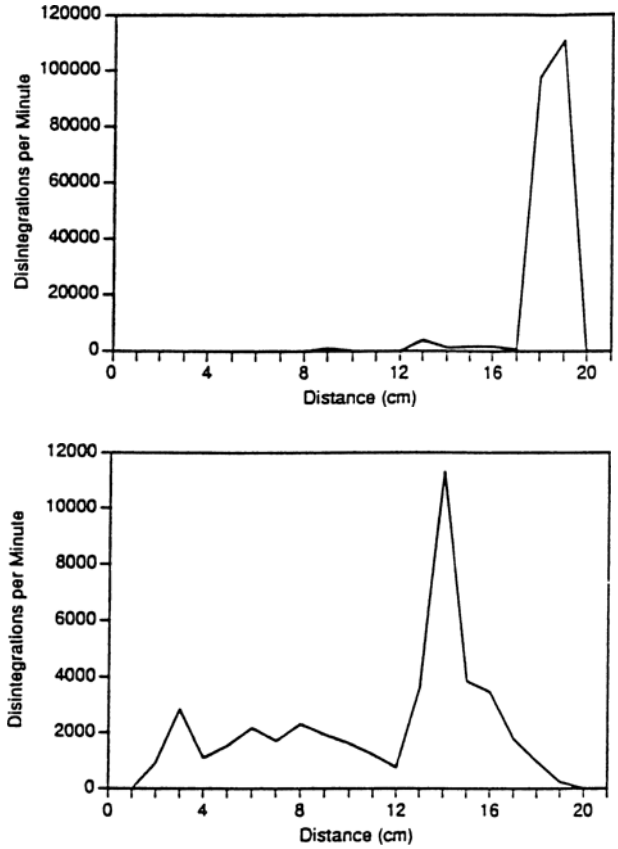


Fig. 5. TLC of [$^{14}$C]-chlorpyrifos before incubation (top) and [$^{14}$C] in methylene chloride extract of cultures of *P. chrysosporium* that were incubated with [$^{14}$C]-chlorpyrifos for 18 d (bottom).

occurred in controls as evidenced by metabolite formation in sterile culture medium (data not shown). Controls also suggested that [$^{14}$C]-chlorpyrifos, [$^{14}$C]-fonofos, and [$^{14}$C]-terbufos may be susceptible to air-stripping, and that volatilization might account for the low mass recovery observed in several experiments and controls. Because of the low mass recoveries, additional experiments (Table 2) were performed in which cultures (20 mL) were incubated in 500-mL Erlenmeyer flasks having a ground-glass joint and fitted with a glass impinger for gas exchange. A trap (Orbo™ 32 tube [Supelco, Bellfonte, PA]) for volatile organics was also placed immediately before the carbon dioxide trap, and the latex tubing used in this apparatus was assayed for absorbed radioactivity. Results were similar to those reported in Table 1 and are consistent with the con-

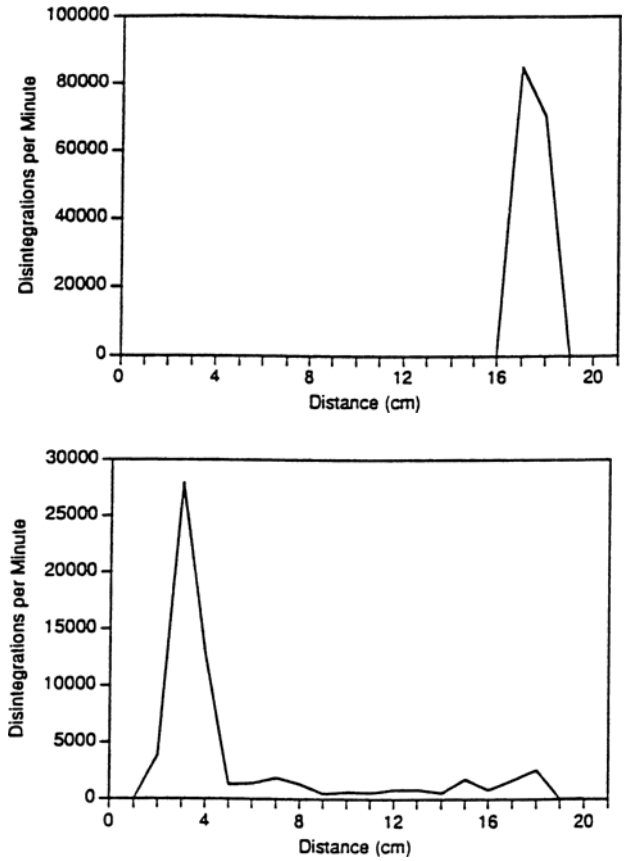


Fig. 6. TLC of [$^{14}$C]-fonofos before incubation (top) and [$^{14}$C] in methylene chloride extract of cultures of *P. chrysosporium* that were incubated with [$^{14}$C]-fonofos for 18 d (bottom).

clusion that substantial amounts of all of these pesticides are degraded by *P. chrysosporium*. In general, mass recoveries were improved using these procedures. Although some radiolabeled material was retained by the volatile traps and some was absorbed by the latex tubing, the authors were still unable to account for substantial amounts of mass in some experiments. We suggest that most of this mass may have been lost during manipulations involved with the methylene chloride extraction. In control experiments, the amount of [$^{14}$C]-labeled material found in [$^{14}$C]-$CO_2$ traps were never $>1.0\%$ of the radioactivity initially present, demonstrating that [$^{14}$C]-$CO_2$ formation was not a major factor in the nonbiological disappearance observed.

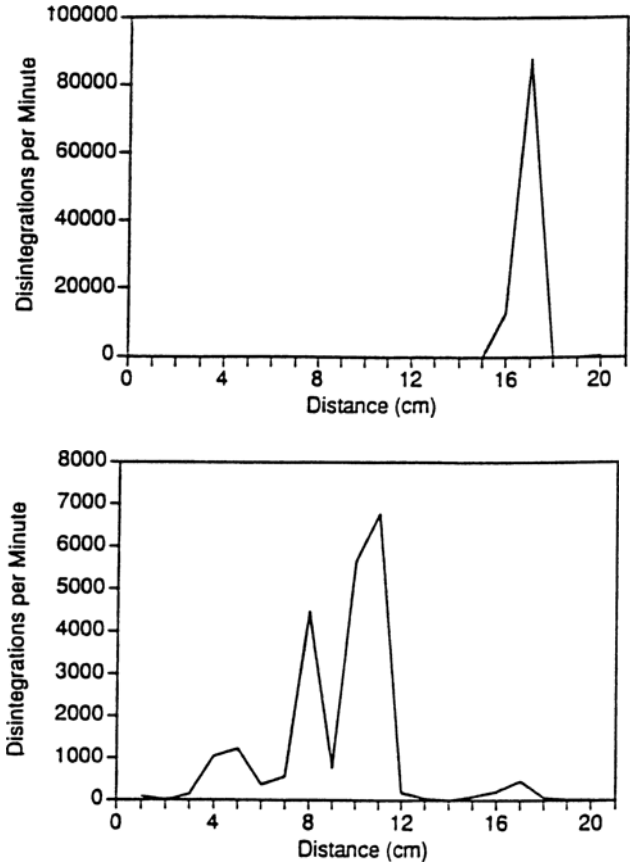


Fig. 7. TLC of [$^{14}$C]-terbufos before incubation (top) and [$^{14}$C] in methylene chloride extract of cultures of *P. chrysosporium* that were incubated with [$^{14}$C]-terbufos for 18 d (bottom).

Table 2
Mass Balance of $^{14}$C-Radioactivity from *P. chrysosporium*
Incubated with $^{14}$C-chlorpyrifos, $^{14}$C-fonofos, and $^{14}$C-terbufos for 21 D[a]

| Compound | %Recovery | Methylene chloride fraction | Aqueous fraction | Particulate | Organic trap | Rubber tubing | $^{14}CO_2$ |
|---|---|---|---|---|---|---|---|
| Chlorpyrifos | 61.1 | 15.1 | 5.5 | 0.4 | 4.0 | 1.7 | 34.4 |
| Fonofos | 78.3 | 10.9 | 50.0 | 1.0 | 0.4 | 0.3 | 15.7 |
| Terbufos | 74.3 | 6.6 | 26.2 | 2.5 | 2.9 | 1.3 | 34.8 |

[a]The initial concentrations of chlorpyrifos, fonofos, and terbufos were 1, 7.1, and 0.14 μM, respectively. All data are expressed as percent.

## DISCUSSION

Organophosphorus insecticides are useful because they are relatively inexpensive, fairly simple to manufacture, and effective against a wide variety of insect pests (*1,2*). As a group, they are relatively quickly degraded in the environment (*1,2*). A number of organophosphorous insecticides do, however, have half-lives on the order of several months and may be thought of as moderately persistent. For example, in a nonsterile Sultan silt loam soil incubated with 50 nmol of [$^{14}$C]-chlorpyrifos for 8 m, 26.1% of the radioactivity was recovered as undegraded [$^{14}$C]-chlorpyrifos, 33% was recovered as [$^{14}$C]-3,5,6-trichloro-2-pyridinol, 33% was not extractable from the soil, 1.1% was converted to unidentified water-soluble metabolites, and 6.6% was degraded to [$^{14}$C]-$CO_2$ (*3*). In other words, after 8 m of incubation, more than 92% of the [$^{14}$C]-chlorpyrifos originally present remained undergraded, as a biologically active metabolite or in a form that was tightly bound to soil.

The biodegradation of organophosphorous insecticides has been studied in soils and in bacterial cultures (*1–4*). Less attention has been given to the biodegradation of these compounds by fungi. Fenitrothion (phosphorothioic acid *O,O*-dimethyl *O*-[3-methyl-4-nitrophenyl] ester) is, however, known to be degraded by *Trichoderma viride, Mortierella isabellina* and *Saprolegnia parasitica* (*13*). The present study showed that *P. chrysosporium* degraded [$^{14}$C]-chlorpyrifos, [$^{14}$C]-fonofos, and [$^{14}$C]-terbufos to [$^{14}$C]-$CO_2$. *T. viride, M. isabellina*, and *P. chrysosporium* are members of the Deuteromycotina, the Zygomycotina, and the Basidiomycotina, respectively, in the Division Amastigomycota, whereas *Saprolegnia parasitica* is a member of the Division Mastigomycota. Although limited, these results suggest that the ability to degrade organophosporous insecticides may be widespread or at least relatively common among the fungi.

Compared to truly persistent environmental pollutants, such as DDT, dieldrin, and chlordane, organophosphorous insecticides in most soils do not present bioremediation problems. Exceptions might exist in cases of inadvertent overapplication, or by repeated application and accumulation in soils containing inordinately low levels of microbial activity. In these cases, it would be logical to amend such soils with microorganisms having the documented ability to degrade the organophosphorous compound in question. If *P. chrysosporium* were used, a suitable, inexpensive growth substrate would also likely be required, because this fungus typically does not use xenobiotics as growth substrates. Growth of *P. chrysosporium* and degradation of environmentally persistent organopollutants in soils have been demonstrated using several inexpensive plant residues, including ground corn cobs, wood chips, peat, and wheat straw (*14,15*).

The ability in lignin-degrading fungi, such as *P. chrysosporium*, to degrade organophosphorous insecticides may be important from another perspective. Pesticide residues or their metabolites are often incorpor-

ated into the insoluble fraction of plants and sls. Some of this insoluble material is undoubtedly lignin or lignin-derived material. Such immobilization represents a form of pesticide inactivation and is sometimes a goal of bioremediation efforts. The immobilized material must eventually undergo turnover and further degradation. Indeed, proponents of immobilization techniques emphasize that release of bound pollutants is expected to occur slowly and at levels that are environmentally insignificant (*16*). *P. chrysosporium* can degrade chlorinated anilines covalently incorporated into lignin (*10*). Thus, in soils, lignin-degrading fungi might accelerate degradation of organopollutants that are covalently bound to lignin or lignin-derived material. This technology is promising, but the microbial ecology and biodegradative ability of lignin-degrading fungi in soil require further scrutiny.

## ACKNOWLEDGMENTS

This research was supported by a Cooperative Research and Development Agreement (C9100300/01) between the Department of Energy (Argonne National Laboratory) and the University of Notre Dame, and by Grant ES 04922 from the National Institute of Environmental Health Sciences.

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