Degradation of Chlorophenolic Compounds by *Trichoderma harzianum* Isolated from Lake Bonney, South-Eastern South Australia

J. A. van Leeuwen,[1] B. C. Nicholson,[1] K. P. Hayes,[1] and D. E. Mulcahy[2]

[1]Australian Water Quality Centre, Private Mail Bag 3, Salisbury, South Australia 5108, Australia

[2]School of Chemical Technology, University of South Australia, The Levels, South Australia 5098, Australia

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ABSTRACT: In a study of the fate of chlorophenolic compounds in Lake Bonney, South-Eastern South Australia, several isolates of the fungus *Trichoderma harzianum* were assessed for their capacities to metabolize these compounds. Lake Bonney receives effluent from two pulp mills, one of which used molecular chlorine for bleaching of pulp between 1966 and September 1991. Effluent and waters of the lake had previously been found to be contaminated with chlorophenolic compounds, including 2,4,6-trichlorophenol and chloroguaiacols. Isolates of *T. harzianum* were obtained from lake water samples collected at locations approximately 10 m, 1, 5, and 9 km from the discharge point of effluent to the lake. The capacity of one isolate (no. 1) collected 10 m from the discharge point, was assessed based on substrate loss in culture media, a corresponding reduction in adsorbable organic halogens (AOX), production of $^{14}CO_2$ from a ^{14}C-labeled chlorophenol, and the release of chloride ions from the dehalogenation of tetrachloroguaiacol. The capacities of several other isolates were briefly assessed based on reductions in concentrations of spiked tetrachloroguaiacol in culture medium and the corresponding AOX.

Trichoderma harzianum (No. 1) mineralized a minor percentage (2–3%) of spiked radiolabeled pentachlorophenol and partially dehalogenated (46%) spiked tetrachloroguaiacol in mineral salts medium. All isolates were found capable of reducing concentrations of tetrachloroguaiacol in a mineral salts medium and the corresponding AOX. Those with the highest capacities were obtained from locations close to the effluent discharge point to the lake.

The presence of this fungus in lake water may account, in part, for the decline in chlorophenolic compounds in Lake Bonney after chlorination at the pulp mill had ceased. Environ Toxicol Water Qual **12:** 335–342, 1997

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INTRODUCTION

The capacities of fungi to degrade halogenated aromatic compounds have been studied in order to determine the fate of these compounds in the environment, for pollution amelioration and wastewater management. Chlorophenolic compounds are important pollutants in effluents of pulp and paper mills and include those that are by-products of chlorine bleaching of pulp (e.g., chloroguaiacols and chlorocatechols) and fungicides used in wood preservation (e.g., 2,3,4,6-tetrachlorophenol and pentachlorophenol). Many studies of the

Correspondence to: J. A. van Leeuwen

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degradation of chlorophenolic compounds by fungi, particularly the basidiomycetes, have been reported. *Phanerochaete chrysosporium* has been shown to degrade 2,4,5-trichlorophenol (Joshi and Gold, 1993) and the degradation of other chlorophenolics by white-rot fungi have been listed by Field *et al.* (1993).

Fungi from other classes have also been reported capable of degrading chlorophenolic compounds. Cserjesi and Johnson (1972) reported the degradation of pentachlorophenol by *Trichoderma virgatum* and Seigle-Murandi *et al.* (1992) reported the degradation of this compound by various species from the class Zygomycetes. Monochlorophenols in spent bleaching effluent were found to be degraded by *Aspergillus* sp., *Aureobasidium* sp., and *Penicillium* sp. (Milstein *et al.*, 1991), and *Aspergillus* degraded pentachlorophenol to tetrachlorodihydroxybenzenes and their methyl ethers (Crosby, 1981).

Phanerochaete chrysosporium and other basidiomycete fungi produce extracellular peroxidases (lignin peroxidases and manganese-dependent lignin peroxidases) which are believed to be involved in the degradation of lignin (Bonnarme and Jeffries, 1990). These extracellular enzymes have also been shown to catalyze the oxidative dechlorination of 2,4,5-trichlorophenol (Joshi and Gold, 1993).

Extracellular peroxidase has been reported to be formed by an isolate of *Trichoderma harzianum* (Freitag and Morell, 1992). However, *Trichoderma* are not ligninolytic organisms (Milstein *et al.*, 1983) and non-ligninolytic microorganisms degrade aromatic xenobiotic compounds intracellularly (Field *et al.*, 1993).

The growth of *Trichoderma* in media with different molecular weight fractions of lignocarbohydrate complexes has been studied by Milstein *et al.* (1983). In media containing a low molecular weight fraction, some decrease in phenolic components was found, whereas in media with a high molecular weight fraction the phenolic part remained intact. This suggests that the degradation of phenolic compounds by *Trichoderma* occurs intracellularly following diffusion of the substrate into the fungal cells.

Chlorophenolic compounds have been found to be widely distributed in the receiving environments of pulp and paper mills (Paasivirta *et al.*, 1980, 1988a, 1988b, 1990; Fogelqvist *et al.*, 1986; Xie *et al.*, 1986; Carlberg *et al.*, 1987; De Sousa *et al.*, 1988; Grimvall *et al.*, 1991, Särkkä *et al.*, 1993). These compounds have also been found to be widely distributed in Lake Bonney, South-Eastern South Australia, which is the receiving environment of a sulphite pulp mill that used chlorine bleaching from 1966 until 1991 (van Leeuwen *et al.*, 1993). Lake Bonney is the largest natural freshwater lake in South Australia with a volume of about 2×10^8 m^3. It is about 23 km long and up to 4.5 km wide, and has a maximum depth of 3.6 m. Concentrations of free chlorophenolics present in the water phase of the lake were found to have declined rapidly following discontinuation of chlorobleaching, while concentrations of bound chlorophenolics declined slowly (van Leeuwen *et al.*, 1996). In assessing the fate of chlorophenolic compounds in Lake Bonney, fungi were isolated from the lake and studied for their capacities to degrade chlorophenolic compounds. In this paper, we report the capacity of *T. harzianum* isolates from Lake Bonney to metabolize several chlorophenolic compounds, including those that had been detected in lake water.

MATERIALS AND METHODS

Chemicals

Eleven chlorophenolic compounds, including one internal standard, were used. These were 4,5-dichloroguaiacol (4,5-DCG), 3,4,5-trichloroguaiacol (3,4,5-TrCG), tetrachloroguaiacol (TeCG), 4,5-dichlorocatechol (4,5-DCC), 3,4,5-trichlorocatechol (3,4,5-TrCC), tetrachlorocatechol (TeCC), 3,4,5-trichloroveratrole (3,4,5-TrCV), tetrachloroveratrole (TeCV), 2,4-dichlorophenol (2,4-DCP), 2,4,6-trichlorophenol (2,4,6-TrCP), and pentachlorophenol (PCP); 2,3,6-trichlorophenol (2,3,6-TrCP) was used as an internal standard. Chlorinated guaiacols, chlorinated veratroles, and tetrachlorocatechol were purchased from Helix Biotech, Corp., Canada. Other chlorophenols were already held by the State Water Laboratory, S.A. or purchased locally. Purchased standards were of 98 to $>99\%$ purity.

Radiolabeled (^{14}C) pentachlorophenol (2.65 mCi/mmol) was obtained from Sigma, Missouri, USA. 4,5-dichlorocatechol and 3,4,5-trichlorocatechol were prepared at the University of South Australia following a procedure described by Neilson *et al.* (1983) and were of greater than 95% purity. Stock solutions of the standards were prepared by dissolving 0.01–0.02 g of each chlorophenolic compound in 50 mL of isopropanol [high performance liquid chromatography (HPLC) grade]. Reagents used were analytical grade unless otherwise stated and solvents were analytical, HPLC or pesticide grade.

Analysis for Chlorophenolic Compounds

Chlorophenolic compounds were analyzed using a method described by Starck *et al.* (1985) with modifications. Aliquots (1 mL) of medium were diluted to

25 mL in Milli-Q water (Millipore, Australia) and adjusted to pH 9 with dilute NaOH. These were spiked with an internal standard (2,3,6-TrCP, 0.545 μg in 0.25 mL isopropanol) and chlorophenolics were acetylated by the addition of 72% potassium carbonate (0.5 mL) and acetic anhydride (0.5 mL). Acetylated compounds were extracted into hexane (2.5 mL) and analyzed by gas chromatography with electron capture detectors (GC-ECD). A Varian 3500 gas chromatograph equipped with two columns of different polarities linked to one injector and two electron capture detectors was used. Columns used were a 30 m $\times$ 0.25 mm i.d. DB5, and a 30 m $\times$ 0.25 mm i.d. DB-1701 (J and W Scientific). The carrier gas was hydrogen (ultra high purity, 1–2 mL/min) and the makeup gas nitrogen (high purity, 30 mL/min). Chromatography column conditions were 60°C for 3 min, 10°C/min to 120°C, 5°C/min to 250°C, and 250°C for 3 min. The injection temperature was 250°C, the detector temperature was 300°C and the split ratio 9 : 1.

Analysis for Adsorbable Halogenated Organic Compounds (AOX)

AOX analysis was performed using the Scan W9:89 method (Scandinavian Pulp, Paper and Board Testing Committee, 1989) with a Euroglas organic halogen analyzer, Model ESC 1000 (Euroglas BV, Delft, The Netherlands). For determination of the halogen content in fungal mycelia, mycelia were washed with Milli-Q water and the halogen content determined using the AOX procedure.

Culture Media

Mineral Salts Medium (MSM)

The constituents of this medium were Na_2HPO_4 (2.4 g), $MgSO_4 \cdot 7H_2O$ (0.1 g), $CaCl_2 \cdot 2H_2O$ (0.0135 g), NH_4NO_3 (0.1 g), and KH_2PO_4 (2.0 g) per liter of distilled water, supplemented with 0.1% (v/v) of a trace element stock solution and 1% of a yeast extract solution (1 g/100 mL distilled water) (Oxoid, Australia, Cat. No. L21). The trace element stock solution consisted of $ZnSO_4 \cdot 7H_2O$ (1.44 g), $MnSO_4 \cdot 4H_2O$ (1.12 g), $CuSO_4 \cdot 5H_2O$ (0.25 g), $CoSO_4 \cdot 7H_2O$ (0.28 g), and boric acid (0.06 g) distilled water (1 L).

Rose Bengal-chloramphenicol broth (RBCB) was also used. The constituents of this medium was the same as for Rose Bengal-chloramphenicol agar except that agar was not included.

Media were sterilized by autoclaving at 121°C for 30 min.

Isolates of *T. harzianum*

Trichoderma harzianum (isolate 1) was obtained from a water sample collected 10 m from a drain (Drain 48) that discharges mill effluent into the lake. Other isolates of *T. harzianum* were obtained from samples collected at approximately 1 km (isolates 2 and 3), 5 km (isolates 4 and 5), and 9 km (isolate 6) from Drain 48.

Experiments Examining the Capacities of *T. harzianum* Isolates from Lake Bonney to Degrade Chlorophenolic Compounds

Assessment for the Capacity of *T. harzianum* (Isolate 1) to Reduce Concentrations of Chloroguaiacols and 2,4,6-Trichlorophenol in Culture Media

(1) Aliquots (15 mL) of Rose Bengal-chloramphenicol broth and mineral salts medium held in McCartney bottles were spiked with tetrachloroguaiacol (2.8 mg/L), inoculated with *T. harzianum* (isolate 1, 0.1 mL of 2 day culture, 15 mL RBCB) and cultured at 20°C for 36 days without agitation. Aliquots of inoculated and control medium (1 mL) were aseptically subsampled during a culture period of 36 days and analyzed for free chlorophenolic compounds and metabolites.

(2) Aliquots (15 mL) of MSM spiked with TeCG (2.9 mg/L) were inoculated with *T. harzianum* [isolate 1; mycelia, equivalent to 0.05 mg dry weight (e.d.w.)] and cultured for 11 days in sterile polystyrene jars (70 mL, Disposable Products, Australia). Subsamples of medium (1.0 mL) were analyzed for chlorophenolic compounds at the start of the experiment and on day 11. Medium was analyzed for AOX at the end of the culture period and the halogen content in mycelia was determined as described.

Dry weights of fungal mycelia were determined following 5 washes with Milli-Q water and heating to constant weight at 70°C.

(3) Aliquots (15 mL) of MSM were spiked with either 2,4,6-TrCP (1.38 mg/L), 4,5-DCG (2.7 mg/L), or 3,4,5-TrCG (1.5 mg/L), inoculated with *T. harzianum* (0.05 mg e.d.w.) and cultured for 20 days in sterile polystyrene jars at 20°C. Subsamples of medium (1 mL) were analyzed for chlorophenolic compounds at the start of the experiment and on day 20. Medium and mycelia were analyzed for AOX at the end of the culture period.

Assessment of *T. harzianum* (Isolate 1) to Mineralize Pentachlorophenol

(1) Aliquots (15 mL) of MSM were spiked with pentachlorophenol (PCP, 1.4 mg/L), inoculated with mycelia (0.08 mg e.d.w.) and incubated for 20 days.

Analyses for PCP in inoculated and control media were performed at the start of the experiment and after culturing for 20 days. AOX analysis was performed after culturing for 20 days.

(2) Aliquots (100 mL) of MSM were spiked with PCP (1.04 mg/L) and ^{14}C-PCP (132,400 counts min^{-1}; measured in 2.0 mL Permafluor E+, [1,2,4-trimethylbenzene (90%) and propyleneglycol(mono)methyl ether (10%), Canberra Packard Pty Ltd., Australia]) and inoculated with mycelia (0.2 mg e.d.w.) of *T. harzianum*. The medium was held in Erlenmeyer flasks (250 mL) into which sterile air (filtered, 0.2 μm, Minisart, Sartorius, Germany) was periodically purged. Expelled air from the culture flasks was slowly bubbled through Carbo-Sorb E (2 × 4 mL, 3-methoxypropylamine, Canberra Packard Pty Ltd., Australia) and 2 M NaOH (2 mL). At the end of the culture period, ^{14}C in the carbon dioxide traps of the inoculated and control flasks were determined.

(3) Aliquots (10 mL) of MSM were spiked with PCP (2.04 mg/L) and ^{14}C PCP (132,400 counts min^{-1}; measured in 2.0 mL Permafluor E+) and inoculated with mycelia (0.04 mg e.d.w.) of *T. harzianum*. The medium was held in sealed McCartney bottles into which sterile air (filtered, 0.2 μm, Minisart, Sartorius, Germany) was periodically purged. Expelled air was bubbled through Carbo-Sorb E (2 mL, ×2) and 2 M NaOH (6.0 mL). Radiolabeled carbon was determined in carbon dioxide traps, in mycelia, and in the culture medium.

Cultures were held at ambient temperatures for a minimum of 20 days.

Radiolabeled carbon was measured using a Packard 2200CA Tricarb scintillation counter with ^{14}C counting efficiency at 85–90% and window settings as follows: lower limit 4.0 keV, upper limit 156 keV.

Radiolabeled carbon was determined in culture medium (0.5 mL) following mixing with isopropanol (2 mL) and 1 mL of Permafluor E+. The presence of ^{14}C in a sodium hydroxide solution (2 M) used to trap $^{14}CO_2$ was determined using the above procedure. Carbo-Sorb E (2 mL) used to trap $^{14}CO_2$ was added to Permafluor E+ (2 mL) and the ^{14}C measured. Radiolabeled carbon in mycelia was determined after washing them with isopropanol and then placing these in isopropanol (1 mL) with Permafluor E+ (3 mL).

Assessment for the Capacity of *T. harzianum* (isolate 1) to Dehalogenate Tetrachloroguaiacol

A mineral salts medium (MSM-Cl) was prepared as previously described except that $CaCl_2$ was replaced by $CaSO_4$. MSM-Cl (50 mL) was initially spiked with TeCG at 8.6 mg/L and inoculated with *T. harzianum* (isolate 1, mycelia 0.1 mg e.d.w.). Aliquots of medium (200 μL and 10 mL) were analyzed for chlorophenolic compounds and chloride ion concentrations, respectively, on days 4, 10, and 15. After 3 weeks, the cultures were spiked twice with TeCG (0.13 mg in 0.2 mL isopropanol), 3 days apart; in the fourth week, all cultures were supplemented with fresh MSM-Cl (25 mL) to encourage continued fungal growth. After a total culture period of 54 days, aliquots (25 mL) of medium were filtered (0.45 μm filters, cellulose nitrate, Sartorius, Germany), each rotary evaporated at 50°C to 5 mL and their chloride ion concentrations determined. Analysis for chloride ion concentrations were determined by potentiometric titration (Clesceri *et al.*, 1989).

Assessment of *T. harzianum* (Isolate 1) to Form Extracellular Peroxidase

Aliquots (15 mL) of MSM were spiked with 4,5-DCG (3.0 mg/L), 3,4,5-TrCG (1.2 mg/L) and TeCG (1.4 mg/L), inoculated with *T. harzianum* (mycelium, 0.1 mg e.d.w.) and cultured for one month. Analyses for chlorophenolic compounds and extracellular peroxidase were performed at the start of the experiment and each week of the culture period. The presence of peroxidase in cell-free medium (filtered, 0.45 μm, cellulose nitrate, Sartorius, Germany) was determined using the method described by Mustranta (1987) with minor variation. Cell-free extract (60 μL) was added to the substrate 2,2′-azino-di-[3-ethylbenzothiazoline-6-sulphonic acid] (20 mM, 0.7 mL) and 0.05 M glycine-HCl buffer, pH 3 (2.2 mL). The reaction was started by the addition of 0.03% hydrogen peroxide (0.1 mL). Enzyme activity was determined by measuring the mean rate of increase in absorbance at 436 nm over 30 min.

Assessment of Other Isolates of *T. harzianum* to Reduce Concentrations of Chloroguaiacols in a Culture Medium

Aliquots of MSM (15 mL) were spiked with TeCG (2.85 mg/L) and inoculated with *T. harzianum* isolates: Nos. 2, 3, 4, 5, or 6 (mycelia, 0.04–0.08 mg e.d.w.). Analysis for chlorophenolic compounds was performed at the start of the experiment and in the third and fifth week of culture. AOX analysis was performed on medium and on mycelia at the end of the experiment.

All experiments, except the first, were performed in duplicate or triplicate. The first experiment was subsequently repeated and similar results were obtained.

RESULTS AND DISCUSSION

Trichoderma harzianum (isolate 1) reduced concentrations of TeCG in MSM and in RBCB (Fig. 1) and predominantly O-methylated this compound in RBCB (Fig. 2). Although TeCG was not detected in RBCB

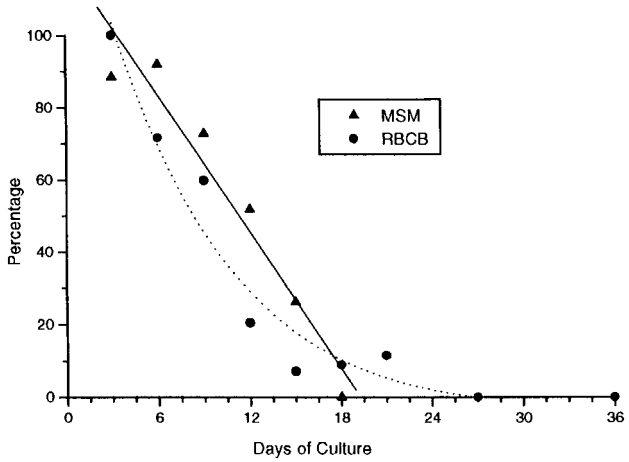

Fig. 1. Percentage of spiked tetrachloroguaiacol detected in MSM and RBCB inoculated with *T. harzianum*.

by day 28, the concentration of tetrachloroveratrole (TeCV) continued to increase to day 36. Subsampling of RBCB for analysis of chlorophenolics could not be done after day 36 due to overgrowth of the fungus. The increase in concentration of TeCV in RBCB from day 28 to day 36 may have been due to its continued release after the complete uptake of TeCG by the fungus. O-methylation of TeCG occurred to a relatively high degree in RBCB with a maximum concentration of 1.6 mg/L of TeCV being detected from an initial concentration of 2.8 mg/L TeCG. In the mineral salts medium, production of TeCV occurred only to a small degree. The greater amount of O-methylation of TeCG in the nutrient rich medium (RBCB) may have been due to a reduced need for the utilization of this compound as an energy source.

Cserjesi and Johnson (1972) found that *T. virgatum* methylated pentachlorophenol to pentachloroanisole in a 2% malt extract medium, although the formation of

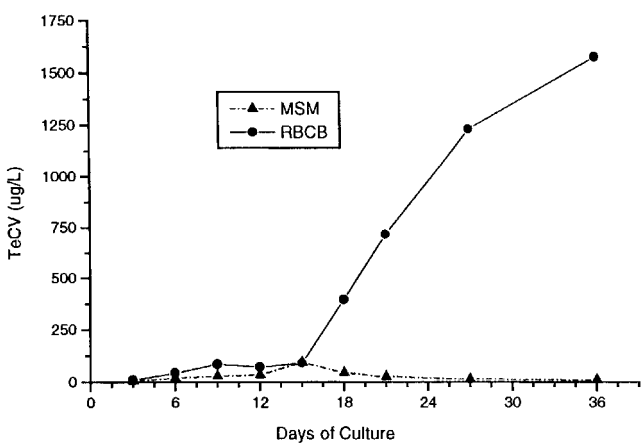

Fig. 2. Formation of TeCV by *T. harzianum* in MSM and RBCB spiked with tetrachloroguaiacol at 2.8 mg/L.

pentachloroanisole did not account for the total reduction in concentration of pentachlorophenol in the medium. These authors suggested that the O-methylation reaction is only the first step in the metabolism of pentachlorophenol or is a parallel reaction to degradation. Cserjesi and Johnson (1972) also found pentachloroanisole to be much less toxic than pentachlorophenol to *T. virgatum*, *Penicillium* sp., and fish in laboratory toxicity tests. In contrast, Allard *et al.* (1988) found O-methylated metabolites of 3,4,5-trichloroguaiacol and tetrachloroguaiacol to be at least as toxic as their precursors using a Zebra fish embryo/larvae test. These authors found bacteria that carry out O-methylation of chloroguaiacols were widely distributed in the environment and postulated that O-methylation of chloroguaiacols occurs under natural conditions.

In MSM spiked with TeCG and inoculated with *T. harzianum* (isolate 1), a high reduction (91%) in AOX was found in cultures after 11 days. Halogen associated with mycelia (1.4%) was negligible compared to the amount spiked, showing that adsorption to mycelia did not account for the reduction of TeCG in the medium.

The high percentage (98%) of TeCG detected in the control samples after 11 days showed that this compound was stable in MSM under the culture conditions used. The mean concentration of AOX (1425 μg/L) found in controls on day 11 was 95% of the total AOX from spiked TeCG. The procedure used to determine the concentration of TeCG also enabled detection of lower chlorinated guaiacols and catechols. However, these compounds and 3,4,5-trichloroveratrole were not detected in any cultures of *T. harzianum* spiked with TeCG. This suggests that they are not metabolic intermediates or are intermediates that are rapidly degraded.

Trichoderma harzianum (isolate 1) decreased concentrations of other chlorophenolics spiked into MSM [compounds that had previously been detected in Lake Bonney waters (Table I)]. Percentages of chloroguaiacols and 2,4,6-TrCP remaining in inoculated MSM were 3.5% or less of the original spiked concentrations after 20 days of culture. Concentrations of AOX in inoculated medium were similarly reduced and halogens were not detected in mycelia. The spiked compounds were found to be stable in MSM controls under the culture conditions used.

Metabolism of PCP in mineral salts medium (MSM) by *T. harzianum* (isolate 1) was initially determined based on GC-ECD and AOX analyses. After 20 days of culture, the concentration of PCP in MSM had decreased by 97.4%, AOX was not detected in the medium, and halogens were not detected in the mycelia.

Radiolabeled carbon in MSM cultures of *T. harzianum* spiked with ^{14}C PCP was detected in the

TABLE I. Percentages of spiked chlorophenolic compounds and AOX levels detected in a mineral salts medium inoculated with *T. harzianum* (isolate 1) and cultured for 20 days[a]

Compounds	%	AOX (μg/L)	% AOX in Mycelia (mg e.d.w.)	Total AOX % Reduction
2,4,6-TrCP				
inoculated	nd	8	nd	98.4
Control	87.0	494	(0.8)	
4,5-DCG				
inoculated	3.5	72	nd	91.9
Control	101.8	883	(1.7)	
3,4,5-TrCG				
inoculated	nd	92	nd	84.1
Control	108.9	578	(1.2)	

[a] nd: not detected.

carbon dioxide traps, in mycelia, and in medium. $^{14}CO_2$ was not detected in 2 M NaOH and minor percentages were detected in Carbo-Sorb E (Table II). A high percentage (43%) of radiolabeled carbon was detected in the medium at the end of the culture period and about 8% of ^{14}C was detected in fungal mycelia. The high percentage of ^{14}C remaining in inoculated MSM and the almost complete reduction in AOX refers that metabolism of PCP by *T. harzianum* (Isolate 1) results in the production of compounds that are not adsorbable to activated carbon, e.g., strongly hydrophilic halogenated compounds, or are adsorbable to activated carbon but are predominantly dehalogenated. A high percentage (46.5%) of the total radiolabeled carbon was not detected in MSM at the end of the culture period and the fate of this is unknown. This loss may be due to the formation of volatile compounds that were not absorbed in the carbon dioxide traps. Dehalogenation of chlorophenolics by *T. harzianum* (isolate 1) is indicated by the assimilation of ^{14}C (8.3%) from ^{14}C PCP and the absence or low detection of halogens in mycelia.

TABLE II. Mean percentages of ^{14}C as trapped $^{14}CO_2$, in mycelia and in mineral salts medium (MSM) spiked with ^{14}C Pentachlorophenol, inoculated with *T. harzianum* (isolate 1) and cultured for a minimum of 20 days[a]

	Percentage		
Exp.	^{14}C as Trapped $^{14}CO_2$	^{14}C in Mycelia	^{14}C in Medium
1	3.2	nt	nt
2	2.2	8.3	43.0

[a] nt: = not tested.

Trichoderma harzianum (isolate 1) dehalogenated spiked TeCG in mineral salts medium, based on chloride ion release into culture medium. After four days of culture, no difference was found in concentrations of chloride ions present in inoculated and control media. On days 10 and 15, chloride ion concentrations in inoculated medium were about 2.5 times those in the control medium. The maximum concentration of chloride ions (14.4 mg/L on day 54) detected in inoculated medium (concentrated) was markedly higher than in concentrated control medium, (2.5 mg/L). This maximum concentration constitutes about 46% of the organically bound chlorine of tetrachloroguaiacol spiked into MSM. The incomplete dehalogenation of tetrachloroguaiacol by the fungus and the reduction in AOX in the culture medium suggests that metabolic by-products may include strongly hydrophilic halogenated compounds.

Extracellular peroxidase was not detected in MSM spiked with chlorophenolic compounds, inoculated with *T. harzianum* (isolate 1) and cultured for one month at 20°C. At the end of the culture period, 4,5-DCG was detected (5.3%) while 3,4,5-TrCG and TeCG were not detected. The lack of detection of extracellular peroxidase in MSM indicates that the degradation of chlorophenolics by *T. harzianum* (isolate 1) occurs by intracellular metabolism.

Isolates of *T. harzianum* obtained from waters collected at approximately 1, 5, and 9 km from the discharge point of effluent to Lake Bonney were also capable of reducing concentrations of TeCG in MSM, with those isolated from locations close to the discharge point showing the greatest capacities (Table III). Reductions in AOX in the inoculated medium correlated with reductions in concentrations of TeCG. In all cultures, only small percentages (0.2–0.9%) of halogens were associated with fungal mycelia.

Lake Bonney is a shallow lake and is subjected to strong coastal winds that result in its waters being well mixed. It could be expected that isolates of varying capacities would be dispersed throughout the lake. However, initial concentrations of free chlorophenolics discharged into the lake when chlorobleaching was still being practiced would have been highest in the immediate receiving waters of the mill's effluent. Concentrations would have rapidly declined along the lake's length by adsorption to sediments and possibly by adsorption to suspended particulate matter and their degradation by microorganisms. The occurrence of isolates with greater capacities to metabolise chlorophenolics is likely to have been greatest at locations close to the effluent discharge point to the lake.

Rapid reductions in concentrations of free chlorophenolic compounds had occurred in Lake Bonney

TABLE III. Mean percentages of spiked TeCG and AOX levels in a mineral salts medium inoculated with various isolates of *T. harzianum* from Lake Bonney[a]

	Percentage of TeCG		AOX μg/L MSM (% red.)	%AOX in Mycelia (mg e.d.w.)
Isolate	Week 3	Week 5	Week 5	
2	nd	nd	63 (94.9)	0.9 (3.3)
3	nd	nd	96 (92.8)	0.7 (3.0)
4	83	73	993 (31.9)	0.2 (1.4)
5	82	78	1051 (28.0)	0.2 (2.0)
6	69	64	862 (40.3)	0.8 (2.2)
Control	114	98	1463	—

[a] nd: not detected.

waters following the discontinuation of chlorine bleaching at the mill (van Leeuwen *et al.*, 1993). However, low concentrations of free chloroguaiacols were found to persist in the water phase for one year. The rapid reductions in concentrations of free chlorophenolics may have been due to adsorption to sediments, as shown by Allard *et al.* (1988) and also by their degradation by *T. harzianum* and other microorganisms. The persistence of low concentrations of free chlorophenolics in lake water after chlorination had ceased was probably due to the slow release of compounds bound in sediments and in the water phase (van Leeuwen *et al.*, 1996). In the water phase, chlorophenolics may have been bound to particulate matter and with other dissolved organics, as micellar aggregates. Jokela and Salkinoja-Salonen (1992) found that by diluting an AOX fraction (> 10,000 MW, obtained by ultrafiltration of bleached Kraft mill effluent), by 100-fold with distilled water, the subsequent retention of AOX in repeated ultrafiltration decreased to one-fourth. According to these authors, chlorinated material present in bleached Kraft mill effluent may occur as micellar aggregates that can dissociate to low molecular weight compounds when the effluent is diluted in distilled water. If chlorophenolic compounds are bound with other organics in pulp and paper mill effluent, then these may be released to their free form following dilution of the effluent in receiving environments such as rivers and lakes. In bound form, the bioavailability of chlorophenolic compounds is reduced (Kookana and Rogers, 1995), which may result in them persisting in the environment for long periods. The release of bound chlorophenolic compounds to their free form is likely to make them susceptible to degradation by microorganisms that are nonligninolytic, such as *T. harzianum*.

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