Fungal Inoculum Properties: Extracellular Enzyme Expression and Pentachlorophenol Removal by New Zealand *Trametes* Species in Contaminated Field Soils

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This study was conducted to improve the ability of indigenous New Zealand white-rot fungi to remove pentachlorophenol (PCP) from contaminated field soil. The effects of different bioaugmentation conditions on PCP removal and extracellular enzyme expression were measured in the laboratory. The conditions were fungal growth substrate and co-substrate composition, culture age, and Tween 80 addition to the contaminated soil. The fungi used were *Trametes versicolor* isolate HR131 and *Trametes* sp. isolate HR577. Maximum PCP removal was 70% after 7 wk from a 1043 mg kg^{-1} PCP-contaminated soil inoculated with an 11-d-old fungal culture of *T. versicolor* isolate HR131. There was minimal production of undesirable pentachloroanisole by the fungi. Tween 80 addition had no affect on PCP removal. Poplar sawdust was more suitable as a fungal growth substrate and a co-substrate amendment for PCP removal and extracellular enzyme expression than the locally available pine and fir sawdust. Pentachlorophenol removal was not necessarily correlated with extracellular enzyme expression. The research results demonstrate that PCP biodegradation was affected by inoculum culture age, by the presence of a co-substrate amendment, and by growth substrate composition after white-rot fungal bioaugmentation into PCP-contaminated field soils.

T HE use of impure technical grade pentachlorophenol (PCP) formulations as biocides for wood treatment has produced a large number of significantly contaminated soils in many countries. Successful field-scale remediation of PCP by white-rot fungi has been demonstrated (Davis et al., 1993; Lamar and Dietrich, 1990; Walter et al., 2005b).

The organisms *Phanerochaete chrysosporium* Burds. and *P. sordida* (Karst.) Erikss. & Ryv. have been used in the majority of white-rot fungal bioremediation studies designed to clean up PCP soils from disused timber treatment facilities (Lamar and Dietrich, 1990; Lamar et al., 1990; Lamar et al., 1993a; Lamar et al., 1993b). These two *Phanerochaete* isolates predominantly express the extracellular enzyme lignin peroxidase. Lignin peroxidase has been reported to degrade a wide range of different anthropogenic organic chemicals (Bumpus and Aust, 1985; Bumpus et al., 1985) and is expressed by the two *Phanerochaete* isolates under nutrient-limiting conditions after the initial rapid growth phase, otherwise known as the stationary phase.

These two fungi are not native to New Zealand, and therefore a screening program was completed to identify suitable white-rot fungal isolates native to New Zealand for their PCP bioremediation potential (Walter et al., 2003). Several fungi of genus *Trametes* were identified as suitable candidates, including *Trametes versicolor* (L.: Fr.) Pilat isolates. *T. versicolor* does not usually express lignin peroxidase and therefore does not degrade lignin as efficiently as *P. chrysosporium* (Leatham and Kent Kirk, 1983). However, among white-rot fungi, *T. versicolor* is one of the most prolific producers of laccase enzyme (Gianfreda et al., 1999). This property has resulted in increasing applications of *T. versicolor* for its PCP bioremediation potential (Lestan and Lamar, 1996; Lestan et al., 1996; Walter et al., 2005a; Walter et al., 2005b).

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Abbreviations: F7, pine sawdust coarse corn grits formulation; F8, pine sawdust kibbled rye formulation; F9, pine sawdust kibbled rye starch formulation; F10, pine sawdust rye flour formulation; F13, poplar sawdust kibbled rye formulation; F14, poplar sawdust corn grits formulation; F15, Douglas fir sawdust corn grits formulation; F26, Douglas fir sawdust; F27, poplar sawdust; IANZ, International Accreditation New Zealand; MEA, malt extra agar; MnP, manganese peroxidase; PCA, pentachloroanisole; PCP, pentachlorophenol; SAFI, co-substrate amendment and fungal inoculum; TPH, total petroleum hydrocarbons; U, one international unit of enzyme as the amount that catalyzes the formation of one micromole of product in 1 min.

Table 1. Composition of sawdust based formulates for fungal inoculum production.

Code	Sawdust†	Grain	Sawdust‡	Grain	Starch	CaCO₃	C/N ratio‡§
				— g kg⁻¹ —			
F7	pine	coarse corn grits	563	387	–	50	85
F8	pine	kibbled rye	563	387	–	50	51
F9	pine	kibbled rye	563	347	40	50	95
F10	pine	rye flour	563	387	–	50	62
F13	poplar	kibbled rye	563	387	–	50	50
F14	poplar	corn grits	750	250	–	–	140
F15¶	fir	corn grits	750	250	–	–	140
F26	fir		–	–	–	–	1013
F27	poplar		–	–	–	–	755

The header "Sawdust–grain fungal growth substrate compositions" spans the Sawdust‡, Grain, Starch, and CaCO₃ columns. Units row "g kg⁻¹".

† Pine = *Pinus radiata*; fir = Douglas fir = *Pseudotsuga menziesii*; poplar = *Populus deltoids*.

‡ Calculated on a dry weight basis and sieved with a 2.80-mm sieve unless stated (2.8-mm aperture, BS410, serial No. 081815); sterilization of substrates was by autoclaving (121°C, 205 kPa, 20 min) final moisture content of formulates before fungal inoculation at 6%. Formulates consisted of 20 g of ingredients and 20 g of water in a 250-mL Erlenmeyer flask (0.1 L scale) sealed with cotton wool plug to maintain sterility unless stated.

§ The C/N of the sawdust and grains was determined at Lincoln University using a LECO, CNS-2000 elemental analyzer.

¶ This sawdust was not sieved. Moisture contents of the sawdust were taken (60°C, 72 h), and moisture levels in the Douglas fir sawdust were adjusted to 62%. The final moisture content after grain and or starch addition was 56%.

There are considerable differences in the approaches taken to study white-rot fungal bioremediation of PCP-contaminated soils. The majority of these studies have incorporated spiking PCP into uncontaminated soil (Lestan and Lamar, 1996; Lestan et al., 1996; Walter et al., 2005a). This approach provides some control over experimental conditions, and co-spiking with ¹⁴C-labeled PCP (Rüttimann-Johnson and Lamar, 1997; Walter et al., 2004) demonstrates PCP mineralization in the soil environment. However, the initial soil contamination in actual field situations may have occurred more than 50 yr ago, and a large portion of the xenobiotic will have aged and may not be available for biodegradation (Luthy et al., 1997). Spiking fresh contaminant into soil has the potential to introduce numerous artifacts into environmental fate and bioremediation experiments (Northcott and Jones, 2000). Examples of these artifacts include sorption to soil organic matter, volatilization, and biodegradation by indigenous soil microbes (Okeke et al., 1993). Although this practice is advantageous for determining specific mechanisms of contaminant behavior in soil (Okeke et al., 1994; Okeke et al., 1997), it is of limited practical value for determining the efficacy of bioremediation processes. Spiking soil with fresh PCP and measuring biodegradation does not simulate the processes predominant in actual field situations (Singleton, 2001). Accordingly, some studies have used aged soil residues from contaminated timber treatment sites (Lamar et al., 1993a; Walter et al., 2005a; Walter et al., 2005b) and have reported such soils as being considerably more toxic and less treatable than soils freshly spiked with PCP.

Only one study has reported that manganese peroxidase was expressed in colonization and biodegradation studies of *T. versicolor* in spiked soils (Lestan et al., 1996). However, there have been no reports of combined laccase and manganese peroxidase (MnP) activity expressed by *T. versicolor* during colonization

and biodegradation of aged PCP residues in field soils. These enzyme assays, or more generic bioassays, have been proposed as alternatives to direct methods of pollutant residue analysis for assessing fungal soil colonization, bioremediation activity, and biodegradation (Singleton, 2001). Therefore, these enzyme assays could provide useful techniques for discriminating the efficacy of different white-rot fungal inoculation conditions on bioremediation potential by measuring enzyme expression over time.

Limited research has been reported on the use of co-substrate amendments to aged contaminant residues as an addition to the bioaugmentation of the fungal inocula. Most studies have reported the use of wood chips or sawdust as a supplement, usually of the hard wood variety such as poplar, oak, or willow (Lamar and Dietrich, 1990; Walter et al., 2005b). One study used alginate-coated pellets (Lestan and Lamar, 1996), with the fungal isolate first colonizing the alginate coat; after bioaugmentation, the fungal mycelium penetrated the interior of the pellet. The material of such a pellet can also be considered an "amendment addition." The choice of the correct amendment or amendments could affect the survival of the fungi for extended periods in soil and enhance biodegradation. Earlier work demonstrated an optimum culture age of 11 d for New Zealand white-rot fungi in colonizing an uncontaminated clay loam soil (Schmidt et al., 2005). However, these data may not apply for soils with aged PCP residue.

The aims of this study were to investigate New Zealand *Trametes* spp. fungal inoculum properties for bioaugmentation into soils contaminated with aged PCP residues. These properties include culture age, inocula composition, co-substrate amendment properties, and whether a co-substrate amendment is necessary under certain conditions. The assessment methods used were PCP residue analysis and assays for the extracellular enzymes laccase, MnP, and lignin peroxidase.

Materials and Methods

Materials

The field soil contaminated with technical grade PCP (aged PCP residue) was obtained from a disturbed environment at a sawmill in the Bay of Plenty region in the North Island of New Zealand. The Temuka clay loam soil ("clay loam") was obtained from Lincoln, New Zealand. The Douglas fir (*Pseudotsuga menziesii* [Mirb.] Franco) and Monterey pine (*Pinus radiata* D. don) sawdust used for fungal inocula production was from a local sawmill, and the poplar sawdust (*Populus deltoides* Batr. Ex Marsh) was from the Hawkes Bay region in the North Island. These were air dried, sieved (2.8 mm aperture; BS410, serial No. 081815), and stored at room temperature. The grains were from local retail outlets. The different grades of corn grits (coarse corn grits and corn grits) were described by the supplier as 320 (corn grits) and 220 mesh (coarse corn grits). Two New Zealand white-rot fungal isolates, *T. versicolor* isolate HR131 and a *Trametes* sp. isolate HR577, were used in all experiments. Both fungi were grown on malt extract agar (Merck, Darmstadt, Germany) at 24 to 29°C for 6 to 7 d. Isolates were

maintained in 20% glycerol (BioLab, Clayton, VIC, Australia) on 1.0-cm-diameter malt extract agar plugs and stored at −80°C. All chemicals used were of analytical grade. The spectrophotometer for the enzyme assays was a Cary 50 Bio UV/VIS spectrophotometer (Varian Australia Pty Ltd). Acetone, hexane, phosphoric acid, methanol, and heptane (Mallinckrodt) were supplied by Biolab New Zealand Ltd. Technical grade PCP was supplied by Sigma Chemical Company (St. Louis, MO)

Methods

Composition and Production of Formulates

The growth substrates used for fungal inocula production for bioaugmentation (formulations F8 and F13) are described in Table 1. The co-substrate amendments (F14, F15, F26, and F27; Table 1), when used, were added to the contaminated soil before fungal inoculum addition. The grains were weighed as they were received from the suppliers. The fungal inocula for bioaugmentation were prepared using the following procedure. Malt extra agar (MEA) was sterilized by autoclaving (121°C, 205 kPa, 20 m) and poured into sterile Petri dishes and allowed to cool. The cooled MEA was inoculated with a 1.0-cm-diameter plug extracted from a MEA agar plate previously inoculated with fungi (<1 mo old) with the mycelial surface facing upward and incubated at 26°C in the dark for 7 d. At the 0.1 L scale, fungal inocula formulates were produced by weighing 20.0 g (air dried weight) of the sawdust–grain–CaCO$_3$ mixes into 250-mL Erlenmeyer flasks and rehydrating by addition of 20.0 g of distilled water to reach 56% moisture content (dry wt. basis, 60°C, 3 d). After hydration and mixing, the formulate flasks were sealed with nonabsorbent cotton wool, covered with a tin foil lid, and sterilized by autoclaving (121°C, 205 kPa, 15 min). Upon cooling, formulates were aseptically inoculated with two 10-mm-diameter mycelial disks extracted from cultures grown on MEA for 2 to 4 wk at room temperature. The prepared flasks were incubated in the dark at 26°C for 7 to 19 d. Moisture was maintained gravimetrically at 7-d intervals. The carbon to nitrogen (C/N) ratio of the sawdust and grains was determined at Lincoln University by a LECO CNS-2000 (Etheridge et al., 1998) elemental analyzer (LECO, St. Joseph, MI)

Unless stated, the following procedure was used to produce the majority of pasteurized co-substrate amendments. Wet Douglas fir sawdust (not sieved), aged <1 mo, was adjusted to 62% moisture content. The final moisture content after grain and/or starch addition was 56%. Each formulate lot (2.04 kg) was sealed in an autoclave bag and sterilized by heat treating in an oven for 5 h at 70°C. The co-substrate amendments were cooled for 43 h before a second heat treatment of 5 h at 70°C and cooled for 24 h before use.

Preparation of PCP–Soil Mix for Bioaugmentation

PCP–Soil Mixes. The contaminated field soil (used as received) and clay loam (air-dried at 23°C) soils were sieved (4.0 mm aperture, BS410/1986, serial No. 5616023, or 2.80 mm aperture, BS410, serial No. 081815). Because of its toxic nature, this contaminated field soil required dilution to enhance fungal survival (Lestan and Lamar, 1996; Lestan et al., 1996; Walter et al., 2005a, 2005b). These two soils were mixed (henceforth known as the PCP–soil mix) in the following proportions: 247 g of clay loam, 36.5 g aged

PCP soil residue, and 60 g distilled water. The dried clay loam was mixed with the aged PCP residue before water was added. The co-substrate for fungal survival and the fungal inoculum for bioaugmentation were then added to this mix (see experiments below for evaluation of these parameters). The prepared mixture was distributed in 157 g lots among three 280-mL containers. The container dimensions were 9.0 × 8.0 × 5.6 cm for the top, base, and depth, respectively, with a 1.0-cm-diameter hole punched in the lid and plugged with cotton wool to provide aeration.

Bioaugmentation Control Cultures. The control cultures for each sieve size (hereafter referred to as PCP conc.) were produced by the addition of 135 g of pasteurized Douglas fir corn grits co-substrate amendment (F15; Table 1) to the PCP–soil mix. Five samples of this material, without fungal inoculum, were analyzed for PCP and lower substituted chlorophenols immediately after mixing (time zero controls). This process was repeated to produce the 7-wk-aged controls without fungal inoculum. The prepared mixture was distributed among three containers (157 g each) for the 4.00-mm sieved soils, and extra PCP–soil mix and co-substrate amendment were produced to provide five containers (157 g each) for the 2.80-mm sieved soils. These containers were incubated for 7 wk (23°C) before PCP and lower substituted chlorophenol analysis.

Compositional Parameters for Bioaugmentation into PCP–Soil Mix. Three different sets of fungal inocula–co-substrate compositional ratios described in the experiments below were prepared for the bioaugmentation treatments into the PCP–soil mix. First, the 135 g of F15-pasteurized co-substrate amendment used in the controls was replaced with 114.5 g of fungal inoculum and 20.5 g of F15 (Table 1) to produce a 15:85 ratio of co-substrate amendment to fungal inocula (SAFI) ratio. This resulted in 38.2 g of fungal inocula per 157 g container. Second, the co-substrate amendment was left out, and only the fungal inoculum was added (114.5 g). The third SAFI ratio was a 50:50 combination of fungal inocula and co-substrate amendment (67.5 g each) added to the PCP–soil mix. This resulted in 22.5 g of fungal inocula per 157-g container.

Experiments for Assessment of Bioaugmentation Parameters

Effect of Fungal Inoculum Culture Age. This experiment was designed to determine the effect of white-rot fungal inoculum culture age on the subsequent colonization of the PCP–soil mix. The culture ages were 7, 9, 11, 15, and 19 d. The two white-rot fungal isolates were grown on the F8 growth substrate (Table 1). The F15 co-substrate amendment was added to the PCP–soil mix before bioaugmentation to provide a 50:50 SAFI ratio. Thirty individual soil microcosm containers of SAFI–PCP–soil mix representing 10 different treatment combinations (composed of five culture ages of the two isolates in triplicate) were prepared as described previously. The prepared microcosms were incubated at 23°C. Sampling for laccase and MnP activity from the soil microcosms was performed as described below. The six PCP–soil mixes inoculated with the 11-d fungal inocula were stored at −20°C after 7 wk of incubation and later analyzed for PCP and lower substituted chlorophenol biodegradation (described below).

A second experiment was conducted to assess the effect of white-rot fungal culture age on colonization of a higher contaminated PCP–soil mix (2.80-mm sieve). The inoculum culture ages

were 9, 11, and 15 d. Two fungal growth substrates (F8 and F13; Table 1) and one fungal isolate (HR131) were assessed. No co-substrate amendment was used before bioaugmentation. Eighteen soil microcosm containers of the fungal inocula–PCP–soil mix from six different treatment combinations (three culture ages for each formulate in triplicate) were prepared as described previously. The soil microcosms were sampled for extracellular enzyme activity as described below, and all 18 containers were stored at −20°C after 7 wk of incubation and later sampled for PCP and lower substituted chlorophenols (described below).

Effect of a Surfactant in Enhancement of Biodegradation of PCP. The aim of this experiment was to increase the bioavailability and thereby enhance the removal of aged PCP in the PCP–soil mix by the addition of the non-ionic surfactant Tween 80 (LabChem, Seven Hills, NSW, Australia), the concentrations being 0, 1, and 2.5 g kg^{-1}. The fungi were grown on the F8 growth substrate (11-d-old cultures). The Tween 80 (1.2 and 0.5 g separately) was added to 60 g of water, which was added to 36.5 g of aged PCP residue before dilution with the clay loam to give the final concentrations quoted.

Eighteen soil microcosm containers of the SAFI–PCP–soil mix from six different treatment combinations (three Tween 80 levels [0, 1, and 2.5 g kg^{-1}] for each isolate in triplicate) were produced as described previously. Sampling for MnP and laccase was performed as described below, and all 18 containers were stored at −20°C after 7 wk of incubation and later sampled for PCP and lower substituted chlorophenols (described below).

Effect of Different Co-substrate Amendments. The aim was to test the effect of different sterile co-substrate amendment (other than F15; Table 1) addition on the production of laccase and MnP after isolate HR577 bioaugmentation into a PCP–soil mix. The fungal inoculum (F8 growth substrate) culture age was 11 d. Twenty-four soil microcosm containers of the fungal inoculum–PCP–soil mix were produced from eight different treatment combinations (a 2^3 factorial design). The factors were grain addition to the co-substrate amendment (corn grits or no corn grits), co-substrate amendment sawdust choice (Douglas fir or poplar), and SAFI ratio (15:85 or 50:50). These sterile co-substrate amendments (F15, F14, F26, and F27) for the different treatments are described in Table 1. These formulates (2.04 kg each) were autoclaved at 117°C for 1 h, rather than being pasteurized, before mixing with the PCP–soil mix.

Effect of Small Variations in Fungal Inocula Formulation. The aim was to assess some compositional changes (to the grain only) in the fungal growth substrate (F8) used in the previous experiments and their effect on the New Zealand *Trametes* isolate colonization of the PCP–soil mix. These new growth substrates, F9 and F10, are described in Table 1. The contaminated field soil and clay loam were sieved with the 2.80-mm screen before mixing. This gave six treatment combinations producing 18 soil microcosms for the two isolates grown on three different growth formulations in triplicate. The SAFI ratio was constant at 15:85. The sterile co-substrate amendment was the Douglas fir corn grits formulate F15. All other experimental design information and production of soil microcosms was as reported previously. Laccase and MnP where assayed from the 18 soil microcosm as described below.

The PCP–soil mixes inoculated with the white-rot fungi grown on the F8 and F9 growth substrates were stored at −20°C after 7 wk of incubation and analyzed for PCP, pentachloroanisole (PCA), and lower substituted chlorophenols (described below).

Corn grits (coarse) was also used as a sawdust supplement instead of kibbled rye (F7; Table 1) for production of fungal inoculum and compared with the F8 growth substrate for colonization of the PCP–soil mix. Triplicate containers were inoculated with 11-d-old *T. versicolor* isolate HR131, F7, and F8 cultures (SAFI = 15:85; Table 1). The fungal soil colonization was assessed visually (3 d) and by laccase and MnP activity (15 d).

Enzyme Assays

Duplicate subsamples of soil-biomass composite (3–5 g) taken with a 1.5-cm-diameter cork borer were extracted with 10 mL of 50 mM malonic acid (BDH Ltd, Palmerston North, New Zealand) at pH 4.5 for 1 h in 25.0-mL universal bottles for the laccase and MnP assays. The universal bottles were hand shaken vigorously three times for 3 s at 20-min intervals during the 1-h extraction procedure to suspend the soil or biomass substrate composite sample and filtered through Whatman No. 5 filter paper (Whatman International Ltd, Maidstone, England). These samples were diluted 10-fold with 50 mM malonate (pH 4.5) immediately before assay measurement. For the MnP assay, 0.5 mM manganese (May and Baker Ltd, Dagenham, England) was added to the diluent. Laccase activity was analyzed as peroxide-independent dimerization of 2,6 dimethoxyphenol (Acros Organics, Geel, Belgium) at λ 469 nm (de Jong et al., 1994). Manganese peroxidase was measured spectrophotometrically by the addition of 0.2 mM H_2O_2 (BDH New Zealand Ltd) by the formation of the Mn III malonate complex at λ 270 nm (Wariishi et al., 1992). Lignin peroxidase was assayed by the standard method (Tien and Kirk, 1983) by oxidation of 3,4 dimethoxybenzyl alcohol (Aldrich, Milwaukee, WI). The Laccase and MnP sampling was performed as part of a multivariate repeated measures factorial design on Days 3, 6, 9, 12, and 15 after bioaugmentation. Lignin peroxidase was assayed on several occasions on Days 12 and 15 after bioaugmentation.

Chemical Analysis

Extraction of Chlorophenols and Anisoles in the Aged PCP Residue. The extraction and analysis of PCP, lower substituted chlorophenols, and their respective ansioles in the aged PCP residue; the extraction and analysis of the former compounds in the aged PCP residue after co-substrate addition; fungal bioaugmentation; and subsequent incubation were completed at HortResearch's International Accreditation New Zealand (IANZ)-certified Food and Biological Chemistry Laboratory under ISO 17025 operating procedures. Briefly, PCP and lower substituted chlorophenol residue analyses were performed by acetone–hexane extraction from the aged field soil in acidic (pH <1.0) conditions (phosphoric acid). The chlorophenols and standards were derivatized with acetic anhydride before analysis by gas chromatography with electron capture detection. Anisole standards were produced from a chlorophenol

mix containing PCP; the three-tetra chlorophenol isomers and four trichlorophenol isomers were derivatized with diazomethane.

Analysis of Other Soil Properties. The total petroleum and diesel hydrocarbons (TPH) were measured by R. J. Hill Laboratories Ltd, New Zealand, which is an IANZ-accredited laboratory. The method was extraction with sonication and quantification by gas chromatography with flame ionization detection (USEPA 8015B) (USEPA, 1986). Analysis of the heavy metals copper, chromium, boron, and arsenic in the aged PCP and pH and organic matter content in the clay loam was completed by R. J. Hill Laboratories Ltd using nitric–hydrochloric acid digestion followed by analysis by inductively coupled plasma spectroscopy–mass spectroscopy (USEPA Method 200.2) (USEPA, 1994).

Data Analysis. The analysis of the repeated measures two- or three-way ANOVA extracellular enzyme data was completed on Genstat 7.1 using restricted maximum likelihood and repeated measures ANOVA for generation of the treatment difference probabilities. The error bars represent the 5% LSD calculated from the standard error of difference among means. The pooled error bounds for the 95% confidence intervals for the factor means from the repeated measures analysis results are given in the figure captions. The PCP residue analysis results (ANOVA) for the different bioaugmentation treatments were tested for statistical significance by the method of Fisher's LSD at the 5 and 10% levels. The ANOVA probabilities and the confidence intervals for the PCP disappearance promoted by the bioaugmentation treatments are also given in the figure captions.

Results

Chlorophenol Levels in Field-Aged Residue Soils and Other Soil Properties

The concentration (mg kg^{-1} dry wt.) of chlorophenols in the PCP-contaminated field soil passed through a 4.00-mm sieve was 6495 ± 1698 for PCP (α = 0.025), 94 ± 24 mg kg^{-1} for 2,3,4,6-tetrachlorophenol, and 3 ± 1.6 mg kg^{-1} for 2,3,4,5-tetrachlorophenol. No trichlorophenols were detected. The concentration of chlorophenols in the soil size fraction passing through a 2.80-mm sieve was 10,743 ± 1259 mg kg^{-1} for PCP and 125 ± 80 mg kg^{-1} for 2,3,4,6 tetrachlorophenol; no 2,3,4,5 tetrachlorophenol or trichlorophenols were detected. The concentration of different petroleum hydrocarbon fractions and TPH in the aged PCP-contaminated soil residue sieved through the 2.80-mm sieve was <80 mg kg^{-1} for C_7 through C_9, 1700 mg kg^{-1} for C_{10} through C_{14}, 14,800 mg kg^{-1} for C_{15} through C_{36}, and 16,400 mg kg^{-1} for TPH. Copper chromium, boron, and arsenic were measured at concentrations of 23, 12, <20, and 10 mg kg^{-1}, respectively, on a dry weight basis. The addition of sterile Douglas fir corn grits to the PCP–soil mixes described previously resulted in average PCP conc. (95% confidence interval) of 697 ± 283 mg kg^{-1} (4.0-mm sieve) and 1043 ± 97 mg kg^{-1} (2.80-mm sieve). The 7-wk aged control PCP conc. were 653 ± 380 mg kg^{-1} (4.0-mm sieve) and 1045 ± 111 mg kg^{-1} (2.80-mm sieve). The pH of the clay loam soil and the PCP-contaminated field soil was 5.6 and
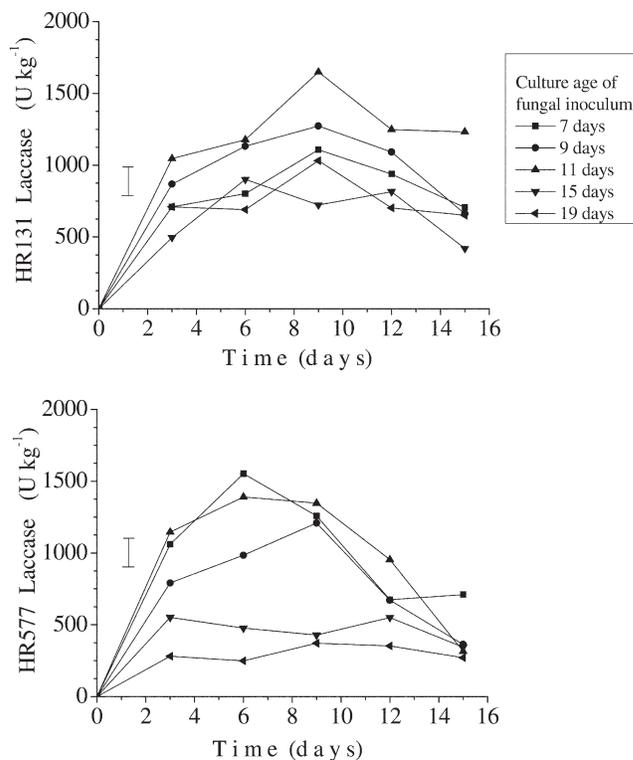

Fig. 1. Laccase activity from the white-rot fungi *Trametes versicolor* HR131 and *Trametes* sp. HR577 after bioaugmentation at various culture ages into a 697 mg kg^{-1} (dry wt.) pentachlorophenol-contaminated field soil. Error bar is the 5% LSD calculated from the SED. Pooled 95% error bounds for the repeated measures ANOVA factor means (U kg^{-1}): culture age main effect ±140; time and isolate effect ±130; culture age and isolate effect ±190.

4.9, respectively; the organic matter content of the clay loam soil was 68 g kg^{-1} (dry wt.).

Effect of Culture Age

White-rot fungal culture age of 11 d provided optimal laccase and MnP expression ($P < 0.001$) from the fungi when inoculated into the PCP–soil mix (Fig. 1 and 2) when compared with the 7-, 9-, 15-, and 19-d-old cultures. This result was confirmed by the experiment testing the effect of fungal culture age inoculated into the higher PCP conc. soil (1043 mg kg^{-1}; Fig. 3) for laccase expression ($P < 0.001$) when compared with the 15-d culture age but not for MnP expression ($P > 0.1$). The 9-d-old inocula cultures in Fig. 3 were not significantly different from the 11-d-old cultures for laccase expression. Visual observations demonstrated that 11-d-old cultures generated more mycelium as a percentage of surface coverage. There was a significant isolate effect ($P = 0.001$; Fig. 3) for laccase expression but not for MnP ($P > 0.1$) because, overall, *T. versicolor* HR131 expressed more laccase than isolate HR577. Laccase expression was significantly higher than MnP production ($P < 0.001$) in all treatments. The MnP expression was significantly higher ($P = 0.081$) in the soils inoculated with the poplar cultures than in the Monterey pine cultures (Fig. 3). There was no laccase or MnP activity detected in the 7-wk-aged controls without fungal inoculum.
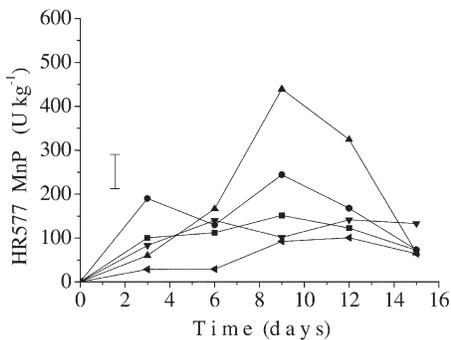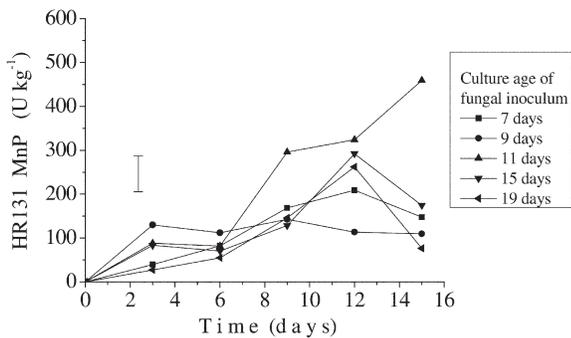
Fig. 2. Manganese peroxidase activity from the white-rot fungal isolates *Trametes versicolor* HR131 and *Trametes* sp. HR577 after bioaugmentation at various culture ages into a 697 mg kg^{-1} (dry wt.) pentachlorophenol-contaminated field soil. Pooled 95% error bounds for the means (U kg^{-1}): culture age main effect ±52; culture age and time ±94; culture age and isolate ±74.
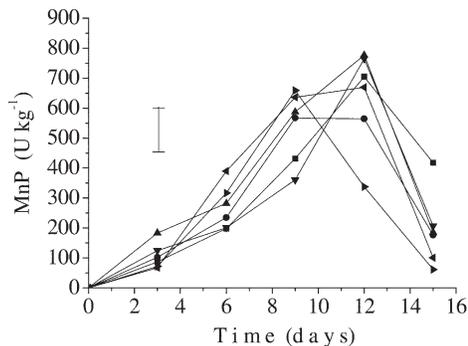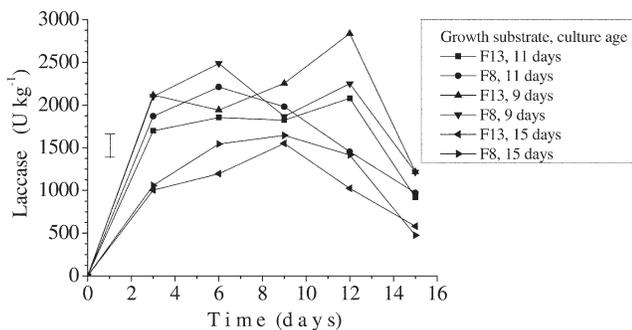

Fig. 3. Laccase and manganese peroxidase (MnP) activity form the white-rot fungus *Trametes versicolor* HR131 after bioaugmentation at various culture ages and two fungal inoculum compositions into a 1043 mg kg^{-1} (dry wt.) pentachlorophenol-contaminated field soil with no co-substrate amendment. Pooled 95% error bounds for the means (U kg^{-1}): MnP culture age main effect ±104 and wood main effect ±85; laccase culture age main effect ±22.
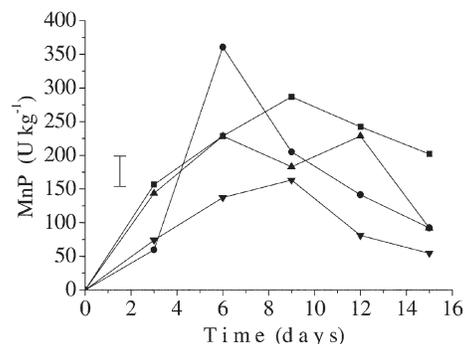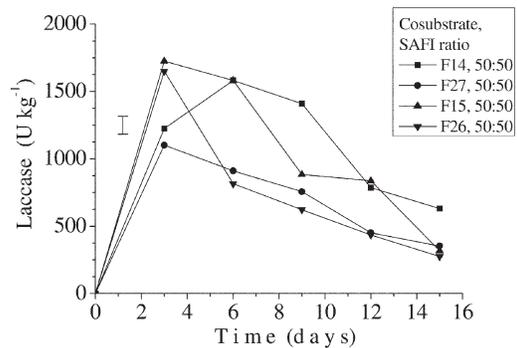

Fig. 4. Laccase and manganese peroxidase (MnP) activity from the white-rot fungus *Trametes* sp. HR577 after bioaugmentation with various co-substrate amendments into a 1043 mg kg^{-1} pentachlorophenol-contaminated field soil at a constant (50:50) co-substrate amendment to fungal inocula ratio (SAFI ratio). Pooled 95% error bounds for the means (U kg^{-1}): MnP and laccase main effects, wood, SAFI ratio and corn grits ±30 and ±90, respectively.

There were some significant two-factor interactions. These included a significant culture age and isolate effect because the older isolate HR131 cultures expressed significantly more laccase ($P = 0.001$) after bioaugmentation than the older isolate HR577 cultures. A significant time and isolate effect ($P = 0.064$) was found because the quicker colonizing isolate HR577 expressed more laccase ($P = 0.064$) and MnP ($P = 0.029$) earlier in the time course; the culture age and time interaction was significant for MnP expression ($P = 0.012$).

Effect of Co-substrate Amendments

The absence of corn grits in the co-substrate amendment (Fig. 4 and 5) lowered laccase expression ($P < 0.001$) but not MnP expression ($P > 0.1$). However, MnP expression varied with time ($P = 0.039$). The poplar sawdust-based formulates produced a higher laccase ($P < 0.001$) and MnP ($P = 0.001$) response from isolate HR577 than the Douglas fir sawdust-based formulates (Stamets and Chilton, 1983). The lower SAFI ratio of 15:85 produced higher laccase and MnP expression for isolate HR577 ($P < 0.001$). From these results, we would expect the poplar, corn grits, and 15:85 SAFI ratio combination treatments to have the highest laccase profile, and this was confirmed by experimental results (Fig. 4 and 5). Significant two-factor interactions for laccase expression were found for time (since bioaugmentation) and SAFI ratio and for time and wood species (poplar or Douglas fir) interaction ($P < 0.001$).
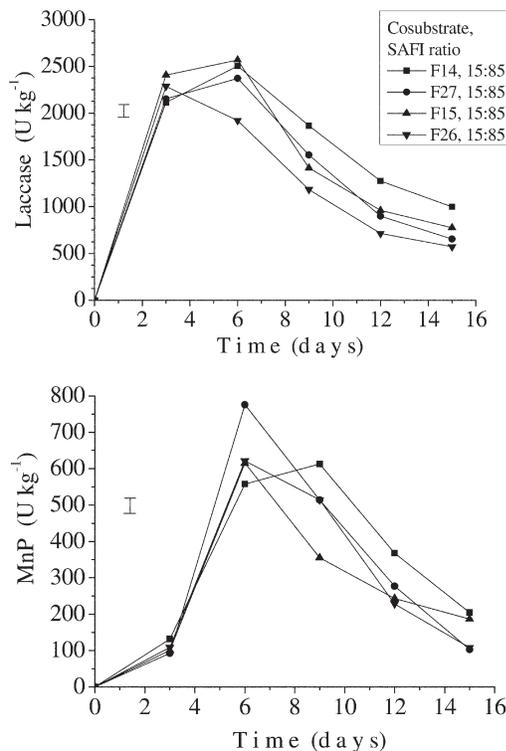
Fig. 5. Laccase and manganese peroxidase (MnP) activity from the white-rot fungus *Trametes* sp. HR577 after bioaugmentation with various co-substrate amendments into a 1043 mg kg^{-1} pentachlorophenol-contaminated field soil at a constant (15:85) co-substrate amendment to fungal inocula ratio (SAFI ratio). Pooled 95% error bounds for the means (U kg^{-1}): MnP and laccase main effects, wood, SAFI ratio and corn grits ±30 and ±90, respectively.
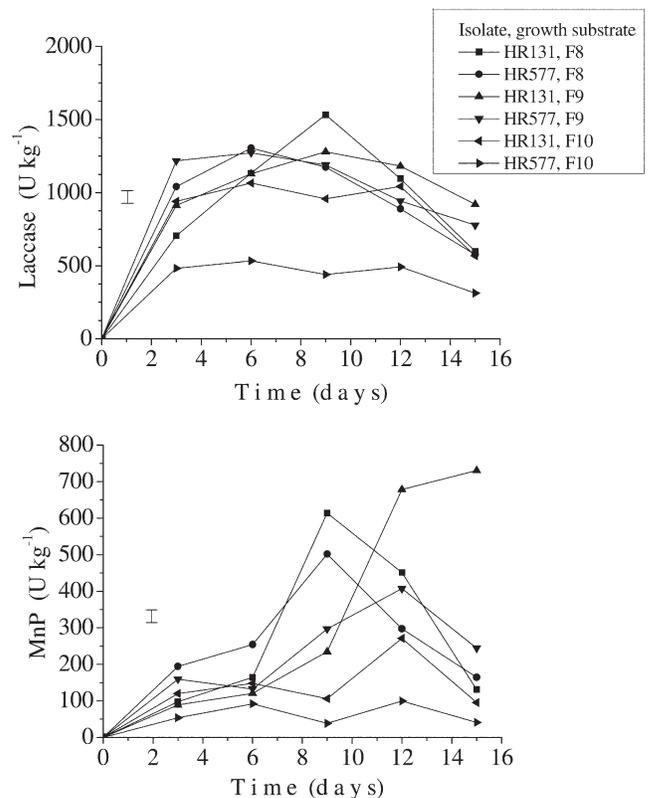
Fig. 6. Laccase and manganese peroxidase (MnP) activity for the white-rot fungi *Trametes versicolor* HR131 and *Trametes* sp. HR577 after bioaugmentation with various fungal inoculum compositions into a 1043 mg kg^{-1} pentachlorophenol-contaminated field soil. Pooled 95% error bounds for the means (U kg^{-1}): MnP isolate and formulate main effects ±18 and ±22, respectively; and laccase isolate and formulate main effects ±48 and ±57, respectively.

Effect of Surfactant in Enhancement of Biodegradation of PCP

The addition of Tween 80 to the PCP–soil mix before bioaugmentation had a significant effect on laccase expression ($P = 0.045$), which also varied with time ($P = 0.05$) but had no significant effect on MnP expression ($P > 0.1$).

Effect of Small Variations in Fungal Inocula Formulation

Figure 6 shows that compositional changes in the growth substrate (F8; Table 1) grain supplement resulted in significant differences in fungal laccase and MnP expression after bioaugmentation. The fungi when grown on the kibbled rye–supplemented sawdust (F8 and F9) produced more laccase than when grown on the rye flour (F10)–supplemented sawdust ($P < 0.001$). Furthermore, *T. versicolor* HR131 produced more laccase than isolate HR577 ($P < 0.001$) after bioaugmentation. Time and all two-factor interactions were significant ($P < 0.001$). The significant improvement in laccase response in F9 (starch addition) over F8 ($P < 0.05$) was not dependent on isolate. The low laccase activity after bioaugmentation when both isolates were grown on F10 was also not isolate dependent; however, laccase expressed by isolate HR577 was still lower than expressed by *T. versicolor* HR131 ($P < 0.001$). The use of coarse corn grits (F7) as a replacement for kibbled rye resulted in less visual fungal colonization and lower

laccase and MnP activity by *T. versicolor* HR131 15 d after bioaugmentation ($P < 0.05$).

Manganese peroxidase expression (Fig. 6) was significantly higher for both New Zealand *Trametes* isolates after bioaugmentation when grown on F9 than when grown on F8 or F10 ($P < 0.001$). This effect was isolate and time dependent because *T. versicolor* produced higher levels of MnP than isolate HR577, especially at the end of the time course (Fig. 6). All main effects (time, formulate, and isolate) and interactions thereof were significant ($P < 0.001$).

Residue Analysis

Significant PCP reduction was observed for all treatments (37–70%) when a sterile Douglas fir corn grits co-substrate was added before fungal bioaugmentation by both white-rot fungal isolates (Fig. 7 and 8). Alternatively, only one treatment combination, the 11-d-old *T. versicolor* HR131 poplar kibbled rye culture, produced significant PCP removal when a co-substrate was not added (Fig. 9). Pentachloroanisole (Lestan et al., 1996) production was <10% of the original PCP conc. in all treatments; there were no significant differences in PCA production between isolates ($P > 0.1$). The addition of Tween 80 had no effect on PCP removal ($P > 0.1$; Fig. 7). The PCP removal re-
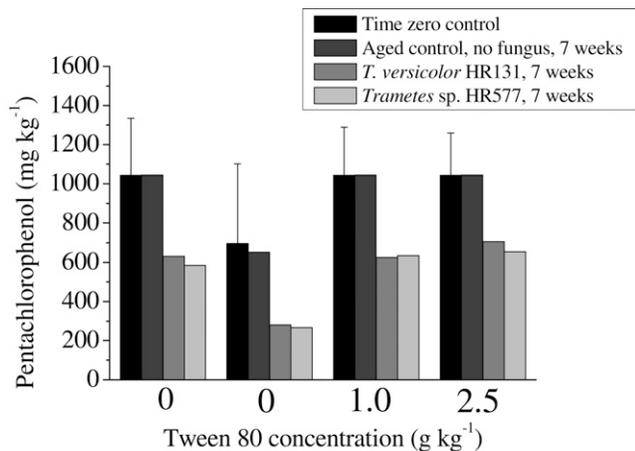
Fig. 7. Pentachlorophenol (PCP) removal after 7 wk by the white-rot fungi *Trametes versicolor* HR131 and *Trametes* sp. HR577 after bioaugmentation into 697 and 1043 mg kg⁻¹ (dry wt.) PCP-contaminated field soils. The latter soil (1043 mg kg⁻¹) was supplemented with two Tween 80 conc. to enhance PCP removal. The error bar represents the 5% Fishers LSD. The 95% confidence intervals were 162 to 397 mg kg⁻¹ PCP (pooled) at a PCP starting concentration of 697 mg kg⁻¹, ANOVA: $P = 0.015$; 536 to 773 and 530 to 718 mg kg⁻¹ PCP (pooled) for isolates HR131 and HR577, respectively, at a PCP starting conc. of 1043 mg kg⁻¹, ANOVA: $P < 0.001$ for both isolates.

sulting from inoculating the F8 and F9 grown white-rot fungi in to the 1043 mg kg⁻¹ aged PCP field soil were all statistically significant ($P < 0.05$) and varied between 37 and 70%. The 70% PCP removal from the 1043 mg kg⁻¹ PCP soil was by *T. versicolor* isolate HR131 when grown on the F8 fungal growth formulation before bioaugmentation (Fig. 8), whereas the lowest value (37%) was the same isolate grown on the F9 formulation. Factorial analysis demonstrated no significant differences for PCP removal between the two kibbled rye formulations (F8 and F9). However, there was a significant interaction between formulate and isolate ($P = 0.013$) because *T. versicolor* HR131
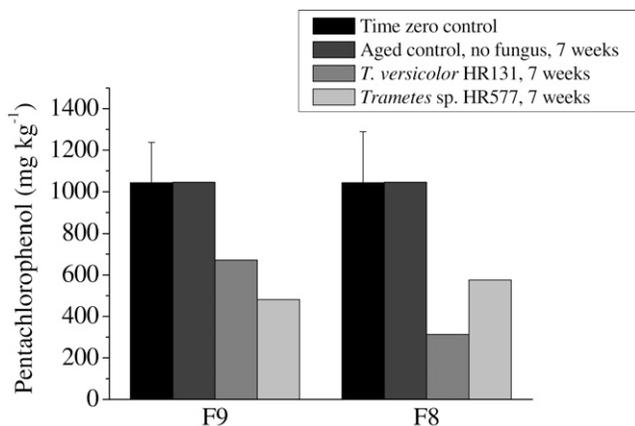

Fig. 8. Pentachlorophenol (PCP) removal after 7 wk by the white-rot fungi *Trametes versicolor* HR131 and *Trametes* sp. HR577 after bioaugmentation with two fungal inocula compositions into a 1043 mg kg⁻¹ (dry wt.) PCP-contaminated field soil. The 90% confidence intervals for the final PCP conc. in the different bioaugmentation treatments (mg kg⁻¹ PCP) were 484 to 812 (HR131, F9), 173 to 515 (HR131, F8), 259 to 620 (HR577, F9), and 155 to 863 (HR577, F8). ANOVA: F9, $P < 0.001$; F8, $P < 0.001$.
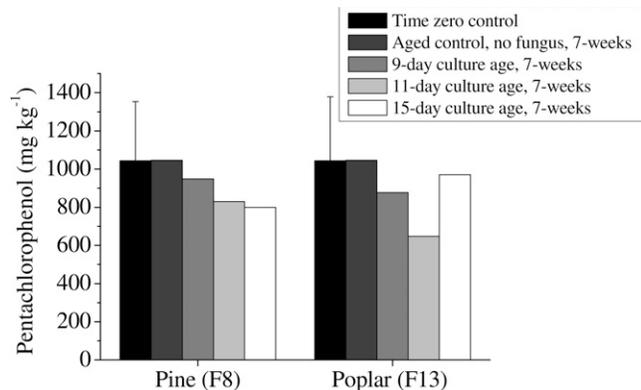

Fig. 9. Pentachlorophenol (PCP) removal after 7 wk by the white-rot fungus *Trametes versicolor* HR131 after bioaugmentation with various culture ages and two fungal inoculum compositions into a 1043 mg kg⁻¹ (dry wt.) PCP-contaminated field soil (no co-substrate amendment). The 90% confidence intervals for the final PCP concentration in the different bioaugmentation treatments (mg kg⁻¹ PCP) were 737 to 1223 (9 d, F8), 541 to 1271 (9-d, F13), 548 to 1163 (11-d, F8), 402 to 936 (11-d, F13), 576 to 1073 (15-d, F8), and 939 to 1071 (15-d, F13). ANOVA: F13, $P = 0.008$; F8, $P = 0.076$; pooled 9-d and 11-d, F8, 785 to 1051.

removed less PCP from the PCP–soil mix when grown on F9, whereas isolate HR577 did not.

Discussion

The higher extracellular enzyme activity after bioaugmentation produced by the 11-d-old fungal inoculum was most noticeable with less fungal inoculum addition (a SAFI ratio of 50:50), which is of interest for the economic aspects of fungal bioremediation because this suggests that more precise control of formulation parameters may allow less fungal inoculum to be produced to obtain the same pollutant degradation (Lestan and Lamar, 1996). However, there is a trade-off against cost. This culture age (11 d) promoted the maximum extent of fungal colonization of the PCP–soil mix in 3 d and promoted a peak in MnP expression after 9 to 15 d (Fig. 2) that was not observed in the bioaugmentation treatments with the other culture ages. These 11-d fungal cultures when inoculated were still in the primary phase of growth (Schmidt et al., 2005). This may have aided fungal survival for longer periods and, hence, may have enhanced extracellular enzyme expression in the PCP-contaminated field soil. Other data (Ford, 2006) demonstrated significant differences between 9- and 11-d-old (isolate HR577) F8 fungal inoculum to degrade PCP at a higher PCP conc. (1920 mg kg⁻¹). The effects of soil:inoculum ratio and time after bioaugmentation (1–7 wk) on extracellular enzyme expression and PCP biodegradation by isolates HR131 and HR577 in the contaminated field soil reported here are covered more comprehensively in another study (Ford et al., 2007). The effects of soil:inoculum ratios on fungal colonization, extracellular enzyme expression, PCP biodegradation, and the practical application in field-scale remediation are also reported in other studies (Davis et al., 1993; Lamar et al., 1993a; Lamar et al., 1993b; Lamar and Dietrich, 1990; Walter et al., 2005b).

Reports in the literature of MnP being expressed in secondary phase cultures in vitro (Reddy and Gold, 2000) apply here

probably because of the lag in peak MnP expression after bio-augmentation compared with laccase for the 11-d-old cultures (Fig. 1–3). In addition, the MnP peak coincides with the appearance of non–white-rot fungi in the soil cultures, which suggests a fungal-mediated detoxification of the soil. Manganese peroxidase has been shown to degrade PCP in vitro to carbon dioxide and water (Reddy and Gold, 2000), whereas laccase can promote polymerization of PCP to potentially less toxic compounds (Ricotta and Bollag, 1996). The presence of both extracellular enzymes is probably necessary for some form of fungal-mediated PCP removal (Evans and Hedger, 2001).

The addition of the co-substrate amendment before fungal bio-augmentation, even though only 15% of the total material added to the PCP-contaminated soil, had a significant effect on whether PCP was removed or not removed under these conditions (Fig. 7–9). The addition of corn grits to the co-substrate amendment formulation (Fig. 4 and 5) did have an effect on increased laccase production (see below). However, the insignificant PCP biodegradation in some treatments (Fig. 9) may not be solely related to co-substrate requirements for fungal survival in soil and extracellular enzyme expression. Previous studies (Gutierrez et al., 2002; Harvey and Thurston, 2001) have reported the presence of unsaturated lipids, which are present in the solvent-extractable portions of most woods and in particular conifers (Gutierrez et al., 2002; Hofrichter, 2002; Stamets and Chilton, 1983), which can act as extracellular enzyme mediators that aid MnP to oxidize compounds with higher oxidation–reduction potentials not usually oxidized by MnP, such as non-phenolic units of lignin and phenanthrene. Such compounds are usually only oxidized by lignin peroxidase (Harvey and Thurston, 2001), which was not detected. However, the extra addition of Tween 80 (Hofrichter, 2002) to the contaminated field soil did not result in significantly more PCP removal (Fig. 7). The only treatment that did not require co-substrate addition for significant PCP removal was growth at optimum culture age (11 d) using poplar sawdust in the growth substrate formulation (Fig. 7). This was expected because *T. versicolor* is principally a degrader of hardwoods such as *Populus* spp. (Stamets, 2000). Higher levels of MnP expression were also recorded for the soils inoculated with the poplar growth substrates and for isolate HR577 grown with a Monterey pine–based growth substrate and inoculated into soils amended with the poplar co-substrate amendments (Fig. 4 and 5). Other data (Ford et al., 2007) for 9-d-old cultures of *Trametes* sp. isolate HR577 grown on the F8 growth substrate also demonstrate significant PCP removal under the same conditions with the Douglas fir corn grits (F15) co-substrate amendment added.

The usefulness of the addition of corn grits to the co-substrate amendment was tested because the previous co-substrate amendments consisted entirely of sawdust or wood chips; the wood chips were typically from hardwoods (Lamar and Dietrich, 1990; Walter et al., 2005b), such as aspen, poplar, or willow. However, corn grits addition may only be suitable for the nitrogen-deregulated *Trametes* genus (Leatham and Kent Kirk, 1983) because the *Phanerochaete* genus expresses peroxidase enzyme systems under conditions of C/N limitation. The Douglas fir co-substrate amendments did not promote as high MnP levels in soil as the poplar co-substrate amendments and would not be preferred in producing fungal

inocula or co-substrate amendment material if there where other options available. However, there may be a trade-off between co-substrate requirements for fungal growth and survival in soil and co-substrate requirements for extracellular enzyme mediation.

The effect of the kibbled rye particles probably aided the survival and colonization of the white-rot fungi in the clay loam–aged residue–co-substrate mix inoculated with the fungal inocula rather than the higher nitrogen levels in the F8 formulate. The F9 (with the kibbled rye starch addition) formulate had a higher C/N ratio than the F10 formulate (with the rye flour addition) and still produced higher extracellular enzyme activity than the F10 (no kibbled rye) formulate after bioaugmentation. The manufacturer reported no preservatives or additives in the rye flour. Completely colonized white-rot fungal pellets have been reported to aid fungal colonization of PCP-contaminated soil (the PCP was spiked) (Lestan and Lamar, 1996). The size of the pellets, which were cylindrical, was 8.0 mm length by 6.0 mm diam., compared with the mean size of the kibbled rye particles of 4.3 mm length by 2.1 mm diam. The addition of starch to the standard F8 kibbled rye growth substrate affected the disappearance of PCP in the two PCP–soil mixes by the two New Zealand *Trametes* isolates differently. However, the enzyme assay results did not correlate with the residue analysis results because MnP expression was higher for *T. versicolor* grown on F9 (Fig. 6) and because less PCP was removed from this treatment. This phenomenon requires further investigation.

The levels of PCP reduction reported here (insignificant to 70%) is lower than that reported for soils freshly spiked with PCP (90%) (Lestan et al., 1996; Walter et al., 2005a) because of the aged nature of the residue and because PCP in oil preservatives had not been used at the site since 1984 (Nadebaum et al., 1992). Moreover, particles of pumice (volcanic ash) were removed from the contaminated field soil during sieving, and their porous nature may contribute to the sequestering of PCP in our experiments (Luthy et al., 1997). A similar result for PCP reduction was reported for *T. hirsuta* (55%) when compared with *P. sordida* and *P. chrysosporium* isolates for bioremediation of a soil where PCP was a co-contaminant with creosote (Lamar et al., 1993a). The level of PCA production was insignificant and is consistent with the results of previous studies with *T. versicolor* (Lestan et al., 1996; Walter et al., 2005a; Walter et al., 2005b).

Conclusions

This is the first report of New Zealand *Trametes* spp. isolates being able to degrade aged PCP residues in contaminated field soils above a PCP conc. of 1000 mg kg^{-1} (dry wt.) with multiple co-contaminants (fuel oil and heavy metals). The maximum PCP removed by the New Zealand *T. versicolor* isolate HR131 was 70% from a 1043 mg kg^{-1} PCP-contaminated soil. The ability of New Zealand white-rot fungi to remove aged PCP residues in field soils with high levels of PCP contamination will be studied further. The optimum culture age for bioaugmentation of New Zealand *Trametes* isolates into the PCP-contaminated field soil that gave the most consistent results in terms of extracellular enzyme expression and visual fungal colonization was 11 d. This did not always translate into more PCP being removed under the experimental

conditions. Tween 80 addition had no affect on PCP removal. Lignin peroxidase was not detected in any of the soil microcosms, whereas laccase and manganese peroxidase were present in all soil microcosms inoculated with the New Zealand *Trametes* isolates. Laccase expression was dominant over manganese peroxidase. The multivariate statistical analysis technique (repeated measures) was useful for determining the bioaugmentation factors or variables that affected extracellular enzyme expression. Kibbled rye was the preferred sawdust grain supplement when compared with corn grits and rye flour. Hardwood sawdust (poplar) negated the use of a co-substrate amendment to achieve significant PCP removal. However, the presence of a Douglas fir corn grits co-substrate amendment may have enhanced the manganese peroxidase oxidation–reduction potential by providing a co-substrate for enzyme mediation as well as for fungal growth and survival. These enzyme mediators were thought to be unsaturated lipids, which are prevalent in the solvent-extractable fraction in coniferous species but not to the same extent in angiosperm species such as poplar. The presence of significant amounts of laccase and manganese peroxidase in the PCP-contaminated field soil did not necessarily correlate with significant PCP removal. This phenomenon will be studied further. There was no significant PCA production in any of the bioaugmentation treatments.

Acknowledgment

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