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Fungal enzyme production and biodegradation of polychlorinated dibenzo-*p*-dioxins and dibenzofurans in contaminated sawmill soil

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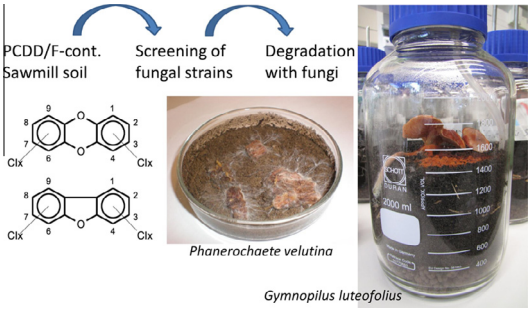
HIGHLIGHTS

- Using non-sterile soil from historically contaminated sawmill area in experiments.
- Measuring enzyme activities directly from soil.
- PCDD/F degradation (64% of WHO-TEQ value) by fungi.
- No degradation with cell free MnP preparation.
- Results support earlier findings of involvement of P450 in biodegradation of PCDD/F.

GRAPHICAL ABSTRACT



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ABSTRACT

The current treatment method for PCDD/F-contaminated soil, which fulfils the requirements for POP soils, is incineration at high temperature. In this study, we investigated if bioaugmentation with fungal inoculum or treatment with manganese peroxidase (MnP) enzyme preparation could be used instead. The main source of PCDD/F contamination in Finland has been the national production and use of a chlorophenol containing wood preservative, which contained PCDD/Fs as impurities. Therefore, historically contaminated soils from three sawmill sites were used in the experiments. In bioaugmentation experiments with living fungal mycelia, enzyme production, CO_2 production and degradation of chlorinated dioxins were measured. When cell free MnP preparation was added to the soil, it was likewise important to follow how enzyme activity was maintained in the soil. As a result of this study, we showed that fungi were able to efficiently degrade PCDD/F, but surprisingly the addition of MnP preparation did not have any effect to the PCDD/F concentration. However, substantial amounts of MnP activity were found in the soil still after 10 d of incubation. Treatment with either *Stropharia rugosoannulata* or *Phanerochaete velutina* resulted in 62–64% decrease in WHO-TEQ value in 3 months. One critical factor for efficient biodegradation was strong growth of fungal mycelia in non-sterile contaminated soil.

1. Introduction

Polychlorinated dibenzo-*p*-dioxins (PCDD) and dibenzofurans (PCDF) belong to the most toxic compounds of persistent organic pollutants (POPs) (Weber et al., 2008). PCDD/Fs are composed of two aromatic rings containing one to eight chlorine atoms. PCDD/Fs are naturally degraded very slowly due to their chemically stable structure and poor bioavailability. In addition, they are highly hydrophobic and become tightly adsorbed on mineral surfaces and absorbed into organic matter in soils and sediments (Srogi, 2008).

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The main source of PCDD/F contamination in Finland has been the national production and use of a chlorophenol containing wood preservative (Ky-5) during 1940–1984. Ky-5 had a specific combination of chlorophenols: 2,3,4,6-tetrachlorophenol (55%), 2,4,6-trichlorophenol (36%), and pentachlorophenol (7%). In addition, PCDD/Fs were found as impurities (Isosaari et al., 2001). During the past several decades, most of the chlorophenols in historically contaminated sawmill soils have volatilized, leached, or biodegraded, but the PCDD/Fs have remained present in the top soil. The main congeners in Ky-5 contaminated soils are hepta- and octachlorodibenzofurans (1,2,3,4,6,7,8-HpCDF and OCDF) (Isosaari et al., 2001).

The current treatment method for soils contaminated with PCDD/Fs, which fulfils the requirements for POP soils, is combustion at high temperature (over 1100 °C). However, combustion is expensive and energy-intensive. Besides, there is not enough capacity in Finland to treat all the contaminated soils. Unlike chlorophenols, PCDD/Fs are not degraded during traditional composting (Laine et al., 1997). However, bioremediation with selected organisms may offer a sustainable alternative, because many bacteria and fungi are able to degrade chlorinated dioxins (Field and Sierra-Alvarez, 2008). Lower chlorinated dioxins can be degraded by aerobic bacteria, e.g. *Sphingomonas* and *Pseudomonas*, which have aromatic ring hydroxylating dioxygenase enzymes (Nam et al., 2005). Anaerobic bacteria can degrade also higher chlorinated dioxins, particularly some *Dehalococcoides* species, which degrade chlorinated dioxins through reductive dechlorination by dehalogenase enzyme (Bunge et al., 2003).

Certain white-rot fungi (WRF) have been shown to degrade all congeners of chlorinated dioxins, even the ones with maximum amount of chlorine atoms (Kamei et al., 2009; Valentín et al., 2013). WRF produce extracellular ligninolytic enzymes and at least some WRF also produce intracellular cytochrome P450 monooxygenase or extracellular aromatic peroxygenase (APO) (Hiratsuka et al., 2005; Stella et al., 2013; Aranda et al., 2010). Ligninolytic enzymes: namely laccase, manganese peroxidase (MnP), lignin peroxidase (LiP), and versatile peroxidase (VP), are non-specific oxidative enzymes. In addition to lignin, they are known to degrade a wide variety of aromatic contaminants (Tuomela and Hatakka, 2011). In bioremediation applications, laccase and MnP are the key fungal enzymes, as in soil environments, LiP activity has been detected only rarely (Wang et al., 2009) and VP production has been found only by *Pleurotus* sp., *Bjerkandera* sp. and *Trametes versicolor* (Tuomela and Hatakka, 2011; Carabajal et al., 2013). P450s are produced by both bacteria and fungi and catalyze the monooxygenation of various lipophilic compounds. Fungal P450 enzymes are probably involved in degradation of lignin, including aromatic contaminants, and they may work together with peroxidases (Hiratsuka et al., 2005). However, the degradation of PCDD/Fs by a fungal P450 has so far only been shown with 2,3-DCDD (Kasai et al., 2010). The extracellular APOs have catalytic similarity to intracellular P450s, but they might have more environmental relevance since the enzymes are secreted outside the fungal mycelia. However, APOs have been described so far only for *Agrocybe aegerita* and *Coprinellus radians*. Aranda et al. (2010) showed that APOs from *A. aegerita* and *C. radians* were able to oxidize non-chlorinated dibenzofuran to mono-, di- and tri-hydroxylated metabolites.

The aim of this study was to explore if soil contaminated with PCDD/F could be bioremediated with fungi or fungal enzymes. The use of enzymes instead of fungal culture in bioremediation could offer a treatment that is easier to control than by any living organism, and the environmental conditions do not need to be optimal for fungal growth and enzyme production (Rao et al., 2010). In our experiments we used non-sterile soil from a historically contaminated sawmill area. Biodegradation of chlorinated dioxins has been studied largely in liquid cultivation conditions with standard compounds (Field and Sierra-Alvarez, 2008). In soil contaminants are less bioavailable than in liquid because they are bound to humic substances. In aged contaminated soil contaminants are even less bioavailable than in spiked soil. Moreover, in non-sterile soil there is competition between inoculated and indigenous microorganisms.

2. Materials and methods

2.1. Soils and soil properties

PCDD/F-contaminated soils from three former sawmill areas, were used in laboratory experiments (Table 1). In all of these areas there has been well documented sawmilling activity for several decades. Two different control soils were used, one in the screening experiments with fungi and another in the degradation experiments with fungal enzymes. Dry matter of soil was determined by drying the fresh soil at 105 °C for 16 h and organic matter content as loss on ignition (mass %) after 4 h at 550 °C. Soil pH was measured in 1 M KCl solution with suspension ratio 1:2.5 (w/v).

Those PCDD/F-congeners, which have chlorine groups in all of the 2,3,7,8-positions (7 PCDDs and 10 PCDFs), are considered as toxic and their toxicity is compared to the most toxic congener, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD), by a TEF-factor (Srogi, 2008). All of these 17 PCDD/F-congeners were analysed from soil samples by isotope dilution, high resolution capillary column gas chromatography (HRGC)/high resolution mass spectrometry (HRMS) (EPA method 1613) by SGS IAC laboratory, Antwerpen, Belgium. The total PCDD/F concentration in soil is given in Table 1 as toxic equivalent (WHO-TEQ), which was calculated by adding up all the 2,3,7,8-TCDD equivalents (WHO 2005 TEF) of all the individual congeners.

2.2. Fungi and fungal enzymes

2.2.1. Fungal strains

Fungal strains were obtained from the Fungal Biotechnology Culture Collection (FBCC) of the Department of Food and Environmental Sciences, University of Helsinki, Finland. Six strains were selected for the screening experiments based on an earlier study (Valentín et al., 2009) (Table 2). The strains were maintained on 2% malt extract agar plates.

Table 1
Properties of soils used in the laboratory experiments.

Soil	Dry matter (%)	Organic matter (% dm)	pH	WHO-TEQ
Sawmill 1	nd	nd	nd	7500–36 000 ng kg^{-1}
Sawmill 2A	78 ± 1	5 ± 1	6.1	14000 ng kg^{-1}
Sawmill 2B	78 ± 1	5 ± 1	6.1	62 000–89 000 ng kg^{-1}
Sawmill 3	66	2.6	6.1	500 ng kg^{-1}
Control soil, screening	nd	0.7	5.7	Not contaminated
Control soil, enzyme exp.	nd	nd	5.5	Not contaminated

nd = not determined.