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Fungal Bioremediation of Creosote-Treated Wood: A Laboratory Scale Study on Creosote Components Degradation by *Pleurotus ostreatus* Mycelium

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Abstract A bioremediation system for creosote-treated wood is proposed, based on the detoxifying capability of *Pleurotus ostreatus*, a ligninolythic fungus. Non-sterilized chipped contaminated wood was mixed at various ratios with wheat straw on which *Pleurotus mycelia* was grown. At 1:2 initial ratio contaminated wood:wheat straw, chemical analyses demonstrated an almost complete degradation of creosote oil components after 44 days, also confirmed by a significant reduction of ecotoxicity. Lower ratios, i.e. higher amount of contaminated wood, lower system efficiency, although a better creosote degradation was obtained by a stepped up wood addition.

Keywords Creosote-treated wood · PAH · Bioremediation · Basidiomycetes

The detoxification of creosote treated wood (CTW) has become more popular since this material has been classified as dangerous waste on the basis of the existing law dispositions (EU Directive 2001/90/CE). Creosote oil is an industrial material obtained by distillation of coal tar, used as a wood preservative owing to its bactericide, fungicide and insecticide properties. It is rich in polycyclic aromatic compounds (PAH) and phenols and has been already recognized as very harmful to humans and environment and potentially cancerogenous (Nylund et al. 1992).

The combustion of CTW in highly protected plants is the main industrial treatment actually in use. Biodegradation can be a less expensive and a more environmentally friendly approach. The great majority of bioremediation experiments concerned with creosote regards polluted soil, where different microorganisms are involved in the oxidation and subsequent mineralization of creosote components: soil bacteria or fungi and white-rot fungi belonging to Basidiomycetes (Eggen et al. 1998; Lau et al. 2003; Atagana et al. 2006; Steffen et al. 2007). The degradation of CTW by means of ligninolythic fungi is described in an US Patent (US Patent 6387689; May 14, 2002), with the species *Antrodia radiculosa* and *Neolentinus lepideus*. The extent of degradation is determined by controlling the dry-weight loss on a time scale. Majcherczyk and Hüttermann (1993) studied the CTW treatment with white-rot fungi: even if a PAH degradation was observed, neither attention was paid to chemical modification of creosote components nor toxicity measurements were reported.

In preliminary works we already reported some data about fungal degradation of creosote-treated wood. The selected fungal mycelium, *Pleurotus ostreatus*, showed a high degradation activity towards creosote components when CTW samples were mixed with a cellulose rich substrate, as wheat straw.

The experiments were performed on a small scale (Petri dishes) in a sterilized environment, in order to prevent the development of competitive microorganisms (Galli et al. 2006).

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The present work describes a more complete set of bioremediation experiments by the same mycelium, *P. ostreatus*, performed on non-sterilized CTW samples. Mycelia were grown on a wheat straw bed to which chipped CTW was added in different amounts. As carcinogenic substances may be formed during PAH biotransformation in living organisms (Pagnout et al. 2006) an accurate analytical study was carried out on the effectiveness of biodegradation and the possible persistence of oxidation products. The results of chemical analyses were compared to ecotoxicological tests.

Materials and Methods

Experiments were performed in three glass boxes with a volume of 5 L and each one was placed in a desiccator. In each box 350 g of dry wheat straw was inoculated with mycelium of *P. ostreatus* (SMR 684) and distilled water was added up to 80% humidity. The glass boxes were incubated at T = 24°C and H = 75%–80%.

After 44 days an abundant mycelium had developed: at this time 175 g of CTW (33%) were added to one of the vessels (sample A). CTW was preliminary cut up to small chips, about 1 × 1 cm, 44 days later (T_1) the contents of sample A box was mixed and weighted. A sample corresponding to 5% of the total weight was taken, as a result of different micro-collections. Furthermore in order to study the effect of subsequent additions of contaminated wood to fungal biomass, an aliquot of 350 g CTW was added both to one of the control vessels (sample B) and to sample A (sample C). These new mixtures (sample B and C) were incubated as stated above. After 60 days (T_2) the final mass was weighted and samples collected and analysed.

The collected fractions from samples A, B and C were pressed in order to eliminate as much as possible the absorbed water. The separated water samples were directly analysed by HPLC for PAH and phenols. PAH separation was performed on a Vydac 251TP54 monomeric C18 column eluted with a gradient of acetonitrile in water from 45% to 97% in 30′ (flow rate 1 mL/min). Phenols were analysed on a Synergy C18 polar embebbed column eluted with a gradient of acetonitrile in water with the addition of acetic acid (pH 2.2) (Galli et al. 2006) Chromatographic elution was detected by both a diode-array (LC610S Shimadzu, Japan) and a fluorescence (Perkin-Elmer S4-Norwalk-CT-USA) detectors.

The solid residues were ground in liquid N_2 and finely milled until a homogeneous powder was obtained. Sample amounts corresponding to 1 g of CTW (W_{1g}) were taken from each of the cryo-milled solid fractions: the exact weight was calculated by the following expression:

$$W_{1\,g} = \frac{W_{cms} - W_{liq}}{0.05 \times W_{ctw}} = 20\frac{W_{cms} - W_{liq}}{W_{ctw}}$$

where W_{cms} corresponds to the collected fraction weight (5% of total mass); W_{liq} is the weight of the separated liquid; W_{ctw} is the total amount of CTW added to the wheat straw in every bioremediation experiment. Each sample was then extracted with 25 mL of methanol in an ultrasonic bath. The methanol extracts were analysed by HPLC for phenols, as described above. PAH were analysed by GC–MS on a 15 m TR-5MS 0.25 mm i.d. capillary column, coated with 5% cross-linked phenyl methyl siloxane as stationary phase (0.25 μm film), with a temperature gradient ranging from 40°C to 300°C (Galli et al. 2006).

In order to validate the previously assessed extraction method on this experimental system, mixtures of CTW–mycelium grown wheat straw were prepared at different ratios, ranging from 10% to 70%. After 30′ "control" samples were prepared and extracted as previously described. In each case PAH were contained in the solid fraction, while phenols were present both in the water fraction and in the residual solid (50%). The recovery of single PAH and phenols in the control samples versus 100% CTW methanol extracts was complete (99 ± 1).

Acute toxicity tests were performed by Microtox analyser (Model 500) with freeze-dried bacteria supplied by Microbics Corporation (Carlsbad, USA) sold in Italy by Ecotox (Milan). Luminescence decrease was determined after 5 min of exposure. After preliminary tests carried out with the negative (solvent control) and positive controls (Phenol standard in the range 5.7–45.6 mg/L), methanol extracts of T_1A, T_2B, T_2C and control T_0A were diluted with water (initial dilution 4%) and analysed in duplicate. The toxicity of the samples was estimated as EC_{50} values on the basis of a dose/response curve (Pagnout et al. 2006).

A genetically homogeneous population of *Daphnia magna* Straus was cultured according to standard methods of the Water Research Institute (IRSA) of the Italian National Research Council (1994); animal growth and reproduction was efficient (72.03 juveniles/female in 21 days, as a mean of all year round). Acute toxicity tests were performed according to standard protocols (CNR-IRSA, 1994). In a preliminary test *Daphnia* were exposed to MeOH (1–1.5–2.0–2.5–3 mL in 75 mL water), to evaluate the possible negative effect of methanol in the extracts: reduction of survival was found only from 2 mL onward (χ^2 analysis), allowing the use of 1.5 mL MeOH in the test. The experiment was repeated both on aqueous fraction and methanol extract of sample A (33% CTW) after mycelium growth (T_1A) and the corresponding T_0 sample. The experimental protocol was the same as in Domji et al (1992) and in Mattei et al. (2006). Data were

analysed by χ^2-test, taking into account all individuals in the three replicas.

Results and Discussion

The effectiveness of *P. ostreatus* to degrade creosote components was examined looking at the possible transfer of the laboratory scale study to a pilot bioremediation plant. With this aim, the experiments were designed to be more cost effective by eliminating the sterilization step, which also gives rise to volatile compounds desorption. Otherwise the effect of an increased CTW/WS amount ratio was tested.

Studies on PAH degradation demonstrated that most PAHs are converted into partially oxidized compounds before decomposed (Bezalel et al. 1996). The partially decomposed compounds include phenols and ketones. In the original creosote these compounds are typically present in small amounts. As they are very harmful, checking of their elimination at the end of the bio-treatment is really important.

Table 1 GC–MS analysis data for PAH and oxy-derivatives in the examined wood samples before (T_0) and after mycelium growth

PAH	T_0 $\mu g/g_W \pm$ S.D.	T_1A $\mu g/g_W \pm$ S.D.	T_2C $\mu g/g_W \pm$ S.D.	T_2B $\mu g/g_W \pm$ S.D.
Naphthalene	42.2 ± 0.5	0.8 ± 0.2	0.7 ± 0.1	1.5 ± 0.0
Methyl-Naphthalenes	54.1 ± 1.0	1.8 ± 0.1	5.8 ± 0.4	9.7 ± 0.4
Acenaphthene	49.6 ± 0.3	1.2 ± 0.3	5.8 ± 0.0	4.4 ± 0.2
Acenaphthylene	1.8 ± 0.0	0.2 ± 0.1	0.5 ± 0.1	0.8 ± 0.0
Biphenyl	5.2 ± 0.1	0.4 ± 0.0	1.8 ± 0.4	4.6 ± 0.2
Methyl-Biphenyl	6.2 ± 0.3	0.2 ± 0.0	1.4 ± 0.1	1.6 ± 0.1
Fluorene	32.5 ± 0.9	0.8 ± 0.0	7.0 ± 0.4	5.8 ± 0.2
Methyl-Fluorene	6.9 ± 0.4	0.0 ± 0.0	1.7 ± 0.2	1.8 ± 0.2
Phenanthrene	636.3 ± 24.8	29.2 ± 1.9	250.7 ± 10.5	320.1 ± 9.9
Anthracene	62.8 ± 3.2	2.7 ± 0.1	30.6 ± 0.1	26.0 ± 1.1
Methyl-Phenantr./Antracen.	222.4 ± 4.4	10.7 ± 0.4	71.6 ± 1.8	95.5 ± 2.0
Fluoranthene	573.4 ± 6.4	43.7 ± 0.8	249.2 ± 0.4	365.7 ± 5.8
Pyrene	272.3 ± 5.0	8.9 ± 0.0	103.7 ± 0.1	133.1 ± 3.2
Methyl-Fluorant./Pyrenes	95.6 ± 5.6	5.3 ± 0.2	45.9 ± 3.9	64.9 ± 1.6
Benzo(c)phenanthrene	12.2 ± 0.3	1.4 ± 0.4	7.3 ± 1.1	11.1 ± 0.8
Benzo(a)anthracene	79.4 ± 5.7	4.7 ± 0.7	40.7 ± 5.0	56.7 ± 2.4
Chrysene + Triphenylene	70.9 ± 0.9	9.1 ± 1.2	40.5 ± 7.4	63.2 ± 4.7
Naphthacene	10.7 ± 0.8	0.7 ± 0.3	3.0 ± 0.0	2.6 ± 0.3
Methyl-(PAH m.w. 228)	21.2 ± 2.9	2.3 ± 0.2	14.0 ± 0.5	22.5 ± 0.4
Benzo(b),(j),(k)fluoranthene	17.6 ± 0.3	3.4 ± 0.6	15.8 ± 1.4	27.5 ± 0.9
PAH m.w. 252	2.8 ± 0.1	0.4 ± 0.1	1.8 ± 0.3	3.1 ± 0.4
Benzo(e)pyrene	7.2 ± 0.1	1.2 ± 0.1	5.9 ± 0.6	8.9 ± 0.3
Benzo(a)pyrene	9.3 ± 0.9	1.1 ± 0.2	6.2 ± 0.5	9.4 ± 0.6
Perylene	2.3 ± 0.0	0.2 ± 0.0	1.3 ± 0.2	2.2 ± 0.4
Indeno(1.2.3-cd)pyrene	12.8 ± 0.9	1.7 ± 1.6	16.2 ± 6.4	16.8 ± 0.9
Benzo(ghi)perylene	3.1 ± 0.9	0.7 ± 0.3	3.9 ± 0.7	3.5 ± 0.5
Total PAH	2310.9 ± 66.7	132.8 ± 9.8	933.0 ± 42.6	1263.1 ± 37.5
9H-Fluorenone	27.5 ± 1.1	4.8 ± 0.4	8.4 ± 0.5	18.8 ± 0.5
Anthraquinone	10.0 ± 0.2	5.6 ± 0.3	8.3 ± 0.8	16.3 ± 0.0
1.8-Naphthalic anhydride	4.5 ± 0.5	0.6 ± 0.0	1.5 ± 0.7	3.0 ± 0.3
Benzo-fluorenones (sum of 3 isomers)	23.2 ± 1.5	4.3 ± 0.5	11.4 ± 2.0	26.2 ± 0.4
Benzanthrone	0.5 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.5 ± 0.1
Acenaphthylenone	1.4 ± 0.2	0.1 ± 0.0	0.2 ± 0.1	0.6 ± 0.1
Total OXY-PAH	67.0 ± 3.5	15.5 ± 1.2	30.0 ± 4.1	65.3 ± 1.4

T_0 = CTW before mycelium treatment; T_1A = 33% mixture of CTW–WS (sample A) after mycelium growth for 44 days (T_1); T_2B = 50% mixture of CTW–WS (sample B) after mycelium growth for 60 days (T_2); T_2C = T_1A with an addition of CTW up to an overall content of 60% (sample C), after 60 days (T_2) of mycelium growth

As demonstrated in previous works, methanol extraction is a good system for both phenols (Becker et al. 2002) and PAH (Portier et al. 1996) desorption from CTW. In the sample preparation for cryo-milling, necessary to homogenize samples and disrupt fibres, an excess of water must be avoided, as the frozen mass would have a vitreous consistence, hampering the milling process. With this aim water was removed by compression.

In the "control" samples phenols are present in both the separated water and methanol extracts of the solid at the concentration of 100 μg/g of CTW. They are absent in both water and methanol extracts of bio-treated samples. PAHs were absent in water corresponding to sample A and found in traces in samples B and C (total amount was less then 1 μg/g CTW). The methanol extracts contain the great majority of pollutants. Analytical evaluation of creosote components is reported in Table 1, summarizing results of GC/MS analyses of methanol extracts before and after bioremediation process.

Chemical analytical methods are used to control contaminated samples and to monitor the efficiency of the remediation process, but they may not be sufficient for environmental risk assessment. Particularly, creosote is constituted by more than 200 compounds and each of them has specific metabolites, so it is really difficult to perform an all-components analysis: a biological approach is appropriate to obtain complementary information (Lau et al. 2003). The results of eco-toxicological tests effectuated on CTW before and after *P. ostreatus* treatment are reported in Fig. 1. The response (EC_{50}) of Microtox assay to the methanol extracts exposure of samples T_1A, T_2B, T_2C and untreated sample T_0A is shown in Fig. 1a. To further state the dramatic decrease in toxicity of bio-treated sample A, the methanol extract and separated water fractions were also utilized in acute toxicity test on Daphnia juveniles. Acute toxicity of methanol extracts before and after the bioremediation process operated by *P. ostreatus* mycelium on CTW (sample A) to Daphnia juveniles is reported in Fig. 1b, as survival rate after 24 h. A dramatic reduction in survival is shown by Daphnia exposed to four different concentration of sample T_0A. No survival reduction is shown by Daphnia exposed to four different concentrations of T_1A sample, after 44 days of bioremediation process: juveniles survived at rates ranging from 87% to 100% when exposed to the same amount of extract as in T_0. No survival reduction was found in Daphnia exposed to T_0A or T_1A separated water fractions (χ^2 analysis).

In the present study eco-toxicological tests are in good agreement with chemical analysis results. Phenols are completely degraded in all bioremediated samples. As regard PAH: sample A is detoxified for more than 90%, sample C for about 60% and sample B for 50%. Some more dangerous compounds, such as methyl-PAH and 5–6 rings

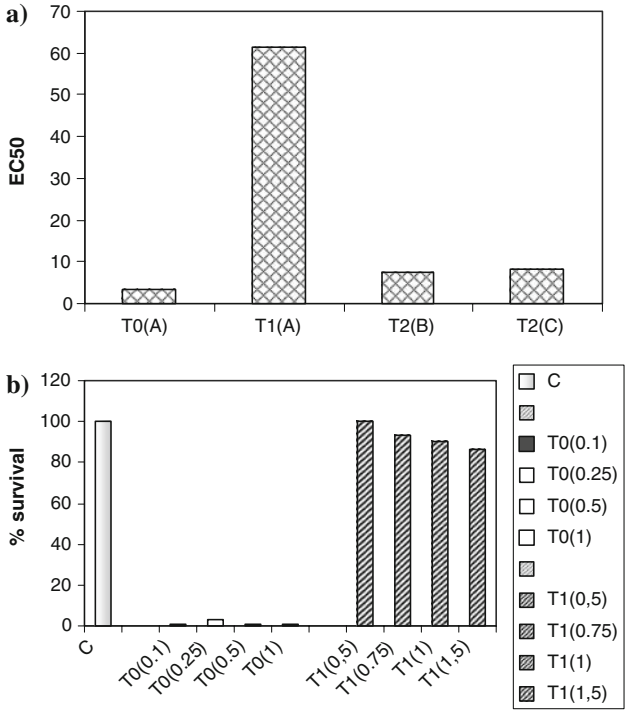

Fig. 1 Results of eco-toxicological test performed on CTW + WS methanol extracts. (**a**) Microtox essay: Y axis: EC_{50} [percentage volume of extracts in the test medium, reducing *Vibrio fischeri* bacterial luminescence by 50% after 5 min; each extract was diluted with double distilled water before testing (4%)]. X axis: T_0A, T_1A, T_2B, T_2C = methanol extracts of mixtures CTW-WS corresponding to 1gr of CTW. T_0A: sample A before bioremediation; T_1A: sample A after 44 days incubation; T_2B: sample B after 60 days incubation; T_2C: sample C after 60 days of incubation. (**b**) *Daphnia Magna* test: Y axis: number of survival of *Daphnia* juveniles after 24 h exposition to the samples under examination. X axis: C = control; T_0 = sample A before bioremediation; T_1 = sample A after 44 days of incubation (0.1–1.5 mL of methanol extracts in 75 mL of water)

PAHs are generally well degraded. Furthermore, the oxy-PAH are efficiently degraded in sample A, they are degraded in some extent in sample C, while in sample B there is no degradation and in some case a little increase: this indicates that the detoxification process is still incompleted.

Accordingly, if compared to T_0 sample, eco-toxicity is lowered in samples B and C and absent in bioremediated sample A, which can be considered almost completely detoxified.

On the basis of these experiments some considerations can be done:

(1) The *P. ostreatus* mycelium efficiently degrades creosote components, confirming results by other authors (Eggen et al. 1998; Atagana et al. 2006). The system could work as well with wood as with soil.
(2) PAH degradation is favoured by the presence of a developed fungal mass at the moment of CTW

addition. In fact, by comparing results from sample C and B (60% and 50%) degradation, there is evidence that CTW addition at different steps makes degradation more efficient than in one single step. As wood is involved in mycelium growth, the more it is added to the mixture, the more the fungal biomass increases, but a more abundant one-step addition determines a creosote concentration causing negative effect on fungal development.

These promising results must still be implemented by deepening our knowledge of the role of several parameters able to promote fungal bioremediation, i.e. composition of the substrate (nutrients), humidity, pollutants substrate ratio: the object of our future work is to choose the most appropriate conditions to improve the detoxification process.

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