PCDD/F formation from chlorophenols by lignin and manganese peroxidases

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Abstract

Polychlorinated dibenzo-p-dioxins and polychlorinated dibenzofurans (PCDD/F) formation was studied, *in vitro*, with two different chlorophenol mixtures (group “di+tri” 2,4-dichlorophenol; 2,3,4-, 2,3,5-, and 3,4,5-trichlorophenols and group “tri+tetra+penta” with 2,4,5-trichlorophenol, 2,3,4,6-tetrachlorophenol and pentachlorophenol) and two different lignolytic enzymes, lignin and manganese peroxidase (LiP and MnP respectively), which can be found during the composting process of sewage sludge. The concentrations of PCDD/F in final samples are compared to the PCDD/F content of the control samples containing the chlorophenols. High increases were observed for experiments with MnP and phosphate buffer. Experiments that contained tri-, tetra- and pentachlorophenol with MnP resulted in more than $8 \cdot 10^8$ ng of OCDD kg^{-1} chlorophenol which was much higher than the initial amount ($1 \cdot 10^7$ ng OCDD kg^{-1} chlorophenol). In relation to LiP experiments, only those at 37 °C showed a moderate increase (from $1.3 \cdot 10^7$ to $2.6 \cdot 10^7$ ng of OCDD kg^{-1} chlorophenol). The results agree with the literature in which high amounts of HpCDD and OCDD were found after a composting process and could explain the biogenic formation suggested by others, but the incidence on the total toxicity is less than that expected.

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Keywords: compost, sewage sludge, manganese peroxidase, lignin peroxidase, enzyme, PCDD/F.

Introduction

Composting is a well-extended practice of waste reduction that consists of a microbial conversion of material in the presence of suitable amounts of air and moisture into a stabilized product, compost, with the general appearance and other characteristics of a fertile soil. Compost is used as a soil amendment and fertilizer since it is a source of macro and micronutrients to plant growth. According to the composting definition, the organic compounds during the process should be reduced or eliminated, but an increase in the PCDD/F content (especially HpCDDs and OCDD) in compost from sewage sludge has been well documented in previous studies (Hamann et al., 1997; Weber et al., 1997; Gómez-Rico et al., 2007). Malloy et al. (1993) also found high concentrations of PCDD/Fs (toxic equivalency mean value of 56 ng I-TEQ kg⁻¹) during the composting process of municipal solid waste (MSW), MSW mixed with sewage sludge and yard waste composting, where 1,2,3,4,6,7,8-HpCDD and OCDD were again the dominant congeners. However, Concejero et al. (2008) after studying three MSW composting facilities found values of 1.53-5.26 ng WHO-TEQ kg⁻¹ (or 2.92-9.22 ng I-TEQ kg⁻¹) which were lower than the initial content.

Therefore, the increase or decrease of the PCDD/F levels during composting seems to depend on the process conditions and initial materials containing PCDD/F precursors. In compost from sewage sludge the precursors necessary for the PCDD/F formation could be introduced to the process through the initial materials, such as sawdust used as a bulking agent that could contain pentachlorophenol (PCP). PCP was used for many years as fungicide to treat wood to avoid woodworm, biocide and herbicide, but nowadays its use is limited because of its relationship with the formation of PCDD/Fs.

It has been observed that PCP is the most contaminated pesticide with these compounds (PCDD/Fs). Sewage sludge used for composting could also contain other kind of chlorophenols (CPs). CPs can be also found in leachates from MSW landfills (Ozkaya, 2005) and therefore they can be present in the MSW compost process.

CPs can be transformed into PCDD/Fs by an enzymatic reaction as reported in literature and this can occur during the composting process of sewage sludge. A group of researchers (Öberg et al., 1987) firstly studied the biological formation of PCDD/Fs from lactoperoxidase (LP) and chlorophenols but without finding PCDD/F formation. Svenson et al. (1989) seem to be the first group in finding that 2,3,7,8-tetrasubstituted congeners were formed in the reaction of 2,4,5-trichlorophenol with horseradish peroxidase (HRP) at 20 °C, mainly tetra-, penta- and hexachloro derivatives. Öberg et al. (1990) studied the reaction of 3,4,5- and 2,4,5-trichlorophenol in the presence of bovine lactoperoxidase (LP) as well as HRP, and hydrogen peroxide at room temperature, where the major products were 1,2,3,6,7,8-HxCDD, 1,2,4,7,8-PeCDD and 1,2,4,6,7,9-HxCDD and agreeing, in part, with Svenson's results. In another study, the same group of researchers tested eight chlorophenols with HRP and LP, all of them resulting in PCDD/Fs formation. Especially a high amount of OCDD was obtained in the experiment with PCP (Öberg et al., 1992). The concentration of PCDD/Fs in their experiments was two- to ten-times higher after the reaction and regarding the initial mixture, depending on the congener. Also Wagner et al. (1990) experimented with 2,4,5-trichlorophenol (triCP) and HRP, finding high amounts of PCDD/F formation. Wittsiepe et al. (2000) studied the transformation of 2,4,5-tri-, 2,3,4,6-tetra-, pentachlorophenol and mixtures of them with plant HP and myeloperoxidase, in the presence of hydrogen peroxide. The yield, the reaction, and the PCDD/F-pattern found depended on the CPs and it could be pointed out that higher yields of OCDD were

obtained in the experiments where pentachlorophenol was involved, as found by Öberg (1992; 1993). The results of Öberg could explain the relatively high concentration of OCDD and HpCDD also found in compost samples studies (Hamann et al., 1997; Weber et al., 1997; Gómez-Rico et al., 2007).

Experiments "in vivo" are also found in literature. Huwe et al. (2000) investigated the formation of dioxins from pentachlorophenol and predioxin nonachloro-2-phenoxyphenol in rats. They concluded that predioxin is the compound which leads to a formation of OCDD instead of pentachlorophenol. Nonachloro-2-phenoxyphenol is an impurity present in pentachlorophenol and other pesticides.

Besides the aforementioned findings, it should be mentioned here that other authors also found PCDD/F degradation by means of biological activity. A group of researchers (Chang, 2008) tried to elucidate the mechanism of the degradation. Suhara et al. (2011) reported the degradation of 2,7-PCDD by some fungi belonging to *genera Coprinus*.

Field et al. (2008) studied the degradation of PCDD by rot-fungi and also reported possible formation of them from chlorophenols. In this study, two different enzymes (not studied before) were used, manganese and lignin peroxidases (MnP and LiP respectively) which can be present in compost. They are lignolytic enzymes typically secreted by White-rot fungi (such as *Phanerochaete chrysosporium*), as reported in literature (Gold et al., 1989; Lankinen et al., 2001; Quintero et al., 2006).

The white-rot fungi are a physiological rather than taxonomic grouping, comprising those fungi that are capable of extensively degrading lignin (Pointing, 2001). They can be commonly found in wood, as reported in literature (Lankinen et al., 2001) and grow at temperatures of 36 – 45 °C (Tuomela et al., 2000). White-rot fungi degrade lignin by means of oxidative enzymes. Because of the nature and size of the lignin molecule, the enzymes responsible for the initial attack must be extracellular and non-specific. The

role of LiP and MnP in lignin degradation has been verified, while that of other enzymes is still uncertain (Hatakka, 1994). The catalytic action of their extracellular enzymes can be used for biodegradation of organic pollutants, such as polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) (Novotný et al., 2004; Oleszczuk, 2007), but the degradation of these compounds could lead to potential precursors for the dioxin formation. These fungi can produce H_2O_2 necessary for the enzyme to produce the activity (Öberg et al., 1992).

Agaricus bisporus is a non-white rot and typically litter degrading fungus known to also produce lignin-degrading peroxidases and typically grows on straw-and-hay based compost under standard conditions of the composting process (Bonnen et al., 1994; Lankinen et al., 2001).

As reported in literature both fungi, *White-rot fungi* and *Agaricus bisporus* can be found in compost (ten Have et al., 2003) and therefore the enzymes LiP and MnP. Alam et al. (2009) produced lignin peroxidase by white-rot fungi using sewage sludge as a major substrate.

Peroxidases are bi-substrate enzymes that require H_2O_2 for the oxidation of CPs into chlorophenoxy radicals. PCDD/Fs are subsequently formed by condensation (Öberg, 1992). H_2O_2 is present in low concentrations in fungal cultures. The oxidation characteristics of these ligninolytic enzymes are reported elsewhere (Mester et al., 2000; Eibes et al., 2005; Eibes et al., 2006). The main function of MnP is to catalyze the oxidation of Mn^{2+} to Mn^{3+} , for which H_2O_2 is required (Feijoo et al., 2008). The Mn^{3+} ion is a strong oxidant and can act on a great variety of phenolic compounds. CPs are good substrates for MnP as previously reported (Sedarati et al., 2003). Its activity is highly increased with malonate buffer and $MnSO_4$ to provide Mn^{2+} . On the other hand,

LiP catalyzes the one-electron oxidation of aromatic compounds by a mechanism involving the formation of substrate aryl cation radical.

In spite of the several studies showing that some other peroxidases such as horseradish and lactoperoxidase catalyze the formation of PCDD/Fs through precursors or predioxins (Öberg et al., 1992; Wittsiepe et al., 2000), no studies have been done with LiP and MnP which can be present in the composting process. The aim of this work was to investigate the "in vitro" formation of PCDD/F from two mixtures of chlorophenols catalyzed by two different enzymes, LiP and MnP in the presence of hydrogen peroxide, thus simulating the reactions which could take place during composting of sewage sludge. The reactions were carried out with two different temperatures and two buffer solutions in order to have the best conditions for the reaction of enzymes involved.

Materials and methods

Reactants

Lignin peroxidase (LiP, EC 1.11.1.14) and manganese peroxidase (MnP, EC 1.11.1.13) were purchased from Sigma-Aldrich. All the chlorophenols were also purchased from Sigma-Aldrich. The following compounds: 2,4,5- , 3,4,5- and 2,3,5-trichlorophenol and 2,3,4,6-tetrachlorophenol were analytical standard grade and these three compounds, 2,3,4-trichlorophenol, pentachlorophenol and 2,4-dichlorophenol were 99 % certified. Stock solutions of the chlorophenols were made in methanol in concentrations ranging 0.10-0.15 mol L⁻¹. The working solutions ranged from 0.006 to 0.015 mol/l.

Control samples

Four control samples were prepared as follows. Two of them, containing either LiP or MnP and all other reactants except the chlorophenols, another one containing the chlorophenol mixture di+tri without enzyme and the last one, containing the chlorophenol mixture tri+tetra+penta and no enzyme (see Table 1). These four control samples were analyzed to determine the initial PCDD/F content in the reactants and compare it with the final samples.

In vitro reactions

For each enzyme four experiments were performed in order to study different conditions and compare the results. The procedure was based on that previously used by other authors with horseradish and myeloperoxidase (Wittsiepe et al., 2000). On the one hand, four experiments were carried out with a mixture of di- and tri-chlorophenols, containing specifically 2,4-dichlorophenol, 2,3,4-trichlorophenol, 2,3,5-trichlorophenol and 3,4,5-chlorophenol (they will be named as “*di + tri*” and correspond to experiments 1 to 4 in Table 1). On the other hand, another group of four experiments consisted of a mixture containing 2,4,5-trichlorophenol, 2,3,4,6-tetrachlorophenol and pentachlorophenol (they will be named as “*tri + tetra + penta*” and correspond to experiments 5 to 8). The experimental temperatures were 25 °C and 37 °C. Temperature 25 °C was chosen as it was the recommended temperature by the supplier for the higher enzymatic activity of MnP and also for MnP formation from *Agaricus bisporus* (Lankinen et al., 2005). Experiments at 37 °C were done at the end of all the experiments and after having seen that there was no enzymatic activity in experiments with LiP at 25 °C. This temperature is also recommended in the literature for experiments with other peroxidases (Wittsiepe et al., 2000). The different

169 experiments and compounds contained in each experiment are summarized in Table 1,
 170 all of them with a total volume of 2 ml..

171 Table 1. Experimental conditions and name of the experiments*. * Experiments are
 172 numbered from 1 to 8. Control samples for MnP and LiP are indicated as MnP and LiP
 173 respectively. Control samples for the chlorophenols are indicated as “di+tri” for the
 174 mixture with 2,4-dichlorophenol, 2,3,4-trichlorophenol, 2,3,5-trichlorophenol and 3,4,5-
 175 chlorophenol and “tri + tetra + penta” for 2,4,5-trichlorophenol, 2,3,4,6-
 176 tetrachlorophenol and pentachlorophenol. A means Acetone and EG ethylene glycol.
 177 Malon means malonate buffer and Phosp means phosphate buffer. “Yes” or “-“ are
 178 written to indicate reactant (MnSO₄ or H₂O₂) was used or not.

	Control			1	2	3	4	Control	5	6	7	8
				di+tri, MnP, Malon, 25 °C	di+tri, MnP, Phosp, 25 °C	di+tri, LiP, Phosp, 25 °C	di+tri, LiP, Phosp, 37 °C		tri+tetra+ penta, MnP, Malon, 25 °C	tri+tetra+ penta, MnP, Phosp, 25 °C	tri+tetra+ penta, LiP, Phosp, 25 °C	tri+tetra+ penta, LiP, Phosp, 37 °C
	MnP	LiP	“di+tri”					“tri+tetra+ penta”				
CPs	-	-	Chlorophenols: “di+tri”					“tri+tetra+penta”				
Buffer	Malon	Phosp	Malon	Malon	Phosp	Phosp	Phosp	Phosp	Malon	Phosp	Phosp	Phosp
T(°C)	25	25	25	25	25	25	37	25	25	25	25	37
Enzyme	MnP	LiP	-	MnP	MnP	LiP	LiP	-	MnP	MnP	LiP	LiP
MnSO4	yes	-	-	yes	yes	-	-	-	yes	yes	-	-
H2O2	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes
Solvent	A	E.G.	A	A	A	EG	EG	A	A	A	EG	EG

179
 180 All reactions were carried out in a buffer solution. The buffer solution used for
 181 experiments numbered from 2 to 4 and from 6 to 8 was phosphate (indicated as Phosp),
 182 KH₂PO₄/K₂HPO₄ (pH = 5.4), and malonate, C₃H₄O₄/NaOH (pH = 4.5) for experiments
 183 1 and 5 (indicated as Malon).

184 Acetone or ethylene glycol was added to the reaction mixture up to a total volume of
 185 2 ml to increase the solubility of chlorophenols due to their low solubility in water
 186 (Czaplicka, 2004). Some solvents, such as methanol which was used to dissolve the
 187 chlorophenols, deactivate the enzyme MnP, therefore acetone is used instead (Eibes et
 188 al., 2005). Ethylene glycol was used for the experiments with LiP as it enhances the
 189 catalytic activity of this enzyme (Yoshida et al., 1997). When MnP was used, MnSO₄ at
 190 a concentration of 20·10⁻⁶ M was also added to the reaction mixture to provide the Mn²⁺

ions which were needed to prevent the dismutation of Mn^{3+} to MnO_2 and Mn^{2+} and to keep Mn^{3+} as a catalytic agent to oxidize the compounds (Feijoo et al., 2008).

The reaction was started when the hydrogen peroxide is added and the incubation time was about 40 hours. The incubation time is much longer than the one suggested by others (Öberg et al., 1987; Wittsiepe et al., 2000), due to the lower enzymatic activity of the experiments in this study.

PCDD/F analysis

After the incubation time, the analytical procedure began. A standard solution containing 17 $^{13}\text{C}_{12}$ labeled PCDD/F congeners was added to the reaction solution and Na_2SO_4 was added to remove the water content. Then the samples were extracted in toluene using accelerated solvent extraction with a Dionex 100 apparatus (Dionex Corp., CA, USA). The extract is solvent exchanged to hexane and extraction with sulfuric acid is done to remove the chlorophenols. The extract was dried by filtering with Na_2SO_4 and then it was cleaned and purified with an automated clean-up system, Power Prep equipment (FMS Inc., Boston, MA).

The purified extract was analyzed using an Autospec Ultima high resolution mass spectrometer (Micromass, UK), with a positive electron impact (EI+) source and interfaced with a Hewlett-Packard (Palo Alto, CA, USA) 6890 Plus gas chromatograph equipped with a PTV (Programmable Temperature Vaporizing) inlet with a septumless head. An Rtx® Dioxin2 (60 m x 0.25 mm x 0.25 μm) fused silica capillary column was used for the separation of the isomer specific analysis. Prior to the injection a recovery standard was added. Identification and quantification of each PCDD/F congener was performed by the isotope dilution method, based on the US EPA Method for PCDD/F determination (US EPA, 1994).

Quality assurance and quality control (QA/QC) criteria included the recovery percentage of the internal standards, the relative retention time of the target compound compared to the internal standard, and the chlorine isotope ratio falling within 15 % of the theoretical chlorine isotope ratio when the two most abundant ions are measured. Additional QA/QC included a laboratory blank that gave results below the detection limit. Regarding the recovery percentage, all the samples were within the established limits of the method (US EPA, 1994). According to previous experiments and laboratory tests carried out by the research group the uncertainty of the measure was estimated in 15 % for values higher than $10 \times 10^4 \text{ ng kg}^{-1}$ and 1 ng kg^{-1} for smaller values. The uncertainty in the TEQ values was estimated in 1 ng kg^{-1} . This uncertainty is based on previous experiments and on the experimental background of the group and comprises the possible errors that can occur during the analytical procedure and also by the equipments.

Results

The control samples containing the enzymes, LiP and MnP respectively, and not containing the chlorophenols resulted in levels near or below the detection limit and were negligible compared with the results of the experiments. On the other hand, the control samples containing the CPs mixtures showed considerable amounts of PCDD/F, thus indicating that CPs were contaminated with PCDD/Fs. Therefore, all the experiments are compared to these control samples in order to show the increase in the concentration after the reaction.

The concentrations in ng kg^{-1} CP and total TEQ concentration for all congeners for initial samples and experiments are shown in Table 2 and Table 3, where $\Sigma 2,3,7,8$ PCDD/Fs refers to the sum of all the 2,3,7,8-tetrasubstituted congeners while Total PCDD/Fs includes the non-2,3,7,8-tetrasubstituted ones. Note that the greater

contribution is mainly due to the toxic congeners. Total 2,3,7,8-PCDD/F concentrations range between $2.4 \cdot 10^6$ and $1.2 \cdot 10^9$ ng kg⁻¹ CP for experiments “di+tri, MnP, Malonate, 25 °C” and “tri+tetra+penta, MnP, Phosphate, 25 °C respectively.

Table 2. Concentrations in ng kg⁻¹ x 10⁴ CP for all the congeners in experiments with “di+tri” mixture (see nomenclature in Table 1).

	Control	1	2	3	4
	di+tri	di+tri, MnP, Malon, 25 °C	di+tri, MnP, Phosp, 25 °C	di+tri, LiP, Phosp, 25 °C	di+tri, LiP, Phosp, 37 °C
2,3,7,8-TCDF	0.2	0.02	0.1	0.08	0.04
1,2,3,7,8-PeCDF	1.4	0.1	0.2	0.28	0.14
2,3,4,7,8-PeCDF	1.4	0.1	0.6	0.32	0.17
1,2,3,4,7,8-HxCDF	1.8	0.1	0.6	0.38	0.23
1,2,3,6,7,8-HxCDF	1.3	0.1	6.4	0.42	0.23
2,3,4,6,7,8-HxCDF	1.9	0.1	116.0	0.44	0.21
1,2,3,7,8,9-HxCDF	1.4	0.2	0.9	0.50	0.17
1,2,3,4,6,7,8-HpCDF	2.3	1.3	37.6	0.99	0.57
1,2,3,4,7,8,9-HpCDF	1.8	0.2	104.1	0.17	0.11
OCDF	2.8	8.8	25.2	2.38	0.85
2,3,7,8-TCDD	0.4	0.2	0.6	0.50	0.20
1,2,3,7,8-PeCDD	1.2	0.5	0.8	0.71	0.56
1,2,3,4,7,8-HxCDD	1.8	0.1	0.4	0.18	0.17
1,2,3,6,7,8-HxCDD	1.7	1.6	2.6	0.69	0.36
1,2,3,7,8,9-HxCDD	1.1	0.5	1.1	0.44	0.21
1,2,3,4,6,7,8-HpCDD	2.8	30	84	1.32	0.93
OCDD	11.3	138	490	6.33	5.65
Σ 2,3,7,8 PCDF	16.4	11	292	6.0	2.7
Σ 2,3,7,8 PCDD	20.2	171	580	10.2	8.1
Σ 2,3,7,8 PCDD/Fs	37	182	872	16	11
TOTAL PCDD/Fs	72	245	1378	36	22
WHO2005 TEQ	3	1.4	17	1.7	1.0

In view of Table 2, it can be observed that the concentration of HpCDD and OCDD increases in both experiments with MnP at 25 °C with an important increase for the OCDD from $11.3 \cdot 10^4 \text{ ng kg}^{-1}$ to $490 \cdot 10^4 \text{ ng kg}^{-1}$ in experiment with Phosphate buffer. It can also be observed a remarkable increment of 2,3,4,6,7,8-HpCDF and 1,2,3,4,7,8,9-HpCDD. OCDF slightly increases in the same experiments. Regarding the concentrations of other congeners and taking into account the estimated uncertainties previously commented, it can be said that their variations are negligible. Experiments with LiP did not reveal variations when compared to the control samples.

With respect to the toxic concentrations, there is a remarkable increase in experiment with MnP and Phosphate, but due to the higher toxicity value of 2,3,4,6,7,8-HxCDF compared to HpCDD and OCDD, the toxicity increase is mainly due to this congener.

From Table 3, it can be deduced that there is an important increase of HpCDD and OCDD in experiments with MnP as it also occurs with “di+tri” mixture. The higher increase is observed in experiment with Phosphate buffer too. On the contrary, no increase is observed for PCDFs as it occurs with “di+tri”, but a remarkable increase is observed in 1,2,3,6,7,8-HxCDD.

Table 3. Concentrations in $\text{ng kg}^{-1} \times 10^4$ CP for all the congeners in experiments with “tri+tetra+penta” mixture (see nomenclature in Table 1).

	Control	5	6	7	8
	tri+tetra+penta	tri+tetra+penta, MnP, Malon., 25 °C	tri+tetra+penta, MnP, Phosp, 25 °C	tri+tetra+penta, LiP, Phosp, 25 °C	tri+tetra+penta, LiP, Phosp, 37 °C
2,3,7,8-TCDF	0.04	0.03	0.3	0.04	0.1
1,2,3,7,8-PeCDF	0.14	0.1	0.9	0.4	0.4
2,3,4,7,8-PeCDF	0.19	0.1	1.1	0.4	0.4
1,2,3,4,7,8-HxCDF	1.88	1.8	3.0	2.2	2.0
1,2,3,6,7,8-HxCDF	0.40	0.3	1.6	0.8	0.6
2,3,4,6,7,8-HxCDF	0.68	0.6	1.5	1.0	1.0
1,2,3,7,8,9-HxCDF	0.39	0.2	1.5	0.7	0.1
1,2,3,4,6,7,8-HpCDF	287	301.0	301.0	283	273
1,2,3,4,7,8,9-HpCDF	13	13.2	18.5	13	13
OCDF	2900	2700	3580	2700	2710
2,3,7,8-TCDD	1.32	1.5	1.6	1.5	1.5
1,2,3,7,8-PeCDD	0.19	0.1	1.5	0.4	0.3
1,2,3,4,7,8-HxCDD	0.14	0.4	12.1	0.4	0.4
1,2,3,6,7,8-HxCDD	7.83	13.3	200.0	8.4	8.1
1,2,3,7,8,9-HxCDD	0.40	1.9	64.7	0.8	0.7
1,2,3,4,6,7,8-HpCDD	243	1020	11900	245	333
OCDD	1350	660	86200	1370	2600
Σ 2,3,7,8 PCDF	3203	3017	3909	3002	3000
Σ 2,3,7,8 PCDD	1603	1697	98380	1627	2944
Σ 2,3,7,8 PCDD/Fs	4806	4715	102289	4628	5945
TOTAL PCDD/Fs	6660	13793	121672	6443	7878
WHO2005 TEQ	9	20	181	10	11

In experiment “tri+tetra+penta, MnP, Phosphate/Malonate and 25 °C” the 2,3,7,8-tetrastituted congeners contribute to more than 70 % to the total concentration in experiments. There is also a considerable increase in the toxicity, expressed as $\text{ng WHO}_{2005} \text{ TEQ/kg chlorophenol}$ in this experiment. This is due to the HpCDD and OCDD congeners. Note also that the concentration of 1,2,3,7,8,9-HxCDD was more than $6 \cdot 10^5 \text{ ng kg}^{-1} \text{ CP}$ which was much higher than the initial concentration ($4 \cdot 10^3 \text{ ng}$

283 kg⁻¹ CP) in experiment “tri+tetra+penta, MnP, Phosphate, 25 °C. Again, experiments
284 with Lip did not report an increase.

285 **Discussion**

286 With the objective of discussing the results, Figure 1 and Figure 2 are presented.
287 These figures show the absolute concentration of the major PCDD/F congeners in ng/kg
288 of chlorophenols (ng kg⁻¹ CPs) for the experiments with “di+tri” and “tri+tetra+penta”
289 chlorophenols group respectively. The increase of each congener can be observed
290 comparing the color bars with the white bars (control sample).

Figure 1. Concentration of the most abundant congeners in ng/kg chlorophenol for the mixtures "di+tri".

Figure 2. Concentration of the most abundant congeners in ng/kg chlorophenol for the mixtures "tri+tetra+penta".

In view of Figure 1 and Figure 2, it can be clearly observed that experiments carried out with MnP show a high increase of HpCDD and OCDD (around 80 % of the total concentration in ng/kg is OCDD) for both mixtures, “di+tri” and “tri+tetra+penta”. Note that, in the experiment with the mixture “di+tri” and phosphate buffer a considerable formation of some furans can also be observed. On the contrary, no considerable variation can be observed with LiP in all the conditions studied, included the one carried out at 37 °C.

Svenson et al. (1989) firstly found the formation of small amounts of 2,3,7,8-tetrasubstituted congeners in the oxidation of 2,4,5-trichlorophenol and horseradish peroxidase. Öberg et al. (1990) found total PCDD/F concentrations of $1.1 \cdot 10^7$ ng kg⁻¹ CP for experiments with lactoperoxidase and 3,4,5-trichlorophenol and $1.0 \cdot 10^7$ ng kg⁻¹ CP with 2,4,5-trichlorophenol with blanks giving no detectable levels. Also an important PCDD/F formation is detected in some experiments of this study with the enzyme MnP.

In experiment “tri+tetra+penta, MnP, Phosphate and 25 °C the concentration of OCDD is more than $8 \cdot 10^8$ ng kg⁻¹ CP while the initial concentration is $1 \cdot 10^7$ ng kg⁻¹ CP resulting in an important increase. On the other hand, OCDD concentrations obtained in experiment “tri+tetra+penta, MnP, Malonate and 25 °C” were more than $6 \cdot 10^7$ ng/kg CP (around 5 times more than initial sample) which is also much higher than the values reported in literature (Öberg et al., 1992). Öberg et al. (1992) found high amounts of OCDD (more than $6 \cdot 10^7$ ng kg⁻¹ CP) in their experiments with PCP and lactoperoxidase.

Regarding the toxicity and as previously commented, only experiments with MnP and Phosphate buffer result in an increase. Congeners profiles for experiments with MnP are shown in Figure 3. The experiment “tri+tetra+penta, MnP and Phosphate”

shows the biggest increase from $9 \cdot 10^4$ to $181 \cdot 10^4$ ng WHO₂₀₀₅-TEQ kg⁻¹ (Figure 3B) which is due to HpCDD and OCDD congeners. Nevertheless, it can be emphasized that the increase in the total toxicity could be much greater if the toxicity factors of HpCDD and OCDD (0.01 and 0.0003 respectively) were close to the unit.

Note how the experiments with the higher chlorinated chlorophenols gave the higher PCDD/F formation with higher chlorinated congeners (HpCDD and OCDD).

Figure 3. Toxic concentrations for the experiments with an important increase (see nomenclature in Table 1).

The results found in this study elucidates a possible enzymatic formation in the compost process at later stages (cooling and maturation stages) as previously reported by Öberg et al. (1994) who found changes in PCDD/Fs in mature garden compost with an increase in the higher chlorinated dioxins. Hamann et al. (1997) also found increasing values in the I-TEQ levels with time in the composting of sewage sludge process and the highest ones were found in the oldest material stored.

Experiments with Manganese Peroxidase, which can be found in compost, show a high increase in PCDD/F concentrations, especially in Phosphate buffer and with “tri + tetra + penta” mixture. The PCDD/F concentration in this experiment increased from $6660 \times 10^4 \text{ ng kg}^{-1} \text{ dm}$ to more than $100000 \times 10^4 \text{ ng/kg dm}$ and from 9×10^4 to $181 \times 10^4 \text{ ng WHO}_{2005} \text{ TEQ ng kg}^{-1}$. The increase in the total toxicity expressed as $\text{ng WHO}_{2005} \text{ TEQ kg}^{-1}$ could be much greater if the toxicity factors of HpCDD and OCDD were close to the unit. The results could explain the biogenic formation previously discussed in the literature during later stages of composting. Consequently, the PCDD/F formation in the compost depends on the specific conditions related to the highest enzymatic activity and precursors present in the initial sample. Due to this and also to minimize a PCDD/F formation during the process, special attention should be paid at the initial materials used for composting.

Acknowledgments

The authors want to thank the University of Alicante for their economic support to perform this research and the project CTQ 2008-05520 of the Spanish Ministry of Science and Innovation, Prometeo 2009/043/FEDER of the Valencian Community of Spain.

- Alam, M. Z.; Mansor, M. and Jalal, K. C. A. (2009) Optimization of lignin peroxidase production and stability by *Phanerochaete Chrysosporium* using sewage-treatment-plant sludge as substrate in a stirred-tank bioreactor. *J Ind Microbiol Biotechnol* 36(5): 757-764.10.1007/s10295-009-0548-5
- Bonnen, A. M.; Anton, L. H. and Orth, A. B. (1994) Lignin-degrading enzymes of the commercial button mushroom, *Agaricus bisporus*. *Appl. Environ. Microbiol.* 60(3): 960-965
- Chang, Y. S. (2008) Recent developments in microbial biotransformation and biodegradation of dioxins. *J Mol Microbiol Biotechnol* 15(2-3): 152-171.000121327 [pii]
10.1159/000121327 [doi]
- Concejero, M.; De la Torre, A.; Sanz, P. and Martinez, A. (2008) PCDD/Fs, PCBs and HCB in leachate and compost from different Spanish landfills. *Organohalogen Compd.* 70: 1773-1775
- Czaplicka, M. (2004) Sources and transformations of chlorophenols in the natural environment. *Sci. Total Environ.* 322(1-3): 21-39.<http://dx.doi.org/10.1016/j.scitotenv.2003.09.015>
- Eibes, G.; Cajthaml, T.; Moreira, M. T.; Feijoo, G. and Lema, J. M. (2006) Enzymatic degradation of anthracene, dibenzothiophene and pyrene by manganese peroxidase in media containing acetone. *Chemosphere* 64(3): 408-414
- Eibes, G.; Lú-Chau, T.; Feijoo, G.; Moreira, M. T. and Lema, J. M. (2005) Complete degradation of anthracene by Manganese Peroxidase in organic solvent mixtures. *Enzyme Microb. Technol.* 37(4): 365-372
- Feijoo, G.; Moreira, M. T.; Alvarez, P.; Lú-Chau, T. A. and Lema, J. M. (2008) Evaluation of the enzyme manganese peroxidase in an industrial sequence for the lignin oxidation and bleaching of eucalyptus kraft pulp. *J. Appl. Polym. Sci.* 109(2): 1319-1327
- Field, J. A. and Sierra-Alvarez, R. (2008) Microbial degradation of chlorinated dioxins. *Chemosphere* 71(6): 1005-1018.10.1016/j.chemosphere.2007.10.039
- Gold, M., H.; Wariishi, H. and Valli, K. (1989) Extracellular peroxidases involved in lignin degradation by the *White Rot Basidiomycete Phanerochaete chrysosporium*. *Biocatalysis in Agricultural Biotechnology* 389(389): 127-140.doi:10.1021/bk-1989-0389.ch009
- Gómez-Rico, M. F.; Font, R. and Fullana, A. (2007) Study of PCDD/Fs formation during composting of sewage sludge in different conditions. *Organohalogen Compd.* 69(P-125): 1570-1573
- Hamann, R.; Weber, H.; Disse, G. and Haupt, H. J. (1997) Determination of the PCDD/F levels in large-scale sewage sludge composting. *Organohalogen Compd.* 32: 400-402
- Hatakka, A. (1994) Lignin-modifying enzymes from selected white-rot fungi: Production and role in lignin degradation. *FEMS Microbiology Reviews* 13(2-3): 125-135
- Huwe, J. K.; Feil, V. J.; Zaylskie, R. G. and Tiernan, T. O. (2000) An investigation of the in vivo formation of octachlorodibenzo-p-dioxin. *Chemosphere* 40(9-11): 957-962.10.1016/s0045-6535(99)00339-2
- Lankinen, P.; Bonnen, A.; Anton, L.; Wood, D.; Kalkkinen, N.; Hatakka, A. and Thurston, C. (2001) Characteristics and N-terminal amino acid sequence of Manganese Peroxidase

407 from solid substrate cultures of *Agaricus Bisporus*. *Appl. Microbiol. Biotechnol.* 55(2):
408 170-176.10.1007/s002530000509

409 Lankinen, P.; Hildén, K.; Aro, N.; Salkinoja-Salonen, M. and Hatakka, A. (2005) Manganese
410 peroxidase of *Agaricus bisporus*: grain bran-promoted production and gene
411 characterization. *Appl. Microbiol. Biotechnol.* 66(4): 401-407.10.1007/s00253-004-
412 1731-2

413 Malloy, T. A.; Goldfarb, T. D. and Surico, M. T. J. (1993) PCDDs, PCDFs, PCBs, chlorophenols
414 (CPs) and chlorobenzenes (CBzs) in samples from various types of composting facilities
415 in the United States. *Chemosphere* 27(1-3): 325-334.10.1016/0045-6535(93)90309-s

416 Mester, T. and Tien, M. (2000) Oxidation mechanism of ligninolytic enzymes involved in the
417 degradation of environmental pollutants. *International Biodeterioration & Biodegradation* 46(1): 51-59

419 Novotný, Č.; Svobodová, K.; Erbanová, P.; Cajthaml, T.; Kasinath, A.; Lang, E. and Šašek, V.
420 (2004) Ligninolytic fungi in bioremediation: extracellular enzyme production and
421 degradation rate. *Soil Biology and Biochemistry* 36(10): 1545-
422 1551.<http://dx.doi.org/10.1016/j.soilbio.2004.07.019>

423 Öberg, L. G.; Glas, B.; Swanson, S. E.; Rappe, C. and Paul, K. G. (1990) Peroxidase-catalyzed
424 Oxidation of Chlorophenols to Polychlorinated Dibenzo-p-dioxins and Dibenzofurans.
425 *Arch. Environ. Contam. Toxicol.* 19: 930-938

426 Öberg, L. G. and Rappe, C. (1992) Biochemical formation of PCDD/Fs from chlorophenols.
427 *Chemosphere* 25(1-2): 49-52

428 Öberg, L. G. and Swanson, S. E. (1987) On the absence of polychlorinated dibenzodioxins and
429 dibenzofurans after lactoperoxidase-catalyzed transformation of chlorophenols. *Bull.*
430 *Environ. Contam. Toxicol.* 38(6): 962-968.10.1007/bf01609081

431 Öberg, L. G.; Wagman, N.; Andersson, R. and Rappe, C. (1993) De novo formation of PCDD/Fs
432 in compost and sewage sludge - a status report. *Organohalogen Compd.* 11: 297-302

433 Öberg, L. G.; Wagman, N.; Koch, M. and Rappe, C. (1994) Polychlorinated dibenzo-p-dioxins,
434 dibenzofurans and non-ortho PCBs in household organic-waste compost and mature
435 garden waste compost. *Organohalogen Compd.* 20: 245-250

436 Oleszczuk, P. (2007) Changes of polycyclic aromatic hydrocarbons during composting of
437 sewage sludge with chosen physico-chemical properties and PAHs content.
438 *Chemosphere* 67: 582-591

439 Ozkaya, B. (2005) Chlorophenols in leachates originating from different landfills and aerobic
440 composting plants. *J. Hazard. Mater.* 124(1-3): 107-
441 112.<http://dx.doi.org/10.1016/j.jhazmat.2005.04.016>

442 Pointing, S. (2001) Feasibility of bioremediation by white-rot fungi. *Appl. Microbiol. Biotechnol.*
443 57(1-2): 20-33.10.1007/s002530100745

444 Quintero, J. C.; Feijoo, G. and Lema, J. M. (2006) Producción de enzimas Lignolíticas con
445 hongos basidiomicetos cultivados sobre materiales lignocelulósicos. *VITAE* 13(2): 61-67

446 Sedarati, M. R.; Keshavarz, T.; Leontievsky, A. A. and Evans, C. S. (2003) Transformation of high
447 concentrations of chlorophenols by the white-rot basidiomycete *Trametes versicolor*
448 immobilized on nylon mesh. *Electronic Journal of Biotechnology* 6: 104-114

449 Suhara, H.; Kamei, I.; Maekawa, N. and Kondo, R. (2011) Biotransformation of polychlorinated
450 dibenzo-p-dioxin by *Coprinellus* species. *Mycoscience* 52(1): 48-
451 52.<http://dx.doi.org/10.1007/S10267-010-0062-7>

452 Svenson, A.; Kjeller, L. O. and Rappe, C. (1989) Enzyme-mediated formation of 2,3,7,8-
453 tetrasubstituted chlorinated dibenzodioxins and dibenzofurans. *Environmental Science*
454 & *Technology* 23(7): 900-902.10.1021/es00065a022

455 ten Have, R.; Wijngaard, H.; Ariës-Kronenburg, N. A. E.; Straatsma, G. and Schaap, P. J. (2003)
456 Lignin degradation by *Agaricus bisporus* accounts for a 30 increase in bioavailable
457 holocellulose during cultivation on compost. *J. Agric. Food Chem.* 51(8): 2242-
458 2245.10.1021/jf021131h

459 Tuomela, M.; Vikman, M.; Hatakka, A. and Itävaara, M. (2000) Biodegradation of lignin in a
460 compost environment: a review. *Bioresour. Technol.* 72(2): 169-183.10.1016/s0960-
461 8524(99)00104-2

462 US EPA (1994). Method 1613. Tetra- through Octa-Chlorinated Dioxins and Furans by Isotope
463 Dilution HRGC/HRMS. SW-846. Washington, D.C., U.S. Environmental Protection
464 Agency.

465 Wagner, H. C.; Schramm, K. W. and Hutzinger, O. (1990) Biogenic polychlorinated dioxin and
466 furan from trichlorophenol. *Organohalogen Compd.* 3: 453-456

467 Weber, H.; Hamann, R.; Disse, G. and Haupt, H. J. (1997) Influence of different treatment
468 methods for sewage sludge on the levels of chlorinated dibenzodioxins and
469 dibenzofurans. *Organohalogen Compd.* 32: 394-399

470 Wittsiepe, J.; Kullmann, Y.; Schrey, P.; Selenka, F. and Wilhelm, M. (2000) Myeloperoxidase-
471 catalyzed formation of PCDD/F from chlorophenols. *Chemosphere* 40(9-11): 963-968

472 Yoshida, S.; Watanabe, T.; Honda, Y. and Kuwahara, M. (1997) Effects of water-miscible organic
473 solvents on the reaction of lignin peroxidase of *Phanerochaete chrysosporium*. *Journal*
474 *of Molecular Catalysis B: Enzymatic* 2(4-5): 243-251

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476

477