**Univerzita Karlova v Praze**

*Přírodovědecká fakulta*

*Environmentální vědy*

**Università degli Studi della Tuscia**

*Dipartimento per l'Innovazione nei sistemi Biologici, Agroalimentari e Forestali*

Title

# Bioremediation of persistent aromatic pollutants

Titolo

# Biorisanamento di contaminanti aromatici persistenti

(BIO/19)

PhD in "Environmental Sciences" (Cycle XXVI)

**MSc. Tatiana Stella**

Tutor and coordinator: Prof. Maurizio Petruccioli
Univesità degli Studi della Tuscia (Viterbo)

Tutor: doc. RNDr. Tomáš Cajthaml, Ph.D.
Přírodovědecká fakulta, Univerzita Karlova v Praze

Viterbo 2014

**Univerzita Karlova v Praze**

*Přírodovědecká fakulta*

*Environmentální vědy*

**Università degli Studi della Tuscia**

*Dipartimento per l'Innovazione nei sistemi Biologici, Agroalimentari e Forestali*

Title

# Bioremediation of persistent aromatic pollutants

Název

# Bioremediace persistentních aromatických polutantů

Disertační práce

**Mgr. Tatiana Stella**

Školitel: Prof. Maurizio Petruccioli
Univesità degli Studi della Tuscia (Viterbo)

Školitel: doc. RNDr. Tomáš Cajthaml, Ph.D.
Přírodovědecká fakulta, Univerzita Karlova v Praze

Praha 2014

**Prohlášení:**

Prohlašuji, že jsem závěrečnou práci zpracoval/a samostatně a že jsem uvedl/a všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze, 07.05.2014

Podpis

***Acknowledgements***

First and foremost I would like to acknowledge my tutors Prof. Maurizio Petruccioli and Assoc. Prof. Tomas Cajthaml, Ph.D for their scientific support, strong encouragement and friendly attitude over the period of my Ph.D.

I would like to express my deep gratitude to my supervisor Alessandro D'Annibale, Ph.D for his insightful advices and the stimulating discussions we had over these years.

My sincere thank goes also to all the members of the laboratory of "Environmental Biotechnology" at the Academy of Science of Czech Republic and the laboratory of "Microbiology and Environmental Biotechnology" at Tuscia University in Viterbo: Stefano Monika, Eva, Zdena, Jana, Alena, Jarka, Brano, Kata, Amelie, Martin and Lenka.

I would like to acknowledge also RNDr. Petr Baldrian, Ph.D and his research group for their contribution to my professional growth and scientific knowledge.

A special thank goes to my "personal" scientific advisor Stefano for the great enthusiasm and the extraordinary patience shown to strongly motivate and support me during my PhD work.

My deepest gratitude goes to my extraordinary family to have been constantly present even though so distant for all these years.

Last but not least, I would like to thank my friends Guglielmo, Fabiana, Simona, Lorena, Samantha, Giorgia, Irene, Salvador, Ruben, Paula and Lucka.

This Ph.D work was supported by grant Competence Center TE01020218 of the Czech Technology Agency, by grant no. 13-28283S of the Czech Science Foundation and by the Ministry of Education, Youth and Sports of the Czech Republic projects CZ.1.07/2.3.00/20.0055 and CZ.1.07/2.3.00/30.0003.

***Abbreviations***

| | |
|---|---|
| 16S rDNA | 16S ribosomal RNA |
| ABTS | 2,2´-azinobis (3-ethylthiazoline-6-sulfonate) |
| APEG | alkaline metal hydroxide/polyethylene glycol |
| ASE | accelerated solvent extraction |
| BCD | base-catalysed decomposition |
| *Bph* | bacterial biphenyl genes |
| BSA | bovine serum album |
| BTEX | benzene, toluene, ethylbenzene, xylenes |
| CBAs | chlorobenzoic acids |
| CEC | cation exchange capacity |
| CO | carbon monoxide |
| CYP420 | cytochrome P420 monooxygenase system |
| CYP450 | cytochrome P450 monooxygenase system |
| DDT | dichlorodiphenyltrichloroethane |
| DMSO | dimethyl sulfoxide |
| DTT | dithiotreitol |
| EDTA | ethylenediaminetetraacetic acid |
| FPLC | fast protein liquid chromatography |
| GC-MS | gas chromatography-mass spectrometry |
| HBT | 1-hydroxybenzotriazole |
| HKC | heat killed control |
| HOPDA | 2-hydroxy-6-oxo-6-(chloro) phenylhexa-2,4-dienoic acid |
| ITS | internal transcribed spacer |
| Koc | organic carbon partition coefficient |
| KPEGTM | potassium metal hydroxide/ polyethylene glycol |
| Lac | laccase |
| LiP | lignin peroxidase |
| MEA | malt extract agar |
| MNA | monitored natural attenuation |
| MnP | manganese-dependent peroxidase |
| MS-MS | tandem mass spectrometry |
| NADPH | reduced nicotinamide adenine dinucleotide phosphate |

| | |
|---|---|
| OC | organic carbon |
| OH-PCBs | hydroxylated polychlorinated biphenyls |
| OTU | operational taxonomic unit |
| PAHs | polycyclic aromatic hydrocarbons |
| PB | piperonyl butoxide |
| PCA | principal component analysis |
| PCBs | polychlorinated biphenyls |
| PCDDs | polychlorinated dibenzo-*p*-dioxins |
| PCDFs | polychlorinated dibenzofurans |
| PCR | polymerase chain reaction |
| PLFA | phospholipid fatty acids |
| PMSF | phenylmethylsulfonyl fluoride |
| QIIME | quantitative insights into microbial ecology |
| RP-HPLC | reversed-phase high performance liquid chromatography |
| SFE | supercritical fluid extraction |
| VP | versatile peroxidase |
| WHC | water holding capacity |

***Abstract***

The remediation of persistent chlorinated aromatic compounds has become a priority of great relevance due to the teratogenic, carcinogenic and endocrine-disrupting properties of these xenobiotics. The use of biological methodologies for the clean-up of contaminated sites, collectively referred to as "bioremediation", has been gaining an increasing interest in recent years because it represents an effective, cost-competitive and environmentally friendly alternative to the physico-chemical and thermal treatments. In this respect, "white rot" fungi, an ecological subgroup of filamentous fungi, display features that make them excellent candidates to design an effective remediation technology ("mycoremediation"). In spite of this, fungi have not been widely exploited for their metabolic capabilities and the mechanism by which they are able to degrade the aforementioned pollutants has not been fully elucidated yet.

Within this frame, the present Ph.D thesis was aimed at:
***i***) assessing the efficiency of different mycoremediation strategies for the clean-up of a polychlorinated biphenyl (PCBs)-contaminated soil;
***ii***) understanding the fungal degradation pathways of polychlorinated biphenyls and their major metabolites, namely chlorobenzoic acids (CBAs) and hydroxylated polychlorinated biphenyls (OH-PCBs).

***i***) The combination of chemical, toxicological and molecular biology techniques provided a comprehensive evaluatation of the technical feasibility of selected remedial strategies. Physico-chemical properties (pH, soil texture, soil organic matter content, *ect*.) as well as the pollutant bioavailability of three different PCB-contaminated soil samples from a dumpsite (bulk soil, topsoil and rhizosphere soil) were assessed before undergoing both bioaugmentation (either with the white rot fungus *Pleurotus ostreatus* or *Irpex lacteus*) and biostimulation (addition of a lignocellulosic substrate) treatment. The inoculation of *P. ostreatus* in the rhizosphere soil was the most effective treatment in terms of PCB degradation and detoxification. The involvement of both intracellular and extracellular fungal enzymes in the biotransformation of PCBs was demonstrated by the identification of several PCB degradation intermediates (*i.e*. chlorobenzoates, chlorobenzaldehydes, chlorocresols, hydroxylated and methoxylated PCBs). Furthermore, new insights into the microbial community structure, diversity and dynamics throughout the bioremediation processes were gained with the combination of two culture-indipendent techniques: phospholipid fatty acids

(PLFA) and 454-pyrosequencing analyses. PLFA analysis showed that either the introduction of allochthonous fungi or the addition of non-inoculated lignocellulosic substrate stimulated the growth of the resident bacterial populations, while the highest fungal concentration was achieved in *P. ostreatus*-topsoil microcosms in the incubation middle phase. Metagenomic analysis of bacterial community revealed that *Firmicutes* relative abundance increased in *Pleurotus ostreatus*-bulk and -rhizosphere soil microcosms; on the other hand, in *I.lacteus*-augmented microcosms, an initial increase of *Proteobacteria* was observed whereas *Bacteroidetes* became dominant at the end of incubation. Analysing the fungal community structure in bioaugmented soils, *P.ostreatus* showed a higher ability than *I. lacteus* to compete with the autochthonous soil mycobiota. Indeed, *P.ostreatus* sequences accounted to more than 90% of the total fungal amplicons along the whole incubation period, thus proving the outstanding capability of this fungus to efficiently grow in PCB-contaminated soils under non-sterile conditions. By contrast, the large majority of fungal sequences in biostimulated microcosms belonged to the phyla *Ascomycota* and *Zygomycota*, with the exception of the topsoil where members of the phylum *Basidiomycota* became predominant in the later phase of the incubation

***ii***) Microsomal fractions rich in cytochrome P450 monooxygenase (CYP450) activities were isolated from the white rot fungi *Lentinus tigrinus* and *Pleurotus ostreatus* to evaluate their involvement in the biotransformation of CBAs and PCBs, respectively. In both cases, CYP450 was firstly detected by carbon monoxide-binding spectrum, and then used to perform *in vitro* degradation tests with selected compounds. Such intracellular enzymatic system was able to degrade either a mixture of CBAs (*L. tigrinus*) or PCBs (*P. ostreatus*). Specifically, the identification of a hydroxylated CBA confirmed the pivotal role of CYP450 in the initial transformation of CBAs. Moreover, a semi-purified laccase obtained from *P. ostreatus* was capable of degrading mono- and dichlorinated hydroxylated biphenyls, at different extent, either under mediated or non-mediated conditions. The chemical structure of chlorinated organic pollutants, namely the number and position of substituents, was the main factor affecting the extent of degradation by both fungal intracellular and extracellular enzymes.

**Keywords**: “white rot” fungi, mycoremediation, polychlorinated biphenyls, ligninolytic enzymes, cytochrome P450 monooxygenases system, 454-pyrosequencing.

***Riassunto***

Il risanamento di matrici ambientali contaminate da composti organoclorurati recalcitranti è divenuto una questione di fondamentale importanza a causa delle proprietà teratogeniche e cancerogene di tali composti, nonchè per le loro caratteristiche di interferenti endocrini. A tal proposito, le tecniche di risanamento biologico (biorisanamento) stanno riscuotendo un crescente interesse negli ultimi anni rispetto ai più convenzionali metodi chimico-fisici in virtù della loro comprovata sostenibilità ambientale ed economica. In particolare, i "funghi del marciume bianco", un sottogruppo ecologico dei funghi filamentosi specializzato nella degradazione della lignina, presentano delle caratteristiche interessanti, tali da renderli degli ottimi candidati per la messa a punto di tecniche di biorisanamento (micorisanamento). Tuttavia, le straordinarie capacità metaboliche di questi funghi non sono ancora state sfruttate appieno per fini di recupero ambientale ed i meccanismi attraverso i quali essi degradano i suddetti contaminanti clorurati non sono stati chiariti in dettaglio.

Detto questo, la presente tesi di dottorato ha avuto come scopo:

***i)*** la valutazione dell'efficacia di diverse tecniche di micorisanamento nel trattamento di suoli contaminati da policlorobifenili (PCB);

***ii)*** l'analisi dei meccanismi attraverso i quali i funghi del marciume bianco degradano (PCB) ed i loro principali metaboliti, acidi clorobenzoici (CBA) e policlorobifenili idrossilati (OH-PCB).

***i***) La fattibilità tecnica delle strategie di risanamento oggetto di studio è stata valutata mediante una combinazione di analisi chimiche, tossicologiche e biomolecolari. In una fase preliminare alle prove di risanamento sono state esaminate le proprietà chimico-fisiche (pH, tessitura, sostanza organica ecc.) di tre diversi campioni di suolo (suolo non di rizosfera, suolo superficiale e suolo di rizosfera) provenienti da un sito di stoccaggio ed inoltre, è stata determinata la biodisponibilità dei contaminanti in essi contenuti. Successivamente, tali matrici sono state sottoposte sia a trattamenti di bioaumento fungino (con *Pleurotus ostreatus* o *Irpex lacteus*, due noti agenti della carie bianca del legno) che di biostimolazione (attraverso l'aggiunta di ammendanti lignocellulosici). L'apporto di *P. ostreatus* nel suolo di rizosfera è risultato il trattamento più efficace in termini di rimozione dei PCB e detossificazione. In generale, l'identificazione di diversi prodotti di degradazione dei PCB (acidi clorobenzoici, clorobenzaldeidi, cresoli clorurati, idrossi- e metossi-PCB) ha dimostrato il coinvolgimento di diversi sistemi enzimatici fungini, intracellulari ed extracellulari, nella biotrasformazione dei suddetti composti. Inoltre, l'utilizzo di due approcci coltura-indipendenti, ovvero l'analisi

degli acidi grassi di membrana (PLFA) ed il pirosequenziamento (sequenziamento 454), ha permesso di ottenere informazioni circa la struttura e l'evoluzione delle comunità microbiche durante le prove di risanamento. L'analisi PLFA ha mostrato che l'aggiunta di inoculi fungini o di semplice substrato lignocellulosico ha avuto un effetto stimolante sulla crescita della popolazione batterica del suolo e che la più alta densità fungina è stata riscontrata nel suolo superficiale bioaumentato con *P. ostreatus* a metà del periodo di incubazione stabilito.

L'analisi della comunità batterica effettuata tramite metagenomica ha rivelato che il bioaumento di *P. ostreatus* sia nel suolo non di rizosfera che di rizosfera ha indotto un aumento dell'abbondanza relativa del phylum *Firmicutes*, mentre nei microcosmi inoculati con *I. lacteus*, i phyla dominanti erano *Proteobacteria* e *Bacteroidetes*, rispettivamente nelle fasi iniziali e finali del trattamento. Dall'analisi della comunità fungina, *P. ostreatus* è risultato migliore di *I. lacteus* nel competere con la microflora autoctona dei suoli. In particolare, *P. ostreatus* ha rappresentato, per l'intero periodo d'incubazione, oltre il 90% delle sequenze fungine nei tre i suoli in cui è stato introdotto, dimostrando di saper crescere in suoli contaminati da PCB in condizioni di non sterilità. Di contro, nei suoli biostimolati l'aggiunta di ammendante in forma sterile ha favorito la crescita di funghi appartenenti ai phyla *Ascomycota* e *Zygomycota*, con la sola eccezione del suolo superficiale in cui il phylum *Basidiomycota* è diventato predominante nella seconda metà del trattamento.

***ii***) Al fine di valutare il coinvolgimento del sistema enzimatico citocromo P450 monossigenasi (CYP450) nella degradazione di CBA e PCB, frazioni microsomali ricche di attività CYP450 sono state isolate dai funghi *Lentinus tigrinus* e *Pleurotus ostreatus*. L'attività del CYP450 è stata quantificata spettrofotometricamente al fine di effettuare successivamente delle prove di degradazione *in vitro* con i composti selezionati. Tale sistema enzimatico intracellulare è stato capace di degradare sia una miscela di CBA (*L. tigrinus*) che di PCB (*P. ostreatus*). In particolare, l'identificazione di una forma idrossilata di un acido clorobenzoico ha confermato il diretto coinvolgimento del CYP450 nelle fasi iniziali di trasformazione dei CBA. Inoltre, la degradazione di forme idrossilate di bifenili mono- e di-clorurati da parte di una laccasi semipurificata, estratta da *P. ostreatus*, è stata dimostrata sia in presenza che in assenza di uno specifico mediatore. La struttura chimica dei suddetti contaminanti ha rappresentato il principale fattore determinante le capacità degradative degli enzimi fungini sia intracellulari che extracellulari.

**Parole chiave**: funghi del marciume bianco, micorisanamento, bifenili policlorurati, enzimi ligninolitici, citocromo P450 monossigenasi, 454-pirosequenziamento.

***Abstrakt***

Remediace persistentních chlorovaných aromatických sloučenin se stala prioritou vzhledem k teratogenním, karcinogenním a endokrinním účinkům těchto xenobiotik. V současné době si získává při sanacích znečištěných lokalit stále větší pozornost použití biologických remediačních technologií, souhrnně označovaných jako bioremediace. Představují efektivní, cenově konkurenceschopnou alternativu šetrnou k životnímu prostředí ve srovnání s fyzikálně-chemickými dekontaminačními postupy. Jako vhodný kandidát pro vytvoření remediační technologie se jeví skupina ligninolytických basidiomycetních hub tzv. houby bílé hniloby (mykoremediace). Doposud však nebyly pro své metabolické schopnosti široce využívány a mechanismus, jakým jsou schopny degradovat výše zmíněné polutanty, není rovněž plně prozkoumán.

Tato disertační práce byla zaměřena na:

***i*)** zhodnocení účinnosti různých mykoremediačních strategií při odstraňování polychlorovaných bifenylů (PCB) z kontaminované půdy,

***ii*)** porozumění mechanismu specifických houbových degradačních drah PCB a jejich hlavních metabolitů (CBA, chlorbenzoové kyseliny a OH-PCB, hydroxylované polychlorované bifenyly).

***i*)** K vyhodnocení technické proveditelnosti vybraných remediačních strategií byla použita kombinace chemických, toxikologických a molekulárně-biologických technologií. Před uskutečněním samotné bioaugmentace (založenou na aplikaci ligninolytických hub *Pleurotus ostreatus* nebo *Irpex lacteus*) a biostimulace (přídavek lignocelulosového substrátu) byly u tří různých vzorků půdních horizontů (A – humusový horizont, B – půdní horizont, C – matečná hornina) ze skládky zeminy kontaminované PCB sloučeninami stanoveny fyzikálně-chemické vlastnosti (pH, struktura půdy, obsah organických látek atd.) a rovněž biodostupnost kontaminantu. Z hlediska schopnosti degradace a detoxifikace PCB byla nejúspěšnější strategie aplikující druh *P. ostreatus* do druhého typu půdního horizontu. Identifikace několika meziproduktů při odbourávání PCB (jako například chlorbenzoáty, chlorbenzaldehydy, chlorokresoly, hydroxylované a methoxylované PCB) demonstrovala zapojení jak intracelulárních, tak extracelulárních houbových enzymů při biotransformaci PCB. Kromě toho, za použití kombinace kultivačně nezávislých metod analýzy fosfolipidových mastných kyselin (PLFA) a 454-pyrosekvenace byl získán nový pohled na strukturu mikrobiální komunity, její divergenci a dynamiku v průběhu celého

bioremediačního procesu. Pomocí PLFA metody se ukázalo, že buď zavedení alochtonních hub nebo přidání lignocelulosového substrátu podporovalo růst bakteriálních populací, zatímco největší houbové koncentrace bylo dosaženo u druhu *P. ostreatus* a prvního typu půdního horizontu, ve střední fázi inkubace. Metagenomická analýza bakteriálního společenstva odhalila, že relativní výskyt kmene *Firmicutes* vzrostl při aplikaci druhu *P. ostreatus* v prostředí druhého a třetího půdního horizontu. Naproti tomu při aplikaci druhu *I. lacteus* byl pozorován zvýšený výskyt kmene *Proteobacteria* v počáteční fázi inkubace a dominantní nástup kmene *Bacteroidetes* na konci inkubace. Co se týče analýzy houbového společenství, *P. ostreatus* vykazoval vyšší schopnost konkurovat původnímu houbovému osazení než *I. lacteus*. Ve skutečnosti více než 90 % analyzovaných sekvencí bylo identifikováno jako *P. ostreatus* po celou dobu inkubace, což dokazuje vynikající schopnost tohoto druhu účinně růst v půdách kontaminovaných PCB za nesterilních podmínek. Naproti tomu velká většina analyzovaných sekvencí v případě biostimulace patřila ke kmenům *Ascomycota* a *Zygomycota*, s výjimkou pozdní inkubační fáze u prvního typu půdního horizontu, kde dominoval kmen *Basidiomycota.*

***ii*)** Mikrosomální frakce bohaté na cytochrom P450 monooxygenasu (CYP450) byly izolovány u hub bílé hniloby *Lentinus tigrinus* a *Pleurotus ostreatus* za účelem posouzení jejich zapojení do biotransformace CBA a PCB. V obou případech, byla nejdříve CYP450 detekována pomocí spektra v komplexu s oxidem uhelnatým, a poté byla použita na in vitro degradační testy s vybranými sloučeninami. Takovýto intracelulární enzymatický systém byl schopný degradovat buď CBA (*L. tigrinus*), nebo PCB (*P. ostreatus*). Konkrétně, klíčová úloha CYP450 v počáteční fázi transformace CBA byla potvrzena identifikováním hydroxylovaných CBA. Kromě toho nedokonale přečištěná lakasa získaná z druhu *P. ostreatus* byla schopna degradovat mono- a dichlorované hydroxylované bifenyly, v různé míře, buď za optimalizovaných, nebo neoptimalizovaných podmínek. Hlavním faktorem, který ovlivňoval rozsah degradace jak intracelulárních, tak extracelulárních houbových enzymů, byla chemická struktura chlorovaných organických kontaminant, jako je počet a poloha substituentů.

**Klíčová slova**: houby bílé hniloby, mykoremediace, polychlorované bifenyly, ligninolytické enzymy, cytochrom P450 monoxygenasový system, 454-pyrosekvenace.

**CHAPTER 1**

**CHAPTER 2**

# CHAPTER 1

# GENERAL INTRODUCTION

## 1.0 Preface

The intensive industrial processes and agricultural practices have been leading to the deliberate or accidental release of potentially toxic chemicals into the environment during the last decades. Nowadays, millions of molecular species of either natural or anthropogenic origin are estimated to be present in the biosphere (Hou *et al*., 2003). These pollutants have become a serious problem worldwide due to their adverse effects on natural ecosystems and human health. They can affect any environmental compartment (air, water and soil): they could be tightly bound into or onto the soil organic matter (SOM) and clay particles, released into the atmosphere by volatilization or leaked into the water. Moreover, living organisms face great complicacies in attempting to degrade most of these compounds. Consequently, they can be accumulated in the food chains and in the environment having a significant impact also on nutrients cycling. They include various organic and inorganic pollutants (Walker *et al*., 2006): petroleum hydrocarbons, halogenated solvents, chlorinated aromatic hydrocarbons, explosives, dioxins, endocrine disrupting compounds, herbicides, pesticide, heavy metals and radionuclides. Organics mostly occur in petrochemical plants, petroleum refineries, gas stations and wood preservative industries, whereas halogenated pollutants are usually found in chemical manufacturing plants, pesticides/herbicides treated fields, marine sediments and landfills. Explosives, such as trinitrotoluene (TNT), contributed to the contamination of military areas and marine sediment, while heavy metals (*i.e.* cadmium, arsenic, chromium and lead) are mainly present in mining sites, marine/river sediments and chemical disposal areas. However, pollutants are commonly present in the environment as complex mixture making the remediation of these co-contaminated sites a challenge to be faced.

## 1.1 Polychlorinated biphenyls

Polychlorinated biphenyls (PCBs), $C_{12}H_{10-n}Cl_n$, are synthetic compounds the structure of which consists of a biphenyl structure (two aromatic rings linked by a C–C bond) that carry from one to ten chlorine atoms. Theoretically, 209 congeners are possible comprising the entire set of PCB homologs and isomers. Based on IUPAC convention, one benzene ring is labelled clockwise and the other counter-clockwise with ordinal numbers assigned to chlorine substituents as shown in *Figure 1.1*. Additionally, a numbering system was developed to assign a sequential number to each of the 209 PCB congeners (Ballschimiter and Zell, 1980).

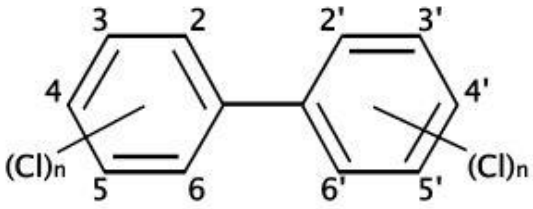



***Fig. 1.1.*** Structure of Polychlorinated Biphenyls molecule.

PCBs were synthesized for the first time in 1881, even though their use at industrial scale was started in 1929 by the Monsanto Company in the United States. Hence, during the mid-twentieth century, polychlorinated biphenyls were produced worldwide as mixtures and sold under different trade names: Aroclor (USA and United Kingdom), Clophen (Germany), Phenoclor (France), Kanechlor (Japan), Fenclor (Italy), Sovol (former URSS), Delor (former Czechoslovakia). These mixtures were manufactured at temperature above 150 °C by direct chlorination of biphenyl with anhydrous chlorine and using iron filings or ferric chloride as catalysts. Due to mechanistic and statistical constraints, about 20 congeners are completely absent in the commercial mixtures: approximately 189 of the 209 possible PCB molecules have been identified in Aroclor and other PCB mixtures (Hutzinger *et al*., 1974).

Most PCBs mixtures are oily, almost transparent liquids, the colour and viscosity of which increase with rising chlorine content. Due to their thermal and chemical stability, flame resistance features and dielectric properties, PCBs were widely used as dielectric liquids in electrical transformers and capacitors, as well as heat-exchange fluids, hydraulic liquids, plasticizers, dust-control agents, adhesive substances and dye carriers in carbonless copy-

paper. The production of PCBs, which was approximately 1000 tons/year in the early 30s, increased up to 200,000 tons/year in 1975 (Abraham *et al*., 2002).

The widespread use of PCBs led to an extensive environmental contamination through accidental releases and/or inappropriate disposal techniques. Even PCBs used in closed systems, such as in electrical equipment, entered the ecosystems due to accidents or leakage. Early reports from Swedish researchers revealed the presence of PCBs in soil and water samples that were being screened for DDT (Jensen, 1966). Later, other studies demonstrated that the biodegradation rate of these synthetic compounds was extremely slow, especially for highly chlorinated biphenyls (Jensen, 1972). Afterwards, two significant events involving direct overexposure of humans to PCBs from contaminated foods firmly endorsed the public perception of PCBs as environmental health risk: clinical manifestations, such as somatic dysfunctions, chloracne and hyperpigmentation, were associated with the ingestion of polychlorinated biphenyls- and dibenzofurans-contaminated rice oil (Rogan *et al*., 1988; Yu *et al*., 2000). Consequently, the Monsanto Company restricted the production of Aroclor PCB mixtures to those ones containing less than 60% of chlorine. Thereafter, the US Environmental Protection Agency (EPA) banned the production and application of PCBs (1979). Consecutively, all the other countries drastically ruled out their manufacture.

Notwithstanding, owing to their aforementioned inertness, PCBs are still present in a number of areas where their production has been carried out for decades. About 1.5 million tons of PCB are still used today (mainly in closed systems) and about 0.5 million tons still reside in the biosphere, mainly in soil and water sediments nearby their former production plants (Vasylieva *et al*., 2010). The range of PCB concentrations in the above-mentioned sites could fluctuate between 10 and $10^4$ mg $Kg^{-1}$ of soil (Vasilyeva & Strijakova, 2007). Such values are several orders of magnitude higher than the limits which range between 0.01 and 50 mg $Kg^{-1}$ depending on the country and the land use.

## 1.2 Environmental fate of PCBs

Once released into the environment, the fate of PCBs is mostly determined by their physical and chemical properties which depend mostly on their degree of chlorination and on the isomeric chlorine substitution pattern (Reid *et al*., 2000; Delle Site, 2001). Octanol-water partition coefficient ($K_{ow}$), water solubility (WS), vapour pressure (Vap P) and soil/sediment organic carbon-water partition coefficients ($K_{oc}$) can significantly affect the transport and the transformation of these chemicals (EPA, 1983; Hawker *et al*., 1988; van Noort, 2009; Cicilio, 2013). Several phenomena, such as partitioning, bioaccumulation processes and chemical

and/or biological transformations can occur over time and, therefore, the composition of PCB mixtures in the environment differs substantially from that of the original commercial mixtures (Cogliano *et al.*, 1998; Robertson & Hanse, 2001).

Generally, PCBs tend to be strongly adsorbed to organic materials, sediments and soils, especially the high-chlorinated congeners that become more resistant to degradation processes (Cortes *et al.*, 1991; CCME, 1999). As a matter of fact, PCBs are mainly transformed through microbial degradation in the soil system. Mono-, di- and tri-chlorinated biphenyls are susceptible to a relatively rapid biodegradation under aerobic conditions (Pieper, 2005; Field & Sierra-Alvarez, 2008), whereas higher-chlorinated biphenyls are slowly transformed (mainly *via* reductive dehalogenation) under anaerobic conditions (Abramowicz, 1990; Wiegel & Wu, 2000). As a consequence of this congener-specific susceptibility to degradation, higher chlorinated biphenyls tend to be accumulated in the soil compartment with the formation of altered persistent PCB mixtures.

However, transport of PCBs to nearby surface water bodies can occur as a result of surface water runoff process. In the aquatic environment, as in the soil system, higher chlorinated and coplanar congeners (mono- and non-*ortho* substituted PCBs) display a strong tendency to be adsorbed to sediments or suspended matter. Thus, the PCB composition in the water will be enriched in the lower chlorinated PCBs due to their greater water solubility and lower $K_{ow}$, while the least water soluble PCBs will remain immobilized on sediments for relatively long periods of time. PCBs are hydrolytically so stable that, even under severe acidic and basic conditions, hydrolysis reactions cannot occur as well as oxidation processes (EPA, 1983). The more highly chlorinated PCBs may undergo photolysis, but this process does not significantly affect their fate (EPA, 1983).

On the other side, due to their high lipophilicity, higher-chlorinated biphenyls tend to partition into the fatty tissues of aquatic organisms (Webster *et al*., 2013). Since the metabolism of these compounds is relatively slow, they may be accumulated over time and concentrated through the food chain, producing residues that are considerably different from the original PCB mixtures (Cogliano *et al.*, 1998; Robertson & Hanse, 2001). Thus, the biomagnification of PCBs leads to greater PCB concentrations along the trophic transfer: PCB amounts will be higher in shellfish than in the plankton on which they feed, and even greater in animals at the top of the food chain such as large predatory fishes or mammals (seals, dolphins, and whales) (Zaranko *et al*., 1997; Berglund *et al*., 2005; Webster *et al*., 2013). The observation of this process in all contaminated aquatic systems (*i.e.* the Baltic Sea and the North American Great Lakes) confirms that biomagnification process represents one of the most important aspects of

PCB environmental fate in the aquatic system (Borga e*t al*., 2005; Burreau *et al*., 2006).

On the other hand, the non-adsorbed PCBs (mostly low chlorinated compounds) can be transported into the atmosphere *via* volatilization from aqueous phase (EPA, 2001; Chiarenzelli *et al.*, 2001; Totten *et al.*, 2003). Although the rate of this process can be low, the total loss by volatilization gradually may be significant because of the stability of PCBs in the air. PCBs primarily exist in the vapour phase and rarely in association with the particulate phase (Hillery *et al*., 1997). In particular, PCBs in the vapour phase are enriched in di- and tri-*ortho* congeners due to their higher vapour pressures, while coplanar and higher chlorinated PCBs tend to bind to aerosols particulate. From a chemical point of view, the only significant atmospheric transformation process could be attributed to the oxidation mediated by hydroxyl radicals or to a lesser extent by ozone (Anderson & Hites, 1996; Totten *et al*., 2002).

PCBs can be also physically removed from the atmosphere by wet deposition (*i.e*. rain and snow scavenging of vapours), by dry deposition of aerosols or by vapour adsorption at the water-air or air-soil interfaces (Gioia *et al*., 2013).

All these processes contribute to the global dispersion of PCBs: small concentrations of these chemicals were detected even in the more remote areas of our planet, such as the Norwegian Sea and the Eastern Arctic (Beyer *et al.*, 2009; Ubl *et al.*, 2011).

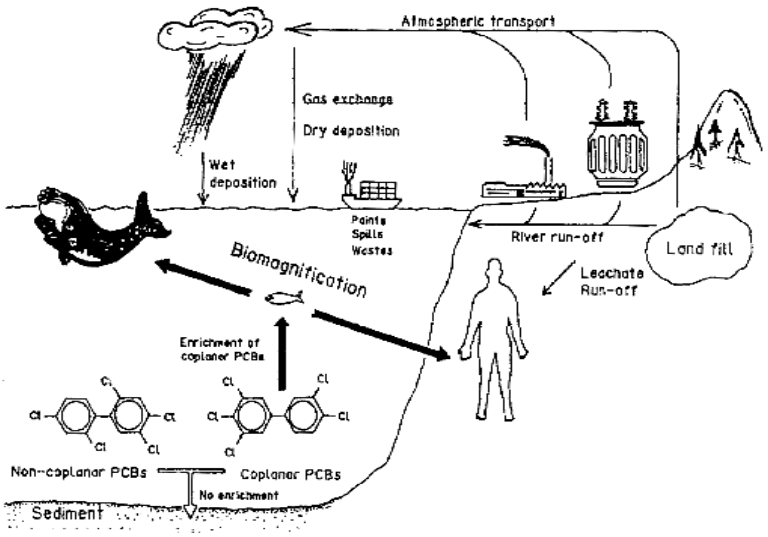



***Fig.1.2.*** Environmental recycling of polychlorinated biphenyls.

## 1.3 PCBs in the soil environment

The amount of PCBs released into the soil has decreased over the years due to the prohibition on their production and the restrictions on processing and reuse of existing PCB-containing materials. However, due to their stability to weathering forces, PCBs are still located in soils and water sediments close to their former production plants (Vasilyeva & Strijakova, 2007). Moreover, accidental leaks or spills from old capacitors, electrical transformers and other equipment as well as releases from containers in landfills and hazardous waste sites still represent a notable source of PCBs, likewise the environmental cycling process involving deposition of atmospheric PCBs to soil surfaces.

Soil acts as a sink as well as a source of PCBs for natural ecosystems: PCBs may enter the aquatic environment, river and lakes and subsequently to the oceans, as already explained above, or the atmosphere through volatilization of lower chlorinated congeners. However, in the soil environment, PCBs fate and behaviour are governed by various factors, such as the chemical properties of contaminants, the soil characteristics and several environmental factors (Reid *et al.*, 2000; Semple *et al.*, 2003). In particular, physico-chemical properties of soil such as organic carbon content (OC), clay content, texture, pH, water holding capacity (WHC), cation exchange capacity (CEC) significantly affect soil sorption/desorption processes and, therefore, the fate of PCBs (NEPI, 2000).

Organic matter content is thought to be the most significant factor dominating organic compound interactions within soil, but also their structure and degree of chlorination can affect the behaviour in soils (Pu *et al.*, 2006). In this respect, PCB binding tendency onto the soil particles increases with raising amounts of organic matter and the higher the hydrophobicity of these chemicals, the greater their sorption potential (Schwarzenbach *et al.*, 2003). Generally, higher chlorinated compounds are more easily adsorbed as demonstrated by Jonker and Smedes (2000): the OC-partition coefficients (Koc) determined from contaminated sediments were lower for highly chlorinated non-planar, *ortho*-substituted PCBs compared to those for planar compounds with low chlorine content.

Furthermore, clays, which are characterized by high surface areas, may enhance sorption through weak physical or electrostatic interactions and also limit the chemical mass transfer due to the formation of clay aggregation and interlayers (Mader *et al*., 1997; Ake *et al*., 2001). As a result, with the raising of organic carbon and clay content, chemical retention increases and rates of chemical release to the aqueous phase decrease.

Upon entering a soil system, generally, pollutants fill up macropores and the surface particles which contain a relatively low number of bacteria (*Figure 1.3*). Afterwards, PCBs can diffuse into smaller pores where the microbial activity is fostered by the specific environmental conditions of these compartments, and thus biotransformation may take place. Thereafter, PCBs tend to slowly diffuse within extremely small pores of the solid organic matter fraction, possibly the lipid fraction (Alexander, 2000) or within the micro- and nano-pores of the mineral fraction (Hatzinger and Alexander, 1995). The slow entrapment of chemicals into these sites of soil matrix, which are not accessible to microorganisms, is called "ageing". This process enables high amounts of pollutants to tightly bind to soil particles and thereby, to form a fraction of contaminants which results to be extremely recalcitrant to any type of process, especially biological transformation processes (Alexander, 2000). Indeed, the sequestration of pollutants, due to ageing, negatively affects their bioavailability, term which refers to that fraction of a chemical in a soil that can be taken up or transformed by living organisms.

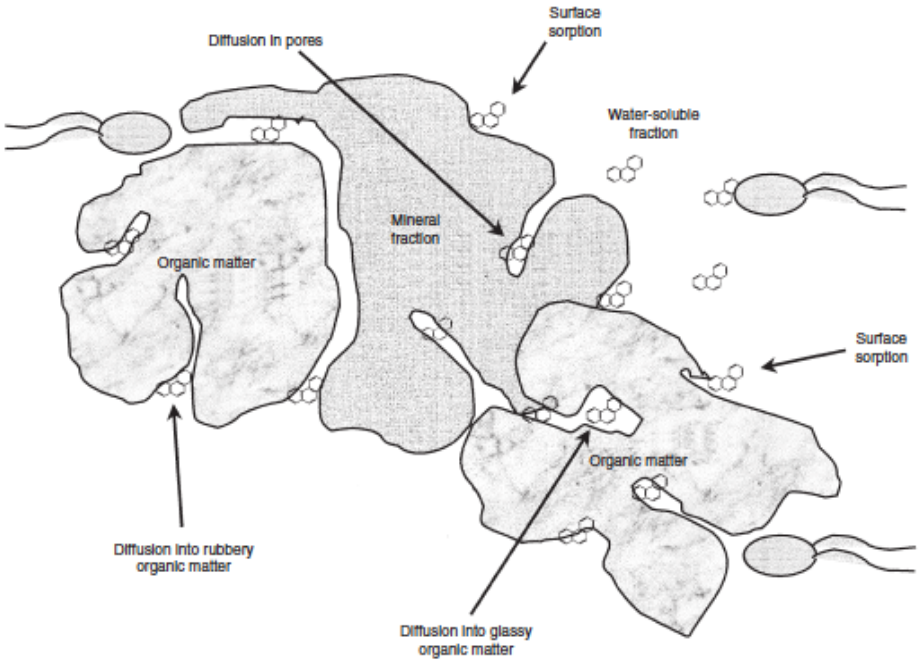



***Fig. 1.3***. Physical behaviour of a contaminant within the soil (from Semple *et al*., 2003).

The influence of ageing on the extractability and bioavailability of organic compounds in soil is shown in *Figure 1.4*. Over time, the readily available fraction (easily extractable or bioavailable fraction) diminishes in a biphasic manner: a portion is biologically degraded or physically/chemically removed, whereas the other one is gradually transformed into the recalcitrant fraction. Thereafter, the latter component may be accessible only by specific aggressive extraction methods or even non-extractable after a considerable time period (Macleod & Semple, 2000).

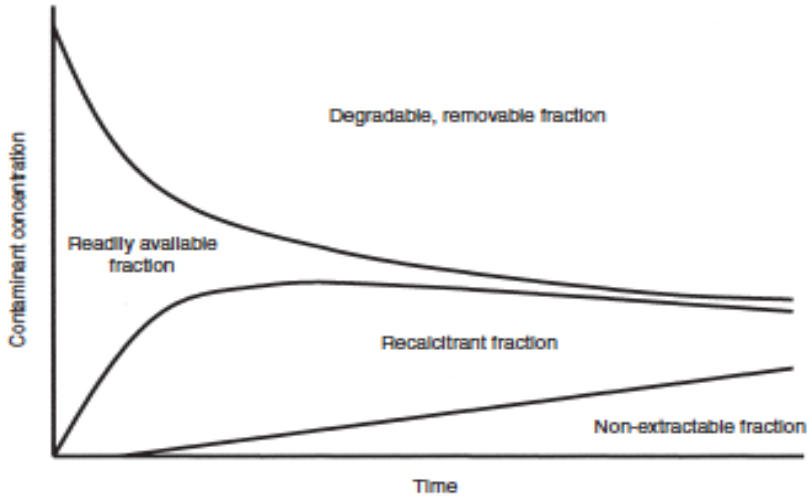



***Fig.1.4***. The influence of contact time on the extractability and bioavailability of a contaminant (from Semple *et al*., 2003).

## 1.4 PCB toxicity

The toxicity of PCBs has been a subject of debate and research for a long time. At present, PCB exposure still raises public health concerns due to the consumption of contaminated food by those populations living close to polluted seas, rivers and lakes, such as the Great Lakes (Turik *et al.*, 2006) and the Baltic Sea (Kiviranta *et al*., 2000) or in areas where PCB producing factories were located, such as Anniston, USA (Silverstone *et al*., 2012), Michalovce, Slovakia (Pavuk *et al*., 2004) and Brescia, Italy (Donato *et al.*, 2006).

The intake of contaminated foods, particularly meat, fish and poultry represents the primary route of exposure to PCBs (ATSDR, 2000). In aquatic environments, the high lipophilicity of PCBs causes these compounds to partition out of the water and to preferentially adsorb onto sediments. Although this phenomenon could prevent the contamination of drinking water supplies, the partitioning of PCBs to sediments contributes to their concentration in the aquatic organisms, especially in bottom-feeding fishes. Moreover, the resistance of PCBs to

biodegradation favours their bioaccumulation moving upward through the food chain (ATSDR 2000).

In addition to dietary exposure, humans are still exposed to some consumer products containing PCBs, including old fluorescent lighting fixtures, electrical devices or appliances. In this case, PCBs can enter the human body through the respiratory tract or dermal route: overheated equipment that contains PCBs can vaporize significant amounts of these compounds, creating an inhalation hazard or PCBs can also be absorbed through the skin following contact with contaminated equipment (ATSDR, 2000). Afterwards, PCBs can be transported by the blood stream to the organs (mainly liver and kidneys), to the muscles and, finally, to adipose tissues or other fat-containing compartments where they are accumulated. Fetuses and neonates are potentially more sensitive to PCBs than adults because the hepatic microsomal enzyme systems that facilitate the metabolism of PCBs are not fully functional (Nieuwenhuijsen *et al*., 2013). Furthermore, considering that PCBs are metabolized mainly in the liver, the health risk related to PCBs increases for those people with impaired hepatic function because of their diminished ability to detoxify and excrete these compounds (ATSDR, 2000).

Nowadays, PCBs, classified as "Persistent Organic Pollutants-POPs" by the Stockholm Convention (2001), are considered among the most hazardous contaminants in the world placing them at the forefront of public health concern (Ross, 2004). The most toxic congeners have a coplanar conformation with chlorine substituents in both *para* positions and at least two *meta* positions, making them stereochemically similar to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (EPA, 2000; Van den Berg *et al*., 2006). Therefore, these congeners exhibit "dioxin-like" toxicity features.

PCB exposure causes overt effects such as acute chloracne, rashes, skin irritation as well as cardiovascular, musculoskeletal and gastrointestinal discomforts (Zani *et al.*, 2013). For a long time, sensory (skin and eye) irritations remained the only consistent health effects that could be definitely attributed to PCBs (Ross, 2004).

However, recent studies indicated that PCBs can affect also the immune, neurological and endocrine systems (Crinnion *et al*., 2011; Kramer *et al*., 2012; El Majidi *et al*., 2013). They are potent inducers of monocytes and thymocytes apoptosis resulting in a reduced number of white blood cells able to initiate an immunological defence (Crinnion *et al*., 2011).

Moreover, alterations in functions of the immune system, such as anomalous antibody production and hypersensitivity, were shown in rodents and primates exposed to higher chlorinated Aroclor mixtures (Carey *et al*., 1998). PCBs can also interact with several

functions of the endocrine system causing reproductive, neurobehavioral and neurodevelopmental disorders (Crinnion *et al.*, 2011; Kramer *et al.*, 2012). These chemicals, in fact, can mimic the action of naturally occurring hormones (*e.g.* thyroid hormones and estrogens) that are essential for normal intellectual and neurologic development: deficits in learning, memory and attentional processes were observed in PCB-exposed women due to the alteration in hormonal binding to the thyroid hormone receptor suggesting a possible mechanism of thyroidotoxicity (EPA, 1998). Thus, PCBs have been classified as "endocrine disruptors".

Furthermore, because of their estrogenic properties, PCBs have been also proposed as possible inducers of breast cancer in PCB-exposed women (Muscat *et al.*, 2003; Helmfrid *et al.*, 2012). An increased incidence of liver, gall bladder, biliary tract and brain in occupationally exposed workers or generally of non-Hodgkin lymphoma has been also reported (WHO-IARC, 1987; ATSDR, 2000; Kramer *et al*., 2012). However, the results of these epidemiologic studies resulted to be inconsistent to ascertain whether the observed effects were directly related to PCB exposure (ATSDR, 2000; Brody *et al.*, 2007). On the contrary, data from animal studies have clearly shown that PCBs cause hepatocarcinomas, pituitary tumors, leukemia, lymphomas, and gastrointestinal tract tumors (Norback *et al.*, 1985; Beebe *et al*., 1993; EPA, 1996; Mayes *et al*., 1998; Knerr *et al*., 2006; NTP, 2011). Accordingly with these results, the US Environmental Protection Agency and the International Agency for Research on Cancer classified PCBs as probable human carcinogen (EPA, 1996; WHO-IARC, 1998).

## 1.5 Remediation technologies

Generally, environmental remediation deals with interventions aimed at removing the pollutants from a given environmental matrix or, at least, at reducing their concentration. The conventional remedial strategies consisted in the excavation of the polluted materials with subsequent burial in dumpsites, or in coating the polluted area with insulating barriers. The former resulted risky, especially during excavation procedures and movement to the dumpsite, whereas the latter represents just a temporary solution, causing expensive monitoring and maintenance.

Latest remedial approaches, which aim at either the complete removal of the contaminants present in the soil or at their transformation into lesser harmful substances, can be divided in:

• *ex situ* treatments: the contaminated material is dug out and transported to the treatment plant, which can be in loco (*on site*) or settled elsewhere (*off site*). Afterwards, the treated matrix could be placed back.
• *in situ* treatments: the contaminated material is treated directly in its natural location, with a variety of advantages with respect to *ex situ* treatments. Wider range of technologies available and more homogeneous removal of the pollutants make *in situ* treatment preferable in many cases.

In addition to the former classification, mainly based on the site where the remedial actions take place, the currently available technologies can be grouped into different kind of treatment: thermal, physicochemical and biological treatments.
Information concerning the site history and topography as well as the physicochemical characteristics of the contaminated soil, sediment or groundwater provides a comprehensive assessment to select the most suitable and site-specific reclamation technology. Moreover, the presence of other contaminants should be assessed due to its impact on the effectiveness of a remediation process.

### 1.5.1 Remediation of PCBs

Polychlorinated biphenyls represent a serious problem worldwide due to their adverse effects on both natural ecosystems and human health. Nowadays, PCBs are still considered among the most hazardous contaminants in the world and the remediation of PCB-polluted sites has become a major issue of concern.
Several technologies that have been used to treat PCBs are well established (*i.e.* incineration) and thus already employed at full-scale level at multiple sites, whereas other remedial approaches (*i.e.* thermal desorption or solvent extraction) have been conducted at pilot- or full-scale in a limited number of sites. Currently, these methods (thermal and chemical treatments) are the most applied ones for the disposal of hazardous chlorinated wastes. In particular, incineration plays the absolute dominant role in the PCB-contaminated matrices remediation management. On the other hand, since these techniques are expensive, not always effective and even dangerous entailing additional risks, the scientific research has been focusing on biological treatments as an environment "friendly" and cost-effective alternative for the clean-up the PCB-contaminated matrices. These emerging technologies have not been shown to consistently recover PCB-polluted soils or sediments at pilot-scale level yet, but the number of bench-scale studies is increasing over time in view of their potential applications.

#### 1.5.1.1 Thermal and physico-chemical treatments of PCBs

Thermal treatments are applied to destroy (*i.e.* incineration) or separate (*i.e.* thermal desorption) contaminants reducing the volume and the mass of the contaminated material and making the hazardous components inert. Physicochemical soil treatment processes are mainly extraction processes aimed at the concentration of contaminants rather than at their removal (*i.e.* solvent extraction). Moreover, some physical procedures are used to stabilize the polluted material (*i.e.* solidification/stabilization) by the conversion of hazardous elements into less soluble, mobile or toxic forms.

##### 1.5.1.1.1 Incineration

The contaminated soil or sediment is excavated and moved to the incinerator (it is usually constructed *on site* for those scenarios where the clean-up process is expected to require several years). After the removal of oversized particles from the matrix to be treated, the hazardous organic contaminants are burnt at high temperatures (greater than 800 °C) in the presence of oxygen which causes volatilization, combustion and destruction of these compounds. The applicability of this process may be limited by the presence of metals which can react to form other metal species that can persist in the treated soil. Moreover, this procedure releases off-gases that need to be treated by the use of cyclones, baghouses, wet and packed scrubbers prior to their release into the atmosphere due to the formation of toxic by-products such as polychlorinated dibenzo-*p*-dioxins (PCDDs) and dibenzofurans (PCDFs).

##### 1.5.1.1.2 Thermal desorption

Thermal desorption is an *ex situ* technique to physically separate organic contaminants from soil, sediment or sludge by heating at temperatures high enough to make them volatile (90-650°C). The temperature achieved and the residence time used by the thermal desorption system will volatilize selected PCB congeners and drive off water (no oxidation or complete breakdown of organic chemicals as by incineration). Off-gases, enriched in volatile organics, are formed and collected on active carbon filter units or in condensation equipment. Once recovered, they can be treated in a catalytic oxidation unit which is integrated into the thermal desorption system, while the treated matrix can be placed back to the original site.

#### 1.5.1.1.3 Solvent extraction

Solvent extraction, as thermal desorption treatment, does not destroy organic contaminants but separate them from soils or sediments, thereby reducing the volume of hazardous waste to be treated. An organic solvent is applied to extract, collect and concentrate PCBs: the polluted solid matrix and solvent are mixed in the extractor and, thus organic contaminants partition into the solvent. Thereafter, the extracted organics are moved in a separator where, changing pressure or temperature, contaminants are separated from the solvent. The solvent can be recycled, whereas the concentrated PCBs require further treatment, such as incineration or dehalogenation.

#### 1.5.1.1.4 Chemical dehalogenation

Dehalogenation is a chemical process to remove halogen atoms (chlorine atoms for PCBs) from organic molecules. This process includes technologies such as base-catalysed decomposition (BCD), alkaline metal hydroxide/polyethylene glycol (APEG) and potassium metal hydroxide/ polyethylene glycol (KPEGTM). The BCD process is deemed to be highly efficient and relatively inexpensive for PCB treatment: the contaminated matrix is mixed with sodium bicarbonate ($NaHCO_3$) and heated at 330 °C in the presence of a hydrogen donor such as sodium hydroxide, sodium bicarbonate or aliphatic hydrocarbons. PCBs are completely dechlorinated, partially volatilized and, thus, condensed to be treated. At the end of the process, the water phase is separated from the soil and treated appropriately and the remediated soil is brought back to its original location.

#### 1.5.1.1.5 Solidification/Stabilization

Solidification/Stabilization (S/S) technology involves the addition of Portland cement as a binder, often augmented with other materials, such as fly ash, cement kiln dust and lime, to convert contaminants into a less soluble, mobile or toxic form. The binding reagent can both solidify (change the physical properties) and stabilize (change the chemical properties) PCBs. Solidification increases the compressive strength, decreases the permeability and encapsulates toxic elements while stabilization converts hazardous elements into less soluble and mobile forms. This process can be applied either *ex situ* or *in situ*: both approaches require the mixing of soil with the binding agent and water in a batch or continuous system and the treatment of off-gases, when needed.

## 1.6 Bioremediation

Bioremediation is a grouping of technologies that use biological systems to degrade or transform hazardous contaminants to carbon dioxide, water, inorganic salts, microbial biomass and other by-products that may be less hazardous than the parent materials (EPA, 2006). In general, bioremediation relies on either microorganisms (bacteria and archea) or their enzymes as contaminant degraders; the specific terms mycoremediation and phytoremediation indicate the main use of fungi and plants, respectively, for the clean-up purposes (Singh & Ward, 2004; Singh, 2006).

Bioremediation technologies may be classified into two categories: *in situ* and *ex situ*. *In situ* processes treat soils, sediments or ground waters in place to regain their original condition, whereas e*x situ* techniques involve the excavation of contaminated samples to be transported to specific facilities for the treatment process. The former approach may be advantageous since the costs of materials handling/transportation are avoid and some inherent environmental impacts may be reduced. In the case of soils, for example, the polluted matrix does not need to be excavated and moved elsewhere and thus, soil properties and functions are better preserved (Mirsal, 2008). However, *in situ* processes may be limited by the ability to control or manipulate the physical and chemical reactions which can occur during the recovery process (EPA, 2006). *Ex situ* technologies represent a suitable alternative to the *in situ* ones especially when dealing with contaminated soils characterized by low permeability and high organic matter content. In this case, *ex situ* processes allow to operate in a manner that facilitates degradation of the contaminant of concern even if its concentration and recalcitrance is high. Moreover, these techniques are preferred when climate conditions can hinder *in situ* treatment processes or when authorities require the remediation to be achieved rapidly (Cookson, 1995; Eweis *et al*., 1998; Robles-Gonzales *et al*., 2008).

*In situ* and *ex situ* treatments may be applied either by supplying chemical amendments such as air (oxygen), organic substrates, nutrients (mainly nitrogen and phosphorous), reducing agents and/or electron donors to stimulate contaminant biodegradation by autochthonous microbial populations (*biostimulation* or *enhanced bioremediation*) or by introducing native or non-native microbes to enhance the degradation process which can not be accomplished by indigenous microbial communities (*bioaugmentation*) (EPA, 2010; Megharaj *et al*, 2011).

Recently, several attempts have been done to promote *monitored natural attenuation* (MNA or *intrinsic bioremediation*) as a clean-up tool for organic and inorganic contaminants: biological, chemical and physical naturally occurring processes can degrade or immobilize

harmful contaminants (EPA, 2008; EPA, 2009). Natural attenuation, which includes a combination of effective sorption, volatilization, dilution and dispersion processes coupled with biodegradation, provides significant benefits in terms of cost and efforts (Sanchez *et al*., 2000). In fact, this approach requires just careful site assessment, ongoing processes monitoring and adjustment of temperature, pH or other factors to enhance the intrinsic remediation process. However, natural bioattenuation is accepted as a suitable method only for treating few classes of contaminants (*e.g.* BTEX) (Atteia & Guillot, 2007). In many cases, this approach turns out to be inadequate and protracted, especially with oligotrophic soils with low microbial density (Megharaj *et al.*, 2011).

The success of any bioremediation technique depends on several factors which characterized the polluted site, such as temperature, pH, oxygen, nutrient concentration and water content (Scullion, 2006) as well as on type, concentration and bioavailability of contaminants of concern, presence of co-contaminants, accumulationof dead-end degradation intermediates and contaminant residues and, thus, possible routes of exposure (Scullion, 2006; EPA, 2006). Thereupon, evaluation of all these factors is needed when bioremediation technologies are preferred to thermal or physico-chemical treatments.

The ideal biological decontamination "machinery" should be able to cope with scarce availability of pollutants, highly toxic environments, lack of nutrients, water or electron acceptors/donors. Despite the ubiquity and the wide catabolic diversity of bacteria, these microorganisms cannot underpin all these conditions. However, the majority of bioremediation strategies developed to date are reliant on bacteria. On the other hand, fungi display many biochemical, metabolic and ecological features that make them excellent candidates to design an effective remediation technology. Nevertheless, the broad metabolic versatility of fungi has not been widely exploited for its potential in bioremediation of hazardous chemicals (Harms *et al.*, 2011). Another alternative to be taken into account is phytoremediation: the use of plants in cleaning up contaminated sites is growing over time, even if some limitations (not applicable to deep and heavily contaminated site) have already been demonstrated. In view of all these consideration, the combination of different approaches may overcome the limitations of each single bioremediation technology enabling the complete site restoration.

### 1.6.1 Bioremediation of PCBs

Microorganisms play a key role in PCB biodegradation processes in both liquid and solid natural environments. Because of their ubiquitous nature, wide diversity and capabilities in catalytic mechanisms and ability to withstand extreme conditions (*i.e*. highly toxic environments, absence of oxygen, *etc*.), the search for pollutant-degrading microorganisms, the understanding of their genetics and biochemistry and the development of methods for their applications at larger scale have become an important endeavour for scientific research. However, despite the abundance of PCB-degrading microorganisms in the environment, PCBs are extremely recalcitrant to any degradation process (Vasilyeva & Strijakova, 2007). The persistency of PCBs is mostly due to the behaviour of these chlorinated compounds. As explained above, when PCBs enter the natural matrices, because of their low water solubility and high hydrophobicity, they can be quickly and tightly adsorbed by solid phase, primarily by organic matter, becoming poorly accessible to bacteria. The use of fungi, in particular filamentous fungi, and/or plants could overcome these limitations in the bacterial removal of PCBs from contaminated materials.

#### 1.6.1.1 Anaerobic bacterial degradation of PCBs

The role of anaerobes in the degradation of PCBs was firstly shown by Brown and co-workers (1987). Analysis of congener patterns in anaerobic sediments of Hudson River revealed that the congener distribution of a known PCB mixture was radically altered over a long period of time resulting in the accumulation of lower chlorinated compounds. Highly chlorinated compounds were extensively removed by reductive dehalogenation reactions giving rise to a higher proportion of lower chlorinated congeners. Afterwards, these findings were confirmed by incubating these sediments with Aroclor PCB mixtures 1242, 1248, 1254 and 1260 in laboratory microcosms (Quensen *et al.*, 1990). In particular, this study demonstrated that the transformations specifically involved the removal of *meta*- and *para*-chlorines resulting in the increase of lower *ortho*-chloro substituted congeners. From a toxicology standpoint, the loss of *para*- and *meta*-chlorines results to be advantageous due to the recalled higher toxicity of these co-planar congeners which are structurally similar to 2,3,7,8- tetrachloro-*p*-dioxin.

In river and marine sediments, reductive dehalogenation is accomplished by anaerobic bacteria, mostly belonging to *Chloroflexi* phylum and the *Dehalococcoides* genus, capable of anaerobic respiration with sulfates, carbonates, nitrates and other oxidized compounds as electron acceptors (Field & Sierra-Alvarez, 2008). In the presence of chlorinated pollutants as PCBs, these anaerobes may switch to the dehalorespiration process where highly oxidized

compounds, particularly higher-chlorinated biphenyls, are used as electron acceptors for energy storage. As a consequence, the dechlorination rate generally decreases with decreasing of chlorine substitution number: as mono- and dichlorinated biphenyls are formed and accumulated in the anaerobic environment, the activity of anaerobes is drastically reduced (Vasilyeva & Strijakova, 2007).

Eight distinct microbial dechlorination routes were observed either in natural environments or laboratory microcosms comparing the patterns of lost and formed congeners (Wiegel & Wu, 2000). Environmental factors (temperature, pH, available carbon source, electron donors as $H_2$ and the presence or absence of different electron acceptors than PCBs) significantly affect the microbial communities determining which kind of dehalogenase activity can be expressed and, thus, which selective route can be undertaken (Wiegel & Wu, 2000). However, as a general rule, dechlorinating enzymes use chlorine as the final electron acceptor, adding one electron to the aryl carbon-chlorine bond (water and hydrogen $H_2$ are used as electron donors) and subsequently, removing chlorine atoms one by one (Vasilyeva & Strijakova, 2007). Moreover, the relatively low abundance of PCB dechlorinators in natural environments results in low dechlorination rates. In view of this evidence, even if natural attenuation process can occur, biostimulation of anaerobic PCB-degrading populations by modification of temperature, pH value or $H_2$ supplementation, or bioaugmentation with enriched PCB-dechlorinating cultures are strongly required to efficiently remediate anaerobic PCB-contaminated sediments (Wiegel & Wu, 2000).

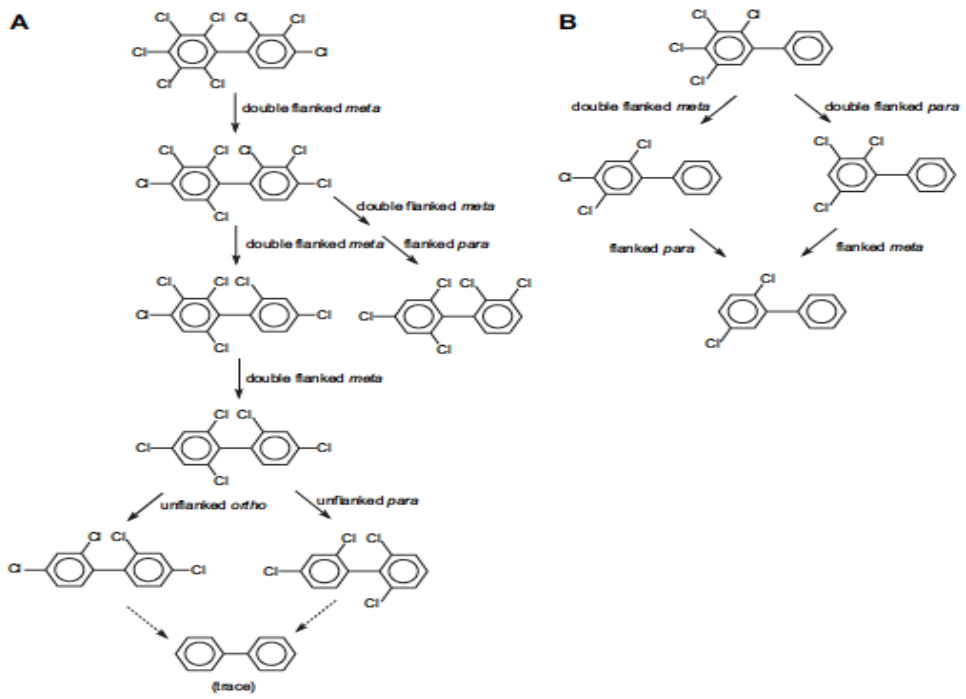



***Fig. 1.5.*** Examples of anaerobic pathways of reductive dechlorination observed in sediment microcosms and anaerobic enrichment cultures. Dotted arrows indicate slow reactions (Field & Sierra-Alvarez, 2008).

### 1.6.1.2 Aerobic bacterial degradation of PCBs

Aerobic PCB degradation was extensively investigated in the last two decades and a large number of PCB-degrading bacteria were found among both Gram negative genera (*Pseudomonas*, *Alcaligens*, *Achromobacter*, *Burkholderia*, *Sphingomonas*, *Comamonas* and *Acinetobacter*) and Gram positive genera (*Rhodococcus*, *Corynebacterium* and *Bacillus*) (Pieper, 2005; Furukawa, 2000; Borja *et al*., 2005).

All these bacteria are capable to co-metabolize lower chlorinated biphenyls PCB using biphenyl as the primary substrate, whereas a more restricted number of strains can grow utilizing mono- or dichlorinated biphenyls as the sole carbon and energy source. The majority of studies report on the co-metabolism of PCBs *via* the biphenyl pathway, a process mediated by four enzymes encoded by biphenyl genes cluster (*Bhp* A, B, C and D) that convert stepwise PCBs to chlorobenzoic acids (CBAs) and to the aliphatic 2-hydroxy-penta-2,4-dienoic acid (Furukawa, 2000; Ohtsubo *et al.*, 2004; Pieper, 2005).

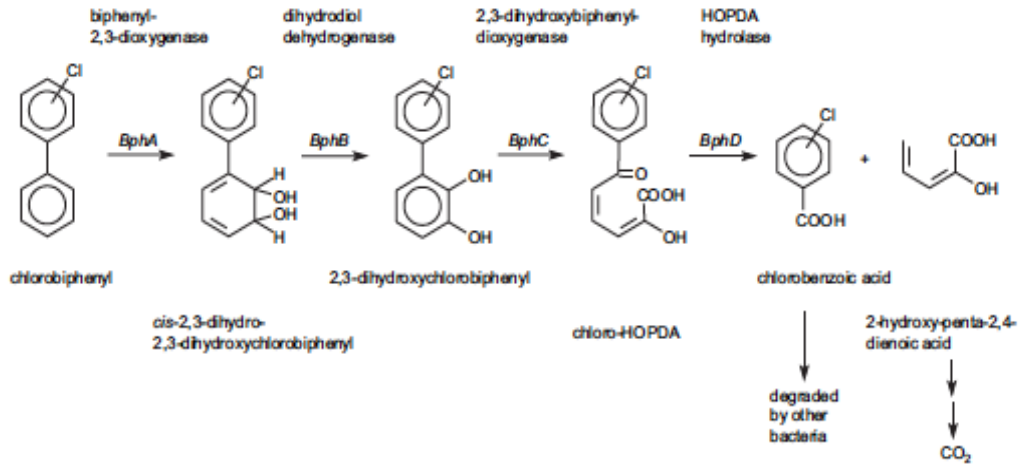



***Fig. 1.6.*** Pathway of aerobic PCB degradation by biphenyl-oxidizing bacteria (Field & Sierra-Alvarez, 2008)

PCBs are oxidized by biphenyl-2,3-dioxigenase (*Bph A*) leading to the formation of their corresponding *cis*-dihydrodiols which are subsequently transformed to dihydroxy-PCBs by a dehydrogenase (*Bph B*). These intermediates are further converted by an oxygenase (*Bph C*) to a *meta* cleavage product 2-hydroxy-6-oxo-6-(chloro) phenylhexa-2,4-dienoic acid (HOPDA). The aliphatic portion of HOPDA is cleaved by a hydrolase (*Bph D*) from the remaining aromatic ring to yield the aliphatic 2-hydroxy-penta-2,4-dienoic acid and CBAs. The former can be metabolized ultimately to $CO_2$ through the tricarboxylic acid cycle, while the latter are generally accumulated as final dead-end products. Consequently, the complete mineralization of PCBs can be merely achieved in consortia composed of PCB-degraders and bacterial strains specialized in chlorobenzoic acid metabolism, as described by Nielsen and co-workers (2000) that demonstrated the efficiency of *Burkholderia* sp. LB400 (capable of metabolizing 3-chlorobiphenyl) and *Pseudomonas* sp. B13(FR1) (capable of mineralizing the chlorobenzoate produced by LB400) co-coltures to completely mineralize PCBs under aerobic conditions.

The rate of microbial PCB degradation depends on the number and position of chlorine atoms on biphenyl rings and principally correlates negatively with the number of chlorine substituents: all the isolated PCB-degrading strains are able to oxidize mono- and di-chloro biphenyls, but as the number of chlorine substituents increases, progressively less and less strains are capable to metabolize PCBs (Vasilyeva & Strijakova, 2007). Moreover, the intracellular localization of PCB-degrading enzymes implies the solubilisation of PCBs in the plasma membrane to enter the cytoplasm. This constitutes a rate-limiting step because PCBs,

due to their high hydrophobicity, are easily entrapped in the hydrophobic components of biological membranes and, thus become less susceptible to enzymatic degradation.

Optimization of PCB-degrading bacteria performances under environmental conditions is strongly demanded for the effective clean-up of contaminated substrates. The required conditions for the active functioning of indigenous microorganisms include maximal oxygenation of polluted matrix, optimal of temperature and supplementation with specific inducers (biphenyl, terpenoids or, preferably cheaper alternative as plant residues which contain large amounts of terpenes) (Abraham *et al*., 2002; Fava *et al.*, 2003; Luo & Hu, 2013). Moreover, to succeed in dealing with PCB-bioavailability constraint, a number of studies was performed to evaluate the effect of the application of chemically synthesized surfactants (Twin, Tritox X-100, Brij 35, sodium dodecyl sulphate, *ect.*), enzymatically produced cyclodextrins, humic substances or biosurfactants of plant origin (lipopeptides, rhamnolipids, saponins, maltothriose esters, *ect.*) on PCB accessibility (Fava & Di Gioia, 1998; Billingsley *et al*., 1999; Mulligan *et al.*, 2001; Fava & Piccolo, 2002; Viisimaa *et al*., 2013.). Most of them have shown that the addition of surfactants enhanced the solubility of PCBs and, thus their removal from the contaminated materials. However, in some cases, chemical surfactants provoked changes in the microbial community resulting in a decrease of PCB degrader population and therefore, in the reduction of PCB degradation rates (Colores *et al*., 2000; Singh *et al.*, 2007).

The scientific research has been focusing also on bioaugmentation approach based on the inoculation of native PCB-degrader enriched cultures (Petric *et al.*, 2007), in the introduction of microbial consortia specialized in both PCBs and CBAs degradation (Ohtsubo *et al.*, 2004) or native consortium of nonspecific microorganisms (Di Toro *et al*., 2006).

Furthermore, attempts to construct genetically modified PCB degrading strains that exhibit enhanced degradation capabilities have been carried out (Ang *et al.*, 2005; Furukawa & Fujihara, 2008; Wasilkowski *et al.*, 2012). Genetic engineering methods allowed the design of either microorganisms with broader substrate specificity by the combination of *Bph* genes from different bacterial strains or microorganisms with multiple sets of genes encoding the enzymes responsible for the degradation of both PCBs and CBAs (Wittich & Wolff, 2007; Rein et al., 2007).

At present, despite several positive outcomes at the laboratory scale, a limited number of both biostimulation and bioaugmentation treatments of PCB contaminated matrices have been carried out under field conditions (Pieper, 2005).

#### 1.6.1.3 Sequential anaerobic-aerobic bioremediation of PCBs

Due to various abovementioned limitations, neither the single anaerobic nor aerobic treatment is adequate for the bioremediation of PCBs, especially of highly chlorinated biphenyls. The sequential application of anaerobic and aerobic treatment offers the prospect of achieving the complete mineralization of PCBs. The two-phase PCB remediation scheme consists in the dehalogenation of higher chlorinated congeners (tetra-, penta- and hexa-BPs) by anaerobes to form less chlorinated compounds (mono-, di- and tri-BPs) which are subsequently oxidized under aerobic conditions.

In laboratory experiments, soil slurry microcosms inoculated with microorganisms extracted from PCB-contaminated Hudson River sediments were used for the anaerobic dechlorination of an aged Aroclor 1248-contaminated soil, followed by exposure to air, addition of biphenyl and inoculation with *Pseudomonas* sp. LB400, an aerobic PCB degrader (Evans *et al.*, 1996). This experiment proved for the first time the feasibility and efficiency of this combined treatment and, thereupon, further laboratory scale studies confirmed this observation. Soil contaminated with either Aroclor 1260 or 1242 were also bioremediated by sequential anaerobic and aerobic laboratory-scale treatment (Master *et al.*, 2002; Rodrigues *et al.*, 2006). In the former study, anaerobic treatment (4 months) with an enriched microbial culture completely or partially transformed all of the major components present in Aroclor 1260 to less chlorinated PCB congeners. Then, the dechlorinated products were degraded during the subsequent aerobic treatment using *Burkholderia* sp. strain LB400 (28 days) (Master *et al.*, 2002). In the other study, river sediments were incubated with the aged-contaminated soil for 1 year under anaerobic conditions to generate lower chlorinated congeners. Subsequently, the soil was treated aerobically by the inoculation with two genetically engineered aerobic bacteria, *Burkholderia xenovorans* LB400 and RHA1, capable of growing on 2-chlorobiphenyl and 4-chlorobenzoate, respectively (Rodrigues *et al.*, 2006).

However, this strategy appears unfeasible to be used at a large scale due to the inherent high costs: it requires the excavation of soil, the mixing of slurries, the long time requirements (ranging from several months to years), maintenance and production of inocula with both anaerobic and aerobic microorganisms.

### 1.6.2 Phytoremediation

Phytoremediation is an emerging technology based on the use of plants and/or associated bacteria for the treatment of contaminated soils and groundwater. This technology encompasses different remediation processes based on the immobilization, removal or degradation of organic compounds: pollutants can be taken up from the soil, transported across plant membranes and released through leaves *via* evapotranspiration (*phytovolatilization*) or accumulated in plant tissues (*phytoextraction*) or degraded by plant enzymes (*phytodegradation* or *phytotransformation*). Moreover, some contaminants can be incorporated and immobilized into soil particles (*phytostabilization*), adsorbed to the plant roots (*rhizofiltration*) or degraded by microbes colonizing the root zone (*rhizoremediation*).

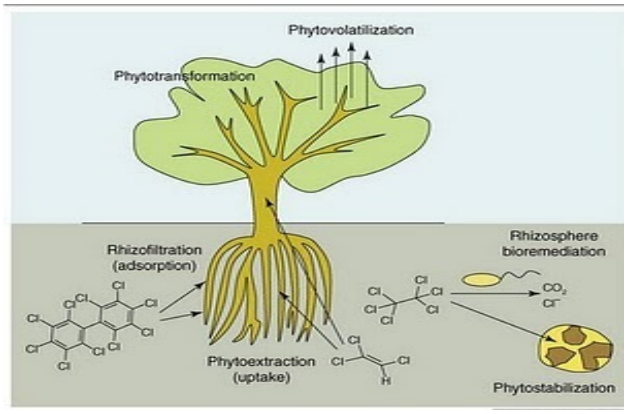



***Fig.1.7.*** Phytoremediation processes (Van Aken & Geiger, 2010).

In general, phytoremediation technologies have shown several advantages over other remediation strategies: *i*) absence of energy-consuming equipment, *ii*) low negative impact on the environment and high potential in improving the soil quality and texture of the remediated sites, *iii*) minimal maintenance costs, *iv*) beneficial side-effects such as erosion control and carbon sequestration, *v*) large acceptance as an attractive green technology (Gerhardt *et al.*, 2009). However, phytoremediation application at the field level can encounter several disadvantages: this biological approach, which is generally more time consuming than other techniques, is restricted to shallow contamination of moderately hydrophobic compounds (Van Aken *et al.*, 2010a, 2010b). Moreover, the remediation mediated by plants can be incomplete because of the lack of biochemical pathways necessary for the complete mineralization of recalcitrant compounds. Thus, toxic intermediates may be formed and

released into the soil, enter the food chain or volatilize into the atmosphere (Pilon-Smits, 2005; Yoon *et al.*, 2006; Mackova *et al.*, 2006). The feasibility of plant-based technologies at large scale faced also several environmental and climatic factors which are negligible in the laboratory or greenhouse applications such as changes in temperature and pH, precipitation, availability of water and nutrients, presence of herbivores and plant pathogens, plant competition in the adaptation to the contaminated site (Chaudhry *et al*., 2002; Gerhardt *et al*., 2009).

#### 1.6.2.1 Phytoremediation of PCBs

Rhizoremediation plays the main role in the biodegradation of polychlorinated biphenyls because their hydrophobic features limit plant uptake and transformation processes. The rhizoremediation process is based on the mutual cooperation between microorganisms and plant roots: in the rhizosphere, the growth and the activity of pollutant-degrading bacteria can be promoted by the release of diverse secondary plant metabolites such as phytohormones/phytoalexins, phytosiderophores, phenols, amino acids or compounds derived from isoprenoid, phenylpropanoid, alkaloid or fatty acids pathways and, concurrently the microbes can provide additional carbon and/or energy sources to plants.

In particular, vegetation can stimulate microbial activity in the soil and differently act to enhance the biodegradation of PCBs:

a) plants secrete extracellular enzymes (*i.e.* peroxidases and dehydrogenases) which can initiate the transformation of PCBs and, thus, facilitate further microbial metabolism (Chu *et al.*, 2006; Schröder & Collins, 2010);
b) plant roots release organic compounds (*i.e.* sugar, organic acids, *ect.*) which can act as electron donors to support either the co-metabolism or the anaerobic dehalogenation of PCBs, or as surfactants increasing PCB solubility, mobility and, thus,, their susceptibility to degradation (Chaudhry *et al.*, 2005; Campanella *et al.*, 2002);
c) plants produce and release also diverse microbial growth factors and inducers (*i.e.* phenolic exudates) that can enhance microbial degradation capabilities (Toussaint *et al.*, 2012; Meggo, 2013);
d) plant roots improve soil permeability and oxygen diffusion in the rhizosphere which potentially stimulate the microbial oxidative transformation pathway (Singh *et al.*, 2003; Chaudhry *et al.*, 2005).

A large number of rhizoremediation studies in micro- and mesocosms were performed to evaluate the efficiency of several plants species in PCB degradation process. Identifying plant species that accelerate microbial PCB degradation is the first step towards the development of a successful rhizoremediation strategy. Several studies have suggested willow as a potentially useful plant for PCB rhizoremediation (de Cárcer *et al.*, 2007a, 2007b; Rein *et al*., 2007; Ionescu *et al.*, 2009). The root zones of willow species have been found to harbour increased numbers of PCB-degrading bacteria relative to other species of bacteria (Leigh *et al*., 2006). Additionally, a diversity of biphenyl dioxygenase genes have been detected in the root zones of certain willows (de Cárcer *et al*., 2007). Willows also are known to produce salicylate, which may induce the expression of the upper biphenyl degradation pathway (Master & Mohn, 2001). Slater and co-workers assessed the potential of two common Alaskan tree species, *Salix alaxensis* (feltleaf willow) and *Picea glauca* (white spruce) to promote PCB degradation in contaminated soil microcosms (2011). The authors observed a significantly higher PCB degradation rate in crushed fine willow roots treated soil than in spruce root chips treated soil. The stimulation of indigenous PCB-degrading bacterial consortia in the rhizosphere of specific plants as well as the degradative ability of the plants themselves was evaluated in both field and pot experiments by Mackova *et al*. (2009). *Nicotiana tabacum* (tobacco), *Solanum nigrum* (black nightshade), *Salix* sp. (willow), *Medicago sativa* (alfalfa) and *Silybum marianum* (thistle) were tested for their capability to support bacterial growth, particularly *Pseudomonas* species which are considered the main candidates for PCB removal. Thistle was the most effective in the stimulation of microbial growth at the field scale. However, tobacco and nightshade treatments decreased the PCB concentration in either field or pot experiments more than other plants.

Poplar is another model plant for phytoremediation of PCBs and several hydroponics studies involving this plant and PCBs were undertaken during the last years (Liu & Schnoor, 2008; Liu *et al.*, 2009). Moreover, the efficiency of poplar in field-scale remediation of soils lightly contaminated by PCBs was recently demonstrated (Meggo, 2013).

Recent investigations have focused also on the application of transgenic plants to optimize plant-microbe bioremediation processes: genetically engineered plants, obtained mainly by the introduction of bacterial biphenyl dioxygenase genes, can initiate the transformation of PCBs and release metabolites for further microbial degradation (Mohammadi *et al*., 2007; Macek *et al*., 2008; Sylvestre *et al.*, 2009). Nevertheless, challenging issues remain to be faced for understanding the expression of heterologous genes and, thus the impact on the rhizobacteria catabolic pathways.

### 1.6.3 Mycoremediation and "white rot" fungi

A specific branch of bioremediation, called "mycoremediation", has been gaining increasing interest in recent years: its name is due to the use of fungi for the remediation of polluted soils or other solid-liquid matrices. Fungi with contaminant-degrading capabilities are mostly wood-degrading basidiomycetes belonging primarily to Agaricales (*i.e. Stropharia rugosoannulata*, *Agrocybe praecox* and *Pleurotus ostreatus*) and Polyporales orders (*i.e. Phanerochaete chysosporium*, *Trametes versicolor*, *Bjerkandera adusta*, *Irpex lacteus*, *ect.*) (Gadd, 2001; Singh, 2006). In addition, several fungi belonging to the phyla Ascomycota (*i.e. Trichoderma* spp.), Zygomycota (*i.e. Mucor* spp.) and anamorphic ascomycetes (*i.e. Aspergillus* spp., *Penicillium* spp., *Paecilomyces* spp.) have also demonstrated the ability to degrade contaminants (Tortella *et al.*, 2005; Tigini *et al.*, 2009; Carvalho *et al.*, 2009).

However, particularly promising appears the use of "white rot" fungi, an ecologically distinct group specialised in lignin breakdown. Indeed, their name derives from the appearance of wood attacked by these organisms, in which lignin removal results in a bleached form. In addition to their natural substrate, these fungi have been shown to degrade and, to some extent mineralize, a wide range of organic and xenobiotic pollutants structurally similar to lignin such as petroleum hydrocarbons, chlorophenols, polycyclic aromatic hydrocarbons, polychlorinated biphenyls, dioxins and furans, pesticides, herbicides and nitroaromatic explosives (Pointing, 2001; Rabinovich *et al*., 2004).

Two important features distinguish these fungi from bacteria and make them excellent candidates for soil bioremediation strategies: the penetration of the fungal hyphae into the polluted matrix and the excretion of oxidative enzymes, mainly laccase, lignin peroxidase and manganese peroxidase. These oxidases exhibit very low substrate specificity and, being active in the extracellular environment, are able to reach and attack scarcely bioavailable contaminants by nonspecific radical-based reactions. In addition to the extracellular enzymatic system, white rot fungi possess also an intracellular one involving cytochrome P450 monooxygenase enzymes- CYP450 (Črešnar & Petrič, 2011). This intracellular pathway, occurring in all eukaryotic organisms, mainly regulates the bioconversion of hormones and the detoxification of drugs and xenobiotics (Bernhardt, 2006). In wood rotting fungi, cytochrome P450 is supposed to cooperate with the ligninolytic system in the general mechanism of xenobiotic degradation (van den Brink *et al*., 1998). Moreover, as already mentioned, "white rot" fungi evolve a spatially extensive hyphal growth enabling them to penetrate across air-filled soil pores, air-water interfaces and even rock matrices (Bornyasz *et*

*al*., 2005) and to act as dispersion vectors of bacteria (Kohlmeier *et al*., 2005; Bonfante & Anca, 2009). Fungi can also tolerate high concentrations of organic contaminants and heavy metals without deleterious effects on their enzyme activities (Baldrian *et al*., 2000; Baldrian, 2003; Tuomela *et al*., 2005).

Unlike bacteria, in spite of their extraordinary degrading capabilities, fungi cannot assimilate contaminants as a source of carbon and energy; for this reason, lignocellulosic residues are used as amendants to support the fungal growth and, thus to improve the mycoremediation performances (Singh, 2006).

Furthermore, it is well known that fungi are involved in soil humification process: in this respect, the use of these organisms in soil remediation could lead, not only to the decontamination, but also to the re-use of the soil for agricultural purposes (Bollag, 1992; Michels, 1998).

#### 1.6.3.1. "White rot" fungi ligninolytic enzymes

White rot fungi variously secrete one or more of three main extracellular lignin degrading enzymes, lignin peroxidase (LiP, E.C. 1.11.1.14), Mn-dependent peroxidase (MnP, E.C. 1.11.1.13), and laccase (Lac, E.C. 1.10.3.2) (Tuor *et al*., 1995). Some authors also reported the expression of a novel Mn-independent peroxidase activity and other versatile peroxidase activities by some white rot fungi genera (Heinfling *et al*., 1998; Camarero *et al.*, 1999; Ruiz-Duenas *et al.*, 2001).

In addition, other enzymes are indirectly involved in lignin modification and, thus associated with lignin degrading enzymes: glyoxal oxidase (E.C. 1.2.3.5), superoxide dismutase (E.C. 1.15.1.1), glucose oxidase (E.C. 1.1.3.4), aryl alcohol oxidase (E.C. 1.1.3.7) and cellobiose dehydrogenase (E.C. 1.1.99.18). They produce $H_2O_2$ which is essential for the catalytic cycle of peroxidases (LiP and MnP) or degrade the non-phenolic substructures of lignin by the formation of reactive hydroxyl radicals •OH (Hatakka, 2001; Leonowicz *et al*., 2001; Lundell *et al*., 2010). All three major ligninolytic enzymes are encoded by gene families that lead to the production of multiple enzyme isoforms (Thurston, 1994; Tuor *et al*., 1995; Martinez, 2002).

Ligninolytic enzyme production by white rot fungi occurs during the secondary metabolism: in *Phanerochaete chrysosporium* cultures, the ligninolytic activities are strongly induced by nitrogen, carbohydrate and sulphur starvation conditions and by the presence of trace metals, $Mg^{2+}$ and $Ca^{2+}$ (Jeffries *et al*., 1981). In contrast, in *Bjerkandera* sp. strain BOS55 the production of lignin degrading peroxidases was significantly improved in N sufficient

conditions (Mester *et al*., 1996). Also growth conditions, such as temperature and agitation, significantly affect the appearance and levels of activity of ligninolytic enzymes in white rot fungi (Podgornik *et al.*, 2001; Bermek *et al*., 2004;). Additionally, recent research has shown that ligninolytic enzyme production by white rot fungi is also affected by the presence/absence of mediators or various chemicals and by specific concentrations of metals $Mn^{2+}$ and/or $Cu^{2+}$ (Scheel *et al*., 2000; Galhaup *et al*., 2002).

#### 1.6.3.1.1 Laccase

Fungal laccases (Lac, E.C. 1.10.3.2; benzendiol:oxygen oxidoreductases) are copper-containing phenoloxidases (Baldrian, 2006). They belong to the group of blue copper oxidases which catalyse the oxidation of different compounds reducing oxygen to water (a four electron reduction reaction). Fungal laccases contain four copper atoms distributed among three different binding sites which play an important role in the catalytic mechanism of the enzyme (*Figure 1.8*).

Laccases are remarkably non-specific: they oxidize different compounds, e.g. phenols, polyphenols and aromatic amines as well as non-phenolic organic substrates, by one electron abstractions resulting in the formation of reactive radicals undergoing further non-enzymatic reactions (e.g., depolymerization, repolymerization, demethylation or quinone formation) (Hatakka, 2001; Baldrian, 2006). Other non-phenolic compounds with high redox potential, including PAHs or other recalcitrant compounds, may also be oxidized by laccase in the presence of either natural mediators derived from oxidized lignin (*i.e. p*-coumaric acid or syringaldehyde; Camarero *et al*., 2008) or synthetic ones (i.e. ABTS (2,2´-azinobis(3-ethylthiazoline-6-sulfonate) or 1-hydroxybenzotriazole (HBT); Baldrian, 2006). Laccases are known to be produced by many fungi in multiple isoforms with typical molecular masses and and isoelectric points ranging from 60 to 80 kDa and from 3.0 to 7.0, respectively (Baldrian, 2006). Laccases from white rot fungi can be intracellular or extracellular and are secreted mostly into the culture media. The research of laccases in other fungi suggested also the presence of laccases associated with fungal cell wall (Zhu *et al*., 2001). The production of laccase activity by white rot fungi can be improved by the addition of $Cu^{2+}$ which regulates laccase production at the gene transcription level (Palmieri *et al.*, 2000; Galhaup & Haltrich, 2001; Soden & Dobson, 2001; Saparrat *et al*., 2002). Several other chemicals such as 2,5-xylidine, veratryl alcohol and guaiacol have also an inducing effect on laccase production (Quaratino *et al*., 2007).

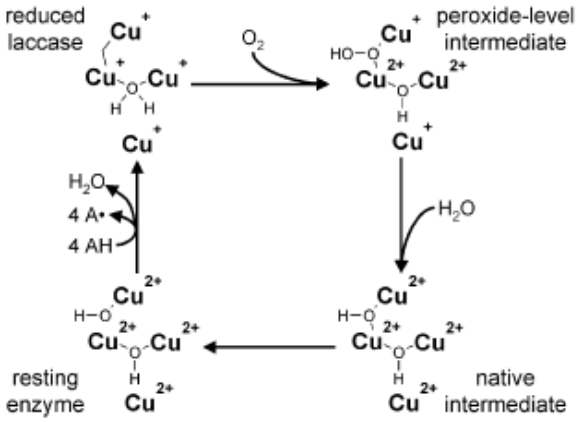



***Fig. 1.8***. The catalytic cycle of fungal laccase (Wesemberg *et al*., 2003).

#### 1.6.3.1.2 Manganese peroxidase

Mn-dependent peroxidase (MnP, E.C. 1.11.1.13) is an extracellular heme-containing peroxidase that catalyses the $H_2O_2$-dependent oxidation of $Mn^{2+}$ to highly reactive $Mn^{3+}$, which is stabilized by fungal chelators such as oxalic and malic acid (Hofrichter, 2002). Thereafter, $Mn^{3+}$ can oxidize phenolic and aromatic amines to phenoxyl and amino radicals, respectively (Wariishi *et al*., 1992; Kuan & Tien, 1993).

The catalytic cycle of MnP involves two oxidative states of the enzyme (*Figure 1.9*): compound I (MnPoxid I) and compound II (MnPoxid II). The cycle is initiated by the binding of $H_2O_2$ to the native ferric enzyme and the formation of an ironperoxide complex (Hofrichter, 2002). The subsequent cleavage of the peroxide oxygen-oxygen bond, which requires a two-electron transfer from the heme, results in the formation of MnP compound I. Afterwards, a water molecule is expelled. A subsequent reduction proceeds through MnP compound II. A monochelated $Mn^{2+}$ ion acts as the one-electron donor for this enzyme intermediate and is oxidized to $Mn^{3+}$. The reduction of MnP compound II proceeds in a similar way and another $Mn^{3+}$ is formed from $Mn^{2+}$ leading to generation of the native enzyme and release of the second molecule of water. Whereas MnP compound I resembles that of LiP and can, besides $Mn^{2+}$, be reduced by other electron donors (ferrocyanide, phenolics), MnP compound II is reduced by other substrates only very slowly and requires $Mn^{2+}$ to complete the catalytic cycle. The $Mn^{3+}$ formed during the cycle is stabilized by carboxylic acids (oxalate, malonate, malate, tartrate or lactate). The chelates of $Mn^{3+}$ cause one-electron oxidations of various substrates. MnP is, similarly to LiP, sensitive to high

concentrations of hydrogen peroxide which cause reversible inactivation of the enzyme by forming MnP compound III.

MnP is often produced in multiple isoforms exhibiting a molecular mass of 45-55 kDa and redox potential higher than 1.0 V (Martinez, 2002). These isoforms differ mostly in their isoelectric points, which are usually rather acidic (pH 3-4), even though near-neutral MnPs have been described in litter-decomposing fungi (Steffen *et al*., 2002).

The expression of MnP in fungal cultures is regulated at the level of gene transcription by hydrogen peroxide and various chemicals including ethanol and 2,4-dichlorphenol as well as by the specific $Mn^{2+}$ concentration (Li *et al.*, 1995; Scheel *et al*., 2000).

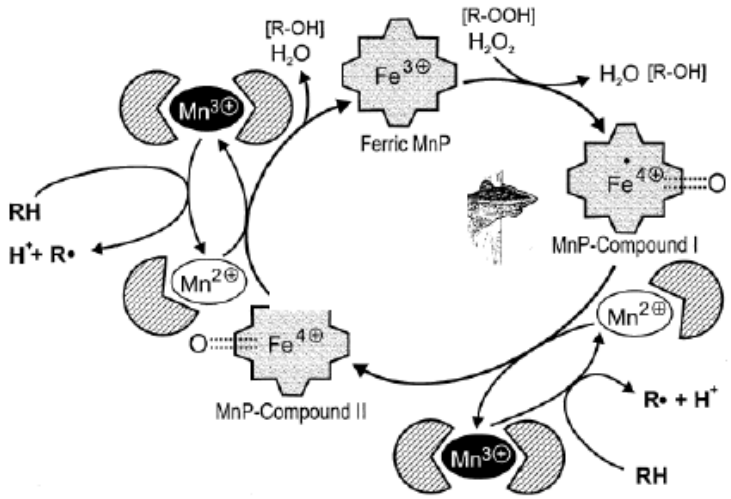



***Fig.1.9***. The catalytic cycle of fungal MnP (Hofrichter, 2002).

**1.6.3.1.3 Lignin peroxidase**

Lignin peroxidase (LiP, E.C. 1.11.1.14) is a glycosylated heme-containing peroxidase with a molecular mass of 40-45 kDa. In the presence of endogenously generated hydrogen peroxide, LiP catalyzes the oxidation of phenolic and nonphenolic aromatic structures generating aryl cation radicals (Hatakka, 2001; Hammel & Cullen, 2008). In detail, during its catalytic cycle (*Figure 1.10*), LiP is oxidized by $H_2O_2$ to form a two-electron intermediate (compound I) which oxidizes substrates by removing one electron and produces a more reduced enzyme intermediate (compound II). This intermediate can then oxidize substrates by one electron, returning the enzyme to its initial state. However, compound II has a very high reactivity with

$H_2O_2$, therefore in the presence of a poor substrate and excess $H_2O_2$, it can be converted to an inactive form of the enzyme (compound III) (Wariishi & Gold, 1989). Compounds such as veratryl alcohol and tryptophan have been shown to have a protective effect against the enzyme inactivation due to the excess of $H_2O_2$ (Collins *et al*., 1997). When present, these molecules are preferred as substrates for compound II and convert it into the resting enzyme, completing the catalytic cycle. An additional role for veratryl alcohol and tryptophane as diffusible mediators in the LiP-catalyzed oxidation of environmental contaminants has also been proposed (Goodwin *et al*., 1995; Collins *et al*., 1997).

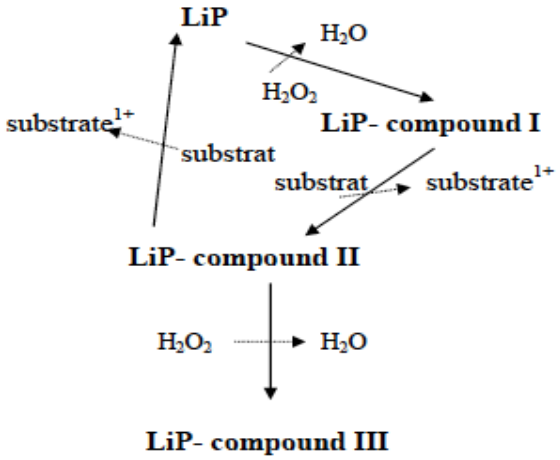



***Fig. 1.10***. The catalytic cycle of LiP (Wariishi & Gold, 1989).

#### 1.6.3.1.4 Versatile peroxidase

Versatile peroxidase (VP) has been discovered in *Bjerkandera* and *Pleurotus* species (Mester & Field 1998; Ruiz-Dueñas *et al*., 2001). VP is able to oxidize both LiP and MnP substrates and, therefore can be considered a hybrid between the two enzymes. VP oxidizes both low and high redox potential compounds, with or without $Mn^{3+}$ mediation (Ruíz-Dueñas *et al*., 2007): it has high affinity for $Mn^{+2}$, hydroquinone and dyes, and also veratryl alcohol, dimethoxybenzene and lignin dimers. However, its catalytic efficiency is much higher in presence of $Mn^{+2}$ than in presence of other aromatic substrates (Heinfling *et al*., 1998). Its optimal pH for oxidation of $Mn^{+2}$ (pH 5) and aromatic compounds or dyes (pH 3) differ, being similar to those of optimal MnP and LiP activity (Ruíz-Dueñas *et al*., 2001). A non-competitive inhibition was proposed for both substrates, which means that VP has, at least, two binding sites (Heinfling *et al*., 1998; Martinez, 2002). The versatility to degrade directly a

wide variety of substrates makes VP an enzyme with a large potential for industrial applications including in the field of contaminant degradation (Pozdnyakova *et al*., 2010)

### 1.6.3.2 Production patterns and cooperation of ligninolytic enzymes

White rot fungi produce extracellular lignin degrading enzymes, the best characterized of which are LiP, MnP and Lac. According to the production patterns of the above three enzymes, white rot fungi could be divided into three main groups, although overlaps and exceptions occur (Hatakka, 1994).

The first group, LiP-MnP group, is represented by the fungi producing mainly LiP and MnP such as *P. chrysosporium*. The two other groups are: white rot fungi producing MnP and laccase (*i.e*. *Dichomitus squalens*, *Ceriporiopsis subvermispora*, *Pleurotus ostreatus*, *Lentinus edodes* and *Panus tigrinus*) and white rot fungi producing LiP and laccase (*i.e., Phlebia ochraceofulva*). LiP, MnP, and laccase are highly non-specific with regard to their substrate range. Indeed, in lignin degradation they act synergistically: LiP catalyzes oxidations in the alkyl side chains of lignin subunits to give benzaldehydes; lignin degraded by LiP thus provides substrates for laccases (Leonowicz *et al*., 2001). MnP cooperation with laccase has been shown to be important for the primary attack on lignin by the fungus *Rigidoporus lignosus* (Galliano *et al*., 1991). Generally, fungi which do not express LiP produce MnP with features similar to LiP, the so called "hybrid MnP" (Mester & Field, 1998). Recently, laccase from *Stropharia rugosoannulata* (litter decomposing fungus) has been shown to oxidize $Mn^{2+}$ to $Mn^{3+}$ in the presence of Mn chelators leading to the production of hydrogen peroxide. The results demonstrated a role of laccase in providing $H_2O_2$ for MnP reactions (Schlosser & Höfer, 2002).

### 1.6.3.3 Mycoremediation of PCBs

Due to their wide metabolic capabilities and, thus to their remarkable potential in organopollutants biodegradation, a large number of white-rot species (*Phanerochaete chrysosporium*, *Trametes versicolor*, *Lentinus edodes*, *Pleurotus ostreatus*, *Grifola frondosa*, *Coriolopsis polyzona*, *Irpex lacteus*, *Bjerkandera adusta*) were tested in laboratory-scale model liquid systems for their ability to degrade chlorinated biphenyls (Vyas *et al*., 1994; Yadav *et al.*, 1995; Dietrich *et al*., 1995; Novotny *et al.*, 1997; Beaudette *et al*., 1998; Koller *et al*., 2000; Ruiz-Aguilar *et al*., 2002; Kamei *et al*., 2006a; Kamei *et al*., 2006b; Cvancarova *et al*., 2012). Generally, all these studies confirmed that the extent of degradation significantly decreases with rising chlorine content. Moreover, some fungal strains (*i.e. Pleurotus*

*ostreatus*) have shown to selectively removed PCB congeners with preference for those compounds with chlorine atom in *ortho* > *meta* > *para* position, while others (*i.e. Phanerochaete chrysosporium*) have not exhibited noticeable specificity for the position of chlorine substitutions (Yadav *et al*., 1995; Kubatova *et al*., 2001). PCB transformation products (methoxylated- and hydroxylated-PCBs, chlorobenzoic acids, chlorobenzaldehydes and chlorobenzylalcohols) were identified in liquid fungal cultures and the involvement of both extracellular ligninolytic system and intracellular cytochrome P450 monooxygenases system in the PCB degradation process was hypothesized (Kamei *et al*., 2006a; Cvancarova *et al*., 2012). Although fungal extracellular phenoloxidases, such as laccase and Mn-dependent peroxidase, have been found to be unable to oxidize PCB congeners (Baudette *et al*., 1998), they are able to perform the breakdown of some degradation intermediates such as their hydroxylated derivatives which can be produced by CYP450 system (Keum *et al*., 2004; Takagi *et al*., 2007). However, any mechanistic interpretation on the effect of the chlorination pattern is prevented by the lack of information concerning the enzymatic basis of the fungal breakdown of these contaminants. Despite the promising outcomes achieved in liquid cultures, few studies investigated the ability of white rot fungi to attack chlorinated biphenyls in artificially contaminated soils (Zeddel *et al*., 1993; Kubatova *et al*., 2001) or in real PCB-contaminated soil (Borazjani, 2005; Federici *et al.*, 2012). Therefore, the fungal application for the treatment of PCB polluted solid matrices requires further investigation to gain insights into the capacity of fungi to grow on contaminated soils where both physico-chemical factors (*i.e.* interaction of PCBs with the soil organic matter) and biological factors (*i.e.* competition with native microorganisms) can affect the efficiency of the treatment.

# CHAPTER 2

# MYCOREMEDIATION OF A LONG TERM PCB-CONTAMINATED SOIL

## 2.1 Introduction

Polychlorinated biphenyls (PCBs) are xenobiotic compounds produced as mixtures and used in the past for several industrial applications because of their thermal and chemical stability, flame resistance and dielectric properties. Due to their inertness, PCBs are still widely distributed in all ecosystems, affecting both natural environments and wildlife. Thus, the clean-up of PCB-contaminated sites has become a priority of great relevance due to the teratogenic, carcinogenic and endocrine-disrupting features of these xenobiotics. Up to now, the removal of PCBs from contaminated environmental matrices has been mainly performed by incineration at high temperature. Since this procedure may entail additional risks, scientific researchers have been focusing also on biological treatments of PCBs. These technologies represent an effective, cost-competitive and environmentally friendly alternative for the removal of PCBs (Šašek *et al*., 2003; Hamman, 2004).

In this respect, filamentous fungi display features that make them excellent candidates to design an effective remediation technology. The hyphal structures, defined as the "fungal highways", allow them to easily penetrate across environmental matrices and to translocate pollutant-degrading bacteria acting as dispersion vectors (Kohlmeier *et al.*, 2005; Bonfante & Anca, 2009). Another important peculiarity of a subgroup of filamentous fungi, namely "white rot" is the secretion of oxidative enzymes characterized by a low substrate specificity that can degrade a wide range of aromatic organopollutants (Pointing, 2001; Rabinovich *et al*., 2004). This nonspecific radical-based system is active in the extracellular environment and thus, fungi can easily explore the contaminated matrix reaching even scarcely bioavailable pollutants.

Nevertheless, fungi have not been widely exploited yet for their metabolic capabilities (Harms *et al*., 2011). As a matter of fact, a relatively small number of white-rot species have been tested on real PCB-contaminated soil (Borazjani, 2005; Federici *et al*., 2012).

Thus, this study was aimed at assessing the technical feasibility of different mycoremediation treatments of a PCB historically contaminated soil. Prior to the application of the selected remedial techniques, physico-chemical properties of soil as well as PCB bioavailability were assessed. Thereafter, degradation capabilities of the white rot fungi *Irpex lacteus* and *Pleurotus ostreatus* and the role of autochthonous microbial communities in the PCB biotransformation process were investigated. Specific attention was paid on the identification of PCB degradation products, and on residual toxicity at the end of each treatment. Furthermore, to gain new insights into the biota composition throughout the remediation

processes, microbial biomass was quantified by phospholipid fatty acids analysis and the diversity and dynamics of both bacterial and fungal communities were estimated *via* high-throughput 454-pyrosequencing method as summarized in *Figure 2.1*.

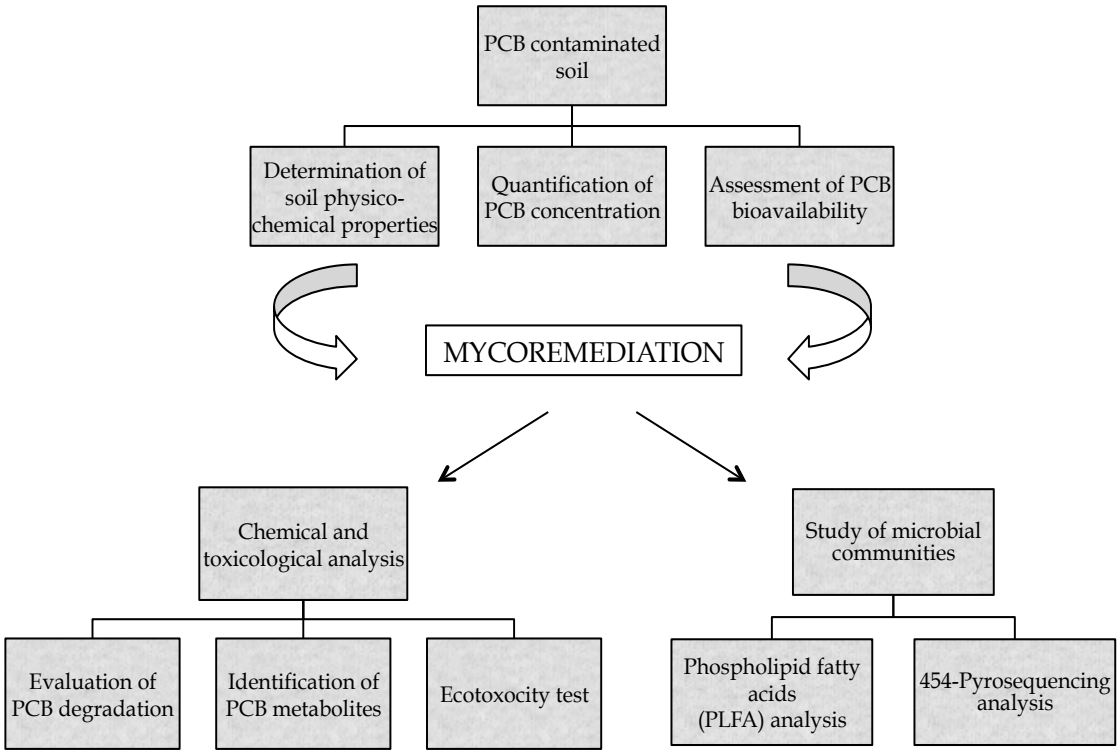



***Fig. 2.1.*** Workflow of Chapter 2.

## 2.2 Materials and methods

### 2.2.1 Soil samples collection

Long-term PCB-contaminated soil was collected from Lhenice dumpsite (South Bohemia, Czech Republic). The soil, originated from a tarmacadam-producing plant (Milevsko, South Bohemia), was moved and stockpiled in this concrete basin more than 20 years ago. The contamination was due to the leakage of the heat-transfer medium Delotherm, a commercial product containing mainly Delor 103 PCB mixture (analogous to Aroclor 1242) that was produced by Chemko Strážske (Slovakia) until 1984. The PCB content is extremely variable and therefore three different soil samples were collected: the bulk soil (referred to as SOIL A), the top soil (referred to as SOIL B) and the rhizosphere soil (referred to as SOIL C). Then the soil samples were sieved (< 2 mm) and homogenized by repeated mixing, air dried for chemical analysis, lyophilized for phospholipid fatty acids (PLFA) analysis and stored at -80 °C for pyrosequencing.

### 2.2.2 Physico-chemical analyses of soil samples

Physico-chemical properties of the three soil samples were evaluated at the Research Institute of Soil and Water Conservation (Prague, Czech Republic). Cation exchange capacity (CEC), water holding capacity (WHC), bulk density, pH and soil texture were assessed as well as the content of organic C, humic and fulvic acids, sulfur, nitrogen and heavy metals (As, Be, Cd, Co, Cr, Cu, Mo, Ni, Pb, V, Zn, Hg).

### 2.2.3 Chemical analysis of contaminants

Polychlorinated biphenyls (PCBs) were extracted by Dionex 200 Accelerated Solvent Extraction (ASE) system (Palaiseau, France). The extractions were performed at 100 °C and 13.8 MPa using a solvent mixture of *n*-hexane:acetone (1:1, v/v). The collected organic extracts were evaporated until dryness, then suspended in *n*-hexane and treated with a mixture of silica gel/sulphuric acid to eliminate interfering compounds, and then applied directly to the chromatography column.

Quantitative analyses of PCBs were performed by gas chromatography-mass spectrometry (GC-MS; 450-GC, 240-MS ion trap detector, Varian, Walnut Creek, CA) using hexachlorobenzene as the internal standard. The GC instrument was equipped with a split/splitless injector maintained at 240 °C. DB-5MS column (Agilent, Prague, Czech Republic) was used for the separations (30 m, 0.25 mm I.D., 0.25 mm film thickness). The

temperature program started at 60 °C and was held for 1 min in the splitless mode. Then the splitter was opened with ratio 1:50. The oven was heated to 120 °C at a rate of 25 °C $min^{-1}$ with a subsequent temperature ramp up to 240 °C at a rate of 2.5 °C $min^{-1}$, where this temperature was maintained for 28 min. The solvent delay time was set at 5 min and the transfer line temperature was set at 240 °C. The mass spectra were recorded at 3 scans $s^{-1}$ under electron impact at 70 eV and mass range 140-410 amu.

PCB metabolites were extracted by ASE system at 150 °C and 11.0 MPa using 1% acetic acid in *n*-hexane:acetone (1:1, V/V). 100 µl of dimethylformamide (DMF) were added to the organic extracts to avoid volatilization of PCB intermediates during the following evaporation step. Once concentrated up to 100 µl, the samples were dissolved in 1 mL of dichloromethane and applied to a gel permeation chromatography (GPC) column (Omnifit ® glass column, 10×500 mm) packed with Bio-beads S-X12 (Bio-Rad, Germany). Metabolites were eluted using dichloromethane as the mobile phase at 0.8 ml $min^{-1}$, collected and then analysed by gas chromatography-mass spectrometry either directly without derivatization or after methylation with diazomethane. PCB products were separated and identified using two different methods: (*i*) the temperature program started at 60 °C and was held for 1 min in the splitless mode. Then the splitter was opened with ratio 1:50. The oven was heated to 100°C at a rate of 25 °C $min^{-1}$ with a subsequent temperature ramp up to 280 °C at a rate of 7.5 °C $min^{-1}$, where this temperature was maintained for 24 min, (*ii*) the oven was heated to 120°C at a rate of 25 °C $min^{-1}$ with a subsequent temperature ramp up to 280 °C at a rate of 2.5 °C $min^{-1}$, where this temperature was maintained for 10 min. The mass spectra were recorded at 3 scans $s^{-1}$ under electron impact at 70 eV and mass range 50-450 amu.

### 2.2.4 Bioavailability of PCBs

Supercritical Fluid Extraction (SFE) was applied to estimate the bioavailability of PCBs in soil A, B and C. SFE was performed with a Labio a.s. extractor (Czech Republic) equipped with a manual restrictor operating at 40 °C and with a downward stream of carbon dioxide. The samples (1.0 g) were placed on the bottom of a 1 mL extraction vessel and the dead volume was filled with sodium sulfate. The PCB analytes were collected on octadecylsilica at -20 °C and eluted within 10 mL of hexane at 25 °C. SFE was performed at 50 °C and 200 bar. Three parallel replicate extractions were carried out and contaminants collected after 5, 10, 20, 40, 60, 80, 120, 160, and 200 min. Individual points of the desorption curves represent means of the three extractions. Data analysis was performed as described by Cajthaml &

Šašek (2005): the chemical release modeled by an empirical two-site model, consisting of the two first-order equations (1):

$$S_t = F \bullet S_o e^{-k1t} + (1 - F) \bullet So \bullet e^{-k2t} \quad (1)$$

where $S_t$ is the residual pollutant concentration in the soil after time $_t$; F is the fraction of chemical rapidly released; $S_o$ is the original concentration of the pollutant in soil; $k_1$ and $k_2$ are the first-order rate constants. The so-called ''F fraction'' is usually assumed to be representative of equilibrium release conditions, and the remaining, slowly released portion is considered to be kinetically rate limited. Therefore, the F fraction represents the portion of the target chemical that is bioavailable in soil (Hawthorne *et al*., 2002; Cajthaml and Šašek, 2005). The fractions were analyzed separately to complete desorption- kinetic profiles. Prism version 4.0 (GraphPad, La Jolla, CA) was used for calculating F values.

### 2.2.5 Microorganisms and inoculum preparation

*Pleurotus ostreatus* 3004 CCBAS 278 and *Irpex lacteus* 617/93 were obtained from the Culture Collection of Basidiomycetes of the Academy of Science of Czech Republic (Prague, CZ). The fungal strains were maintained at 4 °C on Malt Extract Agar plates (MEA) and sub-cultured every month. Five mycelial plugs (0.7 mm Ø) from 7-d-old MEA cultures were used to inoculate 500 mL Erlenmeyer flasks containing 8 g of wheat straw-based pellets previously autoclaved. The moisture of the lignocellulosic substrate (LS) was adjusted to 75% [w/w] with sterile deionized water. Inoculated and non-inoculated LS, used for bioaugmentation and biostimulation treatments respectively, were incubated at 25 °C for 10 days.

### 2.2.6 Microcosms preparation

The three soil samples (A, B and C), the moisture content of which was previously adjusted to 60 % of their respective water holding capacity [w/w], were mixed with either *Pleurotus ostreatus* or *Irpex lacteus* colonized substrate for bioaugmentation treatments to obtain a final soil:ligninolytic substrate mass ratio of 5:1. Soil samples mixed up with the non-inoculated LS were prepared for biostimulation treatments in the same ratios described above. Non-amended and non-inoculated soil samples were prepared and referred to as incubation controls. All experiments were carried out in triplicate under non-sterile conditions. All microcosms were incubated in a ventilated room at approx. 25 °C for 12 weeks and their

moisture content was kept constant by periodical addition of sterile deionized water. Four harvests were set up after 0, 2, 6 and 12 weeks of incubation. Based on the scheduled harvests, the three replicates for each treatment were wholly sacrificed for analyses. The soil samples were air dried for chemical analysis, lyophilized for PLFA analysis and stored at -80 °C for high-throughput sequencing studies.

### 2.2.7 Ecotoxicology test with *Vibrio fischeri* (luminescent bacteria test)

The inhibitory effect of soil extracts on the light emission of *V. fischeri* was measured at the end of each treatments using the Lumino-M90a (ZD Dolni Ujezd, Czech Republic) luminometer according to the normalized ISO 11348-3 protocol 2007 (ISO 11348-3: 2007). Aliquots of the ethyl acetate extracts (0.5 mL) were evaporated to dryness and dissolved in 6% DMSO. The emitted bioluminescence was recorded after 30 min of exposure and percentages of luminescence inhibition (I) were determined as described by Lappalainen et al. (1999).

### 2.2.8 Extraction and analysis of phospholipid fatty acids

Extraction and analysis of phospholipid fatty acids (PLFA) were performed as described by Snajdr et al. (2008). Phospholipids were extracted from 1 g of sample using a mixture of chloroform:methanol:phosphate buffer (1:2:0.8, v/v/v) and then separated by solid-phase extraction cartridges (LiChrolut Si 60, Merck). Samples then underwent mild alkaline methanolysis and the free methyl esters of phospholipid fatty acids were analysed by gas chromatography-mass spectrometry (GC-MS; 450-GC, 240-MS ion trap detector, Varian, Walnut Creek, CA). Fungal biomass was quantified based on 18:2ω6,9 content; bacterial biomass was quantified as a sum of i14:0, i15:0, a15:0, 16:1ω5, 16:1ω7, 16:1ω9, 10Me-16:0, i16:0, i17:0, a17:0, cy17:0, 17:0, 10Me-17:0, 18:1ω7, 18:1ω9, 10Me-18:0, cy19:0 (actinobacteria 10Me-16:0, 10Me-17:0, 10Me-18:0, Gram+ i14:0, i15:0, a15:0, i16:0, i17:0, a17:0 and Gram- 16:1ω7, 16:1ω9, 18:1ω7, cy17:0, cy19:0).

### 2.2.9 DNA extraction, amplification and pyrosequencing analysis

Genomic DNA was extracted from soil samples as described by Sagova-Mareckova et al. (2008). The first amplification step was performed using the eubacterial primers eub530F and eub1100aR to amplify the V4–V6 hypervariable regions of the bacterial 16S rDNA gene and the fungi-specific primers ITS1 and ITS4 to amplify the internal transcribed spacer (ITS) region of fungal rDNA. The three replicates of each soil sample were pooled and purified

using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA). Purified DNA concentration was quantified using ND1000 (Nano-Drop, Wilmington, DE, USA).
In the second amplification step, fusion primers were tailored for tag-encoded 454-Titanium pyrosequencing: different barcode sequences were added at the 5' end of forward primer separated by a trinucleotide spacer. Titanium A adaptor was also used (Roche, Basel, Switzerland). Purification of PCR products was performed using MinElute PCR Purification Kit (Qiagen, Hilden, Germany) and concentration was quantified by ND1000 (Nano-Drop, Wilmington, DE, USA) and Quant-iT Picogreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA). Purified amplicons were used for the subsequent emulsion PCR (emPCR Kit Lib-L, Roche), the products of which were sequenced on the 454 GS Junior platform (Roche) in accordance with manufacturer's instructions (Roche, Basel, Switzerland).

### 2.2.10 Pyrosequencing data analysis

Bacterial and fungal sequences were processed with QIIME 1.6.0 software package. Quality filtering steps were performed to trim off barcodes and primers from the raw sequences and to remove sequences (*i*) < 200 nt in length, (*ii*) with homopolymers longer than 6 nt, (*iii*) with quality score < 25 and < 20 for bacteria and fungi, respectively. Denoising was performed as described by Reeder and Knight (2010). QIIME's implementation of OTUPipe script was applied for chimera checking and OTU picking (Edgar, 2010; Edgar *et al*., 2011). 16S Microbial blast database and UNITE database were used as reference databases for bacterial and fungal chimeric sequences detection, respectively. Resulting chimera-free reads were clustered into OTUs based on their sequence similarity at 97 %. Representative sequences of each OTU were aligned using MUSCLE (Edgar, 2004) and used for taxonomy assignment. Ribosomal Database Project classifier and UNITE database were used in order to taxonomically classify the bacterial and fungal sequences, respectively. Alpha-rarefaction and Alpha-diversity analyses were performed using QIIME 1.6.0 and Beta-diversity was assessed by Principal Component Analysis (PCA).

### 2.2.11 Statistical analysis

Multiple pair-wise comparisons of PCB degradation data were performed by the Tukey test ($P \leq 0.05$) using SigmaStat software version 3.5. Pyrosequencing results were evaluated by Principal Component Analysis (PCA) using Minitab 16 version 2.2.0 (Minitab, Inc., PA, U.S.A.).

## 2.3 Results and Dicussion

### 2.3.1 Physico-chemical characterization of soil samples

Physico-chemical properties of the pristine soils are reported in Table 2.1. The granulometric analysis revealed that the contaminated matrices A, B and C are sandy loamy soils characterized by a content of 59.6, 60.2 and 53.4 % of sand, 31.2, 30.0 and 35.5 % of silt and 9.2, 9.8 and 11.1 % of clay, respectively.

As expected, the amount of organic carbon was higher in the topsoil (soil B) and in the rhizosphere soil (soil C) than in the bulk soil (soil A) (3.46 and 2.4 *vs* 0.98 %). This parameter must to be taken into account considering the biological approaches to remediate contaminated soils: the content of soil organic matter (*i.e.* humic and fulvic acids), as already mentioned, significantly affect the soil sorption/desorption processes and, therefore the fate and the bioavailability of hydrophobic contaminants such as PCBs. Likewise, the cation exchange capacity (CEC) of soil B and C (20.12 and 18.13 mmol (+) $100g^{-1}$) was greater compared to that of soil A (12.67 mmol (+) 100 $g^{-1}$): CEC is closely linked and positively correlated to the organic matter content of the soil as reported in a number of studies (Crovetto, 1997; FAO, 2005). The presence of metals was also assessed in all soil samples resulting in high concentration of chromium (160.1, 123.0 and 174.0 mg $kg^{-1}$ in soil A, B and C, respectively).

***Tab. 2.1.*** Physico-chemical properties of the pristine soil A, B and C.

| | Units | **Soil A** | **Soil B** | **Soil C** |
|---|---|---|---|---|
| pH $H_2O$ | --- | 6.60 | 6.07 | 6.16 |
| pH $CaCl_2$ | --- | 6.37 | 5.81 | 5.74 |
| Exchangeable $H^+$ | mmol+/100g | 0.50 | 2.60 | 2.80 |
| Exchangeable Ca | mmol+/100g | 9.24 | 16.40 | 12.89 |
| Exchangeable Mg | mmol+/100g | 2.73 | 5.56 | 2.21 |
| Exchangeable K | mmol+/100g | 0.26 | 1.25 | 0.70 |
| Exchangeable Na | mmol+/100g | 0.05 | 0.05 | 0.05 |
| CEC | mmol+/100g | 12.67 | 20.12 | 18.13 |
| Sulphur content | mmol+/100g | 12.67 | 17.51 | 15.33 |
| Degree of saturation | % | 100.00 | 87.00 | 85.00 |
| ECEC | mmol+/100g | 12.21 | 17.81 | 13.88 |
| Accessible Ca | mg/kg | 1650.00 | 2649.00 | 2355.00 |
| Accessible Mg | mg/kg | 325.00 | 311.00 | 266.00 |
| Accessible K | mg/kg | 102.00 | 412.00 | 247.00 |
| Accessible P | mg/kg | 45.40 | 111.40 | 79.10 |
| N total | % | 0.07 | 0.23 | 0.17 |
| C (Organic) | % | 0.73 | 2.52 | 1.76 |
| Fulvic acids | % | 0.08 | 0.57 | 0.40 |
| Humic acids | % | 0.17 | 0.37 | 0.24 |
| Hg AMA | mg/kg | 0.04 | 0.05 | 0.05 |
| As (Aqua regia) | mg/kg | 43.91 | 35.86 | 43.93 |
| Be (Aqua regia) | mg/kg | 1.62 | 1.56 | 1.65 |
| Cd (Aqua regia) | mg/kg | 0.22 | 0.34 | 0.25 |
| Co (Aqua regia) | mg/kg | 15.67 | 13.38 | 17.66 |
| Cr (Aqua regia) | mg/kg | 160.10 | 123.00 | 174.00 |
| Cu (Aqua regia) | mg/kg | 31.30 | 20.60 | 29.00 |
| Mo (Aqua regia) | mg/kg | 0.05 | 0.05 | 0.05 |
| Ni (Aqua regia) | mg/kg | 54.10 | 43.70 | 52.60 |
| Pb (Aqua regia) | mg/kg | 24.60 | 22.80 | 22.80 |
| V (Aqua regia) | mg/kg | 40.30 | 41.60 | 44.40 |
| Zn (Aqua regia) | mg/kg | 97.00 | 100.80 | 100.20 |
| Particles <0.001 mm | % | 7.30 | 7.40 | 8.80 |
| Particles <0.002 mm | % | 9.20 | 9.80 | 11.10 |
| Particles < 0.01 mm | % | 18.30 | 19.80 | 22.80 |
| Particles < 0.05 mm | % | 40.40 | 39.80 | 46.60 |
| Particles 0.01-0.05 mm | % | 22.10 | 20.00 | 23.80 |
| Particles 0.05-0.25 mm | % | 24.80 | 26.60 | 22.20 |
| Particles 0.25-2.0 mm | % | 34.80 | 33.60 | 31.20 |
| Bulk density | g/cm3 | 1.33 | 1.21 | 1.17 |
| Water holding capacity (WHC) | % Vol. | 31.80 | 42.40 | 39.90 |
| $S/SO_4$ - $CaCl_2$ leachate | mg/kg | 5.20 | 9.40 | 7.30 |
| Combustion loss | % | 3.81 | 6.15 | 6.46 |

### 2.3.2 Contaminant characterization of soil samples

The three polluted matrices of concern were mostly contaminated with the PCB mixture Delor 103, the composition of which was defined by Taniyasu et al. (2003). Therefore, the chemical characterization of the soil samples was mainly aimed at the quantification of those congeners which are known to be present in the aforementioned mixture. The concentration of PCBs was evaluated in all soil samples and the results are reported in Table 2.2. Among the three soil samples, the bulk soil (soil A) was the most heavily contaminated with an overall PCB concentration of 705.95 mg $Kg^{-1}$, while the least contaminated one (169.36 mg $Kg^{-1}$) was the rhizosphere soil (soil C). In all cases, the contamination mainly consisted of tetrachloro-biphenyls (70, 72 and 74 % in soil A, B and C, respectively) and the congener n. 56 (2,3,3',4'-tetrachlorobiphenyl) was the most abundant one in all samples (17, 21 and 22 % in soil A, B and C, respectively). Interestingly, the congener n. 118 (2,3',4,4',5-pentachlorobiphenyl) was the most abundant among the pentachloro-biphenyls in soil A and B. This congener displays "dioxin-like" properties, referring both to its toxicity and structural features which make it similar to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (EPA).

Moreover, due to the aging of the three contaminated matrices and therefore the possible occurrence of degradation processes along the time, an extensive chemical analysis of PCB degradation intermediates was performed. Among them, only chlorobenzoic acids (CBAs) were detected (Table 2.3). These chlorinated compounds are known either to be produced by fungi as intermediates of PCB mineralization process or to be accumulated as dead-end products during the aerobic upper PCB degradation pathway mediated by bacteria (Cvancarova *et al.*, 2012; Adebusoye *et al.*, 2008). Specifically, the highest concentration of CBAs was observed in soil A (743.6 μg $Kg^{-1}$), while 250.3 and 185.8 μg $Kg^{-1}$ were found in soil B and C, respectively. The presence of CBAs in all soil samples was mainly due to the formation of mono- and di-chlorinated benzoates. Among trichlorinated clorobenzoates, small amount of 2,3,5-CBA and 2,4,6-CBA were detected, while 2,3,6-CBA, tetra- and penta-CBAs were not found.

***Tab. 2.2.*** PCB concentration (mg $Kg^{-1}$) in the pristine soil A, B and C. Data are the mean ± standard deviation of five replicates.

| **PCB congeners** | | **Concentration (mg $Kg^{-1}$)** | | |
|---|---|---|---|---|
| *IUPAC nomenclature* | *Ballschmiter & Zell nomenclature* | **Soil A** | **Soil B** | **Soil C** |
| 2,2',3 + 2,4',6-CB | 16+32 | **11.20** ± 0.15 | **1.97** ± 0.67 | **3.10** ± 1.49 |
| 2,2',4 -CB | 17 | **2.66** ± 2.10 | **0.28** ± 0.17 | **1.15** ± 0.65 |
| 2,2',5 -CB | 18 | **1.49** ± 0.78 | **0.19** ± 0.04 | **0.40** ± 0.01 |
| 2,2',6 -CB | 19 | **0.08** ± 0.05 | **0.00** ± 0.00 | **0.00** ± 0.00 |
| 2,3,4'-CB | 22 | **62.98** ± 0.81 | **13.58** ± 0.87 | **8.92** ± 0.49 |
| 2,3,6 + 2,3',6-CB | 24+27 | **0.17** ± 0.08 | **0.00** ± 0.00 | **0.01** ± 0.01 |
| 2,3',4-CB | 25 | **0.18** ± 0.07 | **0.00** ± 0.00 | **0.00** ± 0.00 |
| 2,3',5-CB | 26 | **0.12** ± 0.06 | **0.00** ± 0.00 | **0.02** ± 0.02 |
| 2,4,4' + 2,4',5-CB | 28+31 | **13.52** ± 0.40 | **4.93** ± 0.21 | **2.24** ± 0.23 |
| 2,2',3,3'-CB | 40 | **0.24** ± 0.05 | **0.09** ± 0.02 | **0.06** ± 0.02 |
| 2,2',3,4 + 2,3,4',6 + 2,3',4',6 + 2,3',5,5'-CB | 41+64+71+72 | **42.10** ± 2.18 | **26.06** ± 1.38 | **12.05** ± 1.42 |
| 2,2',3,4'-CB | 42 | **8.45** ± 0.50 | **6.18** ± 0.50 | **2.54** ± 0.08 |
| 2,2',3,5'-CB | 44 | **67.56** ± 0.98 | **24.64** ± 1.29 | **14.01** ± 0.12 |
| 2,2',3,6-CB | 45 | **21.86** ± 1.02 | **8.37** ± 0.08 | **4.65** ± 0.79 |
| 2,2',3,6'-CB | 46 | **0.15** ± 0.03 | **0.06** ± 0.01 | **0.07** ± 0.04 |
| 2,2',4,4' + 2,2',4,5-CB | 47+48 | **39.62** ± 1.32 | **14.80** ± 0.61 | **6.94** ± 0.08 |
| 2,2',4,5'-CB | 49 | **56.08** ± 0.80 | **23.91** ± 2.69 | **13.25** ± 1.01 |
| 2,2',4,6'-CB | 51 | **0.13** ± 0.02 | **0.06** ± 0.02 | **0.07** ± 0.04 |
| 2,2',5,5'-CB | 52 | **69.45** ± 0.86 | **33.36** ± 1.40 | **17.43** ± 0.67 |
| 2,2',5,6'-CB | 53 | **0.31** ± 0.07 | **0.10** ± 0.03 | **0.07** ± 0.03 |
| 2,3,3',4'-CB | 56 | **117.01** ± 0.16 | **78.61** ± 2.88 | **36.38** ± 1.77 |
| 2,3,4',5-CB | 63 | **0.13** ± 0.02 | **0.07** ± 0.02 | **0.08** ± 0.05 |
| 2,3',4,4'-CB | 66 | **21.53** ± 0.13 | **15.81** ± 0.26 | **6.16** ± 0.80 |
| 2,3',4,5-CB | 67 | **0.11** ± 0.27 | **0.05** ± 0.00 | **0.05** ± 0.02 |
| 2,3',4',5 + 2,3',4',5'-CB | 70+76 | **44.21** ± 0.01 | **38.22** ± 0.72 | **11.34** ± 0.36 |
| 2,4,4',5-CB | 74 | **1.39** ± 0.31 | **0.87** ± 0.04 | **0.84** ± 0.04 |

***Tab. 2.2*** PCB concentration (mg $Kg^{-1}$) in the pristine soil A, B and C. Data are the mean ± standard deviation of five replicates. (*Continued*)

| **PCB congeners** | | **Concentration (mg $Kg^{-1}$)** | | |
|---|---|---|---|---|
| *IUPAC nomenclature* | *Ballschmiter & Zell nomenclature* | **Soil A** | **Soil B** | **Soil C** |
| 2,2',3,4,5'-CB | 87 | **0.25** ± 0.01 | **0.14** ± 0.03 | **0.09** ± 0.00 |
| 2,2',3,4,6 + 2,2',3,5,5' + 2,2',3,5,6'-CB | 89+92+94 | **0.16** ± 0.02 | **0.05** ± 0.01 | **0.03** ± 0.00 |
| 2,2',3,4',6-CB | 91 | **0.11** ± 0.02 | **0.05** ± 0.01 | **0.09** ± 0.01 |
| 2,2',3,5,'6-CB | 95 | **16.40** ± 2.74 | **7.38** ± 0.94 | **5.37** ± 0.30 |
| 2,2',3,4',5'-CB | 97 | **0.14** ± 0.03 | **0.07** ± 0.00 | **0.14** ± 0.00 |
| 2,2',4,4',5 + 2,3,3',5',6-CB | 99+113 | **20.01** ± 0.43 | **10.84** ± 0.48 | **3.98** ± 0.17 |
| 2,2',4,5,5'-CB | 101 | **12.72** ± 2.31 | **11.82** ± 0.53 | **3.80** ± 0.32 |
| 2,3,3',4,4'-CB | 105 | **18.78** ± 1.38 | **12.82** ± 0.76 | **0.00** ± 0.00 |
| 2,3,3',5,5'-CB | 110 | **17.10** ± 0.04 | **15.14** ± 0.77 | **6.66** ± 0.25 |
| 2,3,3',5,5'-CB | 111 | **0.14** ± 0.37 | **0.10** ± 0.03 | **0.12** ± 0.00 |
| 2,3',4,4',5-CB | 118 | **23.16** ± 0.01 | **18.21** ± 0.66 | **5.46** ± 0.58 |
| 2,2',3,4,4',5'-CB | 138 | **6.76** ± 0.56 | **0.68** ± 0.96 | **0.00** ± 0.00 |
| 2,2',3,4',5',6-CB | 149 | **4.46** ± 0.87 | **1.42** ± 0.15 | **1.40** ± 0.65 |
| 2,2',4,4',5,5'-CB | 153 | **1.39** ± 0.41 | **4.68** ± 0.19 | **0.36** ± 0.10 |
| 2,2',3,4,4',5,5'-CB | 180 | **1.65** ± 0.43 | **0.20** ± 0.29 | **0.00** ± 0.00 |
| **overall PCBs** | | **705.95** ± 22.85 | **375.81** ± 19.45 | **169.36** ± 12.63 |

***Tab. 2.3*** CBA concentration (µg $Kg^{-1}$) in the pristine soil A, B and C. Data are the mean ± standard deviation of five replicates.

| **CBA isomers** | **Concentration (µg $Kg^{-1}$)** | | |
|---|---|---|---|
| | **Soil A** | **Soil B** | **Soil C** |
| 2-CBA | **9.2** ± 1.5 | **6.5** ± 0.5 | **4.6** ± 1.7 |
| 3-CBA | **378.4** ± 21.3 | **95.4** ± 9.8 | **85.6** ± 4.2 |
| 4-CBA | **19.0** ± 2.3 | **10.1** ± 1.9 | **0.0** ± 0.0 |
| 2,3-CBA | **24.0** ± 2.6 | **21.6** ± 2.4 | **16.5** ± 1.1 |
| 2,4-CBA | **189.2** ± 15.5 | **62.3** ± 4.3 | **35.0** ± 2.7 |
| 2,5-CBA | **28.3** ± 2.3 | **22.9** ± 2.4 | **16.4** ± 2.8 |
| 2,6-CBA | **6.9** ± 0.8 | **0.0** ± 0.0 | **0.1** ± 0.2 |
| 3,4-CBA | **66.5** ± 6.9 | **22.8** ± 3.1 | **16.1** ± 2.6 |
| 3,5-CBA | **5.1** ± 1.2 | **1.1** ± 1.9 | **0.0** ± 0.0 |
| 2,3,5-CBA | **8.2** ± 1.2 | **3.3** ± 0.7 | **1.1** ± 0.3 |
| 2,3,6-CBA | **0.0** ± 0.0 | **0.0** ± 0.0 | **0.0** ± 0.0 |
| 2,4,6-CBA | **8.9** ± 1.9 | **4.4** ± 0.6 | **10.4** ± 1.6 |
| 2,3,5,6-CBA | **0.0** ± 0.0 | **0.0** ± 0.0 | **0.0** ± 0.0 |
| 2,3,4,5-CBA | **0.0** ± 0.0 | **0.0** ± 0.0 | **0.0** ± 0.0 |
| 2,3,4,5,6-CBA | **0.0** ± 0.0 | **0.0** ± 0.0 | **0.0** ± 0.0 |
| **Overall CBAs** | **743.6** ± 57.2 | **250.3** ± 27.5 | **185.8** ± 17.2 |

### 2.3.3 Bioavailability of PCBs

The bioavailability of pollutants, defined as the amount of compound that could be taken up and transformed by living organisms, is one of the most important factor affecting the success of bioremediation strategies. The fate and the behaviour of organic pollutants in soils and therefore, their bioavailability is influenced by several factors including the physico-chemical characteristics of the soil (*i.e.* soil organic matter and clay content), the concentration and properties of pollutants and their residence time in soil (Reid *et al*., 2000; Bielska *et al.*, 2013). This last factor leading to a phenomenon, referred to as contaminant "ageing", which contributes to the sequestration of pollutants in the soil structure decreasing their accessibility and biodegradability (Alexander, 2000). Hence, due to the prolonged pollutants-soil contact time of our samples, Supercritical Fluid Extraction (SFE) was applied to quantify the bioavailable fraction (F) of PCBs in pristine soil A, B and C and, thus to predict the efficiency of selected remediation approaches towards these contaminated matrices.

Analysis of data indicates that the estimated F fraction of all Delor 103 representative PCBs (where F=1 corresponds to 100 % of biovailability) was higher in the bulk soil A (F=0.94, $r^2$=0.993) than in top soil B (F=0.62, $r^2$=0.997) and rhizosphere soil C (F=0.64, $r^2$=0.977) (*Figure 2.2*). The lower bioavailability of PCBs in soil B and C than in soil A (62 and 64 *vs* 94 %) could be explained by their different soil organic matter content, as above reported. Indeed, the presence of SOM significantly decreases the bioavailability of organic pollutants because of their adsorption onto organic matter and the consequent entrapment within its nanoporous structures (Hatzinger and Alexander, 1995; Kan *et al*., 1998).

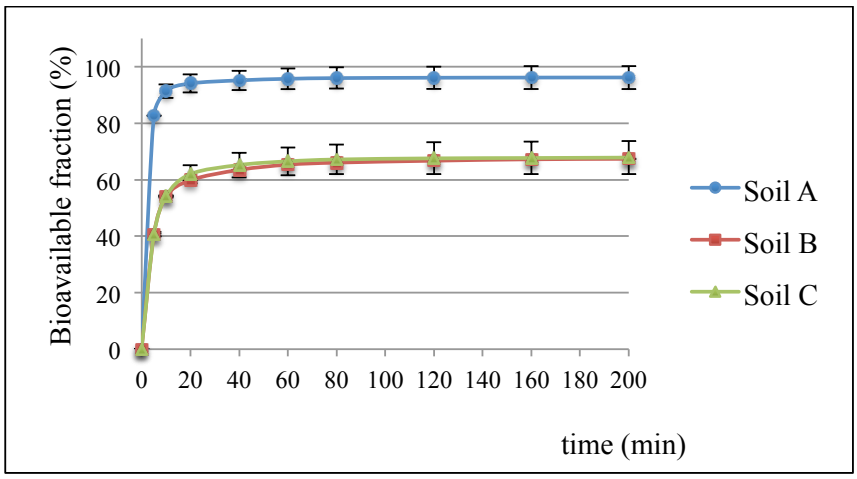



***Fig. 2.2.*** Bioavailability of PCBs in the pristine soil A, B and C. The values, espressed as percentage of bioavailable fraction, are means of three replicates.

Furthermore, the persistence of certain chemicals in soils seems to be related to compound hydrophobicity. In the case of PCBs, hydrophobicity increases with rising chlorine content and thus, the bioavailability of these chemicals was evaluated as a function of their chlorination degree. Results referred to PCB homologs (namely to PCBs bearing the same number of chlorine substituents) showed that the lowest bioavailable fraction was associated with higher chlorinated congeners: the F values of hepta-CBs were 0.79, 0.51 and 0.43 in soil A, B and C, respectively (*Figure 2.3*, Table 2.4). These outcomes confirmed the initial hypothesis that the greater is the hydrophobicity of a chemical, the greater is its sorption potential and, therefore the lower is its availability.

***Tab 2.4.*** F values and $R^2$ of tri-, tetra-, penta-, hexa- and hepta-chlorinated biphenyls (tri-CBs, tetra-CBs, penta-CBs, hexa-CBs and hepta-CBs) in the pristine soil A, B and C. Data are the mean ± standard deviation of three replicates.

| | tri-CBs | tetra-CBs | penta-CBs | hexa-CBs | hepta-CBs |
|---|---|---|---|---|---|
| **Soil A** | | | | | |
| F value | **0.84**±0.01 | **0.93**±0.01 | **0.80**±0.01 | **0.98**±0.02 | **0.79**±0.01 |
| $R^2$† | *0.995* | *0.994* | *0.999* | *0.999* | *0.995* |
| **Soil B** | | | | | |
| F value | **0.65**±0.01 | **0.62**±0.01 | **0.62**±0.02 | **0.62**±0.01 | **0.51**±0.01 |
| $R^2$† | *0.997* | *0.987* | *0.972* | *0.998* | *0.982* |
| **Soil C** | | | | | |
| F value | **0.66**±0.01 | **0.66**±0.02 | **0.64**±0.03 | **0.61**±0.01 | **0.43**±0.01 |
| $R^2$† | *0.996* | *0.968* | *0.956* | *0.993* | *0.995* |

† $R^2$ indicates the coefficient of determination for each fitting equation.

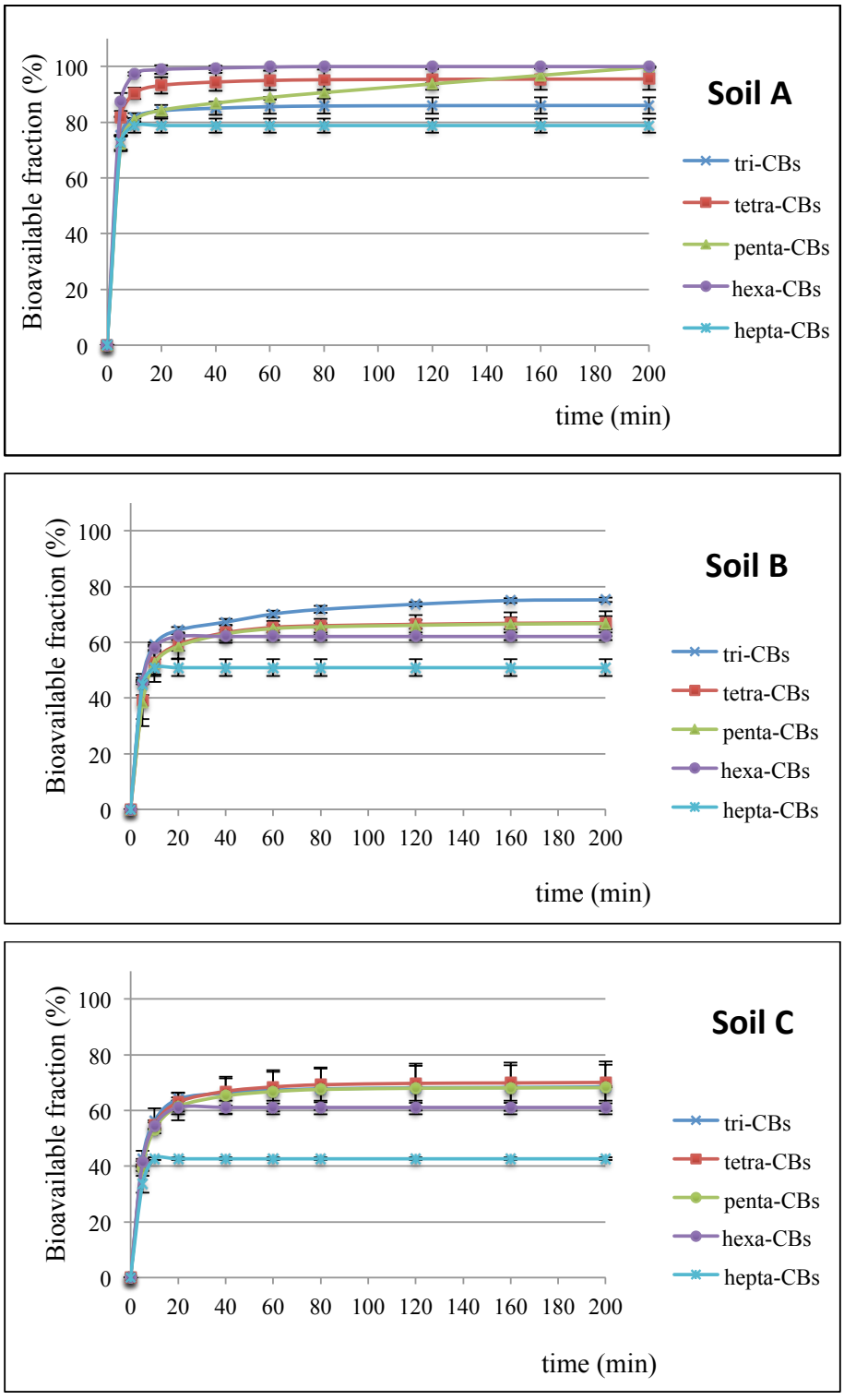



***Fig. 2.3.*** Bioavailability of tri-, tetra-, penta-, hexa- and hepta-chlorinated biphenyls (tri-CBs, tetra-CBs, penta-CBs, hexa-CBs and hepta-CBs) in the pristine soil A, B and C. The values of bioavailable fraction of each homolog group are expressed as percentage of their respective overall contents. Data are means ± standard deviation of three replicates.

### 2.3.4 Degradation of PCBs

With regard to remediation experiment, the time courses of PCB degradation in both myco-augmented and biostimulated microcosms are shown in *Figure 2.4*.

As for the most contaminated soil (soil A), no significant decrease in PCB concentration was observed within the first 6 weeks of incubation, regardless of the treatment applied. A modest removal was obtained only in *P. ostreatus* and *I. lacteus*-augmented microcosms after 12 weeks (18.5 and 19.3 %, respectively).

Concerning soil B, the PCB removal was 41.3 and 39.4 % in the presence of *P. ostreatus* and *I. lacteus,* respectively, at the same time interval (12 weeks). Despite the similar degradation performances of the two bioaugmented fungi, the removal of PCBs in soil B was faster with *I. lacteus* than with *P. ostreatus* within the first 2 weeks (16.9 *vs* 9.3%). On the contrary*, P. ostreatus* was more efficient than *I. lacteus* during the further 4 weeks of incubation (30.4 *vs* 24.3 %).

In the rhizosphere soil (soil C), the highest depletion of PCB (50.5 %) with respect to the original contamination was achieved in *P. ostreatus*-augmented soil, while *I. lacteus* promoted a degradation of 30.3 %. Comparing the amounts of removed PCBs, *P. ostreatus* was more effective in soil B than in soil C (155.2 *vs* 85.5 mg $Kg^{1}$).

The greater degradation capability of *P. ostreatus* than to those of other fungal strains was also previously observed in both liquid and solid systems (Cvancarova *et al*., 2012; Kubatova *et al*., 2001). By using the former system, the degrading efficiency of eight ligninolytic fungi towards the PCB mixture Delor 103 was comparatively evaluated: 98.4 and 99.6 % of PCBs (initial concentration of 10 mg $Kg^{-1}$) were removed by *P. ostreatus* in complex and mineral media, respectively, after 6 weeks of treatment (Cvancarova *et al*., 2012). *P. ostreatus* was also able to degrade about 40% of PCBs in a soil system artificially contaminated with 10 mg $Kg^{-1}$ of Delor 103 after 60 days of incubation (Kubatova *et al*., 2001).

Regardless of the soil typology, the extent of PCB removal by the biostimulation treatments was invariably lower than that achieved in bioaugmented microcosms after 12 weeks of incubation. Generally, even though a modest removal of PCBs was observed in soil B (22.9 %), the stimulation of resident microorganisms *via* the addition of a lignocellulosic substrate was not as successful as the bioaugmentation strategy. This outcome was obtained also in a previous work where the PCB degradation capability of the white rot fungus *Lentinus tigrinus* was compared with the efficiency of a biostimulation treatment in the clean-up of a historically contaminated soil by Aroclor 1260 (Federici *et al*., 2012). In the same study, the

percent depletion of PCBs in *L. tigrinus*-augmented microcosms was higher than that observed in microcosms where maize stalks were added as a lignocellulosic amendant (33.8 *vs* 28.0 %, respectively).

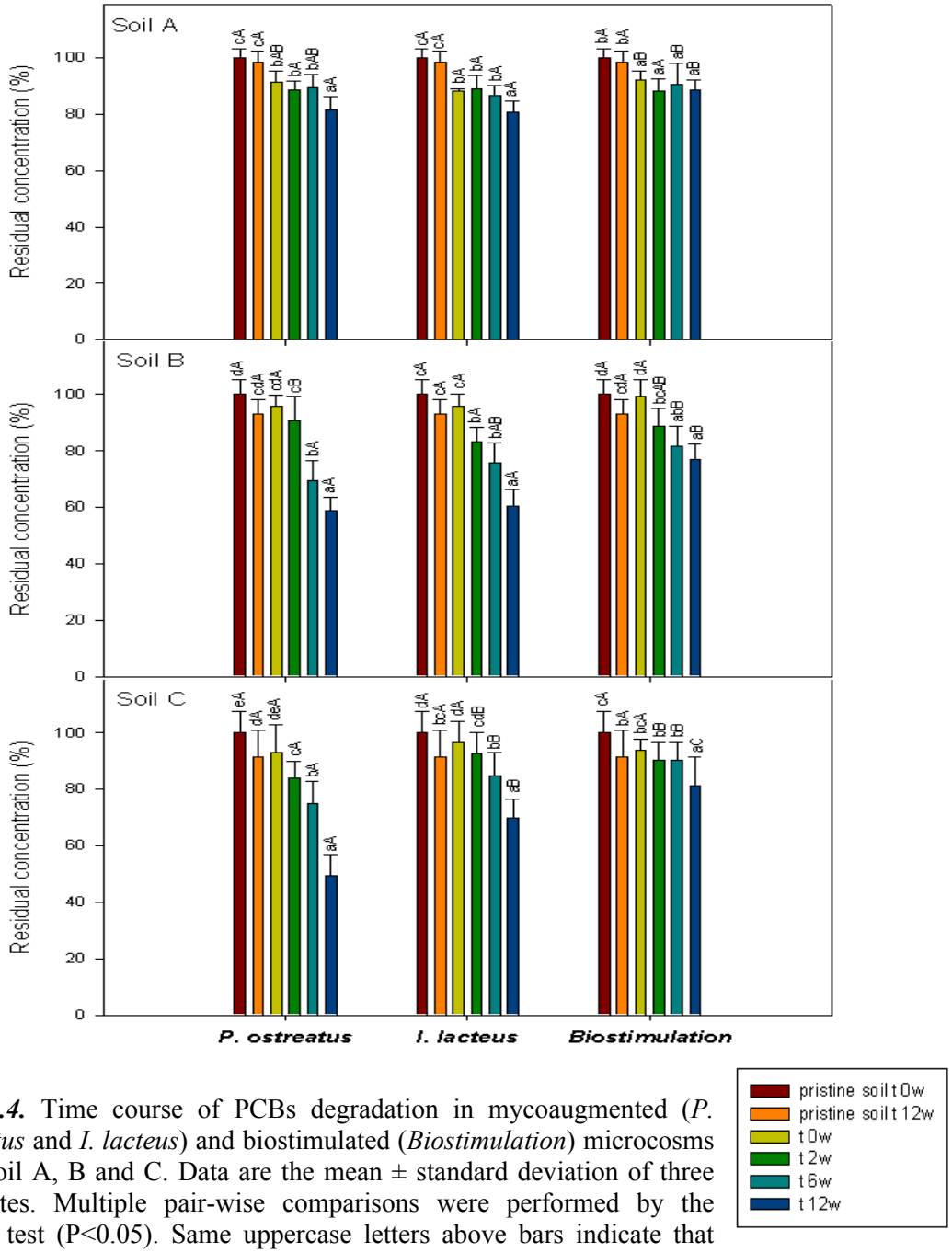



***Fig. 2.4.*** Time course of PCBs degradation in mycoaugmented (*P. ostreatus* and *I. lacteus*) and biostimulated (*Biostimulation*) microcosms with soil A, B and C. Data are the mean ± standard deviation of three replicates. Multiple pair-wise comparisons were performed by the Tukey test (P<0.05). Same uppercase letters above bars indicate that differences among the harvests (time) within the same treatment were not significant. Same lowercase letters indicate lack of statistically significant differences among coeval treatments.

***Tab. 2.5.*** Degradation of PCBs expressed as percentage and mg $Kg^{-1}$ of removed PCBs in mycoaugmented (*P. ostreatus* and *I. lacteus*) and biostimulated (*Biostimulation*) microcosms with soil A, B and C after 12 weeks of incubation.

| Treatment | Soil A | | Soil B | | Soil C | |
|---|---|---|---|---|---|---|
| | PCB degradation (%) | Absolute PCB amount (mg $Kg^{-1}$) | PCB degradation (%) | Absolute PCB amount (mg $Kg^{-1}$) | PCB degradation (%) | Absolute PCB amount (mg $Kg^{-1}$) |
| *P. ostreatus* | 18.5 | 130.5 | 41.3 | 155.2 | 50.5 | 85.5 |
| *I. lacteus* | 19.3 | 136.2 | 39.4 | 148.0 | 30.3 | 51.3 |
| *Biostimulation* | 11.5 | 81.0 | 22.9 | 85.9 | 18.6 | 31.5 |

### 2.3.5 *Vibrio fischerii* toxicity assay

The validity of selected bioremediation methods must be corroborated by the efficiency in the degradation of target pollutants as well as in the toxicity reduction of contaminated matrices. Therefore, toxicological assay, employing the luminescent bacterium *V. fischerii*, were performed to assess the toxicity of soil extracts on this microorganism, before and after the treatments. Data are summarized in *Figure 2.5.*

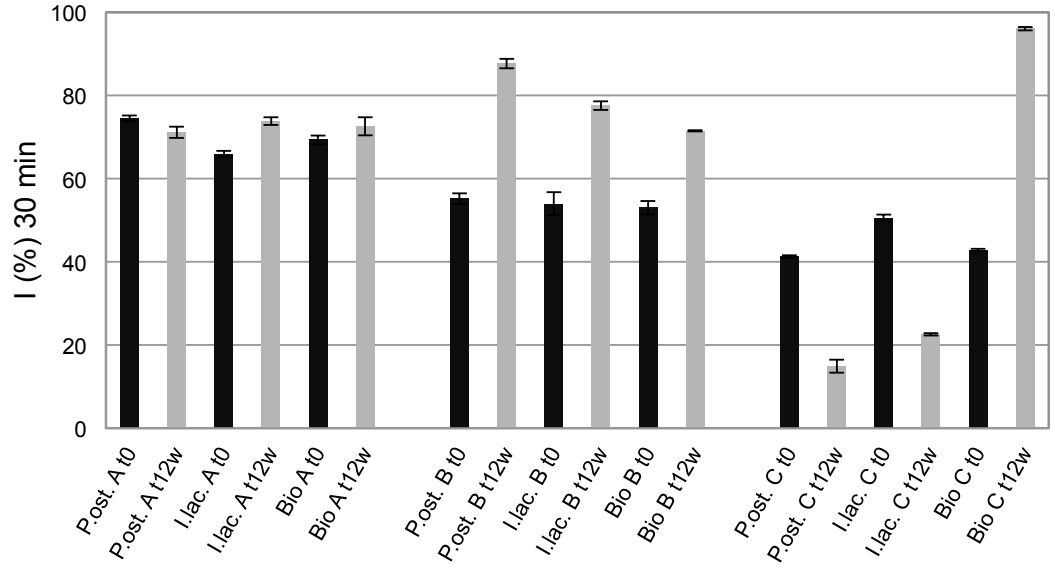



***Fig. 2.5.*** Percentage of luminescence inhibition in mycoaugmented (*P. ost.* and *I. lac.*) and biostimulated (*Bio)* microcosms with soil A, B and C at the beginning (t0) and at the end (t12w) of each treatment.

At the beginning of bioaugmentation or biostimulation treatments, luminescence inhibition was higher in soil A (69.9 %) than in soil B (54.1 %) and C (44.9 %). This result suggested that the higher content of PCBs exerted a greater toxicity towards *V. fischerii*.

Concerning the treatment of soil A, the slight PCB removal in both myco-augmented and biostimulated microcosms did not lead to significant changes in toxicity along the incubation, while an increase in luminescence inhibition was even observed after the treatment of soil B in all cases. Specifically, higher toxicity values were obtained in *P.ostreatus* and *I. lacteus* treated soil (37.0 and 30.4 %, respectively, with respect to the initial percentage of inhibition) than in biostimulated microcosms despite of the capability of these two fungal strains to perform a PCB removal of 41.3 and 39.4 % from soil B. These outcomes suggested that the fungal transformation of PCBs in soil led to the formation of more polar metabolites, which were more toxic than the parent compounds. Conversely, *P. ostreatus* and *I. lacteus* substantially reduced the toxicity (63.9 and 55.3 %. respectively) in soil C after 12 weeks of treatment.

In view of the initial consideration concerning the efficiency of a bioremediation treatment, the objectives in terms of concomitant high PCB degradation and detoxification were achieved with the bioaugmentation strategies, with the best results being obtained with of *P. ostreatus* in the clean-up of the rhizosphere soil (soil C).

### 2.3.6 Detection and identification of PCB degradation intermediates

An extensive set of qualitative GC-MS analysis was undertaken to identify PCB metabolites and to gain insights into the fungal PCB degradation pathways in soil systems. Several intermediates were detected throughout the mycoremediation treatments and their structures were suggested by comparing the data in the NIST 08 library and independently by interpreting the fragmentation patterns (Table 2.6). Product ion scan (MS-MS) was used when necessary to clarify the fragmentation sequence, and all the molecular weights were estimated according to chemical ionization. Derivatization with diazomethane enabled the detection of 3 and 5 isomers of hydroxylated tri- and tetra-chloroBP, respectively, in both bioaugmented and biostimulated microcosms, either in the early phases or at the end of the treatments. Dihydroxylated tri- and tetrachloroBP were also found in soil A and C after the treatment with *I. lacteus*. The formation of the latter compounds can be attributed either to the intracellular CYP450 enzymatic system of *Irpex lacteus* or to the dioxygenases activity of autochthonous bacteria.

Moreover, methoxy-substituted-derivatives of tetrachlorobiphenyls were detected without derivatization in *P. ostreatus* microcosms at the end of incubation. The fungal transformation of PCBs to their corresponding hydroxylated and methoxylated forms was already proved in model liquid systems (Kamei *et al*., 2006a; Cvancarova *et al*., 2012). In particular, both studies proposed a pathway for the biotransformation of PCBs where methoxylated PCBs are formed *via* initial hydroxylation reaction mediated by the cytochrome P450 monooxygenases system (CYP450). Moreover, the formation of hydroxylated compounds when biostimulation treatment was applied suggested that autochthonous fungi took part in the biotransformation of PCBs. In this regard, an early study on PCB microbial degradation reported the capability of the soil fungus *Rhizopus japonicus* to convert 4-chloroBP and 4,4′-dichloroBP to their corresponding hydroxylated forms (Wallnofer *et al*., 1973).

Additionally, trichlorobenzoic acids, dichloro benzylalcohols and trichlorocresol were identified in *P. ostreatus* microcosms with soil A and B after 12 weeks of incubation. The formation of chlorobenzoates (CBAs) from hydroxylated PCBs and their further transformation *via* a reductive pathway was already proposed (Kamei *et al.*, 2006a; Cvancarova *et al.*, 2012). In particular, the concomitant presence of reduced forms of chlorobenzoic acids (dichloro benzylalcohols and trichlorocresol) confirmed the hypothesis that a reductive mechanism operates on the carboxyl group of CBAs in agreement with other studies (Muzikar *et al.*, 2011; Cvancarova *et al*., 2012). Once CYP450 oxidized the aromatic structure of PCBs, ring fission reaction can be mediated by another enzymatic system (*i.e.* ligninolytic system) as suggested by Cajthaml et al. (2006). Thereafter, side-chain reductive reactions of CBAs might proceed *via* a two-step process catalysed by NADPH-dependent aryl-aldehyde dehydrogenase and aryl-alcohol dehydrogenase (Covino, 2010).

All of the detected metabolites were found at trace levels with the exception of 2,3,6-trichlorobenzoic acid. The accumulation of this intermediate is probably due to its recalcitrance as already demonstrated (Covino, 2010; Muzikar *et al*., 2011). The double *ortho*-chlorine substitution and the electron-withdrawing effect of these chlorine atoms adjacent to the carboxyl group can prevent the enzymatic attack towards CBAs.

***Tab. 2.6.*** Degradation intermediates of PCBs in ethyl acetate extracts not derivatized (---) or derivatized diazomethane (Met) with retention times ($t_R$), molecular weights (MW), fragmentation patterns, and treatment (Treat.), harvest (Time) and matrix (Soil) where detected. The structures labeled with one asterisk (*) and two asterisk (**) were identified with method (*i*) and (*ii*), respectively (see *Section 2.3.5*).

| $t_R$ (min) | MW according to CI | *m/z* of fragment ions (relative abundance) | Possible structure | Treat. | Soil | Time | Deriv. |
|---|---|---|---|---|---|---|---|
| 18.471 | 176 | 178 (13.0), 176 (20.9), 143 (18.0), 141 (44.5), 139 (16.3), 115 (24.5), 113 (41.8), 111 (31.5), 77 (100), 75 (41.6) | ?,?-dichlorobenzyl alcohol* | *P. ost.* | A | 12w | --- |
| 21.600 | 238 | 242 (7.1), 240 (19.1), 238 (20.2), 211(30.1), 209 (96.4), 207 (100), 183 (7.1), 181 (13.4), 179(16.2), 143 (16.2), 109 (13.1), 74 (13.7) | 2,3,6-trichlorobenzoic acid* | *P. ost.* | A-B | 12w | Met |
| 22.585 | 210 | 212 (28.4), 210 (39.8), 177 (61.4), 175 (80.3), 173 (25.2), 147 (76.9), 145 (36.4), 111 (100), 109 (177.6) | ?,?,?-trichlorocresol* | *P. ost.* | A | 6w | Met |
| 31.325 | 320 | 322 (83.1), 320 (69.7), 307 (10.1), 305 (44.3), 281 (44.1), 279 (100), 277 (23.4), 207 (65.4) | ?,?,?,?-tetrachloro-?-methoxybiphenyl* | *P. ost.* | A | 12w | --- |
| 31.394 | 320 | 322 (82.2), 320 (68.9), 307 (10.5), 305 (44.5), 281 (43.8), 279 (98.5), 277 (22.4), 207 (65.9) | ?,?,?,?-tetrachloro-?-methoxybiphenyl* | *P. ost.* | B | 12w | --- |
| 30.091 | 286 | 290 (65.7), 288 (88.3), 286 (100), 273 (61.2), 271 (60.3), 245 (58.1), 243 (67.6), 173 (70.2) | ?,?,?-trichloro-?-hydroxybiphenyl* | *I.lac.* | A | 2w | Met |
| 31.420 | 286 | 290 (64.6), 288 (89.9), 286 (100), 273 (51.3), 271 (50.3), 245 (47.1), 243 (44.6), 173 (65.2) | ?,?,?-trichloro-?-hydroxybiphenyl* | *Bio* | A | 6w | Met |
| | | | | *I.lac.* | A | 2w | Met |
| 39.586 | 286 | 290 (66.6), 288 (87.9), 286 (100), 273 (58.3), 271 (61.4), 245 (46.2), 243 (42.5), 173 (67.7) | ?,?,?-trichloro-?-hydroxybiphenyl** | *Bio* | B | 12w | Met |
| | | | | *I.lac.* | A | 2w | Met |
| | | | | *P.ost.* | A | 12w | Met |

(*Continued*)

| $t_R$ (min) | MW according to CI | *m/z* of fragment ions (relative abundance) | Possible structure | Treat. | Soil | Time | Deriv. |
|---|---|---|---|---|---|---|---|
| 32.302 | 320 | 322 (81.1), 320 (67.4), 307 (11.2), 305 (42.1), 281 (42.2), 279 (100), 277 (20.4), 207 (64.4) | ?,?,?,?-tetrachloro-?-hydroxybiphenyl* | *Bio* | C | 12w | Met |
| 33.037 | 320 | 322 (80.3), 320 (66.4), 307 (10.3), 305 (41.1), 281 (44.5), 279 (100), 277 (21.3), 207 (65.0) | ?,?,?,?-tetrachloro-?-hydroxybiphenyl* | *Bio* | C | 12w | Met |
| 33.349 | 320 | 322 (77.1), 320 (73.8), 307 (9.2), 305 (48.8), 281 (48.9), 279 (100), 277 (18.4), 207 (54.8) | ?,?,?,?-tetrachloro-?-hydroxybiphenyl* | *P. ost.* | C | 6w | Met |
| 33.787 | 320 | 322 (69.2), 320 (87.8), 307 (13.4), 305 (50.8), 281 (49.6), 279 (100), 277 (19.9), 207 (58.1) | ?,?,?,?-tetrachloro-?-hydroxybiphenyl* | *Bio* | C | 12w | Met |
| 45.016 | 320 | 322 (72.1), 320 (74.5), 307 (12.2), 305 (49.6), 281 (45.9), 279 (100), 277 (20.3), 207 (55.8) | ?,?,?,?-tetrachloro-?-hydroxybiphenyl** | *P. ost.* | A | 12w | Met |
| 31.433 | 316 | 320 (36.6), 318(100), 316 (95.1), 305(27.0), 303 (63.3), 301 (57.2), 275 (42.8), 273 (72.3), 260 (39.7), 258 (48.6), 240 (30.4), 238 (58.7) | ?,?,?-trichloro-?,?-dihydroxybiphenyl* | *I.lac.* | A | 2w | Met |
| 39.592 | 316 | 320 (38.4), 318(99.5), 316 (85.1), 305(25.6), 303 (61.2), 301 (53.2), 275 (44.8), 273 (78.9), 260 (36.3), 258 (51.2), 240 (34.3), 238 (56.2) | ?,?,?-trichloro-?,?-dihydroxybiphenyl** | *I.lac* | C | 6w | Met |
| 41.206 | 352 | 354 (48.5), 352 (100), 350 (66.3), 339 (17.8), 337 (40.2), 335 (28.4), 294 (24.2), 292 (29.2), 290 (22.7), 240 (10.2), 238 (13.3), 236 (34.5) | ?,?,?,?-tetrachloro-?,?-dihydroxybiphenyl** | *I.lac* | C | 6w | Met |
| 41.601 | 352 | 354 (49.2), 352 (98.5), 350 (64.2), 339 (18.6), 337 (42.5), 335 (19.7), 294 (26.3), 292 (25.4), 290 (20.1), 240 (12.5), 238 (14.7), 236 (32.5) | ?,?,?,?-tetrachloro-?,?-dihydroxybiphenyl** | *I.lac* | A | 6-12w | Met |

### 2.3.7 Phospholipid fatty acids analysis

Phospholipid fatty acids are widely used as taxonomic and phylogenetic biomarkers to describe the structure and the size of the microbial community. Thus, PLFA analysis was performed to evaluate changes occurring in both bacterial and fungal communities during the mycoremediation treatments (*Figure 2.6*). The highest fungal concentrations were observed in myco-augmented microcosms, in particular after 6 weeks of incubation of *P. ostreatus* with soil A (4.02 mg $Kg^{-1}$), B (14.36 mg $Kg^{-1}$) and C (11.38 mg $Kg^{-1}$). The addition of this allochthonous organism seemed to stimulate also the growth of bacteria, the highest concentration of which was reached in soil B (4.20 mg $Kg^{-1}$) and C (1.66 mg $Kg^{-1}$) at the same time. Concerning *I. lacteus* augmentation treatment, the maximum amount of fungal biomass was measured in soil B after 6 weeks of incubation. However, this value was much lower than that found in coeval *P. ostreatus* microcosms (1.34 *vs* 14.35 mg $Kg^{-1}$). The use of *I. lacteus* also stimulated the bacterial growth: an evident increase in the bacterial community size was observed in soil B and C at the end of treatment (1.54 and 2.00 mg $Kg^{-1}$, respectively). Lastly, the addition of the non-inoculated lignocellulosic substrate (biostimulation treatments) affected positively the autochthonous microorganisms: the highest bacterial concentration was reached in soil A, B and C (1.34. 1.86 and 1.48 mg $Kg^{-1}$. respectively) after 6 weeks of incubation. Even though PLFA analysis is one of the most suitable methods for the culture-independent characterization of microbial communities in soil, certain limitations (*i.e.* several fatty acids are common to different microbes) require attention when interpreting the results (Frostegard *et al*., 2010). Thus, the combination of PLFA with molecular techniques (*i.e*. pyrosequencing) describes the changes in microbial community structure more accurately.

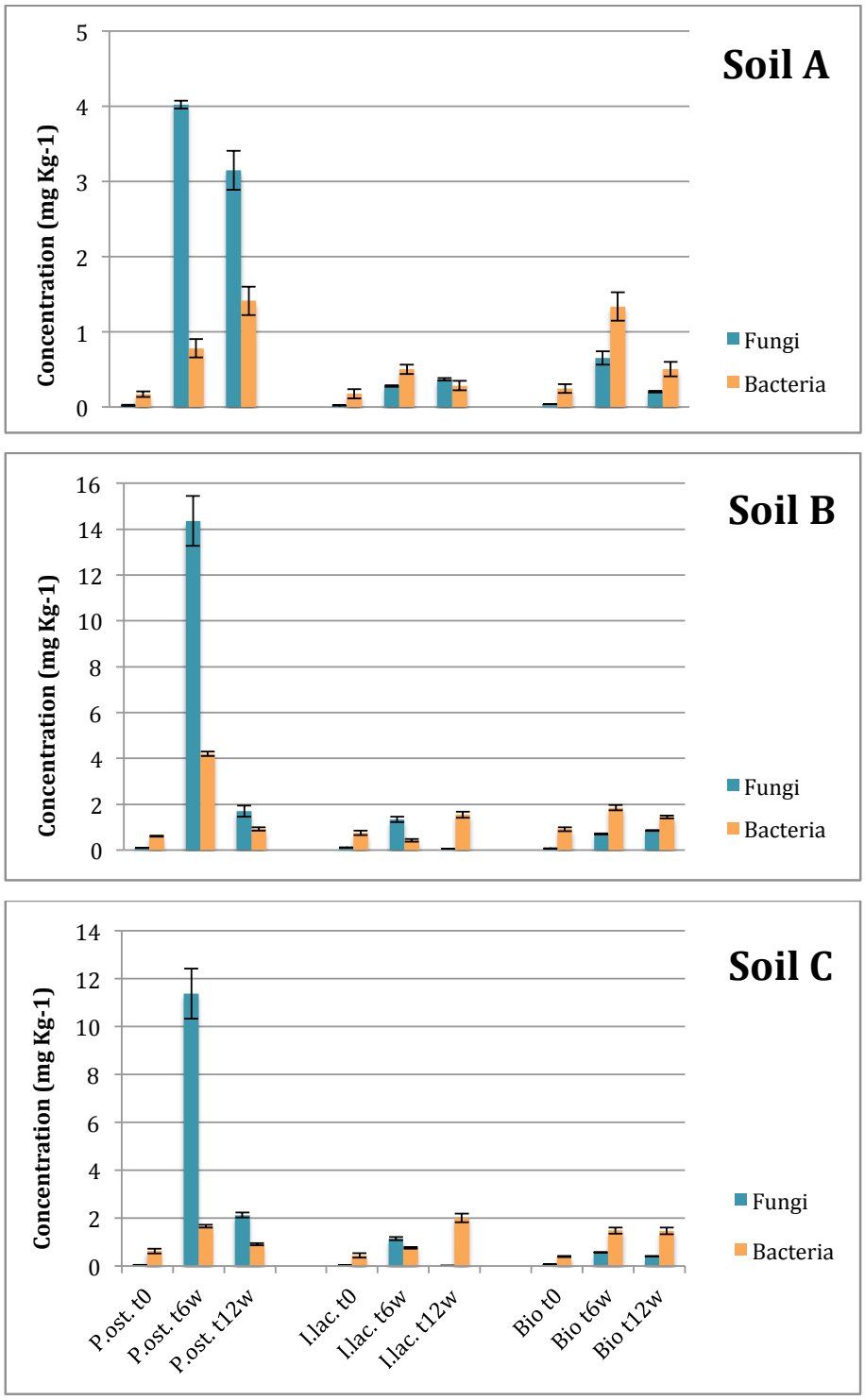



***Figure 2.6.*** Phospholipid fatty acids concentration (mg $Kg^{-1}$) in myco-augmented (*P. ost*. and *I. lac.*) and biostimulated (Bio) microcosms with soil A, B and C at the beginning (t0), and after 6 and 12 weeks (t6w and t12w, respectively).

### 2.3.8 Pyrosequencing

Molecular biology techniques have revolutionized the study of microbial ecology providing a tool for the development of a rapid, culture-independent assessment of microbial communities present in complex ecosystems. Undoubtedly, high-throughput next-generation sequencing methods represent a powerful alternative to traditional approaches (mostly based on cloning and Sanger sequencing) enabling detailed identification and relative quantification of microbial populations from environmental samples. The process involves a long series of steps, from sampling to bioinformatics analysis *via* laboratory handling. Therefore, awareness of methodological biases, potential pitfalls which can yield artificial results and misleading conclusions is required when these innovative techniques are used. In this study, 454 tag-encoded pyrosequencing was selected to gain insights into the dynamic shifts in both bacterial and fungal diversity throughout the mycoremediation treatments.

The pyrosequencing procedure yielded ≈ 186.000 and 163.000 bacterial and fungal sequences, respectively. Upon data processing with the QIIME software, 24 and 47 % of the obtained sequences were omitted since they did not meet stringent quality control criteria. The remaining sequences were aligned and subjected to cluster analysis to ascertain their phylogenetic affiliations. Sequences that could not be assigned to any phylum were assigned as ND (not determined).

Concerning the bacterial population, a considerable decline of *Actinobacteria* and *Chloroflexi* occurred in all cases, while a distinct increase was observed in *Firmicutes* relative abundance when soil A and C were treated with *P. ostreatus* (*Figure 2.7*). This phylum accounted for 94.0 and 64.4 % of the total reads in soil A and C, respectively, after 6 weeks of incubation. Similar raise was found also for *Proteobacteria* phylum in all soil samples augmented with *I. lacteus*. After 6 week of *I.lacteus* treatment, *Proteobacteria* were the most abundant in soil A, B and C making up 90.1, 82.3 and 92.3 %, respectively, of the whole bacterial population. At the end of incubation, this phylum was still the predominant one in soil A, whereas an increase of *Bacteroidetes* was evident in soil B (37.5 %) and C (48.2%).

In biostimulated microcosms, the addition of the lignocellulosic substrate promoted primarily the growth of *Proteobacteria,* the relative abundance of which amounting to 71.1, 64.6 and 60.8 % of total reads in soil A, B and C, respectively.

The other phyla (*Acidobacteria, Gemmatimonadetes* and *Verrucomicrobia*) represented a significantly smaller fraction of bacterial consortia in all microcosms under study.

The presence of sequences belonging to *Chloroflexi* phylum at the beginning of mycoremediation treatments suggested that a certain microflora specialized in PCB degradation has been adapted over time. As mentioned in section 1.6.1.1, anaerobic bacteria belonging to *Chloroflexi* phylum are the main players in the reductive dehalogenation of higher chlorinated biphenyls. These microorganisms can use the chlorine atoms of PCBs as final electron acceptor during the dehalorespiration process leading to the formation of lower chlorinated compounds. Moreover, the hypothesis of the occurrence of adaptation processes was also supported by the initial presence of microorganisms belonging to *Actinobacteria*, *Proteobacteria* and *Firmicutes* phyla. As a matter of fact, these phyla encompass well-known genera with reported PCB-degrading ability, such as *Rhodococcus*, *Bacillus*, *Pseudomonas*, *Achromobacter*, *Burkholderia*, *etc*.

Besides the remarkable shift in bacterial population, augmentation with *P.ostreatus* and *I. lacteus* determined a decrease in both Chao1 and Shannon diversity indices, with the exception of Shannon index when the soil B was treated with *P. ostreatus* (Table 2.7). Conversely, no appreciable changes in bacterial diversity were observed in biostimulated microcosms.

***Tab. 2.7.*** Estimated Chao1 and Shannon indeces for bacterial taxa in myco-augmented (*P. ost.* and *I. lac.*) and biostimulated (Bio) microcosms with soil A, B and C at the beginning (t0), and after 6 and 12 weeks (t6w and t12w, respectively).

| | Soil A | | Soil B | | Soil C | |
|---|---|---|---|---|---|---|
| | Chao1 | Shannon | Chao1 | Shannon | Chao1 | Shannon |
| *P.ost.* t0 | 233.3 | 5.75 | 269.2 | 5.97 | 254.6 | 6.65 |
| *P.ost.* t6w | 97.2 | 3.68 | 276.8 | 6.23 | 143.5 | 4.69 |
| *P.ost.* t12w | 87.0 | 3.47 | 221.8 | 6.13 | 151.9 | 4.25 |
| | | | | | | |
| *I. lac.* t0 | 277.8 | 6.22 | 275.4 | 6.80 | 256.4 | 6.79 |
| *I. lac.* t6w | 41.9 | 2.17 | 113.4 | 4.17 | 82.8 | 3.92 |
| *I. lac.* t12w | 56.0 | 2.91 | 140.3 | 5.40 | 137.8 | 4.53 |
| | | | | | | |
| Bio t0 | 273.2 | 5.63 | 310.1 | 6.85 | 352.4 | 6.85 |
| Bio t6w | 151.2 | 5.49 | 255.7 | 6.33 | 263.1 | 6.08 |
| Bio t12w | 170.7 | 5.63 | 218.0 | 6.45 | 327.0 | 5.94 |

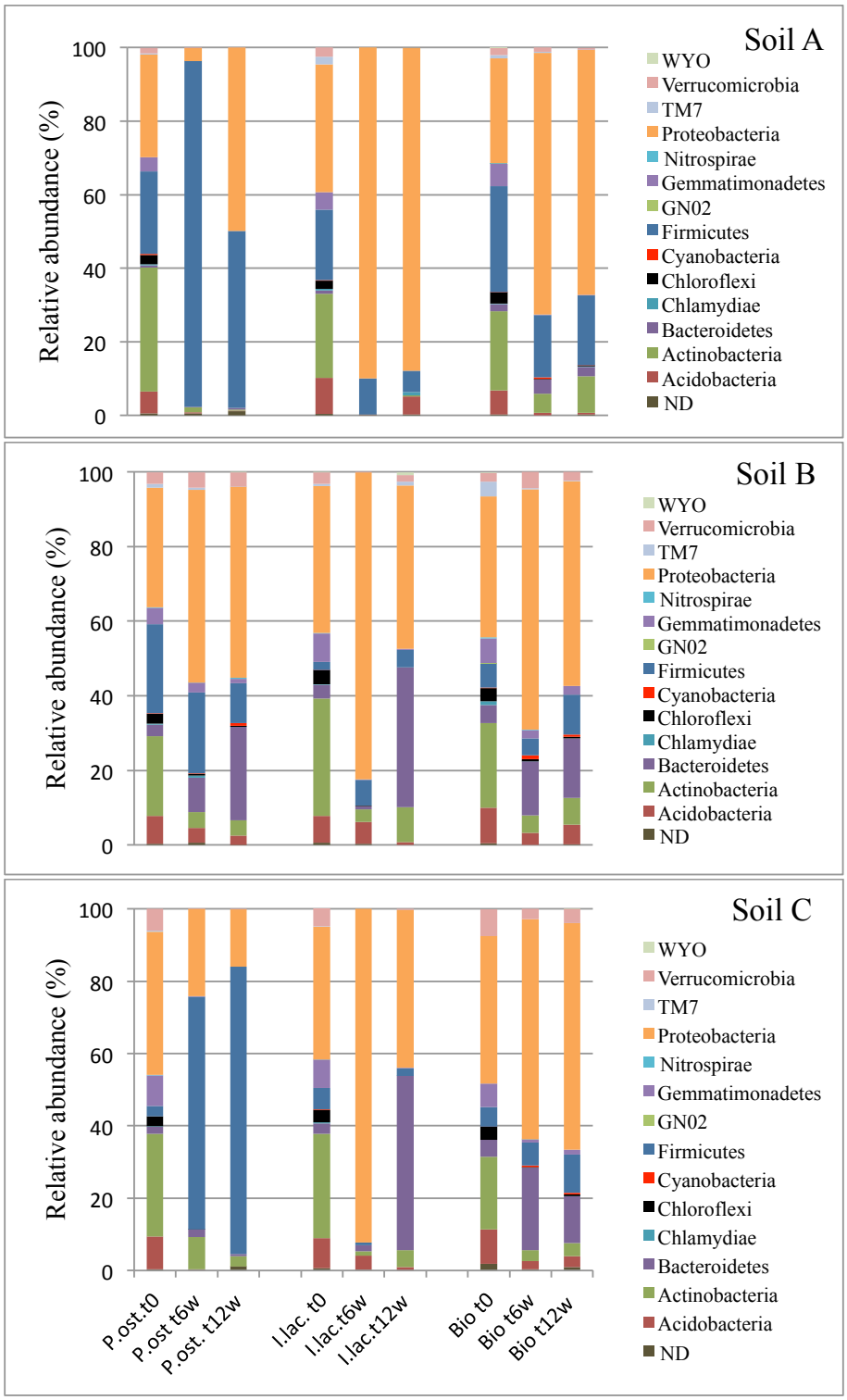



***Fig. 2.7.*** Taxonomic profiles of bacterial community at the phylum level in myco-augmented (*P. ost*. and *I. lac*) and biostimulated (Bio) microcosms with soil A, B and C. The segments composing each bar represent the relative abundance of each phylum, determined by pyrosequencing of 16S rDNA after 0, 6 and 12 weeks (t0, t6w and t12w) of treatment. Sequences that could not be assigned to any phylum were referred to as ND (not determined).

Concerning fungal diversity, the majority of detected fungal sequences belonged to the phyla *Basidiomycota*, *Ascomycota* and *Zygomicota* (*Figure 2.8*). *Basidiomycota* phylum was predominant in all *P. ostreatus*-augmented microcosms along the whole treatment period, while a general decline was observed in *I. lacteus*-augmented soil samples A, B and C reaching 69, 21 and 1 %, respectively, of the total fungi at the end of incubation. Furthermore, genus level analysis established that *Basidiomycota* sequences detected were represented by *P.ostretatus* an *I. lacteus* in their respective microcosms. These results confirmed the ability of the two selected white rot fungi to compete with the autochthonous mycobiota*:* the use of wheat straw-based pellets as lignocellulosic substrate supported the growth of both fungi, and the preparation of fully colonized LS as the inoculum formulation, prior to soil mixing, increased their antagonistic potential as reported in a previous study (Covino *et al*., 2010). Nonetheless, *I. lacteus* sequences abundance drastically decreased during the last 6 weeks of incubation in soil B and C with a concomitant increase of unidentified fungal sequences.

Interestingly, sequences representative of the phylum *Basidiomycota* were also found in biostimulated samples of soil B and C after 12 weeks: the genus-level taxonomy assignment displayed that the *Agrocybe* and *Sphaerobolus* genera were the most abundant Basidiomycetes in soil B (45.5 and 49.7 %, respectively), whereas *Pluteus* and *Cryptococcus* predominated in soil C (27.5 and 7.3 %, respectively).

Concerning the phylum *Ascomycota*, the analysis of fungal sequences in biostimulated microcosms have shown the highest abundance in the soil C (54.4 %), while lower amount of pertinent sequences were found in soil A and B (19.3 and 17.4 % of total fungal sequences) at the beginning of treatments. An increase from 19.3 to 36 % was observed in soil A during the incubation, mainly due to *Penicillium* and *Stachybotrys* growth. Contrary, a descending profile in *Ascomycota* sequences relative abundance was observed in soil B (from 17.4 to 0.1 %) and C (from 54.4 to 31.4 %) throughout the whole incubation. The capacity of *Penicillium* species in the removal of chlorinated biphenyls is well known. Indeed, several species belonging to this genus (*P. chrysogenum*, *P. purpurescens*, *P. digitatum*, *P. aurantiogriseum*) were isolated from PCB-contaminated soils and tested for their ability to degrade PCBs in liquid systems (Tigini *et al*., 2009; Yin *et al*., 2011; Mouhamadou *et al*., 2013): the studies confirmed that these fungi can co-metabolized PCBs when a readily available carbon source was provided.

Regardless of the soil samples, the phylum *Zigomycota* was dominated by members of *Mucor* genus in all biostimulation treatments; the highest relative abundance of Zygomycetes was reached after 6 weeks of incubation in soil A and C (12.4 and 19.6 %, respectively).

Based on the abundance of various taxa within each community, Alpha diversity was also assessed for fungi showing a high variability in Chao1 index in all treatments. On the other hand, Shannon index indicated that biodiversity tend to decrease in *P. ostreatus* microcosms in all cases, with a temporary being observed only in soil A. Moreover, a similar rising was observed also when the same polluted matrix underwent *I. lacteus* and biostimulation treatment.

***Tab. 2.8***. Estimated Chao1 and Shannon indeces for fungal taxa in myco-augmented (*P. ost.* and *I. lac.*) and biostimulated (Bio) microcosms with soil A, B and C at the beginning (t0), and after 6 and 12 weeks (t6w and t12w, respectively).

| | Soil A | | Soil B | | Soil C | |
|---|---|---|---|---|---|---|
| | Chao1 | Shannon | Chao1 | Shannon | Chao1 | Shannon |
| *P.ost.* t0 | 6.5 | 0.66 | 20.3 | 0.90 | 32.3 | 2.45 |
| *P.ost.* t6w | 48.0 | 3.94 | 6.7 | 0.65 | 45.4 | 0.56 |
| *P.ost.* t12w | 7.9 | 0.54 | 5.2 | 0.51 | 6.5 | 0.54 |
| | | | | | | |
| *I. lac*. t0 | 5.6 | 0.96 | 45.5 | 2.81 | 30.9 | 1.47 |
| *I. lac.* t6w | 7.7 | 1.08 | 5.8 | 0.47 | 4.3 | 1.37 |
| *I. lac.* t12w | 10.3 | 1.24 | 23.3 | 2.17 | 19.3 | 1.33 |
| | | | | | | |
| Bio t0 | 36.7 | 2.23 | 47.1 | 4.24 | 4.2 | 0.52 |
| Bio t6w | 42.4 | 3.15 | 43.9 | 2.56 | 60.8 | 3.93 |
| Bio t12w | 49.7 | 3.16 | 5.7 | 0.65 | 59.3 | 3.72 |

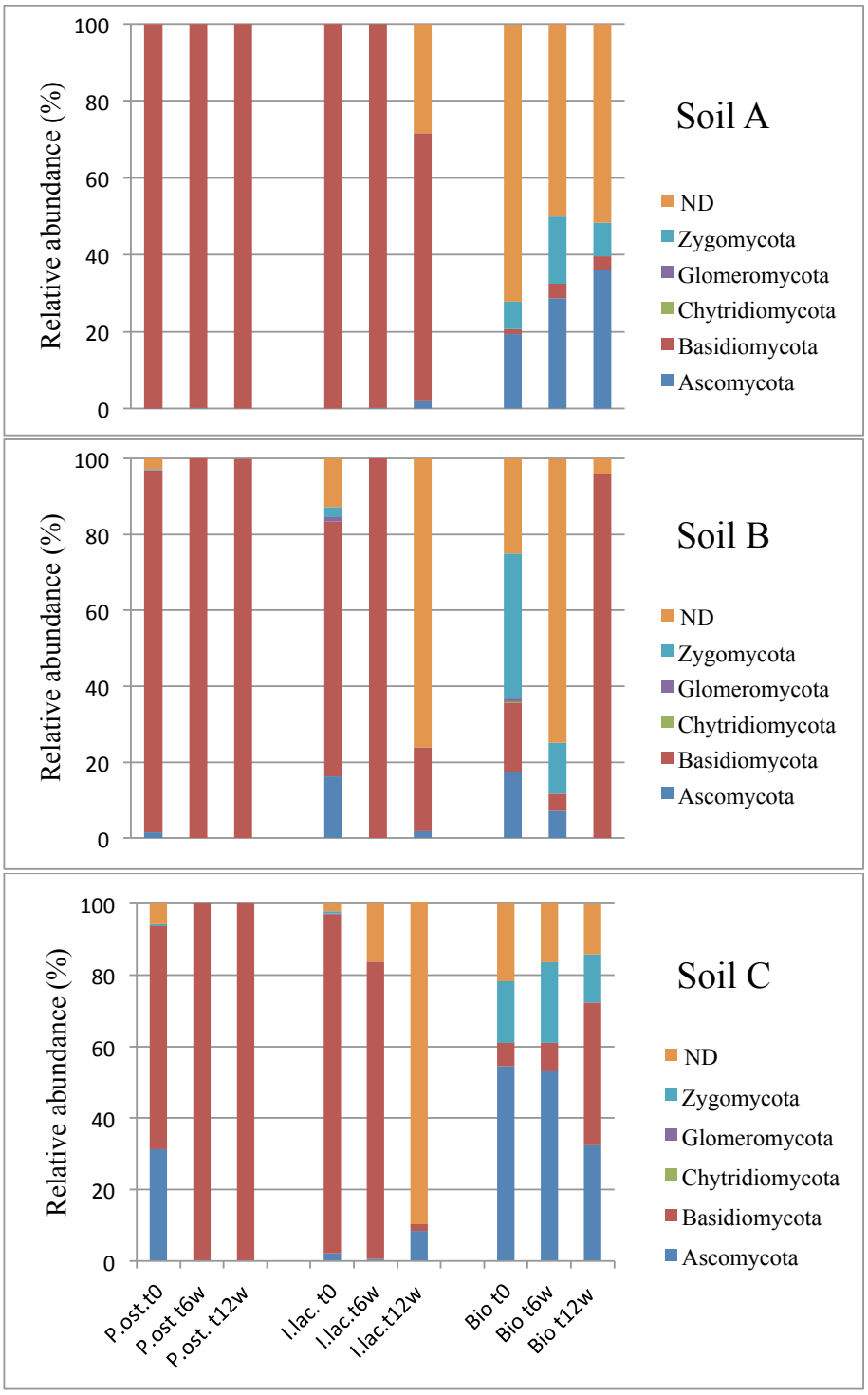
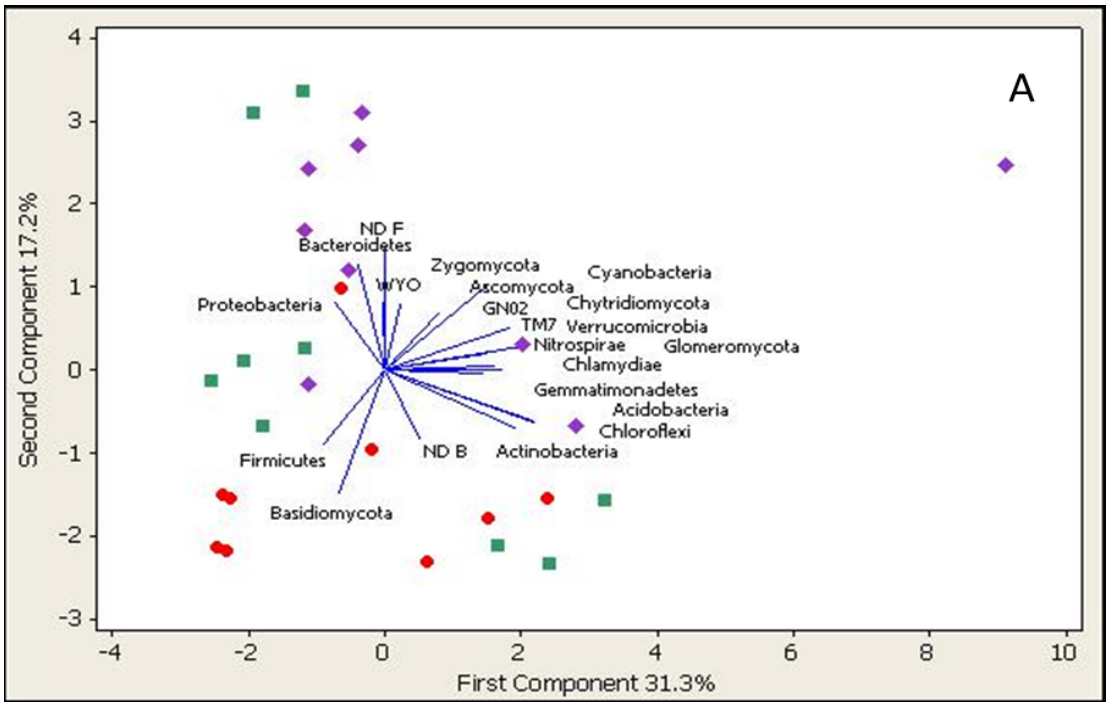



***Fig. 2.8***. Taxonomic profiles of fungal community at phylum level in myco-augmented (*P. ost*. and *I. lac*) and biostimulated (Bio) microcosms with soil A, B and C. The segments composing each bar represent the relative abundance of each phylum, determined by pyrosequencing of the internal transcribed spacer (ITS) after 0, 6 and 12 weeks (t0, t6w and t12w) of treatment. Sequences that could not be classified into any phylum were assigned as ND (not determined).

Lastly, principal component analysis (PCA) was performed in order to provide an overview of similarities in microbial communities among treatments, soil matrix and time of incubation (*Figure 2.9* A, B and C). When the abundances of both fungal and bacterial phyla were analysed, the percentage variance explained by the first and second component amounted to 31.1 and 17.2 %, respectively. Great similarities were observed in augmented-microcosms at the beginning (P0A, P0B, P0C, I0A, I0B and I0C) grouping together in the lower right-hand quadrant (*Fig. 2.9* B). Analogies among *P. ostreatus* treatments in the later phase of incubation with soil A and C (P6A, P12A, P6C and P12C) appeared to be positively correlated with *Basidiomycota* and *Firmicutes* phyla as indicated by the common position of their scores of these treatments with loadings of the aforementioned two variables in the lower left-hand quadrant of the biplot (*Fig. 2.9* A). On the contrary, *I. lacteus* treatments in the middle phase of incubation (I6A, I6B and I6C) segregated along the second component from their respective microcosms at the end of incubation with the exception of soil A (I12A). Indeed, as illustrated above, *Proteobacteria* phylum was the dominant phylum in *Irpex*-augmented microcosms after 6 weeks of incubation and remained the most abundant one at the end of incubation only in soil A, whereas an increase of *Bacteroidetes* appeared in soil B and C. Morever, PCA analysis revealed that the last 2 microcosms (I12B and I12C) grouped with the 6-week-old biostimulated-treatments of soil A, B and C (B6A, B6B and B6C).



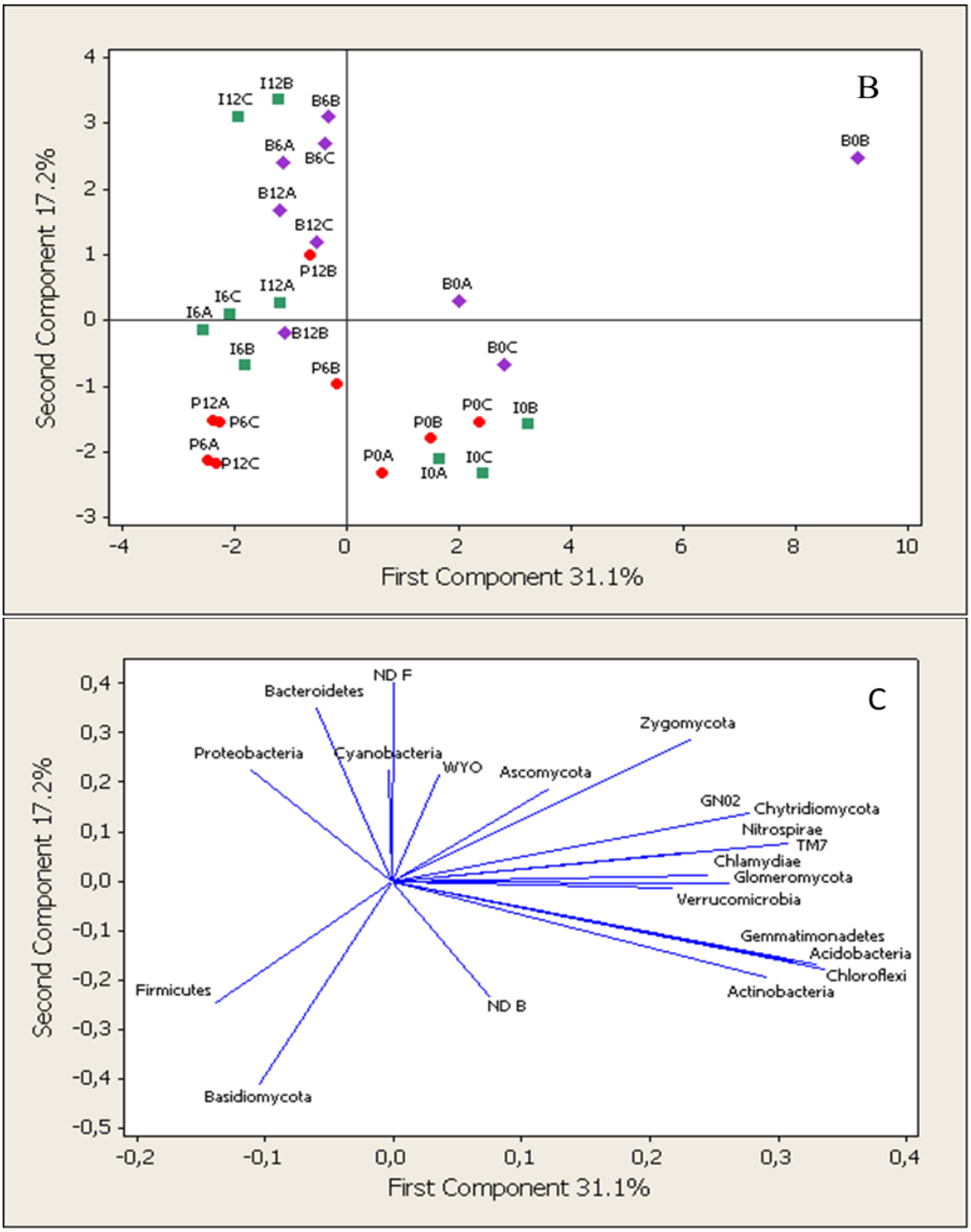



***Fig. 2.9.*** Principal Component Analysis (PCA) biplot (A), scores plot (B) and loadings plot (C) of microbial communities and mycoremediation treatments. In biplot A and scores plot B, red circles, green squares and purple rhombus refer to *Pleurotus ostreatus* augmentation, *Irpex lacteus* augmentation and biostimulation treatments, respectively. In scores plot (B), the first capital letter of the alphanumeric code indicates the treatment type (P= Pleurotus, I= Irpex and B=bistimulation), the Arabic number the weeks of incubation (0, 6 and 12 weeks) and the last capital letter the polluted matrix (A=soil A, B=soil B and C=soil C). The percent variance explained by each component is shown in X and Y axis captions.

## 2.4 Conclusions

The mycoremediation of polychlorinated biphenyls polluted soils represents an effective and environmentally friendly alternative to chemical and thermal procedures. However, the application of this technology requires an accurate evaluation of all the factors which may affect the technical feasibility as well as the efficiency of the selected processes.

In this study, the determination of the physico-chemical properties of the contaminated solid matrices, prior to remediation, revealed that the content of organic matter mainly influenced the bioavailability of PCBs, and thus their accessibility to fungi.

Moreover, the chemical characterization of contaminants showed the concomitant presence of PCBs and CBAs. The latter, well-known intermediates of fungal PCB mineralization process or dead-end products of bacterial aerobic PCB degradation, could have been formed along the time, due to prolonged residence time of the contaminated soil in Lhenice dumpsite.

With regard to the tested remediation approaches, bioaugmentation was more efficient than biostimulation in the removal of PCBs from both topsoil (soil B) and rhizosphere soil (soil C). Particularly promising were *Pleurotus ostreatus* or *I. lacteus* the use of which led to PCB removals of 41.3 and 39.4 % from soil B, respectively, and 50.5 and 30.3 % from soil C after 12 weeks of incubation. Moreover, an evident reduction in toxicity was observed in *P. ostreatus*- and *I. lacteus*- soil C microcosms (63.9 and 55.3 %, respectively) at the end of the treatments.

The identification of hydroxylated and methoxylated PCB derivatives, and the presence of degradation intermediates, such as chlorobenzoates, chlorobenzaldehydes, chlorobenzyl alcohols and chlorocresols confirmed that the degradation process of PCBs involved both fungal intracellular and extracellular enzymes. The detection of mono-hydroxylated-PCB derivatives in biostimulated microcosms suggested that also the resident microbiota can take part in the biotransformation of PCBs.

Furthermore, insights into the microbial community composition, diversity and dynamics throughout the remediation processes were gained with the combination of two different culture-independent techniques: phospholipid fatty acids analysis and 454-pyrosequencing.

The use of the former method showed that the fungal biomass of *P. ostrateus* was much higher than that of *I. lacteus* for the whole incubation time. The introduction of these allochthonous fungi stimulated also the bacterial growth, the biomass of which achieved the maxima values after 6 and 12 weeks in *P. ostreatus*-soil B and *I. lacteus*-soil C microcosms, respectively. Moreover, the addition of non-inoculated lignocellulosic substrate

(biostimulation treatments) evidently promoted the growth of both bacterial and fungal populations, the highest concentrations of which were reached after 6 weeks of incubation in all the soil samples.

On the other hand, the 454-pyrosequencing technique provided a deeper assessment of the microbial changes occurring during the treatments.

*P. ostreatus* augmentation clearly increased the relative abundance of *Firmicutes* phylum in the bulk soil and rhizosphere soil, while a similar raise was observed for *Proteobacteria* phylum in all soil samples treated with *I. lacteus*. However, at the end of incubation, an increase of *Bacteroidetes* sequences was found in *I. lacteus*-soil B and –soil C microcosms.

Additionally, fungal community analysis showed that the phylum *Basidiomycota* was the dominant one in bioaugmentation treatments, the sequences of which belonged to *P. ostreatus* and *I. lacteus* genera in their respective microcosms. As for *I lacteus*, a decrease in its sequences relative abundance was observed during the last 6 weeks of incubation in soil B and C, while *P. ostreatus* was the most abundant one for the whole incubation period. This latter finding proved the outstanding capability of this fungus to compete with the autochthonous mycobiota, and thus to efficiently grow in PCB-contaminated soils under non-sterile conditions.

By contrast, the large majority of fungal sequences in biostimulated microcosms belonged to the phyla *Ascomycota* and *Zygomycota*, with the exception of the topsoil (soil B) where members of the phylum *Basidiomycota* became predominant in the later phase of the incubation. In this case, the presence of *Basidiommycota* sequences, mostly belonging to *Agrocybe* and *Sphaerobolus* genera, prompted us also to hypothesize their potential involvement in PCB transformation process in view also of the best degradation result achieved in soil B among all biostimulated microcosms.

# CHAPTER 3

# FUNGAL DEGRADATION MECHANISMS OF CHLORINATED ORGANIC POLLUTANTS

## 3.1 Introduction

The ability of fungi, in particular "white rot" fungi, to degrade several organopollutants including chlorinated phenols, PCBs, DDT, dioxins, CBAs, PAHs, *etc*., is widely documented. The degrading capability was attributed to their ligninolytic system (Lac, LiP and MnP): as a matter of fact, many of the abovementioned pollutants were transformed by isolated enzymes (Baborová *et al*., 2006; Zhao & Yi, 2010; Covino *et al*., 2010; Fan *et al*., 2013). In the specific case of PCBs, white rot fungi were efficient in the removal of technical PCB mixtures or single PCB congeners, a process that can be undertaken under ligninolytic conditions (*i.e*., nutrient-limited conditions); however, a direct correlation between degradation extent and ligninolytic activities was not proven. Afterwards, a few works indicated that isolated fungal extracellular phenoloxidases and peroxidases were unable to oxidize PCB congeners (Baudette *et al*., 1998; Krcmar *et al*., 1999; Takagi *et al*., 2007). However, they were shown to be able to perform the breakdown of some PCB degradation intermediates, such as hydroxylated derivatives (Keum *et al*., 2004; Kordon *et al*., 2010).

Kamei and co-workers (2006a) investigated the transformation products of 4,4′-dichlorobiphenyl in *Phanerochaete chrysosporium* in the attempt of determining the degradation pathway. Methoxylated- and hydroxylated-PCB derivatives were detected in *P. chrysosporium* liquid cultures and, thus the involvement of cytochrome P450 monooxygenases (CYP450) in the degradation process was hypothesized. Moreover, the addition of a well-known CYP450 inhibitor, namely piperonyl butoxide, to fungal cultures prevented the formation of hydroxylated metabolites supporting the initial hypothesis. Chlorobenzoic acids, chlorobenzaldehydes and chlorobenzylalcohols were also identified as PCB degradation products. In particular, the formation of chlorobenzoates from hydroxylated PCBs and their further transformation *via* a reductive pathway was suggested also by Cvancarova *et al.* (2012). Once CYP450 oxidised the aromatic structure of PCBs, ring fission reaction can be mediated by other enzymatic systems (*i.e.* ligninolytic system) (Cajthaml *et al*., 2006; Cvancarova *et al*., 2012); thereafter, a reductive mechanism can operate on the carboxyl group of CBAs leading to the formation of chlorinated aldehydes and alcohols (Covino, 2010; Muzikar *et al*., 2011).

Concerning CBAs, the degradation capability of laccase and manganese-peroxidase isolated from the white rot fungus *Lentinus tigrinus* (*L. tigrinus*) towards a mixture of chlorobenzoic acids was assessed (Covino, 2010). As for PCBs, these purified enzymes were unable to

oxidize CBAs even under mediated conditions, confirming that other enzymatic systems are involved in the initial attack of chlorobenzoic acids.

However, any mechanistic interpretation on the PCB and CBA degradation processes, especially for the first transformation steps has not been fully elucidated yet. Generally, indications for the pivotal role of fungal CYP450 in the elimination of many xenobiotics (*i.e.*, PCBs, pentachlorophenols, nitroaromatic compounds) were desumed from inhibitory effects exerted by piperonyl butoxide (Teratomo *et al.*, 2004; Kamei *et al*., 2006a; Ning & Wang, 2012) on the enzyme-mediated conversion of pollutants. Because of the high instability and the low expression of CYP450 monooxygenase activities, the proof of the direct involvement of CYP450 enzymes in the transformation of organopollutants under *in vitro* condition has not been provided yet.

In view of these considerations, the aim of this work was the development of a reliable method to extract microsomes rich in CYP450 activities from *P. ostreatus* and *L. tigrinus* cultures, and evaluate their role in the biotransformation process of PCBs and CBAs, respectively.

Moreover, a semi-purified laccase activity was obtained from *P. ostreatus* and used to perform *in vitro* tests with hydroxylated PCBs (OH-PCBs), toxic intermediates that can be accumulated during the degradation process of PCBs.

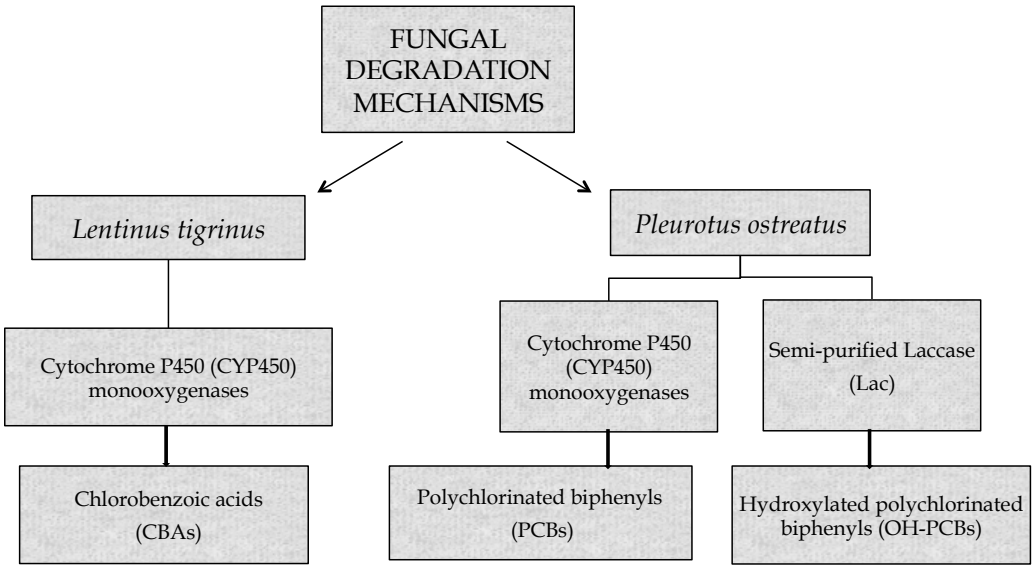



***Fig. 3.1***. Workflow of Chapter 3.

## 3.2 Materials and methods

### 3.2.1 Extraction of fungal microsomal fraction

Microsomal fractions were extracted from 5-day-old static cultures of *P. ostreatus* 3004 CCBAS 278 and *L. tigrinus* CBS 577.79 in malt extract-glucose (MEG) medium. The mycelial pellets were filtered on nylon cloth and washed with cold potassium phosphate buffer (100 mM, pH 7.2), disrupted in liquid nitrogen, and then in a Virtis 45 blender at 375 Hz using the same buffer supplemented with glycerol (200 g $l^{-1}$), bovine serum albumin (BSA, 1.5 g $l^{-1}$), phenylmethylsulfonyl fluoride (PMSF, 0.25 mM), dithiotreitol (DTT, 5 mM) and ethylenediaminetetraacetic acid (EDTA, 1mM) to be subsequently centrifuged (5000 x g, 20 min). The recovered supernatant underwent to two ultracentrifuge steps (100000 x g, 75 min) to separate the cytosolic fraction from the pellets containing microsomes. Pellets were then resuspended and stored in phosphate buffer (50 mM, pH 7.2) containing glycerol (300 g $l^{-1}$) and EDTA (0.1 mM) and, referred to as the microsomal fraction. The total protein concentration was determined by the dye binding method using BSA as the standard (Bradford, 1976).

### 3.2.2 Detection and quantification of cytochrome P450 in the microsomal fraction

The cytochrome P450 monooxygenase activities were measured in microsomal fractions by carbon monoxide (CO)-binding spectrum (Omura and Sato, 1964). Firstly, the sample was diluted to 1 mg $mL^{-1}$ of protein with phosphate buffer, and then dispensed equally into two cuvettes to record spectrophotometrically a baseline spectrum in the range of 400-500 nm. Sodium dithionite (400 µg $mL^{-1}$) was added to each cuvette, and then one was gently gassed with CO for 60 s, while the other one with $N_2$ to the same extent. Thereafter, difference spectra were recorded. The concentration of CYP450 and its respective inactivate form CYP420 were calculated using the extinction coefficients $\varepsilon_{450\text{-}490}$ =91 $mM^{-1}$ $cm^{-1}$ and $\varepsilon_{420\text{-}490}$ = 110 $mM^{-1}$ $cm^{-1}$, respectively.

### 3.2.3 *In vitro* PCB and CBA degradation test with cytochrome P450

*L. tigrinus* CYP450 *in vitro* degradation test was performed using a mixture of CBAs composed of 2-chlorobenzoic acid (2-CBA), 3-chlorobenzoic acid (3-CBA), 4-chlorobenzoic acid (4-CBA), 2,3-dichlorobenzoic acid (2,3-diCBA), 2,4-dichlorobenzoic acid (2,4-diCBA), 2,5-dichlorobenzoic acid (2,5-diCBA), 2,6-dichlorobenzoic acid (2,6-diCBA), 3,4-dichlorobenzoic acid (3,4-diCBA), 3,5-dichlorobenzoic acid (3,5-diCBA), 2,3,5-

trichlorobenzoic acid (2,3,5-triCBA), 2,3,6-trichlorobenzoic acid (2,3,6-triCBA), 2,4,6-trichlorobenzoic acid (2,4,6-triCBA) purchased from Sigma–Aldrich (Germany).

*P. ostreatus* CYP450 *in vitro* degradation test was performed using 2,2′-dichlorobiphenyl (PCB n.4), 2,4′-dichlorobiphenyl (PCB n.8), 2,2′,5-trichlorobiphenyl (PCB n.18). 2,2′,3,6-trichlorobiphenyl (PCB n.45), 2,3,3′,4,4′-tetrachlorobiphenyl (PCB n.105) obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany).

The experiments were carried out in 1 mL reaction mixture containing CBAs (3 mg $l^{-1}$ of each CBA) or PCBs (2 mg $l^{-1}$ of each PCB), 1 mM DTT and 1 mM PMSF in 2% (v/v) dimethyl sulfoxide (DMSO), 0.2 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH) and 1 mM EDTA in Na-phosphate buffer (0.1 M pH 7.2). The reactions, in triplicate, were initiated by the addition of 1 mg of microsomal proteins. Thereafter, all the samples were incubated at 28 °C for 1 h on a rotary shaker (100 rpm).

Five different controls were set up: piperonyl butoxide (PB, 2 mM) and carbon monoxide (CO bubbled for 1 min after sodium dithionite reduction), two well-known CYP450 inhibitors, were selected to carry out CYP450 inhibition controls (PB- and CO-inhibition control, respectively). NADPH dependency of the reaction was verified performing tests in the absence of this co-factor (no-NADPH controls). Moreover, reaction mixtures with inactivated microsomes were set up heating the protein for 1 h at 100 °C (heat-inactivated control) and blank controls were prepared with Na-phosphate buffer. The reactions were stopped by adding 20 mg of NaCl and 1 mM sulphuric acid ($H_2SO_4$) to the mixtures.

### 3.2.4 Semipurification of *Pleurotus ostreatus* laccase

A semi-purified laccase was obtained from 20-day-old *P. ostreatus* solid-state cultures carried out in 3 L Erlenmeyer flasks containing 100 g of wheat straw pellets previously sterilized in autoclave (45 min at 121 °C). The moisture of the lignocellulosic substrate was adjusted to 75% [w/w] with sterile deionized water prior to the inoculation with 10 agar plugs (1.0 cm diameter each) from 7-day-old *P. ostreatus* mycelium.

The fungus-colonized matrix was mechanically homogenized, added with 1 L of 20 mM pH 6.0 Na-phosphate buffer (buffer A), and the extraction was carried out by stirring for 2 h at 4 °C prior to separation of the buffered extract by means of a hydraulic press. The crude extract was then filtered through filter paper and centrifuged at 5000 x g for 15 minutes. Fractional ammonium sulphate precipitation, from 0 to 25% and then from 25 to 80% (w/v) saturation, was carried out to remove colloids and to obtain protein-rich pellets, respectively. The precipitate was resuspended in buffer A and dialyzed at 4 °C against 10 volumes of buffer A

using an Amicon stirred cell (Millipore, Germany) equipped with a 10 kDa cut-off flat membrane. The concentrated extract was then applied to a desalting Hi-Prep column (26/10; GE Healthcare, USA) equilibrated with buffer A at a flow rate of 3 mL $min^{-1}$. The pooled active fractions were separated using a Mono Q 5/50 GL (Amersham Pharmacia, USA) anion exchange column in an AKTA Purifier FPLC device (Amersham Pharmacia, USA). Laccase was eluted by a linear NaCl gradient (from 0 to 0.5 M) in buffer A. Laccase-rich fractions were pooled, desalted (Hi-Prep mini-column), filter-sterilized (0.22 μm) and stored at –20 °C until used.

### 3.2.5 Enzymatic assays

Laccase activity was spectrophotometrically determined by oxidation of 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) as the substrate. The reaction was followed by the absorbance increase at 420 nm and the enzyme activity was expressed in Units defined as the amount of enzyme oxidizing 1 μmol of ABTS per minute ($\varepsilon_{420}$ = 36,000 $M^{-1}cm^{-1}$, Matsumura *et al.*, 1986).

### 3.2.6 *In vitro* hydroxy-PCB degradation test with Lac and MnP

*P. ostreatus* laccase *in vitro* degradation tests were performed in triplicate using 4-hydroxy-4′-chlorobiphenyl (4OH-4′CB), 4-hydroxy-2-chlorobiphenyl (4OH-2′CB), 2-hydroxy-3,5-dichlorobiphenyl (2OH-3,5diCB), 3-hydroxy-2′,5′-dichlorobiphenyl (3OH-2′,5′diCB). 4-hydroxy-2′,5′-dichlorobiphenyl (4OH-2′,5′diCB) obtained from doc. Ing. Ondřej Uhlík, Czech Republic.

The degradation test was conducted in 1 mL reaction mixture containing acetate buffer (100 mM, pH 4.5), single hydroxy-PCB (5 mg $l^{-1}$) and laccase (2 U). In mediated reactions, hydroxybenzotriazole (HBT) was added at final concentration of 1 mM. Relative controls were performed by adding heat-denatured laccase to reaction mixtures (HKC). Incubations were performed at 28 °C for 24 h on a rotary shaker (100 rpm). The reactions were stopped by adding 20 mg NaCl and 1 mM $H_2SO_4$ to the mixtures after 1, 3, 6 and 24 h of incubation.

### 3.2.7 Samples preparation and analytical methods

All the *in vitro* samples from CYP450 and laccase degradation tests were extracted five times with 1.5 mL ethyl acetate aliquots and dried with anhydrous sodium sulphate columns (2 g).
Quantitative analyses of the CBAs were performed by reversed-phase high performance liquid chromatography (RP-HPLC) using a HPLC system consisting of a 2695 Separations Module (Waters) equipped with an X-Bridge C-18 column (4.6 mm × 250 mm, particle Ø 3.5 µm, Waters, MA) according to Muzikář *et al*. (2011).
Detection of CBA degradation products was determined by gas chromatography–mass spectrometry (GC-MS) equipped with a split/splitless injection system (450- GC, 240-MS ion trap detector, Varian. Walnut Creek, CA) without derivatization and after methylation with diazomethane as described by Muzikář *et al*. (2011).
The degradation extent of the PCB mixture and hydroxylated PCBs was evaluated by gas chromatography-mass spectrometry as described in the section 2.2.3.

## 3.3 Results and Discussion

### 3.3.1 Detection of cytochrome P450

Cytochrome P450 monooxygenases are a superfamily of heme-thiolate proteins that catalyse a broad range of reactions such as carbon hydroxylation, heteroatom oxygenation, dealkylation, epoxidation, reduction and dehalogenation (Cabana *et al*., 2007). CYP450, which is present in all eukaryotic organisms, is involved in the biosynthesis of various endogenous compounds (Omura, 1999; Nebert & Russell, 2002; Kelly *et al*., 2005) and in the oxidative detoxification of many xenobiotics including pollutants, drugs and pesticides (Omura, 1999; Stirobova *et a.l*, 2003; Cajthaml *et al*., 2008; Kasai *et al*., 2010; Syed *et al.*, 2010, 2011). Several studies demonstrated the involvement of CYP450 oxygenation reactions in fungal metabolism of recalcitrant xenobiotic compounds. To date, *Phanerochaete chrysosporium* has been the most extensively studied model white rot fungus for the understanding of degradation mechanisms (Ning *et al*., 2010; Kasai *et al*., 2010; Syed *et al.*, 2010, 2011). More than 150 genes coding for different CYP450 enzymes, the amino acid sequences of which are extremely variable, have been identified from this organism. However, the heme binding region at C-terminus of the protein as well as the putative substrate binding region is well conserved in all CYP450 families (van den Brink, 1998; Ning *et al*., 2010).

In this study, microsomal fractions were extracted from two white rot fungi, namely *P. ostreatus* and *L. tigrinus*, wherein CYP450 was detected and quantified (*Figure 3.2* A and B). Spectral scans showed the presence of a major peak at 450 nm: about 2.09 and 2.41 µM of CYP450 were found in *P. ostreatus* and *L. tigrinus* microsomes after 5 days of growth on MEG medium, respectively. Moreover, the detection of a second peak at 420 nm suggested also the presence of the inactive cytochrome form CYP420 amounting to 0.81 and 0.18 µM in *P. ostreatus* and *I. lacteus* microsomes, respectively.

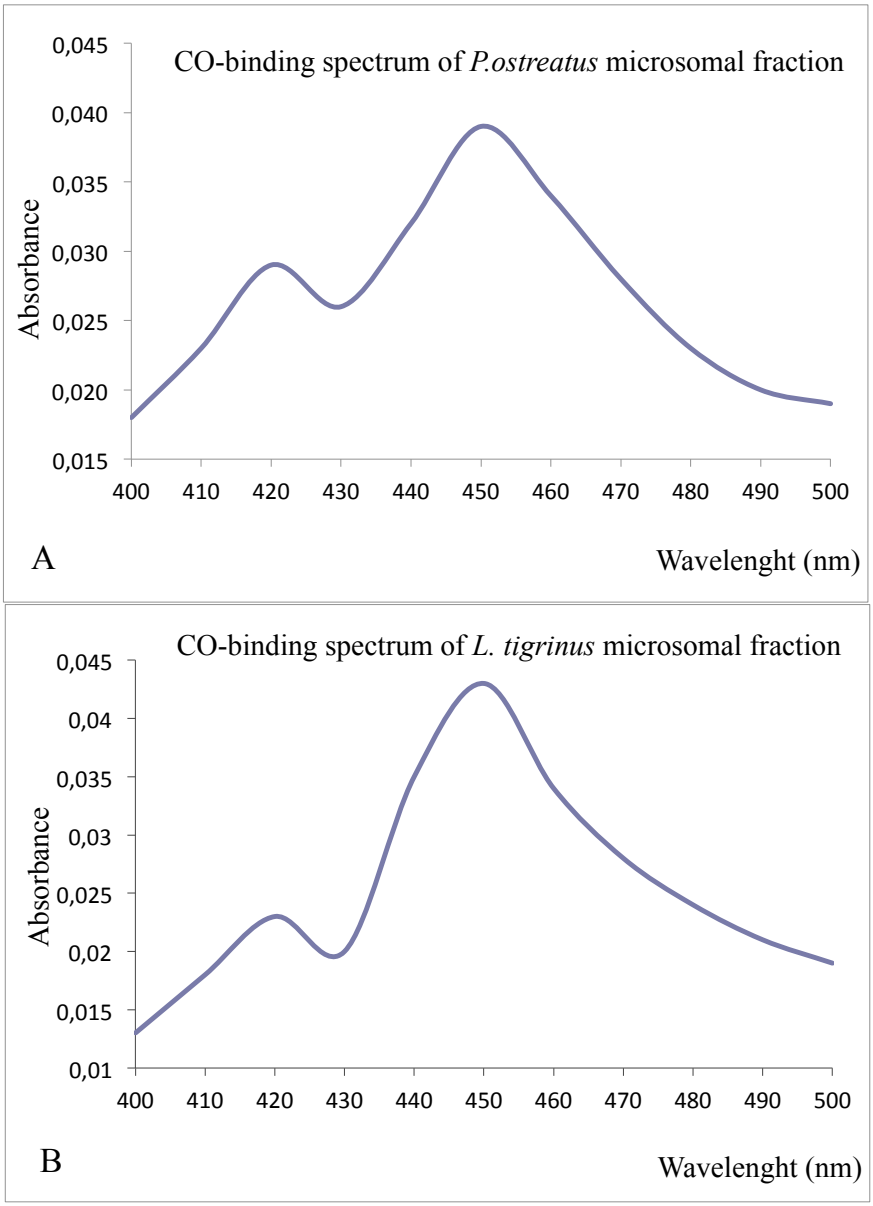



***Fig. 3.2.*** CO-binding spectra of *P. ostreatus* (A) and *L. tigrinus* (B) microsomal fractions from 5-day-old cultures.

### 3.3.2 *In vitro* degradation of CBAs by *Lentinus tigrin*us microsomal fraction

Chlorobenzoic acids (CBAs) are a class of relevant environmental pollutants consisting of a benzoic acid with different degree of chlorination on the aromatic ring. CBAs mainly derive from bacterial biodegradation of polychlorinated biphenyls (PCBs): under aerobic conditions, several bacterial species transform PCBs to chlorobenzoates which tend to be accumulated as dead-end metabolites (Field & Sierra-Alvarez, 2008). Additionally, CBAs can exert an inhibitory effect on the upper biphenyl degradation pathway restricting further PCB transformation (Adebusoye *et al.*, 2008). Due to their high solubility in water, these compounds exhibit a mobility that is several orders of magnitude higher than that of PCBs. Moreover, several CBA isomers are highly toxic toward aquatic organisms (Muccini, 1999; Lee & Chen, 2009), exhibit genotoxicity toward higher plants (Gichner *et al*., 2008) and possess endocrine disrupting activity (Svobodová *et al.*, 2009).

The biotechnological potentiality of the white rot fungus *Lentinus tigrinus* has been clear for many years: this organism was successfully used in the decolourization of textile dyes (Nazareth & Sampy, 2003), ín the biopulping process (Goncalves *et al.*, 2002), in the degradation of chlorophenols (Rabinovich *et al.*, 2004), and in the dephenolization and decolorization of olive mill wastewaters (D´Annibale *et al.*, 2004). In particular, *L. tigrinus* strain CBS 577.79 was reported to be effective in the degradation of PCBs, PAHs, CBAs and other endocrine disrupting compounds such as 17α-ethynylestradiol, bisphenol A and triclosan (Federici *et al*., 2012; Covino *et al*., 2010; Covino, 2010). Moreover, purified laccase and MnP isoenzymes from the same strain were tested for their ability to degrade CBAs: both enzymes were unable to oxidize CBA even under mediated conditions.

For this reason, CYP450 enzymes were extracted from *L. tigrinus* cultures, and their role in the biotransformation process of CBAs was evaluated. The *Figure 3.3* reports the residual concentrations of all CBAs after 1 h incubation with the purified *L. tigrinus* microsomal fraction. HPLC analysis revealed that the recovery of each CBA was higher than 90% in all control samples and that all the tested chlorobenzoate isomers were oxidized at different extent after 1 h of incubation in reaction mixtures with active microsome. In particular, 2,3-diCBA, 2-CBA and 3,5-diCBA were the most degraded ones, the residual concentrations of which were 26.2, 35.5 and 38.8 %, respectively (Tab. 3.1). On the other hand, and in agreement with *in vivo* degradation study (Covino, 2010), the most recalcitrant compounds were 2,6-diCBA and 2,3,6-triCBA, with a residual content of 84.7 and 73.2 %, respectively. Their higher recalcitrance might presumably be due to their inherent properties, namely the

concomitant steric hindrance of the chlorine substituents adjacent to the carboxyl moiety and their electron-withdrawing effect, which may negatively interfere with the reactivity of these compounds.

The NADPH-dependency of CBAs biotransformation was demonstrated by the lack of significant differences between incubations in the absence of this co-factor and those performed with heat-killed microsomal fraction. Likewise, the addition of either piperonyl butoxide or carbon monoxide to the microsomal fraction invariably suppressed CBAs degradation.

| **CBA isomers** | Residual concentration (%) |
|---|---|
| 2-CBA | 35.5 |
| 3-CBA | 71.4 |
| 4-CBA | 48.3 |
| 2,3-diCBA | 26.2 |
| 2,4-diCBA | 55.5 |
| 2,5-diCBA | 59.8 |
| 2,6-diCBA | 84.7 |
| 3,4-diCBA | 68.4 |
| 3,5-diCBA | 38.8 |
| 2,3,5-triCBA | 48.8 |
| 2,3,6-triCBA | 73.2 |
| 2,4,6-triCBA | 63.0 |

***Tab. 3.1.*** Residual concentration (%) of chlorobenzoic acid isomers after 1 h of incubation with *L. tigrinus* microsomal fraction.

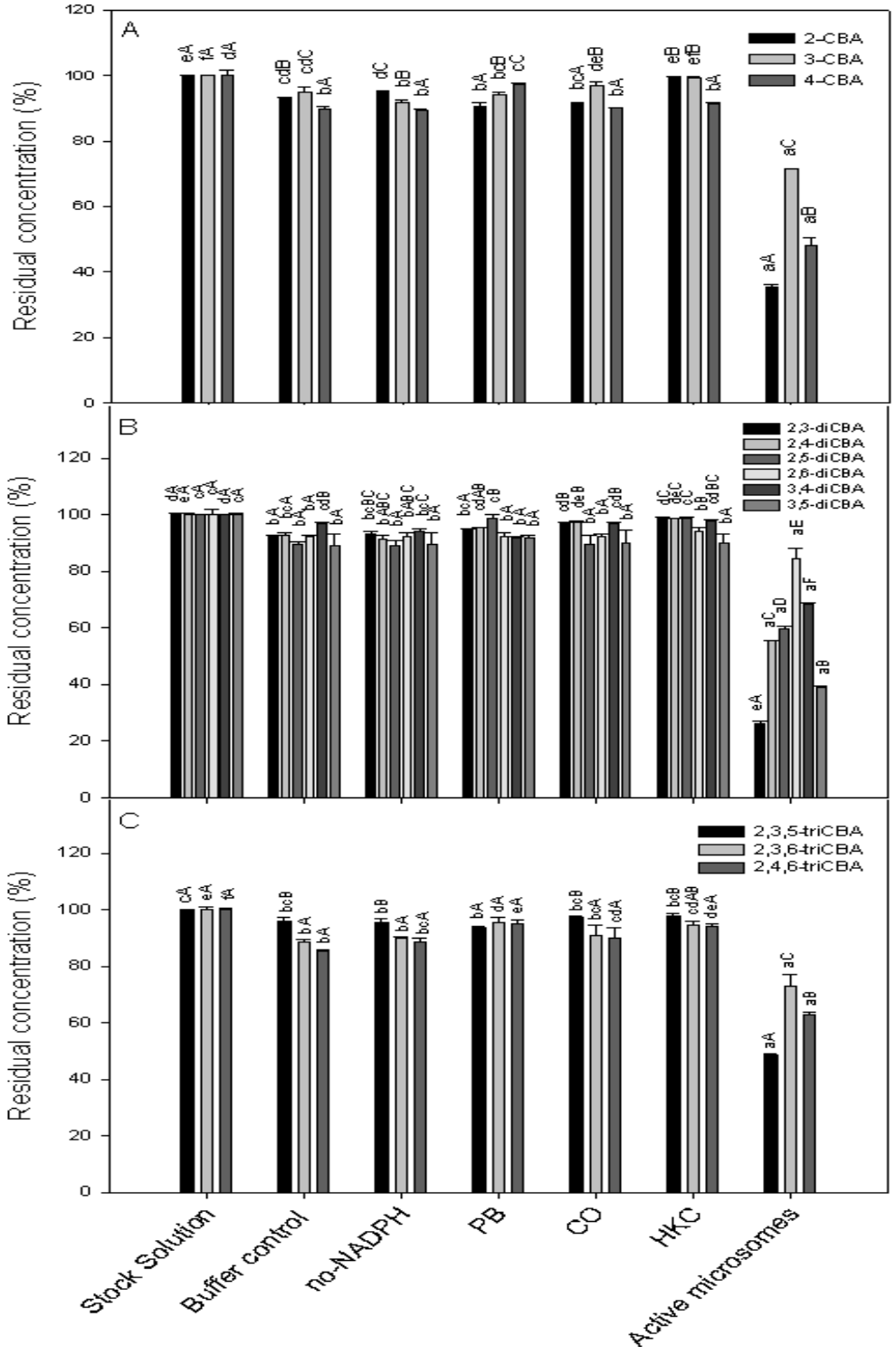



***Fig. 3.3.*** Residual concentration expressed as percentage with respect to the initial amount (Stock Solution) of mono-CBAs (**A**), di-CBAs (**B**) and tri-CBAs (**C**) after 1 h of incubation with *L. tigrinus* microsomal fraction (Active microsomes). Buffer control= reaction mixture without microsomes, no-NADPH= NADPH dependency control, PB= piperonyl butoxide inhibition control, CO= carbon monoxide inhibition control, HKC= heat-killed (-inactivated) control. Data are the mean ± standard deviation of three replicates.

(*continued*)
Multiple pair-wise comparisons were performed by the Tukey test ($P \leq 0.05$). The different lowercase letters above the bars indicate statistically significant differences ($P \leq 0.05$) for the same CBA isomer among different reaction mixtures. The different uppercase letters indicate statistically significant differences ($P \leq 0.05$) among different CBA isomers within the same reaction mixture.

Moreover, cytochrome P450-CBA metabolites were analysed by gas chromatography-mass spectrometry either without derivatization or after methylation with diazomethane. A hydroxylated monochlorobenzoic acid with a GC-$t_R$ of 44.475 min and a molecular mass of 169 was identified after conversion into its respective methyl derivative (*Figure 3.4*).

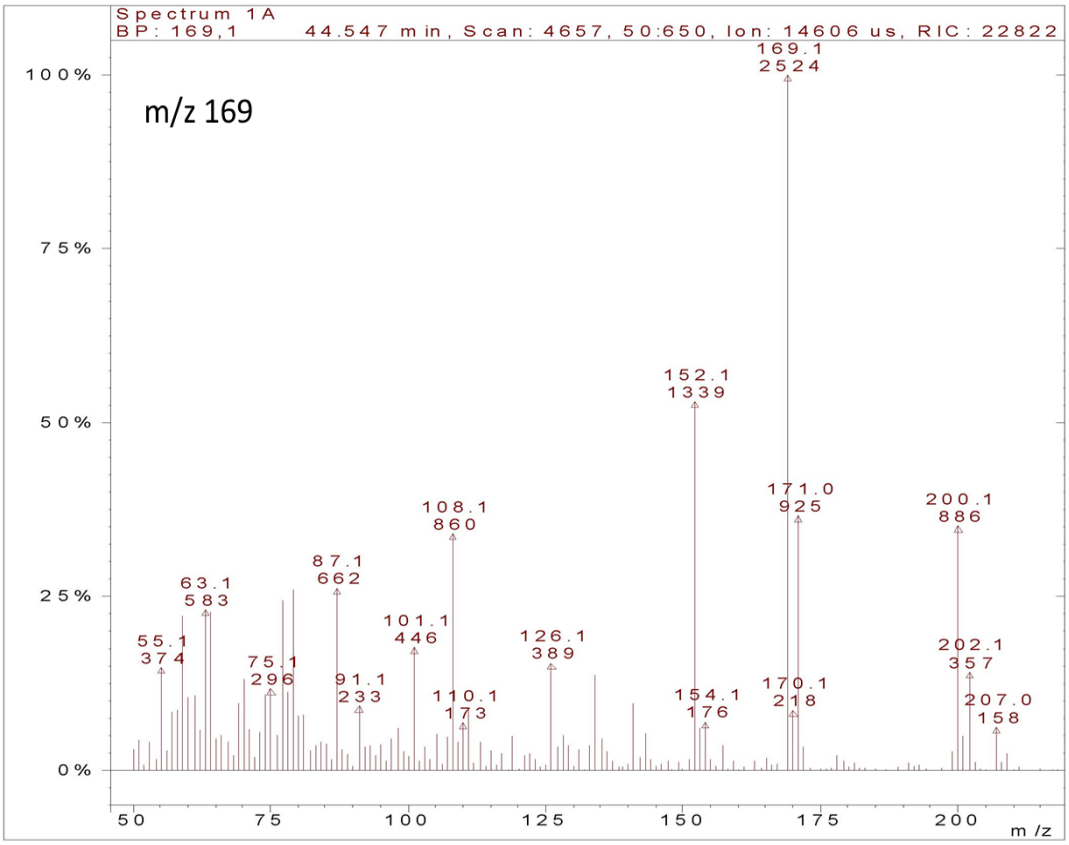



***Fig. 3.4.*** Mass spectrum of a hydroxylated monochlorobenzoic acid detected in *Lentinus tigrinus* CYP450-CBAs reaction mixture after 1 h of incubation. This product was identified after conversion into its respective methyl derivative (m/z 169).

To conclude, this experiment gave a relevant contribution to a more comprehensive understanding of the overall CBA degradation process in *L. tigrinus*. If the involvement of laccase and MnP in the initial transformation steps of CBAs was ruled out due to their inability to bring about oxidation of these compounds, even in the presence of effective mediators, this study provided evidence for the involvement of the *L. tigrinus* cytochrome P450 enzymes in the early phases of CBA degradation process. This could be inferred from the dependency of the reaction on the presence of NADPH as a cofactor, and by its susceptibility to inhibition by piperonyl butoxide and carbon monoxide, two well-known cytochrome P450 inhibitors.

Furthermore, the detection of a hydroxylated intermediate further supported and clearly proved the pivotal role of cytochrome P450 monooxygenase system in the initial CBA bioconversion steps.

### 3.3.3 *In vitro* degradation of PCBs by *Pleurotus ostreatus* microsomal fraction

White rot fungi can degrade both technical PCB mixtures and single congeners, either in liquid or solid systems; however, purified fungal extracellular phenoloxidases have been found to be unable to oxidize PCB congeners (Baudette *et al*., 1998; Krcmar *et al*., 1999; Takagi *et al*., 2007) suggesting the involvement of other enzymatic systems. Nevertheless, to date, the fungal biodegradation mechanism of PCBs has not been fully understood yet.

On the basis of *L. tigrinus* CYP450 degradation experiment with chlorobenzoic acids, this study was also extended to the same enzymatic system isolated from *P. ostreatus* and aimed at assessing its degradation ability towards PCBs.

Five PCB congeners, characterized by different number and position of chlorine substituents, were selected to perform the *in vitro* experiment, the results of which are reported in *Figure 3.5*.

Similarly to CBA- *L. tigrinus* CYP450 degradation test, the recovery of PCBs was higher than 85 % in all controls, the NADPH-dependency of PCB transformation reaction was proved as well as the inhibitory effect exerted by piperonyl butoxide and carbon monoxide.

GC-MS analysis confirmed that PCBs were quantitatively oxidized by active microsomes within 1 h of incubation. In this regard, di-, tri- and tetra-chlorinated biphenyls (congener n. 4, 8, 18 and 45) were degraded at a statistically similar extent with residual contents of 53.9, 48.9, 51.6 and 54.2 %, respectively (Tab. 3.2). On the contrary, the degradation of the congener n. 105 was noticeably lower, the residual concentration of which was 74.9 % at the end of the incubation. The lesser degradation extent of this compound could be explained by

the higher number of chlorine atoms on the aromatic rings with respect to the other congeners under study, and also by the presence of chloro-substituents in both *para* positions.

| PCB congeners | Residual concentration (%) |
|---|---|
| n.4 (2,2′-diBP) | 53.9 |
| n.8 (2,4′-diBP) | 48.9 |
| n.18 (2,2′,5-triCB) | 51.6 |
| n. 45 (2,2′,3,6-tetraBP) | 54.2 |
| n. 105 (2,3,3′,4,4′-pentaBP) | 74.9 |

***Tab. 3.2.*** Residual concentration (%) of PCB congener n. 4, 8, 18, 45 and 105 after 1 h of incubation with *P. ostreatus* microsomal fraction.

These outcomes agree with previous considerations given by Kubatova et al., (2001) regarding the selective PCB degradation mechanism of *Pleurotus ostreatus*. This study, performed on an artificially contaminated soil with Delor 103 PCB mixture, demonstrated that the biodegradation efficiency of *Pleurotus ostreatus* decreased remarkably with the increase of number of chlorine atoms. Moreover, the biotransformation of *ortho* substituted PCBs (halogen atoms in 2, 2′, 6 or 6′ position on biphenyl rings) was preferred to *para* substituted congeners (chlorine atoms in 4 or 4′ positions). The drastically decrease of degradation efficiency of this fungus towards double *para*- substituted congeners was already shown by Zeddel et al. (1993) in a solid state system composed of wood chips (*Picea abies*) and millet seeds. In this case, it was assumed that the selective removal of PCBs could be related to the preferential sorption of some PCB congeners to soil and fungal biomass; however, this assumption should be valid for all fungal systems while other fungi, *i.e. Trametes versicolor* and *Phanerochaete chrysosporium*, have shown different behaviour in straw compartment (Zeddel *et al.*, 1993; Baudette *et al.*, 1998). Therefore, Kubatova and co-workers believed that the degradation performances mostly depended on the specific capability of the fungus to attack these pollutants rather than on behaviour of PCBs in the soil system.

In addition to quantitative analysis, both gas chromatography and liquid chromatography-mass spectrometry analysis were performed to detect PCB metabolites. Unexpectedly, no PCB degradation intermediates, such as hydroxylated derivatives, were identified.

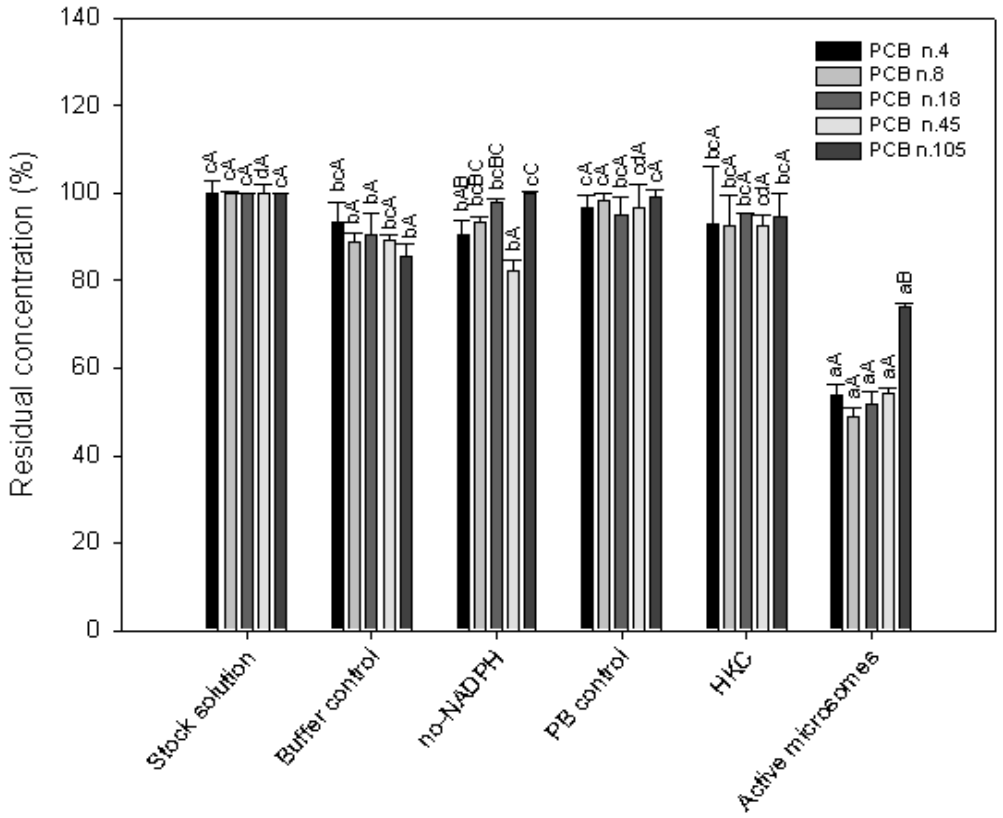



***Fig. 3.5***. Residual concentration expressed as percentage of the original amount (Stock Solution) of PCB congeners n. 4, 8, 18, 45 and 105 after 1 h of incubation with *P. ostreatus* microsomal fraction (Active microsomes). Buffer control= reaction mixture without microsomes, No-NADPH= NADPH dependency control, PB= piperonyl butoxide inhibition control, CO= carbon monoxide inhibition control, HKC= heat-killed (-inactivated) control. Data are the mean ± standard deviation of three replicates. Multiple pair-wise comparisons were performed by the Tukey test ($P \leq 0.05$). The different lowercase letters above the bars indicate that differences among the reaction mixtures considering the same PCB congener were not significant. Same uppercase letters indicate lack of statistically significant differences ($P \leq 0.05$) among congeners in the same reaction mixture.

### 3.3.4 *In vitro* degradation of hydroxy-PCBs by *Pleurotus ostreatus* semi-purified laccase

Hydroxy-PCBs (OH-PCBs), as already mentioned, are toxic metabolites of PCBs which can exert various endocrine disrupting activities: they can influence oestrogen balance by inhibition of sulfotransferase and by binding to oestrogen receptors (Wang *et al.*, 2006; Wang & James, 2007). Moreover, immunosuppression, endometriosis and increased cancer mortality seemed to be related not only to PCB exposure, but also to their derivatives including hydroxylated metabolites (Masuda, 2003). Nevertheless, limited research has been done on biodegradation of these compounds: a few studies dealt with the transformation of hydroxy-PCBs by ligninolytic enzymes of white rot fungi, mainly by phenoloxidases. Besides the oxidation of these toxic molecules, laccases isolated from fungal systems, namely *Pycnoporus cinnabarinus* and *Trametes versicolor*, could transform hydroxy-PCBs producing either chlorinated or dechlorinated dimers (Fujihiro *et al*., 2009; Kordon *et al*., 2010).

In this study, a semi-purified laccase from *P. ostreatus* solid-state cultures (specific activity of 84 U $mg^{-1}$) was used to perform *in vitro* degradation of 2 mono- and 3 di-chlorinated hydroxy biphenyls, either under mediated or non mediated conditions.

Differently from CYP450 experiments, the five selected OH-PCBs were tested separately.

Compounds, such as HBT, ABTS, violuric acid, TEMPO (2,2,6,6-tetramethylpiperidin-1-yl)oxy, can significantly enhance the reaction rates or extend the range of laccase substrates through a redox mediation. Particularly, some synthetic mediators (*i.e.* HBT) characterized by high redox potential enabled the enzyme to oxidize even molecules with high ionization potential such as PAHs (Pozdnyakova *et al*., 2006; Covino *et al*., 2010).

Consequently, the ability of these laccase mediator systems (LMS), as they are commonly termed to, and non mediated systems to degrade OH-PCBs was evaluated.

The presence of HBT markedly accelerated the degradation of all tested compounds which were completely removed within the first 6 h of incubation (*Figure 3.6*, *3.7*).

The performances of laccase in terms of OH-PCB removal under mediated and non mediated conditions were similar in reaction mixtures containing 4OH-2′CB if compared at the end of incubation (*Fig. 3.6* A). However, in the first hour, the addition of HBT led to an increase in the reaction rate enabling a 96 % removal of this compound with respect to its relative control.

By contrast, laccase degradation efficiency towards 4OH-4′CB was noticeably lower than that of mediated reactions (*Figure 3.6* B). At the end of incubation, 50.1 % of this compound was

still found in the reaction. As for the intracellular cytochrome P450 system of *P. ostreatus*, the double *para*-substitutions on the biphenyl skeleton (one chlorine at position 4′ and one hydroxy at position 4) seemed to reduce the capability of laccase to oxidize this monochlorinated hydroxy PCB.

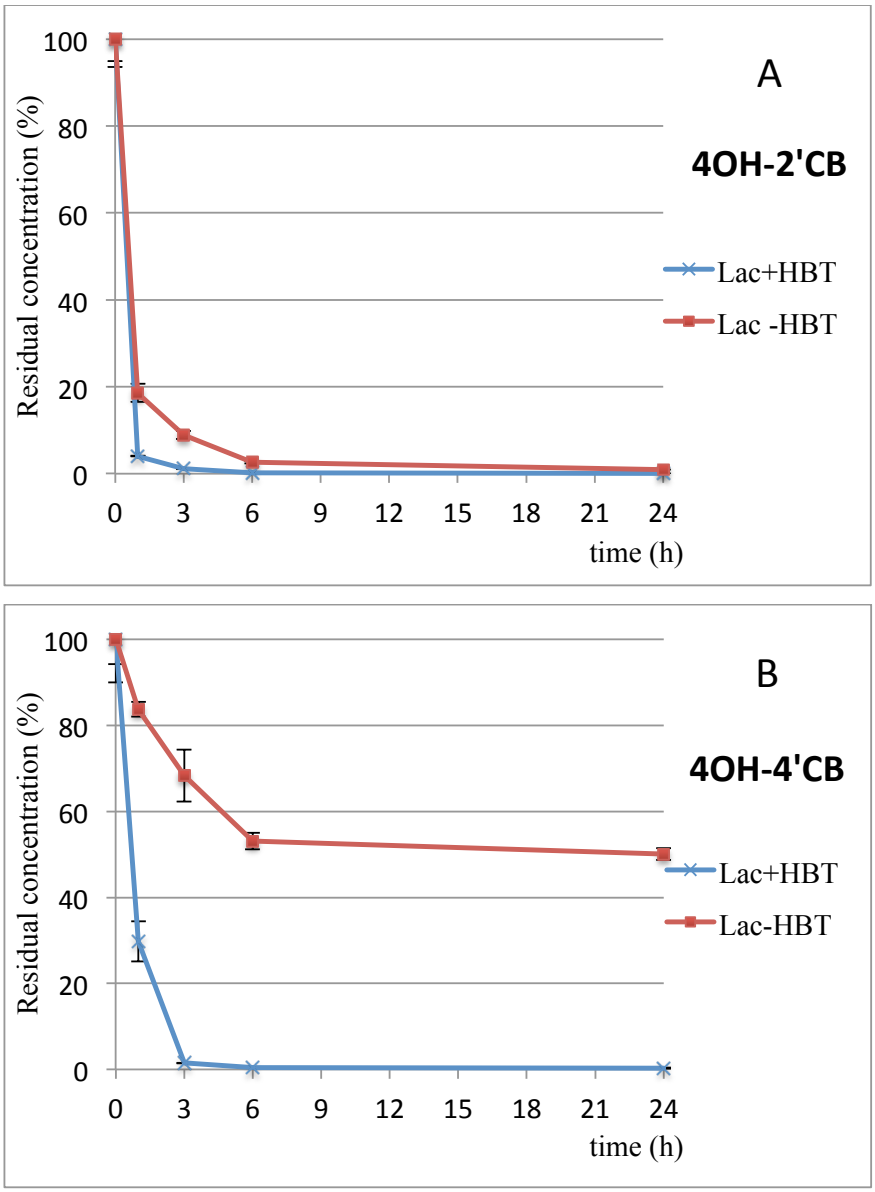



***Fig. 3.6.*** Residual concentration (%) of 4-hydroxy-2-chlorobiphenyl (4OH-2′CB (**A**)) and 4-hydroxy-4′-chlorobiphenyl (4OH-4′CB (**B**)), under mediated (Lac + HBT) or non-mediated (Lac – HBT) conditions.

Among dichlorinated hydroxy-biphenyls, the compound 2OH-3,5diCB was the most extensively degraded under non mediated conditions (*Figure 3.7* A). The higher degradation susceptibility of this compound could be attributed to its chemical structure: the hydroxyl group, which is present on the same ring to which chlorine atoms are bound, polarizes the ring and reduces the electron density facilitating the formation of radicals (Shultz *et al*., 2001). As a consequence, the presence of one chlorine atom (position n. 3) at the *ortho*-position with respect to the hydroxyl substituent (position n. 2) makes this compound even more susceptible to radical attack than others with chlorines in *para*- or *meta*- position (Shultz *et al*., 2001; Keum *et al*., 2004).

Conversely, in the absence of the mediator, the degradation extent of 3OH-2´5´diCB and 4OH-2´5´diCB was lower than that of 2OH-3,5diCB, the residual concentrations of which were 47.9 and 39.6 %, respectively, after 24 h of incubation (*Figure 3.7* B, C). As already mentioned, the reactivity of substrates to laccase is related to their electrostatic properties: 3OH-2´5´diCB and 4OH-2´5´diCB are characterized by high ionization potential (8.9023 and 8.8720 eV, respectively) and thus, hardly oxidized under non mediated conditions. With this regards, Keum and co-workers proved a statistically significant negative correlation between the ionization potential of OH-PCBs and their removal rates.

In this study, the addition of a mediator to the reaction mixture was necessary; the aforementioned compounds, namely 3OH-2´5´diCB and 4OH-2´5´diCB, were completely degraded in the presence of HBT within the first 6 h of incubation (*Figure 3.7* B, C).

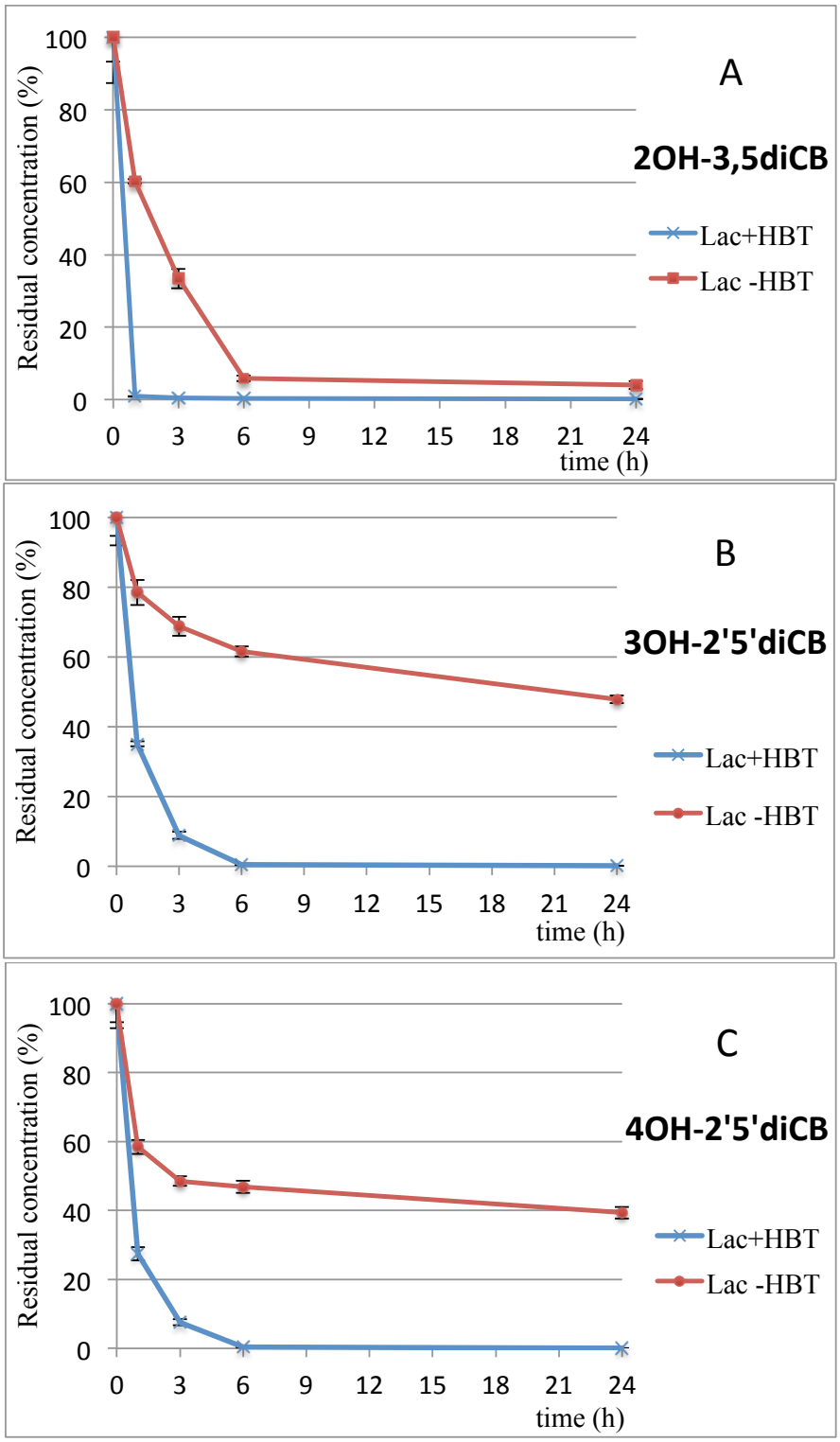



***Fig. 3.7***. Residual concentration (%) of 2-hydroxy-3,5-dichlorobiphenyl (2OH-3,5diCB (**A**)), 3-hydroxy-2′,5′-dichlorobiphenyl (3OH-2′,5′diCB (**B**)) and 4-hydroxy-2′,5′-dichlorobiphenyl (4OH-2′,5′diCB (**C**)), under mediated (Lac + HBT) or non-mediated (Lac – HBT) conditions.

## 3.4 Conclusions

The ability of "white rot" fungi to degrade PCBs and their degradation products, including CBAs and hydroxylated PCBs, is widely proved. However, the degradation mechanisms of these chlorinated pollutants have not been fully elucidated yet.

This study provided more insights into the role of both fungal intracellular cytochrome P450 monooxygenase systems and extracellular ligninolytic enzymes in the degradation process of PCBS, CBAs and OH-PCBs.

*L. tigrinus* microsomal fraction rich in CYP450 were able to degrade, albeit at different extent, all the chlorobenzoic acids (mono-, di- and tri-CBAs) within 1 h of incubation. However, the presence of chlorines in both *ortho*-positions with respect to the carboxyl group negatively affected the depletion levels of 2,6-diCBA and 2,3,6-triCBA.

The involvement of CYP450 system in the CBA biotransformation process was confirmed by the dependency of the reaction on NADPH as well as by its susceptibility to either carbon monoxide or piperonyl butoxide inhibition. Furthermore, the chemical nature of the detected metabolite, namely hydroxylated mono-CBA, clearly proved the pivoltal role of CYP450 in the first steps of CBA degradation.

On the basis of these results with *P. tigrinus* CYP450, this study was also extended to the same enzymatic system isolated from *P. ostreatus* and aimed at assessing its degradation ability towards PCBs.

The *in vitro* degradation study revealed that the concentration of di-, tri- and tetrachlorinated biphenyls (congeners n. 4, 8, 18 and 45) was halved after 1 h of incubation, while the higher number of chlorines and the double chloro-substitution in *para* positions noticeably impaired the degradation of the pentachlorinated biphenyl (congener n. 105). As for *L. tigrinus* CYP450-CBAs *in vitro* degradation test, the involvement of CYP450 in the PCB removal process was proved by either the NADP-dependency, or the PB and CO inhibition of the reaction. However, no PCB degradation intermediates were identified.

Moreover, the experiment conducted with a semi-purified laccase obtained from *P. ostreatus* demonstrated the ability of this fungal phenoloxidase rich extract to degrade mono- and di-chlorinated hydroxyl PCB, either under mediated and non mediated conditions. The presence of HBT as the mediator as well as the chemical structure of OH-PCBs represented the main factors affecting the degradation processes.

# CHAPTER 4

# CONCLUDING REMARKS

The remediation of persistent chlorinated aromatic pollutants has become a priority worldwide due to their adverse effects on natural ecosystems and human health. Particularly, polychlorinated biphenyls (PCBs) represent a serious problem to be faced due to their teratogenic, carcinogenic and endocrine-disrupting properties.

The use of biological systems, referred to as bioremediation, is an eco-friendly and cost-effective alternative to the traditional chemical or thermal treatments for the clean-up of PCB-contaminated matrices. With this regard, filamentous fungi, and particularly the "white rot" fungi display many biochemical, metabolic and ecological features that make them excellent candidates to design an effective PCB remediation technology. Nevertheless, the broad catabolic versatility of these organisms has not been widely exploited yet for its potential in the bioremediation of hazardous chemicals.

Therefore, the main aim of the present Ph.D project was to *i*) assess the efficiency of different mycoremediation strategies for the clean-up of an aged-PCB contaminated soil, and *ii*) clarify the fungal degradation pathways of PCBs and their major metabolites chlorobenzoic acids (CBAs) and hydroxylated polychlorinated biphenyls (OH-PCBs).

The results achieved within the frame of this work are summarized below:

- ✓ Bioavailability of PCBs in soils is negatively correlated with the content of organic matter and the number of chlorine substituents on the biphenyl moiety;
- ✓ Bioaugmentation treatments were more effective than biostimulation ones in the removal of PCBs from soils. Particularly promising seems the use of the white rot fungus *Pleurotus ostreatus* which was able to degrade more than 50 % of PCBs in the rhizosphere soil and to remarkably detoxify the matrix;
- ✓ The chemical structure of PCB metabolites detected throughout the mycoremediation treatments (*i.e.*, hydroxylated and methoxylated PCBs, chlorobenzoates, chlorobenzaldehydes and chlorocresols) suggested the involvement of both intracellular and extracellular fungal enzymes, and also of autochthonous microorganisms in the PCB degradation process;
- ✓ The introduction of allochthonous fungi or the addition of non-inoculated lignocellulosic substrate stimulated the growth of the resident bacterial community;

- ✓ *P. ostreatus* augmentation clearly increased the relative abundance of *Firmicutes* phylum in the bulk soil and rhizosphere soil; a similar raise was observed for the phyla *Proteobacteria* and *Bacteroidetes* in all soil samples treated with *I. lacteus*;

- ✓ The phylum *Basidiomycota* was the dominant one in bioaugmentation treatments, the sequences of which belonged to *P. ostreatus* and *I. lacteus* genera in their respective microcosms. *P. ostreatus* sequences accounted to more than 90 % of the total fungal amplicons along the whole incubation period, proving the outstanding capability of this fungus to compete with the autochthonous microbiota, and thus to efficiently grow in PCB-contaminated soils under non-sterile conditions. By contrast, the majority of fungal sequences in biostimulated microcosms belonged to the phyla *Ascomycota* and *Zygomycota*;

- ✓ Microsomal fractions rich in cytochrome P450 monooxygenase (CYP450) activities isolated from the white rot fungi *Lentinus tigrinus* and *Pleurotus ostreatus* were able to degrade a mixture of CBAs and PCBs, respectively. Specifically, the identification of a hydroxylated CBA confirmed the pivotal role of CYP450 in the initial transformation of CBAs;

- ✓ The semi-purified laccase obtained from *Pleurotus ostreatus* was capable of degrading mono- and dichlorinated hydroxylated biphenyls (OH-PCBs), either under mediated or non mediated conditions;

- ✓ The number and position of substituents were the main factors affecting the extent of degradation by both fungal intracellular cytochrome P450 monooxygenases and extracellular enzymatic systems.

In conclusion, the integration of chemical, toxicological and molecular biology techniques provided a comprehensive evaluation of key parameters affecting the technical feasibility and the efficiency of the mycoremediation process.

The extensive removal of target pollutants in a relatively short time period, the high detoxification of the polluted matrix, the great competition with resident microorganisms, and the remarkable degradation not only of PCBs, but also of their major metabolites (OH-PCBs) clearly proved the outstanding efficiency of the white rot fungus *Pleurotus ostreatus* in the remediation of PCB contaminated soil making its application potentially transferable to a larger scale.

# CHAPTER 5

# BIBLIOGRAPHY

**Abraham W.R., Nogales B., Golyshin P.N., Pieper D.H, Timmis K.N. (2002).** Polychlorinated biphenyl-degrading microbial communities in soils and sediments. *Curr. Opin. Microbiol.* 5: 246–253.

**Abramowicz D.A. (1990).** Aerobic and anaerobic biodegradation of PCBs: A review. *Critical Reviews in Biotechnology*. 10: 241-251.

**Adebusoye S.A., Picardal F.W., Ilori M.O., Amund O.O. (2008).** Influence of chlorobenzoic acids on the growth and degradation potentials of PCB-degrading microorganisms. *World J. Microb. Biot.* 24: 1203-1208.

**Agency for Toxic Substances and Disease Registry (2000).** Toxicological profile for polychlorinated biphenyls (update). Atlanta: US Department of Health and Human Services.

**Ake C.L., Mayura K., Huebner H., Bratton G.R., Phillips T.D. (2001).** Development of porous clay-based composites for sorption of lead from water. *J. Toxicol. Environ. Health*. 63: 459–475.

**Alexander M. (2000).** Aging, bioavailability, and overestimation of risk from environmental pollutants. *Environ. Sci. Technol*. 34: 4259–4265.

**Anderson P.N., Hites R.A. (1996).** OH radical reactions: the major removal pathway for polychlorinated biphenyls from the atmosphere. *Environ. Sci. Technol*. 30: 1756-1763.

**Ang E.L., Zhao H.M., Obbard J.P. (2005).** Recent advances in the bioremediation of persistent organic pollutants via biomolecular engineering. *Enz. Microbial. Technol*. 37: 487-496.

**ATSDR, Agency for Toxic Substances and Disease Registry (2000)**. Polychlorinated biphenyls (PCBs) toxicity. U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES. Agency for Toxic Substances and Disease Registry. Division of Toxicology and Environmental Medicine.

**Atteia O., Guillot C. (2007)**. Factors controlling BTEX and chlorinated solvents plume length under natural attenuation conditions. *J. Contam. Hydrol*. 90: 81–104.

**Baborová P., Möder M., Baldrian P., Cajthamlová K., Cajthaml T. (2006)** Purification of a new manganese peroxidase of the white-rot fungus *Irpex lacteus*, and degradation of polycyclic aromatic hydrocarbons by the enzyme. *Research Microbiol.* 157: 248–253.

**Baldrian P. (2003).** Interactions of heavy metals with white-rot fungi. *Enzyme Microb. Technol.* 32: 78-91.

**Baldrian P. (2006).** Fungal laccases - occurrence and properties. *Fems Microbiology Reviews* 30: 215-242.

**Baldrian P., der Wiesche C.I., Gabriel J., Nerud F., Zadrazil F. (2000).** Influence of cadmium and mercury on activities of ligninolytic enzymes and degradation of polycyclic aromatic hydrocarbons by *Pleurotus ostreatus* in soil. *Appl. Environ. Microbiol*. 66: 2471-2478.

**Ballschmiter K., Zell M. (1980)**. Analysis of polychlorinated biphenyls (PCB) by glass capillary gas chromatography. *Fresenius' Zeitschrift für analytische Chemie*. 302: 20-31.

**Beaudette L.A., Davies S., Fedorak P.M., Ward O.P., Pickard M.A., (1998).** Comparison of gas chromatography and mineralization experiment for measuring loss of selected polychlorinated biphenyl congeners in cultures of white rot fungi. *Appl. Environ. Microbiol.* 64: 2020-2025.

**Beebe L.E., Kim Y.E., Amin S., Riggs C.W., Kovatch R.M., Anderson L.M. (1993)**. Comparison of transplacental and neonatal initiation of mouse lung and liver tumors by N-nitrosodimethylamine (NDMA) and 4-(methylnitrosamino)-1-(3- pyridyl)-1-butanone (NNK) and promotability by a polychlorinated biphenyls mixture (Aroclor 1254). *Carcinogenesis.* 14: 1545-1548.

**Berglund O., Nyström P., Larsson P. (2005).** Persistent organic pollutants in river food webs: influence of trophic position and degree of heterotrophy. *Can. J. Fish. Aquat. Sci*. 62: 2021-2032.

**Bermek H., Gülseren I., Li K., Jung H., Tamerler C. (2004).** The effect of fungal morphology on ligninolytic enzyme production by a recently isolated wood-degrading fungus *Trichophyton rubrum* LSK-27. *World J. Microbiol. Biotechnol*. 20: 345-349.

**Bernhardt R. (2006).** Cytochromes as versatile biocatalists. *J. Biotechnol*. 124:128-145.

**Beyer A., Biziuk M. (2009).** Environmental fate and global distribution of polychlorinated biphenyls. *Rev. Environ. Contam. Toxicol*. 201: 137-158.

**Bielska L., Smidova K., Hofman J. (2013).** Supercritical fluid extraction of persistent organic pollutants from natural and artificial soils and comparison with bioaccumulation in earthworms. *Envir. Pollut*. 176: 48-54.

**Billingsley K.A., Backus S.M., Ward O.P. (1999).** Effect of surfactant solubilization on biodegradation of polychlorinated biphenyl congeners by *Pseudomonas* LB400. *Appl. Microbiol. Biotechnol.* 52: 255-260.

**Bollag J.M.** (1992). Decontaminating soil with enzymes. *Environ. Sci. Technol*, 26: 1876–1881.

**Bonfante P., Anca I.A. (2009).** Plants, mycorrhizal fungi, and bacteria: a network of interactions. *Annual Rev. Microbiol*. 63: 363-383.

**Borazjani H., Wiltcher D., Diehl S. (2005).** Bioremediation of polychlorinated biphenyl and petroleum contaminated soil. In: Lyon WG, Hong JJ, Reddy RK, editors. *Proceedings of the International Conference on Environmental Science and Technology.* New Orleans, USA: American Science Press; p. 502–507.

**Borga K., Fisk A.T., Hargave B., Hoekstra P.F., Swackhamer D., Muir D.C.G. (2005).** Bioaccumulation factors for PCBs revisited. *Environ. Sci. Technol*. 39: 4523-4532.

**Borja J., Taleon D.M., Auresenia J., Gallardo S. (2005).** Polychlorinated biphenyls and their biodegradation. *Process Biochem*. 40: 1999-2013.

**Bornyasz M.A., Graham R.C., Allen M.F.(2005).** Ectomycorrhizae in a soil-weathered granitic bedrock regolith: linking matrix resources to plants. *Geoderma.* 126: 141–160.

**Bradford M.M. (1976).** A rapid and sensitive method for the quantitation of microgram quantitiesof protein utilizing the principle of protein-dye binding. *Anal. Biochem*. 72: 248–254.

**Brody J.G., Moysich K.B., Humblet O., Attfield K.R., Beehler G.P., Rudel R.A. (2007).** Environmental pollutants and breast cancer. Environmental Factors in Breast Cancer, Cancer supplement. 109: 2667-2711.

**Brown J.F., Bedard D.L., Brennan M.J., Carnahan J.C., Feng H, Wagner R.E. (1987).** Polychlorinated biphenyl dechlorination in aquatic sediments. *Science*. 236: 709-712.

**Burreau S., Zebühr Y., Broman D., Ishaq R. (2006).** Biomagnification of PBDEs and PCBs in food webs from the Baltic Sea and the northern Atlantic Ocean. *Science Tota.l Environ.* 366: 659–672.

**Bush B., Kadlec M.J. (1995).** Dynamics of PCBs in the aquatic environment. *Great Lakes Research Review*. 1: 24-30.

**Cabana H., Jones J.P., Agathos S.N. (2007).** Elimination of endocrine disrupting chemicals using white rot fungi and their lignin modifying enzymes: a review. *Eng. Life Sci*. 7: 429-456.

**Cajthaml T., Erbanová P., Kollmann A., Novotný C., Sasek V., Mougin C. (2008).** Degradation of PAHs by ligninolytic enzymes of *Irpex lacteus*. *Folia microbiologica*. 53: 289-294.

**Cajthaml T., Erbanova P., Sasek V., Moeder M. (2006).** Breakdown products on metabolic pathway of degradation of benzo(a)anthracene by a ligninolytic fungus. *Chemosphere*. 64: 560-564.

**Cajthaml T., Erbanová P., Šašek V., Moeder M. (2006).** Breakdown products on metabolic pathway of degradation of benz(*a*)anthracene by a ligninolytic fungus. *Chemosphere*. 64: 560–564.

**Cajthaml T., Šašek V. (2005).** Application of supercritical fluid extraction (SFE) to predict bioremediation efficacy of long-term composting of PAH-contaminated soil. *Environ. Sci. Techn.* 21: 8448-8452.

**Camarero S., Cañas A.I., Nousiainen P., Record E., Lomascolo A., Martínez J., Martínez Á.T., (2008).** *p*–hydroxycinnamic acids as natural mediators for laccase oxidation of recalcitrant compounds. *Environ.Sci.Technol*. 42: 6703–6709.

**Camarero S., Sarkar S., Ruiz-Dueñas F.J., Martinez M.J., Martinez A.T. (1999).** Description of a versatile peroxidase involved in natural degradation of lignin that has both Mn-peroxidase and lignin-peroxidase substrate binding sites. *J. Biol. Chem.* 274: 10324–10330.

**Campanella B.F., Bock C., Schroder P. (2002).** Phytoremediation to increase the degradation of PCBs and PCDD/Fs - Potential and limitations. *Environ. Sci. Pollut.* 9: 73–85.

**Carey J., Cook P., Giesy J., Hodson P., Muir D., Owens W., Solomon K. (1998).** Ecotoxicological risk assessment of the chlorinated organic chemicals. Society of Environmental Toxicology and Chemistry (SETAC), ISBN: 978-1-880611-07-4.

**Carvalho M.B., Martins I., Leitão M.C., Garcia H., Rodrigues C., San Romão V., McLellan I., Hursthouse A., Pereira C.S. (2009).** Screening pentachlorophenol degradation ability
by environmental fungal strains belonging to the phyla Ascomycota and Zygomycota. J. Ind. Microbiol. Biotechnol. 36: 1249–1256.

**CCME, Canadian Council of Ministers of the Environment (1999).** Canadian Environmental Quality Guidelines for the Protection of Environmental and Human Health - Polychlorinated biphenyls.

**Chaudhry Q., Blom-Zandstra M., Gupta S., Joner E.J. (2005).** Utilising the synergy between plants and rhizosphere microorganisms to enhance breakdown of organic pollutants in the environment. *Environ. Sci. Pollut.* 12: 34–48.

**Chaudhry Q., Schröder P., Werck-Reichhart D., Grajek W., Marecik R. (2002).** Prospects and limitations of phytoremediation for the removal of persistent pesticides in the environment. *Environ. Sci. Pollut. Res*. 9: 4- 17.

**Chiarenzelli J., Pagano J., Scrudato R., Falanga L., Migdal K., Hartwell A., Milligan M., Battalagia T., Holsen T.M., Hopke P., Hsu y. (2001).** Enhanced airbone polychlorinated biphenyls (PCB) concentrations and chlorination downwind of Lake Ontario. *Environ. Sci. Technol*. 31: 597-602.

**Chu W.K., Wong M.H., Zhang J. (2006).** Accumulation, distribution and transformation of DDT and PCBs by *Phragmites australis* and *Oryza sativa* L.: I. Whole plant study. *Environ. Geochem. Health*. 28: 159-68.

**Cicilio R. (2013).** Octanol/water partitioning coefficients of PCB mixtures for environmental fate and transport". *Honors Theses*. University of New Hampshire. Paper 131.

**Cogliano V. J. (1998).** Assessing the cancer risk from environmental PCBs. *Environ. Health Perspect.* 106: 317–323.

**Collins P.J., Field J.A., Teunissen P., Dobson A.D.W. (1997).** Stabilization of lignin peroxidases in white rot fungi by tryptophan. *Appl. Environ. Microbiol*. 63: 2543-2548.

**Colores G.M., Macur R.E., Ward D.M., Inskeep W.P. (2000).** Molecular analysis of surfactant-driven microbial population shifts in hydrocarboncontaminated soil. *Appl. Environ. Microbiol.* 66: 2959–2964.

**Cookson J.T. (1995).** Bioremediation Engineering: Design and Application. *McGraw-Hill*, New York, USA.

**Cortes A., Riego J., Paya-Perez A.B., Larsen B. (1991).** Soil sorption of co-planar and non-planar PCBs. *Toxicol. Environ. Chem*. 31/32:79–86.

**Covino S. (2010).** *In vivo* and *in vitro* degradation of aromatic contaminants by white rot fungi. A case study: *Panus tigrinus* CBS 577.79. PhD thesis University of Tuscia, Agrobiology and Agrochemistry Department; [http://hdl.handle.net/2067/1200].

**Covino S., Svobodová K., Kresinová Z., Petruccioli M., Federici F., D'Annibale A., Cvančarová M., Cajthaml T. (2010)**. *In vivo* and *in vitro* polycyclic aromatic hydrocarbons degradation by *Lentinus* (*Panus*) *tigrinus* CBS 577.79 *Biores. Technol*. 101: 3004–3012.

**Črešnar B., Petrič Š. (2011).** Cytochrome P450 enzymes in the fungal kingdom. Review. *Biochim. Biophys. Acta*. 1814: 29–35.

**Crinnion W.J. (2011).** Polychlorinated biphenyls: persistent pollutants with immunological, neurological and endocrinological consequences. *Alternative Medicine Review.* Vol. 16, n. 1.

**Crovetto C. (1997).** La cero labranza y la nutrición del suelo. In: Agricultura sustentable de alta producción. 5´ Congreso Nacional de AAPRESID, Mar del Plata, Argentina. pp. 73–78.
*Curr. Opin. Plant Biol.* 11: 349–355.

**Cvancarová M., Zdena Kresinová Z., Filipová A., Covino S., Cajthaml T. (2012).** Biodegradation of PCBs by ligninolytic fungi and characterization of the degradation products
*Chemosphere*. 88: 1317–1323

**D'Annibale A., Ricci M., Quaratino D., Federici F., Fenice M., (2004).** *Panus tigrinus* efficiently removes phenols, colour and organic load from olive-mill wastewater. *Res. Microbiol.* 155: 596-603.

**de Cárcer D.A., Martín M., Karlson U., Rivilla R. (2007a).** Changes in bacterial populations and in biphenyl dioxygenase gene diversity in a polychlorinated biphenyl-polluted soil after introduction of willow trees for rhizoremediation. *Appl. Environ. Microb*. 73: 6224–6232.

**de Cárcer D.A., Martín M., Mackova M., Macek T., Karlson U., Rivilla R. (2007b).** The introduction of genetically modified microorganisms designed for rhizoremediation induces changes on native bacteria in the rhizosphere but not in the surrounding soil. *ISME J*. 1: 215–223.

**Delle Site A. (2001).** Factors affecting sorption of organic compounds in natural sorbent water systems and sorption coefficients for selected pollutants. A Review. *J. Phys. Chem.* 30 (1): 187-439.

**Di Toro S., Zanaroli G., Fava F. (2006).** Intensification of the aerobic bioremediation of an actual site soil historically contaminated by polychlorinated biphenyls (PCBs) through bioaugmentation with a non acclimated, complex source of microorganisms. *Microbial. Cell Factories*. 6: 1-10.

**Dietrich D., Hickey W.J., Lamar R. (1995).** Degradation of 4,4′-dichlorobiphenyl, 3,3′,4,4′-tetrachlorobiphenyl, and 2,2′,4,4′,5,5′-hexachlorobiphenyl by the white rot fungus *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol*. 61 :3904–3909.

**Donato F., Magoni M., Bergonzi R., Scarcella C., Indelicato A., Carasi S., Apostoli P. (2006).** Exposure to polychlorinated biphenyls in residents near chemical factory in Italy: the food chain as main source of contamination. *Chemosphere.* 64: 1562–1572.

**Edgar R.C. (2004).** MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic acids research*. 32:1792-1797.

**Edgar R.C. (2010).** Search and clustering orders of magnitude faster than BLAST. *Bioinformatics*. 26: 2460-2461.

**Edgar R.C., Haas B., Clemente J.C., Quince C., Knight R. (2011).** UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics.* 27: 2194-2200.

**El Majidi N., Bouchard M., Carrier G. (2013).** Systematic analysis of the relationship between standardized prenatal exposure to polychlorinated biphenyls and mental and motor development during follow-up of nine children cohorts. *Regul. Toxicol. Pharmacol*. 66: 130-146.

**Evans B.S, Dudley C.A., Klasson K.T. (1996).** Sequential anaerobic-aerobic biodegradation of PCBs in soil slurry microcosms. *Appl. Biochem. Biotechnol*. 547-58: 885-894.

**Eweis J.B., Ergas S.J., Chang D.P.Y., Schroeder E.D. (1998).** Bioremediation Principles. *McGraw-Hill*. USA. pp: 207-236.

**Fan B., Zhao,Y., Mo G., Wu J. (2013).** Co-remediation of DDT-contaminated soil using white rot fungi and laccase extract from white rot fungi *J. Soils Sedim*. 13: 1232-1245.

**FAO, Food and Agriculture Organization of the United Nations (2005).** The importance of soil organic matter. Key to drought-resistant soil and sustained food and production. FAO bulletin n.80.

**Fava F., Bertin L., Fedi S., Zannoni D. (2003).** Methyl-beta-cyclodextrin-enhanced solubilisation and aerobic degradation of polychlorinated biphenyls in two aged-contaminated soils. *Biotechnol. Bioeng*. 81: 381-390.

**Fava F., Di Gioia D. (1998).** Effects of Triton X-100 and Quillaya Saponin on the ex situ bioremediation of a chronically polychlorobiphenylcontaminated soil. *Appl. Microbiol. Biotechnol*. 50:623-630.

**Fava F., Piccolo A. (2002).** Effects of humic substances on the bioavailability and aerobic biodegradation of polychlorinated biphenyls in a model soil. *Biotechnol. Bioeng.* 77:204-11.

**Federici E., Giubilei M., Santi G., Zanaroli G., Negroni A., Fava F., Petruccioli M., D'Annibale A. (2012).** Bioaugmentation of a historically contaminated soil by polychlorinated biphenyls with *Lentinus tigrinus*. *Microb. Cell Fact*. 11: 35.

**Field J.A., Reyes Sierra-Alvarez (2008).** Microbial transformation and degradation of polychlorinated biphenyls. Review. *Environmental Pollution*. 155: 1-12.

**Frostegård A., Tunlid A., Bååth E. (2010).** Use and misuse of PLFA measurements in soils. Soil Biol. Biochem. 1-5.

**Fujihiro S., Higuchi R., Hisamatsu S., Sonoki S. (2009).** Metabolism of hydroxylated PCB congeners by cloned laccase isoforms. *Appl. Microbiol. Biotechnol*. 82: 853-860.

**Furukawa K. (2000).** Biochemical and genetic bases of microbial degradation of polychlorinated biphenyls (PCBs). *J. Gen. Appl. Microbiol*. 46: 283-296.

**Furukawa K., Fujihara H. (2008).** Microbial degradation of polychlorinated biphenyls: biochemical and molecular features. *J. Biosc. Bioeng.* 105: 433-449.

**Gadd G.M. (2001).** Fungi in bioremediation. Published for the British Mycological Society. *Cambridge University Press*.

**Galhaup C., Goller S., Peterbauer C.K., Strauss J., Haltrich D. (2002).** Characterization of the major laccase isoenzyme from *Trametes pubescens* and regulation of its synthesis by metal ions. *Microbiol.* 148: 2159-2169.

**Galhaup C., Haltrich D. (2001).** Enhanced formation of laccase activity by the white-rot fungus *Trametes pubescens* in the presence of copper. *Appl. Environ. Microbiol*. 56: 225-232.

**Galliano H., Gas G., Seris J. L., Boudet A.M. (1991).** Lignin degradation by *Rigidoporus lignosus* involves synergistic action of two oxidizing enzymes - Mn peroxidase and laccase. *Enz. Microbial Technol.* 13: 478-482.

**Gerhardt K.E., Huang X.D., Glick B.R., Greenberg B.M. (2009)**. Phytoremediation and rhizoremediation of organic soil contaminants: potential and challenges. A review. *Plant Science*. 176: 20–30.

**Gichner T., Lovecká P., Vrchotová B. (2008).** Genomic damage induced in tobacco plants by chlorobenzoic acids metabolic products of polychlorinated biphenyls. *Mutat. Res.* 657: 140–145.

**Gioia R., Dachs J., Nizzetto L., Lohmann R., Jones K.C. (2013).** Atmospheric transport, cycling and dynamics of polychlorinated biphenyls (PCBs) from source regions to remote oceanic areas. In Occurrence, Fate and Impact of Atmospheric Pollutants on Environmental and Human Health. *ACS Symposium Series, Chapter 1*; American Chemical Society.

**Goncalves A.R., Costa S.M., Esposito E. (2002).** *Panus tigrinus* strains used in delignification of sugarcane bagasse prior to kraft pulping. *Appl. Biochem. Biotechnol.* 98–100: 373–382.

**Goodwin D.C., Aust S.D, Grover T.A. (1995).** Evidence for veratryl alcohol as a redox mediator in lignin peroxidase-catalyzed oxidation. *Biochemistry*. 34: 5060-5065.

**Hammel K.E., Cullen D. (2008).** Role of fungal peroxidases in biological ligninolysis *Curr. Opin. Plant Biol*. 11: 349–355

**Harms H., Schlosser D., Wick L.Y. (2011).** Untapped potential: exploiting fungi in bioremediation of hazardous chemicals. *Nature Reviews Microbiology*. 9: 177-192.

**Hatakka A. (1994).** Lignin-modifying enzymes from selected white-rot fungi - Production and role in lignin degradation. *FEMS Microbiology Reviews*. 13: 125-135.

**Hatakka A. (2001).** Biodegradation of lignin. In *Biopolymers. Biology, Chemistry, Biotechnology, Applications*. Vol 1. Lignin, Humic Substances and Coal. Editors: Hofrichter, M. & Steinbüchel, A. Weinheim: Wiley-VCH. p. 129-180.

**Hatzinger P.B., Alexander M. (1995).** Effect of ageing of chemicals insoil on their biodegradability and extractability. *Environ. Sci. Technol*. 29: 537–545.

**Hawker D.W., Connell D.W. (1988).** Octanol-water partition coefficients of polychlorinated biphenyl congeners. *Environ. Sci. Technol.* 22: 382-387.

**Hawthorne S.B., Poppendieck D.G., Grabanski C.B., Loehr R.C. (2002).** Comparing PAH availability from manufactured gas plant soils and sediments with shemical and biological tests. 1. PAH release during water desorption and supercritical carbon dioxide extraction. *Environ. Sci. Technol*. 36: 4795–4803.

**Heinfling A., Martinez M.J., Martinez A.T., Bergbauer M., Szewzyk, U. (1998).** Purification and characterization of peroxidases from the dye-decolorizing fungus *Bjerkandera adusta*. *FEMS Microbiology Letters*. 165: 43-50.

**Helmfrid I., Berglund M., Löfman O., Wingren G. (2012).** Health effects and exposure to polychlorinated biphenyls (PCBs) and metals in a contaminated community. *Environ. Intern*. 44: 53-58.

**Hillery B.R., Basu I., Sweet C.W., Hites R.A. (1997).** Temporal and spatial trends in a long-term study of gas-phase PCB concentrations near the Great Lakes. *Environ. Sci. Technol*. 31: 1811-1816.

**Hofrichter M., (2002).** Review: lignin conversion by manganese peroxidase. *Enzyme Microb. Technol.* 30: 454-466.

**Hou B.K., Wackett L.P., Ellis L.B. (2003).** Microbial pathways prediction: a functional group approach. *J. Chem. Inf. Comp. Science*. 43: 1051-1057.

**Hutzinger O., Safe S., Zitco V. (1974).** The Chemistry of PCBs. CRC Press, Inc., Cleveland, Ohio. ISBN 0-87819-049-X.

**Integrated Risk Information System, US Environmental Protection Agency (1997).** Polychlorinated biphenyls (PCBs). http://www.epa.gov/iris/subst/0294.htm. Update 2012.

**Ionescu M., Beranova K., Dudkova V., Kochankova L., Demnerova K., Macek T., Mackova M. (2009).** Isolation and characterization of different plant associated bacteria and their potential to degrade polychlorinated biphenyls. *Int. Biodeter. Biodegr*. 63: 667–672.

**ISO 11348-3: 2007.** Water quality – determination of the inhibitory effect of water samples on the light emission of *Vibrio fischeri* (luminescent bacteria test) – Part 3: method using freeze-dried bacteria. ISO (International Organization for Standardization), Geneva, Switzerland.

**Jeffries T.W., Choi S., Kirk T.K. (1981).** Nutritional regulation of lignin degradation by *Phanerochaete chrysosporium. Appl. Environ. Microbiol.* 42: 290-296.

**Jensen S. (1966).** Report of a new chemical hazard. *New Sci*. 32: 612.

**Jensen S. (1972).** The PCB story. *Ambio*.1: 123-131.

**Jonker M.T.O., Smedes F. (2000).** Preferential sorption of planar contaminants in sediments from Lake Ketelmeer, The Netherlands. *Environ. Sci. Technol*. 34: 1620-1626.

**Kamei I., Kogura R., Kondo R. (2006a).** Metabolism of 4,4′-dichlorobiphenyl by white-rot fungi *Phanerochaete chrysosporium* and *Phanerochaete* sp. MZ142 *Appl. Microbiol. Biotechnol.* 72: 566–575.

**Kamei I., Sonoki S., Haraguchi K., Kondo R. (2006b).** Fungal bioconversion of toxic polychlorinated biphenyls by white-rot fungus, *Phlebia brevispora*. *Appl. Microbiol. Biotechnol.* 73: 932–940.

**Kan A.T., Fu G., Hunter M., Chen W., Ward C.H., Tomson M.B. (1998).** Irreversible sorption of neutral hydrocarbons to sediments: experimental observations and model predictions. *Environ. Sci. Technol.* 32: 892-902.

**Kasai N., Ikushiro S., Shinkyo R., Yasuda K., Hirosue S., Arisawa A., Ichinose H., Wariishi H., Sakaki T. (2010).** Metabolism of mono- and dichloro-dibenzo-*p*-dioxins by *Phanerochaete chrysosporium* cytochromes P450. *Appl. Microbiol. Biotechnol*. 86: 773-80.

**Kelly S.L., Kelly D.E., Jackson C.J., Warrilow A.G.S., Lamb D.C. (2005).** Cytochrome P450: Structure, Mechanism, and Biochemistry (3rd Ed.) Ortiz de Montellano, P. R. *Ed. Kluwer Academic / Plenum Publishers*, New York, 585-618.

**Keum Y.S., Li Q.X. (2004).** Fungal laccase-catalyzed degradation of hydroxy polychlorinated biphenyls. *Chemosphere.* 56: 23-30.

**Kiviranta H., Vartiainen T., Verta M., Tuomisto J.T., Tuomisto J.(2000).** High fish-specific dioxin concentrations in Finland. *Lancet.* 355:1883–1885.

**Knerr S., Schrenk D. (2006).** Carcinogenicity of "non-dioxinlike" polychlorinated biphenyls. Critical Reviews in Toxicology. 36: 663-694

**Kohlmeier S., Smits T.M.H., Ford R.M., Keel C., Harms H., Lukas Y.W. (2005).** Taking the fungal highway: mobilization of pollutant-degrading bacteria by fungi. *Environ. Sci. Technol.* 39: 4640–4646.

**Koller G., Moder M., Czihal K. (2000).** Peroxidative degradation of selected PCB: a mechanistic study. *Chemosphere*. 41: 1827-1834.

**Kordon K., Mikolasch A., Schauer F. (2010).** Oxidative dehalogenation of chlorinated hydroxybiphenyls by laccases of white-rot fungi. *Intern. Biodet. Biodegrad*. 64: 203-209.

**Kramer S., Moller Hikel S., Adams K., Hinds D., Moon K. (2012).** Current status of the epidemiologic evidence linking polychlorinated biphenyls and non-Hodgkin lymphoma and the role of immune dysregulation. *Environmental Health Perspectives*. Vol. 120, n. 8.

**Krčmar P., Kubátová A., Votruba J., Erbanová P., Novotny C., Sašek V. (1999).** Degradation of polychlorinated biphenyls by extracellular enzymes of *Phanerochaete chrysosporium* produced in a perforated plate bioreactor. *World J. Microbiol. Biotechnol*. 15: 269-276.

**Kuan I.C., Johnson K., Tien M. (1993).** Kinetic analysis of manganese peroxidase. J. Biol. Chem. 268: 20064-20070.

**Kubátová A., Erbanova P., Eichlerova I., Homolka L., Nerud F., Sasek V. (2001).** PCB congener selective biodegradation by the white-rot fungus *Pleurotus ostreatus* in contaminated soil. *Chemosphere*. 43: 207-215.

**Lappalainen J., Juvonen R., Vaajasaari K., Karp M. (1999).** A new flash method for measuring the toxicity of solid and colored samples. *Chemosphere*. 38: 1069-1083.

**Lee P.Y., Chen C.Y. (2009).** Toxicity and quantitative structure–activity relationships of benzoic acids to *Pseudokirchneriella subcapitata*. *J. Hazard. Mater.* 165: 156–161.

**Leigh M.B., Prouzova P., Mackova M., Macek T., Nagle D.P., Fletcher J.S. (2006).** Polychlorinated biphenyl (PCB)-degrading bacteria associated with trees in a PCB-contaminated site. *Appl. Environ. Microb.* 72: 2331–2342.

**Leonowicz A., Cho N. S., Luterek J., Wilkolazka A., Wojtas-Wasilewska M., Matuszewska A., Hofrichter M., Wesenberg D., Rogalski J. (2001).** Fungal laccase: properties and activity on lignin. *J. Basic Microbiol*. 41: 185-227.

**Li D., Alic M., Brown J. A., Gold M.H., (1995).** Regulation of manganese peroxidase genetranscription by hydrogen peroxide, chemical stress, and molecular oxygen. *Appl. Environ. Microbiol*. 61: 341-345.

**Liu J.Y., Hu D.F., Jiang G.B., Schnoor J.L. (2009).** In vivo biotransformation of 3,3 ',4,4 '-tetrachlorobiphenyl by whole plants-poplars and switchgrass. *Environ. Sci. Technol*. 43: 7503-7509.

**Liu J.Y., Schnoor J.L. (2008).** Uptake and translocation of lesser-chlorinated polychlorinated biphenyls (PCBs) in whole hybrid poplar plants after hydroponic exposure. *Chemosphere.* 73: 1608-1616.

**Lundell T.K., Makela M.R., Hilden K. (2010).** Lignin-modifying enzymes in filamentous basidiomycetes - ecological, functional and phylogenetic review. *J.Basic Microbiol*. 50: 5-20.

**Luo W., Hu C. (2013).** Interaction of plant secondary metabolites and organic carbon substrates affect on biodegradation of polychlorinated biphenyl. *J. Environ. Biol*. 34: 337-343.

**Macek T., Kotrba P., Svatos A., Novakova M., Demnerova K., Mackova M. (2008).** Novel roles for genetically modified plants in environmental protection. Trends Biotechnol. 26: 146-152.

**Mackova M., Dowling D., Macek T. (2006).** Phytoremediation and Rhizoremediation. Focus on Biotechnology, Vol. 9A. *Springer*.

**Mackova M., Prouzova P., Stursa P., Ryslava E., Uhlik O., Beranova K., Rezek J., Kurzawova V., Demnerova K., Macek T. (2009)**. Phyto/rhizoremediation studies using long-term PCB-contaminated soil. *Environ. Sci. Pollut. Res*. 16: 817–829.

**Macleod C.R.J., Semple K.T. (2000).** Influence of contact time on extractability and degradation of pyrene in soils. *Environ. Sci. Technol*. 34: 4952-4957.

**Mader B.T., Uwe-Goss K., Eisenreich S.J. (1997).** Sorption of nonionic, hydrophobic organic chemicals to mineral surfaces. *Environ. Sci. Technol.* 31: 1079-1086.

**Martinez A.T. (2002).** Molecular biology and structure-function of lignin-degrading heme peroxidases .*Enz. Microbial Technol*. 30: 425–444.

**Master E.R., Lai V.W., Kuipers B., Cullen W.R., Mohn W.W. (2002).** Sequential anaerobic-aerobic treatment of soil contaminated with weathered Aroclor 1260. *Environ. Sci. Technol.* 36: 100-103.

**Master E.R., Mohn W.W.(2001).** Induction of *bph*A, encoding biphenyl dioxygenase, in two polychlorinated biphenyl-degrading bacteria, psychrotolerant *Pseudomonas* strain Cam-1 and mesophilic *Burkholderia* strain LB400. *Appl. Environ. Microb*. 67: 2669–2676.

**Masuda Y. (2003).** Health effect of polychlorinated biphenyls and related compounds. *Intern. J. Health Sci.* 49: 333-336.

**Matsumura E., Yamamoto E., Numata A., Kawano T., Shin T., Murao S. (1986).** Structure of the laccase-catalyzed oxidation products of hydroxy-benzoic acids in the presence of ABTS (2,2′-azino-di-(3-ethylbenzothiazoline-6-sulfatic acid)). *Agric. Biol. Chem*. 50: 1355–1357.

**Mayes B.A., McConnel E.E., Neal B.H., Brunner M.J., Hamilton S.B., Sullivan T.M. (1998).** Comparative carcinogenicity in Sprague-Dawley rats of the polychlorinated biphenyl mixtures Aroclors 1016, 1242, 1254 and 1260. *Toxicol. Sci*. 41: 62-76.

**Meggo R.E. (2013).** Cleaning polychlorinated biphenyl (PCB) contaminated garden soil by phytoremediation. *Environ. Sci.* 1: 33 – 52.

**Megharaj M., Ramakrishnan B., Venkateswarlu K., Sethunathan N., Naidu R. (2011).** Bioremediation approaches for organic pollutants: A critical perspective. *Environment International.* 37: 1362–1375.

**Mester T., Field J.A. (1998)**. Characterization of a novel manganese peroxidase-lignin peroxidase hybrid isozyme produced by *Bjerkandera* sp. strain BOS55 in the absence of manganese. *J. Biol. Chem*. 273: 15412-15417.

**Mester T., Pena M., Field J.A., (1996).** Nutrient regulation of extracellular peroxidases in the metal ions. *Microbiology*. 148: 2159-2169.

**Michels J. (1998).** Processes for the bioremediation of soil. Editor: Federal Environmental Agency BMBF Project Management Agency for Waste Management and Remediation of Hazardous Abandoned Sites (PT AWAS). Section III 3.6.

**Mirsal I.A. (2008).**Soil pollution - origin, monitoring and remediation. Springer-Verlag, Berlin (Germany).

**Mohammadi M., Chalavi V., Novakova-Sura M., Laliberte´ J.F., Sylvestre M. (2007).** Expression of bacterial biphenyl-chlorobiphenyl dioxygenase genes in tobacco plants. *Biotechnol. Bioeng*. 97: 496-505.

**Mouhamadou B., Faure M., Sage L., Marcais J., Souard F., Geremia R. (2013).** Potential of autochthonous fungal strains isolated from contaminated soils for degradation of polychlorinated biphenyls. *Fungal Biol*. 117: 268–274.

**Muccini M., Layton A.C., Sayler G.S., Schultz T.W. (1999).** Aquatic toxicities of halogenated benzoic acids to *Tetrahymena pyriformis*. *B. Environ. Contam. Toxicol*. 62: 616–622.

**Mulligan C.N., Yong R.N., Gibbs B.F. (2001).** Surfactant-enhanced remediation of contaminated soils: a review. *Eng. Geology*. 60: 371-380.

**Muscat J.E., Britton J.A., Djordjevic M.V., Citron M.L., Kemeny M., Busch-Devereaux E., Pittman B., Stellman S.D. (2003).** Adipose concentrations of organochlorine compounds and breast cancer recurrence in Long Island, New York. *Cancer Epidemiology*. 12: 1474-1478.

**Muzikar M., Kresinova Z., Svobodova K., Filipova A., Cvancarova M., Cajthamlova K., Cajthaml T. (2011).** Biodegradation of chlorobenzoic acids by ligninolytic fungi. *J. Hazard. Mater.* 196: 386-394.

**Nazareth S.W., Sampy, J.D. (2003).** Production and characterisation of lignocellulases of Panus tigrinus and their application. *Int. Biodeter. Biodegrad.* 52: 207-214.

**Nebert D.W., Russell D.W. (2002).** One of only a handful of CYP reviews that emphasizes the clinical relevance of CYP enzymes, instead of CYP metabolism of drugs or environmental procarcinogens. Clinical importance of the cytochromes P450. *Lancet.* 360: 1155–1162.

**NEPI-National Environmental Policy Institute (2000).** Assessing the bioavailability of organic compounds in soil for use in human health risk assessments. Bioavailability Project Policy, Organics Task Force. NEPI, Washington DC.

**Nielsen A.T., Tolker-Nielsen T., Barken K.B., Molin S. (2000).** Role of commensal relationships on the spatial structure of a surface-attached microbial consortium. *Environ. Micriobiol.* 2: 59-68.

**Nieuwenhuijsen M.J., Dadvand P., Grellier J, Martinez D., Vrijheid M. (2013).** Environmental risk factors of pregnancy outcomes: a summary of recent meta-analyses of epidemiological studies. *Environ. Health*. 12: 6.

**Ning D., Wang H. (2012).** Involvement of cytochrome P450 in pentachlorophenol transformation in a white rot fungus *Phanerochaete chrysosporium. PLoS ONE 7(9): e45887.*

**Ning D., Wang H., Zhuang H. (2010).** Induction of functional cytochrome P450 and its involvement in degradation of benzoic acid by *Phanerochaete chrysosporium*. *Biodegrad*. 21: 297-308.

**Norback D.H.,Weltman R.H. (1985).** Polychlorinated biphenyl induction of hepatocellular carcinoma in the Sprague-Dawley rat. *Environ. Health Perspect*. 60: 97–105.

**Novotny C., Vyas B.R.M., Erbanová P., Kubátová A., Sasek V., (1997).** Removal of PCBs by various white-rot fungi in liquid cultures. *Folia Microbiol.* 42: 136-140.

**NTP, National Toxicology Program, Department of Health and Human Services (2011).** Polychlorinated biphenyls [CAS No. 1336-36-3]. *Report on carcinogens*. 12th edition. 349-353.

**Ohtsubo Y., Kudo T., Tsuda M., Nagata Y., (2004).** Strategies for bioremediation of polychlorinated biphenyls. *Appl. Microbiol. Biotechnol*. 65: 250-258.

**Omura T. (1999).** Forty years of cytochrome. *Biochem. Biophys. Res. Commun*. 266: 690-698.

**Omura T., Sato R. (1964).** The carbon monoxide–binding pigment of liver microsomes. *J. Biol. Chem.* 239: 2370–2378.

**Palmieri G., Giardina P., Bianco C., Fontanella B., Sannia G. (2000).** Copper induction of laccase isoenzymes in the ligninolytic fungus *Pleurotus ostreatus*. *Appl. Environ. Microbiol.* 66: 920-924.

**Pavuk M., Cerhan J.R., Lynch C.F., Schecter A., Petrik J., Chovancova J., Kocan A. (2004).** Environmental exposure to PCBs and cancer incidence in eastern Slovakia. Chemosphere. 54:1509–1520.

**Petric I., Hršak D., Fingler S., Vončina E.,Četkovič H., Begonja Kolar A., and Udikovic Kolic N. (2007).** Enrichment and Characterization of PCB-Degraders. *Food Technol. Biotechnol*. 45: 11–20.

**Pieper D.H. (2005).** Aerobic degradation of polychlorinated biphenyls. *Appl. Microbiol. Biotechnol.* 67: 170-191.

**Pilon-Smits E. (2005).** Phytoremediation. *Annu. Rev. Plant Biol*. 56: 15–39.

**Podgornik H., Podgornik A., Milavec P., Perdih A. (2001).** The effect of agitation and nitrogen concentration on lignin peroxidase (LiP): isoform composition during fermentation of *Phanerochaete chrysosporium*. *J. Biotechn.* 88: 173-176.

**Pointing S., (2001).** Feasibility of bioremediation by white rot fungi. *Appl. Microbiol. Biotechnol*. 57: 20-33.

**Pozdnyakova N.N., Nikiforova S.V.,Turkovskaya O.V. (2010).** Influence of PAHs on ligninolytic enzymes of the fungus *Pleurotus ostreatus* D1. *Cent. Europ. J. Biol*. 5:83-94.

**Pozdnyakova N.N., Rodakiewicz–Nowak J., Turkovskaya O.V., Haber J., (2006).** Oxidative degradation of polyaromatic hydrocarbons catalyzed by blue laccase from *Pleurotus ostreatus* D1 in the presence of synthetic mediators. *Enzyme Microb. Tech*. 39: 1242–1249.

**Pu X.. Lee L.S., Galinsky R.E., Carlson G.P. (2006).** Bioavailability of 2,3′,4,4′,5-pentachlorobiphenyl (PCB118) and 2,2′,5,5′-tetrachlorobiphenyl (PCB52) from soils using a rat model and a physiologically based extraction test. *Toxicology*. 217: 14–21.

**Quaratino D. ,Federici F., Petruccioli M., Fenice M., D'Annibale A. (2007).** Production, purification and partial characterisation of a novel laccase from the white-rot fungus *Panus tigrinus* CBS 577.79. *Antonie van Leeuwenhoek*. 91: 57-69.

**Quensen J.F., Boyd S.A., Tiedje J.M. (1990).** Dechlorination of 4 commercial polychlorinated biphenyl mixtures (Aroclors) by anaerobic microorganisms from sediments. *Appl. Environ.Microbiol.* 56: 2360-2369.

**Rabinovich M.L., Bolobova A.V., Vasilchenko L.G. (2004).** Decomposition of natural aromatic structures and xenobiotics by fungi. *Appl. Biochem. Microbiol*. 40: 5-23.

**Reeder J., Knight R. (2010).** Rapid denoising of pyrosequencing amplicon data: exploiting the rank- abundance distribution. *Nat. Methods*. 7(9): 668-669.

**Reid B.J., Jones K.C., Semple K.T. (2000).** Bioavailability of persistent organic pollutants in soils and sediment-a perspective on mechanisms, consequences and assessment. *Environ. Pollut.* 108: 103-112.

**Rein A., Fernqvist M.M., Mayer P., Trapp S., Bittens M., Karlson U.G. (2007).** Degradation of PCB congeners by bacterial strains. *Appl. Microbiol. Biotechnol*. 77: 469-481.

**Robertson L.W., Hanse L.G. (2001).** PCBs: Recent Advances in Environmental Toxicology and Health Effects. *The University Press of Kentucky*. ISBN 0-8131-2226-0. Pag.429.

**Robles-González I.V., Fava F., Poggi-Varaldo H.M (2008).** A review on slurry bioreactors for bioremediation of soils and sediments. *Microbial Cell Factories*, 7:5.

**Rodrigues J.L.M., Kachel C.A., Aiello M.R., Quensen J.F., Maltseva O.V., Tsoi T.V., Tiedje J.M. (2006).** Degradation of Aroclor 1242 dechlorination products in sediments by Burkholderia xenovorans LB400(ohb) and Rhodococcus sp. strain RHA1(fcb). *Appl. Environ. Microbiol*. 72: 2476-2482.

**Rogan W.J., Gladen B.C., Hung K.L., Kloong S.L., Shih L.Y., Taylor J.S. (1988).** Congenital poisoning by polychlorinated biphenyls and their contaminants. *Taiwan Science*. 241: 334-336.

**Ross G. (2004).** The public health implications of polychlorinated biphenyls (PCBs) in the environment. *Ecotoxicol. Environ. Saf*. 59: 275-291.

**Ruiz-Aguilar G.M.L., Fernandez-Sanchez J.M., Rodrıguez-Vazquez R., Poggi-Varaldo H. (2002).** Degradation by white-rot fungi of high concentrations of PCB extracted from a contaminated soil. *Adv. Environ. Res.* 6: 559-568.

**Ruiz-Dueñas F.J., Camarero S., Pérez-Boada M., Martínez M., Martínez A.T. (2001).** A new versatile peroxidise from *Pleurotus*. *Biochem. Soc. Trans*. 29: 116-122.

**Ruiz- Dueñas F.J., Morales M., Perez-Boada M., Choinowski T., Jesus Martınez M., Piontek K., Martınez A.T. (2007).** Manganese oxidation site in *Pleurotus eryngii* versatile peroxidase: a site-directed mutagenesis, kinetic, and crystallographic study. *Biochemistry*. 46: 66-77.

**Sagova-Mareckova1 M., Cermak L., Novotna J., Plhackova K., Forstova J., Kopecky J. (2008).** Innovative methods for soil DNA purification tested in soils with widely differing characteristics. *App. Environ. Microb*. 74: 2902-2907.

**Sanchez M.A., Campbell L.M., Brinker F.A., Owens D. (2000).** Attenuation the natural way. A former wood-preserving site offers a case study for evaluating the potential of monitored natural attenuation. *Industrial Wastewater.* 5: 37-42.

**Saparrat M.C.N., Guillen F., Arambarri A.M., Martinez A.T., Martinez M.J. (2002).** Induction, isolation, and characterization of two laccases from the white rot basidiomycete *Coriolopsis rigida*. *Appl. Environ. Microbiol*. 68: 1534-1540.

**Šašek V., Glaser J.A., Baveye P. (2003).** The utilization of bioremediation to reduce soil contamination:problems and solution. *Kluwer Academic Publisher*, Amsterdam, The Netherlands.

**Scheel T., Hofer M., Ludwig S., Holker U. (2000).** Differential expression of manganese peroxidase and laccase in white-rot fungi in the presence of manganese or aromatic compounds. *Appl. Environ. Microbiol*. 54: 686-691.

**Schlosser D., Höfer C. (2002).** Laccase-catalyzed oxidation of $Mn^{2+}$ in the presence of natural Mn3+ chelators as a novel source of extracellular $H_2O_2$ production and its impact on manganeseperoxidase. *Appl. Environ. Microbiol*. 68: 3514–3521.

**Schröder P., Collins D.C. (2010).** Organic xenobiotics and plants: from mode of action to ecophysiology. Agriculture. *Springer*.

**Schwarzenbach R.P., Gschwend P.M., Imboden D.M. (2003).** Environmental Organic Chemistry. Wiley-Interscience, Hoboken, NJ, USA.

**Scullion J. (2006).** Remediating polluted soils. *Naturwissenschaften*. 93: 51-65.

**Semple K.T., Morriss A.W.J., Paton G.I. (2003).** Bioavailability of hydrophobic organic contaminants in soils: fundamental concepts and techniques for analysis. *European Journal of Soil Science*. 54: 809-818.

**Shultz A., Jonas U., Hammer E., Schauer F. (2001).** Dehalogenation of chlorinated hydroxybiphenyls by fungal laccase. *Appl. Microbiol. Biotechnol.* 67: 4377-4381.

**Silverstone A.E., Rosenbaum P.F., Weinstock R.S., Bartell S.M., Foushee H.R., Shelton C., Pavuk M. (2012).** Polychlorinated biphenyl (PCB) exposure and diabetes: results from the Anniston community Health Survey. *Environmental Health Perspectives*. Vol. 120, n. 5.

**Singer A.C., Smith D., Jury W.A., Hathuc K., Crowley D.E. (2003).** Impact of the plant rhizosphere and augmentation on remediation of polychlorinated biphenyl contaminated soil. *Environ. Toxicol. Chem.* 22: 1998-2004.

**Singh A, Ward O.P. (2004).** Applied bioremediation and phytoremediation (Soil Biology). Published by Springer.

**Singh A., Van Hamme J.D., Ward O.P. (2007)**. Surfactants in microbiology and biotechnology: Part 2. Application aspects. *Biotechnol. Adv.* 25: 99-121.

**Singh H. (2006).** Mycoremediation:fungal bioremediation. *Wiley-Interscience*.

**Singh O.V, Labana S., Pandey G., Budhiraja R., Jain R.K. (2003).** Phytoremediation: an overview of metallic ion decontamination from soil. *Appl. Microbiol. Biotechnol*. 61: 405-421.

**Slater H., Gouin T., Leigh M.B. (2011).** Assessing the potential for rhizoremediation of PCB contaminated soils in northern regions using native tree species. Chemosphere. 84: 199–206.

**Snajdr J., Valskova V., Merhautova V., Herinkova J., Cajthaml T., Baldrian P. (2008).** Spatial variability of enzyme activities and microbial biomass in the upper layers of *Quercus petraea* forest soil. *Soil Biol. Biochem*. 40: 2068–2075.

**Soden D.M., Dobson A.D.W. (2001).** Differential regulation of laccase gene expression in *Pleurotus sajor-caju. Microbiology* .147: 1755–1763.

**Steffen K.T., Hatakka A., Hofrichter M. (2002).** Degradation of humic acids by the litter-decomposing basidiomycete *Collybia dryophila*. *Appl. Environ. Microbiol*. 68: 3442–3448.

**Stiborová M., Suchá V., Miksanová M., Páca J. Páca J. Jr, (2003).** Hydroxylation of phenol to catechol by *Candida tropicalis*: involvement of cytochrome P450. *Gen. Physiol. Biophys*. 22: 167-79.

**Stockholm Convention on Persistent organic pollutants (2001).** The POPs handbook for the Stockholm Convention on Persistent Organic Pollutants. Written and produced by the participating organisations of the International POPs Elimination Network (IPEN).

**Svobodová K., Placková M., Novotná V., Cajthaml T. (2009).** Estrogenic, androgenic activity of PCBs, their chlorinated metabolites and other endocrine disruptors estimated with two in vitro yeast assays. *Sci. Total. Environ*. 407: 5921–5925.

**Syed K., Doddapaneni H., Subramanian V., Lam Y.W., Yadav J.S. (2010).** Genome-to-function characterization of novel fungal P450 monooxygenases oxidizing polycyclic aromatic hydrocarbons (PAHs). *Biochem. Biophys. Res. Commun*. 399: 492-497.

**Syed K., Porollo A., Lam Y.W., Yadav J.S. (2011).** A Fungal P450 (CYP5136A3) capable of oxidizing Polycyclic Aromatic Hydrocarbons and Endocrine Disrupting Alkylphenols: role of Trp129 and Leu324. PLoS ONE 6(12): e28286. doi:10.1371/journal.pone.0028286.

**Sylvestre M., Macek T., Mackova M. (2009).** Transgenic plants to improve rhizoremediation of polychlorinated biphenyls (PCBs). *Curr. Opin. Biotechnol*. 20: 242–247.

**Takagi S., Shirota C., Sakaguchi K., Suzuki J., Sue T., Nagasaka H., Hisamatsu S., Sonoki S. (2007).** Exoenzymes of *Trametes versicolor* can metabolize coplanar PCB congeners and hydroxy PCB. *Chemosphere*. 67: 54–57.

**Taniyasu S., Kannan K., Holoubek I., Ansorgova A., Horii Y., Hanari N., Yamashita N., Aldous K.M. (2003).** Isomer-specific analysis of chlorinated biphenyls, naphthalenes and dibenzofurans in Delor: polychlorinated biphenyl preparations from the former Czechoslovakia. *Environ. Pollut*. 126 :169-78.

**Teramoto H., Tanaka H., Wariishi H. (2004).** Fungal cytochrome P450s catalyzing hydroxylation of substituted toluenes to form their hydroxymethyl derivatives. *FEMS Microbiology Letters*. 234: 255–260.

**Thurston C.F. (1994).** The structure and function of fungal laccases. *Microbiology*, 140: 19-26.

**Tigini V., Prigione V., Di Toro S., Fava F., Varese G.C. (2009).** Isolation and characterisation of polychlorinated biphenyl (PCB) degrading fungi from a historically contaminated soil. *Microbial Cell Factories*. 8:5 doi:10.1186/1475-2859-8-5.

**Tortella G.R., Diez M.C., Duran N. (2005).** Fungal diversity and use in decomposition of environmental pollutants. *Crit. Rev. Microbiol*. 31: 197-212.

**Totten L.A., Eisenreich S.J., Brunciak P.A. (2002).** Evidence for destruction of PCBs by the OH radical in urban atmospheres. *Chemosphere*. 47: 735-746.

**Totten L.A., Gigliotti C.L., Offenberg J.H., Baker J.E., Eisenreich S.J. (2003).** Reevaluation of air-water exchange fluxes of PCBs in Green Bay and southern Lake Michigan. *Environ Sci Technol.* 37: 1739-43.

**Toussaint J.P., Pham T.T., Barriault D., Sylvestre M. (2012).** Plant exudates promote PCB degradation by a rhodococcal rhizobacteria. *Appl. Microbiol. Biotechnol*. 95: 1589-1603.

**Tuomela M., Steffen K.T., Kerko E., Hartikainen H., Hofrichter M., Hatakka A. (2005).** Influence of Pb contamination in boreal forest soil on the growth and ligninolytic activity of litter-decomposing fungi. *FEMS Microbiology Ecology*. 53: 179–186.

**Tuor U., Winterhalter K., Fiechter A. (1995).** Enzymes of white-rot fungi involved in lignin degradation and ecological determinants for wood decay. *J. Biotech*. 41: 1-17.

**Turyk M., Anderson H.A., Hanrahan L.P., Falk C., Steenport D.N., Needham L.L., Patterson D.G., Freels S., Persky V., Great Lakes Consortium. (2006).** Relationship of serum levels of individual PCB, dioxin, and furan congeners and DDE with Great Lakes sport-caught fish consumption. *Environ. Res.* 100: 173–183.

**Ubl S., Scheringer M., Stohl A., Burkhart J.F., Hungerbuhler K. (2012)**. Primary source regions of polychlorinated biphenyls (PCBs) measured in the Arctic. *Atmospheric Environment*. 62: 391-399.

**US Environmental Protection Agency (1983).** Environmental transport and transformation of polychlorinated biphenyls. EPA 560/5-83-025.

**US Environmental Protection Agency (1996).** PCBs: Cancer Dose-Response Assessment and Application to Environmental Mixtures. EPA/600/P–96/001F.

**US Environmental Protection Agency (1998).** Drinking water criteria document for polychlorinated biphenyls (PCBs). EPA Report N. ECAO-CIN414.

**US Environmental Protection Agency (2000).** Exposure and human health.reassessment of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and related compounds. Part I: Estimating exposure to dioxin-like compounds. Vol. 3EPA/600/P-00/001.

**US Environmental Protection Agency (2001).** Hudson River Sloop Clearwater's Public Comment on EPA's Hudson River PCBs Superfund Reassessment and Proposed Remediation Plan.

**US Environmental Protection Agency (2006).** *In situ* and *ex situ* biodegradation technologies for remediation of contaminated sites. EPA/625/R-06/015.

**US Environmental Protection Agency (2008).** Site characterization to support use of monitored natural attenuation for remediation of inorganic contaminants in ground water. EPA/600/R-08/114.

**US Environmental Protection Agency (2009).** Identification and characterization methods for reactive minerals responsible for natural attenuation of chlorinated organic compounds in ground water. EPA/600/R-09/115.

**US Environmental Protection Agency (2010).** Green Remediation Best Management Practices: Bioremediation. EPA 542-F-10-006.

**Van Aken B., Correa P.A., Schnoor J.L. (2010a).** Phytoremediation of polychlorinated biphenyls: new trends and promises. *Environ. Sci. Technol.* 44: 2767–2776.

**Van Aken B., Geiger S.C. (2010b).** Phytoremediation of Chlorinated Solvent Plumes. *In Situ* Remediation of Chlorinated Solvent Plumes SERDP/ESTCP Environmental Remediation Technology. p.631-675.

**Van den Berg M., Birnbaum L.S., Denison M., De Vito M., Farland W., Feeley M. (2006).** The 2005 World Health Organization reevaluation of human and mammalian toxic equivalency factors for dioxins and dioxin-like compounds. *Toxicol Sci*. 93: 223-241.

**van den Brink H.M., van Gorcom R.F., van den Hondel C.A., Punt P.J. (1998).** Cytochrome P450 enzyme systems in fungi. *Fungal Genet. Biol.* 23: 1–17.

**van Noort P.C.M. (2009).** Estimation of amorphous organic carbon/water partition coefficients, subcooled liquid aqueous solubilities, and n-octanol/water partition coefficients of nonpolar chlorinated aromatic compounds from chlorine fragment constants. *Chemosphere* 74: 1024–1030.

**Vasilyeva G.K., Strijakova E.R. (2007).** Bioremediation of soils and sediments contaminated by polychlorinated biphenyls. *Microbiol*. 74: 725–741.

**Vasilyeva G.K., Strijakova E.R., Nikolaeva S.N., Lebedev A.T., Shea P.J. (2010).** Dynamics of PCB removal and detoxification in historically contaminated soils amended with activated carbon. *Environmental Pollution*. 158: 770–777.

**Viisimaa M., Karpenko O., Novikov V., Trapido M., Goi A. (2013).** Influence of biosurfactant on combined chemical–biological treatment of PCB-contaminated soil. *Chem. Eng. J.* 220: 352–359.

**Vyas B.R.M., Sasek V., Matucha M., Bubner M., (1994).** Degradation of 3,3′,4,4′-tetrachlorobiphenyl by selected white rot fungi. *Chemosphere*. 28: 1127-1134.

**Walker C.H., Hopkin S.P., Sibly R.M., Peakall D.B. (2006).** Principles of ecotoxicology. Fl, USA: CRC Press, Taylor & Francis Group.

**Wallnofer P.R., Engelhardt G., Safe S., Hutzinger O. (1973).** Microbial hydroxylation of 4-chlorobiphenyl and 4,4´-dichlorobiphenyl. *Chemosphere.* 2:69–72.

**Wang L.Q., James M.O. (2007).** Sulfonation of 17ß-estradiol and inhibition of sulfotransferase aktivity by polychlorobiphenyols and celecoxib in channel catfish, *Ictalurus punctatus*. *Aquat. Toxicol*. 81: 286-292.

**Wang L.Q., Lehmler H.J., Robertson L.W., James M.O. (2006).** Polychlorobiphenyols are selective inhibitors of human phenol sulfotransferase 1A1 with 4-nitro-phenol as a substrate. *Chemico-Biolog. Interac.* 159: 235-246.

**Wariishi H., Gold M.H. (1989).** Lignin peroxidase Compound III: formation, inactivation and conversion to the native enzyme. *Febs Letters*. 243: 165-168.

**Wariishi H., Valli K., Gold M.H. (1992).** Manganese(II) oxidation by manganese peroxidase from the basidiomycete Phanerochaete chrysosporium. Kinetic mechanism and role of chelators. *J. Biol. Chem*. 267: 23688-23695.

**Wasilkowski D., Swedziol Z., Mrozik A. (2012).** The applicability of genetically modified microorganisms in bioremediation of contaminated environments. *Chemik*. 66: 817-826

**Webster L., Roose P., Bersuder P., Kotterman M., Haarich M., Vorkamp K. (2013).** Determination of polychlorinated biphenyls (PCBs) in sediment and biota. ICES, International Council for the exploration of the sea. Techniques in marine environmental sciences. N. 53.

**Wesenberg D., Kyriakides I., Agathosv S.N. (2003).** White-rot fungi and their enzymes for the treatment of industrial dye effluents. Biotechnol. Adv. 22: 161–187.

**WHO-IARC, World Health Organization- International Agency for Research on Cancer (1987).** Working group on the evaluation of carcinogenic risks to humans. Polychlorinated biphenyls. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, vol. 18. Subsequent evaluation: Supplement 7. Last updated: February 11, 1998.

**WHO-IARC, World Health Organization- International Agency for Research on Cancer (1998).** "Volume 18: Polychlorinated biphenyls and Polybrominated biphenyls." http://monographs.iarc.fr/ENG/Monographs/vol18/volume18.pdf.

**Wiegel J., Wu Q. (2000).** Microbial reductive dehalogenation of polychlorinated biphenyls. MiniReview. *FEMS Microbiology Ecology*. 32: 1-15.

**Wittich, R.M., Wolff P.(2007).** Growth of the genetically engineered strain Cupriavidus nector RW112 with chlorobenzoate and technical chlorobiphenyls. *Microbiol*. 153: 186–195

**Yadav J.S., Quensen J.F. III, Tiedje J.M., Reddy C.A., (1995).** Degradation of polychlorinated biphenyl mixtures (aroclors 1242, 1254, and 1260) by the white rot fungus *Phanerochaete chrysosporium* as evidenced by congener-specific analysis. *Appl. Environ. Microbiol*. 61: 2560- 2565.

**Yin Y., Guo J., Zheng L., Tian L., Wang X. (2011).** Capability of polychlorinated biphenyl (PCBs) degrading fungi segregated from sediments. *World J. Microbiol. Biotechnol*. 27: 2567-2574.

**Yoon J.M., Van Aken B., Schnoor J.L. (2006).** Leaching of contaminated leaves following uptake and phytoremediation of RDX, HMX, and TNT by poplar. *Int. J. Phytoremediat*. 8: 81–94.

**Yu M.L., Guo Y.L., Hsu C.C., Rogan W.J. (2000).** Menstruation and reproduction in women with polychlorinated biphenyl (PCB) poisoning: long-term follow-up interviews of the women from the Taiwan Yucheng cohort. *International Journal of Epidemiology*. 29: 672-677.

**Zani C., Toninelli G., Filisetti B., Donato F. (2013).** Polychlorinated biphenyls and cancer: an epidemiological assessment. *J. Environ. Sci. Health*. 31: 99-144.

**Zaranko D.T., Griffiths R.W., Kaushik N.K. (1997)**. Biomagnification of polychlorinated biphenyls through a riverine food web. *Environ. Toxicol. Chem*. 16: 1463–1471.

**Zeddel A., Majcherczyk A., Hüttermann A. (1993).** Degradation of polychlorinated biphenyls by white-rot fungi *Pleurotus ostreatus* and *Trametes versicolor* in a solid state systém. *Toxicol. Environ. Chem*. 40: 255-266.

**Zhao Y., Yi X. (2010).** Effects of soil oxygen conditions and soil pH on remediation of DDT-contaminated soil by laccase from white rot fungi *J. Environ. Res. Public Health*. 7: 1612-1621.

**Zhu X. D., Gibbons J., Garcia-Rivera J., Casadevall A., Williamson P. R. (2001).** Laccase of *Cryptococcus neoformans* is a cell wall-associated virulence factor. *Infect. Immun*. 69: 5589-5596.

# CHAPTER 6

# PUBLICATIONS, CONFERENCES, SEMINARS and COURSES

***Publications***

**Stella T**., Covino S., Křesinová Z., D'Annibale A., Petruccioli M., Cvančarová M., Cajthaml T. **(2013)**. Chlorobenzoic acid degradation by *Lentinus* (*Panus*) *tigrinus*: In vivo and in vitro mechanistic study-evidence for P-450 involvement in the transformation. *J. Hazard. Mater*. 260: 975-983.

Křesinová Z., Hostačná L., Medkova J., Cvančarová M., **Stella T**., Cajthaml T. **(2014).** Sensitive GC/MS determination of 15 isomers of chlorobenzoic acids in accelerated solvent extracts of soil hystorically contaminated with PCBs and validation of the entire method. *Int. J. Envir. Analyt. Chem.* In press.

***International conferences*:**

**"FEMS 2013- 5th Congress of European Microbiologists".** Leipzig (Germany), July 21-25, 2013.

- ✓ **T. Stella**, S. Covino, M. Cvančarová, A. D'Annibale, M. Petruccioli, T. Cajthaml. "Mycoremediation of polychlorinated biphenyls aged-contaminated soil from a dumpsite in southern Czech Republic". Poster presentation.

- ✓ S. Covino, **T. Stella**, B. Slavik, T. Cajthaml. "Biodegradation of PAHs and PCBs during different composting phases using poultry litter as substrate". Poster presentation.

**"Environmental Microbiology and Biotechnology in the frame of the Knowledge-Based Bio and Green Economy".** Bologna (Italy), April 10-12, 2012.

- ✓ **T. Stella**, S. Covino, Z. Křesinová, A. D'Annibale, M. Petruccioli, T. Cajthaml. "Mycoremediation of PCBs dead-end metabolites: *in vivo* and *in vitro* degradation of chlorobenzoic acids by the white rot fungus *Lentinus tigrinus"*. In "Environmental Engineering and Management Journal", vol. 11, n.3 supplement. ISSN 1843-3707- S43/P9. Poster presentation.

- ✓ T. Cajthaml, M. Čvančarová, Z.Křesinová, A.Filipová, **T. Stella**, S. Covino. "New insight into fungal degradation of polychlorinated biphenyls". In "Environmental Engineering and Management Journal", vol. 11, n.3 supplement. ISSN 1843-3707- S43/P8. Oral presentation.

- ✓ S. Covino, **T. Stella**, Z. Křesinová, A. D'Annibale, M. Petruccioli, T. Cajthaml. "Degradation of endocrine disrupting chemicals and removal of estrogenic activity by *Lentinus tigrinus* and its extracellular enzymes". In "Environmental Engineering and Management Journal", vol. 11, n.3 supplement. ISSN 1843-3707- S44/P11. Poster presentation.

- ✓ M. Petruccioli, **T. Stella**, E. Carota, A. D'Annibale. "Production of oxidative enzymes by *Trametes ochracea* on the high-molecular weight fraction of olive-mill wastewater". In "Environmental Engineering and Management Journal", vol. 11, n.3 supplement. ISSN 1843-3707- S86/P100. Poster presentation.

**"5th International Symposium on Biosorption and Bioremediation".** Prague (Czech Republic), June 24-28, 2012.

- ✓ **T. Stella**, S. Covino, Z. Křesinová, A. D'Annibale, M. Petruccioli, T. Cajthaml . "A breakthrough in understanding the fungal degradation of chlorobenzoic acids". Poster presentation (poster session "Biodegradation and toxicity of POPs").

**"5th European Bioremediation Conference"**. Chania (Greece), July 4-7, 2011.

- ✓ **T. Stella**, M. Petruccioli, F. Federici, A. D'Annibale. "Detoxification and valorization of the high-molecular weight fraction of olive-mill wastewater via its use as a substrate for fungal fermentation". Oral presentation.

***Seminars***

- ✓ **T. Stella** "Metagenomic investigation of bacterial and fungal communities dynamics during the mycoremediation of PCB contaminated soils" Biotechnology and Biomedicine center (Vestec). February 2014.
- ✓ **T. Stella** "Biodegradation of organic pollutants and bioremediation technologies". Institute of Environmental Studies, Faculty of Science, Charles University (Prague). November 2013.
- ✓ **T. Stella** "Fungal laccases: purification, characterization and applications" Institute of Microbiology, Academy of Sciences of Czech Republic (Prague). September 2013.

***Courses***

- ✓ **"Contaminated site remediation: application of advanced tools to control biological processes"** organized by the Water Research Institute (IRSA-CNR) in collaboration with EU funded project MINOTAURUS and Setac Italian Branch. Rome, 27-29 May 2013.
- ✓ **"Bioremediation of contaminated areas: methodologies, microbial role and investigation techniques"** organized by the Water Research Institute (IRSA-CNR) in collaboration with Setac Italian Branch and Society of Environmental Toxicology and Chemistry. Rome, 30 March -1 April 2011.