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M. MINNA LAINE

# Bioremediation of chlorophenol-contaminated sawmill soil

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## List of original publications

This thesis is based on the following publications, which are referred to by their Roman numerals in the text. In addition, some previously unpublished results are included in the thesis.

I. Laine, M., Jokela, J. and M. Salkinoja-Salonen, 1995. Biomobility of organic halogen compounds from contaminated soil - earthworms as a tool. In: M. Munawar and M. Luotola (eds.) *The Contaminants in the Nordic Ecosystem: Dynamics, Processes & Fate.* Ecovision World monograph Series, 1995, SPB Academic Publishing, Amsterdam, Netherlands.

II. Laine, M. M., and Jørgensen, K. S. 1996. Straw compost and bioremediated soil as inocula for the bioremediation of chlorophenol contaminated soil. Appl. Environ. Microbiol. 62(5), 1507-1513.

III. Laine, M. M., and Jørgensen, K. S. 1997. Effective and safe composting of chlorophenol-contaminated soil in pilot scale. Environmental Science and Technology 30(2), 371-378.

IV. Laine, M. M., Ahtiainen, J., Wågman, N., Öberg, L., and Jørgensen, K. S. 1997. Fate and toxicity of chlorophenols, polychlorinated dibenzo-*p*-dioxins and dibenzofurans during composting of contaminated sawmill soil Environmental Science and Technology 31(11), 3244-3250.

V. Laine, M. M., Haario, H. and Jørgensen, K. S. 1997. Microbial functional activity during composting of chlorophenol-contaminated sawmill soil. J. Microbiol. Methods. 30, 21-32.

VI. Laine, M. M., Jørgensen, K. S., Kiviranta, H., Vartiainen, T., Jokela, J.K., Adibi, A. and Salkinoja-Salonen, M. 199X. Bioaerosols and particle release during composting of chlorophenol-contaminated soil *(submitted manuscript).*

A copy of paper VI is available from the author, address: M. Minna Laine, Finnish Environment Institute, Laboratory, Hakuninmaantie 4-6 B, FIN-00430 Helsinki, Finland.

# Bioremediation of chlorophenol-contaminated sawmill soil

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*Laine M.M. 1998. Bioremediation of chlorophenol-contaminated sawmill soil. Monographs of the Boreal Environment Research No. 9, 1998.*

The present study focused on the biodegradability, mobility and transformation reactions of organic halogen compounds in contaminated sawmill soil during bioremediation by composting. The fate of chlorophenols and microbiology of bioremediation was studied in the laboratory, during pilot-scale composting in the field, as well as during full-scale implementation. Several research tools were used: 1) identification (*metabolic fingerprinting and whole-cell fatty acid composition*) and characterization (*biodegradation tests, community structure*) of the microorganisms that degrade chlorophenols, 2) analyses of the chemical nature and metabolism of organic chlorine compounds in soil (*GC/ECD, HPSEC, radiorespirometry*), 3) environmental conditions affecting bioremediation (*nutrients, temperature, oxygen, pH, humidity*), and 4) both environmental (*toxicity to bacteria, bioavailability to the exposed earthworms*) and occupational health effects (*amounts of polychlorinated dibenzo-p-dioxins and dibenzofurans (PCDD/Fs) and pathogenic microorganisms in soil, composts and airborne particles*) of bioremediation.

The use of straw compost and remediated soil as inocula for bioremediation of chlorophenol-contaminated soil were evaluated. According to laboratory experiments, it was possible to change minor dehalogenation in studied inoculants (consortia in straw compost and bioremediated soil) to very efficient mineralization of pentachlorophenol by three months of enrichment in percolators. During pilot-scale composting in the field, more than 90% of the chlorophenols were removed. The biodegradation of chlorophenols was efficient and fast and not depending on the inocula. Frequent mixing and control of the nutrient level enhanced the chlorophenol degradation activity of the indigenous microbes in the contaminated soil. In a parallel bench-scale experiment, an average of 60% mineralization of radiolabeled pentachlorophenol ([$^{14}$C]PCP) was obtained in four weeks in 1 kg-piles with or without inocula. This result suggested that a major part of the chlorophenols was completely mineralized. Based on the gained experience and good results from the pilot-scale composting, the full-scale implementation was started in July 1995. Although the initial chlorophenol concentration was high, effective degradation was observed (72 to 89% removal of chlorophenols) already after three months of composting. However, during the full-scale composting, the revival of chlorophenol-degrading microorganisms after the winter was not very succe sfull.

The toxicity assessed by luminescent bacteria tests decreased during the composting, and it followed the chlorophenol concentrations in the pilot-scale compost piles. Organic chlorine compounds appeared in high molecular weight fractions indicating that the wood preservative Ky-5 had bound to the soil organic matter during the long exposure time in the contaminated soil. No major polymerization occurred during the composting, but the polymerized fraction was not either degraded or remobilized. Large amounts of polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/Fs), originating from the wood preservative were found in the compost piles, but their concentrations did not significantly change during the bioremediation pro cess.

The bioavailability of organic halogen compounds from contaminated sawmill soils was studied using earthworms from kitchen compost (*Eisenia andrei*) as model organisms. The earthworms incubated in sandy soil accumulated twenty times higher concentrations of tetrahydrofuran extractable halogen compounds than the earthworms incubated in organic soil, although the soils had equal content of organic halogen compounds. This indicated that soil organic matter diminished the bioaccumulation potential of halogen compounds to the earthworms.

Changes in the microbial community during bioremediation were studied using the carbon source utilization patterns in suspensions of compost matrices. The results suggested that fast-growing microbes responsible for utilization of easily available substrates, measured by respiratory activity and substrate utilization patterns, originated mainly from the added bulking agents, straw compost and bark chips. The best indicator of the actual chlorophenol degradation efficiency was the number of microbes growing on plates with pentachlorophenol as the sole carbon source. The chlorophenol-degraders originating from contaminated soil seemed not directly to contribute to the subtrate utilization pattern, but probably had benefited from the enhanced general microbial activity in the composts by cometabolism or synergism.

The release of microbes and contaminants during the mixing of the compost windrows was followed. Air particle samples were collected during the mixing of the full-scale compost windrows using Andersen Non-viable and Viable ambient particle sizing samplers. The concentrations of polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/Fs) were determined in different particle sizes. The results showed that the congener distribution of PCDD/Fs was similar in collected air particle fractions to that found in the compost windrows, and the level of PCDD/Fs was significantly higher than the atmospheric background value. From the collected airborne microbes, 40 bacteria were isolated and identified. Most of the isolated bacteria were gram-positive and spore-forming. None of the identified airborne bacteria is known to demonstrate pathogenic potential. The overall level was low compared to the level found when composting m unicipal waste.

Keywords: polychlorinated phenols, composting, PCDD/Fs, biodegradation, field tests

## Abbreviations

| | |
|---|---|
| Ah-receptor | Aryl hydrocarbon receptor |
| ATCC | American Type Culture Collection |
| ATP | Adenosine 5'-triphosphate |
| AWCD | Average well color development |
| BI | Biolog Identification System (carbon source utilization pattern) |
| BUGM | Biolog Universal Growth Medium |
| CDD/Fs | Chlorinated dibenzo-*p*-dioxins and dibenzofurans |
| CFU | Colony forming unit |
| CMA | Cornmeal-agar |
| CP | Chlorophenol |
| DCP | Dichlorophenol |
| DSM | Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH |
| $EC_{50}$ | The $EC_{50}$ value obtained from the luminescence bacteria test on water extracts corresponds to the concentration of soil in the water extract that gives 50% inhibition in luminescence in comparison to deionized water |
| EOX | Extractable organic halogen |
| FA | MIDI system (whole cell fatty acid analysis) |
| GC | Gas chromatograph |
| GC/ECD | Gas chromatograph interfaced to an electron-capture detector |
| GN | Gram-negative |
| GP | Gram-positive |
| HAMBI | Microbial culture collection at Department of Applied Chemistry and Microbiology, University of Helsinki |
| HP | Hewlett Packard |
| HpCDD | Heptachlorinated dibenzo-*p*-dioxin |
| HpCDF | Heptachlorinated dibenzofuran |
| HPSEC | High performance size exclusion chromatography |
| HRGC-HRMS | High resolution gas chromatograph interfaced to a mass spectrometer |
| HxCDD | Hexachlorinated dibenzo-*p*-dioxin |
| HxCDF | Hexachlorinated dibenzofuran |
| IFO | Institute for Fermentation, Osaka (Japan) |
| I-TEQ | 2,3,7,8-TCDD toxic equivalent |
| $LC_{50}$ | Lethal concentration causing 50% mortality of the tested individuals under given conditions |
| MEA | Malt-extract-agar |
| MIDI | Microbial Identification System (MIDI Inc. Newark DEL) |
| MPN | Most probable number |
| MWD | Molecular weight distribution |
| OCDD | Octachlorinated dibenzo-*p*-dioxin |
| OCDF | Octachlorinated dibenzofuran |
| PAHs | Polynuclear aromatic hydrocarbons |
| PCA | Principal component analysis |
| PCBs | Polychlorinated biphenyls |
| PCDD | Polychlorinated dibenzo-*p*-dioxin |
| PeCDD | Pentachlorinated dibenzo-*p*-dioxin |
| PCDD/Fs | Polychlorinated dibenzo-*p*-dioxins and dibenzofurans |
| PCDF | Polychlorinated dibenzofuran |
| PeCDF | Pentachlorinated dibenzofuran |
| PCP | Pentachlorophenol |

| | |
|---|---|
| PCPPs | Polychlorinated phenoxyphenols |
| PDA | Potato-dextrose-agar |
| TBrP | Tribromophenol |
| TCDD | Tetrachlorinated dibenzo-*p*-dioxin |
| TCDF | Tetrachlorinated dibenzofuran |
| TCP | Trichlorophenol |
| TeCP | Tetrachlorophenol |
| TGY | Tryptone-glucose-yeast-extract medium |
| THF | Tetrahydrofuran |
| TOC | Total organic carbon |
| TSA | Trypticase-soy-agar |
| TSBA | Trypticase-soy-broth-agar |
| [U- $^{14}$C]PCP | Uniformly $^{14}$C-labeled pentachlorophenol |
| UPGMA | Unweighted Pair Group Method with arithmetic Averages is an average linkage cluster analysis method in which the similarity (or distance) at which two groups join is based on the average similarity over all pairs of Operational Taxonomic Units, OTUs, (or organisms) between each group. |

# 1 Introduction

## 1.1 Background

Bioremediation methods can be divided into three categories: *ex situ* treatment when contaminated soil is excavated and treated elsewhere, *on site* treatment when soil is excavated and treated at site, and *in situ* treatment when soil is treated without excavation. Choice of a method for bioremediation of soil depends on chemical characteristics of the pollutant (*e.g.* volatility, biodegradability), and properties of the contaminated soil. Different alternatives exist: composting; land farming or a little more sophisticated solid phase treatment (including control of leachates); *in situ* treatment by circulating either a gas flow (bioventing or biopumping) or water amended with nutrients, often connected to biofilters or bioreactors; and phytoremediation (either plants accumulate pollutants from soil, metabolize them or serve as attachment surface or symbiotic partner for degrading microorganisms). Other, non-biological methods for remediation of contaminated soil include incineration at a waste treatment plant, stabilization, supercritical fluid extraction, chemical oxidation (*e.g.* Fenton's reagent) and soil washing.

Nearly 800 former Finnish sawmill sites are contaminated with chlorophenols, polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/Fs), and phenoxyphenols which originate from the commercial chlorophenol mixture called Ky-5 (Valo, 1984) that was used to impregnate wood until 1988. By the year 1997, 56 of these sites have been remediated, at least 36 of them by composting (Viitasaari and Mikkola, 1995).

Chlorophenolates are soluble in water and may leach from contaminated soil to groundwater. Therefore the contaminated sites must be cleaned up to prevent further spread. Bioremediation offers a feasible method for cleanup, but may be slow or leads to transformation rather than mineralization of the contaminants. The goal of the bioremediation process should always be the complete mineralization of chlorophenols. Degradation and bioremediation of chlorophenols has been extensively studied especially in Finland since the late 1980s (Apajalahti, 1987; Briglia, 1995; Kitunen, 1990; Melin, 1997; Uotila, 1993; Valo, 1990). Yet, rapid, more effective and safer purification methods with no harmful side reactions need to be developed.

## 1.2 Fate of chlorophenols during bioremediation

Chlorophenols are recalcitrant compounds that have been used for decades to impregnate wood, and many residues can be found in the environment (Kitunen et al., 1987) long after the use of the chlorophenols have been discontinued. While some bacteria may mineralize the chlorophenols completely, other microorganisms may O-methylate or polymerize the chlorophenols under certain conditions (Fig. 1). Hence, the chlorophenols in nature are prone to O-methylation into the corresponding chloroanisoles (Häggblom et al, 1988a, 1988b, 1989; Neilson et al., 1988; Suzuki, 1983), dimerization or polymerization (Banerji et al., 1993; Bollag, 1992; Bollag and Loll, 1983; Lamar and Dietrich, 1990; Warith et al., 1993; Öberg and Rappe, 1992) and other metabolic alterations (Boyd et al., 1989; Hague and Ebing, 1988). These biotransformation products may cause ecotoxicological risks and effects on the soil environment.

### *1.2.1 Polymerization/sorption to soil organic matter*

Chlorophenols are incorporated into soil to form so called bound residue, or nonextractable fraction, principally by two mechanisms: sorption or oxidative coupling (Boyd et al., 1989). Sorption can be physical sorption on surfaces of the solid material (adsorption) or partitioning into the solid material (absorption) (Alexander, 1994), whereas oxidative coupling involves covalent binding to form dimers, oligomers or polymerized fraction, and is usually mediated by enzymatic reactions in soil (Boyd et al., 1989).

Sorption of chemicals in soil appears to occur in two stages: an initially rapid stage followed by a slow stage (Loech and Webster, 1996). The slow sorption can be defined as aging: the aged or desorption-resistant fraction of the chemicals in soil may result from a slow

diffusion of these molecules within a solid component in the soil organic matter or small pores of soil aggregates (Alexander, 1994; Hatzinger and Alexander, 1995).

Covalent bonds in polymerization reactions are formed in chemicals that have functional groups similar to humic monomers (Bollag, 1992). It has been demonstrated that a large part of the pentachlorophenol (PCP) present in contaminated soil is covalently bound to the soil organic matter, preferentially to humic acid, by the action of white rot fungi (Rüttimann-Johnson and Lamar, 1996 and 1997). Oxidoreductive enzymes such as manganese peroxidase, lignin peroxidase and laccase are involved in copolymerization reactions of chlorophenols and ferulic acid, a precursor of humic acid (Dec and Bollag, 1994; Hatcher et al., 1993; Rüttimann-Johnson and Lamar, 1996). However, also inorganic chemicals such as birnessite (manganous manganite, Dec and Bollag, 1994) and clay minerals can catalyze oxidative reactions (Alexander, 1994; Verstraete and Devliegher, 1996).

The nature of soil organic matter influences the extent of sorption and desorption of chlorophenols in soil. Benoit et al. (1996) studied sorption of 4-chlorophenol (4-CP) and 2,4-dichlorophenol (2,4-DCP) to wheat straw, chestnut wood, Kraft lignin, composted straw and soil humic acid, and found that the sorption of 4-CP and 2,4-DCP on lignin and composted straw was larger than on humic acid, but desorption was smaller from humificated than fresh organic materials, indicating weaker interactions with fresh materials. The role of biotic and abiotic reactions was further studied (Benoit and Barriuso, 1997) during straw composting, and it was found that the formation of the bound residue or immobilization of chlorophenols in composted straw was mainly due to biological activity. Schäfer and Sandermann (1988) suggested that cell cultures of wheat metabolized PCP to tetrachlorocatechol which in turn was either conjugated or incorporated into the lignin cell wall of wheat. Peat has been shown to sorb PCP irreversibly from wastewater (Viraraghavan and Tanjore, 1994).

### *1.2.2 Remobilization of a polymeric halogenated fraction from soil; surface-active compounds and bioremediation*

Very little is known about the heterogeneous high-molecular-weight chlorinated fraction that is formed in soil during *e.g.* (co)polymerization, and it is not known if polymerization is a safe and an irreversible pathway for removal of contaminants. Organic contaminants incorporated into soil are considered to be resistant against microbial remobilization (Dec et al., 1990) or biodegradation (Robinson and Novak, 1994) and their bioavailability is considered to be insignificant (Bollag, 1991). However, synthetic chlorolignin model compounds have been shown to be bioavailable to rats (Sandermann, 1990). Moreover, recent findings suggest that at least some remobilization may take place due to surfactants.

Surfactants are amphipathic molecules which partition between fluid phases that have different degrees of polarity and hydrogen bonding, and thus reduce surface tension (Desai and Banat, 1997). In the soil environment, the surfactants form micelle pseudo phases that have a hydrophobic core that can attract *e.g.* polynuclear aromatic compounds (PAHs) (Huang et al., 1997). Surfactants can be divided into three groups according to their hydrophilic end: anionic, nonionic and cationic surfactants. As an example of commercially available surfactants, Triton X-100 is an ethoxylated nonionic surfactant which solubilizes naphthalene from sandy soil (Huang et al., 1997).

My interest on surfactants is focused on two aspects: one, does the microbial production of surfactants play a role in bioremediation (see Results and Discussion section), and two, can the polymerized fraction of chlorinated organic compounds or polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/Fs) be remobilized from soil due to surface-active compounds. Certain chemical compounds such as oil, dissolved organic matter in compost, or surface active biologically produced compounds (*e.g.* rhamnolipids), may act as surfactants and release large lipophilic contaminants such as

polychlorinated biphenyls (PCBs) or polynuclear aromatic hydrocarbons (PAHs) from soil and thus increase their bioavailability (Morris and Pitchard, 1994; Raber and Kögel-Knabner, 1995; Schaefer et al., 1995; van Dyke et al., 1993). Simultaneous oil contamination may enhance the mobility of PCDD/Fs in soil-water systems (Först et al., 1995). Thus, the immobilization of *e.g.* chlorophenols into soil as a bound residue, by dimerization or polymerization, is not a favorable remediation event.

### 1.2.3 Bioavailability of chlorophenols to earthworms from contaminated soil

Earthworms have an important role in soil: they contribute to degradation and transport of compounds in soil in addition to soil preparation. They also represent higher organisms in soil. Hence, if earthworms are affected by pollutants in soil, the bioaccumulable compounds may enter a food chain and may therefore have wider hazardous effects in the environment. Haimi et al. (1992) found bioaccumulation of chlorophenols by earthworms to decrease with an increasing content of organic matter in soil. Toxicity of 2,4,6-trichlorophenol (artificial soil test) to *Eisenia fetida* has been reported to be 58 mg (kg dry wt)$^{-1}$ as $LC_{50}$ after 2 weeks of incubation at 20 °C (Neuhauser et al., 1985).

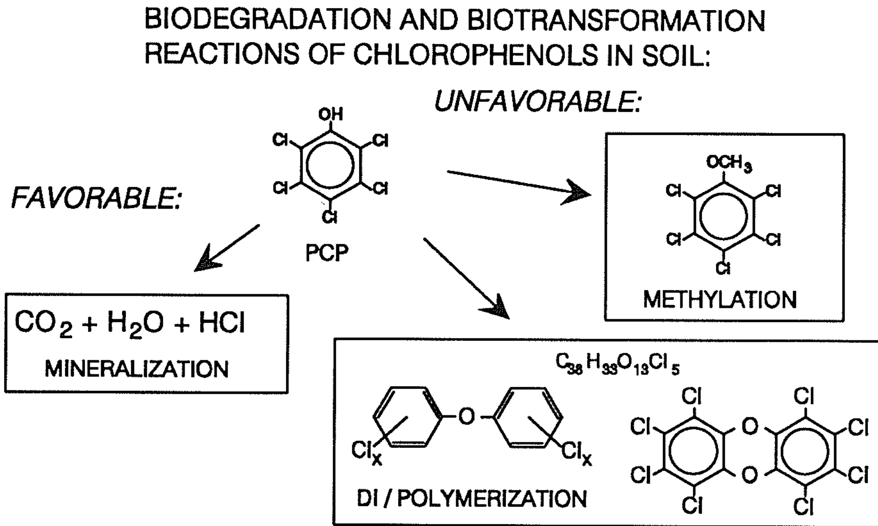


**Fig. 1.** Biodegradation and biotransformation reactions of chlorophenols in contaminated soil. Dimeric compounds presented are imaginary.

### *1.2.4 Impurities of the chlorophenolic wood preservative, Ky-5*

Chlorophenol-contaminated sawmill soil in Finland is often also contaminated with PCDD/Fs as well as with polychlorinated phenoxyphenols (PCPPs), polychlorinated diphenyl ethers and PCBs since the technical wood preservative called Ky-5 that was produced and used in Finland and in other Nordic countries contained these dimeric compounds as impurities (Humppi, 1985 and 1986; Kitunen et al., 1985; Nilsson et al., 1978; Rappe et al., 1978). Chlorinated dioxins and other dimeric compounds may also be biologically generated in contaminated soil. White rot fungi may form PCDD/Fs from chlorophenols using peroxidase enzymes (Öberg and Rappe, 1992) or from chlorinated anisyl compounds that they produce *de novo* (de Jong et al., 1994). Food is the major source of PCDD/Fs for humans (Travis and Hattemer-Frey, 1991). Chickens and cows have been found to accumulate PCDD/Fs from contaminated soil even at a concentration as low of 42 ng $kg^{-1}$ as international 2,3,7,8-TCDD toxic equivalents, I-TEQ (5.6 µg $kg^{-1}$ as a total PCDD/F concentration) (Stephens et al., 1995).

PCDD/Fs are scored as the most toxic man-made compounds in the world. The toxic mechanism of PCDD/Fs and related compounds is based on their binding to an Ah-receptor found in higher organisms. The Ah-receptor-mediated mechanism of action, however, is only one of the several ones suggested as the toxic mechanism of PCDD/Fs (Webster and Commoner, 1994). Researchers' current opinions differ as to how harmful PCDD/F compounds really are, what are their effects in humans, and what is the actual threshold value for the effective dose. Recent studies indicate that chlorophenols rather than the PCDD/Fs are the main cause for detrimental health effects to human in contaminated sawmill areas (Vartiainen et al., 1995a). The PCDD/Fs are less mobile than chlorophenols since they adsorb to the organic matter in soil. However, they may become mobilized from soil via air particles.

Table 1 lists the compounds that may have a potential to cause adverse effects through the Ah-receptor-mediated mechanism of action (Giesy et al., 1994). Many of these compounds, in addition to PCDD/Fs can be found in contaminated sawmill soil. More attention should be paid to the environmental significance of PCPPs, polychlorinated diphenyl ethers and polychlorinated chloroanisoles in contaminated sawmill soils.

**Table 1.** Compounds that may, based on experimental evidence or structure, be expected to have a potential to cause adverse effects through the Ah-mediated mechanism of action (Table adapted from Giesy et al., 1994). The compounds recovered in the wood preservative Ky-5 or in the sawmill soil contaminated with Ky-5 are marked with an asterisk.

| Compound |
|---|
| Polycyclic aromatic hydrocarbons |
| *Polychlorinated anisoles |
| Polychlorinated anthracenes |
| Polychlorinated biphenylenes |
| *Polychlorinated biphenyls |
| *Polychlorinated dibenzofurans |
| *Polychlorinated dibenzo-*p*-dioxins |
| Polychlorinated dibenzothiophenes |
| Polychlorinated dihydroanthracenes |
| *Polychlorinated diphenyl ethers |
| Polychlorinated diphenylmethanes |
| Polychlorinated diphenyltoluenes |
| Polychlorinated fluorenes |
| Polychlorinated naphthalenes |
| Polychlorinated phenylxylylethanes |
| Polychlorinated quaterphenyl ethers |
| Polychlorinated quaterphenyls |
| Polychlorinated xanthenes |
| Polychlorinated xanthones |
| Polybrominated diphenyl ethers |
| Polybrominated azoanthracenes |

An interesting question is, to what compound the Ah-receptor originally served as a binding site? Ah-induced cytochrome P450 enzymes are known to oxidize lipophilic xenobiotics including plant constituents such as flavones in order to subject them to further enzymatic conversions which will enhance water solubility and excretion from the body (Webster and Commoner, 1994). Thus, my wild guess is that the Ah-receptor was originally targeted to plant-excreted toxic compounds. Could plants then also degrade xenobiotics of similar structure, such as PCDD/Fs? If this is true, perspectives on plant-associated bioremediation of PCDD/Fs in

the future would considerably improve.

Although non-chlorinated dibenzo-*p*-dioxin and dibenzofuran biodegrade to some extent (Fortnagel et al., 1990, Joshi and Gold, 1994; Monna et al., 1993; Wittich et al., 1992), only few papers report biological breakdown or dehalogenation of PCDD/Fs (Adriaens and Grbic-Galic, 1994; Adriaens et al, 1996; Barkovskii, 1996; Gold et al., 1994; Takada et al., 1996; Touissant et al., 1995; Halden and Dwyer, 1997). Bioremediation of PCDD/Fs is hindered by their low availability to microorganisms and their strong sorption to soil.

## 1.3 Chlorophenol-mineralizing microorganisms and the real world

### *1.3.1 Chlorophenol-mineralizing microbial species*

Several bacteria and fungi can mineralize chlorophenols (for review, see Häggblom and Valo, 1995). The mineralization of chlorophenols may proceed aerobically (Apajalahti et al., 1986; Crawford and Mohn, 1985; Seech et al., 1991; for review, see Häggblom, 1992) or via reductive dehalogenation (Häggblom et al., 1993; Mikesell and Boyd, 1986; for review, see Mohn and Tiedje, 1992). However, reductive dehalogenation does not always lead to complete mineralization, but to the accumulation of metabolic products (Cole et al, 1994; Smith and Woods, 1994). In summary, several chlorophenol-degrading microorganisms have been isolated and their properties have been carefully investigated in the laboratory (see reviews: Allard and Neilson, 1997; Fetzner and Lingens, 1994; Häggblom, 1992; Häggblom and Valo, 1995; McAllister et al., 1996; Mohn and Tiedje, 1992; van der Meer et al., 1992). The degradation of chlorophenols is usually faster in the laboratory than in the field since the laboratory-scale experiments are performed under more favorable and controlled conditions. In fact, the sometimes poor survival of inoculated pure cultures in contaminated soil may limit their use for effective bioremediation. To promote bioavailability of the chlorophenols and survival of inoculated pure cultures for bioremediation, immobilization of bacteria to *e.g.* polyurethane (Briglia et al., 1990; O'reilly and Crawford, 1989), sodium alginate (Lu et al., 1996) or κ-carrageenan (Cassidy et al., 1997) has been tested and found to improve degradation of chlorophenols, at least in the laboratory scale.

### *1.3.2 Origin and diversity of chlorophenol-degrading enzymes*

Many gram-negative bacteria have a common pathway to degrade PCP and other highly chlorinated phenols. The pathway involves an initial oxygenolytic dechlorination of PCP to tetrachloro-*p*-hydroquinone by PCP-4-monooxygenase. It has been suggested that pentachlorophenol monooxygenase has evolved from a previously existing flavin monooxygenase to accommodate also PCP (Copley, 1997). This dehalogenase of monooxygenase type and the corresponding *pcpB* gene share homology among *Sphingomonas chlorophenolica* ATCC 39723 (Orser and Lange, 1994), *Sphingomonas* sp. RA2 (Radehaus and Schmidt, 1992), *Arthrobacter* sp. ATCC 33790 (Schenk et al., 1989), and *Pseudomonas* sp. strain SR3 (Resnik and Chapman, 1994), as well as other *Pseudomonas* spp. (Leung et al., 1997). According to studies of Ederer et al. (1997), the homology of *pcpB* among these above-mentioned four PCP-degrading microorganisms was exceptionally high, and due to additional culture characteristics, it was suggested that they should be reclassified into the genus *Sphingomonas* of the α-proteobacteria (Nohynek et al., 1995; Karlson et al., 1995). In conclusion, most of the gram-negative bacteria able to degrade PCP appears to belong to the genus *Sphingomonas*, and they have very similar degradation pathways, enzymes and genes to do so.

Chlorinated hydroquinones are the initial metabolites during degradation of PCP and other polychlorinated phenols by gram-positive chlorophenol-mineralizing strains of *Mycobacterium chlorophenolicum* (Apajalahti and Salkinoja-Salonen, 1987; Häggblom et al., 1994), *Mycobacterium fortuitum* (Häggblom et al., 1988b; Nohynek et al., 1993), and *Streptomyces rochei* 303 (Golovleva et al., 1992). In contrast to gram-negative bacteria, these gram-positive bacteria have different PCP-hydroxylating enzymes (Häggblom and Valo, 1995, Karlson et al., 1995) and the further degradation of chlo-

rinated hydroquinone follows a different pathway (Häggblom, 1992). Genetic basis behind reactions is yet unknown.

For less chlorinated phenols, bacteria have a different degradation pathway including oxidation of mono- or dichlorophenols to corresponding chlorocatechols with dehalogenation after ring cleavage (Häggblom, 1992).

White rot fungi have been reported to degrade a wide range of lignocellulosic and xenobiotic compounds (Wittich, 1996). The fungal metabolism of PCP by *Phanerochaeta chrysosporium* may form either pentachloroanisole (Lamar and Dietrich, 1990) or 2,3,5,6,-tetrachloro-2,5-cyclohexadiene-1,4-dione (Mileski et al., 1988). However, it has been shown that one or more bacterial species are always associated and coexist with *Phanerochaeta chrysosporium* (Seigle-Murandi et al., 1996). These bacteria could not be eliminated from the fungus by treatment with either heat, antibiotics, ozone or sodium hypochlorite (Seigle-Murandi et al., 1996). One of these bacteria was *Agrobacterium radiobacter* which has also been isolated together with *Pseudomonas sp.* and *Flavobacterium gleum* from a mixed culture able of degrading PCP synergistically (Yu and Ward, 1997). Since also other bacteria associated with fungus were efficient degraders of aromatic compounds (Seigle-Murandi et al., 1996), it can not be disregarded that degradation of xenobiotic compounds by *Phanerochaeta chrysosporium* may in fact be due to symbiotic (synergetic) relation between bacteria and the fungus.

### *1.3.3 Microbial degradation of chlorophenols under ambient field conditions*

Only a few published experiments have included outdoor field tests (Lamar and Glaser, 1994; Litchfield et al., 1994; Seech et al., 1994). A good review on the results from field tests for the bioremediation of chlorophenol-contaminated soil was presented by Häggblom and Valo (1995). In order to compare the efficiency of chlorophenol degradation in different field studies, the removal rates of chlorophenols need to be estimated. The removal rate expressed as concentration of chlorophenols depleted per time and unit of soil volume is dependent on the starting concentration of chlorophenols and it holds the assumption that degradation rate is constant throughout the bioremediation (following the zero order kinetics), which is hardly ever the case. Hence, the removal rate as such gives a very rough estimate of the efficiency of chlorophenol removal, but it does not take into account either the degree of mineralization or the contamination level reached after bioremediation.

In pilot-scale composting studies by Valo and Salkinoja-Salonen (1986) on an enrichment culture, the removal rate was 2.2 mg CPs (kg dry wt)$^{-1}$ d$^{-1}$ at the level from 280 to 20 mg CPs (kg dry wt)$^{-1}$, and in studies by Mahaffey and Sanford (1990) on a mixed culture in slurry bioreactors, the chlorophenol removal rate was 3.3 mg (kg dry wt)$^{-1}$ d$^{-1}$, at the contamination level from 100 to 0.5 mg CPs (kg dry wt)$^{-1}$ (Table 2). In both cases of Valo and Salkinoja-Salonen (1986), and Mahaffey and Sanford (1990), laboratory studies confirmed that the chlorophenol removal was due to mineralization. The chlorophenol removal rates for mixed culture and pure bacterial strains are in agreement with our pilot-scale studies with a consortium of chlorophenol degraders (Table 2). Considerably higher removal rates could be found in field experiments on inoculation with white rot fungi *Phanerochaete chrysosporium* and *Phanerochaete sordida* (Table 2). Augmentation with a chlorophenol-mineralizing strain *Mycobacterium chlorophenolicum* (Häggblom and Valo, 1995) resulted in chlorophenol removal rates of 0.8 and 9.5 mg (kg dry wt)$^{-1}$d$^{-1}$(contamination levels from 790 to 10 mg (kg dry wt)$^{-1}$, and from 8500 to 18 mg (kg dry wt)$^{-1}$, respectively; Table 2).

Conclusion from these field tests is that augmentation with chlorophenol-degrading pure cultures can significantly improve chlorophenol degradation in soil, provided that mineralization occurs. Relatively good degradation rates can also be achieved by amendment with mixed cultures. However, when the contaminated soil itself has sufficient number of chlorophenol-degrading (mineralizing) microorganisms, further inoculation will not enhance degradation.

**Table 2.** Chlorophenol removal rates in different field-scale experiments.

| Inoculum | Treatment | Chlorophenol removal rate mg (kg dry wt)$^{-1}$ d$^{-1}$ | Starting concentration mg (kg dry wt)$^{-1}$ | Chlorophenol concentration at the end of the treatment mg (kg dry wt)$^{-1}$ | Mechanism of chlorophenol removal | Reference |
|---|---|---|---|---|---|---|
| Mixed culture | Composting | 0.6 | 45 | <10 | Mineralization (2%/d) | Laine & Jørgensen, 1997 |
| Mixed culture | Composting | 2.2 to 4.8 | 1800 | 50 | Mineralization (2%/d) | Laine & Jørgensen, 1997 |
| Enrichment culture | Composting | 2.2 | 280 | 20 | Mineralization | Valo & Salkinoja-Salonen, 1986 |
| Mixed culture | Slurry bioreactor | 3.3 | 100 | 0.5 | Mineralization | Mahaffey & Sanford, 1990 |
| *Pseudomonas resinovorans* | Solid-phase treatment | 3.2 | 680 | 6 | Mineralization (6%/d) | Seech et al., 1994 |
| *Phanerochaete chrysosporium* and *Phanerochaete sordida* | | 4.3 to 9.3 | 1010 | 74 | Methylation, formation of nonextractable compounds, minor miner-alization | Lamar et al., 1990 Lamar & Glaser, 1994 |
| *Phanerochaete chrysosporium* | Composting in large scale | 0.2 (360 to 720 days) | 84 to 200 [a] | 9 to 30 [a] | Not reported | Holroyd & Count, 1995 |
| *Mycobacterium chlorophenolicum* | Composting | 0.8 | 790 | 10 | Mineralization | Häggblom & Valo, 1995 |
| *Mycobacterium chlorophenolicum* | Composting | 9.5 | 8500 | 18 | Mineralization | Häggblom & Valo, 1995 |

a) Sum of 3,4,-DCP; 2,4,5-TCP; 2,3,4,6-TeCP and PCP.

### *1.3.4 Environmental factors affecting chlorophenol degradation*

Many chlorophenol-degrading microbial isolates originate from contaminated soil or sediment (Apajalahti and Salkinoja-Salonen, 1984; Häggblom et al., 1988; Li et al., 1991). In contaminated soil during a long exposure, there has been time for evolution and enrichment of indigenous microorganisms that tolerate and degrade xenobiotic compounds. However, restricting environmental conditions (temperature, low oxygen and nutrient levels, acid pH) in contaminated soil often prevents effective degradation of contaminants. Improvement of environmental conditions has been shown to enhance *e.g.* chlorophenol degradation in soil (Apajalahti and Salkinoja-Salonen, 1984; Valo et al., 1985). Addition of bulking agents such as bark chips provides carbon supply as well as improves aeration. Ammonium and nitrate in added fertilizers may serve as the source of nitrogen. Nitrate may also function as an alternative electron acceptor for oxygen (Häggblom et al., 1993). For active biodegradation, soil microorganisms may need B vitamins, amino acids or other growth factors (Alexander, 1994; Smith and Woods, 1994).

Indigenous soil microorganisms are able to degrade chlorophenols under improved conditions. Microbiological processes occurring in soil during bioremediation are, nonetheless, not fully understood. Is the mechanism for chlorophenol degradation then complete mineralization, cometabolism or synergism? The transformation of an organic compound by a microorganism that is unable to use the substrate as a carbon or energy source to support growth is termed cometabolism (Alexander, 1967). PCP has been found to function as a cometabolite for degradation of persistent di-, tri- and tetrachlorophenols (Liu et al., 1991). Effects of the addition of secondary substrate such as glutamate or glucose to pure cultures of chlorophenol-degrading bacteria has been extensively studied (McAllister et al., 1996). In the presence of dextrose, *Burkholderia (Pseudomonas) cepacia* cometabolizes PCP which it is unable to use as a sole carbon and energy source (Banerji and Bajpai, 1994). Glutamate and glucose stimulates PCP degradation by *Flavobacterium* sp. (Topp et al., 1988; Hu et al., 1994) and may play role in maintaining PCP degradation capacity. *Sphingomonas* RA2 has a preference for PCP over glucose that has no effect on PCP degradation (Radehaus and Schmidt, 1992). In some cases, addition of glucose repress PCP degradation by *e.g.* soil microorganisms (Sato, 1996).

### 1.4 Aims of this study

The aims of this study included the following six points: 1) Development of a composting system to accelerate the bioremediation of chlorophenol-contaminated soil in laboratory, pilot- and full-scale applications. 2) Evaluation of the use of straw compost and remediated soil as inocula. 3) Investigation of fate of chlorophenols and PCDD/Fs during composting. 4) Study on bioaccumulation of halogen containing contaminants from soil into earthworms. 5) Study on microbiology behind chlorophenol degradation; microbial community changes during full-scale bioremediation; the role of cometabolites, other secondary substrates and nutrients on chlorophenol degradation; the mechanism for chlorophenol degradation; complete mineralization, cometabolism or synergism? 6) Evaluation of the occupational health effects during the full-scale bioremediation by quantitating the microbial and chemical release in airborne particles during mixing of the compost windrows.

# 2 Materials and methods

## 2.1 Materials

### *2.1.1 Contaminated soil used in a case study (Papers II-VI)*

The bioremediation study (Papers II through VI) site was a contaminated sawmill where approximately 25 000 kg of a chlorophenol-containing wood preservative (Ky-5) had been used from 1955 to 1977 to impregnate wood. Main components of Ky-5 were 2,4,6-trichlorophenol (2,4,6-TCP, 7 to 15%), 2,3,4,6-tetrachlorophenol (2,3,4,6-TeCP, about 80%) and PCP (6 to 10%) (Valo et al., 1984). Ky-5 contained also up to 1.5% (by weight) of polychlorinated phenoxyphenols, and up to 0.02% (by dry weight) of dibenzo-*p*-dioxins and

dibenzofurans as impurities (Kitunen et al., 1985; Vartiainen et al., 1995b).

### *2.1.2 Straw compost (Papers II-VI)*

Straw compost was studied as inoculum for chlorophenol degradation for several reasons: it is available in most countries, it is produced on a large scale for the cultivation of mushrooms, and it has a constant quality. In addition, it offers a source of nutrients and a surface for attachment of bacteria. This ensures better competition ability and protection against predation and toxic effects in the soil environment.

Straw compost was obtained from a mushroom farm in Finland and it was a so-called phase I compost (Fermor et al., 1985). The straw compost was composed of rye or wheat straw and chicken litter that were pre-composted for approximately three weeks.

### *2.1.3 Remediated soils (Papers I and II-III)*

Earthworms studied in Paper I were exposed to two contaminated soils, one sandy soil and the other one with high organic matter, from the sawmill sites which had been composted for 1 and 2 years, respectively, in order to remove the organic chlorine compounds originating from wood preservation. The ignition loss of sandy soil was 7% (wt / dry wt), organic halogen content was 120 ± 20 (N=2) µg Cl (g dry wt)$^{-1}$, and the pH was 3.9. The ignition loss of organic soil was 55% (wt / dry wt), organic halogen content was 134 ± 43 (N=2) µg Cl (g dry wt)$^{-1}$, and the pH was 6.3.

The chlorophenol degradation potential in successfully bioremediated soil was assessed to see whether the self-remediating capacity of contaminated soil can be maintained and enhanced. Remediated soil that was studied as an inoculum in Papers II and III was obtained from successfully remediated, full-scale three-year composting of chlorophenol-contaminated soil in biopiles where bark chips and nutrients had been added, but no inoculum. For the laboratory experiments, the remediated soil was sampled directly from the compost biopile in the field.

## 2.2 Experimental set-ups in laboratory, bench, pilot and in full scale

### *2.2.1 Adaptation of compost or soil to PCP degradation (Paper II)*

A percolator system was designed to select and enrich microorganisms able to degrade PCP in remediated soil, and to introduce PCP to the microorganisms in straw compost to help their adaptation to chlorophenol degradation (Paper II, Fig. 1). A mineral salts medium, amended with 5 to 10 mg PCP l$^{-1}$, was circulating in the percolators. One third of the volume of circulating liquid was changed weekly to maintain the concentration of PCP in the fluids and to ensure oxygen availability. The percolators operated for three months.

### *2.2.2 Bench-scale piles (Paper III)*

Bench-scale piles were constructed to simulate conditions and material ratios in the pilot-scale composting. Nutrients, lime and bark as well as inocula were added to contaminated soil in the same proportions as to the compost piles. The mini-piles (one kg mass) were stacked in trays, covered with aluminum foil and kept at room temperature in the hood for six months. The piles were mixed twice a month and the moisture maintained by adding distilled water.

### *2.2.3 Outdoor composting of contaminated soil in pilot scale and in full scale (Papers III-VI)*

#### *2.2.3.1 Pilot-scale composting (Papers III and IV)*

The pilot-scale composting was performed in 1994. Four cone-shaped compost piles (3 m × 3 m × 1.5 m) were built in the field (Fig. 2a). The total volume of each pile was about 13 m$^3$. Pile 2 (Straw compost pile) was amended with straw compost, and piles 3 (Remediated soil pile) and 4 (Wood chips pile) were amended with remediated soil (Paper III, Table 1).

Biodegradability of heavily contaminated wooden parts from the bottom of the former wood preservative dip basin was studied in the

Wood chips pile. The contaminated wooden parts (logs) were chipped beforehand, making them easier to mix thoroughly with the soil in pile 4. To pile 1 (Reference pile), only bark was added. Each inoculant was mixed with contaminated soil, pH was adjusted with fine granular lime, nutrients were added in a form of a nitrogen-rich commercial fertilizer, and bark chips (fresh pine) as supporting and aerating material were added to enhance microbial degradation. The chlorophenol-contaminated soil was excavated from 0 to 1 m next to a wood preservation shed, where it had been exposed to rainfall for many years. The soil was homogenized with a crushing machine. The piles were constructed on plastic beds each covered with an insulation layer of bark. After all the materials were piled, the piles were mixed, sampled and covered with tarps. The piles were mixed with a mechanical clamshell every second week for the first two months and then once a month for the following four months. Since the chlorophenol concentrations decreased to less than 10 mg (kg dry wt)$^{-1}$ already after 9 weeks, the chlorophenol concentration in piles 1 to 3 was raised by a second addition of heavily contaminated soil. The soil was excavated from 0 to 1 m depth right beside the wood preserving basin, under the roof of wood preserving building.

#### *2.2.3.2 Composting in full scale (Papers V and VI)*

The full-scale composting was performed 1995-1997. Three windrows were built with the total volume of 520 m$^3$. The windrows were constructed on plastic beds which had an inclination towards separate underdrainage, and which were each covered with an insulation layer of bark (Fig. 2a). All three compost windrows contained chlorophenol-contaminated soil, nutrients and lime, but they differed in amendments and the soil type. Windrows 1 and 2 contained the same batch of contaminated soil that was sandy soil with partly degraded sawdust. The contaminated soil was sieved before adding to the compost windrows. The soil in Windrow 3 was clay, and it had a smaller initial chlorophenol concentration. The soil in Windrow 3 was excavated from 1.5-3 m depth. Windrow 1 was amended with bark chips (pine and spruce) as supporting and aerating material. To enhance chlorophenol degradation, induced straw compost was added as inoculum to windrows 2 and 3. The fresh straw compost was induced for chlorophenol degradation by pre-incubation with composted chlorophenol-contaminated soil obtained from pilot-scale test (Pile 2, Paper II) for 5 weeks before adding to the compost windrows. The ratio of the materials was 2 parts of contaminated soil and 1 part of straw compost or bark by volume. After all the materials were piled, the windrows were mixed by turning and covered with tarps. The windrows were mixed every third week during summer 1995 and once a month during summers 1996 and 1997.

#### *2.2.3.3 Soil sampling (Papers III-VI)*

The pilot-compost piles were mixed every second or third week, and sampled thereafter. A combined sample of 10 to 20 different points from each pile (13 m$^3$) was mixed by shaking and sieved through an 8-mm sieve. The pieces of bark and contaminated wood chips from the sample remained on the sieve. After the full-scale windrows were mixed once a month, they were sampled. Composite samples of 10 to 20 different subsamples per 10-m length (50 to 70 m$^3$ of compost volume) of each windrow were mixed by shaking and sieved through an 8-mm sieve.

A glass jar was filled with the sieved sample and aluminum foil was placed between the jar and the cap. The samples were stored at 4 °C before microbiological measurements and a subsample was frozen from each composite sample for chemical analyses.

**Fig. 2a.** Build-up of composting. Top: Pilot piles were made from soil, bark or straw compost, lime, and nutrients in a sandwich-type (photos: K. Jørgensen). Bottom: Full-scale piles were constructed on on plastic beds which had an inclination towards separate underdrainage, and which were each covered with a 0.5 m insulation layer of bark (photos: R. Hansen).

### *2.2.4 Air sampling (Paper VI)*

The air collection was performed using Andersen Impactor Samplers that are designed to simulate the human respiratory system (Fig. 2b). During the airborne particle sampling, each particle size level of the non-viable Andersen Sampler was loaded with a thin plate of 0.5% agar in water (approximately 6-7 g) on an aluminum foil from which the PCDD/Fs were then extracted and their concentrations determined. For the collection of airborne microorganisms, the Andersen Viable particle sizing samplers were used. Several different selective growth media were used to enumerate bacteria and fungi in air particles (Paper VI; see *Enumeration of bacteria* section).

## 2.3 Fate of chlorophenols

### *2.3.1 Chlorophenol and chloroanisole analysis (Papers II-V)*

During laboratory and pilot-scale experiments (Papers II-IV), chlorophenols and chloroanisoles in solid samples were analyzed by two slightly different acidic acetone extraction methods. Chlorophenol concentrations of the unlabeled replicates were measured after the mineralization test (Paper II) to detect possible metabolites formed by adding acidic acetone and sonicating the samples in a water bath for 10 minutes. Aliquots of acetone extract were transferred to test tubes and neutralized with 2 M NaOH. For chloroanisole analysis, one aliquot was extracted with hexane, which was then dried with $Na_2SO_4$. The other aliquot was derivatized for chlorophenol analysis with 0.1 M $K_2CO_3$ and acetic anhydride.

From the compost samples (Papers III and IV), chlorophenols were extracted by adding acidic acetone, sonicating in a water bath for 15 minutes and shaking on a platform shaker overnight. An aliquot of acetone extract (supernatant) was transferred to a test tube, water was added and the extract was washed with hexane. The hexane phase was recovered and dried with $Na_2SO_4$ for chloroanisole analysis. The residue was neutralized with 2 M NaOH and washed with another aliquot of hexane. The purified extract was derivatized for chlorophenol analysis with 0.2 M $K_2CO_3$ and acetic anhydride. The extract was allowed to acetylate for 10 minutes before the chlorophenols were transferred to a hexane phase, allowed to stay for another 10 minutes, and then dried with $Na_2SO_4$.

From the full-scale composts (Paper V), the chlorophenols were extracted with acidic acetone/hexane and derivatized as described by Kalevi and Jørgensen (1996).

When the chlorophenol degradation ability of isolated gram-positive bacteria was tested, the chlorophenols were analyzed from the growth medium. Five ml samples (two replicates) were pipetted to screw-capped test tubes before 50 µl of internal standard (2,4,6-TBrP, 5.74 µg) and 5 ml of 0.2 M $K_2CO_3$ were added. The chlorophenols were derivatized by adding 98 µl acetic anhydride, shaking and letting the reaction continue for 10 minutes. The chlorophenols were then extracted to 5 ml of hexane, and shaked. An emulsion originating from the growth medium, DSM-65, was removed by adding a few drops of isopropanol. The hexane phase was collected after 10 more minutes and dried with $Na_2SO_4$ before the GC/ECD run.

Chlorinated compounds were analyzed by a Hewlett Packard (HP) 5890 gas chromatograph (GC) with two HP electron capture detectors connected to HP-1 and HP-5 fused-silica capillary columns, respectively. The internal standards used were 2,4,6-tribromophenol and 2,4,6-tribromoanisole. The GC was calibrated with 16 different chlorophenol compounds (di-, tri-, and tetrachlorophenols and PCP) and 7 different chloroanisole compounds (Papers II-V).

### *2.3.2 Mineralization of [U- $^{14}C$]PCP (Papers II and III)*

One sixth of the volume of a 100 ml infusion bottle was filled with remediated soil or compost. PCP with or without [U-$^{14}C$]PCP (specific activity 10.6 mCi $mmol^{-1}$, 14,000 to 140,000 dpm per bottle) was added in either sterile distilled water, phosphate buffer (pH 7.0) or mineral salts medium to make a slurry. Labeled and unlabeled bottles were prepared in duplicate or triplicate. The experiment was performed at room temperature or at 45°C under ambient air, 100% oxygen or nitrogen-flushed atmosphere, and the bottles were shaken on a

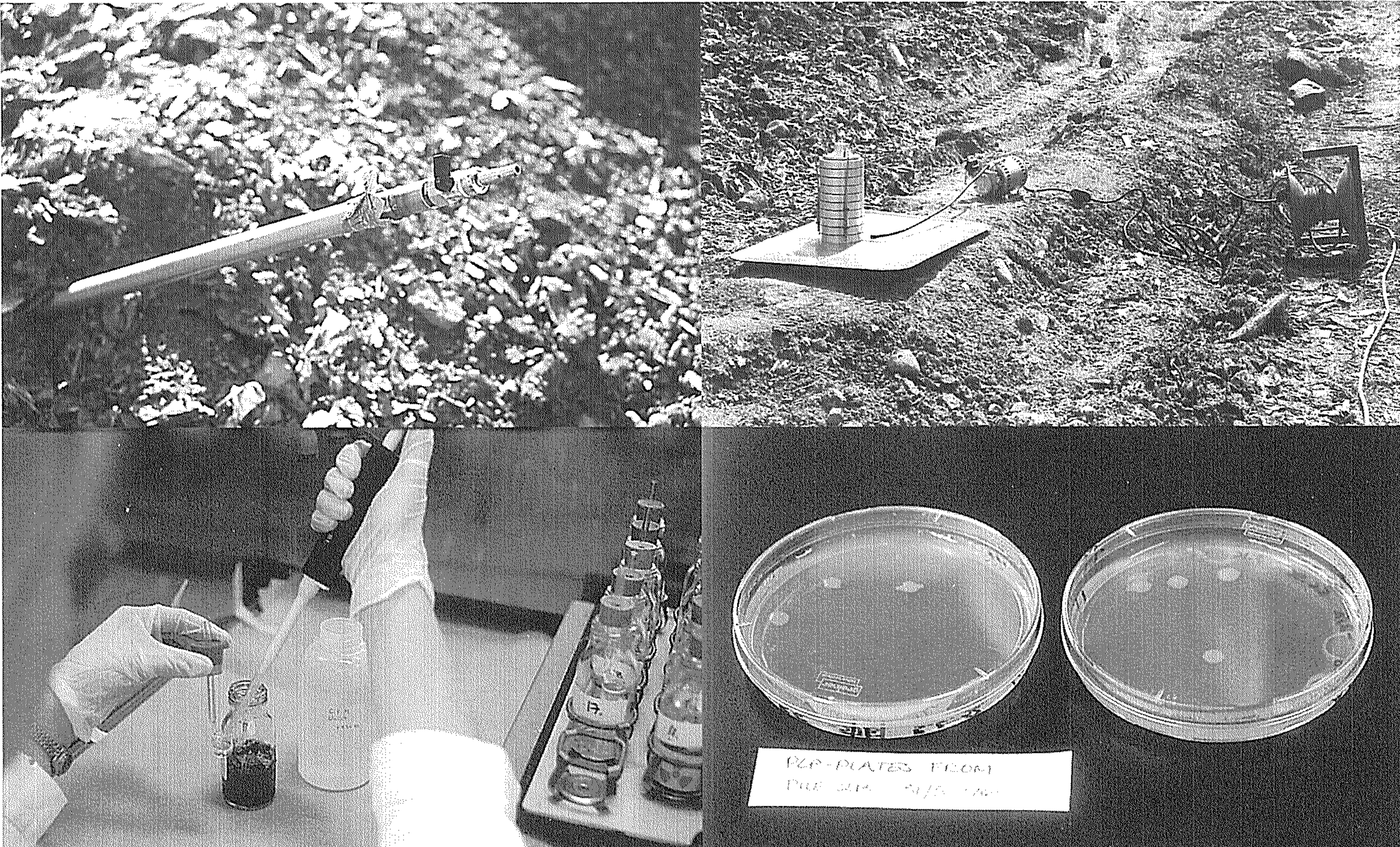


**Fig. 2b.** Follow-up of composting and biodegradation. Top, left: gas collection tube, right: air-particle sampling during mixing (photos: M. M. Laine). Bottom, left: mineralization test (photo: R. Laine), right: colonies on PCP-plates (photo: J. Järvi).

rotary shaker (100 rpm). The bottles were flushed before the experiment and after each sampling. A solution of 1 M NaOH was kept in a cup anchored by the rubber stopper of the bottle (Apajalahti et al., 1986, Fig. 2b). Every third or fourth day, the amount of $^{14}CO_2$ absorbed to 1 M NaOH from the labeled samples was measured using a liquid scintillation counter (Wallac). After sampling, the NaOH in the cup was replaced by 1 ml of fresh NaOH. The mineralization test was terminated after three weeks, if the relative $^{14}CO_2$ evolution between the sampling was less than 20%. The total mineralization was considered significant only, if more than 20% of the [U- $^{14}$C]PCP was converted to $^{14}CO_2$.

### 2.3.3 Bioavailability study with earthworms (Paper I)

Earthworms from kitchen compost *(Eisenia andrei)* were exposed to two remediated sawmill soils as described in Paper I. The earthworms were kept in polyethene containers (two replicates) filled with contaminated soil for 12 or 25 days at 22°C. The earthworms were fed with fresh horse manure and the humidity was maintained by irrigation. Dead earthworms were removed every third day. Containers with soil only (reference soil) were kept under the same conditions to observe any change in soil organic halogen content due to evaporation and/or microbial activities. The soils were not sterilized before the experiment. As a blank, the earthworms not exposed to contaminated soil were analyzed.

### 2.3.4 Extraction of tetrahydrofuran-extractable organic halogen (EOX) from soil, compost and earthworms (Papers I and IV)

Soil, compost and homogenized earthworms were freeze-dried and extracted with tetrahydrofuran (THF) as described in papers I and IV. The earthworms were let to empty their gut content before they were killed with liquid nitrogen. Remediated soil and earthworm extracts (Paper I) were shaken for 3 hours on a platform shaker (200 rpm) before the extracts were allowed to settle. The THF-extract from pilot-scale compost soil (Paper IV) was shaken overnight on a platform shaker (180 rpm), and the extracts were allowed to settle for one hour. THF was used as a blank that followed the extraction procedure after freeze-drying. The supernatant was filtered and the extract was stored at -20 °C under nitrogen gas to prevent oxidation. Since the samples were freeze-dried the volatile halogen compounds were lost.

### 2.3.5 Hydrolysis of extracted halogen compounds in remediated soil and earthworms

#### 2.3.5.1 Mild hydrolysis

THF was evaporated from the extract. The residue was incubated for one hour in a 82 °C water bath with 0.5 ml of KOH solution (pH 10.33). The pH was then adjusted to 5.0-5.5 with 7.4 µl of 0.0144 M $HNO_3$. The hydrolyzed compounds were rediluted with 2 ml of THF and the extract was dehydrated with 1 g of molecular sieve (Sigma, St. Louis, USA). The extract was filtered through a 0.45-µm filter (Nylon Acrodisc, Gelman), and evaporated to approximately 100 µl before HPSEC run.

#### 2.3.5.2 Strong hydrolysis

The strong hydrolysis was adapted from the fatty acid saponification method used in whole cell fatty acid analysis, MIDI (Väisänen et al., 1994). A 200 µl-aliquot of the THF-extract was evaporated to dryness and 0.5 ml of saponification reagent was added (3.75 M KOH in methanol-water, (1:1)). The solution was boiled in a 98 °C water bath for 5 min, mixed, and boiled again for 25 min. After cooling of the solution, the pH was adjusted to 2.0 with $HNO_3$, and 2 ml of THF and 1 g of molecular sieve (Sigma, St. Louis, USA) were added. The dry THF-extract was filtered through a 0.45- µm filter (Nylon Acrodisc, Gelman), evaporated to dryness, and resuspended to 100 µl of THF before HPSEC run.

### *2.3.6 Halogen measurements (Papers I and IV)*

The tetrahydrofuran extractable organic halogen content (EOX) of the extracts was analyzed with an Organic Halogen Analyzer (Euroglas, Delft, The Netherlands) with EOX-equipment as follows: 50 µl of the extract was injected into the oven where it was combusted at 850 °C under the flow of oxygen and argon and the halogen content was measured microcoulometrically. Duplicate or triplicate injections were made from two replicate extracts.

The coefficient of variation between replicate injections from the same extract was from 0 to 3% (average 1%), and between replicate extractions from 0.4 to 33% (average 7%) (Paper IV). The recovery of a chlorophenol standard solution (standard for monomeric chlorinated compounds, see below) after freeze-drying and extraction was approximately 20%. When a blank sample containing uncontaminated sawdust and sand was spiked with the chlorophenol standard solution after freeze-drying, the recovery was approximately 30%.

### *2.3.7 Molecular weight distribution analysis (Papers I and IV)*

The molecular weight distribution (MWD) of organic halogen compounds was analyzed by High Performance Size Exclusion Chromatography (HPSEC). THF was used as the eluent. Four Ultrastyragel (Waters) columns (pore sizes of $10^4$ Å, $10^3$ Å, 500 Å and 100 Å) were used in series. Polystyrene compounds, lignin model compounds erol and bierol, vanillic alcohol and acetone were used as molecular weight standards (Jokela et al., 1993). The standard for monomeric chlorinated compounds was a solution of 16 different di-, tri- and tetrachlorinated phenols and PCP (Paper IV). As a dimeric chlorinated model compound, 4-(2,4,6-trichlorophenoxy)phenol was used. During the HPSEC run the UV-vis-absorption in 225-445 nm and 264 nm was detected. After collecting fractions of 1 ml, the organic halogen content of each fraction was measured by evaporating THF, combusting the residue at 1000 °C under a flow of oxygen and then measuring the halogen content micro-coulometrically. The recovery of the EOX content after the HPSEC run was from 75 to 104%.

## 2.4 Microbiological measurements

### *2.4.1 Enumeration of bacteria (Papers II, III, V, VI)*

Bacteria were extracted from soil or compost by blending 10 g wet wt of the soil or the compost for 2 min with 90 ml of 0.9% NaCl, 1 ml of 10.4% $Na_5P_3O_{10}$ and 100 µl of 2% Tween 80 before serial dilution (Papers II and III).

The total number of bacteria was determined by direct count with acridine orange staining (Paper III). For enumeration of the number of heterotrophic bacteria, 1/5 Tryptone-Glucose-Yeast-extract- (TGY) medium was used (Paper V). For determining the number of culturable bacteria in composts and in straw compost, a rich medium specific for actinomycetes, R8 medium with cycloheximide (Amner et al., 1989), was used (Papers II and III). Bacteria able to grow on mineral salts medium with 1 mM PCP (Paper III) or 2 mM PCP (Paper V) as a sole carbon source were also enumerated in soils from the composts. During the summer of 1996 (five measurements), bacteria able to grow on 100 µM PCP in a mineral medium amended with Na-glutamate (so called Flavomedium) were enumerated. Flavomedium was adapted from Steiert et al. (1987) and it contained, in grams per liter, the following: $K_2HPO_4$, 0.65; $KH_2PO_4$, 0.19; $NaNO_3$, 0.5; $MgSO_4 \times 7\ H_2O$, 0.1; Na-glutamate, 4.0; Bacto-agar, 15.0; as well as 20 µM $FeSO_4$ and 100 µM PCP. Bacteria were grown on 1/5 TGY, R8 and PCP plates with or without extra carbon sources for 5 to 14 days at 30 °C.

Enumeration of PCP-mineralizing microorganisms was performed by most probable number (MPN) calculations (Paper II). The method for MPN counts was modified from the one described by Valo and Salkinoja-Salonen (1987). A dilution series from $10^{-1}$ to $10^{-5}$ was made in the mineral salts medium from the remediated soil after enrichment in the percolator. A mineral salts medium containing PCP and [$^{14}$C]PCP was added to each flask. The MPN of PCP-mineralizing microorganisms was estimated by Thomas' formula (American Public

Health Association, 1985). The mineralization was interpreted as positive if at least 50% of the added [$^{14}$C]PCP was recovered as $^{14}CO_2$ in four weeks.

The following selective growth media were used to enumerate bacteria and fungi in airborne particles (Paper VI): 1/5 Tryptone-Glucose-Yeast-extract- (TGY) medium, ½ Trypticase-Soy-Agar (TSA) with cycloheximide, R8-medium with cycloheximide, Cornmeal-Agar (CMA) with penicillin and streptomycin, Potato-Dextrose-Agar (PDA), pH 3.5; and Malt-extract-Agar (MEA) with penicillin and streptomycin.

### 2.4.2 Identification of the isolated bacteria

Bacteria were isolated from different selective and general growth media and were purified by restreaking at least three times on the original media. The purity of the isolates was followed by the Gram staining.

#### 2.4.2.1 Carbon source utilization pattern

Identification of the isolated strains was performed using Biolog GN and GP MicroPlates™ (Biolog, Inc., Hayward, USA) according to manufacturer's instructions (Biolog, 1993). The Biolog microtitre plates consist of 96 wells prefilled with a carbon source, nutrients and a redox dye, tetrazolium violet (Biolog, 1993). Wells are scored positive by formation of insoluble formazan which precipitates inside the wells upon dehydrogenase activity (Bochner and Savageau, 1977). The bacteria were pre-grown on Tryptic-Soy-agar (TSA) or Biolog Universal Growth Medium (BUGM) amended with 1% of glucose at 30 °C overnight. Biolog GN and GP MicroPlates™ were pre-warmed before inoculation with an appropriate turbidity. The plates were incubated at 30 °C, and the absorbance was read after 4 and 24 hours at 590 nm using a Biolog MicroPlate Reader, and the observed utilization patterns were compared with those in the databases of known bacterial species using Biolog's MicroLog™ 3 computer software. The similarity index must be at least 0.75 after 4 hours, and at least 0.5 after 16-24 hours of incubation to be considered an acceptable species identification by Biolog.

#### 2.4.2.2 Sphingomonas mini-database for user-made library in Biolog

Nine *Sphingomonas* species, two *Pseudomonas* species and *Burkholderia cepacia*, that were obtained from the HAMBI collection at the University of Helsinki were pre-grown on R2A medium at 30 °C for 24 hours before inoculation to Biolog GN MicroPlates™. The absorbances were read after 4, 24 and 48 hours. Three or four replicate inoculations were made and then compiled to form a user-made database (Fig. 3). Since the *Sphingomonas* species often grow slowly, inoculation after 24 hours of pre-growth was occasionally done from three replicate plates. For the future identification purposes, also 48 hours absorbances were read, although 24 hours readings were used for the initial library build-up.

#### 2.4.2.3 Whole cell fatty acid profiling

The whole cell fatty acids were analyzed as described by Väisänen et al. (1994) using the Microbial Identification System (MIDI Inc. Newark DEL). Isolates were pre-grown on TSBA or TSB medium for 24 hours before their fatty acids were saponified, methylated, extracted and finally analyzed using a gas chromatograph. The aerobic and actinomycete libraries of MIDI were used to compare whole cell fatty acid profiles with those of the known bacterial species. Strains with a similarity index of 0.500 or higher with a separation of 0.100 between the first and the second choice are considered good library comparisons by MIDI.

### 2.4.3 Chlorophenol degradation test in liquid culture for gram-positive isolates

The gram-positive isolates were inoculated (two replicates) into 30.0 ml of DSM-65 growth medium (containing, in g per liter, the following: sorbitol, 4.0; malt extract, 4.0; yeast extract, 4.0). The control contained sterile DSM-65 solution. After the isolates had been pre-grown for three to four days at 30 °C shaking on a platform

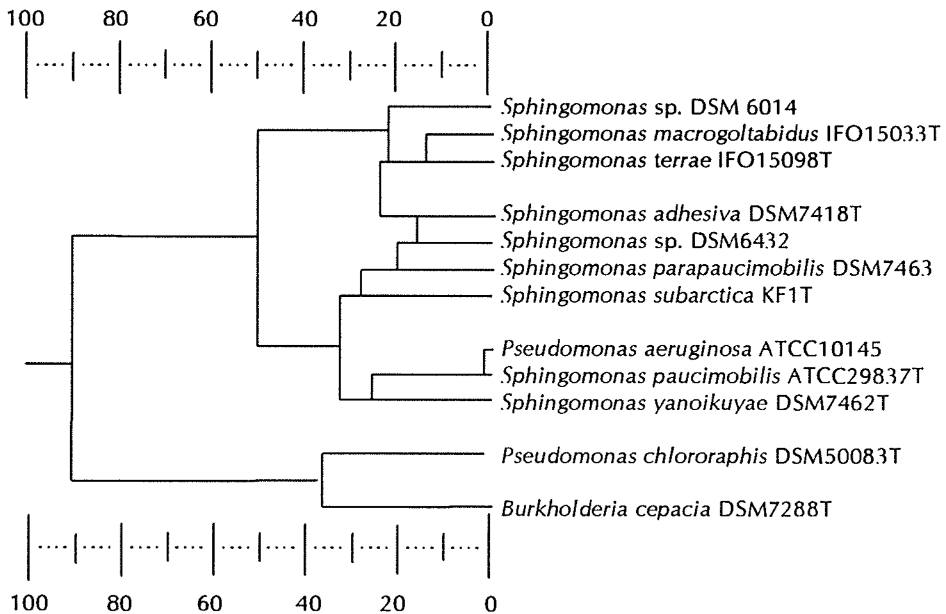


**Fig. 3.** Dendrogram of the nine *Sphingomonas* species, two *Pseudomonas* species and *Burkholderia cepacia* used for compilation of the user-made mini-database of Biolog.

shaker, sterile-filtered 5 µM PCP and the mixture of chlorophenols were added so that the final chlorophenol concentration in liquid was 1.33 mg $l^{-1}$. The mixture of chlorophenols contained 2,4,6-TCP, 2,3,4,6-TeCP and PCP in concentration ratios simulating the ones in the original wood preservative Ky-5. Growth bottles were sampled and the chlorophenols analyzed by GC/ECD (as described in *Chlorophenol and chloroanisole analysis* section) three times: initially after the addition of chlorophenols, after one week, and after two weeks of incubation at 30 °C on the platform shaker.

## 2.4.4 Soil respiration (Papers II, III and V)

### 2.4.4.1 Organic carbon mineralization rates

Organic carbon mineralization rates were determined in the unlabeled bottles during the [$^{14}$C]PCP mineralization experiments as described in Paper II. The amount of $CO_2$ trapped into 0.1 M NaOH was determined as inorganic carbon using a TOC (Total Organic Carbon) analyzer (Shimazu). This amount represented the $CO_2$ evolution over three days (three-day rate). The rates were furthermore calculated as averages of the three-day rates during the first two weeks of the experiment.

### 2.4.4.2 Soil respiration rates

Soil respiration rates were analyzed as described in Papers III and V. Basic and substrate-induced respiration rates were measured with a method modified from the one described by Anderson and Domsch (1978). Two replicate 125 ml closed glass bottles were filled with the amount of natural moist soil equivalent to 10 g by dry weight, and incubated for 2 hours (substrate-induced respiration) and for 20 hours (basic respiration), at a constant temperature of (22 ± 1) °C in a water bath. For measurements of substrate-induced respiration rate, glucose and nutrients were added to the test bottles as a powder, mixed and let to stabilize for 30 minutes. The production of $CO_2$ was measured from the headspace using an Easy-Quant IR carbon analyzer.

### 2.4.5 Community analysis (Paper V)

#### 2.4.5.1 Inoculation of Biolog microtitre plates

Bacteria from three full-scale compost windrows at 8 to 10 different time points were extracted as described in Paper V. The Biolog GN plates were inoculated with the $10^{-2}$ dilution (containing approximately $5 \times 10^5$ to $5 \times 10^6$ CFU of heterotrophic bacteria per ml) to follow the utilization pattern of 95 carbon sources in the microtitre plates. The plates were incubated at 30°C and the absorbance at 590 nm was measured several times during 48 hours of incubation. The utilization of different substrates was also tested separately for contaminated soil, bark and straw compost. The areas below the curve representing the absorbance at 590 nm versus time were integrated for each substrate used during 48 hours incubation, and the area obtained from control well A1 was reduced from the areas obtained from the substrates used. These areas were used in two-dimensional principal component analysis (PCA) to select significant substrates and differences in compost windrow environments.

#### 2.4.5.2 Principal component analysis (V)

The areas gathered as described below and in Paper V were analyzed using the Datana Toolbox package for **Matlab®** mathematical software. The observation matrix consisted of 96 rows giving the areas in four windrows for each of the 95 substrates and control well. The variables of the matrix were the different windrows 1, 2 and 3. Windrow 1 was represented as two separate samples 1B and 1D to evaluate the differences within one windrow. The other selected variables were starting materials, or different time points of one windrow. The observation matrix was centered and scaled before principal component analysis.

A dendrogram was made with Biolog software (UPGMA [Sneath and Sokal, 1973] method, automatic threshold option) for fixed incubation time of 48 hours to compare compost windrows with starting materials.

## 2.5 Other methods used

### 2.5.1 Physical and chemical measurements and follow-up in the field

#### 2.5.1.1 Dry weight, ignition loss and pH (Papers I and III)

The dry weight was determined with an infrared drying balance, after lyophilization or from oven-dried (105 °C) soil. The ignition loss was measured by combusting oven-dry sample (105 °C) at 850 °C for 2.5 hours. The pH was measured with a pH-electrode from deposited soil slurry in a 1 M KCl solution or in Milli-Q-water.

#### 2.5.1.2 Determination of soil gas components and temperature (Papers III and VI)

The soil air composition in the composts was measured before each mixing in perforated gas collection tubes installed inside the composts (Fig. 2b). Oxygen concentrations were measured using a Crowcon Triple 84TR (Crowcon Instruments, UK) gas meter. For $CO_2$ and humidity measurements, gas detector tubes (Dräger, Germany) were used. The temperature inside the composts was measured using a 2-m long temperature probe. The temperature gradient was measured at 0.25-m depth intervals and the average temperature was calculated.

### 2.5.2 Analyses of PCDD/Fs, toxicity, and nutrients

The analyses performed by co-authors and subcontractors are listed in Table 3.

**Table 3.** Methods used by co-authors and subcontractors.

| Parameter | Analysis method | Reference | Analysis performed by |
|---|---|---|---|
| Polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/Fs) of pilot-scale composts | Modified method from the one described by Kjeller *et al.* (1993). Soxhlet extraction with toluene (HRGC-HRMS). | Paper IV | Institute of Environmental Chemistry, Umeå, Sweden |
| PCDD/Fs of soil or compost | Soxhlet extraction with toluene (HRGC-HRMS). | Paper VI | National Public Health Institute, Kuopio, Finland |
| | Soxhlet extraction with toluene (HRGC-HRMS). | Table 9 | JuVe Group Oy, Rovaniemi, Finland |
| | Supercritical fluid extraction (HRGC-HRMS) | Table 9 | National Public Health Institute, Kuopio, Finland |
| PCDD/Fs of airborne particles | The agar plates were extracted with toluene (HRGC-HRMS). | Paper VI | National Public Health Institute, Kuopio, Finland |
| Toxicity tests of chlorophenol-contaminated soil with bacteria | Modified sediment-contact test from the one described by Brouwer et al. (1990). A luminescent bacteria (*Photobacterium phosphoreum*) test on water extracts; the standardized luminescence bacteria test procedure by DIN 38 412 Teil 34. (1991). | Paper IV | Finnish Environment Institute, Laboratory, Environmental Microbiology Unit, Finland |
| Nutrients | The total phosphorus concentration was measured by plasma emission spectroscopy after dry combustion and HCl extraction. Total nitrogen was measured with the Kjeldahl method using a Kjeltec Auto 1030 analyzer. Soluble nitrogen and soluble phosphorus were extracted by shaking for one hour with 0.1 M $K_2SO_4$ solution before analyzing with Kjeltec Auto 1030 analyzer or plasma emission spectroscopy, respectively. | Papers III and V | Novalab Oy, Karkkila, Finland |

# 3 Results and discussion

## 3.1 Fate of chlorophenols during bioremediation

### *3.1.1 Chlorophenol removal*

The composting of chlorophenol-contaminated soil was studied in the field during pilot-scale and full-scale experiments. Over 90% of the chlorophenols were removed during the six months of composting in pilot scale (Paper III, Fig. 2). During the composting, the relative concentration of PCP increased after one week, indicating that 2,3,4,6-TeCP was degraded faster than PCP, but then decreased, showing that also PCP was degraded (Paper III, Table 3; Paper IV, Table 1). The results indicate that tetrachlorinated phenols were degraded more readily than PCP, but PCP did not either accumulate. In full scale, at least 93% of the chlorophenols were removed (Table 4) in all compost windrows which were mixtures of contaminated soil and straw compost (Windrows 1 and 2) or bark chips (Windrow 3).

**Table 4.** Chlorophenol removal in % during full-scale composting 1995-1997.

| Windrow | Chlorophenols at the beginning of composting 1995 mg (kg dry wt)$^{-1}$ [aver ± SE] | Chlorophenol removal | | | | Chlorophenols at the end of composting 1997 mg (kg dry wt)$^{-1}$ [aver ± SE] |
|---|---|---|---|---|---|---|
| | | Annual | | | Total | |
| | | 1995 % | 1996 % | 1997 % | % | |
| Windrow 1 | 960 ± 90 (N = 8) | 96 | 45 | 35 | 98 | 15 ± 1 (N = 8) |
| Windrow 2 | 740 ± 270 (N = 2) | 92 | 70 | 0 | 98 | 18 ± 7 (N = 2) |
| Windrow 3 | 29 ± 6 (N = 2) | 90 | 50 | 0 | 93 | 2 ± 1 (N = 2) |

### *3.1.2 Mineralization*

To evaluate the use of straw compost and remediated soil as inocula for bioremediation of chlorophenol-contaminated soil, the *in situ* biotransformation of PCP and mineralization of radiolabeled [U-$^{14}$C]PCP by straw compost and remediated soil were studied under field simulating conditions before and after three months of enrichment with PCP in a percolator. After PCP adaptation, the straw compost mineralized up to 56% of the [$^{14}$C]PCP (Paper II, Fig. 2). No partial dechlorination of PCP was found. The native straw compost did not mineralize PCP, but partial dechlorination of PCP occurred a) at pH 8 near thermophilic conditions (45°C) and b) at pH 7 in aerobic and mesophilic conditions (Paper II, Fig. 3). No biotransformation reactions occurred at room temperature (25°C) at pH 8 (Paper II, Fig. 3 and Table 2). Enrichment in the percolator enhanced the mineralization of remediated soil to 56% compared to the native remediated soil which mineralized 24% of [$^{14}$C]PCP added (Paper II, Table 3). Both inoculants studied here showed effective mineralization of PCP when they were adapted to PCP in the percolator.

The contaminated soil alone (chlorophenols 850 mg (kg dry wt)$^{-1}$) showed negligible (0.4% in one week) *in situ* mineralization activity for [$^{14}$C]PCP when studied in phosphate buffer (pH 7) (Paper III, p. 374).

In a bench-scale experiment, an average of 60% mineralization of radiolabeled pentachlorophenol ([$^{14}$C]PCP) was obtained in four weeks in 1-kg minipiles imitating composting with or without inocula (Paper III, Fig. 1). This result suggested that a major part of chlorophenols was completely mineralized.

### *3.1.3 Biotransformation; methylation*

Insignificant methylation of chlorophenols to chlorinated anisoles was observed under laboratory conditions that were chosen for pilot-scale and full-scale composting; only trace amounts of chloroanisoles were detected in PCP-adapted remediated soil (Paper II, Table 3). In pilot-

scale compost piles, the chloroanisole concentrations were from 0.1 to 2.4 mg (kg dry wt) $^{-1}$, but the concentrations decreased to less than 0.1 mg (kg dry wt) $^{-1}$ during the composting (Paper III, pp. 374-375).

### 3.1.4 Biotransformation; polymerization

The fate of chlorophenols during composting of sawmill soil and impregnated wood was further studied to see whether chlorophenols, in addition to mineralization, would form any harmful metabolites. Polymerization of chlorophenols was studied by determining the MWD of organic halogen compounds during the bioremediation. Organic chlorine compounds appeared in high molecular weight sizes indicating that binding to soil organic matter had taken place during the long exposure time to the wood preservative Ky-5 in the contaminated soil (Paper IV, Fig. 2). Despite of the same pollutant, Ky-5, the MWDs of halogen compounds differed in different types of soil: in organic soil, the halogen compounds appeared in higher molecular size than in sandy soil (Paper I, Fig. 1).

No major polymerization or dimerization occurred during the pilot-scale composting, but the polymerized fraction was not either degraded or remobilized (Paper IV, Fig. 2). Large amounts PCDD/Fs originating from the wood preservative were found (Paper IV, Table 2). The congener composition of PCDD/Fs resembled the one in original wood preservative (Paper IV, Table 3). However, PCDD/Fs represented only a part of the dimeric fraction of organochlorine compounds in the compost piles (Paper IV, Fig. 3, upper panel), and furthermore, the small molecular weight fraction represented only a minor part of the extractable organic chlorine in contaminated soil (Paper IV, Fig. 3, lower panel). Other dimeric compounds may have consisted of polychlorinated phenoxyphenols, biphenyls and diphenyl ethers, which the original wood preservative Ky-5 also contained. In conclusion, no harmful compounds were formed, but the existing onės such as PCDD/Fs were not removed during the biological treatment of the chlorophenol-contaminated soil.

### 3.1.5 Bioavailability of organic halogen compounds from bioremediated soil

Even though bioremediation did not increase the polymerized fraction of organic chlorine compounds in soil in this case, polymerization had already occurred in contaminated soil with time. In order to study the bioavailability of the polymerized organic chlorine compounds to soil organisms, earthworms from kitchen compost *(Eisenia andrei)* were exposed to two bioremediated soils, one sandy and the other organic soil, for 12 or 25 days at 22°C.

The earthworms accumulated tetrahydrofuran extractable halogen compounds up to 20 times more from sandy soil than from organic soil (Table 5; Paper I, Fig. 2). This indicated that soil organic matter diminished the bioaccumulation potential of halogenated compounds to the earthworms. The MWDs of chlorine compounds in all exposed earthworms followed the same pattern having two major size classes: components with apparent molecular weights of 1000 g mol$^{-1}$ and 6000 g mol$^{-1}$, respectively (Fig. 5; Paper I, Fig. 3). Thus, chlorinated compounds ending up in earthworms from contaminated soil by ingestion or through skin appeared in large molecules, in fact in larger molecules than those found in the corresponding soil, which the earthworms had been exposed to.

In order to study the nature of the bonds in the organic halogen compounds, THF-extracts of contaminated soil and earthworms were subjected to hydrolysis. In the organic contaminated soil, mild hydrolysis did not change MWD of the UV-absorbing compounds, but after more drastic hydrolysis, the UV-absorbing compounds appeared to be smaller suggesting that the humus had become fragmented (Fig. 4). THF-extract of the earthworms exposed to the sandy contaminated soil for 12 days was also subjected to mild hydrolysis. MWD of the halogen compounds in earthworm extracts after hydrolysis showed that halogenated compounds were split into smaller molecules (Fig. 5). In conclusion, organic halogen compounds were bound weaker in earthworms than in soil. However, results did not clearly show whether the

**Table 5.** Extractable organic halogen (EOX) contents in earthworms, contaminated soils and in soil mixture before and after incubation of the earthworms in contaminated soil and approximate mass balance calculations of organic chlorine compounds.

| EOX | Sandy soil | | Organic soil | |
|---|---|---|---|---|
| | Cl, µg (g dry wt)$^{-1}$ | mg Cl[e] | Cl, µg (g dry wt)$^{-1}$ | mg Cl[e] |
| EOX in clean earthworms | 18 | 0.076 | 18 | 0.067 |
| EOX in remediated soil before incubation | 120 | 59 | 130 | 23 |
| EOX in remediated soil after incubation[a] | 74 | 41 | 100 | 20 |
| EOX in soil mixture before incubation[b] | 99 | 28 | 88 | 11 |
| EOX in soil mixture after 12 days of incubation[c] | 57 | 14 | 87 | 8 |
| EOX in the exposed earthworms after 12 days of incubation[d] | 840 | 4.8 | 47 | 0.29 |

a) no earthworms added (reference soil sample). b) Soil mixture consisted of remediated sawmill soil and horse manure. The EOX values were estimated from separate measurements of the remediated soil and the horse manure. c) Earthworms were incubated in soil mixture, fed with horse manure, and the containers were irrigated during incubation. d) Estimated value corresponding to the mass of the earthworms at t = 0, and chlorine concentration in the harvested earthworms at t = 12 d. The gut content of the living earthworm was emptied before measurement. Dead earthworms were removed every third day and not included to the analyses. e) Chlorine content per container.

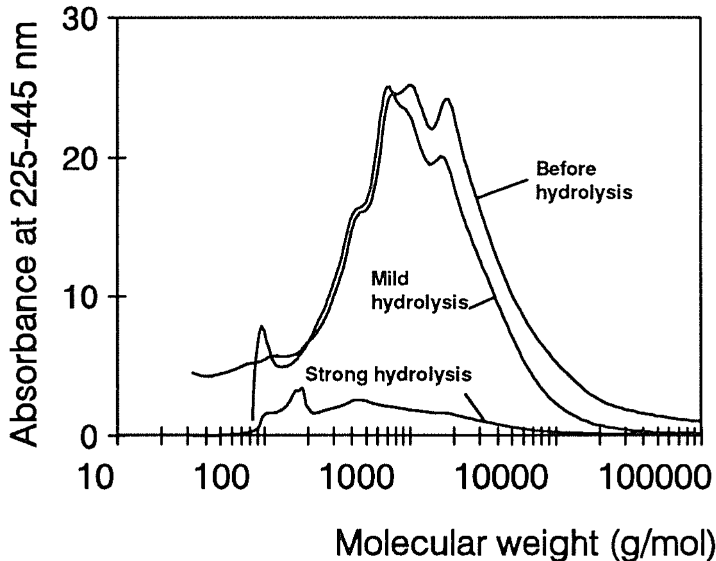


**Fig. 4.** MWDs of the UV-absorbing compounds in the organic contaminated soil before hydrolysis, after mild hydrolysis (KOH, pH 10.3, 82 °C for 1 h) and after strong hydrolysis (3.75 M KOH, water:methanol 1:1, 100 °C for 30 min).

high molecular weight compounds in earthworms were originally large, or did the earthworms form them in their body from smaller molecules.

### *3.1.6 Threshold values for toxic effects of PCP in soil*

The toxicity assessed by luminescent bacteria tests decreased during the pilot-scale composting, and it followed the chlorophenol concentrations in the compost piles (Paper IV, Figures 4 and 5). The threshold value for chlorophenol toxicity appeared to be 200 mg total chlorophenols per kg dry weight. In all the compost piles, there was an apparent decrease in toxicity assessed by the luminescent bacteria test. Salminen et al. (1995) studied the effects of PCP (0, 50 and 500 mg (kg dry wt) $^{-1}$) on soil animal communities and decomposition in a laboratory microcosms. Since sorption to soil organic matter reduces the toxicity of PCP (van Gestel and Ma, 1988), high concentration of spiked PCP was needed before any harmful effects were seen on the soil organisms. The high PCP concentration of 500 mg (kg dry wt) $^{-1}$ reduced the microbial biomass and activity measured by ATP content of the soil and by soil respiration, respectively, as well as enhanced the nutrient mobilization while the low PCP concentration had no effect (Salminen et al., 1995). Reduction of microorganisms due to high concentration of PCP was supposed to reduce densities of microbivorous animals (Salminen, 1996).

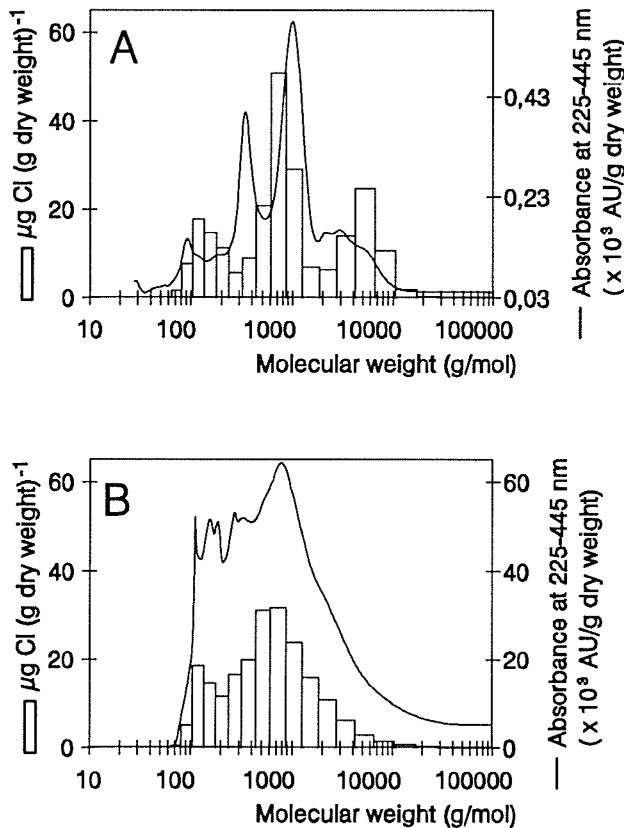


**Fig. 5.** MWD of organic chlorine compounds in THF-extracts of the earthworms exposed to the contaminated sandy soil before (**A**) and after (**B**) mild hydrolysis (KOH, pH 10.3, 82 °C for 1 h).

## 3.2 Microbiology of chlorophenol degradation during bioremediation

### *3.2.1 Mixed cultures: straw compost and remediated soil as inocula*

A pilot-scale composting of chlorophenol-contaminated soil was performed to compare chlorophenol degradation by two different inoculants, straw compost and remediated soil, with that by indigenous soil microorganisms. When enriched in the laboratory, both straw compost and remediated soil showed effective mineralization of PCP (Paper II, Figures 2 and 4). In the field, the biodegradation of chlorophenols was efficient and fast irrespective to the addition of the inocula (Paper III, Fig. 2; Paper IV, Table 1). Frequent mixing and control of the nutrient level enhanced the chlorophenol degradation by the indigenous microorganisms in the contaminated soil.

### *3.2.2 The quest for the identity of chlorophenol-degrading microorganisms*

From the pilot-scale and full-scale composts studied in this paper, 100 bacterial strains were isolated and their ability to degrade chlorophenols has partly been tested. From the different general and selective plates, randomly chosen bacterial colonies were isolated and characterized using the Biolog identification system. Of the isolated strains, 28 were gram-positive and 72 gram-negative bacteria. Since most of the gram-positive isolates did not match any species in the Biolog GP database, identification based on whole cell fatty acid composition was also performed for gram-positive isolates.

#### *3.2.2.1 Gram-positive isolates*

Identification of the gram-positive isolates succeeded either by whole-cell fatty acid analysis or by carbon source utilization patterns for only a few strains (shown in italics in Table 6). Gram-positive isolates originated mainly from R8 and 1/5 TGY plates, and they were not able to grow on PCP as the sole carbon source (Table 6). However, when chlorophenol degradation was tested in liquid culture of pregrown cells in DSM-65 medium, 11 gram-positive isolates were able to remove spiked chlorophenols from the growth medium. A mineralization experiment with [U- $^{14}$C]PCP revealed that less than 2% of the radiolabeled PCP was mineralized in 25 days (Table 6). *Mycobacterium chlorophenolicum* PCP-1, that was used as a reference strain, mineralized 13.5 ± 0.5% of the radiolabeled PCP in 25 days under similar conditions while the rest of the radioactivity was recovered in the growth medium. The radioactivity of the growth medium after mineralization experiment was measured for three isolates, and found to be between 40 and 77% of the total radioactivity (Table 6). The results suggested that gram-positive isolates transformed the chlorophenols, possibly via dechlorination, but no growth-linked metabolism occurred. Another possibility is that chlorophenols sorbed to the isolated gram-positive bacteria which tended to grow as hydrophobic pellets. Brandt et al. (1997) studied sorption of PCP to the cells of *Mycobacterium chlorophenolicum* PCP-1 and suggested that the ionic form of PCP is reversibly adsorbed to cell walls by ion exchange, whereas undissociated PCP is associated with much stronger interactions to hydrophobic side chains of *e.g.* mycolic acids in the cell wall of *Mycobacterium chlorophenolicum* PCP-1. The uptake of hydrophobic compounds by *e.g.* a *Rhodococcus* strain has been shown to occur not only after dissolution in the aqueous phase, but also through direct contact of the *Rhodococcus* with the hydrophobic phase-aqueous phase interface, a mechanism called interfacial uptake (Bouchez et al., 1997).

#### *3.2.2.2 Gram-negative isolates*

Of the 72 isolated gram-negative strains, the majority belonged to *Pseudomonas* sp. according to the Biolog identification system (Table 7, Fig. 6). Most of these Pseudomonads were isolated from PCP plates, and all of them were able to grow on 2 mM PCP as sole carbon source. The most abundant species suggested by Biolog was *Pseudomonas citronellolis*, although colony characteristics of the similarly named 17 isolates varied. With a few exceptions, all of the Pseudomonads used L-glutamic acid as a substrate in Biolog microtitre plates (Table 7). This may have a role on chlorophenol degradation since a common PCP degradation pathway of

gram-negative bacteria involves an initial oxygenolytic dechlorination of PCP to tetrachloro-*p*-hydroquinone. In the following step, tetrachlorohydroquinone dehalogenase converts tetrachloro-*p*-hydroquinone to dichlorohydroquinone in stepwise reactions where two glutathione molecules are needed as reducing equivalents for each chlorine atom removed (Copley, 1997). Essentially, glutamic acid is a precursor of glutathione which is thus required for reductive dechlorination of tetrachloro-*p*-hydroquinone. On the other hand, addition of glutamate increased concentration of NaPCP that *Pseudomonas* spp. UG25 could tolerate and degrade (Leung et al., 1997), possibly by lowering toxic effect of PCP, since PCP when served as the only carbon source have been shown to cause cellular damage to e.g. *Sphingomonas chlorophenolica* (former *Flavobacterium*) ATCC 39723 (Topp et al., 1988). Pseudomonads are abundant in contaminated soil and compost since they are fast growers and they can tolerate contaminants fairly well. Two *Pseudomonas fluorescens* strains has been shown to increase the levels of outer membrane protein expression in the presence of PCP which may explain their tolerance to PCP (Cerda et al., 1997). However, Pseudomonads may not be the effective chlorophenol degraders as individual species.

Since the identification library in the Biolog system was originally designed for clinical isolates, it is far from being perfect for environmental samples unless the user-made additional databases are used. Even a very small database (Fig. 3), consisting of compilation of nine *Sphingomonas* reference strains, two *Pseudomonas* reference strains and *Burkholderia cepacia*, allowed identification of 17 more strains.

Since the isolates were randomly picked up from different selective and general agar plates, they most likely represented the fast-growing bacteria, but still could not at all comprise the whole microbial community involved in bioremediation process. Furthermore, the results suggest that synergism may take place and thus emphasized the need to study the whole microbial community instead of isolated pure organisms.

**Table 6.** Gram-positive isolates. Similarity index is given in parentheses.

| Identification of an isolate | | Growth on | Chlorophenol degradation in liquid culture [d] | | |
|---|---|---|---|---|---|
| Code | Closest species name suggested by BI [a] or FA[b] | PCP plates [c] | Removal | Mineralization | Left in liquid broth |
| 1011 | no ID (FA) | no | 100% PCP, 92% TeCP, 100% TCP | 1.1 ± 0.4% /25 d | nm |
| 1015 | no ID (FA) | no | no | nm[e] | |
| 1017 | no ID (FA) | no | 80-90% PCP, 40-60% TeCP, 30% TCP | 0.9 ± 0.2% / 25 d | nm |
| 1020B | no ID (BI) | no | 60% PCP, 95% TeCP, 100% TCP | nm | |
| 1024 | no ID (FA) | no | 100% PCP, 95% TeCP, 100% TCP | 1.1 ± 0.2% / 25 d | 40% |
| 1033 | no ID (FA) | no | no | nm | |
| 1036 | no ID (FA) | no | no | nm | |
| 1039 | Genus ID: *Streptomyces* (0.585, FA) | no | 100% PCP, 100% TeCP, 100% TCP | 0.6 ± 0.2% / 25 d | nm |
| 1040 | no ID (FA) | no | no | nm | |
| 1041 | Genus ID: *Streptomyces* (0.555, FA) | no | 90% PCP, 80% TeCP, 50% TCP | 1.1 ± 0.1% / 25 d | 77% |
| 1042 | no ID (FA) | no | no | 0.4 ± 0.3% / 25 d | nm |
| 1043 | no ID (FA) | no | no | nm | |
| 1047 | *Curtobacterium flaccumfaciens pv oortii* (0.851, BI) | weak | 70% PCP, 80% TeCP, 100% TCP | nm | |
| 1052 | *Bacillus licheniformis* (0.526, BI) | no | no | nm | |
| 1063 | no ID (BI) | weak | no | nm | |
| 1065 | *Streptomyces violaceusniger* (0.637, BI) | no | no | nm | |
| 1067 | no ID (FA) | no | 100% PCP, 90% TeCP, 100% TCP | 2.1 ± 2% / 25 d | nm |
| 1068 | *Streptomyces violaceusniger* (0.551, FA) | no | 100% PCP, 100% TeCP, 100% TCP | 2.6 ± 0.5% / 25 d | 50 ± 10% |
| 1071 | *Streptomyces violaceusniger* (0.674, FA) | no | 100% PCP, 100% TeCP, 100% TCP | 0.9 ± 0.2% / 25 d | nm |
| 1095 | *Curtobacterium citreum* (0.698, BI) | no | no | nm | |
| 1099 | *Curtobacterium citreum* (0.529, BI) | no | no | nm | |
| 1100 | no ID (FA) | no | no | 0.2 ± 0.04% / 25 d | nm |
| 1101 | *Bacillus polymyxa* (0.791, BI) | no | no | nm | |
| 1110 | no ID (FA) | nm | no | nm | |
| 1111 | Genus ID: *Streptomyces* (0.529, FA) | nm | no | 1 ± 0.03% / 25 d | nm |
| 1117 | *Streptomyces griseoflavus* (0.504, FA) | nm | 30% PCP, 0% TeCP, 0% TCP | nm | |
| 1133 | no ID (BI) | nm | nm | nm | |
| 1134 | no ID (BI) | nm | nm | nm | |

a) BI = Biolog Identification System (carbon source utilization pattern). b) FA = MIDI system (whole cell fatty acid analysis). c) 2 mM PCP (Pentachlorophenol) as a sole carbon source. d) Test conditions: see *Materials and Methods*. PCP = pentachlorophenol; TeCP = 2,3,4,6-tetrachlorophenol; TCP = 2,4,6-trichlorophenol. e) nm = not measured.

**Table 7a.** Gram-negative isolates [a].

| | Identification of an isolate | Source | | | Growth on [d] | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Code | Species or genus suggested by Biolog | Pile [b] no | Medium [c] and dilution | Date | PCP [e] | E1 | B6 | H5 | F10 | Other |
| 941001 | *Pseudomonas citronellolis* | Pilot 1 | 0.2 mM PCP -5 | 6/94 | + | + | + | + | + | |
| 941002 | *Pseudomonas citronellolis* | Pilot 1 | 0.2 mM PCP -5 | 6/94 | + | + | + | — | + | |
| 941005 | *Pseudomonas citronellolis* | Pilot 1 | 1 mM PCP -4 | 7/94 | + | + | + | + | + | |
| 941006 | *Pseudomonas citronellolis* | Pilot 1 | 1 mM PCP -4 | 8/94 | + | + | + | — | + | |
| 941009 | *Pseudomonas citronellolis* | Pilot 1 | 1 mM PCP -4 | 8/94 | + | + | + | — | + | |
| 941025 | *Pseudomonas citronellolis* | Pilot 1 | R8 | 8/94 | + | + | + | + | + | |
| 941016 | *Sphingomonas terrae* | Pilot 1 | 1/5 TGY -6 | 8/94 | — | — | — | — | — | B2, C11 |
| 941048 | *Sphingomonas terrae* | Pilot 2 | R8 -6 | 9/94 | (+) | + | — | — | — | |
| 941018 | *Sphingomonas parapaucimobilis* | Pilot 1 | 1/5 TGY -6 | 9/94 | — | — | + | — | — | |
| 941049 | *Serratia marcescens* | Pilot 2 | R8 -5 | 9/94 | + | + | + | — | — | |
| 941050 | *Sphingobacterium mizutaii* | Pilot 2 | R8 -7 | 6/94 | — | — | + | — | — | |
| 941010 | Genus ID: *Pseudomonas* | Pilot 1 | 1 mM PCP -4 | 9/94 | + | nm [f] | nm | nm | nm | nm |
| 941081 | No ID | Pilot 4 | 1 mM PCP -3 | 7/94 | + | — | + | — | — | |
| 941014 | No ID | Pilot 1 | R8 -6 | 9/94 | — | — | + | — | + | |
| 941034 | No ID | Pilot 2 | 1/5 TGY -6 | 9/94 | + | nm | nm | nm | nm | |
| 941031 | No ID | Pilot 2 | 0.2 mM PCP -5 | 6/94 | + | — | + | — | + | |
| 941013 | No ID | Pilot 1 | R8 -6 | 8/94 | — | nm | nm | nm | nm | nm |
| 941045 | No ID | Pilot 2 | R8 -7 | 9/94 | + | — | + | — | — | |

a) PCP-degradation ability tested in liquid culture (see *Materials and methods*). b) Pilot = Pilot-scale piles. c) PCP = pentachlorophenol as a sole carbon source; R8 = R8 medium (*Amner et al., 1989*); TGY = Trypticase-Soy-Agar. d) E1 = *p*-hydroxy-phenyl-acetic acid (5); B6 = α-D-glucose (42); H5 = phenyl-ethylamine (40); F10 = L-glutamic acid (78); B2 = D-fructose (10); C11 = methyl pyruvate (83). Codes refer to the Biolog wells. e) 2 mM pentachlorophenol as a sole carbon source on agar plates. f) nm = not measured.

**Table 7b.** Gram-negative isolates (Pseudomonads).

| | Identification of an isolate | Source | | | Growth on [c] | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Code | Species or genus suggested by Biolog | Pile [a] no | Medium [b] & dilution | Date | PCP [d] | E1 | B6 | B2 | F10 | E6 |
| 941019 | *Pseudomonas citronellolis* | Pilot 1B | R8 -6 | 8/94 | + | + | (+) | — | + | — |
| 941054 | *Pseudomonas citronellolis* | Pilot 3 | 1 mM PCP -4 | 9/94 | + | + | + | — | + | — |
| 941055 | *Pseudomonas citronellolis* | Pilot 3 | 2 mM PCP -3 | 11/94 | + | + | (+) | — | + | — |
| 941058 | *Pseudomonas citronellolis* | Pilot 3 | 1 mM PCP -4 | 8/94 | + | + | + | — | + | — |
| 941087 | *Pseudomonas citronellolis* | Pilot 4 | 2 mM PCP -4 | 11/94 | + | + | + | — | + | — |
| 951122 | *Pseudomonas citronellolis* | Full 1A | 2 mM PCP -2 | 9/95 | + | + | | + | + | — |
| 951123 | *Pseudomonas citronellolis* | Full 1A | 2 mM PCP -2 | 9/95 | + | + | + | — | + | — |
| 951126 | *Pseudomonas citronellolis* | Full 1B | 2 mM PCP -2 | 9/95 | + | + | + | — | + | — |
| 951131 | *Pseudomonas citronellolis* | Full 1D | 2 mM PCP -4 | 9/95 | + | + | + | — | + | — |
| 951132 | *Pseudomonas citronellolis* | Full 1D | 2 mM PCP -2 | 9/95 | + | nm [e] | nm | nm | nm | nm |
| 951141 | *Pseudomonas citronellolis* | Full 3A | 2 mM PCP -2 | 9/95 | + | + | + | — | + | — |
| 951144 | *Pseudomonas citronellolis* | Full 3B | 2 mM PCP -3 | 9/95 | + | + | + | — | + | — |
| 951125 | *Pseudomonas citronellolis* | Full 1B | 2 mM PCP -3 | 9/95 | + | | + | — | — | — |
| 941032 | *Pseudomonas aeruginosa* | Pilot 2 | 1 mM PCP -4 | 9/94 | + | + | + | — | + | — |
| 941075 | *Pseudomonas aeruginosa* | Pilot 4 | 2 mM PCP -3 | 10/94 | + | + | + | — | — | — |
| 941076 | *Pseudomonas aeruginosa* | Pilot 4 | 1 mM PCP -3 | 10/94 | + | + | + | — | — | — |
| 941077 | *Pseudomonas aeruginosa* | Pilot 4 | 2 mM PCP -4 | 8/94 | + | nm | nm | nm | nm | nm |
| 941083 | *Pseudomonas aeruginosa* | Pilot 4 | 2 mM PCP -3 | 6/94 | + | — | + | — | + | — |
| 951128 | *Pseudomonas aeruginosa* | Full 1C | 2 mM PCP -2 | 9/95 | + | + | + | — | + | — |
| 951129 | *Pseudomonas aeruginosa* | Full 1C | 2 mM PCP -2 | 9/95 | + | — | + | — | + | — |
| 941082 | *Pseudomonas chlororaphis* | Pilot 4 | 2 mM PCP -3 | 10/94 | + | + | + | — | + | — |
| 941079 | *Pseudomonas chlororaphis* | Pilot 4 | 2 mM PCP -5 | 11/94 | + | + | + | — | + | — |
| 951130 | *Pseudomonas nitroreducens* | Full 1C | 2 mM PCP -3 | 9/95 | + | — | — | — | + | + |
| 951136 | *Pseudomonas putida* Type A1 | Full 2A | 2 mM PCP -3 | 9/95 | + | + | + | — | + | — |
| 951138 | *Pseudomonas putida* Type A1 | Full 2B | 2 mM PCP -2 | 9/95 | + | — | + | — | + | — |
| 951145 | *Pseudomonas putida* Type A1 | Full 3B | 2 mM PCP -3 | 9/95 | + | + | + | — | + | — |
| 941028 | *Pseudomonas fluorescens* Type F | Pilot 2 | 0.2 mM PCP -5 | 6/94 | + | — | + | — | + | — |
| 941088 | *Pseudomonas fluorescens* Type B | Pilot 4 | 0.02 mM PCP -5 | 6/94 | + | + | + | — | + | — |
| 941089 | *Pseudomonas fluorescens* Type A | Pilot 4 | 0.02 mM PCP -5 | 6/94 | + | + | + | — | — | — |
| 941056 | Genus: *Pseudomonas* | Pilot 3 | 1 mM PCP -3 | 11/94 | + | + | + | — | + | — |
| 951135 | Genus: *Pseudomonas* | Full 2A | 2 mM PCP -3 | 9/95 | + | + | + | — | — | — |
| 951140 | Genus: *Pseudomonas* | Full 3A | 2 mM PCP -2 | 9/95 | + | + | + | — | + | — |
| 951139 | Genus: *Pseudomonas* | Full 2B | 2 mM PCP -2 | 9/95 | + | + | + | — | + | — |

a) Full = Full-scale windrows; Pilot = Pilot-scale piles. b) PCP = pentachlorophenol as a sole carbon source; R8 = R8 medium (Amner et al., 1989). c) E1 = p-hydroxy-phenyl-acetic acid (5); B6 = α-D-glucose (42); F10 = L-glutamic acid (78); B2 = D-fructose (10); E6 = D,L-lactic acid (45). Codes refer to the Biolog wells. d) 2 mM pentachlorophenol as a sole carbon source on agar plates. e) nm = not measured.

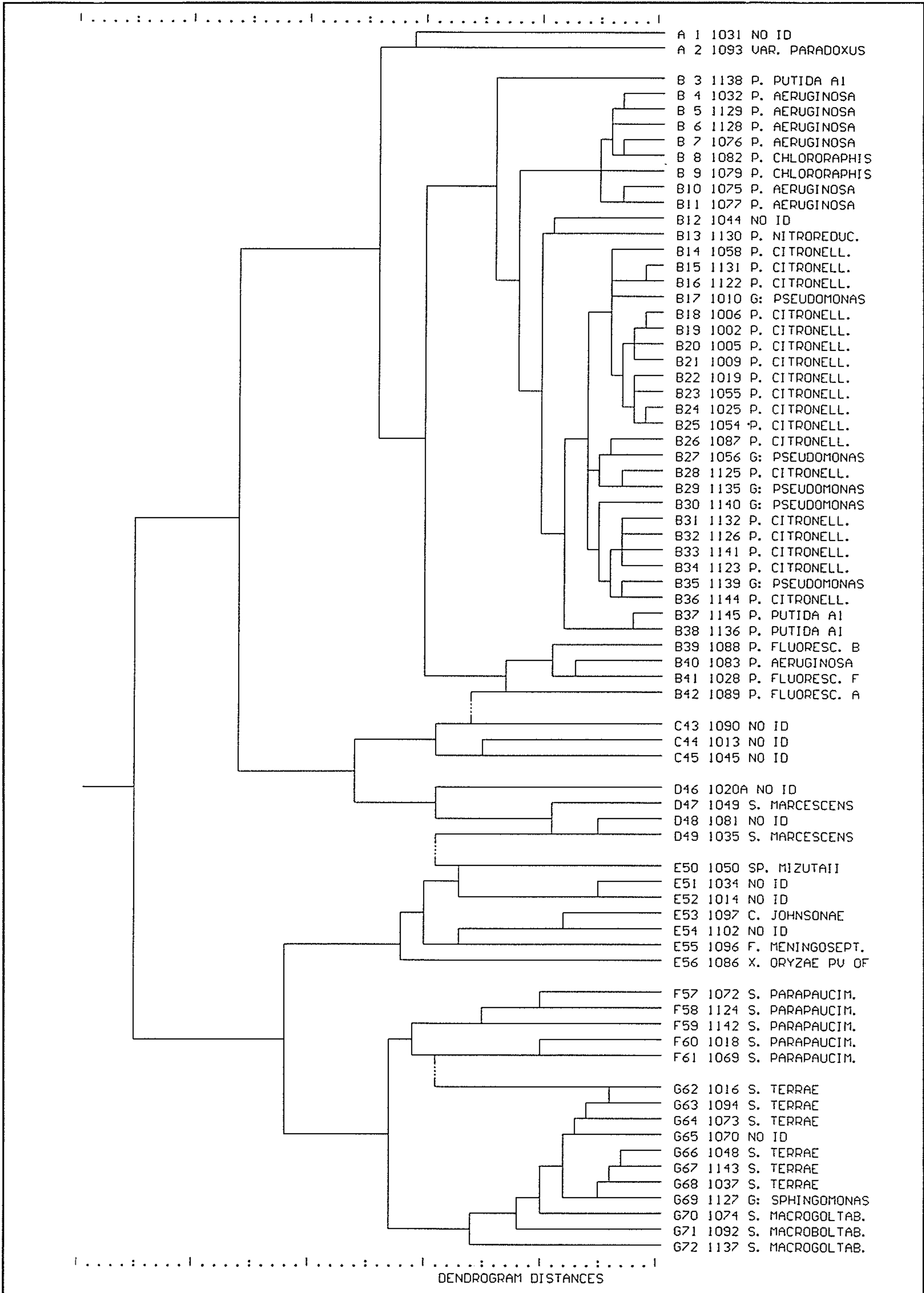


**Fig. 6.** Dendrogram of the isolated gram-negative bacteria.

**Table 7c.** Gram-negative isolates.

| | Identification of an isolate | Source | | | Growth on [c] | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Code | Species or genus suggested by Biolog | Pile [a] no | Medium [b] and dilution | Date | PCP [d] | E1 | B6 | C11 | F10 | Other |
| 941037 | *Sphingomonas terrae* | Pilot 2 | 1/5 TGY -6 | 7/94 | — | — | — | + | — | D11, A5 |
| 941073 | *Sphingomonas terrae* | Pilot 3 | 1/5 TGY -6 | 8/94 | — | — | (+) | — | — | B11 |
| 941094 | *Sphingomonas terrae* | Pilot 4 | 1/5 TGY -6 | 9/94 | — | — | + | — | — | |
| 951143 | *Sphingomonas terrae* | Full 3B | 1/5 TGY -7 | 9/95 | nm [e] | — | — | (+) | — | |
| 941069 | *Sphingomonas parapaucimobilis* | Pilot 3 | 1/5 TGY -6 | 9/94 | — | — | + | — | — | |
| 941072 | *Sphingomonas parapaucimobilis* | Pilot 3 | 1/5 TGY -7 | 9/94 | (+) | — | + | — | — | |
| 951124 | *Sphingomonas parapaucimobilis* | Full 1A | 1/5 TGY -7 | 9/95 | nm | — | + | — | — | |
| 951142 | *Sphingomonas parapaucimobilis* | Full 3A | 1/5 TGY -8 | 9/95 | nm | — | (+) | — | — | |
| 941092 | *Sphingomonas macrogoltabidus* | Pilot 4 | R8 -7 | 11/94 | + | — | — | + | — | D11 |
| 951137 | *Sphingomonas macrogoltabidus* | Full 2B | 1/5 TGY -7 | 9/95 | nm | — | (+) | — | — | |
| 951127 | Genus: *Sphingomonas* | Full 1B | 1/5 TGY -8 | 9/94 | nm | — | — | — | — | A5, H8 |
| 941086 | *Xanthomonas oryzae pv. o.F.* | Pilot 4 | 0.2 mM PCP -5 | 6/94 | + | — | + | — | — | |
| 941093 | *Variovorax paradoxus* | Pilot 4 | 1/5 TGY -4 | 6/94 | — | — | (+) | — | + | D11 |
| 941035 | *Serratia marcescens* | Pilot 2 | 1/5 TGY -8 | 9/94 | — | nm | + | nm | nm | nm |
| 941096 | *Flavobacterium meningosepticum* | Soil | 1/5 TGY -5 | 6/94 | — | — | + | — | — | |
| 941097 | *Cytophaga johnsonae* | Soil | 1/5 TGY -5 | 6/94 | — | — | + | — | — | |
| 941038 | No ID | Pilot 2 | 1/5 TGY -7 | 6/94 | + | — | + | — | — | |
| 941044 | No ID | Pilot 2 | R8 -5 | 9/94 | + | + | (+) | — | + | |
| 941070 | No ID | Pilot 3 | 1/5 TGY -6 | 9/94 | — | — | + | — | — | |
| 941098 | No ID | Soil | 1/5 TGY -5 | 6/94 | — | — | + | — | + | |
| 951102 | No ID | Rem. soil | 1/5 TGY -6 | 6/94 | — | — | + | — | + | |

a) Full = Full-scale windrows; Pilot = Pilot-scale piles; Rem. soil = remediated soil (Papers II & III); Soil = contaminated soil. b) PCP = penta-chlorophenol as a sole carbon source; R8 = R8 medium (Amner et al., 1989); TGY = Trypticase-Soy-Agar. c) E1 = p-hydroxy-phenyl-acetic acid (5); B6 = α-D-glucose (42); C11 = methyl pyruvate (83); F10 = L-glutamic acid (78); D11 = β-hydroxy-butyric acid; A5 = Tween 40 (33); B11 = D-mannitol (82); H8 = 2,3-butanediol (64). Codes refer to the Biolog wells. d) 2 mM pentachlorophenol as a sole carbon source on agar plates. e) nm = not measured.

### 3.2.3 *Microbial community changes during bioremediation*

*3.2.3.1 Methodological aspects of community analysis*

Community structure during a full-scale bioremediation by composting chlorophenol-contaminated soil was studied by the utilization pattern of a large range of substrates using **Biolog®** microtitre plates (Paper V). The data from **Biolog®** microtitre plates was analyzed using the toolbox of **Matlab®** mathematical software. The areas under the substrate utilization curve were integrated for each substrate used, and they were used for principal component analysis. This kinetic analysis has several advantages and an increasing number of users: multiple readings of absorbance over a time course of incubation overcome the effect of nonlinear color development (Kerstens et al., 1997); the integral of the areas under the curve combines several measurements to a single comparable value (Guckert et al., 1996); and the kinetic analysis enables to study behavior of an individual substrate thus generating more information than just clustering different groups as *e.g.* average well color development (AWCD) method when combined with a single-plate reading is restricted to do. However, effective kinetic-type analysis of community structure by Biolog should involve standardized inoculum densities among samples, and/or normalization of the data during analysis (Garland, 1997). In our composts, the number of bacteria growing on *e.g.* TGY plates maintained fairly constant during the composting (Fig. 7.), so it was closely argued to use kinetic-type approach with the constant inoculum size and the normalization of data for PCA.

Although the purpose of Biolog measurement should be an initial metabolism of a given substrate of interest, Winding and Hendriksen (1997) claimed that metabolic fingerprinting of bacterial communities using Biolog plates depends on aerobic growth of a fraction of the community. They showed that cell numbers of inoculated soil bacteria increased from $2 \times 10^7$ to a maximum of $1.6 \times 10^9$ cells $ml^{-1}$ during 96 hours of incubation. The number of cells in the control well reached the same level as in the other wells (Winding and Hendriksen, 1997), possibly due to peptone and yeast extract which are added to all the wells to support growth without formazan formation (Bochner, 1978). The trace of soil nutrients present in the inoculum may also support growth in the control well.

Biolog is a good tool for community analysis if the number of microorganisms is large enough for appropriate starting inoculum size, as it is in a rich compost environment. Mills and Bouma (1997) stated that the use of short-term (less than 24 hours) incubation times provides a profile that is more reflective of *in situ* activity. Indeed, when community level physiological differences are studied in a "poor" environment, long incubation times are needed for color development in Biolog microtitre wells. The cell number of approximately $10^8$ cells $ml^{-1}$ seems to be needed for color development (Winding and Hendriksen, 1997; Haack et al., 1995). Incubation time of several days requires an extended growth on wells. The die-out of one bacterial species may produce carbon source for another one, which may result in formation of formazan. Thus, Biolog plates are no longer indicating either the usage of the peculiar substrate that was added to the well nor the structure of the original bacterial community.

*3.2.3.2 Substrate utilization patterns during bioremediation*

Utilization of different carbon sources in the Biolog microtitre plates, as well as glucose utilized in the substrate-induced respiration measurement, showed the potential of functional capability of the compost community. Fast-growing microorganisms responsible for utilization of easily available substrates, measured by respiratory activity and substrate utilization patterns in Biolog, originated mainly from the added bulking agents, straw compost and bark chips (Paper V, Fig. 3). Studies by Verschuere et al. (1997) on a model microbial community consisting of 2 to 3 known bacterial strains showed that the carbon substrate utilization patterns were dominated by the fastest growing strain, and that the substrate utilization pattern by the slowest growing strain was partially masked by the other ones.

Windrow-specific substrate usage for wind-

rows containing straw compost was seen, but not for windrow containing bark chips (Paper V, Fig. 4). All these characteristic substrates were either amino acids or amines. This amino-favoring guild originated from the straw compost, and it maintained active during composting. In context to isolated gram-negative bacteria, the possible role of glutamate on tolerance and degradation of PCP has already been discussed. It is noteworthy that also on the community level, L-glutamic acid seemed to be important and intensely used substrate throughout the composting period (see substrate number 78 in Fig. 4, Paper V).

The best indicator of the actual chlorophenol degradation efficiency was the number of microorganisms growing on plates with 2 mM PCP as the sole carbon source (Paper V, Table 1). The chlorophenol-degraders ($10^{2}$-$10^{4}$ CFU (g dry wt) $^{-1}$) originating from contaminated soil seemed not directly to contribute to the Biolog utilization pattern, but probably had benefited from the enhanced general microbial activity in the composts by cometabolism or synergism.

### 3.2.4 The role of additives on chlorophenol degradation

#### 3.2.4.1 Nutrients and additional carbon sources

The amendment of bulking agents such as straw compost and bark chips that contains high amounts of easily available carbon sources and active microbial consortium undoubtedly enhances the chlorophenol degradation in contaminated soil as well as it improves the overall microbial functionality and activity. The chlorophenol-degraders originating from contaminated soil seemed not directly use easily available substrates to degrade chlorophenols, but rather had benefited from the enhanced general microbial activity in the composts (Paper V).

The addition of inorganic nutrients in the form of fertilizer or easily available carbon sources such as fresh bark did not enhance the chlorophenol degradation after winter (Paper V). These factors increased the general microbial activity and the amount of biomass, and may also be needed for synergistic action of chlorophenol degradation; the key microorganisms are those chlorophenol-degraders that initiate transformation of chlorophenols to *e.g.* nonchlorinated metabolites which other microorganisms in turn will mineralize.

#### 3.2.4.2 Na-glutamate as a secondary substrate

The number of bacteria growing on general medium, 1/5 TGY, maintained at a level of $10^{7}$-$10^{8}$ CFU (g dry wt) $^{-1}$ during the composting, but the number of microorganisms growing on 2 mM PCP as a sole carbon source gradually reduced during composting (Fig. 7; Paper V, Table 1). The number of bacteria growing on 100 µM PCP amended with Na-glutamate, in turn, was rather constant in 1996 being from $10^{6}$ to $10^{7}$ CFU (g dry wt) $^{-1}$ and thus significantly higher than number of microorganisms growing on 2 mM PCP as the sole carbon source (Fig. 7). On the other hand, the amount of PCP on plates amended with Na-glutamate may have been inadequate to neither support nor inhibit the growth of the microorganisms.

#### 3.2.4.3 Biosurfactants

A variety of microorganisms produce surfactants such as glycolipids, lipopeptides and polymeric carbohydrate-lipid-protein-combinations (Desai and Banat, 1997). In Table 8., some surfactant-producing species are presented that were common in contaminated soil and composts (Tables 6.-7.).

The physiological role of the surfactants is not fully understood, but they are thought to be involved in emulsification of water-insoluble substrates, cell adherence to protect against environmental stress, and antagonistic effect towards other microorganisms (Desai and Banat, 1997). Thus, biosurfactants may play role in survival of microorganisms in contaminated soil and they may also improve biodegradation by enhancing bioavailability. For bioremediation of polychlorinated biphenyls, a consortium of genetically engineered microorganisms capable of utilizing a selective carbon source and expressing foreign genes, have been developed in which

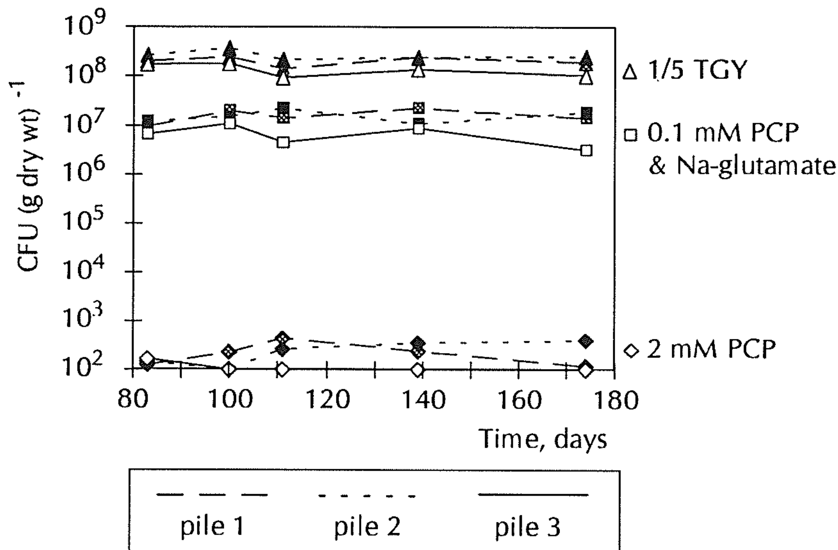


**Fig. 7.** The number of microorganisms grown on general medium (tryptone-glucose-yeast-extract, 1/5 TGY), on plates with 2 mM pentachlorophenol as the sole carbon source (2 mM PCP); or amended with Na-glutamate (0.1 mM PCP + Na-glutamate).

nonionic surfactants are used to both increase the solubility of PCBs and support the growth of surfactant-degrading strains (Lajoie et al., 1997).

Although it is not known whether *e.g. Pseudomonas* species actively produce surface-active compounds such as rhamnolipids during bioremediation, the possibility to do so can be speculated by examining factors affecting biosurfactant production. An interesting feature in regulation of rhamnolipid synthesis is that it seems to be tightly engaged to nitrogen conditions in the environment. In many cases, rhamnolipid is produced under nitrogen-limiting conditions (Ochsner et al., 1995). There was a direct correlation between rhamnolipid production and glutamine synthetase activity in *Pseudomonas aeruginosa* so that increased ammonium and glutamine concentrations repressed both reactions (Mulligan and Gibbs, 1989). Nitrate was superior to ammonium as inorganic nitrogen source (Guerra-Santos et al., 1984). Thus, both inorganic nitrogen compounds and organic ones, such as amino acids and amines (Desai and Banat, 1997) are either needed for surfactant production or are regulating it (nitrogen-glucose feedback inhibition). In conclusion, addition of nutrients and nitrogen source to composts may inhibit the production of biosurfactants and thus weaken bioavailability.

**Table 8.** Some biosurfactant-producing microorganisms (table adapted and modified from a review article by Desai and Banat, 1997).

| Biosurfactant type | Example | Organism | Reference |
|---|---|---|---|
| glycolipid | rhamnolipid | *Pseudomonas aeruginosa* | Robert et al., 1989; Van Dyke et al., 1993 |
| glycolipid | trehalose lipid | *Rhodococcus eryhtropolis* | Rapp et al., 1979 |
| cyclic lipopeptide | surfactin | *Bacillus subtilis* | Arima et al., 1968 |
| lipopeptide antibiotic | polymyxins | *Bacillus polymyxa* | Suzuki et al., 1965 |
| lipopeptide | peptide-lipid | *Bacillus licheniformis* | Yakimov et al., 1995 |
| aminolipid | serratamolide | *Serratia marcescens* | Bar-Ness et al. 1988 |
| polymeric surfactant | carbohydrate-protein-lipid | *Pseudomonas fluorescens* | Persson et al., 1988 |

### 3.2.5 The mechanism for chlorophenol degradation; complete mineralization, cometabolism or synergism?

We were not able to isolate one species that would be responsible for a complete mineralization of chlorophenols even though remarkable (60%) mineralization in intact composts was found (Paper III, Fig. 1). A part of the remaining 30 to 40% of the carbon in chlorophenols was most likely built into the bacterial biomass. Webster et al. (1997) studied carbon transformations during substrate utilization by the microbial community in an organic soil using a solid-state NMR analysis: During 28 days of incubation, the proportion of the added $^{13}C$ from glucose, that remained in soil, gradually decreased from 100 to 34%. Utilization of glucose lead to relative accumulation of O-alkyl- and alkyl-rich compounds, which are typical structural components of polysaccharides, lignin substituents, amino acids and amino sugars (Webster et al., 1997). By contrast, only 12% of the added $^{13}C$ from alanine remained in the soil after 28 days, and was apparently distributed between a range of functionalities (Webster et al., 1997). The conversion of chlorophenols to biomass is remarkably smaller in nutrient-poor environments such as in groundwater; less than 10% (wt/wt) of the chlorophenols was converted to biomass in a laboratory-scale fluidizied-bed bioreactor, where chlorophenols were the only carbon and energy source for the enrichment culture (Mäkinen et al., 1993).

Three bacteria, *Agrobacterium radiobacter*, *Pseudomonas sp.* and *Flavobacterium gleum,* have been isolated from a mixed culture capable to degrade PCP (Yu and Ward, 1997). Individual isolates showed a lower PCP degradation rate than a biodegradation medium inoculated with a combination of the three isolates, which exhibited as efficient PCP degradation as the original mixed culture (Yu and Ward, 1997). This study is an implication of a synergistic degradation of PCP which may also take place in the soil environment.

### 3.2.6 Survival of chlorophenol-degraders during the full-scale composting

The addition of inorganic nutrients in the form of fertilizer or easily available carbon sources such as fresh bark did not enhance the chloro-

phenol degradation after winter. In other words, during the full-scale composting, the revival of chlorophenol-degrading micro-organisms after winter was not very efficient. Valo et al. obtained the same kind of results already in 1985: composting started efficiently and degradation was good until the winter came. After winter, chlorophenol degradation was slow, and the total chlorophenol concentration remained in 15 mg kg dry wt $^{-1}$ at the end of the composting (Valo et al., 1986). Surprisingly similar phenomenon was seen with compost piles augmented with a pure culture of a chlorophenol-degrader (Valo et al., 1986) to that amended with nutrients and bark chips (this study). Why can indigenous chlorophenol-degraders survive for decades in contaminated soil, but not maintain their chlorophenol-degradation activity after winter in compost windrows with nutrients, pH-adjustment and aeration?

There may be many explanations including the following ones:

1) Cold climate (studies in Paper III showed no degradation activity below 10 °C). On the other hand, a high-rate bioremediation of chlorophenol-contaminated groundwater at suboptimal temperatures (4-10 °C) was observed by Järvinen et al. (1994) and Melin et al. (1998). Possibly two types of chlorophenol-degrading microorganisms were present in composts, one adapted to degrade chlorophenols under mesophilic conditions, the other one adapted to cold climate. During the composting, temperatures and community structure changed.
2) Different microbial species degraded chlorophenols in
   - clay soil windrow (Windrow 3) compared to organic soil windrows (Windrows 1 and 2), or
   - at different levels of chlorophenol concentrations (bioavailability and tolerance to high concentrations; properties enhancing attachment to chlorophenols in soil).
3) The compost environment had become more suitable and less toxic for other fast-growing microorganisms that may have conquested and colonized over the chlorophenol-degraders.
4) The chlorophenol-degraders did survive but were inactive under changed, although better, environmental conditions.
5) Bioavailability: Too small a fraction of chlorophenols available for degraders, although the chlorophenol concentration in windrow 3 decreased from 30 to 2 mg (kg dry wt) $^{-1}$ under similar conditions (Table 4),
6) Absence of a possible cosubstrate needed for degradation. The addition of bark chips may have slightly increased the chlorophenol removal (Paper VI, Table 1), maybe because there are phenolic compounds present which may act as cosubstrates for chlorinated phenols, or simply because addition of bark improves the aeration in the compost and chlorophenol degradation seemed to be aerobic.

### 3.3 Bioremediation of contaminated sawmill soil and occupational health

Bioremediation should be performed so that it does not cause further contamination or environmental health effects. Present study included several ways to confirm that chlorophenols were completely degraded during composting. Waters were collected and circulated in compost windrows, the composts were covered with tarps between the samplings and mixing. Occupational health effects were studied by following dioxin content in soil and in airborne particles, by measuring toxicity of compost soils to bacteria, and by determining bacterial release and pathogeneity from the composts to air.

#### *3.3.1 Polychlorinated dibenzo-p-dioxins and dibenzofurans in sawmill soil*

Since the chlorophenols are relatively water-soluble, they may leach into the groundwater and thus cause a threat to the humans and to the environment. PCDD/Fs are less mobile since they adsorb to the organic matter in soil (Adriaens et al., 1995). The main PCDD/Fs exposure from contaminated soil to human is skin contact or via inhalation of dust particles.

Large amounts of polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/Fs) originating from the wood preservative were found in the composts, but their concentrations did not

significantly change during the bioremediation process (Paper IV, Table 2; Paper VI, Fig. 5; and Table 9). The congener composition of PCDD/Fs resembled the one in original wood preservative (Paper IV, Table 3). Since PCDD/Fs were released from the wood chips of the former Ky-5 dipping basin, and not degraded during the bioremediation (Paper IV, Table 2), it is not recommended to treat PCDD/F-contaminated wood chips in biopiles.

### 3.3.2 Quantitation of the microbial and chemical release as airborne particles during mixing of the compost windrows

#### 3.3.2.1 Bioaerosols during bioremediation (Paper VI)

For the collection of airborne microorganisms, Andersen Viable particle sizing samplers were used. Several different selective growth media were used to enumerate bacteria and fungi in airborne particles. Most of the isolated bacteria were gram-positive and spore-forming (Paper VI, Table 1). The amounts of airborne fungi and actinomycetes were 40% higher than the background values (Paper VI, Fig. 2). None of the identified bacteria is known to demonstrate pathogenic potential (Paper VI, Table 1). The viable counts were lower in comparison to those found in municipal waste composts.

#### 3.3.2.2 Airborne PCDD/Fs during bioremediation

The concentrations of PCDD/Fs were determined in different particle sizes. The congener distribution of PCDD/Fs was similar in collected air particle fractions to the one in the compost windrows, and the concentrations of the airborne PCDD/Fs were 1000-fold higher than the previously reported atmospheric background values (Paper VI, Tables 2 and 3).

Particle size fractions were re-grouped into three classes: 9.0-10.0 µm, 1.1-9.0 µm and 0.43-1.1 µm (Fig. 8.). The amounts of PCDD/Fs varied randomly between different size fractions. It seemed, however, that during the first samplings (after the tarps were removed, but the compost windrows were not yet mixed) small particles with diameter of less than 9 µm predominated, and when the windrows were mixed, larger particles (with diameter of 9.0 - 10.0 µm) were released (Fig. 8). Particles of a diameter less than 1.1 µm accounted for 3 to 63% (average 22%) of total PCDD/Fs. Relative concentrations of PCDD/Fs in particles with diameter larger than 1.1 µm were between 37 and 96% (average 78%) of the total PCDD/Fs. According to studies of Kurokawa et al. (1996), particles of diameter less than 1.1 µm accounted for over 50% of total PCDD/Fs in airborne particulates. In our study, the amounts of PCDD/Fs varied randomly between different size fractions, but most of the PCDDs were recovered in fractions larger than 1.1 µm.

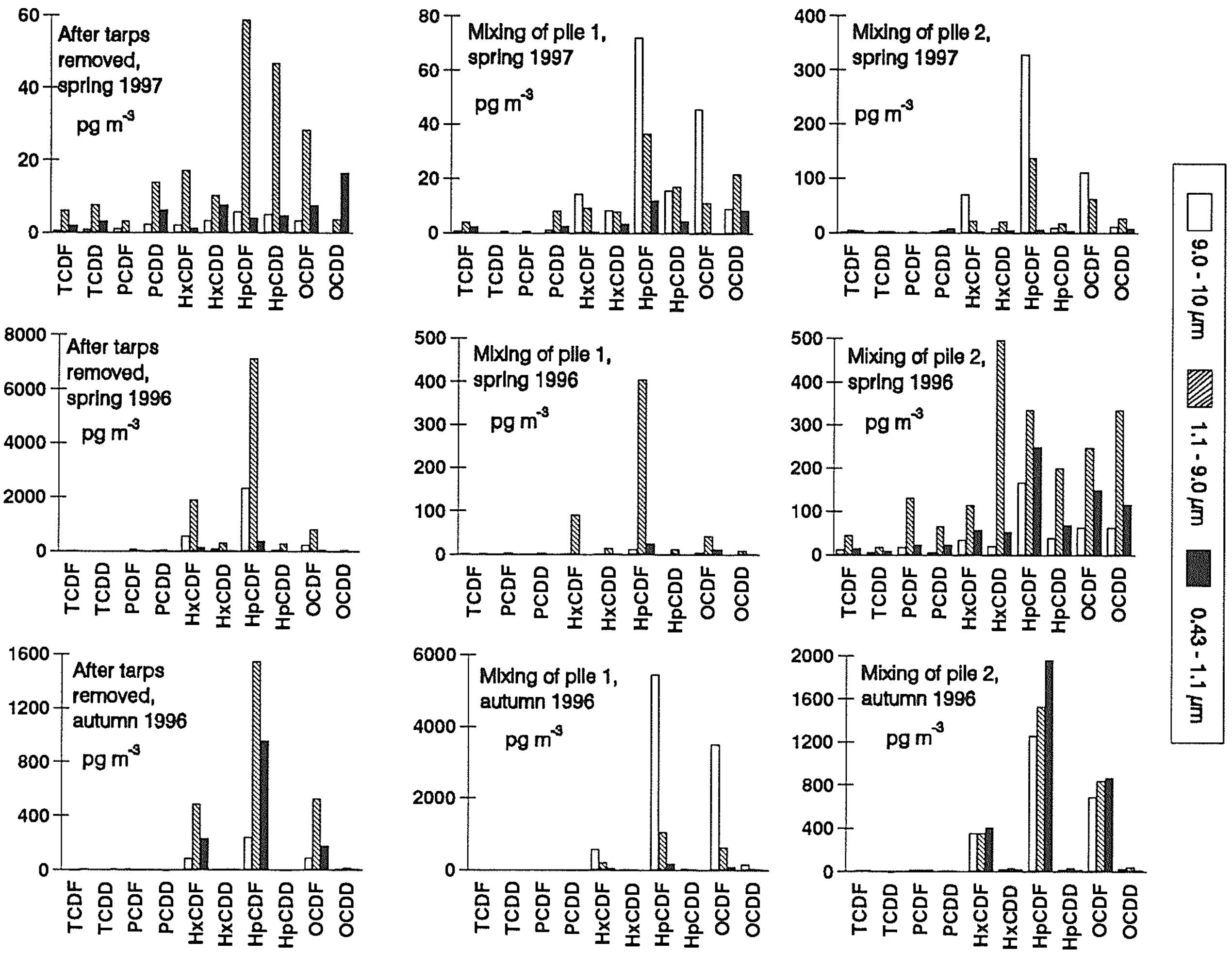


Fig. 8. Total concentrations of the airborne PCDD/Fs in three particle size classes of 9.0 - 10.0 µm, 1.1 - 9.0 µm and 0.43 - 1.1 µm.

**Table 9.** Total concentrations of PCDD/Fs in the compost windrows during the full-scale composting. Analyses were performed at two different laboratories using three different methods. Compost soils were sieved through an 8-mm sieve in 1995, whereas compost soils in 1997 were not sieved before analysis.

| Congener [a] | polychlorinated dibenzo-*p*-dioxins and dibenzofurans [µg (kg dry wt) $^{-1}$] | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Windrow 2B | | | | | Combination samples August 1995 | | | Combination samples August 1997 | | | |
| | 10.7. 1995 | 24.7. 1995 | 14.8. 1995 | 16.10. 1995 | 13.11. 1995 | Windrow 1 | Windrow 2 | Windrow 3 | Windrow 1 | Windrow 2 | Windrow 3 | Stacked soil [c] |
| TCDF | 0.091 | 0.086 | 0.090 | 0.088 | 0.015 | 1.0 | 1.22 | 0.02 | 14.6 | 49.2 | 1.28 | 117 |
| TCDD | 0.027 | 0.026 | 0.022 | 0.034 | 0.025 | 0.60 | 0.44 | 0.03 | 10.8 | 15.0 | 1.43 | 15.5 |
| PeCDF | 1.1 | 0.907 | 0.84 | 1.15 | 0.767 | 7.4 | 7.4 | 0.25 | 32.3 | 45.2 | 3.10 | 159 |
| PeCDD | 2.01 | 1.78 | 1.63 | 1.6 | 1.29 | 20.3 | 16.2 | 0.09 | 35.3 | 58.3 | 5.81 | 82.6 |
| HxCDF | 111 | 113 | 87.9 | 125 | 91.8 | 8940 | 11306 | 53 | 2120 | 2070 | 117 | 17200 |
| HxCDD | 9.6 | 8.57 | 7.56 | 8.52 | 6.87 | 71 | 127 | 2.1 | 141 | 155 | 12.6 | 615 |
| HpCDF | 763 | 639 | 565 | 737 | 604 | 28860 | 61440 | 332 | 5920 | 7540 | 474 | 14030 |
| HpCDD | 11.6 | 9.83 | 7.89 | 10.1 | 8.9 | 48 | 58 | 1.28 | 134 | 174 | 13.9 | 1070 |
| OCDF | 759 | 623 | 600 | 743 | 531 | 2850 | 2250 | 148 | 6090 | 7350 | 329 | 59570 |
| OCDD | 26.6 | 19.2 | 12.7 | 23.5 | 22.1 | 140 | 148 | 2.8 | 214 | 261 | 51.9 | 1740 |
| Total (µg/kg dry wt) | 1680 | 1410 | 1280 | 1650 | 1270 | 40940 | 75350 | 540 | 14720 | 17710 | 1010 | 94600 |
| I-TEQ [b] (µg/kg dry wt) | 17.2 | 16.5 | 13.3 | 18.3 | 13.9 | 89 | 188 | 2 | 46.2 | 48.6 | 3.33 | 139 |

a) T = tetra-, Pe = penta-, Hx = hexa-, Hp = hepta-, and O = octachlorinated dibenzo-*p*-dioxins (CDD) or dibenzofurans (CDF). b) I-TEQ = International 2,3,7,8-TCDD toxic equivalent. c) See Paper VI.

## 4 Summary and conclusions

*Evaluation of the use of straw compost and remediated soil as inocula*

When enriched in the laboratory, both straw compost and remediated soil mineralized 60% of the added PCP. No methylation of chlorophenols to chlorinated anisoles was observed.

*Development of a composting system to accelerate the bioremediation of chlorophenol-contaminated soil in laboratory, pilot and full scale applications*

In the field, the biodegradation of chlorophenols was efficient and fast irrespective to the addition of the inocula. Indigenous soil microorganisms degraded chlorophenols under suitable conditions. At least 93% of the chlorophenols were removed in all compost windrows which were mixtures of contaminated soil and straw compost or bark chips during the full-scale composting 1995-1997. The addition of fertilizer or fresh bark did not enhance the chlorophenol degradation after winter.

*Fate of chlorophenols and PCDD/Fs during composting in pilot scale*

No harmful metabolites were formed during the composting. No major polymerization or dimerization occurred. The polymerized fraction of organic chlorine compounds that was formed in contaminated with time, was not remobilized during the composting. In all the compost piles, there was an apparent decrease in acute toxicity assessed by the luminescent bacteria test. Large amounts of PCDD/Fs up to mg (kg dry wt) $^{-1}$ -range originating from the wood preservative Ky-5 were detected. The congener composition of PCDD/Fs resembled the one in the original wood preservative. PCDD/Fs were neither formed nor degraded during the composting.

*Bioaccumulation of halogen containing contaminants from soil into the earthworms*

The earthworms accumulated tetrahydrofuran extractable halogen compounds up to 20 times more from sandy soil than from organic soil. This indicated that soil organic matter diminished the bioaccumulation potential of halogenated compounds to the earthworms.

*Microbiology of chlorophenol degradation during composting*

The best indicator of the actual chlorophenol degradation efficiency was the number of microorganisms growing on plates with 2 mM PCP as the sole carbon source. Characteristics to the gram-positive isolates was the biotransformation of chlorophenols via other than growth-linked metabolism. The gram-negative isolates were predominated (40 out of 72) by Pseudomonads, which grew on PCP as the sole carbon source so that Na-glutamate seemed to have a role on degradation or tolerance of PCP. None of the isolated 100 compost bacteria alone was responsible for a complete mineralization of chlorophenols even though remarkable (60%) mineralization was found in intact composts. The part of the remaining 30-40% of the carbon in chlorophenols was most likely built into the bacterial biomass. The chlorophenol-degraders originating from contaminated soil seemed not directly to contribute to the substrate utilization pattern, but probably had benefited from the enhanced general microbial activity in the composts by cometabolism or synergism.

*Quantitation of the microbial and chemical release in airborne particles during mixing of the compost windrows*

The congener distribution of PCDD/Fs was similar in collected airborne particle fractions to the one in the compost windrows, and the level of PCDD/Fs was 1000-fold higher than the atmospheric background value. The dose of the airborne PCDD/F during composting equals to the amount of PCDD/Fs taken in by eating one kg of Finnish lake fish. Eighty percent of the isolated airborne bacteria were gram-positive and spore-forming. The amounts of airborne fungi and actinomycetes were 40% higher than the background values. None of the identified bacteria are known to demonstrate pathogenic potential. The viable counts were low compared to those found in municipal-waste composts.

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*In memory of my dear father*
*To all my beloved ones*

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