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The survival of the pentachlorophenol-degrading *Rhodococcus chlorophenolicus* PCP-1 and *Flavobacterium* sp. in natural soil

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Abstract

The survival of two different pentachlorophenol (PCP)-degrading bacteria were studied in natural soil. The PCP-degraders *Rhodococcus chlorophenolicus* and *Flavobacterium* sp., both able to mineralize PCP into CO_2 and chloride in axenic culture, were tested for the capacity to survive and degrade PCP in natural soil. These bacteria were immobilized on polyurethane (PUR) foam and introduced into natural peaty soil to give about 10^9 cells g^{-1} of soil (dry weight). *R. chlorophenolicus* induced PCP-degrading activity in soil remained detectable for 200 days whether or not a carbon source was added (distillery waste or wood chips). Electron microscopic investigation performed almost a year after inoculation, revealed the presence of *R. chlorophenolicus*-like cells in the PUR foam particles. PCP-degrading activity of *Flavobacterium* sp. declined within 60 days of burial in the soil without enhancing the PCP removal. *R. chlorophenolicus* degraded PCP in soil at a mean rate of 3.7 mg of PCP day^{-1} kg^{-1} of soil, which corresponds to ca. 5×10^{-3} pg of PCP degraded per inoculated *R. chlorophenolicus* cell day^{-1}. The solvent extractable organic chlorine contents of the soil decreased stoichiometrically (> 95%) with that of PCP indicating that PCP was essentially mineralized.

Abbreviations: ATCC – American type culture collection, DSM – Deutsche Sammlung für Mikroorganismen, DW – distillery waste, EM – electron microscopy, EOX – extractable organic halogen, GC/ECD – gas chromatograph/electron capture detector, GC/MS – gas chromatograph/mass spectrometer, PCP – pentachlorophenol, WC – wood chips, d.wt. – dry weight, w.wt. – wet weight, d.s. – dry soil, d.H_2O – distilled water, PCA – polychlorinated aromatics

Introduction

Microbes play an important role in degrading synthetic chemicals in soil (Alexander 1981). For instance, several microorganisms are capable of transforming chloroaromatic compounds both in pure (Schraa et al. 1986; Ryan & Bumpus 1989) and mixed culture (Valo et al. 1985; Liu 1989), of which some degrade also in heterogeneous systems like soil (Edgehill & Finn 1983; Kilbane et al. 1983; Valo & Salkinoja-Salonen 1986; Ryan & Bumpus 1989), sludge (Boyd & Shelton 1984; Mikesell & Boyd 1985) or groundwater (Valo et al. 1990).

In contrast to the wealth of information on the

biodegradation of xenobiotics in pure culture, little is known about the degrading activity of microbes under field conditions. Rather often the researchers have been protagonists of laboratory successes and spectators of failures in field while attempting practical application of microbes, known of degrading capability in pure culture. The failure may result for instance from the die off of the degrading organisms by starvation, by inhibition by antibiotic-producing soil microbes (Goldstein et al. 1985; Acea et al. 1988) or predation (Henis 1987; Acea & Alexander 1988; Casida 1989).

Our study was directed to understanding the factors affecting the externally introduced xenobiotic degrading activity and the survival of such degraders in the natural soil. For this study two different degraders of one chemical, pentachlorophenol (PCP) were chosen, *Rhodococcus chlorophenolicus* PCP-1 (Apajalahti et al. 1986; Apajalahti & Salkinoja-Salonen 1986) and *Flavobacterium* (Saber & Crawford 1985). The first one is a Gram-positive actinomycete, sensitive in axenic culture to PCP concentrations above 2 ppm, and the second degrader is a Gram-negative rod that tolerates as high as 100 ppm PCP in liquid culture. Both strains mineralize PCP into CO_2 and chloride.

The present paper compares the survival of these two degraders in natural soil under identical conditions.

Materials and methods

Soil

Peaty soil was collected from the experimental forest farm of Kettula (Suomusjärvi, Finland), a forest area that had not been exposed to either pesticides or fertilizer for the past 40 years. Description of the chemical, physical and microbiological features of this soil is given in Tables 1 and 2. Samples of approximately 40 kg fresh weight were collected from 4 sites 25 m apart and the four samples blended. The top 10 cm layer, containing plant material, was discarded. Soil was collected to a depth of 35 cm, and sieved (16 mm grid). The soil was stored with natural moistness at + 4° C in the dark until use.

The soil moisture content was calculated from weight loss after drying to constant weight at 105° C. The field capacity was calculated either as percentage of weight loss after drying water saturated soil at 105° C or by capillary imbibition (Italian Society for Soil Science 1985). Soil pH was measured in a 1 : 5 (d.wt. v^{-1}) mixture of soil either in d.H_2O or in 1 M KCl after 1 h. Ash content was determined after combustion of the dry soil for 16 h at 375° C and the organic carbon content was estimated from the ignition loss (Ball 1964). The content of the acid soluble (50% HNO_3) inorganic elements in the soil (Anonymous 1980) was determined by plasma emission spectrometry. Total N was determined by the Kjeldahl method (Bremner 1965a); exchangeable ammonium and combined nitrite plus nitrate were determined in 2 M KCl by titrating the extracts made by alkaline steam distillation, before and after addition of Devarda's alloy (Bremner 1965b).

Inocula

Rhodococcus chlorophenolicus PCP-1 (DSM 43826) was grown in DSM-65 medium at 28° C on an orbital shaker, (140 rpm) in the dark. *Flavobacterium* sp. strain (ATCC 39723) was grown in Minimal Salts Medium (Steiert & Crawford 1985) under the same conditions. A stationary culture of *R. chlorophenolicus* was induced to degrade PCP by daily adding increasing amounts (10 to 20 μM) of filter sterilised PCP in acetylation buffer for three days. The *Flavobacterium* sp., was induced by adding PCP, (in 0.1 M NaOH) to 0.2 mM, after the culture reached midlogarithmic phase.

PUR foam was used to prepare immobilized inoculum. Before use the 24 g (w.wt.) of PUR foam was washed with d.H_2O, sterilised in 250 ml of the respective growth media in 300 ml bottles at 110° C for 20 min, inoculated and incubated for two days at 28° C in the dark. PUR foam did not inhibit growth of either strain. The PUR immobilized biomass was harvested by centrifugation for 15 min at

2800 × g. The PUR foam (24 g w.wt.) containing either 7×10^{11} cells of *R. chlorophenolicus* or 9×10^{11} *Flavobacterium* was blended with moist soil (1 kg d.wt.), yielding about 10^9 cells g^{-1} of soil.

Media and chemicals

DSM-65 medium contained ($g\,l^{-1}$ d.H_2O) : glucose (4.0); yeast extract (4.0); malt extract (10.0). KN-Salt medium (Sundman 1964) supplemented with 0.5 g of yeast extract per liter was used for MPN counting of PCP-mineralizing bacteria in soil inoculated with *Rhodococcus*.

Pentachlorophenol (97% purity) was from E. Merck (Darmstadt, Germany), 2,4,6-tribromoanisole was synthesized from 2,4,6-tribromophenol (98% purity from Fluka AG Buchs, Switzerland) by methylating with dimethylsulfate (Vogel 1956). Uniformly ^{14}C-labelled PCP (96% radiochemical purity, 10.57 mCi $mmol^{-1}$) was obtained from Pathfinder Laboratories Inc. (St. Louis, Mo, USA). Acetylation buffer, pH 9.9, consisted of 182 ml 0.1 M NaOH and 1000 ml 0.05 M $NaHCO_3$. All solvents used for chlorophenol extraction were from Rathburn Chemicals Ltd. (Walkerburn, Scotland) and were of glass-distilled grade. Polyurethane foam, PUR 90/16 type with active carbon, was from Bayer (Leverkusen, Germany).

Experimental set up

The combinations of soil and bacteria studied are shown in Table 3. Sterile PUR foam was used in place of immobilized biomass for the blank assay. Aliquots of 2.6 kg soil (1 kg d.wt.) were used. PCP (750 mg kg^{-1} d.s.) was added as a 15 mg ml^{-1} solution in 0.1 M NaOH which did not affect soil pH measurably. Additional carbon sources optionally were applied to the soils: DW (*Triticum vulgaris*, from Alko Koskenkorva Distilleries), added (sterilised 10 min, 110° C) 8% w v^{-1} aqueous suspension, 60 ml kg^{-1} (d.s.) and WC, a blend of Norway spruce, (*Picea abies*) and Scots pine (*Pinus sylvestris*), (oven-heated, 100° C, 24 h) 10 g per kg (d.s.). The soil, with the inoculum and carbon sources, was sealed in polyethylene (0.05 mm) bags (20 cm × 60 cm) and incubated at room temperature (23° C ± 2° C) in the dark. Polyethylene is relatively permeable to O_2 and CO_2 but prevents evaporation of water (Clarholm et al. 1981).

Electron microscopy

For transmission electron microscopy, the PUR foam samples were prefixed for 3.5 h by adding glutaraldehyde (Leiras, Finland) to the culture to give a final concentration of 3% (w v^{-1}), and washed 3 times in 0.1 M sodium phosphate buffer (pH 7.2). The specimens were postfixed and examined as described earlier (Smolander et al. 1988).

Chemical analysis

Soil subsamples of 100 g were analysed for chlorophenols and methylated phenols as described elsewhere (Middeldorp et al. 1990). Tribromoanisole and tribromophenol were used as internal standards. The detection limit of pentachlorophenol and the methylated chlorophenols was 0.1 mg kg^{-1} peaty soil (d.wt.). The standard deviation (S_x) was 15% of the mean value ($n = 7$).

The extractable organic halogen (EOX) was measured from an acetone/hexane extract of 50 g of soil as described elsewhere (Middeldorp et al. 1990).

Microbial counts

The MPN method (Koch 1981) was used to estimate microbial counts. For total aerobic heterotrophs, the serial dilutions were prepared in DSM-65, incubated at 28° C in the dark on a gyratory shaker and the tubes were scored after one week. To estimate the number of PCP-mineralising cells the serial dilutions were prepared either in KN-Salt medium (*Rhodococcus*) or in the Basic medium (*Flavobacterium*). Triplicate 10 ml aliquots of each dilution

were made, PCP was added to 15 μM of which ^{14}C-PCP to 4000 cpm and placed in stoppered 100 ml bottles provided with glass cups containing 0.5 ml of 1 M NaOH. After three weeks of incubation at 28° C, duplicate 0.2 ml aliquots of the NaOH solution were analysed for absorbed ^{14}C in 4.5 ml of scintillation liquid by using a LKB Wallace 1215 Rackbeta liquid scintillation counter (LKB, Stockholm). 10% yield of $^{14}CO_2$ or more of the added ^{14}C-PCP was interpreted as positive indication of mineralization.

The total aerobic heterotrophic count (Pochon & Tardieux 1962), the number of colony forming of filamentous fungi (Pochon & Tardieux 1962) and actinomycetes (Briglia 1986) were estimated by using plate count methods (Table 2).

Results

We studied the behaviour of externally introduced PCP-degrading bacteria, *Rhodococcus chlorophenolicus* and *Flavobacterium* sp. in natural soil. Pristine spruce forest soil (*Oxalis-Myrtillus* type) was used. The soil contained neither organic chlorine compounds (EOX < 0.1 mg kg^{-1}) nor microbes capable of mineralising pentachlorophenol (PCP) (< 10 degrading cells g^{-1} d.s.) as it is shown in the Tables 1 and 2. The survival of the strains was followed for 200 days both in the presence and in the absence of added carbon source and of PCP.

Table 1. Description of the physical and chemical characteristics of the soil employed.

Texture	Peaty
Moisture content (%w w^{-1})	62
Field capacity (gH_2O 100 g^{-1} soil)	216.5
WHC (capillary imbibition %)	146.5
Organic carbon (%w w^{-1})	30.3
Cation Exchange Capacity (meq 100 g^{-1} d.s.)	24.4
PH (H_2O)	6.0
pH (KCl)	4.7
Sum of chlorophenols (mg kg^{-1} d.s.)	< 0.1
Total N (%W w^{-1})	1.95
C/N ratio	15.5
EOX (mgCl kg^{-1} d.s.)	< 0.1
Acid soluble elements (mg kg^{-1} d.s.)	
Fe	42600
Mg	2400
Ca	9300
Mn	550
NH_4-N	72
(NO_3 + NO_2)-N	1.8

Table 2. Microbiological characteristics of the peaty soil used.

Type of microbes	cfu g^{-1} d.s.
Total aerobic bacteria	1.1×10^8
Actinomycetes	8.7×10^4
Filamentous fungi	2.4×10^5
PCP-mineralising microbes	< 10*

*MPN-count = cells g^{-1} d.s.

The PCP-mineralising activity of the inoculated soils was MPN-counted ten days after the inoculation and then after 60, 140 and 200 days. The results, reported in Figs 1, 2, suggest that on day 10 the count of PCP degrading cells in the soils corresponded to that inoculated on day = 0. The PCP-mineralising activity in the *R. chlorophenolicus* inoculated soils remained relatively constant for 200 days both with and without PCP (Fig. 1). After 60 days of incubation, the PCP-degrading MPN-count of the soils inoculated with the *Flavobacterium* strain had declined by seven orders of magnitude, similarly with or without PCP, with or without a second carbon source (Fig. 2). The presence of PCP or other carbon sources had no effect on the maintenance of PCP degrading activity in the soil.

Figure 3 shows electron micrographs of the PUR foam before (a, b) and after (c, d) soil burial. The

Table 3. Set up of soil experiment.

1. Pristine soil
2. Idem + PCP + PUR
3. Idem + PCP + *R. chlorophenolicus*
4. Idem + *R. chlorophenolicus*
5. Idem + PCP + *Flavobacterium*
6. Idem + *Flavobacterium*
7. Idem + PCP + DW + *R. chlorophenolicus*
8. Idem + PCP + DW + *Flavobacterium*
9. Idem + PCP + WC + *R. chlorophenolicus*
10. Idem + PCP + WC + *Flavobacterium*
11. Idem + PCP stored at − 20° C

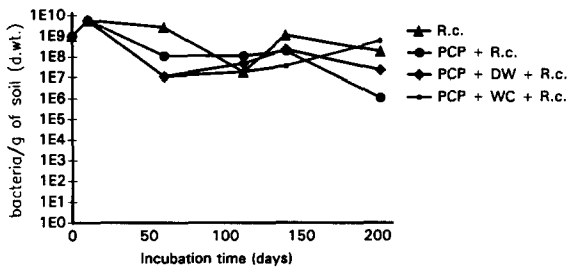


Fig. 1. MPN-count of PCP-mineralizing microbes in soil inoculated with immobilized *R. chlorophenolicus* PCP-1 cells in pristine and in PCP contaminated soil, with and without a second carbon source (DW and WC).

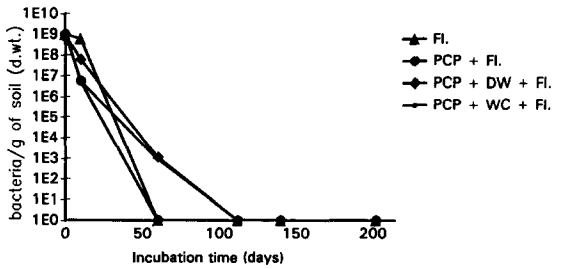


Fig. 2. MPN-count of PCP-mineralizing microbes in soil inoculated with immobilized *Flavobacterium* cells in pristine and in PCP contaminated soil, with and without a second carbon source (DW and WC).

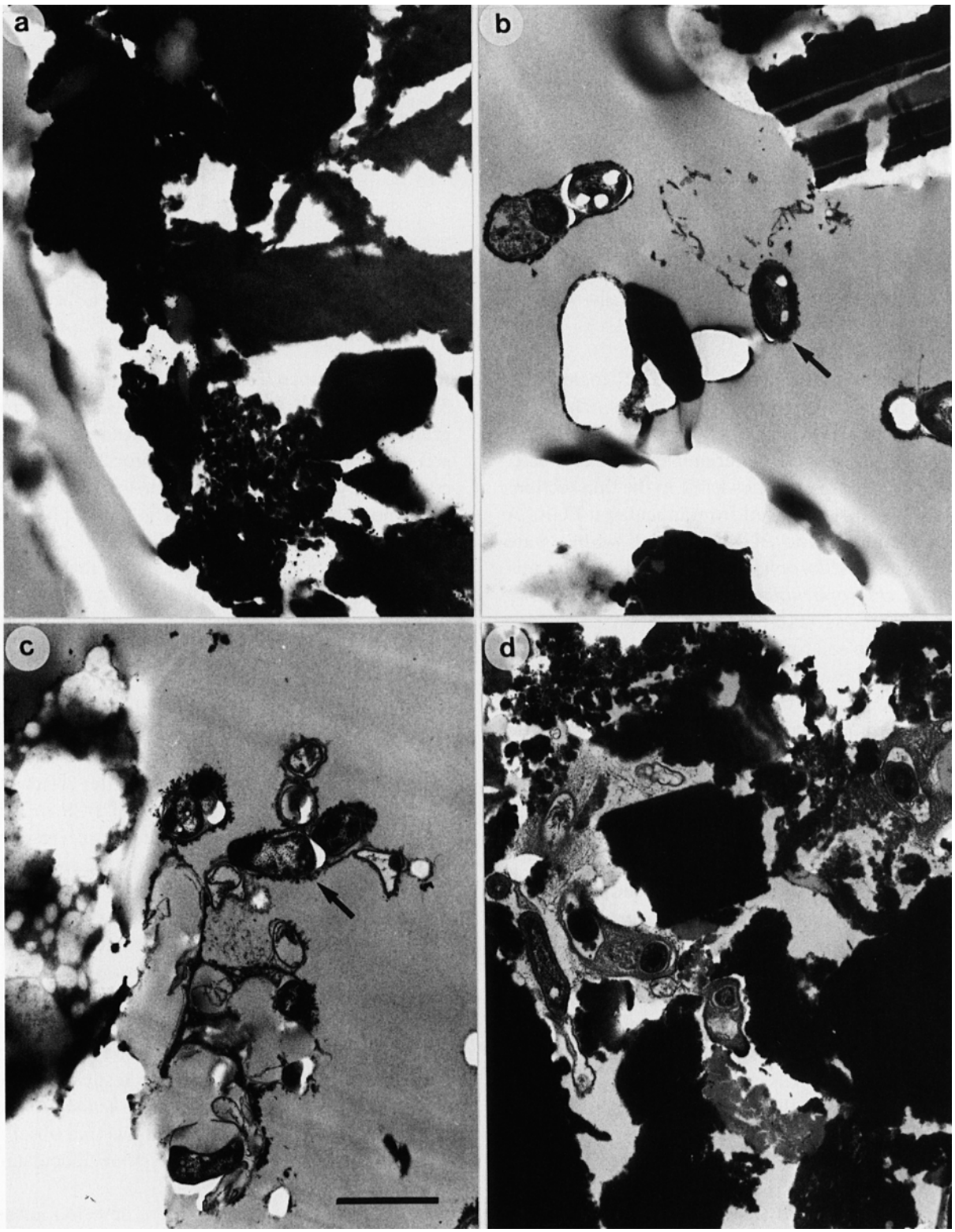
micrograph (a) shows the sterile PUR foam structure with no bacterium-like structures. The PUR foam cannot be cut evenly, because of differences in the hardness of the material. Nevertheless bacterial structures are clearly visible in the thin sections (Figs 3b, c, d) prepared from inoculated PUR. A thin section of the PUR colonized with a pure culture of *R. chlorophenolicus* (Fig. 3b) shows several *R. chlorophenolicus* cells. The picture shown in Fig. 3c was taken after the foam pads were incubated in soil for 290 days, bacterial colony can be seen. These bacteria appear morphologically similar to the pure culture (Fig. 3b). Figure 3d shows the appearance of the PUR foam pads after 290 days of incubation in soil with added DW. In this micrograph many bacterial cells lying in the cavities of the PUR are depicted. Most of these bacteria have an ultrastructure different from *R. chlorophenolicus* PCP-1.

We counted the number of *R. chlorophenolicus*-like cells immobilized onto PUR foam also by electron microscopy on the base of the dimensions of the thin sections (7.5 μm length, 5.46 μm breadth and 0.2 μm width) and of the amount of the micrographs (46 photos). Patterns with cell wall resembling structures were counted as cells. Ca. 9×10^{12} cells per 50 cm^3 PUR w.wt. were found from the micrographs of PUR inoculated with pure culture of *R. chlorophenolicus* PCP-1. This corresponds approx. to 9×10^9 cells g^{-1} d.wt. of soil. Similar counts, made on PUR foam particles after 290 days of incubation in soil (Fig. 3c) resulted to 6×10^9 cells g^{-1} of soil. In addition we observed ghosts of *R. chlorophenolicus* resembling cells numbered ca. 3×10^9 dead cells g^{-1} soil (Fig. 3c). In the micrographs prepared from PUR particles incubated in soil with PCP and distillery waste for 290 days, we counted 8×10^{12} cells per 50 cm^3 of PUR w.wt. corresponding to 8×10^9 cells g^{-1} soil, plus the debris of 3×10^9 cells g^{-1} soil (Fig. 3d).

The results of the microscopic count thus coincided with the radioisotopic MPN-counts of ^{14}C-PCP degraders, suggesting that the inoculated morphotype (*R. chlorophenolicus*) indeed had survived in natural soil for about a year.

Inoculation with *R. chlorophenolicus* resulted in 60 days in 38% of PCP removal in the absence of DW, 63% in the presence of DW and 80% when WC were added to the soil (Fig. 4). After 200 days, there was a 3% to 23% residual of PCP in soil without or with a second carbon source respectively. The PCP was degraded in natural soil by *R. chlorophenolicus* at the mean rate of 3.7 mg of PCP per day per kg of soil, which corresponds to ca. 5×10^{-3} pg of PCP degraded per inoculated *R. chlorophenolicus* cell per day. Inoculation of the soil with *Flavobacterium* sp. did in 60 days not result in a PCP removal rate beyond that observed in uninoculated soil whether amended with a second carbon source or not (Table 4). The results thus indicate that inoculation of *R. chlorophenolicus* into soil enhanced PCP degradation above that observed in uninoculated or *Flavobacterium* inoculated soil.

Methylated chlorophenols were detected in the GC/ECD and GC/MS chromatograms of the peaty


a
b
c
d

←

Fig. 3. Electron micrographs of thin sections of PUR foam containing active carbon before and after colonization by *R. chlorophenolicus* PCP-1. (a) sterile PUR foam; (b) idem immediately after colonization by a pure culture of *R. chlorophenolicus* PCP-1; (c) a similar PUR foam pads as in b, but sampled after 290 days burial in PCP contaminated soil, showing *R. chlorophenolicus*- like cells (arrow), and (d) as picture b, but after 290 days burial in PCP contaminated soil, where distillery waste was added. Extracellular material attached to cell wall is visible (arrows). The bar represents 1 μm.

soil at a concentration of 1% or less of the PCP input (< 3 mg kg^{-1}). There was no indication on the accumulation of lower chlorinated phenols. In order to assess for the mineralization of PCP-bound chlorine, we assayed for solvent extractable organic chlorine (EOX). The results in Table 5 show that the EOX decreased from the input of 400 mg kg^{-1} soil to below 20 mg kg^{-1} in *R. chlorophenolicus* inoculated soils, indicating that the PCP bound chlorine had essentially become mineralised (> 95%).

Discussion

The aim of our present work was to study the activity and the survival of different PCP-degraders in soil ecosystem in order to answer to the question why externally introduced xenobiotic degrading microbes seldom perform well under field conditions.

Our results showed that *R. chlorophenolicus*

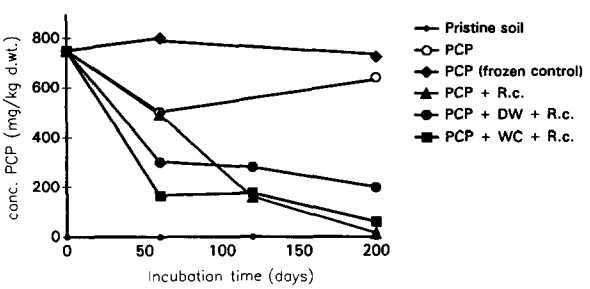


Fig. 4. Removal of PCP (750 mg kg^{-1} d.s. input) from peaty soil inoculated with PUR immobilized *R. chlorophenolicus* PCP-1 (R.c.) cells. PCP and other carbon sources (DW, WC) were added into the soil as indicated.

Table 4. Removal of PCP from the peaty soil inoculated with PUR immbilized *Flavobacterium* (Fl) cells.

Soil treatment	[PCP] in soil mg kg^{-1} d.s.	
	Incubation time (days)	
	0	60
Pristine soil	0	0
Pristine soil + PCP	750	510
Pristine soil + PCP + Fl	750	700
Pristine soil + PCP + DW + Fl	750	620
Pristine soil + PCP + WC + Fl	750	600
Pristine soil + PCP (frozen control)	750	810

PCP-1 inoculant maintained PCP degrading activity for 200 days in pristine and in PCP containing natural soil whether or not a second carbon source was added (Fig. 1). *R. chlorophenolicus* PCP-1 tolerated and degraded 600 ppm PCP in soil (Salkinoja-Salonen et al. 1989). The PCP degrading activity of the inoculated *Flavobacterium* cells declined within 60 days in natural soil under the same conditions (Fig. 2) as above. During this period it did not express any PCP-degrading activity (Table 4). Crawford & Mohn (1985) also observed that even after several inoculations the activity of the *Flavobacterium* sp. in soil declined in 3 or 4 weeks.

In liquid culture *Flavobacterium* tolerates much higher concentrations (100 ppm) of PCP than *R. chlorophenolicus* PCP-1 (2 ppm) (Saber & Crawford 1985; Apajalathi & Salkinoja-Salonen 1986). Thus it seems that behaviour in liquid culture does not predict behaviour in soil.

The soil survival differences between these two

Table 5. Concentrations of extractable organic halogen (EOX), PCP-Cl and PCA-Cl in the soil, inoculated with PUR immobilized *Rhodococcus chlorophenolicus* (R.c.), after 200 days of incubation.

	EOX	PCP-Cl (mg/kg d.wt.)	PCA-Cl
Pristine soil	< 0.1	0.0	< 0.06
Pristine soil + PCP + R.c.	7.7	1.1	0.30
Pristine soil + PCP + DW + R.c.	13.5	18.9	1.10
Pristine soil + PCP + WC + R.c.	19.3	12.2	1.70
Pristine soil + PCP (frozen control)	404.0	570	< 0.06

PCP-degrading bacteria indicate different metabolic properties. *Rhodococcus* showed to behave as autochthonous soil bacteria whereas *Flavobacteria* behave as zymogenous bacteria (classification by Winogradsky, cited by Henis 1987). It has been shown that an *Arthrobacter* strain was 100% viable after having starved for 30 days (Boylen & Ensign 1970). *Nocardia corallina* has also a slow rate of endogenous metabolism, fivefold lower than *E. coli,* which allows longer survival (Robertson & Batt 1973). *R. chlorophenolicus* is an actinomycete that undergoes rod to coccus cycle (Apajalathi et al. 1986) like also *Arthrobacter* sp. and *Nocardiae* do. The coccoid cells may differ from the rods in their survival properties. A layer of extracellular material was found to coat the cell after 2 weeks of incubation (Apajalahti et al. 1986). This layer can also be seen in Figs 3b, c, around *R. chlorophenolicus*-like cells (arrow). The presence of extracellular material may protect the cell against protozoal attack (Dudman 1977).

Autochthonous bacteria grow very slow in soil. This may explain why the numbers of PCP degrading cells remained virtually unchanged for almost a year also when there was selective advantage in form of high PCP concentration (Fig. 1)

It was interesting that also EM revealed in the PUR foam pads the presence of great numbers of bacteria morphologically similar to the *R. chlorophenolicus* strain inoculated, as long as after 290 days of burial in soil. We are not aware of any other study where inoculated microbes would be microscopically detected after such an extended period in soil. In soil enriched with distillery waste there were also other types of cells, indicating an invasion of bacteria from soil into the PUR foam. This shows that soil enrichment with degradable carbon is not necessarily beneficial to the cleanup, at least in the case of peaty forest soil.

The stability of PCP-degrading activity of *R. chlorophenolicus* in soil showed to be useful in remediating PCP-contaminated soil and groundwater also under field conditions (Salkinoja-Salonen et al. 1989; Valo & Salkinoja-Salonen 1986; Valo et al. 1990; Valo, Ph.D. thesis 1990, Department of General Microbiology, University of Helsinki, Finland).

Laboratory experiments with ^{14}C-labelled PCP have shown that *R. chlorophenolicus* mineralised PCP-carbon in natural soil into $^{14}CO_2$ (Middeldorp et al. 1990). In this paper we show that also the PCP-bound and the organically bound halogen (EOX) was essentially (95%) mineralized. EOX analysis is more suitable than radiolabelled PCP to monitor mineralisation of PCP under field conditions.

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