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Accelerated Mineralization of Pentachlorophenol in Soil upon Inoculation with *Mycobacterium chlorophenolicum* PCP1 and *Sphingomonas chlorophenolica* RA2

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Mineralization of pentachlorophenol (PCP) was studied in nonsterile soil from a PCP-contaminated site upon inoculation with two PCP-degrading bacterial strains. At spiked [^{14}C]PCP concentrations of 30 and 100 mg/kg, the effects of organism type, different inoculation techniques, including structural amendment with sawdust and cell attachment to polyurethane (PU), as well as the effect of different inoculum sizes of 10^4 to 10^8 cells per g (dry weight) of soil were compared with PCP mineralization by indigenous bacteria. Gas chromatographic analysis was used to monitor PCP disappearance and to check mass balances. The survival and activity of the released bacteria were examined by immunofluorescence microscopy and respiking experiments. Noninoculated soil completely mineralized 30 mg of PCP per kg within 7 months but showed no or only low degradation activity at 100 mg/kg in the same period. Structural amendment with PU or sawdust initiated slow mineralization after half a year. Soil inoculation with *Sphingomonas chlorophenolica* RA2 shortened the mineralization time drastically to 1 month at 30 mg of PCP per kg using 10^8 cells per g, with approximately 80% of the added radioactivity being converted to CO_2. The inoculated cells disappeared rapidly, with a count of 2×10^6 cells per g after 2.3 months and nondetectability after 7 months. At 100 mg/kg, mineralization was slower because of PCP toxicity but approached completion within 7.5 months. The inhibition could be overcome by addition of sawdust (1 g/kg of soil), resulting in a mineralization rate of 3 to 4 mg/kg · d. PU had the opposite effect. Lower inoculum densities resulted in prolonged lag phases and lower rates, although mineralization was still enhanced over the background level. At 30 mg of PCP per kg, inoculation with *Mycobacterium chlorophenolicum* PCP1 increased mineralization slightly over the indigenous bacterial activity, regardless of inoculum size, but only when the organisms were attached to PU. At 100 mg of PCP per kg, only 27% were mineralized within 7.5 months. After 7 months, the original strain PCP1 inoculum of 10^8 cells per g was recovered at 5×10^6 to 3×10^7 cells per g, depending on the PCP concentration, but independent of PU amendment. Amendment with sawdust had no effect on the performance of this organism. Possible reasons for the poor performance of this strain include its sensitivity to PCP and its preference for slightly acidic soil conditions.

Pentachlorophenol (PCP) has been used primarily as a wood preservative, with additional applications in agriculture and industry (17). The U.S. Environmental Protection Agency has listed PCP as a priority pollutant and considers materials contaminated with PCP to be hazardous (35). Because of its persistence, PCP has accumulated in the environment and caused serious pollution of soil and groundwater (10, 17).

Although PCP is described as an uncoupler of oxidative phosphorylation, several PCP-mineralizing microorganisms have been isolated (1, 7, 13, 25, 27–29, 32–34, 36). However, studies on bioremediation of actual contaminated soil involving inoculated PCP-degrading organisms were successful to only a minor extent (9, 12, 14, 30). In several cases, noninoculated controls were missing or proved to be effective as well (17, 22). Reasons for slow or absent biodegradation in the environment may include an insufficient number of PCP degraders and inhibition by toxic concentrations of PCP or other contaminants. Therefore, inoculation with PCP degraders may, in some cases, be the only way for microbial cleanup of contaminated sites. However, the success of bioaugmentation is affected by several factors, including the type of inoculum, soil properties, and the presence of substances other than the target pollutant.

Two PCP-degrading bacterial species have been studied in much detail: *Mycobacterium chlorophenolicum* PCP1 (1, 4, 16), which has been tested in pristine soils artificially contaminated with PCP at up to 630 mg/kg (5, 24), and *Sphingomonas chlorophenolica* RA2 (23, 26, 27), which has been studied for PCP degradation in liquid culture (27) and recently was tested for degradation of artificial PCP contamination at up to 1,200 mg/kg in previously noncontaminated sandy loam (8). This study focused on the enhancement of PCP mineralization under laboratory conditions by inoculation of soil from an actual PCP-contaminated site and compared the activity and survival of gram-positive and gram-negative PCP degraders on the background of indigenous degradation activity. Factors influencing the applicability and limitations of the inoculation technique for remediation purposes were also investigated.

MATERIALS AND METHODS

Soils. Soil was obtained from a PCP-contaminated site in Skalborg, Denmark, that had been excavated for containment. The average field contamination was ca. 30 mg of PCP per kg. In the sample obtained, PCP had been degraded during storage, transport, and processing to a residual concentration of 0.2 to 2 mg/kg. A portion of sampled soil was sieved (2 mm) and stored field moist at 4°C until use. The soil characteristics were as follows: origin and texture, sandy subsoil;

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water-holding capacity, 250 g/kg; total organic C, 3.4 g/kg; total organic N, 0.25 g/kg; pH (KCl) 7.9.

A second soil, used for comparison, was obtained from M. Salkinoja-Salonen. It has been described earlier (5) as follows: noncontaminated surface soil; texture, sandy loam; water-holding capacity, 210 g/kg; total organic C, 7 g/kg; total organic N, 0.3 g/kg; pH (KCl) 4.6.

Experimental setup. Ten-gram soil samples were spiked with PCP, labeled with 5×10^{10} Bq of [U-^{14}C]PCP (Sigma, St. Louis, Mo.), and dissolved in 0.5 M NaOH from a stock of 10 g of PCP per liter to final concentrations of 30 and 100 mg of PCP per kg. The radiochemical purity of the [U-^{14}C]PCP was >90% by high-pressure liquid chromatography–radiochemical detection (data provided by the manufacturer). The PCP spike did not affect the soil pH measurably. The water content of the soil was adjusted to 40% of its water-holding capacity with sterilized water or a mineral salt buffer and to 60% for the sandy loam soil type. Four replicates of each treatment were incubated at 15°C in the dark.

Mineralized [^{14}C]PCP was trapped as $^{14}CO_2$ in a sampling vial containing 400 μl of 1 M KOH and quantified by liquid scintillation (Beckman Ready Gel, Beckman LS-1801 counter). The average efficiency of the traps was determined to be 91% ± 5% by using ^{14}C-labeled $NaHCO_3$. However, the efficiency was not considered when calculating CO_2 production. Based on CO_2 production rates, the equivalent amount of PCP mineralized was calculated and spot checked by gas chromatography (GC) analysis.

Amendment with structural materials. When applicable, cell suspensions were incubated overnight at room temperature with 1-mm pieces of polyurethane (PU; Bayvitec C2200; Bayer, Leverkusen, Federal Republic of Germany) at 1 g/kg of soil, allowing the bacteria to attach prior to inoculation into the soil. This procedure led to immobilization of about 10 and 30% of 10^8 cells of *M. chlorophenolicum* PCP1 and *S. chlorophenolica* RA2, respectively, per g as determined by acridine orange direct counts of the bacterial suspensions prior to and after immobilization. Autoclaved beechwood sawdust (250- to 850-μm particle size) was added to the soil samples at 1 g/kg of soil after PCP spiking and before inoculation.

Inocula. Induced cells were harvested by centrifugation, suspended in a mineral salts medium, counted by acridine orange direct counting, and inoculated at the appropriate cell density into soil with or without structural materials. Cultivation and induction of *M. chlorophenolicum* PCP1 (DSM 43826) prior to inoculation was performed as described by Karlson et al. (22).

S. chlorophenolica RA2 (DSM 8671) was grown in a mineral salts medium (27) with 100 mg of PCP per liter as the sole source of carbon and energy. Additional PCP at the same concentration was fed after PCP disappearance, as determined by measuring the A_{320} of the culture supernatant. The culture was harvested after three subsequent feedings, counted, and diluted to the appropriate cell number with fresh, PCP-free medium.

Cell suspensions of *M. chlorophenolicum* PCP1 could be stored in M9 buffer (6 g of Na_2HPO_4 per liter, 3 g of KH_2PO_4 per liter, 0.5 g of NaCl per liter, 1 g of NH_4Cl per liter, pH 7.4) at 4°C for up to 5 months without loss of activity, although storage time for this study did not exceed 1 week. Cells of *S. chlorophenolica* RA2 were used within 3 days after cultivation. The PCP-mineralizing activity of all cells was reconfirmed with [^{14}C]PCP at 30 μmol of PCP per liter at the time of soil inoculation.

Extraction of bacteria. Bacteria were extracted from soil by using polyvinylpyrrolidone in phosphate buffer, pH 7.4 (modified as described in reference 11). The recovery efficiencies, determined by extraction immediately after soil inoculation, were 78% ± 13% and 98% ± 9% for *M. chlorophenolicum* PCP1 and *S. chlorophenolica* RA2, respectively. For extraction of *M. chlorophenolicum* PCP1, $Ca(OH)_2$ and $MgCO_3$ (0.3 and 0.6 g per 100 ml of extract) were added for flocculation of soil particles. For *S. chlorophenolica* RA2, the extract was centrifuged at 300 × *g* for 2 min to avoid precipitation of cells together with soil particles. The samples were conserved in 2.7% formaldehyde and stored at 4°C.

Immunofluorescence microscopy. Polyclonal antibodies against *M. chlorophenolicum* PCP1 and *S. chlorophenolica* RA2 were raised in rabbits as previously described (6, 23). Crude sera were used as obtained without further purification. Bacterial extracts were filtered through black polycarbonate filters (0.2-μm pore size; Nucleopore) and stained with rabbit serum and fluorescein-conjugated swine anti-rabbit antibodies (Dako A/S, Copenhagen, Denmark) in accordance with standard procedures (20). Bacteria on the stained filters were counted with an Axioplan fluorescence microscope (Zeiss) at a magnification of ×1,000. The detection limit was 10^5 cells per g of soil. Tests with different, related bacterial species revealed no cross-reaction, except for cross-reactions between several *Mycobacterium* spp. and between four *S. chlorophenolica* strains (23). Furthermore, blank extractions of experimental soil yielded zero counts above the detection limit.

Extraction and determination of PCP. After acetone extraction and acetylation, soil PCP was quantified by capillary GC with a ^{63}Ni electron capture detector (HP5890 series II, HP-5 fused silica column) by using 2,4,6-tribromophenol at 10 mg/kg as an internal standard (adapted from reference 24). The following temperature program was used: 0.2 min at 40°C, 30°C/min to 70°C, 4.5°C/min to 165°C, holding for 2 min, and then 50°C/min to 300°C. Chlorophenols were quantified by using response factors of multilevel calibration corrected by the internal standard. When applicable, an aliquot of the acetone extract was used for ^{14}C mass balance and analyzed by liquid scintillation.

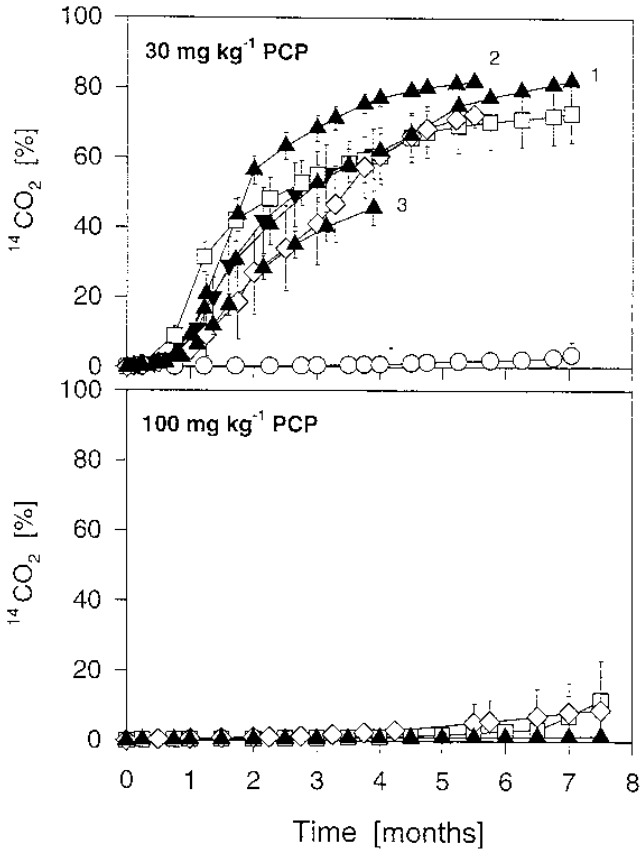

FIG. 1. Mineralization of [^{14}C]PCP in noninoculated soil from a contaminated site at spiked PCP concentrations of 30 and 100 mg kg^{-1}. Symbols: ▲, M9 buffer, different batches 1 to 3; ▼, water; □, soil amended with PU; ◇, soil amended with sawdust; ○, autoclaved control.

RESULTS AND DISCUSSION

For results of $^{14}CO_2$ measurements of soil at initial spiked PCP concentrations of 30 and 100 mg/kg, see Fig. 1, 2, 3, and 5. Data are expressed in percent [^{14}C]PCP recovery in the form of $^{14}CO_2$, and each point represents the mean of four replicates and the bars indicate the standard deviation.

Noninoculated soil. PCP mineralization was observed in noninoculated soil (Fig. 1) at a spiked PCP concentration of 30 mg/kg with maximum rates of 0.3 to 0.5 mg/kg · day. Within 7 months, 82% of the added PCP was recovered as $^{14}CO_2$. The corresponding residual PCP concentration was 0.95 mg/kg. Addition of nutrients (M9 buffer supplying N and P) and structural amendments with sawdust and PU did not significantly influence the activity at 30 mg of PCP per kg in an observed variation range of different batches. At 100 mg of PCP per kg, less than 2% of the added PCP was mineralized in nonamended soil over a period of 7.5 months. But single replicates amended with PU or sawdust started to mineralize PCP after 5 months (>5% $^{14}CO_2$), raising the amount of mineralized PCP in the following 2.5 months to 10%, on average, with single samples containing up to 30% $^{14}CO_2$. Reasons for the effect of PU and sawdust may include a reduction of the toxic concentration of PCP due to adsorption of the pollutant (2, 19, 21, 39).

In contrast, a sandy loam that had never been exposed to PCP before and was used for comparison responded to a PCP spike of 30 mg/kg with no mineralization (data not shown), and

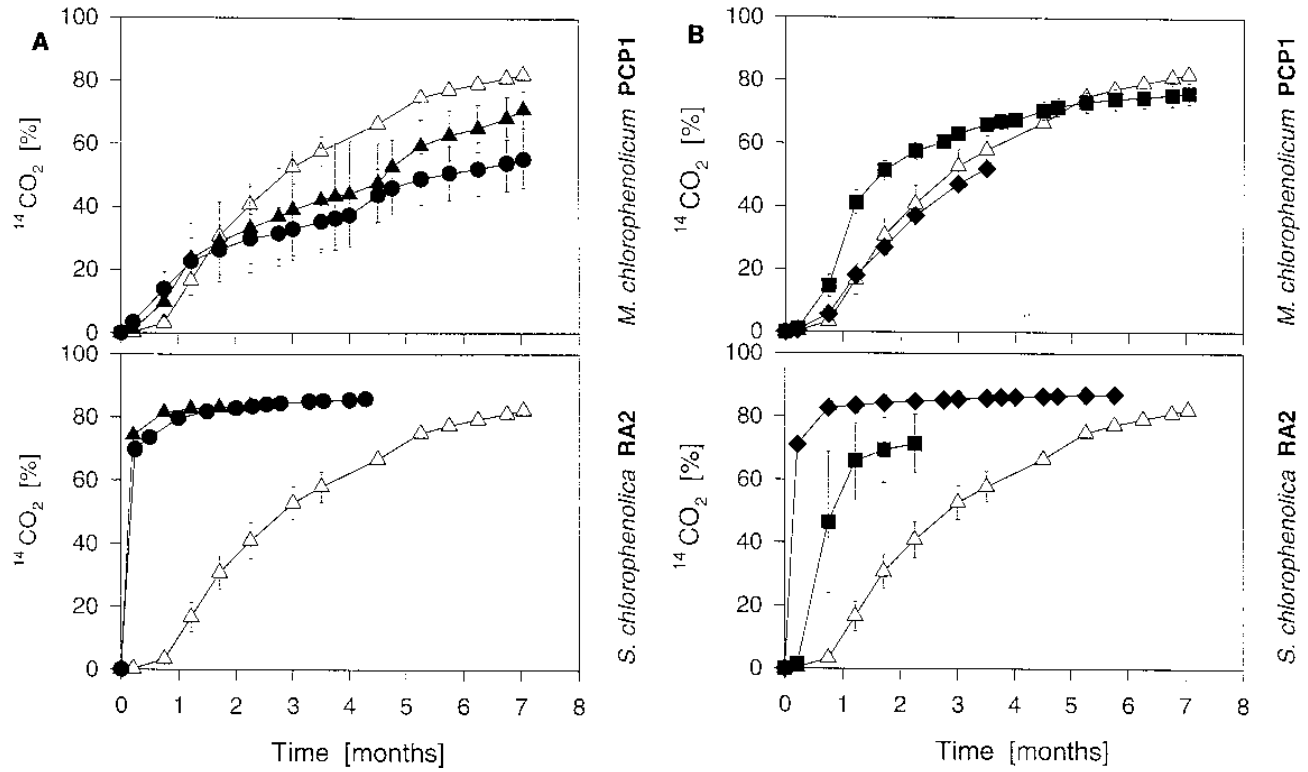

FIG. 2. (A) Effect of inoculation on mineralization of [^{14}C]PCP at a spiked concentration of 30 mg kg^{-1} in natural and autoclaved soil from a contaminated site. Symbols: △, noninoculated soil; ▲, 10^8 cells per g of natural soil; ●, 10^8 cells per g of autoclaved soil. (B) Effect of PU and sawdust on mineralization of [^{14}C]PCP upon inoculation (10^8 cells per g) at a spiked concentration of 30 mg of PCP kg^{-1} in soil from a contaminated site. Symbols: △, noninoculated control; ■, soil with PU as a matrix for the inoculum; ◆, soil amended with sawdust prior to inoculation.

a pristine peaty soil mineralized only 13% of a 30-mg/kg spike within 4 months (24). The high mineralization potential in our sandy soil can be explained by adaptation and enrichment processes during the field exposure to an average of 30 mg of PCP per kg which had been degraded during sample preparation and storage prior to the laboratory experiment. The CO_2 measurements indicate slow further adaptation and enrichment in response to the PCP spikes. This was supported by bacterial plate counts of soil extracts on a complex medium (tryptic soy agar), showing an increase in the total number of bacteria from 3.85×10^6 to 7.4×10^6/g and an increase in carbenicillin-resistant bacteria from 1.5×10^5 to 4.1×10^6/g during 4 months of soil incubation with an initial PCP concentration of 30 mg/kg. On the basis of available data, a correlation between PCP degradation and carbenicillin resistance can be neither postulated nor excluded.

Effect of inoculation and structural amendments. Both of the PCP degraders tested showed mineralization activity in soil at a PCP level of 30 mg/kg when applied to sterilized soil (Fig. 2A), indicating the degradation potential of these inoculants. Mineralization rates upon inoculation into nonsterile soil were not significantly different, indicating that competition with the indigenous microbiota was not a relevant factor. The degree of stimulation by inoculation into natural soil depended on the organism type. While inoculation with *M. chlorophenolicum* PCP1 did not enhance mineralization over the indigenous rate, inoculation with *S. chlorophenolica* RA2 resulted in strong stimulation. With 10^8 cells of strain RA2 per g, the 80% level of $^{14}CO_2$ was achieved within 1 month; this took 7 months in noninoculated soil. This corresponded to a residual PCP concentration of 0.3 mg/kg (GC analysis) and a maximum mineralization rate of 3.5 to 4 mg/kg · day. The inoculated cells disappeared rapidly, reaching 2×10^6 cells per g after 2.3 months and becoming nondetectable ($<10^5$ cells per g) after 7 months (data not shown). Lower inoculation densities of strain RA2 (10^6 and 10^4 cells per g) resulted in strongly reduced mineralization rates, with 10^4 cells per g not significantly differing from the control. However, at higher PCP concentrations, at which the indigenous activity was zero, even 10^4 cells per g stimulated mineralization to 2.4 mg/kg · day after a lag phase of ca. 1 month (data not shown). Soil amendment with PU or sawdust did not have a positive effect on the performance of *S. chlorophenolica* RA2 at 30 mg of PCP per kg (Fig. 2B). To the contrary, PU resulted in a lag phase of 1 week, which was not observed in nonamended inoculation.

Nonamended inoculation with *M. chlorophenolicum* PCP1 resulted in decreased CO_2 production compared with the noninoculated background (Fig. 2A). GC analyses, however, did not show a significant difference between the residual PCP concentrations resulting from inoculated and noninoculated treatment at 3.85 and 7.5 months. While amendment with sawdust had no effect, addition of *M. chlorophenolicum* PCP1 attached to PU raised mineralization above the background level with a maximum rate of 0.6 to 0.7 mg/kg · day, regardless of the inoculum size tested (10^8, 10^6, or 10^4 cells per g of soil). These results are in the same range as those reported for artificially contaminated pristine soil upon inoculation with *M. chlorophenolicum* PCP1 immobilized on PU (24). Carriers like PU should improve the conditions for the released bacteria by protecting them from predation and contaminant toxicity (15, 18). However, our results do not indicate improved survival, despite the higher degradation activity, as cell densities be-

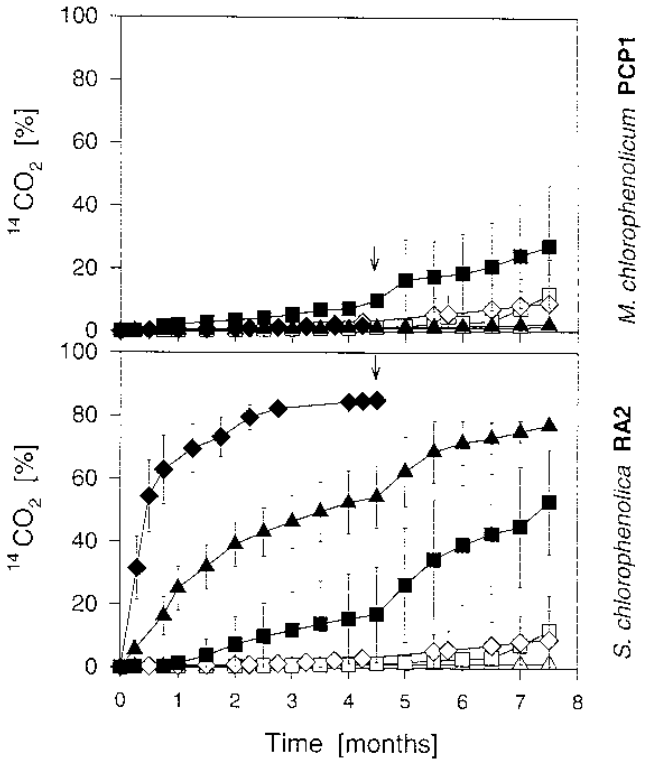

FIG. 3. Mineralization of [^{14}C]PCP at a spiked PCP concentration of 100 mg kg^{-1} in soil from a contaminated site. Open symbols, noninoculated soil; filled symbols, soil inoculated with 10^8 cells per g. Symbols: △, ▲, no amendment; □, ■, PU as a matrix for the inoculum; ◇, ◆, soil amended with sawdust; ↓, soil remoistened.

tween 4.6×10^6 and 8.6×10^6 cells per g, regardless of PU amendment, were detected by immunofluorescence microscopy after 7 months of incubation (initial cell density, 10^8 cells per g; initial PCP concentration, 30 mg/kg).

At 100 mg of PCP per kg, both strains were inhibited (Fig. 3). Inoculation with nonamended *S. chlorophenolica* RA2 cells resulted in slow mineralization with a maximum rate of 1.4 mg/kg · day, the activity remaining measurable over a period of 10.75 months until the initial PCP had been degraded to an average level of 1.5 mg/kg (not all data shown). Addition of sawdust prior to inoculation with *S. chlorophenolica* RA2 accelerated the mineralization by up to threefold, such that rates of 3.7 to 4 mg/kg · day, as with 30 mg of PCP per kg and no amendment, were achieved. GC analysis of single replicates showed mineralization of 83% PCP within 35 days and a residual PCP concentration of 3.7 mg/kg. Colores et al. (8) recently reported that in remediation experiments with sandy loam, 2×10^7 cells of *S. chlorophenolica* RA2 per g mineralized 600 mg of PCP per kg in less than 2 months without any amendments. Factors contributing to this higher performance may include adaptation to a high PCP concentration prior to the soil experiment (300 mg/liter in shake flasks cultures) and the PCP availability situation in the soil, influenced by higher water contents (70% of the water-holding capacity), but also by soil properties, e.g., PCP adsorption to the soil and a soil pH of 7.0. In our study, when *S. chlorophenolica* RA2 was inoculated for comparison into sandy loam with a pH of 4.6, PCP mineralization was observed only after pH adjustment to 7.0 with $Ca(OH)_2$ (data not shown), indicating the importance of a neutral pH for this strain.

Inoculation with nonamended *M. chlorophenolicum* PCP1 cells at 100 mg of PCP per kg failed to produce a measurable effect (Fig. 3). Addition of PU facilitated some PCP mineralization, but only with a very low rate of 0.25 mg/kg · day, which resulted in overall mineralization of 27% ± 19% within 7.5 months, although previously the strain, immobilized on PU, had been used for simulated cleanup of a sandy loam artificially contaminated with up to 600 mg of PCP per kg (5). Our inoculum size was at the same level, and we counted 2.9×10^7 cells per g by immunofluorescence microscopy after 7.5 months of incubation, indicating that low survival was not a factor. However, the 10-fold lower PU concentration used in this study may have contributed to the poor performance of the inoculum at a high PCP concentration. *M. chlorophenolicum* PCP1 is strongly inhibited at PCP concentrations above 15 mg/liter in liquid culture (38). Equilibrium measurements for PCP in this soil without PU indicated that at 100 mg of PCP per kg, the concentration of PCP dissolved in soil water is in excess of 800 mg/liter at the soil moisture content applied in this study. The low adsorption of PCP is attributable to this soil's texture and low organic carbon content. Amendment with a larger amount of PU, acting as a buffer for dissolved PCP, due to the affinity for organic compounds of its large inner surface (19, 21), could have been used to protect *M. chlorophenolicum* PCP1. However, application of more than 1 g of PU per kg was deemed impractical with respect to soil bioremediation. Another inhibiting factor may have been the soil pH of 7.9, considering that the strain has its pH optimum between 5 and 7 (38).

The effects of the soil amendments PU and sawdust on the activities of strains PCP1 and RA2 cannot be fully explained, as both adsorb PCP and reduce its concentration in the soil water (2, 3, 21, 39). In a study with pure cultures of a phenoxyalkanoate-degrading *Flavobacterium* sp. (19), the bacteria were able to degrade higher concentrations of the toxic herbicides, and with a reduced lag phase, when in the presence of PU. This was attributed to the herbicides' reduced initial concentrations due to reversible adsorption, which did not, however, prevent their final complete degradation. While PU increased the mineralization activity of *M. chlorophenolicum* PCP1 and sawdust did not, the opposite was observed for *S. chlorophenolica* RA2 (Fig. 2B). However, PCP mineralization was still faster with strain RA2 and PU than with strain PCP1 and PU. With the identical structural materials, the PCP adsorption capacity of the PU was found to be 10 times higher than that of sawdust, whereas desorption occurred seven times more slowly than from PU (39). One might speculate that considering the high potential mineralization rate of strain RA2, the rate of desorption of PCP from PU was limiting while that from sawdust was not. For strain PCP1, the more PCP-sensitive and more slowly metabolizing organism, the adsorption of PCP to sawdust was not strong enough to reduce toxicity and the rate of desorption from PU was high enough not to be limiting. In general, sawdust would be preferable over PU as a structural amendment for environmental reasons.

Mass balances. Mass balances based on GC-electron capture detector analysis, ^{14}C counting in soil extracts, and cumulative evolution of $^{14}CO_2$ over time at a spiked PCP concentration of 30 mg/kg are presented in Fig. 4. Depletion of PCP was indicated by $^{14}CO_2$ levels of 80 to 85% for all setups. GC analysis showed no accumulation of lower chlorinated phenols or chloroanisols in detectable amounts during degradation and indicated final PCP concentrations below 1 mg/kg. Nonchlorinated metabolites, determined by scintillation counting of the acetone extracts appeared only temporarily and in small percentages. Considering incorporation of ^{14}C in biomass with a

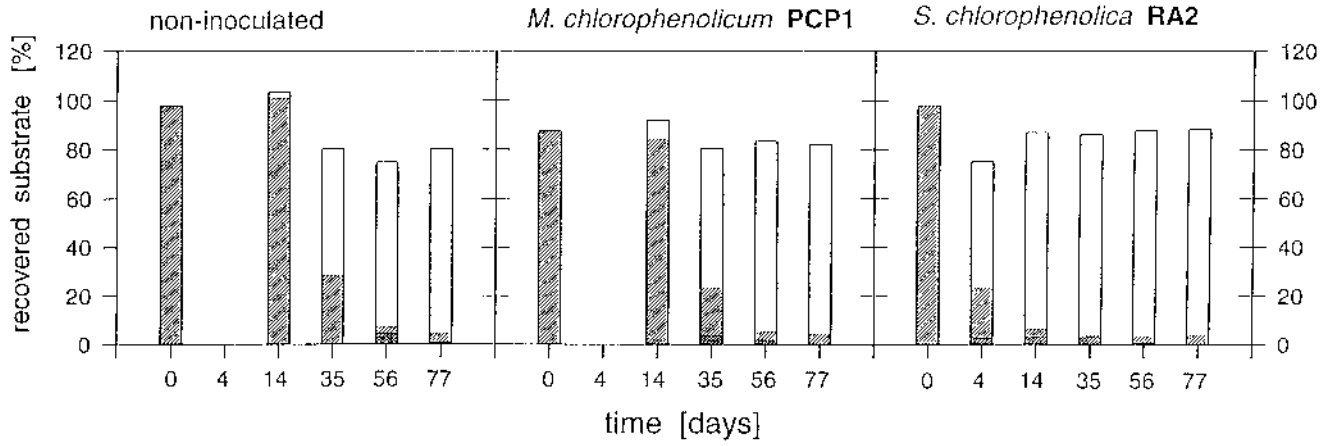

FIG. 4. Mass balances of residual soil PCP (measured by GC analysis), metabolized PCP (measured by the evolution of $^{14}CO_2$), and extractable non-PCP ^{14}C in soil from a contaminated site with an initial spiked PCP concentration of 30 mg kg^{-1}, with and without inoculation at 10^8 cells per g. *M. chlorophenolicum* PCP1 was attached to PU. ▨, PCP; ▩, extractable non-PCP ^{14}C; ▭, captured $^{14}CO_2$.

yield of about 10 to 15% (determined in liquid culture experiments in this study [data not shown] and in earlier studies [27, 28]) and the trap efficiency of ca. 91%, the balances add to approximately 100%.

Respiking experiments at 30 mg of PCP per kg. Subsequent addition of 30 mg of [^{14}C]PCP per kg after depletion of the original PCP spike resulted in mineralization rates of 1 to 1.5 mg/kg · day in all of the variations tested (Fig. 5), except that presterilized soil inoculated with strain RA2 mineralized at only 0.3 mg/kg · day, which is consistent with the low survival of RA2 after complete PCP mineralization measured in the other experiments. This indicated that the observed mineralization in nonsterile soil was additive from both the inoculum and stimulated indigenous microbes. The magnitude of the soil biomass of active PCP degraders has been shown to correlate well with the rate of PCP mineralization in spiked soil (31). Application of those findings to our data suggests that the initial PCP spike approximately tripled the number of active indigenous PCP degraders, that these degraders remained active for up to 7 months, and that consecutive spikes did not result in a further increase of their number. Watanabe (37), who investigated the changes in microbial populations in field plots during annual applications of PCP, found 100 to 1,000 times more (potentially) PCP-degrading microorganisms after PCP exposure. Since our soil had already been exposed to PCP earlier, the effect of the experimental PCP spike on the indigenous microbiota was not so pronounced.

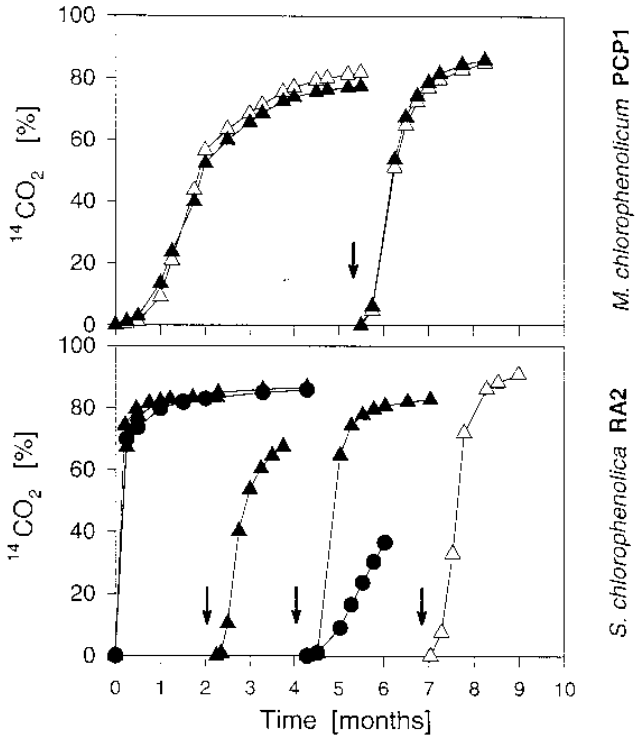

FIG. 5. Respiking experiments. PCP was added to a level of 30 mg kg^{-1} after complete mineralization of the first PCP spike in soil from a contaminated site. Symbols: ↓, PCP respike; ▲, soil inoculated at time zero; ●, autoclaved soil inoculated at time zero; △, noninoculated soil.

In conclusion, the results indicate that when the requirements of the individual strain were met, inoculation of PCP degraders enhanced biodegradation above the high indigenous activity of a soil with a history of PCP contamination, especially at a high PCP concentration. Structural amendments amplified the effect of inoculation. However, general soil categories are insufficient to predict the success of bioremediation upon inoculation, even when the same microbial strain is used. The activity of inoculated bacteria is multifactorially influenced and needs to be investigated case by case before application in the field.

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