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Chlorophenol removal from soil suspensions: effects of a specialised microbial inoculum and a degradable analogue

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Abstract

Two soils of different contamination history were tested in slurry for their self-remediability towards mono-, di- and trisubstituted chlorophenols. The landfill soil showed poor ability in removing the compounds. Instead, the soil from the golf course, treated for many years with a 2,4,6-trichlorophenol derivative (Prochloraz), remediated different concentrations of the same 2,4,6TCP, 2,4-dichlorophenol and monochlorophenol isomers, singly and in mixtures, at varying degradation rates. *Ralstonia eutropha* TCP, a specialised microorganism capable of degrading 2,4,6TCP, proved highly efficient in removing the compound from both tested soils. The same microbial inoculum allowed total removal of the ternary mixture of monochlorophenol isomers from the golf course soil, but it did not accelerate the removal of the same compounds when singly supplied. The addition of phenol as a degradable analogue was more effective in co-metabolically removing not only the single monochlorophenols,but also their mixtures, the removal occurring faster and independently of the presence of the microbial inoculum. From the golf course soil, a microorganism, phenotypically and genetically identical to *R. eutropha* TCP, was isolated and classified as *R. eutropha* TCP II.

Abbreviations: 2,4,5T – 2,4,5-trichlorophenoxyacetate; 2,4D – 2,4-dichlorophenoxyacetate; 2,4,6TCP – 2,4,6-trichlorophenol.

Introduction

Chloroorganic contaminants are present in the environment due to their extensive use in agriculture and industry. Thus the bioremediation of ecosystems polluted with such compounds is an important goal of basic and applied research. Among such contaminants are the chlorophenols, used widely as biocides, wood preservatives and organic precursors of pesticides like 2,4D, 2,4,5T and Prochloraz. Chlorophenols are notoriously persistent and toxic towards different biological systems; some of these compounds have been included in the Environmental Protection Agency list. Chlorophenol toxicity towards microorganisms is mostly dependent on the physico-chemical properties of the chlorophenol, as well as on the position and number of chlorine substituents on the aromatic ring (Beltrame et al. 1987; Boyd et al. 2001). However, each microorganism presents different tolerance towards chlorophenol isomers of different substitution grade, in relation to the concentration, the type of isomer and the degree of substitution of the compound tested (Ruckdeschel et al. 1987; Kiyohara et al. 1992).

Recently, the aspects related to chlorophenol biodegradation and toxicity as well as the problems of treating contaminated sites were reported (Leung et al. 1997; Litchfield & Rao 1998). Despite chlorophenol toxicity, different microorganisms have been found to degrade different chlorophenol isomers, with particular regard to 2,4,6TCP and pentachlorophenol, more than monochlorophenols, which are mainly degraded through co-metabolism in the presence of an easily degradable carbon source (Hill et al. 1996; Dapaah & Hill 1992; Lu & Tsai 1993; Kim & Hao 1999; Wang

& Loh 2001). The use of microorganisms with appropriate degrading properties for the biorestoration of haloaromatic contaminated sites has long been studied on the laboratory scale and in field studies (Karns et al. 1984; Balfanz & Rehm 1991; Comeau et al. 1993; Litchfield et al. 1994; Valenzuela et al. 1997; Andreoni et al. 1998; Fava et al. 2000).

One current approach to the biotreatment of soil pollutants concerns the microbial removal of mixed contaminants from ecosystems, notoriously more difficult than that of the single compounds (Baggi 2000). Chlorophenol mixtures of low substitution grade may be present as pollutants in different ecosystems, resulting from industry and agriculture through the chlorination of processing waters and effluents, the bleaching of pulp and paper, and the reductive biotic and abiotic dechlorination of highly substituted chlorophenols in soil (Evans et al. 1971a, b; Karns et al. 1983; Salkinoja et al. 1984; Armenante et al. 1993; Juteau et al. 1995; Bouchard et al. 1996). In this regard, data on the removal of chlorophenol mixtures are not exhaustive and concern mainly binary mixtures (Schmidt 1987; Klecka & Maier 1988; Dapaah & Hill 1992; Menke & Rehm 1992). More recently, the co-metabolic removal of binary and ternary mixtures of the three monochlorophenol isomers, phenol being the carbon source, brought about by a *Pseudomonas aeruginosa* strain and *Ralstonia eutropha* TCP (formerly *Alcaligenes eutrophus* TCP) in batch experiments, was reported (Baggi et al. 2002).

In the present paper, the removal of chlorophenols was investigated in soil suspensions, simulating slurry-phase conditions that better reflect the actual biodegrading potential of soil. The aims of the work were: (i) to assess two soils of different contamination history for their intrinsic chlorophenol degradation properties and (ii) to evaluate the effects of the aforementioned *R. eutropha* TCP as an inoculum and of an additive such as phenol to obtain information for the improvement of chlorophenol bioremediation strategies.

Materials and methods

Soils

The soils used for the suspensions were collected from a landfill, Cavenago, Italy, and from a golf-course, Como, Italy. The golf course soil had been treated with Prochloraz for a long time. Both soils were characterised, see Table 1. For control samples of the two soils were autoclaved three times at 24-hr intervals, at 120 °C for 20 min.

Table 1. Characteristics of the two soils

	LS[a]	ST[b]
Microbial load (CFU g^{-1} dry soil)	4×10^{6} [c]	4×10^{7} [c]
	7×10^{3} [d]	5×10^{5} [d]
pH	7.75	7.05
Organic content	1.1%	9.3%
Water content	6.4%	2.3%

[a]Landfill soil.
[b]Prochloraz-treated soil.
[c]On Plate Count Agar.
[d]On Malt Extract Agar.

Organism and culture conditions

The strain used as the inoculum for the soil suspensions was *R. eutropha* TCP, previously isolated from the activated sludge of a wastewater plant and capable of utilising phenol and 2,4,6TCP as sole carbon and energy source. A mineral medium supplemented with phenol or 2,4,6TCP (300 mg l^{-1}) as carbon source was used for growth (Andreoni et al. 1998).

Preparation of microbial inocula

The inocula were obtained from liquid cultures of *R. eutropha* TCP grown aerobically at 30 °C on the appropriate substrate (phenol and 2,4,6TCP at 300 mg l^{-1}, benzoate and glutamate at 1 g l^{-1}). The optical density at 540 nm ($OD_{540\,nm}$) was adjusted to 0.04, corresponding to about 3.8×10^{7} CFU ml^{-1} determined by plate counts on Plate Count Agar (Difco, Detroit, Michigan, USA). The size of the inocula is given in detail in the Results and Discussion.

Preparation of soil suspensions

The experiments were performed in 750 ml Erlenmeyer flasks, suspending the dry, sieved (2 mm mesh) soil in distilled H_2O (40:100, w/w). After shaking the flasks, 15 ml of each suspension was taken and each diluted to a total volume of 150 ml using mineral medium amended with the tested carbon source (phenol or chlorophenols) and, when required, with phenol together with monochlorophenol isomers singly or mixed, at the concentrations given in the Results and discussion. Each experiment consisted of two sets: soil and autoclaved soil as control. In the assays requiring the microbial inoculum, two other sets were

performed: soil inoculated with *R. eutropha* TCP, and autoclaved, inoculated soil as control. At appropriate time intervals, 5 ml of each soil suspension was withdrawn, filtered (0.22 μm) to remove soil particles and microbial cells, and subjected to analytical determinations.

Isolation of a 2,4,6TCP-degrading organism

Through enrichment techniques, a 2,4,6TCP-degrading strain was isolated by plating on Plate Count Agar a transfer of the original suspension performed with the Prochloraz-treated soil in the presence of 2,4,6TCP (300 mg l^{-1}) as sole carbon source.

Phenotypic and molecular characterisation of the bacterial strain

The organism was characterised by conventional procedures (Collins et al. 1989) and submitted to Apisystem 20NE (BioMèrieux, Marcy L'Etoile, France) for classification. The characterisation at the molecular level was performed through DNA extraction (i) and PCR amplifications and sequence analysis (ii).

(i) DNA extraction was carried out on the cell suspensions with an OD_{600} of 2.0 in 100 μl; the cells were centrifuged at 13,000 *g* for 7 min and suspended with 100 μl of sterile MilliQ water, 100 μl of 10 mM Tris-HCl (pH 8.0), and 13 μl of Proteinase K (1 mg ml^{-1}). The mixture was incubated for 2 hr at 55 °C, then boiled for 10 min and centrifuged at 13,000 *g* for 5 min. The supernatant containing DNA was drawn and put in sterile Eppendorf microtube.

(ii) Amplification of the small ribosomal subunit gene (16S rDNA) was performed using eubacterial universal primers P27f and P1495r referred to the *E. coli* nucleotide sequence of the 16S rDNA gene. It was carried out in a reaction volume of 50 μl containing: 2.5 mM $MgCl_2$, 0.2 mM of each nucleotide dATP, dTTP, dCTP and dGTP (Amersham Biosciences, Sweden), 0.2 μM forward primer P27f (5′-AGA GTT TGA TCC TGG CTC AG-3′) (Invitrogen, UK) and 0.2 μM reverse primer P1495r (5′-CTA CGG CTA CCT TGT TAC GA-3′) (Invitrogen), 1 U of Taq (Bioline, UK) and 2 μl of extracted DNA. The thermal profile, performed in an Hybaid PCR Sprint apparatus (Hybaid, UK), was as follows: denaturation at 95 °C for 3 min, 35 cycles at 94 °C for one min, 55 °C for one min, 72 °C for 2 min, then a final extension step at 72 °C for 15 min. Five μl of the amplified products was analysed on 1.5% agarose gels, visualised by a standard procedure (Sambrook et al. 1989), and photographed by a Geldoc apparatus (Biorad, UK).

The sequencing reaction was performed by PCR amplification in a final volume of 20 μl using 100 ng of PCR products, 5 pmol of primer and 9.5 μl of Dye-Terminator premix according to ABI-Applied Biosystems (California, USA) protocol. Primers used in the PCR reaction of sequencing products were the same as in normal PCR. After heating to 94 °C for 2 min, the reaction was cycled as follows: 25 cycles of 30 s at 94 °C, 30 s at 55 °C, and 4 min at 60 °C (2400 thermal cycler, ABI-Applied Biosystems). Removal of excess Dye-Terminators was performed using Quick Spin columns (Boehringer Mannheim). The forward and reverse samples were run on an ABI Prism 310A genetic analyser (ABI – Applied Biosystems). The new partial sequences were aligned with published sequences from Genbank, using ClustalW.

Analytical methods

The chlorophenol and phenol concentration in the soil suspensions was determined by HPLC using a Jasco ternary gradient unit (model LG 980-02, Jasco, Tokyo, Japan) with a UV/VIS detector (model UV-975 Intelligent, Jasco). Detection was by UV light at 254 nm. A Merck Lichrosorb RP8 column was utilised. Free chloride concentration was determined turbidimetrically at 460 nm using the mercury (II) thiocyanate method (Florence & Farrar 1971).

Chemicals

Organic and inorganic reagents were from Aldrich (Milan, Italy).

Results and discussion

Self-remediability of the soils

The intrinsic capability of the two soils to degrade chlorophenols differing in substitution grade and chlorine position on the aromatic ring (monochlorophenol isomers, 2,4-dichloro- and 3,5-dichlorophenol, 2,4,6TCP and 2,3,6-trichlorophenol) was evaluated in experiments conducted using soil suspensions to which chlorophenols tested at different concentrations (from 20 to 300 mg l^{-1}) had been added. The control used for the degradation activity of the autochthonous microorganisms was phenol at 300

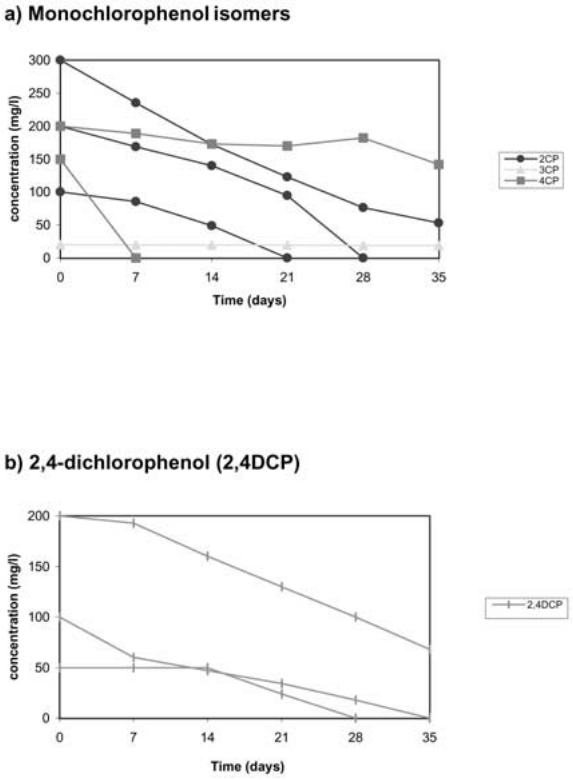

Figure 1. Removal rate of chlorophenols at different concentrations in suspensions performed with the Prochloraz-treated soil.

mg l^{-1}. The decrease in chlorophenols and phenol was followed for up to 42 days of incubation. In both soils, the complete degradation of phenol occurred within 4–7 days of incubation. Among the chlorophenols tested in the landfill soil suspensions, there was the slow decrease of only 2-chlorophenol at 300 mg l^{-1} (but not beyond 75%) with a non-stoichiometric chloride release (only 20%). In contrast, 2,4,6TCP at 300 mg l^{-1} was completely removed, with stoichiometric dechlorination and within 14 days of incubation, from the golf course soil. In the same soil 2,4-dichlorophenol and the monochlorophenol isomers at different concentrations and degradation rates were also degraded (Figure 1). 3,5-Dichlorophenol and 2,3,6-trichlorophenol were not removed in either of the soils within the monitored times, even at the lowest concentration tested (20 mg l^{-1}). No chlorophenol degradation occurred in abiotic controls containing autoclaved soil.

The different intrinsic degradative capability towards chlorophenols of the two soils likely depends on their different contamination history: the landfill soil, long exposed to undifferentiated contamination, showed poor degradation ability. In contrast, the golf-course soil which had been treated for many years with Prochloraz, a fungicide whose breakdown generates 2,4,6TCP (Bock et al. 1996), exhibited an intrinsic ability to degrade the chlorophenol in short times at the highest concentration tested, as well as monochlorophenol isomers and 2,4-dichlorophenol, although at lower concentrations and degradation rates.

Isolation of a 2,4,6TCP-degrading organism

Through subsequent transfers of the original suspension performed with the golf course soil in the presence of 300 mg l^{-1} 2,4,6TCP, the biodegradative process was accelerated. After some transfers, a microorganism, capable of utilising 2,4,6TCP as the sole carbon source, was isolated in pure culture by plating on complete medium. The strain showed a phenotype identical to *R. eutropha* TCP, which we had previously isolated from the activated sludge of a wastewater plant (Andreoni et al. 1998). Partial sequence of the 16S rRNA gene (600 bp of the forward reaction and 600 bp of the reverse reaction) of the new isolate showed 99.9% sequence homology to the 16S rDNA sequence of *R. eutropha* strain TCP (Andreoni et al. 1998), 99.7% DNA to *R. eutropha* (D88004) and lower homologies to other Ralstonia-related genera and species present in EMBL and GenBank databases (Figure 2). On the basis of these data, the strain was identified as *R. eutropha* TCP II. The new isolate showed the same degradative properties towards chlorophenols of *R. eutropha* TCP (data not shown). This result is in accord with the principles of the natural selection of the microorganisms and confirms the involvement of this species in 2,4,6TCP degradation, independently of its source, as supported also by previous literature data. In fact, among chlorophenol-degrading organisms, *R. eutropha* JMP 134 is well known for its wide degradation ability towards different xenobiotics such as 2,4,6TCP and other chlorophenols (Clément et al. 1995). Instead, *R. eutropha* TCP and the new isolate (*R. eutropha* TCP II) are highly specialised in growing only on 2,4,6TCP as the carbon source and co-metabolising monochlorophenols singly and in mixture in the presence of another carbon source (Andreoni et al. 1998; Baggi et al. 2002).

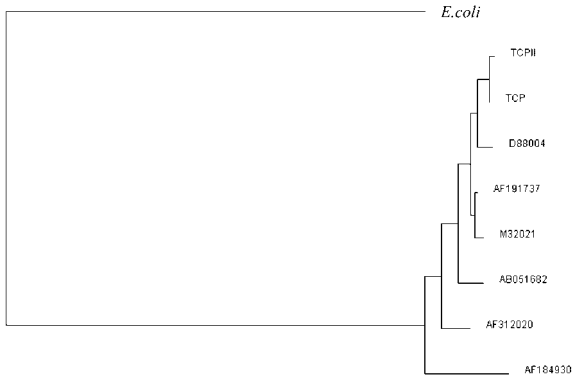

Figure 2. Phylogenetic position of strain TCP II with respect to previously analysed *Ralstonia eutropha* strain TCP and strains present in GenBank. The analysis was based on 16S rDNA nucleotide sequence comparison. D88004: *Ralstonia eutropha*; AF191737: *Cupriavidus necator*; M32021: *Alcaligenes eutrophus*: AB051682: *Ralstonia* sp. HAB08; AF312020: *Ralstonia campinensis*; AF184930: 2,4-D degrading bacterium. *E. coli* 16S rDNA sequence was used as an out group organism. The distance matrix to build the phylogenetic tree was analysed by using the Neighbour Joining coefficient.

Efficiency of a specialised inoculum in 2,4,6TCP removal

Suspensions of the two kinds of soil added with 300 mg l^{-1} of 2,4,6TCP were inoculated with *R. eutropha* TCP to study the degradation rate of this chlorophenol in the presence of a specialised strain and the effect of the size (from 10 to 0.1 ml, corresponding to about 3.8×10^7 CFU ml^{-1}) and the growth substrate (2,4,6TCP and phenol at 300 mg l^{-1} or benzoate and glutammate at 1 g l^{-1}) of the inoculum on the degradative process. In all the inoculated assays, there was the total removal of 2,4,6TCP within 40–48 hr of incubation, regardless of inoculum size and growth substrate. In the controls containing autoclaved inoculated soils, 2,4,6TCP was completely degraded within the same time. No 2,4,6TCP degradation was observed in the abiotic controls.

The rapid removal of the haloaromatic compound in both soils, regardless of their different intrinsic potential, demonstrated the fitness of the strain in conditions simulating slurry-phase treatments. In addition, the degradation process proceeded well, being accomplished with inocula at low cell density and independently of the growth substrate of the inoculated microbial strain, and without any evidence of competitive phenomena with the autochthonous microorganisms of the tested soils.

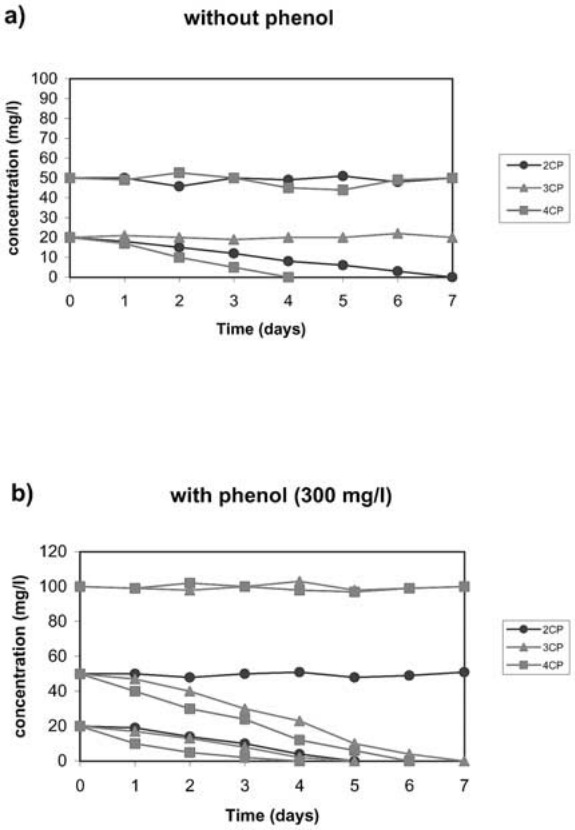

Figure 3. Effect of phenol on the removal rate of monochlorophenols singly supplied in suspensions performed with the Prochloraz-treated soil inoculated with *R. eutropha* TCP.

Co-metabolic removal of monochlorophenols in golf-course soil suspensions inoculated with R. eutropha *TCP*

To evaluate the effects of *R. eutropha* TCP as the microbial inoculum and a degradable analogue like phenol in promoting or accelerating monochlorophenol removal, golf course soil suspensions were inoculated with the strain grown on phenol (size of the inoculum, 0.1 ml cell suspension of an $O.D._{540}$ nm = 0.04 per flask) and added with monochlorophenol isomers at different concentrations (from 20 to 100 mg l^{-1}), singly and in mixtures, and, when required, with phenol at 300 mg l^{-1}. Non-inoculated and autoclaved controls, with or without phenol, were performed for each case.

Monochlorophenols supplied as single co-substrates

Monochlorophenols were added at concentrations ranging from 20 to 100 mg l^{-1} in (i) the absence and

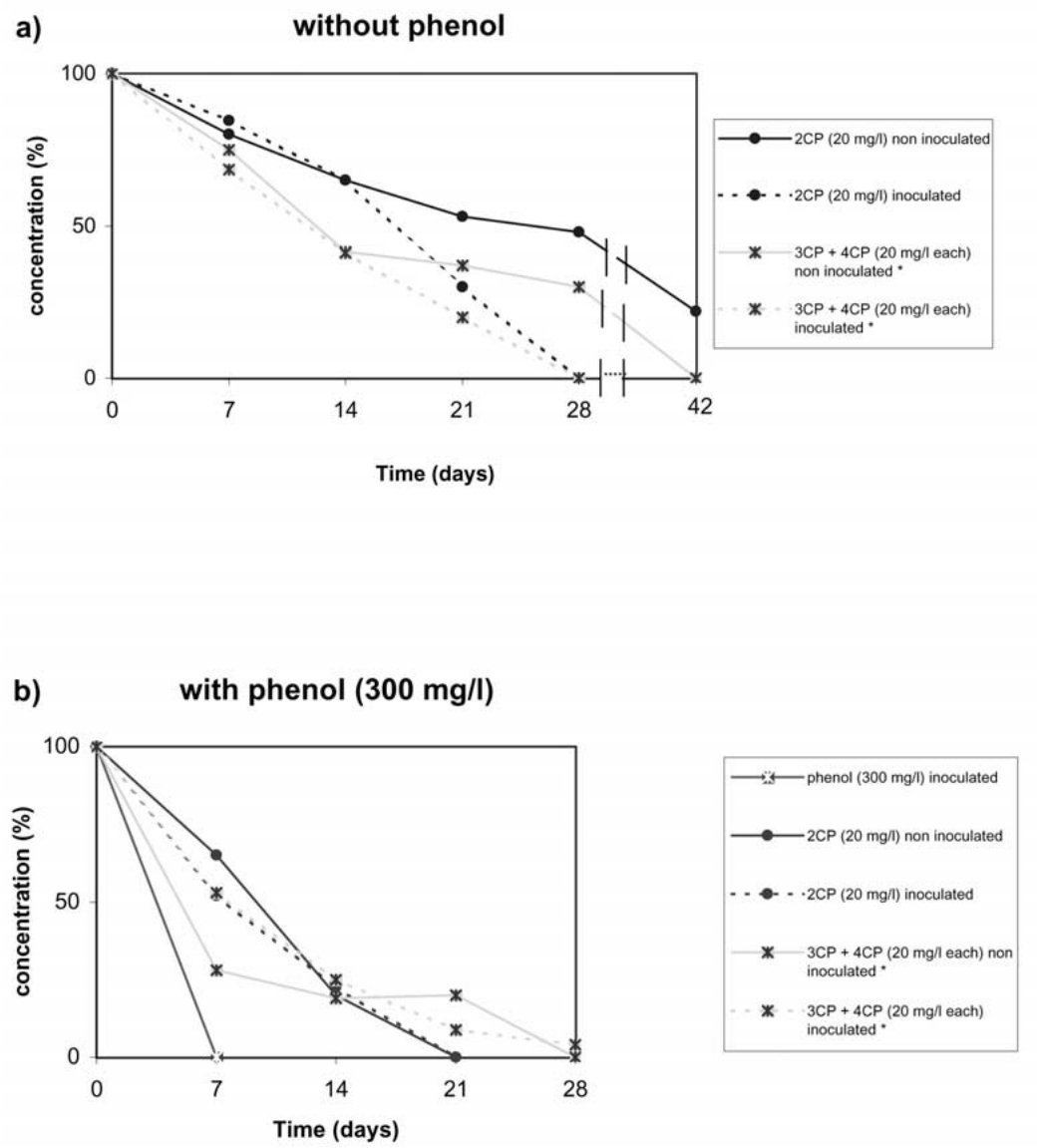

Figure 4. Effects of phenol and *R. eutropha* TCP as microbial inoculum on degradation rate of monochlorophenol ternary mixture in suspensions performed with the Prochloraz-treated soil. *3CP and 4CP are not separable in HPLC analyses.

(ii) the presence of phenol (300 mg l^{-1}). (i) In inoculated suspensions, the three monochlorophenol isomers supplied at 20 mg l^{-1} were all removed, but the degradation rate of each isomer differed little compared with the removal times obtained with the same non-inoculated soil: 7 days for 2-chlorophenol and 4-chlorophenol, 42 days for 3-chlorophenol (Figure 3a). (ii) At 20 mg l^{-1}, each isomer was removed within only 4–5 days of incubation, together with phenol. There was the persistence, over 7 days of incubation, of 50 mg l^{-1} of 2-chlorophenol, as well as the inhibition of phenol degradation, whereas at the same concentration the other two isomers were co-metabolically removed in the course of phenol degradation in 7 days. When added at 100 mg l^{-1}, 3-chlorophenol and 4-chlorophenol were not removed and phenol degradation was also inhibited (Figure 3b). Identical results were obtained in non-inoculated controls. In autoclaved controls with or without phenol, neither monochlorophenols at each concentration tested nor phenol, when present, was degraded.

These results show the irrelevant effect of the inoculum in the removal of each isomer at low concentration from a soil having a discrete self-remediability towards these compounds. In contrast, the addition of phenol as an easily degradable compound had a more significant influence on markedly accelerating the removal of 3-chlorophenol, the most persistent isomer in the soil without phenol, In fact, the compound was removed faster and at higher concentrations than in the presence of only the inoculum. In contrast, the removal rate of 4-chlorophenol and 2-chlorophenol did not seem significantly influenced. It is noteworthy that when the monochlorophenols were supplied at increasing concentrations (50 mg l^{-1} for 2-chlorophenol and 100 mg l^{-1} for 3-chloro- and 4-chlorophenol), presumably toxic for the soil microflora and/or microbial inoculum, phenol degradation was also inhibited. An enhanced removal of 4-chlorophenol in the presence of phenol as a supplemental carbon source has been already reported (Bae et al. 1996).

Monochlorophenols supplied in ternary mixture

Similar golf course soil suspensions were performed by furnishing monochlorophenol isomers (each at 20 mg l^{-1}) in ternary mixture in (i) the absence and (ii) the presence of 300 mg l^{-1} of phenol. (i) In the presence of the microbial inoculum, the ternary mixture disappeared in 28 days; in the controls without inoculum, 42 days were required for the removal of two components of the mixture (3-chlorophenol and 4-chlorophenol), whereas about 25% of 2-chlorophenol persisted even after 50 days of incubation (Figure 4a). (ii) The co-metabolic removal of the ternary monochlorophenol mixture occurred within the same time in inoculated and non-inoculated suspensions (28 days of incubation), whereas phenol degradation was completed in 7 days in both cases (Figure 4b). No removal of the monochlorophenol mixture or phenol, when added, occurred in autoclaved controls.

These results show that addition of the microbial inoculum allowed removal of all the components from the mixture completely (also 2-chlorophenol) and quickly (28 days). Within the same time, the removal of the mixture was completed also in the presence of phenol, independently of the inoculum, demonstrating the non-cumulative effect of the two factors. To our knowledge, this is the first report on the complete removal of a monochlorophenol ternary mixture, even though in a laboratory-scale system.

Conclusions

On the whole, these results confirm the difficulty in the degradation of mixed pollutants and highlight the importance of the presence of an easily degradable compound and, secondarily, of a specialised microbial inoculum to favour the co-metabolic removal of the monochlorophenol isomers singly and in mixture. Such indications could be useful to improve the setting up of biorestoration strategies for soils containing noxious and persistent compounds.

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