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Degradation of chlorinated and non-chlorinated aromatic solvents in soil suspensions by pure bacterial cultures

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Summary. Several strains that utilize aromatic solvents were isolated and tested for their ability to degrade chlorinated and non-chlorinated aromatic hydrocarbons. The effect of inoculation with pure bacterial cultures on the degradation of benzene, toluene, *o*-, *m*- and *p*-xylene, chlorobenzene, *o*-dichlorobenzene and 1,3,5-trichlorobenzene in soil slurries was studied. The compounds for which organisms were added were rapidly degraded. Without inoculation, however, degradation of benzene, toluene, *m*- and *p*-xylene and chlorobenzene was slow, while *o*-xylene and *o*-dichlorobenzene were only slightly degraded. The results showed that degradation was due to growth of the inoculated cells using the aromatic compounds as sources of carbon and energy. Addition of activated sludge did not stimulate degradation. The degradation rate of aromatic solvents by the added bacteria in soil slurries was similar or higher than that observed in liquid cultures of the same organisms.

Introduction

Biological treatment of contaminated soil is receiving increasing interest. It is an attractive alternative to expensive physical or chemical cleanup methods, when rapid degradation of the compounds concerned can be obtained (Wilson and Ward 1987). Environmental factors such as oxygen limitation and moisture content can cause severe reduction in biodegradation rates. This can be improved by a suitable technical approach. Treatment of contaminated soil in a slurry reactor system could be used to optimize oxygen supply and contact between microorganisms and contaminating compounds. For recalcitrant pollutants like chlorinated compounds, however, biodegradation rates may be limited by the low numbers of microorganisms that are capable of degrading these chemicals. Inoculation with specific microbial cultures may be useful to obtain accelerated degradation (Finn 1983).

In laboratory experiments, inoculation of soils with bacteria that can degrade pesticides has been shown to stimulate biodegradation. A strain of *Pseudomonas cepacia*, capable of utilizing 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), could degrade 2,4,5-T in contaminated soil and removed more than 99% of 2,4,5-T present at 1 mg/g soil within a week (Chatterjee et al. 1982). Addition of pentachlorophenol (PCP)-utilizing *Arthrobacter* cells in soil reduced the half life of the pesticide from 2 weeks to less than 1 day (Edgehill and Finn 1983). Crawford and Mohn (1985) have found that it is possible to remove PCP from contaminated soil by inoculation with cells of a PCP-degrading *Flavobacterium*.

In industry, large amounts chlorinated and non-chlorinated aromatic hydrocarbons are used as solvents in various processes. Due to improper treatment of waste streams, these compounds are a frequent cause of pollution of groundwater and soils. Although several monocyclic aromatic hydrocarbons, including chlorinated derivatives, have been found to be biodegradable (Cerniglia 1984, Gibson and Subramanian 1984), long adaptation periods were often required, especially for chlorinated benzenes (Schraa et al. 1986; Reineke and Knackmuss 1984; Marinucci and Bartha 1979). Since soil pollution is often caused by mixtures of these compounds which vary in degradability, it would be advantageous if inoculation with specific cultures could stimulate degradation

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rates. Here, we describe the effect of addition of pure bacterial cultures to soil slurries contaminated with chlorinated and non-chlorinated aromatic solvents. Soil suspensions were used in order to optimize mass transfer.

Materials and methods

Organisms. The organisms used in this study were *Pseudomonas* strains GJ31, GJ40, GJ60 and OE8. The toluene degrading strain GJ40 was isolated from sediment of the river Rhine by batch enrichment with 1 mM toluene as carbon source. Strain OE8 was isolated as a *p*-xylene utilizing organism from a mixed culture provided by Prof. S. P. P. Ottengraf, Technical University of Eindhoven, The Netherlands. Strain GJ31 utilized benzene, toluene and chlorobenzene and was obtained by conventional batch enrichment from sediment samples taken from the river Rhine, using 1 mM chlorobenzene as sole carbon source. Prolonged adaptation times as observed by Reineke and Knackmuss (1984) for strain WR1306 were not required. Strain GJ60 was also isolated from samples of sediment from the river Rhine, using 1 mM 1,2 dichlorobenzene as sole carbon source. A long adaptation period, as observed by van der Meer et al. (1987), was again not required since growth and chloride liberation were detected within 6 days after inoculation of a 100 ml enrichment with about 1 g of sediment.

Identification tests, such as Gram reaction, oxidase and catalase assays, and flagella staining were performed according to standard procedures (Smibert and Krieg 1981; Doetsch 1981).

Media and growth conditions. NB medium contained 8 g nutrient broth (Difco) per liter. Mineral salts medium (MMY) contained per l: 5.37 g $Na_2HPO_4 \cdot 12H_2O$, 1.36 g KH_2PO_4, 0.5 g $(NH_4)_2SO_4$, 0.2 g $MgSO_4 \cdot 7H_2O$, 5 ml trace elements solution, as described previously (Janssen et al. 1984), and 10 mg yeast extract. The pH after sterilization was 7.0. Cultures in liquid MMY medium were grown aerobically at 30° C in closed serum flasks filled maximally to one-fifth of their volume with medium. Carbon sources were added at concentrations of 1–5 mM.

For solid media, 1.5% agar was added. Volatile carbon sources (20 µl) were applied to pieces of filter paper which were placed in the lids of the petri-dishes. These were sealed with Parafilm and incubated at 30° C.

Soil incubations. All tests on the biodegradation of the aromatic compounds were carried out at 30° C with unsterilized soil slurries that were prepared from garden soil (clay) in MMY-medium to a moisture content of 60%, pH 7.0. Slurries were supplemented with 500 µM each of benzene, toluene, *o*-, *m*- and *p*-xylene, chlorobenzene, *o*-dichlorobenzene and 1,3,5-trichlorobenzene and stored for 2 months at 4° C to allow adsorption to take place before the start of the experiments. Because of partitioning of the solvents between the gas and liquid phase, initial concentrations were below 500 µM.

To prevent evaporation of the highly volatile solvents, a gas-tight system was used for incubation. Soil slurries were placed in closed glass flasks, filled to one-fifth of their volume. The flasks were equipped with a three-way valve in a teflon lined screwcap. All incubations took place at 30° C in a rotating carousel (8 rpm) to ensure sufficient mixing.

Cultures of strains GJ31, GJ40, GJ60 and OE8 were grown in nutrient broth. Cells were harvested by centrifugation (10 min at 5000 *g*), suspended in MMY-medium and added to soil suspensions as indicated. Soil suspensions were also inoculated with activated sludge from a wastewater treatment plant (10 g dry weight per l slurry). Controls supplemented with 1% sodium azide were carried out to ensure that physical loss or adsorption did not significantly contribute to the observed decrease in concentration of the aromatic compounds.

Growth of bacteria in slurries was determined by viable counts. In order to release bacteria from the soil matrix, soil samples were vortexed vigorously before dilutions were made. Colonies were counted on MMY agar plates, supplemented with aromatic solvents as carbon source. Replica plating was used for further identification of added strains.

Analyses. Degradation of aromatic solvents was measured by gas chromatography after extraction with pentane. Slurry samples of 4 ml were withdrawn periodically and added to 2 ml pentane containing 0.5 mM trichloroethylene as an internal standard. After shaking for 24 h the pentane layer was separated by centrifugation (3 min at 3000 *g*) and analyzed by capillary gas chromatography as described previously (Janssen et al. 1988).

Chloride levels in culture fluids and in slurries were determined with an ion-selective electrode (Orion type 97-17).

Oxygen consumption experiments were performed with cells washed with and suspended in 20 mM potassium phosphate buffer (pH 7.0). Incubations were carried out in the presence of 3.3 mM substrate at 30° C in a biological oxygen monitor.

Materials. Halogenated and non-halogenated aromatics were obtained from E. Merck AG, Darmstadt, Federal Republic of Germany, from Janssen Chimica, Beerse, Belgium and from J. T. Baker Chemicals B. V., Deventer, The Netherlands. The purity of these chemicals was cheked by gas chromatography.

Results and discussion

Characteristics of the strains used for inoculation in soil slurries

Pure bacterial cultures that are capable of degrading aromatics were isolated from various samples of sediment or sludge as described under materials and methods. All strains shared the following characteristics: Gram-negative, rod shaped, motile, pale pigmented, oxidase positive and catalase positive. They probably all belong to the genus *Pseudomonas*. Strain GJ31 was further investigated for classification. It was a motile rod with monopolar peritrichous flagellar insertion. Fluorescent pigments were produced during growth on NB agar plates. Nitrogen fixation and nitrate reduction were negative. There was no hydrolysis of gelatin. The organism did not accumulate poly-β-hydroxybutyrate. Acid production from glucose was only observed under aerobic conditions.

Table 1. Some properties and aromatic substrates of microorganisms used for biodegradation of aromatics in soil suspensions

Strain	GJ31	GJ40	GJ60	OE8
Isolated on	chlorobenzene	toluene	*o*-dichlorobenzene	*p*-xylene
Doubling time[a] (h)	3.0	1.5	5.5	2.5
Doubling time[b] (h)	2.3	1.8	3.0	2.1
Nitrate reduction	neg	neg	pos	pos
Aromatic substrates[c]:				
benzene	+	+	O	O
toluene	+	+	O	+/O
o-xylene	O	O	O/−	O
m-xylene	O	O	O	+
p-xylene	O	O	O	+
chlorobenzene	+	−	+	O
o-dichlorobenzene	O	O	+	O
1,3,5-trichlorobenzene	O	O	O	O

[a] Doubling time in MMY medium supplemented with 1 mM carbon source used for isolation of the culture
[b] Doubling time in soil slurry supplemented with 1 mM carbon source used for isolation of the culture
[c] The compounds indicated were tested as sole carbon and energy source. Symbols: +, growth with liberation of chloride from chlorinated substrates; O, no growth; −, no growth and the compound caused inhibition of growth on the carbon source used for enrichment

Strain GJ31 was not capable of autotrophic growth with a mixture of 70% H_2, 20% O_2 and 10% CO_2. These observation suggested that strain GJ31 is a strain of *Pseudomonas putida*.

The ability of the isolates to degrade aromatic compounds is summarized in Table 1. Replica plating and tests with liquid cultures gave the same results. Doubling times of the microorganisms with the substrates on which they were enriched varied from 1.5 to 5.5 h (Table 1).

Strain GJ40, which utilized toluene, also grew on ethanol, 1-butanol, 1-octanol and benzene but not on the other aromatics shown in Table 1. Unlike strain GJ31, strain GJ40 did not grow on toluene or benzene in liquid culture in the presence of 1 mM chlorobenzene as a second substrate.

Strain GJ31, enriched on chlorobenzene, tolerated up to 3 mM chlorobenzene in liquid culture. During growth of GJ31 on chlorobenzene all organic chlorine was liberated as inorganic chloride. Strain GJ31 was not able to grow with phenol, *o*-, *m*- or *p*-xylene as sole carbon source and cometabolism of the xylenes was not detected during growth of GJ31 on chlorobenzene. Unlike the chlorobenzene degrading bacterium described by Reineke and Knackmuss (1984), strain GJ31 utilized also toluene and benzene as carbon sources. When toluene or benzene was present in the culture medium in combination with chlorobenzene, none of the substrates was detectable after growth. It was found that strain GJ31 has constitutive catechol 2,3-dioxygenase activity toward catechol (data not shown). Washed cells of strain GJ31 grown with citrate showed no significant oxygen uptake with chlorobenzene or toluene as substrates. For chlorobenzene- or benzene-grown cells, oxygen uptake was found with chlorobenzene, toluene or benzene as substrates and varied from 70–190 μmol O_2 min^{-1} mg^{-1} cell dry weight, showing that the catabolic pathway of aromatics by strain GJ31 was inducible. Strain GJ31 apparently differs form strain WR1306 (Reineke and Knackmuss 1984), which had lost catechol 2,3-dioxygenase activity after several passages of growth with chlorobenzene and therefore was no longer able to use benzene as growth substrate.

The *o*-dichlorobenzene degrading bacterial strain GJ60 was easily obtained by batch enrichment from river sediment. Strain GJ60 also utilized chlorobenzene and 1,2,4-trichlorobenzene but not benzene, toluene or any of the xylenes, and is in this respect similar to the organism recently described by Haigler et al. (1988).

The *p*-xylene degrading organism strain OE8 was isolated from a three member consortium obtained from activated sludge. It also utilized *m*-xylene but not *o*-xylene as sole carbon source.

Isolation of an o-xylene degrading bacterium

From inoculated soil slurries in which biodegradation of *o*-xylene was observed (see below), an *o*-xylene degrading pure culture was obtained by batch enrichment, using 1 mM *o*-xylene as sole carbon source. This *o*-xylene degrading organism, designated strain GJ100, also utilized benzene,

toluene, ethylbenzene, *n*-propylbenzene and 1,2,4-trimethylbenzene as sole carbon sources but not *p*-xylene, *m*-xylene or *o*-dichlorobenzene. GJ100 had a doubling time of 16 h with 1 mM *o*-xylene as substrate.

Strain GJ100 was characterized as a Gram-positive, acid-fast positive and rod shaped bacterium which probably belongs to the genus *Nocardia*.

Degradation of (chlorinated) aromatic compounds in soil suspension

The effect of addition of specific bacteria on the degradation of benzene, toluene, *o*-, *m*- and *p*-xylene, chlorobenzene, *o*-dichlorobenzene and 1,3,5-trichlorobenzene in soil slurries was studied. Different conditions were tested: soil slurry inoculated with GJ31, GJ40, GJ60 and OE8, soil slurry inoculated with activated sludge, soil slurry without inoculation and liquid medium (without soil) inoculated with GJ31, GJ40, GJ60 and OE8. All tests were carried out at 30° C.

Without inoculation, the indigenous microflora needed about 30 h for adaptation to degrade benzene, toluene, *m*- and *p*-xylene and chlorobenzene, and 100 h for complete removal of these compounds. *o*-Xylene and *o*-dichlorobenzene, however, were only partially degraded when no microorganisms that utilize these compounds as carbon source were added (Fig. 1A). The partial degradation of *o*-xylene and *o*-dichlorobenzene stopped when the other aromatics had disappeared. This suggests that removal of *o*-xylene and *o*-dichlorobenzene can occur through cometabolic conversion by toluene, benzene or xylene degrading bacteria that were already present in the soil.

Upon addition of only 5 mg cell dry mass per liter of cultures that degrade benzene, toluene, *m*- and *p*-xylene, chlorobenzene and *o*-dichloroben-

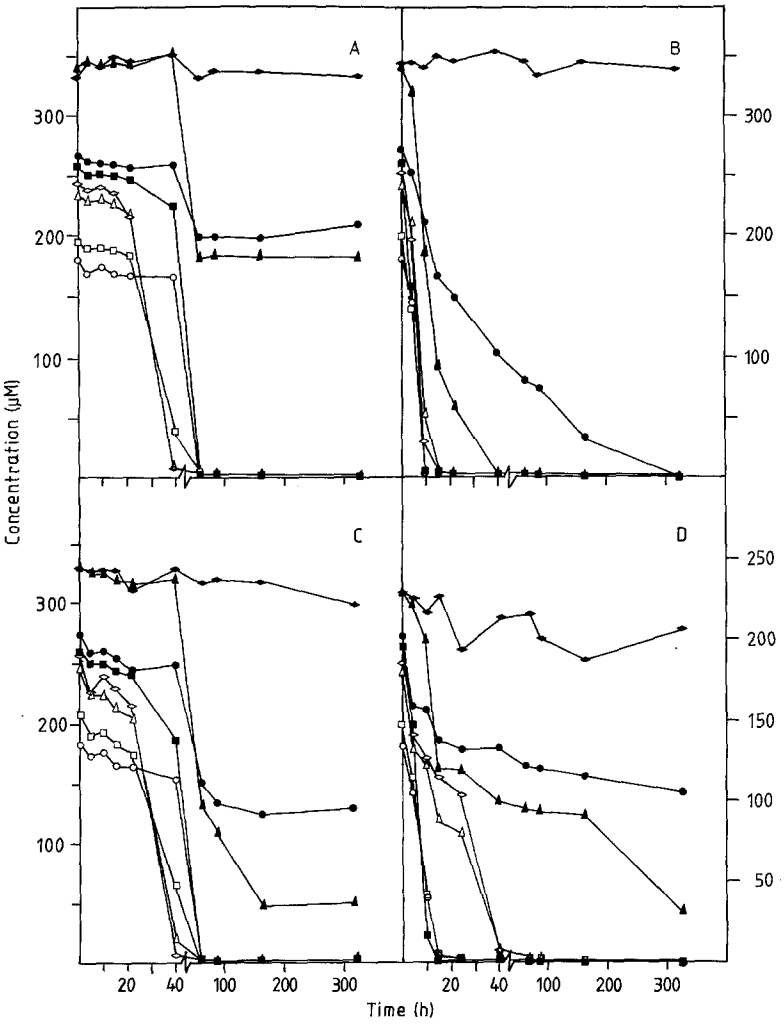

Fig. 1 A–D. Effect of inoculation on the degradation of aromatic solvents in soil slurries. Slurries containing 500 µM aromatic compounds were incubated at 30° C, as described in materials and methods. **A.** No inoculation. **B.** Inoculated with cultures of strains GJ31, GJ40, GJ60 and OE8 for the degradation of benzene, toluene, *p*-xylene, *m*-xylene, chlorobenzene and *o*-dichlorobenzene, respectively. An amount of 5 mg dry weight per l of slurry was added for each strain. **C.** Inoculated with activated sludge (10 g dry weight per l). **D.** Mineral medium, without soil, inoculated with pure bacteria cultures (50 mg dry weight per l for each strain). Benzene (○), toluene (□), *o*-xylene (●), *m*-xylene (◇), *p*-xylene (△), chlorobenzene (■), *o*-dichlorobenzene (▲) and 1,3,5-trichlorobenzene (◆)

zene to soil slurries, these compounds were rapidly degraded and disappeared completely within 50 h (Fig. 1B). For the relatively easily degradable compounds benzene, toluene, *m*-xylene and *p*-xylene, inoculation caused degradation to start immediately, no adaptation period of more than a few hours being required. *o*-Dichlorobenzene, which was only partially removed without inoculation, was degraded within 50 h. Moreover, increased biodegradation of *o*-xylene was observed. On the other hand, 1,3,5-trichlorobenzene, for which no specific organisms were added, was not degraded. From the observation that addition of activated sludge did not stimulate degradation, even at a concentration of 10 g dry weight per l (Fig. 1C), it can be concluded that the increased degradation rates are caused by the specific capabilities of the introduced strains and are not a result of a general increase in biomass concentration.

The data presented in Fig. 1 indicate that *p*- and *m*-xylene are more easily degraded than *o*-xylene, the latter compound disappearing only 300 h of incubation.

Increasing the amount of cells added from 5 to 50 mg of cell dry weight per l soil slurry for each strain did not stimulate biodegradation further

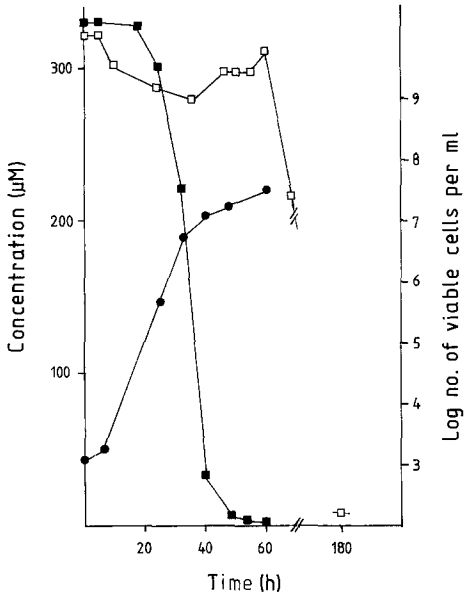

Fig. 2. Degradation of chlorobenzene and bacterial growth in soil slurries, with and without inoculation with strain GJ31. Slurries containing 500 µM chlorobenzene were incubated at 30° C, as described in materials and methods, and the degradation of chlorobenzene was followed. The slurry was inoculated with 0.25 µg cells/l (about 10^3 viable cells/ml), and the viable cells count was followed during the incubation (●). Chlorobenzene without (□) and chlorobenzene with prior inoculation (■)

(data not shown). By measuring cell numbers at different timepoints after inoculation with a much smaller amount of cells (10^6 viable cells per liter), it was found that all four strains of added bacteria could actively grow in soil slurries, contaminated with the relevant carbon sources. This is illustrated for the biodegradation of chlorobenzene and growth of the chlorobenzene degrading bacterium strain GJ31 in a soil slurry (Fig. 2). Since non-sterilized soil was used, growth apparently was not inhibited by the indigenous microflora. The data also show that the added microorganisms did not disappear rapidly when chlorobenzene was depleted. Instead, they survived for at least 50 times their generation time (Fig. 2). Inoculation with other strains showed essentially the same results. It appeared that the doubling time of the added bacteria in the presence of soil suspensions was the same or even shorter that in liquid culture (Table 1).

Biodegradation of a mixture of aromatic solvents by introduced bacteria was more rapid in soil slurries than in liquid culture. Although no lag time was observed in liquid culture, much lower degradation rates were found for the xylenes and *o*-dichlorobenzene when compared to soil slurries (Fig. 1B, D). It appears that soil (clay) had a protecting effect on microorganisms with respect to toxicity of a combination of organic solvents.

Degradation of pollutants by added bacteria could possibly be limited by the inability of introduced microorganisms to compete in the soil environment with indigenous organisms, especially when other easily degradable carbon compounds are present. Addition of the non selecting carbon source peptone, however, did neither affect the rate of biodegradation of chlorobenzene nor the growth rate of chlorobenzene degrading bacteria in soil suspensions (Fig. 3). This also indicates that an easily available carbon source does not necessarily repress the degradation of aromatics by added bacteria.

The above results show that inoculation with low amounts of specific microbial strains (10^6 cells per l of slurry) was sufficient to initiate rapid degradation of (chloro)benzenes. The degradation rate was up to 2 mg l^{-1} h^{-1} for the aromatics for which bacteria were added, at which point the slurries contained 10^6–10^7 viable cells per ml. This rate was obtained under conditions that were optimal with respect to environmental factors such as mass transfer, temperature, availability of nutrients, and aeration. Under these conditions, degradation rates in soil slurries apparently can

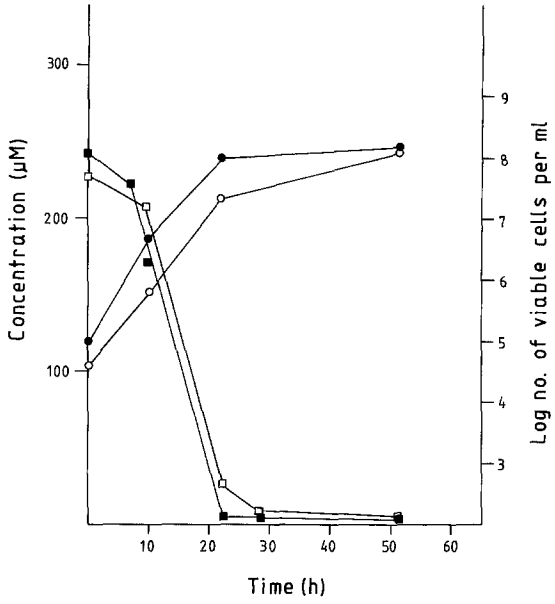

Fig. 3. Effect of peptone on growth of chlorobenzene degrading bacteria and chlorobenzene degradation in soil slurries after inoculation with strain GJ31. Slurries containing 500 µM chlorobenzene were incubated at 30°C, as described in materials and methods, and the degradation of chlorobenzene was followed. The slurry was inoculated with 15 µg cells/l (about $4.5 * 10^4$ viable cells/ml). Peptone was added to a final concentration of 100 mg per liter slurry. Cell density without (○) and with peptone (●), chlorobenzene without (□) and with peptone (■)

be equal to or higher than rates found in liquid cultures with pure strains. Degradation velocities were mainly limited by the capacity of microorganisms to degrade the pollutants. Inoculation will therefore be most significant for the (highly) chlorinated benzenes, for which microorganisms that are able to utilize them are not present in soil or only in very small numbers.

There are many factors involved in the survival of introduced strains in natural ecosystems. It has been suggested (Goldstein et al. 1985) that too low substrate levels, growth-inhibiting compounds, susceptibility to predators, or inaccessibility of the substrates may cause failure of inoculation to stimulate degradation. It was shown in our experiments that inoculation was successful on laboratory scale, at relatively high substrate levels. For optimization of environmental factors that influence biodegradation rates, it seems advantageous to make slurries in which desorption and solubilization of pollutants are optimized, since mass transfer could be a limiting step during treatment of dry soil. Degradation of pollutants in dry soil probably requires colonization of numerous separate microenvironments by microorganisms possessing suitable degradative abilities. At high water content, contact between microorganisms and pollutants is facilitated. It seems possible to obtain such conditions in large scale installations of several hundred liters (R. H. Kleijntjens, personal communication). Mixing of soil and water to form a homogeneous slurry is easier than homogenization of moist or dry soil. Furthermore, a temperature increase to 25–30°C may be obtained during extensive mixing as a result of mechanical heat release. Nevertheless, the energy costs for the cleanup of solid wastes with a process involving a slurry bioreactor may be considerably higher than for composting, land disposal or injection procedures for in situ bioreclamation.

Under conditions where the presence of suitable microorganisms is limiting, inoculation should be useful in decreasing adaptation periods and obtaining high degradation rates. Inoculation permits a more sophisticated technological approach, to the development of slurry reactors designed for optimization of environmental factors (Kleijntjens et al. 1987). Such reactors may be expected to be economically competitive with thermal treatment or physical extraction techniques for the cleanup of contaminated soil.

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