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MICROBIAL DEGRADATION OF TETRACHLORO-ETHYLENE AND TOLUENE IN POLLUTED SOIL

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ABSTRACT

A contaminated area situated at Eppelheim (Germany) is a dump for volatile hydrocarbons wastes [mainly perchloroethylene (PCE); benzene, toluene, ethylbenzene and xylene (BTEX-aromatics)]. The present work was carried out to investigate the efficiency of indigenous soil microorganisms present in successive soil layers up to 8 m to degrade PCE and toluene under anaerobic and aerobic conditions, respectively. The incorporation of carbon from PCE or toluene into cell mass of indigenous soil microorganisms was studied as well. PCE was completely dechlorinated via reductive dechlorination within 56 days of incubation. Two patterns of dechlorination, depending on soil depth, could be distinguished: (i) PCE--> trichloroethylene (TCE) --> *cis-1,2*-dichloroethylene *(cis*DCE*)* --> vinyl chloride (VC) --> ethane [unpolluted upper soil] and (ii) PCE--> TCE--> *cis*DCE [moderately pollted lower soil], suggesting that two different anaerobic metabolic microbial groups participated in the dechlorination process. Experiments with ^{14}C-[1,2]-PCE indicated that [^{14}C]ethane was the principal product of biodegradation in unpolluted upper soil reaching up to 75.9 %. On the other hand, in moderately polluted lower soil the major biodegradation products were ^{14}C-less-chlorinated hydrocarbons but not ^{14}C-ethane. $^{14}CO_2$ and $^{14}CH_4$ were observed in only low concentrations (2.2 to 9.6 % and 0.0 to 5.0 %, respectively). However, the production of $^{14}CO_2$ from [^{14}C]PCE indicated that at least partial mineralization of PCE could occur. The assimilated PCE-C in the cell mass accounted only for 0.4 to 4.4 %, indicating that soil microorganisms were unable to use PCE as a growth substrate. Experiments with [^{14}C]-uniform-toluene demonstrated that toluene was biologically mineralized in all soil depths with different ratios. Indeginous soil microorganisms converted toluene to CO_2 (45.5 - 60.4 %) and cellular material (16.0 - 27.7 %). This relatively high percentage of radioactive biomass formation indicated that toluene was assimilated by the soil microorganisms as an energy and carbon substrate. The $^{14}CO_2$ formation activities ranged between 0.074 and 0.183 mg $^{14}CO_2$-C/kg soil dry wt. h^{-1} and were *ca*. 4.4 times lower than the corresponding elimination of unlabelled toluene (0.17 and 0.93 mg toluene-C/ kg soil dry wt. h^{-1}).

Keywords*:* biodegradation, mineralization, hydrocarbons, perchloroethylene (PCE), trichloroethylene (TCE), *trans-1,2*-dichloroethylene (*trans-1,2*-DCE), *cis-1,2*-dichloroethylene (*cis-1,2*-DCE), vinyle chloride (VC), toluene, bioremediation.

INTRODUCTION

Tetrachloroethylene (perchloroethylene, PCE) is an organic solvent that is widely used for dry cleaning of textiles and degreasing of machines and metal parts. This highly toxic compound has been released into the environment for decades, and therefore, it has become one of the most

common contaminants of soils and groundwater. PCE is persistent in the environment due to its resistance to microbial degradation and to its toxicity to microorganisms. Under aerobic conditions, PCE is considered nonbiodegradable. It have been reductively dechlorinated under anaerobic conditions by mixed cultures to less-chlorinated ethenes (Vogel et al., 1987) and, under certain conditions, to the nontoxic products ethene (DiStefano et al., 1991; Fennell et al., 1997) and ethane (deBruin et al., 1992).

Soil, sediment and groundwater are frequently contaminated with petroleum products as a result of leaks in underground storage tanks, improper disposal techniques, and inadvertent spills. Of the many constituents of petrolium, the nonoxygenated, homocyclic aromatic compounds that include benzene, toluene, ethylbenzene and xylenes (BTEX) are of particular concern because they are confirmed or suspected carcinogenes, even at very low concentrations (Dean, 1985). The microbial degradation of compounds such toluene and benzene under aerobic conditions has been studied in great detail (Gibson and Subramanian, 1984; Smith, 1990). In contrast, the fate of homocyclic aromatic compounds in anaerobic environments is poorly investigated. Certain monoaromatic hydrocarbons, most frequently toluene, have since been shown to be degraded by microorganisms under denitrifying (Zeyer et al., 1990; Evans et al., 1991), iron-reducing (Lovley and Lonergan, 1990), sulfate-reducing (Beller et al., 1992) and methanogenic conditions (Wilson et al.,1987).

A contaminated area situated at Eppelheim (Germany) is an abandoned dump containing comparatively low concentrations of hazardous waste (volatile chlorinated hydrocarbons [mainly chloroethenes] and BTEX-aromatics [mainly toluene, ethylbenzene and xylene]). One important demand for the biotechnological concepts established at the dump was the microbial mineralization of the pollutants to CO_2 and/or CH_4. Such mineralization is of important concern, since no toxic intermediates are formed. The aim of the present study was to exploit the ability of of the indiginous soil microorganisms to mineralize the more important pollutants PCE and toluene. The biodegradation pathway should be studied as well. The capability of soil microorganisms to transform some of the carbon in these pollutants into cell material was also investigated.

MATERIALS AND METHODS

Description of the studied area and soil Sampling:

The contaminated area under investigation is located at Eppelheim near Heidelberg (State of Baden-Würtemberg, Germany). It is an abandoned dump established in 1950 and characterized by high contamination with volatile hydrocarbons mainly chloroethenes as well as BTEX-aromatics. After about 40 years of using the dump for different hazardous wastes, the area was enlisted as a proposed testing site by the State Authority for Environmental Protection of the State of Baden-Würtemberg. Some mechanical methods were tested for their efficiency in decontamination of soil. Among methods applied was the partial removal of organic pollutants by leaching out with phosphate-buffered mineral solution under high pressure

through a special steel column inserted vertically to a depth of 10 m. For detailed information, see von Reis and Meyer (1995). To investigate the efficiency of indigenous soil microorganisms present in the treated soil column to degrade PCE, soil samples at different depths from soil profile were withdrawn throughout special openings along the steel column by means of clean samplers. Corresponding set of samples were similarly taken from a calibration untreated column used as a control to study the biodegradation of toluene.

Biodegradation assay :

1. Chemicals:

Unlabelled PCE, trichloroethylene (TCE), *trans-1,2*-dichloroethylene *trans*DCE, *cis-1,2*-dichloroethylene (*cis*DCE) and toluene were obtained in neat liquid form (analytical grade) for use as analytical standards and in biodegradation experiments.They were purshased from Merck (Darmstadt, Germany). Vinyl chloride (VC), Methane, ethene and ethane were obtained as gases in steel tanks from Linde (Unterschließen, Germany). ^{14}C[1-2]-PCE with a specific activity of 12.7 mCi/mmol (117322 dpm = 100%) and ^{14}C-uniform- toluene (55707 dpm = 100%) were obtained from Sigma (Deisenhofen, Germany).

2. Medium:

The mineral medium proposed by Meyer and Schlegel (1983) was used in the biodegradation experiments. It consisted of (g/l): $Na_2HPO_4.12H_2O$, 9; KH_2PO_4, 1.5; NH_4Cl, 1.5; $MgSO_4.7H_2O$, 0.2; $CaCl_2.2H_2O$, 0.02; F^{+++}- ammonium citrate, 0.001 plus 1 ml of trace element solution consisting of (mg/l): $ZnSO_4.7H_2O$, 100; $MnCl_2.4H_2O$, 30; H_3BO_3, 300; $CoCl_2.6H_2O$, 200; $CuCl_2.2H_2O$, 10; $NiCl_2.6H_2O$, 20; $Na_2MoO_4.2H_2O$, 30; Na_2SeO_3, 20 (final pH, 7.0). In experiments conducted for PCE biodegradation, the medium was enriched with 0.2 % sucrose as a co-substrate (Meyer et al., 1993).

3. Experimental:

The biodegradation of PCE and toluene under anaerobic and aerobic conditions, respectively, was assessed in two separate experiments using non-radioactive as well as ^{14}C-labelled chemicals. In both experiments, soil-medium suspensions were incubated in dark at room temperature (*ca*. 20 °C).

a. Experiment with non-radioactive chemicals:

Two soils representing the 4 and 8 m depth of the column partially leached with mineral solution under high pressure (355 bar) [for PCE treatment] or the untreated column (for toluene treatment) were used in this experiment. The first soil was a normal unpolluted one, while the second one was moderately polluted with PCE (15.8 mg /kg soil dry wt.) [see Refae, 1994].

For anaerobic biodegradation of PCE, 100-ml serum bottles were provided with 10 ml mineral medium enriched with 0.2 % sucrose. After sterilization at 121 °C for 15 min., each bottle was provided with 3g fresh soil

sample and tightly sealed with tefloncoated butyl rubber septa (CS-Chromatography Service, Langerwehe, Germany). To ensure anaerobic conditions, the air inside bottles was replaced by N_2, in addition providing the soil-medium mixture with 0.02 % of *L*-cystein to reduce the redox potential. Each bottle was supplemented with 490 µg PCE added by injection using Hamilton syring. For each soil layer, a sufficient number of bottles was prepared and covered with aluminium crimps caps.

Chemical analysis was carried out at intervals up to 56 days. At each sampling date, two bottles were taken at random for gas chromatographic analysis (Chrompack 438 A GC) of residual PCE and its dechlorinated compounds namely volatile chlorinated hydrocarbons (VCH), e. g. TCE, *cis*DCE, *trans*DCE, VC as well as ethene, ethane and methane present in the headspace of the bottle.

The aerobic biodegradation of toluene was determined by providing a set of serum bottles each with 10 ml sucrose-free mineral medium and after sterilization each bottle was provided with 3g fresh soil sample plus 125 µg toluene. Bottles were gently shaken to ensure even distribution of toluene and coated with teflon-coated butyl rubber septa. Residual toluene was determined at intervals (up to 56 days) using two bottles taken at random at each sampling date (1 week).

In both biodegradation assessments, abiotic controls were similarly prepared in which soils were autoclaved before added to the bottles.

b. Experiment with radio-labelled ^{14}C-chemicals:

Ability of indigenous soil microorganisms present in successive soil layers to degrade PCE and toluene was assessed using isotopic technique. The experiment was designed to be similar to the previous one except that 490 µg uniform labelled ^{14}C-[1,2]-PCE or 125 µg labelled ^{14}C-uniform-toluene was added to serum bottles containing soil-medium mixture. Moreover, chemical analysis was carried out once at the end of 56-day incubation.

For anaerobic biodegradation of PCE, prepared bottle was provided with two small cylinders. The first was supplemented with 2.5 ml KOH (20 %) added from the beginning to absorb $^{14}CO_2$ produced throughout microbial activity. The second cylinder was provided with 3 ml ethylene glycol monomethyl ether (EGME) injected at the end of incubation to absorb VCH resulted from microbial activity on PCE. At the end of incubation, 1 ml of 5N H_2SO_4 was injected and mixed with soil suspension to change HCO^-_3 + CO_3^{--} to CO_2 which will be absorbed in KOH solution. Analysis comprised the determination of ^{14}C in CO_2 absorbed, remaining PCE, VCH absorbed in EGME solution plus methane, ethene and ethane in the headspace. ^{14}C in microbial cells and in residual VCH adsorbed on soil particles was determined as well.

Aerobic biodegradation of toluene was carried out by providing the bottles containing soil-medium mixture with an cylinder containing KOH solution to absorb $^{14}CO_2$ evolved. Each bottle was covered with teflon-coated butyl rubber septa and incubated for 56 days, after which carbonates were converted to CO_2 as mentioned before. Analysis comprised the determination

of residual toluene in headspace, ^{14}C in CO_2, residual toluene absorbed on soil and cell biomass.

c. Analysis:

1. Gaschromatographic analysis. The headspace technique (Bellar and Lichtenberg, 1974; Kolb et al., 1979) was employed. Samples (100 µl) of the headspace (total volume ca. 88.5 ml) from each vial were withdrawn periodically by a gas tight Hamilton syring and analyzed by the gas chromatography-technique. Headspace concentrations of PCE remained and its dechlorination products were measured by gas chromatography with a Chrompack 438 A GC coupled with a laboratory computing integrator. The GC was equipped with a fused silica capillary column (Amchro, Sulzbach) [60 m by 0.53 mm; stationary phase chemical bonded silikon; 3µm film thickniss] connected to a flame ionization detector which was employed with hydrogen and air flows of 30 and 250 ml/min, respectively. The column was operated in the splitless injection mode at 35 °C for 10 min, ramped 4 °C/min to 160 °C. At a carrier gas (N_2) flow of 30 ml/min, retention times were as follows: PCE (33.7 min), TCE (26.26 min), *cis*DCE (20.02 min), VC (6.97 min). More refined separation of methane, ethene and ethane was obtained on a stainless-steel column (2.5 m by 0.25 mm) packed with porapack R (50 - 80 A°), operated isothermally at 175 °C.

Residual toluene in the headspace was monitored gaschromatography as in case of PCE.

Headspace of each bottle was accurately determined and calculations were carried out as µmol of the estimated compound/g oven dry soil.

2. Isotopic analysis:

a. Methane, ethene and ethane. The ^{14}C-gaseous compounds in the headspace (methane, ethene and ethane) were separated from each other and counted directly using the method of Zehnder et al. (1979). The separation of the three gases was as follows: A 0.5-ml headspace sample was injected onto a GC column (2.0 m by 0.3 mm) packed with porapack R (80 - 100 A°), operated isothermally at 22 °C. The three gases in the GC column effluent were trapped together between the following times (minutes): 0.5 (CH_4), 1.2 (ethene) and 1.9 (ethane).

b. Water soluble intermediates, VCH adsorbed on soil and biomass. The remaining ^{14}C in the soil suspensions (water soluble intermediates, VCH adsorbed on soil particles and cell mass) was determined as follows: The soil suspension was centrifugated (15.800 x g) where the supernatant contained water soluble non-volatile ^{14}C-by-products, e.g. alcohols, acids, etc. The sediment contained microorganisms and solid soil particles. The detergent sodium dodecyl sulfate (SDS; 10 %) was added for disruption of microbial cells (Bhaduri and Demchick, 1983). After recentrifugation (15.800 x g), the radioactivity in both the supernatant (assimilated PCE-C in the cell mass) and the sediment (adsorbed compounds and/ or by-products on the soil particles.) was measured.

In case of toluene mineralization, ^{14}C in CO_2, cell biomass and toluene adsorbed on soil particles were determined similarly as in PCE. Residual toluene in the headspace was determined by absorption in EGME.

All samples were counted with a Packard Tri-Carb liquid Scintillation counter. Counts per minute were converted to disintegrations per minute by using an efficiency plot for known ^{14}C quench standards according to Cooper (1981).

RESULTS AND DISCUTION

1.Time course and sequence for the dechlorination of PCE by the indigenous microorganisms in soil:

The dechlorination of perchloroethylene (PCE) in two soil samples with different levels of pollution taken at depths of 4 m (nonpolluted) and 8 m (polluted mainly with PCE) from the column partially decontaminated under high pressure was evaluated to (i) exploit the ability of the indigenous soil microorganisms to degrade PCE and (ii) to demonstrate the mechanism of biodegradation. Sucrose was chosen as a co-substrate for the dechlorination of PCE, since in earlier experiments (Meyer et al., 1993) sucrose was found to be the most effective co-substrate for the dechlorination of chloroethenes.

The soil harboured microorganisms capable of dechlorinating PCE under anaerobic conditions via a reductive mechanism, i.e. the replacement of chlorine with hydrogen. PCE was rapidly and completely disappeared within the 13-day incubation period in both soil samples (Fig. 1). The reductive dechlorination of PCE occurred with no apparent lag time. The degradation of PCE was sequential and showed transient accumulation of dechlorination products. Two patterns of dechlorination, depending on soil depth, could be distinguished: (i) perchloroethylene (PCE) --> trichloroethylene (TCE) --> *cis*-1,2-dichloroethylene (*cis*DCE) --> vinyl chloride (VC) --> ethane (4m depth) and (ii) PCE--> TCE--> *cis*DCE (8m depth). The formation of dechlorination products was seen only in the active bottles. Neither the disappearance of PCE nor the formation of dechlorination products was observed in the autoclaved control bottles, showing that the dechlorination process is indeed of biological origin.

TCE did not accumulate in any of tested soils. It could not be detected in 4m-depth sample. In connection with this point, it is worth to mention that the accuracy and detection limit of the method applied for detection of chloroethenes ranges from 97 to 100 % recovery. Consequently, it is quit possible that trace amount of TCE is still present but not detected by the method used in the present study. In 8m-depth sample, TCE was formed after 4 days and then rapidly disappeared (Fig. 1). These observations together with the formation of *cis*DCE in both soils indicate that TCE may be adsorbed on the soil depending on the chemical and physical properties of this soil (similar adsorption of 10 - 15 % of TCE on the soil was noticed by Kleopfer et al., 1985), or suggesting that TCE was rapidly transformed to *cis*DCE as has been observed in other studies using thermophilic anaerobic inoculants from various anaerobic environments (Kengen et al., 1999).

As shown in Fig. 1, *cis* 1,2-DCE is formed, while *trans* 1,2-DCE did not appear. Similar observations were found (Scholz-Muramatsu et al., 1990; Kengen et al., 1999). Other workers have demonstrated that the predominant isomer was *trans* 1,2-DCE with anaerobic enrichment cultures supporting the methanogenesis (Freedman and Gossett, 1989). Kleopfer et al. (1985) on the other hand could only identify one isomer which was 1,2-DCE.

The microbial degradation activity towards *cis*DCE was lower than of those towards TCE. The *cis*DCE formation rate was much more than the *cis*DCE transformation, indicating that *cis*DCE accumulates more than TCE. *cis*DCE was transformed either after a lag time of 5 days (4 m depth) or at negligible rates (8 m depth) [Fig. 1].

Figure 1

Figure 1: Time course for PCE degradation by the indigenous microorganisms in soil samples taken at 4 m (A) and 8 m (B) from the soil column partially decontaminated under high pressure.

VC was formed only in 4m-deph sample during the disappearance of *cis*DCE, but at substoichiometric concentration (maximum 50 % of initial PCE) [Fig. 1a]. The observed dechlorination of VC was slow (only 8.1 % was transformed in 20 days). However, the formation of ethane, an Cl-free intermediate, indicates that this formation was the consequence of VC dechlorination. The ethane concentration began to appear after 25 days and the rate of appearance increased till the end of the experiment, while VC began to decrease after 37 days (Fig. 1a). After 56 days of incubation, 1.21 µmol of PCE was consumed; 0.57 µmol of VC and 0.54 µmol of ethane were formed. The difference (0.1 µmol) between PCE consumption and VC and ethane production suggest that an undetectable product (for example CO_2) was formed from PCE degradation. Similar transient accumulation of VC during the reductive dechlorination of PCE was also found by Vogel and McCarty (1985).

The initial steps of PCE dechlorination (PCE --> TCE --> *cis*DCE) did not coincide with the pattern of CH_4 formation in both tested soil samples. CH_4 was formed first after 18 days whereas PCE was disappeared within 13 days (Fig. 1), suggesting that CH_4 biosythesis was not necessary for PCE and TCE reductive dechlorination to occur and other anaerobic microorganisms other than methanogens were involved in the depletion of PCE. Different metabolic microbial groups (Damborsky, 1999), e.g. halorespirators (e.g. *Desulfitobacterium dehalogenans* JW/IU-DC1; *Desulfomonile tiedjei* DCB-1), acetogenes (e.g. *Acetobacterium woodii* WB1) and facultative anaerobes (e.g. *Pantoea agglomerans* ATCC 27993) in addition to methanogenes (e.g. *Methanosarcina sp*. strain DCM; Fathepure and Boyd 1988) are able to metabolize PCE. However, there are some findings suggest a possible role of methanogenisis in the dechlorination of the less-chlorinated hydrocarbons, i.e. *cis*DCE and VC: (i) *cis*DCE began to decrease as methane formed and (ii) *cis*DCE dechlorination was more higher in unpolluted soil (4m depth; higher methane formation) than those in polluted soil (8m depth; lower methane formation). The observed low CH_4 formation in 8m-depth sample may be due to the inhibitory effect of pollutants existing in the lower part of the dump on methanogenic bacteria. From these results it can be concluded that at least two different microbial groups were participating in the transformation of PCE and its dechlorination products in the tested soil. Only methanogenic bacteria are known to produce methane. These bacteria exclusively metabolize simple one- or two-carbon compounds and hydrogen. Therefore, the methanogenic degradation of chloroethenes observed using sucrose as a co-substrate is believed to carried out by the cooperative interaction of several groups of bacteria.

As shown in Figure (1), the rate of product formation and transformation slowed as the less chlorinated products appeared. Fathepure et al. (1987) have pointed out that, in anaerobic systems, the fewer chlorine atoms remaining on an alkene, the more difficult they are to remove. As a result of this, the less chlorinated products (*cis*DCE and VC), which are known to be toxic than the parent compounds PCE and TCE, tend to persist. Although ethane was formed in 4m-depth soil at substoichiometric concentration (Figure 1a), the formation of this product provides evidence

that the indigenous microorganisms in the soil are able to dechlorinate PCE completely to ethane. This finding is significant because, unlike VC, ethane is an environmentally acceptable biotransformation product. Ethane is sparingly soluble in water, and it has not been associated with any long-term toxicological problems (Autian 1980). For anaerobic bioremediation to be a useful method, PCE and TCE must be completely dechlorinated to nonchlorinated products. Few studies have shown the complete dechlorination of chlorinated alkene. Freedman and Gossett (1989) found that ethene was the final product of the PCE transformation sequence in the sediment. They also observed that the rate limiting step was the conversion of VC to ethene and that the presence of an electron donor was necessary. Strain *Dehalococcoides ethenogenes* 195 was reported to dehalogenate PCE completely to ethene (Maymo-Gatell et al., 1997).

2. Evidence for complete biodegradation of PCE:

[^{14}C]-*1,2*-PCE experiments on 6 successive soil layers were undertaken to (i) obtain information about whether the observed PCE conversion sequence (PCE --> TCE --> *cis*DCE--> VC --> ethane) can lead to the complete mineralization of these compounds, i.e. formation of $^{14}CO_2$ and/or $^{14}CH_4$ and (ii) to determine whether the ethane formed was a consequence of PCE degradation. The described method for measuring of radioactivity gave a very good mass balance for the ^{14}C remaining in the bottles. The sum of radioactivity measured (recovered) [chloroethenes, CO_2, CH_4, ethene, ethane, water soluble intermediates, PCE adsorbed on soil, and cell mass] ranged from 99.2 to 102.8 % (average 101.0 %) of the total radioactivity added (Table, 1).

Table (1) presents the distribution of ^{14}C in each of the bottles at the end of its incubation period (56 d). The principal product of [^{14}C]PCE degradation by the indigenous microorganisms in unpolluted upper soil was [^{14}C]ethane reaching up to 75.9 %. On the other hand, in moderately polluted lower soil the major biodegradation products were ^{14}C-less-chlorinated hydrocarbons but not ^{14}C-ethane. However, ^{14}C-ethene was occasional formed (4 m depth) in low concentration (1.8 %), indicating that ethene was further reduced to ethane. $^{14}CO_2$ and $^{14}CH_4$ were observed in only low concentrations (2.2 to 9.6 % and 0.0 - 5.0 %, respectively; Table, 1). However, the production of $^{14}CO_2$ from [^{14}C]PCE indicates that at least partial mineralization of PCE can occur. No $^{14}CH_4$ was detected in 7 and 8m-depth soils. Furthermore, methane formation from sucrose in 8m-depth was very low (Figure 1b). These findings suggest the formation of $^{14}CH_4$ from [^{14}C]PCE dependent on methane formation from the co-substrate sucrose. CO_2 is a typical substrate for methanogenic bacteria which involved in methane formation (Zeikus, 1977). Under methanogenic conditions, the $^{14}CO_2$ formed from [^{14}C]PCE may be reduced to $^{14}CH_4$. Thus, the $^{14}CH_4$ formed may not be a direct degradation product from PCE, but rather a transformation product of $^{14}CO_2$. In a study using two columns under methanogenic conditions, Vogel and McCarthy (1985) reported 24 and 27 % conversions of low concentrations of [^{14}C]PCE and [^{14}C]VC, respectively, to $^{14}CO_2$. In another study using enrichment cultures under methanogenic conditions (Freedman

and Gossett, 1989), the quantity of $^{14}CO_2$ observed did not exceed 2.7 % of the total radioactive [^{14}C]PCE used.

The dechlorination grade depended on soil depth and amounted to 83.9 % in 3m-depth soil (Table 1). The portion of [^{14}C]PCE recovered in $^{14}CH_4$, $^{14}CO_2$, and cell mass was considered as mineralization grade. It ranged from 8.0 to 13.8 %. The ethane formed from PCE can be degraded easily under aerobic conditions to CO_2, so the sum from anaerobic mineralization and ethane formation in Table (1) represents the actual mineralization. It is obvious from these results that PCE served as electron acceptor and not as electron donor. Electrons transferred from sucrose were diverted to PCE. As a result, sequential reductive dechlorinaion of PCE to ethane occured. PCE is a relatively strong electron acceptor (Vogel et al., 1987).

Table 1: Distribution of ^{14}C in bottles spiked with [^{14}C]-*1,2*-PCE and amended with soil samples taken from the soil column partially decontamenated under high pressure.

% of radioactivity recovered after 56 days incubation											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
Depth (m)	Volat. chlorin. hydroc.	CO_2	CH_4	C_2H_4	C_2H_6	Water soluble interm.	Cell mass	PCE-Adsorb on soil	Dechl-orinat. grade	Mineral i-zation grade	Total recov-ery
3	0.3	2.2	3.8	0	75.9	15.9	2.0	0.6	83.9	8.0	100.8
4	46.0	8.4	2.4	1.8	23.7	18.8	0.9	0	37.2	11.7	102.0
5	60.4	6.7	5.0	0	11.8	17.9	0.8	0.2	24.3	12.5	102.8
6	7.2	3.9	4.8	0	70.7	13.7	0.4	0	79.8	9.1	100.7
7	70.4	9.6	0	0	0	17.4	1.1	0.7	10.7	10.7	99.2
8	64.9	9.4	0	0	0	21.4	4.4	0.6	13.8	13.8	100.7

(1) radioactivity in ethylene glycole monomethyl ether (PCE remained and its dechlorination products TCE, *cis*DCE and VC), (2) radioactivity in KOH, (3, 4, 5) radioactivity in headspace, (6) radioactivity in 15800 x g centrifugation supernatant, (7) radioactivity in SDS centrifugation supernatant, (8) radioactivity in SDS centrifugation sediment, (9) $\sum$ 2+3+4+5+7, (10) $\sum$ 2+3+7 and (11) $\sum$ 1 to 8.

The suggested degradation pathway is a completely reductive process and did not include any oxidative steps. The mechanism, from which $^{14}CO_2$ and $^{14}CH_4$ were produced from PCE, is not clear. Imaginable is the formation of ethene, ethane, chloroethane, CO_2, CH_4, and/or chloromethane as a result of VC mineralization.

The assimilated PCE-C in the cell mass accounted only for 0.4 to 4.4 % (Table 1). This agreed with the observation of the nearly complete transformation of PCE to ethane and indicate that soil microorganisms were unable to use PCE as a growth substrate, i.e. partially to oxidize and partially to reduce PCE. On the other hand, the formation of $^{14}CO_2$ from [^{14}C]PCE indicate that the soil harboures microorganisms which were able to transform PCE oxidatively. It should be realized, that the measured cell-mass did not represent the actual cell-mass production, since the added co-substrate sucrose was responsible for the whole cell yield.

3. Elimination and mineralization of toluene:

The previuos study of Meyer et al. (1993) indicated that the soil at Eppelheim is anaerobic (characterized with methane formation) at least from 1 m depth. The present study, however, showed the presence of facultative anaerobic microorganisms which were capable of transformation of toluene under aerobic conditions. The rate of unlabelled toluene removal (disappearance from headspace) ranged between 0.17 and 0.93 mg toluene-C/ kg soil dry wt. h^{-1}, depending on soil depth. No decrease of toluene concentration in the autoclaved control bottles was observed over the course of 56 days incubation, showing that removal process is indeed of biological origin.

Under aerobic conditions, toluene is initially converted via alcohols and aldehydes to benzoate which is then converted to catechol. The ring cleavage is carried out through oxygen. The end products of aerobic degradation of toluene are CO_2 and cell mass. Experiments with [^{14}C]-uniform-toluene demonstrated that toluene was biologically mineralized in all soil depths with different ratios. Indigenous soil microorganisms converted toluene to $^{14}CO_2$ (45.5 - 60.4 %) and cellular material (16.0 - 27.7 %) [Table 2]. This relatively high percentage of radioactive biomass formation indicates that toluene was assimilated by the soil microrganisms as an energy and carbon substrate. The $^{14}CO_2$ formation activities ranged between 0.074 and 0.183 mg $^{14}CO_2$-C/kg soil dry wt. h^{-1}. These activities were in avearage of about 4.4 times lower than the corresponded elimination of unlabelled toluene (0.17 and 0.93 mg toluene-C/ kg soil dry wt. h^{-1}). These results suggest that the speed limiting step for mineralization of toluene is not the elimination, but is one of the subsequent ractions resulting in CO_2 formation.

The mineralization grades of toluene ($\sum$ CO_2 + cell mass) were obviously higher (66.3 to 81.0 %), than those of PCE ($\sum$ CO_2 + CH_4 + cell mass) [8.0 - 13.8 %]. This is not surprisingly, since toluene was shown to be a growth substrate for different bacteria (Vecht et al., 1988; Hubert et al., 1999).

The radioactivity in the volatile fraction recovered (residual toluene) did not exceed 0.2 % of the total radioactivity added after 56 days incubation, indicating that nearly all toluene was biodegraded (Table 2).

Thus, it can be concluded that the main products of toluene mineralization were CO_2 and biomass. Aromate ring fission took place, since about 50 % of the toluene carbon was found as CO_2. The observed high percentage of assimilated toluene-C into biomass indicated that toluene could serve as a suitable substrate for the soil microorganisms. This is of important concern, since the cost of a co-substrate required for a biological soil decontamination should be considered.

Results obtained in the present research work obviously indicate that indeginous soil microorganisms present in the hydrocarbon-polluted or unpolluted soil are quite capable of bioremediating soils polluted with hydrocarbons. High efficient rates of mineralization of both PCE and toluene are recorded. Application of the obtained results deserves to be investigated under field conditions.

Table 2: Distribution of ^{14}C in bottles spiked with [^{14}C]-uniform-toluene and amended with soil samples taken from the untreated soil column.

% of radioactivity recovered after 56 d incubation							
	1	2	3	4	5	6	7
Depth (m)	Toluen Resedual	CO_2	Water soluble intermediates	Cell mass	Toluene-C adsorbed on soil	Mineralization grade	Total recovery
2	0.1	51.1	8.8	16.4	12.8	67.5	89.2
3	0.2	48.6	4.1	23.1	20.5	71.7	96.5
4	0.2	45.5	2.9	27.7	11.4	73.2	87.7
5	0.1	51.1	3.8	27.5	9.5	78.6	92.0
6	0.1	60.4	3.1	20.6	12.8	81.0	97.0
7	0.1	49.0	3.9	17.3	13.7	66.3	84.0
8	0.2	52.7	2.3	16.0	9.1	68.7	80.3

(1) radioactivity in ethylene glycole monomethyl ether (residual toluene in headspace), (2) radioactivity in KOH, (3) radioactivity in 15.800 x g centrifugation supernatant, (4) radioactivity in SDS centrifugation supernatant, (5) radioactivity in SDS centrifugation sediment, (6) $\sum$ 2+4, (7) $\sum$ 1 to 5.

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التحليل الميكروبى لمركبى رابع كلوريد الايثيلين والتولوين فى تربه ملوثه

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توجد منطقه ملوثه بمخلفات المركبات الهيدروكربونيه الطياره (بصفه أساسيه المركب الأليفاتى رابع كلوريد الايثيلين PCE ومركبات البنزين ، التولوين، اثيل بنزين والزيلين العطريه) وتقع هذه المنطقه بالقرب من بلدة Eppelheim (ألمانيا الغربيه). الهدف من هذا البحث هو دراسة قدرة الميكروبات الطبيعيه الموجوده فى طبقات تربه متتاليه (حتى ٨ متر) على تكسير مركبى PCE والتولوين. أيضا تم دراسة قدرة هذه الميكروبات على الاستفاده من الكربون الموجود فى هذين المركبين فى بناء الخليه. مركب ال PCE تحلل ميكروبيا (نزع منه الكلور) تماما عن طريق عملية الازاله الاختزاليه للكلور وذلك فى خلال ٥٦ يوما من التحضين. أمكن تمييز ميكانيكيتين (تبعا لعمق التربه) لعملية التحلل: (١) رابع كلوريد الايثيلين -> ثالث كلوريد الايثيلين --> ثانى كلوريد الايثيلين --> أحادى كلوريد الايثيلين (كلوريد الفينايل) --> ايثان (طبقات التربه العلويه الغير ملوثه)، (٢) رابع كلوريد الايثيلين --> ثالث كلوريد الايثيلين --> ثانى كلوريد الايثيلين (طبقات التربه السفليه الملوثه) مما قد يدل على مشاركة ميكروبات (مجموعات ميكروبيه) مختلفه فى عملية التحلل. أوضحت التجارب باستخدام النظائر المشعه لمركب ال PCE (^{14}C[1,2]PCE) أن الايثان المشع كان هو الناتج الرئيسى لعملية التحلل mineralization فى طبقات التربه العلويه الغير ملوثه حيث بلغت نسبته حتى ٧٥,٩% . من ناحيه أخرى فانه فى طبقات التربه السفليه الملوثه كان الناتج النهائى لعملية التكسير مركبات كلورونيه مشعه وليس الايثان المشع. تكون كل من ثانى أكسيد الكربون و الميثان المشعين بتركيزات قليله (٢,٢ - ٩,٦ % و ٠ - ٥ % على التوالى). ومع ذلك فان تكون ثانى أكسيد الكربون المشع من ^{14}C[1,2]PCE يدل على حدوث تحلل كامل-لحد ما على الأقل- لمركب ال PCE. الكربون المشتق من ال PCE والمستفاد منه داخل الخلايا الميكروبيه بلغ فقط ٤,. - ٤,٤ % مما يدل على أن ميكروبات التربه كانت غير قادره على استعمال ال PCE كمصدر نمو. أوضحت التجارب باستخدام النظائر المشعه لمركب التولوين (^{14}C-uniform-toluene) أن التولوين قد تحلل ميكروبيا mineralizedفى كل أعماق التربه بدرجات مختلفه. ميكروبات التربه الطبيعيه أمكنها تحليل التولوين الى $^{14}CO_2$ (٥, ٤٥- ٤, ٦٠ %) و مكونات الخلايا (١٦ - ٢٧,٧ %). هذه النسبه العاليه من مكونات الخلايا المشعه تدل على أن ميكروبات التربه استفادت من التولوين كمصدر للكربن والطاقه. تراوحت معدلات تكون $^{14}CO_2$ من التولوين المشع بين ٠,٠٧٤ الى ١٨٣,. ميلليجرام $^{14}CO_2$-C/ كجم تربه جافه/ساعه وكانت أقل بمقدار ٤, ٤ مرات عن معدلات تحلل (النقص فى تركيز) التولوين الغير مشع (٠,١٧ و ٠,٩٣) ميلليجرام Toluene-C/ كجم تربه جافه/ساعه.