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ASSESSMENT OF PCE BIODEGRADATION
BY MICROBES ENDOGENOUS TO
MONITORING WELLS AT THE
USDOE OAK RIDGE Y-12 PLANT

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ABSTRACT

Perchloroethylene (PCE) is a persistent environmental contaminant whose chemical stability and hydrophobicity have made it difficult to remove from contaminated groundwater. PCE is also toxic and has been implicated as a carcinogen. This study was aimed at assessing methods for biological degradation of PCE. As a part of the study, we have characterized possible products of the degradation of PCE, and have determined the effects of detergents and solvents on the water solubility of PCE and on the toxic effects of PCE on bacteria. We have also isolated PCE-resistant microorganisms from monitoring wells at Y-12. To date all of the PCE-resistant bacteria isolated from the monitoring wells have been of the genus Bacillus. One of these isolates appears to be able to degrade PCE, as indicated by the disappearance of PCE from cultures of growing cells. The organism does not grow on PCE as the sole carbon source, so degradation of the solvent must occur by cometabolism.

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INTRODUCTION

Contamination of soils and sludges by hazardous wastes is a problem of serious economic consequence. There are currently more than 800 sites identified as hazardous waste sites (proposed and final listing) by the US Environmental Protection Agency (1985). These sites contain a variety of chemical wastes, and the same pollutants may appear in many waste sites. In many cases hazardous organics find their way into drinking water supplies, presenting a hazard to human health. The frequency of occurrence of some hazardous wastes in drinking water supplies (Council on Environmental Quality, 1980) is shown in Table 1. Contamination of groundwater by hazardous organic compounds has caused the closing of many public wells in the last few years (Craun, 1984).

TABLE 1. ORGANIC COMPOUNDS REPORTED
IN DRINKING WATER SUPPLIES

Compound	Percent of sites
Dichloroethylenes	23
1,1,1-Trichloroethane	19
1,1-Dichloroethane	18
Carbon tetrachloride	18
Trichloroethylene	14
Perchloroethylene	13
1,2-Dichloroethylene	7
Vinyl chloride	6
Methylene chloride	2

Hazardous wastes accumulate predominantly as a result of various industrial and agricultural activities. They may occur in waste water from manufacturing processes or in sludges and residues, in soil contaminated by spillage, run-off, or direct application of compounds, or in aquifers and other water supplies (Leisinger, 1983). Detoxifying contaminated sites by physical or chemical means is extremely costly. Biological detoxification may be less costly, but may also be very slow. Therefore it is worthwhile to develop methods for enhancing biodegradation of organic pollutants and bioconcentration or bioconversion of toxic metals, so that polluted sites might be detoxified more economically.

The cost of waste site remediation by conventional means depends on the system used, but all are extremely costly.

Immediate solutions include removing contaminated soil to another location, but that is not an ultimate solution because the wastes have not been eliminated, merely moved. Other techniques include washing the soil with water, extracting it with organic solvents, or heating it to moderately high temperatures (Noland and Koltuniak, 1987). Washing and extracting the soil with solvents may not remove the contaminants because of their solubility properties and affinity for soil particles, extraction and incineration destroy the usefulness of soil, and all of these techniques require digging up and processing large amounts of soil. Waste water or contaminated groundwater may be stripped of volatile organics by pumping the water through a spray nozzle and letting the contaminants evaporate (Allen and Parmele, 1983; Lamarre et al., 1983). However, this technique also releases the contaminants to the environment and is limited by the volatility of the contaminants and the extent of their partitioning from soil into groundwater. Washing, incineration, and air stripping are all expensive. In addition, if the wastes are in soil, and especially if they are hydrophobic, they are likely to be tightly bound to soil particles and thus are not readily removed with the groundwater.

An alternative treatment of contaminated waste sites is provided by the development of biological degradation and immobilization systems. Soil microorganisms are often found associated with soil particles, whose surfaces they may colonize. Many kinds of microorganisms have cell surfaces which can interact with hydrophobic compounds, and these organisms may be able to incorporate the compounds. In many cases the compounds can then be altered biochemically by the microorganisms. Thus, in situ biodegradation can be an effective means of removing organic compounds from soil, by complete degradation to inorganic constituents or by metabolic transformation to non-hazardous compounds. Microbes in the groundwater can migrate to localized concentrations of pollutants and attack them in situ, decontaminating the soil particles that serve as a reservoir for hazardous organic compounds.

A variety of technologies are currently being developed for the delivery of organisms and nutrients to contaminated zones in soil. Microbial degradation of chemical wastes has been an important process for many years, being the principal means of treating sewage. A number of studies have been carried out to determine whether hazardous organic wastes can be degraded by microbes. The results have been varied, but many studies have shown that the majority of organic compounds listed as being hazardous wastes can be degraded biologically. In some cases the degrading organisms have been found in contaminated soils and in other cases the organisms in ordinary municipal sewage treatment systems were found to be able to degrade complex organic compounds.

Systems for testing hazardous organic wastes to assess

their biodegradability have been proposed and discussed by a number of authors (e.g., Freitag et al., 1979; Gerike and Fischer, 1979, 1981; Gilbert, 1979; Shelton and Tiedje, 1984), and model and field assessments of groundwater transport and degradation of hazardous organics have also been reported (Castro and Belser, 1968; Heyse et al., 1985; Pignatello, 1986; Roberts et al., 1980; Scow et al., 1986; Wetzel et al., 1985; Wilson et al., 1981; Wilson and Noonan, 1984; Wilson and Wilson, 1985). Organisms capable of degrading organic pollutants not ordinarily present at significant concentrations have been found in municipal sewage (Clark et al., 1979; Cook et al., 1983; Ebing and Schuphan, 1979; Gerike and Fischer, 1979, 1981; Ghisalba, 1983; Leisinger, 1983; Tabak et al., 1981). Microbial treatment systems have also been described for removing nitrogen (Focht and Chang, 1975), phosphate (Timmerman, 1979), and various metals (Gale and Wixson, 1979; Hansen et al., 1984; Shumate et al., 1978; Strandberg et al., 1981; Zajic and Chiu, 1972).

EPA investigators studied the biodegradation of most of the organic compounds on the EPA priority pollutant list (Keith and Telliard, 1979) by microbial inocula taken from sewage treatment systems (Tabak et al., 1981). They found that most of the phenolic compounds and all of the phthalate esters and naphthalenes tested were degraded. Singly substituted benzenes were degraded but multiply substituted benzenes were not. Most nitrogenous organics and halogenated aliphatics evaluated were degraded. Polycyclic aromatic hydrocarbons, halogenated ethers and polychlorinated biphenyls were variably degraded, and organochlorine pesticides were not degraded during the test incubation. In many cases degradative ability increased with successive subcultures, indicating that adaptation to more effective degradation of hazardous wastes can occur. In other cases successive subculture resulted in decreased degradation of the test compounds, indicating that a cumulative toxicity can also result from exposure to the hazardous organics. These findings were essentially confirmed by Patterson and Kodukala (1981), who also reported that biological treatment systems could in some cases degrade compounds better than laboratory flask cultures. This was particularly true when cumulative toxicity occurred upon subculture of flask cultures. It appears that field biological treatment systems are better able to protect the organisms from toxic effects of the hazardous organics.

Numerous other examples of degradation of toxic organic chemicals by microorganisms have been reported. For example, chlorinated biphenyls can be degraded microbially by a number of organisms (for example, Ahmed and Focht, 1973; Furukawa et al., 1978; Massé et al., 1984; Sayler et al., 1977; Shiaris and Sayler, 1982; Shields et al., 1985; Yagi and Sudo, 1980). Microbial capabilities to degrade a number of other organochlorine compounds have been reviewed by Motosugi and Soda (1983). In some cases degradative processes are preferentially aerobic, as with *o*-phenyl-phenol (Hutchins et al., 1984),

whereas in other cases anaerobic degradation has been shown to be successful, as with halogenated hydrocarbons (Bouwer and McCarty, 1983, 1985; Stucki et al., 1983) or methyl carbamate pesticides (Kiene and Capone, 1986). Many studies have been carried out in static flask culture, in which conditions can range from aerobic to anaerobic at different levels in the medium and at different times during the growth period. Similarly, in a microbially based treatment system in contaminated soil, there would be both aerobic and anaerobic microhabitats, providing the opportunity for both kinds of metabolism of the hazardous organics.

Detailed laboratory data about the mechanisms of biodegradation and physiological conditions under which the organisms can most efficiently degrade solvents are very informative. However, many studies have shown that upon exposure to degradable substrates, bacteria with the appropriate metabolic capabilities will metabolize those compounds, even if the growth conditions are not optimal. Furthermore, complex nutritional and physiological conditions may not readily be economically achieved in contaminated groundwater. Therefore, it is appropriate to consider also what nutritional conditions will merely increase the numbers of the organism, with an eye to applying those conditions in situ, as is often done in bioremediation systems.

In the study described here, environmental samples from a series of monitoring wells at the USDOE Oak Ridge Y-12 Plant were evaluated for potential to degrade a series of hazardous organic compounds, and a bacterial strain capable of growing in the presence of high concentrations of perchloroethylene (PCE) was isolated and identified. Preliminary experiments were carried out to assess the ability of this bacterial strain to degrade PCE, and to determine under what conditions the organism can be propagated.

RATIONALE

The samples provided by Y-12 came from wells which have been shown to contain significant levels of PCE, trichloroethylene (TCE), and dichloroethylene (DCE), whereas the tanks leaking into the soil near these wells contained only PCE. PCE is stable to chemical decomposition. Therefore it seems reasonable to assume that some biological activity in the soil is responsible for dechlorination of the compounds. The goal of this project was to isolate bacteria which are capable of complete mineralization of PCE, TCE, DCE, and vinyl chloride. Direct selection for the ability to use PCE as the sole carbon source was applied to the samples. However, the occurrence of complete mineralization could not be assured by the ability to tolerate PCE and to gain energy from its metabolism. In the absence of complete mineralization, complete dechlorination of the solvent is an acceptable remedial alternative, ethylene being considerably less toxic than its halogenated derivatives. Therefore we also assessed the dechlorination of compounds which are derivatives of other compounds shown to be acceptable substrates for bacterial metabolism. These compounds were: monochloroacetic acid, which should yield acetate, a readily metabolized carbon source; 3-chlorobenzoic acid, which can be metabolized by a halogenase-related plasmid-coded pathway in *Pseudomonads*; and 4-chlorobenzoic acid, which can be metabolized by a chromosomal pathway in *Pseudomonads*.

METHODS

In these studies it was necessary to analyze samples for the presence of several chemical species, including PCE, TCE, chloroform, and trichloroacetic acid (TCA). The latter compounds could be generated by metabolism of PCE by dechlorination (Reaction 1), possibly followed by oxidation or epoxidation and rearrangement (Reaction 2). Two methods were used for the measurement of PCE and its metabolites. These were dynamic headspace gas chromatography and direct automatic injection gas chromatography. These methods are described below. The same conditions were used for quantitation of TCE, chloroform, and other reaction products.

Reaction 1

Reaction 2

This section also includes a description of growth media and enumeration methods used for culturing bacteria from the well samples.

PERCHLOROETHYLENE

Formula: C_2Cl_4

M.W. 165.8

B.P. 121°

d. 1.623 g/ml

SYNONYMS: TETRACHLOROETHYLENE

Instrumentation: TRACOR MODEL 550 GAS CHROMATOGRAPH
TEKMAR MODEL 4000 DYNAMIC HEADSPACE
CONCENTRATOR

Technique: DYNAMIC HEADSPACE GAS CHROMATOGRAPHY,
FLAME IONIZATION DETECTOR

Analyte: PERCHLOROETHYLENE

Injection volume: 2-100 ul

Trap: Tekmar Tenax trap

Temperature: Injection 230°
Detector 250°
Column 60° 4 min
8°/min to 235°
235° 10 min (more as necessary)

Carrier: Ne , flowmeter on 9.5

Column: Supelco
Stainless steel, 1/8 in x 8 ft
1% SP-1000 on 60/80 Carbopack B

Calibration: Solutions of PERCHLOROETHYLENE in METHANOL

Range: Lower, 2 ug/sample @ Atten.= 8
Upper, 100 mg/sample @ Atten.= 1024

REAGENTS:

1. Perchloroethylene, chromatographic quality
2. Methanol, chromatographic quality
3. Nitrogen, 99.999%
4. Hydrogen, purified
5. Compressed air, zero grade

EQUIPMENT:

1. 5-ml capacity screw-cap tubes or 1-dram vials
2. Teflon-lined caps for tubes or vials
3. Volumetric flasks, 5 ml and 50 ml
4. Syringes, 10 ul and 250 ul gas-tight

SAMPLE PREPARATION:

1. Prepare two clean 5-ml screw-cap tubes or 1-dram sample vials for each sampler. Rinse each twice with methanol and allow to drain.

CALIBRATION AND QUALITY CONTROL:

2. Prepare a calibration standard solution by adding 50 mg (30.8 ul) PCE to a 50-ml volumetric flask and filling to the mark with methanol (1 mg/ml). Mix and transfer 0.25 ml (using a large gas-tight syringe) to a 5-ml volumetric flask. Fill to the mark with methanol (0.05 mg/ml).
3. Calibrate daily with at least five working standards including (for attenuator set at 8) 0.05, 0.15, 0.3, 0.45, and 0.6 ug (using a standard solution of 0.05 mg/ml in methanol, add 1.0, 3.0, 6.0, 9.0, or 12 ul to 2 ml H₂O).
4. Prepare a calibration graph of peak height vs amount of perchloroethylene.

MEASUREMENT:

5. Set the gas chromatograph as indicated above. Purge time is 15 min. With a 15-min purge, there is a 4-min dry purge to remove almost all of the methanol from the Tenax trap.
6. Rinse syringe by filling and discarding the sample solution three times. Waste sample can be discarded into activated charcoal in a beaker or flask. Add the desired volume of sample to 2 ml of water in a clean 13x100 mm screw-cap tube and attach to the dynamic headspace concentrator.
7. Run analysis cycle and determine peak height. Under the conditions listed, methanol elutes at about 2 min at 60°, and perchloroethylene elutes at about 16 min, at 210°.

CALCULATIONS:

8. Using the calibration chart, determine the mass (ug) of perchloroethylene in each peak. Calculate the mass in each sample by dividing by volume injected and multiplying by the volume of extract, e.g.:

$$W \text{ (mg)} = [\text{Sample ug/sample ul}] \times 5.0 \text{ ml}$$

PERCHLOROETHYLENE

Formula: C_2Cl_4

M.W. 165.8

B.P. 121°

d. 1.623 g/ml

SYNONYMS: TETRACHLOROETHYLENE

Instrumentation: HEWLETT-PACKARD MODEL 5880 GAS
CHROMATOGRAPH

Technique: DIRECT AUTOMATIC INJECTION GAS
CHROMATOGRAPHY,

^{63}Ni ELECTRON CAPTURE DETECTOR

Analyte: PERCHLOROETHYLENE

Injection volume: 2-100 μ l

Temperature: Injection 230°
Detector 230°
Column 120° 2 min
10°/min to 240°
240° 4 min

Carrier: N_2 , flow 20 cc/min

Column: Supelco
Stainless steel, 1/8 in x 8 ft
1% SP-1000 on 60/80 Carbopack B

Calibration: Solutions of PERCHLOROETHYLENE in METHANOL

Range: Lower, 2 μ g/sample
Upper, 100 mg/sample

REAGENTS:

1. Perchloroethylene, chromatographic quality
2. Methanol, chromatographic quality
3. 95% Argon/5% Methane

EQUIPMENT:

1. 5-ml capacity screw-cap tubes or 1-dram vials
2. 1-cc crimp-top sample bottles with Teflon septa
3. Teflon-lined caps for tubes or vials
4. Volumetric flasks, 5 ml and 50 ml
5. Syringes, 10 μ l and 250 μ l gas-tight

SAMPLE PREPARATION:

1. Pipette samples into sample bottles and seal with Teflon septa.

CALIBRATION AND QUALITY CONTROL:

2. Prepare a calibration standard solution by adding 50 mg (30.8 μ l) PCE to a 50-ml volumetric flask and filling to the mark with methanol (1 mg/ml). Mix and transfer 0.25 ml (using a large gas-tight syringe) to a 5-ml volumetric flask. Fill to the mark with methanol (0.05 mg/ml).
3. Calibrate daily with at least five working standards including (for attenuator set at 8) 0.05, 0.15, 0.3, 0.45, and 0.6 μ g (using a standard solution of 0.05 mg/ml in methanol, add 1.0, 3.0, 6.0, 9.0, or 12 μ l to 2 ml H_2O).
4. Prepare a calibration graph of peak height vs amount of perchloroethylene.

MEASUREMENT:

5. Set the gas chromatograph as indicated above. Set autosampler to rinse the syringe three times, and pump five times. Sample size is 1-2 μ l.
6. Analyze standards and unknowns, using automatic baseline reset mode (Mode 0). Construct a calibration chart, using the peak areas observed for each standard concentration.

CALCULATIONS:

7. Using the calibration chart, determine the mass (μ g) of perchloroethylene in each sample. Calculate the concentration in each sample by dividing by volume injected.

The characteristic retention times of the various compounds are given in Table 2.

TABLE 2.
RETENTION TIMES OF HALOGENATED
HYDROCARBONS IN TWO GAS CHROMATOGRAPHIC
ANALYTICAL SYSTEMS

COMPOUND	RETENTION TIME (min)	
	SYSTEM 1 ¹	SYSTEM 2 ²
DICHLOROETHYLENE	9.8	4.3
CHLOROFORM	10.4	4.7
TRICHLOROETHYLENE	15.4	8.2
PERCHLOROETHYLENE	20.9	12.7

¹DYNAMIC HEADSPACE CHROMATOGRAPHY
(TRACOR 550)

²DIRECT INJECTION (HP 5880)

TRICHLOROACETIC ACID

A method to measure TCA in urine by dynamic headspace gas chromatography was described by Senft (1985). This method relies on the conversion of TCA by heat to chloroform and CO₂, followed by gas chromatographic determination of the chloroform concentration. This method was adapted for the measurement of TCA in culture supernates after growth of bacteria in the presence of PCE.

Samples containing TCA are placed in screw-cap tubes with Teflon-faced seals. The samples are heated in a water bath for 105-120 min at 85°C, chilled, and analyzed for chloroform by gas chromatography by the techniques described above. Figure 1 shows the rate of conversion of TCA to chloroform when solutions containing 50 ug TCA were heated at 85°C. Figure 2 gives the concentration response of this system. In this procedure, chloroform peak heights were proportional to amount of TCA added to reaction tubes before heating; no chloroform was observed in unheated tubes with the same amounts of TCA.

GROWTH MEDIA

R2A is a semi-defined growth medium designed for the enumeration of bacteria from natural samples (Reasoner and Geldreich, 1985). It contains (grams per liter):

Yeast extract	0.5
Proteose peptone #3	0.5
Casamino acids	0.5
Glucose	0.5
Soluble starch	0.5
Sodium pyruvate	0.3
K ₂ HPO ₄	0.3
MgSO ₄ · 7H ₂ O	0.07
* Distilled water	1 l
Adjust pH to 7.2	

MSVT is a defined salts medium made as follows:

Prepare and sterilize separately

MS base (autoclave):

1.0 g	FeSO ₄
NH ₄ Cl	0.5 g
KCl	0.2 g
MgSO ₄	0.2 g
NaCl	0.1 g
KH ₂ PO ₄	0.44 g
K ₂ HPO ₄	2.0 g
Distilled water	996 ml

Vitamin stock solution (filter-sterilize):

Calcium pantothenate	4.0 mg
Thiamine	4.0 mg
Pyridoxine	4.0 mg
Nicotinic acid	4.0 mg
p-Amino benzoic acid	2.0 mg
Riboflavin	2.0 mg
Distilled water	100 ml

Trace element stock solution (filter-sterilize): KNO₃

50.0 mg	
MnCl ₂	10.0 mg
Co(NO ₃) ₂	38.0 mg
ZnCl ₂	10.0 mg
CaCl ₂	2.0 mg
H ₃ BO ₃	1.9 mg
Na ₂ MoO ₄	1.0 mg
Na ₂ SeO ₃	2.8 mg
NiCl ₂	5.0 mg
Distilled water	100 ml

Biotin stock solution (filter-sterilize):

Biotin	24.0 mg
Distilled water	100 ml

Calcium-copper stock solution (filter-sterilize):

CaCl ₂	0.15 g
CuSO ₄	2.0 ml of
	2.5 mg/100 ml solution
Distilled water	98 ml

Mix aseptically

MS base	996 ml
Trace elements stock	0.2 ml
Vitamin stock	2.0 ml
Biotin stock	164 ul
Calcium-copper stock	2.0 ml

SM is another defined salts medium (Billen et al., 1971), which contains (per liter):

K ₂ HPO ₄	14.0 g
KH ₂ PO ₄	6.0 g
Na citrate	1.0 g
NH ₄ Cl	0.8 g
MgCl ₂ ·6H ₂ O	0.33 g
Na ₂ SO ₄	14.0 mg
Distilled water	1 l

ENUMERATION OF BACTERIA

Bacteria in the water samples were enumerated by plate-count techniques. The samples were diluted in sterile water and spread on agar plates containing R2A agar medium (Reasoner and Geldreich, 1985). Colonies were counted after 24 to 48 hours incubation at room temperature.

GROWTH OF BACTERIA

Samples from the wells containing PCE and other organic pollutants were inoculated into selective media and indicator media to select for the ability to metabolize chlorinated organics as sole carbon sources and for the ability to co-metabolize chlorinated organics. Selection was done for utilization of PCE, monochloroacetic acid (MCA), 3-chloro- and 4-chlorobenzoic acid (3-CBA and 4-CBA), and 4-nitrochlorobenzene (CNB).

The protocol for inoculation of the bacteria under different conditions is given below:

Dilute 200 ml of water samples with 22 ml of ten-fold concentrated MSVT stock and dispense into tubes or bottles containing the desired substrates as follows:

1. Control

Add 20 ml to a serum bottle containing no other additions.

2. YE Control

Add 20 ml to a serum bottle with 0.02 ml of 10% yeast extract.

3. Acetate-PCE cometabolism

Add 20 ml to a serum bottle with 0.02 ml of

10% yeast extract and 0.4 ml 10% sodium acetate, and 20 ul PCE.

4. PCE utilization - YE supplement

Add 20 ml to a serum bottle with 20 ul PCE and 0.02 ml 10% yeast extract.

5. MCA utilization - YE supplement

Add 20 ml to a serum bottle with 0.4 ml of 10% MCA and 0.02 ml 10% yeast extract.

6. 3-CBA utilization

Add 20 ml to a serum bottle with 0.02 ml of 10% yeast extract and 0.2 ml of 10% 3-CBA in ethanol.

7. 2-CBA utilization

Add 20 ml to a serum bottle with 0.02 ml of 10% yeast extract and 0.2 ml of 10% 2-CBA in ethanol.

8. Dechlorination of CNB

Add 5 ml to a tube containing 0.1 ml 10% sodium acetate, 5.0 ul 10% yeast extract, and 50 ul 0.10% CNB in ethanol.

9. Acetate enrichment

To 20 ml add 0.4 ml 10% sodium acetate and 0.02 ml 10% yeast extract. Incubate until growth is observed. Use this culture to reinoculate for tests above if necessary.

EXPERIMENTAL

CHARACTERIZATION OF POTENTIAL DEGRADATION PRODUCTS OF PCE

The goal of these experiments was to isolate and characterize microorganisms which could carry out partial or complete biodegradation of halogenated organic solvents. Initial consideration was given to the kinds of compounds which might appear in growth medium as a result of degradation of PCE. Potential pathways for biodegradation of PCE might include successive dechlorination (to TCE, DCE, vinyl chloride, and ethylene), oxidation of the double bond with formation of alcohol or diol, oxidation with elimination of chloro groups, or addition of acid or base to the double bond. Oxidation of TCE by mammalian cells has been observed to lead to formation of a diol product, followed by rearrangement, with the ultimate formation of TCA (Senft, 1985). Therefore TCA was also considered to be a potential degradation product of PCE, by a reaction which would have to include elimination of one of the chloro groups.

In order to be sure that we could identify a variety of degradation products of PCE in subsequent experiments, an initial study was done to characterize reaction products of PCE. A number of potential degradation products and intermediates were characterized chromatographically so they could be identified if they appeared during experiments on biodegradation of PCE. Standards containing PCE, TCE, and DCE were mixed and chromatographed as described in the Methods section for the analysis of PCE. Figure 3 shows a tracing of a typical chromatographic analysis of these standards. PCE, TCE, and DCE are well separated in this analysis, but vinyl chloride elutes in the solvent peak. Oxidation products of PCE were generated by incubating PCE with concentrated sulfuric or hydrochloric acid at 60° C for 48 hours. The aqueous fraction was then analyzed by dynamic headspace gas chromatography. The results showed that a substantial amount of the PCE was converted to an apparently more polar compound, probably sulfate, alcohol, or diol, which eluted later than PCE, and a small amount of an early-eluting material was also formed (Figure 4).

Similarly, a mixture of PCE and water was irradiated with approximately 5×10^4 rads of radiation from a ^{60}Co gamma source, and a mixture of PCE and 1 M KMnO_4 was incubated for 7 days at room temperature. The aqueous layers of these mixtures were then analyzed by dynamic headspace gas chromatography. In the case of radiation, radiolysis of water would be expected to yield a number of reactive species; the likely addition product would be alcohol or diol, formed by the addition of hydroxyl radical to the double bond of the PCE molecule. As a result of permanganate reaction, epoxide formation would be expected to occur, followed by formation of a diol and perhaps subsequent rearrangement and elimination of chloro groups. Figure 4 shows

the results of these experiments. In this figure, the tracings are offset to facilitate comparison of the profiles. In each case, addition products were observed, although in very small amounts in the case of radiolysis products. Two early-eluting products were formed as a result of permanganate reaction, one of which chromatographed identically with the early-eluting addition product of acid treatment.

The characteristic retention times of chlorinated ethylenes and the observed addition products as measured by dynamic headspace gas chromatography are listed in Table 3.

TABLE 3.
RETENTION TIMES OF HALOGENATED
HYDROCARBONS AND PCE REACTION PRODUCTS
ANALYZED BY GAS CHROMATOGRAPHY

COMPOUND	RETENTION TIME (min)
DICHLOROETHYLENE	9.4
CHLOROFORM	10.4
TRICHLOROETHYLENE	15.2
PERCHLOROETHYLENE	20.9
PCE ADDITION PRODUCTS	
ACID ADDITION	3.9, 9.4, 27
PERMANGANATE ADDITION	3.45, 3.9

These experiments demonstrated that products of addition to the double bond of PCE can be detected. Acid addition appears to have formed small amounts of dichloroethylene and an unidentified early-eluting material, as well as a substantial amount of slow-eluting adduct, probably an alcohol or a sulfate. Permanganate addition formed two early-eluting compounds, one of which appears to be identical to the early-eluting acid product. Conditions which would have allowed detection of the slow-eluting addition compounds were included in subsequent analyses of PCE biodegradation products.

SITE CHARACTERIZATION

Water samples were removed by Y-12 personnel from eleven

monitoring wells at the Y-12 plant on March 10, 1986. Measurements of temperature, pH, dissolved oxygen, and conductivity were made in the field and reported to us when the water samples were delivered. These values are reported in Table 4, along with our assignment of sample numbers.

Water samples were diluted and plated on R2A agar plates (Reasoner and Geldreich, 1985) for the enumeration of total aerobic heterotrophic bacteria. These values ranged from undetectable at a 10^{-2} dilution to 10^4 organisms per ml. (By definition, clean water contains fewer than 10^3 total heterotrophs per ml.) There was no apparent statistical correlation of the number of bacteria or the logarithm of the number of bacteria with other measured parameters in the water samples. Significant concentrations of PCE and TCE were not detected in the samples by gas chromatography (dynamic headspace concentration method), but trace amounts of these compounds could have been present initially and lost by adsorption to the walls of the sample bottle.

TABLE 4.
CHARACTERIZATION OF WATER SAMPLES FROM
MONITORING WELLS AT Y-12

SAMPLE NO.	WELL NO.	D.O.	TEMP (°C)	pH	CONDUCTIVITY (uS/cm)	AEROBES per ml
1	GW-8	2.5	15.6	6.8	0	1.50x10 ⁴
2	GW-14	3.2	12.8	7.5	0.2	5.95x10 ³
3	GW-15	3.4	10.4	7.2	0.3	8.39x10 ³
4	BG-18	3.1	17.4	7.4	0.3	3.50x10 ³
5	GW-20	2.7	15.3	7.9	0.1	1.00x10 ⁴
6	GW-27	3.1	16.9	6.7	0.4	1.60x10 ⁴
7	DR-29	3.2	14.0	6.8	0.8	8.65x10 ⁴
8	GW-59	5.7	14.8	7.6	0.1	7.45x10 ⁴
9	GW-71	2.5	14.1	11.8	0.7	<1.00x10 ³
10	GW-79	4.5	14.7	7.8	0.1	4.46x10 ³
11	GW-94	4.1	15.9	9.3	0	1.14x10 ³
MEAN		3.45	14.72	7.89	0.27	2.36x10 ³
STD DEV		0.92	1.85	1.42	0.25	3.46x10 ³

SOLUBILITY OF PCE IN DETERGENT SOLUTIONS

PCE and similar organic solvents are extremely poorly soluble in water, so disposal or degradation processes that are based on reactions in water solutions are not very efficient. In particular, biological degradation systems are hampered by low availability of poorly soluble substrates. In addition, it is difficult to make accurate measurements of rates of degradation if the total amount of substrate is greater than the soluble concentration. Therefore methods of increasing the solubility of PCE in water would be helpful in studies of biodegradation. In addition, bacterial cell membranes contain high quantities of lipids, which are essential for the proper structure and functioning of the membranes and thus for the survival and metabolism of the cell. PCE and similar solvents are soluble in lipids and could dissolve in cell membranes, disrupting their structure enough to kill the cells or disrupt their energy metabolism or other metabolic processes. The experiments described here were done to determine whether the addition of various organic solvents and detergents might increase the solubility of PCE in water-based media without increasing the toxicity of the PCE-containing media.

We hypothesized that in addition to increasing the availability of PCE to the cells, solubilizing agents might also provide the additional benefit of reducing the toxicity of the solvent to the growing cells. In this study a number of agents were used that are known to increase the miscibility of polar and nonpolar compounds and are not highly toxic to bacterial cells. These compounds were ethanol, dimethylsulfoxide (DMSO), Triton X-100, and Tween-80.

The growth medium used was MSVT + sodium acetate + 0.01% yeast extract. Various organic solvents or detergents were added, along with 0.1% (v/v) PCE. Parallel cultures were incubated either uninoculated or inoculated with subcultures from sampling wells (Table 4). The inoculated cultures were assessed for toxicity and in some cases for degradation of PCE. Cultures containing the test compounds but not PCE were also inoculated with bacteria to assess toxicity of the test compound alone.

PCE concentrations were measured by dynamic headspace gas chromatography, as described in the Methods section, except that the temperature was held constant at 210° C. Samples of 1 ul were diluted in 2.0 ml of glass-distilled water in screw-cap tubes. The tubes were then attached to the head space concentrator, purged with nitrogen for 6 min, and injected into the column by thermal desorption from the concentrator's trap. Under these conditions an injection peak was observed at 1.0 min, and PCE eluted at 4.5-4.8 min. With an output attenuator setting of 16, 1 ul of PCE-saturated water gave a peak area of 86.8 sq mm (height x width at 1/2 max height) and 1 ug of PCE gave a peak area of 392 sq mm. The

latter value was used to calculate the concentration of PCE in the experimental samples.

The effects of the various additions on solubility of PCE are given in Table 5.

TABLE 5.
SOLUBILITY OF PCE IN GROWTH MEDIUM
WITH VARIOUS SOLVENT ADDITIONS

ADDITION	CONC. (v/v)	PEAK HT (mm) ¹	HALF MAX WIDTH (mm)	AREA (sq mm)	ppm PCE ²
None		28.0	3.1	86.8	233
DMSO	5.0%	29.3	3.4	99.8	268
Ethanol	5.0%	23.8	3.4	80.9	218
Tween-80	0.5%	56.0	3.2	179.2	482
Triton X-100	0.5%	123.5	3.1	382.9	1030

¹Peak height was normalized to the height of the injection peak.

²Calculated relative to a standard containing 1 ug PCE in methanol. No peaks were observed at the position of PCE when solvent controls containing no PCE were chromatographed.

These results demonstrate that the solubility of PCE in aqueous media can be significantly increased by the addition of certain detergents to the medium. The increase in solubility could make PCE in water more available to bacteria for biodegradation. In addition, the increased solubility might be important in situations where PCE is hydrophobically bound, for example to soil particles. Addition of the appropriate detergent to water being used to flush contaminated soil could be expected to increase the amount of PCE leached from a spill site.

ISOLATION OF PCE-RESISTANT BACTERIA

The purpose of this project was to isolate and characterize naturally occurring microorganisms which are capable of degrading PCE. Water samples from monitoring wells in the Y-12 plant were used as the source of the organisms; these wells show contamination with PCE and other volatile organics. The rationale was first to select organisms resistant to the toxic effects of PCE, and then to screen these for the ability to degrade PCE, either as a nutrient source or by cometabolism.

Selective and indicator media were inoculated from the well samples as indicated in the Methods section. Cultures were grown in 60-cc serum bottles sealed with Teflon septa. Initially, 20-ml samples were incubated at room temperature for 10-14 days. Cultures in which the bacteria grew were saved for isolation and further analysis. Some cultures in which 3-chlorobenzoate was the primary carbon source turned dark after several days, indicating degradation of the benzoate. The ability to grow with the indicated substrates as the major carbon source was confirmed by subculture of the bacteria. In addition, several of the samples were subsequently shown to be able to degrade biphenyl, the parent compound of polychlorinated biphenyls. The results of these experiments are summarized in Table 6.

Except for the control (#1), all cultures contained 10 ppm yeast extract and 100 ppm of the indicated substrate. All cultures showed little or no growth in the controls, indicating minimal carryover of nutrients, and sparse growth in all yeast extract controls (#2).

1. Control - no addition
2. YE Control - 10 ppm yeast extract only
3. Acetate-PCE, 100 ppm each
4. Perchloroethylene, 100 ppm
5. Monochloroacetate, 100 ppm
6. 3-Chlorobenzoic acid, 100 ppm
7. 2-Chlorobenzoic acid, 100 ppm
8. Chloronitrobenzene + acetate, 100 ppm each
9. Acetate, 100 ppm

TABLE 6.
GROWTH OF BACTERIA FROM MONITORING WELLS
IN SELECTIVE AND INDICATOR MEDIA

SAMPLE NO.	WELL NO.	EXTENT OF GROWTH IN CONDITION NUMBER:						
		3	4	5	6	7	8	9
1	GW-8	±	±	-	+	+	+++ YLO	+
2	GW-14	++	+++	+++	++ BR	++	++	++
3	GW-15	+++ BK	++	++ YLO	+	++ BK	++ BK	++ BK
4	BG-18	++	++	- YLO	-	±	+	+
5	GW-20	++	+	±	+	-	+++	-
6	GW-27	++	+++	-	-	-	++	-
7	DR-29	++	-	-	++ YLO	-	+++	-
8	GW-59	++	+	-	-	±	±	-
9	GW-71	++	±	±	± YLO	± YLO	++ YLO	-
10	GW-79	++	± YLO	± YLO	± YLO	± YLO	±	+
11	GW-94	++	-	+	-	±	±	-

Pigments and colored products produced as indicated:
BR = brown, BK = black, YLO = yellow

- = No growth; ± = barely turbid; + = sparse growth;
++ = moderate growth; +++ = heavy growth

Bacteria were streaked onto R2A agar and individual colonies were observed for morphological types. Cultures grown in the presence of excess PCE yielded mainly flat, matte colonies characteristic of the genus Bacillus. Several of the colonies were restreaked, and the isolated bacterial strains were tested further. Bacterial strains were identified on the basis of morphology, gram character, motility, and biochemical properties, using Bergey's Manual (Sneath et al., 1986) as the reference.

PROTECTIVE EFFECT OF DETERGENT FOR PCE-SENSITIVE CELLS

The potential application of detergents to increase solubility and thus possible biodegradation of PCE depends in part on the toxicity of PCE to the cells. The suggestion was made above that the addition of detergent to bacterial culture media might reduce the potential partitioning of PCE to the lipids of the bacterial cell membrane, and thus reduce the toxicity of PCE to the cells. To test this hypothesis, an experiment was done to test the protective effects of one of the detergents against inhibition of growth of bacterial strains found in the monitoring wells. In this experiment, strains of bacteria from the sampling wells that were shown not to grow rapidly in R2A with 0.1% PCE were incubated in R2A alone or with 0.5% Tween-80, 0.1% PCE, or both PCE and Tween-80. The extent of growth was measured by determining the optical density of each culture. After 48 hours at room temperature, the results were as follows:

TABLE 7.
PROTECTIVE EFFECTS OF TWEEN-80:
GROWTH OF BACTERIA IN R2A WITH ADDITIONS

Well #	OPTICAL DENSITY (600 nm)			
	CONTROL	TW-80	PCE	TW-80+PCE
2	1.23	1.09	0.27	0.70
5	1.12	0.97	0.62	0.60
6	1.54	1.90	0.50	0.60
8	0.44	0.54	0.11	0.17
9	0.46	0.64	0.06	0.18
11	1.46	1.79	0.30	0.60

These results indicate that in general, Tween-80 was not significantly toxic to the bacteria. Tween-80 had some

protective effect against the toxic effects of PCE when the bacteria were sensitive to PCE, but that the protection was somewhat limited. The bacteria from Well 5 grew reasonably well in the culture containing PCE, and this culture did not grow more extensively when Tween-80 was added along with the PCE. This may indicate that PCE-resistant strains are not as susceptible as PCE-sensitive strains to solubilization of essential cell components by PCE, and are thus not protected additionally by the presence of detergent. Overall, the protection of cells by detergent from the toxic effects of PCE was only marginal, indicating that the method will have limited practical application for growth of PCE-sensitive cells in the presence of high concentrations of PCE.

IDENTIFICATION OF BACILLUS W8

One of the PCE-resistant isolates, designated W8, was chosen as a representative and examined in some detail. Studies were done to determine its growth requirements and substrates. Its general properties are summarized in Table 8. The organism appears to be a variety of Bacillus, and some of its properties match those of some of the Bacillus species described in Bergey's Manual. However, none of the species of Bacillus listed in Bergey's Manual matched all of the properties of W8. It proved to be able to grow in a simple mineral salts medium without added trace elements or vitamins, and to grow on mineral salts with citrate as the sole carbon and energy source. Glucose is a poor but usable energy and carbon source; yeast extract and vitamin-free Casamino acids are better substrates. Inspection of old nutrient agar streaks of the organism revealed the presence of spores, indicating that it belongs to the genus Bacillus. The organism grew in nutrient broth containing saturating concentrations of PCE, indicating that it is resistant to the toxic effects of that compound.

TABLE 8.
PROPERTIES OF Bacillus W8

- * Gram-positive spore-forming rod
- * Facultatively anaerobic
- * Catalase-positive, oxidase-negative, urease-negative
- * Nitrate reduced to nitrite but not to N_2
- * Grows on glucose, citrate, or yeast extract
- * Vitamins not required for growth
- * Does not grow at 50° C
- * Grows in the presence of excess PCE

RESISTANCE OF Bacillus W8 TO PCE

The toxicity of PCE to Bacillus W8 was compared to its toxicity to Escherichia coli B/R and a Pseudomonas species. Toxic effects were measured on cells grown to stationary phase in tryptone broth. The cells were diluted and plated on nutrient agar to determine the initial viable titer. A saturating amount of PCE (100 $\mu\text{l/ml}$) was added to each culture, and the cell suspensions were vigorously mixed for 15 seconds. The cells were sampled again, diluted, and plated on nutrient agar to determine the surviving viable titer. The results (Table 9) showed that the resistance of Bacillus W8 is several orders of magnitude greater than that of the other bacterial strains tested. E. coli and Pseudomonas are Gram-negative and therefore have a significantly different cell wall composition and structure than the Gram-positive Bacillus W8. This difference contributes to differential sensitivity to antibiotics and detergents, and could result in a difference in sensitivity to PCE. However, because another strain of Bacillus, Bacillus subtilis 168, has been observed not to grow in the presence of excess PCE, the resistance of Bacillus W8 is not likely to have been a result of the Gram-positive cell wall per se. It appears likely that the resistance is a property selected for in the PCE-containing environment of the organism.

TABLE 9.
COMPARISON OF RESISTANCE OF *Bacillus* W8
AND OTHER BACTERIAL STRAINS TO PCE

STRAIN	CFU/ml		SURVIVAL (%)
	INITIAL	FINAL	
<u><i>Escherichia coli</i> B/R</u>	2.3×10^7	< 50	$< 2.0 \times 10^{-4}$
<u><i>Pseudomonas</i> sp.</u>	6.7×10^8	1.5×10^8	2.2×10^{-4}
<u><i>Bacillus</i> W8</u>	2.2×10^7	2.5×10^6	11.3

BIODEGRADATION OF PCE

Cultures were grown in 20 ml of MSVT with 20 ul PCE and various carbon sources, sodium acetate, 3-chlorobenzoate (3CBA), and 2-chlorobenzoate (2CBA). The chlorobenzoates were used to induce halogenases, which would be necessary for degradation of the aromatic ring. Cultures that showed growth were subcultured, and PCE degradation was determined as the disappearance of PCE measured by gas chromatography. Two samples have shown reduced levels of PCE after incubation. One, from sampling well GW-59, showed degradation of PCE in MSVT + 0.01% yeast extract + 0.1% 3CBA and 0.1% PCE. Another, from sampling well GW-20 showed degradation in chloride-free MSVT + 0.002% yeast extract + sodium acetate + 0.1% PCE. In this case it is possible that growth of the organism depends on dechlorination of PCE to release Cl^- for cell metabolism. This possibility should be investigated further.

As described above, *Bacillus* W8 appeared to be able to degrade PCE. The experiments described below were carried out with this strain to evaluate further its potential to degrade halogenated solvents. Cells of *Bacillus* W8 were grown overnight in MSVT containing 0.1% yeast extract as the carbon source. They were centrifuged and washed with MSVT, and then resuspended at a 1:20 dilution in serum bottles containing 20 ml of MSVT + 0.01% yeast extract + 0.1% 3-CBA. PCE was added to a final concentration of 100 ppm, and the serum bottles were sealed with Teflon septa. Controls contained either no cells or no PCE. The bottles were incubated at 30° C, and at weekly intervals 200 ul samples were taken and diluted into 2 ml of water for analysis by dynamic headspace gas chromatography. The results of this experiment (Figure 5) indicated that after about two weeks of incubation, the concentration of PCE in the medium decreased, until at 4 weeks it was not detected. In

contrast, the bottles containing no bacteria did not show a reduction in PCE concentration.

In a similar experiment, the effect of carbon source on degradation of PCE and TCE was tested. Cells of Bacillus WB were grown overnight in SM + 0.1% yeast extract. They were then centrifuged and washed with SM, and resuspended at a 1:20 dilution in serum bottles containing 20 ml of tryptone broth, SM alone, or SM + 0.1% yeast extract or casamino acids. PCE or TCE was added at a final concentration of 100 ppm in methanol (final methanol concentration 1000 ppm). The bottles were sealed with Teflon-lined septa and incubated at 30° C. At weekly intervals 200 ul samples were taken and diluted into 2 ml of water for analysis by dynamic headspace gas chromatography. The results showed degradation of the solvents in all of the media. Examples of the degradation of PCE and TCE in the various media are given in Figures 6 and 7. These results show that degradation of the compounds occurred more rapidly in the presence of a rich energy source such as the amino acid mix (CAA), yeast extract (YE), or Tryptone broth than in SM alone. The results indicated that both PCE and TCE were degraded in a mineral salts medium (SM) containing only citrate as the only additional carbon source. No degradation of chlorinated hydrocarbons has been observed without an additional carbon source.

In none of the gas chromatographic analyses was there evidence of volatile intermediate metabolites, particularly dehalogenated intermediates. It has been shown that halogenated aromatics and TCE may be degraded by epoxidation, followed by hydrolysis, which results in hydroxyl groups being added to the double bond to yield a glycol. This could then rearrange, perhaps with a spontaneous dechlorination to TCA. If this or a similar mechanism is responsible for the observed degradation of PCE, a buildup of toxic vinyl chloride should not be expected to occur during degradation of PCE.

A method to measure TCA in urine by dynamic headspace gas chromatography was described by Senft (1985). This method relies on conversion of TCA by heat to chloroform and CO_2 , followed by chromatographic determination of chloroform concentration. The application of this method to the determination of TCA concentrations in bacterial culture is described in the Methods section.

Preliminary analysis of samples suggested that after a sample of the culture that had been grown in medium with PCE was heated, the amount of chloroform increased, as would occur if the cells or medium contained TCA. Surprisingly, the amount of PCE observed in these samples also increased (data not shown). A further experiment was performed, in which the culture was centrifuged and chromatographic analysis was carried out on both the supernatant and the resuspended cells (Table 10). Both cells and medium appeared to contain significant amounts of chloroform even without heating to convert TCA. The heated supernatant showed an increase in the amounts of both chloroform and PCE, whereas the resuspended cells appeared to contain less precursor of chloroform (presumably TCA). In this experiment, relatively little PCE was observed in the heated cells, although the amount of PCE purged from the cells increased by a factor of 5 as it did in the unfractionated culture. These experiments suggest that the cells accumulate some amount of PCE which is held in a non-volatile form, perhaps as a lipid complex in the cell membrane or as an excreted lipid or lipid-protein complex, and that PCE may be metabolized to a variety of products, including chloroform and probably TCA.

TABLE 10.
DISTRIBUTION OF PCE AND CHCl₃ IN
A BACTERIAL CULTURE

CONDITION	PCE ppm	CHCl ₃ ug
Culture ¹ , unheated	14.2	1.14
Culture, 85° 150 min	77.2	0.99
Supernatant ²		
Unheated	0.3	1.12
Heated 85° 150 min	14.1	2.07
Cells resuspended		
Unheated	1.1	1.03
Heated 85° 150 min	5.5	1.27

¹A culture of *Bacillus* W8 growing in MSVT + 0.1% yeast extract and 0.1% 3-chlorobenzoate was diluted 50-fold into MSVT with 0.1% yeast extract and 100 ppm PCE. After two weeks of incubation at 30° C, a 200 ul sample of the culture was diluted with 2 ml H₂O and analyzed by dynamic headspace gas chromatography, with the attenuator set at 32.

²1 ml of culture was centrifuged to pellet the cells, and the cells were resuspended in 1 ml MSVT. Cells and supernatant were analyzed by dynamic headspace gas chromatography.

CONCLUSIONS

Monitoring wells in the Y-12 Plant in which PCE had been detected were screened to determine whether they contained bacteria resistant to the toxic effects of PCE and with the metabolic capability to degrade PCE and TCE. All of the wells surveyed contained some bacteria capable of growing in the presence of PCE. These bacteria were grown in mixed cultures in mineral salts media with various carbon sources, and several of them showed the ability to metabolize other chlorinated substrates. Most of the bacteria isolated in the presence of PCE had morphological characteristics indicating that they were probably of the genus Bacillus. One of these isolates was positively identified and named Bacillus W8. It was shown to be resistant to saturating concentrations of PCE, and measurements of PCE concentrations in culture media indicated that the organism is capable of degrading PCE.

Compounds formed by oxidation or irradiation of PCE were characterized by gas chromatography. These compounds had longer retention times than the chloroethylenes, implying that they were more polar, as would be expected of alcohol or diol addition products. None of these compounds were observed in cultures of PCE-degrading bacteria in the presence of PCE. However, accumulation of chloroform was observed and accumulation of small amounts of trichloroacetic acid was inferred, suggesting a degradative pathway that includes either dehalogenation followed by hydration/hydroxylation and rearrangement, or hydration/hydroxylation followed by rearrangement and elimination of a chloro group, yielding a precursor (perhaps a diol) for further degradation to trichloroacetic acid and chloroform.

We conclude that PCE-resistant organisms can be selected for in well water (and probably soils) in which the concentration of PCE is high, and that bacteria can be isolated from these sources which have the capability of degrading PCE and TCE. It should be possible to enhance the ability of these organisms to degrade halogenated solvents in situ by a variety of methodologies, including nutritional enhancement to increase the total number of bacteria, in vitro cultivation of solvent-degraders and their reinoculation into the contaminated wells, or use of the bacteria as biological sorbents or filters. There is no evidence that bacterial spores can degrade PCE, but it is clear that sporulating organisms, in particular Bacillus W8, can survive exposure to high concentrations of PCE and thus can maintain viability in groundwater or soil during periods of exposure to high solvent concentrations, so that resumption of growth and metabolism could occur after the solvents had dispersed to a sufficiently low concentration.

Detergents added to growth media increased the solubility of PCE and to some extent protected PCE-sensitive bacteria from the toxic effects of the solvent. Detergent addition did not appear to increase the degradation of PCE by bacteria in vitro.

When PCE was readily available in solution. However, it is possible that in situ, detergents might increase microbial degradation of PCE by enhancing the dissolution of PCE bound hydrophobically to soil particles, making the solvent more readily accessible to the bacteria.

RECOMMENDATIONS

On the basis of the observations described above, we propose that further studies should be done to maximize the capacity of appropriate microbes to degrade PCE and TCE:

1. Experiments to determine the identity of degradation products. This information is crucial to assessment of the effectiveness of biodegradative systems. To date, no direct degradation products have been identified after incubation of Bacillus WB with PCE, although the production of TCA was inferred from the appearance of chloroform in the growth medium after a PCE-containing culture was incubated and then heated. Possible mechanisms of degradation could include step-wise dehalogenation, reduction of the double bond, or hydrolytic or oxidative cleavage. Under the conditions used for the studies reported above, TCE and dichloroethylenes, products of step-wise dehalogenation, would have been detected if they had been produced. Additional studies should be carried out to determine whether tetrachloroethane or alcohol derivatives are formed by this organism upon incubation in the presence of PCE and TCE. Identification of degradation products would be facilitated by the use of radiolabeled PCE, if budgetary constraints so allow. The presence or absence of the potential degradation products listed above should be established by gas chromatographic measurements, but precise identification of products is likely to require mass spectroscopy as well.
2. Determination of the kinetics of degradation of PCE and TCE by Bacillus WB. The rates of degradation of both high and low concentrations of PCE and TCE by the organism should be determined to make estimates of bioremediation times feasible.
3. Determination of the minimal nutritional conditions required for PCE and TCE degradation. Studies should be done to identify possible inducers of PCE and TCE degradation. These studies would facilitate choice of remediation conditions, and would be essential to devising an effective treatment strategy.
4. Determination of nutritional conditions for development of adequate cell populations in situ. Many studies of biodegradation have indicated that organisms present in

nature can biodegrade contaminants by cometabolism, so providing adequate nutrients for their growth may be one of the most effective methods of enhancing biodegradation. Bacillus W8 has been shown to grow in mineral salts media with no added vitamins and citrate as the sole carbon source. Studies should be done to determine the minimal concentration requirements for phosphate and nitrogen sources, and whether acetate or other carbon sources will substitute for citrate. These studies would allow treatment conditions in situ to be optimized for growth of the biodegradative organisms.

5. Studies of sporulation. The formation of endospores is characteristic of the genus Bacillus. Because endospores are metabolically dormant, it is important to know whether conditions inhibiting sporulation can be applied to the treatment system. Nutritional conditions under which sporulation occurs or is inhibited should be determined for Bacillus W8.
6. Sorption of PCE and TCE to cells and spores of Bacillus W8. Elsewhere, we have described studies aimed at developing treatment systems for heavy metals which feature the reversible adsorption of metal ions to cells or spores. Similar studies should be done to determine whether PCE and TCE can sorb to or dissolve in the membranes of vegetative cells or in the outer coat of spores. These studies should include nutritional manipulation of the membrane fatty acid composition to increase solubility of PCE and TCE in the membrane. If these studies were successful, a method might be devised to remove PCE and TCE from contaminated water by sorption to microbial cells and regenerate the sorbent by stripping the PCE or TCE into a suitable solvent. Such a method would probably be quicker than biodegradation but would not be a method for destroying the pollutant.

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Kinetics of Conversion of TCA to Chloroform

Fig.1. Kinetics of conversion of TCA to chloroform. 50 ug of TCA in 2 ml H₂O were heated in a water bath at 85° in screw-cap tubes sealed with Teflon liners. At the indicated times tubes were removed and chilled in ice. They were analyzed by dynamic headspace gas chromatography, with an attenuator setting of 128. Symbols represent duplicate analyses.

Concentration Response of Conversion of TCA to Chloroform

Fig.2. Concentration response of conversion of TCA to chloroform. The indicated amounts of TCA were sealed in tubes with 2 ml H_2O and heated at $85^{\circ}C$ for 150 min. The tubes were chilled in ice and then analyzed by dynamic headspace chromatography, with an attenuator setting of 128. Symbols represent duplicate analyses.

Gas Chromatography of Chlorinated Ethylenes

Fig 3

PCE Addition Products

Analysis by GC

Retention Time (min)

Degradation of PCE by Bacillus W8 in MSVT

Fig.5. Cells of Bacillus W8 growing in sealed culture bottles in MSVT + 0.01% yeast extract + 0.1% 3-CBA and 100 ppm PCE were sampled at intervals and analyzed for PCE concentration as described in the text. Controls contained no cells or no PCE.

Degradation of PCE by Bacillus W8 with Added Carbon Sources

Fig. 6. Cells of Bacillus W8 growing in sealed culture bottles in SM, SM + 0.1% yeast extract or casamino acids, or tryptone (all with 100 ppm PCE) were sampled at intervals and analyzed for PCE concentration as described in the text.

Degradation of TCE by Bacillus W8 with Added Carbon Sources

Fig. 7. Cells of Bacillus W8 growing in sealed culture bottles in SM, SM + 0.1% yeast extract or casamino acids, or tryptone (all with 100 ppm TCE) were sampled at intervals and analyzed for PCE concentration as described in the text.