

Degradation of Pentachlorophenol by Anaerobic

Subsurface Microorganisms

by

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(ABSTRACT)

Microbial populations from subsurface soil collected from a hydrocarbon contaminated site and a pristine site with no history of contamination had the ability to degrade pentachlorophenol (PCP) in anaerobic enrichment cultures. Increasing concentrations of PCP in nitrate, sulfate and yeast extract-mineral salts media were used to acclimate the cultures. Nitrate enrichments, previously incubated in an anaerobic phenol-mineral salts medium, showed 23% degradation in medium containing $40 \mu\text{g ml}^{-1}$ PCP during a 32 d incubation period. Cultures not adapted to phenol degradation did not degrade PCP at concentrations over $20 \mu\text{g ml}^{-1}$. Enrichment cultures grown in the anaerobic yeast extract-mineral salts medium did not degrade PCP at concentrations over $20 \mu\text{g ml}^{-1}$ and phenol adaptation did not enhance PCP degradation. The sulfate reducing enrichment containing $1 \mu\text{g ml}^{-1}$ PCP showed 71.3% degradation after 32 d incubation. No degradation occurred at or above $5 \mu\text{g ml}^{-1}$ PCP. PCP intermediates, 2,4,6-trichlorophenol (TCP) and 3,4,5 TCP were found in the spent culture of the nitrate reducing enrichment. In the spent culture of the sulfate reducing enrichment, 3,4,5 TCP and 2,3,4,5-tetrachlorophenol were found. Attempts to obtain a pure culture of an anaerobic PCP degrading bacterium were unsuccessful.

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Introduction

Pentachlorophenol (PCP) is a broad spectrum biocide used commercially since 1936 (Ahlborg and Thunberg, 1980). In 1977, PCP was the second most heavily used pesticide in the U.S. (Conklin and Fox, 1978). The U.S. Environmental Protection Agency has placed PCP on the Priority Pollutant List because of its toxicity and extensive environmental contamination (Federal Register, 1978). The environmental chemistry of pentachlorophenol has been reviewed by Arsenault (1976), Alborg and Thunberg (1980), Crosby (1981), and Engelhardt *et al* (1986).

The potential of microorganisms to degrade PCP has been the subject of several investigations during the last several years. The capacity of microorganisms to degrade PCP has been established although it is less clear if that potential can be realized in the environment. The various studies on the subject are presented in the literature review section. At the start of this study, public attention was focused on the PCP contamination of subsurface and groundwater systems. Several independent groundwater studies have documented the presence of a significant microbial biomass in the subsurface, thereby destroying the myth that there were no microorganisms in subsurface soil (Ventullo and Larson, 1985; Webster *et al*, 1985; Wilson *et al*, 1983). However, there are significant differences in the species diversity between the microbiology of surface and subsurface soils. Therefore, this study was directed at the question, is there a PCP degrading microflora in subsurface habitats? The emphasis of this study was placed primarily on anaerobic PCP degrading

microorganisms because studies of aerobic aspects of the problem were proceeding at other laboratories. When this study was initiated, subsurface samples from pristine and contaminated habitats were used because the distribution of PCP degrading microorganisms in the subsurface habitat was unknown. The first objective of this work was to isolate the PCP degrading enrichment cultures from subsurface habitats. The second objective of this thesis was to isolate the PCP degrading members of those enrichment cultures in pure culture in order to initiate physiological and genetic studies of PCP degrading microorganisms.

Literature Review

Introduction

Pentachlorophenol (PCP) was the second most heavily used pesticide in the U.S. in 1977. Approximately 80% of the 23 million kg produced was used by the wood preserving industry (Cirelli, 1978). PCP prevents the degradation of wood by reducing the activity of bacteria, fungi and wood boring insects (Benevise and Beckman, 1967). Other uses of PCP include: fungicide or bactericide in the processing of cellulosic products, adhesives, leather, paints, rubber, and oil; mildew control in rug shampoos and textiles; mold and slime control in food manufacturing plants (Benevise and Beckman, 1967); pesticide use in pineapple and sugar cane fields; and molluscicide to control schistosomiasis (Ahlborg and Thunberg, 1980). Between 3 and 6 million people in the U.S. are exposed to PCP each year in their homes or places of work (Donnelly *et al* 1987). The Environmental Protection Agency has placed PCP on the Priority Pollutant List because of its toxicity and extensive environmental contamination (Federal Register, 1978). Because of these contamination problems, Japan and Sweden have restricted the use of PCP (Ahlborg and Thunberg, 1980).

PCP in the Environment

PCP is widespread in the environment as evidenced by its occurrence in water (Fountain *et al* 1976), sewage (Buhler *et al* 1973), soil, and food (Crosby, 1981). Bottom sediment and leaf litter in water systems may permit the accumulation of PCP in those habitats and this contamination may persist for over 2 years (Pierce and Victor, 1978). Even pristine mountain habitats (Crosby, 1981) and centuries old lake sediments (Salkinoja-Salonen, 1988) contain PCP. The ubiquitous distribution of PCP may be due to air transport and natural formation of PCP. The production of chlorinated phenols during fungal metabolism (Arsenault, 1976), the combustion of wood through forest fires (Salkinoja-Salonen, 1988), and the municipal chlorination of water (Arsenault, 1976) illustrate formation of chlorinated phenols in open ecosystems. PCP is persistent in the environment (Crawford and Mohn, 1985) and residues have been measured in some soils 12 months after initial exposure (Benevise and Beckman, 1967).

Toxicity of PCP

PCP is a potent uncoupler of oxidative phosphorylation (Weinbach, 1954). Other deleterious effects of PCP include: mutagenicity to *Saccharomyces cerevisiae* (Grisham, 1986); embryotoxicity to rats (Crosby, 1981); and disruption of membrane organization in mammalian cells leading to membrane lipid degradation by phospholipase C (Duxbury and Thompson, 1987). PCP acts as a parasite substrate in the ATP phosphoribosyltransferase reaction (Dall-Larsen *et al* 1976). Increased frequency of chromosomal damage has been detected in personnel in a pentachlorophenol plant (Schmid *et al* 1983). The minimal lethal human dose is 29 mg kg^{-1} PCP (Ahlborg and Thunberg, 1980). Four families became sick from drinking well water which contained $12.5 \text{ } \mu\text{g ml}^{-1}$ PCP (Benevise and Beckman, 1967). Fish are sensitive to PCP and many species die in water containing $0.6 \text{ } \mu\text{g ml}^{-1}$ PCP (Edgehill and Finn, 1983). Massive fish kills have occurred in streams below wood preservation treatment plants when PCP was spilled (Pierce *et al* 1977; Renberg *et al* 1983). Fish in slightly contaminated streams can bioaccumulate PCP 500-8000 times that of the surrounding water (Ahlborg and Thunberg, 1980).

The toxicity of PCP against bacteria can vary greatly. While some genera of *Salmonella* can grow in the presence of $1000 \mu\text{g ml}^{-1}$ PCP (Ruckdeschel *et al* 1987), methanogens are inhibited at concentrations of $0.2 \mu\text{g ml}^{-1}$ PCP (Guthrie *et al* 1984). Some gram positive aerobic bacteria and obligate anaerobic bacteria are more sensitive to PCP than some gram negative aerobic or facultative bacteria (Table 1).

PCP Degradation in Aerobic Soil and Aquatic Systems

PCP is mineralized in aerobic soils. Soils with a history of PCP exposure have a shorter lag period for PCP degradation than pristine soils (Kuwatsuka and Niki, 1976). The population of PCP degraders in PCP amended soils may be two logs greater than the population in non-amended soils (Watanabe, 1977). Increasing organic matter and moisture content increases the rate of PCP degradation in soil (Engelhardt *et al* 1986). In clay loam soil amended with 100 mg kg^{-1} PCP, Baker and Mayfield (1980) demonstrated 80% loss of PCP (20% loss in sterile controls) over 160 d of incubation.

The literature of PCP degradation in subsurface soils is more limited than surface soils but PCP degradation has been shown with subsurface material. Smith and Novak (1986) demonstrated PCP degradation in subsurface soil microcosms. PCP (15 ppm) was degraded in two different subsurface soil microcosms microcosms in 37 and 57 d respectively. Lee *et al* (1984) observed PCP degradation in subsurface microcosms from contaminated and uncontaminated sites. The available literature supports the hypothesis that the potential for PCP degradation is present in contaminated and uncontaminated soils from surface and subsurface sites.

PCP in groundwater may persist for a long period in that habitat (Valo *et al* 1985b). PCP has a strong affinity for colloids and its low solubility may restrict its movement in an aquifer (Johnson *et al* 1985). The low species diversity (Swindoll *et al* 1988), low temperatures, and low organic matter status of the groundwater ecosystem may reduce the capacity of subsurface microorganisms to decompose PCP *in situ*.

PCP can be decomposed in streams by photolysis and microbial degradation (Pignatello *et al* 1983). In a pristine artificial stream, PCP degradation was observed after a 3 week lag. The optimum pH for degradation was between 6.8 and 8.2 and the MPN of PCP degraders increased by 1 order of magnitude after acclimation. Over 35% of the PCP in an aerobic recirculating trickling filter containing bark, wood chips, and $53 \mu\text{g ml}^{-1}$ PCP was biodegraded (Valo *et al* 1985a). The addition of ammonium salts stimulated PCP degradation in this study. The optimum pH for PCP degradation was 6.4 to 7.2. Degradation was inhibited above a pH of 8.4. Liu *et al* (1981) added PCP adapted sludge to a cyclone fermenter containing a mineral salts medium with $2 \mu\text{g ml}^{-1}$ PCP. The half-life of PCP was 0.36 d under aerobic conditions and the addition of ammonium salts did not stimulate degradation.

PCP Degradation in Anaerobic Soil and Aquatic Systems

Anaerobic conditions may reduce the extent of PCP degradation in mixed culture systems unless there is a potential for anaerobic respiration. In the study of Baker and Mayfield (1980) anaerobic conditions reduced the amount of PCP loss to 7% (5% loss in sterile control) over 160 d. In a microcosm of the artificial pristine stream where aerobic PCP degradation was observed, PCP degradation was inhibited when the O_2 concentration was less than 0.3 to 0.7 mg l^{-1} (Brown *et al* 1986). And in a recirculating trickling filter - bark and wood chip system, PCP degradation ceased and the biomass died when the O_2 concentration was below 0.002% (Valo *et al* 1985a). The PCP half-life in a cyclone fermenter containing a mineral salts medium increased from 0.36 d to 192 d when conditions were changed from aerobic to anaerobic (Liu *et al* 1981).

Mikesell and Boyd (1986) were able to mineralize PCP in an anaerobic methanogenic sludge system. The major intermediates in degradation were 3,5-dichlorophenol (DCP) and 3,4,5-trichlorophenol (TCP). The PCP acclimated sludge in this study could degrade PCP at a concentration of $11 \mu\text{g ml}^{-1}$ PCP. Guthrie *et al* (1984) showed that methanogenesis in unacclimated sludge could be inhibited at $0.2 \mu\text{g ml}^{-1}$ PCP. Reductive dechlorination has been proposed as one of the major mechanisms for anaerobic PCP degradation (Ide *et al* 1972). The

ortho and *para* positions of PCP are more susceptible to reductive dechlorination (Kaufman, 1978). On the other hand, chlorobenzoates are *ortho* and *para* stable and reductive dechlorination is more probable at the *meta* position (DeWeerd *et al* 1986). The identification of intermediates is important in PCP degradation studies because some intermediates such as 3,4,5 TCP are generally more toxic towards microorganisms than PCP, while some intermediates such as 2,4,6 TCP are generally less toxic (Table 1).

Microbial population changes in marsh mud exposed to PCP were studied (Nelson *et al* 1984). At a concentration of 1000 mg kg⁻¹ PCP, total numbers of aerobic, nitrate reducing, and PCP-resistant bacteria increased. total numbers of anaerobic and sulfate reducing bacteria populations weren't affected. The sodium salt of PCP had a more pronounced effect than PCP on microbial populations possibly due to its increased solubility in water. At 100 mg kg⁻¹ PCP, only PCP-resistant bacteria were enhanced by the presence of PCP.

Microbial Degradation of PCP

Several genera of microorganisms capable of aerobic PCP degradation in pure culture have been identified (Engelhardt *et al* 1986). One strain of *Flavobacterium* was able to degrade PCP in a glutamate amended medium containing 600 µg ml⁻¹ PCP (Brown *et al* 1986). In medium containing glutamate and 53.3 µg ml⁻¹ PCP, the degradative pathway was determined to be PCP → tetrachlorohydroquinone (TCHQ) → trichlorohydroquinone → 2,6-dichlorohydroquinone (2,6 DCHQ) (Steiert and Crawford, 1986). The initial hydroxylation utilized ¹⁸O derived chiefly from H₂¹⁸O, but neither PCP nor TCHQ degraded under anaerobic conditions. Amendments of either glutamate or glucose greatly enhanced degradation, but collectively they repressed PCP degradation (Topp *et al* 1988). Several *Pseudomonas* spp. have been isolated which degrade PCP (Watanabe, 1973; Suzuki, 1977; Karns *et al* 1983; Rott *et al* 1979). The *Pseudomonas* sp. isolated by Watanabe degraded PCP in medium containing 40 µg ml⁻¹ PCP after an 8 d lag and degradation was inhibited when (NH₄)₂SO₄ was substituted for NaNO₃. PCP degradation by this bacterium was enhanced by yeast extract but repressed by glucose. PCP degradation was inhibited in medium

containing $200 \mu\text{g ml}^{-1}$ PCP. The *Pseudomonas* sp. isolated by Suzuki produced TCHQ and tetrachlorocatechol (TCC) as intermediates in degradation. An *Arthrobacter* sp. isolated by Stanlake and Finn (1982) achieved optimal growth in batch culture (pH 7.2) containing 10 to $135 \mu\text{g ml}^{-1}$ PCP as a sole carbon source and this culture degraded PCP up to a concentration of $300 \mu\text{g ml}^{-1}$ PCP. In a chemostat employing $915 \mu\text{g ml}^{-1}$ the *Arthrobacter* sp. utilized PCP as a sole carbon source (Edgehill and Finn, 1982). This strain was unable to metabolize phenol and benzoate as sole carbon sources. The length of the lag growth phase of this culture decreased when the pH was increased from 6.8 to 7.8. At pH 6.5, PCP degradation was obtained by this *Arthrobacter* sp. in media containing $50 \mu\text{g ml}^{-1}$ PCP but not $100 \mu\text{g ml}^{-1}$ PCP, but at pH 6.0, degradation did not occur in a medium containing $50 \mu\text{g ml}^{-1}$ PCP. Chu and Kirsch (1972) isolated a saprophytic coryneform (KC-3) similar to the *Arthrobacter* sp. isolated by Stanlake and Finn. KC-3 degraded PCP in a medium containing $26.6 \mu\text{g ml}^{-1}$ PCP as a sole carbon source and in trypticase soy broth medium containing $200 \mu\text{g ml}^{-1}$ PCP. KC-3 utilized 2,6 DCP and tri- and tetrachlorophenols with chlorine substituents in both the 2 and 6 positions (Chu and Kirsch, 1973). Phenol, mono- and dichlorophenols (2,6 DCP not included) did not support the growth of KC-3. PCP and 2,4,6 TCP were the only substrates that could induce the PCP degradative enzyme system of this culture. Intermediates observed in PCP degradation by KC-3 included TCHQ and 2,6 DCHQ (Engelhardt *et al* 1986). *Rhodococcus chlorophenolicus* isolated by Apajalahti *et al* (1986) could not tolerate concentrations of PCP above $10 \mu\text{g ml}^{-1}$ in liquid culture (Salkinoja-Salonen, 1988). TCHQ is the first intermediate in PCP degradation in the presence of O_2 . However the hydroxylation derives the ^{18}O from H_2^{18}O and this has important ecological ramifications for anaerobic PCP degradation (Apajalahti and Salkinoja-Salonen, 1987a). This strain degrades TCHQ anaerobically to trihydroxybenzene through two hydrolytic and three reductive steps (Apajalahti and Salkinoja-Salonen, 1987b). Trichlorotrihydroxybenzene is the first intermediate after TCHQ and trihydroxybenzene is mineralized to CO_2 in the presence of O_2 . The PCP as well as all tri- and tetrachlorophenols serve as inducers for the entire PCP degradative system. Toluene and benzene may be anaerobically degraded by a similar mechanism (Vogel and

Grbic-Galic, 1986). There are no reports in literature of anaerobic PCP degradation by microorganisms in pure culture

Several genera of fungi capable of PCP degradation have been identified. *Trametes versicolor*, a wood invading basidiomycete, secretes the enzyme laccase into medium which dechlorinates PCP (Engelhardt *et al* 1986) and *Trichoderma virgatum* methylates PCP thereby removing its uncoupling ability (Cserjesi and Johnson, 1971). *Coriolus versicolor* produces a laccase which oxidizes PCP to form quinones (Engelhardt *et al* 1986). Mileski *et al* (1988) isolated a strain of *Phanerochaete chrysosporium* capable of PCP degradation. Spores of this species were repressed when added to medium containing over $4 \mu\text{g ml}^{-1}$ PCP, however, mycelium remained viable when immersed in medium containing $500 \mu\text{g PCP ml}^{-1}$. The ligninase from this strain was purified and incubated in a solution of PCP. After ten minutes of incubation, 78% of the PCP was converted to TCHQ.

Use of Microorganisms for Soil Decontamination

R. chlorophenolicus has been used to decontaminate soil containing $10,000 \text{ mg kg}^{-1}$ PCP (Salkinoja-Salonen, 1988). This illustrates the problem of predicting *in situ* success of introduced organisms because this organism does not tolerate more than $10 \mu\text{g ml}^{-1}$ PCP in a medium under laboratory conditions. An inoculum of 10^6 cells of this actinomycete was required to achieve significant PCP degradation and the soil temperature must be above 8°C . *Flavobacterium* sp. have also been used for soil decontamination (Crawford and Mohn, 1985). Soil containing 298 mg kg^{-1} PCP required multiple inoculations of this bacterium and no degradation was observed at a concentration of 500 mg kg^{-1} PCP. After a 2 d lag period, an inoculum of 3.1×10^3 *Flavobacterium* cells mineralized PCP at an equal rate as an inoculum of $> 10^6$ cells in soil containing 100 mg kg^{-1} PCP. PCP degradation by this strain is inhibited below 12°C . An *Arthrobacter* sp. was also being used to decontaminate soils containing 200 mg kg^{-1} PCP (Edgehill and Finn, 1983).

Nitrate reducing bacteria have the ability to anaerobically degrade a number of aromatic compounds including *p*-cresol (Bossert *et al* 1986), phthalate, phenol, benzoate, hydroxybenzoate (Sleat and Robinson, 1984), *o*- fluorobenzoate (Schennen *et al* 1985) and *p*-fluorobenzoate (Taylor *et al* 1979). Sulfate reducing bacteria have the ability to degrade benzoate, phenylacetate, and hydroxybenzoate (Sleat and Robinson, 1984). It is desirable to isolate a microorganism in pure culture capable of anaerobic PCP degradation for possible use in decontamination of anaerobic soil and aquatic systems. Furthermore, more knowledge is needed about the physiology and genetics of such organisms under controlled laboratory conditions.

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**Degradation of Pentachlorophenol by Enrichment
Cultures of Ground Water Bacteria Including
Nitrate and Sulfate Reducing Bacteria**

Introduction

Groundwater contamination by pentachlorophenol (PCP) has become a serious environmental problem. There is a need to understand the potential of indigenous subsurface microorganisms to biodegrade PCP in both contaminated and pristine subsurface habitats. Microcosms of subsurface soil from both contaminated and pristine sites have demonstrated PCP degradation (Smith and Novak, 1986; Lee *et al* 1984) as well as other xenobiotic compounds (Sulflita and Miller, 1985; Swindoll *et al* 1988). However, PCP in subsurface habitats may persist for long periods of time (Valo *et al* 1985). The microbial groups responsible for PCP degradation need to be characterized and, if possible, isolated into pure culture.

PCP is wide spread in the environment as evidenced by its occurrence in soil, water, sewage, and food (Crosby, 1981). Phenolic compounds with a high degree of chlorination are generally persistent in the environment (Crawford and Mohn, 1985). PCP is mineralized in aerobic soils and aquatic systems through both oxidative and reductive pathways (Kaufman, 1978). In soils, increased organic matter and moisture content enhanced PCP degradation (Kuwatsuka and Niki, 1976). The population of PCP degraders in PCP amended soils may be two logs greater than in non-amended soil (Watanabe, 1977).

Anaerobic conditions may reduce the extent of PCP degradation in mixed culture systems unless there is a potential for anaerobic respiration (Kaufman, 1978). Flooded soils show a higher rate of PCP degradation but little mineralization (Kuwatsuka and Niki, 1976; Murthy *et al* 1979). PCP was completely mineralized in anaerobic sludge (Mikesell and Boyd, 1986). Chlorinated phenols were mineralized by microorganisms in microcosms of pristine, methanogenic aquifer material but tended to persist in non-methanogenic material from the same site (Sulflita and Miller, 1985). Reductive dechlorination has been proposed as one of the major mechanisms for anaerobic PCP degradation (Ide *et al* 1972).

Several genera of microorganisms capable of aerobic PCP degradation in pure cultures have been identified (Engelhardt *et al* 1986) and three of these strains have been applied in the bioremediation of PCP contaminated aerobic soils. *Rhodococcus chlorophenolicus* is capable of decontaminating soil containing 10,000 mg kg⁻¹ PCP (Salkinoja-Salonen, 1987). Strains of *Flavobacterium* (Crawford and Mohn, 1985) and *Arthrobacter* (Edgehill and Finn, 1983) have been used to decontaminate soils containing 298 and 182 mg kg⁻¹ PCP respectively. No microorganism capable of anaerobic PCP degradation in pure culture has been reported in the literature.

The low species diversity (Swindoll *et al* 1988), low organic matter, and low temperatures may reduce the capacity for subsurface microorganisms to decompose PCP *in situ*. The objectives of this research were to evaluate the potential for subsurface microorganisms from pristine and hydrocarbon contaminated sites to degrade PCP in anaerobic respiring enrichment media and isolate microorganisms capable of anaerobic PCP degradation.

Materials and Methods

Soil: Subsurface soils were collected from a Philadelphia gas refinery site at a depth of 15.5 m and a pristine site in Dumfries, Virginia at a depth of 31.6 m. The collection methods and soil characteristics have been previously described (Novak *et al* 1985). Surface soil was collected from the utility pole storage area on the Virginia Tech campus. Soil samples were refrigerated at 7° C until used.

Enrichment culture selection

Sample pretreatment: Soil was added to an anaerobic phenol-mineral salts medium containing 50 $\mu\text{g ml}^{-1}$ phenol and incubated for one year to acclimate the microorganisms to anaerobic phenol degradation. The phenol adapted Philadelphia and Virginia enrichment cultures were designated A and B respectively. Philadelphia subsurface soil which received no pretreatment was designated C. Virginia Tech surface soil was designated D. Philadelphia soil added to a tert-butyl alcohol-sulfate reducing enrichment was designated E. The tert-butyl alcohol-sulfate reducing enrichment contained (per liter distilled deionized H_2O): 20 mg disodium EDTA, 12 mg FeSO_4 , 200 mg MgSO_4 , 75 mg CaCl_2 , 1.0 g NaCl , 1.0 g $(\text{NH}_4)_2\text{SO}_4$, 1 mg thiamine HCl, and 1 mg biotin. After autoclaving the medium, 2 ml of sterile phosphate buffer was added aseptically. The phosphate buffer contained (per 200 ml distilled deionized H_2O): 1.6 g KH_2PO_4 and 2.4 g K_2HPO_4 . One ml of filter sterilized trace element solution was added aseptically and anaerobically to 1 liter of the above. The trace element solution contained (per liter distilled deionized H_2O): 50 g disodium EDTA, 22 g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 2.27 g CaCl_2 , 5.06 g $\text{MnCl}_2 \cdot \text{H}_2\text{O}$, 4.99 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 1.1 g $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$, 1.57 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, and 1.61 g $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$. These five samples were used as the inocula for the enrichment media described below.

Nitrate reducing enrichment medium (NR medium): Ten ml of medium (Jeter and Ingraham, 1981) containing 4 g l^{-1} yeast extract (Difco, Detroit, MI) and 5 $\mu\text{g ml}^{-1}$ PCP (Aldrich

Chemical Co.) was dispensed in screw-cap tubes with Teflon seals. The tubes were autoclaved and then stored in an anaerobic chamber (Coy Laboratories, Ann Arbor, MI) until anoxic as determined by a resazurin control. The pH of the uninoculated medium was 6.75. One ml of cultures A and B and 1 g of soil sample C were each inoculated into the tubes. Six serial transfers using a 0.5 ml inoculum were made during a total incubation period of 220 d. The PCP concentration in the enrichment series media was increased in the following manner for each serial transfer: 5 $\mu\text{g ml}^{-1}$, 5 $\mu\text{g ml}^{-1}$, 20 $\mu\text{g ml}^{-1}$, 40 $\mu\text{g ml}^{-1}$, 40 $\mu\text{g ml}^{-1}$, and 80 $\mu\text{g ml}^{-1}$. All cultures were incubated in the anaerobic chamber at room temperature (21 to 25°C) in the dark.

The second 40 $\mu\text{g ml}^{-1}$ PCP culture group was used as the inoculum for subsequent experiments. Each of the three samples (A, B, C) were inoculated into NR medium containing 40 $\mu\text{g ml}^{-1}$ PCP. The PCP concentration of the spent medium was measured after 4, 8, 16, and 32 d of incubation by high performance liquid chromatography (HPLC). Five replicate culture tubes were selected at random for analysis on each sample date and sacrificed. Two replicates of uninoculated medium served as a control at time 0 and on each sample date. The chloride ion concentration in the medium was measured after 40 d. The presence of nitrate was tested at the end of the incubation by method 2 of Smibert and Krieg (1981).

Five tubes NR medium supplemented with 400 $\mu\text{g ml}^{-1}$ phenol (to promote cometabolism) and 40 $\mu\text{g ml}^{-1}$ PCP were also inoculated. The PCP concentration was measured after 32 d of incubation by HPLC. Uninoculated medium served as a control.

To isolate nitrate reducing PCP degraders, culture samples were plated onto NR medium with 1.5% agar (Difco, Detroit, MI). Plating and incubation experiments were carried out in the anaerobic chamber. Isolates were picked and inoculated into NR medium containing 40 $\mu\text{g ml}^{-1}$ PCP. PCP was measured after 40 d of incubation by HPLC.

Yeast extract-Mineral Salts medium (YE medium): The YE medium contained (per liter distilled H_2O) 0.5 g K_2HPO_4 , 0.2 g KH_2PO_4 , 1.0 g NH_4NO_3 , 0.1 g FeSO_4 , 0.1 g MgSO_4 , and 5.0

g yeast extract. Ten ml of YE medium containing $5 \mu\text{g ml}^{-1}$ PCP was dispensed into capped test tubes. One half of the culture tubes were stored aerobically, while the remaining one half were placed in the anaerobic chamber to deoxygenate. One ml of cultures A and B and 1 g of soil samples C and D were each inoculated into the aerobic and anaerobic tubes. Four serial transfers using a 0.5 ml inoculum were made during a total incubation period of 140 d. The PCP concentration in the enrichment series medium was increased in the following manner for each serial transfer: $5 \mu\text{g ml}^{-1}$, $5 \mu\text{g ml}^{-1}$, $20 \mu\text{g ml}^{-1}$, and $20 \mu\text{g ml}^{-1}$. The last transfer culture groups (both aerobic and anaerobic) were screened for PCP loss after 26 d incubation by gas chromatography with electron capture detection (GC-EC). All cultures were incubated in the dark at room temperature (21 to 25°C).

The fourth anaerobic transfer cultures A, B and C were transferred again into enrichment medium containing $20 \mu\text{g ml}^{-1}$ PCP. This culture was used as an inoculum for subsequent experiments. Ten tubes of YE medium containing $40 \mu\text{g ml}^{-1}$ PCP in screw-cap tubes were inoculated. The PCP concentration of the spent medium was measured after 16 and 32 d of incubation by HPLC. Five replicate culture tubes were selected at random on each sample date and sacrificed. Uninoculated medium served as a control at time 0 and on each sample date. Five tubes of Y-MS medium supplemented with $400 \mu\text{g ml}^{-1}$ phenol (to promote cometabolism) and $40 \mu\text{g ml}^{-1}$ PCP was also inoculated and analyzed after 32 d incubation by HPLC. Uninoculated medium served as a control.

Methyloleptotroph enrichment medium (ME medium): Twenty ml of medium (Zhao and Hanson, 1984) containing $5 \mu\text{g ml}^{-1}$ PCP was dispensed into 70 ml serum vials stoppered with butyl rubber septum stoppers and autoclaved. The headspace of the vials was adjusted to 20% CH_4 , 3% O_2 and a balance of N_2 with sterile syringes. One ml of culture A and 1 g of soil sample C were each inoculated into the vials. Four serial transfers using a 0.5 ml inoculum were made during a total incubation period of 105 d. The PCP concentration in the enrichment series media was increased in the following manner for each serial transfer: $5 \mu\text{g ml}^{-1}$, $5 \mu\text{g ml}^{-1}$, $20 \mu\text{g ml}^{-1}$,

and $20 \mu\text{g ml}^{-1}$. The fourth transfer cultures of each sample were screened for PCP and CH_4 loss after 26 d incubation by GC-EC and GC with flame ionization detection (GC-FID) respectively. Incubations were in the dark at room temperature (21 to 25°C).

Sulfate reducer enrichment medium: Postgate's medium B (Postgate, 1979) was deoxygenated by boiling and purging with O_2 -free N_2 gas. The ascorbic acid, thioglycollic acid and PCP were added after cooling. Twenty ml of medium containing $1 \mu\text{g ml}^{-1}$ PCP was dispensed anaerobically into 70 ml serum vials, stoppered with butyl rubber septum stoppers and autoclaved. After the medium cooled, the headspace of the serum vials was adjusted to 20% H_2 , 79% N_2 and 1% CO_2 by use of sterile syringes and FeSO_4 was added from a sterile, anaerobic stock solution. The vials were inoculated with 1 ml of culture E. Five serial transfers using a 0.05 ml inoculum were made during a total incubation period of 567 d.

The last enrichment culture of the series was used as the inoculum for subsequent experiments. Twenty vials of Postgate's medium containing $1 \mu\text{g ml}^{-1}$ PCP were inoculated. The PCP of the spent medium was measured after 4, 8, 16, and 32 d of incubation by GC-EC. Five replicate vials were selected at random for analysis on each sample date and sacrificed. Uninoculated medium served as a control at time 0 and on each sample date. Ten vials supplemented with $100 \mu\text{g ml}^{-1}$ sodium benzoate (to promote cometabolism) and $1 \mu\text{g ml}^{-1}$ PCP and 10 vials supplemented with 1 mM Na_2MoO_4 and $1 \mu\text{g ml}^{-1}$ PCP were inoculated and analyzed at 16 and 32 d. Five vials were selected at random on each sample date and sacrificed. All cultures were incubated in the dark at room temperature (21 to 25°C).

The initial $1 \mu\text{g ml}^{-1}$ PCP enrichment was also serially transferred into medium containing $10 \mu\text{g ml}^{-1}$ PCP. This $10 \mu\text{g ml}^{-1}$ culture was the inoculum for 5 vials containing $5 \mu\text{g ml}^{-1}$, $10 \mu\text{g ml}^{-1}$, $15 \mu\text{g ml}^{-1}$, $20 \mu\text{g ml}^{-1}$, and $25 \mu\text{g ml}^{-1}$ PCP. PCP was measured in the final set after 32 and 67 d of incubation. Uninoculated medium served as a control.

To isolate sulfate reducing PCP degraders, 2 similar methods were used. One drop of culture was placed (under anaerobic and aseptic conditions) on the bottom of an anaerobic culture tube (Hungate, 1969) containing 20 ml of Postgate's medium E (Postgate, 1979). The tube was placed in a spindle and rotated. While rotating, an inoculating loop was used to streak the sample spirally up the agar inside of the tube. For the second method, 2 drops of culture were inoculated into the anaerobic culture tubes containing Postgate's medium E immediately after autoclaving the media when its temperature cooled to 45 to 50°C. The tubes were then spun until the agar solidified on the inner wall of the tube. After 5 to 10 d incubation, black colonies were selected and the isolation procedures were repeated.

Analytical

PCP analysis: All culture samples and uninoculated controls were acidified to pH 1.5 with 6N HCl and extracted three times with cyclohexane (Fisher Scientific). For HPLC analysis, a Varian 5000 equipped with a 15" MCH-5 reverse phase column was used. The mobile phase consisted of 75:25 acetonitrile:5% acetic acid. Detection was at 300 nm by a Varian 2050 UV detector. Chlorophenolic intermediates were identified at 280 nm using a 55:45 mobile phase ratio.

For GC analysis, culture extracts were derivatized with diazomethane and analyzed with a Microtek 220 GC equipped with a 6', 1/4" ID Supelcoport 3% SP-2100 80/100 mesh packed glass column with a ⁶³Ni electron-capture detector. The column, inlet and detector temperatures were 180°C, 230°C and 330°C respectively. N₂ was the carrier gas at 85 psi.

Chlorophenol standards to identify PCP intermediates were obtained from Aldrich (Milwaukee, Wis.) except 2,4,5- and 2,4,6-trichlorophenol (TCP) (Fluka; Buchs, Switzerland) and 2,3,5,6-tetrachlorophenol (TeCP) (Custom Chem lab; Riverside, CA).

Methane analysis: Methane was measured with a Varian 3700 GC equipped with a 6' Porapak Q 150-200 mesh packed glass column with a flame-ionization detector. The column,

injector and detector temperatures were 120°C, 120°C and 250°C respectively. N₂ was the carrier gas at 12 ml/min.

Chloride analysis: Spent cultures and uninoculated medium were filtered through a membrane filter (Gelman, GS-8; 0.45µ pore diameter). CuNO₃ (0.03g) was added to 4 ml of medium to complex NH₄⁺ and PO₄³⁻ ions to reduce electrode interference. Chloride was measured with a halide specific electrode (Orion model 96-17b) and quantified using standard solutions of NaCl (Fisher) prepared in our laboratory.

Results

The concentrations of PCP and several PCP degradation intermediates which inhibited cell growth of various bacteria are shown in Table 1. Gram positive aerobic or facultative bacteria and some anaerobic bacteria are generally more sensitive to PCP than gram negative aerobic bacteria. The concentration of PCP which inhibited PCP degradation in the enrichment cultures used in this study is also shown in Table 1. A summary of the samples used and enrichment media prefixes are described in Table 2. Enrichment cultures in the nitrate reducing, yeast extract-mineral salts, methylotrophic and sulfate reducing media were given the prefixes NR-, YE-, ME-, and SR- respectively.

NR enrichment cultures: The degradation of PCP by enrichment groundwater cultures of nitrate reducing bacteria growing in NR medium containing $40 \mu\text{g ml}^{-1}$ PCP is shown in Figure 1. There was a 23% loss of PCP in the NR-A and NR-B enrichment culture media during the 32 d incubation period as compared to the uninoculated control. HPLC Chromatograms illustrating PCP loss are shown in Figure 2. The final pH of the spent culture medium was 7.4. There was slightly more PCP degradation in the NR-C culture than there was PCP loss in the uninoculated medium. The NR-C culture demonstrated over 22.4% PCP degradation in NR medium containing $20 \mu\text{g ml}^{-1}$ PCP during the second transfer of the enrichment series after 26 d of incubation. Since the NR-A and NR-C cultures were derived from the same subsurface sample and their responses to different concentrations of PCP were different, the preliminary enrichment of the NR-A culture in an anaerobic phenol-mineral salts medium appeared to have a significant effect on the capacity of the microbial population in the culture to metabolize different concentrations of PCP.

The 14.3% loss of PCP from the uninoculated medium is thought to be PCP loss by volatilization during the experimentation. None of the cultures demonstrated microbial degradation at $80 \mu\text{g ml}^{-1}$ PCP.

Table 1. Minimum inhibitory concentrations (MIC) of PCP ($\mu\text{g ml}^{-1}$) and three degradative intermediates for selected bacteria.

	<u>PCP</u>	<u>3,5 DCP</u>	<u>3,4,5 TCP</u>	<u>2,3,4,5 TeCP</u>
Aerobic conditions¹				
<i>Pseudomonas putida</i>	532	10	12	232
<i>Salmonella typhimurium</i>	1065	82	99	116
<i>Escherichia coli</i>	266	10	6	29
<i>Bacillus subtilis</i>	33	10	12	14
<i>Staphylococcus aureus</i>	133	41	12	14
<i>Mycobacterium phlei</i>	17	10	3	4
<i>Streptomyces achromogenes</i>	17	5	0.8	0.9
Anaerobic conditions				
<i>Clostridium perfringens</i>	17	99	20	14
<i>Desulfovibrio gigas</i> ²	16			
<i>Desulfovibrio vulgaris</i> ³	50			
<i>Desulfovibrio desulfuricans</i> ⁴	35			
<i>Desulfotomaculum nigrificans</i> ⁵	5			
Methanogenic sludge ^{6, 7}	0.2 to 11			
Conditions of this study⁸				
NR-A, NR-B	80			
NR-C	40			
YE-A, YE-B, YE-C	40			
SR-E	5			

¹ Colony formation on Standard Agar I (Merck); Ruckdeschel *et al* (1987).

² Lactate-Sulfate medium; Barton *et al* (1983).

³ Growth in lactate-yeast extract-sulfate broth; Postgate (1979).

⁴ Microbistatic minimum concentration (MMC). Colony formation on meat extract powder, neutral meat peptone, D-glucose agar; Paulus and Genth (1983).

⁵ Growth in lactate-yeast extract-sulfate broth, Saleh *et al* (1964).

⁶ Guthrie *et al* (1984).

⁷ Mikesell and Boyd (1986).

⁸ Concentration that inhibited anaerobic degradation of PCP in enrichment culture.

Table 2. Sources and history of subsurface soil samples used in this study, and enrichment media used to grow enrichment cultures.

<u>Sample</u>	<u>Comments</u>
A	Subsurface soil sample collected from the Philadelphia refinery site with a history of hydrocarbon contamination incubated for one year in an anaerobic phenol-mineral salts medium.
B	Subsurface soil sample collected from a pristine site in Dumfries, Virginia and incubated for one year in an anaerobic phenol-mineral salts medium.
C	Subsurface soil collected from a Philadelphia refinery site with a history of hydrocarbon contamination. This sample is not adapted to anaerobic phenol degradation.
D	Surface soil collected from the utility pole storage area on the Virginia Tech campus. This soil did not receive any pretreatment and was used as a surface control.
E	Subsurface soil sample from the Philadelphia refinery site incubated one year in a tertiary butyl alcohol degrading microcosm.
Designated medium code	
NR-	Sample was enriched in nitrate reducing medium.
YE-	Sample was enriched in the yeast extract-mineral salts medium.
ME-	Sample was enriched in the methylotroph enrichment medium.
SR-	Sample was enriched in the Postgate's sulfate reducer enrichment medium.

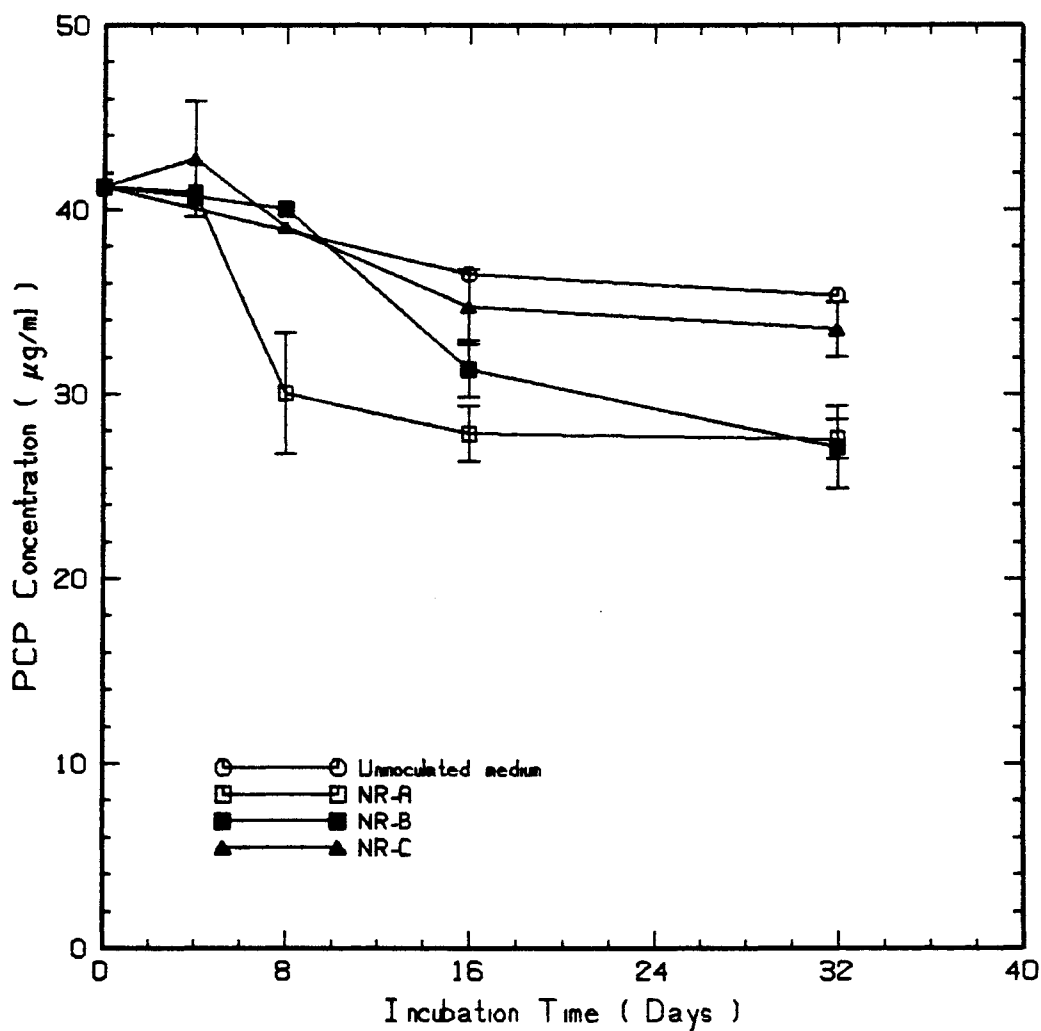


Figure 1. PCP degradation by subsurface enrichment cultures in a nitrate reducing medium after 32 days of incubation at 23°C. The cultures were incubated in an anaerobic chamber. The inoculum was acclimated to PCP by increasing the PCP concentration during six serial transfers. The A, B and C cultures were from contaminated (phenol acclimated), pristine (phenol acclimated), and contaminated sites. Error bars represent 1 standard deviation.

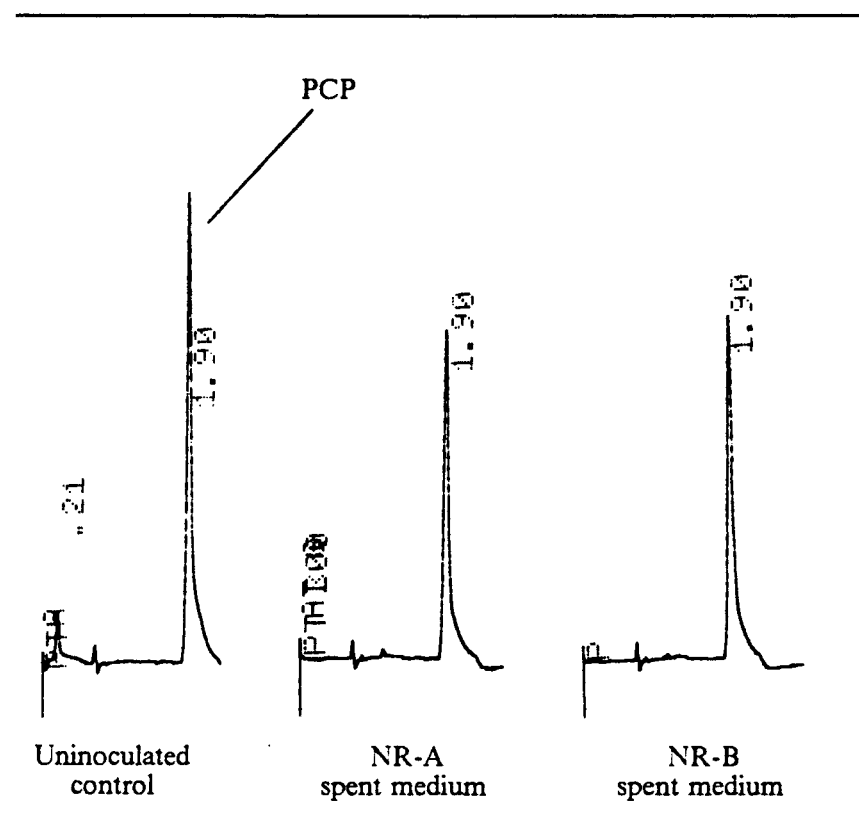


Figure 2. HPLC chromatograms of spent medium of subsurface enrichment cultures after 32 d of incubation in nitrate reducing medium containing $40 \mu\text{g ml}^{-1}$ PCP. The A culture was from the contaminated site, the B culture was from the pristine site.

A typical HPLC chromatogram used to measure PCP intermediates in the NR-A culture is shown in Figure 3. The appearance of the PCP degradation intermediates of 3,4,5-TCP and 2,4,6-TCP at concentrations of 3.1 and 0.6 $\mu\text{g ml}^{-1}$ respectively provides further evidence that PCP was degraded. These intermediates may have also contributed to a reduction in microbial activity during the latter part of the incubation period. The increase of the chloride ion concentration in the spent media (Table 3) represents 24 to 39% of the available chloride from PCP in the medium as compared to 23% PCP loss determined by HPLC analysis.

Cultures supplemented with 400 $\mu\text{g ml}^{-1}$ phenol demonstrated no visible cell turbidity nor PCP degradation. The subsurface microorganisms may have been sensitive to the high amount of phenol or a synergistic toxicity occurred that inhibited growth.

Based upon microscopic and colony observations, each of the enrichment cultures shown in Figure 1 are mixed cultures consisting of two or three different microorganisms. These enrichments were streaked on NR agar medium containing 40 $\mu\text{g ml}^{-1}$ PCP. Colonies which grew on this medium were transferred to NR medium containing 40 $\mu\text{g ml}^{-1}$ PCP. Nine isolates were obtained which grew in the NR medium that were resistant to the toxic effects of PCP. However, the isolates displayed neither nitrate reduction nor PCP degradation when inoculated into NR medium.

YE enrichment cultures: PCP degradation in the YE-A, YE-B, YE-C, and YE-D enrichment cultures containing 20 $\mu\text{g ml}^{-1}$ PCP is shown in Table 4. Substantial degradation was observed in all the enrichments with the exception of the aerobic YE-C culture. PCP degradation was greatest in the YE-D surface aerobic and anaerobic enrichments.

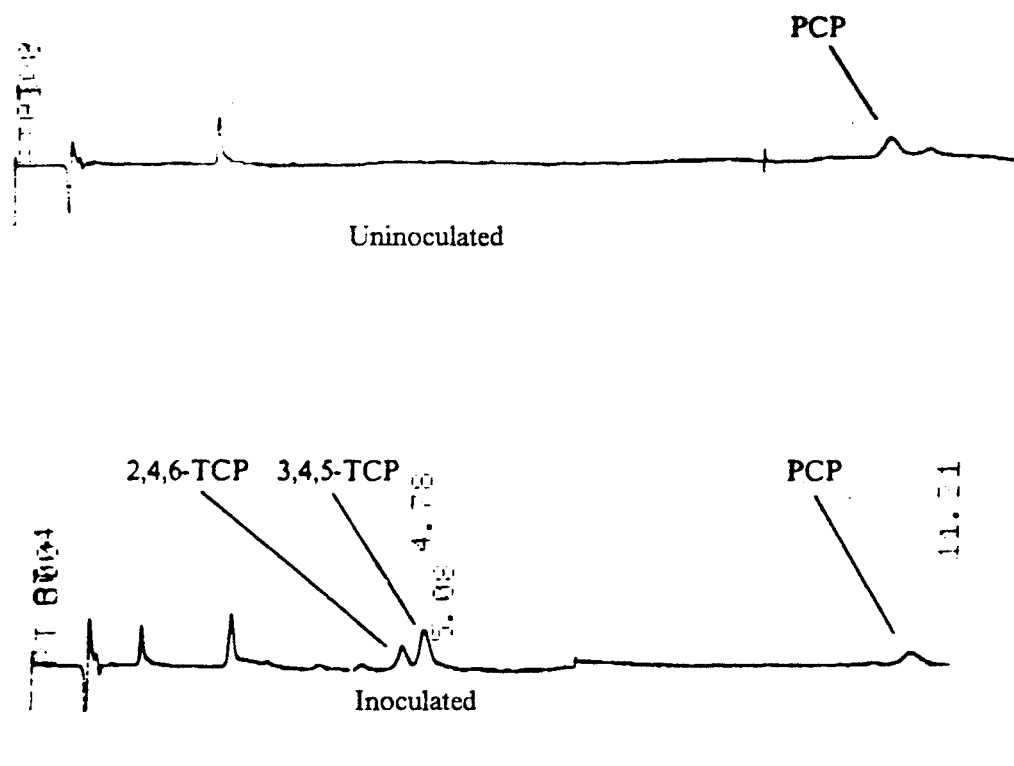


Figure 3. HPLC chromatograms showing PCP degradation intermediates in spent NR-A nitrate enrichment culture after 32 d of incubation at 23°C in an anaerobic glove box. The initial concentration of PCP was 40 $\mu\text{g ml}^{-1}$.

Table 3. The increase of chloride ion was measured in spent cultures of subsurface enrichment cultures in nitrate reducer medium after 40 d of incubation at 23°C. Chloride was measured by halide selective electrode and quantified by comparison to standard solutions of NaCl.

<u>Sample</u>	<u>Total Chloride (μM)</u>	<u>Accumulated Chloride (μM)¹</u>	<u>% of Total PCP Chloride</u>
Uninoculated	0.85 \pm 0.08		
NR-A	1.04 \pm 0.04	0.19	24.54
NR-B	1.15 \pm 0.10	0.30	38.74

¹Total chloride ion concentration in spent cultures subtracted from total chloride concentration in the uninoculated control.

Table 4. PCP degradation by subsurface enrichment cultures after 26 d of incubation at 23°C in a yeast extract- mineral salts medium containing 20 $\mu\text{g ml}^{-1}$ PCP. The cultures were acclimated to PCP by passing the inoculum through four serial transfers in medium with increasing PCP concentration. The anaerobic cultures were incubated in an anaerobic glove box.

<u>Sample</u>	<u>Aerobic</u>		<u>Anaerobic</u>	
	<u>PCP ($\mu\text{g ml}^{-1}$) remaining</u>	<u>% PCP loss</u>	<u>PCP ($\mu\text{g ml}^{-1}$) remaining</u>	<u>% PCP loss</u>
Uninoculated	20.5	---	19.9	---
YE-A	13.8	32.7	7.0	64.8
YE-B	9.7	52.7	12.5	37.2
YE-C	20.0	2.4	7.9	60.3
YE-D	2.2	89.3	4.6	76.9

Substantial anaerobic PCP degradation was observed in both subsurface soils, but the culture derived from the contaminated site was more active than the culture from the pristine site.

There was neither PCP degradation nor visible cell turbidity observed in the YE-A, YE-B, nor YE-C enrichment cultures in the anaerobic YE medium containing $40 \mu\text{g ml}^{-1}$ PCP nor in the $400 \mu\text{g ml}^{-1}$ phenol supplemented medium. These enrichment cultures were more sensitive to PCP than the NR enrichment cultures. These data are consistent with our hypotheses that (i) PCP can be degraded by anaerobic groundwater cultures and (ii) the extent of that degradation is affected by the microbial diversity of the original subsurface soil and the environmental conditions of the enrichment culture.

Methylobacterium enrichments: The ME-C enrichment culture did not oxidize any methane during the 26 d incubation period, although loss of PCP in the medium was 16.6%. The inoculum may have contained a low quantity of trace organic matter which permitted sufficient microbial metabolism to cause a minimal degradation of PCP. There was no PCP degradation nor methane utilization in the ME-B enrichment. In this preliminary experimentation, the incubation time in the methylobacterium study may have been too brief and the culture conditions too restrictive to permit the optimum development of methylobacteriums or methylobacteriums may have been absent. Further studies will be required to prove the involvement of methylobacteriums in PCP degradation in subsurface systems.

Sulfate reducer enrichment cultures: The degradation of PCP in an enrichment culture of sulfate reducing bacteria containing $1 \mu\text{g ml}^{-1}$ PCP is shown in Figure 4. During the 32 d incubation period, 71.4% of the PCP was degraded. PCP degradation intermediates 3,4,5 TCP and 2,3,4,5 TeCP were identified in the spent culture medium following 16 d of incubation.

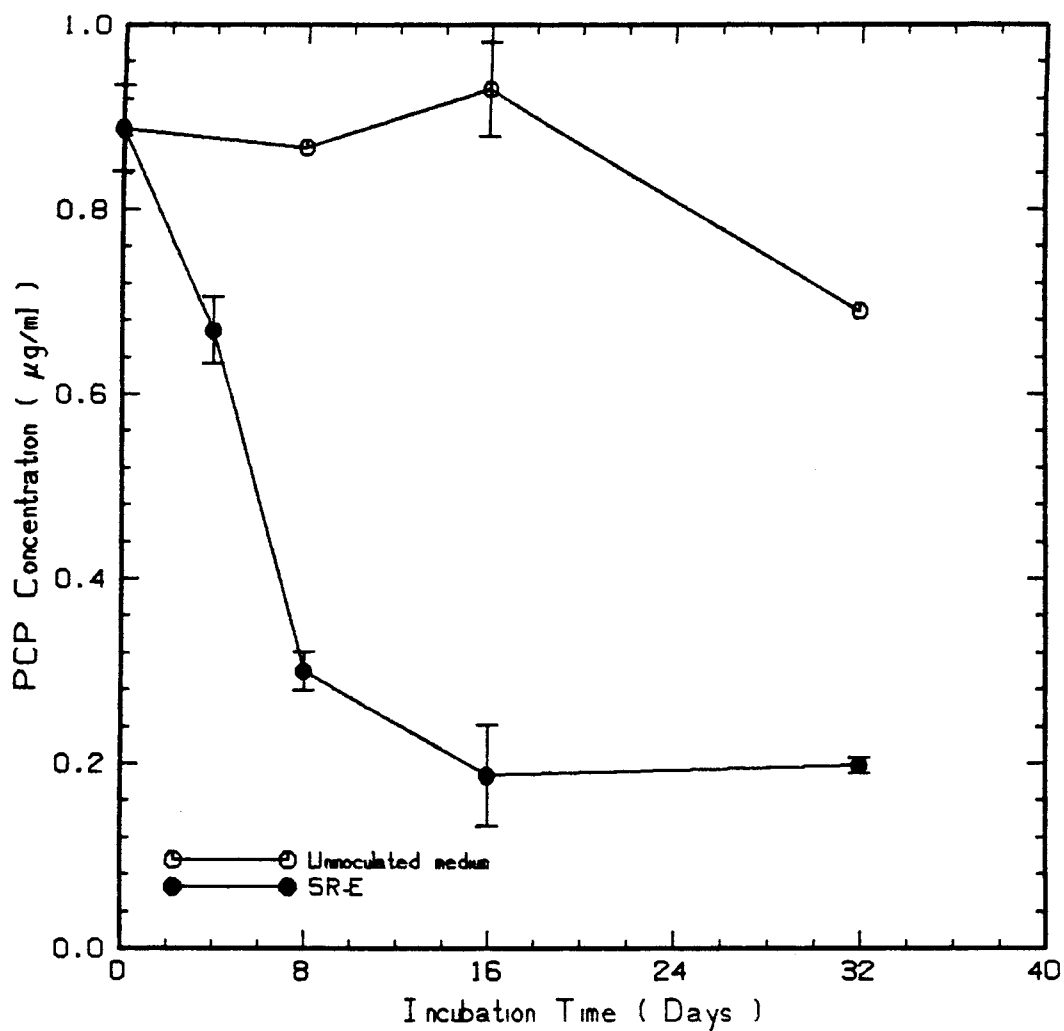


Figure 4. PCP degradation by a subsurface soil, sulfate reducing enrichment culture growing in Postgate's medium B at 23°C. Visible FeS production appeared at the 8th day of incubation. Error bars represent 1 standard deviation.

The incorporation of benzoate into the medium did not enhance PCP degradation. Rapid PCP degradation without an apparant lag occurred early in the incubation period. The onset of sulfide production after 8 d of incubation coincided with the cessation of PCP degradation. The addition of 1 mM Na_2MoO_4 inhibited sulfate reduction and a different intermediate, 2,3,6 TCP, was identified (Figure 5).

The addition of $1 \mu\text{g ml}^{-1}$ PCP to sulfate reducing medium increases the lag time for sulfide production from 4 d in PCP-free medium to 8 d in PCP-amended medium. Increasing the concentration of PCP to 5 to $25 \mu\text{g ml}^{-1}$ did not inhibit sulfide production. No PCP degradation was observed in medium containing 5 to $25 \mu\text{g ml}^{-1}$ PCP even after 67 d of incubation.

Throughout the isolation procedure, approximately 2 dozen black colonies were selected and restreaked or reinoculated into anaerobic culture tubes in succession. Non-sulfate reducing contaminants were always present. After approximately 3 successive transfers, only non-sulfate reducing bacteria were observed. The addition of niacin, biotin, thiamine, vitamin B_{12} , and trace elements to the isolation medium was not useful in obtaining an isolated sulfate reducer. Varying the PCP concentration in the range from 0 to $10 \mu\text{g ml}^{-1}$ in the agar did not aid culture isolation. Non-sulfate reducing isolates obtained from the anaerobic culture tubes did not degrade PCP when reinoculated into Postgate's medium B.

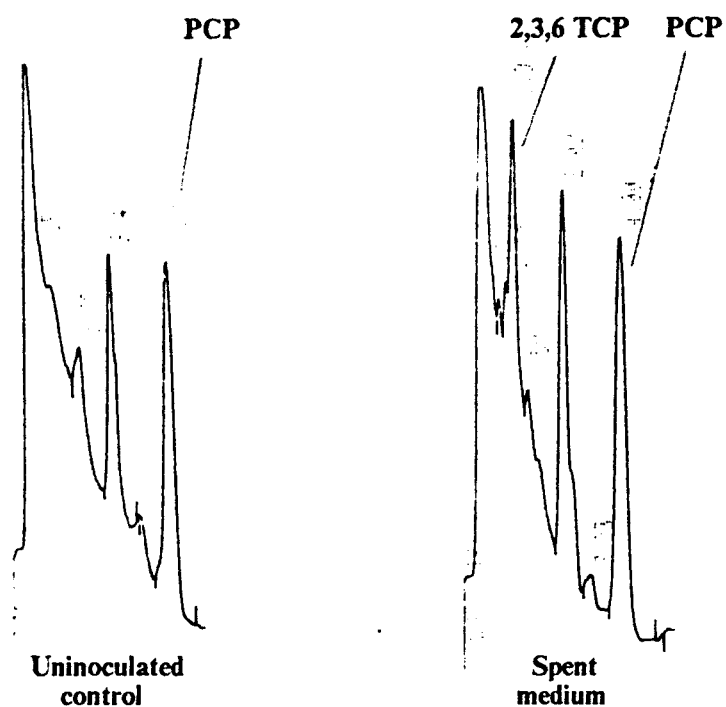


Figure 5. GC chromatograms of the sulfate reducing enrichment SR-E growing in Postgate's medium B supplemented with 1mM Na_2MoO_4 and $1 \mu\text{g ml}^{-1}$ PCP after 16 d of incubation.

Discussion

PCP can be degraded by subsurface microorganisms obtained from pristine and hydrocarbon contaminated sites in anaerobic enrichment cultures. Smith and Novak (1986) showed PCP degradation in microcosms of pristine subsurface soil. Although the environmental history of these subsurface soils was different, the microbial potential to degrade PCP was evident. Chlorinated phenols can be produced naturally through the combustion of wood and fungal metabolism (Arsenault, 1976). The exposure of the subsurface microflora to these chlorinated phenols may produce a natural enrichment of microorganisms with a potential to dechlorinate aromatic compounds. The examination of more subsurface soils is needed to confirm this hypothesis.

Under anaerobic conditions, PCP should theoretically be degraded more easily than under aerobic conditions since the increased degree of chlorination of the aromatic ring decreases the electron density of the aromatic nucleus and the electrophilic attack of oxygen is more difficult. A decreased electron density should enhance anaerobic enzymatic attack by a reductive (nucleophilic) mechanism (Sahm *et al* 1986). Fourty $\mu\text{g ml}^{-1}$ in the nitrate reducer enrichment is the highest concentration of PCP which PCP degrading microorganisms have been able to tolerate thus far reported in the literature. Preadaptation to anaerobic phenol degradation prior to the PCP enrichment phase enhanced the ability of the enrichment culture to degrade PCP at higher concentrations in the nitrate reducer medium. The nitrate reducer enrichment which was not adapted to anaerobic phenol degradation was able to degrade PCP at $20 \mu\text{g ml}^{-1}$ but not at $40 \mu\text{g ml}^{-1}$. Preadaptation to phenol has been shown by Shimp and Pfeander (1987) to enhance *p*-chlorophenol degradation by aquatic microorganisms.

Microorganisms capable of dissimilatory nitrate reduction have been shown to be effective in the dehalogenation of halogenated aromatic compounds (Schennen *et al* 1985; Taylor *et al* 1979). In this study, the nitrate reducing enrichment culture obtained was able to tolerate high PCP con-

centrations, possibly because some members in the mixed enrichment culture detoxified the PCP sufficiently to limit the toxic effects of PCP.

The extensive degradation of PCP by enrichment cultures under anaerobic conditions in the yeast extract medium is further evidence that several physiological groups of groundwater bacteria can degrade PCP. Substantially more degradation occurred in the surface soil control enrichments than in the subsurface soil enrichments. Lee *et al* (1984) showed PCP degradation in microcosms of contaminated and uncontaminated subsurface soil with surface soil as a control. Substantial degradation was shown in all microcosms with greater degradative activity in the surface soil. When Liu *et al* (1981) inoculated sludge adapted to PCP degradation into an anaerobic mineral salts medium containing $2 \mu\text{g ml}^{-1}$ PCP and no organic matter, the half-life for PCP was 192 d. Under aerobic conditions the half-life of PCP in this ecosystem was 0.32 d.

Although PCP is persistent in groundwater, microcosms consisting of subsurface soil and soil-free groundwater show substantial PCP degradation at ambient temperatures. In soil decontamination studies, Crawford and Mohn (1985) report inhibition of PCP degradation below 12°C and Valo and Salkinoja-Salonen (1986) reported inhibition below 8°C . PCP degradation by subsurface microorganisms *in situ* may possibly be restricted by the cool environment among other factors.

The methylotroph enrichment was attempted because of the capacity of this group to degrade recalcitrant organic compounds (Anthony, 1986). Methylotrophs capable of PCP degradation may be present in the soil samples investigated. The inability to provide proper culture conditions may have inhibited methylotroph growth.

One of the characteristics of the sulfate reducers is their sensitivity to PCP. Inhibition values range from 5 to $50 \mu\text{g ml}^{-1}$ PCP (Table 1). The sulfate reducing enrichment cultures in used this study produced sulfide in the presence of $25 \mu\text{g ml}^{-1}$ PCP following successive serial transfers and acclimation. However, the capacity of this enrichment culture to degrade PCP was restricted

to media which contained PCP concentrations below $5 \mu\text{g ml}^{-1}$ PCP. Gibson and Sulflita (1986) demonstrated that the presence of sulfate severely lowers the potential for microbial populations to degrade chlorinated phenols in anoxic environments. Whether sulfate or increased PCP concentrations inhibited PCP degradation in the sulfate reducing enrichment in this study is not known. The PCP degradation of an aerobic PCP degrading pseudomonad isolated by Watanbe (1973) was inhibited when NaNO_3 in the medium was replaced by $(\text{NH}_4)_2\text{SO}_4$.

The failure to isolate an anaerobic bacterium which can hydroxylate the aromatic ring of PCP may not be limited by the absence of oxygen per se. *Rhodococcus chlorophenolicus*, was able to *para*-hydroxylate PCP with an ^{18}O derived from H_2^{18}O (Apajalahti and Salkinoja-Salonen, 1987a), and that product, tetrachlorohydroquinone, was then degraded anaerobically to trihydroxybenzoate (Apajalahti and Salkinoja-Salonen, 1987b). In this study, attempts to isolate microorganisms in pure culture capable of anaerobic PCP degradation were not successful. Considering the sensitivity of anaerobes to PCP, the relatively high concentrations of PCP may have been toxic to the cultures. Also, the formation of toxic intermediates during PCP degradation in these products may have adversely affected microbial metabolism. Some PCP intermediates such as 3,4,5 TCP and 2,3,4,5 TeCP, which are more toxic than the parent compound, were detected in this study. The high concentrations of PCP used in this study may have selected for microbial consortia which can detoxify or degrade PCP in concert, but, no single member of the consortia can degrade PCP in pure culture at that PCP concentration. These data support the hypothesis that media with high concentrations of PCP are more likely to select microorganisms which are PCP tolerant than microorganisms that degrade PCP.

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Appendix A. Media components used throughout this study

Nitrate reducing enrichment medium: Three solutions are autoclaved separately.

Solution A: 0.87 g K_2HPO_4 , 0.54 g KH_2PO_4 , 5 g KNO_3 , 1.0 g $(\text{NH}_4)_2\text{SO}_4$, 4 g yeast extract, 5 to 80 mg PCP, and 980 ml distilled H_2O .

Solution B: 2.0 g MgSO_4 and 100 ml distilled H_2O .

Solution C: 0.2 g CaCl_2 , 0.1 g FeSO_4 , 0.05 g MnSO_4 , 0.01g Na_2MoO_4 , 0.01 g CuSO_4 , and 100 ml distilled H_2O .

Aseptically add 10 ml of solution B and 10 ml of solution C to 980 ml of solution A.

Sulfate reducing enrichment medium: 0.5 g KH_2PO_4 , 1.0 g NH_4Cl , 1.0 g CaSO_4 , 2.0 g MgSO_4 , 3.5 g sodium lactate, 1.0 g yeast extract, and 1 liter distilled H_2O .

Boil the mixture while purging with O_2 -free N_2 gas. After cooling add: 0.1 g ascorbic acid, 0.1 g thioglycollate, and desired concentration of PCP. Dispense 20 ml of media into 70 ml serum vials, stopper with septum stoppers and autoclave. Add 0.1 ml FeSO_4 from an anaerobic, sterile stock solution (0.1 g ml^{-1}) for a final concentration of 0.5 g l^{-1} FeSO_4 .

Methylotroph enrichment medium: 1.0 g KNO_3 , 0.26 g KH_2PO_4 , 0.74 g $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 1.0 g MgSO_4 , 0.2 g CaCl_2 , 4.0 mg FeSO_4 , 4 mg CuSO_4 , 0.3 mg MnSO_4 , 0.34 mg ZnSO_4 , 0.24 mg Na_2MoO_4 , 0.01 g EDTA, 0.5 g NaCl , 5 to 20mg PCP, and 1 liter distilled H_2O .

Appendix B. Sulfate reduction by enrichment culture from sample E in Widdel & Pfennig's sulfate reducer medium with alternative carbon substrates

To determine a preferential medium for the growth of culture E, the tert-butyl alcohol enrichment was used as an inoculum in Widdel and Pfennig's medium with various carbon substrates. The results are listed in Table 1a. Sulfate reduction was determined by visual production of FeS.

A defined medium was desired as an alternative to Postgate's medium B which contains a substantial amount of yeast extract. Yeast extract interferes with HPLC analysis of PCP and extraction is required for accurate quantification. Culture samples grown in Widdel and Pfennig's medium can be injected directly. PCP can be quantified accurately by HPLC-UV analysis at a concentration of $5 \mu\text{g ml}^{-1}$ PCP.

Table 1a. Sulfate reduction in Widdel and Pfennig's medium¹ containing various carbon substrates and inoculated with sample E.

Acetate	+	Lactate	+
Benzoate	-	Malate	-
Dextrose	-	Pyruvate	-
Formate	+		

¹ Krieg, N.R. 1981. Enrichment and Isolation. *In* P. Gearhardt *et al* (eds), *Manual of Methods for General Bacteriology*. ASM. Wash. p.139.

Appendix C. Inhibition of sulfate reduction in SR-E cultures by PCP

SR-E was inoculated into Postgate's medium B containing different concentrations of PCP to determine the sensitivity of subsurface strains of sulfate reducing bacteria to PCP (Table 2a).

The sulfate reducing enrichment used in this study ultimately showed sulfide production in media containing $25 \mu\text{g ml}^{-1}$ PCP but with no degradation. This acclimation may have been due to tolerance of the sulfate reducer. However, the extreme sensitivity of the two-member consortium gives evidence that selection of other members in the culture may be responsible for protecting the sulfate reducing member of the population.

Table 2a. Inhibition of sulfate reduction in sulfate reducer enrichment cultures of SR-E by PCP (lag of sulfate reduction in d).

Inoculum	PCP ($\mu\text{g ml}^{-1}$)									
	0	0.2	0.5	0.8	1.0	2.0	3.0	5.0	7.0	10.0
SR-E not acclimated to PCP ¹	5	5	5	43	44	--	--	NR	NR	NR
SR-E acclimated to PCP ²	4	4	7	7	7	8	--	26	26	26
Two member consortium from SR-E ³	--	--	4	--	5	8	25	NR	--	--
Pandapas Pond Isolate ⁴	--	--	3	--	3	3	3	3	5	20

¹ An anaerobic microcosm of Philadelphia subsurface soil containing tertiary butyl alcohol which showed sulfide production after 3 mo of incubation was used as the inoculum.

² The unacclimated culture which showed sulfide production after 44 d of incubation in medium containing $1 \mu\text{g ml}^{-1}$ PCP was used as the inoculum for the PCP acclimated treatment.

³ The sulfate reducer and a contaminant were isolated from acclimated SR-E cultures isolated in anaerobic culture tubes.

⁴ A sulfate reducing bacterium isolated from the sediment of Pandapas Pond, Montgomery County, VA.

NR-no sulfide production observed.

Appendix D. Volatilization of PCP from various culture chambers

An experiment was conducted to determine which culture chamber would allow the least volatilization of PCP during culture incubation. The test medium contained: 0.3 g K_2HPO_4 , 0.2 g KH_2PO_4 , 0.5 g KNO_3 , 0.4 g NH_4SO_4 , 0.01 g $FeSO_4$, 300 ml distilled H_2O , and 12 mg PCP ($40 \mu g \text{ ml}^{-1}$). The pH of the medium was 7.2. The four types of media chambers were: Hungate tubes (anaerobic culture tubes) with butyl rubber stoppers (40 ml capacity), screw-cap tubes with Teflon seal caps (25 ml capacity), serum vials with septum stoppers (70 ml capacity), and test tubes with Kaputs (25 ml capacity). The Hungate tubes and serum vials contained 20 ml of medium while the screw-top tubes and test tubes contained 10 ml of medium. The PCP was measured in the medium before and after autoclaving in each container. There was no loss of PCP in the medium during autoclaving. The PCP in the test medium was measured after 13 d of incubation at 23°C. All tests were in duplicate. PCP was measured by HPLC analysis. The results are listed in Table 3a.

Table 3a. Recovery of PCP from different types of culture containers containing a mineral salts-PCP ($40 \mu\text{g ml}^{-1}$) medium, after 13 d of incubation. The medium pH was 7.2. HPLC analysis was used. Values are average of 2 replicates. The PCP in the control medium was measured immediately after autoclaving.

<u>Type of medium container</u>	<u>Change in PCP concentration (%)</u>
Hungate tubes	-2.39
Hungate tubes incubated upside down	-47.49
Screw top tubes	+ 1.63
Screw top tubes incubated upside down	-7.89
Serum vials	-11.85
Serum vials incubated upside down	-73.53
Culture tubes w/Kaputs	-0.44

Appendix E. Chloride ion concentration standard curve

To quantify the concentration of free chloride ion in the nitrate reducer enrichment culture media, a standard curve was developed using known concentrations of NaCl and measuring the electrode response with a halide selective electrode. (Figure 1a). The chloride ion concentrations of the spent culture media were calculated from the measured electrode responses of those cultures.

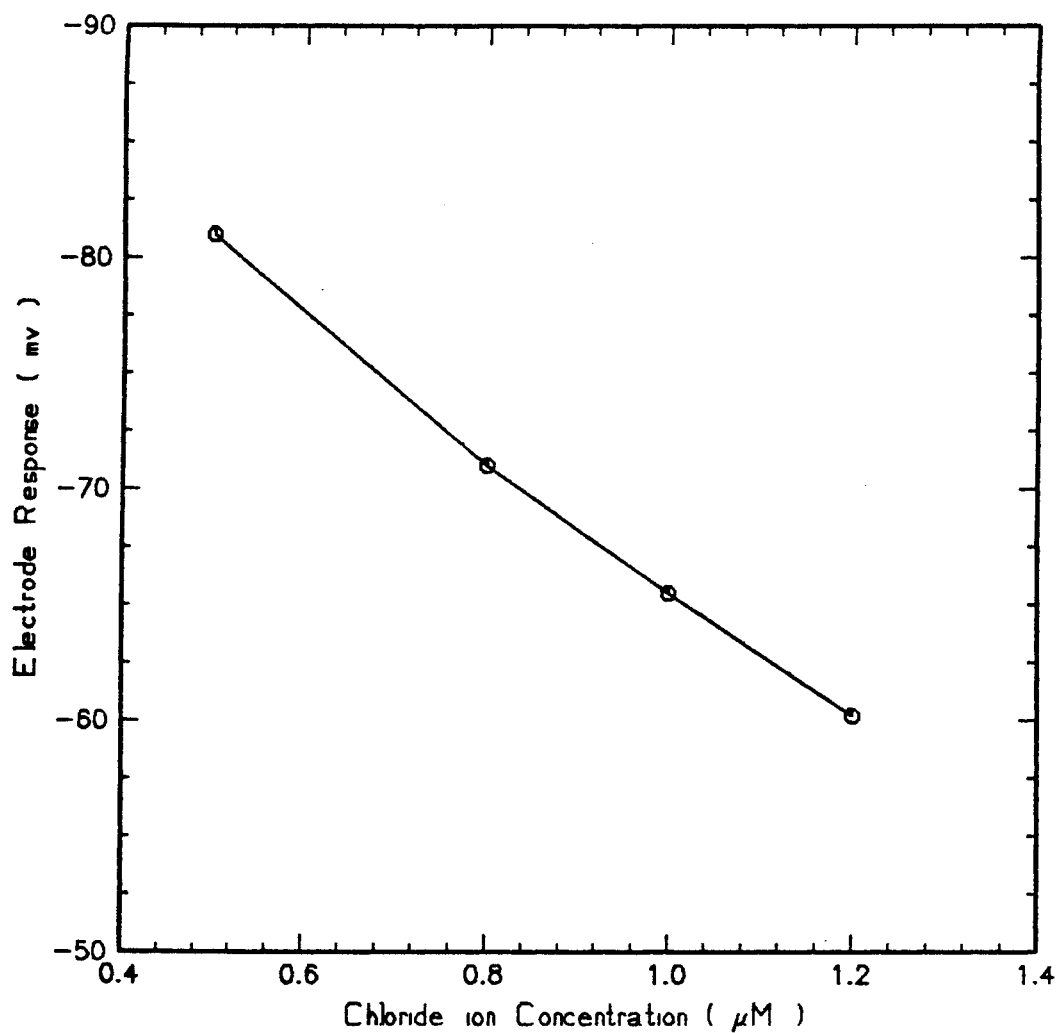


Figure 1a. Chloride ion concentration standard curve. An Orion halide selective electrode was used as described in the text to measure the electrode responses of known concentrations of NaCl.

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