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Degradation of 2,3,4,6-tetrachlorophenol at low temperature and low dioxygen concentrations by phylogenetically different groundwater and bioreactor bacteria

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Abstract

Effects of low temperature and low oxygen partial pressure on the occurrence and activity of 2,3,4,6-tetrachlorophenol degrading bacteria in a boreal chlorophenol contaminated groundwater and a full-scale fluidized-bed bioreactor were studied using four polychlorophenol degrading bacterial isolates of different phylogenetic backgrounds. These included an α-proteobacterial *Sphingomonas* sp. strain MT1 isolated from the full-scale bioreactor and three isolates from the contaminated groundwater which were identified as β-proteobacterial *Herbaspirillum* sp. K1, a Gram-positive bacterium with high G + C content *Nocardioides* sp. K44 and an α-proteobacterial *Sphingomonas* sp. K74. The *Sphingomonas* strains K74 and MT1 and *Nocardioides* sp. K44 degraded 2,4,6-trichlorophenol and 2,3,4,6-tetrachlorophenol as the sole carbon and energy sources. Close to stoichiometric inorganic chloride release with the 2,3,4,6-tetrachlorophenol removal and the absence of methylation products indicated mineralization. Tetrachlorophenol degradation by the *Herbaspirillum* sp. K1 was enhanced by yeast extract, malate, glutamate, pyruvate, peptone and casitone. At 8 °C, *Sphingomonas* sp. K74 had the highest specific degradation rate ($\mu_{max} = 4.9 \times 10^{-12}$ mg h^{-1} $cell^{-1}$) for 2,3,4,6-tetrachlorophenol. The *Nocardioides* strain K44 had the highest affinity ($K_s = 0.46$ mg l^{-1}) for tetrachlorophenol. K1 and MT1 grew microaerophilically in semisolid glucose medium. Furthermore, the growth of MT1 was inhibited in liquid glucose medium at high oxygen partial pressure indicating sensitivity to accumulating toxic oxygen species. On the other hand, trichlorophenol degradation was not affected by oxygen concentration (2–21%). The isolates K44, K74 and MT1, with optimum growth temperatures between 23 and 25 °C, degraded tetrachlorophenol faster at 8 °C than at room temperature indicating distinctly different temperature optima for chlorophenol degradation and growth on complex media. These results show efficient polychlorophenol degradation by the isolates at the boreal groundwater conditions, i.e., at low temperature and low oxygen concentrations. Differences in chlorophenol degradation and sensitivities to chlorophenols and oxygen among the isolates indicate that the phylogenetically different chlorophenol degraders have found different niches in the contaminated groundwater and thus potential for contaminant degradation under a variety of saturated subsurface conditions.

Introduction

The use of a technical chlorophenol (CP) mixture of 2,4,6-trichlorophenol (TCP), 2,3,4,6-tetrachlorophenol (TeCP) and pentachlorophenol (PCP) at hundreds of sawmills has led to several extensive groundwater contaminations (Kitunen et al. 1987). Large-scale contamination by CPs was discovered in 1987 at Kärkölä groundwater aquifer, in Southern Finland (Lampi et al. 1990). In addition to high CP concentration, the groundwater in Kärkölä is characterized by low dissolved oxygen (typically < 1 mg l^{-1}) and high ferrous

iron concentrations (6–10 mg l^{-1}). Since 1995 an aerobic full-scale fluidized-bed bioremediation system has treated the contaminated groundwater (Puhakka & Melin 1998) and the CP concentrations have levelled to approximately 10 mg l^{-1} in the groundwater.

The ability to degrade polychlorophenols is common and widely distributed among different genera in the Kärkölä groundwater (Männistö et al. 1999, 2001). Polychlorophenol-degraders from the contaminated groundwater belonged to α,β,γ-Proteobacteria, *Cytophaga/Flexibacter/Bacteroides* branch and Gram-positives with high G + C content (Männistö et al. 1999). In contrast to the groundwater, the full-scale bioreactor has a very low species diversity as analysed by DGGE, LH-PCR and RFLP of the 16S rDNA (Tiirola et al. manuscript in preparation). The dominant chlorophenol degrader in the bioreactor was isolated and designated *Sphingomonas* sp. MT1 (Tiirola et al. manuscript in preparation). Polychlorophenol degradation at low temperatures has mainly been studied using mesophilic organisms (for review, see Puhakka & Melin 1998) and a strong decrease in degradation rates at low temperatures has been reported (e.g., Melin et al. 1998; Wittmann et al. 1998).

In this research, 2,3,4,6-TeCP degradation was studied using four psychrotolerant isolates from the groundwater and the bioreactor. Polychlorophenol degraders from alpha and beta *Proteobacteria* and a Gram-positive bacterium with high G + C content were selected to investigate the impact of phylogenetic diversity on chlorophenol degradation in the environment. Tetrachlorophenol degradation kinetics were studied at ambient groundwater temperature (8 °C) and rates of degradation were compared to those at room temperature. Growth and chlorophenol degradation was studied at various oxygen concentrations to better understand the effect of oxygen on the activity of the groundwater and bioreactor bacteria. The effect of supplementary organic and inorganic compounds on CP degradation were additionally investigated using the β-proteobacterial and Gram-positive isolates.

Materials and methods

Organisms and culture conditions

The bacterial strains used in this study were as listed in Table 1. Three of the strains, K1, K44 and K74 originated from contaminated groundwater containing approximately 10 mg l^{-1} of CPs (75% 2,3,4,6TeCP, 15% 2,4,6-TCP and 10% PCP). The isolation, whole cell fatty acid compositions and partial 16S rDNA sequences of these isolates have been described by Männistö et al. (1999). The strain MT1 was isolated from the effluent of the full-scale fluidized-bed bioreactor which treats the contaminated groundwater. MT1 represents the dominant species in the bioreactor and is a member of the genus *Sphingomonas*, as characterized by Tiirola et al. (manuscript in preparation). The studied isolates were psychrotolerant growing at temperatures between 2 and 30–33 °C with optimum growth between 23 and 26 °C (Männistö & Puhakka 2001).

The bacterial strains were maintained on R2A agar (BBL, Becton Dickinson, Cockeysville, Md., USA) or peptone yeast extract glucose medium (PYGV) containing 0.05% w/v of each and 5 ml l^{-1} of vitamin solution (Staley 1968). For the experiments, the strains were grown in liquid PYGV medium on a gyratory shaker (100–150 rpm) at 8 °C or room temperature (23 ± 2 °C). Prior to CP degradation experiments the cells were induced by addition of 2,3,4,6-TeCP (3–20 mg l^{-1}) twice at 24 h intervals.

Chemicals

Degradation of 2,3,4,6-TeCP was assayed using the highest purity product available (>99%, Riedel de Häen, Germany). For induction of TeCP degradation a technical grade TeCP was used (80% pure with 20% PCP; Tokyo Kasei Kogyo, Tokyo, Japan). 2,4,6-TCP (99% purity) was purchased from Fluka Chemie (Buchs, Switzerland). All other reagents were of the highest purity available.

Identification of the strains

Partial 16S rRNA sequences (~440 bp) for the isolates K1, K74 and K44 and MT1 were obtained earlier (Männistö et al. 1999; Tiirola et al. manuscript in preparation). In this study, nearly complete 16S rDNA genes of the strains were sequenced to confirm the identification. Bacterial DNA was extracted after lysis with proteinase K and purified with phenol-chloroform extractions and isopropanol precipitation as described by Wilson (1990), except no CTAB (hexadecyltrimethyl ammonium bromide) precipitation was used. PCR-amplified fragments (fragments corresponding the *Escherichia coli* numbering positions 319–1058 and 968–1541) were cloned to pGEM-T-vector (Promega, Madison, WI) and analyzed by

Table 1. Identification of the bacterial strains and kinetic parameters of 2,3,4,6-TeCP degradation by isolates K44, K74 and MT1

Isolate	Source	Identification	μ_{max} 10^{-12} mg h^{-1} $cell^{-1}$	K_s mg l^{-1}	K_i mg l^{-1}	r^2
K1	Groundwater	*Herbaspirillum* sp.	ND	ND	ND	
K44	Groundwater	*Nocardioides* sp.	0.19	0.5	5.1	0.97
K74	Groundwater	*Sphingomonas* sp.	4.9	2.4	0	0.9
MT1	Bioreactor	*Sphingomonas* sp.	0.52	2.1	0	0.93

ND, not determined.

bidirectional sequencing using LI-COR DNA4200 sequencer (LI-COR, Lincoln, NE) with universal sequencing primers SP6 and T7. 16S rDNA sequences were compared to sequences in GenBank database using the BLAST program (Altschul et al. 1997). Sequences were deposited in the GenBank database with accession numbers AJ009709 (K74), AJ001384 (K1), AJ009714 (K44) and AJ303009 (MT1).

Chlorophenol degradation

Rates of CP degradation were studied at ambient groundwater and bioreactor conditions using autoclaved effluent from the bioreactor as the basal medium. The effluent DOC, PO_4^{3-} and NO_3^- concentrations were 5.2–7.1 mg l^{-1} (Langwaldt et al. 2000), 0.6–5.5 mg l^{-1} and 2.4–4.8 mg l^{-1}, respectively. TeCP degradation was studied at 8 (±1) °C and at room temperature (23 ± 2 °C). The cells were grown at 8 °C or room temperature for at least 4 generations prior to the tests. Kinetics of 2,3,4,6-TeCP degradation was studied in 8 ml screw cap vials with a liquid volume of 2 ml. PYGV grown cells were centrifuged (8000 g/20 min) and washed once with phosphate buffered saline. The cells (approximately 10^9 cells ml^{-1}) were suspended in autoclaved effluent water from the bioreactor and CPs were added in to the suspension. Two ml of this suspension was pipetted to each of 25–30 vials and the vials were incubated in a gyratory shaker (150 rpm). At 5–90 min intervals, duplicate vials were removed from the shaker, the reaction was stopped by addition of formaldehyde (2%) and the whole content of the vial was sacrificed for CP analysis. Inorganic chloride releases were measured after 5 day incubation with 5 mg l^{-1} (strain K44) or 13 mg l^{-1} (strains K1, K74 and MT1) of TeCP and used as an indication of chlorophenol mineralization (Järvinen et al. 1994).

Modelling TeCP degradation

The maximum TeCP degradation rate was calculated from the linear part of the degradation curve and the modified Haldane equation was used to calculate the kinetic parameters:

$$\mu = \frac{\mu_{max}}{1 + \frac{K_s}{S} + \left(\frac{S}{K_1}\right)^n}, \quad (1)$$

where μ is the substrate removal rate, μ_{max} is maximum specific substrate removal rate, K_s is half-saturation constant, K_i is inhibition constant and n is inhibition response coefficient. When no inhibition was observed the Michaelis–Menten equation was used. Model parameters (μ_{max}, K_s, K_i, n) were determined by non-linear curve fitting by minimizing the sum of residual squares.

Effect of oxygen on growth and chlorophenol degradation

Microaerophilic growth was assayed in test tubes containing semisolid (0.2% agar) PYGV or glucose medium (0.2% glucose + 0.02% yeast extract in mineral salts medium). The tubes were inoculated with 30 μl of PYGV grown strains (approximately 10^8 cells ml^{-1}) to the liquid medium (45 °C). The tubes were rolled vigorously and cooled immediately in ice water. The test tubes were viewed after 3–7 days of incubation at room temperature. Growth of K1 in semisolid nitrogen free medium was tested on NFb medium designated for the microaerophilic diazotrophs (Döbereiner 1992).

The effect of oxygen on growth on liquid glucose medium was studied in serum bottles (125 ml) containing 20 ml of the medium and capped with rubber stoppers. The oxygen levels in the headspace of the bottles were adjusted to 2%, 6% or left at 21%. The bottles with low levels of oxygen were flushed

with filter sterilized N_2 and O_2 was adjusted with filter sterilized air. Triplicate bottles with 10^4–10^5 cells ml^{-1} were incubated at room temperature on a gyratory shaker (130 rpm). Low cell densities were used because high cell density can increase the oxygen tolerance of microaerophilic organisms thereby overcoming oxygen toxicity (Krieg and Hoffman, 1986). Growth was monitored by measuring optical density at 600 nm. The headspace oxygen concentration was monitored daily. Oxygen was adjusted by injecting sterile air through the rubber stopper. During the incubations, the oxygen concentration varied between 0.5–2.5% and 4.5–6.5% in the 2% and 6% bottles, respectively.

The effect of oxygen concentration on CP degradation was studied at 8 °C in 120 ml serum bottles. The medium (autoclaved effluent from the bioreactor) was boiled and flushed with nitrogen to remove the oxygen. Fifty ml of the medium was pipetted in the bottles with simultaneous N_2 flushing and the bottles were sealed with rubber stoppers. Oxygen in the headspace was adjusted to 2–21% by injecting filter sterilized air through the rubber stoppers. Duplicate bottles were inoculated with 10^7 cells ml^{-1} and incubated on a gyratory shaker (150 rpm).

Analyses

Chlorophenols were extracted and analyzed as described earlier (Männistö et al. 1999). Oxygen was measured from the gas phase using a Perkin Elmer gas chromatograph equipped with a thermal conductivity detector and a Supelco 1-3823 column ($6' \times 1/8''$) packed with molecular sieve 5A with mesh size of 60/80. The oven, injector and detector temperatures were 60, 120 and 120 °C, respectively. Inorganic chloride, PO_4^{3-} and NO_3^- were measured with a Dionex (Dionex, Sunnyvale, CA) DX-120 ion chromatograph. The optical density was measured at 600 nm using a Shimadzu (Shimadzu, Kyoto, Japan) UV-1601 spectrophotometer. The biomass was estimated by direct microscopic counting of cells stained with 4′,6′-diamidino-phenylindole (DAPI, 3 μg ml^{-1}) using a Zeiss Axioskop 2 epifluorescence microscope.

Results

The strain K1 had 97% sequence identity to the type strain of *Herbaspirillum seropedicae* (ATCC 35892) indicating a close relation to this microaerophilic N_2 fixing genus. Moreover, when grown in nitrogen-free semi-solid malate medium, K1 formed thin pellicles 2–10 mm below the surface within 5 day incubation at room temperature indicating that it is a microaerophilic nitrogen fixer. The Gram-positive isolate K44 had 97% sequence identity to *Nocardioides pyridinolyticus* OS4 (KCTC 9974BP), *N. nitrophenolicus* NSP41 (KCTC 0457BP), *Nocardioides* sp. C190 and *N. jensenii* (DSM 20461^T). Strains K74 and MT1 were members of the genus *Sphingomonas* with 97% sequence identity to *S. xenophaga* BN6 (DSM 6383^T) and 95% sequence identity to *S. aromaticivorans* (IFO16084), respectively. Based on the 16S rDNA gene sequences and whole cell fatty acid compositions reported earlier (Männistö et al. 1999) the groundwater isolates were identified as species of *Herbaspirillum* (K1), *Nocardioides* (K44) and *Sphingomonas* (K74 and MT1) (Table 1).

Effect of oxygen on growth of the isolates

Microaerophilic growth of the isolates was tested in test tubes with semisolid media. Growth of the strains K1, MT1 and K74 after 3 days incubation is shown in Figure 1A. The strain K74 grew on the surface of the tube indicating aerobic growth. On glucose and PYGV medium the other two Gram-negative strains K1 and MT1 formed a band of growth 4–6 and 4–8 mm below the surface, respectively, indicating that they were microaerophilic. Strain K44 grew from the surface to approximately 20 mm below it (results not shown). K44 was non-motile and, therefore, no aerotactic movement to the preferred oxygen concentration occurred. Band movement of the strains K1 and MT1 was observed as the incubation was continued. After 3 weeks of incubation growth of K1 and MT1 occurred down to 2 cm below the surface (Figure 1B)

The effect of different oxygen partial pressures on growth in liquid cultures was tested with K1, K44 and MT1 using glucose-yeast extract medium and the results are shown in Figure 2. The *Herbaspirillum* strain K1 grew similarly at all oxygen partial pressures. The *Nocardioides* strain K44 grew slightly slower at the lowest oxygen concentration than with ambient air. The bioreactor isolate *Sphingomonas* sp. MT1 grew best at the lowest oxygen partial pressure. At ambient air oxygen concentration the growth of MT1 slowed after 3 days indicating possibly the accumulation of toxic oxygen species in the medium. The bioreactor strain MT1 thus showed microaerophilic growth in both semisolid and liquid media while K1

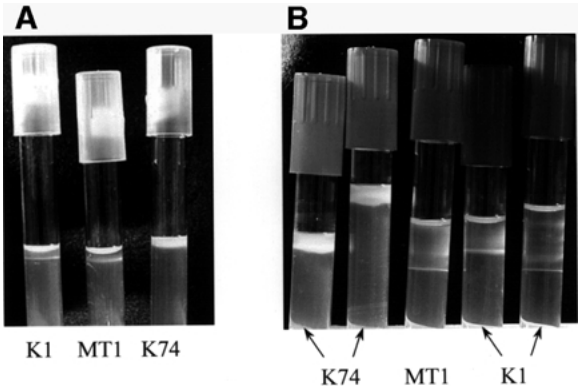

Figure 1. Growth of the isolates K1, MT1 and K74 in semisolid glucose medium after 3 days of incubation (A), and in semisolid PYGV medium after 3 weeks of incubation (B).

grew microaerophilically only in the semisolid medium. These results indicate different oxygen optima for the isolates.

Effect of oxygen on 2,4,6-TCP degradation

To investigate whether oxygen concentration affects 2,4,6-TCP degradation by K44, K74 and MT1 they were incubated in batch bottles at different headspace oxygen concentrations (Figure 3). After the second spike of 2,4,6-TCP, degradation by the *Sphingomonas* isolates K74 and MT1 was similar at all oxygen concentrations. This showed that although growth of MT1 was inhibited at elevated oxygen partial pressures the chlorophenol degradation was not. The lowest oxygen concentration slightly slowed down TCP degradation by the isolate K44. These results show that oxygen concentration (2–21% in the headspace) did not substantially affect TCP degradation by these isolates.

Kinetics of 2,3,4,6-TeCP degradation

Kinetics of 2,3,4,6-TeCP degradation by strains K44, K74 and MT1 was studied at 8 °C. The specific degradation rates with different TeCP concentrations are shown in Figure 4. Inorganic chloride releases for K44, K74 and MT1 were 74%, 73% and 79% of the stoichiometric chloride release, respectively. Further, no methylation products appeared indicating mineralization of the 2,3,4,6-TeCP. K1 degraded TeCP poorly under the test conditions (data not shown) although TeCP was readily degraded in PYGV medium. The TeCP degradation by the *Sphingomonas* isolates K74 and MT1 was typical for Michaelis–Menten kinetics whereas the degradation by K44 was

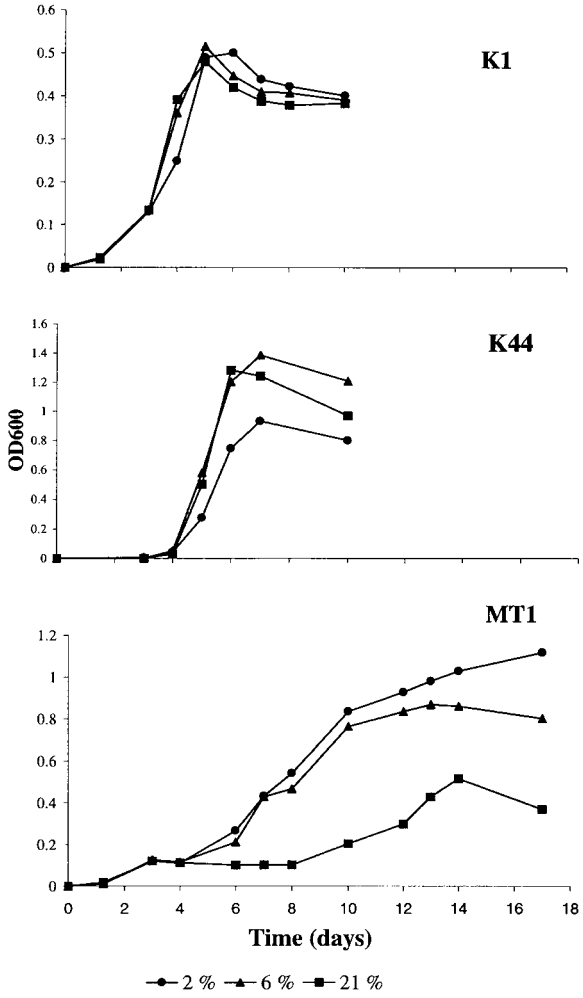

Figure 2. Effect of different headspace oxygen concentrations on growth of K1 (3.1×10^4 cells ml^{-1}), K44 (2.9×10^5 cells ml^{-1}) and MT1 (4.2×10^4 cells ml^{-1}) on glucose. Data points are means of three replicates.

partially inhibited above 3 mg l^{-1} of TeCP. The Michaelis–Menten equation was used to describe the kinetics of the isolates K74 and MT1. TeCP degradation by K44 was fitted in to the modified Haldane model. Best fit for the Haldane model was obtained when n = 3.1. The maximum specific degradation rates (μ_{max}) of the *Sphingomonas* isolates K74 and MT1 were 4.9 and 0.52 (10^{-12} mg h^{-1} cell^{-1}), respectively (Table 1). The K_s values were similar for the *Sphingomonas* isolates namely 2.4 mg l^{-1} for K74 and 2.1 mg l^{-1} for MT1. The Haldane equation gave the following values for *Nocardioides* sp. K44: μ_{max} = 0.19 (10^{-12} mg h^{-1} cell^{-1}), K_s = 0.46 mg l^{-1}, K_i = 5.14 mg l^{-1}. These results indicate that at high TeCP concentrations, such as in

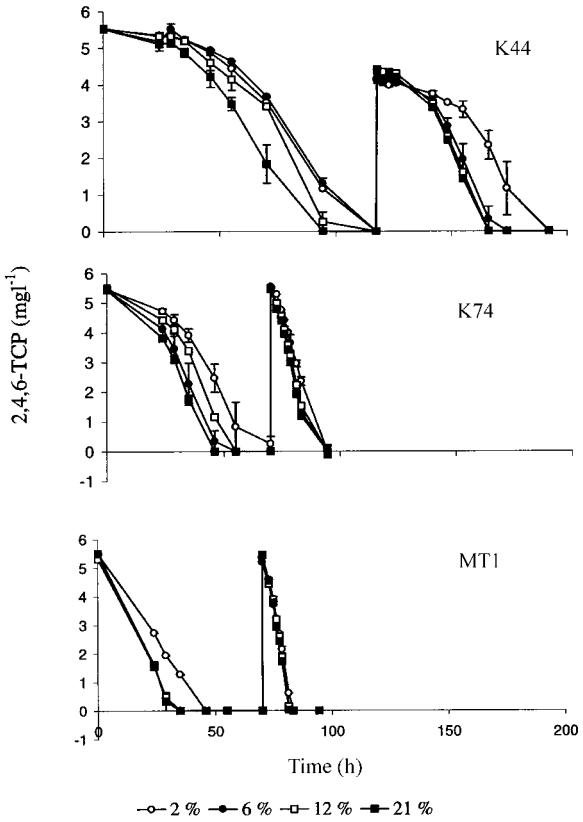

Figure 3. Effect of different oxygen concentrations on 2,4,6-TCP degradation by K44 (7×10^7 cells ml^{-1}), K74 (3.5×10^7 cells ml^{-1}) and MT1 (4.1×10^7 cells ml^{-1}).

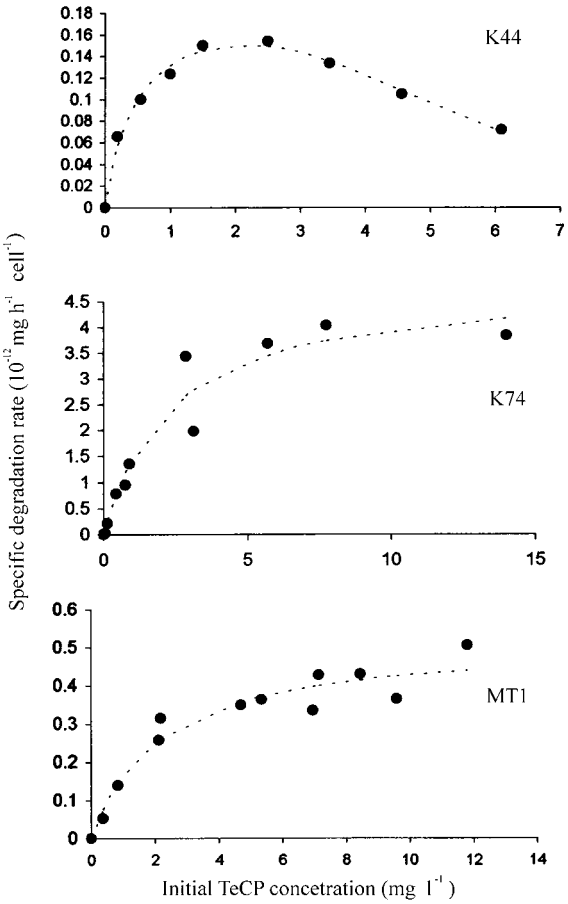

Figure 4. Specific 2,3,4,6-TeCP degradation by isolates K44, K74 and MT1 at different initial TeCP concentrations.

the contaminated groundwater, the higher μ_{max} of the groundwater *Sphingomonas* sp. K74 provides competitive advantage over the bioreactor isolate MT1. The low K_s of the *Nocardioides* sp. K44 as compared to the sphingomonads and its sensitivity to high TeCP concentrations indicate that the ecological niche for K44 may be the lightly contaminated parts of the aquifer. The kinetic characteristics of the isolates did not, however, explain the predominance of MT1 in the bioreactor as specific affinities (μ_{max}/K_s) of both K74 (2.04×10^{-12} l h^{-1} $cell^{-1}$) and K44 (0.41×10^{-12} l h^{-1} $cell^{-1}$) were higher than that of MT1 (0.25×10^{-12} l h^{-1} $cell^{-1}$).

Influence of supplementary compounds on TeCP degradation by K1 and K44

TeCP degradation by K1 was very slow when the TeCP was the only carbon and energy source although TeCP was readily degraded in the PYGV medium. The effect of supplementary carbon sources, vitamins and nutrients were, therefore, investigated and the results are shown in Table 2. The degradation of 5 mg l^{-1} of TeCP was considerably enhanced by yeast extract, pyruvate, glutamate, malic acid, peptone and casitone. Addition of glucose did not affect TeCP degradation. For efficient degradation the supplementary organic compound additions at 20 times higher concentrations than the TeCP was required. When added at 10 mg l^{-1}, TeCP degradation was enhanced but with lower rates (Table 2; Figure 5). Addition of vitamins or mineral salts medium with macro and micro nutrients did not affect the degradation showing that slow TeCP degradation was not due to inorganic nutrient limitations.

The effect of supplementary glucose (100 mg l^{-1}) and yeast extract (100 mg l^{-1}) on TeCP degradation by the actinobacterial isolate K44 was also studied. In contrast to the isolate K1, supplemental substrates did not affect the TeCP degradation by K44 (Figure 5).

Table 2. Effect of supplementary compounds on degradation of 2,3,4,6-TeCP (5 mg l^{-1}) by the isolate K1. Results are shown as percentage of TeCP removed; standard deviations are in the brackets

Supplementary compound	mg l^{-1}	Percentage of TeCP removed[1] After 6 h	After 24 h
DL-malic acid	100	100 (±0)	100 (±0)
Na-pyruvate	10	49 (±3.8)	87 (±0.5)
	100	91 (±0.9)	100 (±0)
Yeast extract	10	21 (±0.7)	72 (±28)
	100	87 (±0.8)	100 (±0)
Na-glutamate	10	31 (±1.6)	72 (±1.1)
	100	94 (±3.9)	100 (±0)
Casitone	10	22 (±0.5)	51 (±0)
	100	71 (±2.9)	100 (±0)
Peptone	10	17 (±0.9)	47 (±0.3)
	100	60 (±2.7)	100 (±0.5)
Glucose	10	13 (±1.9)	24 (±0.9)
	100	9 (±1.9)	20 (±2.4)
Balch vitamin solution[2]		18 (±0.5)	46 (±2.8)
Mineral salt solution[3]		23 (±0.4)	51 (± 29)
None[4]		11 (±2.7)	30 (±11.8)

[1] As determined by GC-ECD analysis. Test vials contained autoclaved effluent water from the bioreactor, 1.2×10^8 cells ml^{-1}, 4 mg TeCP l^{-1} and supplementary compounds as indicated.
[2] Cote & Gherna (1994).
[3] Vials were amended with 10% of DSM 465 mineral salt solution.
[4] Average of two separate experiments.

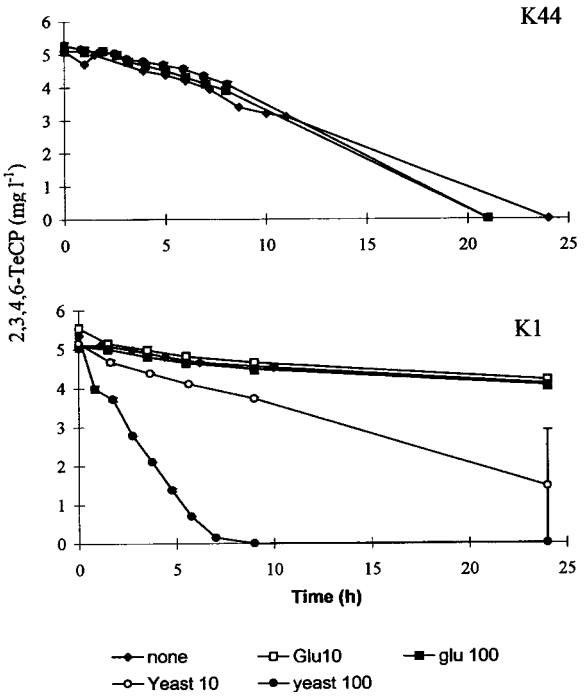

Figure 5. Effect of glucose and yeast extract (10 and 100 mg l^{-1}) on 2,3,4,6-TeCP degradation by the strains K44 and K1.

Effect of temperature on TeCP degradation

To compare CP degradation rates at ground water and optimum growth temperatures (23–26 °C), TeCP degradation was studied at 8 °C and at room temperature. Degradation of 2.5–5 mg l^{-1} of TeCP at room temperature (23 ± 2 °C) and 8 °C by K44, K74 and MT1 is shown in Figure 6. TeCP was degraded faster at 8 °C than at room temperature by all isolates. Rates of TeCP degradation by the sphingomonads K74 and MT1 were slightly slower at room temperature. *Nocardioides* sp. K44 did not significantly degrade the TeCP within 5 h at room temperature whereas at 8 °C TeCP was completely degraded. These results show that the optimum temperature for CP degradation was significantly lower than that for growth and is closer to ambient groundwater temperature than the optimum growth temperature.

Discussion

Bacterial polychlorophenol degradation mechanisms and the use of microbes in bioremediation have been extensively studied [for reviews see e.g., McAllister et al. (1996); Häggblom & Valo (1995); Puhakka & Melin (1996)] whereas the factors affecting chlorophenol degradation under saturated subsurface conditions are much less understood. Polychlorophenol degrading bacteria have mainly been described among the genera *Sphingomonas* (Nohynek et al. 1995, 1997; Ederer et al. 1997; Radehaus & Schmidt 1992) and *Mycobacterium* (Häggblom et al. 1994; Wittmann et al. 1998) and consequently CP degradation by pure cultures has mainly been studied by members of these genera. Furthermore, polychlorophenol degradation has mainly been studied at 20 °C or higher under oxygen saturated conditions and only a few reports exists on the effect of low temperature and oxygen concentrations on CP degradation. Long-term contamination of the aquifer studied here has resulted in a diverse community of TeCP degrading bacteria as indicated by analysis of 52 and 17 isolates for whole cell fatty acid compositions and partial 16S rDNA sequences, respectively (Männistö et al. 1999, 2001).

In order to better understand the effects of saturated subsurface and bioreactor conditions on chlorophenol biodegradation potential four different polychlorophenol degrading isolates representing α and β proteobacteria and Gram-positives with high

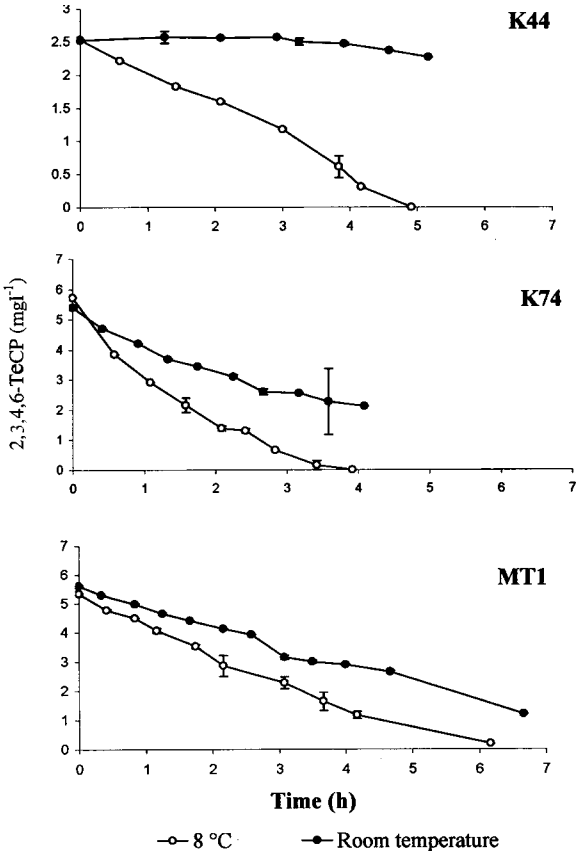

Figure 6. Effect of temperature on TeCP degradation by K44, K74 and MT1. The cell densities at 8 °C and at room temperature were as follows: K44, 3.3×10^9 (8 °C) and 3.6×10^9 cells ml^{-1} (RT); K74, 2.7×10^9 cells ml^{-1} at both temperatures; MT1 2.7×10^9 (8 °C) and 2.9×10^9 cells ml^{-1} (RT). The optimum growth temperatures on PYGV for K44, K74 and MT1 were 25 °C, 25 °C and 23 °C, respectively (Männistö & Puhakka 2001).

G + C (Männistö et al. 1999) were selected for this study. The isolates K74 and MT1 were clearly members of the genus *Sphingomonas* as indicated by their phylogenetic relatedness to the other sphingomonads and their cellular fatty acid compositions (Männistö et al. 1999; Tiirola et al. manuscript in preparation). The 16S rDNA sequence of the isolate K1 together with its ability to grow on nitrogenfree semi-solid malate medium (NFb) indicates close relatedness to *Herbaspirillum* species, which are diazotrophs commonly isolated from the roots and rhizosphere soil samples of various plants (Baldani et al. 1992, 1996). To our knowledge, xenobiotic-degrading organisms have never before been described among this genus. The actinobacterial isolate, strain K44 was related to xenobiotic degrading *Nocardioides* strains including pyridine degrading *N. pyridinolyticus* (Yoon et al. 1997) and *p*-nitrophenol degrading *N. nitrophenolicus* (Yoon et al. 1999). *Nocardioides* is thus an important xenobiotic degrading genus. Seven of the 58 chlorophenol degraders isolated from the contaminated Kärkölä groundwater had similar nocardioform morphology and cellular fatty acid composition as strain K44 indicating that this species is an important CP degrader in the groundwater (Männistö et al. 1999, 2001).

Degradation of TeCP by the *Sphingomonas* isolates K74 and MT1 and the *Nocardioides* sp. K44 was associated with 73–79% inorganic chloride release. This together with the absence of methylation products gave evidence of mineralization. Highest affinity for TeCP was observed for the *Nocardioides* sp. K44. This strain was also sensitive to TeCP showing partial inhibition for TeCP above 3 mg l^{-1} of 2,3,4,6-TeCP. This indicates that this organism may better compete for TeCP in the less contaminated parts of the aquifer. Nevertheless this organism survives in the highly polluted groundwater as shown by its frequent isolation from the bioreactor influent (Männistö et al. 1999, 2001). The groundwater *Sphingomonas* sp. K74 showed the highest specific degradation rate of TeCP. Polychlorophenol degrading species of *Sphingomonas* are usually remarkably tolerant to PCP and able to degrade it at high concentrations (Radehaus & Schmidt 1992; Resnick & Chapman 1994; Wittmann et al. 1998) whereas *Actinobacteria* such as members of mycobacteria are more sensitive to polychlorophenols (Wittmann et al. 1998). The ability of strain K1 to degrade 2,3,4,6-TeCP was poor as compared to the other isolates studied here. Addition of intermediates of the citric acid cycle such as glutamate and malate or pyruvate enhanced TeCP degradation. The relatedness of K1 to plant associated *Herbaspirillum* species indicate that it originates from the root zone. Plants provide the root zone bacteria various compounds for growth including amino, aliphatic and aromatic acids, amides, sugars and amino sugars (Paul & Clark 1989). Growth and CP degradation of K1 may be enhanced in such environments. Glucose or yeast extract supplementation did not affect the TeCP degradation by K44 indicating different catabolic mechanisms in these two strains.

High recycling rate in the fluidized-bed bioreactor ensures that the reactor CP concentrations are close to those of the effluent (0.002–0.1 mg l^{-1}) (Puhakka et al. 2000). The bioreactor microbes benefit therefore from a high affinity for 2,3,4,6-TeCP which is the main contaminant in the groundwater. The spe-

cific affinity (μ_{max}/K_s) for TeCP was higher for both groundwater isolates K44 an K74 than for the bioreactor isolate MT1 indicating that factors other than chlorophenol degradation kinetics have resulted in the predominance of MT1 in the bioreactor.

Due to high concentrations of Fe(II) and DOC the oxygen concentrations in the KärköLä groundwater are low (down to 0.1 mg l^{-1}). The presence of microaerophilic bacteria in the groundwater is therefore likely. Screening the growth of 39 isolates on semisolid media indicated that microaerophily is common among the groundwater bacteria (Männistö and Puhakka 2001). Of the four tested strains, K1 and MT1 grew microaerophilically on semi-solid glucose and PYGV media. In liquid glucose medium growth of MT1 slowed down after 3 days in bottles with ambient air. Oxygen concentration had, however, no effect on the CP degradation of MT1 or the other isolates. The strain MT1 lacked catalase activity and growth in rich medium likely results in accumulation of toxic oxygen species. In low substrate environments the metabolic activity is low and consequently the formation of toxic oxygen species is less significant. For this reason oligotrophic organisms are proposed to lack catalases and may therefore not be cultivable in rich media (Poindexter 1981; Morita 1997). Due to aeration, the oxygen concentration in the bioreactor is approximately 5 mg l^{-1}. The bioreactor isolate MT1 showed, however, highest sensitivity of the isolates to O_2. This indicates that oxygen sensitivities of the isolates and differences between the oxygen concentrations in the groundwater and bioreactor do not explain the predominance of MT1 in the bioreactor. Oxygen concentration did not affect the growth rate of the *Herbaspirillum* sp. strain K1 in liquid glucose medium, but the strain showed similar aerotactic behaviour by band movement in semi-solid medium as reported in species of *Herbaspirillum* (Döbereiner 1992). Species of *Herbaspirillum* fix nitrogen only under microaerophilic conditions, but can grow aerobically in the presence of fixed nitrogen (Krieg & Hoffmann 1986). Microaerophily may therefore be an adaptive mechanism for K1 that guides it to optimal oxygen concentrations for nitrogen fixation as proposed for the microaerophilic nitrogen fixing *Azospirillum brasilense* (Zhulin et al. 1996). The attraction of low oxygen environment to *Azospirillum brasilense* has been attributed to maximal proton motive force at this O_2 concentration which coincides with the optimal O_2 concentration for nitrogen fixation. The change in proton motive force was proposed to act as a signal for aerotaxis which guides these free-living diazotrophs to the optimal niche for nitrogen fixation in the rhizosphere.

Polychlorophenol degradation at low temperatures has mainly been studied using mesophilic organisms (for review see, Puhakka & Melin 1998) and strong decreases in degradation rates due to lowering temperature have been reported (e.g., Melin et al. 1998; Wittmann et al. 1998). The effect of temperature on chlorophenol degradation in Kärkölä groundwater has previously been studied by Melin et al. (1998) using activated sludge enriched bacteria. An unusually strong effect of temperature on CP degradation in fluidized-bed reactors was reported at temperatures ranging from 4 to 16.5 °C. Highest degradation rate was at the highest temperature (16.5 °C) and a 10 °C decrease in temperature generally decreased CP degradation rates by a factor of seven. The biomass in the fluidised-bed reactors was enriched from activated sludge at room temperature (Melin et al. 1998) and therefore the temperature optima is likely different from the temperature optima of the indigenous groundwater bacteria.

Wittmann et al. (1998) studied the well known PCP degraders *Sphingomonas chlorophenolica* RA2 and *Mycobacterium chlorophenolicum* PCP-1 which have optimum growth temperatures on glucose at 33 °C and 30 °C, respectively. Optimum temperature for PCP degradation by *S. chlorophenolica* was similar to the optimum growth temperature, but *M. chlorophenolicum* exhibited highest PCP degrading activity at 41 °C, which is 10 °C higher than the optimum temperature for growth.

The effect of temperature on CP degradation by the cold-adapted groundwater isolates studied here was unique. The optimum growth temperatures of strains K44, K74 and MT1 on PYGV medium are 25 °C, 25 °C and 23 °C respectively (Männistö & Puhakka 2001), yet TeCP was degraded faster at 8 °C than at room temperature by all of these isolates indicating distinctly different temperature optima for chlorophenol degradation.

In summary, the boreal groundwater isolates K1, K44 and K74 and the bioreactor isolate MT1 represent distinctly different organisms both phylogenetically and physiologically. High TeCP degradation rates at low temperature and O_2 concentrations indicate that the isolates are well adapted to the existing groundwater conditions. Degradation kinetics and sensitivity to chlorophenols indicate, however, different niches for the isolates. The *Sphingomonas* isolates may compete

better in the more contaminated groundwater while the environmental niche for the *Nocardioides* sp. K44 may be in the less contaminated part of the aquifer. The niche for the *Herbaspirillum* sp. K1, on the other hand, may be in the root zone. Factors other than TeCP degradation kinetics and oxygen sensitivity have been the reason for MT1 enrichment in the full-scale bioreactor.

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