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Chlorophenol dechlorination and subsequent degradation in denitrifying microcosms fed low concentrations of nitrate

Robert A. Sanford[1] & James M. Tiedje[2,*]
[1] *Department of Civil Engineering, University of Illinois at Urbana-Champaign Urbana, Illinois 61801, USA:*
[2] *Center for Microbial Ecology and Department of Microbiology, Michigan State University, East Lansing, Michigan 48824-1325, USA; (* corresponding author)*

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Abstract

The potential of using nitrate as a terminal electron acceptor to stimulate anaerobic degradation of mixtures of monochlorophenols (MCPs) or dichlorophenols (DCPs) was evaluated. Contaminated and non-contaminated soils were added to water saturated anaerobic microcosms supplemented with 1 mM or 5 mM nitrate. Denitrification and dechlorination activity were present in three diverse soil types and were maintained upon refeeding both nitrate and the appropriate chlorophenol. However, dechlorination activity could only be serially transferred in enrichments with an added electron donor such as acetate. Dehalogenation activity in enrichments from four of the primary microcosms showed at least five different dechlorination reactions, each mediated by different microbial communities. Three of these are distinct *ortho*-dechlorinating paths; two are *meta*-dechlorinating and one is the *para*-dechlorination of 3,4-DCP. Simultaneous dechlorination and denitrification was observed and both activities could be maintained in microcosms but only in the presence of low nitrate concentrations. Dechlorination and denitrification were mediated by two separate microbial communities; one that dechlorinates without use of nitrate and one that denitrifies while oxidizing the dechlorinated aromatic ring. There was no evidence that dechlorination is mediated by the denitrifying community, however the maintenance of a denitrification potential using low (< 1 mM) nitrate concentrations may be useful for completing the food chain by stimulating the mineralization of phenol and benzoate.

Introduction

Chlorophenolic compounds (CPs) have been used extensively in several industries and there has been considerable effort to study the degradation of these compounds under aerobic and strictly anaerobic conditions (Reineke & Knackmuss 1988; Häggblom 1992; Mohn & Tiedje 1992). Several polychlorinated phenols and monochlorophenols (MCPs) are degraded under aerobic and anaerobic conditions (Boyd et al. 1983; Gibson & Suflita 1986; Hrudey et al. 1987; Genthner et al. 1989; Dietrich & Winter 1990; Hale et al. 1990; Zhang & Wiegel 1990; Madsen & Aamand 1992). Only two such studies reported the use of denitrifying conditions with chlorophenols, however no simultaneous dechlorination and nitrate reduction was observed (Genthner et al. 1989; Häggblom et al. 1993). Nitrate is attractive as an electron acceptor because of its solubility and because complete mineralization of some aromatic compounds under denitrifying conditions occurs at reasonably rapid rates. Also, reductive dechlorination of chlorinated phenolic compounds, an exergonic and potentially an electron accepting reaction (Dolfing & Harrison 1992) may not be inhibited under low nitrate concentrations as it is likely to be by oxygen. This is true for the *o*-MCP dechlorinating isolate 2CP-3, which continues to dechlorinate in the presence of nitrate (Sanford 1996). Other studies have shown that dechlorination of MCPs does occur, presumably once nitrate has been depleted, but efforts to transfer these cultures to nitrate-containing media resulted in no dechlorination activity (Genth-

ner et al. 1989). Nitrate-dependent chlorobenzoate-degrading enrichments, however, have been observed (Genthner et al. 1989b; Häggblom et al. 1993). This would indicate that there may be differences between the microbial communities that degrade these two types of compounds.

Anaerobic chlorophenol dechlorination studies have predominantly used anaerobic sediments and sludges as inocula for enrichments or microcosms (Boyd et al. 1983; Gibson & Suflita 1986; Genthner et al. 1989; Kohring et al. 1989; Hale et al. 1991; Hendriksen et al. 1992; Häggblom et al. 1993). In contrast, the best source of denitrifying populations may be surface soils where the presence of nitrate and the fluctuating oxygen status provides regular selection for denitrifiers. Soils high in litter residue may also harbor reservoirs of naturally occurring chlorinated aromatic compounds. Several MCPs and dichlorophenols (DCPs) are known to be produced naturally (Berger 1972; Siuda & Debernardis 1973; Gribble 1992) and white rot fungi are known to produce up to 75 mg/kg litter of chlorinated anisyl metabolites in nature (de Jong et al. 1994).

We evaluated the degradation of mixtures of MCPs and DCPs under low nitrate concentrations (< 5 mM) in microcosms seeded with contaminated and relatively pristine soils. Mixtures were used to model the complex nature of wastes that occur in contaminated material and to broaden the selective pressure for dechlorinating microorganisms. Enrichment cultures were developed from microcosms that exhibited both denitrification and dechlorination activity. Our objectives were to determine the relationship of nitrate and denitrifiers to the degradation activity and to evaluate the substrate range and specificity of the dechlorination reactions mediated by these enriched populations. We observed at least five different dechlorination reactions in these microcosms and subsequent enrichments thought to be carried out by different populations.

Materials and methods

Enrichment inocula

Fourteen different soil samples were obtained from several sources. Material from contaminated sites was obtained from Remediation Technology and Ecova Corp. in Seattle, Washington. Sediment sludge samples from a paper pulp mill effluent treatment system in Ontario, Canada were provided by Dr. Roberta Fulthorpe. Soil samples from the Kellogg Biological Station (KBS) were obtained from agricultural plots being treated with different levels of 2,4-D. A compost soil, high in organic matter, was sampled at the base of a compost pile in Lansing, Michigan. Two tropical soil samples were collected in the lowland rainforest of equatorial Cameroon by Dr. Roland Weller.

Microcosms

Duplicate microcosms were started in 160 ml serum bottles with 100 ml of boiled degassed medium and approximately 10 g of soil or sludge. Glass beads were used as a negative control. One microcosm set received a mixture of *o*-MCP, *m*-MCP and *p*-MCP at 200 µM each. The other set was supplemented with 125 µM each of 2,3-DCP, 2,4-DCP, 2,5-DCP and 3,4-DCP. Those MCPs and DCPs degraded during incubation were replenished at a concentration of 125 µM. Nitrate (as KNO_3) was provided at 5 mM or 1 mM to the microcosms. Nitrate was replenished to initial levels once it was depleted. After several additions only 1-2 mM nitrate was added to active microcosms. Serum bottles were closed with butyl rubber stoppers and incubated at 30 °C.

Medium formulation

The following mineral salts medium (Stevens et al. 1992) was used for all cultures: 2 mM potassium phosphate buffer (pH 7.2 - 7.5), and per liter $CaCl_2 \cdot 2H_2O$ 0.015 g, $MgCl_2 \cdot 6H_2O$ 0.02 g, $FeSO_4 \cdot 7H_2O$ 0.007 g, and Na_2SO_4 0.005 g. A trace metals solution was added to give the following final concentration per liter: $MnCl_2 \cdot 4H_2O$ (5 mg), H_3BO_3 (0.5 mg), $ZnCl_2$ (0.5 mg), $CoCl_2 \cdot 6H_2O$ (0.5 mg), $NiSO_4 \cdot 6H_2O$ (0.5 mg), $CuCl_2 \cdot 2H_2O$ (0.3 mg), and $NaMoO_4 \cdot 2H_2O$ (0.1 mg). NH_4Cl was added to a concentration of 8 mM and 10 mM $NaHCO_3$was added to buffer the headspace which contained a N_2:CO_2 ratio of 95:5. A vitamin solution (Wolin et al. 1963) was provided after cooling.

Enrichment cultures

Aliquots from microcosms were transferred to fresh medium in serum bottles or anaerobic culture tubes with butyl rubber stoppers for the purpose of enriching the dechlorinating and denitrifying activities. Duplicate enrichments received nitrate at concentrations of 0, 1 or 5 mM. Those without nitrate received 1 mM

Table 1. Summary of dechlorinating and denitrifying microcosms that were active within a year of incubation. Microcosms received mixtures of MCPs and DCPs and nitrate at 5 mM or 1 mM.

Soil or sludge	Substrates	Denitrification[a]	Dechlorination[b]	Products
Compost	MCPs; NO_3^-(5)[c]	+DC	++(*o*-MCP)	Phenol·benzoate
	DCPs; NO_3^-(5)	+DC	++(all DCPs)	Phenol, benzoate, *m*-MCP, *p*-MCP
Trop I	MCPs; NO_3^-(1)	+DC	++(*o*-MCP)	Phenol
	MCPs; NO_3^-(5)	+D	+(*o*-MCP)	Phenol
Trop II	MCPs;NO_3^-(1)	+D	++(*o*-MCP)	Phenol
O1C	MCPs; NO_3^-(5)	+D	++(*o*-MCP, *m*-MCP)	Phenol
	DCPs; NO_3^-(5)	+D	+(all DCPs)	Phenol, *m*-MCP, *p*-MCP

[a] +DC indicates denitrification occurred concurrently with dechlorination. +D indicates that denitrification occurred, and nitrate was depleted prior to any other activity. Denitrification was determined by measuring N_2 or N_2O in the microcosm headspace after NO_3^- and NO_2^- disappearance.
[b] + or - indicates presence of dechlorination acitivity. Substrates dechlorinated are indicated in parentheses. ++ indicates a relatively higher level of dechlorination activity.
[c] NO_3^- is given in (mM).

acetate or a mixture of volatile fatty acids (formate 625 µM, succinate 125 µM, propionate 125 µM, and butyrate 125 µM) as electron donors. The media were reduced with 0.2 mM cysteine and 0.2-0.5 mM Na_2S. Resazurin (1 µg/l) was added as a redox indicator. Individual MCPs and DCPs were used as substrates at a concentration of 125 µM. Cultures were incubated at 30 °C.

Chemical analysis

Nitrate and nitrite were analyzed by using a Whatman Partisil 10 SAX column on a Schimadzu HPLC. The eluent was a 50 mM phosphate buffer (pH = 3.0) pumped at a rate of 1 ml/min. UV adsorption at 210 nm was used for detection. Samples from primary enrichments were diluted 1:100 in deionized H_2O prior to nitrate and nitrite analysis.

MCPs, DCPs and aromatic products of dechlorination were analyzed on a Hewlett Packard 1050 HPLC with a Chemstation analysis package. The eluent was phosphoric acid (0.1%) and methanol pumped at 1.5 ml/min using a gradient from 48% to 55% methanol. A Hibar RP-18 (10 µm) column was used. Peaks were quantified at 218 nm on a UV multiwavelength detector. Samples (1 ml) from the enrichments were taken, made basic with 10 µl of 2N NaOH, centrifuged for 6 min in a microfuge and filtered through Acrodisc LC13 PVDF 0.45 µm filters prior to HPLC analysis.

The headspace of the microcosms and enrichments was analyzed for CH_4, N_2, H_2 and CO_2 using a Carle gas chromatograph equipped with a 1.83 m Porapak Q column and a thermal conductivity detector. Headspace pressure was normalized to atmospheric by venting with a needle prior to removing 0.3 ml of gas for injection into the GC. N_2O was quantified on a Perkin Elmer 910 GC with a Porapak Q column and Ni 63-ECD detector. Denitrification products were measured after exchanging the headspace with Ar.

Chemicals

Chlorophenols and dichlorophenols were obtained from Aldrich Chemical Co. Phenol was purchased from Malinkrodt and benzoate from Sigma Chemical Co.

Results

Dechlorinating denitrifying microcosms

Only seven of the 30 microcosms had both dechlorination and denitrification activities after one year of incubation (Table 1). No activity was observed with glass beads in the abiotic control. Microcosms with MCPs exhibited either *ortho*-dechlorination alone or simultaneously with *meta*-dechlorination. DCP microcosms, in contrast, showed considerable dechlorination of all DCPs at all positions. Extensive gas production was observed indicating complete reduction of nitrate to nitrogen gas (Table 1). This was confirmed by GC

Table 2. Rates of concurrent dechlorination and nitrate reduction observed at different nitrate concentrations in duplicate microcosms.[a]

Microcosm	[Nitrate] mM	Dechlorination rate μM d^{-1}	Nitrate reduction rate μM d^{-1}
Compost MCP	2.2	13.3[b]	422
	6.8	0.0	338
Compost DCP	1.6	31.0[c]	329
	6.8	0.0	326
Trop I MCP	1.0	3.9[a]	121
	7.0	0.1	66

[a] Rates were determined from data taken only when both nitrate and CPs were measureable.
[b] Rate of *o*-MCP dechlorination reported.
[c] Rate of total DCP dechlorination reported. A mixture of 2,3-DCP; 2,4-DCP; 2,5-DCP; and 3,4-DCP was used. All were dechlorinated at similar rates.

analysis of the headspace gases, done in an Ar background, which showed increased concentrations of N_2 and N_2O after nitrate and nitrite depletion.

Significant dechlorination rates were observed in the presence of low nitrate in the compost soil microcosms (Table 2). The compost soil microcosm with MCPs exhibited specificity for *o*-MCP and showed no evidence of *m*-MCP or *p*-MCP disappearance over a 200 day period. Phenol was the predominant product observed, but it was rapidly degraded in the presence of nitrate (Table 1). Benzoate was also detected at lower concentrations as a transient product in this microcosm. When excess nitrate (6.8 mM) was amended to the microcosm, the dechlorination rate was considerably reduced, but the nitrate reduction rate was unaffected (Table 2). Similar effects of nitrate concentration on dechlorination rates were observed with the DCP amended microcosms (Table 2). However, when subsequent additions of only 2 mM nitrate were made to both MCP and DCP microcosms after nitrate depletion, both dechlorination and denitrification occurred. Since sampling times were more infrequent at this stage it was not possible to determine if nitrate reduction and dechlorination activity were concurrent. Nitrite was not detected in any of the denitrifying microcosms.

The compost soil microcosms amended with mixtures of DCPs showed little selectivity for which DCPs were dehalogenated. Several aromatic dechlorination products were observed in the microcosms, including all of the MCPs (Table 1). Phenol, *o*-MCP, *m*-MCP and benzoate appeared transiently after several subsequent additions of the dichlorophenol mixture. *Para*-chlorophenol continued to accumulate in the compost DCP microcosm, but to only 28% of the theoretical concentration that would occur if no *para*-dechlorination of the isomers had occurred.

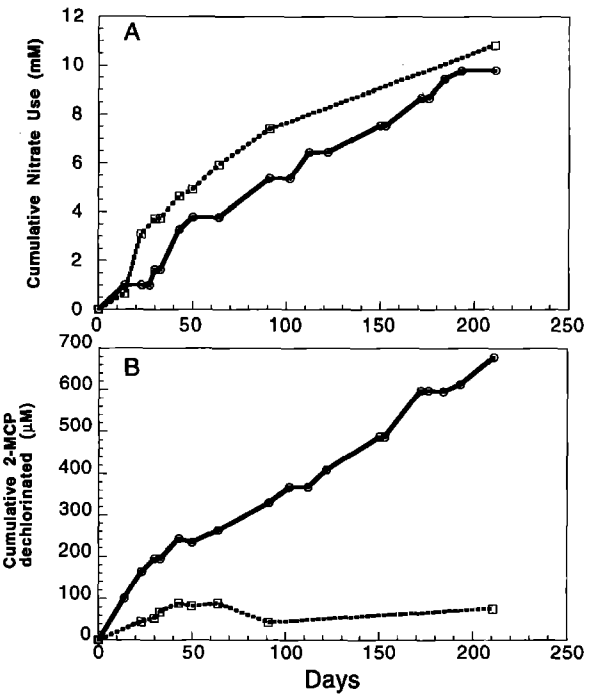

Figure 1. The effect of nitrate concentration on cumulative nitrate reduced (*A*) and cumulative *o*-MCP dechlorinated (*B*) with time in the tropical soil (Trop I) microcosm. Nitrate was added in 1 mM increments (solid line) or 5 mM increments (dashed line).

Two sets of microcosms containing soils collected in Cameroon, Trop I and Trop II, were very sensitive to the nitrate concentration with significant rates of dechlorination only occurring with denitrification when the nitrate concentration was 1 mM or lower (Tables 1, 2). With the 5 mM Trop I microcosms, nitrate was slowly depleted and dechlorination did not occur until nitrate had been removed, after 200 days. Furthermore, in the Trop I microcosms fed 5 mM nitrate *o*-MCP dechlorination was negligible, while significant dechlorination occurred in those fed 1 mM nitrate (Figure 1 B). There was little difference in nitrate reduced under the two nitrate regimes (Figure 1 A).

The O1C microcosms with the MCP mixture dechlorinated both *o*-MCP and *m*-MCP stoichiometrically to phenol only when nitrate had been depleted (Table 1). This activity took over 140 days to develop. Upon refeeding both *o*-MCP and *m*-MCP were dechlorinated simultaneously (Figure 2). The addition of

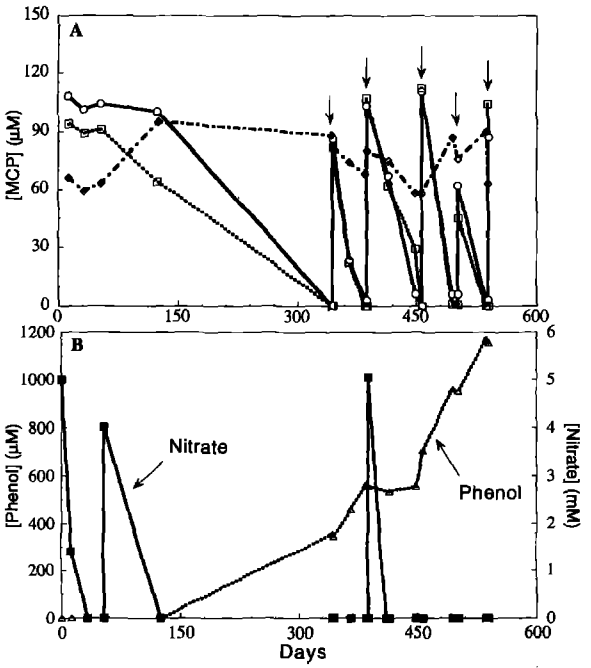

Figure 2. Dechlorination of MCPs in the O1C MCP microcosm. The disappearance of ○-MCP (m), *m*-MCP (□), and *p*-MCP (▲) (*A*) is shown relative to time with the appearance of phenol and dissappearance of nitrate (*B*). Nitrate and MCPs were added after each was depleted as denoted by the arrows in the graph.

nitrate after 380 days had an apparent inhibitory affect on dechlorination, but nitrate was rapidly consumed and N_2 was detected in the Ar-purged headspace. This indicated that an active denitrifying population was present despite the absence of nitrate for nearly 300 days in the microcosm. The persistence of *p*-MCP suggests that abiotic loss of CPs was not a factor in this culture.

Characterization of enrichments derived from microcosms

Since the microcosm experiments suggested dechlorination occurred prior to mineralization and that nitrate above 5 mM was inhibitory to dechlorination, we tested whether nitrate at lower concentrations was required for dechlorination using enrichments derived from the microcosms. Degradation of MCPs to phenol occurred in Trop I derived enrichments when acetate and no nitrate was supplied as an electron donor, however no degradation occurred when nitrate only was added (Figure 3A). With acetate as an electron donor, this *o*-MCP enrichment stoichiometrically dechlorinated *o*-

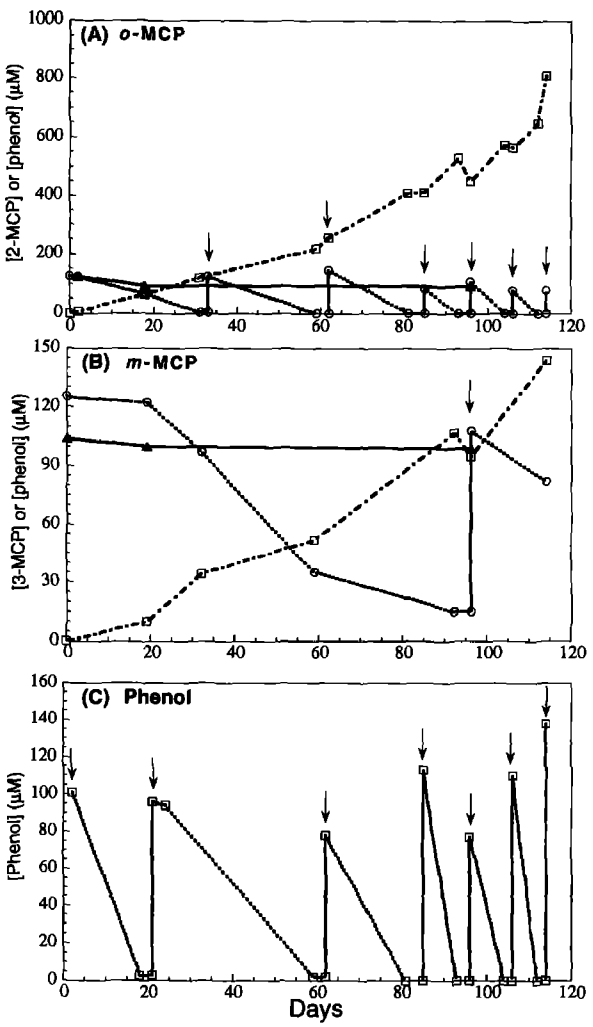

Figure 3. Substrate consumption and phenol production in dechlorinating and denitrifying enrichments fed MCP plus acetate (1 mM) (○) or plus nitrate (1 mM) (▲). (*A*) *o*-MCP enrichment from Trop I microcosm that shows that dechlorination only occurs in the presence of acetate. (*B*) DCP enrichment culture from compost microcosm that shows dechlorination of *m*-MCP also only occurs when nitrate was not present. Phenol (□) production was stoichiometric in both (A) and (B). (*C*) Enrichment with phenol and nitrate (1 mM) from compost microcosm shows that phenol (□) was readily degraded under denitrifying conditions. Arrows denote addition of substrate.

MCP to phenol, which reached concentrations greater than 2 mM. The stoichiometry of acetate consumption and the reductive dechlorination of *o*-MCP suggests that a microorganism was gaining energy from this process. Of the available reducing equivalents from acetate, 65% were used for reductive dechlorination (data not shown). Similar results were obtained in enrichments inoculated from the compost soil micro-

Table 3. Characteristics of active enrichment cultures. At least five different dechlorination patterns are observed in the enrichments. Shown are the chlorophenolic substrate specificities of the different enrichment cultures. Also indicated is the sensitivity of dechlorination to nitrate and whether methane is detected in the enrichment cultures.

	Enrichments[a]				
Substrate(s)[b]	*o*-MCP culture	2,3-DCP	*o*- and *m*-MCP	*m*-MCP	3,4-DCP
o-MCP	+	-	+	-	-
m-MCP	-	+	+	+	+
p-MCP	-	-	-	-	-
2,3-DCP–>*m*-MCP	-	+	-	-	-
2,4-DCP–>*p*-MCP	+	-	+	-	-
2,5-DCP–>phenol	-	-	+	-	-
2,5-DCP–>*m*-MCP	+	-	+	-	-
2,6-DCP–>phenol	+	-	-	-	-
2,6-DCP–>*o*-MCP	+	+	-	-	-
3,4-DCP–>*m*-MCP	-	-	-	-	+
Dechlorination with					
NO_3^-[c]	+	-	-	-	-
methanogenesis[d]	-	+	-	+	+

[a] Enrichments are identified by the substrate used for dechlorination. Since *m*-MCP is a product in the 2,3-DCP and 3,4-DCP cultures the *m*-MCP dechlorinating enrichment probably is a subset microbial population of these other dechlorination enrichments.
[b] Substrates are chlorinated phenolic compounds monitored for dechlorination in cultures amended with acetate as and electron donor.
[c] 5 mM nitrate was added to media containing the primary chlorinated substrate of the enrichment and acetate. A positive result indicates that nitrate is not inhibitory to dechlorination.
[d] Positive indicates that CH_4 was detected in the headspace of enrichment cultures showing dechlorination activity.

cosms exhibiting only *o*-MCP dechlorinating activity. Since in the above experiment acetate or nitrate were added separately, we also added them together to the enrichments to determine if nitrate was inhibitory or if there might be a nitrate-linked respiratory dechlorinating organism. Four of the five dechlorination activities were inhibited by 5 mM nitrate (Table 3), but dechlorination did occur in the *o*-MCP enrichment. The rate of dechlorination did not increase in the presence of nitrate indicating that nitrate respiration was not involved.

DCP microcosms with *meta*-dechlorination activity were transferred to media containing only *m*-MCP with and without nitrate. *Meta*-chlorophenol was dechlorinated to phenol with acetate present as an electron donor. The culture with nitrate and *m*-MCP did not have any activity (Figure 3B). *Meta* dechlorination was considerably slower than *ortho* dechlorination, taking almost 100 days. Similar results were obtained with enrichments containing 2,3-DCP which exhibited *ortho*-dechlorination in the presence of acetate. Subsequent *meta*-dechlorination was also observed in these enrichments after a considerable lag time. Nitrate was preferentially reduced to nitrite when it was added to the 2,3-DCP enrichment and no dechlorination was observed during nitrate reduction.

To test if anaerobic phenol degradation occurred in the presence of nitrate, transfers were done from microcosms to a phenol plus nitrate medium. Phenol was completely degraded (Figure 3C). These cultures exhibited extensive gas production in each culture and nitrate was completely depleted, indicating that denitrifiers were capable of degrading phenol. Cultures with phenol alone also showed degradation activity with the appearance of methane, however this activity was considerably slower than was exhibited with nitrate.

Specificity of enrichments for substrates

Although dechlorination activity in all enrichments was dependent on the presence of acetate as an electron donor, there appeared to be different responses to the MCPs and DCPs added as substrates. A possible explanation was that different microbial populations were mediating reductive dechlorination in each enrichment

culture. To test this, each enrichment was tested for its ability to dechlorinate the MCPs and DCPs originally used in the microcosms. In addition 2,6-DCP was used as a test substrate. Each of the five enrichments showed a different substrate specificity, indicating that as many as five different microbial populations were responsible for dechlorination (Table 3). This physiological diversity between the enrichments was confirmed by testing a variety of electron donors for reductive dechlorination. Although all enrichments used acetate as an electron donor, subsequent enrichment transfers showed that this was not the best carbon and energy source for all of the dechlorinating populations. Several of the enrichments exhibited higher levels of dechlorination activity when formate, H_2 or butyrate were used as electron donors (data not shown). This would be expected among diverse microbial populations. Methanogenesis also occurred in some enrichments and it was not resolved whether this activity was correlated to the dechlorination observed in these cultures. The addition of BESA to the *m*-MCP enrichment, however did not inhibit dechlorination (data not shown).

Discussion

Dechlorination of MCPs and DCPs in the microcosms occurred in the presence of low concentrations (< 5 mM) of nitrate, however initial chlorine removal was not mediated by denitrifiers. Although concurrent denitrification and reductive dechlorination did occur, these activities were clearly not coupled, since equivalent dechlorination was sustained in enrichments in the absence of nitrate. Reductive dechlorination activity in these enrichments continued as long as a suitable electron donor was present. When high nitrate concentrations (> 5 mM) were present, there was little effect on denitrification activity, however significant inhibition of dechlorination did occur. The reason other studies have not observed concurrent dechlorinating and denitrifying activity in chlorophenol enrichments may be because excessive nitrate concentrations were used, generally 15 mM or greater (Genthner et al. 1989; Häggblom et al. 1993). High nitrate concentrations are contrary to what would naturally be present in the environment, and low nitrate levels would provide a more permissive environment for these two processes. Indeed nitrate additions made in low concentration increments may be best for optimizing CP degradation rates, since the products of dechlorination may be removed by the denitrifiers present. The enrichment cultures were even more sensitive to nitrate, suggesting that the microcosm environment has a buffering effect on its inhibitory activity allowing dechlorination to continue. *o*-MCP seems to be unique in that its dechlorination is less sensitive to nitrate concentration. This is consistent with recent pure culture studies which have shown *o*-MCP dechlorination in the presence of 1 mM nitrate (Cole et al.; Sanford 1996).

The appearance of phenol and chlorophenols as products of dechlorination in the microcosms indicate reductive dechlorination (Suflita et al. 1982). This is further supported by the enrichment culture results where stoichiometric concentrations of dechlorinated phenolic products are observed in the medium if electron donors (i.e. acetate) are provided. There is precedence for reductive dechlorination to be coupled to respiratory processes in anaerobic microorganisms (Dolfing 1990; Mohn & Tiedje 1990; Cole et al. 1994). This halorespiration would be occurring in the microcosms and enrichments when the CPs are used as terminal electron acceptors. Whether individual dechlorinating populations are halorespiring the CPs has yet to be determined in these cultures. The significant proportion of reducing equivalents from acetate used for reductive dechlorination of *o*-MCP, however suggests that the MCP is the likely physiological electron acceptor. Halorespiration is a reasonable possibility for two reasons. First the free energy available from the dechlorination of MCPs and DCPs is about -160 kJ/rxn (Dolfing & Harrison 1992), which is similar to that available from the reduction of nitrate to nitrite (- 163 kJ/rxn) (Thauer et al. 1977). Second, the ability for acetate to serve as an electron donor in the enrichments is suggestive of halorespiration since the oxidation of acetate to H_2 and CO_2 is not exergonic (+ 105 kJ/rxn) unless it is coupled to the reduction of a suitable electron acceptor like nitrate or CP (- 134 kJ/reductive dechlorination). Recently Cole and co-workers isolated an *o*-MCP reductively dechlorinating culture (strain 2CP-1) which grows with acetate as an electron donor, establishing that chlorophenol respirers exist (Cole et al. 1994). The microbial populations in the current study mediate this dechlorination only when suitable electron donors were present, a feature consistent with halorespiration.

Recently several DCP dechlorinating isolates have been characterized. The trichlorophenol and DCP dechlorinating anaerobic spore former, *Desulfitobacterium hafniense* DCB-2, was isolated (Madsen & Licht 1992) and recently named (Christiansen &

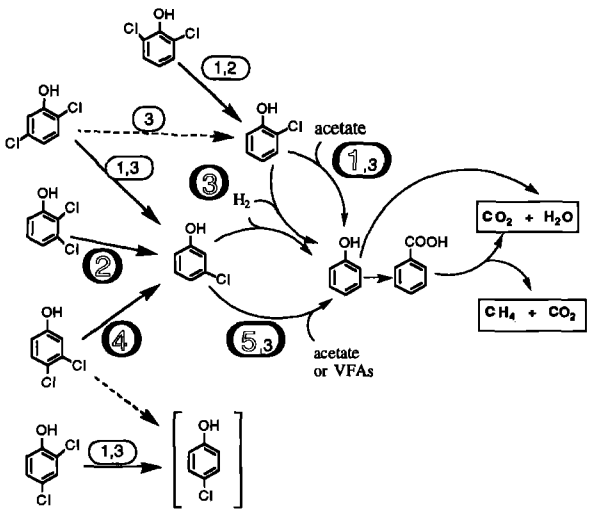

Figure 4. Schematic representation that summarizes the different dechlorination and degradation activities observed in the enrichments. Large numbers indicate the predominant reaction mediated by a particular enrichment and small numbers indicate additional dechlorination reactions that are observed in these cultures. (Numbers correspond to the following enrichments: reaction 1 = *o*-MCP; reaction 2 = 2,3-DCP; reaction 3 = *o*- and *m*-MCP; reaction 4 = *m*-MCP and reaction 5 = 3,4-DCP) Reactions 1, 2 and 3 represent distinct *ortho* dechlorinating pathways. Cultures mediating reaction 1 do not dechlorinate 2,3-DCP. *Meta* dechlorination is mediated by at least two different communities depicted by reactions 3 and 5. *Para* dechlorination is only observed for 3,4-DCP as shown by reaction 4. The fate of phenol is summarized in two possible pathways; denitrifying and methanogenic through benzoate.

Ahring 1996). Another gram-positive 2,4-DCP dechlorinating bacterium, *Desulfitobacterium dehalogenans*, was isolated from enrichments that had been heat-treated, implying the presence of spores (Utkin et al. 1994). However, it was not shown whether these two cultures will grow using the chlorinated phenolic compounds as their sole physiological electron acceptor. A third *Desulfitobacterium*, strain PCE1, was shown to reductively dechlorinate tetrachloroethene and couple growth to the reductive dechlorination of *ortho*-chlorinated phenols (Gerritse et al. 1996). Microscopic examinations of one of our dechlorinating enrichments (2,3-DCP) did show the presence of spore- formers. Although the data here are not sufficient to determine if this spore-former was responsible for dechlorination, subsequent work resulted in isolation of such an organism, *Desulfitobacterium chlororespirans* (Sanford et al. 1996).

The dechlorination activities exhibited by the different enrichments are summarized in Figure 4. Our evidence suggests that each of these activities may be carried out by a different population, indicating considerable diversity in chlorophenol dechlorinating organisms. Figure 4 suggests possible degradation pathways for the MCPs and DCPs in the enrichments studied. Even though some chlorophenols are used by more than one enrichment culture, there are substrate specificities that differentiate these cultures so that they could co-exist in an environment where a mixture of substrates exist. Those specific reductive dechlorinations mediated by different enrichment cultures would potentially cross-feed each other in the overall microbial community. Enrichment culture specific dechlorination reactions are numbered to indicate each potentially distinct microbial population. The compost soil enrichments are able to mediate four of the five suggested dechlorination reactions as well as both denitrifying and methanogenic degradation pathways for phenol. Step one, involving *o*-MCP dechlorination to phenol, has been shown to be independent of the dechlorination of *m*-MCP (step 5) or the dechlorination of the 3,4-DCP (step 4), which involves an initial *para* dechlorination. It might be reasoned that the *ortho* dechlorinating population would dechlorinate all the DCPs, however the *o*-MCP (step 1) cultures were not able to dechlorinate 2,3-DCP (Table 2). As would be expected a separate enrichment culture exhibits an alternative *ortho* dechlorination pathway which did dechlorinate 2,3-DCP (step 2). This 2,3-DCP enrichment is not able to use *o*-MCP, which indicates that the microbial populations in the enrichments that mediate step one and two could coexist when both substrates are present. Also included in Figure 4 is a third *ortho* dechlorination reaction, which exhibits the simultaneous dechlorination of *ortho*- and *meta*-substituted MCPs and 2,5-DCP (step 3) with hydrogen as an electron donor. This latter suggestion is derived from the transient appearance of H_2 in the headspace of enrichments in the absence of methanogenesis, suggesting a direct coupling to dechlorination. In summary the evidence suggests that there are three different *ortho* dechlorinating populations, two different *meta* dechlorinating populations and at least one population capable of *para*-dechlorination.

The rate of dechlorination from substitution position, namely *ortho*> *meta*> *para*, was similar to those previously observed, although surface soil populations and CP mixtures have not generally been studied. (Boyd & Shelton 1984; Hrudey et al. 1987; Kohring et al. 1989; Genthner et al. 1989b; Dietrich & Winter 1990; Hale et al. 1991; Häggblom et al. 1993). One difference in our study was that the *para* rather than *meta*

chlorine was removed from 3,4-DCP. However, this is the predicted activity based on the electronegativity of the chlorine in the *para* position which makes it the better leaving group (Cozza & Woods 1992). Previous enrichments studied had shown that *meta* dechlorination of 3,4-DCP is the apparent predominant biological dechlorination activity (Mikesell & Boyd 1985; Woods et al. 1989).

One of the interesting aspects of the results is the influence of source material on the presence of both denitrification and dechlorination. In contrast to other studies, mostly surface soils were used in our microcosms in order to favor the recovery of denitrifiers. In general microcosms seeded with non- contaminated soils exhibited more activity than those with contaminated soil. Four of the five observed different dechlorination reactions were recovered from the compost soil microcosms. The reductive removal of chlorines from *o*-MCP was recovered in both compost and tropical soil derived enrichments. A possible selective basis for the indigenous dechlorination ability in these soils may be the presence of naturally occurring chlorinated phenols (Siuda & Debernardis 1973; Gribble 1992).

De Jong et al. (1994) established the chlorinated anisyl metabolites produced by white rot fungi can easily be transformed by bacteria into chlorophenols, in this case 3-chloro-anisaldehyde and 3,5-dichloroanisaldehyde to *o*-MCP and 2,6-DCP, respectively. This implies that high organic matter soils with a potentially large fungal population could be reservoirs for significant amounts of CPs.

Although dechlorination and denitrification in the microcosms are not mediated by the same bacterial population it is possible that the degradation of phenol, the main product of dechlorination, may be stimulated through the addition of low concentrations of nitrate. This could be advantageous in formulating remediation strategies for contaminated sites. Chlorophenolic compounds would continue to be anaerobically dechlorinated and the products will be subsequently mineralized by the denitrifiers present. In contrast additions of high nitrate concentrations would inhibit the initial reductive dechlorination, resulting in reduced CP mineralization.

We have shown that dechlorination and denitrification activity can be concurrently or sequentially maintained in an active microcosm, but that these processes are mediated by physiologically different populations. We found no evidence for the existence of denitrifiers that dechlorinate MCPs or DCPs under nitrate reducing conditions. Anaerobic dechlorination of MCPs and DCPs is complex, with at least five different types of reactions observed.

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