

**Bacterial oxidation
of low-chlorinated compounds
under anoxic conditions**

Promotor: Prof. dr. ir. A.J.M. Stams
Persoonlijk hoogleraar bij het laboratorium voor Microbiologie
Laboratorium voor Microbiologie, Wageningen Universiteit

Copromotoren: Dr. G. Schraa
Universitair docent
Laboratorium voor Microbiologie, Wageningen Universiteit

Dr. J. Gerritse
Onderzoeks- en projectleider
Milieubiotechnologie, TNO Milieu, Energie en Procesinnovatie

Promotiecommissie: Prof. dr. D.B. Janssen
Rijksuniversiteit Groningen

Prof. dr. W. Reineke
Bergische Universitat Wuppertal, Duitsland

Prof. dr. ir. S.E.A.T.M. van der Zee
Wageningen Universiteit

Dr. T.N.P. Bosma
TNO-NITG, Utrecht

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J.A. Dijk

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J.A. Dijk

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*Progress is a debt we all must pay
Its convenience we all cherish
Its pollution we disdain
(Bad Religion – Progress)*

General introduction

1.1 Introduction

Human activities have led to contamination of the environment throughout the world. As a result of spillage, leakage, improper waste disposal policies, accidents and their intended use, xenobiotics entered soil and groundwater. Here, they form an increasing threat towards ecosystems and affect human health.

An important group of contaminants consists of chlorinated hydrocarbons. These compounds belong to the most frequently found contaminants in soil and groundwater. Industrial applications range from degreaser and solvent to biocide and precursor. For example, 1,2-dichloroethane and vinyl chloride are used for the production of poly(vinyl chloride) (PVC) and are among the most produced bulk organic chemicals in the U.S. (40). In Table 1.1 the emission and source of emission of the most important chlorinated aliphatic hydrocarbons in the Netherlands in 1998 are listed. The amount of a chlorinated compound that will eventually end up in the environment depends mainly on its use and its chemical properties. The total amount of chlorinated aliphatic hydrocarbons and chlorinated aromatic hydrocarbons that reached surface waters in the Netherlands in 2001 was 11.232 and 1.890 ton/year, respectively (Datawarehouse Emission Inventory, 2003).

Besides being formed via anthropogenic production, many chlorinated compounds are naturally produced (51,52). An example of a chlorinated hydrocarbon of biogenic origin is chloromethane, which is synthesized by evergreen trees, potato tubers, mushrooms, the iceplant, marine algae and giant kelp, a bryozan and certain Basidiomycetes (51,71,72).

Many chlorinated hydrocarbons are toxic and recalcitrant, which increases their potential threat to the environment. Therefore, it is of great importance to clean-up contaminated sites.

A possible clean-up technique is bioremediation (bioremediation) in which biological processes are used to solve an environmental problem. Microorganisms are important in this respect, considering their abundance, phylogenetic diversity, broad biodegradative capabilities, high metabolic activity and evolutionary potential. Bioremediation by means of microorganisms can include: 1) a method of monitoring the natural progress of degradation to ensure that the contaminant concentration decreases with sampling time (bioattenuation), 2) the intentional stimulation of resident xenobiotic-degrading bacteria by addition of electron acceptors, water, nutrients, or electron donors (biostimulation), or 3) the addition of (laboratory grown) bacteria that have the appropriate degradative abilities (bioaugmentation) (118). To achieve successful bioremediation, knowledge about *in situ* biodegradation processes is of utmost importance. On the one hand it is essential to know whether or not and under which conditions *in situ* biodegradation can occur at a significant rate. In this respect, the environmental conditions (temperature, pH, soil structure,

soil humidity, bioavailability, nutrients etc.) are crucial. On the other hand physiological information about the microorganisms is needed in order to know how to stimulate the process. Biochemical and genetic information is important for monitoring *in situ* biodegradation and for bioaugmentation. Therefore, extensive research on microbial transformation of chlorinated hydrocarbons has been done, especially on aerobic degradation and anaerobic reductive dehalogenation processes.

Table 1.1 Emission and source of chlorinated aliphatic hydrocarbons in the Netherlands in 1998.

Chlorinated aliphatic hydrocarbon	Abbreviation	Emission ¹⁾ (ton/yr)	Major use ¹⁾
dichloromethane (methylene dichloride)	DCM	984	rubber-, medicine industry
trichloromethane (chloroform)	CF	43.7	solvent
tetrachloromethane (carbon tetrachloride)	CT	14.8	solvent, fiber industry solvent, extraction
1,2-dichloroethane (ethylene dichloride)	DCA	35.7	PVC production
1,1,1-trichloroethane (methylchloroform)	TCA	206	metal degreasing
chloroethene (vinyl chloride)	VC	51	PVC production
trichloroethene	TCE	1220	solvent, dry cleaning
tetrachloroethene (perchloroethene)	PCE	2030	metal degreasing

¹⁾ Report series Environmental Monitor (2002). Inspectorate of Housing, Spatial Planning and the Environment.

1.2 Microbial degradation of chlorinated hydrocarbons.

Microbial degradation is one of the key processes that determine the fate of chlorinated hydrocarbons in the environment. The cleavage of the carbon-chlorine bond is often the crucial step in the degradation pathway. Once the chlorine substituent has been removed, the hydrocarbon can be more easily assimilated in the organisms' central metabolism (60). Microorganisms have evolved a variety of metabolic strategies for degrading chlorinated hydrocarbons.

Table 1.2 Possible microbial energy-yielding reactions for 1,2-dichloroethane (DCA) degradation and the corresponding Gibbs free energy values.

Electron donor / acceptor	Proposed reaction equation	ΔG° /1,2-DCA transformed (kJ/mol)
<i>Energy yielding oxidations</i>		
DCA/oxygen	$2 \text{C}_2\text{H}_4\text{Cl}_2 + 5 \text{O}_2 \rightarrow 4 \text{CO}_2 + 4 \text{Cl}^- + 4 \text{H}^+ + 2 \text{H}_2\text{O}$	-1295
DCA/nitrate	$\text{C}_2\text{H}_4\text{Cl}_2 + 2 \text{NO}_3^- \rightarrow 2 \text{CO}_2 + 2 \text{Cl}^- + \text{N}_2 + 2 \text{H}_2\text{O}$	-1229
DCA/manganese oxide	$\text{C}_2\text{H}_4\text{Cl}_2 + 5 \text{MnO}_2 + 8 \text{H}^+ \rightarrow 2 \text{CO}_2 + 2 \text{Cl}^- + 5 \text{Mn}^{2+} + 6 \text{H}_2\text{O}$	-896
DCA/ferrihydrite	$\text{C}_2\text{H}_4\text{Cl}_2 + 10 \text{Fe}(\text{OH})_3 + 18 \text{H}^+ \rightarrow 2 \text{CO}_2 + 2 \text{Cl}^- + 10 \text{Fe}^{2+} + 26 \text{H}_2\text{O}$	-249
<i>Energy yielding reduction</i>		
Formate/DCA	$\text{C}_2\text{H}_4\text{Cl}_2 + \text{CHOOH} \rightarrow \text{C}_2\text{H}_4 + \text{CO}_2 + 2 \text{Cl}^- + 2 \text{H}^+$	-234
<i>Fermentation</i>		
DCA/DCA	$6 \text{C}_2\text{H}_4\text{Cl}_2 + 4 \text{H}_2\text{O} \rightarrow 5 \text{C}_2\text{H}_4 + 2 \text{CO}_2^- + 12 \text{Cl}^- + 12 \text{H}^+$	-185

Gibbs free energy values were calculated according to the formula $\Delta G^\circ = \sum \Delta G_f^\circ$ (products) - $\sum \Delta G_f^\circ$ (substrates). The ΔG_f° values were taken from (59). The standard conditions were: T = 25°C; pH = 7; O₂, H₂, N₂, CO₂, DCA and ethene as gaseous compounds at a pressure of 1 atm, Cl⁻, NO₃⁻, Mn²⁺, Fe²⁺ and formate at aqueous concentrations of 1 M and MnO₂ and Fe(OH)₃ as solid compounds (1 mol).

These overall-metabolisms can be divided broadly into five categories; energy-yielding oxidation, co-metabolic oxidation, fermentation, energy-yielding reduction and co-metabolic reduction reactions (40,60,80,81,114). Proposed reaction equations and Gibbs free energy values for the energy-yielding reactions of 1,2-dichloroethane degradation are depicted in Table 1.2.

1.2.1 Energy-yielding oxidation reactions

A wide range of chlorinated hydrocarbons can be used as sole energy and carbon source in the presence of oxygen as electron acceptor. Chlorinated aliphatic hydrocarbons that support microbial growth include chloromethanes, chloroethanes, chloropropanes and chloroethenes (97,114). For example, *Xanthobacter autotrophicus* GJ10 can grow on 1,2-dichloroethane, 1-chloropropane and 1,3-dichloropropane as sole carbon and energy source in the presence of oxygen (70). Chlorinated aromatic hydrocarbons can also be used as sole energy and carbon source. Monochlorobenzene is an example of a growth substrate for several aerobic bacteria (95,98,101).

Energy-yielding oxidations in the absence of oxygen rely on alternative electron acceptors, like nitrate, iron(III), manganese(IV), humic acids and sulfate. Chloromethane and dichloromethane can be used as sole carbon and energy source under denitrifying conditions (43,44,77). Anaerobic oxidation of other chlorinated aliphatic hydrocarbons has been observed under different electron-accepting conditions. For example, dichloroethene can be completely oxidized to carbon dioxide under iron(III)-, sulfate-, manganese(IV)- and humic acids-reducing conditions (15,20,21). However, due to lack of pure cultures or even defined mixed cultures it is not yet known if these oxidations are energy-yielding. The anaerobic oxidation of chlorinated hydrocarbons will be further discussed in section 1.4.

Finally, facultative anaerobic bacteria, like most known denitrifiers, might benefit from the combination of oxygen and other electron acceptors in environments where oxygen is limiting. It was speculated that under mixed oxic/denitrifying conditions biodegradation of aromatic hydrocarbons may occur through a combination of oxygen and nitrate utilization (120). Very low levels of oxygen might result in oxidation of the benzene ring, followed by further degradation of the formed intermediates with nitrate as electron acceptor. However, it is not yet clear whether oxygen and nitrate are used simultaneously or sequentially and which mechanism(s) are involved.

1.2.2 Co-metabolic oxidation reactions

The original definition of co-metabolism is the transformation of an organic compound by a microorganism that is unable to use the substrate as a source of energy or of one of its constituent elements (2). This transformation is merely a fortuitous

modification by enzymes or cofactors, which normally catalyze other reactions (65). Since microorganisms do not benefit from this co-metabolic transformation they need another substrate for their growth. Co-metabolism is considered to be a consequence of broad enzyme specificity, imprecise induction specificity and other processes (116). A variety of aerobic bacteria is capable of oxidizing chlorinated hydrocarbons co-metabolically. This co-metabolic oxidation is particularly catalyzed by mono- and dioxygenase systems (40). The chlorinated hydrocarbon competes with the growth substrate for the active site of the enzyme. For example, several chlorinated alkenes, including trichloroethene, *cis*- and *trans*-1,2-dichloroethene, vinyl chloride, 1,3-dichloropropene and 2,3-dichloropropene can be co-metabolically degraded by alkene monooxygenase from *Xanthobacter* strain Py2 (38).

1.2.3 Fermentation reactions

In fermentation reactions chlorinated hydrocarbons serve as electron donor and a dechlorinated intermediate (or the chlorinated hydrocarbon itself) serves as electron acceptor. This microbial metabolism is different from the above mentioned process, as no externally supplied electron acceptor is needed.

There are indications for the fermentation of tetrachloromethane, 1,2-dichloroethane, vinyl chloride and trichloroethene, but the underlying mechanisms are not known yet (16-18,31,48).

Two homoacetogenic bacteria have been described that use chlorinated hydrocarbons as sole carbon and energy source by means of fermentation, namely *Acetobacterium dehalogenans* and *Dehalobacterium formicoaceticum*. *Acetobacterium dehalogenans* ferments chloromethane to acetate and carbon dioxide and *Dehalobacterium formicoaceticum* ferments dichloromethane to acetate and formate as end products (85,111). These latter two fermentation processes could also be seen as an energy-yielding oxidation since carbon dioxide from the medium is reduced and incorporated in the acetyl-CoA synthase reaction to yield acetate. In this introduction we refer to this metabolism as fermentation since in literature the term "fermentation" is also commonly used. The fermentation pathways will be further discussed in section 1.4. There are also indications that acetogenesis is involved in the fermentation of vinyl chloride (17,18,31).

1.2.4 Energy-yielding reduction reactions

During energy-yielding reduction, also known as halorespiration or dehalorespiration, a chlorinated compound is used as terminal electron acceptor for growth. Several bacterial pure cultures have been described that are able to utilize chlorinated hydrocarbons as terminal electron acceptors. For an overview see (12,68,92,103). Especially the respiration with chlorinated ethenes, which are of particular environmental concern, is well studied. Several bacteria have been described that

can reductively dechlorinate tetrachloroethene to trichloroethene (*Desulfitobacterium frappieri* PCE1 and *Desulfitobacterium* sp. Viet1), dichloroethene (several *Sulfurospirillum*, *Desulfitobacterium*, *Dehalobacter* and *Desulfuromonas* species and *Enterobacter* strain MS-1) or vinyl chloride (*Dehalococcoides ethenogenes* 195) (12,64,83). The reductive dechlorination of chlorinated ethenes at polluted sites is often incomplete and leads frequently to the accumulation of dichloroethene and vinyl chloride. Only recently it has been demonstrated that also the reductive dechlorination of vinyl chloride to ethene can be coupled to growth by *Dehalococcoides* strain BAV1 (62). Other low-chlorinated aliphatic hydrocarbons that can be used as terminal electron acceptor include, 1,2-dichloroethane, 1,1,1-trichloroethane, 1,2-dichloropropane, 1,2-dichlorobutane, 2,3-dichlorobutane and dichloroethene (62,82,87,88,109,119).

1.2.5 Co-metabolic reduction reactions

Co-metabolic reduction of chlorinated hydrocarbons is widespread under fermentative, methanogenic, acetogenic, sulfate-reducing and iron-reducing bacteria (for a definition of co-metabolism see section 1.2.2). C1 and C2 chlorinated hydrocarbons like tetrachloromethane, 1,2-dichloroethane, 1,1,1-trichloroethane and tetrachloroethene can be co-metabolized by a broad spectrum of bacteria (36,65). For instance, 1,2-dichloroethane is reductively dechlorinated to chloroethane and ethene by several methanogenic bacteria (6,34,66).

Compared with energy-yielding reductive dechlorination, anaerobic co-metabolic dechlorination reactions proceed at much lower rates.

Enzyme systems that are involved in co-metabolic reduction processes include protein-bound tetrapyrrole cofactors (iron(II) porphyrins, corrinoids or cofactor F₄₃₀), flavoprotein-flavin complexes and ferredoxins (45,64,67,78,79).

1.3 Anaerobic oxidative degradation of low-chlorinated hydrocarbons.

In contaminated anoxic subsurface systems, polychlorinated ethenes like tetrachloroethene and trichloroethene are often reductively dechlorinated to dichloroethene and vinyl chloride (12,92). Vinyl chloride however, is a bigger threat to human health than tetrachloroethene and trichloroethene, since it is known to be carcinogenic and more mobile than the higher chlorinated ethenes. So besides direct release, low-chlorinated hydrocarbons can also arise in soil and groundwater systems as a result of partial dechlorination of higher chlorinated hydrocarbons.

The fate of those low-chlorinated hydrocarbons in the environment is strongly dependent on microbial processes. The tendency of chlorinated hydrocarbons to

undergo oxidative dehalogenation generally increases upon a decrease in the number of chlorine substituents (Fig. 1.1) (113,114).

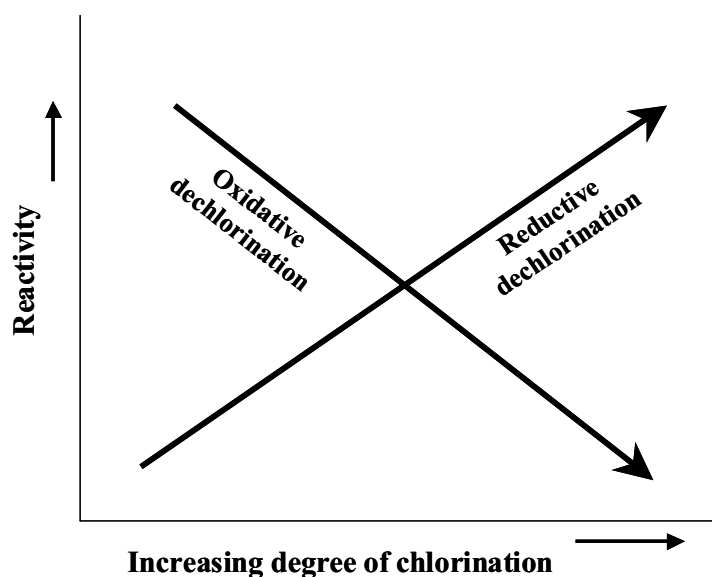


Figure 1.1 Relative trends of oxidative versus reductive dechlorination as a function of the degree of chlorination (adapted from (1,10,114))

For example, reductive dechlorination of vinyl chloride is often slow, whereas higher chlorinated ethenes are readily dechlorinated under anoxic conditions (3,4,5,11,23,25,30,39,41,89,96,115,122). Whether low-chlorinated hydrocarbons are more likely to be degraded by oxidation than by reduction processes under anoxic conditions depends primarily on environmental conditions (e.g. redox conditions, availability of electron acceptors/donors, present microorganisms).

Oxygen is often not present in soil and groundwater that are contaminated with chlorinated hydrocarbons. This implies that their microbial degradation relies on anaerobic microorganisms and that anaerobic oxidation is potentially an important process for bioremediation of the low-chlorinated compounds.

The anaerobic oxidation of low-chlorinated hydrocarbons by bacteria is the process in which electrons, released during their oxidation, are transferred to another electron acceptor than oxygen (Fig. 1.2). Some important electron acceptors with their corresponding redox couple and standard redox potential are listed in Table 1.3. The information on low-chlorinated hydrocarbons that can serve as electron donors in the absence of oxygen is however restricted.

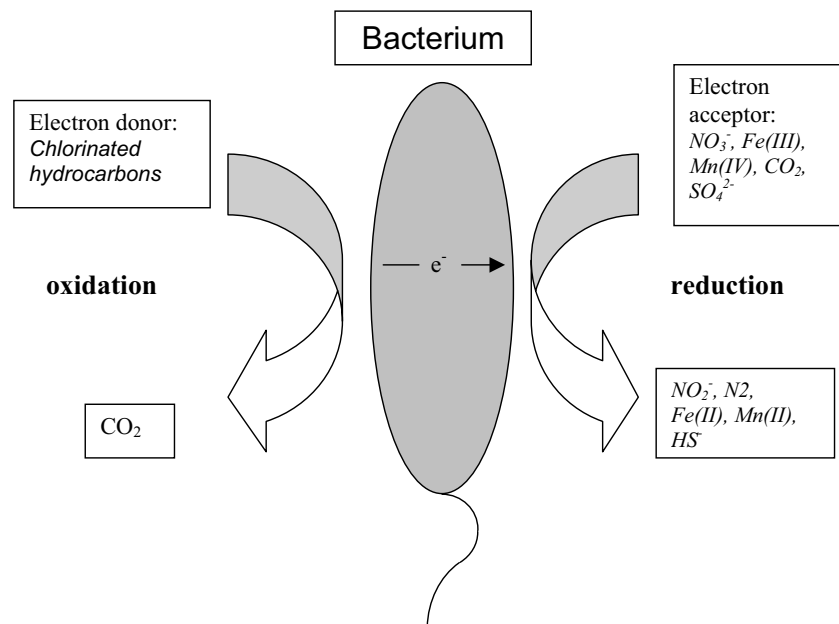


Figure 1.2 Schematic representation of the anaerobic oxidation of chlorinated hydrocarbons linked to respiration with different electron acceptors.

Table 1.3 Redox potentials of some electron acceptors and their corresponding redox couples.

Electron acceptor	Redox couple	Redox potential (mV) ¹⁾
Oxygen	O ₂ /H ₂ O	+820
Iron(III) (soluble)	Fe ³⁺ /Fe ²⁺	+770
Nitrate	NO ₃ ⁻ /N ₂	+740
Nitrate	NO ₃ ⁻ /NO ₂ ⁻	+430
Manganese(IV)	MnO ₂ /Mn ²⁺	+380
Fe(OH) ₃ (amorph)	Fe(OH) ₃ /Fe ²⁺	+59 ²⁾
Fumarate	Fumarate/succinate	+33
Iron(III) (solid)	Fe ³⁺ /Fe ²⁺	0
2,6-AQDS	2,6-AQDS/2,6-ADHS	-184 ³⁾
Humic acids		-200 to +300 ⁴⁾
Sulfate	SO ₄ ²⁻ /HS ⁻	-230
Carbon dioxide	CO ₂ /CH ₄	-240

¹⁾ Standard redox potential at pH 7.0 and 25°C (94,110).

²⁾ (63,93)

³⁾ (7)

⁴⁾ (105)

Table 1.4 An overview of anaerobic oxidation or fermentation of low-chlorinated hydrocarbons.

Compound	Electron accepting conditions	End product	Pure or mixed culture	Reference
Chloromethane	Acetogenic	Acetate	Strain MC	(111)
	Nitrate-reducing	n.d. ¹⁾	<i>Pseudomonas aeruginosa</i> strain NB1	(44)
Dichloromethane	Nitrate-reducing	n.d. ¹⁾	<i>Acinetobacter</i> sp., <i>Hyphomicrobium</i> sp. DM2	(43,77)
	Acetogenic	Formate + acetate	<i>Dehalobacterium formicoaceticum</i>	(86)
	Methanogenic	Carbon dioxide + acetate (+ formate)	Enrichment cultures, packed-bed reactor	(9,42,106)
Tetrachloromethane	Acetogenic	Carbon dioxide + acetate + formate	Mixed culture	(22)
1,2-Dichloroethane	Sulfate-reducing	Carbon dioxide + acetate	Packed bed reactor	(9)
	Acetogenic?	n.d. ¹⁺²⁾	Packed bed reactor	(8)
	Nitrate-reducing	n.d. ¹⁾	Microcosms/soil columns	(27,48)
	Nitrate-reducing	Carbon dioxide	Enrichment culture	(29)
Vinyl chloride	Iron(III)-,sulfate- and humic acids-reducing	Carbon dioxide	Microcosms	(13,15,20)
	Methanogenic	Carbon dioxide + methane	Microcosms	(14,16-18,115)

Table 1.4 Continued

Compound	Electron accepting condition	End product	Pure or mixed culture	Reference
Dichloroethene (29% <i>trans</i> and 71 % <i>cis</i> isomers)	Iron(III)-, sulfate-, humic- acid- and manganese(IV)-reducing	Carbon dioxide	Microcosms	(15,20,21)
	Methanogenic	Carbon dioxide ³⁾	Microcosms	(14)

¹⁾ n.d. = not determined

²⁾ Sometimes chloroform and dichloromethane found as transformation products

³⁾ Also detection of vinyl chloride, ethene and ethane

Six low-chlorinated alkanes and alkenes have been described that can be degraded anaerobically with different terminal electron acceptors without the addition of other electron donors (9,13,14,15-18,20,21,22,27,29,43,44,48,77,86,111,115). Because the division between anaerobic oxidation and fermentation processes is indistinct in some of these observations due to possible involvement of acetogens and/or methanogens, these fermentation processes are included (see also section 1.2.3). In Table 1.4 an overview is given of these low-chlorinated hydrocarbons that can be oxidized or fermented under iron(III)-reducing, manganese(IV)-reducing, nitrate-reducing, sulfate-reducing, humic acids-reducing, methanogenic or acetogenic conditions. *Dehalobacterium formicoaceticum* and *Acetobacterium dehalogenans* are the only pure cultures of strictly anaerobic bacteria that have been isolated so far that use aliphatic chlorinated hydrocarbons as carbon and/or energy source for growth. Both bacteria ferment the chlorinated substrate in the presence of carbon dioxide (see section 1.4). Two facultative anaerobic bacteria; *Acinetobacter* sp. and *Hyphomicrobium* sp. DM2 have been described that can oxidize dichloromethane with nitrate as electron acceptor. Recently, another facultative anaerobic bacteria; *Pseudomonas aeruginosa* strain NB1 was described that uses chloromethane as its sole source of carbon and energy under nitrate-reducing conditions (44). The other mentioned anaerobic degradation processes of low-chlorinated hydrocarbons under different terminal electron accepting conditions are observed in non-pure cultures (Table 1.4).

Even though for some degradation processes only carbon dioxide is found as end product it is not known if these processes are direct oxidations by a single organism or net oxidations by multiple organisms. Due to the lack of pure cultures or defined mixed cultures, it is not yet known if these oxidations are energy-yielding.

Anaerobic oxidation of low-chlorinated aromatic hydrocarbons has not yet been described, although there are some indications that the oxidation of monochlorobenzene in the absence of oxygen might be possible (74,117). Chlorinated phenols and benzoates on the other hand are known to be degraded under different electron-accepting conditions. Chlorinated benzoates and phenols are dechlorinated under methanogenic conditions to benzoate and further metabolized to methane and carbon dioxide (46,47,49,50,69,92,108). The initial step of degradation under methanogenic conditions is generally reductive dechlorination (65,92). Syntrophic associations between the dehalogenating and other microorganisms are important for the overall degradation. For example, in a defined mixed-culture *Desulfomonile tiedjei* reductively dechlorinated 3-chlorobenzoate to benzoate, which was further converted to acetate and methane by a benzoate fermenting rod-shaped bacterium and a hydrogen-consuming methanogen. The hydrogen produced by the benzoate fermenting bacterium was partially used as electron donor for the reductive dechlorination of 3-chlorobenzoate (28,32,102).

Chlorinated benzoates and phenols can also be anaerobically degraded in the presence of sulfate, iron(III) or nitrate as electron acceptor (54,55,56,57,75,76,104). Complete oxidation of these compounds to carbon dioxide may be coupled to processes such as denitrification, iron(III)-reduction and sulfate reduction. Several pure cultures of Proteobacteria have been isolated with 3-chlorobenzoate as sole carbon and energy source under denitrifying conditions (58,104). Attempts to isolate chlorophenol-oxidizing bacteria under denitrifying conditions are unsuccessful so far. Since no pure cultures or defined mixed cultures have been obtained under sulfate- and iron(III)-reducing conditions, it remains unclear whether a single bacterium is sufficient or that a consortium is needed for the complete oxidation of chlorinated benzoates and phenols. Reductive dechlorination was found to be the first step of degradation under sulfate- and iron(III)-reducing conditions like under methanogenic conditions which suggests the involvement of a consortium (53,83).

Several strains of *Rhodopseudomonas palustris*, a purple nonsulphur phototrophic bacterium, have been reported to utilize 3-chlorobenzoate as sole carbon source under anoxic conditions with light as the energy source (33,73,121). A study on the degradation of 3-chlorobenzoate by *Rhodopseudomonas palustris* under anoxic conditions suggested that 3-chlorobenzoate was first converted to 3-chlorobenzoyl-coenzyme A (3-chlorobenzoyl-CoA), followed by reductive dechlorination to benzoyl-CoA, which is further degraded to acetyl-CoA and carbon dioxide (33).

1.4 Biochemistry of anaerobic oxidation or fermentation of low-chlorinated aliphatic hydrocarbons.

Only limited information about the biochemistry of the anaerobic oxidative degradation or fermentation of low-chlorinated hydrocarbons is available. A few bacteria have been isolated that can (partly) oxidize or ferment low-chlorinated hydrocarbons in the absence of oxygen (Table 1.4). The methylotrophic homoacetogenic bacterium *Acetobacterium dehalogenans* ferments chloromethane and forms three moles of acetate per four moles of chloromethane fermented by the incorporation of carbon dioxide. The remaining mol of chloromethane is oxidized to carbon dioxide (Fig. 1.3 A) (111). The first step of this fermentation reaction is catalyzed by a chloromethane dehalogenase. This enzyme transfers the methyl group of chloromethane onto tetrahydrofolate, yielding inorganic chloride and methyl tetrahydrofolate (91). Experiments with crude cell extracts established the further metabolism of methyl tetrahydrofolate to acetate via the reactions of the acetyl-CoA pathway (90). For dichloromethane (DCM) degradation in the strictly anaerobic *Dehalobacterium formicoaceticum* it has been found that tetrahydrofolate was also involved (85). Dichloromethane was converted to methylene tetrahydrofolate, of

which two-thirds was oxidized to formate while one-third gave rise to acetate by incorporation of carbon dioxide from the medium in the acetyl-CoA synthase reaction (Fig. 1.3 B). The methylene group of dichloromethane is transferred onto tetrahydrofolate by a dichloromethane dehalogenase. There are, however, major differences between this dichloromethane dehalogenase activity and the chloromethane dehalogenase activity. The enzyme system of *D. formicoaceticum* must cleave two carbon-chlorine bonds to yield a C₁ tetrahydrofolate product, whereas chloromethane dehalogenase of strain MC only has to cleave a single carbon-chlorine bond. Mägli *et al.* (85) suggest that a reduced Co(I) corrinoid can activate dichloromethane that is much less reactive to nucleophilic displacement than chloromethane.

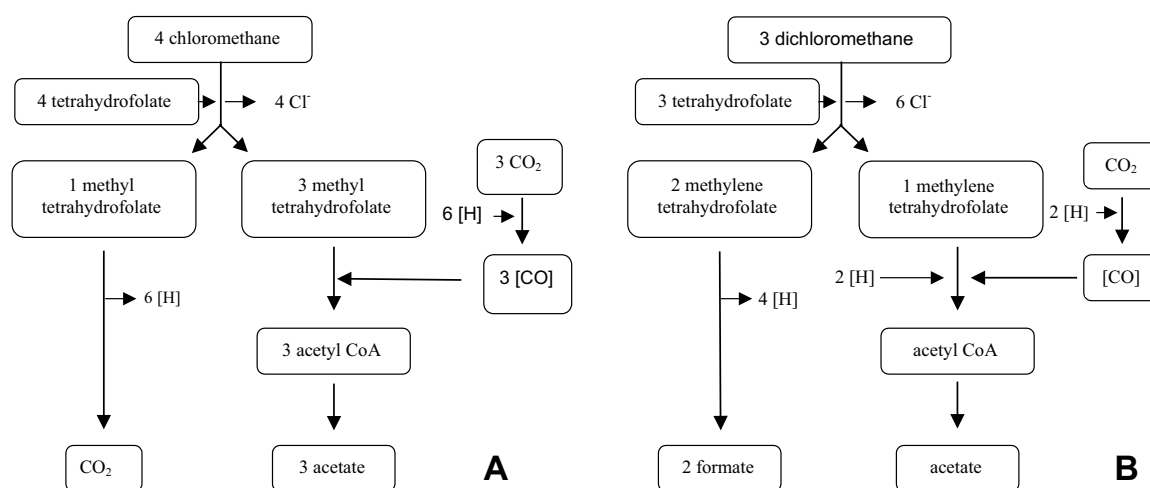


Figure 1.3 A and B. Scheme of the anaerobic metabolism of chloromethane by *Acetobacterium dehalogenans* (A) and the anaerobic metabolism of dichloromethane by *Dehalobacterium formicoaceticum* (B). Not all steps are mentioned in these schematic representation.

The degradation of dichloromethane by *Hyphomicrobium* sp. strain DM2 and *Acinetobacter* sp. under denitrifying conditions is glutathione dependent like under oxic conditions (43,77). Dichloromethane dehalogenase, the key enzyme of aerobic dichloromethane degradation, was induced in *Hyphomicrobium* sp. strain DM2 under both oxic and denitrifying conditions (77). This dehalogenase catalyzes the first step of dichloromethane utilization that leads to the formation of inorganic chloride and S-chloromethyl glutathione. The latter intermediate is unstable and decomposes to

glutathione, inorganic chloride and formaldehyde, a central metabolite of methylotrophic growth.

The anaerobic degradation pathway(s) of chlorinated ethenes, like vinyl chloride and dichloroethene, must be different from known aerobic pathways since oxygen-requiring monooxygenases are involved in the aerobic degradation pathways (26,61,112). The microorganisms responsible for anaerobic chloroethene oxidations are still unknown and the involved oxidation processes have not been fully characterized.

However, four hypothetical mechanisms for the net anaerobic oxidation of chloroethenes by microorganisms have been proposed: 1) oxidation to carbon dioxide by a single microorganism (13), 2) net oxidation with chloroethanol as intermediate (115), 3) syntrophic oxidation with acetate as intermediate (16-18) and 4) syntrophic oxidation with ethene as intermediate (12,19) (Fig. 1.4).

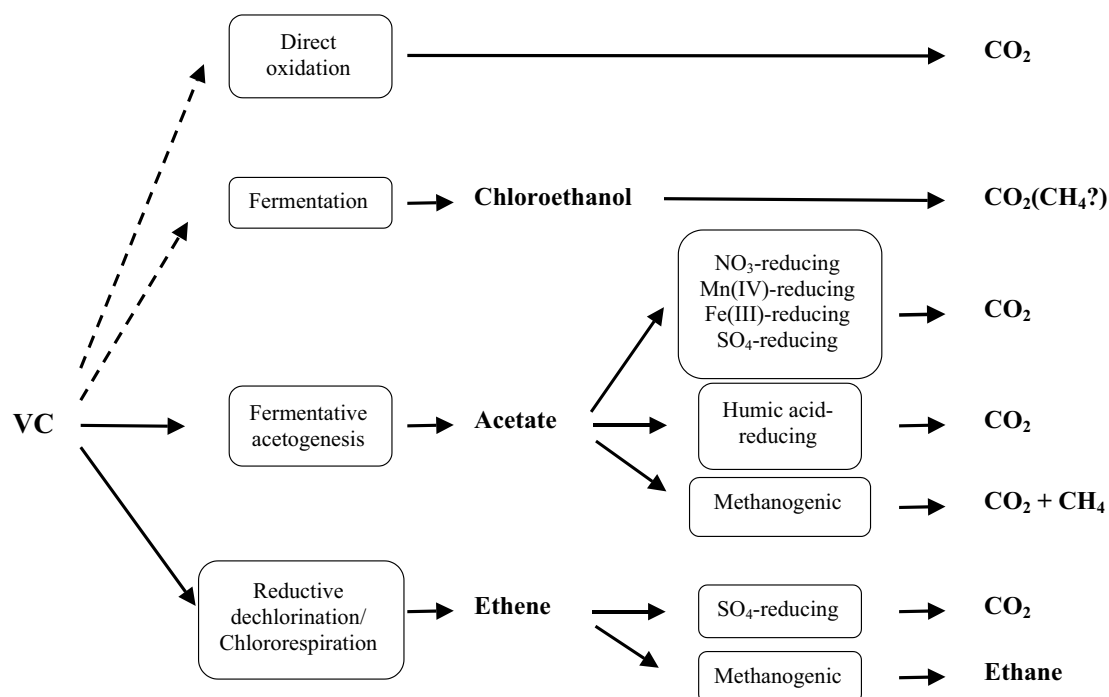


Figure 1.4 Possible model for the anaerobic degradation of VC. The dashed lines indicates pathways that have not been established (adapted from (12)).

Oxidation of vinyl chloride to carbon dioxide has been proposed since no production or accumulation of volatile organic compounds such as ethene could be detected during oxidation of [1,2- ^{14}C] vinyl chloride to ^{14}C labeled carbon dioxide (13). However, subsequent research by Bradley and Chapelle showed that potential

intermediates such as acetate can be quickly metabolized and therefore may not be detected (16-18). In the absence of a pure culture that can anaerobically oxidize vinyl chloride to carbon dioxide this mechanism remains hypothetical.

The second mechanism is based on the aerobic mineralization of 1,2-dichloroethane where the first step is a hydrolytic dechlorination of 1,2-dichloroethane to chloroethanol (70,107). Vogel and McCarty (115) found that [1,2-¹⁴C] vinyl chloride was transformed to ¹⁴C labeled carbon dioxide and they hypothesized that the oxidation of vinyl chloride may involve the addition of water to form chloroethanol, followed by oxidation to chloroacetaldehyde. The aldehyde may be further oxidized to carbon dioxide and perhaps methane (115). However, none of the proposed intermediates was detected in this study and no other evidence was given for this degradation pathway.

Direct evidence for the third mechanism was provided by Bradley and Chapelle, who reported syntrophic anaerobic oxidation of vinyl chloride to carbon dioxide by fermentative acetogens and acetate oxidizing anaerobic microorganisms (16-18). Their studies demonstrated the degradation of [1,2-¹⁴C] vinyl chloride and simultaneous production of ¹⁴C labeled carbon dioxide and ¹⁴C labeled methane with temporary accumulation of ¹⁴C labeled acetate. The acetate was rapidly fermented to ¹⁴C labeled carbon dioxide and ¹⁴C labeled methane and it was concluded that methanogens were responsible for this conversion based on BES (2-bromoethanesulfonic acid, an inhibitor of methanogenesis) addition (18). This finding and the finding that acetate accumulated during vinyl chloride degradation led to the hypothesis that acetogens are responsible for the initial anaerobic degradation of vinyl chloride to a nontoxic product. Thus, the microbial degradation of vinyl chloride to methane and carbon dioxide probably involves fermentative acetogenesis followed by methanogenesis (18). Subsequently, Bradley and Chapelle suggested that fermentative acetogenesis might also be the initial step in the net oxidation of vinyl chloride to carbon dioxide under iron(III)-reducing, sulfate-reducing and humic acids-reducing conditions. The underlying process of acetogenic fermentation is not yet known. The mechanism proposed by Vogel and McCarty (115), in which vinyl chloride is hydrated to chloroethanol, cannot yet be ruled out (12). Simple and substituted alcohols can be easily fermented to acetate (24,35,37,99,100).

Similarly, there is evidence for syntrophic vinyl chloride oxidation with ethene as intermediate (mechanism 4). Studies have demonstrated that respiratory reductive dechlorination of vinyl chloride can be significant even at H₂ concentrations that are characteristic for sulfate-reducing conditions (84,123). Bradley and Chapelle (19) demonstrated that [1,2-¹⁴C] ethene can be completely mineralized to ¹⁴C labeled carbon dioxide under sulfate-reducing conditions. These results suggest that anaerobic degradation of vinyl chloride can result from the reductive dechlorination of vinyl chloride to ethene, followed by an anaerobic oxidation of ethene to carbon

dioxide (19). Thermodynamic calculations indicate that anaerobic ethene oxidation is favorable provided that there is an efficient sink for H₂ (31). The role of H₂ sink may be fulfilled by sulfate-reducing or by the vinyl chloride-respiring bacteria (19,84). The net oxidation of dichloroethene under iron(III)- and sulfate-reducing and methanogenic conditions appeared to involve an initial, rate-determining reduction to vinyl chloride followed by the oxidation of vinyl chloride to carbon dioxide or carbon dioxide and methane (15). Only under manganese(IV)-reducing conditions microorganisms directly oxidized dichloroethene to carbon dioxide without an apparent initial reduction to vinyl chloride (21).

1.5 Outline of this thesis

The aim of the research presented in this thesis was to get a better insight in anaerobic oxidation processes of low-chlorinated hydrocarbons. Two main research questions were addressed, namely can anaerobic oxidation processes of low-chlorinated hydrocarbons occur and if so which organisms and enzymes are involved in these oxidation processes?

Batch and column experiments that were performed to investigate the potential of anaerobic oxidation processes for natural and stimulated degradation of chlorinated and non-chlorinated hydrocarbons are described in chapter 2. Soil from sites contaminated with chlorinated and non-chlorinated hydrocarbons was used.

The isolation and characterization of *Pseudomonas stutzeri* strain JJ, a bacterium that can degrade 2-chloroethanol under denitrifying conditions, are described in chapter 3. This bacterium has been isolated from a 1,2-dichloroethane degrading enrichment culture with nitrate as electron acceptor. Chapter 4 deals with the degradation pathway of 2-chloroethanol in this organism in the absence of oxygen.

The attempt to characterize anaerobic oxidation of monochlorobenzene under nitrate reducing conditions in an up-flow soil column is described in chapter 5. This soil column was used during the experiments described in chapter 2 and was continued with higher concentrations of monochlorobenzene.

The results from the different chapters are summarized and discussed in chapter 6.

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Anaerobic oxidation of (chlorinated) hydrocarbons

**John A. Dijk, Jan A. M. de Bont, Xiaoxia Lu, Petra M. Becker, Tom N. P.
Bosma, Huub H. M. Rijnaarts and Jan Gerritse**

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Abstract

Batch and soil column experiments were performed to investigate the potential of anaerobic oxidation processes for natural and stimulated degradation of chlorinated and non-chlorinated hydrocarbons. Model pollutants which included 1,2-dichloroethane, 1,1,1-trichloroethane, vinyl chloride, *cis*-1,2-dichloroethene, *trans*-1,2-dichloroethene, trichloroethene, monochlorobenzene, 2-chloroethanol and octane were supplied as electron donor under iron (III), manganese (IV) or nitrate reducing conditions. Evidence was obtained that 1,2-dichloroethane, vinyl chloride, monochlorobenzene, 2-chloroethanol and octane can be oxidized under anoxic conditions. Particularly nitrate-reducing conditions may have great potential for natural or stimulated remediation of sites contaminated with these particular hydrocarbons.

Introduction

Low-chlorinated and non-chlorinated hydrocarbons enter soil and groundwater systems through accidental spills, for example at industrial sites or petrol stations, and belong to the most important pollutants. In addition, they are formed by partial dechlorination of highly chlorinated hydrocarbons. In contaminated soil and groundwater systems that are anoxic, microbial degradation routes are based on processes for which oxygen is not needed. Reductive dechlorination is a well-established anaerobic biotransformation pathway for many highly chlorinated solvents such as tetrachloroethene (PCE) or hexachlorobenzene. Recent research indicated that contaminants such as vinyl chloride (VC), dichloroethene, 1,1,1-trichloroethane (TCA), dichloromethane and n-alkanes (C₅-C₁₂) can also be removed through anaerobic oxidative pathways (2-4,7,9,10). Oxidized iron, manganese or nitrate may serve as alternative electron acceptor for the bacteria involved in these anaerobic oxidations. Knowledge on the role and potential of anaerobic oxidation processes for natural or stimulated degradation of aliphatic hydrocarbons is still limited.

Objectives.

The aim of this paper is to identify the potential for anaerobic oxidation processes for natural and stimulated degradation of chlorinated and non-chlorinated hydrocarbons. Both batch and soil column experiments were performed with material obtained from five different polluted sites. Model-pollutants that were supplied as electron donors included: 1,2-dichloroethane (DCA), TCA, VC, *cis*-1,2-dichloroethene (CIS), *trans*-

1,2-dichloroethene (TRANS), trichloroethene (TCE), monochlorobenzene (MCB), 2-chloroethanol (CE) and/or octane. Biotransformation of these contaminants was investigated in the presence of nitrate, ferrihydrite ($\text{Fe}(\text{OH})_3$) or manganese oxide (MnO_2) as electron acceptor.

Materials and Methods

Batch cultures.

Batches were prepared under N_2 atmosphere in 120-ml bottles which were crimp-sealed with butyl or viton rubber stoppers and contained 50 ml media. The growth media consisted of the following components: $\text{Na}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ buffer (20 mM, pH 7), 1.0 g $\text{NH}_4\text{Cl}/\text{l}$, 0.1 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}/\text{l}$, 0.05 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}/\text{l}$, 0.1 g yeast extract/l, 0.1 % resazurin, 1 ml trace elements/l (5) and vitamin solution (6). For the batches grown with chloroethanol, NH_4Cl was replaced by $(\text{NH}_4)_2\text{SO}_4$ and 500-ml bottles were used containing 250 ml media. Vitamins were filter sterilized and added after autoclaving the media. Electron donors were added separately from autoclaved stock solutions to final concentrations of 1–10 mM. Electron acceptors were added to final concentrations of 5–10 mM (NaNO_3 , MnO_2 or $\text{Fe}(\text{OH})_3$). Amorphous $\text{Fe}(\text{OH})_3$ was synthesized by neutralising a 0.4 M FeCl_3 solution with 1 M NaOH until a pH of 7 was achieved. Manganese oxide was prepared by mixing equal amounts of 0.4 M MnO_4 and 0.6 M MnCl_2 and adjusting the pH to 10 with 1 M NaOH . After preparing, the solutions were washed with milli-Q water by centrifugation. All solutions were prepared under N_2 atmosphere. Inoculum material was obtained from six different polluted sites in The Netherlands (Rotterdam, Arnhem, Tilburg, Uden, Groningen and Wageningen). Batches were incubated horizontally at 20°C under continuous shaking (100 rpm.).

Soil column set-up.

Four glass columns (35 cm length x 3.6 cm internal diameter) were packed in an anaerobic glove box with sediment from four different contaminated sites (Rotterdam, Arnhem, Tilburg and Uden). The sites were mainly polluted with DCA, chloroethenes, BTEX and/or gasoline. A layer of glass wool and glass beads were put at the bottom of the column to avoid clogging of the inlet. Sampling ports were sealed with both butyl rubber and viton rubber septa. The columns were percolated (up-flow) with media at a flow-rate of 5.8 ml/h (liquid retention time \pm 18 hours) by a peristaltic pump (505S, Watson Marlow Ltd., UK). Influent medium was continuously stirred and flushed with N_2/CO_2 (90:10 v/v) and consisted of $\text{Na}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ buffer (2 mM, pH 7), 0.1 g $\text{NH}_4\text{Cl}/\text{l}$, 0.01 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}/\text{l}$, 0.005 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}/\text{l}$ and 0.1 ml trace

elements/l. A substrate mixture with 10 mM DCA, 10 mM CIS, 10 mM TRANS, 5 mM TCE, 1mM MCB, 1 mM TCA and 1 mM VC was injected (58 μ l/h) into the medium flow by a syringe pump. The substrate cocktail was mixed and diluted 100-fold with the medium in a stainless steel coil construction just before entering the column. Fe(OH)₃ (about 80 mmol) and MnO₂ (about 50 mmol) were mixed with the soil material before filling the columns. Nitrate was supplied by a syringe pump (final concentration 5 mM) with the same pumping-rate as the substrate pump. The columns were operated in a 20°C climate room. For detailed information about the soil column set-up see chapter 5.

Analytic methods.

DCA, TCA, CIS, TRANS, TCE, MCB, VC, ethene, ethane and methane were determined by gas chromatography (GC). Liquid samples of 0.5 ml were taken from the column inlet or outlet and injected into 22 ml vials containing 7.5 ml milli-Q water and 0.1 ml HgCl₂ (50 g/l). HgCl₂ was added to prevent further microbial degradation. The vials were automatically sampled (Tekmar headspace autosampler) and 100 μ l headspace was injected in a Varian Genesis GC equipped with a Porabond-Q column (Chrompack B.V., Middelburg, The Netherlands, i.d. 0.32 mm, length 25 m) and a flame ionization detector (300°C). The temperature program of the column oven ranged from 35°C (3 min) to 250°C (5.5 min) and increased with a rate of 10°C /min. Helium was used as carrier gas. Concentrations of octane were determined by analyzing 100 μ l headspace samples on a Chrompack CP9000 gas chromatograph equipped with a Chrompack CP-Sil 8B column (length 25 m, i.d. 0.22 mm). The oven temperature was kept at 75°C and the detector (FID) temperature was 270°C. Optical densities were determined at 660 nm in a Perkin-Elmer 55 A UV-VIS spectrophotometer. Chloride, nitrate, nitrite, manganese and iron were all determined colorimetrically. Chloride was measured according to (1). In batch cultures with chloroethanol, chloride production was used as a measure for the chloroethanol consumption. Nitrate was measured with a 6% brucine solution in methanol and sulfuric acid. Nitrite was measured with a 1% sulfanilamide solution in 2.5 N HCL and a 0.02% N-(1-naphtyl) ethylenediamine solution in water. A Dr. Lange Pipetting test (Dr. Lange Nederland B.V.) was used for measuring manganese. Iron was determined according to (8).

Calculations.

In batch experiments, concentrations of (chlorinated) hydrocarbons were determined by comparing integration areas with a calibration curve and ignoring headspace-liquid partitioning. In the column experiments, no headspace was present. Half-lives were calculated according to $t_{1/2} = \ln 2/k$, where $k = (\ln c_1 - \ln c_2)/(t_2-t_1)$ (day⁻¹). For the soil

columns $\Delta t = (\pi d^2 \theta [l_2 - l_1]) / 4K_p$ (Where l_1 and l_2 are the distances to the bottom of the column; d is the inner diameter; θ is the effective porosity and K_p the medium-pump rate). The effective porosity was assumed to be 0.3.

Results

Batch cultures.

To reveal the potential for anaerobic oxidation of chlorinated solvents, soil samples obtained from five polluted locations were incubated with different chlorinated hydrocarbons in the presence of either nitrate, $\text{Fe}(\text{OH})_3$ or MnO_2 . The studied compounds were VC, CIS, TRANS, TCE, DCA, MCB, CE and octane. Enrichment cultures were obtained with the following electron donor / electron acceptor combinations: CE plus nitrate, DCA plus nitrate, octane plus nitrate, and DCA plus MnO_2 . In batch cultures with other electron donor / electron acceptor combinations no degradation was observed within 500 days.

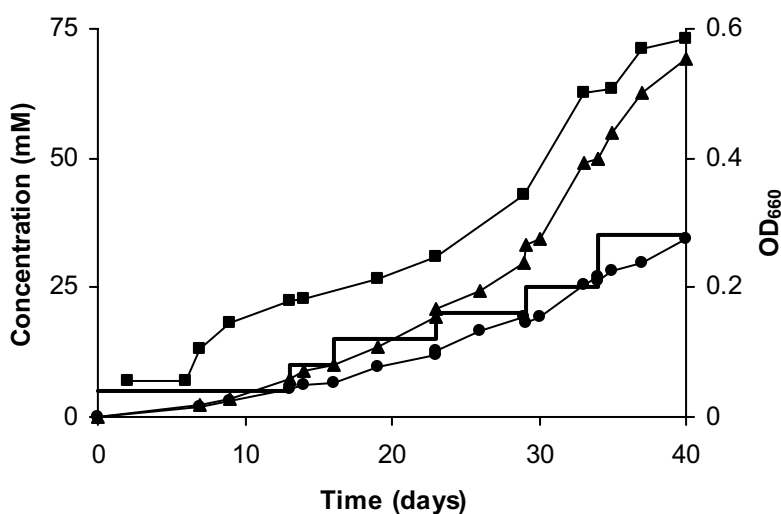


Figure 2.1 Degradation of 2-chloroethanol under nitrate reducing conditions. Chloride produced (●), nitrate consumed (▲), OD_{660} (■) and chloroethanol supplied (-)

The batch culture with CE and nitrate was obtained from a transfer of a batch culture growing on DCA and nitrate. The DCA enrichment culture was described previously (4), and the first order degradation rate constants (k -values) of DCA ranged from 0.15 to 1.68 day^{-1} (unpublished). Because the DCA-degrading capacity of this culture decreased after repeated transferring, CE (suspected intermediate) was used as

electron donor instead of DCA to further enrich and isolate the DCA degrading bacteria. After a lag phase of 6 days, CE degradation started (Fig. 2.1).

The total amount of CE added was 35 mM, which resulted in a chloride production of 34 mM and a nitrate consumption of 69.6 mM. Nitrite accumulated temporarily to concentrations of about 1 mM but subsequently was further reduced, possibly to nitrogen. The mass balance calculation indicates that nitrate-coupled CE oxidation proceeded according to following reaction:



The rate of chloride production showed that CE degradation proceeded at a first-order decay rate of 0.066 day^{-1} . This rate was relatively low compared to the k -values of the DCA degrading culture from which it originated. The increase of the OD_{660} indicated that CE degradation was coupled to growth, corresponding to an μ of approximately 0.04 day^{-1} . After 5 transfers, the CE degrading enrichment culture had lost the capacity to transform DCA in the presence of nitrate.

Under manganese(IV) reducing conditions, DCA was degraded with a maximum transformation rate of 0.012 day^{-1} (data not shown). As a result of manganese reduction, 0.46 mM Mn(II) was formed. However, according to a complete oxidation reaction 1 mol DCA would require as much as 5 mol manganese. Further research is needed to explain this discrepancy. Compared to denitrifying conditions, the DCA-transformation rate obtained under manganese reducing conditions was relatively low.

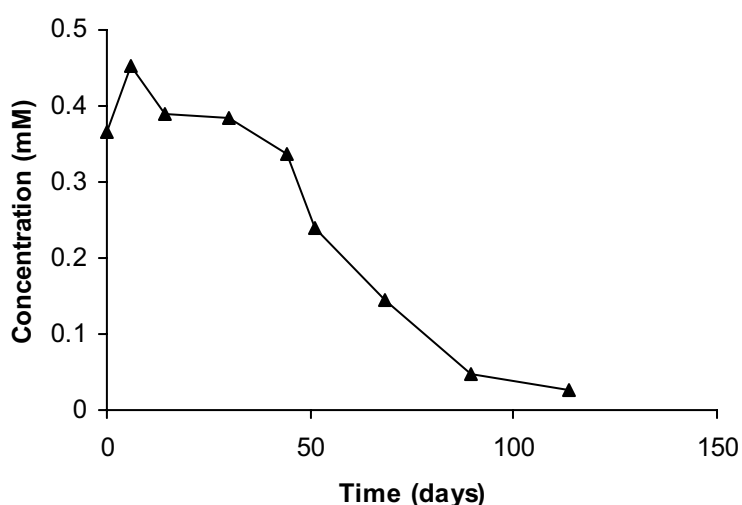


Figure 2.2 Degradation of octane (▲) under nitrate reducing conditions.

Octane was degraded under denitrifying conditions (Fig. 2.2). The observed maximum transformation rate of 0.018 day^{-1} , was in the same order as the transformation rate obtained for nitrate coupled CE oxidation. Nitrate was reduced (2.1 mM) and no nitrite was detected. Theoretically, 10 mol nitrate is required to oxidize 1 mol octane. Since approximately $400 \mu\text{M}$ octane was consumed, 4 mM nitrate would be needed for complete oxidation to carbon dioxide. The fact that only 2.1 mM nitrate was used suggests that octane was not completely oxidized to carbon dioxide and that microbial biomass and organic acids (e.g. acetate) may have been produced.

Soil columns.

Soil column experiments were performed with a mixture containing the following chlorinated compounds in the influent medium (concentrations ranging from 10 to $100 \mu\text{M}$): VC, CIS, TRANS, TCE, DCA, TCA and MCB. To enhance the potential for oxidative degradation of the chlorinated compounds three different columns were supplied with either $\text{Fe}(\text{OH})_3$ ($\pm 0.22 \text{ mol/g}$ of soil), MnO_2 ($\pm 0.14 \text{ mol/g}$ of soil) or NO_3^- (influent concentration 5mM) as external electron acceptors. One control column was run to which no external electron acceptor was added. The columns were operated at a hydraulic retention time of about 1 day, and the influent and effluent concentrations of the chlorinated compounds were monitored over a period of approximately 200 days. In the column supplied with $\text{Fe}(\text{OH})_3$ only DCA was instantly removed ($> 75 \%$) (Fig. 2.3A). This DCA-removal could not be contributed to adsorption, because compared to some of the other compounds such as TCA and MCB, the adsorption potential of DCA is much lower. Both DCA and VC were degraded (75 % and 100 % respectively) in the column amended with MnO_2 (Fig. 2.3B). The molar ratio of manganese (II) versus manganese (IV) increased from zero to 0.12 over the length of the column, indicating the occurrence of manganese reduction. The nitrate reducing soil column also showed removal of DCA and VC ($>90\%$), and MCB concentrations were reduced to less than 50% of the influent (Fig. 2.3C).

Nitrite was formed (about $6 \mu\text{M}$) and the nitrate concentration decreased (from 5 to 3 mM). Interestingly, in the control column (i.e. no additional electron acceptors supplied) more than 70% of DCA, VC and MCB were removed (Fig. 2.3D).

The lag-time for DCA degradation was about 2 weeks for all DCA-degrading columns while for VC it was about 3 months. Reductive dechlorination products such as ethane, ethene and benzene were not detected in any of the columns, indicating that VC, DCA and MCB degradation proceeded through anaerobic oxidation pathways. It is not clear which electron acceptors present in the soil may have been involved in the degradation of DCA, VC and MCB in the control column.

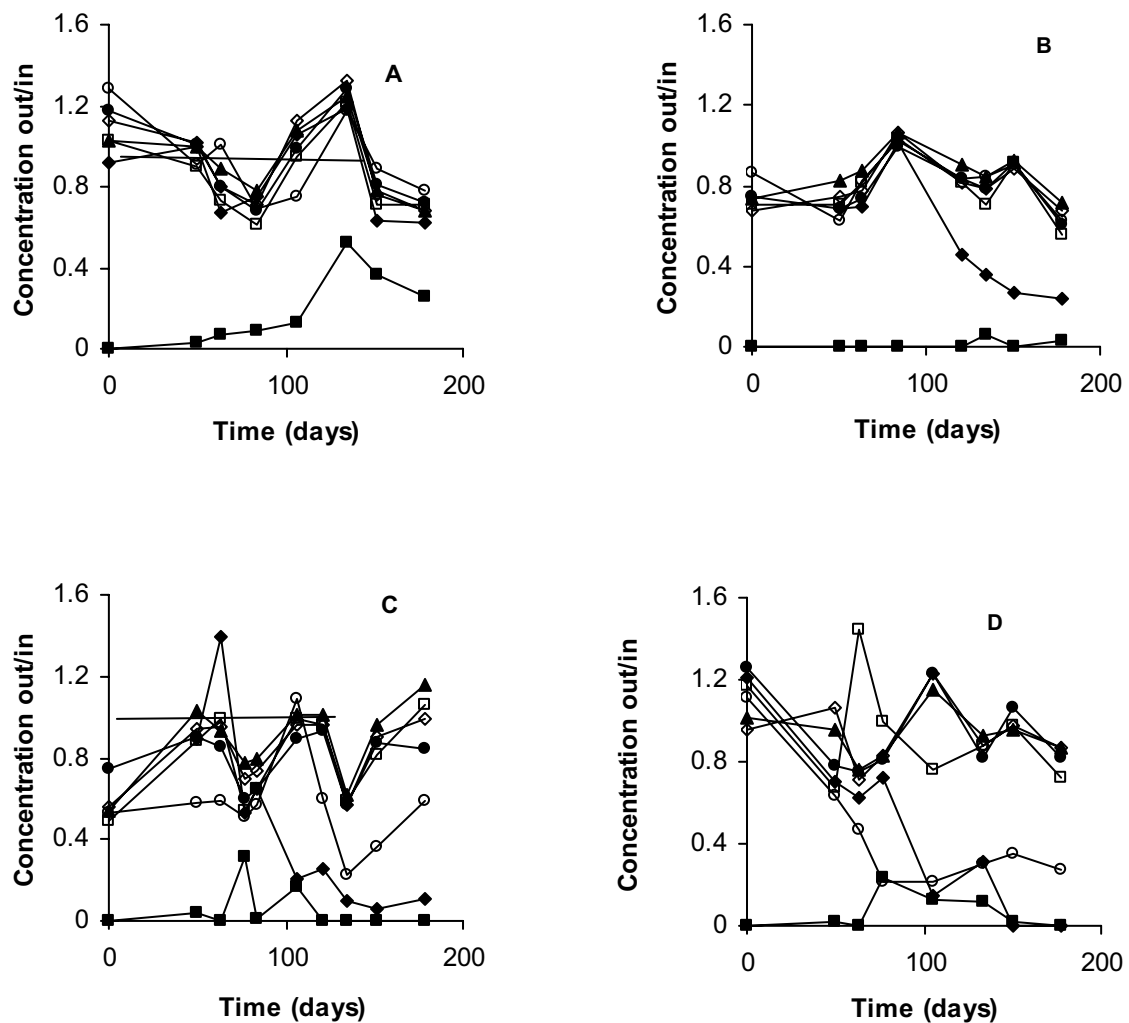


Figure 2.3 Degradation of a mixture of chlorinated compounds in soil columns supplied with A: Fe(OH)₃, B: MnO₂, C: NO₃⁻ and D: no external electron acceptor supplied. VC (◆), DCA (■), TCA (□), cDCE (▲), tDCE (◇), TCE (●), MCB (○).

Table 2.1 Half-lives obtained for the (possible) oxidation of (chlorinated) hydrocarbons in batch cultures and soil columns

Pollutant	Abbreviation	Half-life (days)		
		<i>Electron acceptors</i>		
		NO_3^-	MnO_2	$Fe(OH)_3$
Monochlorobenzene	MCB	0.8 ^b	-	-
Octane		17 ^a	n.d.	n.d.
Vinyl chloride	VC	0.2 ^b	0.32 ^b	-
<i>trans</i> -1,2-Dichloroethene	TRANS	-	-	-
<i>cis</i> -1,2-Dichloroethene	CIS	-	-	-
Trichloroethene	TCE	-	-	-
1,2-Dichloroethane	DCA	0.14–4.6 ^{a,b}	0.09–25 ^{a,b}	0.25 ^b
1,1,1-Trichloroethane	TCA	-		
2-Chloroethanol	CE	11 ^a	n.d.	n.d.

^aobtained in batch experiments^bobtained in soil column experiments

Discussion

The results obtained both from the batch and soil column experiments reveal that it is possible to have anaerobic oxidation of DCA, CE, VC, MCB and octane (Table 2.1). DCA was degraded under all the three different electron accepting conditions studied (i.e. nitrate, iron and manganese-reduction) and the half-lives ranged from several hours to weeks. Chloroethanol and octane oxidation were only tested in nitrate reducing batch cultures and proceeded with half-lives of 11 and 17 days, respectively. VC degradation was observed under both manganese and nitrate reducing conditions with half-lives of 0.2 and 0.3 days, respectively. Further research is required to definitely prove that the half-life of less than one day for MCB, observed in the control and the nitrate reducing columns, can be attributed to biological degradation processes.

Particularly nitrate-reducing conditions may have great potential for natural or stimulated remediation of sites contaminated with (low-chlorinated) hydrocarbons, since several of the chlorinated compounds were degraded under denitrifying conditions. Besides, denitrifiers are widely distributed in soil and sediments (11). Especially for stimulated remediation it is of big advantage that nitrate is highly soluble in water and can therefore be easily supplied to the soil or groundwater to enhance degradation.

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**Anaerobic oxidation of 2-chloroethanol
under denitrifying conditions by
Pseudomonas stutzeri strain JJ**

**John A. Dijk, Alfons J. M. Stams, Gosse Schraa, Hendrik Ballerstedt,
Jan A. M. de Bont and Jan Gerritse**

Abstract

A bacterium that uses 2-chloroethanol as sole energy and carbon source coupled to denitrification was isolated from 1,2-dichloroethane-contaminated soil. Its 16S rDNA sequence showed 98 % similarity with the type strain of *Pseudomonas stutzeri* (DSM 5190) and the isolate was tentatively identified as *Pseudomonas stutzeri* strain JJ. Strain JJ oxidized 2-chloroethanol completely to carbon dioxide with nitrate or oxygen as electron acceptor, with a preference for oxygen if supplied in combination. Optimum growth on 2-chloroethanol with nitrate occurred at 30°C with a μ_{\max} of 0.14 h⁻¹ and a yield of 4.4 g protein per mol 2-chloroethanol metabolized. Under oxic conditions the μ_{\max} was 0.31 h⁻¹. Nitrite also served as electron acceptor, but reduction of iron(III) hydroxide, manganese(IV) oxide, sulfate, fumarate or chlorate was not observed. Another chlorinated compound used as sole energy and carbon source under oxic and denitrifying conditions, was chloroacetate. Various different bacterial strains, including some closely related *Pseudomonas stutzeri* strains, were tested for their ability to grow on 2-chloroethanol as sole energy and carbon source under oxic and denitrifying conditions, respectively. Only three strains, *Pseudomonas stutzeri* strain LMD 76.42, *Pseudomonas putida* US2 and *Xanthobacter autotrophicus* GJ10, grew aerobically on 2-chloroethanol. This is the first report of oxidation of 2-chloroethanol under denitrifying conditions by a pure bacterial culture.

Introduction

2-Chloroethanol (CE) is used in industry mainly for the synthesis of insecticides and as a solvent. Very little is known about the emission and fate of CE in the environment due to the fact that CE is not included in routine analyses for soil pollution. CE is metabolized both *in vivo* and *in vitro* by mammalian alcohol dehydrogenase to 2-chloroacetaldehyde, which is considered to be mutagenic (23). Several bacteria have been described that can degrade CE aerobically, these included *Pseudomonas* sp. strain CE1, *Pseudomonas putida* US2, *Pseudomonas* sp. strain DCA1, *Xanthobacter autotrophicus* GJ10, *Pseudomonas* sp. strain GJ1, *Ancylobacter aquaticus* strains AD20 and AD25 and *Mycobacterium* sp. strain GP1 (12,16,17,30,36,37,40). Usually, degradation proceeds via the intermediates 2-chloroacetaldehyde and chloroacetate to glycolic acid. Recently, Poelarends *et al.* (30) showed that *Mycobacterium* sp. strain GP1 degraded CE via ethylene oxide, without production of the 2-chloroacetaldehyde intermediate.

Although the aerobic CE-metabolizing *Pseudomonas* sp. strain GJ1 and *Xanthobacter autotrophicus* GJ10 are able to reduce nitrate, nothing is known about the degradation of CE under denitrifying conditions. Vogel and McCarty (38) proposed a mechanism for the anaerobic mineralization of vinyl chloride to carbon dioxide under methanogenic conditions in which vinyl chloride is hydrated to CE, followed by an oxidation to form the aldehyde. The aldehyde may be further oxidized to eventually carbon dioxide. However, none of the proposed intermediates were detected in this study and no other evidence was given for this degradation pathway. This report describes the isolation and characterization of *Pseudomonas stutzeri* strain JJ, the first microorganism known to grow anaerobically on CE with nitrate as electron acceptor. This is especially interesting, since many soils that are contaminated with chlorinated aliphatics are anoxic and nitrate is often present in the groundwater (6,10).

Materials and Methods

Organisms.

Pseudomonas stutzeri strain LMD 76.42 (28) and *Pseudomonas stutzeri* strain LMD 26.48 (34) were purchased from The Netherlands Culture Collection of Bacteria (NCCB, Utrecht, The Netherlands). *Pseudomonas mendocina* (DSM 50017, type strain) (35), *Pseudomonas stutzeri* (DSM 5190, type strain) (35), (25), *Xanthobacter autotrophicus* GJ10 (17), *Pseudomonas stutzeri* strain KC (7) *Pseudomonas putida* US2 (36) and *Alcaligenes xylosoxidans* subsp. *denitrificans* (DSM 6505) (3) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). *Pseudomonas* sp. strain P51 (24), *Pseudomonas putida* strain C 3024, *Pseudomonas stutzeri* strain 346B (DSM 6538) (1) and *Pseudomonas aeruginosa* (ATCC 17933) (5) were obtained from the culture collection of the Laboratory of Microbiology of Wageningen University. The new isolate, *Pseudomonas stutzeri* strain JJ, has been deposited in the DSM as strain DSM 15012.

Media and cultivation techniques.

Batches were prepared under a nitrogen atmosphere in 120-ml serum bottles that were crimp-sealed with butyl-rubber stoppers and contained 50 ml medium. Standard medium consisted of the following components: Na₂HPO₄/KH₂PO₄ buffer (20 mM, pH 7), 0.7 g (NH₄)₂SO₄/l, 0.1 g MgSO₄·7H₂O/l, 0.05 g CaCl₂·2H₂O/l, 1 ml trace elements/l (11) and 1 ml vitamin solution/l (14). Vitamins were filter sterilized (0.2 µm) and added to the media after autoclaving. Phosphate buffer was also added separately after autoclaving. Unless mentioned otherwise, CE (5 mM) was added as

electron donor and NaNO₃ (10 mM) as electron acceptor from separately sterilized stock solutions. Batches were transferred weekly to fresh media to maintain actively growing cultures. Batch enrichments were initially incubated at 20°C, but after isolation, incubations of strain JJ were carried out at 30°C unless mentioned otherwise. Agar plates consisted of the above mentioned components plus 15 g Bacto-agar/l (Difco Laboratories, Detroit, Mich., USA). The plates were incubated in a nitrogen-flushed anaerobic jar or in air.

Enrichment, isolation and purity control.

Strain JJ originates from an anaerobic enrichment culture growing on 1,2-dichloroethane as sole energy and carbon source in the presence of nitrate (10). One ml of the enrichment culture was transferred to standard medium, containing 1 mM CE and 5 mM NaNO₃. Upon growth, samples were plated on anoxic agar plates. Colonies were isolated from the plates and transferred to liquid medium. This process was repeated several times, until a pure culture was obtained.

Purity was tested by phase-contrast microscopy and by aerobic growth on both solid and liquid standard media with glucose (5 mM) and yeast extract (5 g/l). Oxidase, catalase and Gram-type tests were performed at The Netherlands Culture Collection of Bacteria (Utrecht, The Netherlands).

Identification.

Physiological tests. The API 20NE and BIOLOG GN analyses were carried out at The Netherlands Culture Collection of Bacteria (Utrecht, The Netherlands).

DNA isolation, amplification, sequence determination and analysis of 16S rDNA. Genomic DNA was isolated from whole cells by a combined method of SDS-treatment and bead beating in the presence of phenol, followed by subsequent phenol-chloroform-isoamylalcohol (25:24:1) extraction (9,31). The final DNA-extracts were further purified, using the Wizard DNA Clean-Up System (Promega, Leiden, The Netherlands). PCR amplification of the 16S rRNA-gene was done using 1 µl DNA-extract as template, bacteria-specific primers FD1 and RP2 (39) (*Escherichia coli* numbering 8-27, and 1512-1492 (4)), AmpliTaq LD DNA-polymerase (Perkin Elmer Life Sciences, Zaventem, Belgium) and a thermal cycler temperature program as follows: denaturation at 95°C for 5 min; touch down PCR step: 10 cycles of 94°C for 30 s, 65°C - 55°C for 30 s, 72°C for 1 min; 27 similar cycles at 55°C annealing temperature and with a 20 s extension of elongation phase per cycle. PCR product was purified via agarose gel separation stained with 1 x Nuclistain (National Diagnostics, Manville, USA) and subsequent DNA extraction using a DNA extraction kit (MBI Fermentas, St. Leon-Rot, Germany). The purified 16S rDNA was ligated into a pGEM-T Easy Vector (Promega) and transformed into *E.coli* XL 1-Blue-MRF' cells (Stratagene, La Jolla, Calif., USA). The transformed cells were subjected to blue-

white color screening, randomly picked white colonies were grown overnight in selective LB-medium as described elsewhere (33), and recombinant plasmids were subsequently isolated using QIAprep Spin Miniprep kit (Qiagen, Westburg B.V., Leusden, The Netherlands). Cloned inserts were reamplified using pGEM-T-Easy Vector binding primers PG1f and PG2r (13), and subsequently digested with restriction enzymes *MspI* and *RsaI* (Promega) overnight at 37°C. The restriction patterns were monitored to confirm uniformity of the inserts using 4 % agarose gels in 1 x TBE-buffer (33). Bi-directional sequence was determined by MWG Biotech AG (Ebersberg, Germany) using iniversal SP6 and T7 primers and primers 984f (15) and 907r (27). The determined sequence was compared with 16S rDNA sequences obtained from the EMBL data library using the Fasta3 program (29).

Characterization of the CE-degrading strain JJ. To determine the optimum growth temperature, batch cultures of strain JJ with 5 mM CE and 10 mM nitrate were incubated at 4, 12, 20, 25, 30, 35 and 45°C, respectively. Liquid samples (1,5 ml) were taken at different time points and the OD₆₆₀ was determined. Specific growth rates were obtained by plotting the natural logarithm of the OD₆₆₀ against time. The determination of the optimum pH was done in the same way. Batch cultures of strain JJ were incubated at pH 6, 6.5, 7, 7.5, 8, 8.5, 9 and 9.5.

Electron donor utilization.

Initially, utilization of chlorinated electron donor was screened in 10-ml vials containing 5 ml standard medium and crimp-sealed with butyl-rubber stoppers. Chlorinated electron donors were added from autoclaved stock solutions to a final concentration of 1 mM. Media were incubated anaerobically with 5 mM nitrate, or under an air atmosphere. After 8 weeks of incubation the chloride concentration was determined. Batches with a chloride production > 0.2 mM were transferred to standard media in 120-ml serum bottles with 5 mM electron donor and incubated anaerobically with 10 mM nitrate, or under air, respectively. After 3 weeks, growth was determined by visual detection of increased turbidity.

Growth on non-chlorinated electron donors was tested in 120 ml-serum bottles with 50 ml standard medium, containing 10 mM electron donor with 10 mM nitrate, or under air, respectively. After 3 weeks, growth was determined by visual detection of increase in turbidity.

Electron acceptor utilization.

Electron acceptors were added according to the theoretical amounts of reduction equivalents needed for the complete oxidation of 5 mM CE: NaNO₃ (10 mM), NaNO₂ (17 mM), MnO₂ (25 mM), Fe(OH)₃ (50 mM), Na₂SO₄ (6 mM), NaClO₃ (8 mM), O₂ (air atmosphere; 12 mmol O₂/l medium) and fumarate (25 mM). Amorphous Fe(OH)₃ was prepared by neutralizing an 0.4 M FeCl₃ solution with 1 M NaOH until a pH of 7 was

achieved (20). MnO_2 was prepared by mixing equal amounts of 0.4 M MnO_4^- and 0.6 M MnCl_2 and adjusting the pH to 10 with 1 M NaOH (21,26). After preparation, the $\text{Fe}(\text{OH})_3$ and MnO_2 suspensions were washed four times with milli-Q water by centrifugation. All stock solutions were flushed with N_2 before use. After 4 weeks of incubation, the concentration CE of the batches with the respective electron acceptors was determined. When more than 2 mM of CE had been consumed, the electron acceptor was considered to be utilized.

Growth on CE.

Batch cultures of strain JJ were grown in 1-l screw cap flasks with butyl-rubber stoppers, containing 500 ml medium with 5 mM CE and 10 mM nitrate. The flasks were inoculated with 1-10 ml of an exponentially growing batch culture and incubated at 30°C in a shaking waterbath (90 r.p.m.). The optical densities and the CE, nitrate, nitrite, ammonium and chloride concentrations were monitored over time.

Experiments with nitrate and oxygen present at the same time were carried out in 500-ml screw cap flasks with butyl-rubber stoppers. The flasks contained 250 ml medium with 10 mM CE, 1 mM oxygen and 2 mM nitrate. The inoculum was pre-grown on CE and a combination of nitrate and oxygen. The optical densities and the CE, nitrate and oxygen concentrations were monitored over time.

Comparison of the newly isolated strain JJ with other bacterial strains.

Aerobic growth on CE of different bacterial strains was tested in standard medium containing 2.5 mM CE. After 3 weeks the OD_{660} was determined. For anaerobic growth experiments with CE, batch cultures were pre-grown on medium containing: 27 g sodium succinate/l, 1 g KNO_3 /l, 1 g K_2HPO_4 /l, 1 g $(\text{NH}_4)_2\text{SO}_4$ /l, 0.1 g MgSO_4 /l, 0.05 g CaCl_2 /l and 1 ml trace-elements/l (see Media and cultivation techniques) to induce nitrate reduction. Subsequently, the batch cultures were transferred to anoxic standard medium (2% inoculum) containing 2.5 mM CE and 5 mM nitrate. After 3 weeks, growth was determined by visual detection of increase in turbidity.

Analytical methods.

OD_{660} was determined by a Perkin-Elmer 55 A UV-VIS spectrophotometer. Chloride, nitrate and nitrite were determined by suppressor-mediated ion chromatography (Dionex, Breda, The Netherlands) and conductivity detection. Eluent consisted of 1.8 mM Na_2CO_3 and 1.7 mM NaHCO_3 at a flow rate of 1 ml/min. The chromatograph was equipped with an IonPac AS9-SC column (Dionex, Breda, The Netherlands). Ammonium was determined colorimetrically according to the Berthelot reaction (32). CE was measured by extracting 2-ml samples with 1 ml diethylether (containing 0.1 mM 1-butanol as internal standard) and injecting 2 μl into a Hewlett Packard HP 6890 gas chromatograph, equipped with a CPWax 52 CB column (length, 30 m; diameter,

0.32 mm; Chrompack, Middelburg, The Netherlands), using an FID detector (300°C). The oven temperature was 80°C isothermal and the detection limit was 50 µM. CE concentrations are presented as nominal concentrations in the batch cultures, assuming that all CE was present in the liquid phase. Oxygen and nitrogen were measured on a Chrompack CP9000 gas chromatograph equipped with a CP Molsieve 5Å column (length, 30 m; diameter, 0.53 mm; Chrompack, Middelburg, The Netherlands) and a TCD detector (150°C). The oven temperature was kept at 50°C. Protein was measured with the Lowry reagent with bovine serum albumin as a standard (22)

Chemicals.

All chemicals were of analytic grade with a purity of more than 97% and obtained from commercial sources.

Nucleotide sequence accession numbers.

The nucleotide sequence was deposited in the GenBank database under accession number AF411219.

Results

Enrichment and isolation of strain JJ.

A 1,2-dichloroethane (DCA)-degrading, denitrifying enrichment culture was previously obtained from DCA contaminated soil (10). Upon sub-culturing, the enrichment culture lost the ability to degrade DCA. However, after supplying this culture with CE, a presumed intermediate of DCA degradation, a well growing enrichment culture on CE (5 mM) and nitrate (10 mM) was obtained (8). From this enrichment strain JJ was isolated.

Identification of strain JJ.

Strain JJ was identified by using the API 20NE, the ID 32 GN and BIOLOG GN systems and by sequencing the 16S rDNA. The API 20NE, ID 32 GN and BIOLOG GN tests identified strain JJ as *Pseudomonas stutzeri* (99.5 % probability). The 16S rDNA sequence was compared with the BLAST database and strain JJ showed 98 % similarity with the type strain of *Pseudomonas stutzeri* (DSM 5190; U26262) (2). Therefore, the isolate was tentatively designated as *Pseudomonas stutzeri* strain JJ. The general characteristics of strain JJ are represented in Table 3.1.

Table 3.1 Characteristics of *Pseudomonas stutzeri* strain JJ.

<i>Pseudomonas stutzeri</i> strain JJ characteristics	
Gram stain	+
Cell morphology	rod shaped
Cell size	1-2.5 x 0.6-1.3 μm
Colony morphology	two types; wrinkled and smooth
Oxidase	+
Catalase	+
Motility	+
Spore formation	-
pH optimum	± 7.5
Temp. optimum (°C)	30
Growth at 4 °C	-
Growth at 40 °C	+
Growth substrates	glycogen, glucose, maltose, 3-methylglucose, α -methyl-D-glucoside, β -methyl-D-glucoside, α -methyl-D-mannoside, palatinose, stachyose, D-trehalose, turanose, D-sorbitol, xylitol, dextrin, β -cyclodextrin, inulin, proline, L-alanine, lactamide, acetate, α -hydroxybutyrate, β -hydroxybutyrate, caprate, citrate, gluconate, L-glutamate, itaconate, α -ketoglutarate, α -ketovalerate, D-lactate-methylester, D-malate, L-malate, propionate, pyruvate and methylpyruvate
No growth	amygdalin, L-arabinose, D-arabitol, arbutin, cellobiose, D-fructose, D-galactose, gentiobiose, mannose, D-melibiose, rhamnose, D-ribose, salicin, sedoheptulosan, D-sucrose, D-tagatose, D-xylose, inositol, mannitol, mannan, γ -hydroxybutyrate, ρ -hydroxyphenylacetate, L-lactate, mono-methylsuccinate, succinate, N-acetyl L-glutamate, D-galacturonate, alaninamide, D-alanine, L-alanine, L-alanyl-glycine, L-asparagine, glycyl-L-glutamate, L-pyroglutamate, L-serine, putrescine, 2,3-butanediol, glycerol, adenosine, 2'-deoxy adenosine, thymidine, uridine, histidine, α -cyclodextrin, adenosine-5'-monophosphate, uridine-5'-monophosphate, fructose-6-phosphate, glucose-1-phosphate, glucose-6-phosphate, D-L- α -glycerol phosphate, 2-ketogluconate, N-acetyl glucosamine, adipate, suberate, 3-hydroxybenzoate, 4-hydroxybenzoate and phenyl-acetate

Electron donor and acceptor utilization.

No difference in the use of electron donor substrates was observed between aerobic and denitrifying conditions. Under both conditions strain JJ grew on ethanol, propanol, butanol, acetate, pyruvate, glucose, chloroethanol and chloroacetate. The following substrates were not utilized by strain JJ: methanol, lactate, 1,1-dichloroethane, 1,2-dichloroethane, 1,1,1-trichloroethane, 1-chloropropane, 2-chloropropane, 1,2-dichloropropane, 1,3-dichloropropane, 1,3-dichloro-2-propanol, 2-chloropropanol, vinyl chloride, 1,1-dichloroethene, *cis*-1,2-dichloroethene, *trans*-1,2-dichloroethene, trichloroethene and tetrachloroethene.

During growth on CE strain JJ reduced nitrate, nitrite and oxygen. Not utilized as electron acceptor were sulfate, iron(III) hydroxide, manganese(IV) oxide, chlorate and fumarate.

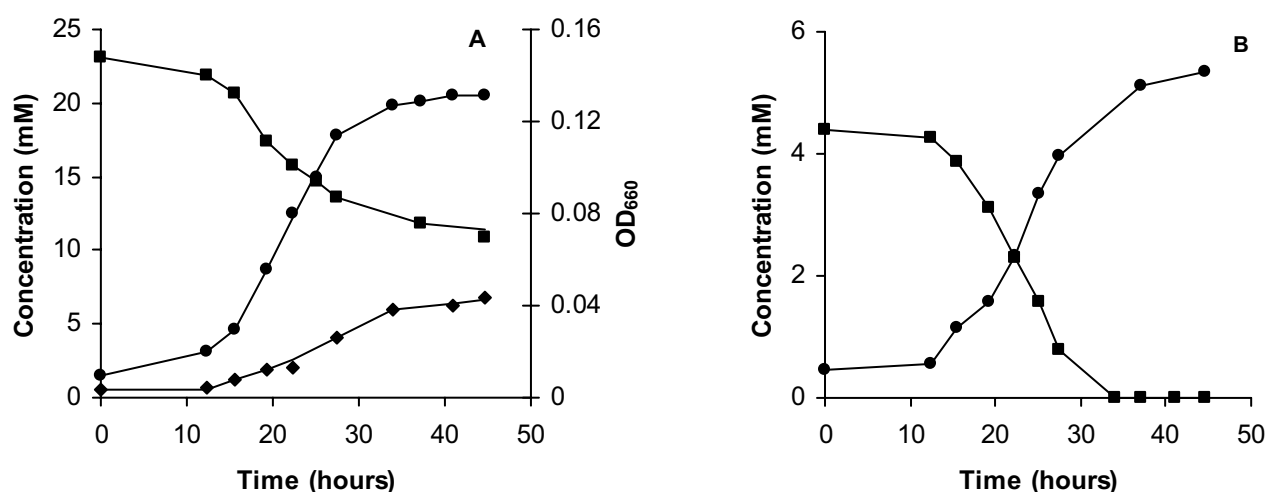


Figure 3.1 A and B. Degradation of 2-chloroethanol (5 mM) with nitrate (10 mM) as electron acceptor by *Pseudomonas stutzeri* strain JJ. **A** increase in optical density at 660 nm (●), the consumption of NO₃⁻ (■) and the formation of nitrite (◆). **B** degradation of 2-chloroethanol (■) and the release of chloride (●)

Growth on CE.

Growth on CE with nitrate started after a lag-phase of about 12 hours and proceeded according to a maximum specific growth rate (μ_{max}) of 0.14 h⁻¹ (Fig. 3.1A). After 45 hours, 4.8 mM of chloride was produced and 4.4 mM CE had been consumed (Fig. 3.1B). Concurrently, 12.2 mM of nitrate was reduced, mainly to nitrite (6.2 mM) and presumable to nitrogen gas (not measured), since ammonium was not produced. The total amount of protein formed was 19.4 mg/l, corresponding to a yield of 4.4 g protein per mol CE

metabolized. When grown on CE and nitrate, strain JJ displayed a temperature optimum at 30°C. No growth was observed at 4°C and at 45°C.

When both oxygen and nitrate were simultaneously present in a batch culture, strain JJ had a preference for oxygen. The μ_{\max} during the aerobic growth phase on CE was 0.31 h⁻¹. When the oxygen concentration dropped to below approximately 500 μM , strain JJ started to reduce nitrate to nitrite. Nitrite was subsequently reduced to most likely nitrogen gas, since no ammonium formation was detected.

Comparison of newly isolated strain JJ with other bacterial strains.

Eleven bacterial strains were tested for their ability to grow on CE under both oxic and nitrate reducing conditions (Table 3.2). *Pseudomonas* sp. strain P51 and *Pseudomonas putida* strain P42 could not grow on succinate with NO_3^- as electron acceptor. *Pseudomonas stutzeri* strain LMD 76.42, *Pseudomonas putida* US2 and *Xanthobacter autotrophicus* GJ10 were able to oxidize CE under aerobic conditions. The other tested strains were able to use nitrate as electron acceptor, but did not oxidize CE. Only strain JJ was able to oxidize CE under both aerobic and denitrifying conditions.

Table 3.2 The ability of different *Pseudomonas* strains, *Xanthobacter autotrophicus* GJ10 and *Alcaligenes xylosoxidans* subsp. *denitrificans* to grow on 2-chloroethanol under denitrifying or oxic conditions.

Species	Growth conditions		
	Succinate + NO_3^-	Chloroethanol + NO_3^-	Chloroethanol + O_2
Strain JJ	+	+	+
<i>Pseudomonas stutzeri</i> type strain	+	-	-
<i>Pseudomonas stutzeri</i> strain LMD 76.42	+	-	+
<i>Pseudomonas stutzeri</i> strain LMD 26.48	+	-	-
<i>Pseudomonas stutzeri</i> strain KC	+	-	-
<i>Pseudomonas stutzeri</i> strain 346B	+	-	-
<i>Pseudomonas mendocina</i> type strain	+	-	-
<i>Pseudomonas</i> sp. strain P51	-	-	-
<i>Pseudomonas putida</i> strain C 3024	-	-	-
<i>Xanthobacter autotrophicus</i> GJ10	+	-	+
<i>Pseudomonas aeruginosa</i>	+	-	-
<i>Pseudomonas putida</i> US2	+	-	+
<i>Alcaligenes xylosoxidans</i> subsp. <i>denitrificans</i>	+	-	-

Discussion

This report describes the isolation and characterization of a bacterium that is able to grow on CE under both oxic and denitrifying conditions. The isolated strain was designated as *Pseudomonas stutzeri* strain JJ and is the first pure bacterial culture described that is capable of anaerobic oxidation of CE with nitrate as electron acceptor.

Based on the present findings, the reaction equation for growth of strain JJ on CE plus nitrate is: $CE + 2HNO_3 \rightarrow 2CO_2 + HCl^- + N_2 + 3H_2O$ and $CE + 5HNO_3 \rightarrow 2CO_2 + HCl^- + 5HNO_2 + 2H_2O$. When nitrate was supplied in excess (23.1 mM nitrate + 4.4 mM CE), nitrite accumulated (up to 6.2 mM) in batches of strain JJ. It is known that nitrate can have an inhibitory effect on nitrite reduction by *Pseudomonas stutzeri* (18,19). Also the nitrate concentration in the pre-culture is important, since nitrate starvation inactivates the existing capacity of nitrite reduction (41).

Various other bacterial strains that were tested, including some closely related *Pseudomonas stutzeri* strains, did not grow on CE with nitrate, indicating that the ability to grow on CE with nitrate is an exceptional feature of *Pseudomonas stutzeri* strain JJ. Possibly, strain JJ expresses a 2-chloroethanol dehydrogenase or a hydrolytic 2-chloroethanol dehalogenase, enabling it to grow on CE under denitrifying conditions. However, it is remarkable that *Xanthobacter autotrophicus* GJ10 cannot grow on CE with nitrate. This strain is able to reduce nitrate, to grow on CE aerobically, and it contains both dehydrogenases and dehalogenases, which do not require oxygen (16,17). More insight into the regulation of the CE degradation pathway under denitrifying conditions may clarify this observation.

The ability of strain JJ to grow on CE and chloroacetate under denitrifying conditions may be important for survival in its natural habitat. The aquifer from which strain JJ was obtained was anoxic and contained small amounts of nitrate (< 10 μ M), chlorinated aliphatics, including DCA (up to 3.5 mM), vinyl chloride and various chlorinated methanes and propanes (10). The ability to use low-chlorinated aliphatics as substrates under denitrifying conditions may add significantly to the competitive strength of strain JJ in the environment. In addition, if oxygen and nitrate are both present in the field, strain JJ can easily switch from aerobic growth to denitrification, when oxygen becomes limited, and *vice versa*.

It remains unclear, which chlorinated compound(s) served as growth substrate for strain JJ in the field. CE and chloroacetate are degradation products from DCA. Unfortunately, CE and chloroacetate are not covered in the standard analyses of the pollutants at this location. Another possibility would be that strain JJ could grow on DCA in a mixed culture, since the enrichment from which strain JJ originates was able to degrade DCA under denitrifying conditions (10). Finally, strain JJ may possibly have lost the ability of degrading DCA during isolation and cultivation on CE in the laboratory. The transfer and expression

of a DCA dehalogenase, e.g. from *Xanthobacter autotrophicus* GJ10, might enable strain JJ to grow on DCA (16).

The observation that denitrifying bacteria can use chlorinated aliphatics, may have important implications for the natural attenuation and bioremediation of contaminated aquifers. The presence of nitrate in the groundwater may enable biodegradation of chlorinated aliphatics that entered the soil directly or have been formed *in situ* via partial dechlorination. Anaerobic oxidation pathways are of particular importance when chlorinated contaminated plumes spread from reduced (e.g. methanogenic, sulfate-reducing) to more oxidized (e.g. iron-reducing and denitrifying) redox zones. These groundwater situations frequently occur at polluted sites in Western Europe.

Nitrate-coupled bioremediation has several advantages because nitrate-reducers grow rapidly, nitrate dissolves well in water and it does not form precipitates in anoxic groundwater, which frequently clog infiltration wells during aerobic bioremediation.

Oxidation of vinyl chloride and DCA has also been demonstrated under denitrifying conditions (6,8,10). Since no microorganisms that are able to oxidize vinyl chloride or DCA with nitrate have been described thus far, the isolation of such bacteria would provide useful information for natural attenuation and bioremediation.

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**Degradation pathway of
2-chloroethanol in
Pseudomonas stutzeri strain JJ
under denitrifying conditions**

John A. Dijk, Jan Gerritse, Gosse Schraa and Alfons J. M. Stams

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(with slight modifications)**

Abstract

The pathway of 2-chloroethanol degradation in the denitrifying *Pseudomonas stutzeri* strain JJ was investigated. In cell free extract activities of a phenazine methosulfate (PMS)-dependent chloroethanol dehydrogenase, an NAD-dependent chloroacetaldehyde dehydrogenase and a chloroacetate dehalogenase were detected. This suggested that the 2-chloroethanol degradation pathway in this denitrifying strain is the same as found in aerobic bacteria that degrade chloroethanol. Activity towards primary alcohols, secondary alcohols, diols and other chlorinated alcohols could be measured in cell free extract with chloroethanol dehydrogenase activity. PMS and phenazine ethosulfate (PES) were used as primary electron acceptors, but not NAD, NADP or ferricyanide. Cells of strain JJ cultured in a continuous culture under nitrate limitation, exhibited a twelve times higher chloroethanol dehydrogenase activity than cells grown in batch culture. However, under chloroethanol-limiting conditions, chloroethanol dehydrogenase activity was in the same range as in batch culture. Cells grown on ethanol did not exhibit chloroethanol dehydrogenase activity. Instead, NAD-dependent ethanol dehydrogenase activity and PMS-dependent ethanol dehydrogenase activity was detected.

Introduction

Pseudomonas stutzeri strain JJ is a recently isolated bacterium that is able to grow on 2-chloroethanol (CE) under denitrifying conditions (6). Aerobic CE-degrading bacteria convert CE by two known degradation pathways. The most common degradation pathway of CE is via chloroacetaldehyde, chloroacetate and glycolate, where the responsible enzymes are CE dehydrogenase (CE-DH), chloroacetaldehyde dehydrogenase and chloroacetate dehalogenase, respectively (13,24). Poelarends *et al.* (21) described another degradation pathway for CE in a 1,2-dibromoethane degrading bacterium. They showed that *Mycobacterium* sp. strain GP1 converts CE to ethylene oxide with a haloalcohol dehalogenase. The pathway by which ethylene oxide is further degraded is still unclear.

The degradation pathway of CE in the absence of oxygen has not been described yet. Vogel and McCarty (29) proposed a mechanism for the anaerobic mineralization of vinyl chloride to carbon dioxide, in which vinyl chloride is hydrated to CE. They suggested that CE may be further oxidized to chloroacetaldehyde and eventually to carbon dioxide. However, none of the proposed intermediates were detected in this study and no other evidence was given for this degradation pathway.

Reductive dehalogenation under anoxic conditions has been reported for many different chlorinated aliphatic compounds (11,12,19). However, very little is known on the anaerobic oxidation of chlorinated aliphatic compounds and the enzymes that are involved. The potential of anaerobic oxidation processes for bioremediation of sites polluted with low-chlorinated aliphatics was already shown for 1,2-dichloroethane, vinyl chloride and dichloroethene (3-5,8). For understanding such processes, knowledge on the anaerobic degradation pathways of low-chlorinated aliphatics would be of importance. In this study we describe the degradation pathway of CE in *Pseudomonas stutzeri* strain JJ under denitrifying conditions.

Materials and methods

Organisms and growth conditions.

Pseudomonas stutzeri strain JJ (DSM 15012) was recently isolated (6). *Xanthobacter autotrophicus* GJ10 (DSM 3874) was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). Routine cultivation was done in 120-ml serum bottles that were crimp-sealed with butyl rubber stoppers and contained 50 ml medium and a gas phase of nitrogen (> 99.999 % pure). Oxidic batches were prepared in the same way, but in 250-ml bottles with a headspace of air. Strain JJ was grown in mineral medium as previously described (6). The pH was 7.5 instead of 7.0. Unless mentioned otherwise, CE (5 mM) was added as electron donor and NaNO₃ (10 mM) as electron acceptor. These compounds were added from separately autoclaved stock solutions. *Xanthobacter autotrophicus* GJ10 was cultured as described by Janssen *et al.* (14). All experiments were carried out at 30°C.

Chemostat culture.

Cells were grown continuously in a chemostat (Applikon Dependable Instruments B.V., Schiedam, The Netherlands.) with a volume of 1000 ml under constant stirring (300 rpm). The temperature was kept at 30°C and the pH was monitored and automatically adjusted to 7.5 with 1 M NaOH. The headspace of the vessel was continuously flushed (300 ml/h) with technical grade nitrogen passed through a reduction column (Glasgeraetebau Ochs GmbH, Bovenden/Lenglern, Germany) to remove traces of oxygen. The medium composition was the same as used for the batch cultures. The dilution rate was 0.01 h⁻¹ when cells were grown for preparation of crude cell free extract. CE or ethanol concentrations in the reservoir medium were 10 mM and the nitrate concentration was either 15 mM (nitrate limitation) or 25 mM (chloroethanol limitation).

Preparation of crude cell free extract.

Cells were harvested from batch cultures (250 ml) during late exponential phase when the optical density at 660 nm had reached 75 % of the final optical density. Cells from chemostat cultures were obtained by collecting the effluent overnight on ice. All further operations were carried out at 0-4°C. Cells were harvested by centrifugation (10 min, 10.000 rpm at 4°C), washed twice with 10 mM Tris.H₂SO₄ buffer, pH 7.5 and resuspended in the same buffer. After addition of 2 mM β-mercaptoethanol the cells were disrupted by sonication (Branson Ultrasonic Corporation, Danbury, CT) for 30 s followed by cooling on ice for 30 s. This cycle was repeated six times. Crude cell free extract was obtained after centrifugation (10 min at 13.000 rpm).

Enzyme assays.

CE-DH activity was measured by following the CE-dependent reduction of 2,6-dichlorophenol indophenol (DCPIP) spectrophotometrically at 570 nm in a coupled assay with PMS or PES as electron mediator. The incubation mixture contained in a final volume of 1 ml: 38 mM Tris.H₂SO₄ (pH 8.5), 15 mM NH₄Cl, 0.30 mM PMS or PES, 0.09 mM NaCN, 0.25 mM DCPIP and a suitable amount of crude cell free extract. The reaction was started by the addition of substrate (25 mM final concentration unless mentioned otherwise) after equilibration of the other compounds and was carried out at 30°C. NAD-dependent chloroacetaldehyde dehydrogenase was measured by following the NAD reduction spectrophotometrically at 340 nm. Crude cell free extract was incubated with 100 mM pyrophosphatebuffer (pH 9.0) and NAD (1 mM) at 30°C. After addition of chloroacetaldehyde (final concentration of 5 mM), the formation of NADH was monitored at 340 nm. Activity was corrected for NADH oxidase activity by measuring the disappearance of NADH (0.1 mM) when incubated with crude cell free extract. NAD-dependent alcohol dehydrogenase activity was measured as described above, but the buffer was replaced by 100 mM Tris.H₂SO₄ (pH 7.5 or 9.0) and 25 mM ethanol was used as substrate. Chloroacetate dehalogenase activity was measured by following the chloride release colorimetrically at 30°C in 3 ml 50 mM glycine. NaOH buffer (pH 9.0) by taking samples at different time intervals. The final substrate concentration was 5 mM. Chloroacetate dehalogenase activity was corrected for the chloride release in the absence of cell free extract. Ferricyanide reductase activity was assayed by following the disappearance of ferricyanide (1 mM) at 420 nm in 50 mM Tris.H₂SO₄ (pH 8.0) with a suitable amount of crude cell free extract and 25 mM substrate.

Analysis.

The pyrroloquinoline quinone (PQQ) content was determined enzymatically using crude membranes from *Escherichia coli* K-12 according to the method of (7).

OD₆₆₀ was determined in a Perkin-Elmer 55 A UV-VIS spectrophotometer.

Nitrate, nitrite and chloride were determined by suppressor mediated ion chromatography (Dionex, Breda, The Netherlands) and conductivity detection. Eluent consisted of 1.8 mM Na₂CO₃ and 1.7 mM NaHCO₃ at a flow rate of 1 ml/min. The chromatograph was equipped with an IonPac® AS9-SC column (Dionex, Breda, The Netherlands). 10 mM Mannitol was added to the samples for stabilization and 1 mM sodium bromide was added as internal standard (both final concentrations).

Alcohols were measured on a Chrompack CP9001 gas chromatograph equipped with a Chrompack Sil 5 CB capillary column (length, 25 m; diameter, 0.32 mm; df, 1.2 µm). 15 mM 2-propanol was used as internal standard.

Colorimetric chloride determination was done according to the method of (1).

Protein in crude cell free extract was determined with the Bio-Rad D_c protein assay (Bio-Rad Laboratories, Richmond, California) and cell protein in cultures was measured according to (16). Bovin serum albumin was used as standard.

Chemicals.

All chemicals were of analytical grade with a purity of more than 97% and obtained from commercial sources.

Results

Degradation pathway of CE in *Pseudomonas stutzeri* strain JJ under denitrifying conditions.

To reveal the degradation pathway of CE under denitrifying conditions, crude cell free extracts of *Pseudomonas stutzeri* strain JJ were tested for specific enzyme activities. The extracts of chloroethanol/nitrate grown cells showed a PMS-dependent CE-DH activity (0.08 µmol/mg protein/min), an NAD-dependent chloroacetaldehyde dehydrogenase activity (0.05 µmol/mg protein/min) and a chloroacetate dehalogenase activity (9.02 µmol/mg protein/min).

CE dehydrogenase.

CE-DH activity was observed with PMS and PES, but not with NAD, NADP or ferricyanide as primary electron acceptor. Addition of NH₄Cl had a slightly stimulating effect (+28 %) on the enzyme activity and the optimal pH was 8.5. Enzyme activity was completely inhibited by EDTA (5 mM). No measurable difference in CE-DH activity was found when the preparation of cell free extract and the enzyme assays were done under oxic or anoxic conditions. The CE-DH activity was induced during growth on CE, but not during growth on acetate and ethanol (after six serial transfers). The enzyme activity found in crude cell free extract from batch grown cells

was relatively low. However, higher activities were detected in cells of strain JJ grown continuously in a chemostat. Cells grown in the chemostat under nitrate-limiting conditions showed an CE-DH activity about fourteen times higher than cells grown under CE-limiting conditions (Table 4.1). The CE-DH activity in cells grown continuously under CE limitation was in the same range as in cells from batch cultures (Table 4.1). The direct effect of nitrate and nitrite on the CE-DH activity was investigated by adding nitrate and/or nitrite to the enzyme assay mixture in different concentrations (0, 10, 20 and 50 mM). Only nitrite had a clear negative effect on the enzyme activity at a concentration of 50 mM. At this concentration the enzyme activity was reduced by 50 %.

To investigate whether the observed CE-DH activity can account for the rate of CE degradation during growth in batch and chemostat cultures, CE degradation rates in cell cultures and CE-DH activities in cell free extracts were compared (Table 4.1). In cell free extract from both batch and chemostat grown cells, the CE-DH activity was much lower than the calculated degradation rates of CE in batch and chemostat cultures under comparable conditions

Table 4.1 Chloroethanol degradation rates in cell cultures and chloroethanol dehydrogenase (CE-DH) activities in crude cell free extract. Cells were cultured in a chemostat or in batch culture under different conditions.

Condition		Growth rate (h ⁻¹)	Chloroethanol degradation rate or CE-DH activity (μmol/mg protein/min)
<i>Cell culture</i>			
Chemostat	Chloroethanol limitation	0.02	0.14
	Chloroethanol limitation	0.04	0.10
	Chloroethanol limitation	0.06	0.20
Batch		0.14	0.53
<i>Crude cell free extract</i>			
Chemostat	Chloroethanol limitation	0.01	0.006
	Nitrate limitation	0.01	0.084
Batch		0.14	0.007

Strain JJ was grown in a chemostat with ethanol under nitrate-limiting conditions, to investigate whether the CE-DH is a non-specific ethanol dehydrogenase (E-DH). In crude cell free extract of ethanol grown cells no PMS-dependent E-DH activity or PMS-dependent CE-DH activity was found. Instead, PMS-dependent E-DH activity was detected in the pellet that remained after centrifugation of the sonified cell suspension. In washed whole cells, pre-grown on ethanol and nitrate, a PMS-dependent E-DH activity of 0.11 $\mu\text{mol}/\text{mg}$ protein/min was detected. When these cells were disrupted by means of sonication or French Press, no activity remained in the cell free extract. In crude cell free extract an NAD-dependent E-DH activity of 0.15 $\mu\text{mol}/\text{mg}$ protein/min was detected. No NAD-dependent CE-DH activity could be found in crude cell free extract of ethanol grown cells.

Different substrates were tested for PMS-dependent alcohol dehydrogenase activity in cell extract from cells grown on ethanol and nitrate and in cell free extract from cells grown on CE and nitrate (Table 4.2). In cell free extract from cells grown on CE, PMS-dependent activity was found for primary alcohols, secondary alcohols, diols and some chlorinated alcohols. In cell extract from cells grown on ethanol, only activity was measured for various primary and secondary alcohols.

In batch cultures supplied with ethanol and nitrate and inoculated with strain JJ pre-grown on CE and nitrate, CE-DH activity was still found after two serial transfers. After six serial transfers no CE-DH activity could be found and only an NAD-dependent E-DH activity was observed in cell free extracts.

Chloroacetate dehalogenase.

Chloroacetate dehalogenase activity was also tested for 2-chloropropionate, 3-chloropropionate and 4-chlorobutyrate. Only chloroacetate dehalogenase activity for chloroacetate and 2-chloropropionate was found (5.1 and 0.7 $\mu\text{mol}/\text{mg}$ protein/min, respectively). Rapid chloride release was observed with 4-chlorobutyrate as substrate, but this activity was probably due to chemical conversion, since a similar chloride release was observed in the absence of cell free extract. Chloroacetate is a growth substrate for strain JJ, but 2-chloropropionate is not.

Table 4.2 PMS-dependent alcohol dehydrogenase activities for different substrates (25 mM) in crude cell free extract (chloroethanol grown cells) or cell suspension (ethanol grown cells). Strain JJ was grown in a chemostat under nitrate-limiting conditions with chloroethanol or ethanol as sole carbon/energy source respectively. The enzyme activity for the growth substrate was considered 100 % (0.084 $\mu\text{mol/mg protein/ml}$ for chloroethanol-grown cells and 0.11 $\mu\text{mol/mg protein/ml}$ for ethanol-grown cells, respectively).

Substrate	PMS –dependent alcohol dehydrogenase activity	
	Growth substrate	
	chloroethanol	ethanol
methanol	20	< 1
ethanol	78	100
1-propanol	54	55
2-propanol	29	57
1-butanol	29	33
2-butanol	26	70
1-hexanol	23	19
2-hexanol	11	8
1-nonanol	11	1
1,2-propanediol	31	< 1
1,2-butanediol	74	< 1
1,3-butanediol	18	< 1
chloroethanol	100	< 1
1-chloro-2-propanol	54	< 1
1-chloro-3-propanol	35	< 1
1,3-dichloro-2-propanol	< 1	< 1
2,3-dichloro-1-propanol	8	< 1
formaldehyde	11	< 1
chloroacetaldehyde	54	< 1

CE degradation.

Xanthobacter autotrophicus GJ10 was chosen as model organism for aerobic CE degraders that can reduce nitrate since its aerobic CE degradation pathway is well

known. The capacity of strain GJ10 to grow on CE under nitrate-reducing conditions was investigated in the presence of various additions. Batch cultures with nitrate (10 mM) and chloroethanol (5 mM) were supplied with PQQ (3 μ M), filter sterilized anaerobic cell free extract of strain JJ (grown on CE and nitrate) or oxygen (4 mM). As a control, batch cultures were incubated with only CE and nitrate. Under oxic conditions CE was completely consumed. Batch cultures with PQQ and cell free extract as addition and the control batch culture did not differ significantly with respect to nitrate consumption, CE consumption and nitrite production. About 0.4 mM CE and 1.4 mM nitrate were consumed and about 1.1 mM of nitrite was produced. Under mixed oxic/nitrate reducing conditions 1.8 mM CE and 2.6 mM nitrate were consumed and 2 mM nitrite was formed.

Growth on succinate and nitrate was observed but not on methanol and nitrate.

Discussion

During growth of *Pseudomonas stutzeri* strain JJ on CE and nitrate, activity was found for a PMS-dependent CE-DH, an NAD-dependent chloroacetaldehyde dehydrogenase and a chloroacetate dehalogenase. Strain JJ is also able to grow on the corresponding intermediates (2-chloroacetate and glycolate), except for chloroacetaldehyde. However, since halogenated aldehydes are considered to be very toxic (15,18), it is not surprising that chloroacetaldehyde cannot be used as growth substrate. These results suggest that the degradation pathway of CE under denitrifying conditions in *Pseudomonas stutzeri* strain JJ is the same as found in aerobic bacteria (13,23,24). According to this pathway CE is first oxidized to chloroacetaldehyde, then chloroacetaldehyde is oxidized to chloroacetate and subsequently chloroacetate is dehalogenated to glycolate, which enters the central metabolic pathways (Fig. 4.1).

CE-DH activity is inducible, since no CE-DH activity was found when strain JJ was grown on acetate or on ethanol (after six serial transfers). The measured CE-DH activity was too low to account for the CE degradation rates observed in batch cultures during growth. This might be caused by a non-optimal enzyme assay or loss of activity during cell free extract preparation.

During growth on ethanol in a chemostat, crude cell free extract contained NAD-dependent E-DH activity but no PMS-dependent E-DH activity. In whole cells, PMS-dependent E-DH activity was detected, but this activity was lost after disrupting the cells. Loss of enzyme activity on preparation of cell extracts was also observed by (17).

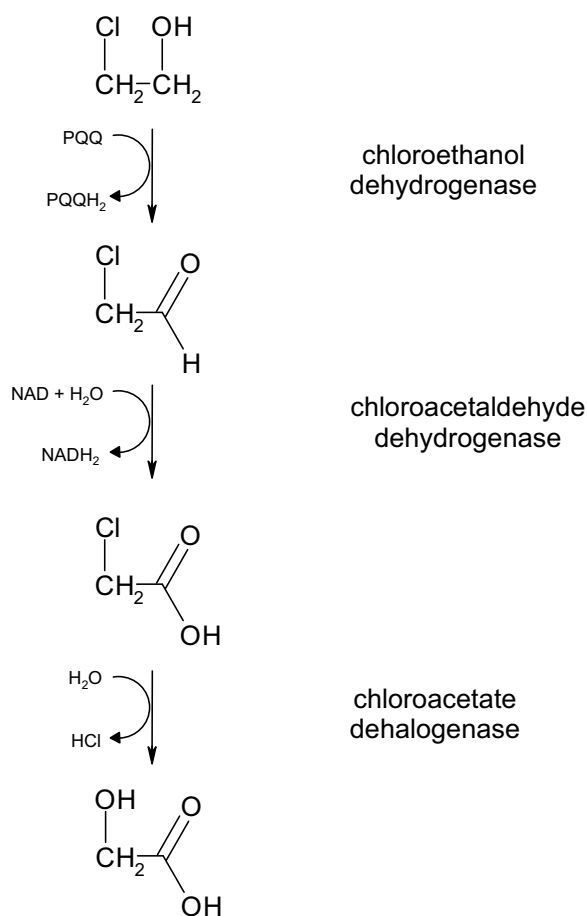


Figure 4.1. Proposed route of chloroethanol degradation under denitrifying conditions in *Pseudomonas stutzeri* strain JJ.

It is known for other *Pseudomonas* strains that different alcohol dehydrogenases can be expressed simultaneously in one organism when grown on one particular alcohol and that several alcohol dehydrogenases can be expressed during growth on different alcohols (26,27). Two observations suggest the presence of two different PMS-dependent alcohol dehydrogenases in strain JJ. Firstly, crude cell free extract from cells grown on CE showed PMS-dependent alcohol dehydrogenase activity towards different substrates than cell extract from cells grown on ethanol. Secondly, cell extract and cell free extract from ethanol-grown cells showed no activity with CE. CE conversion is therefore likely catalyzed by a CE-DH and not by a non-specific alcohol dehydrogenase that due to its broad specificity can convert CE like the CE-DH found in *Xanthobacter autotrophicus* GJ10 (13). This is supported by the finding that the highest PMS-dependent alcohol dehydrogenase activity in crude cell free extract from cells grown on CE, was found for chloroethanol as substrate.

The characteristics of the CE-DH activity (high pH optimum, the positive effect of NH_4Cl addition, inhibition by EDTA, the ability to use methanol as substrate and the fact that ferricyanide cannot be used as electron acceptor in the dye-linked assay), suggest that a type I quinoprotein alcohol dehydrogenase is responsible for the observed PMS-dependent CE-DH activity (9). These kinds of enzymes contain non-covalently bound PQQ as prosthetic group. Indeed, we could detect PQQ in cells and medium during aerobic growth on CE and the addition of PQQ enhanced the yield of strain JJ. However, no PQQ could be detected in cells and medium of a denitrifying culture of strain JJ. From research on PQQ-dependent glucose dehydrogenases it is known that several bacteria cannot produce PQQ under anoxic conditions. For example, *Pseudomonas aeruginosa* and *Rhodopseudomonas sphaeroides* produce both PQQ and the apo-enzyme from glucose dehydrogenase under oxic conditions, while under denitrifying conditions only the apo-enzyme is produced (20,22). Oxygen is probably required for the last step of the PQQ synthesis (25,28). It is unknown how this last step is catalyzed under denitrifying conditions in for example *Paracoccus* or *Hyphomicrobium* during growth on methanol or in strain JJ during growth on CE. Although no PQQ could be detected during growth of strain JJ under denitrifying conditions, it does not exclude the possibility that PQQ is in fact synthesized. It is possible that only very low levels of PQQ are produced in the absence of oxygen so that all PQQ is bound to the apo-enzyme. The non-covalently bound PQQ can not be detected by the enzymatic method for PQQ determination. The fact that PQQ addition had a stronger effect on the yield of strain JJ under denitrifying conditions than under oxic conditions might be an indication that PQQ is indeed produced in the absence of oxygen, but only in very low quantities.

In a previous publication we showed that several aerobic CE degraders can not degrade CE under nitrate-reducing conditions (6). The current experiments with *Xanthobacter autotrophicus* GJ10, a well-known aerobic CE degrader, suggest that under nitrate-reducing conditions the degradation of CE stops, because a certain factor becomes limiting. Probably this factor is needed for the degradation of CE that strain GJ10 is able to synthesize only under oxic conditions. However, no evidence was found that PQQ might be this factor. The addition of PQQ did not have an effect on the CE degradation under anoxic conditions by strain GJ10 while it is known that exogenously added PQQ is able to restore alcohol dehydrogenase activity when the quinoprotein apo-enzyme is produced, but no PQQ is synthesized (2,10). Another possibility is that one of the intermediates, like chloroacetaldehyde, or nitrite accumulates to toxic levels under nitrate-reducing conditions.

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**Monochlorobenzene degradation
in a nitrate-reducing
up-flow soil column**

John A. Dijk, Jan Gerritse, Gosse Schraa and Alfons J. M. Stams.

Submitted for publication

Abstract

Monochlorobenzene removal was observed in a nitrate-reducing up-flow soil column. Monochlorobenzene was supplied as the sole carbon and energy source and nitrate was added as electron acceptor. Of the ingoing 120 μM monochlorobenzene, 73 μM had been removed at the end of the column. Chloride formation and nitrate reduction were observed, but the concentrations were not in accordance with the amount of monochlorobenzene removed if total oxidation of monochlorobenzene to carbon dioxide is assumed. When oxygen was supplied to the column, the extent of monochlorobenzene removal increased. After an aerobic period for eight weeks, the oxygen was replaced by nitrate. As a result the removal of monochlorobenzene completely stopped. Monochlorobenzene sorption onto the soil column matrix was low and could not account for the monochlorobenzene disappearance. Based on these observations we assume that the monochlorobenzene disappearance in the nitrate-reducing column was the result of anaerobic biodegradation. However, clear evidence could not be obtained.

Introduction

Monochlorobenzene (MCB) is one of the most widely used chlorinated benzenes. In the past it was mainly applied in the manufacture of chlorinated pesticides, especially DDT and in the production of phenol and aniline. Nowadays, MCB is an important intermediate in the synthesis of chemicals such as nitrochlorobenzenes and diphenyl oxide and is used as solvent. Due to its widespread use in the industry it has become an ubiquitous environmental pollutant. In addition, MCB can occur in anoxic groundwater systems as a result of reductive dechlorination of higher chlorinated benzenes (hexachlorobenzene (28)), lower chlorinated benzenes (trichlorobenzene (5)) and hexachlorocyclohexane (3,21,26).

MCB is used as sole energy and carbon source by several aerobic bacteria (24,29,32), but is considered persistent under anoxic conditions. Reductive dechlorination of MCB has been reported, though benzene was either not measured or only found in very low quantities compared to the MCB that had disappeared (19,25). There are indications that MCB can be oxidized anaerobically with nitrate as electron acceptor (8,15,34). The aim of this research is to further investigate this transformation in soil columns and batch cultures.

Materials and methods

Soil column set-up.

A glass column (35 cm length x 3.6 cm internal diameter) was packed in an anoxic glove box with sediment from four different contaminated sites (depths varying from 5 to 30 m). These sites were mainly polluted with 1,2-dichloroethane, chloroethenes, BTEX and/or gasoline. A layer of glass wool and glass beads was put at the bottom of the column to avoid clogging of the inlet. Sampling ports were sealed with both butyl rubber and viton rubber septa, in which the viton rubber septum was in contact with the soil material. The columns were percolated (up-flow) with media at a flow-rate of 4.5 ml/h (liquid retention time \pm 30 hours) by a peristaltic pump (101U, Watson Marlow Ltd., UK). Influent medium was continuously stirred and flushed with N₂ that was led through a titanium citrate solution to remove traces of oxygen (Fig. 5.1). Titanium citrate solution was prepared as described by Zehnder and Wuhrmann (35). The medium consisted of the following components: Na₂HPO₄/KH₂PO₄ buffer (2 mM, pH 7), 0.07 g (NH₄)₂SO₄/l, 0.01 g MgSO₄·7H₂O/l, 0.006 g CaSO₄·2H₂O/l and 0.1 ml trace element solution/l (11). Unless mentioned otherwise, 5 mM NaNO₃ was added as electron acceptor. Oxygen was supplied by flushing the medium with oxygen gas (medical grade). The desired concentration was achieved by adjusting the flow rate of oxygen and N₂ separately.

In the case of mixed oxic/nitrate reducing conditions, separately adjustable oxygen and nitrogen flows were combined, just before entering the medium vessel.

An aqueous solution of 1.95 mM MCB was injected into the medium by a syringe pump (KD Scientific, model 200). The substrate was mixed with the medium in a stainless steel coil construction just before entering the column (Fig. 5.1). By adjusting the pump rate of the syringe pump, the preferred concentration was established. The columns were operated in the dark in a 20°C climate room.

Batch cultures.

Batch cultures consisted of 50 ml soil column effluent in 120-ml crimp-sealed bottles with viton rubber stoppers. In some experiments, soil material from the column was also added. Bottles were filled in an anaerobic glove box with a N₂/H₂ gas phase in a 95% / 5% ratio. Afterwards, the bottles were flushed with nitrogen gas (> 99.999 % pure). Unless mentioned otherwise, 50 µM MCB and 1 mM NaNO₃ were added (both final concentrations). Batches were incubated under continuous shaking (100 rpm) at 20°C in the dark.

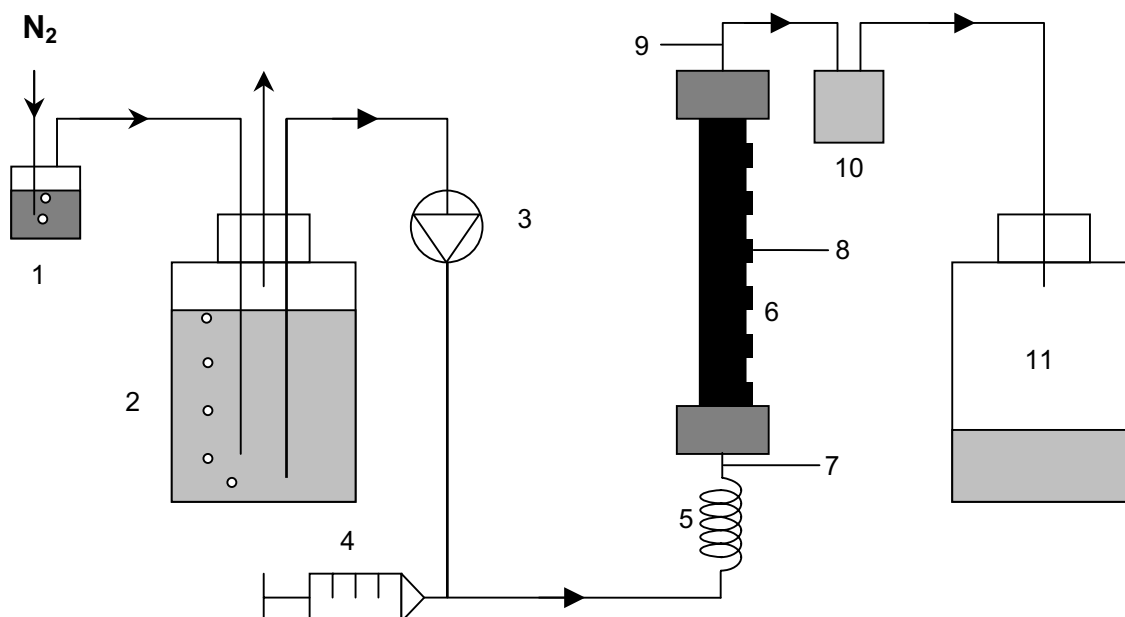


Figure 5.1 Schematic representation of the soil column set-up. Nitrogen gas was first led through a titanium citrate solution (1) and then flushed the reservoir medium (2). The medium was pumped up-flow through the soil column (6) by a peristaltic pump (3). MCB (stock solution in water) was added to the medium by a syringe pump (4) and mixed with the media in a coil like structure (5). Before the medium entered the waste vessel (11) it was let through a replaceable vial (10), which was used for collecting effluent. Influent and effluent samples were taken from (7) and (9), respectively. Sample ports in the column (8) were used for profile measurements.

Analyses.

MCB was measured on a Packard model 436 gas chromatograph (Packard Instrument B.V.), equipped with a Sil 5CB capillary column (length; 25 m, diameter; 0.32 mm, df; 1.20 μm , Chrompack, Middelburg, The Netherlands) using a FID detector. The oven temperature was 70°C isothermal. 0.5 ml Liquid samples from the column were directly injected into a 10 ml vial containing 4.5 ml 27.5 mM NaOH (preservation) and crimp-sealed with viton rubber stoppers. Headspace samples from these vials were injected into the GC after one-hour incubation at 70°C. Batch cultures were analyzed for MCB by taking 0.2 ml headspace samples. The detection limit was $\pm 2 \mu\text{M}$ MCB.

Cl^- , NO_3^- and NO_2^- were determined by suppressor mediated ion chromatography (Dionex, Breda, The Netherlands) and conductivity detection. Eluent consisted of 1.8 mM Na_2CO_3 and 1.7 mM NaHCO_3 at a flow rate of 1 ml/min. The chromatograph was equipped with an IonPac[®] AS9-SC column (Dionex, Breda, The Netherlands). 10 mM

mannitol was added to the samples for stabilization and 1 mM sodium bromide was added as internal standard (both final concentrations).

O₂ was measured on the same gas chromatograph, but with a CP-Molsieve 5A column (length; 30 m, diameter; 0.53 mm, df; 15 µm, Chrompack) at 50°C.

Determining ¹²C/¹³C isotope composition.

MCB was extracted from 250 ml influent and effluent samples (containing 1 g NaOH/l for preservation) with 4 ml pentane (extra pure). Analysis was done by Dr. Hans H. Richnow at the Sektion Sanierungsforschung, Umweltforschungszentrum Leipzig-Halle (Germany) (30).

MCB extraction from soil material.

Soil material (5 g) was put in a 10 ml glass vial together with 2 ml *n*-pentane (for organic trace analysis) and capped with teflon-coated stoppers. The vials were put in a sonic bath for 15 minutes and then incubated for 1 hour in a end-over-end-shaker. Soil that had been extracted two times with pentane was used for making a calibration curve. The pentane fraction was analyzed on a gas chromatograph equipped with a Sil 5CB capillary column (as described).

Results

Soil column.

An up-flow soil column has been operated continuously under anoxic conditions for more than four years with monochlorobenzene as sole carbon and energy source and nitrate as electron acceptor. Initially, this soil column was supplied with a mixture of chlorinated hydrocarbons and nitrate (8). During those four years, removal of MCB and reduction of nitrate was consistently observed. The concentration of MCB supplied to the soil column was gradually increased during the four years from 10 µM to 120 µM. The removal of MCB over this period increased from about 6 ± 2 µM to 73 ± 3 µM. At the highest MCB concentration chloride increase in the effluent varied from 35 µM to 140 µM and nitrate consumption varied from 88 µM to 1200 µM. Nitrite could not be detected (< 25 µM) and intermediates of MCB degradation (e.g. benzene) were not found.

To investigate the role of nitrate as electron acceptor and the influence of oxygen on the MCB degradation both with and without nitrate, different terminal electron accepting conditions were applied (Table 5.1).

Table 5.1 MCB removal in a soil column supplied with 121 μM MCB under different electron accepting conditions.

Electron acceptor	MCB in (μM)	MCB out (μM)	MCB disappearance (%)
NO_3^- (5 mM)	121	48	60 ± 3
No electron acceptor	121	86	29 ± 7
NO_3^- (1 mM) + O_2 (50 μM)	121	75	38 ± 8
O_2 (690 μM)	121	16	87 ± 3

When nitrate was omitted from the medium, removal of MCB was still observed, but it was reduced from 60 ± 3 % to 29 ± 7 % of the influent concentration. Subsequently, mixed oxic/nitrate-reducing conditions were applied with 1 mM nitrate and 50 μM oxygen and MCB degradation was 38 ± 8 %. The MCB removal under mixed oxic/nitrate-reducing conditions was significantly lower than under nitrate-reducing conditions. After the mixed oxic/nitrate-reducing conditions the oxygen concentration was increased from 50 μM to 690 μM and nitrate was omitted from the medium. The amount of supplied oxygen was theoretically sufficient for the oxidation of 82 % of the ingoing MCB to carbon dioxide (oxygen-limiting conditions). As a result the MCB degradation increased to 87 ± 3 % of the ingoing MCB concentration. No oxygen could be detected in the effluent.

After these oxygen-limiting conditions, the column was switched over to nitrate-reducing conditions (5 mM nitrate) again. However, MCB disappearance decreased and degradation stopped completely after two weeks.

To test the degree of absorbance of MCB to the soil material, samples were taken from different positions in the column and extracted with *n*-pentane for MCB analysis. The lower part of the column contained 0.0019 ± 0.0003 μmol MCB/g soil, the middle part 0.0022 ± 0.0011 μmol MCB/g soil and the upper part 0.0428 ± 0.0032 μmol MCB/g soil.

Batch cultures.

Batch cultures with column effluent as medium were incubated to determine the mass balance of the MCB degradation. Effluent was collected when MCB degradation was observed under nitrate-reducing conditions. Nitrate (1 mM), oxygen (oxic) or a combination of both (1 mM nitrate + 25 μM oxygen), were supplied as electron acceptor with MCB (50 μM) as sole carbon and energy source. In different batches, additional compounds were used to exclude the possibility that traces of oxygen would be present in the batch culture. Ascorbate (4 mM) was used as reducing agent in the denitrifying batches. Ethanol (100 μM) was added to some

batches so that (facultative) aerobic bacteria could consume ethanol together with traces of oxygen that might be present.

Rapid degradation of MCB was only observed under oxic conditions (Fig. 5.2). Within three days \pm 98% of the MCB was degraded. After the second addition of MCB (50 μ M), 95 % was degraded within two days (not shown).

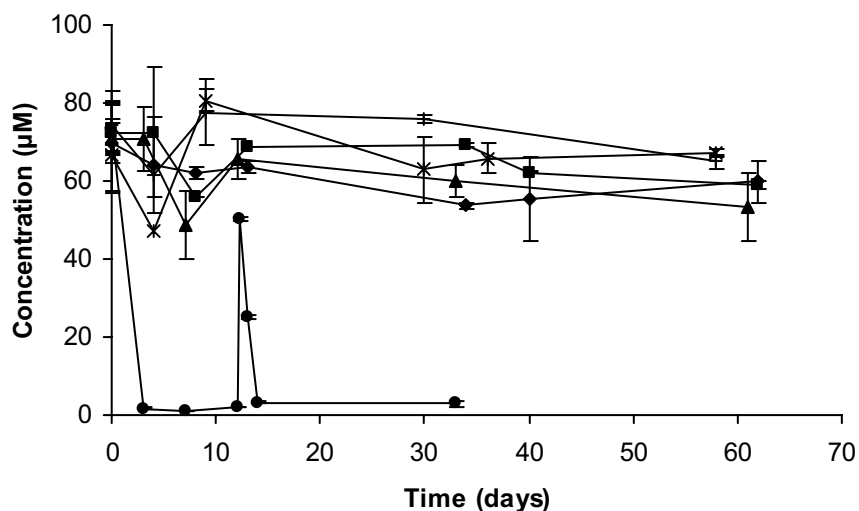


Figure 5.2 Concentration of monochlorobenzene (MCB) in batch cultures consisting of soil column effluent with addition of 50 μ M MCB and 1 mM nitrate. After 12 days, MCB (50 μ M) was added to the aerobic batch culture (●). Humic acids (1 g/l) were added after 30 days. As electron acceptor oxygen (●), nitrate (■), nitrate [with ascorbate] (◆), nitrate [with ethanol] (*), nitrate with oxygen (▲) and no electron acceptor (-) were used.

Humic acids (1 g/l) were added after about thirty days, when still no MCB disappearance had been observed in the different anoxic batch cultures. Humic acids were supplied because of their ability to serve as electron shuttle or electron acceptor in the anaerobic oxidation of organic compounds (16). It was also observed that humic acids greatly accelerate the rate of benzene degradation in aquifer sediments in which Fe(III) is the terminal electron acceptor (17) However, they had no effect on the behavior of MCB (Fig. 5.2). A significant decrease in the MCB concentration was not observed, even after 450 days (data not shown).

More batch experiments were carried out with different concentrations of MCB (50 μ M and 100 μ M) and nitrate as electron acceptor. These batches were inoculated with column effluent and soil material from the column. MCB removal was not observed in a total of thirty batches after 100 days (data not shown). Only in one batch culture an almost complete removal of MCB (> 98 %) was found under anoxic conditions in the presence of nitrate. After the third addition of extra MCB and nitrate,

the degradation stopped (Fig. 5.3). Transfer to fresh medium (10 % inoculum) did not result in restoration of the degradation activity.

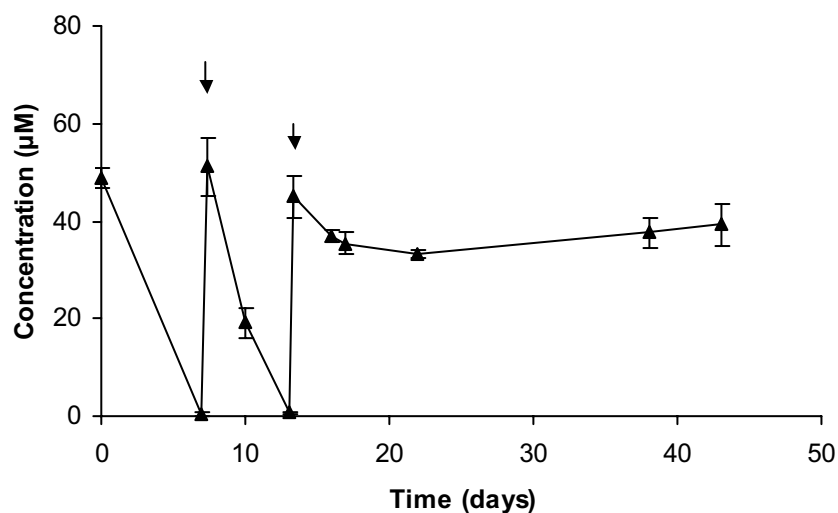


Figure 5.3 Removal of monochlorobenzene (MCB) in a batch culture supplied with 50 µM MCB and 5 mM nitrate. Re-addition of 50 µM MCB is indicated by arrows

Stable isotope fractionation.

Stable carbon isotope fractionation was used to demonstrate biodegradation. Laboratory studies have shown that aromatic compounds can undergo isotopic fractionation during microbial degradation (see also: Discussion) (1,18,20,22). The $^{12}\text{C}/^{13}\text{C}$ isotope compositions of inlet and outlet samples from the soil column were determined, but a statistically relevant alteration of the isotope signature was not found between the inlet and outlet samples.

Discussion

In a soil column that was operated for more than four years, removal of MCB was consistently observed under nitrate-reducing conditions. The amount of nitrate that was reduced varied too much to determine accurate mass balances in case of a possible oxidation of MCB to carbon dioxide. Higher nitrate consumption than expected on basis of complete oxidation of MCB with nitrate as electron acceptor might be explained by the anaerobic oxidation of ammonium with nitrate/nitrite as electron acceptor. The chloride detected was higher than expected according to the

amount of MCB that had disappeared. Benzene formation could not be detected. Attempts to obtain degradation in anoxic batch cultures with nitrate as electron acceptor and inoculated with soil material, were not successful. Only MCB removal under oxic conditions was observed.

The disappearance of the MCB in the soil column and the observed nitrate reduction and chloride formation suggest degradation of MCB. The observation that MCB removal with nitrate as electron acceptor stopped completely after an oxic period of about eight weeks, also suggests that the MCB removal was of anaerobic microbiological origin.

MCB removal was also observed under conditions without an added electron acceptor, under oxic conditions and under mixed nitrate-reducing/oxygen-limiting conditions in the soil column. This corresponds with earlier results, where MCB disappearance was observed in soil columns with nitrate as electron acceptor and with no electron acceptor added, but not in columns with manganese(IV) oxide or iron(III) hydroxide as electron acceptor (8). It is unclear why MCB removal occurred in the absence of a possible electron acceptor, nevertheless the MCB removal was only half of the MCB removal in the presence of nitrate.

Carbon isotope fractionation can be used to demonstrate biodegradation however, aerobic degradation of hydrocarbons is not necessarily accompanied by a stable carbon isotope fractionation. For example, anaerobic toluene degradation was accompanied by carbon isotope fractionation, but isotope fractionation during aerobic biodegradation depends on the enzymatic pathway involved (20,22,23). Carbon isotope fractionation was observed for the aerobic toluene degradation via the methyl oxidation pathway (*Pseudomonas putida* mt-2) (20), but no carbon isotope fractionation occurred during degradation via ring oxidation by *P. putida* F1 (23). For benzene it was found that carbon isotopic enrichment factors for anaerobic and aerobic biodegradation are comparable (18). Recently, it was found that anaerobic degradation of monochlorobenzene can be demonstrated by isotopic fractionation (15). However, isotopic fractionating could not be measured during MCB removal in the soil column, but we assume that a concentration gradient of 60 % between the inlet and outlet of the column (72 μM MCB removal) was too small to detect isotope fractionating.

The MCB removal cannot be explained by sorption of the MCB to the soil. We summed up all the MCB that has disappeared during the running period of the column and assume that the soil material has absorbed all this MCB. In this case, assuming an even distribution, one gram of soil material should contain $\pm 14 \mu\text{mol}$ MCB to account for the MCB disappearance during more than four years. We measured at the end of the column experiment between 0.0019 (at the bottom) and 0.043 μmol (at the top) MCB/g soil. The difference in the measured MCB concentration at the bottom and the top of the column, may be explained by the fact

that the samples of the top of the column contained more liquid medium. Since the MCB removal had stopped at the time of measuring, the medium contained relatively high concentrations of MCB.

The participation of oxygen in the removal of MCB can be excluded under the conditions in which the column was operated anaerobically. For a complete aerobic oxidation of one mole MCB to carbon dioxide seven moles oxygen would be required. In the absence of oxygen 73 μM MCB was degraded with nitrate, which would require 511 μM oxygen in the case of complete mineralization. This oxygen concentration is higher than the maximum solubility of oxygen at 20°C (assuming 21 % oxygen in the air).

If oxygen would only be involved in an initial oxygenation with a dioxygenase, one mole of oxygen is needed per mole MCB. This would imply that for the degradation of 73 μM MCB, 73 μM oxygen would be required. It is very unlikely that such an amount of oxygen was present in the medium as nitrogen gas (>99.9 % pure) was led through a titanium citrate solution to remove traces of oxygen, before it flushed the medium.

Reductive dechlorination of MCB is also not very likely since there is no possible electron donor added to the medium (besides MCB itself). No benzene was detected in both the column and batch cultures.

These observations and calculations lead to the conclusion that MCB was oxidized with nitrate in the absence of oxygen. Anaerobic biodegradation is of increasing interest for intrinsic and stimulated bioremediation processes. The biodegradation of compounds like BTEX in groundwater is often limited by the supply of dissolved oxygen (4,6). Nitrate is therefore a suitable alternative to oxygen, since its energy yield is close to oxygen, it is highly water soluble, it does not precipitate oxides, it is inexpensive and it is non-toxic to aquifer microorganisms at concentrations below 500 mg/l (14). However, addition of nitrate to the subsurface should be well controlled to prevent drinking water contamination and because of its potential to contribute to eutrophication of surface waters.

Several bacteria have been described that can degrade homocyclic aromatic hydrocarbons, such as xylene, ethylbenzene and toluene, with nitrate as electron acceptor (2,9,10,27,31,33). For a long time there was only one article that mentioned the anaerobic degradation of benzene (13), but recently two strains of *Dechloromonas* were isolated that can couple the anaerobic benzene oxidation to nitrate reduction (7,13). Given this development of knowledge on the anaerobic benzene degradation, it is not unthinkable that in the near future also anaerobic monochlorobenzene degrading microorganism(s) will be isolated.

Gottschal *et al.* (12) also observed MCB degradation in anoxic soil columns, but they too could not demonstrate complete MCB oxidation to carbon dioxide or repeat these results in batch cultures. Although data are not yet completely conclusive, we have

clear indications for the degradation of MCB under nitrate-reducing conditions. Repeated transfer of degradation activity in batch cultures and enrichment of the responsible bacteria is essential to unequivocally demonstrate anaerobic oxidation of MCB.

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Summary and concluding remarks

Chlorinated hydrocarbons belong to the most frequently encountered contaminants in soil and groundwater. Many of them were found to be toxic and recalcitrant, which causes a potential threat to the environment. Therefore, it is of great importance that sites contaminated with chlorinated hydrocarbons are remediated. Bioremediation refers to the use of living organisms (mainly microorganisms) to solve an environmental problem such as contaminated soil or groundwater. Under controlled conditions, bioremediation can be a practical, cost effective and safe method to remove hydrocarbons from contaminated soils.

In this thesis we focus on anaerobic microbial oxidation of low-chlorinated hydrocarbons. These low-chlorinated hydrocarbons can enter soil and groundwater by direct release or by partial dechlorination of higher chlorinated hydrocarbons. Their fate strongly depends on microbial processes in soil and groundwater systems. Since many contaminated aquifers contain large volumes that are dominated by anaerobic processes it is apparent that anaerobic microorganisms play an important role in the degradation of contaminants (1).

The tendency of low-chlorinated hydrocarbons to undergo oxidative dehalogenation increases when the number of chlorine substituents decreases (38,39). Whether low-chlorinated hydrocarbons are more likely to be degraded by oxidation than by reduction processes under anoxic conditions depends primarily on environmental conditions (e.g. redox conditions, availability of electron acceptors/donors, present microorganisms).

For low-chlorinated ethenes it has been demonstrated that anaerobic oxidation can occur in microcosms inoculated with aquifer and stream sediment in the presence of electron acceptors such as nitrate, iron(III), manganese(IV), in a methanogenic bioreactor and in iron(III)-reducing sediment (4,5,6,7,39). The ability of microorganisms that are present at polluted sites to oxidize low-chlorinated ethenes in the absence of oxygen has important implications for natural attenuation of these contaminants. However, knowledge on anaerobic oxidation of low chlorinated hydrocarbons is still limited.

The aim of the research, presented in this thesis, was to investigate whether anaerobic oxidation processes can occur at sites contaminated with low chlorinated hydrocarbons and to investigate the microorganisms responsible for these processes.

Chapter 1 is a summary of the knowledge on anaerobic microbial oxidation of low-chlorinated hydrocarbons. The difference between aerobic and anaerobic oxidation and between anaerobic oxidation and anaerobic reduction is explained. Subsequently, an overview is given of the different low-chlorinated hydrocarbons that are known to be oxidized and/or fermented in the absence of oxygen and the organisms that are involved. This chapter also summarizes the anaerobic oxidative dechlorination pathways and fermentative pathways that are known.

The potential for anaerobic oxidation of different pollutants is presented in **chapter 2**. Four up-flow soil columns, filled with a mixture of soil from different sites, mainly polluted with 1,2-dichloroethane (DCA), chloroethenes, BTEX and/or gasoline were supplied with a mixture of different chlorinated hydrocarbons. They were operated in the absence of oxygen under nitrate-, iron(III)- or manganese(IV)-reducing conditions or in the absence of an externally supplied electron acceptor. A series of batch cultures inoculated with contaminated soil were incubated with different chlorinated hydrocarbons under the same terminal electron accepting conditions. It was found that DCA, 2-chloroethanol (CE), vinyl chloride (VC), monochlorobenzene (MCB) and octane could be removed in the absence of oxygen. DCA was removed in soil columns under all three different electron-accepting conditions and in the absence of an added electron acceptor. DCA was degraded under nitrate- and manganese(IV)-reducing conditions in batch cultures. Half-lives for DCA removal/degradation ranged from several hours to days. VC removal was observed in soil columns under both manganese(IV)- and nitrate-reducing conditions with half-lives of 0.2 and 0.3 days, respectively. MCB removal was observed in soil column experiments under nitrate-reducing conditions. In batch cultures, CE and octane degradation was detected under nitrate reducing conditions.

An attempt was made to isolate bacteria responsible for the removal of DCA under nitrate-reducing conditions in batch cultures (17). However, we were not able to maintain the DCA-degrading activity upon sub-culturing. Subsequently, several possible intermediates of the DCA degradation pathway were tested as growth substrate instead of DCA. CE turned out to be a good growth substrate and we obtained a well growing enrichment culture on 5 mM CE and 10 mM nitrate. From this enrichment culture *Pseudomonas stutzeri* strain JJ was isolated. This bacterium, which is described in **chapter 3**, is the first isolate known that can grow anaerobically with CE as sole carbon and energy source and nitrate as electron acceptor. Besides nitrate, also oxygen and nitrite could be used as electron acceptors, but not manganese(IV) oxide, iron(III) hydroxide, sulfate, chlorate or fumarate. No difference in the spectrum of substrates was observed between oxic and denitrifying conditions. However, the μ_{\max} during growth on CE under denitrifying conditions was significantly lower than under oxic conditions (0.14 h^{-1} and 0.31 h^{-1} , respectively).

The only other chlorinated compound found that could be used as growth substrate was chloroacetate.

The aquifer from which strain JJ was obtained was anoxic, contained small amounts of nitrate ($< 10 \mu\text{M}$) and contained chlorinated aliphatics, including DCA (up to 3.5 mM), VC and various chlorinated methanes and propanes (17). The ability to use low-chlorinated aliphatics as substrates under denitrifying conditions may add significantly to the competitive strength of strain JJ in the environment.

It remains unclear, which (chlorinated) compound(s) served as growth substrate for strain JJ *in situ*. CE and chloroacetate are degradation products from DCA. Unfortunately, CE and chloroacetate were not covered in the standard analyses of the pollutants at this location. Another possibility would be that strain JJ could grow on DCA in a mixed culture, since the enrichment from which strain JJ originates was able to degrade DCA under nitrate-reducing conditions (17). Finally, strain JJ may have lost the ability of degrading DCA during isolation and cultivation on CE in the laboratory. For example, strain JJ might have lost the DCA dehalogenase or the dehalogenase is not induced by DCA, but possibly by another chlorinated compound in the environment. A PCR reaction with primers of different dehalogenases using DNA from the original DCA-degrading enrichment culture and from a pure culture of strain JJ might give us more information whether or not a DCA dehalogenase gene was/is present in strain JJ.

The ability to grow on CE and nitrate was also tested with various other bacterial strains, including some closely related *Pseudomonas stutzeri* strains and aerobic CE degraders. None of these strains grew on CE and nitrate. This indicates that the ability to grow on CE and nitrate is an exceptional feature of *Pseudomonas stutzeri* strain JJ. The degradation pathway of CE under denitrifying conditions by strain JJ is presented in **chapter 4**. The results represented in this chapter suggest that the CE degradation pathway in strain JJ is the same as found in aerobic bacteria that degrade CE (24,32,33). According to this pathway, CE is first oxidized to chloroacetaldehyde, then chloroacetaldehyde is oxidized to chloroacetate and subsequently chloroacetate is dehalogenated to glycolate, which enters the central metabolic pathways.

The CE-dehydrogenase (CE-DH) activity was further investigated and appeared to be inducible, since no activity was found when strain JJ was grown on acetate or ethanol (after six serial transfers). Two observations suggest the presence of two different PMS-dependent alcohol dehydrogenases in strain JJ. First, crude cell free extract from cells grown on CE showed PMS-dependent alcohol dehydrogenase activity towards other substrates than cell extract from cells grown on ethanol. Second, cell (free) extracts from ethanol-grown cells showed no activity with CE. CE conversion is therefore likely catalyzed by a CE-DH and not by a non-specific alcohol dehydrogenase that due to its broad specificity can convert CE like the CE-DH from *Xanthobacter autotrophicus* GJ10 (24). This is supported by the finding that the highest PMS-dependent alcohol dehydrogenase activity in crude cell free extract from cells grown on CE, was found for CE as substrate.

Unfortunately, we were not able to reveal why strain JJ can grow on CE with nitrate, while other aerobic CE degraders that can reduce nitrate cannot grow on CE and nitrate. The addition of pyrroloquinoline quinone (PQQ) to the media of *Xanthobacter autotrophicus* GJ10 did not result in growth on CE and nitrate, while it is known that

exogenously added PQQ is able to restore alcohol dehydrogenase activity when the quinoprotein apo-enzyme is produced, but no PQQ is synthesized. Despite this observation it cannot be ruled out that PQQ plays a role in the inability of aerobic CE degraders that can reduce nitrate to grow on CE and nitrate. It has been suggested that oxygen is required for the last step of PQQ synthesis (35,36). However, strain JJ can grow on CE and *Paracoccus* and *Hyphomicrobium* species can grow on methanol under denitrifying conditions with the use of a PQQ-dependent alcohol dehydrogenase. Knowledge on how the last step of PQQ synthesis is catalyzed under denitrifying conditions may also help us to find an explanation why only strain JJ is able to grow on CE under denitrifying conditions.

Our assumption that the degradation pathway of CE under denitrifying conditions in *Pseudomonas stutzeri* strain JJ is similar to the degradation pathway under oxic conditions was also observed for the degradation of dichloromethane (DCM). DCM dehalogenase, the key enzyme of DCM degradation under oxic conditions, was induced in *Hyphomicrobium* sp. strain DM2 under both oxic and anoxic (in the presence of nitrate) conditions (26).

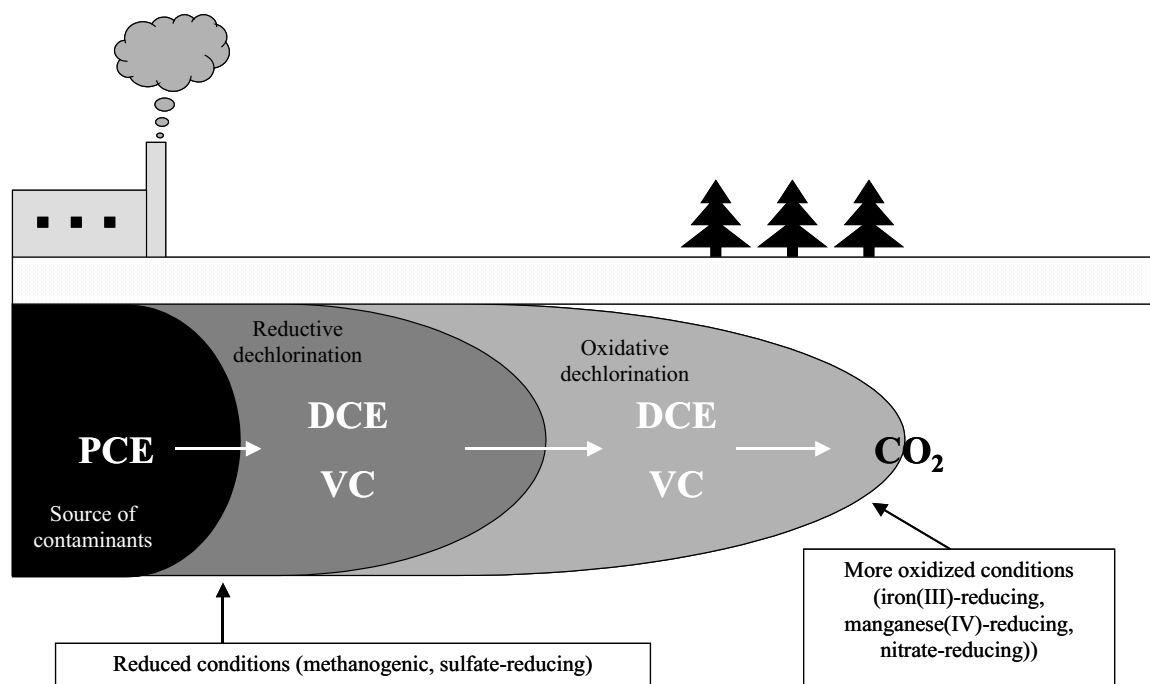
The last chapter, **chapter 5** describes the removal of monochlorobenzene (MCB) in a nitrate-reducing up-flow soil column. This column was the nitrate-reducing column used for the experiments described in chapter two. The substrate cocktail with different chlorinated compounds that was supplied to the column was replaced by a solution with only MCB. To further investigate the MCB transformation that we observed, both soil column and batch experiments were carried out. In the soil column that was operated for more than four years, partial removal of MCB was consistently observed under nitrate-reducing conditions. Of the ingoing 120 μM MCB, typically 73 μM had been removed at the end of the column. The amount of nitrate that was reduced varied too much to obtain accurate mass balances for the possible oxidation of MCB to CO_2 . Higher nitrate consumption than expected on basis of complete oxidation of MCB with nitrate as electron acceptor might be explained by the anaerobic oxidation of ammonium with nitrate/nitrite as electron acceptor. The chloride detected was higher than expected according to the amount of MCB that had disappeared. Benzene formation could not be detected. MCB degradation (two times 50 μM) was observed in one denitrifying batch culture, but this process ceased after the third supply of 50 μM MCB. Attempts to transfer MCB degradation in anoxic batch cultures with nitrate as electron acceptor with and without additional soil column inocula were not successful. Only MCB degradation under oxic conditions was consistently observed.

The disappearance of the MCB in the soil column and the observed nitrate reduction and chloride formation suggest degradation of MCB. The fact that MCB removal with nitrate as electron acceptor stopped completely after an oxic period of about eight weeks, also suggests that the MCB removal was caused by biotransformation. In

addition, it was proven that the observed MCB removal cannot be explained by sorption of the MCB to the soil matrix. These observations and calculations can only be explained by assuming that MCB was oxidized in the absence of oxygen with nitrate as electron acceptor. MCB degradation in anoxic soil columns has also been observed by other researchers, but they also could not demonstrate complete MCB oxidation to carbon dioxide or obtain anaerobic degradation in batch cultures (18,25).

In conclusion, this research shows anaerobic removal of several chlorinated hydrocarbons (CE, DCA, VC and MCB) under different electron accepting conditions in soil-columns inoculated with soil samples from polluted sites. No possible intermediates of reductive dechlorination processes were detected. A denitrifying CE-oxidizing bacterium was isolated from an enrichment culture and further characterized. These results underline the potential importance of anaerobic oxidation processes for the clean-up of sites polluted with chlorinated hydrocarbons as pointed out by others (30,37,38). Anaerobic oxidation pathways can be of particular importance when plumes contaminated with chlorinated compounds spread from reduced (e.g. methanogenic, sulfate-reducing) to more oxidized (e.g. iron-reducing and denitrifying) redox zones (Fig. 6.1). These redox situations frequently occur at polluted sites in Western Europe.

Figure 6.1. Idealized distribution of terminal electron accepting processes and associated dechlorination processes in a tetrachloroethene(PCE)-contaminated aquifer.



From these findings the question arises, what the consequences for bioremediation of low-chlorinated hydrocarbons may be? In the final part of this chapter, an attempt is made to translate the results obtained from laboratory studies to practice. Especially nitrate-reducing conditions seem to have potential, since we found removal of CE, DCA, VC, MCB and octane under these conditions. DCA degradation under nitrate-reducing conditions was also observed by others (9,13,17). Furthermore, chloromethane and dichloromethane can be used as sole carbon and energy source under denitrifying conditions (15,16,26). Mean nitrate levels in groundwater bodies in Western Europe from 1993-2001 are above the Drinking Water level guide (<25 mg/l) (European Environment Agency). In the Netherlands the percentage of measurements exceeding the EU-norm of 50 mg/l nitrate in 2003 was 20% for shallow groundwater and 6% of medium deep groundwater (RIVM/MC/Aug04/0608). These data implicate that nitrate is an abundant electron acceptor in groundwater bodies in Western Europe. Besides, denitrifying bacteria are widely distributed in soil and sediments (34).

When natural attenuation is inadequate because of nitrate limitation, introducing additional nitrate can enhance the process. Several studies show that the addition of nitrate to groundwater systems can enhance the *in situ* biodegradation of TEX compounds (2,11,12,22,23,31). However, the sites described by Barbaro, Hutchin and Schreiber are sandy aquifers in the US and Canada, where the aqueous nitrate concentration is below 0.05 mg/l. In Western Europe nitrate concentrations are generally much higher as mentioned above and therefore addition of nitrate for possible biostimulation is in most places unnecessary and even unwanted. Here the natural attenuation under denitrifying conditions brings the extra advantage of nitrate removal.

On one hand nitrate-reducing conditions may be promising for anaerobic *in situ* oxidation of chlorinated hydrocarbons as shown above. However, on the other hand nitrate can have an inhibitory effect on microbial reductive dechlorination as a result of electron donor competition or a facultative metabolic shift toward nitrate reduction (8,27,28,40). So it depends on the conditions (which chlorinated compound(s), which microbial dechlorination process) whether or not nitrate-reducing conditions are favorable for bioremediation.

When sites are polluted with chlorinated ethenes, nitrate-reducing conditions are unwanted because of the inhibitory effect of nitrate on reductive dechlorination. Other electron-accepting conditions are more favorable in this case. The potential of natural attenuation of chlorination ethenes under manganese(IV)-reducing, iron(III)-reducing, sulfate-reducing and methanogenic conditions has been demonstrated by field and laboratory studies. Laboratory studies showed also that chlorinated ethenes like tetrachloroethene and trichloroethene can be degraded to non-toxic and non-chlorinated products under the same terminal electron-accepting conditions (either by

reductive dechlorination alone or by a combination of reductive, oxidative and fermentative processes) (For an overview see: (3)). Complete reductive dechlorination of trichloroethene or tetrachloroethene to ethene in the field as a result of bioaugmentation or of a combination of biostimulation and bioaugmentation, respectively have also been demonstrated (14,29). Reductively-dechlorinating enrichment cultures, like the commercial available KB-1, were used for bioaugmentation. These cultures can completely dechlorinate chlorinated ethenes to ethene and contain phylogenetic relatives of *Dehalococcoides ethanogenes* (10,14,19,21). Also the recently isolated *Dehalococcoides* strain BAV1 can reductively dechlorinate dichloroethene and VC to ethene and couples this process to growth (20).

When sites are polluted with DCA, nitrate-reducing conditions may be promising for bioremediation. We demonstrated fast dechlorination of DCA in a nitrate-reducing soil column and in a batch culture. Also others observed the dechlorination of DCA under nitrate-reducing conditions (9,17). Recently, an enrichment culture was described that is able to mineralize DCA to carbon dioxide under nitrate-reducing conditions (13). From this culture two bacteria were isolated that were able to degrade DCA under nitrate-reducing conditions but at slower rates than the parent enrichment culture. It is not yet sure whether the dominant DCA-degrading organism from the enrichment culture was isolated. Nevertheless, these results are a big step forward, since biochemical and genetic knowledge of anaerobic DCA-degrading bacteria is now within reach. Because of unsuccessful attempts to enrich and/or isolate anaerobic oxidizing bacteria (e.g. this thesis) the knowledge on anaerobic oxidation processes and their potential for bioremediation remains limited. Physiological information about a bacterium is especially important for biostimulation and bioaugmentation. Genetic and biochemical knowledge is important for example for the development of gene probes. These probes can help us to confirm the presence or absence of DCA-degrading bacteria at polluted sites and to monitor these bacteria. Such information is essential to determine the potential of *in situ* anaerobic oxidation at polluted sites.

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Samenvatting

Gechloreerde koolwaterstoffen behoren tot de meest voorkomende verontreinigingen in de bodem en het grondwater. Van veel van deze verbindingen is bekend dat ze toxisch zijn en moeilijk afbreekbaar, wat hun potentiële gevaar voor flora en fauna vergroot. Het is daarom van groot belang om locaties die verontreinigd zijn met deze gechloreerde verbindingen schoon te maken.

Bioremediatie of biologische sanering is een techniek waarbij levende organismen (vooral micro-organismen) worden gebruikt om de vervuiling “op te ruimen”.

In het onderzoek beschreven in dit proefschrift hebben we gekeken naar de anaërobe microbiële oxidatie van laaggechloreerde koolwaterstoffen. Hierbij wordt de gechloreerde koolwaterstof door de bacterie als elektronendonor gebruikt in de afwezigheid van zuurstof, maar in de aanwezigheid van een andere geschikte elektronenacceptor. Deze laaggechloreerde koolwaterstoffen kunnen in het milieu terecht komen door bijvoorbeeld lekkage of onzorgvuldig gebruik en door gedeeltelijke dechlorering van hoger gechloreerde koolwaterstoffen. Wat er met deze verbindingen gebeurt in het milieu hangt vooral af van microbiële processen ter plekke. Aangezien veel vervuilde grondwatersystemen voor een groot deel anoxisch zijn, spelen anaërobe micro-organismen een grote rol in de afbraak van deze vervuilingen (1).

Laaggechloreerde koolwaterstoffen hebben de neiging om eerder geoxideerd te worden dan te worden gereduceerd (19,20). Of dit ook werkelijk gebeurt in het milieu hangt af van omgevingsfactoren zoals redox condities, beschikbaarheid van elektronendonoren en elektronenacceptoren en de aanwezigheid van de juiste micro-organismen.

Voor laaggechloreerde ethenen is het aangetoond dat ze anaëroob geoxideerd kunnen worden in de aanwezigheid van elektronenacceptoren zoals nitraat, ijzer(III) en mangaan(IV) en onder methanogene omstandigheden (3-6,20). Het vermogen van micro-organismen die aanwezig zijn op vervuilde locaties, om laaggechloreerde ethenen te oxideren in de afwezigheid van zuurstof heeft grote implicaties voor de natuurlijke afbraak van deze verbindingen. Echter de kennis van anaërobe oxidatie van laaggechloreerde koolwaterstoffen is beperkt.

Het doel van het onderzoek beschreven in dit proefschrift was om te onderzoeken of anaërobe oxidatie processen kunnen plaatsvinden op plekken die vervuild zijn met laaggechloreerde koolwaterstoffen en om uit te zoeken welke micro-organismen verantwoordelijk zijn voor deze processen.

Hoofdstuk 1 is een samenvatting van wat er op dit moment bekend is over de anaërobe microbiële oxidatie van laaggechloreerde koolwaterstoffen. Daarnaast wordt het verschil tussen aërobe en anaërobe oxidatie en tussen anaërobe oxidatie en anaërobe reductie uitgelegd. Er is een overzicht gegeven van de verschillende

laaggechloreerde koolwaterstoffen waarvan bekend is dat ze kunnen worden geoxideerd of gefermenteerd in de afwezigheid van zuurstof en welke micro-organismen hierbij betrokken zijn (voor zover bekend). Ten slotte wordt er aan het eind van het hoofdstuk de anaërobe oxidatieve dechloreringroutes en fermentatieve routes beschreven die tot nu toe bekend zijn.

Het potentieel van anaërobe oxidatie van verschillende (gechloreerde) verontreinigingen is weergegeven in **hoofdstuk 2**. Aan vier “up-flow” grondkolommen, gevuld met een mengsel van grond van verschillende locaties, voornamelijk verontreinigd met 1,2-dichloorethaan (DCA), chloorethenen, BTEX en/of benzine, werd een oplossing van verschillende gechloreerde koolwaterstoffen toegevoegd. De kolomproeven werden uitgevoerd in de afwezigheid van zuurstof onder nitraat-, ijzer(III)- of mangaan(IV)-reducerende omstandigheden of in de afwezigheid van een extern toegevoegde elektronenacceptor. Een serie batchcultuurproeven werd onder vergelijkbare condities ingezet.

We vonden dat DCA, vinyl chloride (VC), monochloorbenzeen (MCB) en octaan werden verwijderd in de afwezigheid van zuurstof. DCA werd verwijderd in alle vier grondkolommen en in batchculturen onder nitraat- en mangaan(IV)-reducerende omstandigheden. Half-waarde tijden voor DCA verwijdering/afbraak varieerden van enkele uren tot dagen. VC verwijdering trad op in de grondkolommen onder mangaan(IV)- en nitraatreducerende omstandigheden met half-waarde tijden van respectievelijk 0,2 en 0,3 dagen. MCB verwijdering werd waargenomen in de grondkolom met nitraat als elektronenacceptor. In batchculturen werd de afbraak van octaan onder nitraatreducerende omstandigheden waargenomen.

Er werd geprobeerd om de bacteriën verantwoordelijk voor de afbraak van DCA onder nitraatreducerende omstandigheden te isoleren (7). We waren echter niet in staat om de afbraakactiviteit te behouden na doorenting in nieuw medium. We hebben vervolgens verschillende mogelijke intermediairen van DCA afbraak getest als mogelijk groeisubstraat in plaats van DCA. Het bleek dat 2-chloorethanol (CE) een geschikt groeisubstraat was, waarmee we een goed groeiende ophopingscultuur in handen kregen. Uit deze ophopingscultuur hebben we uiteindelijk *Pseudomonas stutzeri* stam JJ geïsoleerd. Deze bacterie, beschreven in **hoofdstuk 3**, is het eerste beschreven isolaat dat anaëroob kan groeien met CE als enige koolstof- en energiebron en met nitraat als elektronenacceptor. Naast nitraat kan stam JJ ook zuurstof en nitriet als elektronenacceptor gebruiken, maar niet mangaan(IV) oxide, ijzer(II) hydroxide, sulfaat, chloraat of fumarate. Er werd géén verschil gevonden in het spectrum van groeisubstraten dat gebruikt kon worden door stam JJ onder oxische en denitrificerende omstandigheden. Echter de μ_{\max} tijdens groei op CE onder denitrificerende omstandigheden was significant lager dan onder oxische omstandigheden (respectievelijk $0,14 \text{ h}^{-1}$ en $0,31 \text{ h}^{-1}$).

De enige andere geteste gechloreerde verbinding die door stam JJ als groeisubstraat gebruikt kon worden was chlooracetaat.

De aquifer waar stam JJ uit verkregen is, was anoxisch, bevatte lage concentraties nitraat ($< 10 \mu\text{M}$) en was verontreinigd met gechloreerde alifaten, waaronder DCA (tot 3,5 mM), VC en verschillende gechloreerde methanen en propanen (7). Het vermogen van stam JJ om laag gechloreerde alifaten als groeisubstraat te gebruiken onder denitrificerende condities levert misschien een competitief voordeel op.

Het is niet bekend welke (gechloreerde) verbinding(en) als groeisubstraat dienden voor stam JJ *in situ*. CE en chlooracetaat zijn beide afbraakproducten van DCA. Echter, CE en chlooracetaat werden niet meegenomen in de standaard analyses van verontreinigingen op deze locatie. Een andere mogelijkheid is dat stam JJ kan groeien op DCA in een mengcultuur, aangezien de ophopingscultuur waar stam JJ uit is geïsoleerd in staat was om DCA af te breken onder nitraatreducerende omstandigheden (7). Tenslotte kan het zo zijn dat stam JJ het vermogen om DCA af te breken heeft verloren tijdens de isolatie en cultivatie op CE onder laboratorium omstandigheden. Stam JJ kan bijvoorbeeld het DCA dehalogenase zijn verloren of het dehalogenase wordt niet geïnduceerd door DCA, maar door een mogelijk andere gechloreerde verbinding in het milieu. Een PCR reactie met primers van verschillende dehalogenase genen en DNA van de originele DCA-afbrekende ophopingscultuur en van een reïncultuur van stam JJ kan ons misschien meer informatie geven of er een DCA dehalogenase aanwezig was/is in stam JJ.

Ook andere bacterie soorten, waaronder enkele nauw verwante *Pseudomonas stutzeri* stammen zijn getest op het vermogen om op CE en nitraat te groeien. Géén van deze stammen groeide op CE en nitraat. Dit is een aanwijzing dat het vermogen om op CE en nitraat te groeien een uitzonderlijke eigenschap van stam JJ is.

De afbraakroute van CE onder denitrificerende condities in stam JJ staat beschreven in **hoofdstuk 4**. De resultaten in dit hoofdstuk suggereren dat de afbraakroute van CE in stam JJ dezelfde is als die beschreven voor verschillende aërobe bacteriën die op CE kunnen groeien (10,15,16). Volgens deze afbraakroute wordt CE eerst geoxideerd tot chlooracetaldehyde, vervolgens wordt chlooracetaldehyde geoxideerd naar chlooracetaat, wat op zijn beurt wordt gedehalogeneerd naar glycolaat. Glycolaat komt tenslotte in centrale metabolische routes terecht.

De CE-dehydrogenase (CE-DH) activiteit werd verder onderzocht en bleek induceerbaar te zijn, aangezien er géén activiteit werd gevonden wanneer stam JJ op acetaat of ethanol (na zes seriële overentingen) werd gekweekt. Twee waarnemingen suggereren de aanwezigheid van twee verschillende phenazine methosulfaat(PMS)-afhankelijke alcohol dehydrogenases in stam JJ. Ten eerste, ruw celvrij extract van cellen gekweekt op CE vertoonden PMS-afhankelijke alcohol dehydrogenase activiteit met andere substraten dan celextract van cellen gekweekt op ethanol. Ten tweede, cel(vrij) extract van cellen gekweekt op ethanol vertoonden

géén activiteit met CE. Het is daarom waarschijnlijk dat de omzetting van CE wordt gekatalyseerd door een CE-DH en niet door een niet-specifiek alcohol dehydrogenase dat door zijn brede substraat specificiteit CE kan omzetten zoals het CE-DH van *Xanthobacter autotrophicus* GJ10 (10). Dit wordt ondersteund door de waarneming dat de hoogste PMS-afhankelijke alcohol dehydrogenase activiteit in ruw celvrij extract van cellen gekweekt op CE werd gevonden met CE als substraat. We waren echter niet in staat om op te helderen waarom stam JJ op CE en nitraat kan groeien, terwijl andere aërobe CE afbrekers die nitraat kunnen reduceren niet op CE en nitraat kunnen groeien. Uit literatuur is bekend dat onder anoxische omstandigheden vaak alleen het apo-enzym van het quinoproteïn dehydrogenase wordt gesynthetiseerd, maar niet de cofactor pyrroloquinoline quinone (PQQ), die nodig is voor een actief dehydrogenase (13,14). Eveneens is bekend dat extern toegevoegd PQQ de dehydrogenase activiteit kan herstellen wanneer het quinoproteïn apo-enzym wel wordt geproduceerd, maar PQQ niet (2,9). De toevoeging van PQQ aan het medium van *Xanthobacter autotrophicus* GJ10 resulteerde echter niet in groei op CE en nitraat. Ondanks deze waarneming kan het niet worden uitgesloten dat PQQ toch een rol speelt bij het onvermogen van aërobe bacteriën die nitraat kunnen reduceren, om op CE groeien met nitraat als elektronenacceptor. Onderzoek wijst erop dat zuurstof is vereist voor de laatste stap in de PQQ synthese (17,18). Echter stam JJ kan groeien op CE en *Paracoccus* en *Hyphomicrobium* soorten kunnen groeien op methanol onder denitrificerende omstandigheden in de afwezigheid van zuurstof. Kennis over hoe de laatste stap van de PQQ synthese wordt gekatalyseerd in deze bacteriën in de afwezigheid van zuurstof stelt ons misschien ook in staat om een verklaring te vinden over waarom tot nu toe alleen gevonden is dat stam JJ kan groeien op CE onder denitrificerende omstandigheden.

Onze bevinding dat de afbraakroute van CE onder denitrificerende omstandigheden in *Pseudomonas stutzeri* stam JJ gelijk is aan de afbraakroute onder oxische omstandigheden is ook waargenomen voor de afbraak van dichloormethaan (DCM). DCM dehalogenase, het sleutelenzym van aërobe DCM afbraak, werd geïnduceerd in *Hyphomicrobium* sp. stam DM2 in zowel de aanwezigheid als in de afwezigheid van zuurstof (in de aanwezigheid van nitraat) (12).

Het laatste hoofdstuk, **hoofdstuk 5**, beschrijft de verwijdering van monochloorbenzeen (MCB) in een nitraatreducerende “up-flow” grondkolom. Deze kolom was de nitraatreducerende kolom die gebruikt werd voor de experimenten beschreven in hoofdstuk 2. De oplossing met verschillende gechlorideerde verbindingen die aan de kolom werd toegevoegd, werd vervangen door een oplossing met alleen MCB. Om de door ons waargenomen MCB verwijdering verder te onderzoeken, werden er zowel grondkolomproeven als batchcultuurproeven uitgevoerd. In de grondkolom, die voor meer dan vier jaar draaide, trad gedurende

deze periode gedeeltelijke verwijdering van MCB op. Van de ingaande 120 μM MCB was gemiddeld 73 μM verdwenen aan het eind van de kolom. De hoeveelheid gereduceerd nitraat varieerde te sterk om een nauwkeurige massabalans op te stellen voor de mogelijke oxidatie van MCB naar CO_2 . Hogere nitraatconsumptie dan verwacht op basis van complete oxidatie van MCB met nitraat als elektronenacceptor kan mogelijk verklaard worden door anaërobe oxidatie van ammonium (medium component) met nitraat/nitriet als elektronenacceptor. De geproduceerde chloride was hoger dan verwacht op basis van de hoeveelheid MCB dat was verdwenen. Er werd geen productie van benzeen waargenomen.

MCB afbraak (twee keer 50 μM) werd ook waargenomen in één denitrificerende batchcultuur, maar deze afbraak stopte na de derde toevoeging van 50 μM MCB. Pogingen om de MCB afbraak activiteit over te brengen in anoxische batchculturen met nitraat als elektronenacceptor, met en zonder toegevoegd grondkolommateriaal, waren niet succesvol. We vonden alleen herhaaldelijk MCB afbraak in batchculturen onder oxische omstandigheden.

De verwijdering van MCB in de grondkolom samen met de waargenomen nitraatreductie en chlorideproductie suggereren afbraak van MCB. Het feit dat MCB afbraak met nitraat als elektronenacceptor volledig stopte na een oxische periode van ongeveer acht weken is een extra aanwijzing dat de MCB verwijdering werd veroorzaakt door biotransformatie. Bovendien kon de gevonden MCB verwijdering niet worden verklaard door adsorptie van de MCB aan de grondmatrix. Deze waarnemingen kunnen alleen verklaard worden door aan te nemen dat MCB werd geoxideerd in de afwezigheid van zuurstof met nitraat als elektronenacceptor. Andere onderzoekers hebben eveneens MCB afbraak in anoxische grondkolommen waargenomen, maar ook zij konden de volledige oxidatie van MCB naar koolstofdioxide niet bewijzen en/of namen géén anaërobe MCB afbraak waar in batchculturen (8,11).

Samengevat laat dit onderzoek de anaërobe (oxidatieve) verwijdering zien van verschillende gechlloreerde koolwaterstoffen (DCA, VC en MCB) en van CE onder verschillende elektronenaccepterende omstandigheden in grondkolommen met grond van vervuilde locaties. Mogelijke intermediären van reductieve dechloreringsprocessen werden niet waargenomen wat wijst op oxidatieve processen.

Uit een DCA-afbrekende ophopingscultuur werd een denitrificerende CE-oxiderende bacterie geïsoleerd, waarvan de afbraakroute werd gekarakteriseerd. Deze resultaten onderstrepen het potentiële belang van anaërobe oxidatie processen voor het schoonmaken van locaties verontreinigd met gechlloreerde verbindingen.

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Curriculum Vitae

Johan Anton (John) Dijk werd geboren op 9 maart 1975 te Wijhe. In 1993 behaalde hij zijn VWO-diploma aan het Florens Radewijns College te Raalte en besloot hij vervolgens biologie te studeren aan de Rijksuniversiteit Groningen. Al gauw werd zijn interesse voor de microbiologie gewekt wat resulteerde in een eerste afstudeeronderzoek bij de vakgroep Microbiële Ecologie. Hier deed hij onderzoek naar de interacties tussen de dechlorerende *Desulfitobacterium* sp. strain TCE1 en de sulfaatreducerende stam SULF1. Het tweede afstudeeronderzoek werd uitgevoerd bij de vakgroep Biochemie, waar hij de specificiteit en kinetiek van het haloalkaan dehalogenase DhaA onderzocht voor gechloreerde propanen. In 1998 studeerde hij af als bioloog met als specialisatie milieubiologie en microbiologie. In datzelfde jaar begon hij aan zijn promotieonderzoek bij de sectie Industriële Microbiologie aan de Wageningen Universiteit. Wegens opheffing van deze vakgroep in 2000 werd het onderzoek voortgezet bij het Laboratorium voor Microbiologie. De resultaten van het promotieonderzoek staan beschreven in dit proefschrift.

In januari 2005 is hij begonnen als Postdoc bij het Laboratorium voor Bodem- en Waterbeheer aan de Katholieke Universiteit Leuven. Hier onderzoekt hij de samenstelling van de microbiële gemeenschap in riviersediment verontreinigd met gechloreerde alifatische koolwaterstoffen.