Application of *Pseudomonas* sp. strain DCA1 for the removal of chlorinated hydrocarbons

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Het was een onbekende weg
Die ik heb afgelegd
Na het licht
Op zoek naar het donker

(Frank Boeijen, De Verzoening)

1

General Introduction

INTRODUCTION

Chlorinated aliphatic hydrocarbons (CAHs) are widely used as solvents, cleaners, degreasing agents and in the production of chemicals. As a result of spills and improper disposal practices, CAHs have been released into the environment, resulting in contamination of the subsurface, including soil and groundwater. Although chronic industrial pollution has been reduced over recent years, major accidents still occur (Timmis and Pieper, 1999). Moreover, new polluted sites are continually discovered. Contamination of soil and groundwater with CAHs is of great concern, because of the toxicity, persistence and bioaccumulation of these compounds. Furthermore, groundwater is of great importance for our drinking water supply. In Europe and in the USA more than 75%, respectively 51% of the drinking water is prepared from groundwater (Morris et al., 2003). In an extensive survey, Westrick et al. (1984) showed a widespread occurrence of 19 different CAHs in drinking water supplies from groundwater sources in the USA.

The transport characteristics of CAHs after their release into the subsurface largely depend on the solubility, the density and the relative tendency to remain dissolved in water rather than to be adsorbed onto soil organic carbon. The latter is expressed in the soil-water coefficient (K_{oc}). In Table 1.1 these parameters for some common CAHs are listed.

Table 1.1 Characteristics of common CAHs[a]

Chlorinated hydrocarbon	Density (kg m^{-3})	Solubility in water (kg m^{-3})	K_{oc} (l kg^{-1})	Mobility class
1,2-Dichloroethane	1.25×10^3	8.69	19-36	very high
cis-1,2-Dichloroethene	1.27×10^3	3.50	36-49	high - very high
1,2-Dichloropropane	1.16×10^3	2.70	51	high
Trichloroethene	1.46×10^3	0.11	152	moderate
1,1,1-Trichloroethane	1.34×10^3	0.70	155	moderate
Tetrachloroethene	1.62×10^3	0.15	303	moderate

[a] From: World Health Organization (1984, 1985, 1992, 1993, 1995); Fetter (1994); US Environmental Protection Agency (http://www.epa.gov/safewater/dwh/t-voc/12-dich2.html).

The density of CAHs is generally higher than that of water; therefore pure CAH will sink to the bottom of the aquifer after a spill has occurred. CAHs such as tetrachloroethene and trichloroethene are only slightly soluble in water and may remain at the bottom of the aquifer as a dense nonaqueous-phase liquid (DNAPL). Some of the CAH will go into solution so that there will also be a plume of groundwater with dissolved CAH. Contaminants such as 1,2-dichloroethane and *cis*-

1,2-dichloroethene have a relatively high solubility and a low K_{oc} value, resulting in a (very) high mobility (Fetter, 1994).

GROUNDWATER REMEDIATION

A well-known way to remediate contaminated groundwater is by pump-and-treat. Pump-and-treat systems for groundwater remediation came into wide use in the early to mid-1980s (US Environmental Protection Agency, 1996). These systems generally consist of a series of extraction wells to pump the contaminated groundwater to the surface. Besides removal of the contaminant, pump-and-treat systems are also used for hydraulic containment of contaminant plumes, i.e. to prevent the continued expansion of the contaminated zone.

Once extracted from the subsurface, the groundwater has to be treated before it can be reinjected or disposed on the surface waters. This treatment can be physical (e.g. air stripping, steam stripping or adsorption onto activated carbon), chemical (e.g. chemical oxidation) or biological. From an environmental perspective, biological treatment of contaminated groundwater is attractive, since the contaminant is not just transferred to another compartment (e.g. adsorbed onto activated carbon), but is really decomposed. Moreover, biological treatment can be economically favorable when compared to expensive methods such as adsorption onto activated carbon. Biological on-site treatment of contaminated (ground)water is discussed below.

Although pump-and-treat is still one of the most widely applied groundwater remediation technologies, the effectiveness is sometimes questioned (Travis and Doty, 1990; US Environmental Protection Agency, 1996). For some applications pump-and-treat is expensive and ineffective since the operations are on the scale of decades, multiple plume volumes need to be extracted and concentrations are not reduced to the target levels (Travis and Doty, 1990; Duba et al., 1996). In these cases, in situ bioremediation may be a good alternative. This technology is based on microorganisms biodegrading the contaminants in the subsurface. “In situ” (Latin for “in its place”) indicates that the groundwater (and/or the soil) are treated while staying in their place; e.g. no groundwater extraction and above-ground treatment take place. Biodegradation of contaminants requires the following: an energy and carbon source (in some cases the contaminant), an electron acceptor (e.g. oxygen or nitrate), nutrients (nitrogen, phosphorous) and a microbial community capable of degrading the contaminant. In many situations one of these requirements is missing or limiting. Injecting one of the requirements mentioned above can then stimulate

biodegradation. Injection of microorganisms, a process called 'bioaugmentation', is discussed in Chapter 5.

BIOLOGICAL ON-SITE TREATMENT

In subsurface environments, where the groundwater is often depleted of oxygen, anaerobic biodegradation plays an important role in the removal of CAHs (Ferguson and Pietari, 2000). However, most systems for biological on-site treatment of contaminated groundwater are based on aerobic biodegradation (Langwaldt and Puhakka, 2000). Two types of aerobic oxidations are discussed below:
- Energy-yielding oxidations;
- Co-metabolic oxidations.

Energy-yielding oxidations of CAHs

A selection of CAHs can be converted aerobically by bacteria, and serve as the sole carbon and energy source. These CAHs include chloromethanes, chloroethanes, chloropropanes and chloroethenes. Some examples are given in Table 1.2.

Table 1.2 Examples of aerobic utilization of chlorinated alkanes and alkenes as the sole carbon and energy source by pure bacterial cultures

Compound	Bacterial strain	Reference
Chloromethane	*Hyphomicrobium* MC1	Hartmans et al., 1986
Dichloromethane	*Pseudomonas* DM1	Brunner et al., 1980
1,2-Dichloroethane	*Pseudomonas* DE2	Stucki et al., 1983
Chloroethene (Vinyl chloride)	*Mycobacterium* L1	Hartmans and de Bont, 1992
1-Chloropropane	*Xanthobacter autotrophicus* GJ10	Janssen et al., 1985
1,3-Dichloropropane	*Xanthobacter autotrophicus* GJ10	Janssen et al., 1985
1,3-Dichloropropene	*Pseudomonas cichorii* 170	Poelarends et al., 1998

Highly chlorinated alkanes and alkenes are generally not degradable under aerobic conditions. For these compounds anaerobic biodegradation is favored. As the degree of chlorination decreases, aerobic degradation becomes more favorable (Vogel et al., 1987). To date no bacterial strains that utilize 1,2-dichloropropane under aerobic conditions have been isolated.

Co-metabolic oxidations

In co-metabolic oxidations, a primary substrate is oxidized by a mono- or dioxygenase and, due to the aspecific nature of these enzymes, chlorinated alkanes

and alkenes are oxidized simultaneously. Co-metabolic oxidations of CAHs are fortuitous reactions that have no beneficial effects for the cell (Horvath, 1972; Perry, 1979). Actually, these reactions require reducing equivalents, thereby reducing the amount of energy available for growth. This can result in a disadvantage in relation to competition with other bacteria in mixed cultures (Mars et al., 1998).

Two strains that have been extensively studied for their co-metabolic oxidation of chlorinated ethenes and ethanes are *Burkholderia cepacia* G4 and *Methylosinus trichosporium* OB3b (Semprini, 1997). Strain G4 uses phenol and toluene as a primary substrate, whereas strain OB3b is a methane-utilizing bacterium. Strain OB3b expresses two types of a methane monooxygenase: a particulate form (pMMO) and a soluble monooxygenase (sMMO). The latter is capable of oxidizing trichloroethene at very high rates (Fox et al., 1990; Oldenhuis et al., 1989; Sullivan et al., 1998).

Tetrachloroethene, which is fully halogenated, has long been considered non-degradable under aerobic conditions. In 2000, however, Ryoo et al. reported aerobic co-metabolic oxidation of tetrachloroethene by the strain *Pseudomonas stutzeri* OX1, which expresses a toluene-*o*-xylene monooxygenase.

Many co-metabolic oxidations of CAHs result in the production of toxic intermediates. For example, trichloroethene and 1,1-dichloroethene are oxidized to their corresponding epoxides. Rearrangement or hydrolysis of these epoxides results in acyl chlorides, which have a strong negative effect on cell viability (van Hylckama Vlieg et al., 1996, 1997; Dolan and McCarty, 1995). Co-metabolic oxidation of 1,2-dichloropropane by resting cells of strain OB3b was reported to yield the toxic intermediate 2,3-dichloro-1-propanol (Bosma and Janssen, 1998).

Bioreactors

Aerobic treatment of contaminated groundwater that has been pumped to the surface is faced with two problems. First, groundwater is often anaerobic and has to be oxygenated. Direct oxygenation, however, results in stripping of (volatile) compounds. For example, Freitas dos Santos and Livingston (1993) showed that more than 30% of the 1,2-dichloroethane supplied to a conventional air-lift reactor was lost to the exit gas stream. Besides, groundwater often contains iron and direct oxygenation then results in coagulation of iron oxides, having detrimental consequences for the operating systems such as clogging. Second, influent contaminant concentrations in groundwater are generally one or two orders of magnitude lower than in wastewater (Langwaldt and Puhakka, 2000). Therefore, biomass build-up is relatively slow and bioreactor systems are therefore limited to

attached-growth systems, i.e. biofilm reactors. Using a membrane bioreactor can solve these problems. A membrane offers a surface onto which a biofilm can attach. Furthermore, the combination of a membrane with a biofilm allows oxygenation without the effect of stripping the contaminant and coagulation of iron oxides, since oxygen and the substrate (i.e. the contaminant) are supplied from two different sides. Below, membrane bioreactors are discussed in more detail.

MEMBRANE BIOREACTORS

Two types of membrane bioreactors (MBRs) based on attached growth can be discerned for the treatment of water (Casey et al., 1999):

1) membrane-aerated biofilm reactor (MABR);
2) extractive membrane bioreactor (EMB).

Both reactor types are discussed below. Since both the MABR and the EMB are biofilm systems, some important aspects related to biofilms are reviewed as well.

Membrane-aerated biofilm reactor (MABR)

In order to avoid stripping of the contaminant, bubble-free oxygenation is required. This can be obtained in an MABR (Brindle and Stephenson, 1996). The principle of an MABR is schematically drawn in Figure 1.1.

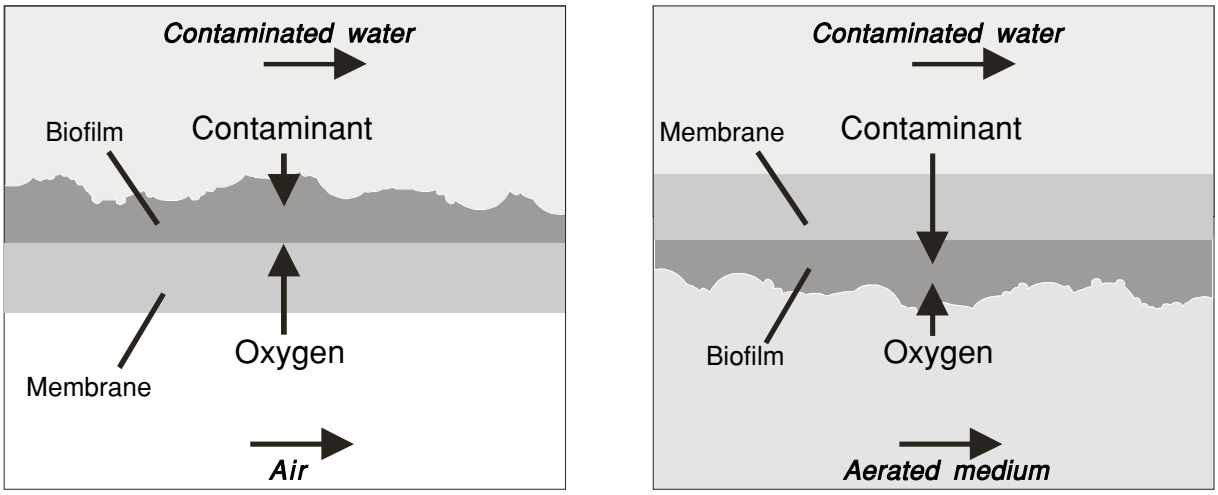

Figure 1.1 Principle of treatment of contaminated water in an MABR (A) and an EMB (B)

At one side of the membrane a gas phase is present for the supply of air or oxygen. The biofilm is located at the other side of the membrane, where the contaminant is present in the liquid phase. Contaminant diffuses into the biofilm, where

biodegradation takes place. Both hydrophobic microporous membranes and dense gas-permeable membranes (such as silicone-rubber) can be used to obtain bubble-free oxygenation (Côte et al., 1988). In case of a hydrophobic microporous membrane, the pores are filled with gas. Mass transfer is facilitated by the diffusion through these gas-filled pores and the mass transfer coefficient is directly proportional to the porosity of the membrane. However, if liquid penetrates into the pores, e.g. due to a high pressure at the liquid side, the mass transfer coefficient will decrease severely (Casey et al., 1999). Debus and Wanner (1992) used a silicone-rubber membrane for the removal of xylene. Besides the high permeability of silicone-rubber for oxygen, CO_2 and volatile organic compounds, these membranes are also very resistant to mechanical and chemical stress.

Extractive membrane bioreactor (EMB)

In an EMB both sides of the membrane consist of a liquid phase. Unlike in the MABR, the biofilm in an EMB is situated at the membrane side opposite to the contaminant. The contaminant is selectively transported through the membrane into the biofilm, where degradation takes place (Casey et al., 1999). At the biofilm side of the membrane, a liquid phase containing (bio)medium is circulated. This biomedium is aerated and conditions for biodegradation can be optimized. The principle of an EMB is shown in Figure 1.1. Selective transport of contaminants through the membrane is especially useful when the water that has to be treated is not amenable to direct biological treatment. This can be due to the inorganic composition, such as a high salt concentration, a low pH and/or the presence of heavy metals (Livingston, 1993; Liu et al., 2001; Chuichulcherm et al., 2001). Based on the properties mentioned above (high permeability for oxygen and volatile organic carbons), silicone-rubber membranes are generally used in EMBs. EMBs have been used in lab-scale studies for the removal of 1,2-dichloroethane, monochlorobenzene and mixtures of *cis*- and *trans*-1,3-dichloropropene from wastewaters (Freitas dos Santos and Livingston, 1994, 1995[a]; Freitas dos Santos et al., 1997, Katsivela et al., 1999).

Biofilms

Until the 90s, the main focus of microbiologists has been on planktonic bacteria. However, in most ecosystems the majority of the bacteria are present in a biofilm (Costerton et al., 1995). A biofilm can be described as a collection of microorganisms and their associated extracellular products (matrix) at an interface and generally attached to a biotic or abiotic surface (Palmer Jr and White, 1997; Davey and O'Toole, 2000). This support is often referred to as substratum, and the matrix is also called glycocalyx. A biofilm can exist of up to 97% of water (Sutherland, 2001). The main components of a biofilm are summarized in Table 1.3. Generally, biofilms in

nature do not only include bacteria, but also contain eukaryotic organisms. In fact, pure prokaryotic biofilms are rare in nature (Palmer Jr and White, 1997).

Table 1.3 Main components of a biofilm[a]

Component	% of matrix
Water	up to 97%
Microbial cells	2-5%
Polysaccharides	1-2%
Proteins	<1-2%
DNA and RNA	<1-2%

[a]From: Sutherland (2001)

Biofilms have long been considered as homogeneous structures. This has changed, however, since the application of new, non-invasive techniques for the study of biofilms, such as confocal scanning laser microscopy (Caldwell et al., 1992). Biofilms are now often considered as mushroom- or tower-shaped structures, exhibiting channels and large (fluid-filled) internal void spaces (Costerton et al., 1994; de Beer et al., 1994; Massol-Deyá, 1995; Palmer Jr and White, 1997; Sutherland, 2001; Donlan and Costerton, 2002). It was shown that liquid could flow through these voids, but was stagnant in cell clusters (Stoodley et al., 1994; de Beer and Stoodley, 1995). Consequently, mass transfer in cell clusters is determined by diffusion only, whereas in the voids both convective and diffusive mass transfer takes place.

In the process of biofilm formation, different stages have been proposed. First, cells weakly adhere to a surface (Characklis, 1981; van Loosdrecht, 1988). This is considered to be a random process, based on surface free energy and propinquity of the cells (Jenkinson and Lappin-Scott, 2001). Once cells have attached, the surface is colonized by cell division and attachment of additional cells (Palmer Jr and White, 1997). Finally, the microcolonies differentiate into an exopolysaccharide-encased, mature biofilm (Costerton et al., 1999; Costerton, 1999). The least-studied biofilm process is detachment. Cells are released from the biofilm as single cells or small portions of the biofilm (erosion) or by "rapid, massive loss" (sloughing) (Characklis, 1990; Stoodley et al., 2001). Detachment can be due to cell growth and division within biofilms, an increase in shear stress (e.g. change of direction or rate of flow) or changes in substrate concentration (Donlan and Costerton, 2002).

Biofilm thickness is an important parameter for MBR performance. On the one hand, the presence of a biofilm is desired as a diffusion barrier to avoid stripping of volatile contaminants (Freitas dos Santos, 1995[c]; Debus and Wanner, 1992). Furthermore,

the presence of a biofilm increases the flux across the membrane, since contaminant degradation results in a steeper local concentration profile at the membrane-biofilm interface (in case of an EMB) (Freitas dos Santos, 1995[c]). On the other hand, however, an increased biofilm thickness can reduce the flux across the membrane. In EMBs and MABRs, contaminant and oxygen diffuse into the biofilm from opposite directions (counterdiffusion) and, due to oxygen diffusion limitation, biodegradation may only take place in a small part of the entire biofilm volume and inactive zones may exist (Nicolella et al., 2000). An increased biofilm thickness will then only result in an increased mass transfer resistance. Other disadvantages of a thicker biofilm are increased hydraulic resistances, clogging problems, and (in large-scale applications) the required treatment and disposal of excess biofilm (Strachan et al., 1996; Freitas dos Santos, 1995[c]). Based on these considerations, an optimal biofilm thickness exists. Pavasant et al. (1996) developed a model to predict this optimal biofilm thickness for an EMB treating 1,2-dichloroethane-contaminated wastewater.

Biofilm dry weight contents normally range between 10 and 130 kg m^{-3} (Characklis and Marshall, 1990). Freitas dos Santos and Livingston (1995[b]) reported an average dry weight content of 60 kg m^{-3} for a biofilm of strain *Xanthobacter autotrophicus* GJ10.

1,2-DICHLOROETHANE

The main contaminant studied in this work is 1,2-dichloroethane (DCA). The chemical properties and use, environmental fate and possible ways of biodegradation are discussed below. DCA is biodegraded under both aerobic and anaerobic conditions. Anaerobic biodegradation is discussed briefly, whereas aerobic biodegradation is discussed in more detail.

Chemical properties, use and environmental fate of DCA

DCA is a synthetic chemical that has no known natural sources. It is mainly used as a feedstock for the production of vinyl chloride, which is used to make a variety of plastic and vinyl products including polyvinyl chloride (PVC). The annual production of DCA was estimated to be over 8 million tonnes in 1993, making it the chlorinated solvent produced in the largest quantity (Cox et al., 2000). As was stated above, DCA has a very high mobility due to the high solubility and the low sorption coefficient. Some characteristics of DCA are listed in Table 1.4.

Table 1.4 Characteristics of 1,2-dichloroethane[a]

Aspect	
Trade names and synonyms	EDC; 1,2-DCE, Ethylene dichloride; Brocide; Borer-Sol; Destruxol; Dichlor-mulsion; Dutch Liquid; Dutch Oil; ENT 1656; Freon 150; Gaze
CAS registry number	Olefiant
Chemical structure	107-06-2
Molecular weight (g mole^{-1})	$C_2H_4Cl_2$
	98.96
Vapor pressure at 20°C (kPa)	8.5
Sorption coefficient (log K_{oc})	1.28
Density at 20°C (kg m^{-3})	1,253
Solubility in water at 20°C (kg m^{-3})	8.69
Water-air distribution ratio at 25 °C	20

[a]From: World Health Organization (1995); Amoore and Hautala (1983).

Aerobic biodegradation

Aerobic biodegradation of DCA was first demonstrated by Stucki et al. (1983). Their isolate, *Pseudomonas* sp. strain DE2, is able to grow on DCA as the sole carbon and energy source. No DCA degradation was observed in cell extracts of strain DE2, however, the extracts did contain an NAD-dependent 2-chloroacetaldehyde dehydrogenase and a 2-chloroacetate halidohydrolase activity. Hydroxylation of DCA yielding the unstable intermediate 1,2-dichloroethanol was suggested as the first step in DCA metabolism followed by spontaneous formation of 2-chloroacetaldehyde. The latter compound was thought to be oxidized to 2-chloroacetic acid and subsequently dehalogenated to glycolate.

A strain studied in more detail is *Xanthobacter autotrophicus* GJ10 (Janssen et al., 1985). The degradation pathway is shown in Figure 1.2. The first step in DCA metabolism in this strain consists of a hydrolytic dehalogenation to 2-chloroethanol, which is subsequently converted to chloroacetaldehyde by an alcohol dehydrogenase. Chloroacetaldehyde is further dehydrogenated to form chloroacetic acid. Subsequently, chloroacetic acid is dehalogenated by chloroacetate dehalogenase to glycolic acid, which can enter the central metabolic pathways.

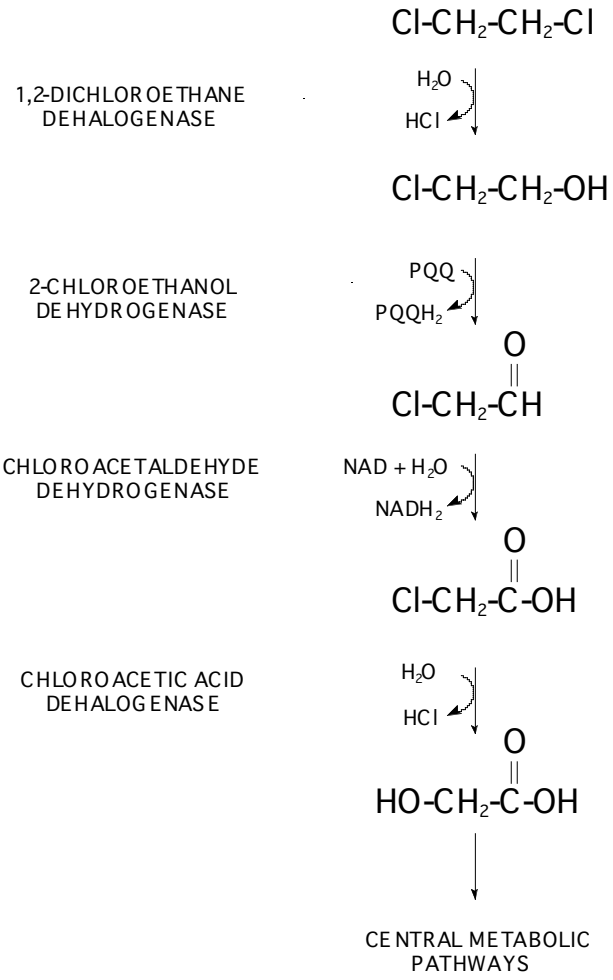

Figure 1.2 DCA degradation pathway for *X. autotrophicus* GJ10

The genes encoding for the enzymes responsible for degradation of DCA have been isolated and the responsible enzymes have been characterized (Keuning et al., 1985; van der Ploeg et al., 1991; van der Ploeg et al., 1994 Janssen et al., 1987). Both the alcohol dehydrogenase and the chloroacetic acid dehalogenase are chromosomally encoded and appear to be common in nature. The haloalkane dehalogenase and the aldehyde dehydrogenase are plasmid encoded and are believed to be specifically adapted for degradation of xenobiotic compounds (Tardif et al., 1991; Janssen et al., 1994).

Since the affinity of the haloalkane dehalogenase of strain GJ10 for DCA is rather low (K_m value of 571 µM), van den Wijngaard et al. (1992) tried to isolate microorganisms with a higher affinity for DCA. This resulted in the isolation of the bacterial strain *Ancylobacter aquaticus* AD25. It was shown that this strain expresses the same dehalogenase as strain GJ10, however, at much higher levels. This results in an

apparent K_s value of 24 µM for this strain, compared to 260 µM for strain GJ10 (van den Wijngaard et al., 1993[a]).

All three strains mentioned above required vitamins for optimal growth (Stucki, 1983; van den Wijngaard et al., 1993[b]).

Recently, Olaniran et al. (2004) isolated 5 different strains from contaminated sites in Nigeria and South Africa. All these strains could utilize DCA as the sole carbon and energy source with growth rates varying between 0.04 en 0.07 h^{-1}. The isolated strains were tentatively identified as *Corynebacterium*, *Bacillus*, *Burkholderia*, *Micrococcus* and *Pseudomonas* species.

Anaerobic biodegradation

Under anoxic conditions, DCA can undergo different dechlorination reactions. Dihaloelimination of DCA results in the formation of ethene (Holliger et al., 1990; Egli et al., 1987; Belay and Daniels, 1987; de Wildeman et al., 2003[b]). Hydrogenolysis of DCA results in the formation of chloroethane, which is converted to ethane in a consecutive hydrogenolysis step (Holliger et al., 1990). These dechlorination reactions are based on co-metabolism and are therefore slow and incomplete. Only two strains have been reported to dehalorespire DCA, i.e. convert DCA into ethene in an energy-conserving reaction. The first isolate *Dehalococcoides ethenogenes* 195, however, produces up to 1% of the carcinogenic vinyl chloride (Maymó-Gatell et al., 1999). Moreover, strain 195 is dependent on undefined bacterial extracts. Recently, de Wildeman et al. (2003[a]) isolated another dehalorespiring strain designated *Desulfitobacterium dichloroeliminans* strain DCA1. No formation of toxic intermediates such as vinyl chloride was reported (de Wildeman et al., 2004). Anaerobic oxidation of DCA to CO_2 under nitrate-reducing conditions was described by Gerritse et al. (1999).

Application of DCA-degrading strains in bioremediation

The DCA-degrading strains GJ10 and DE2 have been applied in bioremediation. Stucki and Thüer (1995) described the treatment of groundwater that contained DCA as the single contaminant in the range of 20 – 150 µM and that had to be treated to DCA levels below 0.1 µM. The treatment plant consisted of two sand filters, followed by two activated carbon filters. The treatment plant was inoculated with *X. autotrophicus* GJ10 and *Pseudomonas* sp. DE2. An earlier lab-scale study showed that inoculation of granular activated carbon with these strains resulted in a lower carbon consumption as a result of biological activity (Stucki and Thüer, 1994). In the field-scale application a total of 5.000 kg of activated carbon was consumed during the first month of operation. However, after 2 years of operation and with the

implementation of a Rotating Biological Contactor (RBC) the exchange of carbon became redundant. During the fifth year, most of the DCA was removed by the RBC, resulting in effluent concentrations of DCA that were usually much lower than 0.1 µM. The initially black surface of the RBC had by then changed to a yellow color, suggesting the presence of *X. autotrophicus* GJ10 as dominating DCA degrader. Reisolation of DCA-degrading microorganisms confirmed the presence of *Xanthobacter* sp. with an identical gene encoding for the DCA dehalogenase.

Strain GJ10 has also been used in the lab-scale studies aimed at the biological removal of DCA from synthetic wastewaters (Freitas dos Santos and Livingston, 1994, 1995[a]). In these studies removal efficiencies of up to 99% were achieved in an EMB. However, the synthetic wastewater contained DCA in concentrations of 16 mM and 20 mM, which are levels not often encountered in groundwater remediation.

OUTLINE OF THIS THESIS

The general objective of the research described in this thesis was to gain more insight in several aspects of bioremediation of DCA and other CAHs. The research is directed towards both microbiological and engineering aspects of bioremediation. More specifically, two major objectives were postulated:

1) isolation of a DCA-degrading strain with a high affinity for DCA and without requirements for additional organic nutrients for optimal growth;
2) application of the new DCA-degrading strain in an MABR, which allows aerobic biodegradation of DCA without the need for direct oxygenation of the contaminated (ground)water

In Chapter 2 the search for DCA-degrading microorganisms with a high affinity for DCA is described. A new isolate, designated *Pseudomonas* sp. strain DCA1, is characterized and the DCA degradation pathway elucidated. It is shown that the degradation of DCA is mediated by a monooxygenase.

In Chapter 3 the application of strain DCA1 in an MABR for the removal of DCA from (ground)water is described. The use of an MABR allows aerobic biodegradation without undesired oxygenation of the water phase (causing coagulation of iron oxides), and avoids stripping of the contaminant to the gas phase. A model is used to elucidate the key parameters that define the removal rates at low contaminant concentrations.

Since the degradation of DCA by strain DCA1 is mediated by a monooxygenase, and these enzymes are generally aspecific, the potential of strain DCA1 to (co-metabolically) oxidize other chlorinated compounds is assessed (Chapter 4). The research is focussed on the degradation of 1,2-dichloropropane, since this compound shows structural analogy to DCA but is recalcitrant to aerobic biodegradation.

In Chapter 5 the focus is shifted from *on-site* bioremediation to *in situ* bioremediation. In this chapter an overview is given of the use of non-indigenous microorganisms for in situ bioremediation; a process called bioaugmentation. The pros and cons of this technology are discussed, based on the results of recent field and lab trials.

2

Monooxygenase-mediated 1,2-dichloroethane degradation by *Pseudomonas* sp. strain DCA1

J.C. Hage
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ABSTRACT

A bacterial strain, designated *Pseudomonas* sp. strain DCA1, was isolated from a 1,2-dichloroethane (DCA)-degrading biofilm. Strain DCA1 utilizes DCA as the sole carbon and energy source and does not require additional organic nutrients, such as vitamins, for optimal growth. The affinity of strain DCA1 for DCA is very high, with a K_m value below the detection limit of 0.5 µM. Instead of a hydrolytic dehalogenation, as in other DCA utilizers, the first step in DCA degradation in strain DCA1 is an oxidation reaction. Oxygen and NAD(P)H are required for this initial step. Propene was converted to 1,2-epoxypropane by DCA-grown cells, and competitively inhibited DCA degradation. We concluded that a monooxygenase is responsible for the first step in DCA degradation in strain DCA1. Oxidation of DCA probably results in the formation of the unstable intermediate 1,2-dichloroethanol, which spontaneously releases chloride, yielding chloroacetaldehyde. The DCA degradation pathway in strain DCA1 proceeds from chloroacetaldehyde via chloroacetic acid and presumably glycolic acid, which is similar to degradation routes observed in other DCA-utilizing bacteria.

INTRODUCTION

1,2-Dichloroethane (DCA) is a synthetic chemical which has no known natural sources. DCA is mainly used as an intermediate in the synthesis of vinyl chloride, but it is also used in the production of chlorinated solvents such as trichloroethene, tetrachloroethene, and 1,1,1-trichloroethane. Based on the low sorption coefficient (log K_{oc} = 1.28) and the good solubility in water, leaching of DCA to groundwater is expected when DCA is released to the environment (World Health Organization, 1995). Abiotic degradation of DCA does occur, however, very slowly. Under conditions similar to those in groundwater (15°C, pH 7, and in the presence of 1 mM total sulfide) the half-life of DCA is 23 years (Barbash and Reinhard, 1989).

Aerobic biodegradation of DCA was first demonstrated by Stucki et al. (1983). The isolated *Pseudomonas* sp. strain DE2, is able to grow on DCA as the sole source of carbon and energy with a growth rate of 0.08 h^{-1} at 30°C. A strain studied in more detail is *Xanthobacter autotrophicus* GJ10 (Janssen et al., 1984, 1985). This strain was isolated from a mixture of activated sludge and contaminated soil. The first step in DCA metabolism in *X. autotrophicus* GJ10 is a hydrolytic dehalogenation to 2-chloroethanol, which is subsequently converted to chloroacetaldehyde by an alcohol dehydrogenase (Janssen et al., 1985). Chloroacetaldehyde is further dehydrogenated to form chloroacetic acid. The latter compound is presumably dehalogenated by chloroacetate dehalogenase to glycolic acid, which can enter the central metabolic pathways (Janssen et al., 1985).

X. autotrophicus GJ10 has been used in several studies aimed at biological treatment of synthetic wastewaters containing DCA (Freitas dos Santos and Livingston, 1993, 1994, 1995[a]). In most of these studies, however, DCA concentrations were in the millimolar range whereas, for practical application, DCA in groundwater has to be removed at concentrations in the micromolar range. However, the kinetic parameters determined for suspended cells of strain GJ10 (van den Wijngaard et al., 1993[a]) indicate that the affinity of this strain for DCA it too low for the efficient removal of DCA at these low concentrations. In an attempt to isolate microorganisms with a higher affinity for DCA, van den Wijngaard et al. (1992) isolated the bacterial strain *Ancylobacter aquaticus* AD25. They reported a K_s value of 24 µM for this strain compared to 260 µM for strain GJ10. Interestingly, strain AD25 expressed the same dehalogenase as strain GJ10, but at much higher levels, which explains the higher apparent affinity of strain AD25 for DCA (van den Wijngaard et al., 1992, 1993[a]). However, *A. aquaticus* AD25, as well as *X. autotrophicus* GJ10 and *Pseudomonas* sp. strain DE2, require vitamins for optimal growth (Stucki et al., 1983; van den Wijngaard et al., 1993[b]). Therefore, we set out

to isolate DCA-degrading bacteria with a high affinity for DCA but without the dependency on vitamins for optimal growth. In this report we describe the initial characterization of a new isolate, *Pseudomonas* sp. strain DCA1, which has a high affinity for DCA and utilizes a novel pathway for DCA degradation.

MATERIALS AND METHODS

Isolation of bacteria

Enrichments in batch cultures were set up in closed bottles containing mineral salts medium (Hartmans et al., 1992), with mixtures of soil and water from various sources as inocula. Incubations were carried out with 1 mM DCA at different temperatures (21°C and 30°C).

In a second approach, a 2-liter fermenter containing 1 liter of mineral salts medium (Hartmans et al., 1992) was used for the enrichment. The mineral salts medium was initially supplemented with 0.02 g of yeast extract per liter. The dilution rate was 0.03 h^{-1}. DCA was supplied at 1.2 mmol h^{-1} via the gas phase. A biofilm sample from a groundwater purification plant, as described by Stucki and Thüer (1995), was used as the inoculum. The temperature of this contaminated groundwater varied between 8 and 12°C. For practical reasons, the fermenter used for the enrichment was kept at 21°C. From the mixed culture which developed in the fermenter, a pure culture was isolated by using serial dilutions in mineral salts medium with DCA and yeast extract.

Analytical methods

Concentrations of DCA were determined by analyzing 100-µl headspace samples on a Chrompack CP9000 gas chromatograph equipped with a CP-Sil 8CB column (Chrompack B.V., Middelburg, The Netherlands). The oven temperature was kept at 100°C. All experiments were performed at 25°C unless stated otherwise. The partition coefficient for DCA between the gas phase and the liquid phase at this temperature is 0.05 (Amoore and Hautala, 1983). Concentrations of CO_2 were measured by injecting 100-µl gas phase samples into a Hewlett-Packard 6890 gas chromatograph containing a Chrompack Poraplot Q column. Qualitative determination of the chiral composition of the formed 1,2-epoxypropane was performed by headspace analysis on a Carlo Erba Strumentazione (series 4200) gas chromatograph with a -cyclodextrin 225 column (Supelco, Zwijndrecht, The Netherlands) at 50°C. Samples were heated to 60°C and 500-µl headspace was injected into the column. Retention times were 8.9 and 9.3 min for the *R*- and *S*-enantiomers, respectively.

Batch cultures

Growth experiments in batch cultures were performed in 250-ml serum bottles containing 50 ml of mineral salts medium (Hartmans et al., 1992). In the case of volatile compounds, such as DCA, Boston bottles with Teflon caps (Mininert; Phase Separations, Waddinxveen, The Netherlands) were used in order to prevent substrate loss by evaporation. In the case of DCA as the carbon and energy source, the substrate concentration was 1 mM unless stated otherwise. Growth rates were assessed by measuring CO_2 production during the exponential phase.
In the comparative DCA degradation experiment between *Pseudomonas* sp. strain DCA1 and *X. autotrophicus* GJ10, the phosphate buffer concentration in the medium was 2.5 times higher than in other experiments. The initial DCA concentration was 5 mM. Incubations with *X. autotrophicus* GJ10 were supplemented with 12 µg of biotin per liter, which was filter sterilized by using 0.2-µm-pore-size disposable filters (Schleicher & Schuell, Dassel, Germany). Incubations with strain GJ10 were performed at 30°C. The growth of strain DCA1 was tested on chloroacetaldehyde, chloroacetic acid, 2-chloroethanol, and ethanol at different concentrations (1, 2 and 5 mM). Substrates were added to the medium in pure form, except for chloroacetic acid, which was added as a filter-sterilized solution. The CO_2 production and turbidity increase after 5 days of incubation at 21°C were used as an indication of growth on the substrate.

Continuous culture of strain DCA1

Strain DCA1 was grown continuously in a 2-liter fermenter containing 1 liter of mineral salts medium (Hartmans et al., 1992). The pH was maintained at 7.0 by the addition of sterile 2N NaOH. The dilution rate was 0.05 h^{-1}, and the temperature was 25°C. Air was bubbled through a column containing pure DCA at a rate of 5 ml min^{-1}. This air stream was diluted by mixing with a second stream of air (2,000 ml min^{-1}), resulting in an ingoing DCA concentration in the gas phase of 1 mg l^{-1} which was bubbled through the liquid phase. The biomass density was monitored by determining the optical density at 660 nm (OD_{660}). Under steady-state conditions, the OD_{660} was approximately 0.5.

Preparation of cell extracts

Cells were centrifuged for 10 min at 16,000 x *g* and then washed in an equal volume of 50 mM Tris-HCl buffer (pH 7.5). The pellet was resuspended in a 100-times smaller volume, and the cells were disrupted by sonication for 1 min on ice followed by centrifugation (20 min at 39,000 x *g*). The supernatant was used immediately for the determination of enzyme activities. The protein content was determined by using BCA Protein Assay Reagent (Pierce, Illinois, USA).

Enzyme assays

DCA monooxygenase activity in cell extracts of strain DCA1 was determined in 25-ml glass vials (Supelco, Zwijndrecht, The Netherlands) closed with Teflon valves (Mininert). Cell extract was diluted in 50 mM Tris-HCl (pH 7.5) to a final volume of 2 ml. NADH or NADPH was added to a final concentration of 2 mM. DCA was added at an initial concentration of 100 µM unless stated otherwise, and DCA degradation was followed by headspace analysis as described above. For anaerobic incubations, 35-ml serum bottles with rubber septa were used. Bottles were flushed with nitrogen for 10 min before the DCA was added. A control incubation showed that no significant loss of DCA occurred during the time scale of the experiments. Monooxygenase activity in washed whole cells was determined with resuspended cells in a total volume of 2 ml of mineral salts medium. Monooxygenase activities in cell extracts as well as in whole cells were determined at 25°C.

Chloroacetaldehyde dehydrogenase activity was determined spectrophotometrically. Cell extract was incubated in 50 mM Tris-HCl (pH 7.5) with NAD in a final concentration of 1 mM. After the addition of chloroacetaldehyde (final concentration, 5 mM), the formation of NADH was monitored at 340 nm and at 30°C. Activities were corrected for NADH oxidase activity by measuring the disappearance of NADH (0.1 mM) when incubated with cell extract.

Chloroacetic acid dehalogenase activity was assayed at 30°C by measuring the release of chloride. Cell extract was incubated in 50 mM Tris-HNO_3 (pH 9.0) containing 5 mM chloroacetic acid. At different times, 100-µl samples were taken and chloride concentrations were determined by using a colorimetric method (Bergmann and Sanik, 1957).

Effect of growth substrate on enzyme activities in strain DCA1

To determine the effect of different growth substrates on enzyme activities, cells of strain DCA1 were grown at 21°C in stirred batch cultures by using 5-liter Erlenmeyer flasks containing 1 liter of mineral salts medium (Hartmans et al., 1992). The concentration of phosphate buffer was 2.5-times higher than in the standard mineral salts medium, and growth substrates were added at a concentration of 5 mM. Flasks were inoculated with cells that were pregrown on the same substrate. Cells were harvested in the exponential phase. One part of the cell suspension was washed in mineral salts medium (Hartmans et al., 1992) and used immediately for the determination of monooxygenase activity in whole cells. The other part of the cell suspension was washed and concentrated in 50 mM Tris-HCl buffer (pH 7.5) and stored at -20°C until the preparation of cell extracts, and the determination of enzyme activities was performed as described above.

Competition experiments

Competitive inhibition of the monooxygenase was measured with DCA-grown cells of strain DCA1 harvested from the fermenter. Cells were diluted in mineral salts medium (Hartmans et al., 1992) to a final OD_{660} of 0.13. Incubations were performed in 135-ml serum bottles with an initial DCA concentration of 250 µM. Different amounts of propene were added to the bottles. Based on the partition coefficient of 0.12 between liquid and air phases at 25°C (Mackay and Shiu, 1981), propene concentrations in the liquid phase were 0, 8, and 40 µM. Bottles were shaken vigorously at 25°C, and DCA degradation was measured as described above.

RESULTS

Isolation of DCA-degrading bacteria

In order to isolate bacteria with a high affinity for DCA, a fermenter was inoculated with a biofilm sample, kindly provided by G. Stucki, that originated from a rotating biological contactor treating groundwater polluted with DCA as the sole contaminant (Stucki and Thüer, 1995). Within 3 weeks, a DCA-degrading mixed culture was obtained. This mixed culture did not grow very well on mineral salts medium agar plates with DCA vapor present as carbon and energy source; therefore, serial dilutions in liquid cultures were used to obtain a pure culture. The obtained bacterial culture gave one colony type on rich (yeast-glucose) medium agar plates. The new isolate was identified by the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany) as a bacterium belonging to the genus *Pseudomonas*. The partial sequence of the 16S ribosomal DNA showed 99.4% similarity with *P. fluorescens* and 99.1% similarity with *P. viridiflava*. However, important characteristics such as fluorescense and hydrolysis of gelatin, which are typical for these subspecies, were all negative for the new isolate. The new isolate was designated *Pseudomonas* sp. strain DCA1.

Temperature

In preliminary experiments with strain DCA1 it was observed that, when precultured at 25°C, inocul herefore, the optimum tempe d. As can be seen in Figure growth rate of strain DCA1. Th . At 30°C, the maximum grow occurred at 35°C.

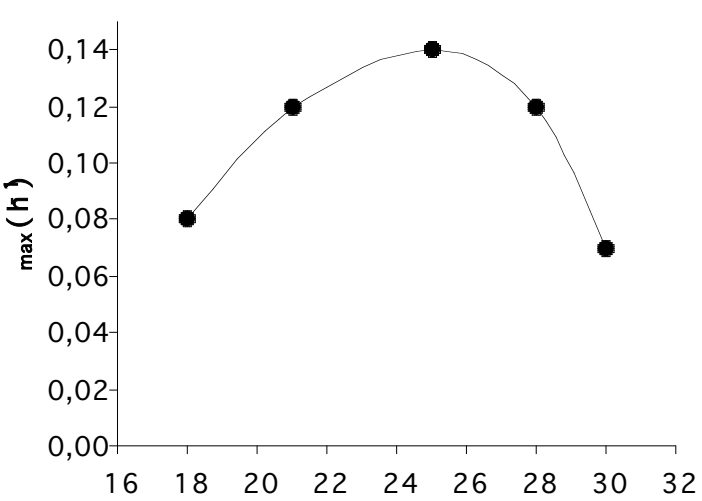

Figure 2.1 Influence of temperature on maximal specific growth rates of *Pseudomonas* sp. strain DCA1 growing on 1,2-dichloroethane (1 mM).

Growth kinetics

We could not determine the K_m value for DCA with washed whole cells of strain DCA1 since this value was below the detection limit of approximately 0.5 µM. Therefore, a comparative growth experiment between *Pseudomonas* sp. strain DCA1 and *X. autotrophicus* GJ10 was performed to study the kinetic differences between these two strains at low substrate concentrations. Substrate depletion curves in closed batch cultures were determined for both strains while they were growing on DCA at an initial concentration of 5 mM. In Figure 2.2, the last part of the DCA depletion curves is shown.

It is evident that the DCA degradation rate of strain GJ10 decreased dramatically at lower concentrations. Strain DCA1, however, did not show any decrease in the DCA depletion rate, even when the substrate concentration became very low, indicating a much higher affinity for DCA.

First step in DCA degradation by *Pseudomonas* sp. strain DCA1

In the absence of a cofactor, no DCA degradation was observed in cell extracts of DCA-grown cells of strain DCA1 (Figure 2.3). This suggests that a hydrolytic DCA dehalogenase, as is involved in the DCA degradation pathway of *X. autotrophicus* GJ10, is apparently not present in strain DCA1. Alternatively, the first step in DCA degradation could be an oxidation reaction by a monooxygenase (Yokota et al., 1986). In that case, oxygen and a source of reducing power (NADH or NADPH) would be required for DCA degradation in cell extracts. Degradation of DCA in cell extract did indeed occur when NADH was added to the reaction mixture (Figure 2.3).

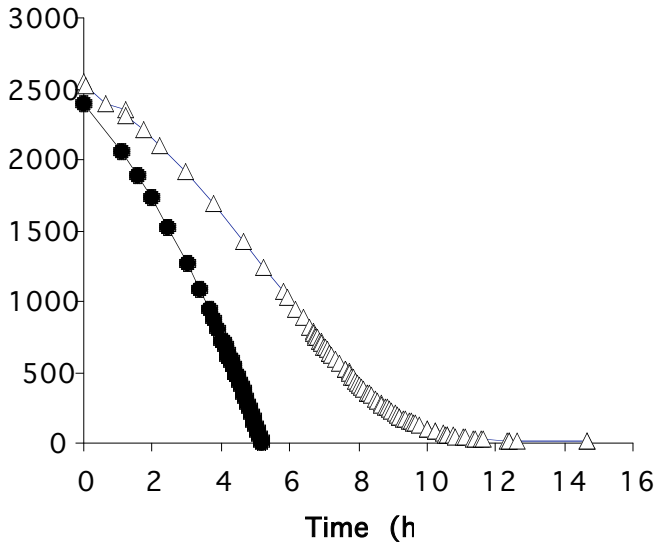

Figure 2.2 DCA depletion curves of growing cells of *Pseudomonas* sp. strain DCA1 ( ) and *X. autotrophicus* GJ10 (_).

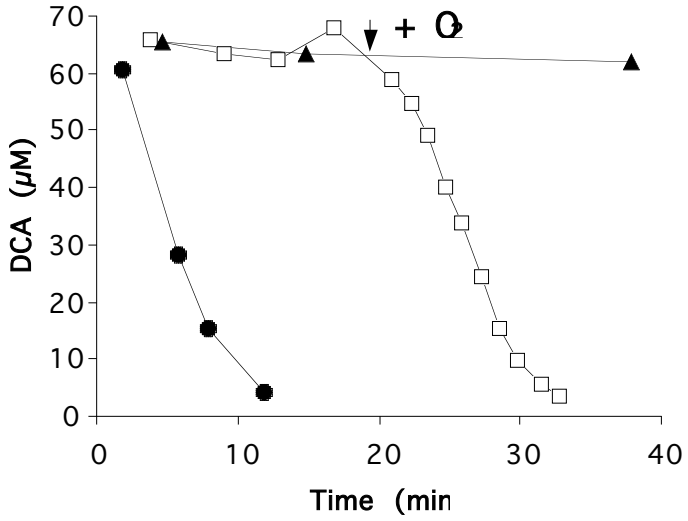

Figure 2.3 Oxygen and NADH dependency of DCA degradation in cell extract of *Pseudomonas* sp. strain DCA1. Symbols: , aerobic incubation in the presence of NADH; _, nitrogen-flushed cell extract in the presence of NADH; , aerobic incubation without NADH. Arrow indicates the replacement of 3 ml of headspace by air.

An oxygen dependency of DCA degradation was first observed with whole cells of strain DCA1 (results not shown). In the absence of oxygen, no DCA degradation occurred. However, after addition of air to the incubation mixture, DCA degradation was restored. Similarly, DCA was only degraded after the addition of air when

nitrogen-flushed cell extract was used (Figure 2.3). These results indicate that the first step in DCA degradation by *Pseudomonas* sp. strain DCA1 requires molecular oxygen and NAD(P)H, suggesting that DCA is oxidized by a monooxygenase.

Competitive inhibition of DCA degradation

Alkane monooxygenases generally have a broad substrate specificity and are often capable of performing both epoxidation and hydroxylation reactions (Hartmans et al., 1989). Oxidation of alkenes by cells expressing alkane monooxygenase activity often results in the accumulation of the corresponding epoxides. To determine whether the DCA-oxidizing enzyme would act similarly, DCA-grown cells of strain DCA1 were incubated with propene. This resulted in the formation of 1,2-epoxypropane, confirming the presence of monooxygenase activity in whole cells. Analysis of headspace samples of the reaction mixture on a chiral column revealed that both enantiomers of 1,2-epoxypropane were formed.

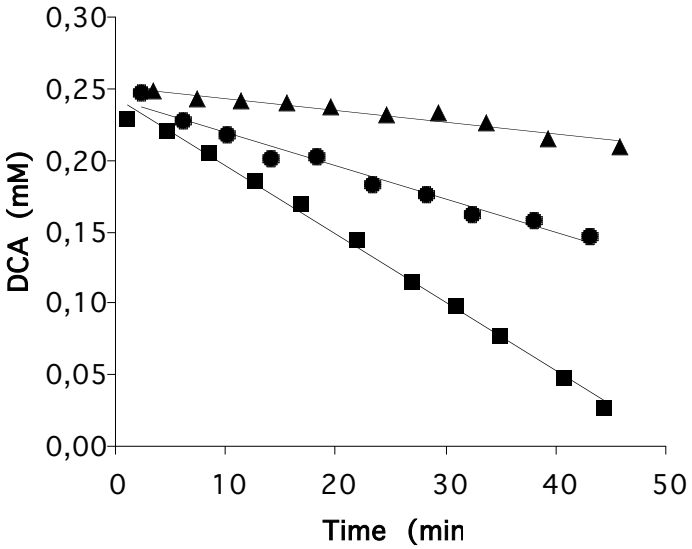

Figure 2.4 Effect of different propene concentrations on DCA degradation rates of whole cells of *Pseudomonas* sp. strain DCA1. Symbols: , no propene; , 8 µM propene; , 40 µM propene.

It is expected that, if one and the same monooxygenase oxidizes both DCA and propene, the presence of a mixture of these substrates would lead to lower reaction rates. Since DCA degradation could be measured more accurately than propene depletion, the effect of different concentrations of propene on DCA conversion rates was determined. The results of this competition experiment, performed with whole cells of strain DCA1, clearly show that at higher propene concentrations the DCA degradation rates are lower (Figure 2.4).

Influence of protein content on monooxygenase activity

Many monooxygenases are multicomponent enzymes. This can often be illustrated by determining the enzyme activity as a function of the protein content in the assay. Figure 2.5 shows that the relation between protein content and DCA monooxygenase activity is nonlinear, resulting in lower specific activities at protein concentrations below approximately 0.4 mg ml^{-1}.

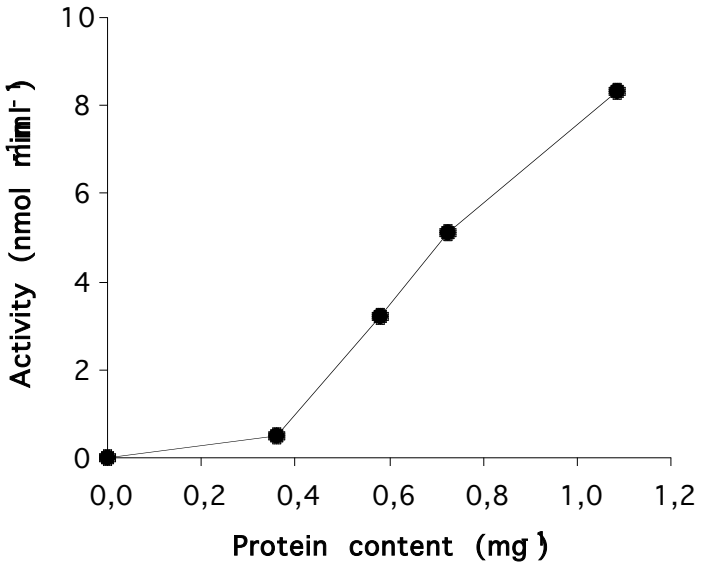

Figure 2.5 Relationship between protein content in the reaction mixture and monooxygenase activity.

The maximum specific DCA monooxygenase activity in cell extract, calculated from this experiment, is about 7 nmol min^{-1} mg^{-1} of protein. When NADPH was used instead of NADH, this activity was threefold lower.

Enzymes involved in DCA degradation pathway

To further study the DCA degradation pathway, *Pseudomonas* sp. strain DCA1 was tested to see if it could grow on the chlorinated intermediates in the proposed DCA degradation pathway shown in Figure 2.6.

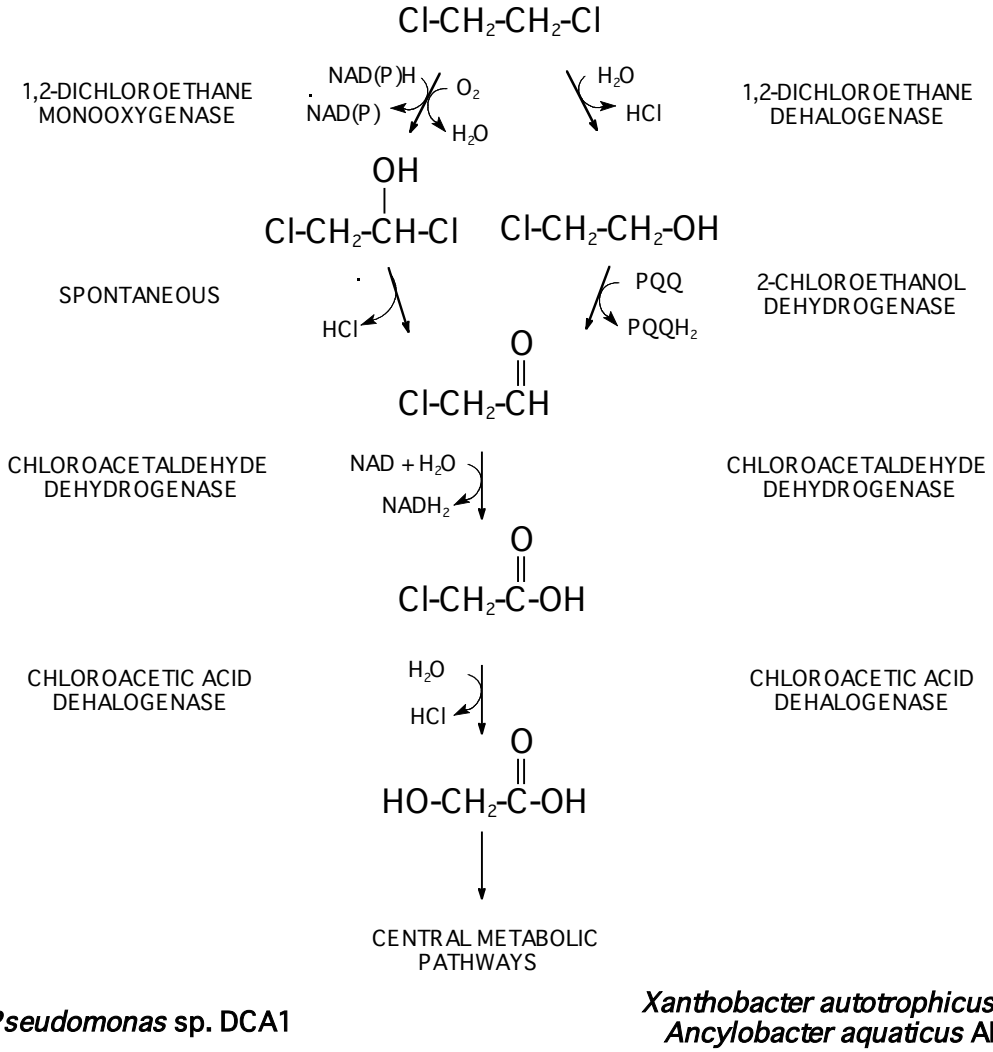

Figure 2.6 Different pathways of DCA degradation by *Pseudomonas* sp. strain DCA1 (proposed), *X. autotrophicus* GJ10, and *A. aquaticus* AD25.

Strain DCA1 could grow on chloroacetic acid, but no growth was observed on chloroacetaldehyde. This is probably due to the toxicity of this compound, since growth on DCA was inhibited in the presence of 1 mM chloroacetaldehyde. Strain DCA1 could also grow on 2-chloroethanol. The expression of DCA monooxygenase, chloroacetaldehyde dehydrogenase, and chloroacetic acid dehalogenase was determined in cells of strain DCA1, grown on DCA, chloroacetic acid, and ethanol as a control (Table 2.1).

Table 2.1 Enzyme activities in *Pseudomonas* sp. strain DCA1 grown on various carbon sources.

Carbon source	Activities of:		
	DCA monooxygenase[a]	Chloroacetaldehyde dehydrogenase[b]	Chloroacetic acid dehalogenase[b]
DCA	62	21	1,008
Chloroacetic acid	<0.5	8	907
Ethanol	<0.5	20	0

[a] Specific activities of DCA monooxygenase were determined in washed whole cells and are expressed as nanomoles per minute per milligram (dry weight) of cells.

[b] Activities were measured in cell extracts and are expressed as nanomoles per minute per milligram of protein.

DCA monooxygenase activities were determined in washed whole cells instead of cell extracts, since DCA monooxygenase is a rather unstable enzyme. DCA monooxygenase activity was found in DCA-grown cells, whereas negligible activities were found in cells grown on chloroacetic acid or ethanol. Chloroacetaldehyde dehydrogenase activity was found in cells grown on all of the tested substrates, with the lowest activity in cells grown on chloroacetic acid. Cells grown on DCA and chloroacetic acid expressed high chloroacetic acid dehalogenase activities, whereas no dehalogenase activity was present in ethanol-grown cells of strain DCA1.

DISCUSSION

To obtain new DCA-degrading microorganisms, we set up batch enrichment cultures with 1 mM DCA at different temperatures with various inocula. However, no DCA degradation was observed in any of these cultures. Apparently, DCA-degrading bacteria are not widespread in the environment.

Stucki and Thüer (1995) described the treatment of groundwater that contained DCA as the sole contaminant. The groundwater treatment plant consisted of activated carbon filters and a rotating biological contactor (RBC) and was inoculated with *X. autotrophicus* GJ10 and *Pseudomonas* sp. strain DE2. Initially, the RBC had little effect on DCA removal. However, during the fourth year of operation, DCA elimination by the RBC increased continuously. During the fifth year, most of the DCA was removed by the RBC, resulting in effluent concentrations of DCA which were usually much lower than 10 µM. The initially black surface of the RBC had by then changed to a yellow color, suggesting the presence of *X. autotrophicus* GJ10

as the predominant DCA degrader. Reisolation of DCA-degrading microorganisms confirmed the presence of *Xanthobacter* sp. with an identical gene encoding for the DCA dehalogenase (Stucki and Thüer, 1995). The average DCA concentration in the effluent, however, was much lower than could be expected, based on the kinetic parameters determined for suspended cells of *X. autotrophicus* GJ10 (van den Wijngaard et al., 1993[a]). We therefore anticipated that another DCA-degrading microorganism with a high affinity for DCA must be present in the RBC biofilm. To isolate this strain, a sample of the RBC biofilm was used to inoculate a fermentor. Enrichment in a continuous culture under DCA limitation was used to select the microorganism with the highest affinity for DCA (lowest K_s value). The isolated *Pseudomonas* sp. strain DCA1 did indeed exhibit a much higher affinity for DCA than strain GJ10, as was shown by the DCA depletion curves determined in closed cultures (Figure 2.2). Considering the high affinity of strain DCA1 and also the relatively high growth rate with DCA as a carbon source, it is somewhat surprising that strain GJ10 was still present in the RBC biofilm. However, besides these kinetic differences, other factors such as decay rates and biofilm-forming capabilities, will affect the performance of the microorganisms in the biofilm.

The first DCA-utilizing pseudomonad described in the literature is *Pseudomonas* sp. strain DE2 (Stucki et al., 1983), which was also used to inoculate the RBC (Stucki and Thüer, 1995). The physiological similarities between strains DE2 and DCA1 are striking; however, there are some distinctions. Strain DE2 does not form colonies on nutrient agar or other solidified media (Stucki et al., 1983). Strain DCA1 does not grow on mineral salt agar plates either, but it does form small colonies on rich-medium agar plates. The growth rates of both strains at 30°C are comparable. Strain DE2 is reported to be dependent on vitamins, whereas we found that strain DCA1 does not require any additional organic nutrients for optimal growth. Further studies, e.g., 16S sequence analysis, are required to establish whether these two strains are intrinsically different.

In contrast to the hydrolytic dehalogenation of DCA in *X. autotrophicus* GJ10 and *A. aquaticus* AD25 (Janssen et al., 1985; van den Wijngaard, 1992), an oxidation reaction seems to be the first step in DCA degradation in both *Pseudomonas* strains DE2 and DCA1. No DCA degradation was observed in the cell extracts of strain DE2, and a hydroxylation of DCA, yielding the unstable intermediate 1,2-dichloroethanol, was suggested as the first step in DCA metabolism (Stucki et al., 1983). We also did not observe degradation of DCA in cell extracts of strain DCA1 unless NAD(P)H was added to the incubation mixture. In the presence of NAD(P)H, DCA degradation in cell extract was oxygen dependent, suggesting the involvement of a monooxygenase (Figure 2.3). Oxygen-dependent dehalogenation of long-chain

(C_5 to C_{12}) _,_-dichloroalkanes was recently observed in *Pseudomonas* sp. strain 273 (Wischnak et al., 1998), and hexadecane-grown cells of *Rhodococcus erythropolis* Y2 showed oxygenase-type dehalogenation activity towards C_4 to C_{18} _-chloroalkanes (Armfield et al., 1995). Conversion of DCA by a monooxygenase was suggested by Yokota et al. (1986). They described the conversion of DCA by resting cells of two different methanotrophic strains.

Additional proof that a monooxygenase activity was responsible for DCA degradation in our strain was provided by conversion of propene to 1,2-epoxypropane and the results of competition experiments (Figure 2.4). Propene competitively inhibited DCA degradation in DCA-grown cells of strain DCA1, suggesting that both substrates compete for the same active site. In general, alkane monooxygenases yield racemic mixtures of epoxides (Weijers et al., 1988). Therefore, the observed conversion of propene to both enantiomers of 1,2-epoxypropane was expected. Interestingly, at a DCA concentration of 250 µM, the presence of 8 µM propene already inhibited DCA degradation by the monooxygenase for more than 50%, indicating an even higher affinity of the monooxygenase for propene than for DCA.

The nonlinear relationship between the protein content in the enzyme assay and the specific activity of DCA monooxygenase (Figure 2.5) could indicate that DCA monooxygenase is a multicomponent enzyme, which is not uncommon for monooxygenases (Hartmans et al., 1991). Significant levels of DCA monooxygenase activity were only present in DCA-grown cells of strain DCA1 (Table 2.1). Apparently, DCA monooxygenase is an inducible enzyme, and DCA itself or chloroacetaldehyde could be the inducer.

The next enzymatic step in DCA degradation is probably the dehydrogenation of chloroacetaldehyde. Chloroacetaldehyde dehydrogenases are reported to have broad substrate specificities and are also induced during growth with ethanol (Janssen et al., 1987; Strotmann et al., 1990). Chloroacetaldehyde dehydrogenase activities in cell extracts of strain DCA1 were rather low, probably due to nonoptimal assay conditions. The activity in cell extracts was higher in DCA- or ethanol-grown cells of strain DCA1 than in cells grown on chloroacetic acid (Table 2.1).

The product of chloroacetaldehyde dehydrogenase is chloroacetic acid, which subsequently has to be dehalogenated. Dehalogenation of chloroacetic acid by halidohydrolases was observed in all DCA-utilizing bacteria described in the literature (Janssen et al., 1985; Stucki et al., 1983; van den Wijngaard et al., 1992). Chloroacetic acid dehalogenase in strain DCA1 is obviously an inducible enzyme,

since no activity was found in ethanol-grown cells. Dehalogenation of chloroacetic acid will most likely result in the formation of glycolic acid (Strotmann, 1990).

The complete proposed DCA degradation pathway of *Pseudomonas* sp. strain DCA1 is shown in Figure 2.6. Only the initial attack of the DCA molecule seems to be different from the DCA degradation pathway in *X. autotrophicus* GJ10 and *Ancylobacter aquaticus* AD25 (Janssen et al., 1985; van den Wijngaard et al., 1992).

The isolation of a DCA-degrading bacterial strain with a high affinity for DCA offers promising opportunities for the efficient biological removal of this compound from groundwater.

3

Membrane-aerated biofilm reactor for the removal of 1,2-dichloroethane by *Pseudomonas* sp. strain DCA1

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ABSTRACT

A membrane-aerated biofilm reactor (MABR) with a biofilm of *Pseudomonas* sp. strain DCA1 was studied for the removal of 1,2-dichloroethane (DCA) from water. A hydrophobic membrane was used to create a barrier between the liquid and the gas phase. Inoculation of the MABR with cells of strain DCA1 grown in a continuous culture resulted in the formation of a stable and active DCA-degrading biofilm on the membrane. The maximum removal rate of the MABR was reached at a DCA concentration of approximately 80 µM. Simulation of the DCA fluxes into the biofilm showed that the MABR performance at lower concentrations was limited by the DCA diffusion rate rather than by kinetic constraints of strain DCA1. Aerobic biodegradation of DCA present in anoxic water could be achieved by supplying oxygen solely from the gas phase to the biofilm grown on the liquid side of the membrane. As a result, direct aeration of the water, which leads to undesired coagulation of iron oxides, could be avoided.

INTRODUCTION

1,2-Dichloroethane (DCA) is an anthropogenic chemical that is mainly used in the production of vinyl chloride, the monomer of PVC (World Health Organization 1995). DCA has a low sorption coefficient and a good solubility in water. Consequently, spillage of DCA onto soil often results in contamination of the groundwater (US Environmental Protection Agency, http://www.epa.gov/safewater/dwh/t-voc/12-dichl.html). Since groundwater is the major source for the preparation of drinking water (Centraal Bureau voor de Statistiek, 2000; Morris et al., 2003), the significance of groundwater treatment is evident.

Biodegradation can offer a good alternative to the existing chemical and physical technologies to remove xenobiotic compounds from groundwater. Several bacterial strains, such as *Xanthobacter autotrophicus* GJ10 (Janssen et al., 1985) and *Ancylobacter aquaticus* AD25 (van den Wijngaard et al., 1992) are able to degrade DCA aerobically. In both of these strains, the first step in the DCA-degradation pathway is performed by an identical haloalkane dehalogenase. This enzyme, however, has a rather low affinity for DCA, with a K_m value of 571 µM (van den Wijngaard et al., 1993). In comparison, the Intervention Value, a quality criterion linked to remediation urgency, is 4 µM for DCA in groundwater in The Netherlands (Lijzen et al., 2001; Swartjes 1999). To efficiently remove DCA in this low concentration range, we previously searched for bacteria with a higher affinity for this compound. This resulted in the isolation of *Pseudomonas* sp. strain DCA1, which utilizes DCA as sole carbon and energy source. The first step in DCA metabolism in this strain is a monooxygenase-mediated oxidation. Strain DCA1 has a very high affinity for DCA; the K_m value is below 0.5 µM (Hage and Hartmans 1999).

Another important issue that must be addressed for the successful (aerobic) treatment of groundwater is the supply of oxygen. Since groundwater is often anaerobic and iron-containing, aeration can result in the coagulation of iron oxides, with detrimental consequences for the operating systems. Moreover, aeration can cause stripping of DCA from groundwater to the air. Freitas dos Santos and Livingston (1994) used a silicone-rubber extractive membrane to prevent stripping of DCA from wastewater. The membrane provided a barrier between DCA-containing wastewater on one side, and recirculated aerated medium on the other side, where a biofilm of *X. autotrophicus* GJ10 was present. Debus and Wanner (1992) used a different configuration for the degradation of xylene. Oxygen was supplied through a gas-permeable silicone membrane, on the outside of which a biofilm was grown.

The use of membrane bioreactors for the treatment of wastewater has been reviewed by Brindle and Stephenson (1996) and Casey et al. (1999) and for the treatment of wastegas by Reij et al. (1998).

In this work, we explored the biofilm formation and subsequent application of *Pseudomonas* sp. strain DCA1 in a membrane-aerated biofilm reactor (MABR). We used a hydrophobic membrane as a barrier between the gas phase and the (anaerobic) groundwater (Figure 3.1). On the groundwater side of the membrane, degradation of DCA occurs in a biofilm attached to the surface. Oxygen diffuses from the opposite side through gas-filled pores to the biofilm, where it is consumed. As a result, direct contact between oxygen and the groundwater is minimized to prevent the coagulation of iron oxides and stripping of DCA.

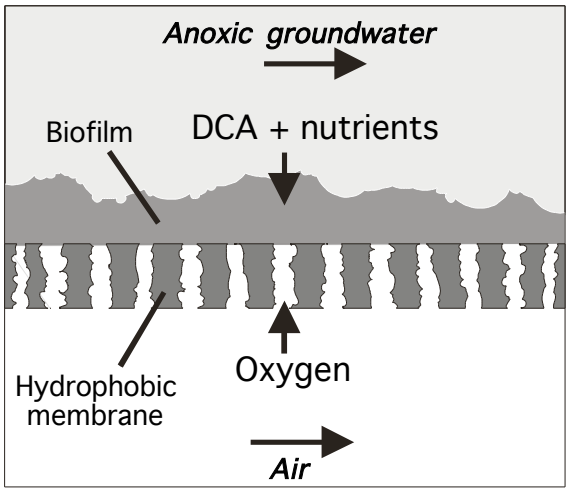

Figure 3.1 Principle of aerobic biodegradation of 1,2-dichloroethane (DCA) in groundwater using a hydrophobic membrane.

MATERIALS AND METHODS

Bacteria and growth conditions

Pseudomonas sp. strain DCA1 was previously isolated in our laboratory from a DCA-degrading biofilm (Hage and Hartmans 1999) and is deposited in the Industrial Microbiology Culture Collection of Wageningen University (CIMW no. 412B). Strain DCA1 was grown continuously in a 2-l fermenter containing 1 l of mineral salts medium (MM), which contained (per liter): 1.55 g K_2HPO_4, 0.85 g $NaH_2PO_4.H_2O$, 2 g $(NH_4)_2SO_4$, 100 mg $MgCl_2.6H_2O$, 10 mg EDTA, 2 mg

$ZnSO_4.7H_2O$, 1 mg $CaCl_2.2H_2O$, 5 mg $FeSO_4.7H_2O$, 0.2 mg $Na_2MoO_4.2H_2O$, 0.2 mg $CuSO_4.5H_2O$, 0.4 mg $CoCl_2.6H_2O$ and 1 mg $MnCl_2.4H_2O$. The pH was maintained at 7.0 by the addition of sterile 2N NaOH. The dilution rate was 0.05 h^{-1}, and the temperature was 25 °C. Air was bubbled through a column containing pure DCA at a rate of 10 ml min^{-1}. The bubble column was kept at a constant temperature of 23 °C. This air stream was diluted by mixing with a second stream of air (2000 ml min^{-1}). Based on the partition coefficient of 0.05 between air and liquid phases at 25°C (Amoore and Hautala 1983), the ingoing DCA concentration in the gas phase was 2 mg l^{-1}.

Analytical methods

Concentrations of DCA were determined by analyzing 100-µl headspace samples on a Chrompack CP9000 gas chromatograph, equipped with a CP-Sil 8CB column (Chrompack, Middelburg, The Netherlands). The oven temperature was 100 °C and the vials were kept at 30 °C. Chloride concentrations were determined using a colorimetric assay (Bergmann and Sanik 1957).

Operation of MABR

The MABR is shown schematically in Figure 3.2 and consisted of a hydrophobic membrane (pore size 0.22 µm; Millipore Durapore, Bedford, Mass.) clamped between two Perspex halves, creating two compartments of 8 ml each. The porosity of the membrane was 75% and the thickness was 125 µm, as stated by the supplier. The effective membrane area between the two compartments was 40 cm^2. Through the upper compartment, MM was circulated at a rate of 50 ml min^{-1}. The mean fluid velocity was 2.1 cm s^{-1} and the Reynolds number (Re) was 88. Air containing DCA was blown through the lower compartment of the MABR, concurrent with the liquid flow. The air was bubbled through a column containing pure DCA (23 °C) at 1 ml min^{-1} and diluted with a stream of air at 100 ml min^{-1} before entering the MABR, resulting in an ingoing DCA concentration in the gas phase of 4 mg l^{-1} and a concentration in the liquid phase of 808 µM at equilibrium. Sterile 0.2-µm PTFE filters (Midisart 2000; Sartorius, Goettingen, Germany) were placed at the entrance and exit of the MABR. The MABR was operated aseptically until the first sampling took place. The membrane and tubing were sterilized for 30 min at 121 °C. The Perspex module, which was not heat-resistant, was sterilized by soaking in 70% ethanol and subsequent air-drying in a flow cabinet.

The MABR was inoculated by connecting the outlet of a continuous culture containing *Pseudomonas* sp. strain DCA1 to the MABR. The excess liquid from the loop exited via a vertical tube (1 m tall; Figure 3.2, point 5). This tube also allowed the removal of air bubbles from the liquid entering the MABR. In this way damage to

the biofilm, due to shear caused by the air/liquid interface (An and Friedman 1997), was avoided.

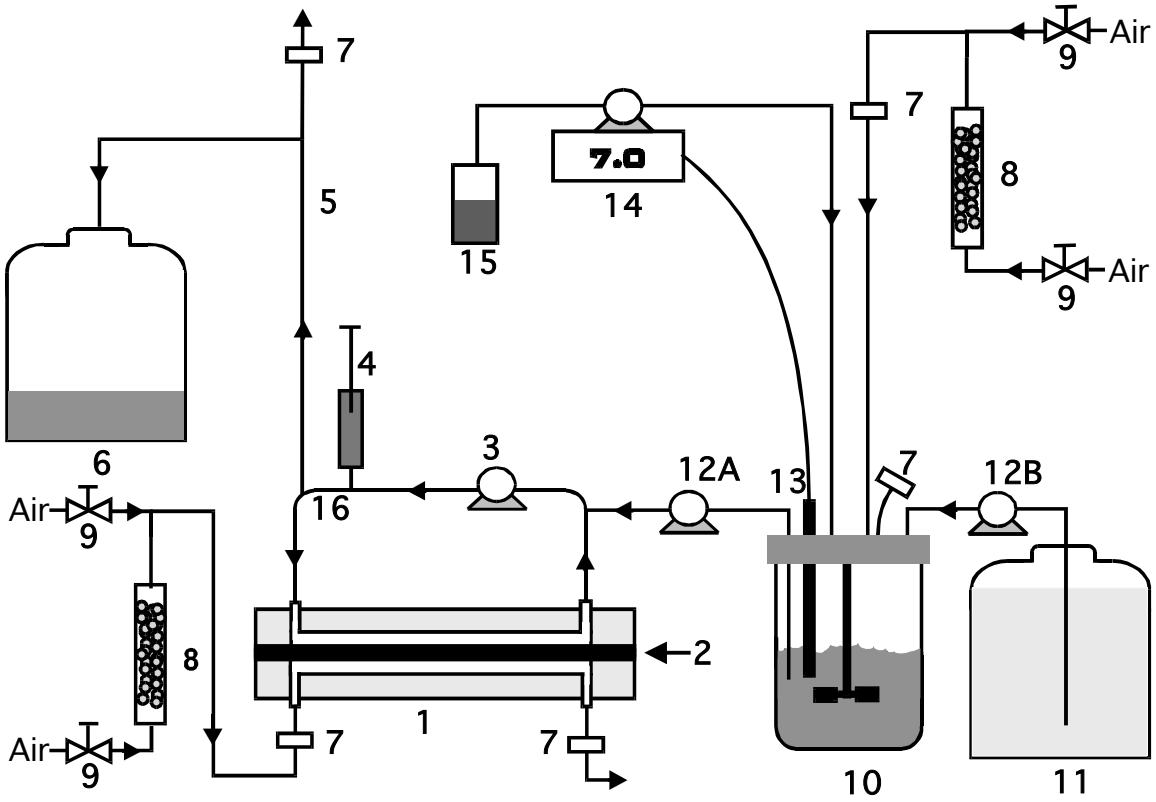

Figure 3.2 Experimental set-up of membrane-aerated biofilm reactor (MABR) and fermenter (not to scale): (1) Perspex module; (2) membrane; (3) peristaltic pump (50 ml min^{-1}); (4) sampling point; (5) vertical tube; (6) waste vessel; (7) sterile air filter; (8) column containing DCA; (9) mass flow controller; (10) fermenter; (11) vessel; (12A, 12B) pumps (50 ml h^{-1}); (13) pH electrode; (14) pH control unit; (15) sterile 2N NaOH; (16) place to close loop.

Closed-loop experiments

At the end of the inoculation stage, the continuous culture (Figure 3.2, fermenter 10) was disconnected from the MABR and replaced by a vessel containing fresh MM. The MABR was then rinsed with 300 ml MM to remove suspended cells and HCl produced during DCA degradation. Sampling from the MABR was done by closing the loop at point 16, and using a 2-ml syringe (sampling port 4) to take a 1-ml sample. During the closed-loop experiments, DCA was supplied via the gas phase at the same concentration as during the inoculation stage and the liquid phase was circulated at a rate of 50 ml min^{-1}.

MABR performance at different DCA concentrations in the aqueous phase

To assess the performance of the MABR at different DCA concentrations, a biofilm was allowed to form during 3 days of non-stop inoculation, as described above. The DCA supply via the gas phase was then shut down and the MABR was rinsed. The rinsing procedure was the same as in the closed-loop experiments. However, to reduce background levels of chloride an alternative mineral medium (designated MM_{low}) was used. The composition of MM_{low} was the same as MM, except that $MgCl_2.6H_2O$ was replaced by $MgSO_4.7H_2O$ (120 mg l^{-1}). After rinsing the MABR with MM_{low}, the feed vessel was replaced by a feed vessel containing MM_{low} with approx. 1 mM DCA. The actual DCA concentration in the MABR could be varied by changing the feed rate of this solution (Figure 3.2, pump 12A). The exact flow rate was determined by collecting and weighing the effluent over time. In order to obtain steady-state DCA concentrations below 20 µM, a feed vessel containing 120 µM DCA in MM_{low} was used. Each time after changing the DCA supply rate, the system was allowed 30 min to reach a new steady-state. Subsequently, three 2-ml samples were taken from the MABR (Figure 3.2, sampling port 4) at 15 min intervals. Then, 1 ml of each sample was transferred to a 25-ml glass vial (Supelco, Zwijndrecht, The Netherlands) closed with a Teflon valve (Mininert; Phase Separations, Waddinxveen, The Netherlands) for determination of the DCA concentration as described above. The vial contained 100 µl of a 10% H_3PO_4 (w/v) solution to inactivate all microbial activity. The remaining part of the sample was stored at –20 °C until the chloride concentration was determined, as described above.

The DCA removal rate was calculated using the chloride concentration and the flow rate of the DCA solution and assuming a stoichiometry of two chloride ions formed per DCA removed. The chloride concentration was corrected for the background level present in the medium.

Removal of DCA from anoxic water

To minimize oxygen diffusion into the MABR during the anoxic experiment, all silicone tubing was replaced by Tygon (Saint-Gobain Performance Plastics, Charny, France). Anoxic water was prepared by bubbling nitrogen through a 10-l vessel containing 9 l of MM_{low} overnight. Subsequently, DCA was added, followed by flushing of the headspace for 1 h. The pumping rate of the DCA-containing MM_{low} into the loop was 450 ml h^{-1}. A plastic bag filled with nitrogen was attached to the vessel to allow the pressure to equalize during the pumping, without the entry of oxygen. Each time after a parameter was changed, the MABR was allowed 1 h to

reach a new steady-state. Subsequently, five samples were taken from the MABR at 10-min intervals. These samples were treated and analyzed as described above.

Simulation program BIOSIM

Some of the experimentally determined DCA-fluxes were fitted using the simulation program BIOSIM, which numerically calculates substrate consumption rates in a flat layer of biomass (biofilm). These calculations are based on Michaelis-Menten kinetics combined with diffusion rates, according to:

$$D\frac{\partial^2 C}{\partial x^2} = V_{max}\frac{C}{C + K_m} \tag{1}$$

with the boundary condition:

$$\frac{dC}{dx} = 0 \qquad \text{at } x = L \text{ (or } x = x_f\text{)} \tag{2}$$

where D is the diffusion coefficient ($m^2\ s^{-1}$), C is the substrate concentration in biofilm (mol m^{-3}), x is the distance to biofilm surface (m), V_{max} is the maximum volumetric activity of the cells (mol $m^{-3}\ s^{-1}$), K_m is the Michaelis-Menten constant (mol m^{-3}), L is the biofilm thickness and x_f is the distance from the biofilm surface where the substrate concentration approaches zero (m). A more detailed description of BIOSIM is given by De Gooijer et al. (1989) and Reij et al. (1995).

An optimal fit of the experimentally determined DCA fluxes was obtained by varying the value of the biofilm thickness in BIOSIM. The method of least squares was used to determine at which biofilm thickness the calculated DCA fluxes best fitted the experimentally determined data points, assuming a constant bulk DCA concentration. The sum of squared errors (SSE) was calculated from:

$$SSE = \sum (y_i - \hat{y}_i)^2 \tag{3}$$

with: y_i = measured DCA flux (mol $m^{-2}\ s^{-1}$) and
$\hat{y}_i$ = DCA flux generated with BIOSIM

For the simulation of reactor performance, the axial mixing of the liquid flow in the MABR was assumed to be comparable with a series of ten ideally mixed stirred-tank reactors (Hartmans and Tramper, 1991). At the interface of the biofilm and the bulk liquid phase, a zone is present where both biodegradation and convective transport occur simultaneously (Massol-Deyá et al., 1995). Therefore, the external mass-transfer resistance at the interface is negligible and not taken into account in the simulation of the MABR performance.

RESULTS AND DISCUSSION

Biofilm formation and stability

Previous studies showed that *Pseudomonas* sp. strain DCA1 is able to utilize DCA as sole carbon and energy source. Moreover, strain DCA1 has a very high affinity for DCA (Hage and Hartmans 1999). However, for a successful application in a MABR, strain DCA1 also has to form a stable and active DCA-degrading biofilm. Strain DCA1 does not grow on mineral salts agar plates (Hage and Hartmans 1999). However, we observed biofilm formation on glass and stainless steel parts of a fermenter after a prolonged time of running (data not shown). Fitch et al. (1996) reported that a pure culture of *Methylosinus trichosporium* OB3b, which is capable of co-metabolic degradation of trichloroethene, did not attach to plastic, metal, glass or diatomaceous earth, although it did grow on agar plates. Clapp et al. (1999), however, did obtain a biofilm of this strain under different conditions.

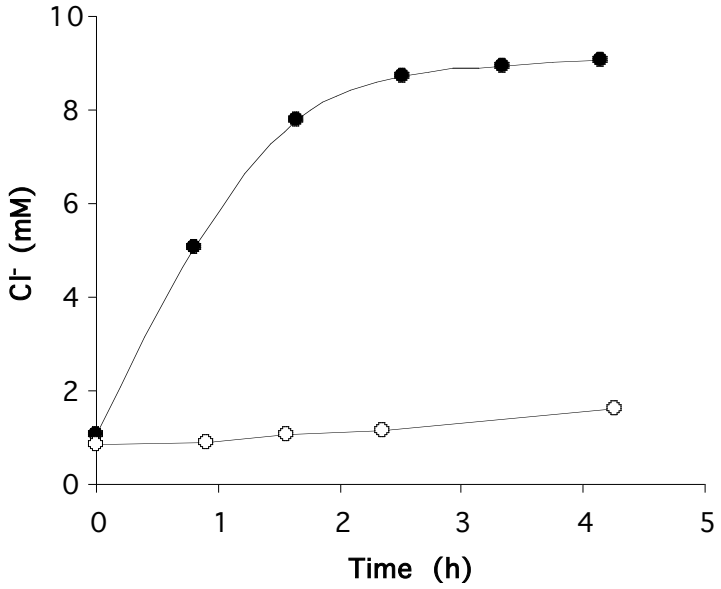

Figure 3.3 Chloride production in MABR resulting from DCA degradation by a *Pseudomonas* sp. strain DCA1 biofilm. Symbols: , biofilm activity after 6 days of inoculation; , chloride production after replacing the Perspex module and membrane by a non-inoculated, identical one.

Biofilm formation was tested by inoculating the MABR during 6 days with cells of strain DCA1 growing in a fermenter. The fermenter was then disconnected and the MABR was rinsed with fresh MM. Subsequently, the chloride production resulting from the oxidation of DCA, which was supplied via the gas phase, was measured in

a closed loop. As can be seen in Figure 3.3, DCA was indeed degraded in the MABR in the absence of suspended cells. From this, it was concluded that strain DCA1 is able to form a mono-culture biofilm with DCA as the sole substrate. After approximately 2 h, the production of chloride stopped. This is probably caused by a decrease in pH, which had by then dropped to pH 5 due to the formation of more than 8 mM of hydrochloric acid as a result of DCA degradation.

After the last sample was taken, the background activity resulting from possible biofilm formation in the tubing of the MABR was determined. The Perspex module, including the membrane, was replaced by an identical but clean one, and another closed-loop experiment was performed. The results in Figure 3.3 show that biofilm activity in the tubing is not significant.

These results show that strain DCA1 is capable of forming an active and stable biofilm on a hydrophobic membrane with DCA as the sole substrate.

Biofilm development over time

To get more insight into the formation of the biofilm over time, DCA-degrading activities were measured on 4 days consecutively, using two different inoculation protocols. In one experiment, the continuous culture was reconnected to the MABR after the daily activity measurement, to resume inoculation with fresh cells of strain DCA1. In the other experiments, the MABR was inoculated during 1 day only. After the activity had been determined on day 1, the fermenter was not reconnected. Instead, fresh MM was supplied to the MABR at a rate of 50 ml h^{-1}, which equaled the rate that was used to supply cells from the fermenter. In these experiments the pH in the MABR did not drop below pH 6.5 during the inoculation stage.

The daily biofilm activity measurements were performed in closed-loop mode as described above. Typical examples of chloride formation are shown in Figure 3.4. From these results, DCA-degradation rates were calculated (Figure 3.5). Based on the results presented in Figure 3.5, biofilm formation was not significantly enhanced by reconnecting the fermenter after initial biofilm formation had occurred. The increase in biofilm activity during days 2–4 was similar for both situations. Apparently, the attachment of additional cells was negligible, compared with the growth of cells that had already become attached to the membrane surface on the first day. Between day 1 and day 2, activity did not increase. Possibly, the activity measurements had a negative effect on the immature biofilm. All of the following experiments were performed with biofilms that had been grown during 3 days of non-stop inoculation, with 4 mg l^{-1} DCA in the gas phase.

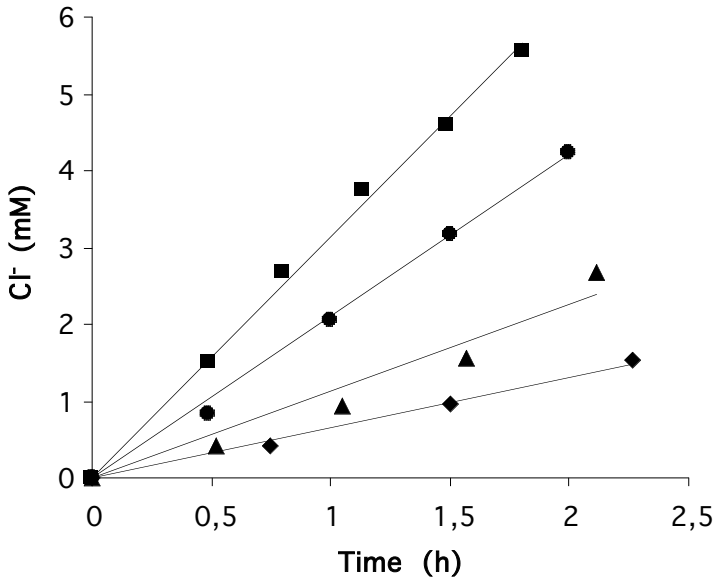

Figure 3.4 A typical example of chloride production on different days. Concentrations were corrected for the chloride concentration present at t=0 (approximately 1 mM). Symbols: , day 1; , day 2; , day 3; , day 4.

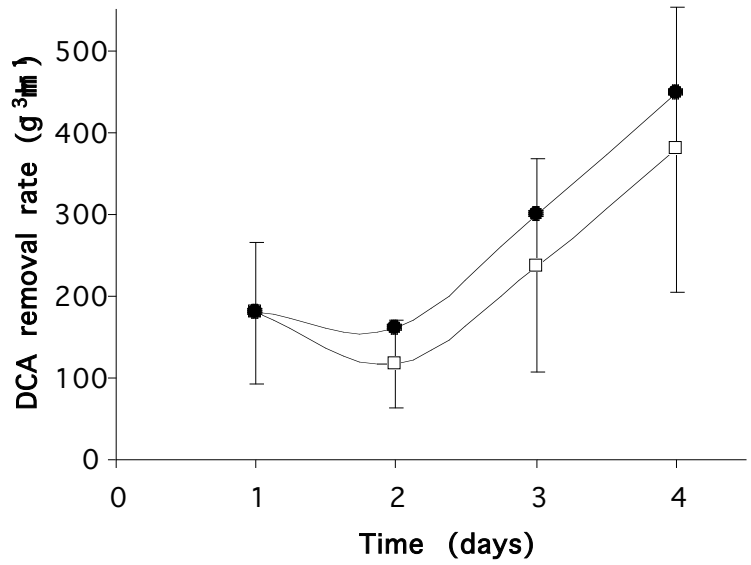

Figure 3.5 Development of biofilm activity over time. Symbols: , MABR was reinoculated after daily measurements; _, MABR was only inoculated during one day at the start of the experiment. Error bars indicate standard deviation between measurements of two separate experiments without re-inoculation after day 1. The activity (and error bar) given on day 1 represents the average value of the three experiments.

MABR performance at low DCA concentrations in the aqueous phase

Since pollutants often have to be removed down to very low concentrations, the removal efficiency at these substrate concentrations is an important parameter in bioremediation processes. We therefore assessed the effect of the DCA concentration in the aqueous phase on the performance of the MABR. The experimental set-up allowed a variable supply rate of MM_{low} containing DCA, without affecting the flow rate in the MABR. During the experiment, the DCA concentrations in the circulating liquid phase in the MABR were determined. To account for possible effects of biofilm growth or decay during the experiment, the measurements were started at the highest DCA concentration (340 µM), followed by a stepwise decrease to the lowest concentration. Finally, the concentration was increased again to the higher concentration ranges. However, no time-related change in biofilm activity was observed in these experiments, which were all performed on 1 day. The DCA removal rates were calculated based on chloride formation, using the flow rates and chloride concentrations in the outlet of the system (Figure 3.6).

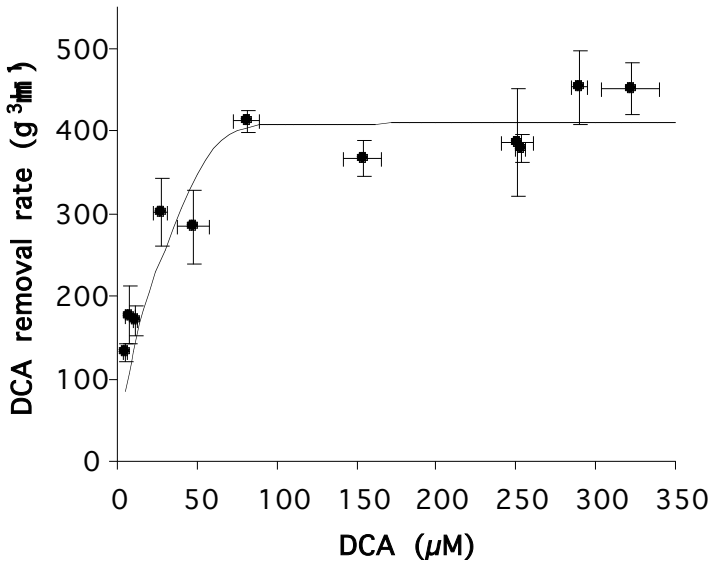

Figure 3.6 DCA removal rates at different inlet concentrations. Data points represent the average value of three different samples per steady-state. Error bars indicate standard deviation. The line represent the removal rates calculated with the BIOSIM reactor model, assuming a biofilm thickness of 48 µm.

At DCA concentrations above 100 µM, the DCA degradation rate was more or less constant, with an average value of 410 g m^{-3} h^{-1} which is in rather good agreement with the DCA removal rate determined in the closed-loop experiments with a biofilm

that resulted from 3 days of inoculation (Figure 3.5). The slightly lower removal rate in that experiment is probably the result of a negative effect of the performed experiments, as discussed above. Apparently, at DCA concentrations above 100 µM the complete biofilm participated in the degradation of DCA.

The simulation program BIOSIM was used to model the DCA fluxes into the biofilm that correspond to the measured removal rates shown Figure 3.6. The parameters used in the model were as follows. The K_m value used in BIOSIM was 0.5 µM, which was previously determined as the upper limit value for strain DCA1 (Hage and Hartmans 1999). A diffusion coefficient of DCA in water (D_w) of $1.15*10^{-9}$ m^2 s^{-1} (at 25°C) was calculated with the empirical correlation determined by Nakanishi (Reid et al., 1987). As a biofilm consists of microbial cells, extracellular polymers and water (Sutherland 2001), the effective diffusion coefficient (D_{eff}) in a biofilm is lower than D_w (Stewart 1998; Rittmann and McCarty 1980). We took 80 % of the diffusion coefficient in pure water (Chang and Rittmann 1987; Hinson and Kocher 1996), which is $0.92*10^{-9}$ m^2 s^{-1}, as D_{eff}.

Using these parameters, fluxes into the biofilm at different DCA concentrations were calculated with BIOSIM for different biofilm thicknesses. The V_{max} value used in the model was adjusted at each biofilm thickness to obtain a maximum flux of 2.3 µmol m^{-2} s^{-1} corresponding to a removal rate of 410 g m^{-3} h^{-1}. The best fit (the lowest SSE) was obtained at a biofilm thickness of 48 µm (Figure 3.7A) and a corresponding V_{max} of 48 mmol m^{-3} s^{-1}. To illustrate the effect of biofilm thickness on performance, fluxes that were calculated at two other biofilm thicknesses are also shown in Figure 3.7A.

The maximum specific DCA degradation rate (v_{max}) of cells of strain DCA1 grown on DCA was 49 nmol min^{-1} mg (dry weight) of $cells^{-1}$ (Hage et al., 2001). With the calculated V_{max} of 48 mmol DCA m^{-3} s^{-1}, the biofilm density would be about 58 kg m^{-3}. This value is in good agreement with biofilm densities reported in the literature, which normally range between 10 kg m^{-3} and 130 kg m^{-3} (Characklis and Marshall 1990). Freitas dos Santos and Livingston (1995) reported an average density of 60 kg m^{-3} for a biofilm of strain GJ10.

BIOSIM was used to assess the effect of different parameters on DCA flux into the biofilm. Figure 3.7B shows the sensitivity of the flux towards the K_m value. As can be seen, lowering the K_m value to 0.005 µM, which is 100-fold lower than the value used for strain DCA1, did not have a significant effect on the calculated DCA flux. However, at higher K_m values, such as a value of 571 µM (which was determined for *X. autotrophicus* GJ10; van den Wijngaard et al., 1993), this parameter has a big impact on the DCA flux into the biofilm within this concentration range. Based on the

limited effect of decreasing the K_m value at concentrations below 0.5 µM, some parameter other than the kinetic properties must become limiting at these substrate concentrations. We therefore calculated the influence of the DCA diffusion coefficient in the biofilm (D_{eff}) on the DCA flux into the biofilm (Figure 3.7C).

As can be seen, changing the value of D_{eff} had a significant effect. It was shown earlier that small changes in the estimation of D_{eff} resulted in a significant effect on predicted removal rates (Ergas et al., 1999). In the same figure, the results of calculations using the K_m value of strain GJ10 are shown. For this situation, the effect of changing D_{eff} is minimal at DCA concentrations well below the K_m value of strain GJ10. From these figures, it can be concluded that the decreased activity at DCA concentrations below 80 µM was caused by DCA diffusion limitation rather than the kinetic properties of strain DCA1.

Freitas dos Santos and Livingston (1994) described the efficient removal (>99%) of DCA from wastewater in an extractive membrane bioreactor with a biofilm consisting of *X. autotrophicus* GJ10. Their study, however, was performed at an initial DCA concentration of 16 mM. When strain GJ10 would be used at DCA concentrations that are in the micromolar range, e.g. at the Intervention Value of 4 µM, the removal efficiency would decrease dramatically. Figure 3.7B indicates that, at a DCA concentration of 4 µM, the DCA flux into a strain GJ10 biofilm is less than 5% of the flux generated into a biofilm consisting of strain DCA1. However, it must be noted that, in these calculations, the assumption was made that a biofilm of strain GJ10 would, besides the different K_m value, have the same characteristics (V_{max}, D_{eff}) as a biofilm of strain DCA1. It would be interesting to compare the biofilm formation characteristics of these two strains and to perform competition studies, as the coexistence of strains DCA1 and GJ10 in a biofilm was suggested in earlier studies (Stucki and Thüer 1995; Hage and Hartmans 1999).

DCA removal from anoxic water

For practical reasons, the experiments described above were performed under oxic conditions. The goal of this research, however, was to remove DCA by aerobic degradation while keeping the contaminated groundwater (largely) anoxic. To put the system to the test, we wanted to demonstrate that DCA present in anoxic water could be degraded aerobically by supplying oxygen solely from the gas phase. After a biofilm was grown as described above, DCA removal was first determined by supplying oxic MM_{low} containing DCA to the MABR. Subsequently, the oxic water was replaced by anoxic MM_{low} containing the same concentration of DCA.

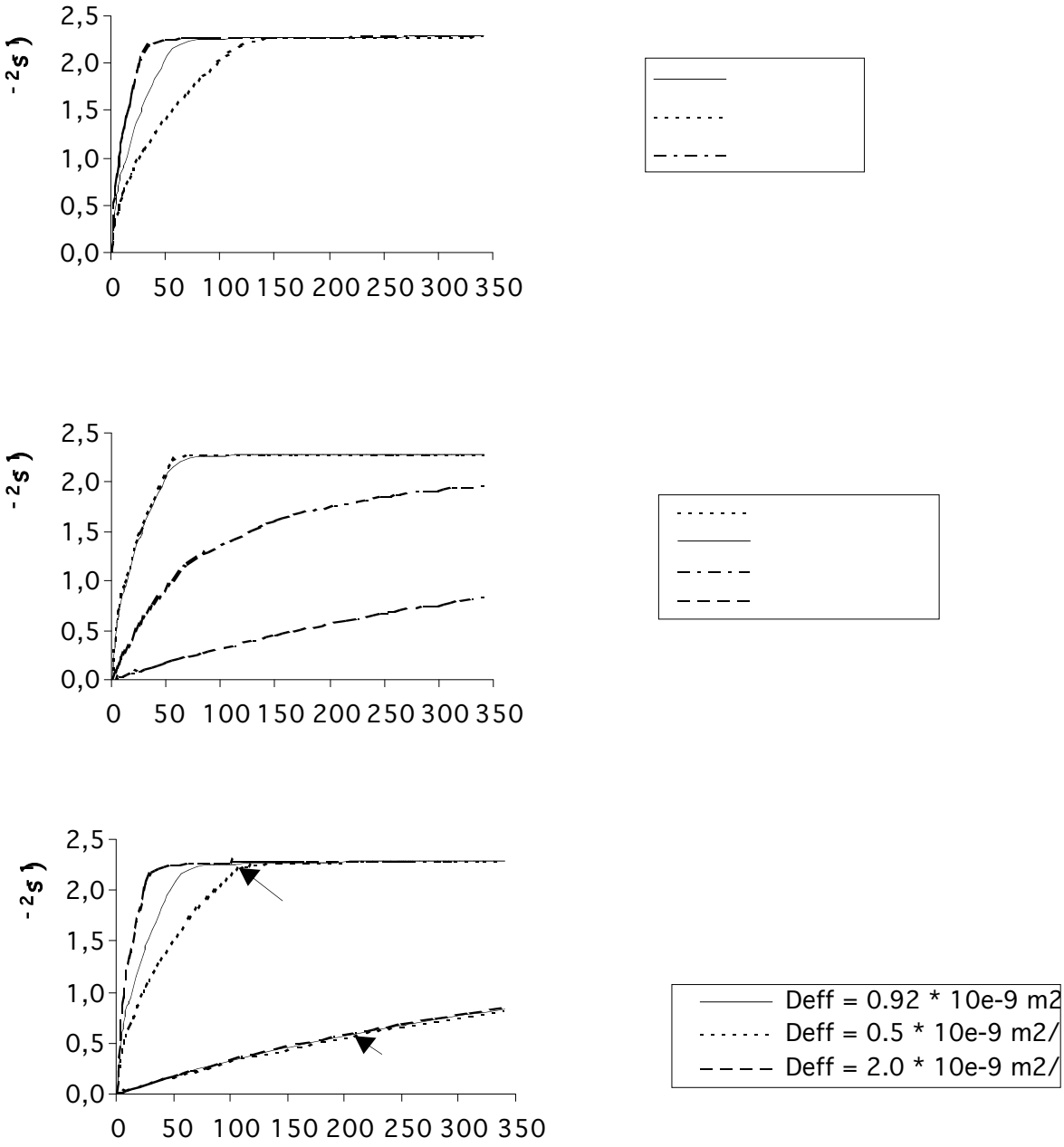

Figure 3.7A-C Simulations with BIOSIM. The three simulations show the effect of changing the biofilm thickness (*L*, Figure 3.7A), the K_m value (Figure 3.7B) and the diffusion coefficient of DCA in the biofilm (D_{eff}, Figure 3.7C) on the DCA fluxes into a biofilm. The effect of changing the diffusion coefficient was calculated for two different K_m values (0.5, 571 µM).

As can be seen in Figure 3.8, degradation of DCA also occurred under these conditions. As a control, the air supply was then switched off and nitrogen was blown through the gas compartment instead. Since oxygen is required for the first step in the degradation of DCA by strain DCA1 (Hage and Hartmans 1999), no DCA removal was expected in the MABR under anoxic conditions. However, a low chloride formation rate could still be detected under these conditions, indicating that trace amounts of oxygen could enter the MABR. The last column in Figure 3.8 shows that DCA removal returned to the original level when air was supplied again, demonstrating that the biofilm was still active. The average DCA concentration under both oxic and anoxic conditions in these experiments was 380 µM, which is well above the concentration at which diffusion limitation occurred (Figure 3.7C). The results presented here show that anoxic water can be treated aerobically in the MABR by supplying oxygen solely from the gas phase.

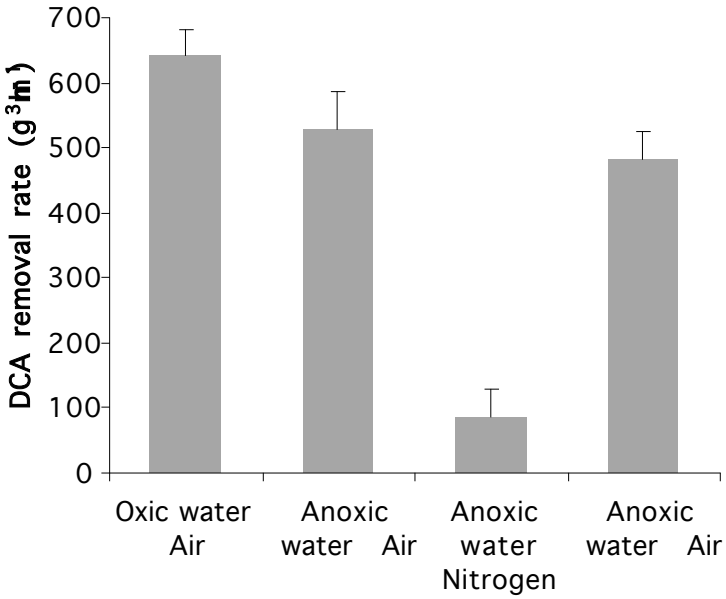

Figure 3.8 DCA removal from water in the MABR with *Pseudomonas* sp. strain DCA1. The first column represents the removal of DCA from oxic groundwater, while air was blown through the gas compartment of the MABR. The oxic water was then replaced by nitrogen-flushed water (second column). Subsequently, the air phase was replaced by nitrogen (third column). Finally, nitrogen was replaced by air again as a control to confirm that the biofilm was still active (last column). The DCA removal rates are average values of five measurements. Error bars show the standard error between these measurements.

CONCLUSIONS

The DCA-degrading bacterial strain *Pseudomonas* sp. strain DCA1 was capable of forming a stable (mono-culture) biofilm in a MABR with DCA as the sole substrate. Inoculation of the MABR with cells of strain DCA1 was important during the first stage of the biofilm formation. But, as soon as a biofilm was formed, prolonged inoculation had no significant beneficial effect. This suggests that biofilm growth was largely due to already attached cells, growing on DCA supplied via the gas phase.

The maximal DCA removal rate was reached at a DCA concentration in the liquid phase in the MABR of approximately 80 µM. Simulations with the program BIOSIM showed that, at lower concentrations, biofilm activity was limited by the DCA diffusion rate in the biofilm, rather than by the substrate affinity of strain DCA1.

The MABR allowed the aerobic degradation of DCA present in anoxic water by supplying oxygen solely from the gas phase. In this way, groundwater can be kept (largely) anoxic, which is important since aeration of anoxic groundwater can result in undesired coagulation of iron oxides. For successful operation of the MABR, the amount of oxygen added via the gas phase must be regulated. On the one hand, enough oxygen must be provided to avoid oxygen limitation in the biofilm, while on the other hand, supplying too much oxygen results in oxygenation of the groundwater. A program like BIOSIM can be a useful tool to calculate the optimal dosage of oxygen. Further research is required to determine the effect of coagulation of iron oxides within the biofilm. Iron present in the groundwater might enter the biofilm and then come into contact with oxygen. The long-term operation of the system should also be evaluated, since biological systems are often faced with clogging problems. Since DCA can serve as sole carbon and energy source for strain DCA1 and accumulation of toxic intermediates is not expected (Hage and Hartmans 1999), the biodegradation of DCA by strain DCA1 is an intrinsically stable process.

Another possible application of the MABR with strain DCA1 is the co-metabolic degradation of chlorinated hydrocarbons (Hage et al., 2001). The required co-substrate could then be supplied together with oxygen via the gas phase.

4

Co-metabolic degradation of chlorinated hydrocarbons by *Pseudomonas* sp. strain DCA1

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Applied Microbiology and Biotechnology (2001) 57:548-554

ABSTRACT

Pseudomonas sp. strain DCA1, which is capable of utilizing 1,2-dichloroethane (DCA) as sole carbon and energy source, was used to oxidize chlorinated methanes, ethanes, propanes and ethenes. Chloroacetic acid, an intermediate in the DCA degradation pathway of strain DCA1, was used as a co-substrate since it was readily oxidized by DCA-grown cells of strain DCA1 and did not compete for the monooxygenase. All of the tested compounds except tetrachloroethene (PCE) were oxidized by cells expressing DCA monooxygenase. Strain DCA1 could not utilize any of these compounds as a growth substrate. Co-metabolic oxidation during growth on DCA was studied with 1,2-dichloropropane. Although growth on this mixture occurred, 1,2-dichloropropane strongly inhibited growth of strain DCA1. This inhibition was not caused by competition for the monooxygenase. It was shown that the oxidation of 1,2-dichloropropane resulted in the accumulation of 2,3-dichloro-1-propanol and 2-chloroethanol.

INTRODUCTION

Large quantities of chlorinated aliphatic hydrocarbons have been released to soil and groundwater, by spills, from wastewater, or because of improper disposal. Contamination of groundwater is of serious concern, since in the Netherlands, as well as in the USA, more than 50% of the drinking water is obtained from groundwater (Centraal Bureau voor de Statistiek, 2000; Solley et al., 1998). An extensive survey of drinking water supplies from groundwater sources in the USA revealed a widespread occurrence of not less than 19 different chlorinated aliphatic hydrocarbons (Westrick et al., 1984).

A groundwater pollutant of particular interest is 1,2-dichloropropane (DCP). DCP has been released into the soil and groundwater in agricultural areas, due to the application of the soil fumigant 'Mix D/D' as a nematocide before planting. The major and active ingredient of this mixture is 1,3-dichloropropene (50-80 % of the total). However, 20 to 40 % of the mixture consists of 1,2-dichloropropane, which has no known nematocidal activity (Krijgsheld and van der Gen, 1986; World Health Organization, 1993). DCP is recalcitrant to microbial degradation, and to date, no aerobic utilization of DCP as a carbon source has been reported.

In our research into the degradation of 1,2-dichloroethane (DCA), we isolated the bacterial strain *Pseudomonas* sp. strain DCA1, which is capable of growing on DCA as sole carbon and energy source. The first step in DCA metabolism in this strain is a monooxygenase-mediated oxidation (Hage and Hartmans, 1999). Monooxygenases generally have a broad substrate spectrum (Hartmans et al., 1989), and therefore strain DCA1 may also be capable of (co-metabolic) degradation of other chlorinated hydrocarbons. Co-metabolic degradation of chlorinated alkenes and alkanes has been described in the literature. A strain studied in detail is the methane oxidizer *Methylosinus trichosporium* OB3b, which expresses a monooxygenase capable of degrading trichloroethene (TCE) at very high rates (Fox et al., 1990; Oldenhuis et al., 1989; Sullivan et al., 1998). Besides methane monooxygenases, there are also reports of other monooxygenases capable of oxidizing TCE, such as ammonia monooxygenase (Arciero et al., 1989), propane-monooxygenase (Wackett et al., 1989), and toluene mono- and dioxygenase (Shields et al., 1989; Wackett and Gibson, 1988).

Although co-metabolic oxidation processes offer possibilities for (aerobic) degradation of a wide variety of chlorinated compounds (Semprini, 1997), co-metabolic processes are fortuitous reactions that have no beneficial effects for the cell (Horvath, 1972; Perry, 1979). In fact, the ability to co-oxidize (chlorinated)

compounds can be a disadvantage with respect to the competitive capacity of bacteria in mixed cultures (Mars et al., 1998). The co-metabolic oxidations require reducing equivalents, thereby reducing the amount of energy available for growth. Furthermore, the formation of possibly toxic intermediates can be detrimental for growth.

In this study, we explored the possibilities of *Pseudomonas* sp. strain DCA1 in the degradation of various chlorinated alkanes and alkenes.

MATERIALS AND METHODS

Continuous culture of *Pseudomonas* sp. strain DCA1

Pseudomonas sp. strain DCA1 was previously isolated in our lab from a DCA-degrading biofilm (Hage and Hartmans, 1999) and is deposited in the Industrial Microbiology Culture Collection of Wageningen University (CIMW no. 412B). Strain DCA1 was grown continuously in a 2-liter fermenter containing 1 liter of mineral salts medium (Hartmans et al., 1992). The pH was maintained at 7.0 by the addition of sterile 2N NaOH. The dilution rate was 0.05 h^{-1}, and the temperature was 25°C. Air was bubbled through a column containing pure DCA at a rate of 5 ml min^{-1}. The bubble column was kept at 25°C. This air stream was diluted by mixing with a second stream of air (2000 ml min^{-1}), resulting in an in-going DCA concentration in the gas phase of 1 mg l^{-1}, which was bubbled through the liquid phase. The biomass density was monitored by determining the optical density at 660 nm (OD_{660}). At steady-state conditions, the OD_{660} was approximately 0.5. For all batch experiments with strain DCA1, cells harvested from the continuous culture were used as the inoculum.

Analytical methods

Concentrations of chlorinated hydrocarbons were determined by analyzing 50- or 100-µl headspace samples on a Chrompack CP9000 gas chromatograph, equipped with a CP-Sil 8CB column (Chrompack B.V., Middelburg, The Netherlands). The oven temperature was kept at 100°C. 1,2-Epoxypropane concentrations were measured on the same column, at an oven temperature of 50 °C. Concentrations of CO_2 were measured by injecting 100-µl gas phase samples into a Hewlett Packard 6890 gas chromatograph, containing a Chrompack Poraplot Q column.

Detection of 2,3-dichloro-1-propanol was carried out on a Hewlett Packard 6890 gas chromatograph, equipped with a CP Wax 52 CB column (Chrompack). The oven temperature was kept at 45°C for 3 min, followed by an increase to 200°C at 10°C min^{-1}. Culture samples were filtered by using 0.2-µm-pore-size disposable filters

(Schleicher & Schuell, Dassel, Germany). Subsequently, 4.5-ml aliquots were extracted with 1.5 ml diethyl ether, containing 0.1 mM 1-butanol as the internal standard, and 1 µl of the ether phase was injected in the gas chromatograph.

For GC-MS analysis the Wax column was transferred to a Hewlett Packard 6890 gas chromatograph connected to a HP 5973 mass-selective detector. The carrier gas was helium and the temperature programme described above was used. Samples for GC-MS analysis were prepared as described above, followed by concentration of the ether phase and splitless injection in the GC-MS. The retention times of the products formed in the culture samples were compared with those of commercially purchased 2,3-dichloro-1-propanol (Fluka, Buchs, Switzerland) and 2-chloroethanol and were confirmed with GC-MS.

Determination of co-metabolic oxidation rates

Co-metabolic oxidation rates were determined by measuring the disappearance of chlorinated hydrocarbons by headspace analysis on a GC as described above. Oxidation rates were determined using washed whole cells harvested from the fermenter. Cells from the fermenter were centrifuged for 10 min at 16,000 x *g* and then washed in an equal volume of mineral salts medium (Hartmans et al., 1992). The pellet was resuspended in a 100 times smaller volume of mineral salts medium and was stored on ice. All experiments were performed at 25 °C in 25-ml glass vials (Supelco, Zwijndrecht, The Netherlands) closed with Teflon valves (Mininert; Phase Separations, Waddinxveen, The Netherlands). The 2-ml reaction mixture contained mineral salts medium and chloroacetic acid (2 mM) as the co-substrate. Chlorinated hydrocarbons were added from a stock solution to a final concentration of 0.5 mM in the liquid phase, calculated with partition coefficients from the literature (Amoore and Hautala, 1983; Leighton and Calo, 1981; Nirmalakhandan and Speece, 1988). Since the DCA monooxygenase is a rather unstable enzyme, a fresh cell suspension was prepared for the determination of the oxidation rate of each chlorinated hydrocarbon. Oxidation rates were determined in triplicate. Each assay was performed within 20 minutes. To correct for variations in the monooxygenase activity between the different batches of cell suspensions, the DCA degradation rate of each cell suspension was also determined (in the presence of 2 mM chloroacetic acid). Oxidation rates of the different chlorinated hydrocarbons were related to the average DCA degradation rate of all cell suspensions, which was 49±9 nmol min^{-1} mg (dry weight) $cells^{-1}$. Cell dry weights were determined by weighing dried (24 h, 105 °C) cell suspensions. These dry weights were corrected for the dry weight of the mineral salts medium in which the cells were suspended.

1,2-Epoxypropane production rates were determined in 35-ml serum bottles closed with rubber septa. Cell suspensions were prepared as described above, and were incubated with 1 ml propene in a total volume of 2 ml mineral salts medium, resulting in a liquid phase concentration of 131 µM (Mackay and Shiu, 1981).

Growth experiments

Utilization of different chlorinated compounds by strain DCA1 was measured in 250-ml serum bottles containing 50 ml of mineral salts medium (Hartmans et al., 1992); 50, 100, and 200 µmol of each chlorinated compound were tested. The CO_2 production and turbidity increase after 10 days of incubation at 25°C were used as indicators of growth.

Growth experiments on mixtures of substrates were carried out in 250-ml Boston bottles closed with Teflon valves (Mininert; Phase Separations, Waddinxveen, The Netherlands). In these experiments, the concentration of phosphate buffer was 2.5-times higher than in the standard mineral salts medium. Initial concentrations of DCA and DCP were 4.4 mM and 146 µM, respectively.

Effect of DCP oxidation

The effect of the accumulation of possibly toxic intermediates in the medium due to the conversion of DCP was tested by reinoculating filter-sterilized medium in which strain DCA1 had grown on a mixture of DCA and DCP. This incubation on DCA and DCP was performed as described above. As soon as all DCA was consumed, the culture fluid was filtered to remove the cells, and the pH was readjusted to 7.0 by adding 10N NaOH. In order to remove all DCP from the liquid, the medium was flushed with air. Subsequently, 35 ml of this liquid was filter-sterilized by using 0.2-µm-pore-size disposable filters (Schleicher & Schuell, Dassel, Germany) and transferred into a sterile Boston bottle followed by reinoculation with strain DCA1. DCA was added as the carbon and energy source (5.6 mM). Headspace analysis on the gas chromatograph confirmed that all DCP had been removed from the medium. The control incubation consisted of reinoculated medium in which strain DCA1 had grown on DCA only. The medium was treated similarly as described above.

The effect of 2,3-dichloro-1-propanol on growth of strain DCA1 was tested in Boston bottles containing 35 ml mineral salts medium and 5.6 mM DCA. In these experiments the concentration of phosphate buffer was 2.5-times higher than in the standard mineral salts medium. 2,3-Dichloro-1-propanol was added as a filter sterilized solution to concentrations of 0, 5, 9, 19 and 45 µM, respectively. The production of CO_2 was measured to determine the effect on the growth of strain DCA1.

RESULTS

Effect of chloroacetic acid on monooxygenase activity

Co-metabolic oxidations require reduction equivalents. DCA is not suitable as a co-substrate since it would compete for the monooxygenase. Therefore, the potential of different compounds to serve as a co-substrate in co-metabolic oxidations was tested by measuring initial CO_2 production after addition of the substrate to DCA-grown cells harvested from a continuous culture. Tested substrates included glucose (10 mM), succinate (5 mM), sodium acetate (5 mM), ethanol (2 mM), chloroethanol (2 mM) and chloroacetic acid (2 mM). Based on the observed CO_2 production, only chloroacetic acid was readily oxidized (results not shown).

To demonstrate the effect of chloroacetic acid on co-metabolic oxidations by DCA-grown cells, the conversion of propene to 1,2-epoxypropane was used as a model reaction. As described earlier (Hage and Hartmans, 1999), the monooxygenase of DCA-grown cells of strain DCA1 oxidizes propene to 1,2-epoxypropane, which is not further degraded. The effect of different chloroacetic acid concentrations (1, 2 and 4 mM) on 1,2-epoxypropane production rates was determined (Figure 4.1).

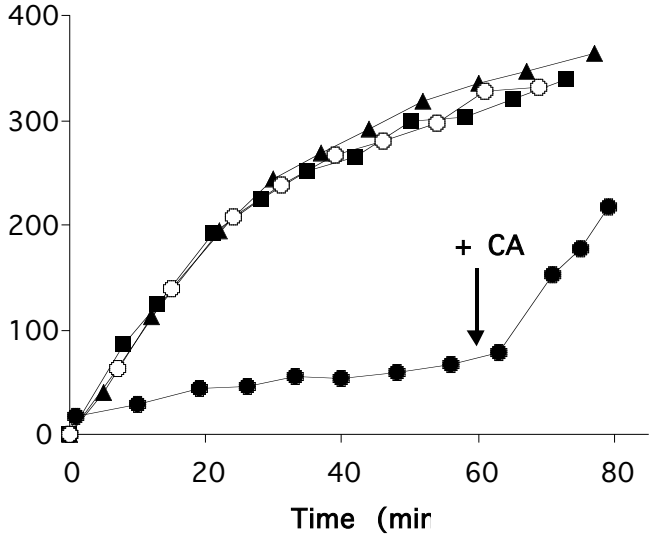

Figure 4.1 Effect of chloroacetic acid (CA) on the conversion of propene to 1,2-epoxypropane by DCA-grown cells of *Pseudomonas* sp. strain DCA1. Symbols: , 0 mM CA; , 1 mM CA; , 2 mM CA; , 4 mM CA. Arrow indicates the addition of 2 mM CA.

The initial specific oxidation rates in the presence of chloroacetic acid were 42 nmol min^{-1} mg (dry weight) $cells^{-1}$. In the absence of the co-substrate, the initial rate was only 4 nmol min^{-1} mg (dry weight) $cells^{-1}$. Addition of 2 mM chloroacetic acid after 1 h resulted in an increase of the 1,2-epoxypropane formation to 42 nmol min^{-1} mg (dry

weight) cells^{-1}, demonstrating that the monooxygenase was relatively stable under these conditions.

Competitive inhibition of propene oxidation by chloroacetic acid (0-4 mM) was not observed, even when very low propene concentrations (1 µM in the liquid phase) were applied (results not shown). No inhibition of the DCA degradation rate due to the presence of 2 mM chloroacetic acid could be measured either.

Degradation of chlorinated hydrocarbons

Co-oxidation rates of a variety of chlorinated alkanes and alkenes were determined with DCA-grown cells of strain DCA1 in the presence of 2 mM chloroacetic acid as energy source (Table 4.1).

Table 4.1 Relative co-metabolic oxidation rates of chlorinated hydrocarbons by DCA-grown cells of *Pseudomonas* sp. strain DCA1.

Chlorinated hydrocarbon[a]	Relative oxidation rates[b] (%)
1,2-Dichloroethane	100[c]
Dichloromethane	33
1,1-Dichloroethane	12
1,1,1-Trichloroethane	4
1-Chloropropane	12
2-Chloropropane	6
1,2-Dichloropropane	14
1,1-Dichloroethene	11
cis-Dichloroethene	21
trans-Dichloroethene	14
Trichloroethene	11
Tetrachloroethene	0

[a] Concentration in the liquid phase was 0.5 mM.

[b] Relative oxidation rates were determined in washed whole cells in the presence of 2 mM chloroacetic acid and were corrected for variations in activity between different batches of cell suspensions by determining the DCA oxidation rates.

[c] 100 % corresponds to the average DCA oxidation rate of all batches of cell suspensions which was 49 9 nmol per minute per milligram (dry weight) of cells.

Since a wide variety of chlorinated compounds were oxidized, the ability of *Pseudomonas* sp. strain DCA1 to also utilize these chlorinated hydrocarbons as sole carbon and energy source for growth was tested (Table 4.2). However, DCA was the only chlorinated alkane that was utilized for growth by strain DCA1. Strain DCA1 did

grow on all mono- and dichlorinated acids analyzed. No growth was observed on trichloroacetic acid at any of the concentrations tested.

Table 4.2 Growth of *Pseudomonas* sp. strain DCA1 on chlorinated compounds.

Substrate[a]	Growth[b]
Chlorinated alkanes	
Dichloromethane	-
Chloroform	-
Tetrachloromethane	-
1,1-Dichloroethane	-
1,2-Dichloroethane	+
1,1,1-Trichloroethane	-
1-Chloropropane	-
2-Chloropropane	-
1,2-Dichloropropane	-
1,3-Dichloropropane	-
1,2,3-Trichloropropane	-
2-Chlorobutane	-
Chlorinated alcohols	
2-Chloroethanol	+
1-Chloro-2-propanol	-
3-Chloro-1-propanol	-
(R)-(-)-2-Chloro-1-propanol	+
(S)-(+)-2-Chloro-1-propanol	+
Chlorinated acids	
Chloroacetic acid	+
Dichloroacetic acid	+
Trichloroacetic acid	-
2-Chloropropionic acid	+
3-Chloropropionic acid	+

[a] Substrates were supplied at 50, 100 and 250 µmol in 250-ml serum bottles containing 50 ml mineral salts medium.

[b] Growth was assessed by measuring CO_2 production after 10 days of incubation.

Degradation of 1,2-dichloropropane

One of the tested compounds of special interest is 1,2-dichloropropane (DCP). Of all chlorinated hydrocarbons tested, DCP has the most structural resemblance to DCA,

with two chlorine atoms present on adjacent carbon atoms. DCP was oxidized by resting cells of strain DCA1 (Table 4.1); however, growth on this compound was not observed (Table 4.2). Possibly, the oxidation of DCP does not yield enough energy to support growth. Therefore it was determined whether strain DCA1 could grow on a mixture of ethanol (7 mM) and DCP (146 µM). Strain DCA1 is able to utilize ethanol as carbon and energy source (Hage and Hartmans, 1999). Although strain DCA1 did grow on this mixture, no significant degradation of DCP occurred (results not shown). As a control, growth of strain DCA1 on mixtures of ethanol (7 mM) and different concentrations of DCA (0.1, 0.6 and 1.0 mM) was tested. In these incubations, DCA was degraded completely (results not shown).

To induce the DCA monooxygenase, growth on a mixture of DCA (4.4 mM) and 146 µM DCP was monitored, with DCA serving as both the energy source and the inducer of the monooxygenase. The DCA and DCP depletion curves are shown in Figure 4.2.

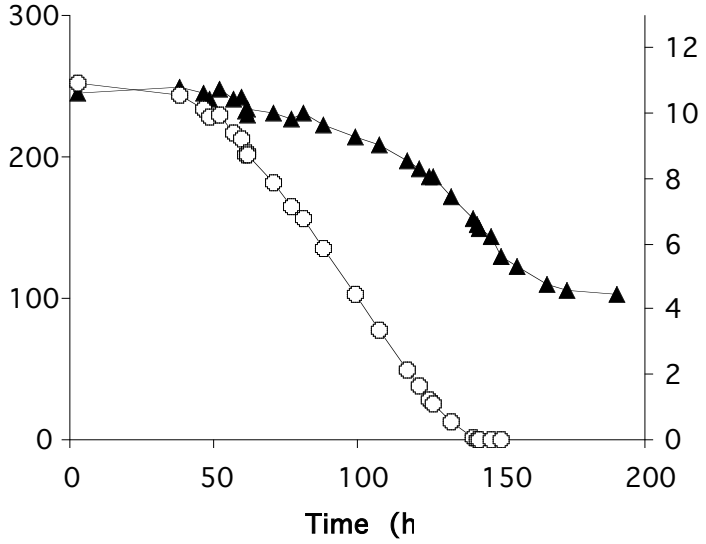

Figure 4.2 Degradation of a mixture of 1,2-dichloroethane and 1,2-dichloropropane by *Pseudomonas* sp. strain DCA1. Symbols: , 1,2-dichloropropane (DCP); , 1,2-dichloroethane (DCA). Initial amounts correspond to a concentration of 146 µM DCP and 4.2 mM DCA in the liquid phase.

It can be seen that simultaneous degradation of DCA and DCP occurred, although DCP degradation ceased soon after all the DCA had been consumed. Moreover, when growth of strain DCA1 in the presence of DCP was compared to a control incubation without DCP (Figure 4.3), it was obvious that the presence of DCP strongly inhibited the growth of strain DCA1 on DCA, quantified as CO_2 formation. A possible effect of competition between DCA and DCP for the monooxygenase was

determined by measuring the initial oxidation rates of 1 mM DCA by DCA-grown cells in the presence of different concentrations DCP. However, no effect of 50 or 100 µM DCP could be measured either in the presence or absence of 2 mM chloroacetic acid (results not shown).

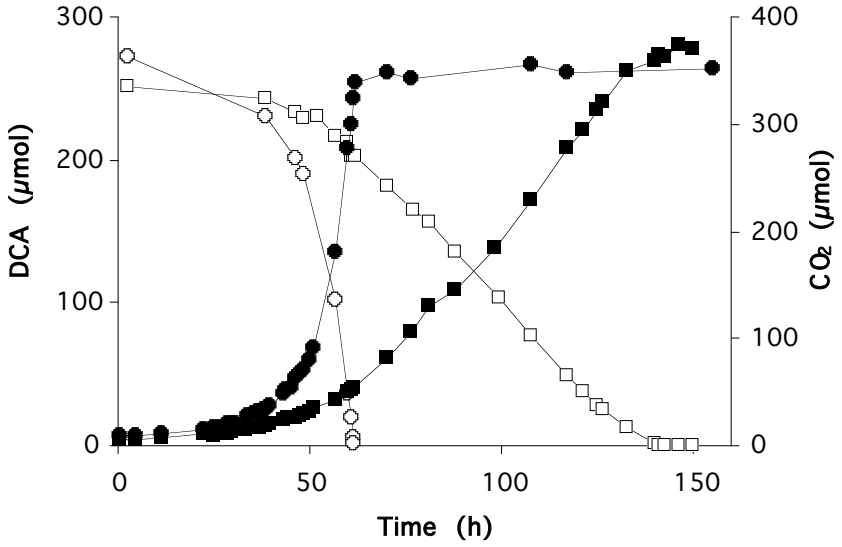

Figure 4.3 Effect of the presence of 1,2-dichloropropane (DCP) on CO_2 production (closed symbols) and DCA depletion (open symbols) by *Pseudomonas* sp. strain DCA1. Symbols: (,_), 146 µM DCP present; (,), no DCP present. The initial DCA concentrations in the liquid phase were 4.2 and 4.5 mM, respectively.

Since inhibition of the growth of strain DCA1 in the presence of DCP could not be explained by competitive inhibition of the monooxygenase, toxicity of DCP oxidation products was examined. First, it was tested whether toxic intermediates accumulated in the medium. A culture of strain DCA1 grown on a mixture of DCA and DCP was filter-sterilized after all DCA had been consumed. After reinoculation, growth of strain DCA1 was followed by measuring DCA depletion and CO_2 formation. As a control, a culture grown without DCP was treated in the same manner.

As can be seen in Figure 4.4, the presence of oxidation products in the medium slightly inhibited growth of strain DCA1 on DCA. Analysis of culture fluid on a gas chromatograph after growth of strain DCA1 on a mixture of DCA and DCP revealed the formation of 2,3-dichloro-1-propanol (2,3-DCP-1-ol). The conversion of 85 µM DCP resulted in a concentration of 15 µM 2,3-DCP-1-ol. However, subsequent growth experiments with different concentrations of 2,3-DCP-1-ol showed that the presence of this compound up to a concentration of 45 µM did not have any effect on the growth of strain DCA1 on DCA (results not shown). To further identify the reaction products resulting from DCP oxidation, the culture fluid of a batch culture grown on

DCA (4.4 mM) and DCP (146 µM) was analyzed after 75 % of the DCA was consumed. GC-MS analysis revealed the presence of 2,3-DCP-1-ol and 2-chloroethanol. These intermediates were not detected in the control incubation, which was grown on DCA only.

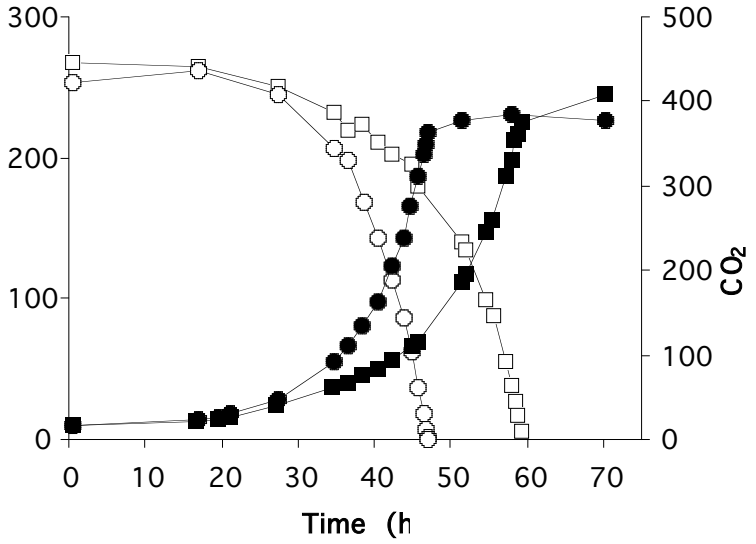

Figure 4.4 Effect of the presence of 1,2-dichloropropane (DCP) oxidation products in medium on CO_2 production (closed symbols) and DCA depletion (open symbols) by *Pseudomonas* sp. strain DCA1. Symbols: (,_), DCP oxidation products present in medium; (,), control incubation. The initial DCA concentrations in the liquid phase were 5.7 and 5.4 mM, respectively.

DISCUSSION

For co-metabolic oxidation of chlorinated hydrocarbons, reduction equivalents are required. Therefore, a suitable co-substrate has to be present for cells carrying out co-metabolic oxidation processes. We showed earlier that the DCA monooxygenase is induced during growth on DCA as the carbon source (Hage and Hartmans, 1999). Therefore, DCA was used as the growth substrate for strain DCA1. However, using DCA as a co-substrate might slow down co-metabolic oxidation rates, since competition for the monooxygenase is likely to occur, especially when the very high affinity of this enzyme for DCA is considered. Competitive inhibition was clearly demonstrated in earlier studies in which DCA and propene were both present (Hage and Hartmans, 1999). Therefore, we searched for a co-substrate that did not compete for the monooxygenase. Chloroacetic acid, an intermediate in the degradation pathway of DCA in strain DCA1 (Figure 4.5), was readily oxidized by

DCA-grown cells and did not competitively inhibit the DCA monooxygenase. Chloroacetic acid is thus a suitable energy source to supply sufficient reducing equivalents to study co-metabolic oxidations by DCA-grown cells of strain DCA1. As shown in Figure 4.1, the presence of chloroacetic acid significantly enhanced propene oxidation rates. It must be noted that after approximately 20 minutes, the 1,2-epoxypropane-production rates decreased. Since all three incubations in the presence of chloroacetic acid showed exactly the same pattern, this decrease was not an effect of chloroacetic acid depletion. Probably, 1,2-epoxypropane accumulation inhibited the monooxygenase or had an effect on chloroacetic acid metabolism.

From the results presented in Table 4.1, it is evident that DCA-grown cells of strain DCA1 can oxidize a broad range of compounds. Tetrachloroethene (PER) was the only compound tested that was not oxidized. This is not unexpected as aerobic degradation of PER was only recently reported in cells expressing toluene-o-xylene monooxygenase activity (Ryoo et al., 2000). By far the highest activity was measured for the degradation of the growth substrate DCA, followed by the oxidation rate of dichloromethane, which was three-fold lower. An important aspect of the co-oxidation of chlorinated compounds is the formation of (toxic) oxidation products. For example, TCE can be converted to the very toxic and reactive TCE-epoxide (Alvarez-Cohen and McCarty, 1991; Hyman et al., 1995; Oldenhuis et al., 1991), and other chlorinated ethenes can also be transformed into reactive epoxides (Dolan and McCarty, 1995; van Hylckama Vlieg, 1999). Rasche et al. (1991) discussed the formation of acyl chlorides resulting from the hydroxylation of a dichlorinated carbon followed by elimination of one of the chlorines. Acyl chlorides can act as protein modifying agents. The degradation of DCA by the monooxygenase of strain DCA1 is also based on a hydroxylation reaction followed by elimination of chlorine (Hage and Hartmans, 1999). For the chlorinated hydrocarbons listed in Table 4.1, formation of acyl chlorides can be expected when 1,1-dichloroethane and dichloromethane are oxidized. However, during the short-term assays used to determine oxidation rates of the different chlorinated hydrocarbons, we did not observe any decline in degradation rates. Apparently, the amounts of potentially toxic intermediates produced were too low to significantly inactivate the co-metabolic oxidations. The constant oxidation rates that we observed showed that the concentrations of 0.5 mM were well above the K_m values of the different substrates and also demonstrated that there was sufficient reducing power available due to the oxidation of chloroacetic acid. Hence, maximum oxidation rates were measured.

During growth on a mixture of ethanol and DCP, no DCP degradation was observed, whereas during growth on a mixture of ethanol and DCA, all DCA was degraded.

This demonstrates that the DCA monooxygenase can be induced during growth on ethanol, as long as an appropriate inducer is present. Apparently, DCP does not induce the DCA monooxygenase.

It was shown that DCP did not competitively inhibit the monooxygenase and hence DCA oxidation. Therefore, the strong inhibition of growth of strain DCA1 on DCA in the presence of DCP (Figure 4.3) could indicate that the oxidation of DCP results in the formation of one or more toxic reaction products. The results presented in Figure 4.4 suggest an effect of the accumulation of one or more these products: however, the growth inhibition was much less than observed in Figure 4.3. This suggests that a toxic intermediate was formed transiently, or that the inhibiting intermediate was not very stable. Bosma and Janssen (1998) reported the formation of 2,3-DCP-1-ol resulting from DCP oxidation by the methane monooxygenase of resting cells of *Methylosinus trichosporium* OB3b. This compound was also formed in the oxidation of DCP by strain DCA1. However, based on the results of the growth experiments with *Pseudomonas* sp. strain DCA1 in the presence of 2,3-DCP-1-ol, this compound was not directly responsible for the observed inhibition of the growth.

Analogous to the DCA degradation pathway (Hage and Hartmans, 1999), oxidation of DCP by the DCA monooxygenase was expected to yield 2-chloropropionaldehyde as an intermediate (Figure 4.5). Unfortunately, we could not test the direct effect of 2-chloropropionaldehyde, since it is not commercially available. Besides 2,3-DCP-1-ol, also 2-chloroethanol was detected with GC-MS during growth of strain DCA1 on a mixture of DCA and DCP. This could be an indirect result of 2-chloropropionaldehyde formation. 2-Chloropropionaldehyde probably interferes with the oxidation of chloroacetaldehyde, which is an intermediate in DCA metabolism. If chloroacetaldehyde is not oxidized directly to chloroacetic acid, it will be reduced to 2-chloroethanol. A similar situation was observed in the degradation of styrene in which phenylacetaldehyde, the intermediate of styrene metabolism, was oxidized to phenylacetic acid but also reduced to 2-phenylethanol (Hartmans, 1995; Itoh et al., 1996). Probably, in our experiments 2-chloropropionaldehyde formation was sufficient to affect chloroacetaldehyde metabolism, but the concentration was too low to result in the accumulation of detectable amounts of 2-chloro-1-propanol.

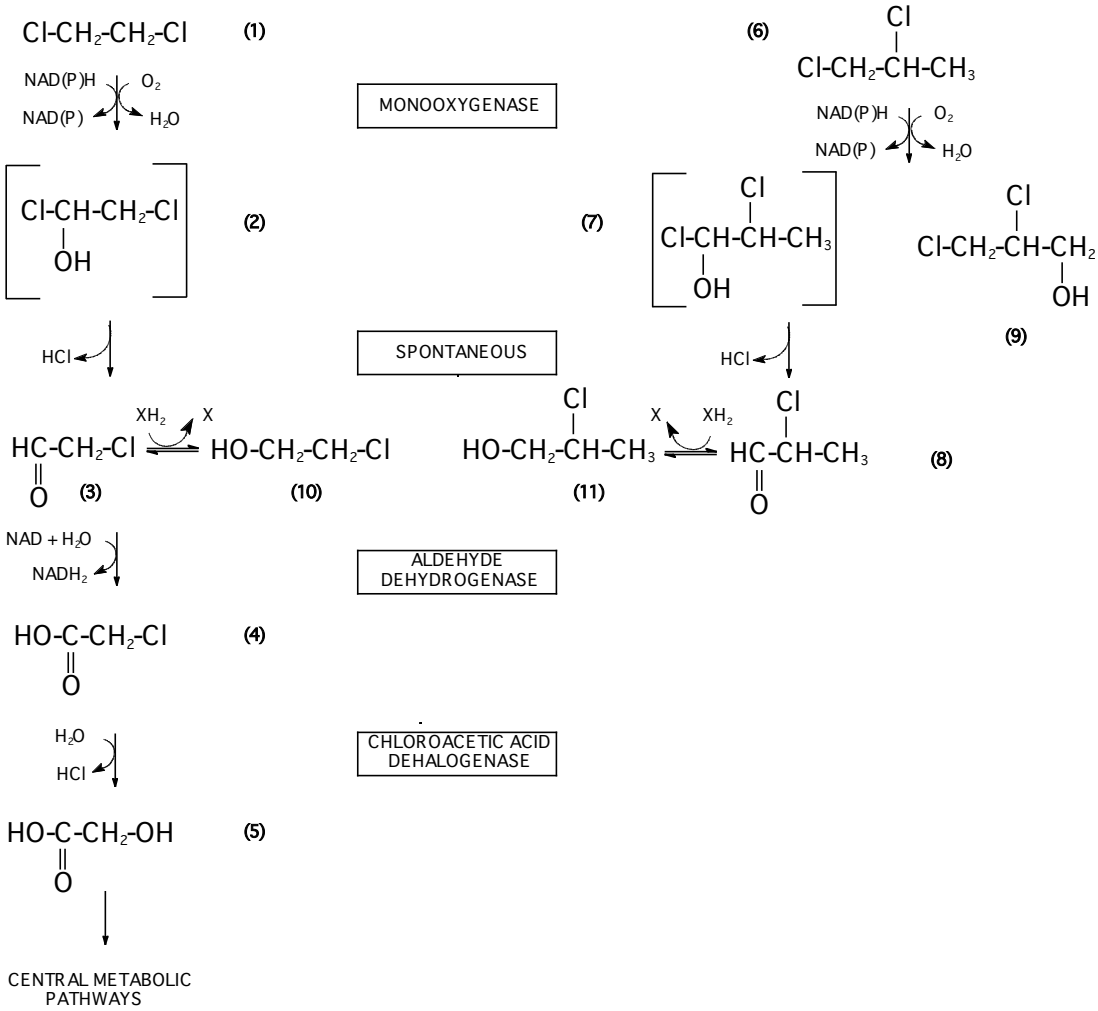

Figure 4.5 Degradation pathway of DCA by *Pseudomonas* sp. strain DCA1 as previously elucidated (Hage and Hartmans, 1999). DCA (1) is oxidized by a monooxygenase to the unstable 1,2-dichloroethanol (2), which is converted to chloroacetaldehyde (3), chloroacetic acid (4) and glycolic acid (5), respectively. On the right hand side, a tentative DCP oxidation pathway is shown. DCP (6) is oxidized by the monooxygenase to 1,2-dichloro-1-propanol (7), followed by a spontaneous conversion to 2-chloropropionaldehyde (8). Oxidation of DCP also results in the production of 2,3-dichloro-1-propanol (9). Inhibition of the aldehyde dehydrogenase may result in formation of 2-chloroethanol (10) from chloroacetaldehyde and 2-chloro-1-propanol (11) from 2-chloropropionaldehyde.

Interestingly, strain DCA1 is capable of growing on 2-chloro-1-propanol (Table 4.2), which is presumably metabolized via 2-chloropropionaldehyde and 2-chloropropionic acid. Therefore, strain DCA1 is capable of synthesizing all the catalytic activities required for complete DCP metabolism. Nevertheless, we have not been able to identify the conditions that result in simultaneous expression of these enzymes and hence growth of strain DCA1 with DCP as sole carbon source.

5

Bioaugmentation: getting the right bugs on the right spot

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To be submitted

ABSTRACT

Bioaugmentation, the injection of non-indigenous microorganisms into the subsurface, can be applied for in situ bioremediation of contaminated sites in case the indigenous microorganisms are:
- degrading the contaminant at rates that are too low;
- inhibited by the presence of multiple contaminants;
- killed as a result of drastic (abiotic) remediation techniques;
- not capable to carry out the desired reactions.
The latter case seems to be the most promising field for application of bioaugmentation. A good example is the incomplete reduction of tetrachloroethene, which often stalls at *cis*-1,2-dichloroethene. Bioaugmentation with members of the *Dehalococcoides* group has proven its success, resulting in complete reduction of tetrachloroethene to the harmless end product ethene. To efficiently bioaugmentate a contaminated site, the introduced microorganisms have to be distributed in the subsurface and come into close contact with the contaminant(s). Transport of cells in the subsurface is often limited because injected cells are filtered by soil particles, but can be enhanced by using solutions of low ionic strength, surfactants and bacteria with limited adhesive properties. The survival of injected bacteria depends on factors such as competition for electron donors and acceptors, contaminant toxicity and the availability of inorganic nutrients. The use of genetically engineered microorganisms (GMOs) for bioaugmentation has not made much progress over the past decade due to regulatory constraints and public adversity. It is important to accurately assess the effect of bioaugmentation on the biodegradation. Molecular techniques have evolved rapidly, allowing the determination of both numbers and types of bacteria present. Future research on bioaugmentation should focus on large-scale field studies and more attention has to be paid to proper control plots and assessment of bioaugmentation efficacy.

INTRODUCTION

Large quantities of contaminants have been released to soil and groundwater, by spills, from wastewater, or because of improper disposal. These contaminants include chlorinated and non-chlorinated hydrocarbons, volatile organics and polycyclic aromatic hydrocarbons (PAH). Contamination of groundwater is of serious concern, since in Europe and the USA, 75% respectively 51% of the drinking water is prepared from groundwater (Morris et al., 2003).

In situ bioremediation (Box 5.1) can offer a good alternative for the existing chemical and physical technologies to remove contaminants from groundwater. The success of bioremediation depends on the presence of the right community of microorganisms carrying out the desired reactions and the (bio)availability of sufficient electron donor, electron acceptor and nutrients. Enhanced biodegradation, also referred to as *biostimulation*, is the process of satisfying the requirements for the biodegradation processes by addition of electron donor, acceptor and/or nutrients. *Bioaugmentation* is the process where non-indigenous microorganisms are injected into the soil. Generally, biostimulation is preferred over bioaugmentation, where exogenous microorganisms have to be distributed, survive and compete in the subsurface. However, in some cases the indigenous microorganisms are:

1) not capable to carry out the desired reactions;
2) degrading the contaminant at rates that are too low;
3) inhibited by the presence of multiple contaminants;
4) killed as a result of drastic (abiotic) remediation techniques.

This chapter focuses on the role of bioaugmentation in in situ bioremediation. The goal is to point out what aspects play a role in bioaugmentation and what the status of research is of these topics. The results of recent bioaugmentation field trials and lab-scale studies are discussed to establish the status of bioaugmentation as a bioremediation approach.

Box 5.1 Definition of bioremediation

Dasappa and Loehr (1991) defined bioremediation as: “a managed treatment process that uses microorganisms to degrade and transform organic chemicals in contaminated soil, aquifer material, sludges and residues”. This definition is still valid, although inorganics (such as heavy metals) can also be bioremediated. “In situ” (Latin for “in its place”), indicates that the soil and/or groundwater are treated while staying in their place; e.g. no excavation or groundwater extraction and above-ground treatment takes places.

APPLICATION OF BIOAUGMENTATION

Incomplete contaminant degradation

Hendrickson et al. (2002) examined 24 contaminated sites, scattered throughout Europe and North America, where degradation of chloroethenes occurred. At 21 of these sites, members of the *Dehalococcoides* group (Box 5.2) were present and the chloroethenes were completely reduced to ethene. At the other 3 sites, no members of the *Dehalococcoides* group were detected and dechlorination stalled at cDCE. This research showed a direct relationship between the presence of members of the *Dehalococcoides* group and a complete reduction. As a result, incomplete reduction to cDCE or VC is expected when members of the *Dehalococcoides* group are absent. In this case the relevance of bioaugmentation is evident.

Box 5.2 Biodegradation of chloroethenes

The following abbreviations are used throughout this chapter:

Tetrachloroethene	PCE
Trichloroethene	TCE
cis-Dichloroethene	cDCE
Vinyl chloride	VC

Due to their use as dry-cleaning solvent and as degreasing agent, tetrachloroethene (PCE) and trichloroethene (TCE) are the most ubiquitous of the chlorinated hydrocarbons released to the environment. These (and some other) chloroethenes can serve as sole terminal electron acceptors in an energy-conserving, growth-coupled metabolism. This process is called dehalorespiration and was first described by Holliger et al. (1993). Dehalorespirers capable of metabolizing PCE and TCE to cDCE or VC are rather common. However, the halorespirer *Dehalococcoides ethenogenes* strain 195 is the only strain that is known to complete the degradation of PCE via TCE, cDCE, and VC to the harmless product ethene (Maymó-Gatell et al., 1997, 1999).

A successful field study was done at a site where TCE dechlorination stalled at cDCE (Ellis et al., 2000; Harkness et al., 1999). Soil column studies were carried out to test the effect of the addition of a wide variety of electron donors as well as supplemental nutrients and vitamins, which did not result in dechlorination of cDCE. Addition of a selected bacterial culture, however, resulted in complete dechlorination of TCE to ethene (Harkness et al., 1999). Subsequent injection of the same culture at field-scale also resulted in the complete conversion of TCE and cDCE to ethene (Ellis et al., 2000). A similar result was obtained after bioaugmentation with a dehalorespiring

microbial consortium at another site where TCE dechlorination had stalled at cDCE (Major et al., 2002). The consortium contained the strain *Dehalococcoides ethenogenes*, which was not detected at the site prior to the addition. At the completion of the study, *D. ethenogenes* was detected in all monitoring and extraction wells.

Enhancement of biodegradation rates

In some cases, microorganisms capable of executing the desired biodegradation reactions are present on the site, but the degradation rates are too low. For example, the fuel additive MTBE (methyl tertiary-butyl ether) can be degraded by naturally occurring microorganisms, however, the growth rate is often low. As a result, bacterial enrichment of MTBE degraders may not be sufficient for significant MTBE-degradation. Salanitro et al. (2000) did a field-scale bioaugmentation test. An MTBE-degrading consortium was injected into the aquifer. Since this consortium degrades MTBE aerobically, O_2 gas was injected as well. Controls consisted of a biostimulated area, where only O_2 gas was injected, and an area that was not treated at all. Both the bioaugmentation and the biostimulation resulted in significant MTBE degradation to concentrations on day 261 of 0.001-0.01 mg l^{-1} and 0.01-0.1 mg l^{-1}, respectively. Biostimulation, however, resulted in significant MTBE degradation only after 173-230 days after start-up, while for the bioaugmentation area the lag time was only 30 days.

Bioaugmentation of co-contaminated sites

Another problem is the presence of contaminants that are toxic to the microorganisms involved in the biodegradation of another contaminant. For example, many sites are contaminated with both metals and organics (co-contamination). Toxicity of metals inhibits the biodegradation of organics (Said and Lewis, 1991). Research on bioaugmentation as a remedial approach for these sites has focused on the introduction of microorganisms that are both metal resistant and capable of degrading the organic contaminants (Roane et al., 2001). Field studies, however, were unsuccessful. Roane et al. (2001) suggested that the energy requirements to maintain concurrent metal resistance and organic degradation are too high. They therefore inoculated co-contaminated soil with both metal-detoxifying and organic-degrading bacteria. In this pilot field study it was shown that addition of a cadmium-detoxifying bacterial strain indeed enhanced the degradation of 2,4-dichlorophenoyacetic acid by another introduced strain.

Bioaugmentation in combination with abiotic remediation technologies

Bioaugmentation may also be needed when bioremediation is preceded by or combined with abiotic remediation technologies. These abiotic technologies, which include in situ chemical oxidation and thermal soil treatment (Box 5.3), are

progressively used for the treatment of, especially, source zones. Due to the drastic nature of these technologies, microbial life is likely to be (partly) killed off (Stroo et al., 2003). We could not find reports of field studies describing the effect of this treatment on microbial activity in (subsequent) bioremediation. It must be noted that (lab scale) experiments revealed positive effects of *simultaneous* abiotic and biotic transformation processes. This enhanced biodegradation was probably caused by better conditions due to the abiotic treatment, such as a lowered toxicity and better degradability of the contaminant, selective advantage of biodegrading microorganisms or a higher oxygen availability (Miller et al., 1996; Howsawkeng et al., 2001; Buyuksonmez et al., 1998, 1999; Nadarajah et al., 2002). These studies indicate the need for more research on combined remediation technologies in order to elucidate the benefit of bioaugmentation.

Box 5.3 Abiotic remediation technologies

Besides bioremediation, abiotic remediation techniques such as in situ chemical oxidation are used for the treatment of contaminants. A well known method is the chemical oxidation based on Fenton-like reactions. In this reaction, hydrogen peroxide (H_2O_2) is decomposed in the presence of ferrous iron, which results in the generation of hydroxyl radicals:

$H_2O_2 + Fe^{2+} \quad Fe^{3+} + OH^- + OH^{\cdot}$

The formed hydroxyl radical is a strong and non-specific oxidant that reacts with most organics, resulting in partial mineralization, lowered toxicity or increased biodegradability. For example, PCE reacts to dichloroacetic acid, which is a readily biodegradable compound (Buyuksonmez et al., 1999). Potassium permanganate ($KMNO_4$) is another common oxidising agent.

Another example of an abiotic remediation technique is soil heating. For example, Six-Phase Soil Heating™ uses electricity to heat contaminated soil to the boiling point of water (100°C). This elevated temperature results in an increased volatility of the contaminants. The produced steam strips volatile and semi-volatile contaminants from the soil, which are extracted in extraction wells (Anonymous, 1999).

DISTRIBUTION OF MICROORGANISMS IN THE SUBSURFACE

To efficiently bioaugmentate a contaminated site, the introduced microorganisms have to be distributed in the subsurface and come into close contact with the contaminant(s). However, transport of cells in the subsurface is often limited because

injected cells are filtered by soil particles (Camesano and Logan, 1998; Li and Logan, 1999). Cells attach to soil grains and, as a result, the concentration of suspended bacteria can decrease by several orders-of-magnitude within a short distance (e.g. 0.1 to 1 m) from the injection well (Li and Logan, 1999). Growth of attached cells and biofilm formation can result in a permanent decrease in soil permeability, causing well clogging. The mechanisms and impact of biological clogging are extensively reviewed by Baveye et al. (1998).

The use of solutions of low ionic strength and surfactants can enhance bacterial transport (Camesano and Logan, 1998; Li and Logan, 1999). However, the effects are limited and for field applications, when cells have to be transported over distances >1 m, other methods will be needed (Li and Logan, 1999).

Another solution is the use of microorganisms that have limited adhesive properties. An adhesion-deficient MTBE-degrading variant of the bacterial strain *Hydrogenophaga flava* ENV735 was selected by sequential passage of cells through columns of sterile sediment (Streger et al., 2002). However, no results of bioaugmentation field studies with this variant have been reported yet. A similar method was used to obtain a stable adhesion-deficient variant of *Burkholderia cepacia* G4, designated ENV435, which was used in a bioaugmentation field study (DeFlaun et al., 1999). This strain travelled the 12 m from injection to recovery well with an average linear velocity of 0.37-0.54 m day^{-1}. This velocity is close to the velocity observed for a bromide tracer, which was 0.53-0.68 m day^{-1} (Steffan et al., 1999).

Other methods that have been suggested to enhance bacterial transport, are encapsulation of cells within polymeric gel microbeads (Moslemy et al., 2002[a,b], 2003), applying the microorganisms with microbubble foams (Rothmel et al., 1998) and electrokinetic transport (DeFlaun and Condee, 1997). These methods, however, have not been tested in field studies yet.

Bacterial chemotaxis, the movement of bacteria under the influence of a chemical gradient, may also play a role (Pandey and Jain, 2002). It was shown that induced cells of the toluene-degrading bacterium *Pseudomonas putida* F1 are chemotactically attracted to cDCE, TCE and also slightly to PCE (Parales et al., 2000). Strain *Pseudomonas stutzeri* KC, which is capable of co-metabolizing carbon tetrachloride (CT) under anoxic conditions, was shown to be chemotactic towards acetate and nitrate. In a model aquifer column, cells migrated at a velocity exceeding that of the groundwater, removing both aqueous and sorbed CT (Witt et al., 1999).

FATE OF INTRODUCED MICROORGANISMS

Once introduced into soil, bacteria have to survive in a hostile environment. The survival of added microorganisms depends on both biotic and abiotic factors (van Veen et al., 1997). Biotic factors include competition with indigenous populations for electron donors and acceptors. For example, it was shown that hydrogen plays a key role in the activity of dehalorespirers. Due to a very high affinity for hydrogen, these bacteria can outcompete other bacteria under certain anaerobic conditions (Yang and McCarty, 1998; Ballapragada et al., 1997).

Another biotic factor is predation by protozoans, a process called ‘grazing’. Protozoan grazing can have a significant effect on bacterial community structure (Rønn et al., 2002; Travis and Rosenberg, 1997). Kota et al. (1999) showed that the presence of protozoa resulted in decreased rates of BTEX (benzene, toluene, ethylbenzene and xylenes) biodegradation.

An abiotic factor that could influence the survival of introduced microorganisms is toxicity of contaminants present in the subsurface. Toxicity of metals and toxicity of reaction products formed during co-metabolic oxidations have already been mentioned above. No general rule concerning toxicity of contaminants can be given. For example, whereas CT degradation is inhibited by substrate toxicity (Devlin and Müller, 1999; Sponza, 2003), dechlorination of PCE was observed for cultures even in the presence of free-phase PCE (dense nonaqueous-phase liquids; DNAPLs) (Adamson et al., 2003; Yang and McCarty, 2000). In fact, the presence of these DNAPLs was shown to be a competitive advantage for the dechlorinating bacteria, since the other microorganisms were inhibited (Yang and McCarty, 2000).

Other abiotic factors include subsurface conditions like pH, inorganic nutrients (N and P) and temperature (van Veen et al., 1997). In some cases preparation of a niche is required. Some microorganisms, for example, are sensitive to oxygen (Lendvay et al., 2003). If the aquifer is aerobic in its natural state, pre-reduction can be achieved by addition of substrates like lactate (Lendvay et al., 2003; Ellis et al., 2000) or acetate (Major et al., 2002), creating a strongly reducing environment. Dybas et al. (1998, 2002) amended groundwater with bases to create favourable pH conditions (>7,6), needed for strain KC (described above) to grow and degrade CT.

GENETICALLY ENGINEERED MICROORGANISMS

The bacterial strains discussed so far are all strains selected from the environment. However, microorganisms have not had enough time to evolve appropriate catabolic pathways to deal with all contaminants present in the environment. For example, contaminants like pentachlorophenol (PCP) and polychlorinated biphenyls (PCBs) remain highly recalcitrant to microbial degradation (Garbisu and Alkorta, 1999). Genetic engineering enables the construction of bacterial strains with desired catabolic pathways (Furukawa, 2003; Dua et al., 2002; Timmis and Pieper, 1999).

However, the use of genetically engineered microorganisms (GMOs) for bioremediation purposes has not made much progress over the past decade (Sayler and Ripp, 2000). In fact, field testing of bioaugmentation with GMOs has only been done once, with strain *Pseudomonas fluorescens* HK44, capable of degrading polyaromatic hydrocarbons (Ripp et al., 2000). This study did show that also GMOs can be viably maintained after release to the environment (in this case 660 days), and are not necessarily unable to compete under field conditions due to energy-consuming foreign genetic elements (Sayler and Ripp, 2000).

The main reasons for the limited applications are the regulatory constraints on the release of GMOs into the environment and public adversity (Morrissey et al., 2002; Watanabe, 2001; Sayler and Ripp, 2000; Garbisu and Alkorta, 1999; Drobník, 1999; Miller, 1997). Different ways of biological containment of released GMOs have been proposed, including so-called ‘suicide genes’, which are activated when the contaminant is no longer present and result in death of the microorganisms. However, a substantial fraction of the GMOs escapes suicide, even under laboratory conditions (Garbisu and Alkorta, 1999).

The relevance of the outcome of a discussion held more than 10 years ago (Box 5.4) as well as the performance of only one field release since, indicates that the last decade has not been very fruitful for the use of GMOs in bioremediation. However, the development of molecular tools for detection of microorganisms did evolve significantly during this period, possibly enabling a better monitoring during field releases. The future role of GMOs in bioremediation is looked upon differently. Whereas some expect that the use of GMOs in bioremediation will most likely become one of the most important applications of GMOs (Garbisu and Alkorta, 1999), others say that the future of GMOs in bioremediation is uncertain and markets for GMOs in bioremediation may well be small (Sayler and Ripp, 2000; Watanabe, 2001).

Box 5.4 GMOs and bioremediation: the debate goes on…

In 1992 a meeting was held on the Use of Natural and Genetically Engineered Microorganisms to Combat Pollution. The last paragraph of the meeting report (Broda, 1992) was:

In the final discussion, there was concern that political pressure could prevent or delay legitimate remediation projects. Those involved with regulatory processes emphasized that properly worked out proposals for release of GMOs in specific situations analogous to those required for other agents (e.g. pesticides and herbicides) was the only way in which precedents and case-law could be established by governments. Another strongly held view was that it was the duty of scientists to explain and justify their plans to a concerned public.

ASSESSMENT OF BIOAUGMENTATION EFFICACY

To justify the cost of bioaugmentation it is important to accurately assess the effect of bioaugmentation on the biodegradation. Besides biodegradation, processes like dilution, sorption and volatilization will also determine the fate of the contaminant. The effect of bioaugmentation can therefore not be measured by contaminant concentrations alone. Ideally, complete mass balances would have to be made, but due to the heterogeneity of the subsurface this is often difficult to achieve. We will first discuss ways to assess the effect of biodegradation in general, and then focus on assessment of bioaugmentation efficacy.

Assessment of biodegradation

Injection of liquids containing bacteria and/or nutrients displaces the contaminated groundwater, possibly resulting in dilution of the contaminant. Tracers such as bromide are commonly used to follow injected liquids during bioremediation, in order to discriminate between biodegradation and dilution. To evaluate the effect of biodegradation, Steffan et al. (1999) distinguished between chlorinated hydrocarbons that could be degraded by the introduced bacterial strain ENV435 (TCE, cDCE, VC) and those that could not be biodegraded (PCE, dichloroethane, trichloroethane). In this way, the nondegradable chlorinated hydrocarbons present at the site served as an internal standard.

Analysis of kinetic isotope fractionation was used to assess the biodegradation of PCE, TCE and MTBE (Hunkeler et al., 1999, 2001; Kirtland et al., 2003; Sherwood Lollar et al., 2001). This method is based on the change in $^{13}C/^{12}C$ ratio during (bio)degradation. The bonds formed by the lighter ^{12}C isotope are usually weaker

(and thus more reactive) than the bonds formed by the heavier ^{13}C isotope. Biodegradation therefore results in an enrichment of ^{13}C isotopes in the precursor (i.e. the contaminant), while the degradation products become depleted in ^{13}C isotopes.

Assessment of bioaugmentation

When biodegradation is in fact established, the next question to be asked is whether this is really due to bioaugmentation and not, for example, a result of biostimulation. This question is not always answered satisfactorily. For example, the bioaugmentation study done by Ellis (described above) was preceded by a 9-month biostimulation period, while Major et al. (2002) did not carry out a biostimulation test. Good side-by-side comparisons between biostimulation and bioaugmentation were made by Salanitro et al. (2000) and Lendvay et al. (2003), clearly demonstrating the beneficial effect of bioaugmentation.

The effect of bioaugmentation can also be expressed on the level of microbial community structures. Molecular techniques have evolved rapidly, allowing the detection of both numbers and types of bacteria. These molecular techniques are essential, since traditional microbiological methods (i.e. incubation in the laboratory) are time-consuming and indirect. Molecular analysis has become an essential part of bioremediation research in general, and bioaugmentation research in particular. An extensive review of the available molecular techniques is given by Widada et al. (2002).

CONCLUSIONS AND FUTURE PROSPECTS

Based on the research discussed above, bioaugmentation can be a useful bioremediation tool, especially in case of incomplete dechlorination of PCE and TCE. It has been proven unequivocally that introduced microorganisms can occupy a niche in the subsurface and survive for a prolonged period of time. The number of comprehensive, large-scale field studies, however, is still limited. More studies as done by Salanitro et al. (2000) and Lendvay et al. (2003), which demonstrate the additional effect of bioaugmentation compared to biostimulation, are required to establish bioaugmentation. Many important aspects concerning distribution and survival of microorganisms after introduction into the soil, have only been studied on lab scale. As a result, the individual significance of these aspects remain difficult to predict and are still often a matter of ‘trial and error’. Future research on bioaugmentation should focus on large-scale field studies, including good control

plots and assessment of bioaugmentation efficacy. The use of molecular techniques is a must for this assessment.

The role of GMOs in bioaugmentation remains uncertain due to regulatory constraints and public adversity, resulting in reluctance at the side of investors and researchers. The amount of field release studies will therefore probably remain limited, making risk assessment difficult. Consequently, regulations are not likely to become less stringent: a catch-22.

6

Concluding remarks

Two major objectives were proposed at the start of this thesis. The first objective was to isolate 1,2-dichloroethane-degrading microorganisms with a high affinity for 1,2-dichloroethane (DCA) and without requirements for additional organic nutrients for optimal growth. The second objective was to apply the new strain in an MABR that allows aerobic biodegradation of DCA, without direct oxygenation of the contaminated groundwater.

ISOLATION AND CHARACTERIZATION OF A NEW DCA-DEGRADING STRAIN

The existing DCA-degrading strains *Xanthobacter autotrophicus* GJ10 and *Ancylobacter autotrophicus* AD25 do not have the desired characteristics for treatment of DCA-contaminated groundwater at low concentration ranges. Although strain AD25 does have a much higher affinity for DCA than GJ10, both strains require additional organic nutrients for optimal growth (van den Wijngaard et al., 1993[b]). We therefore set out to isolate new DCA-degrading strains.

Batch enrichment cultures with various inocula did not result in DCA degradation. However, when a specific inoculum, originating from an RBC that efficiently degraded DCA, was transferred to a fermenter operated at a dilution rate of 0.03 h^{-1}, a DCA-degrading mixed culture was obtained within 3 weeks. From this mixed culture, strain DCA1 was isolated. The use of a substrate-limited continuous culture was shown to be an effective way to select for a strain with a high affinity, since strain DCA1 has indeed a very high affinity for DCA. The implications of this high substrate affinity are discussed below. Strain DCA1 does not need any additional organic nutrients for optimal growth. Hence, with the isolation of strain DCA1, both prerequisites mentioned above have been fulfilled.

Comparison of DCA-degrading strains

The DCA degradation pathway of strain DCA was elucidated and is shown in Figure 6.1. The first two reaction steps in strain DCA1 are different from those in GJ10 and AD25. The presence of different catabolic enzymes has consequences for the kinetic properties of the DCA-degrading strains.

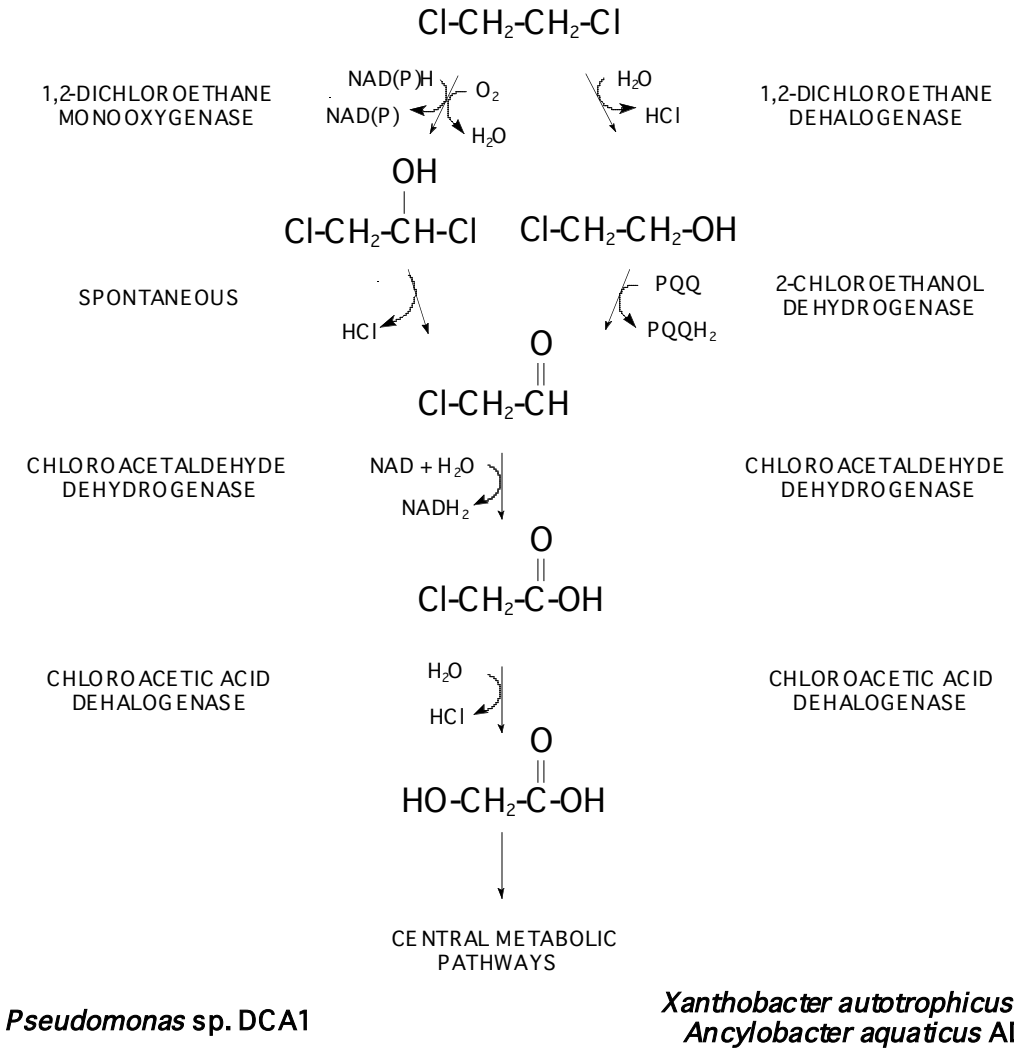

Figure 6.1 DCA degradation pathways of *Pseudomonas* sp. strain DCA1, *X. autotrophicus* GJ10, and *A. aquaticus* AD25.

In Table 6.1 some parameters of the different DCA-degrading strains are summarized. Strain DCA1 has the highest maximum specific growth rate (0.14 h^{-1}). It must be noted that this growth rate was determined at the optimum growth temperature of 25°C (Chapter 2). The growth rates of the other strains were determined at 30°C, which may not be the optimum growth temperature for these strains.

The yield of strain DCA1 on DCA was calculated from the cell densities that were obtained in the continuous cultures (Chapter 2 and 4). The OD_{660} of 0.5 in the fermenter corresponded to a dry weight of approximately 200 mg l^{-1}, resulting in a yield of strain DCA1 of 0.08 g biomass/g DCA. This yield is lower than the yield

reported for strain GJ10 and AD25. This is expected, based on the different initial steps in DCA degradation. Oxidation of DCA by the DCA monooxygenase requires reducing equivalents, whereas the oxidation of 2-chloroethanol in strain GJ10 and AD25 yields energy. The low yield of strain DCA1 is an advantage for bioremediation purposes, since less biomass results in less clogging. Moreover, less nutrients are required per kg DCA removed.

Table 6.1 Parameters determined for strains DE2, GJ10, AD25 and DCA1

Strain	μ_{max} (h^{-1})	Yield ($g\ g^{-1}$)	K_s (µM)	K_m (µM)	Reference
Ps. DE2	0.08	nd[a]	nd	nd	Stucki et al. (1983)
X. autotrophicus GJ10	0.104	0.24	260	571[c]	van den Wijngaard et al. (1993[a])
A. aquaticus AD25	0.098	0.16	24	571[c]	van den Wijngaard et al. (1993[a])
Ps. DCA1	0.14[b]	0.08	nd	< 0.5[d]	Hage and Hartmans (1999)

[a] nd = not determined; [b] μ_{max} at 25°C; [c] determined for purified enzyme; [d] determined for whole cells.

There is a striking difference between the K_s values of strain GJ10 and AD25. The K_s value represents the affinity of a microorganism for the growth substrate. Since DCA is a rather apolar compound, transport across the membrane is not expected to be rate limiting. The K_s value is therefore determined by the affinity of the first catabolic enzyme: the haloalkane dehalogenase (Janssen en de Koning, 1995). Although both strains express the same haloalkane dehalogenase, the K_s value of strain AD25 is more than 10-fold lower than of strain GJ10. This is caused by a much higher expression of this enzyme by strain AD25 (van den Wijngaard, 1993[a]). The K_s value of strain DCA1 could not be determined because it was too low.

Like many monooxygenases, the DCA monooxygenase is not a very stable enzyme. DCA degradation was shown in cell extracts, however, the highest specific activity measured was only 7 nmol min^{-1} mg^{-1} of protein, while the highest specific activity determined in whole cells was 62 nmol min^{-1} mg^{-1} of dry weight (Chapter 2). Based on the nonlinear relationship between the protein content in the assay and the specific activity of the DCA monooxygenase (Figure 2.5), this enzyme may well be a multicomponent enzyme. These enzyme characteristics complicate experiments with cell extracts. In fact, even during experiments with washed whole cells, a (temporary) loss of DCA-degrading activity was observed after storage on ice for more than 3 hours (data not shown). Based on these considerations, no attempts were made to

purify the DCA monooxygenase and most experiments related to the DCA monooxygenase were done with washed whole cells.

The K_m value of strain DCA1 could not be determined since it is lower than our detection limit for DCA (< 0.5 µM). This K_m value is much lower than the value determined for the dehalogenase of strains GJ10 and AD25. For the practical application of bacterial strains in biodegradation, not only the K_m value is important, but the relation of this value with the maximum specific activity V_{max} (Janssen and de Koning, 1995). The V_{max} of the haloalkane dehalogenase was determined by Keuning et al. (1985) and was 10 µmol min^{-1} mg^{-1} of (purified) protein. As was mentioned above, we did not determine the V_{max} of the DCA monooxygenase but determined the specific activity (v_{max}) in washed whole cells. To compare the kinetic properties of strains GJ10, AD25 and DCA1, the v_{max} values of whole cells of strains GJ10 and AD25 were calculated from the haloalkane dehalogenase content in the cells and the V_{max} value determined for purified dehalogenase (van den Wijngaard et al., 1993[a]; Keuning et al., 1985). Alternatively, we calculated the theoretical specific activities ($v_{max,th}$) for the three strains from the maximum growth rates and the yields reported in Table 6.1. The $v_{max,th}$ represents a minimal specific activity that is required to support the maximum growth rates. The measured and calculated v_{max} values and the calculated $v_{max,th}$ values are shown in Table 6.2.

Table 6.2 Measured and calculated maximum specific activities of haloalkane dehalogenase of strains GJ10 and AD25 and DCA-monooxygenase of strain DCA1.

Strain	Haloalkane dehalogenase concentration in cells (mg of protein mg^{-1} of cells)	V_{max} (nmol min^{-1} mg^{-1} of purified protein)	v_{max} (nmol min^{-1} mg^{-1} of cells)	$v_{max,th}$ (nmol min^{-1} mg^{-1} of cells)
GJ10	0.0075	10,000	75[a]	73
AD25	0.105	10,000	1,050[a]	103
DCA1	-	-	62[b]	295

From: Keuning et al. (1985); van den Wijngaard et al. (1993[a]); Hage and Hartmans (1999).
[a] calculated; [b] measured in whole (washed) cells.

The v_{max} and $v_{max,th}$ for strain GJ10 are in rather good agreement. Due to the elevated expression level of the haloalkane dehalogenase in strain AD25, the v_{max} value is much higher than the $v_{max,th}$ value. Consequently, the dehalogenation by the haloalkane dehalogenase is not the rate limiting step in this strain. A DCA degradation rate exceeding 103 nmol min^{-1} mg^{-1} of cells will therefore result in the accumulation of DCA degradation products. The difference between the v_{max} and

$v_{max,th}$ for strain DCA1 is probably a result of inactivation of the DCA monooxygenase during the preparation of the (washed) cell suspension.

The v_{max} / K_m and $v_{max,th}$ / K_m ratios of strains GJ10, AD25 and DCA1 are shown in Table 6.3. From these ratios it is concluded that strain DCA1 is the most efficient DCA degrader at low concentrations of DCA, while strain AD25 theoretically has the highest DCA degradation capacity at high DCA concentrations. Based on the v_{max} and K_m values, DCA degradation rates of strain DCA1 and AD25 are expected to be similar at a concentration of approximately 35 µM. At a concentration of 4 µM, which is the *intervention value* (a quality criterion linked to remediation urgency) for DCA in The Netherlands (Chapter 3), the DCA degradation rate of strain DCA1 would be more than 7 times higher than that of strain AD25 and more than 100 times higher than that of strain GJ10.

Table 6.3 Comparison of kinetic properties of DCA-degrading strains.

Strain	v_{max}/K_m	$v_{max,th}/K_m$
X. autotrophicus GJ10	0.13	0.13
A. aquaticus AD25	1.84	0.18
Ps. DCA1	124	590

In two separate studies in continuous cultures, strain GJ10 was outcompeted by strain AD25 (van den Wijngaard et al., 1993[a]) and DCA1 (results not shown), respectively, indicating the impact of kinetic properties on competitive behavior. As was mentioned in Chapter 2, based on the kinetic properties of strain DCA1 and GJ10, it seems somewhat unexpected that strain GJ10 was still present in the DCA-degrading RBC biofilm, which served as an inoculum for our enrichment. However, the behavior of microorganisms in a biofilm can be very different from that in a continuous culture (Soda et al., 1999). Stewart et al. (1996) reported a stable coexistence of *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* in a labaratory-grown biofilm, even though the growth rate of the latter is twice that of *P. aeruginosa* under planktonic growth conditions. They suggested that this was due to different rates of bacterial attachment and detachment, as well as structural heterogeneity of the biofilm. Banks and Bryers (1991) showed that when two different biofilm-forming bacterial strains were deposited simultaneously on a clean glass surface, the faster-growing strain became the dominant biofilm species, but the slower-growing strain remained established within the biofilm and continued to increase in number over time.

Co-metabolic degradation of CAHs

In Chapter 4, the co-metabolic oxidation of a wide variety of CAHs by strain DCA1 was tested. Chloroacetic acid, an intermediate in the DCA degradation pathway, was used as the energy source. DCA-grown cells of strain DCA1 oxidized all of the tested compounds, except for tetrachloroethene. The relative oxidation rates of the CAHs were much lower than of DCA. As constant oxidation rates were obtained during the assays, this is not due to toxicity of intermediates, a low substrate concentration or energy depletion. Interestingly, the conversion rate of propene into 1,2-epoxypropane was 42 nmol min^{-1} mg (dry weight) $cells^{-1}$, which is comparable to the oxidation rate of DCA. As was shown in competition experiments (Chapter 2) the affinity of strain DCA1 for propene is even higher than for DCA. At a DCA concentration of 250 µM, the presence of 8 µM propene already inhibited DCA degradation for more than 50%. Propene is not only emitted due to industrial activity, but is also produced and emitted by vegetation (Sanderson, 2002). Therefore, it can not be excluded that this compound is the natural substrate of the DCA-monooxygenase. However, no growth of strain DCA1 on propene was observed (results not shown).

It must be noted that, since the oxidation rates were determined in whole cell assays, it cannot be completely ruled out that one or more other oxygenases may be present in strain DCA1. To prove that indeed the DCA monooxygenase is responsible for the oxidation of the CAHs tested, purification of this enzyme is required. Experiments with purified DCA monooxygenase can also exclude effects such as (lack of) transport and uptake of the different CAHs on the observed substrate specificity of this enzyme in whole cells. Indirect evidence for the oxidation of the CAHs by the DCA monooxygenase can be obtained with competition experiments between the individual CAHs and DCA, as was successfully done with propene (Chapter 2).

Even in case other oxygenases turn out to be (partly) responsible for the oxidation of the CAHs, the broad substrate spectrum is still an important feature of strain DCA1 and has consequences for application of this strain in bioremediation. On the one hand, a broad substrate spectrum is useful, as strain DCA1 can then also be used for the biodegradation of other CAHs. On the other hand, a broad substrate spectrum may be a disadvantage in relation to the competitive behavior towards other strains, in case the substrates that are oxidized do not support growth.

Oxidation of 1,2-dichloropropane

The oxidation of 1,2-dichloropropane (DCP) by strain DCA1 was studied in more detail. DCP is an important contaminant, but pure cultures capable of utilizing DCP as the sole carbon source under aerobic conditions have not been isolated. Although DCP was oxidized by DCA-grown resting cells of strain DCA1, DCP was not utilized

as the sole carbon and energy source. We therefore studied the co-metabolic oxidation of DCP during growth on DCA. Contamination of sites with both DCA and DCP is not likely to occur frequently, based on the use of these compounds in industrial processes and agriculture, respectively. However, DCA was added since it is the only compound known so far that both serves as carbon and energy source, as well as an inducer of the DCA monooxygenase in strain DCA1. As is observed with many co-metabolic reactions, degradation of DCP also resulted in the formation of one or more toxic intermediates. We were not able to identify these intermediates, but there are indications that 2-chloropropionaldehyde played a role. This could not be verified, since this compound is not commercially available. The formation of 2,3-dichloro-1-propanol was confirmed by GC-MS analysis, but this compound did not inhibit growth of strain DCA1. The tentative DCP oxidation pathway shown in Figure 4.5 suggests the subsequent formation of 2-chloropropionaldehyde and 2-chloro-1-propanol. Strain DCA1 could utilize 2-chloro-1-propanol as the sole carbon and energy source. If both DCP and 2-chloro-1-propanol are catabolized via 2-chloropropionaldehyde, strain DCA1 is capable of synthesizing all the catalytic activities required for complete DCP metabolism. However, since it seems difficult to create the right conditions, resulting in the simultaneous expression of these enzymes, strain DCA1 will probably not have a practical application in bioremediation of DCP.

MEMBRANE-AERATED BIOREACTOR

The second objective proposed in this thesis, is the application of the new DCA-degrading strain in an MABR. This MABR is used to allow aerobic degradation, whilst direct oxygenation of the (ground)water is avoided. At the same time stripping of the contaminant as a result of oxygenation is prevented.

Biofilm formation and stability

A prerequisite for application of strain DCA1 in an MABR is that this strain is capable of forming a stable and active DCA-degrading biofilm. Strain DCA1 does not grow on mineral salt agar plates (in the presence of DCA in the vapor phase) and only forms small colonies on rich-medium agar plates (Chapter 2). However, strain DCA1 did form a biofilm on the glass and stainless steel parts of the fermenter after a prolonged time of running a continuous culture. In Chapter 3 it was shown that strain DCA1 is also able to form a stable and active biofilm on a hydrophobic microporous membrane in an MABR. After initial biofilm formation had occurred, the supply of fresh cells from the fermenter did not significantly enhance biofilm formation. We did not test whether the inoculation phase can be reduced to less than one day; maybe a

couple of hours is long enough for initial biofilm formation to occur. Although the available surface of the tubing in the experimental set-up was approximately the same as the membrane surface area (assuming a total tube length of 250 mm and an internal diameter of 5 mm), the control showed that no significant biofilm formation occurred on the tubing surface. This may be due to a decreased availability of DCA and/or oxygen, the properties of the tubing material, or the two-fold higher flow velocity in the tubing, resulting in more shear.

All biofilm experiments with strain DCA1 were done on a time-scale of days. Future research should focus on long-term stability of a biofilm of strain DCA1 in an MABR. The estimated biofilm thickness after 3 days of inoculation was approximately 50 µm. It is not known what the maximum thickness of a biofilm of strain DCA1 can be under these circumstances. As was discussed in Chapter 1, an increased biofilm thickness has disadvantages, such as an increased mass transfer resistance and clogging problems. In this respect, the rather low yield of strain DCA1 on DCA compared to strain AD25 and GJ10 (see above) may be an advantage. Moreover, strain GJ10 is known to produce a high amount of extracellular polymers (Freitas dos Santos, 1995[b]).

DCA removal rates

The maximum DCA removal rate (RR_{max}) in the MABR was 410 g m^{-3} h^{-1}. In Table 6.4, this removal rate is compared to those obtained in two EMBs with biofilms of strain GJ10 (Freitas dos Santos et al., 1994; 1995[a]). The removal rates are calculated from the data reported by the authors.

Table 6.4 Comparison of MABR performance with EMBs

Reference	Reactor Type	V_{mbr} (m^3)	A_m (m^2)	$A_{m,spec}$ (m^2 m^{-3})	$RR_{max,spec}$ (g m^{-2} h^{-1})	RR_{max} (g m^{-3} h^{-1})
Freitas dos Santos et al. (1994)	EMB	1.7×10^{-4}	0.3	1,765	1.20	2,112
Freitas dos Santos et al. (1995[a])	EMB	9.8×10^{-5}	0.157	1,600	1.44	2,310
Hage et al. (2004)	MABR	8.0×10^{-6}	0.004	500	0.82	410

(V_{mbr}) Volume of liquid phase compartment that contains biofilm and DCA-contaminated water; (A_m) membrane surface; ($A_{m,spec}$) specific membrane area (m^2 membrane per m^3 liquid volume); ($RR_{max,spec}$) maximum DCA removal rate expressed per m^2 membrane area; (RR_{max}) maximum DCA removal rate per m^3 liquid phase compartment that contains biofilm and DCA-contaminated water.

As can be seen, the RR_{max} observed by Freitas dos Santos et al. is 5 to 6 times higher than we observed for our MABR. This is partly due to the much higher specific membrane area ($A_{m,spec}$), however, also the maximum DCA removal rate expressed per m^2 membrane area ($RR_{max,spec}$) is higher. It must be noted that the experiments with the EMBs were done with a DCA concentration varying between 16 en 20 mM, which is about 50 times higher than the maximum concentration tested in our studies. The EMBs may have a higher RR_{max} at this DCA concentration range, however, at the low concentrations encountered in groundwater remediation (micromolar range) the RR_{max} is expected to be much lower.

To determine the effect of bacterial kinetics on reactor performance, we simulated the performance of strain GJ10 in our MABR. With strain DCA1 the RR_{max} was reached at a DCA concentration of 80 µM. At this concentration the simulated DCA flux into a strain GJ10 biofilm is more than 8 times lower. At a concentration of 4 µM (the *intervention value*), the flux into a biofilm of strain GJ10 is less than 5% of the flux generated into a biofilm of strain DCA1. The use of different bacterial strains and DCA concentrations makes a good comparison between the EMBs and the MABR rather difficult. It would therefore be interesting to repeat the experiments done by Freitas dos Santos et al. with strains GJ10, AD25 and DCA1 at a DCA concentration that is closer to those encountered in groundwater remediation.

DCA removal from anoxic water

For practical reasons, the experiments with the MABR were done under oxic conditions. Since the objective was to remove DCA by aerobic degradation while keeping the contaminated (ground)water anoxic, this was tested in the final experiment described in Chapter 3. From the results it is concluded that aerobic degradation of DCA present in anoxic (ground)water is indeed possible by supplying oxygen solely from the gas phase. It must be noted, however, that the DCA removal rate with oxic water was almost 20% higher than measured with anoxic water and air supplied from the gas phase. Based on the standard error between the measurements, this difference seems to be significant. This may be caused by oxygen limitation in the biofilm. If this is indeed the case, this implies that a part of the biofilm (at the interface between the biofilm and the liquid phase) does not participate in DCA degradation. As a result, no oxygen is released into the liquid phase, which was one of the reasons for application of an MABR for the treatment of DCA-contaminated water. However, we did not verify if oxygen penetrated into the liquid phase. Experiments with resazurin as the redox indicator were not successful since it strongly inhibited MABR performance (results not shown). Therefore, oxygen measurements in the liquid phase have to be carried out. Additional research is also needed to determine what the optimum biofilm thickness is, i.e. under what

conditions does the largest part of the biofilm participate in DCA degradation, without oxygen penetrating into the liquid phase. A simulation program like BIOSIM can be a very useful tool for this research.

A relatively small decrease (<10%) of the DCA removal rates before and after the supply of anoxic water in combination with nitrogen from the gas phase (Figure 3.8, second and fourth column, respectively) was observed. Apparently, switching from oxic to anoxic conditions does not have a strong negative effect on biofilm activity. This is important for practical application, since the capacity to cope with fluctuations adds to the robustness of the system.

We did not test the effect of precipitation of iron oxides in the biofilm. We could not find reports of this effect on biofilm performance in the literature. However, since liquid flow in biofilms occurs within the internal void spaces (Stoodley et al., 1994; de Beer and Stoodley, 1995), penetration of oxygen and subsequent iron oxidation is expected to occur within the biofilm.

BIOAUGMENTATION

Although pump-and-treat systems are widely applied for groundwater remediation, this technology is in some cases expensive and ineffective. In situ bioremediation, a technology based on microorganisms biodegrading the contaminants in the subsurface, may then be a good alternative. Biodegradation can be enhanced by injecting electron donor, acceptor and/or nutrients. This process, *biostimulation*, has been reviewed extensively in the literature. However, data on the impact of *bioaugmentation*, the introduction of non-indigenous bacteria into the soil, remain fragmentary. We therefore carried out a literature study to establish the current status of bioaugmentation as a bioremediation approach. Important issues such as distribution and fate of microorganisms after their introduction into the subsurface, the use of genetically engineered microorganisms and assessment of bioaugmentation efficacy were included.

From the results of recent field trials and lab-scale studies, it can be concluded that bioaugmentation can, in specific cases, be a useful tool for in situ bioremediation. For easily degradable contaminants such as fuel-associated aromatic hydrocarbons (benzene, toluene, ethylbenzene and xylenes) or mineral oil, bioaugmentation is not likely to enhance the bioremediation process. In these cases, the indigenous microorganisms are generally capable of degrading the contaminants at sufficient rates, but (one of the) other prerequisites for biodegradation (e.g. oxygen) are

limiting. However, bioaugmentation can enhance bioremediation in case the indigenous microorganisms are :
- degrading the contaminant at rates that are too low;
- inhibited by the presence of multiple contaminants (e.g. organic contaminant and heavy metals);
- killed as a result of drastic (abiotic) remediation techniques;
- not capable of carrying out the desired reactions.
The latter case seems to be the most promising field for application of bioaugmentation. A good example is the reduction of tetrachloroethene to ethene, which often stalls at *cis*-1,2-dichloroethene. Bioaugmentation with members of the *Dehalococcoides* group has proven its success, resulting in complete reduction of tetrachloroethene to the harmless end product ethene. In fact, the absence of members of the *Dehalococcoides* group seems to be a sign that complete reduction is not to be expected at that particular site.

The role of genetically engineered strains in bioaugmentation is uncertain, due to public adversity and regulatory constraints.

Future research on bioaugmentation should focus on large-scale field studies. More attention has to be paid to proper control plots and assessment of bioaugmentation efficacy, including the use of molecular detection techniques. To elucidate the role of bioaugmentation, it is important to clearly distinguish between the effects of bioaugmentation and biostimulation (addition of electron donor, acceptor and/or nutrients), two techniques that are often applied in combination.

Bioaugmentation of DCA-contaminated sites

Under aerobic groundwater conditions, DCA-degrading microorganisms do not always occur naturally, even when the subsurface has a known history of exposure to DCA (Kle_ka et al., 1998). Therefore, bioaugmentation with aerobic DCA degraders is discussed first.

A problem of strain DCA1 for potential application for bioaugmentation is its stability. As was discussed above, possibly due to the monooxygenase-mediated DCA degradation, strain DCA1 is a rather sensitive microorganism. On the other hand, we observed that DCA-grown batches of strain DCA1 that were kept at room temperature for weeks after the DCA was depleted, could easily be revitalized by adding DCA (results not shown).

Once introduced into the subsurface, the DCA-degrading microorganisms have to be distributed and come into close contact with the contaminant. We did not study the

distribution of cells of strain DCA1 in soil columns or in the subsurface; therefore no comments can be made on the transport characteristics of this strain. The high extracellular polymer production by strain GJ10 is likely to result in problems with clogging of the injection wells and limited distribution in the subsurface.

After injection into the subsurface, the DCA degraders have to compete with the indigenous microorganisms. Competition for the substrate (DCA) is not an issue, since bioaugmentation is not needed if other DCA degraders are already present. Competition for oxygen is more likely to be important. No data on the affinity of the DCA-degrading strains for oxygen are available. In case DCA is not present as the sole contaminant, but other CAHs are present as well, competition for the catabolic enzymes may occur. The DCA monooxygenase of strain DCA1 has a very broad substrate spectrum, therefore different CAHs will compete for this enzyme The effect of this competition on DCA degradation depends on the affinity of the DCA monooxygenase for these compounds and the relative concentrations in which they occur. The effect of competition between the CAHs and DCA was not tested, but a strong competitive inhibition of the DCA monooxygenase by propene was observed (Chapter 2). Moreover, these co-metabolic oxidations cost reducing equivalents, as these substrates do not serve as growth substrate for strain DCA1. As a result, the amount of energy available for growth is reduced. Furthermore, the production of toxic intermediates can inhibit growth of strain DCA1, as was shown for DCP (Chapter 4). Janssen et al. (1985) showed that the haloalkane dehalogenase of strain GJ10 is also able to dehalogenate other CAHs beside DCA. An important difference with strain DCA1 is that all of the compounds that were dehalogenated could also serve as a growth substrate for strain GJ10 and therefore yield energy.

Hunkeler and Aravena (2000) showed that substantial isotope fractionation occurred during DCA mineralization by strain GJ10. This may be a useful tool to monitor biodegradation in field trials (Chapter 5). A strong isotope fractionation was observed as a result of the initial enzymatic transformation of DCA, suggesting that a similar effect may be observed during DCA mineralization by strain AD25. Isotope fractionation during degradation of DCA by strain DCA1 is yet to be investigated. As the first step in DCA degradation by strain DCA1 is monooxygenase-mediated, an effect of isotope fractionation during DCA mineralization by strain DCA1 may be very different than observed for GJ10, if present at all.

Bioaugmentation with anaerobic DCA degraders

Under anaerobic conditions, DCA-degrading strains are generally present (Kle_ka et al., 1998; Cox et al., 2000; Dyer et al., 2003). However, anaerobic DCA degradation by most strains is based on co-metabolic reactions, which are often slow and

incomplete. Only two strains, *Dehalococcoides ethenogenes* 195 and *Desulfitobacterium dichloroeliminans* strain DCA1, are able to dehalorespire DCA (i.e. convert DCA into ethene in an energy-conserving reaction). Strain 195 is suitable for bioaugmentation, as was shown for PCE-contaminated sites (Chapter 5). However, during degradation of DCA this strain produces up to 1% of the carcinogenic vinyl chloride. Recently, de Wildeman et al. (2004) reported promising (lab-scale) results concerning *Desulfitobacterium dichloroeliminans* strain DCA1. This strain degraded DCA at a very high rate (> 350 nmol chloride released per min per mg total bacterial protein) and did not produce any toxic byproducts. Based on these preliminary results, *Desulfitobacterium dichloroeliminans* strain DCA1 seems to be a good candidate for large-scale bioaugmentation studies under anaerobic conditions.

It should be noticed that for anaerobic strains, such as *Desulfitobacterium dichloroeliminans* strain DCA1, DCA serves as an electron acceptor. Consequently, an electron donor has to be present in the subsurface or must be added besides the microorganisms (i.e. a combination between bioaugmentation and biostimulation is required). Lactate was used as an electron donor for the removal of DCA by *Desulfitobacterium dichloroeliminans* strain DCA1. For the aerobic DCA degraders, DCA serves as an electron donor and oxygen as the electron acceptor. If oxygen is not present it has to be supplied. However, oxygen (air) is cheaper and generally easier to distribute in the subsurface than substrates like lactate.

Pump-and-treat versus in situ bioremediaton

Although in situ bioremediation may in some cases be a good alternative, pump-and-treat systems will still be applied at many sites in the future, as it has great value for (hydraulic) containment of contaminant plumes and contaminant mass reduction.

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SUMMARY

The large-scale application of chlorinated aliphatic hydrocarbons (CAHs) has resulted in many cases of groundwater contamination. Contaminated groundwater can be remediated by pump-and-treat: the groundwater is pumped to the surface and treated. The groundwater can be treated in bioreactors, in which the contaminants are biodegraded. Since extracted groundwater is often anaerobic, it has to be oxygenated in order to allow aerobic biodegradation of the contaminants. However, direct oxygenation of (anaerobic) groundwater may result in two undesired effects: stripping of volatile contaminants and coagulation of iron oxides. The use of a membrane-aerated biofilm reactor (MABR) can solve these problems. At one side of the membrane a gas phase is present for the supply of oxygen. A biofilm is located at the other side of the membrane, where the contaminant is present in the liquid phase. The contaminant diffuses into the biofilm, where biodegradation takes place. The combination of a membrane with a biofilm allows oxygenation and biodegradation without stripping the contaminant and coagulation of iron oxides.

The main contaminant studied in this thesis is 1,2 dichloroethane (DCA), a synthetic chemical that is mainly used as a feedstock for the production of vinyl chloride. Known bacterial strains that are able to aerobically degrade DCA, *Xanthobacter autotrophicus* GJ10 and *Ancylobacter autotrophicus* AD25, do not have the desired characteristics for treatment of DCA-contaminated groundwater. Both strains require additional organic nutrients, such as vitamins, for optimal growth. Moreover, strain GJ10 has a very low affinity for DCA (K_m value of 570 µM).

Two major objectives were proposed at the start of this research. The first objective was to isolate DCA-degrading microorganisms with a high affinity for DCA and without requirements for additional organic nutrients for optimal growth. The second objective was to apply the isolated microorganisms in a MABR that allows aerobic biodegradation of DCA, without direct oxygenation of the contaminated groundwater.

In Chapter 2, the isolation and characterization of a bacterial strain, designated *Pseudomonas* sp. strain DCA1, is described. Strain DCA1 utilizes DCA as the sole carbon and energy source and does not require additional organic nutrients for optimal growth. The maximum specific growth rate of strain DCA1 on DCA is 0.14 h^{-1}. The affinity of strain DCA1 for DCA is very high, with a K_m value below the detection limit of 0.5 µM. Instead of a hydrolytic dehalogenation, as was shown in other DCA utilizers, the first step in DCA degradation in strain DCA1 is an oxidation reaction. Oxygen and NAD(P)H are required for this initial step. Propene was converted to 1,2-epoxypropane by DCA-grown cells, and competitively inhibited DCA degradation. We

concluded that a monooxygenase is responsible for the first step in DCA degradation in strain DCA1. Oxidation of DCA probably results in the formation of the unstable intermediate 1,2-dichloroethanol, which spontaneously releases chloride, yielding chloroacetaldehyde. The DCA degradation pathway in strain DCA1 proceeds from chloroacetaldehyde via chloroacetic acid and glycolic acid, which is similar to degradation routes observed in other DCA-utilizing bacteria.

Strain DCA1 was applied in a MABR for the removal of DCA from (ground)water (Chapter 3). A hydrophobic membrane was used to create a barrier between the liquid and the gas phase. Inoculation of the MBR with cells of strain DCA1 grown in a continuous culture resulted in the formation of a stable and active DCA-degrading biofilm on the membrane. The maximum DCA removal rate in the MABR was 410 g m^{-3} h^{-1}. This maximum removal rate was reached at a DCA concentration of approximately 80 µM. Simulation of the DCA fluxes into the biofilm showed that the MABR performance at lower concentrations was limited by the DCA diffusion rate rather than by kinetic constraints of strain DCA1. To determine the effect of bacterial kinetics on reactor performance, we also simulated the performance of strain GJ10 in our MABR. At 80 µM the simulated DCA flux into a strain GJ10 biofilm is more than 8 times lower. At a concentration of 4 µM (the *intervention value* for DCA in The Netherlands), the simulated flux into a biofilm of strain GJ10 is less than 5% of the flux generated into a biofilm of strain DCA1. Aerobic biodegradation of DCA present in anoxic water could be achieved by supplying oxygen solely from the gas phase to the biofilm grown on the liquid side of the membrane.

Since the degradation of DCA by strain DCA1 is mediated by a monooxygenase, and these enzymes generally have a broad substrate spectrum, strain DCA1 was used to co-metabolically oxidize chlorinated methanes, ethanes, propanes and ethenes (Chapter 4). Chloroacetic acid, an intermediate in the DCA degradation pathway of strain DCA1, was used as a co-substrate since it was readily oxidized by DCA-grown cells of strain DCA1 and did not compete for the monooxygenase. All of the tested compounds except tetrachloroethene were oxidized by cells expressing DCA monooxygenase. Strain DCA1 could not utilize any of these compounds as a growth substrate. Co-metabolic oxidation during growth on DCA was tested with 1,2-dichloropropane, since this compound shows structure analogy to DCA but is recalcitrant to aerobic biodegradation. Although growth on this mixture occurred, 1,2-dichloropropane strongly inhibited growth of strain DCA1. This inhibition was not caused by competition for the monooxygenase. It was shown that the oxidation of 1,2-dichloropropane resulted in the accumulation of 2,3-dichloro-1-propanol and 2-chloroethanol.

An alternative to pump-and-treat is in situ bioremediation. This technology is based on microorganisms biodegrading the contaminants in the subsurface. Besides addition of electron donors, electron acceptors and/or nutrients to stimulate the biodegradation ('biostimulation'), also non-indigenous microorganisms can be injected into the subsurface, a process called 'bioaugmentation' (Chapter 5). Bioaugmentation can be applied in case the indigenous microorganisms are:
- degrading the contaminant at rates that are too low;
- inhibited by the presence of multiple contaminants;
- killed as a result of drastic (abiotic) remediation techniques;
- not capable to carry out the desired reactions.

The latter case seems to be the most promising field for application of bioaugmentation. A good example is the incomplete reduction of tetrachloroethene, which often stalls at *cis*-1,2-dichloroethene. Bioaugmentation with members of the *Dehalococcoides* group has proven its success, resulting in complete reduction of tetrachloroethene to the harmless end product ethene. To efficiently bioaugmentate a contaminated site, the introduced microorganisms have to be distributed in the subsurface and come into close contact with the contaminant(s). Transport of cells in the subsurface is often limited because injected cells are filtered by soil particles, but can be enhanced by using solutions of low ionic strength, surfactants and bacteria with limited adhesive properties. The survival of injected bacteria depends on factors such as competition for electron donors and acceptors, contaminant toxicity and the availability of inorganic nutrients. The use of genetically engineered microorganisms (GMOs) for bioaugmentation has not made much progress over the past decades due to regulatory constraints and public adversity. To justify the cost of bioaugmentation it is important to accurately assess the effect of bioaugmentation on the biodegradation. Molecular techniques have evolved rapidly, allowing the determination of both numbers and types of bacteria present. Future research on bioaugmentation should focus on large-scale field studies and more attention has to be paid to proper control plots and assessment of bioaugmentation efficacy.

SAMENVATTING

De grootschalige toepassing van gechloreerde alifatische koolwaterstoffen (CKWs) heeft geleid tot veel gevallen van bodemverontreiniging (grond en grondwater). Verontreinigd grondwater kan behandeld worden door middel van 'pump-and-treat': het grondwater wordt opgepompt en bovengronds behandeld. Deze behandeling kan plaatsvinden in bioreactoren, waarin de verontreinigingen biologisch worden afgebroken. Het onttrokken grondwater is vaak anaëroob waardoor beluchting noodzakelijk is om aërobe biodegradatie mogelijk te maken. Directe beluchting van (anaëroob) grondwater kent echter twee ongewenste effecten: strippen van vluchtige verontreinigingen en coagulatie van ijzeroxiden. Het gebruik van een membraanbeluchte biofilm reactor (MABR) kan deze problemen oplossen. Aan de ene kant van het membraan is een gasfase aanwezig voor de toevoer van zuurstof. Een biofilm bevindt zich aan de andere zijde van het membraan, waar de verontreiniging aanwezig is in de waterfase. De verontreiniging diffundeert in de biofilm, waar biodegradatie plaatsvindt. De combinatie van een membraan met een biofilm maakt beluchting en biodegradatie mogelijk, zonder dat de verontreiniging gestript wordt en zonder de coagulatie van ijzer oxiden.

Het onderzoek beschreven in dit proefschrift heeft zich vooral gericht op 1,2-dichloorethaan (DCA), een synthetische verbinding die voornamelijk wordt toegepast als grondstof voor de productie van vinyl chloride. Bestaande bacteriestammen die DCA aëroob kunnen afbreken, *Xanthobacter autotrophicus* GJ10 and *Ancylobacter autotrophicus* AD25, hebben niet de gewenste eigenschappen voor de behandeling van grondwater dat is verontreinigd met DCA. Beide stammen hebben extra organische nutriënten, zoals vitaminen, nodig voor optimale groei. Stam GJ10 heeft bovendien een zeer lage affiniteit voor DCA (K_m waarde van 570 µM).

Het doel van het onderzoek beschreven in dit proefschrift was tweeledig. Het eerste doel was om DCA-afbrekende micro-organismen te isoleren met een hoge affiniteit voor DCA, zonder dat extra organische nutriënten nodig zijn voor optimale groei. Het tweede doel was het toepassen van de nieuwe isolaten in een MABR om aërobe biodegradatie mogelijk te maken, zonder het verontreinigde grondwater daarbij direct te beluchten.

De isolatie en karakterisatie van een bacteriestam, genaamd *Pseudomonas* sp. stam DCA1, is beschreven in Hoofdstuk 2. Stam DCA1 kan DCA gebruiken als enige koolstof- en energiebron en heeft geen extra organische nutriënten nodig voor optimale groei. De maximum specifieke groeisnelheid van stam DCA1 op DCA is 0.14 h^{-1}. De affiniteit van stam DCA1 voor DCA is zeer hoog, met een K_m waarde die

lager is dan de detectielimiet van 0.5 µM. In plaats van een hydrolytische dehalogenering, zoals aangetoond in andere DCA-afbrekers, bestaat de eerste stap in de DCA afbraak in stam DCA1 uit een oxidatie reactie. Zuurstof and NAD(P)H zijn benodigd voor deze eerste stap. Propeen werd omgezet in 1,2-epoxypropane door DCA-gekweekte cellen en leidde tot competitieve remming van de DCA afbraak. We concludeerden dat een monooxygenase verantwoordelijk is voor de eerste stap in de DCA afbraak in stam DCA1. Oxidatie van DCA resulteert waarschijnlijk in de vorming van het instabiele intermediair 1,2-dichloorethanol, dat spontaan chloride afsplitst, waarbij chlooraceetaldehyde gevormd wordt. De DCA afbraakroute verloopt vervolgens via chloorazijnzuur en glycolaat, zoals eerder is aangetoond in andere DCA-afbrekende bacteriën.

Stam DCA1 werd toegepast in een MABR voor de verwijdering van DCA uit (grond)water (Hoofdstuk 3). Met een hydrofoob membraan werd een scheiding tussen de vloeistof- en gasfase gecreëerd. Inoculatie van de MABR met cellen van stam DCA1 gekweekt in een continu-culture leidde tot de vorming van een stabiele en actieve DCA-afbrekende biofilm op het membraan. De maximum DCA verwijderingssnelheid in de MABR was 410 g m^{-3} u^{-1}. Deze maximale waarde werd bereikt bij een DCA concentratie van ongeveer 80 µM. Uit simulaties van de DCA fluxen in de biofilm, bleek dat de omzettingssnelheid bij lagere concentraties gelimiteerd werd door de DCA-diffusiesnelheid en niet door kinetische eigenschappen van stam DCA1. Het effect van kinetische eigenschappen op de DCA flux werd gesimuleerd met parameters van stam GJ10. Bij een DCA-concentratie van 80 µM was de DCA flux in een GJ10 biofilm meer dan 8 maal zo laag als in een DCA1 biofilm. Bij een concentratie van 4 µM (de *interventiewaarde* voor DCA in Nederland) was de gesimuleerde flux in een GJ10 biofilm minder dan 5% van de flux gegeneerd in een DCA1 biofilm. Aërobe biodegradatie van DCA in anoxisch water kon worden gerealiseerd door zuurstoftoediening via uitsluitend de gasfase.

Omdat bij de DCA afbraak door stam DCA1 een monooxygenase is betrokken en deze enzymen in het algemeen een breed substraatspectrum hebben, werd stam DCA1 gebruikt om gechloreerde methanen, ethanen, propanen en ethenen co-metabolisch om te zetten (Hoofdstuk 4). Chloorazijnzuur, een intermediair in de DCA afbraakroute van stam DCA1, werd gebruikt als een co-substraat omdat het direct werd geoxideerd door DCA-gekweekte cellen van stam DCA1 en niet competeerde voor het monooxygenase. Al de geteste verbindingen behalve tetrachlooretheen werden geoxideerd door het monooxygenase. Stam DCA1 kon geen van deze verbindingen gebruiken als een groeisubstraat. Co-metabolische oxidatie tijdens groei op DCA werd onderzocht met 1,2-dichloorpropaan omdat deze verbinding qua structuur sterk lijkt op DCA maar niet aëroob wordt afgebroken. Hoewel groei op dit

mengsel plaatsvond, werd de groei van stam DCA1 sterk geremd door 1,2-dichloorpropaan. Deze remming werd niet veroorzaakt door competitie voor het monooxygenase. De oxidatie van 1,2-dichloorpropaan resulteerde in de ophoping van 2,3-dichloor-1-propanol en 2-chloorethanol.

Een alternatief voor pump-and-treat is in situ bioremediatie. Deze techniek is gebaseerd op biodegradatie van verontreinigingen in de bodem. Behalve de toevoeging van elektronendonoren en -acceptoren en/of nutriënten om de biodegradatie te stimuleren ('biostimulatie'), kunnen ook micro-organismen in de bodem worden geïnjecteerd, een proces dat 'bioaugmentatie' wordt genoemd (Hoofdstuk 5). Bioaugmentatie kan worden toegepast als de in de bodem aanwezige bacteriën:
- niet in staat zijn om de verontreinigingen snel genoeg om te zetten;
- geremd worden door de aanwezigheid van meerdere verontreinigingen
- gedood zijn als gevolg van drastische (abiotische) saneringstechnieken
- niet in staan zijn om de gewenste omzettingen uit te voeren.

Laatstgenoemd geval lijkt het meest kansrijk voor de toepassing van bioaugmentatie. Een goed voorbeeld is de onvolledige afbraak van tetrachlooretheen, die vaak stopt bij *cis*-1,2-dichlooetheen. Bioaugmentatie met bacteriën behorende tot de *Dehalococcoides* groep is succesvol gebleken, resulterend in een complete omzetting tot het onschadelijke etheen. Om een verontreinigde locatie effectief te saneren met behulp van bioaugmentatie, dienen de toegevoegde micro-organismen verspreid te worden in de bodem en in nauw contact te komen met de verontreinigingen. De verspreiding van cellen in de bodem wordt vaak beperkt doordat cellen gefilterd worden door bodemdeeltjes, maar kan bevorderd worden door de toepassing van oplossingen met een lage ionische sterkte, surfactants en bacteriën die zich minder hechten. De overleving van geïnjecteerde bacteriën hangt af van factoren zoals competitie voor elektronendonoren en -acceptoren, toxiciteit van de verontreiniging(en) en de beschikbaarheid van anorganische nutriënten. Het gebruik van genetisch gemodificeerde micro-organismen (GMOs) voor bioaugmentatie heeft weinig vooruitgang geboekt in de laatste decennia door regelgeving en publieke bezorgdheid. Het is van belang om het effect van bioaugmentatie op de biodegradatie vast te stellen. Door de ontwikkeling van moleculaire technieken kunnen zowel aantallen als soorten bacteriën bepaald worden. Toekomstig onderzoek op het gebied van bioaugmentatie dient gericht te zijn op grootschalige veldproeven. Meer aandacht dient besteed te worden aan goede blanco's en het vaststellen van de effectiviteit van bioaugmentatie.

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To be submitted

CURRICULUM VITAE

Jacobus Cornelis (Ko) Hage werd geboren op 20 augustus 1972 te Rotterdam. In 1990 werd aan de Scholengemeenschap ‘De Lage Waard’ te Papendrecht het VWO diploma behaald. In datzelfde jaar begon hij aan de studie ‘Bioprocestechnologie’ aan de toenmalige Landbouwuniversiteit Wageningen. Na afstudeervakken bij de sectie Industriële Microbiologie, TNO-Voeding te Zeist en een stage bij het Department of Chemical Engineering, University of Sydney, Australië werd de studie in september 1996 afgerond. In oktober van datzelfde jaar begon hij aan een promotieonderzoek bij de sectie Industriële Microbiologie van Wageningen Universiteit, waarvan de resultaten staan beschreven in dit proefschrift. Sinds februari 2002 is hij werkzaam bij Groundwater Technology B.V. te Delft.

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It's a beautiful day
Don't let it get away

(U2, Beautiful day)