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in Ingegneria Chimica, dell'Ambiente e della Sicurezza

XIX Ciclo

**CHLORINATED ALIPHATIC AND AROMATIC HYDROCARBONS  
BIODEGRADATION:  
BIOAUGMENTATION TESTS IN SLURRY MICROCOSMS AND STUDY  
OF THE CATABOLIC POTENTIAL OF MICROBIAL COMMUNITY IN  
THE INTERFACE BETWEEN GROUNDWATER AND SURFACE  
WATER**

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## ***INTRODUCTION AND PROBLEM DEFINITION***

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Especially in the last 50 years of industrial development, the amount and variety of hazardous substances has drastically increased. It is estimated that the human-made chemicals presently in use amount to 100.000, and hundreds of new ones are produced every year. Due to the increase in industrial and agricultural activities and exports of wastes, not only the traditional industrialized countries, but all nations are confronted with widespread soil pollution. A significant number of synthetic compounds persist in the environment, particularly those with no relation to natural ones.

Essentially, there are three major categories of sites with polluted soils: (a) sites that have been polluted by either spillage or leakage during production, handling or use of industrial material (including mining and oil drilling); (b) locations that have been used as disposal sites for diverse waste; (c) farmlands that have been excessively exposed to pesticides.

Contaminated land sites are seriously dangerous for human beings and therefore unsuitable for housing or agriculture. The downward migration of pollutants from the soil into the groundwater is especially problematic in developing countries, where groundwater is often directly drunk, without any prior treatment.

The halocarbons, both halogenated compounds and solvents, are widespread air, water, soil, and sediment pollutants; they are recalcitrant molecules resistant to mineralization due to the stability of their carbon-halogen bond. The stability and chemical inertness of many halogenated compounds is part of their appeal in many industrial processes, but it also makes their degradation extremely slow.

Halogenated compounds have been used for a variety of purposes for hundreds of different industrial processes over the last 50 years, although they are dangerous for human health and include known toxins and potential carcinogens

as dioxins, pesticides and PCBs. One prevalent example of a halogenated organic compound is the widely used pesticide DDT, which has been shown to bioaccumulate in animal fat tissue, disrupt hormone function, and damage ecosystems. PCBs, polychlorinated biphenyls, are another type of halogenated organic compound widely used for industrial applications as coolants, lubricants, plasticizers, and dyes. PCBs are toxic chemicals and their carcinogenicity has been shown through laboratory studies. PCBs may also adversely affect human health by contributing to neurological, immune system, reproductive system, and other organs damage.

Among halogenated compounds, chlorinated solvents and their natural transformation products are prevalent ground water organic contaminants in the U.S. These solvents, consisting primarily of chlorinated aliphatic hydrocarbons (CAHs), have been widely used in factory processing as fumigants, pesticides, degreasing agents and solvents. They may pollute the environment through accidental spills and leaks or illegal dumping. Their relative solubility in water and their poor sorption, causes them to migrate downward through soils, thus contaminating groundwater. Many CAHs and their transformation products are defined as possible human carcinogens by the United States Environmental Protection Agency (U.S. EPA).

A major problem with halogenated compounds is that they belong to a class of molecules known as *persistent organic pollutants*, which tend to biodegrade very slowly. It was originally thought that there were no natural sources of halogenated compounds in the environment, hence no organisms had evolved to exploit them. On the contrary, it has recently been shown that this assumption was incorrect (organisms as well as volcanic eruptions can produce these compounds), and that natural production of chlorinated phenols may actually be greater than anthropogenic sources. Since these compounds have in fact existed for millions of years, there are naturally occurring strains of bacteria which have evolved to break down halogenated compounds, thus opening up the possibility for bioremediation treatment of contaminated sites.

### **Bioremediation principles**

Biological cleaning procedures make use of the fact that most organic chemicals are subject to enzymatic attack by living organisms. These activities are summarized under the term biodegradation. However, the end products of these enzymatic processes might differ drastically. For instance, an organic substance might be mineralized (i.e. transformed to carbon dioxide and water), but it might also be converted to a product that binds to natural materials in the soil, or to a toxic substance.

Bioremediation refers to the productive use of micro-organisms to remove or detoxify pollutants contaminating soils, water or sediments and threatening public health. Bioremediation is not new: micro-organisms have been used to remove organic matter and toxic chemicals from domestic and manufacturing waste discharge for many years. Indeed, micro-organisms are frequently the only means, biological or non-biological, to convert synthetic chemicals into inorganic compounds. What is new is the emergence of bioremediation as an industry that is driven by its particular usefulness for sites contaminated with petroleum hydrocarbons.

Over the past decade, opportunities for applying bioremediation to a much broader set of contaminants have been identified. Indigenous and enhanced organisms have been shown to degrade industrial solvents, polychlorinated biphenyls (PCBs), explosives, and many different agricultural chemicals. Pilot, demonstration, and full-scale applications of bioremediation have been carried out on a limited basis. Equally importantly, microorganisms that transform and sequester heavy metals and radionuclides have been identified and employed, to a limited extent, for in situ bioremediation. However, the full benefits of bioremediation have not been realized, because processes and organisms effective in controlled laboratory tests are not always equally effective in full-scale applications. The failure to perform optimally in the field setting stems from a lack of predictability due, in part, to inadequacies in the fundamental scientific understanding of how and why these bioremediation processes work.

## Content and objectives

Chlorinated aliphatic and aromatic hydrocarbons are among the most common contaminants of soils, groundwaters and sediments, and most of them are known or suspected carcinogen. Several studies showed that most of these pollutant can be biodegraded by single bacterial strains or mixed microbial populations via aerobic direct metabolism or cometabolism using aromatic and aliphatic hydrocarbons such as methane, propane, butane, phenol or toluene as growth substrates. In general, applications of *in situ* bioremediation can be grouped into *enhanced bioremediation* and *intrinsic bioremediation*. In enhanced bioremediation amendment such as oxygen, nutrients or even exogenous microorganisms (*bioaugmentation*) are provided to manipulate the microbial environment and facilitate biodegradation of contaminants. Conversely, intrinsic bioremediation depends on indigenous microflora to degrade contaminants without any amendments, exclusively relying on natural physical, chemical, and biological processes to reduce or attenuate contaminant concentrations.

In this thesis, two studies have been carried out concerning different situations where bioremediation processes of chlorinated hydrocarbons were involved. The first one dealt with an enhanced bioremediation situation while the second was related to intrinsic bioremediation.

- 1) The first experimental work (enhanced bioremediation) consisted in the **study of microbial consortia able to degrade a mixture of 6 CAHs** (chlorinated aliphatic hydrocarbons) via aerobic cometabolism. Several aspects of the long-term growth of these consortia were investigated. Biomass was grown in batch bioreactors for 150 days and, during this period, the maintenance of the ability to degrade the 6 CAHs mixture was tested. Furthermore the effectiveness of these consortia as inocula for the bioaugmentation of different types of aquifers was investigated. The reason why we were interested in the characterization of the behavior of these consortia was their potential usefulness as inocula in bioaugmentation treatment (in field scale or in pilot scale).
- 2) The second study (intrinsic bioremediation) dealt with **monochlorobenzene biodegradation in the interface between groundwater and surface water**.

It aimed at investigating the natural pollutant degradation capacity of the aquifer zone representing this interface. The interface can be considered a zone with changing redox conditions characterized by specific degradation potential for pollutants passing through as a result of steep physico-chemical gradients. Molecular techniques (PCR-DGGE) were also applied to characterize the structure of the microbial community harboured in the interface.

### **1. VOLATILE ORGANIC COMPOUNDS**

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#### **1.1 INTRODUCTION**

Within a physico-chemical context volatile organic compounds (VOCs) are defined as “any chemical compound based on carbon chains or rings (and also containing hydrogen) with a vapour pressure greater than 2 mm Hg at 25°C. These compounds may contain oxygen, nitrogen and other elements. Substances that are specifically excluded are: carbon dioxide, carbon monoxide, carbonic acid, carbonate salts, metallic carbides and methane” (Australian Department of Environment and Heritage, 2003). Within a regulatory context, USEPA provides this definition (under the Clean Air Act, published in the Code of Federal Regulation): “any compound of carbon, excluding carbon monoxide, carbon dioxide, carbonic acid, metallic carbides or carbonates, and ammonium carbonate, which participates in atmospheric photochemical reactions”. The IUPAC naming convention identifies two classes of VOCs: aliphatic hydrocarbons, having an open chain of carbon atoms (alkanes and alkenes) and aromatic hydrocarbons, characterized by an alternating carbon-carbon single and double bonds arranged in a ring structure. An alkane is a straight chain or cyclic (ring-like, such as cycloalkane) structure that consists of carbon-carbon and carbon-hydrogen single bonds. An alkene is typically a straight-chain structure that contains at least one carbon-carbon double bond. These double bonds impart more stability to the compound than the single bond in an alkane compound. A chlorinated alkane or alkene also contains at least one chlorine-carbon single bond while chlorinated aromatic compounds also contain one chlorine-carbon single bond (for example, chlorobenzene). Aromatic compounds are typically more resistant to degradation (more stable) than the alkane and alkene compounds.

The aliphatic and the aromatic hydrocarbons are commonly sub grouped even further based on the presence of attached halogen atoms (chlorine as chloro, bromine as bromo, or fluorine as fluoro) or functional groups including, but not limited to, alkyl radicals. The VOC subgroups include the alkyl benzenes (such as methylbenzene), chlorinated alkanes (such as 1,2-dichloroethane), chlorinated alkenes (such as 1,1-dichloroethene), and the chlorinated aromatics (such as 1,2-dichlorobenzene).

Halogenated or alkylated aromatics such as chlorobenzene or toluene are more easily degraded than benzene in aerobic and anaerobic ground water because the stability of the benzene ring is reduced and the ring is weakened (Borden and others, 1997). Adding halides or alkyl groups to the ring structure disperses the electrical charges from the carbon-carbon bonds on the ring and weakens that bond.

## ***1.2 VOLATILE ORGANIC COMPOUNDS DETECTED IN GROUNDWATER***

A relatively large amount of literature exists that describes VOCs in groundwater at specific, known areas of contamination. Few documents, however, describe VOC contamination in a regional or national context. One report by Arneft and others (1989) lists the top 15 VOCs detected in ground water near landfills in the United States and in Germany (table 1.1). This list shows that the VOCs contaminating groundwater near landfills are similar in both countries. Most of these VOCs are chlorinated solvents and gasoline compounds. Furthermore, the frequency of VOCs detected in representative studies completed on national, regional, and site-specific scales in the United States show a remarkable similarity to those in table 1.1 (table 1.2; Delzer and Ivahnenko, 2003; Moran, 2006; Zogorski and others, 2006). Although the number of VOCs analysed in ground-water samples is large for national and regional studies, the most commonly detected compounds, primarily chlorinated solvents and gasoline compounds, are similar to those at site-specific studies completed at U.S. Department of Defence installations (table 1.3).

The ten most commonly detected VOCs in the studies summarized in tables 1.2 and 1.3 are methyl *tert*-butyl ether (MTBE), tetrachloroethene (PCE), 1,1,2-trichloroethene (TCE), methylbenzene (toluene), 1,1,1-trichloroethane, (1,1,1-TCA), benzene, *cis*-1,2-dichloroethene (1,2-cDCE), 1,1-dichloroethane (1,1-DCA), *trans*-1,2-dichloroethene (1,2-tDCE), the dimethylbenzenes (*m*-, *o*-, *p*-xylenes).

**Table 1.1. Volatile organic compounds ranked by those frequently detected in groundwater near landfills and hazardous waste dumps in the United States and the Federal Republic of Germany<sup>1</sup>.**

Rank	United States of America		Federal Republic of Germany	
	IUPAC name <sup>2</sup>	Common or alternative name	IUPAC name <sup>2</sup>	Common or alternative name
1	1,1,2-trichloroethene	1,1,2-trichloroethylene, TCE	tetrachloroethene	perchloroethylene, tetrachloroethylene, PCE
2	tetrachloroethene	perchloroethylene, tetrachloroethylene, PCE	1,1,2-trichloroethene	1,1,2-trichloroethylene, TCE
3	<i>cis</i> -1,2-dichloroethene	<i>cis</i> -1,2-DCE	<i>trans</i> -1,2-dichloroethene	<i>trans</i> -1,2-DCE
4	benzene	benzene	trichloromethane	—
5	chloroethene	vinyl chloride	1,1-dichloroethene	1,1-dichloroethylene, DCE
6	trichloromethane	—	dichloromethane	methylene chloride
7	1,1,1-trichloroethane	methyl chloroform	1,1,1-trichloroethane	methyl chloroform
8	dimethylbenzene	xylene	1,1-dichloroethane	ethylene dichloride
9	<i>trans</i> -1,2-dichloroethene	<i>trans</i> -1,2-dichloroethylene	1,2-dichloroethane	ethylene dichloride
10	methylbenzene	toluene	phenol	—
11	ethylbenzene	ethylbenzene	acetone	dimethyl ketone, 2-propanone, and beta-ketopropane
12	dichloromethane	methylene chloride	toluene	methyl benzene
13	dichlorobenzene, total	—	bis-(2-ethylhexyl)-phthalate	—
14	chlorobenzene	chlorobenzene	benzene	benzene
15	tetrachloromethane	carbon tetrachloride	chloroethene	vinyl chloride

<sup>1</sup>Arneth and others, 1989, p. 399  
<sup>2</sup>International Union of Pure and Applied Chemistry, 2006

Table 1.2. Volatile organic compounds detected in regional and national groundwater studies in the United States.

[µg/L, micrograms per liter; [12], percentage of samples above the analytical reporting limit; <, less than; ND, not detected above analytical reporting level]

Rank	Statewide, ground water in Wisconsin <sup>1</sup>	Ground water in the Santa Ana River Basin, California <sup>2</sup>	Ground-water and drinking-water supply wells in the United States (concentrations greater than 0.2 µg/L) <sup>3</sup>		
			Aquifer studies <sup>4</sup>	Domestic water-supply wells <sup>5</sup>	Public water-supply wells <sup>6</sup>
1	dichloromethane [16.3]	1,1,2-trichloroethene (TCE) [12]	tetrachloroethene (PCE) [3.7]	2-methoxy-2-methylpropane (MTBE) [2.9]	2-methoxy-2-methylpropane (MTBE) [5.4]
2	1,1-dichloroethane [13.6]	1,1,1-trichloroethane [10.5]	2-methoxy-2-methylpropane (MTBE) [2.8]	tetrachloroethene (PCE) [2.0]	tetrachloroethene (PCE) [5.3]
3	<i>cis</i> -1,2-dichloroethene, 1,1-dichloroethane [13.6]	tetrachloroethene (PCE) [9.1]	1,1,2-trichloroethene (TCE) [2.6]	1,1,1-trichloroethane [1.4]	1,1,2-trichloroethene (TCE) [4.3]
4	1,1,2-trichloroethene (TCE) [13.3]	1,1-dichloroethene [5.7]	methylbenzene [1.9]	methylbenzene [1.0]	1,1,1-trichloroethane [2.2]
5	methylbenzene [11.6]	2-methoxy-2-methylpropane (MTBE) [5.3]	1,1,1-trichloroethane [1.7]	chloromethane [0.97]	1,1-dichloroethane [2.0]
6	tetrachloroethene (PCE) [9.8]	<i>cis</i> -1,2-dichloroethene [4.3]	chloromethane [1.1]	1,1,2-trichloroethene (TCE) [0.92]	<i>cis</i> -1,2-dichloroethene [1.5]
7	benzene [8.5]	methylbenzene [3.8]	<i>trans</i> -1,2-dichloroethene [0.91]	dichloromethane [0.67]	1,1-dichloroethene (DCE) [1.3]
8	chloroethene [8.0]	1,1-dichloroethane [2.9]	dichloromethane [0.89]	1,2,4-trimethylbenzene [0.32]	<i>trans</i> -1,2-dichloroethene [1.0]
9	1,3- and 1,4-dimethylbenzenes [7.9]	benzene [1.4]	1,1-dichloroethane [0.86]	1,1-dichloroethane [0.29]	methylbenzene [1.0]
10	1,1,1-trichloroethane [7.8]	1,2-dimethylbenzene [1.4]	1,1-dichloroethene [0.66]	benzene, 1,2-dichloroethane [0.21]	tetrachloromethane [0.73]
11	ethylbenzene [7.6]	1,3- and 1,4-dimethylbenzene [1.4]	benzene [0.63]	tetrachloromethane [0.21]	1,3- and 1,4-dimethylbenzene [0.60]
12	1,2,4-trimethylbenzene [7.1]	<i>trans</i> -1,2-dichloroethene [<1]	1,2,4-trimethylbenzene [0.63]	1,1-dichloroethene [0.21]	1,2-dichloroethane [0.56]
13	1,2-dimethylbenzene [6.8]	dichloromethane [<1]	1,2-dichloroethane [0.47]	total xylenes [0.21]	1,2-dimethylbenzene [0.48]
14	chloromethane [6.7]	ethylbenzene [<1]	<i>cis</i> -1,2-dichloroethene [0.42]	<i>cis</i> -1,2-dichloroethene [0.18]	benzene, dichloromethane, ethylbenzene [0.46]
15	naphthalene [6.5]	naphthalene [<1]	total xylenes [0.38]	naphthalene [0.15]	chloromethane [0.38]
16	chloroethane [6.3]	tetrachloromethane [<1]	tetrachloromethane [0.31]	ethylbenzene [0.12]	1,2,4-trimethylbenzene [0.32]
17	chlorobenzene [4.3]	1,2,4-trimethylbenzene [<1]	chloroethane [0.29]	chloroethane [0.093]	chloroethane [0.28]
18	1,2-dichloroethane [3.7]	chlorobenzene [ND]	chloroethene [0.26]	chloroethene [0.083]	vinyl benzene [0.19]
19	<i>trans</i> -1,2-dichloroethene [3.3]	chloroethane [ND]	ethylbenzene [0.26]	<i>trans</i> -1,2-dichloroethene [0.045]	chlorobenzene, 1,2-dichlorobenzene [0.18]
20	1,1-dichloroethene (DCE) [2.6]	chloromethane [ND]	chlorobenzene [0.17]	chlorobenzene [0.042]	chloroethene [0.18]
21	1,2-dichlorobenzene [2.4]	chloroethene [ND]	naphthalene [0.16]	1,2-dichlorobenzene [0.042]	naphthalene [0.10]
22	2-methoxy-2-methylpropane (MTBE) [2.3]	1,2-dichlorobenzene [ND]	1,2-dichlorobenzene [0.12]	vinyl benzene [ND]	1,2,4-trichlorobenzene [ND]
23	tetrachloromethane [1.8]	1,2-dichloroethane [ND]	vinyl benzene [ND]	1,2,4-trichlorobenzene [ND]	1,3-dichlorobenzene [ND]
24	vinyl benzene [1.2]	1,2,3-trichlorobenzene [ND]	1,2,3-trichlorobenzene [ND]	1,1,2-trichloroethane [ND]	1,1,2-trichloroethane [ND]
25	1,2,4-trichlorobenzene, 1,1,2-trichloroethane [<1.0]	vinyl benzene [ND]		1,2,3-trichlorobenzene [ND]	1,2,3-trichlorobenzene [ND]

<sup>1</sup>1,305–4,086 samples (Wisconsin Department of Natural Resources, 2000)  
<sup>2</sup>9–112 samples (Hamlin and others, 2002)  
<sup>3</sup>Zogorski and others, 2006  
<sup>4</sup>1,710–3,498 samples  
<sup>5</sup>1,190–1,208 samples  
<sup>6</sup>828–1,096 samples

**Table 1.3. Volatile organic compounds detected in groundwater case studies at selected U.S. Department of Defence installations.**

[[43.3], percentage of samples with a detected concentration; ND, not detected above analytical reporting level]				
Rank	Dover Air Force Base, Maryland <sup>1</sup>	U.S. Army Armament Research and Development Center, Picatinny, New Jersey, 1958–85 <sup>2</sup>	U.S. Naval Undersea Warfare Center, Washington, D.C. <sup>3</sup>	Wright-Patterson Air Force Base, Ohio, 1993–94 <sup>4</sup>
1	2-methoxy-2-methylpropane (MTBE) [25.5]	1,1,2-trichloroethene (TCE) [58.5]	chloroethene [64]	1,1,2-trichloroethene (TCE) [12.5]
2	<i>cis</i> -1,2-dichloroethene [21.7]	tetrachloroethene (PCE) [24.9]	<i>cis</i> -1,2-dichloroethene [59]	tetrachloroethene (PCE) [5.8]
3	1,1,2-trichloroethene (TCE) [20.3]	<i>trans</i> -1,2-dichloroethene (DCE) [18.6]	<i>trans</i> -1,2-dichloroethene [44.8]	1,1,1-trichloroethane [2.3]
4	tetrachloroethene (PCE) [13.7]	1,1,1-trichloroethane [16.8]	1,1,2-trichloroethene (TCE) [40.4]	chloromethane [2.3]
5	benzene [10.4]	1,1-dichloroethane [9.6]	total BTEX compounds [40.1]	<i>cis</i> - and <i>trans</i> -1,2-dichloroethene [1.2]
6	methylbenzene [6.6]	<i>cis</i> -1,2-dichloroethene (DCE) [9.6]	1,1-dichloroethane [37.2]	chloroethene [9]
7	dimethylbenzenes ( <i>m</i> -, <i>p</i> -xylene) [3.7]	methylbenzene (toluene) [4.4]	chloroethane [33.9]	dichloromethane [9]
8	ethylbenzene [2.3]	benzene [2.6]	1,1-dichloroethene [31.3]	methylbenzene [6]
9	chloroethene [ND]	—	tetrachloroethene (PCE) [9.6]	benzene [3]
10	—	—	1,1,1-trichloroethane [6.9]	chloroethane [3]
11	—	—	—	tetrachloromethane [3]

<sup>1</sup>212 samples (Barbaro and Neupane, 2001; Guertal and others, 2004)  
<sup>2</sup>607 samples (Sargent and others, 1986)  
<sup>3</sup>121–179 samples (Dinicola and others, 2002)  
<sup>4</sup>343 samples (Schalk and others, 1996)

### 1.3 CHLORINATED ALIPHATIC HYDROCARBONS (CAHs)

#### 1.3.1 Sources of CAHs

CAHs are manmade organic compounds, typically manufactured from methane, ethane, ethene and chlorine through various processes that substitute one or more hydrogen atoms with a chlorine atom, or selectively dechlorinate chlorinated compounds to a less chlorinated state.

CAHs are typically used in the manufacturing of industrial, chemical, electronic, and consumer goods (Smith and others, 1988; U.S. Environmental Protection Agency, 2005b). In addition, these compounds are heavily used as solvents in cleaning and degreasing products. For example, 1,1,1-TCA is used as a solvent for adhesives and in metal degreasing, pesticides, textile processing, cutting fluids, aerosols, lubricants, cutting oil formulations, drain cleaners, shoe polishes, spot cleaners, printing inks, and stain repellents. Carbon tetrachloride

(CTET) was used as feedstock for the production of chlorofluorocarbon gases, such as dichlorodifluoromethane (F-12) and trichlorofluoromethane (F-11), which were used as aerosol propellants in the 1950s and 1960s (Holbrook, 1992). During 1974, the U.S. Food and Drug Administration (FDA) banned the sale of CTET in any product used in the home and the USEPA regulated the use of chlorofluorocarbon gases as aerosols or propellants. By 2000, CTET production for no feedstock purposes was phased-out completely.

Chemical manufacturing is the largest use of 1,1-DCA and 1,2-dichloroethane (1,2-DCA). Both compounds serve as an intermediate during the manufacture of chloroethene (vinyl chloride, VC), 1,1,1-TCA, and to a lesser extent high-vacuum rubber. Both DCA isomers also are used as a solvent for plastics, oils, and fats, and in cleaning agents and degreasers (Agency for Toxic Substances and Disease Registry, 1990c, p. 51; 2001, p.160). About 98% of the 1,2-DCA produced in the United States is used to manufacture VC. Smaller amounts of 1,2-DCA are used in the synthesis of vinylidene chloride, TCE, PCE, aziridines, and ethylene diamines, and in other chlorinated solvents (U.S. Environmental Protection Agency, 1995).

1,1,1-TCA was initially developed as a safer solvent to replace other chlorinated and flammable solvents. The compound is used as a solvent for adhesives (including food packaging adhesives) and in metal degreasing, pesticides, textile processing, cutting fluids, aerosols, lubricants, cutting formulations, drain cleaners, shoe polishes, spot cleaners, printing inks, and stain repellents, among other uses (Agency for Toxic Substances and Disease Registry, 2004, p. 181). The other TCA isomer, 1,1,2-trichloroethane (1,1,2-TCA), has limited use as a common, general-use solvent but is used in the production of chlorinated rubbers (Archer, 1979). In some cases, 1,1,2-TCA may be sold for use in consumer products (Agency for Toxic Substances and Disease Registry, 1989, p.59).

Before 1979, the single largest use of chloroethane was in the production of tetraethyl lead. As recently as 1984, the domestic production of tetraethyl lead accounted for about 80 percent of the chloroethane consumed in the United States, whereas about 20 percent was used to produce ethyl cellulose, and used in

solvents, refrigerants, topical anaesthetics, and in the manufacture of dyes, chemicals, and pharmaceuticals. Since the 1979 ban on tetraethyl lead in gasoline and its subsequent phase out in the mid-1980, the production of chloroethane in recent years has declined substantially in the United States (Agency for Toxic Substances and Disease Registry, 1998, p. 95).

Among the chloroethenes, PCE and TCE are two of the most widely used and distributed solvents in the United States and Europe. The textile industry uses the largest amount of PCE during the processing, finishing of raw and finished textiles, and for industrial and consumer dry cleaning (U.S. Environmental Protection Agency, 2005b, Web page: [http://www.epa.gov/opptintr/chemfact/f\\_perchl.txt](http://www.epa.gov/opptintr/chemfact/f_perchl.txt), accessed May 23, 2006). Most of the TCE used in the United States is for vapour degreasing of metal parts and some textiles (U.S. Environmental Protection Agency, 2005b, Web page: <http://www.epa.gov/OGWDW/dwh/t-voc/trichlor.html>, accessed May 23, 2006). Other uses of PCE and TCE include manufacturing of pharmaceuticals, other organic compounds, and electronic components, and in paint and ink formulations (Smith and others, 1988).

Historical management of wastes containing CAHs has resulted in contamination of soil and groundwater, with CAHs present at many contaminated groundwater sites in the United States. TCE and PCE are the most prevalent of those contaminants (U.S. Air Force 1998). In addition, CAHs and their degradation products, including dichloroethane (DCA), dichloroethene (DCE), and vinyl chloride (VC) tend to persist in the subsurface. Table 1.4 lists the CAHs more commonly identified as environmental contaminants, their abbreviations, their common names, and the types of waste from which they commonly originate. Figure 1.1 presents the molecular structure of those CAHs.

Table 1.4. CAHs commonly identified as environmental contaminants

Name	Common Name(s)	Abbreviation <sup>1</sup>	Common Waste Sources
<b>CHLORINATED ETHENES</b>			
Tetrachloroethene(-ethylene)	<i>Perchloroethene</i>	PCE	Solvent waste
<i>Trichloroethene(-ethylene)</i>	None	TCE	Solvent waste, degradation product of PCE
<i>cis-1,2-Dichloroethene(-ethylene)</i>	Acetylene dichloride	cis-DCE	Solvent waste, degradation product of PCE and TCE
<i>trans-1,2-Dichloroethene(-ethylene)</i>	Acetylene dichloride	trans-DCE	Solvent waste, degradation product of PCE and TCE
<i>1,1-Dichloroethene(-ethylene)</i>	Vinylidene chloride	1,1-DCE	Solvent waste, degradation product of 1,1,1-TCA
Chloroethene(-ethylene)	<i>Vinyl chloride</i>	VC	Polyvinyl chloride production waste, degradation product of PCE and 1,1,1-TCA
<b>CHLORINATED ETHANES</b>			
<i>1,1,1-Trichloroethane</i>	Methyl chloroform	1,1,1-TCA	Solvent waste
<i>1,1,2-Trichloroethane</i>	Vinyl trichloride	1,1,2-TCA	Solvent waste
<i>1,2-Dichloroethane</i>	Ethylene chloride	1,2-DCA	Solvent waste, degradation product of 1,1,2-TCA
<i>1,1-Dichloroethane</i>	Ethylidene chloride	1,1-DCA	Degradation product of 1,1,1-TCA
<i>Chloroethane</i>	None	CA	Refrigerant waste, tetraethyl lead manufacturing waste, degradation product of 1,1,1-TCA and 1,1,2-TCA
<b>CHLORINATED METHANES</b>			
Tetrachloromethane	<i>Carbon tetrachloride</i>	CT	Solvent waste, fire extinguisher waste
Trichloromethane	<i>Chloroform, methane trichloride</i>	CF	Solvent waste, anesthetic waste, waste degradation product of CT
Dichloromethane	<i>Methylene chloride, methylene dichloride</i>	MC	Solvent waste, degradation product of CT
<i>Chloromethane</i>	Methyl chloride, monochloromethane	CM	Refrigerant waste, degradation product of CT
<p><u>Notes:</u>  <sup>1</sup>Abbreviations are based on the names in bold italic type.  Sources: Sawyer and others 1994; Merck 1989</p>			

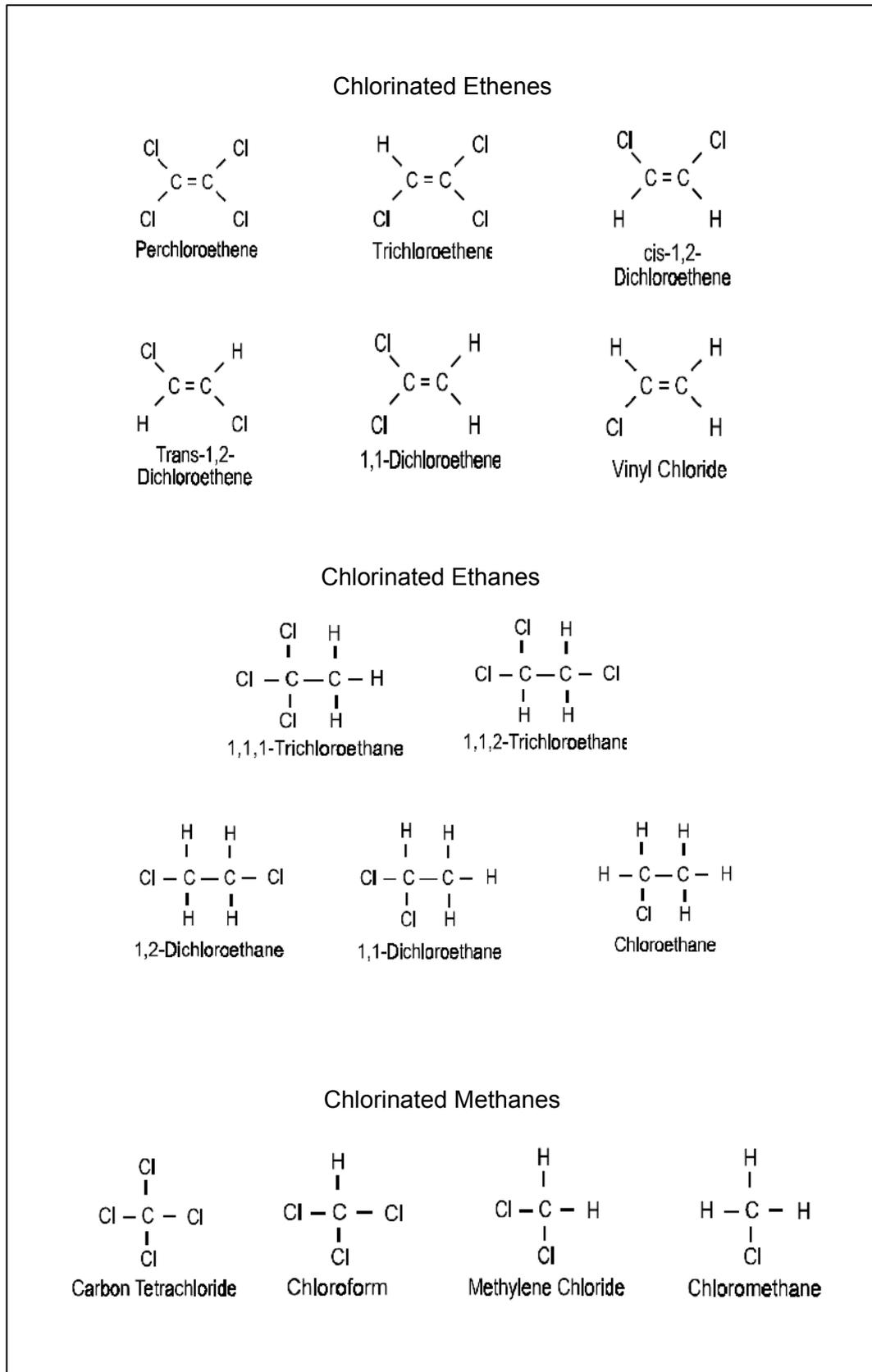


Figure 1.1. Molecular structures of common CAHs

### 1.3.2 Physical and chemical properties

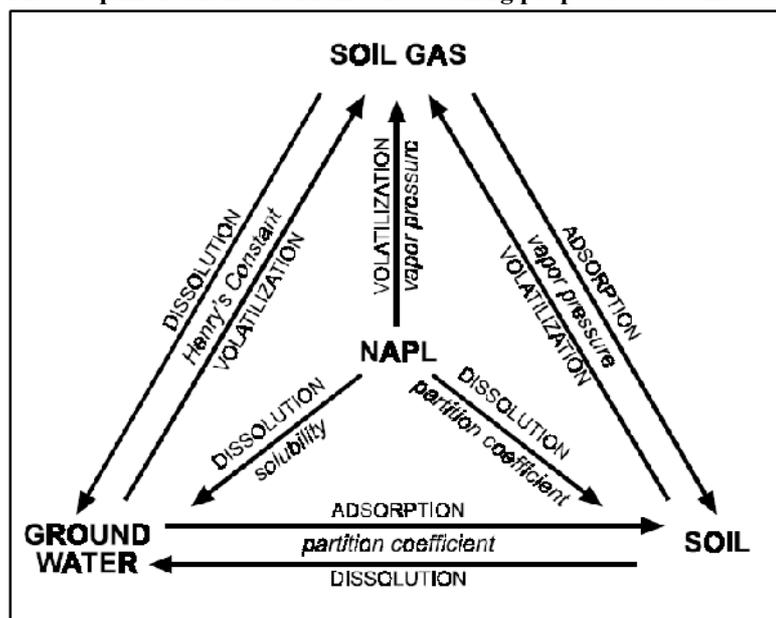
The physical and chemical properties of CAHs govern their fate and transport in the subsurface environment; the number of chlorine atoms directly affects the physical and chemical behaviour of the compound: as the number of substituted chlorine atoms increases, molecular weight and density generally increases, and vapour pressure and aqueous solubility generally decreases. In table 1.5 the major physical and chemical data for the CAHs commonly identified as subsurface contaminants are listed.

### 1.3.3 Transport processes

The extent of the contaminant spreading into the environment is affected by the physical and chemical properties of the compound (in particular solubility, volatility and density), besides the specific characteristics of the site.

A CAH released to the subsurface as a pure organic liquid (NAPL – Non Aqueous Phase Liquid) will reach phase equilibrium, and it will remain as a NAPL, adsorb to soil, dissolve in groundwater, or volatilise to soil gas to the extent defined by the physical and chemical properties of the individual CAH and the subsurface environment. Figure 1.2 shows the mechanisms by which CAHs transfer phases in the attempt to reach equilibrium conditions.

Figure 1.2. Phase equilibrium mechanisms and defining properties of CAHs



In particular it can be observed that:

- partition coefficient (related to the hydrophobicity and the solubility) define the extent to which a CAH will partition between NAPL and soil, and NAPL adsorbed to soil and the groundwater;
- the aqueous solubility of a CAH defines the equilibrium between NAPL and groundwater;
- CAHs dissolved in the groundwater will partition themselves between the dissolved phase and the vapour phase, as defined by the Henry's constant;
- vapour pressure describes the equilibrium between NAPL or NAPL adsorbed to soil and the soil gas.

Most of the CAHs are denser than water (referred to as dense non-aqueous phase liquids - DNAPLs) and tend to sink through both unsaturated and saturated permeable soils until they reach the impermeable layer at the bottom of the aquifer. Those kinds of free phases are located in the deepest layers and are therefore extremely difficult to identify and locate, thus making both biological and physico-chemical remediation more difficult. Moreover the dense non-aqueous phase (NAPL or DNAPL) acts as a local polluting source gradually releasing the contaminant in the aquifer. The contaminant concentrations observed next to NAPL (next to water solubility) could then be toxic for microorganisms, thus making a biological intervention more difficult or even impossible.

In addition to transferring phases in an attempt to reach equilibrium conditions, CAHs can migrate in the subsurface in their non-aqueous, aqueous and vapour phase by both active and passive processes. In active processes such as advection and dispersion, CAHs migrate along with the flow of the groundwater or soil gas to which they are partitioned. Passive processes, such as diffusion, are the result of concentration gradients, which cause the CAH to seek phase and concentration equilibrium with its surrounding environment. Typically, releases of CAH to the groundwater result in the formation of a plume and advection is one of the most important processes affecting the transport of the contaminants.

Table 1.5. Chemical and physical properties of CAHs.

Compound	Number of Substituted Chlorine Atoms	Molecular Weight (g/mole) <sup>1</sup>	Liquid Density (g/ml @ 20 °F/4 °C) <sup>1</sup>	Aqueous Solubility (mg/L @ approx. 25 °C) <sup>2</sup>	Vapor Pressure (mm Hg @ 25 °C) <sup>2</sup>	Log K <sub>ow</sub> (Octanol/Water Partition Coefficient) <sup>3</sup>	Henry's Law Constant (atm-m <sup>3</sup> /mol) <sup>3</sup>
<b>Chlorinated Ethenes</b>							
PCE	4	165.8	1.62	150	17.8	2.60	0.0153
TCE	3	131.4	1.46	1,100	57.9	2.38	0.0091
cis-DCE	2	96.9	1.28	3,500	208	0.70	0.0037
trans-DCE	2	96.9	1.28	6,300	324	0.48	0.0072
1,1-DCE	2	96.9	1.21	2,250	600	1.84	0.018
VC	1	62.5	gas	2,670	2,660	1.38	0.315 (5)
<b>Chlorinated Ethanes</b>							
1,1,1-TCA	3	133.4	1.34	1,500	123	2.50	0.008
1,1,2-TCA	3	133.4	1.44	4,500	30	2.47	0.0012
1,2-DCA	2	99.0	1.26	8,520	64	1.48	0.00098
1,1-DCA	2	99.0	1.18	5,500	182	1.79	0.0059
CA	1	64.5	gas	5,700	1,064	1.52 to 2.16 (4)	0.0085
<b>Chlorinated Methanes</b>							
CT	4	153.8	1.59	757	90	2.64	0.0304
CF	3	119.4	1.48	8,200	151	1.97	0.00435
MC	2	84.9	1.33	20,000	362	1.30	0.00268
CM	1	50.5	gas	6,500	4,310	0.95	0.0452 (5)

**Notes:**

1. Data from Merck Index 1989
2. Data from EPA 1986
3. Data from Gossett 1987
4. Data from EPA 1998
5. Data from EPA 1998a

"Gas" is indicated for liquid density of VC, CA, and CM because they are pure compounds that are gasses under typical environmental conditions.

### 1.3.4 Transformation of CAHs

In the natural environment CAHs may undergo chemical and biological transformations. The chlorinated alkanes can be degraded by *abiotic processes* through hydrolysis or dehydrohalogenation (with no external transfer of electrons) or by *biotic processes* through reductive dechlorination or (direct and cometabolic) aerobic oxidation; (oxidation-reduction reactions, requiring an external transfer of electrons). These degradation processes can proceed under either aerobic or anaerobic conditions (Vogel and McCarty, 1987a; Vogel, 1994). According to McCarty (1997), 1,1,1-TCA is the only chlorinated compound that can be degraded in groundwater within 20 years under all likely groundwater or aquifer conditions. Oxidation-reduction reactions are the dominant mechanisms driving VOC degradation and most of these reactions are catalyzed by microorganisms (Wiedemeier and others, 1998; Azadpour-Keeley and others, 1999). Substitution reactions that can remove chlorine atoms, such as hydrolysis, can degrade some chlorinated alkanes (trichloroethane) to nonchlorinated alkanes (ethane) with or without a microbial population catalyzing the reaction (Vogel and others, 1987; Olaniran and others, 2004).

While aerobic oxidation and anaerobic reductive dechlorination can occur naturally under proper conditions, enhancements such as the addition of electron donors, electron acceptors, or nutrients help to provide the proper conditions for aerobic oxidation or anaerobic reductive dechlorination to occur. In general, highly chlorinated CAHs degrade primarily through reductive reactions, while less chlorinated compounds degrade primarily through oxidation (Vogel and others 1987b). Highly chlorinated CAHs are reduced relatively easily because their carbon atoms are highly oxidized. During direct reactions, the microorganism causing the reaction gains energy or grows as the CAH is degraded or oxidized. During cometabolic reactions, the CAH degradation or oxidation is caused by an enzyme or cofactor produced during microbial metabolism of another compound. CAH degradation or oxidation does not yield any energy or growth benefit for the microorganism mediating the cometabolic reaction. The degradation mechanisms that typically occur in the degradation of

each CAH are summarized in table 1.6 while table 1.7 shows biological degradation mechanisms.

Table 1.6. Common abiotic and biotic reactions involving halogenated aliphatic hydrocarbons.

Reaction	Mechanism	Scheme
I. Substitution	(a) solvolysis, hydrolysis	$RX + H_2O \Rightarrow ROH + HX$
	(b) conjugation and other nucleophilic reactions	$RX + N^- \Rightarrow RN + X^-$
II. Dehydrohalogenation		$\begin{array}{c}   \quad   \quad \backslash \quad / \\ -C-C- \Rightarrow C=C + HX \\   \quad   \quad / \quad \backslash \\ \quad \quad X \quad H \end{array}$
III. Oxidation	(a) $\alpha$ -hydroxylation	$\begin{array}{c}   \\ -C-X + H_2O \Rightarrow -C-X + 2H^+ + 2e^- \\   \quad \quad \quad   \\ H \quad \quad \quad OH \end{array}$
	(b) halosyl oxidation	$\begin{array}{c}   \\ -C-X + H_2O \Rightarrow -C-X^+O^- + 2H^+ + 2e^- \\   \quad \quad \quad   \end{array}$
	(c) epoxidation	$\begin{array}{c} X \quad \quad \quad O \quad X \\ \backslash \quad / \quad \quad \backslash \quad / \\ C=C + H_2O \Rightarrow C-C + 2H^+ + 2e^- \\ / \quad \backslash \quad \quad / \quad \backslash \end{array}$
	(d) biohalogenation	$\begin{array}{c} \quad \quad \quad \quad \quad \quad OH \quad X \\ \backslash \quad / \quad \quad \quad \quad \quad \quad   \quad   \\ C=C + X^- + H_2O \Rightarrow C-C + H^+ + 2e^- \\ / \quad \backslash \quad \quad \quad \quad \quad \quad   \quad   \end{array}$
IV. Reduction	(a) hydrogenolysis	$RX + H^+ + 2e^- \Rightarrow RH + X^-$
	(b) dihalo-elimination	$\begin{array}{c}   \quad   \\ -C-C- + 2e^- \Rightarrow C=C + 2X^- \\   \quad   \\ X \quad X \end{array}$
	(c) coupling	$2RX + 2e^- \Rightarrow R-R + 2X^-$

Table 1.7. Chemical and physical properties of CAHs.

CAH	Aerobic Oxidation		Anaerobic Reductive Dechlorination	
	Direct	Cometabolic	Direct	Cometabolic
<b>CHLORINATED ETHENES</b>				
PCE	X	X	●	●
TCE	X	●	●	●
cis-DCE	X	●	●	●
trans-DCE	X	●	●	●
1,1-DCE	X	●	●	●
VC	●	●	●	●
<b>CHLORINATED ETHANES <sup>1</sup></b>				
1,1,1-TCA	X	●	X	●
1,2-DCA	●	X	X	●
1,1-DCA	●	X	X	●
CA	X	X	X	X
<b>CHLORINATED METHANES</b>				
CT	X	X	X	●
CF	X	●	X	●
MC	●	●	●	●
CM	●	●	X	X

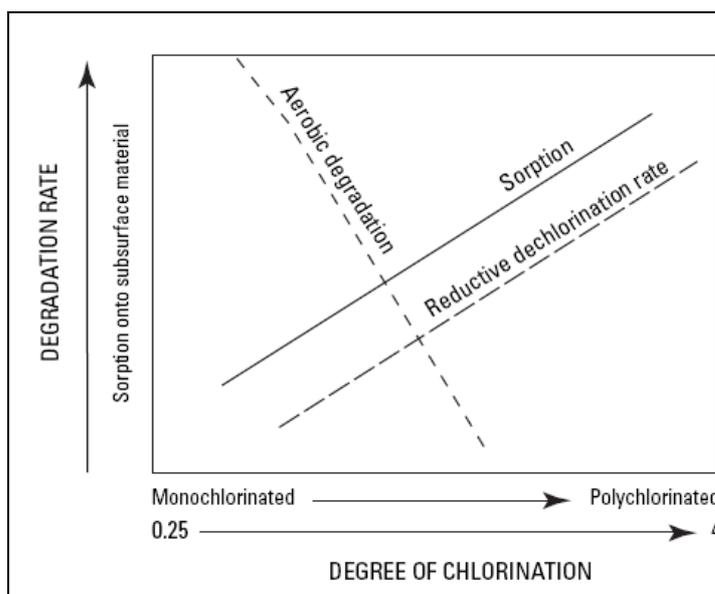
<sup>1</sup>Insufficient information was available for 1,1,2-TCA.  
**KEY:**  
 ● Typically occurring  
 X Not typically occurring  
 Sources: RTDF 1997; IIRC 1998; EPA 1998

### 1.3.4.1 Abiotic degradation processes

The abiotic processes occurring most frequently under either aerobic or anaerobic conditions are hydrolysis and dehydrohalogenation. Abiotic transformations generally result only in a partial transformation of the compounds that are either more readily or less readily biodegraded by microorganisms.

Hydrolysis and dehydrohalogenation are two abiotic processes that may degrade chlorinated ethanes under either aerobic or anaerobic conditions. The tendency for a chlorinated ethane to degrade by hydrolysis depends on the ratio of chlorine to carbon atoms (figure 1.3) or the location of chlorine atoms on the number 2 carbon in the compound. Chlorinated alkanes are more easily hydrolyzed when the chlorine-carbon ratio is less than two or when chlorine atoms are only located on the number 1 carbon atom (Vogel and McCarty, 1987b; Vogel,

1994). For example, chloroethane and 1,1,1-TCA have half-lives that are measured in days or months (Vogel and others, 1987; Vogel, 1994; table 1.8). Conversely, the more chlorinated ethanes such as 1,1,1,2-tetrachloroethane (PCA) and those with chlorine atoms on the number 2 carbon tend to have half-lives measured in decades or centuries (table 1.8). Dehydrohalogenation is the removal of one or two halogen atoms from an alkane (Vogel and McCarty, 1987a). The dehydrohalogenation of two chlorine atoms is called dichloroelimination.



**Figure 1.3. Relation between degree of chlorination and anaerobic reductive-dechlorination, aerobic degradation and sorption onto subsurface material. Degree of chlorination is number of chloride atoms divided by number of carbon atoms.**

**Table 1.8. Laboratory half-lives and by-products of the abiotic degradation (hydrolysis or dehydrohalogenation) of chlorinated alkane compounds detected in groundwater**

[IUPAC, International Union of Pure and Applied Chemistry; —, not applicable]			
Compound (IUPAC name) <sup>1</sup>	Degradation by-products	Half-life	Literature reference
chloroethane	ethanol	44 days	Vogel and others, 1987
1,1-dichloroethane	—	61 years	Jeffers and others, 1989
1,2-dichloroethane	—	72 years	Jeffers and others, 1989
1,1,1-trichloroethane	acetic acid; 1,1-dichloroethane	1.1–2.5 years	Mabey and Mill, 1978; Jeffers and others, 1989; Vogel and McCarty, 1987a,b
1,1,2-trichloroethane	1,1-dichloroethane	140 years	Jeffers and others, 1989
1,1,1,2-tetrachloroethane	trichloroethene	47–380 years	Mabey and Mill, 1978; Jeffers and others, 1989
1,1,2,2-tetrachloroethane	1,1,2-trichloroethane; trichloroethene	146–292 days	Mabey and Mill, 1978; Jeffers and others, 1989

<sup>1</sup>International Union of Pure and Applied Chemistry, 2006

Chen and others (1996) show that PCA can be abiotically transformed to TCE under methanogenic conditions (figure 1.4). In addition, the abiotic degradation of 1,1,1-TCA has been well studied in the scientific literature (figure 1.5; Jeffers and others, 1989; McCarty and Reinhard, 1993; Chen and others, 1996; McCarty, 1997). McCarty and Reinhard (1993) indicate that the transformation of 111-TCA by hydrolysis is about four times faster than by dehydrochlorination. During abiotic degradation, about 80 percent of 1,1,1-TCA is transformed to acetic acid by hydrolysis (McCarty, 1997), and the remaining 20 percent is transformed to 1,1-DCE by dehydrochlorination (Vogel and McCarty, 1987b; McCarty, 1997). The presence of 1,1-DCE in contaminated groundwater is probably the result of the dehydrochlorination of 1,1,1-TCA (McCarty, 1997).

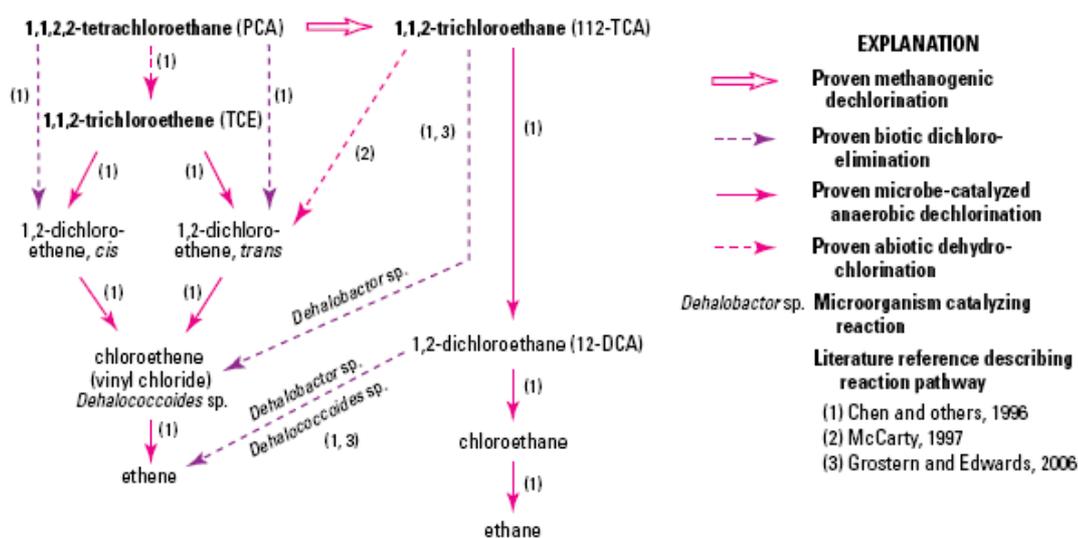


Figure 1.4. Laboratory-derived pathway for the abiotic degradation, anaerobic and methanogenic biodegradation of 1,1,2-tetrachloroethane; 1,1,2-trichloroethane; and 1,1,2-trichloroethane.

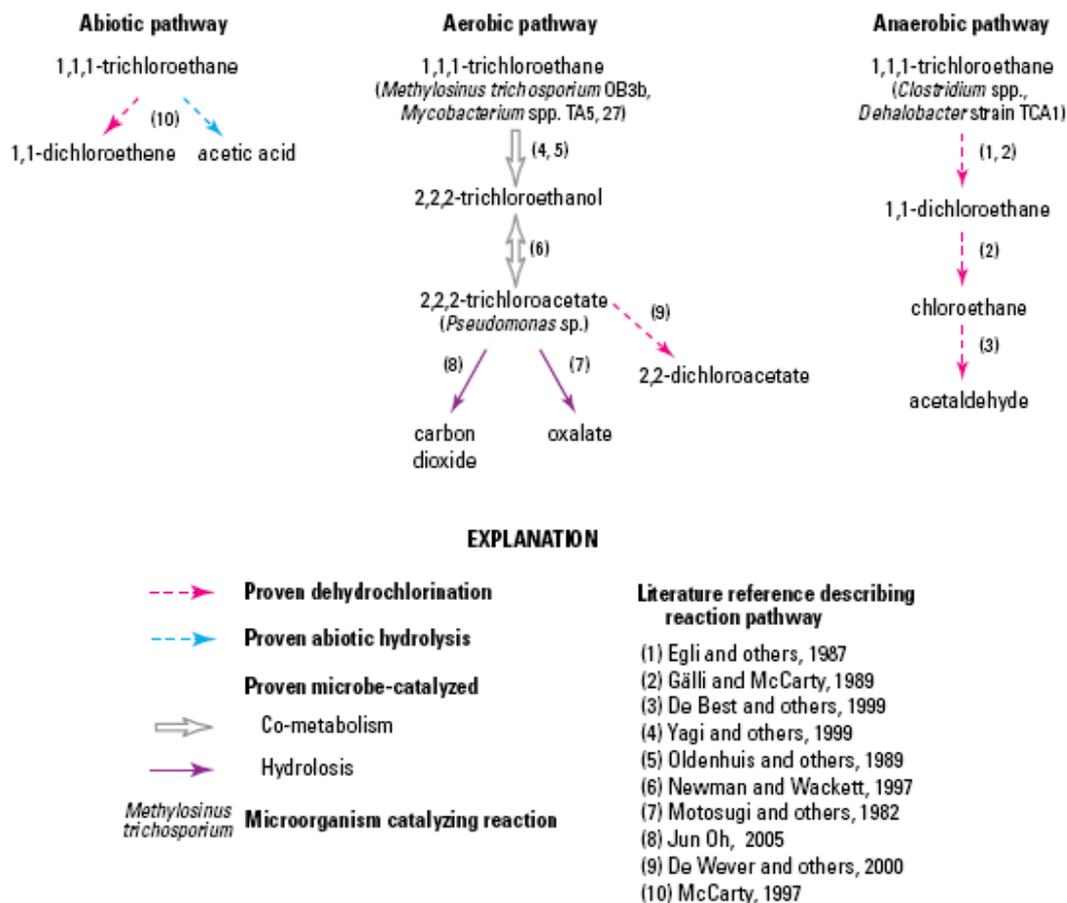


Figure 1.5. Laboratory-derived pathway for the abiotic degradation, anaerobic and methanogenic biodegradation of 1,1,1-trichloroethane.

#### 1.3.4.2 Biotic degradation processes

Bacteria transform environmentally available nutrients to forms that are useful for incorporation into cells and synthesis of cell polymers. Biotic transformations occur through reactions involving a transfer of electrons between the chlorinated solvents and an external agent; energy is made available when an electron donor transfers its electrons to a terminal electron acceptor. The energy gained is stored as high energy compounds, such as ATP and low-energy compounds, such as nicotinamide adenine dinucleotide (NAD). A portion of the stored energy is used to conduct biological processes necessary for cell maintenance and reproduction. In addition, cell building-block materials are required in the form of carbon and other nutrients (such nitrogen and phosphorus). The terminal electron acceptor used during metabolism is important for establishing the redox conditions, and therefore the type of zone that will dominate in the subsurface. Common terminal electron acceptors include oxygen under aerobic conditions and nitrate, Mn(IV), Fe(III), sulphate and carbon dioxide under anaerobic conditions.

The typical electron-acceptor classes of bacteria are listed in table 1.9 in the order of those causing the largest energy generation during the redox reaction to those causing the smallest energy generation during the redox reaction. A bacteria electron acceptor class causing a redox reaction generating relatively more energy will dominate over a bacteria electron acceptor class causing a redox reaction generating relatively less energy (Table 1.9). Aerobic biotic transformations generally are oxidations: they are classified as “hydroxylations”, in the case of a substitution of a hydroxyl group on the molecule, or “epoxidations”, in the case of unsaturated CAHs. The anaerobic biotic processes generally are reductions that involve either hydrogenolysis, the substitution of a hydrogen atom or chlorine on the molecule, or dehaloelimination, where two adjacent chlorine atoms are removed, leaving a double bond between the respective carbon atoms.

Table 1.9. Typical electron-acceptor classes of bacteria

Dominance (as determined by relative energy generation)	Bacteria Electron Acceptor Class	Predominant CAH Biodegradation Mechanism	Approximate Redox Potential (volts) <sup>1</sup>
Most dominant ↓ Least dominant	Oxygen-reducing (aerobes)	Aerobic oxidation	+0.82
	Nitrate-reducing	Reductive dechlorination	+0.74
	Manganese (IV)-reducing		+0.52
	Iron (III)-reducing		-0.05
	Sulfate-reducing		-0.22
	Carbon dioxide-reducing (methanotrophs)		-0.24

<sup>1</sup> Standard redox potentials at pH of 7

#### 1.3.4.2.1 Anaerobic biodegradation

Organic compounds can be transformed by microorganisms through two basically different processes: **direct metabolism** and **cometabolism**. In the first process the organism consumes the organic compound as a primary substrate to satisfy its energy and carbon needs. The compound serves as an electron donor and as a primary growth substrate for the microbe mediating the reaction. Electrons that are generated by the oxidation of the compound are transferred to an electron acceptor such as oxygen. In addition a microorganism can obtain energy for cell maintenance and growth from the oxidized compound. In general only the less chlorinated CAHs (with one or two chlorine atoms) can be used directly by microorganism as electron donors. The CAHs are oxidized into carbon

dioxide, water, chlorine and electrons, in conjunction with the reduction of oxygen to water.

Few CAHs have been shown to serve as primary substrates for energy and growth. Pure cultures have been isolated that can grow aerobically on dichloromethane (DM) as sole carbon and energy source. VC and 1,2-DCA have also been shown to be available as primary substrates under aerobic conditions (Hartmans et al, 1992, Verce et al, 2000, Klier et al, 1998). Other CAH that can oxidized directly include DCE, DCA, CA, MC and CM (Bradley 1998; RTDF 1997; Harknessvand others 1999). These few exceptions suggest that only the less halogenated one- and two-carbon CAHs might be used as primary substrates, and that the organisms that are capable of doing this are not necessarily widespread in the environment. Figure 1.6.shows an example of aerobic oxidation of a CAH.

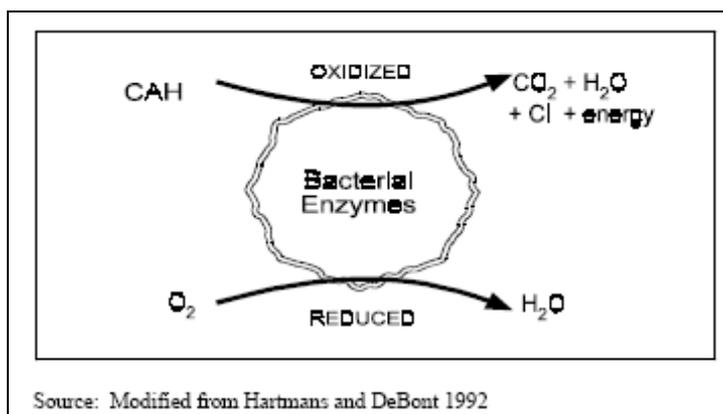


Figure 1.6. Aerobic oxidation (direct).

Most of the CAHs can be biologically transformed by the process of **cometabolism**. Cometabolism is the fortuitous transformation of an organic compound by non specific enzymes, produced for other purposes during microbial metabolism of another compound. Most of the enzymes involved in the degradation of chlorinated solvents determine a sequence of reactions that oxidize NADH to NAD<sup>+</sup>, but do not catalyse the opposite reduction process of NAD<sup>+</sup> to NADH. Thus, in the cometabolic transformation the microorganisms do not get energy or carbon from the process; The transformation does not provide the

organisms any direct benefit, indeed it may be harmful to them, resulting in increased maintenance requirements and decay rates (Criddle, 1992). A primary growth substrate must be at least intermittently available to prevent the depletion of energy and maintain a viable microbial population.

The CAHs that have been observed to be oxidized cometabolically under aerobic conditions include TCE, DCE, VC, TCA, DCA, CF and MC (Munakata-Marr 1997; McCarty and others 1998; RTDF 1997; Edwards and Cox 1997; McCarty 1997a; Bradley and Chapelle 1998; Travis and Rosenberg 1997). The electron donors observed in aerobic cometabolic oxidation include methane, ethane, ethene, propane, butane, aromatic hydrocarbons (such as toluene and phenol), and ammonia. Under aerobic conditions a monooxygenase enzyme mediates the electron donation reaction. That reaction has the tendency to convert CAHs into unstable epoxides (Anderson and Lovley 1997). Unstable epoxides degrade rapidly in water to alcohols and fatty acids, which are readily degradable. Figure 1.7 shows an example of aerobic cometabolic oxidation of a CAH.

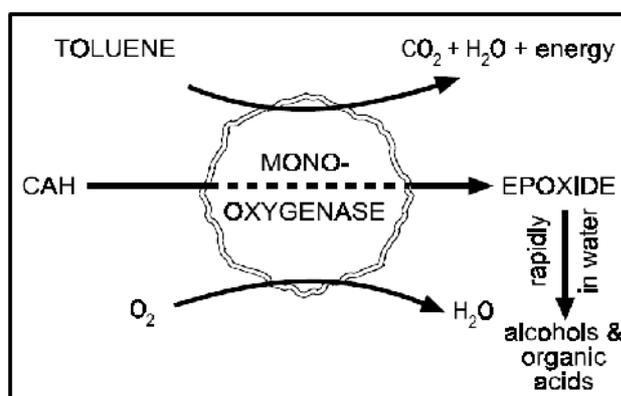
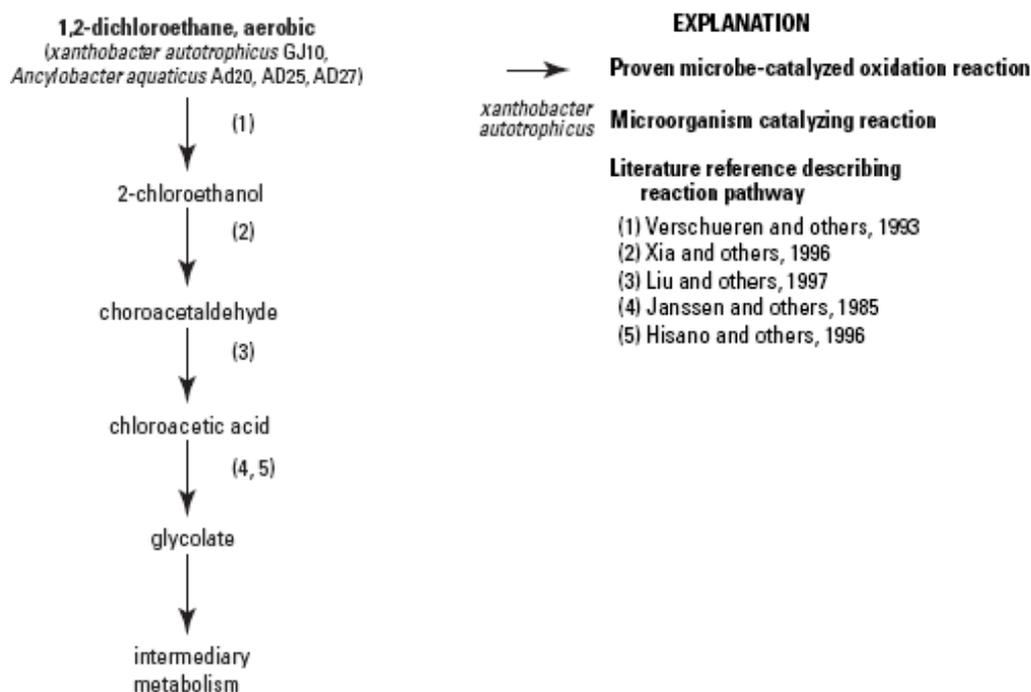


Figure 1.7. Aerobic oxidation (cometabolic).

**Aerobic biodegradation of chlorinated alkanes.** According to the degradation pathway constructed by Sands and others (2005) and Whittaker and others (2005), the dichloroethanes are not a by-product of 1,1,1-TCA or 1,1,2-TCA biodegradation under aerobic conditions (figure 1.5). Apparently, the only source of 1,1-DCA and 1,2-DCA via a degradation pathway is the reductive

dechlorination of 1,1,1-TCA and 1,1,2-TCA, respectively, under anaerobic conditions (figures 1.4 and 1.5). Under aerobic conditions, however, 1,2-DCA can be degraded when used as a carbon source by microorganisms. The intermediate by-product of this degradation is chloroethanol, which is then mineralized to carbon dioxide and water (figure 1.8; Stucki and others, 1983; Janssen and others, 1985; Kim and others, 2000; Hage and others, 2001).



**Figure 1.8.** Laboratory-derived pathway for the aerobic biodegradation of 1,2-dichloroethane.

**Aerobic biodegradation of chlorinated alkenes.** Several studies have shown that chlorinated ethenes, with the exception of PCE, can degrade under aerobic conditions by oxidation (Hartmans and De Bont, 1992; Klier and others, 1999; Hopkins and McCarty, 1995; Coleman and others, 2002) and by co-metabolic processes (Murray and Richardson, 1993; Vogel, 1994; McCarty and Semprini, 1994). Studies describing the degradation of PCE under aerobic conditions were not found in the peer-reviewed literature. In one study, aerobic biodegradation of

PCE was not measurable beyond analytical precision after 700 days of incubation (Roberts and others, 1986). Furthermore, Aronson and others (1999) indicate that PCE is not degraded when dissolved oxygen (DO) is greater than 1.5 mg/L, the approximate boundary between aerobic and anaerobic conditions (Stumm and Morgan, 1996). Chen and others (1996) suggest the structure and oxidative state of PCE prevents its aerobic degradation in water.

According to the aerobic biodegradation pathway constructed by Whittaker and others (2005), the dichloroethenes are not a by-product of TCE degradation under aerobic conditions (figure. 1.5). Rather, TCE is degraded along three different pathways by different microorganisms (figure. 1.9). These pathways do not form any of the dichloroethene compounds and the only apparent source of 1,2-DCE is by the reductive dechlorination of TCE under anaerobic conditions (figures. 1.4 and 1.12). The compounds 1,2-DCE and VC, however, can be degraded under aerobic conditions by microorganisms utilizing the compounds as a primary carbon source (figure. 1.8; Bradley and Chapelle, 1998). Although PCE is not known to degrade through cometabolism under aerobic conditions, co-metabolism is known to degrade TCE, the dichloroethenes, and VC. The rate of cometabolism increases as the degree of chlorination decreases on the ethene molecule (Vogel, 1994). During aerobic cometabolism, the chlorinated alkene is indirectly dechlorinated by oxygenase enzymes produced when microorganisms use other compounds, such as BTEX compounds, as a carbon source (Wiedemeier and others, 1998). The co-metabolic degradation of TCE, however, tends to be limited to low concentrations of TCE because high concentrations in the milligram per litre range are toxic to microbes catalyzing this reaction (Wiedemeier and others, 1998). In field studies by Hopkins and McCarty (1995), VC is shown to degrade by co-metabolism under aerobic conditions when phenol and toluene were used as a carbon source.

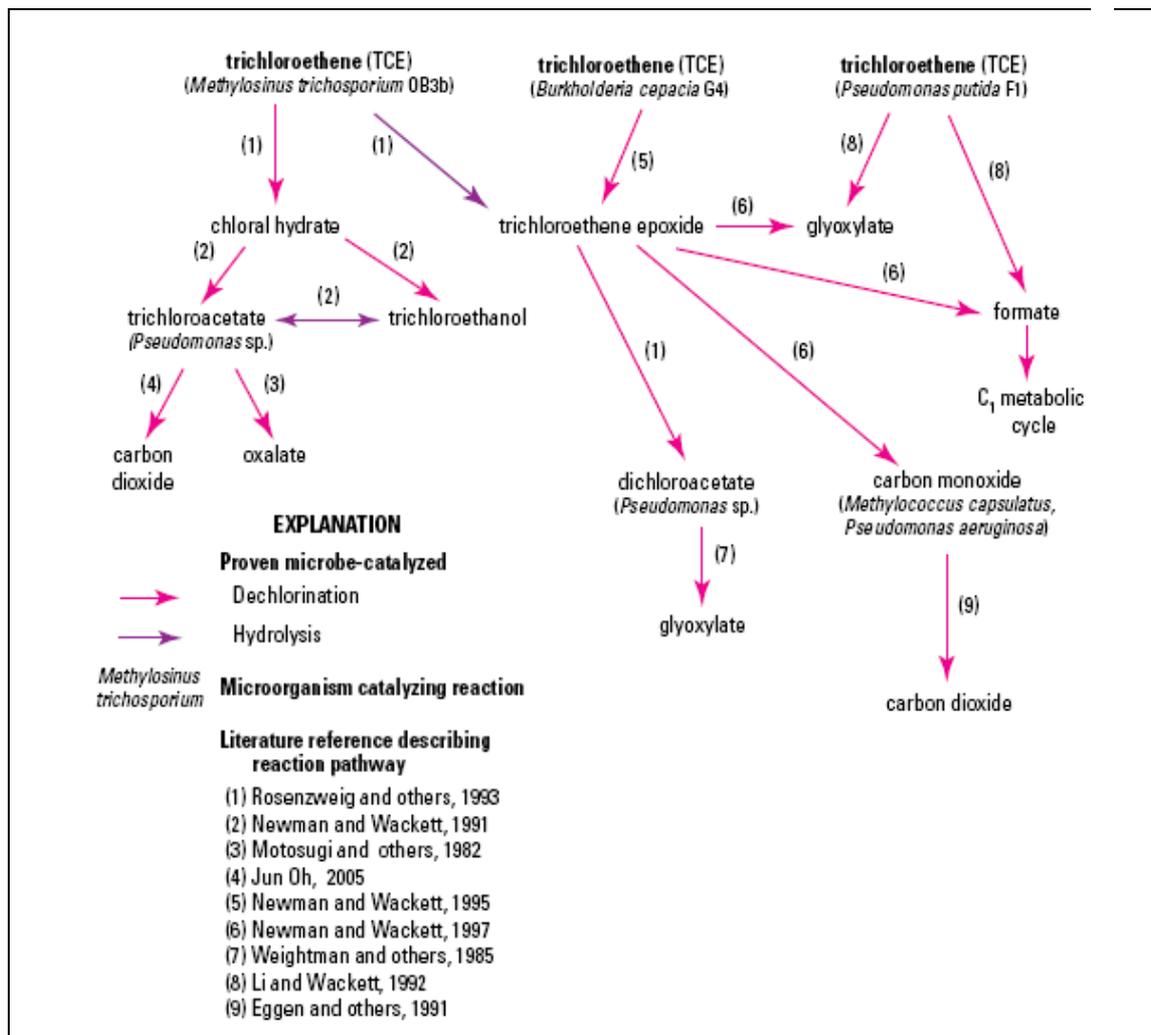


Figure 1.9. Laboratory-derived pathway for the aerobic biodegradation of trichloroethane.

### 1.3.4.2.2 Anaerobic biodegradation

Under anaerobic conditions, reductive dechlorination mechanisms can effectively biodegrade CAHs. This process generally involves a series of decarboxylations and oxidation-reduction (redox) reactions catalyzed either by single microorganisms or by a consortium of microorganisms (Dolfing, 2000). In **direct anaerobic reductive dechlorination** the mediating bacteria use the CAH directly as an electron acceptor in energy-producing redox reactions. **Cometabolic anaerobic reductive dechlorination** occurs when bacteria

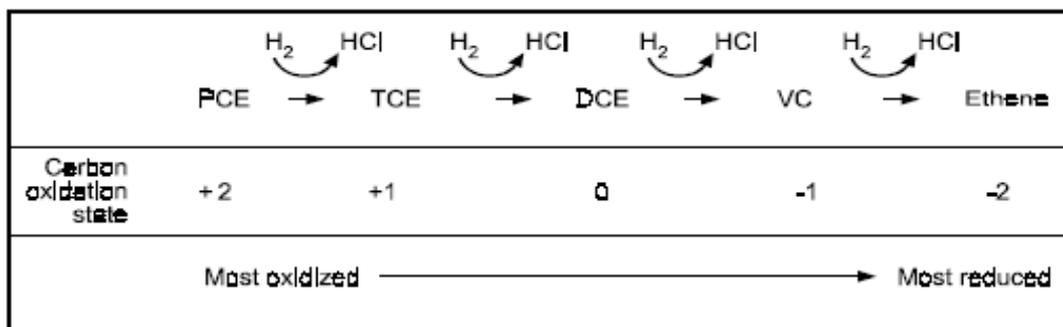
incidentally dechlorinate a CAH in the process of using another electron acceptor to generate energy.

Theoretically, reductive dechlorination is the sequential replacement of one chlorine atom on a chlorinated compound with a hydrogen atom. The replacement continues until the compound is fully dechlorinated. For example, PCE can undergo reductive dechlorination to less-chlorinated compounds, such as TCE or 1,2-DCE, or to nonchlorinated compounds such as ethene, ethane, or methane (methanogenesis). Each successive step in the dechlorination process is theoretically slower than the preceding step. The dechlorination process slows because as chlorines are removed the energy costs to remove another chlorine atom increases (free energy of the reaction decreases; Dolfing, 2000). As a result, biodegradation may not proceed to completion in some aquifers leaving intermediate compounds (for example, dichloroethenes and vinyl chloride) to accumulate in ground water (Azadpour-Keeley and others, 1999). Other constraints on biodegradation such as a reduction in or loss of primary substrate, or microbial suppression also can play a role in the accumulation of intermediate compounds. This is a particular concern with VC because it is a known human carcinogen (Agency for Toxic Substances and Disease Registry, 2005) and its accumulation may create a health issue that might not be a concern during the early stages of groundwater contaminated by TCE.

Reductive dechlorination theoretically is expected to occur under most anaerobic conditions, but has been observed to be most effective under sulfate-reducing and methanogenic conditions (EPA 1998). As in the case of aerobic oxidation, the direct mechanism may biodegrade CAHs faster than cometabolic mechanism (McCarthy and Semprini, 1994).

In direct anaerobic reductive dechlorination bacteria gain energy and grow as one or more chlorine atoms on a chlorinated hydrocarbon are replaced with hydrogen. In that reaction, the chlorinated compound serves as electron acceptor, and hydrogen as electron donor (Fennel and others 1997). Hydrogen used in the reaction typically is supplied indirectly through the fermentation of organic substrates (lactate, acetate, methanol, glucose, toluene). The reaction is also referred to *halorespiration* or *dehalorespiration* (Gosset and Zinder 1997). Direct

anaerobic reductive dechlorination has been observed in anaerobic systems in which PCE, TCE, DCE, VC and DCA are used directly by a microorganism as an electron-acceptor in their energy-producing redox reactions. The mechanism generally results in the sequential reduction of a chlorinated ethene or chlorinated ethane to ethene or ethane. Figure 1.10 shows the step-by-step dechlorination of PCE.



Source: Modified from DeStephano and others 1992

Figure 1.10. Anaerobic reductive dechlorination of PCE.

Several CAHs have been observed to be reductively dechlorinated by cometabolic mechanisms. In those instances, the enzymes that are intended to mediate the electron-accepting reaction “accidentally” reduce and dehalogenate the CAH. Cometabolic anaerobic reductive dechlorination has been observed for PCE, TCE, DCE, VC, DCA and CT under anaerobic conditions (Fathepure 1987; Workman 1997; Yager and others 1997).

**Anaerobic biodegradation of chlorinated alkanes.** While researching the scientific literature for their report, Wiedemeier and others (1998) did not find published studies describing anaerobic biodegradation of chlorinated ethanes in ground water. Since the publication of Wiedemeier and others (1998), however, numerous published studies describe the anaerobic biodegradation of chlorinated ethanes. McCarty (1997) indicates that carbon tetrachloride was transformed to chloroform under denitrifying conditions and mineralized to carbon dioxide and water under sulfate-reducing conditions (figure 1.11). Adamson and Parkin (1999) show that under anaerobic conditions, carbon tetrachloride and 1,1,1-TCA tend to inhibit the degradation of each other. Adamson and Parkin (1999) also show that

carbon tetrachloride was rapidly degraded by cometabolism when acetate was the carbon source.

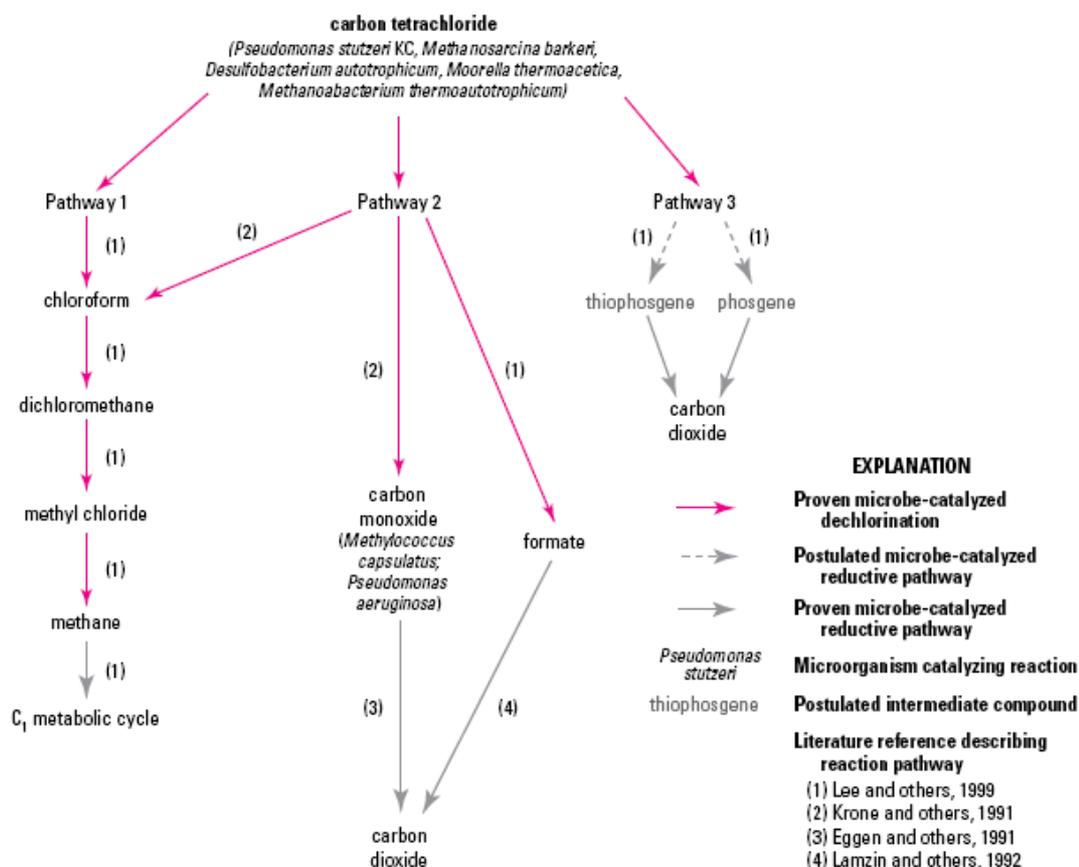
Chen and others (1996) describe how methanogenic conditions in a municipal sludge digester allowed the degradation of TeCA to 1,1,2-TCA, and 1,1,2-TCA to 1,2-DCA through dehydrohalogenation (figure 1.4). De Best and others (1999) report that cometabolic transformations of 1,1,2-TCA will occur under methanogenic conditions. In this study, 1,1,2-TCA was degraded to chloroethane when sufficient amounts of the carbon source were present (figure 1.4). This transformation was inhibited by the presence of nitrate, but not nitrite.

Dolfing (2000) discusses the thermodynamics of reductive dechlorination during the degradation of chlorinated hydrocarbons and suggests that fermentation of chloroethanes to ethane or acetate may be energetically more favorable than “classic” dechlorination reactions. Moreover, polychlorinated ethanes may degrade preferentially by reductive dechlorination under strongly reducing conditions. Dichloroelimination, however, may actually be the dominant degradation reaction for polychlorinated ethanes because more energy is available to microorganisms than is available during reductive dechlorination (Dolfing, 2000). During anaerobic biodegradation, the mean half-lives of the chloroethane compounds can be as short as three days, in the case of 1,1,1-TCA, or as long as 165 days, in the case of 1,2-DCA (table 1.10).

**Table 1.10. Mean half-life in days for the anaerobic biodegradation of selected chlorinated alkane and alkene compounds.**

[(27), number of samples used to derive the mean value; —, not available]		
Compound	All studies	Field/in situ studies
chloroethene	0.018 (27)	0.0073 (19)
1,2-dichloroethane	63–165 (2)	63–165 (2)
tetrachloroethene (PCE)	239–3,246 (36)	239 (16)
tetrachloromethane	47 (19)	40 (15)
1,1,1-trichloroethane	2.3–2.9 (28)	—
1,1,2-trichloroethane	47–139 (1)	—
trichloroethene (TCE)	1,210 (78)	277 (30)

<sup>1</sup>Aronson and Howard, 1997, p. 111



**Figure 1.11.** Laboratory-derived pathway for the anaerobic biodegradation of tetrachloromethane.

**Anaerobic biodegradation of chlorinated alkenes.** Many laboratory and field studies have shown that microorganisms degrade chlorinated ethenes under anaerobic conditions (Bouwer and others, 1981; Bouwer, 1994, Dolfing, 2000). Groundwater is considered anoxic when the dissolved oxygen concentration falls below 1.0–1.5 mg/L (Stumm and Morgan, 1996; Christensen and others, 2000). Under anoxic conditions, anaerobic or facultative microbes will use nitrate as an electron acceptor, followed by iron (III), then sulphate, and finally carbon dioxide (methanogenesis; Chapelle and others, 1995; Wiedemeier and others (1998). As the concentration of each electron acceptor sequentially decreases, the redox

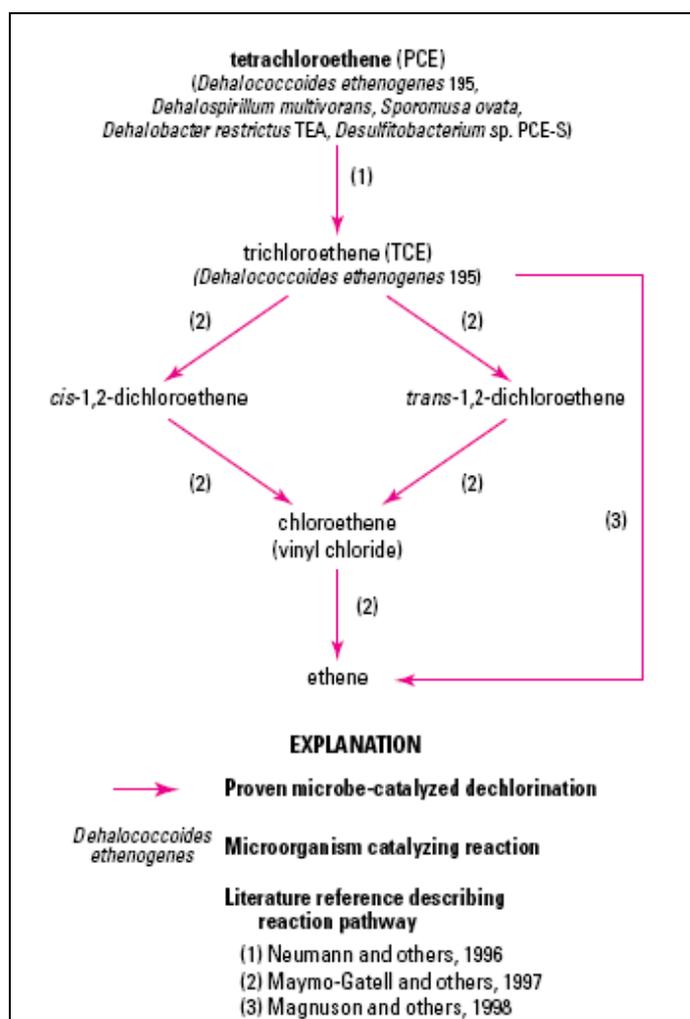
potential of the ground water becomes greater (more negative) and biodegradation by reductive dechlorination is favoured.

Anaerobic conditions in ground water can be determined by measuring the vertical and spatial concentrations of oxygen, iron (II), manganese (II), hydrogen sulfide or methane in groundwater and using that data as a qualitative guide to the redox status (Stumm and Morgan, 1996; Christensen and others, 2000). Other measurements of anaerobic conditions involving microorganism biomarkers include volatile fatty acids, ester-linked phospholipid fatty acid (PLFA), deoxyribonucleic acid (DNA), and ribonucleic acid (RNA) probes, and TEAP bioassay (Christensen and others, 2000). The reduction of iron (III) to iron (II), manganese (IV) to manganese (II), sulfate to hydrogen sulfide, and carbon dioxide to methane during the microbial reduction of CAHs can have a major influence on the distribution of iron (II), manganese (II), hydrogen sulfide, and methane concentrations in ground water (Stumm and Morgan, 1996; Lovley, 1991; Higgs and others, 1996; Braun, 2004).

The highly chlorinated alkenes are commonly used as electron acceptors during anaerobic biodegradation and are reduced in the process (Vogel and others, 1987). The primary anaerobic process driving degradation of CAHs, except VC, is reductive dechlorination (figures 1.4 and 1.12; Bouwer and others, 1981; Bouwer, 1994). Tetrachloroethene and TCE are the most susceptible to reductive dechlorination because they are the most oxidized of the chlorinated ethenes; however, the more reduced (least oxidized) degradation by-products such as the dichloroethenes and vinyl chloride are less prone to reductive dechlorination. The main by-product of anaerobic biodegradation of the polychlorinated ethenes is VC (figure 1.12), which is more toxic than any of the parent compounds (Agency for Toxic Substances and Disease Registry, 2004). The rate of reductive dechlorination tends to decrease as the reductive dechlorination of daughter products proceeds (Vogel and McCarty, 1985; Bouwer, 1994). Murray and Richardson (1993) suggest that the inverse relation between the degree of chlorination and the rate of reductive dechlorination may explain the accumulation of 1,2-DCE and VC in anoxic groundwater contaminated with PCE and TCE. In addition, the anaerobic reduction of VC to ethene is slow and

inefficient under weak reducing conditions, which favours the persistence of VC in anoxic groundwater (Freedman and Gossett, 1989).

Reductive dechlorination has been demonstrated under nitrate- and iron-reducing conditions (Wiedemeier and others, 1998). Reductive dechlorination of the CAHs, however, may be more rapid and more efficient when oxidation-reduction (redox) conditions are below nitrate-reducing levels (Azadpour- Keeley and others, 1999). Sulfate-reducing and methanogenic groundwater conditions create an environment that facilitates not only biodegradation for the greatest number of CAHs, but also more rapid biodegradation rates (Bouwer, 1994). Reductive dechlorination of DCE and VC is most apparent under sulfate reducing and methanogenic conditions (Wiedemeier and others, 1998). Anaerobic biodegradation rates for the chlorinated alkenes can be as short as 45 minutes, in the case of VC, to as long as 9 years for PCE (table 1.10).



**Figure 1.12. Laboratory-derived pathway for the anaerobic biodegradation of tetrachloroethene.**

## **1.4 CHLORINATED BENZENES**

### **1.4.1 Sources of chlorinated benzenes**

Four chlorinated benzenes commonly detected in groundwater contamination studies include chlorobenzene (CB), 1,2-dichlorobenzene (1,2-DCB), and two isomers of trichlorobenzene, 1,2,3-trichlorobenzene (1,2,3-TCB) and 1,2,4-trichlorobenzene (1,2,4-TCB; tables 1.2 and 1.3). Chlorobenzene is commonly used as a solvent for pesticide formulations, in the manufacturing of di-isocyanate, as a degreaser for automobile parts, and in the production of nitrochlorobenzene. Solvent uses accounted for about 37 percent of chlorobenzene consumption in the United States during 1981 (Agency for Toxic Substances and Disease Registry, 1990a, p. 45). The compound 1,2-DCB is used primarily to produce 3,4-dichloroaniline herbicides (Agency for Toxic Substances and Disease Registry, 1990b, p. 263). The two trichlorobenzene isomers are primarily used as dye carriers in the textile industry. Other uses include septic tank and drain cleaners, the production of herbicides and higher chlorinated benzenes, as wood preservatives, and in heat-transfer liquids (U.S. Environmental Protection Agency, 2005b).

### **1.4.2 Biodegradation of chlorinated benzenes**

Several studies have shown that chlorinated benzene compounds containing up to four chlorine atoms can be degraded by microorganisms under aerobic conditions (Reineke and Knackmuss, 1984; Spain and Nishino, 1987; Sander and others, 1991). Under aerobic conditions, 1,2,4-trichlorobenzene (1,2,4-TCB; Haigler and others, 1988) and chlorobenzene (CB; Sander and others, 1991) are used as a primary carbon source during biodegradation by microorganisms such as *Burkholderia* and *Rhodococcus* species (Rapp and Gabriel-Jürgens, 2003). During biodegradation, these compounds are completely mineralized to carbon dioxide (CO<sub>2</sub>) (van der Meer and others, 1991). Rapp and Gabriel-Jürgens (2003) also indicate that all of the dichlorobenzene isomers were

biodegraded by the *Rhodococcus* bacterium. The biodegradation pathways for 1,2,4-TCB, 1,4-DCB, 1,2-DCB, and CB, under aerobic conditions are shown in figures 1.13 to 1.15, respectively. These pathways are similar to that of benzene, except that one chlorine atom is eventually eliminated through hydroxylation of the chlorinated benzene to form a chlorocatechol, then ortho cleavage of the benzene ring (Van der Meer and others, 1998).

Calculated and published degradation half-lives for the chlorobenzenes under aerobic conditions are shown in table 1.11. The compounds 1,2,4-TCB, 1,2-DCB, and CB lose 50 percent of their initial mass within 180 days (table 1.11). Conversely, Dermietzel and Vieth (2002) show that chlorobenzene was rapidly mineralised to CO<sub>2</sub> in laboratory and in situ microcosm studies, with complete mineralisation ranging from 8 hours to about 17 days. In addition, the compound 1,4-DCB was completely mineralised within 25 days. Nevertheless, under the aerobic conditions of Dermietzel and Vieth (2002) study, 1,2,4-TCB, 1,2-DCB, and 1,3-DCB were only partially degraded after 25 days. In another laboratory-microcosm study by Monferran and others (2005), all isomers of DCB were mineralised to CO<sub>2</sub> within 2 days by the aerobe *Acidovorax avenae*.

Although Wiedemeir and others (1998) indicate that few studies existed that described the anaerobic degradation of the chlorobenzene compounds, a study by Ramanand and others (1993) did suggest that 1,2,4-TCB could be biodegraded to chlorobenzene with 1,4-DCB as an intermediate compound under anaerobic conditions. Moreover, Middeldorp and others (1997) show that 1,2,4-TCB was reductively dechlorinated to 1,4-DCB, then to chlorobenzene in a methanogenic laboratory microcosm in which chlorobenzene-contaminated sediment was enriched with lactate, glucose, and ethanol. These compounds served as carbon sources. Furthermore, the microbial consortia facilitating the dechlorination of 1,2,4-TCB also was able to degrade isomers of tetrachlorobenzene to other isomers of TCB and 1,2-DCB. More recent studies show that a strain of *Dehalococcoides*, can reductively dechlorinate 1,2,4-TCB under anaerobic conditions (Holscher and others, 2003; Griebler and others, 2004a). In addition, Adrian and others (1998) suggest that fermentation is the primary degradation process for the chlorobenzenes under anaerobic conditions. This study also

showed that the cometabolism of 1,2,4-TCB was inhibited by the presence of sulfate, sulfite and molybdate.

Furthermore, Ramanand and others (1993) show that 1,2,4-TCB had declined by 63% within 30 days under anaerobic conditions. Dermietzel and Vieth (2002) show that the anaerobic biodegradation of 1,4-DCB was markedly slower under iron-reducing conditions than under aerobic conditions. In general, it appears that the biodegradation of the chlorinated benzenes is slower under anaerobic than under aerobic conditions.

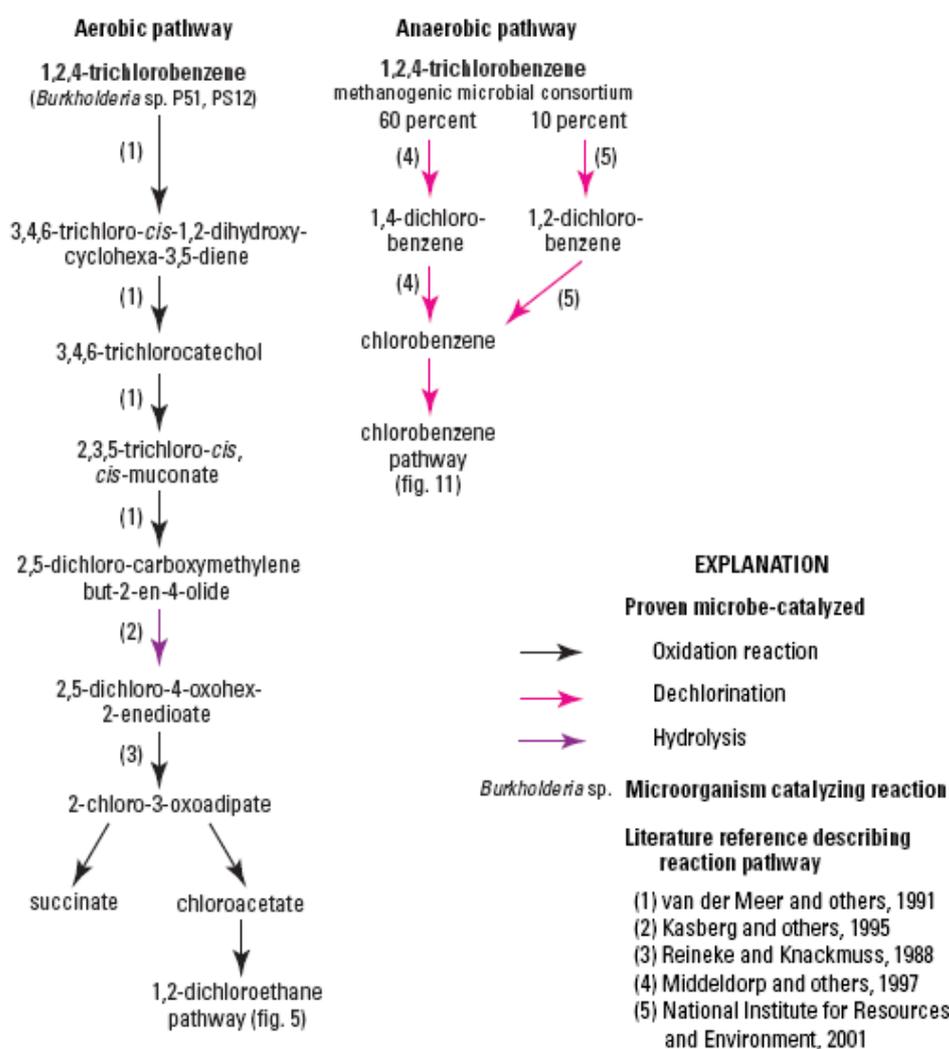
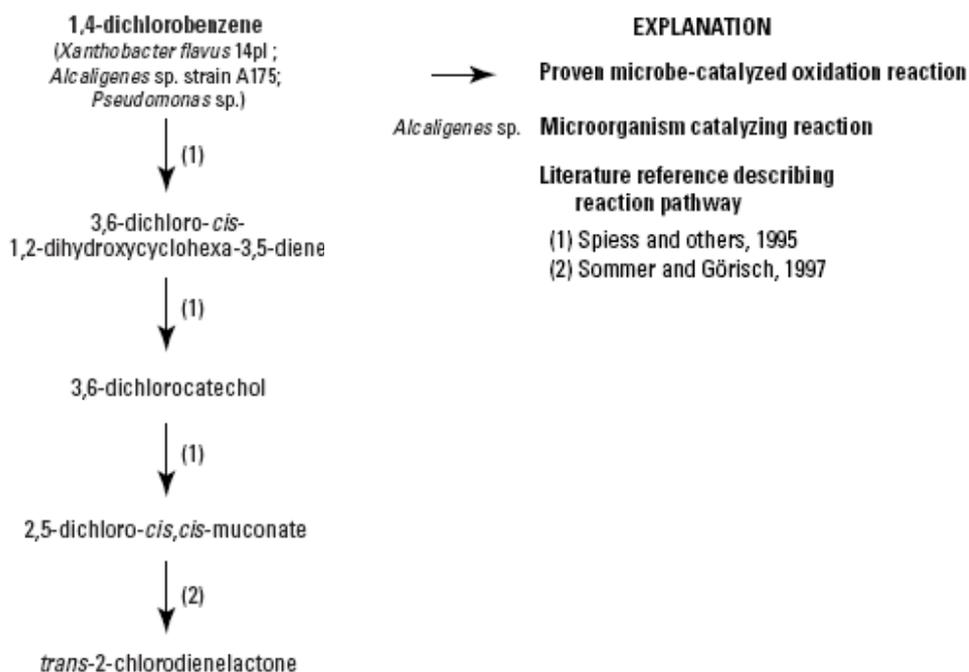


Figure 1.13. Laboratory-derived pathway for the aerobic and anaerobic biodegradation of 1,2,4-trichlorobenzene.



**Figure 1.14.** Laboratory-derived pathway for the aerobic biodegradation of 1,4-dichlorobenzene.

**Table 1.11.** Laboratory or environmental half-lives and by-products for the aerobic and anaerobic biodegradation of selected chlorinated benzene compounds detected in groundwater.

[IUPAC, International Union of Pure and Applied Chemistry; CO<sub>2</sub>, carbon dioxide; DCB, dichlorobenzene]

Compound (IUPAC name) <sup>1</sup>	Degradation by-products	Half-life (days)	Literature reference
Aerobic conditions			
chlorobenzene	3-chlorocatechol, CO <sub>2</sub>	69–150	Rathbun, 1998; McLeish, 2005
1,2-dichlorobenzene	chlorobenzene	28–180	Rathbun, 1998
1,4-dichlorobenzene	chlorobenzene	28–180	Rathbun, 1998
1,2,4-trichlorobenzene	succinate, chloroacetate	28–180	Rathbun, 1998; Renhao, 2005; Yao, 2006
Anaerobic conditions			
chlorobenzene	CO <sub>2</sub>	280–580	Rathbun, 1998; Monferran and others, 2005
1,2-dichlorobenzene	CO <sub>2</sub>	119–722	Rathbun, 1998
1,4-dichlorobenzene	chlorobenzene	112–722	Rathbun, 1998; Yao, 2006
1,2,4-trichlorobenzene	1,4-DCB, chlorobenzene	112–722	Rathbun, 1998; Yao, 2006

<sup>1</sup>International Union of Pure and Applied Chemistry, 2006

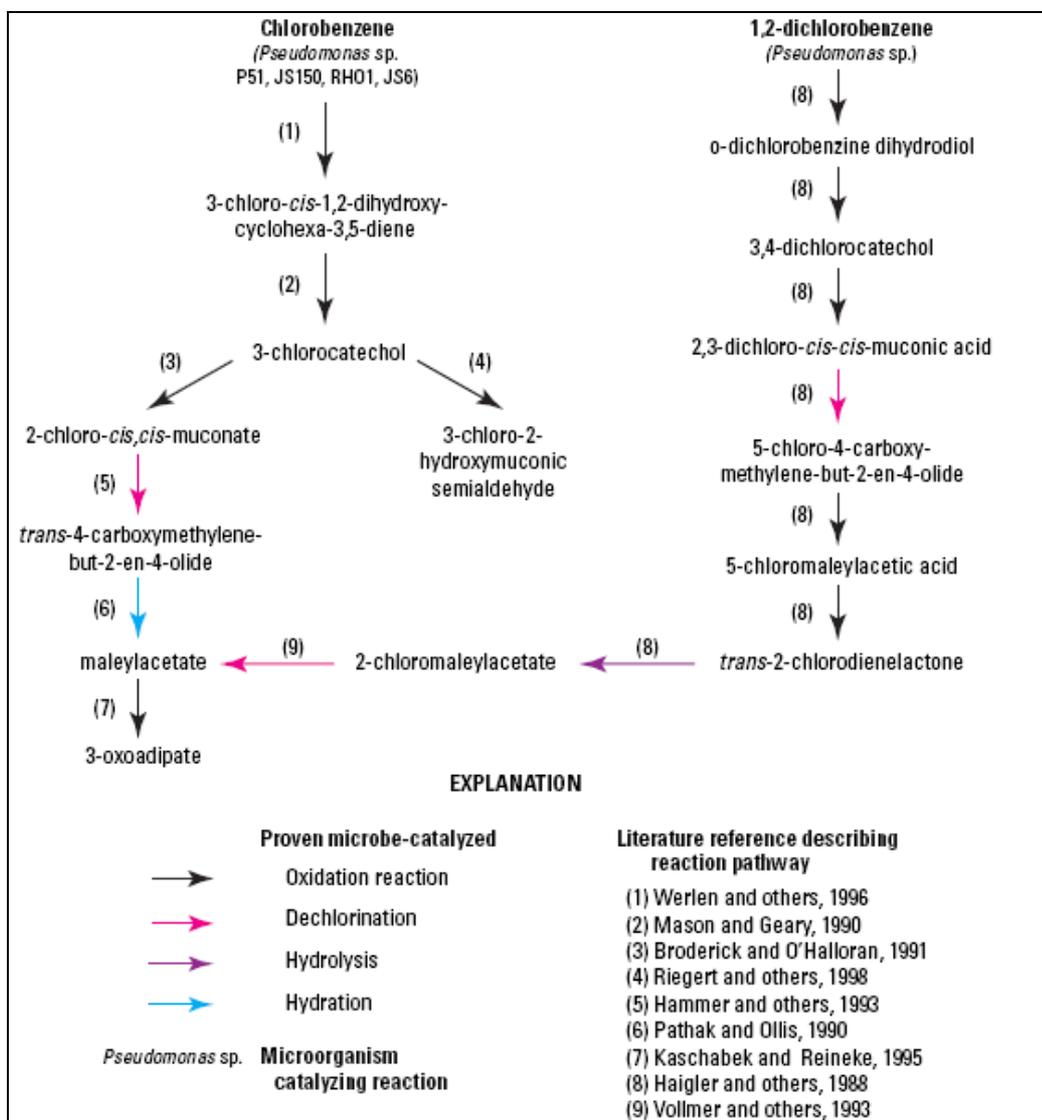


Figure 1.15. Laboratory-derived pathway for the aerobic biodegradation of chlorobenzene and 1,2-dichlorobenzene.

### 1.4.3 Bioegradation of monochlorobenzene

In four billion years, micro-organisms have evolved an extensive range of enzymes and control mechanisms to be able to degrade a wide array of naturally occurring aromatic compounds including chlorobenzene. The rate of naturally occurring biodegradation is often limited by either the concentration of an appropriate electron-acceptor or the availability of nutrients for cell growth. Although chlorobenzene can be metabolized aerobically (Nishino *et al.*, 1992; Reineke *et al.*, 1984; Rochkind *et al.*, 1986; Van der Meer *et al.*, 1992), it has not been reported to be degraded through the use of other electron acceptors (Bouwer *et al.*, 1983; Nishino *et al.*, 1992; Reineke *et al.*, 1984). Molecular oxygen appears necessary for ring fission. Some removal through reductive dechlorination may occur in conducive environments with excess chlorobenzene (Montgomery *et al.*, 1994). In general, intermediary metabolites of chlorobenzene appear similar to those documented for unhalogenated aromatic compounds. Biodegradation under microaerophilic conditions has also been reported (Vogt *et al.*, 2003).

The main enzymes involved in chlorobenzene's catabolic reactions are oxygenases. Oxygen can be incorporated immediately into organic products by reactions catalyzed by enzymes such as oxygenases or hydroxylases (Gibson *et al.*, 1982; Harayama *et al.*, 1992). These enzymes use metals to activate dioxygen that is not reactive in its original state. During these processes oxygen is metabolized into very reactive forms like singlet oxygen and hydroxyl radicals. Oxygenase enzymes play an important role in aromatic catabolic pathways. They initiate the degradation of aromatic compounds by hydroxylation of the aromatic ring for preparation of the ring fission and are involved in ring fission. Oxygenases of different organisms catalyzing similar reactions share similar features, structures, and reaction mechanisms. By means of the amino acid sequence, oxygenases can be divided into different families. Comparisons of the amino acid sequence of related enzymes can give information on amino acids of essential function including active sites, and on the evolution of the enzymes.

Oxygenases involved in initial attack of the aromatic compound can be divided in either mono-oxygenases or dioxygenases. Oxygenases that incorporate

only one oxygen atom into the structure of the substrate are called mono-oxygenases. In that case, the remaining oxygen atom of the oxygen molecule is reduced into H<sub>2</sub>O. Mono-oxygenases are multi-component enzyme complexes. They consist of a combination of the following components, i.e., a hydroxylase component consisting of an  $\alpha$ -,  $\beta$ - and  $\gamma$ -subunit, a ferredoxin component, a small oxygenase subunit, and a flavo-iron-sulfur NADH-oxidoreductase component. Some mono-oxygenases contain additional polypeptides with unknown function. The hydroxylase component is the protein that activates molecular oxygen and binds it to the substrate.

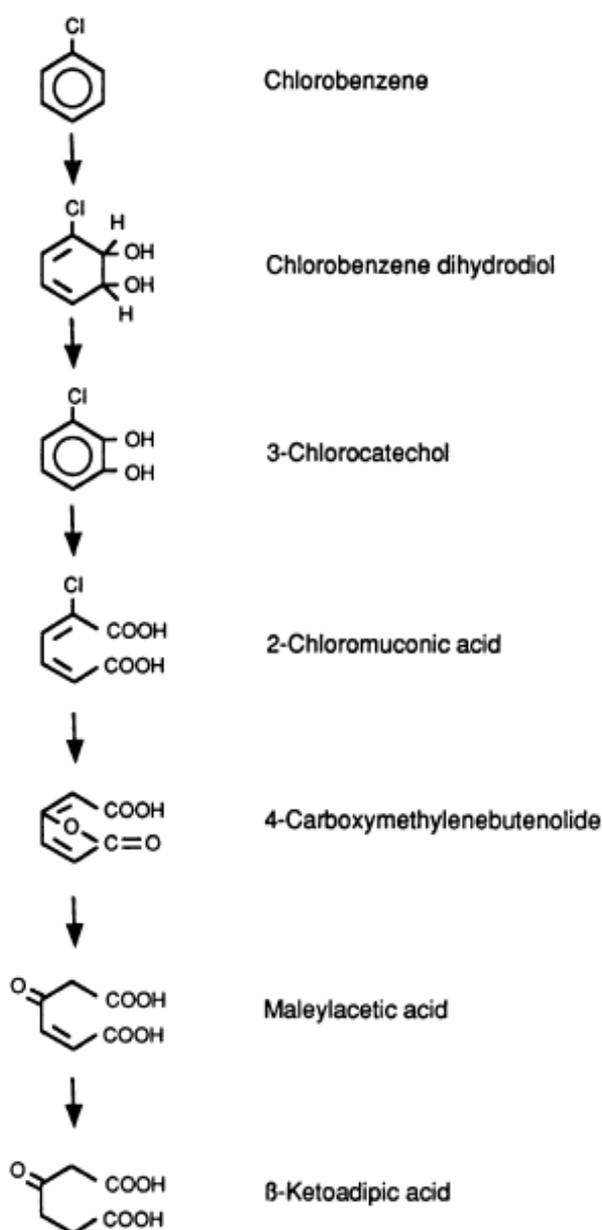
Oxygenases that incorporate two oxygen atoms into the substrate are called dioxygenases. They are also multi-component enzyme complexes, and in most cases consist of four components including an iron-sulfur oxidase large  $\alpha$ -subunit, a ferredoxin component, an iron-sulfur oxidase small  $\beta$ -subunit and a reductase component.

#### 1.4.3.1 Degradation via the *ortho*-cleavage pathway

The majority of the microorganisms able to mineralize chlorinated aromatics do not possess enzyme systems capable of initial dechlorination. They transform chloroaromatics to chlorocatechols, which are further metabolized via the enzyme of the *ortho*-cleavage pathway, and dechlorination occurs after ring-cleavage (Schlömman, 1984).

In 1983, Reineke *et al.* isolated a bacterium (strain **WR136**) able to grow on monochlorobenzene and proposed a degradative pathway on the basis of the enzyme activities found (Figure 1.16). 3-Chlorocatechol is subject to *ortho* cleavage with formation of 2-chloro-cis,cis-muconic acid. This is cycloisomerized with coincident or subsequent elimination of chloride yielding 4-carboxymethylenebut-2-en-4-olide, which is further converted by use of a hydrolase. The resulting maleylacetate is reduced in an NADH-dependent reaction to 3-oxoadipate. This modified *ortho* pathway is the only pathway currently known for the aerobic degradation of catechol formed from chlorobenzene. Analogous pathways have been described for the dichlorocatechols derived by the transformation of dichlorobenzenes (de Bont *et al.*, 1986; Haigler *et al.*, 1988;

Schraa *et al.*, 1986; Spain *et al.*, 1987). In each instance, the initial attack is by a dioxygenase. The initial oxidation results in the formation of a *cis*-dihydrodiol. Subsequent ring fission and elimination of chloride leads to the detoxification and mineralization of these compounds. The key enzyme is the pyrocatechase II (Dorn *et al.*, 1978; Reineke *et al.*, 1984) that converts chlorocatechols to chloro-*cis,cis*-muconic acids. Absence of this enzyme in organisms with initial oxygenases with broad substrate specificities may lead to the accumulation of chlorocatechols or to the misrouting of chlorocatechol down the *meta* cleavage pathway, ultimately resulting in cell death.



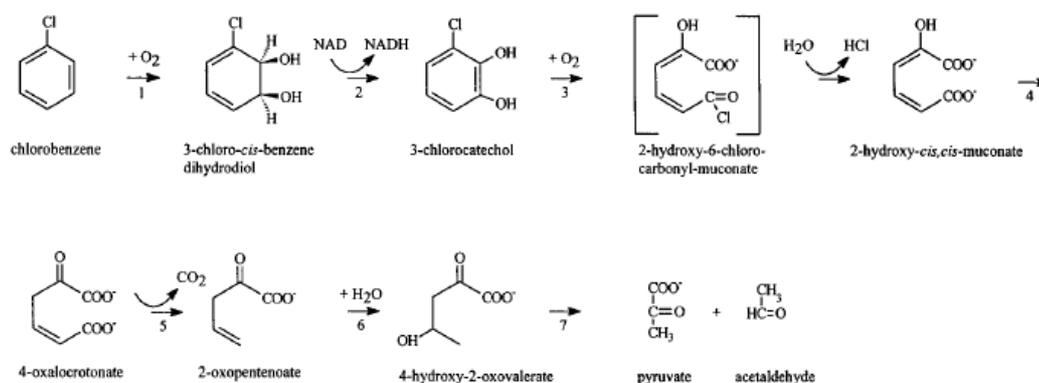
In another study (Nishino *et al.*, 1992), bacterial isolates were obtained from groundwater and soils contaminated with chlorobenzene. The isolates were tested to determine whether the natural community could remove the groundwater contaminants. These isolates were identified and characterized as to their ability to grow on chlorobenzene and related aromatic compounds. The complete consortium could mineralize approximately 54% of the chlorobenzene within 7 days, with no accumulation of 3-chlorocatechol. Metabolic pathways were evaluated for several isolates. One phenotype was characterized by the ability to degrade chlorobenzene by the modified *ortho* pathway. One strain also degraded *p*-dichlorobenzene by using the same pathway. Isolates exhibiting a second phenotype degraded *p*-cresol, benzene, and phenol by the classical *ortho* pathway and accumulated 3-chlorocatechol when grown in the presence of chlorobenzene. Strains of the third phenotype grew on complex media in the presence of chlorobenzene but did not transform any of the aromatic compounds tested.

#### 1.4.3.2 Degradation via the *meta*-cleavage pathway

It is generally accepted that degradation of chloroaromatics does not proceed via the *meta*-cleavage pathway (Knackmuss, 1981; Pettigrew *et al.*, 1991; Rojo *et al.*, 1987). An explanation for this has been found in the production of an acylchloride from 3-chlorocatechol by the catechol 2,3-dioxygenase of the *meta*-cleavage pathway, which leads to rapid suicide inactivation of the enzyme (Bartels *et al.*, 1984). Therefore, *meta*-cleavage is considered to be unsuitable for the mineralization of haloaromatics that are degraded via halocatechols. Whereas chlorocatechols are mineralized via *ortho*-cleavage pathways, methylaromatics are commonly mineralized via *meta*-cleavage routes. Simultaneous metabolism of chloro- and methylcatechols often creates biochemical anarchy. *Meta*-cleavage leads to substrate misrouting in the case of 4-chlorocatechol or formation of a suicide product in the case of 3-chlorocatechol. Formation of dead-end methylactones can occur when the *ortho*-cleavage pathway is dealing with methylcatechols.

Consequently, only a few strains which can grow on mixtures of methylated and chlorinated aromatics are known (Haigler *et al.*, 1992; Pettigrew

*et al.*, 1991). They all use a modified *ortho*-cleavage pathway for the conversion of the chlorinated substrate. *Pseudomonas putida* GJ31 (Oldenhuis *et al.*, 1989) and *Pseudomonas* sp. strains JS6 (Pettigrew *et al.*, 1991) and JS150 (Haigler *et al.*, 1992) are the only strains known to grow on a mixture of chlorobenzene and toluene. Mars *et al.* (1997) showed that *Pseudomonas putida* GJ31 grows on chlorobenzene via a *meta*-cleavage pathway which allows the simultaneous utilization of toluene (Figure 1.17). In addition, 3-chlorocatechol was found to be the ring cleavage substrate formed from chlorobenzene, which was dehalogenated during ring cleavage to produce 2-hydroxymuconic acid. The authors observed that the enzymes of the modified *ortho*-cleavage pathway were never present, while the enzymes of the *meta*-cleavage pathway were detected in all cultures. Apparently, *Pseudomonas putida* GJ31 has a *meta*-cleavage enzyme (catechol 2,3-dioxygenase) which is resistant to inactivation by the acylchloride, providing this strain with the exceptional ability to degrade both toluene and chlorobenzene via the *meta*-cleavage pathway.



**Figure 1.17.** Proposed catabolic pathway of chlorobenzene by *P.putida* GJ31 by analogy to the known *meta*-cleavage pathway. Enzymes: 1, chlorobenzene dioxygenase; 2, chlorobenzene dihydrodiol dehydrogenase; 3, catechol 2,3-dioxygenase; 4, oxalocrotonate isomerase; 5, oxalocrotonate decarboxylase; 6, 2-oxopent-4-enoate hydratase; 7, 4-hydroxy-2-oxovalerate aldolase.

#### 1.4.3.3 Biodegradation of mixtures of substituted benzenes

The degradation of a wide range of substituted aromatic compounds by a strain of *Pseudomonas* has been observed (Haigler et al., 1992). *Pseudomonas* sp. strain JS150 was isolated as a nonencapsulated variant of *Pseudomonas* sp. strain JS1 that contains the genes for the degradative pathways of a wide range of substituted aromatic compounds. *Pseudomonas* sp. strain JS150 grew on phenol, ethylbenzene, toluene, benzene, naphthalene, benzoate, p-hydroxybenzoate, salicylate, chlorobenzene, and several 1,4-dihalogenated benzenes. Enzyme assays with cell extracts showed that the enzymes of the meta, ortho, and modified ortho cleavage pathways can be induced in strain JS150. Strain JS150 contains a nonspecific toluene dioxygenase with a substrate range similar to that found in strains of *Pseudomonas putida*. Chlorobenzene-grown cells of strain JS150 degraded mixtures of chlorobenzene, benzene, toluene, naphthalene, trichloroethylene, and 1,2- and 1,4-dichlorobenzenes in continuous culture. Results indicated that induction of appropriate biodegradative pathways in strain JS150 permits the biodegradation of complex mixtures of aromatic compounds.

#### 1.4.3.4 Degradation under oxygen-limited conditions

Vogt et al. (2002) studied the monochlorobenzene degradation at low oxygen concentration by five bacterial strains (*Acidovorax facilis* B517, *Cellulomonas turbata* B529, *Pseudomonas veronii* B547, *Pseudomonas veronii* B549, and *Paenibacillus polymyxa* B550) isolated on chlorobenzene as the sole source of carbon and energy. These strains were screened for the accumulation of the putative metabolic intermediate 3-chlorocatechol during growth on chlorobenzene under oxygen-limited conditions in the presence and absence of nitrate (1 mM). 3-Chlorocatechol accumulated in the growth media of all five strains, but accumulation was significantly less in cultures of *A. facilis* B517 compared to the other four strains. The presence of nitrate did not influence the biological conversion pattern. For *P. veronii* B549, a clear relationship between the presence of 3-chlorocatechol in the medium and low oxygen concentrations was demonstrated. The authors made the assumption that accumulation of 3-chlorocatechol was due to the low enzymatic turnover of the 3-chlorocatechol cleaving enzyme, catechol-1,2-dioxygenase, at low oxygen concentrations.

Other strains able to degrade monochlorobenzene as a sole carbon source include *Escherichia Hermanii* (Kiernicka *et al.*, 1999) and *Acidovorax avenae* (Monferràn *et al.*, 2005). *Escherichia Hermanii* was isolated from sludge of an industrial wastewater treatment plant. High chlorobenzene concentrations (up to 394 mg l<sup>-1</sup>) had low toxic effects towards this strain, which was able to degrade chlorobenzene without any previous adaptation. *Acidovorax avenae* was isolated in a polluted site of Suquia River (Argentina) from a subsurface microbial community acclimated during 15 days using 1,2-dichlorobenzene as the sole carbon source (aerobic conditions). *Acidovorax avenae* was able to perform the complete biodegradation of 1,2-dichlorobenzene in two days affording stoichiometric amounts of chloride. This pure strain was also tested for biodegradation of chlorobenzene, 1,3-dichlorobenzene and 1,4-dichlorobenzene, giving similar results to the experiments using dichlorobenzene. The aromatic-ring-hydroxylating dioxygenase (ARHDO)  $\alpha$ -subunit gene core, encoding the catalytic site of the large subunit of chlorobenzene dioxygenase, was detected by PCR amplification and confirmed by DNA sequencing. These results suggest that the isolated strain of *A. avenae* could use a catabolic pathway, via ARHDO system, leading to the formation of chlorocatecols during the first steps of biodegradation, with further chloride release and subsequent paths that showed complete substrate consumption.

**Cometabolic biodegradation** has also been reported (Jeckorek *et al.*, 2002). The degradation of chlorobenzene was investigated with the specially chosen strain *Methylocystis* sp. GB 14 DSM 12955, in 23 ml headspace vials and in a soil column filled with quaternary aquifer material from a contaminated location in Bitterfeld (Germany). A long-term experiment was carried out in this column: groundwater polluted by chlorobenzene was continuously fed through the column, bubbled with a 4% CH<sub>4</sub>-96% air mixture. Chlorobenzene was oxidized by up to 80% under pure culture conditions in the model experiments and was completely degraded under the mixed culture conditions of the column experiments. The enzyme responsible for this ability was the sMMO (soluble methane monooxygenase)



**2. BIODEGRADATION TECHNOLOGIES FOR REMEDIATION OF CONTAMINATED SITES**

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**2.1 INTRODUCTION**

Bioremediation is a grouping of technologies that use microbiota to degrade or transform hazardous contaminants to compounds such as carbon dioxide, water, inorganic salts, microbial biomass, and other byproducts that may be less hazardous than the parent compounds. Numerous application of bioremediation are nowadays widely accepted as a remedial alternative and are in wide use at site contaminated with petroleum products and/or hazardous wastes. Some bioremediation technologies, such as cometabolic bioventing, are still in development and should be considered innovative. Other bioremediation technologies, such as anaerobic bioventing, are current topic of research.

The following contaminants have been bioremediated successfully at many sites:

- Halogenated and non-halogenated volatile organic compounds (VOCs)
- Halogenated and non-halogenated semi-volatile organic compounds (SVOCs).

Contaminants with a more limited bioremediation performance include:

- Polycyclic aromatic hydrocarbons (PAHs)
- Organic pesticides and herbicides
- Polychlorinated biphenils (PCBs).

Bioremediation remains an active field of technology research and development at both the laboratory and field scale. For example, applications to chlorinated aliphatic hydrocarbons (CAHs), perchlorate and methyl-tert-butyl ether (MTBE) were developed rapidly in recent years.

The field of bioremediation can be divided into several broad categories. For example, bioremediation technologies may be applied to *in situ* or *ex situ* media. *In situ* processes treat soils and groundwater in place without removal while *ex situ* processes involve the removal of the contaminated media to a treatment area.

Another way to divide the bioremediation field is based on additives to environmental media. *Intrinsic bioremediation* depends on indigenous microflora to degrade contaminants (EPA, 2000). This approach is used *in situ* and takes advantage of pre-existing processes to degrade hazardous wastes. Intrinsic bioremediation requires careful site assessment and monitoring to make sure that the ongoing processes are protective of environmental receptors. Alternatively, *enhanced bioremediation* facilitates biodegradation by manipulating the microbial environment, typically by supplying chemical amendments such as air, organic substrates, electron donors, nutrients and other compounds that affect metabolic reactions (EPA, 2000). Enhanced bioremediation may also be called *biostimulation* when only chemical amendments are added. Examples include bioventing, land farming, biopiles, composting. Biostimulation can be applied *in situ* or *ex situ*, to treat soil and other solids, groundwater and surface water. Sometimes *bioaugmentation* (addition of microbial cultures) is used to enhance biotreatment. Bioaugmentation (almost always performed in conjunction with biostimulation) may be needed for specific contaminants that are not degraded by the indigenous microorganisms.

In bioremediation, fundamental biological activities are exploited to degrade or transform contaminants of concern. The biological activity to be exploited depends on the specific contaminants of concern and the media where the contamination is located. For example in *aerobic environments* many microbes are able to degrade organic compounds, such as hydrocarbons. These microbes gain energy and carbon for building cell materials from these biochemical reactions. At many sites with fuel contamination, the amount of oxygen present limits the extent of biotreatment. Thus, by adding oxygen in the form of air, contaminant degradation proceeds directly. In *cometabolism*, microbes do not gain energy or carbon from degrading a contaminant. Instead, the contaminant is degraded via a side reaction. Cometabolic bioventing is an example, where

microbes may be fed with propane and degrade trichloroethylene (TCE) or less chlorinated ethenes.

Depending on the contaminant of concern and the media, a technology may exploit aerobic or anaerobic metabolism. Aerobic metabolism is more commonly exploited and can be effective for hydrocarbons and other organic compounds. Many organisms are capable of degrading hydrocarbons using oxygen as the electron acceptor and the hydrocarbons as carbon and energy sources. In some cases, contaminants are aerobically degraded to carbon dioxide and water, but in other cases the microbes do not completely degrade contaminants. Aerobic technologies may also change the ionic form of metals. If a site contains mixed metal and organic wastes, it is necessary to consider whether the oxidized forms of the metal species will be environmentally acceptable.

*Anaerobic metabolism* involves microbial reactions occurring in the absence of oxygen, and encompasses many processes including fermentation, methanogenesis, reductive dechlorination, sulfate-reducing activities, and denitrification. Depending on the contaminant of concern, a subset of these activities could be cultivated.

In anaerobic metabolism, nitrate, sulfate, carbon dioxide, oxidized metals, or organic compounds may replace oxygen as the electron acceptor. For example, in anaerobic reductive dechlorination, chlorinated solvents may serve as the electron acceptor.

When selecting a bioremediation technology, it is important to consider the contaminants of concern, contaminated matrix, potential biological pathways to degrade a contaminant, and current condition of a site. For example, TCE can be degraded via aerobic and anaerobic mechanisms. If groundwater is contaminated with TCE current groundwater conditions may be helpful in deciding which biological mechanism to exploit. If groundwater is already anaerobic, then anaerobic reductive dechlorination may be the best approach. However, if the TCE plume is diffuse and the groundwater is aerobic, it may be possible to use cometabolic technologies.

A key concept in evaluating all bioremediation technologies is microbial availability: if the contaminant is so tightly bound up in the solid matrix that

microorganism cannot access it, then it cannot be bioremediated. However, low microbial availability does not imply an absence or risk; compounds may be available to environmental receptors depending on the receptors and routes of exposure.

Thus, when selecting a bioremediation technology for a specific site, it is prudent to consider the contaminants of concern, potential degradation intermediates and residual of the contaminants, co-contaminants, environmental receptors, routes of exposure, and buffer zones between contamination and receptors. Bioremediation technologies have proven to be protective and cost-effective solutions at many sites. However, conditions at a specific site may not be appropriate.

## **2.2 IN SITU BIOREMEDIATION**

There are two major types of *in situ* bioremediation: intrinsic and enhanced. Both rely on natural processes to degrade contaminants with (enhanced) or without (intrinsic) amendments.

In recent years, *in situ* bioremediation concepts have been applied in treating contaminated soil and groundwater. Removal rates and extent vary based on the contaminant of concern and site-specific characteristics. Removal rates also are affected by variables such as contaminant distribution and concentrations; indigenous microbial populations and reaction kinetics; and parameters such as pH, moisture content, nutrient supply and temperature. Many of these factors are a function of the site and the indigenous microbial community and, thus, are difficult to manipulate.

When *in situ* bioremediation is selected as a treatment, site monitoring activities should demonstrate that biologically mediated removal is the primary route of contaminant removal. Sampling strategies should consider appropriate analytes and tests, as well as site heterogeneity. In some cases, extensive sampling may be required to distinguish bioremediation from other removal mechanisms or statistical variations. Small-scale treatability studies using samples from the contaminated site may also be useful in the demonstrating the role that biological activity plays in contaminant removal (EPA, 1995B; EPA, 1998a; EPA, 2000).

### 2.2.1 Intrinsic *in situ* bioremediation

Intrinsic bioremediation relies on natural processes to degrade contaminants without altering current conditions or adding amendments. Intrinsic bioremediation may play a role in monitored attenuation (MNA) sites. Natural attenuation (NA) relies on natural physical, chemical and biological processes to reduce or attenuate contaminant concentrations. Under favorable conditions, NA will reduce the concentrations, mass, toxicity, mobility, and/or volume of contaminants in soil and groundwater. Natural processes in NA include dilution, dispersion, sorption, volatilization, chemical reactions such as oxidation and reduction, biological reactions and stabilization. Some processes have undesirable results, such creation of toxic degradation products or the transfer of contaminants to other media.

Implementing natural attenuation requires a thorough site assessment and development of a conceptual model of the site. After determining the presence of a stable shrinking plume, site-specific, risk-based decisions using multiple lines of evidence may facilitate implementation of MNA at a site. While MNA is somewhat passive in that nothing is being added to the contamination zone, it requires active monitoring, which should be included as part of the design plan for a site. In some cases, such long-term monitoring may be more expensive than active remediation. MNA is only applicable to carefully controlled and monitored sites and must reduce contaminant concentrations to levels that are protective of human health and the environment in reasonable time frames (EPA, 1998a). Depending on site-specific conditions, MNA may be a reasonable alternative for petroleum hydrocarbons as well as chlorinated and non chlorinated VOCs and SVOCs (EPA, 1999a; EPA, 1999b).

Important observations related to the performance of natural attenuation technology are:

- it is a relatively simple technology compared to other remediation technologies;
- it can be carried out with little or no site disruption;
- it often requires more time to achieve cleanup goals than other conventional remediation methods;

- it requires a long-term monitoring program; program duration affects the costs;
- if natural attenuation rates are too slow, the plume could migrate;
- it is difficult to predict with high reliability the performance of natural attenuation;

Sites must meet one or more of the following criteria:

- it must be located in an area with little risk to human health or the environment;
- the contaminated soil or groundwater must be located an adequate distance from potential receptors;
- there must be evidence that natural attenuation is actually occurring at the site.

### **2.2.2 Enhanced *in situ* bioremediation**

Enhanced *in situ* bioremediation can be applied to groundwater, vadose zone soils or, more rarely, aquatic sediments. Exogenous microorganisms may be added where organisms able to degrade specific contaminants are absent (bioaugmentation). Additives such as oxygen (or other electron acceptors), nutrients, biodegradable carbonaceous substrates, bulking agents, and/or moisture are added to enhance the activity of natural occurring or indigenous microbial populations:

*Bioaugmentation:* involves the addition of supplemental microbes to the subsurface where organisms able to degrade specific contaminants are deficient. Microbes may be “seeded” from populations already present at a site and grown in aboveground reactors or from specially cultivated strains of bacteria known to degrade specific contaminants. The application of bioaugmentation technology is highly site-specific and highly dependent on the microbial ecology and physiology of the subsurface (EPA 1998).

*Nutrient addition:* involves the addition of key biological building blocks, such as nitrogen and phosphorus and other trace nutrients necessary for cell growth. Addition of nutrients generally is applied as a supplement to

bioaugmentation or addition of electron donors or electron acceptors, so that concentrations of nutrients in the subsurface do not become a limiting factor for an *in situ* bioremediation application.

*Electron donor addition:* involves the addition of a substrate that acts as a reductant in the redox reaction used by the CAH-degrading microbe to produce energy. A substrate such as toluene, propane, or methane may be added to act as a cometabolic oxidant, when the CAH also is oxidized. A substrate such as hydrogen, a source of hydrogen, or a hydrogen release compound may be added to act as a direct reductant, when the CAH is reduced.

*Electron acceptor addition:* involves the addition of oxygen (for aerobic mechanisms) or an anaerobic oxidant such as nitrate (for anaerobic mechanisms), which is used by the CAH-degrading microbes present in the subsurface.

#### **2.2.2.1 Vadose zone soil remediation**

While the fundamental biological activities exploited by *in situ* bioremediation may occur naturally, many sites will require intervention to facilitate cleanup. For example the addition of organic substrates, nutrients or air will provide the appropriate environment for specific microbial activities or enhanced removal rates. In general, hydrocarbons and lightly chlorinated contaminants may be removed through aerobic treatment while highly chlorinated species are degraded primarily through anaerobic treatment. Both anaerobic and aerobic treatment may occur through direct or cometabolic pathways (see 1.3.4.2).

The primary *in situ* biological technology applicable is bioventing (aerobic, cometabolic, or anaerobic).

**Aerobic bioventing** is useful in treating aerobically degradable contaminants such as fuels. Contaminated unsaturated soils with low oxygen concentrations are treated by supplying oxygen to facilitate aerobic microbial biodegradation. Oxygen is typically introduced by air injection wells that push air into the subsurface (figure 2.1); vacuum extraction wells, which draw air through the surface, may also be used. Extracted gases may require treatments since volatile compounds may be removed from the ground. Compared with soil vapor extraction bioventing employs lower air flow rates that provide only the amount of oxygen required to enhance removal. Operated properly the injection of air does

not result in the release of the contaminants to the atmosphere through volatilization because of these low flow rates. Bioventing is designed primarily to treat aerobically degradable contaminants, such as non-chlorinated VOCs and SVOCs, that are located in the vadose zone of the capillary fringe. In addition to fuels treatment, aerobic bioventing has treated a variety of other contaminants including non-halogenated solvents such as benzene, acetone, toluene, and phenol; lightly halogenated solvents such as 1,2-dichloroethane, dichloromethane, and chlorobenzene; and SVOCs such as low-molecular-weight PAHs. Nevertheless bioventing has some limitations involving the ability to deliver oxygen to the contaminated soil. For example, soils with extremely high moisture content may be difficult to biovent because of reduced soil gas permeability.

While it is relatively inexpensive, bioventing can take a few years to clean up a site depending on contaminant concentrations and site-specific removal rates.

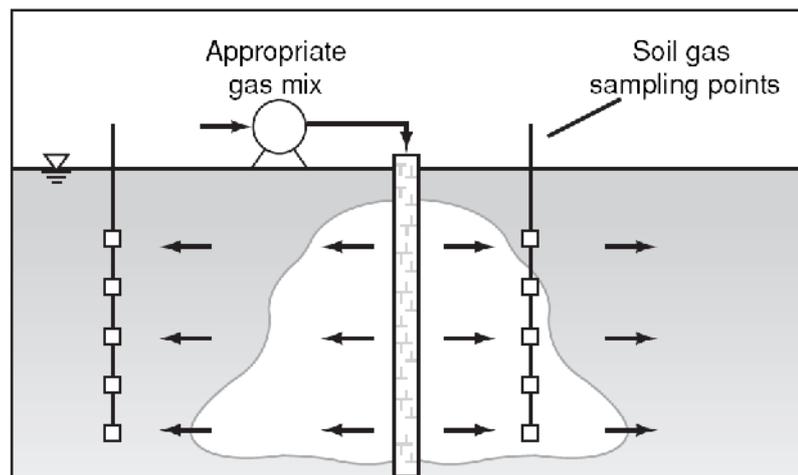


Figure 2.1. Aerobic bioventing in injection mode.

**Cometabolic bioventing** has been used at a few sites to treat chlorinated solvents such as trichloroethylene (TCE), trichloroethane (TCA) and dichloroethene (DCE). Similar to bioventing, cometabolic bioventing involves the injection of gases into the subsurface; however cometabolic bioventing injects both air and a volatile organic substrate, such as propane. This technology exploits competitive reactions mediated by monooxygenase enzymes which catalyze the oxidation of hydrocarbons, often through epoxide intermediates. These enzymes can also catalyze the dechlorination of chlorinated hydrocarbons. Thus, by

supplying an appropriate organic substrate and air, production of monooxygenases may be stimulated resulting in the contaminants breakdown (see 1.3.4.2.1).

In addition to the variable discussed for the aerobic bioventing, the degradation rate and design of cometabolic bioventing systems are dependent on many factors including soil gas permeability, organic substrate concentration, type of organic substrate selected, and oxygen supply and radius of influence. As with aerobic bioventing, difficulty in distributing gases in the subsurface may make the application of this technology more complicated.

**Anaerobic bioventing.** While aerobic and cometabolic bioventing are useful for degrading many hydrocarbons and lightly chlorinated compounds, some chlorinated species are not effectively treated aerobically. Microbes may degrade these contaminants directly via anaerobic reductive dechlorination or through anaerobic cometabolic pathways. Anaerobic reductive dechlorination is a biological mechanism typically marked by sequential removal of chlorine from a molecule (see 1.3.4.2.2). Microbes possessing this pathway do not gain energy from this process. Anaerobic cometabolism is similar to aerobic cometabolism in that microbes fortuitously degrade contaminants while reducing other compounds (cometabolites). Anaerobic bioventing may use both biological mechanisms to destroy the contaminants of concern.

Anaerobic bioventing uses the same type of gas delivery system as the other bioventing technologies, but injects nitrogen and an electron donor, instead of air, to establish reductive anaerobic conditions. The nitrogen displaces the soil oxygen, and small amounts of an electron donor gas (such as hydrogen and carbon dioxide) produce reducing conditions in the subsurface, thereby facilitating microbial dechlorination. Volatile and semi-volatile compounds may be produced during anaerobic bioventing. Some of these compounds may be slow to degrade under anaerobic conditions. These compounds may be treated in two ways. Volatile compounds may diffuse into the soils surrounding the treatment zone, where aerobic degradation may occur. SVOCs and VOCs remaining in the treatment zone may be treated by following anaerobic bioventing with aerobic bioventing. Since aerobic and anaerobic bioventing share similar gas delivery systems, the switch can be made by simply changing the injected gas.

Anaerobic bioventing is an emerging technology that has been demonstrated in several laboratory and field studies. This process may be useful in treating highly chlorinated compounds such as tetrachloroethene (PCE), TCE, RDX, pentachlorophenol, and pesticides such as lindane and dichlorodiphenyltrichloroethane (DDT). As with the other bioventing technologies, the ability to deliver gases to the subsurface is important. Soils with high moisture content or low gas permeability may require careful system design to deliver appropriate levels of nitrogen and the electron donor. Sites with shallow contamination or nearby buildings are also a challenge since this technology is operated by injecting gases. In addition, anaerobic bioventing can take a few years to clean up a site depending on the contaminant concentrations and site-specific removal rates.

#### **2.2.2.2 Surficial soil remediation**

If contamination is shallow, soil may be treated in place using techniques similar to land treatment or composting. Variations of these technologies involve tilling shallow soils and adding amendments to improve aeration and bioremediation. Since these treatments do not include an impermeable sublayer, contaminant migration may be a concern depending on the contaminants of concern and treatment amendments. A more prudent approach would be to excavate soils and treat them in lined beds.

### 2.2.2.3 Groundwater and saturated soil remediation

*In situ* bioremediation techniques applicable to ground water and saturated soil include dechlorination using anaerobic reducing conditions, enhanced aerobic treatment, biological reactive barriers that create active remediation zones, and bioslurping/biosparging techniques that promote aerobic degradation.

**Anaerobic reductive dechlorination** has been used at many sites where the ground water has been contaminated with chlorinated solvents, such as TCE or PCE. In this treatment, organic substrates are delivered to the subsurface where they are fermented. The fermentation creates an anaerobic environment in the area to be remediated and generates hydrogen as a fermentation byproduct. The hydrogen is used by a second microbial population to sequentially remove chlorine atoms from chlorinated solvents (see 1.3.4.2.1). If PCE were degraded via reductive dechlorination, the following sequential dechlorination would be observed: PCE would be converted to TCE, then to DCE, vinyl chloride (VC), and/or dichloroethane (EPA, 1998a).

Anaerobic dechlorination may also occur via cometabolism where the dechlorination is incidental to the metabolic activities of the organisms. In this case, contaminants are degraded by microbial enzymes that are metabolizing other organic substrates. Cometabolic dechlorination does not appear to produce energy for the organism. At pilot- or full-scale treatment, cometabolic and direct dechlorination may be indistinguishable, and both processes may contribute to contaminant removal. The microbial processes may be distinguished in the more controlled environment of a bench-scale system (EPA, 1998a).

Anaerobic reductive dechlorination is primarily used to treat halogenated organic contaminants, such as chlorinated solvents. As well as the variables discussed initially, the treatment rate and system design are dependent on several factors including site hydrology and geology, type and concentration of organic substrates, and site history. As with cometabolic bioventing, the selection of organic substrate and the concentration used are controllable and can be important to the removal rate. Treatability or bench-scale testing can be useful in selecting the best organic substrate and concentration for a site. In addition, small-scale testing can demonstrate that full dechlorination is possible at a site. In some cases,

dechlorination may stall at DCE despite the presence of sufficient electron donors. If a site does not demonstrate full dechlorination (either as part of site assessment or in microcosm testing), a combined treatment strategy, such as anaerobic treatment followed by aerobic treatment, may be successful. Alternatively, bioaugmentation may improve the dechlorination rate.

**Aerobic Treatment.** Similar to bioventing, enhanced in situ aerobic ground water bioremediation processes are used in situations where aerobically degradable contaminants, such as fuels, are present in anaerobic portions of an aquifer. In these situations, air or other oxygen sources are injected into the aquifer near the contamination (figure 2-2). As the oxygenated water migrates through the zone of contamination, the indigenous bacteria are able to degrade the contaminants (EPA, 1998a; EPA, 2000).

Aerobic treatment may also be used to directly or cometabolically degrade lightly chlorinated species, such as DCE or VC. In the direct aerobic pathway, air is injected into the aquifer. The microbes appear to generate energy by oxidizing the hydrocarbon backbone of these contaminants, resulting in the release of chloride (EPA, 2000). This process has been used to complete contaminant removal following anaerobic treatment at several sites (EPA, 1998a; EPA, 2000).

Cometabolic aerobic treatment is founded on the same biological principles as cometabolic bioventing and involves the addition of oxygen and organic substrates, such as methane, to the aquifer. As with other cometabolic processes, these organic substrates are metabolized by enzymes that incidentally degrade the contaminant. In this treatment, sufficient oxygen must be present to fuel the oxidation of both the substrate and contaminant.

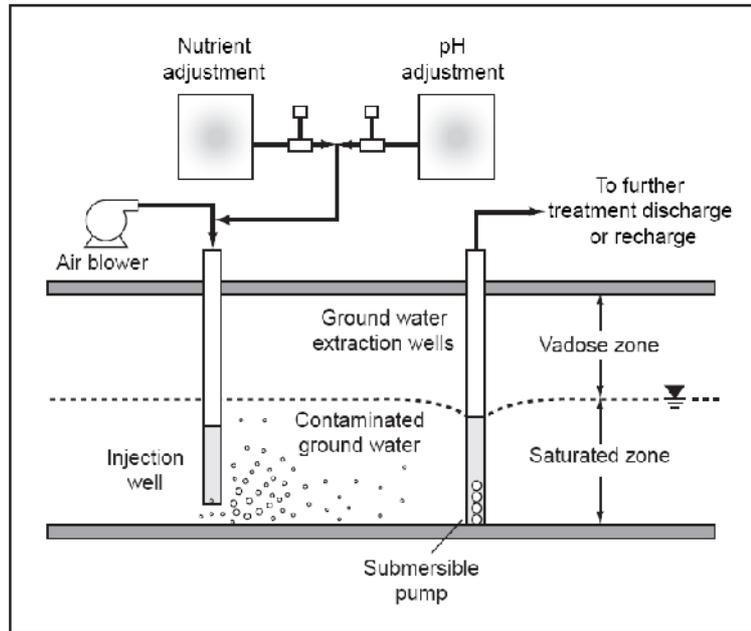


Figure 2.2. Aerobic treatment.

**Amendment Delivery.** In situ groundwater treatment, either aerobic or anaerobic, may be configured as direct injection of air or aqueous streams or as ground water recirculation. In direct injection, amendments, such as organic substrates, oxygen sources, or nutrients, are directly injected into the aquifer. For example, oxygen may be sparged into the aquifer as a gas. Lactate or hydrogen peroxide may be injected as a liquid stream; when using hydrogen peroxide, caution should be used as it may act as a disinfectant. In some cases, both liquids and gases are added. The ground water recirculation configuration involves extracting ground water, amending it as needed, and then re-injecting it back into the aquifer. Recirculation may also be conducted below the ground surface by extracting ground water at one elevation, amending it in the ground, and re-injecting it into another elevation (EPA, 1998a; EPA, 2000).

In addition to the variables discussed initially, the treatment rates and system design are the result of several factors including site hydrology and geology, amendment to be added, solubility of air or oxygen sources, and site

history. The low solubility of air in water often limits reaction rates and may make this process impractical if cleanup time is short.

**Biological reactive barriers** consist of an active bioremediation zone created in the contamination zone. The use of in situ treatment walls for remediation is an emerging technology that has been developed and implemented only within the last few years. Treatment walls are structures installed underground to treat the contaminated groundwater found at hazardous waste sites (figure 2.3). Treatment walls rely on the natural movement of water to carry the contaminants through the wall structure. As contaminated groundwater passes through the treatment wall, the contaminants are either trapped by the treatment wall or transformed into harmless substances that flow out of the wall (USEPA, 1996d). Target contaminant groups for passive treatment walls are VOCs, SVOCs, and inorganics. The specific filling chosen for the wall is based on the contaminant found at the site. Wall fillings work through different chemical processes, of which the three most common are (USEPA, 1996d; Birke et al., 2003):

- *Sorption barriers* contain fillings that remove contaminants from the groundwater by physically removing contaminants from the groundwater and holding them on the barrier surface. Zeolites and activated carbon are two examples of sorption barriers.
- *Precipitation barriers* contain fillings that react with contaminants in the groundwater as they pass through the treatment wall. The reactions cause the contaminants dissolved in groundwater to become insoluble and to precipitate out. The barrier traps the insoluble products and clean groundwater flows out the other side.
- *Degradation barriers* cause reactions that break down the contaminants in the groundwater into harmless products. Filling walls with iron granules helps to degrade certain VOCs, and walls filled with a mixture of nutrients and oxygen sources can stimulate the activity of the microorganisms found in the groundwater.

Two main types of treatment walls exist:

- *Permeable reactive trench*: this is the simplest form of treatment walls and it consists of a trench that extends across the entire width of the plume. The system is installed by digging a trench and filling it with permeable material. As the contaminant plume moves through the wall, contaminants are removed by various mass transfer processes such as air stripping, SVE, and adsorption.
- *Funnel and gate systems*: used primarily when contaminated plumes are too large or too deep to dig a trench across its width. To overcome this problem, a system consisting of low permeability cut-off walls are installed to funnel contaminated groundwater to a smaller reactive wall to treat the plume. When dealing with funnel and gate systems, the gate is used to pass contaminated groundwater through the reactive wall, and the funnel is integrated into the system to force water through its gates. Plumes which contain a mixture of contaminants are funnelled through a gate with multiple reactive walls in series

Important observations related to the performance of passive/reactive treatment technology are:

- It is limited to a subsurface lithology that has a continuous aquitard at a depth that is within the vertical limits of the trenching equipment.
- Passive treatment walls have a tendency to lose their reactive capacity over time, and require replacement of the reactive medium.
- Large and deep plumes are more difficult to remediate than small and shallow plumes. The complete cost of using treatment walls to remediate contaminated groundwater is not available. However, the cost is believed to be dependent on the reactive media and the contaminant concentration in the groundwater.

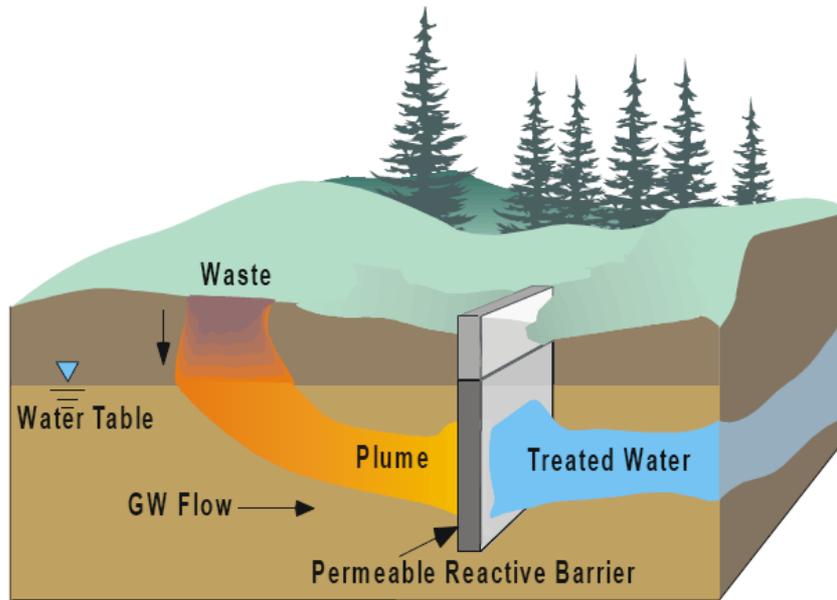


Figure 2.3. Permeable reactive barrier.

**Biosparging and bioslurping.** Biosparging (similar to air sparging) involves the injection of a gas (usually air or oxygen) and occasionally gas-phase nutrients, under pressure, into the saturated zone to promote aerobic biodegradation. In air sparging, volatile contaminants also can be removed from the saturated zone by desorption and volatilization into the air stream. Emphasis on the biological degradation rate over physical removal, as well as lower rates of air injection, are what distinguishes this technology from air sparging.

Typically, biosparging is achieved by injecting air into a contaminated subsurface formation through a specially designed series of injection wells. The air creates an inverted cone of partially aerated soils surrounding the injection point. The air displaces pore water, volatilizes contaminants, and exits the saturated zone into the unsaturated zone. While in contact with groundwater, oxygen dissolution from the air into the groundwater is facilitated and supports aerobic biodegradation.

A number of contaminants have been successfully addressed with biosparging technology, including gasoline components such as benzene, toluene, ethylbenzene, and xylenes (BTEX) and SVOCs. Biosparging is most often recommended at sites impacted with mid-weight petroleum hydrocarbon contaminants, such as diesel and jet fuels. Lighter contaminants, such as gasoline,

tend to be easily mobilized into the unsaturated zone and physically removed. Heavier contaminants, such as oils, require longer remedial intervals because of reduced microbial bioavailability with increasing carbon chain length (EPA, 2004b). Care must be taken to determine whether contaminant concentrations in soil gas and released vapors resulting from biosparging require treatment. For this reason, biosparging may be implemented along with SVE or bioventing as a remedy for increased contaminant concentrations in the unsaturated zone. The SVE wells are designed to capture the introduced air and contaminant vapors (EPA, 2004b). Figure 2.4 depicts a typical biosparging system with optional SVE system. Alternatively, a lower-flow bioventing system may be added to facilitate bioremediation of volatilized contaminants in the vadose zone.

One specialized form of biosparging involves the injection of organic gases into the saturated zone to induce cometabolic biodegradation of chlorinated aliphatic hydrocarbons (analogous to cometabolic bioventing). The injection of gases below the water table distinguishes biosparging from bioventing. In contrast to cometabolic bioventing, the solubility of organic gases in water limits delivery of the primary substrate during cometabolic biosparging applications.

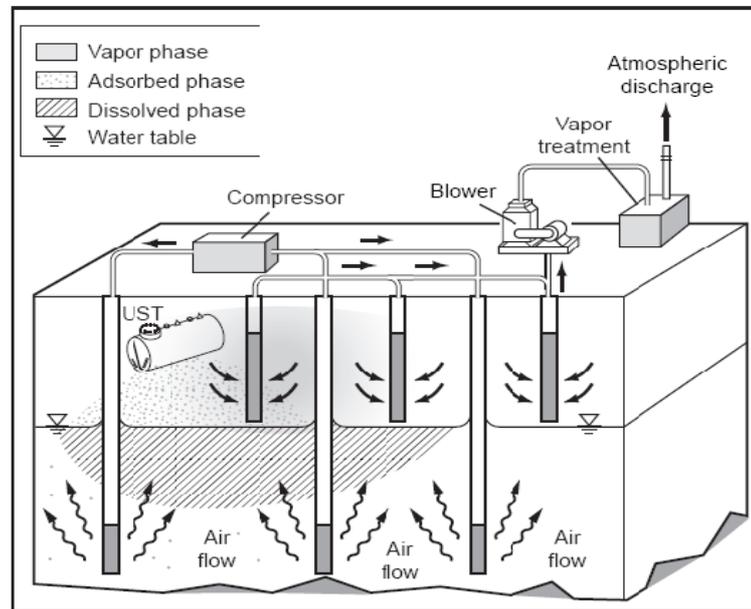


Figure 2.4. Biosparging system.

Bioslurping (also known as multi-phase extraction) is effective in removing free product that is floating on the water table (Battelle, 1997). Bioslurping combines the two remedial approaches of bioventing and vacuum-enhanced free-product recovery. Bioventing stimulates aerobic bioremediation of contaminated soils in situ, while vacuum-enhanced free-product recovery extracts light, nonaqueous-phase liquids (LNAPLs) from the capillary fringe and the water table. A bioslurping tube with adjustable height is lowered into a ground water well and installed within a screened portion at the water table (see Figure 2.5). A vacuum is applied to the bioslurping tube and free product is “slurped” up the tube into a trap or oil water separator for further treatment. Removal of the LNAPL results in a decline in the LNAPL elevation, which in turn promotes LNAPL flow from outlying areas toward the bioslurping well. As the fluid level in the bioslurping well declines in response to vacuum extraction of LNAPL, the bioslurping tube also begins to extract vapors from the unsaturated zone. This vapor extraction promotes soil gas movement, which in turn increases aeration and enhances aerobic biodegradation (Miller, 1996).

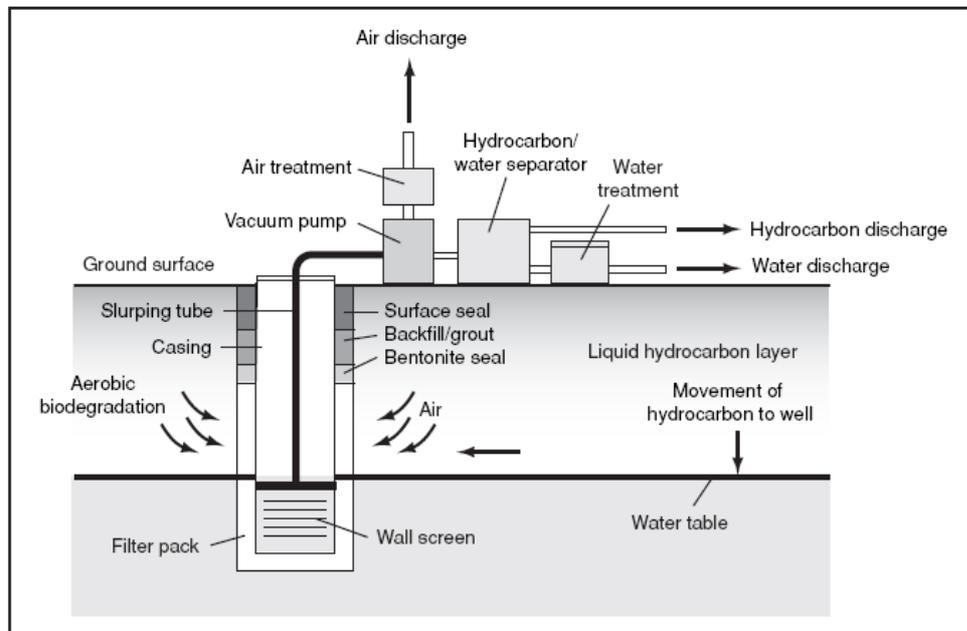


Figure 2.5. Bioslurping technology.

## **2.3 EX SITU BIOREMEDIATION**

Ex situ bioremediation technologies can most easily be classified by the physical state of the medium to which they are typically applied: solids, solid–liquid mixtures, and liquids. Also common to the ex situ remediation technologies are the processes for removing contaminated materials for treatment. Contaminated media are excavated or extracted (e.g., ground water removal by pumping) and moved to the process location, which may be within or adjacent to the contamination zone.

### **2.3.1 Solids**

The most common types of solids bioremediation are (1) land farming or land treatment, (2) composting, and (3) biopiles, cells, or mounds.

#### **2.3.1.1 Land Treatment**

Land treatment, also called land farming, is useful in treating aerobically degradable contaminants. This process is suitable for non-volatile contaminants at sites where large areas for treatment cells are available. Land treatment of site-contaminated soil usually entails the tilling of an 8-to 12-inch layer of the soil to promote aerobic biodegradation of organic contaminants. The soils are periodically tilled to aerate the soil, and moisture is added when needed. In some cases, amendments may be added to improve the tilth of the soil, supply nutrients, moderate pH, or facilitate bioremediation. Typically, full-scale land treatment would be conducted in a prepared-bed land treatment unit (see Figure 2.6)—an open, shallow reactor with an impermeable lining on the bottom and sides to contain leachate, control runoff, and minimize erosion and with a leachate collection system under the soil layer (EPA, 1993). In some cases, hazardous wastes (such as highly contaminated soils) or process wastes (such as distillate residues) may be treated in land treatment units. In these cases, the waste may be applied to a base soil layer.

The performance of land treatment varies with the contaminants to be treated. For easily biodegradable contaminants, such as fuels, land treatment is

inexpensive and effective. Contaminants that are difficult to degrade, such as PAHs, pesticides, or chlorinated organic compounds, are topics of research.

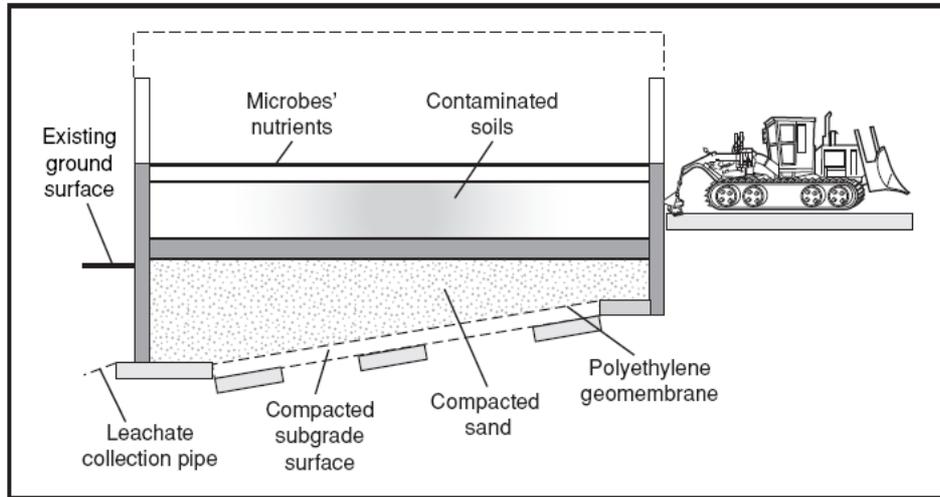


Figure 2.6. Land treatment.

### 2.3.1.2 Composting

Composting is a controlled biological process that treats organic contaminants using microorganisms under thermophilic conditions (40°–50°C). For some practitioners, the creation of thermophilic conditions is the primary distinction between composting and biopiles (which operate at less than 40°C), although others use composting as a term that encompasses both temperature ranges.

In composting, soils are excavated and mixed with bulking agents and organic amendments, such as wood chips and vegetative wastes, to enhance the porosity of the mixture to be decomposed. Degradation of the bulking agent heats up the compost, creating thermophilic conditions. Oxygen content, moisture levels, and temperatures are monitored and manipulated to optimize degradation. Oxygen content usually is maintained by frequent mixing, such as daily or weekly turning of windrows. Surface irrigation often is used to maintain moisture content. Temperatures are controlled, to a degree, by mixing, irrigation, and air flow, but are also dependent on the degradability of the bulk material and ambient conditions.

There are three designs commonly applied for composting:

- *aerated static piles*: compost is formed into piles and aerated with blowers or vacuum pumps;
- *mechanically agitated in-vessel composting*: compost is placed in a reactor vessel, in which it is mixed and aerated;
- *windrow composting*: compost is placed in long, low, narrow piles (i.e., windrows) and periodically mixed with mobile equipment.

Windrow composting is the least expensive method, but has the potential to emit larger quantities of VOCs. In-vessel composting is generally the most expensive type, but provides for the best control of VOCs. Aerated static piles, especially when a vacuum is applied, offer some control of VOCs and are typically in an intermediate cost range, but will require offgas treatment. Berms may also be needed to control runoff during composting operations. Runoff may be managed by retention ponds, provision of a roof, or evaporation.

Composting has been successfully applied to soils and biosolids contaminated with petroleum hydrocarbons (e.g., fuels, oil, grease), solvents, chlorophenols, pesticides, herbicides, PAHs, and nitro-aromatic explosives (EPA, 1998b; EPA, 1997; EPA, 2004b). Composting is not likely to be successful for highly chlorinated substances, such as PCBs, or for substances that are difficult to degrade biologically (EPA, 1998b).

### **2.3.1.3 Biopiles**

Biopiles involve the mixing of excavated soils with soil amendments, with the mixture placed in a treatment area that typically includes an impermeable liner, a leachate collection system, and an aeration system. Biopiles are typically 2–3 meters high, and contaminated soil is often placed on top of treated soil (see figure 2.7). Moisture, nutrients, heat, pH, and oxygen are controlled to enhance biodegradation. This technology is most often applied to readily degradable species, such as petroleum contaminants. Surface drainage and moisture from the leachate collection system are accumulated, and they may be treated and then recycled to the contaminated soil. Nutrients (e.g., nitrogen and phosphorus) are often added to the recycled water. Alkaline or acidic substances may also be

added to the recycled water to modify or stabilize pH to optimize the growth of select microbes capable of degrading the contaminants of concern.

An air distribution system is buried in the soil as the biopile is constructed. Oxygen exchange can be achieved utilizing vacuum, forced air, or even natural draft air flow. Low air flow rates are desirable to minimize contaminant volatilization. If volatile constituents are present in significant concentrations, the biopile may require a cover and treatment of the offgas.

Biopile treatment lasts from a few weeks to a few months, depending on the contaminants present and the design and operational parameters selected for the biopile. Biopiles are typically mesophilic (10°–45°C).

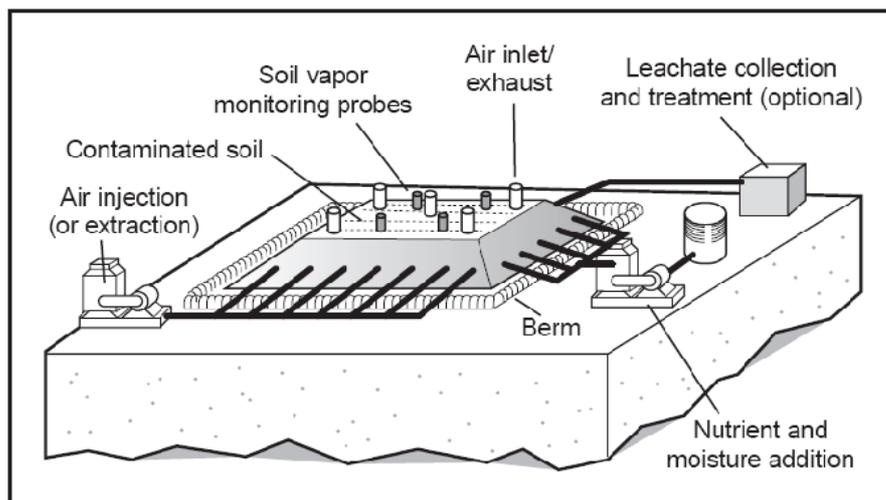


Figure 2.7. Typical biopile system.

### 2.3.2 Solid–liquid mixtures

Solid-liquid mixtures consist of materials such as slurries and sludges. One technology for treating such mixtures is discussed below.

#### 2.3.2.1 Slurry Bioreactors

Slurry bioreactors are utilized for soil, sediments, sludge, and other solid or semi-solid wastes. Slurry bioreactors are costly and, thus, are likely to be used for more difficult treatment efforts.

Typically, wastes are screened to remove debris and other large objects, then mixed with water in a tank or other vessel until solids are suspended in the liquid phase. If necessary, further particle size reduction can be accomplished before the addition of water (by pulverizing and/ or screening the wastes) or after the addition of water (through use of a sheering mixer). Suspension and mixing of the solids may increase mass transfer rates and may increase contact between contaminants and microbes capable of degrading those contaminants (EPA, 1990). Mixing occurs in tanks or lined lagoons. Mechanical mixing is generally conducted in tanks. Typical slurries are 10–30% solids by weight. Aeration, with submerged aerators or spargers, is frequently used in lagoons and may be combined with mechanical mixing to achieve the desired results. Nutrients and other additives, such as neutralizing agents, surfactants, dispersants, and co-metabolites (e.g., phenol, pyrene) may be supplied to improve handling characteristics and microbial degradation rates. Indigenous microbes may be used or microorganisms may be added initially to seed the bioreactor or may be added continuously to maintain proper biomass levels. Residence time in the bioreactor varies with the matrix as well as the type and concentration of contaminant (EPA, 1990).

Once contaminant concentrations reach desired levels on a dry-weight basis, the slurry is dewatered. Typically, a clarifier is utilized to dewater the slurry by gravity. Other dewatering equipment may be used depending on slurry characteristics and cost considerations (Olin et al., 1999). Water, air emissions from all process steps, and oversize materials may require additional treatment.

### **2.3.3 Liquids**

Liquids, such as surface water, groundwater, mine drainage, and effluent from other treatment operations, can undergo *ex situ* bioremediation in constructed wetlands. Note that surface water and groundwater have important differences, such as concentrations of contaminants and degradable organic material, than may be found in waste streams from other treatment operations.

#### **2.3.3.1 Constructed wetlands**

Constructed wetlands provide for biological assimilation, breakdown, and transformation of contaminants; chemical breakdown and transformation of contaminants; and physical sedimentation and filtration (USDA and EPA 1994a), as shown in figure 2.8. Biological processes associated with wetlands include bioremediation (microbially-based remediation) and phytoremediation (plant-based remediation). Microbes attached to the surfaces of plants, plant litter, and the wetland substrate degrade and/or sorb the organic substances present in the water undergoing treatment (USDA and EPA, 1994a). Phytoremediation uses plants to remove, transfer, stabilize, or destroy contaminants through biological, chemical, and physical processes that are influenced by plants and their roots (i.e., rhizosphere) that include degradation, extraction through accumulation in plant roots/shoots/leaves, metabolism of contaminants, and immobilization of contaminants at the interface of roots and soil (EPA, 2004a).

Wetlands inherently have a higher rate of biological productivity/activity than many other natural ecosystems and are thus capable of efficiently and economically transforming many common contaminants to harmless byproducts (Kadlec and Knight, 1996). Constructed wetlands have been applied successfully to remove contaminants such as metals, petroleum hydrocarbons, and glycols; to decrease metal concentrations via chemical or microbial precipitation; and to neutralize acidity. Recent research also has demonstrated applicability to explosive-contaminated water (Bader, 1999). However, wetlands are sensitive to high ammonia levels, herbicides, and contaminants that are toxic to the plants or microbes.

Constructed wetlands are well suited for the treatment of contaminated groundwater emerging from surface and mine seeps, pump-and-treat waste streams with low concentrations of easily biodegradable contaminants, and contaminated surface waters (EPA, 2001c). Constructed wetlands may also be used to pretreat contaminated water prior to conventional treatment or to further treat a waste stream prior to disposition or discharge (USDA and EPA, 1994b). However, applicability to highly acidic waste streams may not be cost-effective (USDA and EPA, 1994b). Discharges must meet applicable effluent limitations and related regulatory requirements. Discharges that do not meet these

requirements may be required to undergo further treatment or may be found suitable for recycling into the wetland as a supplemental water source.

There are various types of constructed wetlands, depending on the type of flow (surface or subsurface), contaminant of concern, or type of substrate, which can include limestone, organic material such as compost, or gravel. The chemical and microbial processes may proceed either in an anaerobic or aerobic environment.

Since constructed wetlands function both as macroscopic and microscopic ecosystems to promote contaminant treatment, the biological characteristics of the system must be taken into account during the design phase. The chemistry of the waste stream and how the passive chemical, physical, and biological processes affect this or are, in turn, affected by the waste stream are important design factors. The chemical characteristics of the waste stream can affect sizing of the system for adequate retention time and whether the waste stream may require pretreatment to (1) address concentration, ammonia, nutrient, and organic loads that may damage vegetation, or (2) remove solids or materials, such as grease, that may clog the wetland (USDA and EPA, 1994a). In addition, pH adjustment may be necessary, either prior to waste stream treatment or through use of limestone substrate (USDA and EPA, 1994b). Climatic and seasonal circumstances as well as waste stream characteristics are important considerations when selecting the types of plants to use in a constructed wetland. Salinity, either in the waste stream or as a result of treatment, can harm or destroy the wetland vegetation if the plants are not salt tolerant. In addition, cold weather can reduce microbial activity, and hail or other weather events can damage the plants (USDA and EPA, 1994a).

The low cost, passivity (i.e., lack of dependence on power or mechanical components), and efficacy for treating many common contaminants are key advantages of constructed wetland treatment systems. Constructed wetlands are often visually attractive, but can require more space than other remedial systems. The wetlands should be sized with an understanding that both plant-based and bacterial-based remediation will decline during colder seasons. A key design element is sizing to achieve adequate retention time to enable the biological, chemical, and physical processes to be effective (USDA and EPA, 1994a).

Seasonal, climatological, and waste stream factors that control the water balance in the wetland also must be considered during design to achieve project goals.

Constructed wetlands require a continuous supply of water. While tolerant of fluctuating flows, constructed wetlands cannot withstand complete drying. A slow water flow must be maintained to prevent the development of stagnant water that can lead to performance and vector difficulties. Recycling wetland water can supplement inflow, but this can increase salinity over time, which can affect design and cost (USDA and EPA, 1994a).

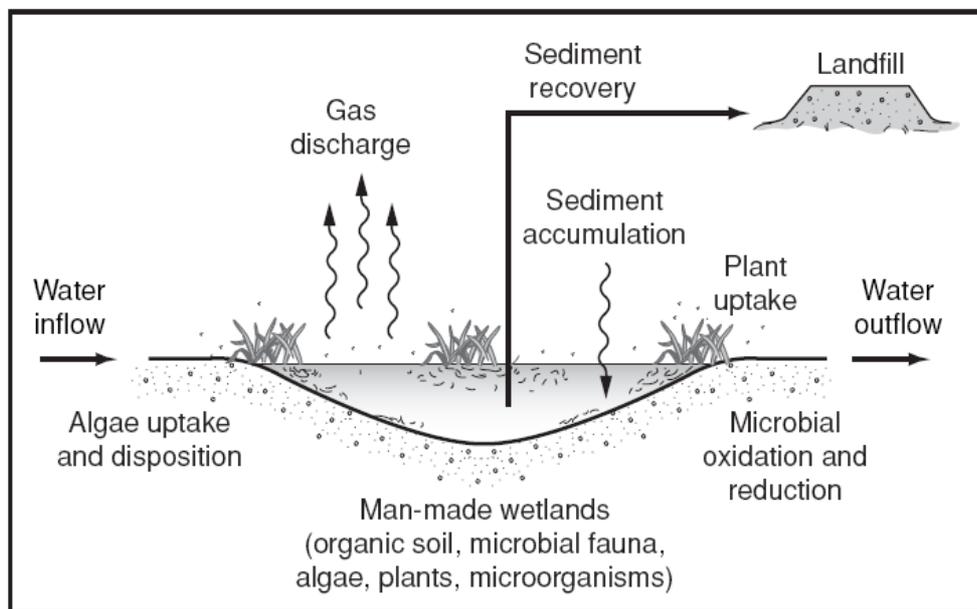


Figure 2.8. Constructed wetland.

## CHAPTER 3

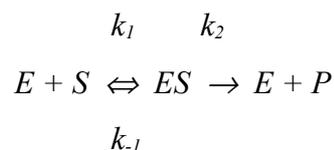
### 3. KINETIC MODELS FOR COMETABOLISM

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Cometabolic biotransformation models most often stem from Michaelis-Menten and Monod enzyme kinetics. These expressions have been expanded to include processes such as substrate inhibition (Broholm et al., 1992; Ely et al., 1995a; and Kim et al., 2002a), product toxicity (Chang and Criddle, 1997; Alvarez-Cohen and McCarty, 1991; Kim et al., 2000) and reducing energy limitations (Chang and Alvarez-Cohen, 1995a; Sipkema et al., 2000). Alvarez-Cohen and Speitel (2001) provided a review and discussion of these processes and the models representing them.

#### 3.1 ENZYMATIC REACTIONS: MICHAELIS-MENTEN KINETICS

In 1913 Michaelis and Menten proposed a kinetic model to describe enzyme catalyzed reactions: with some simplifications, this model can be adopted to simulate most of the reactions occurring in the biological systems with a limited number of parameters. The model is based on the hypothesis that the free enzyme (E) and the substrate (S) bind in an activated complex (ES), generating the product (P) and the enzyme (E):



The parameters  $k_1$ ,  $k_2$ ,  $k_{-1}$  represent the reaction constants; the first reaction is assumed at equilibrium, while the second is considered irreversible.

Assuming that the process of formation and disruption of the activated complex follow a second order and a first order kinetics respectively, the reaction rates can be expressed by the following equations:

$$\frac{d[P]}{dt} = k_2 \cdot [ES] \quad (3.1)$$

$$\frac{d[ES]}{dt} = k_1 \cdot [E] \cdot [S] - (k_{-1} + k_2) \cdot [ES] \quad (3.2)$$

where the expressions in the square brackets represent the concentrations of the corresponding reagents.

The enzyme mass balance assumes the following form, where  $E_0$  is the total enzyme concentration:

$$[E] + [ES] = E_0 = \text{const} \quad (3.3)$$

The system can be analytically solved with the hypothesis that the mass of the activated complex is conservative, as expressed by the following equation:

$$\frac{d[ES]}{dt} = k_1 \cdot [S] \cdot [E] - (k_{-1} + k_2) \cdot [ES] = 0 \quad (3.4)$$

This assumption, known as *quasi-stationary state approximation*, is correct if the ratio of the total enzyme concentration  $E_0$  to the initial substrate concentration  $S_0$  is sufficiently low. This condition is satisfied in most of the practical applications, with the exception of a short initial transitory state. With these hypotheses, the analytical solution for the product formation rate, corresponding to the substrate degradation rate, assumes the form:

$$\frac{d[P]}{dt} = \frac{k_2 \cdot [ES] \cdot [S]}{K_s + [S]} \quad (3.5)$$

where  $K_s$ , known as “affinity constant” or “half saturation constant, is defined:

$$K_s = \frac{k_{-1} + k_2}{k_1} \quad (3.6)$$

For the application of this model to the microbial mediated reactions,  $[E_0]$  can be assumed as a constant among different microorganisms; thus,  $[E_0]$  is proportional to the microbial concentration  $X$ . The substrate degradation velocity can be rewritten, omitting the brackets for simplicity, as:

$$r_s = \frac{dS}{dt} = - \frac{k_{\max, s} \cdot X \cdot S}{K_s + S} \quad (3.7)$$

The substrate specific degradation velocity per unit of biomass, also known as “degradation rate” ( $q_s = r_s / X$ ), is expressed by the following equation:

$$q_s = \frac{dS}{dt \cdot X} = - \frac{k_{\max, s} \cdot S}{K_s + S} \quad (3.8)$$

The parameter  $k_{\max,s}$  accounts for both the constant  $k_2$  and the factor of proportionality between  $E_0$  and  $X$ ; this constant is generally recognized as “maximum specific degradation rate” of substrate consumption, and corresponds to the maximum degradation velocity that can be achieved by a unitary mass of microorganisms. This condition, expressed by Eq. 2.9, is verified when the affinity constant  $K_s$  can be neglected in comparison to the substrate concentration  $S$ ; in this concentration range, the substrate degradation follow a zero order kinetic:

$$\frac{dS}{dt} \cdot X = -k_{\max, s} \quad (3.9)$$

The kinetic parameters are specific for the microbial culture and the substrate. When the degradation is sustained by a microbial consortium, the kinetic constants  $k_{\max}$  and  $K_s$  are obtained from a weighed average of the characteristic values of each species.

The following equation represents the microbial growth ( $\text{mg L}^{-1} \text{d}^{-1}$ ) according to the Monod model, and introduces the cell growth yield  $Y$  ( $\text{mg cell/mg substrate}$ ) and the cell decay coefficient  $b$  ( $\text{d}^{-1}$ ), representing the endogenous cell inactivation:

$$r_x = \frac{dX}{dt} = -Y \frac{dS}{dt} - bX \quad (3.10)$$

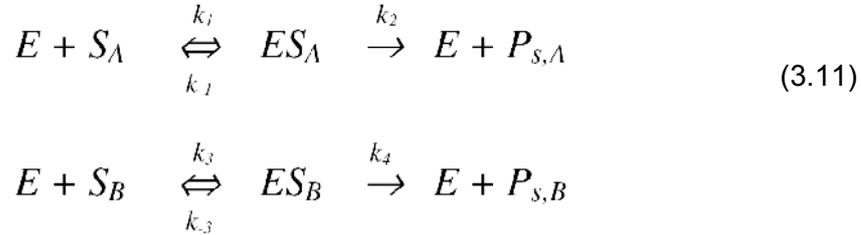
### 3.2 SUBSTRATE INHIBITION

Microorganisms capable of transforming CAHs through aerobic cometabolism have catabolic oxygenases that catalyze the initial oxidation step of their respective primary growth substrates and have potential for initiating the oxidation of CAHs. The oxygenases are often non specific and fortuitously initiate oxidation of a variety of compounds including most of the CAHs. In general, oxygenases act on unsaturated CAHs such as TCE by adding oxygen across the double bond to form an epoxide. With saturated CAHs such as CF or TCA, a hydroxyl group is generally substituted for one of the hydrogen atoms in the CAH molecule. Frequently, the resulting products from CAHs oxidation are chemically unstable: they decompose yielding products that are further metabolized by other microorganisms present in nature.

Because a single enzyme is responsible for the oxidation of both types of substrates, the presence of the growth substrate can inhibit the oxidation rate of the non beneficial substrate and vice versa. Substrate inhibition describes the hindrance of substrate transformation or utilization due to the competition for, or alteration of degradative enzymes. There are several types of inhibition, including self , competitive, noncompetitive, and mixed-inhibition. *Self inhibition* may result when the growth substrate itself is inhibitory at high concentrations. When an enzyme lacks specificity, *competitive inhibition* may occur in which one substrate binds to the catalytic site of the enzyme, thus preventing another substrate from reacting. A substrate may also bind to a non-reactive site on the enzyme, altering its conformation and creating *noncompetitive inhibition* which reduces the utilization of another substrate. Competitive and noncompetitive inhibition may occur simultaneously, causing a condition termed *mixed inhibition* (Rittman and McCarty, 2001). Competition between the growth substrate and the cometabolic substrate for oxygenase enzymes may significantly affect cometabolic degradation rates.

A single enzyme may thus be able to catalyze the degradation of two or more substrates; this scenario, which includes the cometabolic processes, can be

described by the following reaction scheme, where A and B represent distinct substrates:



With the same assumptions as presented in the case of one substrate, the following degradation rates are obtained for the two substrates:

$$\begin{aligned}
 \frac{dS_A}{dt} &= -\frac{k_{\max, A} \cdot S_A \cdot X}{K_{s, A} \cdot \left(1 + \frac{S_B}{K_{c, B}}\right) + S_A} \\
 \frac{dS_B}{dt} &= -\frac{k_{\max, B} \cdot S_B \cdot X}{K_{s, B} \cdot \left(1 + \frac{S_A}{K_{c, A}}\right) + S_B}
 \end{aligned}$$

The half-saturation portion of each equation becomes a function of the inhibitor competitive inhibition constant ( $K_{c,A}$  and  $K_{c,B}$  respectively). This model is known as “competitive inhibition” and describes the most frequent inhibition type included in mathematical modeling of cometabolic biotransformations (Broholm et al., 1992; Chang and Alvarez-Cohen, 1995; Chang and Criddle, 1997; Lee et al., 2000). The competitive inhibition constant has often been approximated with the competing substrate half-saturation constant. Kim et al. (2002), however, noted various studies where this appeared to be an incorrect assumption and, in response, presented a method for determining the inhibition type and the respective constants. Kim focused on butane utilization by a mixed culture with cometabolic transformation of 1,1-DCE, 1,1-DCA, and 1,1,1-TCA and observed that competitive and mixed inhibition occurred. CAHs competitively inhibited the

degradation of the other CAHs and butane, while butane showed mixed inhibitory effects toward the CAHs.

Noncompetitive inhibition more specifically influences the maximum degradation rate, and equation 2.7 may be transformed to:

$$\frac{dS}{dt} = -\frac{k_{\max} \cdot X}{\left(1 + \frac{I_u}{K_{I,u}}\right)} \cdot \left(\frac{S}{K_s + S}\right) \quad (3.12)$$

where  $I_u$  = aqueous concentration of noncompetitive inhibitor (mg/L)

$K_{I,u}$  = constant for noncompetitive inhibition (mg inhibitor/L)

In the case of mixed inhibition, the equation assumes a combined form of 2.11 and 2.12, resulting in:

$$\frac{dS}{dt} = \frac{-k_{\max} \cdot X}{1 + \frac{I_u}{K_{Iu}}} \cdot \left( \frac{S}{\frac{K_s}{1 + \frac{I_u}{K_{Iu}}} \cdot \left(1 + \frac{I_c}{K_{Ic}}\right) + S} \right) \quad (3.13)$$

Competitive and non competitive inhibition may or may not be caused by the same inhibitor. Terms for competitive and non competitive inhibition are additive and equation 2.13 may be extended to include several inhibitors.

### 3.3 CAHs DEGRADATION PRODUCT TOXICITY

The oxidation of chlorinated organics by oxygenase enzymes generates short lived toxic intermediate products that may damage cells, causing cellular inactivation: this phenomenon is known as “product toxicity”. Alvarez-Cohen and McCarty verified that some oxidation products of chlorinated aliphatics, such as fosgene in the oxidation of chloroform, and epoxides in oxidation of chlorinated ethenes, irreversibly bind to proteins and lipids, thus inactivating some cellular functions.

The capability of microbial cultures to oxidize compounds which exert product toxicity can be quantified using the “transformation capacity” ( $T_C$ ) parameter, defined as the maximum mass of solvent that can be transformed by a given amount of cells before they are completely inactivated.

In the cometabolic processes, after the initial oxidation step, growth substrates are further degraded to regenerate reducing energy (NADH), which promotes more substrate oxidation. In the absence of growth substrates, methane oxidizers are capable of using both internal energy sources, such as polyhydroxybutirate (PHB), and methane catabolic intermediates, such as formate, to regenerate NADH; oxidation of non beneficial substrates can be carried out in the absence of growth substrate as long as some source of NADH regeneration is available. As oxygenase expressing cultures, such as propane and phenol oxidizers, may also exhibit similar responses since they have similar enzyme mechanisms. However, the oxidation of non beneficial substrates in the absence of growth substrates can cause the depletion of NADH in cells, since NADH is not regenerated. Organisms in the absence of growth substrate are referred as “resting cells”.

When cometabolic substrates such as chlorinated organics are oxidized by resting cells, the degradation may be limited by both the depletion of endogenous cellular reducing energy and the product toxicity; it follows that  $T_C$  measured in these conditions is a function of both NADH level and toxicity. If the external reducing energy runs out before the cells are completely inactivated by toxicity, the reaction is interrupted because of the absence of NADH, resulting in an artificially low  $T_C$ .

To estimate the effects of toxicity alone,  $T_C$  should be measured in the presence of a NADH regenerant; a comparison of  $T_C$  measured in the two conditions may reflect the amount of endogenous reducing energy available to cells. However some NADH regenerant may exert additional effect on cells which may also bias the measured  $T_C$ : for example, growth substrates would promote additional enzyme production, whereas other amendments may be toxic themselves.

Chang and Alvarez-Cohen (1995b) tested the effect of amendments on the transformation capacities of four oxidizing cultures. The  $T_C$  values of chlorinated organics could be significantly increased by the addition of low concentrations of growth substrates; this effect was overcome at higher concentrations for two of the cultures, presumably by toxicity of the growth substrates. No better results were obtained providing as amendments catabolic intermediates of the growth substrate. These results suggest that although  $T_C$  may be a good tool for the comparison of the toxic effects of chlorinated organic degradations, care must be taken to minimize the effects of reducing energy limitations and amendment interference. The measurement of  $T_C$  in the presence of growth substrate avoids errors due to energy limitations, but the potential for confounding factors such as substrate toxicity and enzyme regeneration should be considered. The use of nontoxic NADH regenerant which is not a growth substrate may result in  $T_C$  measurements which more directly reflect the effect of degradation toxicity.

Further research performed by Chang and Alvarez-Cohen gave deeper insight into the phenomenon of toxicity:

- All the experiments demonstrated that toxicity is caused by the degradation products rather than the solvent themselves; this was verified in batch microcosms with four CAHs and three different substrates. In the first phase, the solvent degradation was inhibited, thus exposing the cells to the solvent in the absence of their degradation products; in the second phase the solvent were stripped. The growth substrate degradation was evaluated prior and after the exposure to the solvent: the measured value did not differ significantly;

- the transformation capacity of a given chlorinated solvent changes with the primary substrate used for growing the culture; thus  $T_c$  is a good parameter for comparing the potential of different substrates to address a specific contaminant;
- in mixed bacterial consortia, the transformation capacity may change in time, following shifts in the composition of the microbial population;
- the transformation capacity relative to a specific chlorinated solvent and a growth substrate is independent from the initial concentrations of both biomass and contaminant;
- the transformation capacity of cells grown on a specific substrate decreases with the chlorine- to carbon- atoms numerical ratio in the solvent molecule;
- the transformation yield  $T_y$  is proportional to the transformation capacity  $T_c$  through the growth yield  $Y$ :  $T_y = T_c \times Y$ .

Alvarez-Cohen and Speitel (2001) reviewed the interpretations for approximating inactivation, separating them into two classes. One class (1) represents loss of full cellular function, while the other class (2) assumes the loss of specific enzyme activity. Among the second class models, important contributions were provided by Ely in 1995. The model incorporates enzyme inhibition, caused by the presence of a cometabolic compound, inactivation, resulting from toxicity of a cometabolic product, and recovery associated with bacterial synthesis of new enzyme in response to inactivation. The first class is the most commonly used in modelling biodegradation.

In 1991 Alvarez-Cohen and McCarty proposed the kinetic model introducing the transformation capacity in order to account for the toxic effects of the degradation products on the biomass. The transformation capacity  $T_c$  was thus defined as the quantity of a compound that a specific mass of microorganisms can degrade before it is inactivated by toxicity from transformation products. Units of transformation capacities are typically mass of degraded substrate per mass of cell.

Assuming that the degradation of the chlorinated solvents follows the Michaelis-Menten kinetics, as described in 3.1, and that the biomass concentration decreases in time as a consequence of both product toxicity and endogenous decay, the following model can be applied in the absence of growth substrate (Alvarez-Cohen and McCarty, 1991):

$$r_x = \frac{dX}{dt} = \left( -b + \frac{1}{T_c} \cdot r_c \right) \cdot X \quad (3.14)$$

where:

$r_x$  (mg protein/L/h), cell decay rate

$r_c$  (□g/mg protein/h), contaminant degradation rate

$b$  (h<sup>-1</sup>), cell endogenous decay coefficient

$T_c$  (mg/mg), contaminant transformation capacity

$X$  (mg protein/L), specialized cell concentration

Successive studies (Chang and Alvarez, 1995a, b) evidenced that the oxidation of chlorinated organics by resting cells is also limited by the depletion of reducing energy. Cometabolic degradation rates were observed to increase with the addition of external energy sources; on the other hand, the degradation performances were found to be affected by substrate inhibition, when the external energy was provided through the addition of growth substrate.

A modification of Michaelis-Menten/Monod kinetics was proposed to describe the kinetics of cometabolic degradation, incorporating the effects of product toxicity, depletion of oxygen and reducing energy, competition between growth substrate and cometabolic substrate. The model, summarized in the equations 3.15 – 3.17, was able to predict the experimental results. The factors  $R/(R+K_R)$  and  $O_2/(K_{O_2}+ O_2)$ , included in the growth substrate and contaminant degradation rate equations, take into account oxygen and reducing energy sources

as possibly limiting reactants. When they are supplied in large excess, such that  $R \gg K_R$  and  $O_2 \gg K_{O_2}$ , the corresponding Monod terms approach 1 and the specific degradation rates become functions of substrate, contaminant and biomass concentration alone.

$$r_s = \left( \frac{O_2}{K_{O_2} + O_2} \right) \cdot \left( \frac{R}{K_R + R} \right) \cdot \left( \frac{k_{\max, s} \cdot S}{K_{s, s} \cdot \left( 1 + \frac{C}{K_{I, c}} \right) + S} \right) \cdot X$$

$$r_c = \left( \frac{O_2}{K_{O_2} + O_2} \right) \cdot \left( \frac{R}{K_R + R} \right) \cdot \left( \frac{k_{\max, c} \cdot C}{K_{s, c} \cdot \left( 1 + \frac{S}{K_{I, s}} \right) + C} \right) \cdot X$$

$$r_X = \left( -Y \cdot r_s - b + \frac{1}{T_c} \cdot r_c \right) \cdot X$$

Where:

$O_2$  (mg/L) dissolved oxygen concentration

$K_{O_2}$  (mg/L) half saturation constant of dissolved oxygen

$R$  (mmol e<sup>-</sup>/L) reducing energy electron equivalent concentration

$K_R$  (mmol e<sup>-</sup>/L) half saturation constant of reducing energy

$C$  (mg/L) contaminant concentration

$K_{s, c}$  (mg/L) contaminant half saturation constant

$K_{s, s}$  (mg/L) substrate half saturation constant

$K_{I, c}$  (mg/L) contaminant competitive inhibition constant

$K_{I, s}$  (mg/L) substrate competitive inhibition constant

and all the other symbols as previously defined

## **CHAPTER 4**

### **4. MOLECULAR BIOLOGY TECHNIQUES USED IN THE ANALYSIS OF THE MICROBIAL DIVERSITY IN CONTAMINATED SOILS**

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Identification of microorganisms by conventional methods requires the isolation of pure cultures followed by laborious characterization experiments. These procedures are therefore inadequate for study of the diversity of a natural or engineered ecosystem. A new set of molecular techniques developed during the 1990s revolutionized microbial ecology research. Among these techniques, cloning and the creation of a gene library, denaturant gradient gel electrophoresis (DGGE) and fluorescent *in situ* hybridization with DNA probes (FISH) stand out. Cloning provides very precise taxonomical information, but it is time consuming and requires specialized personnel whereas DGGE is a rapid and simple method that provide characteristic band patterns for different samples, allowing quick sample profiling, while retaining the possibility of a more thorough genetic analysis by sequencing of particular bands. FISH makes possible to identify microorganisms at any desired taxonomical level, depending on the specificity of the probe used. It is the only quantitative molecular biology technique, although quantification is either complex or tedious and subjective. Combination with a confocal laser-scanning microscope allows the visualization of three-dimensional microbial structures. These methods have deepened our understanding of the microbiology of contaminated soils. Both DGGE and FISH have been extensively employed.

## **4.1 MICROBIAL ECOLOGY OF CONTAMINATED SOILS**

### **4.1.1 Phenotypic analysis of soil bacterial communities**

The diversity of bacterial communities in contaminated soils has been based in the 1980-1990's on CFU counting and colony morphology typing. Colony morphology typing relies on grouping of bacterial colonies cultured on plates containing relevant media according to their colony appearance (Haldeman and Amy, 1993). Latest years, community-level physiological profiles (CLPPs) or sole-carbon-source utilization profiles have been used as an indicator of community structure and function (Becker and Stottmeister, 1998; Degens, 1998; Gamo and Shoji, 1999; Garland and Mills, 1991; Garland and Mills, 1994; Smalla et al., 1998; Wünsche et al., 1995). CLPP is based on metabolic response patterns of communities extracted from environmental samples, inoculated into a 96 wells BIOLOG<sup>®</sup> plate. The 96-well microtiter plates contain nutrients and a tetrazolium dye. When a bacterial community is capable of oxidizing the carbon substrate, the dye turns purple, and a spectrophotometric plate reader quantifies the response. However, these patterns not always reflect the organisms directly involved in the mainstream energy flux of the ecosystem (Boon, 2002). Wünsche et al. (1995) applied these techniques to examine the effect of hydrocarbon contamination on microbial community structure and function and found that characteristic shifts of the substrate utilization patterns followed changes in hydrocarbon content in soils. Furthermore, the altered patterns of substrate utilization corresponded to similar changes in abundance of hydrocarbon-utilizing bacteria determined by plate counts. Strong-Gunderson and Palumbo (1994) adapted this CLPP method for rapidly screening the metabolic potential of bacteria to oxidize semi-volatile and volatile compounds as a sole carbon source.

Although the CLPP assay has been proposed as a measure of functional diversity, assay responses are attributed mainly to a small subset of heterotrophic bacteria in the tested environmental sample (Ibekwe et al., 2001). Difficulties in analyzing the complexity of bacterial communities by classic methods of

cultivation and subsequent physiological characterization have necessitated the development of new approaches for community and functional analysis.

#### **4.1.2 Culture-independent analysis of microbial communities by lipid-based tools**

White and Findlay (1988) developed a community-level approach to characterize microbial community structure by evaluating shifts in phospholipid fatty acids (PLFA) from environmental samples. Different groups of bacteria are characterized by specific PLFA profiles. Therefore, a change in the phospholipid pattern in soil would indicate a change in the bacterial composition of that soil. The polar lipid fraction of environmental samples is composed primarily of PLFA of “viable” micro-organisms present in the sample (assuming rapid degradation of intact phospholipids after cell death) (Ringelberg et al., 2001; White and Findlay, 1988). For complex matrices such as soil, PLFA analysis has been shown to be a valuable tool for detecting changes in microbial communities in response to pollution of alkanes (Ringelberg et al., 1989) and chlorinated hydrocarbons (Phelps et al., 1988). Recently, analysis of  $^{13}\text{C}$ -labeled PLFAs resulting from incorporation of  $^{13}\text{C}$  during cell growth from  $^{13}\text{C}$ -labeled C substrates has been used to define the groups of organisms utilizing those substrates (Boschker et al., 1998; Padmanabhan et al., 2003). Therefore,  $^{13}\text{C}$ -labeled substrates are introduced into the soil where bacteria use them as a C-source and after PLFA extraction the active micro-organisms are identified by analyzing  $^{13}\text{C}$ -labeled PLFA. Hanson et al. (1999) identified the indigenous population(s) responsible for toluene degradation in Yolo silt loam, by employing both traditional culture-based approaches and PLFA and  $^{13}\text{C}$ -PLFA analysis. After 119 h of incubation with  $^{13}\text{C}$ -toluene, 96% of the incorporated  $^{13}\text{C}$  was detected in only 16 of the total 59 PLFAs (27%) extracted from the soil. Of the total  $^{13}\text{C}$ -enriched PLFAs, 85% were identical to the PLFAs contained in a toluene-metabolizing bacterium isolated from the same soil, showing that this strain was one of the main toluene degraders in that soil. In contrast, the majority of the soil PLFAs (91%) became labeled when the same soil was incubated with  $^{13}\text{C}$ -glucose. In laboratory

microcosms, Pelz et al. (2001a, 2001b) incubated sediments from a petroleum hydrocarbon (PHC) contaminated aquifer and a nearby pristine aquifer under anoxic sulfate-reducing conditions with methyl-<sup>14</sup>C-toluene to determine the <sup>14</sup>C-mass balances and with methyl-<sup>13</sup>C-toluene to follow the flow of carbon from toluene into PLFA. <sup>14</sup>C quantification revealed that 61.6% of the methyl-<sup>14</sup>C-toluene was mineralized and 2.7% was assimilated, while <sup>13</sup>C-labeled PLFA analysis linked toluene degradation to the metabolic activity of *Desulfobacter*-like populations. These populations could play an important role in the clean-up of aromatic PHC contaminated aquifers.

#### **4.1.3 Culture-independent analysis of the bacterial communities by nucleic acid-based tools**

Conventional microbiological techniques, based on isolation of pure cultures and morphological, metabolic, biochemical and genetic assays, have provided extensive information on the biodiversity of microbial communities in natural and engineering systems. However the drawbacks of the existing conventional methods, such as incomplete knowledge about their physiological needs and the complex syntrophic and symbiotic relations, which are abundant in nature, make impossible to obtain pure cultures of most microorganisms in natural environments. Moreover, most culture media tend to favor the growth of certain groups of microorganisms, whereas others that are important in the original sample do not proliferate. It is therefore generally accepted nowadays that the number of known prokaryotic species (including the two domains *Bacteria* and *Archaea*) is very small compared to the diversity of microorganisms and illustrates how difficult it is to get a full picture of the bacterial diversity of an ecosystem by relying only on conventional methodology. At present, about 7000 bacterial species have been described, but according to molecular and ecological estimates, the real number must be several orders of magnitude higher (Amman et al, 1995). This small known fraction does not reflect the composition and diversity of a microbial community.

One suitable solution to this problem is to use molecular biology approaches. The techniques are based on the RNA of the small ribosomal subunit (16S rRNA for prokaryotes) or their corresponding genes, considering it as a “molecular clock” or “evolutionary chronometer”. This molecule was chosen because of its universality and abundance in all living beings ( $10^3$  to  $10^5$  ribosomes/cell) and the fact that it is a highly conserved molecule throughout evolution although bears some highly variable regions. These features allow comparison of organisms within the same domain, as well as differentiation of strains of the same species. Moreover, the gene sequence is sufficiently long to generate statistically relevant data and can be easily sequenced with current technology.

As a consequence of the necessity to also address non-culturable members of the bacterial community, more and more molecular gene probe methods have been developed that are based on the analysis of nucleic acids extracted from soil (Akkermans et al., 1995; Amann et al., 1995; Holben and Harris, 1995; Sayler and Layton, 1990; Shi et al., 1999; Stapleton et al., 1998; Trevors and van Elsas, 1995). The development of methods for directly extracting DNA from environmental samples bypassed the need to culture organisms, thus providing a more representative sampling of microbial constituents within a complex community (Akkermans et al., 1995; Leahy and Colwell, 1990; Shi et al., 1999; Stapleton et al., 1998; Trevors, 1992; Trevors and van Elsas, 1995).

**Cloning and sequencing** of the gene that codes for 16S rRNA is still the most widely used molecular technique in the field of microbial ecology. This methodology implies the extraction of nucleic acids, amplification and cloning of the 16S rRNA genes, followed by sequencing and, finally, identification and affiliation of the isolated clone with the aid of phylogenetic software. While amplicons generated from pure cultures of bacteria could be sequenced directly, in the case of genomic DNA extracts from microbial communities, the cloning step has to be included. This is necessary in order to separate the different copies of 16S rDNA, as a mixed template cannot be sequenced. Because this approach is so widespread, half of the approximately 240000 sequences deposited in the 16S rDNA NCBI-database (April 2006), belong to non-cultured and unknown

organisms, that is, organisms detected by 16S rDNA cloning. This illustrates how extensively and successfully the cloning strategy has been employed since its introduction in the beginning of the 1990s (Ward et al, 1990). However cloning is time consuming and so less apt for analyzing larger sets of samples, for example, when monitoring changes in natural or engineered microbial communities over time, particularly if several time points are required. The main advantages and disadvantages of this approach can be summarized as follows:

- Advantages:
  - complete 16S rRNA sequencing allows:
    - very precise taxonomic studies and phylogenetic trees of high resolution to be obtained;
    - design of primers (for PCR) and probes (for FISH);
  - if time and effort is not a limiting factor, the approach covers most microorganisms, including minority groups, which would be hard to detect with genetic fingerprinting methods;
  - identification of microorganisms that have not been yet cultured or identified.
  
- Disadvantages:
  - very time consuming and laborious, making it unpractical for high samples throughput;
  - extraction of a DNA pool representative of the microbial community can be difficult when working with certain sample types (e.g. soil, sediments).
  - many clones have to be sequenced to ensure most of individual species in the sample are covered;
  - it is not quantitative. The PCR step can favor certain species due to differences in DNA target site accessibility.

**Denaturing gel electrophoresis (DGGE)** is based on the different mobility on a gel of denaturated DNA-fragment of the same size but with different nucleic acid sequences, thus generating band patterns that directly reflect

the genetic biodiversity of the sample. The number of bands corresponds to the number of dominant species. Coupled with sequencing and phylogenetic analysis of the bands, this method can give a good overview of the composition of a given microbial community. DGGE is the method of choice when the desired information does not have to be as phylogenetically exhaustive as that provided by cloning, but still relatively precise to determine the dominant members of a microbial community with medium phylogenetic resolution.

The most important application of DGGE is monitoring dynamic changes in microbial communities, especially when many samples have to be processed. For community analysis, the variable areas of the 16S rRNA gene are often used as bioindicator (Maidak et al., 1997; Stackebrandt et al., 1993; Woese, 1987). A great number of 16S rRNA gene sequences are accessible via databases and sequence comparisons allows the identification of areas with unique sequences for specific bacterial groups (Maidak et al., 1997; van Elsas et al., 1998).

As such, primers can be designed for PCR detection of specific groups of bacteria by amplifying the corresponding 16S rRNA gene. The obtained PCR fragments can then be cloned and sequenced (Amann et al., 1995; Hugenholtz and Pace, 1996) or they can be separated and visualized by fingerprinting techniques allowing direct diversity analysis (Dejonghe et al., 2001). Community fingerprints are generated by separating the amplified nucleic acid fragments based on their sequence variability as in Denaturing Gradient Gel Electrophoresis/Temperature Gradient Gel Electrophoresis (DGGE/TGGE) (Muyzer et al., 1993) and Single Strand Conformation Polymorphism (SSCP) analysis (Lee et al., 1996), or by their size as in Terminal Restriction Fragment Length Polymorphism (T-RFLP) (Liu et al., 1997) and Amplified Ribosomal DNA Restriction Analysis (ARDRA) (Massol-Deya et al., 1995). Using DGGE/TGGE, Muyzer et al. (1993) showed that it is possible to identify constituents of the microbial population which represent only 1% of the total population. There are unfortunately some constraints related to these techniques, i.e., the occurrence of multiple bands relating to one bacterium (e.g. multiple 16S rDNA sequences) or one band corresponding to more than one strain. Ralebitso et al. (2000) enriched and isolated in the presence of different selection pressures, particularly based on pH and electron donor

concentration, indigenous microbial associations which catabolize selected petroleum hydrocarbon components (benzene, toluene and *o*-, *m*- and *p*-xylene (BTX), from a petroleum hydrocarbon contaminated sandy soil. PCR and 16S rDNA fingerprinting by DGGE were employed to explore the diversities and analyse the structures of the isolated microbial associations. Pearson product-moment correlation indicated that different, but chemically similar, petroleum hydrocarbon molecules, effected the isolation of different associations. However, some similar numerically-dominant bands characterized the associations. A 30% similarity was evident between the *m*- and *o*-xylene catabolizing associations regardless of the molecule concentration and the enrichment pH. PCR-DGGE was also used to complement conventional culture-based microbiological procedures for environmental parameter optimisation. Band pattern differences indicated profile variations of the isolated associations, which possibly accounted for the growth rate changes recorded in response to pH and temperature perturbations. Currently, no reports are available concerning TGGE, SSCP or T-RFLP application to follow up the microbial diversity at petroleum hydrocarbon contaminated sites polluted with prevalently BTEX. Massol-Deya et al. (1997) used ARDRA to compare community composition, succession, and performance in fluidized bed reactors (FBR) treating BTX contaminated water. One reactor was inoculated with the toluene degrading strains *P. putida* mt-2 (PaW1), *B. cepacia* G4, and *B. pickettii* PKO1. Strain mt-2 was found to outcompete the other two strains. When groundwater strains were allowed to challenge the steady-state biofilm developed by the inoculated strains, they readily displaced the inoculated strains and further reduced the toluene effluent concentration. ARDRA of 16S rRNA gene amplicons from the reactor community showed a succession of populations into a pattern that was stable for at least 4 months of operation. The convergence of communities to the same composition from three different starting conditions and their constancy over several months suggested that a rather stable community was selected.

Alternatively, 16S rDNA group specific probes can be designed for DNA:DNA hybridization targeting 16S rRNA. However, the polymerase chain reaction technique is much more sensitive (by 3 orders of magnitude), permitting

the detection of 1 cell per g of sediment sample (Steffan and Atlas, 1988; Leahy et al., 1990). To quantify hybridization signals and determine the amount of DNA in each sample, a regression equation is generated from hybridization signal intensities of known DNA standards, included on each vacuum blot. Signal intensities are obtained by computer-aided analysis of autoradiogram images (Guo et al., 1997; Stapleton et al., 1998; Shi et al., 1999). The relative abundance of domains or subgroups is determined by normalizing hybridization signals to the signal generated from hybridization to a universal 16S rDNA probe (Zheng et al., 1996). Shi et al. (1999) used phylogenetic probes in hybridization analysis to (i) determine *in situ* microbial community structures in regions of a shallow sand aquifer that were oxygen depleted and fuel contaminated (FC) or aerobic and non-contaminated (NC) and (ii) examine alterations in microbial community structures resulting from exposure to toluene and/or electron-acceptor supplementation (nitrate). The latter objective was addressed by using the NC and FC aquifer materials for anaerobic microcosm studies in which phylogenetic probe analysis was complemented by microbial activity assays. Domain probe analysis of the aquifer samples showed that the communities were predominantly Bacteria, Eucarya and Archaea were not detectable. At the phylum and subclass levels, the FC and NC aquifer material showed similar relative abundance distributions of 43 to 65%  $\beta$ - and  $\gamma$ -Proteobacteria (B+G), 31 to 35%  $\alpha$ -Proteobacteria (ALF), 15 to 18% sulfate-reducing bacteria, and 5 to 10% high G+C gram positive bacteria. Compared to that of the NC region, the community structure of the FC material differed mainly in an increased abundance of B+G relative to that of ALF. The microcosm communities were similar to those of the field samples. Addition of nitrate and/or toluene stimulated microbial activity in the microcosms, but only supplementation of toluene alone significantly altered community structure. For the NC material, the dominant subclass shifted from B+G to ALF, while in the FC microcosms 55 to 65% of the Bacteria community was no longer identifiable by the phylum or subclass probes used. The latter result suggested that toluene exposure fostered the proliferation of phylotype(s) that were otherwise minor constituents of the FC aquifer community. These studies demonstrated that alterations in aquifer microbial communities resulting from specific anthropogenic

perturbances can be inferred from microcosm studies integrating chemical and phylogenetic probe analysis and in the case of hydrocarbon contamination may facilitate the identification of organisms important for *in situ* biodegradation processes.

The main advantages and disadvantages of this technique are summarizing as follows:

- Advantages:
  - Permits rapid and simple monitoring of the spatial-temporal variability of microbial populations if just band patterns are considered;
  - it is relatively easy to obtain an overview of the dominant species of an ecosystem;
  - it is adequate for analysis of a large number of samples (far more than cloning).
  
- Disadvantages:
  - depending on the nature of the sample, extraction and amplification of representative genomic DNA can be difficult (as in cloning);
  - after the PCR amplification, the DNA copy number – which depends on abundance of a particular microorganism and the ease of amplification of the 16SrRNA – can be very different (as in cloning). The intensity the bands obtained on a DGGE gel may therefore vary (not quantitative);
  - the number of detected bands is usually small, which implies:
    - the number of identified species is also small;
    - the bands correspond, although not necessarily, to the predominant species in the original sample;
  - the sequences of the bands obtained from a gel correspond to short DNA fragments (200 – 600 bp), and so phylogenetic relations are less reliably established than with cloning of the whole 16SrRNA gene. In addition, short sequences are less useful for designing new specific primers and probes.

The latest advance in molecular technology is the use of **nucleic acid microarrays** or DNA chips in which the probes are immobilized (Blohm and Guiseppi-Elie, 2001; Cho and Tiedje, 2001; Koizumi et al., 2002; Sergei et al., 2001; Small et al., 2001; Urakawa et al., 2003; Wilson et al., 2002). This method allows the simultaneous study of thousands of genes or messenger RNAs under various physiological states. Both methods place a variety of single strand DNA probes of interest on glass computer chips (or microscope slides). However the use of this technique for environmental samples is still limited. Recently, Koizumi et al. (2002) characterized a mesophilic, sulfate-reducing, toluene-degrading consortium (TDC) and an ethylbenzene-degrading consortium (EDC) by DGGE fingerprinting of PCR amplified 16S rRNA gene fragments, followed by sequencing. The sequences of the major bands were affiliated with the family *Desulfobacteriaceae*. Another major band from EDC was related to an uncultured non-sulfate-reducing soil bacterium. Oligonucleotide probes specific for the 16S rRNAs of target organisms corresponding to the major bands were designed, and hybridization conditions were optimized for two analytical formats, membrane and DNA microarray hybridization. Both formats were used to characterize the TDC and EDC, and the results of both were consistent with the DGGE analysis. In order to assess the utility of the microarray format for analysis of environmental samples, oil-contaminated sediments from the coast of Kuwait were analyzed. The DNA microarray successfully detected bacterial nucleic acids from these samples, but probes targeting specific groups of sulfate-reducing bacteria did not give positive signals.

Application of **quantitative PCR techniques** such as competitive PCR and real-time PCR allow to obtain a quantitative picture of the specific groups of bacteria. Recently, due to the development of methods for total extraction of RNA from environmental samples of different origin RT-PCR can be used to amplify cDNA derived from 16S rRNA from a RNA extract. This allows to amplify 16S rRNA from metabolically active populations in a community and to get information about the active members of a community. However, no RT-PCR studies concerning 16S rRNA diversity to detect the active bacterial community present in BTEX contaminated soils are reported.

In case sequence information exist about functional genes involved in degradation of xenobiotic compounds, this information can be used to design specific primers or DNA probes for direct PCR detection of or hybridization with the corresponding genes. Application of such techniques can provide a more detailed picture of the catabolic gene structure and sequence diversity in environmental samples, which will increase significantly our knowledge of the functional potential of the microbial community in the studied environment. Moreover, shifts in catabolic gene structure allow the deduction of the evolutionary fitness of catabolic genes, operons and their respective hosts (Junca and Pieper, 2004). Recently, many studies reported the design of PCR primers to detect and/or quantify by PCR the presence of genotypes, encoding key steps in bacterial BTEX biodegradation pathways in soil DNA extracts. Moreover, RT-PCR on RNA extracts allows to see if the genes are actively transcribed in the sample. Ogram et al. (1995) designed primer pairs to specifically detect the genes encoding the  $\alpha$ -subunit of the hydroxylase component (TmoA) of the toluene 4-mono-oxygenase of *P. mendocina* KR1 and the iron-sulfur oxidase  $\alpha$ -subunit (TodC1) of the toluene dioxygenase of *P. putida* F1. The primers were used for the detection of the expression of these genes and hence their activity in low-biomass deep subsurface BTEX contaminated sediments by employing RT-PCR on mRNA extracted from the aquifer. They detected *tmoA* homologous RNA transcripts. Recently, Baldwin et al. (2003) designed degenerate primers to specifically detect genes encoding two groups of  $\alpha$ -subunits of the diiron hydroxylase component of different multi-component mono-oxygenases and the gene encoding the hydroxylase component (XylM) of the side chain xylene mono-oxygenase. The primer sets were used for detection and enumeration of aromatic oxygenase genes by multiplex and real-time PCR on pure cell DNA, but they have no data available on aquifer samples. Primer sets for the detection of C23O genes specific for the *meta*-cleavage of the aromatic catechol structure in fluorescent *Pseudomonas* or *Sphingomonas* were reported by Hallier-Soulier et al. (1996), Okuta et al. (1998), Meyer et al. (1999), Mesarch et al. (2000) and Junca and Pieper (2004). Cavalca et al. (2003) analyzed the functional and phylogenetic

biodiversity of bacterial communities in a BTEX-polluted aquifer treated by air-sparging. Five months of air injection reduced species diversity in the cultivable community (as calculated by the Shannon-Weaver index), while little change was noted in the degree of biodiversity in the total bacterial community, as monitored by DGGE analysis of PCR amplified 16S rRNA genes. BTEX-degrading isolates belonged to the genera *Pseudomonas*, *Microbacterium*, *Azoarcus*, *Mycobacterium* and *Bradyrhizobium*. The degrading capacities of three strains in batch liquid cultures were also studied. In some of these micro-organisms different pathways for toluene degradation seemed to operate simultaneously. *Pseudomonas* strains of the P24 operational taxonomic unit, able to grow only on catechol and not on BTEX, were the most abundant, and were present in the groundwater community at all stages of treatment, as evidenced both by cultivation approaches and by DGGE profiles. The presence of different *tmo*-like genes in phylogenetically distant strains of *Pseudomonas*, *Mycobacterium* and *Bradyrhizobium* suggested recent horizontal gene transfer in the groundwater. Junca et al. (2004) used PCR-SSCP for determining the diversity of C23O genes in environmental samples. These PCR-SSCP results were assessed by comparing sequence data from PCR-DNA clone libraries and C23O sequences and metabolic performance of micro-organisms exhibiting C23O activity. PCR-SSCP was demonstrated to be a reliable and rational tool to rapidly determine sequence diversity within a catabolic gene family in environmental samples obtained from a BTEX contaminated site. In another study, Junca et al. (2003) used an approach identical to ARDRA or amplified functional DNA restriction analysis (AFDRA) to rapidly characterize C23O subfamily I.2.A genes, known to be of crucial importance for aromatic degradation. Restriction of the genes by *Sau3A1* theoretically produced characteristic profiles from each subfamily I.2.A member and their similarities reassembled the main divergent branches of C23O gene phylogeny. Cluster analyses of the restriction fragment profiles obtained from isolates from a BTEX contaminated site showed patterns with distinct similarities to the reference strain profiles, allowing to distinguish four different groups. Sequences of PCR fragments from isolates were in close agreement with the phylogenetic correlations predicted with the amplified functional DNA restriction analysis

(AFDRA) approach. AFDRA thus provided a quick assessment of C23O diversity in a strain collection and insights of its gene phylogeny affiliation among known family members, but may also define the predominant polymorphism of a functional gene present in environmental DNA extracts. This approach may be useful to differentiate functional genes also for many other gene families.

The microbial diversity or presence and activity of functional genes may also be assessed by techniques like hybridization by application of specific DNA probes using the whole microbial DNA, DNA amplified by PCR or even at the rRNA level. Guo et al. (1997) studied concentrations of selected genes, including several involved in the degradation of BTEX (*tmoABCDE*, *todC1C2BA*, *xylE*) by quantitative DNA:DNA hybridization on DNA extracted from subsurface soil samples collected along a gradient of BTEX concentrations at a fuel oil-contaminated site. DNA from contaminated samples was significantly enriched in most of the catabolic genes, relative to DNA from the non-contaminated site. Hybridization of *tmo* and *xylE* were significantly higher in the contaminated samples than in the non-contaminated samples. The level of hybridization was, in descending order, as follows: *xylE* > *tmoABCDE*. No hybridization was observed with *todC1C2BA*. Hybridization of *xylE* increased with increasing aromatic concentration up to approximately 100 mg aromatics g<sup>-1</sup> soil. Above that concentration, the hybridization of *xylE* generally decreased. In an assessment of the microbiological potential for the natural attenuation of petroleum hydrocarbons in a shallow aquifer system, Stapleton and Sayler (1998) performed a quantitative DNA:DNA hybridization molecular analysis on 60 uncontaminated aquifer samples (only 15 had previous exposure to low levels of hydrocarbons) using DNA probes targeting genes encoding degradative enzymes such as toluene dioxygenase (*todC1C2*), toluene mono-oxygenase (*tomA*), and xylene mono-oxygenase (*xylA*). Each target sequence was present in nearly all samples. Hydrocarbon degrading genotypes from previously exposed samples did not differ from the other 45 samples that had no prior contaminant exposure, suggesting that the microbial community of previously exposed sediments had re-equilibrated. The level of hybridization was, in descending order, as follows: *todC1C2* > *tomA* > *xylA*. The genotype consistently found in the lowest abundance was *xylA*. From

the same samples, they isolated and characterized 26 indigenous micro-organisms capable of biodegrading fuel-related compounds such as BTEX (Stapleton et al., 2000). Only one isolate hybridized with the *todC1C2* gene probe and two isolates hybridized with the *xylA* gene probe, while no isolates hybridized with the *tomA* gene probe. To monitor changes in the molecular microbial ecology as well as stimulation of natural biodegradative processes under transient field study conditions, Stapleton and Sayler (2000) introduced a large, synthetic “model” jet fuel mixture containing BTEX compounds and naphthalene in a decane carrier into the subsurface. Over time they took subsurface samples at different places of the spreading petroleum hydrocarbon plume and monitored changes in subsurface catabolic gene frequencies during natural attenuation of the petroleum hydrocarbons by quantitative DNA:DNA hybridization using the same DNA probes. Each of the target genotypes showed significant responses to hydrocarbon exposure. At first only significant enrichment for degradative micro-organisms was seen for the *todC1C2* genotype. Then both degradative genotypes *todC1C2* and *xylA* significantly increased in samples collected from the source. After reaching a certain peak population level, both genotypes underwent significant decreases followed by a stabilization of both the plume front and degradative genotypes.

Methods allowing the direct (whole cell hybridization, applied on the sample without nucleic acid extraction as preceding step) characterization of microbial communities and specific nucleic acid sequences have been long awaited and include ***in situ* (RT-)PCR** (Amann and K uhl, 1998), and **fluorescent *in situ* hybridization (FISH)** (Amann et al., 2001). FISH is a technique where fluorescent oligonucleotides (16-20 nucleotides) recognize 16S rRNA sequences in fixed cells and hybridize with them *in situ* (DNA-RNA matching). It involves: (i) fixation and permeabilisation of the sample, (ii) hybridization by fluorescently labeled, rRNA targeted oligonucleotide probes, (iii) washing steps to remove unbound probe, and (iv) detection of labeled cells by microscopy (epifluorescence or confocal laser scanning microscopy) or flow cytometry (Boon, 2002). The fluorescence signal emitted by a cell also indicates the physiological state of the

cell. The more active a cell, the more ribosomes are present that can serve as a target for the oligonucleotides.

Microorganisms can be identified, localized and quantified in almost every ecosystem with hybridization (Amann et al, 1990). The specificity of the probe enables detection/identification of any desired taxonomic level, from domain down to a resolution suitable for differentiating between individual species. The main shortcoming of this technique lies in the lack of availability of probes targeting the desired bacterial taxon or group. Although it is possible, in theory, to design the most apt probe for each application thanks to the growing rRNA sequence database (16/18S and 23/28S rRNA), it may be impossible to develop a probe that specifically detects certain groups of microorganisms that share metabolic properties (for example, sulfate-reduction or halo-respiration). Furthermore, some previous knowledge of the expected microorganisms in the sample is often required to apply this method successfully. To target a particular species, a specific probe must be ready or its 16S rRNA sequence must be available. FISH is exclusively a taxonomic method that is most commonly used to examine whether members of a specific phylogenetic affiliation are present in a sample. It cannot, however, reveal information about the function or metabolic features of the microorganisms detected with phylogenetically-related bacteria.

***In situ* (RT-)PCR** involves amplification of specific nucleic acid sequences inside intact prokaryotic cells followed by color or fluorescence detection of the localized PCR product via bright-field or epifluorescence microscopy (Chen et al., 2000; Hodson et al., 1995). Chen et al. (2000) coupled prokaryotic *in situ* RT-PCR with flow cytometry to detect mRNA transcripts of the toluene dioxygenase (*todC1*) gene in intact cells of the bacterium *P. putida* F1. The combination of flow cytometry and a prokaryotic *in situ* RT-PCR approach allowed the rapid detection and enumeration of functional populations of microbial cells. Tani et al. (2002) injected *Ralstonia eutropha* KT1, which degrades TCE, into an aquifer after activation with toluene, and then monitored the number of bacteria by *in situ* PCR targeting the phenol hydroxylase gene and by FISH targeting 16S rRNA. Recently, a combination of FISH and

microautoradiography was developed to determine *in situ* the identities, activities and specific substrate uptake profiles of individual bacterial cells within complex microbial communities, but to our knowledge, this has not been applied for detection of BTEX degrading bacteria in environmental samples (Lee et al., 1999).

As an alternative to DGGE as a community profiling method, terminal restriction fragment length polymorphism (tRFLP) can be applied when treating complex, species-rich samples. This technique is also PCR based but the further procedure differs from PCR/DGGE or PCR/cloning. In tRFLP the 16S gene is amplified with universal primers, one of them being fluorescently labelled, and the product is digested with frequently cutting restriction enzymes. Given that each species in the sample has differences in the amplified gene sequences, the terminal restriction fragment will differ in size, so can be separated electrophoretically. Furthermore, it is possible to sequence and identify the generated fragments via comparison with a sequence database. The strength of the fluorescent signal yields additional information on the abundance of different species, though this feature should be regarded with caution, just like the band intensity in patterns of a DGGE gel.

**5. GROWTH OF CHLORINATED SOLVENT-DEGRADING MICROBIAL CONSORTIA IN METHANE- AND PROPANE-FED BIOREACTORS AND TESTING OF THEIR EFFECTIVENESS AS INOCULA FOR THE BIOAUGMENTATION OF DIFFERENT TYPES OF AQUIFERS**

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**ABSTRACT**

In this work we studied the long-term growth process of two microbial consortia effective in the aerobic cometabolic biodegradation of a mixture of 6 chlorinated aliphatic hydrocarbons (CAHs), and the effectiveness of these consortia as inocula for the bioaugmentation of different types of microcosms. The main goals of the study were to verify the maintenance of the consortia's capacity to degrade a CAH mixture during a prolonged growth process in the absence of the CAHs, and to verify the consortia's effectiveness in CAH biodegradation upon inoculation in slurry microcosms set up with different types of aquifer materials. The propane-utilizing consortium generally proved the most effective one, being able to biodegrade vinyl chloride, cis- and trans-1,2-dichloroethylene, trichloroethylene, 1,1,2-trichloroethane and 1,1,2,2-tetrachloroethane at all the CAH concentrations tested. Both consortia maintained unaltered CAH degradation capacities during a 300-day growth period in the absence of the CAHs and were effective in inducing the rapid onset of CAH depletion upon inoculation in slurry microcosms set up with 5 types of aquifer materials. A consortium developed in microcosms supplied with both methane and propane combined the best degradation capacities of the two single-substrate consortia. The degree of conversion of the organic Cl to chloride ion was equal as an average to 90%.

## **5.1 INTRODUCTION**

Chlorinated solvents, or chlorinated aliphatic hydrocarbons (CAHs), are among the most common contaminant of soils, groundwaters and wastewaters, and most of them are known or suspected carcinogens (Gossett, 2005). Of over 1200 hazardous waste sites included in the U.S. EPA 2002 National Priority List, 47% are contaminated by trichloroethene (TCE), 42% by perchloroethylene (PCE) and 37% by 1,1,1-trichloroethane (1,1,1-TCA) (U.S. EPA, 2002). Since the 1980s, numerous literature studies documented the successful biodegradation of CAHs by means of both aerobic and anaerobic processes. In particular, research on aerobic CAH cometabolism tested primarily the utilization of methane (Andersen and McCarty, 1996; Chang and Alvarez-Cohen, 1996), toluene, phenol (Hopkins and McCarty, 1995; McCarty *et al.*, 1998) and ammonia (Ely *et al.*, 1997; Keener and Arp, 1993) as growth substrates, whereas a limited number of studies focused on propane. These studies showed that propane-grown single-strains (Wackett *et al.*, 1989; Wilcox *et al.*, 1995) and mixed cultures can transform TCE, cis- and trans-1,2-dichloroethylene (cis- and trans-DCE), 1,1-dichloroethylene (1,1-DCE), vinyl chloride (VC), 1,1,2,2-tetrachloroethane (1,1,2,2-TeCA), 1,1,2- and 1,2,2-trichloroethane (1,1,2- and 1,2,2-TCA), 1,1- and 1,2-dichloroethane (1,1- and 1,2-DCA) and chloroform (CF).

Despite the encouraging results of the experimental studies, practitioners are still reluctant to utilize aerobic cometabolism for the full-scale remediation of CAH-contaminated sites (Semprini, 2001). One of the reasons for this is represented by the long lag-time that is sometimes required for the onset of the aerobic cometabolic process by the indigenous biomass of CAH-contaminated sites (Frasconi *et al.*, 2006). As a result of an extended lag period, a fraction of the contaminated plume may pass through the treatment zone and reach sensitive targets. When preliminary lab-scale investigations indicate the presence of long lag-phases, bioaugmentation, consisting of the introduction of a suitable microbial inoculum into the treatment system, can represent a very effective tool (Gentry *et al.*, 2004; Vogel, 1996). Several studies of lab-scale and in-situ biodegradation of CAHs report the successful application of this technology (Jitnuyanont *et al.*,

2001; Harkness *et al.*,1999; Munakata *et al.*,1996; Steffan *et al.*,1999). Bioaugmentation can be performed both with a single bacterial strain and with a microbial consortium; if a site is polluted with a complex contaminant mixture, the latter solution may be preferred, the combined action of different microorganisms being required to achieve a complete clean-up. In this case, in order to perform a full-scale bioaugmentation, it is necessary to develop a suitable initial microbial inoculum, and to grow it in a bioreactor so as to produce the amount of biomass required to colonize an appropriate fraction of the contaminated area. The inoculum growth process should be operated in an economically feasible way, but at the same time it must guarantee the stability of the microbial consortium, and consequently the maintenance of its degradation capacities. The characteristics required by the growth process in order to satisfy these two conditions represent an important theme of investigation. For example, growing the inoculum on the primary substrate in the absence of the target contaminants would represent a significant simplification of the production plant and a reduction of the fixed and operational costs; however, this solution might lead to a loss of the consortium's degradation abilities. Moreover, inoculated strains often survive poorly and may lose their in mixed microbial ecosystems. Several factors have been implicated in the survival, activity and maintenance of introduced strains. Some factors are of physicochemical nature, such as presence or absence of oxygen, pH or temperature. Other factors reflect the physiological adaptability of the bacterium, such as kinetics of substrate utilization, nutrients and trace elements scavenging. Some bacteria may be particularly prone to predation by protozoa when they maintain a freely suspended state rather than attach themselves easily to surfaces or form sticky material (McClure *et al.*, 1991). Most inoculation studies have shown that the population size of introduced strains decline strongly in mixed microbial ecosystems (McClure *et al.*, 1989; Watanabe *et al.*, 1998). On the other hand, some strains were shown to be particularly effective in colonizing and maintaining themselves (McClure *et al.*, 1991; Megharaj *et al.*, 1997), which was attributed to the fact that the strains were "preadapted" to the prevailing conditions or originating from the same environment. Some concern also exists that under some conditions the genetic

information for degradation of the pollutants may not be stable in the introduced organism and be lost or altered after some time (Kuar and Schugerl, 1990; Proctor, 1994). Thus, effective assessment of the capabilities of bacteria requires the study of their maintenance and activity. Therefore we were very interested in evaluating the maintainance of degradation abilities of mixed consortia after long-term growth in batch bioreactors and in assessing their capacity to quickly start and maintain the contaminant's degradation process in environments differing from the one they had been isolated from.

This study represents the continuation of a previous microcosm study (Frasconi *et al.*, 2006) where we had developed a methane-utilizing and a propane-utilizing microbial consortium able to perform the long-term biodegradation of a mixture of VC, trans- and cis-DCE, TCE, 1,1,2-TCA and 1,1,2,2-TeCA (a high-chlorinated solvent generally considered non-biodegradable in aerobic conditions) via aerobic cometabolism in slurry conditions.

The goals of the study were (i) to study the long-term aerobic biodegradation of the CAH mixture above mentioned; (ii) to investigate the efficacy of bioaugmentation with two types of internal inocula obtained from the indigenous biomass of the studied site; (iii) to identify the CAH-degrading bacteria. VC, methane and propane were utilized as growth substrates. The biodegradation process was investigated at both 25 and 17°C by means of bioaugmented and non-bioaugmented sediment-groundwater slurry microcosm tests. The non-bioaugmented microcosms were characterized, at 25 °C, by an average 18-day lag-time for the direct metabolism of VC (accompanied by the cometabolism of cis- and trans-DCE) and by long lag-times (36–264 days) for the onset of methane or propane utilization (associated with the cometabolism of the remaining CAHs). In the inoculated microcosms the lag-phases for the onset of growth substrate utilization and CAH cometabolism were significantly shorter (0–15 days at 25 °C). Biodegradation of the 6-CAH mixture was successfully continued for up to 410 days. The low-chlorinated solvents were characterized by higher depletion rates. The composition of the microbial consortium of a propane-utilizing microcosm was determined by 16s rDNA sequencing and phylogeny analysis.

This study showed that the indigenous biomass of the investigated aquifer material proved able to grow on VC 18–43 days (depending on the temperature) after the establishment of aerobic conditions, and to degrade via cometabolism *cis*- and *trans*-DCE but not TCE, 1,1,2-TCA or 1,1,2,2-TeCA present. Conversely, the supply of methane or propane led to the biodegradation of the entire 6-CAH mixture. Moreover the bioaugmentation treatments, performed with internal inocula obtained from the site's indigenous biomass, were highly effective in reducing the long and variable lag-phases required for the onset of propane or methane uptake in the non-augmented microcosms. In all the propane- or methane-fed microcosms the biodegradation of each CAH rapidly reached a stationary condition with higher rates in the low-chlorinated solvents. Besides this was, to the best of our knowledge, the first study that documented the long-term aerobic biodegradation of 1,1,2,2-TeCA.

In this work we investigated several aspects relative to the feasibility of utilizing the two above-mentioned microbial consortia as inocula for bioaugmentation treatments of CAH-contaminated sites with different physical-chemical characteristics. In particular, the goals of the study were:

i) to verify the maintenance of the consortia's capacity to degrade the 6-CAH mixture during a prolonged process of microbial growth in the presence as well as in the absence of the 6-CAH mixture;

ii) to verify the consortia's ability – after a prolonged growth process - to lead to the rapid onset of biodegradation of the CAH mixture upon inoculation in slurry microcosms set up with aquifer materials taken from sites with different physical-chemical characteristics;

iii) to develop a third consortium able to combine the best characteristics of the methane-utilizing and of the propane-utilizing consortia object of the study: in fact, the previous study had shown that, while both consortia were effective in the aerobic cometabolic biodegradation of VC and *cis*-DCE, the methane-utilizing biomass had a higher capacity to transform *trans*-DCE, whereas the propane-utilizing one was more effective towards 1,1,2-TCA, 1,1,2,2-TeCA and, secondarily, TCE;

iv) to characterize in terms of specific CAH depletion rates and degree of mineralization of the organic C1 the best methane-utilizing and the best propane-utilizing consortium obtained as a result of the inoculation in the microcosms set up with different aquifer materials.

In order to evaluate the consortia's ability and the maintenance of their degradation capacities during the prolonged growth process, we chose to start with a "black box" empirical method consisting in the inoculation – at different times during the growth process – of small amounts of the two consortia in slurry microcosms containing aquifer materials from different sites, and in the subsequent evaluation of the lag-times for the onset of biodegradation of the CAH mixture and of the long-term CAH depletion rates obtained.

## **5.2 MATERIALS AND METHODS**

### **5.2.1 Overview of the experimental scheme**

This paragraph provides a short description of the experimental scheme, whereas the details relative to the set-up and operation of each microcosm and growth reactor are presented in the following paragraphs. The experimental scheme of the study is shown – limitedly to the bioaugmented microcosms and growth reactors – in Figure 5.1. In addition, a non-bioaugmented control microcosm was set up for each type of aquifer material and for each growth substrate, and two sterile control microcosms were set up to monitor abiotic reactions, losses through caps and losses due to the microcosm sampling procedures.

As explained afterwards the study involved the following steps:

- **inoculation of growth bioreactors** and set up of Time 0 slurry microcosms in order to characterize the biodegradation abilities of the initial inoculum;
- **inoculation at different times of slurry microcosms** with biomass sampled from the growth bioreactors in order to verify the maintenance of the consortia's capacity to degrade the 6-CAH mixture during the

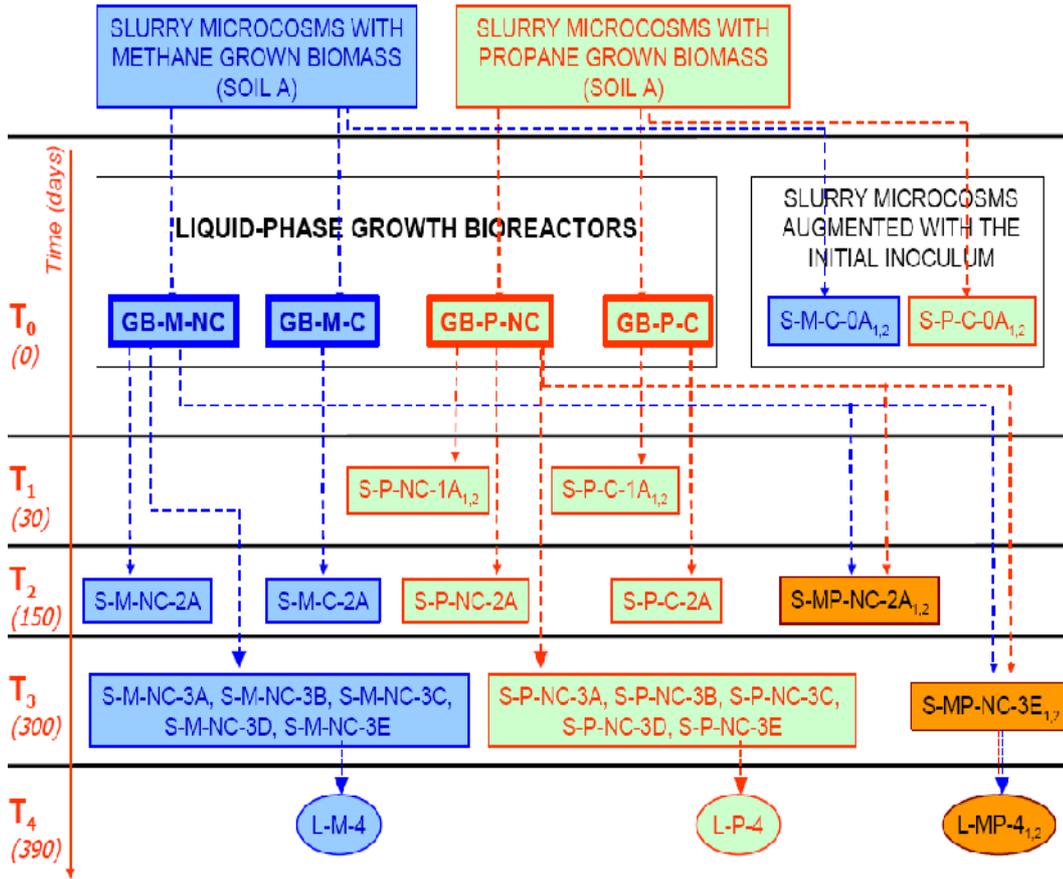
prolonged growth process in the presence as well in the absence of the 6-CAH mixture;

- **inoculation of slurry microcosms set up with aquifer materials taken from sites with different physical-chemical-biological characteristics** with biomass sampled from the growth reactors, in order to evaluate the effectiveness of bioaugmentation in different types of aquifers;
- **development of a new consortium able to combine the best characteristics of the two studied consortia** by inoculation of slurry microcosms with both the methane-utilizing and the propane-utilizing biomass;
- **inoculation of liquid-phase microcosms** in order to characterize in terms of specific CAH depletion rates and degree of mineralization of the organic Cl the best consortia obtained in the microcosms set up with different aquifer materials.

As explained in section 5.2.4, the performance of the studied consortia was evaluated in terms of:

- **lag-time** required for the onset of the aerobic cometabolic biodegradation of the 6-CAH mixture;
- **long term degradation rate** of the 6-CAH mixture.

Figure 5.1. Graphical representation of the experimental scheme of the bioaugmented



microcosms. The first group of letters indicates the type of bioreactor: GB, growth bioreactor; S, slurry microcosm; L, liquid-phase microcosm. The second group of letters indicates the type of biomass initially inoculated as well as the growth substrate supplied: M, inoculation of methane-utilizing biomass and supply of methane; P, inoculation of propane-utilizing biomass and supply of propane; MP: inoculation of both types of biomass and supply of both substrates. The third group of letters indicates the condition of biomass growth (in the case of the growth bioreactors) or the type of inoculum introduced (in the case of the slurry microcosms)(this group of letters is not included in the labelling of the liquid-phase microcosms): NC, inoculum growth in the absence of the CAH mixture; C, inoculum growth in the presence of the CAH mixture. The number indicates the time the microcosm was set up and inoculated (the time is not specified for the growth bioreactors, which were set up only at time zero). The last letter – present only in the labelling of the slurry microcosms – indicates the type of aquifer material and groundwater utilized for microcosm set up. Duplicate microcosms are indicated with the subscript “1,2”.

With regard to the methane-utilizing consortium, the initial inoculum object of the study was obtained by sampling 25 mL of suspension from each of three methane-fed slurry microcosms of the previous study (Fracari *et al.*, 2006) with a sterile syringe after 30 seconds of intense agitation, whereas the propane-utilizing initial inoculum was obtained from three propane-fed microcosms of the above-mentioned study, following the same procedure. The initial concentration of both inocula was equal to  $2.7 \times 10^7$  colony forming units (CFU) per mL.

At time zero (onset of this study), the methane-grown inoculum was introduced into two liquid-phase methane-fed growth bioreactors where the consortium was grown for 150-300 days in the presence (bioreactor GB-M-C) and in the absence (bioreactor GB-M-NC) of the same 6-CAH mixture object of the previous study (VC, trans- and cis-DCE, TCE, 1,1,2-TCA and 1,1,2,2-TeCA). The growth process was continued in reactor GB-M-C for 150 days and in reactor GB-M-NC for 300 days, by supplying methane pulses corresponding to an average feed of 37 mmol<sub>c</sub>/week. In addition, in order to characterize the initial methane-grown inoculum in terms of its ability to lead to the rapid onset of biodegradation of the 6-CAH mixture upon inoculation in slurry microcosms set up with the same aquifer material object of the previous study (soil + groundwater type A), two duplicate slurry microcosms (S-M-C-0A) set up with the same aquifer material were bioaugmented with the same inoculum introduced in the growth bioreactors. These microcosms were then spiked with methane and with the 6-CAH mixture, at the same initial concentrations utilized in the previous study and typical of aquifer A (methane 125 μM; VC 25 μM; trans-DCE 3.4 μM; cis-DCE 3.1 μM; TCE 1.9 μM; 1,1,2-TCA 0.30 μM; 1,1,2,2-TeCA 0.15 μM).

Similarly, at time zero the propane-grown inoculum was introduced into two propane-fed bioreactors where the consortium was grown in the presence (bioreactor GB-P-C) and in the absence (bioreactor GB-P-NC) of the 6-CAH mixture, and two slurry microcosm (S-P-C-0A) set up with soil and groundwater type A were bioaugmented with the same inoculum, spiked with propane and with the 6-CAH mixture (propane 46 μM; same CAH initial concentrations as in methane-fed microcosms S-M-C-0A) and subsequently operated by adding

consecutive propane and CAH pulses to evaluate the propane and CAH lag-times and the CAH depletion rates.

After 30 days of growth of the two consortia in the four bioreactors (time 1), four slurry microcosms were set up with soil and groundwater from site A and spiked with propane and with the 6-CAH mixture (at the same initial concentrations utilized for the previous propane-fed microcosms). Of these, two duplicates (S-P-NC-1A) were augmented with 2 mL of biomass suspensions sampled from bioreactor GB-P-NC, and two (S-P-C-1A) were augmented with 2 mL from bioreactor GB-P-C.

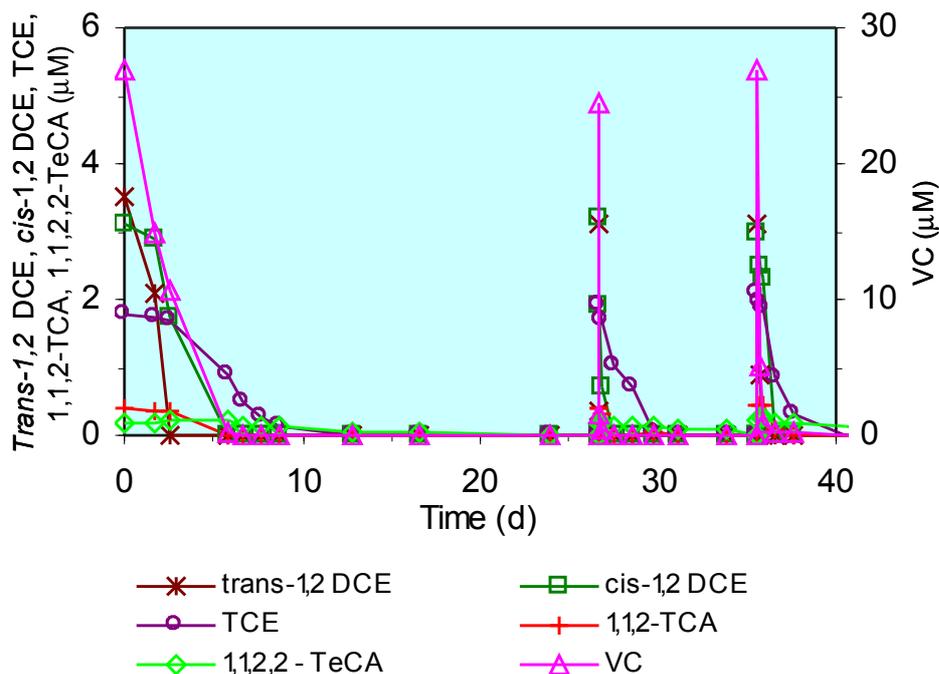
After 150 days of growth of the two consortia (time 2), six slurry microcosms were set up with aquifer material from site A and spiked with the 6-CAH mixture (at the same initial concentrations utilized for the previous microcosms). Of these, two (S-M-NC-2A and S-M-C-2A) were spiked with methane (125  $\mu\text{M}$ ) and augmented respectively from bioreactors GB-M-NC and GB-M-C, and two (S-P-NC-2A and S-P-C-2A) were spiked with propane (46  $\mu\text{M}$ ) and augmented from bioreactors GB-P-NC and GB-P-C; finally, in the attempt to develop a third consortium able to combine the best characteristics of the methane-utilizing and of the propane-utilizing consortia object of the study, two duplicate microcosms (S-MP-NC-2A) were spiked with both methane (62.5  $\mu\text{M}$ ) and propane (23  $\mu\text{M}$ ) and augmented from both GB-M-NC and GB-P-NC.

All the slurry microcosms set up at times 0, 1 and 2 with aquifer material type A were operated by adding consecutive pulses of methane or propane (or both in the case of S-MP-NC-2A) and of the 6-CAH mixture, at the same concentration supplied for each compound in the initial pulse, for a total of 5-7 CAH pulses. As an example, the plot of CAH aqueous phase concentration versus time relative to the first 4 days of operation of one of the two duplicate microcosms S-M-C-0A is shown in Figure 5.2. The growth substrate and CAH concentrations measured in these microcosms were utilized to evaluate the lag-times for the onset of substrate utilization and of biodegradation of the entire CAH mixture and the long-term CAH depletion rates, as explained in detail in section 5.2.4. The results were compared with those obtained respectively in the methane-fed and propane-fed non-bioaugmented slurry microcosms set up with

the same aquifer material, described in the previous study (Frasconi *et al.*, 2006) and utilized as the source of the initial inoculum. With the exception of S-MP-NC-2A, the microcosms of time 2, as well as the following ones, were not set up as duplicates, as the results from the microcosms set up at times 0 and 1 indicated a high reproducibility of the data obtained in the duplicate microcosms (as reported in more detail in section 5.3.2 and in Figure 5.3). Conversely S-MP-NC-2A, as well as the double-substrate microcosms set up at later times, was set up in duplicate, in order to have the possibility, after a consistent number of CAH pulses sustained by the supply of both substrates, to perform a final period of operation characterized by the supply of only methane in one duplicate and only propane in the other one.

On the basis of the positive results obtained in the microcosms set up at times 1 and 2 with regard to the possibility to grow either consortium in the absence of the CAH mixture and to utilize it to bioaugment slurry microcosms identical - in terms of type of aquifer material as well as type and concentration of contaminants - to those where the two consortia had initially been developed (see section 5.3.3), at time 3 (300 days of consortium growth in the bioreactors) we set up a further group of microcosms (S-M-NC-3A, B, C, D and E; S-P-NC-3A, B, C, D and E) aimed at investigating the potential of utilization the two consortia as inocula for the bioaugmentation of different CAH-contaminated aquifers. These microcosms, inoculated respectively from growth reactors GB-M-NC and GB-P-NC (consortia grown in the absence of the CAH mixture), were constructed with soil and groundwater taken from different sites: aquifers C and D, similarly to A, are CAH-contaminated sites containing sandy/silty soils, whereas aquifers B and E are uncontaminated sites containing respectively a sandy soil and a humic soil characterized by a high fraction of organic carbon (1.5%). The purpose of utilizing aquifer materials from uncontaminated sites was to simulate a bioremediation treatment conducted right after the occurrence of the contamination with the CAH mixture: in this condition, the site's indigenous biomass has not been subjected to the selective pressure of the chlorinated compounds. In order to give a more general scope to this section of the work, the microcosms of time 3 were spiked both at set-up and in the subsequent pulses

with an equal molar concentration (set to 4  $\mu\text{M}$ ) of the different CAHs. Besides, VC was not included in the CAH mixture, as numerous studies showed that this compound is easily biodegraded by several bacteria via both direct metabolism and cometabolism (Frasconi *et al.*, 2006; Verce *et al.*, 2002; Coleman *et al.*, 2005; Hartmans *et al.*, 1992).



**Figure 5.2.** Aqueous phase concentrations of the 6 CAHs versus time during the first 40 days of operation of microcosm S-M-C-0A<sub>1</sub>. For higher clarity the methane pulses, supplied daily at 125  $\mu\text{M}$  and rapidly consumed, are not represented.

In addition to the ones above-listed, a further microcosm (S-MP-NC-3E) was set up in duplicate at time 3 with aquifer material from site E, bioaugmented from both GB-M-NC and GB-P-NC and spiked with the 5-CAH mixture (no VC) at 4  $\mu\text{M}$  as well as with both methane (62.5  $\mu\text{M}$ ) and propane (23  $\mu\text{M}$ ). Besides, a non-bioaugmented control microcosm was set up for each substrate and for each aquifer material, for a total of 10 tests (K-M-A, B, C, D and E; K-P-A, B, C, D and E), and additioned with the 5-CAH mixture at 4  $\mu\text{M}$  and with methane (125  $\mu\text{M}$ ) or propane (46  $\mu\text{M}$ ).

The microcosms of time 3, similarly to the previous ones, were operated by addition of consecutive pulses of methane or propane (or both in the case of S-

MP-NC-3E) and of the 6-CAH mixture, at the same concentration supplied for each compound in the initial pulse, for a total of 3 CAH pulses (7 for the E-type microcosms). The results, elaborated in terms of substrate and CAH mixture lag-time and of CAH depletion rates, were compared to those obtained in the corresponding non-bioaugmented control microcosms.

Finally, at time 4 (390 days: 300 days of consortium growth in bioreactors GB-M-NC and GB-P-NC in the absence of CAHs + 90 days of consortium growth in the slurry microcosms of time 3 in the presence of the 5-CAH mixture), four liquid-phase microcosms were set up with a chloride-free mineral medium and spiked with the 5-CAH mixture (4  $\mu\text{M}$  for each compound): one (L-M-4) was inoculated from slurry microcosm S-M-NC-3E and spiked with methane (125  $\mu\text{M}$ ), one (L-P-4) was inoculated from S-P-NC-3E and spiked with propane (46  $\mu\text{M}$ ), whereas two duplicates (L-MP-4) were augmented from S-MP-NC-3E and spiked with both methane (62.5  $\mu\text{M}$ ) and propane (23  $\mu\text{M}$ ). The purpose of operating microcosms in the absence of soil was to obtain an evaluation of the long-term CAH *specific* depletion rates (in the slurry microcosms, given the difficulty of performing a precise valuation of active biomass, specific depletion rates cannot be evaluated with high accuracy). The reason why the liquid-phase microcosms were inoculated from the slurry microcosms containing soil from a site not characterized by a historical CAH contamination (site E) is that we were interested in investigating the characteristic of the consortia obtained from the interaction between the CAH-degrading methane-utilizing or propane-utilizing inoculated consortium and an indigenous biomass not affected by a previous selective pressure due to CAH contamination. The liquid-phase microcosms, similarly to the slurry ones, were operated by addition of consecutive pulses of growth substrate (methane or propane or both) and of the 5-CAH mixture, at the same concentration of each compound as in the initial pulse.

### **5.2.2 Growt bioreactors set up and operation**

Growth bioreactors (Figure 5.3) consisted of 5-l glass bottles sealed with Teflon-lined septa and containing 1 L of sterile mineral medium (Table 5.1).

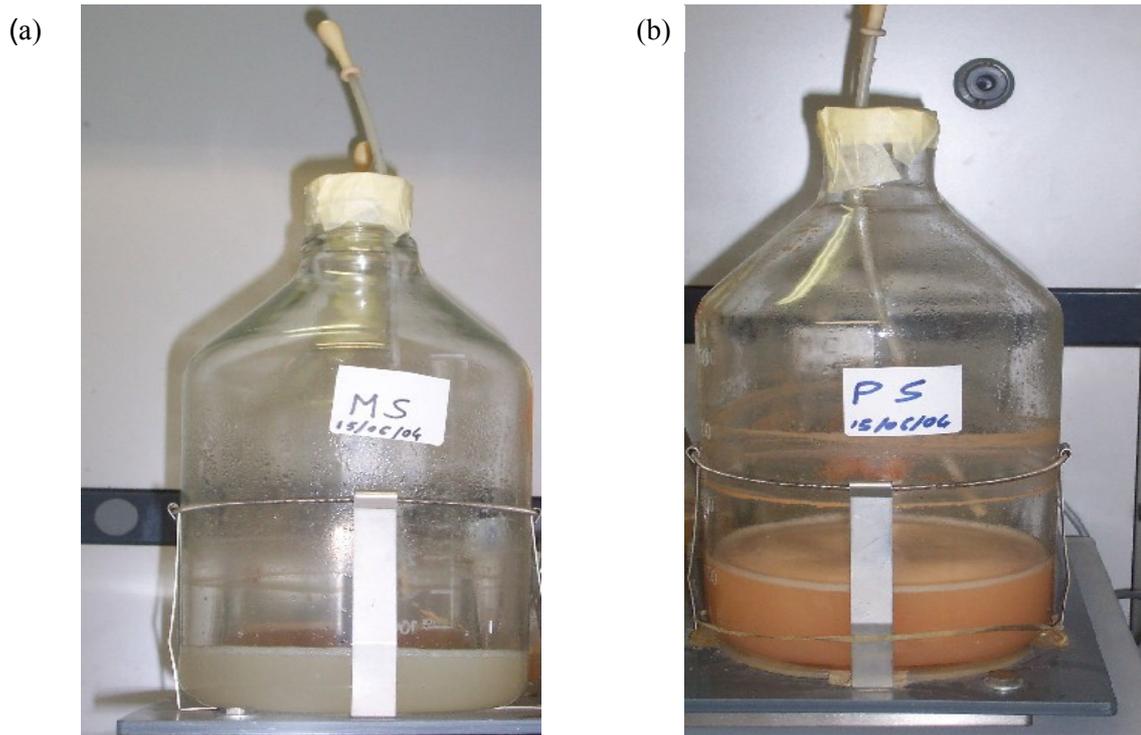


Figure 5.3. Batch growth reactors with the two different biomasses: a) metanotrophs; b) propanotrophs

Table 5.1. Mineral medium composition

Compound	Concentration ( $\mu\text{M}$ )
$(\text{NH}_4)_2\text{SO}_4$	797
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	244
$\text{CaCl}_2$	132
$\text{K}_2\text{HPO}_4$	8902
$\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$	5355
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	22.6
$\text{NaNO}_3$	9000
$\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$	1.52
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	0.510
$\text{H}_3\text{BO}_3$	1.00
$\text{Na}_2\text{MO}_4 \cdot 2\text{H}_2\text{O}$	0.450
$\text{NiCl}_2 \cdot 2\text{H}_2\text{O}$	0.144
$\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$	0.100
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	0.100

As shown in Table 5.2, each bioreactor was inoculated with 30 ml of the starting inoculum ( $2.7 \times 10^7$  CFU/mL). The primary substrate (methane or propane) was supplied in consecutive spikes (at the initial liquid-phase concentration in each pulse of 54  $\mu$ M for methane and 20  $\mu$ M for propane.) corresponding to an average feed rate of 37 mmol<sub>C</sub>/week. The 6 chlorinated solvents were introduced into bioreactors GB-M-C and GB-P-C by spiking 7.4 ml of gaseous VC and 900  $\mu$ L of a concentrated aqueous solution of trans-DCE (14.8 mM), cis-DCE (8.1 mM), TCE (7.9 mM), 1,1,2-TCA (0.83 mM) and 1,1,2,2-TeCA (0.12 mM) each time all of them had been completely degraded. The CAH initial concentrations in each pulse were : VC 25  $\mu$ M; trans-DCE 3.4  $\mu$ M; cis-DCE 3.1  $\mu$ M; TCE 1.9  $\mu$ M; 1,1,2-TCA 0.30  $\mu$ M; 1,1,2,2-TeCA 0.15  $\mu$ M. As a result of this procedure, the CAH overall feed rate was equal to 0.1 mmol/week. Aerobic conditions were maintained by adding pure oxygen each time the primary substrate was added, and the reactor aqueous phase was periodically air-stripped in order to remove CO<sub>2</sub> and possible volatile toxic degradation products A nutrient solution containing ammonium (as NH<sub>4</sub>Cl 918 mM) and phosphate (as KH<sub>2</sub>PO<sub>4</sub>: 38 mM and K<sub>2</sub>HPO<sub>4</sub>: 45 mM) was periodically provided so as to maintain a C:N:P molar ratio in the bioreactors feed equal to 220:11:1. Every 120 days the culture medium was centrifuged and biomass resuspended in fresh medium. The growth reactors, equipped with baffles in order to increase the gas-liquid mass transfer rate, were kept in continuous agitation in an orbital shaker (130 rpm) at 25 °C.

**Table 5.2. Growth reactors set-up and operational data**

Reactor	Growth substrate		CAH mixture	Initial inoculum	
	Type	Initial aq.concentration ( $\mu$ M)		Volume (ml)	Concentration ( $10^7$ CFU/ml)
GB-M-NC	Methane	54	Not present	30	2,7
GB-M-C			Present		
GB-P-NC	Propane	20	Not present	30	2,7
GB-P-C			Present		

### **5.2.3 Microcosms set up and operation**

Slurry and liquid phase microcosms were set up using 119 ml amber serum bottles sealed with Teflon-lined rubber septa. Each microcosm contained 20 g of soil and 50 ml of groundwater. In the microcosms set up with soil from aquifers B and E, in the absence of groundwater from the site, we introduced the same mineral medium utilized for the growth bioreactors (Table 5.3). The liquid-phase microcosms contained 50 ml of a Cl-free mineral medium having the same composition as that of the bioreactors, but containing CaSO<sub>4</sub> instead of CaCl<sub>2</sub> (Table 5.4). Each inoculated microcosm was augmented with 2 mL of biomass suspension. The biomass concentrations in the growth bioreactors at the time of each inoculation are reported in Table 5.5. The primary substrate was supplied in consecutive spikes (initial liquid-phase concentration in each pulse: methane 125 μM, propane 46 μM, methane 62.5 μM + propane 23 μM in the double-substrate tests), corresponding to an average feed rate of 1.1 mmol<sub>C</sub>/week. The CAH were introduced by spiking gaseous VC (except for the tests of times 3 and 4) and an aqueous solution of the remaining 5 CAHs (solution utilized for the tests of time 0, 1 and 2: trans-DCE 11.1 mM, cis-DCE 9.1 mM, TCE 5.9 mM, 1,1,2-TCA 1.8 mM, 1,1,2,2-TeCA 0.32mM; solutions for the tests of times 3 and 4: trans-DCE (7.9 mM), cis-DCE (6.5 mM), TCE (8 mM), 1,1,2-TCA (5.9 mM) and 1,1,2,2-TeCA (5.7 mM)

Aerobic conditions were maintained by adding pure oxygen (9 ml) with a frictionless glass syringe prior to each primary substrate supply and the microcosms aqueous phase was air-stripped each time a new pulse of CAHs was added, in order to remove CO<sub>2</sub> and possible volatile toxic degradation products. A nutrient solution containing ammonium (as NH<sub>4</sub>Cl 289 mM) and phosphate (as KH<sub>2</sub>PO<sub>4</sub>: 12 mM and K<sub>2</sub>HPO<sub>4</sub>: 14 mM) was periodically provided so as to maintain a C:N:P molar ratio in the feed equal to 220:11:1. The microcosms were maintained in agitation in a roller (3.3 rpm) at 25 ° C. To evaluate abiotic CAH depletion rates and losses through caps, four control microcosms were set up and sterilized with NaN<sub>3</sub> 57 mM: two (ST-A<sub>1,2</sub>) contained E-type aquifer materials and were spiked with the CAH mixture at the initial concentrations similar to those typical of site A (and utilized in the microcosms of times 0, 1 and 2), whereas two

(ST-E<sub>1,2</sub>) contained E-type aquifer materials and were spiked with TCE, 1,1,2-TCA and 1,1,2,2-TeCA at 4 µM.

**Table 5.3. Set up data of the slurry microcosms**

Time	Label	Composition	Growth substrate	Parent bioreactor	Duplicates	
T <sub>0</sub>	S-M-C0A	Soil A + water A	Methane		2	
	S-P-C0A		Propane		2	
T <sub>1</sub> (30 d)	S-P-NC-1A	Soil A + water A	Propane	GB-P-NC	2	
	S-P-C-1A			GB-P-C	2	
T <sub>2</sub> (150 d)	S-M-NC-2A	Soil A + water A	Methane	GB-M-NC	1	
	S-M-C-2A			GB-M-C	1	
	S-P-NC-2A		Propane	GB-P-NC	1	
	S-P-C-2A			GB-P-C	1	
	S-MP-NC-2A		Methane + propane	GB-P-NC/GB-M-NC	2	
T <sub>3</sub> (300 d)	S-M-NC-3A	Soil A + water A	Methane	GB-M-NC	1	
	S-M-NC-3B	Soil B + mineral medium		GB-M-NC	1	
	S-M-NC-3C	Soil C + water C		GB-M-NC	1	
	S-M-NC-3D	Soil D + mineral medium		GB-M-NC	1	
	S-M-NC-3E	Soil E + mineral medium		GB-M-NC	1	
	S-P-NC-3A	Soil A + water A	Propane	GB-P-NC	1	
	S-P-NC-3B	Soil B + mineral medium		GB-P-NC	1	
	S-P-NC-3C	Soil C + water C		GB-P-NC	1	
	S-P-NC-3D	Soil D + mineral medium		GB-P-NC	1	
	S-P-NC-3E	Soil E + mineral medium		GB-P-NC	1	
	S-MP-NC-3E	Soil A + water A		Methane + Propane	GB-P-NC/GB-M-NC	2
Non inoculated microcosms	KMA	Soil A + water A	Methane	-	2	
	KMB	Soil B + mineral medium	Methane	-	2	
	KMC	Soil C + water C	Methane	-	2	
	KMD	Soil D + mineral medium	Methane	-	2	
	KPA	Soil A + water A	Propane	-	2	
	KPB	Soil B + mineral medium	Propane	-	2	
	KPC	Soil C + water C	Propane	-	2	
	KPD	Soil D + mineral medium	Propane	-	2	
		KMPE	Soil E + mineral medium	Methane + propane	-	2

**Table 5.4. Set up data of the liquid-phase microcosms**

Label	Growth substrate	Parent microcosm	Duplicates
L-MP-4	Methane + propane	S-MP-NC-3E	2
L-M-4	Methane	S-M-NC-3E	1
L-P-4	Propane	S-P-NC-3E	1

**Table 5.5. Viable biomass concentrations in the growth bioreactors at the times of inoculation of the slurry microcosms**

Bioreactor	Time (day)			
	0	30	150	300
GB-M-NC	7.7E+05	1.7E+07	1.7E+08	4.5E+08
GB-M-C	7.7E+05	4.9E+06	4.9E+07	-
GB-P-NC	7.7E+05	7.2E+06	7.2E+07	3.1E+08
GB-P-C	7.7E+05	4.0E+06	2.0E+07	-

#### 5.2.4 Estimation of lag times, degradation rates and rate/concentration ratios

The lag-times for the onset of growth substrate consumption and CAH degradation were obtained by the intersection of the maximum slope line of the concentration-time curve with the horizontal line passing through the initial concentration value. The lag-times are reported in the Results in terms of substrate lag-time and additional lag-time relative to the CAH mixture, measured from the onset of substrate consumption and defined as the longest of the additional lag-times relative to the single CAHs.

Each CAH pulse was characterized by the maximum degradation rate relative to each compound, calculated by dividing the maximum slope of the mass-time curve by the volume of the liquid phase. Each degradation rate was associated with the aqueous phase concentration corresponding to the initial value of the portion of the mass-time curve utilized to calculate the degradation rate. The CAH depletion rates were elaborated according to the method described in detail in the previous study (Frascari *et al.*, 2006): the rates obtained for each compound in each microcosms were plotted versus the mass of growth substrate consumed; Figure 5.4 shows as an example the plot degradation rate versus consumed carbon mass for the first 5 pulses of VC in duplicate microcosms S-M-C-0-A<sub>1</sub> and S-M-C-0-A<sub>2</sub>. This elaboration indicated that, after about 4 - 5 pulses of CAH mixture depletion, each microcosm reached a roughly stationary condition in terms of CAH depletion rates; therefore we calculated the average of the depletion rates evaluated – for each compound and for each microcosm – in

the stationary phase; this average rate was divided by the average of the concentrations associated with the depletion rates included in the average. The rate/concentration ratio obtained – indicate in the following with  $k^*$  - was utilized as an index to compare the depletion rates obtained in a given microcosm relatively to the different CAHs, and in different microcosms relatively to the same CAH. This simplified approach allowed to compare, within each microcosm, the depletion rates obtained for the different CAHs at different initial concentrations; it also allowed to compare the rates obtained for a given CAH at time 3 (when all the CAHs were supplied at 4  $\mu$ M) with those obtained for the same CAH at earlier times (when the different CAHs were supplied at the concentrations typical of site A).

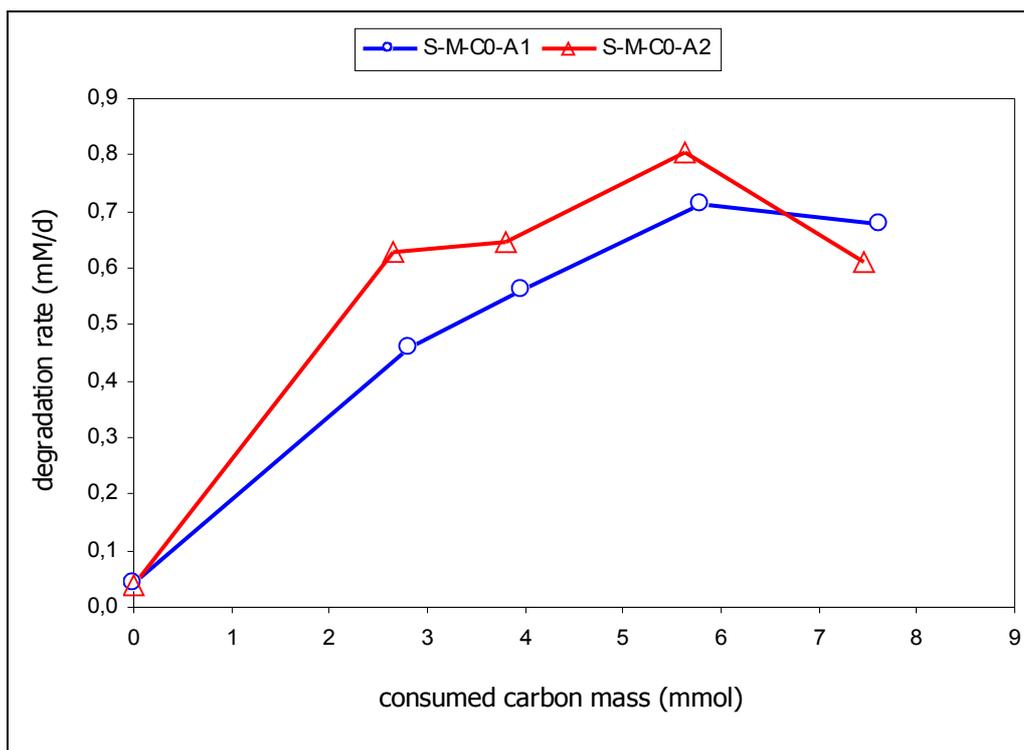


Fig 5.4. Plot of the degradation rate-growth substrate consumed for the first 5 VC pulses in duplicate microcosms S-M-C-0-A<sub>1</sub>, S-M-C-0-A<sub>2</sub>.

The ratio of depletion rate to concentration ( $k^*$ ) can be considered a pseudo first-order constant that includes biomass concentration), in the hypothesis that the biodegradation of each CAH followed a first-order kinetic model within the concentration ranges tested for each compound. This hypothesis was confirmed – although within concentration ranges lower than those of this study, in particular for 1,1,2-TCA and 1,1,2,2-TeCA – by the results of the previous study (Frasconi *et al.*, 2006), where it was shown that, in each slurry microcosm, the depletion rates obtained for each CAH at a roughly constant biomass concentration were proportional to the corresponding initial concentrations in the pulses. The assumption of first-order kinetic is in agreement with the observation that the CAH half-saturation constants reported in the literature are typically higher than the concentration ranges investigated in this study (Alvarez-Cohen *et al.*, 2001; Arp *et al.*, 2001; Oldenhuis *et al.*, 1991).

The only exception to the above-described procedure for the elaboration of the CAH depletion rates was made for the slurry microcosms set up at time 3 with aquifer materials from sites A, B, C and D: because these tests were operated only for 3 CAH pulses, the plots of the depletion rates versus substrate mass consumed indicated that the stationary condition had not been achieved. Therefore, to characterize the rates achieved in these microcosms we chose, for each compound, the ratio of the depletion rate of the third CAH pulse to the corresponding aqueous-phase concentration. Consequently, the microcosms set up at time 3 with aquifer material from site E (which were operated for 7 CAH pulses) were characterized according to this criterion in the elaborations where they were compared with the corresponding microcosms containing materials from sites A, B, C and D, whereas in the elaborations where they were compared with other microcosms that had achieved the stationary condition, they were characterized according to the general criterion of the stationary CAH depletion rate.

In the case of the liquid-phase microcosms, the procedure described with regard to the slurry microcosms was applied to the CAH specific degradation rates, obtained by dividing each CAH rate by the corresponding biomass concentration measured at the beginning of the CAH pulse. The ratio of specific depletion rate to concentration is indicated in this study with  $k_{sp}^*$

The lag-times and depletion rates reported in the Results relatively to the duplicate microcosm are averages of the corresponding values estimated in the two duplicates.

### 5.2.5 Analytical methods

#### Gas Chromatography Analysis

The gas-phase concentrations of methane, propane and CAHs were measured with a HP6890 gas chromatograph equipped with a capillary HP-VOC column connected to a Flame Ionisation Detector (FID) for the analysis of methane, propane and VC and to a micro Electron Capture Detector ( $\mu$ -ECD) for the analysis of the remaining CAHs; the instrument and the method characteristics are reported in Table 5.6. Detection limits were ( $\mu$ M in the aqueous phase): methane and propane, 0.007; VC, 1.2; *trans*-DCE, 0.08; *cis*-DCE, 0.15; TCE,  $4 \times 10^{-6}$ ; 1,1,2-TCA, 0.01; 1,1,2,2-TeCA, 0.02.

**Table 5.6. GC Method for methane, propane and the 6 CAHs mixture's analysis**

Instrument	HP 6890 serie II plus
Column	HP-VOC capillary column
Column I.D.	0.32 mm
Column length	30 m
Liner	Splitless
Split ratio	10:1
Front detector	$\mu$ ECD
Back detector	FID
Carrier gas	Helium
Make up gas	Nitrogen
Flow	0.9 ml/min
Injection volume	500 $\mu$ L
Pressure	1.6 Bar
Injector temperature	250°C
Detector temperature	250°C
Initial temperature	60°C (3 min)
Ramp	20°C/min to 230°C
Final temp	230°C (5 min)
Run time	16.5 min

Total masses and aqueous phase concentrations in standards and slurry microcosms were calculated utilizing the gas/liquid and solid/liquid equilibrium constants estimated at 25°C. The following equations describe the equilibrium:

$$m_i = (c_{L,i} \bullet V_L + c_{G,i} \bullet V_G) \quad (5.1)$$

$$c_{G,i} = H_i \bullet c_{L,i} \quad (5.2)$$

that implies:

$$c_{L,i} = m_i / (V_L + H_i \bullet V_G) \quad (5.3)$$

where:

$V_L, V_G$ : liquid and gas volume in the standard

$H_i$ : dimensionless Henry constant for the compound  $i$  (Sanders, 1999)

$m_i$ : mass of the compound  $i$  (mg)

$c_{G,i}$ , gas and liquid concentration of compound  $i$

Concerning the evaluation of the compound concentration into the microcosms, these have been based on the gas-phase concentration by headspace analysis. Soil and liquid-phase concentration have been evaluated by assuming equilibrium between the phases. The calculation has been based on equation 5.2 and on the following linear adsorption isotherm (Semprini, 2000):

$$c_{S,i} = K_{d,i} \bullet c_{L,i} \quad (5.4)$$

where:

$c_{S,i}$ : concentration of compound  $i$  in the solid phase (soil) related to dry soil mass (mg/kg dry soil)

$K_{d,i}$ : adsorption constant of compound  $i$  (L/kg)

The adsorption constant has been evaluated on the basis of the soil organic carbon content ( $f_{oc}$ , dimensionless) and of the value of the carbon-water partition constant ( $K_{oc}$ , L/kg):

$$K_{d,i} = f_{oc} \bullet K_{oc,i} \quad (5.5)$$

The amount of compound (substrate or contaminant) in the microcosm has then been measured (starting from the gas-phase concentration) through the following relation:

$$m_i = c_{L,i} \cdot (V_L + H_i \cdot V_G + f_{oc} \cdot K_{oc,i} \cdot M_T) \quad (5.6)$$

where  $M_T$  (kg) is the soil mass in the microcosm, while  $c_{L,i}$  has been evaluated through the relation (5.2).

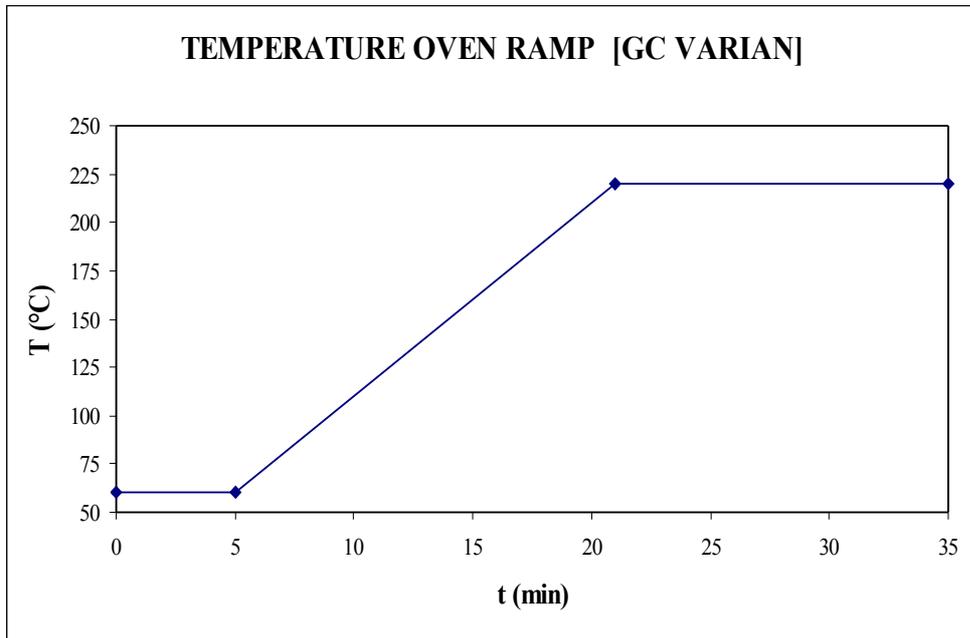
### **Bacterial counts**

Serial dilution of biomass suspension sampled from growth bioreactors were plated on Petri dishes containing R2A medium. Bacterial colonies grown on R2A agar plates were grouped in different clusters and were counted on the basis of their different morphologies after a 5-7 days incubation at 30°C; the viable cells concentration was expressed as colonies forming units per ml of suspension (CFU/ml). R2A medium contained: yeast extract (500 mg/L), caseine hydrolyzed (500 mg/L), thiotone/peptone (500 mg/L), Glucose (500 mg/L), Sodium Piruvate (300 mg/L),  $\text{Na}_2\text{HPO}_4$  (300 mg/L),  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (30 mg/L); Agar (gelificant agent, 15 g/L).

### **Measure of CO<sub>2</sub> and O<sub>2</sub> concentration in the headspace**

In order to evaluate the consumption of O<sub>2</sub> and the release of CO<sub>2</sub> correlated with the consumption of primary substrate, 1 ml of microcosms headspace gas has been sampled and analysed in a VARIAN 3300 gas chromatograph equipped with a TCD detector (Thermal Conductivity Detector) and a packed column CARBONSIEVE SII SS. Injector temperature was 150°C, filament temperature, 250°C and detector temperature, 220°C. The temperature program was as follows (Figure 5.5): 5 min at 60 °C; ramp to 220°C at 10°C/min; 14 min at 220°C.

Figure 5.5. Temperature oven ramp for CO<sub>2</sub> and O<sub>2</sub> analyses



**Total protein concentration** was evaluated as described by Peterson (1977).

**Concentration of Cl<sup>-</sup>** was measured by Ion Chromatography with the method described by Frascari *et al.*(2006).

### 5.3 RESULTS

#### 5.3.1 CAH depletion in the sterilized controls

The ratios of CAH initial abiotic depletion rate to initial concentration ( $k_{st}^*$ ) evaluated in the sterile controls are reported in Table 5.7. The comparison between these rates and the corresponding rates obtained in the viable microcosms is reported and commented in sections 5.3.3 and 5.3.4. CAH depletion in the sterilized controls followed a first-order kinetic. The best estimates of the first-order constants, reported in Table 2, correspond to abiotic half-lives varying between 2 months (for TCE and 1,1,2,2-TeCA) and 2.5 years (for *cis*-DCE).

**Table 5.7. Abiotic rate/concentrations ratios ( $k_{st}^*$ ) estimated in the sterilized controls ST-A<sub>1,2</sub> and ST-E<sub>1,2</sub> operated at 25°C (average values).**

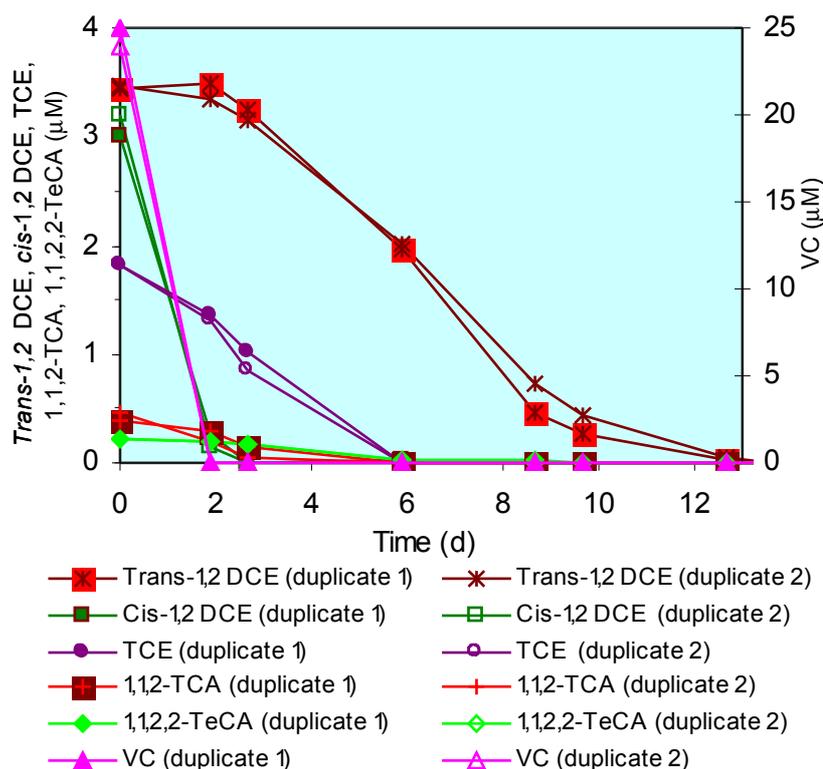
CAH	Microcosms ST-A <sub>1,2</sub>		Microcosms ST-E <sub>1,2</sub>	
	C <sub>i</sub> <sup>a</sup> (μM)	k <sub>st</sub> <sup>* b</sup> (year <sup>-1</sup> )	C <sub>i</sub> <sup>a</sup> (μM)	k <sub>st</sub> <sup>* b</sup> (year <sup>-1</sup> )
VC	30	1.6	-	-
trans-DCE	3.6	1.4	-	-
cis-DCE	3.7	0.28	-	-
TCE	2.2	4.1	4	4.6
1,1,2-TCA	0.62	0.44	4	0.55
1,1,2,2-TeCA	0.37	4.1	4	7.9

<sup>a</sup> Initial aqueous phase concentration.

<sup>b</sup> Ratio of initial depletion rate to initial concentration.

### 5.3.2 Behaviour of the duplicate slurry microcosms

All the duplicate slurry microcosms led to equal results (with deviations < 10%) in terms of both lag-times for the onset of CAH biodegradation and CAH depletion rates. As an example, the plot of CAH concentration versus time relative to the first 13 days of operation of microcosms S-P-NC-1A<sub>1</sub> and S-P-NC-1A<sub>2</sub> is shown in Figure 5.6.



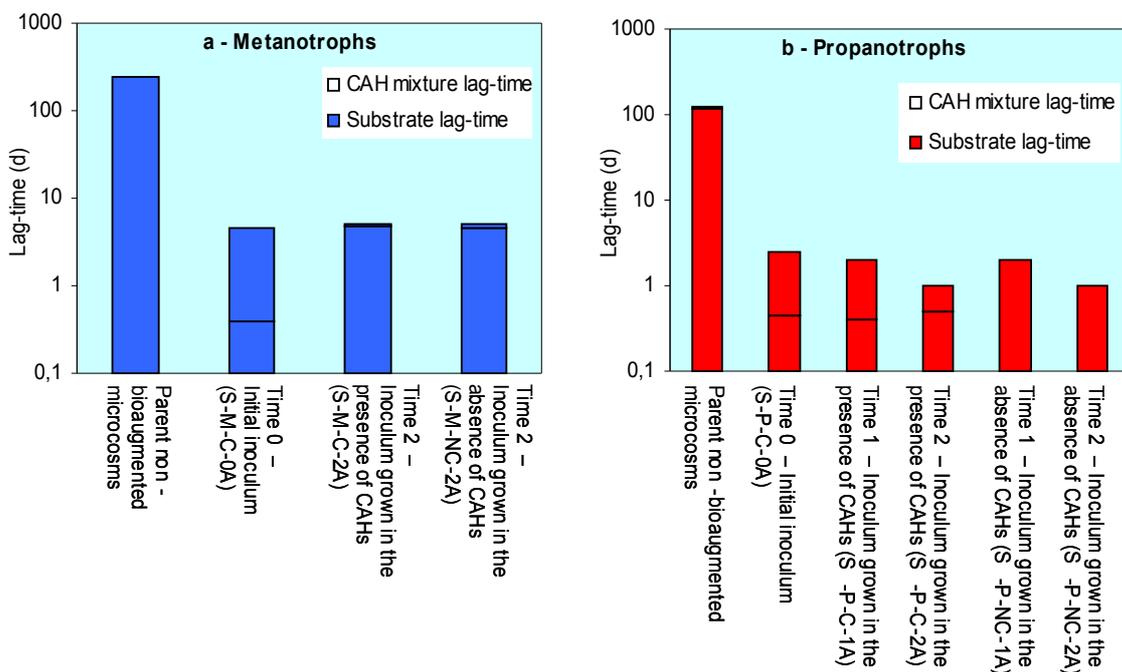
**Figure 5.6.** Aqueous phase concentrations of the 6 CAHs versus time during the first 13 days of operation of duplicate microcosms S-P-NC-1A<sub>1</sub> and S-P-NC-1A<sub>2</sub>. For higher clarity the propane pulses, supplied daily at 46  $\square$ M and rapidly consumed, are not represented.

### 5.3.3 Effect of inoculum growth time and condition (presence/absence of CAHs)

The lag-times for the onset of substrate utilization and CAH mixture biodegradation obtained in the parent non-bioaugmented microcosms relative to the previous study (Frasconi *et al.*, 2006) and in the microcosms set up at times 0,

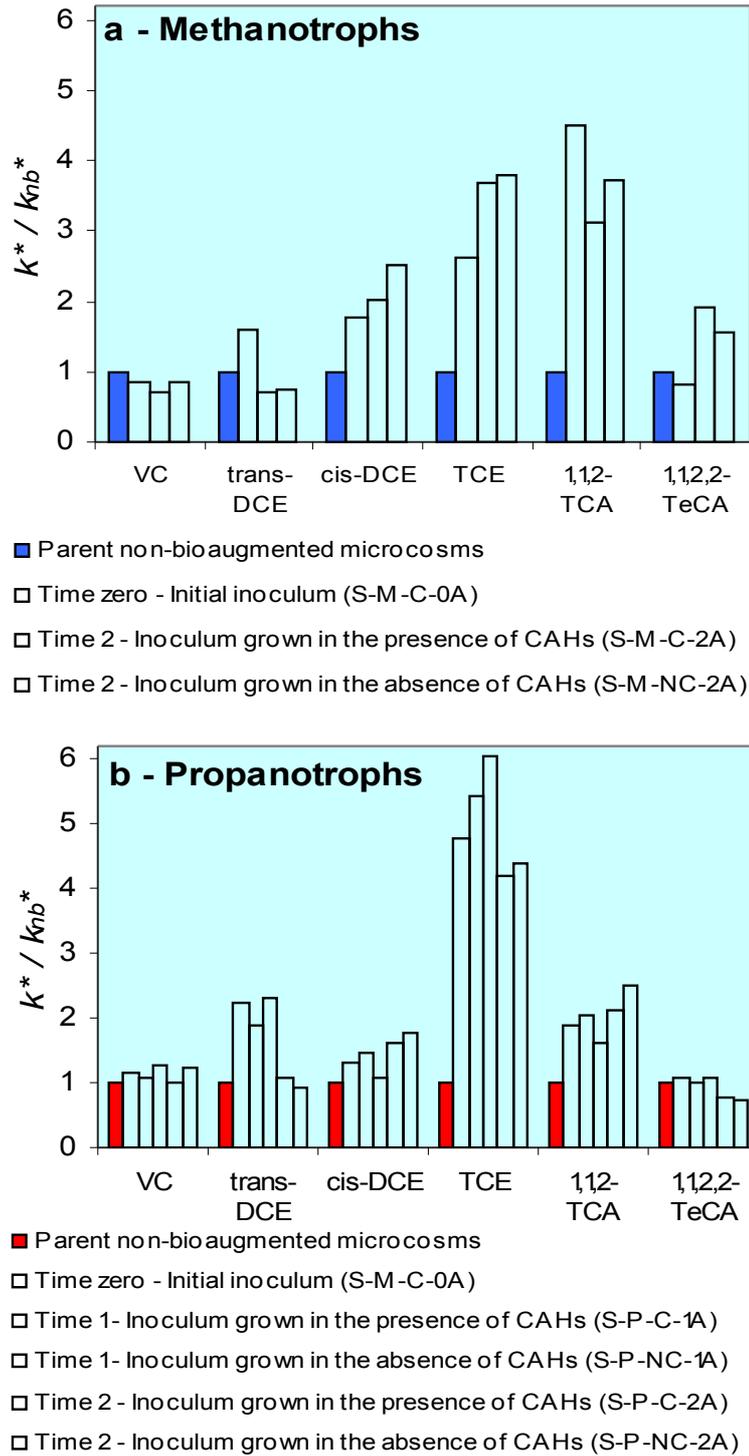
1 and 2 with aquifer materials from site A are reported in Figure 5.7, whereas the corresponding  $k^*$  are shown in Figure 5.8 in normalized form, having equalized to 1 the values corresponding to the parent non-bioaugmented microcosms. The actual ratios  $k^*$  relative to these microcosms are reported in the left-hand part of Table 5.8.

Figure 5.7 shows in the first place that the both inocula utilized at time zero to set up the growth bioreactors led to a drastic decrease of the lag-time for the onset of biodegradation of the entire 6-CAH mixture upon introduction in the microcosms containing aquifer materials from site A (microcosms S-M-C-0A and S-P-C-0A: from 100-200 days in the non-bioaugmented tests to 2-4 days in the inoculated ones). This important result indicates that, in case of an in-situ cometabolic bioremediation of site A, the utilization of either inoculum is fundamental in order to rely on a fast onset of the remediation process and on a significant saving on substrate costs. Figure 5.7 also shows that, for both consortia, the 150-day growth process in the presence as well as in the absence of the selective pressure exerted by the CAH mixture did not lead to any significant loss of the capacity to induce the rapid onset of biodegradation of the CAH mixture, indicating that an eventual process of production of large amounts of biomass to utilize for a real-scale bioaugmentation treatment can be operated in the absence of the CAHs, with a significant simplification of the production plant and a reduction of the fixed and operational costs. A possible explanation for this experimental result is the fact that the inocula initially supplied to the growth bioreactors had previously been subjected to a prolonged period of CAH biodegradation in the slurry microcosms operated within the previous study (410 days for the methanotrophs, 310 days for the propanotrophs), during which they had been strongly stabilized by the CAH selective pressure. Lastly, it can be observed in Figure 5.7 that in the non-bioaugmented microcosms the lag-time basically coincides with the time required by the site's indigenous biomass to start growing on the primary substrate, whereas in the inoculated tests the short lag-time is in some cases a substrate lag, and in others a CAH lag.



**Figure 5.7.** Lag-times for the onset of primary substrate utilization and CAH mixture biodegradation by methane-utilizing (a) and propane-utilizing (b) biomasses in the parent non-bioaugmented microcosms, in the microcosms bioaugmented with the initial inoculum (time 0) and in those bioaugmented with biomass sampled from the growth reactors at times 1 (30 days) and 2 (150 days). All the data refer to microcosms containing aquifer materials from site A, and to the CAH initial concentration typical of site A (Table 5.8).

The  $k^*$  reported in Figure 5.8 show that, for both consortia, the prolonged growth process in the presence as well as in the absence of the CAH mixture did not lead to any decrease of the long-term CAH depletion rates obtained in the inoculated microcosms set up with materials from site A, in comparison with the rates obtained inoculating the same type of microcosms with the initial inoculum (tests set up at time zero: S-M-C-0A and S-P-C-0A). Besides, with regard to trans- and cis-DCE, TCE and 1,1,2-TCA, in the microcosms inoculated at time zero we obtained depletion rates higher than those measured in the parent non-bioaugmented microcosms: this result may be explained by considering that each of the initial inocula was obtained by mixing biomass samples from three slurry microcosms, which may have resulted in the formation of two consortia combining the best degradation capacities of the biomasses they originated from.



**Figure 5.8.** Normalized ratios of depletion rate to concentration ( $k^*$ ) relative to the biodegradation of the 6 CAH mixture by methane-utilizing (a) and propane-utilizing (b) biomasses in the parent non-bioaugmented microcosms, in the microcosms bioaugmented with the initial inoculum (time 0) and in those bioaugmented with biomass sampled from the growth reactors at times 1 (30 days) and 2 (150 days). The  $k^*$  relative to the parent non-bioaugmented microcosms ( $k_{nb}^*$ ) were equalized to 1, and their actual value is reported in the left-hand part of Table 5.8. All the data refer to microcosms containing aquifer materials from site A, and to the CAH initial concentrations typical of site A (Table 5.8).

Combining the results shown in Figures 5.7 and 5.8, it can be stated that the prolonged growth process of both inocula in the absence of CAHs allowed to attain, as a result of the inoculation in type-A slurry microcosms, CAH biodegradation rates equal or higher than those obtained in the non-bioaugmented microcosms, with drastically shorter lag-times for the onset of the bioremediation process.

As evidenced in our previous study (Frasconi *et al.*, 2006), the  $k^*$  obtained in the non-bioaugmented microcosms and reported in the left-hand part of Table 5.8 are characterized by a tendency to a decrease as the number of chlorine atoms in the solvent increases, with – in the case of the methanotrophs – a difference of two order of magnitude passing from VC to 1,1,2,2-TeCA. This tendency was maintained during the consortia growth process in the four bioreactors.

**Table 5.8. CAH depletion rate / concentration ratios relative to the microcosms utilized as references for the normalized data reported in Figures 5.8 and 5.10 (day<sup>-1</sup>).**

CAH	Parent non-bioaugmented microcosms (Frasconi <i>et al.</i> , 2006) <sup>a</sup>		Slurry microcosms set up at time 3 with soil E and inoculated from growth bioreactors GB-M-NC or GB-P-NC <sup>b,c</sup>	
	Methane-fed	Propane-fed	S-M-NC-3E (methane-fed)	S-P-NC-3E (propane-fed)
VC	29	24	<sup>d</sup>	<sup>d</sup>
trans-DCE	10	0.59	1.7	1.1
cis-DCE	3.1	8.3	1.6	12
TCE	0.40	1.6	1.8	6.1
1,1,2-TCA	0.44	5.7	1.5	9.2
1,1,2,2-TeCA	0.29	1.1	0	1.0

<sup>a</sup> Initial concentration in the pulses: VC 25 μM; trans-DCE 3.4 μM; cis-DCE 3.1 μM; TCE 1.9 μM; 1,1,2-TCA 0.30 μM; 1,1,2,2-TeCA 0.15 μM.

<sup>b</sup> Initial concentration in the pulses: 4 μM for all the 5 CAHs.

<sup>c</sup> The CAH depletion rates utilized for the microcosms set up at time 3 are - unlike those utilized for the parent non-bioaugmented tests - those of the third CAH pulse, when the microcosms were not yet in a stationary conditions in terms of CAH rates.

<sup>d</sup> VC was not included in the microcosms set up at time 3.

### **5.3.4 Effect of the type of aquifer material contained in the inoculated microcosms**

Figure 5.9 reports the substrate and CAH mixture lag-times obtained in the non-bioaugmented methane-fed and propane-fed microcosms containing aquifer materials from sites A, B, C, D and E (and spiked with the 5-CAH mixture, with each compound at 4  $\mu\text{M}$ ) and in the corresponding microcosms inoculated at time 3 from bioreactors GB-M-NC and GB-P-NC (300 days of consortia growth in the absence of CAHs). The CAH lags relative to the methanotrophs (Figure 5.9a) do not include 1,1,2,2-TeCA as, after a monitoring time of at least 100 days, the biodegradation of this solvent (at the initial concentration of 4  $\mu\text{M}$ ) was observed neither in the non-bioaugmented microcosms nor in the inoculated ones. Figure 5.9 shows that the inoculation of the two consortia led to drastic lag-time reductions in the case of aquifers A and E, and to less marked – but not negligible – reductions in the case of aquifers B, C and D (in the worst case, occurred with the inoculation of the propanotrophs in aquifer material type B, we observed a 2-fold lag-time reduction). The short lag-times obtained in the inoculated microcosms indicate that the prolonged growth process of the two consortia in the absence of CAHs did not lead - with the exception of 1,1,2,2-TeCA for the methanotrophs - to any significant loss of their ability to induce the rapid onset of CAH biodegradation. The lack of 1,1,2,2-TeCA biodegradation in the methane-fed inoculated microcosms is probably to be ascribed to the higher initial concentration of this compound in the microcosms of times 3 with respect to those inoculated at previous times (4 versus 0.15  $\mu\text{M}$ ), rather than to a loss of 1,1,2,2-TeCA degradation capacity of the methane-utilizing consortium during the growth process in GB-M-NC. This hypothesis, although not fully demonstrated within this study, is partly supported by the observation that, while in the non-inoculated microcosms containing A-type aquifer material and 1,1,2,2-TeCA at 0.15  $\mu\text{M}$  we observed the biodegradation of this compound shortly after the onset of methane consumption, in the non-inoculated tests containing the same aquifer material and 1,1,2,2-TeCA at 4  $\mu\text{M}$  no biodegradation of this solvent occurred, indicating that the microbial capacity to transform 1,1,2,2-TeCA is affected by its concentration.

The results shown in Figure 5.9 relatively to the non-bioaugmented microcosms containing soil E confirm the observation that when particularly long overall lag-times are observed, they are due mainly to the time required by the site's indigenous biomass to start growing on the primary substrate.

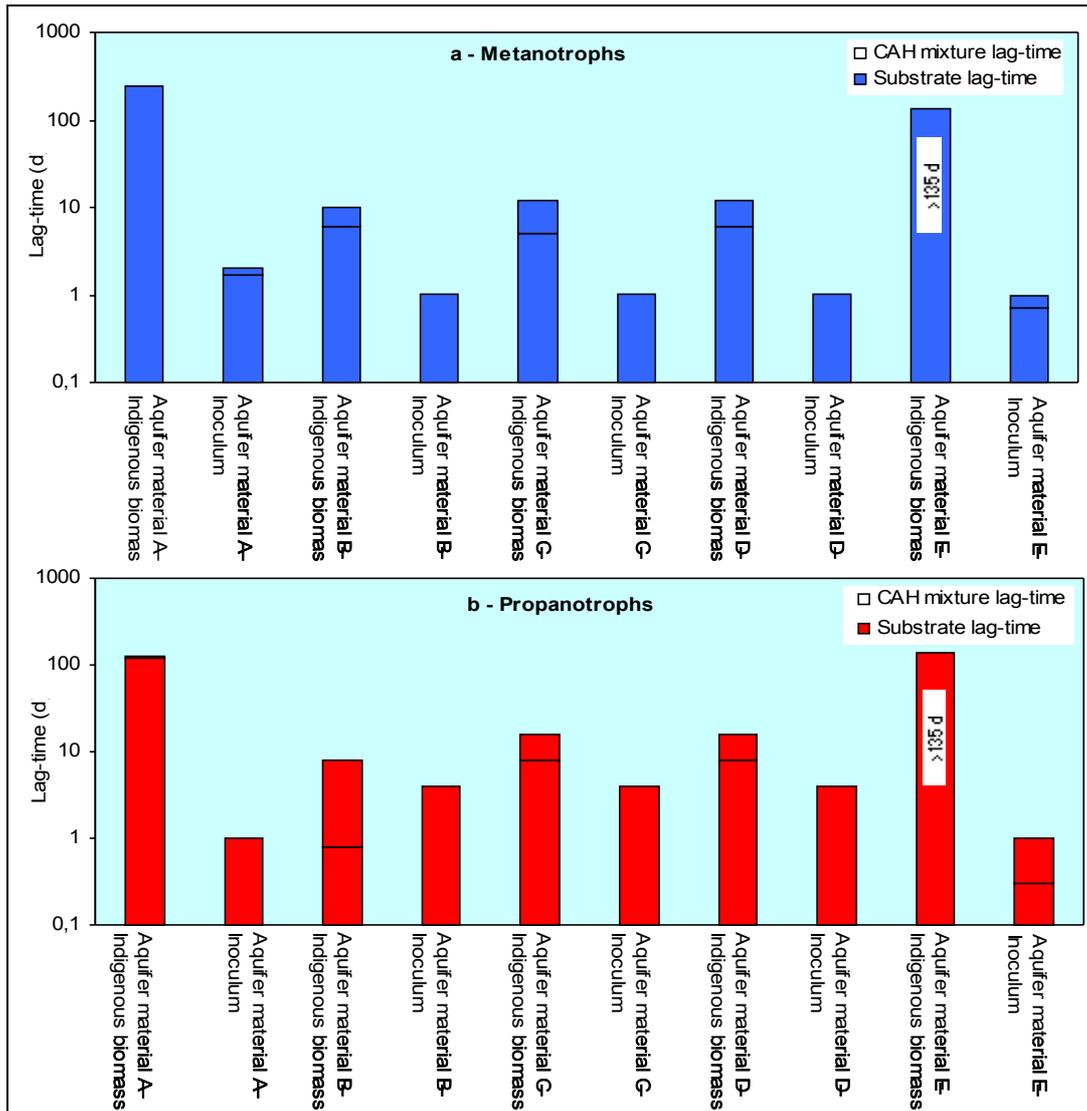


Figure 5.9. Lag-times for the onset of primary substrate utilization and CAH mixture biodegradation by methane-utilizing (a) and propane-utilizing (b) biomasses in non-bioaugmented microcosms set up with five different aquifer materials and in the corresponding microcosms bioaugmented with biomass sampled in growth reactors GB-M-NC and GB-P-NC (microbial growth in the absence of CAHs) at time 3 (300 days of biomass growth). VC was not spiked in the microcosms of time 3. In part (a) the CAH lag-times refer

**to the time for the onset of the transformation of trans- and cis-DCE, TCE and 1,1,2-TCA, as no 1,1,2,2-TeCA biodegradation was observed in the methane-fed tests of time 3.**

Figure 5.10 reports the  $k^*$  relative to the methane-fed and propane-fed microcosms set up with the different aquifer materials and inoculated at time 3 from GB-M-NC and GB-P-NC. Given the wide ranges of variations obtained, the rate/concentration ratios relative to the microcosms containing E-type materials were made equal to 1, and their actual value is reported in the right-hand side of Table 5.8. As explained in section 5.2.4, the CAH depletion rates utilized to build Figure 5.10 are - unlike those of Figure 5.8 - those of the third CAH pulse, when the microcosms were not yet in a stationary condition in terms of CAH rates. The data summarized in Figure 5.10 and in Table 5.3 show that – with the exception of 1,1,2,2-TeCA for the methanotrophs – the two inocula, after the prolonged growth process in the absence of CAHs, were able to induce in all the aquifer materials tested CAH depletion rates analogous to those obtained in the parent non-bioaugmented microcosms. Overall, the bioaugmented microcosms containing E-type materials resulted in the highest CAH degradation rates; for this reason, as well as for our interest in site E as a non historically contaminated site, these microcosms were operated for a significantly longer time than the other microcosms of time 3 (7 versus 3 CAH pulses), and were eventually utilized as the source of the inocula for the liquid-phase microcosms of time 4.

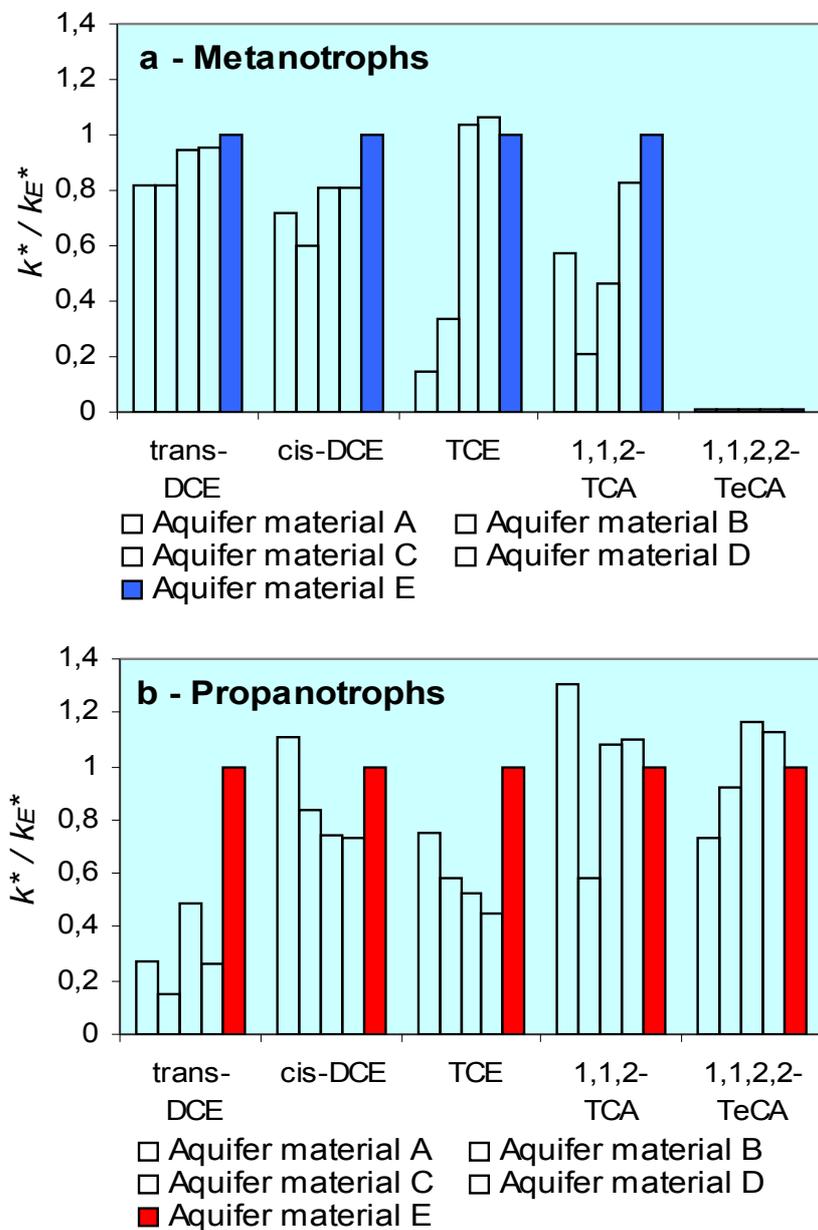


Figure 5.10. Normalized depletion rate to concentration ratios ( $k^*$ ) for the biodegradation of the 5-CAH mixture by methane-utilizing (a) and propane-utilizing (b) biomasses in the microcosms set up with different aquifer materials and bioaugmented with biomass sampled from growth reactors GB-M-NC and GB-P-NC at time 3. The  $k^*$  relative to the E-type microcosms ( $k_E^*$ ) were equalized to 1 and their actual value is reported in the right hand part of Table 5.8.

The ratio of the  $k_{st}^*$  evaluated in sterile controls ST-E<sub>1,2</sub> (spiked with TCE, 1,1,2-TCA and 1,1,2,2-TeCA at 4  $\mu$ M) to the corresponding  $k^*$  obtained in the microcosms of time is < 1% for 1,1,2-TCA, < 3% for 1,1,2,2-TeCA and < 5% for TCE. This result confirms the minor contribution of abiotic reactions and losses through the caps to the depletion rates observed in the viable microcosms.

The combined results shown in Figures 5.9 and 5.10 indicate that both the inocula object of this study have a high potential for the bioaugmentation of CAH-contaminated sites, even in cases where the site's indigenous biomass has not been previously affected by the selective pressure due to a historical CAH contamination. The lack of 1,1,2,2-TeCA biodegradation at 4  $\mu$ M by the methanotrophs indicates, in agreement with numerous literature studies (Kim *et al.*, 2000; U.S. E.P.A, 2000; Chen *et al.*, 1996), that the aerobic biodegradation of TeCA is a problematic process and that propane is more effective than methane in inducing the microbial degradation of this compound.

### 5.3.5 Effect of the type of microbial consortium inoculated and growth substrate supplied

Figure 5.11a shows the  $k^*$  relative to the three types of slurry microcosms set up with type-A aquifer material and inoculated at time 2 with biomass grown in the absence of the CAH mixture: S-M-NC-2A (inoculated with methane-grown biomass and fed with methane), S-P-NC-2A (inoculated with propane-grown biomass and fed with propane) and S-MP-NC-2A (inoculated with both methane-grown and propane-grown biomass and fed with both substrates). Similarly, Fig. 11b shows the ratios of depletion rate to concentration relative to the three types of slurry microcosms set up with type-E aquifer material and inoculated at time 3 with biomass grown in the absence of the CAH mixture: S-M-NC-3E, S-P-NC-3E and S-MP-NC-3E. The CAH rates utilized to build Figure 5.11b, unlike those utilized for Figure 5.10 and for the right-hand part of Table 5.8, are long-term rates obtained when the microcosms were in a stationary condition in terms of CAH depletion rates.

It can be observed in the first place that the “single-substrate” consortia (methane-fed and propane-fed) are characterized by significantly different biodegradation capacities with respect to the various CAHs. In particular, while the methanotrophs, at both inoculation times and with both aquifer materials, were 14 times more effective on trans-DCE than the propanotrophs, the latter were significantly more effective than the methanotrophs on 1,1,2-TCA (with a 9-10 fold advantage), 1,1,2,2-TeCA (with a 2-fold advantage at time 2, when this compound was supplied at 0.15  $\mu\text{M}$ , and with the lack of TeCA biodegradation by the methanotrophs at time 3, when it was supplied at 4  $\mu\text{M}$ ) and – limitedly to the A-type microcosms – TCE (with a 5-fold advantage). As for VC, while it was not supplied in the microcosms of time 3, at time 2 it was characterized by the similar ratio of depletion rate to concentration in the methane-fed and in the propane-fed consortia, as can be evinced by comparing the last VC bars in Figure 5.8a and b.

The different but complementary degradation capacities of the two single-substrate consortia suggested the idea to test the characteristics of a double-substrate consortium. It can be observed in both Figure 5.11a and 5.11b that, for all the CAHs characterized by a different degradation ability of the two single-substrate consortia, the mixed propane/methane-fed consortium behaved like the best single-substrate consortium.

Interestingly, the  $k^*$  obtained, for each CAH and for each type of substrate, by inoculating A-type microcosms at time 2 (Figure 5.11a) and E-type microcosms at time 3 (Figure 5.11b), are approximately the same, with the exception of TCE and 1,1,2,2-TeCA for the methanotrophs (a probable consequence of the increase of concentration, as discussed for TeCA in section 5.3.4). This result indicates that neither the additional 150-day growth period of the inocula in the absence of the CAH mixture, nor the different type of aquifer material (and consequently of indigenous biomass) contained in the inoculated microcosms led – in particular for the propane-fed and methane/propane-fed consortia – to any significant difference in the type of consortium obtained as a result of the interaction between the inoculum and the microcosms’ indigenous biomass. Besides, considering that the A-type microcosms were spiked with CAH initial concentrations in the 0.15-3.4  $\mu\text{M}$  range (excluding VC), while the E-type

tests contained each CAH at 4  $\mu\text{M}$ , the similarity of the  $k^*$  represents a confirmation of the validity of the utilization of the pseudo first-order constant  $k^*$  as an index of CAH degradation capacity of the different consortia.

In order to further investigate the effect of the type of growth substrate on the CAH degradation capacity, in microcosms S-M-NC-3E, S-P-NC-3E, S-MP-NC-2A<sub>1,2</sub> and S-MP-NC-3E<sub>1,2</sub>, after 7 pulses of biodegradation of the CAH mixture we operated a change of the type of substrate supplied: in particular, in the first two microcosms we inverted the growth substrate, feeding S-M-NC-3E with propane (46  $\mu\text{M}$ ) and S-P-NC-3E with methane (125  $\mu\text{M}$ ), whereas in each double-substrate test we started to feed one duplicate (S-MP-NC-2A<sub>1</sub> and S-MP-NC-3E<sub>1</sub>) with only methane (125  $\mu\text{M}$ ), and the other (S-MP-NC-2A<sub>2</sub> and S-MP-NC-3E<sub>2</sub>) with only propane (46  $\mu\text{M}$ ). In each of these microcosm, after one week of feed with the new substrate in the absence of CAHs, we monitored the biodegradation of one further pulse of the 5- or 6-CAH mixture (supplied at the same concentration of each compound as in the previous pulses). In the double-substrate microcosms (S-MP-NC-2A and S-MP-NC-3E), the supply of only one substrate did not lead - limitedly to the single CAH pulse monitored - to any significant change of the CAH depletion rates (data not shown). Conversely, in microcosms S-M-NC-3E and S-P-NC-3E the inversion of substrate led each consortium to attain CAH depletion rates analogous to those observed in the corresponding double-substrate microcosm S-MP-NC-3E: in other words, the supply of methane in S-P-NC-3E resulted in a marked increase of the trans-DCE rate and in the maintenance of the depletion rates relative to the other CAHs, whereas the supply of propane in S-M-NC-3E led to a marked increase of the 1,1,2-TCA rate, to the onset of 1,1,2,2-TeCA biodegradation and, as in S-P-NC-3E, to no significant loss of the degradation capacity relative to the other CAHs (data not shown). While the rapid development of new degradation capacities as a result of the inversion of substrate represents an interesting result, the maintenance of the degradation abilities characteristic of the previous substrate cannot be considered a definitive result: in fact, because it was observed only relatively to one CAH pulse, it might be a residual of the CAH-transformation capacities acquired during the period of feed with the initial substrate.

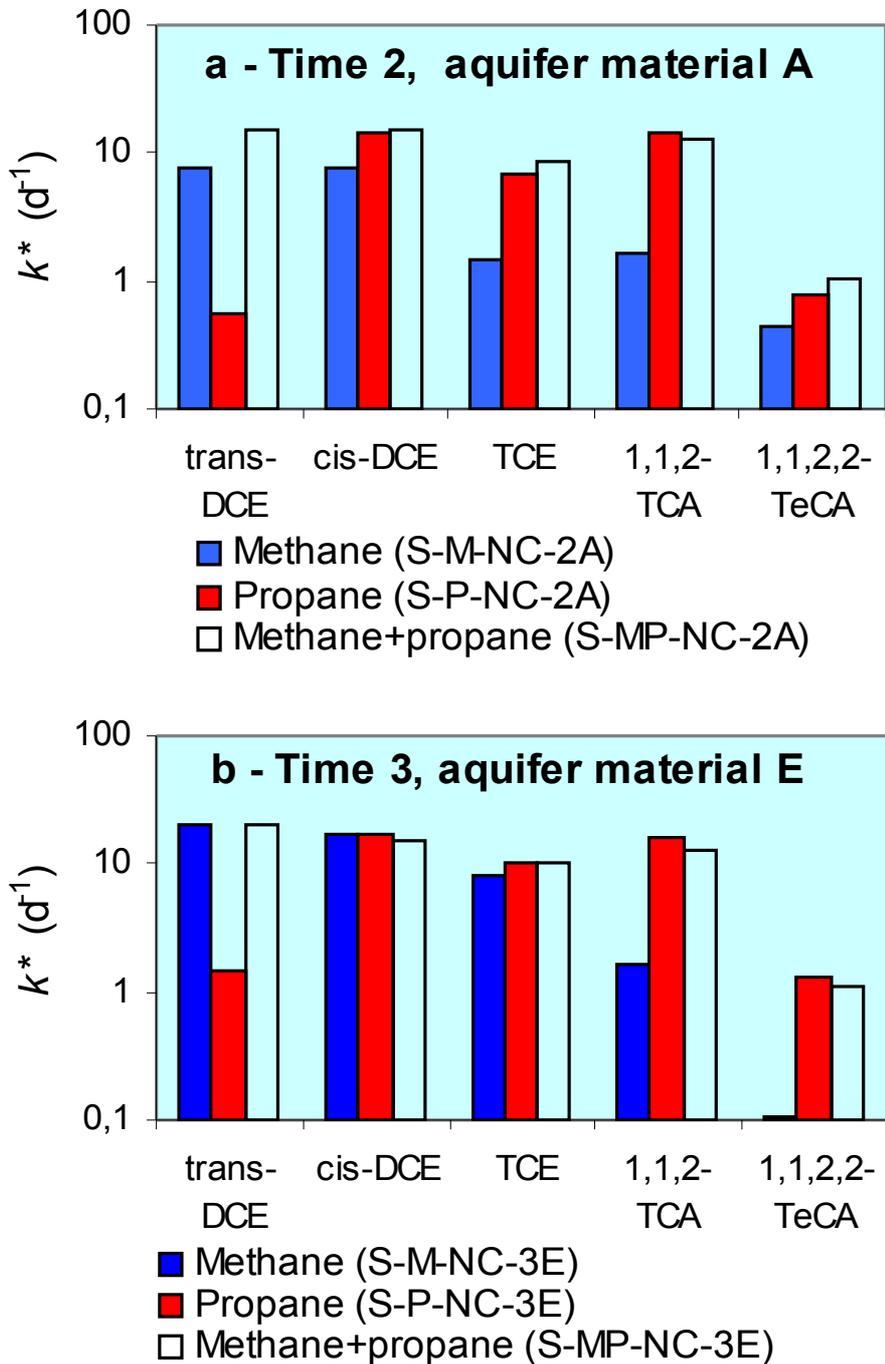


Figure 5.11. Depletion rate to concentration ratios ( $k^*$ ) for the biodegradation of the 5-CAH mixture by biomasses grown on methane, propane and methane + propane in microcosms set up at time 2 with aquifer material A (a) and at time 3 with aquifer material E (b), and bioaugmented with biomass sampled from growth reactors GB-M-NC and GB-P-NC (microbial growth in the absence of CAHs).

### **5.3.6 Results relative to the liquid-phase microcosms**

Fig. 5.12 shows the ratios of specific CAH rate to concentration ( $k_{sp}^*$ ) relative to the methane-, propane- and methane/propane-fed liquid-phase microcosms, inoculated with biomass suspension sampled from the corresponding E-type microcosms after 90 days of CAH biodegradation and spiked with the 5-CAH mixture at 4  $\mu$ M for each compound. The results obtained in the liquid-phase tests are highly similar to those of the E-type microcosms. In particular, with regard to each compound, the mixed propane/methane-fed consortium (L-MP-4) behaved like the best single-substrate consortium. Besides, for each CAH, the ratio of the  $k_{sp}^*$  of propane-fed L-P-4 to the corresponding  $k_{sp}^*$  of methane-fed L-M-4 is about equal to the same ratio calculated for slurry microcosms S-P-NC-3E and S-M-NC-3E, as well as for microcosms S-P-NC-2A and S-M-NC-2A, in terms of non-specific depletion rates: 0.07–0.11 for trans-DCE, about 1 for cis-DCE and TCE, 9–10 for 1,1,2-TCA, infinite for 1,1,2,2-TeCA (except the A-type slurries, possibly due to the lower TeCA concentration). This result suggests that the different degradation abilities shown in slurry tests – for any given CAH – by the methane-utilizers in comparison with the propane-utilizers are not the mere result of the attainment of different concentrations of active biomass in the two types of microcosms. On the contrary, they reflect actual differences in the specific CAH transformation capacities of the two consortia.

In the liquid-phase microcosms, the utilization of a Cl-free mineral medium allowed a precise evaluation of the increase in Cl concentration as a result of CAH dechlorination. The resulting ratios of the Cl<sup>-</sup> moles actually produced to the Cl<sup>-</sup> moles corresponding to the complete dechlorination of the total amount of CAHs depleted, evaluated in correspondence of the degradation of 6 CAH pulses, are equal to 0.88 for L-MP-4, 0.92 for L-P-4 and 0.90 for L-M-4. The missing 10% of Cl<sup>-</sup> moles indicates that further research is needed to evaluate the type of degradation products as a result of the aerobic cometabolism of CAHs.

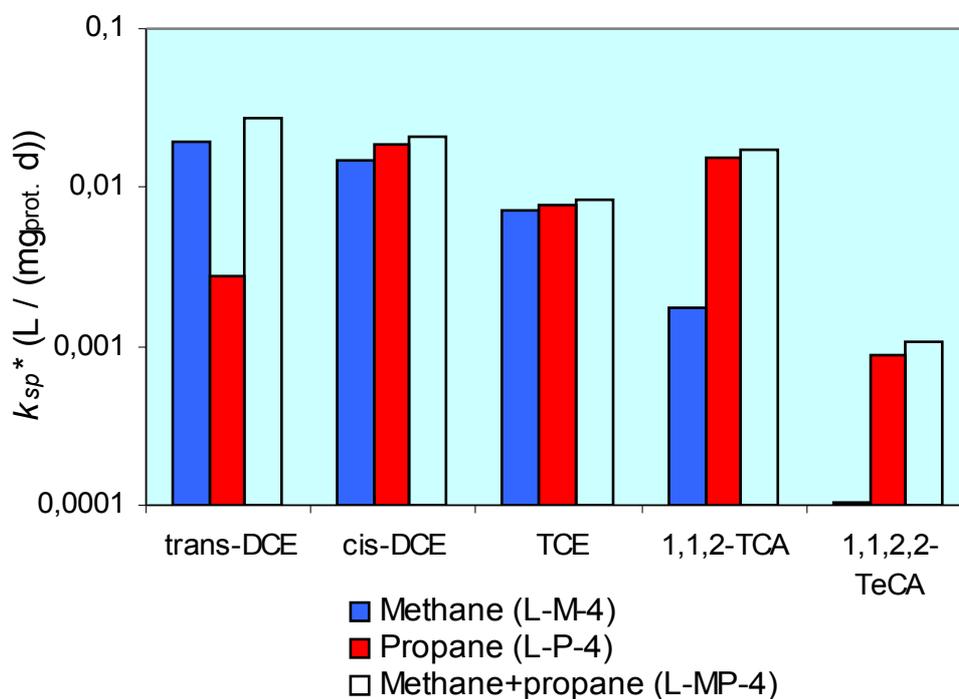


Figure 5.12. Ratios of specific depletion rate ( $k_{sp}^*$ ) for the biodegradation of the 5-CAH mixture by biomasses grown on methane, propane and methane + propane in the liquid-phase microcosms,

#### 5.4 DISCUSSION AND CONCLUSIONS

In this work we studied the long-term growth process of two CAH-degrading microbial consortia under different experimental conditions, and we investigate the effectiveness of these consortia as inocula for the operation of bioaugmentation treatments in different types of aquifers.

Our results show in the first place that methane and propane are effective growth substrates for the aerobic cometabolism of CAH mixtures. In particular, the propane-grown biomass proved able to degrade VC, trans- and cis-DCE, TCE, 1,1,2-TCA and 1,1,2,2-TeCA at all the concentrations tested, whereas the methanotrophs failed to deplete 1,1,2,2-TeCA when its concentration was raised from 0.15 to 4  $\mu\text{M}$ .

We consider the long-term biodegradation of 1,1,2,2-TeCA up to 4  $\mu\text{M}$  by the propane-utilizing biomasses a result of particular significance. In fact, this high-chlorinated compound has been generally considered in the literature as non-

biodegradable by means of aerobic processes (U.S. E.P.A., 2000; Chen *et al.*, 1996). Its aerobic cometabolic biodegradation by methane-oxidizing cultures was evidenced for the first time by Chang and Alvarez-Cohen (1996), whereas Frascari *et al.* (2006) had documented in a previous study its long-term biodegradation by methane-fed and propane-fed biomasses in the 0-0.65  $\mu\text{M}$  range.

This study also evidenced that, in the case of a large-scale bioremediation with bioaugmentation, the production of large amounts of biomass starting from the two inocula object of the investigation can be operated in the absence of the CAH mixture, with a significant simplification of the production plant and a reduction of the fixed and operational costs. A possible explanation of this experimental result is the fact that the inocula initially supplied to the growth bioreactors had previously been subjected to a prolonged period of CAH biodegradation in the slurry microcosms operated within the previous study (410 days for the methanotrophs, 310 days for the propanotrophs), during which they had been strongly stabilized by the CAH selective pressure.

The CAH lag-times and depletion rates obtained in the non-bioaugmented microcosms set up with different types of aquifer materials indicate that the indigenous biomasses of different sites can have significantly diverse capacities to grow on the primary substrate supplied and to start degrading the CAH mixture: in fact, out of five aquifer materials tested, two resulted in lag-phases of over three months for the onset of substrate utilization (both with methane and with propane), whereas in the remaining three the overall lag-times (substrate + CAH mixture) were equal to two weeks at the most. Conversely, the introduction of either inoculum led in all the five types of aquifers to very short lag-times (< 4 days) for the onset of CAH degradation. This result indicates, in agreement with the findings of the previous study (Frascari *et al.*, 2006), that bioaugmentation can play a crucial role in the successful bioremediation of CAH-contaminated sites and that, consequently, further research on the production and the stability of CAH-degrading inocula and on their interaction with the indigenous biomasses of different aquifers is needed.

Interestingly, in the inoculated microcosms, while the lag-times were almost independent of the type of aquifer material utilized, the depletion rates obtained for each CAH with the different aquifer materials differed in some cases by one order of magnitude, indicating that the chemical, physical and biological characteristics of the bioaugmented site play a significant role in the long-term CAH depletion rates achieved. Besides, the short lag-times and the high CAH depletion rates obtained in the inoculated E-type microcosms show that bioaugmentation can be successful even in sites whose indigenous biomass has not been exposed to the selective pressure due to a previous CAH contamination and is not capable to grow on any of the primary substrates supplied.

Lastly, in the microcosms supplied with both methane and propane we obtained a microbial consortium combining the degradation capacities of the two single-substrate consortia. This result suggests that the double-substrate approach – a novel technique not previously reported to the best of our knowledge in any study of cometabolic biodegradation – can find useful applications for the degradation of complex CAH mixtures.

**6. BIODEGRADATION OF CHLOROBENZENE: STUDY OF THE CATABOLIC POTENTIAL AND THE STRUCTURE OF THE MICROBIAL COMMUNITY IN THE INTERFACE BETWEEN GROUNDWATER AND SURFACE WATER**

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**ABSTRACT**

The catabolic potential and the structure of the microbial community present in the interface between groundwater and surface water were studied. The main goal of this study was to find out whether bacteria present in the interface are involved in pollutants degradation. Therefore batch degradation tests and molecular analyses (PCR-DGGE analysis of 16S rRNA gene, catabolic genes, *dsrA* gene) were carried out on aquifer material extracted at different depths in the interface in three locations characterized by different monochlorobenzene contamination levels. Batch tests were performed under oxygen-limited conditions in order to study chlorobenzene degradation under the *in situ* conditions. The position in the interface did not have any effect on the process and biodegradation was exclusively limited by a lack of oxygen. Up to 50 mg/l of monochlorobenzene were consumed in 20 days in both aquifers, and also in groundwater and surface water, when sufficient oxygen was available (1.5–2 mg/l). 16S rRNA PCR-DGGE analysis were carried out on undisturbed sediment cores extracted from the three studied locations in different seasons. Results indicated that the structure of the microbial community changed in function of depth. Moreover the structure of the community appeared different in the three locations while significant similarities were observed in samples extracted in each location in different seasons. Cloning and sequencing allowed to identify the dominant bands in the DGGE pattern as belonging to the group of *Proteobacteria*. It is still unclear if bacteria corresponding to these bands play a role in

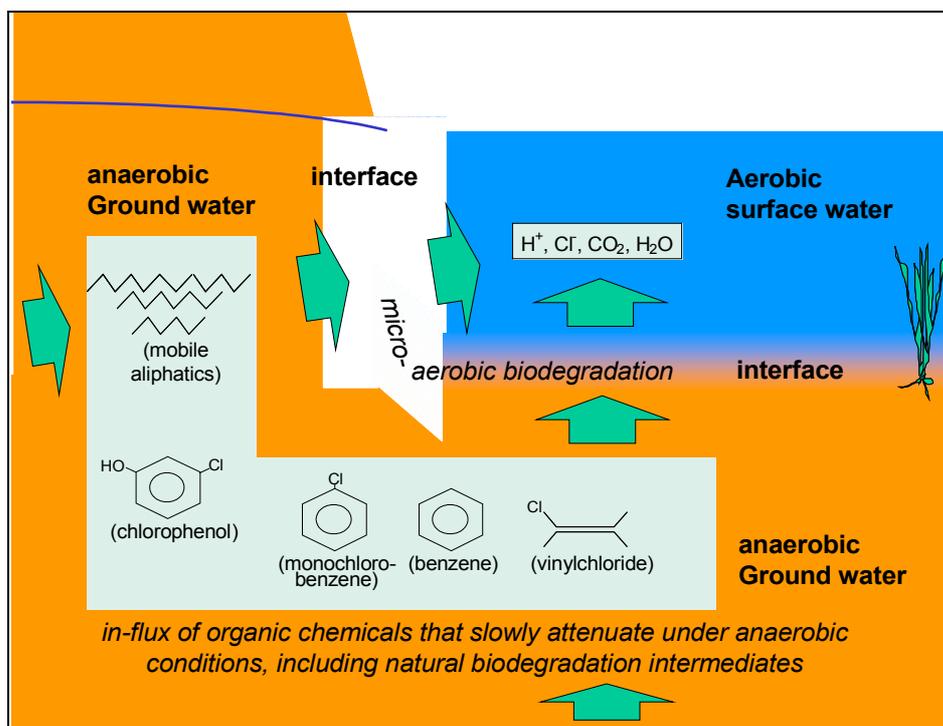
chlorobenzene degradation. The only degradative gene detected until now is the mono-oxygenase *tmoA*. This gene is involved in the degradation of BTEX, which are structurally similar to monochlorobenzene, thus being probably involved in its degradation. This hypothesis seemed to be confirmed by the observation that, after 200 days of incubation, some bands became more visible in *tmoA*-DGGE analyses of samples taken from the batch degradation tests.

## **6.1 INTRODUCTION**

The widespread use of chlorobenzenes during the last decades led to their common occurrence in the environment. Chlorobenzenes are of great concern because of their toxicity, persistence and accumulation in the food chain (Aelion *et al.*, 1987). Monochlorobenzene has been identified as priority pollutant by the U.S. Environmental Protection. Chlorinated aromatic compounds are non-degradable or slowly degradable by microorganism (van der Meer, 1997), thus being considered among the most problematic categories of environmental pollutants. Nevertheless, bacteria that are able to use these compounds as sole source of carbon and energy have been isolated from polluted environments (Schraa *et al.*, 1986; Spain and Nishino, 1987; van der Meer *et al.*, 1987; Haigler *et al.*, 1988; Sander *et al.*, 1991; Spiess *et al.*, 1995). Chlorobenzenes are readily mineralized under appropriate conditions in the laboratory by bacteria isolated from soil and water (de Bont *et al.*, 1986; Haigler *et al.*, 1988; Reineke *et al.*, 1984; Schraa *et al.*, 1986; Spain and Nishino, 1987). Field studies on contaminated sites have shown that river sediments exposed to chlorobenzenes degraded them faster than sediments from unpolluted sites (Aelion *et al.*, 1987). Furthermore, chlorinated benzenes are chemically stable in nature, their photochemical degradation does not play an important role in soil and aquatic environment. Biological degradation could therefore be considered a feasible process to eliminate these compounds.

Polluted groundwater in urban and industrial areas often represents a continuous source of (diffuse) contamination of surface waters. However there are

strong indications that the interface between groundwater and surface water plays an important role in the natural degradation of organic contaminants. This is especially the case for mobile contaminants (such as monochlorobenzene) that are persistent in anaerobic subsurface environment, but relatively easily mineralised under more oxidized environmental conditions (Figure 6.1).



**Figure 6.1. Natural attenuation at the reactive interface between groundwater and surface water**

The interface is a dynamic ecotone where active exchanges of water and dissolved material between the stream and groundwater in many porous sand- and gravel-bed rivers occur (Karaman, 1935; Orghidan, 1959; Sabater and Vila, 1991). It contains a unique invertebrate fauna (Williams and Hynes, 1974) next to many forms of fungi and microbes that transfer, release, and stabilise different forms of transient nutrients (Hendricks, 1993). Interfaces are important storage zones for organic carbon (Bretschko and Moser, 1993) and are generally characterised by sharp physical and chemical gradients (Fraser and Williams, 1998), thus enabling a broad spectrum of metabolic processes to occur within small spatial scales. As a consequence the interfaces are often hot spots in productivity and diversity of organisms (Pusch *et al.*, 1998) and may substantially

contribute to the carbon, nutrient and energy flow through the river system (Naegeli and Uehlinger, 1997).

Although nitrogen (Valett *et al.*, 1997) and dissolved organic carbon biogeochemistry (Vervier and Naiman, 1992) have received much attention in relation to streambed hydrologic retention and surface/subsurface exchange, not much is known about the effect of the interface on the degradation of pollutants. Lendvay and Adriaens (Lendvay and Adriaens, 1999) observed, using multilevel arrays, that concentrations of methane and chloroethene decreased as groundwater became increasingly oxidised along the groundwater-surface water interface in sample points impacted by infiltration of oxygenated surface water. Schwarzenbach *et al.* (1983) observed the enhanced removal of alkylated and chlorinated benzenes in the interface. Since under the conditions typical for the groundwater environment these aromatic compounds do not undergo chemical reactions at significant rates and since these compounds are also weakly sorbed, these authors presumed that any elimination must be attributed to biological transformation and/or mineralisation. Fuller and Harvey (Fuller and Harvey, 2000) observed an enhanced metal uptake in the interface by the analysis of dissolved-metal streambed profiles and conservative solute tracers.

Although previous studies (Schwarzenbach *et al.*, 1983; Lendvay and Adriaens, 1999) presumed that the decrease in the concentration of pollutants was due to the activity of the microbial community present in the interface, abiotic processes (such as dispersion, adsorption, convection) may as well have been responsible for this decrease, which is in fact only a dilution of the pollutant, not resulting in a reduced risk for humanity and ecosystems.

This study aims at investigating the presumed involvement of bacteria present in the interface in monochlorobenzene degradation in order to understand if interface can act as a biobarrier towards the infiltrating contaminants. If such condition really exists in the field and residence times of the polluted groundwater in the transitional zones are sufficient, the “naturally occurring biobarrier” could provide a valuable guarantee that pollution is dealt with adequately, thus not requiring “active” and expensive remediation technology (such as pump and threat or air-sparging).

To investigate these issues we therefore carried out batch degradation tests using sediment material extracted at two different depths in the interface, from three sampling locations characterized by different level of contamination. Tests were operated under oxygen-limited conditions similar to those observed *in situ*. Furthermore we used different molecular techniques (Polymerase chain reaction-Denaturing Gradient Gel Electrophoresis (PCR-DGGE)) to study the structure of the interface microbial community in three different locations throughout the year (analysis of seasonal data). We chose three sampling locations with different levels of contamination to investigate the effect of the presence/absence of the contaminant on the microbial community, and we analyzed samples extracted in different times of the year to evaluate the effect of seasonal variations. Due to the structural similarity between chlorobenzene and BTEX compounds we chose to investigate if BTEX catabolic genes were present in our samples. In addition we studied the diversity of sulfate-reducing bacteria in the same samples. Cloning and sequencing of the dominant DGGE-bands eventually enabled us to identify the dominant species in the microbial community.

## **6.2 MATERIALS AND METHODS**

### **6.2.1 TEST SITE AND SAMPLING**

We used three sampling places located in an industrial site in the Port of Amsterdam (The Netherlands), mainly polluted with monochlorobenzene at different contamination levels (Figure 6.2). The first one is a ditch constructed by TNO (Toegepast Natuurwetenschappelijk Onderzoek, The Netherlands) and characterized by high monochlorobenzene concentration in groundwater (5–10 mg/l), the second one (Leendertgracht) is a canal representing a natural interface situation with lower monochlorobenzene concentration in groundwater (0,3 mg/l), the third one (Vijver) is a pond chosen as negative control, due to the lack of monochlorobenzene in the sediment of the interface and in surface water.

(a)



(b)



(c)



**Figure 6.2.** The three sampling places in the industrial site in the Port of Amsterdam (The Netherlands); (a), Artificial ditch; (b), Leendergracht; (c), Vijver.

Groundwater was sampled as close as possible to the interface sampling point. Three different boreholes were used: Q-32 (artificial ditch), A-31/1 (Leendertgracht) and A-18/2 (Vijver). Due to the lack of a closer one we chose borehole A-18/2 for the Vijver; nevertheless this is one of the wells beside the canal.

Sediment and water samples were used for molecular tests and batch degradation tests; physico-chemical parameters (pH, redox potential, dissolved oxygen concentration, conductivity, temperature) were also measured and water was analysed to determine the concentration of chlorobenzenes, benzene, chlorides, electron acceptors and metals.

Samplings were performed on 24/11/2004, 22/03/2005, 31/05/2005, 31/08/2005, 20/04/2006, 22/11/2006 in order to have sediment samples representative for each season in the year and to study the microbial community seasonal variations. Table 6.1 shows the tests carried out for each sampling.

**Table 6.1. Tests carried out on sediment cores**

Date	Sampling point		
	Artificial ditch	Leendertgracht	Vijver
24/11/2004 (Autumn)	Molecular tests	-	-
22/03/2005 (Winter)	Molecular tests + batch degradation tests	Molecular tests + batch degradation tests	Molecular tests + batch degradation tests
31/05/2005 (Spring)	Molecular tests	Molecular tests	Molecular tests
31/08/2005 (Summer)	Molecular tests	Molecular tests	Molecular tests
20/04/2006 (Spring)	Batch degradation tests (only water)	Molecular tests + batch degradation tests	Molecular tests + batch degradation tests
22/11/2006	-	-	-

### **6.2.2 Retrieval of the samples**

Undisturbed sediment samples were obtained by hammering a 4 cm diameter plastic tube into the interface (Figure 6.3). Fresh aquifer material obtained from these cores was used in batch degradation tests: they were cut into two parts (top and bottom) which were homogenized and used in the setup of the microcosms. Some of the cores were frozen on dry ice immediately after sampling in the field in order to be used for molecular analyses. In the laboratory, these undisturbed frozen samples were cut into slices of approximately 1 cm by using an electrical saw. Great care was taken in the sterilisation of the saw between each different cut using ethanol. From each core all the slices from the first 7–10 cm were selected and those from 10 cm downwards were analysed every 4–5 cm. From each selected slice sub-samples were used for molecular tests (Figures 6.4 and 6.5) and for the analysis of chlorobenzenes concentration (extraction with methanol followed by GC-MS analysis). Groundwater was withdrawn by means of a peristaltic pump (Eijkelkamp, Agrisearch Equipment BV, Giesbeek, The Netherlands); dissolved oxygen, pH, redox, conductivity and temperature were measured on site using a multimeter (WTW Multiline P4, Weilheim, Germany) in a flow through cell (Eijkelkamp Agrisearch Equipment BV, Giesbeek, The Netherlands) (Figure 6.6). The groundwater level was monitored by means of an Interface Meter (Eijkelkamp Agrisearch Equipment BV, Giesbeek, The Netherlands). Groundwater and surface water were collected in 2,5 l bottles and in 40 ml tubes containing 2 g of ascorbic acid. Chlorobenzenes and benzene concentrations were measured in the laboratory by GC-MS headspace analysis by pouring 5 g of water from the 40 ml tubes in 10 ml vials and adding 100  $\mu$ l of 85% *ortho*-phosphoric acid, as explained in paragraph 6.2.5. In the laboratory, the pellets of a 150 ml sample obtained after centrifugation during 20 min at 7500 rpm were re-suspended in 2 ml of the same water and used in the molecular analyses described in paragraph 6.2.6.



**Figure 6.3. Extraction of an undisturbed sediment sample**

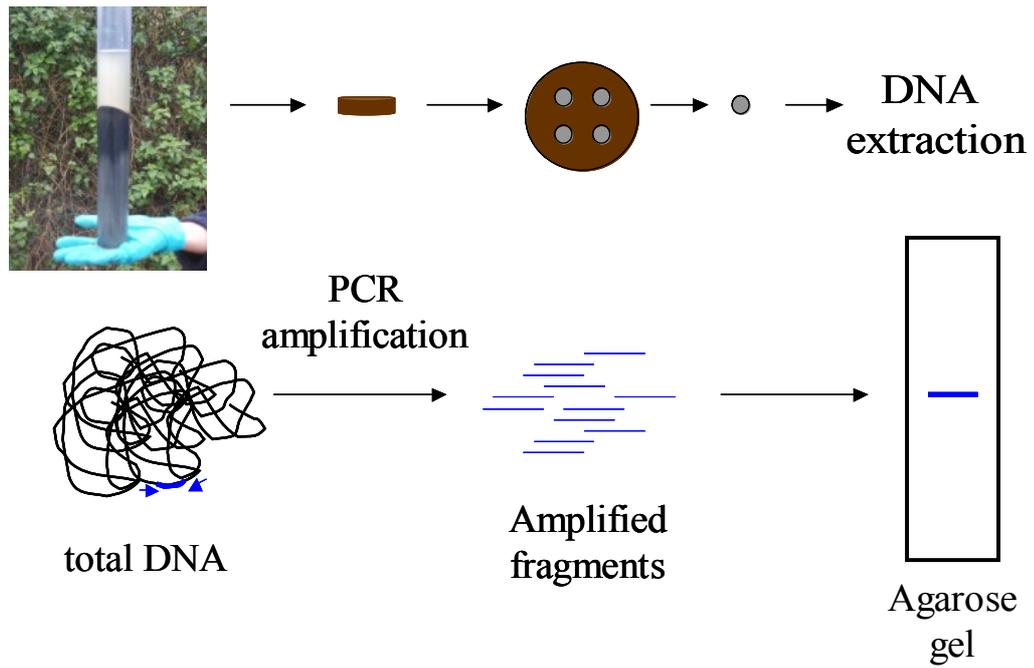


Figure 6.4. Presentation of the strategy followed to study the presence of catabolic genes, 16S rRNA gene and *dsrB* gene at different depths in the interface.



Figure 6.5. Extraction of sub-samples for PCR-DGGE analyses from frozen sediment slices.

(a)



(b)



(c)



(d)



**Figure 6.6.** Sampling of surface water (a) and groundwater (b) and measure of pH, temperature, redox potential, dissolved oxygen and conductivity in groundwater (c) and surface water (d).

### **6.2.3 Batch degradation tests of monochlorobenzene**

Two groups of microcosms were set up using aquifer material and water sampled on 22/03/2005 and on 20/04/2006. Tests were performed in 160 ml glass bottles. 37,5 g of aquifer material were suspended in 70 ml of surface water or groundwater (Figure 6.7). Both kinds of aquifer materials (top and bottom, obtained as explained above) were used in order to evaluate the effect of the position in the interface on the monochlorobenzene degradation potential. Each type of sediment tests were performed in two different conditions using surface water or groundwater. Degradation by the community present in groundwater and in the surface water was investigated in bottles containing just water (85 ml) and no aquifer material. In the first group of tests (22/03/2005) microcosms flasks were filled in addition with filter-sterile or non-filter-sterile groundwater to study monochlorobenzene degradation by bacteria present in the aquifer only. In order to consider the a-biotic removal of monochlorobenzene control tests were set up by adding 35 ml of groundwater and 35 ml of surface water to 19 g of each kind of sediment (top and bottom) and by poisoning bacteria with 800  $\mu$ l of formaldehyde. Monochlorobenzene was added to the microcosms where a concentration lower than 1 mg/l was detected, in order to reach a final concentration of 3 to 4 mg/l (the *in situ* monochlorobenzene concentration) and the degradation process was followed in function of time by GC-analysis of the headspace. Microcosms were set up using materials sampled from each of the studied locations and incubated statically at room temperature; all tests were performed in duplicate. Details of the setup are showed in Table 6.2.

Microcosm tests were carried out under anaerobic conditions in an anaerobic glove box thus avoiding oxygen concentration raising in water and sediment and starting the test at the same conditions present in the field.

Flasks of the first group of microcosms were incubated for 215 days. Since monochlorobenzene concentration was constant in all the bottles (except the ones containing only water) oxygen was added to try to stimulate biodegradation and the process was monitored for 200 further days. The second group of microcosms

(20/04/2006) was set up to study the degradation process more in detail (specifically regarding the effect of oxygen concentration). No tests were performed with sediment from the Artificial Ditch, since on 20/04/06 it was impossible to take samples from the interface; from this location tests were carried out using only surface water or groundwater. Monochlorobenzene and oxygen concentrations were monitored during the whole incubation period and, since the former remained stable for more than 30 days, small amounts of oxygen were added to one of the two duplicate flasks in order to reach the level of 2,5–3 mg/l, whereas the second duplicate was used as negative control.

Each time monochlorobenzene was completely consumed, microcosms were re-spiked at the initial concentration. Concentration was then gradually increased up to 50 mg/l to test the microbial community capacity to degrade high contaminant concentration. At the end of the experiment DNA extraction followed by PCR–DGGE analysis was performed on sediment material present in the bottles and on groundwater/surface water (for tests containing only water), as explained below. The results obtained were compared with the ones from PCR – DGGE analysis of sediment and water used to set up the microcosms (Time 0) in order to evaluate the differences in catabolic genes and changes in the microbial community, due to the exposure and degradation of monochlorobenzene during the 200 days.

**Table 6.2. Setup of the batch degradation tests**

<b>Aquifer</b>	<b>Water</b>
-	Surface water
-	Groundwater
Top	Surface water
	Groundwater
Bottom	Surface water
	Groundwater
Top	Filter-sterile groundwater
Bottom	(only in 23/03/2005 tests)
Top + bottom	Groundwater + surface water + formaldehyde



**Figure 6.7. Batch degradation test**

#### **6.2.4 GC-analysis of monochlorobenzene concentration in the microcosms**

Monochlorobenzene concentrations were measured by headspace analysis on a CP 3800 Varian gas chromatograph connected with a flame ionization detector (FID) (type 1079 at a temperature of 250°C) equipped with a Rtx-502,2:30 m x 0,53 mm x 3µm and a DB-1:30 m x 0,53 x 5 µm column. Split injection was implemented at an inlet temperature of 250 °C. Helium was used as carrier gas at a constant flow rate of 11,9 ml/min. Analyses were carried out using the following temperature gradient: 2 min at 50°C, ramp to 155°C at 10°C/min, ramp to 190°C at 20°C/min, 3 min at 190°C. An external standard calibration curve (one point) was used to calculate the concentrations of the analytes.

#### **6.2.5 Analysis of dissolved oxygen concentration in the microcosms**

Dissolved oxygen was monitored by injecting a 300 µL sample in an oxygen meter (Strath Kelvin Instruments, Glasgow, Scotland) and waiting 2 min for the stabilization of the instrument (Figure 6.8).



**Figure 6.8.** Analysis of dissolved oxygen concentration

### **6.2.6 Methanol extraction of VOCs and GC-MS analyses**

2,5 g of sediment were suspended in 2,5 g of methanol in 10 ml vials; internal standard (D4-1,2-dichloroethane, D8-toluene, D6-benzene, D4-1,2-dichlorobenzene) was added to the vials before putting them into an ultrasonic bath for 30 min. Methanol containing the extracted VOCs was then diluted by putting 0,5 g of supernatant in 4,5 g water in new 10 ml vials. 100  $\mu$ l of 85 % *ortho*-phosphoric acid were also added to kill biomass.

Chlorobenzenes and benzene concentration was determined by GC-MS headspace-analysis using a Thermo GC-MS equipped with a DB-5ms 60 m x 0,25 mm x 0,25  $\mu$ m column. The samples were injected into a split/splitless injector at 220°C and put on to the column with a constant flow of 9 ml/min Helium. The GC-oven program works as follows: 3 min at 38°C, ramp to 175°C at 5°C/min. The analytes are then detected by the MS-detector. The concentration of each analyte was calculated through internal standard calibration curve (including eight different concentrations).

### **6.2.7 DNA extraction from sediment and water samples**

Total genomic DNA from soil and water samples was extracted and purified as previously described by Hendrickx *et al.* (2006). Two g of sediment or the pellet of 2 ml watersample was suspended in 4 ml Tris-glycerol buffer (10 mM Tris, 15 % glycerol, pH = 7). The cells were mechanically lysed by beating with glass beads (diameter: 0,10 – 0,11 mm) for 2 x 30 sec in a MK4 bead beater apparatus (Braun Biotech International GmbH, Melsugen, Germany). Before lysis with proteinase K (32  $\mu$ l, 20 mg/ml) and 20 % sodium dodecyl sulfate (120  $\mu$ l) during 30 min at 50°C, cells were subjected to an enzymatic lysing step with lysozyme (160  $\mu$ l, 50 mg/mL) in Tris-glycerol buffer (30 min at 37°C). This was followed by an addition of 2 ml of NaKPO<sub>4</sub> buffer (conc.: 1,12 M, pH = 8) and a second bead-beating step for 2 x 30 sec. Glass beads, soil and cell debris were removed by centrifugation (10 min at 7000 rpm) and the DNA contained in the aqueous phase was extracted twice with 5 ml phenol/chloroform/isoamylalcohol (25:24:1), and purified with 5 ml chloroform/isoamylalcohol (24:1). 0,1 g of polyvinylpyrrolidone (Sigma-Aldrich, Germany) were added to the DNA solution

and vials were rotated on a rotating shaker for 30 min. Polyvinylpyrrolidone was eliminated by centrifugation (10 min at 6000 rpm) and the DNA was precipitated with 2 volumes of 100% ethanol (Merck KGaA, Darmstadt, Germany) at  $-20^{\circ}\text{C}$  overnight. The crude DNA pellet was suspended in 500  $\mu\text{L}$  of sterile water and purified over a Wizard column (Wizard DNA Clean-Up System, Promega Corporation, Madison, USA). The purified DNA was recovered in 50  $\mu\text{L}$  of TE buffer (10 mM Tris, 50 mM EDTA, pH 9) and stored at  $-20^{\circ}\text{C}$

### **6.2.8 PCR amplification**

Polymerase chain reaction on the extracted DNA was performed in a volume of 50  $\mu\text{L}$ . A 495 bp eubacterial 16S rRNA gene fragment was amplified using the primer set GC-63F/518R, described by Marchesi *et al.* (1998). 1  $\mu\text{L}$  of 1:10 or 1:50 dilution of template DNA was added to 49  $\mu\text{L}$  of PCR mix consisting of 5  $\mu\text{L}$  of 10x *exTaq* reaction buffer (20 mM  $\text{MgCl}_2$ ), 0,25  $\mu\text{L}$  *exTaq* Polymerase ( $5\text{ U } \mu\text{L}^{-1}$ ), 4  $\mu\text{L}$  dNTP (deoxynucleoside triphosphate; 2,5 mM each), 0,25  $\mu\text{L}$  of both primers and 39,25  $\mu\text{L}$  sterile demineralised water. The *exTaq* Polymerase, dNTPs and PCR reaction buffer were purchased from TaKaRa (TaKaRa Shuzo Co., Biomedical Group, Japan). The PCR profile consisted of an initial denaturation of 5 min at  $94^{\circ}\text{C}$ , followed by 35 further denaturation cycles of 1 min at  $94^{\circ}\text{C}$ , annealing of 1 min at  $55^{\circ}\text{C}$ , and elongation for 1 min at  $65^{\circ}\text{C}$ . The last step included an extension for 5 min at  $65^{\circ}\text{C}$ .

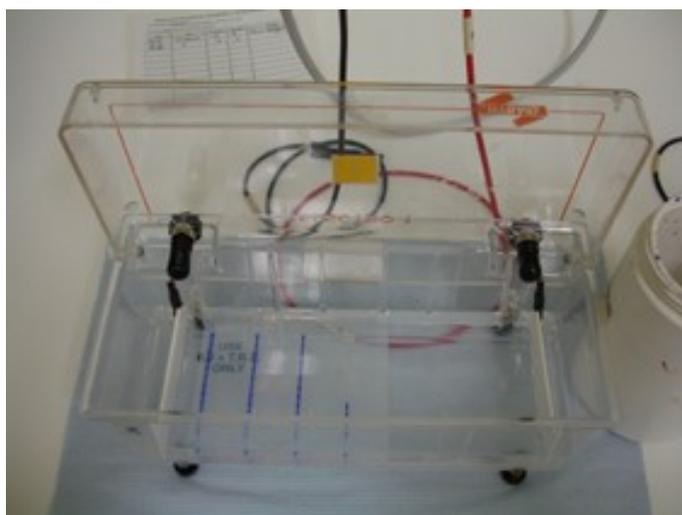
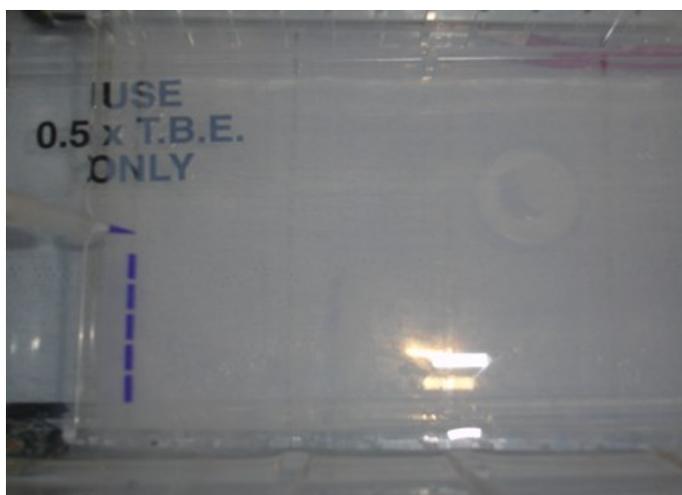
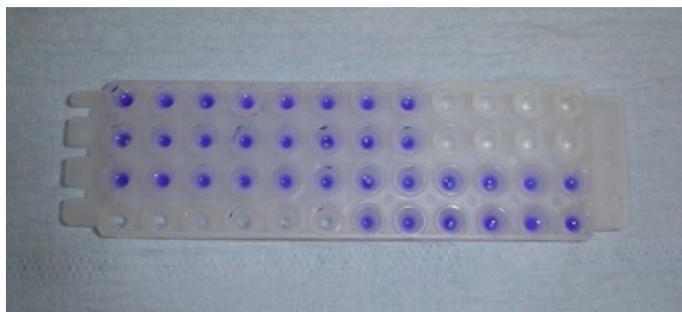
The primer sets for detection of the genotypes *tmoA*, *xylE*, *todC1*-like genes, *cdo*, *tbuE*, and *todE* are reported in Table 6.3 and were applied in PCR as described by Hendrickx *et al.*, (2006). To allow DGGE analysis of the amplicons, PCR products obtained with the catabolic primer set *tmoA*-F/*tmoA*-R were submitted to a semi nested PCR by using the same primer with a GC clamp at the forward primer (*tmoA*-F). These primer sets are used for the detection of catabolic genes involved in the aerobic degradation of BTEX compounds. Due to the homology between the structure of the BTEX compounds and the structure of monochlorobenzene the same primers were also used to detect genes involved in the degradation of the last one. Primer sets GC-P2060F/DSR4R, described by

J.Geets *et al.* (2006), amplifies a 350 bp fragment of the  $\beta$ -subunit dissimilatory sulfite reductase (*dsrB*) gene of sulfate-reducing bacteria. The PCR profile consisted of an initial denaturation of 5 min at 94°C, followed by 40 further denaturation cycles of 1 min at 94 °C, annealing of 1 min at 55°C, and elongation for 1 min at 72°C. The last step included an extension for 8 min at 72°C. PCR was performed on a Biometra thermocycler (Biometra, Göttingen, Germany). 10  $\mu$ L of the PCR products were analysed by agarose gel electrophoresis to evaluate their size and quality, (1,5 % agarose (Invitrogen, Paisley, Scotland, UK), 1 x EY running buffer (10 x EY-buffer: 0,4 M Tris, 0,02 M EDTA, on pH = 7,9 with acetate in H<sub>2</sub>O), 1 hour at 85 V). DNA bands were visualized by ethidium bromide staining (1 mg/l) (Figure 6.9).

**Table 6.3. PCR primer sets used in this study.**

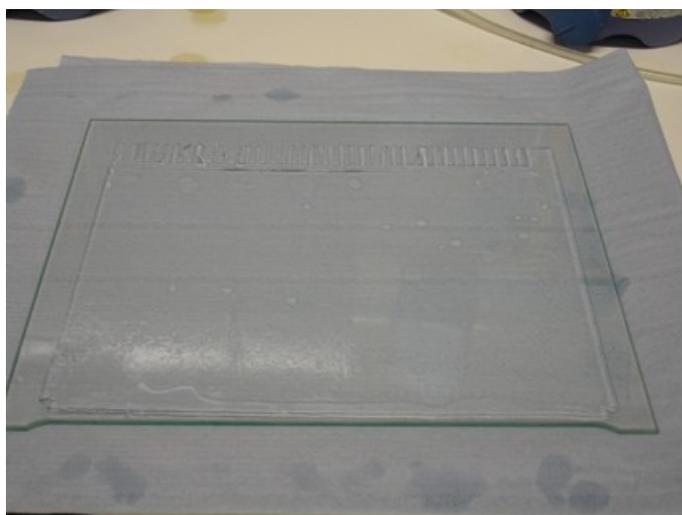
\*A 40 bp GC clamp (5'-CGCCCGCCGCGCGCGGGCGGGGCGGGGGCACGGGGG-3') was attached to the 5' end of forward primers TMOA-F, DSRp2060F and 63F (Muyzer et al., 1993)

Primer pair	Proteins targeted	Sequence	Amplicon size (bp)	PCR annealing temp (°C)	Reference
TBMD-F/TBMD-R	Subfamily 1 of $\alpha$ -subunits of hydroxylase component of multi-component mono-oxygenases	5'-GCCTGACCATGGATGC(C/G)TACTGG-3' 5'-CGCCAGAACCACTTGTC(A/G)(A/G)TCCA-3'	640	65.5	(Hendrickx <i>et al.</i> , 2006)
(GC-)TMOA-F/TMOA-R*	Subfamily 2 of $\alpha$ -subunits of hydroxylase component of multi-component mono-oxygenases	5'-CGAAACCGGCTT(C/T)ACCAA(C/T)ATG-3' 5'-ACCGGGATATTT(C/T)TCTTC(C/G)AGCCA-3'	505	61.2	(Hendrickx <i>et al.</i> , 2006a)
TOL-F/TOL-R	Subfamily 5 of hydroxylase component of two-component side chain mono-oxygenases	5'-TGAGGCTGAACTTTACGTAGA-3' 5'-CTCACCTGGAGTTGCGTAC-3'	475	55	(Baldwin <i>et al.</i> , 2003)
XYLA-F/XYLA-R	Electron transfer component of two-component side chain mono-oxygenases	5'-CCAGGTGGAATTTTCAGTGGTTGG-3' 5'-AATTAAC TCGAAGCGCCACCCCA-3'	291	64	(Hendrickx <i>et al.</i> , 2006)
TODC1-F/TODC1-R	Subfamilies D.1.B + D.1.C + D.2.A + D.2.B + D.2.C of $\alpha$ -subunits of Type D iron-sulfur multi-component aromatic dioxygenases	5'-CAGTGCCGCCA(C/T)CGTGG(C/T)ATG-3' 5'-GCCACTTCCATG(C/T)CC(A/G)CCCA-3'	510	66	(Hendrickx <i>et al.</i> , 2006)
XYLE1-F/XYLE1-R	Subfamily I.2.A of catechol extradiol dioxygenases	5'-CCGCCGACCTGATC(A/T)(C/G)CATG-3' 5'-TCAGGTCA(G/T)CACGGTCA(G/T)GA-3'	242	61.5	(Hendrickx <i>et al.</i> , 2006)
XYLE2-F/XYLE2-R	Subfamily I.2.B of catechol extradiol dioxygenases	5'-GTAATTCGCCCTGGCTA(C/T)GTICA-3' 5'-GGTGTTACCGTCATGAAGCG(C/G/T)TC-3'	906	64	(Hendrickx <i>et al.</i> , 2006)
CDO-F/CDO-R	<i>cdo</i> (U01826) of subfamily I.2.C of catechol extradiol dioxygenases	5'-CATGTCAACATGCGCGTAATG-3' 5'-CATGTCTGTGTTGAAGCCGTA-3'	255	58	(Hendrickx <i>et al.</i> , 2006)
TBUE-F/TBUE-R	<i>tbuE</i> (U20258) of subfamily I.2.C of catechol extradiol dioxygenases	5'-CTGGATCATGCCCTGTTGATG-3' 5'-CCACAGCTTGCTTCACTCCA-3'	444	60	(Hendrickx <i>et al.</i> , 2006)
TODE-F/TODE-R	<i>todE</i> (Y18245), <i>todE</i> (Y18245), <i>tobE</i> (AF180147) of subfamily I.3.B of catechol extradiol dioxygenases	5'-GGATTTCAAAC TGGAGACCAG-3' 5'-GCCATTAGCTTGCAGCATGAA-3'	246	58	(Hendrickx <i>et al.</i> , 2006)
(GC-)63F/518R*	Eubacterial 16S rRNA gene	5'-CAGGCCTAACACATGCAAGTC-3' 5'-TTACCGCGGCTGCTGG-3'	455	55	Marchesi <i>et al.</i> (1998)



**Figure 6.9. Loading of an agarose gel**  
**6.2.9 Denaturing gradient gel electrophoresis**

Bacterial diversity was examined by denaturing gradient gel electrophoresis. In DGGE analysis DNA fragments of the same length but with different base-pair sequences can be separated, thus obtaining a band pattern in a denaturing polyacrylamide gel in which each band theoretically corresponds to one type of bacterium. Eubacterial 16S rRNA gene PCR products obtained with the primer set GC-63F/518R were analysed in 8% polyacrylamide gels with a denaturing gradient of 35% to 65% urea-formamide (100% denaturant gels contain 7 M urea and 40% formamide). PCR products obtained with the GC-P2060F/DSR4R primer set were analysed in 8% polyacrylamide gels with a denaturing gradient (40% to 70%). In both cases, DGGE was performed at a constant voltage of 120 V for 15 h in 1 x TAE (Tris-acetate-EDTA) running buffer at 60°C. PCR products obtained with the GC-TMOA-F/TMOA-R primer set were analysed in 6% polyacrylamide gels with a denaturing gradient (40% to 70%) at a constant voltage of 110 V for 16 h 40 min in 1 x TAE running buffer at 60°C. In all cases electrophoresis was performed on an INGENY phorU-2 DGGE apparatus (INGENY International BV, Goes, The Netherlands). After electrophoresis, the gels were stained in a 1 x TAE buffer containing 1 x SYBR Gold nucleic acid stain (molecular Probes Europe BV, Leiden, The Netherlands) and photographed under UV light with a Pharmacia digital camera system with Liscap Image Capture 1.0, Pharmacia Biotech, UK). Photo files were processed and analysed with Bionumerics software (version 2.5, Applied Maths, Kortrijk, Belgium) (Figure 6.10).

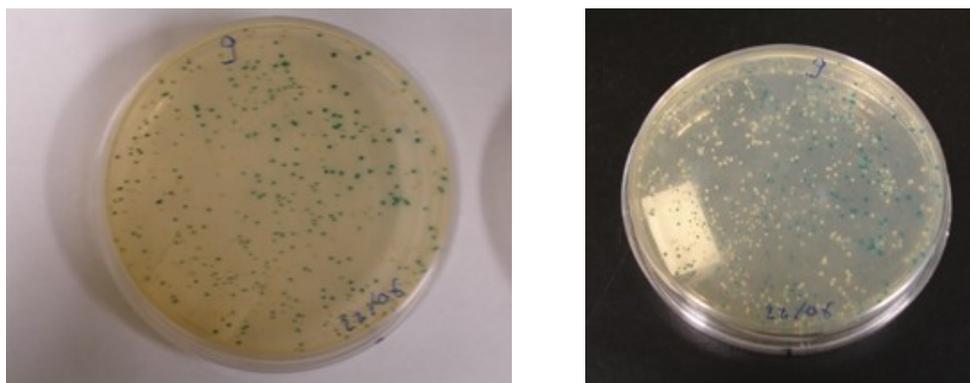


**Figure 6.10. DGGE (Denaturing Gradient Gel Electrophoresis). Electrophoresis, polyacrylamide gel and capture of the DGGE image.**

### **6.2.10 Cloning, sequencing and analysis of PCR amplified 16S rRNA, *tmoA* and *dsrB* gene**

PCR products obtained with the 16S rRNA gene primer set 63F/518R and with primer sets TMOA-F/TMOA-R and P2060F/DSR4R were cloned into plasmid vector pCR2.1-TOPO using the TOPO TA cloning kit with the TOP10 One Shot Electrocompetent cells (N.V. Invitrogen SA, Merelbeke, Belgium) as described in the kit's protocol. Clones containing recombinant vectors (blue colonies, Figure 6.11) and forming white colonies on selective agar medium (LB plates containing 50 – 100 µg/ml ampicillin and 40 mg/ml X-gal 2%), were examined for the presence of the exact insert by PCR using first M13 primer (delivered with the kit) which confirms whether the exact insert had been found, followed by a semi nested primer with GC-63F/518R or TMOA-F/TMOA-R or GC-P2060F/DSR4R.

Cloned fragments were compared with the original soil sample fingerprint by using DGGE. A selection of clones with different DGGE patterns was sequenced by VIB Genetic Service Facility (University of Antwerp, Belgium).



**Figure 6.11. White and blue colonies on selective agar medium. White colonies contain the exact insert**

## **6.3 RESULTS**

### **6.3.1 Monitoring of the *in situ* physico-chemical parameters**

Tables 6.4, 6.5 and 6.6 show the physico-chemical characteristics of groundwater and surface water in the three studied locations measured at each sampling. Most of the parameters, except water temperature and oxygen concentration, remained quite stable in the three locations.

The temperature of the surface water was the lowest in November 2004 in the Artificial Ditch (5.3°C) while the maximum value was reached in August 2005 (20°C). At this time the difference was also maximum between the three locations (3°C between the Leendertgracht and the Vijver) while only very slight differences were noticed in Autumn (Figure 6.12a). In groundwater the lowest temperature was measured in Winter while in Summer values around 20°C were measured in all the three locations (Figure 6.12b). In groundwater anaerobic or microaerophilic conditions prevailed: the highest oxygen concentration was measured in November 2006 in the Vijver (2.33 mg/l) and in the Artificial Ditch (2 mg/l). In surface water the oxygen level ranged from 2.15 mg/l (November 2004) to 7.56 mg/l (April 2006) in the Artificial Ditch, from 1.76 mg/l (November 2006) to 9.64 mg/l (March 2005) in the Leendertgracht and from 3.75 mg/l (November 2006) to 13.09 mg/l (May 2005) in the Vijver. A very high chloride concentration (up to 3000 mg/L) was measured in the Artificial Ditch and in the Vijver (surface water). No significant concentrations of N were present in any of the locations (often behind the detection limit), while significant amounts of SO<sub>4</sub><sup>2-</sup> (up to 450 mg/l) were found in the Artificial Ditch and in the Vijver (surface water). Tables 6.4 and 6.5 show that the main pollutant was monochlorobenzene (up to 10000 µg/L in the Artificial Ditch's groundwater) and, to a lesser extent, 1,4-dichlorobenzene and benzene. No VOC's contamination was present in surface water in the Vijver.

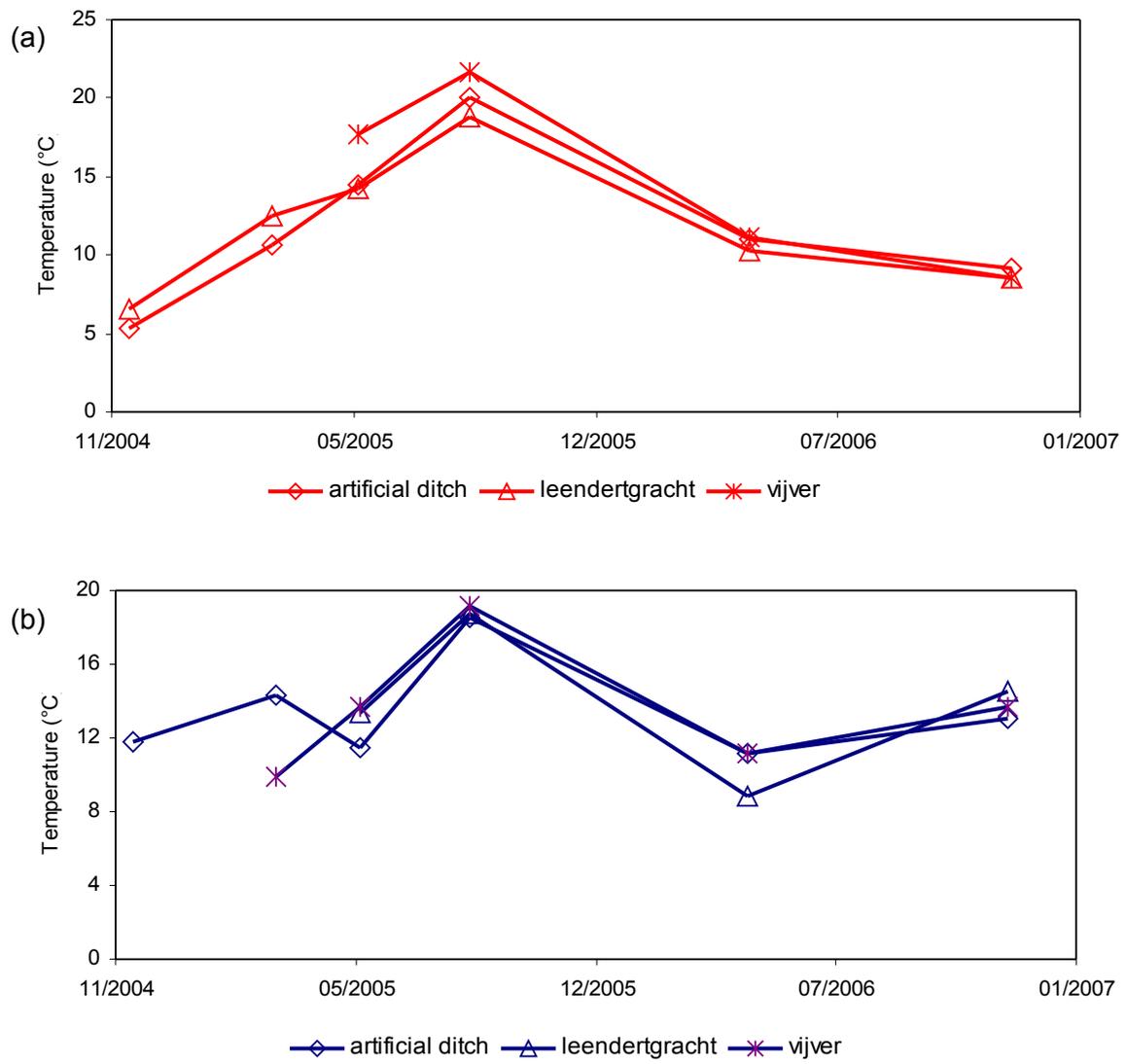


Figure 6.12. Monitoring of the temperature in surface water (a) and in groundwater (b) in different seasons in the three studied locations

**Table 6.4. Physico-chemical parameters of groundwater and surface water in the Artificial Ditch. ND: not detected.**

Parameter	Artificial Ditch											
	Groundwater (borehole Q-32)						Surface water					
	November 24, 2004	March 22, 2005	May 31, 2005	August 31, 2005	April 20, 2006	November 22, 2006	November 24, 2004	March 22, 2005	May 31, 2005	August 31, 2005	April 20, 2006	November 22, 2006
Surface water level (m)	-	-	-	-	-	-	0.3	0.3	-	0.4	>1	>1
Groundwater level (m-mv)	2	-	1.83	1.55	-	1	-	-	-	-	-	-
O <sub>2</sub> concentration (mg L <sup>-1</sup> )	0.84	0.23	0.95	1.57	0.88	2	2.15	6.1	4.25	7.42	7.56	3.4
Temperature (°C)	11.8	14.3	11.5	18.5	11.2	13.1	5.3	10.6	14.5	20	12.2	9.1
pH	6.46	6.97	7.08	6.84	7.1	7.37	7.13	6.90	7.89	7.42	8.49	7.65
Redox (mV)	-33	-129	-158	-	-131	-	89	-58	3	-	3	-
Conductivity (µS cm <sup>-1</sup> )	8020	-	1644	7830	1680	1810	2070	2490	2060	8490	1709	2220
Cl <sup>-</sup> (mg L <sup>-1</sup> )	2370	2400	1800	2200	1100	280	3590	1700	2600	2400	810	360
NO <sub>3</sub> <sup>2-</sup> (mg L <sup>-1</sup> )	1.45	3.6	<0.23	<0.23	<0.23	<0.23	0.31	0.56	<0.23	<0.23	<0.23	<0.23
NO <sub>2</sub> <sup>2-</sup> (mg L <sup>-1</sup> )	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02
SO <sub>4</sub> <sup>2-</sup> ( mg L <sup>-1</sup> )	399	390	450	410	240	150	580	409	360	370	280	200
SO <sub>3</sub> <sup>2-</sup> ( mg L <sup>-1</sup> )	<0.1	0.34	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Fe (µg L <sup>-1</sup> )	3580	3240	2460	1040	<50	380	192	1220	845	2050	548	1000
monochlorobenzene (µg L <sup>-1</sup> )	5661	7251	10041	-	4493	-	6	162	209	-	-	-
1,4-dichlorobenzene (µg L <sup>-1</sup> )	88	83	ND	ND	ND	-	ND	ND	ND	ND	ND	-
benzene (µg L <sup>-1</sup> )	788	271	ND	ND	ND	-	ND	ND	ND	ND	ND	-

**Table 6.5. Physico-chemical parameters of groundwater and surface water in the Leendertgracht.**

Parameter	Leendertgracht											
	Groundwater (borehole A-31/1)						Surface water					
	November 24, 2004	March 22, 2005	May 31, 2005	August 31, 2005	April 20, 2006	November 22, 2006	November 24, 2004	March 22, 2005	May 31, 2005	August 31, 2005	April 20, 2006	November 22, 2006
Surface water level (m)	-	-	-	-	-	-	0.5	0.7	-	-	-	0.35
Groundwater level (m-mv)	0.1	-	1.18	0.63	0.63	0.99	-	-	-	-	-	-
O <sub>2</sub> concentration (mg L <sup>-1</sup> )	-	-	-	-	0.15	0.62	3.35	9.64	-	4.13	2.8	1.76
Temperature (°C)	-	-	13.4	18.7	8.8	14.5	6.6	12.5	14.2	18.8	10.3	8.6
pH	-	-	7.3	7.15	7.59	7.29	7.06	8.07	7.56	7.3	7.4	7.53
Redox (mV)	-	-	-104	-	-203	-	50	108	-108	-	93	-
Conductivity (µS cm <sup>-1</sup> )	-	-	2250	2170	1151	1787	1390	1052	1415	1672	1415	1760
Cl <sup>-</sup> (mg L <sup>-1</sup> )	-	-	780	410	170	260	175	120	200	260	210	200
NO <sub>3</sub> <sup>2-</sup> (mg L <sup>-1</sup> )	-	-	<0.23	<0.23	<0.23	<0.23	<0.23	<0.23	<0.23	<0.23	<0.23	<0.23
NO <sub>2</sub> <sup>2-</sup> (mg L <sup>-1</sup> )	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	0.027
SO <sub>4</sub> <sup>2-</sup> ( mg L <sup>-1</sup> )	-	-	5.7	26	34	62	43.2	53	100	4.9	28	58
SO <sub>3</sub> <sup>2-</sup> ( mg L <sup>-1</sup> )	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	1.25	<0.1	<0.1	<0.1
Fe (µg L <sup>-1</sup> )	0	0	536	<50	1870	4400	1030	587	7620	3660	1480	3700
monochlorobenzene (µg L <sup>-1</sup> )	-	86	287	-	15	-	159	ND	1	ND	2	-
1,4-dichlorobenzene (µg L <sup>-1</sup> )	-	-	-	-	-	-	0.7	ND	ND	ND	ND	ND
benzene (µg L <sup>-1</sup> )	-	-	-	-	-	-	2.2	ND	-	-	-	-

**Table 6.6. Physico-chemical parameters of groundwater and surface water in the Vijver**

Parameter	Vijver									
	Groundwater (borehole A-18/2)					Surface water				
	March 22, 2005	May 31, 2005	August 31, 2005	April 20, 2006	November 22, 2006	March 22, 2005	May 31, 2005	August 31, 2005	April 20, 2006	November 22, 2006
Surface water level (m)	-	-	-	-	-	-	0.86	0.55	0.43	0.65
Groundwater level (m-mv)	-	-	-	-	0.4	-	-	-	-	-
O <sub>2</sub> concentration (mg L <sup>-1</sup> )	0.06	-	0.27	0.52	2.33	7.2	13.09	9.47	8.08	3.75
Temperature (°C)	9.9	13.7	19.2	11.2	13.7	13.5	17.7	21.6	11.2	8.6
pH	7.46	7.4	7.15	7.19	7.15	7.78	8.11	7.93	7.83	7.81
Redox (mV)	-222	-143	0	-162	-	-	-10	-	60	-
Conductivity (µS cm <sup>-1</sup> )	1678	1604	1833	1555	1567	-	2420	7940	1834	8780
Cl <sup>-</sup> (mg L <sup>-1</sup> )	160	180	170	202	130	-	2800	2400	2000	2600
NO <sub>3</sub> <sup>2-</sup> (mg L <sup>-1</sup> )	<0.23	<0.23	<0.23	<0.23	<0.23	<0.23	<0.23	<0.23	1.4	<0.23
NO <sub>2</sub> <sup>2-</sup> (mg L <sup>-1</sup> )	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02
SO <sub>4</sub> <sup>2-</sup> (mg L <sup>-1</sup> )	15	3.1	3.7	2.4	11	-	440	370	330	410
SO <sub>3</sub> <sup>2-</sup> (mg L <sup>-1</sup> )	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Fe (µg L <sup>-1</sup> )	97	71	110	233	360	-	852	1060	379	450
monochlorobenzene (µg L <sup>-1</sup> )	86	62	-	92	-	ND	ND	ND	ND	ND

### 6.3.2 Pollutants concentration at different depths in the sediment cores

High concentrations of monochlorobenzene were measured in cores extracted from the Artificial ditch. Concentration ranged from 50 to 1000  $\mu\text{g}/\text{kg}$  in November 2004, from 60 to 6000  $\mu\text{g}/\text{kg}$  in March 2005, from 500 to 18000  $\mu\text{g}/\text{kg}$  in May 2005 and from 20 to 10000  $\mu\text{g}/\text{kg}$  in August 2005. The lowest values were observed in slices close to the surface, and concentration increased depending on depth (Figure 6.13a). Not negligible but significantly lower concentrations of 1,4-dichlorobenzene were also observed (up to 1000  $\mu\text{g}/\text{kg}$ ), showing the same trend in function of depth. Other chlorobenzenes (1,2- 1,3-dichlorobenzene; 1,2,3- 1,2,4 and 1,3,5-trichlorobenzene) were present in low concentrations (mostly one order of magnitude lower). In March and May low concentrations of benzene were also measured (up to 100  $\mu\text{g}/\text{kg}$ ). In the Leendertgracht (Figure 6.13b) monochlorobenzene level generally decreased from the top to the bottom of the core. The lowest values were measured in cores sampled in August 2005 (0-150  $\mu\text{g}/\text{kg}$ ), while highest ones were observed in May 2005 (10 – 10000  $\mu\text{g}/\text{kg}$ ). 1,4-dichlorobenzene did not exceed 400  $\mu\text{g}/\text{kg}$  while the other compounds (1,2- 1,3-dichlorobenzene; 1,2,3- 1,2,4 and 1,3,5-trichlorobenzene and benzene) ranged between 10 and 50  $\mu\text{g}/\text{kg}$ . In general the other pollutant followed the same trend as monochlorobenzene, thus increasing in function of depth. No chlorobenzenes or benzene were detected in sediments cores extracted from the Vijver.

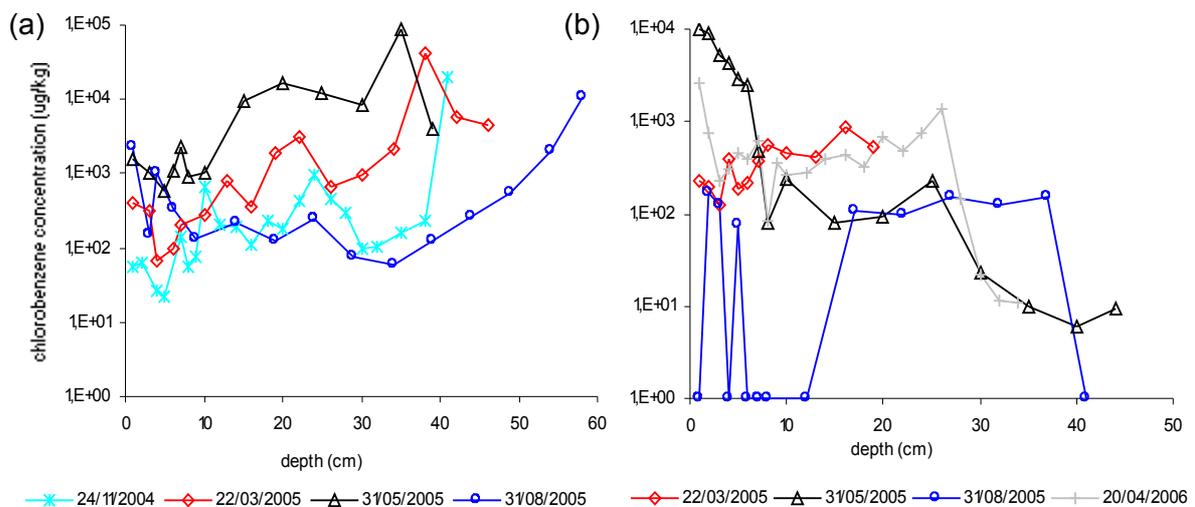
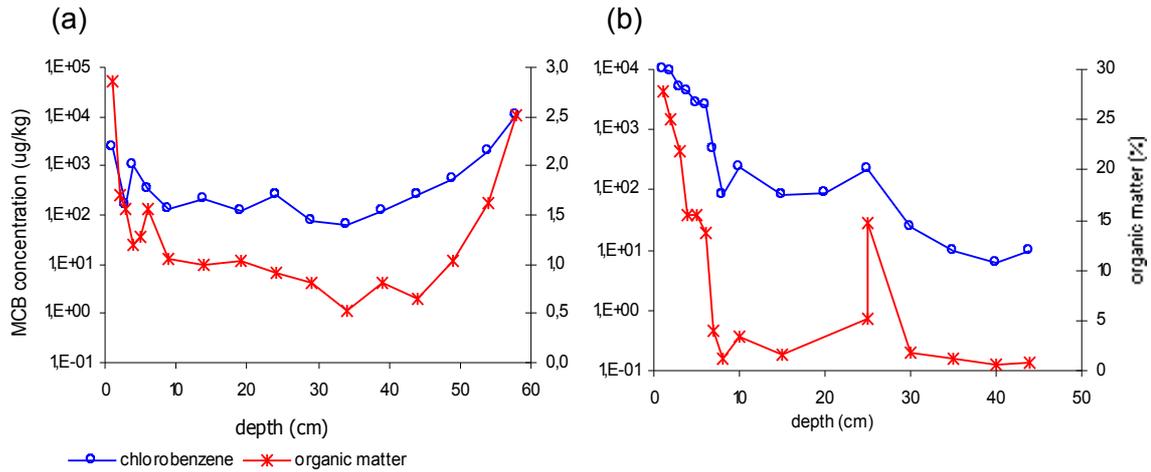


Figure 6.13. Monochlorobenzene concentration in undisturbed sediment cores extracted from the Artificial Ditch (a) and the Leendertgracht (b).

A direct relation was moreover observed between the concentration of monochlorobenzene adsorbed to the sediment and the total organic matter. As an example Figure 6.14 shows organic matter and monochlorobenzene concentration in function of depth in two cores extracted from the Artificial Ditch and the Leendertgracht.



**Figure 6.14 Total organic matter and monochlorobenzene concentration at different depths in cores extracted from the Artificial Ditch on 31/08/05 (a) and from the Leendertgracht on 31/05/05 (b).**

### 6.3.3 Degradation of monochlorobenzene under oxygen-limited conditions in batch degradation tests

Monochlorobenzene degradation was studied under the *in situ* conditions by bringing the aquifer material (top or bottom) in contact with groundwater or surface water sampled *in situ*.

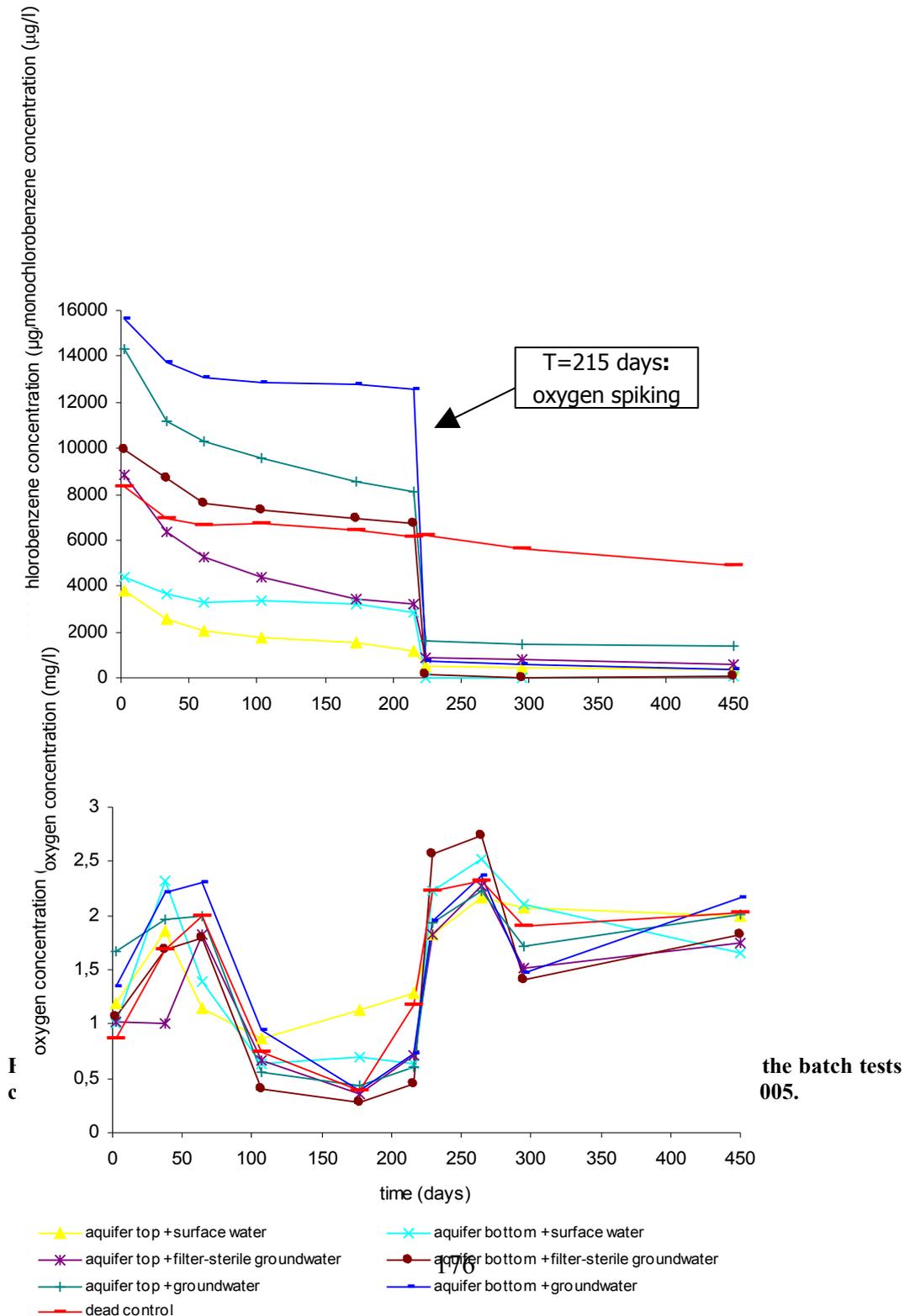
#### Tests set up with material sampled on 23/03/2005

Figure 6.15 shows the time-concentration profiles of monochlorobenzene and oxygen for the microcosms constructed with sediment and water from the Artificial ditch.

A small decrease in monochlorobenzene concentration was noticed in these microcosms during the first 30 days of incubation both in the living and dead control conditions; after this initial period the degradation stopped and the concentration did not change significantly for 215 days. On day 215, after microcosms were spiked with oxygen, biodegradation readily started again in all

living tests but not in the dead controls. The pollutant was in some cases (aquifer bottom + surface water and aquifer bottom + filter-sterile groundwater) even completely consumed. In the following 250 days no further oxygen was added and the contaminant's concentration remained stable.

Analogous results were observed in the tests set up with material sampled from the other two locations (Leendertgracht and Vijver).



Tests set up with material sampled on 20/04/2006

The initial oxygen concentration in the microcosm tests containing both water and sediment ranged between 2 and 2,5 mg/l. Figure 6.16 shows monochlorobenzene concentration in function of time in the tests carried out with material sampled from the Leendertgracht, during the first 35 days of incubation. Limited monochlorobenzene biodegradation took place during the first 6 days in all microcosm, then it stopped; from day 6 to day 36 monochlorobenzene concentration remained stable and oxygen concentration stabilized at 1,5 – 2 mg/l.

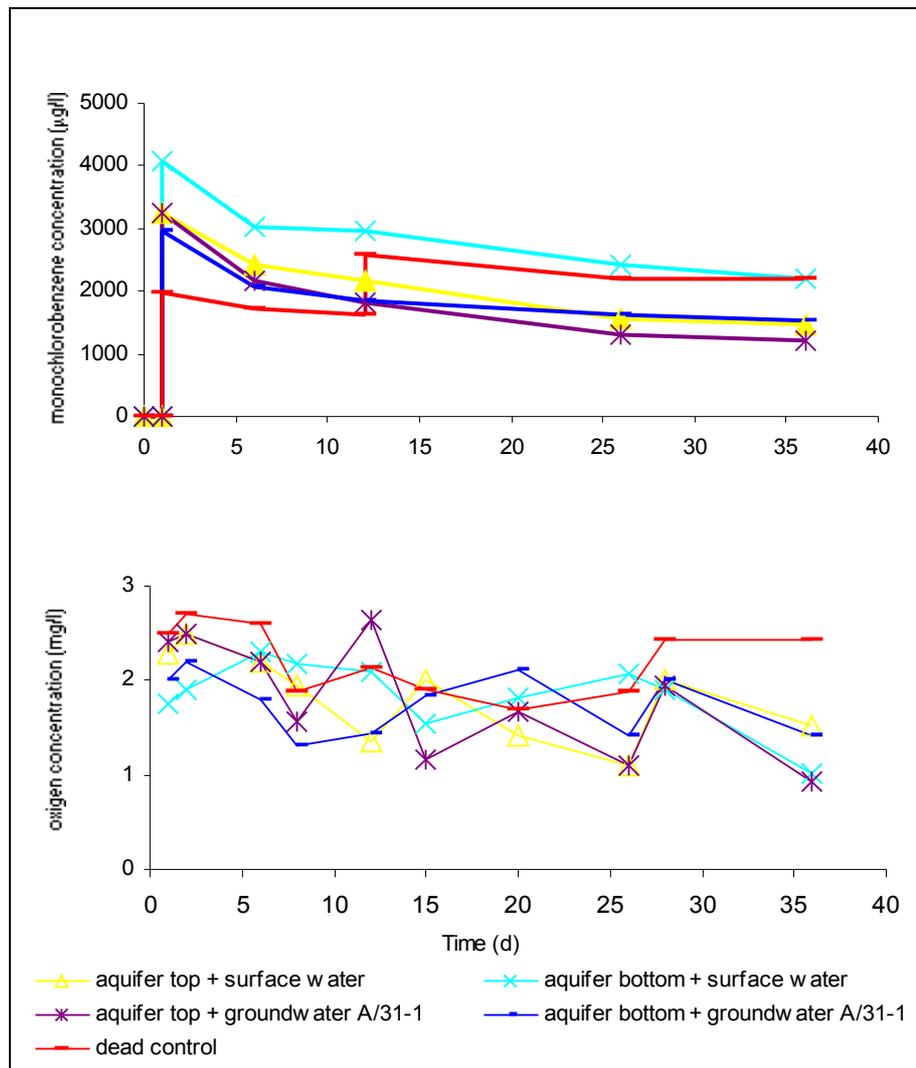


Figure 6.16. Monochlorobenzene and oxygen aqueous phase concentration in the batch tests carried out with sediment and water sampled in the Leendertgracht on 20/04/2006 (first 35 days of incubation).

On day 37 oxygen was therefore added (2.5-3 mg/l) to the first duplicate of each type of microcosm (duplicate 1), resulting in an immediate onset of the biodegradation, which stopped when the oxygen concentration dropped to 1,5–2 mg/l. Each time degradation stopped the spiking with a low volume of oxygen (to increase the concentration of 1 ppm) proved to be effective to stimulate biodegradation. Surprisingly, in the condition “aquifer top+groundwater” we observed a slow but total degradation also in duplicate 2, with oxygen concentrations below 1.5 mg/l. The complete degradation of the monochlorobenzene originally present required the addition of 17 mg (average value of the 4 microcosms aquifer top/bottom + groundwater, aquifer top/bottom + surface water) of oxygen altogether. Considering the total amount of monochlorobenzene provided throughout the 200 days of incubation, 16 mg of oxygen were needed for each mg of monochlorobenzene added (average value of the microcosms mentioned above). During this incubation period monochlorobenzene was re-spiked each time it was completely degraded and concentrations were gradually increased up to 45–50 mg/l. Even at these concentrations the contaminant was consumed with no evident inhibition effects.

Oxygen concentration was apparently the only factor limiting biodegradation: the process stopped each time oxygen concentration dropped under 1,5–2 mg/l, regardless the position in the interface (top or bottom of the sediment) and the type of water (groundwater or surface water). On the contrary, no degradation was observed in the living microcosms without oxygen spiking, where the same behaviour as in the a-biotic controls was observed. (Figure 6.17).

Similar results were obtained in tests performed using samples from the non polluted location (Vijver), except for the smaller oxygen amount required to biodegrade monochlorobenzene: 3.3 mg of oxygen were necessary to oxidize the initial amount of monochlorobenzene and  $2.5 \text{ mg}_{\text{oxygen}}/\text{mg}_{\text{monochlorobenzene}}$  were consumed on the whole. In the condition “aquifer top+groundwater” degradation only started when oxygen concentration was increased to 3.5-4 mg/l.

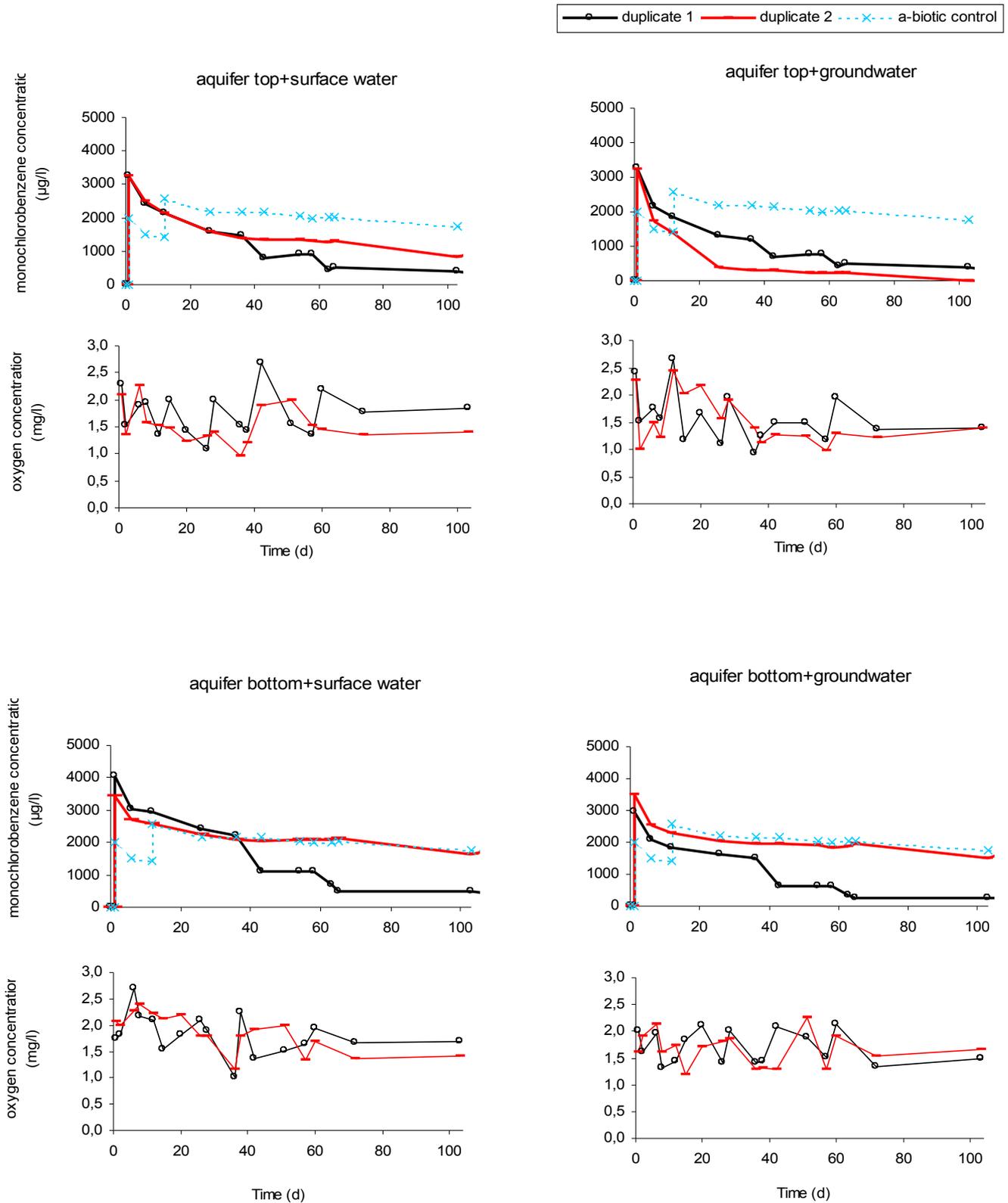
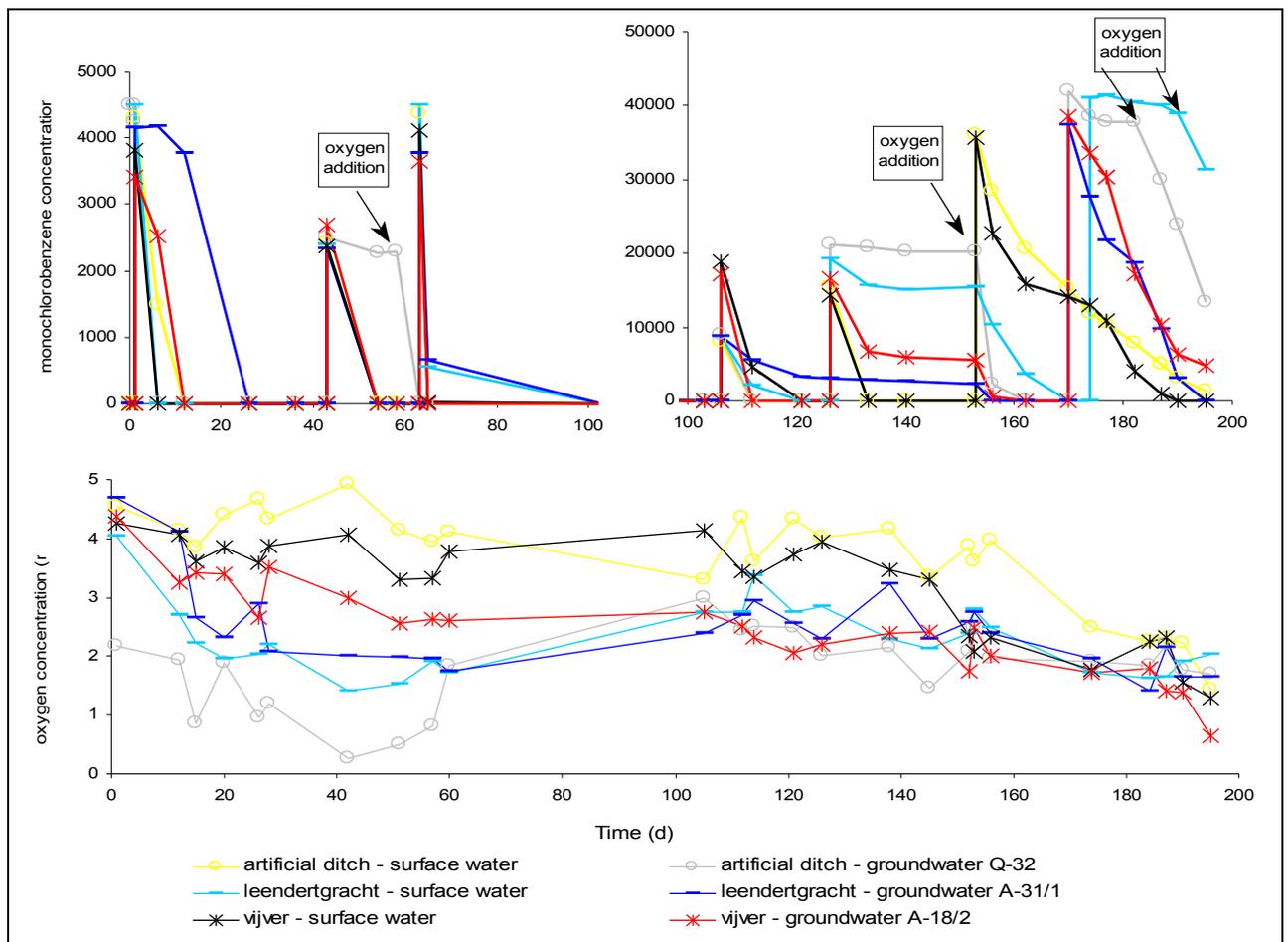


Figure 6.17. Monochlorobenzene and oxygen aqueous phase concentration in the duplicate batch tests carried out with sediment and water sampled in the Leendertgracht on 20/04/2006 (100 days of incubation).

Tests carried out with groundwater or surface water (with no sediment), show that initial oxygen concentration was higher than in microcosms with sediment (2,5–5,5 mg/l) and oxygen was consumed more slowly, thus inducing very fast degradation of the added monochlorobenzene in water sampled from all the studied locations: 5 mg/l of monochlorobenzene were degraded in less than 5–6 days; in most of the cases several monochlorobenzene pulses were degraded before the process stopped, with oxygen concentration of 1,5–2 mg/l. When monochlorobenzene concentration was increased to 40–45 mg/l degradation was fast anyway: it took 20-30 days, if enough oxygen was present to support biodegradation. (Figure 6.18).  $1.3 \text{ mg}_{\text{oxygen}}/\text{mg}_{\text{monochlorobenzene}}$  were in average necessary to oxidize monochlorobenzene. No remarkable differences were noticed depending on the location (Artificial ditch, Leendertgracht and Vijver) or the type of water used (groundwater or surface water).



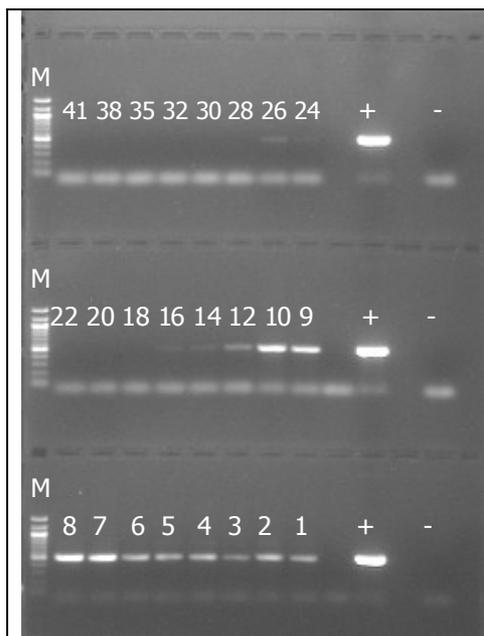
**Figure 6.18. Monochlorobenzene and oxygen aqueous phase concentration in the batch tests carried out with groundwater and surface water sampled in the Artificial Ditch, Leendertgracht and Vijver on 20/04/2006. No degradation was observed in any of the dead controls (data not shown).**

A high monochlorobenzene degradation potential seems to be present in both sediment and water: apparently groundwater and surface water and both aquifer materials (top and bottom) contained a microbial community able to degrade monochlorobenzene very quickly. The aquifer material sampling depth in the interface did not influence the biodegradation of monochlorobenzene, and, during the 200 incubation days, the only limiting factor appeared to be the oxygen concentration.

#### **6.3.4 Detection of catabolic genes at different depths of the interface**

DNA extracts were used to investigate the spread of catabolic genes over the interface in the three studied locations, at different times of the year. Among all the studied catabolic genes (*tmoA*, *xylE*, *todC1*-like genes, *cdo*, *tbuE*, and *todE*) only the *tmoA* gene was systematically detected. This gene encodes the initial attack in the degradation of toluene and benzene, and has already been detected in soils polluted with BTEX compounds (Hendrickx *et al.*, 2005).

As an example Figure 6.19 shows the gel image of the PCR products obtained from a sediment core sampled on 24/11/2004 in the Artificial ditch. The fragment of the expected length (505 bp) is present with a bright signal down to approximately 10 cm; from 12 to 16 cm the signal becomes weaker and disappears completely at higher depths. The bright bands corresponding to the positive controls (+) confirmed the success of the reaction; the absence of signal corresponding to the negative controls shows that no interference had biased the amplification process. The universal marker (100 bp ladder) is necessary to know the exact size of the amplified amplicon.



**Figure 6.19, Gel image of the PCR products obtained with TMOA primer from a sediment core sampled in the artificial ditch on 24/11/2004. Numbers represent the depth of the analysed slice in the interface (cm), M indicates the universal marker, + and – the positive and negative controls.**

Figures 6.20, 6.21, 6.22 shows the results of the PCR analyses carried out on sediment cores extracted respectively from the Artificial ditch, Leendertgracht and Vijver in the different seasons. In most cases, the *tmoA* gene is present at least down to 15-20 cm in the interface and is not detected at greater depths. However in some cases depths of even 35–40 cm were reached (in particular in Vijver in all seasons, except Winter).

Apparently a relation also exists between the presence of the gene and the season, since in Winter *tmoA* has been detected in the Artificial ditch only down to the depth of 10 cm and is completely absent in any other location. The highest depths are apparently reached in the samples collected in Summer. These results are summarized in Table 6.7 PCR targeting the *tmoA* gene never gave positive results for groundwater samples, whereas in surface water the gene was present only in two cases (both sampled in Summer 2005).

Among the other catabolic genes, only *tbmD*, *cdo* and *tbuE* were occasionally detected in a few samples (data not shown).

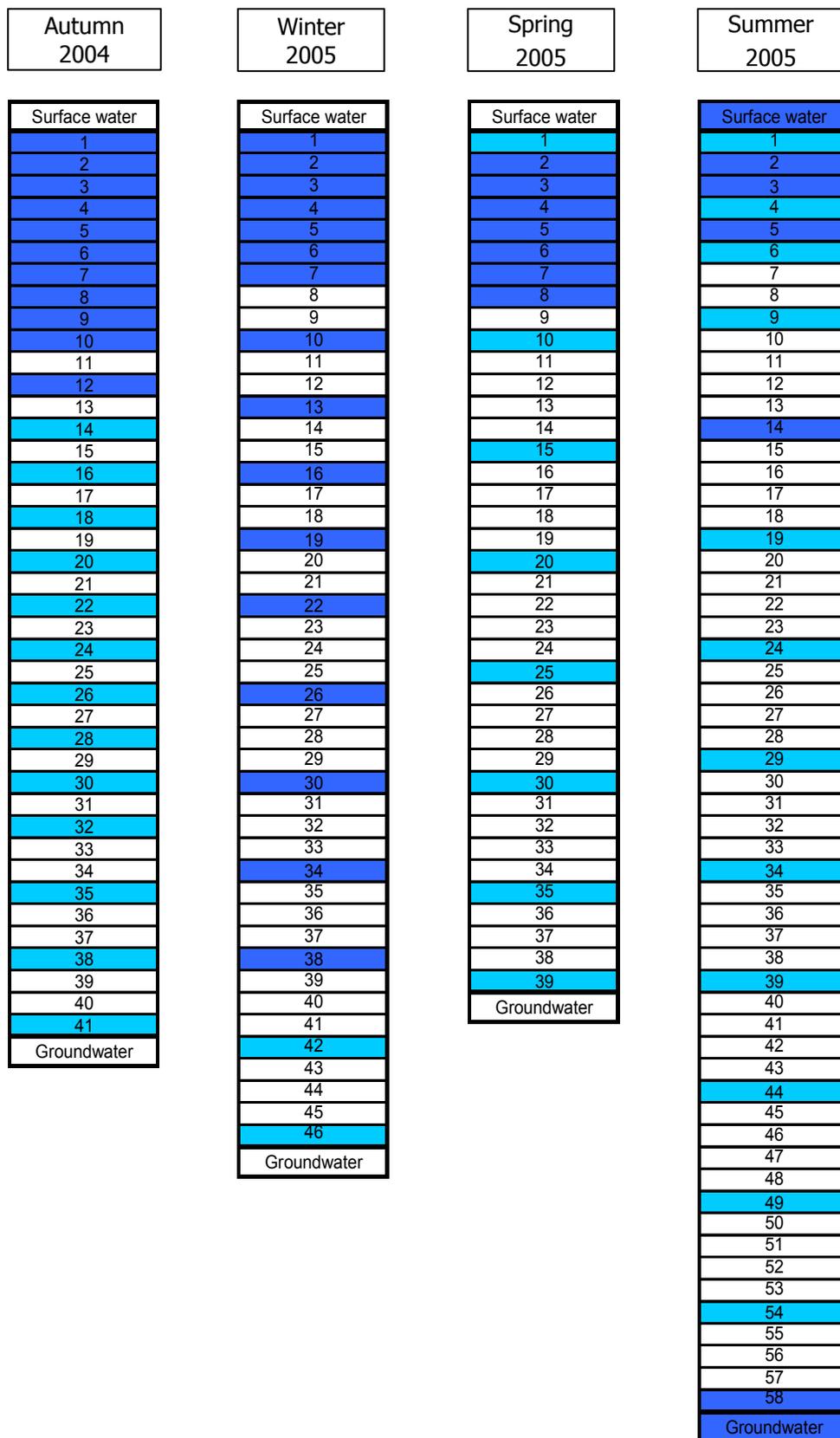


Figure 6.20. PCR results of the detection of the *tmoA* gene in sediment cores extracted from the Artificial ditch at different sampling times. Boxes represent sediment slices obtained from the frozen sediment core, groundwater or surface water; White: not analysed samples, blue: strong amplification, light blue: weaker amplification, shaded: no amplification.

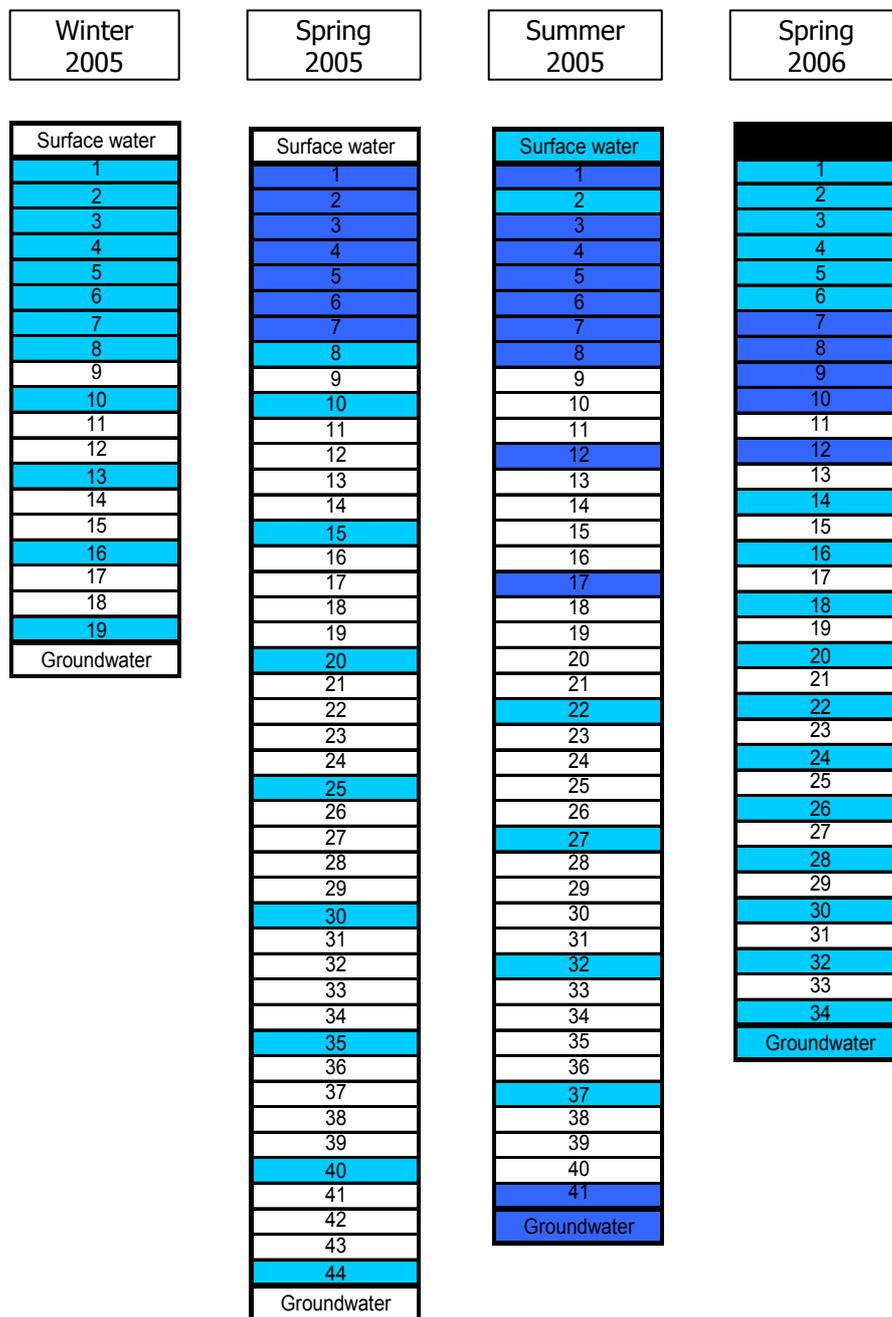


Figure 6.21. PCR results of the detection of the *tmoA* gene in sediment cores extracted from the Leendertgracht at different sampling times. Boxes represent sediment slices obtained from the frozen sediment core, groundwater or surface water; White: not analysed samples, blue: strong amplification, light blue: weaker amplification, shaded: no amplification.

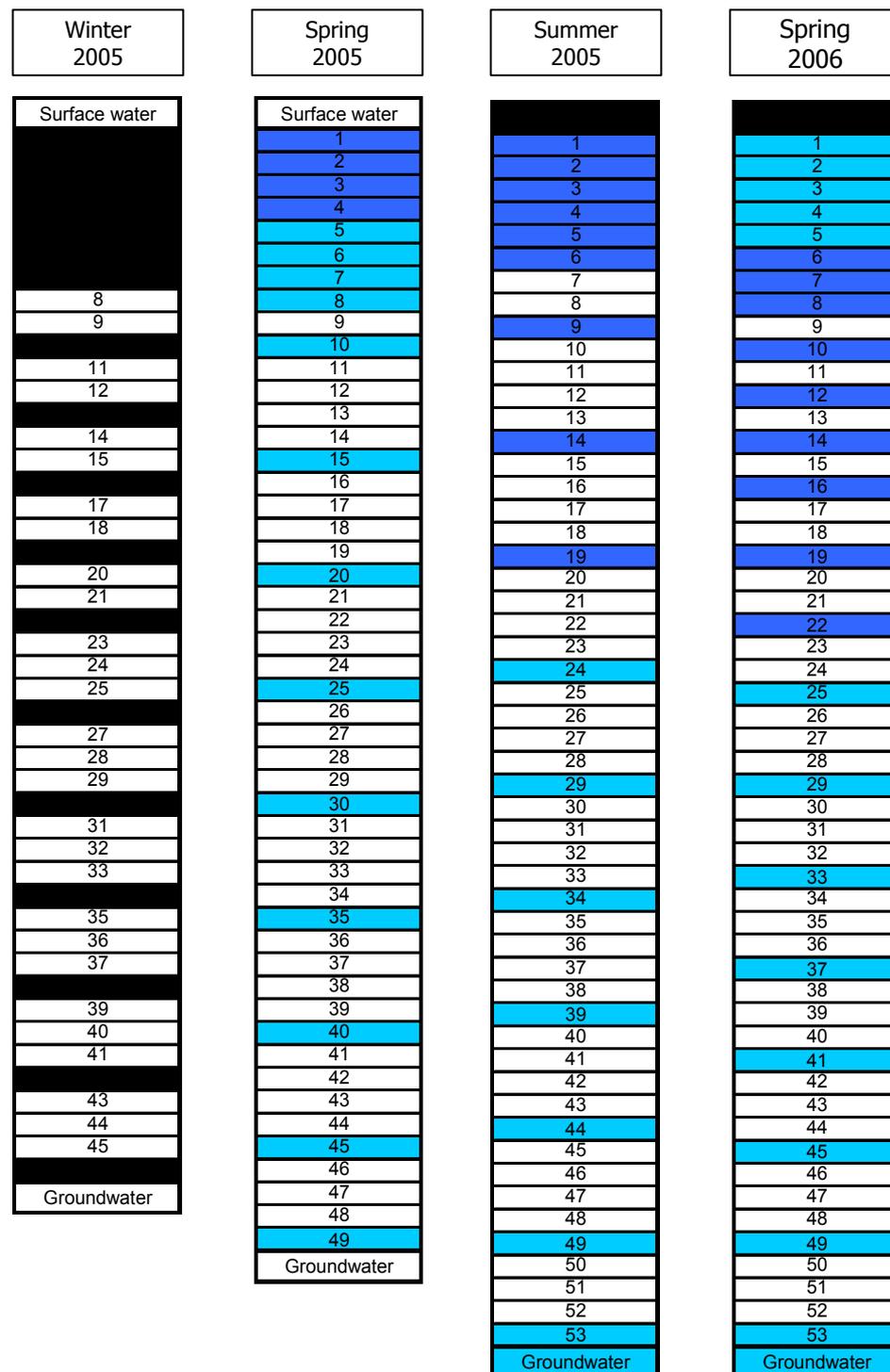


Figure 6.22. PCR results of the detection of the *tmoA* gene in sediment cores extracted from the Vijver at different sampling times. Boxes represent sediment slices obtained from the frozen sediment core, groundwater or surface water; White: not analysed samples, blue: strong amplification, light blue: weaker amplification, shaded: no amplification.

**Table 6.7. Maximum depth of the *tmoA* gene in sediment cores from the three studied locations at different sampling times; NA: not analysed, ND: not detected.**

	Autumn 2004	Winter 2005	Spring 2005	Summer 2005	Winter 2006
Artificial ditch	16 cm	10 cm	15 cm	29 cm	NA
Leendertgracht	NA	ND	10 cm	27 - 32 cm	14 cm
Vijver	NA	ND	35 – 45 cm	34 cm	29 cm

### 6.3.5 Study of the structure of the eubacterial community by 16S rRNA gene PCR - DGGE analysis

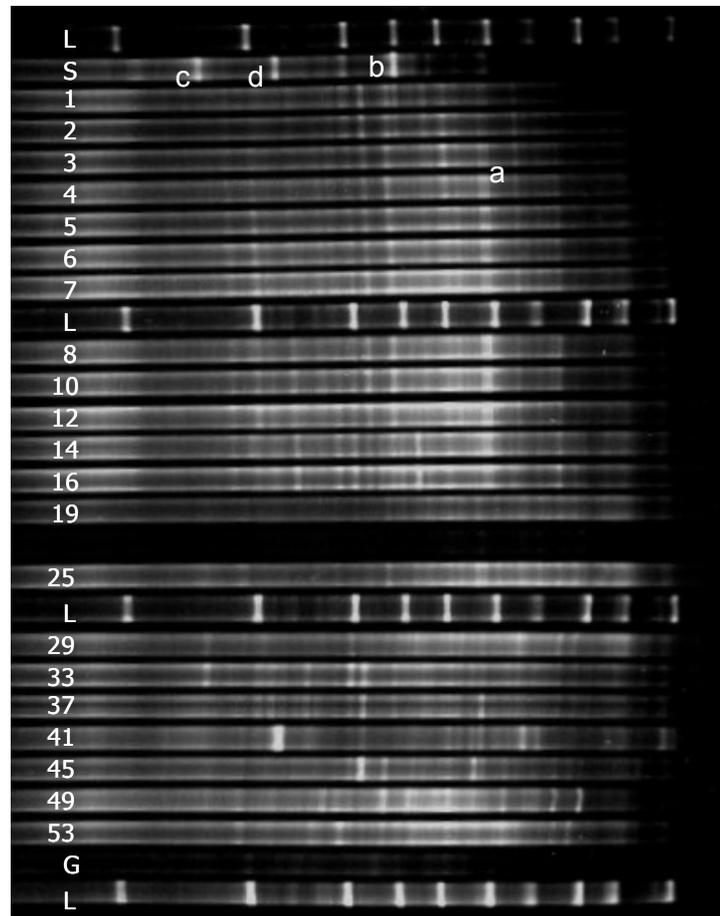
*Diversity of the eubacterial community at different depths in the interface:*

Figure 6.23 shows the PCR–DGGE results of a sediment core sampled on 20/04/2006 from the Vijver as an example.

A complex DGGE pattern was obtained for slices from the different positions in the interface, thus indicating that different types of *Eubacteria* were present and a high diversity of species exists in these sediments; however the degree of dominance of the bacteria seemed to be related with depth: a DGGE pattern mostly observed in the first 10 – 15 cm slightly changed in function of depth.

Some bands appeared starting from the depth of 3 cm, sometimes becoming brighter as the depth increased (for example band “a”) down to 19 cm and suddenly disappearing. None of the bands seemed to be present at every depth of the interface. In addition, the dominance of certain bacterial species of the microbial community diminished depending on depth and a DGGE pattern with a smaller number of bright and so dominant bands occurred in the slices obtained from a deeper position in the interface (from 19 cm to the bottom) and profiles differ from each other. Surface water showed a less complex DGGE pattern compared to that of sediment slices, characterized by the presence of 3 – 4 bright bands. Some of the bands present in these fingerprints were also observed in the underlying sediment slices. For instance band “b” was also present in slices 1 and 2, thus indicating that surface water infiltrates down to 2 – 3 cm. On the other hand bands “c” and “d” seemed to be present only in surface water, thus indicating the difference between the two microbial communities present in the

surface water and the interface. In groundwater only a few and faded bands were observed, showing that this microbial community is much less diverse than those of the sediment and surface water with a very limited presence of bacteria.



**Figure 6.23.** Eubacterial 16S rRNA gene PCR-DGGE profiles of the surface water (S), groundwater (S) and slices of an undisturbed sediment core sampled in the Vijver on 20/04/2006. Numbers indicate the depth (cm) in the interface; L: DGGE marker.

*Study of the microbial community in different locations.*

Very low similarities were observed in the DGGE patterns obtained from sediment samples taken at the same time from the three different sampling locations Artificial ditch, Leendert gracht and Vijver. In Figure 6.24 the results are shown from sediment cores sampled on 22/03/2005.

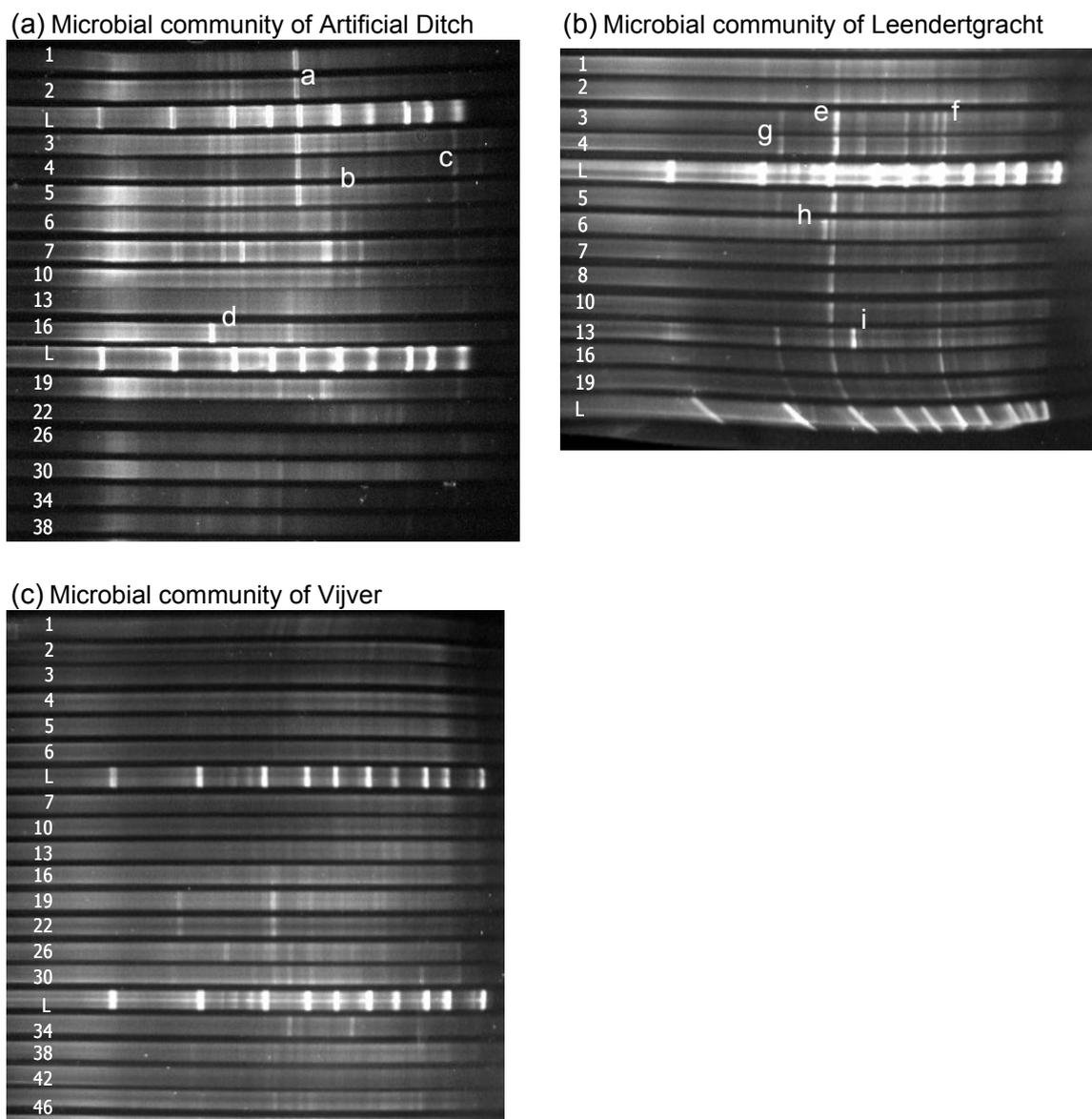
A high diversity was present in slices from the Artificial ditch (Figure 6.24a), we observed the appearance and disappearance of bands going from the

top to the bottom of the core. Band “a” was an example of a very bright band present down to 5 cm and disappearing downwards; band “b” appeared at the depth of 3 cm and was present down to 7 – 8 cm but its abundance increased suddenly at 7 cm; band “c” was present at a depth from 3 to 10 cm, while a very bright band (“d”) was observed only at 16 cm. This band could correspond to the “d” band of the surface water. Apparently more dominant bands could be observed in the first 10 cm of the core. From 20 cm to the bottom, a few dominant bands were observed. Several faint bands have been observed over the total core thus demonstrating considerable diversity.

In the core from Leendertgracht (Figure 6.24b), two bands (“e” and “f”) were observed almost throughout the whole sample, while a third one (“g”) disappeared at 7 cm and returned at 13 cm; the dominance of a few bacteria was higher in the first layers where 8 – 9 dominant bands were present and decreased starting from the depth of 5–6 cm; in the bottom layers only very thin bands seemed to be present. Two bands (“h” and “i”) were unique at depths of 6 and 13 cm respectively.

The observations we did in the two previous locations were confirmed in the third one (Vijver). The bacterial dominance in the DNA samples from Vijver appeared very low in the first 10 cm (only very thin bands) and slightly increased from 19 cm to the bottom (Figure 6.24c).

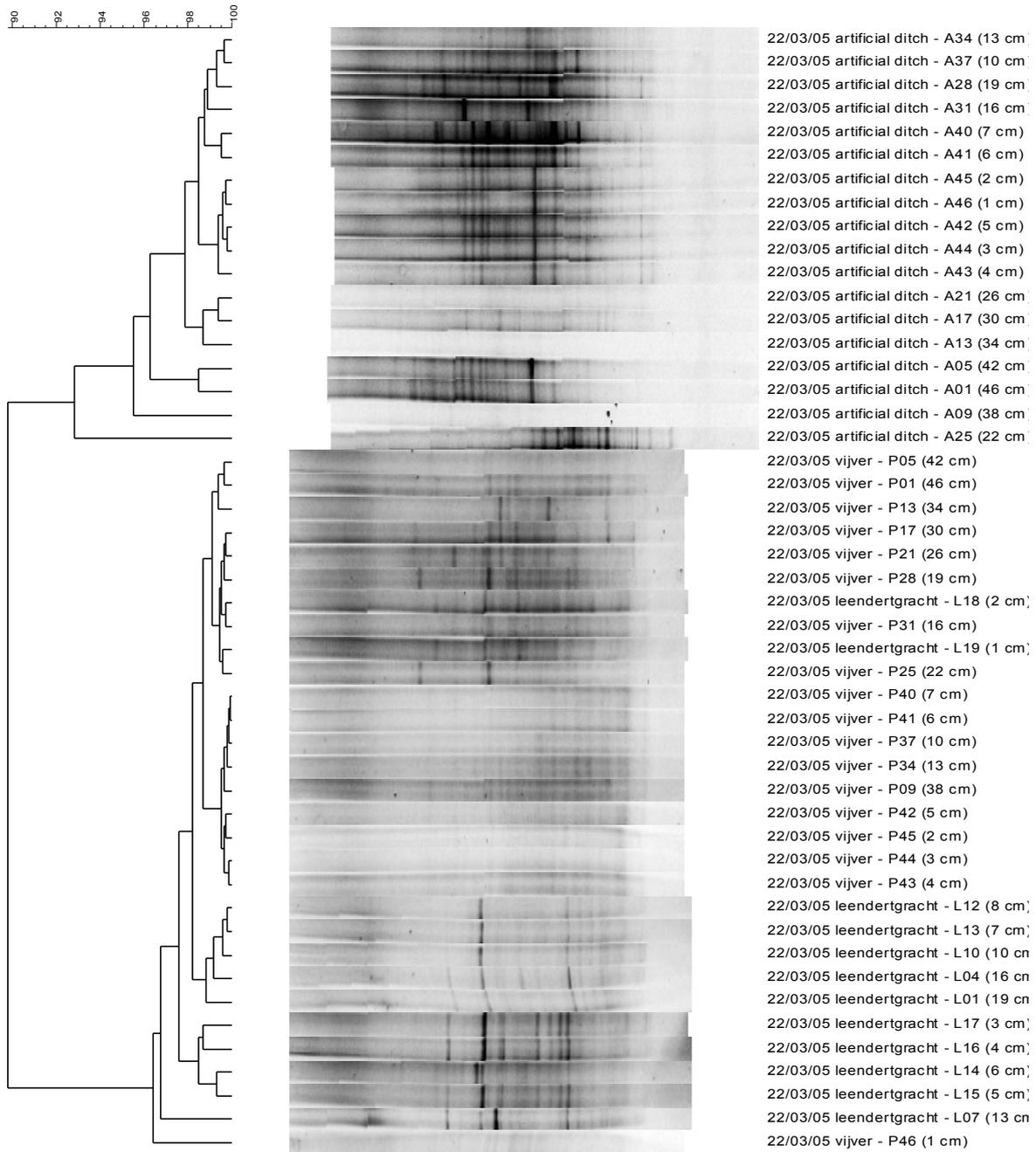
Results of PCR-DGGE analyses of undisturbed sediment cores demonstrated the very low similarity observed on 16SrRNA level when the three different sampling locations are considered. The highest microbial diversity was observed in samples from the Artificial ditch, whereas a much less diverse microbial community was present in the Leendertgracht, but almost present in the whole core. On the other hand samples from the Vijver showed in general a quite high diversity from a certain depth downwards.



**Figure 6.24.** Eubacterial 16S rRNA gene PCR-DGGE profiles of undisturbed sediment cores slices obtained from Artificial ditch (a), Leendertgracht (b) and Vijver (c) on 22/03/2005. Numbers represent the depth in the interface (cm); L: DGGE marker.

The cluster analysis of the community fingerprints (Figure 6.25) confirms that these communities are mostly different in the three places. Three main groups can thus be observed. The greatest differences can be observed between Artificial Ditch and Leendertgracht while a less marked difference can be observed between bacterial communities of Leendertgracht and Vijver. Moreover, Figure 6.25 shows that, for each location, the samples can be divided into two groups: the first one is composed by the slices from the first 5 – 10 cm, the second one is made up of the deeper slices, thus confirming that the structure of the community changes clearly depending on depth.

Cosine coefficient (Tol 1.0%-1.0%) (H>0.0% S>0.0%) [0.0%-100.0%]  
**16 S** **16 S**



**Figure 6.25. Eubacterial 16S rRNA gene; UPGMA clustering of the DGGE fingerprints of undisturbed sediment core slices obtained from Artificial ditch (a), Leendertgracht (b) and Vijver (c) on 22/03/2005. Numbers represent the depth in the interface (cm); L: DGGE marker. Clustering was performed using the Cosine similarity coefficient (processed by Bionumerics, Applied Maths, Belgium).**

*Study of the microbial community at different times.*

The comparison between PCR-DGGE profiles of sediment cores taken from the Artificial ditch throughout the year (Autumn, Winter, Spring, Summer) offered the chance to follow the evolution of the microbial community structure over time. For example, by comparing banding patterns referring to Autumn and Winter (Figure 6.26 a and b) some similarities can be observed: in both situations a dominant band is present from the surface to the depth of 5 cm; also profiles comprised between 5 and 10 cm showed significant similarities: bands b and c and region d appeared in both situations approximately at the same depth and a less complex pattern occurred in the slices from the approximate depth of 15 cm to 40 cm. In addition the same band “a” seemed to be present also in the profiles from sediment cores sampled in Spring and Summer. On the other hand also in Figure 6.26b (Winter) and c (Spring) similar elements are certainly present: the fingerprints corresponding to the first 10 cm in Figure 6.26c (rectangle and region d) showed a considerable similarity even with the samples referring to Autumn and Winter, but the latter appeared brighter. In the deeper slices (starting from 15 cm) diversity was much higher and more bands absent in the two other seasons clearly appeared. In Autumn, Winter and Spring the most important activity was certainly at the top of the core. In Spring, for example, we observed two blocks of bands: in DGGE fingerprints derived from the top of the core the high GC% bands were especially missing (lower denaturation range of the gel), while they were present in the cores from 15 cm to 39 cm ( approximately 50% denaturation range).

In sediment cores extracted in Summer (Figure 6.26d) we observed only a few bright bands, but probably the diversity is so high that we only found thin bands. The “d” group of the summer sample is unique, but when we compared the gel with the others it was quite clear that the migration of this gel was different and probably we lost that group in the other cases. Even at the depth of 60 cm there was an important population present during Summer. Here the bands are dominant but fewer.

In undisturbed sediment cores from the Leendertgracht interesting analogies between the different seasons were also observed and at least 3-4 common bands (sometimes at similar depths) were present in cores extracted in different periods. The dominance of a couple of bacteria in the microbial diversity was in general lower than in the Artificial ditch. In particular in Spring samples very few and thin bands were detected, whereas microbial community was very diverse in Summer samples, mostly down to the depth of 10 cm.

In cores extracted in Spring 2005 in the Vijver a low dominance of certain bacterial species (presented by very bright bands) was observed in the superficial layers and it increased from 15 cm downwards, while in Spring 2006 microbial dominance seemed lower in the first 3-4 cm than in the deeper layers and a sudden change in the banding pattern was observed from 20 cm downwards. Moreover a greater degree of microbial dominance was detected in Summer: a very bright band was observed in the first slice (the same band, probably coming from surface water, was also present in Spring 2005), and only slight changes down to 5-6 cm. From this depth downwards the DGGE profile changed very frequently (at least every 4 cm) and diversity remained very high down to 54 cm, with an extremely high number of very thin bands.

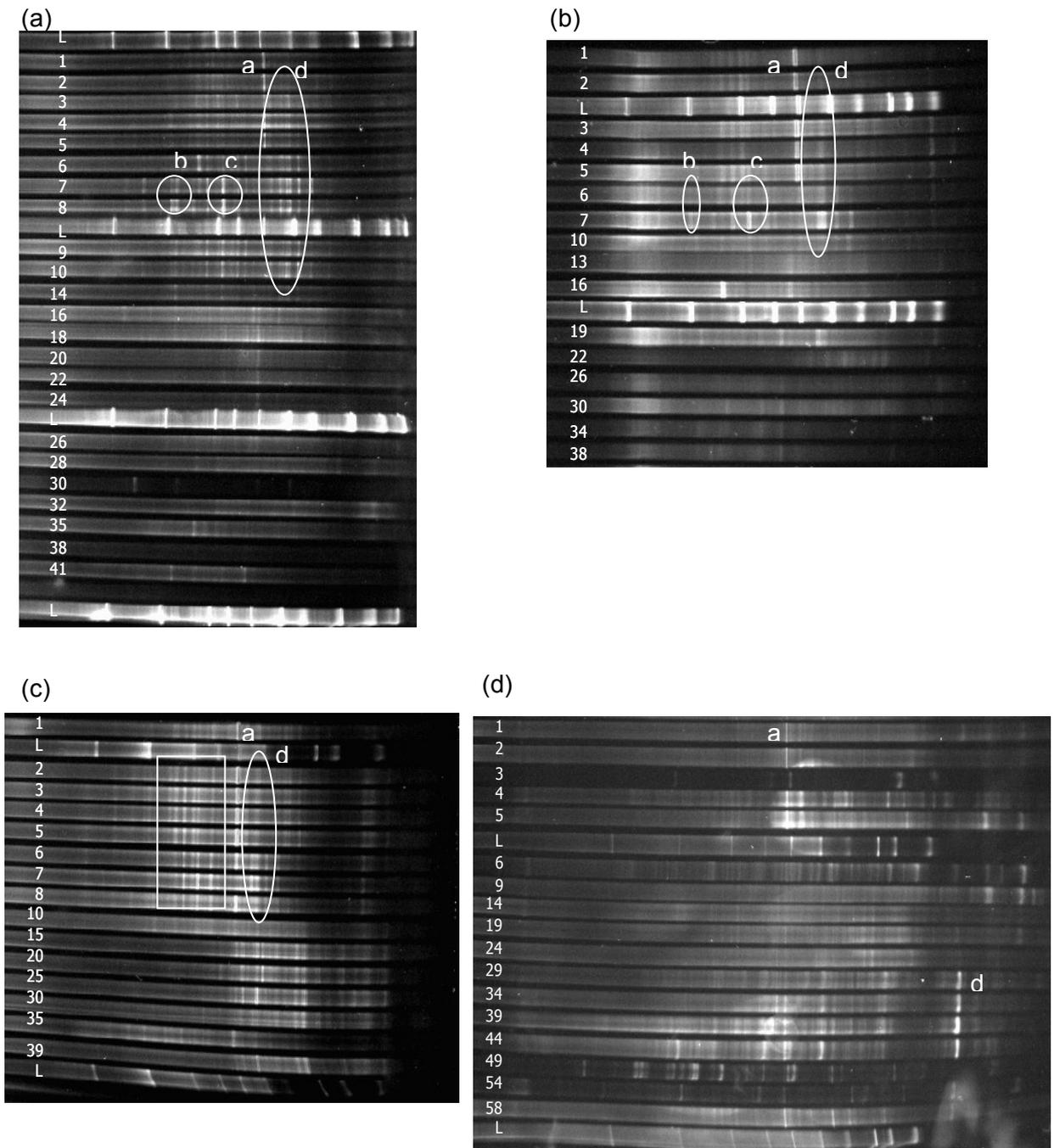


Figure 6.26. *Eubacterial 16S rRNA* gene PCR-DGGE profiles of undisturbed sediment cores slices obtained from Artificial ditch in Autumn 2004 (a), Winter 2005 (b), Spring 2005 (c) and Summer 2005(d). Numbers represent the depth in the interface (cm); L: DGGE marker.

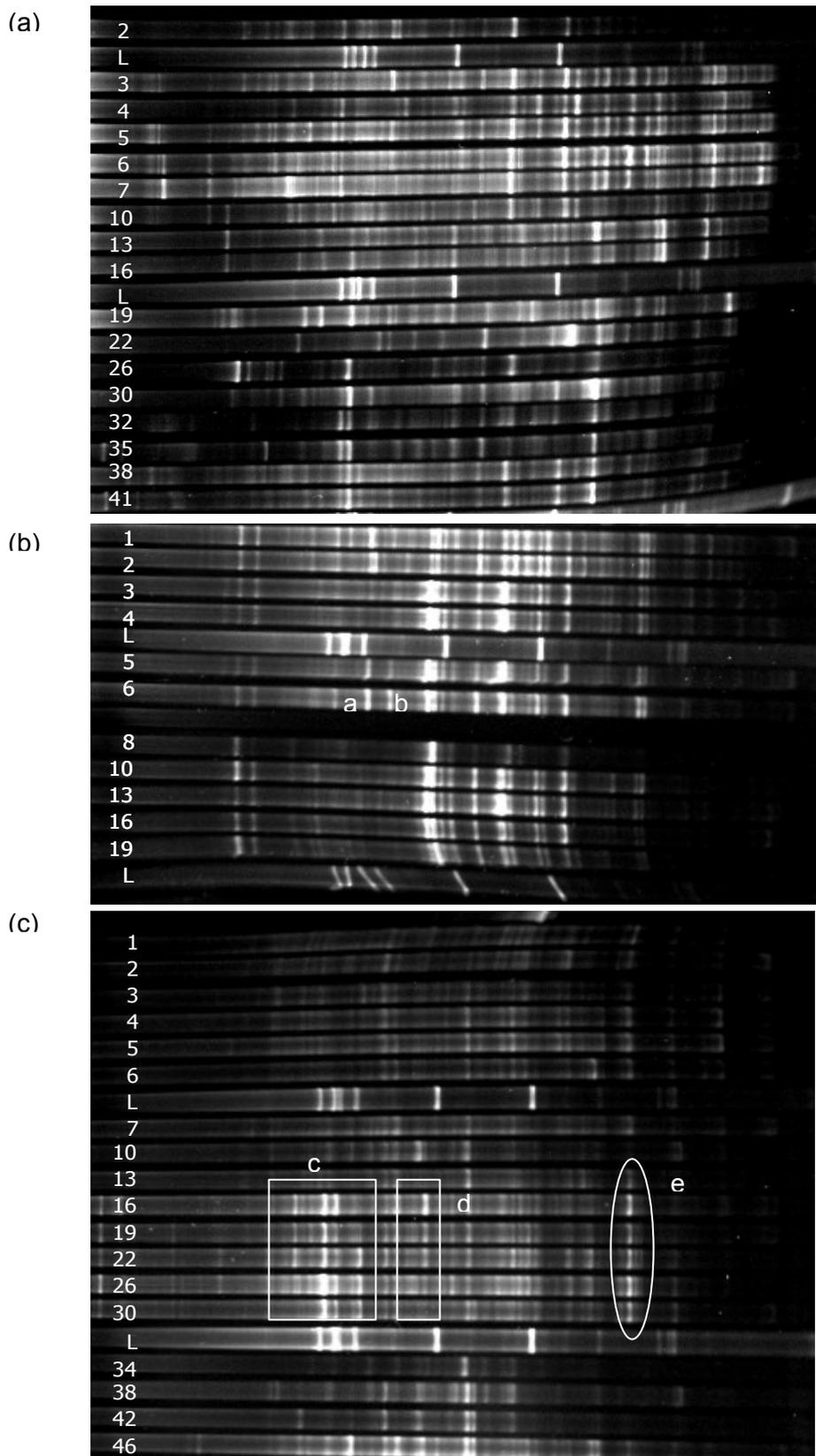
### **6.3.6 Study of the structure of the sulfate-reducing bacterial community by PCR - DGGE analysis**

*Study of the microbial community in different locations.*

In the first place PCR-DGGE analysis showed that sulfate-reducing bacteria were present in all sediment cores extracted from the studied locations, regardless of their depth in the interface or of the sampling time.

The comparison between the results obtained from the three sampling locations on 22/03/05 shows that a very diverse community was present especially in the Artificial Ditch (Figure 6.27a) and mostly down to 7-10 cm, whereas at greater depths different fingerprints were observed and diversity significantly decreased. Moreover the structures of the bacterial communities observed in the three positions appeared markedly different from each other. In the sediment core extracted from the Leendertgracht (Figure 6.27b) 3-4 dominant bands were present throughout the core with only slight changes in the DGGE profile (for example bands “a” and “b” disappeared from 6 cm downwards). Several very faint bands were observed in DGGE patterns obtained from the core sampled in the Vijver (Figure 6.27c); even if the profile did not change significantly depending on depth some very dominant bands appeared starting from 16 cm (groups “c” and “d”), while other bands became more important (for example “e”). All these bands suddenly disappeared at the depth of 34 cm.

Similar results were obtained - from samples extracted in the other seasons too: it was clear that sediments from the three studied locations harboured different sulfate-reducing bacterial communities.



**Figure 6.27.** Sulfate reducing bacteria PCR-DGGE profiles of undisturbed sediment cores slices obtained from Artificial ditch (a), Leendertgracht (b) and Vijver (c) on 22/03/2005. Numbers represent the depth in the interface (cm); L: DGGE marker.

*Study of the microbial community at different times:*

Figure 6.28 shows the comparison between sulfate reducing bacteria DGGE profiles obtained from sediment cores taken from the Artificial Ditch in different seasons. Microbial diversity was very high in Autumn and Winter and gradually decreased in Spring and Summer, when fewer and thinner bands were observed. In all these cores the banding patterns suddenly changed (sometimes thoroughly) from 10-15 cm downwards and the same happened in the cores extracted in the other two locations. Two or three dominant bands were present in the surface layers and they completely disappeared when the above cited depths were reached. Several similar bands were present in the profiles from cores extracted in different seasons, mainly concerning those extracted in Winter and Autumn. For example band “a” was present in both Autumn and Winter, but also in Spring, from 1 to 10 cm deep. Also band “b” could be observed in Autumn and Winter. Band “c” appeared in Autumn, in Winter and in Spring, band “d” in Winter, in Spring and in Summer, “e” was present in Spring and in Summer only and “f” in Winter and in Autumn, while “g” appeared in Spring, in Summer, in Autumn and in Winter throughout the whole core. Data regarding the other two locations were similar (data not shown).

The comparison between sulphate reducing bacteria PCR-DGGE profiles of undisturbed sediment cores slices obtained from Artificial ditch over four seasons indicated that a large diversity in bacteria exists, and several species were present during the whole year.

The selected regions represented our findings. Band “c” for example was present in nearly every slice, even in the surface water.

Sometimes their appearance was more visible in one season than in another (bands “g” and “d”). During Winter we observed the clearest diversity with the presence of strong bands in the upper part of the core. In the rest of the year fainter fingerprints were found in the upper part and some bright bands appeared downwards.

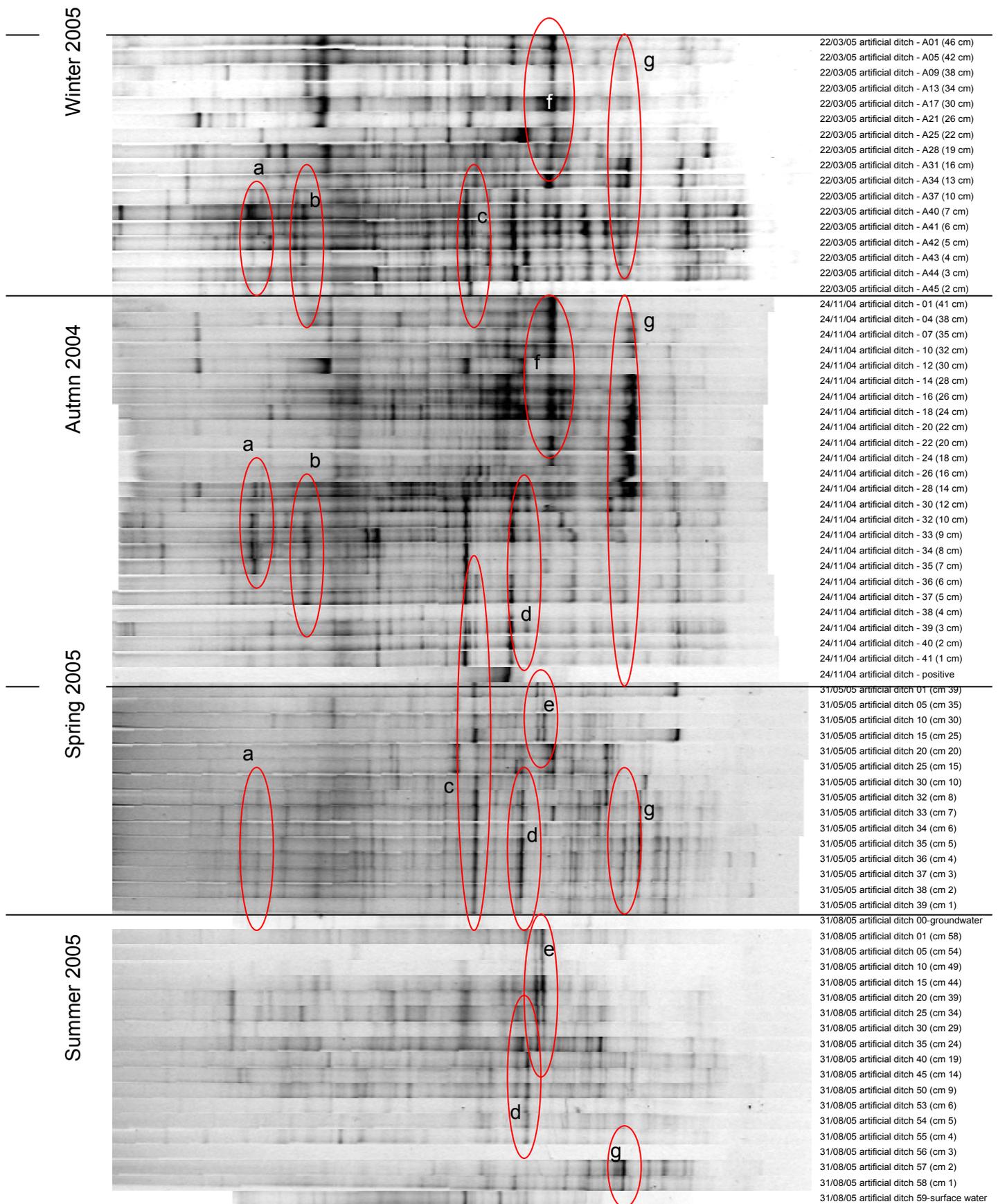


Figure 6.28. Comparison between sulfate reducing bacteria PCR-DGGE profiles of undisturbed sediment cores slices obtained from Artificial ditch in Autumn 2004, Winter 2005, Spring 2005 and Summer 2005 (processed by Bionumerics, Applied Maths, Belgium). Depths are indicated in brackets

### **6.3.7 PCR - DGGE analysis of *tmoA*-like genes**

*Study of the microbial community in different locations:*

Figure 6.29 shows that in Summer 2005 the communities carrying *tmoA*-like genes were characterized by a rather low microbial diversity. In addition significant differences were observed in the three sampling locations: in the Artificial Ditch two dominant bands were present, starting from the top of the core down to the depth of 24 and 59 cm (rectangle). These bands seemed to be present also in the Leendertgracht but only at the depths of 5, 7 and 42 cm. It also appeared at 14 cm in the Vijver. In the analysis of *tmoA*-like gene band (or group of bands) “a” seemed very important and it was interestingly present in the Vijver too and very feebly in the Artificial ditch, in all cases approximately down to 30 cm. In addition some further thin bands appeared in Leendertgracht, down to a depth of 7-8 cm.

In the Vijver a series of very close bands can be noticed, while three further isolated ones were observed at the depth of 14 cm.

Despite the similarities among the dominant bands of these communities we observed important differences between their fingerprints.

In undisturbed sediment cores extracted in other seasons a very low diversity of *tmoA*-like genes and only slight differences between the sampling places were observed. In both Spring 2005 and Spring 2006 band “a” was the only significant one (present in all the locations) while in Winter 2005 *tmoA* gene was absent in the Leendertgracht and Vijver (data not shown).

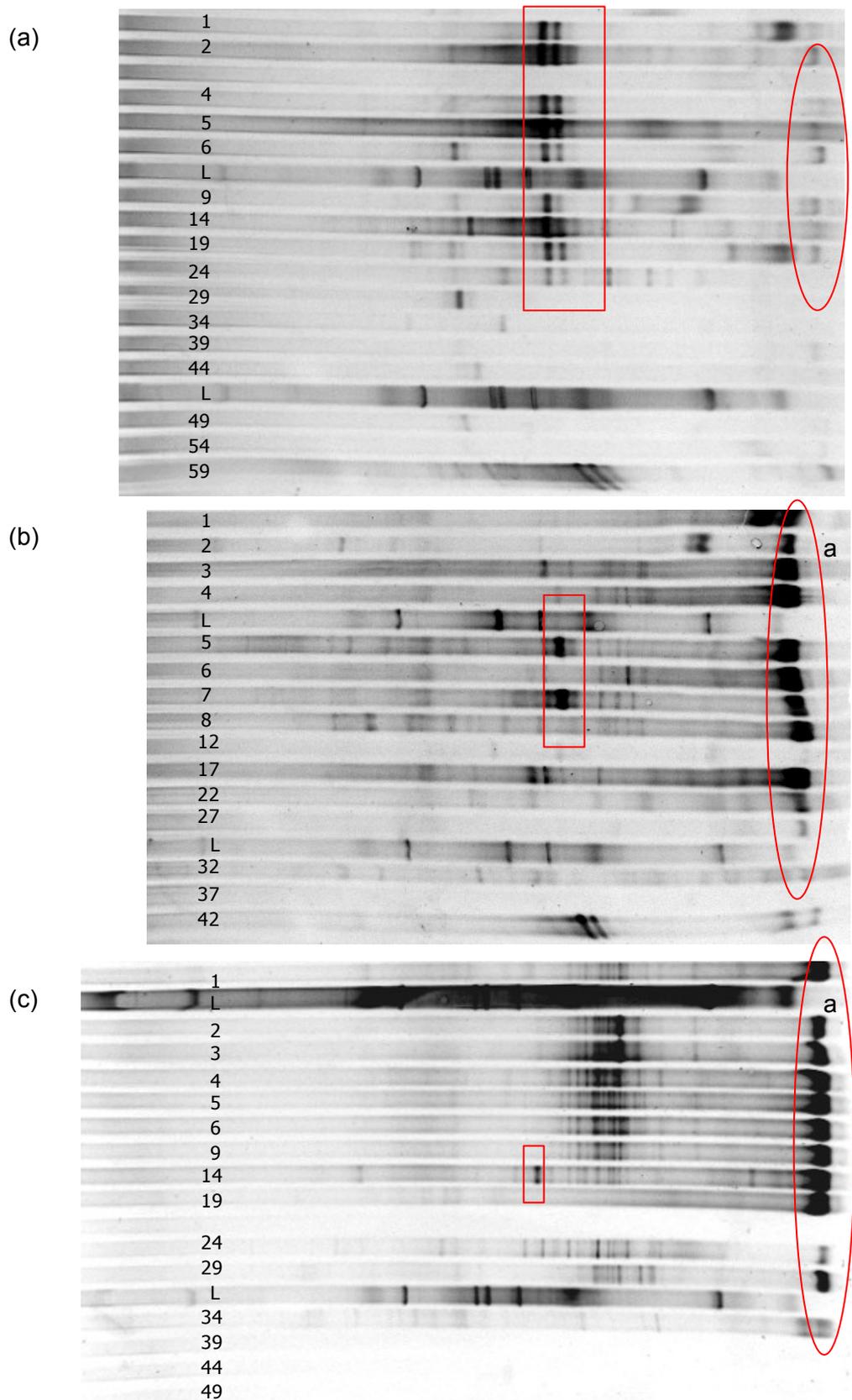


Figure 6.29. *TmoA* gene PCR-DGGE profiles of undisturbed sediment cores slices obtained from Artificial ditch (a), Leendertgracht (b) and Vijver (c) on 31/08/2005. Numbers represent the depth in the interface (cm); L: DGGE marker.

*Study of the microbial community at different seasons:*

Comparing the *tmoA* results obtained from the Artificial Ditch in cores extracted in different seasons we observed that two dominant bands which appeared in Autumn (Figure 6.30a) were also present in Winter (Figure 6.30b) and in Summer (Figure 6.30c), but not in Spring. In the Spring samples another double band appeared and it was different from the one we observed in the other seasons.

These observations, concerning the *tmoA*-like genes in function of the seasons showed us a very poor bacterial activity with some single extra bands in Summer and an extra double band in Autumn at the depths of 24 and 26 cm.

In the Leendertgracht and Vijver the diversity was higher in Summer 2005 than in Spring 2005 and in Spring 2006, when the only dominant band was “a”, already shown in Figure 6.29 and only few and faint bands were observed (data not shown).

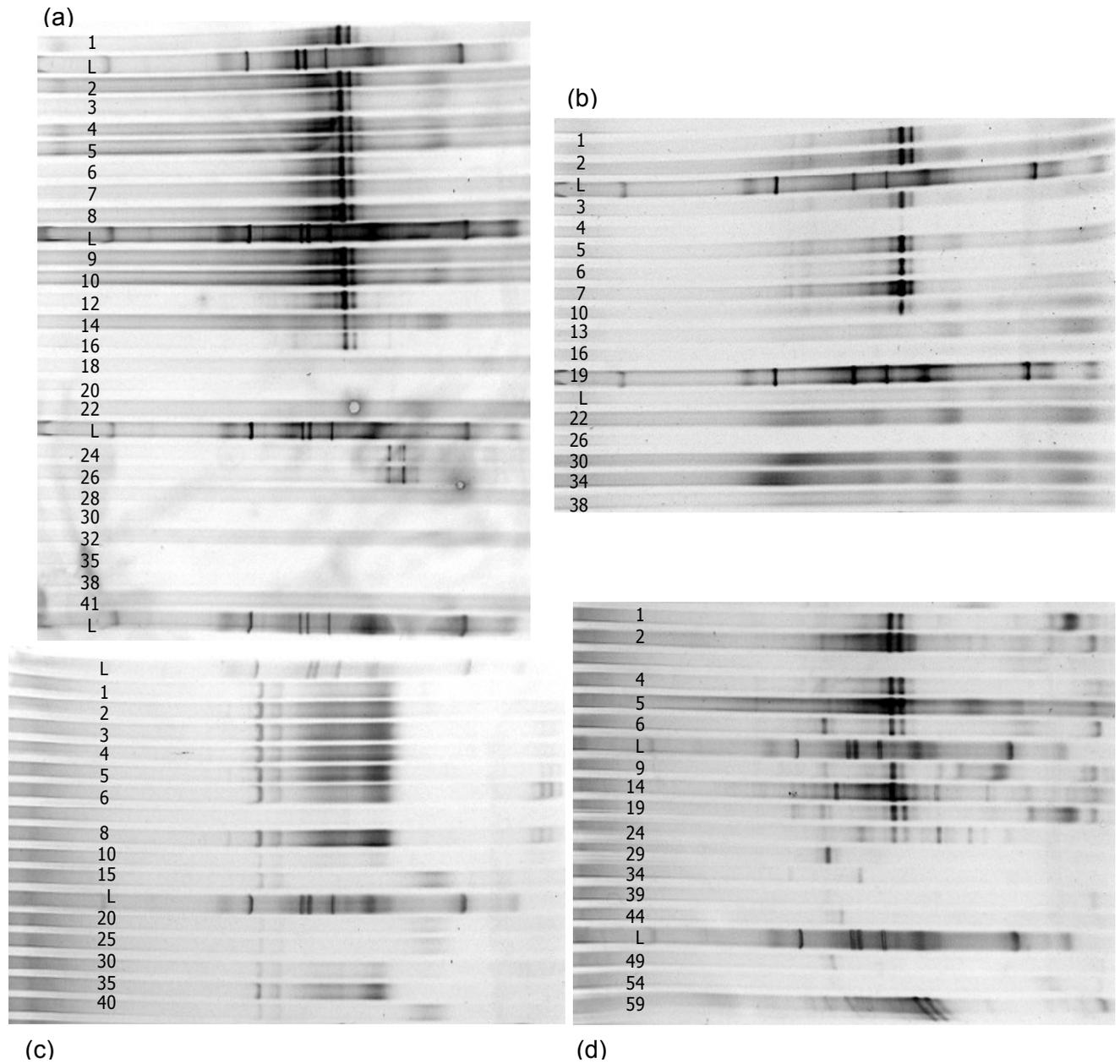


Figure 6.30. *TmoA* gene PCR-DGGE profiles of undisturbed sediment cores slices obtained from Artificial ditch in Autumn 2004 (a), Winter 2005 (b), Spring 2005 (c) and Summer 2005(d). Numbers represent the depth in the interface (cm); L: DGGE marker.

### **6.3.8 Batch degradation tests: study of the evolution of the microbial community during the incubation period**

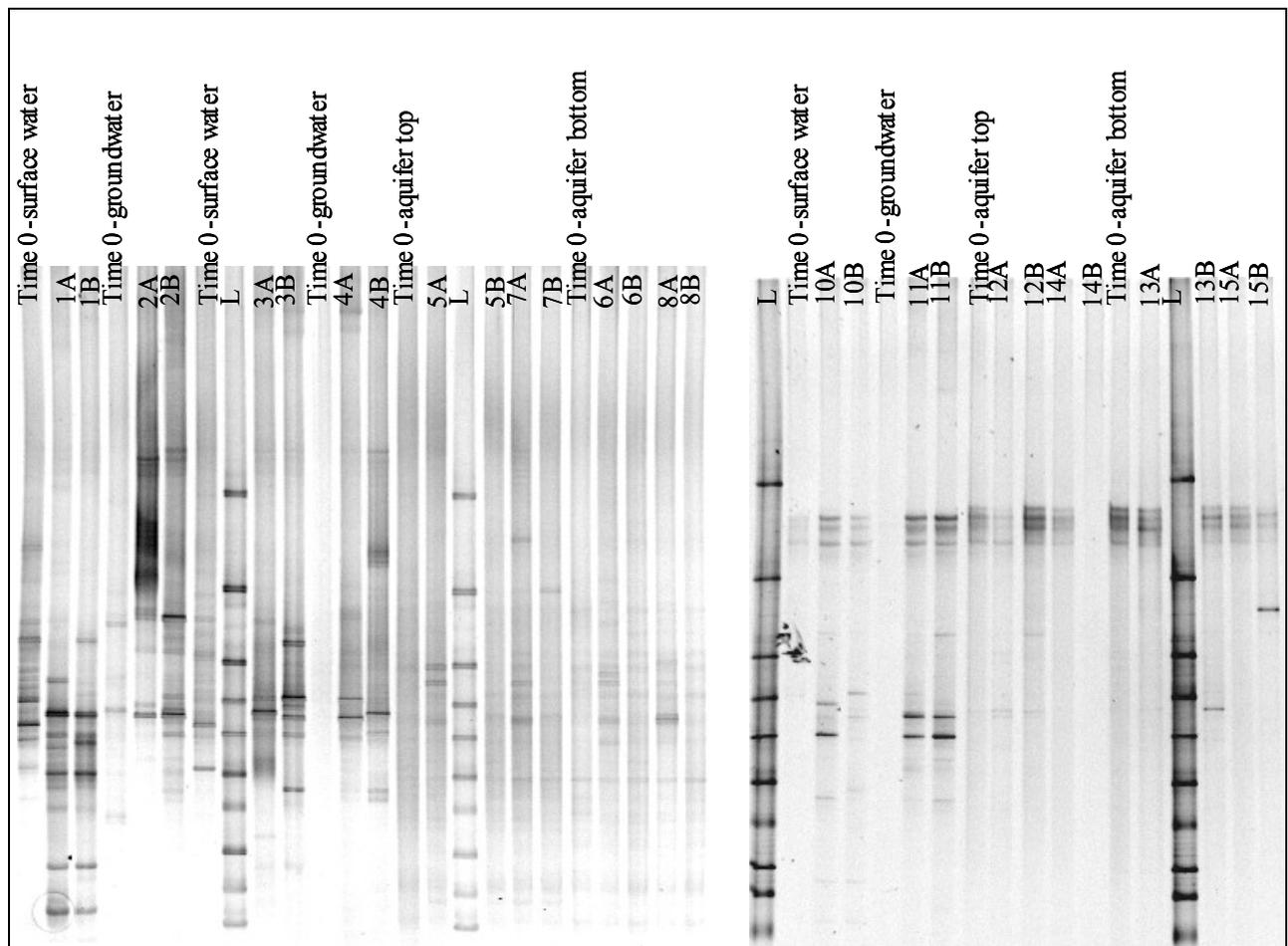
Figure 6.31 shows 16S rRNA gene DGGE profiles obtained from sediment and water samples, taken from the microcosms at the end of the incubation, compared with samples used in the set up at time 0. It was clear that in the tests where monochlorobenzene biodegradation occurred, new DGGE bands appeared.

Moreover the intensity of the band was often related to the mass of pollutant consumed. For example in tests set up with surface water from the Artificial Ditch (1A and 1B), four very strong bands appeared at the end of the incubation, after 7 and 5 mg of monochlorobenzene were degraded respectively.

These bands were apparently absent in the time 0 water sample, thus indicating that these bacteria probably grew on monochlorobenzene. The same has been observed in groundwater samples (2A and 2B: 4.5 and 6.5 mg of monochlorobenzene consumed) where only two very faint bands were detected in the time 0 sample, while 7 – 8 bands appeared at the end. Also in surface water and groundwater samples from the Leendertgracht (3A and 3B: 6.5 and 4.5 mg of monochlorobenzene; 4A and 4B: 5.5 and 3.5 mg of monochlorobenzene) new bands appeared. On the other hand in tests containing also sediment, some faint bands appeared in microcosms where oxygen was added to stimulate biodegradation (6A and 8A: 1.2 and 1.7 mg of monochlorobenzene), while no bands or even weaker ones (compared to time 0 samples) appeared where biodegradation was not stimulated (6B and 8B: 0.2 and 0.3 mg of monochlorobenzene). Batch tests carried out with sediment and water sampled in the Vijver gave DGGE patterns different from both the Artificial Ditch and the Leendertgracht. New bands were detected in microcosms set up with surface water (10A: 5 mg of monochlorobenzene) and groundwater (11A and 11B: 11 mg and 8 mg) and one band appeared in the conditions “aquifer bottom+surface water” and “aquifer bottom+groundwater” (13B and 15B: 6 mg of monochlorobenzene), where degradation was stimulated with oxygen, while no differences were observed with time 0 sediment samples, where degradation was not stimulated (13A and 15A: 1.5 mg of monochlorobenzene). The new bands

observed in the samples analysed at the end of the incubation (especially where considerable amounts of monochlorobenzene had been consumed) were mostly different from the ones observed in time 0 samples, where, in some cases, the signal was even absent.

These findings could indicate that species different from the ones dominant in time 0 samples probably grew utilising monochlorobenzene as growth substrate. No positive signals were obtained in the PCR performed with DNA extracted from the a-biotic control, thus indicating the inhibition of the microbial activity by formaldehyde.



**Figure 6.31.** 16S rRNA gene PCR-DGGE profiles of sediment samples obtained from batch degradation tests at the end of the incubation period. Numbers represent the labels of the microcosms (Table 6.8); L: DGGE marker.

Label	Description
1A	artificial ditch – surface water
1B	
2A	artificial ditch – groundwater
2B	
3A	leendertgracht – surface water
3B	
4A	leendertgracht – groundwater
4B	
5A	leendertgracht – aquifer top + surface water
5B	
6A	leendertgracht – aquifer bottom + surface water
6B	
7A	leendertgracht – aquifer top + groundwater
7B	
8A	leendertgracht – aquifer bottom + groundwater
8B	
10A	vijver – surface water
10B	
11A	vijver – groundwater
11B	
12A	vijver – aquifer top + surface water
12B	
13A	vijver – aquifer bottom + surface water
13B	
14A	vijver – aquifer top + groundwater
14B	
15A	vijver – aquifer bottom + groundwater
15B	

**Table 6.8. Labels used for batch degradation tests in Figures 6.31 and 6.32**

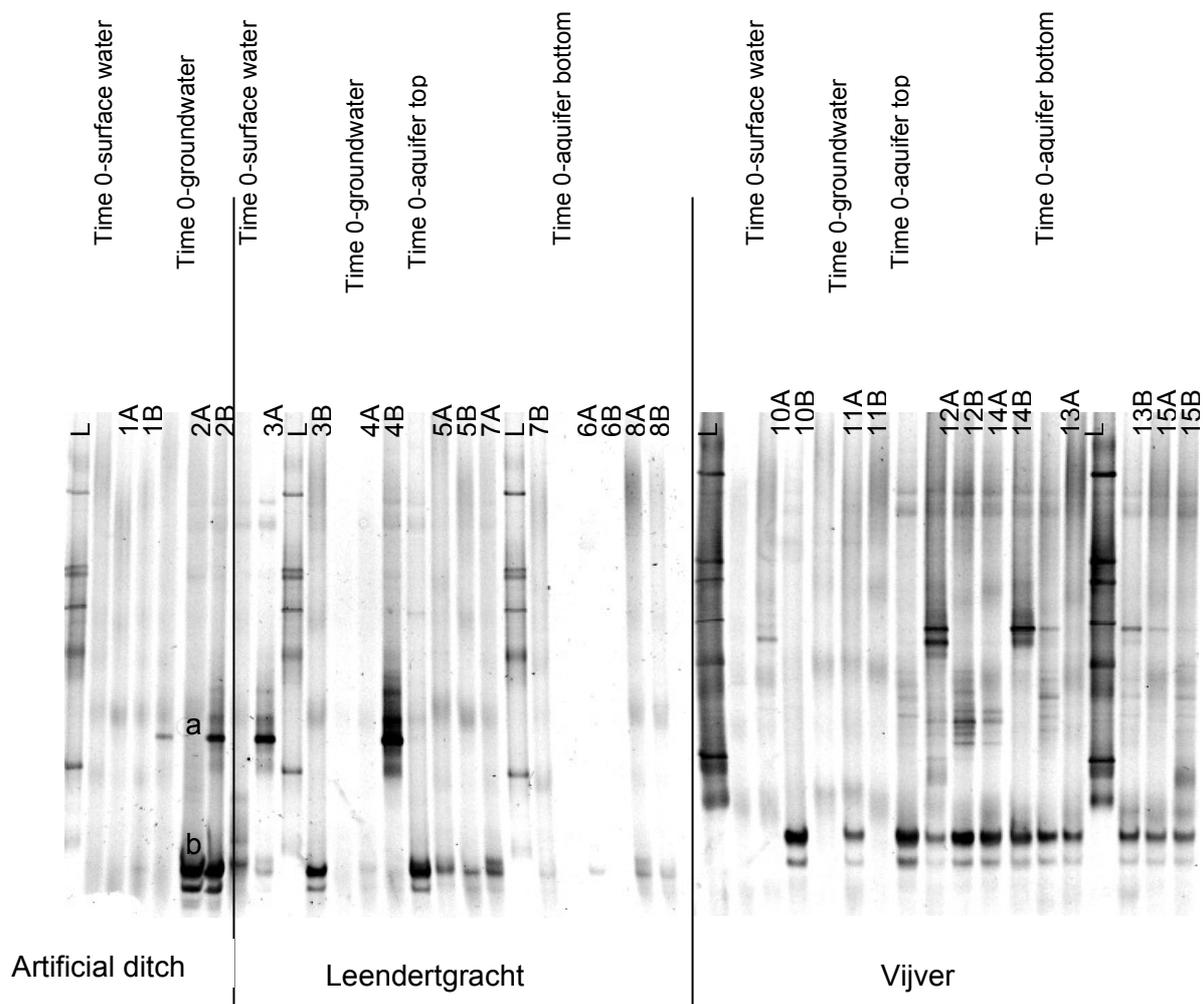
Figure 6.32 shows the fingerprints of *tmoA*-like genes in the time 0 samples used to set up the microcosms (water and sediment) and in samples collected from the flasks at the end of the incubation.

- **Artificial ditch:** in surface water the gene was not present neither at the beginning nor at the end of the incubation, while in groundwater a faint band is present and became very thick at the end (band “a”). Besides two new strong bands appeared after the 200 days incubation (samples 2A and 2B, band “b”).
- **Leendertgracht:** band “a” appeared in one duplicate in surface water (3A) while band “b” appeared in the second (3B). Band “a” was also present in groundwater at the end of the incubation. Band “b” was observed again in the time 0 top sediment and apparently became less important at the end of the

experiment while it was almost absent in the bottom sediment (end of incubation and time 0).

- **Vijver:** band “b” appeared in surface water (10B) and groundwater (11A) at the end of the experiment and two dominant bands were noticed in two cases in microcosms set up with the top sediment (12A and 14B). Apparently the diversity in the sediment from the Vijver was greater than the one of the Leendertgracht.

These results showed that in most cases new and thick bands appeared at the end of the incubation in the flasks where considerable amount of monochlorobenzene had been consumed (2A: 4.5 mg, 2B and 3A: 6.5 mg, 4B: 3.5 mg, 12A: 11.5 mg, 12B: 6 mg), while very limited differences were observed when comparing the time 0 samples with the ones where small amounts of monochlorobenzene had been degraded (5B: 0.3mg; 6B, 8B, 14A: 0.2 mg; 15A: 0.1 mg).



**Figure 6.32. *TmoA* gene PCR-DGGE profiles of sediment samples obtained from batch degradation tests at the end of the incubation period. Numbers represent the labels of the microcosms (Table 6.8); L: DGGE marker.**

### 6.3.9 Cloning and sequencing of *tmoA* gene fragments

Among the obtained samples extracted on 31/05/2005 and 31/08/2005 we selected the following ones for further research on cloning and sequencing of the *tmoA* gene:

- 31/05/05 – Artificial Ditch – 6 cm deep;
- 31/05/05 – Leendertgracht – 7 cm deep;
- 31/08/05 – Leendertgracht – 17 cm deep;
- 31/08/05 – Vijver – 4 cm deep.

*TmoA* gene amplicons were then cloned to determine the gene sequences corresponding to the DGGE bands and those clones were matched with the corresponding DGGE profiles obtained from sediment slices. For all samples, almost all dominant bands were recovered in the clone libraries.

Figure 6.33 shows DGGE profiles obtained with sample “31/08/05 - Leendertgracht – 17 cm deep” (rectangles) and fingerprints resulting from the cloning. Clones E3, E5, E11 and E12 were selected for sequencing and the deduced nucleotides sequences were blasted against the NCBI bank.

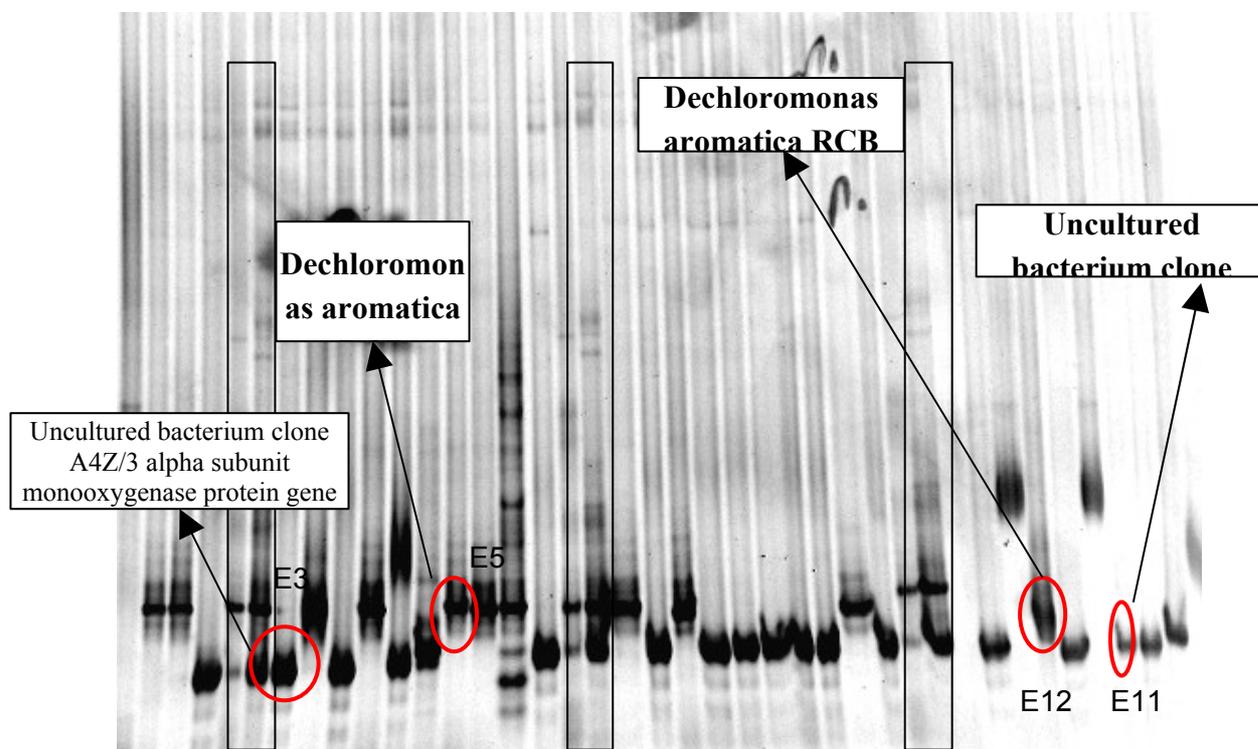


Figure 6.33. DGGE profiles obtained with sample “31/08/05 - Leendertgracht – 17 cm deep” (rectangles) and fingerprints resulting from the cloning. (*tmoA* gene).

As an example Figure 6.34 shows the result of the blast analysis for clone E5. Lines represent the sequences producing the best alignment.

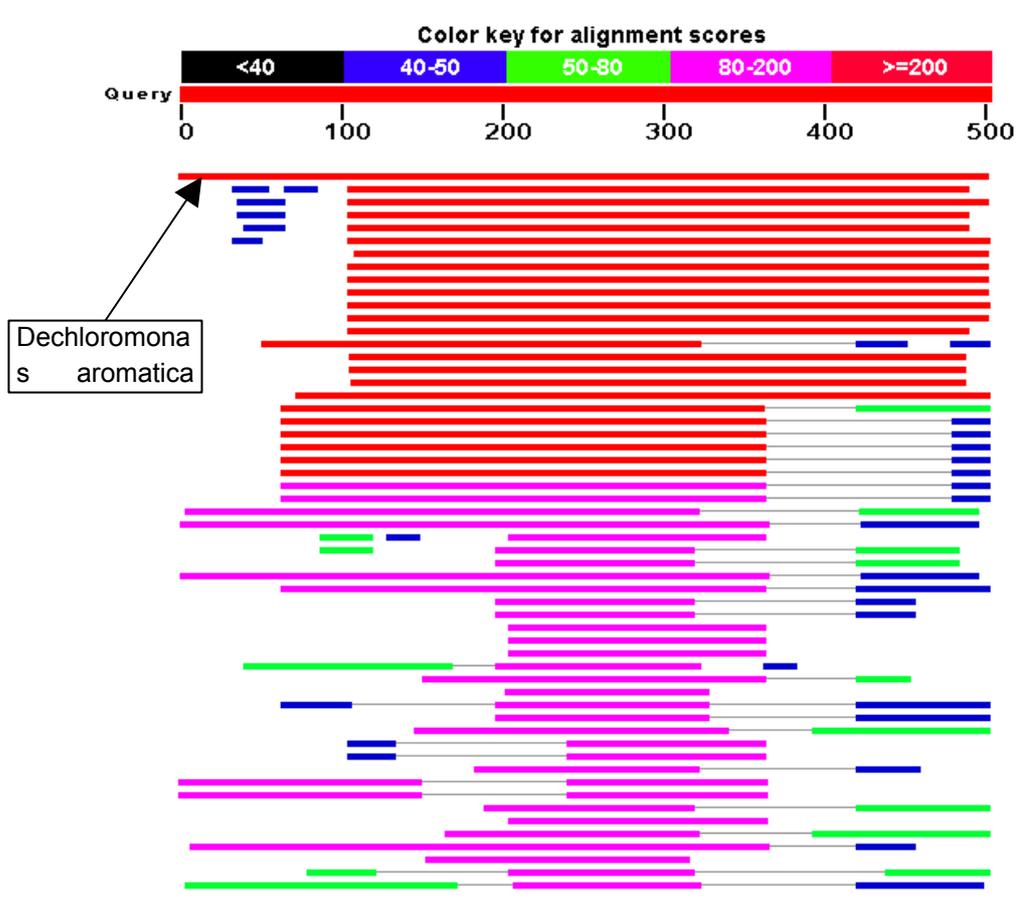


Figure 6.34. Result of the blast analysis for clone E5

Table 6.9 summarizes the results of blast analysis for our sequences and shows the nearest nucleotide matches based on blast analysis of the cloned sequences.

In the Artificial Ditch samples sequenced clones were similar to *Pseudomonas Mendocina KRI* (a *Pseudomonas* strain able to metabolise toluene) while in the samples from Leendertgracht *tmoA* sequences were similar to those of *Ralstonia Pickettii PKO1* (clone E3), also able to grow on BTEX. In several cases, clones obtained from samples taken in the Leendertgracht and in the Vijver seemed related to uncultured bacteria clones obtained from samples recovered from

BTEX contaminated sites (clones E3, E12, F5, F6, F8, F10). Clone E3 was also related with *Pseudomonas* JS150, which is able to degrade a wide range of substituted aromatic compounds, including chlorobenzene. *TmoA* sequences of clones E5, E11 and F2 were related (90% of similarity) to the sequence of *Dechloromonas aromatica* strain RCB.

*Dechloromonas aromatica* strain RCB is the only organism in pure culture that can oxidize benzene in the absence of oxygen. It can also oxidize aromatics such as toluene, benzoate, and chlorobenzoate. *D. aromatica* couples growth and benzene oxidation to the reduction of either O<sub>2</sub>, or chlorate, or nitrate. These results showed that clones obtained in the three locations were mostly different.

Moreover, also in the not contaminated location (Vijver) sequences related with the metabolism of aromatics were found. Clones strongly similar to *Dechloromonas aromatica* RCB were recovered from both the Leendertgracht and the Vijver.

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Origin	Clone designation	Nearest matches in BLAST analysis			
		Nucleotide (Accession no.)	Host	Nucleot. identities	
31/05/05 – Artificial Ditch – 6 cm	11	Toluene-4-monooxygenase gene cluster (AY552601)	<i>Pseudomonas mendocina KRI</i>	79%	
		Monooxygenase alpha subunit gene (AY504976)	<i>Arthrobacter polychromogenes AI-69</i>	80%	
	12	Toluene-4-monooxygenase gene cluster (AY552601)	<i>Pseudomonas mendocina KRI</i>	80%	
		Monooxygenase alpha subunit gene (AY504976)	<i>Pseudomonas mendocina KRI</i>	80%	
31/05/05 – Leendertgracht – 7 cm	16	seq. too short			
31/08/05 – Leendertgracht – 17 cm	E3	Alpha subunit monooxygenase protein gene (AY450333)	Uncultured bacterium clone A4Z/3	99%	
		Toluene-para-monooxygenase gene cluster (AY541701)	<b>Ralstonia pickettii strain PKO1</b>	87%	
	E5	Tbc2 gene cluster (AF282898) CP000089	<i>Burkholderia cepacia JS150</i>	87%	
		Gene for putative benzene monooxygenase (AB274231) CP000089	<i>Dechloromonas aromatica RCB</i>	90%	
	E11	Gene for putative benzene monooxygenase (AB274231) CP000089	Uncultured bacterium	93%	
		Gene for putative benzene monooxygenase (AB274231) CP000089	<i>Dechloromonas aromatica RCB</i>	90%	
	E12	Gene for putative benzene monooxygenase (AB274231) CP000089	Uncultured bacterium	92%	
		Alpha subunit monooxygenase protein gene (AY450315)	Uncultured bacterium clone A1Z/7	100%	
31/08/05 – Vijver – 4 cm	F2	Alpha subunit monooxygenase protein gene (AY450318)	Uncultured bacterium clone A1Z/10	99%	
		CP000089	<b>Dechloromonas aromatica RCB</b>	91%	
	F4	(CP00431) seq. too short (36 bp)	<i>Rhodococcus RHA1</i>	96%	
		Alpha subunit monooxygenase protein gene (AY450323)	Uncultured bacterium clone A3Z/3	97%	
	F6	Alpha subunit monooxygenase protein gene (AY450322)	Uncultured bacterium clone A3Z/2	94%	
		Toluene-4-monooxygenase gene cluster (AY552601)	<i>Pseudomonas mendocina KRI</i>	99%	
	F8	Alpha subunit monooxygenase protein gene (AY450323)	Uncultured bacterium clone A3Z/3	98%	
		Alpha subunit monooxygenase protein gene (AY450323)	Uncultured bacterium clone A3Z/3	92%	

**Table 6.9.** Sequences from clones isolated from sediment slices (*tmoA* gene).

### 6.3.10 Cloning and sequencing of *dsrB* and 16SrRNA gene fragments

*DsrB* and 16SrRNA gene amplicons obtained from sediment cores extracted from the Leendertgracht on 31/08/2005 (7 cm and 32 cm) were cloned. Concerning *dsrB* gene amplicons, at least 40 different clones were obtained. Among these clones, the ones corresponding to the most dominant bands of the original samples were sequenced. Therefore 11 samples were selected: 7 for “31/08/05 – Leendertgracht – 32 cm deep” (clones C1, C5, C9, C10, C12, C19, C20 – Figure 6.35) and 4 for “31/08/05 – Leendertgracht – 7 cm deep” (clones D5, D7, D8 and D12). Rectangles indicate fingerprints of the original samples the clones came from.

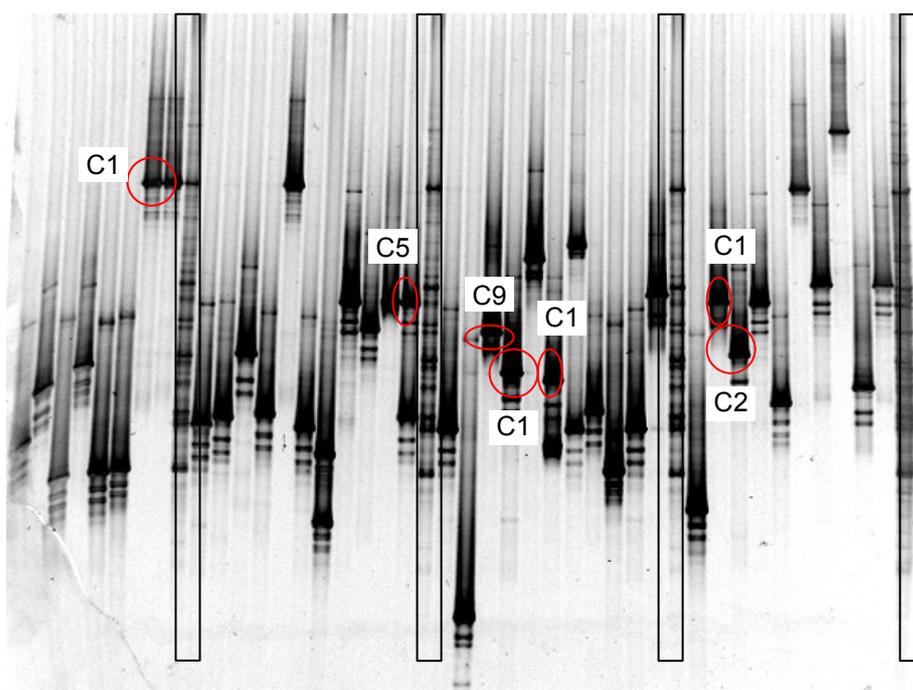


Figure 6.35. DGGE profiles obtained with sample “31/08/05 - Leendertgracht – 32 cm deep” (rectangles) and fingerprints resulting from the cloning. (*dsrB* gene).

The results of the sequencing are summarized in Table 6.10. All the recovered clones were related to uncultured sulfate-reducing bacteria.

Due to the high diversity of bands present in the original DNA sample, several different clones were obtained resulting in a great differentiation of sequences (except clones C20 and C10).

**Table 6.10. Sequences from clones isolated from sediment slices (*dsrB* gene).**

Origin	Clone designation	Nearest match in BLAST analysis			
		Nucleotide (Accession no.)	Host	Nucleot. identities	
31/08/05 Leendertgrachtde pth: 32 cm	C1	Dissimilatory sulfite reductase beta subunit ( <i>dsrB</i> ) gene (EF064998)	Uncultured sulfate-reducing bacterium isolate DGGE gel band 09	94%	
	C5	Dissimilatory sulfite reductase beta subunit (AY753141)	Uncultured bacterium clone ng7d1139	83%	
	C9	Dissimilatory sulfite reductase alpha subunit ( <i>dsrA</i> ) and dissimilatory sulfite reductase beta subunit ( <i>dsrB</i> ) genes (EF065029)	Uncultured sulfate-reducing bacterium clone LGWG24	97%	
	C10	Dissimilatory sulfite reductase alpha subunit ( <i>dsrA</i> ) and dissimilatory sulfite reductase beta subunit ( <i>dsrB</i> ) genes (EF065024)	Uncultured sulfate-reducing bacterium clone LGWG08	94%	
	C12	Seq. too short for sequencing			
	C19	Dissimilatory sulfite reductase alpha subunit-like ( <i>dsrA</i> ) gene (DQ250756)	Uncultured sulfate-reducing bacterium clone G-77	92%	
	C20	Sulfite reductase alpha subunit ( <i>dsrA</i> ) and dissimilatory sulfite reductase beta subunit ( <i>dsrB</i> ) genes (EF065024)	Uncultured sulfate-reducing bacterium clone LGWG08	93%	
31/08/05 Leendertgracht depth: 7 cm	D5	Dissimilatory sulfite reductase alpha subunit ( <i>dsrA</i> ) and dissimilatory sulfite reductase beta subunit ( <i>dsrB</i> ) genes (EF065066)	<b>Uncultured sulfate-reducing bacterium clone LGWK15</b>	95%	
	D7	Dissimilatory sulfite reductase subunit B ( <i>dsrB</i> ) gene (AY015596)	Uncultured sulfate-reducing bacterium clone UMTRAdsr624-8	88%	
	D8	Dissimilatory sulfite reductase alpha subunit-like ( <i>dsrA</i> ) gene (DQ250781)	Uncultured sulfate-reducing bacterium clone I-70	91%	
	D12	Seq. too short for sequencing			

With regard to 16SrRNA gene amplicons, 6 among the more than 40 obtained clones were sequenced (Table 6.11). Apparently *Proteobacteria* were the main group of resident bacteria, with 96% similarity (clone B15) and 98% (clone B19).

**Table 6.11. Sequences from clones isolated from sediment slices (16S rRNA gene).**

Origin	Clone designation	Closest relative in blast analysis (Accession no.) (Class)	Similarity
31/08/05 Leendertgracht depth: 32 cm	A7	Uncultured <i>Desulfosarcina</i> sp. clone CBII140 (DQ831553)( <i>Proteobacteria</i> )	90%
	A16	uncultured bacterium (AY711541)	93%
	A18	uncultured actinobacterium (AY307865)	95%
31/08/05 Leendertgracht depth: 7 cm	B15	Uncultured beta proteobacterium clone JG36-GS-10 (AJ582037)	96%
	B16	Uncultured delta proteobacterium clone Hyd89-52 (sequence too short)	-
	B19	AY221613.1	98%

#### **6.4 DISCUSSION**

This work deals with the study of monochlorobenzene degradation in the interface between groundwater and surface water. The catabolic potential of the microbial community present in the interface was studied in batch degradation tests setup with sediments sampled from three different location operated at low oxygen concentrations. The diversity of the microbial community was also studied using molecular techniques (PCR/DGGE) and the presence of catabolic genes was investigated.

Our results indicate that aquifer material (top and bottom) from the three studied locations are characterised by a high degradation potential. Monochlorobenzene biodegradation was only limited by a lack of oxygen: in the batch tests the indigenous microorganisms were able to degrade up to 50 mg/l monochlorobenzene when sufficient oxygen was available, with no need to add

nutrients or other amendments. Apparently, no degradation occurred below oxygen concentrations of 1.5-2 mg/l. Moreover, the catabolic potential seemed not to be related with depth: degradation took place indifferently in the microcosms containing both top and bottom aquifer material. The fact that significantly different amounts of oxygen were required to degrade approximately the same amounts of monochlorobenzene in tests setup with different aquifer materials (from the Leendert gracht and the Vijver) could be explained considering that the total organic matter content was rather different in the two situations. Sediment material used to setup microcosms contained on average 2.45% organic matter in the Leendertgracht while only 0.95% in the Vijver. The probable presence of other potential carbon sources and their preferential use by microorganisms could have resulted in a fast oxygen consumption preceding monochlorobenzene degradation. For the same reason oxygen concentrations in microcosms containing only water remained higher and monochlorobenzene degradation went on quickly, requiring only a few oxygen spikings. The specific amount of oxygen ( $\text{mg}_{\text{oxygen}}/\text{mg}_{\text{monochlorobenzene}}$ ) needed was thus even lower compared to the sediment tests.

Surprisingly, a high degradation potential was present in sediment material from the Vijver too. This is in contrast with other studies (Van der Meer *et al.*, 1998; Dermietzel and Vieth, 2001), isolating CB-degrading bacteria only within the contaminated zone, while degradation potential was very low or even absent in uncontaminated subsurface material. Possibly, CB-degrading bacteria arose elsewhere and was transported to the Vijver zone because of the closeness of the two locations.

Unfortunately, the chloride concentration of groundwater and surface water samples was very high (up to 3000 mg/l) making it impossible to use chloride release analysis to determine whether complete monochlorobenzene mineralization had occurred. Furthermore, a study (Vogt *et al.*, 2003) reporting chlorobenzene degradation in microaerophilic conditions by five strains isolated on chlorobenzene as sole carbon source, showed the accumulation of the toxic intermediate 3-chlorocatechol. In one of these strains a clear relationship was demonstrated between the presence of 3-chlorocatechol in the medium and low

oxygen concentration. However, no 3-chlorocatechol was detected under oxygen-limited reactor operation in liquid samples of the Bitterfeld pilot plant *in situ* reactor (Vogt *et al.*, 2004).

*TmoA* gene-PCR-DGGE analysis carried out at the end of the microcosms incubation on sediment contained in the flasks showed a different DGGE pattern between the aquifer materials from the two locations (Leendertgracht and Vijver), but also the presence of some common bands (band "b"-Figure 6.32). New bands appeared at the end of the incubation in some microcosms probably as a result of monochlorobenzene degradation by these microorganisms. Some of these bands are absent or not dominant in DNA extracted from the analyzed cores from the Leendertgracht and the Vijver. This could indicate that microorganisms present *in situ* are not active in monochlorobenzene degradation. Nevertheless this is not surprising, because no chlorobenzene was detected in the Vijver, while its concentration in the Leendertgracht was maybe too low to stimulate and sustain the growth of microorganisms. These findings seem confirmed by the observation that also in 16S rRNA DGGE pattern new bands (absent or very faint at Time 0) appeared after the incubation period were, thus probably indicating that these bacteria were present at concentrations below the detection limit and significantly grew using chlorobenzene as energy source.

Besides, the low oxygen concentration measured in groundwater and surface water seems to suggest that chlorobenzene degradation is scarcely probable in the sediment: results from batch degradation tests indicated that at least 1.5-2 mg/L of oxygen are required to sustain the degradation process but the contribution given by surface water is probably insufficient due to its low oxygen concentration (often below 4 mg/l). Furthermore oxygen consumption by sediments has to be taken into account. This consumption, also depending on the organic matter concentration, can reach high levels and significantly reduce the oxygen amount available for chlorobenzene degradation. The prevailing redox conditions at different depths in the interface need investigation through further research. The very low chlorobenzene concentrations detected in surface water compared to groundwater are probably a result of dilution and sorption in the organic matter of the sediment (up to 15 mg/kg of MCB; Figures 6.13 and 6.14).

Cloning and sequencing of *tmoA* sequences showed that cloned sequences were mostly related with microorganisms involved in the metabolism of BTEX. One sequence was related in third similarity with *Pseudomonas* JS150, which can grow on a wide variety of aromatic compounds, including monochlorobenzene. Possibly, these microorganisms have monooxygenases catalyzing the first step of the reaction, but unable to completely mineralize chlorobenzene. Initial steps of chlorobenzene degradation give rise to 3-chlorocatechol which is usually degraded via the *ortho* pathway described by Reineke and Knackmuss (1984). The initial attack is by a dioxygenase acting like toluene dioxygenase and benzene dioxygenase. The initial oxidation results in the formation of a *cis*-dihydrodiol. Subsequent ring fission and elimination of chloride leads to the mineralization of these compounds. The key enzyme is the pyrocatechase II (Dorn *et al.*, 1978; Reineke *et al.*, 1984) that converts chlorocatechols into chloro-*cis,cis*-muconic acids. The absence of this enzyme in organisms with initial oxygenases with broad substrate specificities may lead to the accumulation of chlorocatechols or to the misrouting of chlorocatechol down the *meta* cleavage pathway, ultimately resulting in cell death. Further research should focus on isolating and characterizing chlorobenzene-degraders present in the interface.

PCR-DGGE analysis of the eubacterial 16S rRNA gene showed, in the first place, that in general the structure of the microbial community is very diverse and changes depending on depth., significant changes were frequently observed from 5-10 cm downwards. Koizumi *et al.* (2003) also observed vertical changes of a bacterial community structure in a mesophilic lake sediment by DGGE analysis of amplified 16S rDNA and reversely transcribed 16S rRNA fragments. They noticed that the diversity indices obtained from the 16S rDNA-based DGGE-profiles were greater than those obtained from the 16S rRNA-based DGGE profiles. The diversity of inactive bacteria (DNA level) did not change drastically in function of depth since they were only influenced by bacteria that accumulated in association with sedimentation. In contrast, the diversity of active bacteria (RNA level) decreases with sediment depth. More specifically, the rRNA-based dendrogram showed a significant difference between the upper layers (0-2, 2-5, and 5-8 cm) and the lower ones (8-11, 11-14, 14-17, and 17-20 cm).

In most cases a huge number of very faint bands was observed together with some dominant thick bands, thus confirming that a very diverse bacterial community, dominated by a few species, is harboured in the interface. The depending-on-depth relevant changes in the community's structure are probably related with the gradient in the redox conditions. The microbial communities from the three sampling locations appeared considerably different although some similar bands were observed. In the Artificial ditch diversity seemed higher and more dominant bands were present; this could be caused by the high concentration of chlorobenzene. The presence of high concentration of the contaminant could have stimulated the development and growth of microorganisms able to use it as carbon and energy source (evidenced by the presence of the dominant bands). This is in contrast with another study carried out in a BTEX-contaminated site (Hendricks *et al.*, 2005), demonstrating that the uncontaminated area was characterized by a much more diverse bacterial community than the contaminated one. Alfreider *et al.*, (2002) studied the microbial diversity in an *in situ* reactor system treating MCB-contaminated groundwater. They observed that the significance of specific pollutants for the structure within the bacterial assemblages in contaminated groundwater ecosystems is hard to assess, because

various physical, chemical and biological factors may often mask anthropogenic effects. They also observed differences in the microbial community structure between sediments and groundwater samples (as we did) and they explained it referring to the differences between these two habitats, attached and free-living bacteria. Very little is known about the differences in the microbial community structure between original sediment and groundwater. Roling *et al.*, (2001) found that pollution in a landfill leachate-contaminated aquifer did not affect the particle-bound microorganisms, but groundwater community structure was clearly affected by pollution and redox processes, thus supporting the hypothesis that bacteria attached to sediment particles and forming biofilms usually consists of stable communities which are less influenced by changing environmental factors.

Conversely the Leendertgracht and the Vijver were characterized by a great diversity and a more uniform distribution of vanishing bands.

The characterization of the bacterial community revealed the presence of sequences related to *Proteobacteria* in agreement with Alfreider *et al.*, (2002) who found this bacteria in chlorobenzene-contaminated groundwater and sediment samples from their *in situ* reactor.

PCR-DGGE analyses of 16S rRNA gene and *tmoA* gene showed a greater diversity in cores extracted in Summer. This is probably due to the more intense microbial activity depending on higher temperature (Figures 6.12, 6.26 and 6.30).

*DsrB* gene fragments cloning and sequencing confirmed that a very high diversity of sulfate-reducing bacteria is present in each of the three locations, in every season, regardless of depth. This seems in contrast with the presence of bacteria carrying *tmoA* like genes in the first 10-20 cm sediment layers. The presence of anaerobic obligate sulfate reeducing bacteria can be explained assuming that the sediment might contain both aerobic and anaerobic micro-niches. The co-occurrence of sulfate-reducing bacteria and aerobic organisms has been shown before in aerobic wastewater biofilms (Ito *et al.*, 2002b; Ito *et al.*, 2002a; Kühl and Jorgensen, 1992; Okabe *et al.*, 1999). Recently, Shi *et al.* (1999) showed the co-occurrence of these organisms in a fuel contaminated aquifer, where the conditions were micro-aerophilic to anaerobic.

Anaerobic bacteria can be responsible of the reduction of highly chlorinated benzenes to chlorobenzene and of the accumulation of monochlorobenzene in the aquifer. This could also provide an explanation for the detection of di- and especially tri-chlorobenzenes concentrations which were significantly lower if compared to monochlorobenzene.

Studies on the effect of the interface between groundwater and surface water on the degradation of pollutants is limited. Lendvay *et al.* (1998, 1999) studied the biogeochemical effects of a large surface water on a chloroethene contaminated anaerobic groundwater at the groundwater/surface water interface (GSI) using spatially discretized multilevel arrays. Concentrations of methane and chloroethene decreased as the groundwater became increasingly oxidized along the GSI in shallow sample points impacted by infiltration of oxygenated lake water. Cis-1,2-dichloroethene remained unchanged or increased at the same locations indicating that the decrease in methane and chloroethene was not due to dilution effects from lake water infiltration. Schwarzenbach *et al.* (1983) investigated, by the installation of a network of observation wells, the transport and fate of chlorinated hydrocarbons, alkylated benzenes, and chlorinated phenols during natural infiltration of river water to groundwater. Biotransformation was observed in the interface for all alkylated C1-C4-benzenes, naphthalene, the methylnaphthalenes, and 1,4-dichlorobenzene. Alkylated benzenes were always eliminated within the first few meters of infiltration, even at temperatures below 5°C. The biotransformation of 1,4-dichlorobenzene occurred at a lower rate while chloroform, 1,1,1-trichloroethane, trichloroethylene, and tetrachloroethylene were not degraded in the interface. With respect to these last compounds, bank infiltration is thus ineffective as a first step in the treatment of river water for water supplies. Feris *et al.* (2003) investigated through the use of microbial techniques (DGGE, 16S rRNA phylogeny, phospholipid fatty acid analysis, direct microscopic enumeration, and quantitative PCR) the effect of a range of sediment metal loads on the microbial community inhabiting the hyporheic zone of six different rivers. They found that metal stress in fluvial environments does not reduce biomass, diversity, or productivity rather the structure of microbial communities changes. It appeared that the hyporheic-zone communities exhibited

a decrease in  $\alpha$ -proteobacteria but no significant change in  $\beta$ -proteobacteria and an increase in  $\gamma$ -proteobacteria with increasing metal contamination. They concluded from their study that more studies are needed to define or describe the taxa that comprise the communities in the interface that are affected by pollutants and that this could be mainly achieved by the use of molecular techniques.

## **6.5 CONCLUSIONS**

In this study we investigated the biodegradation of chlorobenzene in the interface between groundwater and surface water. Main goal was to understand if the microbial community present in the interface was involved in the degradation of pollutants passing through the sediment layer and if the interface can therefore have an active role in the breakdown of pollutant in groundwaters reaching surface waters.

Therefore we carried out batch degradation tests and we applied molecular techniques (PCR-DGGE), in order to study the catabolic potential of the microbial community present in the interface, using sediment material extracted from the interface in three different locations and in different seasons.

Results of batch tests carried out at low oxygen concentrations (such as the presumed *in situ* conditions) showed that a high chlorobenzene degradation potential is present, regardless of depth in the interface. Biodegradation of chlorobenzene was only limited by a lack of oxygen. Furthermore we found the catabolic gene *tmoA* (involved in the initial step of the degradation of BTEX compounds, similar to monochlorobenzene) everywhere. This gene was only present down to a depth of 10-20 cm, indicating that a shortage of oxygen possibly prevents its presence in the microbial population of the deeper layers. Further research will have to investigate whether mineralization of chlorobenzene is complete or if it is a partial transformation, possibly resulting in the accumulation of the toxic metabolite chlorocatechol. Further research will also have to investigate the real *in-situ* situation in order to assess the presence of the right conditions for biodegradation.

Molecular tests also indicated that the structure of the microbial community significantly changed depending on depth. Besides, communities from the three studied locations were mostly different, while similarities were observed in each location throughout the whole year.

## **CONCLUSIONS**

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Especially in the last 50 years of industrial development, the amount and variety of hazardous substances has drastically increased. Among them, halogenated compounds, are widespread air, water, soil, and sediment pollutants; they are recalcitrant molecules resistant to mineralization due to the stability of their carbon-halogen bond. Since these compounds have existed for millions of years, there are naturally occurring strains of bacteria which have evolved to break down halogenated compounds, thus opening up the possibility for bioremediation treatment of contaminated sites.

Chlorinated aliphatic and aromatic hydrocarbons are among the most common contaminants of soils, groundwaters and sediments. Several studies showed that most of these pollutant can be biodegraded by single bacterial strains or mixed microbial populations via aerobic direct metabolism or cometabolism.

In this thesis, two studies have been carried out concerning different situations where bioremediation processes of chlorinated hydrocarbons were involved.

The first experimental work consisted in the **study of microbial consortia able to degrade a mixture of 6 CAHs** (chlorinated aliphatic hydrocarbons) via aerobic cometabolism. We studied the long-term growth process of two microbial consortia using different primary substrates (methane and propane) and effective in the aerobic cometabolic biodegradation of a mixture of 6 chlorinated aliphatic hydrocarbons (CAHs), and the effectiveness of these consortia as inocula for the bioaugmentation of different types of aquifer materials. The main goals of the study included:

- to verify the maintenance of the consortia's capacity to degrade the 6-CAH mixture during a prolonged process of microbial growth in the presence as well as in the absence of the 6-CAH mixture;

- to verify the consortia's ability – after a prolonged growth process - to lead to the rapid onset of biodegradation of the CAH mixture upon inoculation in slurry microcosms set up with aquifer materials taken from sites with different physical-chemical characteristics;
- to develop a third consortium able to combine the best characteristics of the methane-utilizing and of the propane-utilizing consortia object of the study: in fact, a previous study had shown that, while both consortia were effective in the aerobic cometabolic biodegradation of VC and cis-DCE, the methane-utilizing biomass had a higher capacity to transform trans-DCE, whereas the propane-utilizing one was more effective towards 1,1,2-TCA, 1,1,2,2-TeCA and, secondarily, TCE;
- to characterize in terms of specific CAH depletion rates and degree of mineralization of the organic Cl the best methane-utilizing and the best propane-utilizing consortium obtained as a result of the inoculation in the microcosms set up with different aquifer materials.

The propane-utilizing consortium generally proved the most effective one, being able to biodegrade vinyl chloride, cis- and trans-1,2-dichloroethylene, trichloroethylene, 1,1,2-trichloroethane and 1,1,2,2-tetrachloroethane at all the CAH concentrations tested.

Both consortia maintained unaltered CAH degradation capacities during a 300-day growth period in the absence of the CAHs and were effective in inducing the rapid onset of CAH depletion upon inoculation in slurry microcosms set up with 5 types of aquifer materials.

A consortium developed in microcosms supplied with both methane and propane combined the best degradation capacities of the two single-substrate consortia.

The degree of conversion of the organic Cl to chloride ion was equal as an average to 90%.

These results indicated that a large amount of inoculum potentially useful in bioaugmentation treatments of CAH-contaminated sites could be grown in liquid-

gas bioreactors in the absence of CAHs and soil, starting from small amounts of biomass suspension. The use of a methane-propane-utilizing consortium would result in the best degradative performances.

The second study dealt with **monochlorobenzene biodegradation in the interface between groundwater and surface water**. Soil remediation in practice often consists of the application of extensive techniques for the active removal of the contamination source and remediation of the plume. Objects of risk are often surface water systems. There are strong indications that the interface between groundwater and surface water plays an important role in the natural degradation of organic contaminants. This is especially the case for mobile contaminants that are persistent in anaerobic subsurface environment, but mineralized relatively easy under more oxidized environmental conditions (e.g. chlorobenzene or vinyl chloride).

Main goal was investigating the natural pollutant degradation capacity of the aquifer zone representing this interface. The interface can be considered a zone with changing redox conditions characterized by specific degradation potential for pollutants passing through as a result of steep physico-chemical gradients. Thus the catabolic potential and the structure of the microbial community present in the interface between groundwater and surface water were studied to find out whether bacteria present in the interface are involved in pollutants degradation. Therefore batch degradation tests and molecular analyses (PCR-DGGE) were carried out on aquifer material extracted at different depths in the interface in three locations characterized by different monochlorobenzene contamination levels. Chlorobenzene degradation was studied in batch tests under oxygen-limited conditions in order to simulate the *in situ* conditions. 16S rRNA PCR-DGGE analysis were carried out on undisturbed sediment cores extracted from the three studied locations in different seasons to detect the presence of catabolic genes at different depth in the interface and to study the structure of the microbial community.

Results from batch degradation tests indicated that the position in the interface did not have any effect on the chlorobenzene degradation and the process was exclusively limited by a lack of oxygen. Up to 50 mg/l of monochlorobenzene were consumed in 20 days in both aquifers, and also in groundwater and surface water, when sufficient oxygen was available (1.5–2 mg/l)

The structure of the microbial community changed in function of depth. Moreover the structure of the community appeared different in the three locations while significant similarities were observed in samples extracted in each location in different seasons. Cloning and sequencing allowed to identify the dominant bands in the DGGE pattern as belonging to the group of *Proteobacteria*. Bacteria carrying *tmoA*-like genes were mostly related to BTEX degraders: *Pseudomonas Mendocina KRI*, *Ralstonia Pickettii PKO1*, *Dechloromonas aromatica* strain RCB.

It is still unclear if bacteria corresponding to these DGGE bands play a role in chlorobenzene degradation. The only degradative gene detected until now is the mono-oxygenase *tmoA* (involved in the degradation of BTEX, structurally similar to monochlorobenzene) thus being probably involved in its degradation.

## BIBLIOGRAPHY

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Adamson, D.T. and Parkin, G.F., 1999, Biotransformation of mixtures of chlorinated aliphatic hydrocarbons by an acetate-grown methanogenic enrichment culture, *Water Research*, Volume 33, Number 6, p. 1482–1494.

Adrian, L., Manz, W., Szewzyk, U. and Gorisch, H., 1998, Physiological characterization of a bacterial consortium reductively dechlorinating 1,2,3- and 1,2,4-trichlorobenzene, *Applied and Environmental Microbiology*, Volume 64, Number 2, p. 496–503.

Adrian, L. and Görisch, H., 2002, Microbial transformation of chlorinated benzenes under anaerobic conditions, *Research in Microbiology*, 153, p. 131-137.

Aelion, M.C., Swindoll, C.M. and Pfaender, F.K., 1987, Adaptation to and biodegradation of xenobiotic compounds by microbial communities from a pristine aquifer, *Applied and Environmental Microbiology*, 53, p. 2212-2217.

Agency for Toxic Substances and Disease Registry, 1990b, Toxicological profile for 1,4-dichlorobenzene, U.S. Department of Health and Human Services, Public Health Service, Atlanta, Georgia, p. 478, online at <http://www.atsdr.cdc.gov/toxprofiles/tp10.html>.

Agency for Toxic Substances and Disease Registry, 1990c, Toxicological profile for 1,1-dichloroethane, U.S. Department of Health and Human Services, Public Health Service, Atlanta, Georgia, p. 115, online at <http://www.atsdr.cdc.gov/toxprofiles/tp133.htm>.

Agency for Toxic Substances and Disease Registry, 1998, Toxicological profile for chloroethane, U.S. Department of Health and Human Services, Public Health Service, Atlanta, Georgia, p. 162, online at <http://www.atsdr.cdc.gov/toxprofiles/tp105.html>.

Agency for Toxic Substances and Disease Registry, 2004, Draft toxicological profile for 1,1,1-trichloroethane, U.S. Department of Health and Human Services, Public Health Service, Atlanta, Georgia, p. 382, online at <http://www.atsdr.cdc.gov/toxprofiles/tp70.html>.

Agency for Toxic Substances and Disease Registry, 2005, Draft toxicological profile for benzene, U.S. Department of Health and Human

Services, Public Health Service, Atlanta, Georgia, p. 147, online at <http://www.atsdr.cdc.gov/toxprofiles/tp3.html>.

Akkermans, A.D.L., van Elsas, J.D. and de Bruijn F.J., 1995, Molecular Microbial Ecology Manual. Kluwer Academic Publishers, Dordrecht, The Netherlands, p. 1-488.

Alfreider, A., Vogt, C. and Babel, W., 2002, Microbial diversity in an *in situ* reactor system treating monochlorobenzene contaminated groundwater as revealed by 16S ribosomal DNA analysis, *System. Appl. Microbiol.* 25, p. 232-240.

Alfreider, A., Vogt, C. and Babel, W., 2003, Expression of chlorocatechol 1,2-dioxygenase and chlorocatechol 2,3-dioxygenase genes in chlorobenzene-contaminated subsurface samples, *Applied and Environmental Microbiology*, Volume 69, Number 3, p. 1372-1376.

Alvarez-Cohen, L. and McCarty, P.L., 1991, A Cometabolic Biotransformation Model for Halogenated Aliphatic Compounds Exhibiting Product Toxicity, *Environmental Science and Technology*, Volume 25, Number 8, p. 1381-1387

Alvarez-Cohen, L. and Speitel, G.E., 2001, Kinetics of Aerobic Cometabolism of Chlorinated Solvents, *Biodegradation*, Volume 12, p.105-126.

Amann, R., Binder, B.J., Olson, R.J., Chisholm, S.W., Devereux, R. and Stahl, D.A., 1990, Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations, *Applied and Environmental Microbiology*, 56, p. 1919-1915.

Amann, R., Ludwig, W. and Schleifer, K.H., 1995, Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation, *Microbiol. Rev.*, 59, p. 143-169.

Amann, R. and Kuhl, M., 1998, *In situ* methods for assesment of microorganisms and their activities, *Current Opinion in Microbiology*, Volume 1, p. 352-358.

Amann, R., Fuchs, B.M. and Behrens, S., 2001, The identification of microorganisms by fluorescence *in situ* hybridization, *Current Opinion in Biotechnology*, Volume 12, p. 231-236.

Anderson, J.E. and McCarty, P.L., 1996, Effect of three chlorinated ethenes on growth rates for a methanotrophic mixed culture, *Environ. Sci. Technol.*, 30, p. 3517-3524.

Anderson, R.T. and Lovley, D.R., 1997, Ecology and Biogeochemistry of In Situ Groundwater Remediation, *Advances in Microbial Ecology*, Volume 15, Plenum Press, New York, p. 289-350.

Archer, W.L., 1979, Other chloroethanes, in Kirk-Othmer Encyclopedia of chemical technology, 3rd ed., Volume 5, p. 722–742.

Arneth, J.D., Milde, G., Kerndorff, H. and Schleyer, R., 1989, The 15 most frequently detected organic compounds in ground water at waste disposal sites in Germany and the U.S, in Baccini, Peter, ed., *The Landfill — Reactor and Final Storage*, Swiss Workshop on Land Disposal of Solid Wastes Gerzensee, March 14 –17, 1988: Lecture Notes in Earth Sciences, New York, N.Y., Springer-Verlag, Inc., p. 399.

Aronson, D., Citra, M., Shuler, K., Printup, H. and Howard, P.H., 1999, Aerobic biodegradation of organic chemicals in environmental media — A summary of field and laboratory studies, prepared for U.S. Environmental Protection Agency by Environmental Science Centre, Syracuse Research Corporation, North Syracuse, N.Y., SRC TR 99-002, p. 189.

Arp, D.J., Yeager, C.M. and Hyman, M.R., 2001, Molecular and cellular fundamentals of aerobic cometabolism of trichloroethylene, *Biodegradation*, 12, p. 81-103.

Arturo, S.M., Tamanai, S.Z., Thomas, D., and Cormier, M., 1996, Variations in R-plasmid DNA concentrations of *Escherichia coli* during starvation in sewage and brackish waters, *J. Appl. Bacteriol.*, 80, p. 117–123.

Australian Department of Environment and Heritage, 2003, National Pollutant Inventory, volatile organic compound definition and information, version 2.4, Department of Environment and Heritage, Commonwealth of Australia, p. 4, online at <http://www.npi.gov.au/handbooks/pubs/voc.pdf>.

Azadpour-Keeley, A., Russell, H.H. and Sewell, G.W., 1999, Microbial processes affecting monitored natural attenuation of contaminants in the subsurface, Ada, Okla., National Risk Management Research Laboratory, Subsurface Protection and Remediation Division, Robert S. Kerr Environmental Research Centre, EPA/540/S-99/001, p. 18.

Bader, D., 1999, The Use of Constructed Wetlands to Phytoremediate Explosives-Contaminated Groundwater at the Milan Army Ammunition Plant, Milan, Tennessee (abstract), Arlington, VA, Environmental Security Technology Certification Program (ESTCP), online at <http://oaspub.epa.gov/eims/eimsapi.dispdetail?deid=1875>.

Baldwin, B.R., Nakatsu, C.H. and Nies, L., 2003, Detection and enumeration of aromatic oxygenase genes by multiplex and real-time PCR, *Appl. Environ. Microbiol.*, Volume 69, p.3350-3358.

Balcke, G.U., Turunen, L.P., Geyer, R., Wenderoth, D.F. and Schlosser, D., Chlorobenzene biodegradation under consecutive aerobic-anaerobic conditions, *FEMS Microbiology Ecology*, 49, p. 109-120.

Battelle, 1997, Engineering Evaluation and Cost Analysis for Bioslurper Initiative (A005), Brooks AFB, TX, AFCEE, online at <http://www.afcee.brooks.af.mil/products/techtrans/download/bioslu01.pdf>.

Becker, P.M. and Stottmeister, U., 1998, General (Biolog GN) versus site-relevant (pollutant-dependent) sole-carbon-source utilization patterns as a means to approaching community functioning, *Can. J. Microbiol.*, Volume 44, p. 913-919.

Birke, V., Burmeier, H. and Rosenau, D., 2003, Design, construction and operation of tailored permeable reactive barrier, *Practice Periodical of Hazardous, Toxic and Radioactive Waste Management*, Volume 7, Number 4, p. 264-280.

Blohm, D.H., and Guiseppi-Elie, A., 2001, New developments in microarray technology, *Current Opinion in Biotechnology*, Volume 12, p.41-47.

Borden, R.C., Melody, J.H., Shafer, M.B. and Barlaz, M.A., 1997, Anaerobic biodegradation of BTEX in aquifer material, U.S. Environmental Protection Agency, EPA/600/S-97/003, p. 9.

Boon, N., 2002, Bioaugmentation of activated sludge reactors to enhance chloroaniline removal, PhD-thesis, Faculty of Agricultural en Applied Biological Sciences, Ghent University, Ghent.

Boschker, H.T.S., Nold, S.C., Wellsbury, P., Bos, D., de Graaf, W., Pel, R., Parkes, R.J. and T.E. Cappenberg, 1998, Direct linking of microbial populations to specific biogeochemical processes by <sup>13</sup>C-labelling of biomarkers, *Nature*, 392, p.801-805.

Bouwer, E.J., Rittman, B.E. and McCarty, R.L. 1981, Anaerobic degradation of halogenated 1- and 2-carbon organic compounds, *Environmental Science and Technology*, Volume 15, Number 5, p. 596–599.

Bouwer, E.J. 1994, Bioremediation of chlorinated solvents using alternate electron acceptors, *Handbook of Bioremediation*, Lewis Publishers, Inc. Boca Raton, Florida.

Bradley, P.M. and Chapelle, F.H., 1998, Effect of contaminant concentration on aerobic microbial mineralization of DCE and VC in stream-bed sediments, *Environmental Science and Technology*, Volume 32, Number 5, p. 553–557.

Braun, C.L., 2004, Reductive dechlorination of chlorinated ethenes under oxidation-reduction conditions and potentiometric surfaces in two trichloroethene-

contaminated zones at the Double Eagle and Fourth Street Superfund Sites in Oklahoma City, Oklahoma, U.S. Geological Survey Scientific Investigations Report 2004–5050, p.20.

Bretschko, G. and Moser, H., 1993, Transport and retention of matter in riparian ecotones, *Hydrobiologia* 251, p. 95-102.

Broholm, K., Christensen, T.H. and Jensen, B.K., 1992, Modeling TCE Degradation by a Mixed Culture of Methane-Oxidizing Bacteria, *Water Research*. Volume 26, Number 9, p.1177-1185.

Cavalca, L., Dell'Amico, E. and Androni, V., 2003, Intrinsic bioremediability of an aromatic hydrocarbon-polluted groundwater: diversity of bacterial population and toluene monooxygenase genes, *Appl. Microbiol. Biotechnol.* In press.

Chang, H-L. and Alvarez-Cohen, L., 1995a, Model for the Cometabolic Biodegradation of Chlorinated Organics, *Environmental Science and Technology*, Volume 29, Number 6, p. 2357-2367.

Chang, H-L., Alvarez-Cohen, L., 1995b, Transformation Capacities Of Chlorinated Organics By Mixed Cultures Enriched On Methane, Propane, Toluene or Phenol, *Biotechnology and Bioengineering*, Volume 45, p.440-449.

Chang, H.L. and Alvarez-Cohen, L., 1996, Biodegradation of individual and multiple chlorinated aliphatic hydrocarbons by methane-oxidizing cultures, *Appl. Environ. Microbiol.*, 62, p. 3371-3377.

Chang, W. and Criddle, S.C., 1997, Experimental Evaluation of a Model for Cometabolism: Prediction of Simultaneous Degradation of Trichloroethylene and Methane by a Methanotrophic Mixed Culture, *Biotechnology and Bioengineering* Volume 56, Number 5, p.492-501.

Chapelle, F.H., McMahon, P.B., Dubrovsky, N.M., Fujii, R.F., Oaksford, E.T. and Vroblesky, D.A., 1995, Deducing the distribution of terminal electron-accepting processes in hydrologically diverse groundwater systems, *Water Resources Research*, Volume 31, p. 359–371.

Chen, C., Puhakka, J.A. and Ferguson, J.F., 1996, Transformation of 1,1,2,2-tetrachloroethane under methanogenic conditions, *Environmental Science and Technology*, Volume 30, Number 2, p. 542–547.

Chen, F., Binder, B. and Hodson, R.E., 2000,. Flow cytometric detection of specific gene expression in prokaryotic cells using *in situ* RT-PCR, *FEMS Microbiol. Lett.* 184, p. 291-295.

Cho, J.-C. and Tiedje, J.M., 2001, Bacterial species determination from DNA-DNA hybridization by using genome fragments and DNA microarrays, *Applied and Environmental Microbiolog*, 67, p.3677-3682.

Christensen, T.H., Bjerg, P.L., Banwart, S.A., Jakobsen, R., Heron, G. and Albrechtsen, H., 2000, Characterization of redox conditions in groundwater contaminant plumes, *Journal of Contaminant Hydrology*, Volume 45, p. 165–241.

Coleman, N.V., Mattes, T.E., Gossett, J.M. and Spain, J.C., 2002, Phylogenetic and kinetic diversity of aerobic vinylchloride- assimilating bacteria from chlorinated-ethene contaminated sites, *Applied and Environmental Microbiology*, Volume 68, Number 12, p. 6162–6171.

Connon, S.A., Tovanabootr, A., Dolan, M., Vergin, K., Giovannoni, S.J. and Semprini, L., 2004, Bacterial community composition determined by culture-independent and –dependent methods during propane-stimulated bioremediation in trichloroethene-contaminated groundwater, *Environ. Microbiol.*, 7, p. 165-178.

Criddle, C.S., 1992, The Kinetics of Cometabolism, *Biotechnology and Bioengineering*, Volume 41, p. 1048-1056.

De Best, J.H., Hage, A., Doddema, H.J., Janssen, D.B. and Harder, W., 1999, Complete transformation of 1,1,1-trichloroethane to chloroethane by a methanogenic mixed population, *Applied Microbiology and Biotechnology*, Volume 51, p. 277–283.

De Bont, J.A.M., Vorage, M.J.A.W., Hartmans, S. and van den Tweel, W.J.J., 1986, Microbial degradation of 1,3-dichlorobenzene, *Applied and Environmental Microbiology*, 52, p. 677-680.

Degens, B.P., 1998, Microbial functional diversity can be influenced by the addition of simple organic substrates to soil, *Soil Biol. Biochem.* 30, p.1981-1988.

Dejonghe, W., Boon, N., Seghers, D., Top, E.M. and Verstraete, W., 2001, Bioaugmentation of soils by increasing microbial richness: missing links, *Environ. Microbiol.* 3, p. 649-657.

Delle Site, A., 2001, Factors affecting sorption of organic compounds in natural sorbent/water systems and sorption coefficients for selected pollutants. A review, *J. Phys. Chem. Ref. Data*, 30, p. 187-439.

Delzer, G.C. and Ivahnenko, T., 2003, Occurrence and temporal variability of methyl tert-butyl ether (MTBE) and other volatile organic compounds in select sources of drinking water — Results of the focused survey: U.S. Geological Survey Water-Resources Investigations Report 02-4084, p. 65.

Dermietzel, J. and Vieth, A., 2002, Chloroaromatics in groundwater — Chances of bioremediation, *Environmental Geology*, Volume 41, Number 6, p. 683–689.

Dolfing, J., 2000, Energetics of anaerobic degradation pathways of chlorinated aliphatic compounds, *Microbial Ecology*, Volume 40, p. 2–7.

Edwards, E.A. and Cox, E.E., 1997, Field and laboratory evidence of sequential aerobic chlorinated solvent biodegradation, In situ and On Site Bioreclamation, Volume 3, Battelle Press, Columbus, Ohio.

Ely, R.L., Williamson, K.J., Guenther, R.B., Hyman, M.R. and Arp, D.J., 1995a, A Cometabolic Kinetics Model Incorporating Enzyme Inhibition, Inactivation, and Recovery: I. Model Development, Analysis, and Testing, *Biotechnology and Bioengineering*, Volume 46, p. 218-231.

Ely, R.L., Hyman, M.R., Arp, D.J., Guenther, R.B. and Williamson, K.J., 1995b, A Cometabolic Kinetics Model Incorporating Enzyme Inhibition, Inactivation, and Recovery: II. Trichloroethylene Degradation Experiments, *Biotechnology and Bioengineering*, Volume 46, p. 232-245.

Ely, R.L., Williamson, K.J., Hyman, M.R. and Arp, D.J., 1997, Cometabolism of chlorinated solvents by nitrifying bacteria: kinetics, substrate interactions, toxicity effects and bacterial response, *Biotech. Bioeng.*, 54, p. 520-534.

Fathepure, B.Z. et al., 1987, Anaerobic bacteria that dechlorinate perchloroethene, *Applied and Environmental Microbiology*, Volume 53, p. 2771-2674.

Fennell, D.E. et al., 1997, Comparison of butyric acid, ethanol, lactic acid, and propionic acid as hydrogen donors for the reductive dechlorination of trichloroethene, *Environmental Science and Technology*, Volume 31, p. 918-925.

Fascari, D., Pinelli, D. and Nocentini, M., 2002, Aerobic cometabolic degradation of chloroform with butane: influence of system features on biomass adaptation, in: Proceedings of the Third International Conference on Remediation of Chlorinated and Recalcitrant Compounds (Eds: A.R. Gavaskar, A.S.C. Chen), Battelle Press, Columbus 2002, paper 2B-32 (on CD).

Fascari, D., Kim, Y., Dolan, M.E. and Semprini, L., 2003, A kinetic study of aerobic propane uptake and cometabolic degradation of chloroform, cis-dichloroethylene and trichloroethylene in microcosms with groundwater/aquifer solids, *Water Air Soil Pollution: Focus* 2003, 3, p. 285-298.

Fascari, D., Zannoni, A., Fedi, S., Pii, Y., Zannoni, D., Pinelli D. and Nocentini, M., 2005, Aerobic cometabolism of chloroform by butane-grown microorganisms: long-term monitoring of depletion rates and isolation of a high-performing strain, *Biodegradation*, 16, p. 147-158.

Fascari, D., Zannoni, A., Pinelli, D., Nocentini, M., Baleani, E., Fedi, S., Zannoni, D., Farneti, A. and Battistelli, A., 2006, Long-term aerobic cometabolism of a chlorinated solvent mixture by vinyl chloride-, methane- and

propane-utilizing biomasses, *J. Haz. Mat.*, in press. DOI:10.1016/j.jhazmat.2006.05.009.

Fraser, B.G. and Williams, D.D., 1998, Seasonal boundary dynamics of a groundwater/surface water ecotone, *Ecology* 79, p. 2019-2031.

Freedman, D.L. and Gossett, J.M., 1989, Biological reductive dechlorination of tetrachloroethylene and trichloroethylene to ethylene under methanogenic conditions, *Applied and Environmental Microbiology*, Volume 55, p. 2144-2151.

Fuller, C.C., and Harvey, J.W., 2000, Reactive uptake of trace metals in the hyporheic zone of a mining-contaminated stream, Pinal Creek, Arizona, *Environ. Sci. Technol.* 34, p. 1150-1155.

Gamo, M. and Shoji, T., 1999, A method of profiling microbial communities based on a most-probable-number assay that uses BIOLOG plates and multiple sole carbon sources, *Applied and Environmental Microbiology*, 65, p. 4419-4424.

Garland, J.L. and Mills, A.L., 1991, Classification and characterization of heterotrophic microbial communities on the basis of patterns of community-level sole-carbon-source utilization, *Applied and Environmental Microbiology*, 57, p. 2351-2359.

Garland, J.L. and Mills, A.L., 1994, A community-level physiological approach for studying microbial communities., p. 77-83, *In* K. Ritz, et al., eds. *Beyond the biomass: composition and functional analysis of soil microbial communities*, Wiley, Chichester, United Kingdom.

Geets J., Borremans, B., Diels, L., Springael, D., Vangronsveld, J., van der Lelie, D., et al., 2006, DsrB gene-based DGGE for community and diversity surveys of sulfate-reducing bacteria, *J. Microbiol. Meth.*, p. 194-205.

Gentry, T.J., Rensing, C. and Pepper, I.L., 2004, New approaches for bioaugmentation as a remediation technology, *Crit. Rev. Env. Sci. Tec.*, 34, p. 447-494.

Gossett, J.M., and Zinder, S.H., 1996, Microbiological aspects relevant to natural attenuation of chlorinated ethenes, proceedings, US EPA Symposium in Natural Attenuation of Chlorinated Organics in Ground Water, Dallas, Texas, Sept 11-13. 1996, EPA/540/R-97/504/1997, p. 12-15.

Gossett, J.M., 2002, Fishing for microbes, *Science*, 298, p. 974-975.

Griebler, C., Lorenz, A., Meckenstock, R.U. and Richnow, H.H., 2004a, Stable carbon isotope fractionation during aerobic and anaerobic transformation of trichlorobenzene, *FEMS Microbiology Ecology*, Volume 48, Number 3, p. 313-321.

Guo, C., Suo, W., Harsh, J.B. and Ogram, A., 1997, Hybridization analysis of microbial DNA from fuel oil-contaminated and noncontaminated soil, *Microb. Ecol.* 34, p. 178-187.

Haigler, B.E., Nishino, S.F. and Spain, J.C., 1988, Degradation of 1,2-dichlorobenzene by a *Pseudomonas* sp., *Applied and Environmental Microbiology*, 54, p. 294-301.

Haldeman, D. and Amy, P., 1993, Diversity within a colony morphotype: implications for ecological research, *Applied and Environmental Microbiology*, 59, p. 933-935.

Hage, J.C., Kiestra, F.D. and Hartmans, S., 2001, Cometabolic degradation of chlorinated hydrocarbons by *Pseudomonas* sp. strain DCA1, *Applied Microbiology and Biotechnology*, Volume 57, Number 4, p. 548-554.

Hallier-Soulier, S., Ducrocq, V., Mazure, N. and Truffaut, N., 1996, Detection and quantification of degradative genes in soils contaminated by toluene, *FEMS Microbiol. Ecol.* 20, p. 121-133.

Haigler, B.E., Nishino, S.F. and Spain, J.C., 1988, Degradation of 1,2-dichlorobenzene by a *Pseudomonas* sp., *Applied and Environmental Microbiology*, Volume 54, Number 2, p. 294-301.

Haigler, B.E., Pettigrew, C.A. and Spain, J.C., 1992, Biodegradation of mixtures of substituted benzenes by *Pseudomonas* sp. Strain JS150, *Applied and Environmental Microbiology*, Volume 58, Number 7, p. 2237-2244.

Hanson, J.R., Macalady, J.L., Harris, D. and Scow, K.M., 1999, Linking toluene degradation with specific microbial populations in soil, *Applied and Environmental Microbiology*, 65, p. 5403-5408.

Harkness, M.R. et al, 1999, Use of bioaugmentation to stimulate complete reductive dechlorination of trichloroethene in Dover soil columns, *Environmental Science and Technology*, Volume 33, p. 1100-1109.

Hartmans, S. and De Bont, J.A.M., 1992, Aerobic vinyl chloride metabolism in *Mycobacterium aurum* L1, *Applied and Environmental Microbiology*, Volume 58, Number 4, p.1220-1226.

Hendricks, S.P.,1993, Microbial ecology of the hyporheic zone: a perspective integrating hydrology and biology, *J. N. Am. Benthol. Soc.* 12, p. 70-78.

Hendrickx, B., Dejonghe, W., Boëne, W., Brennerova, M., Cernik, M., Lerdeer, T., Bucheli-Witscel, M., Bastiaens, L., Verstraete, W., Top, E.M., Diels, L. and Springael, D., 2005, Dynamics of oligotrophic bacterial aquifer community

during contact with a groundwater plume contaminated with benzene, toluene, ethylbenzene and xylenes: an in situ mesocosm study, *Applied and Environmental Microbiology*, Volume 71, Number 7, p. 3815-3825.

Hendrickx, B., Junca, H., Vosahlova, J., Lindner, A., Rüegg, I., Bucheli-Witschel, M., Faber, F., Egli, T., Mau, M., Schlömann, M., Brennerova, M., Brenner, V., Pieper, D.H., Top, E.M., Dejonghe, W., Bastiaens, L. and Springael, D., 2006, Alternative primer sets for PCR detection of genotypes involved in bacterial aerobic BTEX degradation: distribution of the genes in BTEX degrading isolates and in subsurface soils of a BTEX contaminated industrial site, *Journal of Microbiological Methods*, 64, p. 250-265.

Hendrickx, B., Dejonghe, W., Faber, F., Boëne, W., Bastiaens, L., Verstraete, W., Top, E.M. and Springael, D., 2006a, PCR-DGGE method to assess the diversity of BTEX mono-oxygenase genes at contaminated sites, *FEMS Microbiol. Ecol.* 55, p. 262-273.

Higgo, J.J.W., Nielsen, P.H., Bannon, M.P., Harrison, I. and Christensen, T.H., 1996, Effect of geochemical conditions on fate of organic compounds in groundwater, *Environmental Geology*, Volume 27, Number 4, p. 335–346.

Holbrook, M.T., 1992, Carbon tetrachloride, in Kroschwitz, J.I., and M. Howe-Grant, eds., *Kirk-Othmer encyclopedia of chemical technology*, Volume 5, 4th ed., New York, N.Y.

Hodson, R.E., Dustman, W.A., Garg, R.P. and Moran, M.A., 1995, *In-situ* PCR for visualization of microscale distribution of specific genes and gene-products in prokaryotic communities, *Applied and Environmental Microbiology*, 61, p. 4074-4082.

Holben, W. and Harris, D., 1995, DNA-based monitoring of total bacterial community structure in environmental samples, *Mol. Ecol.* 4, p. 627-631.

Holscher, T., Gorisch, H. and Lorenz, A., 2003, Reductive dehalogenation of chlorobenzene congeners in cell extracts of *Dehalococcoides* sp., strain CBDB1, *Applied and Environmental Microbiology*, Volume 69, Number 5, p. 2999–3001.

Hopkins, G.D. and McCarty, P.L., 1995, Field evaluation of in situ aerobic cometabolism of trichloroethylene and three dichloroethylene isomers using phenol and toluene as the primary substrates, *Environmental Science and Technology*, Volume 29, Number 6, p. 1628–1637.

Hugenholtz, P. and Pace N.R., 1996, Identifying microbial diversity in the natural environment: a molecular phylogenetic approach, *Trends Biotechnol.* 14, p. 190-197.

Ibekwe, A.M., Papiernik, S.K., Gan, J., Yates, S.R., Yang, C.-H. and Crowley, D.E., 2001, Impact of fumigants on soil microbial communities, *Appl. Environ. Microbiol.* 67, p. 3245-3133.

Janssen, D.B., Scheper, A., Dijkhuizen, L. and Witholt, B., 1985, Degradation of halogenated aliphatic compounds by *Xanthobacter autotrophicus* GJ10, *Applied and Environmental Microbiology*, Volume 49, Number 3, p. 673–677.

Jechorek, M., Wendlandt, K.-D. and Beck, M., 2003, Cometabolic degradation of chlorinated aromatic compounds, *Journal of Biotechnology*, 102, p. 93-98

Jeffers, P.M., Ward, L.M., Woytowitch, L.M. and Wolfe, N.L., 1989, Homogeneous hydrolysis rate constants for selected chlorinated methanes, ethanes, ethenes, and propanes, *Environmental Science and Technology*, Volume 23, Number 8, p. 965–969.

Jitnuyanont, P., Sayavedra, L.A. and Semprini, L., 2001, Bioaugmentation of butane-utilizing microorganisms to promote cometabolism of 1,1,1-trichloroethane in groundwater microcosms, *Biodegradation*, 12, p. 11-22.

Junca, H. and Pieper, D.H., 2003, Amplified functional DNA restriction analysis to determine catechol 2,3-dioxygenase gene diversity in soil bacteria, *J. Microbiol. Meth.* 55, p. 697-708.

Junca, H. and Pieper, D.H., 2004, Functional gene diversity analysis in BTEX contaminated soils by means of PCR-SSCP DNA fingerprinting: comparative diversity assessment against bacterial isolates and PCR-DNA clone libraries, *Environ. Microbiol.* 6, p. 95-110.

Kadlec, R. H. and Knight, R.L., 1996, *Treatment Wetlands*, CRC Lewis Publishers, Boca Raton.

Kahl, S. and Hofer, B., 2003, A genetic system for the rapid isolation of aromatic-ring-hydroxylating dioxygenase activities, *Microbiology*, 149, p. 1475-1481.

Karaman, S., 1935, Die Fauna unterirdischen gewässer Jugoslawiens, Internationale Vereinigung für theoretische und angewandte Limnologie Verhandlungen. 7, p. 46-73.

Keener, W.K. and Arp, D.J., 1993, Kinetic studies of ammonia mono-oxygenase inhibition in *Nitrosomonas europea* by hydrocarbons and halogenated hydrocarbons in an optimized whole-cell assay, *Applied and Environmental Microbiology*, 59, p. 2501-2510.

Kiernika, J., Seignez, C. and Peringer, P., 1999, *Escherichia hermannii* – a new bacterial strain for chlorobenzene degradation, *Letters in Applied Microbiology*, 28, p. 27-30.

Kim, Y., Arp, D.J. and Semprini, L., 2000, Chlorinated solvent cometabolism by butane-grown mixed culture, *Journal of Environmental Engineering*, Volume 126, p. 934–942.

Kim, Y., Arp, D.J. and Semprini, L. 2002a, A Combined Method for Determining Inhibition Type, Kinetic Parameters, and Inhibition Coefficients for Aerobic Cometabolism of 1,1,1-Trichloroethane by a Butane-Grown Mixed Culture, *Biotechnology and Bioengineering*, Volume 77, Number 5, p.564-576

Klier, N.J., West, R.J. and Donberg, P.A., 1999, Aerobic biodegradation of dichloroethylenes in surface and subsurface soils, *Chemosphere*, Volume 38, Number 5, p. 1175–1188.

Koizumi, Y., Kelly, J.J., Nakagawa, T., Urakawa, H., El-Fantroussi, S., Al-Muzaini, S., Fukui, M., Urushigawa, Y. and Stahl, D.A., 2002, Parallel characterization of anaerobic toluene- and ethylbenzene-degrading microbial consortia by PCR-Denaturing Gradient Gel Electrophoresis, RNA-DNA membrane hybridization and DNA microarray technology, *Applied and Environmental Microbiology*, 68, p. 3215-3225.

Koizumi, Y., Kojima, H. and Fukui, M., 2003, Characterization of depth-related microbial community structure in lake sediment by denaturing gradient gel electrophoresis of amplified 16S rDNA and reversely transcribed 16S rRNA fragments, *FEMS Microbiol. Ecol.*, 1573, p. 1-11.

Leahy, J.G. and Colwell, R.R., 1990, Microbial degradation of hydrocarbons in the environment, *Microbiol Rev.*, 54, p. 305-315.

Lee, C.C. and Caskey, C.T., 1990, cDNA cloning using degenerated primers, p. 46-53 In: PCR protocols: a guide to methods and applications, Academic Press, Inc.

Lee, D.H., Zo, Y.G. and Kim, S.J., 1996, Nonradioactive method to study genetic profiles of natural bacterial communities by PCR single-strand-conformation polymorphism, *Applied and Environmental Microbiology*, 62, p. 3112-3120.

Lee, N., Nielsen, P.H., Andreasen, K.H., Juretschko, S., Nielsen, J.L., Schleifer, K.H. and W. M., 1999, Combination of fluorescent *in situ* hybridization and microautoradiography - a new tool for structure-function analyses in microbial ecology, *Applied and Environmental Microbiology*, 65, p. 1289-1297.

Lee, S.B., Strand, S.E, Stensel, H.D., 2000, Sustained Degradation of Trichloroethylene in a Suspended Growth Gas Treatment Reactor by an

Actinomycetes Enrichment, *Environmental Science and Technology*, Volume 34, Number 15, p. 3261- 3268.

Lendvay, J.M. and Adriaens, P., 1999, Laboratory evaluation of temporal trends in biogeochemical conditions at a groundwater-surface water interface, *Phys. Chem. Earth* 24, p. 511-516.

Liu, W.T., Marsh, T.L., Cheng, H. and Forney, L.J., 1997, Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA, *Applied and Environmental Microbiology*, 63, p. 4516-4522.

Lovley, D.R., 1991, Dissimilatory Fe (III) and Mn (IV) reduction, *Microbiological Review*, Volume 55, Number 2, p. 259–287.

Maidak, B.L., Olsen, G.J., Larsen, N., Overbeek, R., McCaughey, M.J. and Woese, C.R., 1997, The RPD (Ribosomal Database Project), *Nucl. Acids. Res.* 25, p. 109-111.

Mars, A.E., Kasberg, T., Kascabek, R., van Agteren, M.H., Janssen, D.B. and Reineke, W., 1997, Microbial degradation of chloroaromatics: use of the meta-cleavage pathway for mineralization of chlorobenzene, *Journal of Bacteriology*, Volume 179, Number 14, p. 4530-4537.

Martin, A., 1999, *Biodegradation and Bioremediation*, 2nd ed., Academic Press, S. Diego.

Massol-Deya, A.A., Odelson, D.A., Hickey, R.F. and Tiedje, J.M., 1995, Bacterial community fingerprinting of amplified 16S and 16-23S ribosomal DNA gene sequences and restriction endonuclease analysis (ARDRA), p.1-8, *In* A.D.L. Akkermans, J.D. van Elsas and F.J. de Bruijn, eds. *Molecular Microbial Ecology Manual*, Kluwer Acad Pub, Dordrecht.

Massol-Deya, A., Weller, R., Rios-Hernandez, L., Zhou, J.Z., Hickey, R.F. and Tiedje, J.M., 1997, Succession and convergence of biofilm communities in fixed-film reactors treating aromatic hydrocarbons in groundwater, *Applied and Environmental Microbiology*, 63, p. 270-276.

Marchesi J.R., Sato, T., Weightman, A.J., Martin, T.A., Fry, J.C., Hiom, S.J. and Wade, W.G., 1998, Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA, *Applied and Environmental Microbiology*, 64, p. 795-799.

McCarty, P.L. and Reinhard, M., 1993, Biological and chemical transformations of halogenated aliphatic compounds in aquatic and terrestrial environments in the biochemistry of global change, in Oremland, R.S., ed., *The biogeochemistry of global change — Radioactive trace gases*, New York, N.Y., Chapman and Hall, Inc., p. 839–852.

McCarty, P.L. and Semprini, L., 1994, Ground-water treatment for chlorinated solvents, in Norris, R.D., R.E. Hinchee, R. Brown, P.L. McCarty, L. Semprini, J.T. Wilson, D.H. Kampbell, M. Reinhard, E.J. Bouwer, R.C. Borden, T.M. Vogel, J.M. Thomas, and C.H. Ward, eds., *Groundwater cleanup through bioremediation, Handbook of Bioremediation*, Boca Raton, Fla., Lewis Publishers, Inc., p.87.

McCarty, P.L., 1997, Biotic and abiotic transformations of chlorinated solvents in ground water, in *Proceedings of the Symposium on Natural Attenuation of Chlorinated Organics in Ground Water*, Dallas, Texas, September 11–13, 1996: U.S. Environmental Protection Agency, EPA/540/R-97/504, p. 7–11.

McCarty, P.L., Goltz, M.N., Hopkins, G.D., Dolan, M.E., Allan, J.P., Kawakami, B.T. and Carrothers, T.J., 1998, Full scale evaluation of in situ cometabolic degradation of trichloroethylene in groundwater through toluene injection, *Environmental Science and Technology*, Volume 32, p. 88–100.

McClure, N.C., Fry, J.C. and Weightman, A.J., 1991, Survival and catabolic activity of natural and genetically engineered bacteria in a laboratory-scale activated-sludge unit, *Applied and Environmental Microbiology*, 57, p. 366–373.

McClure, N.C., Weightman, A.J. and Fry, J.C., 1989, Survival of *Pseudomonas putida* UWC1 containing cloned catabolic genes in a model activated-sludge unit, *Applied and Environmental Microbiology*, 55, p. 2627–2634.

Megharaj, M., Wittich, R.-M., Blasco, R., Pieper, D.H. and Timmis, K.N., 1997, Superior survival and degradation of dibenzo-*p*-dioxin and dibenzofuran in soil by soil-adapted *Sphingomonas* sp. Strain RW1, *Appl. Microbiol. Biotechnol.*, 48, p. 109–114.

Mesarch, M.B., Nakatsu, C.H. and Nies, L., 2000, Development of catechol 2,3-dioxygenase-specific primers for monitoring bioremediation by competitive quantitative PCR, *Applied and Environmental Microbiology*, 66, p. 678-683.

Meyer, S., Moser, R., Neef, A., Stahl, V. and Kämfer, P., 1999, Differential detection of key enzymes of polyaromatic-hydrocarbon-degrading bacteria using PCR and gene probes, *Microbiology* 145, p. 1731-1741.

Middeldorp, P.J.M., De Wolf, J., Zehnder, A.J.B. and Schraa, G., 1997, Enrichment and properties of a 1,2,4-trichlorobenzene-dechlorinating methanogenic microbial consortium, *Applied and Environmental Microbiology*, Volume 63, Number 4, p. 1225–1229.

Miller, R. R., 1996, Bioslurping, Technology Overview Report TO-96-05, Groundwater Remediation Technologies Analysis Centre, October.

Monferran, M.V., Echenique, J.R. and Wunderlin, D.A., 2005, Degradation of chlorobenzenes by a strain of *Acidovorax avenae* isolated from a polluted aquifer, *Chemosphere*, Volume 61, Number 1, p. 98–106.

Moran, M.J., 2006, Occurrence and implications of selected chlorinated solvents in ground water and source water in the United States and in drinking water in 12 Northeast and Mid-Atlantic States, 1993–2002: U.S. Geological Survey Scientific Investigations Report 2005–5268, p. 70.

Munakata-Marr, J., McCarty, P.L., Shields, M.S., Reagin, M. and Francesconi, S.C., 1996, Enhancement of trichloroethylene degradation in aquifer microcosms bioaugmented with wild type and genetically altered Burkholderia (*Pseudomonas*) cepacia G4 and PR1, *Environ. Sci. Technol.*, 33, p. 2045-2052.

Munakata-Marr, J. et al., 1997, Long-term biodegradation of trichloroethylene influenced by bioaugmentation and dissolved oxygen in aquifer microcosms, *Environmental Science and Technology*, Volume 31, p. 786-791.

Murray, W.D., and Richardson, M., 1993, Progress toward the biological treatment of C1 and C2 halogenated hydrocarbons, *Critical Reviews in Environmental Science and Technology*, Volume 23, Number 3, p. 195–217.

Muyzer, G., de Waal, E.C. and Uitterlinden, A., 1993, Profiling of complex microbial populations using denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA, *Applied and Environmental Microbiology*, 59, p. 695-700.

Naegeli, M.W. and Uehlinger, U., 1997, Contribution of the hyporheic zone to ecosystem metabolism in a prealpine gravel-bed river, *J. N. Am. Benthol. Soc.* 16, p. 794-804.

Nishino, S.F., Spain, J.C., Belcher, L.A. and Lichtfield, C.D., 1992, Chlorobenzene degradation by bacteria isolated from contaminated groundwater, *Applied and Environmental Microbiology*, Volume 58, Number 5, p. 1719-1726.

Olaniran, A.O., Pillay, D. and Pillay, B., 2004, Chloroethenes contaminants in the environment — Still a cause for concern, *African Journal of Biotechnology*, Volume 3, Number 12, p. 675–682.

Oldenhuis, R., Oedzes, J.Y., van der Waarde, J.J. and Janssen, D.B., 1991, Kinetics of chlorinated hydrocarbon degradation by *Methylosinus trichosporium* OB3b and toxicity of trichloroethylene, *Applied and Environmental Microbiology*, 57, p. 7-14.

Olin, T. J., Bailey, S. E., Mann, M. A., Lutes, C. C., Seward, C. A. and Singer, C. F., 1999, Physical Separation (Soil Washing) Equipment for Volume Reduction of Contaminated Soils and Sediments, EPA-905-R99-006. Chicago, IL, U.S. EPA, Great Lakes National Program Office.

Ogram, A., Sun, W., Brockman, F.J. and Frederickson, J.K., 1995, Isolation and characterization of RNA from low-biomass deep-subsurface sediments, *Applied and Environmental Microbiology*, 61, p. 763-768.

Okuta, A., Ohnishi, K. and Harayama, S., 1998, PCR isolation of catechol 2,3-dioxygenase gene fragments from environmental samples and their assembly into functional genes, *Gene* 212, p. 221-228.

Orghidan, T., 1959, Ein neuer Lebensraum des unterirdischen Wassers, der hyporheische Biotop, *Archiv für Hydrobiologie* 55, p. 392-414.

Padmanabhan, P., Padmanabhan, S., DeRito, C., Gray, A., Gannon, D., Snape, J.R., Tsai, C.S., Park, W., Jeon, C. and Madsen, E.L., 2003, Respiration of <sup>13</sup>C-labeled substrates added to soil in the field and subsequent 16S rRNA gene analysis of <sup>13</sup>C-labeled soil DNA, *Applied and Environmental Microbiolog*, 69, p. 1614-1622.

Pelz, O., Chatzinotas, A., Andersen, N., Bernasconi, S.M., Hesse, C., Abraham, W.-R. and Zeyer, J., 2001a, Use of isotopic and molecular techniques to link toluene degradation in denitrifying aquifer microcosms to specific microbial populations, *Microbiology* 175, p. 270-281.

Peterson, G.L., 1977, A simplification of the protein assay method of Lowry et al. which is more generally applicable, *Anal. Biochem.*, 83, p. 346-356.

Pettigrew, C.A., Hailgler, B.E. and Spain, J.C., 1991, Simultaneous biodegradation of chlorobenzene and toluene by a *Pseudomonas* strain, *Applied and Environmental Microbiology*, Volume 57, Number 1, p. 157-162.

Phelps, T.J., Ringelberg, D., Hedrick, D., Davis, J., Fliermans, C.B. and White, D.C., 1988, Microbial biomass and activities associated with subsurface environments contaminated with chlorinated hydrocarbons, *Geomicrobiol. J.* 6, p. 157-170.

Proctor, G.N., 1994, Mathematics of microbial plasmid instability and subsequent differential growth of plasmid-free and plasmidcontaining cells, relevant to the analysis of experimental colony number data, *Plasmid*, 32, p. 101–130.

Pusch, M., Fiebig, D., Brettar, I., Eisenmann, H., Ellis, B.K., Kaplan, L.A. et al., 1998, The role of micro-organisms in the ecological connectivity of running waters, *Freshwater Biology* 40, p. 453-495.

Ralebitso, T.K., Roling, W.F., Braster, M., Senior, E. and van Verseveld, H.W., 2000. 16S rDNA-based characterization of BTX-catabolizing microbial associations isolated from a South African sandy soil, *Biodegradation* 11, p. 351-357.

Ramanand, K., Balba, M.T. and Duffy, J., 1993, Reductive dehalogenation of chlorinated benzenes and toluenes under methanogenic conditions, *Applied and Environmental Microbiology*, Volume 59, Number 10, p. 3266–3272.

Rapp, P. and Gabriel-Jürgens, L.H.E., 2003, Degradation of alkanes and highly chlorinated benzenes, and production of biosurfactants, by a psychrophilic *Rhodococcus* sp. And genetic characterization of its chlorobenzene dioxygenase, *Microbiology*, Volume 149, Number 10, p. 2879–2890.

Reineke, W. and Knackmuss, H.J., 1984, Microbial metabolism of haloaromatics — Isolation and properties of a chlorobenzene-degrading bacterium, *Applied and Environmental Microbiology*, Volume 47, Number 2, p. 395–402.

Ringelberg, D.B., J.D. Davis, G.A. Smith, S.M. Pfiffner, P.D. Nichols, J.S. Nickels, J.M. Henson, J.T. Wilson, M. Yates, D.H. Kampbell, H.W. Read, T.T. Stocksdale, and D.C. White, 1989, Validation of signature polarlipid fatty acid biomarkers for alkane-utilizing bacteria in soils and subsurface aquifer materials. *FEMS Microbiol. Ecol.* 62, p. 39-50.

Ringelberg, D.B., J.W. Talley, E.J. Perkins, S.G. Tucker, R.G. Luthy, E.J. Bouwer, and H.L. Frederickson, 2001, Succession of phenotypic, genotypic and metabolic community characteristics during in vitro bioslurry treatment of polycyclic aromatic hydrocarbon-contaminated sediments, *Applied and Environmental Microbiology*, 67, p. 1542-1550.

Rittmann, B.E, McCarty, P.L. 2001, *Environmental Biotechnology: Principles and Applications*, McGraw-Hill Companies, Inc. New York City, NY.

Roberts, P.V., Goltz, M.N. and Mackay, D.M., 1986, A natural gradient experiment on solute transport in a sand aquifer — III. Retardation estimates and mass balances for organic solutes, *Water Resources Research*, Volume 22, Number 13, p. 2047–2058.

Röling, W.F., van Breukelen, B.M., Braster, M., Lin, B. and van Verseveld, H.W., 2001, Relationships between microbial community structure and hydrochemistry in a landfill leachate-polluted aquifer, *Applied and Environmental Microbiology*, 67, p. 4619-4629.

Remediation Technologies Development Forum, 1997, *Natural Attenuation of Chlorinated Solvents in Groundwater: Principles and Practices*. Version 3.0. August.

Sabater, F. and Vila, B., 1991, The hyporheic zone considered as an ecotone, *Oecologia aquatica* 10, p. 35-43.

Sander, P., Wittach, R.M., Fortnagel, P., Wilkes, H., and Francke, W., 1991, Degradation of 1,2,4-trichloroand 1,2,4,5-tetrachlorobenzene by *Pseudomonas* strains, *Applied and Environmental Microbiology*, Volume 57, Number 5, p. 1430–1440.

Sander, R., 1999, Compilation of Henry's law constants for inorganic and organic species of potential importance in environmental chemistry, online at <http://www.mpch-mainz.mpg.de/~sander/res/henry.html>.

Sands, T., Dodge, T. and Fitzgerald, M., 2005, 1,1,1-trichloroethane (an/aerobic) pathway map — University of Minnesota biocatalysis/biodegradation database, online at [http://umbbd.msi.umn.edu/tca/tca\\_map.html](http://umbbd.msi.umn.edu/tca/tca_map.html).

Sayler, G.S., and Layton, A.C., 1990, Environmental application of nucleic acid hybridization, *Annu. Rev. Microbiol.* 44, p. 625-648.

Schmidt, E. and Knackmuss, H.-J., 1980, Chemical structure and biodegradability of halogenated aromatic compounds, *Biochem. J.*, 192, p. 339-347.

Schraa, G., Boone, M.L., Jetten, M.S.M., van Neerven, A.R.W., Colberg, P.J. and Zehnder, A.J.B., 1986, Degradation of 1,4-dichlorobenzene by *Alcaligenes* sp. Strain A175, *Applied and Environmental Microbiology*, 52, p. 1374-1381.

Schwarzenbach, R.P., Giger, W., Hoehn, E. and Schneider, J.K., 1983, Behavior of organic compounds during infiltration of river water to groundwater. Field studies, *Environ. Sci. Technol.* 17, p. 472-479.

Semprini, L., 2001, Editorial, *Biodegradation*, 12, p. 79-80.

Sergei, G.B., J.P. Akowski, V.M. Zakhariyev, V.E. Barsky, A.N. Perov, and A.D. Mirzbekov, 2001, Portable system for microbial sample preparation and oligonucleotide microarray analysis, *Applied and Environmental Microbiology*, 67, p. 922-928.

Shi, Y., M.D. Zwolinski, M.E. Schreiber, J.M. Bahr, G.W. Sewell, and W.J. Hickey, 1999, Molecular analysis of microbial community structures in pristine and contaminated aquifers: field and laboratory microcosm experiments, *Applied and Environmental Microbiology*, 65, p. 2143-2150.

Sipkema, E.M., de Konig, W., Ganzeveld, K.J., Janssen, D.B., Beenackers, A., 2000, NADH-Regulated Metabolic Model for Growth of *Methylosinus trichosporium* OB3b. Model Presentation, Parameter Estimation, and Model Validation, *Biotechnology Program*, Volume 16, p.176-188.

Small, J., D.R. Call, F.J. Brockman, T.M. Straub, and D.P. Chandler, 2001, Direct detection of 16S rRNA soil extracts by using oligonucleotide microarrays, *Appl. Environ. Microbiol.* 67, p. 4708-4716.

Smalla, K., U. Wachtendorf, H. Heuer, W.T. Liu, and L. Forney, 1998, Analysis of BIOLOG GN substrate utilization patterns by microbial communities, *Applied and Environmental Microbiology*, 64, p. 1220-1225.

Smith, J.A., Witkowski, P.J. and Fusillo, T.V., 1988, Manmade organic compounds in the surface waters of the United States — A review of current understanding, U.S. Geological Survey Circular 1007, p. 52.

Spain, J.C. and Nishino, S.F., 1987, Degradation of 1,4-dichlorobenzene by a *Pseudomonas* sp., *Applied and Environmental Microbiology*, Volume 53, Number 3, p. 1010–1019.

Spiess, E., Sommer, C. and Gorisch, H., 1995, Degradation of 1,4-dichlorobenzene by a *Xanthorbacter flavus* 14pl., *Applied and Environmental Microbiology*, 61, p. 3884-3888.

Stackebrandt, E., Liesack, W. and Goebel, M.B., 1993, Bacterial diversity in a soil sample from a subtropical Australian environment as determined by 16S rDNA analysis, *FASEB J.* 7, p. 232-236.

Stapleton, R.D. and Saylor, G.S., 1998, Assessment of the microbiological potential for the natural attenuation of petroleum hydrocarbons in a shallow aquifer system, *Microb. Ecol.* 36, p. 349-361.

Stapleton, R.D., S. Ripp, L. Jimenez, S. Cheol-Koh, J.T. Fleming, I.R. Gregory, and G.S. Saylor, 1998, Nucleic acid analytical approaches in bioremediation: site assessment and characterization, *J. Microbiol. Meth.* 32, p. 165-178.

Stapleton, R.D. and Saylor, G.S., 2000, Changes in subsurface catabolic gene frequencies during natural attenuation of petroleum hydrocarbons, *Environ. Sci. Technol.* 34, p. 1991-1999.

Stapleton, R.D., N.G. Bright, and G.S. Saylor, 2000, Catabolic and genetic diversity of degradative bacteria from fuel-hydrocarbon contaminated aquifers, *Microb. Ecol.* 39, p. 211-221.

Steffan, R.J., and R.M. Atlas, 1988, DNA amplification to enhance detection of genetically engineered bacteria in environmental samples, *Applied and Environmental Microbiology*, 54, p. 2185-2191.

Steffan, R.J., Sperry, K.L., Walsch, M.T., Vainberg, S. and Condee, C.W., 1999, Field-scale evaluation of in-situ bioaugmentation for remediation of chlorinated solvents in groundwater, *Environ. Sci. Technol.*, 33, p. 2771-2781.

Stucki, G., Krebsler, U. and Leisinger, T., 1983, Bacterial growth on 1,2-dichloroethane, *Experientia*, Volume 39, Number 11, p. 1271–1273.

Stumm, W. and Morgan, J.J., 1996, *Aquatic Chemistry*: 3rd ed., New York, N.Y., John Wiley & Sons, Inc., 1022 p. Suzuki, Masahiko, Takahiko.

Tani, K., M. Muneta, K. Nakamura, K. Shibuya, and M. Nasu, 2002, Monitoring of *Ralstonia eutropha* KT1 in groundwater in an experimental bioaugmentation field by *in situ* PCR, *Applied and Environmental Microbiology*, 68, p. 412-416.

Tchelet, R., Meckenstock, R., Steinle, P. and van der Meer, J.R., 1999, Population dynamics of an introduced bacterium degrading chlorinated benzenes in a soil column and in sewage sludge, *Biodegradation*, 10, p. 113-125.

Tovanabootr, A. and Semprini, L., 1998, Comparison of TCE transformation abilities of methane- and propane-utilizing microorganisms, *Bioremed. J.*, 2, p. 105–124.

Travis, B.J. and Rosenberg, N.D., 1997, Modeling in situ bioremediation of TCE at Savannah River: Effects of product toxicity and microbial interactions on TCE degradation, *Environmental Science and Technology*, Volume 31, p. 3093-3102.

Trevors, J.T., 1992, DNA extraction from soil, *Microb. Release* 1, p. 3-9.

Trevors, J.T., and van Elsas, J.D., 1995, *Nucleic acids in the environment: methods and applications.*, p. 256 Springer-Verlag, Heidelberg, Germany. ISBN3-540-58069-7.

Urakawa, H., El-Fantroussi, S., Smidt, H., Smoot, J.C., Tribou, E.H., Kelly, J.J., Noble P.A. and Stahl, D.A., 2003, Optimization of single-base-pair mismatch discrimination in oligonucleotide microarrays, *Applied and Environmental Microbiology*, 69, p. 2848-2856.

U.S. Air Force, 1998, Installation Restoration Program, Aerobic Cometabolic In Situ Bioremediation Technology Guidance Manual and Screening Software User's Guide, Centre for Environmental Excellence, Environmental Restoration Division. Brooks Air Force Base, Texas, June.

U.S. Environmental Protection Agency, 1990, Engineering Bulletin: Slurry Biodegradation, EPA/540/2-90/016, Washington DC: Office of Solid Waste and Emergency Response.

U.S. Environmental Protection Agency, 1993, Bioremediation Using the Land Treatment Concept: Environmental Regulation and Technology, EPA/600-R93/164.

U.S. Department of Agriculture and U.S. Environmental Protection Agency, 1994(a), A Handbook of Constructed Wetlands: A Guide to Creating Wetlands for Agricultural Wastewater, Domestic Wastewater, Coal Mine Drainage, and Stormwater in the Mid-Atlantic Region. Volume 3, Agricultural Wastewater, Publication Number 843F00002, National Environmental Publications Information System, online at <http://nepis.epa.gov>.

U.S. Department of Agriculture and U.S. Environmental Protection Agency, 1994(b), A Handbook of Constructed Wetlands: A Guide to Creating Wetlands for Agricultural Wastewater, Domestic Wastewater, Coal Mine Drainage, and Stormwater in the Mid-Atlantic Region. Volume 4, Coal Mine Drainage. Publication Number 843F00003, National Environmental Publications Information System, online at <http://nepis.epa.gov>.

U.S. Environmental Protection Agency, 1995, Contaminant specific fact sheets, Volatile organic chemicals — Technical version: U.S. Environmental Protection Agency, EPA 811-F-95-004-T, online at <http://www.epa.gov/safewater/dwh/tvoc.html>.

U.S. Environmental Protection Agency, 1995b, Manual: Bioventing Principles and Practice. Volume II: Bioventing Design, EPA/625/xxx/001, Office of Research and Development, September, online at <http://www.epa.gov/ord/NRMRL/pubs/bioreem/pdf/iibiov.pdf>.

U.S. Environmental Protection Agency, 1996d, A citizen's guide to treatment walls, EPA542-F-96-016, Washington DC, Office of Solid Waste and Emergency Response, online at <http://www.epa.gov/ord/NRMRL/pubs/bioreem/pdf/iibiov.pdf>.

U.S. Environmental Protection Agency, 1997, Innovative Uses of Compost. Composting of Soils Contaminated by Explosives, EPA/530/F-997-045, online at <http://www.epa.gov/epaoswer/non-hw/compost/explos.pdf>.

U.S. Environmental Protection Agency, 1998a, Seminar Series on Monitored Natural Attenuation for Ground Water, EPA/625/K-98/001. Washington, DC: Office of Solid Waste and Emergency Response, online at [http://www.epa.gov/OUST/oswermna/mna\\_epas.htm](http://www.epa.gov/OUST/oswermna/mna_epas.htm).

U.S. Environmental Protection Agency, 1998, Use and distribution of MTBE and ethanol: Washington, D.C., Office of Underground Storage Tanks, U.S. Environmental Protection Agency, MTBE Fact Sheet #3, EPA 510-F-97-016, p. 3, online at <http://www.epa.gov/swerust1/mtbe/mtbef3.pdf>.

U.S. Environmental Protection Agency, 1999a, Monitored Natural Attenuation of Chlorinated Solvents, EPA/600/F-98/022. Office of Research and Development.

U.S. Environmental Protection Agency, 1999b, Monitored Natural Attenuation of Petroleum Hydrocarbons, EPA/600/F-98/021. Office of Research and Development.

U.S. Environmental Protection Agency, 2000, Engineered Approaches to In Situ Bioremediation of Chlorinated Solvents: Fundamentals and Field Applications, EPA-542-R-00-008, Washington DC, Office of Solid Waste and Emergency Response.

U.S. Environmental Protection Agency, 2001c, Constructed Wetlands: Passive Systems for Wastewater Treatment, Technology Status Report, August, online at [http://clu-in.org/download/studentpapers/constructed\\_wetlands.pdf](http://clu-in.org/download/studentpapers/constructed_wetlands.pdf).

U.S. Environmental Protection Agency's National Priorities List, 2 October 2002, online at [www.epa.gov/superfund/sites/npl/](http://www.epa.gov/superfund/sites/npl/).

U.S. Environmental Protection Agency, 2004a, Treatment Technologies for Site Cleanup: Annual Status Report, Eleventh Edition, EPA-542-R-03-009, Washington DC, Office of Solid Waste and Emergency Response, online at <http://www.epa.gov/tio/download/remed/asr/11/asr.pdf>.

U.S. Environmental Protection Agency, 2004b, How to Evaluate Alternative Cleanup Technologies for Underground Storage Tank Sites. A Guide for Corrective Action Reviewers, EPA 510-R-04-002, Washington DC, Office of Solid Waste and Emergency Response, online at <http://www.epa.gov/swrust1/pubs/tums.htm>.

U.S. Environmental Protection Agency, 2005b, references complete index, online at [http://www.epa.gov/enviro/html/emci/chemref/complete\\_index.htm](http://www.epa.gov/enviro/html/emci/chemref/complete_index.htm).

Valett, H.M., Dahm, C.N., Campana, M.E., Morrice, J.A. and Baker, M.A., 1997, Hydrologic influences on groundwater-surface water ecotones: heterogeneity in nutrient composition and retention, *J. N. Am. Benthol. Soc.* 16, p. 239-247.

Van der Meer, J.R., Roelofsen, W., Schraa, G. and Zehnder, A.J.B., 1987, Degradation of low concentrations of dichlorobenzenes and 1,2,4-trichlorobenzene by *Pseudomonas* sp. P51 in nonsterile soil columns, *FEMS Microbiol. Ecol.*, 45, p. 333-341.

Van der Meer, J.R., Van Neerven, A.R., de Vries, E.J., de Vos, W.M. and Zehnder, A.J., 1991, Cloning and characterization of plasmid-encoded genes for the degradation of 1,2-dichloro-, 1,4-dichloro-, and 1,2,4-trichlorobenzene of *Pseudomonas* sp. strain P51, *Journal of Bacteriology*, Volume 173, Number 1, p. 6-15.

Van der Meer, J.R., 1997, Evolution of novel metabolic pathways for the degradation of chloroaromatic compounds, *Anton. Leeuw.* 71, p. 159-178.

Van der Meer, J.R., Werlen, C., Nishino, S.F. and Spain, J.C., 1998, Evolution of a pathway for chlorobenzene metabolism leads to natural attenuation in contaminated groundwater, *Applied and Environmental Microbiology*, Volume 64, Number 11, p. 4185–4193.

Van Elsas, J.D., Duarte, G.F., Rosado, A.S. and Smalla, K., 1998, Microbiological and molecular biological methods for monitoring microbial inoculants and their effects in the soil environment, *J. Microbiol. Meth.* 32, p. 133-154.

Verce, M.F., Ulrich R.L. and Freedman, D., 2000, Characterisation of an isolate that uses vinyl chloride as growth substrate under aerobic conditions, *Applied and Environmental Microbiology*, Volume 66, Number 8, p. 3535-3542.

Verce, M.F., Gunsch, C.K., Danko, A.S. and Freedman, D.L., 2002, Cometabolism of cis-1,2-dichloroethylene by aerobic cultures grown on vinyl chloride as the primary substrate, *Environ. Sci. Technol.*, 36, p. 2171-2177.

Vervier, P. and Naiman, R.J., 1992, Spatial and temporal fluctuations of dissolved organic carbon in subsurface flow of the Stillaguamish River (Washington, USA), *Arch. Hydrobiol.* 123, p. 401-412.

Vogel, T. and McCarty, P.L. 1987a, Abiotic and Biotic Transformations of 1,1,1-Trichloroethane under Methanogenic Conditions, *Environmental Science and Technology*, Volume 21, Number 12, p.1208-1213.

Vogel, T. M. and McCarty, P. L., 1987b, Rate of Abiotic Formation of 1,1-Dichloroethylene and 1,1,1-Trichloroethane in Groundwater, *Journal of Contaminant Hydrology*, Volume 1, Number 3, p. 299-308

Vogel, T. M., Criddle, C. S. and McCarty, P. L., 1987, Transformation of Halogenated Aliphatic Compounds, *Environmental Science and Technology*, Volume 21, Number 8, p. 722-736.

Vogel, T.M., 1994, Natural bioremediation of chlorinated solvents, in Norris, R.D., R.E. Hinchee, Robert Brown, P.L. McCarty, Lewis Semprini, J.T. Wilson, D.H. Kampbell, Martin Reinhard, E.J. Bouwer, R.C. Borden, T.M. Vogel, J.M. Thomas, and C.H. Ward, eds., *Handbook of Bioremediation*: Boca Raton, Fla., Lewis Publishers, p. 201–222.

Vogel, T.M., 1996, Bioaugmentation as a soil bioremediation approach, *Curr. Opin. Biotech.*, 7, p. 311-316.

Vogt, C., Simon, D., Alfreider, A. and Babel, W., 2003, Microbial Degradation of chlorobenzene under oxygen-limited conditions leads to accumulation of 3-chlorocatechol, *Environmental Toxicology and Chemistry*, Volume 23, Number 2, p. 265-270.

Vogt, C., Alfreider, A., Lorbeer, H., Hoffmann, D., Wünsche, L. and Babel, W., 2004, Bioremediation of chlorobenzene-contaminated groundwater in an in situ reactor mediated by hydrogen peroxide, *Journal of Contaminant Hydrogen*, 68, p. 121-141.

Wackett, L.P., Brusseau, G.A., Housholder, S.R. and Hanson, R.S., 1989, Survey of microbial oxygenases: trichloroethylene degradation by propane-oxidizing bacteria, *Applied and Environmental Microbiology*, 55, p. 2960-2964.

Ward, D.M., Weller, R., Bateson, M.M., 1990, 165 rRNA sequences reveal numerous uncultured microorganisms in a natural community, *Nature*, 345, p. 63-65.

Wenderoth, D.F., Rosenbrock, P., Abraham, W.R., Pieper, D.H., Höfle, M.G., 2003, *Microbial Ecology*, 46, p. 161-176.

Werlen, C., Kohler, H.-P. E. and van der Meer, J.R., 1996, The broad substrate chlorobenzene dioxygenase and *cis*-chlorobenzene dihydrodiol dehydrogenase of *Pseudomonas* sp. Strain P51 are linked evolutionarily to the enzymes for benzene and toluene degradation, *The Journal of Biological Chemistry*, Volume 271, Number 8, p. 4009-4016.

White, D.C. and Findlay, R.H., 1988, Biochemical markers for measurement of predation effects on the biomass, community structure, nutritional status, and metabolic activity of microbial biofilms, *Hydrobiologia* 159, p. 119-132.

Whittaker, M., Monroe, D., Jun Oh, D. and Anderson, S., 2005, Trichloroethylene pathway map — University of Minnesota biocatalysis/biodegradation database, online at [http://umbbd.msi.umn.edu/tce/tce\\_map.html](http://umbbd.msi.umn.edu/tce/tce_map.html).

Wiedemeier, T.H., Swanson, M.A., Moutoux, D.E., Gordon, E.K., Wilson, J.T., Wilson, B.H., Kampbell, D.H., Haas, P.E., Miller, R.N., Hansen, J.E. and Chapelle, F.H., 1998, Technical protocol for evaluating natural attenuation of chlorinated solvents in ground water, Cincinnati, Ohio, U.S. Environmental Protection Agency, National Risk Management Research Laboratory, Office of Research and Development, EPA/600/R-98/128, p. 244.

Wilcox, D.W., Autenrieth, R.L. and Bonner, J.S., 1995, Propane-induced biodegradation of vapor phase trichloroethylene, *Biotechnol. Bioeng.*, 46, p. 333-342.

Williams, R.A., Shuttle, K.A., Kunkler, J.L., Madsen, E.L. and Hooper, S.W., 1997, Intrinsic bioremediation in a solvent-contaminated alluvial groundwater, *Journal of Industrial Microbiology and Biotechnology*, 18, p. 177-188.

Wilson, K.H., Wilson, W.J., Radosevich, J.L., DeSantis, T.Z., Viswanathan, V.S., Kucsmarski, T.A. and Andersen, G.L., 2002, High-density microarray of small-subunit ribosomal DNA probes, *Applied and Environmental Microbiology*, 68, p. 2535-2541.

Woese, C.R., 1987, Bacterial Evolution, *Microbiol. Rev.* 51, p. 221-271.

Workman, D.J. et al., 1997, Microbial reduction of Vitamin B12 by *Shewanella alga* Strain BrY with subsequent transformation of carbon tetrachloride, *Environmental Science and Technology*, Volume 31, p. 2292-2297.

Wünsche, L., Brüggermann, L. and Wolfgang, B., 1995, Determination of substrate utilization patterns of soil microbial communities: an approach to assess population changes after hydrocarbon pollution, *FEMS Microbiol. Ecol.* 17, p. 295-306.

Yager, R.M. et al., 1997, Metabolic and in situ attenuation of chlorinated ethenes by naturally occurring microorganisms in a fractured dolomite aquifer near Niagara Falls, New York, *Environmental Science and Technology*, Volume 31, p. 3138-3147.

Zheng, D., Alm, E.W., Stahl, D.A. and Raskin, L., 1996, Characterization of universal small-subunit rRNA hybridization probes for quantitative molecular microbial ecology studies, *Applied and Environmental Microbiology*, 62, p. 4504-4513.

Zogorski, J.S., Carter, J.M., Ivahnenko, T., Lapham, W.W., Moran, M.J., Rowe, B.L., Squillace, P.J. and Toccalino, P.L., 2006, The quality of our Nation's waters — Volatile organic compounds in the Nation's ground water and drinking-water supply wells, U.S. Geological Survey Circular 1292, p. 101, online at <http://pubs.usgs.gov/circ/circ1292/>.