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Halogenated Aliphatic and Aromatic Aerobic Biodegradation Via Direct Metabolism and Cometabolism: Batch and Continuous Tests

by

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Abstract of "Halogenated Aliphatic and Aromatic Aerobic Biodegradation Via Direct Metabolism and Cometabolism: Batch and Continuous Tests" by Andrea Meniconi, Ph.D., University of Bologna, March 2007.

In this thesis the application of biotechnological processes based on microbial metabolic degradation of halogenated compound has been investigated. Several studies showed that most of these pollutants can be biodegraded by single bacterial strains or mixed microbial population via aerobic direct metabolism or cometabolism using as a growth substrates aromatic or aliphatic hydrocarbons. The enhancement of two specific processes has been here object of study in relation with its own respective scenario described as follow:

1st) the bioremediation via aerobic cometabolism of soil contaminated by a high chlorinated compound using a mixed microbial population and the selection and isolation of consortium specific for the compound.

2nd) the implementation of a treatment technology based on direct metabolism of two pure strains at the exact point source of emission, preventing dilution and contamination of large volumes of waste fluids polluted by several halogenated compound minimizing the environmental impact.

In order to verify the effect of this two new biotechnological application to remove halogenated compound and purpose them as a more efficient alternative continuous and batch tests have been set up in the experimental part of this thesis.

Results obtained from the continuous tests in the second scenario have been supported by microbial analysis via Fluorescence *in situ* Hybridisation (FISH) and by a mathematical model of the system. The results showed that both process in its own respective scenario offer an effective solutions for the biological treatment of chlorinate compound pollution.

This dissertation by Andrea Meniconi is accepted in its present form by Department of Chemical Mining and Environmental Engineering as satisfying the dissertation requirement for the degree of Doctor of Philosophy

Date…….. …….
Prof. Ing. Massimo Nocentini, Advisor

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The Vita of Andrea Meniconi

Andrea Meniconi was born in October 3rd, 1977, in Montepulciano, Siena, Italy. He pursued a graduate degree in chemical engineering at University of Bologna, Bologna, in 2003. In December he got admission to Ph.D. program at the department of chemical mining and environmental technology engineering, University of Bologna, Bologna, Italy.

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Publications and Conferences

Journal Publications:

1. A.Meniconi, D Frascari, D. Pinelli e M .Nocentini, 2005 "Biodegradazione aerobica cometabolica del 1,1,2,2-tetracloroetano: prove di bioaugmentation in microcosmi slurry ed in mezzo liquido." In: L.Morselli (a cura di), Tecnologie Innovative per l'Industrializzazione del Sistema Ambiente. Atti dei Seminari di Ecomondo 05 (Rimini, Ottobre 2005), Maggioli Editore, Rimini, pp. 412-419.

2. A.Meniconi, D Frascari, D. Pinelli e M .Nocentini, 2006. "Aerobic Cometabolic biodegradation of 1,1,2,2-tetrachloroethane: a microcosm study." In: 7th UK Meeting – IWA UK International Researchers Conference (Bath, UK, April 4-5, 2006). p.12

3. M. Koutinas, I.I.R. Baptista, A.Meniconi, L.G. Peeva[1], A.Mantalaris, P.M.L. Castro and A. G. Livingston, "The Use of an Oil-Absorber-Bioscrubber System During Biodegradation of Sequentially Alternating 1,2-Dichloroethane and Fluorobenzene in Waste Gas". Submitted for review to Chemical Engineering Science.

4. M. Koutinas, I.I.R. Baptista, A.Meniconi, L.G. Peeva[1], P.M.L. Castro and A. G. Livingston, 2006. "Microbial dynamics and bioreactor stability in an oil-absorber-bioscrubber system exposed to an alternating sequence of 1,2-dichloroethane and fluorobenzene" Poster presentation n. 229, in AICHE Conference – San Francisco Novembre 2006.

Conference Proceedings:

1. *Ecomondo RIMINI 2005* – "Biodegradazione aerobica cometabolica del 1,1,2,2-Tetracloroetano: prove di bioaugmentation in microcosmi slurry ed in mezzo liquido." (Oral presentation)

2. IWA conference BATH 2006 – "Aerobic co-metabolic biodegradation of 1,1,2,2-tetrachloroethane: a microcosm study" (Oral presentation and Poster) - *First Prize Award for the best poster presentation.*

To my Family
Marcella, Arnaldo
and Eva
(Who still doesn't want to be called "the grandma")

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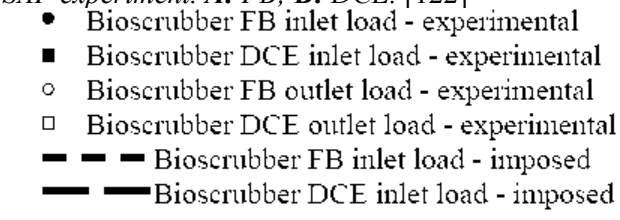

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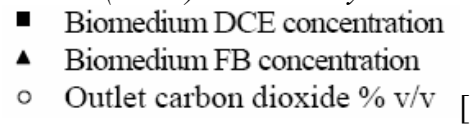

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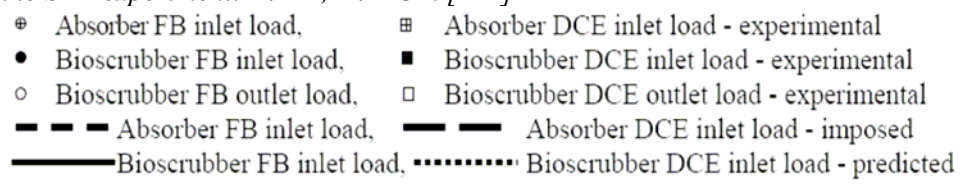

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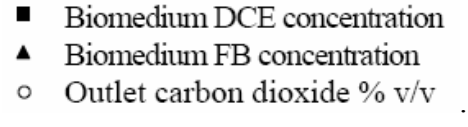

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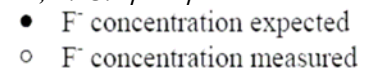

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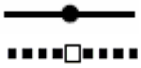

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Chapter 1

Introduction

1.1 Problem definition

Biosphere is supporting now over six billions of people, resulting in an increasing demand of fuel, industrial chemicals, processed food, textiles and similar essential products in order to meet the society's desire to improve the quality of life. To satisfy such demands, industrialization was offering commodities and services to the public, leading to an increasing sophistication of the chemicals industries in the past century and particularly over the last thirty years.

The first side-effect of this industrial expansion have been an increasing amount and complexity of toxic waste effluents which are released into the environment through wastewaters, gaseous emissions and solid residues causing environmental pollution and a deterioration of natural resources.

As the same time, fortunately, regulatory authorities have been paying more attention to the environmental contamination problems taking drastic measures in several industrial sectors to achieve a sustainable development; for this reason industrial companies are becoming increasingly aware of the political, social, environmental and regulatory pressures to prevent escape of effluent into the environment.

In a parallel way, the occurrence of major incidents (such as the Exxon Valdez oil spill, the Union-Carbide (Dow) Bhopal disaster, large-scale contamination of the

Rhine River, the progressive deterioration of the aquatic habitats and conifer forests in the Northeastern US, Canada, and parts of Europe, or the release of radioactive material in the Chernobyl accident, etc.) and the subsequent massive publicity due to the resulting environmental problems has highlighted the potential for imminent and long-term disasters in the public's conscience.

Even though policies and environmental efforts should continue to be directed towards applying pressure to industry to reduce toxic waste production, a number of strategies for industrial pollutant control have been implanted during the past few decades in order to prevent environmental pollution and achieve sustainable utilization of natural resources.

In this scenario, biotechnology appears as a valid opportunity to detoxify industrial toxic effluents even if the bioprocesses must compete with existing methods in terms of efficiency and economy.

However, the biotechnological solutions to the problem require only moderate capital investment, a low energy input, are environmentally safe, generate a limited amount of waste, and are self-sustaining.

Biotechnological methods of toxic waste treatment are likely to play an increasingly key role both as a displacement for existing disposal methods and for the detoxification of novel xenobiotic compounds.

On the other hand, however, it is important to limit the generation of both hazardous and non-hazardous waste as much as possible, and utilize recycling methods wherever possible.

Over the last few years, the increasingly stringent regulatory requirements have been accompanied by the raise of companies already established to develop and commercialize biodegradation technologies.

The interest of commercial businesses in utilizing microorganisms to detoxify effluents, soils and wastewaters is reflected in "bioremediation" having become a common buzzword in waste management.

1.2 Bioremediation as a possible solution

Offering a good option to destroy or render harmless various contaminants using natural biological activity, bioremediation is one of the most suitable solution which can often be carried out on site for cleaning soil wastewater and waste stream from the presence of xenobiotic compounds because of its potential of completely degrading waste material with little or no toxic by-products coupled with the advantage of a lower costs whit a generally high public acceptance.

Bioremediation will not always be suitable, however, as the range of contaminants on which it is effective is limited, the time scales involved are relatively long, and the residual contaminant levels achievable may not always be appropriate.

Although the methodologies employed are not technically complex, considerable experience and expertise may be required to design and implement a successful bioremediation program, due to the need to thoroughly assess a site for suitability and to optimize conditions to achieve a satisfactory result.
Bioremediation has been used at a number of sites worldwide, including Europe, with varying degrees of success.

Techniques are improving as greater knowledge and experience are gained, and there is no doubt that bioremediation has great potential for dealing with certain types of site contamination.

Unfortunately, the principles, techniques, advantages, and disadvantages of bioremediation are not widely known or understood, especially among those who will have to deal directly with bioremediation proposals, such as site owners and regulators.

1.3 Motivation and objectives

Halogenated compounds form a class of chemicals extensively used as solvents, pesticides, fuel additives, pharmaceuticals, etc.

Despite their usefulness, these chemicals are toxic and carcinogenic, so their release to the environment has to be restricted and controlled.

Nevertheless this kind of compounds, with particular regard to the subclass of chlorinated aliphatic hydrocarbons, (CAHs) is widespread contaminants that are often present in polluted site as a complex mixture.

Several studies showed that most of these pollutants can be biodegraded by single bacterial strains or mixed microbial population via aerobic direct metabolism or cometabolism using, as a growth substrates, aromatic and aliphatic hydrocarbons such as methane, propane, butane, phenol or toluene.

In this thesis two different scenarios where biotechnological processes based on microbial metabolic degradation of halogenated compound found their application, have been studied:

1st) the bioremediation via aerobic cometabolism of soil contaminated by a high chlorinated compound using a mixed microbial population and the selection and isolation of consortium specific for the compound.

2nd) the implementation of a treatment technology based on direct metabolism of two pure strains at the exact point source of emission, preventing dilution and contamination of large volumes of waste fluids polluted by several halogenated compound minimizing the environmental impact *[122]*.

Special regards has been given to one of the major drawback of this strategy due to the fact that these emissions often result from batch processes: the typical fluctuating loads and sequentially alternating pollutants (S.A.P.) in waste streams.

The objective of this thesis is to investigate and verify the effect of new biotechnological processes to remove halogenated compound in this two scenarios and purpose them as an alternative and more efficient in clean-up situation.

1.4 Outline

This thesis is organized as follows.

Chapter 2 present a literature review on the volatile organic compounds paying particular attention to the subclass of halogenated and chlorinated VOC. Sources, basic properties and degradation pathways have been object of this section.

Chapter 3 constitutes an overview on the different approaches in removing chlorinated compound. Bioremediation is presented here as an important option to achieve remarkable results and a short review on in situ remediation technology alongside with bioreactors applications is here presented.

Chapter 4 aims to illustrate the aerobic biodegradation mechanism and the kinetic models for cometabolism and direct metabolism object of the two section of this thesis.

Chapter 5 constitutes the first section of the experimental work of the present study: the object was to investigate the possibility of biodegradation of 1,1,2,2 tetrachloroethane via aerobic cometabolic degradation. All the tests have been carried out in batch conditions and the microbial part (selection and isolation of the pure strain and consortium) has been realized in collaboration with the Department of Experimental and Evolutionary Biology of the University of Bologna.

Chapter 6 and the following two have been based on the enhancement of a biotechnological process object of study of the Department of Chemical Engineering of the Imperial College London. The use of a system absorber

bioscrubber in scenario typical of the chemical batch processes (sequentially alternating pollutant loads) has been object of study of this chapter: the pure microbial strains inoculated in the bioreactor were able of direct metabolism on the tested chlorinated substrates*[122]*.

Chapter 7 aims to design a mathematical model able to describe the performance of the oil absorber bioscrubber system investigated in the previous chapter the model is based on unstructured biological kinetics has been developed for the system based on Chang's model.

Chapter 8 compare different approaches in determining the microbial growth kinetic parameters: batch and fed batch method have been investigated in order to understand what could be the most suitable method to obtain accurate values. The need for this last section of the work finds the roots in the sensitive analysis of the model previous chapter indicating the kinetic parameters as defective.

Chapter 2

Volatile Organic Compound

2.1 Introduction on VOCs

The compounds object of bio-degradation tests in this thesis belong to the class of organic chemicals called volatile organic compounds (VOCs). Depending on the source, a VOC has two definitions — one within a physic-chemical context and the other within a regulatory context.

The physic-chemical definition of a VOC as stated by Australia's National Pollutant Inventory is:

Any chemical compound based on carbon chains or rings (and also containing hydrogen) with a vapour pressure greater than 2 mm of mercury (mm Hg) at 25 degrees Celsius (°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).

A physic-chemical definition of VOC as explicit as that from Australia and originating in the United States was not found during extensive Internet searches. In the United States, the regulatory definition of VOC is provided by the USEPA under the Clean Air Act and published in the Code of Federal Regulations — *Volatile organic compound (VOC) means any compound of carbon, excluding carbon*

monoxide, carbon dioxide, carbonic acid, metallic carbides or carbonates, and ammonium carbonate, which participates in atmospheric photochemical reactions (U.S. Environmental Protection Agency, 2000a).

Under the IUPAC naming convention, VOCs are commonly assigned to two general groups: (1) aliphatic hydrocarbons (alkanes, alkenes), and (2) aromatic hydrocarbons (Brown and LeMay, 1977).

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 indicate stronger covalent bonds between two carbon atoms and 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.

In contrast to the aliphatic compounds, aromatic compounds are those with alternating carbon-carbon single and double bonds arranged in a ring structure (Benzene is the most commonly recognized aromatic compound as stated by Brown and LeMay, 1977). 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 subgrouped 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).

The alkyl radicals are the lower molecular weight alkanes minus one hydrogen atom and are highly reactive compounds that can easily displace a hydrogen atom on another molecule.

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.

The presence of volatile organic compounds (VOCs) in ground water is a major concern to all who use ground water as a drinking water source because many of these compounds can adversely affect human health.

For this reason is important to describe the basic chemical properties of selected VOCs by subclass, and (3) describe the various pathways and chemical by-products associated with the degradation of selected VOCs in ground water.

2.2 Source of VOCs detected in ground water

A relatively large amount of literature exists that describes VOCs in ground water at specific, known areas of contamination.

Few documents, however, describe VOC contamination in a regional or national context. One report by Arneth and others (1989) lists the top 15 VOCs detected in ground water near landfills in the United States and in Germany (table 4).

This list shows that the VOCs contaminating ground water near landfills are similar in both countries. Most of these VOCs are chlorinated solvents (CVOCs) and gasoline compounds (gVOCs). 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 2.1 (table 6; Delzer and Ivahnenko, 2003; Moran, 2006; Zogorski and others, 2006).

Rank	United States of America		Federal Republic of Germany	
	IUPAC name[2]	Common or alternative name	IUPAC name[2]	Common or alternative name
1	1,1,2-trichloroethene	1,1,2-trichloroethylene, TCE	tetrachloroethene	perchloroethylene, tetrachloro-ethylene, PCE
2	tetrachloroethene	perchloroethylene, tetrachloroethylene, PCE	1,1,2-trichloroethene	1,1,2-trichloroethylene, TCE
3	*cis*-1,2-dichloroethene	*cis*-1,2-DCE	*trans*-1,2-dichloroethene	*trans*-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	*trans*-1,2-dichloroethene	*trans*-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

[1]Arneth and others, 1989, p. 399
[2]International Union of Pure and Applied Chemistry, 2006

Table 2.1 - *Volatile organic compounds ranked by those frequently detected in ground water near landfills and hazardous waste dumps in the United States and the Federal Republic of Germany.*

Rank	Dover Air Force Base, Maryland[1]	U.S. Army Armament Research and Development Center, Picatinny, New Jersey, 1958–85[2]	U.S. Naval Undersea Warfare Center, Washington, D.C.[3]	Wright-Patterson Air Force Base, Ohio, 1993–94[4]
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	*cis*-1,2-dichloroethene [21.7]	tetrachloroethene (PCE) [24.9]	*cis*-1,2-dichloroethene [59]	tetrachloroethene (PCE) [5.8]
3	1,1,2-trichloroethene (TCE) [20.3]	*trans*-1,2-dichloroethene (DCE) [18.6]	*trans*-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]	*cis*- and *trans*-1,2-dichloroethene [1.2]
6	methylbenzene [6.6]	*cis*-1,2-dichloroethene (DCE) [9.6]	1,1-dichloroethane [37.2]	chloroethene [.9]
7	dimethylbenzenes (*m*-, *p*-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]

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

Table 2.2 - *Volatile organic compounds detected in ground-water case studies at selected U.S. Department of Defense installations.*

Although the number of VOCs analyzed in ground-water samples is large for national and regional studies, the most commonly detected compounds, primarily CVOCs and gVOCs, are similar to those at site-specific studies completed at U.S. Department of Defense installations (table 2.2).

[ug/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[1]	Ground water in the Santa Ana River Basin, California[2]	Ground-water and drinking-water supply wells in the United States (concentrations greater than 0.2 µg/L)[3]		
			Aquifer studies[4]	Domestic water-supply wells[5]	Public water-supply wells[6]
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	*cis*-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 [.97]	1,1-dichloroethane [2.0]
6	tetrachloroethene (PCE) [9.8]	*cis*-1,2-dichloroethene [4.3]	chloromethane [1.1]	1,1,2-trichloroethene (TCE) [.92]	*cis*-1,2-dichloroethene [1.5]
7	benzene [8.5]	methylbenzene [3.8]	*trans*-1,2-dichloroethene [0.91]	dichloromethane [.67]	1,1-dichloroethene (DCE) [1.3]
8	chloroethene [8.0]	1,1-dichloroethane [2.9]	dichloromethane [0.89]	1,2,4-trimethylbenzene [.32]	*trans*-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 [.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 [.21]	tetrachloromethane [.73]
11	ethylbenzene [7.6]	1,3- and 1,4-dimethylbenzene [1.4]	benzene [.63]	tetrachloromethane [.21]	1,3- and 1,4-dimethylbenzene [0.60]
12	1,2,4-trimethylbenzene [7.1]	*trans*-1,2-dichloroethene [<1]	1,2,4-trimethylbenzene [.63]	1,1-dichloroethene [.21]	1,2-dichloroethane [.56]
13	1,2-dimethylbenzene [6.8]	dichloromethane [<1]	1,2-dichloroethane [.47]	total xylenes [0.21]	1,2-dimethylbenzene [.48]
14	chloromethane [6.7]	ethylbenzene [<1]	*cis*-1,2-dichloroethene [.42]	*cis*-1,2-dichloroethene [.18]	benzene, dichloromethane, ethylbenzene [.46]
15	naphthalene [6.5]	naphthalene [<1]	total xylenes [.38]	naphthalene [.15]	chloromethane [.38]
16	chloroethane [6.3]	tetrachloromethane [<1]	tetrachloromethane [.31]	ethylbenzene [.12]	1,2,4-trimethylbenzene [0.32]
17	chlorobenzene [4.3]	1,2,4-trimethylbenzene [<1]	chloroethane [.29]	chloroethane [.093]	chloroethane [.28]
18	1,2-dichloroethane [3.7]	chlorobenzene [ND]	chloroethene [.26]	chloroethene [.083]	vinyl benzene [.19]
19	*trans*-1,2-dichloroethene [3.3]	chloroethane [ND]	ethylbenzene [.26]	*trans*-1,2-dichloroethene [.045]	chlorobenzene, 1,2-dichlorobenzene [.18]
20	1,1-dichloroethene (DCE) [2.6]	chloromethane [ND]	chlorobenzene [.17]	chlorobenzene [.042]	chloroethene [.18]
21	1,2-dichlorobenzene [2.4]	chloroethene [ND]	naphthalene [.16]	1,2-dichlorobenzene [.042]	naphthalene [.10]
22	2-methoxy-2-methylpropane (MTBE) [2.3]	1,2-dichlorobenzene [ND]	1,2-dichlorobenzene [.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]

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

Table 2.3 -*Volatile organic compounds detected in regional and national ground-water studies in the United States.*

The 10 most commonly detected VOCs in the studies summarized in tables 2.2 and 2.3 are methyl *tert*-butyl ether (MTBE), tetrachloroethene (PCE), 1,1,2-trichloroethene (TCE), methylbenzene (toluene), 1,1,1-trichloroethane (111-TCA), benzene, *cis*-1,2-dichloroethene (12-cDCE), 1,1-dichloroethane (11-DCA), *trans*-1,2-dichloroethene (12-tDCE), the dimethylbenzenes (*m*-, *o*-, *p*-xylenes).

2.2.1 Sources of chlorinated alkanes

The chlorinated solvents within the alkane group are listed in table 2.4. The CVOCs 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 non-feed-stock purposes was phased-out completely.

Chemical manufacturing is the largest use of 11-DCA and 1,2-dichloroethane (12-DCA). Both compounds serve as an intermediate during the manufacture of chloroethene (vinyl chloride, VC), 111-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 percent of the 12-DCA produced in the United States is used to manufacture VC. Smaller amounts of 12-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).

The compound 111-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 (112-TCA), has limited use as a common, general-use solvent but is used in the production of chlorinated rubbers (Archer, 1979). In some cases, 112-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 anesthetics, 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).

[IUPAC, International Union of Pure and Applied Chemistry; CAS, Chemical Abstract Services; —, not applicable]

IUPAC name[1]	Common or alternative name (synonyms)[2]	Other possible names[2]	Predominant source	CAS number[1]
		Alkyl benzenes		
1,2-dimethylbenzene	*o*-xylene	The X in BTEX, dimethyltoluene, Xylol	gasoline	95-47-6
1,3-dimethylbenzene	*m*-xylene			108-38-3
1,4-dimethylbenzene	*p*-xylene			106-42-3
ethylbenzene	—	The E in BTEX, Ethylbenzol, phenylethane	gasoline	100-41-4
methylbenzene	toluene	The T in BTEX, phenylmethane, Methacide, Toluol, Antisal 1A	gasoline	108-88-3
1,2,4-trimethylbenzene	pseudocumene	pseudocumol, asymmetrical trimethyl-benzene	gasoline	95-63-6
		Aromatic hydrocarbons		
benzene	—	The B in BTEX, coal naphtha, 1,3,5-cyclohexatriene, mineral naphtha	gasoline	71-43-2
naphthalene	naphthene	—	gasoline, organic synthesis	91-20-3
styrene	vinyl benzene	phenethylene	gasoline, organic synthesis	100-42-5
		Ethers		
2-methoxy-2-methylpropane	methyl *tert*-butyl ether, MTBE	*tert*-butyl methyl ether	fuel oxygenate	1634-04-4
		Chlorinated alkanes		
chloroethane	ethyl chloride, monochloroethane	hydrochloric ether, muriatic ether	solvent	75-00-3
chloromethane	methyl chloride	—	solvent	74-87-3
1,1-dichloroethane	ethylidene dichloride	—	solvent, degreaser	75-34-3
1,2-dichloroethane	ethylidene dichloride	glycol dichloride, Dutch oil	solvent, degreaser	107-06-2
tetrachloromethane	carbon tetrachloride	perchloromethane, methane tetrachloride	solvent	56-23-5
1,1,1-trichloroethane	methyl chloroform	—	solvent, degreaser	71-55-6
		Chlorinated alkenes		
chloroethene	vinyl chloride	chloroethylene, monochloroethene, monovinyl chloride (MVC)	organic synthesis, degradation product	75-01-4
1,1-dichloroethene	1,1-dichloroethylene, DCE	vinylidene chloride	organic synthesis, degradation product	75-35-4
cis-1,2-dichloroethene	*cis*-1,2-dichloroethylene	1,2 DCE, Z-1,2-dichloroethene	solvent, degradation product	156-59-2
trans-1,2-dichloroethene	*trans*-1,2-dichloroethylene	1,2 DCE, E-1,2-dichloroethene	solvent, degradation product	156-60-2
dichloromethane	methylene chloride	—	solvent	74-09-2
		Chlorinated alkenes		
tetrachloroethene	perchloroethylene, PCE, 1,1,2,2-tetrachloroethylene	ethylene tetrachloride, carbon dichloride, PERC®, PERK®	solvents, degreasers	127-18-4
1,1,2-trichloroethene	1,1,2-trichloroethylene, TCE	acetylene trichloroethylene	solvents, degreasers organic synthesis	79-01-6
		Chlorinated aromatics		
chlorobenzene	monochlorobenzene	benzene chloride, phenyl chloride	solvent, degreaser	108-90-7
1,2-dichlorobenzene	o-dichlorobenzene	ortho dichlorobenzol	organic synthesis	95-50-1
1,2,3-trichlorobenzene	1,2,6-trichlorobenzene	—	organic synthesis	87-61-6
1,2,4-trichlorobenzene	1,2,4-trichlorobenzol	—	organic synthesis	102-82-1

[1]International Union of Pure and Applied Chemistry, 2006
[2]U.S. Environmental Protection Agency, 1995

Table 2.4 - *Names and synonyms of volatile organic compounds commonly detected in ground water.*

2.2.2 Sources of chlorinated alkenes and benzenes

The chlorinated alkenes listed in table 2 include two of the most widely used and distributed solvents in the United States and Europe. These solvents, PCE and TCE, also are among the most common contaminants in ground water (tables 5 and table 6). 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*, 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).

Four chlorinated benzenes commonly detected in groundwater contamination studies include chlorobenzene (CB), 1,2-dichlorobenzene (12-DCB), and two isomers of trichlorobenzene, 1,2,3-trichlorobenzene (123-TCB) and 1,2,4-trichlorobenzene (124-TCB; tables 5 and 6).

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 12-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, Web page: *http://www.epa.gov/OGWDW/dwh/t-voc/t-124-tric.html*, accessed May 23, 2006).

2.3 Basic properties of selected volatile organic compounds

Volatile organic compounds have a number of unique properties that both inhibit and facilitate ground-water contamination. Tables 2.5 through 2.9 list basic physical properties of 27 VOCs detected in ground water. Physical properties unique to each compound typically are governed by the number of carbons and the covalent bonding in the compound, the number and location of chlorine atoms, and the number, location and type of alkyl groups.

The physical properties addressed in this report include the Henry's Law constant (H), water solubility, density, octanol-water partitioning (Log K_{ow}), and organic carbon partitioning (Log K_{oc}) of the non-aqueous phase liquid (NAPL).

Models that estimate the fate and transport of VOCs in ground water depend on the accuracy and reliability of physical property measurements. Some models, such as the fugacity models, also use these properties to predict a compound's rate of movement into and out of environmental compartments (soil, water, air, or biota; Mackay, 2004).

Predicting the environmental fate of a compound in ground water depends on data that quantifies: (1) the compound's tendency to volatilize (gaseous phase), (2) to dissolve in water (aqueous phase), (3) to float on or sink beneath the water surface, (4) to dissolve in or to sorb to other organic compounds (including natural organic matter), and (5) the compound's affinity for ionically charged surfaces such as clay or soil particles.

Fugacity models of varying complexity are in common use and rely on the physical properties of these compounds to estimate plume migration and persistence, and to guide the remediation of contaminated ground water (Mackay and others, 1996; Institute for Environmental Health, 2004; Saichek and Reddy, 2005).

IUPAC name[1]	Common or alternative name[2]	Henry's Law[3] constant (H) (kPa m^3 mol^{-1} at 25°C)
tetrachloromethane	carbon tetrachloride	2.99
chloroethene	vinyl chloride, chloroethylene	2.68
1,1-dichloroethene	1,1-dichloroethylene, DCE	2.62
1,1,1-trichloroethane	methyl chloroform	1.76
tetrachloroethene	perchloroethylene, tetrachloroethylene, PCE	1.73
chloroethane	ethyl chloride, monochloroethane	[4]1.11
1,1,2-trichloroethene	1,1,2-trichloroethylene, TCE	1.03
trans-1,2-dichloroethene	*trans*-1,2-DCE, *trans*-1,2-dichloroethylene	.960
chloromethane	methyl chloride	[5].920
ethylbenzene	—	.843
1,3-dimethylbenzene	*m*-xylene	.730
1,4-dimethylbenzene	*p*-xylene	.690
methylbenzene	toluene	.660
1,1-dichloroethane	1,1-ethylidene dichloride	.630
benzene	—	.557
1,2-dimethylbenzene	*o*-xylene	.551
1,2,4-trimethylbenzene	pseudocumene	.524
cis-1,2-dichloroethene	*cis*-1, 2-dichloroethylene, *cis*-1,2-DCE	.460
chlorobenzene	monochlorobenzene	.320
styrene	vinyl benzene	.286
1,2,4-trichlorobenzene	1,2,4-trichlorobenzol	.277
1,2,3-trichlorobenzene	1,2,6-trichlorobenzene	.242
1,2-dichlorobenzene	o-dichlorobenzene	.195
1,2-dichloroethane	1,2-ethylidene dichloride, glycol dichloride	.140
1,1,2-trichloroethane	methyl chloroform	.092
2-methoxy-2-methylpropane	methyl *tert*-butyl ether, MTBE	.070
naphthalene	naphthene	.043

Increasing tendency for a compound to move from the water phase to the vapor phase when in equilibrium with pure water

[1]International Union of Pure and Applied Chemistry, 2006
[2]U.S. Environmental Protection Agency, 1995
[3]Lide, 2003
[4]Gossett, 1987
[5]National Center for Manufacturing Sciences, 2006

Table 2.5 - *Names and synonyms of volatile organic compounds commonly detected in ground water*.

IUPAC name[1]	Common or alternative name[2]	Water solubility[3] (mg/L at 25°C)
2-methoxy-2-methylpropane	methyl *tert*-butyl ether, MTBE	36,200
1,2-dichloroethane	1,2-ethylidene dichloride, glycol dichloride	8,600
chloromethane	methyl chloride	[4]5,320
chloroethane	ethyl chloride, monochloroethane	[5]6,710
cis-1,2-dichloroethene	*cis*-1,2-dichloroethylene	6,400
1,1-dichloroethane	1,1-ethylidene dichloride	5,000
1,1,2-trichloroethane	methyl chloroform	4,590
trans-1,2-dichloroethene	*trans*-1,2-dichloroethylene	4,500
chloroethene	vinyl chloride, chloroethylene	2,700
1,1-dichloroethene	1,1-dichloroethylene, DCE	2,420
benzene	—	1,780
1,1,1-trichloroethane	methyl chloroform	1,290
1,1,2-trichloroethene	1, 1, 2-trichloroethylene, TCE	1,280
tetrachloromethane	carbon tetrachloride	1,200
methylbenzene	toluene	531
chlorobenzene	—	495
styrene	vinyl benzene	321
tetrachloroethene	perchloroethylene, tetrachloroethylene, PCE	210
1,2-dimethylbenzene	*o*-xylene	207
1,4-dimethylbenzene	*p*-xylene	181
1,3-dimethylbenzene	*m*-xylene	161
ethylbenzene	—	161
1,2-dichlorobenzene	o-dichlorobenzene	147
1,2,4-trimethylbenzene	pseudocumene	57
1,2,4-trichlorobenzene	1,2,4-trichlorobenzol	37.9
naphthalene	naphthene	[6]31.0
1,2,3-trichlorobenzene	1,2,6-trichlorobenzene	30.9

[1]International Union of Pure and Applied Chemistry, 2006

[2]U.S. Environmental Protection Agency, 1995

[3]Lide, 2003

[4]National Center for Manufacturing Sciences, 2006

[5]Horvath, 1982

[6]Lyman, 1982

Increasing amount of non-aqueous phase liquid that can dissolve in water

[IUPAC, International Union of Pure and Applied Chemistry; mg/L, milligrams per litre; °C, degrees Celsius; —, not applicable]

Table.2.6 - *Water-solubility data for selected volatile organic compounds detected in ground water.*

IUPAC name[1]	Common or alternative name[2]	Density[3] (g/cm³, 20°C)
1,2,3-trichlorobenzene	1,2,6-trichlorobenzene	[4]1.690
tetrachloroethene	perchloroethylene, tetrachloroethylene, PCE	1.623
tetrachloromethane	carbon tetrachloride	1.594
1,1,2-trichloroethene	1,1,2-trichloroethylene, TCE	1.464
1,2,4-trichlorobenzene	1,2,4-trichlorobenzol	[4]1.45
1,1,2-trichloroethane	methyl chloroform	1.44
1,1,1-trichloroethane	methyl chloroform	1.339
1,2-dichlorobenzene	o-dichlorobenzene	1.306
cis-1,2-dichloroethene	*cis*-1,2-dichloroethylene	1.284
trans-1,2-dichloroethene	*trans*-1,2-dichloroethylene	1.256
1,2-dichloroethane	1,2-ethylidene dichloride, glycol dichloride	1.235
1,1-dichloroethene	1,1-dichloroethylene, DCE	1.213
1,1-dichloroethane	1,1-ethylidene dichloride	1.176
chlorobenzene	monochlorobenzene	1.106
pure water at 20°C		1.000
naphthalene	naphthene	.997
chloromethane	methyl chloride	.991
chloroethane	ethyl chloride	.920
chloroethene	vinyl chloride, chloroethylene	[4].910
styrene	vinyl benzene	.906
1,2-dimethylbenzene	*o*-xylene	.880
benzene	—	.876
ethylbenzene	—	.867
1,2,4-trimethylbenzene	pseudocumene	.876
methylbenzene	toluene	.867
1,3-dimethylbenzene	*m*-xylene	.864
1,4-dimethylbenzene	*p*-xylene	.861
2-methoxy-2-methylpropane	methyl *tert*-butyl ether, MTBE	.740

Increasing density (heavier than water)

Decreasing density (lighter than water)

[1]International Union of Pure and Applied Chemistry, 2006

[2]U.S. Environmental Protection Agency, 1995

[3]Lide, 2003

[4]Chiou and others, 1983

[IUPAC, International Union of Pure and Applied Chemistry; g/cm, grams per centimetre; °C, degrees Celsius; —, not applicable]

Table 2.7 - *Density of selected volatile organic compounds detected in ground water compared to the density of water at 20 degrees Celsius.*

IUPAC name[1]	Common or alternative name[2]	Octanol/ water partition coefficient[3] (Log K_{ow})
1,2,3-trichlorobenzene	1,2,6-trichlorobenzene	[4]4.07
1,2,4-trichlorobenzene	1,2,4-trichlorobenzol	[4]4.04
1,2,4-trimethylbenzene	pseudocumene	3.65
1,2-dichlorobenzene	o-dichlorobenzene	3.46
naphthalene	naphthene	3.36
1,3-dimethylbenzene	*m*-xylene	3.20
ethylbenzene	ethylbenzene	3.15
1,4-dimethylbenzene	*p*-xylene	3.15
1,2-dimethylbenzene	*o*-xylene	3.12
styrene	vinyl benzene	3.05
tetrachloroethene	perchloroethylene, tetrachloroethylene, PCE	2.88
chlorobenzene	monochlorobenzene	[4]2.84
methylbenzene	toluene	2.73
tetrachloromethane	carbon tetrachloride	[4]2.64
1,1,2-trichloroethene	1,1,2-trichloroethylene, TCE	[3]2.53
1,1,1-trichloroethane	methyl chloroform	[4]2.49
1,1,2-trichloroethane	methyl chloroform	2.38
1,1-dichloroethene	1,1-dichloroethylene, DCE	2.13
benzene	—	2.13
trans-1,2-dichloroethene	*trans*-1,2-dichloroethylene	1.93
cis-1,2-dichloroethene	*cis*-1,2-dichloroethylene	1.86
1,1-dichloroethane	1,1-ethylidene dichloride	[4]1.79
1,2-dichloroethane	1,2-ethylidene dichloride, glycol dichloride	[4]1.48
chloroethane	ethyl chloride	1.43
chloroethene	vinyl chloride, chloroethylene	1.38
2-methoxy-2-methylpropane	methyl *tert*-butyl ether, MTBE	.94
chloromethane	methyl chloride	.91

Increasing affinity for organic matter and lipids

[1]International Union of Pure and Applied Chemistry, 2006

[3]U.S. Environmental Protection Agency, 1995

[3]Sangster, 1989

[4]Mackay and others, 1992a

[IUPAC, International Union of Pure and Applied Chemistry; K_{ow}, octanol-water partition coefficient; —, not applicable]

Table 2.8 - *Octanol-water partition coefficients for selected volatile organic compounds detected in ground water.*

IUPAC name[1]	Common or alternative name[2]	Soil-sorption coefficient (Log K_{oc} in soil)
1,2,4-trimethylbenzene	pseudocumene	[3]3.34
1,2,3-trichlorobenzene	1,2,6-trichlorobenzene	[4]3.18–[3]3.42
naphthalene	naphthene	[3]2.98
1,2,4-trichlorobenzene	1,2,4-trichlorobenzol	[5]2.94
vinyl benzene	styrene	[2]2.72–2.74
1,2-dichlorobenzene	o-dichlorobenzene	[6]2.46–[5]2.51
tetrachloroethene	perchloroethylene, tetrachloroethylene, PCE	[7]2.37
ethylbenzene	—	[5]2.22
1,1-dichloroethene	1,1-dichloroethylene, DCE	[2]2.18
1,3-dimethylbenzene	*m*-xylene	[7]2.11–2.46
1,1,1-trichloroethane	methyl chloroform	[8]2.03
1,1,2-trichloroethene	1,1,2-trichloroethylene, TCE	[7]2.00
chlorobenzene	monochlorobenzene	[5]1.91
1,1,2-trichloroethane	methyl chloroform	[7]1.78–2.03
tetrachloromethane	carbon tetrachloride	[8]1.78
methylbenzene	toluene	[7]1.75–[10]2.28
chloroethene	vinyl chloride, chloroethylene	[2]1.75
1,2-, 1,4-dimethylbenzene	*o*-xylene, *p*-xylene	[2]1.68–1.83
chloroethane	ethyl chloride	[4]1.62
cis-1,2-dichloroethene	*cis*-1,2-dichloroethylene	[2]1.56–1.69
1,2-dichloroethane	1,2-ethylidene dichloride, glycol dichloride	[6]1.52
trans-1,2-dichloroethene	*trans*-1,2-dichloroethylene	[2]1.56–1.69
1,1-dichloroethane	1,1-ethylidene dichloride	[12]1.52
benzene	—	[5]1.49–[7]1.73
methyl *tert*-butyl ether	MTBE	[11]1.09
chloromethane	methyl chloride	[3].778

Increasing affinity for soil organic matter

[1]International Union of Pure and Applied Chemistry, 2006

[2]U.S. Environmental Protection Agency, 1995

[3]Boyd and others, 1990

[4]Schwarzenbach and Westall, 1981

[5]Chiou and others, 1983

[6]Chiou and others, 1979

[7]Seip and others, 1986

[8]Friesel and others, 1984

[9]Kile and others, 1996

[10]Garbarini and Lion, 1986

[11]U.S. Environmental Protection Agency, 1994

[12]U.S. Environmental Protection Agency, 2005b

[IUPAC, International Union of Pure and Applied Chemistry; K_{oc}, soil organic carbon partition coefficient; —, not applicable]

Table 2.9 - *Soil-sorption partition coefficients for selected volatile organic compounds detected in ground water.*

2.4 Degradation of selected Volatile Organic Compounds in ground water

Under specific conditions, most organic compounds degrade at a particular rate during a given length of time.

The speed of the degradation depends on the presence and activity of microbial consortia (bacteria and fungi species), environmental conditions (temperature, aquifer materials, organic matter content), and the availability and concentration of carbon sources (primary substrate) available to the microbial consortia.

The primary substrate can be a VOC or organic carbon found dissolved in water or sorbed to aquifer sediments. When primary substrate concentrations are small, the microbial population is small and biodegradation rates are relatively slow.

As the substrate concentrations increase, the microbial population grows and the degradation rate increases concomitantly (Bradley and Chapelle, 1998) until the microbial population will reach a maximum growth rate (Aronson and others, 1999).

The degradation of VOCs in ground water is a transformation of a parent compound to different compounds commonly called daughter products, degradates, or degradation by-products. These transformations can be grouped into two general classes: (1) those that require an external transfer of electrons, called oxidation-reduction reactions; and (2) those that do not involve a transfer of electrons, called substitutions and dehydrohalogenations (Vogel and others, 1987). Table 2.10 summarizes these reactions.

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).

Typically, the polychlorinated compounds (for example, PCE and TCE) easily degrade under anaerobic conditions and are less mobile in soil and aquifer materials than the di- and mono-chlorinated compounds (figure 2.1).

Degradation pathways are illustrated in figures 2.2 through 2.10 for a subset of the compounds listed in table 2.4. These figures are modifications of pathways described in the University of Minnesota's biodegradation/biocatalysis database (Ellis and others, 2006) accessible via the Internet at *http://umbbd.msi.umn.edu*, accessed May 23, 2006.

Aquifer conditions (aerobic and anaerobic) and microbial metabolism (respiration, fermentation, and co-metabolism) control the environmental degradation of VOCs in ground water.

In aerobic environments, oxygen serves as the terminal electron acceptor (TEA) and compounds such as MTBE and BTEX are subsequently degraded (oxidized) to other compounds (Azadpour-Keeley and others, 1999). Furthermore, under aerobic conditions CVOCs can be inadvertently degraded (co-metabolized) via nonspecific enzymes (oxygenases) produced by microorganisms during the metabolism of other compounds serving as primary substrates (for example, BTEX, methane, propane, toluene, ammonia, ethene, ethane).

Although the aerobic mineralization of most VOCs ultimately yields carbon dioxide and water, co-metabolic biodegradation of CVOCs generally proceeds via an unstable epoxide intermediate that spontaneously decomposes to carbon dioxide, chloride, or other organic by-products such as acetate (Roberts and others, 1986).

Anaerobic degradation is typically a series of decarboxylations and oxidation-reduction (redox) reactions catalyzed either by single microorganisms or by a consortium of microorganisms (Dolfing, 2000).

During anaerobic degradation, CVOCs function as terminal electron acceptors in a process called reductive dechlorination (Vogel and others, 1987). 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 12-DCE, or to nonchlorinated compounds such as ethene, ethane, or methane (methanogenesis).

Reactions	Potential reaction products
Substitution	
abiotic hydrolysis	alcohol then an acid or diol (chloroethanol → chloroacetic acid)
biotic hydrolysis	alcohol then an acid or diol via microbial enzymes (hydrolases or glutathione S-transferases; (chloroethanol → chloroacetic acid)
conjugation or nucleophilic reactions (biotic)	free halide plus a new compound with the nucleophile or conjugate
Dehydrohalogenation	
dehydrohalogenation	halogenated acid (chloroacetic acid), alkane to alkene (dichloroethane → chloroethane)
Oxidation	
α-hydroxylation	monochlorinated alkane to a monochlorinated alcohol (chloroethane → chloroethanol)
halosyl oxidation	monohalogenated alkane to a nonhalogenated alkane (chloroethane → ethane + Cl)
epoxidation	halogenated epoxide compound
biohalogenation	nonhalogenated alkene to a monohalogenated alcohol (ethene + Cl → chloroethanol)
Reduction	
hydrogenolysis	free halide and nonhalogenated compound (chloroethane → Cl + ethane)
dihaloelimination	dihalogenated alkane to a nonhalogenated alkene (dichloroethane → ethene)
coupling	combining of two halogenated compounds into one halogenated compound

[1]Vogel and others, 1987, figure 1

[+, plus; Cl, chloride]

Table 2.10 - *Common abiotic and biotic reactions involving halogenated aliphatic hydrocarbons.*[1]

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 ground-water contaminated by TCE.

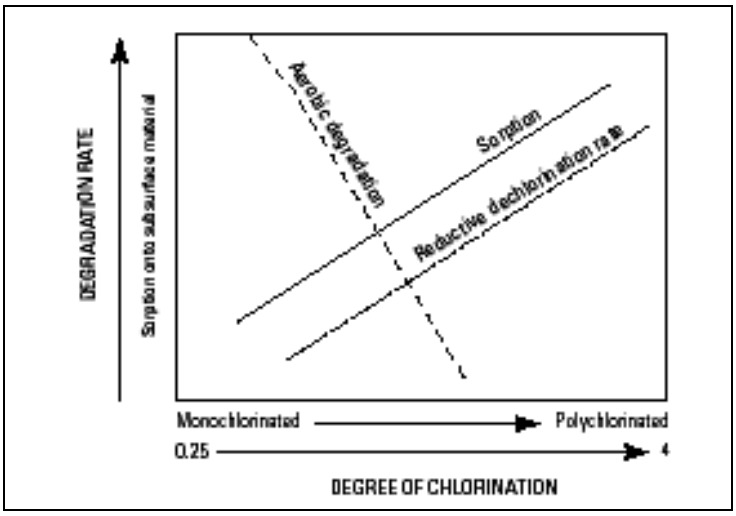


Figure 2.1 - *Relation between degree of chlorination and anaerobic reductive-dechlorination, aerobic degradation and sorption onto subsurface material (modified from Norris and others, 1993, p. 10–19). Degree of chlorination is number of chloride atoms divided by number of carbon atoms.*

2.5 Degradation of the chlorinated alkanes

The degradation of chlorinated VOCs is fundamentally different from that of BTEX compounds (Wiedemeier and others, 1995). The chlorinated alkanes can be degraded by abiotic processes through hydrolysis or dehydrohalogenation or by biotic processes through reductive dechlorination or dichloroelimination.

These degradation processes can proceed under either aerobic or anaerobic conditions (figs. 3–6; Vogel and McCarty, 1987a; Vogel, 1994). According to McCarty (1997), 111-TCA is the only chlorinated compound that can be degraded in ground water within 20 years under all likely ground-water or aquifer conditions.

2.5.1 Abiotic transformation

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 2.1) 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 111-TCA have half-lifes that are measured in days or months (Vogel and others, 1987; Vogel, 1994; table 14).

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-lifes measured in decades or centuries (table 2.11).

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.

Chen and others (1996) show that PCA can be abiotically transformed to TCE under methanogenic conditions (figure 2.2).

In addition, the abiotic degradation of 111-TCA has been well studied in the scientific literature (fig. 4; 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.

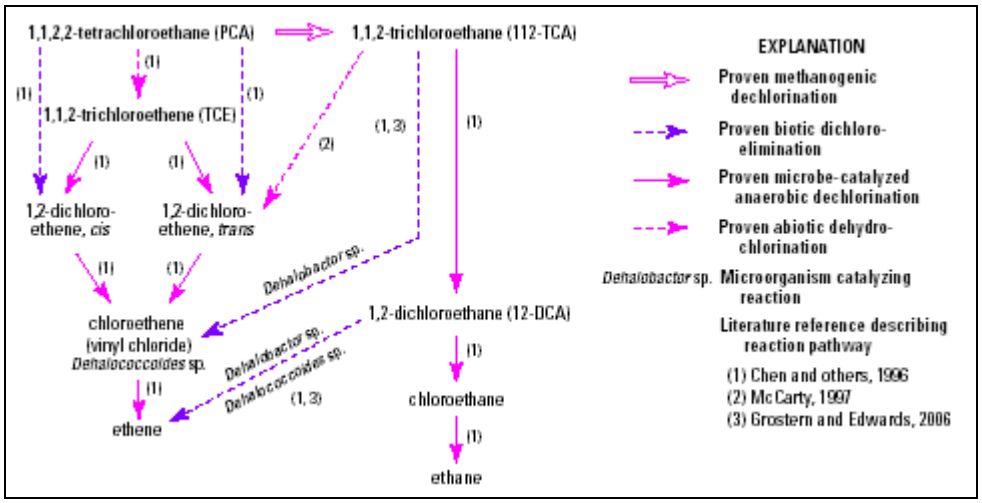


Figure 2.2 - *Laboratory-derived pathway for the abiotic degradation, anaerobic, and methanogenic biodegradation of 1,1,2,2-tetrachloroethane; 1,1,2-trichloroethene; and 1,1,2-trichloroethane (modified from Chen and others, 1996).*

During abiotic degradation, about 80 percent of 111-TCA is transformed to acetic acid by hydrolysis (McCarty, 1997), and the remaining 20 percent is transformed to 11-DCE by dehydrochlorination (table 7; Vogel and McCarty, 1987b;

McCarty, 1997). The presence of 11-DCE in contaminated ground water is probably the result of the dehydrochlorination of 111-TCA (McCarty, 1997).

Compound (IUPAC name)[1]	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

[1]International Union of Pure and Applied Chemistry, 2006

[IUPAC, International Union of Pure and Applied Chemistry; —, not applicable]

Table 2.11 - *Laboratory half-lifes and by-products of the abiotic degradation (hydrolysis or dehydrohalogenation) of chlorinated alkane compounds detected in ground water.*

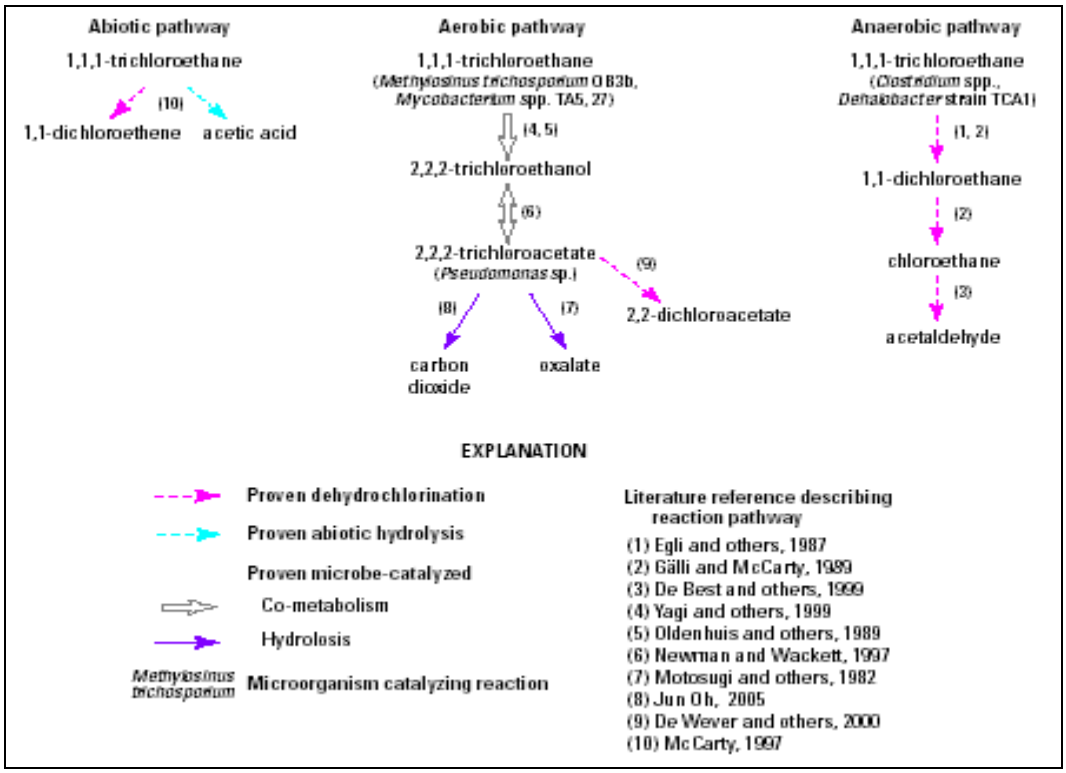


Figure 2.3 - *Laboratory-derived pathway for the abiotic, aerobic, and anaerobic biodegradation of 1,1,1-trichloroethane (modified from Sands and others, 2005; Whittaker and others, 2005)*

2.5.2 Aerobic biodegradation

According to the degradation pathway constructed by Sands and others (2005) and Whittaker and others (2005), the dichloroethanes are not a by-product of 111-TCA or 112-TCA biodegradation under aerobic conditions (figure 2.3).

Apparently, the only source of 11-DCA and 12-DCA via a degradation pathway is the reductive dechlorination of 111-TCA and 112-TCA, respectively, under anaerobic conditions (figures 2.2 and 2.3). Under aerobic conditions, however, 12-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 2.4 ; Stucki and others, 1983; Janssen and others, 1985; Kim and others, 2000; Hage and others, 2001).

2.5.3 Anaerobic biodegradation

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 2.5).

Adamson and Parkin (1999) show that under anaerobic conditions, carbon tetrachloride and 111-TCA tend to inhibit the degradation of each other. Adamson and Parkin (1999) also show that carbon tetrachloride was rapidly degraded by co-metabolism when acetate was the carbon source.

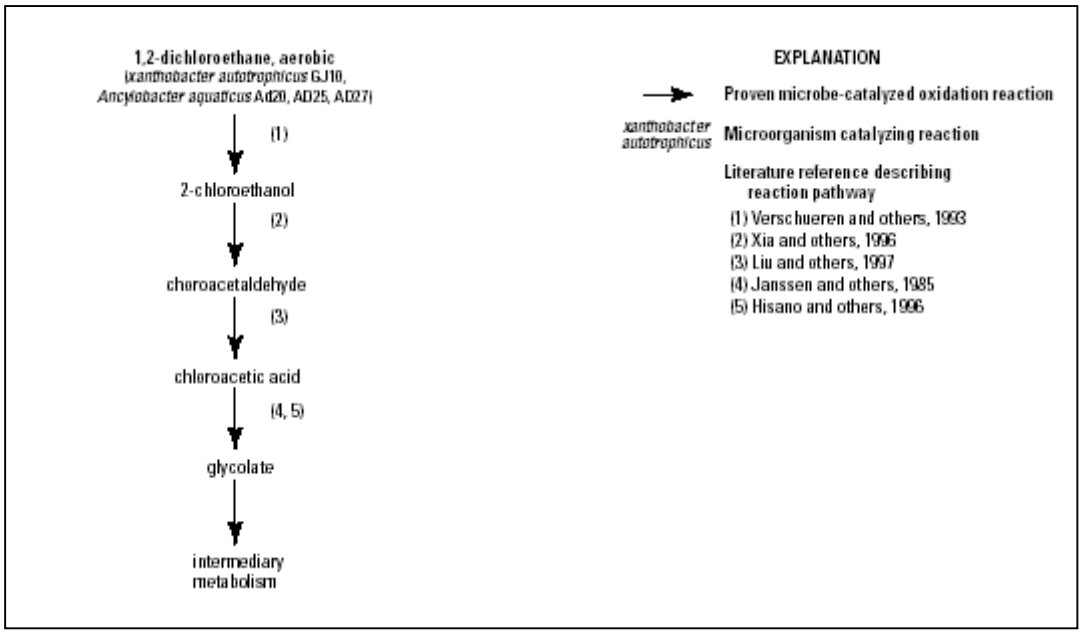


Figure 2.4 - *Laboratory-derived pathway for the aerobic biodegradation of 1,2-dichloroethane (modified from Renhao, 2005).*

Chen and others (1996) describe how methanogenic conditions in a municipal sludge digester allowed the degradation of PCA to 112-TCA, and 112-TCA to 1,2-DCA through dehydrohalogenation (figure 2.2).

De Best and others (1999) report that co-metabolic transformations of 112-TCA will - occur under methanogenic conditions. In this study, 112-TCA was degraded to chloroethane when sufficient amounts of the carbon source were present (fig. 3). 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 favourable 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-life of the chloroethane compounds can be as short as three days, in the case of 111-TCA, or as long as 165 days, in the case of 12-DCA (table 2.12).

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)

[1]Aronson and Howard, 1997, p. 111

[(27), number of samples used to derive the mean value; —, not available)

Table 2.12 - *Mean half-life in days for the anaerobic biodegradation of selected chlorinated alkane and alkene compounds.*

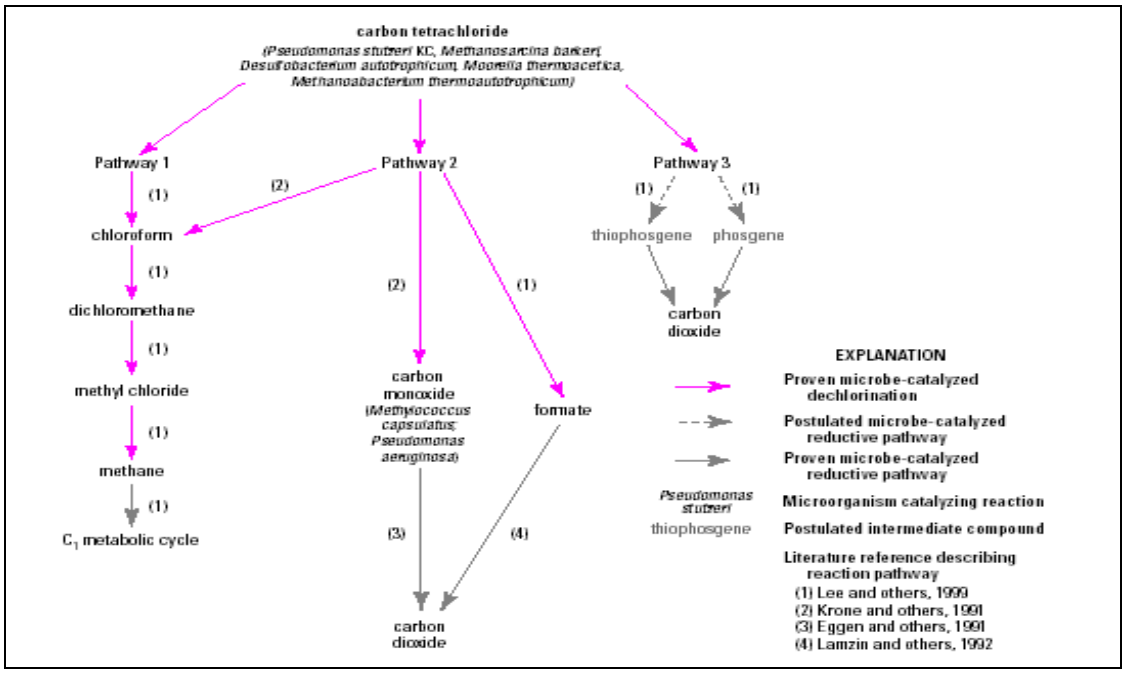


Figure 2.5 - *Laboratory-derived pathways for the anaerobic biodegradation of tetrachloromethane (carbon tetrachloride; modified from Ma and others, 2005; Sands and others, 2005).*

2.6 Degradation of the chlorinated alkenes

The primary degradation of the most common chlorinated alkenes is microbial reductive dechlorination under anaerobic conditions.

However, biodegradation of certain chlorinated compounds — such as trichloroethene, the dichloroethenes, vinyl chloride, or chloroethane — can also proceed via oxidative pathways under aerobic conditions. Two forms (isomers) of dichloroethene occur in ground water as chemical by-products of PCE and TCE biodegradation (Wiedemeier and others, 1998; Olaniran and others, 2004). Abiotic degradation of PCA to TCE can occur in PCA-contaminated ground water (fig. 3; Chen and others, 1996).

2.6.1 Aerobic biodegradation

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).

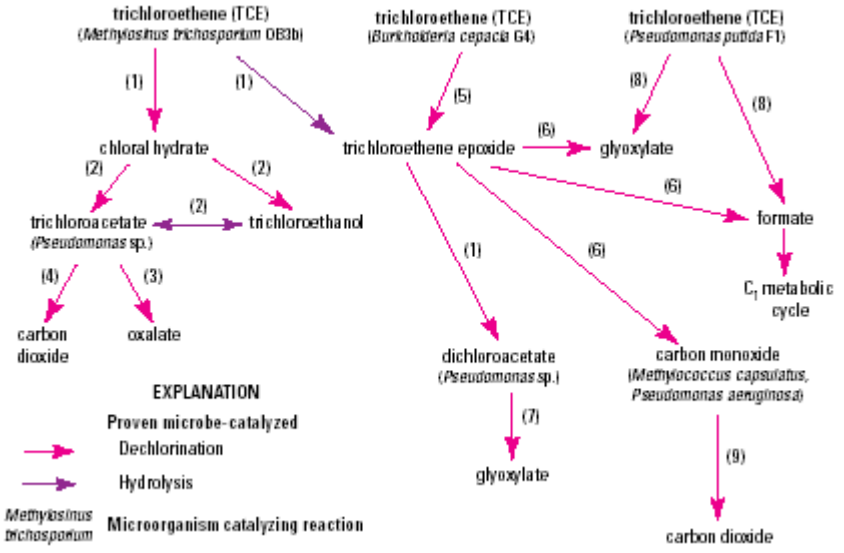
Studies describing the degradation of PCE under aerobic conditions were not found in the peer-reviewed literature.

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 2.3). Rather, TCE is degraded along three different pathways by different microorganisms (figure 2.6).



Literature reference describing reaction pathway
(1) Rosenzweig and others, 1993 (2) Newman and Wackett, 1991 (3) Motosugi and others, 1982 (4) Jun Oh, 2005 (5) Newman and Wackett, 1995 (6) Newman and Wackett, 1997 (7) Weightman and others, 1985 (8) Li and Wackett, 1992 (9) Eggen and others, 1991

Figure 2.6 - *Laboratory-derived pathways for the aerobic biodegradation of trichloroethene (modified from Whittaker and others, 2005).*

These pathways do not form any of the dichlorothene compounds and the only apparent source of 12-DCE is by the reductive dechlorination of TCE under anaerobic conditions (figs. 2.4 and 2.7).

The compounds 12-DCE and VC, however, can be degraded under aerobic conditions by microorganisms utilizing the compounds as a primary carbon source (fig. 5; 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.

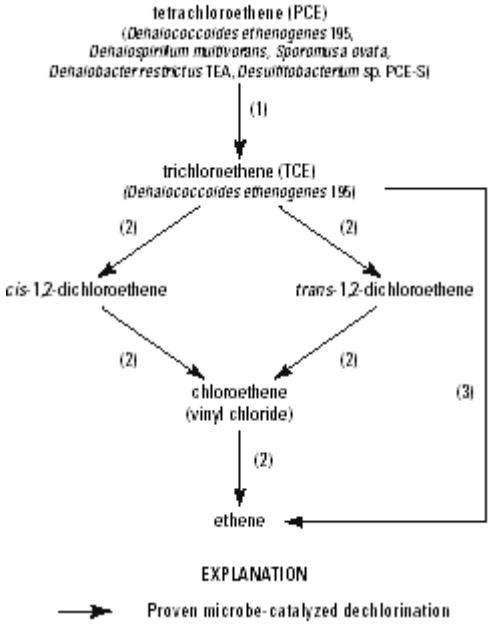
2.6.2 Anaerobic biodegradation

Many laboratory and field studies have shown that microorganisms degrade chlorinated ethenes under anaerobic conditions (Bouwer and others, 1981; Bouwer, 1994, Dolfing, 2000). Ground water 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 sulphide, or methane in ground water and using that data as a qualitative guide to the redox status (Stumm and Morgan, 1996; Christensen and others, 2000).



- Literature reference describing reaction pathway: (1) Neumann and others, 1996 (2) Maymo-Gatell and others, 1997 (3) Magnuson and others, 1998.
- Microorganism catalyzing reaction: *Dehalococcoides ethenogenes*

Figure 2.7 - *Laboratory-derived pathway for the anaerobic biodegradation of tetrachloroethene (modified from Ellis andAnderson, 2005).*

Other measurements of anaerobic conditions involving microorganism biomarkers include volatile fatty acids, ester-linked phospholipids' 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), sulphate to hydrogen sulphide, and carbon dioxide to methane during the microbial reduction of chlorinated VOCs can have a major influence on the distribution of iron (II), manganese (II), hydrogen sulphide, and methane concentrations in ground water (Stumm and Morgan, 1996; Lovley, 1991; Higgo 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 CVOCs, except VC, is reductive dechlorination (figure 2.2 and 2.7; 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 2.7), 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 12-DCE and VC in anoxic ground water 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 ground water (Freedman and Gossett, 1989).

Reductive dechlorination has been demonstrated under nitrate- and iron-reducing conditions (Wiedemeier and others, 1998). Reductive dechlorination of the CVOCs, however, may be more rapid and more efficient when oxidation-reduction (redox) conditions are below nitrate-reducing levels (Azadpour- Keeley and others, 1999). Sulphate-reducing and methanogenic ground-water conditions create an environment that facilitates not only biodegradation for the greatest number of CVOCs, 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 15).

2.7 Degradation of the 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 (124-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 *Burkholdia* and *Rhodococcus* species (Rapp and Gabriel-Jürgens, 2003).

During biodegradation, these compounds are completely mineralized to carbon dioxide (CO2) (van de 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 124-TCB, 14-DCB, 12-DCB, and CB, under aerobic conditions are shown in figures 2.8 to 2.10, 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-lifes for the chlorobenzenes under aerobic conditions are shown in table 2.12.

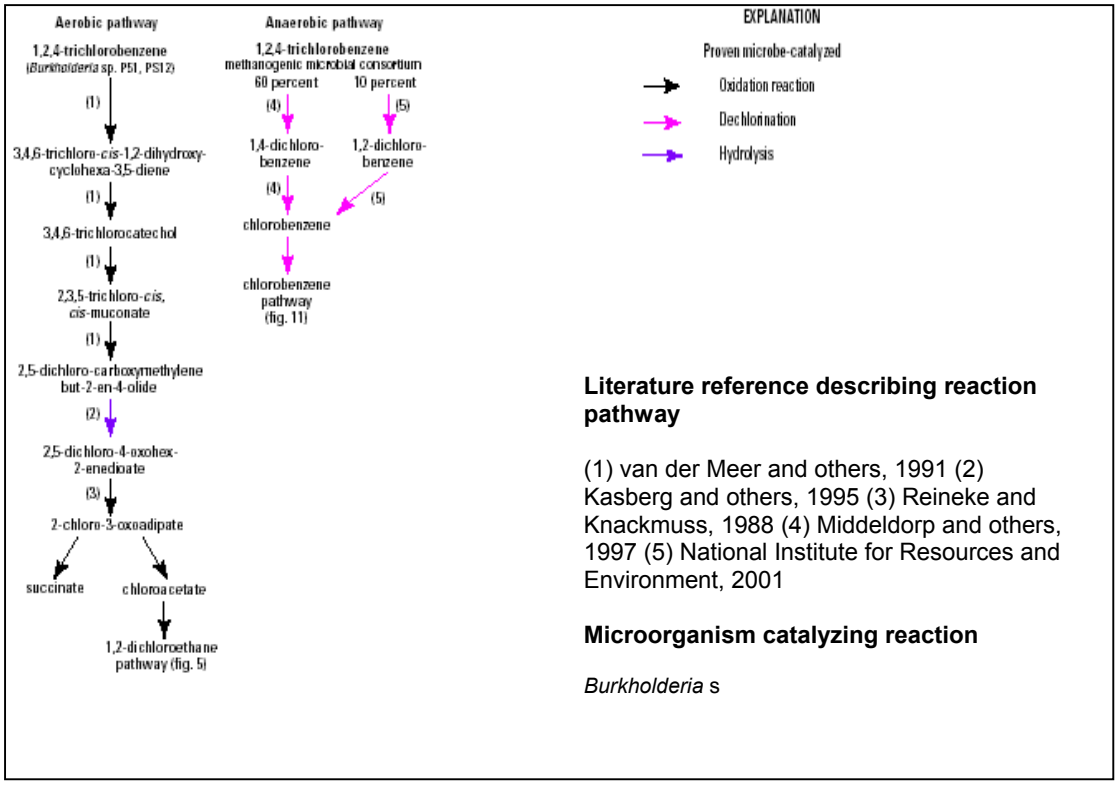


Figure 2.8 - *Laboratory-derived pathways for the aerobic and anaerobic biodegradation of 1,2,4 trichlorobenzene (modified from Yao, 2006)*

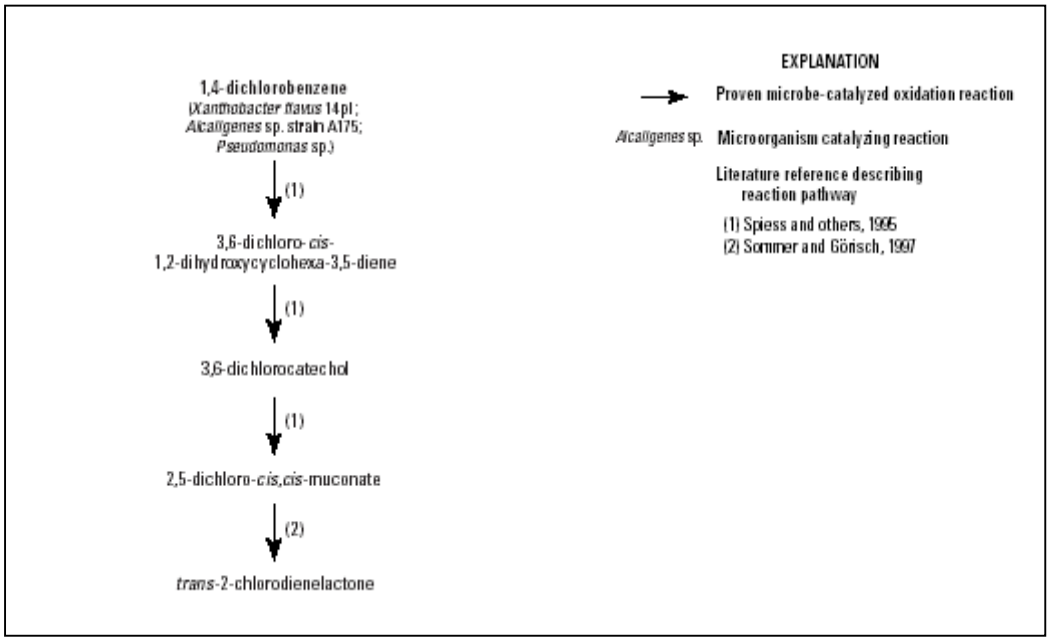


Figure 2.9 - *Laboratory-derived pathway for the aerobic biodegradation of 1,4-dichlorobenzene (modified from Liu, 2006).*

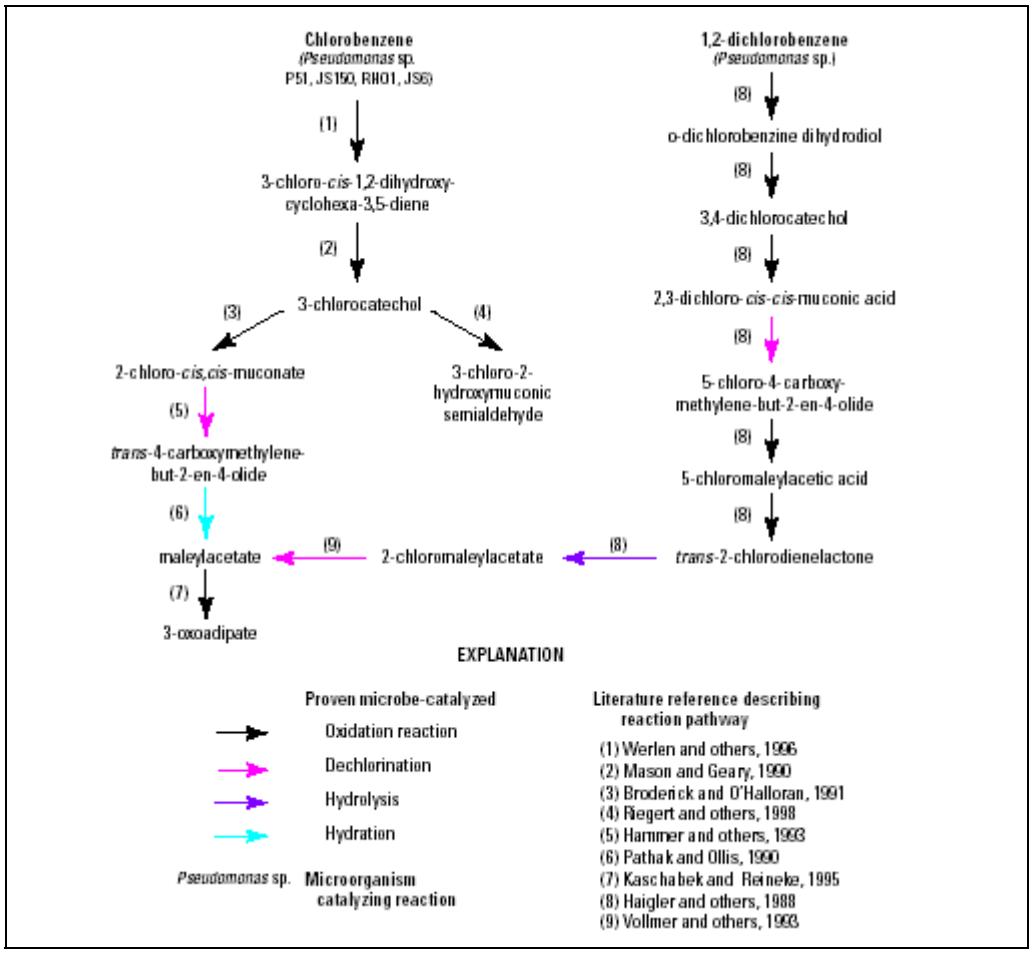


Figure 2.10 - *Laboratory-derived pathway for the aerobic biodegradation of chlorobenzene and 1,2-dichlorobenzene (modified from McLeish, 2005).*

The compounds 124-TCB, 12-DCB, and CB lose 50 percent of their initial mass within 180 days (table 2.12). Conversely, Dermietzel and Vieth (2002) show that chlorobenzene was rapidly mineralized to CO2 in laboratory and in situ microcosm studies, with complete mineralization ranging from 8 hours to about 17 days. In addition, the compound 14-DCB was completely mineralized within 25 days.

Nevertheless, under the aerobic conditions of Dermietzel and Vieth (2002) study, 124-TCB, 12-DCB, and 13-DCB were only partially degraded after 25 days. In another laboratory-microcosm study by Monferran and others (2005), all isomers of DCB were mineralized to CO2 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 124-TCB could be biodegraded to chlorobenzene with 14-DCB as an intermediate compound under anaerobic conditions.

Moreover, Middeldorp and others (1997) show that 124-TCB was reductively dechlorinated to 14-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 124-TCB also was able to degrade isomers of tetrachlorobenzene to other isomers of TCB and 12-DCB. More recent studies show that a strain of the bacterium, *Dehalococcoides,* can reductively dechlorinate 124-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 co-metabolism of 124-TCB was inhibited by the presence of sulphate, sulphite, and molybdate. Furthermore, Ramanand and

others (1993) show that 124-TCB had declined by 63 percent within 30 days under anaerobic conditions.

Dermietzel and Vieth (2002) show that the anaerobic biodegradation of 14-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.

Chapter 3

Biotechnological processes to remove Chlorinated Volatile Organic Compound

3.1 Biological technologies for biodegradation of chlorinated Volatile Organic Compounds: different approaches

As seen previously, groundwater and soil contamination by various anthropogenic organic compounds is a widespread problem in agricultural and industrialized environments and, between the organic environmental contaminants, chlorinated compound are very persistent in the subsurface environment.

Moreover groundwater pollution often results in further migration of contaminants and contamination of drinking water supplies. Contaminated groundwater is of concern due to the toxicity towards humans and the ecotoxicity.

Because the principle of bioremediation is to utilize microbial degradation processes in technical and controlled treatment systems, in this thesis, two techniques based on different approaches to chlorinated compound biodegradation have been investigated:

i) Bioaugmentation as in-situ remediation of soil and groundwater polluted by cVOC.

ii) The use and implementation of a bioreactor based technique for air pollution control technologies to prevent release of cVOC.

Within this chapter a particular regard has been given to the reviews of this two approaches.

3.2 Bioremediation: application as on site technology

The mechanism to biodegrade CAHs in contaminated soil and groundwater have been enhanced by the application of in-situ bioremediation technologies. These technologies, commercially available since the 1970s, employ engineered system to improve the effects of naturally occurring degradation processes.

It has gained increasing acceptance and publicity since the U.S. EPA and Exxon Co. demonstrated its effectiveness on Alaskan beaches contaminated by the *Valdez* oil spill.

Most of the early literature on bioremediation, in a great part derived from the petroleum industry practice, has been concentrated on biostimulation, i.e., artificial creation of an environment able to promote the growth of indigenous, naturally occurring microorganisms capable of degrading the target contaminants.

Between the major categories of on-site system, *in-situ/ex-situ* bioremediation could involve pumping groundwater to the surface for treatment in an aboveground reactor or, in a variation of this technique not include aboveground biotreater, application of nutrients, oxydizers (nitrate, peroxides, oxygen, etc.) and other compounds directly into the soil or groundwater to provide biostimulation.

In the first option the effluent, from the bioreactor, containing oxygen, nutrients and acclimated microorganisms is then percolated or injected back into the ground to remediate the contaminated soil associated with groundwater. This process combines ex situ groundwater treatment with in situ soil treatment.

The second option is based on engineered system designed to include several classes of technologies:

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.

The components of *in-situ* bioremediation technologies can be implemented by different general configuration, for example, direct injection, groundwater recirculation, permeable reactive barrier and bioventing chapter 3.2.5 will focus on this aspect.

3.2.2 Key geochemical and biological indicators of in-situ degradation

A key element in evaluating potential use of these two remedial technologies is subsurface conditions. For a technology to be cost-effective, it must be matched with geochemical and biological conditions of the subsurface. These conditions, which should be assessed during site evaluation activities, will indicate if aerobic or anaerobic degradation is naturally occurring and/or if conditions could be enhanced through the addition of biostimulating or bioaugmenting media.

Key geochemical and biological indicators useful in the evaluation of the occurrence/favourability of in-situ degradation of chlorinated ethenes by Anaerobic Reductive Dechlorination or Aerobic Cometabolism are listed below in table 3.1.

Parameter	Anaerobic Reductive Dechlorination	Aerobic Cometabolism
Oxidation-Reduction Potential (ORP)	!	!
pH	!	!
Temperature	!	!
Competing Terminal Electron Acceptors *(Nitrate, Sulfate, Iron (III), Manganese (IV), carbon dioxide)*	!	
Dissolved Oxygen *(DO)*	!	!
Contaminants and their breakdown products *(PCE, TCE, cis-1, 2-DCE, trans-1, 2-DCE, 1, 1-DCE, VC)*	!	!
Dissolved gases *(methane, ethane, ethane, sulfide)*	!	
Volatile fatty acids *(lactic, butyric, pyruvic, propionic, acetic, formic)*	!	
Electron Donor *(Hydrogen)*	!	
Electron Donor *(TOC and/or BTEX)*	!	!
BioDechlor CENSUS *(Dehalococcoides sp.)*	!	
Total Aerobic Microorganisms Plate Count		!
Pseudomonas sp. population and morphology		!

Table 3.1 - *Key geochemical and biological indicators useful in the evaluation of the occurrence/favourability of in-situ degradation of chlorinated ethenes.*

Each of these can be evaluated through collection of field samples for lab analysis and/or direct field measurement. It is recommended that DO, ORP, and pH be field tested.

In general microbiological terms, DO is the electron acceptor of choice for microorganisms in the subsurface which degrade organic substrates. More energy is gained from aerobic respiration than from denitrification, iron reduction, etc. If DO is present at concentrations greater than 1mg/l, aerobic bacteria will likely be plentiful. Microorganisms which utilize alternative electron acceptors (i.e., nitrate, sulphate, manganese [Mn(IV)], iron [Fe (III)], carbon dioxide and chlorinated ethenes) cannot survive in aerobic conditions.

Another key measure of the presence of aerobic or anaerobic conditions is ORP. ORPs of key reduction reactions are presented on Figure 3.1 below. Zones of aerobic and anaerobic conditions are denoted. As oxygen is depleted, indigenous microorganisms then utilize nitrate (by denitrification), manganese (IV) (by manganese reduction), iron (III) (by iron reduction), sulphate (by sulphate reduction), and carbon dioxide (by methanogenesis) in turn as terminal electron acceptors. When conditions are favourable for the reactions above, the microorganisms responsible for their performance are likely abundant.

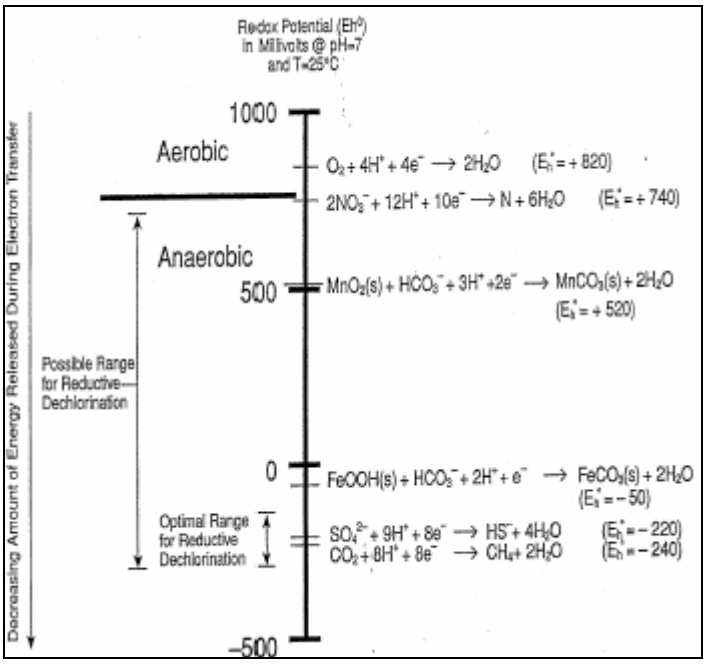


Figure 3.1 – *Oxidation-reduction potentials for various reactions (modified from Bouwer, 1994).*

As shown above, when aerobic conditions are present, a theoretical ORP level of approximately 750 mV could be anticipated if equilibrium conditions have been attained.

However, in the subsurface, equilibrium is generally not present; ORPs under aerobic conditions in the range of 300 to 400 mV are more common. As oxygen becomes depleted and other terminal electron acceptors become favoured, ORPs decrease to as low as -430 mV and anaerobic conditions are considered to be present. After depletion of oxygen, nitrate is the preferred electron acceptor, followed by bioavailable manganese (IV), bioavailable iron (III), sulphate, and carbon dioxide (and/or acetate). As shown above, the optimal range for reductive dechlorination is estimated at approximately -130 mV to -300 mV.

ORP and DO measurements, taken together with concentrations of the indicators listed previously, give an indication of the geochemical and biological conditions present at a site.

They indicate which of subsurface processes mentioned above are most active and permit evaluation of the most favourable means of further degradation of chlorinated ethenes. If aerobic conditions are determined to be present, aerobic cometabolism would likely be the technology of choice, while the presence of anaerobic conditions would favour the application/enhancement of reductive dechlorination. Otherwise, use of the other technology would first require treatment of the subsurface environment to create the required conditions for effectiveness, adding to the cost of the remediation.

3.2.3 Mechanism of anaerobic reductive dechlorination

In reductive chlorination of PCE, TCE, DCE, and VC, hydrogen serves as the electron donor and the chlorinated ethenes as the electron acceptor/carbon source/energy source for the microorganisms performing the degradation.

The oxidation-reduction reactions taking place are as follows:

$$C_2Cl_4 + H_2 \rightarrow C_2HCl_3 + H^+ + Cl^- \quad (1)$$

$$C_2HCl_3 + H_2 \rightarrow C_2H_2Cl_2 + H^+ + Cl^- \quad (2)$$

$$C_2H_2Cl_2 + H_2 \rightarrow C_2H_3Cl + H^+ + Cl^- \quad (3)$$

$$C_2H_3Cl + H_2 \rightarrow C_2H_4 + H^+ + Cl^- \quad (4)$$

The presence of hydrogen is a key factor in whether or not reductive dechlorination will occur.

Commercially available remediation compounds for biostimulation of reductive dechlorination result in the generation of hydrogen. As an example, Hydrogen Release Compound (HRC) of Regenesis, Inc. consists of a polylactate ester which, when hydrated, produces lactic acid. The lactic acid then serves as the electron donor for anaerobic microorganisms, yielding sequential degradation to lower volatile fatty acids and producing hydrogen. When enhancing reductive dechlorination, the competing electron acceptors (i.e. nitrate, sulphate, iron (III), and manganese (IV)) exert a hydrogen demand.

Thus, sufficient biostimulant needs to be added to meet the hydrogen demand of the alternative electron acceptors plus that of the chlorinated solvents requiring remediation.

While reductive dechlorination may occur under anaerobic conditions along the entire range of oxidation-reduction potentials, it is observed to be most rapid when sulphate-reducing or slightly methanogenic conditions exist [1]. The optimal ORP range for the reactions to occur is approximately -150 mV to -300 mV. A hydrogen concentration between 1 and 4 nanomole (nM) is most favourable. As the hydrogen level increases beyond 4 nM, reductive chlorination becomes less favourable and methanogenesis becomes the predominant reaction.

Degradation of cis-1,2-DCE and VC to ethenes may be stalled if hydrogen levels become too elevated. Kean et al [2] report on a site in Florida where hydrogen levels, after injection of HRC, were so elevated that methanogenesis became the dominant reaction, effectively halting further degradation of cis-1,2-

DCE and VC. When hydrogen levels had been reduced to a “threshold” level, reductive dechlorination recommenced.

Debate has ensued over the years on whether the “right” bacteria are needed for reductive dechlorination, especially for the complete degradation of cis-1,2-DCE and VC to ethene [3,4]. Sites have been observed where these compounds tend to accumulate without further degradation.

Various strains of the Dehaloccocoides spp. (Dehalococcoides ethenogenes strain 195, Dehalococcoides sp. strain VS, Dehalococcoides sp. strain FL2, and Dehalococcoides sp. Strain BAV1) have been determined to be capable of degradation of PCE to ethene [5]. However, not all of the above strains are able to perform all of the sequential degradations (i.e. PCE to TCE to DCE to VC). Recent studies have isolated specific microorganisms responsible for cis-1,2-DCE and VC degradation to ethene [5, 6, 7]. Specifically, the BAV-1 and VS strains have been shown to perform the degradation of VC to ethene, utilizing VC for growth as an electron acceptor with hydrogen as the electron donor.

Even though the right geochemical conditions may exist, if the specific strains of Dehalococcoides sp. are not present or plentiful enough, degradation of cis-1,2-DCE and VC may not occur. On the basis of these findings, Regenesis, Inc. has developed BioDechlor INOCULUM (BDI), a consortium of bacteria containing BAV-1 that can be injected in-situ to enhance the degradation of cis-1,2-DCE and VC.

3.2.4 Degradation mechanism of aerobic cometabolism

When levels of dissolved oxygen are greater than 1.0 mg/l, reductive dechlorination cannot occur because the microorganisms required do not grow in the presence of oxygen. Under these conditions, aerobic cometabolism could potentially be used to degrade the chlorinated ethenes.

Aerobic cometabolism involves the degradation of chlorinated VOCs using oxygenase enzymes produced by aerobic microorganisms when they

degrade a suitable organic substrate. Aerobic cometabolism of TCE, DCE and VC has been reported.

Sequential degradation results in the production of chlorinated oxides, aldehydes, ethanols and epoxides [1]. Vandenburgh and Kunka [8] report on laboratory headspace studies which investigate aerobic cometabolism of TCE and other chlorinated hydrocarbons by Pseudomonas Fluorescens (PFL12). Subsequent studies were conducted in which different strains of Pseudomanas were utilized for aerobic cometabolism of PCE even if intermediate and final products of aerobic cometabolism were not determined in any of these studies.

The aerobic cometabolic reactions require the presence of sufficient organic substrate and dissolved oxygen concentrations greater than 1.0 mg/l [9]. Higher DO levels are preferred. Air sparging and/or the addition of an oxygen-releasing compound are commonly used to enhance oxygen levels at a site.

An example of a commercially available technology utilizing aerobic cometabolism is CLSolutions' CL-Out.

CL-Out is a blend of Pseudomonas sp. contained in a liquid with sugar supplements. Upon injection, sugar amendments provide the organic substrate required for growth of the microbial population and generation of the oxygenase enzymes which degrade the chlorinated VOCs. Note that organic substrate additions should be limited so that oxygen levels are not totally depleted. Otherwise, the microbial population would cease to grow.

3.2.5 A short review of in-situ bioremediation technologies

In-situ soil and groundwater bioremediation involves a series of technology that encourages growth and reproduction of indigenous microorganisms to enhance biodegradation of organic constituents in the saturated zone based on chemical/physical and biological processes. In-situ groundwater bioremediation can effectively degrade organic constituents which are dissolved in groundwater and adsorbed onto the aquifer matrix and within this paragraph a brief report on the available bioremediation technologies is presented

SOIL VAPOUR EXTRACTION (SVE), (also known as *soil venting* or *vacuum extraction*), reduces concentration of volatile constituents in petroleum products adsorbed to soils in the unsaturated (vadose) zone. In this technology, a vacuum is applied through wells near the sources of contamination in the soils. Volatile constituents of the contaminant mass "evaporate" and the vapours are drawn towards the extraction wells. Extracted vapour is then treated as necessary (commonly with carbon adsorption) before being released to the atmosphere. The increased air flow through the subsurface can also stimulate biodegradation of some of the contaminants, especially those that are less volatile. Wells may be either vertical or horizontal. In areas of high groundwater levels, water table depression pumps may be required to offset the effect of upwelling induced by the vacuum.

BIOVENTING uses indigenous microorganisms to biodegrade organic constituents adsorbed to soils in the unsaturated zone. Soils in the capillary fringe and the saturated zone are not affected. In Bioventing, the activity of the indigenous bacteria is enhanced by inducing air (or oxygen) flow through the unsaturated zone (using extraction or injection wells) and, if necessary, by adding nutrients. When extractions wells are used for bioventing, the process is similar to soil vapour extraction. However, while SVE removes constituents primarily through volatilization, bioventing systems promote biodegradation of constituents and minimize volatilization (generally using lower flow rate than for SVE). In practice some degree of volatilization and biodegradation occurs when either SVE or bioventing is used.

BIOPILES (a.k.a. biocells, bioheaps, biomounds, and compost piles) are used to reduce concentrations of petroleum constituents in excavated soils through the use of biodegradation. This technology involves heaping contaminated soils into piles (or "cells") and stimulating aerobic microbial activity within the soils through the aeration and/or addition of minerals, nutrients, and moisture. The enhanced microbial activity results in degradation of adsorbed petroleum-product constituents through microbial respiration. Biopiles are similar to landfarms , also known as land treatment or land application, in that they are both above-ground, engineered systems that use oxygen, generally from air, to stimulate the growth and reproduction of aerobic bacteria which, in turn, degrade the petroleum constituents adsorbed to soil. While landfarms are aerated by tilling or plowing, biopiles are aerated most often by forcing air to move by injection or extraction through slotted or perforated piping placed throughout the pile.

LOW-TEMPERATURE THERMAL DESORPTION (LTTD), (a.k.a. low-temperature thermal volatilization, thermal stripping, and soil roasting), is an ex-situ remedial technology that uses heat to physically separate petroleum hydrocarbons from excavated soils. Thermal desorbers are designed to heat soils to temperatures sufficient to cause constituents to volatilize and desorb (physically separate) from the soil. Although they are not designed to decompose organic constituents, thermal desorbers can, depending upon the specific organics present and the temperature of the desorber system, cause some of the constituents to

completely or partially decompose. The vaporized hydrocarbons are generally treated in a secondary treatment unit prior to discharge to the atmosphere.

AIR SPARGING (a.k.a. in situ air stripping and in situ volatilization) is an in situ remedial technology that reduces concentrations of volatile constituents in products that are adsorbed to soils and dissolved in groundwater. This technology involves the injection of contaminant-free air into the subsurface saturated zone, enabling a phase transfer of hydrocarbons from a dissolved state to a vapor phase. The air is then vented through the unsaturated zone. Air sparging is most often used together with SVE: in this case SVE system creates a negative pressure in the unsaturated zone through a series of extraction wells to control the vapor plume migration. This combined system is called AS/SVE.

BIOSPARGING is an in-situ remediation technology that uses indigenous microorganisms to biodegrade organic constituents in the saturated zone. In biosparging, air (or oxygen) and nutrients (if needed) are injected into the saturated zone to increase the biological activity of the indigenous microorganisms and it can be used to reduce concentrations of pollutants that are dissolved in groundwater, adsorbed to soil below the water table, and within the capillary fringe. When volatile constituents are present, biosparging is often combined with SVE or bioventing and can also be used with other remedial technologies. When biosparging is combined with vapour extraction, the vapour extraction system creates a negative pressure in the vadose zone through a series of extraction wells that control the vapour plume migration.

MONITORED NATURAL ATTENUATION (MNA) refers to the reliance on natural attenuation processes (within the context of a carefully controlled and monitored site cleanup approach) to achieve site-specific remediation objectives under long-term performance monitoring within a time frame that is reasonable compared to that offered by other more active methods MNA is often dubbed “passive” remediation because natural attenuation processes occur without human intervention to a varying degree at all sites. It should be understood, however, that this does not imply that these processes necessarily will be effective at all sites in meeting remediation objectives within a reasonable time frame. The fact that some natural attenuation processes are occurring does not preclude the use of “active” remediation or the application of enhancers of biological activity (e.g., electron acceptors, nutrients, and electron donors). In fact, MNA will typically be used in conjunction with, or as a follow-up to, active remediation measures, and typically only after source control measures have been implemented.

DUAL-PHASE EXTRACTION (DPE) (a.k.a. multi-phase extraction, vacuum-enhanced extraction, or sometimes bioslurping) is an in-situ technology that uses pumps to remove various combinations of contaminated groundwater, separate-phase petroleum product, and hydrocarbon vapour from the subsurface. Extracted liquids and vapour are treated and collected for disposal, or re-injected to the subsurface (where permissible under applicable state laws).

THERMAL TREATMENT uses steam forced into an aquifer through injection wells to vaporize volatile and semivolatile contaminants. Vaporized components rise to the unsaturated (vadose) zone where they are removed by vacuum extraction and then treated. Hot water or steam-based techniques include Contained Recovery of Oily Waste (CROW), Steam Injection and Vacuum Extraction (SIVE), In Situ Steam-Enhanced Extraction (ISEE), and Steam-Enhanced Recovery Process (SERP). Hot water or steam flushing/stripping is a pilot-scale technology. In situ biological treatment may follow the displacement and is continued until ground water contaminants concentrations satisfy statutory requirements. The process can be used to remove large portions of oily waste accumulations and to retard downward and lateral migration of organic contaminants. The process is applicable to shallow and deep contaminated area, and readily available mobile equipment can be used.
Hot water/steam injection is typically short to medium duration, lasting a few weeks to several months.

3.2.6 Pilot testing, remedial design and monitoring

Prior to full-scale implementation of either enhanced reductive dechlorination or aerobic cometabolism, pilot-scale testing is necessary. Pilot tests are normally designed in areas where existing monitoring wells can be used to evaluate results. After pre-injection sampling in the well(s) of concern for the appropriate parameters presented earlier, the biostimulating and/or bioaugmenting compounds are introduced into the subsurface.

Then, post-injection samples at the wells of concern are collected over a three- to six-month period. Periods closer to six months are required to yield data sufficient to evaluate the effectiveness of the technology.

If pilot testing shows success, the remedial design may consist of source zone treatment, hot spot treatment and/or injection in the form a “barrier” perpendicular to groundwater flow.

In addition to chlorinated VOC concentrations, location and spacing of injection points will depend on liquid filled porosity of the subsurface, groundwater flow direction, hydraulic conductivity of the treatment zone and groundwater velocity.

With anaerobic reductive dechlorination and aerobic cometabolism, the ability to disperse the biostimulant and/or bioaugmenter is crucial. Tighter formations (i.e. silt and clays) will likely require closer spacing of injection points, while more permeable formations (i.e. sands and gravels) will likely require less closely spaced injection points to achieve effective distribution of the compound injected.

Remedial monitoring may consist of monitoring for the appropriate parameters presented earlier.

It is recommended that monitoring be performed on a quarterly basis. As needed based on monitoring results, additional injection of the biostimulants and/or bioaugmenters may be required.

At the present bioremediation several processes are tested as pilot scale technology; here follows a short review of novel technology recently purposed:

HYDROFRACTURING is a pilot-scale technology in which pressurized water is injected to increase the permeability of consolidated material or relatively impermeable unconsolidated material. Fissures created in the process are filled with a porous medium that can facilitate bioremediation and/or improve extraction efficiency. Fractures promote more uniform delivery of treatment fluids and accelerated extraction of mobilized contaminants. The fracturing process begins with the injection of water into a sealed borehole until the pressure of the water exceeds the overburden pressure and a fracture is created. A slurry phase composed of coarse-grained sand and guar gum gel or a similar substitute is then injected as the fracture grows away from the well. After pumping, the sand grains hold the fracture open while an enzyme additive breaks down the viscous fluid. The thinned fluid is pumped from the fracture, forming a permeable subsurface channel suitable for delivery or recovery of a vapour or liquid. The hydraulic fracturing process can be used in conjunction with soil vapour extraction technology to enhance recovery. Hydraulically-induced fractures are used to deliver fluids, substrates and nutrients for in situ bioremediation applications.

IN-WELL AIR STRIPPING technology air is injected into a vertical well that has been screened at two depths. The lower screen is set in the groundwater saturated zone, and the upper screen is in the unsaturated zone, often called as vadose zone. Pressurized air is injected into the well below the water table, aerating the water. The aerated water rises in the well and flows out of the system at the upper screen. Contaminated groundwater is drawn into the system at the lower screen. The volatile organic compounds (VOCs) vaporize within the well at the top of the water table, as the air bubbles out of the water. The vapours are drawn off by SVE system. The partially treated ground water is never brought to the surface; it is forced into the unsaturated zone, and the process is repeated as water follows a hydraulic circulation pattern or cell that allows continuous cycling

of ground water. As ground water circulates through the treatment system in situ, contaminant concentrations are gradually reduced. Modifications to the basic in-well stripping process may involve additives injected into the stripping well to enhance biodegradation. In addition, the area around the well affected by the circulation cell (radius of influence) can be modified through the addition of certain chemicals to allow in situ stabilization of metals originally dissolved in ground water.

CIRCULATING WELLS (CWs) provide a technique for subsurface remediation by creating a three-dimensional circulation pattern of the ground water. Ground Water is drawn into a well through one screened section and is pumped through the well to a second screened section where it is reintroduced to the aquifer. The flow direction through the well can be specified as either upward or downward to accommodate site-specific conditions. Because ground water is not pumped above ground, pumping costs and permitting issues are reduced and eliminated, respectively. Also, the problems associated with storage and discharge are removed. In addition to ground water treatment, CW systems can provide simultaneous vadose zone treatment in the form of bioventing or SVE. CW systems can provide treatment inside the well, in the aquifer, or a combination of both. For effective in-well treatment, the contaminants must be adequately soluble and mobile so they can be transported by the circulating ground water. Because CW systems provide a wide range of treatment options, they provide some degree of flexibility to a remediation effort.

PASSIVE/REACTIVE TREATMENT WALLS is a technology based on a permeable reaction wall installed across the flow path of a contaminant plume, allowing the water portion of the plume to passively move through the wall. These barriers allow the passage of water while prohibiting the movement of contaminants by employing such agents as zero-valent metals, chelators, sorbents, microbes, and others. The contaminants will either be degraded or retained in a concentrated form by the barrier material. The wall could provide permanent containment for relatively benign residues or provide a decreased volume of the more toxic contaminants for subsequent treatment.

FUNNEL AND GATE system for in situ treatment of contaminated plumes, modifications to the basic passive treatment walls, consists of low hydraulic conductivity (e.g., 1E-6 cm/s) cut-off walls (the funnel) with a gate that contains in situ reaction zones. Ground water primarily flows through high conductivity gaps (the gates). The type of cut-off walls most likely to be used in the current practice are slurry walls or sheet piles. Innovative methods such as deep soil mixing and jet grouting are also being considered for funnel walls. It may involve a funnel-and-gate system or an iron treatment wall.

IRON TREATMENT WALL, another modification of the basic passive treatment walls, involves the use of iron granules or other iron bearing minerals for the treatment of chlorinated contaminants such as TCE, DCE, and VC. As the iron is oxidized, a chlorine atom is removed from the compound by one or more reductive dechlorination mechanisms, using electrons supplied by the oxidation of

iron. The iron granules are dissolved by the process, but the metal disappears so slowly that the remediation barriers can be expected to remain effective for many years, possibly even decades. Two types of barriers have been the focus of initial application, permeable reactive barriers and in-place bioreactors.

3.3 Bioremediation of waste stream: Bioreactors application

In the past decades technological short-term depollution solutions have been developed and applied to reduce the amplitude of these emissions.

Concerning the VOC emissions problem, existing depollution measures are of three orders:

1. to ban the utilization of certain VOC in industrial processes
2. to review and modify existing processes to reduce their associated VOC emissions and to make them less polluting (by installation of recycling systems, for example);
3. to install depollution systems downstream of the emitting sites.

Due both to the diversity and to the quantities of VOC released into the atmosphere, a variety of air pollution control technologies (APCT) have been developed. The operating process for all of these APCT involves either one or several physicochemical air/contaminant separation steps. The main differences between the APCT are related to the treatment of pollutants, following the separation procedure for their recovery (recycling) or destruction. The main APCT giving rise to recovery and recycling of VOC are the following:

- *phase transfer technologies:* adsorption and absorption.
-*VOC concentration technologies:* condensation, cryocondensation and membrane processes.

These technologies permit the extraction of VOC from the initial gas phase, and to then concentrate them.

Processes leading to the partial or total elimination of VOC involve the use of more or less drastic oxidative ways:

- *combustion processes:* incineration and catalytic oxidation.
- *chemical or photochemical oxidation technologies.*

- *biotechnologies*: biotrickling filter, bioscrubber, and biofilter.

The choice of a well-adapted process depends on the operating conditions (flow rate, VOC concentrations, temperature, humidity, etc.) and on the pollutants' physic-chemical characteristics (solubility, phase transition points, biodegradability level, in flammability, etc.) (Crocker and Schnelle, 1998).

Figure 3.2 presents the application limits (flow rate—VOC concentration) of the different APCT (Juteau, 1997; Crocker and Schnelle, 1998; Devinny et al., 1999).

Biological processes constitute pertinent ways for the elimination of biodegradable VOC (alcohols, ketones, aldehydes, aromatic compounds, etc.) emitted at low to moderate concentrations (1 ppm to 1000 ppm) (Devinny and Hodge,1995; Kim, 2004). The industries mainly concerned with depollution biotechnologies are chemistry, petrochemistry, pulp and paper, metallurgy, mining and energy production (Bailey and Ollis, 1986; Robert and Pilon, 2000).

In the following sections, bioprocesses (bioscrubber, biotrickling filter and biofilter) are generally described, with a more detailed section dedicated to biofiltration.

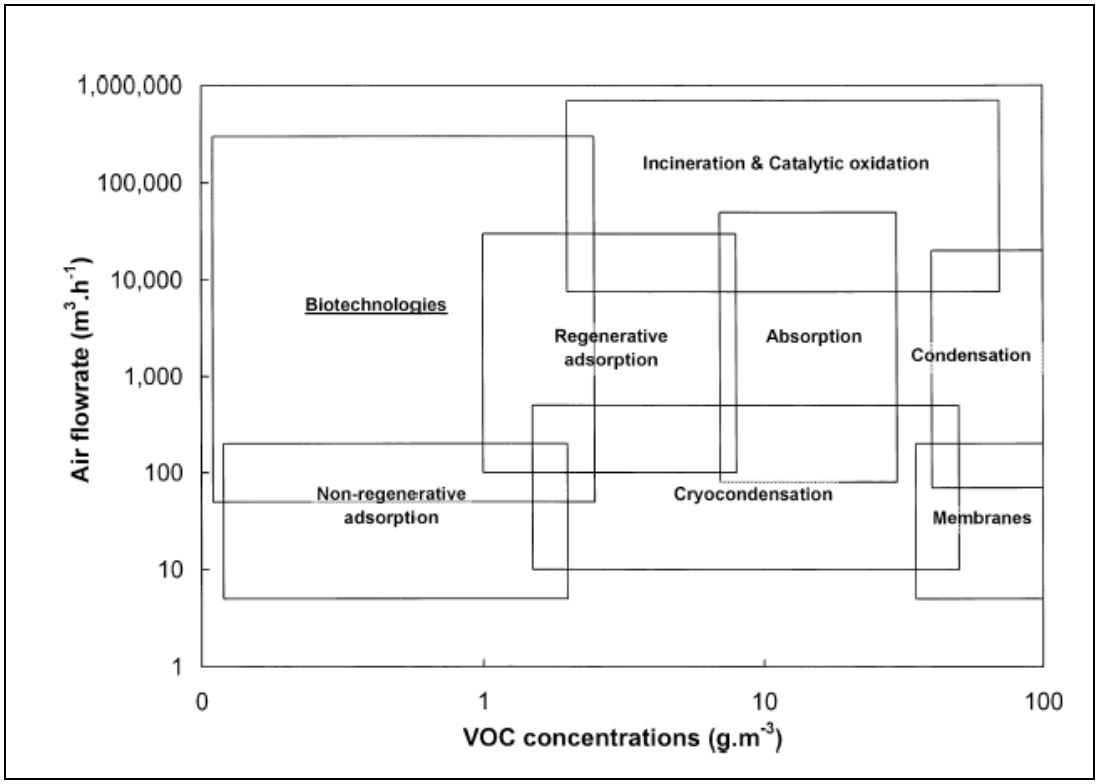


Figure 3.2 - *Application limits (flow rate—VOC concentration) of different APCT, based on references of Crocker and Schnelle, 1998; Juteau, 1997, and Devinny et al., 1998.*

The particular interest of these biotechnologies is that they do not utilize energy other than the capacity of microorganisms to metabolize a wide range of VOC: biocatalytic oxidation of pollutants.

The catalysts are heterotrophic microbial strains (bacteria, fungi) that are able to utilize VOC in two ways:

- contaminants, oxidized in the course of the catabolic pathway (respiratory chain) are a source of energy;
- contaminants are also a source of available carbon for the anabolic processes, such as cell growth.

The products of the biological reactions engaged in these bioreactors are essentially: carbon dioxide, water, inorganic byproducts (e.g. HCl, SOX) related to the presence of heteroatoms in the VOC skeleton (Cl, S, N, etc.), new cellular matter, and organic byproducts metabolites such as exopolymers).

Biooxidations are exothermic reactions (some kcal/mol VOC oxidized), thus the associated heat release is also a biodegradation reaction product (Scriban, 1993).

The effective use of biocatalysts requires strict control of their environment, normally the biological growth medium.

There are some critical parameters that are common to the 3 types of bioreactors:

(a) temperature optimum between 20 and 35 °C for a mesophilic microflora) (Leson and Winer, 1991; Swanson and Loehr,1997),

(b) pH (optimum at about 7) (Leson and Winer,1991; Leson, 1998),

(c) moisture content in the growth medium, and

(d) availability of essential, non-carbon nutrients (N, P, K, S and micronutrients).

The main differences between the three bioreactors come from their design and mode of operation: microorganisms conditioning, respective disposal of fluid phases (gas and liquid), presence or absence of stationary solid phases.

Even if bioreactors have been used for treatment of wastewater for over 100 years (for reviews, see Czysz et al.,1989; Degremont, 1991; Tchobanoglous and Burton 1991), during the last two decades different groundwater cleanup technologies have been developed. In many applications, prior to treatment, contaminated groundwater is pumped above ground. One benefit of this approach is that pumping may prevent further migration of contaminants.

Bioreactor processes can be distinguished on the basis of biomass retardment mechanism. Biomass grows on a carrier in attached growth, i.e. biofilm systems or as a suspension in sludge processes. Early bioreactor tests were done by Alexander Mueller in 1865 and resulted later in the trickling filter design, which was based on attached growth on a support material in trickling filters (Corbett, 1903). Rotating biological contactor was patented by Weigand in Hoechst (1900).

The first suspended bioreactor was the activated sludge processes introduced by Ardern and Lockett (1914). Rotating biological contactors have been used in Germany since the 1920s (Glaze et al., 1986). The next steps in bioreactor development were completely mixed stirred tank reactors in the late 1950s (Bazyakina, 1948), fluidized-bed reactors in the 1970s (Jeris et al., 1974) and the upflow anaerobic sludge blanket reactor in 1978 (Lettinga et al., 1980).

Different bioreactors can be operated under aerobic or anaerobic conditions. Anaerobic bioreactors have been used since the 1880s to treat wastewater with high organic solid concentrations (Admussa and Korus, 1996).

The continuous flow system can be plug-flow or completely mixed (a scheme of bioreactors in current use are presented in Figure 3.3).

These basic bioreactor types have also been applied for groundwater treatment.

The process designs, however, are very different from those applied in wastewater treatment.

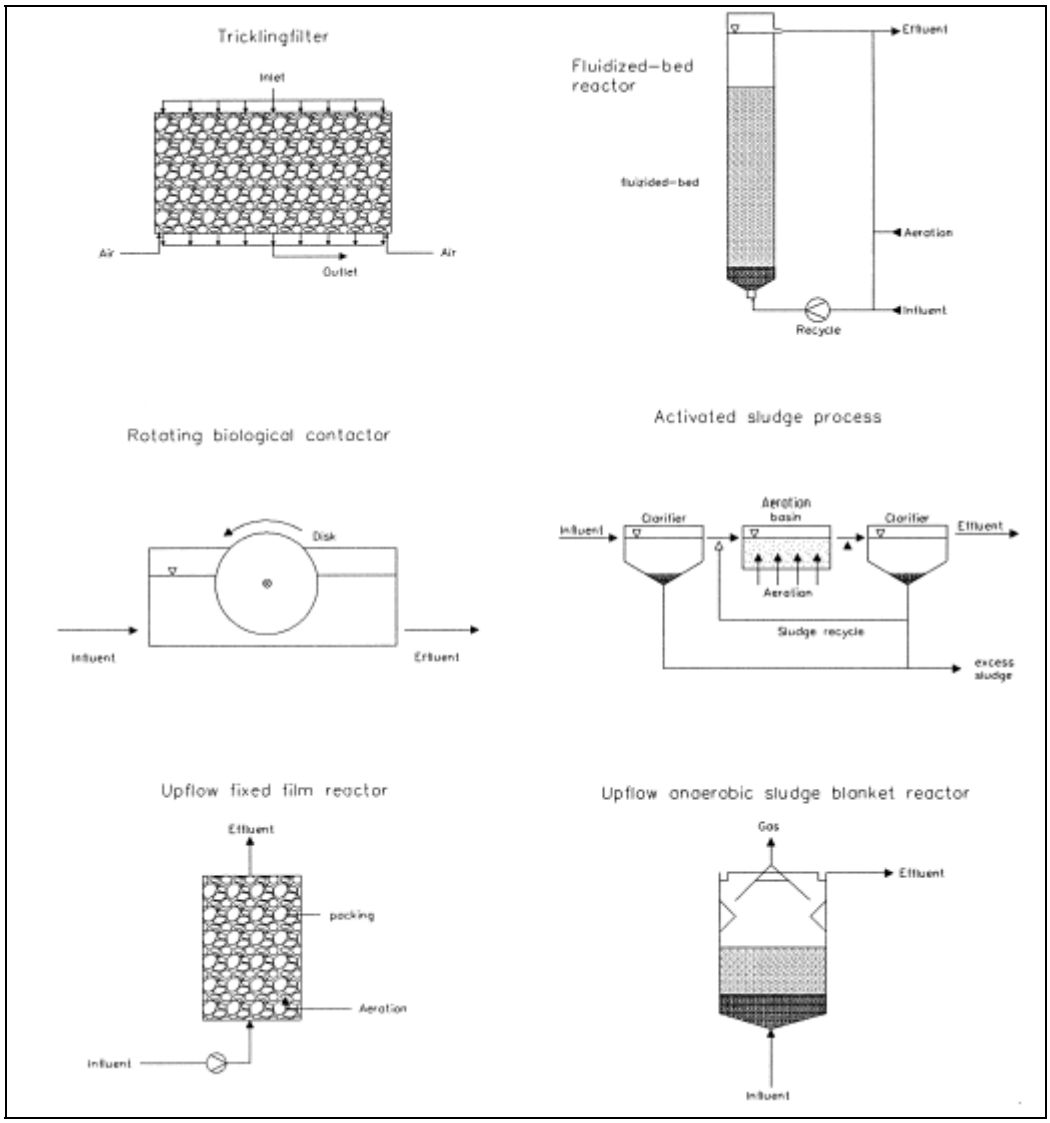


Figure 3.3 – *Schemes of bioreactors.*

For waste-stream remediation, bioreactors require specific designs to fulfil the technical, environmental and economical requirements (Table 4). The process must function at low contaminant concentration, which causes slow biomass accumulation and possible start-up problems.

Very low contaminant concentrations have to be achieved to meet regulatory goals. The system must reliably operate under varying conditions, especially at very low feed concentrations towards the end of remediation action. Operational costs have to be low to allow long-term operation.

Process	Aerobic/anaerobic	Full-scale experience in groundwater treatment	Major benefits	Major shorthands
Attached growth				
Trickling filter	Aerobic	Yes	Simple to design, operate and maintain	Poor effluent quality
Rotating biological contactor	Aerobic	Yes	Simple and inexpensive design, low energy consumption	Poor effluent quality
Upflow fixed-film reactor	Aerobic	Yes	Adjustable retention time	
	Anaerobic	No	Reductive dehalogenation of contaminants possible	High temperature and high organic carbon supplementation needed
Fluidized-bed reactor	Aerobic	Yes	Reliable operation, low effluent concentrations, dilution of toxic contaminant, easy to start-up	Relatively high energy consumption
Suspended growth				
Activated sludge	Aerobic	Yes	None	Difficult to generate and maintain the biomass, high energy consumption
Upflow anaerobic sludge blanket	Anaerobic	No	Reductive dehalogenation of contaminants possible	High temperature and high organic carbon supplementation needed

Table 3.2 – *Benefits and shorthand of different bioreactors designs in wastestream treatments.*

3.3.1 Bioreactors based on attached microbial growth

Bacteria have a tendency to grow in interfaces. In flowing environments, this interfacial growth gives a growth benefit. For attachment, bacteria produce exo-polymer-based gelatinous material which together with cells forms a biofilm on the carrier material. Bacteria use specific cell surface structures, i.e. pili and fimbriae, to stick to surfaces. Dissolved contaminants and oxygen diffuse throughout the biofilm. The outer layers of the biofilm protect the inner cells from toxicity and reduce soluble contaminant concentrations by adsorption (Hu et al., 1994).

Bacteria attached to a support material usually show higher specific activity than those observed in suspended growth. Holladay et al. (1978) and Rittmann (1982) showed that from different biofilm reactor type fluidized-bed reactors operated at the highest loading rates and achieved the lowest effluent concentrations. The modern understanding of biofilm structure describes it as a non-homogenous porous matrix which allows non-uniform liquid flows through the matrix, as shown in Figure 3.4 (Costerton et al., 1995).

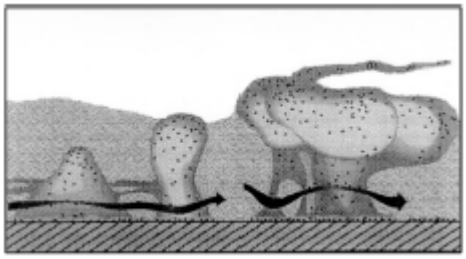


Figure 3.4 – *Schemes of biofilm structure with non uniform liquid flow.*

The principle of a trickling filter is to support attached growth of bacteria by allowing the contaminated water to trickle through the support material (inert packing) due to gravity. Trickling filter is aerated from the bottom mostly by natural draught, but in some cases also with air blower. Trickling filters can be categorized on the basis of the support material. Typical support materials include traditional fill, like lumps of crushed rocks, slag or pumice, and plastic fills (Admussa and Korus, 1996).

Bench-scale tests with a trickling filter were carried out by van der Hoek et al. (1989) to remove polycyclic aromatic hydrocarbons, benzene, toluene, ethylbenzene, xylene and phenols from groundwater. The test showed that trickling filters are less effective than upflow fixed-film reactors. Trickling filters have been used to treat dichloroethylene-contaminated groundwater under aerobic conditions in laboratory-scale by Koziollek et al. (1998). Trickling filter treatment has been used in full-scale, but to our knowledge, not reported in scientific periodicals.

Rotating biological contactors: In these bioreactors microorganisms grow on the surface of rotating disks. The disks are partially immersed into the water and are partially in contact with the atmosphere. Rotation of the disks brings the attached biomass alternately in contact with the water to be treated and the air.
Use of rotating biological contactors for treatment of contaminated groundwater has been rare. Rotating biological contactors were compared with upflow fixed-film reactors in a laboratory study, showing that the aromatic hydrocarbons and phenols removal efficiencies were lower than in an upflow fixed-film reactor (van der Hoek et al., 1989). Enrichment of 1,2-dichloroethane mineralizing biomass on the rotating disks was a long-term process shown also in a full-scale application

where biomass build-up required 1 year (Stucki and Thuèer,1995). A rotating biological contactor has also been used to treat vinyl chloride contaminated groundwater under methanotrophic conditions in an air-tight pilot-scale system (Belcher et al., 1997).

Upflow fixed-film reactor: In an upflow fixed-film reactor bacteria are growing on submerged inert packing. The upflowing water provides microorganisms in the biofilm with substrates and nutrients. If aerobic conditions are required oxygen is often added by air sparging. The hydraulic conditions in the reactor may cause some expansion of the packing, but not fluidization. Upflow reactors are widely used for cleanup of contaminated groundwater with mixed or pure cultures. Especially, fixed-film bed reactors with granular-activated carbon used as packing provide benefits for microbial growth due to sorption of contaminants on the granular-activated carbon (for reviews, see Weber et al., 1970; Bouwer and McCarty, 1982). In laboratory-scale studies anaerobic upflow fixed-film reactors were also used, but not at ambient groundwater temperatures (Hendriksen et al., 1991; Juteau et al., 1995; Komatsu et al., 1997). Aerobic-fixed film reactors are common reactors for groundwater treatment (Lenzo and Ward, 1994; Skladany, 1994).This bioreactor design has been successfully applied at bench, pilot and full scale (Marsman et al., 1993).

Fluidized-bed reactor: In fluidized-bed reactors the biomass grows on granular support material. The main principle of this reactor type is the fluidization of the bed by high recirculation rates of the water to be treated. Recirculation of the water dilutes the concentration of the influent to a non-toxic level for bacteria and provides completely mixed conditions. Aeration of the groundwater is often designed to the recycle line. High biomass concentrations can be achieved in this process. Fluidized-bed reactors are successfully used for cleanup of groundwater (Ahmadvand et al., 1995; Gandee et al., 1995; Massol-DeyaÂ et al., 1997), also at low groundwater temperatures (JaÈ rvinen et al., 1994; Puhakka and Melin, 1998). Especially, fluidized-bed reactors with granular-activated carbon as support material have the advantage of establishing rapid

microbial growth due to sorption of contaminants on the granular-activated carbon (for reviews, see Edwards et al., 1994; Sutton and Mishra, 1994). In laboratory scale, fluidized-bed processes have been also used for anaerobic treatment (Ballapragada et al., 1997; Magaret al., 1999).

3.3.2 Bioreactors based on suspended microbial growth

Microorganisms grow in suspension in concentrated substrate solutions: in this way, bacteria form flocks or granules which can be separated from the purified water by sedimentation. In most groundwater contamination cases the contaminant concentration is too low to allow biomass build-up in suspended systems.

Therefore, the suspended growth applications are limited to situations where contaminated groundwater is treated ex-situ/off-site in existing wastewater treatment plants. In these applications groundwater contaminants are minor constituents of the organic feed and, therefore, the process performance cannot be optimized for contaminant degradation.

Activated sludge process: The basic design of an activated sludge process includes an aeration basin and a mechanical clarifier to separate the sludge from the effluent for recycle. Aeration of the water can be done by various types of air blower and aeration nozzles. The design of the settling tank is adapted from wastewater cleaning processes. An example of municipal activated sludge experience in groundwater treatment was described by Ettala et al.(1992) confirming that, as shown in laboratory-and pilot-scale studies (Petrasek et al., 1983; Melcer and Bedford, 1988), the trace toxic organic removal is low and cannot be optimized.

Upflow anaerobic sludge blanket reactor: In upflow anaerobic sludge blanket (UASB) reactors suspended granular biomass degrades the contaminants by production of methane and carbon dioxide. Main limitations of the technology

for groundwater treatment are, so far, the requirements of elevated temperatures (528 °C) (Hendriksen et al., 1992; Mohn and Kennedy, 1992) and high organic carbon concentration (Woods et al., 1989) which were not the conditions for groundwater treatment. Therefore, treatment of contaminated groundwater has not yet been reported in pilot or full scale. New bioreactors like the expanded granular sludge bed reactors allow anaerobic treatment of low strength wastewater at low temperatures (Lettinga et al., 1997) but, so far, are not applied for groundwater treatment.

3.3.4 Bioscrubbers

The bioscrubber, as represented in Figure 3.5, consists of two subunits: 1) an absorption tower and 2) a bioreactor. In the absorption unit, input gaseous contaminants are transferred to a dispersed liquid phase (aerosol). Gas and liquid phases flow counter-currently within the column, which may contain a packing.

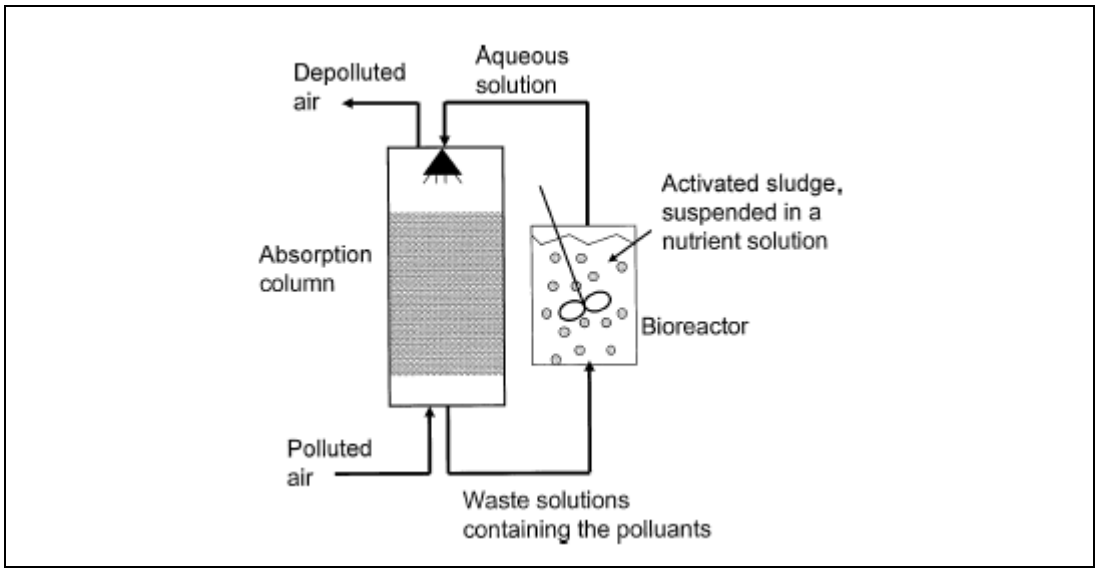


Figure 3.5 – *Bioscrubber description.*

Nevertheless, the addition of inert packing (structured ceramic for example) provides for increased transfer surface between the VOC and the aqueous phase (Kellner and Flauger, 1998). The washed gaseous phase .

The advantages of bioscrubbers are as follows:

- operational stability and good control of the biological parameters (pH, nutrients);
- bioscrubbers do not generate high pressure drops (Rho, 2000);
- their installation does not require large spaces.

However, the major limitations of bioscrubbers are:

- bioscrubbers are adapted to treat readily soluble VOC (alcohols, ketones), with low Henry coefficients(<0.01), and at concentrations in the gaseous phase of less than 5 g ·m−3 (Kelner and Flauger, 1998);
- only quite low specific surface areas are available for gas/liquid transfer (generally <300 m−1);
- production of a sedimented sludge at the bottom of the bioreactor;
- production of waste water.

Some recent studies show that the addition of emulsifying agents (silicon oil, phthalate) in the aqueous solution can significantly improve the elimination of less soluble compounds, because they favor the VOC mass transfer from gas to liquid phases (Mortgat, 2001). Despite this progress, the utilization of bioscrubbers still remains rare.

3.3.5 Bioreactors in pump-and-treat systems: contaminant retardation and bioprocess design limitations

Pump-and-treat systems for remediating groundwater came into wide use in the early to mid-1980s.

The traditional pump-and-treat system contains a series of recovery (extraction) wells or interceptor trenches to pump the contaminated groundwater

from the subsurface. The contaminated groundwater is usually treated chemically, physically or biologically.

The purified groundwater is re-injected through injection wells to the aquifer. Continuous pumping of contaminated groundwater provides hydraulic control of subsurface contaminants to prevent their migration.

The contaminant concentrations in groundwater are typically one to two orders of magnitude lower than those of the wastewater treatment influents (Bouwer et al., 1988). Effluent concentrations from groundwater treatment should fulfil drinking water standards at $\mu g\ l^{-1}$ level as compared to $mg\ l^{-1}$ level from wastewater treatment. Therefore, groundwater treatment process designs differ considerably from those used for wastewater in order to achieve low effluent concentrations.

The contaminant availability in bioreactors is limited to the water-soluble fraction. Pump-and-treat groundwater remediation has frequently proven inefficient due to slow desorption, diffusion and dissolution processes of NAPLs and residual contaminants trapped in soil (Mackay and Cherry, 1989; Travis and Doty, 1990; Nyer,1993). Slow desorption of contaminants like polycyclic aromatic hydrocarbons (PAHs) correlate with the organic carbon content of the soil and the properties of the organic compounds.

Slow diffusion of contaminants is of concern especially if low-permeability layers or lenses are present and the site has been contaminated for long periods prior to start-up of remediation (Grathwohl, 1998; Griffoen and Hetterschijt, 1998). Slow dissolution, especially by chemicals such as PAHs and NAPLs (Freeze and Cherry, 1989), cause immiscible plumes of contaminants. In heterogeneous aquifers, the groundwater flows mainly in the high-permeability zone which reduces the flushing of low-permeability layers and lenses (Berglund and Cvetkovic, 1995). These mass transfer limiting processes require a large number of pore volumes groundwater has to be pumped up to meet cleanup requirements resulting in long pumping times (Borden and Kao, 1992; Bouwer et al., 1988).

Therefore, most contaminated groundwater sites cannot be remediated with this approach alone and regulatory cleanup standards cannot be achieved in a reasonable time and cost frame.

The design development of bioreactors to clean up contaminated groundwater has similar trends with the design development for wastewater treatment. Simple bioreactors like trickling filters and rotating biological contactors were used in the 1980s with limited success resulting in low removal efficiency, poor effluent quality and sensitivity to shock loadings. Application of more efficient designs like upflow fixed-film and fluidized-bed reactors improved cleanup efficiencies, but did not eliminate the general limitations associated with the groundwater pumping.

The main limitation of the bioprocesses applied for groundwater treatment has been thought to be the groundwater temperature which causes slow degradation rates (Melin et al., 1998), low loads and slow biomass build-up.

Heating of the groundwater is not economic-ally reasonable (Valo et al., 1990; Stinson et al., 1991;Piotrowski et al., 1994). Therefore, bioprocesses operating at low temperatures have been developed (Puhakka and Melin, 1996, 1998).

Anaerobic bioreactors are believed to be difficult to operate at low groundwater temperatures and limited experiences of this approach exist, so far.

Enrichment of contaminant-degrading microorganisms from contaminated groundwater can be a long-term process, because 97±99% of subsurface bacteria is attached on soil particles (Harvey et al., 1984; Bouwer, et al., 1988). Use of pure cultures to degrade harmful organic compounds has had limited success.

Indigenous bacteria have been shown to degrade lower contaminant concentrations than pure cultures (Massol-Deyaà et al., 1997).

Chapter 4

Aerobic biodegradation mechanisms and kinetic models

4.1 Biodegradation mechanisms in bioremediation of chlorinated compounds

Bioremediation of CAHs can occur through natural mechanisms (intrinsic bioremediation) or by enhancing the natural mechanisms (enhanced bioremediation). Even if for few CAHs degradation can also occur by abiotic (non-biological) mechanisms, biological degradation tends to dominate.

Although a number of biological degradation mechanisms have been identified theoretically and observed on a laboratory scale, the bioremediation mechanisms carried out by bacteria that typically are used for enhanced bioremediation of CAHs, generally can be classified into one of the following mechanism categories:

- Aerobic oxidation (direct and cometabolic)
- Anaerobic reductive dechlorination (direct and cometabolic)

As seen before, while aerobic oxidation and anaerobic reductive dechlorination can occur naturally under proper conditions, enhancements 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 and, usually, 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 whereas in cometabolic reactions, the CAH degradation or oxidation is caused by an enzyme or cofactor produced during microbial metabolism of another compound.

4.2 Microbial biodegradation and ecology

CAH degradation or oxidation does not yield any energy or growth benefit for the microorganism mediating the cometabolic reaction.

For the most part, the microorganisms that carry out the bioremediation of the CAHs are single-celled prokaryotic bacteria. As living organisms, the bacteria require a source of food to survive and propagate. This requirement, or the bioenergetics of a bacterial system, is defined by the thermodynamics of the processes used by the microbes to derive energy and raw materials from substrates and to use them to carry on biological processes and reproduce. Figure 4.1 depicts the basic bioenergetics of a typical microbial system.

In general, the mediating bacteria collect energy in the form of electrons by a chemical reduction-oxidation (redox) reaction (or photosynthesis). The energy is generated in a redox reaction from the transfer of electrons from an electron donor (the organic contaminant in aerobic oxidation (direct)) to an electron acceptor (oxygen in an aerobic reaction). 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 the 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 as nitrogen and phosphorus). The thermodynamics of a given system defines the energy that is available from a substrate, the energy transfer efficiency losses that will occur, and the portion of the available energy that will be used for reproduction versus the portion that will be used for cell maintenance.

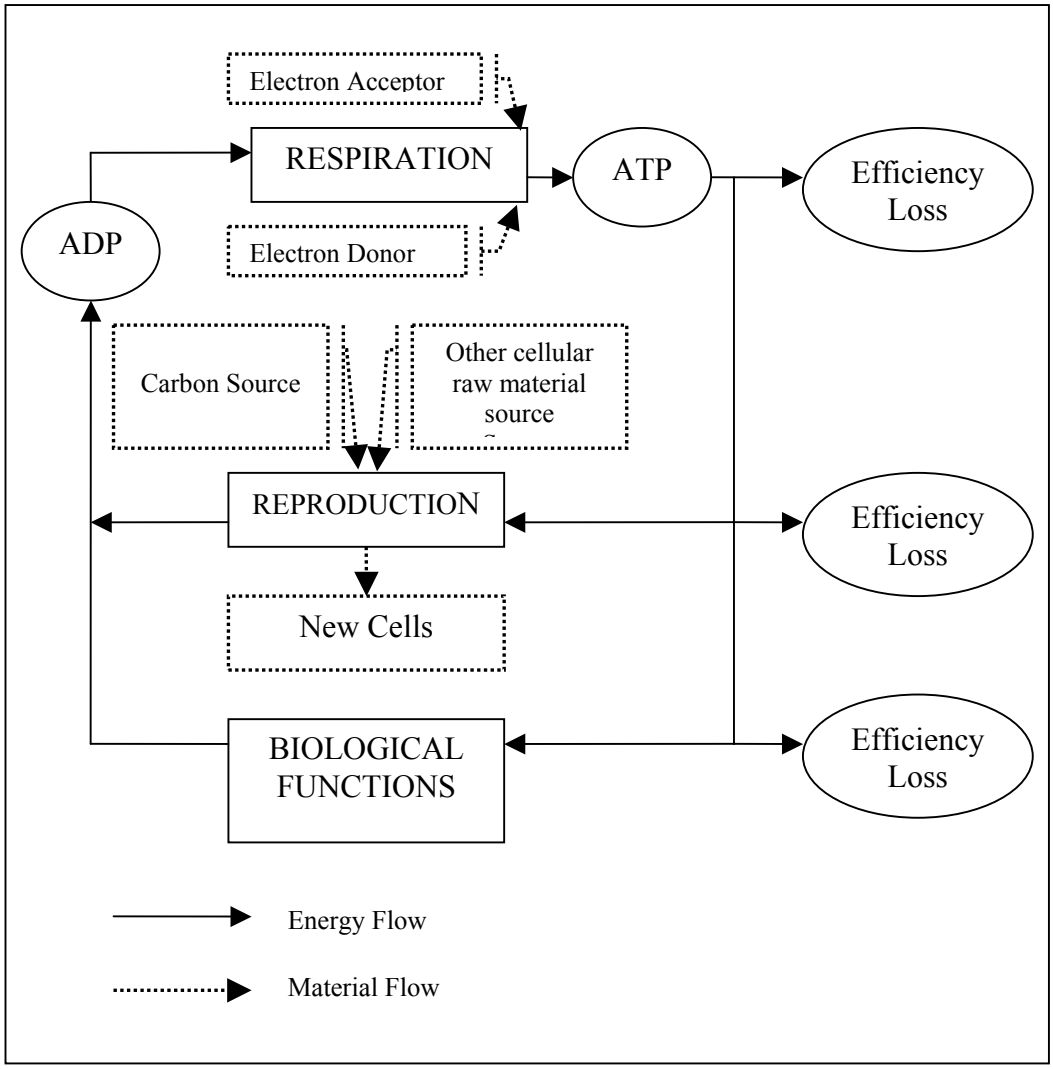


Figure 4.1 – *Diagram of basic bioenergetics of a typical microbial system*

Bacteria generally are categorized by 1) the means by which they derive energy, 2) the type of electron donors they require, or 3) the source of carbon that they require.

Typically, bacteria that are involved in the biodegradation of CAHs in the subsurface are chemotrophs (bacteria that derive their energy from chemical redox reactions) and use organic compounds as electron donors and sources of organic carbon (organoheterotrophs). However, lithotrophs (bacteria that use inorganic electron donors) and autotrophs (bacteria that use carbon dioxide as a carbon source) also may be involved in degradation of CAHs. CAH-degrading bacteria are classified further by the electron acceptor that they use, and therefore the type

of zone that will dominate in the subsurface (for example, an aerobic zone will dominate when aerobes are present). The typical electron-acceptor classes of bacteria are listed below 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.

4.3 Aerobic biodegradation of chlorinated compound

In aerobic zones of the subsurface (zones of the subsurface where oxygen is present), certain CAHs can be oxidized to carbon dioxide, water, and chloride by direct and cometabolic mechanisms. Direct mechanisms are more likely to occur with the less chlorinated CAHs (mono- and di-chlorinates).

In general, the more chlorinated CAHs can be oxidized by cometabolic mechanisms, but no energy is provided to the organism. Incidental oxidation is caused by enzymes intended to carry out other metabolic functions. Generally, direct oxidation mechanisms degrade CAHs more rapidly than cometabolic mechanisms (McCarty and Semprini 1994)

- Aerobic oxidation (direct) is the microbial breakdown of a compound in which 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 one or more electron acceptors (for example, oxygen, nitrate, manganese (IV), iron (III), sulphate, or carbon dioxide).In addition a microorganism can obtain energy for cell maintenance and growth from the oxidized compound (the compound acts as the reductant). In general, only the less chlorinated CAHs (CAHs with one or two chlorines) can be used directly by microorganisms as electron donors. CAHs that can be oxidized

directly under aerobic conditions include DCE, DCA, VC, CA, MC, and CM. The CAHs are oxidized into carbon dioxide, water, chlorine, and electrons, in conjunction with the reduction of oxygen to water.

- Aerobic oxidation (cometabolic) is the microbial breakdown of a contaminant in which the contaminant is oxidized incidentally by an enzyme or cofactor produced during microbial metabolism of another compound. In such a case, the oxidation of the contaminant does not yield any energy or growth benefit for the microorganism involved in the reaction. The CAHs that have been observed to be oxidized cometabolically under aerobic conditions include TCE, DCE, VC, TCA, DCA, CF, and MC. The electron donors observed in aerobic oxidation (cometabolic) include methane, ethane, ethene, propane, butane, aromatic hydrocarbons (such as toluene and phenol), and ammonia. Under aerobic conditions, a monooxygenase (methane monooxygenase in the case of methanotrophic bacteria) enzyme mediates the electron donation reaction. That reaction has the tendency to convert CAHs into unstable epoxides rapidly converted in water to alcohols and fatty acids, which are readily degradable.

4.4 Kinetic of aerobic direct metabolism of chlorinated solvents

Michaelis-Menten enzymatic kinetics could be used to describe the aerobic and anaerobic biodegradation processes and is the basis of the related kinetic models.

Named for Leonor Michaelis and Maud Menten, this kinetic model is valid only when the concentration of enzyme is much less than the concentration of substrate (i.e., enzyme concentration is the limiting factor), and when the enzyme is not allosteric and it postulated that enzyme (catalyst) and substrate (reactant)

are in fast equilibrium with their complex, which then dissociates to yield product and free enzyme.

For many enzymes the rate of catalysis varies with the substrate concentration and the Michaelis-Menten model proposes a specific complex as a necessary intermediate in the catalysis described as follow:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES \xrightarrow{k_2} E + P \tag{4.4.1}$$

An enzyme E combines with a substrate S to form an ES complex with rate constant k_1. Te complex could dissociate to E and S with rate constant k_{-1} or it can proceed and form a product P with rate constant k_2; it's important to note that this enzymatic reaction is supposed to be irreversible, and the product does not rebind the enzyme

In steady state, assuming the concentrations of the intermediates as constant, their time derivatives are zero and that leads to:

$$\frac{d[ES]}{dt} = k_1[E][S] - k_{-1}[ES] - k_2[ES] = 0 \tag{4.4.2}$$

Once expressing the initial concentration of the enzyme $[E]_0$, and thus the total concentration of enzyme, as the sum of that which is free in the solution [E] and that which is bound to the substrate [ES]

$$[E_0] = [E] + [ES] \tag{4.4.3}$$

the conclusion is the following expression for the complex [ES] concentration

$$[ES] = \frac{([E_0] - [ES])[S]}{K_m} \tag{4.4.4}$$

or rearranging it in

$$[ES] = [E_0]\frac{1}{1 + \frac{K_m}{[S]}} \tag{4.4.5}$$

where Km, Michaelis-Menten constant, is defined as:

$$K_m = \frac{k_{-1} + k_2}{k_1} \tag{4.4.6}$$

Based on the previous consideration the rate of product [P] expressed by

$$\frac{d[P]}{dt} = k_2[ES] \tag{4.4.7}$$

could be, accounting of (4.4.5), written as

$$\frac{d[P]}{dt} = k_2[E_0]\frac{[S]}{K_m + [S]} = V_{max}\frac{[S]}{K_m + [S]} \quad = V \tag{4.4.8}$$

where V is the reaction rate V_{max} is the maximum reaction rate. The typical diagram reaction rate V, Substrate concentration [S] could be represented as in Figure 4.1. When [S] equals K_m, [S]/ (K_m + [S]) equals 0.5. In this case, the rate of product formation is half of the maximum rate (1/2 V_{MAX}).

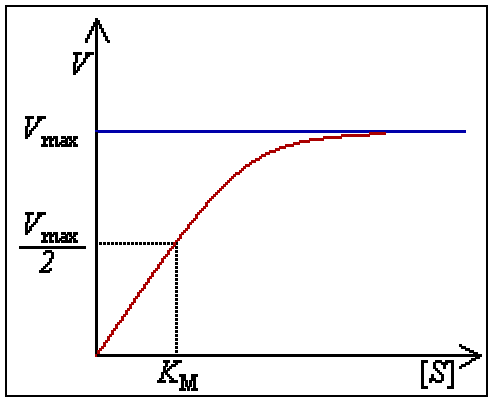


Figure 4.2 – *Diagram of reaction rate and Michaelis-Menten constant*

Notice that if [S] is consistent compared to K_m, [S]/ (K_m + [S]) approaches 1. Therefore, the rate of product formation is equal to $k_2[E_0]$ in this case.

In other words when the substrate concentration is much greater than Km the rate of catalysis is equal to k2.However, most enzymes are not normally saturated with substrate. When [S] << Km, the enzymatic rate is much less than k2 because most of the active sites are unoccupied and (4.4.8) becomes

$$V = \frac{k_2[E_0][S]}{K_M} \qquad (4.4.9)$$

where the reaction rate is linear in the substrate concentration whereas, when [S] >> Km , all the enzyme is present in the enzyme-substrate complex excluding every effect in adding more substrate on the reaction rate and equation (4.4.8) becomes

$$V = k_2[E_0] \qquad (4.4.10)$$

By plotting V_0 against [S], one can easily determine V_{max} and Km. Note that this requires a series of experiments at constant E0 and different substrate concentration [S].

The expression derived from (4.4.8) could be analyzed with the Lineweaver-Burk plot (or double reciprocal plot) in Figure 4.1that is a graphical representation of enzyme kinetics, described by Hans Lineweaver and Dean Burk in 1934.

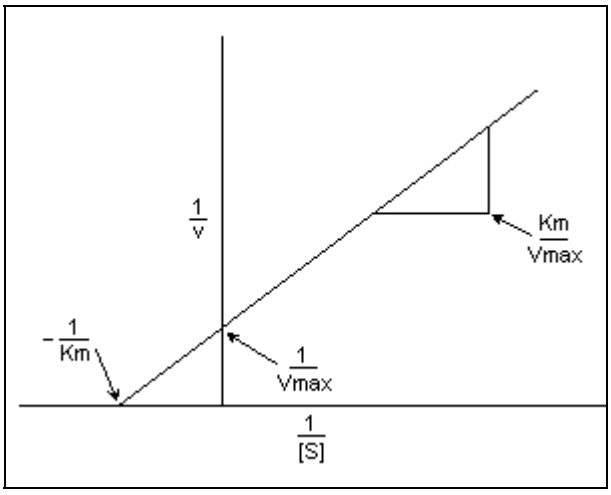


Figure 4.3 – *Lineweaver-Burk plot*

The plot provides a useful graphical method for analysis of the Michaelis-Menten equation: indeed, taking the reciprocal of (4.4.8) gives

$$\frac{1}{V} = \frac{K_m + [S]}{V_{max}[S]} = \frac{K_m}{V_{max}}\frac{1}{[S]} + \frac{1}{V_{max}} \qquad (4.4.11)$$

The plot provides a useful graphical method for analysis of the Michaelis-Menten equation and was widely used to determine important terms in enzyme kinetics, such as Km and Vmax before the wide availability of powerful computers and non-linear regression software, as the y-intercept of such a graph is equivalent to the inverse of Vmax; the x-intercept of the graph represents -1/Km. It also gives a quick, visual impression of the different forms of enzyme inhibition.

The double reciprocal plot distorts the error structure of the data, and it is therefore unreliable for the determination of enzyme kinetic parameters. Although it is still used for representation of kinetic data, non-linear regression or alternative linear forms of the Michaelis-Menten equation such as the Eadie-Hofstee plot are generally used for the calculation of parameters.

Because the rate of bacterial growth is a function of substrate concentration, an equation, formally equivalent to (4.4.8) used in enzyme kinetic analysis, could be written:

$$\mu = \mu_{MAX}\frac{[S]}{K + [S]} \qquad (4.4.12)$$

where μ_{MAX} is the growth rate (µmol or mg $L^{-1}d^{-1}$) at substrate saturation, [S] is the substrate concentration (µmol or mg L^{-1}); k, is a saturation constant (µmol or mg substrate (mg cells)$^{-1}$ d^{-1}) that is numerically equivalent to the substrate concentration at which $\mu = 1/2\ \mu_{MAX}$.

4.5 Introduction and background on aerobic co-metabolism

Cometabolic transformations are reactions that are catalyzed by existing microbial enzymes and that yield no carbon or energy benefits to the transforming cells (Horvath 1972). Therefore, a growth substrate must be available at least periodically to grow new cells, provide an energy source, and induce production of the cometabolic enzymes.

Cometabolism may occur relatively slowly in comparison to metabolism of growth substrates (Alexander 1994). Therefore, the kinetics of cometabolism can be an important consideration in bioremediation applications. For example, the likelihood of degradation kinetics, in contrast to mass transfer rates, controlling the overall contaminant removal rate is greater with cometabolism than with metabolism-based treatment schemes. In addition, the requirement for growth substrates in addition to cometabolic substrates and the related stoichiometry make predictions of cometabolic kinetics complex.

The aerobic cometabolic transformation of chlorinated solvents involves oxygenase enzymes, molecular oxygen, and a source of reducing equivalents, typically NAD(P)H. The oxygenase reaction generates chlorinated solvent oxidation products that may react with cellular macromolecules or may be hydrolyzed spontaneously into carbon dioxide, chloride, or other non-volatile products that are easily mineralized by microorganisms. As is discussed in detail below and depicted in Figure 4.1, some of the factors that may adversely affect the cometabolic degradation of chlorinated solvents by oxygenase-expressing microorganisms include enzyme inhibition by growth or other cometabolic substrates, chlorinated solvent product toxicity, and reducing
energy or reductant shortages.

- *Enzyme competition*: Since enzymes that catalyze cometabolic reactions have active sites that can react with a number of different substrates, including the primary substrate and perhaps a wide range of cometabolic substrates, competition for the active site may occur when multiple substrates are simultaneously available (Figure 4.1a), resulting in an apparent decrease in

enzyme affinity for each substrate (competitive inhibition). Consequently, competition between growth substrate and cometabolic substrate, or among different growth or cometabolic substrates, can result in overall decreased transformation rates of each substrate and in decreased degradation rates for each compound. Other types of inhibition have also been observed to occur during the cometabolic oxidation of solvents. Non-competitive inhibition of growth substrate was also observed involves the independent binding of substrate and inhibitor to different sites on the enzyme causing a decrease in maximum reaction rate without an associated decrease in substrate affinity for the enzyme. Inhibition patterns that did not fit the classical models but that suggested the presence of a secondary binding site were observed for highly chlorinated solvents.

- *Reducing energy consumption*: Oxygenase enzymes generally consume molecular oxygen and reductants such as NAD(P)H or ubiquinone during the oxidation of both energy generating and cometabolic substrates. Energy generating substrates, however, regenerate the reductant during subsequent metabolic steps, while cometabolic substrates such as chlorinated organics do not. The simultaneous availability of primary substrate can be beneficial during cometabolism from the viewpoint of regenerating reductant, but primary substrate can also be detrimental to the cometabolism rate because of competitive inhibition between the primary and cometabolic substrates. The utilization of an alternate energy substrate for the regeneration of reductant allows cometabolic oxidations to be carried out without limitations due to either reducing energy depletion or competitive inhibition. Eventually, the growth substrate must be provided again, however, because oxygenase enzyme levels may diminish and biosynthesis cannot proceed in the absence of enzyme-inducing growth substrates.

- *Product toxicity*: The cometabolic oxidation of chlorinated solvents by a wide range of oxygenase enzymes can result in product toxicity (Figure 1c) Although the specific chlorinated solvent products responsible for the observed product toxicity are not known, toxic effects have been shown to include damage directly to the oxygenase enzymes as well as to general cellular constituents.

Studies conducted with a wide range of oxygenase-utilizing cultures suggest that both the extent of product toxicity and the mode of action are highly variable across species and genera resulting in activity and viability decrease in proportion to the amount of compound degraded.

4.6 Kinetic of aerobic co-metabolism of chlorinated solvents

Saturation kinetics

The kinetics of cometabolic degradation reactions have been described by a number of different models, ranging from simple first-order reaction models to complex multi-substrate mixed order models.

The most commonly applied approach to cometabolic modelling involves modifications to the saturation kinetic expression originally derived from Michaelis-Menten enzyme kinetics:

$$V = k_C[X]\frac{[S_C]}{K_{SC} + [S_C]} \qquad (4.5.1)$$

where V is the rate of cometabolic reaction (μmol or mg $L^{-1}d^{-1}$); S_C, the cometabolic substrate concentration (μmol or mg L^{-1}); k_c, the maximum specific rate of cometabolic substrate degradation (μmol or mg substrate (mg cells)$^{-1}$ d^{-1}); X, the active microbial concentration (mg cells L^{-1}); K_{SC}, the half-saturation constant for the cometabolic substrate (μmol or mg L^{-1}).

With this approach, the rate of cell growth is typically expressed as a function of growth substrate consumption and cell decay as follows:

$$r_X = \mu[X] = [Y]r_g - b[X] = [Y]\frac{k_g[X][S_g]}{K_{Sg} + [S_g]} - b[X] \qquad (4.5.2)$$

where r_x is the net cellular growth rate (μmol or mg L^{-1} d^{-1}); μ, the net specific cellular growth rate (d^{-1}); r_g, the rate of growth substrate consumption (μmol or mg L^{-1} d^{-1}); Y , the cellular yield of growth substrate (mg cells (μmol or mg

growth substrate)$^{-1}$); S_g, the growth substrate concentration (μmol or mgL^{-1}); kg, the maximum specific rate of growth substrate degradation (μmol or mg substrate (mg cells)$^{-1}$ d^{-1}); K_{sg}, the half-saturation constant for growth substrate (μmol or mg L^{-1}); b, the cell decay rate (d^{-1}).

Pseudo first order model

The pseudo-first order rate model for cometabolism is a simplification of saturation kinetics predicated on the assumption that substrate concentrations (S_c) are significantly lower than half-saturation constants K_{sc}):

$$rc = -k_1 X S_c, \qquad (4.5.3)$$

where k_1 is the pseudo-first-order cometabolic degradation rate constant (L (mg cells)$^{-1}$ d^{-1}). In this expression, k_1 is equivalent to k_C / K_{SC} in Equation (4.5.1).

Incorporation of k_C and K_{SC} into a single term can be especially useful in the common situation where these two parameters cannot be determined independently because of toxicity problems associated with the high concentrations needed to achieve saturation kinetics.

Many researchers have applied the pseudo-first-order model to describe the cometabolic oxidation of chlorinated solvents when concentrations are relatively low and both competitive inhibition and product toxicity are not of concern.

Modelling product toxicity

Product toxicity associated with cometabolic oxidations has been observed to cause cell activity to decrease in proportion to the amount of compound degraded.

This product toxicity is different from classically modelled product inhibition, in which cell activity decreases in proportion to the accumulation of inhibitory products.

With some exceptions, the products of chlorinated solvent oxidation are transient, so they do not accumulate appreciably. Rather, they exert toxicity either

while being formed or immediately after formation. Product toxicity associated with the cometabolic oxidation of chlorinated solvents has been quantified in a number of different ways. Oldenhuis et al. (1991) introduced an inactivation constant, p, that related the change in the maximum rate of cometabolic substrate degradation to the amount of cometabolic substrate being degraded as follows:

$$p = \frac{dk_C}{d[S_C]} \tag{4.5.4}$$

where p is the inactivation constant (L (mg cells)$^{-1}$ d^{-1}).

The implication of this approach is that the toxic effects function to decrease enzyme activity specifically rather than affecting overall cellular functions.

A similar approach introduced by Alvarez-Cohen & McCarty (1991b) utilized a transformation capacity (Tc) term, a constant representing the amount of compound degraded divided by the amount of cells inactivated:

$$Tc = \frac{d[S_C]}{d[X]} \tag{4.5.5}$$

where Tc is the transformation capacity for the cometabolic substrate (μmol or mg cometabolic substrate (mg cells)$^{-1}$); dS_C, the change in cometabolic substrate concentration during the reaction (μmol or mg L^{-1}); dX, the change in active cell concentration during the cometabolic reaction (mg L^{-1})

The implication of this approach is that the toxic effects function to decrease overall cellular functions rather than affecting specific enzyme activity alone.

A related term, the transformation yield (Ty), was defined as the amount of cometabolic substrate degraded prior to cell inactivation divided by the amount of primary substrate required to grow the cells, and is calculated as [Y] Tc, the cellular yield of growth substrate (mg cells (μmol or mg growth substrate)$^{-1}$) multiplied by the transformation capacity.

Ty is of considerable practical significance in that it provides an indication of the amount of growth substrate needed, which directly affects operating costs.

The distinction between the inactivation constant and transformation capacity approaches is primarily conceptual, with the inactivation constant representing the observed decrease in degradation activity as a decrease in the maximum enzyme degradation rate and the transformation capacity representing it as a decrease in the active cell concentration.

The transformation capacity representation of product toxicity has been incorporated into the saturation kinetic expression to describe cometabolic reaction rates occurring in the presence or absence of growth substrate by defining the net specific cell growth rate (μ) as a function of cell growth due to consumption of growth substrate (Sg) and cell inactivation due to product toxicity and cellular decay as follows:

$$\mu = \frac{r_X}{[X]} = Y\frac{r_g}{[X]} - \frac{1}{T_C}\frac{r_C}{[X]} - b \qquad (4.5.6)$$

An important aspect of this modelling expression is that cometabolic oxidations may continue temporarily in the absence of growth substrate until the active cell mass (that is, cells with active oxygenase enzymes) has been depleted due to the combined effects of product toxicity and cell decay. Modifications of the above model incorporating the concept of a transformation capacity have been used by a number of researchers to effectively describe the cometabolic degradation of chlorinated solvents by a number of oxygenase expressing cells both in the presence and absence of growth substrate.

Ely et al. (1995a) derived a general cometabolic model from enzyme kinetics that incorporated the concept of an enzyme inactivation constant introduced by Oldenhuis et al. (1991). This model explicitly includes terms representing the potential recovery of enzyme activity following inactivation into the expression for maximum degradation rate and a simplification of this model was also used by Yang et al. (1999) to describe TCE degradation by a nitrifying enrichment culture.

Although this model explicitly includes a term for enzyme recovery from toxic damage, it does not include an expression for cell growth. Interestingly, while the model was designed to incorporate cell repair rather than cell re-growth in response to product toxicity, the resulting recovery term is mathematically equivalent to the cell yield term used for cell growth in the previously described models but the major conceptual difference between the model of Ely approach and the other models is that enzyme recovery is de-coupled from cellular growth, allowing for the possibility that enzyme recovery following toxic inactivation may occur more rapidly and with less energy consumption than cellular re-growth.

Chapter 5

Aerobic Cometabolic Biodegradation of 1,1,2,2-Tetrachloroethane: Batch tests

5.1 Abstract

1,1,2,2-tetrachloroethane (TeCA) is a widespread toxic contaminant whose main sources are industrial production processes and it is commonly used as a degreaser and solvent in the treatment of raw material. Among the numerous studies on the biodegradation of chlorinated aliphatic hydrocarbons (CAHs, only few focused on the aerobic degradation of the halogenated compounds by pure or mixed cultures) [Alvarez-Cohen and McCarty 1991; Oldenhuis et al., 1991].

The objective of this work was to study a process of TeCA biodegradation via aerobic cometabolism by methane-utilizing and propane-utilizing microbial cultures.

Both cultures, selected in a previous work [Frascari et al., 1998], were studied by means of slurry microcosm tests and the performance of the propane-utilizing culture was further studied in liquid/gas bioreactors.

TeCA was biodegraded by both microbial cultures via aerobic cometabolism in the 0-600 μg/L concentration range. The process was successfully continued for several months without any apparent inhibitory or toxic effect on the microbial cultures tested. In bio-augmented microcosms, the inoculation of the propane-utilizing culture developed in the slurry microcosms led to a marked reduction of the lag-period required for the onset of TeCA degradation. The positive results obtained from the liquid/gas reactors show that it is possible to grow the isolated propane-utilizing culture in a liquid/gas bioreactor

without any observable loss of TeCA degradation capacity. This conclusion is relevant with respect to the production of significant amounts of inoculum to utilize in bioaugmentation treatments.

5.2 The reasons behind this study

In a previous work, the aerobic cometabolic biodegradation of a mixture of chlorinated aliphatic hydrocarbons (CAHs) including vinyl chloride (VC), cis- and trans-1,2-dichloroethylene (cis-DCE, trans-DCE), trichloroethylene (TCE), 1,1,2-trichloroethane (1,1,2-TCA) and 1,1,2,2-tetrachloroethane (1,1,2,2- TeCA) was investigated at both 25 and 17 ◦C by means of bioaugmented and non-bioaugmented sediment-groundwater slurry microcosm tests.

The goals of the study were (i) to study the long-term aerobic biodegradation of a CAH mixture including a high-chlorinated solvent (1,1,2,2-TeCA) generally considered non-biodegradable in aerobic conditions; (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 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 phylotype analysis. To

the best of our knowledge, this is the first study that documents the long-term aerobic biodegradation of 1,1,2,2-TeCA.

The conclusions of this study stated that the indigenous biomass of the aquifer material investigated in this study 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-TeCApresent. 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.

The long-term aerobic cometabolic transformation of 1,1,2,2-TeCA by the two biomasses have been considered a remarkable achievement and because cometabolism induced by bioaugmentation could be a suitable process used by micro-organisms to degrade chlorinated solvents, this result have been found of particular interest for an in depth investigation during this Ph.D. thesis.

5.3 1,1,2,2-tetrachloroethane: an introduction.

1,1,2,2-Tetrachloroethane (TeCA) is a colourless, dense liquid that has a sweet, chloroform-like odour and, based on a report realized for the U.S. Government by the NIOSH (National Institute for Occupational Safety and Health) on the occupational exposures, it could be considered one of the first chlorinated solvent produced in large amount before the Great War and, even if it was replaced at the end of the World War II by other chlorinated compound thought less toxic, could be classified as a relatively common groundwater contaminant [13].

In the present the production of TeCA as an end product has decreased significantly [14] in comparison with the past years when its production in large quantities was also justified in order to produce trichloroethylene, tetrachloroethylene e 1,2- dichloroethylene, as a solvent, in cleaning and degreasing metals, in paint removers, varnishes and lacquers, in photographic films, as an extractant for oils and fats and in pesticides. Other applications of this halogenated compound were as a solvent for waxes, cellulose acetate, sulphur and rubber and rarely [15] as a carrier or reaction solvent in manufacturing processes for other chemicals and as an analytical reagent for polymers.

TeCA have been used as a major component of a decontaminating agent produced in the past by the U.S. military and was used in an organic solvent process to manufacture chemical-agent-resistant clothing [16]

As it is no longer widely used in the U.S. as an end product, present sources of TeCA are fugitive emissions or discharges when is generated as a by-product and during chemical production activities in which it is an intermediate product but, as the majority of the chlorinated hydrocarbons, it has became widely distributed environmental contaminants through discharge of industrial wastewater, seepage from landfills and leakage from underground storage tanks.

Although use and improper disposal of this chlorinated compound have resulted in groundwater contamination at several military bases and other industrial sites and low level of TeCA have been detected in groundwater and surface water, a nationwide survey of drinking water supplies in 1980s in U.S. did not find evidence contamination and even if a drinking water standard has not been set-up for 1,1,2,2-tetrachloroethane, a low-risk based concentration of 0.05 mg/L has been established because of possible carcinogenic effect [17].

In respect with the previous consideration, although available data are incomplete, it has been hypothesized that the carcinogenicity of 1,1,2,2-tetrachloroethane may be associated with the formation of free radicals, lipid peroxydation, or hepatic damage. Therefore, on the basis of data currently available, it is not possible to draw any firm conclusions with respect to the potential carcinogenicity of 1,1,2,2-tetrachloroethane in humans [18,19] and EPA

(Environmental Protection Agency) has classified 1,1,2,2-Tetrachloroethane as a *Group C* possible human carcinogenic [20].

Moreover, as stated by Arnold et Al. [21], the impact of a pollutant is determined not only by its persistence in the environment but by the lifetimes of any products formed via its transformation. Degradation products usually are more toxic than parent compound as for the human carcinogenic vinyl chloride (VC) released during the reductive dehalogenation of the trichloroethylene (TCE); for this reason the knowledge of the final product distribution could be considered important as the reaction rates especially when multiple products may result from a single reaction pathway.

5.4 1,1,2,2- tetrachloroethane: non-cometabolic biodegradation pathways.

Whereas low chlorinated compound - as cholorethane (CA), 1,1-dichloroethane (1,1-DCA) and 1,2-dichloroethane (1,2-DCA) - could be involved in biodegradation processes as a growth substrates by aerobic microorganisms but only co-metabolized by anaerobic microorganisms, high chlorinated compounds - as 1,1,1-trichloroethane (1,1,1-TCA), 1,1,2-trichloroethane (1,1,2-TCA) tetrachloroethane (TeCA), pentachloroethane and hexachloroethane (HCA) biodegraded by cometabolic processes or mainly under aerobic condition.

Because of his recalcitrant behaviour in aerobic condition, the biodegradation of 1,1,2,2- tetrachloroethane has been studied under anaerobic conditions. The increasing probabilities that this chlorinated compound biodegrades via reductive reaction are due to the high level of oxydation and stability assured by the four high electronegative chloride atoms in comparison with its not halogenated partner.

While few information are available about TeCA transformation, as shown in Figure 5.1 reductive hydrogenolysis, dichloroelimination and

dehydrochlorination are classified as three possible pathways for 1,1,2,2-TeCA degradation [15].

- *Reductive hydrogenolysis* or *reductive dechlorination* is a common transformation of 1- and 2- carbon chlorinated aliphatics under methanogenic conditions. Reductive dechlorination is a two-electron transfer reaction and: for example 1,1,1-TCA is converted in 1,1-DCA and PCE is successively converted to TCE, *cis*DCE, VC and ethene. Bouwer et Al. [22] suggested 1,1,2-TCA as an intermediate of TeCA transformation in continuous-flow column experiment via reductive dechlorination and Chen et Al. [15] showed 1,2-DCA as the result of the hidrogenolysis of TeCA e if the amount of the two compounds is not so relevant to justify more than the 5% of the depletion of the TeCA.

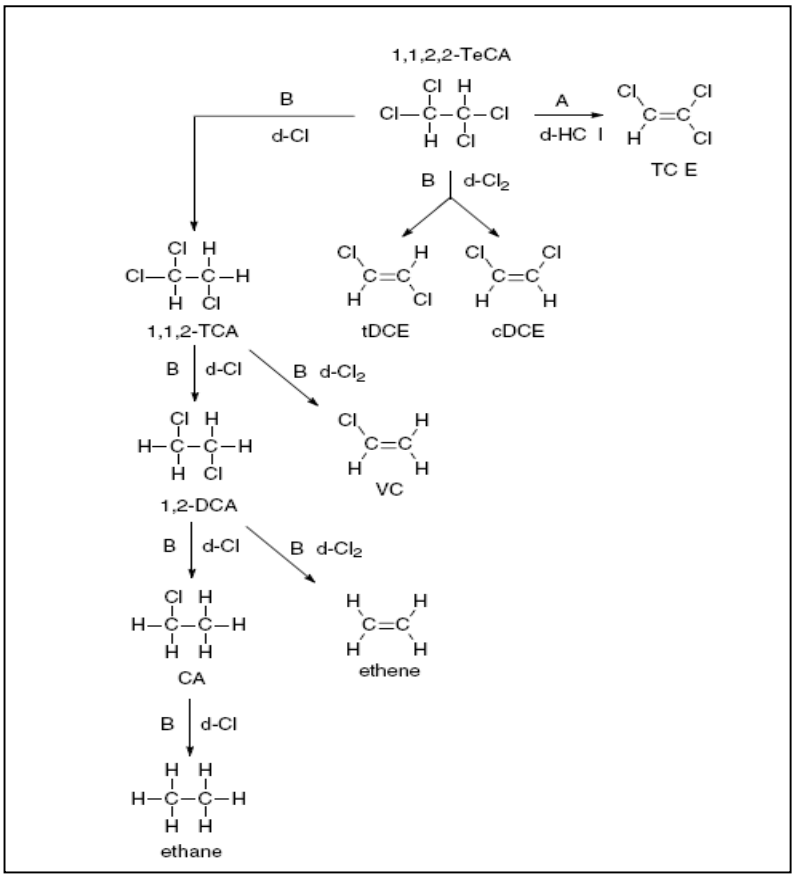


Figure 5.1 – *Non cometabolic pathways of 1,1,2,2-TeCA in anaerobic sludge (Chen et al., 1996; van Eckett et al. 1999). A=abiotic; B= biotic; d-Cl= reductive hydrogenolysis; d-HCl= dehydroclhoroelimination; d-Cl= diclhoroelimination*

- *Dehydrochlorination*, which is not classified as a redox reaction, has been seen, for example, in the abiotic conversion of pentachloroethane to PCE and 1,1,1-TCA conversion to 1,1-DCE and in abiotic and biotic transformation of TeCA together with the production of TCE .

- *Dichloroelimination*, which is another type of reductive dechlorination reaction, releases two adjacent chlorine atoms simultaneously, forming an alkene. Dichloroelimination of TeCA could produce cis- and trans-1,2-DCE in methanogenic conditions. 1,1,2-TCA , a TeCA hydrogenolysis product, could produce VC via dichloroelimination.

Although these three biodegradation pathways of TeCA have been noticed in bioremediation studies, the occurrence and dominance pathways of in situ degradation of TeCA in groundwater or soil are largely unknown. Laboratory batch and column experiments constructed with anaerobic mineral medium or glass beads constitutes the few reported studies [15,22] where the methanogenic mixed cultures were obtained from laboratory-scale municipal sludge digester or wastewater filters, whereas Schanke et Al. [23] use in a laboratory study an abiotic aqueous mixture of transition metal coenzymes.

Even though laboratory studies have helped to elucidate mechanisms by which TeCA can be degraded, they provide little information on the preferred degradation pathways in groundwater and soil for this halogenated compound.

The fact that field evidence of degradation reactions related to this chloroethane has been lacking may be in part because TCE (able to release contemporarily the same possible daughter product of TeCA and TeCA daughter product itself) was frequently used and disposed of at the same sites of TeCA.

Lorah et Al. [16] report both field and laboratory evidence of TeCA degradation for a freshwater tidal wetland that receives groundwater discharge from a contaminated sand aquifer with results indicating natural attenuation of TeCA trough complete anaerobic biodegradation before sensitive surface water receptors are reached. The depletion of TeCA in the wetland anaerobic sediment was related to the reductive declhorination products formation as 1,1,2-TCA and 1,2-DCA, transformed in VC in a second step. Sequentially the removal of the chlorinated compound was totally achieved utilizing the biomass under

methanogenic conditions. Data shows how the impact of the intermediate products could affect the performance of the bioremediation process and that a sequentially treatment is needed to reach the complete depletion.

The capability of some bacteria in utilizing a specific halogenated compound as electron acceptor in an anaerobic process of respiration, was recently reported (Hollinger, 1994 and 1998). This process, known as halorespiration or dehalorespiration, is confirmed in the biodegradation of PCE by *Desulfitobacterium* sp.Y51 and could be used to quickly convert pentachloroetanes and TeCA to dichloroethanes (Suyama, 2001).

5.5 1,1,2,2- tetrachloroethane: aerobic cometabolic biodegradation pathways

Only little information is available in literature especially in relation with the intermediate reaction products of the TeCA involving monoxygenasys enzymes in the biodegradation. Rasche et Al. (1991) published data related to the biodegradation by nitrifying bacteria specie, *Nitrosomonas europeaea* where chloral, C_2Cl_3HO, has been found as the main intermediate product of the co-oxidization of 1,1,2,2-TeCA and TCA in both isomeric state (Figure 5.2).

Also the P450 cytochrome class demonstrated the capability to degrade the 1,1,2,2-Tetrachloroethane: P450BM3 isolated from *Bacillus megaterium*, for instance, shows the capability to catalyze the Oxydative dehalogenation of the TeCA (Alworth, 1997), whereas eukaryotic cytochrome P450 from rat microsomes could transform TeCA in dichloroacetic acid (Halews et Al. 1987).

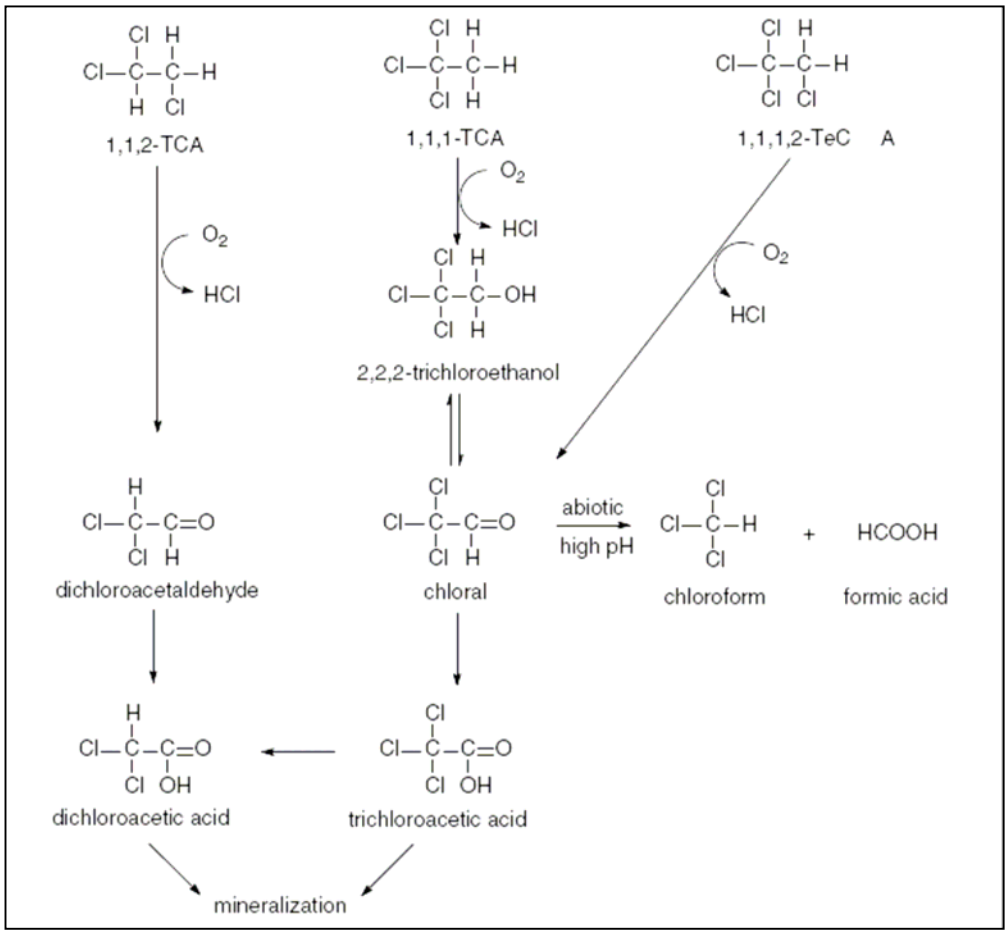


Figure 5.2 – *Aerobic co-oxydization pathways of 1,1,2-TCA, 1,1,1-TCA and 1,1,2,2-TeCA*

Table 5.1 shows a collection of kinetic data available in literature on the cometabolic degradation of the two isomer of TeCA: all the references are related with anaerobic cometabolic degradation.

COMPOSTO	REDOX CONDITION	COLTURE	ACTIVITY (mg g^{-1} dwt d^{-1})	REFERENCE
1,1,1,2-TeCA	CoM AN M	Mrthanogenic Biofilm	3	Van Eckert et al. (1999)
	CoM AN H	*Desullfobacterium* strain Y51	11626	Suyama et al. (2001)
1,1,2,2-TeCA	CoM AN M	Mrthanogenic Biofilm	1	Van Eckert et al. (1999)
	CoM AN M	Municipal sludge digester	10	Chen et al. (1996)
	CoM AN H	*Desullfobacterium* Strain Y51	3879	Suyama et al. (2001)

Table 5.1 – *Kinetic data related to the two TeCA isomers available in literature. (CoM=cometabolism; AN=anaerobic; M=methanogenic; H=halorespiration)*

It is possible to notice how there are no data available for the aerobic cometabolic pathways and the experiments set up in this thesis aim to fill this gap as highlighted in Table 5.2.

<table>
<tr><td rowspan="5">1,1,2,2-TeCA degradation pathways</td><td rowspan="4">Biological processes</td><td rowspan="2">Cometabolism</td><td>Oxidization</td><td>Present study</td></tr>
<tr><td rowspan="2">Reduction</td><td>Dechlorination</td></tr>
<tr><td>Dichloroelimination</td></tr>
<tr><td rowspan="2">Direct metabolism</td><td>Oxydization</td><td>Dehalorespiration</td></tr>
<tr><td>Reduction</td><td>Pathway not available</td></tr>
<tr><td>Abiotic processes</td><td colspan="3">Dehydrohalogenation</td></tr>
</table>

Table 5.2 – *1,1,2,2 Tetrachloroethane pathways and collocation of this present study*

5.9 Materials and methods

5.9.1 Experimental scheme and microcosm set up

The study has been conducted in aerobic slurry batch microcosms, prepared using 119-mL bottles with Teflon-coated rubber septa.

Each microcosm of every series contained 20 g of soil from the relative site (see Table 5.3) and 50mL of brackish groundwater taken from the same site.

When the latest was not available, the same volume of mineral media has been used to substitute the groundwater (composition displayed in table 5.4).

Microcosms Series	Soil composition	water
A2P	Silt , sand	Mineral Media
D2P	Superficial soil	Mineral Media
M2P, TP, TM	Sand , silt, clay	Brackish groundwater
P2P	Sand	Mineral Media
R2P	Superficial soil (high organic carbon content)	Mineral Media

Table 5.3 – *soil composition and water used in the microcosms set up*

Compound	Concentration	
	(mg/L)	(mmol/L)
KH_2PO_4	1211,43	8,9016
NaH_2PO_4	760,21	5,3551
NH_4Cl	426,31	7,9697
$NaNO_3$	13,62	0,1603
$MgCl_2 \cdot 6H_2O$	49,70	0,2444
$CaCl_2 \cdot 2H_2O$	14,70	0,1324
$FeCl_2 \cdot 4H_2O$	4,49	0,0226
$MnCl_2 \cdot 4H_2O$	0,3008	0,0015
$ZnCl_2$	0,0695	0,00051
H_3BO_3	0,0618	0,0010
$Na_2MoO_4 \cdot 2H_2O$	0,1089	0,00045
$NiCl_2 \cdot 6H_2O$	0,0238	0,00014
$CuCl_2 \cdot 2H_2O$	0,0170	0,0001
$CoCl_2 \cdot 6H_2O$	0,0238	0,0001
Na_2SO_4	725	5,5618
K_2SO_4	875	4,7059
SO_4^{2-}	996,0	10,2677
NO_3^-	9,93	0,1602

Table 5.4 – *Mineral media composition*

The 59-mL headspace was filled with air. To avoid bacterial contamination, bottles, caps and all the tools used for microcosm preparation were autoclaved (121 °C, 20 min).

The experimental tests have been carried on in three different steps:

1) **TMX-TPX step**: This part of the investigation concerned the effectiveness of the bio-augmentation tests performed in slurry batch reactors (microcosms).

The effectiveness has been evaluated in terms of:

- **Lag-time** required for the onset of the aerobic cometabolic transformation of 1,1,2,2-TeCA

- **Long term degradation rate of 1,12,2-TeCA** in slurry conditions up to the concentration of 600 μg/L.

Both of the two biomasses selected from the previous experimental work (and, so far, for each growth substrate), after a preliminary test, have been utilized as inocula in three different set of triplicate tests.

Each microcosm have been maintained in aerobic condition, continuously mixed and tested at the temperature of 25°C.

As described in table 5.4, three different scenario of initial concentration of 1,1,2,2-tetrachloroethane in aqueous phase have been analyzed and, with subsequent pulses, the same initial concentration of pollutant have been reset once the TeCA decreased under the sensibility of the analytical instruments.

In adding to the 18 microcosms (every reactor was initially inoculated with either a propane utilizing or methane utilizing biomass), six sterile control batch reactors were set up in order to evaluate the rate of TeCA depletion due to abiotic processes (hydrolysis, absorption in the rubber septa, losses due to sampling procedures).

Microcosms Series	Tests Name	TeCA: initial concentration in liquid phase (μg/l)	Main growth substrate		Inocula		
			Alcane	initial concentration in liquid phase (mg/l)	Source	Volume (ml)	Conc. (10^6 CFU/ ml)
TM Inoculated by methanotrophic biomass	TM 1, 2, 3	50	CH_4	2,0	3 *slurry* microcosms, containing metanotrophic biomass, from a previous experimental work	1.5	1.6
	TM 4, 5, 6	150					
	TM 7, 8, 9	600					
TP Inoculated by methanotrophic biomass	TP 1, 2, 3	50	C_3H_8	2,0	3 *slurry* microcosms, containing propanotrophic biomass, from a previous experimental work	1.5	7.4
	TP 4, 5, 6	150					
	TP 7, 8, 9	600					
KTA Control microcosms [1]	KTA 1, 2	50	No substrate	-	-	-	-
	KTA 3, 4	150					
	KTA 5, 6	600					
[1] Chemically sterilized by 4 g/l di NAN_3 adding							

Table 5.5 – *TMX-TPX experimental scheme*

2) **ADMPR step**: the **effectiveness of bioaugmentation in different scenario** by the propane utilizing biomass has been tested in microcosm scale in this second part of the experimental work. Five soils collected in different sites have been selected for the tests: soil A and M belong to two historically contaminated sites (more info available in appendix A), whereas soils D, P and R have never been exposed to chlorinated compound contamination in advance.

The only biomass tested was the propane utilizing one because of its better performance (in terms of lag-times and 1,1,2,2-TeCA degradation rate) and it was injected as inocula following the scheme presented in Table 5.6

For every soil a microcosm have been inoculated with the pre-selected biomass and the results have been compared with the behaviour of two non inoculated control microcosms and a sterile control (obtained by adding NaN_3).

Each microcosm have been maintained in aerobic condition, continuously mixed and tested at the temperature of 25°C and exposed to a 1,1,2,2-tetrachloroethane initial concentration in aqueous phase of 150 μg/L reset by subsequential pulses once the TeCA decreased under the sensibility of the analytical instruments.

Microcosms Series	Tests specification		TeCA: initial concentration in liquid phase (μg/l)	Main growth substrate	
				Alkane	initial concentration in liquid phase (mg/l)
A2P	I	Inoculated[1]	150	C_3H_8	2,0
	1, 2	Control			
	C	Control sterile[2]			
D2P	I	Inoculated[1]	150	C_3H_8	2,0
	1, 2	Control			
	C	Control sterile[2]			
M2P	I	Inoculated[1]	150	C_3H_8	2,0
	1, 2	Control			
	C	Control sterile[2]			
P2P	I	Inoculated[1]	150	C_3H_8	2,0
	1, 2	Control			
	C	Control sterile[2]			
R2P	I	Inoculated[1]	150	C_3H_8	2,0
	1, 2	Control			
	C	Control sterile[2]			
([1]) Inocula: 1.5 ml from TP5-6-7 @ Conc. (10^6 CFU/ml)					
([2]) Chemically sterilized by 4 g/l di NAN_3 adding					

Table 5.6 – *ADMPR experimental scheme*

3) **Isomer and high concentration tests**: in this step, the capability of the previously selected propane utilizing biomass has been tested on aerobic cometabolic biodegradation of the isomeric form of the TeCA mainly investigated in this work: 1,1,1,2 tetrachloroethane. The set up of the microcosms has been following the same protocol of the previous tests and the operational condition are displayed in table 5.7 where have been reported also the experimental condition for a different series aimed to investigate the possible toxic effect of higher concentration of 1,1,2,2 tetrachloroetane.

Microcosms Series	Tests name	Main growth substrate		Pollutant	
		Alkane	initial concentration in liquid phase (mg/l)	Isomeric form	initial concentration in liquid phase (μg/l)
TI ([1]) [isomer]	TI 1, 2, 3	C_3H_8	2,0	1,1,1,2 TeCA	400
TH ([1]) [high concentration]	TH 1, 2, 3	C_3H_8	2,0	1,1,2,2 TeCA	900-2500
(1) Inocula: 1.5 ml from TP5-6-7 @ Conc. (10^6 CFU/ml); Soil: from site M; Water: brackish ground water from site M.					

Table 5.7 - *TI (isomer)and TH (high concentration) experimental scheme*

4) LA-LB step: The last part of this work aimed to understand the possibility to grow the isolated propane utilizing culture in a liquid gas batch bioreactor (microcosm) testing the maintenance of 1,1,2,2-TeCA degradation capacity.

An inoculum of biomass was extracted from the microcosms containing propane utilizing biomass and let grow in a set of four liquid-gas microcosms in order to achieve a "dilution" with the intent to minimize the amount of soil present in the batch and to maintain the biodegradative activity

Each microcosms of the series LA, containing 60 ml of sterile mineral media have been maintained in aerobic condition, continuously mixed and tested at the temperature of 25°C and exposed to an 1,1,2,2-tetrachloroethane initial concentration in aqueous phase of 150 μg/L reset by subsequential pulses once the TeCA decreased under the sensibility of the analytical instruments.
A further "dilution", following the same experimental operating condition of the previous one, was subsequently performed by sampling an inoculum from these liquid-gas microcosms and injecting it into a second series of liquid-gas microcosms, series LB. Table 5.8 shows a scheme of the experimental set up of this step.

Microcosms Series	**Tests name**	TeCA: **initial concentration in liquid phase (μg/l)**	**Main growth substrate**		**Inocula**		
			Alkane	**initial concentration in liquid phase (mg/l)**	**Source**	**Vol. (ml)**	**Conc. (10^6 CFU/ml)**
LA (1^{st} dilution in liquid media)	LA 1, 2, 3, 4	150	C_3H_8	2,0	TP 5, 6, 7	1,5	21
LB (2^{nd} dilution in liquid media)	LB 1, 2, 3, 4	150	C_3H_8	2,0	LA 1, 2, 3, 4	2,0	30

Table 5.8 – *LA-LB experimental scheme*

5.6.2 Bacterial strains

Object of this work has been the testing of the "performance" of two different biomasses and the consequent isolation of a pure strain (Rhodococcus*)* and a consortium of microorganisms (able to biodegrade tetrachloroethane in both isomeric forms via aerobic co-metabolism) from slurry phase microcosms.

5.6.3 Gas-chromatographic analysis

GC analysis was employed for determination of the chlorinated compounds and both substrates (methane and propane) in the gaseous samples from the headspace of the microcosms. An Agilent 6850 Series II Gas Chromatograph with a FID (*Flame Ionization Detector*) and a μ-ECD (*Electron Capture Detector*) detector and a 'J&W Scientific' (Agilent Technologies UK Limited, UK) column with HP-1 stationary phase (30 m × 0.32 mm × 0.25 μm) have been used. For gaseous samples, 25 μL were injected into the GC and the temperature program run at 40 ºC for 2 min and then increased to 70 ºC at a rate of 20 ºC min-1.The measure of the concentration

Substrates and pollutant standard concentrations in gaseous and liquid phase have been calculated on the basis of the injected volumes assuming the equilibrium have been reach between the two phase. The following equations describe the equilibrium:

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

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

Leading to:

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

Where:

V_L, V_G: standard (L) liquid and gas volumes

H_i : dimensionless Henry constant for the compound *i* (@20°C, $H_{propano}$ = 27.3; $H_{methane}$ = 29.2; $H_{1,1,2,2TeCA}$ = 0.02; $H_{1,1,1,2TeCA}$ = 0.017;) (Mackay e Shiu, 1981)

m_i : mass of the compound *i* injected into the standard (mg)

$c_{G,i}$, $c_{L,i}$: gaseous and liquid concentration for the compound *i* (mg/L).

Concerning the evaluation of the compound concentration into the microcosms, these have been based on the headspace analysis, whereas liquid and soil concentration have been evaluated by assuming equilibrium between the phases. The calculation has been based on equation 5.2 and on the linear isothermal absorption (Semprini, 2000).

$$c_{S,i} = K_{d,i} \cdot c_{L,i} \qquad (5.4)$$

Where:

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

$K_{d,i}$: absorption constant for the compound *i* (L/kg)

The absorption constants have been evaluated on the basis of the organic carbon content for the tested soil (f_{oc}, dimensionless) and on the value of the partition coefficient between organic carbon and water (K_{oc}, L/kg):

$$K_{d,i} = f_{oc} \cdot K_{oc,i} \qquad (5.5)$$

The value assumed for the partition coefficient K_{oc} have been based on US EPA (2000): $K_{oc,1,1,2,2\ TeCA}$ = 102 L/kg, $K_{oc,1,1,12\ TeCA}$ = 525 L/kg, $K_{oc,methane}$ = 8 L/kg; $K_{oc,propano}$ = 144 L/kg.

The amount of the compounds (pollutant and substrate) into the microcosm Have been determined (starting from the headspace gas analysis) on the basis of the following equation:

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

Where M_T (kg) is the mass of the soil contained into the microcosm while equation 5.2 allows an estimation of $c_{L,i}$.

5.6.4 Methane and propane analysis

In order to estimate the methane and propane concentration an Agilent 6850 Series II Gas Chromatograph with a FID (*Flame Ionization Detector*) has been used. Table 5.9 shows method and device parameters

The calibration curve for the two substrate have been created injecting defined volumes of the gas in 119 mL microcosm sealed with rubber septum containing 60 ml of de-ionized water. Before every series of analysis, the calibration has been checked by a standard containing a defined amount of substrate (corresponding to the liquid concentration of 2.0 mg/L).

DEVICE	HP 6890 series II plus
COLUMN TYPE	Capillary column HP-VOC
COLUMN I.D.	0.32 mm
COLUMN LENGHT	30 m
LINER	Splitless
SPLIT RATIO	10:1
FRONT DETECTOR	μECD
BACK DETECTOR	FID
CARRIER GAS	Helium
MAKE-UP GAS	Nitrogen
INJECTOR PRESSURE	404 Kpa
FLOW	2.8 ml/min
RATE	59 cm/s
INJECTION VOLUME	200μL
PRESSURE	1.6 Bar
ANALYTICAL TIME	2.0 min
INJECTOR TEMPERATURE	250°C
DETECTOR TEMPERATURE	250°C
COLUMN TEMPERATURE	130°C

Table 5.9 – *Operational conditions for the HP 6890 series II*

A new calibration has been found necessary every time the discrepancy with standard was exceeding the 5% of the desired amount.

Liquid phase calibration curve for propane is represented in Figure 5.3, where it could be appreciated the high linearity in the feedback from the FID.

5.6.5 Chlorinated solvent Analysis

The analysis of the chlorinated compounds has been performed by the μECD detector mounted on the same device described in the previous paragraph (the specifications have been shown in table 5.8).

The calibration have been carried on by standard at fixed concentration(ranging between 5μg/L e 5000 μg/L in liquid phase)in 60 mL of de-ionized water in 119 mL microcosms sealed with Teflon coated rubber septa.

Before every series of analysis, the calibration has been checked by a standard containing a defined amount of substrate (corresponding to the liquid concentration of 2.0 mg/L).

A new calibration has been found necessary every time the discrepancy with standard was exceeding the 5% of the desired amount.

Liquid phase calibration curve for propane is represented in Figure 3.3, where it could be appreciated the high linearity in the feedback from the μECD.

5.6.6 CO_2 and Oxygen Analysis

In order to evaluate the consumption of O2 and the generation of CO2 correlated with the consumption of main substrate, the headspace of the microcosms has been sampled and the analysis have been performed by VARIAN gas chromatograph via a TCD detector (Thermal conductivity Detector) and a packed column.

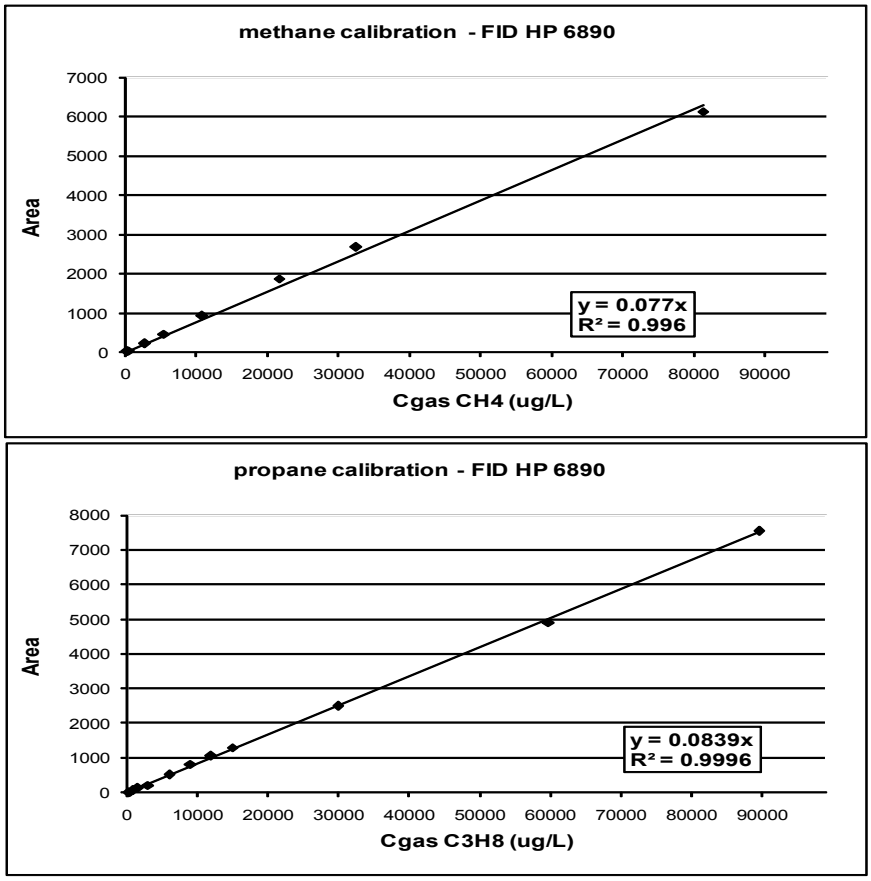


Figure 5.3 – *Substrates calibration curve*

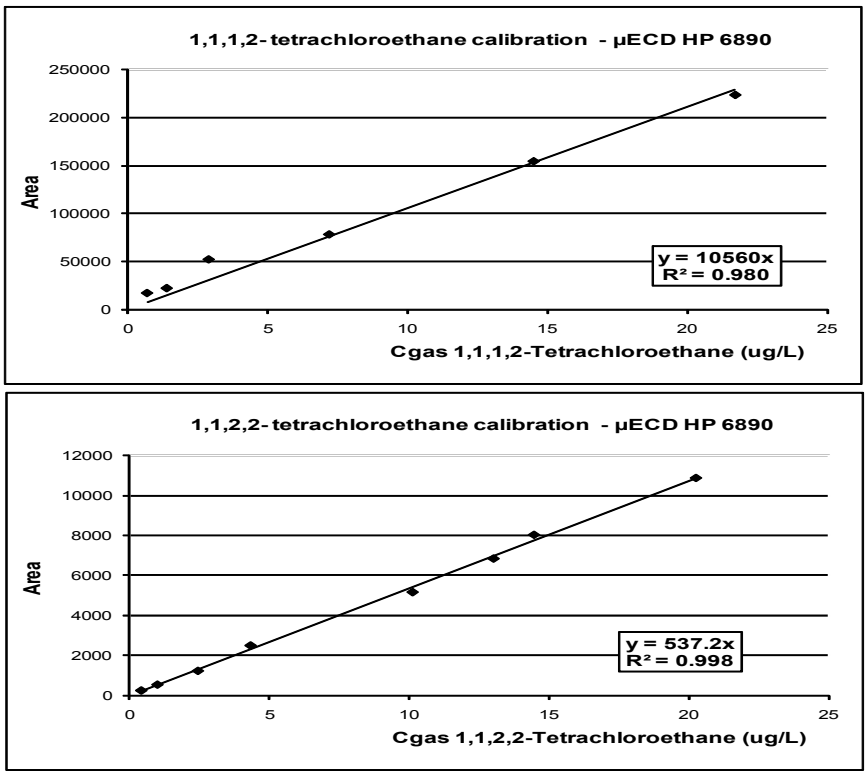


Figura 5.4 – *1,1,2,2- tetrachloroethane calibration curve*

The characteristics of this device and the method used have been shown in Table 5.10 whereas Figure 5.5 shows the temperature oven ramp.

DEVICE	VARIAN 3300	
COLUMN TYPE	Packed Column CARBONSIEVE SII SS	
COLUMN LENGHT	3 m	
DETECTOR	TCD	
INJECTOR TEMPERATURE	150°C	
FILAMENT TEMPERATURE	250°C	
DETECTOR TEMPERATURE	220°C	
TEMPERATURE OVEN RAMP SPECIFICATION		
INITIAL TEMPERATURE	60 °C	Hold for 5 min
RATE	10 °C/min	Until 220°C
FINAL TEMPERATURE	220 °C	Hold for 14 min

Table 5.10 – *VARIAN gas chromatograph configuration for O2 and CO2 analysis*

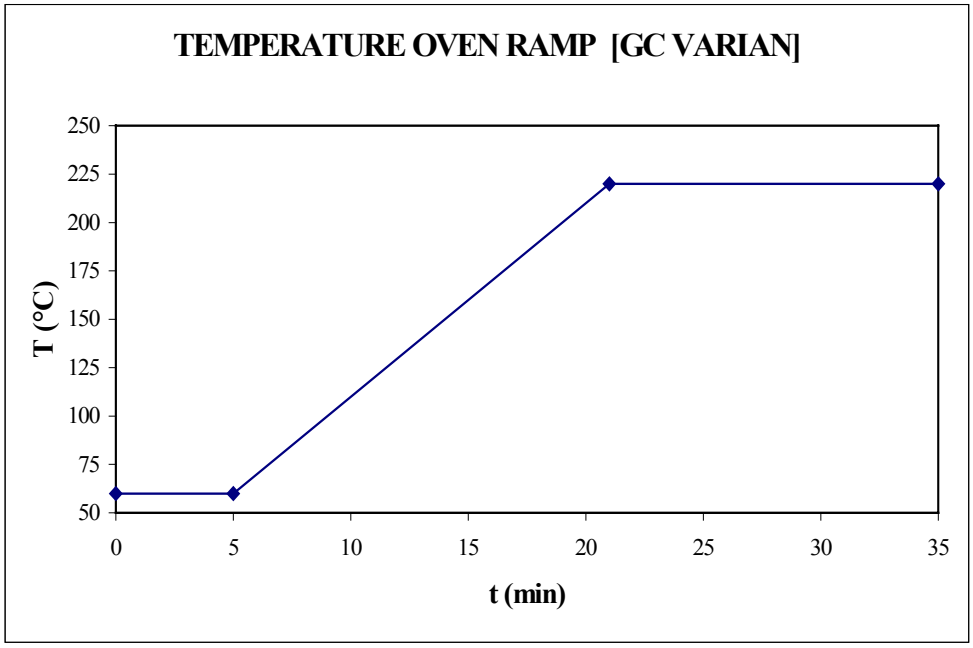


Figure 5.5 – *Temperature* oven ramp

5.6.7 Media preparation

In order to allow the growth and a quantification of the biomass during each step of the experimental work, several liquid and solid media have been selected and their composition has been shown in Table 5.11 and Table 5.12.

SOLID MEDIA COMPOSITION	
R2A (Difco, Detroit MI, USA)	
Yeast extract	500 mg/L
Caseine hydrolyzed	500 mg/L
Thiotone/Peptone	500 mg/L
Glucose	500 mg/L
Sodium Piruvate	300 mg/L
Na_2HPO_4	300 mg/L
$MgSO_4 \cdot 7H_2O$	50 mg/L
SOIL LB (Luria Bertani)	
Yeast extract	5 g/L
Tryptone	10 g/L
NaCl	10 g/L
Agar (gelificant agent)	15 g/L

Table 5.11 – *Solid media composition*

LIQUID MEDIA COMPOSITION	
MINERAL MEDIA	
K_2HPO_4	1550.4 mg/L
$NaH_2PO_4 \cdot H_2O$	739 mg/L
$(NH_4)_2SO_4$	105.2 mg/L
$NaNO_3$	765 mg/L
$MgSO_4 \cdot 7H_2O$	60.2 mg/L
$CaCl_2$	14.7 mg/L
MICRO-ELEMENT SOLUTION	
$FeSO_4 \cdot 7H_2O$	6283 µg/L
$MnCl_2 \cdot 4H_2O$	300.8 µg/L
$ZnSO_4 \cdot 7H_2O$	146.6 µg/L
H_3BO_3	61.8 µg/L
$Na_2MO_4 \cdot 2H_2O$	108.9 µg/L
$NiCl_2 \cdot 2H_2O$	23.8 µg/L
$CuCl_2 \cdot 2H_2O$	17 µg/L
$CoCl_2 \cdot 6H_2O$	23.8 µg/L

Table 5.12 – *Liquid media composition*

Whereas solid media contain source of carbon, mineral media is a minimum media and requires the adding of propane or methane as a main substrate and tetrachloroethane as a secondary substrate.

5.6.8 Biomass concentration estimation

The calculation of biomass concentration has been obtained using either the evaluation of optical density at fixed wave-length (660 nm) via spectrophotometric analysis or via plate count on R_2A after serial dilution.

The above mentioned technique, that is a method of estimating the viable population of a sample, is based on the serial dilution of the suspensions (with sterile water to give a range of concentrations from 1x10-4 to 1x10-8) sampled with a sterile syringe from a microcosm after 5 minutes centrifugation at 180rpm.

The serial dilutions (100μl of sample in 900 μl of sterile water containing 0.85% of NaCl) should be mixed thoroughly with a vortex mixer before each dilution to ensure that bacterial clumps are dispersed. Different pipettes should be used for each dilution.

A sample of each dilution is then spread on an empty sterile Petri dish containing the solid media and the plates with the inoculum are then incubated (30 ºC) for 48-36 hours.

After a selection of the plates containing between 10 and 200 colonies, is possible count, for each single species (based on the morphological aspect) the number of colonies present. From this count the viable population of the original sample can be calculated.

Biomass concentration could be then expressed as the logarithm of CFU (colony forming unit) on millilitre of suspension.

Whereas plate counting is suitable for liquid samples, when used for the slurry microcosm this method gives an estimation of the concentration of the suspended biomass (2-5 % of the biomass attached to the soil): under this point of view the value obtained could be assumed as an index of the variation on time of the biomass concentration.

5.6.9 Microcosms set-up and operational condition

Soil and water samples taken from each site have been stocked in container without headspace at1°C and homogenised before the microcosms set up. The brackish water contains 13 g/L of chloride (80% of the average concentration of chloride ion in seawater) and N-NH3 30mg/L.

Microcosms have been set up in 119 mL amber serum bottles gas tight sealed with teflon coated rubber septa in order to avoid loss of substrate, CO_2 and chlorinated compounds and, as described previously, they could be classified as:

"*slurry"* phase - Each microcosm contains 20 g of soil from the relative site and 50mL of brackish groundwater taken from the same site. When the latest was not available, the same volume of mineral media has been used to substitute the ground-water.

"liquid-gas" phase - Each microcosm contains 60 ml of sterile mineral media.

All the materials, glassware and bench have been previously sterilized via autoclave thermal treatment (40 minutes at 120ºC).

In every microcosm series, after the onset of methane or propane utilization, these substrates were supplied in consecutive spikes (5.4mL of methane or 1.85mL of propane, at a room temperature of 20–22 ◦C) corresponding to average feed rates of about 4mgC/week.

The typical trends for the microcosms are shown in Figure 5.6 where is possible to note the daily pulses of the main substrate (propane or methane) and stoichoimetric oxygen and the weekly pulses of the chlorinated compound at concentrations similar to those at the initial set-up. Each time the TeCA was completely degraded, it was re-introduced by spiking 25 µL of a sterile concentrated aqueous solution of 1,1,2,2 tetrachloroethane. During each pulse of pollutant, the value of TeCA lost as a result of the headspace samplings for GC analysis was estimated around 0.15% of the total amount depleted.

Substrates and the stoichiometric amount of oxygen has been added, as well as chlorinated compound, in sterile condition.

Ammonium (as NH_4Cl) and phosphate (as KH_2PO_4 and K_2HPO_4 at 0.65:1 weight ratio) were periodically added via sterile solution, so as to prevent N and P from being limiting factors for bacterial growth.

In order to renew the headspace, remove the exceeding CO_2 and co-metabolic toxic product, microcosms have been opened and stripped with filtered air under fume cupboard for 15 minutes.

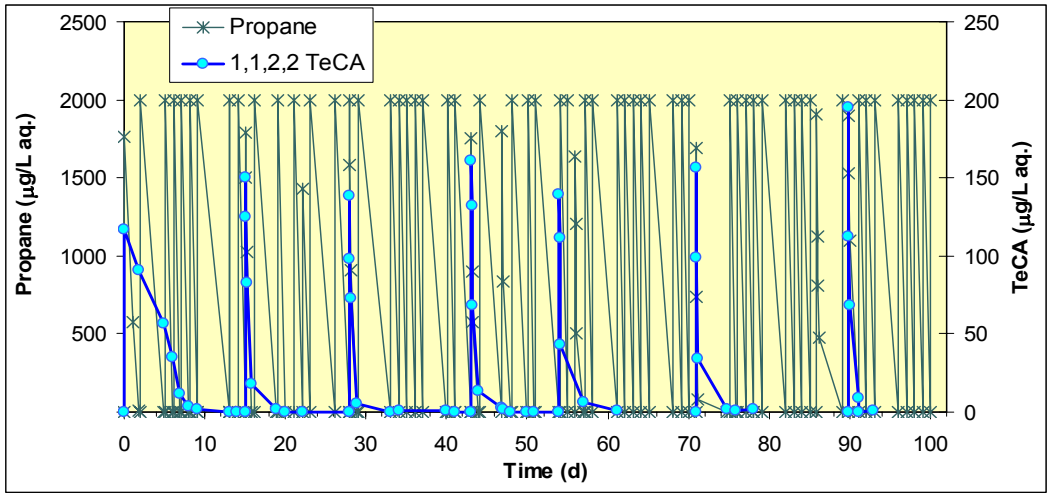


Figure 5.6 – *typical concentration profile for propane and TeCA in liquid phase in a slurry microcosm*

Microcosms were kept in continuous agitation in a roller operated at 3.3 rpm and maintained at 25±0.5 and were operated for a time variable between 60 and 450 days.

The lag-times for the onset of primary 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.

Each pulse was characterized by the maximum degradation rate relative to each substance, 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 uncertainties relative to average lag-times, degradation rates and total amounts degraded are reported as 95% confidence intervals.

5.7 Results and discussion

5.7.1 TeCA degradation and substarte consumption lag-times in slurry microcosms Series TM - TP

In a previous study, performed at the microcosm scale, with groundwater and soil from the same site but without the bioaugmentation treatment, a primary substrate (methane or propane) and TeCA lag-times of the order of several months and, in some cases, even longer than 1 (36-450 days) year have been observed.

In contrast, in this present work, obviously as a consequence of the bioaugmentation treatment, a drastic reduction of the TeCA and substrate lag times has been registered.

In every microcosms of both series the onset of the growth substrates have been noticed in few hours after the set-up operation, not dependently on the biomass and on the initial concentration of TeCA. Results show that lag times for the propane utilizing microbial culture are significantly lower than for the methane-utilizing culture.

The onset of VC degradation was characterized by a lag-time not longer than 7 days with value shorter two days for the propane utilizing biomass: in Figure 5.7 have been shown the average value of the lag times for the onset of TeCA degradation in every triplicate tests for each biomasses.

Results show that lag times for the propane utilizing microbial culture are significantly lower than for the methane-utilizing culture and the initial concentration of the chlorinated compound has no effect on the reduction of the

lag time for the methane utilizing biomass, whereas for the propane utilizing biomass, it seems that the lag time decreases with the concentration.

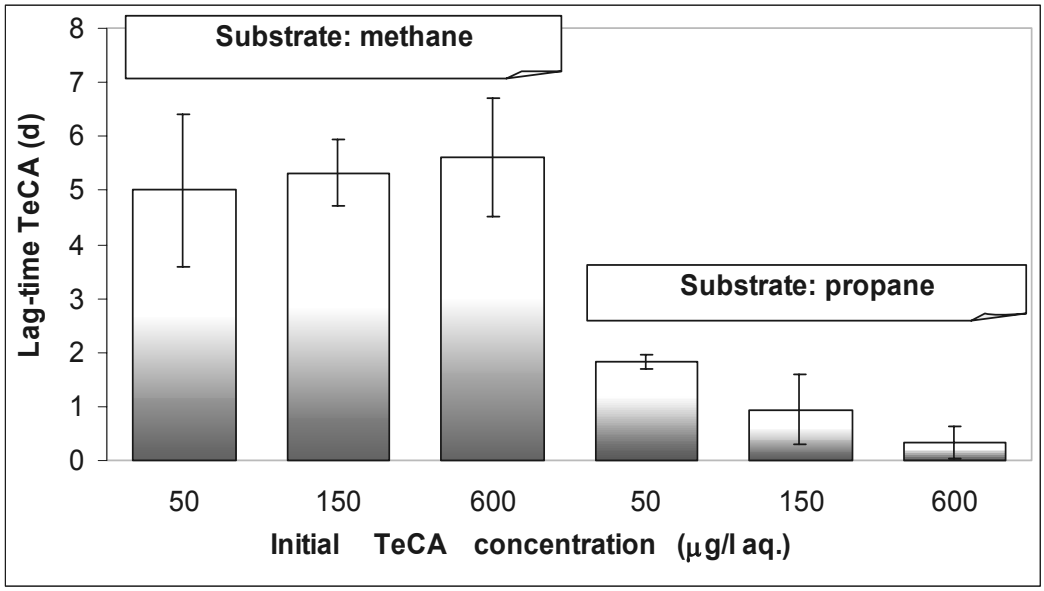


Figure 5.7 - *onset TeCA degradation Lag-times in slurry phase microcosms series TM (substrate methane) –TP (substrate propane)*

5.7.2 TeCA degradation rate and substrate consumption rate in slurry microcosms Series TM - TP

Figure 5.8 shows the trend, for each concentration of TeCA, of the degradation rate where the X-axis represents the mass of substrate consumed (and thus, also represent time).

During this series of experiment (lasting for more than one year for the propane utilizing biomass) has been noticed that the rate increases with time until a stable value is reached, which occurs after a long period of time (4months) and moreover the stable value reached by the propane utilizing biomass is more than twice that of the value reached by the methane utilizing biomass for each TeCA concentration.

Based on this experiment it's possible to assume that, in the TeCA concentration range tested, the "stable" value of the degradation rate reached for both substrates is a linear function of the initial pollutant concentration, excluding the toxic effect of the pollutant on the microorganisms selected and for each

concentration of TeCA tested, the long term degradation rate for the propane utilizing biomass was double than the methane utilizing.

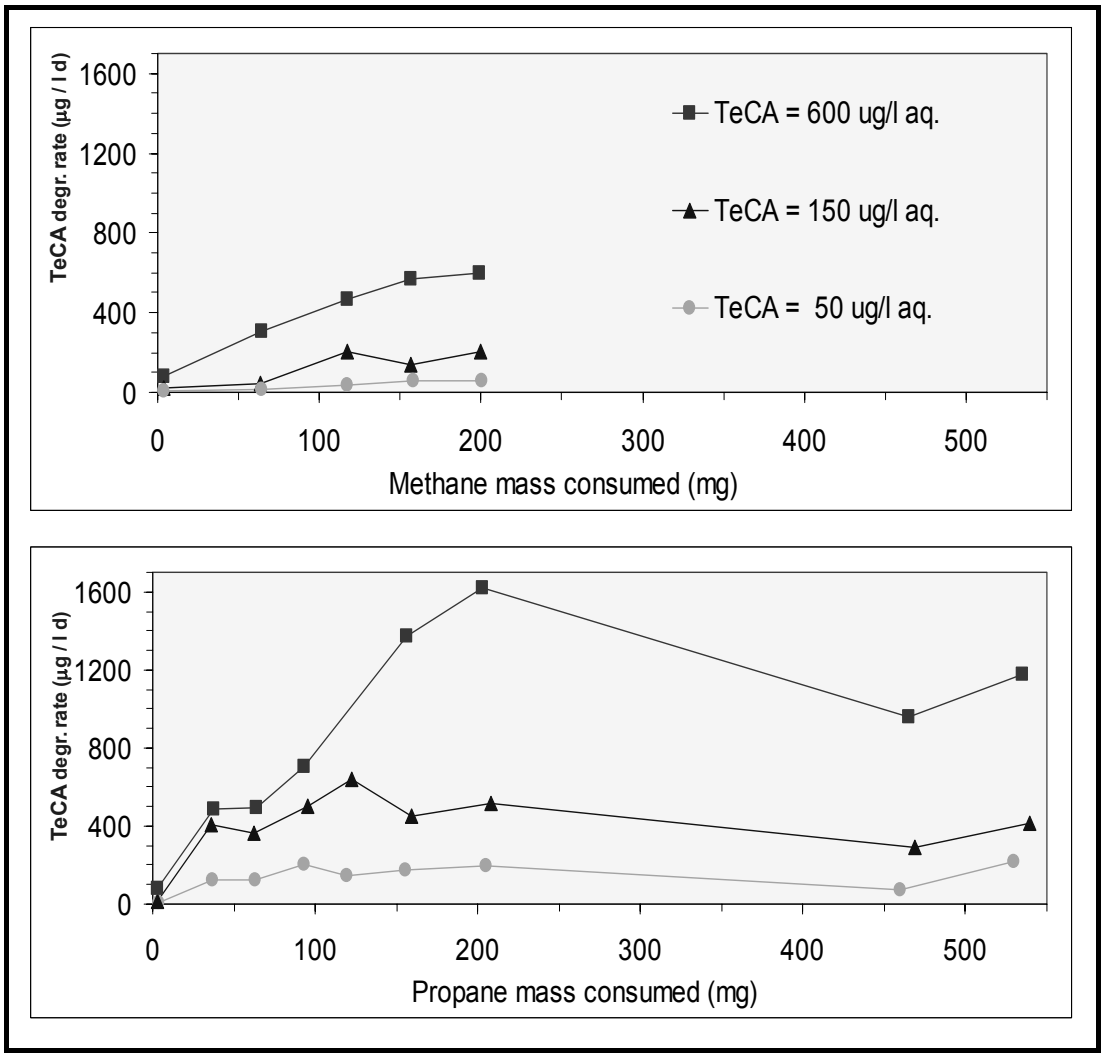


Figure 5.8 *-degradation rate for each initial concentration:methane utilizing biomass (TM) and propane utilizing biomass(TP): average values in each triplicate tests.*

Similarly the long term substrate consumption rate for the propane utilizing biomass was double than of the methane utilizing biomass (200 mg/(l d) for the propane utilizing biomass and 100 mg/(l d) for the methane utilizing) and this values have been not influenced by the initial concentration of TeCA in the subsequent pulses.

5.7.3 Bioaugmentation effectiveness tests in different soils in slurry microcosms: series ADMPR

During the microcosms tests series TM and TP better performances, in terms of lag-times and TeCA degradation rate, have been found for the propane utilizing biomass and this remarkable achievement suggested to focus the following part of the experimental tests on the propanotrophic inoculum.

In this second part of the experimental work, the effectiveness of the bioagmentation has been tested on five soils where aerobic cometabolic biodegradation of the inoculated propane utilizing biomass has been monitored at the initial concentration of 150 µg/L.

Results, shown in Figure 5.9 and in figure 5.10, indicate that in every soil tested, bioaugmentation allowed a significantly reduction of the lag-times related to the consumption of the growth substrate and to the TeCA degradation.

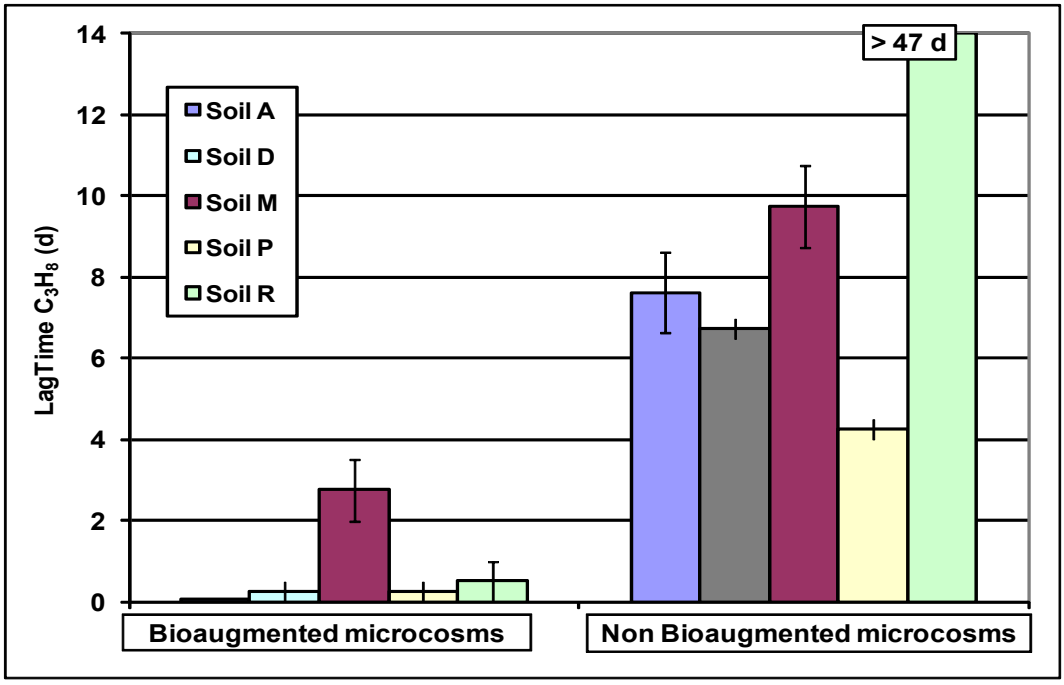


Figure 5.9 - *Propane consumption lag-times*

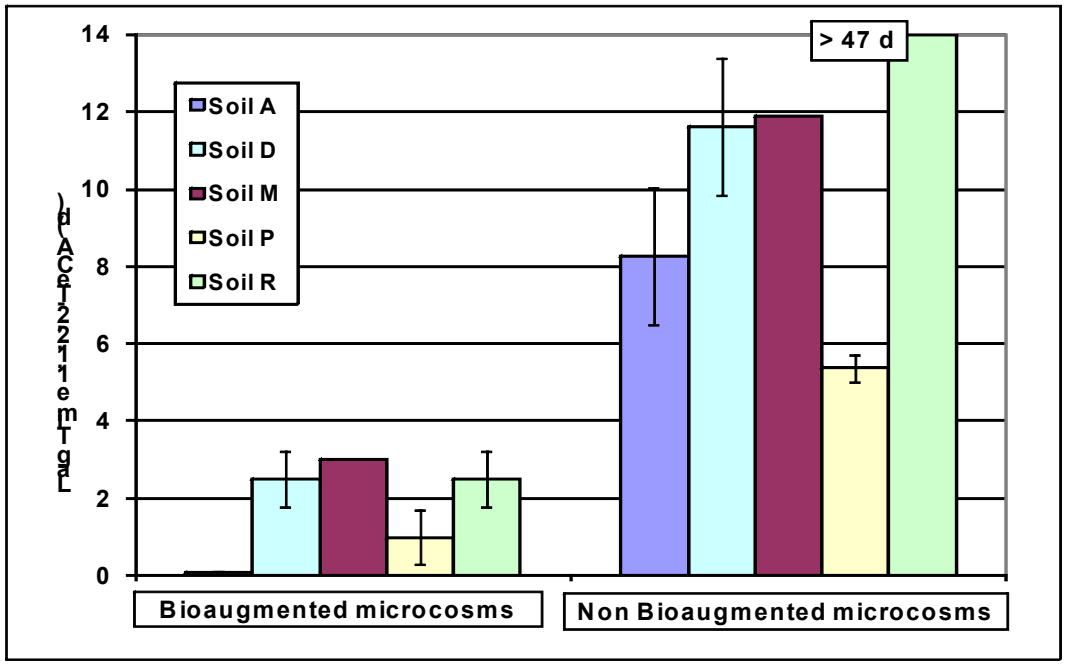


Figure 5.10 - *1,1,2,2-TeCA biodegradation lag-times*

Moreover the test carried on the soil R indicates that without the introduction of the inoculum of biomass, for the whole period of monitoring, no biodegradative process has started.

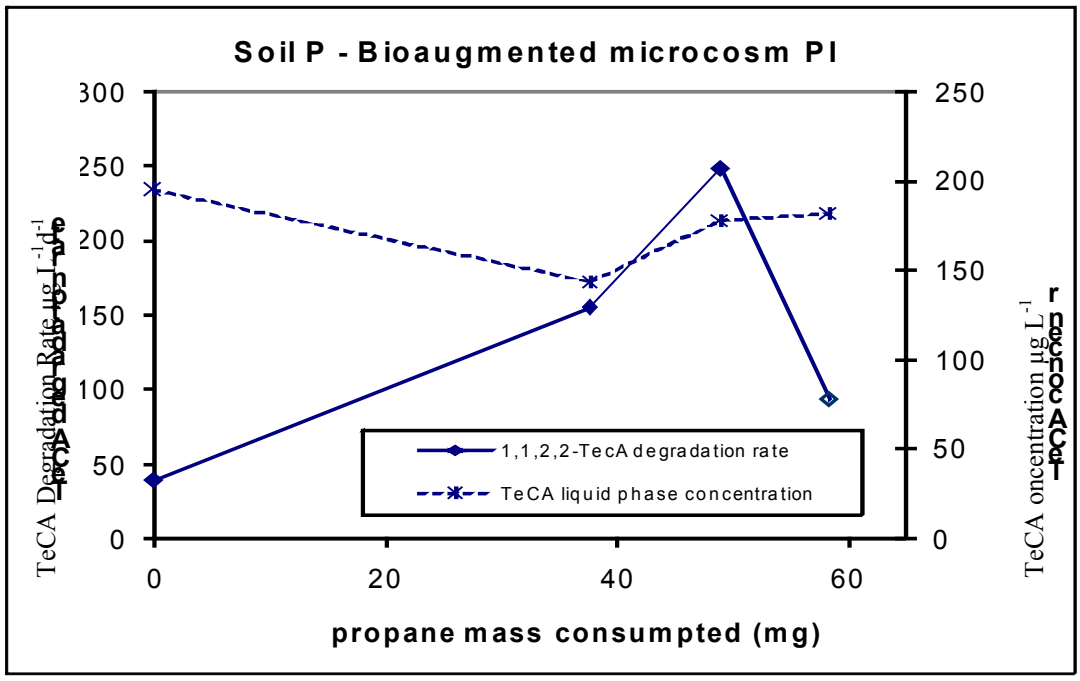


Figure 5.11 - *degradation rate of 1,1,2,2-TeCA in a bioaugmented microcosm (the last data point for the degradation rate have been obtained in absence of main substrate)*

Typical trend of the initial 1,1,2,2 TeCA degradation rate during subsequential pulses has been rappresented in Figure 5.11. for a bioaugmented microcosms and, in Figure 5.12, the different result obtained without inoculation of propane utilizing biomass.for a non inoculated could be appreciated.

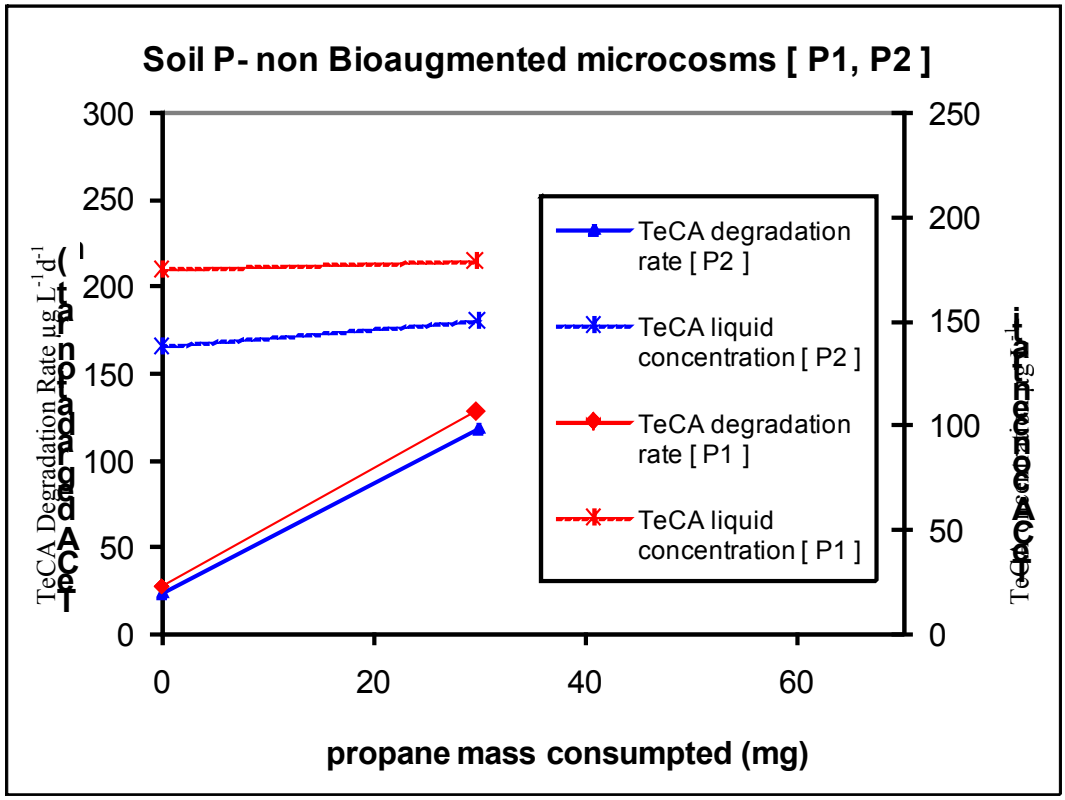


Figure 5.12 - *degradation rate of 1,1,2,2-TeCA in a non bioaugmented microcosm*

Based on the result obtained, the 1,1,2,2-tetrachloroethane degradation rate increase with the consumption of propane due to the increasing of the biomass into the microcosm.

Althought the degradation rates for chlorinated compound reach the same order of magnitude without dependance on the adding of inoculum, the bioaugmentation allows, during the same interval of time, significantly bigger amount of TeCA in comparison with the non inoculated tests.

Another interesting aspect of the bioaugmentation tests is related with the maximum TeCA degradation rate obtained in different soil.

Figure 5.13 report the values obtained taking account of the "hystory" affecting the soils tested: Soils A and M , previously contaminated by a mixtures

of several chlorinate compounds after bioaugmentation develop a biomass with better performances in terms of degradation rate in comparison with soil D, P and R never contaminated by CAHs before and this is more evident in Figure 5.14 where the average long term TeCA degradation rate at the concentration of 150 μg/L in each microcosms is reported .

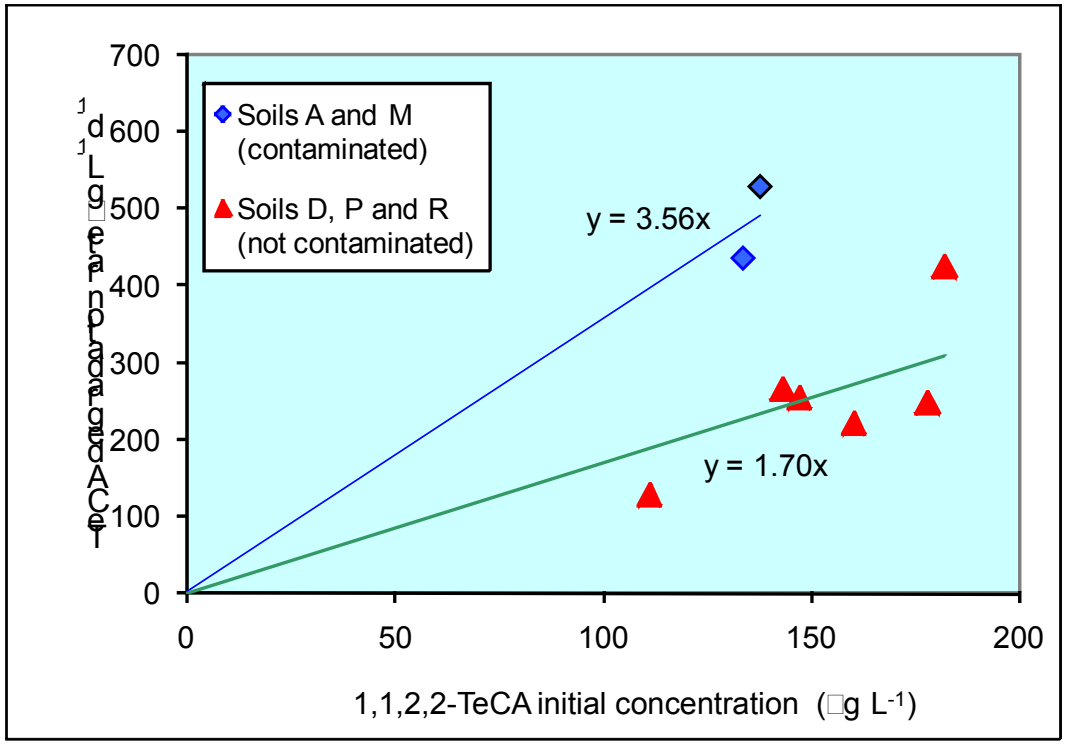


Figure 5.13 - *comparison between soils with respect on the previous "contamination hystory".*

Considering this result it's possible to appreciate a similarity in value**,** even if the biomasses growth attached to different soils starting from the same inoculums source.

Moreover the degradation rate registered for the soil M is very close in value to the one registered for the TP series because contain soil sampled in the close proximity of site M.

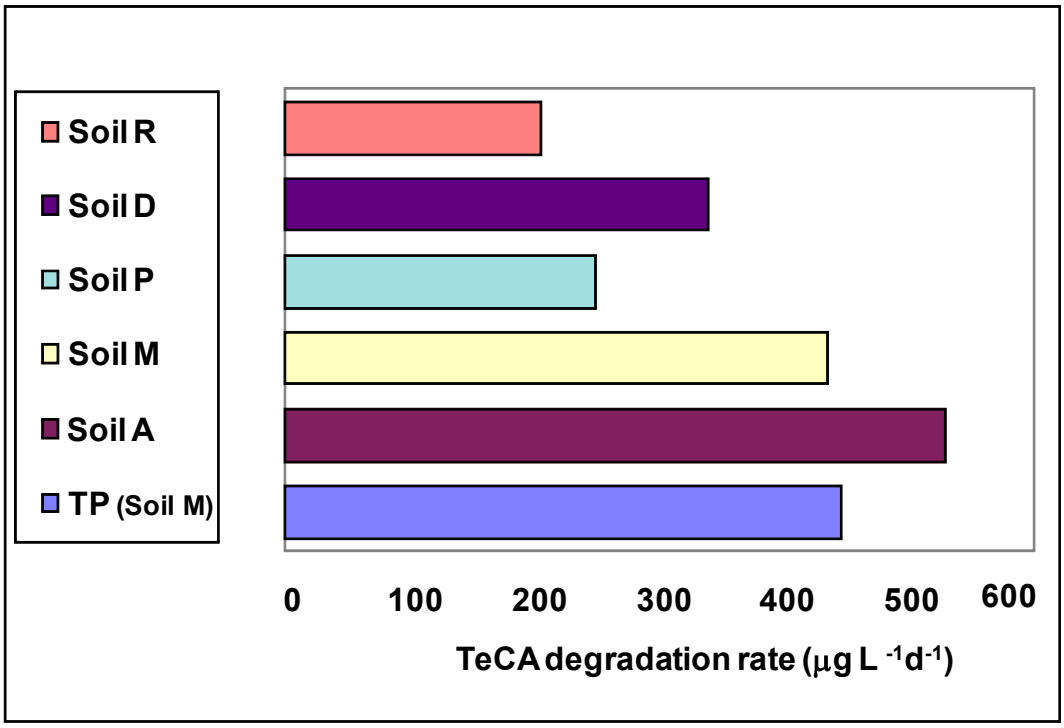


Figure 5.14 - *comparison betweenthe maximum degradation rate in different soils.*

5.7.4 1,1,2,2-tetrachloroetane high concentration tests: series TH

This paragraph describes the experimental work focused on finding evidence of a toxic effect due to the high concentration of the pollutant 1,1,2,2 tetrachloroetanne in the range between 900 and 2500 μg/L in liquid phase.

In order to describe the degradation rate of the biomass selected the model of Monod reported in Equation 5.7.1 and derived from 4.5.1 has been adopted:

$$R_{1,1,2,2-TeCA} = Q_{MAX}^{1,1,2,2-TeCA} \frac{C_{1,1,2,2-TeCA}[X]}{K_S^{1,1,2,2-TeCA} + C_{1,1,2,2-TeCA}} \qquad (5.7.1)$$

Where $R_{1,1,2,2-TeCA}$ is the TeCA degradation rate referred to the liquid phase (μg $L^{-1}d^{-1}$), [X] is the active microbial concentration (mg cells L^{-1}), $Q_{MAX}^{1,1,2,2-TeCA}$ is the maximum specific degradation rate (μg substrate (mg cells)$^{-1}$ d^{-1}), $K_S^{1,1,2,2-TeCA}$

is the the half-saturation constant for the TeCA (μg L^{-1}), $C_{1,1,2,2-TeCA}$ the cometabolic substrate concentration in liquid phase(μg L^{-1}).

Figure 5.15 shows the trend of the degradation rate on pollutant concentration for data points collected during the TP and the TH series experiments: the values fit with a good confidence a linear trend-line and this results allows to consider the range investigated in this thesis 50-2500 μg/L as the low concentration range of the Monod equation (5.7.1) where the reaction rate is linear in the pollutant concentration and to exclude the effect of the toxicity due to high pollutant concentration.

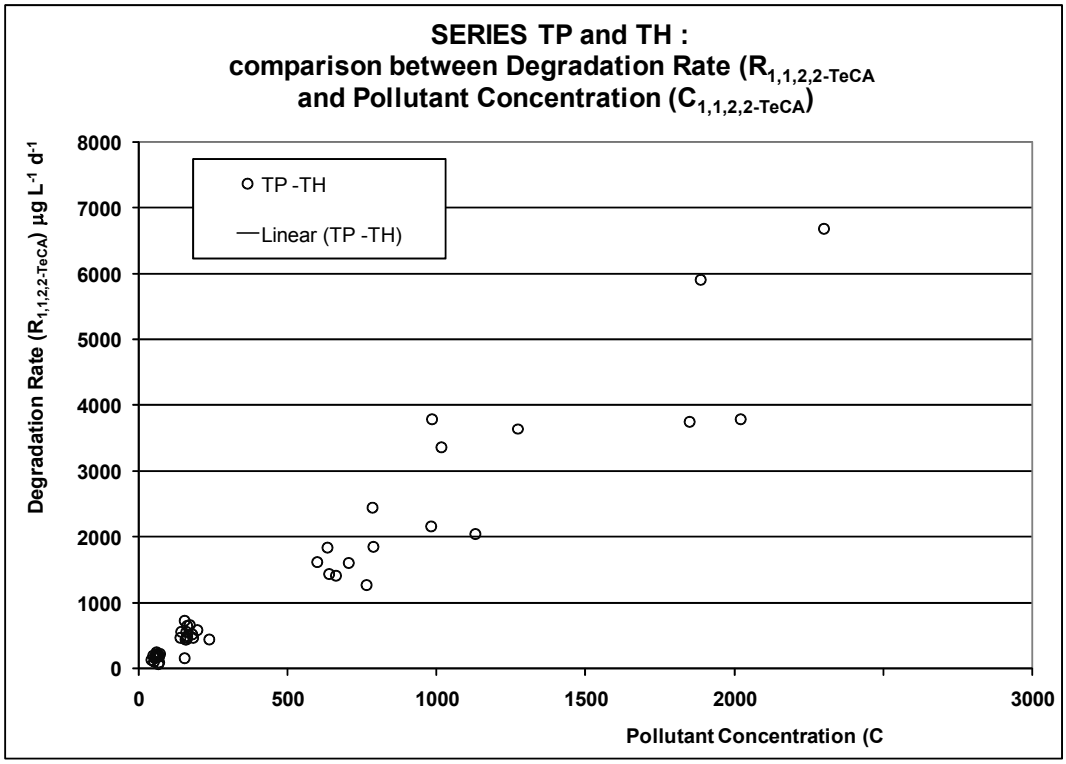


Figure 5.15 - *comparison between 1,1,2,2-TeCA degradation rate and pollutant concentration.*

5.7.5 1,1,1,2-tetrachloroethane degradation tests: series TI

To give a short background on this chlorinated haliphatic compound, 1,1,1,2-tetrachloroethane is an intermediate in one process for the manufacture of trichloroethylene and tetrachloroethylene and has been reported to occur as an impurity in these widely used products. It has been detected at low levels in ambient air and in drinking-water.

Even if no epidemiological data relevant to the carcinogenicity of 1,1,1,2-tetrachloroethane were available,here is *limited evidence* in experimental animals for the carcinogenicity of this compound because it induced gene mutations, sister chromatid exchanges and aneuploidy, but not chromosomal aberrations, in rodent cell cultures and gene mutations in bacteria.

Based on these previous considerations, 1,1,1,2-Tetrachloroethane is *not classifiable as to its carcinogenicity to humans (Group 3)*.

In this explorative test the aim was to evaluate the capability of the biomass propane utilizing selected in the series TP on biodegradation of the isomer 1,1,1,2 tetrachlorotane at the concentration of 400 μg/L in liquid phase anfd if a change in performace (long terms degradation rate, lag times) could occour because of the gene mutation induced by this compound.

Typical concentration profiles for substrate (propane) and 1,1,1,2 TeCA has been reported in Figure 5.16 where is possible to notice the operational condition similar to the ones adopted in the previous step of the sperimentation: dayly pulses of substrate and stoichiometric oxygen ahve been granted and each time the TeCA was completely degraded, it were re-introduced by spiking 25 μL of a sterile concentrated aqueous solution of 1,1,1,2 tetrachloroethane.

During the monitoring period the lag time for the onset o substrate consumption and Pollutant degradation in this series of microcosm have been shorter than two hours and the long term degradation have reached a stable average value of 200 $\mu g L^{-1} d^{-1}$ as shown un figure 5.17.

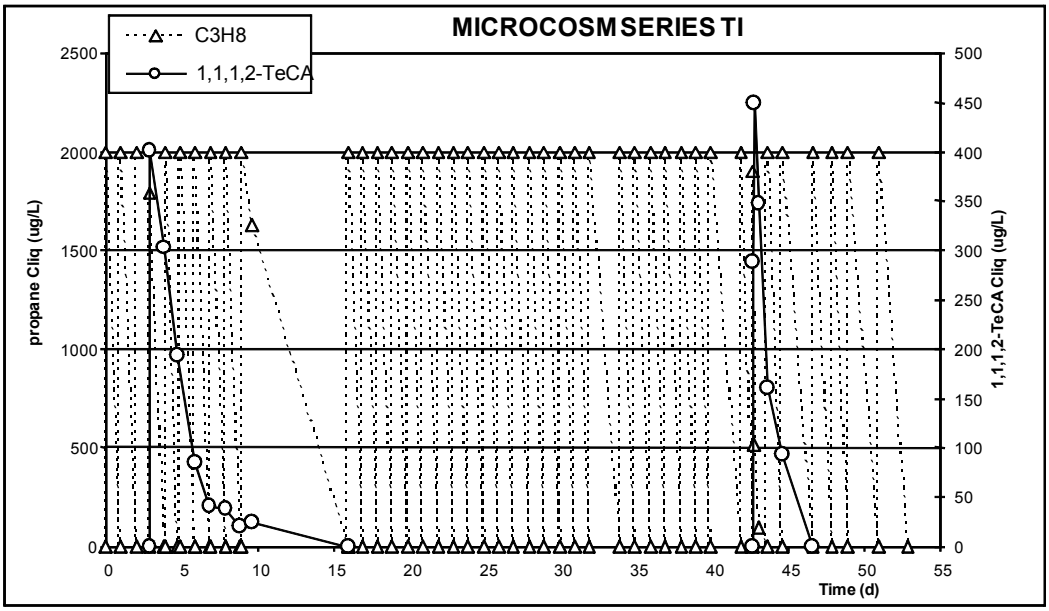


Figure 5.16 - *Propane and 1,1,1,2 tetrachloroethane concentration profile.*

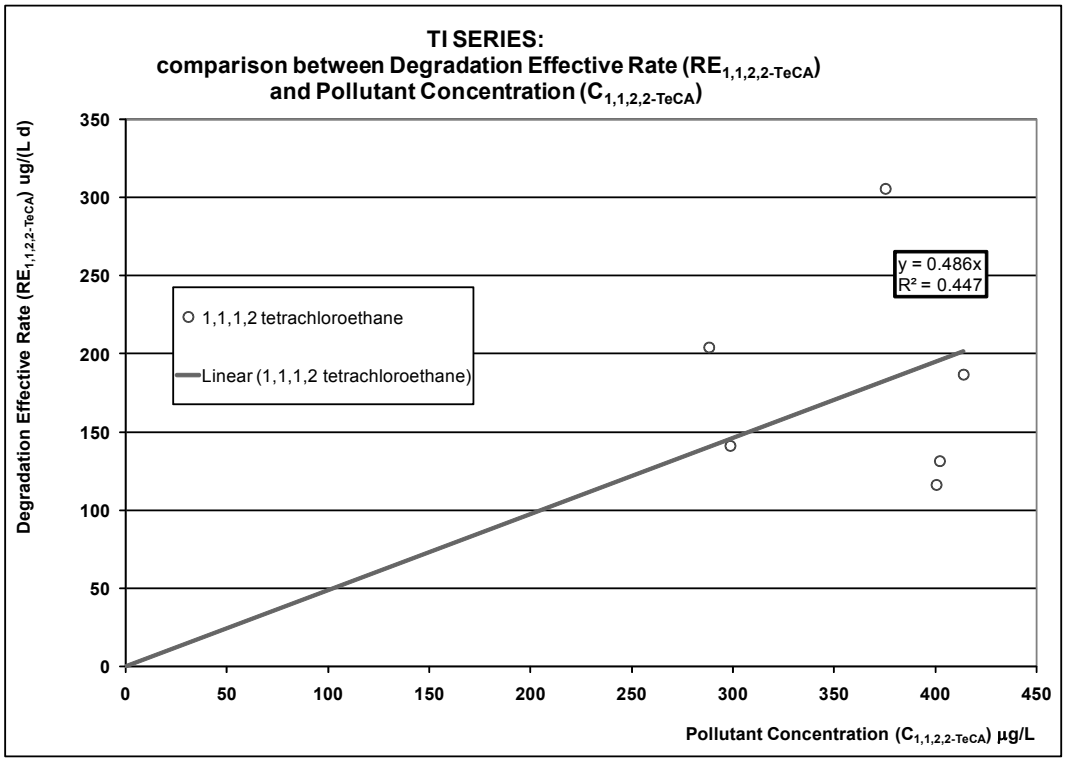


Figure 5.17 - *comparison between 1,1,1,2-TeCA degradation rate and pollutant concentration.*

5.7.6 Selection of the consortium in liquid-gas microcosms: Series LA and LB

In this section, as said previously, an inoculum of biomass was extracted from the microcosmsa containing propane utilizing biomass and let grow in four liquid-gas bioreactors in order to minimize the amount of soil present and to verify the maintainanace of the biodegradative activity.

Following this protocol a "diluition" a futher one has been subsequentially performed by sampling an inoculum from the first series of liquid-gas microocosms (LA) and injecting it into a second series (LB) of liquid-gas bioreactors: a scheme of the protocol used is represented in figure 5.18

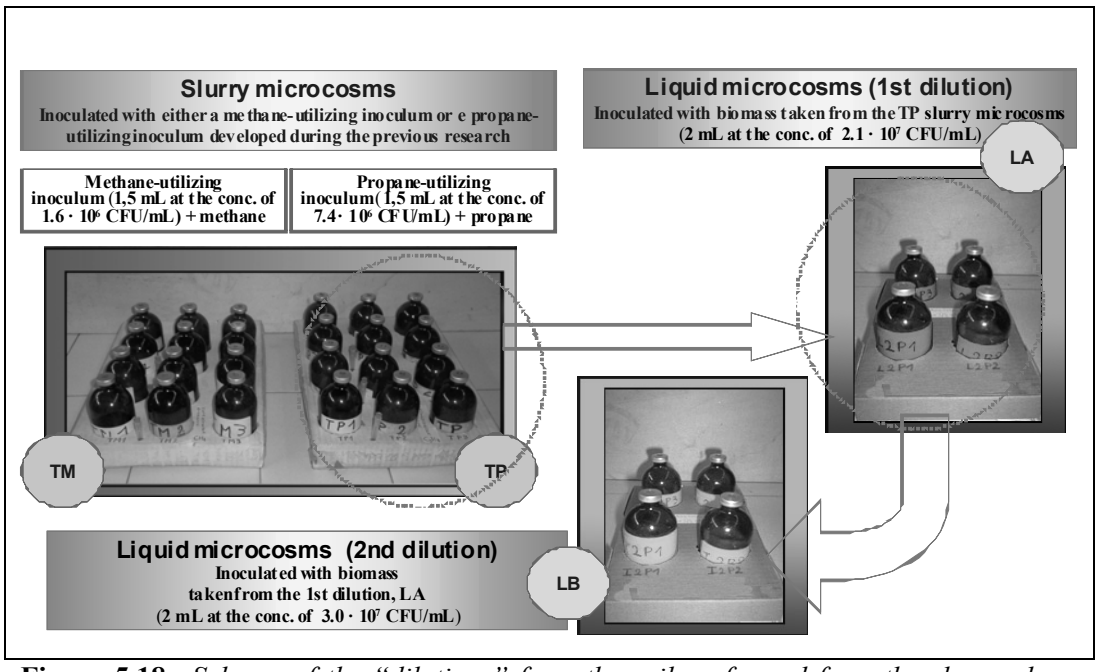


Figure 5.18 *–Scheme of the "dilutions" from the soil performed from the slurry phase microcosms series TP.*

Typical trends for the liquid gas tests are shown in Figure 5.19 where is possible to notice the daily pulses of propane and oxygen, and the weekly pulses of TeCA at the same concentration that were initially added to the bioreactors.

In both series, LA and LB, the immediate onset of propane degradation and TeCA lag-time shorter than two hours has been observed in all the quadruplicate tests.

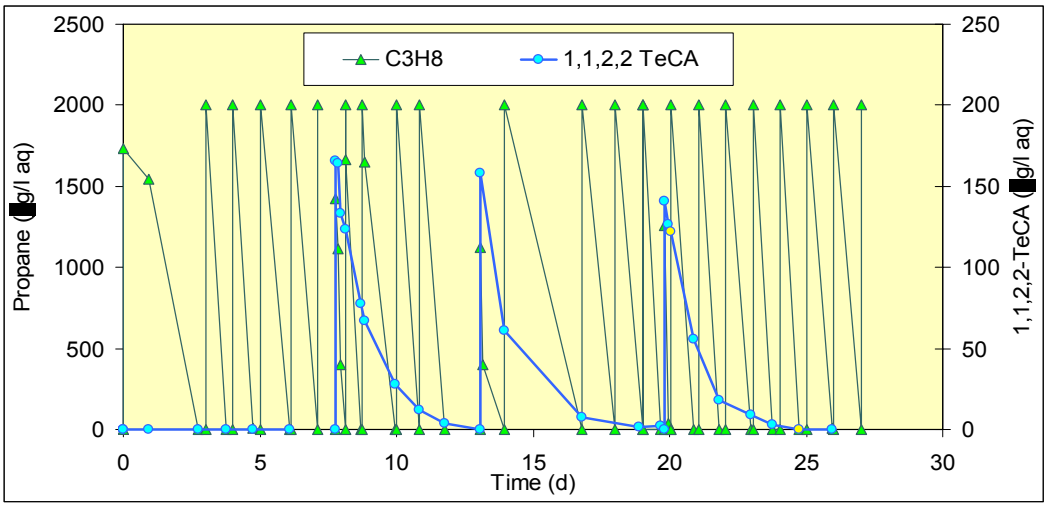


Figure 5.19 – *typical concentration profile for propane and TeCA in liquid phase in a liquid-gas microcosm*

The results indicate that the inoculation from slurry reactors and subsequent growth of the inoculated biomass led to the maintenance of the TeCA degradation capability.

In the graph represented in Figure 5.20 has been reported, for each kind of microbial culture, a comparison between the average value for the initial 1,1,2,2-TeCA degradation rate for the slurry microcosms at concentrations of 150 µg/L and the values obtained from both series of the liquid-gas microcosms.

The value observed for the propane utilizing biomass is significantly higher than the value for the methane utilizing biomass in slurry phase, whereas, paying attention to the fact that we are not estimating specific rates due to the fact that there are different concentrations of biomass in the two different bioreactors, the TeCA biodegradative process by aerobic cometabolism was also performed successfully in the liquid-gas microcosms, with a degradation rate of the same order of magnitude of the value obtained for the slurry bioreactors

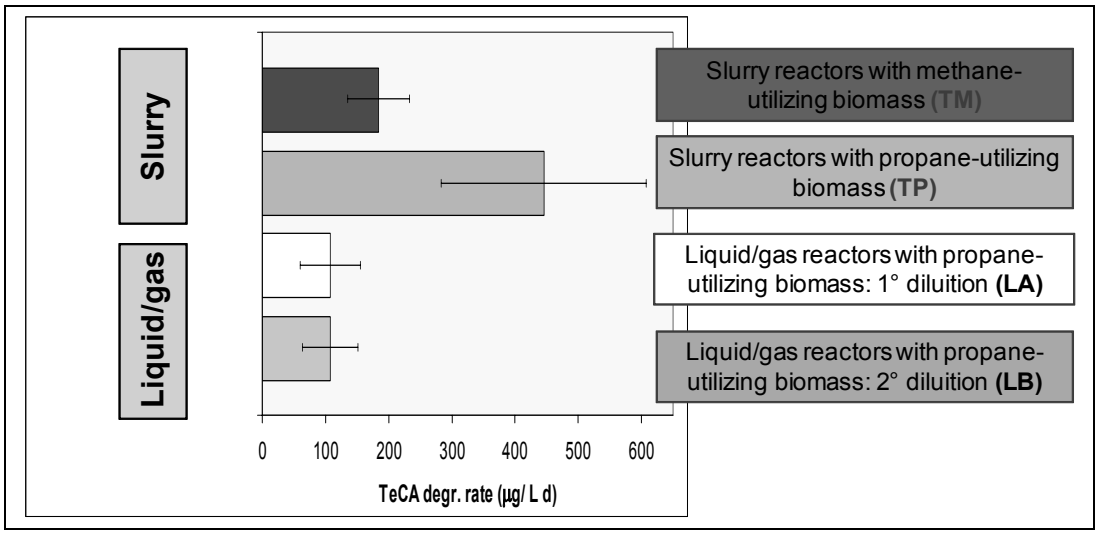


Figure 5.20 – *Comparison of the average 1,1,2,2-TeCA degradation rate obtained at 150 µg/L in the slurry (TM and TP) and in the liquid-gas (LA and LB) microcosms.*

5.7.7 Isolation of the pure strain from the liquid-gas microcosms

In collaboration with the Department of Evolutionary and Experimental Biology of the University of Bologna, using a sample taken from the second "dilution" from soil in liquid gas microcosms (LB), a small amount of active biomass has been selected in order to characterize the microbial consortium forming the previously called "propane utilizing biomass".

The aim it was to isolate the pure strain responsible of the biodegradative processes and verify the maintenance of this biodegradative activity after the isolation procedures.

Figure 5.21 represent the result of the isolation of the bacterial strain "*Rhodococcus*" on an agar plate.

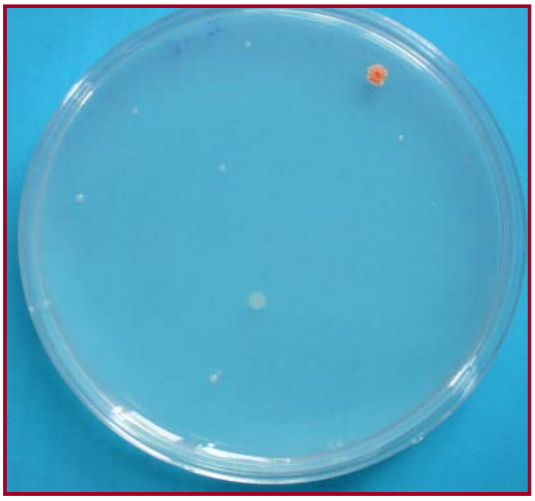


Figure 5.21 *–Agar plate with the isolated pure strain "Rhodococcus" visible as an Orange dot on the top right of the picture*

Pure strain tests have been performed in ***resting cells***, a special protocol were the cell investigated are non active by a mitotycal point of view (i.e. the amount of biomass could be assumed as a constant value), but active in the metabolic process of the chlorinated compound degradation.

This methodology , that allows the extimation the specific degradation rate for the pure strain, has been used in a test performed in aerobic condition in liquid gas microcosms containing 20 mL of mineral media with an headspace of 19 mL, continuosly agitated and at 25°C.

The initial concentration of 1,1,2,2- TeCA investigated in liquid phase was 200 mg/L, the selected substrate was, obviously, propane and the tests have been performed in triplicate whit two controls containing thermally inactivated inocula and one abiotic control.

Typical trend of the concentration profile of 1,1,2,2 tetrachloroethane is represented in Figure 5.22 and , based on this method, the estimated degradation rate for the pure strain has been evaluated in **5 mmol L^{-1} d^{-1}** with a transformation capacity for the pure strain *Rhodococcus* of **2.01 mmol $_{degraded\ TeCA}$ $mg^{-1}_{deactivated\ protein}$** .

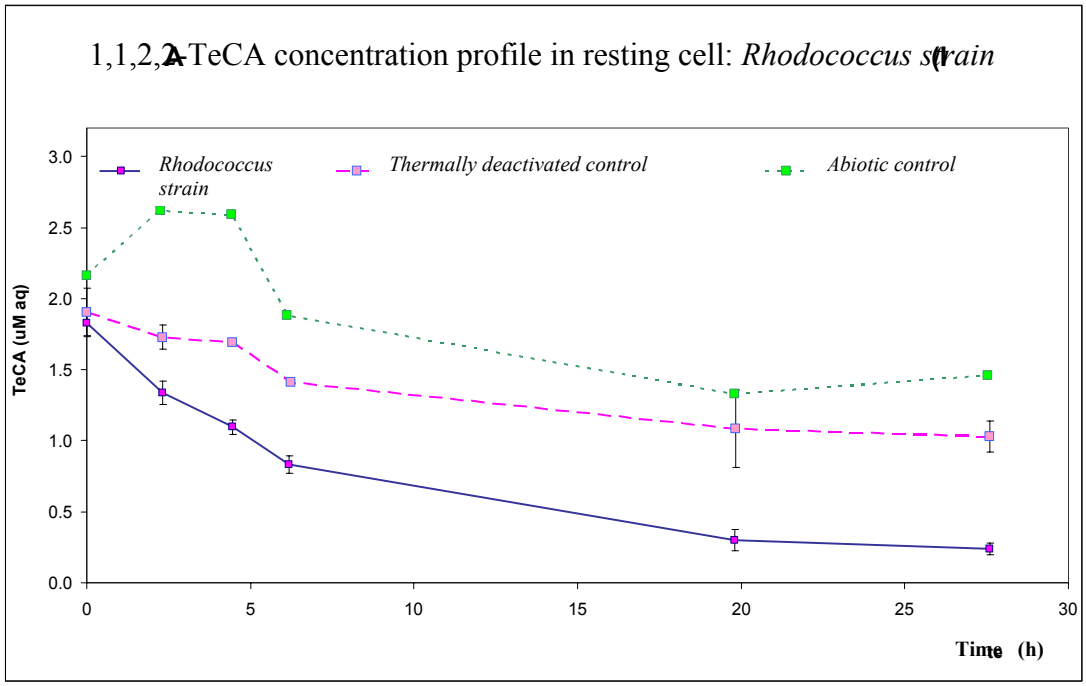


Figure 5.22 –*typical trend of the concentration profile in a resting cell test.*

5.8 Conclusion

The results of this study indicate that:

1,1,2,2 tetrachloroethane can be biodegraded via aerobic cometabolism by means of both methane-utilizing and propane-utilizing biomasses whereas an explorative tests carried only with the biomass propane utilize proved the possibility to biodegrade via aerobic cometabolism the isomer 1,1,1,2 tetrachloroethane.

The biodegradative cometabolic process of the 1,1,2,2- tetrachlorothane can be effectively maintained for several months in slurry reactors without any apparent sign of toxic effects on the biomasses in the range of concentration tested for the pollutant (50-600 µg L^{-1}) and the propane utilizing biomass has been proved to maintain biodegradative capability for several months at increasing concentration of pollutant in the range (900-2500 µg L^{-1})

In the aquifer studied, the addition of a suitable inoculum of biomass methane utilizing or propane utilizing induced a drastic reduction of the lag-times required for the onset of TeCA degradation (from several months to a few days). The biomass propane utilizing has been tested in different soils and, even if in the majority of the soil investigated the cometabolic biodegradative process has started in less than 12 days without dependence on the injection of the inocula, the bioaugmentation allowed a sensible reduction in terms of lag times for the onset of the depletion of the pollutant.

The best results were obtained with the propane-utilizing biomass with

- long-term 1,1,2,2-TeCA degradation rates of 1.2 – 1.4 mg L^{-1} d^{-1} at 0.6 mg L^{-1} in the liquid phase
- highest 1,1,2,2-TeCA degradation rate of 6.0 - 6.5 mg L^{-1} d^{-1} at 2.5 mg L^{-1} in the liquid phase
- 1,1,1,2-TeCA degradation rate of 200 μg L^{-1} d^{-1} at 0.4 mg L^{-1} in the liquid phase

The transfer of inocula from slurry reactor to sterile liquid/gas reactor and the subsequent growth of the inoculated biomass led to the maintenance of the TeCA degradative capacity. Positive results obtained from the liquid-gas tests demonstrates that, starting from few millilitre of propane utilizing biomass from the slurry microcosms, an high amount of inoculum could be generated without presence of soil via growth bioreactor. No evidence of decreasing in biodegradative performance has been observed during the diluition from the soil and the degradative activity has been granted in resting cell test even after the isolation of the pure strain *Rhodococcus*.

Chapter 6

Stability of an Oil-Absorber-Bioscrubber System During Biodegradation of Sequentially Alternating 1,2-Dichloroethane and Fluorobenzene in Waste Gas

The work in this chapter and part of the data elaboration has been realized in collaboration with Doctor Michalis Koutinas. Chapter 6 contains results, discussions and comments of both authors and some parts are from a co-authored paper that is as yet unpublished. All the tests have been performed using the facilities of the Imperial College London under the supervision of Professor Andrew G. Livingston

6.1 Abstract

The aim of the second part of this was to study was to study the microbial strain dynamics in a novel absorber-bioreactor system dealing with sequentially alternating loads of inhibitory pollutants in waste gas streams undergoing biological treatment the bioreactor.

The bioscrubber, as a biological waste gas treatment technology, has been operating downstream to an absorber acting as buffer for dynamic conditions, such as i) shock loadings, ii) starvation periods and iii) sequentially alternating pollutants.

The results showed the beneficial effect of the absorber on maintaining an active microbial culture during sequentially alternating loads of DCE and fluorobenzene (FB) [122].

The stability of the OAB system was then compared to that of a Bioscrubber Only (BO) system when each was subjected to dynamic pollutant loadings.

Results obtained with Fluorescence in situ Hybridisation (FISH) indicate that significant changes occurred in the physiological states of the microbial culture during the dynamic conditions on both systems, and the communities formed were also highly dynamic. Also, any negative effects of dynamic loading on the microbial culture were significantly reduced with the use of the absorber in the process.

The results showed that the OAB system offers an effective solution to the biological treatment of waste gas during dynamic loading conditions [122].

"6.2 Sequentially Alternating Pollutants (SAP)

A critical characteristic of industrial waste streams [122], is the random variations in the VOC concentration profiles. Although past studies have extensively described the responses of biological systems to changes in VOC concentration, there has been limited work performed to study responses during periodic switches in carbon source (Ferreira Jorge and Livingston, 2000a).

This extreme scenario of sequential changes in the waste stream chemical composition is reported in the present thesis as "sequentially alternating pollutants" (SAP), and describes the case when the pollutant composition is alternating sequentially from one set of compounds to another over cycle periods of days or weeks.

The first study introducing a waste stream with a sequentially alternating composition of pollutants in a bioreactor was performed by Goodall, et al. (1998).

The biodegradation of an alternating sequence of two nitrobenzoate isomers (meta- and para-nitrobenzoic acid) by two specific microbial strains in a series of immobilised cell airlift reactors, was investigated in this work.

During the period when the specific substrate was not present in the process inlet, the microbial strains were retained in the system through immobilisation into sodium alginate-beads.

However, apart from the physical retention of cells in the bioreactor, the two strains were also maintained active during the non-supply period, due to the

presence of a common intermediate produced from the catabolic pathway of the two isomers.

Two different bioreactor configurations were tested, either immobilising both microbial strains into the same beads or restricting each strain immobilisation to different beads[122].

The comparison of the two configurations showed that when the microbial strains were immobilised into the same bead, the system could respond faster to the re-introduction of each isomer, due to interactions occurring between the two microorganisms in the co-immobilised system.

Furthermore, the same group of researchers developed a mathematical model to describe the co-immobilised configuration in a separate study (Goodall and Peretti, 1998). The metabolic behaviour of the mixed co-immobilised culture was predicted, based on measurements of pathways and biomass activities of the two microbial strains. The model predictions were in agreement with the experimental results, indicating that when detailed mechanistic pathway information is not available, oxygen kinetics can be applied successfully to describe complex systems[122].

Ferreira Jorge and Livingston, (2000a) investigated the aerobic biodegradation of an alternating mixture of organic compounds in a CSTB. They used a mixed culture in suspension, consisting of Pseudomonas sp. JS150 and *Xanthobacter autotrophicus* GJ10 which utilised MCB and DCE respectively.

In the experiments performed, they aimed (i) to characterise the microbial dynamics resulting from the degradation of the alternating sequence of the two organic compounds and (ii) to enhance the CSTB performance [122].

During the experiments, there was no MCB accumulation observed when MCB was re-introduced to the CSTB; though, GJ10 could not deal with DCE for the first few hours after the pollutant was re-introduced to the system and additional re-acclimation periods were required. The authors concluded that JS150 cells were maintained more active in the CSTB when only DCE was fed to the system, due to microbial interactions between the two species. A continuous flow of a ‘maintenance feed’ consisting of 12 % and 6 % of the total MCB and DCE

fed in the CSTB at steady state respectively, was introduced to the system to prevent the undesirable DCE accumulation[122].

After the addition of the maintenance feed, GJ10 responded faster to the transition from MCB to DCE, and DCE accumulated at lower levels[122].

Overall, the maintenance feed enhanced the CSTB performance when switching from MCB to DCE[122]. However, it was not a feasible solution due to the excessive amounts of DCE required to reduce the organic mass discharged to background levels.

For this reason, Ferreira Jorge and Livingston, (2000a) suggest the retention of the biomass in the bioreactor via immobilisation as a better option. Ferreira Jorge and Livingston, (2000b) also performed a follow-up study of their previous work, investigating the performance of an Extractive Membrane Bioreactor (EMB) challenged with sequentially alternating pollutant loads.

They compared the previous feeding strategy of the 'maintenance feed' to cell immobilisation, using the same model strain-compound system. The biofilm formed on the membrane improved the response of the system to changes in the composition of the wastewater[122].

Both strains were retained active on the membrane surface as a biofilm and this was explained due to microbial interactions in the biofilm during the degradation of the compound fed. This work showed that biofilms can maintain active concentrations of microbial strains independent of the compound fed to the system[122].

The studies presented above dealing with the SAP scenario in biological systems indicate that the greater the variability of pollutants in the waste stream, the greater the constraints of bioreactors treating such waste streams and highlight the need for a control strategy in order to enhance the performance of bioreactors fed with waste streams containing SAP .

6.3 Fluorinated Organic Compounds

Fluorinated compounds currently have various applications in the agricultural, pharmaceutical or other industries, due to a series of useful chemical and physical characteristics (Natarajan, et al. 2005). The carbon-fluorine [122] bond has one of the largest bond energies in nature (eg. CH_3-F, 116 kcal mol^{-1}), contributing significantly to the stability of fluorinated molecules.

Furthermore, for many synthetic fluorinated organics their stability is probably related to their unique molecular structure, which is unlike anything currently known in nature (Key, et al. 1997). However, fluorine chemistry is almost entirely synthetic and fluorinated chemicals are considered as xenobiotic compounds.

Although fluorinated organics can support the growth of microbial cultures as a sole carbon and energy source (Carvalho, et al. 2002; Carvalho, et al. 2005), most of the research curried out for the biotransformation of halogenated organics has been focused on brominated and chlorinated organic (Janssen, et al. 2001).

Also, the reason why less attention has been given to the environmental fate of fluorinated organics, is that they are regarded as more inert biologically, and so are expected not to have a major impact on human and environmental health. However, as discussed by Key, at al. (1997) fluorinated organics can cause a series of significant biological effects.

Due to the fact that limited research has been performed up to date on the biotransformation of fluorinated organics, it would be very interesting to focus our research on these compounds as well. Fluorobenzene (FB) is a recalcitrant fluoroaromatic compound persistent in air that is mainly used as an insecticide, as a solvent in the pharmaceutical industry, as a reagent for the production of plastics and resin polymers and as a starting material for the industrial production of drugs (Carvalho, et al. 2006).

The biodegradation of FB has received very little attention and only recently has complete mineralization by a microbial consortium been achieved (Carvalho, et al. 2002). Furthermore, Carvalho, et al. (2005) reported the isolation of a pure

bacterial strain, possibly belonging to a new class related to the order Rhizobiales (strain F11), capable of FB degradation as a sole carbon and energy source. Finally, strain F11 has been recently applied to the removal of FB in an up-flow *fixed bed reactor (Carvalho, et al. 2006)* [122].

6.4 Objective

Because the Oil-Absorber-Bioscrubber configuration can successfully retain an active microbial community during starvation periods of pollutants [122] and, with its smoothening effect when dealing with variations in the process inlet concentration, the absorber might be a successful alternative in overcoming the unfavourable effects caused by SAP conditions during treatment.

The strain-compound model system chosen for the experiments presented below was *Xanthobacter autotrophicus* GJ10 able to degrade DCE, and strain F11 able to degrade FB. This model system was chosen because:

1) each of the microbial strains is capable of metabolising only one of the organic pollutants used,

2) the two microbial strains and the FISH protocol followed for the identification and monitoring of the two microbial strains were available within our research group[122].

The objectives of the work presented in this Chapter was to investigate the response of the microbial culture, containing the specific degrading strains Xanthobacter autotrophicus GJ10 and strain F11, during the sequentially alternating DCE and FB loads in both systems, Oil Absorber Bioscrubber (OAB) and Bioscrubber Only (BO), by monitor the dynamics of the microbial culture using FISH detected by fluorescent microscopy (general stains as DAPI and PI have been used to distinguish difference in the physiological states of cells).

6.5 Materials and methods

6.5.1 Cultivation of Microorganisms

Subcultures of *X. autotrophicus* GJ10 (ATCC no. 43050) and strain F11 were grown in mineral medium containing 200 mg L^{-1} DCE, incubated at 30 °C and used for bioscrubber inoculation. The composition of the mineral medium was 1360 mg L^{-1} KH_2PO_4, 2130 mg L^{-1} Na_2HPO_4, 3000 mg L^{-1} $(NH_4)_2SO_4$, 200 mg L^{-1} $MgSO_4.7H_2$ O, 5 mL L^{-1} trace elements solution (Table 6.1) and 1 mL L^{-1} vitamin solution (Table 6.2).

Chemical	Concentration [mg L^{-1}]
$CaCl_2$	530
$FeSO_4.7H_2O$	200
$ZnSO_4.7H_2O$	10
H_3BO_3	10
$CoCl_2.6H_2O$	10
MnSO4.5H2O	4
$Na_2MoO_4.2H_2O$	3
$NiCl_2.6H_2O$	2

Table 6.1 –*Composition of trace element solution.*

Chemical	Concentration [mg L^{-1}]
Biotin	12
Choline Chloride	1000
Calcium (D)-Pantothenate	1000
i-Inositol	2000
Nicotinic Acid	1000
Pyridoxine Chloride	1000
p-Aminobenoic Acid	200
Cyanocobalamin	10
Thiamine Chloride	1000

Table 6.2 –*Composition of vitamins solution.*

The trace elements and vitamin solutions were derived from Janssen, et al. (1984) [122].

6.5.2 Experimental Set-Up

The experimental set up used is presented in schematic diagram reported in Figure 6.1. The total flow rate of air influent to the system was 0.3 L min-1 consisted of three different gas streams (G1-3), giving a volumetric gas flow rate per bioscrubber volume of 0.2 min-1. Streams G1 and G2 were enriched with FB and DCE respectively by passing via a sintered glass sparger through two saturation vessels containing pure compounds, while stream G3 comprised air. Gas Direction I was followed when the absorber was not used and the waste gas was introduced directly to the bioscrubber.

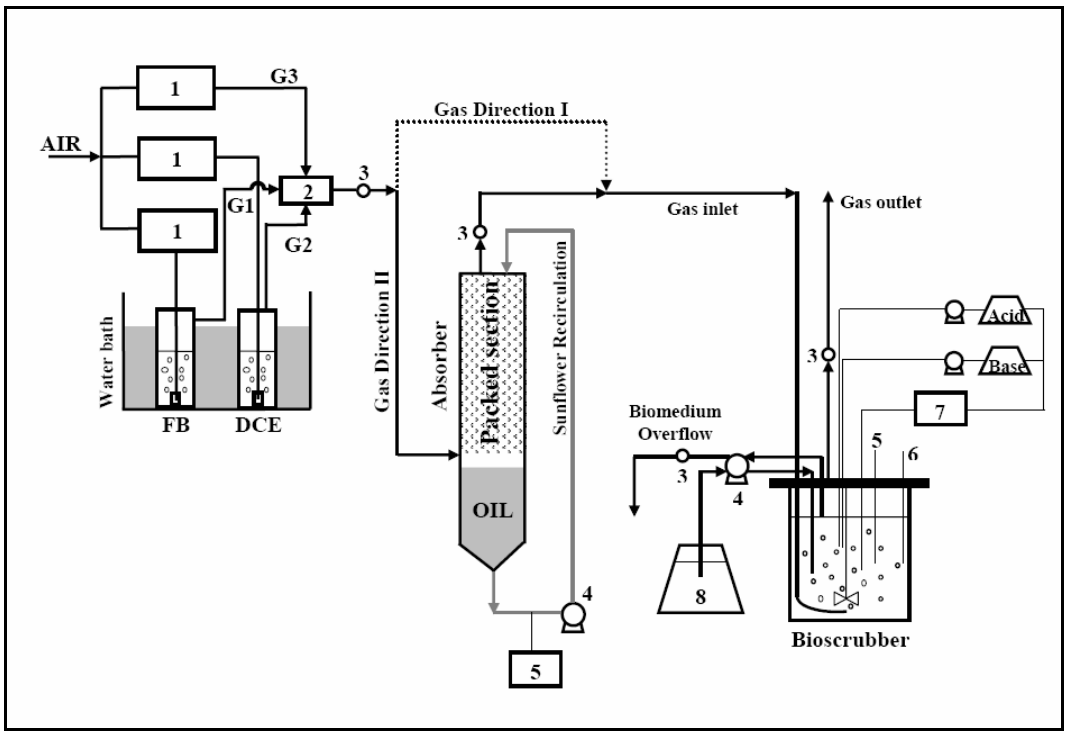


Figure 6.1 – *Experimental set-up. Gas direction I was followed when the waste-gas was introduced directly to the bioscrubber, bypassing the absorber. In gas direction II the waste-gas passed through the absorber and was fed to the bioscrubber. 1) mass flow controller; 2) mixing vessel; 3) sampling port; 4) pump; 5) temperature controller; 6) dissolved oxygen meter; 7) pH controller; 8) mineral medium; G1, G2, G3: gas streams;[122]*

The gas was distributed in the biomedium aqueous phase via a stainless steel sparger. Two impellers, rotating at 800 rpm (a 'marine' impeller at the bottom of the shaft and a Rushton impeller at the middle of the biomedium height), provided optimal mixing of the two phases.

The bioscrubber was an SGI "30/SET002" (SGE, France) model with a total volume of 1.8 L, which was operated as a continuous stirred tank bioreactor (CSTB) at 1,5 L working volume. The mineral medium was prepared in 10 L batches and has been fed to the bioreactor continuously via a Watson Marlow 205S (Watson-Marlow Bredel Products, UK) peristaltic pump giving a dilution rate of 0.023 h-1.

The pH was controlled at 7 ± 0.05 by the addition of 1M solutions of H_2SO_4 or NaOH, temperature was kept constant at 25 °C and the dissolved oxygen concentration was monitored with an Ingold (Mettler Toledo Ltd, UK) oxygen probe.

The absorber was a glass column (50 cm height, 5 cm i.d.) divided in two sections:

i) a packed section with a 27 cm high bed of pall rings

ii) an oil reservoir.

The gas stream inlet was between the two sections at 10 cm column height and the two streams (gas and oil) flowed counter-currently. The oil (Pure Sunflower Oil, Tesco Stores Ltd, UK) was recirculated via a Watson-Marlow 603S peristaltic pump at flow rate of 2 L min-1.

The temperature of the column was controlled via a temperature controller, utilising a thermocouple and a heating coil to maintain the column temperature constant at 30°C. The absorber effluent gas was fed to the bioscrubber at the same conditions as described above[122].

6.5.3 Chemicals

Al chemicals used were obtained from Merck (UK) and were of ANALAR grade. DCE and FB were obtained from Sigma (UK) 99 % pure[122].

6.5.4 Analysis

GC analysis was employed for determination of the DCE concentration in the gaseous and aqueous samples. An Agilent 6850 Series II Gas Chromatograph with an FID detector and a 'J&W Scientific' (Agilent Technologies UK Limited, UK) column with HP-1 stationary phase (30 m × 0.32 mm × 0.25 μm) was used.

For gaseous samples, 25 μL were injected into the GC and the temperature program run at a constant temperature of 30 °C for 8 min. However, the flow rate of the mobile phase (He) was reduced to 1 ml min^{-1} in order to achieve satisfactory separation between the peaks of DCE and FB.

Biomedium samples were centrifuged for 10 min at 13000 rpm and the supernatant solution was filtered through 0.2 μm filters to remove any remaining solids. 8 ml of the centrifuged sample was vortexed for 1 min with 2.5 ml of n-decane and finally 1 μL of the n-decane solution was injected into the GC.

For analysis of these biomedium samples, the GC column temperature was kept constant for 8 min at 30°C and then increased by 40 °C min-1 to 260°C. The coefficients of variation for 6 samples were 2.3 % for DCE and 3.7 % for FB at concentration levels of 7.6 mg_{DCE} L^{-1} and 1.1 mg_{FB} L^{-1}.

Carbon dioxide concentration of the bioscrubber gas effluent was determined using an isothermal GC (Shimadzu GC-14A, Shimadzu, UK) fitted with a thermal conductivity detector (TCD). Samples were injected to a Porapak N column packed with DVB (divinylbenzene)/vinylpyrrolidinone at 28 °C. The coefficient of variation for 5 samples was 2.6 % at a concentration level of 0.03 % v/v carbon dioxide.

Biomass concentration was determined by absorbance at 660 nm on a UV-VIS spectrophotometer (Spectronic Unicam, UK) interpolating from a previously established dry weight calibration curve. The coefficient of variation for 5 samples was 2.3 % at a concentration level of 0.5 $mg_{biomass}$ L^{-1}.

Total Organic Carbon (TOC) was measured with a Shimadzu 5050 (Shimadzu, UK) total organic carbon analyser. The biomass and any remaining

solids were removed from the biomedium with centrifugation and filtration as described before.

The samples were diluted with distilled water at an organic carbon concentration lower than 1000 mg L-1. The coefficient of variation for 3 samples was 0.5 %.

The anion and cation concentrations were also estimated. The analysis was performed with an Ion Chromatograph Dionex DX-120 (Dionex (UK) Ltd, UK) coupled to a Dionex AS40 automated sampler. The anion column was an IonPac AS14 (4×250 mm) and the carrier liquid 3.5 mM Na_2CO_3 / 1 mM $NaHCO_3$.

The cation column was an IonPac CS12A (Dionex (UK) Ltd, UK) and the carrier liquid was 19 mM CH_4O_3S (methanesulphonic acid). The anions that could be detected were: fluoride, acetate, chloride, nitrite, bromide, nitrate, phosphate and sulphate. The cations that could be detected were: lithium, sodium, ammonium, potassium, magnesium and calcium[122].

6.5.5 Initial Tests in Shake Flasks

Prior to the operation of the BO and OAB systems, the degradation capabilities of F11 and GJ10 for the two substrates were tested under different conditions. GJ10 and F11 were first inoculated in separate shake flasks containing 100 ml of mineral medium and 400 ml headspace under sterile conditions. 2 mM of FB (192 mg L^{-1}) were added in the GJ10 containing shake flask and 2 mM of DCE (198 mg L^{-1}) were added in the F11 containing shake flask.

Furthermore, two additional shake flasks were prepared containing the same ratio between mineral medium and headspace volume. This time both pollutants were added in each shake flask at a concentration of 2 mM each and one of the two strains was inoculated in each flask, in order to test whether the microorganisms could degrade their specific substrate under the presence of the second substrate[122].

6.5.6 Bacterial Population Analysis

A GJ10 specific oligonucleotide probe [5'-TGT GTG CAG GTC CAT TGC TG-3'] was used for the identification of GJ10 cells within the microbial community: it was labelled at the 5' end with Cy3 fluorochrome (Thermo Electron GmbH, Germany). For the identification of F11 cells an oligonucleotide probe [5'-TTT GGA GAT TTG CTA AGG G-3'] labelled at the 5' end with Cy3 fluorochrome was used.

The probes targeting the 16S rRNA were designed using Primrose computer software (Ashelford, et al. 2002). DAPI was used for determining the total number of cells present and PI, which is membrane impermeable and is generally excluded from viable cells, was used for identifying dead cells in the population. Propidium Iodide (PI) has been used for determining the dead cells in the population. Active cells were also measured using the EUB338I oligonucleotide probe.

For the FISH analysis, cells harvested from the bioreactor in 1 mL biomedium samples (diluted to OD~0.2) were centrifuged at 13000 rpm for 10 min and washed twice in 1 mL PBS buffer (1040 mg L-1 Na2HPO4, 332 mg L^{-1} NaH2PO4 and 754 mg L^{-1} NaCl)and 10 µL of 0.1 % (w/w) Igepal solution to permebilize the cells. The cells were then fixed for 1 h at 4 °C in 4 % paraformaldehyde and stored in a 1:1 mixture of ethanol and PBS.

Teflon coated slides with eight wells (VWR), were pre-coated with gelatine coating solution - 0.1 % (w/v) gelatine and 0.01 % (w/v) chromium potassium sulphate in hot distilled water (70 °C). 6 µL of the cell suspension were added to each spot of the slides. After air-drying, the slides were dehydrated in a series of increasing ethanol concentrations (50, 80 and 100 % (v/v), for 3 minutes each step).

A 9 µL aliquot of hybridisation buffer (900 mM NaCl, 20 mM Tris-HCl, 0.1 % (v/v) sodium dodecyl sulphate (SDS), 30 % (v/v) formamide) and 1 µL of GJ10 specific probe were applied on each spot. The slides were then placed to hybridise for 2 h at 35 °C in an equilibrated humidity chamber. After hybridisation, the slides were rinsed with distilled water and placed for 15 min in

washing buffer (112 mM NaCl, 20 mM Tris-HCl and 0.1 % (v/v) SDS) at 35 °C, then rinsed again with distilled water and air-dried. Prior to microscopic analysis, 9 μL of DAPI aqueous solution (1 mg L-1) were added on each spot for 2-3 minutes. For the "live-dead" cells analysis a similar procedure was applied.

However, the cells were re-suspended in a PBS buffer only. Igepal was not used to avoid additional permeabilization of the cells. 6 μL of the cell suspension were added to each spot of the slides and after the air-drying step, without any dehydration, DAPI (1 mg L^{-1}) and PI (0.3 mg L^{-1}) aqueous solutions (6 μL each) were applied to the spot for 2-3 min.

Finally, the slides were washed with distilled water, air-dried, mounted with Citifluor and analysed using an Olympus BX51 epifluorescence microscope (Olympus Optical Co., Germany) equipped with an attached Olympus DP 50 digital photographic camera. Images were acquired and analysed using specialised imaging software (AnalySIS – Soft Imaging System, version 3.2, Germany).

However, the two specific probes utilised the same fluorochrome. Therefore, each of the specific probes was added at separate wells in order to visualise one of the two strains under the microscope each time[122].

6.5.7 Two Sample t-Test

Quantitative FISH was used to monitor changes concerning the dynamics or the physiological states of the microbial population.

The significance of such changes was measured using a hypothesis or significance test [122], which shows if the difference between two statistical population means is significantly different.

The starting hypothesis is that no change is apparent in the means of the two populations, while the alternative to the starting hypothesis is that the mean values are different.

A critical threshold probability is selected against which to test the starting hypothesis, and the values below the threshold indicate that the difference between the mean values is significant. Typical threshold values for rejection of

the starting hypothesis are P= 0.05 (level of significance), P = 0.01 (highly significant) and P = 0.001 (very highly significant).

The method used for analysing the difference between two means (and accepting or rejecting the starting hypothesis) was *t-test*. The t-test was performed using MS Excel™ [122].

6.6 Results and discussion

6.6.1 Bioscrubber Only (BO) scenario

In this first part of the experiment [122] the contaminated gas stream was fed directly to the bioscrubber (Figure 6.1) and the absorber was disconnected. During period BO.I, FB (Figure 6.2A) and DCE (Figure 6.2B) were fed to the BO configuration continuously at average loading rates of 14 g_{FB} m^{-3} h^{-1} and 97 g_{DCE} m^{-3} h^{-1}. Constant DCE and FB removal efficiencies were observed (more than 80 % removal for both substrates), given by outlet loading rates of 1 g_{FB} m^{-3} h^{-1} , 14 g_{DCE} m^{-3} h^{-1}, and biomedium concentrations of 0 g_{FB} m^{-3} and 15 g_{DCE} m^{-3} (Figure 6.3).

During period BO.II, the feeding of FB ceased for a period of 72 h (flow rate G1 was set to 0 L min^{-1}, Figure 6.1) and only DCE was fed to the system at the same loading rate as in BO.I. Due to the lack of FB the carbon dioxide concentration was significantly reduced between 0 and 3 h (Figure 6.3) from an average value of 0.68 % v/v for period BO.I to 0.45 % v/v. The reduction of the carbon dioxide concentration continued, but more slowly, between 3 and 72 h, reaching a minimum of 0.37 % v/v (time 71 h).

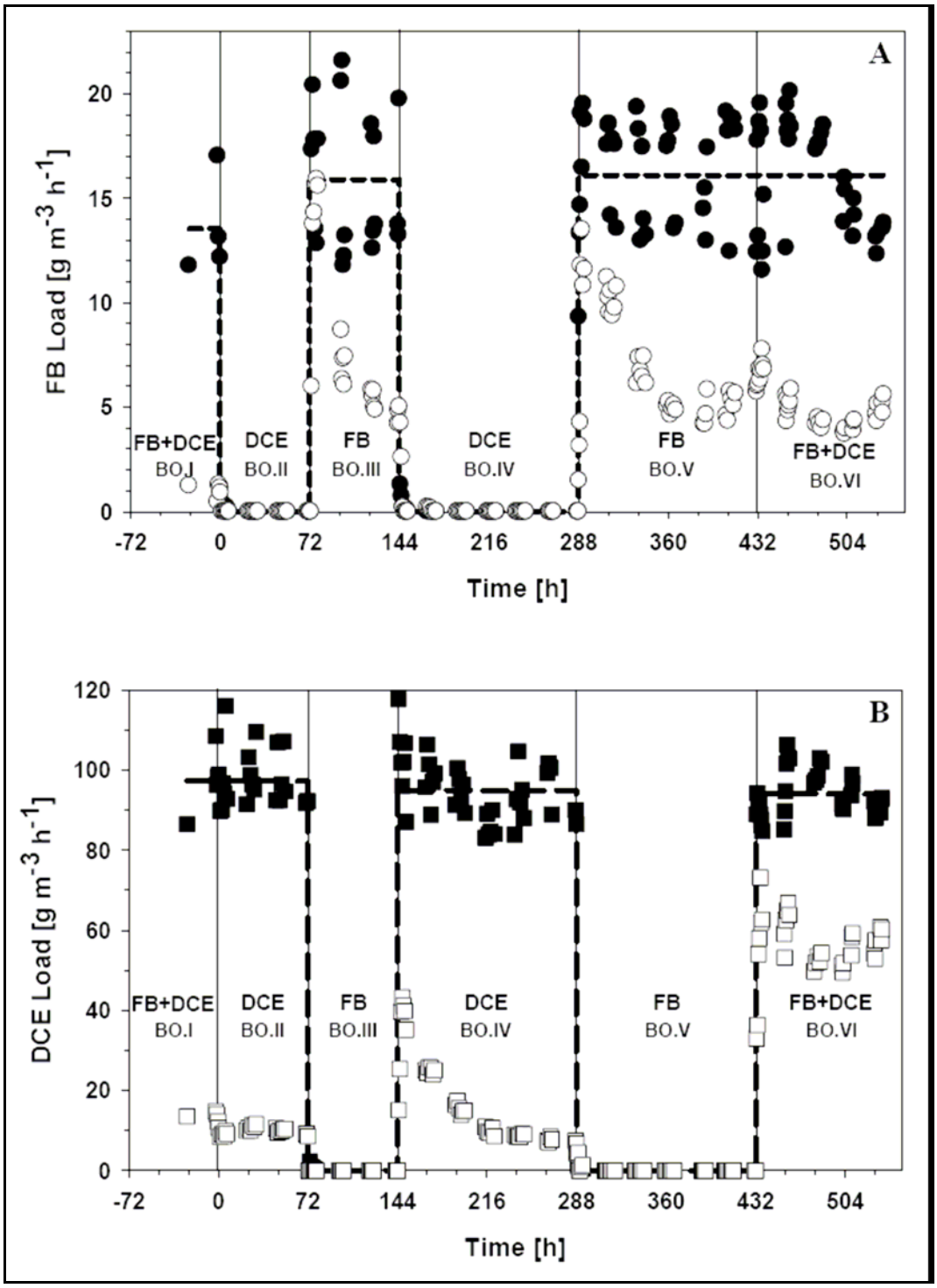


Figure 6.2 - *Evolution of the FB and DCE inlet and outlet loads in the BO system during the SAP experiment.* ***A:*** *FB;* ***B:*** *DCE. [122]*

- Bioscrubber FB inlet load - experimental
- Bioscrubber DCE inlet load - experimental
- Bioscrubber FB outlet load - experimental
- Bioscrubber DCE outlet load - experimental
- Bioscrubber FB inlet load - imposed
- Bioscrubber DCE inlet load - imposed

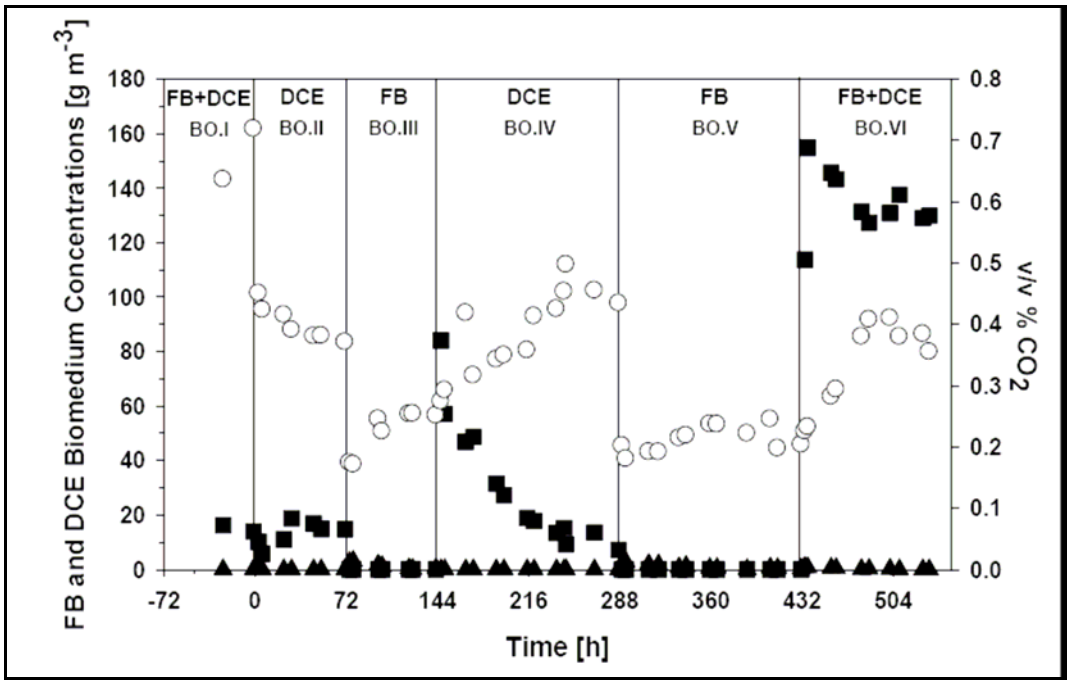


Figure 6.3 - *Evolution of DCE and FB biomedium concentrations and outlet carbon dioxide (% v/v) in the BO system during the SAP experiment. [122]*

At the beginning of period BO.III, the FB load was re-introduced at an average value of 16 g_{FB} m^{-3} h^{-1} and the DCE feed was stopped for 72 h (flow rate G2 was set to 0 L min-1, Figure 6.1). During period BO.III (Figure 6.2A) the FB bioscrubber outlet load increased to 16 g_{FB} m^{-3} h^{-1} ^{in} just 4 h after the re-introduction of FB (time 76 h), thus reducing the removal efficiency to 0 %.

The FB bioscrubber outlet load reduced slowly over time to reach 5 g_{FB} m^{-3} h^{-1} at time 128 h and the FB removal efficiency increased to reach a maximum of 75 % at time 144 h. Therefore, the FB removal efficiency did not recover to the level of period BO.I (> 90 % FB removal) even 3 d after the re-introduction of FB. The carbon dioxide concentration was significantly reduced from 0.37 % v/v (time 71 h) to 0.17 % v/v 3 h after FB re-introduction, and increased slowly over time to 0.25 % at the end of BO.III.

Although the biomedium FB concentration during BO.III remained lower than 3 g_{FB} m-3 (Figure 6.3), due to the severe increase of the bioscrubber FB outlet load a significant amount of FB was released untreated to the environment.

Moreover, during the first few hours following FB re-introduction foaming occurred, indicating that the culture was under stress conditions. During period BO.IV the FB feed was stopped for a period of 6 d (144 h) and DCE was re-introduced at an average loading rate of 95 g_{DCE} m^{-3} h^{-1}. 4 h after DCE re-introduction the bioscrubber DCE outlet load increased to a maximum of 43 g_{DCE} m^{-3} h^{-1}, the biomedium DCE concentration increased to 84 g_{DCE} m^{-3} and the DCE removal efficiency dropped to a minimum of 55 %.

The system required 2 d to recover to the initial performance of periods BO.I-II, reducing the bioscrubber DCE outlet load to 15 g_{DCE} m^{-3} h^{-1} and increasing the DCE removal efficiency to 85 %. During period BO.IV the carbon dioxide concentration increased from 0.25 % v/v at the beginning of the period to 0.45 % v/v at the end (time 288 h). However, the carbon dioxide concentration required 3 d to increase to the same level of period BO.II (0.36 % v/v), indicating that the activity of GJ10 cells was reduced during period BO.III when DCE was not supplied.

The feeding of the waste gas was again switched from DCE to FB during period BO.V for 6 d. The bioscrubber FB inlet load was re-introduced at an average value of 16 g_{FB} m^{-3} h^{-1} (time 288 h) and the bioscrubber FB outlet load was increased to 14 g_{FB} m^{-3} h^{-1} after 3 h, causing the FB removal efficiency to drop to a minimum of 18 %.

The FB removal efficiency increased to 60 % 2 d after the re-introduction of FB (time 335 h); though it varied between 60-70 % for the rest of the monitoring of the experiment (periods BO.V-VI), failing to re-establish the initial removal efficiency of period BO.I (more than 90 % FB removal).

The carbon dioxide concentration was reduced immediately from 0.45 to 0.18 % v/v 5 h after the re-introduction of FB, and it increased slowly over time to a maximum of 0.24 % v/v, similarly to period BO.III (0.25 % v/v).

However, during period BO.III the carbon dioxide concentration required 3 d to increase to that level, while during period BO.V the increase required 5 d. This fact suggests that after the 6 d of FB starvation, F11 needed a longer re-acclimation period to recover after re-introduction of FB, than was required after 3 d of FB starvation.

Following 6 d of DCE starvation, the DCE feed was re-introduced at an average value of 94 g_{DCE} m^{-3} h^{-1} (time 432 h) maintaining the feeding of FB at an average value of 16 g_{FB} m-3 h^{-1}. Upon the re-introduction of DCE, the bioscrubber DCE outlet load increased to a maximum value of 73 g_{DCE} m^{-3} h^{-1} reducing the DCE removal efficiency to 18 %, 4 h after the restart of the feed.

However, for the rest of period BO.VI the DCE removal efficiency showed a relatively small increase to a level between 30-50 %, failing to re-establish the initial DCE removal efficiency of period BO.I (90 %). Also, the biomedium DCE concentration followed a similar profile to the bioscrubber DCE outlet load during period BO.VI, increasing to a maximum value of 155 g_{DCE} m-3, 5 h after the DCE re-introduction.

The biomedium DCE concentration was maintained at high values for the rest of the experiment (~130 g_{DCE} m^{-3}). The carbon dioxide concentration was increased from 0.24 % v/v at the end of period BO.V to a maximum value of 0.41 % v/v 3 d after the re-introduction of DCE.

Nevertheless, the carbon dioxide concentration did continue to increase, failing to restore the average concentration of period BO.I (0.68 % v/v). Comparing the performance of the system during periods BO.IV and BO.VI, following 3 d of DCE starvation (BO.IV) the bioscrubber was able to recover the initial DCE removal of periods BO.I-II, 2 d after the re-introduction of the substrate.

However, after 6 d of DCE starvation the bioscrubber improved its performance only during the first two days after the re-introduction and remained stable with low DCE removal efficiency for the rest of the experiment.

The expected and measured concentrations of F- and Cl- anions in the bioscrubber during the SAP experiment are displayed in Figure 6.4. The expected concentrations over time were calculated based on the amount of the two substrates biodegraded (according to analysis of the influent and effluent streams) and the rate that the anions were washed out of the bioscrubber. Figure 6.4 shows that the expected concentrations were in agreement with the measured values, implying complete dehalogenation of the two substrates removed was taking place at all times [122].

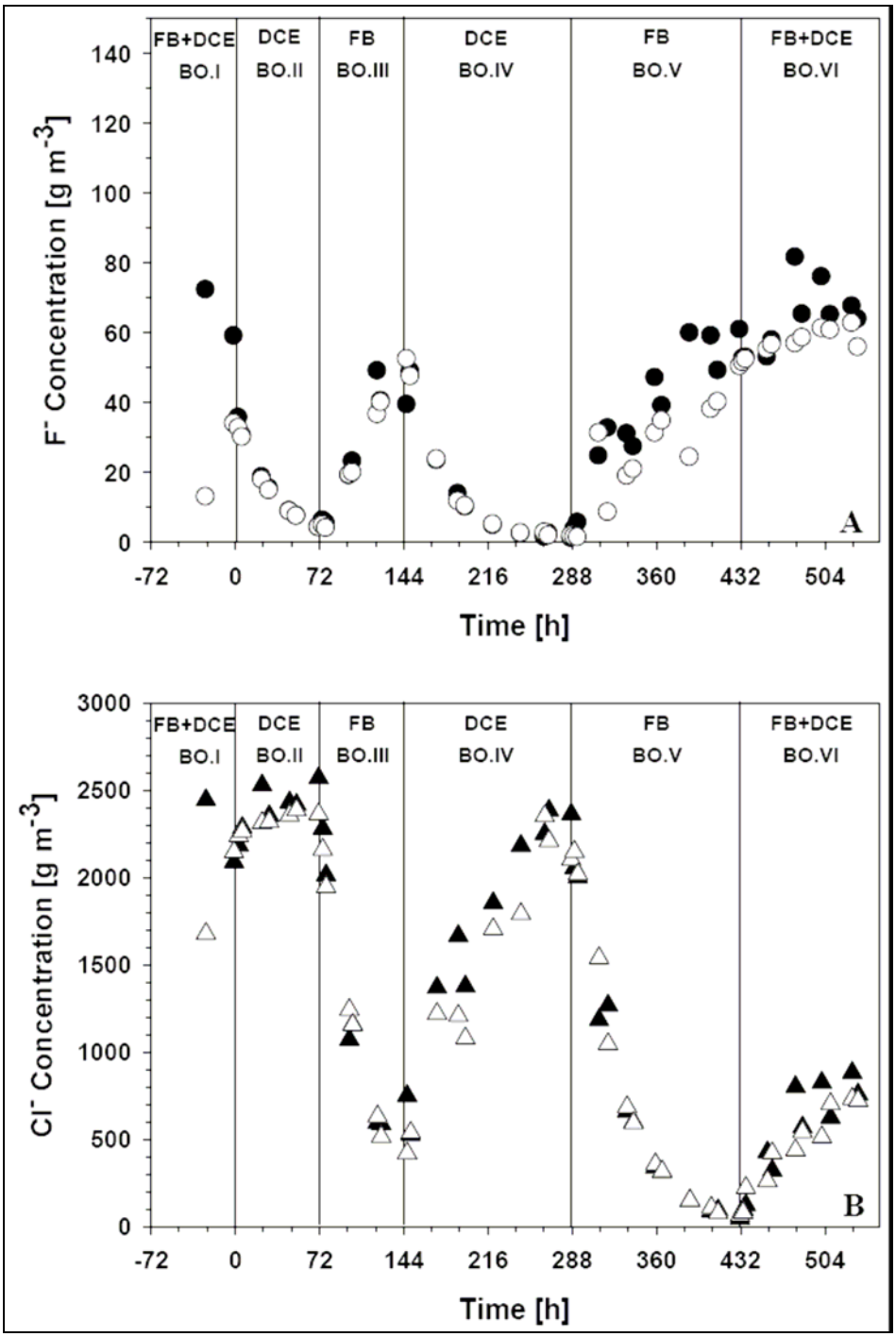


Figure 6.4 - *Evolution of expected and measured F^- and Cl^- concentrations in the BO system during the SAP experiment. The F^- and Cl^- concentration expected were calculated based on the amount of F^- and Cl^- released into the biomedium, due to the biodegradation of the influent FB and DCE, and the removalefficiency achieved for each substrate over time. The F^- and Cl^- concentrations measured were determined by Ion Chromatograph analysis **A**: F^-;**B**: Cl^- [122]*

6.6.2 Oil-Absorber-Bioscrubber (OAB) scenario

In the second part of this work [122] the absorber has been connected upstream of the bioscrubber to act as a buffer for alternating DCE and FB (Figure 6.1). The absorber was fed with average FB and DCE inlet loads of 16 g_{FB} m^{-3} h^{-1} and 104 g_{DCE} m^{-3} h^{-1} respectively, resulting in average bioscrubber inlet loads of 13 g_{FB} m^{-3} h^{-1} and 100 g_{DCE} m^{-3} h^{-1} during period OAB.I (Figure 6.5). The average values of parameters such as the bioscrubber outlet loads and the removal efficiencies were 2 g_{FB} m^{-3} h^{-1} and 86 % for FB, and 24 g_{DCE} m^{-3} h^{-1} and 77 % for DCE respectively.

Moreover, the average biomedium concentrations of the two compounds during period OAB.I were 0 g_{FB} m-3 and 45 g_{DCE} m-3, while the average value of the carbon dioxide concentration was 0.51 % v/v (Figure 6.6).

At the beginning of period OAB.II, the FB feed ceased for 3 d (flow rate G1 was set to 0 L min-1, Figure 6.1) while DCE was fed to the system at the same loading rate as in OAB.I. Thus, during the 3 d period that FB was not fed to the system, the FB absorbed in sunflower oil was desorbed, and the bioscrubber FB inlet load reduced over time to reach a minimum value of 1 g_{FB} m^{-3} h^{-1} at the end of OAB.II.

The bioscrubber continued to remove the FB fed with an average FB removal efficiency of 92 %, although the carbon dioxide concentration did not decrease proportionally to the decrease of the bioscrubber FB inlet load as expected.

At 0 h the biofilm on the walls of the bioscrubber was sampled for microbial analysis, which resulted in a large proportion of the biofilm being suspended in the bioscrubber and washed out over the next few hours of operation.

Therefore, although stable DCE removal efficiency and smooth reduction of the carbon dioxide concentration were expected during period OAB.II, these two parameters were reduced abruptly to 60 % and 0.30 % v/v for the first 48 h.

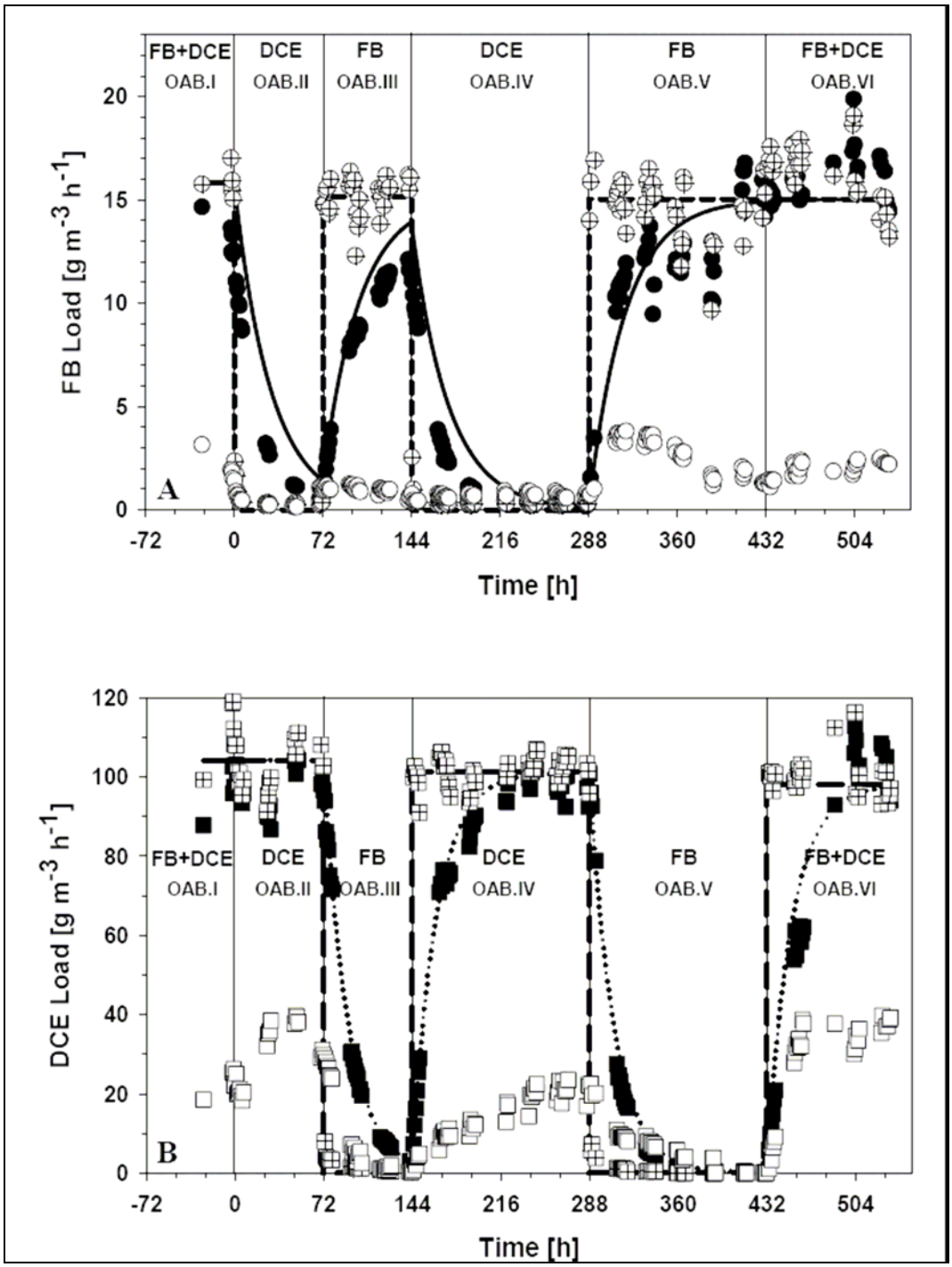


Figure 6.5 - *Evolution of the FB and DCE inlet and outlet loads in the OAB system during the SAP experiment.* ***A:*** *FB;* ***B:*** *DCE. [122]*

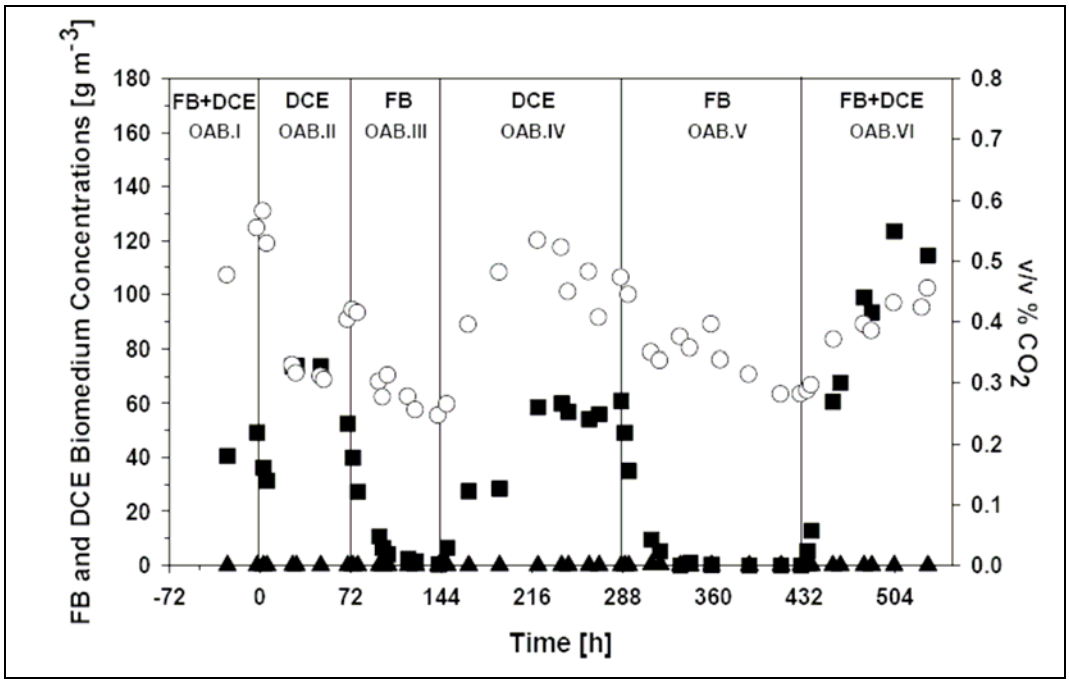


Figure 6.6 - *Evolution of DCE and FB biomedium concentrations and outlet carbon dioxide (% v/v) in the OAB system during the SAP experiment. [122]*

However, they recovered on the last day of period OAB.II, reaching 70 % DCE removal efficiency and 0.42 % v/v carbon dioxide concentration at the end of OAB.II.

At 72h, the FB feed was re-introduced at an average load of 15 g_{FB} m^{-3} h^{-1} and the DCE feed ceased for 3 d (period OAB.III). Due to desorption, the bioscrubber FB inlet load increased slowly over time to reach a maximum value of 12 g_{FB} m^{-3} h^{-1} at time 144 h. Thus, the slow increase of the bioscrubber FB inlet load allowed sufficient time for strain F11 to grow in the bioscrubber, resulting in low values of bioscrubber FB outlet load during period OAB.III.

The bioscrubber FB outlet load did not increase more than 1 g_{FB} m^{-3} h^{-1} showing that strain F11 was remained active during the 3 d of FB starvation. Therefore, the bioscrubber was able to restore similar FB removal efficiency to period OAB.I during the whole duration of OAB.III period (up to 95 % FB removal efficiency achieved), contrary to the poor FB removal efficiency achieved in the BO system (period BO.III).

Furthermore, due to the lack of DCE from the process inlet, the bioscrubber DCE inlet load was reduced over time to reach a minimum of 3 g_{DCE} m^{-3} h^{-1}at time 144 h. The bioscrubber DCE outlet load and the biomedium DCE concentration were reduced to 0 g_{DCE} m^{-3} $h^{-1}$and 0 g_{DCE} m^{-3} respectively. Also, the carbon dioxide concentration was reduced over time to reach a minimum of 0.25 % v/v.

After 3 d of DCE starvation, the DCE feed was re-introduced at an average loading of 101 g_{DCE} m^{-3} h^{-1} and the FB feed was simultaneously stopped for 6 d (OAB.IV).

The bioscrubber DCE inlet load increased slowly over time and required approximately 3 d to increase to the same level as the absorber DCE inlet load. Due to the absorber, the system responded to the DCE re-introduction (period OAB.IV) similarly to the re-introduction of FB (period OAB.III). The bioscrubber DCE outlet load increased slowly over time during period OAB.IV, to reach a maximum value of 23 g_{DCE} m^{-3} h^{-1} at the end of OAB.IV. The biomedium DCE concentration followed the same profile as the bioscrubber DCE outlet load, reaching a maximum value of 61 g_{DCE} m^{-3}.

The DCE removal efficiency was higher than 77 % during OAB.IV, showing that the re-introduction of DCE did not affect the performance of the bioscrubber comparing to period OAB.I. On the other hand, the bioscrubber FB inlet load was reduced over time to 1 g_{FB} m^{-3} h^{-1}, approximately 3 d after the interruption of the FB feed. However, during the last 3 d of period OAB.IV, FB was still detected in the bioscrubber inlet and was fed into the bioscrubber at loads ranging between 0.2-1 g_{FB} m^{-3} h^{-1}. The carbon dioxide concentration increased over time due to the re-introduction of DCE and reached a maximum value of 0.53 % v/v.

At time 288 h, the FB feed was resumed at an average loading rate of 15 g_{FB} m^{-3} h^{-1} and the DCE feed was stopped for a period of 6 d. The bioscrubber FB inlet load increased slowly over time due to the absorption of FB in the oil and was equal to the absorber FB inlet load after approximately 3 d. Once again the effect of the absorber was beneficial for strain F11, showing that even a very low load of FB fed constantly to the bioscrubber during the starvation period (less than

1 g_{FB} m^{-3} h^{-1}), was able to maintain the strain active. Therefore, the bioscrubber FB outlet load increased to 4 g_{FB} m^{-3} h^{-1} for the first two days after the re-introduction of FB and was reduced to a level between 1-2 g_{FB} m^{-3} h^{-1} by the end of period OAB.V. The biomedium FB concentration was 0 g_{FB} m^{-3} at all times and the FB removal efficiency had a minimum value of 66 % during the first 2 d; though, the FB removal efficiency increased to a maximum of 92 % at the end of period OAB.V.

The bioscrubber DCE inlet load was reduced over time during period OAB.V and reached 1 g_{DCE} m^{-3} h^{-1} approximately 3 d after the interruption of the DCE feed. However, DCE was still supplied to the bioscrubber at a very low loading that ranged between 0.3-1 g_{DCE} m^{-3} h^{-1}, for the remaining 3 d of DCE starvation. The carbon dioxide concentration was reduced over time and reached a minimum value of 0.28 % at the end of OAB.V. During period OAB.VI, the DCE feed was re-introduced at 98 g_{DCE} m^{-3} h^{-1} and the FB feed was maintained at the same loading rate as in OAB.V (15 g_{FB} m^{-3} h^{-1}).

After the re-introduction of DCE, the bioscrubber DCE inlet load increased over time to reach the level of the absorber DCE inlet load after approximately 3 d. The bioscrubber DCE outlet load increased to a maximum value of 38 g_{DCE} m^{-3} h^{-1} 1 d after the restart of the DCE feed and maintained that level for the next days of monitoring.

The biomedium DCE concentration increased to a maximum value of 123 g_{DCE} m^{-3} and the DCE removal efficiency decreased to a minimum value of 33 % at time 461 h. However, the DCE removal efficiency increased for the next days of monitoring to a level between 60-70 %. The carbon dioxide concentration increased over time to 0.45 % v/v after 4 d, while for the whole duration of OAB.VI period the bioscrubber maintained similar FB removal as in period OAB.V.

Complete dehalogenation of the two substrates removed was taking place at all times in the OAB system, a fact that is in agreement with the results obtained in the BO system[122]. This is clear from Figure 6.7, which shows that the expected F- and Cl- concentrations were in general close to the measured values during the SAP experiment.

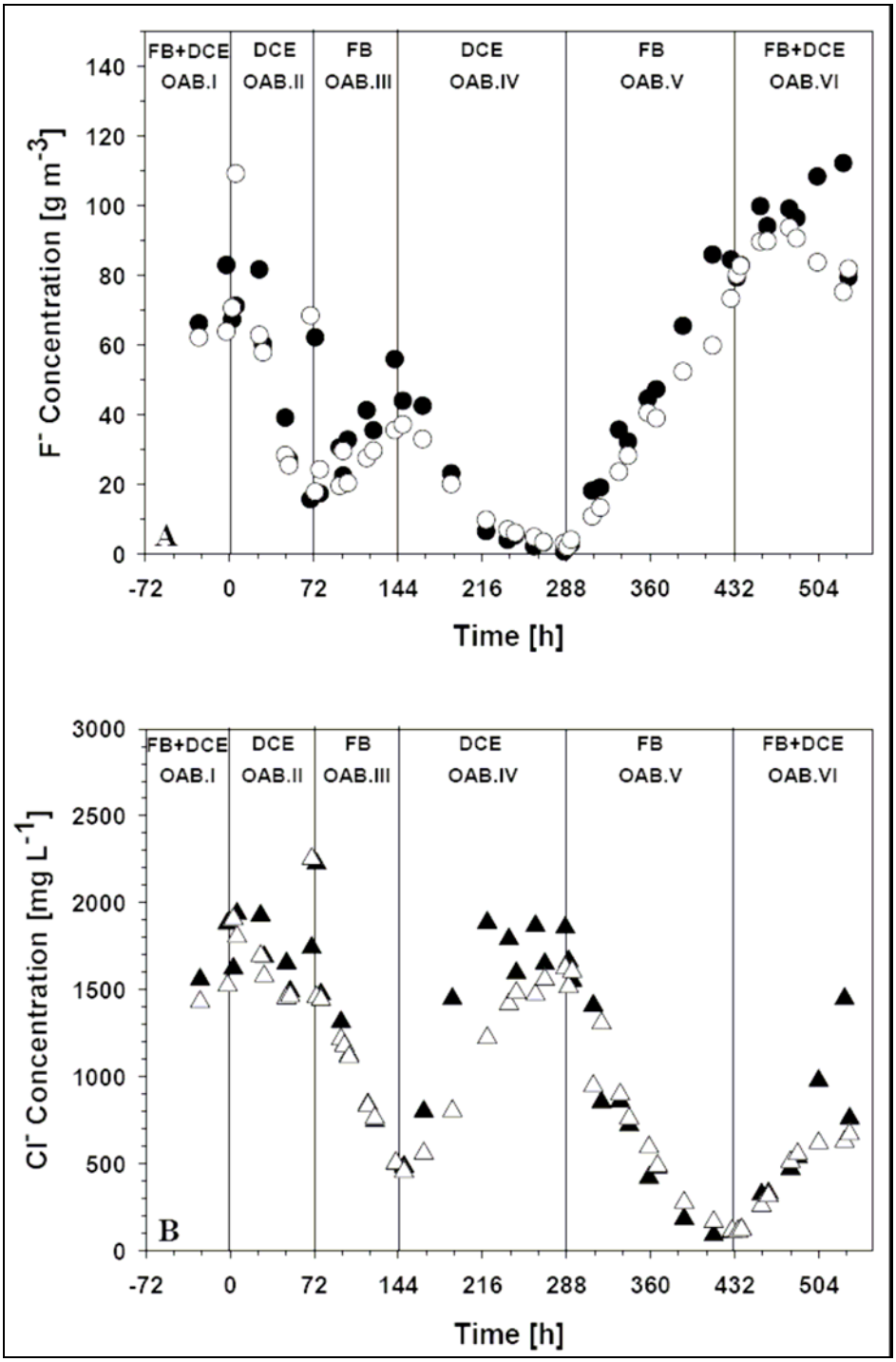


Figure 6.7 - *Evolution of expected and measured F^- and Cl^- concentrations in the OAB system during the SAP experiment. The F^- and Cl^- concentration expected were calculated based on the amount of F^- and Cl^- released into the biomedium, due to the biodegradation of the influent FB and DCE, and the removal efficiency achieved for each substrate over time. The F^- and Cl^- concentrations measured were determined by Ion Chromatograph analysis **A**: F^-; **B**: Cl^- [122]*

Figure 6.8 shows the total organic mass discharged as FB (TOD_{FB}) and DCE (TOD_{DCE}) from the BO and OAB systems during each period. During the BO operation, TOD_{FB} was significantly higher than in the OAB system during the FB re-introduction periods (BO.III, BO.V, BO.VI and OAB.III, OAB.V, OAB.VI). During periods BO.III, BO.V and BO.VI, the TOD_{FB} was 557, 979 and 504 g_{FB} m^{-3} respectively.

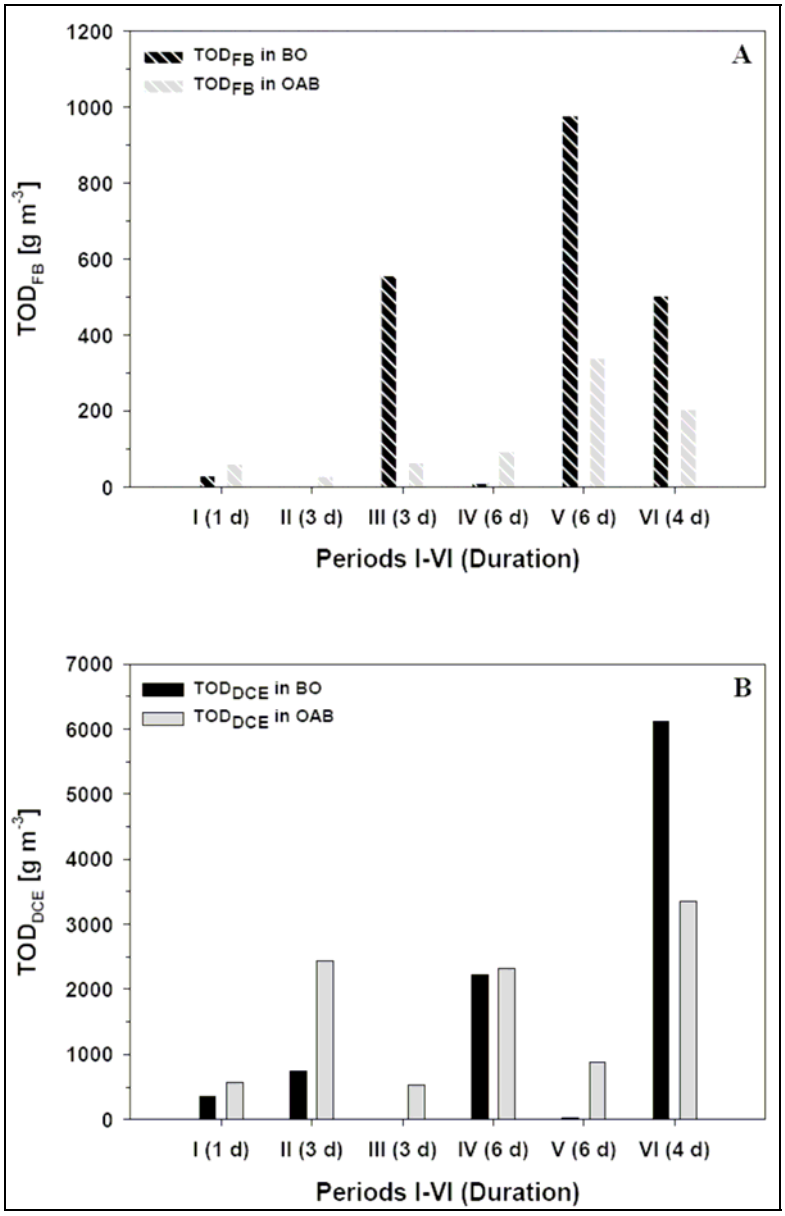


Figure 6.8 - *Total organic discharged during each period of the BO and OAB systems. The mass of FB and DCE discharged was calculated for each period by integrating the effluent FB and DCE concentrations multiplied by the flowrates of the gas and biomedium outlets of the bioscrubber and normalised by the bioscrubber volume. The duration of each period is displayed in brackets. A: TOD_{FB}; B: TOD_{DCE}. [122]*

Thus, the addition of the absorber prior to the bioscrubber significantly reduced the discharges of FB (up to 9 times for period OAB.III) and during periods OAB.III, OAB.V and OAB.VI, the TODFB was 65, 339 and 205 g_{FB} m^{-3} respectively.

Nevertheless, as shown in Figure 6.8B the TOD_{DCE} discharged during the operation of the OAB system was similar to the TOD_{DCE} discharged from the BO system[122].

Overall, the absorber was able to maintain strain F11 active in the OAB system during the SAP experiment, reducing significantly the TOD_{FB} during the re-introduction periods of FB. However, the discharges of DCE were not reduced during the OAB operation, perhaps due to a possible inhibitory effect on GJ10 caused by metabolic products from FB degradation and due to the presence of FB for longer time periods in the bioscrubber of the OAB, than in the BO system.

Thus, although the absorber reduced the peak concentrations of FB influent to the bioscrubber during the FB re-introduction periods, it is possible that the concentration reduction was not sufficient to avoid the inhibitory effect.

Furthermore, the presence of FB in the bioscrubber inlet persisted for prolonged time periods in the OAB system due to the effect of the absorber, thus inhibiting the degradation of DCE for a longer period and the TOD_{DCE} was not reduced [122].

6.6.3 Microbial Dynamics Analysis

The relationship between the operational stability of the two systems (BO and OAB) [122] and the behaviour of the microbial population was monitored during the SAP experiments. Figure 6.9 displays the evolution of active cells (% of active cells = 100 % × EUB stained cells / DAPI stained cells) for both systems tested and indicates that significant changes in cellular activity occurred during the SAP experiment. When the FB feed was stopped at 0 h, the percentage of

active cells increased from an average value of 62% during period BO.I (Figure 6.9A) to a maximum value of 91% at the end of BO.II.

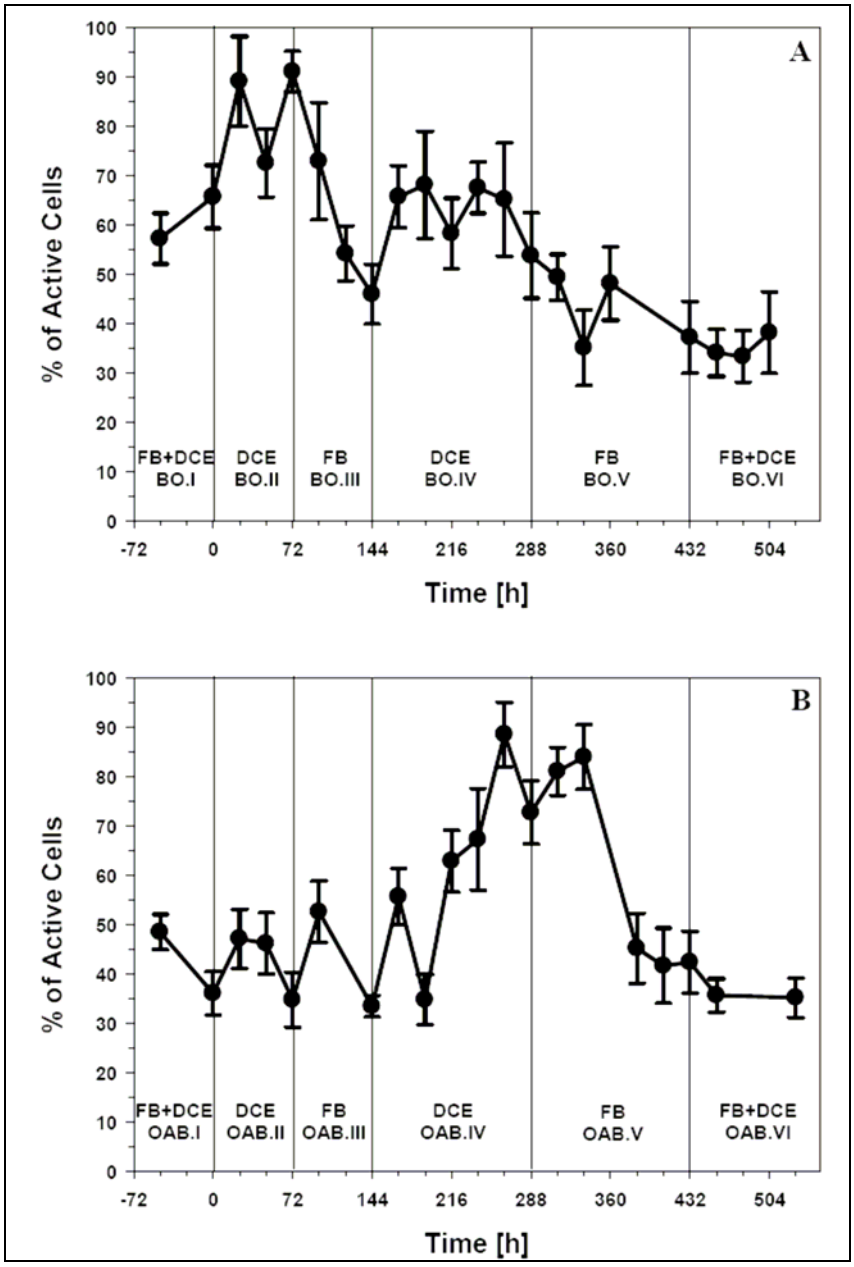


Figure 6.9 - *Evolution of active cells in the BO and OAB configurations. The results are obtained as an average from 9 individual measurements at each point and the error bars are calculated for confidence interval of 95 %. A: BO; B: OAB.*

% of active cells = 100 % × EUB stained cells / DAPI stained cells [122]

Switching the substrate feed from DCE to FB in period BO.III caused a significant reduction of cellular activity which decreased to a minimum of 46% at 144 h. However, cellular activity increased slightly during period BO.IV when

only DCE was fed to the bioscrubber, and decreased again to a minimum value of 37% during period BO.V when only FB was fed. During the last period of the SAP experiment (BO.VI), the percentage of active cells remained constant at an average value of 36%.

The activity of the biofilm formed in the bioscrubber due to the wall growth was also measured before and after the SAP experiment. Therefore, at time 0 h the cellular activity in the biofilm was 66% and at time 528 h it was 45%.

The activity of cells was significantly reduced in the biofilm during the SAP experiment, which is in agreement with the overall reduction in the suspended cell activity at the same time. The evolution of active cells in the BO system shows that although the activity of cells increased during the periods when only DCE was fed to the system (BO.II and BO.IV), a significant reduction in the cell activity occurred when only FB was fed (BO.III and BO.V).

The response of cell activity during the SAP experiment was different for the OAB system (Figure 6.9B). The average value of the activity prior to the first starvation cycle (OAB.I) was 42 % and switching the feed sequentially to only FB (OAB.II) or only DCE (OAB.III) for 3 d did not change this value significantly.

However, the cellular activity started to increased at time 216 h, 3 d after stopping the FB feed for a second time (OAB.IV), reaching a value of 84 % at time 336 h. The activity dropped again 4 d after switching to FB at period OAB.V to a level of 42 % at time 432 h. The feeding of both substrates during period OAB.VI did not change the activity significantly, from an average value of 36 %.

The activity of cells in suspension before the SAP experiment did not change significantly after the sequential switches in the substrate feed. This is in agreement with the activity of the cells growing as a biofilm on the walls of the bioscrubber which was also maintained relatively constant during the SAP experiment (24 % at time 0 h and 30 % at time 528 h). In contrast to the BO operation, the results presented above show that during the 3 d sequential periods of DCE and FB starvation (OAB.II-III) the activity of cells did not change significantly. This was attributed to the absorber continuing to feed both substrates to the bioscrubber at significant rates during the whole duration of periods OAB.II-III, maintaining the activity of the microbial culture constant.

However, during the 6 d sequential periods of DCE and FB starvation (OAB.IV-V) the activity of cells increased when only DCE was fed and decreased when only FB was fed. These changes occurred 3-4 d after switching the substrate feed, in contrast to the BO experiment when they occurred immediately after changing the conditions.

In general, the activity of the microbial culture in both systems shifted up during the periods that only DCE was fed and shifted down during the periods that only FB was fed [122].

This fact also indicates the possible inhibitory effect of metabolites produced from FB degradation (such as F- anions) to the microbial culture reducing the cells activity when FB was fed. However, during the periods that FB was not fed to the bioscrubber, the activity of cells increased, most probably due to the absence of the inhibitory FB degradation metabolites [122].

In figure 6.10, the measured viability of cells during the SAP experiments was reported using the following formula: % of viable cells = 100% × (DAPI stained cells - PI stained cells) / DAPI stained cells. In the BO experiment, the viability was constant during period BO.I at an average value of 50%. This percentage decreased slightly during the 3 d period that only DCE was fed reaching a minimum value of 41% at the end of BO.II. However, during BO.III the viability decreased substantially reaching a minimum value of 9% at time 144 h.

Switching the substrate from FB to DCE in period BO.IV increased the viability of cells to 36 % at the end of the period. The viability did not change significantly over the last two periods of operation (BO.V-VI) maintaining an average value of 36 % for the rest of the experiment.

During the first 120 h of the SAP experiment in the OAB system (Figure 6.10, periods OAB.I-III) the viability of cells was not monitored due to a problem encountered with the PI staining of cells. However, the evolution of cell viability during periods OAB.IV-VI indicates that the viability remained relatively constant and at higher values than in the BO system.

Overall, significant changes occurred in the viability of cells during the SAP experiment, when the absorber was not used. Though, a direct comparison of

the cells viability in the two systems could not be made for the first three periods of each experiment and no conclusions could be made for the 3 d sequential alternation of the substrate feed between DCE and FB [122].

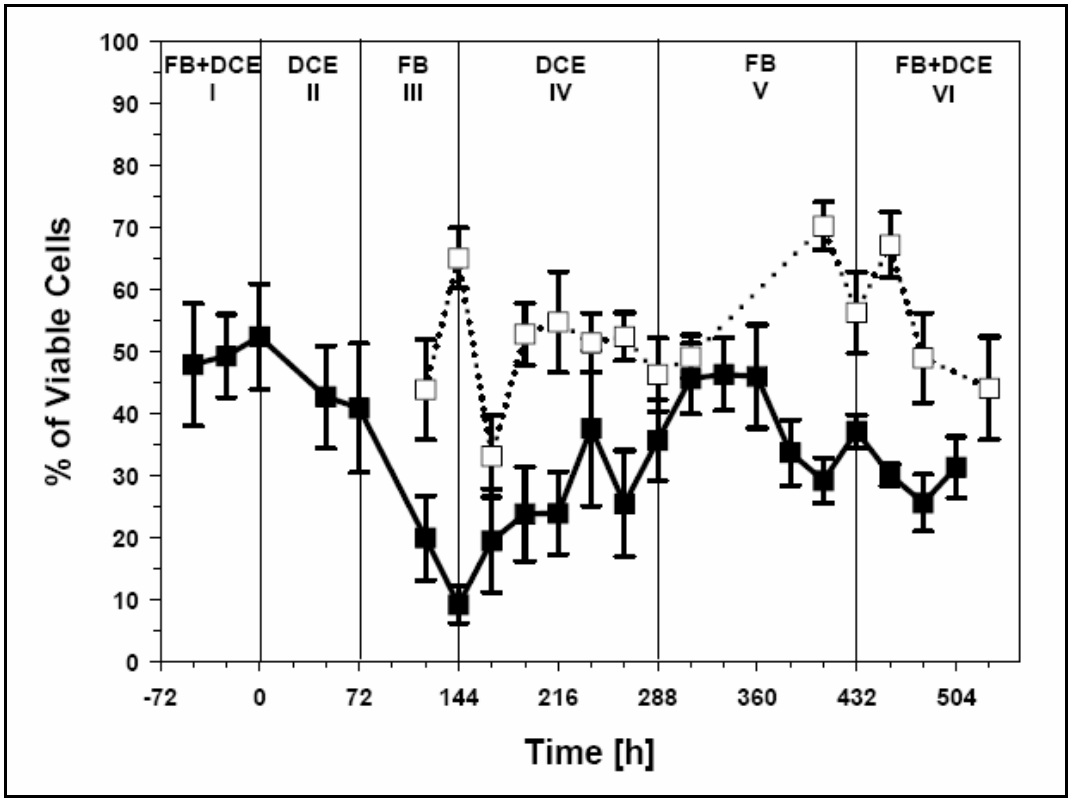


Figure 6.10 -*Evolution of cell viability in the BO and OAB configurations. % of viable cells = 100 % × (DAPI stained cells - PI stained cells) / DAPI stained cells. The results are obtained as an average from 9 individual measurements at each point and the error bars are calculated for confidence interval of 95 %. [122]*

Evolution of viable cells in the BO system

Evolution of viable cells in the OAB system

The evolution of strain F11 and *Xanthobacter autotrophicus* strain GJ10 cells were monitored during the SAP experiments in the BO and OAB configurations (Figure 6.11). The percentage of each specific strain was calculated using the following formula: % of GJ10 or F11 = 100 % × GJ10 or F11 cells / EUB stained cells.

The percentage of each strain was expected to decrease during the periods that the specific substrate was not supplied and to increase respectively during the feeding periods of the specific substrate.

However, it is obvious that the percentages of each specific strain did not follow that trend and were rather scattered over time in both systems.

The percentage of the specific degraders (added together) was rather low compared to the total number of active cells in the bioscrubber and did not increase more to than 55 % in the BO system or 64 % in the OAB system.

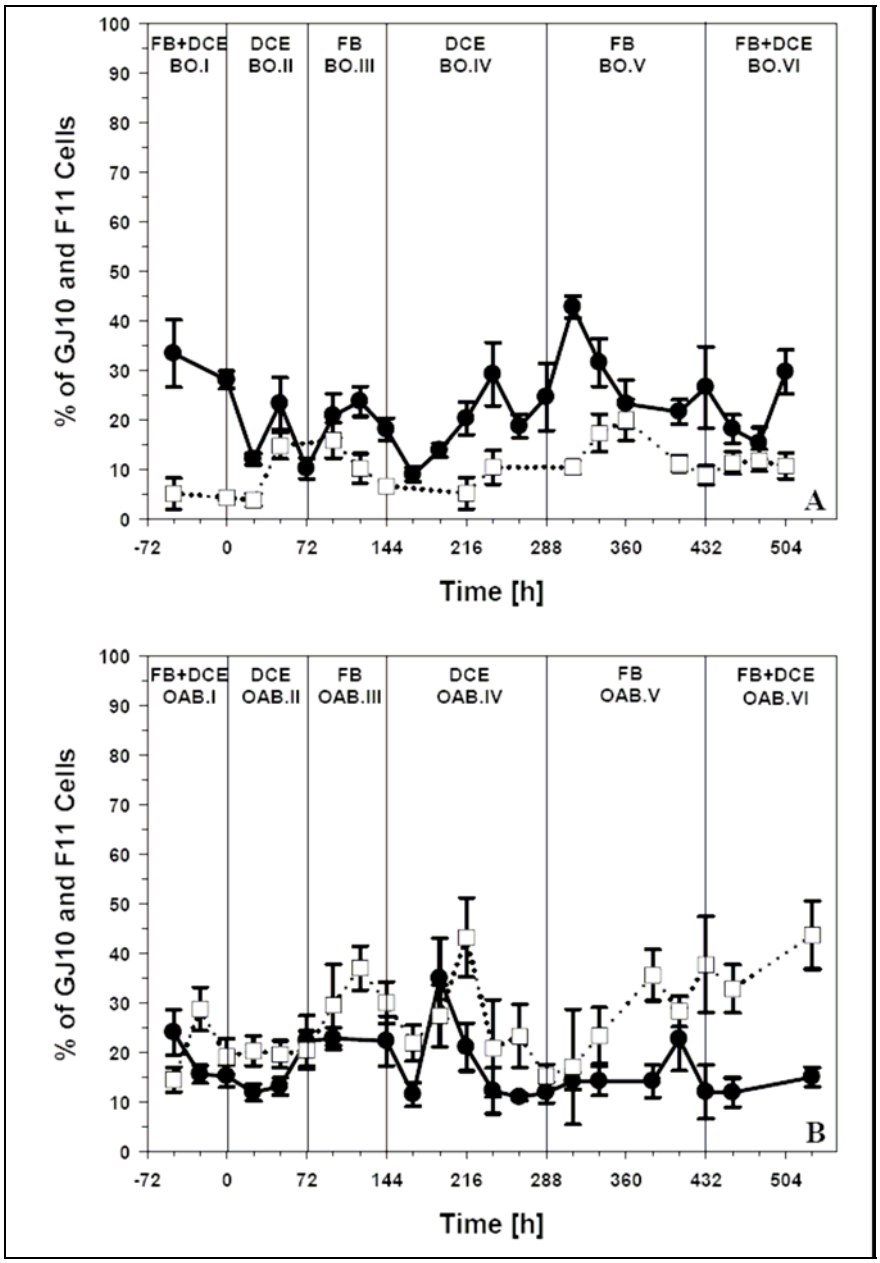


Figure 6.11 - *Evolution of strain F11 and Xanthobacter autotrophicus strain GJ10 in the BO and OAB configurations. The results are obtained as an average from 9 individual measurements at each point and the error bars are calculated for confidence interval of 95 %. A: BO; B: OAB. of cell viability in the BO and OAB configurations. [122]*

% of GJ10 = 100 % × GJ10 cells / EUB stained cells

% of F11 = 100 % × F11 cells / EUB stained cells

The low percentages of the two specific degraders indicate that dynamic microbial communities were formed in the bioscrubber during the operation of the two systems and microorganisms apart from the two specific degraders might have been growing in the microbial culture.

Therefore, if other bacterial strains than F11 and GJ10 were growing in the bioscrubber, these might have been involved in the degradation of DCE or FB and were able to tolerate the inhibitory effect. This is in agreement with the findings of Carvalho, et al. (2006) who identified the presence of nine different bacterial strains as well as the original inoculum (strain F11) growing in a GAC bioreactor used for the degradation of FB.

Furthermore, the distribution of the two specific degraders in the biofilm growing on the walls of the bioscrubber was similar to the percentages of the two degraders in the suspended culture for both systems. Thus, in the BO system the percentages of GJ10 and F11 in the biofilm were 13 % and 4 % respectively at time 0 h, while at time 528 h these percentages were 12 % and 7 %. In the OAB system, the percentages of GJ10 and F11 in the biofilm were 20 % and 24 % at time 0 h, while at time 528 h GJ10 and F11 were 28 % and 48 % respectively. The percentages of the two specific strains in the biofilm and in suspension were similar[122].

6.7 Conclusions

In the OAB system [122] the TOD_{DCE} was not reduced after the re-introduction of the DCE feed (compared to the BO system), as would be and this could be attributed to the possible presence of inhibitory metabolites produced from FB degradation, which persisted in the bioscrubber for longer time periods in the OAB system due to the effect of the absorber.

Evidence for this inhibitory effect was also apparent from the evolution of activity in the bioscrubber, which was reduced during periods that FB only was fed and increased when the feed was switched to DCE.

FISH results also suggest that a dynamic community developed in the bioscrubber during SAP conditions, and the percentage of the main degraders remained relatively low.

Compared to the BO system, the removal efficiency of FB was maintained at higher levels in the OAB, during the re-introduction of FB in the substrate feed. Due to the effect of the absorber, the TOD_{FB} was substantially reduced after the 6 d that FB was not supplied, while following the 3 d that FB was not supplied the TOD_{FB} was maintained at background levels and any re-acclimation was not required. Due to the fact that the absorber failed to reduce the DCE discharged, a different compound-strain model system, without any inhibitory effect[122], would be more appropriate to study the effect of the absorber during SAP conditions." [122]

Chapter 7

A Mathematical Model for the Oil-Absorber-Bioscrubber System During Biodegradation in a Sequentially Alternating Pollutant Scenario

All the tests have been performed using the facilities of the Imperial College London under the supervision of Professor Andrew G. Livingston

7.1 Abstract

A mathematical model has been purposed in this section for the Oil-Absorber-Bioscrubber system presented in the previous chapter and suitable for the biodegradation of solvent during biodegradation of waste gas in a sequentially alternated pollutant scenario.

The model has been developed for a set of two couple strain/substrate and then it was used to predict the behaviour of the same system but operating with a different set of couple.

Thus, once known the microbial growth kinetic of the strains involved, the model could be applied to study the stability of the system Oil-Absorber-Bioscrubber using phase plane plots.

The reasons for modelling this particular system are various: first of all the need in finding a useful design tool to help the scale-up of the process then, on the other hand, to understand in what scenario the OAB system is suitable to decrease the emission in the environment of untreated pollutants.

The scenarios could vary in terms of

- *set strains/substrates*: in the previous chapter it has been suggested the possibility of an inhibitory effect on the growth kinetic of one of the two

strains probably due to the cometabolic by-product generated from the other. In some case the substrate for one of the strain of the set could be inhibitory for the other.

- *peak concentration of the pollutant*: the high concentration of one of the pollutant during the S.A.P. scenario could create a shock load.
- frequency in the alternation of the pollutant feeding: a frequency to high or too low in the sequencing of the pollutant could create a remarkable instability in the system resulting in long recovery period for the biomass in the bioscrubber

To overcome those problems, the need of a well designed the oil absorber highly request a mathematical model for the OAB system.

7.2 The reasons for a mathematical model.

In the present work, mathematical modelling for the OAB system constitutes a useful tool to describe and to understand the process.
Modelling the absorber and the bioscrubber behaviour:

- improves the understanding of the dynamic biodegradative process considering all the relation cause-effect and the complex interaction between the involved variables such as growth kinetic, biodegradation rate, mass transfer.
- helps in the experimental design, finding the important parameters and evaluating with a sensible analysis the ones affecting the system and the negligible ones, especially in the set up of an OAB system in a different scenario.
- could be used in a predictive mode, after the validation of the model itself, reducing the number of experiment to optimize the process.

7.3 Mathematical Model purposed

At first iteration a mathematical model based on unstructured biological kinetics has been developed for the system based on Chang's model (Chang, et al. 2005) to describe the behaviour of the Oil-Absorber-Bioscrubber System operating S.A.P. condition with the two set of strain GJ10 and F11 capable to use as a sole source of carbon and energy respectively 1,2-Dichloroethane and Fluorobenzene. The nomenclature for the model has been reported at the end of this chapter (section 7.6).

The following assumptions were made:

Oil Absorber

- The partition coefficient for DCE and FB between sunflower oil and air is temperature dependent;
- The liquid and gas phases in the oil absorber are perfectly mixed;
- No volume change occurs upon absorption;

Bioscrubber

- Unstructured model, therefore there is no division between different types and ages of cells;
- The liquid phase in the reactor is well mixed;
- Biomass can grow in suspension and as a biofilm on the walls of the reactor;
- The biofilm is considered as a uniform, homogenous and non-porous solid;
- The concentration of cells in the biofilm, for both strains, (X_f^{GJ10}, X_f^{F11}) is uniform.
- The biofilm occupies a small fraction of the bioreactor volume ($V_v \sim V_b$);
- The mineral medium fed to the reactor is sterile;

- Ferreira Jorge and Livingston (1999) showed that Luong kinetics (Luong, 1986) describe the microbial kinetics for *Xanthobacter autotrophicus* strain GJ10 growing on DCE;
- In a previous work we find that the microbial kinetics for the strain F11 growing on FB follow the Luong kinetics (Luong, 1986) as described in literature in the work of Carvalho and Ferreira Jorge (2005), but with different values for the kinetic parameters
- The kinetics of the wall-attached biomass is assumed to be growth-limited by DCE, FB and oxygen. Thus, the specific growth rate in the biofilm is described by the following equation:

$$\mu_f^{GJ10} = \frac{\mu_{\max}^{GJ10}\ S_f^{DCE}}{K_s^{GJ10} + S_f^{DCE}} \left(1 - \frac{S_f^{DCE}}{S_m^{DCE}}\right) \frac{C_f}{C_f + K_{O_2}^{GJ10}} \tag{7.1}$$

$$\mu_f^{F11} = \frac{K_{\max}^{F11} S_f^{F11}}{K_s^{F11} + S_f^{F11}} \left(1 - \frac{S_f^{F11}}{S_m^{F11}}\right)^n \frac{C_f}{C_f + K_{O_2}^{F11}} \tag{7.2}$$

- During the experimental runs, no oxygen limitation was observed for the cells in suspension. Therefore, in order to simplify the mathematical model, DCE is considered as the only source of carbon and energy and as the only growth-limiting substrate for the *Xanthobacter autotrophicus* strain GJ10 in suspension and FB is considered as the only source of carbon and energy and as the only growth-limiting substrate for the strain F11;
- The biomass yield coefficients for DCE, FB and oxygen are assumed to be constant;

The OAB can be described by the following system of equations. The DCE mass balance in the gas stream and the accumulation of DCE in the sunflower oil

contained in the oil absorber unit are expressed by Equations 7.3 and 7.4 respectively.

The FB mass balance in the gas stream and the accumulation of FB in the sunflower oil contained in the oil absorber unit are expressed by Equations 7.5 and 7.6 respectively.

Also, the sunflower oil partition coefficient for DCE and for the FB (Equation 7.7 and 7.8 respectively) is temperature dependent as described previously (Koutinas, et al. 2006).

$$G\left(C_{a,in}^{DCE} - C_{a,out}^{DCE}\right) = \left(K_L a\right)_{oil}\left(C_{a,out}^{DCE} P_{sun,oil}^{DCE} - C_{sun,oil}^{DCE}\right)V_{oil} \tag{7.3}$$

$$\left(K_L a\right)_{oil}\left(C_{a,oil}^{DCE} P_{sun,oil}^{DCE} - C_{sun,oil}^{DCE}\right) = \frac{dC_{sun,oil}^{DCE}}{dt} \tag{7.4}$$

$$G\left(C_{a,in}^{FB} - C_{a,out}^{FB}\right) = \left(K_L a\right)_{oil}\left(C_{a,out}^{FB} P_{sun,oil}^{FB} - C_{sun,oil}^{FB}\right)V_{oil} \tag{7.5}$$

$$\left(K_L a\right)_{oil}\left(C_{sun,oil}^{FB} P_{sun,oil}^{FB} - C_{sun,oil}^{FB}\right) = \frac{dC_{sun,oil}^{FB}}{dt} \tag{7.6}$$

$$P_{sun,oil}^{DCE} = -12.1T + 1049.6 \tag{7.7}$$

$$P_{sun,oil}^{FB} = -19.7T + 1622.3 \tag{7.8}$$

For the bioscrubber unit of the OAB system, the DCE and FB mass balance in the gas stream is expressed by Equation 9 and 10 respectively, while the DCE and FB balances are expressed by Equations 7.11 and 7.12 respectively. The third term on the right hand side of Equations 7.11 and 7.12 accounts for the DCE and FB consumption in the biofilm.

Moreover the biomass balances for both species GJ10 and F11 are expressed by Equations 7.13 and 7.14. The last term of Equations 7.13 and 7.14 expresses the rate of biomass detachment from the biofilm.

The microbial kinetics for *Xanthobacter autotrophicus* strain GJ10 growing on DCE follow Luong kinetics (Luong, 1986) and include an inhibitory effect with increasing DCE concentrations up to 1080 g_{DCE} m^{-3} (Equation 7.15). The microbial kinetics for the strain F11 growing on FB follow Luong kinetics (Luong, 1987) and include an inhibitory effect with increasing FB concentrations up to 3748 g_{FB} m^{-3} (Equation 7.16)

$$G\left(C_{a,out}^{DCE} - C_{b,out}^{DCE}\right) = \left(K_L a\right)_{DCE}\left(\frac{C_{b,out}^{DCE}}{H_{DCE}} - S_b^{DCE}\right)V_b \tag{7.9}$$

$$G\left(C_{a,out}^{FB} - C_{b,out}^{FB}\right) = \left(K_L a\right)_{FB}\left(\frac{C_{b,out}^{FB}}{H_{FB}} - S_b^{FB}\right)V_b \tag{7.10}$$

$$\left(K_L a\right)_{DCE}\left(\frac{C_{b,out}^{DCE}}{H_{DCE}} - S_b^{DCE}\right)V_b = MS_b^{DCE} + \frac{\mu_b^{GJ10}}{Y_{DCE}}X_b^{GJ10}V_b + k_f^{DCE}A\left(S_b^{DCE} - S_f^{DCE}\Big|_{z=L_f}\right) + \frac{dS_f^{DCE}}{dt}V_b \tag{7.11}$$

$$\left(K_L a\right)_{F11}\left(\frac{C_{b,out}^{FB}}{H_{FB}} - S_b^{FB}\right)V_b = MS_b^{FB} + \frac{\mu_b^{F11}}{Y_{FB}}X_b^{F11}V_b + k_f^{FB}A\left(S_b^{FB} - S_f^{FB}\Big|_{z=L_f}\right) + \frac{dS_f^{FB}}{dt}V_b \tag{7.12}$$

$$\frac{dX_b^{GJ10}}{dt}V_b = \left(\mu_b^{GJ10} - b_d^{GJ10}\right)X_b^{GJ10}V_b - MX_b^{GJ10} + Ab_s^{GJ10}L_f X_b^{GJ10} \tag{7.13}$$

$$\frac{dX_b^{F11}}{dt}V_b = \left(\mu_b^{F11} - b_d^{F11}\right)X_b^{F11}V_b - MX_b^{F11} + Ab_s^{F11}L_f X_b^{F11} \tag{7.14}$$

$$\mu_b^{GJ10} = \frac{\mu_b^{GJ10}S_b^{DCE}}{K_s^{GJ10} + S_b^{DCE}}\left(1 - \frac{S_b^{DCE}}{S_m^{DCE}}\right) \tag{7.15}$$

$$\mu_b^{F11} = \frac{K_{\max}^{F11}S_b^{FB}}{K_s^{F11} + S_b^{FB}}\left(1 - \frac{S_b^{FB}}{S_m^{FB}}\right)^n \tag{7.16}$$

The variations in DCE, FB and oxygen concentrations in the biofilm are described by Equations 7.16 and 7.18 which are subjected to the boundary conditions given by Equations 7.17 and 7.19. At z=0, the consumption of DCE, FB and oxygen is not permitted due to the non-porous solid (interface between the walls of the reactor and the biofilm).

$$\frac{dS_f^{DCE}}{dt} = D_f^{DCE}\frac{d^2S_f^{DCE}}{dz^2} - \mu_f^{GJ10}\frac{X_f^{GJ10}}{Y_{DCE}}; \qquad 0<z<L_f \tag{7.17}$$

$$\frac{dS_f^{DCE}}{dz} = 0 \text{ , at z=0;} \quad D_f^{DCE}\frac{dS_f^{DCE}}{dz} = k_f^{DCE}\left(S_b^{DCE} - S_f^{DCE}\right), \quad \text{at } z=L_f \tag{7.18}$$

$$\frac{dS_f^{FB}}{dt} = D_f^{FB}\frac{d^2S_f^{FB}}{dz^2} - \mu_f^{F11}\frac{X_f^{F11}}{Y_{FB}}; \qquad 0<z<L_f \qquad (7.19)$$

$$\frac{dS_f^{FB}}{dz} = 0 \text{, at z=0;} \qquad D_f^{FB}\frac{dS_f^{Fb}}{dz} = k_f^{FB}\left(S_b^{FB} - S_f^{FB}\right), \qquad \text{at z=}L_f \qquad (7.20)$$

$$\frac{dC_f}{dt} = D_{of}\frac{d^2C_f}{dz^2} - \mu_f^{GJ10}\frac{X_f^{GJ10}}{Y_{O_2}^{GJ10}} - \mu_f^{F11}\frac{X_f^{F11}}{Y_{O_2}^{F11}}; \qquad 0<z< L_f \qquad (7.21)$$

$$\frac{dC_f}{dz} = 0 \text{, at z=0;} \qquad D_{of}\frac{dC_f}{dz} = k_{of}\left(C_B - C_f\right), \qquad \text{at z=}L_f \qquad (7.22)$$

Cells attached to the biofilm grow due to the utilisation of the substrate, can get inactive and can be removed as a result of the biomedium in motion. Taking into account the above, Equation 7.13 can describe the biomass balance in the biofilm, as well as the biofilm thickness over time.

$$\frac{dL_f}{dt} = \int_0^{L_f}\left[\mu_f^{GJ10} + \mu_f^{F11} - b_d - b_s\right]dz \qquad (7.23)$$

Where it has been assumed:

$$b_s^{GJ10} = b_s^{F11} = b_s / 2 \qquad (7.24)$$

$$b_d^{GJ10} = b_d^{F11} = b_d / 2 \qquad (7.25)$$

Equations 7.1 - 7.25 were solved using gPROMS (Process Systems Enterprise, UK). The parameters used in the model are presented in Table 7.I. The input

values of the bioscrubber inlet DCE and FB gas concentration ($C_{a,out}^{DCE}, C_{a,out}^{FB}$) for System I operation and the oil absorber inlet DCE and FB gas concentration ($C_{a,in}^{DCE}, C_{a,in}^{FB}$) for System II operation, were estimated according to the experimental loading introduced to the system each time.

The biofilm shear loss coefficient (b_s) and the coefficient for cell death (b_d^{GJ10}, b_d^{F11}) were derived from the literature for the detachment and decay of sludge cells from a biofilm.

The mass transfer coefficients for DCE, FB and oxygen in the biofilm ($k_f^{DCE}, k_f^{FB}, k_{of}$) were also taken from the literature. k_f^{DCE} and k_f^{FB} were obtained for the transport of trichloroethylene in a GJ10 biofilm and k_{of} for the transport of oxygen in a mixed microbial culture biofilm degrading monochlorobenzene.

Finally, the dissolved oxygen concentration in the bulk biological liquid (C_B) was measured experimentally and it was observed that did not vary significantly with time during the experimental runs. Therefore, the experimental value monitored was used for the simulations.

7.4 Results and Discussion

7.4.1 Modeling of the OAB-BO Experiment during S.A.P feed condition: set [GJ10 on DCE] / [F11 on FB]

In Figure 7.1 and Figure 7.2 the fitting between the experimental data and the model during the Oil Absorber Bioscrubber experiment (OAB) for the FB load profile and DCE load profile respectively has been reported.

Whereas the model fits with good results the performance of the load profile of the feeding from the Bioscrubber (Bioscrubber inlet) coming from the Oil Asorber for each halogenatod volatile compound, a little discrepancy has to be noticed for the load profile in the Bioscrubber outlet.

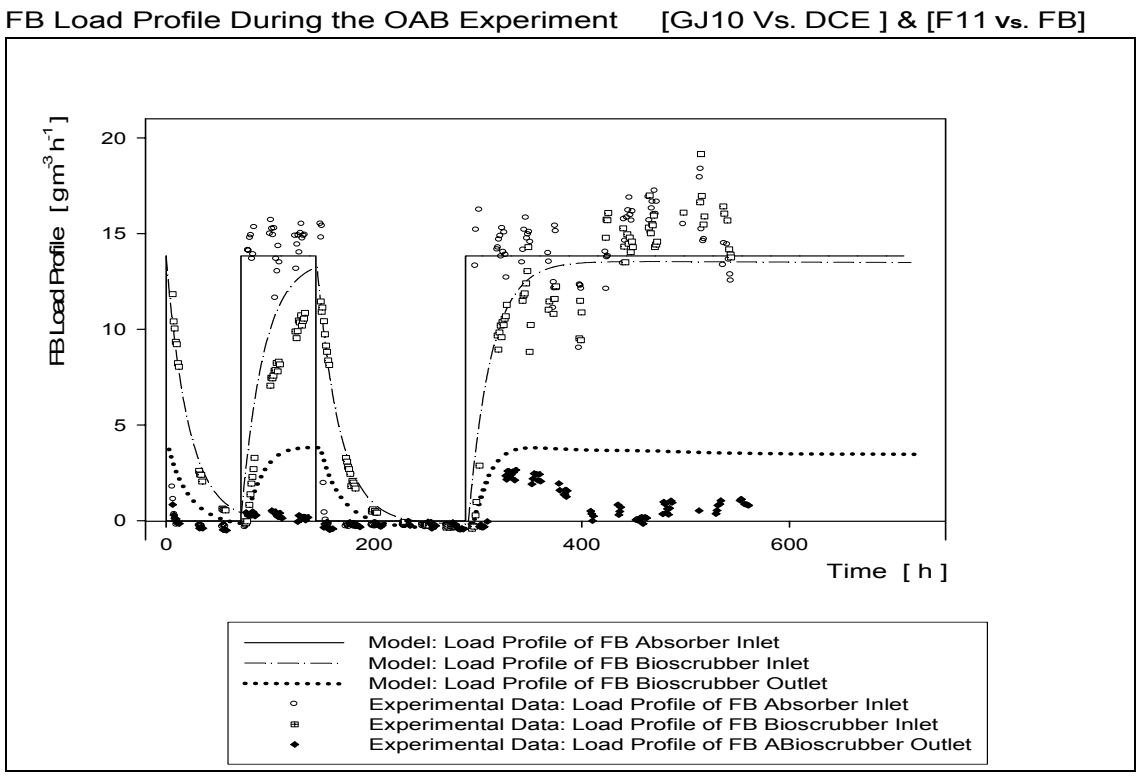


Figure 7.1 - *Comparison between model and experimental data for the FB load profile during the OAB Experiment*

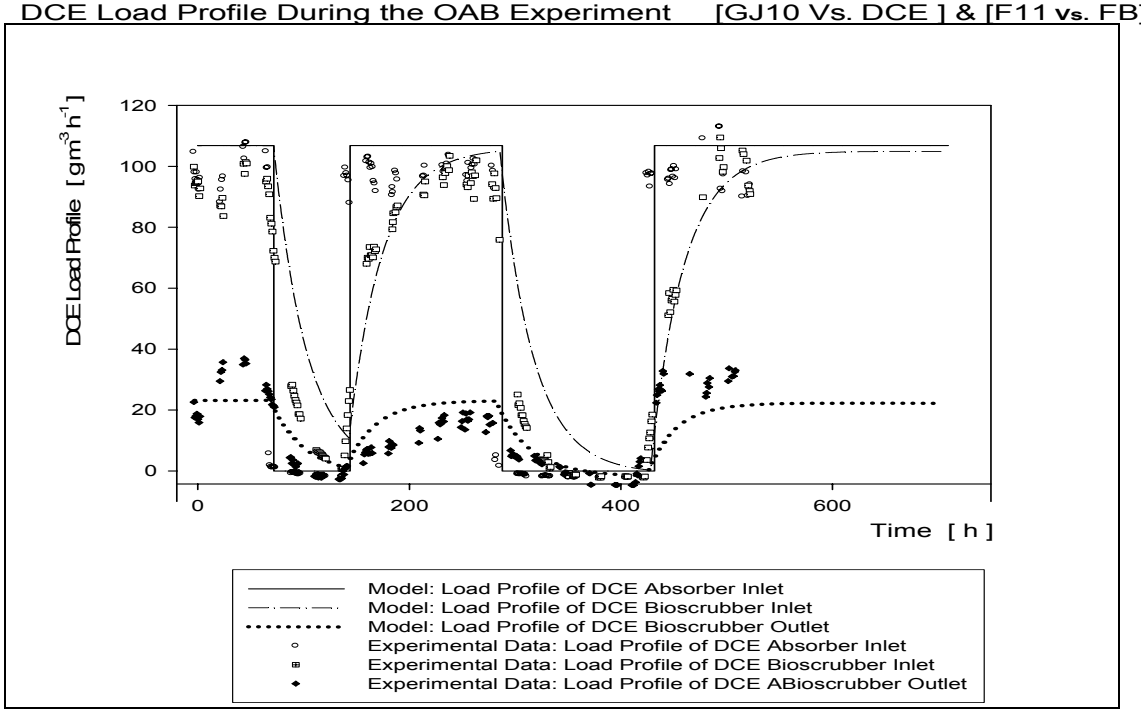


Figure 7.2 - *Comparison between model and experimental data for the DCE load profile during the OAB Experiment*

The values for the FB load profile in the Bioscrubber outlet obtained by the model overestimates the amount of fluorinated compound released untreated in the environment, while for the DCE load profile in the Bioscrubber outlet the model curve fits satisfactorily the data point.

Figure 7.3 and Figure 7.4 show respectively load profile of FB and DCE in comparison with the measured data point ands the results obtained by the model for the same system operating without the Oil-Absorber (BO Bioscrubber Only configuration) with the same condition of load of the previous experiment.

In this particular case it seems that the curves purposed by the model don't fit in a proper with the data points, especially with the load profile of DCE this time.

A role in this discrepancy could be due to the cloudy distribution of data points related with the bioscrubber inlet profile: apparently it's very difficult to mantain the set point value for the halogenated compound in the gas stream and this could affect the fitting.Once more is evident the importance of the oil absorber upstream to the bioscrubber because of its smoothening effect on the load profile in the bioscrubber inlet.

Has to be noticed that the model predict the small peak in the DCE load profile due to the re-introduction at time 432 h of the fluorinated compound.

During the evaluation of the model describing the bioscrubber performance it was found the kinetics parameters for the strain F11 playing a very sensible role and for this reason they were object of a further investigation described in the following chapter.

In relation with that aspect, the model helped to find a consistent error in the methodology adopted for obtaining the kinetic parameters obtained from the literature regarding the strain F11.

7.4.2 Evaluation of inhibitory effect on the strain GJ10

The possibility of an inhibition effect on the strain JG10 due to ion fluoride in the biomedium of the bioscrubber has been hypothesized and a tested

in a fed-batch system. Several tests at different fluoride ion in the mineral media have been set up to evaluate the effect on the growth rate.

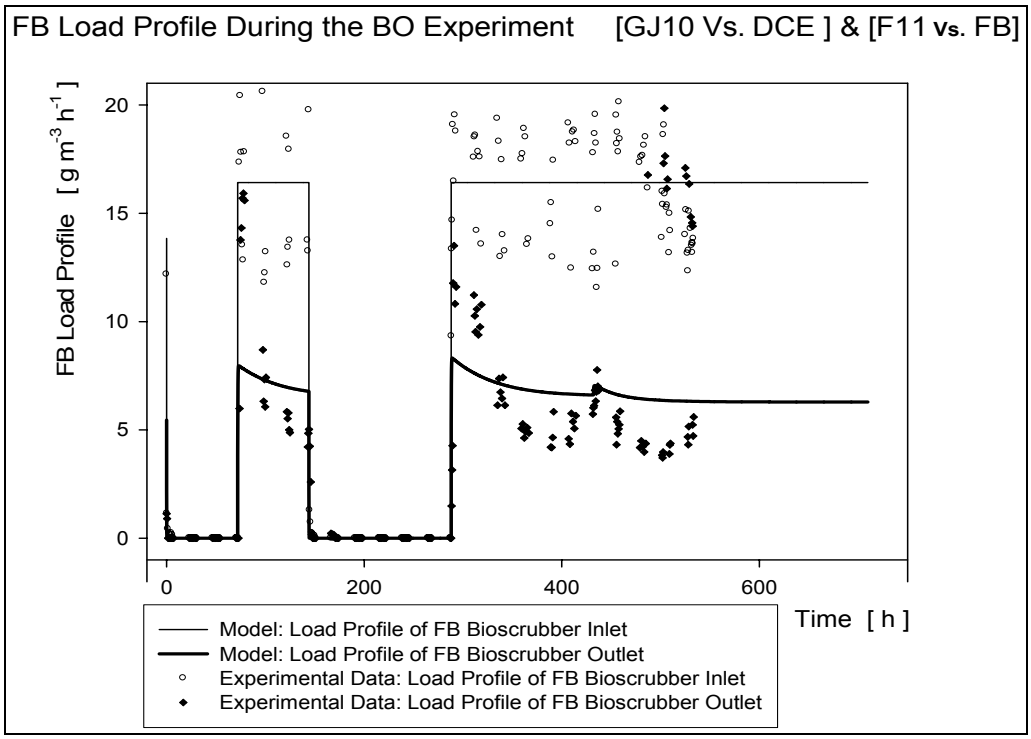


Figure 7.3 - *Comparison between model and experimental data for the FB load profile during the BO Experiment*

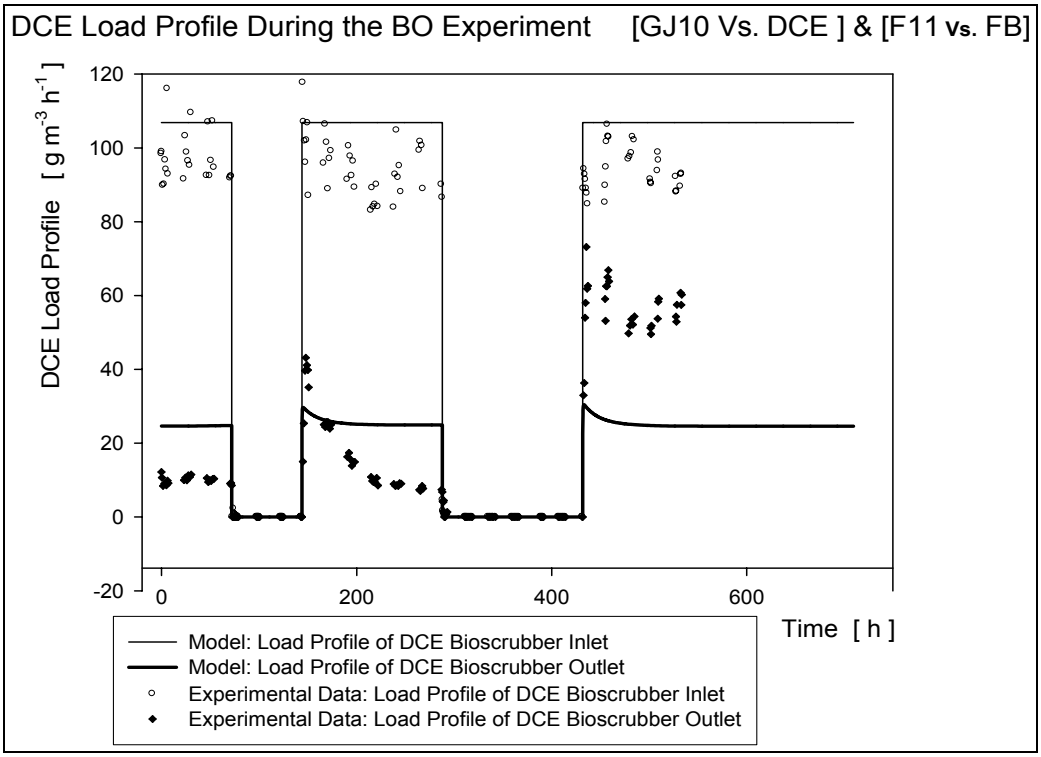


Figure 7.4 - *Comparison between model and experimental data for the DCE load profile during the BO Experiment*

In figure 7.5 have been shown the results of this experiment where it's possible to appreciate the inhibitory effect on the strain GJ10 Analyzing the trend of the growth rate at different ion fluoride concentration the inhibitory effect is evident even at low concentration (200 ppm).

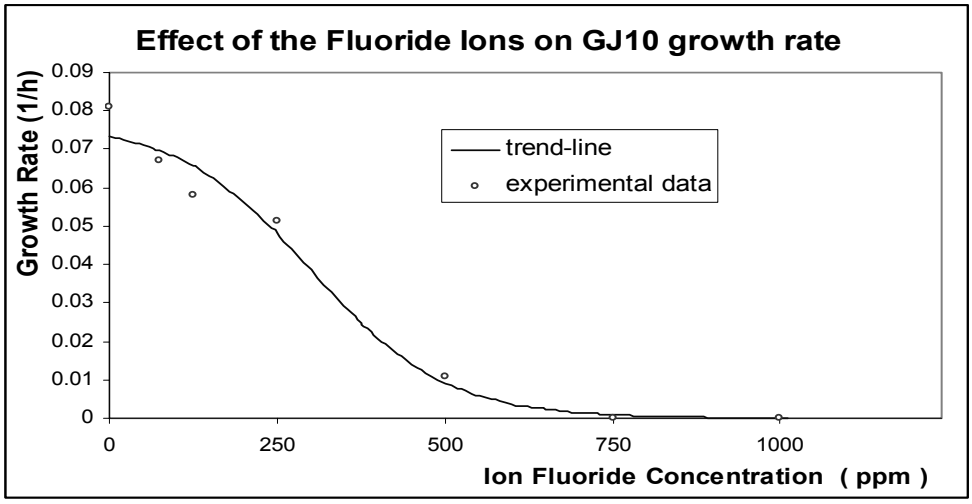


Figure 7.5 - *trend of the growth rate at different ion fluoride concentration*

The experimental data suggest a trend line which could be normalized in order to find a correction factor for the purposed GJ10 growth rate as reported in figure 7.6: this term changes the μ_b^{GJ10} as it follows, where s^{Cl^-} is the concentration of ion fluoride in the biomedium.

$$\mu_b^{GJ10} = \frac{\mu_b^{GJ10} S_b^{DCE}}{K_s^{GJ10} + S_b^{DCE}} \left(1 - \frac{S_b^{DCE}}{S_m^{DCE}}\right) \left(\frac{1}{0.953 + 8.8e^{0.01 S^{Cl^-}}}\right) \tag{7.26}$$

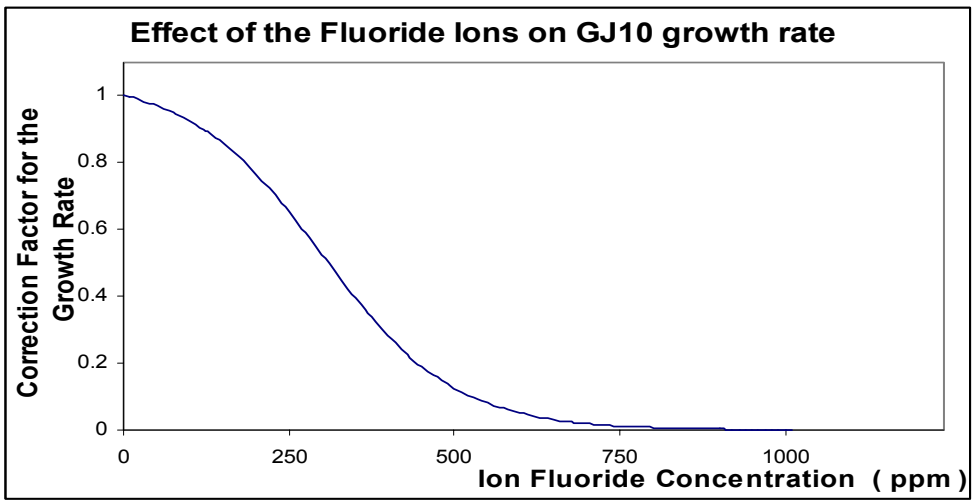


Figure 7.6 – *Correction factor for GJ10 growth rate*

Nevertheless as shown in Figure 7.7 for the whole duration of the BO experiment the concentration of fluoride 1on in the biomedium, evaluated by ion cromatograhic analysis and supported by the result of the model, is in a range of value below 100 ppm, where the inhibition effect on GJ10 growth rate is not evident.

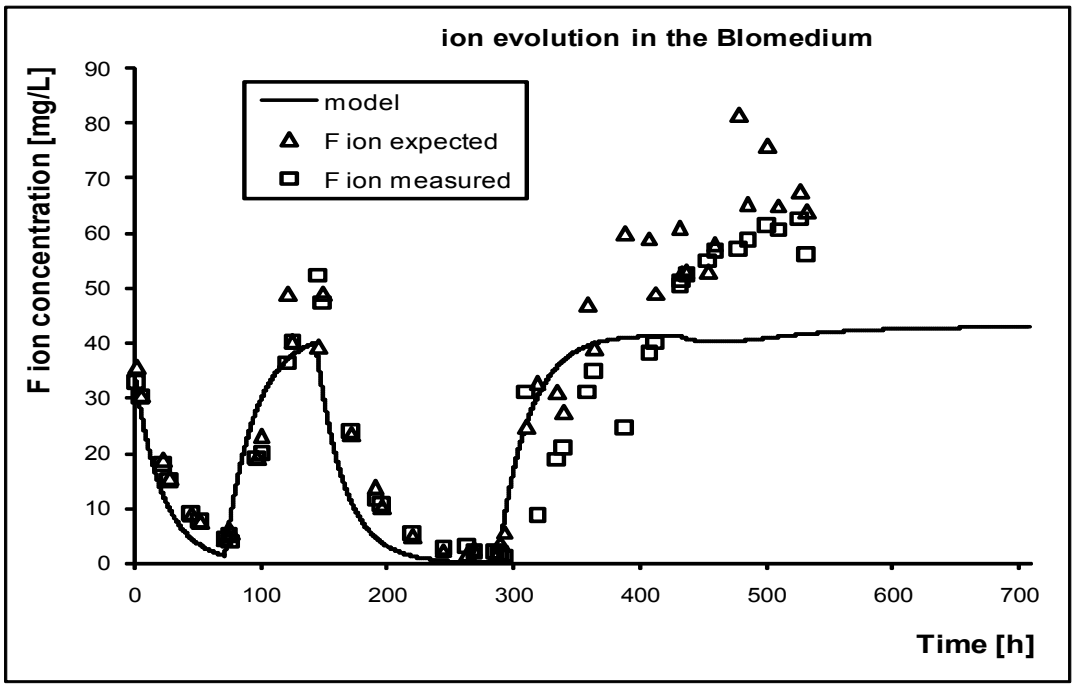


Figure 7.7 - *Concentration of Fluoride Ion in the biomedium during the BO experiment*

7.5 Conclusions

The model purposed describes satisfactorily the behaviour of the OAB system and has been proved as a powerful tool to individuate the sensittive parameters that affect the process.

The result obtained excludes the possibility of an hinibitory effect due to the concentratrion of the ion fluoride in the biomediumindicating a different factor for the decrease in performance of the bioreactor.

7.6 Nomenclature

A	Surface area available for biofilm attachment and growth [dm^2]
b_d	Coefficient for cell death [s^{-1}]
b_d^{GJ10}	Coefficient for cell death for the strain GJ10 [s^{-1}]
b_d^{F11}	Coefficient for cell death for the strain F11 [s^{-1}]
b_s	Biofilm shear loss coefficient [s^{-1}]
b_s^{GJ10}	Biofilm shear loss coefficient for the strain GJ10 [s^{-1}]
b_s^{F11}	Biofilm shear loss coefficient for the strain F11 [s^{-1}]
$C_{a,in}^{DCE}$	Oil absorber inlet DCE gas phase concentration [mg L^{-1}]
$C_{a,out}^{DCE}$	Oil absorber outlet (or bioscrubber inlet) DCE gas phase concentration [mg L^{-1}]
$C_{a,in}^{FB}$	Oil absorber inlet FB gas phase concentration [mg L^{-1}]
$C_{a,out}^{FB}$	Oil absorber outlet (or bioscrubber inlet) FB gas phase concentration [mg L^{-1}]
C_B	Dissolved oxygen concentration in bulk biological liquid [mg L^{-1}]
$C_{b,out}^{DCE}$	Bioscrubber outlet DCE gas phase concentration [mg L^{-1}]
$C_{b,out}^{FB}$	Bioscrubber outlet FB gas phase concentration [mg L^{-1}]
C_f	Dissolved oxygen concentration in the biofilm [mg L^{-1}]
$C_{sun,oil}^{DCE}$	DCE concentration in sunflower oil [mg L^{-1}]
$C_{sun,oil}^{FB}$	FB concentration in sunflower oil [mg L^{-1}]
D_f^{DCE}	Diffusion coefficient of DCE in the biofilm [dm^2 s^{-1}]
D_f^{FB}	Diffusion coefficient of FB in the biofilm [dm^2 s^{-1}]
D_{of}	Diffusion coefficient of oxygen in the biofilm [dm^2 s^{-1}]
G	Gas phase flow rate [L s^{-1}]
H_{DCE}	DCE Henry's law coefficient based on concentration ratio [-]

H_{FB}	FB Henry's law coefficient based on concentration ratio [-]
k_f^{DCE}	Mass transfer coefficient for DCE in the biofilm [dm s^{-1}]
k_f^{FB}	Mass transfer coefficient for FB in the biofilm [dm s^{-1}]
k_{of}	Mass transfer coefficient for oxygen in the biofilm [dm s^{-1}]
$(K_La)_{DCE}$	DCE volumetric mass transfer coefficient [s^{-1}]
$(K_La)_{FB}$	FB volumetric mass transfer coefficient [s^{-1}]
$(K_La)_{oil}$	Volumetric mass transfer coefficient in the oil absorber [s^{-1}]
$K_{O_2}^{GJ10}$	Monod rate constant for oxygen for the strain GJ10 [mg L^{-1}]
$K_{O_2}^{F11}$	Monod rate constant for oxygen for the strain GJ10 [mg L^{-1}]
K_s^{GJ10}	Substrate saturation constant for the strain GJ10 [mg L^{-1}]
K_s^{F11}	Substrate saturation constant for the strain F11 [mg L^{-1}]
L_f	Biofilm thickness [dm]
M	Mineral medium flow rate [L s^{-1}]
n	Indicative parameter of the relation between the growth rate and the substrate concentration for the strain F11 [-]
$P_{sun,oil}^{DCE}$	Partition coefficient for DCE between sunflower oil and air [-]
$P_{sun,oil}^{MCB}$	Partition coefficient for MCB between sunflower oil and air [-]
$P_{sun,oil}^{FB}$	Partition coefficient for FB between sunflower oil and air [-]
S_b^{DCE}	DCE concentration in the biomedium [mg L^{-1}]
S_m^{DCE}	DCE biomedium concentration for complete inhibition [mg L^{-1}]
S_f^{DCE}	DCE concentration in the biofilm [mg L^{-1}]
S_b^{FB}	FB concentration in the biomedium [mg L^{-1}]
S_f^{FB}	FB concentration in the biofilm [mg L^{-1}]
S_m^{FB}	FB biomedium concentration for complete inhibition [mg L^{-1}]
T	Oil absorber temperature [^oC]
t	Time [s]
V_b	Bioscrubber volume [L]
V_{oil}	Volume of sunflower oil [L]
V_v	Volume of void space in the bioscrubber [L]

X_b^{GJ10}	Suspended biomass concentration for the strain GJ10 [mg L^{-1}]
X_b^{F11}	Suspended biomass concentration for the strain F11 [mg L^{-1}]
X_f^{GJ10}	Cell concentration in the biofilm for the strain GJ10 [mg L^{-1}]
X_f^{F11}	Cell concentration in the biofilm for the strain F11 [mg L^{-1}]
Y_{DCE}	Yield coefficient for biomass on DCE [$g_{biomass}$ g_{DCE}^{-1}]
Y_{FB}	Yield coefficient for biomass on DCE [$g_{biomass}$ g_{DCE}^{-1}]
$Y_{O_2}^{GJ10}$	Yield coefficient for biomass on oxygen related to the strain GJ10 [$g_{biomass}$ g_{O2}^{-1}]
$Y_{O_2}^{F11}$	Yield coefficient for biomass on oxygen related to the strain F11 [$g_{biomass}$ g_{O2}^{-1}]
z	Distance in the biofilm for the solid-biofilm interface [dm]

Greek letters

μ_b^{GJ10}	Specific growth rate for cells in suspension for the strain GJ10 [s^{-1}]
μ_b^{F11}	Specific growth rate for cells in suspension for the strain F11 [s^{-1}]
μ_f^{GJ10}	Specific growth rate for cells in the biofilm for the strain GJ10 [s^{-1}]
μ_f^{F11}	Specific growth rate for cells in the biofilm for the strain F11 [s^{-1}]
μ_{max}^{GJ10}	Maximum specific growth rate for the strain GJ10 [s^{-1}]
μ_{max}^{F11}	Maximum specific growth rate for the strain F11 [s^{-1}]

Table 7.1: Values of parameters used in the model.

Parameter	***Value***	***Reference***
A	11.04 dm^2	Present study
b_d	2.14×10^{-7} s^{-1}	Lin and Lee, 2001
b_s	3.322×10^{-6} s^{-1}	Fouad and Bhargava, 2005
C_B	2.5 mg L^{-1}	Present study
D_f^{DCE}	6.66×10^{-7} dm^2 s^{-1}	Freitas dos Santos and Livingston, 1995
D_f^{FB}	6.66×10^{-7} dm^2 s^{-1}	Assumed
D_{of}	1.54×10^{-7} dm^2 s^{-1}	Freitas dos Santos and Livingston, 1995
G	0.005 L s^{-1}	Present study
H_{DCE}	0.0562 [-]	Present study
H_{FB}	0.0387 [-]	Sander,1999
K_s^{GJ10}	7.8 mg L^{-1}	Ferreira Jorge, 2000
K_s^{F11}	153,7 mg L^{-1}	Carvalho, 2005
k_f^{DCE}	2.2×10^{-4} dm s^{-1}	Zhang,1998
k_f^{FB}	2.2×10^{-4} dm s^{-1}	Assumed
k_{of}	9.4×10^{-4} dm s^{-1}	Nicolella, et al. 2000
$(K_La)_{DCE}$	7.486×10^{-4} s^{-1}	Present study
$(K_La)_{FB}$	2.985×10^{-3} s^{-1}	Present study
$(K_La)_{oil}$	5×10^{-5} s^{-1}	Koutinas, et al. 2006
$K_{O_2}^{GJ10}$	0.01 mg L^{-1}	Freitas dos Santos and Livingston, 1995
$K_{O_2}^{F11}$	0.01 mg L^{-1}	Assumed

M	1.906×10^{-5} L s^{-1}	Present study
n	2.3	Carvalho, 2005
S_m^{DCE}	1080 mg L^{-1}	Ferreira Jorge, 2000
S_m^{FB}	3748 mg L^{-1}	Carvalho, 2005
V_b	1.4 L	Present study
V_{oil}	0.56 L	Present study
X_f^{GJ10}	5.7×10^4 mg L^{-1}	Zhang, et al. 1998
X_f^{F11}	5.7×10^4 mg L^{-1}	Assumed
Y_{DCE}	0.23 g_{biom} g_{DCE}^{-1}	Ferreira Jorge, 2000
Y_{FB}	1.66 g_{biom} g_{FB}^{-1}	Present study
$Y_{O_2}^{GJ10}$	0.304 g_{biom} g_{O2}^{-1}	Freitas dos Santos and Livingston, 1995
$Y_{O_2}^{F11}$	1.375 g_{biom} g_{O2}^{-1}	Present study
μ_{max}^{GJ10}	4.167×10^{-5} s^{-1}	Ferreira Jorge, 2000
μ_{max}^{F11}	2.167×10^{-5} s^{-1}	Carvalho, 2005

Chapter 8

An Attempt to Compare Different Approaches to Evaluate Kinetic Parameters: parallel analysis of Batch and Fed-Batch Method

All the tests have been performed using the facilities of the Imperial College London under the supervision of Professor Andrew G. Livingston

8.1 Abstract

The evaluation of growth kinetic parameters of microorganisms is commonly performed by batch methods involving shaking flasks experiments.

This approach, widely used because of low cost and simple handling, could be affected by limitations such as inadequate supplies of substrate and oxygen and inhibition effects due to the increasing concentration of metabolites or biodegradation products: these restrictions are able to directly alter kinetic measurements and biomass performances.

This chapter purpose a comparison between the results obtained with batch experiments based on shaking flask and a different fed-batch method that could be easily applied to determine kinetic parameter avoiding these limitations.

The parallel evaluation of growth kinetic parameters of two pure strains of microorganisms by both methods suggest a possible range of condition where these different approaches are equivalent and in which case the fed-batch method is applicable as the most suitable one.

8.2 Introduction

The batch method based on shaking flasks is one of the most common approaches used to determine the growth kinetics of the pure strains and for the biosynthesis of microbial products.

Unfortunately this method is affected by limitations during the whole depletion process typically related with the batch operating conditions, such as: changing of pH, decreasing of concentration of oxygen and substrate and increasing of metabolite or biodegradation product concentration [24].

These problems could be avoided using a different fed-batch method that involves a C.S.T.R. bioreactor used in a bio-scrubber configuration with a continuous gas feeding at a constant concentration of oxygen and substrate.

This novel method was developed for the determination of microbial growth kinetic on hydrophobic volatile organic compound (Ferreira et al. [25])

A comparison between this two different approaches has been studied on the scale-up of fermentation process, from shaking flask to fermentors, where it was evident how a non-homogeneous variations (include temperature, dissolved oxygen, and fluid shear) could affect the stability of the strains [26].

These variations can cause strain degradation and failure of a strain to perform satisfactorily over long fermentation times in large-scale vessels, whereas in the experiments set-up to determine the kinetic parameters, the shorter operating times (1-3 days) and the smaller volume of the bioreactor (2.5 L) required, avoid this category of mixing problems.

In order to define the suitability of the two different methods, the results obtained during the growth kinetic characterisation of two systems strain/substrate (Alpha Proteobacteria sp. strain F11 [AY362040] on fluorobenzene and *Pandoreae pnomenusa* sp. strain MCB032 [AY686701] on monochlorobenzene) have been analysed in a parallel way and, where is possible, compared with result available in literature.

Several examples of studies involving shaking flask experiment are available in litterture (M.F. Carvalho P.Castro) [27,28] and an evaluation of different solutions to the problems related with the shaking flask method have

been suggested as the generally applied substrate addition protocol or different feeding scheme [29].

This work aims to compare the results obtained using two different methods and the results suggest the suitability of the batch method only in a restricted range of substrate concentrations, highly dependent on the growth kinetic investigated, whereas the fed-batch method allows a better description of the growth phenomena in the whole range of the interesting substrate concentration, overcoming the problem related with the limitation intrinsic to the use of shaking flask

8.3 Materials and methods

8.3.1 Cultivation of Microorganisms and Mineral Medium composition

Alpha Proteobacteria sp. strain F11 [AY362040] was kindly provided by Prof P. M. L. Castro for use in this work and is capable of utilising Fluorobenzene (FB) as a sole source of carbon and energy via direct metabolism under aerobic condition.

Pandoreae pnomenusa sp. strain MCB032 [AY686701] is capable to utilise monochlorobenzene (MCB) as a sole source of carbon and energy and was isolated as a new dominant strain that overtook the bioreactor out-competing specific strain GS150 as previously reported [30].

Subcultures of both strains have been prepared and maintained in mineral medium with substrate concentration in aqueous phase of 200 mg L^{-1} of FB for the strain F11 and 150 mg L^{-1} of MCB for the strain MCB032 respectively.

The concentration of the mineral medium in batch (shake flasks) and fed-batch experiments was 1360 g m^{-3} KH_2PO_4, 2130 g m^{-3} Na_2HPO_4, 500 g m^{-3} $(NH_4)_2SO_4$, 200 g m^{-3} $MgSO_4 \cdot 7H_2O$ g m^{-3} O, 5 mL L^{-1} trace elements solution and 1 mL L^{-1} vitamin solution as described in chapter 6 (Table 6.1 and 6.2).

8.3.2 Experimental Set-Up

The experimental set up used is presented in schematic diagram reported in Figure 8.1. The total flow rate of air influent to the system was 0.4 L min-1 consisted of two different gas streams, giving a volumetric gas flow rate per bioscrubber volume of 0.4 min-1. Stream 18 was enriched with the halogenated substrate by passing via a sintered glass sparger through the saturation vessel containing pure compounds, while stream 17 comprised of air.

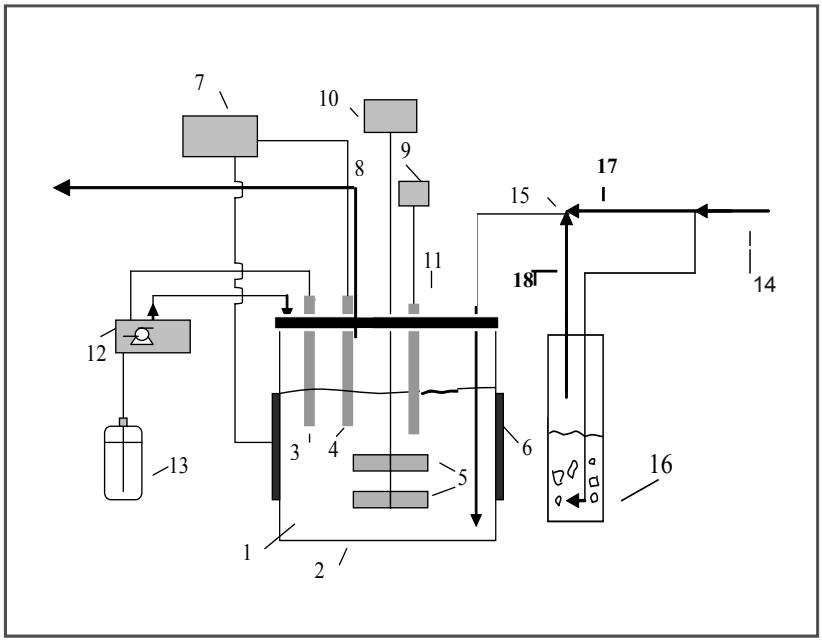


Figure 8.1 - *Experimental set-up for the fed-batch reactor.1)biomedium; 2)bioreactor; 3)pH probe; 4)thermocouple; 5)mixing shaft; 6)heating jacket; 7) temperature controller; 8) gas outlet; 9) dissolved oxygen probe 10) mixing control unit 11) sampling port; 12) pH controller 13) 1M NaOH solutiρn; 14) air line; 15) bioscrubbergas inlet; 16) saturation vessel; 17) air make- up; 18) air line saturated in halogenated compound;*

The gas was distributed in the biomedium aqueous phase via a stainless steel sparger. Two impellers, rotating at 300 rpm (a 'marine' impeller at the bottom of the shaft and a Rushton impeller at the middle of the biomedium height), provided optimal mixing of the two phases.

The bioscrubber was an SGI "30/SET002" (SGE, France) model with a total volume of 1.8 L, which was operated as a continuous stirred tank bioreactor (CSTB) at 1,5 L working volume.

The bioscrubber was a Bioflow reactor with a total volume of 2.0 L, which was operated as a continuous stirred tank bioreactor (CSTB) at 1 L working volume.

The pH was controlled at 7 ± 0.05 by the addition of 1M solutions of HCl or NaOH, temperature was kept constant at 30 °C and the dissolved oxygen concentration was monitored with an Ingold (Mettler Toledo Ltd, UK) oxygen probe.

8.3.3 Analysis

GC analysis was employed for determination of the DCE concentration in the gaseous and aqueous samples. An Agilent 6850 Series II Gas Chromatograph with an FID detector and a 'J&W Scientific' (Agilent Technologies UK Limited, UK) column with HP-1 stationary phase (30 m × 0.32 mm × 0.25 μm) was used.

For gaseous samples, 25 μL were injected into the GC and the temperature program run at a constant temperature of 30 °C for 8 min. However, the flow rate of the mobile phase (He) was reduced to 1 ml min-1.

Biomedium samples were centrifuged for 10 min at 13000 rpm and the supernatant solution was filtered through 0.2 μm filters to remove any remaining solids. 8 ml of the centrifuged sample was vortexed for 1 min with 2.5 ml of n-decane during the experiments with the set strain substrate F11 / fluorobenzene or with 2.5 ml of dichloromethane during the experiments with the set strain substrate MCB032 / monochlorobenzene and finally 1 μL of the n-decane or dichloromethane solution was injected into the GC.

For analysis of these biomedium samples, the GC column temperature was kept constant for 8 min at 30 °C and then increased by 40 °C min^{-1} to 260 °C.

The coefficients of variation for 6 samples were 3.4 % for MCB and 3.7 % for FB at concentration levels of 5.5 mg_{MCB} L^{-1} and 1.1 mg_{FB} L^{-1}.

Carbon dioxide concentration of the bioscrubber gas effluent was determined using an isothermal GC (Shimadzu GC-14A, Shimadzu, UK) fitted with a thermal conductivity detector (TCD). Samples were injected to a Porapak N column packed with DVB (divinylbenzene)/vinylpyrrolidinone at 28 ^{o}C.

The coefficient of variation for 5 samples was 2.6 % at a concentration level of 0.03 % v/v carbon dioxide.

Biomass concentration was determined by absorbance at 660 nm on a UV-VIS spectrophotometer (Spectronic Unicam, UK) interpolating from a previously established dry weight calibration curve. The coefficient of variation for 5 samples was 2.3 % at a concentration level of 0.5 $mg_{biomass}L^{-1}$.

The anion and cation concentrations were also estimated. The analysis was performed with an Ion Chromatograph Dionex DX-120 (Dionex (UK) Ltd, UK) coupled to a Dionex AS40 automated sampler. The anion column was an IonPac AS14 (4×250 mm) and the carrier liquid 3.5 mM Na_2CO_3 / 1 mM $NaHCO_3$.

The cation column was an IonPac CS12A (Dionex (UK) Ltd, UK) and the carrier liquid was 19 mM CH_4O_3S (methanesulphonic acid). The anions that could be detected were: fluoride, acetate, chloride, nitrite, bromide, nitrate, phosphate and sulphate. The cations that could be detected were: lithium, sodium, ammonium, potassium, magnesium and calcium.

Biomass concentrations were determined by filtering a known volume of a sample through a pre-weighed and dried 0.2-µm-pore nitrate cellulose membrane (Whatman, UK). These filters were then dried at 105 °C for 48 h, and the weight increase measured.

For each sample the absorbance of the solution was also measured at 660 nm and a calibration curve was generated.

8.3.4 Chemicals

All chemicals used in the preparation of media were obtained from BDH (UK) and were of ANALAR grade. All the organic solvents were obtained from Sigma, UK, and were 99% pure.

8.3.5 Estimation of the parameters for the microbial growth kinetic for the strain F11 and MCB032 by fed-batch method

The growth kinetic of the strain F11 was determined using the method described by Ferreira Jorge and Livingston (1999): using the operating mode described above results in a constant concentration of VOC substrate in the reactor liquid phase for low biomass concentrations. To see why this is so, consider the mass-balance equation for the VOC in the reactor aqueous phase:

$$\frac{dS}{dt} = -\mu \frac{X}{Y} + K_L a(S^* - S) \qquad (8.1)$$

where S is the substrate liquid concentration, μ is the specific growth rate, X is the biomass concentration, Y is the yield coefficient, K_L is the overall mass-transfer cofficient, S* is the substrate liquid-phase concentration in equilibrium with the bulk gas phase, and a is the gas-liquid interfacial area.

When the biomass concentration is less than around 400 mg L^{-1}, the substrate uptake by the cells is negligible.The total gas flow is high enough (1.5 vol vol^{-1} min^{-1}) that changes in the gas-phase concentration of the VOC across the aqueous phase are neglible.

With vigorous agitation to provide high KLa values, the VOC concentration in the aqueous phase is approximately equal to its concentration at the gas-liquid interface (S = S*). Therefore, dS/dt = 0 and substrate concentration in the aqueous phase will be constant at S. This was confirmed experimentally. By independently changing the ratio of VOC-laden to clean air flows, different and controllable values of S were obtained. Samples were periodically taken for biomass and chloride concentration determinations.All the work was carried out

under sterile condition and the purity of the culture was checked by growth at 35ºC on NB plates.

The experimental tests covered the range between 0.10 mM and 8.10 mM of Fluorobenzene concentration in the biomedium (typical profile has been shown in Figure 8.2**)** and for each experiment the specific growth rate has been calculated as the slope of the straight-line ln (biomass concentration/ initial biomass concentration) Vs. time (as reported in Figure 8.3) in the exponential-growth phase of the microorganisms into the bioreactor.

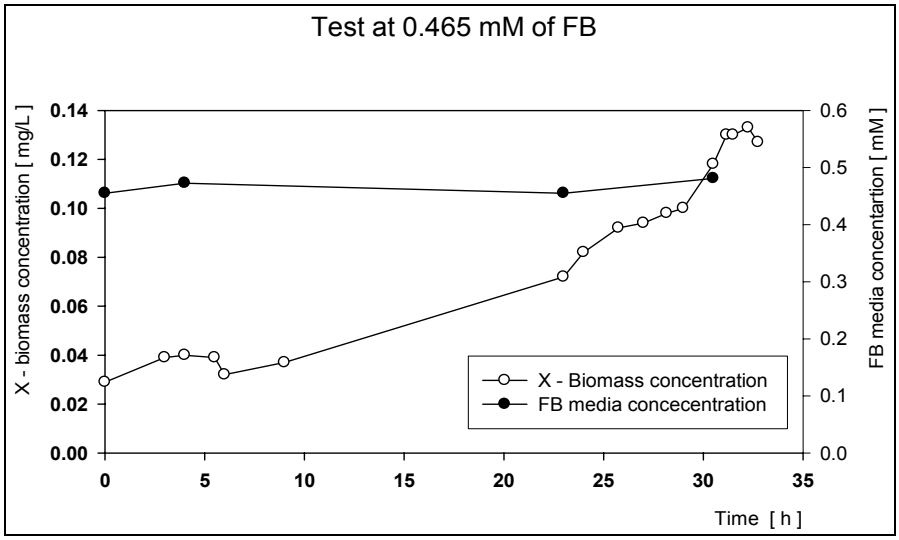


Figure 8.2 – *Typical profile of biomass concentration for the fed batch experiment at constant concentration of substrate.*

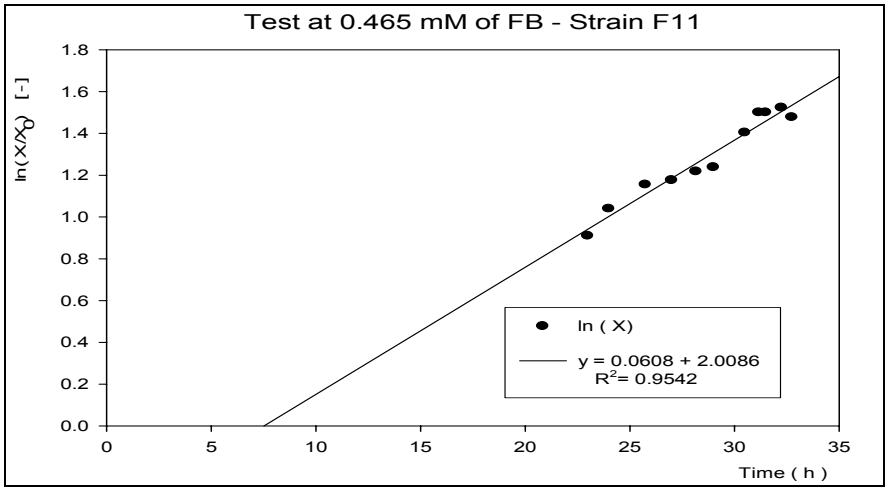


Figure 8.3 – *Determination of the specific growth rate using the linear regression analysis.*

8.4 Results and discussion

8.4.1 Microbial growth kinetic for the strain F11 by fed-batch method

Based on the results obtained from the fed batch method a determination of the kinetic parameter of the growth rate expression of F11 is possible by non linear regression analysis in order to obtain the kinetic model that best fit the experimental data.

A first comparison between the results obtained with the fed-batch method and the parameters available in literature [27] based on batch cultures in shake flasks, showed a consistent discrepancy of values, as reported in Table 8.1 and the effect of this difference in values could be evaluated in figure 8.7 where the two curves (the continuous line is based on the data from the literature whereas the dashed line is obtained by the present study data).

The evidence of an underestimation, in therms of lower growth rates in the range 0-3.9 mM of FB concentration in liquid phase and lower susbstrate inhibition value, is clear and this was confirming the defectiveness of the kinetic growth parameters as the model suggested in the sensitive analysis.

Method	μMax	Ks	N	Sm
	[1/h]	[mM]	[-]	[mM]
Batch[(a)]	0.078	0.16	2.3	3.9
Fed-batch	0.095	0.005	1.8	4.5

[(a)] *M. F. Carvalho et Al. (6)*

Table 8.1 - *Comparison between the kinetic parameter obtained using different method.*

The effect of the growth kinetic parameters obtained via the fed-batch method on the model presented is mainly evident in the FB load profile reported in Figure 8.4 for the OAB experiment and in figure 8.5 for the BO experiment.

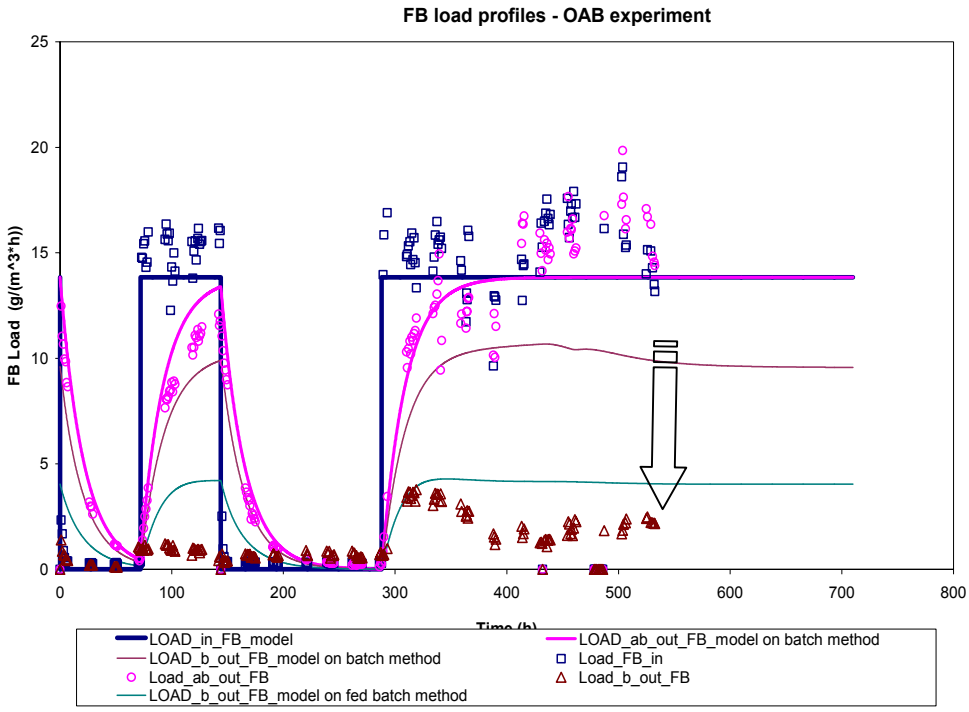


Figure 8.4.1 - *FB load profile during the OAB Experiment:parameters obtained via Fed-Batch metod modify the bioscrubber outlet profile curve (see arrow)*

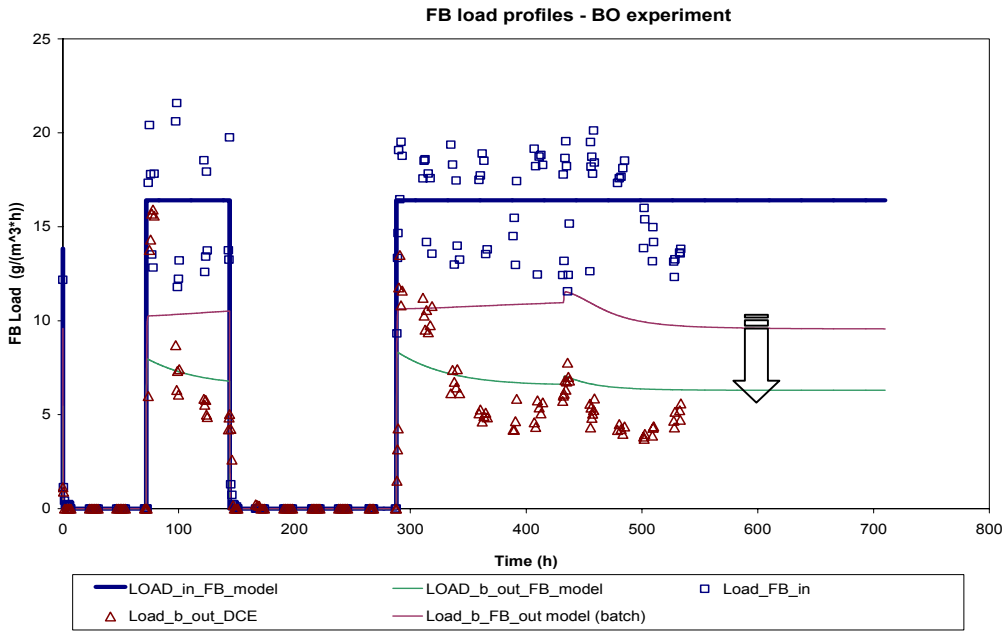


Figure 8.4.2 - *FB load profile during the BO Experiment:parameters obtained via Fed-Batch metod modify the bioscrubber outlet profile curve (see arrow)*

8.4.2 Microbial growth kinetic for the strain MCB032 by fed-batch method

The growth kinetic of the strain MCB032 was investigated applying the fed-batch method in a previous study of Baptista et Al. (private communication).

A possible interpretation of the experimental data has been suggested in the present work by Equation 8.3 where we applied the method of the least squares for the determination of the dimensionless inhibition parameters *a* and *b*.

$$\mu_{(MCB)}^{MCB032} = \frac{\mu_{\max}^{MCB032} C_{MCB}}{K_s + C_{MCB} + \dfrac{e^{aC_{MCB}}}{b}} \tag{8.3}$$

Figure 8.5 shows the fitting between the model suggested by the equation and the experimental data that cover a substrate concentration range between 0.07mM and 1.27 mM of monochlorobenzene in liquid phase.

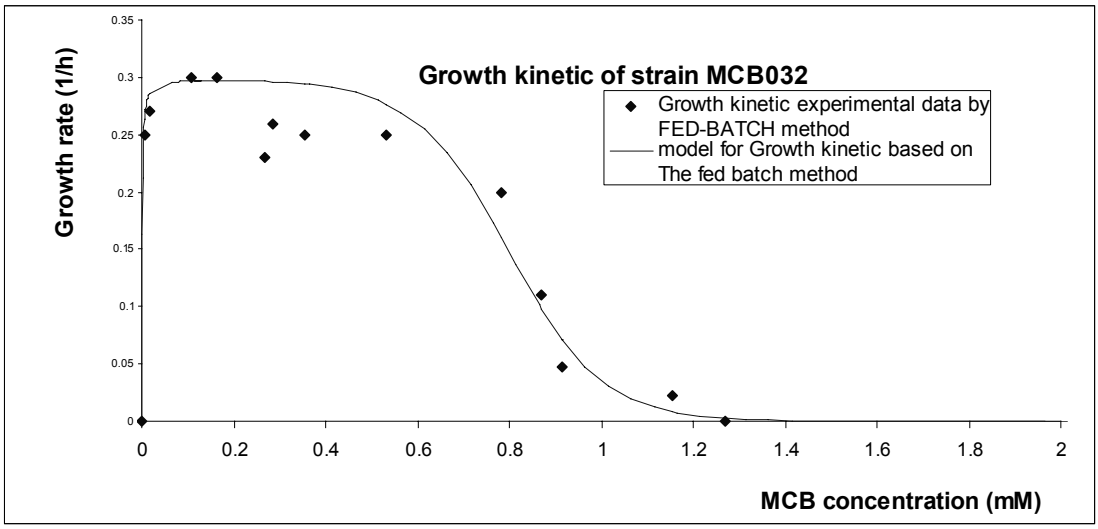


Figure 8.5 - *Growth kinetic of strain MCB032: fitting between model and experimental data based on the fed-batch method experiment. The solid line shows the kinetic equation plotted with* $\mu_{MAX}^{MCB032} = 0.3\ h^{-1}$, $K_s = 0.000706$ *mM , a = 11 and b = 8000.*

8.4.3 Microbial growth kinetic for the strain F11 by batch method

In order to investigate the discordance previously mentioned, a new series of batch experiment were prepared.

For each substrate initial concentration tested, four shake flasks have been set up at the same initial concentration of FB and one control cultures were investigated in order to quantify physical losses.

The batch reactors were provided with a special lid, internally Teflon coated with a stainless steel sampling port sealed with a Teflon coated rubber septum.

For each flask the concentration of FB, the fluoride ions released and the optical density were analysed and typical profile has been represented on Figure 8.6 and the specific growth rate has been calculated as described previously in the fed- batch case tests.

The results obtained from literature have been compared with the fed batch and batch experiment and reported on Figure 8.7 where is possible notice that the discrepancy between the data and the previous literature is still confirmed.

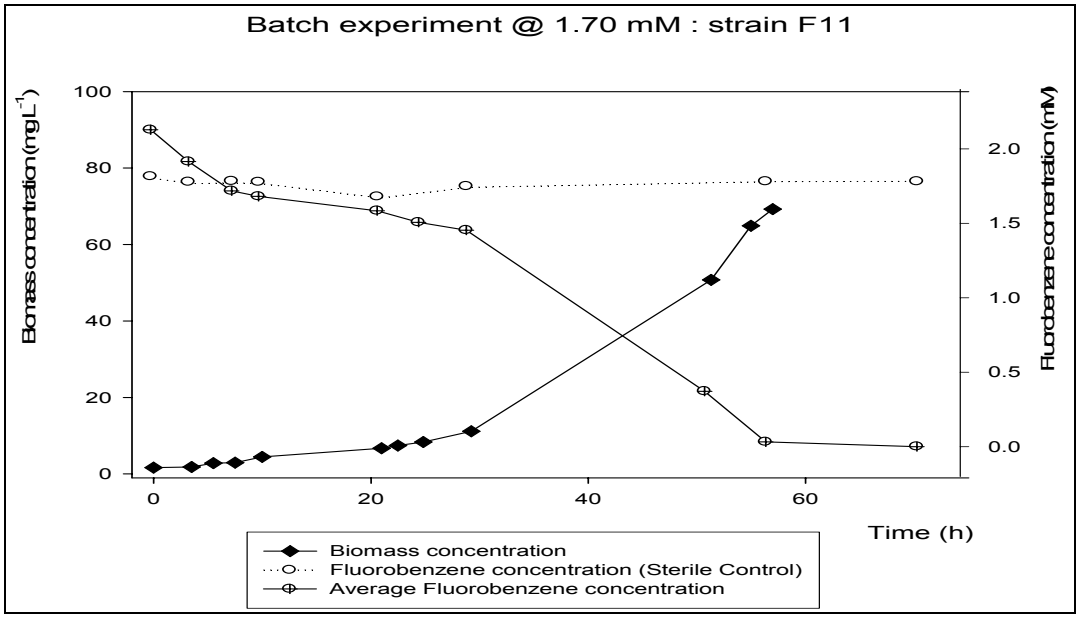


Figure 8.6 – *Typical profile of biomass concentration for the batch experiment at constant concentration of substrate*

The results demonstrate the equivalence of the two methods in the limited range of Fluorobenzene initial concentration between 0.25 mM and 1.25 mM, whereas out of this range the two approaches show a remarkable dissimilarity.

This because in the range of low concentrations, the small amount of substrate injected into the shaking flask is not sufficient to allow a proper investigation of the growth phenomenon, while the microorganisms fully biodegrade via direct metabolism the halogenated compound before showing the typical trend of the exponential growth phase.

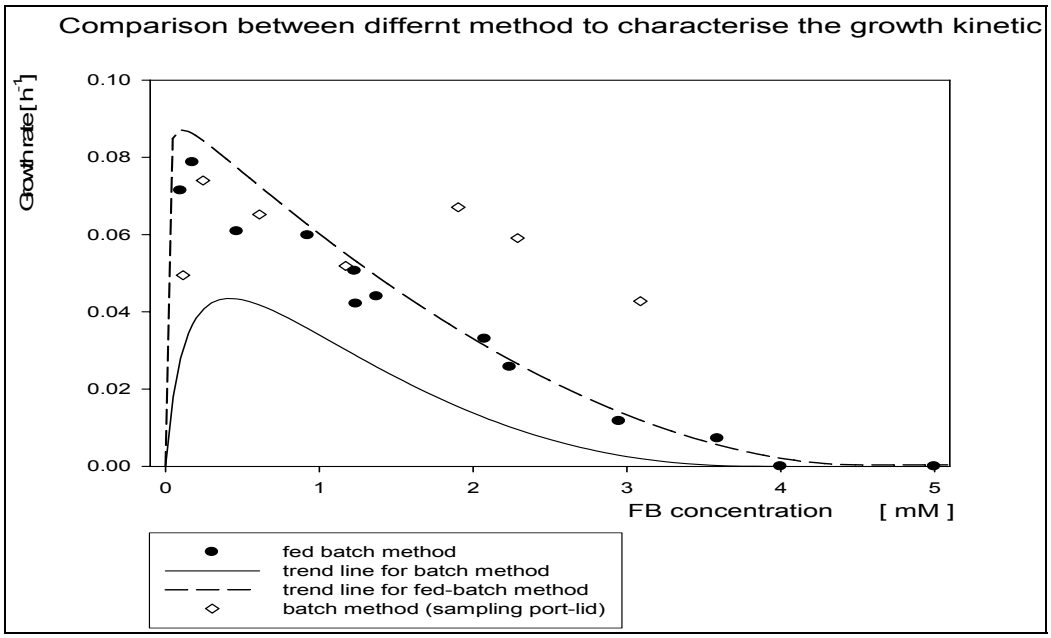


Figure 8.7 - *Growth Kinetics of F11: comparison between experimental data based on the fed-batch method and the profile of the growth kinetic based on the data from the batch method.*

A different event occurs in case of high substrate concentration: a sort of "right-shift" of the experimental data has been observed in comparison with the one obtained by the fed-batch method.

This fact, due to the long exponential phase could be described by the Figure 8.8, where we could appreciate how at a Fluorobenzene initial concentration of 2.3 mM, the exponential growth phase is three days long and, during this period, the concentration of pollutant into the shake flask decreases because of the biodegradation process.

Because of the substrate consumption, if we related the estimated growth rate of every single day with the average fluorobenzene concentration of the whole period, the data pairs hardly fit the trend line curve obtained from the fed-batch method.

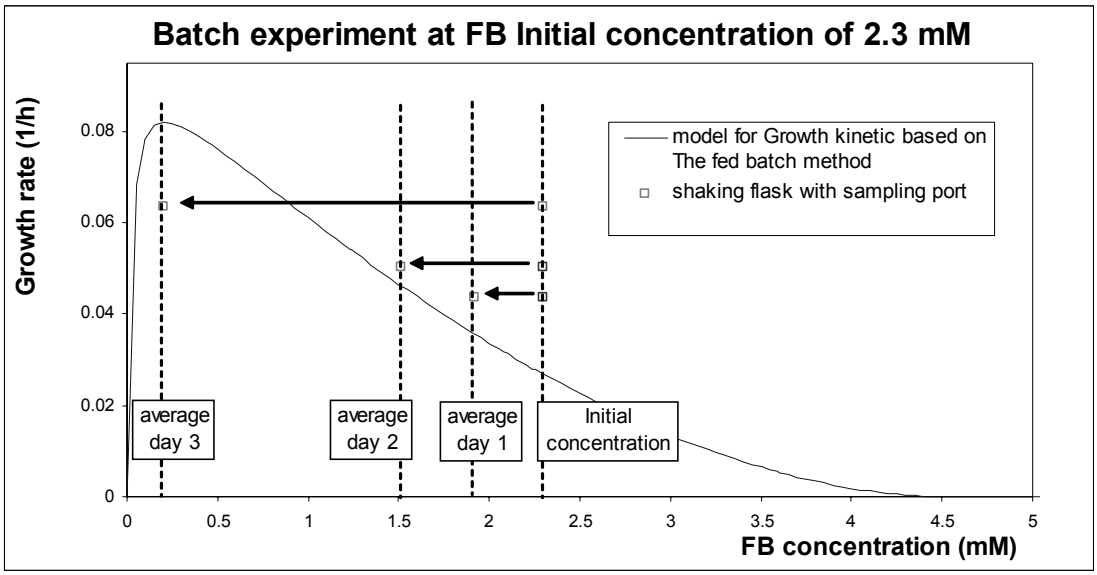


Figure 8.8 - *Comparison between two different approaches to interpret the batch results (right shift phenomenon)*

On the other hand, if we assume to relate the values of the growth rate in every single day of the exponential growth phase with the daily average substrate concentration values, the fitting improves drastically.

That means that in a batch method the best value for the fluorobenzene concentration value to be assumed in a "substrate concentration / growth rate" data pair in a batch experiment is really debatable.

Both the average value of the substrate concentration in the overall period of the experiment or the average concentration during the exponential growth phase only, could give more reliable results than using the values of the initial concentration.

8.4.4 Microbial growth kinetic for the strain MCB032 by batch method

Batch experiments on the strain MCB032 have been set up to confirm the results obtained with the strain F11: the purpose was to compare the effect of the different approaches on a strain with a growth kinetic substantially different from the one described by the model purposed by Luong.

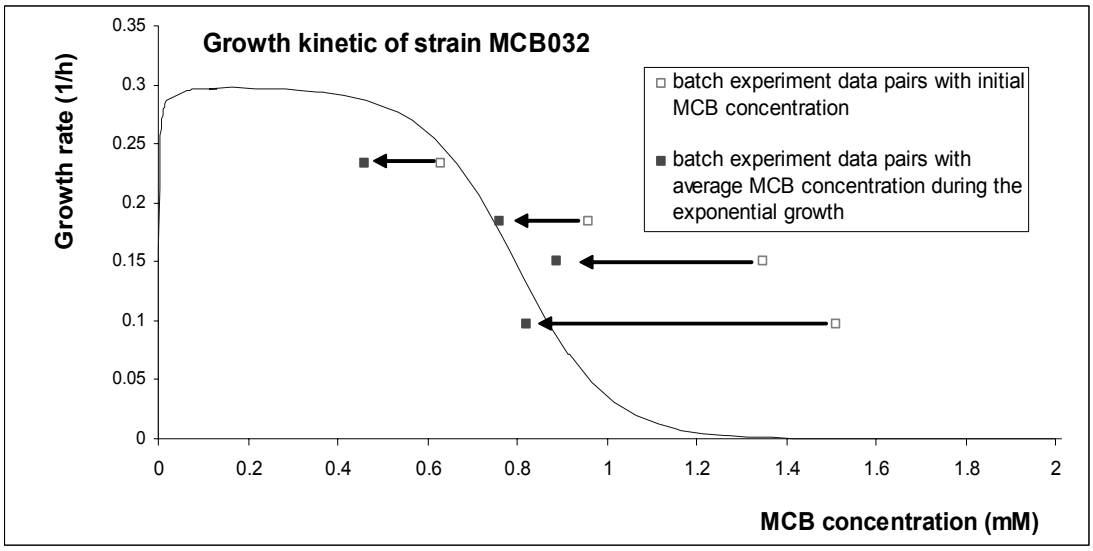


Figure 8.9 - *Comparison between two different approaches to interpret the batch results for the strain MCB032*

In Figure 8.9 is possible to notice how the two methods could be equivalent for the whole range of MCB concentration between 0.1 mM and 0.7 mM, where the growth rate reaches the highest value. But when the growth rate starts to decrease the difference of result obtained between the fed-batch and the batch method is evident.

As for the case of the F11 kinetic estimation, also with the strain MCB032 the assumption of the average values of concentration during the exponential growth phase in the data pair with the measured growth rate gives a better fitting with the curve suggested by the fed-batch method. This gives strength to the consideration that the fed batch method could be the most suitable.

Is important to pay attention to the fact that the error due to the assumption of the batch method as suitable for the determination of a parameters for the

microbial kinetic is highly dependent on the microbial kinetic and on the range of substrate concentration selected.

For Luong-like microbial kinetic the error could hardly be avoided whereas in kinetic with a relatively big plateau at constant value of maximum growth kinetic it does exist an interval of concentration where the two method are almost equivalent giving the same results.

An example is the case of the strain MCB032 where in the substrate concentration range 0-0.5 mM the growth rate assume the constant value 0.29 h^{-1} so, even if the substrate concentration in the batch decreases during the experiment, the value obtained for the growth kinetic is still the same and confirmed as the plateau value.

8.5 Conclusions

The results of this section allow to individuate the fed batch method as the most suitable for the determination of the growth kinetic in case of strain growing on VOC substrate even if, in the concentration range corresponding to the maximum growth rate, the two could give the same results.

Moreover the data obtained in this part of the experimental work help to confirm the growth kinetic parameters as the most sensitive one in the model purposed in the previous chapter and, moreover, to find their correct values.

Chapter 9

Conclusions

9.1 Introduction

This last chapter summarizes the results obtained in the present thesis and the reached achievements according with the general objectives as described in Chapter 1. Each part of the experimental section will be discussed with particular regard to its own scenario suggesting future investigation objectives and possible application in order to outline the importance of this work.

9.2 Project review

The research objectives of this project (presented in section 1.2) have been achieved. The main conclusions derived from the results of the present study are presented below.

SECTION ONE

Aerobic Cometabolic Biodegradation of 1,1,2,2-TeCA: Batch tests

The first objective of this part of the project was to study a process of 1,1,2,2-Tetrachloroethane biodegradation via aerobic cometabolism by methane-utilizing and propane-utilizing microbial cultures.

Results indicate that 1,1,2,2 tetrachloroethane can be biodegraded via aerobic cometabolism by means of both methane-utilizing and propane-utilizing biomasses whereas an explorative tests carried only with the biomass propane

utilizer proved the possibility to biodegrade via aerobic cometabolism the isomer 1,1,1,2 tetrachloroethane.

The biodegradative cometabolic process of the 1,1,2,2- tetrachlorothane can be effectively maintained for several months in slurry reactors without any apparent sign of toxic effects on the biomasses in the range of concentration tested for the pollutant (50-600 μg L^{-1}) and the propane utilizing biomass has been proved to maintain biodegradative capability for several months at increasing concentration of pollutant in the range (900-2500 μg L^{-1})

In the aquifer studied, the addition of a suitable inoculum of biomass methane utilizing or propane utilizing induced a drastic reduction of the lag-times required for the onset of TeCA degradation (from several months to a few days). The biomass propane utilizing has been tested in different soils and, even if in the majority of the soil investigated the cometabolic biodegradative process has started in less than 12 days without dependence on the injection of the inocula, the bioaugmentation allowed a sensible reduction in terms of lag times for the onset of the depletion of the pollutant.

The best results were obtained with the propane-utilizing biomass with

- long-term 1,1,2,2-TeCA degradation rates of 1.2 -1.4 mg L^{-1} d^{-1} at 0.6 mg L^{-1} in the liquid phase
- highest 1,1,2,2-TeCA degradation rate of 6.0 - 6.5 mg L^{-1} d-1 at 2.5 mg L^{-1} in the liquid phase
- 1,1,1,2-TeCA degradation rate of 200 μg $L^{-1}d^{-1}$ at 0.4 mg L^{-1} in the liquid phase

The transfer of inocula from slurry reactor to sterile liquid/gas reactor and the subsequent growth of the inoculated biomass led to the maintenance of the TeCA degradative capacity. Positive results obtained from the liquid-gas tests demonstrates that, starting from few millilitre of propane utilizing biomass from the slurry microcosms, an high amount of inoculum could be generated without presence of soil via growth bioreactor. No evidence of decreasing in biodegradative performance has been observed during the

diluition from the soil and the degradative activity has been granted in resting cell test even after the isolation of the pure strain Rhodococcus.

SECTION TWO

Part one: **Stability of an Oil-Absorber-Bioscrubber System During Biodegradation of Sequentially Alternating 1,2-Dichloroethane and Fluorobenzene in Waste Gas [122]**

The aim of the first part of this section was to study the microbial strain dynamics in a novel absorber-bioreactor system dealing with sequentially alternating loads of inhibitory pollutants in waste gas streams undergoing biological treatment the bioreactor.

In the oil absorber bioscrubber configuration (OAB) the possible presence of inhibitory metabolites produced from FB degradation (which persisted in the bioscrubber for longer time periods in the system due to the effect of the absorber) results in a performance reduction in terms of TOD_{DCE} removal after the re-introduction of the DCE feed (compared to the BO system) [122].

Evidence for this inhibitory effect was also apparent from the evolution of activity in the bioscrubber, which was reduced during periods that FB only was fed and increased when the feed was switched to DCE.

FISH results also suggest that a dynamic community developed in the bioscrubber during SAP conditions, and the percentage of the main degraders remained relatively low[122].

Compared to the BO system, the removal efficiency of FB was maintained at higher levels in the OAB, during the re-introduction of FB in the substrate feed. Due to the effect of the absorber, the TOD_{FB} was substantially reduced after the 6 d that FB was not supplied, while following the 3 d that FB was not supplied the TOD_{FB} was maintained at background levels and any re-acclimation was not required. Due to the fact that the absorber failed to reduce the DCE discharged, a

different compound-strain model system, without any inhibitory effect, would be more appropriate to study the effect of the absorber during SAP conditions [122].

This first part of the work including data elaborations has been carried on in collaboration with Doctor Michalis Koutinas and parts are from a co-authored paper that is as yet unpublished.

Part two: **A Mathematical Model for the Oil-Absorber-Bioscrubber System During Biodegradation in a Sequentially Alternating Pollutant Scenario**

A mathematical model has been purposed in this part for the oil absorber bioscrubber system presented in the previous chapter and suitable for the biodegradation of solvent during biodegradation of waste gas in a sequentially alternated pollutant scenario.

The model has been developed for a set of two couple strain/substrate and it could be used to predict the behaviour of the same system but operating with a different set of couple.

Althought the heavy assumption imposed to the mathematical model purposed the results describe satisfactorily the behaviour of the OAB system and the model has been proved as a powerful tool to individuate the sensitive parameters that affect the process.

The result obtained excludes the possibility of an inhibitory effect due to the concentratrion of the ion fluoride in the biomediumindicating a different factor for the decrease in performance of the bioreactor and the sensitivity analysis indicates the microbial kinetic parameters used in the equation set (with particular regard to the strain F11) as the possible defective ones.

Part three: **An Attempt to Compare Different Approaches to Evaluate Kinetic Parameters: parallel analysis of Batch and Fed-Batch**

This last part of the second section purpose a comparison between the results obtained with batch experiments based on shaking flask and a different fed-batch method that could be easily applied to determine kinetic parameter

avoiding inadequate supplies of substrate and oxygen and inhibition effects due to the increasing concentration of metabolites or biodegradation products.

The parallel evaluation of growth kinetic parameters of two pure strains of microorganisms by both methods suggest a possible range of condition where these different approaches are equivalent and in which case the fed-batch method is applicable as the most suitable one.

The results allow to individuate the fed batch method as the most suitable for the determination of the growth kinetic in case of strain growing on VOC substrate even if, in the concentration range corresponding to the maximum growth rate, the two methodologies could give the same results.

Moreover the data obtained in this part of the experimental work help to confirm the growth kinetic parameters as the most sensitive one in the model purposed in the previous chapter and, moreover, to find their correct values.

9.3 Implications and future works

Regarding the first section of this thesis , batch tests have been set up in the present work in order to understand the possibility of a biodegradative process of the 1,1,2,2-TeCA via aerobic cometabolism, but not some operational conditions need a further investigation.

The temperature is an important factor, affecting the microbial kinetics and thus the performances of the consortium in terms of lag times, TeCA degradation rate and substrate consumption rate. All the tests have been performed at 25°C, because of their explorative nature, but the real temperature condition on site is obviously lower.

This consideration suggest the importance of a series of tests set up at different temperatures, in order to understand the role of this parameter, the tests could be performed in *slurry* phase conditions using the inoculum from the Series LB (or directly the consurtium selected from this second diluition from the soil), but it could be interesting study the the pure strain (or consortium) behaviour in liquid gas tests as well.

Another important aspect of the tetrachloroethane biodegradation is the mass balance on chloride that could confirm the complete mineralization of the halogenated compound object of this study in both its isomeric forms.

At the present moment a series of tests is object of a current research line investigating the chloride release in the mineral medium during the aerobic co-metabolic degradation of 1,1,2,2-tetrachloroethane. In a private comunication the results of this long term study confirm the effective biodegradation process, satisfying with a good approximation the relation between the release of ion chloride into the biomedia and the 1,1,2,2 TeCA depletion, as suggested by the results from control tests.

In this above metioned ongoing study, the abiotic consumption of TeCA due to the sampling pocedures and absorption on the rubber septa have been evaluated in order to correct the degradation rates obtaind in this present thesis.

Considering the results obtained via the batch tests in the first section of the present work the need for a scale-up of the process is evident and the challenge of setting up a continuous test is an important step in order to better understand the problem of a bioremediation treatement on site.

"In relation with the second part of the experimental work of the thesis, the performance analysis of the oil absorber bioscrubber (OAB) system, althought the technology exhibits his great potential for reduction or even elimination of the instability induced during dynamic treatment conditions, prolonged high organic loading or starvation periods should be carefully considered"[122].

"Following long periods of high organic loading fed to the system, the equilibrium obtained between the waste gas and the absorbent VOC concentration may have a negative effect on the bioscrubber. Therefore, although the absorber is expected to absorb the high organic loads fed to the bioscrubber, it might stop being effective and biomass inhibition may occur "[122].

"The above indicate that the design of the absorber is very critical for the process. In cases of prolonged high organic loadings or starvation periods applied to the system, the absorber scale-up should consider a sufficiently long period for

these unstable conditions in order to buffer efficiently any changes in substrates concentration or composition"[122].

Even if the results from the mathematical description of the sistem oil absorber bioscrubber under SAP condition demonstrate a satisfactory fitting, the model needs to be confirmed by a second experiment on bioreactor system fed in different SAP condition and inoculated with different bacteria without inhibitory interaction (in therms of substrate and cometabolic or degradation products).

In order to describe more accurately the biodegradative process, the model could be improved by adding equation related to the effect of the biofilm grown on the surface of the wall of bioscrubber and, to have more consistent predictions of the performance of the two systems (OAB and BO) during dynamic treatment conditions, the variations of different physiological states of microbial strains and the microbial strain dynamics obtained by different molecular techniques could be included in the mathematical models as well as information on the attached biomass.

The model, once tested, could be used to understand the range of possible application of this technology in relation with the real case accounting:

- *peak concentration of the pollutants*: the high concentration of one of the pollutant during the S.A.P. scenario could create a shock load that.
- frequency in the alternation of the pollutant feeding: a frequency too high or too low in the sequencing of the pollutant could create a remarkable instability in the system resulting in long recovery period for the biomass in the bioscrubber.

Based on a positive result of the model, with th knowledge of the suggested design parameters, an economical analysis could be then performed to confirm the suitability of the system with the real scenario because, in the end, the OAB system results in a complication of the basic BO configuration and a detailed economic analysis should be performed to give a more accurate estimation of the cost of the technology chosen.

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Appendix

A.1 Fluorescent *In Situ* Hybridization - F.I.S.H.

Identification of microorganisms by conventional methods requires the isolation of pure cultures followed by laborious characterization experiments but these procedures are therefore inadequate for study of the biodiversity 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 is time consuming and requires specialized personnel and so its introduction in wastewater treatment has been slow.

DGGE is a rapid and simple method that provides 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 (granules, biofilms). The methods discussed have deepened our understanding of the microbiology of biological wastewater treatment.

PCR-based methods (cloning and DGGE) have proved suitable for identifying the microorganisms that form the sludge. Both DGGE and FISH have been extensively employed. FISH is currently being used for elucidation of the composition, quantification and distribution of different bacterial groups in granules and biofilms, as well as their structure and architecture.

An excellent way to overcome some of the problems of studying microbial populations of a microcosm without resorting to traditional methodology is to use fluorescent probes.

These are short sequences of DNA (16–20 nucleotides) labelled with a fluorescent dye. These sequences recognize 16SrRNA sequences in fixed cells and hybridize with them in situ (DNA–RNA matching) (Figure A.1).

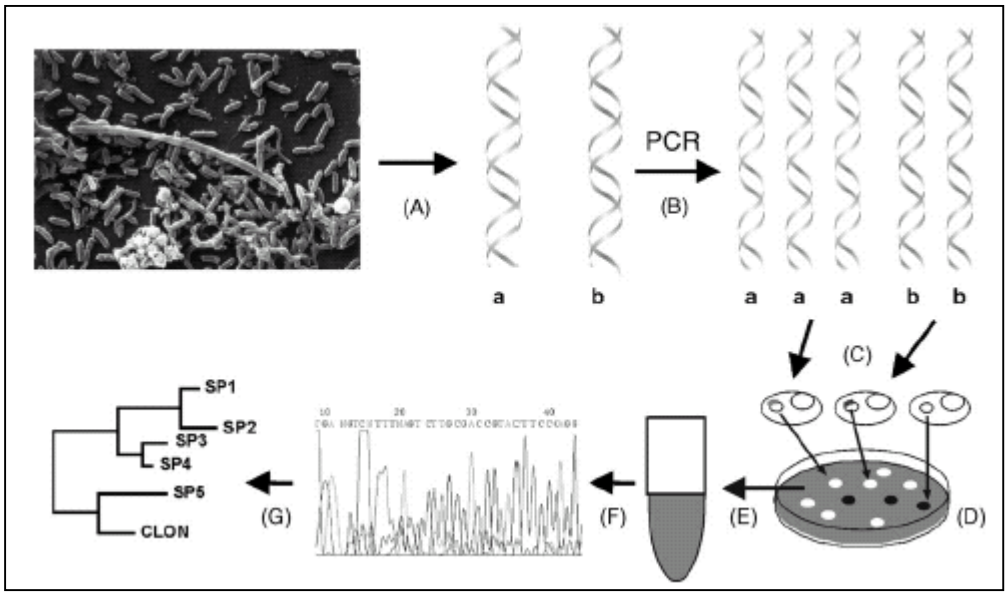

Figure A.1 - *Outline of the cloning procedure for studying a microbial community. The work cycle is as follows: (A) direct nucleic acid extraction, without the need for previous isolation of microorganisms; (B) amplification of the genes that code for 16S rRNA by polymerase chain reaction (PCR), commonly using universal primers for bacteria or archaea, resulting in a mixture of rDNA copies corresponding to the microorganisms present in the sample; (C) cloning of the PCR products obtained into a suitable high copy number plasmid and transformation of competent E. coli cells with this vector; (D) selection of transformed clones with an indicator contained in the plasmid (the white colonies in the figure); (E) extraction of plasmid DNA; (F) sequencing of the cloned gene, creating a clone library; (G) determination of the phylogenetic affiliation of the cloned sequence with the help of dedicated computer programs (ARB, Seqlab, PAUP, PHYLIP).*

Microorganisms can be identified, localized and quantified in almost every ecosystem with hybridization. The specificity of the probe enables detection/identification on 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 databases (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.

The use of oligonucleotide probes targeting 16S rRNA presents a revolution in microbial ecology, both for basic research and practical applications.

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, although these characteristics can sometimes be deduced from comparison of the microorganisms detected with phylogenetically-related bacteria.

Another closing remark refers to the quantitative aspects of FISH. The possibility of quantitative results represents a big advantage over the other molecular techniques, but this only applies to homogeneous and evenly distributed samples.

The bacterial count per region of the microscopic grid should lie between 30 and 150. Between 10 and 20 regions should be counted to ensure statistically significant cell counts.

Non-ideal samples and fluorescent background (a common phenomenon with environmental and sludge samples) can make cell counting by fluorescence microscopy a tedious and time-consuming process that can be influenced by the judgment of the operator and his or her experience.

A standardized and automatic procedure would be preferred. This is possible with a laser confocal microscope or an epifluorescence microscope coupled to a digital camera and a computer workstation to analyze the
Pictures as we use in the monitoring of the bioscrubber microbial stability.

The system usually requires expensive software, which makes it less accessible, although free simple software can be found on the Internet (ImageJ, Image Pro Plus, Figure A.2 shows typical results). Confocal microscopy on its own is of course already an extraordinarily powerful tool for examining the three-dimensional structure and texture of the microbial aggregates (granules, biofilms) that develop in wastewater treatment systems.

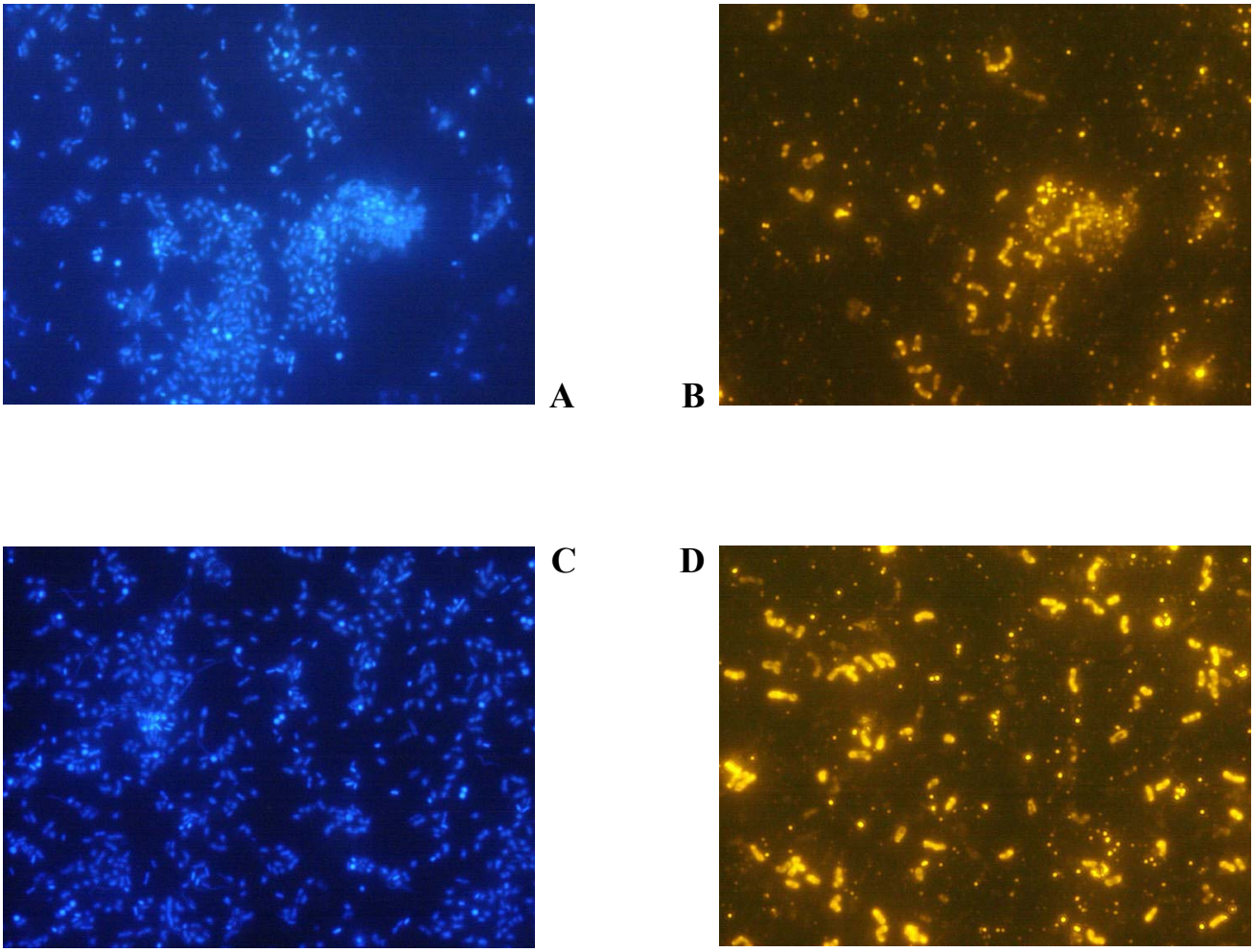

Figure A.2 - *Bacterial cells stained with the GJ10 specific probe and with DAPI during Pulse II.3. DAPI determines the total number of cells present and the GJ10 specific probe identifies GJ10 cells within the microbial community. (DAPI and GJ10 specific probe pictures pairs show the same spot of bacteria through different filters).* ***A.*** *Bacterial cells stained with DAPI before Pulse II.3;* ***B.*** *Bacterial cells stained with the GJ10 specific probe before Pulse II.3;* ***C.*** *Bacterial cells stained with DAPI 3 d after Pulse II.3;* ***D.*** *Bacterial cells stained with the GJ10 specific probe 3 d after Pulse II.3* [122].

In short, the main advantages and disadvantages of FISH can be summarized as follows:

Advantages:

- easy and fast if required probes are available (a wide array has already been described);
- allows direct visualization of non-cultured microorganisms;
- generally quantitative;
- quantification of specific microbial groups is also possible,in contrast to conventional techniques (most probable number, plate counts) or other molecular techniques;
- differential/preferential detection of active microorganisms;
- apt for routine use, highly trained and specialized personnel is not necessary, only a basic knowledge of microscopy and laboratory experience are required.

Disadvantages:

- a priori knowledge of the ecosystem under study and themicroorganisms most likely to be detected is necessary (combined use with other techniques may be necessary);
- if a particular microorganism has to be detected and quantified, its rRNA sequence must be known (if the corresponding probe has not yet been published);
- the design of a specific and unambiguously restrictive probe for a certain group of microorganisms is not always possible, especially if metabolic criteria are applied (e.g. nitrifying bacteria, halo-respiring bacteria);
- the design and optimization of hybridization conditions for a new probe is a difficult process that requires experience and dedication, and the results may not always be satisfactory;
- quantification can be tedious and subjective (manual counting) or complex (image analysis);
- structural analysis of aggregates (granular sludge, biofilms) requires a confocal microscope and an image analysis environment (expensive, trained personnel necessary).

A.2 Possible strategy to sample the biomass from the bioscrubber during the S.A.P. scenario

In order to sample the multi-species bio-film, it is possible to use a stainless steel stick constantly in contact with the bio-medium during the whole experiment.
On the tip of the stick I created four cavities: this particular shape is specially designed with the purpose of allow an easy attachment of the biomass and a relatively simple sampling operation (Figure A.3), whereas Figures A.4 and A.% report a picture of the final assembly and the preliminary sketch, respectively.
For example in relation with an "OAB-BO-like" experiment, the idea is to sample the biomass by the following timetable:

Day 0 (only DCE load after the start-up period)
- sampling the port A in order to know the initial composition of the biomass

Day 3 (only FB load)
- sampling the port A in order to know the composition of the new biomass grew on the stick only during the load of DCE for 3 days
- sampling the port B in order to know the composition of the initial biomass after the DCE load

Day 6 (only DCE load)
- sampling the port B in order to know the composition of the new biomass grew on the stick only during the load of FB for 3 days
- sampling the port C in order to know the composition of the initial biomass after the DCE and the FB load

Day 12 (only FB load)
- sampling the port C in order to know the composition of the new biomass grew on the stick only during the load of DCE for 6 days

- sampling the port A in order to know the composition of the biomass after the DCE and the FB load

Day 18 (FB and DCE load)

- sampling the port A in order to know the composition of the new biomass grew on the stick only during the load of FB for 6 days
- sampling the port B in order to know the composition of the biomass after the DCE and the FB load

Day 21 (end of the experiment)

- sampling the port B in order to know the composition of the new biomass grew on the stick only during the load of FB and DCE together for 3 days
- sampling the port D in order to know the composition of the initial biomass after whole experiment.

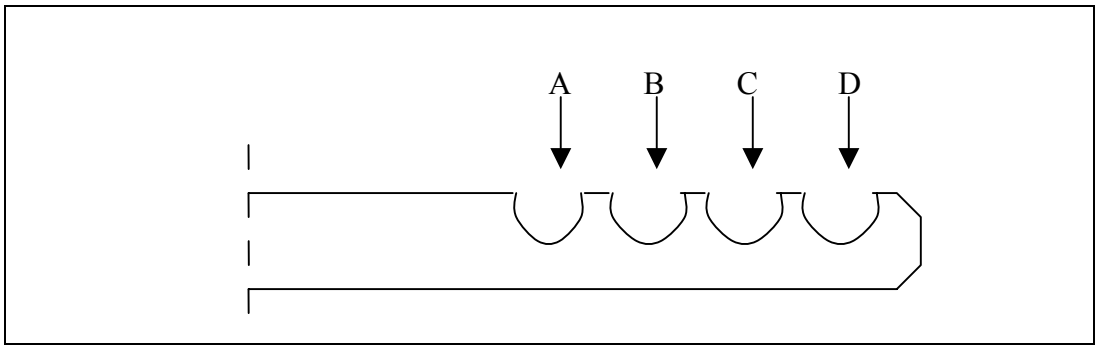

Figure A.3 – *Scheme of the sampling rod cavities for sampling the biomass in the OAB-BO experiment.*

Figure A.4 – *picture of the sampling rod.*

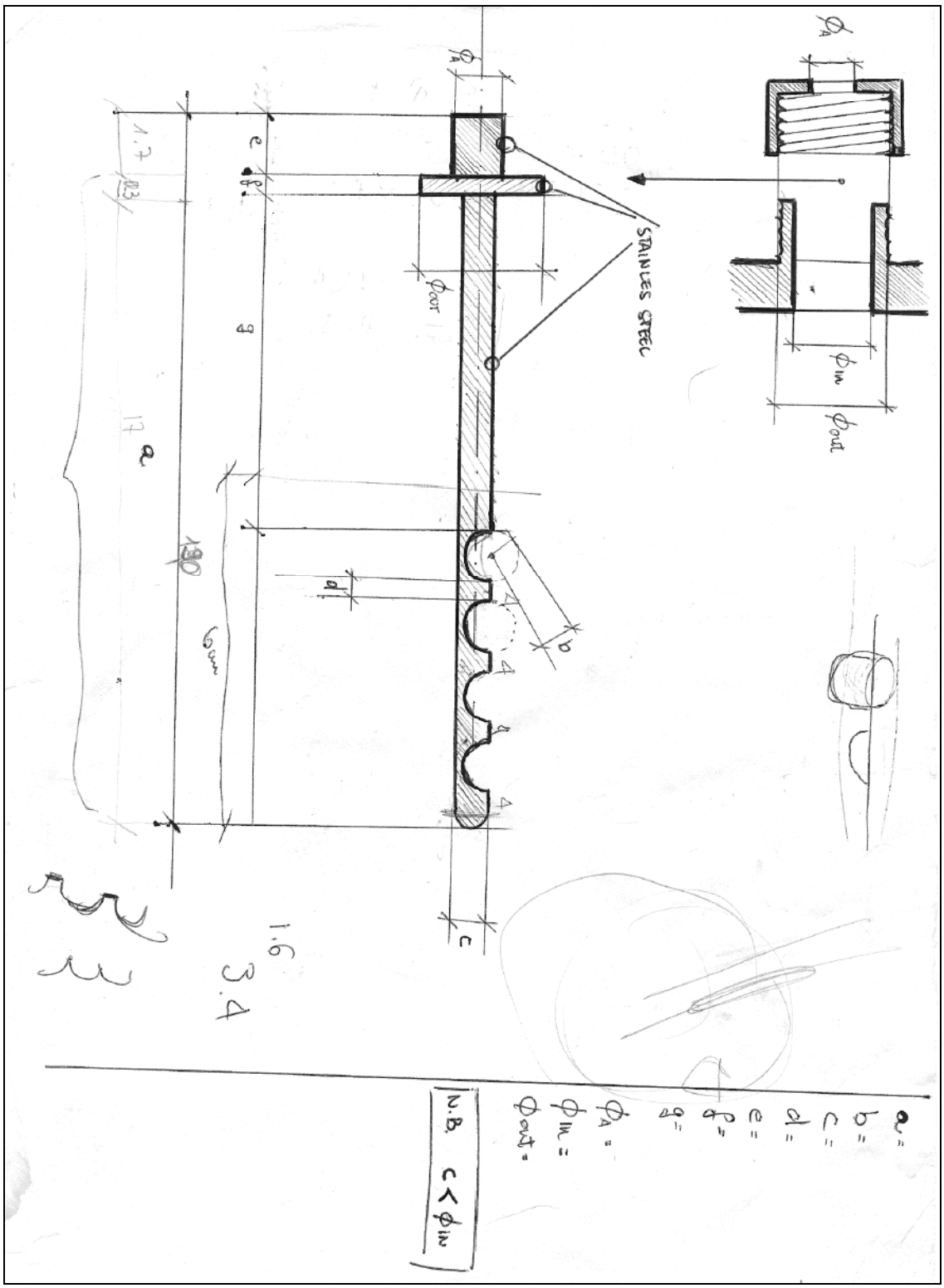

Figure A.5 – *preliminary scheme of the sampling rod.*

A.3 Integrated Risk Information System (I.R.I.S) on Tetrachloroethane

In the following paragraphs have been reported the two reports on both the isomer of tetrachloroethane provided by Environmental Protection Agency and available on-line in the Integrated Risk Information System

1,1,1,2-Tetrachloroethane (CASRN 630-20-6)

1,1,1,2-Tetrachloroethane; CASRN 630-20-6

Health assessment information on a chemical substance is included in IRIS only after a comprehensive review of chronic toxicity data by U.S. EPA health scientists from several Program Offices and the Office of Research and Development. The summaries presented in Sections I and II represent a consensus reached in the review process. Background information and explanations of the methods used to derive the values given in IRIS are provided in the Background Documents.

STATUS OF DATA FOR 1,1,1,2-Tetrachloroethane

Category (section)	Status	Last Revised
Oral RfD Assessment (I.A.)	on-line	12/01/1996*
Inhalation RfC Assessment (I.B.)	no data	
Carcinogenicity Assessment (II.)	on-line	01/01/1991*

I. Chronic Health Hazard Assessments for Noncarcinogenic Effects

I.A. Reference Dose for Chronic Oral Exposure (RfD)

Substance Name—1,1,1,2-Tetrachloroethane
CASRN — 630-20-6
Last Revised — 12/01/1996

The oral Reference Dose (RfD) is based on the assumption that thresholds exist for certain toxic effects such as cellular necrosis. It is expressed in units of mg/kg-day. In general, the RfD is an estimate (with uncertainty spanning perhaps an order of magnitude) of a daily exposure to the human population (including sensitive subgroups) that is likely to be without an appreciable risk of deleterious effects during a lifetime.

Please refer to the Background Document for an elaboration of these concepts. RfDs can also be derived for the noncarcinogenic health effects of substances that are also carcinogens. Therefore, it is essential to refer to other sources of information concerning the carcinogenicity of this substance. If the U.S. EPA has evaluated this substance for potential human carcinogenicity, a summary of that evaluation will be contained in Section II of this file.

I.A.1. Oral RfD Summary

Critical Effect	Experimental Doses*	UF	MF	RfD
Mineralization of the kidneys in males, hepatic clear cell change in females Rat, Chronic Oral Gavage Study NTP, 1983	NOAEL: none LOAEL: 125 mg/kg/day (converted to 89.3 mg/kg/day)	3000	1	3E-2 mg/kg/day

*Conversion Factors -- Dose adjusted for gavage schedule (5\days/week).

I.A.2. Principal and Supporting Studies (Oral RfD)

NTP (National Toxicology Program). 1983. Carcinogenesis studies of 1,1,1,2-tetrachloroethane in F344/N rats and B6C3F1 mice. NTP, Washington, DC.

The NTP (1983) treated groups of 50 male and 50 female F344/N rats by gavage with doses of 0, 125, or 250 mg/kg/day of technical grade 1,1,1,2- tetrachloroethane (>99% pure) in corn oil 5 days/week for 103 weeks. Mortality, body weights, and clinical signs were noted, and comprehensive histopathologic examinations were performed on rats from all groups.

Mean body weights of treated and control rats were similar throughout the study. During weeks 44-103, signs of CNS effects, inactivity, and incoordination were observed in high-dose rats of both sexes. A statistically significant reduction in survival in high-dose males occurred. Eleven control and 7 low-dose males apparently died from heat stress. In addition, 20 male rats (14 control, 3 low-dose, and 3 high-dose) and 15 female rats (2 control, 5 low-dose, and 8 high-dose) died from gavage error. Male rats showed treatment-related increased incidences of mineralization of the kidneys (control 12/48, low dose 19/50, high dose 26/48). Hepatic clear cell changes in female rats were also increased in a dose-related manner (0/48 control, 3/49 low dose, 9/44 high dose). The low dose, 125 mg/kg/day, was considered the LOAEL.

In the NTP (1983) study, groups of 50 male and 50 female B6C3F1 mice were treated by gavage with 0, 250, or 500 mg/kg/day of technical 1,1,1,2- tetrachloroethane (greater than 99% pure) in corn oil 5 days/week for 103 weeks (control and low-dose mice) or 65 weeks (high-dose mice). A statistically significant decrease in mean body weights was observed in high- dose mice. Beginning at week 34, CNS involvement was noted in high-dose mice. The mice appeared sluggish after treatment and by week 51 appeared uncoordinated and weak, and breathed rapidly after treatment. A statistically significant reduction in survival occurred in high-dose mice of both sexes and low-dose female mice, as compared with controls. At week 65, surviving high- dose mice were sacrificed because they were moribund. Incidences of nonneoplastic alterations of the liver

(inflammation, necrosis, fatty metamorphosis, and hepatacytomegaly) were greatly increased in high-dose mice, but not in low-dose groups.

I.A.3. Uncertainty and Modifying Factors (Oral RfD)

UF — An uncertainty factor of 3000 was used: 10 to extrapolate from a LOAEL, 10 for interspecies extrapolation and 10 to provide additional protection for unusually sensitive individuals, and an additional factor of 3 for lack of adequate supporting reproductive and chronic toxicity studies.
MF — None

I.A.4. Additional Studies/Comments (Oral RfD)

The only oral reproductive study of 1,1,1,2-tetrachloroethane was part of a long-term oral study conducted by Truhaut et al. (1974). In this study, male and female rats were treated by gavage at 0 or 300 mg/kg/day, 5 days/week for up to 10 months. Treated females had reduced growth, and treated males and females had increased mortality. Reproductive function was not impaired, but all of the pups from treated rats died within 48 hours of birth. Hepatic fatty vacuolization was observed in adults and pups, and centrilobular necrosis was observed in adults.
1,1,1,2-Tetrachloroethane has not been tested for teratogenicity.

I.A.5. Confidence in the Oral RfD

Study — Low
Database — Low
RfD — Low
Because a NOAEL in rats was not identified, confidence in the NTP (1983) study is low. Confidence in the database is low because, although two chronic and one reproductive bioassays are available, no NOAELs were established, the effects seen at the LOAELs were significant, and only a few doses were given. Low confidence in the RfD follows.

I.A.6. EPA Documentation and Review of the Oral RfD

Source Document — U.S. EPA, 1983
Other EPA Documentation — None
Agency Work Group Review — 04/16/1987
Verification Date — 04/16/1987
A comprehensive review of toxicological studies published through August 2006 was conducted. No new health effects data were identified that would be directly useful in the revision of the existing RfD for 1,1,1,2-Tetrachloroethane and a change in the RfD is not warranted at this time. For more information, IRIS users may contact the IRIS Hotline at hotline.iris@epa.gov or (202)566-1676.

I.A.7. EPA Contacts (Oral RfD)

Please contact the IRIS Hotline for all questions concerning this assessment or IRIS, in general, at (202)566-1676 (phone), (202)566-1749 (FAX) or hotline.iris@epa.gov (internet address).

I.B. Reference Concentration for Chronic Inhalation Exposure (RfC)

Substance Name — 1,1,1,2-Tetrachloroethane
CASRN — 630-20-6
Not available at this time.

II. Carcinogenicity Assessment for Lifetime Exposure

Substance Name — 1,1,1,2-Tetrachloroethane
CASRN — 630-20-6
Last Revised — 01/01/1991
Section II provides information on three aspects of the carcinogenic assessment for the substance in question; the weight-of-evidence judgment of the likelihood that the substance is a human carcinogen, and quantitative estimates of risk from oral exposure and from inhalation exposure. The quantitative risk estimates are presented in three ways. The slope factor is the result of application of a low-dose extrapolation procedure and is presented as the risk per (mg/kg)/day. The unit risk is the quantitative estimate in terms of either risk per ug/L drinking water or risk per ug/cu.m air breathed. The third form in which risk is presented is a drinking water or air concentration providing cancer risks of 1 in 10,000, 1 in 100,000 or 1 in 1,000,000. The rationale and methods used to develop the carcinogenicity information in IRIS are described in The Risk Assessment Guidelines of 1986 (EPA/600/8-87/045) and in the IRIS Background Document. IRIS summaries developed since the publication of EPA's more recent Proposed Guidelines for Carcinogen Risk Assessment also utilize those Guidelines where indicated (Federal Register 61(79):17960-18011, April 23, 1996). Users are referred to Section I of this IRIS file for information on long-term toxic effects other than carcinogenicity.

II.A. Evidence for Human Carcinogenicity

II.A.1. Weight-of-Evidence Characterization

Classification — C; possible human carcinogen.
Basis — increased incidence of combined hepatocellular adenomas and carcinomas in female mice; inadequate evidence from human studies.

II.A.2. Human Carcinogenicity Data

Inadequate. Norman et al. (1981) reported a study of the effects on military personnel of exposure to tetrachloroethane used as a solvent on clothing during World War II. It was not specified which isomer or mixture of isomers was used. One isomer, 1,1,2,2-tetrachloroethane, has been classified as C, possible human carcinogen. Of 3859 exposed men, 833 deaths were reported during the period 1946-1976 compared with 1821 deaths among 9396 nonexposed men. Results were reported by race; further analyses were restricted to white males. Risks for leukemia, lymphoma, and cancers of the prostate and testis were shown to be slightly elevated among the exposed group, but these increases were not significant. It should be noted that there were possible concomittant exposures to dry cleaning solvents.

II.A.3. Animal Carcinogenicity Data

Limited. One chronic study has been reported (NTP, 1983). 1,1,1,2- tetrachloroethane was administered in corn oil by gavage to 50 each male and female F344/N rats and

B6C3F1 mice per dose group. Rats received 0, 125, or 250 mg/kg/day 5 days/week for 103 weeks, and mice were similarly treated with 0, 250, or 500 mg/kg/day. Primarily as a consequence of heat stress during week 62, 27 male rats died (14/50 controls, 10/50 low-dose group and 3/50 high-dose group) and were excluded from statistical analysis of survival. In addition, 15 female rats were accidentally killed during the study (2 control, 5 low-dose, and 8 high-dose). Cumulative toxic effects with signs of CNS involvement were noted from week 44. There were no significant increases in tumor incidence as a consequence of treatment in female rats. Mortality was significantly increased in high-dose male rats. Male rats were also observed to have a significant dose-related trend for only the combined incidence of neoplastic nodules and carcinomas of the liver in the life table test (0/49 controls; 1/49 low dose; 3/48 high dose). While NTP concluded that carcinogenicity was not demonstrated in F344 rats, an increased proportion of male rats with liver tumors that may have been associated with treatment was observed. Accidental killing of 27 male and 15 female rats decreased the sensitivity of this assay.
High-dose mice were sacrificed at 65 weeks as signs of CNS toxicity were observed. The low-dose and control mice were killed at 104-105 weeks. Increased incidences of hepatocellular adenomas and carcinomas were noted in female mice, and a dose-related trend was observed. A statistically significant dose-related increase in the incidence of hepatocellular adenomas occurred in male mice; a significant increase in hepatocellular carcinomas was not observed. Incidence of hepatocellular adenomas were 4/49, 8/46, and 24/48 in the control, low- and high-dose females and 6/48, 14/46, and 21/50 in the control, low- and high-dose males, respectively. Incidences of hepatocellular carcinomas were 1/49, 5/46, and 6/48 in the control, low-, and high-dose females and 12/48, 13/46, and 6/50 in the control, low-, and high-dose males, respectively.
The NTP study authors concluded that carcinogenicity was not demonstrated for rats, but that the sensitivity of the assay was compromised by the accidental killing of animals. They concluded that although the MTD for mice was exceeded at the high dose and survival was decreased, carcinogenicity was demonstrated for mice.

II.A.4. Supporting Data for Carcinogenicity

1,1,1,2-Tetrachloroethane was not mutagenic for Salmonella typhimurium (Simmon et al., 1977) and was negative in a rat liver focus initiation/promotion assay (Story et al., 1986).

II.B. Quantitative Estimate of Carcinogenic Risk from Oral Exposure

II.B.1. Summary of Risk Estimates

Oral Slope Factor: 2.6E-2 per (mg/kg)/day
Drinking Water Unit Risk: 7.4E-7 per (ug/L)
Extrapolation Method — Linearized multistage procedure, extra risk
Drinking Water Concentrations at Specified Risk Levels:

Risk Level	Concentration
E-4 (1 in 10,000)	1E+2 ug/L
E-5 (1 in 100,000)	1E+1 ug/L
E-6 (1 in 1,000,000)	1E+0 ug/L

II.B.2. Dose-Response Data (Carcinogenicity, Oral Exposure)

Tumor Type: hepatocellular adenoma or carcinoma
Test animals: mouse/B6C3F1, female
Route: gavage
Reference: NTP, 1983

Administered Dose (mg/kg)/day	Human Equivalent Dose (mg/kg)/day	Tumor Incidence
0	0	5/49
250	14.8	13/46
500	27.6	30/48

II.B.3. Additional Comments (Carcinogenicity, Oral Exposure)

Human equivalent doses were calculated using animal body weights of 40 g for the control and low-dose groups and 32 g for the high-dose group. Although the high-dose animals were killed at 65 weeks, no adjustment was made for early termination of the high-dose group; animals were moribund; and currently accepted adjustments were inconsistent with the dose-response data.
The unit risk should not be used if the water concentration exceeds 1E+4 ug/L, since above this concentration the unit risk may not be appropriate.

II.B.4. Discussion of Confidence (Carcinogenicity, Oral Exposure)

Adequate numbers of mice were treated, and low-dose animals only were observed for a period approximating their natural life span. Since the high- dose animals were terminated at 65 weeks, the incidence of carcinomas that may have been developed in a lifetime study is not known, but the adenoma incidence was statistically increased and was dose related in both males and females.

II.C. Quantitative Estimate of Carcinogenic Risk from Inhalation Exposure

II.C.1. Summary of Risk Estimates

Inhalation Unit Risk: 7.4E-6 per (ug/cu.m)
Extrapolation Method — Linearized multistage procedure, extra risk
Air Concentrations at Specified Risk Levels:

Risk Level	Concentration
E-4 (1 in 10,000)	1E+1 ug/cu.m
E-5 (1 in 100,000)	1 ug/cu.m
E-6 (1 in 1,000,000)	1E-1 ug/cu.m

II.C.2. Dose-Response Data for Carcinogenicity, Inhalation Exposure

This inhalation risk estimate was derived from oral data presented in II.B.2.

II.C.3. Additional Comments (Carcinogenicity, Inhalation Exposure)

The unit risk should not be used if the air concentration exceeds 1E+3 ug/cu.m, since above this concentration the unit risk may not be appropriate.

II.C.4. Discussion of Confidence (Carcinogenicity, Inhalation Exposure)

See II.B.4.

II.D. EPA Documentation, Review, and Contacts (Carcinogenicity Assessment)

II.D.1. EPA Documentation

Source Document — U.S. EPA, 1987

II.D.2. EPA Review (Carcinogenicity Assessment)

Agency Work Group Review — 02/24/1988, 03/23/1988, 05/04/1988, 10/19/1988
Verification Date — 05/04/1988
A comprehensive review of toxicological studies published through August 2006 was conducted. No new health effects data were identified that would be directly useful in the revision of the existing carcinogenicity assessment for 1,1,1,2-Tetrachloroethane and a change in the assessment is not warranted at this time. For more information, IRIS users may contact the IRIS Hotline at hotline.iris@epa.gov or (202)566-1676.

II.D.3. EPA Contacts (Carcinogenicity Assessment)

Please contact the IRIS Hotline for all questions concerning this assessment or IRIS, in general, at (202)566-1676 (phone), (202)566-1749 (FAX) or hotline.iris@epa.gov (internet address).

III. [reserved]

IV. [reserved]

V. [reserved]

VI. Bibliography

Substance Name — 1,1,1,2-Tetrachloroethane
CASRN — 630-20-6
Last Revised — 08/01/1991

VI.A. Oral RfD References

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U.S. EPA. 1983. Health Hazard Profile on 1,1,1,2-Tetrachloroethane. Pre- pared by the Office of Health and Environmental Assessment, Environmental Criteria and Assessment Office, Cincinnati, OH for the Office of Solid Waste, Washington, DC.

VI.B. Inhalation RfD References

None

VI.C. Carcinogenicity Assessment References

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1,1,2,2-Tetrachloroethane (CASRN 79-34-5)

1,1,2,2-Tetrachloroethane; CASRN 79-34-5

Health assessment information on a chemical substance is included in IRIS only after a comprehensive review of chronic toxicity data by U.S. EPA health scientists from several Program Offices and the Office of Research and Development. The summaries presented in Sections I and II represent a consensus reached in the review process. Background information and explanations of the methods used to derive the values given in IRIS are provided in the Background Documents.

STATUS OF DATA FOR 1,1,2,2-Tetrachloroethane

Category (section)	Status	Last Revised
Oral RfD Assessment (I.A.)	no data	
Inhalation RfC Assessment (I.B.)	no data	
Carcinogenicity Assessment (II.)	on-line	02/01/1994

I. Chronic Health Hazard Assessments for Noncarcinogenic Effects

I.A. Reference Dose for Chronic Oral Exposure (RfD)

Substance Name — 1,1,2,2-Tetrachloroethane
CASRN — 79-34-5
Not available at this time.

I.B. Reference Concentration for Chronic Inhalation Exposure (RfC)

Substance Name — 1,1,2,2-Tetrachloroethane
CASRN — 79-34-5
Not available at this time.

II. Carcinogenicity Assessment for Lifetime Exposure

Substance Name — 1,1,2,2-Tetrachloroethane
CASRN — 79-34-5
Last Revised — 02/01/1994
Section II provides information on three aspects of the carcinogenic assessment for the substance in question; the weight-of-evidence judgment of the likelihood that the substance is a human carcinogen, and quantitative estimates of risk from oral exposure and from inhalation exposure. The quantitative risk estimates are presented in three ways. The slope factor is the result of application of a low-dose extrapolation procedure and is presented as the risk per (mg/kg)/day. The unit risk is the quantitative estimate in terms of either risk per ug/L drinking water or risk per ug/cu.m air breathed. The third form in which risk is presented is a drinking water or air concentration providing cancer risks of 1 in 10,000, 1 in 100,000 or 1 in 1,000,000. The rationale and methods used to develop the carcinogenicity information in IRIS are described in The Risk Assessment Guidelines of 1986 (EPA/600/8-87/045) and in the IRIS Background Document. IRIS summaries developed since the publication of EPA's more recent Proposed Guidelines for Carcinogen Risk Assessment also utilize those Guidelines where indicated (Federal Register 61(79):17960-18011, April 23, 1996). Users are referred to Section I of this IRIS file for information on long-term toxic effects other than carcinogenicity.

II.A. Evidence for Human Carcinogenicity

II.A.1. Weight-of-Evidence Characterization

Classification — C; possible human carcinogen
Basis — Increased incidence of hepatocellular carcinomas in mice

II.A.2. Human Carcinogenicity Data

None.

II.A.3. Animal Carcinogenicity Data

In a bioassay undertaken by NCI (1978) 50 each male and female Osborne- Mendel rats and B6C3F1 mice were gavaged with technical grade (90% pure) 1,1,2,2,-tetrachloroethane in corn oil, 5 days/week. Treatment was over 78 weeks, followed by observation periods of 32 weeks for the rats and 12 weeks for the mice. The high and low average doses (incorporating varying dosage levels throughout the treatment period) were, respectively, 108 and 62 mg/kg/day for male rats, 76 and 43 mg/kg/day for female rats, and 282 and 142 mg/kg/day for mice of both sexes. Control groups consisted of 20 animals/sex and species. Vehicle controls received corn oil at the same rate as the high-dose animals; untreated controls were not intubated. Ten of the high-dose female rats died within the first 5 weeks of the study, but the association between increased dosage and elevated mortality was not statistically significant for male rats. Significantly increased mortality was also evident in the high-dose mice of both sexes. No statistically significant incidence of neoplasms was observed in rats. A highly significant dose-related increase in the incidence of hepatocellular carcinomas was observed in both male and female mice.

II.A.4. Supporting Data for Carcinogenicity

1,1,2,2-Tetrachloroethane is mutagenic for the Salmonella typhimurium missense mutants TA1530 and TA1535 and selectively inhibits growth of E. coli polA (Rosenkranz 1977; Brem et al., 1974).

II.B. Quantitative Estimate of Carcinogenic Risk from Oral Exposure

II.B.1. Summary of Risk Estimates

Oral Slope Factor — 2.0E-1 per (mg/kg)/day
Drinking Water Unit Risk — 5.8E-6 per (ug/L)
Extrapolation Method — Linearized multistage procedure, extra risk
Drinking Water Concentrations at Specified Risk Levels:

Risk Level	Concentration
E-4 (1 in 10,000)	2E+1 ug/L
E-5 (1 in 100,000)	2E+0 ug/L
E-6 (1 in 1,000,000)	2E-1 ug/L

II.B.2. Dose-Response Data (Carcinogenicity, Oral Exposure)

Tumor Type: hepatocellular carcinoma
Test animals: Mouse/B6CC3F1
Route: gavage
Reference: NCI, 1978

Administered Dose (mg/kg)/day	Human Equivalent Dose (mg/kg)/day	Tumor Incidence
2	0	0/20
87	6.56	30/48
174	13.12	43/47

II.B.3. Additional Comments (Carcinogenicity, Oral Exposure)

Administered doses are TWAs, adjusted for frequency (5/7 days) and length of exposure (546 days of an assumed lifespan of 637). Control group received vehicle (corn oil) by stomach tube. Weight of animals was assumed to be 0.030 kg. Human equivalent dose was adjusted by (0.03/70)**1/3 for body weight.
The unit risk should not be used if the water concentration exceeds 2E+3 ug/L, since above this concentration the unit risk may not be appropriate.

II.B.4. Discussion of Confidence (Carcinogenicity, Oral Exposure)

An adequate number of animals was treated. Malignancies increased as a function of treatment dose, and their incidence was significantly increased at both doses.

II.C. Quantitative Estimate of Carcinogenic Risk from Inhalation Exposure

II.C.1. Summary of Risk Estimates

Inhalation Unit Risk — 5.8E-5 per (ug/cu.m)
Extrapolation Method — Linearized multistage procedure, extra risk
Air Concentrations at Specified Risk Levels:

Risk Level	Concentration
E-4 (1 in 10,000)	2E+0 ug/cu.m
E-5 (1 in 100,000)	2E-1 ug/cu.m
E-6 (1 in 1,000,000)	2E-2 ug/cu.m

II.C.2. Dose-Response Data for Carcinogenicity, Inhalation Exposure

The inhalation risk estimates were calculated from the oral exposure data in II.B.2.

II.C.3. Additional Comments (Carcinogenicity, Inhalation Exposure)

The unit risk should not be used if the air concentration exceeds 2E+2 ug/cu.m, since above this concentration the unit risk may not be appropriate.

II.C.4. Discussion of Confidence (Carcinogenicity, Inhalation Exposure)

See II.B.4.

II.D. EPA Documentation, Review, and Contacts (Carcinogenicity Assessment)

II.D.1. EPA Documentation

Source Document — U.S. EPA, 1980
The values in the Ambient Water Quality Criteria Document for Chlorinated Ethanes (1980) received extensive peer and public review.

II.D.2. EPA Review (Carcinogenicity Assessment)

Agency Work Group Review — 06/26/1986
Verification Date — 06/26/1986
Screening-Level Literature Review Findings — A screening-level review conducted by an EPA contractor of the more recent toxicology literature pertinent to the cancer assessment for 1,1,2,2-Tetrachloroethane conducted in September 2002 did not identify any critical new studies. IRIS users who know of important new studies may provide that information to the IRIS Hotline at hotline.iris@epa.gov or (202)566-1676.

II.D.3. EPA Contacts (Carcinogenicity Assessment)

Please contact the IRIS Hotline for all questions concerning this assessment or IRIS, in general, at (202)566-1676 (phone), (202)566-1749 (FAX) or hotline.iris@epa.gov (internet address).

III. [reserved]
IV. [reserved]
V. [reserved]

VI. Bibliography

Substance Name — 1,1,2,2-Tetrachloroethane
CASRN — 79-34-5
Last Revised — 08/01/1989

VI.A. Oral RfD References

None

VI.B. Inhalation RfD References

None

VI.C. Carcinogenicity Assessment References

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