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IN SITU BIOREMEDIATION OF SOIL CONTAMINATED WITH MONOCHLOROBENZENE AND ANILINE

A microbiological study

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Samenvatting
Dit is de rapportage van het binnen het NOBIS-project 'Biologische in situ sanering met een combinatie van afbreekbare verontreinigingen' uitgevoerde microbiologische onderzoek. In dit project zijn de mogelijkheden van en de optimale condities voor de microbiologische afbraak van monochloorbenzeen en aniline en de combinatie van beide stoffen onderzocht.

De resultaten van dit onderzoek hebben geleid tot een voorstel voor een saneringsstrategie voor een deel van de site van de chemische industrie ICI Holland BV. In deze aanpak is het gebruik voorzien van zowel de van nature in de bodem aanwezige biologische afbraakpotentie als van gestimuleerde biologische afbraak.

Trefwoorden
Gecontroleerde termen:
aniline, biological degradation, chlorobenzene

Vrije trefwoorden:
remediation strategy, research strategy

Titel project
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Abstract
This is the report of the microbiological study of the NOBIS project 'In situ bioremediation of soil contaminated with a combination of aniline and monochlorobenzene'. In this project, the possibilities of microbiological degradation of monochlorobenzene and aniline, and the optimum conditions for the process have been investigated.

The results of this research have led to a proposal for a remediation strategy for an area on the site of the chemical industry ICI Holland BV. Point of departure was the development of a low intensity, biological remedial and/or containment alternative.

Keywords
Controlled terms:
aniline, biological degradation, chlorobenzene

Uncontrolled terms:
remediation strategy, research strategy

Project title
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Dutch Research Programme In-Situ Bioremediation (NOBIS)

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SAMENVATTING

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A microbiological study

Dit rapport is het verslag van het binnen het NOBIS-project 'Biologische in situ sanering met een combinatie van afbreekbare verontreinigingen' uitgevoerde microbiologische onderzoek. Hoofddoelstelling van dit project is het vaststellen van de mogelijkheden voor biologische afbraak van monochloorbenzeen en aniline en de optimale omstandigheden voor dit afbraakproces.

Op een deel van de locatie van de chemische industrie ICI Holland BV te Rozenburg bevindt zich een bodemverontreiniging die hoofdzakelijk bestaat uit monochloorbenzeen (MCB) en aniline (AN). Om de vraag te kunnen beantwoorden of in situ bioremediatie in dit geval een geschikt saneringsconcept kan zijn, was (microbiologisch) onderzoek noodzakelijk. Het microbiologisch onderzoek richt zich op de afbraakprocessen van MCB en AN en de invloed van verschillende redoxomstandigheden op deze afbraakprocessen.

Het gehele project is verdeeld in drie subfasen:

1. karakterisatie en monstername door middel van veldonderzoek;
2. literatuuronderzoek en laboratoriumexperimenten naar de omstandigheden waaronder biologische afbraak kan optreden;
3. interpretatie van resultaten en vertaling naar saneringsmogelijkheden.

In dit rapport wordt alleen ingegaan op de resultaten van de tweede subfase:

a. Resultaten van het literatuuronderzoek
Een overzicht van de huidige beschikbare informatie over biologische afbraakprocessen van monochloorbenzeen, aniline en hieraan gerelateerde (halo)aromatische verbindingen, de organismen die hierbij betrokken zijn en de omstandigheden waaronder afbraak kan plaatsvinden.

b. Resultaten van het laboratoriumonderzoek
Nadat biologische activiteit van de van nature in de bodem aanwezige micro-organismen was aangetoond, is de invloed van verschillende omstandigheden (nutriënten, elektronenacceptoren, temperatuur, enz.) op de afbraakkinetiek van monochloorbenzeen onderzocht in batch slurries, batch enrichments, grondkolommen en continue systemen. Het onderzoek was hoofdzakelijk gericht op de invloed van zuurstof, nitraat en aniline op de afbraaksnelheid van monochloorbenzeen.

a. Literatuuronderzoek
Chloorbenzenen
Onder anoxische condities worden chloorbenzenen door anaërobe bacteriën gebruikt als elektronenacceptoren. Reductie van polygehalogeneerde benzenen resulteert in lager gehalogeneerde benzenen: chlooratomen worden vervangen door waterstofatomen. Hoger gehalogeneerde benzenen kunnen makkelijker worden gereduceerd dan lager gehalogeneerde benzenen als gevolg van een negatiever Gibbs-vrij energieverschil ($\Delta G^{0'}$) bij reductieve dechlorinering. Reductieve dechlorinering van gechloreerde benzenen wordt zowel onder methanogene als sulfidogene condities gevonden. Er zijn veel reincultures beschreven die in staat zijn om gechloreerde benzenen tot CO_2 en celmateriaal onder oxische omstandigheden te mineraliseren. Oxische afbraak van benzeen gaat via het tussenproduct catechol door de *ortho*- of *meta*-afbraakroute, terwijl afbraak van chloorbenzeen plaatsvindt via een aangepaste *ortho*-afbraakroute. Ophoping van (toxische) intermediairen kan worden verwacht als benzeenafbrekende organismen per ongeluk chloor-

benzeen omzetten via de *ortho*- of de *meta*-route. Bij monochloorbenzeenconcentraties boven 3 - 4 mM (0,3 - 0,4 g/l) zijn veelvuldig problemen met toxiciteit waargenomen in reincultures.

Aniline
Mineralisatie van aniline is aangetoond onder oxische, denitrificerende, ijzerreducerende en sulfaatreducerende omstandigheden. Aniline kan dienen als koolstof-, energie-, en stikstofbron. Lange lag-perioden worden waargenomen in anaërobe enrichments van aniline-afbrekende bacteriën (6 - 18 maanden). Toxiciteit is waargenomen bij anilineconcentraties van 0,5 mM. Onder oxische condities wordt aniline door een initieel aniline-oxygenase omgezet in catechol wat verder wordt afgebroken via ofwel de *ortho*- ofwel de *meta*-route.

Biologische afbraak van mengsels van xenobiotische aromatische verbindingen
Mengsels van aromatische xenobiotische verbindingen kunnen zowel een stimulerend als een remmend effect hebben op elkaars biodegradatie. Metabolische afbraakroutes kunnen worden geremd en alternatieve afbraakroutes van substraten kunnen leiden tot vorming van toxische producten waaraan het organisme uiteindelijk zelf sterft. Aan de andere kant kunnen additionele substraten biodegradatie van primaire of secundaire substraten versnellen, doordat afbraak nu via specifieke geïnduceerde routes zal plaatsvinden.

Biologische oxidatie van (halo)aromaten onder verschillende redoxcondities
Biologische afbraak van aromaten is aangetoond onder denitrificerende condities (zowel in afwezigheid als in aanwezigheid van kleine hoeveelheden zuurstof), onder metaalreducerende condities, sulfidogene en methanogene condities. Er zijn echter geen gevallen gerapporteerd over de oxidatie van monochloorbenzeen onder anoxische condities.

Biologische behandeling van monochloorbenzeen
Biologische afbraak van met monochloorbenzeen verontreinigd materiaal is aangetoond in afvalgassen, grond en grondwater. In grond verontreinigd met monochloorbenzeen en 1,2-, 1,3-, en 1,4-dichloorbenzeen is gestimuleerde afbraak verkregen door gebruik te maken van beluchting. Kleine hoeveelheden organisch materiaal (stro) en nutriënten zijn toegevoegd aan de bodem om de hier aanwezige bacteriepopulaties te stimuleren. Binnen een periode van 5 weken was meer dan 90 % van de chloorbenzenen afgebroken.

b. Laboratoriumonderzoek
De belangrijkste onderzoeksvragen en de in het laboratoriumonderzoek gevonden antwoorden worden hieronder gegeven.

1. Zijn er levende micro-organismen op de verontreinigde locatie aanwezig en zijn deze organismen in staat om monochloorbenzeen en/of aniline af te breken?

   Op de locatie is microbiologische activiteit gevonden. Hieronder bevonden zich zowel aniline- als monochloorbenzeenafbrekende organismen.

2. Hoe toxisch zijn monochloorbenzeen en aniline voor deze micro-organismen?

   Monochloorbenzeen en aniline zijn niet toxisch tot concentraties van 10 mM.

3. Verhoogd toevoeging van extra koolstofbronnen en nutriënten de afbraaksnelheid van monochloorbenzeen?

   Noch een positief noch een negatief effect kon worden waargenomen op de afbraaksnelheid van monochloorbenzeen bij extra toevoeging van een koolstofbron of nutriënten.

4. Wat is het effect van de nevenverontreiniging aniline op de afbraaksnelheid van monochloorbenzeen?

   Aniline heeft een wisselend en soms inhibiterend effect op de afbraaksnelheid van monochloorbenzeen.

5. Vindt afbraak van monochloorbenzeen plaats onder anaërobe omstandigheden in de aan- of afwezigheid van alternatieve elektronenacceptoren, zoals nitraat, metalen, sulfaat en koolstofdioxide?

   Er vindt geen afbraak plaats van monochloorbenzeen onder anaërobe omstandigheden. Niet in de aanwezigheid van alternatieve elektronenacceptoren en niet in de aanwezigheid van alternatieve elektronendonoren.

6. Wordt de afbraaksnelheid van monochloorbenzeen beïnvloed door lagere partiële zuurstofspanningen?

   Afnemende afbraaksnelheden van monochloorbenzeen zijn gevonden bij afnemende zuurstofconcentraties.

7. Stimuleren additionele alternatieve elektronenacceptoren, zoals nitraat en sulfaat, de afbraak van monochloorbenzeen onder lage zuurstofspanningen?

   Additionele alternatieve elektronenacceptoren, zoals nitraat en sulfaat, stimuleerden de afbraaksnelheid van monochloorbenzeen bij lage zuurstofspanning niet, ondanks het feit dat wat nitraat werd gereduceerd.

SUMMARY

In situ bioremediation of soil contaminated with monochlorobenzene and aniline

A microbiological study

Within the framework of the Dutch NOBIS research program for *in situ* bioremediation, a microbiological study has been performed to answer the question whether *in situ* bioremediation is a suitable method for cleaning up a site contaminated with monochlorobenzene and aniline. The study therefore focuses on biodegradation of monochlorobenzene and aniline, and the influence of different redox conditions on the degradation processes.

This report presents:

a. Results of a literature research
An overview of currently available information on biodegradation processes of monochlorobenzene, aniline and related (halo)aromatic compounds, the organisms that are involved in these processes, and the conditions under which degradation takes place.

b. Results of a laboratory study
Once biological activity of the indigenous microbes was shown, the influence of environmental conditions (nutrients, electron acceptors, temperature, etc.) on the degradation kinetics of monochlorobenzene has been investigated in batch-soil slurries, batch enrichments studies, soil columns and continuous systems. The main focus was to study the influence of oxygen, nitrate and aniline on the degradation rate of monochlorobenzene.

a. Literature research

Chlorobenzenes
Under anoxic conditions, chlorobenzenes are used as electron acceptors by anaerobic bacteria. Reduction of polyhalogenated benzenes results in lower halogenated benzenes: chlorine substituents are replaced by hydrogen atoms. Higher halogenated benzenes are more easily reduced than lower halogenated benzenes due to a more negative Gibbs free energy difference ($\Delta G^{0'}$) upon reductive dechlorination. Reductive dechlorination of chlorinated benzenes is reported both under methanogenic and sulphidogenic conditions. Many pure cultures are described which are able to mineralize chlorinated benzenes to CO_2 and cell carbon under oxic conditions. Oxic degradation of benzene proceeds via catechol through the *ortho*- or *meta*-pathway whereas chlorobenzene degradation proceeds via a modified *ortho*-pathway. Accumulation of (toxic) intermediates can be espected if benzene degrading organisms accidentally degrade chlorobenzene via the *ortho*-pathway or the *meta*-pathway. If the monochlorobenzene concentration exceeds 3 - 4 mM (0.3 - 0.4 g/l) toxicity problems were observed in pure cultures.

Aniline
Mineralization of aniline has been shown under oxic, denitrifying, iron reducing and sulphate reducing conditions. Aniline can serve as carbon-, energy-, and nitrogen source. Long lag periods are observed in anaerobic enrichments of aniline degrading bacteria (6 - 18 months). Toxicity was observed above aniline concentrations of 0.5 mM. Under oxic conditions an initial aniline-oxygenase converts aniline into catechol which is further degraded via either the *ortho*- or the *meta*-pathway.

Bacterial degradation of mixtures of xenobiotic aromatic compounds
Mixtures of aromatic xenobiotic compounds can have both stimilatory and inhibitory effects on their biodegradation. Metabolic pathways can be inhibited and misrouting of substrates may lead

to toxic (suicidal) products. On the other hand additional substrates may enhance biodegradation of primary or secondary substrates by improved induction of particular pathways.

Bacterial oxidation of (halo)aromatics under various redox conditions
Biodegradation of aromatics has been shown under denitrifying conditions in the absence and presence of small amounts of oxygen, under metal reducing conditions, sulphidogenic and methanogenic conditions. However, no examples were found reporting on the oxidation of monochlorobenzene under anoxic conditions.

Biological treatment of monochlorobenzene
Biological treatment of monochlorobenzene contaminated waste has been shown in waste gas, soil, and groundwater. In soil contaminated with monochlorobenzene and 1,2-, 1,3-, and 1,4-dichlorobenzene degradation by indigenous microbes was obtained by using biostimulation and aeration. Low levels of organic material (straw) and nutrients were added to the soil to stimulate the indigenous population to degrade the chlorobenzenes. Within a period of 5 weeks more than 90 % of the chlorobenzenes (1 - 6 mg/kg) was degraded.

b. Laboratory study
The main questions asked for the laboratory study and their answers are shortly described below.

1. Are viable microbes present at the polluted site, and are these indigenous microbes capable of degrading monochlorobenzene?

   At the polluted site microbial activity was found, as well as aniline and monochlorobenzene degrading bacteria.

2. How toxic are monochlorobenzene and aniline for these bacteria?

   Monochlorobenzene and aniline are not toxic up to concentrations of 10 mM.

3. Do additional carbon sources and nutrients enhance the degradation rate of monochlorobenzene?

   Neither a positive nor a negative effect of additional carbon sources and nutrients on the degradation rate of monochlorobenzene was observed.

4. What is the effect of the co-contaminant aniline on the degradation rate of monochlorobenzene?

   Aniline does have a variable, sometimes inhibiting effect on the degradation rate of monochlorobenzene.

5. Does monochlorobenzene degradation take place under anaerobic conditions in the absence or presence of alternative electron acceptors such as nitrate, metals, sulphate and carbon dioxide?

   No degradtion of monochlorobenzene takes place under anaerobic conditions. Not in presence of alternative electron acceptors and not in the presence of alternative electron donors.

6. Is the degradation rate of monochlorobenzene influenced by low partial pressures of oxygen?

   Decreasing monochlorobenzene degradation rates were found with decreasing oxygen concentrations.

7. Do additional alternative electron acceptors such as nitrate and sulphate enhance the monochlorobenzene degradation under low oxygen concentrations?

   Additional alternative electron acceptors such as nitrate and sulphate did not enhance the monochlorobenzene degradation rate with low partial pressure of oxygen, despite the fact that some nitrate was reduced.

CHAPTER 1

INTRODUCTION

For the chemical company ICI Holland BV there exists a need to remediate a contaminated site comprising of soil polluted with a mixture of monochlorobenzene and aniline. Dispersion of these contaminants should be prevented. One method to prevent horizontal and vertical transport of the contaminants is the extraction of groundwater. An other method is *in situ* bioremediation based on the use of the biodegradative capacities of indigenous microbes. Possibly dispersion of the contaminants can be prevented and even clean up of the site can be established by making the environmental conditions as favourable as possible for microbial degradation of the pollutants. **To answer the question whether *in situ* bioremediation will be a suitable method for cleaning up the polluted site, it is important to know which organisms are involved and under which conditions degradation occurs**. The biodegradation of monochlorobenzene and aniline and the influence of different redox conditions on the degradation processes have been investigated by means of a literature review and a laboratory investigation.

Main questions to be solved
To answer the question whether *in situ* bioremediation will be a suitable method for cleaning up a polluted site contaminated with monochlorobenzene and aniline, the main questions to be solved are the following:

1. Are viable microbes present at the polluted site, and are these indigenous microbes capable of degrading monochlorobenzene?
2. How toxic are monochlorobenzene and aniline for these bacteria?
3. Do additional carbon sources and nutrients enhance the degradation rate of monochlorobenzene?
4. What is the effect of the co-contaminant aniline on the degradation rate of monochlorobenzene?
5. Does the degradation of monochlorobenzene take place under anaerobic conditions with alternative electron acceptors such as nitrate, metals, sulphate and carbon dioxide?
6. Is the degradation rate of monochlorobenzene influenced by low partial pressures of oxygen?
7. Do additional alternative electron acceptors such as nitrate and sulphate enhance the monochlorobenzene degradation under low oxygen concentrations?

Chapter 2 will present the results of the literature review.
Chapter 3 gives the practical approach followed for the laboratory research.
Chapter 4 presents the results of the laboratory research.
In chapter 5 conclusions for the laboratory results will be drawn.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

The chemical company ICI Holland BV is confronted with the task to clean up a site contaminated with a mixture of monochlorobenzene and aniline. In any case, spreading of these contaminants must be prevented. One method to prevent horizontal and vertical migration of the contaminants is groundwater extraction. Another method is *in situ* bioremediation based on the use of biodegradative indigenous microbes. By making the environmental conditions as favourable as possible for microbial degradation of the pollutants, the contamination can possibly both be prevented from spreading and even cleaned up. To answer the question whether *in situ* bioremediation is a suitable method for cleaning up the polluted site, microbiological research is necessary. For that purpose, first an overview must be obtained of current knowledge on biodegradation processes of monochlorobenzene, aniline and related (halo)aromatic compounds. It is important to know what organisms are involved and under which conditions degradation takes place. The applicable information that was found is described below.

2.2 Monochlorobenzene

The haloaromatic compound monochlorobenzene is an aromatic ring in which one hydrogen atom is replaced by a chlorine atom. The chemical structure and main characteristics of monochlorobenzene are listed in table 1. The critical step in microbial degradation of halogenated aromatic compounds is cleavage of the halogen-carbon bond. For monochlorobenzene this can be established via two different processes: 1) reductive dehalogenation (see 2.2.1), and 2) dioxygenation reactions (see 2.2.2). After removal of the halogen, a natural non-halogenated substrate is formed which can be metabolized relatively easily. In general, successful biodegradation and the fate of haloaromatic compounds in nature depend on a) physico-chemical conditions at the contaminated site favouring a particular dehalogenation process, or a combination of dehalogenation processes, and b) the presence of bacteria capable of metabolizing these contaminants.

Table 1. Characteristics of monochlorobenzene.

structural formula:	
molecular weight (g/mol):	112.6
melting point (°C):	-45.6
boiling point (°C):	132.0
relative density (water = 1):	1.0058
relative vapor density (air = 1):	3.9
vapor pressure (mbar at 20 °C):	12
water solubility (g/l at 20 °C):	0.4
log P octanol/water:	2.8

2.2.1 *Reductive dehalogenation of monochlorobenzene under anaerobic conditions*

During reductive dehalogenation the halogen attached to the aromatic ring is replaced by a hydrogen atom (see fig. 1). This process occurs almost exclusively under anoxic conditions (i.e. sulphidogenic and methanogenic) in which the halogenated benzene serves as a (terminal) electron acceptor for anaerobic bacteria [Commandeur and Parsons, 1990; Häggblom, 1990].

Fig. 1. Reductive dehalogenation of halobenzene to benzene.

The more halogens are attached to the aromatic nucleus, the more it is thermodynamically favourable for the reductases, and hence the better they can dehalogenate the compound. The Gibbs free energy difference ($\Delta G^{0'}$) upon reductive dechlorination of higher chlorinated benzenes is more negative than the difference in Gibbs free energy of reductive dehalogenation of lower chlorinated chlorobenzenes. Moreover, the ease with which microbiologically catalysed dehalogenations take place corresponds with the redox potential of the redox couples of the substrates involved. Redox couples which evoke the highest energy yield are used preferentially. Therefore, more highly halogenated aromatics, which have higher redox potentials relative to H^+/H_2 than the reference system, are more easily reduced than lower halogenated aromatics [Beurskens et al., 1994; Dolfing and Harrison, 1993; Holliger et al., 1992]. As a result, dichlorobenzenes and monochlorobenzene often accumulate as end products from reductions of higher chlorinated benzenes, because it is less energetically favourable for bacteria to further reduce these compounds. For these reasons, mineralization of higher halogenated benzenes can best be established by a combination of anoxic and oxic degradation. Fathepure and Vogel [1991] described the reductive dechlorination and mineralization of hexachlorobenzene (HCB) by a combination of anaerobes and aerobes in a two-stage biofilm reactor. The extent of dechlorination was maximal when the anaerobic biofilm was supplied with acetate as the primary carbon source. HCB was dechlorinated to trichlorobenzenes. Trichlorobenzene was metabolized by the aerobic biofilm. Holliger et al. [1992] showed the reductive dechlorination of higher chlorinated benzenes (hexa- and pentachlorobenzene, all three isomers of tetrachlorobenzene, 1,2,3-trichlorobenzene (1,2,3-TCB) and 1,2,4-TCB) in methanogenic mixed cultures. Hexa- penta- and tetrachlorobenzene were converted to trichlorobenzene; 1,2,4-trichlorobenzene was not dechlorinated and 1,3-dichlorobenzene was the main end product of the reductive dechlorination of 1,2,3-trichlorobenzene. The dechlorinating activity was only maintained as long as an electron donor was added. The best electron donors were lactate, ethanol, and hydrogen. The optimal temperature for dechlorination was 30 °C and the optimum pH was 7.2 [Holliger et al., 1992]. Beurskens et al. [1994] showed reductive dechlorination of hexachlorobenzene in anoxic methanogenic sediment originating from lake Ketelmeer and the river Rhine. Lactate served as the electron donor in these slurries.

The optimal temperature for dechlorination was around 30 °C, but at 3 °C dechlorinating activity was still observed. Ramanand et al. [1993] described the reductive dechlorination of hexa- and pentachlorobenzene and a mixture of tetra- , tri- and dichlorobenzenes to monochlorobenzene in methanogenic soil. Monochlorobenzene accumulated as the end product. Biotransformation of alfa-, beta-, gamma-, and delta-hexachlorocyclohexane in a methanogenic flow-through column also yielded monochlorobenzene as the main product in the effluent [Middeldorp et al., 1996]. Nevertheless, reductive dechlorination of monochlorobenzene to benzene by a methanogenic mixed culture has been shown by Nowak and co-workers [1996]. However, the reduction of monochlorobenzene to benzene was very poor and took place only in the presence of trichloro-

benzene as electron acceptor. The reductive process was stimulated by the addition of pyruvate, methanol, ethanol, acetone and acetate. Transformations of higher halogenated benzenes to lower halogenated benzenes were also shown under sulphidogenic conditions. Monochlorobenzene was also the end product of the reductive dechlorination of trichlorobenzenes under anoxic conditions in columns packed with river Rhine sediment. High concentrations of sulphate (20 mM) did not inhibit the dechlorinating activity. Denitrifying conditions in these soil columns however prevented reductive dechlorination [Bosma et al., 1988]. Beurskens [1995] showed the reductive dechlorination of HCB to 1,2,4-trichlorobenzene and 1,3,5-trichlorobenzene after a lag period of 30 weeks in the presence of 5 mM sulphate. Only 1,3,5-trichlorobenze was further dechlorinated to 1,3-dichlorobenzene. Table 2 presents an overview from the literature of enrichments that reductively dehalogenated chlorinated benzenes.

Table 2. Reductive dechlorination of chlorinated benzenes and related compounds to lower chlorinated benzenes under anoxic conditions.

organism	electron acceptor	product	condition	reference
enrichment	HCB[a]	TCBs	anoxic	Fathepure and Vogel [1991]
enrichment	HCB, PCB[b], tetra, 1,2,3-TCB[c]	TCBs, 1,3-DCB[d]	methanogenic	Holliger et al. [1992]
enrichment	HCB		methanogenic	Beurskens et al. [1994]
soil slurries	hexa-, penta-, tetra-, tri- and dichlorobenzenes	CB[e]	methanogenic	Ramanand et al. [1993]
sediment	*α*-, *β*-, *γ*-, and *δ*-hexachlorocyclohexane	CB	methanogenic	Middeldorp et al. [1996]
mixed culture	CB	benzene	methanogenic	Nowak et al. [1996]
sediments	TCBs + sulphate	CB	sulphidogenic	Bosma et al. [1988]
sediments	HCB + sulphate	1,2,4-TCB, 1,3-DCB	sulphidogenic	Beurskens [1995]

[a] : HCB = hexachlorobenzene;
[b] : PCB = pentachlorobenzene;
[c] : TCB = trichlorobenzene;
[d] : DCB = dichlorobenzene;
[e] : CB = monochlorobenzene.

2.2.2 *Aerobic dehalogenation of monohlorobenzene*

Lower chlorinated benzenes, common products of anaerobic reductive processes, are more easily degraded by aerobic micro-organisms than higher chlorinated benzenes. Several aerobic bacteria have been isolated which are able to degrade mono-, di-, tri- and even tetrachlorobenzene. The general picture for aerobic degradation of chlorobenzenes is that these compounds are metabolized via chlorocatechols. Initial attack of a dioxygenase, which incorporates molecular oxygen into the aromatic ring, leads to the formation of chlorinated dihydrodiols. Subsequently, a chlorinated catechol is formed by the action of a dihydrodiol dehydrogenase, which is further metabolized via the *ortho*-cleavage pathway via the initial action of catechol-1,2-dioxygenase. The chlorine atom is released during the formation of muconolactone (see fig. 2) [Haigler et al., 1992; Nishino et al., 1992; Reineke and Knackmuss, 1984]. The *meta*-pathway is inactivated because chlorocatechols have an inhibiting effect on the enzyme catechol-2,3-dioxygenase [Bartels et al., 1984; Klecka and Gibson, 1981]. If chlorobenzenes are degraded accidentally via the *meta*-pathway, toxic intermediates, such as 5-chloroformyl-2-hydroxypenta-2,4-dionic acid, are produced which are suicidal for the cells.

Fig. 2. Oxic degradation pathway of monochlorobenzene [Haigler et al., 1992; Nishino et al., 1992; Reineke and Knackmuss, 1984].

Gibson et al. [1968] showed that *Pseudomonas putida,* grown on toluene as a source of carbon, converted chloro-, bromo-, iodo-, and fluorobenzenes to their corresponding 3-halocatechol. Reineke and Knackmuss [1984] described a bacterium, strain WR1306, that could use monochlorobenzene as sole source of carbon and energy. The bacterium was isolated by continuous culture enrichment. The enrichment was initially fed with benzene and over a period of nine months monochlorobenzene had gradually replaced benzene as the growth substrate. Enzyme measurements revealed that this strain metabolized monochlorobenzene via the modified *ortho*-pathway. In the modified *ortho*-pathway the substituted catechol is cleaved by the pyrocatechase II-enzyme, resulting in the formation of chloro-*cis,cis*-muconate. In the classical *ortho*-pathway, pyrocatechase I is the ring-fission enzyme, which is not active on substituted catechols [Dorn and Knackmuss, 1978]. Therefore, problems can be expected when organisms possess the pyrocatechase I-enzyme and not the II-enzyme. Substituted benzenes can be converted to their corresponding halocatechols by the initial dioxygenases of the classical *ortho*-pathway. These enzymes do often have a broad substrate range. The halocatechols formed cannot be cleaved by the pyrocatechase I-system, leading to accumulation of toxic chlorocatechols or accidental misrouting via the *meta*-pathway by the action of catechol-2,3-dioxygenase. Misrouting via the *meta*-pathway will ultimately result in cell death. Nishino et al. [1992] did HPLC analyses on culture fluid of glucose + monochlorobenzene grown cells of strain 12A7A and showed chlorobenzene-dihydrodiol and 3-chlorocatechol accumulation. The initial enzymes could convert monochlorobenzene, but the organism possessed the pyrocatechase I-system leading to the accumulation of chlorocatechols. Accumulation of chlorocatechols could easily be recognized by the appearance of a black/brownish colour, due to auto-oxidation. However, the presence of organisms possessing the classical *ortho*-pathway on a site contaminated with chlorinated benzenes should not necessarily be a problem as long as there are also organisms present that do possess the modified *ortho*-pathway.

Pyrocatechase II is also known to be a non-specific enzyme, so, accumulating chlorocatechols will readily be metabolized in such a mixed community. Additionally, the range of xenobiotic compounds that a mixed community can succesfully mineralize may even increase [Slater and Lovatt, 1984]. The presence of organisms possessing pyrocatechase II in microbial communities at such a contaminated site is therefore essential to establish mineralization.

Monochlorobenzene can also be transformed into 4-chlorophenol. Probably this is due to induction and activity of monooxygenases or dehydratases. Burback and Perry [1993] demonstrated that propane grown cells of *Mycobacterium vaccae* catabolized monochlorobenzene to 4-chlorophenol which was not further metabolized. In this way no mineralization of monochlorobenzene is achieved, but such co-oxidative and catabolic transformations can result in intermediates that are more amendable to biodegradation.

Dichlorobenzenes are metabolized via the same modified *ortho*-pathway as monochlorobenzene. A *Pseudomonas* sp. capable of growth on 1,2-DCB converted it via a dihydrodiol into 3,4-dichlorocatechol. The dichlorocatechol was degraded via *ortho*-cleavage to 2,3-dichloro-*cis,cis*-muconate, and chloride was eliminated during subsequent lactonization followed by hydrolysis to form 5-chloromaleylacetate [Haigler et al., 1988]. De Bont et al. [1986] described an *Alcaligenes* sp. capable of growth on monochlorobenzene, 1,3-DCB, and 1,4-DCB. This bacterium degraded 1,3-DCB through the dihydrodiol into 3,5-dichlorocatechol which was also degraded via the *ortho*-pathway yielding 2,4-dichloromuconic acid. Several strains of *Pseudomonas* and *Alcaligenes* degraded 1,4-DCB via 3,6-dichlorocatechol which is subsequently metabolized via *ortho*-cleavage to 2,5-dichloromuconic acid [Oltmanns et al., 1988; Schraa et al., 1986; Spain and Nishino, 1987].

Chlorobenzenes and benzene are known to be inhibitory to several bacteria but can be used as carbon sources when dispensed at a low concentration or in the gaseous form [De Bont et al.,1986; Haigler et al., 1988; Nishino et al., 1992; Reineke and Knackmuss, 1984; Schraa et al., 1986]. When benzene and monochlorobenzene were supplied in the liquid form it even prevented growth of a chlorobenzene degrading bacterium, strain WR1306 [Reineke and Knackmuss, 1984]. Fritz et al. [1992] showed that concentrations exceeding 3.5 mM monochlorobenzene were toxic to *Pseudomonas* sp. strain RHO1, due to co-operative toxicity of the substrate and the metabolite 3-chlorocatechol. *Pseudomonas putida* GJ31 and strain WR1306 had toxic treshold concentrations of 3 mM [Oldenhuis et al., 1989] and 100 mg/l [Reineke and Knackmuss, 1984], respectively. Utilization of high concentrations of halogenated benzenes was shown for *Rhodococcus opacus* GM14, a Gram positive actinomycete [Zaitsev et al., 1995]. This organism utilized a very wide range of haloaromatic compounds. It grew at very high concentrations: in saturated aqueous solutions of benzene, monochlorobenzene, and 1,3-, and 1,4-dichlorobenzene (up to 13, 3, 0.5 and 0.5 g/l, respectively). Specific growth rates on benzene and chlorobenzene (0.4 g/l) were 0.2 h^{-1}, and a growth yield of 40 to 50 g (dry weight) per mol substrate used was achieved. Benzene derivatives could be introduced into the medium as liquids, crystals or vapors.

Chlorinated benzenes can also be degraded by fungi. Yadav et al. [1995] described the degradation of monochlorobenzene and *o-, m-,* and *p*-dichlorobenzenes by *Phanerochaete chrysosporium*, a white rot fungus. The rate of chlorobenzene degradation was 5.13 mg/liter/day, and at concentrations of 10 mg/l chlorobenzene substantial amounts of growth and degradation were observed.

Under nutrient-rich culture conditions the degradation rate was higher than under nutrient-poor conditions. Oxygen depletion from the cultures resulted in slowing down of chlorobenzene degradation. In table 3 organisms known to utilize chlorinated benzenes under oxic conditions have been listed.

Table 3. Organisms utilizing monochlorobenzene (MCB) or dichlorobenzenes (DCB) as a substrate under oxic conditions.

organism	substrate	product	reference
Pseudomonas putida	halobenzenes	halocatechols	Gibson et al. [1968]
strain WR1306	MCB	CO_2, HCl, biomass	Reineke and Knackmuss [1984]
strain 12A7A	glucose + MCB	chlorobenzene-dihydro-diol + 3-chlorocatechol	Nishino et al. [1992]
Mycobacterium vaccae	propane + MCB	4-chlorophenol	Burback and Perry [1993]
Pseudomonas sp.	1,2-DCB	CO_2, HCl, biomass	Haigler et al. [1988]
Alcaligenes sp.	MCB, 1,3-, 1,4-DCB	CO_2, HCl, biomass	De Bont et al. [1986]
constructed bacterium	1,4-DCB	CO_2, HCl, biomass	Oltmanns et al. [1988]
Alcaligenes strain A175	1,4-DCB	CO_2, HCl, biomass	Schraa et al. [1986]
Pseudomonas sp.	1,4-DCB	CO_2, HCl, biomass	Spain and Nishino [1987]
Pseudomonas sp. strain RH01	MCB	CO_2, HCl, biomass	Fritz et al. [1992]
Pseudomonas putida GJ31	MCB	CO_2, HCl, biomass	Oldenhuis et al. [1989]
Rhodococcus opacus GM14	benzene, CB, 1,3-, 1,4-DCB	CO_2, HCl, biomass	Zaitsev et al. [1995]
Phanerochaete chrysosporum	MCB	CO_2, HCl, biomass	Yadav et al. [1995]
Pseudomonas sp. JS150	MCB, 1,2-, 1,4-DCB	CO_2, HCl, biomass	Haigler et al. [1992]
Pseudomonas sp.	MCB	CO_2, HCl, biomass	Pettigrew et al. [1991]
Pseudomonas putida sp.	MCB	CO_2, HCl, biomass	Bartels et al. [1984]
Pseudomonas putida sp.	MCB	CO_2, HCl, biomass	Klecka and Gibson [1981]

2.3 **Aniline**

Aniline is a naturally occurring primary aromatic amine. The main characteristics of aniline are shown in table 4.

Table 4. Characteristics of aniline.

structural formula:

molecular weight (g/mol):	93.1
melting point (°C):	-6
boiling point (°C):	184.0
relative density (water = 1):	1.02
relative vapor density (air = 1):	3.2
vapor pressure (mbar at 20 °C):	0.4
water solubility (g/l at 20 °C):	34
log P octanol/water:	0.9

Aniline can be degraded, both under oxic and anoxic conditions, by bacteria that can use aniline as energy, carbon and nitrogen source. However, during the manufacture of industrial products environmental concentrations of aniline can rise dramatically. On account of its toxicity, this compound therefore poses an obvious environmental risk.

2.3.1 *Anaerobic degradation of aniline*

Only very few examples are known of anaerobic degradation of aniline. Anaerobic transformation of aniline and some halogen-substituted derivatives were shown for a *Parococcus* sp., a facultative anaerobic denitrifying bacterium [Bollag and Russel, 1976]. The decomposition of aniline was related to growth of the bacterium. Complete mineralization of aniline to CO_2 and NH_3 with sulphate as an external electron acceptor was shown by Schnell et al. [1989]. *Desulfobacterium anilini* oxidized aniline with stoichiometric reduction of sulphate to sulphide as shown in the equation below:

$$2C_6H_5NH_2 + 7SO_4^{2-} + 8H_2O \rightarrow 12HCO_3^- + 7HS^- + 2NH_4^+ + 3H^+$$

Enrichment of anaerobic aniline degrading bacteria, however, resulted in long lag periods. From freshwater sediment, even after 12 months, neither sulphide production nor bacterial growth was observed. Enrichments with marine sediment samples and 0.5 mM aniline formed detectable sulphide production after 6 months. *Desulfobacterium anilini*, isolated from such a marine enrichment, had a doubling time of 72 hours. Initial concentrations of aniline exceeding 0.5 mM retarded growth, probably due to toxicity of the substrate. The amino nitrogen was released quantitatively as ammonia and could also cover the nitrogen need for synthesis of cell material. Degradation of aniline depended on the presence of CO_2. Initial carboxylation to 4-aminobenzoate probably occurred [Schnell and Schink, 1991]. Kazumi et al. [1995] reported the anaerobic degradation of aniline under iron reducing conditions in sediment enrichments.

However, the activity in the enrichments could not be maintained with refeeding, possibly due to an inhibitory effect of accumulated metabolic products, or the lack of a cosubstrate which was subsequently depleted. Myers et al. [1994] studied the microbial potential for the anaerobic degradation of simple aromatic compounds in river sediments. After 18 months significant losses of aniline were shown in sediments amended with multiple electron acceptors, including manganese(IV) and Fe(III) oxides, indicating the presence of anaerobic aniline degrading microbes. Swindoll et al. [1995] showed aniline degradation under denitrifying conditions in soil slurries from a contaminated wetland. Aniline concentrations decreased on average from 44 mg/l to 5 mg/l in 19 days after an acclimation period of 16 days. Aniline degradation did not occur in slurries incubated under sulphate reducing and methanogenic conditions over a 103-day period.

2.3.2 *Aerobic degradation of aniline*

Oxic degradation of aniline proceeds via catechol, which is further metabolized via either the *ortho*-, or the *meta*-pathway, similar to the degradation routes found for oxic degradation of benzene. Ammonia is released during the initial steps leading to the formation of catechol. Probably an initial aniline-oxygenase is involved in the conversion of aniline and some substituted derivatives into catechol. This was shown for a *Nocardia* sp. [Bachofer et al.,1975] , *Rhodococcus* sp. AN117 [Janke et al., 1988], a *Pseudomonas* sp. JL2 [Latorre et al., 1984], and *Moraxella* sp. G [Zeyer et al., 1985]. Kaminski et al. [1983] did a study on the degradation of aniline by *Rhodococcus* sp. strain AN117 and *Pseudomonas* sp. strain SB3. The first organism metabolized aniline exclusively by conversion to and *ortho*-cleavage of catechol. The pseudomonad degraded aniline through the *meta*-pathway by the activity of a constitutive *meta*-cleavage pyrocatechase. The *meta*-pathway was also used by *Pseudomonas* sp. strain CIT1 which posessed high activities of the catechol-2,3-dioxygenase [Anson and Mackinnon 1984; Meyers, 1994]. Loidl et al. [1990] described the degradation of aniline and monochlorinated anilines by soil-born *Pseudo-*

monas acidovorans strains. Initial aniline concentrations of 2.14 mM (0.20 g/l) were degraded within 30 hours by *Pseudomonas acidovorans* CA28 which used aniline as carbon-, energy-, and nitrogen source. The generation time was 3.0 hours and a growth yield of 0.6 g/g carbon was achieved. No catechol-2,3-dioxygenase activity could be detected, but high activities of the catechol-1,2-dioxygenase were present, indicating the *ortho*-pathway to be used for the degradation. Table 5 gives an overview of reports describing the oxidation of aniline under various redox conditions.

Table 5. Organisms mineralizing aniline under various conditions.

organism	condition	reference
Parococcus sp.	denitrifying	Bollag and Russel [1976]
Desulfobacterium anilini	sulphate reducing	Schnell et al. [1989]
enrichment	iron reducing	Kazumi et al. [1995]
sediments	multiple electron accepting	Myers et al. [1994]
soil slurries	denitrifying	Swindoll et al. [1995]
Nocardia sp.	oxic	Bachofer et al. [1975]
Rhodococcus sp. AN117	oxic	Janke et al. [1988]
Pseudomonas sp. JL2	oxic	Latorre et al. [1984]
Moraxella sp. G	oxic	Zeyer et al. [1985]
Pseudomonas sp. SB3	oxic	Kaminski et al. [1983]
Pseudomonas sp. CIT1	oxic	Anson and Mackinnon [1984]
Pseudomonas acidovorans CA28	oxic	Loidl et al. [1990]

2.4 Bacterial degradation of mixtures of xenobiotic aromatic compounds

Organic pollutants can interact and this can have both stimilatory and inhibitory effects on their biodegradation. Such interactions can be very important since most sources of pollution discharge a whole spectrum of substances. Whether multiple substrates can be degraded simultaneously depends on the pollutant concentration, active biomass concentration, temperature, pH, availability of nutrients and electron acceptors, microbial diversity (presence of appropiate metabolic pathways), and the toxicity of the individual substrates or intermediates. Especially when the mixture consists of structure analogues, simultaneous biodegradation of pollutants can be problematic. One substrate can inhibit a metabolic pathway that is needed for biodegradation of the other. Additionally, accidental conversion of structure analogues by enzyme systems (misrouting) may lead to decreased biodegradation, accumulation of intermediates, or production of suicidal products resulting in cell death. The simultaneous degradation of chloro- and methyl-substituted aromatic substrates is for many bacteria impossible as the catabolic pathways are incompatible. Methyl-substituted aromatic substrates are generally degraded via the *meta*-ring-fission pathway, catalysed by catechol-2,3-dioxygenase. Chloro-substituted aromatic substrates are degraded via the modified *ortho*-pathway [Dorn and Knackmuss, 1978; Knackmuss, 1984]. Bartels et al. [1984] and Klecka and Gibson [1981] showed the incompatibility of these pathways for *Pseudomonas putida* strains. The catechol-2,3-dioxygenase of the *meta*-pathway was inactivated by chlorocatechols. For the degradation of haloaromatics by genetically engineered microbes the removal of the *meta*-pathway was needed [Reineke et al., 1982]. Methyl-substituted aromatics are misrouted via the modified *ortho*-pathway if the *meta*-pathway is absent, resulting in accumulation of methyl-muconolactone [Knackmuss et al., 1976]. Burback and Perry [1993] studied the biodegradation and biotransformation of groundwater pollutant mixtures by *Mycobacterium vaccae*. When toluene and benzene were present concomitantly, toluene was

catabolized and benzene oxidation was delayed. Toluene promoted the degradation of styrene, but the degradation rate of toluene was lowered by styrene. 4-chlorophenol had an antagonistic effect on the ability of *Mycobacterium vaccae* to degrade toluene, benzene and chlorobenzene. Burback et al. [1994] showed that metabolites of ethylbenzene, propylbenzene and chlorobenzene biotransformation (4-chlorophenol, 4-ethylphenol and propylphenol) inhibited toluene degradation by *Mycobacterium vaccae* at concentrations of 0.2 mM, 0.4 mM and 0.4 mM, respectively. However, the authors did not speculate about inhibition or induction of specific enzymes involved. Yet, beneficial substrate interactions can occur by enhanced induction of specific catabolic pathways by additional substrates. For example, Arvin et al. [1989] showed that bacteria grown on toluene- and *o*-xylene were able to degrade benzene, whereas naphtalene-, 1,4-dimethylnaphtalene-, and phenantrene-degrading bacteria did have no or very little benzene-degrading ability. However, the stimulating effect of toluene and *o*-xylene was only true if the two compounds were present alone. If both were present an antagonistic effect was observed. Pettigrew et al. [1991] studied the simultaneous degradation of monochlorobenzene and toluene by a *Pseudomonas* strain. This bacterium degrades monochlorobenzene via the modified *ortho*-pathway and toluene is metabolized via the *meta*-pathway. However, when the organism was exposed to a mixture of monochlorobenzene and toluene, the *meta*-pathway was strongly inhibited by the intermediate 3-chlorocatechol and toluene was metabolized via the same modified *ortho*-pathway used for the degradation of monochlorobenzene.

Pseudomonas sp. strain JS150 is capable of synthesizing at least four ring-fission pathways and three separate initial dioxygenases when grown on the appropriate single aromatic substrates [Haigler et al., 1992]. When strain JS150 was continuously grown on monochlorobenzene, it degraded mixtures of monochlorobenzene, benzene, toluene, naphtalene, trichloroethylene, and 1,2- and 1,4-dichlorobenzenes. Phenol-grown cells degraded a mixture of phenol, 2-chloro-, 3-chloro-, and 2,5-dichlorophenol and 2-methyl- and 3-methylphenol. Some compounds that could not serve as growth substrates (chloro- and methyl-substituted phenols, 1,3-dichlorobenzene and 4-chlorotoluene) were readily metabolized by chlorobenzene-grown cells without accumulation of transformation products. Probably enzymes are induced by chlorobenzene which allows the organism to derive carbon and energy from substrates that can not serve as primary growth substrates. Thus the induction of appropriate biodegradative pathways in strain JS150 permits the biodegradation of complex mixtures of aromatic compounds. Alvarez and Vogel [1991] described enhanced benzene degradation by the presence of low amounts (0.1 - 1 mg/l) of toluene by *Pseudomonas* sp. strain CFS215. Strain CFS215 might utilize the same enzyme to degrade both benzene and toluene. The enhanced microbial activity is probably caused by induction of toluene dioxygenase with a broad substrate specificity. The presence of *p*-xylene inhibited benzene degradation. However, when both toluene and *p*-xylene were present with benzene, the enhancement of toluene outweighed the inhibition by *p*-xylene resulting in a faster degradation of benzene than when benzene was present alone. In the same report these authors describe cometabolic degradation of toluene and *p*-xylene by *Arthrobacter* sp. strain HCB during growth on benzene. When benzene was omitted from the medium, no further disappearance of toluene and *o*-xylene was observed [Alvarez and Vogel, 1991]. The white rot fungus *Phanerochaete chrysosporium* can degrade toluene and monochlorobenzene when presented as a mixture [Yadav et al., 1995]. No mutual inhibition of degradation of these compounds was observed. They suggest that the chlorobenzene- and toluene degradation pathways share certain enzymes.

2.5 Bacterial oxidation of (halo)aromatics under various redox conditions

Aromatic hydrocarbons are readily degradable in the presence of oxygen. Oxygen plays a dual role during aerobic degradation. It serves as the final electron acceptor, but also as a cosubstrate in initial reactions catalysed by mono- or dioxygenases. These enzymes incorporate one (monooxygenase) or two oxygen (dioxygenase) atoms derived from molecular oxygen into the aromatic ring. Under anaerobic conditions, nitrate, sulfate or metals, like iron(III), manganese (IV) or Cr(IV) can substitute oxygen and serve as the final electron acceptor. The preferential use of other inorganic electron acceptors depends on the redox potential. The electron acceptor with the highest redox potential is used first, which is generally oxygen, then nitrate, metals, sulphate and carbon dioxide (see fig. 3).

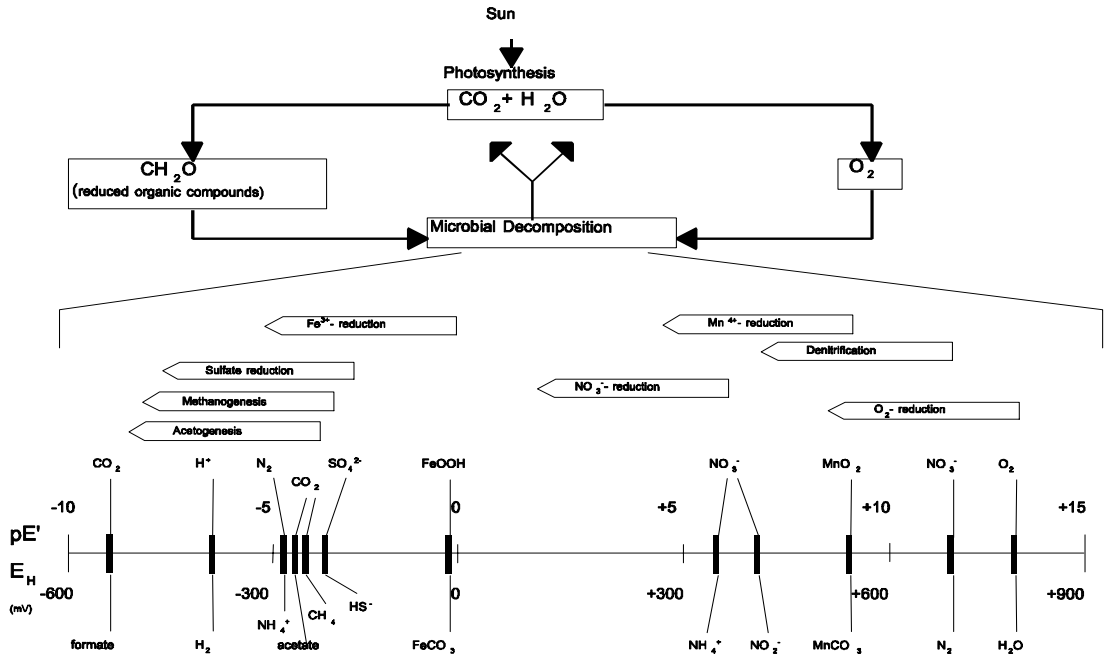

Fig. 3. Microbial decomposition of organic compounds and the preferentially used electron accepting process according to the actual redox potential.

However, such electron acceptors as nitrate, metals, sulphate or carbon dioxide can not act as a cosubstrate for the initial reactions. Another mechanism is probably involved to establish breakdown of the aromatic ring under anoxic conditions. Vogel and Grbíc-Galíc [1986] showed that under methanogenic conditions, activation of the aromatic ring involves the addition of an oxygen atom derived from water to the ring.

Since hydrocarbons entering the environment will move through the soil and/or the groundwater where the level of dissolved oxygen decreases, resulting in decreasing redox potentials, natural aerobic biodegradation of the hydrocarbons will be limited. Therefore, mineralization of (halo)aromatics in such environments will not only depend on the availability of oxygen, but also on the availability of alternative electron acceptors. There are a few recent reports describing the oxidation of (halo)aromatic compounds in the presence of alternative electron acceptors.

2.5.1 *Biodegradation of (halo)aromatics under denitrifying conditions*

Dolfing et al. [1990] isolated and characterized a *Pseudomonas* sp. that mineralizes toluene in the absence of molecular oxygen. The organism mineralizes toluene and *m*-xylene in pure culture, and nitrate served as the electron acceptor and was reduced to nitrite. Nitrite was reduced further only after nitrate depletion. Conversion of 1 mM toluene resulted in production of about

3 mM nitrite, which appeared growth inhibitory. When nitrous oxide served as the electron acceptor, this inhibitory effect could be avoided and therefore a higher cell yield was obtained. However, in FeS reduced medium, toluene degradation coupled to the reduction of nitrous oxide was severely inhibited. Sulphide probably inhibits the nitrous oxide reductase. This problem was not seen when nitrate was supplied as the primary electron acceptor. The authors do not speculate about the pathway used for the anaerobic degradation of toluene. Coschigano et al. [1994] reported on the metabolism of 4-chlorobenzoate and toluene by a constructed bacterial strain. This bacterium obtained the ability to convert 4-chlorobenzoate hydrolytically to 4-hydroxybenzoate. The latter could be degraded with nitrate as an electron acceptor. Nitrate was reduced to nitrite and finally to nitrogen gas. Probably, Coenzyme A thioesters are intermediates in the degradation pathway. These thioesters are formed due to the action of benzoylCoA-ligase. The constructed bacterial strain could also degrade a mixture of 4-chlorobenzoate and toluene under denitrifying conditions. However, toluene was metabolized by the transconjugant at an initial rate of 95 $\mu mol \cdot l^{-1} \cdot h^{-1}$ if provided as the sole substrate. The initial degradation rate decreased dramatically to 15 $\mu mol \cdot l^{-1} \cdot h^{-1}$ when 4-chlorobenzoate was also present at a concentration of 300 μM. The initial rate of 4-chlorobenzoate degradation was 57 $\mu mol \cdot l^{-1} \cdot h^{-1}$, which decreased to 29 $\mu mol \cdot l^{-1} \cdot h^{-1}$ in the presence of toluene. Su and Kafkewitz [1994] described a *Pseudomonas maltophilia* species able to utilize toluene and xylenes under low oxygen and anoxic conditions in the presence of nitrate. In the presence of low oxygen concentrations (2 %) and nitrate better growth was observed than in the presence of nitrate alone. Nitrate was completely reduced to nitrogen gas. The presence of benzene (100 μM) did not affect the extent of the degradation of toluene (500 μM). The presence of low amounts of oxygen in combination with nitrate seems to be beneficial for the degradation, possibly because the presence of oxygen increases the cell numbers. Biegert and Fuchs [1995] reported the anaerobic oxidation of toluene to benzoate by a denitrifying *Thauera* species. The bacterium used nitrate as electron acceptor. Nitrate was first completely reduced to nitrite before nitrite was reduced to N_2O and dinitrogen during growth on toluene. Cell suspensions of anaerobically toluene grown cells degraded 4-, 3-, and 2-fluorotoluene, 4-, 3-, and 2-chlorotoluene, *p*-cresol, *p-, m-,* and *o*-xylene and *p-, m-,* and *o*-methylbenzoic acid with nitrate as electron acceptor to their benzoate analogues. The oxidation of the methyl group of toluene was coupled to the reduction of nitrate, and was inhibited by the presence of oxygen.

The benzoate formed is probably further metabolized via benzoylCoA. Jorgensen et al. [1995] investigated the stoichiometry and kinetics of microbial degradation of toluene under denitrifying conditions by bacterial communities derived from a mixture of sludges from sewage treatment plants. The culture was able to degrade toluene in the presence of nitrate, nitrite, nitric oxide, nitrous oxide or oxygen. The degradation rate of toluene obtained under denitrifying conditions was approximately the same as under oxic conditions. The rates of nitrate and nitrite consumption were proportional to the rate of toluene degradation. The maximum specific rate of degradation was estimated to be 0.71 mg toluene per mg protein per hour, and the yield coefficient was estimated to be 0.14 mg protein per mg toluene. Maximum specific growth rates of 0.10 h^{-1} were acheived. Rabus and Widdel [1995] reported on the anaerobic degradation of ethylbenzene and other related aromatic compounds by new denitrifying bacteria, related to *Thauera selenatis*. One of the isolates, strain EbN1, degraded ethylbenzene with stoichiometric reduction of nitrate to nitrogen gas, see equation below:

$$C_8H_{10} + 8.4NO_3^- + 0.4H^+ \rightarrow 8HCO_3^- + 4.2N_2 + 1.2H_2O$$

No growth under oxic conditions could be obtained on ethylbenzene, propylbenzene, toluene and *m*-xylene, either in the presence or absence of nitrate. However, most of the polar aromatic substrates, such as benzoate and phenylacetate, on which anaerobic growth was obtained could also be degraded under oxic conditions. The mechanism of the initial reaction during aromatic

hydrocarbon degradation in the absence of oxygen remains unclear. Olsen et al. [1994] and Kukor and Olsen [1996] studied the biodegradation of toluene by some *Pseudomonas* sp. under both low (2 %) and high (air saturation) oxygen concentrations. Three pseudomonads showed enhanced nitrate dependent growth on toluene under oxygen-limiting conditions. These pseudomonads (*Pseudomonas* sp. strain PKO1, *Pseudomonas* sp. strain W31 and *Pseudomonas fluorescens* CFS215) were isolated from hypoxic (i.e. O_2-limiting) petroleum contaminated aquifers and were compared with pseudomonads originating from non-hypoxic aerobic environments that did not show enhanced nitrate dependent growth on toluene under hypoxic conditions. The toluene degradation rate was significantly enhanced by the presence of nitrate for strains PKO1, W31 and CSF215. These strains seemed to have a catechol-2,3-dioxygenase with higher affinities than those strains that could not grow with low oxygen concentrations.This suggests that a group of micro-organisms has evolved which is for the most part indistuinguishable from closely related species, but which has adapted to growth and metabolism in low oxygen environments. Such organisms may facilitate the *in situ* bioremediation of xenobiotics because often low oxygen concentrations and nitrate are present in soils and/or groundwater.

2.5.2 *Biodegradation of (halo)aromatics under metal reducing conditions*

Anaerobic degradation of monoaromatic compounds with iron(III) as the sole electron acceptor by *Geobacter metallireducens* was shown by Lovley et al. [1993].

Under iron reducing conditions toluene, benzoate, benzaldehyde, benzylalcohol, *p*-hydroxybenzoate, *p*-hydroxybenzaldehyde, *p*-hydroxybenzylalcohol, phenol and *p*-cresol were utilized by this organism. Kazumi et al. [1995] demonstrated the degradation of monochlorinated and non-chlorinated aromatic compounds under iron reducing conditions in anoxic sediment enrichments. Benzoate, phenol and 2-,3-, and 4-chlorophenol degradation coupled to stoichiometric reduction of Fe^{3+} to Fe^{2+} could be obtained and maintained after refeeding of the sediment enrichments. Anaerobic degradation of 3-chlorobenzoate and aniline was initially observed but could not be maintained after refeeding of the enrichments. The stoichiometry of the anaerobic degradation of phenol and chlorophenol are shown in the equations below:

phenol:

$$C_6H_6O + 28Fe^{3+} + 17H_2O \rightarrow 6HCO_3^- + 28Fe^{2+} + 34H^+$$

chlorophenol:

$$C_6H_5OCl + 27Fe^{3+} + 17H_2O \rightarrow 6HCO_3^- + 27Fe^{2+} + 33H^+ + Cl^-$$

Coates et al. [1996] isolated five organisms from freshwater aquatic sediments, a pristine deep aquifer, and a petroleum-contaminated shallow aquifer, which could obtain energy for growth by dissimilatory Fe(III) reduction. All the isolates belong to the genus *Geobacter*. Three of the five isolates could use hydrogen gas and benzoate as electron donors for Fe(III) reduction. One strain could use toluene as electron donor in the presence of soluble Fe(III) as electron acceptor. Rapid anaerobic benzene oxidation with a variety of chelated Fe(III) forms was demonstrated by Lovely et al. [1996]. Fe(III) chelated to compounds like EDTA, *N*-methyliminodiacetic acid, ethanol diglycine, humic acids and phosphates stimulated benzene oxidation coupled to Fe(III) reduction in anaerobic sediments from a petroleum-contaminated aquifer. So, many forms of chelated Fe(III) might be essential for remediation of aquifers. In microcosms and enrichments Shen et al. [1996] achieved bacterial degradation of benzoate with the transport of electrons to Cr(IV).

Nitrate or oxygen were used as initial stimulators, and after depletion of oxygen or nitrate microcosms and enrichments still retained the capacity of benzoate oxidation linked to Cr(IV) reduction to Cr(III). Benzoate degradation was strongly dependent on the presence of Cr(IV). The addition of nitrate up to a concentration of 5.0 mM did not inhibit Cr(IV) reduction but enhanced benzoate degradation in the enrichments.

2.5.3 *Bacterial oxidation of aromatics coupled to sulphate reduction*

Haag et al. [1991] reported on the degradation of toluene and *p*-xylene in anaerobic microcosms. They found evidence for sulphate acting as a terminal electron acceptor. Complete degradation of toluene and xylene by aquifer micro-organisms coupled to the reduction of sulphate was also shown by Edwards et al. [1992].

Toluene and *o-, m-,* and *p*-xylene were completely mineralized to CO_2 and biomass under strictly anoxic sulphate reducing conditions. The doubling time of the culture was approximately 20 days and the cell yield 0.1 to 0.14 g of cells (dry weight) per g of substrate. Sulphide production as a result of sulphate reduction was inhibitory for the degradation of the aromatic hydrocarbons. Beller et al. [1992] investigated microbial degradation of toluene under sulphate reducing conditions in anaerobic microcosms and the influence of iron on the biodegradative process. Addition of amorphic iron ($Fe(OH)_3^-$) to microcosms and enrichment cultures either greatly facilitated the onset of toluene degradation or accelerated the rate once degradation had begun. In microcosms and enrichments amended with iron, the reduction of ferric iron to ferro iron proceeded concurrently with toluene degradation and sulphate reduction. Probably abiotic reduction of iron with hydrogen sulphide originating from sulphate reduction occurred. Rabus et al. [1993] isolated a bacterium from marine sediment, strain Tol2, under strictly anoxic conditions. The organism degrades toluene under sulphate reducing conditions and seems to belong to the genus *Desulfobacterium*. Toluene concentrations exceeding 0.5 mM were toxic for the bacterium. Doubling times of 72 hours could be achieved. Lovley et al. [1995] showed benzene degradation coupled to the reduction of sulphate. With tracer element studies they demonstrated that [^{14}C]benzene was for 92 % degraded to $^{14}CO_2$. Sulphate was depleted from the medium during benzene metabolism. Benzene degradation immediately stopped when when sediments became sulphate limiting and benzene uptake resumed when sulphate was added again. Molybdate, an inhibitor of sulphate reduction resulted in ceased benzene degradation. The stoichiometry of benzene metabolism coupled to the reduction of sulphate is shown in the equation below:

$$4C_6H_6 + 15SO_4^{2-} + 12H_2O \rightarrow 24HCO_3^- + 15HS^- + 9H^+$$

2.6 **Bacterial oxidation of (halo)aromatics under methanogenic conditions**

Grbíc-Galíc and Vogel [1987] were the first to report on the anaerobic degradation of benzene under methanogenic conditions. At least 50 % of the carbon of both toluene and benzene were degraded to CO_2 and methane. Detection of intermediates revealed that both benzene and toluene were initially oxidized by ring-hydroxylation (benzene) or methyl oxidation (toluene). Based on other detectable intermediates a degradation pathway is proposed for the degradation of toluene and benzene. The tentative sequences of anaerobic methanogenic toluene and benzene degradation to methane and carbon dioxide is shown in figure 4.

Fig. 4. Tentative sequences of anaerobic toluene and benzene degradation by mixed methanogenic cultures [Grbíc-Galíc and Vogel, 1987].

Anaerobic degradation of toluene and *o*-xylene by a methanogenic consortium, derived from an aquifer contaminated with creosote and pentachlorophenol was shown by Edwards and Grbíc-Galíc [1994]. Toluene and *o*-xylene were completely mineralized to CO_2, CH_4 and biomass after very long lag periods (100 - 120 days for toluene degradation, 200 - 255 days for *o*-xylene degradation). Growth inhibitory concentrations were 700 μM for xylene and 1,800 μM for toluene. Alternate electron acceptors, such as oxygen, nitrate and sulphate, inhibited the degradation. The same was true when additional preferred substrates were present (acetate, hydrogen, propionate, methanol, acetone, glucose, amino acids, fatty acids, peptone and yeast extract). This suggests that the presence of natural organic substrates or co-contaminants may inhibit anaerobic degradation of pollutants such as toluene and *o*-xylene at contaminated sites. Chaudri and Wiesmann [1995] detected benzoate as an intermediate in anaerobic enrichment cultures during anaerobic benzene degradation. Although methane was produced, it was doubted whether the methanogens themselves degraded the benzene. When micro-aerobic conditions were established in the enrichment, the growth and benzene degradation rates were noticed to increase only slightly but the production of methane reduced.

Table 6 summarizes the (halo)aromatic oxidizing organisms under various redox conditions.

Table 6. Organisms oxidizing (halo)aromatic compounds under denitrifying, metal reducing, sulphate reducing and methanogenic conditions.

organism	substrate(s)	products	condition	reference
Pseudomonas sp.	toluene, *m*-xylene	CO_2, NO_2^- and N_2	denitrifying	Dolfing et al. [1990]
constructed bacterial strain	4-chlorobenzoate. toluene	CO_2, NO_2^- and N_2	denitrifying	Coschigano et al. [1994]
Pseudomonas maltophilia sp.	toluene, xylenes	CO_2, N_2	denitrifying and oxic	Su and Kafkewitz [1994]
Thauera spp.	toluene	CO_2, NO_2^-, N_2O, N_2	denitrifying	Biegert and Fuchs [1995]
communities	toluene	CO_2, N_2	denitrifying and oxic	Jorgensen et al. [1995]
Thauera selenatis EbN1	ethylbenzene, propylbenzene, toluene, xylene, benzoate, phenylacetate	CO_2, N_2	denitrifying	Rabus and Widdel [1995]
Pseudomonas spp.	toluene	CO_2	oxic + NO_3^-	Kukor and Olsen [1996]; Olsen et al. [1994]
Geobacter metallireducens	toluene, benzoate, benzaldehyde, benzylalcohol, *p*-hydroxybenzoate, *p*-hydroxybenzaldehyde, *p*-hydroxybenzylalcohol, phenol, *p*-cresol	CO_2 , Fe(II)	Fe(III) reducing	Lovley et al. [1993]
sediment enrichments	benzoate, phenol, 2-, 3-chlorophenol	CO_2, Fe(II), HCl	Fe(III) reducing	Kazumi et al. [1995]
Geobacter spp.	benzoate, toluene	CO_2, Fe(II)	Fe(III) reducing	Coates et al. [1996]
sediments	benzene	CO_2, Fe(II)	Fe(III) reducing	Lovley et al. [1996]
enrichments	benzoate	CO_2, Cr(III)	Cr(IV) reducing	Shen et al. [1996]
microcosms	toluene, *p*-xylene	CO_2, H_2S	sulphate reducing	Haag et al. [1991]
enrichments	toluene, xylenes	CO_2, H_2S	sulphate reducing	Edwards et al. [1992]
microcosms	toluene	CO_2, H_2S	sulphate reducing	Beller et al. [1992]
Desulfobacterium sp. strain Tol2	toluene	CO_2, H_2S	sulphate reducing	Rabus et al. [1993]
sediments	benzene	CO_2, H_2S	sulphate reducing	Lovley et al. [1995]
sediments	benzene, toluene	CO_2, CH_4	methanogenic	Grbíc-Galíc and Vogel [1987]
consortium	toluene, *o*-xylene	CO_2, CH_4	methanogenic	Edwards and Grbíc-Galíc [1994]
enrichments	benzene	CO_2, CH_4	methanogenic	Chaudri and Wiesmann [1995]

2.7 Biological treatment of monochlorobenzenes

There are only a few known examples of bioremediation of monochlorobenzene contaminations at this moment. The biological treatment of waste gas contaminated with monochlorobenzene and 1,2-dichlorobenzene was shown by Oh and Bartha [1994]. A maximum removal rate of 200 g/m^3/h of these chlorobenzenes (1.8 g/m^3) was obtained by leading the gas stream via a biological trickling filter (1.6 l) which was inoculated with a bacterial culture (mainly *Pseudomonas* sp.). The air velocity was 102 m/h and the flow of the liquid phase was 3.2 l/h. In soil contaminated with monochlorobenzene and 1,2-, 1,3-, and 1,4-dichlorobenzene degradation by indigenous microbes was obtained by using biostimulation and aeration [Peck et al., 1995]. From laboratory experiments it was concluded that the indigenous microbes were able to degrade the contaminants. Low levels of organic material (straw) and nutrients were added to the soil to stimulate the indigenous population to degrade the chlorobenzenes. Within a period of 5 weeks more than 90 % of the chlorobenzenes (1 - 6 mg/kg) was degraded. Folsom et al. [1995] reported on the remediation of groundwater contaminated with a mixture of pollutants. One of the main contaminants, 1,2-dichlorobenzene and some other dichlorobenzenes (0.1 - 2 mg/l), were degraded for more than 92 % in a fluidized-bed bioreactor (250 l). The flow rate of the reactor was 7.6 l/min (hydraulic retention time of 30 minutes).

2.8 Concluding remarks

Biodegradation of chlorobenzenes under both oxic and anoxic conditions was shown by many researchers. Under anoxic conditions, chlorinated benzenes serve as electron acceptors: chlorine substituents are replaced by hydrogen atoms. Higher halogenated benzenes are more easily reduced than lower halogenated benzenes. Under oxic conditions many bacteria are able to mineralize chlorinated benzenes to CO_2 and cell carbon. Lower halogenated benzenes are more easily degraded than higher halogenated benzenes under oxic conditions.

Biological treatment of chlorobenzenes contaminated waste has been shown in waste gas, soil, and groundwater.

Aniline can be mineralized both under oxic and anoxic conditions and can serve as carbon-, energy-, and nitrogen source. However, under anoxic conditions very long lag-periods (6 - 18 months) were observed.

Mixtures of aromatic xenobiotic compounds can have both stimulatory and inhibitory effects on their biodegradation. Metabolic pathways can be inhibited and misrouting of substrates may lead to toxic (suicidal) products. On the other hand additional substrates may enhance biodegradation of primary substrates by improved induction of particular pathways.

Degradation of (halo)aromatic compounds under denitrifying, metal reducing, sulphidogenic and methanogenic conditions has been shown in pure culture-, mixed culture- and microcosm studies.

CHAPTER 3

PRACTICAL APPROACH

The biodegradative capacities of the indigenous microbes will be studied. Therefore the presence of viable microbes at the contaminated site which are capable of degrading monochlorobenzene and to a lesser extent aniline will be shown first. Next, the optimum physico-chemical conditions for the biodegradation of monochlorobenzene and aniline will be determined. Experiments will be done in soil slurries, batch enrichments, and bioreactors. Field measurements at the contaminated site revealed that monochlorobenzene and aniline are indeed the main contaminants, although high concentrations of di-aminodiphenylmethane were also observed. Therefore the main focus will be on the kinetics of monochlorobenzene degradation and the influence of aniline on this degradation process. Once good degradation is obtained in soil slurries and soil columns, the influence of diaminophenylmethane could be tested additionally. At some sampling sites the pH seemed to be very high (≈ 13), but the general picture is that the pH value lies between 7 and 9.

3.1 Activity of indigenous microbes

Viable monochlorobenzene and aniline degrading microbes must be present at the contaminated site to establish *in situ* bioremediation.

Soil samples with groundwater (1 : 1) of the various contaminated layers (top sandy layer (0 - 5 m depth), clay (5 - 15 m depth), and the deeper sand layer (15 - 23 m depth)) will be incubated at both the *in situ* temperature (≈ 12 °C) and 30 °C without additional nutrients and substrates (monochlorobenzene and aniline). Decrease of the *in situ* concentrations of monochlorobenzene and aniline and the production of carbon dioxide will be monitored in time.

3.2 Batch incubations (soil slurries)

The influence of environmental factors on the degradation rates of monochlorobenzene and aniline in a semi-natural system (presence of heterogenic soil or sediment) can be studied in these batch slurry incubations.

Soil samples will be suspended in groundwater (1 : 1) and additions of substrates, electron acceptors and nutrients can be made in these soil slurries. The degradation rate of monochlorobenzene will be monitored under oxic conditions.

To determine the toxicity of monochlorobenzene, incubations will be done with increasing monochlorobenzene concentrations.

If there are inhibitory or stimulatory effects of the additional contaminant aniline on the degradation rate of monochlorobenzene, incubations with monochlorobenzene as a substrate in the presence of increasing concentrations aniline (range 0 - 10 mM) will be carried out. To see whether additional carbon sources and nutrients will enhance the degradation rate, the decrease of monochlorobenzene in soil slurries will be followed in the presence of different amounts of yeast extract and nutrients The degradation of monochlorobenzene under anoxic conditions in the presence of alternative electron acceptors will also be studied, since oxidation of (halo)aromatics under such conditions has been shown previously. Whether there are microbes present capable of dechlorination of monochlorobenzene to benzene will also be tested in anoxic incubations in the presence of mixtures of electron donors. The main parameters to be tested are listed in table 7.

Table 7. Conditions to be tested for their influence on the degradation rate of chlorobenzene in soil slurries at both the in situ temperature (12 °C) and 30 °C.

oxic:	different concentrations CB (range 0 to 10 mM)
	CB + different concentrations aniline (range 0 - 10 mM)
	CB + different amounts of yeast extract/nutrients
anoxic:	CB + nitrate (+ or - aniline, and + or - nutrients)
	CB + Fe(III) (+ or - aniline, and + or - nutrients)
	CB + sulphate (+ or - aniline, and + or - nutrients)
	CB + HCO_3^- (+ or - aniline, and + or -nutrients)
	CB (electron acceptor) + mixture of electron donors: formate, lactate, acetate, yeast extract and nutrients

3.3 Soil column experiments

In soil columns the natural habitat of the indigenous micro-organisms can be maintained by packing the columns with undisturbed soil samples of the contaminated site. The response of varying environmental conditions on the degradation kinetics of monochlorobenzene in such a system will be closely related to the natural situation.

The most favourable conditions for biodegradation of monochlorobenzene obtained in batch-slurry experiments will be applied to these soil columns. A flow through of groundwater with particular additions can be established and the degradation of the contaminants will be monitored in time.

3.4 Batch enrichment studies

Oxygen plays a dual role in the degradation of aromatic compounds. Oxygen serves as the final electron acceptor, but it is also a cosubstrate in initial reactions catalysed by enzymes like dioxygenases or mono-oxygenases which incorporate molecular oxygen into the aromatic ring. However, in soils and sediments the oxygen concentration is often low. Therefore, mineralization of (halo)aromatics may depend on the presence of alternative electron acceptors or combinations of various electron acceptors.

In soil slurries it is **not possible** to study the influence of low oxygen concentrations on the degradation rates of monochlorobenzene, because of the heterogeneity caused by the presence of sediment. The influence of low oxygen concentrations on the degradation rate can be studied in batch enrichments, in which microbial consortia can be studied in homogenous cultures without any sediment.

The degradation rate of monochlorobenzene will be determined in the presence of oxygen varying from 1 to 20 % O_2. Under low oxygen conditions also the influence of nitrate will be determined, since there are a few examples in the literature in which enhanced degradation of aromatics was shown with oxygen plus nitrate as electron acceptors (see table 8).

Table 8. The influence of various oxygen concentrations in the absence and presence of nitrate on the degradation rate of monochlorobenzene that will be tested in batch enrichment studies.

MCB + 1 % O_2*	+ or - nitrate
MCB + 2 % O_2	+ or - nitrate
MCB + 5 % O_2	+ or - nitrate
MCB + 10 % O_2	+ or - nitrate
MCB + 20 % O_2	+ or - nitrate

* 1 % O_2 in the gas phase, represents 0.9 mM O_2 available for monochlorobenzene degradation in the liquid phase (liquid = 100 ml, gas phase = 210 ml).

3.5 Continuous culture experiments

In continuous cultures the environmental conditions can be manipulated most effectively. Therefore the influence of pH, temperature, substrate concentrations, additional substrates, oxygen supply, and additional electron acceptors on the degradation of monochlorobenzene can be investigated with high accuracy. Moreover, the influence of **very low** oxygen concentrations can hardly be examined in **batch enrichment** studies. In addition, studies in chemostats will be a useful tool to investigate the effect of oxygen-limitation on the biodegradative capacities of microbial consortia.

Also the effects of combinations of various environmental conditions (low O_2 + different pH, various temperatures, additional carbon sources, etc.) on the degradation rates can be studied in continuous culture experiments.

3.6 Isolations and characterizations

It is obvious from literature that when it is known which organisms do degrade the contaminants and which pathways are most important for the metabolism of the substrates, predictions can be made about which factors are important to establish good degradation. Therefore isolations will be done from the contaminated site.

At first dilution series have to be made. From these dilution series the most abundant monochlorobenzene degrading organism(s) will be isolated and characterized. Effects of environmental conditions on the degradation kinetics of these monochlorobenzene degrading bacteria can be studied in detail. Whether attempts will be made to isolate monochlorobenzene or aniline oxidizing organisms under anoxic conditions will depend on the success of the anoxic incubations of the soil slurry experiments.

3.7 Inocula

For the laboratory experiments soil samples of the various contaminated layers (top sandy layer (0 - 5 m depth), clay (5 - 15 m depth), and the deeper sand layer (15 - 23 m depth)) will be used as inocula. In addition, biosludge from an activated sludge water treatment plant will be used. The influent of the activated sludge water treatment plant contains aniline and monochlorobenzene, so the biosludge is adapted to these substrates. Therefore, long lag periods can be avoided.

CHAPTER 4

RESULTS

4.1 Activity of indigenous microbes

In soil slurry experiments it was proven that there is activity of indigenous microbes, responsible for the degradation of monochlorobenzene under aerobic conditions. A decrease in monochlorobenzene concentration, consumption of oxygen and production of CO_2 could be measured.

Soil samples from filters at the MDI location, filters 601, 602 and 603, were incubated under aerobic conditions (20 % oxygen) in the presence of 0.5 mM monochlorobenzene (*in situ* concentration) (T = 30 °C). Disappearance of monochlorobenzene was followed in time (see table 9 and plotted in fig. 5 (filter 603)).

Table 9. Degradation of monochlorobenzene in soil slurries from filters 601, 602, and 603.

filter	degradation of MCB	activity (O_2-consumption; CO_2-production)	degradation rate of CB (μmol·kg^{-1}·day^{-1})	degradation rate of CB (mg·kg^{-1}·day^{-1})
601	+	+	155	17
602	+	+	207	23
603	+	+	207	23

\+ = complete disappearance of monochlorobenzene.

Degradation of monochlorobenzene started after one month of incubation. Once degradation started, monochlorobenzene degradation rates between 155 and 207 μmol per kilogram soil per day were established. After approximately two months all the monochlorobenzene was disappeared in the soil slurry from filter 603. Soil slurries from filters 601 and 602 needed three months for complete monochlorobenzene degradation, including the lag-period (adaptation period micro-organisms need to start degradation, see fig. 5).

Fig. 5. Degradation of chlorobenzene in a soil slurry at 30 °C (20 % oxygen).

4.2 Toxicity of monochlorobenzene and aniline to monochlorobenzene and aniline degrading bacteria

Incubations in batch slurries inoculated with sludge originating from the waste water treatment system with various concentrations monochlorobenzene or aniline, revealed that biodegradation takes place up to 10 mM monochlorobenzene or aniline, added as sole C/E source.

So, based on these results, no toxicity problems of the primary contaminants are to be expected to play a role. However, the lag-phase before growth occurred increased with increasing substrate concentrations.

In addition, degradation rates of monochlorobenzene may be influenced positively or negatively by the presence of the co-contaminant aniline (see 4.4).

4.3 Effect of additional carbon sources and nutrients on monochlorobenzene degradation

In batch slurries, inoculated with soil (filters 601, 602, 603), incubated at 30 °C with 2 mM monochlorobenzene showed degradation rates between 155 and 207 μmoles/kg per day (see 4.1). Additions of 0.5 g/l yeast extract (a complex mixture of organic nutrients) or vitamins did not enhance the degradation rate of monochlorobenzene. More carbon dioxide was produced and higher concentrations of oxygen were consumed, both due to biodegradation of organic carbon present in yeast extract.

4.4 Effect of the co-contaminant on monochlorobenzene degradation

The effect of the co-contaminant aniline on the monochlorobenzene degradation was tested in enrichments from both soil and sludge at 30 °C. The initial MCB concentration in these slurries was 1 mM, whereas the initial aniline concentration varied from 0 to 2 mM.

4.4.1 *Effects of aniline on monochlorobenzene degradation in batch-soil enrichments*

The influence of the co-contaminant aniline on the degradation kinetics of monochlorobenzene in soil was tested in batch enrichments. The enrichments were made from soil slurries originating from filters 601, 602, and 603. After vigorous shaking of a soil-slurry, made from soil and groundwater (1 : 1), and sedimentation of soil particles, the above liquid was filtered through a 5 μm filter to get rid of protozoa. The filtrate was used as inoculum for batch enrichments, which contained synthetic growth medium with 1 mM monochlorobenzene as the growth substrate. After MCB degradation was complete, the culture was transferred and subdivided into fresh media with 2 mM monochlorobenzene and either 0, 0.5, 1 or 2 mM aniline. With increasing concentrations of aniline decreasing degradation rates of monochlorobenzene were measured, from 624 μmol·h^{-1}·g protein^{-1} in cultures with only monochlorobenzene as growth substrate to 92 μmol·h^{-1}·g protein^{-1} if 2 mM aniline was present (see table 10 and fig. 6A-D). These cultures were all incubated aerobically (air saturating conditions), at 30 °C, in the dark.

Table 10. Effect of aniline on MCB degradation rate in soil enrichments.

additional aniline (mM)	MCB degradation rate (μmol·h^{-1}·g protein^{-1})
0	624
0.5	417
1	128
2	92

Fig. 6A-D. Effect of aniline on chlorobenzene degradation rate in soil enrichments.
A: 1 mM CB without aniline; B: 1 mM CB with 0.5 mM aniline; C: 1 mM CB with 1 mM aniline; D: 1 mM CB with 2 mM aniline.

4.4.2 *Effect of aniline on monochlorobenzene degradation in batch-sludge enrichments*

The influence of the co-contaminant aniline on the degradation kinetics of monochlorobenzene in sludge was tested both in batch slurries and batch enrichments. The effect of additional aniline is not significant in slurries made from sludge: neither a positive effect nor a negative effect was observed. However, in sludge enrichement cultures (after 5 transfers), a strong negative influence on the specific rate of monochlorobenzene degradation was observed. This decreased from 595 to 21 μmol·h^{-1}·g protein^{-1} with increasing aniline concentrations (see table 11 en fig. 7A-H). Yet, significant variation in the outcome of these experiments was noted.

Table 11. Effect of aniline on monochlorobenzene degradation rate in sludge enrichments and sludge slurries.

additional aniline (mM)	monochlorobenzene degradation rate	
	sludge slurry (μmol·h^{-1}·liter sludge^{-1})	sludge enrichment (μmol·h^{-1}·g protein^{-1})
0	240	595
0.5	220	96
1	200	24
2	180	21

A B

C D

Fig. 7A-D. Effect of aniline on chlorobenzene degradation rate in sludge enrichments.
A: 1 mM CB without aniline; B: 1 mM CB with 0.5 mM aniline; C: 1 mM CB with 1 mM aniline; D: 1 mM CB with 2 mM aniline.

E F

G H

Fig. 7E-H. Effect of aniline on chlorobenzene degradation rate in sludge slurries.
E: 1 mM CB without aniline; F: 1 mM CB with 0.5 mM aniline; G: 1 mM CB with 1 mM aniline; H: 1 mM CB with 2 mM aniline.

In the very first enrichment transfer from active sludge a positive effect of aniline on the monochlorobenzene degradation rate was observed (data not shown). Upon further transfer of this enrichment into the fresh media, with various concentrations of aniline, the results were obtained as shown in table 11. This effect may be explained by substantial changes in the composition of the selection of microbial species which outcompeted the initial population with respect to monochlorobenzene degradation but were more sensitive to aniline.

4.5 Monochlorobenzene degradation in soil-, and sludge slurries incubated under anaerobic conditions

In batch slurries, both from soil (filter 801 and 802, 16 - 17 m depth) and sludge, the capacity of the indigenous microbes to degrade monochlorobenzene under anaerobic conditions was tested. Alternative electron acceptors such as nitrate, iron(III) and sulphate, were added to these slurries. In none of theses slurries monochlorobenzene degradation occurred within 7 months. If monochlorobenzene itself was supplied as electron acceptor in combination with a range of electron donors, such as lactate, formate and acetate, no disappearance of monochlorobenzene

could be detected. So, under these anaerobic conditions no degradation of monochlorobenzene could be detected (see table 12).

Table 12. Degradation of monochlorobenzene from filters 801 and 802.

filter	electron donor	electron acceptor	CB degradation
801, 802	monochlorobenzene	oxygen	+
801, 802	monochlorobenzene	nitrate	-
801, 802	monochlorobenzene	iron(III)	-
801, 802	monochlorobenzene	sulphate	-
801, 802	lactate/formate/acetate	monochlorobenzene	-

4.6 Degradation kinetics of monochlorobenzene under reduced partial pressures of oxygen

In batch experiments the degradation rate of monochlorobenzene at varying oxygen concentrations was determined at 30 °C, both for enrichments from activated sludge and soil samples, and at the *in situ* temperature for sludge enrichments (12 °C).

4.6.1 *Degradation kinetics of monochlorobenzene at 30 °C*

In soil enrichments with low oxygen concentrations, 2 % and 0,5 % O_2 respectively, no chloride was formed, due to incomplete monochlorobenzene degradation. Probably accumulation of chlorocatechols occurred, since the media turned brownish. The specific monochlorobenzene degradation rates decreased with decreasing oxygen concentrations (see table 13). In figure 8A, B, C and D the results of the complete (A + B) and incomplete (C + D) degradation in soil enrichments of monochlorobenzene are shown.

Monochlorobenzene degradation in enrichments of activated sludge showed the same pattern as soil enrichments, that is decreasing monochlorobenzene degradation rates with decreasing oxygen concentrations. However, the monochlorobenzene degradation at low oxygen concentrations was complete. Both total disappearance of monochlorobenzene and full chloride recovery could be measured (see table 13).

Table 13. Degradation of monochlorobenzene at various oxygen concentrations in sludge- and soil enrichments at 30 °C.

inoculum	% O_2	mM CB degraded	mM Cl^- formed	degradation rate (μmol·h^{-1}·g $protein^{-1}$)
sludge	20	1.8	1.6	442
	2	1.6	0.9	210
	1.5	2.0	1.6	207
soil	20	1.0	1.2	150
	10	1.7	1.6	121
	2	1.1	0.1	83
	0.5	0.6	0	69

A

B

C

D

Fig. 8A-D. Degradation of chlorobenzene at various oxygen concentrations in soil enrichments. A: 20 % O_2; B: 10 % O_2; C: 2 % O_2; D: 0.5 % O_2.

4.6.2 *Degradation kinetics of monochlorobenzene at 12 °C*

Sludge- and soil enrichments were also incubated with monochlorobenzene at various oxygen concentrations at the *in situ* temperature (12 °C). The MCB degradation rate in sludge enrichments (12 °C) was significantly lower than the degradation rate achieved at 30 °C. At high oxygen concentrations the degradation rate was also higher than at low oxygen concentrations, a comparable tendency was found at 30 °C.

In batch-soil enrichments complete degradation occurred at high oxygen concentrations. No degradation was observed within 2 months under reduced partial pressures of oxygen (see table 14 and fig. 8A and B).

At higher oxygen concentrations a higher MCB degradation rate was observed, both at 30 °C and 12 °C.

Table 14. Degradation of monochlorobenzene at various oxygen concentrations in sludge- and soil enrichments at 12 °C.

inoculum	$\%O_2$	mM CB degraded	mM Cl^- formed	degradation rate (μmol·h^{-1}·g protein^{-1})	degradation rate (μmol·day^{-1})
sludge	20	0.8	0.6	61	-
	3	0.7	0.6	57	-
soil	20	0.5	0.5	no degradation	< 8
	3	0.5	no degradation	no degradation	-

A B

Fig. 9A-B. Degradation of chlorobenzene at various oxygen concentrations (12 °C) in sludge enrichments. A: 20 % O_2; B: 3 % O_2.

4.7 Effect of additional alternative electron acceptors on monochlorobenzen degradation at low oxygen concentrations

The effect of additional alternative electron acceptors on the monochlorobenzene degradation at low oxygen concentrations (3 % O_2) at 30 °C and 12 °C was studied in both soil- and sludge enrichments.

In sludge enrichments a slight decrease in the initial nitrate concentration (from 10 to 7 mM) was observed. The sludge enrichments in which sulphate was added no decrease of sulphate was observed. In soil enrichments a comparable effect was shown for incubations with nitrate as additional electron acceptor. Since no brownish colour appeared in the medium during incubations in both soil- and sludge enrichments probably no catechol was produced as an intermediate.

The MCB degradation rates in both sludge- and soil enrichments are presented in table 15 and figures 10A, B, C and D (sludge), and figure 11.

Table 15. Degradation of monochlorobenzene with alternative electron acceptors at low oxygen concentration (3 %) (2.8 mM available for CB degradation, headspace = 210 ml, liquid = 100 ml).

source enrichment	incubation temperature (°C)	additional electron acceptor	monochlorobenzene degradation rate (μmol·h^{-1}·g protein^{-1})
sludge	30	nitrate	109
	12	sulphate	95
		nitrate	57
		sulphate	74
soil	30	nitrate	93
	12	nitrate	no degradation

A

B

C

D

Fig. 10A-D. Degradation of chlorobenzene with alternative electron acceptors at low oxygen concentrations in sludge enrichments.
A: nitrate (30 °C); B: sulphate (30 °C); C: nitrate (12 °C); D: sulphate (12 °C).

Fig. 11. Degradation of chlorobenzene with nitrate as alternative electron acceptor in a soil enrichment (30 °C).

4.8 Monochlorobenzene degradation in soil columns

Procedure
The soil columns were packed with 1 kg of a mixture of soil originating from filters 601, 602, 603, 801 and 802.The column is recirculated (flow rate is 540 ml/hr) with 230 ml ground water (see fig. 12). A monochlorobenzene concentration of 1.0 mM and 10 mM nitrate is injected into the bottle. To establish saturation of monochlorobenzene attached to the soil particles, the columns were respiked several times with 1.0 mM MCB to a final nominal concentration equivalent to 23 mM in the water phase and 0.6 mg per kg of soil. As soon as a stable level in the water phase of MCB was established (about 10 μM) the column was fed with peroxide (0.1 mM/day), to create an aerobic environment.

Results
After 150 days of recirculation of the water phase, only between 0.16 and 0.24 mg MCB/kg soil (comparable with 6 - 9 mM in the water phase) remained in both the aerobic and anaerobic soil column according to GC-MS measurements (mean values). High concentrations of chloride could also be detected at the end of incubation. Therefore degradation of MCB occurred both under anaerobic and aerobic conditions. In the anaerobic column, no benzene could be detected as a product of anaerobic dehalorespiration (see table 16).

The observation of CB disappearance in the anoxic column is unexpected if compared with the negative results obtained with anoxic batch incubations (see table 12). Both oxygen and redox analysis of the circulating medium confirmed that the conditions in the column were strictly anoxic. The incomplete recovery of the chlorine could indicate the accumulation of undegraded chlorine containing intermediates not detected in the GC-analysis employed in this study. Yet, in spite of these incomplete balances we may conclude that monochlorobenzene is not only degraded under oxic conditions. It can only be speculated that that presence of soil structures and surfaces may explain the observed degradation in soil columns and the absence of breakdown in batch cultures.

Fig. 12. Overview soil column experiment.

Table 16: MCB consumption and chloride production in aerobic and anaerobic soil column (room temperature about 22 °C).

column	MCB (t = 0)		MCB (t = 150 days)		MCB disappeared	Cl^- produced
	mM	g MCB/kg soil	μM	mg MCB/kg soil	mM	mM
aerobic	23	0.6	9	0.24	23	11.1
anaerobic	23	0.6	5.9	0.16	23	11.4

4.9 **Monochlorobenzene degradation in continuous culture**

A continuous culture was set up as a tool to study the effects of environmental conditions in an accurate way on the MCB degradation kinetics.

A continuous culture was started (dilution rate 0.01 h^{-1}, pH 7 - 7.3, $Sr_{chlorobenzene}$ 1 mM, $Sr_{nitrate}$ 10 mM, 1% O_2) with an inoculum from a soil slurry. The incubation temperature was 12 °C. No degradation of monochlorobenzene could be detected during one month. After reinoculation with activated sludge material, good MCB degradation was observed. However, as soon as the bio-reactor became limited for several days, monochlorobenzene degradation ceased. The same phenomenon was observed at a temperature of 30 °C. So, no continuous enrichment culture could be obtained.

Toxic products accumulating during MCB limited growth can be ruled out since stable MCB degrading enrichments were able to grow in the supernatant of the chemostat culture.

Wash out of growth factors present in the inoculum (activated sludge) or disturbance of formed bacterial aggregates by stirring may be responsible for the interrupted MCB degradation.

CHAPTER 5

CONCLUSIONS

Degradation of monochlorobenzene occurred both in soil- and sludge slurries and in enrichments.

Incomplete degradation of monochlorobenzene was observed under conditions with restricted oxygen availability. In soil enrichments chlorocatechols accumulated during MCB degradation under low oxygen concentrations. This same effect was not observed during MCB degradation at low oxygen concentration in sludge enrichments.

Decreasing MCB degradation rates were found with decreasing oxygen concentrations for both soil-, and sludge enrichments

Additional alternative electron acceptors such as nitrate and sulphate did not enhance the monochlorobenzene degradation rate of low partial pressure of oxygen, despite the fact that some nitrate was reduced.

The effect of the co-contaminant aniline appears to be somewhat unpredictable: initial stimulatory effects were no longer observed upon further transfers. Instead, decreased rates of degradation were observed, possibly due to changes in community composition.

Degradation of monochlorobenzene in sludge slurries occurred at 30 °C and at the *in situ* temperature (12 °C) in the presence of low oxygen concentrations.

No MCB degradation was observed under denitrifying, iron reducing, sulphate reducing and dehalorespiring conditions in batch slurries and enrichments but in anoxic soil columns indications for breakdown of monochlorobenzene have been obtained.

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