Cometabolic degradation of thiophene with benzene as primary substrate

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Ph.D. thesis

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PREFACE

This study was carried out at the Department Environment & Resources DTU (E&R DTU) and at the department of Chemistry at the Technical University of Denmark from February 1998 to May 2001.

I would like to thank my advisors professor Erik Arvin E&R DTU and associated professor Hans Mosbæk for their help and guidance during this project. I want to thank all colleagues of the E&R DTU for help and support, especially Anders Torp Gundersen and Susanne Kruse for technical assistance in the laboratory, Grete Hansen and Helle Offenberg (Library), Birte Brejl and Torben Dolin (Drawing), Ph.D. Eberhard Morgenroth and Ph.D. student Roald Kommedal for many discussions about biodegradation studies.

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Lyngby, July 2001

Isabelle M. Rivas

ABSTRACT

Thiophene is a tar component and is often present at sites contaminated by benzene that is one of the most widespread contaminants in soil and groundwater. Earlier research shows that thiophene is resistant to biological degradation, but it can be degraded cometabolically with benzene or other aromatic hydrocarbons as a primary substrate. However, little is known about thiophene degradation. This thesis summarises the available knowledge about thiophene and its biological degradation.

During this thesis work, the conversion of thiophene with benzene as primary substrate was investigated with suspended and attached bacterial cultures. The study showed that the transformation of thiophene was characterised by competitive inhibition between both substrates. Thiophene conversion was stimulated by benzene. It also lead to the loss of bacterial activity which could be recovered by increasing the benzene concentration. Microbial death could also be observed during thiophene conversion. Cells that were not fed with benzene as growth substrate but had previously been activated with benzene were able to degrade thiophene.

A review of the mathematical models for cometabolism was performed. The model thought to be the most suitable for the simulation of the thiophene conversion with benzene as primary substrate was compared to the experimental data. This model which included inactivation by loss of energy sources and microbial death due to toxicity of metabolites could describe the transformation of thiophene qualitatively. However, it failed in describing it quantitatively. This study did not result in a mathematical model which fully could describe the cometabolic conversion of thiophene with benzene as primary substrate.

Thiophene was transformed into metabolites that were not degraded further. Mass spectrometry and nuclear magnetic resonance analysis allowed the identification of the two main thiophene conversion products called C4 (78% of the converted thiophene) and C5 (20% of the converted thiophene). C4 and C5 are two diastereoisomers of thiophene sulphoxide dimer which is the result of a Diels-Alder condensation between two thiophene S-oxides. Two minor metabolites (C2 and C3) were partially identified. C2 and C3 were shown to be the adducts of a Diels-Alder condensation of thiophene or thiophene sulphoxide derivatives. A fifth metabolite (C1) could not be identified because it was lost during the identification procedures. About 0.05% of the converted thiophene was transformed into benzothiophene. The presence of, or the concentration of benzene did not have any influence on the distribution of the metabolites.

Dose-response experiments showed that the compounds C1-C5 had no toxic effect on the benzene-degrading ability of the microorganisms. Consequently, microbial death resulting from thiophene degradation could not be due to the toxicity of these compounds. Thiophene itself or other more toxic metabolic intermediates might be responsible for the microbial death.

Acute toxicity tests with the fresh water algae *Selenastrum capricornutum* and the marine bacterium *Vibrio fischeri* showed that the compounds C2-C5 are more toxic than thiophene.

RESUMÉ

Cometabolisk nedbrydning af thiophen med benzen som primært substrat af Isabelle M. Rivas. Ph.D. afhandling, Miljø & Ressourcer DTU, Danmarks Tekniske Universitet, 2001. Afhandlingen er skrevet på engelsk med følgende titel: Cometabolic conversion of thiophene with benzene as primary substrate.

Thiophen er et tjærestof og findes tit på lokaliteter forurenet med benzen, en af de mest udbredte forurenings komponenter i jord og grundvand. Tidligere forskning har vist at thiophen ikke er bionedbrydelig, men stoffet kunne nedbrydes ved cometabolisme med benzen eller andre aromatiske kulbrinter som primært substrat. Viden om thiophens nedbrydning er imidlertidig meget lille. Denne Ph.D. afhandling sammenfatter viden om thiophen og dets bionedbrydning.

Under Ph.D. arbejdet blev thiophens nedbrydning med benzen som primært substrat undersøgt med suspenderet og adhæreret biomasse. Undersøgelsen viste, at thiophens nedbrydning er karakteriseret ved kompetitiv inhibering mellem begge substrater. Benzen stimulerede thiophens omdannelse. Thiophens omdannelse førte til tab af den bakterielle aktivitet, der dog kunne genoprettes gennem en forøgelse af benzens koncentration. Der blev også iagttaget mikrobiel død. Celler, der ikke voksede på benzen men som tidligere havde vokset på benzen, kunne også nedbryde thiophen.

Afhandlingen indholder en oversigt over matematiske modeller for cometabolisme. Modellen, der blev betragtet som den bedste til at beskrive thiophens nedbrydning med benzen som primært substrat, blev sammenlignet med de eksperimentelle resultater. Modellen omfattede inaktivering af cellerne på grund af energitab og mikrobiel død, der skyldes toksicitet af omdannelsesprodukterne. Modellen kunne beskrive thiophens nedbrydning på en kvalitativ måde. Den kunne dog ikke beskrive den på en kvantitativ måde. Undersøgelsen kunne ikke foreslå en matematisk model, der fuldt ud kan beskrive den cometaboliske nedbrydning af thiophen med benzen som primært substrat.

Thiophen blev omdannet til produkter, der ikke kunne nedbrydes videre. Analyse med mass spektrometri og nuklear magnetisk resonans førte til identificering af to større thiophen omdannelsesprodukter C4 (78% af det omdannede thiophen) og C5 (20% af det omdannede thiophen). C4 og C5 er to diastereoisomerer af thiophen S-oxid dimeren, der er resultat af en Diels-Alder reaktion mellem to thiophen S-oxider. To mindre omdannelsesprodukter (C2 og C3) blev delvist identificeret. De viste sig at være resultater af en Diels-Alder reaktion mellem thiophen eller thiophen S-oxid derivativer. Et femte omdannelsesprodukt (C1) kunne ikke identificeres, fordi stoffet blev tabt i analyseprocedurerne. Omkring 0.05% af det omdannede thiophen blev til benzothiophen. Tilstedeværelse af, eller koncentrationen af benzen, indvirkede ikke på fordelingen af omdannelsesprodukter.

Ved hjælp af dosis-respons undersøgelser blev det vist, at produkterne C1-C5 ikke var toksiske i relation til bakteriernes evne til at nedbryde benzen. Den mikrobielle død, der fandt sted, når thiophen blev omdannet, kan derfor ikke forklares med toksicitet af disse produkter. Thiophen eller andre mere toksiske omdannelsesprodukter må være ansvarlig for den mikrobielle død.

Akut toksicitetsundersøgelser med ferskvandsalgen *Selenastrum capricornutum* og den marine bakterie *Vibrio fischeri* viste, at produkterne C2-C5 var mere toksiske end thiophen.

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List of publications

This thesis is based on the four following publications:

Rivas, I. M. and Arvin, E. (2000). Biodegradation of thiophene by cometabolism in a biofilm system**.** *Wat. Sci. Tech.* **41** (4), 461-468.

Rivas, I. M.; Mosbæk, H.; Jensen, K. J. and Arvin, E. (2002a). Identification of products from microbial oxidation of thiophene**.** Submitted to Environmental Science & Technology.

Rivas, I. M.; Mosbæk, H. and Arvin, E. (2002b). Product formation from thiophene by a mixed bacterial culture - Influence of benzene as growth substrate**.** Submitted to Water Research.

Rivas, I. M.; Mosbæk, H. and Arvin, E. (2002c). Toxicity of thiophene oxidation products**.** Submitted to Environmental Toxicology and Chemistry.

The papers are not included in this www-version but can be obtained from the Library at Environment & Resources DTU, Bygningstorvet, Building 115, Technical University of Denmark, DK-2800 Kgs. Lyngby (library@er.dtu.dk).

PART I

1 Introduction

More than 99% of drinking water and freshwater resources in Denmark originate from groundwater. Any kind of contamination of the groundwater is therefore of primary concern; groundwater quality is receiving considerable attention. As soon as contamination has been detected, it is important to be able to evaluate its extent, its consequences, the possibility of natural attenuation, as well as the likelihood of bioremediation.

The first considerable chemical pollution of soils and groundwater started in the 19th century, with the production of combustible gas from coal, coke, and oil for urban heating, cooking, and lighting. Approximately 3000 gasworks were reported in the United Kingdoms (Wilson, 1981), and there were 1000 to 2000 in the United States (Luthy et al., 1994). In Denmark, 112 gasworks have been registered (Upton-Hansen et al., 1983). Traditionally, coal was extracted, then treated to make combustible gas. On sites where coal mining was not possible for economical or environmental reasons, underground coal gasification took place (Stuermer et al., 1982). The production of gas continued until the 1950´s, at which time coal gas was replaced by natural gas, and most of the gasification plants were closed (Wilson, 1981).

The gasification of coal, coke and oil resulted in the production of secondary products such as tar and ammonia-rich process water. The produced tar was mostly managed off site or disposed of in wells, or sewers near pits or streams (Luthy et al., 1994). As a result, tar constituents contaminated the soils and aquifers.

The secondary products produced during the gasification of coal, coke, or oil were valuable products that were used further. The tar was used for road construction (McNeil, 1983), roof covering, or wood impregnation (Environment Canada, 1993; Mueller et al., 1989). The ammonia-rich water was used for fertilising purposes (Dyreborg et al., 1999). Many of these materials were also used in the pharmaceutical, pesticide, explosive, dye, cleaning and sweetening industries (Bollag and Kaiser, 1991; Kuhn and Suflita, 1989). As a consequence of the industrial use of these products, the contamination by tar constituents was not only located at gasworks but was more widespread.

Tar contains a large number of organic compounds, of these, only a few have been identified (Environment Canada, 1993; McNeil, 1983). These organic compounds can be grouped into monoaromatic hydrocarbons (e.g. BTEX), polyaromatic hydrocarbons (PAH), phenols, and heterocyclic aromatic carbons. The tar composition varies with the gas production process (IARC, 1984), but it is only slightly influenced by the origins of the coal (Kleffner et al., 198; Novotny et al., 1981). In general, tar contains 85% PAH, 1-10% phenolic compounds, a few percent monoaromatic hydrocarbons, and 5-13% heterocyclic aromatic compounds (Arvin and Flyvbjerg, 1992; Dyreborg et al., 1999; Johansen et al., 1998). Heterocyclic aromatic carbons are organic compounds that contain at least one aromatic ring and one heteroatom which can be nitrogen, sulphur, or oxygen. These compounds are also called NSO-compounds. The ratio of nitrogen-, sulphur- and oxygen-compounds in

the NSO-fraction of tar is approximately 7:2:1 (Collin and Zander, 1982; Mueller et al., 1989).

In case of a subsurfacial tar spill, gravity leads the tar constituents to migrate downwards through the unsaturated zone to the saturated zone of the contaminated ground. Because tar is very viscous and has a density which is slightly higher than water (Dyreborg et al., 1999; Johansen et al., 1998), the tar constituents penetrate the saturated zone. This results in the dissolution of tar constituents in the groundwater (Dyreborg et al., 1999; Luthy et al., 1994). The concentration of the different tar components in the groundwater will depend on the tar composition, the water solubility and sorption capacity of each compound (Dyreborg et al., 1999). Heterocyclic aromatic compounds are, due to their structure, often more polar and water soluble than their homocyclic counterparts (Adrian and Suflita, 1994). They have therefore a greater tendency to be transported to the subsurface environment. Compounds such as phenols, monoaromatic hydrocarbons, and heterocyclic aromatic compounds are dominant in the water-soluble fraction because of their relatively high solubility. Naphthalene is also dominant because of its high concentration in tar. Typically the water-soluble fraction of coal tar will contain up to 38% of phenolic compounds and about 25% NSO-compounds (Arvin, 1993; Arvin and Flyvbjerg, 1992; Dyreborg et al., 1999; Godsy et al., 1987). Johansen et al. (1997) reported that heterocyclic aromatic compounds could constitute up to 50-75% of an aqueous creosote contamination.

NSO-compounds are often toxic even at low concentration (Bollag and Kaiser, 1991), and most have a strong smell and taste (Buttery et al., 1977; Maga, 1981). Their presence therefore reduces the quality and the potability of water.

The subject of this thesis work was the microbial degradation of one of these NSO-compounds, thiophene. This compound is part of the benzene fraction of coal tar distillate and is therefore present at sites contaminated with benzene. Thiophene was shown to be resistant to biological degradation when it is present as the sole carbon source. However, it could be degraded in the presence of other organic substrates (e.g. benzene) (Dyreborg et al., 1997; Dyreborg et al., 1998; Dyreborg et al., 1996). The knowledge about its degradation is, however, very scarce.

The aim of this thesis work was to study the microbial transformation of thiophene in the presence of benzene, because these two compounds usually occur together. A further goal of this study was to investigate the formation of metabolites from thiophene and the evolution of toxicity during this conversion. The third objective of this work was to find a mathematical model that can predict the fate of thiophene.

The major aim of this report is to review the international literature on the studied subject and to demonstrate how this work has contributed to the increase in the state of knowledge.

This report is organised in eight chapters including the introduction. Chapter 2 deals with thiophene, its origin, physical properties, toxicity, and the extent of contamination by this compound. The third chapter gives an overview of the knowledge about the cometabolic conversion of thiophene and chapter 4 deals with the thiophene conversion products and proposes a pathway for the microbial transformation of thiophene. The fifth chapter deals with the toxicity increase during the microbial conversion of thiophene. The sixth chapter reviews mathematical

models describing the process of cometabolism and intends to find a model describing the transformation of thiophene. Finally, the seventh chapter summarises the results obtained during this thesis.

2 Thiophene, a tar and petroleum component

2.1 Physical and chemical properties

Thiophene is a five atom heterocyclic aromatic compound consisting of four carbon atoms and one sulphur atom. This compound is part of the NSO-fraction of coal tar. Victor Meyer discovered it in 1885 (Meyer, 1888) as an impurity in the benzene fraction of coal tar distillate. Meyer isolated this compound from the benzene fraction of coal tar and determined its structure (Fig. 1).

Fig. 1. Thiophene

Thiophene and benzene have very similar physical and chemical characteristics (Table 1). As a result of their close boiling points (84.16 °C for benzene and 80.10 °C for thiophene), these two compounds are difficult to separate by distillation. This explains why thiophene is almost always present in the crude benzene fraction of coal tar distillate to the extent of app. 0.5 to 1.5 wt % (Collin and Kleffner, 1983).

Thiophene and its derivatives are also found in carbonaceous deposits of lignite, peat, shale, coal and certain crude oils (Buchholz, 1983; Collin and Kleffner, 1983; Pratt, 1995). Thiophene derivatives are also naturally produced by some plants (Buitelaar et al., 1991).

Table 1. Physical data for thiophene and benzene

	Unit	Thiophene	Benzene
Molar weight	$g.mol^{-1}$	84.14	78.11
Water solubility at 18°C [a]	$g.m^{-3}$	3600	1760
Boiling point at 101.3 kPa [b]	°C	84.16	80.10
Volatility v.p. at 25°C [c]	kPa	10.70	12.70
Henry's low constant at 25°C [c]	-	0.10	0.22
Partition coefficient log K_{ow}		1.81	2.13

a: (Verschueren, 1996); b: (Lide, 1993), c: (Mackay and Shiu, 1981)

2.2 Thiophene as an industrial intermediate

2.2.1 Industrial interests of thiophene

Thiophene chemistry proved to be very interesting for several industrial branches. Its use has developed widely, which is witnessed by the large number of patents and literature references describing applications or synthesis of thiophene derivatives.

The thiophene moiety found a broad application in the fine chemical industry as an intermediate for medicinal, veterinarian, agrochemical, dyestuff, and flavouring

applications (Fuller et al., 1978; Zaretskii et al., 1989). The list of pharmaceutical products containing the thiophene moiety is extensive. A few of the applications in which the thiophene moiety is present are anti-inflammatory compositions, analgesic agents, treatments for cardiovascular diseases, antipsychotic medicine, and antibiotics (Fuller, 1997; Fuller et al., 1978). In agrochemistry, thiophene derivatives are used in herbicidal compositions (Fuller, 1997).

New developments in the polymer industry, with the production of polyorganosiloxanes, allowed thiophene to be used for electronic applications. The polyorganosiloxanes are components with unique electronic properties (Zaretskii et al., 1989; Zhogin et al., 1998).

2.2.2 Production and sources of thiophene

Methods for the chemical synthesis of thiophene were investigated, since the separation of thiophene from benzene is too difficult. The first semi-commercial thiophene production unit was started up in 1947 in the United States by Socony Vacuum Company (the present Exxon Mobil Corporation). The process used was the "Socony-Vacuum" process that consisted of a continuous high temperature (560ºC), non-catalytic reaction of butane and sulphur (Buchholz, 1983). However, this process allowed a yield of only 40% based on butane and produced highly odorous thiophene tars that were difficult to dispose of. It was therefore abandoned in the 1960´s. Today, thiophene is prepared according to different methods that are all using a variety of C4 raw materials with a sulphur source in continuous, vapour phase processes with special metal oxide catalyst. The catalysts enhance the reaction rates and yields, and avoid the formation of thiophene tars (Buchholz, 1983). The C4 raw material and sulphur source combination may be furan with hydrogen sulphide, 1-butene, butadiene, n-butyl aldehyde or crotonaldehyde with carbon disulphide, or butane with sulphur (Buchholz, 1983).

In Russia however, no synthetic methods for the production of thiophene have been developed (Zaretskii et al., 1989). Until recently, the main source of thiophene was the benzene fraction of the products of coking hard coals of the Donetsk Basin that have a thiophene content of 1.3-1.7% (Zhogin et al., 1998). The dissolution of the Soviet Union forced the Russian industry to find new sources of thiophene and to improve the separation processes of thiophene from the benzene fraction in order to continue to meet industrial demands.

The world annual industrial production of thiophene was estimated to be between 1500 and 3000 tonnes in 1982 (Buchholz, 1983). In 1997, the production was estimated at several hundreds of tonnes, which implies a reduced production (Fuller, 1997). Other sources report, however, that the demand for thiophene - for the production of polyorganosiloxanes - reached 1500 tons a year in 1998 in Russia (Zhogin et al., 1998).

2.3 Thiophene contamination

Risk assessment studies of contaminated sites are typically limited to mono- and polyaromatic hydrocarbons, as well as phenols. Although NSO-compounds frequently have a strong smell and a high solubility, they are seldom reported. When NSO-compounds are measured, thiophene is most often not included in the analysis of the

environmental samples. Therefore, only a few reports on thiophene contamination are available (Table 2).

The lack of documentation does not mean that thiophene is not present as an environmental contaminant. Thiophene is expected to be present as a minor contaminant on sites contaminated by tar. The industrial production of thiophene and its use in organic synthesis or as a solvent might lead to its release in waste streams. Thiophene might also be present as a major contaminant at industrial sites (Pratt, 1995).

As a consequence, the exposure to thiophene might take place at production sites and through the ingestion of contaminated food or water.

Table 2. Reports of thiophene contamination

Reference	Concentration	Location
(Johansen et al., 1997)	8.0 μg/L	Groundwater at Ringe Asphalt Factory, Denmark (at the source)
(Johansen et al., 1997)	1.1 μg/L	Groundwater at Holte Gasworks, Denmark
(Johansen et al., 1997)	6.7 μg/L	Groundwater at Fredericia gasworks, Denmark (25 m from the source)
(Frandsen et al., 1999)	16.0 μg/L	Groundwater at Valby gasworks, Denmark
(Mironov and Scekaturina, 1986)	2.3 x the concentration of a moderately polluted area in mussels	Black sea

2.4 Toxicity of thiophene

Thiophene was shown to be moderately toxic (Table 3). The toxicity of thiophene can be compared to the toxicity of tienilic acid that contains the thiophene moiety (Imler et al., 1987). Tienilic acid can lead to jaundice with the presence of anti-endoplasmic reticulum antibodies (Imler et al., 1987). Thiophene lead to similar symptoms in women, who had received this compound during medical treatment (Imler et al., 1987). Several other pharmaceutical applications containing the thiophene moiety have also shown similar effects on patients. For other applications containing the thiophene moiety, hepatotoxicity has never been mentioned (Imler et al., 1987).

Table 3. Toxicity data for thiophene

Species		Concentration
Mice	LC_{50} inhalation (2h)	9.5 mg/L [a]
Mice	LC_{10} inhalation (unspecified exposure period)	30 mg/L [b]; 2.9 mg/L [d]
Mice	LD_{50} oral	420 mg/kg [c]
Rats	LD_{50} oral	1400 mg/kg [c]
Redwing blackbird	LD_{50} oral	101 mg/kg [e]
Orange spotted sunfish	LC (1h)	27 mg/L [f]
Fish *Oryzias latipes*	LC_{50} (48h)	15.6 mg/kg [g]
Daphnia magna	EC_{50}	13 mg/kg [h]
Green algae	EC_{95} (48h)	10 mg/L [i]
Vibrio fischeri	EC_{50} (30 min)	180 mg/L [j]

a: (Fuller, 1997); b: (Lewis and Tatken, 1979; Weast, 1973); c: Mikhailets, Gig. Tr. Prof. Zabol. 10, 57 (1966); d: (Deichmann, 1969); e: (Schafer et al., 1983); f: (McKee and Wolf, 1963); g: (Elf Atochem, 1994); h: (Synthetic Chemicals Ltd., 1994); i: (Giddings, 1979); j: (Kaiser and Palabrica., 1991).

2.5 Smell of thiophene

Like benzene, thiophene has an aromatic smell. The concentration threshold in the air for the smell was measured to be 2.6 $\mu g/m^3$ (Ruth, 1986) and 1-20 mg/m^3 (Verschueren, 1996). This compound is therefore not welcome in groundwater used for drinking.

3 Microbial degradation

Contaminants can be subject to different natural attenuation mechanisms. They can be attenuated through abiotic processes such as dilution, chemical conversion, photodegradation, as well as physical processes such as volatilisation, or by biotic processes such as aerobic or anaerobic microbial degradation.

The extent of each of these processes may be different depending on the environment of the contaminated site. Photodegradation will for example, only take place in the atmosphere or very near to the water and soil surfaces. Volatilisation is considered as relatively unimportant for the removal of hydrocarbon in surface water (Morgan and Watkinson, 1989). Microbiological degradation is the process believed to contribute the most to contaminant attenuation in groundwater (Dyreborg et al., 1999; Johansen et al., 1998).

3.1 Microbiological degradation of organic compounds

The microbial degradation of organic contaminants depends on the physical and microbial conditions present at the polluted site. Their degradation pattern may differ from compound to compound and site to site. BTEX (benzene, toluene, ethylbenzene and xylenes) for example, are reported to be degraded by soil bacteria that have not been exposed to these organic contaminants before (Armon et al., 2000), while other pollutants were resistant to microbial conversion under the same circumstances. Another example of diversity in degradability is the duration of the acclimation period of soil microorganisms to a single compound, which might differ from site to site at similar concentrations (Alexander, 1994a).

This diversity in contaminant degradability can be explained by different factors such as the ability of the microbial population to degrade the contaminants, the availability of the contaminants, the presence of electron acceptors and inorganic nutrients, the redox conditions, pH and temperature conditions, the level of moisture of the ground, as well as the presence of toxic compounds at the contaminated site (Alvarez, 1991; Armon et al., 2000; Arvin and Flyvbjerg, 1992; Mueller et al., 1989).

3.1.1 Degradability of organic contaminants

The ability of microorganisms to degrade organic compounds depends on the presence of specific enzymes that catalyse the corresponding degradation reactions. Single enzymes are often able to catalyse the degradation of several compounds. Therefore, the ability of a microbial community to degrade one substrate frequently results in the simultaneous degradation of some, but not all, structurally related molecules. Cells include in general two groups of enzymes: constitutive and inducible enzymes. The constitutive enzymes are generated regardless of whether the substrates for the enzymes are present. Inducible enzymes are first produced in appreciable amounts when the substrate or structurally related compound or metabolites are present (Alexander, 1994a). A specific contaminant is degradable if the necessary enzyme is present or can be induced in the available microbial community. In the case that the contaminant is not able to induce the required enzyme, its degradation will rely on the presence of another substrate (inducer) that is able to induce the enzyme. If

the inducer is not present in the system, the contaminant will be resistant to microbial degradation.

The availability of a contaminant also controls its microbiological degradation. The contaminant has to be accessible to the bacteria to be submitted to biodegradation. The accessibility of the pollutant depends on its physical property. Its solubility in water facilitates the access of the bacteria to the compound. A strong sorption capacity, the presence of the contaminant in non-aqueous-phase liquids or its entrapment within the soil matrix will hinder its contact with the bacteria (Alexander, 1999).

Minimum and maximum threshold concentrations of a compound might restrain its degradability. Some contaminants have to be present at a certain minimum threshold concentration in order to induce the enzyme necessary for their microbial degradation. If this concentration is not reached, the contaminants remain persistent. In the case of the degradation of 3- and 4-chlorobenzoates by *Acinetobacter calcoaceticus*, for example, the induction of the enzymes involved with the first metabolic steps occurs at concentrations above but not below 1 μmole/L (Reber, 1982). As there exists threshold contaminant concentration, under which no enzymatic activity can be observed, there may also exist a threshold concentration above which the contaminant is toxic to the microorganisms and the enzymatic activity. Degradation studies have shown for example that benzene was not degraded at concentrations of 250 mg/L (Alvarez, 1991). This was attributed to the toxicity of benzene to the bacteria.

The toxicity of other pollutants present at the polluted site might affect the degradability of a specific compound, for example, by hindering the enzyme induction and activity. Benzene is easily degradable by microorganisms present in groundwater under aerobic conditions. Its degradation, however, was inhibited in the presence of organic compounds like thiophene, benzothiophene, benzofuran and 1-methylpyrrole (Dyreborg et al., 1996b). This might explain why benzene could be detected at creosote contaminated sites (Kiilerich and Arvin, 1994).

3.1.2 Lagphase prior to microbiological degradation

Many organic contaminants are not degraded as soon as they are brought in contact with microorganisms. The period between the first contact of the bacterium with the organic compound and the time point when a reduction in contamination concentration can be observed is called lagphase or acclimation phase. It can vary from minutes to months. This depends on the compound, the bacterial population and the polluted site investigated (Alexander, 1994a). Such variations in the acclimation phase have been reported e.g. for 4-nitrophenol added to fresh and marine waters and IPC and 2,4-D added to lake waters (Alexander, 1994a).

Enzyme induction is considered as having a minor influence on the lag-phase, as it usually is completed in minutes or hours (Richmond, 1968) and the acclimation phase varies from minutes to months. Only the first period of the acclimation time can be attributed to the enzyme induction (Alexander, 1994a).

The population size is believed to be the major factor that influences the lagphase length (Alexander, 1994a). The lagphase reflects the time it takes for the population to become large enough to ensure a detectable change in the contaminant concentration.

It can therefore be shortened or lengthened by any factor enhancing or inhibiting the microbial growth rate. The growth rate might be influenced by the contaminant concentration, the presence of other compounds and the general factors previously mentioned, that influence microbial degradation.

The growth rate of the microorganisms is frequently mathematically described by a Monod rate (Alvarez, 1991) and is dependent on the growth substrate concentration. A higher growth substrate concentration increases the growth rate of the microorganisms. Consequently, the lagphase for this specific substrate or compounds degraded by this bacterial population will be shortened. A lower concentration would lead to a longer lag-phase.

On the other hand, high concentrations might falsely indicate longer lag-phases due to poor analysis conditions. The beginning of microbial activity is not detected since the relative change concentration is small compared to the absolute concentration level (Alexander, 1994a). The analysis of product formation instead of the analysis of the decrease in contaminant concentration might overcome these biased lag-phases. High contaminant concentrations might also have a toxic effect on the bacterium and would slow the growth rate (Alexander, 1994a; Arvin et al., 1990).

Other contaminants present in the system might have an influence on the growth rate of the degrading species. They might act as toxicants and reduce the growth rate. In this case, the lagphase will tend to be longer (Alexander, 1994a). NSO-compounds can be taken as examples of toxicants for the benzene degradation. Their presence influences the benzene degradation rate and hence increases the lagphase prior to degradation (Arvin et al., 1996; Arvin et al., 1989; Dyreborg, 1996a; Dyreborg et al., 1996b). Other contaminants might have a stimulating effect, in which case the growth rate is enhanced and the lagphase is shortened. Benzene, for example, was reported to stimulate the degradation of anthracene (Bauer and Capone, 1988) and the presence of either toluene or o-xylene was shown to enhance the degradation of benzene (Arvin et al., 1989).

3.1.3 Cometabolism

Cometabolism is a specific microbial degradation process that has a considerable impact on the environment because it allows the removal of otherwise persistent contaminants. Since its discovery, cometabolism has been shown to allow the transformation of numerous compounds which were thought to be persistent.

Dalton and Stirling defined this process as *the transformation of a non-growth substrate in the obligate presence of a growth substrate or another transformable compound* and called fortuitous cometabolism the transformation of a non-growth substrate in the absence of growth substrate (Dalton and Stirling, 1982). Horvath et al. (1972) defined cometabolism as the degradation of any substances without utilisation of the energy derived from the oxidation to support microbial growth. According to Horvath, this process does not infer the presence or absence of a growth substrate. In this thesis, cometabolism is taken in the sense defined by Horvath and includes fortuitous cometabolism. The non-growth substrate may also be called cosubstrate or secondary substrate while the growth substrate may be called primary substrate. This process can be defined by the four following features (Horvath, 1972):

- The cosubstrate does not support microbial growth;
- The metabolites formed during the cosubstrate conversion are not degraded further by the microorganisms;
- The cosubstrate conversion involves the utilisation of existing enzymes;

3.1.3.1 Energy supply

The transformation of the cosubstrate neither supports growth, nor produces energy. In order to transform the cosubstrate, the cells need energy that is generated during the transformation of the growth substrate. Resting cells are cells that are not supplied with growth substrate (they are also called non-growing cells). If resting cells have previously been activated by the growth substrate, they contain the necessary enzymes and energy sources in the form of reducing equivalents (NADH) (Bae and Rittmann, 1995; Oldenhuis et al., 1989) or storage compounds (e.g. poly-β-hydroxybutyrate (PHB) (Dalton and Stirling, 1982; Henrysson and McCarty, 1993; Thomson et al., 1976). Such cells are able to convert the cosubstrate. The cosubstrate conversion, however, does not lead to the energy source regeneration. It will therefore lead to the depletion of storage compounds and reducing equivalents and subsequently to the cell inactivation. The ability of cells to perform cometabolic reactions was shown to depend on their ability to build up energy reserves such as PHB (Higgins et al., 1979; Stirling and Dalton, 1979).

3.1.3.2 Accumulation of metabolites

The cosubstrate conversion leads to organic products that cannot further be transformed by the microorganisms into metabolic intermediates used for biosynthesis or energy production (Alexander, 1994). If these transformation products are not degradable by the bacterial population present at the polluted site, they accumulate in the system. Cometabolic conversions can result in the production of toxic compounds from relatively non-toxic substrates (Dalton and Stirling, 1982; Wackett and Gibson, 1988) and lead to an increased toxicity of the environmental sample. The transformation products though are not always more toxic than the original compound.

3.1.3.3 Enzymes

The cosubstrate does not induce the enzymes necessary for its degradation. Its degradation will therefore rely on the unspecificity of enzymes that are induced by the growth substrate or are otherwise present in the cells (Alexander, 1994b; Dalton and Stirling, 1982). If the same enzyme is responsible for the conversion of growth and non-growth substrate, both compounds compete for the same enzyme when they are present at the same time (Folsom et al., 1990; Strand et al., 1990b). Competitive inhibition between growth and non-growth substrate therefore often characterises the kinetics of cometabolism.

3.2 The microbiological transformation of thiophene

3.2.1 Literature review

Several investigations on the aerobic and anaerobic thiophene conversion reported the persistency of this compound while subjected to microbial attack. Thiophene was shown to be persistent under methanogenic conditions in the presence of anaerobic digesting sludge (Battersby and Wilson, 1989). It was also reported to be persistent in the presence of aquifer slurries under anoxic conditions (no removal after 1 year incubation) (Adrian and Sulfita, 1994) and anaerobic conditions (no removal after 3 months incubation) (Kuhn and Suflita, 1989). Several investigations reported that thiophene was resistant to aerobic degradation as sole carbon source (Amphlett and Callely, 1969; Cripps, 1973; Dyreborg et al., 1996b; Dyreborg et al., 1998; Dyreborg et al., 1996).

Thiophene degradation was achieved under anaerobic conditions with microorganisms from sludge collected near an oil well or in the bottom of a crude oil reservoir and a mineral medium supplemented with polypeptone and lactic acid (Kurita et al., 1971). This conversion lead to non-identified organic compounds and to H_2S release. This lead Kurita et al. (1971) to suggest that the thiophene conversion was a reductive reaction.

Thiophene degradation was achieved aerobically in combination with other aromatic carbons (Dyreborg, 1996; Dyreborg et al., 1996b; Moriya and Horikoshi, 1993). Thiophene was shown to be readily degradable in the presence of benzene, toluene and ethylbenzene. The conversion of thiophene by a pure strain of *Pseudomonas putida* was reported without further details about substrate conditions (Boyd et al., 1996a; Boyd et al., 1996b).

3.2.2 Aerobic biotransformation of thiophene in the presence of benzene

The biotransformation of thiophene in the presence of benzene is of special interest because thiophene is usually found in combination with benzene at polluted sites. This process was investigated in batch experiments (Andersen, 1998; Dyreborg et al., 1998; Rivas et al., 2002b), as well as in a biofilm reactor run continuously (Rivas and Arvin, 2000) with an inoculum originating from a coal tar contaminated site (Fredensborg, Denmark).

3.2.2.1 Cometabolic transformation

Thiophene was shown not to be degraded as a sole carbon source if the micro-organisms have not first been activated with benzene (Andersen, 1998; Dyreborg et al., 1998; Rivas and Arvin, 2000; Rivas et al., 2002b). The microorganisms were able to degrade thiophene in the presence of benzene, but also in the absence of benzene if they previously had been activated by benzene (Rivas and Arvin, 2000). Close to 100% of the converted thiophene was transformed into metabolites that were not further degraded (Andersen, 1998; Rivas and Arvin, 2000; Rivas et al., 2002b). This implies that thiophene did not support growth. These features suggest that the conversion of thiophene is a cometabolic process, as proposed by Dyreborg et al. (1998).

3.2.2.2 The lagphase

The lagphase prior to conversion of benzene was increased by thiophene in batch experiments (Andersen, 1998; Rivas et al., 2002b). It increased exponentially with rising ratio of the thiophene to benzene concentrations. As proposed by Alexander (1994a), the lagphase mostly reflects the biomass growth rate. The longer lagphase imposed by thiophene indicates that this compound inhibited microbial growth and that the inhibition increased with the thiophene concentration.

3.2.2.3 Competitive inhibition

Competitive inhibition between thiophene and benzene was shown to play a role in the microbiological degradation of these two compounds (Rivas and Arvin, 2000). This is often the case between growth and cosubstrate in cometabolic processes (Alexander, 1994b; Folsom et al., 1990; Strand et al., 1990a). As explained earlier (paragraph 3.1.3), competitive inhibition might be due to the fact that the same enzyme(s) is (are) involved in the first degradation steps for thiophene and benzene.

3.2.2.4 Conversion rate of thiophene

The initial thiophene conversion rate in batch experiments was shown to depend linearly on the initial benzene concentration in the range investigated (Rivas et al., 2002b). The thiophene degradation rate was shown not to depend on the thiophene concentration, which implies a zero order rate for thiophene concentrations between 2 and 40 mg/L (Rivas et al., 2002b).

3.2.2.5 Inactivation of the bacteria

As mentioned above, resting cells that previously have been activated with benzene were able to degrade thiophene. However, this process lead to cell inactivation. The inactivation of the cells was measured as the reduction of the microbiological benzene degradation rate. Experimental investigations have been performed in a continuous biofilm reactor, during which the benzene influent was stopped, while the thiophene influent supply was maintained (Rivas and Arvin, 2000). It was shown that the inactivation of the microorganisms depended on the ratio of thiophene to benzene concentrations in the reactor inlet prior to the change in benzene supply (Rivas and Arvin, 2000). A lower ratio between thiophene and benzene concentrations lead to less inactivation of the biomass. The depletion of energy sources necessary to maintain microbiological activity might explain this phenomenon.

Biomass inactivation also took place during thiophene conversion by cells growing on benzene (Rivas and Arvin, 2000). Experimental investigations were done with a continuous biofilm reactor during which thiophene was added to the reactor inlet after steady state operation with benzene as sole carbon source. The addition of thiophene lead to partial or complete reduction of the biofilm activity. The inactivation rate depended on the ratio between the inlet concentrations of thiophene and benzene (Rivas and Arvin, 2000).

Regeneration of the biofilm activity took place when thiophene was removed from the influent. The benzene concentration time course for one of these continuous biofilm experiments (benzene inlet concentration=1.7 mg/L, thiophene inlet concentration during step=5.9 mg/L) is represented in Fig. 2. When thiophene was

removed from the reactor inlet, the benzene concentration decreased in the reactor, which means that the biofilm activity increased. The dotted curve (called no inactivation - no inhibition curve) represents the time course of the benzene concentration in the reactor calculated with a biofilm activity equal to the activity existing before the introduction of thiophene in the system and with no inhibition of the bacterial activity by thiophene. The measured benzene concentration differed considerably from the concentration calculated in the case of no inactivation and no inhibition by thiophene (Fig. 2). The regeneration of the benzene degradation activity occurred in two steps. In the first phase (part I, Fig. 2), a fraction of the previous activity is regained at a certain rate. During this period, thiophene was still present in the reactor. It was first completely washed out at the end of phase I. The difference between the calculated benzene concentration in case of no inactivation - no inhibition and the measured benzene concentration might be due to competitive inhibition or inhibition of the bacterial activity by thiophene or its metabolites. It might also be related to the regeneration of bacteria inactivated due to depletion of their energy sources. In the second phase of the regeneration (part II, Fig. 2), thiophene was no longer present in the reactor. Therefore, a faster increase in the degradation rate of benzene was expected. However, the benzene concentration decreased at a slower rate compared to part I. The increase in degradation rate corresponded to the increase in case of biomass growth (data not shown). This suggests that part of the biomass had been killed while thiophene was fed to the reactor. This experiment suggests that the reduction in benzene degradation activity occurring during the presence of thiophene in the reactor was due to death of microorganisms as well as to inhibition by thiophene and/or its metabolites and/or to reducing power depletion.

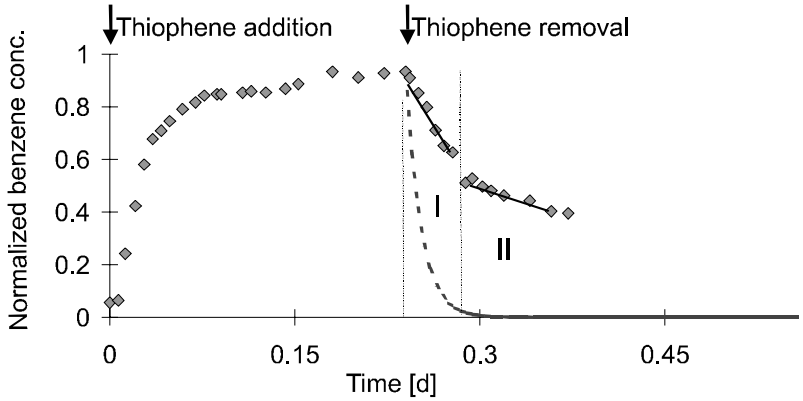

Fig. 2. Time course of the benzene concentration during steps changes of the thiophene concentration. ♦ measured benzene concentration. ------ calculated benzene concentration with the bacterial activity of time 0. Phases I and II explained in the text

Batch experiments showed complementary results (Rivas et al, 2002b). The benzene degradation activity of the bacteria as well as the bacterial concentration was monitored during the conversion of thiophene and benzene. Figure 3 shows the evolution of the specific bacterial activity during this process. The benzene degradation activity of the microorganisms decreased drastically with the presence and the conversion of thiophene. This fast decrease at the beginning of the thiophene and benzene degradation phase is very probably not due to the energy source

depletion because the amount of transformed thiophene when the activity dropped abruptly was relatively small (<1 mg/L). It may find its explanation in the damage of cells by thiophene or its conversion products.

3.2.2.6 A critical threshold

The ratio between the concentrations of thiophene and benzene was shown to dominate the concomitant conversion of thiophene and benzene (Andersen, 1998; Dyreborg et al., 1998). The degradation of these two compounds in batch cultures took place only when the ratio of thiophene to benzene was below a critical value reported to vary between 10 and 20 mg thiophene/mg benzene. This phenomenon was also observed during the continuous biofilm reactor investigation reported in Rivas and Arvin (2000) and referred to in paragraph 3.2.2.5. However, the ratios at which complete inactivation of the biofilm took place were lower than those reported in the batch experiments (2 mg thiophene/mg benzene in the reactor bulk phase). The critical ratio might be explained by the depletion of reducing power, as the amount produced from benzene degradation is not sufficient to sustain the degradation activity.

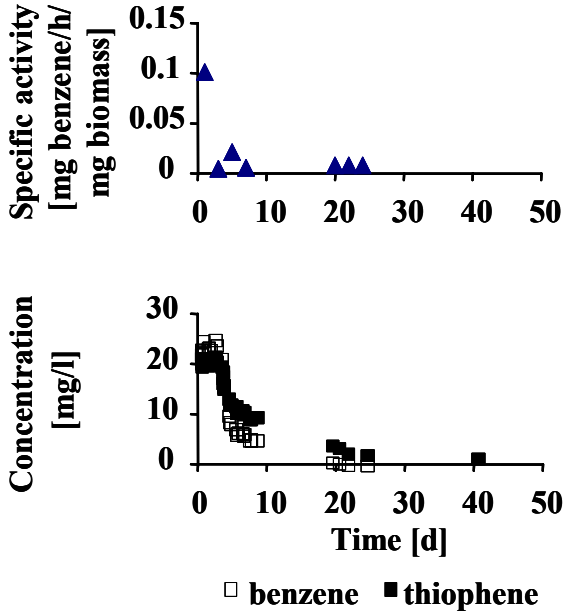

Fig. 3. Time course of the benzene and thiophene concentrations and the microbiological benzene-degrading activity during a batch investigation (Rivas, 2000).

3.2.2.7 Transformation capacity

The amount of thiophene degraded in the investigated biofilm reactor depended on the available amount of benzene (Rivas and Arvin, 2000). The transformation capacity of thiophene per amount of benzene was calculated to be between 0.2 and 2.0 mg thiophene/mg benzene. This transformation capacity is comparable to the one obtained by Dyreborg et al. (1998) and Andersen et al. (1998) in suspended cultures. It is much higher than the transformation capacities reported for trichloroethylene (TCE) or chlorophenols (CP) (Table 4).

Table 4. Transformation capacities for different cometabolic processes

Primary/Secondary substrate	Transformation capacity	References
CH_4/TCE	0.155 mg TCE/mg CH_4	(Oldenhuis et al., 1991)
CH_4/TCE	0.11 to 0.28 mol TCE/mol CH_4	(Broholm et al., 1992)
CH_4/TCE	0.013 mg TCE/mg CH_4 without formate 0.026 mg TCE/mg CH_4 with formate	(Alvarez-Cohen and McCarty., 1991b)
Phenol/TCE	0.029-0.060 mg TCE/mg phenol in batch	(Segar et al., 1994b)
Phenol/TCE	0.003-0.004 mg TCE/mg phenol in situ	(Segar, 1994a)
Phenol/TCE	0.016 mgTCE/mg phenol	(Folsom and Chapman, 1991)
Toluene/TCE	0.007 to 0.014 mol TCE/mol toluene	(Jensen, 1994)
Phenol/4-CP	0.1 mg 4-CP/mg phenol	(Saez and Rittmann, 1993)
Benzene/thiophene	2.2 mg thiophene/mg benzene = 2 mol thiophene/mol benzene 1.5-3.3 mol thiophene/mol benzene	(Dyreborg et al., 1998)
Benzene/thiophene	0.88-5.15 mg thiophene/mg benzene	(Andersen, 1998)
Benzene/thiophene	0.20-2.00 mg thiophene/mg benzene	(Rivas, 2000)

4 Metabolite formation

The microbiological transformation of thiophene by cells growing on benzene and by resting cells was shown to lead to cell inactivation and death. A hypothesis put forth to explain the microbiological death is the formation of toxic metabolites from thiophene. This chapter deals with the formation of metabolites from benzene and thiophene during the concomitant degradation of these two compounds.

4.1 Benzene metabolites

Microbial degradation of benzene was reported first in 1913 by Söhngen (1913). Benzene was shown to be transformed to catechol by a strain of *Nocardia corallina* (Haccius and Helfrich, 1958; Wieland et al., 1958). Wieland et al. (1958) assumed that phenol was the metabolic intermediate between benzene and catechol. Gibson et al. (1968, 1984) however, showed that benzene was first transformed to cis-benzene dihydrodiol by the incorporation of two atoms of oxygen derived from molecular oxygen, which suggests a dioxygenase reaction. Cis-benzene dihydrodiol was further converted to catechol by a strain of *P. putida* (Gibson et al., 1968; Gibson and Subramanian, 1984). Similar results were obtained by Högn and Jaenicke (1972) with a *Moraxella* species. Gibson et al. (1968, 1984) excluded the formation of benzene epoxide as metabolic intermediate between benzene and cis-benzene dihydrodiol and proposed benzene peroxide as the intermediate between these two compounds. However, benzene peroxide could not be isolated. The further degradation of catechol lead to the meta-fission of the aromatic ring and the formation of 2-hydroxymuconic semialdehyde (Ribbons and Eaton, 1982) with *P. putida* and to the ortho-fission and the formation of β-ketoadipate via cis-cis-muconic acid with the *Moraxella* species (Högn and Jaenicke, 1972).

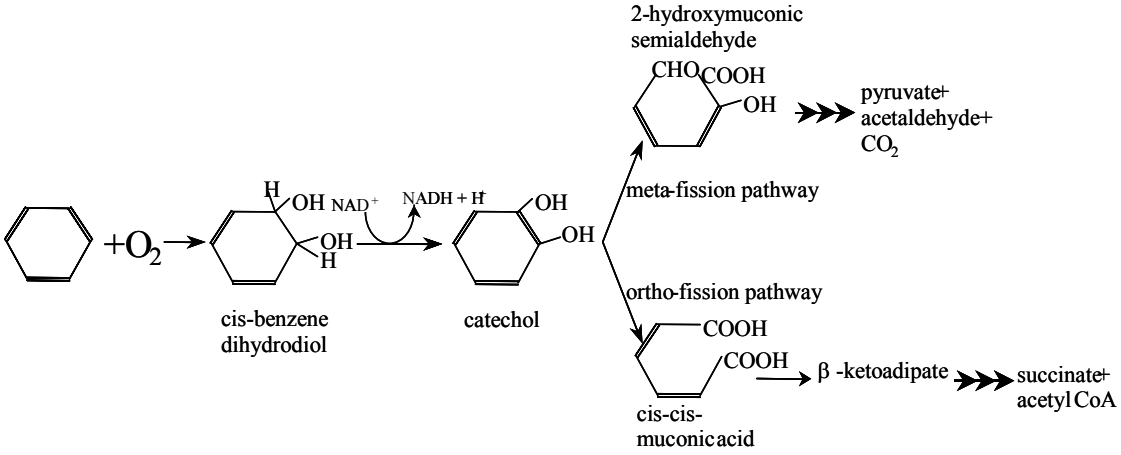

Fig. 4. Aerobic degradation pathways of benzene

Numerous biodegradation studies with benzene alone or in mixture with other aromatic compounds (for example BTEX) have been conducted on the microbiological degradation of benzene since these early reports. A motivation for

such studies can be found in the threat this compound represents to humans and the environment. Benzene was shown to be readily degradable by aerobic bacteria (Smith 1990; Zylstra 1994) and numerous reports including kinetic data of its biodegradation are available.

The benzene degradation was investigated in a biofilm reactor with and without thiophene (Rivas and Arvin, 2000). In the absence of thiophene, benzene was metabolised to biomass and CO_2. The biomass production yield from benzene was calculated as 0.52 g biomass COD / g benzene COD related to the consumption of O_2 (3.61 mole) and the production of CO_2 (2.19 mole) per mole of benzene degraded. The reaction equation used for calculation in Rivas and Arvin (2000) is:

$$C_6H_6 + (7.5 - 5 \cdot Y_B) \cdot O_2 + Y_B \cdot NH_3 \rightarrow Y_B \cdot C_5H_7NO_2 + (6 - 5 \cdot Y_B) \cdot CO_2 + (3 - 2 \cdot Y_B) \cdot H_2O \quad (1)$$

where Y_B is the yield in mole biomass/mole benzene.

The introduction of thiophene in the system lead to a slight decrease of the CO_2 production from benzene. This implies that the presence of thiophene and its conversion changed the benzene degradation metabolism. Part of the converted benzene (about 5%) was not completely mineralised, but transformed into organic metabolites analysed as non-volatile organic compounds (Rivas and Arvin, 2000). These metabolites have not been identified.

4.2 Thiophene metabolites

4.2.2 Literature review

4.2.2.1 Oxidation products of thiophene

Few investigations on thiophene degradation products have been conducted. The main oxidation products are represented in Fig. 5.

In *P. putida* UV4, the oxidation of thiophene lead to the formation of a mixture of cis- and trans-2,3-dihydroxy-2,3-dihydrothiophene (**I**, ca. 1% of the converted thiophene), cis-3a,4,7,7a-tetrahydro-cis-4,7-epithio-1 benzothiophen-syn-8-oxide (cycloadduct monosulphoxide **II**, 11% of the converted thiophene) and cis-3a,4,7,7a-tetrahydro-cis-4,7-epithio-1 benzothiophen-syn-8-dioxide (thiophene sulphoxide dimer **III**, 45% of the converted thiophene) (Boyd et al., 1996a; Boyd et al., 1996b). The structure of compound **II** and **III** were deduced from proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy and crystallographic analysis.

In rabbits and rats, the transformation of thiophene lead to the formation of 2,5-dihydrothiophene sulphoxide bearing a N-acetyl-cysteinyl group on position 2 which is 2-thienylmercapturic acid **IV**, and to the formation of a premercapturic acid (3-hydroxy-2,3-dihydro-2thienylmercapturic acid, **V**) (Bray and Carpanini, 1968; Bray et al., 1971). Dansette et al. (1992) reported that about 30% of the thiophene doses administrated to rats was retrieved in their urine in the form of compound **IV**. The authors considered the formation of **IV** as a first evidence for the formation of thiophene sulphoxide **VI** as metabolite intermediate from thiophene in vivo. Thiophene Sulphoxideis a very reactive molecule and binds to glutathione to give

metabolite **IV**. Treiber et al. (1997) showed that about 2% of the thiophene doses administrated to rats was transformed into thiophene sulphoxide dimer **III**. The in vitro metabolism with liver microsomes of rats lead to compound **III** and its diastereoisomer **VII** that differ by the position of the oxygen on the non-bridging sulphoxide.

Chemical oxidation of thiophene with trifluoroacetic acid and hydrogen peroxide lead to compounds **III** and **VII** (Treiber et al., 1997). Chemical oxidation of thiophene also lead to 4:7:8:9-tetrahydro-4:7-sulphinylthionaphthen 1:1-dioxide (thiophene sesquioxide **VIII**) (Davies and James, 1954; Melles and Baker, 1953; Merrill and Sherwood, 1977; Treiber et al., 1997).

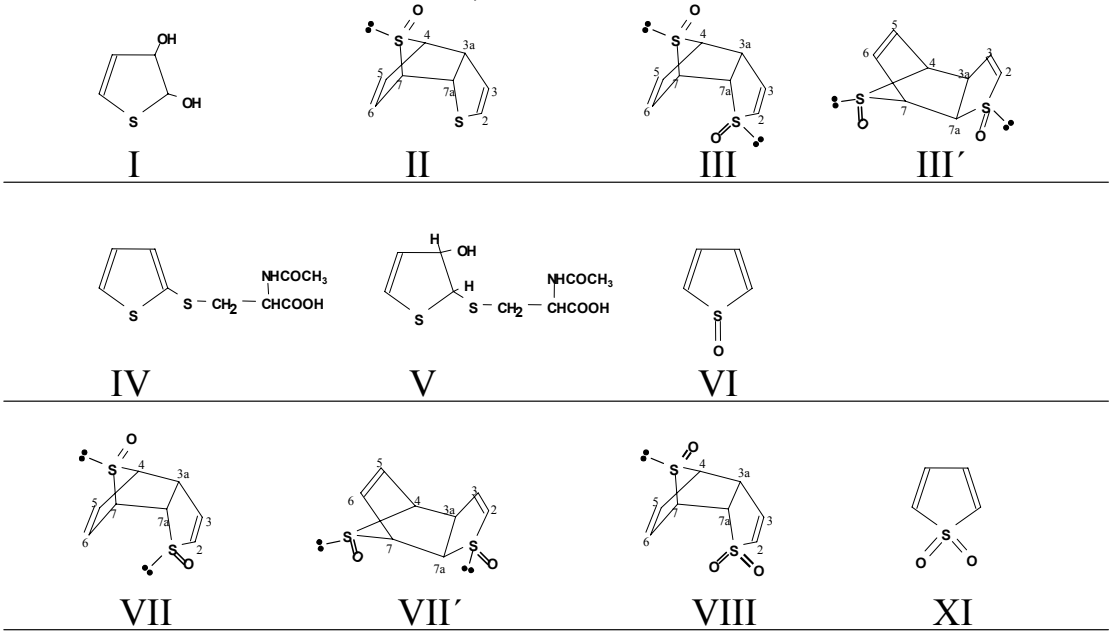

Fig. 5. Thiophene oxidation products

The cycloadduct monosulphoxide **II** was suggested to be the result from a Diels-Alder reaction between thiophene and thiophene sulphoxide **VI** (Boyd et al., 1996a; Boyd et al., 1996b), while the thiophene sulphoxide dimers **III** and **VII** were proposed to be the adduct of a Diels-Alder reaction between two thiophene S-oxides **VI** (Boyd et al., 1996a; Boyd et al., 1996b). The thiophene sesquioxide **VIII** was proposed to be the result of a Diels-Alder reaction between a thiophene sulphoxide **VI** and thiophene sulphone **IX** by Melles and Baker (1953). Treiber et al. (1997) reported that the sesquioxide **VIII** was the result of the further oxidation of **III** and **VII**.

4.2.2.2 Oxidation products of thiophene related compounds

Two major objectives have been driving the studies of microbiological metabolism of organosulphur compounds. One was to understand the fate of sulphur compounds when released into the environment. The other was to develop processes for the desulphurisation of fossil fuels and coals in order to reduce the environmental problem caused by sulphur emissions that result during the combustion of fuels and coals. This explains why numerous investigations have been performed on the microbiological conversion of thiophene related compounds such as benzothiophene

(Eaton and Nitterauer, 1994; Fedorak and Grbić-Galić, 1991; Kropp et al., 1994), dibenzothiophene (Constantí, 1994; Kodoma et al., 1973; Kropp et al., 1997a; Laborde and Gibson, 1977; Mormile and Atlas, 1989), naphthothiophene (Kropp et al., 1997b), alkyl thiophenes and alkylbenzothiophenes (Brown and Espenson, 1996; Fedorak and Grbić-Galić, 1991; Fedorak and Peakman, 1992; Kropp et al., 1996).

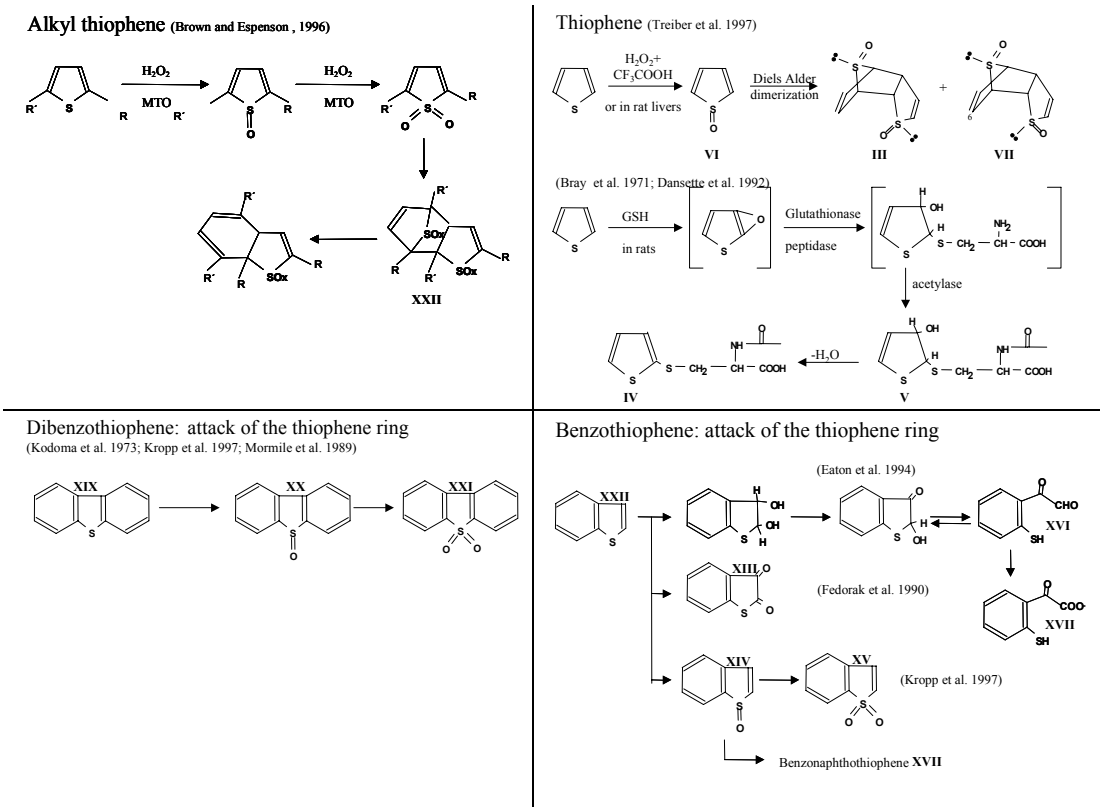

Fig. 6. Oxidation pathways of thiophene and thiophene derivatives

The aerobic biodegradation of benzothiophene **XII** was initiated at the benzene or the thiophene ring (Eaton and Nitterauer, 1994; Fedorak and Grbić-Galić, 1991; Kropp et al., 1994) (Fig. 6). The attack on the thiophene ring lead to the formation of benzothiophene-2,3-dione **XIII** (Bohonos et al., 1977; Fedorak and Grbić-Galić, 1991), benzothiophene sulphoxide **XIV** and sulphone **XV** (Kropp et al., 1994) or to the ring opening and the formation of 2´-mercaptomandelaldehyde **XVI** and 2-mercaptophenylglyoxalate **XVII** (Eaton and Nitterauer, 1994). Benzothiophene-2,3-dione **XIII** was shown to be produced from 2-mercaptophenylglyoxalate during the extraction procedure (Eaton and Nitterauer, 1994). Kropp et al. (1994) reported that about 7% of the initial benzothiophene amount was transformed to benzo[*b*]naphtho[1,2-*d*]thiophene **XVIII**. This product was proposed to be the result of an abiotic, Diels-Alder type reaction of benzothiophene sulphoxide with formal loss of 2H+S+2O.

Dibenzothiophene **XIX** (fig. 6) was used as a model compound for the study of desulphurisation of coals (Kilbane, 1990) and similarly to benzothiophene, its degradation was initiated at the benzene ring and at the thiophene ring (Constantí,

1994; Kodoma et al., 1973; Kropp et al., 1997b; Laborde and Gibson, 1977; Mormile and Atlas, 1989). The attack on the thiophene ring lead to the formation of dibenzothiophene sulphoxide **XX** and sulphone **XXI** (Kodoma et al., 1973; Kropp et al., 1997b; Mormile and Atlas, 1989). Constantí (1994) reported the formation of sulphate during dibenzothiophene degradation.

The aerobic degradation of alkyl thiophenes or alkyl benzothiophenes mainly occurred by the attack on the alkyl group and it lead to carboxylic acids according to Fedorak and Peakman (1992) and Kropp et al. (1996). Traces of sulphoxides, sulphones and diones, 3(2H)ones and 2(3H)ones resulting from the attack on the thiophene ring as well as Diels-Alder cycloadducts **XXII** of the sulphoxides and the sulphones were also identified (Boyd et al., 1996b; Brown and Espenson, 1996; Fedorak and Grbić-Galić, 1991; Fedorak and Peakman, 1992; Kropp et al., 1996).

4.2.3 Metabolites expected from the microbiological oxidation of thiophene

According to the presented literature, the metabolites expected from the microbiological thiophene conversion are sulphoxides, sulphones. The production of sulphoxide might lead to the Diels-Alder condensation and the formation of the corresponding sulphoxide dimers.

4.2.4 Diels-Alder association

4.2.4.1 Definition

In 1928 two German scientists Otto Diels and Kurt Alder, developed a 1,4-cycloaddition of dienes that has since come to bear their names, the Diels-Alder association (Solomons and Fryhle, 2001). In general terms, this association consists of a reaction between a conjugated diene (a 4π-electron system, **XXIII** in Fig. 7) and a compound that contain a double bond (a 2π-electron system, **XXIV** in Fig. 7) called a dienophile (diene + Greek: phile in love). The dienophile might be olefinic (**XXIII**) or acetinilic (**XXV**). The product of the reaction is called an adduct. In the reaction, two new σ-bonds are formed at the expense of two π-bonds of the diene and the dienophile. The adduct contains a new six-membered ring with one double bond. σ-bonds are usually stronger than π-bonds, therefore formation of the adduct is usually favoured energetically. However, most Diels-Alder reactions are reversible (Solomons and Fryhle, 2001). Alder stated that the Diels-Alder reaction is favoured in the presence of electron withdrawing groups in the dienophile and electron releasing groups in the diene. High temperature and high pressure were found to enhance the rate of the Diels-Alder reaction (Solomons and Fryhle, 2001).

By engaging the lone pairs of the thiophene sulphur bond formation, the oxidation of thiophene destroys the aromatic stabilisation and the double bonds receive a more diene character (Torssell, 1976). Thiophene sulphoxide has both diene and ene properties and may undergo a Diels-Alder dimerization. Moreover, Napertskov (1997) reported that in addition to the Diels-Alder reactivity of thiophene S-oxide, the oxidation of thiophene was difficult to stop at the thiophene sulphoxide **VI** stage and usually produced the corresponding thiophene sulphone **XI**. Thiophene sulphoxide is more reactive as diene than the corresponding sulphone (Nakayama and Sugihara,

1997). A Diels-Alder reaction between the sulphoxide **VI** and the sulphone **XI** therefore leads to thiophene sesquioxide **VIII** (Davies and James, 1954; Melles and Backer, 1953).

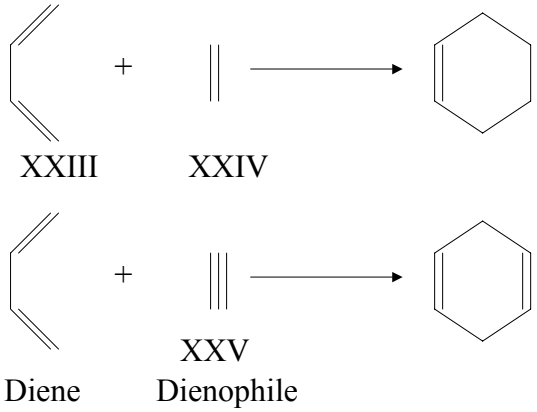

Fig. 7. Diels-Alder reactions

4.2.4.2 Stereochemistry

For dienes and dienophiles that are part of aromatic rings several associations are possible: the endo- or exo-configuration of the adduct or the syn-addition. The Diels-Alder association is stereospecific (Solomons and Fryhle, 2001).

4.2.4.2.1 Endo-exo

A cyclic diene such as thiophene sulphoxide can act as a diene and a dienophile for a Diels-Alder reaction. Two configurations are possible for the Diels-Alder adduct of such a cyclic diene: the endo- and the exo-configuration (Fig. 8). There are fewer steric reactions in the exo-adduct than in the endo-adduct, which implies that the exo-adduct is more stable than the endo-adduct (Solomons and Fryhle, 2001). Though, the transition state for the endo-product is of lower energy because of favourable orbital interactions, which implies that the exo-configuration is formed slower than the endo-configuration (Solomons and Fryhle, 2001). The Diels-Alder reaction occurs therefore rather in an endo- than in an exo-configuration when the reaction is kinetically controlled. The endo-form is the kinetic and major product of the Diels-Alder reaction.

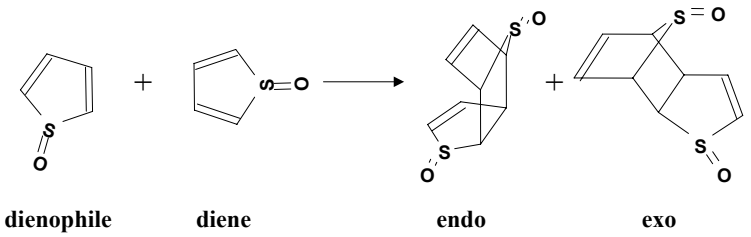

Fig. 8. Diels-Alder condensation between two thiophene S-oxides

4.2.4.2.2 Syn-addition

Diels-Alder reactions show a π-facial diastereoselectivity, where the addends have two different faces (Nakayama and Sugihara, 1997). In S-oxides, the oxygen

atom may have two different positions on the sulphur atom (Fig. 9). These compounds have therefore two different faces: the syn-face (the sulphur-oxygen bearing face) and the anti-face (the lone pair bearing face). The Diels-Alder reactions have been conducted with a series of dienophiles and sulphoxide compounds as dienes. In all cases, the syn-adducts (with respect to the sulphoxide oxygen) were formed exclusively, revealing that the dienophiles added to the syn oxygen-face of the diene (Nakayama and Sugihara, 1997).

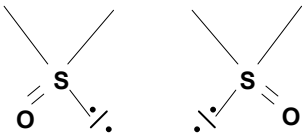

Fig. 9. Position of oxygen on a sulphur atom

It was reported that the cyclo-diene thiophene sulphoxide reacted with exclusive facial control (syn-face) to the dienophile (Napertskow et al., 1989; Torssell, 1976). The oxygen has therefore obligatory a syn-position on the bridging sulphoxide of the Diels-Alder adducts of two thiophene S-oxides.

The configuration of the dienophile is retained in the Diels-Alder adduct.

4.2.4.2.3 Racemic mixture

The stereospecificity of the Diels-Alder reaction of a cyclic diene results in the formation of predominantly one stereoisomeric form (endo with retention of the original configuration of the dienophile) and the dienophile reacts exclusively with the syn-face of the diene with respect to the position of the sulphur oxygen. However, either face of the dienophile can interact with the diene (Solomons and Fryhle, 2001). The adduct therefore is formed as a racemic mixture of two enantiomers. The reaction of the diene with one face of the dienophile results in one enantiomer while the bonding of the diene to the other face of the dienophile results in the other enantiomer (Fig. 10). In the absence of chiral influences, both faces of the dienophile are equally likely to be attacked.

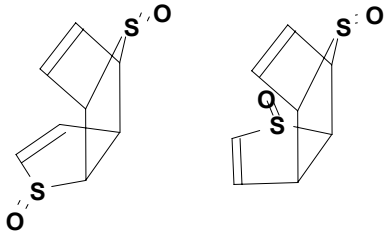

Fig. 10. Enantiomer formation

4.2.4.2.4 Conclusion

The Diels-Alder association between thiophene S-oxides is an exclusive syn-addition. The oxygen on the non-bridging sulphoxide can take two positions. The endo-syn Diels-Alder cyclo-addition between thiophene sulphoxides leads to two diastereoisomers that differ by the position of the oxygen on the non-bridging sulphoxide. The two enantiomers of each of these diastereoisomers are expected to be produced in a racemic mixture as reported in Boyd et al. (1996). The same is valid for

the exo-syn Diels-Alder cyclo-addition. The endo-configuration of the adduct is favoured compared to the exo-configuration. Therefore, the main products of the Diels-Alder reaction are expected to be the diastereoisomers with endo-configuration.

In the case of the Diels-Alder addition between thiophene and thiophene S-oxide, a similar result is expected. However, the monosulphoxide resulting during the microbiological oxidation of thiophene, was found to have an excess of one enantiomer (77%) by chiral stationary phase analysis (Boyd et al., 1996b). Boyd et al. (1996b) proposed that this could arise if the cycloaddition occurred in a chiral environment (e.g. the toluene dioxygenase system or via an enzyme-catalysed kinetic resolution).

4.2.5 Transformation pathway of thiophene in a mixed bacterial culture

Several investigations showed that thiophene was not mineralised during its cometabolic conversion with benzene as the primary substrate (Andersen, 1998; Rivas and Arvin, 2000; Rivas et al., 2002b). Close to 100% of the converted thiophene was transformed to five metabolites that were analysed as non-volatile organic carbon (NVOC) and by high performance liquid chromatography (HPLC) (Rivas and Arvin, 2000; Rivas et al., 2002b). These compounds are called **C1**, **C2**, **C3**, **C4** and **C5**. About 0.05% of the converted thiophene was transformed to benzothiophene (Rivas et al., 2002a).

The production of oxidation products from thiophene did not depend on the presence or the concentration of benzene nor on the concentration of thiophene (Rivas et al., 2002b). Invariably 78% of the converted thiophene on a mg carbon/L basis was converted into compound **C4** (**III**, **III´**), while 20% was transformed into **C5** (**VII**, **VII´**) (Rivas et al., 2002a; Rivas et al., 2002b). The remaining 2% was retrieved into compounds **C1** to **C3**.

The compounds **C1-C5** were not further degraded as sole carbon source or in the presence of benzene and/or thiophene (Rivas et al., 2002b).

*4.2.5.1 Identification of **C1-C5***

The five metabolites **C1-C5** were separated by preparative HPLC and analysed by ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) as well as mass spectrometry (MS) (Rivas et al., 2002a).

The major metabolite of thiophene, **C4** was identified to be the thiophene sulphoxide dimer **III** resulting from the endo-syn Diels-Alder addition of thiophene sulphoxide or more precisely a mixture of **III** and its enantiomer **III´** (Rivas et al., 2002a). The second major metabolite of the thiophene conversion, **C5** was identified to be a mixture of the thiophene sulphoxide **VII** and its enantiomer **VII´**, differing from **III** and **III´** by the position of the oxygen on the non-bridging sulphoxide (Rivas et al., 2002a).

Compounds **C2** and **C3** were not completely identified. ^{1}H- and ^{13}C-NMR showed that the structure of these two compounds was similar to the one of **III** and **VII**, which indicated that they also are the result of a Diels-Alder condensation of two thiophene derivatives (Rivas et al., 2002a). Several possibilities are open for them. They might be the cyclo-adduct between thiophene sulphoxide **VI** and thiophene sulphone **XI** (sesquioxide **VIII**). They also can be the result of the exo-syn cyclo-

addition of two thiophene sulphoxides. The cyclo-adduct monosulphoxide **II** is very unlikely because of the divergence of the ^{1}H-NMR chemical shift between **C2** and **C3** and **II** (Haughey, 1996; Rivas et al., 2002a). The fragmentation resulting from MS analysis of **C2** and **C3** did not allow complete identification.

Compound **C1** could not be produced in sufficient amount to be analysed successfully by MS and NMR.

4.3 Summary

The product identification provided strong evidence that the microbial oxicdation of thiophene by a mixed culture originating from a creosote contaminated site lead first to the formation of thiophene sulphoxide **VI.** Although VI was never detected in the cultures its fleeting presence was indicated by the presence of Diels-Alder condensation to compounds **III** and **III´,** and **VII** and **VII´** and three further non identified adduct. A small amount of compounds **III** and **III´,** or **VII** and **VII´** (0.05% of the converted thiophene) was further transformed into benzothiophene **XII** by the formal loss of 2H+S+2O. The metabolites **C2** and **C3** were determined to be adducts of a Diels-Alder condensation between thiophene derivatives but could not be completely identified. The pathway for the microbially mediated transformation of thiophene is illustrated in Fig. 11.

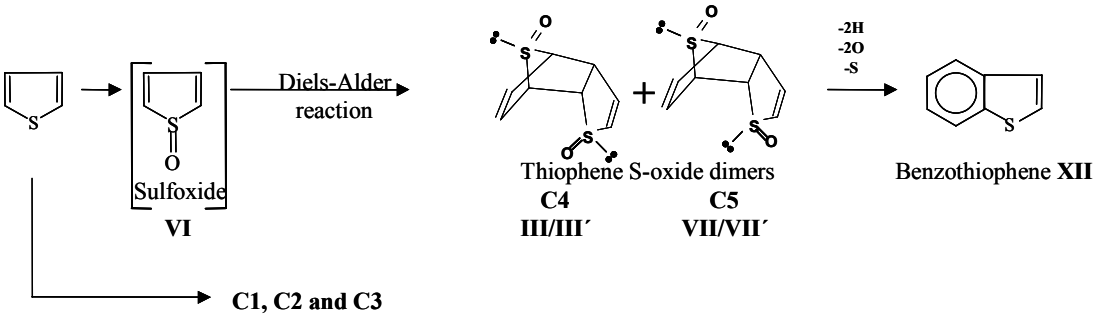

Fig. 11. Pathway proposed for the microbially mediated conversion of thiophene

5 Toxicity arising from the cometabolic transformation of thiophene

5.1 Biological conversion as a detoxification reaction?

Microbial organisms as well as mammals or other animals like fish, birds, etc … initiate detoxification reactions by the use of enzymes in order to reduce the toxicity represented by pollutants such as aromatic hydrocarbons (Cerniglia, 1980; Ribbons and Eaton, 1982). However, these detoxication reactions lead very often to highly reactive electrophilic compounds, known as potent carcinogenic and/or mutagenic compounds (Heidelberger, 1975; Jakoby, 1978; Ribbons and Eaton, 1982; Sims and Grover, 1974). This has been the case for polyaromatic hydrocarbons (Heidelberger, 1975; Sims and Grover, 1974) or chlorinated hydrocarbons.

The cometabolic degradation of chlorinated hydrocarbon, for example trichloroethylene (TCE), leads to unstable metabolic intermediates that affect the activity of the microorganisms (Henry and Grbić-Galić, 1991; Shields and Reagin, 1992; Wackett and Householder, 1989). Janssen and Koning (1995) proposed that the epoxides formed from the chlorinated compounds were responsible for this inactivation. These compounds may have affected the enzymes (Janssen and Koning, 1995) but they also damaged the bacteria through intracellular reactions (Janssen and Koning, 1995; Oldenhuis et al., 1991). In the case of TCE, different species of microorganisms presented different sensitivity to its conversion either because of a different degradation pathway or because the bacteria did not have the same sensitivity to the damages caused by the degradation products (Landa et al., 1994).

The cometabolic oxidation of thiophene was shown to lead to loss of degradation activity and also to microbial death (Rivas and Arvin, 2000). It lead to the formation of products **C1**-**C5** which were not degraded further (Rivas and Arvin, 2000; Rivas et al., 2002a; Rivas et al., 2002b). The microbial death could be due to the toxicity of thiophene conversion end-products. Therefore, the toxicity of **C1**-**C5** towards the benzene-degrading bacteria used in Rivas and Arvin (2000) was investigated (Rivas et al., 2002c).

Furthermore, the aquatic toxicity of **C1**-**C5** towards the environment was investigated with two acute toxicity tests to determine whether the conversion of thiophene is expected to lead to a reduced or increased toxicity of environmental samples: the microbiological test with *Vibrio fischeri* bacteria and the phytotoxicity test with *Selenastrum capricornutum* (Rivas et al., 2002c).

5.2 Toxicity towards benzene-degrading bacteria

Toxicity of the thiophene transformation products C1-C5

The analysis of the toxicity of the thiophene metabolites towards benzene-degrading bacteria was performed by following the benzene degradation activity of microorganisms in dose response experiments. The metabolites **C1**-**C5** were shown to have no effect on the benzene degradation activity of the bacterial culture used (Rivas et al., 2002c).

The bacterial death occurring during the transformation of thiophene must have its explanation elsewhere. It is possible that thiophene acts as a toxicant towards the

microorganisms or that unstable metabolic intermediates between thiophene and **C1-C5** are responsible for this toxic effect.

Thiophene sulphoxide as potential toxicant

Thiophene sulphoxide was proposed to be a metabolic intermediate in the conversion of thiophene (Rivas et al., 2002a). This compound is very reactive. In studies on the metabolism of thiophene in rat livers, thiophene sulphoxide was also a metabolic intermediate and it reacted with glutathione (GSH) in the liver of rats (Dansette et al., 1992; Mansuy et al., 1991; Treiber et al., 1997). GSH S-transferases is an enzyme present in most aerobic microorganisms, plants and animals. It catalyses the nucleophilic addition of the tripeptide GSH to substrates that have electrophilic functional groups. Its primary function is generally considered to be the detoxication of both endogenous and xenobiotic alkylating agents such as epoxides, α-β-unsaturated aldehydes and ketones, alkyl and aryl halides and others (Armstrong, 1991) that otherwise would react with cell proteins and lead to damage in the cells. The reaction of GSH with toxicants containing an electrophilic centre leads to the formation of thioethers (Ketterer and Mulder, 1990) that are, in mammals, excreted into the bile or that are further metabolised to mercapturates that are excreted by the kidney (Jakoby, 1978; Ketterer and Mulder 1990). This last step is the one observed with thiophene sulphoxide during in vivo thiophene metabolism in rat livers (Dansette et al., 1992; Mansuy et al., 1991; Treiber et al., 1997). The instability of thiophene sulphoxide indicates that this compound is a potential toxicant for the microorganisms.

However no toxicity test could be done with thiophene sulphoxide because of the high reactivity of this compound. The oxidation of thiophene could not be stopped at thiophene sulphoxide and went further to the Diels-Alder dimerization products identified as microbiological metabolites of thiophene.

Benzothiophene as potential toxicant

Rivas et al. (2002a) showed that 0.05% of the converted thiophene was transformed to benzothiophene. Benzothiophene therefore also has to be considered as being potentially responsible for the microbial death. This compound however could not be traced during the experiments showing death of microorganisms because its concentration was probably under the detection limit. The amount of thiophene converted during these experiments was always below 2 mg/L. The distribution of the thiophene metabolites was shown not to depend on the concentration of thiophene or benzene. One can therefore estimate that the maximum benzothiophene concentration reached during the experiments was 0.05% of 2 mg/L, which is 1 µg/L. Seymour et al. (1997) used the Microtox tets and measured the EC50 of benzothiophene to be 1.7 mg/L. At such low concentration, the responsibility of benzothiophene in the microbial death can be rejected.

5.3 Toxicity towards *Vibrio fischeri* and *Selenastrum capricornutum*

5.3.1 Definition of toxicity tests

To determine the aquatic toxicity of pure compounds, mixtures or of wastewater, various test organisms have been chosen for different ecological groups. The marine

bacterium *Vibrio fischeri* (or *Photobacterium phosphoreum*) was for example chosen to represent microbiological populations. The fresh water algae *Selenastrum capricornutum* represents the phytoplancton. The choice of organisms was based on the wish of standardisation and does not mean that these organisms have the same behaviour that all other organisms of their ecological group.

The principle of a test on aquatic toxicity consists in placing the test organism in the presence of the test compound for a certain time period and to determine subsequently a biological parameter specific for the test organism.

The tests are distinguished according to the relation between the exposure duration (**t**) and the organism generation time (**g**). If (**t**) is shorter than (**g**) the test is considered as an acute toxicity test and if (**t**) is longer than (**g**) the test is called a chronic or reproductive toxicity test.

The toxicity tests are performed with a dilution series of the compounds to investigate. The outcome of the tests are parameters such as the EC_x that is the compound concentration that affects X% of the test population after a specified exposure time, or LD_x that is the dose of toxicant lethal to X% of the population. The EC_x concentrations (where EC stands for effect concentration) generally reflect parameters other than mortality, as for example the growth rate, development abnormality or deformity of the test population.

5.3.2 Toxicity tests of thiophene oxidation products

To determine the toxicity of thiophene oxidation products towards the aquatic environment, the acute toxicity tests with the marine bacterium *V. fischeri* and the fresh water algae *S. capricornutum* were chosen.

V. fischeri is a light-emitting bacterium. The biological parameter for the stress level determination is the luminescence which is measured with a Luminometer. The toxicity of a sample on this species is directly proportional to the decrease in light emission. The EC_{50} (EC_{20}) value for a compound is the concentration producing a reduction of the light emission of 50% (20%) after a 5, 15 or 30 min exposure period. The test is performed according to ISO/DIS 11348-3 (International Organisation for Standardisation, 1996).

The biological parameter for the algae *S. capricornutum* is its growth rate. The toxicity of a sample on *S. capricornutum* is determined by analysing the reduction in growth rate of the algae over a 48-h exposure time. The EC_{50} (EC_{20}) concentration is the concentration at which the algal growth rate is reduced by 50% (20%) over 48 h. The test is performed according to ISO8692 (International Organisation for Standardisation, 1989).

5.3.3 Results

In contrast to the benzene-degrading bacterial community, *V. fischeri* and *S. capricornutum* were shown to be sensitive to the oxidation products of thiophene. They were more sensitive to **C2**-**C5** than to thiophene (Rivas et al., 2002c). This means that the microbiological conversion of thiophene to the compounds **C1**-**C5** lead to an increased toxicity of the sample towards *V. fischeri* and *S. capricornutum*.

The *V. fischeri* and *S. capricornutum* did not show the same sensitivity to compound **C4** and **C5**, though these two compounds only differ by the position of the

oxygen on the non-bridging sulphoxide (Rivas et al., 2002c). The EC_{50} values for **C4** were three to five times lower than those for **C5** (Rivas et al., 2002c). A similar phenomenon has been reported for the drug tienilic acid (TA) and its isomer (Bonierbale et al., 1999). Despite the similarity of their chemical structure, TA and its isomer produced very different alkylation patterns. TA lead to an immune mediated hepatitis in humans while its isomer triggered direct hepatitis in rats.

5.4 Summary

The metabolites of thiophene **C2-C5** were shown to be acutely toxic to *V. fischeri* and *S. capricornutum*. However they did not affect the benzene degradation activity of the microorganisms used in this study. The microbial death which could be determined during conversion of thiophene can therefore not be explained by the toxicity of these metabolites. Further research is necessary to determine the cause of this microbial death.

6 Model of the cometabolic transformation of thiophene

The aim of this chapter is to develop a model describing the cometabolic conversion of thiophene using benzene as primary substrate with the objective of predicting the fate of thiophene as a contaminant.

Models featuring cometabolism will be reviewed and analysed with regard to the characteristics of the thiophene conversion. The most suitable models will be compared with data obtained from continuous biofilm reactor experiments (Rivas and Arvin, 2000) and batch experiments (Andersen, 1998).

6.1 Review of mathematical models describing cometabolism

Cometabolism models are predominantly based on a Monod type degradation rate like most of the mathematical models for microbiological processes. Cometabolism is furthermore often characterised by competitive inhibition between growth and non-growth substrate, inhibition by the non-growth substrate or its metabolites and stimulation by the growth substrate.

Michaelis-Menten/Monod rate

Numerous mathematical models have been proposed to describe the process of cometabolism. Most of them are based on a Michaelis-Menten/Monod type expression for the conversion rate of growth substrate and cosubstrate (Alvarez-Cohen and McCarty, 1991a; Arcangeli and Arvin, 1995; Arcangeli and Arvin, 1997a; Chang and Alvarez-Cohen, 1995):

$$r_g = -k_g \cdot X \cdot \frac{S_g}{S_g + K_{sg}} \tag{1a}$$

$$r_c = -k_c \cdot X \cdot \frac{S_c}{S_c + K_{sc}} \tag{1b}$$

The symbols are defined in Table 7.

Competitive inhibition

Competitive inhibition between growth and non-growth substrate often controls cometabolism. Bailey et al. (1977) proposed a modification of the Monod kinetic to describe competitive inhibition, as shown in expressions 2a and 2b.

$$r_g = -k_g \cdot X \cdot \frac{S_g}{S_g + K_{sg} \cdot \left(1 + \frac{S_c}{K_{ic}}\right)} \tag{2b}$$

$$r_c = -k_c \cdot X \cdot \frac{S_c}{S_c + K_{sc} \cdot \left(1 + \frac{S_g}{K_{ig}}\right)} \tag{2b}$$

The competitive inhibition constants K_{ij} were approximated by the half saturation constant K_{sj} in Broholm et al. (1992), Chang and Alvarez-Cohen (1995), while Landa (1994) found different values for inhibition and half saturation constants.

Inhibition by the non-growth substrate (other than due to competition)

The inhibition by the cosubstrate can mostly be explained by the reduction of reducing equivalents (e.g. NADH) and the toxicity of the metabolites produced

through the cosubstrate conversion. Alvarez-Cohen and McCarty (1991) introduced the term of transformation capacity Tc (Tables 5 and 6) to describe the availability of reducing equivalents for the cometabolic conversion as well as the toxicity of metabolites. Tc represents the maximum mass of cosubstrate that can be transformed per unit mass of resting cells. Once the biomass has transformed this Tc amount of cosubstrate per unit mass of resting cells, the biomass is inactivated or dead. This means that Tc describes the transformation of the active cells into dead or inactive biomass during the cosubstrate conversion. The inactivation of the biomass takes place through the depletion of reducing equivalents in the cells (Alvarez-Cohen and McCarty, 1991a). The death of the cells might take place because of the toxicity of the cosubstrate and its transformation products (Alvarez-Cohen and McCarty, 1991b; Wackett and Gibson, 1988; Wackett and Householder, 1989). Therefore, Tc is a function of the reducing power availability, and of the toxicity of cosubstrate and its transformation products (Alvarez-Cohen and McCarty, 1991b; Wackett and Gibson, 1988; Wackett and Householder, 1989).

The availability of reducing power (e.g. NADH) may also be described by a switching function in the degradation rates of growth and cosubstrate as described in Chang et al (1995) (Tables 5 and 6). Chang and Alvarez-Cohen (1995) introduced the concentration of NADH as a variable in the cometabolic model, in which NADH is produced during the degradation of growth substrate and consumed during the degradation of the cosubstrate. Bae and Rittmann (1995) introduced the NADH/NAD ratio in order to account for the energetical situation of the cells and predict the degradation rate of the cosubstrate. The reducing power of the cells, R_p, defined as a linear function of log (NADH/NAD), and the enzyme content of the cells were introduced in the cosubstrate degradation rate (Tables 5 and 6). Log (NADH/NAD) was itself defined as a function of the growth substrate degradation rate and the electron acceptor concentration.

A further way of describing the utilisation of reducing equivalents and the toxicity of the cosubstrate or its metabolites was to relate the maximum specific degradation rates of growth and co-substrates with the cosubstrate conversion product concentration (Ely et al. 1997).

Stimulation by the growth substrate

Alvarez-Cohen and McCarty (1991) introduced the term of transformation yield Ty with the intention to describe the stimulation effect of the growth substrate on the cometabolic conversion. It represents the maximum mass of cosubstrate transformed per unit mass of growth substrate and per unit mass of biomass (Tables 5 and 6). Ty is also mentioned as growth substrate transformation capacity (Criddle, 1993).

Arcangeli (Arcangeli and Arvin, 1995; Arcangeli and Arvin, 1997b) introduced in the Monod type degradation rate of the cosubstrate a switching function depending on the concentration of the growth substrate $\frac{S_g}{S_g + K_{sg}}$. The function describes the same stimulation effect of the growth substrate on the degradation of the cosubstrate. Ely et al. (1997) introduced the concentration of the growth substrate transformation products in the maximum specific degradation rate of growth and co-substrates to describe the stimulation of the bacterial activity by the growth substrate degradation (Table 5-7).

Critical ratio between co- and growth substrate

Dyreborg et al. (1998) reported that the thiophene biotransformation with benzene as growth substrate did not take place when the ratio of concentrations between thiophene and benzene reached a certain critical value. To describe this phenomenon, Dyreborg et al. (1998) introduced in the degradation rates of growth and co-substrates a switching function depending on the concentration ratio of thiophene to benzene. This switching function may describe the depletion of energy intermediates or reducing equivalents as well as the toxicity of the cosubstrate and its transformation products.

The stoichometry and reaction rates for these different models are listed in Tables 5 and 6 for the cometabolic conversion of thiophene with benzene as primary substrate. The general formulation for the differential equations describing this process follows equation (3).

$$\frac{d}{dt}\begin{bmatrix} x_1 \\ . \\ . \\ . \\ x_N \end{bmatrix} = \begin{bmatrix} \nu_{11} & . & . & . & \nu_{1M} \\ . & . & & & \\ . & & . & & \\ . & & & . & \\ \nu_{N1} & & & & \nu_{NM} \end{bmatrix} \bullet \begin{bmatrix} r_1 \\ . \\ . \\ . \\ r_N \end{bmatrix} \qquad (3)$$

where X_i are the concentrations of the different component considered (biomass, thiophene, benzene, CO_2, thiophene metabolites …), ν_i are the stoichiometric coefficients and r_i are the reaction rates.

Table 5. Reaction rates and stoichiometry for the degradation of the growth substrate benzene according to the cometabolic models cited above

Reference	Growth substrate degradation rate	X	Xs	Sg	Sr	S_{CO2}
1 (Alvarez-Cohen et al., 1991a)	$k_g \cdot X \cdot \frac{S_g}{S_g + K_{sg} \cdot \left(1 + \frac{S_c}{K_{sc}}\right)}$	Y_g		-1		$-0.3 + 0.37 \cdot Y_g$
2 (Chang et al., 1995)	$k_g \cdot X \cdot \frac{S_g}{S_g + K_{sg} \cdot \left(1 + \frac{S_c}{K_{sc}}\right)} \cdot \frac{S_r}{S_r + K_{sr}}$	Y_g		-1	α_g (production of reduction equivalents)	$-0.3 + 0.37 \cdot Y_g$
3 (Dyreborg et al., 1998)	$k_g \cdot X \cdot \frac{S_g}{S_g + K_{sg} \cdot \left(1 + \frac{S_c}{K_{sc}}\right)} \cdot \left(1 - \frac{1}{K_{cgc}} \cdot \frac{S_c}{S_g}\right)^{n_g}$	Y_g		-1		$-0.3 + 0.37 \cdot Y_g$
4 (Arcangeli et al., 1995)	$kg \cdot X \cdot \frac{S_g}{S_g + K_{sg} \cdot \left(1 + \frac{S_c}{K_{sc}}\right)}$	Y_g		-1		$-0.3 + 0.37 \cdot Y_g$
5 (Ely et al., 1997)	$\left(k_g - k_{inact} \cdot S_{met} + k_{rec} \cdot S_{CO2}\right) \cdot X \cdot \frac{S_g}{S_g + K_{sg} \cdot \left(1 + \frac{S_c}{K_{sc}}\right)}$	Y_g		-1		$-0.3 + 0.37 \cdot Y_g$
6 (Landa et al., 1994)	$k_g \cdot X \cdot \frac{S_g}{S_g + K_{sg} \cdot \left(1 + \frac{S_c}{K_{sc}}\right)}$	Y_g		-1		$-0.3 + 0.37 \cdot Y_g$

Table 6. Reaction rates and stoichiometry for the degradation of the cosubstrate thiophene according to the cometabolic models cited above

Reference	Non-growth substrate degradation rate	X	Xs	St	Sr	Smet
1 (Alvarez-Cohen et al., 1991a)	$\left(T_y \cdot \frac{q_g}{X} + k_c\right) \cdot X \cdot \frac{S_c}{S_c + K_{sc} \cdot \left(1 + \frac{S_g}{K_{sg}}\right)}$	$-\frac{1}{T_c}$	$\frac{1}{T_c}$	-1		-0.25
2 (Chang et al., 1995)	$kc \cdot X \cdot \frac{S_c}{S_c + K_{sc} \cdot \left(1 + \frac{S_g}{K_{sg}}\right)} \cdot \frac{S_r}{S_r + K_r}$	$-\frac{1}{T_c}$	$\frac{1}{T_c}$	-1	α_c (consumption of reduction equivalents)	-0.25
3 (Dyreborg et al., 1998)	$k_c \cdot X \cdot \frac{S_c}{S_c + K_{sc} \cdot \left(1 + \frac{S_g}{K_{sg}}\right)} \cdot \left(1 - \frac{1}{K_{cgc}} \cdot \frac{S_c}{S_g}\right)^{nc}$			-1		-0.25
4 (Arcangeli et al., 1995)	$k_c \cdot X \cdot \frac{S_c}{S_c + K_{sc} \cdot \left(1 + \frac{S_g}{K_{sg}}\right)} \cdot \left(\frac{S_g}{S_g + K_{sg}}\right)$			-1		-0.25
5 (Ely et al., 1997)	$\frac{k_c}{k_g}\left(k_g - k_{inact} \cdot S_{met} + k_{rec} \cdot S_{CO2}\right) \cdot X \cdot \frac{S_c}{S_c + K_{sc} \cdot \left(1 + \frac{S_g}{K_{sg}}\right)}$			-1		-0.25
6 (Landa et al., 1994)	$k_c \cdot X \cdot \frac{S_c}{S_c + K_{sc} \cdot \left(1 + \frac{S_g}{K_{ig}}\right)}$			-1		-0.25
7 {Bae & Rittmann 1995 ID: 89}	$k_c \cdot X \cdot \frac{S_c}{S_c + K_{sc}} \cdot \frac{S_a}{S_a + K_{sa}}[E] \cdot R_p$ $R_p = \log\left(\frac{NADH}{NAD}\right) + M$ $\log\left(\frac{NADH}{NAD}\right) = f(q_g, S_a)$			-1		-0.25

Table 7. Definition of the variables and parameters

α_c	net stoichiometric coefficient of NAD(P)H regeneration from the oxidation of thiophene	[ ]
α_g	net stoichiometric coefficient of NAD(P)H regeneration from the degradation of benzene	[ ]
[E]	Enzyme content in the cells	
kc	maximum specific degradation rate of thiophene	g COD thiophene/g COD biomass/d
Kcgc	critical concentration ratio between thiophene and benzene	[g COD/g COD]
kg	maximum specific degradation rate of benzene	g COD benzene/g COD biomass/d
Kic	inhibition coefficient of thiophene	[g COD/m^3]
Kig	inhibition coefficient of benzene	[g COD/m^3]
Ksa	half saturation coefficient of the electron acceptor	[g/m^3]
Ksc	half saturation coefficient of thiophene	[g COD/m^3]
Ksg	half saturation coefficient of benzene	[g COD/m^3]
Ksr	half saturation coefficient of external NAD(P)H regenerant	[mol e-/m^3]
M	constant	
rc	degradation rate of thiophene	
rg	degradation rate of benzene	
Rp	reducing power	
Sa	Electron acceptor concentration in the reactor	[g/m^3]
Sc	thiophene concentration in the reactor	[g COD/m^3]
Sg	benzene concentration in the reactor	[g COD/m^3]
S_{met}	Metabolites of thiophene	[g C/m^3]
Sr	NAD(P)H concentration	[mol e-/m^3]
Tc	biomass transformation capacity	g COD thiophene/g COD biomass
Ty	growth substrate transformation capacity	g COD thiophene/g COD benzene
X	active biomass concentration	[g COD/m^3]
Xs	slowly degradable suspended solids concentration	[g COD/m^3]
Yg	yield coefficient of biomass to benzene	g COD biomass/g COD benzene

6.2 Choice of a model for the cometabolic transformation of thiophene

From the preceding paragraphs can be seen that different cometabolic models exist. These models are, in the next paragraphs, analysed with the purpose to determine which are the best suited to describe the thiophene conversion.

Andersen (1998) investigated the model of Dyreborg et al. (1998) to describe the cometabolic conversion of thiophene with benzene as primary substrate in batch experiments. The estimation of the model parameters for these data (performed with the AQUASIM software from EAWAG, Dübendorf, Switzerland) did not lead to a unique set of parameters satisfying the data of all the batches. Especially the critical ratio K_{cgc} showed an important deviation from batch experiment to batch experiment. This would suggest that the model of Dyreborg et al. (1998) can not describe in a satisfying way the conversion of thiophene with benzene as primary substrate.

The characteristic features of the cometabolic transformation of thiophene with benzene as primary substrate revealed by the experiments of Rivas and Arvin (2000, 2002b) are following:

1. The biomass grows on benzene
2. Thiophene does not support growth
3. Competitive inhibition between thiophene and benzene exists
4. Resting cells are able to degrade thiophene
5. Thiophene or its conversion into products reduce the bacterial degradation activity
6. Thiophene or its conversion into products lead to bacterial death
7. Benzene stimulates the bacterial degradation activity

Dyreborg et al. (1998) reported that the microbiological conversion of thiophene and benzene stopped as soon as the ratio of concentration between thiophene and benzene reached a critical value. The inactivation of the biomass due to reducing equivalents depletion or to microbial death caused by the toxicity of thiophene or its conversion products can explain this phenomenon. This peculiarity is therefore included in the characteristic features 5 and 6.

The features 1-7 have to be included in the mathematical model. The different models considered are listed in Table 8 with their ability to satisfy these different features. The models proposed by Dyreborg et al. (1998) and Arcangeli and Arvin (1995) do not allow the transformation of thiophene by resting cells or the death of microorganisms during the conversion of thiophene. These features are relevant in the conversion of thiophene. These two models will therefore not be considered. The model of Landa et al. (1994) only including competitive inhibition was proposed by Albrechtsen and Arvin (1996) to describe cometabolism. This model however does not include the stimulation of the cometabolic activity by benzene or microbial death. The model of Ely et al. (1997) includes the stimulation by benzene, the inactivation by thiophene as well as the cometabolic activity of resting cells. It lacks nevertheless the bacterial death due to thiophene conversion.

Table 8. Comparison of the proposed models regarding to the characteristics of thiophene degradation

		1. growth on benzene	2. no growth on thiophene	3. Competitive inhibition	4. Resting cell degradation	5. Reduction of degradation activity (other than by competitive inhibition)	6. Killing of the microorganisms	7. Stimulation of the activity by benzene
1	(Alvarez-Cohen and McCarty, 1991a)	+	+	+	+	+	+	+
2	(Chang and Alvarez-Cohen, 1995)	+	+	+	+	+	+	+
3	(Dyreborg et al., 1998)	+	+	+	-	+	-	+
4	(Arcangeli and Arvin, 1995)	+	+	+	-	-	-	+
5	(Ely et al., 1995)	+	+	+	+	+	-	+
6	(Landa et al., 1994)	+	+	+	+	-	-	-
7	(Bae and Rittmann, 1995)	+	+	-	+	+	-	+

It appears that the only models satisfying all the characteristics of the cometabolic transformation of thiophene are the models of Alvarez-Cohen and McCarty (1991) and Chang and Alvarez-Cohen (1995). The model of Chang and Alvarez-Cohen (1995) includes the concentration of reducing equivalent NADH and two additional parameters α_g and α_c. In the present problem the concentration of reduction equivalents was not investigated and would therefore be an important uncertainty on the parameters α_g and α_c.

The model of Bae and Rittmann (1995) accounts for the reduction of microbiological activity due to depletion of reducing power as well as for the stimulation of the degradation by benzene and might therefore also be of interest for describing the cometabolic conversion of thiophene. It introduces however at least five additional parameters linked to the reducing equivalent concentration. For the same reason than for the model of Chang and Alvarez-Cohen (1995), this model will be disregarded in this study.

The model of Alvarez-Cohen and McCarty (1991) was therefore chosen to model the conversion of thiophene with benzene as primary substrate.

6.3 Modelling of experimental data

The data used for modelling are results of batch experiments (Andersen, 1998) and data obtained during the continuous biofilm investigation described in Rivas and Arvin (2000).

6.3.1 Description of the data

Batch experiments of Andersen (1998)

The investigation performed by Andersen (1998) included nine microcosm experiments of a volume of 2 l with the same medium and inoculum as in Rivas and Arvin (2000). The batches differed from each other by the initial concentrations of benzene that varied between 1.5 and 16.0 g COD/m^3 (between 0.9 mg/L and 9.4

mg/L), while the thiophene initial concentration was set to 11.0 g COD/m^3 (4.8 mg/L).

Biofilm continuous reactor data of Rivas and Arvin (2000)

During the experiments used for modelling, the biofilm reactor was run with a flow of 4 l/h of mineral medium containing benzene and thiophene. The experiments consisted of step changes applied to the benzene or the thiophene concentration, after which the concentration profiles of both compounds were followed.

Experiment 1: the benzene inlet concentration was kept constant at 7.0 g COD/m^3 while the thiophene inlet concentration was changed from 0.0 g COD/m^3 to 1.8 g COD/m^3, further to 5.0 g COD/m^3 and back to 0.0 g COD/m^3.

Experiment 2: the thiophene inlet concentration was kept constant at 3.6 g COD/m^3 while the benzene inlet concentration was changed from 5.2 g COD/m^3 to 0.0 g COD/m^3.

Experiment 3: the thiophene inlet concentration was kept constant at 1.5 g COD/m^3 while the benzene inlet concentration was changed from 11.0 g COD/m^3 to 0.0 g COD/m^3.

6.3.2 Parameter estimation

The modelling program AQUASIM offers two different algorithms for the parameter estimations: the secant algorithm and the simplex algorithm. Both possibilities were used in the following parameter estimations when the model of Alvarez-Cohen and McCarty (1991) were compared to the experimental data.

The parameter estimation for the model of Alvarez-Cohen and McCarty (1991) and the data of Andersen (1998) lead to an unique set of values which described the nine batch experiments. These parameter values are listed in Table 9.

The estimation of the model parameters with the data of the biofilm reactor experiments 1-3 was conducted separately as well as for the three experiments together. The biomass situation at the beginning of each experiment (initial biomass concentration) was also estimated during the parameter estimation. The model parameter values obtained with a satisfying data fits are listed in Table 9 and the corresponding data fits are represented in Fig. 12-15 for the thiophene and benzene concentrations.

Table 9. Parameter resulting from the parameter estimations performed with batch experiment data originating from Andersen et al. (1998) and with data of experiment 1, 2 and 3 with the continuous biofilm reactor

	b	k_c	k_g	K_{sc}	K_{sg}	K_{ic}	K_{ig}	T_c	T_y
Andersen et al. (1998)	0.36	5.49	9.17	19.23	0.014	0.01	10.00	6.11	0.00
EXP. 1	0.32	10.00	15.00	0.08	0.005	0.011	0.005	0.20	0.00
EXP. 2	0.32	3.80	10.00	0.015	10.0	10.00	10.00	0.007	0.00
EXP. 3	0.32	5.90	10.00	0.013	10.0	10.00	10.00	0.05	0.00

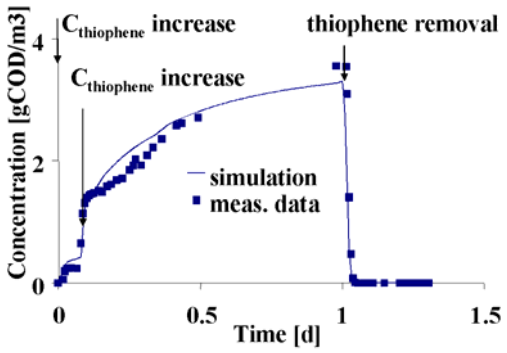

Fig. 12. Time course of the thiophene concentration in EXP. 1

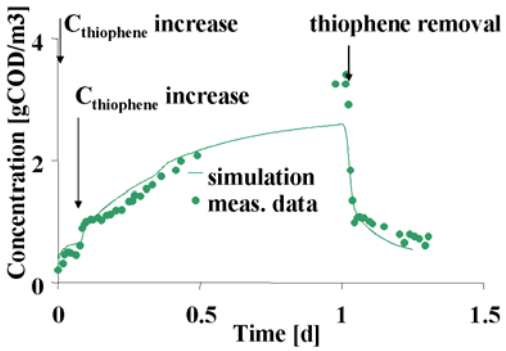

Fig. 13. Time course of the benzene concentration in EXP. 1

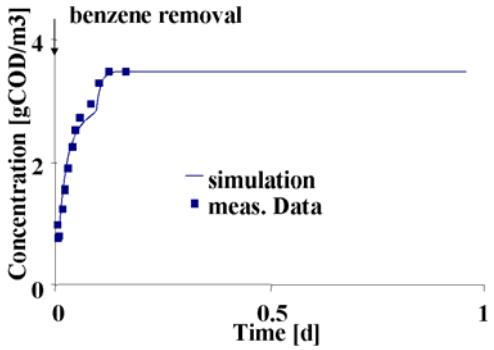

Fig. 14. Time course of the thiophene concentration in EXP. 2

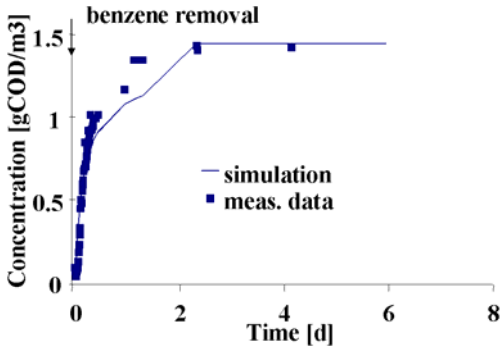

Fig. 15. Time course of the thiophene concentration in EXP. 3

6.3.3 Results

Batch investigation

The data of the nine microcosm experiments of Andersen (1998) could be described qualitatively and quantitatively with an unique set of parameters (Table 9). The model of Alvarez-Cohen and McCartyis more efficient to describe the cometabolic conversion of thiophene in batch experiments than the model of Dyreborg (1998) (Andersen, 1998). Rivas et al. (2002b) however reported that the thiophene degradation rate was of zero order in a concentration range between 2 and 40 mg/L for thiophene. The value obtained by parameter estimation for the thiophene half saturation constant Ksc (19.23 g COD/m^3) does not reflect this result.

Biofilm reactor investigation

The data of the experiments 1-3 of Rivas and Arvin (2000) were fitted in a satisfying way with the parameter values listed in Table 9 for each experiment separately (Fig. 12-15). The parameter values resulting from the parameter estimation of the data from experiment 2 and 3 are very close to each other while they are quite different from the parameter values obtained for experiment 1. The parameter estimation run on the data of experiment 1-3 together did not allow the experimental

data to be approached in a satisfying way. It was not possible to obtain an unique set of parameter fitting the data of the three experiments.

6.4 Discussion

The cometabolic model of Alvarez-Cohen and McCarty (1991) was chosen for modelling the cometabolic conversion of thiophene because it satisfied theoretically the characteristic features of this process. The model was compared with data from degradation investigation with suspended and attached biomass with help of the computer program AQUASIM.

The model of Alvarez-Cohen and McCarty (1991) was capable to reflect qualitatively the concentrations of thiophene and benzene during dynamic changes of these concentrations in the reactor inlet. However, this model could not quantitatively describe the conversion of thiophene as it was not possible to obtain an unique set of parameters to represent the experimental data considered. The model of Alvarez-Cohen and McCarty does not appear to be appropriate to describe the cometabolic conversion of thiophene with benzene as primary substrate.

The four sets of parameters obtained for the batch experiments and the three dynamic biofilm reactor experiments have one feature in common. The transformation yield Ty was found to have a value of 0 by performing parameter estimations on these data. Apparently the transformation yield Ty does not play a role in the cometabolic conversion of thiophene. This could indicate that the stimulation of the cometabolic conversion by benzene only occurs because benzene supports the growth of the microorganisms. This is however in contradiction with the experimental results that showed that benzene stimulated the transformation of thiophene (Rivas et al., 2002b).

Before rejecting this model completely, a more thorough study should be performed relatively to the identifiability of the model parameters. Such an investigation would give information on the dependency between parameters and whether it is possible to determine them as done in the present report.

One of the parameters with a considerably uncertainty influence in the model is the biomass concentration in the biofilm reactor. The only data available for the biomass is the biofilm thickness. This however, does not reflect the concentration of active biomass in the biofilm. The initial biomass concentration for each experiment (1-3) was estimated together with the model parameters. The uncertainty on this variable might explain the lack of success of the Alvarez-Cohen and McCarty model. In order to avoid this uncertainty, the biomass concentration should be determined independently. This can be performed by a direct measurement of the biomass concentration applying microbiological methods such as fluorescent in–situ hybridisation, which would account for the active benzene degrader population. It can also be performed by indirect tests such as respirometric assays.

During the conversion of thiophene, the biomass is inactivated or killed. In the applied model, the biomass is transformed to inactive biomass that is further hydrolysed, or into inert biomass. There should be made a distinction between biomass that is inactivated due to the depletion of reducing power and that can be reactivated by the addition of benzene, and dead biomass which is further hydrolysed or stays inert. This is possible with the model of Chang and Alvarez-Cohen (1995) and Bae and Rittmann (1995). To use these models, the reducing power situation has to be defined and investigated by the measurement of NADH/NAD.

For engineering purposes it might not be advisable to have a model with too many variables and parameters to determine. A simpler model like the one that was proposed by Arcangeli and Arvin (1995) might be sufficient to describe the conversion of thiophene by growing cells.

7 Conclusions

The microbiological oxidation of thiophene in the presence of benzene as primary substrate under aerobic conditions has been investigated in suspended culture microcosms as well as in a biofilm system. Following conclusions could be derived from this study:

- Thiophene can be oxidized by cells growing on benzene as well as by resting cells if these cells have been activated with benzene prior to their contact with thiophene. If the cells have not previously been activated, thiophene remains resistant to microbiological oxidation.

- Kinetic experiments conducted in a continuous biofilm reactor indicated that the conversion of thiophene with benzene as primary substrate is characterised by competitive inhibition, and that it leads to a reduction of the benzene degradation activity. The activity loss was supposed to be caused by the depletion of energy sources as well as by microbial death due to the toxicity of thiophene or its transformation products. Benzene was shown to stimulate the microbiological transformation of thiophene.

- Suspended culture microcosm experiments as well as continuous biofilm experiments showed that thiophene is converted into non-volatile products which were not further degraded.

- A chemical investigation showed that the converted thiophene was transformed into six metabolites. Two major products **C4** and **C5** represented 78% and 20% of the converted thiophene on a mg C/L basis. They were identified to be the result of a Diels-Alder reaction between two thiophene sulphoxides: two diastereoisomers of thiophene sulphoxide dimer. Two minor products **C2** and **C3** were also identified to be the result of a Diels-Alder condensation between thiophene derivatives, but their complete identity could not be determined. Benzothiophene (0.05% of the converted thiophene) was identified to be produced from thiophene. It was supposed to result from the further reaction of thiophene sulphoxide dimer by the formal loss of 2H+S+2O. The sixth product **C1** could not be identified because it was produced in a too small amount and because it reacted further during the identification procedures.

- The influence of benzene on the formation of the thiophene transformation products was investigated in suspended culture microcosms. This study showed that the metabolite distribution did not depend on the presence of benzene, or on the concentration of benzene or thiophene.

- The metabolites **C1**-**C5** were not further converted by the microbiological culture as sole carbon source or in the presence of thiophene and/or benzene.

- The toxicity of the five thiophene transformation products **C1**-**C5** towards the microorganisms was investigated by performing dose respons experiments and by

following the microbiological benzene uptake. This investigation showed that **C1-C5** do not affect the benzene degradation activity of the microorganisms. Thus, they are not responsible for the microbial inactivation that occurred during the conversion of thiophene. The conversion products of thiophene **C2-C5** were toxic to the bacterium *V. Fischeri* and the algae *S. Capricornutum*.

- The mathematical model proposed by Alvarez-Cohen and McCarty (1991) for cometabolism was applied to the experimental data resulting from the biofilm reactor investigation. This model was able to describe the conversion of thiophene in the presence and the absence of benzene in a qualitative way. Though it was not able to describe this process quantitatively.

It was not possible to determine in this study what was responsible for the microbial inactivation during the conversion of thiophene. Thiophene or unstable intermediates between thiophene and **C1-C5** could be responsible for it.

No model could be proposed that could describe quantitatively the microbiological conversion of thiophene. A more thorough model investigation with regards to the identifiability of the model parameters needs to be performed in order to determine whether the chosen model of Alvarez-Cohen and McCarty (1991) is suited to describe this cometabolic process. Further, other models will have to be compared with the experimental data obtained.

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