Anaerobic biodegradation of polycyclic aromatic hydrocarbons

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Seneca et hoc genus omne,
das schreibt und schreibt sein unausstehlich weises Larifari
als gält es primum scribere,
deinde philosophari.

Friedrich Nietzsche, Die fröhliche Wissenschaft 34

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Anaerobic degradation of polycyclic aromatic hydrocarbons

Michael Safinowski

Aromatic hydrocarbons belong to the most important ground water contaminants. Especially in the absence of molecular oxygen their degradation proceeds very slowly, leading to the formation of long contaminant plumes and pollutant transport over long distances. Such processes endanger the drinking water supply in many areas.

The objective of this work was the examination of microbial degradation pathways and enrichment of new cultures. Based on laboratory results, conclusions should be drawn about potential natural attenuation in contaminated ground water.

The sulphate-reducing culture N47 can utilise naphthalene or 2-mnethylnaphthalene as the sole carbon source and electron donor. Anaerobic degradation of 2-methylnaphthalene is initiated by an addition of fumarate to the methyl group producing the first intermediate, naphthyl-2-methyl-succinate. In a subsequent β-oxidation of the original methyl atom, the central metabolite 2-naphthoic acid is generated. In the following pathway, the aromatic ring system is reduced, cleaved, and finally oxidized to CO_2.

The activities of two enzymes involved in the upper degradation pathway of 2-methylnaphthalene, succinyl-CoA:naphthyl-2-methyl-succinate CoA-transferase and naphthyl-2-methyl-succinyl-CoA dehydrogenase, were measured in crude cell extracts. Succinyl-CoA:naphthyl-2-methyl-succinate CoA-transferase was not inhibited by sodium borohydride or hydroxylamine indicating that this enzyme belongs to the family III of CoA-transferases similar to the corresponding enzyme in the anaerobic toluene degradation pathway.

The enzymatic activity of naphthyl-2-methyl-succinyl-CoA dehydrogenase could only be detected using phenazine methosulphate (PMS) as electron acceptor. No activity was observed with natural electron acceptors such as nicotinamide adenine dinucleotide (NAD^+) or flavin adenine dinucleotide (FAD).

Also the activation mechanism of naphthalene was investigated. Contrary to previous reports postulating a carboxylation, it could be demonstrated that the initial reaction in the anaerobic degradation of naphthalene is methylation to 2-methylnaphthalene. Naphthyl-2-methyl-succinate and naphthyl-2-methylene-succinate, specific metabolites occurring exclusively during anaerobic degradation of 2-methylnaphthalene were detected during growth on naphthalene. Additionally, enzymes of the anaerobic 2-methylnaphthalene degradation pathway could be detected in naphthalene-grown cells with similar activities. When the cells were transferred from 2-methylnaphthalene to naphthalene, the lag-phase lasted for almost 100 days indicating that additional catabolic enzymes have to be activated in this case. In the opposite case, after the transfer from naphthalene to 2-methylnaphthalene, the cells grew immediately demonstrating that no new enzymes had to be induced.

As contaminant plumes in ground water contain normally mixtures of numerous substances, the influence of diverse polycyclic and heterocyclic compounds on growth on naphthalene or on 2-methylnaphthalene was investigated. Cometabolic transformation of the majority of the offered cosubstrates was observed, whereas they were converted to the corresponding carboxylic acids,

frequently to several isomers. Some compounds were methylated and transformed to the corresponding methyl-succinic acids. In few cases, a partial or total inhibition of growth was detected. As one of the principal obstacles in the investigation of anaerobic degradation of aromatic compounds is the lack of appropriate cultures, new enrichments with different substrates and electron donors were prepared. Amongst others, a novel sulphate-reducing biphenyl degrading enrichment culture was obtained. 4-biphenylic acid was detected in culture supernatants as a putative metabolite. The culture was also able to convert cometabolically 4-fluorobiphenyl to 4-flourobiphenyl-carboxylic acid.
The data obtained during this work contribute significantly to a better understanding of anaerobic degradation of polycyclic aromatic compounds.

Anaerober Abbau polyzyklischer aromatischer Kohlenwasserstoffe

Michael Safinowski

Aromatische Kohlenwasserstoffe gehören zu den häufigsten Schadstoffen im Grundwasser. Besonders in Abwesenheit vom molekularen Sauerstoff werden sie extrem langsam abgebaut, was zur Ausbildung langer Schadstofffahnen und ihrem Transport über lange Distanzen führt. So gefährden sie die Trinkwasserversorgung in vielen Gebieten.

Das Ziel dieser Arbeit war die Untersuchung mikrobieller Abbauwege und Anreicherung neuer Kulturen. Auf Grundlage der Laborergebnisse sollten Aussagen über den potentiellen Schadstoffabbau im kontaminiertem Grundwasser getroffen werden.

Die sulfatreduzierende Kultur N47 kann mit Naphthalin oder 2-Methylnaphthalin als einziger Kohlenstoffquelle und Elektronendonor wachsen. Der anaerobe Abbau von 2-Methylnaphthalin beginnt mit der Addition von Fumarat an die Methylgruppe, wodurch das erste Intermediat, Naphthyl-2-methyl-succinat, entsteht. Bei der folgenden β–Oxidation am ursprünglichen Methyl-Atom entsteht der zentrale Metabolit, 2-Naphthoesäure. Während des weiteren Abbaus wird das aromatische Ringsystem reduziert, gespalten und dann zu CO_2 oxidiert.

Die Aktivitäten zweier Enzyme des oberen Abbauweges von 2-Methylnaphthalin, succinyl-CoA:naphthyl-2-methyl-succinat CoA-Transferase und Naphthyl-2-methyl-succinyl-CoA Dehydrogenase, wurden in Zellextrakten gemessen. Succinyl-CoA:naphthyl-2-methyl-succinate CoA-Transferase wurde nicht durch Natrium Borhydrid oder durch Hydroxylamin inhibiert, was ein Hinweis auf die Zugehörigkeit dieses Enzyms zur Familie III der CoA-Transferasen ist, zu der auch das analoge Enzym des anaeroben Abbauweges von Toluol gehört.

Die Aktivität der Naphthyl-2-methyl-succinyl-CoA Dehydrogenase konnte nur mit dem künstlichen Elektronenakzeptor Phenazinmethosulfat (PMS) gemessen werden. Keine Enzymaktivität war mit natürlichen Elektronenakzeptoren wie Nikotinamid-Adenindinukleotid (NAD^+) oder Flavinadenin-Dinukleotid (FAD) messbar.

Es wurde auch der Aktivierungsmechanismus von Naphthalin untersucht. Entgegen früheren Berichten, die eine Carboxylierung postulierten, konnte in der vorliegenden Arbeit nachgewiesen werden, dass die Initialreaktion im anaeroben Naphthalinabbau eine Methylierung zu 2-Methylnaphthalin ist. Naphthyl-2-methyl-succinat und Naphthyl-2-methylen-succinat, zwei spezifische Metabolite, die ausschließlich während des anaeroben Abbaus von 2-Methylnaphthalin entstehen, wurden während des Wachstums auf Naphthalin in Kulturüberständen nachgewiesen. Zusätzlich konnten die Enzyme des anaeroben Abbauweges von 2-Methylnaphthalin während des Wachstums auf Naphthalin mit ähnlichen Aktivitäten nachgewiesen werden. Nachdem die Zellen von 2-Methylnaphthalin auf Naphthalin übertragen worden waren, dauerte die Lag-Phase nahezu 100 Tage, was darauf hindeutete, dass zusätzliche Enzyme aktiviert werden müssen. Im umgekehrten Fall, nach der Übertragung von Naphthalin auf 2-Methylnaphthalin, setzte das Wachstum sofort ein, wobei offensichtlich keine neuen Enzyme induziert werden mussten.

Da die Schadstofffahnen im Grundwasser normalerweise Gemische zahlreicher Substanzen enthalten, wurde der Einfluss verschiedener polyzyklischer und heterozyklischer Substanzen auf das Wachstum auf Naphthalin oder 2-Methylnaphthalin untersucht. Es konnte kometabolische Umsetzung der

meisten eingesetzten Substanzen beobachtet werden, wobei die Kosubstrate zu den entsprechenden Carboxylsäuren umgewandelt wurden, oft zu mehreren Isomeren. Manche Substanzen wurden methyliert und zu den entsprechenden Methylsuccinaten verstoffwechselt. In einigen Fällen konnte eine partielle oder völlige Inhibierung des Wachstums beobachtet werden.
Da bei der Untersuchung des anaeroben Abbaus aromatischer Substanzen der Mangel an entsprechenden Kulturen eines der Haupthindernisse ist, wurden Anreicherungen mit verschiedenen Substraten und Elektronenakzeptoren durchgeführt. Unter anderen konnte eine neue sulfatreduzierende, biphenyl-abbauende Kultur angereichert werden. 4-Biphenylsäure wurde als potentieller Metabolit in den Kulturüberständen entdeckt. Die Kultur konnte ebenfalls 4-Fluorobiphenyl zu 4-Flourobiphenyl-Carboxylsäure kometabolisch umwandeln.
Die während der vorliegenden Arbeit gewonnenen Daten tragen wesentlich zum Verständnis des anaeroben Abbaus polyzyklischer aromatischer Kohlenwasserstoffe bei.

Contents

1 General introduction

1.1 Aromatic compounds in the environment

Petroleum and the products derived from it are of fundamental importance for our industrialised civilisation. It plays a dominant role as raw material for fuel production and for a wide range of chemical syntheses. The second very important fossil energy carrier is coal, which can also be refined to coal tar that serves as a source of a huge amount of chemical compounds. Both, petroleum and coal tar are mixtures of numerous chemical substances, predominantly of aliphatic and aromatic hydrocarbons as well as heterocyclic compounds.

As hydrocarbons consist only of carbon and hydrogen, the lack of functional groups and polarised bonds renders them chemically inert at moderate temperature range. Cyclic molecules, in which all C-atoms are sp^2-hybridised and which posses a delocalised π-electron system over the ring, are termed aromatic hydrocarbons. Their chemical stability is even higher due to the mesomeric delocalisation energy.

The ubiquitous use of petroleum and charcoal products leads inevitably to the input of aromatic hydrocarbons into the environment. One can distinguish diffuse emissions through combustion of coal or gasoline, which cause a widespread pollution of air and soil, especially close to main traffic roads, and massive punctual contaminations due to spills during transport and at sites of storage and refinement of petroleum and coal products. Especially in such cases when large amounts of aromatic hydrocarbons enter environments with limited oxygen supply such as ground water, the enhanced microbial respiration leads to a rapid oxygen depletion and to the development of anoxic conditions. The lack of molecular oxygen retards massively the degradation of aromatic compounds so that long pollutant plumes are transported in aquifers endangering drinking water supply in many areas.

However, there are also natural sources of aromatic hydrocarbons in the environment. Anaerobic bacteria produce benzene and toluene during fermentation of aromatic aminoacids (Jüttner and Henatsch, 1986; Jüttner, 1988; Fischer-Romero et al., 1996). Naphthalene is used by certain termite species to fumigate their nests (Chen et al., 1998). Additionally, a wide spectrum of especially polynuclear aromatic hydrocarbons is generated by incomplete combustion of organic material, for example during forest fires (Hirner et al., 2000). One has to mention also natural petroleum spills. This continuous natural release of aromatic hydrocarbons into the environment, however, did not result in substantial damage of soil or water, which demonstrates the high efficiency of natural attenuation. As these compounds were present in the biosphere since millions of years, microorganisms could develop pathways for their degradation under different redox conditions.

The profound understanding of such natural attenuation processes and of microbial degradation pathways of aromatic hydrocarbons is absolutely important for the measures of remediation of contaminated areas. Hereby, it is necessary to consider that in complex environments such as ground water several bacterial strains using different electron acceptors can apply varying degradation pathways for one compound that are enhanced or inhibited by different factors. A huge spectrum of chemical substances occurring in petroleum and coal products (Franck, 1963) makes clear that beside the degradation pathways for single compounds also different mutual inhibition and cometabolism effects should be considered, as already observed by some authors (Bouchez et al., 1995; Wackett and Ellis, 1999).

However, there are also situations where a degradation of aromatic hydrocarbons is highly undesirable. It was recently shown that the degradation of petroleum compounds in subsurface reservoirs leads to oil alteration with major economic consequences (Aitken et al., 2004).

This short summary over the occurrence and role of aromatic hydrocarbons in the environment shows that understanding of their degradation is not only of academic interest but has also many practical application areas. The increasing knowledge about the conditions of natural attenuation can provide possibilities to exert influence on microbial processes in the underground in order to secure our drinking water and energy supply.

1.2 Microbial degradation of aromatic hydrocarbons

Many processes can contribute to a concentration decrease in areas polluted with aromatic compounds. But unlike sorption or dilution, only bacterial degradation can remove them totally from the environment by converting them to carbon dioxide. The initial step in the aerobic degradation of aromatic hydrocarbons is mono- or dioxygenation resulting in a direct incorporation of one or two oxygen atoms from molecular oxygen and leading to hydroxylated products (Cerniglia, 1984; Gibson and Subramanian, 1984; Cerniglia, 1992). However, many ecosystems, most of them in the subsurface, have only a limited supply with molecular oxygen. Increased microbial activity can lead to a rapid oxygen depletion and development of anaerobic conditions, which makes the use of oxygenases impossible. Therefore, aromatic hydrocarbons were for a long time considered to be recalcitrant under anoxic conditions.

First in the 1980's the anaerobic degradation of aromatic hydrocarbons was definitively demonstrated to occur (Kuhn et al., 1985). Detailed studies of the biochemistry have shown that anaerobic bacteria use previously unknown mechanisms of hydrocarbon activation that differ totally from that of aerobic microorganisms.

Toluene turned out to be the easiest degradable aromatic hydrocarbon under anoxic conditions and many nitrate-reducing (Dolfing et al., 1990; Evans et al., 1991; Song et al., 1999), iron-reducing (Lovley and Lonergan, 1990), sulphate-reducing (Rabus et al., 1993; Beller et al., 1996; Meckenstock, 1999), and phototrophic (Zengler et al., 1999) strains were isolated that can grow on toluene as the sole carbon source and electron donor. It was also demonstrated that anaerobic degradation of toluene can be performed by defined, syntrophic cultures (Meckenstock, 1999) or under methanogenic conditions (Beller and Edwards, 2000). It was shown that the first step in the anerobic toluene degradation pathway is the addition of an unsaturated dicarboxylic acid, *i.e.*, fumarate to the methyl group resulting in benzylsuccinate (Biegert et al., 1996; Beller and Spormann, 1997a; Beller and Spormann, 1997b). In this way, the poorly reactive methyl carbon atom is converted to a methylen carbon in *β*–position to a carboxylic group, which allows the subsequent oxidation. Benzylsuccinate synthase was purified from *Thauera aromatica* (Leuthner et al., 1998) and *Azoarcus* sp. strain T (Krieger et al., 2001) and identified as a new glycyl radical enzyme. It possesses high sequence similarities with other known glycyl radical enzymes, pyruvate formate lyase and anaerobic ribonucleotide reductase. Native benzylsuccinate synthase is a heterohexamer with the structure $\alpha_2\beta_2\gamma_2$. The subunits consist of 860, 73 and 57 aminoacids, as predicted from the gene sequence. A gene located directly upstream to the genes encoding for benzylsuccinate synthase shows homology to the pyruvate formate lyase activating enzyme (Coschigano et al., 1998;

Leuthner et al., 1998). The activation mechanism involves a cleavage of S-adenosylmethionine to methionine and adenosyl radical, which on his part abstracts a hydrogen atom from glycine generating a glycyl radical. The presence of a stable organic free radical was detected in partially purified benzylsuccinate synthase from anaerobically grown toluene-induced cells of *Azoarcus* sp. with electron paramagnetic resonance spectroscopy (Krieger et al., 2001). The reaction of benzylsuccinate synthase begins with the homolytic cleavage of a C-H bond in the methyl group of toluene, whereby a hydrogen atom is transferred to the enzyme and a benzyl radical is formed that attacks the double bond of fumarate. Finally, the hydrogen atom is transferred back to benzylsuccinate leaving the enzyme in the active, radical form. The reaction is highly stereospecific and yields exclusively R-(+)-benzylsuccinate (Beller and Spormann, 1998). In the following degradation steps, benzylsuccinate is activated to a CoA-ester with succinyl-CoA serving as the donor of the CoA-moiety and undergoes a β-oxidation on the original methyl-atom resulting in the central metabolite benzoate. The genes that code for enzymes of the β-oxidation of benzylsuccinate to benzoate are localised on one single operon (Leutwein and Heider, 1999). Succinyl-CoA:(R)-benzylsuccinate CoA-transferase and (R)-benzylsuccinyl-CoA dehydrogenase were purified and characterized. Succinyl-CoA:(R)-benzylsuccinate CoA-transferase is fully reversible and forms only 2-(R)-benzylsuccinyl-CoA. In the presence of (S)-benzylsuccinate, the enzyme activity is significantly lower. The enzyme consists of two subunits in $\alpha_2\beta_2$ configuration and is not inactivated by sodium borohydride or hydroxylamine. This finding and kinetic investigations indicate that succinyl-CoA:(R)-benzylsuccinate CoA-transferase belongs to the family III of CoA-transferases and that the enzyme reaction proceeds through the formation of a ternary complex without an enzyme-bound CoA intermediate (Leutwein and Heider, 2001). The first oxidative step in the anaerobic toluene degradation is catalysed by the (R)-benzylsuccinyl-CoA dehydrogenase. This enzyme is a homotetramere with a subunit size of 45 kDa. Every subunit contains one FAD cofactor. As succinyl-CoA:(R)-benzylsuccinate CoA-transferase, also (R)-benzylsuccinyl-CoA dehydrogenase is highly stereospecific and is inhibited by (S)-benzylsuccinyl-CoA. The natural electron acceptor of the (R)-benzylsuccinyl-CoA dehydrogenase reaction could not be determined so far (Leutwein and Heider, 2002).

The product of the β-oxidation on the original methyl atom of toluene, benzoyl-CoA, undergoes finally reductive dearomatisation and ring cleavage. The reaction of benzoyl-CoA reductase is coupled with the hydrolysis of two molecules ATP to ADP in order to overcome the high mesomeric energy of the aromatic ring. The redox centre of the benzoyl-CoA reductase consists of three cysteine-ligated [4Fe-4S] clusters with redox potential more negative than –500 mV (Boll et al., 2001). The product of the reaction, cyclohex-1,5-diene-1-carbonyl-CoA undergoes a ring reduction, hydroxylation and cleavage to 3-hydroxypimelyl-CoA that yields three acetyl-CoA, one CO_2 and six reducing equivalents (Harwood et al., 1999).

Anaerobic degradation of xylene proceeds analogous to that of toluene. Several cultures that grow on toluene can also utilise *meta* or *orto*-xylene (Dolfing et al., 1990; Rabus and Widdel, 1995; Hess et al., 1997; Harms et al., 1999; Morasch et al., 2004). In contrary to this, there are almost no cultures that can degrade *p*-xylene anaerobically (Häner et al., 1995). Analogously to toluene, also the initial step in the anaerobic xylene degradation is the

addition of fumarate yielding a methylbenzylsuccinate isomer, which is further oxidised to the corresponding methylbenzoate (Krieger et al., 1999; Morasch et al., 2004).

In sulphate-reducing bacteria, also the anaerobic degradation of ethylbenzene begins with fumarate addition to the benzyl carbon atom of the side chain (Kniemeyer et al., 2003), as concluded from metabolites, (1-phenylethyl)succinate and 4-phenylpentanoate, found in culture supernatants. Unlike in toluene or xylene degradation, a direct oxidation to benzoyl-CoA is not possible as the fumarate addition yields a tertiary carbon atom in the benzyl position. Thus, a rearrangement of the carbon skeleton was proposed to take place, but no closer insights could be obtained so far.
The activation mechanism of ethylbenzene under nitrate-reducing conditions is totally different. In this case, ethylbenzene is directly oxidised to 1-phenylethanol that is subsequently oxidised to acetophenone, carboxylated and activated to 3-oxo-3-phenylpropionyl-CoA and cleaved to benzyl-CoA and acetyl-CoA (Ball et al., 1996; Johnson and Spormann, 1999). Ethylbenzene dehydrogenase was identified as a new molybdenium/iron-sulphur/haem protein in the bacterial periplasm. It consists of three subunits with molecular masses of 96 kDa, 43 kDa and 23 kDa, respectively, in $\alpha\beta\gamma$ structure. It is the first enzyme that oxidises a nonactivated hydrocarbon without molecular oxygen (Kniemeyer and Heider, 2001).

Despite anaerobic catabolism of benzene was detected in many cases (Coates et al., 2002), only one nitrate-reducing pure culture was isolated so far (Coates et al., 2001). The biochemistry of the initial reaction in anaerobic benzene degradation remains unclear. Although benzoate was reported as an intermediate of anaerobic biodegradation of benzene (Caldwell and Suflita, 2000; Phelps et al., 2001), a direct carboxylation was considered as rather improbable (Phelps et al., 2001). Some authors favoured methylation and further catabolism *via* the anaerobic toluene degradation pathway but without delivering stringent proofs (Coates et al., 2002).

Only few cultures have been isolated so far that can degrade polycyclic aromatic hydrocarbons such as naphthalene (Zhang and Young, 1997; Galushko et al., 1999; Meckenstock et al., 2000; Rockne et al., 2000), 2-methylnaphthalene (Annweiler et al., 2000) or phenanthrene (Zhang and Young, 1997).
Naphthoic acid and phenanthroic acid were detected as central metabolites in anaerobic degradation of naphthalene or phenanthrene, respectively. It was shown that the C-atom of the carboxylic group of 2-naphthoic acid is derived from bicarbonate of the culture buffer (Zhang and Young, 1997; Meckenstock et al., 2000). The best studied anaerobic degradation pathway for polycyclic aromatic hydrocarbons is the one of 2-methylnaphthalene. Naphthyl-2-methyl-succinate (NMS) and naphthyl-2-methylene-succinate (NMeS) were found in culture supernatants during growth on 2-methylnaphthalene indicating that the degradation pathway is analogous to that of toluene.
The initial reaction in anaerobic 2-methylnaphthalene catabolism, the addition of fumarate to the methyl group could be detected in an enzyme test in dense cell suspension (Annweiler et al., 2000).

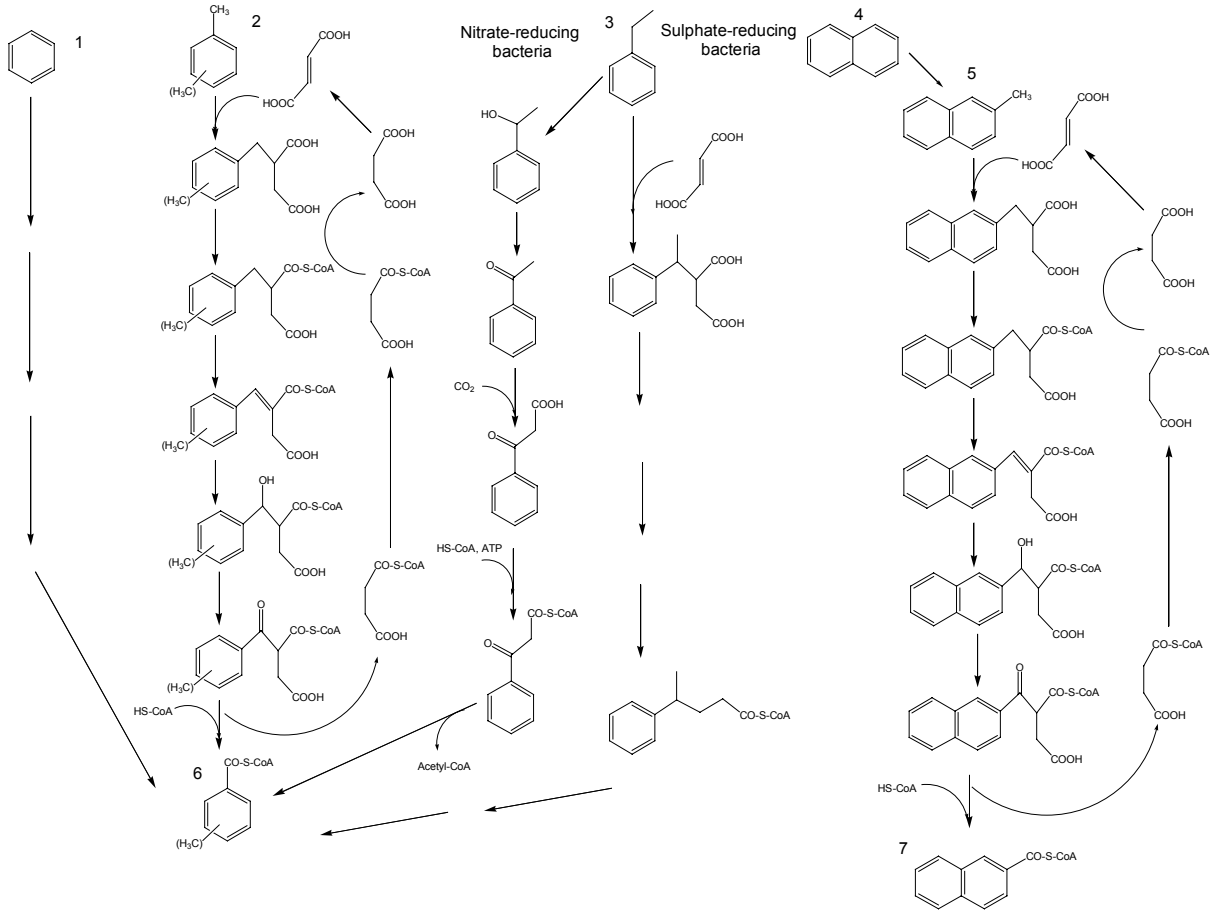

Figure 1. Proposed pathways and intermediates in the anaerobic degradation of benzene (1) toluene or xylene (2), respectively, ethylbenzene (3), naphthalene (4) and 2-methylnaphthalene (5). Benzoic acid or methylbenzoic acid (6), respectively, is the central metabolite in the upper anaerobic degradation pathway of all BTEX compounds. The exact conversion mechanism of benzene to benzoic acid is not yet elucidated. Toluene and xylene undergo an addition of fumarate to the methyl group yielding benzylsuccinate, which is later oxidised at the original methyl atom. In sulphate-reducing bacteria, the initial step in anaerobic ethylbenzene degradation is also an addition of fumarate to the benzyl C-atom, followed by oxidation and C-skeleton rearrangement. In nitrate-reducing bacteria, ethylbenzene is directly oxidised to α-phenylethanol that is subsequently converted to benzoic acid.
The anaerobic degradation of 2-methylnaphthalene (5) proceeds analogous to that of toluene with a fumarate addition as the initial step and subsequent β–oxidation to 2-naphthoic acid (7). According to the results of this work, naphthalene (4) is methylated to 2-methylnaphthalene (5) and further degraded through this pathway.

The naphthyl-2-methyl-succinate synthase assay was the first reaction in the anaerobic PAHs degradation pathways performed in an *in vitro* test. The upper anaerobic 2-methylnaphthalene degradation pathway was proposed to occur through the addition of fumarate to the methyl group, an activation of the resulting NMS to the corresponding CoA-ester and the following β-oxidation leading to the central metabolite 2-naphthoic acid.
The further fate of 2-naphthoic acid was elucidated based on metabolites found in culture supernatants. The metabolite pattern of reduced 2-naphthoic acid derivates was identical during growth on naphthalene or 2-methylnaphthalene. It was shown that 2-

naphthoic acid is reduced to 5,6,7,8-tetrahydro-2-naphthoic acid. No 1,2,3,4 –tetrahydro-2-naphthoic acid could be detected. Further degradation of tetrahydronaphthoic acid continues through octahydronaphthoic acid. Some authors found also decahydronaphthoic acid in supernatants of naphthalene-grown cultures, however it seems to be a dead-end metabolite (Zhang et al., 2000; Annweiler et al., 2002). Octahydronaphthoic acid is presumably hydroxylated and oxidised to β-oxo-decahydro-2-naphthoic acid that allows a thiolytic ring cleavage. A dicarboxylic acid with the total formula $C_{11}H_{16}O_4$ and 2-carboxycyclohexylacetic acid were identified as ring cleavage products (Annweiler et al., 2002). This study also showed that degradation of naphthalene and its derivatives proceeds through saturated intermediates and not through monoaromatic compounds.

1.3 Anaerobic microbial degradation of heterocyclic compounds

Substances that contain heteroatoms such as oxygen, nitrogen or sulphur in their ring system are called heterocycles. Only nitrogen-heterocycles such as quinoline or indole were found to be sufficiently biodegradable under anoxic conditions. *Desulfobacterium indolicum*, a strain that can utilise quinoline, indole and their methylated analogs as sole carbon source and electron donor, was isolated already in 1986 (Bak and Widdel, 1986). The initial step in anaerobic degradation of nitrogen-containing heterocycles is a hydroxylation of a carbon atom in the α-position to the nitrogen (Johansen et al., 1997a; Johansen et al., 1997b). Ring reduction and cleavage were proposed as following degradation steps. Sulphur-containing heterocycles such as dibenzothiophene can undergo a reductive desulphurisation (Kim et al., 1990; Marcelis et al., 2003), however, no degradation of resulting aromatic hydrocarbons was observed in this case. In other studies, sulphur and oxygen heterocycles were persistent under anoxic conditions (Dyreborg et al., 1997). Nonetheless, benzothiophene was shown to be cometabolically converted to 2-carboxybenzothiophene and 5-carboxybenzothiophene by a sulphate-reducing naphthalene degrading culture (Annweiler et al., 2001), even though it could not be utilised as primary substrate. This fact indicated that the anaerobic degradation of polycyclic and heterocyclic compounds under natural conditions can occur cometabolically and be a result of interactions of several bacterial strains with different metabolic capacities, which was also proposed by other authors (Wackett and Ellis, 1999).

1.4 Objectives of this work

As outlined above, the elucidation of degradation pathways of aromatic compounds is of major importance for understanding the microbial strategies to utilise them as growth substrate and to assess the potential for natural attenuation processes in the environment. Recently the enrichment culture N 47 was obtained from a contaminated aquifer (Meckenstock et al., 2000). It is the first culture originating from a fresh water habitat that can degrade polycyclic aromatic compounds, *i.e.* naphthalene and 2-methylnaphthalene under anoxic conditions. The initial reaction in the anaerobic degradation pathway of 2-methylnaphthalene was measured in dense cell suspension (Annweiler et al., 2000).
The primary goal of my thesis was the further elucidation of PAH degradation pathways with the sulphate-reducing, naphthalene and 2-methylnaphthalene degrading culture N47 as model organism.

Reactions in the upper degradation pathway should by measured in enzymatic tests in order

to obtain detailed information about the conversion of 2-methylnaphthalene to the central metabolite 2-naphthoic acid. Another important point was to elucidate the initial activation mechanism of naphthalene in anaerobic catabolism. Some authors suggested a carboxylation (Zhang and Young, 1997), however this hypothesis could never be verified by a direct proof. The profound understanding of the initial reaction in anaerobic degradation of an unsubstituted aromatic compound could not only reveal interesting biochemistry, but also open new possibilities for remediation of contaminated sites.

The second goal of this work was to study the influence and cometabolic transformation of diverse polycyclic and heterocyclic substances during the naphthalene or 2-methylnaphthalene degradation. In most cases, contaminant plumes contain several hundreds or even thousands of compounds. However, microbiologic research was focussed more or less exclusively on the degradation of single compounds by single bacterial strains so far. Possible inhibitory effects and cometabolic transformations occurring in the environment could not be investigated in this way, even if one can assume that they play an important role under natural conditions.

As there are only few cultures worldwide that can degrade polycyclic compounds anaerobically, new enrichment cultures should be obtained and described, especially under aspect of the biochemistry of the degradation process.

For this reason, soil material from contaminated sites should be incubated in bottles containing medium with different electron acceptors and an added substrate. In order to keep the substrate concentration low and to prevent eventual inhibitory effects by other compounds in applied soil material, the culture bottles should be supplied with adsorber resin XAD-7.

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2 Enzymatic reactions in anaerobic 2-methylnaphthalene degradation pathway

2.1 Introduction

Aromatic compounds were for a long time believed to be recalcitrant in the absence of molecular oxygen. The anaerobic degradation of aromatic hydrocarbons was demonstrated for the first time in 1985 with xylene (Kuhn et al., 1985). Since then, anaerobic degradation was shown for a large spectrum of PAHs (Coates et al., 1997; Rothermich et al., 2002), but only few microbial cultures using PAH as the sole carbon source and electron donor could be obtained. For methylated aromatic compounds, the addition of fumarate to the methyl group was described as activation mechanisms (Biegert et al., 1996; Müller et al., 1999; Annweiler et al., 2000),. Recently, several cultures that are able to grow with naphthalene and nitrate or sulphate as the electron acceptor, were reported (Zhang and Young, 1997; Galushko et al., 1999; Meckenstock et al., 2000; Rockne et al., 2000). A carboxylation of naphthalene to 2-naphthoic acid was postulated as the initial activation reaction based on the identification of 2-naphthoic acid as a major metabolite and the incorporation of isotope-labelled bicarbonate into the carboxyl group of 2-naphthoic acid (Zhang and Young, 1997; Meckenstock et al., 2000). During further metabolism, 2-naphthoic acid is subsequently reduced and undergoes a ring cleavage, as was shown on the basis of metabolites found in culture supernatants (Annweiler et al., 2000; Meckenstock et al., 2000; Zhang et al., 2000; Sullivan et al., 2001). A sulphate-reducing enrichment culture N 47 previously obtained from contaminated aquifer sediment (Meckenstock et al., 2000) is able to grow on naphthalene and 2-methylnaphthalene. In supernatants of cultures fed with this substrate, naphthyl-2-methyl-succinate (NMS) and naphthyl-2-methylene-succinate (NMeS) were found (Annweiler et al., 2000), suggesting an addition of fumarate to the methyl group as the initial activating reaction. This reaction could be identified with enzyme assays in dense cell suspension (Annweiler et al., 2000). The two metabolites, NMS and NMeS, also suggested that the upper anaerobic degradation pathway of 2-methylnaphthalene occurs through an oxidation of the original methyl-atom to a carboxyl-group to form the central metabolite, 2-naphthoic acid. This sequence of reactions would be similar to the anaerobic degradation of toluene (Leutwein and Heider, 1999). However, the initial fumarate addition to 2-methylnaphthalene was the only enzyme reaction in the anaerobic degradation of PAHs measured *in vitro* so far.

Here, two subsequent enzymatic reactions in the anaerobic metabolism of 2-methylnaphthalene could be measured in cell extract: the formation of naphthyl-2-methyl-succinyl-CoA from naphthyl-2-methyl-succinate and succinyl-CoA, and its subsequent oxidation to naphthyl-2-methylene-succinyl-CoA.

2.2 Experimental procedures

2.2.1 Cultivation and harvest of bacteria

The naphthalene and 2-methylnaphthalene degrading sulphate-reducing culture N 47 was enriched from sediments of a tar oil-contaminated aquifer and cultivated under anoxic conditions in half-filled 120 ml serum bottles as described earlier (Meckenstock et al., 2000). Naphthalene or 2-methylnaphthalene were added with a syringe as a 1.5% solution in heptamethylnonane (1 ml per bottle).

The preparation of crude cell extract was performed under strict anoxic conditions. The cells were centrifuged for 30 min at 13,680×*g*. In a glove box filled with N_2/H_2 (95/5), cell pellets were resuspended with enzyme test buffer (see below), transferred with a syringe into nitrogen-flushed vials closed with butyl rubber stoppers and broken with a french press at 137 MPa.

2.2.2 Synthesis of naphthyl-2-methylsuccinyl-CoA and naphthyl-2-methylenesuccinyl-CoA

Naphthyl-2-methyl-succinic acid (NMS) and naphthyl-2-methylene-succinic acid (NMeS) were synthesized as described previously (Annweiler et al., 2000). The CoA esters used in the enzymatic reactions were synthesized from internal anhydrides of NMS and NMeS. For the synthesis of internal NMS-anhydride, 50 mg of NMS was dissolved in 800 µl acetic acid at 80°C. 50 µl acetic anhydride was added and the temperature was increased to 120°C. After 1.5 hours all acetic anhydride reacted and the acetic acid was evaporated. The remaining internal NMS-anhydride formed a yellow-brownish drop and solidified by cooling to room temperature. The pellet was dissolved in 1 ml acetonitrile. The synthesis was followed by thin layer chromatography (POLYGRAM SIL G/UV254, Machery-Nagel, Düren, Germany) and with a mixture of heptane/diethylether/acetic acid (1/1/0.1, vol/vol) as the running solvent.
For the synthesis of NMS-CoA, 10 mg of coenzyme A (Sigma) was dissolved in 1 ml $KHCO_3$ (0.5 M). The NMS-anhydride solution in acetonitrile was then added in ten aliquots of 100 µl. After each addition, the reaction mixture was shaken and incubated for 30 seconds. After all the NMS-anhydride solution was added, the reaction mixture was put on ice and acidified with 1 M HCl to pH 1.5. The reaction of NMS-anhydride with coenzyme A was followed by reversed phase thin layer chromatography on Alugram RP-18W/UV254 (Machery-Nagel) plates with a mixture of water/acetonitrile/acetic acid (2.5/2.5/0.1, vol/vol) as the running solvent.
NMeS-anhydride was synthesized analogously. As NMeS was less soluble, 2 ml of acetic acid were used to dissolve 50 mg NMeS. The synthesis of the internal anhydride was identical to the procedure described for NMS. The NMeS internal anhydride precipitated as bright yellow crystals, which were dissolved in 5 ml acetonitrile and added to the coenzyme A solution in ten aliquots of 500 µl, as described above.
Because the internal anhydrides hydrolysed spontaneously at neutral pH, a 15-fold molar excess of NMS or NMeS internal anhydride was used to synthesize the CoA-ester of NMS or NMeS. To remove the remaining free acid from the acidic reaction mixture, it was extracted three times with 10 ml diethyl ether.
The stability of the synthesized CoA esters against hydrolysis was tested in 50 mM Tris/HCl buffer, pH 7.0, at room temperature. The CoA ester concentration was 40 µM. The buffer conditions and compound analysis were analogous to the succinyl-CoA:napthyl-2-methyl-succinate CoA-transferase test. The measured half-life times were 96.5 min for NMS-CoA and 5.05 min for NMeS-CoA.

2.2.3 Enzyme assays

The reactions of the succinyl-CoA:naphthyl-2-methyl-succinate CoA-transferase und of the naphthyl-2-methyl-succinyl-CoA
dehydrogenase were first measured simultaneously in one assay in crude cell extracts. As the activity of the succinyl-CoA:naphthyl-2-methyl-succinate CoA-transferase was measured as a backwards reaction and that of the naphthyl-2-methyl-succynil-CoA dehydrogenase as a forwards reaction, both enzymes could utilize the same substrate, NMS-CoA, producing two different

products, succinyl-CoA and NMeS-CoA, respectively. To avoid any competition, NMS-CoA was added in huge excess. In more detailed studies performed in order to determine the actual electron acceptor and the influence of $NaBH_4$ and NH_2OH, the enzymatic activities were measured separately. The enzyme assays were performed at 30°C in 50 mM Tris/HCl-buffer, pH 7.0, under strict anoxic conditions. 1 ml cell extract was injected in a 5 ml vial flushed with N_2 and closed with a butyl rubber stopper. The enzyme reaction buffer contained: Tris/HCl (50 mM), disodium succinate (2 mM), NAD^+ (0.5 mM), FAD (0,5 mM), PMS (0.5 mM), Titanium(III) citrate (100 µM) (Zehnder and Wuhrmann, 1976), NMS-CoA (200 µM). All stock solutions and the reaction buffer were vacuum degassed. The reaction was started by the addition of the NMS-CoA solution. To neutralize the pH shift caused by the strongly acidic NMS-CoA solution, the pH was measured with pH-indicator and adjusted with 1M NaOH. Samples of 100 µl were taken discontinuously with a Hamilton precision syringe through the stopper for 40 minutes. The reaction was stopped by addition of ethanol (80% final concentration).

After removal of precipitated salts by centrifugation (10 min, 15,000*g), the samples were analysed by HPLC with a C_{18} reversed-phase column (GROM-SIL 120 ODS-5 ST, 5 µm, column length 250 mm, GROM, Herrenberg, Germany) using 100 mM ammonium phosphate buffer and acetonitrile as eluents. The analysis of succinyl-CoA:naphthyl-2-methyl-succinate CoA-transferase assay occurred under the following HPLC conditions: 13 min gradient from 5% to 45% acetonitrile, 5 min isocratic at 45% acetonitrile, 1 min gradient to 5% acetonitrile, 6 min isocratic at 5% acetonitrile. The compounds were detected at 210 nm with a UV/vis spectrometer. The observed elution times were: succinyl-CoA - 6.3 min; NMS-CoA - 13.3 min; NMS - 16.4 min.

The analysis of samples of the naphthyl-2-methyl-succinyl-CoA dehydrogenase assay was performed by isocratic elution at 40% acetonitrile. The elution times were: NMS - 7.9 min; NMeS - 8.3 min.

The protein concentrations were determined with the bio-rad RC DC Protein Assay.

2.3 Results

2.3.1 Succinyl-CoA:naphthyl-2-methyl-succinate CoA-transferase activity

The first enzymatic reaction in the anaerobic degradation of 2-methylnaphthalene is an addition of fumarate to the methyl group, thereby generating naphthyl-2-methyl-succinic acid. Through the analogy with anaerobic toluene degradation (Leutwein and Heider, 1999), it was proposed that NMS becomes thereafter activated to the corresponding CoA ester for further β-oxidation to 2-naphthoic acid.

The activity of the succinyl-CoA:naphthyl-2-methyl-succinate CoA-transferase was measured as the backwards reaction, *i.e.* the transfer of the CoA-moiety from naphthyl-2-methyl-succinyl-CoA to succinate. This was done to measure both enzymatic reactions described here simultaneously as the cell material was limited due to the extremely slow growth of the culture. The substrate NMS-CoA was added in excess to avoid a possible competition of the two measured enzymatic activities for their common substrate. As the ΔG of CoA-transfer reactions is usually close to 0, the reactions are fully reversible. The analogous enzyme in the anaerobic toluene degradation pathway was also measured as the backwards reaction (Leutwein and Heider, 2001). An increase of the succinyl-CoA concentration was observed in the first 15-20 minutes of the experiment (Figure 2.1a).

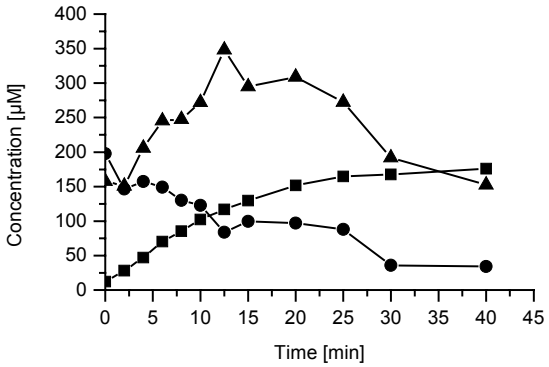

Figure 2.1a. Succinyl-CoA:naphthyl-2-methyl-succinate CoA-transferase assay with cell free extract of the sulphate-reducing enrichment culture N 47. The CoA-moiety is transferred from naphthyl-2-methyl-succinyl-CoA (●) to succinate generating succinyl-CoA (▲). The other reaction product is naphthyl-2-methyl-succinate (■). This figure shows a single experiment out of two independent repetitions

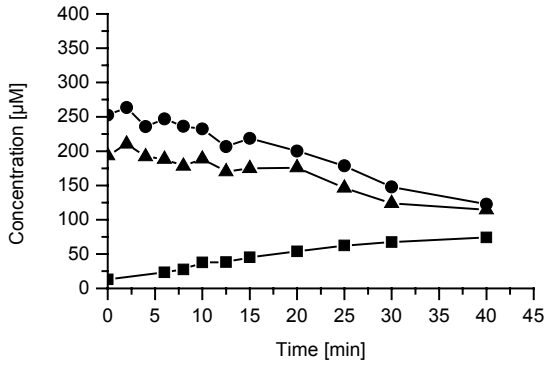

Figure 2.1b. Cell-extract free control of the succinyl-CoA:naphthyl-2-methyl-succinate CoA-transferase assay. Only spontaneous hydrolysis of naphthyl-2-methyl-succinyl-CoA (●) and succinyl-CoA (▲) can be observed. The concentration of free NMS (■) increases due to the hydrolysis of NMS-CoA.

Then, the concentration decreased because of the spontaneous hydrolysis of both, succinyl-CoA and NMS-CoA. Concomitantly, the concentration of naphthyl-2-methyl-succinic acid increased continuously. No formation of succinyl-CoA was observed in the absence of cell extract (Figure 2.1 b). Also, no transfer of the CoA-moiety from NMS-CoA to acetate or the formation of NMS-CoA in presence of NMS, CoA, and ATP could be detected. The compounds were identified with HPLC by coelution with the reference compounds. The specific activity of succinyl-CoA:naphthyl-2-methyl-succinate CoA-transferase in two independent experiments was 51.3±4.6 nmol× (min×mg protein)$^{-1}$ in extracts of 2-methylnaphthalene grown cells. The measured activities represent 74.6-fold the *in vivo* activity, which was calculated from growth experiments. This reaction could also be measured in the presence of oxygen without any significant loss of activity. In the oxic experiments, the used solutions were not degassed and no titanium(III) citrate was added.

Three different groups of CoA-transferases can be distinguished (Heider, 2001). Several inhibitors were tested to determine to which group the succinyl-CoA:naphthyl-2-methyl-succinate CoA-transferase belongs to. The tests were performed according to measurements of the cinamoyl-CoA:(R)-phenyllactate CoA-transferase (Dickert et al., 2000). The cell extract was incubated with 200 µM NMS-CoA and 10 mM $NaBH_4$ for 10 minutes. Then, the reaction was started by adding succinate. No inhibition of succinyl-CoA:naphthyl-2-methyl-succinate CoA-transferase could be observed in this case. Incubation of the cell extract with 200 µM NMS-CoA for 10 minutes followed by 10 mM NH_2OH for 20 minutes resulted in a residual activity of 65% compared to a control assay without inhibitor.

No activity could be observed after incubation with 200 mM NH_2OH.

2.3.2 Naphthyl-2-methyl-succinyl-CoA dehydrogenase activity

The first oxidative reaction in the anaerobic degradation pathway of 2-methylnaphthalene was proposed to be the oxidation of naphthyl-2-methyl-succinyl-CoA to naphthyl-2-methylene-succinyl-CoA. The enzymatic activity was detected in the same assays as the one of the succinyl-CoA:naphthyl-2-methyl-succinate CoA-transferase. Because of the rapid autohydrolysis of the reaction product, naphthyl-2-methylene-succinyl-CoA to NMeS and CoA (half-life time $t_{1/2} \approx 5$ min), the formation of its hydrolysis product, naphthyl-2-methylene-succinic acid was measured (Figure 2.2). A continuous formation of naphthyl-2-methylene-succinic acid was observed only in the presence of cell extract. The specific activity of the naphthyl-2-methyl-succinyl-CoA dehydrogenase in two independent experiments was 0.103 ± 0.015 nmol× (min×mg protein)$^{-1}$. These activities represented 11.5% of the calculated *in vivo* activity. Similar to the succinyl-CoA:naphthyl-2-methyl-succinate CoA-transferase, this enzyme was also insensitive to oxygen.

After the first tests, the electron acceptor of the naphthyl-2-methyl-succinyl-CoA dehydrogenase reaction was determined. No activity was observed with NAD^+ or FAD. In the presence of PMS only, the activity was as high as in presence of all three electron acceptors (data not shown).

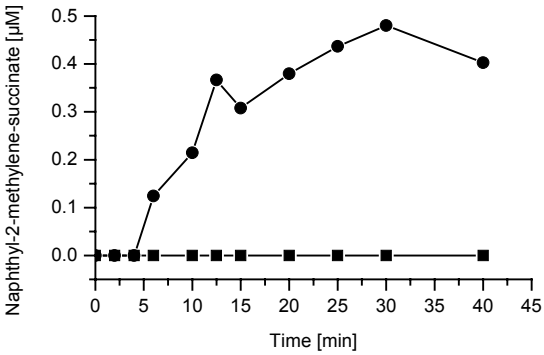

Figure 2.2. Naphthyl-2-methyl-succinyl-CoA dehydrogenase assay with cell free extract of the sulphate-reducing enrichment culture N 47. Naphthyl-2-methylene-succinate is generated in samples with cell extract (●), no naphthyl-2-methylene-succinate production could be observed without cell extract (■). The initial concentration of the substrate (NMS-CoA) was 200 µM. This figure shows a single experiment out of two independent repetitions.

2.4 Discussion

In the present study two enzymatic reactions in the anaerobic 2-methylnaphthalene degradation pathway of the sulphate-reducing enrichment culture N 47 were elucidated. It was shown that naphthyl-2-methyl-succinate, which is generated by the addition of fumarate to 2-methylnaphthalene, becomes activated to the corresponding CoA-ester with succinyl-CoA as the CoA donor. This is followed by an oxidative step at the original methyl atom leading to the formation of naphthyl-2-methylene-succinyl-CoA. By these two reactions, the *β*-oxidation at the original methyl-atom is initiated and leads to the formation of the central metabolite 2-naphthoyl-CoA, which enters then the reductive ring cleavage pathway (Annweiler et al., 2002) . The findings obtained in this study confirm the upper degradation pathway of 2-methylnaphthalene that was proposed earlier (Annweiler et al., 2000). Concerning the fact that the fumarate addition to a methyl group of an aromatic compound with a subsequent *β*-oxidation at the original methyl atom was detected for other substances such as toluene, xylene and cresol (Beller and Spormann, 1997; Müller et al., 1999), it can be postulated that this is a common reaction pattern in the anaerobic degradation of methylated aromatic hydrocarbons.

The sulphate-reducing culture N47 was transferred for at least 20 times over four years

from the original inoculum, before the experiments were performed. Only one type of cells could be observed with phase contrast microscopy indicating a homogenous, although not pure culture. All attempts to obtain a pure culture failed so far, as the cells did not grow in dilutions higher than 10^{-4}.

The investigation of catabolic pathways in PAHs-degrading anaerobic organisms is generally limited by the extremely low cell yield. The sulphate-reducing culture N 47 needs for example 3 months to reach an OD of 0.15 and for unknown reasons, the bacteria refuse to grow in bottles larger than 150 ml. Therefore the total amount of protein that was used in this study for one enzyme assay was in the range of 1 mg. Surprisingly, the specific activities measured in the performed enzyme assays are close to those measured in studies of anaerobic toluene degradation by denitrifying bacteria (Leutwein and Heider, 2001).

The enzyme assays performed with the sulphate-reducing culture N47 are so far the only measured reactions of anaerobic degradation pathways of PAHs. No similar experiments were carried out with other anaerobic, 2-methylnaphthalene degrading cultures. Also, organisms or enzymes known for anaerobic toluene degradation pathways were not reported to convert two-ring aromatic hydrocarbons such as 2-methylnaphthalene or naphthalene.

The substrates used in our enzyme assays were probably mixtures of different isomers, which might have influenced the measured enzyme activities. The synthesized NMS-CoA was probably a mixture of the two CoA-esters 2-carboxymethyl-3-naphthyl-propionyl-CoA and 3-carboxy-4-naphthyl-butyryl-CoA. Only the first of these CoA-esters is proposed to be the biologically active compound (Annweiler et al., 2000). By synthesis of the analogous monoaromatic compound, benzylsuccinyl-CoA, other authors obtained a ratio of 40% 3-carboxy-4-phenylbutyryl-CoA and 60% of 2-carboxymethyl-3-phenylpropionyl-CoA (Leutwein and Heider, 1999). In a later publication, it was shown that purified succinyl-CoA:(R)-benzylsuccinate CoA-transferase produces only 2-carboxymethyl-3-phenylpropionyl-CoA from R-benzylsuccinate and succinyl-CoA (Leutwein and Heider, 2001). The naphthyl-2-methyl-succinate used in our experiments was probably also a mixture of two enantiomeres. In the anaerobic toluene degradation pathway, succinyl-CoA:(R)-benzylsuccinate CoA-transferase is inhibited by (S)-benzylsuccinyl-CoA and its enzymatic activity is lowered by 30% in the presence of racemic benzylsuccinyl-CoA (Leutwein and Heider, 2001). This could indicate that in the 2-methylnaphthalene degradation pathway the maximal activity of succinyl-CoA:naphthyl-2-methyl-succinate CoA-transferase is even higher than measured in the performed assays due to the inhibition through racemic NMS-CoA. The finding that the succinyl-CoA:naphthyl-2-methyl-succinate CoA-transferase was not or only partially inhibited by $NaBH_4$ and NH_2OH, indicates that the enzyme belongs to the same enzyme family (III) of CoA transferases as the analogous enzyme in the anaerobic toluene degradation pathway, *i.e.* succinyl-CoA: (R)-benzylsuccinate CoA-transferase.

The activity of the naphthyl-2-methyl-succinyl-CoA dehydrogenase was only detected in the presence of the artificial electron acceptor PMS and not with possible natural electron acceptors such as NAD^+ or FAD. The lack of the natural electron acceptor in the enzymatic assay could have negatively influenced the measured activity of this enzyme. Also the activity of the corresponding enzyme in anaerobic toluene degradation, benzylsuccinyl-CoA dehydrogenase, was measured with artificial electron acceptors such as ferricenium hexaflourophosphate, dichlorophenol indophenol or PMS. Also in

this case, no activity was observed using NAD^+ or FAD (Leutwein and Heider, 2002).
NMS-CoA and NMeS-CoA hydrolyse spontaneously in neutral, aqueous solutions. Their half-life times (96.5 min and 5.05 min respectively) are comparable with those of the analogous CoA-thioesters from toluene degradation pathway: approximately one hour for benzylsuccinyl-CoA and several minutes for phenylitaconyl-CoA (Johann Heider, personal communication). There are no evidences, if NMeS resulting from the spontaneous NMeS-CoA hydrolysis *in vivo* can be activated with CoA and re-introduced into the degradation pathway. However, other authors observed that the oxidation of benzylsuccinate in cell extracts does not take place in tests with lower protein concentrations, which was interpreted that the short-living metabolites are passed quickly from one enzyme to another (Biegert et al., 1996). The enzymatic oxidation of benzylsuccinyl-CoA to benzoyl-CoA was also measured in one single assay without development of detectable intermediates suggesting a fast sequence of reactions (Leutwein and Heider, 2001).
The reactions presented here show that the anaerobic degradation of 2-methylnaphthalene proceeds via β-oxidation on the original methyl-atom. This is analogous to the upper degradation pathway of toluene (Leutwein and Heider, 1999) and suggests that the fumarate molecule, which is added to the methyl group of 2-methylnaphthalene can be recycled in a later enzymatic reaction by cleavage of a succinyl-CoA moiety generating 2-naphthoyl-CoA. Other authors demonstrated also that 2-methylnaphthalene is oxidized to 2-naphthoic acid in the upper degradation pathway (Sullivan et al., 2001). However, they could not find the metabolites NMS and NMeS, which can be explained by the derivatisation method for GC-MS samples. NMS and NMeS could be detected only as methyl esters, but not as trimethylsilyl derivates, which was the derivatisation method used by other authors (Sullivan et al., 2001). The results of the present study prove also that the metabolites NMS and NMeS, which were previously found in culture supernatants, are intermediates of the degradation pathway and not dead end products.

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3 The initial reaction in anaerobic naphthalene degradation

3.1 Introduction

Aromatic hydrocarbons are important constituents of petroleum and its products and cause important environmental problems due to their adverse health effects, chemical stability and their ubiquitous use and occurrence. Especially if larger amounts of aromatic compounds enter the subsurface, increased microbial respiration leads to a rapid depletion of oxygen in the groundwater. This in turn massively slows down the degradation kinetics of the contaminants. As a consequence, extended anoxic plumes are formed wherein aromatic compounds can be transported over long distances endangering drinking water supplies. As anaerobic degradation of aromatic hydrocarbons is comparably slow, they were believed to be recalcitrant in absence of molecular oxygen. The first reports about anaerobic degradation of xylene were published in 1985 (Kuhn et al., 1985). Since then, many cultures able to degrade a wide spectrum of monoaromatic compounds, naphthalene, 2-methylnaphthalene and phenanthrene were isolated (Spormann and Widdel, 2000; Widdel and Rabus, 2001). The anaerobic degradation of several higher condensed, polycyclic aromatic hydrocarbons was demonstrated in microcosm experiments with various terminal electron acceptors (Meckenstock et al., 2004). A lot of evidence was collected indicating that the anaerobic degradation of aromatic compounds contributes significantly to the natural attenuation in oil-contaminated aquifers (Elshahed et al., 2001; Gieg and Suflita, 2002; Griebler et al., 2004). Recently, it was discovered that anaerobic degradation of aromatic hydrocarbons is one of the major processes contributing to the aging of oil reservoirs with important economic consequences (Aitken et al., 2004).

The biochemistry of the anaerobic catabolism of aromatic compounds has been well studied with toluene as the model substance (Leuthner et al., 1998; Leutwein and Heider, 1999; Boll et al., 2002; Leutwein and Heider, 2002). Also the anaerobic degradation pathways of xylene (Beller and Spormann, 1997; Krieger et al., 1999; Morasch et al., 2004), ethylbenzene (Ball et al., 1996; Kniemeyer and Heider, 2001; Kniemeyer et al., 2003) and the common central metabolite, benzoic acid (Boll et al., 2001), have been elucidated. In addition, the anaerobic catabolism of polycyclic aromatic compounds such as 2-methylnaphthalene and 2-naphthoic acid has been studied in detail (Annweiler et al., 2000; Meckenstock et al., 2000; Annweiler et al., 2002; Safinowski and Meckenstock, 2004). However, the first step in the anaerobic degradation of unsubstituted compounds still remains unclear, even though a few pure cultures degrading naphthalene or benzene have been isolated (Galushko et al., 1999; Rockne et al., 2000; Coates et al., 2001). For anaerobic naphthalene degradation, some authors demonstrated that stable isotope labelled bicarbonate from the buffer was incorporated in the carboxylic group of detected 2-naphthoic acid and concluded that a carboxylation is the initial activation reaction (Zhang and Young, 1997).

In the following, results are presented that lead to the conclusion that the first step in the anaerobic degradation of naphthalene by sulphate-reducing bacteria is a methylation to 2-methylnaphthalene. The methyl group might possibly be generated from bicarbonate *via* a reverse CO-dehydrogenase pathway. The resulting 2-methylnaphthalene is then oxidised to the central metabolite 2-naphthoic acid.

3.2 Experimental procedures

Cultivation of the sulphate-reducing culture N47 was performed as previously described (Meckenstock et al., 2000). The substrates, naphthalene or 2-methylnaphthalene, were added as a 1.5% solution in 2,2,4,4,6,8,8-heptamethylnonane with a syringe through the stopper. Substrate utilisation was followed by increasing sulphide concentrations measured according to (Cline, 1969). The enzyme assays for naphthyl-2-methyl-succinate synthase, succinyl-CoA:naphthyl-2-methyl-succinate CoA-transferase and naphthyl-2-methyl-succinyl-CoA dehydrogenase were performed as described previously (Annweiler et al., 2000; Safinowski and Meckenstock, 2004). CO-dehydrogenase was measured under anoxic conditions in 50 mM potassium phosphate buffer (pH 7.5) containing 100 mM methyl viologen in a stoppered quartz cuvette. The reaction progress was followed by the change of optical density at 578 nm. The reaction was started by addition of 500 µl CO with a syringe through the cuvette stopper.
For the detection of metabolites, the culture supernatants were acidified with HCl to pH 1 and extracted three times with diethylether. The organic phase was collected, dried over water-free sodium sulphate and evaporated under a continuous nitrogen stream. The residue was redissolved in 1.8 ml methanol. 200 µl trimethylchlorsilane was added and the samples were derivatized for 1 hour at 75°C in order to transform the carboxylic acids into methyl esters. After cooling down, the samples were dried under a continuous nitrogen stream and dissolved with 1 ml ethyl acetate. The detection of the metabolites was performed with GC-MS (GC, HP 6890 with a DB-5MS column, 0.25 µm film thickness 0.2 mm i.d., 30 m length, J&W Scientific; MS, HP 5973). Sample injection was splitless (1µl) and the flow rate of the carrier gas helium was 0.9 ml/min. The oven temperature was 43°C for 5 minutes, then ramped at a rate of 4°C/min to 280°C and was held for 5 minutes. The MS was operated at 315 °C in the scan mode.

3.3 Results

3.3.1 Growth of the sulphate-reducing culture N47 on deuterated naphthalene

In order to study metabolites of anaerobic naphthalene degradation that could provide indications about the degradation pathway, the cells were cultivated on fully deuterated naphthalene as the sole carbon source and electron donor. During growth, deuterated metabolites of the anaerobic 2-methylnaphthalene degradation pathway, naphthyl-2-methyl-succinic acid (NMS) and naphthyl-2-methylene-succinic acid (NMeS) could be detected in culture supernatants (figure 3.1). These compounds were seven mass units heavier than the same metabolites appearing during the anaerobic degradation of non-labelled 2-methylnaphthalene. This difference in molecular mass results from seven remaining deuterium atoms at the aromatic ring system of naphthalene-d_8. The two metabolites, NMS and NMeS, are very specific indicators of anaerobic degradation of 2-methylnaphthalene and do not occur in any other known biochemical pathway investigated so far nor are they produced in any industrial process. The concentration of these metabolites in the culture supernatants steadily increased during growth, demonstrating their continuous generation in the cells (figure 3.2).

3.3.2 Enzyme activities of the proposed naphthalene degradation pathway

Several enzymatic reactions of the anaerobic 2-methylnaphthalene degradation pathway were detected also in naphthalene grown cells. The enzymes naphthyl-2-methyl-succinate synthase, succinyl-CoA:naphthyl-2-methyl-

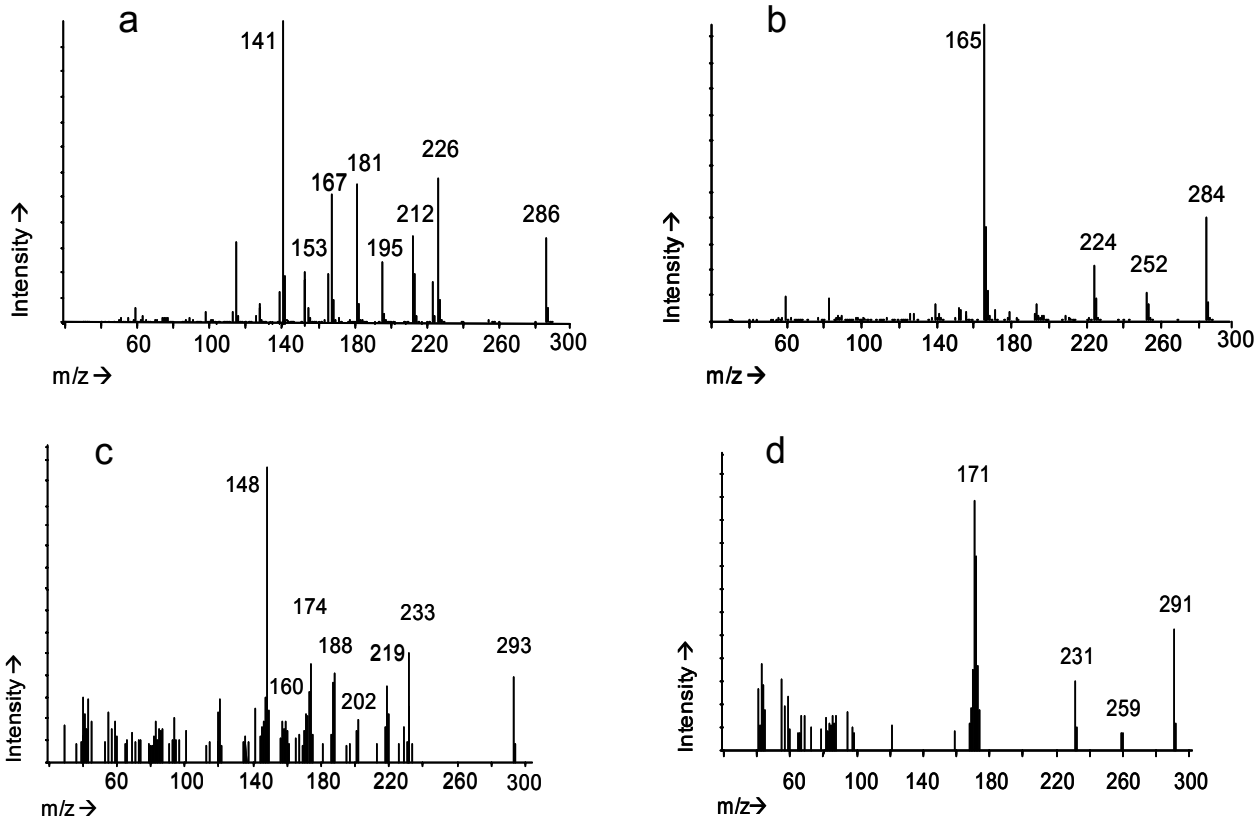

Figure 3.1. Mass spectra of naphthyl-2-methyl-succinic acid methyl ester (a) and naphthyl-2-methylene-succinic acid methyl ester (b), metabolites occurring during anaerobic degradation of 2-methylnaphthalene. Deuterated naphthyl-2-methyl-succinic acid (c) and naphthyl-2-methylene-succinic (d) acid were found in the supernatants of cultures growing on deuterated naphthalene.

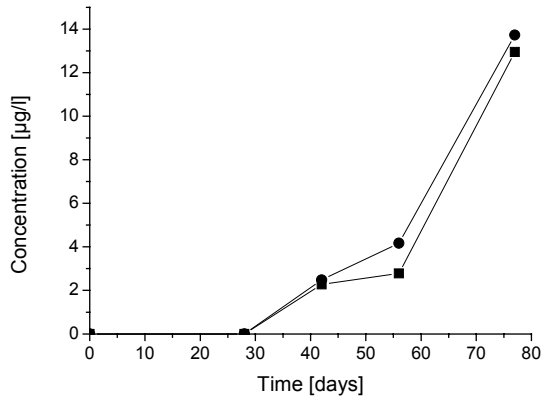

Figure 3.2. Concentrations of ring-deuterated naphthyl-2-methyl-succinic acid (■) and naphthyl-2-methylene-succinic acid (●) in culture supernatants during growth of the culture N47 on naphthalene-d_8.

succinate CoA-transferase, and naphthyl-2-methyl-succinyl-CoA dehydrogenase, which are the only enzymes catalysing reactions in the anaerobic degradation of polycyclic aromatic compounds that could be measured *in vitro* so far, were also present in cells grown on naphthalene with similar activities as in 2-methylnaphthalene-grown cells (table 3.1). The cells taken for these experiments were cultivated on naphthalene as the only carbon source since several transfers in order to avoid any possible enzyme induction by residual 2-methylnaphthalene.

In order to demonstrate that a methyl group can be generated from bicarbonate, the activity of CO-dehydrogenase was measured photometricaly at 578 nm using methylviologen as electron acceptor. The calculated specific activity was 0.0974 ± 0.007 µmol×(min× mg protein)$^{-1}$ as a mean of two independent experiments.

Table 3.1. Specific enzyme activities in naphthalene and 2-methylnaphthalene-grown cells of the culture N47, respectively. The activities are given in nmol×min^{-1}× mg protein^{-1} as the means of two independent replicates

.

Enzyme name	Activity in naphthalene-grown cells	Activity in 2-methylnaphthalene-grown cells
Naphthyl-2-methyl-succinate synthase	0.03 ± 0.001	0.020 ± 0.003
Succinyl-CoA:naphthyl-2-methyl-succinate CoA-transferase	71.8 ± 44.2	51.3 ± 4.6
Naphthyl-2-methyl-succinyl-CoA dehydrogenase	0.13 ± 0.05	0.103 ± 0.015

3.3.3 Induction of catabolic pathways

The sulphate-reducing culture N47 can utilise naphthalene or 2-methylnaphthalene as the sole carbon source and electron donor. The growth behaviour of the culture, monitored as sulphide production, was studied when transferred from one substrate to another (figure 3.3). Naphthalene and 2-methylnaphthalene were utilized with similar growth kinetics, when the parental culture grew on the same substrate. When the culture was transferred from naphthalene to 2-methylnaphthalene, the growth continued immediately, even if it was slightly slower compared with the cultures that were transferred to naphthalene. Contrary to this, after a transfer of cells from 2-methylnaphthalene to naphthalene, the lag phase lasted for almost 100 days. After this, the culture started to grow with the same growth kinetics as in other cases.

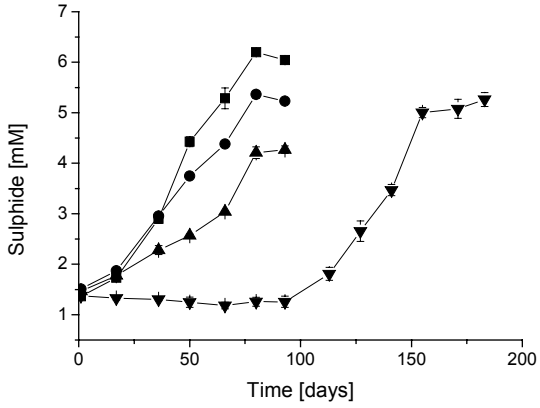

Figure 3.3. Substrate utilisation of the culture N47 monitored as sulphide formation when transferred from naphthalene to naphthalene (■), from naphthalene to 2-methylnaphthalene (▲), from 2-methylnaphthalene to 2-methylnaphthalene (●) and from 2-methylnaphthalene to naphthalene (▼).

3.4 Discussion

The presented results show that the initial reaction of anaerobic naphthalene degradation is a methylation to 2-methylnaphthalene. Ring-deuterated metabolites that are highly specific to the anaerobic 2-methylnaphthalene degradation pathway, *i.e.* naphthyl-2-methyl-succinic acid and naphthyl-2-methylene-succinic acid, were detected in supernatants of cultures growing on deuterated naphthalene. The only plausible explanation of their occurrence is an initial conversion of naphthalene-d_8 to 2-methylnaphthalene-d_7, as these compounds occur exclusively during the anaerobic catabolism of 2-methylnaphthalene and are not produced in any other known metabolic pathway. NMS and NMeS also appeared during growth on non-labelled

naphthalene. However, commercially available naphthalene always contents traces of 2-methylnaphthalene that might possibly by the reason for their appearance. This is not the case in our experiments with the deuterated naphthalene where no traces of ring-deuterated 2-methylnaphthalene could be detected with GC-MS. In the following sequence of reactions, the resulting 2-methylnaphthalene is oxidized to the central metabolite 2-naphthoic acid which was also detected in our experiments in the deuterium labelled form. Earlier publications showed that the carboxyl group of the 2-naphthoic acid formed during anaerobic naphthalene degradation derived from the carbonate buffer (Zhang and Young, 1997; Meckenstock et al., 2000). Therefore, some authors postulated a carboxylation of naphthalene to 2-naphthoic acid as the initial reaction in the anaerobic degradation pathway based on this finding (Zhang and Young, 1997). However, this observation can be perfectly explained by our finding of a methylation as the initial reaction, as the methyl group could be generated from bicarbonate *via* a reversed CO-dehydrogenase pathway. CO-dehydrogenase was detected in enzyme tests with cell-free extracts of culture N47.

The detection of the enzymes of the anaerobic 2-methylnaphthalene degradation pathway in naphthalene-grown cells with similar activities as in 2-methylnaphthalene-grown cells is a clear evidence that during growth on naphthalene, the enzymes for the catabolism of 2-methylnaphthalene are induced and active. Considering the low energy yield of sulphate-reducing bacteria degrading a chemically stable compound such as naphthalene (- 44.3 kJ/mol SO_4^{2-}) (Spormann and Widdel, 2000), it would be very unlikely that the organisms spend a lot of energy to synthesize several enzymes that are not required for the actual catabolism.

Also the lack of a longer lag-phase when the cells were transferred from naphthalene to 2-methylnaphthalene supports the assumption that all the enzymes for 2-methylnaphthalene degradation are present and active in naphthalene growing cells. In the case of the transfer from 2-methylnaphthalene to naphthalene, the lag-phase lasted for nearly one hundred days. This indicates clearly that the specific enzymes for naphthalene degradation must be expressed after the transfer from 2-methylnaphthalene to naphthalene as substrate. This observation is also a strong indication that naphthalene is first methylated and then catabolised *via* the 2-methylnaphthalene degradation pathway in sulphate-reducing bacteria.

As the anaerobic degradation pathways of 2-methylnapthalene and toluene are analogous to each other, some interesting parallels might arise by the comparison of anaerobic degradation of naphthalene and benzene. Benzene is the simplest, but also the most stable and most water soluble unsubstituted aromatic hydrocarbon. Although benzoate was reported as an intermediate of anaerobic biodegradation of benzene (Caldwell and Suflita, 2000; Phelps et al., 2001), a direct carboxylation was considered as rather improbable also in this case (Phelps et al., 2001; Coates et al., 2002).

Other authors proposed a hydroxylation to phenol as the initial step in anaerobic benzene degradation (Grbic-Galic and Vogel, 1987; Caldwell and Suflita, 2000). Even though a direct hydroxylation of ethylbenzene to 1-phenylethanol has been shown for the denitrifying *Azoarcus* strain EB1 (Ball et al., 1996), a similar reaction seems to be unlikely for benzene due to of the different chemical properties of the alkyl side chain and the aromatic ring. The difference in bond enthalpy between a benzylic C-H bond and an aromatic C-H bond is more than 100 kJ/mol, which was already mentioned by other authors (Widdel

and Rabus, 2001). As the aromatic ring exhibits a high electron density, an electrophilic substitution would be a favourable reaction type. In organic chemistry, the Friedel-Crafts alkylation of aromatic compounds is a common and exergonic reaction, in which an alkyl cation replaces a proton at the aromatic ring. Also from other biological systems, methylation of aromatic hydrocarbons was reported. Biologically mediated, S-adenosyl-methionine dependent methylation of monoaromatic compounds was observed in bone marrow cells (Flesher and Myers, 1991). Also higher condensed substances such as benz[a]pyrene, benz[a]anthracene and anthracene were methylated in rat liver and lung cells (Myers and Flesher, 1991).

The data presented here show that methylation is the initial activation reaction in anaerobic catabolism of naphthalene. Combined with several indications for the methylation of benzene to toluene as the initial reaction in anaerobic catabolism (Coates et al., 2002), it can be proposed that methylation is a novel, general initial reaction in anaerobic degradation of unsubstituted aromatic compounds.

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4 Cometabolic transformation of polycyclic and heterocyclic aromatic hydrocarbons

4.1 Introduction

Polycyclic aromatic hydrocarbons (PAHs) including heterocyclic compounds are wide spread pollutants in the environment. In most cases, they originate from natural and anthropogenic pyrolysis of organic material such as forest fires, coal-refining processes and oil industry. Resulting mixtures of products often consist of an enormous number of individual substances; coal tars for example are suggested to contain more than 10,000 single compounds (Franck, 1963).

Microbial degradation of PAHs under oxic conditions has been investigated since a long time (Cerniglia, 1992) and lots of strains capable to grow an aromatic compounds with up to four rings have been isolated (Müller et al., 1990; Wiesenfels et al., 1990; Boldrin et al., 1993). To date, no pure bacterial culture was shown to utilise any PAH compound containing more than four rings as the sole carbon source and electron donor. All reported biotransformations of five-ring compounds such as benz[a]pyrene or dibenz[a,h]anthracene are based on cometabolism (Ye et al., 1996; Juhasz et al., 1997).

Recently, also several anaerobic PAH-degrading cultures have been enriched and purified with nitrate or sulphate as electron acceptor (Zhang and Young, 1997; Galushko et al., 1999; Meckenstock et al., 2000; Rockne et al., 2000). Detailed information on anaerobic degradation of PAHs is scarce. Pathways have been described partially for the anaerobic degradation of naphthalene and 2-methylnaphthalene (2-MN) under sulphate-reducing conditions (Annweiler et al., 2000; Annweiler et al., 2002; Safinowski and Meckenstock, 2004). Firstly, naphthalene was reported to be carboxylated to 2-naphthoic acid (2-NA) (Zhang and Young, 1997), while 2-MN was shown to be activated *via* an addition of fumarate to the methyl group and the formation of naphthyl-2-methylsuccinic acid (Annweiler et al., 2000) followed by a β-oxidation to 2-naphthoic acid (Safinowski and Meckenstock, 2004). Later experiments with the sulphate-reducing enrichment culture N47 indicated that in the anaerobic degradation pathway of naphthalene, 2-naphthoic acid (2-NA) is not a direct product of a carboxylation, but naphthalene may first be methylated to 2-MN, which subsequently is degraded to central metabolite 2-NA (previous chapter). 2-NA then undergoes a sequential reduction and finally a cleavage of the ring system (Annweiler et al., 2002).

Little is known also regarding anaerobic degradation of heterocyclic aromatic hydrocarbons (HAH). It seems that nitrogen-containing heterocycles such as indole and quinoline can be relatively easy metabolised by in the absence of molecular oxygen. The first metabolic step in anaerobic degradation of indole, quinoline and their methylated analogs by *Desulfobacterium indolicum* is the hydroxylation of a carbon atom next to the nitrogen atom (Johansen et al., 1997; Fetzner, 1998). In contrary, some studies showed oxygen and sulphur heterocycles to be persistent to anaerobic degradation (Dyreborg et al., 1997). Other studies showed sulphur heterocycles to be desulphurised under anoxic conditions by *Desulfovibrio desulfuricans* M6 (Kim et al., 1990; Marcelis et al., 2003). However, such partial transformation of HAHs do not lead to a complete mineralisation of the resulting aromatic hydrocarbon. In a previous study, a cometabolic conversion of

benzothiophene to 2-carboxybenzothiophene and 5-carboxybenzothiophene and a further reduction to dihydrocarboxybenzothiophene by the sulphate-reducing enrichment culture N47 was shown (Annweiler et al., 2001). This study indicated that benzothiophene is converted by the same enzyme system as naphthalene.

As mentioned above, PAHs are present in the environment as complex mixtures of many substances. It is thus important not to study the degradation of single compounds only, but also the degradation of substrate mixtures in order to understand the processes of natural attenuation under environmental conditions. Most common phenomena in this context are the inhibition of the utilisation of one substrate in presence of a structurally similar compound or the cometabolic conversion of substances that cannot serve as a sole carbon and energy sources, when offered simultaneously with a substrate that supports growth (Evans et al., 1991; Bouchez et al., 1995; Phelps and Young, 1999; Chang et al., 2002; Meckenstock et al., 2004).

In this study, the cometabolic transformation of different PAHs and HAHs by the naphthalene- and 2-methylnaphthalene-degrading, sulphate-reducing enrichment culture N-47 is reported. Furthermore, a field survey was conducted at the tar oil-contaminated site from where N47 was originally enriched to investigate whether divers PAHs and HAHs are converted in field in similar way as observed in the batch experiments.

4.2 Experimental procedures

4.2.1 Cultivation of bacteria

The naphthalene and 2-methylnaphthalene-degrading sulphate-reducing enrichment culture N47 was enriched from a tar oil-contaminated aquifer and cultivated under strictly anoxic conditions as described earlier (Meckenstock et al., 2000). New cultures were routinely inoculated at a ratio of 1:10 into 120-ml serum bottles, half-filled with bicarbonate-buffered, sulphide-reduced medium with 10 mM Na_2SO_4 and a pH of about 7.4. Bottles were flushed with N_2/CO_2 (80/20) and closed with butyl rubber stoppers. Naphthalene or 2-methylnaphthalene were added with a sterile syringe as 1 ml of a 1.5% solution in heptamethylnonane. The concentration of the cosubstrates in heptamethylnonane was 0.1% in the case of benzofuran, benzothiophene, indane, indene, indole, 1-methylnaphthalene, quinoline and biphenyl, and 0.5% in the case of acenaphthene, acenaphthylene, fluorene, phenanthrene and anthracene. Bacterial activity was monitored via analysis of accumulating sulphide (Cline, 1969).

4.2.2 Field investigation site and sampling

The field survey took place at a former gasworks site located in southwest-Germany (Testfeld-Sued). It is characterized by multiple contaminant sources, *i.e.* tar pits in the southern part as well as further BTEX and PAH sources in the central and western part, close to and within the investigated area (figure 4.1). Non-aqueous phase liquids (NAPLs) are locally present in both the saturated and the unsaturated zones (Zamfirescu and Grathwohl, 2001). The resulting contaminant plume has an estimated width of 120 m and low molecular weight PAHs were still detected at a distance of 280 m downstream of the NAPL zone (Bockelmann et al., 2001). The overall length of the plume is unknown so far, as no monitoring wells are available further downstream.

The actual investigation took place in November 2002 and focused on a 7,500 m^2 section within the area of the proposed PAH plume (figure 4.1). Here more than 20 monitoring wells equipped with filter screens through the entire aquifer have been installed

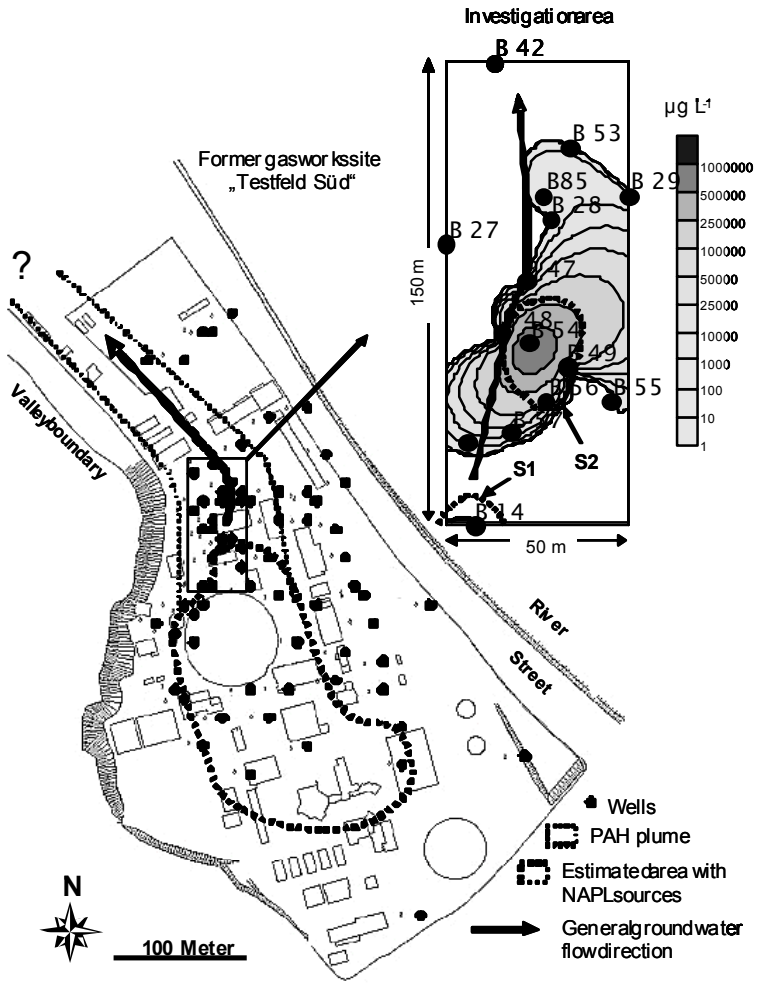


Figure 4.1 Schematic overview of the investigated site 'Testfeld Süd'. The insert shows the position of the sampled observation wells and the general distribution of aromatic contaminants in the investigation area given as sum of the 16 EPA PAHs. The two source zones S1 and S2 are highlighted by dotted lines.

of which 15 wells have been selected for sampling and measurements. Within the investigated area two point sources were detected, one southwest (termed source 1; S1) and another located in the central part of the section (termed source 2; S2; figure 4.1). Detailed information on the physical-chemical situation at the investigation site is given elsewhere (Griebler et al., 2004).

The well water was replaced at least once by means of a submersible pump (MP1, Grundfos Corp.) before groundwater was sampled and filled into pre-cleaned brown glass bottles which were closed without headspace by screw caps with teflon-coated silicon septa. Samples for analysis of PAHs, heterocyclic aromatic hydrocarbons (HAHs) and metabolites received the addition of NaOH (100 mM final concentration) to stop biological activity. Samples were transported and stored cold (4 °C) until further processing.

4.2.3 Analysis and identification of metabolites

The volatile compounds indane and indene were analysed directly from water without prior concentrations by Purge and Trap/GC-MS following the protocol given in Zamfirescu & Grathwohl (Zamfirescu and Grathwohl, 2001). PAHs were analysed after liquid/liquid extraction with cyclohexane by GC-MS as described in Griebler et al. (Griebler et al., 2004). For detection of metabolites, NaOH amended field samples (1L, pH ≥ 12) were extracted twice with 20 mL of cyclohexane (Merck Corp.) to remove nonpolar aromatic hydrocarbons. Thereafter, the samples were acidified with HCl (37%, Merck Corp.) to pH ≤ 2 and extracted twice with diethylether (Merck Corp.). The combined extracts were dried with anhydrous sodium sulphate before transferred into a new round flask, concentrated with a vacuum rotary-evaporator to a final volume of about one mL and subsequently dried under a continuous nitrogen gas stream. In contrary, the aqueous phase of cultures were acidified immediately with HCl to a pH of <2. Here, the metabolites were extracted 3 times with diethyl ether. The extracts were combined in a glass vial and dried with anhydrous sodium sulphate. The liquid phase was then transferred into a new vial and the solvent was removed under a gentle stream of nitrogen gas. The residues of both, culture and field samples were resolved in one mL methanol (chromatographic grade, Merck Corp.), diluted with trimethylchlorosilane (TMCS) to a ratio of 9:1 (methanol:TMCS, v:v) and derivatised for one hour at 75°C. The solution containing the methylated aromatic carboxylic acids was again dried under a continuous nitrogen stream and resolved in one mL ethylacetate (Merck Corp.) for subsequent separation and identification via gas chromatography – mass spectrometry (GC: HP 6890, MS: HP 5973; Agilent Corp.). The GC column for separation of target compounds was a DB-5MS (film thickness: 0.25 µm; inner diameter: 0.2 mm; length: 30 m; J. & W. Scientific). Samples were injected splitless and flow rate of the carrier gas (Helium) was 0.9 mL min^{-1}. The oven temperature was held at 43°C for 5 min, then ramped at a rate of 4°C min^{-1} to 280°C and held for 5 min. The MS was operated at 315°C in the scan mode and acquired data from 29 to 400 mass units (m/z). Here the detection limit for individual methylated polar organic acids ranged from 0.1 µg L^{-1} to 1 µg L^{-1}. For identification of metabolites, instrumental library searches of the NIST (National Institute of Standard and Technology) database, comparison with published mass spectra, and co-injections with commercially available authentic reference compounds was used. However, only few reference substances were commercially available, *i.e.* 2-benzofuranoic acid, 2-indolic acid, 3-indolic acid, 5-indolic acid and 6-indolic acid. In the cases where no reference substances or reference spectra were available, the putative metabolites in culture samples were identified with following criteria: (i) the substance of concern did not appear when the culture N47 grew only on naphthalene or 2-methylnaphthalene, (ii) it was also not present in the substrates applied and (iii) its mass spectrum was analogous to the mass spectra of the metabolites appearing in the anaerobic naphthalene degradation, such as 2-naphthoic acid, for example. All experiments were repeated twice to exclude any possibility of contingent contamination. Mass spectra obtained from culture studies were used as references for the subsequent investigation of field samples.

For quantitative estimations of metabolites concentration in field samples, available authentic standards were dissolved in one litre ultrapure water (Millipore) and treated similar to groundwater samples (*i.e.* liquid-liquid extraction with cyclohexane, acidification,

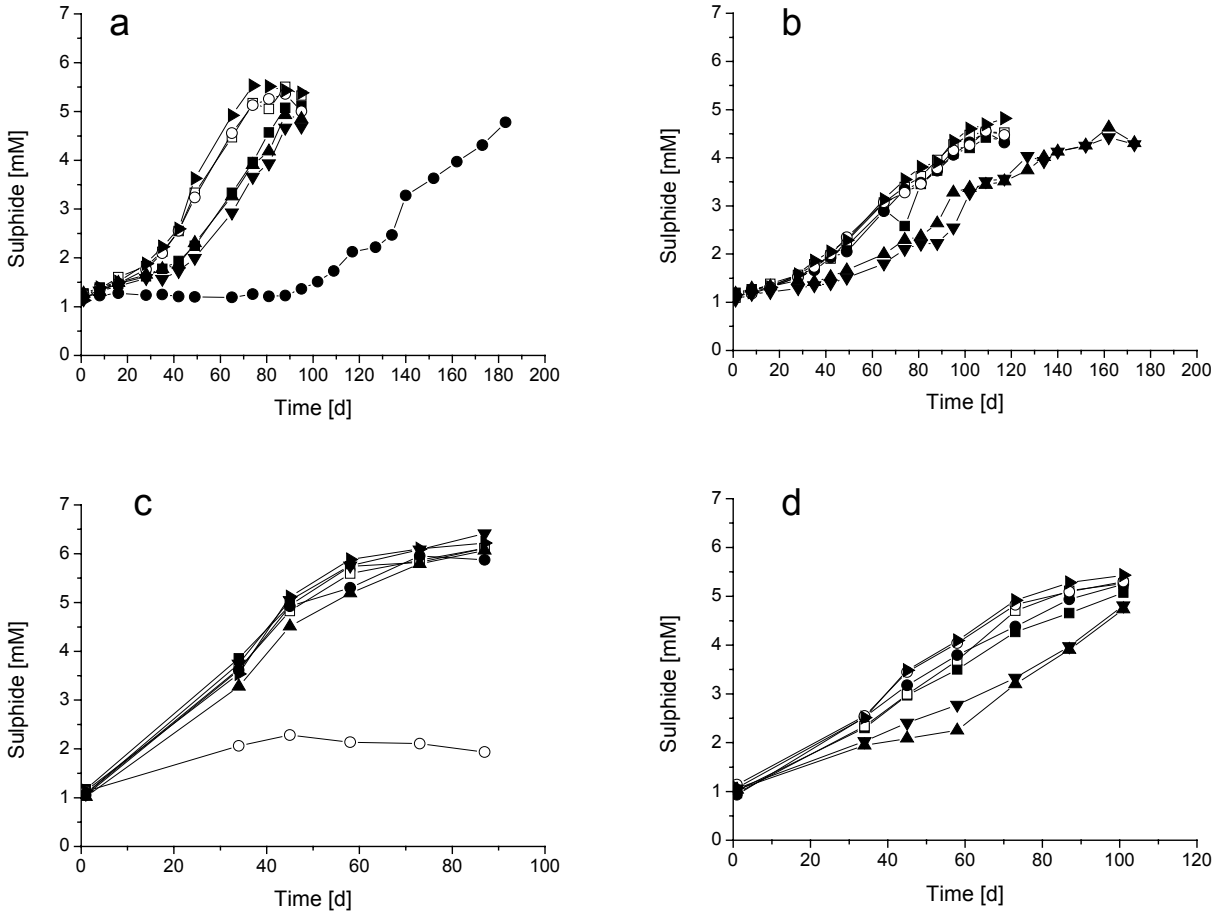


Figure 4.2 Substrate utilisation of the sulphate-reducing enrichment culture N 47 monitored as sulphide production. The following combinations of main substrate and cosubstrate were used; 4.2a) main substrate: naphthalene, cosubstrates: benzofuran (■), benzothiophene (□), indene (●), indane (○), indole (▲), quinoline (▼), no cosubstrate (►); 4.2b) main substrate: 2-methylnaphthalene, cosubstrates as in 4.2a; 4.2c) main substrate: naphthalene, cosubstrates: : biphenyl (■), 1-methylnaphthalene (□), acenaphthene (●), acenaphthylene (○), fluorene (▲), phenanthrene (▼), anthracene (►); 4.2d) main substrate: 2-methylnaphthalene, cosubstrates as in 4.2c.

extraction with diethylether, derivatization and GC-MS analysis) to obtain calibration curves. If no authentic standards were available concentrations of compounds were estimated using calibration curves of substances with similar properties, particularly with similar retention time in the GC column. Additionally, duplicate samples of ultrapure water, groundwater from well B42 (less contaminated) and well B54 (highly contaminated) were spiked with two compounds which have not been detected in samples from these wells, *i.e.* 1-napthylacetic acid and naphthyl-2-methylensuccinic acid (NMeS), to evaluate the recovery efficiency of the applied extraction-derivatisation protocol. Results showed that for these two compounds the recovery efficiency varied between 50-150% in natural samples in relation to the contaminant-free reference sample (spiked ultrapure water). However, the presented data analysis for metabolites focuses on relative concentration trends rather than on absolute concentration values.

4.3 Results and discussion

4.3.1 Growth of N47 with varying cosubstrates

In order to examine the influence of different polycyclic and heterocyclic compounds on the growth of the enrichment culture N47, the microbial activity was monitored by measuring sulphide concentration. With few exceptions, the addition of a cosubstrate had no significant influence on the growth of the enrichment culture N47 with naphthalene or 2-methylnaphthalene (figure 4.2). However, with 2-methylnaphthalene as main substrate, the nitrogen heterocycles indole and quinoline slightly retarded bacterial activity (figure 4.2b). A more pronounced inhibitory effect was observed when naphthalene was served together with indene, *i.e.* growth was inhibited for nearly 100 days (figure 4.2a). The amendment of naphthalene fed cultures with acenaphthylene resulted in a total inhibition of growth (figure 4.2c). Interestingly, no negative effects occurred when indene and acenaphtylene were added to N47 growing on 2-methylnaphthalene (figure 4.2d). Also no inhibition appeared, when the corresponding reduced cosubtrates indane and acenaphthene were added. A slight retardation of bacterial activity also occurred when 2-methylnaphthalene was offered together with fluorene and phenanthrene (figure 4.2d). No effects were observed with naphthalene as main substrate (figure 4.2c).

Results from the growth experiments clearly demonstrate that the degradation of aromatic compounds under field conditions faces much more difficulties then in laboratory experiments. The wide spectrum of substances occurring in contaminant plumes may display serious effects on indigenous biodegradation. Not only high concentrations of single compounds can act inhibiting or even toxic, but also a specific combination of substrate and cosubstrate, both at moderate concentrations, may hamper bacterial activity. While indene and acenaphthylene had no adverse influence, when the bacteria grew with 2-methylnaphthalene, these compounds caused an inhibition with naphthalene as the main substrate. It can be assumed that the common structural feature of these compounds, *i.e.* a benzene-ring condensed with a C_5-ring containing a double bond, strongly restrain the methylation as the initial reaction in the anaerobic naphthalene degradation pathway (previous chapter). Additionally, it has to be mentioned that the culture N47, which can grow on naphthalene or 2-methylnaphthalene as sole carbon source and electron donor, cannot utilize these two substrates when added simultaneously.

4.3.2 Cometabolic conversion of polycyclic and heterocyclic aromatic compounds

It was investigated, if the enrichment culture N47 can cometabolically transform polycyclic and heterocyclic compounds during growth on naphthalene or 2-methylnaphthalene. Most of the offered cosubstrates offered to strain N47 when growing on naphthalene or 2-MN were transformed to the corresponding carboxylic acids, often to several isomers (table 4.1). For some of the resulting carboxylic acids we could detect further metabolites partially reduced on the aromatic ring (table 4.1). This reaction pattern is homologous to the degradation pathways of naphthalene and 2-MN both of which fuels into the major metabolite 2-naphthoic acid (2-NA) that then undergoes a stepwise ring reduction to octahydro-2-naphthoic acid (Annweiler et al., 2002). No cometabolic conversion was observed with the 3-ring compounds fluorene, phenanthrene and anthracene as cosubstrates as well as with biphenyl. It also remains unclear if a cometabolic conversion of quinoline took place, as the peak of the hypothetic quinolinic acid in the GC chromatogram was too small to

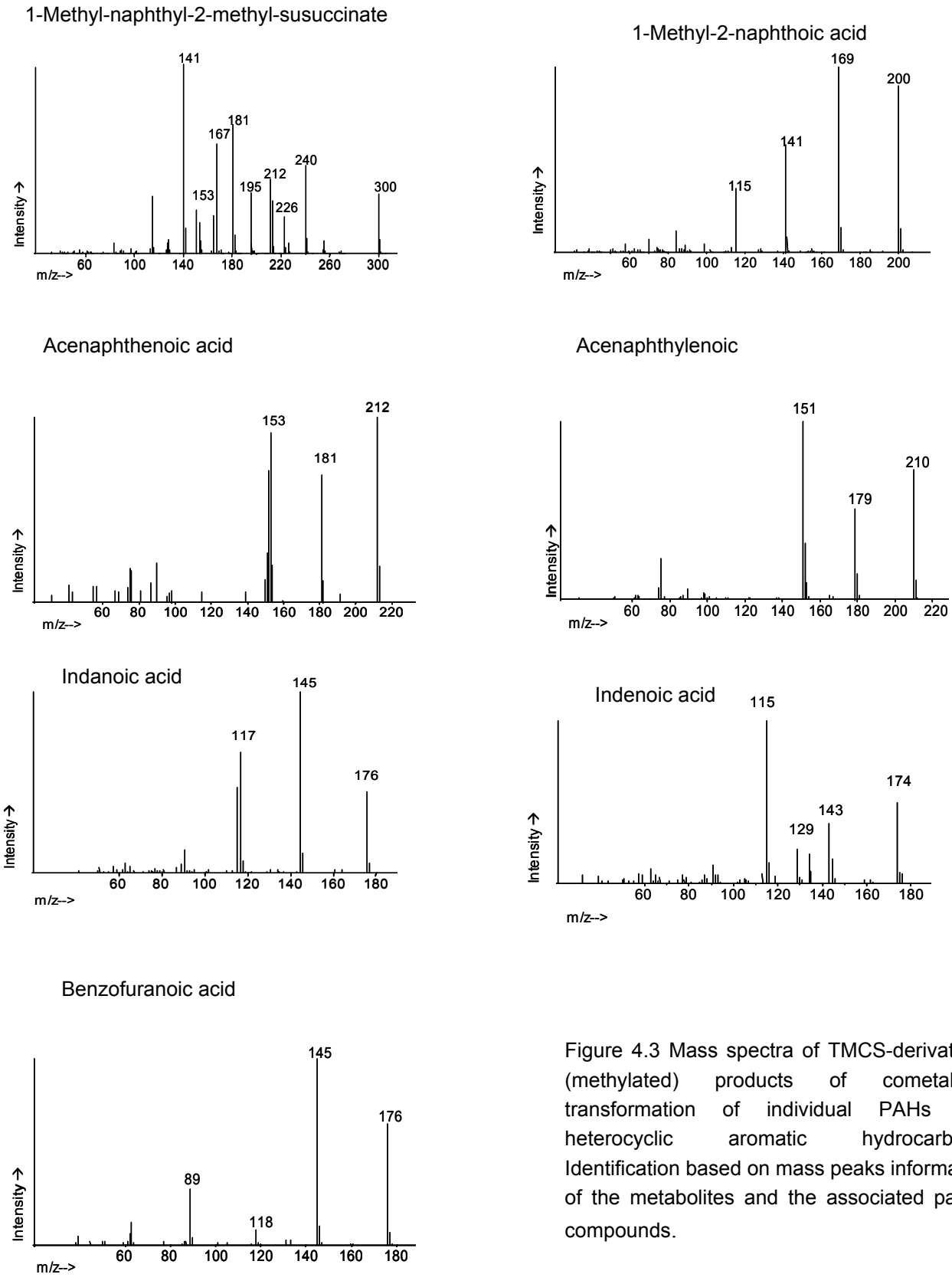


Figure 4.3 Mass spectra of TMCS-derivatised (methylated) products of cometabolic transformation of individual PAHs and heterocyclic aromatic hydrocarbons. Identification based on mass peaks information of the metabolites and the associated parent compounds.

allow a clear identification. The cometabolic conversion of 1-methylnaphthalene provides some interesting insights into the biochemistry of the anaerobic degradation of naphthalene and 2-methylnaphthalene. Unlike in the case of 2-methylnaphthalene, no addition of fumarate to the methyl group occurred during cometabolic conversion of 1-methylnaphthalene. However, two metabolites with a similar mass fragment pattern and a total mass 14 units higher then naphthyl-2-methyl-succinate were found (figure 4.3). The 14 mass units correspond to an additional methyl group. In order to evaluate this observations, additional batch experiments were carried out with dimethylnaphthalenes as cosubstrates. The intention of the experiment was to generate specific metabolites occurring during dimethylnaphthalene cometabolims and to compare their mass spectra with that found in the culture supernatants, where 1-methylnaphthalene was the cosubstrate. Metabolites with identical mass spectra were found indeed when 1,2-dimethylnaphthalene was the cosubstrate. As no traces of dimethylnaphthalene could be detected in substances added to the bottles amended with 1-methylnaphthalene, this finding indicates that 1-methylnaphthalene can be methylated in the 2 position, which matches with recent findings, that a methylation is the initial step in the anaerobic degradation of naphthalene (previous chapter). It further indicates that the naphthyl-2-methyl-succinate synthase accepts 2-methylnaphthalene and 1,2-dimethylnaphthalene as substrates, but not 1-methylnaphthalene.

A methylation as initial step of transformation seems also likely in experiments with benzothiophene and benzofuran as cosubstrates where metabolites were detected with mass spectra analogous to the mass spectra of naphthyl-2-methyl-succinic acid and naphthyl-2-methylene-succinic acid, but 10 mass units lighter or 6 mass units heavier. These mass differences correspond exactly to the mass difference between naphthalene and benzofuran, or naphthalene and benzothiophene, respectively.

The enrichment culture N47 obviously can transform benzofuran and benzothiophene to very specific metabolites such as benzofuran-methyl-succinate and benzofuran-methylene-succinate, or benzothiophene-methyl-succinate and benzothiophene-methylene-succinate, respectively.

This observation corroborates the hypothesis that methylation is the initial reaction in anaerobic naphthalene degradation pathway. In a previous study was shown that ring-deuterated naphthyl-2-methyl-succinate d_7 is produced during catabolism of naphthalene d_8 indicating a former methylation (previous chapter). The results of the present study indicate that also other compounds like 1-methylnaphthalene, benzofuran and benzothiophene are methylated by the culture N47. This shows that methylation could be a general initial reaction in anaerobic degradation pathways of many aromatic and heterocyclic substances.

Methylation of aromatic compounds can also be used to explain observations made by other authors, who found methyl-naphthoic acid as a putative metabolite in the anaerobic 2-methylnaphthalene degradation (Sullivan et al., 2001). They concluded the existence of an alternative 2-methylnaphthalene degradation pathway with direct carboxylation of 2-methylnaphthalene as initial reaction. Concerning the methylation of divers aromatic compounds, the finding of methyl-naphthoic acids can be explained by an accidental, additional methylation of 2-methylnaphthalene.

The wide spectrum of the compounds that can be cometabolically converted by the culture N47 shows that the *in situ* degradation of organic pollutants may proceed mainly as a metabolic cooperation between several

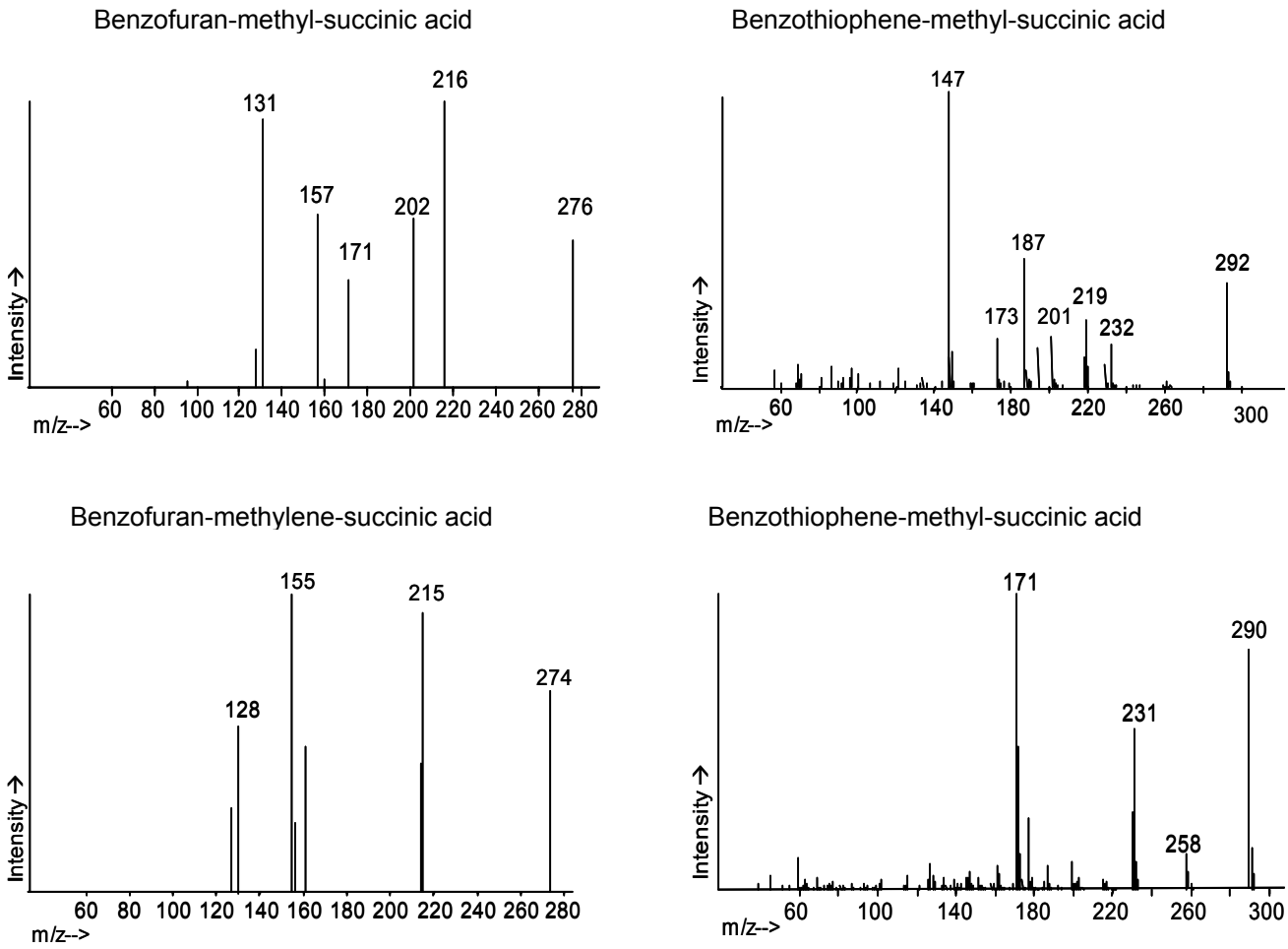
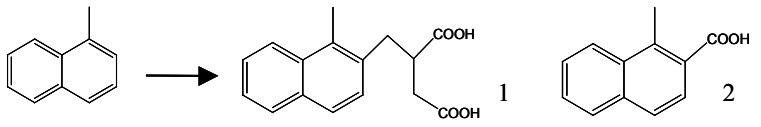
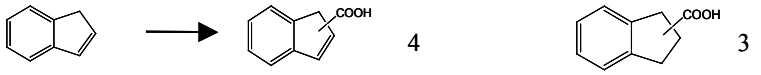
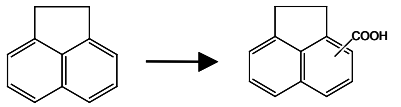
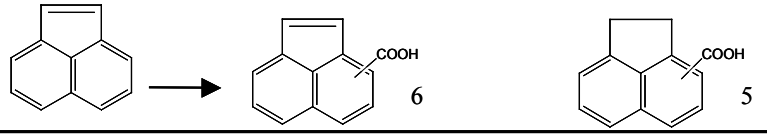
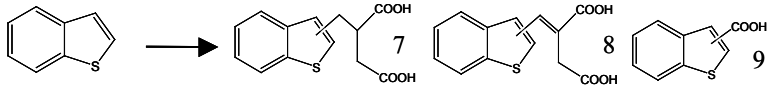
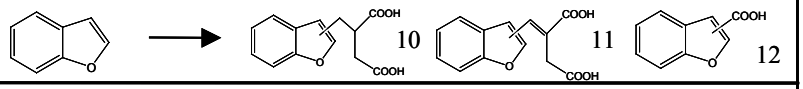
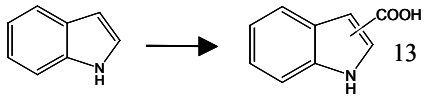


Figure 4.4 Mass spectra of TMCS-derivatised (methylated) products of cometabolic transformation of benzofuran and benzothiophene. Identification based on mass peaks information of the metabolites and the associated parent compounds.

bacterial strains. More than half of the offered polycyclic and heterocyclic compound were transformed to the corresponding carboxylic acids. Primarily this transformation has an undesirable effect concerning contaminated aquifers. The mobility of the daughter compounds might be increased due to higher solubility, but on a long-term base it might strongly enhance and accelerate natural attenuation. The introduction of a carboxylic group into a molecule and a partial ring reduction weaken the aromatic system that is responsible for the chemical stability of aromatic compounds. This can result in higher bioavailability and degrability.

Inhibition and cometabolism phenomena were already reported, when aerobic, PAHs degrading strains were exposed to a mixture of aromatic substances (Bouchez et al., 1995). Phenanthrene strongly inhibited the degradation of anthracene, whereas fluorene was cometabolised without being inhibitory. In another strain, an addition of fluorene enhanced the utilisation of phenanthrene, but an addition of fluoranthene had strong inhibitory effects. Polycyclic aromatic compounds are also cometabolised by organisms representing a totally different metabolic type, like for example by the nitrifying bacterium *Nitrosomonas europaea* (Chang et al., 2002). Under anaerobic conditions, an inhibition of toluene degradation by BTEX-compounds, alkanes and gasoline was observed (Phelps and Young, 1999).

Table 4.1 Structures of the contaminants and metabolites identified after anaerobic cometabolic transformation in batch experiments with the sulphate-reducing enrichment culture N47 grown either on naphthalene or 2-methylnaphthalene: 1) 1-methyl-naphthyl-2-methyl-susuccinate, 2) 1-methyl-2-naphthoic acid, 3) indanoic acid, 4) indenoic acid, 5) acenaphthenoic acid, 6) acenaphthylenoic acid, 7) benzothiophene-methyl-succinic acid , 8) benzothiophene-methyl-succinic acid, 9) benzothiophenoic acid, 10) benzofuran-methyl-succinic acid, 11) benzofuran-methylene-succinic acid, 12) benzofuranoic acid, 13) indolic acid

Substrate		Cosubstrate	Metabolites identified
Naph	2-MN		
X	X	**1-MN**	
X	X	**Indane**	
X	X	**Indene**	
X	X	**Acenaphthene**	
	X	**Acenaphthylene**	
X	X	**Benzothiophene**	
X	X	**Benzofuran**	
X	X	**Indole**	

Contrary, the presence of toluene can also suppress the anaerobic degradation of *o*-xylene (Meckenstock et al., 2004).

It has been stressed in the literature that degradation pathways are described now for only approximately 1000 compounds, while more then 10 million organic compounds are thought to be biodegradable (Wackett and Ellis, 1999). The same authors proposed that some compounds may be metabolised only collectively and sequentially be different bacterial strains under certain environmental conditions. Such interactions between prokaryotic cells are well known for example in the synthrophic degradation of fatty acids and alcohols under methanogenic conditions (Schink, 2002). In such communities, the microbial partners share the resulting energy and exchange metabolites and reduction equivalents between each other. Similar models should be considered also for the *in situ* degradation of xenobiotics.

4.3.3 Occurrence of metabolites in a tar oil-contaminated aquifer

A selected area of a tar oil-contaminated aquifer from which the enrichment culture N47 was originally isolated was screened for the presence of the metabolites detected in batch experiments. The investigated field site is characterized by multiple BTEX and PAH sources, of which two were located within the investigated area (figure 4.1). According to the redox potential measurements, only the wells B27, B42 and B53 might contain traces of oxygen as positive redox values were detected. Other investigated wells exhibited E_H values between -45 mV to –240 mV with lowest values found in the source zones. Dissolved sulphide and ferrous iron concentrations indicated sulphate-reducing conditions to prevail in and close to the source zone with sulphide concentrations in the range of 1 to 17 mg L^{-1} and dissolved iron concentrations were mostly below the detection limit. In the less contaminated wells, Fe(II) concentrations were in the range of 0.3 to 6.3 mg L^{-1} accompanied by low sulphide concentrations between 35 to 140 µg L^{-1}. The prevailing of sulphate-reducing conditions was also supported by results from several years of intense field investigations in that area (Bockelmann et al., 2001; Zamfirescu and Grathwohl, 2001; Griebler et al., 2004). At the time of sampling (November 2002), groundwater samples from the wells B54 and B56 (source zone 2, figure 4.1) contained NAPL (non aqueous phase liquids) phase. Total concentrations of 390 mg L^{-1} and 219 mg L^{-1} were determined for naphthalene and 2-methylnaphthalene (2-MN), respectively, by far exceeding theoretical water solubility values. The general distribution pattern of aromatic contaminants is highlighted in figure 4.1 where the concentrations of the 16 EPA PAHs are shown. With increasing distance to the source zones S1 and S2 all investigated PAHs and heterocyclic aromatic hydrocarbons showed a strong decline in concentrations along the groundwater flow path to less than 1µg L^{-1} at the wells B42 and B53 (figure 4.1). Only acenaphthene, which is typically one the most persistent aromatic compounds under anoxic conditions, exhibited comparable high concentrations of 50 to 300 µg L^{-1} at the wells B27, B28, and B29 where most other compounds of interest were already below 1 µg L^{-1}. At the most distant wells, *i.e.* B42 and B53, even the acenaphthene concentration decreased under 1µg L^{-1}.

Most of the putative metabolites from cometabolic transformation of heterocyclic aromatic compounds identified in batch experiments with N47 could also be detected in groundwater from the tar oil-contaminated aquifer (table 4.2). Highest metabolite concentrations of up to 700 µg L^{-1} were observed for the degradation products of benzothiophene, *i.e.* two carboxybenzothiophene isomers and a further reduced dihydrocarboxybenzothiophene isomer, respectively (table 4.2). Similarly high concentrations were only found for indanoic acid, a proposed metabolite in the anaerobic conversion of indane, with more than 300 µg L^{-1} at well B14 (table 4.2). Other metabolites of interest did not exceed concentrations of 100 µg L^{-1}, but were typically found in the lower microgram per liter range. Spatial distribution patterns indicate that highest metabolite concentrations occured in the direct vicinity of the source zones. However, no samples for metabolite analysis could be obtained from the source wells B54 and B49 during this sampling survey.

A few of the metabolites observed in batch culture experiments could not be detected *in situ*. These include methyl-NMS, benzofuran-methyl-succinic acid, benzofuran-methylene-succinic acid, benzothiophene-methyl-succinic acid, and benzothiophene-methylene-succinic acid.

Table 4.2 Concentrations [µg L^{-1}] of putative metabolites from transformation of heterocyclic aromatic hydrocarbons in samples from individual wells at the 'Testfeld Süd' site.

Putative metabolite	**Retention time [min]**	**B14**	**B27**	**B28**	**B29**	**B42**	**B44**	**B47**	**B48**	**B53**	**B55**	**B56**	**B57**	**B85**
Benzofuranoic acid (isomer 1)	29.1	11	-	-	-	-	3	-	≤ 1	-	-	≤ 1	-	-
Benzofuranoic acid (isomer 2)	29.31	19	-	-	-	-	3	≤ 1	2	-	-	≤ 1	-	-
Indanoic acid	31.1	332	-	-	-	-	50	7	14	-	≤ 1	13	9	≤ 1
Indenoic acid (isomer 1)	31.3	24	-	-	-	-	3	≤ 1	4	-	-	2	4	≤ 1
Indenoic acid (isomer 2)	31.5	37	-	-	-	-	6	2	5	-	-	3	6	2
2-Carboxybenzothiophene (isomer 1)	34.32	9	≤ 1	≤ 1	-	-	4	3	8	-	≤ 1	3	4	2
Carboxybenzothiophene (isomer 2)	34.8	92	-	-	-	-	9	-	-	-	2	5	-	-
Carboxybenzothiophene (isomer 3)	35.04	5	-	-	-	-	9	-	-	-	2	5	-	-
5-Carboxybenzothiophene (isomer 4)	35.44	150	≤ 1	≤ 1	-	-	73	44	69	-	4	13	92	20
Dihydrocarboxybenzothiophene	37.71	703	3	5	-	-	403	226	326	-	21	131	433	132
Methyl naphthoic acid (isomer 1)	37.8	-	-	2	3	-	≤ 1	≤ 1	2	-	2	-	3	3
Methyl naphthoic acid (isomer 2)	38.1	36	-	≤ 1	≤ 1	-	20	3	6	-	2	-	11	≤ 1
Methyl naphthoic acid (isomer 3)	38.3	12	≤ 1	≤ 1	2	-	4	2	4	-	4	9	6	3
Methyl naphthoic acid (isomer 4)	38.7	63	-	-	-	-	29	2	4	-	15	-	6	≤ 1
Acenaphthenoic acid	40.3	20	2	5	6	-	11	10	12	-	12	-	18	9
Acenaphthylenoic acid	41.6	12	≤ 1	≤ 1	5	-	4	2	10	-	8	25	22	10

The last two decades several studies focused on the occurrence of intermediates from anaerobic degradation of petroleum hydrocarbons in contaminated aquifers (Beller, 2000; Elshahed et al., 2001; Beller, 2002; Gieg and Suflita, 2002; Reusser and Field, 2002; Reusser et al., 2002; Martus and Püttmann, 2003; Namocatcat et al., 2003; Alumbaugh et al., 2004). However, to date only a few studies concentrated on degradation products of PAHs and HAHs (Johansen et al., 1997; Fetzner, 1998; Annweiler et al., 2001; Ohlenbusch et al., 2002; Phelps et al., 2002; Zwiener et al., 2003; Griebler et al., 2004; Young and Phelps, 2005). The main reason is probably the lack of information on anaerobic degradation pathways of these compounds. Concerning PAHs anaerobic degradation pathways are elucidated to a large extent for naphthalene, 2-methylnaphthalene and phenanthrene (Zhang and Young, 1997; Annweiler et al., 2000; Meckenstock et al., 2000; Annweiler et al., 2002; Safinowski and Meckenstock, 2004), concerning HAHs some details are available for the anaerobic degradation of indole, quinoline and benzothiophene (Johansen et al., 1997; Fetzner, 1998; Annweiler et al., 2001). With a few exceptions, most intermediates from cometabolic transformation of HAHs as described here for the first time from batch experiments with the naphthalene-degrading enrichment culture N47 have not been looked for in the field, before. Carboxybenzothiophene isomers and dihydrocarboxybenzothiophene have been detected already two times in previous field surveys at the investigated site (Annweiler et al., 2001; Griebler et al., 2004). Although slight variations in metabolite concentrations could be observed in samples from individual wells when compared with data from the previous studies, recent data confirmed their distribution patterns in the tar oil-contaminated aquifer. Samples from several wells also revealed the presence of four isomers of methyl-2-naphthoic acid, a putative intermediate in the cometabolic anaerobic degradation pathway of 1-methylnaphthalene and dimethylnaphthalenes. However, this degradation pathway is still poorly understood.

Since the enrichment culture N47 was isolated from the Testfeld Süd aquifer, it was used to study the anaerobic degradation pathways of naphthalene and 2-methylnaphthalene (Annweiler et al., 2000; Meckenstock et al., 2000; Annweiler et al., 2002; Safinowski and Meckenstock, 2004) and the cometabolic conversion of benzothiophene (Annweiler et al., 2001) as well as further PAHs and HAHs (this study). The ‘from field to lab and from lab to field’ approach now revealed strong evidence that degradation pathways elucidated in batch experiments are also active *in situ*.

This study also indicates that natural degradation processes in complex pollutant mixtures may proceed more complicated than assumed so far with a lot of inhibitory effects and cometabolic interactions between different bacterial strains

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5 Anaerobic biphenyl degradation by a novel sulphate-reducing enrichment culture

Polycyclic aromatic hydrocarbons (PAH) are among the most important ground water contaminants. Nonetheless, little information about anaerobic PAH degradation could be obtained so far and only few microbial cultures are described in the literature that can utilise polycyclic compounds as sole carbon source and electron donor (Zhang and Young, 1997; Galushko et al., 1999; Meckenstock et al., 2000). Biphenyl was shown to be biodegradable under anoxic conditions (Rockne and Strand, 1998; Natarajan et al., 1999; Rockne and Strand, 2001), but no anaerobic biphenyl-degrading culture could be enriched so far that shows enduring growth after serial transfers into new, sterile, sediment-free medium. Apart from the environmental problem of recalcitrant biphenyl, a more prominent public concern is focussed on polychlorinated biphenyls (PCBs), which have much more severe health effects then biphenyl itself. PCBs accumulate in the food chain due to their hydrophobicity (MacDonald et al., 2000). Certain congeners are carcinogenic (Cogliano, 1998) or act as environmental estrogens leading to decreased fertility (Nicolopoulou-Stamati and Pitsos, 2001). Under anoxic conditions, the biodegradation of PCBs occurs *via* reductive dechlorination leading to congeners with fewer chlorine atoms, or at best, to biphenyl (Wiegel and Wu, 2000). Lower halogenated PCB's and biphenyl are readily degraded by aerobic bacteria (Gibson et al., 1993), but turned out to be recalcitrant under anoxic conditions in many cases (Abraham et al., 2002).

The novel sulphate-reducing culture BiphS1 that can utilize biphenyl as the sole carbon source and electron donor was enriched from contaminated soil material near a former coking plant in Gliwice, Poland, and was since then transferred 12 times into new, sterile, sediment-free medium. The cells were cultivated in 120 mL serum bottles, half-filled with bicarbonate-buffered fresh water medium, pH 7.1, containing 10 mM Na_2SO_4 and reduced with 1 mM Na_2S (Widdel et al., 1983; Widdel and Bak, 1992). 3 mM $FeCl_2$ was added in order to scavenge free sulphide, which was toxic for the cells. No growth could be observed without addition of Fe^{II}. Substrate was added as solid crystals, 5-10 mg per bottle, combined with 0.3 g absorber resin XAD-7 in order to keep the actual biphenyl concentration low but providing sufficient amount of substrate (Morasch et al., 2001). Alternatively, the substrate could be added as a 1.5% solution in heptamethylnonane with a syringe through the bottle stopper. The bottles were flushed with N_2-CO_2 (80:20), closed with butyl rubber stoppers and incubated in the dark at 30 °C. New cultures were inoculated with 10 ml of parental cultures in 2 months intervals. Bacterial growth was monitored indirectly by measuring the increasing sulphide concentration (Cline, 1969). The maximal sulphide concentration produced never exceeded the added 3 mM Fe^{II}. However, even if 6 mM Fe^{II} was added, the final sulphide concentration did not reach much higher levels (figure 5.1).

To study metabolites of anaerobic biphenyl degradation, the culture supernatants were acidified with HCl to pH 1 and extracted three times with diethyleter. The organic phase was collected, dried over water-free sodium sulphate and evaporated under a continuous nitrogen stream. The residue was redissolved in 1.8 mL methanol. 200 µL trimethylchlorsilane was added and the samples were derivatized for 1 hour at 75°C in order to transform the carboxylic acids into methyl esters. After cooling, the samples were

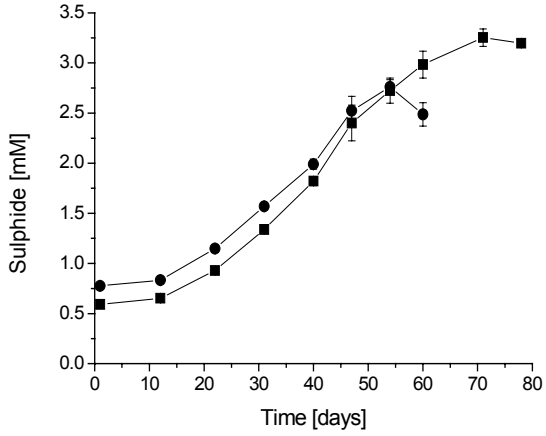

Figure 5.1. Growth of a sulphate-reducing enrichment culture BiphS1 with biphenyl as the sole carbon source and electron donor. Sulphide formation was monitored in the presence of 3 mM Fe^{II} (●) and 6 mM Fe^{II} (■).

dried under a continuous nitrogen stream and dissolved with 1 mL ethyl acetate. The detection of the metabolites was performed with GC-MS (GC, HP 6890 with a DB-5MS column, 0.25 µm film thickness, 0.2 mm i.d., 30 m length, J&W Scientific; MS, HP 5973). Sample injection was splitless (1µl) and the flow rate of the carrier gas helium was 0.9 ml/min. The oven temperature was 43°C for 5 minutes, then ramped at a rate of 4°C/min to 280°C and was held for 5 minutes. The MS was operated at 315 °C in the scan mode.

In order to obtain indications on the anaerobic biphenyl degradation pathway, culture supernatants were analysed for putative metabolites. Biphenyl-4-carboxylic acid was the only putative intermediate that could be identified by coelution with commercially available standard and comparison of the mass spectrum by GC-MS analysis. No other isomer of biphenyl-carboxylic acid could be found. The mechanism of biphenyl-4-carboxylic formation remains unclear. However, this finding corresponds with the general pattern in the anaerobic degradation of many aromatic hydrocarbons. Carboxylic acids were found to be central metabolites in the anaerobic catabolism of benzene (Caldwell and Suflita, 2000; Phelps et al., 2001), naphthalene (Zhang and Young, 1997; Meckenstock et al., 2000), 2-methylnaphthalene (Annweiler et al., 2000; Annweiler et al., 2002), phenanthrene (Zhang and Young, 1997) and many other compounds (Harwood et al., 1999).

It was also tested if halogenated biphenyls can be degraded by the enrichment culture BiphS1 with 4-fluorobiphenyl as a model compound. No microbial activity was observed when 4-fluorobiphenyl was offered as the sole carbon source. However, it could be transformed cometabolically with biphenyl. In this experiment, 1 mL 1.5% biphenyl solution in heptamethylnonane containing 0.1% 4-fluorobiphenyl was injected in each inoculated serum bottle. After 3 months, culture supernatants were extracted as described above. In addition to the biphenyl-4-carboxylic acid, a novel metabolite appeared that was 18 mass units heavier (figure 5.2). This corresponds with the mass difference between a fluorine and a hydrogen atom. This finding deserves special attention, because of the parallels to the degradation of polychlorinated biphenyls. Under oxic conditions, PCBs are oxygenated, followed by the cleavage of the aromatic ring system and the subsequent removal of chlorine. Under anoxic conditions, only reductive dehalogenation of PCBs to biphenyl by a microbial consortium was observed (Natarajan et al., 1996), leaving the aromatic ring system intact. The known sediment-free enrichment cultures that dechlorinate PCBs are not even able to remove all the chlorine atoms (Wu et al., 2000; Cutter et al., 2001). The cometabolic transformation of 4-fluorobiphenyl to the corresponding carboxylic acid demonstrates that the complete degradation of halogenated biphenyls to CO_2 might be possible also under anoxic conditions after most of the halogen atoms have been removed by reductive dehalogenation. The introduction of a carboxylic group weakens the

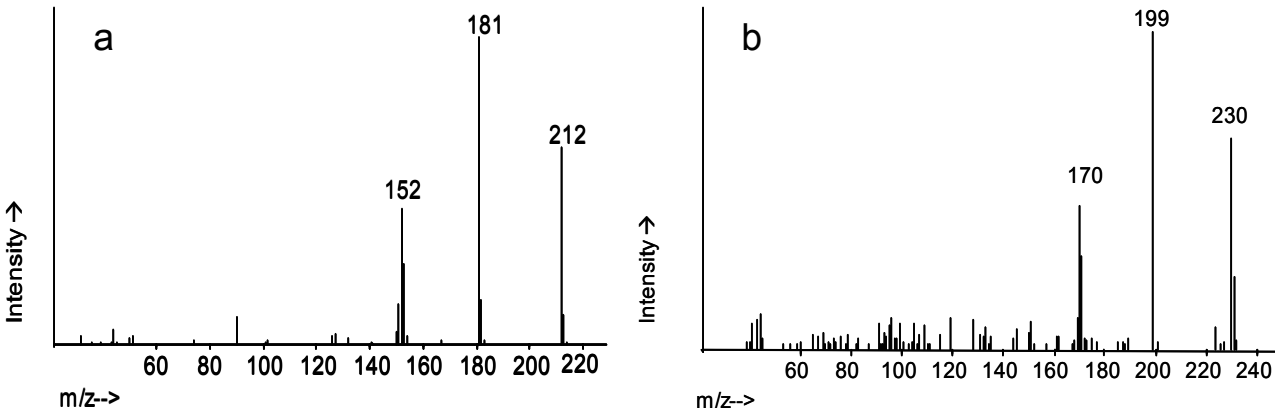

Figure 5.2. Mass spectrum of biphenyl-4-carboxylic acid (as methyl ester) that was found in supernatants of the enrichment culture BiphS1 (a). When 4-fluorobiphenyl was added as cosubstrate, 4-fluorobiphenyl-carboxylic acid could be detected (b).

aromatic ring system. In many anaerobic degradation pathways of aromatic compounds examined so far, the transformation of a substance into a carboxylic acid takes place in the first phase of the catabolism followed by ring reduction and cleavage. Also not fully dehalogenated PCBs could be transformed to carboxylic acids and further degraded to harmless compounds by such kind of cometabolic interactions. This could be an interesting example of a "modular degradation pathway" as proposed by other authors (Wackett and Ellis, 1999).

Biphenyl is also a product of another anaerobic microbial process, i.e. the reductive desulphurisation of dibenzothiophene (Kim et al., 1990; Marcelis et al., 2003). The removal of sulphur is opportune in the case of fuel refinement, but insufficient concerning bioremediation of contaminated sites. This could be an example how combined activities of desulphurising and biphenyl-degrading bacteria could contribute to natural attenuation of toxic compounds in the environment.

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6 General conclusions and outlook

Despite the great importance of polycyclic aromatic hydrocarbons (PAH) as fuel constituents and xenobiotics, their fate in the environment is by far not sufficiently elucidated yet. Until twenty years ago, aromatic compounds were considered to be recalcitrant in the absence of molecular oxygen (Kuhn et al., 1985). Even if some sulphate-reducing bacterial strains able to utilise naphthalene or phenanthrene as sole carbon source and electron donor have been isolated in the recent years (Zhang and Young, 1997; Galushko et al., 1999; Meckenstock et al., 2000), detailed information about the catabolic pathways were very scarce due to the very slow growth of the PAH degrading bacteria. Only one reaction in the anaerobic degradation pathways of polycyclic compounds could be measured in dense cell suspension (Annweiler et al., 2000) demonstrating that the initial step in anaerobic catabolism of 2-methylnaphthalene is the addition of fumarate to the methyl group, which is a common reaction pattern for methylated aromatic substances. (Biegert et al., 1996; Beller and Spormann, 1997a; Beller and Spormann, 1997b; Leuthner et al., 1998; Krieger et al., 1999). Based on metabolites found in culture supernatants, it was demonstrated that the resulting naphthyl-2-methyl-succinate is oxidised to 2-naphthoic acid that undergoes a stepwise ring reduction and cleavage (Annweiler et al., 2000; Zhang et al., 2000; Annweiler et al., 2002). However, no further reaction could be verified in an enzyme test.

In the present work two enzyme reaction in the anaerobic 2-methylnaphthalene degradation pathway could be measured in cell extracts: the activation of naphthyl-2-methyl-succinate to the corresponding CoA-ester with succinyl-CoA as the CoA donor and the following oxidative step at the original methyl atom leading to the formation of naphthyl-2-methylene-succinyl-CoA. These two reactions initiate the β-oxidation at the original methyl atom leading to the formation of the central metabolite 2-naphthoyl-CoA. The results demonstrated that in-depth investigation of anaerobic catabolism of PAHs is feasible despite bacterial doubling times of nearly one month and protein yields of a few milligrams per litre. Nonetheless, the specific activities measured in the performed enzyme assays are similar to those measured in studies of anaerobic toluene degradation by denitrifying bacteria (Leutwein and Heider, 2001; Leutwein and Heider, 2002).

Also the behaviour of the succinyl-CoA:naphthyl-2-methyl-succinate CoA-transferase in the presence of certain inhibitors indicated parallels to the succinyl-CoA: (R)-benzylsuccinate CoA-transferase, which is the analogous enzyme in the anaerobic toluene degradation pathway. Further enzyme reactions such as transformation of naphthyl-2-methylene-succinate to 2-naphthoic acid or the stepwise ring reduction of 2-naphthoic acid were attempted to be measured. However, after unsuccessful trials, they had to be abandoned due to lack of cell material.

One very important aspect in the research on anaerobic degradation of aromatic hydrocarbons was the elucidation of the initial step in catabolism of unsubstituted compounds. Earlier, carboxylation was proposed to be the first step in the anaerobic naphthalene degradation pathway (Zhang and Young, 1997). However, the results presented by the authors could be explained alternatively. Furthermore, a direct carboxylation of unsubstituted aromatic compounds is an uncommon reaction in biochemistry, whereas alternative mechanisms such as methylation were reported for human cells (Flesher and Myers, 1991; Myers and Flesher, 1991). Methylation of aromatic compounds is a very

common reaction in organic chemistry known as Friedel-Crafts alkylation.

It turned out that the initial step in the anaerobic degradation of naphthalene is a methylation producing 2-methylnaphthalene. Similar as in the degradation pathways of methylated aromatic compounds, the initial step is not a redox reaction, and in this case, it does not even introduce a carboxyl or any other polar group into the molecule. However, after the methylation, naphthalene can be further degraded *via* the 2-methylnaphthalene pathway.

One important aspect for future investigations should be to find out, if methylation is also the initial step in other anaerobic naphthalene degrading strains and anaerobic catabolic pathways of other unsubstituted aromatic compounds such as benzene. A profound understanding of the activation mechanisms could be the first step to infulence degradation processes in the environment and provide applicable approaches for remediation of polluted areas.

A very promising observation for practical application was the discovery of the high cometabolic potential of the sulphate-reducing culture N47. A wide spectrum of polycyclic and heterocyclic compounds was converted to the corresponding carboxylic acids. The aspect of cometabolism was not investigated exhaustively so far with regard to natural attenuation of aromatic substances. But especially in complex mixtures of xenobiotics that are generally found in pollutant plumes in ground water (Franck, 1963; Zamfirescu and Grathwohl, 2001), cometabolic interactions could play a decisive role in mineralisation of numerous compounds. Such interactions were already proposed by other authors (Wackett and Ellis, 1999) and in the light of the results of this work, further investigation seems very significant. It should not be neglected that also several inhibitory effects observed can play a major role in natural environments. In order to be able to apply findings of laboratory studies under field conditions, one should investigate also the behaviour and metabolic activity of bacterial strains when exposed to a mixture of contaminants, in order to simulate conditions in their natural habitat.

Despite the enormous structural diversity of polycyclic aromatic compounds, only naphthalene, 2-methylnaphthalene and phenanthrene were shown to be degraded by transferable cultures under anoxic conditions (Zhang and Young, 1997; Galushko et al., 1999; Meckenstock et al., 2000).

The enrichment of a sulphate-reducing culture that can grow on biphenyl as the sole carbon source and electron donor enlarges the spectrum of PAHs, whose anaerobic degradation can be studied in laboratory. I succeeded to keep this strain in a transferable culture, even though efforts in order to enhance the growth showed less success. Also this culture exhibited a cometabolic potential and transformed fluorinated biphenyl to the corresponding carboxylic acid. This finding can be regarded as a model for cometabolism of lower halogenated aromatic compounds that are often recalcitrant under anoxic conditions (Wiegel and Wu, 2000).

Generally it can be said that the research field of the anaerobic degradation of polycyclic aromatic hydrocarbons is still nearly a terra incognita so far. Even if the anaerobic degradation pathways of naphthalene and 2-methylnaphthalene are at least roughly elucidated, these results should be compared with other cultures utilising the same substrates. It should be kept in mind that the bacteria can exhibit a different behaviour under natural conditions than in the laboratory culture. This aspect should be considered in future investigations of natural attenuation. The new insights could largely contribute to the evaluation and management of contaminated sites in general and for the

protection of drinking water reservoirs in subsurface.

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Appendix

Succinyl-CoA:naphthyl-2-methyl-succinate CoA-transferase enzyme assays

Concentrations [µM] measured in a succinyl-CoA:naphthyl-2-methyl-succinate CoA-transferase assay with 2-methylnaphthalene grown cells.

Time [min]	Succinyl-CoA	NMS	NMS-CoA
0	57.37	24.75	209.56
5	102.85	69.60	180.40
10	130.73	105.06	122.60
15	116.95	133.70	92.55
25	108.15	164.40	71.86

Measured protein concentration : 157.03 mg/L

The enzyme activity was calculated using the increase of succinyl-CoA concentration between 0 and 10 minutes.
Calculated activity : 46.75 nmol*min^{-1}*mg protein

Concentrations [µM] measured in a succinyl-CoA:naphthyl-2-methyl-succinate CoA-transferase assay with 2-methylnaphthalene grown cells.

Time [min]	Succinyl-CoA	NMS	NMS-CoA
0	157.67	12.27	197.71
2	150.82	28.18	146.56
4	205.84	47.15	157.44
6	245.52	70.15	149.21
8	247.58	85.37	130.22
10	272.24	102.25	122.93
12,5	348.24	116.76	83.92
15	294.75	129.61	99.55
20	308.82	151.79	97.12
25	272.51	164.83	87.96
30	191.88	167.51	35.98
40	152.40	175.87	34.12

Measured protein concentration : 134.96 mg/L

The enzyme activity was calculated using the increase of succinyl-CoA concentration between 0 and 20 minutes.
Calculated activity : 55.99 nmol*min^{-1}*mg protein

These data were used for the figure 2.1a

Control experiment for the a succinyl-CoA:naphthyl-2-methyl-succinate CoA-transferase assay without cells. These data were used for the figure 2.1b

Time [min]	Succinyl-CoA	NMS	NMS-CoA
0	193.25	252.51	13.08
2	210.58	263.70	n. d.
4	191.93	235.73	n. d.
6	188.50	246.96	23.43
8	178.23	236.32	27.71
10	188.66	232.48	37.64
12.5	170.16	206.50	38.21
15	174.96	218.48	45.26
20	175.91	200.21	53.91
25	146.13	178.56	62.58
30	123.79	147.91	67.33
40	114.56	122.51	74.23

Concentrations [µM] measured in a succinyl-CoA:naphthyl-2-methyl-succinate CoA-transferase assay with naphthalene grown cells.

Time [min]	Succinyl-CoA	NMS	NMS-CoA
0	114.15	29.44	993.83
15	205.41	186.17	393.53
30	131.83	242.04	232.88
60	96.06	273.07	117.46

Measured protein concentration : 220.67 mg/L

The enzyme activity was calculated using the increase of succinyl-CoA concentration between 0 and 15 minutes.
Calculated activity : 27.57 nmol*min^{-1}*mg protein

Concentrations [µM] measured in a succinyl-CoA:naphthyl-2-methyl-succinate CoA-transferase assay with naphthalene grown cells.

Time [min]	Succinyl-CoA	NMS	NMS-CoA
0	274.93	35.16	385.54
2.5	294.11	44.87	371.69
5	255.17	36.18	421.89
7,5	328.47	116.08	300.50
10	351.82	148.79	245.91
15	441.83	199.15	163.90
20	530.26	296.53	125.31

Measured protein concentration : 110.33 mg/L

The enzyme activity was calculated using the increase of succinyl-CoA concentration between 0 and 20 minutes.
Calculated activity : 115.7 nmol*min^{-1}*mg protein
Concentrations [µM] measured in a succinyl-CoA:naphthyl-2-methyl-succinate CoA-transferase assay (oxic)

Time [min]	Succinyl-CoA	NMS	NMS-CoA
0	311.36	25.06	398.58
2.5	367.11	84.96	266.91
5	481.76	166.38	204.83
7,5	437.58	188.54	177.25
10	418.16	191.95	137.33
15	399.36	243.27	143.58
20	359.39	272.95	134.33

Measured protein concentration : 435 mg/L

The enzyme activity was calculated using the increase of succinyl-CoA concentration between 0 and 5 minutes.
Calculated activity : 78.16 nmol*min^{-1}*mg protein

Influence of hydroxylamin at the activity of the succinyl-CoA:naphthyl-2-methyl-succinate CoA-transferase: Formation of succinyl-CoA in relative units

Time [min]	without hydroxylamin	20 mM hydroxylamin
0	20.3	32.1
3	37.5	47.2
6	75.3	61.9
9	88.2	86.9
12	103.8	87.4

Influence of borohydride at the activity of the succinyl-CoA:naphthyl-2-methyl-succinate CoA-transferase: Formation of succinyl-CoA in relative units

Time [min]	without borohydride	10 mM borohydride
0	39	27
3	77	49
6	96	67
9	104	74
15	102	87

Influence of borohydride at the activity of the succinyl-CoA:naphthyl-2-methyl-succinate CoA-transferase: Formation of succinyl-CoA in relative units

Time [min]	without borohydride	10 mM borohydride
0	37.6	37.4
3	43.6	38.0
6	55.1	39.4
9	60.8	51.8
12	79.0	62.2
15	85.6	71.6

Naphthyl-2-methyl-succinyl-CoA dehydrogenase enzyme assays

NMeS concentrations measured in a naphthyl-2-methyl-succinyl-CoA dehydrogenase assay with 2-methylnaphthalene grown cells.

Time [min]	NMeS [µM]
0	0
5	0.072
10	0.138
15	0.141
25	0.192

Measured protein concentration : 157.03 mg/L

The enzyme activity was calculated using the increase of NMeS concentration between 0 and 10 minutes.
Calculated activity : 0.0879 nmol*min^{-1}*mg protein

NMeS concentrations measured in a naphthyl-2-methyl-succinyl-CoA dehydrogenase assay with 2-methylnaphthalene grown cells.

Time [min]	NMeS [µM]
0	0
2	0
4	0
6	0.124
8	n. d.
10	0.214
12,5	0.366
15	0.308
20	0.379
25	0.436
30	0.480
40	0.402

Measured protein concentration : 134.96 mg/L

The enzyme activity was calculated using the increase of NMeS concentration between 0 and 30 minutes.
Calculated activity : 0.119 nmol*min^{-1}*mg protein
These data were used for the figure 2.2

NMeS concentrations measured in a naphthyl-2-methyl-succinyl-CoA dehydrogenase assay with naphthalene grown cells.

Time [min]	NMeS [µM]
0	0
15	0.586
30	0.627
60	0.637

Measured protein concentration : 220.67 mg/L

The enzyme activity was calculated using the increase of NMeS concentration between 0 and 15 minutes.
Calculated activity : 0.177 nmol*min^{-1}*mg protein

NMeS concentrations measured in a naphthyl-2-methyl-succinyl-CoA dehydrogenase assay with naphthalene grown cells.

Time [min]	NMeS [µM]
0	0
2.5	0
5	0
7.5	0.11
10	0.052
15	0
20	0.166
30	0.286

Measured protein concentration : 110.33 mg/L

The enzyme activity was calculated using the increase of NMeS concentration between 0 and 30 minutes.
Calculated activity : 0.086 nmol*min^{-1}*mg protein

NMeS concentrations measured in a naphthyl-2-methyl-succinyl-CoA dehydrogenase assay (oxic)

Time [min]	NMeS [µM]
0	0
2.5	0.28
5	1.16
7.5	1.60
10	1.79
15	2.66
20	2.79
25	2.88
30	2.92

Measured protein concentration : 435 mg/L

The enzyme activity was calculated using the increase of NMeS concentration between 0 and 20 minutes.
Calculated activity : 0.32 nmol*min^{-1}*mg protein

Electron acceptor test for thenaphthyl-2-methyl-succinyl-CoA dehydrogenase. NMeS concentrations are given in µM.

Time [min]	PMS + FAD + NAD	PMS	FAD + NAD
0	0	0	0
5	2.878	0.844	0
10	3.156	2.276	0
15	3.368	2.818	0
20	4.498	5.213	0
30	5.137	6.122	0

Naphthyl-2-methyl-succinate synthase enzyme assays with naphthalene grown cells

time [h]	NMS [µM]
0	0
1	1.007
2	1.338
3	1.739
4	1.746
5	2.021
6	1.394
7	2.943
9	4.260

Measured protein concentration : 537 mg/L

The maximal enzyme activity was calculated using the increase of NMS concentration between 0 and 1 hours.
Calculated activity : 0.029 nmol*min^{-1}*mg protein

time [h]	NMS [µM]
0	0.366
0,5	1.102
1	2.461
2	4.499
3	5.096
5	6.232

Measured protein concentration : 1110 mg/L

The maximal enzyme activity was calculated using the increase of NMS concentration between 0 and 2 hours.
Calculated activity : 0.031 nmol*min^{-1}*mg protein

Growth of the culture N47 monitored as sulphide concentration when transferred from naphthalene to naphthalene (bottles 1 and 2), from naphthalene to 2-methylnaphthalene (bottles 3 and 4), from 2-methylnaphthalene to 2-methylnaphthalene (bottles 5 and 6) and from 2-methylnaphthalene to naphthalene (bottles 7 and 8).

These data were used for the figure 3.3

days	1	2	3	4	5	6	7	8
1	1.33	1.40	1.47	1.46	1.48	1.54	1.34	1.42
17	1.76	1.70	1.82	1.74	1.86	1.89	1.30	1.35
36	2.96	2.84	2.34	2.22	2.92	2.99	1.29	1.32
50	4.50	4.36	2.59	2.54	3.75	3.75	1.18	1.32
66	5.43	5.14	3.05	3.03	4.38	4.38	1.14	1.23
80	6.18	6.21	4.13	4.29	5.31	5.41	1.20	1.32
93	6.06	6.03	4.22	4.32	5.22	5.24	1.18	1.34
113							1.72	1.90
127							2.51	2.80
141							3.39	3.55
155							5.07	4.93
171							5.21	4.94
183							5.36	5.16

Excretion of ring-deuterated metabolites napthyl-2-methyl-succinate (NMS) and naphthyl-2-methylene-succinate (NMeS) into the supernatant by culture N47 during growth on fully deuterated naphthalene d_8.

These data were used for the figure 3.2

weeks	NMS [µg/L]	NMeS [µg/L]
4	n.d.	n.d.
6	2.272	2.478
8	2.783	4.168
11	12.948	13.725

Substrate utilisation of the sulphate-reducing enrichment culture N 47 monitored as sulphide production. The following combinations of main substrate and cosubstrate were used; naphthalene + benzofuran (1), 2-methylnaphthalene + benzofuran (2), naphthalene + benzothiophene (3), 2-methylnaphthalene + benzothiophene (4), naphthalene + indene (5), 2-methylnaphthalene + indene (6), naphthalene + indane (7), 2-methylnaphthalene + indane (8), naphthalene + indole (9), 2-methylnaphthalene + indole (10), naphthalene + quinoline (11), 2-methylnaphthalene + quinoline (12), naphthalene (13), 2-methylnaphthalene (14).

These data were used for the figures 4.2a and 4.2b

days	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	1.271	1.194	1.239	1.093	1.187	1.161	1.251	1.139	1.268	1.178	1.151	1.050	1.131	1.154
8	1.393	1.271	1.359	1.219	1.228	1.248	1.347	1.174	1.306	1.285	1.327	1.169	1.392	1.210
16	1.468	1.340	1.604	1.385	1.275	1.311	1.473	1.310	1.492	1.330	1.415	1.212	1.511	1.309
28	1.621	1.556	1.831	1.569	1.238	1.532	1.765	1.522	1.656	1.450	1.585	1.291	1.879	1.580
35	1.775	1.711	2.125	1.787	1.249	1.661	2.094	1.733	1.773	1.421	1.569	1.350	2.229	1.861
42	1.935	1.978	2.549	1.905	1.206	1.924	2.555	1.994	1.868	1.526	1.733	1.392	2.594	2.052
49	2.246	2.168	3.332	2.268	1.201	2.052	3.236	2.351	2.304	1.667	1.997	1.508	3.628	2.290
65	3.331	2.929	4.472	3.068	1.190	2.888	4.555	3.075	3.262	2.000	2.925	1.803	4.922	3.135
74	3.962	2.584	5.172	3.392	1.258	3.340	5.128	3.275	3.905	2.297	3.659	2.105	5.530	3.549
81	4.567	3.453	5.050	3.616	1.211	3.486	5.258	3.456	4.185	2.372	3.933	2.211	5.514	3.807
88	5.077	3.733	5.506	3.953	1.227	3.728	5.359	3.778	4.928	2.639	4.667	2.226	5.432	3.912
95	5.112	4.087	5.326	4.279	1.363	4.058	5.003	4.156	4.847	3.280	4.688	2.550	5.384	4.349
102		4.202		4.476	1.511	4.321		4.261		3.378		3.268		4.600
109		4.418		4.534	1.732	4.538		4.555		3.440		3.511		4.692
117		4.367		4.523	2.126	4.315		4.473		3.513		3.567		4.819
127					2.218					3.741		4.036		
134					2.467					4.015		3.929		
140					3.279					4.131		4.124		
152					3.632					4.259		4.247		
162					3.972					4.629		4.425		
173					4.311					4.307		4.277		

Substrate utilisation of the sulphate-reducing enrichment culture N 47 monitored as sulphide production. The following combinations of main substrate and cosubstrate were used; naphthalene + : biphenyl (1), naphthalene + 1-methylnaphthalene (2) naphthalene + acenaphthene (3) naphthalene + acenaphthylene (4) naphthalene + fluorene (5), naphthalene + phenanthrene (6), naphthalene + anthracene (7), 2-methylnaphthalene + biphenyl (8) 2-methylnaphthalene + 1-methylnaphthalene (9), 2-methylnaphthalene + acenaphthene (10), 2-methylnaphthalene + acenaphthylene (11), 2-methylnaphthalene + fluorene (12), 2-methylnaphthalene + phenanthrene (13), 2-methylnaphthalene + anthracene (14)

These data were used for the figures 4.2c and 4.2d

days	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	1.170	1.079	1.054	1.131	1.021	1.081	1.141	1.021	0.965	0.934	1.141	1.041	1.059	1.074
34	3.856	3.640	3.615	2.061	3.278	3.747	3.535	2.299	2.337	2.521	2.548	1.943	2.032	2.514
45	4.946	4.828	4.925	2.283	4.513	5.048	5.115	2.969	2.988	3.175	3.447	2.086	2.408	3.482
58	5.745	5.590	5.300	2.137	5.192	5.758	5.881	3.498	3.683	3.796	4.042	2.253	2.774	4.095
73	5.821	5.865	5.957	2.110	5.791	6.082	6.100	4.271	4.702	4,380	4.828	3.201	3.329	4.917
87	6.113	6.117	5.876	1.938	6.067	6.414	6.217	4.654	5.114	4,938	5.091	3.904	3.976	5.283
101								5.077	5.266	5,247	5.300	4.737	4.811	5.432

Growth of the sulphate-reducing enrichment culture BiphS1 with biphenyl as sole carbon source and electron donor monitored as sulphide concentration [mM] in presence of 3 mM Fe^{II} (1 and 2) and 6 mM Fe^{II} (3 and 4)

These data were used for the figure 5.1

days	1	2	3	4
1	0.782	0.769	0.593	0.593
12	0.825	0.843	0.626	0.678
22	1.174	1.121	0.949	0.909
31	1.564	1.576	1.357	1.319
40	2.027	1.954	1.852	1.790
47	2.625	2.423	2.526	2.276
54	2.812	2.710	2.812	2.635
60	2.404	2.569	3.079	2.889
71	1.992	2.114	3.315	3.192
78	--	--	3.203	3.191